Algae Meets Fungi: Microalgae-Fungi Co-Pelletization for Biofuel Production

Claire Scrimini and Vanessa Scrimini
Grade 11 | BC, Canada

Microalgae-fungi biofuel has significantly less CO_2 emissions than fossil fuels, making it much more environmentally friendly. As well, unlike traditional biofuel, microalgae-fungi does not require large masses of agricultural land for production. Thus, microalgae-fungi is an optimal option for biofuel production. This is a cost-effective renewable energy source that can be used in place of regular gas in cars and other means of transportation. By determining the most effective fungi for biofuel production, the threat of the impending environmental damage from pollution can be diminished.

This novel experiment determines which fungi: *Aspergillus niger*, *Rhizopus stolonifer* or *Saccharomyces cerevisiae*, is the most effective bioflocculant in the microalgae-fungi co-pelletization process for biofuel production.

We hypothesize that when paired with the microalgae *Chlorella vulgaris*, *Rhizopus stolonifer* will be the most effective. It has a high lipid content which could enhance the overall production of biofuel. Furthermore, its negative charge will aid with attracting and neutralizing the *C. vulgaris* colloidal particles resulting in an easier and more efficient removal of microalgae particles.

Through the process of bioflocculation, pelletization, esterification and transesterification, the most effective fungi paired with *C. vulgaris* was determined.

This experiment was carried out thoroughly and precisely resulting in a cost-effective solution for the world's current pollution crisis.

INTRODUCTION

The UN Report on Climate Change states that mankind has 12 years to change our ways of polluting and damaging the environment before it is too late [1]. One important step that the global community can do to address this is to reduce our dependence on the use of fossil fuels. The microalgae-fungi biofuel developed in this project could be an excellent solution to the climate change crisis. It is carbon neutral, making it much more environmentally friendly than fossil fuels. Moreover, unlike traditional biofuels, microalgae does not require plots of agricultural land for production making microalgae-fungi biofuel an optimum option for clean energy. Background

The following scientific processes were used to produce biofuel.

BIOFLOCCULATION

A process in which a selected biological agent is mixed into an aqueous solution to enhance particle collisions in order to allow for easier removal of biomass [2].

PELLETIZATION

The productivity of filamentous fungi and its ability to act as a bioflocculant in a bioreactor is highly interlinked with its ability to form mycelial pellets during pelletization [3]. The algae and fungi attach and create large spherical shaped masses that allow the microalgae to be more easily extracted from water.

ESTERIFICATION AND TRANSESTERIFICATION

Esterification and transesterification is a two-step reaction which results in raw biofuel and raw glycerol. Esterification is a reaction in which an alcohol and a carboxylic acid form an ester and water. Transesterification is a necessary process to create fatty acid methyl esters (FAMEs) for biofuel production. This process occurs when an alcohol, catalyst and fatty acid are mixed after esterification.

PURPOSE

The purpose of this experiment is to determine which fungi: *Aspergillus niger*, *Rhizopus stolonifer* or *Saccharomyces cerevisiae*, paired with the microalgae *Chlorella vulgaris* serves as the most effective bioflocculant for microalgae-fungi co-pelletization for biofuel production. Subsequently, resulting in a microalgae-fungi combination that produces the largest amount and highest overall quality of biofuel.

HYPOTHESIS

We hypothesize that when paired with *C. vulgaris*, *R. stolonifer* will be the most effective bioflocculant for biofuel production. *R. stolonifer* has a high lipid content [4] which will contribute to the overall amount of oil produced for biofuel. Furthermore, this fungi has a negative charge [5] which will aid in attracting and neutralizing the *C. vulgaris* colloidal particles. This will lead to an easier and more efficient removal of the colloidal microalgae particles, resulting in a higher oil yield.

PREPARATIONS

All equipment were sterilized prior to use. Then, Chlorella vulgaris was cultivated for 18 days in flasks on a 16-hour light/8-hour dark cycle at 22 °C. Each had aeration hoses providing CO_2. To ensure that each sample received the same amount of algae, a secchi tube was used.

The Fungi: *Aspergillus niger*, *Rhizopus stolonifer* and *Saccharomyces cerevisiae* were also cultivated during this time by plating each fungal culture into 3 additional nutrient agar plates that were subsequently stored in an incubator at 30°C for the 18 day cultivation period.

