Proceedings of GREAT Day

Volume 2017 Article 7

2018

Investigation into Cost Effective Cultivation and Biofuel Production from *Chlorella* Algal Species

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Abstract

As resources for petroleum-based fuel become increasingly scarce, third generation biofuels, which utilize algae as a renewable feedstock, offer a promising solution. The problem hindering widespread marketability, however, is that current biodiesel production processes are expensive and lipid yields are inconsistent. Our research worked to make algae cultivation more feasible by focusing on both the growth of algae and its conversion to crude biodiesel. To decrease the cost of cultivation, a modified Bold's Basal medium was created using low cost chemicals. This treatment was compared to commercial Alga-gro© and a water control. *Chlorella*, a fast growing microalgae with high lipid content, was then introduced to all three conditions and cell growth was monitored for 35 days. After harvesting the cells, the non-polar lipids were extracted using a 2:1 chloroform-methanol ratio, which showed yields (18.01%) consistent with previous work. Samples next underwent a transesterification reaction upon which IR spectroscopy was used to detect the presence of fatty acid methyl esters (FAME).

Introduction

Petroleum resources are finite; it is only a matter of time before these dwindling supplies disappear. In order to minimize our dependence on fossil fuels, the search for alternative sources of fuel has been of critical importance. Three generations of biodiesel have been explored. In the first generation, crops, such as corn and soybeans, were initially used to make ethanol and biodiesel. This strategy, however, is unsustainable due to the high energy and land requirements for harvesting, not to mention the competition with food markets in an age when the United Nations Food and Agriculture Organization (2015) estimates about 795 million people worldwide are undernourished. Aiming to combat some of these issues, second-generation biofuels are produced using organic agricultural waste, including corn husks. Here, high processing costs limit productivity. The third generation, biodiesel production using photosynthetic microalgae, offers a promising solution not only to these fuel limitations, but also to the rising concerns of global warming. While growing, microalgae such as *Chlorella* sequester CO_2 from the atmosphere, remove nitrogen and phosphorous from wastewater, and help to reduce our carbon footprint. Microalgae do not compete with the food market and have lower processing costs; however, it is the expense of cultivation and inconsistency of lipid yields that is currently preventing widespread marketability. With our work, we hope to take steps toward minimizing this cost through the creation of our own medium for the cultivation of *Chlorella* and testing the resulting lipid yields, ultimately contributing to a greener, more sustainable future.

Methods

1) Making modified Bold's Basal medium

The following stock solutions were created by adding the measured-out solute to volumetric flasks and diluting them with distilled water. The EDTA/KOH solution was heated up on a hot plate and stirred with a stir bar in order to dissolve the EDTA. The acidified water that was used to make the $FeSO_4 \cdot 7H_2O$ solution was created by slowly adding 1 mL of H_2SO_4 to

Published by KnightScholar, 2018
Zoe Marr, Colleen Steward, & Barnabas Gikonyo.
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Chemical Compound	Solute to Water Ratio	Mass of Compound
$NaNO_3$	2.5/100 mL H_2O	2.5066g
$MgSO_4 \cdot 7H_2O$	0.5 g/400 mL H_2O	.5061g
K_2HPO_4	1g/100 mL H_2O	1.0238g
KH_2PO_4	1.5/100 mL H_2O	1.5339g
$CaCl_2$	.25g/100 mL H_2O	.2581g
NaCl	.25g/100mL H_2O	.25130g
EDTA KOH	50g EDTA and 31g KOH/1 L H_2O	50.0650g 31.0339g
$FeSO_4 \cdot 7 H_2O$	.498g/100mL acidified H_2O	.4990g

Figure 1. Chemical compounds and ratios used to create Bold's Basal medium.

999 mL of distilled water. All of the solutions were made in advance and stored in poly bottles.

The Bold's Basal medium was created by adding 1 mL of the $NaNO_3$ solution, 10 mL of the $MgSO_4 \cdot 7H_2O$ solution, 10 mL of the K_2HPO_4 solution, 10 mL of the KH_2PO_4 solution, 10 mL of the $CaCl_2$ solution, 10 mL of the NaCl solution, and 1 mL of the EDTA/KOH solution to a 2.0 L Erlenmeyer flask filled with 936 mL of distilled water. The acidic $FeSO_4 \cdot 7H_2O$ solution was slowly added and the entire solution was mixed for several minutes with a stir bar.

