RESEARCH ARTICLE

Stress-induced production of lipids in oleaginous microalgae for biofuel optimization

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Abstract

Emerging concerns over energy security and accelerating climate change have led to a global interest in developing a sustainable biofuel. Recently, it was determined that algal biofuels can be possibly cultured in a wastewater medium due to high levels of nutrients nitrogen, phosphorus, and potassium (NPK), reducing economic costs. Further efforts are needed in strain selection for cultivation in wastewater or inexpensive fertilizer. Common algal strains Nannochloropsis sp. and Chlorella sp. were studied to determine effects. Both strains were exposed to standard nutrients, which acted as controls, and high levels of NPK to simulate wastewater. The results revealed that NPK had a positive effect on turbidity and dissolved oxygen while decreasing the lipid productivities (measured via hexane solvent extraction) by an average of 36.5 percent. Specifically, Nannochloropsis sp. had overall higher values for all dependent variables. It is believed that under nutrient limiting conditions the cells deposited fatty acids in a triacylglycerol pathway. A follow-up experiment was performed using salinity as a novel method of decreasing biomass while maintaining lipid viability. Results revealed that freshwater algae can be cultivated in salinity levels between 10-15 ppt in order to increase lipid production. It is believed that while salinity osmotically interfered with the cell, lipid content increased, acting as a protective mechanism. Future research should focus on optimizing lipid production under stressed conditions using genetic manipulation of the TAG biosynthesis pathway.

Keywords: Biofuel; Lipids; Algae; Nitrogen, Phosphorus, and Potassium; Biomass; Salinity Stress; Nannochloropsis; Chlorella; Novel; Optimization

Introduction

Human life in the 21st century is related to the availability of energy sources, specifically non-renewable fossil fuels. Due to their non-fossil fuel based nature, biofuels have the potential to act as an alternative. Previous attempts to create a 1st generation "biofuel" with fats, oil, and cellulolytic enzymes have been unsuccessful as they are limited in reaching targets for oil-product substitution, climate change mitigation, and economic growth [1]. As a result, scientists have transitioned to next-generation microalgal biofuel in an attempt to reduce land requirements for production while sustaining a high energy yield. Second generation biofuels are those made from non-food crops. After realizing such yield potential, scientists often term algal biofuels 3rd or 4th generation fuels. Third generation biofuels are those made from non-arable land (ex. algae), but require the destruction of biomass. Fourth generation biofuels are made using non-arable land (ex. algae) and do not require

biomass destruction. Specifically, these biofuels can achieve targets for production, offset carbon emissions, and stimulate industry growth [1].

However, numerous improvements (i.e. growing parameters) are needed in order to make algal biofuels viable. Due to high levels of nutrients nitrogen, phosphorus, and potassium (NPK), algal biofuels can possibly be cultured in a wastewater medium. Thus, the use of wastewater in algal production could lower economic costs while producing significant biomass. Further efforts are needed in strain selection with the use of "nutrient-rich wastewater or inexpensive fertilizer"" containing the NPK nutrients [1]. Nannochloropsis sp. and Chlorella sp., seawater and freshwater strains respectively, are commonly studied in biofuel research due to generally high lipid and biomass productivities. Microalgal biofuels can potentially be used for bio-mitigative purposes, transferring excess carbon out of the atmosphere while producing high levels of oxygen. Under certain conditions, O2 output may differ affecting the bio-mitigative capability for each strain. Thus, it is imperative to determine how these two strains develop in nutrient conditions (high levels of NPK) similar to wastewater and if increased biomass equates to increased lipid and O2 productivity.

Because algal cultivation is not directly correlated to human use and does not require a large production area, it is a prime source for biofuels. The production of an algal biofuel is related to the cellular lipid production per dry weight of biomass. Thus, the prime concern in algal biofuel development is the yield per input cost. Overall "success" of algal strain is determined by growth rate, cell density and lipid content, so "optimization of each parameter affects this downstream processing efficiency" [2]. The relation between biomass and lipid content is of extreme importance as these parameters will ultimately affect productivity. Overall algal yield is inherently controlled by environmental and genetic factors that are currently not fully understood. If, however, the cultivation process can become economical, such as using low-quality sources (including the presence of waste or increased salinity), this may result in further progress in creating commercial biofuel production in combination with optimizing downstream harvesting, extraction, and conversion to biofuel. Thus, this study attempts equating "algal yield" with economic efficiency in order to optimize growing procedures.

