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The Potential of Microalgae for Cellulose Degradation and Utilization for Biofuel Application

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ABSTRACT

As society is becoming more reliant on fuels, a more sustainable form of energy must be investigated. Another presiding issue is the output of cellulose left over from other plants that are used for biofuels, such as corn. However, the microalgae, *Chlamydomonas Reinhardtii*, a unicellular organism, is an ideal source of energy, as there is evidence that it contains the genes that are responsible for the encoding of Cellulases, which allow for the degradation of cellulose, such as endoglucanases. *Chlamydomonas Reinhardtii* typically lives in both soil and water environments, a photosynthetic organism that utilizes light as an energy source. The uncommon trait for microalgae to express cellulase allows for external sources of carbon to be utilized by the microorganism, which could affect the biological output of macromolecules common in biofuels such as lipids and carbohydrates. The study aims to compare not only the cellulase expression levels of *Chlamydomonas Reinhardtii*, but also see how the lipid output of the microalgae compares to other microorganisms used in the biofuel industry such as *Chlorella Vulgaris*, another phototrophic microalgae, which is used for direct fuel. Additionally, *Trichoderma Reesei* will also be compared, which is another microorganism that is used for biofuel production. However, the industry utilizes Trichoderma Reesei's ability to produce cellulase, rather than just taking directly from the microorganism. The conclusions unfortunately did not show any cellulase expression, and biofuel output favored algae.

Introduction

As society is becoming more reliant on fuels, a more sustainable form of energy must be investigated. Another presiding issue is the output of cellulose left over from other plants that are used for biofuels, such as corn. However, the microalgae, *Chlamydomonas Reinhardtii*, is an ideal source of energy, as there is evidence that it contains the genes that are responsible for the encoding of Cellulases, which allow for the degradation of cellulose.

Chlamydomonas Reinhardtii typically lives in both soil and water environments, a photosynthetic organism that utilizes light as an energy source. The uncommon trait for microalgae to express cellulase allows for external sources of carbon to be utilized by the microorganism, which could affect the biological output of macromolecules common in biofuels such as lipids and carbohydrates.

The study aims to compare not only the cellulase expression levels of *Chlamydomonas Reinhardtii*, but also see how the lipid output of the microalgae compares to other microorganisms used in the biofuel industry such as *Chlorella Vulgaris*, another phototrophic microalgae, which is used for direct fuel. Additionally, *Trichoderma Reesei* will also be compared, which is another microorganism that is used for biofuel production.

The conclusions indicate no cellulase expression, and all assays indicate a significance level of $p>0.05$, meaning that the data is not statistically significant.

Society's reliance and overuse of traditional energy sources calls for alternatives to be researched. Current types of energy include Wind, Solar, Fossil Fuels, and Biofuels. Fossil fuels have dominated, with 80%

of domestic consumption originating from these fuels, they are rapidly becoming an issue with their sustainability. Alternatives such as biofuels have the potential to overtake fossil fuels.

Biofuels have emerged as a popular alternative to traditional forms of energy. Biofuels are energy fuels originated from living organisms. This includes ethanol, green diesel, and biogasses. The understanding and operations of biofuels have acted as a restraint. Currently, biofuels are too inefficient and too expensive to implement and run consistently. One aspect of biofuels is the operation of cellular assimilation, or degradation. Plants have lipids and carbohydrates extracted for biofuel use. This leaves cellulose a waste product. In the United States, corn is a very popular plant species to act as a biofuel, with approximately 37% of all ethanol production using corn, as it contains the lipids and carbohydrates needed to create ethanol, and the process of ethanol extraction is more efficient compared to the majority of other plants (1).

The process mainly extracts the lipids and carbohydrates from the plant, leaving cellulose as an unused product. This is a difficult material to break down, and a leftover product from the procedure.
With a lack of an efficient waste disposal system for the cellulose, it becomes an issue. Currently, about 40 million tons of cellulose from biofuel production is being produced a year. (2)

The costs of production in general biofuel production is also an issue. The cost difference between production of biodiesel and fossil fuels is 130% (3). This is higher than the current price of regular diesel. In addition to cost drawbacks, the efficiency of biofuels causes doubts about its future.
The costs directly correlate with the efficiency of production. So finding a solution that helps to fix both issues is ideal.

