An Analysis of Diatom Growth Rate and the Implications for the Biodiesel Industry

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ABSTRACT

The need for an economically-feasible, carbon-neutral fuel source rises as rates of carbon emissions increase and climate change persists. Diatom biodiesel is currently being researched as a carbon-neutral alternative to petroleum. Biochemical engineering, a method utilized to increase lipid accumulation in microalgae for harvest, works by stressing algal cultures in order to bias their metabolism towards lipid (fat) production. Although Nitrogen (N) is the most common limiting-nutrient in research, Silicon (Si) is also vital to diatom cell growth and division, and therefore its limitation would also cause an accumulation of lipids in cells. In fact, Si-limitation has yielded higher lipid content in diatoms than N-limitation without any of the severe physiological damage, making it a prime candidate for biochemical engineering of microalgae biodiesel. Two different species of diatom, Thalassiosira rotula and Coscinodiscus radiatus, were cultured and grown in a silicon-deficient media (20µM-Si) and in a silicon-rich media (80 µM-Si) to observe how differences in environmental Si affected frustule1 size, diatom growth rates, and lipid accumulation. We also stained the cells with dyes to observe Si frustules and lipid content, then took photographs with a fluorescent microscope for qualitative analysis. Our results suggested that diatom species had a greater impact on growth rate than Si treatment. Since all diatom species have different rates of growth, Si uptake and metabolism, it could be more prudent to focus on which species of diatom, rather than which nutrient-limitations, are best suited for biodiesel production.

BIOCHEMICAL ENGINEERING

A method utilized to increase lipid accumulation in microalgae for harvest. This involves limiting nutrients or manipulating environmental factors, such as salinity, temperature, pH and light intensity in order to bias the metabolism of the cells towards lipid production.
INTRODUCTION

The combustion of fossil fuels has released more than 350 billion metric tons of carbon into the atmosphere since the beginning of the Industrial Revolution, and about 36% of that is a direct result of burning petroleum for transportation purposes (Levitan et al. 2014). This high input of carbon dioxide into the atmosphere over a relatively short period of time has had unintended negative consequences, such as global climate change, which has drastic effects on the world. We must develop an economically-feasible, carbon-neutral fuel to commercially replace petroleum (Levitan et al. 2014) in order to mitigate future effects of climate change. The production of biodiesel has been successful worldwide and is currently a hopeful source of renewable transportation fuels (Courchesne et al. 2009; Yang et al. 2014; Chisti 2008).

Although there is ongoing research regarding biodiesel, microalgae biodiesel is considered the only known option with the potential to completely replace petroleum-derived transportation fuels (Chisti 2008; Courchesne et al. 2009).

It is noteworthy that algal lipids are a major contributor to the formation of petroleum (Levitan et al. 2014). Since our current fuel sources are partially derived from ancient algae, it stands to reason that an attempt to harvest the energy from lipids in living algae may be very successful (Levitan et al. 2014).

Biochemical engineering is a method used to maximize the accumulation of lipids in microalgae for harvest (Courchesne et al. 2009). This involves limiting nutrients or manipulating environmental factors, such as salinity, temperature, pH and light intensity in order to bias the metabolism of the cells towards lipid production (Courchesne et al. 2009; Coombs et al. 1967; Pal et al. 2011; Znachor et al. 2013).

When the photosynthetic capacity of cells under stress is exceeded by the carbon supply, algae will synthesize energy-rich molecules (often lipids) in order to survive nutrient starvation or other growth-limiting conditions (Pal et al, 2011). Thus, the excess energy that would normally be used for growth or other metabolic processes is stored for later use during more favorable conditions (Pal et al. 2011). However, biochemical engineering is not without its drawbacks.

THE PRODUCTION OF BIODIESEL HAS BEEN SUCCESSFUL WORLDWIDE AND IS CURRENTLY A HOPEFUL SOURCE OF RENEWABLE TRANSPORTATION FUELS.

Nitrogen starvation, the most popular experimental nutrient limitation, yields higher lipid content, but also severely limits cell growth and division (Yang et al. 2014; Pal et al. 2014; Coombs et al. 1967; Flynn and Martin-Jézéquel 2000; Levitan et al. 2014, Courchesne et al. 2009; Znachor et al. 2013). When it comes to nutrient starvation, the very mechanism that initially stimulates lipid production in cells will eventually lead to an overall decrease in lipid productivity due to severely impeded photosynthesis and cell growth (Pal et al. 2011; Coombs et al. 1967; Courchesne et al. 2009).