MATERIALS AND METHODS

BIOFLOCCULATION

For the bioflocculation process a 3:1 algal-fungal ratio was used. To ensure that all beakers had the same amount of algal biomass, a secchi tube was used to determine the turbidity of the solution. The process of using the secchi tube was repeated 3 times to ensure accurate results. We then used a magnetic stirrer to mix each solution at 250 rotations per minute (rpm) for 1 minute to allow for effective fungal distribution, then the speed was reduced to 125 rpm to allow for efficient mixing for 72 hours at 35°C. Every 24 hours, a hemocytometer and the trypan blue exclusion dye method were used to observe the dead and alive cells. A secchi tube was also used to test the flocculation effectiveness. After 72 hours we let each solution sit for another 2 hours.

EXTRACTION OF LIPIDS

To extract the algal-fungal biomass, a 0.2-5 micron filter was used. For the algal-fungal biomass drying, a rotary evaporator was set at 64°C to evaporate the water content until only the dry biomass remained. Then, 2 mL of methanol (MeOH) was added to the biomass from the large flask by dissolving it. This substance was then transferred into a vial where the remainder of the biomass in the initial flask was mixed and repeated with 1 mL of MeOH again. The algal-fungal MeOH substance was then freeze dried to remove the MeOH and water. Next, 10mg of each vial's dry biomass was weighed out and 1.5 mL chloroform ($CHCl_3$): MeOH, (2:1) was added and subsequently vortexed for 2 minutes. Then, 0.4 mL of deionized water was added and vortexed again for 20 seconds. The phases separated within 10 minutes, allowing us to then filter out the cell debris using glass wool. The water content was then withdrawn from the vial. A nitrogen stream was used to evaporate the $CHCl_3$ and MeOH content from the lipids.

ESTERIFICATION

100 mL of MeOH was added as the alcohol and 10mL of BF3 diethyl-etherate as a catalyst for the reaction. We then let the reactions stand overnight.

TRANSESTERIFICATION

Water was added to quench the solution. Then 1 mL of hexane and 1 mL of ether were added to create FAMEs. After transferring the FAMEs into a new vial, another 1 mL of hexane was added into the old vial, then the extracted lipids were removed and transferred into a new vial.

GAS CHROMATOGRAPHY AND MASS SPECTROMETERY (GS AND MS)

Lipids were washed to remove the H20 content, then a nitrogen stream was used to concentrate the solution until only 1 mL was left in the vial. The stock solutions were then diluted (10μL) by a factor of 100 into a new 1 mL of hexane. Lastly, 10μ7 of internal standard (17:0) was added, making the

samples ready for analysis. To determine the lipid content in micrograms per 10mg of dry weight, a GC and MS machine were used to analyze the results. The following calculations were used: *Internal standard (IS) = counts at around 18 minutes*

$$\text{Calculation for 20µL of IS:} \quad \frac{Counts}{Internal\ Standard} = x \cdot 1000 = \mu g$$

$$\text{Calculation for 10µL of IS:} \quad \frac{Counts \cdot 2}{Internal\ Standard} = x \cdot 1000 = \mu g$$

SPEED OF IGNITION ANALYSIS

A wick was placed at the bottom of a beaker with 1mL of microalgae-fungi oil. The wicks were lit holding a lighter ½ cm away. A stopwatch was used to time how long it took for the candle to ignite (hold its own flame).

BURNING TIME TEST

This was determined by timing the moment the wick catches fire to the time it goes out. The most effective microalgae-fungi oil is the one that burns the longest.

FATTY ACID (FFA) ANALYSIS

This is calculated through an acid value of oils by titration test. To do so, 10 mL of titrant (isopropyl alcohol) was added into a flask, then 1 drop of liquified turmeric powder was also combined. 1 mL of oil was poured into the solvent then mixed with KOH until there was a definite colour change (must persist for at least 15 seconds.) The amount of titrant consumed in milliliters was recorded then evaluated to determine the FFA % using the following formula:

$$\frac{Volume\ titrant\ (mL)\ Normality\ of\ KOH\ (N) x\ 56.1}{Mass\ of\ sample\ (g)}$$

STATISTICAL ANALYSIS

Differences were considered statistically significant when the P value was 0.05 or less. Statistics were carried out through the analysis of variance (ANOVA) and a two-tailed t-test. Standard deviations were also calculated.

