Optimization of *Schizochytrium limacinum* SR21Growth using Different Carbon Sources and Copper Concentrations

by
Steven W. Soo

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Chemical Engineering
(Honors Scholar)

Presented February 14, 2017
Commencement June 2018

AN ABSTRACT OF THE THESIS OF

Steven W. Soo for the degree of Honors Baccalaureate of Science in Chemical Engineering presented on February 14, 2017.
Title: Optimization of *Schizochytrium limacinum* SR21Growth using Different Carbon Sources and Copper Concentrations

Abstract approved:_____

Zhenglun "Glen" Li

The purpose of this project was to optimize the biomass growth of *Schizochytrium limacinum* SR21. This was achieved by studying the effect of various concentrations of glucose, use of pure glycerol and purified crude glycerol as alternative carbon sources, and inhibition of cell growth with the presence of copper. The studied glucose concentrations were 10 g/L, 20 g/L, and 50 g/L. It was found that higher concentrations of glucose resulted in greater cell growth. Pure glycerol and purified crude glycerol, both at 9 wt%, were compared with the glucose concentrations. SR21 growth was greatest in glucose media, followed by purified crude glycerol, and lowest in the pure glycerol media. Copper inhibition at 0 mM, 0.5 mM, and 1.0 mM was investigated. Copper did inhibit biomass growth, but it was very minimal in the tested concentrations. This study showed that it is possible to utilize purified crude glycerol for the growth of *Schizochytrium limacinum* SR21.

Key Words: *Schizochytrium limancinum* SR21, Glucose, Glycerol, Crude Glycerol, Copper Inhibition, DHA

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APPROVED:

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Zhenglun "Glen" Li, Mentor, representing Bioresource Research Program

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Toni Doolen, Dean, Oregon State University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Steven W. Soo, Author

Tables of Contents

List of Figures

Abstract

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Keywords: *Schizochytrium limancinum* SR21, Glucose, Glycerol, Crude Glycerol, Copper Inhibition, DHA

Introduction

The disposal or utilization of crude glycerol has become a serious financial and environmental issue for biodiesel companies. Glycerol is a common byproduct of biodiesel manufacture, and is also a byproduct of corn ethanol production [1]. The biodiesel industry has been rapidly growing all over the world, resulting in a large surplus of glycerol. For every 100 pounds of biodiesel produced, approximately 10 pounds of crude glycerol are created. In 2013, approximately 1.8 billion gallons of biodiesel was produced in the United States alone [2]. Various methods for utilization have been attempted, including composting, combustion, anaerobic digestion, animal feeds, and thermochemical/biological conversions [1].

Docosahexaenoic acid (DHA) has beneficial health effects associated with the brain and retina [3]. Therefore, the demand is high, and a common mode of production is by microbial fermentation. Microbial DHA can be produced using bacteria [4], algae [5], fungi [6], and protists [6]. Increasing the production yield of microbial cell mass containing DHA has huge economic benefits. *Thraustochytrium* protists are efficient producers of DHA since they contain a high percentage of DHA in their total lipids and a low amount of polyunsaturated fatty acids that are similar to DHA structurally [7].

The species of the order Thraustochytriale have been reported to produce a considerable amount of DHA. *Schizochytrium limacinum* SR21 belongs to the order Thraustochytriale and is a unicellular organism [8]. Unicellular microorganisms have been preferred for study as they are easier to cultivate than filamentous microbes. *Schizochytrium limacinum* SR21 has may advantages over other species of genus *Thraustochytrium*, such as faster growth rate, higher tolerance levels to high shear stress and high glucose concentrations, the ability to grow in media with higher salinities, and potential to convert residues and by-products like glycerol, giving high DHA productivity [9]. *Schizochytrium limacinum* SR21 was selected for this study for all these advantages.

The accumulation of cellular biomass during *S. limacinum* cultivation is greatly dependent on the physiological conditions and nutrient composition of the medium, most notably, the carbon source [10]. *Schizochytrium limacinum* SR21 can grow on different sugars, but there is most rapid growth and production of DHA when grown on glucose and fructose [11].

Interestingly, glycerol can be used in media as a carbon source for SR21. However, crude glycerol must first be purified to minimize inhibitors and simplify DHA production process controls. Before purification, crude glycerol has a purity of 15-80%. It contains contaminants such as water, methanol, soap (sodium or potassium salt of free fatty acids), and unused reactants from biodiesel processes. Salts add to osmotic stress during fermentation. In addition, crude glycerol also contains a variety of elements such as calcium, magnesium, phosphorous, and sulfur [12]. The common practice of using alkaline catalysts during the biodiesel manufacturing process results in a high pH (above 10). The presence of contaminants creates challenges for the conversion process, as they could plug reactors, deactivate catalysts, and inhibit metabolic activities in DHA production [13]. Copper is a known inhibitor to the fermentation of microorganisms and is commonly found in crude glycerol. Additionally, sugars can be produced from catalytic processes where copper ions are introduced [14]. Another challenge for the utilization of crude glycerol is the inconsistency of its composition, which would alter feedstock and production procedures. Therefore, crude glycerol must be purified so that it does not complicate process controls.

Different purification processes have been developed. One common process is acidification, followed by neutralization and solvent extraction [15]. Addition of acid to crude glycerol causes separation of the crude glycerol into three phases rich in fatty acid, glycerol, and salt, respectively. The acidification process was used in this study.

In the present work, glucose and glycerol were used as carbon sources. The concentration of glucose was varied to analyze cell growth. Crude glycerol was purified using acidification. The cell growth in pure glycerol and purified crude glycerol was compared to see if the byproduct of the biodiesel industry could be used. To quantify the inhibition tolerance of *Schizochytrium limacinum* SR21, known concentrations of copper were added to the glycerol and purified crude glycerol.

Materials and Methods

Microorganism and Storage

The organism *Schizochytrium limacinum* SR21 was obtained from American Type Culture Collection (ATCC-MYA-1381). The cells were maintained in media containing 5 g/L glucose, 1 g/L yeast extract, 1 g/L peptone in artificial sea water (ATCC 790). The composition of artificial sea water used for media preparation was: 2.44 g/L $MgSO_4 \cdot 7\ H_2O$, 0.60 g/L KCl, 1.00 g/L $NaNO_3$, 0.30 g/L $CaCl_2 \cdot 2\ H_2O$, 0.05 g/L KH_2PO_4, 1.00 g/L Tris Buffer, 0.267 g/L NH_4Cl, 10.0 mL/L PI Metal Solution, and 3.00 mL/L Chelated Iron Solution. Chemicals used in the study and media preparation were purchased from Sigma Aldrich (St. Louis, MO). The PI metal solution and chelated iron solution were prepared according to the standard method available at UTEX's website (www.utex.org). The medium was autoclaved at 250 °F for 15 minutes before use.

Inoculum Preparation and Cultivation Conditions

Seed culture was prepared by growing 0.5 mL ATCC *Schizochytrium limacinum* SR21 in medium containing 5 g/L glucose, 1 g/L yeast extract, 1 g/L peptone in artificial sea water for one day in a 250 mL conical flask with 100 mL of working volume. *S. limacinum* growth medium was then inoculated with 10% (10 mL) of seed medium. Flasks were then incubated at 20 °C on a rotary shaker for one week or until growth decreased. Production medium contained 10 mL of seed inoculum, 10 g/L yeast extract, carbon sources, copper sulfate, and artificial sea water with 250 mL of working volume in a 500 mL conical flask.

Quantitative Analysis of Cell Growth Curve

A growth curve of the organism was generated by plotting the cell optical density (OD) against the culturing time (hours). Optical density was measured from 600-750 nm in 25 nm increments using a spectrophotometer. OD was plotted using values at

650 nm [10]. All experiments were run in duplicates. The average optical density at 650 nm was graphed versus growth time, with error bars equal to the standard deviation. The specific growth rate (μ) was calculated by taking the natural log of optical density, selecting points during the growth phase, plotting those points, and then fitting a linear equation to the points. The specific growth rate is the slope of the linear equation. Raw data, average and standard deviation data, and specific growth rate data are given in appendices A, B, and C, respectively.

Purification Process of Crude Glycerol

The crude glycerol used in this study was obtained from PDX Biofuels (Portland, OR). Purification of crude glycerol was done using the acidification method described by Nanda et al [13]. 1 L of crude glycerol was adjusted to a pH of 1 at room temperature using phosphoric acid while stirring. The crude glycerol was left to allow the formation of three separate layers, a fatty acid phase (top), a glycerol rich phase (middle), and an inorganic salt phase (bottom). The glycerol was vacuum filtered to separate the salt. The glycerol was again allowed to separate into two distinct phases. The fatty acid phase was separated using a pipette. The remaining glycerol was neutralized by adding 12 M KOH while stirring. The neutralized glycerol was placed into an oven for 2 hours to allow water to evaporate. The glycerol was removed from the oven and vacuum filtered again to remove the precipitated salt. The product is now the purified crude glycerol.

Optimization of Concentration of Glucose in Growth Media

To optimize the concentration of glucose for cell growth, cells were cultured in a production medium with different concentrations of glucose. Production medium contained 10 mL seed culture, glucose, 10 g/L yeast extract, and artificial sea water with a working volume of 250 mL in a 500 mL conical flask, where glucose concentration was equal to 10, 20, and 50 g/L. Flasks were inoculated with 10% (10 mL) inoculum from seed culture grown for about 1 day and incubated at 20 °C for one week.

Effect of Concentrations of Copper

To study the effect of copper inhibition on cell growth, different concentrations of copper were added to identical production medium. Production medium consisted of 10 mL seed culture, 9 wt% glycerol, 10 g/L yeast extract, and artificial sea water with a working volume of 250 mL in a 500 mL conical flask. Copper was added in the

form of anhydrous copper sulfate. The different concentrations of copper studied were 0 mM, 0.5 mM, and 1 mM.

Effect of Pure versus Purified Crude Glycerol

To study the effect of pure versus purified crude glycerol, growth media was kept constant with the glycerol either being pure or purified. Media was composed of 10 mL seed culture, 9 wt% glycerol, 10 g/L yeast extract, Cu (concentrations specified previously), and artificial sea water with a working volume of 250 mL in a 500 mL conical flask.

