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PRODUCTION OF BIODIESEL FROM OLEAGINOUS ORGANISMS USING

UNDERUTILIZED WASTEWATER RESOURCES

by

Valerie Godfrey

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biochemistry

Approved:

Dr. Lance C. Seefeldt Major Professor	Dr. Scott A. Ensign Committee Member
Dr. Joan M. Hevel Committee Member	Dr. Mark R. McLellan Vice President for Research and Dean of the School of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2012

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ABSTRACT

Production of Biodiesel from Oleaginous Organisms

Using Underutilized Wastewaters

by

Valerie Godfrey, Master of Science

Utah State University, 2012

Major Professor: Lance C. Seefeldt
Department: Chemistry and Biochemistry

Driven by the rising costs, decreasing convenience, and increased demand of fossil fuels, the need for alternative, sustainable energy sources has caused a spark in interest in biomass-based fuels. Oleaginous organisms such as yeast, algae, and bacteria have been considered as microscopic biofactories for oils that can be converted into biodiesel. The process of growing such organisms using current technology requires an alarming amount of freshwater, which is another resource of growing concern. The research detailed within explains how several sources of underutilized wastewater can serve as growth medium in the biodiesel production process. Using only nitrogen and in one case phosphorus as external supplements, algae were shown to grow on produced water from oil and gas industry waste, local municipal wastewater, environmental brackish water from the Great Salt Lake, and wastewater from the potato processing industry. In each case, growth and biodiesel production in wastewaters was as good as or better than laboratory media. The bacterial organism *Rhodococcus opacus* PD630 and

the yeast organism *Cryptococcus curvatus* were also used to grow on the dairy manufacturing wastewater whey permeate, a large source of underutilized fixed carbon, with successful lipid production. *C. curvatus* was also used to successfully grow and form large amounts of biodiesel from ice cream factory wastewater and from wheat straw hydrolysate. In each case, the need for freshwater and outside nutrients was nearly entirely replaced, with the exception of some nitrogen supplementation, with a wastewater nutrient source, thus adding to the sustainability of biomass-based fuels.

(152 pages)

PUBLIC ABSTRACT

As part of the BioEnergy Team at Utah State Univeristy, my research objectives have been centered around the common theme of innovating new methods and technology for producing biological compounds and, by means of chemical conversion and/or extraction, isolating new forms of biodiesel available for transportation fuel. Within the broad scope of this project, I focused on the replacement of freshwater in cultivating such biological systems with wastewaters. If accomplished and correctly applied, such research would reduce the environmental impact of biodiesel production by reducing the demand for freshwater. It also would reduce production costs by reducing the amount of supplemented nutrients to growth media. In some cases of investigated wastewater types, the biological growth of such organisms hold the potential to clean up, or remediate, pollutants in the water, making it environmentally cleaner, safer, and less expensive than current technologies for disposal.

The research of produced water as a wastewater resource in algal biodiesel production was a 12-18 month proposed project costing $80,000. Other wastewaters were considered as part of a larger biofuels project funded through one earmark of a multimillion dollar grant, the portion of which was devoted to my resources is difficult to quantify but could be approximated at $30,000 over another approximately 12-16 month period.

Valerie Godfrey

Lance Seefeldt Laboratory, Utah State University

ACKNOWLEDGMENTS

I would like to thank the “Carbon Dioxide and Waste Water Remediation” project established through the Uintah Impact Mitigation Special Service District, with special thanks to Rob Behunin for funding of the produced water experiments. I also wish to thank the Department of Energy and the Utah Science and Technology Research (USTAR) for continued funding for biofuels research.

Appreciation is expressed to each of the following entities for wastewater collection: Anadarko for the access to Uintah Basin produced water, the City of Logan for access to the Logan Lagoon Wastewater Treatment Facility for municipal wastewater acquisition, Glanbia Foods® for acquisition of “Delac” whey permeate, J.R. Simplot for acquisition of each of the four wastewaters from their potato factory, and Blue Bunny® for acquisition of both wastewaters from their ice cream factory.

I extend special thanks to all colleagues and the advisory faculty of the Department of Chemistry and Biochemistry, particularly Dr. Lance Seefeldt, Dr. Brad Wahlen, Robert Willis, and Alex McCurdy. Undergraduate researcher Lynsey Talbot was invaluably instrumental in assisting with early experimentation with produced water, and later in working with Delac and the yeast organism *Cryptococcus curvatus*. Other members of the BioEnergy Center have provided a great network of motivation and ingenuity, with particular thanks to Kevin Shurtleff.

I greatly appreciate the continued support and encouragement of my dear husband, Aaron Godfrey. This degree would never have been possible without his unending belief in and desire for my ability to succeed. Support from other family

members has been overwhelming and greatly appreciated. Thank you to everyone who made this degree possible.

Valerie Godfrey

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CHAPTER 1

INTRODUCTION

At the 1900 World's Fair in Paris, Rudolph Diesel, the mechanical engineer who created the diesel engine, displayed an engine running on peanut oil so well that, according to his own words, "worked so smoothly that very few people were aware of it [the peanut fuel source]." Diesel was a large proponent of agricultural products as fuels in his engines. He stated,

> It has been proved that Diesel engines can be worked on earth-nut oil without any difficulty... similar successful experiments have also been made in St. Petersburg with castor oil; and animal oils, such as train-oil, have been used with excellent results. The fact that fat oils from vegetable sources can be used may seem insignificant today, but such oils may perhaps become in course of time of the same importance as some natural mineral oils and the tar products are now… In any case, they make it certain that motor-power can still be produced from the heat of the sun, which is always available for agricultural purposes, even when all our natural stores of solid and liquid fuels are exhausted (Nitske & Wilson, 1965).

Although a grand idea at that time, the subject of agricultural-based biodiesel lay dormant for decades due to the prosperity of petroleum during most of the twentieth century. Some mention of using palm oil as a diesel fuel occurred in the 1920s (Shay 1993), but interest faded during World War II when vegetable oil fuels were pronounced 12.5 times the cost of diesel fuels (Baker and Sweigert, 1947). During the late 1970s and early 1980s, the United States became more concerned about energy and reinvestigated the properties of vegetable oil (Bruwer et al., 1980; Goering et al., 1987). The recent cost rises in fossil fuels and the increasing rise in energy demands have led to a renewed interest in alternative sources of energy. Harvesting energy from agricultural products not only provides energy, but does so in a theoretically carbon-neutral cycle as the carbon

dioxide released from biofuel combustion is consumed by the crops, thus appeasing environmental concerns about the global accumulation of greenhouse gases.

With the increase in production of bio-ethanol from corn feedstock in the United States, one of the growing concerns about biofuels is the competition of agricultural land space between fuel and food. Microalgae have been considered as a feedstock for biofuel production because of its ability to grow on non-aerable land and produce higher densities of extractable oil at faster rates than a field of corn (Hu et al., 2008). Microalgae, however, require much more water than corn (up to 14 times) for growth medium (Clarens et al., 2010). The Energy Independence and Security Act of 2007 requires that 57 billion liters of ethanol be produced per year by the year 2015, which implicates that, if 44% of national corn produced is used for ethanol production and irrigiation demands remain unchanged from 2003 estimates, then the policy mandates 6 billion m^3 $year^{-1}$, or 3% of the total national irrigation water. This water demand exceeds the entire water usage of the state of Iowa (Dominguez-Faus et al., 2009).

Water scarcity across the globe is becoming as much a growing topic of concern as climate changes (Johnson et al., 2001). The World Resources Institute claimed that by 2025, 3.5 billion people, or approximately half the world population, will live in water-stressed basins where annual per capita water availability falls below 1700 m^3 (Revenga, 2000). If biofuels are to replace even a fraction of the petroleum energy demand and sustain itself for generations to come, it must do so relying on waste water streams instead of freshwater.

This thesis contains many insights into the process of taking a waste water stream and using it for the production of biofuel, specifically biodiesel. While much of the

content refers directly to microalgae as a feedstock for fuel, later experiments show that other oleaginous organisms such as yeast and bacteria also hold great potential for large scale fuel production, especially when a sufficient carbon waste stream is used. With the research contained herein and the countless other discoveries on the subject of sun-grown biodiesel, the ideas so eloquently anticipated by Rudolph Diesel over a century ago are finally being realized.

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CHAPTER 2

INITIAL EXPERIMENTATION WITH PRODUCED WATER

Abstract

Produced water is currently considered a waste product of great expense from the oil and natural gas industry. Shown here is a demonstration of the ability of microalgae to grow for the purpose of lipid production for biofuel conversion on produced water with optimization of nutrient supplementation of only 150-300 mg L^{-1} sodium nitrate. Hydrocarbon removal is necessary for higher lipid yield, and can be done by settling over months of time, centrifugation, or use of activated carbon filtration. Nutrient spiking at the end of growth does not increase lipid productivity. The process has successfully been scaled up to a 200-L raceway with 1.2 g L^{-1} dry weight and 9% total lipid content over a period of three weeks. The effects of remediation are also shown, with successful reductions in most elements to make it suitable for traditional disposal with the exception of salinity. The inability of microalgae to reduce the salinity of produced water makes remediation efforts futile. Overall, the process of growing algae on produced water for the production of biofuels is a great substitute to freshwater and purchased chemicals.

1. Introduction

1.1. Produced Water

Produced water is the largest byproduct of the oil and natural gas extraction process. Any given extraction site goes down miles into the earth’s crust to bring up oil and natural gas, and inevitably water will come up with the desired product. This water

is collected in large tanks on the site and must be trucked off daily. This water is contaminated, and of greatest worry is its salt content, hydrocarbons and other organic compounds, other heavy metals, and in some cases radioactive materials (DOE, 2009).

The water is disposed of using various techniques. It can be reinjected into the ground, generally into saline aquifers about 2000 feet underground. Evaporation sites also exist to drive off the water, leaving behind contaminants to be concentrated and dealt with at a future date. A personal visit to an evaporation pond near Vernal, Utah, showed produced water being added to ponds lined with heavy plastic, filled repeatedly, with the salted residue concentrating more and more with time. The liner is eventually pulled away to the barren land adjacent the pond and left there, indefinitely. Efforts have been made to chemically treat the water as well, although this generally a much more expensive option. In the state of Utah, 98% of produced water is reinjected into Class II wells (aquifers), close to 2% is evaporated in ponds, and negligible amounts are treated chemically (Utah DEQ, 2010, personal communication). According to Clark and Veil (2009), approximately 21 billion barrels of produced water are generated each year in the United States alone. That is equivalent to roughly 2.4 billion gallons per day. This is outside the 50 billion barrels generated each year from wells outside the United States. The cost of treating one barrel of produced water ranges, depending on the area, anywhere from $0.40 to $1.50, or in some areas and cases upwards of $10.00, this becomes a huge expense to the oil and natural gas industry (and eventually out of the pockets of the consumer) (Boysen et al., 2001).