The introduction of fungi into this process has potential to increase biofuel output. It possesses the ability to release cellulase to break down the cellulose. It can also act as a source of carbohydrates and lipids itself. Traditional processes to process the fungi include fermentation and deconstruction in order to extract the components that are wanted from the organism. They can later be repurposed into components to create a biofuel.

As the demand for biofuels is rising, the processes in the production also rise to keep up with demand. Traditional processes pose an array of issues. These include cost, labor, and efficiency of current methods of extraction and maintenance of the microorganisms. Additionally, they take up a substantial amount of resources to properly maintain. Examples are mediums, temperature, and proper storage to avoid contamination.

The carbohydrates and lipids from fungi have potential for biofuel production. The issues stem with cost and efficiency. Up to 75% of the total costs for producing microbial lipids were for feedstock or carbon sources (4). The drawbacks from fungi usage in the industry has prevented fungi from being used widely.

One of the alternatives to fungi that has been researched is algae. *Chlamydomonas Reinhardtii* is a microalgae is an unicellular green algae that possesses two flagella. The algae takes its energy from typical sources. When in an environment with minimal or no access to carbon dioxide and a source of light, the algae begins to utilize cellulose as an energy. This occurs due to the *Chlamydomonas Reinhardtii* requiring a source of carbon. Without a ready source of cellulose, it must change its pathways in order to utilize cellulose instead. By placing the algae in an environment of high-salt minimal medium (MM) and air levels of carbon dioxide it was able to express cellulase and grow. Without having a source of carbon, the microalgae attempts to utilize the cellulose as the source of carbon (5). In the presence of a crystalline cellulose and low carbon levels, the organism is able to assimilate the carbon in the cellulose and use its source of carbon.

The second trait of the algae is its lipids and carbohydrates. It is similar to the fungi in the lipid and carbohydrates production. It could also be repurposed as a component in a biofuel. The main difference between algae and fungi is the efficiency and cost. The algae being able to grow better in multiple environments, it could be more appealing in comparison to the fungi. The cost of algae is also substantially lower than the cost of fungi. The lipid output of certain microalgae, such as Chlorella Vulgaris hold potential to outperform fungi. Chlorella Vulgaris, has high amounts of lipids which could be extracted using proper techniques (6).

In the experiment, there was an inclusion of three separate microorganisms in order to test cellulase production and lipid output to simulate commercial use. They were *Trichoderma Reesei*, *Chlamydomonas Reinhardtii*, and *Chlorella Vulgaris*. used The purpose of the study was to compare the cellulase and lipid output of fungi and microalgae, and determine which is more favorable. Under a week of study, and attempting to replicate similar environments to past studies, a hypothesis was created that the microalgae would outperform the fungi. Both the C. Reinhardtii and T. Reesi have cellulase expression, but Chlorella Vulgaris was tested due to its lipid output.

Hypothesis #1: Chlamydomonas Reinhardtii will be able to express more cellulase in comparison to the Trichoderma Reesei, and will also be able to utilize the cellulose

Hypothesis #2: The microalgae will be able to outperform the fungi in lipid output

Experimental Procedure

Bioreactor Set Up

Mediums for Inside of the Bioreactors

- *Chlamydomonas reinhardtii*: Sueoka's high salt medium (10% sodium acetate solution), (Blifernez-Klassen, Klassen, Doebbe, Kersting, Grimm, Wobbe, Kruse) Distilled water, Cellulose
- *Trichoderma reesei*: Voguel's modified minimum medium (10% sucrose), Distilled water, Cellulose
- *Chlorella Vulgaris*: Fritz A + B (10%) , Distilled water, Cellulose
- Each contained 90 mls of their respective medium, and 10 mls of the microorganisms to grow
- 5 grams of methyl cellulose was added to each
- It was contained in a shaker that was set to 150 rpm at 30 degrees celsius

Materials

- Trichoderma reesei (T)
- Chlamydomonas Reinhardtii (CR)
- Chlorella Vulgaris (CV)
- Clear, breathable Erlenmeyer flasks
- Methyl cellulose
- 3,5 Dinitrosalicylic acid
- Sodium hydroxide
- Rochelle salts
- Phenol
- Sodium metabisulfite
- Citric acid monohydrate
- D-Glucose Assay Kit (GOPOD Format, Megaenzyme)
- Spectrophotometer
- Methanol
- Chloroform
- Water bath
- Sonicator
- Vogel's salts
- Fritz A and B
- Sueoka's high salts