Results and Discussion

Inoculum Preparation and Cultivation Conditions

Due to a lab change in the middle of this study, the cultivation conditions were slightly different and must be noted. All the glucose experiments were performed in a shaker with temperature control. The temperature was precisely controlled at 20 °C. All the glycerol experiments, pure and purified, were performed in a shaker without temperature control. The temperature of the room was warm and ice was used once daily to lower the temperature of the water bath to 20 °C. This means that the temperature was above 20 °C at times, which may explain why the seed took longer to grow during the glycerol trials even though the media was not changed. Ideally, the temperature would be more precisely controlled during the glycerol trials to reflect a better side by side comparison between glucose and glycerol trials.

Crude Glycerol Purification Process

The crude glycerol was purified using a standard method of acidification. However, the final product was never characterized to ensure that the glycerol was pure, or to see what the composition actually was. It is possible that some contaminants from the biodiesel process remained in the purified product. Also, it is important to recognize that crude glycerol obtained from different biodiesel companies may have different compositions, so this is an area of further study.

Optimization of Concentrations of Glucose

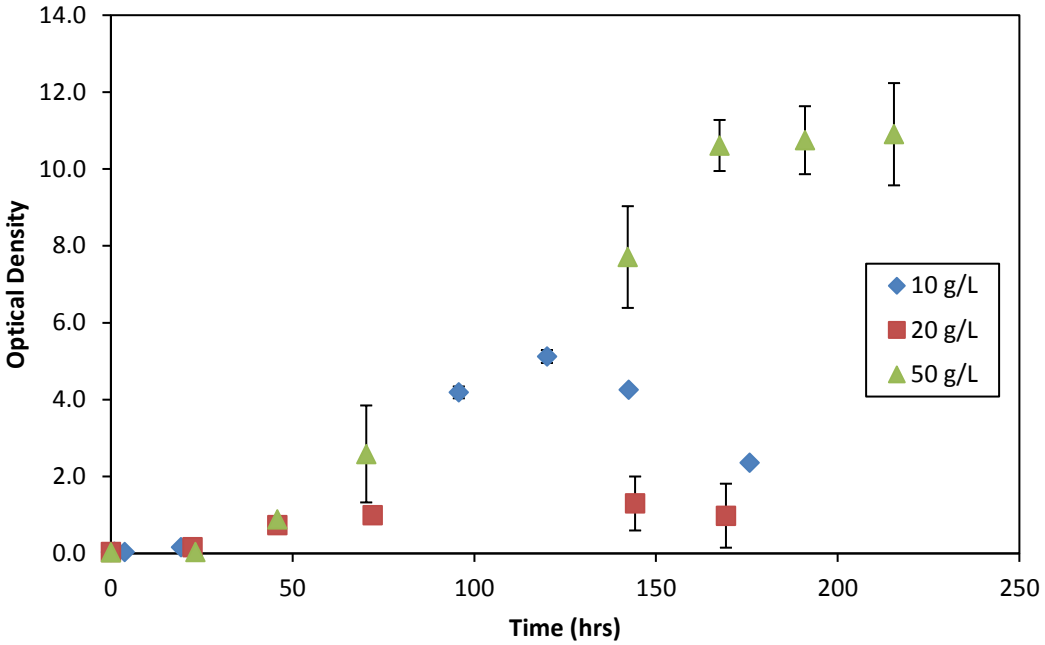

Figure 1: Effect of Glucose Concentration on Cell Optical Density. Schizochytrium limacinum SR21 grown in media containing different concentrations of glucose. Optical density measured at 650 nm. Error bars are equal to standard deviation.

The effect of various glucose concentrations are shown on *Figure 1*. For the first 2 days (48 hours), the growth was about the same across all concentrations. This was the lag phase, and it was the same duration for all concentrations. After that, the cells entered the log phase. The 20 g/L optical densities seemed low, and should be treated as an outlier because there was unusual cell mass in the flasks. There was a thick, dark grey contaminant at the bottom of each flask. The growth flasks were potentially contaminated in sampling during the pipetting procedure. Alternatively, since both flasks had low but similar growth, the seed activity could have been unusually low. From the figure, the greater the maximum cell growth, the longer it takes to reach the maximum. The 10 g/L and 20 g/L glucose concentrations began their decay phase after about 5 days (120 hours). The 50 g/L glucose concentration plateaus at approximately day 7 and does not start decaying even after 9 days. From the two non-anomalous glucose concentration trials, 10 g/L and 50 g/L, the higher the concentration, the greater the growth. Therefore, maximum cell growth can be increased by increasing the glucose concentration in the medium.

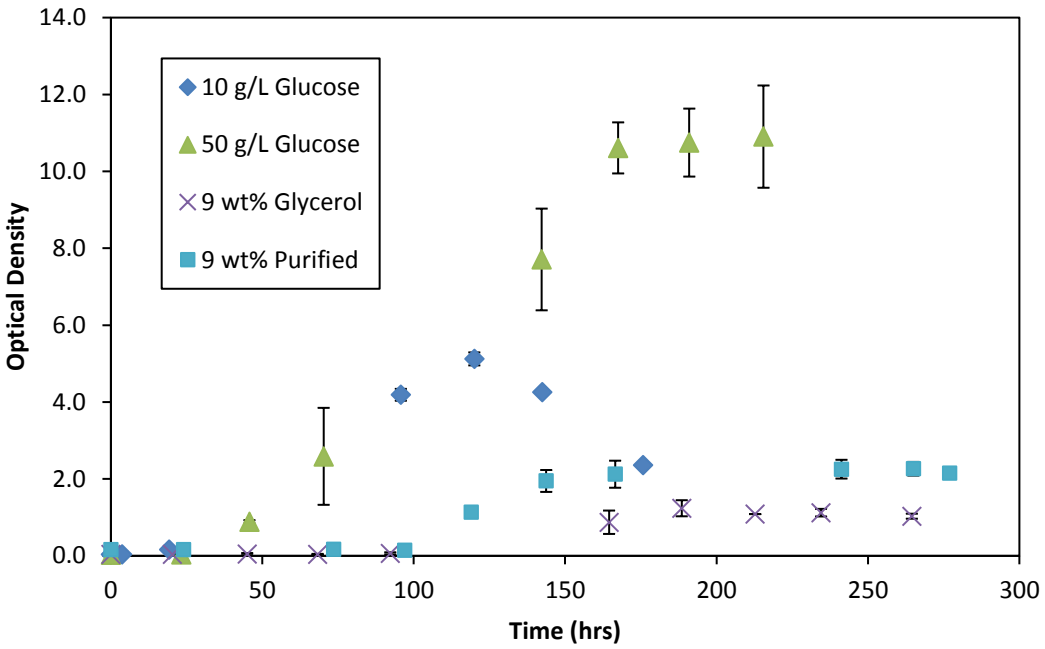

Figure 2: Effect of Different Carbon Sources without Copper Inhibition on Cell Optical Density. Schizochytrium limacinum SR21 grown in media containing different carbon sources and concentrations with no copper. Optical density measured at 650 nm. Error bars are equal to standard deviation.

From *Figure 2*, glucose was the best carbon source out of the three studied for maximum optical density. The glycerol, both pure and purified, had less optical density than the studied glucose concentrations. The lag phase for glycerol as the carbon source was slightly longer than when glucose was used. Furthermore, the log phase for glycerol was not as rapid and the maximum was significantly less than the maximum for any concentration of glucose. A figure of all the different carbon sources without copper is below along with the fitted linear lines that represent the specific growth rate.

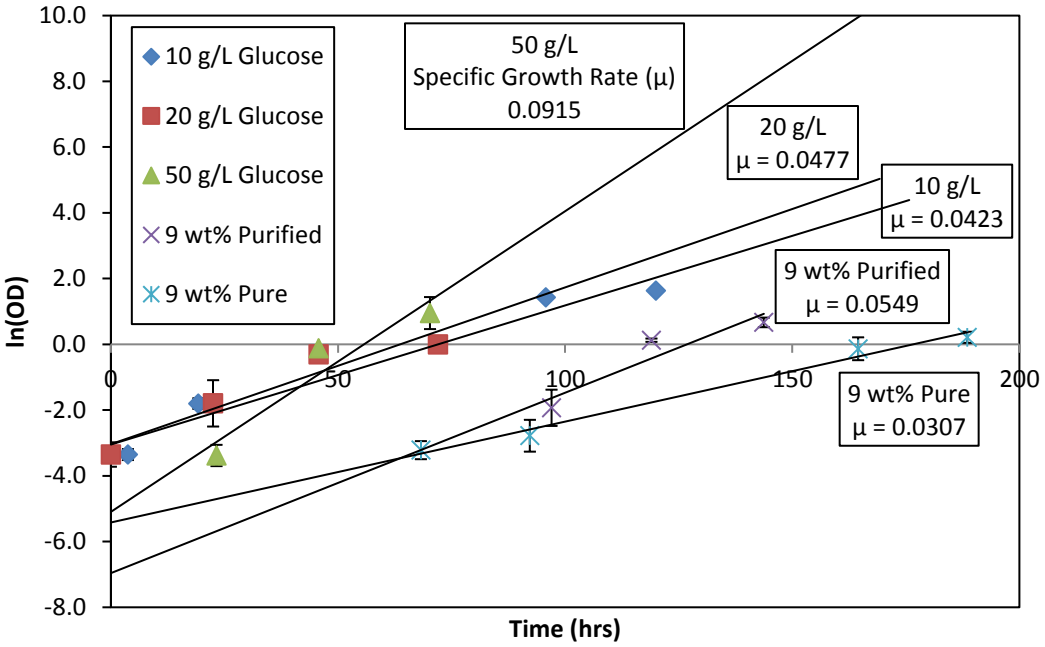

Figure 3: Specific Growth Rates of Different Carbon Sources with No Copper. Schizochytrium limacinum SR21 grown in media containing different carbon sources and concentrations with no copper. Optical density measured at 650 nm. Error bars are equal to standard deviation of the OD measurement divided by the OD value. Complete linear trendline equations and R^2 values can be found on Figure 13 in Appendix C.

Although the 20 g/L glucose media had lower OD values than the 10 g/L glucose media, the specific growth rate in the 20 g/L was slightly greater. For the glucose containing media, the specific growth rate was greatest for the 50 g/L concentration and lowest for the 10 g/L. The 9 wt% glycerol specific growth rate was lower than all the other media. Most interestingly, the specific growth rate of the 9 wt% crude glycerol was not only greater than the 9 wt% pure glycerol, but also greater than the 10 and 20 g/L glucose.