It is already established that increased NPK levels result in increased biomass [1]. However, these previously studied nutrient levels were not representative of those in wastewater. Consequently, research has recently focused on the utilization of wastewater in biofuel production to limit economic expenses. Initial experimentation has assessed variables for maximizing algal production under various types of wastewater-like conditions [3]. However, the comparison of biomass and dissolved O2 (DO) with lipid productivities of specific algal strains has been only partially reviewed. If increased biomass with NPK amounts similar to wastewater equates with higher lipid productivities, this would provide an attractive advantage for biofuels with regard to limiting expenses. A high NPK level in wastewater may have an adverse effect and inhibit algal growth in certain species. This study attempts to simulate wastewater levels of NPK by creating a growing medium similar to the proportions found in residential wastewater, the first of its kind to do so [3].

The purpose of this study is to determine the effects of NPK levels similar to wastewater conditions on the lipid, DO, and biomass productivity (turbidity) of

microalgal strains Chlorella sp. and Nannochloropsis sp. The experiment has a total of two treatment levels, positive and negative presence of fertilizer in two species. The negative presence of fertilizer in each strain will act as controls. The presence of fertilizer, specifically 50.19 mg of nitrogen and 18.75mg of phosphorus per liter, the primary nutrients, was decided upon in order to simulate wastewater-like nutrients [4]. Two different types of fertilizer will be mixed in order to achieve the desired NPK levels based on the percentage of NPK in each. It is believed that if Chlorella sp. is exposed to levels of NPK, then it will have the greatest growth after its respective growing period, amount of lipids present, and amount of DO. This is based on previous experiments where Chlorella sp. had the both the highest biomass and lipid productivities under optimal experimental conditions [5].

Materials and Methods

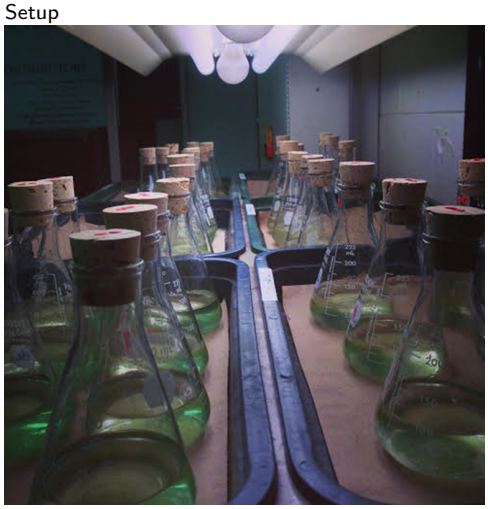
Five stock cultures of Chlorella sp. and Nannochloropsis sp. (Carolina Bio) were swirled in order to ensure equal distribution. In order to obtain a 1:10 ratio between stock culture and medium, 228 mL of the stock culture for each strain was transferred into 2280 mL of its respective growing medium. After stirring the subculture, 100mL of the Chlorella sp. subculture were pipetted into 10 cleaned and autoclaved 250mL flasks. Then, 1500mL of the Chlorella sp. subculture were poured and mixed thoroughly into another beaker containing 118mg of "24N-25P-4K Scotts Turf Builder Starter Food," and 168mg of "32N-0P-10K Scotts Turf Builder WinterGuard Fall Lawn Fertilizer." Next, 100mL of the Chlorella sp. containing NPK were pipetted into another 15 of the same flasks with the letter "F," indicating that these cultures were to have the presence of NPK. These above steps were repeated for the Nannochloropsis sp. strain.

A sample of 3mL from each flask was placed into a Vernier colorimeter, calibrated to 470nm, the optimal absorption wavelength for chlorophyll a. The respective growing medium was used as a blank, and the Absorption Units (AU) were recorded for an initial turbidity reading. The initial DO content was measured in mg/L using a "Lab Quest" device with an "Optical DO" probe. All flasks were corked and placed 7cm below a "white light" area, emitting an electromagnetic wavelength appropriate for photosynthesis, where the temperature was 22 C, the optimal growing condition for strains of algae used. The light was switched on and it was confirmed that no other forms of direct light were present (figure 1). After 10 days, the flasks were removed from the light and the DO content and turbidity were recorded in the same manner.