A newly established oil well will produce one barrel of water for every barrel of oil. The amount of water increases with the lifespan of the well, sometimes producing 50

barrels of water per barrel of oil. The well is shut down when the cost of disposing the water exceeds the value of the oil.

1.2. Produced Water and Algae

Microalgae have been used previously for wastewater treatment and show a high tolerance to heavy metals and other contaminants (Yu et al., 1999). They also hold great potential as a source of renewable energy through the production of lipids convertible into fatty acid methyl esters, which can be used as biodiesel. If microalgae could be shown to grow and produce a reasonable amount of lipids to be converted into biodiesel on produced water, production costs could be mitigated to both oil/gas companies and algae biofuels projects.

2. Materials and Methods

2.1. Collection and Storage of Wastewaters

Produced Water. The produced water used in this study was obtained from the Anadarko injection site located in the Uintah Basin, near Vernal, Utah. This site collects produced water from hundreds of wells throughout the Basin and pre-treats it by running it through a carbon filter (to remove hydrocarbons) prior to re-injection. Our two barrel sample was collected prior to carbon filter treatment. The barrel was centrifuged in 400-mL aliquots as needed during the first two months for hydrocarbon removal. After about two months, however, the gray color of the water began to dissipate as the hydrocarbons settled with time in a black mass on the bottom of the barrel. At this point, without greatly disturbing the barrel, produced water was removed from the top of the barrel.

Later experimentation involved testing the use of activated carbon as a carbon filter to substitute centrifugation. Activated Filter Carbon by Aquarium Pharmaceuticals® was purchased from a local pet store, and is designed for removal of organic wastes, colors, and odors using small pebbles to act as a filter. An equivalent of one-half cup of pebbles was used to treat less than 40 liters of produced water. Bench-scale experiments ran produced water through an activated carbon-packed column, secured with cheesecloth. Outdoor raceway experiments utilized a specially-made device to pass water through the activated carbon pebbles while being pumped into the raceway. This filter apparatus had been built to hold enough activated carbon for 80 L of produced water to pass through, and was therefore changed out twice for filling of the raceway. It was observed that water exiting the filter had a black grainy substance that was washing off of the activated carbon pebbles, and that rinsing the pebbles in water prior to loading in the filter reduced this effect.

Municipal Wastewater. The municipal wastewater used in the final remediation study was collected from the lagoon-style Logan City wastewater treatment facility on January 5, 2011, at a point just prior to chlorination and release from the facility. Water was collected in plastic containers, centrifuged for debris removal, and autoclaved for sterility until inoculation. Elemental analysis was performed by Utah Veterinary Diagnostics Laboratory (UVDL), which included nitrate analysis (Biospec-1601, Shimadzu, Columbia, MD) and ICP analysis (ELAN 6000, Perkin Elmer, Waltham, MA) of the following elements: Ag, Al, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Li, K, Mg, Mn, Mo, Na, Ni, P, Pb, Sb, Se, Si, Sn, Sr, Tl, V, and Zn. Sodium chloride was supplemented at 180 mg L^{-1} for algal growth.

Great Salt Lake Water. The environmental, brackish water used in the final remediation study was collected from the Great Salt Lake at a point two miles from a freshwater inlet on January 19, 2011. Upon collection in plastic containers, the water was centrifuged for debris removal and autoclaved until inoculation. The compositional analysis was performed identically to that of municipal wastewater. Because nitrate and phosphorus were found to be so low, sodium nitrate and potassium phosphate were supplemented to equal that of produced water N:P content.

2.2. Strain Selection and Stock Cultures

Amphora coffeaformis UTEX 2039, *Chaetoceros gracilis* UTEX LB 2658, *Phaeodactylum tricornutum* UTEX 640, BA116 (isolated from Great Salt Lake, or GSL), BA117 (isolated from GSL), BA118 (isolated from GSL), BA050 (isolated from GSL), and USU080 (formerly known as GA080, isolated from GSL) were maintained within the laboratory in agar plates and 250 mL stock cultures in 500-mL baffled flasks. Liquid cultures were rotated at 140 rpm with lighting at 180 µmol m^{-2-} s^{-1} on a light:dark photoperiod of 14:10. Broad Seawater (BS) medium was used for culture maintenance and inoculum, and consists of the following ingredients per liter: NaCl (18 g), $NaHCO_3$ (1 g) (none for *Chaetoceros gracilis* cultures), KCl (0.6), $MgSO_4$•$7H_2O$ (1.3 g), $CaCl_2$•$2H_2O$ (100 mg), K_2HPO_4 (250 mg), Na_2SiO_3•$9H_2O$ (70 mg) (none used for USU080 cultures), ferric ammonium citrate (5 mg). Trace metals were also added at 1 mL per L of media: H_3BO_3 (600 mg L^{-1}), MnCl•$4H_2O$ (250 mg L^{-1}), $ZnCl_2$ (20 mg L^{-1}), $CuCl_2$•$2H_2O$ (15 mg L^{-1}), Na_2MoO_4•$2H_2O$ (15 mg L^{-1}), $CoCl_2$•$6H_2O$ (15 mg L^{-1}), $NiCl_2$•$6H_2O$ (10 mg L^{-1}), V_2O_5 (2 mg L^{-1}), KBr (10 mg L^{-1}).

2.3. Compositional Analysis of Produced Water

Samples of produced water were delivered to Utah State University Analytical Labs (USUAL) for ICP analysis (Thermo Electron, Marietta, OH) of the following elements: Al, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Si, Sr, and Zn. In addition, USUAL was also utilized to determine pH, electrical conductivity (for calculation of total dissolved solids, or TDS), and nitrate, chloride, carbonate, and bicarbonate concentrations. A total of eleven samples of produced water were submitted to USUAL, which included one sample from the barrel upon receipt (prior to hydrocarbon settling), two samples following settling (to observe the range of variability from sample to sample), one sample of produced water without inoculation but still treated with tube illumination and gas bubbling, 2 samples including before and after growth of *A. coffeaformis*, 3 samples from 2 different batch growths of *C. gracilis* including 1 before and 2 after samples from each batch, and 1 following growth of USU080.

Testing of the nutrients nitrogen, phosphorus, and silicate concentrations during the activated carbon experiments was performed by QuikChem 8500, Series 2 (Lachat, Loveland, CO).

2.4. Growth Conditions and Measurements

Tube Reactors. Growths were carried out in two different tube reaction vessels, unless otherwise noted in the section about scale-up. The first to be used consisted of a rack of ten 450-mL autoclaved tubes illuminated by fluorescent light bulbs at approximately 300 µmol m^{-2} s^{-1}, and was used only in initial experiments with strain

selection on produced water and nitrate and phosphate studies in *A. coffeaformis*. The second tube reaction system consisted of a rack of 14 1.3-L autoclaved tubes similarly illuminated and was used for the remainder of tube reaction systems. During experiments, ten percent inoculum was added to either Broad Seawater or produced water, with the nutrient supplements (i.e. sodium nitrate, potassium phosphate) as specified in each experiment. Cultures were sparged with air with 1% CO_2.

Growth Measurements. Daily optical density readings (300-900 nm) were taken (UV-2401PC, Shimadzu, Colombia, MD) and charted based on the 600 nm reading. Cultures were harvested once OD_{600} showed signs of peaking. Cultures were centrifuged at 8000 rpm for 15 minutes, with collected cells frozen and subsequently lyophilized (Free Zone 4.5, Lanconco, Kansas City, MO). Any supernatants were kept frozen until submitted for analysis. Dry weight was determined from lyophilized cell mass and cells were kept frozen until lipid analysis.

USU080 "Wild" Strain Experiments. In 2 500-mL tubes, 400 mL Broad Seawater Bicarb. media was added with 70 mL of USU080 wild strain culture from the greenhouse raceway. Daily monitoring of optical density was the critical information which dictated when to dilute the cultures. During the first week, growth was allowed to level off to observe the maximum density capacity for the strain. On day 6, fifty percent of the cultures were harvested and to one tube was added an equivalent volume of fresh Broad Seawater media, and to the other tube was added the supernatant media from the harvested volume (i.e. the "spent" media). The cells had a tendency to clump, which meant that, without more efficient mixing prior to harvesting, the optical density would not necessarily fall by half, which was the case every time cells were harvested. On day

16, 50% of cell cultures were again harvested and replaced with either fresh media or spent media, as before. Two days later, upon returning from a tour of facilities in Vernal, the gas capillary tube in the vessel receiving fresh media was found completely blocked and the culture dead. Though the cells in the second vessel receiving spent media had been treated differently, on day 20, 100 mL cells were taken from it to act as inoculum to restart the vessel to receive fresh media at each harvest. This tube receiving fresh media grew quickly, and was harvested and filled with fresh media on days 23, 25, 27, 28, and 30. Meanwhile, the tube receiving spent media did not grow sufficiently, appearing to be nutrient limited. On day 32, both 500-mL tubes were dumped into larger 1.3-L tubes. Both were filled to 1.2 L using fresh Broad Seawater media. In the larger vessels, the tube receiving spent media grew fastest and was harvested on day 36 and refilled with the spent media. This tube on spent media did not grow very well for the remainder of the 53 day experiment. The tube receiving fresh media was harvested and refilled with fresh media on day 37 and again on day 42.

Two additional 500mL tubes containing 400 mL produced water and 70 mL USU080 “wild” inoculum from the greenhouse raceway were also monitored for rapid growth. Cultures were diluted with fresh produced water when OD levels peaked, and diluted again when cultures reached that same OD.