The cells grown in glycerol have not begun the death phase, as there was a plateau in optical density, so the cells never moved out of the stationary phase. This was interesting as it could mean that the cells were more self-sustaining when grown in glycerol. Of course, it is important to remember that the cultivation conditions for the glucose and glycerol were different, so it could just be that the glycerol cells had a smaller population so that the stationary phase was longer for glycerol.

Effect of Concentrations of Copper

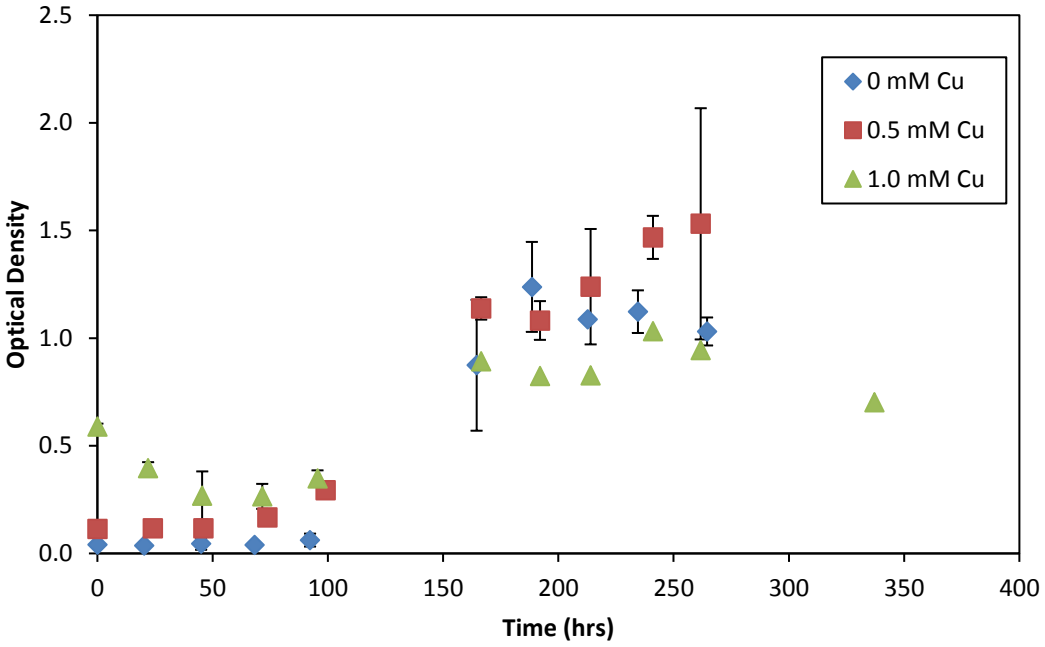

Figure 4: Effect of Various Concentrations of Copper in Pure Glycerol Media on Cell OD. Schizochytrium limacinum SR21 grown in pure glycerol media containing different concentrations of copper. Optical density measured at 650 nm. Error bars are equal to standard deviation.

Looking at *Figure 4*, the maximum growth and growth patterns were similar when the media contained different concentrations of copper. It is important to note that when there is 1 mM Cu present, the optical density was higher initially, even though copper is a known inhibitor. This is because measurements were taken from 600-750 nm, which is well within 500-900 nm, the absorption range of copper itself. However, it was interesting that when there was copper, the optical density dropped after time zero. This suggests that the cells quenched the absorption of the copper and then (or simultaneously) multiplied, which was why the OD increased at around 75 hours. Or, perhaps the cells died from the high copper concentration. The lag phase was about 75 hours regardless of copper concentration. The log phase seemed to begin after 75 hours. The maximum optical density for all concentrations was reached between 150 and 250 hours. And finally, the death phase begun after 250 hours.

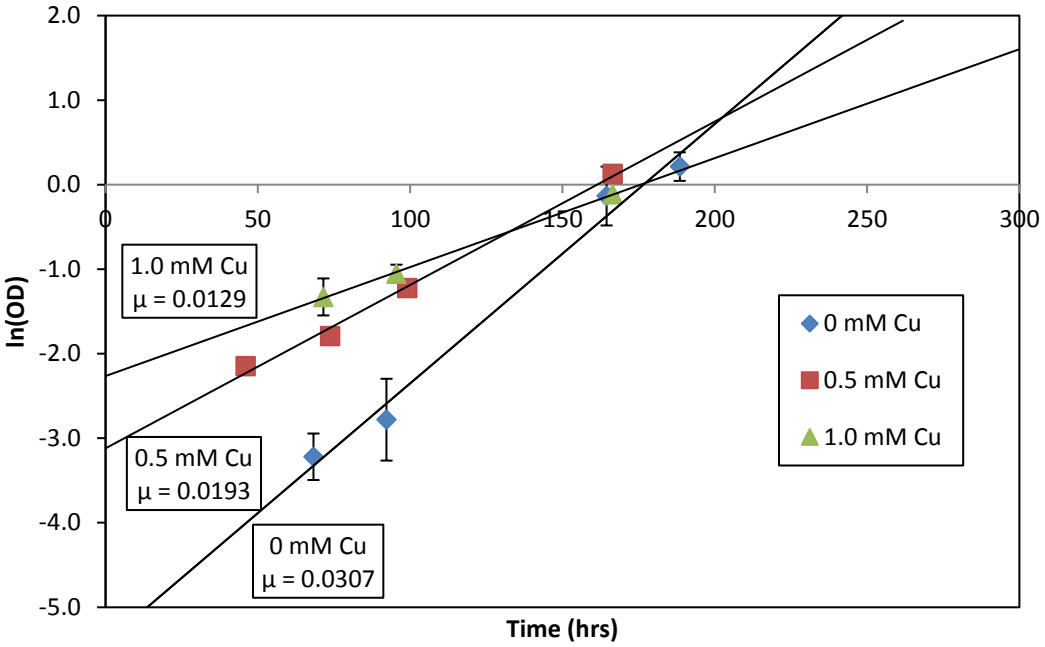

Figure 5: Specific Growth Rates of Pure Glycerol Media with Different Copper Concentrations. Schizochytrium limacinum SR21 grown in pure glycerol media containing different concentrations of copper. Optical density measured at 650 nm. Error bars are equal to standard deviation of the OD measurement divided by the OD value. Complete linear trendline equations and R^2 values can be found on Figure 14 in Appendix C.

From *Figure 5*, the greater the copper concentration, the slower the growth rate. Although the growth for all copper concentrations was similar in shape and magnitude (*Figure 4*), which suggested that copper did not inhibiting growth at the given concentrations, the presence of copper did slow the specific growth rate.

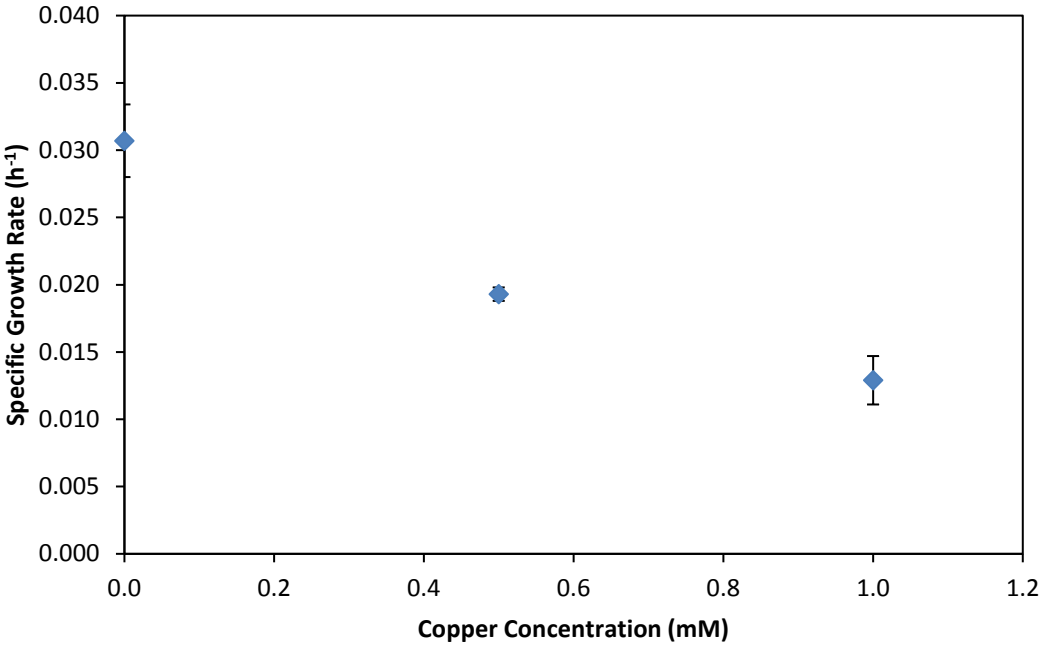

Figure 6: Copper Concentration vs Specific Growth Rate in Pure Glycerol Media. Schizochytrium limacinum SR21 grown in pure glycerol media containing different concentrations of copper. Optical density measured at 650 nm. Error bars are equal to the minimum and maximum specific growth rates calculated from the error of the natural log of optical density values.

Figure 6 shows that as the concentration of copper increased, the specific growth rate decreased.

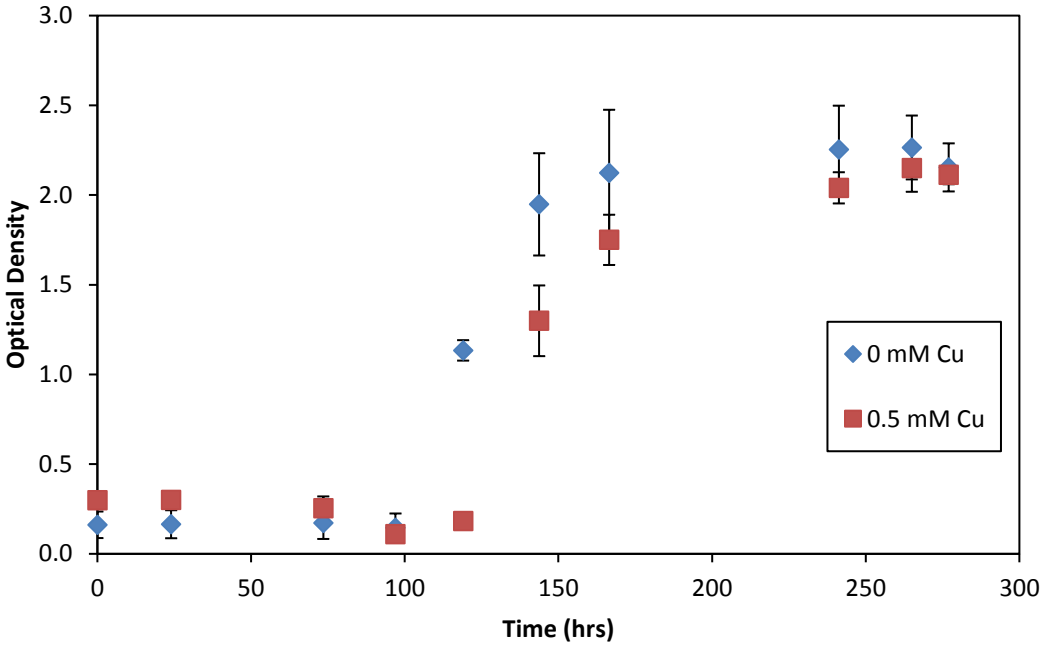

Figure 7: Effect of Multiple Concentrations of Copper in Purified Glycerol Media on Cell OD. Schizochytrium limacinum SR21 grown in purified crude glycerol media containing different concentrations of copper. Optical density measured at 650 nm. Error bars are equal to standard deviation.