In order to successfully produce microalgae biodiesel on a large scale, optimal growth rate and lipid accumulation must be attained. To achieve this end, and to produce biodiesel in a manner that is economically competitive with oil, less severe nutrient-limitation options must be explored across multiple algal genera to identify the best strains for biodiesel production (Levitan et al. 2014; Courchesne et al. 2009).

Diatoms are successful in oceans, have a relatively high resistance to pathogens, and tend to out-compete other algal species in cell cultures, giving diatoms favorable

FRUSTULE
The hard, silica-derived exoskeleton unique to diatoms (a type of algae).
Diatoms may be highly favored for biodiesel production because they have relatively high lipid content under normal growth conditions. Furthermore, in the 1980s, 3000 species of microalgae were screened for their potential to produce lipids, only 50 were deemed potentially fit for biodiesel production (Levitan et al. 2014). Of those 50 species, about 60% were diatoms (Levitan et al. 2014). Diatoms may be highly favored for biodiesel production because they have relatively high lipid content under normal growth conditions: about 15-25% of their cell biomass (Levitan et al. 2014). With nutrient and environmental manipulations, this percentage can be pushed as high as 61% dry weight (Levitan et al. 2014).

The hard, Si-derived exoskeleton unique to diatoms, known as the frustule, may be the key to their high lipid accumulation under stress (Annekov et al. 2013; Znachor et al 2013; Leblanc and Hutchins 2005; Coombs et al. 1967; Martin-Jézéquel et al. 2000; Flynn and Martin-Jézéquel 2000; Levitan et al. 2014). Silicon must be absorbed from the surrounding seawater in order for it to be incorporated into diatom frustules (Annekov et al. 2013; Znachor et al. 2013; Leblanc and Hutchins 2005; Coombs et al. 1967). Therefore, silicate metabolism in diatoms is undisputedly linked to cell growth and division, making silicon a major controlling factor in lipid productivity (Coombs et al. 1967; Martin-Jézéquel et al. 2000; Flynn and Martin-Jézéquel 2000; Levitan et al. 2014). Interestingly, diatoms often accumulate more total lipids under Si-starvation than they do under N-starvation (Levitan et al. 2014). While Si-starvation disrupts the diatom cell cycle and reduces growth, the physiological damage is not nearly as severe as N-limiting conditions (Levitan et al. 2014). More experimentation with Si-limitation in diatoms must be completed in order to gain full insight into the extent to which biochemical engineering can be utilized for biodiesel production.

Again, two different species of diatoms, *T. rotula* and *C. radiatus*, were separately cultured and grown in both a silicon-poor media (20µM-Si) and a silicon-rich media (80µM-Si). We predict (1) that growth rates for cells in the 80µM-Si treatment will be significantly higher compared to the 20µM-Si treatment, independent of species, (2) that there will be a significant difference between the growth rates of *T. rotula* and *C. radiatus*, independent of Si treatment, and (3) that the difference in diatom growth rate observed between the 20µM-Si and 80µM-Si treatments will be significantly different between the two species. We also predict that cellular lipid content will be greater in the 20µM-Si treatment than in the 80µM treatment, independent of species. However, the overall lipid content will be higher in *C. radiatus* because it is much larger than *T. rotula*. We will first test each of the above hypotheses for equality, or as a null hypothesis. If we find that the data are statistically significant, then we will reject the null hypothesis and will note how each of the variables is statistically different, therefore accepting the above alternative hypotheses.

**Null Hypothesis:** This is a hypothesis of equality used for statistics that postulates no statistical significance among given sets of data. In other words, the variation observed in a set of data is due to chance events rather than actual differences among the variable being studied. If the null hypothesis is accepted (or not rejected) then we are assuming that the data being compared are the same. If the null hypothesis is not supported by statistics (in this case, if there is a greater than 5% chance that the differences are NOT due to chance), then we reject the null hypothesis and move on to an alternative hypothesis.
MATERIALS AND METHODS

Experimental Set-up

We inoculated six bottles with *T. rotula* (three replications for each Si media) and six other bottles with *C. radiatus* (3 reps for each Si media).