Lipid Quantification Assay

Sonication-Assisted Bligh and Dyer Method

1. Collect 2mls of culture and add to tubes
2. Sonicate each culture at 24 amp for 20 seconds
3. Create a solution of 1:2 of chloroform:methanol, this is solution 1
4. Add 7.5 mls of solution 1 to each culture
5. Add 2.5 mls of chloroform to each culture
6. Add 2.5 mls of distilled water to each culture
7. Centrifuge each culture for 5 minutes at 10 rpm
8. Collect the bottom layer, and transfer to another tube. This is now called tube 2
9. Place tube 2 under a fume hood to allow for the evaporation of the chloroform
10. After evaporation, weigh the products against an empty tube to find the weight of the lipids remaining

Cellulase Quantification Assay

Cellulase Filter Paper Assay

This assay will be done to conclude the rate of cellulase expression, by analyzing the amount of cellulase in the environment.

Preparing the DNS reagents

1. Combine the 1416 mLs of the distilled water, 10.6 grams of the 3,5 Dinitrosalicylic acid, and 19.8 grams of Sodium hydroxide. Stir till completely dissolved.
2. Then add the 306 grams of Rochelle salts (sodium potassium tartrate), 7.6mls of phenol, and 8.3 grams of Sodium metabisulfite.

Filter paper assay procedure

3. Cut 6 x 1 cm filter paper strips and place into each test tube
4. Add 1.0 mL of the 0.05 M Na-citrate into each tube
5. Equilibrate tubes to 50 degrees celsius in a water bat
6. Create a stock solution, one of 1.9 mg glucose and another of 2.1 mg of glucose
7. Add 0.5 mls of enzyme diluted to each tube
8. Put in water bath for 60 minutes exactly
9. Add 3 mls of DNS reagent to each tube to stop reaction
10. Add the tubes to a bath of vigorous boiling water for 5 minutes exactly
11. Add the tubes to an ice cold bath till the filter paper settles
12. Measure each of the test tubes at a 510 nm

D-Glucose Assay (Megaenzyme)

Preparation of Reagent Assay

1. Dilute bottle 1 of the kit to 1 liter of distilled water, this is now solution 1
2. Add the contents of bottle 2 to 20 mls of solution 1
3. Add bottle 2 and the 20 mls of solution one back into the remainder of solution 1
4. Wrap in aluminum foil and store at 2-5 celsius

D-glucose Assay Procedure

1. Adding 3mls of the reagent for every 0.1 ml of the sample size, incubate at 40-50 celsius for approximately 20 minutes
2. Read absorbances at 510 nm
3. Read the absorbance against the reagent blank at 510 nm

Disposal Procedure

For fungi: Fungal plates and flasks will be autoclaved to kill any bacteria, and then will be disposed in the contamination bin. For algae: Algae tanks and cultures will be treated with bleach in order to kill any algae. They will be then disposed in the contamination bin

Results

Abbreviations: NC: No culture, NCCV: No cellulose Chlorella Vulgaris, NCT: No cellulose Trichoderma Reesei, NCCR: No cellulose Chlamydomonas reinhardtii, T: Trichoderma Reesei, CV: Chlorella Vulgaris, CR: Chlamydomonas Reinhardtii.