A titration was set up in order to neutralize the medium. 31.0406g of KOH were added to 1 L of distilled water and used as the titrant. A Vernier and a pH probe were used to measure the pH of the medium. The KOH solution was added dropwise until the pH was around 7.

2) Autoclaving and establishing a culture

Figure 2. Populations of growing Chlorella.

250 mL of distilled water, Alga-gro©, and the Bold's Basal medium were autoclaved for 20 minutes each. The solutions were kept covered and allowed to cool.

The *Chlorella* was purchased from Carolina Biological Supply Company and stored in the fridge until use. The *Chlorella* was added by carefully removing some of the autoclaved medium with a sterilized pipette and adding it to the algae cultures. The *Chlorella* was then removed from the proteose agar with the pipette and transferred to the medium.

3) Monitoring the growth

In order to measure the growth, ~0.5 mL of medium was removed from the flasks with an autoclaved pipette. The culture was observed using a hemocytometer. The average number of cells per square mm was measured and then used to calculate the cell concentration. The cell count was measured over a period of 35 days.

4) Harvesting

The *Chlorella* was allowed to settle to the bottom and the top layer of medium was removed. The remaining medium and algae, now highly concentrated, was placed in centrifuge tubes and weighed out so the mass of each tube evenly matched another tube. The tubes of *Chlorella* were centrifuged at 2000 rpm for 10 minutes until a pellet at the bottom formed. The excess medium was removed and the pellets of algae were allowed to dry in the sun.

5) Lipid extraction

After the *Chlorella* cells were harvested and dried, the non-polar lipids were extracted from the microalgae using a method previously tested on dried *Chlorella* samples. In this process, organic solvents break open cell membranes to expose the desired lipids and sepa-

rate out other cellular structures. Because the algae samples were hardened to the bottom of the test tubes, 3 mL of chloroform and 1.5mL of methanol were carefully measured and added to each centrifuge tube to establish the desired 2:1 chloroform-methanol ratio. These tubes were then vigorously shaken for 10–15 minutes. Another 1 mL of methanol was added to lower the specific gravity before the tubes were centrifuged at 3000 rpm for 30 minutes. The supernatant was poured into a separatory funnel, the pellet consisting of unwanted cellular components discarded, and 2 mL of chloroform added to reestablish the 2:1 chloroform-methanol ratio. Additionally, a 0.73% sodium chloride (NaCl) was added to the separatory funnel to induce clearer layer separation between the lower non-polar lipid-chloroform layer and upper water-methanol layer. The bottom layer was then drained into a preweighed round bottom flask and rotary evaporated in a 50°C water bath for 1.5 hours. The round bottom flask was again weighed and a small sample was used for IR analysis.

6) Transesterification

Following lipid extraction, a transesterification reaction was conducted to convert the triglycerides into fatty acid methyl esters (FAME), otherwise known as biodiesel. Our setup consisted of a water bath, thermometer, and reflux condenser. A ratio of 12 mL of methanol and 3% HCl to 100 mg of harvested lipids was added to the round bottom flask and the transesterification reaction left to carry out at 65°C for 6 hours.

Results

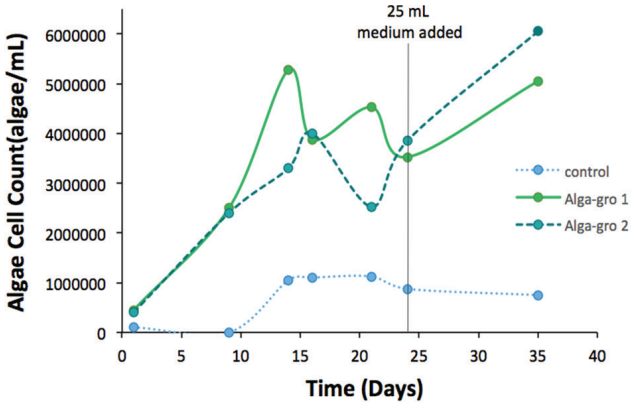

Figure 3. Growth pattern of Chlorella in Alga-gro© over a -35day period. Increasing growth for the first 13–15 days, then leveling off until the addition of 25 mL medium.