RESULTS

The average biomass was weighed in milligrams (mg) using a fine balance. The weight correlates to the greatest quantity of biofuel. The more biomass there is, the larger the amount of biofuel produced. The error bars represent standard errors of the means at a 95% confidence level. All of the tests were statistically significant when tested at an alpha level of 0.05.

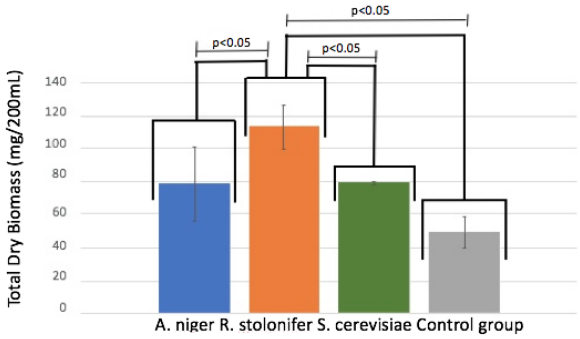

Figure 1: Average dry biomass

The total lipid yield of the biofuel was measured using a GC-MS machine. Before analysis, the H_20 content was removed from the samples and the internal standard (17:0) was added to the stock solutions. The GC-MS analyzes the molecules of the lipids and indicates the overall quality of the samples. The error bars represent standard errors of the means at a 95% confidence level. All of the tests were statistically significant when tested at an alpha level of 0.05.

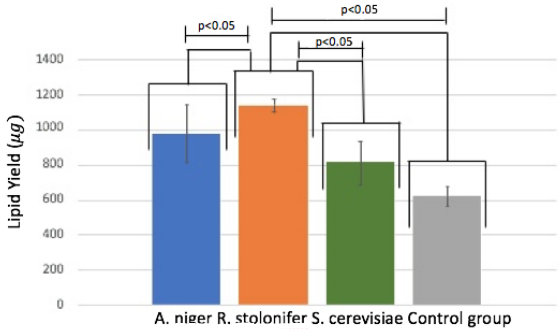

Figure 2: Average lipid yield

The speed of ignition is defined as the time taken for a candle to ignite (hold a constant flame on its own) and was determined using a stopwatch. The purpose of this test is to correlate the speed of ignition of a wick with the combustion time of the biofuel in a car (cetane number). The error bars represent standard errors of the means at a 95% confidence level. All of the tests were statistically significant when tested at an alpha

level of 0.05.

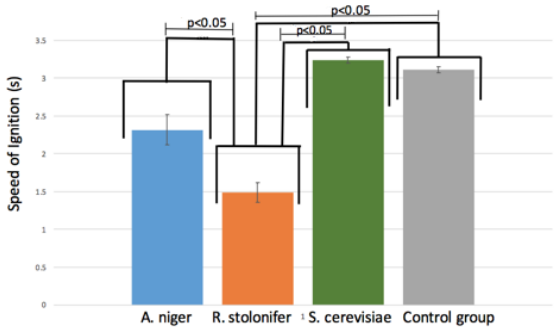

Figure 3: Average speed of ignition

For the burning time test, the most effective microalgae-fungi oil is the one that burns the longest. The purpose of doing this test is to determine how long the biofuel will last. The error bars represent standard errors of the means at a 95% confidence level. All of the tests were statistically significant when tested at an alpha level of 0.05.

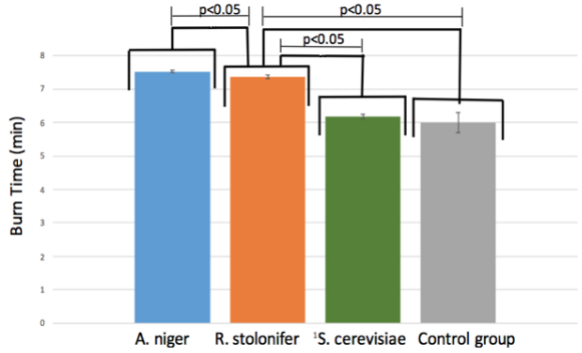

Figure 4: Average burning time

Acid value by titration allows for analysis of the amount of FFAs within the biofuel. The total acid number determines the fuels "shelf life" before oxidation by measuring the amount of FFAs within the biofuel. For biofuel, it is ideal to have as low FFA levels as possible. The error bars represent standard errors of the means at a 95% confidence level. All of the tests were statistically significant when tested at an alpha level of 0.05.