Copper did not have a significant impact on cell growth in purified crude glycerol (*Figure 7*). As before, it is important to note that when there was copper present, the optical density was higher initially, even though copper is a known inhibitor. This is because copper itself has an absorption. Again, the optical density dropped after time zero when there was copper present. The lag phase was about 100 hours regardless of copper concentration; this was slightly longer than with pure glycerol. The log phase began after about 100 hours. The maximum optical density for all concentrations was around 250 hours. And finally, the decay phase began after 300 hours. The growth for the two copper concentrations was similar in shape and magnitude, which suggested that copper was not inhibiting growth at the given concentrations.

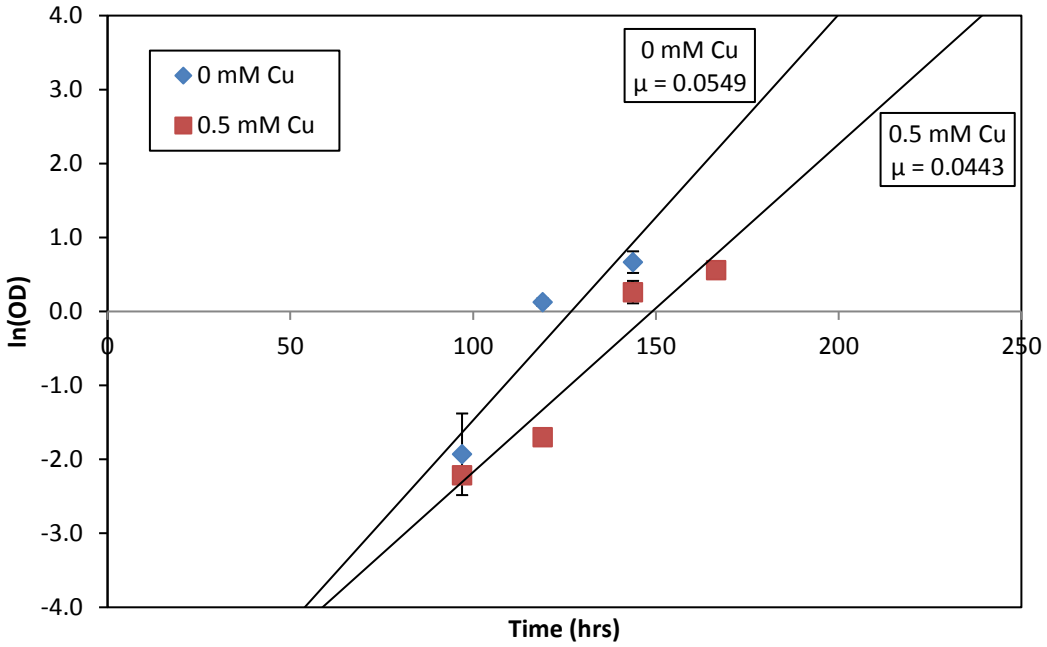

Figure 8: Specific Growth Rates of Purified Glycerol Media with Various Copper Concentrations. Schizochytrium limacinum SR21 grown in purified crude glycerol media containing different concentrations of copper. Optical density measured at 650 nm. Error bars are equal to standard deviation of the OD measurement divided by the OD value. Complete linear trendline equations and R^2 values can be found on Figure 15 in Appendix C.

Looking at the specific growth rates for purified crude glycerol (*Figure 8*), there is no significant difference in specific growth rate. Additionally, since there were only two concentrations studied, no general growth patterns could be determined. In the concentration range of the two copper concentrations studied, the concentration had no effect on specific growth rate.

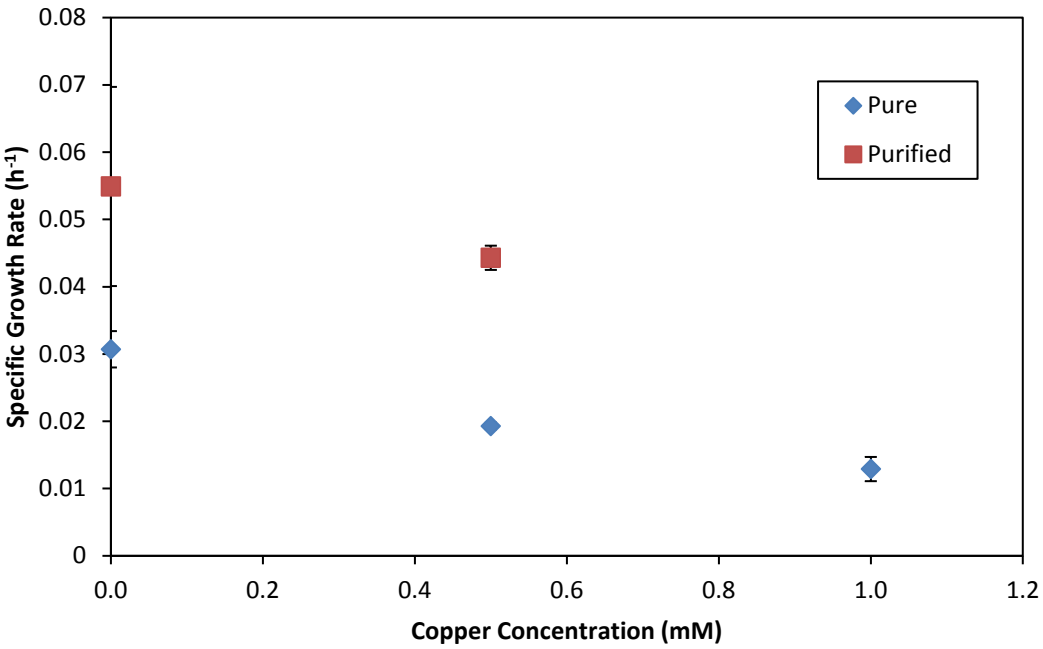

Figure 9: Copper Concentration vs Specific Growth Rate in Pure and Purified Glycerol Media. Schizochytrium limacinum SR21 grown in pure glycerol and purified crude glycerol media containing different concentrations of copper. Optical density measured at 650 nm. Error bars are equal to the minimum and maximum specific growth rates calculated from the error of the natural log of optical density values.

For the pure glycerol media, the specific growth rate decreased as the concentration of copper increased. For the purified glycerol media, there was an insufficient amount of data points to make conclusions about the effect of copper concentration on specific growth rate. However, looking at *Figure 9*, the purified crude glycerol growth pattern seemed similar to that of the pure glycerol media, with the only difference being that the specific growth rate values were shifted up by about 0.01 from 0 mM Cu to 0.5 mM Cu. Therefore, if there were additional copper concentrations for the purified crude glycerol media, it is likely that the specific growth rate would decrease with an increase in copper concentration.

Effect of Pure versus Purified Glycerol

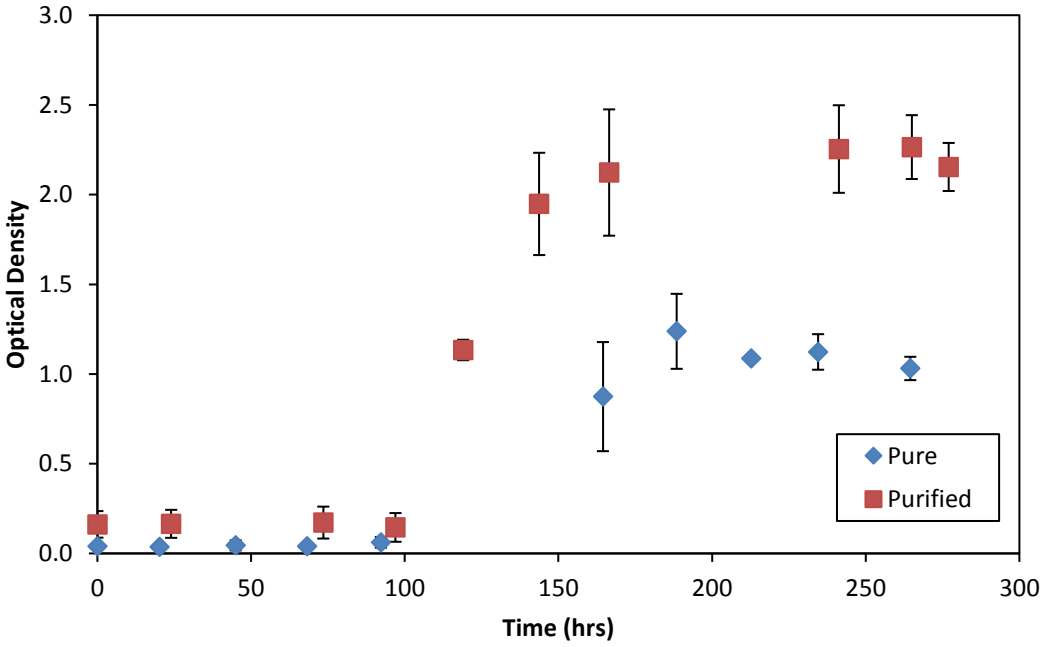

Figure 10: Effect of Pure vs Purified Crude Glycerol Media without Copper on Cell OD. Schizochytrium limacinum SR21 grown in pure glycerol and purified crude glycerol media containing no copper. Optical density measured at 650 nm. Error bars are equal to standard deviation.