Data Collection

After each bottle was inoculated with cells, we took fluorescence measurements every one to two days for twelve days in order to observe the lag and exponential growth stages. Around day 10, the PDMPO stain for Si was prepped and added to the cultures on day 11 (Spillane 2014). Photographs were taken with a fluorescence microscope for each species/Si treatment combination.

RESULTS

We failed to reject our null hypothesis regarding growth rate observed in the 80µM-Si treatment compared to the 20µM-Si treatment, independent of species (Table 1). There was no significant difference in the growth rates observed between the 20µM-Si treatment and the 80µM-Si treatment alone (Table 1).

However, we rejected our null hypothesis regarding the difference between the growth rates of the two species (Table 2). The growth rate of *T. rotula* is significantly higher than that of *C. radiatus*, independent of treatment type (Table 2).

We rejected our null hypothesis regarding the differences in growth rates between the two species, dependent on treatment type (p-value=0.042) (Figure 2). The difference in diatom growth rate observed between the two treatments
FIGURE 2.
The observed mean diatom growth rate of T. rotula and C. radiatus cultured in 20µM-Si and 80µM-Si media with error bars indicating SE for n=3 replicates of each species/treatment combination.

TABLE 1.
The diatom growth rate (mean±SE) observed in n=6 replicates of the 20µM-Si treatment and n=6 replicates of 80µM-Si treatments, independent of diatom species. Analysis of variance (ANOVA) test results (indicated by lowercase letters) are shown.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Growth Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>20µM-Si</td>
<td>0.257±0.004 a</td>
</tr>
<tr>
<td>20µM-Si</td>
<td>0.113±0.012 a</td>
</tr>
<tr>
<td>80µM-Si</td>
<td>0.238±0.011 a</td>
</tr>
<tr>
<td>80µM-Si</td>
<td>0.139±0.009 a</td>
</tr>
<tr>
<td>ANOVA p-value</td>
<td>0.712</td>
</tr>
</tbody>
</table>

TABLE 2.
The diatom growth rate (mean±SE) observed in n=6 replicates of species T. rotula and n=6 replicates of species C. radiatus, independent of Si-treatment. Analysis of variance (ANOVA) test results (indicated by lowercase letters) are shown.

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. rotula</td>
<td>0.257±0.004 a</td>
</tr>
<tr>
<td>T. rotula</td>
<td>0.238±0.011 a</td>
</tr>
<tr>
<td>C. radiatus</td>
<td>0.113±0.012 b</td>
</tr>
<tr>
<td>C. radiatus</td>
<td>0.139±0.009 b</td>
</tr>
<tr>
<td>ANOVA p-value</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>
in *C. radiatus* was significantly higher than difference in growth rate observed between the two treatments in *T. rotula* (Figure 2).

It appears that there is less silicon present in *T. rotula* frustules grown in the 20µM-Si media than those grown in the 80µM-Si media (Figure 3). It also appears there is less chlorophyll fluorescence in *T. rotula* cells grown in the 20µM-Si media than those grown in the 80µM-Si media (Figure 3).

Similarly, it is difficult to say whether the growth of the diatom frustule in *C. radiatus* was reduced in the 20µM-Si solution compared to the 80µM-Si (Figure 4). However, it appears that there is reduced PDMPO fluorescence, and therefore less silicon present, around the exterior of *C. radiatus* frustules grown in the 20µM-Si media than those grown in the 80µM-Si media (Figure 4). Nothing conclusive can be said about chlorophyll fluorescence in either treatment (Figure 4).

There is a higher lipid content in *T. rotula* cells grown in the 20µM-Si media than those grown in the 80µM-Si media (Figure 5). It also appears that there is less chlorophyll fluorescence in *T. rotula* cells grown in the 20µM-Si media than those grown in the 80µM-Si media (Figure 5).
It is difficult to say whether there is higher lipid accumulation in *C. radiatus* grown in the 20µM-Si solution compared to the 80µM-Si (Figure 6). Nothing conclusive can be said about lipid content in either of the treatments (Figure 5). It does appear, however, that there is less chlorophyll fluorescence in *C. radiatus* cells grown in the 20µM-Si media than those grown in the 80µM-Si media (Figure 5).