Scale-up Methods. Scale-up cultures included three additional forms of bioreactors. A 5-L bottle was equipped with magnetic stir-plate mixing rotating at 50 rpm and air and CO_2 sparging and lighting from two sides opposite each other on a 14:10 light:dark cycle. A 50-L bag was hung outdoors with air and CO_2 sparging. Although plastic bags are routinely used for growth using lab media, initial experiments were

required to prove that a plastic bag could hold produced water alone without causing corrosion or deformation of the bag. Using a smaller scale, approximately 8-L bag, produced water was shown to successfully hold for several weeks with no sign of deformation or leakage. Sparging in 5-L and bag reactors was similar to that in tube reactors described above. A 220-L raceway was the largest bioreactor tested, equipped with a paddlewheel and pH control via a CO_2 injection system. The raceway was filled with 150 L produced water using the activated carbon filter apparatus and pump, and was inoculated with 50 L of either *C. gracilis* or USU080 from a bag. Daily measurements including optical density, pH, dissolved oxygen, and fluorescence (a measure of cell fitness) were taken on the raceway scale.

The scale-up process requires starting from a healthy shaker flask at 200 mL, followed by a 1.3-L tube, then a 5-L reactor, to a 50-L bag, and if the culture appears healthy enough it can then be used to inoculate the raceway. Produced water supplemented with nitrate was used as the medium during all stages of *C. gracilis* growth but the 200-mL shaker flask, and for the 50-L bag, the newly acquired, unsettled produced water was used before testing on the activated carbon filter had been completed. USU080 inoculum was grown on nitrate-supplemented produced water for all stages except the 200-mL flask and 50-L bag, where Broad Seawater Bicarb. Edition was substituted.

2.5. Neutral Lipid Analysis

200 mg dry, homogenized biomass was weighed into a clean 16 x 125-mm test tube and mixed with 5 mL solvent mixture of tetrahydrofuran, chloroform, and hexane

(1:1:1). Test tube contents were sonicated 3 times in 10 second intervals to release TAG from cells. Upon centrifugation, lipid and solvent mix is extractable from pelleted cell debris and removed by syringe into a 40-mL I-Chem bottle, pre-marked to 15 mL using 15 g distilled water. Solvent addition, sonication, centrifugation, and extraction of lipid-solvent mix was repeated twice more. The final extracted volume is brought to 15 mL with solvent mixture, and 1 mL is removed into a GC vial with Teflon septum. Standards were prepared using tripalmitin at concentrations of 0.065, 0.13, 0.26, 0.39, 0.52, and 0.65 mg mL^{-1}. 10 μL octacosane (10 mg mL^{-1}) and 50 μL MSTFA were added to each sample prior to GC analysis.

2.6. FAME analysis

Where indicated, FAME analysis was also performed, as described in Wahlen et al. (2011). This included mixing 100 mg dry biomass with 2 mL acidified chloroform (with 1.8% v/v H_2SO_4) in a commercial scientific microwave (Discover S, CEM USA, Matthews, NC) at 90°C for 20 minutes to complete transesterification. Chloroform was added to reaction vessel and rinsed with water twice to remove residual methanol, acid, and glycerol. The chloroform-FAME layer was removed by syringe into a 10-mL volumetric flask and rinsed with chloroform twice more to remove maximal lipids from the biomass. The final volume was brought to 10 mL with chloroform, and 1 mL was removed into a GC vial, capped with a Teflon septum. FAME standards were prepared using a mixture of methyl myristate, methyl palmitoleate, and methyl oleate at final concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg mL^{-1}. 10 μL octacosane (10 mg mL^{-1}) was added to each sample and standards prior to GC analysis.

2.7. Gas Chromatography

Lipid content, both TAG and FAME, were determined by gas chromatography (Model 2010, Shimadzu Scientific, Columbia, MD) coupled with programmable temperature vaporizer (PTV) and flame ionization detector (FID). The carrier gas used was helium, set at a constant flow rate of 50 cm s^{-1}. A 1-µL sample was injected into the PTV in direct mode, with programmed temperature set to match the column. The column used to separate analytes was an RTX-Biodiesel column, 15 m long, 0.32 mm ID, and 0.1 µm film thickness (Restek, Bellefont, PA). The oven temperature was set to 60°C for 1 minute, followed by an increase of 10°C min^{-1} to 370°C for 6 minutes. The FID was set to 370 °C. GCsolution postrun 2.3 (Shimadzu) was used for lipid peak integration.

3. Results and Discussion

3.1. Growth of Algae on Produced Water

Original efforts on this project were aimed to prove that microalgae could grow on produced water. After centrifuging the produced water to remove visible black

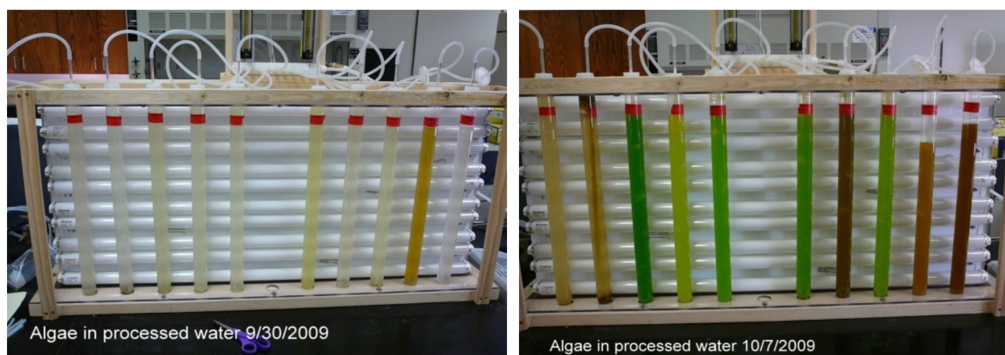

Figure 2.1. Photographs of algal strains at beginning (left) and end (right) of growth on produced water.

Table 2.1. Dry weight and TAG results of initial strains on produced water.

Strain	**Dry Weight (g L^{-1})**	**% TAG Content**
A. coffeaformis	1.80	10%
P. tricornutum	1.40	1%
C. gracilis	1.32	3%
BA116	2.22	1%
BA117	1.84	1%
BA050	2.42	2%
BA118	0.48	12%

hydrocarbons, ten strains were selected as inoculum to 450-mL tubes. Sodium nitrate was added shortly after inoculation, followed by potassium phosphate addition. As seen above in Figure 2.1, growth was achieved on produced water for all strains tested. Neutral lipids (TAG) were analysed and reported as a percent of the dry weight for seven of the strains and are presented in the table below.

Table 2.1 shows that several strains grew successfully on produced water, with few strains producing significant amounts of TAG. With the premise that several strains of algae are capable of growing and producing lipid on produced water, efforts to optimize growth and lipid production parameters became the new focus.

3.2. Elemental Analysis of Produced Water

In order to hypothesize supplementation of produced water to optimize it for algal growth, an elemental analysis was performed. The elemental composition is presented below in Table 2.2.

Aside from carbon and water, the next most important elements to a phototrophic organism are nitrogen and phosphorus (Shuler and Kargi, 2002). The above nutrient analysis shows that produced water already contains acceptable concentrations of many

Table 2.2. Compositional analysis of produced water.

Element	**mg L^{-1}**
Nitrate-N	29.4
Chloride	83500
Aluminum	<0.12
Arsenic	0.06
Boron	3.23
Calcium	590
Cadmium	<0.001
Cobalt	<0.005
Chromium	0.01
Copper	<0.008
Iron	0.118
Potassium	98.8
Magnesium	66.3
Manganese	0.59
Molybdenum	<0.15
Sodium	7235
Nickel	<0.003
Phosphorus	6.24
Lead	<0.03
Sulfur	321
Selenium	<0.04
Silica	34.7
Strontium	27.7
Zinc	<0.005
Carbonate/Bicarbonate	19.9
pH	8.12

elements, but nitrate and phosphorus were both found to be much lower than expected for growth. This is why nitrate and phosphate were added to the initial experiment.

Knowing that several strains had the capacity to grow on produced water with additional nitrate and phosphate addition, the next attempts focused on optimizing the precise amount of these 2 nutrients for maximal growth and lipid production.

3.3. Determination of N and P Supplementation for Lipid Production in A. coffeaformis

Phosphate. Because *A. coffeaformis* yielded high TAG content and acceptable dry weight, original studies focused on the performance of this strain for experimentation. Phosphate concentration was first optimized using various concentrations of added potassium phosphate, ranging from no addition to addition 230 mg L^{-1} (approaching the BS recipe which contains 250 mg L^{-1} potassium phosphate). Sodium nitrate (150 mg L^{-1}) was added to match that of the BS recipe. The results from the experiment are shown below in Figure 2.2. As shown in Figure 2.2b, the highest lipid production occurred when phosphate concentration was lowest, with no addition of phosphate. Algal growth (Figure 2.2a) was greatest at the highest phosphate concentration, and no significant difference was found in biomass production at all other concentrations. It was concluded that algae could produce sufficient lipid and biomass without the addition of phosphate.

Nitrate. Using the above finding that addition of phosphate stimulates no advantage in the biofuels objective, trials proceeded to optimize the concentration of

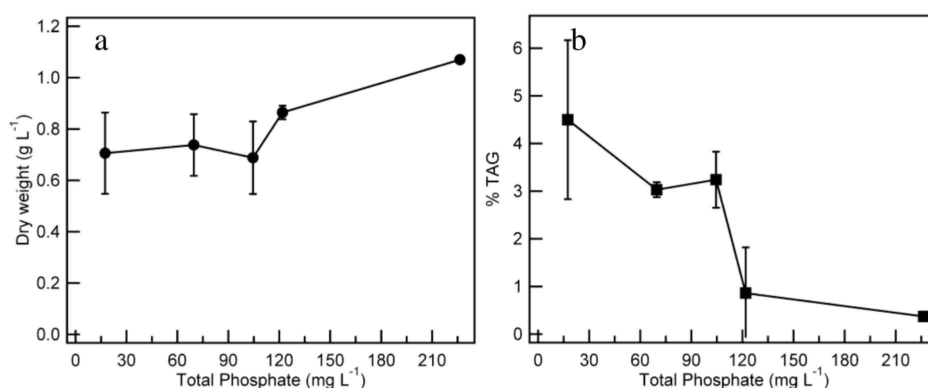

Figure 2.2. Results of growing *A. coffeaformis* on produced water with supplementation of various concentrations of phosphate. (a) Dry weight of cells increased when addition of phosphate exceeded 105 mg L^{-1}. (b) Percent of the dry mass extracted as TAG was found to decrease as phosphate concentration increased.