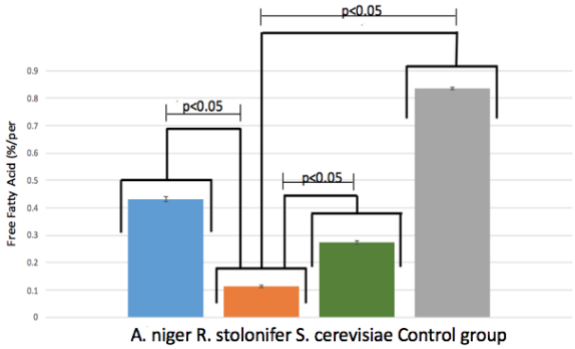

Figure 5: Average acid value by titration

CONCLUSION

The most effective fungi for biofuel production was determined to be *Rhizopus stolonifer.* It had the best overall results in comparison to the other fungi and control group based on its ability as a bioflocculant in the microalgae-fungi co-pelletization process. The average yield of all trials of *R. stolonifer* was 186.87ug and the average biomass was 103 mg, which is a high extraction rate, especially in comparison to the other trials. Moreover, our results also indicate that *R. stolonifer* had the longest burn time of an average of 7.51 minutes. It also had an average FFA value of 0.1131% which is excellent. This correlates to the amount of oxidation, or in other words, its "shelf life". Furthermore, *R. stolonifer* exceeds the American Society For Testing and Materials (ASTM) standards in its FFA test. Although, *R. stolonifer* did not have the fastest speed of ignition, it was a close second, having an average of 2.39 seconds speed of ignition, compared to *S. cerevisiae* which had an average of 1.79 seconds. Statistical analysis, using ANOVA and T-tests indicate that our results have a p-value of $p<0.05$, which means that we can reject the null hypothesis and therefore conclude that for these test there is indeed statistical significance. *After this experiment, we can conclude that R. stolonifer* had the best overall results, thus making it the most effective fungal bioflocculant for biofuel production.

APPLICATION

Using microalgae-fungi biofuel produced by C. *vulgaris* and *R. stolonifer* paired with bioflocculation is an excellent option for the climate change crisis currently threatening the globe. Although other types of biofuel have been

considered, microalgae-fungi is by far the most promising. The issue with other types of biofuel including soybean and vegetable oil is that they require large plots of healthy farmland, whereas algae can easily be cultivated in wastewater, vertical farms and other unusable ground. It can even be paired with a bioremediation plant for a dual function. Microalgae-fungi biofuel production is also classified as carbon neutral because the carbon dioxide that is absorbed by the algae is equal to the carbon dioxide that is released when the fuel is burned. As well, microalgae-fungi biofuel with C. *vulgaris* and *R. stolonifer* is a cost effective biofuel that can be used for backup generators and medical equipment in third world countries as it can even be produced in homes.

Greenhouse gas emissions from commercial air travel poses a huge threat to the environment and are one of the main contributors to greenhouse gases [6]. In using our microalgae-fungi biofuel, a planes large carbon-footprint would be non-existent. The findings of this project could revolutionize, and potentially solve global warming in the near future with these two simple organisms.

IMPROVEMENTS AND FUTURE EXTENSIONS

There are still many measures that can be taken to further enhance the overall strength of this experiment. Initially, we did plan to test more samples, however, were unable to do so due to the cost of purchasing the algae and the amount of time it takes for the subculture to effectively cultivate. Furthermore, as we had to adapt the procedure to fit our lab standards, upon doing so we lost one sample from each trial (leaving us with a total of three samples for each trial, as opposed to four.)

Through increasing our sample size, we would be able to test more samples of each fungi to further confirm their efficiency. Furthermore, it would allow for a more effective evaluation at a larger scale.

In the future we intend on pursuing further investigations with the hopes of industrializing our novel microalgae-fungi biofuel. Within the bioflocculation process, we would like to test different concentrations of *R. stolonifer*. In doing so**,** we would be able to examine which concentration allows for an enhanced amount of particle collisions, resulting in more effective pelletization.

We will also be testing our biofuel in a diesel engine, to make a direct comparison between biofuel and gasoline. Moreover, we also intend on using a cetane engine to determine the fuels speed of combustion. All of which would bring us one step closer to industrialization.

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