Figure 10 shows that without copper, there was more cell growth in purified glycerol than pure glycerol. The specific growth rate of purified crude glycerol was also greater than that of pure glycerol, being 0.0549 compared to 0.0307. This was very surprising as one would think that contaminants in crude glycerol would inhibit growth. In the best case scenario, the cell would grow the same in pure and crude glycerol. In this case, the higher optical density was probably from cells other than *Schizochytrium limacinum* SR21. This would make more sense as the contaminants, especially phosphate, in crude glycerol potentially make sufficient feedstock for other organisms. Therefore, the purified glycerol should be characterized in future study.

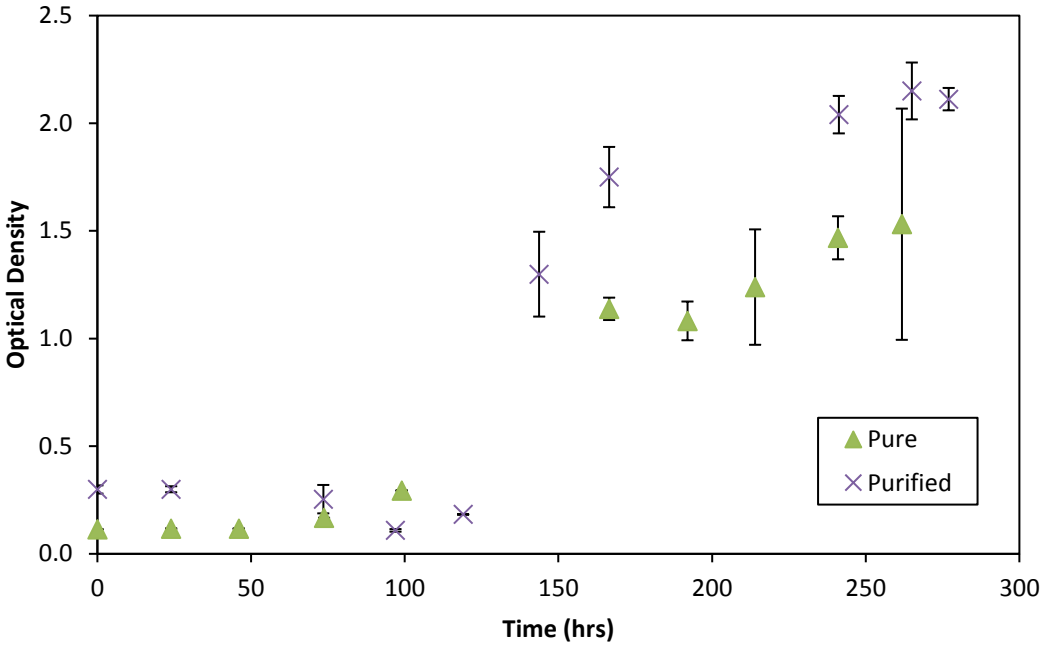

Figure 11: Effect of Pure vs Purified Crude Glycerol Media with 0.5 mM Copper on Cell OD. Schizochytrium limacinum SR21 grown in pure glycerol and purified crude glycerol media containing 0.5 mM of copper. Optical density measured at 650 nm. Error bars are equal to standard deviation.

With copper, as shown in *Figure 11*, the result was the same as the previous figure with no copper, there was more cell growth in the crude glycerol than pure glycerol. Again, the specific growth rate of the crude was also greater, being 0.0443 compared to 0.0193. The higher optical density was probably from cells other than *Schizochytrium limacinum* SR21. This would make more sense as the contaminants in crude glycerol would make sufficient feedstock for other organisms. However, it was also possible that contaminants in crude glycerol, such as phosphate, assist *Schizochytrium limacinum* SR21 growth.

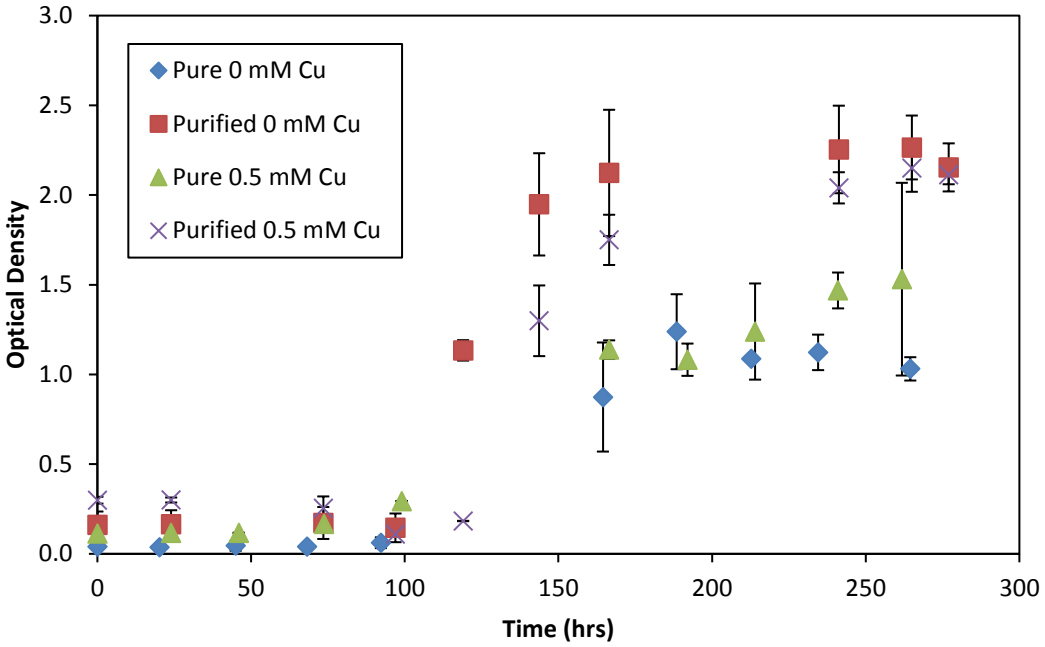

Figure 12: Effect of Pure vs Purified Crude Glycerol Media with Multiple Concentrations of Copper on Cell OD. Schizochytrium limacinum SR21 grown in pure glycerol and purified crude glycerol media containing different concentrations of copper. Optical density measured at 650 nm. Error bars are equal to standard deviation.

Figure 12 shows the effect of no copper (*Figure 10*), and with 0.5 mM copper (*Figure 11*) on cell growth in pure and purified crude glycerol media on a single figure. To identify the cause of higher cell growth in crude glycerol, the composition of the purified glycerol needs to be analyzed. Additionally, the cell growth needs to be characterized to verify that all the optical density is from the growth of *Schizochytrium limacinum* SR21 and not other organisms.

Concluding Remarks

The present work has shown that it is possible to optimize biomass growth. Future work will include measuring DHA production directly to see if more optimization is needed since greater cell growth does not necessarily mean more DHA production. *Schizochytrium limacinum* SR21 was grown best when using glucose as a carbon source, but glycerol and purified crude glycerol are also viable carbon sources. The presence of copper, a known inhibitor, did not seem to affect biomass growth at the concentrations studied. Other copper concentrations will need to be studied before a complete conclusion can be made, but at this point, for industries that would like to utilize crude glycerol, small concentrations of copper can be present. This is a positive result as removing all copper from crude glycerol would be inconvenient and more costly than targeting a specific concentration. Also, in future work, the composition of the purified crude glycerol needs to be characterized. The study showed that biomass growth was greater in crude glycerol than in pure glycerol, which was surprising. Studying the composition of the crude glycerol is key to understanding if *Schizochytrium limacinum* SR21 actually grows better in crude glycerol, potentially due to some contaminant that the strain can use for growth. Furthermore, characterizing the cells would also verify that no other organism has grown in the media, which would contribute to the larger optical density reading. Regardless of potential improvements, the results found in this study are promising. The utilization of crude glycerol, after simple purification, is possible and viable, and is a low cost feedstock compared to glucose. It can be used as a carbon source for the growth of *Schizochytrium limacinum* SR21, which can produce DHA, a profitable product. In addition, the strain had copper tolerance. This study offers a solution to the issue of the large surplus of crude glycerol, a common byproduct of the biodiesel industry and corn grain processes. The financial and environmental burden of crude glycerol could potentially be reversed into a financial gain by utilizing it to produce high value products, such as DHA.

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Appendices

Appendix A: Raw Data

All times are in hours.

Glucose (10 g/L) OD

t = 0			
Wavelength (nm)	A	B	C
600	0.056	0.061	0.060
625	0.044	0.046	0.047
650	0.034	0.037	0.037
675	0.026	0.027	0.028
700	0.022	0.023	0.023
725	0.017	0.017	0.018
750	0.014	0.015	0.015
t = 1			
Wavelength (nm)	A	B	C
600	0.101	0.063	0.074
625	0.084	0.049	0.058
650	0.064	0.037	0.048
675	0.059	0.029	0.038
700	0.050	0.024	0.033
725	0.036	0.019	0.028
750	0.035	0.016	0.024
t = 3.75			
Wavelength (nm)	A	B	C
600	0.056	0.061	0.059
625	0.043	0.047	0.046
650	0.033	0.036	0.035
675	0.017	0.019	0.019
700	0.012	0.014	0.013
725	0.008	0.010	0.009
750	0.004	0.006	0.005
t = 19.25			
Wavelength (nm)	A	B	C
600	0.231	0.198	0.174
625	0.212	0.180	0.156
650	0.193	0.163	0.139
675	0.175	0.147	0.123
700	0.167	0.129	0.114
725	0.153	0.122	0.104
750	0.139	0.121	0.091
t = 95.75			
Wavelength (nm)	A	B	C
600	3.792	5.120	4.428
625	3.616	5.008	4.304

650	3.496	4.900	4.172
675	3.336	4.696	4.060
700	3.264	4.536	4.004
725	3.156	4.436	3.880
750	3.052	4.368	3.700
t = 120			
Wavelength (nm)	A	B	C
600	5.976	6.272	4.528
625	5.640	6.128	4.432
650	5.256	5.880	4.232
675	5.240	5.352	3.896
700	4.792	4.864	3.656
725	4.568	4.608	3.336
750	4.344	4.400	3.184
t = 142.5			
Wavelength (nm)	A	B	C
600	4.432	5.616	0.768
625	4.112	4.712	0.680
650	3.856	4.656	0.600
675	3.608	4.320	0.560
700	3.328	4.208	0.496
725	3.064	4.104	0.448
750	2.856	4.000	0.408
t = 175.75			
Wavelength (nm)	A	B	C
600	2.028	5.076	0.804
625	1.884	4.900	0.720
650	1.732	4.720	0.624
675	1.572	4.540	0.568
700	1.480	4.364	0.548
725	1.400	4.148	0.504
750	1.284	3.968	0.456