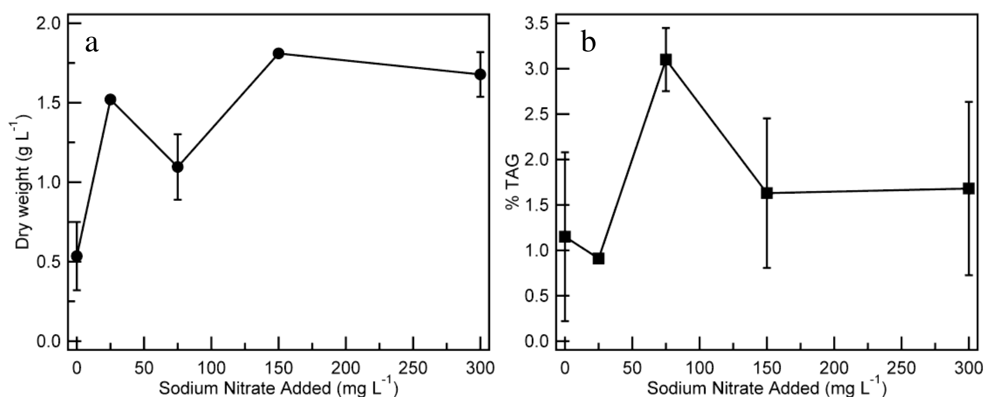

Figure 2.3. Results of growing *A. coffeaformis* on produced water with supplementation of various concentrations of nitrate. (a) Dry weight of cells vs. concentration of supplemented sodium nitrate. (b) TAG content as a percent of the dry weight of the cell vs. concentration of supplemented sodium nitrate.

nitrate addition for *A. coffeaformis* growing on produced water. Sodium nitrate was supplemented at 0, 25, 75, 150, and 300 mg L^{-1}.

As shown above, neutral lipid formation (Figure 2.3b) was highest when 75 mg L^{-1} sodium nitrate was supplemented, yet still high when 150 mg L^{-1} and 300 mg L^{-1} nitrate was added. It appeared that adding less than 75 mg L^{-1} nitrate was insufficient to trigger lipid formation, as they did not contain as high amounts of neutral lipids nor did they grow much biomass (Figure 2.3a). The highest dry weight was achieved when 150 mg L^{-1} nitrate was added to the produced water. Because biomass production was highest at this concentration and a reasonable concentration of neutral lipids was formed, 150 mg L^{-1} (1.8 mM) sodium nitrate was selected as the amount to add to produced water as an algal medium.

3.4. Additional Strain Selection

Having more autonomy in the laboratory and with an apparatus to design and perform personal experiments, it was possible to once again look at the results from the first

experiment of ten strains grown on produced water and reevaluate strain selection. *A. coffeaformis* did perform well, but working with this strain presented difficulties—for example, its tendency to clump on the surfaces of the growth vessel (exhibiting benthic growth) during early stages of growth can impact the gas bubbling consistently from tube to tube. Thus, it becomes difficult to draw definite conclusions from experiments knowing that some conditions tested had unmeasured variables. One more attempt was made to identify a strain easy to observe experimentally that could produce high yield of lipids and biomass, and whether that strain required addition of phosphate to do so. The three strains *A. coffeaformis*, BA118, and *C. gracilis* were used and were tested with and without phosphate addition.

As seen in Figure 2.4a, all organisms tested exhibited growth on produced water, although some of the *A. coffeaformis* and BA118 tubes did grow benthically during initial growth stages which obstructed air flow until corrected. *C. gracilis* reported the darkest average optical density throughout the experiment (data not shown), both with and

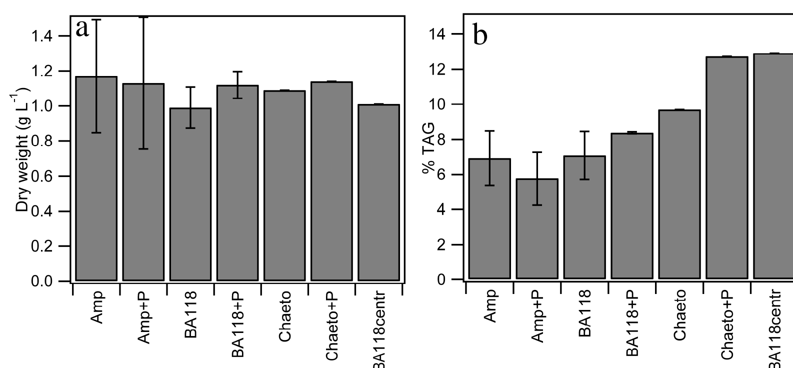

Figure 2.4. Bar graph representing the growth and lipid production of three algal strains on produced water with and without phosphate addition. Amp = *A. coffeaformis*, Chaeto = *C. gracilis*, and centr = centrifuged. The effect of additional centrifugation on the growth of BA118 is also shown. (a) Dry weight and (b) percent of the dry weight extracted as TAG of each sample is shown.

without phosphate addition. *C. gracilis* also reported the highest average dry weight of 1.45 g L^{-1} when phosphate was added. The sample of BA118 grown on centrifuged produced water without phosphate addition had the highest reported percent TAG content (Figure 2.4b), with 12.9% TAG. *C. gracilis* with phosphate addition was close with 12.7% TAG.

Addition of phosphate did not have a uniform effect on the different organisms tested. Both dry weight and TAG content in *A. coffeaformis* actually were higher when no phosphate was added. However, the opposite was seen in BA118, with a slight increase in dry weight and TAG content with phosphate addition. Although samples in *C. gracilis* were not duplicated, it did appear that phosphate addition increased both growth and lipid production.

Figure 2.5 shows data of the total TAG concentration in g L^{-1}, a combination of the percent TAG content and the dry weight. As shown, *C. gracilis* was the organism that produced the most TAG, and produced even more when phosphate is supplemented to the medium. Also shown is the resulting increase in TAG in BA118 when produced

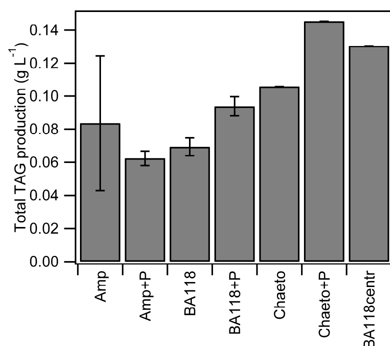

Figure 2.5. Total neutral lipid production of three algal strains on produced water with or without phosphate addition. Calculated from dry weight and % TAG of each sample. Amp = *A. coffeaformis*, Chaeto = *C. gracilis*, and centr = centrifuged.

water is additionally centrifuged. Extra care to remove hydrocarbons increases the TAG accumulation in the biomass.

3.5. Optimization of N and P supplementation in C. gracilis

Phosphate. With proof that *C. gracilis* can grow well and produce lipids in produced water medium, efforts to optimize the nutrients necessary for maximal biomass and lipid productivities within this organism began with understanding the effect of supplementing essential nutrients (i.e. phosphate and nitrate) and comparing that with a baseline media recipe. Although some previous experiments determined that phosphate addition was not necessary in *A. coffeaformis*, only two samples of *C. gracilis* were used to detect the effect of phosphate addition and no experiment compared the performance of produced water against standard lab media at similar phosphate concentrations. In order to determine the effect of using produced water at any phosphate concentration, and which would produce maximal growth and lipid production, this experiment was necessary. Dibasic potassium phosphate was supplemented at 0, 90, and 175 mg L^{-1}, with additional supplementation of 150 mg L^{-1} sodium nitrate.

As shown in Figure 2.6a, *C. gracilis* observed higher optical densities on produced water than BS media. Dry weight (Figure 2.6b) from any given phosphate concentration was also always higher in produced water than BS, and increased in both media as phosphate concentration increased. Neutral lipid content (Figure 2.6c) in BS increased as phosphate concentration increased, whereas in produced water it was highest with 90 mg L^{-1} phosphate, though not much lower with no phosphate addition. Lipid

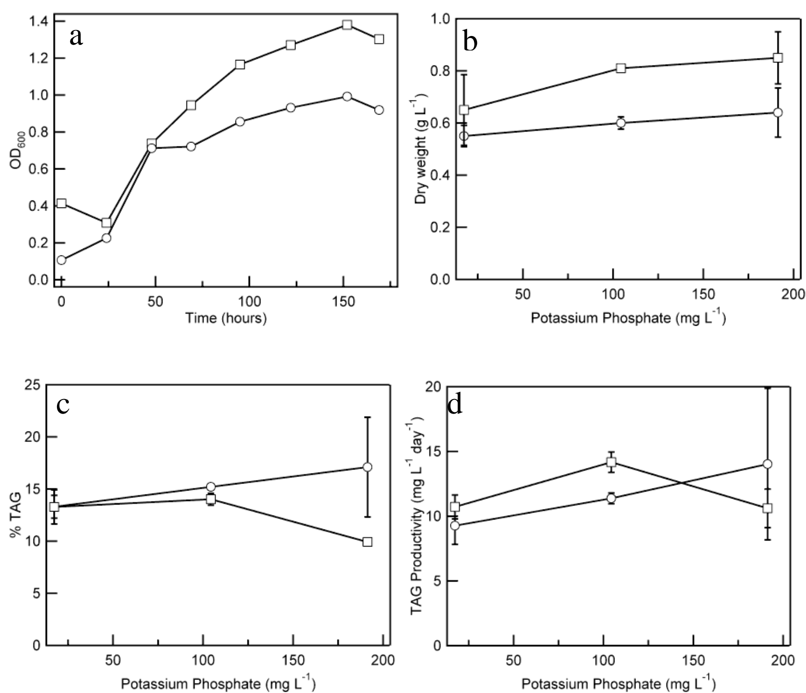

Figure 2.6. Results of growing *C. gracilis* at various potassium phosphate concentrations within produced water (□) and BS media (○). (a) Average optical density measurements of all produced water and BS cultures taken at 600 nm throughout the batch growth. (b) Harvested dry weight of cells at each phosphate concentration. (c) Neutral lipid content, as percent of the cell dry weight, vs. phosphate concentration. (d) TAG productivity vs. phosphate concentration.

productivity, which takes into account both biomass production and lipid content (Figure 2.6d), was seen highest when grown on produced water with 90 mg L^{-1} phosphate, at an average of 14.2 mg TAG L^{-1} day^{-1}. For BS, lipid productivity increased as phosphate concentration increased; in produced water it seemed optimized at 105 mg L^{-1} potassium phosphate. These results show that produced water is a sufficient growth medium for *C. gracilis* and can outperform BS in growth and lipid production. It also shows that phosphate addition can increase both lipid and growth productivities. However, sufficient growth can be achieved without phosphate addition if production costs are desired to be kept minimal.