Prior to lipid extraction, Chlorella sp. cultures were subjected to 3g of NaCl per 100mL flask and the Nannochloropsis sp. were subjected to 46.5g. The Chlorella sp. was heated to 70 C for two hours and the Nannochloropsis sp. was heated to 70 C for five hours using a water bath to lyse the cells. The purpose of adding the salt and heat was to degrade the cellular membranes and walls and provide for cytoplasmic material to exit the cell. Because the Nannochloropsis sp. thrives in a salt water medium, a greater amount of salt was needed in order to achieve the same effects as in the freshwater strain. The cells were viewed under a microscope to determine when they were lysed. The extra-cellular material was filtered out using Fisher P8 filter paper, filtering particulates from 20 to 25 micrometers. The turbidity of all

cultures were equilibrated to the average of the control group with the lowest turbidity (.064 AU) as a standard for lipid comparison. To prevent any growth from contaminants, the cultures were refrigerated at 2 C. Seven milliliters of hexane (C6H14) were added to 100mL of the lysate and swirled for four minutes. Eye protection/gloves were worn during all parts that involved hexane. The lysate/hexane solution was then placed into a 500mL separation funnel and the bottom water layer was drained into a 100mL beaker. Three grams of sodium sulfate anhydrous were added to the hexane mixture (top layer) and the solution was filtered, using P8 filter paper, into another beaker. The initial mass of the beaker was determined before filtering. This was repeated for the same water layer three times with new sodium sulfate to extract the maximum amount of lipids. The hexane solution was then placed under a fume hood in order to evaporate the hexane (time varied). The difference between the initial and final mass of the beaker was determined. This extraction method was repeated for all treatment levels. At completion, the flasks/beakers were cleaned and the lipids/algae were disposed of properly.

Figure 1 Algal Culture Setup



Results

The effects of high concentrations of NPK on the DO content, turbidity, and lipid productivities of Chlorella sp. and Nannochloropsis sp. were studied and the results of the statistical analysis are shown in figures 1-3. A research hypothesis was formed that if Chlorella sp. is exposed to levels of NPK, then it will have the greatest growth after its respective growing period and amount of lipids and DO. The mean change in turbidity, DO, and lipid mass was determined for each treatment group. The comparison of both strains with the presence of NPK to the controls implies that this specific growing parameter does have an effect on the turbidity, DO content, and lipid productivity. Upon further analysis, it is shown that the NPK treatment groups had the greatest positive change in turbidity/DO and that these nutrients have a positive effect (figures 2 and 3). Specifically, after the 10 day period, the Nannochloropsis sp. NPK group had a greater change in turbidity/DO compared to the Chlorella sp. NPK. In addition, it is shown that the control groups had the

greatest production of lipids in both strains. Specifically, after the 10 day period, the Nannochloropsis sp. control group had a greater lipid production compared to the Chlorella sp. control group (figure 4). Due to these results, the research hypothesis was not supported. The standard deviation was low for all levels of IV implying that the data sets were tight and precise.

A single variable ANOVA test was performed on the data with a level of significance of 0.01 with appropriate degrees of freedom. The null hypothesis was that there would be no significant difference in the change in lipid productivities, turbidity, and DO content between Nannochloropsis sp. and Chlorella sp. exposed to levels of NPK and the controls. The calculated value for almost all levels of independent variable was greater than the critical F values. This implies that the null hypothesis should be rejected and there is a significant difference between most treatment groups (except between Nannochloropsis sp. control and Chlorella sp. NPK for DO content as shown in an overlapping confidence interval). The probability of the results being due to chance is less than 0.01 for turbidity, DO, and lipids and implies that the results are most likely due to the independent variable.