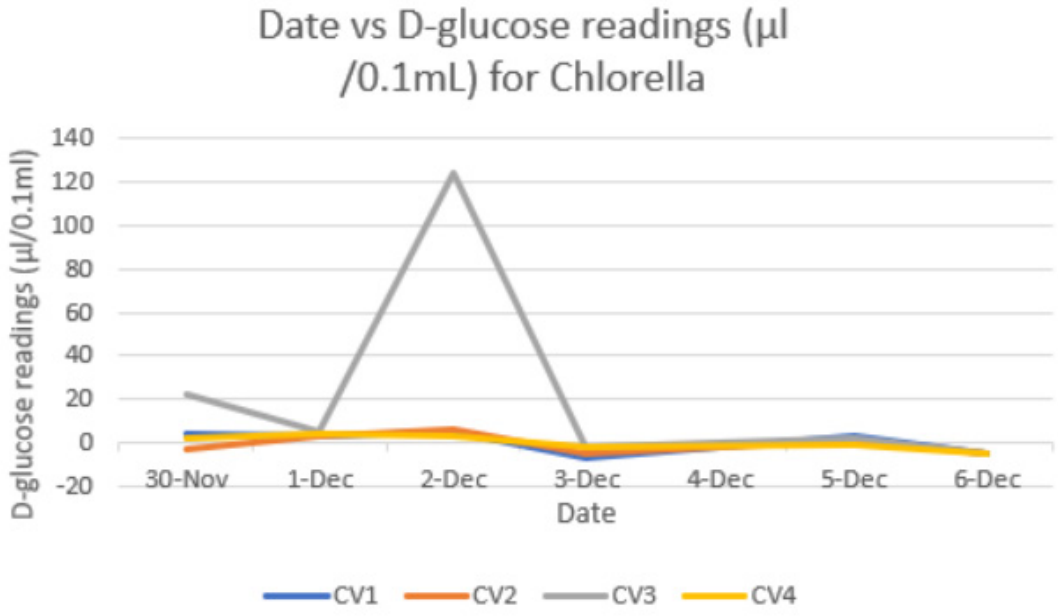

Figure 1. Chlorella D-glucose results

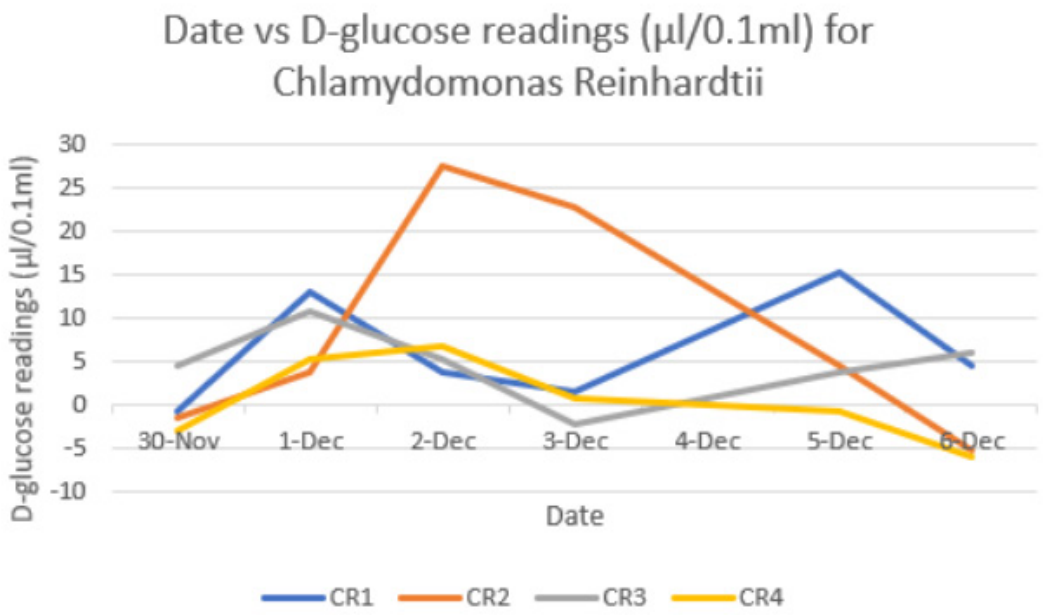

Figure 2. Chlamydomonas D-glucose results

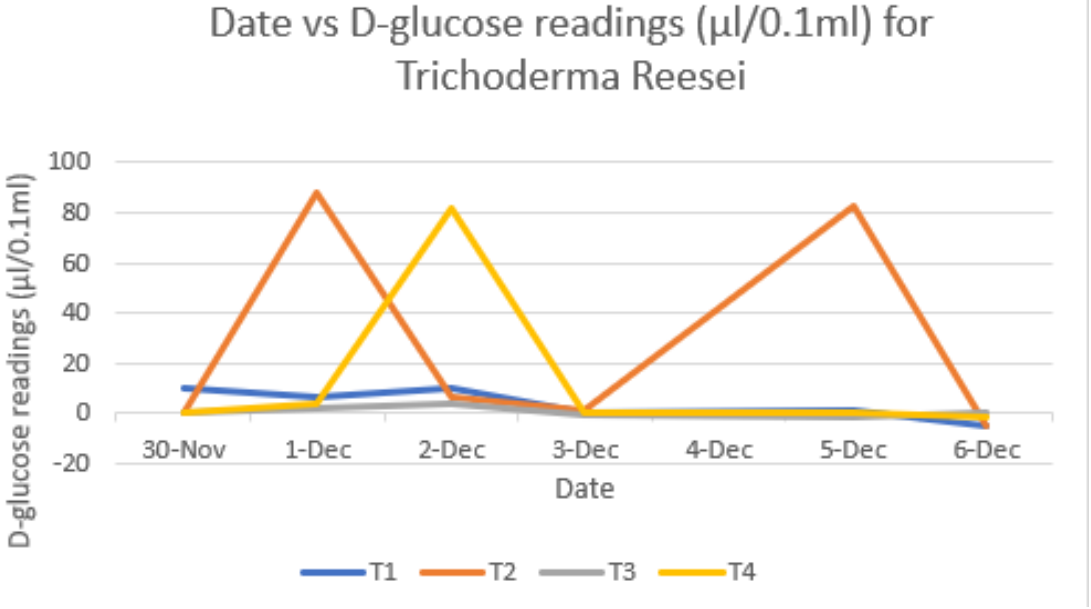

Figure 3. Trichoderma D-glucose results

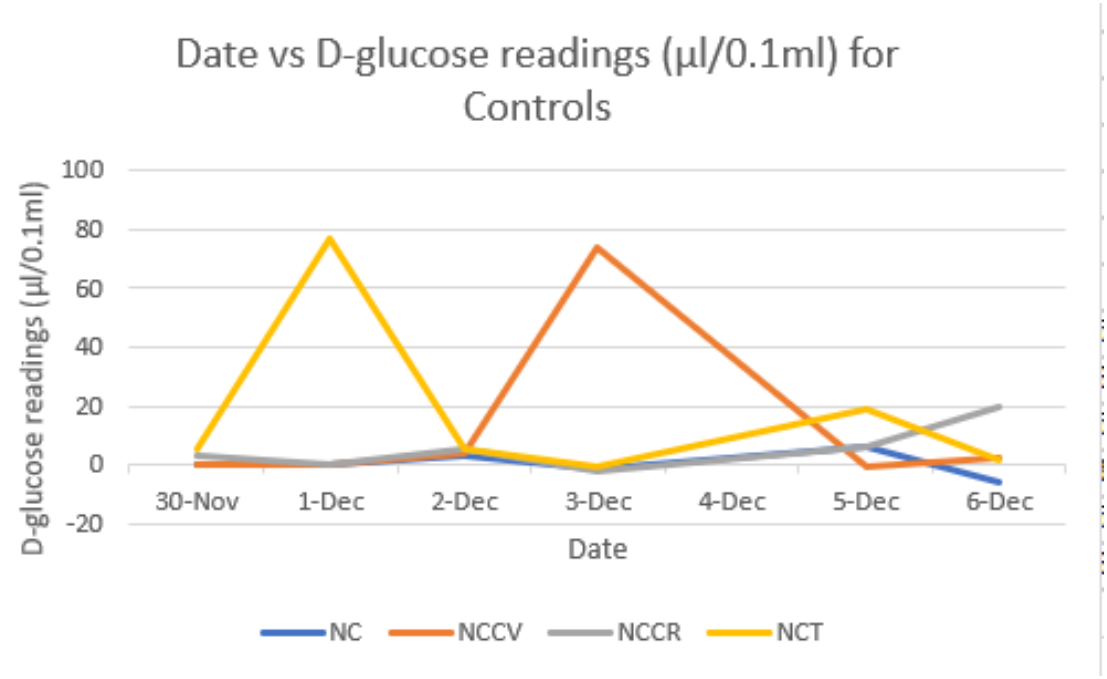

Figure 4. Controls D-glucose results

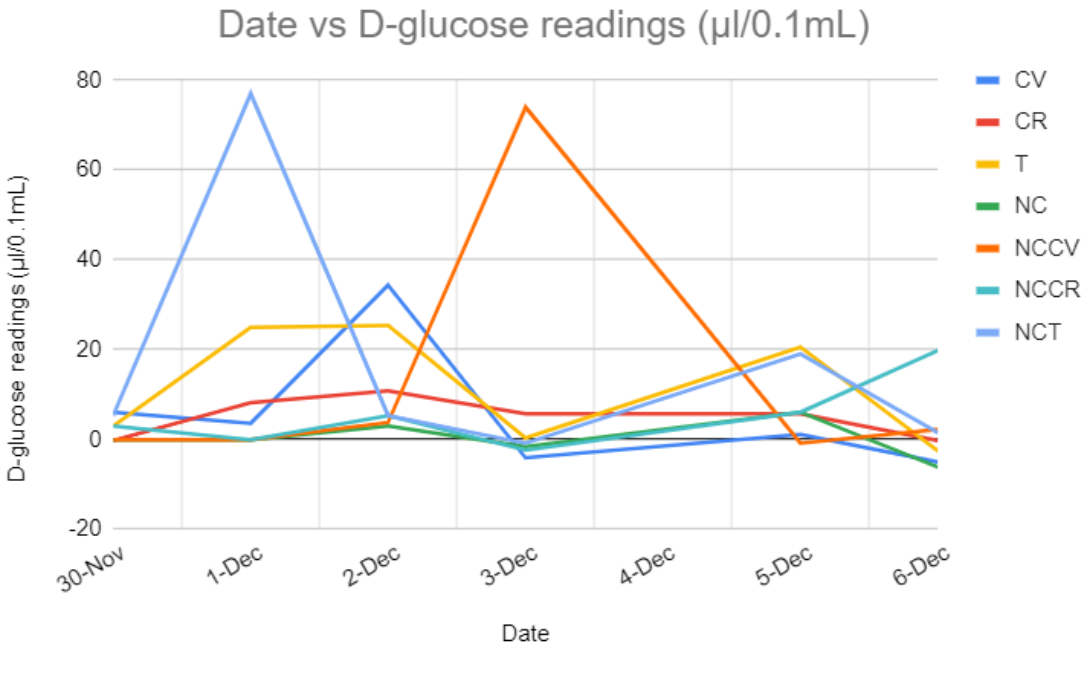

Figure 5. Complete D-glucose results

From the D-glucose assay, data was received which showed no significant change, only peaking at certain times in times which is assumed that error such as actual organism causing a change in the results. This shows that there was no constant change between the dates interval.

Cellulase Filter Paper Assay

From the cellulase filter paper assay, the data shows spikes in changes between absorbance levels, which indicates changes in glucose levels, which indicates a cellulase catalyzing the glucose added.

	CR	CV	T	NC	NCCV	NCCR	NCT	Glucose dilution
1-Dec	0.92	0.80	0.38	0.00	0.07	0.00	0.00	0.19
	0.00	0.00	1.82	0.34	0.00	3.35	0.96	0.21
3-Dec	0.00	0.00	0.00	0.34	0.00	0.00	0.00	0.19
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.21
5-Dec	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.19
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.21

Figure 6. Cellulase filter paper assay results

For the Cellulase filter paper assay, the glucose that ended up being expressed does not indicate any cellulase expression from any culture.

Lipid Quantification Assay

From the lipid quantification assay, the algae seemed to have a higher average in comparison to the Fungi.