Glucose (20 g/L) OD

t = 0			
Wavelength (nm)	A	B	C
600	0.054	0.076	0.046
625	0.041	0.061	0.034
650	0.031	0.050	0.025
675	0.025	0.043	0.020
700	0.021	0.039	0.016

725	0.017	0.035	0.013
750	0.014	0.031	0.010
t = 22.5			
Wavelength (nm)	A	B	C
600	0.349	0.155	0.095
625	0.320	0.149	0.086
650	0.299	0.122	0.077
675	0.280	0.117	0.066
700	0.265	0.105	0.059
725	0.255	0.098	0.057
750	0.242	0.092	0.054
t = 45.75			
Wavelength (nm)	A	B	C
600	0.725	0.865	0.868
625	0.696	0.823	0.830
650	0.649	0.776	0.787
675	0.618	0.746	0.751
700	0.589	0.713	0.718
725	0.560	0.682	0.689
750	0.531	0.652	0.658
t = 72			
Wavelength (nm)	A	B	C
600	1.055	1.125	1.190
625	1.000	1.055	1.125
650	0.940	0.990	1.055
675	0.865	0.945	1.000
700	0.820	0.890	0.950
725	0.795	0.830	0.890
750	0.755	0.795	0.850
t = 144.25			
Wavelength (nm)	A	B	C
600	1.336	1.464	1.664
625	1.256	1.384	1.584
650	1.160	1.272	1.464
675	1.096	1.208	1.392
700	1.056	1.168	1.344
725	1.008	1.096	1.264
750	0.952	1.032	1.200
t = 169.25			
Wavelength (nm)	A	B	C
600	0.920	1.220	1.280
625	0.865	1.140	1.205
650	0.790	1.045	1.110
675	0.755	1.000	1.060
700	0.720	0.945	1.005
725	0.680	0.895	0.950
750	0.655	0.855	0.910

Glucose (50 g/L) OD

t = 0			
Wavelength (nm)	A	B	C
600	0.058	0.052	0.050
625	0.044	0.040	0.039
650	0.032	0.029	0.027
675	0.027	0.024	0.023
700	0.021	0.017	0.017
725	0.020	0.013	0.014
750	0.016	0.012	0.013
t = 23.25			
Wavelength (nm)	A	B	C
600	0.069	0.055	0.047
625	0.051	0.037	0.030
650	0.046	0.031	0.025
675	0.034	0.020	0.015
700	0.030	0.016	0.011
725	0.025	0.012	0.007
750	0.023	0.009	0.005
t = 43.25			
Wavelength (nm)	A	B	C
600	0.972	0.948	0.888
625	0.945	0.921	0.861
650	0.921	0.897	0.837
675	0.902	0.873	0.811
700	0.890	0.863	0.802
725	0.872	0.844	0.778
750	0.856	0.831	0.769
t = 70.25			
Wavelength (nm)	A	B	C
600	2.256	8.544	3.720
625	1.952	8.184	3.528
650	1.696	7.520	3.48
675	1.632	6.704	3.064
700	1.416	6.104	3.032
725	1.408	5.600	2.920
750	1.176	5.128	2.792
t = 142.25			
Wavelength (nm)	A	B	C
600	6.880	8.640	8.768
625	6.640	8.528	8.664
650	6.184	8.392	8.552
675	5.808	8.176	8.480
700	5.584	7.992	8.368
725	5.360	7.896	8.256
750	4.784	7.392	8.152
t = 167.5			

Wavelength (nm)	A	B	C
600	10.984	10.080	11.464
625	10.760	10.000	11.400
650	10.632	9.936	11.264
675	10.488	9.840	11.160
700	10.280	9.776	11.088
725	10.104	9.704	10.992
750	9.936	9.616	10.568
t = 191			
Wavelength (nm)	A	B	C
600	11.256	11.968	10.296
625	10.816	11.872	10.200
650	10.416	11.752	10.080
675	9.992	11.648	9.968
700	9.560	11.584	9.904
725	9.280	11.496	9.824
750	8.984	11.432	9.744
t = 215.5			
Wavelength (nm)	A	B	C
600	12.056	11.456	9.536
625	12.000	11.408	9.520
650	11.952	11.352	9.408
675	11.872	11.280	9.392
700	11.800	11.248	9.384
725	11.696	11.184	9.328
750	11.616	11.136	9.264

Pure Glycerol (9 wt% with 0 mM Cu) OD

t = 0		
Wavelength (nm)	A	B
600	0.058	0.074
625	0.045	0.058
650	0.034	0.047
675	0.026	0.036
700	0.021	0.030
725	0.017	0.023
750	0.012	0.018
t = 20.25		
Wavelength (nm)	A	B
600	0.052	0.077
625	0.038	0.059
650	0.026	0.046
675	0.021	0.039
700	0.014	0.030
725	0.012	0.024
750	0.006	0.020
t = 45		
Wavelength (nm)	A	B
600	0.049	0.105
625	0.035	0.087
650	0.025	0.065
675	0.018	0.053
700	0.012	0.042
725	0.009	0.037
750	0.007	0.035
t = 68.25		
Wavelength (nm)	A	B
600	0.056	0.076
625	0.041	0.059
650	0.032	0.048
675	0.023	0.036
700	0.018	0.030
725	0.014	0.024
750	0.012	0.019
t = 92.25		
Wavelength (nm)	A	B
600	0.162	0.071
625	0.132	0.056
650	0.079	0.044
675	0.059	0.035
700	0.059	0.027
725	0.044	0.021
750	0.041	0.017
t = 164.5		

Wavelength (nm)	A	B
600	1.176	0.731
625	1.122	0.686
650	1.089	0.659
675	1.068	0.632
700	1.031	0.608
725	1.011	0.588
750	0.976	0.571
t = 188.5		
Wavelength (nm)	A	B
600	1.467	1.181
625	1.418	1.133
650	1.385	1.090
675	1.347	1.042
700	1.318	0.999
725	1.288	0.691
750	1.259	0.930
t = 212.75		
Wavelength (nm)	A	B
600	1.206	1.177
625	1.144	1.126
650	1.088	1.086
675	1.033	1.040
700	0.984	1.007
725	0.932	0.972
750	0.889	0.938
t = 234.5		
Wavelength (nm)	A	B
600	1.186	1.297
625	1.118	1.243
650	1.053	1.193
675	0.985	1.149
700	0.929	1.108
725	0.873	1.064
750	0.825	1.028
t = 264.5		
Wavelength (nm)	A	B
600	1.113	1.202
625	1.051	1.140
650	0.985	1.077
675	0.930	1.026
700	0.874	0.972
725	0.830	0.927
750	0.787	0.879

Pure Glycerol (9 wt% with 0.5 mM Cu) OD

t = 0		
Wavelength (nm)	A	B
600	0.149	0.149
625	0.130	0.131
650	0.113	0.114
675	0.097	0.098
700	0.086	0.086
725	0.077	0.075
750	0.061	0.061
t = 24		
Wavelength (nm)	A	B
600	0.156	0.156
625	0.132	0.131
650	0.117	0.117
675	0.099	0.098
700	0.084	0.084
725	0.075	0.076
750	0.062	0.062
t = 46		
Wavelength (nm)	A	B
600	0.151	0.150
625	0.135	0.135
650	0.116	0.117
675	0.100	0.101
700	0.089	0.090
725	0.076	0.077
750	0.067	0.068
t = 73.75		
Wavelength (nm)	A	B
600	0.168	0.166
625	0.147	0.147
650	0.131	0.131
675	0.114	0.114
700	0.101	0.100
725	0.090	0.090
750	0.079	0.078
t = 99		
Wavelength (nm)	A	B
600	0.332	0.330
625	0.310	0.308
650	0.295	0.293
675	0.281	0.279
700	0.269	0.268
725	0.260	0.259
750	0.251	0.249
t = 166.5		

Wavelength (nm)	A	B
600	1.254	1.169
625	1.204	1.134
650	1.174	1.101
675	1.154	1.079
700	1.132	1.059
725	1.115	1.032
750	1.102	1.020
t = 192		
Wavelength (nm)	A	B
600	1.077	1.188
625	1.041	1.176
650	1.018	1.145
675	0.986	1.118
700	0.963	1.099
725	0.947	1.080
750	0.931	1.050
t = 214		
Wavelength (nm)	A	B
600	1.465	1.104
625	1.441	1.078
650	1.428	1.049
675	1.393	1.016
700	1.375	1.001
725	1.354	0.979
750	1.338	0.969
t = 241		
Wavelength (nm)	A	B
600	1.418	1.655
625	1.405	1.648
650	1.397	1.538
675	1.380	1.516
700	1.360	1.508
725	1.313	1.420
750	1.298	1.400
t = 261.75		
Wavelength (nm)	A	B
600	1.246	1.992
625	1.169	1.973
650	1.151	1.911
675	1.104	1.890
700	1.068	1.865
725	1.036	1.839
750	1.003	1.800
t = 337.15		
Wavelength (nm)	A	B
600	0.671	1.085
625	0.618	1.072

650	0.570	1.041
675	0.530	1.007
700	0.492	0.963
725	0.456	0.930
750	0.426	0.889

Pure Glycerol (9 wt% with 1.0 mM Cu) OD

t = 0		
Wavelength (nm)	A	**B**
600	0.712	0.698
625	0.646	0.637
650	0.599	0.579
675	0.553	0.535
700	0.508	0.488
725	0.472	0.442
750	0.427	0.415
	t = 24	t = 20.25
Wavelength (nm)	A	**B**
600	0.458	0.504
625	0.410	0.458
650	0.376	0.416
675	0.339	0.380
700	0.305	0.343
725	0.277	0.311
750	0.249	0.281
	t = 46	t = 45
Wavelength (nm)	A	B
600	0.234	0.422
625	0.213	0.383
650	0.189	0.348
675	0.170	0.314
700	0.150	0.283
725	0.132	0.256
750	0.117	0.234
	t = 73.75	t = 68.25
Wavelength (nm)	A	B
600	0.265	0.376
625	0.244	0.340
650	0.224	0.306
675	0.203	0.276
700	0.187	0.249
725	0.169	0.225
750	0.153	0.205
	t = 99	t = 92.25
Wavelength (nm)	A	B
600	0.405	0.348
625	0.389	0.354