Figure 2 Average change in turbidity of algal strains exposed to varying conditions
Average change in turbidity (with 95 percent confidence error) of two different algal strains exposed to either control conditions or the presence of nitrogen, phosphorus, and potassium. n=10 for control and n=15 for experimental conditions. ANOVA is significant at α=0.01 and p-value= $7.84*10^{-14}$

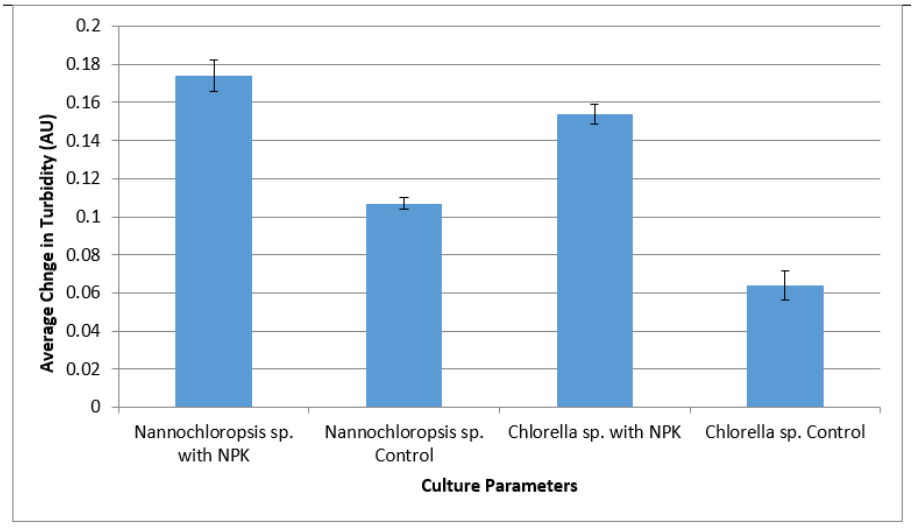


Figure 3 Average change in dissolved oxygen of algal strains exposed to various conditions
Average change in dissolved oxygen (with 95 percent confidence) of two different algal strains exposed to either control conditions or the presence of nitrogen, phosphorus, and potassium. n=10 for control and n=15 for experimental conditions. ANOVA is significant at α=0.01 and p-value= $2.53*10^{-19}$

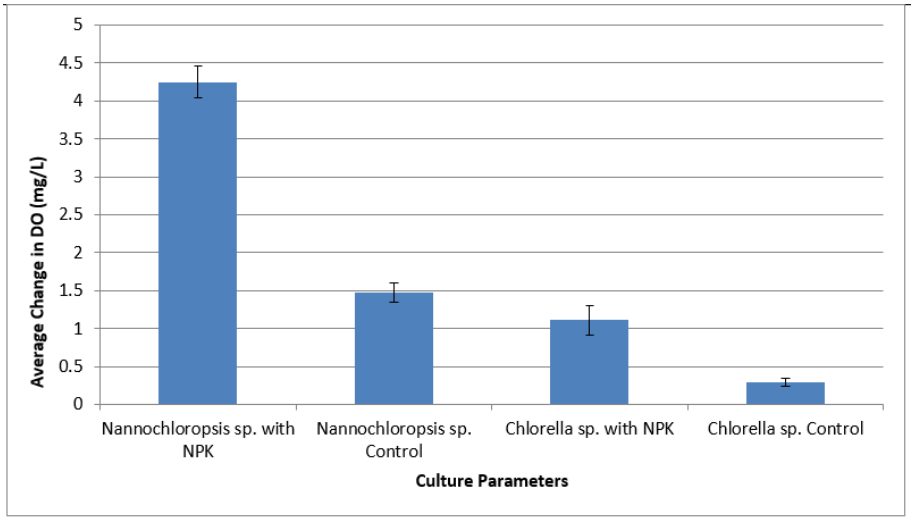
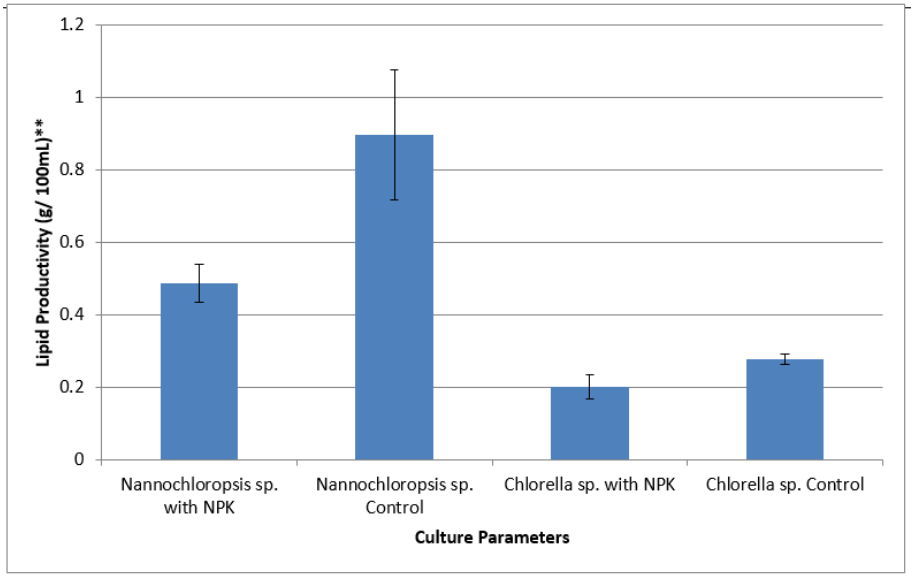