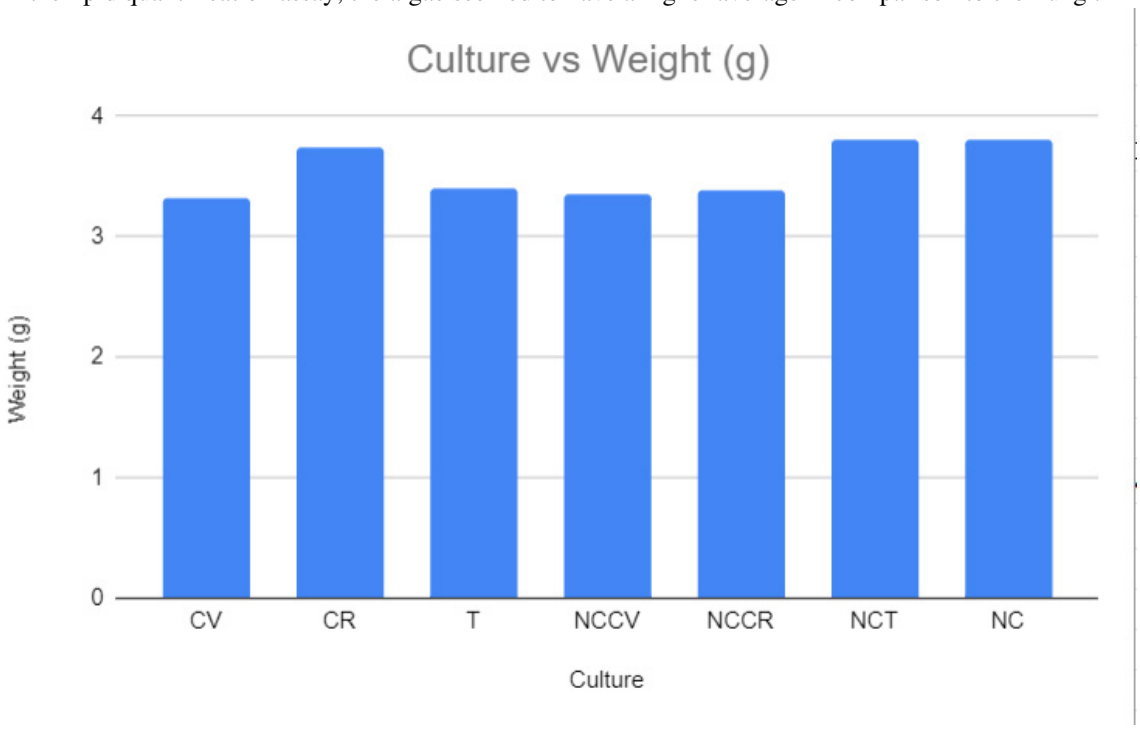

Figure 7. Lipid quantification assay.

Discussion

Overall, the data does support the hypothesis of the experiment, however it showed very little signs of cellulase production in any culture. The first assay, the D-glucose assay, did not show any consistent result, most likely

due to not only the organisms causing errors in the data, the data has peaked in certain areas, but also there is a possibility that there was a significant change in the D-glucose concentration, indicating a presence of a cellulase or another factor affecting the d-glucose concentration. My second assay, the cellulase filter paper assay, also showed results that indicate that the microorganism did not express cellulase. Even though there is a release of glucose, the results of the microorganisms that were aiming to express cellulase and the positive control had a similarity that could not be ignored. Spikes in data were due to error in such a manner that were not consistent. However, Trichoderma without cellulose culture showed results that could show promise in evidence of cellulase production. Statistical analysis shows p-values greater than 0.05, which indicates that the data was not significant. This indicates that the levels of glucose released are not significant enough to warrant a plausible assumption that there was cellulase expression. However, the third assay, the lipid quantification assay, resulted in an average weight of lipid from the algae being greater than the average in the fungi. This indicates that the microalgae would be a more appealing form of biofuel, accepting hypothesis #2.

Limitations and Error Analysis

Some limitations that could have caused the cellulase not to be expressed could be factors such as a lack of limitation on carbon for the *Chlamydomonas Reinhardtii*, which was a component of other experiments, and a non-optimal environment for cellulase expression in the *Trichoderma Reesei.* Future research done would have to try and attempt to deal with this limitation, to attempt to address this issue. Additionally, the type of cellulose could have also acted as a limitation for the culture. Methyl cellulose was used in this experiment, and so for future experiments, other cellulose types such as lignocelluloses or carboxymethyl cellulose could be used. Random errors also should be in consideration during the experiment, as small errors in measurements. There were errors with the use of the micropipette due viscosity issues. Additionally, there were slight temperature changes from 30 degrees celsius during the usage of the water bath that could have contributed to differences in results.

Future Research

For future research, fixes could be made to the design of the experiment, changing some of the data gathering procedures and factors such as the cellulose type being optimized for the cultures to break down. With society relying on fossil fuels heavily, this research could help to pave a way towards a more sustainable future.

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Gave insight into the enzymes secreted by T. Reesei, and gave the conditions of the culture that optimized the conditions.
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Background on T. Reesei and the cellulase produced, Also insight into cellulase production in the fungi
Blifernez-Klassen, O., Klassen, V., Doebbe, A. *et al.* Cellulose degradation and assimilation by the unicellular phototrophic eukaryote *Chlamydomonas reinhardtii*. *Nat Commun* **3,** 1214 (2012). https://doi.org/10.1038/ncomms2210
Within the article, many topics are discussed, including cellular assimilation, and the eukaryote Chlamydomonas reinhardtii. In specific, the article goes into the different factors and variables of Chlamydomonas Reinhardtii. For example, when under limited carbon environments, the algae is shown to secrete endo-β-1,4-glucanases, which digest cellulose. Not only this, but the algae is able to grow in many different situations, which allow for the algae to be very versatile. Another example of a factor they discussed was cellodextrins. In which are products of cellular degradation. Furthermore, they aimed to show the conversion between Cellulose to Cellodextrins.
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