650	0.375	0.322
675	0.362	0.294
700	0.352	0.265
725	0.341	0.241
750	0.333	0.220
t = 166.5		
Wavelength (nm)	A	
600	0.962	
625	0.928	
650	0.893	
675	0.857	
700	0.827	
725	0.804	
750	0.779	
t = 192		
Wavelength (nm)	A	
600	0.890	
625	0.854	
650	0.824	
675	0.795	
700	0.769	
725	0.747	
750	0.724	
t = 214		
Wavelength (nm)	A	
600	0.879	
625	0.855	
650	0.827	
675	0.794	
700	0.767	
725	0.742	
750	0.726	
t = 241		
Wavelength (nm)	A	
600	1.114	
625	1.082	
650	1.033	
675	1.003	
700	0.968	
725	0.941	
750	0.911	
t = 261.75		
Wavelength (nm)	A	
600	1.016	
625	0.980	
650	0.945	
675	0.918	
700	0.882	

725	0.850	
750	0.837	
t = 337.15		
Wavelength (nm)	A	
600	0.801	
625	0.752	
650	0.703	
675	0.669	
700	0.623	
725	0.591	
750	0.558	

Purified Crude Glycerol (9 wt% with 0 mM Cu) OD

t = 0		
Wavelength (nm)	A	B
600	0.270	0.141
625	0.239	0.125
650	0.214	0.109
675	0.188	0.086
700	0.183	0.074
725	0.159	0.061
750	0.150	0.053
t = 24		
Wavelength (nm)	A	B
600	0.273	0.142
625	0.244	0.123
650	0.220	0.110
675	0.190	0.090
700	0.180	0.080
725	0.155	0.070
750	0.148	0.062
t = 73.5		
Wavelength (nm)	A	B
600	0.293	0.146
625	0.261	0.122
650	0.235	0.109
675	0.211	0.094
700	0.191	0.087
725	0.174	0.076
750	0.163	0.072
t = 97		
Wavelength (nm)	A	B
600	0.257	0.122
625	0.225	0.100
650	0.201	0.088
675	0.183	0.074
700	0.162	0.063
725	0.146	0.056
750	0.135	0.049
t = 119		
Wavelength (nm)	A	B
600	1.245	1.154
625	1.206	1.123
650	1.174	1.094
675	1.162	1.070
700	1.139	1.049
725	1.108	1.031
750	1.101	1.019
t = 143.75		

Wavelength (nm)	A	B
600	1.794	2.207
625	1.772	2.179
650	1.746	2.149
675	1.727	2.132
700	1.707	2.116
725	1.669	2.102
750	1.651	2.090
t = 166.5		
Wavelength (nm)	A	B
600	1.935	2.394
625	1.905	2.390
650	1.874	2.372
675	1.856	2.353
700	1.839	2.328
725	1.827	2.316
750	1.821	2.310
t = 241.25		
Wavelength (nm)	A	B
600	2.140	2.471
625	2.106	2.447
650	2.081	2.426
675	2.056	2.411
700	2.049	2.406
725	2.025	2.395
750	2.016	2.378
t = 265		
Wavelength (nm)	A	B
600	2.205	2.416
625	2.164	2.400
650	2.139	2.391
675	2.112	2.379
700	2.098	2.360
725	2.080	2.342
750	2.071	2.329
t = 277		
Wavelength (nm)	A	B
600	2.105	2.299
625	2.084	2.265
650	2.059	2.248
675	2.031	2.222
700	2.019	2.205
725	2.001	2.195
750	1.989	2.178

Purified Crude Glycerol (9 wt% with 0.5 mM Cu) OD

t = 0		
Wavelength (nm)	A	B
600	0.360	0.390
625	0.332	0.353
650	0.285	0.312
675	0.258	0.261
700	0.227	0.250
725	0.195	0.224
750	0.178	0.199
t = 24		
Wavelength (nm)	A	B
600	0.365	0.380
625	0.332	0.353
650	0.290	0.310
675	0.277	0.261
700	0.240	0.255
725	0.220	0.228
750	0.199	0.201
t = 73.5		
Wavelength (nm)	A	B
600	0.397	0.271
625	0.345	0.232
650	0.300	0.207
675	0.275	0.182
700	0.243	0.155
725	0.222	0.140
750	0.212	0.123
t = 97		
Wavelength (nm)	A	B
600	0.158	0.151
625	0.132	0.125
650	0.113	0.105
675	0.093	0.085
700	0.071	0.064
725	0.054	0.048
750	0.042	0.034
t = 119		
Wavelength (nm)	A	B
600	0.233	0.240
625	0.209	0.212
650	0.182	0.184
675	0.162	0.161
700	0.140	0.139
725	0.127	0.124
750	0.112	0.109
t = 143.75		

Wavelength (nm)	A	B
600	1.494	1.216
625	1.464	1.188
650	1.438	1.160
675	1.404	1.126
700	1.391	1.105
725	1.358	1.077
750	1.339	1.060
t = 166.5		
Wavelength (nm)	A	B
600	1.922	1.721
625	1.884	1.688
650	1.849	1.651
675	1.828	1.631
700	1.792	1.595
725	1.771	1.577
750	1.763	1.568
t = 241.25		
Wavelength (nm)	A	B
600	2.179	2.043
625	2.135	2.010
650	2.101	1.978
675	2.072	1.954
700	2.050	1.942
725	2.025	1.915
750	1.998	1.896
t = 265		
Wavelength (nm)	A	B
600	2.318	2.121
625	2.277	2.084
650	2.243	2.056
675	2.211	2.028
700	2.179	2.002
725	2.150	1.983
750	2.129	1.963
t = 277		
Wavelength (nm)	A	B
600	2.202	2.111
625	2.176	2.098
650	2.149	2.075
675	2.130	2.059
700	2.111	2.040
725	2.101	2.021
750	2.089	2.007

Appendix B: Average and Standard Deviation Data

Glucose OD at 650 nm

10 g/L		
t (hrs)	Average	Std Dev
0	0.036	0.002
1	0.05	0.014
3.75	0.035	0.002
19.25	0.165	0.027
95.75	4.189	0.702
120	5.123	0.832
142.5 (No C)	4.256	0.566
175.75	2.359	2.119
20 g/L		
t (hrs)	Average	Std Dev
0	0.035	0.013
22.5	0.166	0.117
45.75	0.737	0.077
72	0.995	0.058
144.25	1.299	0.154
169.25	0.982	0.169
50 g/L		
t (hrs)	Average	Std Dev
0	0.029	0.003
23.25	0.034	0.011
45.75	0.885	0.043
70.25 (No B)	2.588	1.261
142.25	7.709	1.323
167.5	10.611	0.664
191	10.749	0.884
215.5	10.904	1.33

Pure Glycerol (9wt%) OD at 650 nm

0 mM Cu		
t (hrs)	Average	Std Dev
0	0.041	0.009
20.25	0.036	0.014
45	0.045	0.028
68.25	0.040	0.011
92.25	0.062	0.030
164.5	0.874	0.304
188.5	1.238	0.209
212.75	1.087	0.001
234.5	1.123	0.099
264.5	1.031	0.065
0.5 mM Cu		
t (hrs)	Average	Std Dev
0	0.114	0.001
24	0.117	0.000
46	0.117	0.001
73.75	0.167	0.000
99	0.294	0.001
166.5	1.138	0.052
192	1.082	0.090
214	1.239	0.268
241	1.468	0.100
261.75	1.531	0.537
1.0 mM Cu		
t (hrs averaged)	Average	Std Dev
0	0.589	0.014
22	0.396	0.028
45.5	0.269	0.112
71.5	0.265	0.058
95.5	0.349	0.037
166.5	0.893	
192	0.824	
214	0.827	
241	1.033	
261.75	0.945	
337.15	0.703	

Purified Crude Glycerol (9wt%) OD at 650 nm

0 mM Cu		
t (hrs)	Average	Std Dev
0	0.162	0.074
24	0.165	0.078
73.5	0.172	0.089
97	0.145	0.080
119	1.134	0.057
143.75	1.948	0.285
166.5	2.123	0.352
241.25	2.254	0.244
265	2.265	0.178
277	2.154	0.134
0.5 mM Cu		
t (hrs)	Average	Std Dev
0	0.299	0.019
24	0.300	0.014
73.5	0.254	0.066
97	0.109	0.006
119	0.183	0.001
143.75	1.299	0.197
166.5	1.750	0.140
241.25	2.040	0.087
265	2.150	0.132
277	2.112	0.052

Appendix C: Specific Growth Rate Data

For each carbon source, the natural log of the average OD was taken. However, only the values used to calculate the specific growth rate (slope) are shown. The linear equation was calculated by using Microsoft Excel's linear trend line fit function. The graph with the full linear equation and R squared values are shown. The uncertainty of natural log values were calculated by dividing the standard deviation by the average OD (see Appendix B for standard deviation values). Error bars (uncertainty) are not shown on the graphs but can be seen in the Results and Discussion section.