Figure 4 Average lipid productivity of algal strains exposed to varying conditions
Average lipid productivity (per 0.064 AU of biomass with 95 percent confidence) of two different algal strains exposed to either control conditions or the presence of nitrogen, phosphorus, and potassium. n=10 for control and n=15 for experimental conditions. ANOVA is significant at α=0.01 and p-value= $6.15*10^{-6}$



Discussion

The purpose of this study was to determine the effects of NPK on the turbidity, DO content, and lipid productivity of Chlorella sp. and Nannochloropsis sp. The experiment incorporated a blocked design with each species being exposed to NPK.

After 10 days of exposure, the appropriate dependent variables were measured. A research hypothesis was formed that if Chlorella sp. is exposed to levels of NPK, then it will have the greatest growth after its growing period, amount of lipids present, and amount of DO. It was found that the presence of NPK had a positive effect on the turbidity and DO content, but had a negative effect on the lipid production in the strains, specifically Nannochloropsis sp. Due to these results, the research hypothesis was not supported. An ANOVA test revealed statistical significance. A Sudan III stain verified lipid presence.

Other researches are investigating the effects of different growing parameters on microalgae suitable for biofuel use. Sharma et al. studied both urea and phosphorus limitational stress on numerous strains, finding both similar and contrary results. The study found that Nannochloropsis sp. lipid content increased by 15.31 percent via nitrogen limitation yet, phosphorus limitation resulted in decreased lipid content in Nannochloris atomus. However, this study focused on the specific ratio of nitrogen to phosphorus based on residential wastewater, thus if a higher phosphorus concentration and a different method of lipid quantification was used, most likely the results from Sharma et al. would be obtained [6]. Wang and Lan also found when studying the effects of NPK on Chlorella sp. in wastewater conditions, increased biomass production occurred with a growth pattern characterized by exponential phases in the first three days followed by stationary phases in the next six days [7]. Visual algal observation in this experiment supports this claim by Wang and Lan.

There are a number of ways that this study could have been improved. Although it was attempted to view the lysed cells under a microscope, there was no exact way to determine if the cells were completely lysed. In addition, it was difficult to determine when the hexane evaporation was completed. Although sodium sulfate was used as a drying agent, some water may have remained in the hexane mixture. Lastly, salt contents used to lyse the cells were varied during the experiment in order to find the optimal salinity for lysing, a possible source of error.

Conclusions

Statistical evidence supports a positive correlation between nutrient starvation and increased lipid productivity. Previous research characterizes environmental stress when nutrients are limited, causing a decline in cell division. Remarkably, active biosynthesis of fatty acid chains and lipid polymers are maintained in such conditions. When the rate of cell division decreases (decreasing biomass yield), cells deposit and redirect fatty acids into a triacylglycerol (TAG) pathway. Thus, the production of TAGs may serve as a protective mechanism and the lipids extracted increase with the deprivation of NPK, a possible explanation for the results received [6]. Although Chlorella sp. had fast, exponential growth, Nannochloropsis sp. efficiently utilized (long term) the supplied nutrients resulting in a higher biomass yield after the 10 days. Specifically, experimental cultures utilized NPK to increase the cell division rate thus increasing the turbidity. Because of this greater photosynthetic rate, experimental cultures had greater DO contents than their respective controls.