Discussion and Conclusion

The cell growth of *Chlorella* in three different medias was closely monitored for 35 days using a hemocytometer. The cell count was then utilized to calculate the average cell concentration. Graphs of cell concentration vs time were plotted in order to compare and visualize the varying growth rates. Figure 3 shows that the *Chlorella* samples in Alga-gro© began immediate growth once added to the medium and continued growing until around day 13–15. Between day 15–24, the algae reached a stagnant period in which their growth plateaued due to decreasing availability of nutrients in the medium. The fluctuations in these values are likely due to the error associated with averaging small sample sizes when using the hemocytometer. After the addition of 25 mL of medium, cell growth again increased, further indicating that the growth was dependent on the availability of nutrients and followed the expected trends observed in algae cultivation. Compared to samples in Alga-gro©, the *Chlorella* in modified Bold's Basal medium showed little growth initially (Figure 4). The cell count remained constant until approximately day 17 when an additional 25 mL of medium was added. As seen in the other sample, after this addition, growth increased rapidly. Despite its slower start, the *Chlorella* in this experimental medium successfully reached the same concentration as those in Alga-gro©. Therefore, it was determined that the modified Bold's Basal medium was effective. In order to increase efficiency, adjustments to future procedures and nutrient concentrations will be taken to stimulate growth through more frequent addition of medium.

After centrifuging the algae in a 2:1 chloroform to methanol ratio, the lipids and the chloroform were subsequently separated from the methanol using a separatory funnel extraction. The excess chloroform was evaporated off and then the mass of the lipids were measured to calculate the percent yield. In the Alga-gro© sample, the volume decreased almost immediately with vigorous bubbling upon rotary evaporation, leading to low lipid yields. However, the chloroform evaporated off slowly in the samples grown in the modified Bold's Basal medium, and an 18.01% lipid yield was calculated. This value falls within the expected 14–22% range for *Chlorella* (Milano et al., 2016).

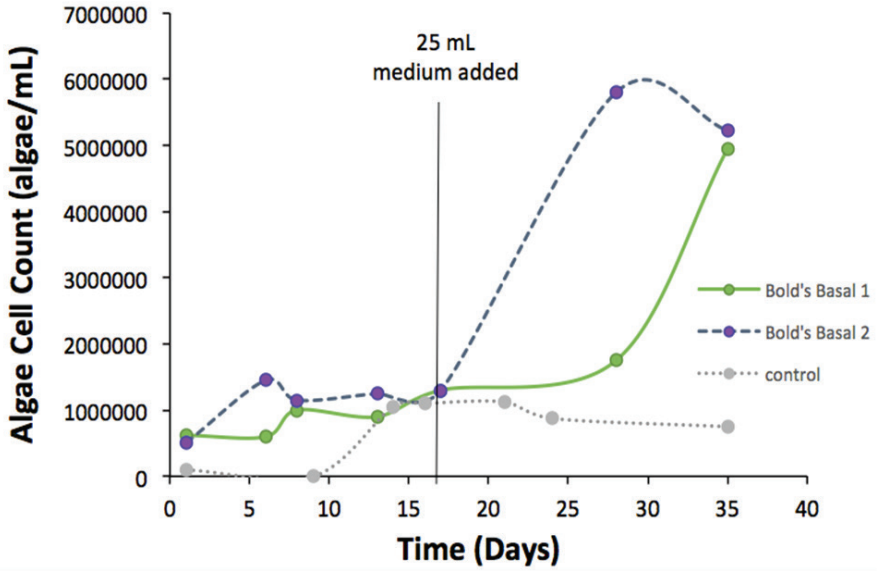

Figure 4. Growth pattern of Chlorella in Bold's Basal medium for 35 days. Algae had little growth within the first 17 days but grew rapidly after 25 mL medium were added.