Glucose at 650 nm

10 g/L			
t (hrs)	Average OD	ln(average OD)	Uncertainty
0	0.036		
1	0.05		
3.75	0.035	-3.352	0.057
19.25	0.165	-1.802	0.164
95.75	4.189	1.432	0.166
120	5.123	1.634	0.162
142.5	4.256		
175.75	2.359		
20 g/L			
t (hrs)	Average OD	ln(average OD)	Uncertainty
0	0.035	-3.352	0.371
22.5	0.166	-1.796	0.705
45.75	0.737	-0.305	0.104
72	0.995	-0.005	0.058
144.25	1.299		
169.25	0.982		
50 g/L			
t (hrs)	Average OD	ln(average OD)	Uncertainty
0	0.029		
23.25	0.034	-3.381	0.324
45.75	0.885	-0.122	0.049
70.25	2.588	0.951	0.487
142.25	7.709		
167.5	10.611		
191	10.749		
215.5	10.904		

Pure Glycerol (9 wt%) at 650 nm

0 mM Cu			
t (hrs)	Average OD	ln(average OD)	Uncertainty
0	0.041		
20.25	0.036		
45	0.045		
68.25	0.040	-3.219	0.275
92.25	0.062	-2.781	0.484
164.5	0.874	-0.135	0.348
188.5	1.238	0.213	0.169
212.75	1.087		
234.5	1.123		
264.5	1.031		
0.5 mM Cu			
t (hrs)	Average OD	ln(average OD)	Uncertainty
0	0.114		
24	0.117		
46	0.117	-2.146	0.009
73.75	0.167	-1.790	0
99	0.294	-1.224	0.003
166.5	1.138	0.129	0.046
192	1.082		
214	1.239		
241	1.468		
261.75	1.531		
1.0 mM Cu			
t (hrs)	Average OD	ln(average OD)	Uncertainty
0	0.589		
22	0.396		
45.5	0.269		
71.5	0.265	-1.328	0.219
95.5	0.349	-1.053	0.106
166.5	0.893	-0.113	0
192	0.824		
214	0.827		
241	1.033		
261.75	0.945		
337.15	0.703		

Purified Crude Glycerol (9 wt%) at 650 nm

0 mM Cu			
t (hrs)	Average OD	ln(average OD)	Uncertainty
0	0.162		
24	0.165		
73.5	0.172		
97	0.145	-1.931	0.552
119	1.134	0.126	0.050
143.75	1.948	0.667	0.146
166.5	2.123		
241.25	2.254		
265	2.265		
0.5 mM Cu			
t (hrs)	Average OD	ln(average OD)	Uncertainty
0	0.299		
24	0.300		
73.5	0.254		
97	0.109	-2.216	0.055
119	0.183	-1.698	0.005
143.75	1.299	0.262	0.152
166.5	1.750	0.560	0.080
241.25	2.040		
265	2.150		

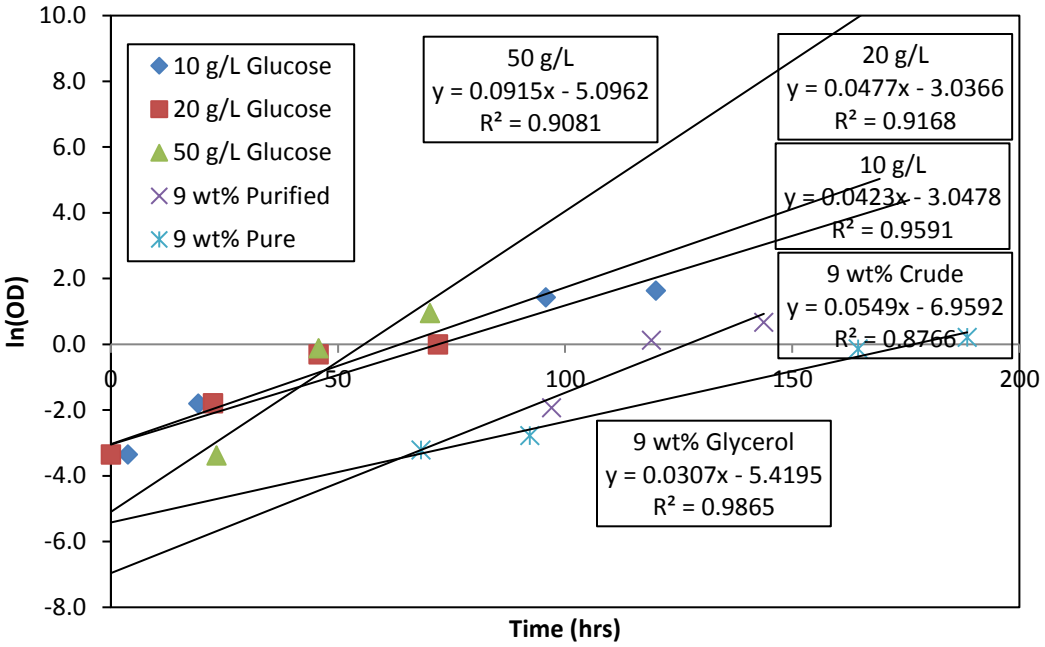

Figure 13: Specific Growth Rates of Different Carbon Sources with No Copper. Schizochytrium limacinum SR21 grown in media containing different carbon sources and concentrations with no copper. Optical density measured at 650 nm. Error bars were not included due to spacing, but can be found on Figure 3. Linear trendline equations and R^2 values calculated on Microsoft Excel 2016.

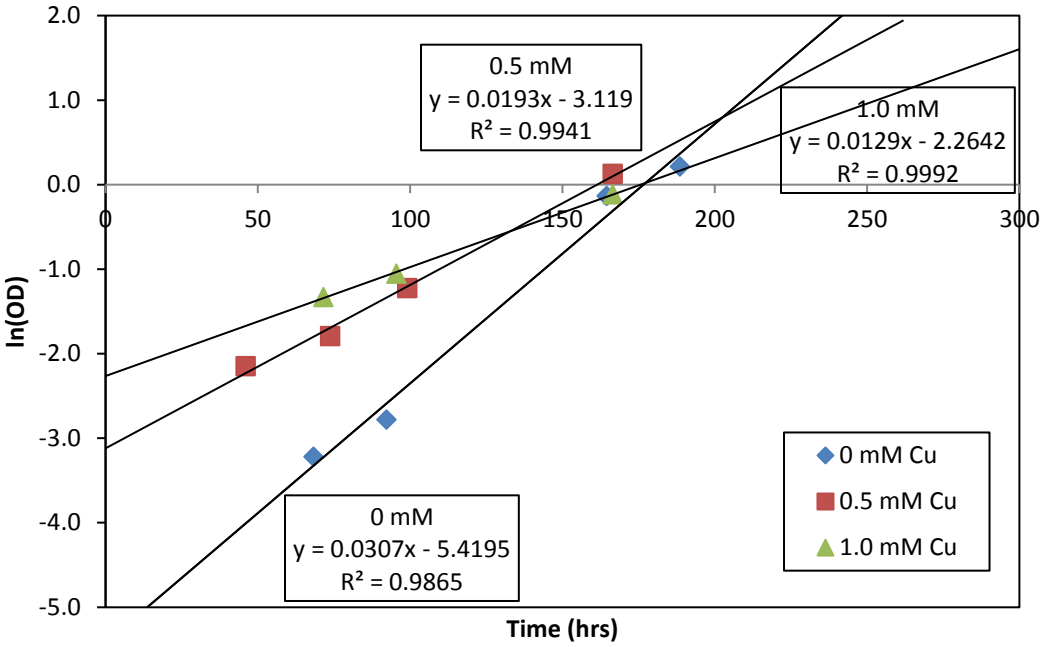

Figure 14: Specific Growth Rates of Pure Glycerol Media with Multiple Copper Concentrations. Schizochytrium limacinum SR21 grown in pure glycerol media containing different concentrations of copper. Optical density measured at 650 nm. Error bars were not included due to spacing, but can be found on Figure 5. Linear trendline equations and R^2 values calculated on Microsoft Excel 2016.

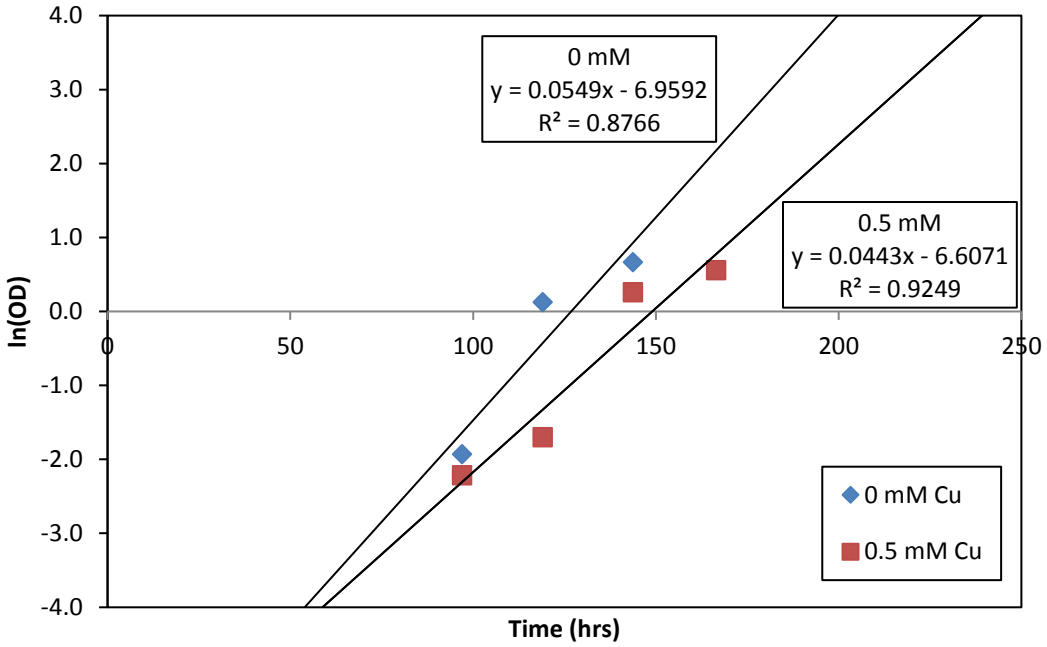

Figure 15: Specific Growth Rates of Purified Glycerol Media with Multiple Copper Concentrations. Schizochytrium limacinum SR21 grown in purified crude glycerol media containing multiple concentrations of copper. Optical density measured at 650 nm. Error bars were not included due to spacing, but can be found on Figure 8. Linear trendline equations and R^2 values calculated on Microsoft Excel 2016.

The error of the specific growth rate was calculated by finding the maximum and minimum specific growth rate (slope) using the first natural log minus uncertainty and last natural log plus uncertainty, and first natural log plus uncertainty and last natural log minus uncertainty, respectively. The maximum and minimum specific growth rates are in the tables below, as well as the difference between the reported specific growth rate.

Pure Glycerol Specific Growth Rate Error

0 mM Cu	Min/Max SGR	Difference from SGR
Max	0.0334	0.0027
Min	0.028	0.0027
0.5 mM Cu	Min/Max SGR	Difference from SGR
Max	0.0198	0.0005
Min	0.0189	0.0004
1.0 mM Cu	Min/Max SGR	Difference from SGR
Max	0.0147	0.0018
Min	0.0111	0.0018

Purified Crude Glycerol Specific Growth Rate Error

0 mM Cu	Min/Max SGR	Difference from SGR
Max	0.0696	0.0147
Min	0.0401	0.0148
0.5 mM Cu	Min/Max SGR	Difference from SGR
Max	0.0461	0.0018
Min	0.0426	0.0017