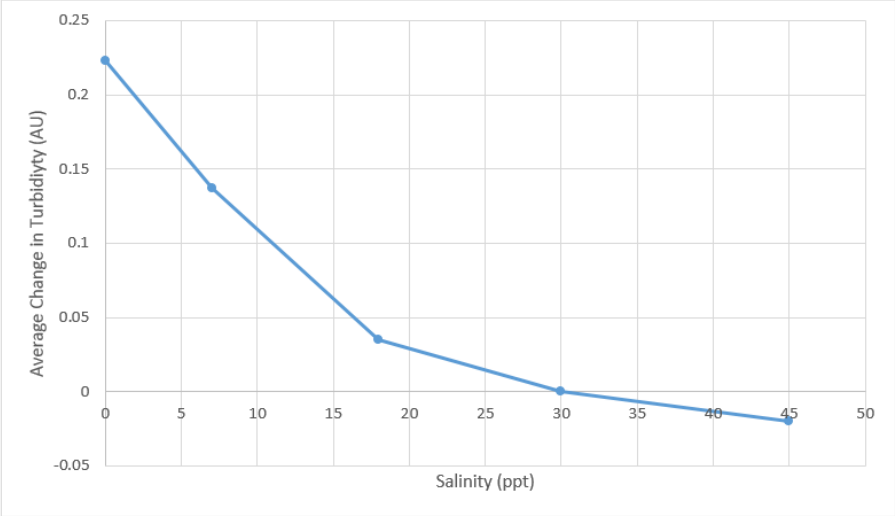
Based on the results of NPK having a positive effect on the turbidity and DO of microalgae, specifically Nannochloropsis sp., and having an adverse effect on the

overall lipid productivity, many conclusions can be made. Although the main focus of this study was to determine the effects of NPK in a ratio simulating wastewater, other findings were made regarding constants such as salinity, temperature, refrigeration, and lysing time in each strain. This study confirms that NPK will increase the biomass of Nannochloropsis sp. and Chlorella sp. Nannochloropsis sp. responds best to these nutrients during long term growth and is recommended for extended lipid production. It can be concluded that in the long term, Nannochloropsis sp. is a more sustainable strain, as it has the greater oxygen output offsetting carbon emissions. However, this study recommends the Chlorella sp. for a quick biomass yield. For future study, the growing period of both algal strains can be reduced as this may produce different results and gas chromatography can be used to identify specific lipids. Although culturing Nannochloropsis sp. in wastewater nutrient levels will produce greater biomass with reduced economic expenditures, the lipid productivity per cell ultimately decreases. The economic costs and benefits of culturing algae for biofuel using this method must be determined before industrialization. Further research should focus upon increasing lipid productivity via the genetic manipulation of acetyl-coenzyme A when these strains are cultured with increased nutrients in order to optimize the biofuel production process.

Follow-Up Research

Currently, follow-up research is nearing completion regarding increased lipid content and environmental stressed conditions resulting in decreased growth, a correlation determined from this study. Microalgal species can differ in their halotolerance capability. Chlorella sp. is currently being studied in relation to its halotolerance and lipid yield. If this strain proves to have increased lipid yield with biomass viability under salinity-stressed conditions, this could mark a major breakthrough in biofuel efficiency and industrialization. With exposure to a range of salinity levels (representing levels from fresh to brine water) of 0, 7, 18, 30,and 45 ppt, Chlorella sp. can be cultivated in salinity levels between 10 to 15 ppt in order to increase lipid production, but not in lieu of dramatically decreasing biomass production (figure 5). A brackish medium will prove useful in biofuel optimization.

Figure 5 Average Change in Turbidity of Chlorella sp. After 7 Days with Varying Salinities
Average change in turbidity of Chlorella sp. exposed to varying concentrations of salinity. n=10 for all control and experimental levels. ANOVA is significant at alpha level of 0.01 and p-value= $5.78*10^{-27}$



Competing interests
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Author's contributions
Eric Teichner designed all procedures, performed all experimentation, and wrote this manuscript. A mentor was **not** utilized.

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