The lipids underwent a transesterification process to convert the triglycerides to methyl esters. In Figure 5, the product from the transesterification is displayed with the major peaks represented by the C=O stretch at 1716 cm^{-1} and several peaks from various C-H stretches around 3000 cm^{-1}. Compared to previous values, the C=O was more similar to the stretching frequency of the triglyceride than the methyl ester. This indicates that the transesterification reaction likely did not run to completion and consisted of some triglycerides. One complication that could have led to an incomplete reaction was the imprecise temperature measurement. Due to the narrow condenser, only the temperature of the water bath could be taken, not the temperature of the solution itself. In the future, the temperature of the reaction rather than the water bath will be measured to eliminate this source of error. Another possible explanation of this difference in frequencies could be that biodiesel was produced and the R groups of the fatty acid methyl esters were α,β-unsaturated, shifting the carbonyl peak into the 1715–1730 cm^{-1} range. However, more analysis is needed to confirm this premise.

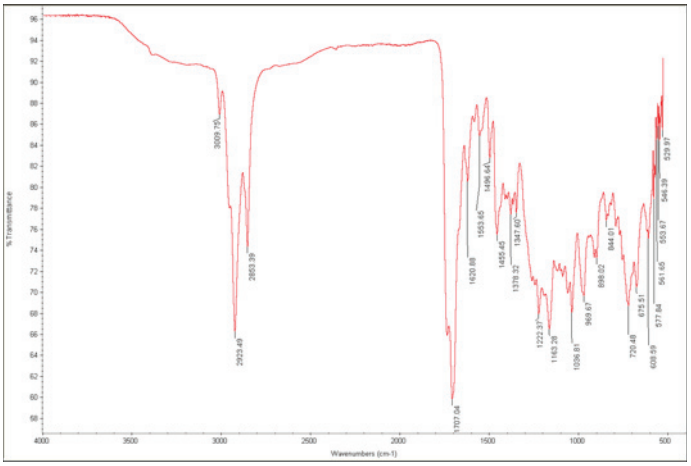

Figure 5. IR spectroscopy of lipids previously extracted from dried Chlorella samples; carbonyl peak 1707 cm^{-1}.

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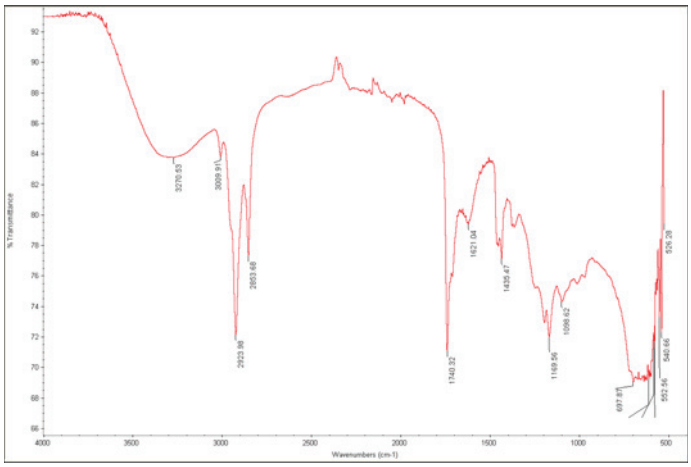

Figure 6. IR spectroscopy of previous transesterification product from dried carbonyl peak Chlorella; carbonyl peak 1740 cm^{-1}.

Overall, expanding upon previous studies with dried samples, we were able to successfully grow *Chlorella* not only in an Alga-gro© medium that cost $21.50 a quart, but also in a modified Bold's Basal medium at $0.10 a liter. Lipids were successfully extracted with expected yields and procedures to create biodiesel performed.