Nitrate. Having understood the effect of phosphate addition to *C. gracilis* in two different nutrient media environments, it became necessary to also optimize the nitrate concentration in solution. Similar to the above mentioned experiment, produced water supplemented with three different concentrations of sodium nitrate (75, 150, and 300 mg L^{-1}, each run as duplicates) were compared to BS at the same sodium nitrate concentrations as growth media. This would show both how produced water compares as a growth medium for *C. gracilis* and how much nitrate is necessary for optimal growth and lipid production.

As shown in the Figure 2.7, growth of *C. gracilis* in produced water medium exceeded that of BS, as measures both by optical density (Figure 2.7a) throughout the

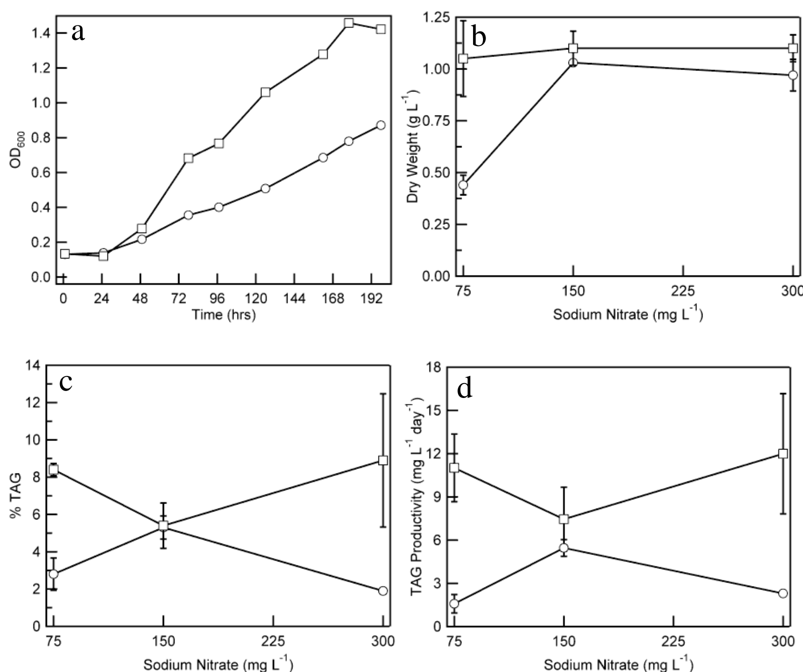

Figure 2.7. Results of growing *C. gracilis* at various sodium nitrate concentrations within produced water (□) and BS media (○). (a) Average optical density measurements of all produced water and BS cultures taken at 600 nm throughout the batch growth. (b) Harvested dry weight of cells at each nitrate concentration. (c) Neutral lipid content, as percent of the cell dry weight, vs. nitrate concentration. (d) TAG productivity vs. nitrate concentration.

growth and final dry weight (Figure 2.7b). It is also seen from these graphs that adding only 75 mg L^{-1} nitrate did not produce as much growth as the other two higher nitrate concentrations. In produced water, the dry weight was equal for 150 mg L^{-1} and 300 mg L^{-1} nitrate, whereas in BS dry weight actually decreased slightly in the higher 300 mg L^{-1} nitrate media. Lipid content (Figure 2.7c) of cells grown in produced water was also higher than in BS. BS seemed to optimize lipid production at 150 mg L^{-1} nitrate.

Although produced water seemed to show the opposite trend, with higher average lipid content at 75 mg L^{-1} and 300 mg L^{-1} nitrate than at 150 mg L^{-1} nitrate, the margin of error within these readings leads one to believe that lipid production can occur at all concentrations of sodium nitrate added. The above graph showing TAG productivity similarly shows that growth and lipid production of *C. gracilis* in produced water exceed that in Broad Seawater, and that nitrate addition was optimized in BS at 150 mg L^{-1} and inconclusive for produced water given the margin of error.

3.6. Effect of Nutrient Spiking

First attempt. From the previous two experiments, it was determined that *C. gracilis* was capable of producing lipid from growth on produced water better than BS with only addition of 150 mg L^{-1} sodium nitrate. Research has shown that lipid production is triggered within the algal cell in low nitrogen solutions, but that some minimal level of nitrogen is necessary for proper growth (Guschina and Harwood, 2006) and that a proper C:N:P ratio will yield greatest lipid productivity in algae (Redfield, 1934). It was hypothesized that growing algae on the optimized nitrate concentration

until growth reached a peak and then spiking the medium with extra nutrients could induce even greater cell growth and lipid production.

Originally, twelve cultures were inoculated to be supplemented with additional nutrients at the end of the growth. Soon into the growth, however, it became apparent that nine cultures were different from the remaining three, and that the cause was differing ages of inoculum cells. The experiment continued, with the intent to treat the nine cultures, which were spiked with various micronutrients including iron, silicate, trace metals, and phosphate, separately from the three, spiked with nitrate only.

Although it can be difficult to extrapolate definite conclusions from the data in Table 2.3 given the errors in experimental design, some assumptions can be made which can be useful in designing further experiments. Tubes 10-12 show that spiking with nitrate at the end of a growth can increase both growth (as seen in the increase in optical density versus the control) and lipid production, doubled from 15% to 31% when 50 mg L^{-1} sodium nitrate was spiked. Adding iron and silicate of all other nutrients also seemed

Table 2.3. Results from first spike experiment. Includes how much the optical density increased from the day following the nutrient spike, the total number of days grown, the amount of cellular TAG as percent of the dry weight, and the dry weight of the cell.

Tube #	**Spiked with**	**% Increase OD after spike**	**Days grown**	**% TAG**	**Dry weight ($g L^{-1}$)**
1	0.6 mM PO_4^{3-}	68%	21	8.2%	1.11
2	0.6 mM PO_4^{3-}	75%	21	9.3%	1.12
3	1.2 mL trace metals	40%	20	9.0%	1.05
4	1.2 mL trace metals	48%	21	9.6%	0.96
5	17.3 M SiO_3^{2-}	68%	21	17.8%	1.26
6	17.3 M SiO_3^{2-}	57%	21	20.3%	1.12
7	1.4 M Fe	72%	21	24.8%	1.34
8	1.4 M Fe	94%	21	23.8%	1.42
9	all of above	47%	20	18.8%	1.09
10	1.8 mM NO_3^-	47%	16	26.1%	1.32
11	None	0%	12	15.3%	1.04
12	0.6 mM NO_3^-	30%	16	31.5%	1.51

to encourage growth and lipid accumulation, though there is no control to compare with. All that is shown is that once the tube was spiked with the nutrient, the final optical density was, in the case of tube 8 spiked with iron, 94% greater than just before the spike. The neutral lipid content also reached nearly 24%, which is much higher than about 9% in tubes spiked with phosphate or trace metals. Clearly, further explanation is necessary to confirm any further conclusions.

Second attempt. From the first spike experiment, it was shown that spiking with 50 mg L^{-1} and 150 mg L^{-1} nitrate worked well to increase dry weight and TAG content. The effect of spiking with phosphate remained undetermined due to a lack of controls, but the importance of the C:N:P ratio drove the need to understand the effect of spiking primarily with phosphate and nitrate. Using these preliminary results, it was decided to use 75 mg L^{-1} sodium nitrate and 90 mg L^{-1} potassium phosphate as a final spike concentration for the next experiment. The effect of spiking cultures with either nitrate or phosphate, or both, was compared against the controls of unspiked produced water and BS. As shown in Table 2.4, adding phosphate alone did not improve optical density or

Table 2.4. Results from second spike experiment. Includes the averages (and standard deviations) of total number of days grown, the dry weight of each sample, how much the optical density increased from the day following the nutrient spike expressed as a percent, the amount of cellular TAG as a percent of the dry weight, the FAME content as a percent of the cell dry weight, and the productivity of FAME.

Media + spiked nutrient	**Days grown**	**Dry weight (g L^{-1})**	**% Increase in OD after spike**	**% TAG**	**% FAME**	**FAME Productivity (mg L^{-1} day^{-1})**
BS	13	0.88	1.7	18.0	24.9	15.7
PW	18	1.35	5.3	23.9	30.6	21.7
PW+N	19.5 (±2.1)	1.50 (±0.19)	16.4 (±6.5)	23.0 (±0.1)	27.7 (±1.4)	21.2 (±0.6)
PW+P	17 (±1.4)	1.49 (±0.37)	1.79 (±1.7)	19.1 (±2.7)	22.1 (±3.3)	19.2 (±3.4)
PW+N+P	18 (±0)	1.45 (±0.11)	0.6%	23.1 (±1.3)	25.9 (±0.7)	20.9 (±1.0)

lipid content as compared to both controls, although one tube was harvested much earlier than most, at day 15, had the highest reported lipid productivity. Spiking with nitrate did not increase the lipid productivity although it improved the optical density. It is likely that the excess nitrogen was used to synthesize chlorophyll within the cell, which would cause an increase in optical density without affecting the dry weight or lipid accumulation much (Li et al., 2008). Unless the biodiesel is purified, such chlorophyll could cause damage to a diesel engine. Spiking with both nitrate and phosphate did not present any advantages either, to either growth or lipid productivity. Thus it was concluded that nutrient spiking is unnecessary for optimal lipid production.

3.7. Growth of USU080

A new green alga organism was identified within the laboratory, isolated from the Great Salt Lake, and seen to accumulate high amounts of lipid and grow to greater densities than *Chaetoceros* species. USU080 was tested for its ability to grow and produce lipids on produced water supplemented with sodium nitrate.

According to Table 2.5, it was shown that not only can USU080 grow to high densities (2.5 g L^{-1} compared to 1.35 g L^{-1} *C. gracilis*), it also has superior lipid productivities. Containing 40% FAME and a lipid productivity rate of 40 mg L^{-1} day^{-1}, this organism outperformed any past sample of any organism yet tested on produced water. Table 2.5 contains the data from duplicate cultures.

Table 2.5. Values of average (and standard deviation) growth and lipid production of USU080 on produced water.