The overall lipid content of *C. radiatus* seems higher than the overall lipid content of *T. rotula* (Figures 5 and 6). However, we were unable to determine the magnitude of this difference and whether or not it was significant.

**DISCUSSION**

We evaluated changes in growth rates as well as visual differences in diatom frustules, chlorophyll content, and lipid content between both species type and the growth limiting (20µM-Si) or non-limiting (80µM-Si) Si treatments. Unexpectedly, there was no difference in growth rate based solely on Si content. This means that, considering the Si media alone, all diatoms grew at the same rate, even though one treatment was supposedly growth-limiting while the other was not. This result is inconsistent with current literature, which states that Si is a major limiting
factor in diatom growth, especially when it comes to cell division (Coombs et al. 1967; Martin-Jézéquel et al. 2000; Flynn and Martin-Jézéquel 2000; Levitan et al. 2014). Martin-Jézéquel et al. (2000) observed a large uptake of Si in diatoms immediately preceding cell division in order to adequately form new frustule valves. In fact, all components of the frustule are deposited around the time of cell division (Martin-Jézéquel et al. 2000). This suggests that diatoms may not be able to divide as often without sufficient Si for frustule formation. Research conducted by Coombs et al. (1967) supports this idea, as they observed a reduced growth rate in Si-starved cells. Although most literature agrees that nutrient deficiency decreases microalgal growth rates, including Si in diatoms, Levitan et al. (2014) declares that Si-starvation is not nearly as physiologically damaging to diatoms as other nutrient deficiencies, namely N. We may not have observed differences in growth rates based solely on Si treatment because Si-starvation did not affect diatom growth rate as much as anticipated. If this is true, even though 20µM-Si is considered growth-limiting and 80µM-Si is considered non-limiting, there may not have been enough of a discrepancy in the Si treatments to observe clear differences in diatom growth rates. Another potential reason we may not have seen a difference was because our method of gathering data was not sensitive enough. For example, if we had analyzed cell count data over time we may have seen different trends. In addition, it is a possibility that differences in growth rates would have been observed if we had recorded data over a longer period of time, potentially until cells hit their deceleration or stationary growth phase.

Either way, *T. rotula* grew twice as quickly as *C. radiatus*. *T. rotula*’s smaller cell size, about 25µm in length compared to *C. radiatus*’ 75-100µm diameter (Figures 4 and 5), may allow it to divide at a higher rate. These results suggest that the Si treatment may not be as important in determining cell growth and metabolism as the specific species of diatom. The individual cell cycle, metabolism, and Si needs of each diatom species may have more of an impact on growth rate than the Si available in the environment. Consistent with this supposition is our finding that the change in growth rate between the 20µM-Si and 80µM-Si treatment was higher in *C. radiatus* than in *T. rotula*. Potentially, this difference is due to the large cell size of *C. radiatus*, which may require more Si than the smaller frustules seen in *T. rotula*. Martin-Jézéquel et al. (2000) observed that cell size as well as frustule thickness can contribute to the extent of silification in diatoms and Levitan et al. (2014) observed variations in diatom metabolism, such as cellular lipid content. This result further supports the idea that the species of diatom, and the specific metabolic needs associated with that species, has more of an influence on growth rate than does Si availability. If this is so, it has important implications for the biodiesel industry, which will be discussed in the conclusion.

In the fluorescence microscopy photographs of *T. rotula*, we observed a reduction in the Si frustule and chlorophyll content of the cells cultured in the Si-limiting treatment; while cellular lipid content seemed to be higher in the limiting-treatment compared to the non-limiting treatment. This was expected and is consistent with the literature. When diatoms are grown in unfavorable conditions, such as a Si-limited media, reduced rates of growth and photosynthesis are observed as well as a negative net synthesis of chlorophyll, while lipid production increases as a means of storing excess energy for later use (Coombs et al. 1967; Yang 2014; Pal et al. 2011; Martin-Jézéquel et al. 2000). Reduced Si frustules were observed because there was less Si in the media for
the cells to absorb. We observed reduced chlorophyll in the Si-stressed cells because their photosynthesis rates decreased, rendering a positive net production of chlorophyll unnecessary. Since the cells could not grow or perform normal metabolic processes, they stored the unused energy as lipids, resulting in the higher lipid content observed in the 20µM-Si treatment. It would have been preferable if both of these photographs had been taken at the same magnification with similar resolutions so that more accurate comparisons could be made between them.