Future directions

We plan to increase the scale of our project by moving the growing process into a greenhouse in the SUNY Geneseo E-Garden. Another goal is to cultivate different algae strains that would maximize the lipid yields as well as cultivate higher algae concentrations. In order to make counting cells more efficient, we will utilize ImageJ so that the count will be computerized and more accurate. In terms of processing the algae and converting the lipids into biodiesel, we plan on altering the transesterification reaction conditions to ensure that biodiesel is formed. To verify biodiesel production, we will utilize ^{1}H NMR, ^{13}C NMR, and possibly gas chromatography as supplements to the IR spectroscopy, further verifying the identity of the fatty acid methyl esters and specific side chains. Once the current transesterification process has been solidified, we will use another procedure that is *in situ.*

Acknowledgements

We would like to thank Dr. Barnabas Gikonyo for his advisement, the Office of Sponsored Research for their financial support, and the SUNY Geneseo

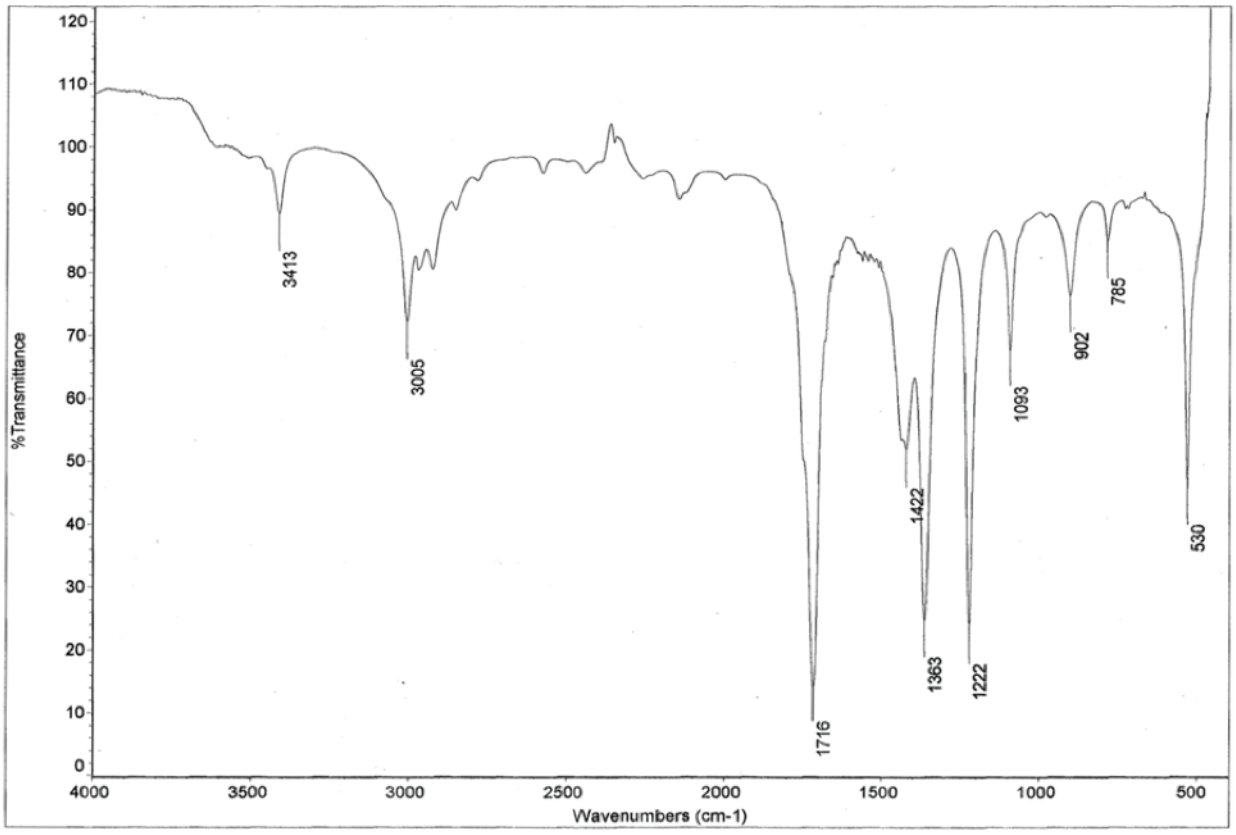

Figure 7. Infrared spectroscopy of transesterification product from extracted Chlorella lipids grown in the experimental Bold's Basal medium.

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Chemistry Department for their logistical and material support.

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Further reading

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