Days grown	**Dry Weight (g L^{-1})**	**% TAG**	**% Biodiesel**	**Productivity (mg L^{-1} day^{-1})**
20.5 (±0.7)	2.51 (±0.04)	13.85% (±0.04)	40.0 (±2.3)	48.9 (±2.0)

3.8. Natural Selection of USU080 "Wild" Strain

Other researchers in the biofuels team were conducting experiments in the greenhouses of the new Solar Innovations (Outdoor) Facility growing both USU080 and *Chaetoceros gracilis* in open raceways to see which could outperform the other from rapid growth and lipid productivity perspectives. The open nature of the raceway steps away from sterility and other precautions to maintain strain purity. At the end of the experiment, the lime green color of USU080 appeared morphed into a dark olive green and questions were raised as to its true identity. Termed the "USU080 wild strain," our team was interested in the performance of this organism, hypothesizing that if it was not truly USU080, then whatever strain did take over would grow even better. If one could find an organism that grew to the same or higher density in half the batch time, the productivity would be at least doubled.

"USU080 wild strain" was brought into the laboratory for testing in a closed environment in the tube vessels. The objective was to decrease batch growth time by eliminating cells from the tube once it reached a certain optical density, then refill the tube with fresh media, attempting to naturally select for fast growing cells within the population, eventually forming a culture of cells with accelerated growth time. The experiment was also designed to learn the advantage of using fresh media versus the spent media from which cells were just harvested, eventually understanding what the minimal nutrient requirement would be for a semicontinuous culture.

The USU080 wild strain was also tested for growth on produced water, which was done successfully in two different cultures. However, growth was much slower, taking 3 weeks to grow to the density that was achieved on BS in just 1 week. These

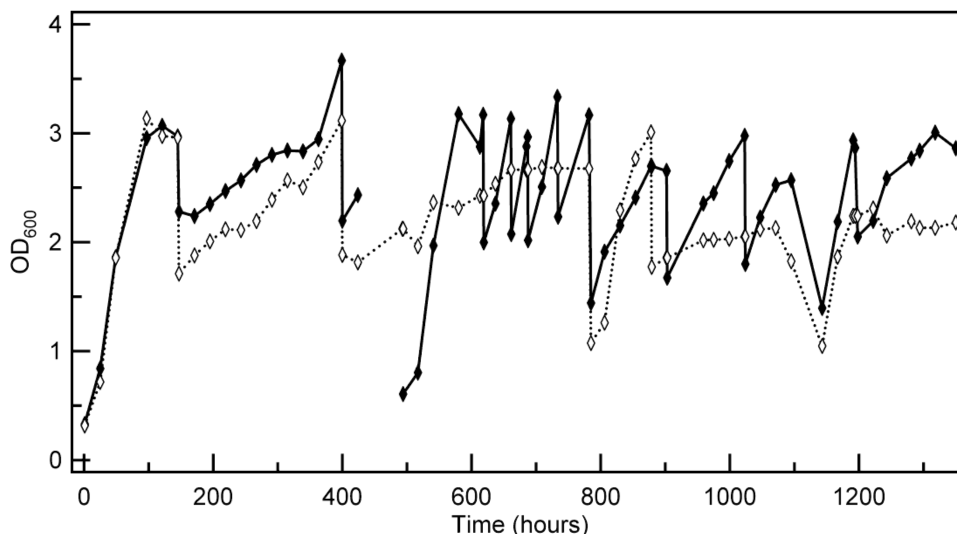

Figure 2.8. Optical density readings of USU080 “wild” strain receiving fresh (♦) and spent (◊) BS-Bicarb. media upon dilution. The culture receiving fresh media grew much faster than that receiving spent media. Optical density shown at 600 nm.

cultures were not selected for fast growth, just a proof-of-concept that this “wild” strain can also grow on produced water.

The USU080 wild strain was found to grow rapidly in tubes (Figure 2.8), being able to grow back thirty percent of its cell mass in just one day. The “wild strain” did not seem to behave any differently in tubes than the original USU080 strain, and the assumption that this strain was indeed different than USU080 was highly questioned. The “wild” strain was captured in an agar plate for long-term storage for future experiments, but interest was lost in the natural selection process of a fast-growing culture.

3.9. Effect of Pretreatment Using Activated Carbon

A visit to oil production facilities near Vernal, Utah, and the new Solar Innovations (outdoor) Facility equipped with various sizes of raceways sparked great interest in being

able to grow algae on produced water at a much larger scale than small tubes on a light bank. In order to make the idea of growing algae on produced water commercially viable, demonstrations were necessary for a proof of concept. One 220-L raceway was purchased for the exclusive use of produced water experiments. A new stock of produced water in three 55-gallon barrels was acquired to provide enough water to perform larger scaled experiments, but these barrels did not have 6 months to settle out hydrocarbons, and in 3 to 4 months the sunny, summer weather would turn too cold for an outdoor experiment. Early experiments showed that, at least in BA050, lipid production greatly increased when extra care was taken to remove hydrocarbons by centrifugation; however, centrifugation in a large, commercial process is too costly. For this raceway alone, the only means to centrifuge 200 L of produced water was a continuous centrifuge used for harvesting algae that would have been contaminated by the potent, black sludge left behind by the crude produced water. For these reasons, the need to find a more commercially viable means to remove hydrocarbons from large volumes of produced water without altering other nutrients such as phosphorus essential for microbial growth was essential before scaling up growth reactors.

Activated carbon was identified as a possible agent for hydrocarbon removal, however, its effect in removing important nutrients required for algal growth, specifically nitrogen, phosphorus, and silica, was uncertain. Experimentation needed to demonstrate

Table 2.6. Produced water nutrient concentrations before and after activated carbon filtration.

	Phosphorus (mg L^{-1})	Silica (mg L^{-1})	Nitrogen (mg L^{-1})
Before	4.97	44.2	21.1
After	1.90	31.7	14.0

the effectiveness of activated carbon as a hydrocarbon filter for produced water.

As shown in Table 2.6, each major nutrient was reduced by the activated carbon filter, with phosphorus being reduced the most. Without supplementing with phosphorus, this could become a problem. Experimentation was designed to see how growing USU080 on nitrogen-supplemented produced water passed through an activated carbon filter compared to produced water centrifuged for hydrocarbon removal.

As seen in Figure 2.9, the optical densities are nearly indistinguishable. The final dry weight of cells in centrifuged media was 1.48 g L^{-1} versus 1.39 g L^{-1} for the activated carbon filter. The centrifuged media yielded 29.0% FAME versus 36.2% FAME from activated carbon. Both methods of hydrocarbon removal proved effective in growing algae for lipid production, although activated carbon yielded more lipid than centrifugation. Activated carbon is an acceptable method of hydrocarbon removal for large scale algal biodiesel production.

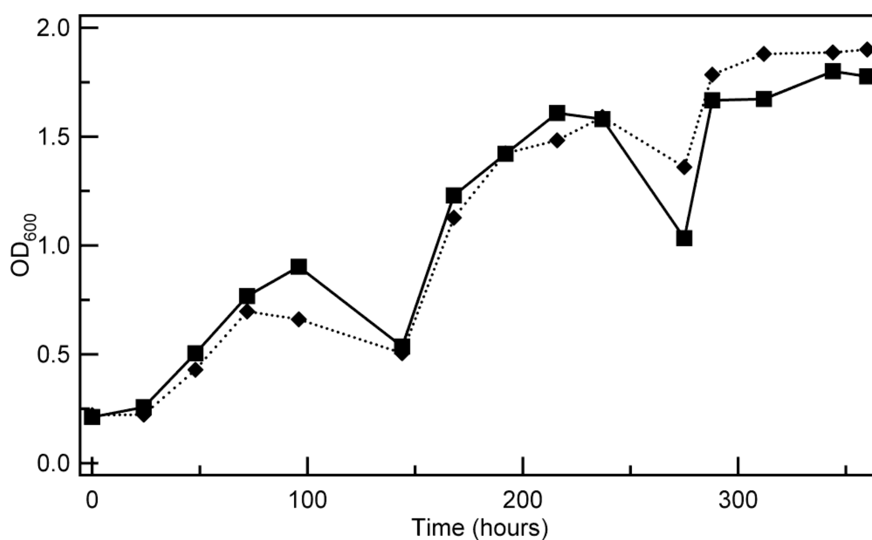

Figure 2.9. Optical density readings of USU080 on produced water with centrifuged (♦) and activated carbon filtration (■) pretreatment. Optical density shown at 600 nm.

3.10. Scale-up

Successful growth of algae in a raceway using produced water media was attempted twice. Because it takes over a month to produce sufficient inoculum for a 220-L raceway, *C. gracilis* was attempted first as USU080 had not yet been grown on produced water. This bag grew outside for one month which included one night of frost. Although the cells were not of ideal health, they still showed signs of life and were therefore used as inoculum into the raceway. The raceway appeared a murky gray color and had the typical petroleum smell of produced water, and after the inoculum was poured in gave off a light orange color. Over several days, growth did not improve and was determined dead.

USU080 was used in the second attempt at produced water raceway growth. Within the first couple of days following raceway inoculation, the measurements indicated cell death, as seen in a disappearance of the chlorophyll peak in the optical density, drop in dissolved oxygen, and undetectable fluorescence. The raceway was left unattended for several days until reports from a fellow worker came that the raceway was darkening in color. After three weeks, the raceway had shown successful growth and 1 L was harvested and analyzed for dry weight and FAME content.

Table 2.7. Results of USU080 grown in a 220-L raceway of produced water.

Total Volume (L)	202
Days grown	21
Dry Weight (g L^{-1})	1.21
Total dry weight (g)	243.4
% Biodiesel	9.13
Biodiesel (g L^{-1})	0.110
Productivity (mg L^{-1} day^{-1})	5.24

Aside from achieving growth in a 220-L raceway (see Table 2.7), USU080 was found to contain 9.13% FAME and had a dry weight of 1.2 g L^{-1} (biodiesel productivity rate of 5.24 mg FAME L^{-1} day^{-1}). These values are lower than seen in closed tube systems, but are quite acceptable for an open raceway without any of the same controls and sterility as found inside the laboratory. It was concluded that algal growth could indeed be achieved in larger scale systems using produced water supplemented only with nitrate.

3.11. Pre-publication Quality Experiments

Attempts to refine the data from previous experiments have each led to the need to include more controls and reoptimize parameters, along with the discovery of more detailed behavior of algae with produced water. This experiment was designed to confirm the following hypotheses:

1. Algae are capable of growing on produced water as well as or better than on laboratory media.
2. 150 mg L^{-1} is the only necessary supplement to produced water for optimal algal growth and lipid production.
3. Produced water can be remediated by the growth of microalgae.