In the fluorescence microscopy photographs C. radiatus, we observed a reduction in the exterior edges of the Si frustules and chlorophyll content of the cells cultured in the Si-limiting treatment, but were unable to draw any conclusions about the differences in lipid content between the two treatments. The reasoning for the reduced frustule and chlorophyll are the same as stated above. Since reduced frustule and chlorophyll content is indicative of reduced cell growth, we can infer that the lipid content of the cell in the Si-limiting treatment was higher than that of the non-limiting Si treatment. It is important to note that all of the above interpretations regarding the fluorescence microscopy photographs are based off purely visual, qualitative data and therefore are not backed by statistics. The diatoms in this study are three-dimensional cells. Since the photographs taken by the microscope only offer a two-dimensional view, they will not be an entirely accurate representation of the cells; we hope this has not altered our results severely. Our data would have been more concrete and conclusive if we had been able to perform a quantitative analysis, such as mass spectrometry.

CONCLUSION

Perhaps the most important conclusion drawn from our data is that the diatom species has more of an influence on growth rate than does Si limitation. As mentioned above, this has important implications for the biodiesel industry.

SI TREATMENT MAY NOT BE AS IMPORTANT IN DETERMINING CELL GROWTH AND METABOLISM AS THE SPECIFIC SPECIES OF DIATOM.

If different morphologies and metabolic rates are unique to each diatom species, then it stands to reason that maximum lipid accumulation is also unique to each species. Therefore, it is more prudent to focus research efforts on determining which diatom species are most suitable for commercial biodiesel production rather than which nutrient-limitation will produce the highest lipid content (Levitan et al. 2014).

Currently, biodiesel is not competitive with oil in terms of cost-effectiveness; significant economic challenges must be overcome before the large-scale production of biodiesel becomes feasible (Levitan et al. 2014; Courchesne et al. 2009). However, the benefits of a successful biodiesel industry, such as a carbon-neutral fuel source, carbon dioxide mitigation, waste heat utilization, and other novel bioproducts, make this a worthy cause (Courchesne et al. 2014). To overcome these economic obstacles, other methods of increasing lipid accumulation must be identified and extensively researched. In this experiment, we focused on biochemical engineering as a method of increasing lipid production in diatoms, but there are two other methods currently in their infancy. The first, genetic engineering, involves altering the algal genome to achieve the overproduction of lipids (Courchesne et al. 2009). While only a few studies have been published
using this method, genetic engineering has been shown to successfully increase lipid production in diatoms (Levitan et al. 2014). The second method is transcription factor (TF) engineering. TFs are proteins that recognize specific DNA sequences and can either cause or prevent the expression of the gene(s) with which they are associated (Courchesne et al. 2009). The concept of TF engineering involves overexpressing TFs known to up- or down-regulate the pathways involved in the production of lipids (Courchesne et al. 2009). This approach has had success by demonstrating improved production of valuable metabolites (Courchesne et al. 2014). Both genetic engineering and TF engineering are promising methods of lipid overproduction in the long-term perspective (Courchesne et al. 2009; Levitan et al. 2014). The next step in microalgal biodiesel research is to determine which diatom species and methods yield the highest cellular lipid content. It may be valuable to attempt to combine methods in order to efficiently produce the highest lipid yield possible. A combination of two or three of the above methods could potentially be the most productive way to continue biodiesel research. The more methods we test, the closer we come to replacing petroleum transportation fuels on a large scale.

ALTERNATIVE HYPOTHESIS
This is a hypothesis of inequality used for statistics that postulates a statistical significance among given sets of data. In other words, the variation observed among a set of data is not due to chance events, but rather is due to actual differences among the variable being studied. If the alternative hypothesis is accepted (or not rejected), then we are assuming that the data being compared are statistically different in some way.

LAG PHASE
A stage of bacterial growth where no apparent cell division is occurring, but the bacteria are undergoing physiological changes to promote cell growth due to favorable conditions.

LOG PHASE
A stage of bacterial growth in which favorable conditions result in exponential colony growth via cell division.

References