Unexpectedly, the graphs in Figure 2.10a show that algal growth for both strains is optimized at 300 mg L^{-1} nitrate, not 150 mg L^{-1} as hypothesized. FAME content (Figure 2.10b) did seem optimized when 150 mg L^{-1} nitrate was added, but because of the better growth performance of *C. gracilis*, both medias showed highest lipid productivity rates (Figure 2.10c) when 300 mg L^{-1} nitrate was added. For both organisms, growth was

better on produced water than Broad Seawater at all nitrate concentrations. Although lipid content was slightly higher in USU080 grown on Broad Seawater verses produced water when nitrate was added, the outperformance of USU080 growth on produced water caused the lipid productivity rate to be much higher than that from Broad Seawater. For *C. gracilis*, produced water with 300 mg L^{-1} nitrate added generated the most growth, lipid content, and lipid productivity rate.

3.12. Remediation Studies

One of the original interests in produced water was bioremediation. Because it is such a large resource, with an estimated 21 billion barrels generated each year in the U.S. alone, produced water holds value as a replacement for fresh water and nutrient source in the algae-to-biofuels project. However, its heavy contamination prevents easy disposal, sometimes costing as much as $4 per barrel (Clark and Veil, 2009). Algae have been used in wastewater treatment facilities for the very purpose of remediating water streams; their ability to absorb various heavy metals (Wilde and Benemann, 1993; Yu et al., 1999;

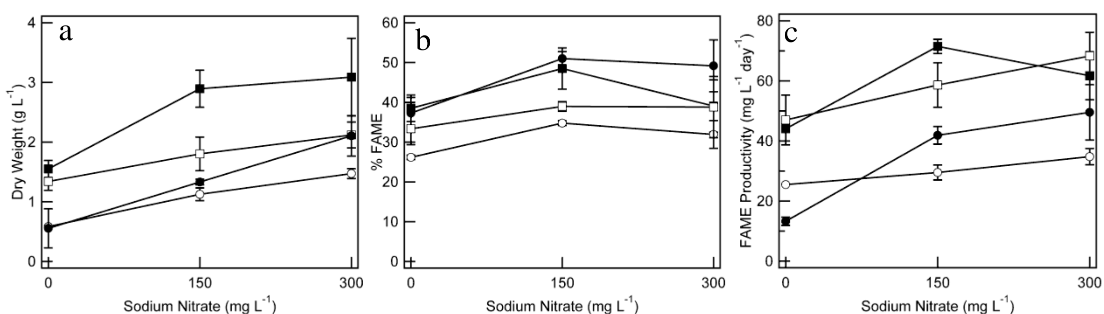

Figure 2.10. Results of pre-publication experiment, including growth and lipid production of *C. gracilis* (open symbols) and USU080 (filled symbols) in produced water (squares) and media (circles). (a) Final dry weight, (b) percent FAME content of the biomass, and (c) FAME productivity of cultures represented across three different nitrate concentrations. *C. gracilis* grown on BS and USU080 grown on BS Bicarb.

Davis et al., 2003) is well known. If it could be shown that algae have the capacity to reduce concentrations of harmful heavy metals and other substances to make it environmentally stable for municipal or irrigation disposal, the algae-to-biofuel project as

Table 2.8. Results from two samples of produced water sent for elemental analysis by USUAL. Includes actual concentrations within each sample, average, and standard deviation

Produced Water Analysis						
Element	Symbol	PW before 1	PW before 2	Average	Standard deviation	
Nitrogen	NO_3^-	<0.1	<0.1	<0.1		mg L^{-1}
Chloride	Cl^-	14900	15100	15000	141	mg L^{-1}
Carbonate	CO_3^{2-}	0.0	0.0	0	0.00	mg L^{-1}
Bicarbonate	HCO_3^-	15.1	18.0	16.55	2.05	mg L^{-1}
Aluminum	Al	<	<	<		mg L^{-1}
Arsenic	As	0.02	0.02	0.02	0.00	mg L^{-1}
Boron	B	3.14	3.14	3.14	0.00	mg L^{-1}
Barium	Ba	2.21	2.23	2.22	0.01	mg L^{-1}
Calcium	Ca	499	505	502	4.24	mg L^{-1}
Cadmium	Cd	<	<	<		mg L^{-1}
Cobalt	Co	<	<	<		mg L^{-1}
Chromium	Cr	<	0.008	0.007		mg L^{-1}
Copper	Cu	<	<	<		mg L^{-1}
Iron	Fe	1.04	1.07	1.055	0.02	mg L^{-1}
Potassium	K	136	131	133.5	3.54	mg L^{-1}
Magnesium	Mg	70.9	71.3	71.1	0.28	mg L^{-1}
Manganese	Mn	0.62	0.63	0.625	0.01	mg L^{-1}
Molybdenum	Mo	<	<	<		mg L^{-1}
Sodium	Na	10890	9001	9945.5	1336	mg L^{-1}
Nickel	Ni	<	0.003	0.003		mg L^{-1}
Phosphorus	P	6.26	6.31	6.285	0.04	mg L^{-1}
Lead	Pb	<	<	<		mg L^{-1}
Sulfur	S	349	351	350	1.41	mg L^{-1}
Selenium	Se	<	<	<		mg L^{-1}
Silicon	Si	36.6	36.3	36.45	0.21	mg L^{-1}
Strontium	Sr	30.1	30.2	30.15	0.07	mg L^{-1}
Zinc	Zn	<	<	<		mg L^{-1}
Total Dissolved Solids	TDS	$2.58*10^4$	$2.60*10^4$	$2.59*10^4$	141	ppm

well as the company owning the oil well would both be beneficiaries.

Variability of USUAL Readings. The following table shows the results from two identical untreated (other than settling of hydrocarbons with time) produced water samples submitted to USUAL, thus demonstrating the margin of error found within

Table 2.9. Best remediation values reported for *A. coffeaformis*, *C. gracilis*, and USU080. Reported as a percent of the original concentrations. ND=not detected, i.e. values prior to algal growth were already below detection.

	% Reduction		
	A. coffeaformis	*C. gracilis*	USU080
Nitrate-N	57%	61%	84%
Chloride	90%	15%	0%
Aluminum	ND	ND	ND
Arsenic	33%	33%	0%
Boron	6%	64%	9%
Barium	51%	34%	33%
Calcium	58%	60%	26%
Cadmium	ND	ND	ND
Cobalt	ND	ND	ND
Chromium	< 40%	ND	ND
Copper	ND	ND	ND
Iron	93%	33%	74%
Potassium	34%	67%	-11%
Magnesium	18%	60%	2%
Manganese	93%	65%	74%
Molybdenum	ND	ND	ND
Sodium	13%	27%	8%
Nickel	ND	ND	25%
Phosphorus	66%	72%	54%
Lead	ND	ND	ND
Sulfur	18%	54%	6%
Selenium	ND	ND	ND
Silica	99%	94%	12%
Strontium	33%	62%	16%
Zinc	ND	ND	ND
Bicarbonate (mmolc L^{-1})	69%	1%	51%
Carbonate (mmolc L^{-1})			15%

USUAL readings.

For most readings, the margin of error is quite small. Sodium reports the greatest deviation, although the salinity, represented by TDS, does not vary much between samples. It was concluded that USUAL contained some margin of error from sample to sample, but that variability was small.

Remediation Comparison of Three Algal Strains. Table 2.9 above presents the data reported of produced water before and after growth of three algal strains: *A. coffeaformis*, *C. gracilis*, and USU080. In cases when multiple samples of growth from the same organism were submitted, the highest remediation values are shown. Actual concentration values, as well as the detection limit of the method, are shown in addition to the percent of reduction from the original solution.

All organisms were fairly successful in decreasing the concentration of many heavy metals, such as iron, silica (in the case of diatoms), manganese, calcium, barium, and also many elements that are more potently harmful to the environment such as arsenic, phosphorus, and strontium. The diatoms *A. coffeaformis* and *C. gracilis* generally absorbed more from produced water than did USU080, although USU080 reduced the nitrate lower than the other two algal strains.

Water Quality Standards. To best judge the potential of any of these organisms to be used industrially to remediate produced water, one must understand the water quality standards to be reached for traditional wastewater streams. Under the Clean Water Act of 1972, the National Pollutant Discharge Elimination System (NPDES), which is managed by the Environmental Protection Agency (EPA), distributes permits for the discharge of all wastewaters. The standards are dictated for each specific industry, area/region, and in

the case of produced water, each individual well can be taken into consideration for the quality required for discharge. Individual states have the right to set regulations for their own regions, but the EPA maintains the right to oversee all state regulations and alter them as necessary. It can be difficult, therefore, to find one quality standard to reach when aiming to remediate produced water for more traditional forms of wastewater disposal. In the state of Utah, each estuary/reservoir is held to different standards, determined by the technology that is currently in place at each location. Across this state, major concerns arise only from TDS, total P, Se, dissolved oxygen, and algae biomass which cause eutrophication. Aside from state criteria, Ayers and Westcot (1985) have published certain standards for irrigation waters, some as regulatory values and other recommended for proper balance of agricultural practices. Below is a table of the various listed water quality standards with source, the concentrations of produced water before algal growth and the most favorable value of the medium after growth of USU080 and *C. gracilis*. Also listed is an indication of whether or not the water after algal growth met the listed water quality standard.

According to Table 2.10, produced water would meet water quality standards for most pollutants without algal remediation. Components that are above regulatory values include TDS, sodium, magnesium, calcium, phosphate, boron, and manganese. Algal growth decreased the concentrations of three pollutants that thereby met water quality standards: phosphate, calcium, and manganese, and in one case with *C. gracilis*, magnesium. However, TDS, sodium, and chloride are hopelessly well above the target concentrations, each of them being orders of magnitude away from meeting state and textbook irrigation regulations. Algae can absorb heavy metals well, but are incapable of

removing that much salt from a solution, making produced water too salty to meet these water quality standards.

From the above findings, it was concluded that produced water can be a useful means of substituting fresh water and most chemical nutrients to grow algae for lipid production; however, efforts to remediate the water for easier, less costly disposal is not advised due to the high salt content of produced water.

Influence of Tube Reactors on Remediation. The ICP results from the pre-

Table 2.10. Water quality standard concentrations and their respective concentrations in produced water before and after algal growth of USU080 and *C. gracilis*. A statement on whether or not the algal growth caused an unacceptable element to reach water quality standards is listed along with the source of the water standard. Irrigation water (IW) regulations and recommendations are listed according to Ayers and Westcot (1985).

Pollutant of concern	Reg. Target	Produced Water	After USU080	After *C.gracilis*	Algae met target?	Source
TDS (mg L^{-1})	1200	25900	25900	27400	no	state of Utah criterion
Na (meq L^{-1})	40	432	379	432	no	IW regulations
Cl (meq L^{-1})	30	423	451	460	no	IW regulations
Mg (meq L^{-1})	5	6.1	6.0	2.29	yes for *C.g.*	IW regulations
As (mg L^{-1})	0.1	0.02	0.02	0.02	already low	IW recommendations
Ca (meq L^{-1})	20	25	9	12	yes	IW regulations
CO_3 (meq L^{-1})	0.1	0	0	0		IW regulations
HCO_3 (meq L^{-1})	10	0.27	0.01	0.01	already low	IW regulations
NO_3-N (meq L^{-1})	10	0	0	0.06		IW regulations
PO_4 (mg L^{-1})	0.025	0.04	0	0	yes	Deer Creek reservoir TDML (UT)
B (mg L^{-1})	2	3.15	2.64	3.05	no	IW regulations
Al (mg L^{-1})	5	0				IW recommendations
Cd (mg L^{-1})	0.01	0				IW recommendations
Co (mg L^{-1})	0.05	0				IW recommendations
Cr (mg L^{-1})	0.1	0.008				IW recommendations
Cu (mg L^{-1})	0.2	0				IW recommendations
Fe (mg L^{-1})	5	1.05	0.07	0.2	already low	IW recommendations
Mn (mg L^{-1})	0.2	0.62	0.05	0.05	yes	IW recommendations
Mo (mg L^{-1})	0.01	0				IW recommendations
Ni (mg L^{-1})	0.2	0.003				IW recommendations
Pb (mg L^{-1})	5	0				IW recommendations
Se (µg L^{-1})	5	0				Ashley Valley TDML (UT)
Zn (mg L^{-1})	2	0				IW recommendations

publication experiment included two samples of produced water before growth (see above on variability of USUAL) and two after growth of *C. gracilis* and USU080. Also included in this batch was a sample of produced water that was treated the same as algal growths but without inoculation. This last of the five total samples revealed the effect of processing produced water in tube reactors on remediation. Results from ICP and other nutrient analysis of produced water before and after treatment were very insightful.

Table 2.11 below shows by what percentage each element or ion changed from the original produced water solution ((i.e. (before-after)/before). It shows the remediation of many components, from bicarbonate to iron, seems to come from the treatment *process* of bubbling in tubes and harvesting, not from the growth of algae.

Table 2.11. Results from pre-publication produced water experiment. Reported is the percent reduction (reduction in each component as a percent of the original concentration) seen in elemental concentrations of produced water as a result from algal growth (*C. gracilis* or USU080) or the tube treatment ("No algal treatment").

	Percent Reduction from Before		
Symbol	***C. gracilis***	**USU080**	**No algal treatment**
Cl^-	-10.0%	-6.7%	-31.3%
HCO_3^-	95.9%	95.9%	92.3%
As	0%	0%	0.0%
B	2.8%	15.9%	5.3%
Ba	43.2%	68.1%	23.4%
Ca	52.9%	63.0%	27.4%
Fe	79.1%	93.6%	87.3%
K	34.4%	12.7%	11.5%
Mg	-8.5%	-9.2%	-6.9%
Mn	92.3%	91.6%	50.0%
Na	0.0%	12.2%	3.6%
P	62.8%	72.8%	50.4%
S	10.9%	15.5%	7.6%
Si	93.3%	14.5%	-0.6%
Sr	31.5%	41.7%	22.0%

While an explanation as to where the elements are escaping in a closed system reactor is difficult to provide, it can be useful to understand that at least to some degree the "bioremediation" seen in tubes following algal growth could be owed to the process of growth.

The Influence of Inoculum Ingredients on Remediation. During preparation for publication-quality results, efforts were still being directed toward showing the nutrient-

Table 2.12. Percent reduction in elements from produced water, municipal wastewater, and Great Salt Lake water resulting from growth of *C. gracilis* and USU080.

	% Reduction					
	C. gracilis			**USU080**		
	Produced water	**Municipal wastewater**	**Great Salt Lake**	**Produced water**	**Municipal wastewater**	**Great Salt Lake**
PO_4^{3-}	97.4%			100.0%		
NO_3^-						
HCO_3^-	95.9%			95.9%		
Al		97.1%	96.6%		84.3%	92.1%
As	3.0%	50.0%	33.3%	6.0%		66.7%
B	2.8%	-1.9%	13.0%	15.9%	-7.5%	52.9%
Ba	43.2%	57.6%	52.7%	68.1%	60.6%	92.7%
Ca	52.9%	-0.3%	8.2%	63.0%	2.1%	87.0%
Cl	-10.0%			-6.7%		
Cr		33.3%	-900.0%		16.7%	-900.0%
Cu		-228.6%	-375.0%		-257.1%	-316.7%
Fe	79.1%	-61.2%	10.0%	93.6%	7.1%	82.3%
K	34.4%	-661.1%	-516.6%	12.7%	-738.3%	-390.1%
Li		21.1%	27.3%		26.3%	29.5%
Mg	-8.5%	-77.7%	-77.2%	-9.2%	-70.0%	-32.2%
Mn	92.3%	91.7%	96.3%	91.6%	50.0%	92.6%
Mo		0.0%	-25.0%		-50.0%	-50.0%
Na	0.0%	-1802.4%	-503.7%	12.2%	-2062.9%	-484.8%
Ni		-33.3%	-33.3%		0.0%	33.3%
P	62.8%	-120.4%	-5541.8%	72.8%	90.6%	53.3%
Pb		0.0%				
S	10.9%			15.5%		
Se						50.0%
Si	93.3%	97.4%	96.8%	14.5%	28.3%	70.1%
Sn						
Sr	31.5%	30.7%	30.8%	41.7%	42.2%	92.0%
V		-1100.0%	-300.0%		-1700.0%	-225.0%
Zn		72.7%	60.0%		90.9%	80.0%

absorptive capacity of microalgae to remediate wastewaters. In a similar fashion as shown in experiments above, supernatant from the growth of *C. gracilis* and USU080 on three different wastewaters, namely produced water, municipal wastewater, and Great Salt Lake water, were tested for compositional analysis and compared to composition before algal growth. Table 2.12 shows the percent reduction of each given nutrient.

Table 2.12 shows that many nutrients actually increased in concentration from the growth of algae. A mass balance of individual nutrients would dictate that this is impossible, and so an explanation of the results must validate the results seen. After careful consideration, it was determined that, especially for municipal wastewater and Great Salt Lake water, where nutrients prior to inoculation were already so low compared to the inoculation medium (BS), the addition of even 10% of the volume as not just algal cells but the nutrient-rich medium can vastly change the nutrient profile of the wastewater prior to absorption by algae. Thus, the true concentration of nutrients before the growth is undetermined. The negative percent reduction values in Table 2.12 reflect the introduction of these nutrients by BS from the inoculum. In future remediation studies, the method of inoculation should be altered so that the cells grown in lab media are spun to a pellet and resuspended in the wastewater, removing the introduction of lab media nutrients to the wastewater.

4. Conclusions

Produced water is an underutilized, abundant resource that has the potential of replacing the need of micronutrients and fresh water in algal growth. Eight strains of microalgae were shown to successfully grow and produce some amount of neutral lipids.

Growth and lipid production through nitrate and phosphate addition were initially optimized in the diatom organism *A. coffeaformis*. This organism showed greatest lipid productivity with no phosphate addition and 150 mg L^{-1} sodium nitrate addition. Because of difficulties with benthic properties during batch growth, other strains were reconsidered. Most consistently, the diatom strain *C. gracilis* and the green alga *Chlorella* sp. USU080 have been shown to have high lipid and growth productivities with only addition of 300 mg L^{-1} sodium nitrate. While potassium phosphate was considered as an additional nutrient supplement in *C. gracilis*, it was shown to be beneficial for *C. gracilis* but not necessary for growth and lipid production if production costs are desired to be kept minimal. Efforts to pretreat the produced water for hydrocarbon removal also increase growth and lipid productivities, whether by centrifugation, activated carbon filtration, or settling over months' time.

USU080 was shown to scale up to grow on a 220-L produced water raceway, and although efforts to scale up growth of *C. gracilis* on produced water were fruitless, care to avoid overnight outdoor frost could increase chances of success.

Finally, growth of microalgae did cause a reduction in several compositional elements of produced water, as expected because of the absorptive qualities exhibited by microalgae in other wastewater environments. Upon further analysis, however, the decrease of many elements seems to be at least partially due to the handling and growth procedures rather than the microalgae. When comparing the final concentration of elements against water quality standards, microalgae were only successful in reducing calcium, phosphate, manganese, and, for *C. gracilis* growth, magnesium to below acceptable concentration ranges. Most other elements were in an acceptable range prior

to microalgal growth. Total dissolved solids (TDS), sodium, and chlorine concentrations were found way above water quality standards and were hardly affected by microalgal growth. For this reason, bioremediation of produced water using microalgae should not be considered realistic until improvements in salinity removal are made. It should be noted that media recipes for microalgal growth contain similar salinity concentrations and therefore would share this problem of disposal as produced water. Efforts to commercialize this process must address the downstream processing of saline water disposal during scale up.

The effect of inoculum media contents on the wastewater elemental analysis was exploited in wastewaters with relatively low nutrient concentrations, such as municipal wastewater and Great Salt Lake water. Remediation in these low-nutrient wastewaters is unknown, as many elements were seen up to 55 times greater after algal growth than before. This theoretically impossible outcome is explained by the introduction of high concentration elements from the media accompanying inoculum, and could be avoided in the future with centrifugation of inoculum cells and resuspension in the intended nutrient medium for the batch growth.

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CHAPTER 3

THE STUDY OF USING PRODUCED WATER, MUNICIPAL WASTEWATER, AND ENVIRONMENTAL BRACKISH WATER IN THE PRODUCTION OF ALGAL BIODIESL USING TWO ALGAL SPECIES[a]

Abstract

Because of increased interest in renewable, carbon-neutral energy sources, processing biodiesel from microalgae has become the objective for many researchers and companies. To create the process on a large scale without drawing on already-taxed freshwater ecosystems, and to help mitigate production costs, natural waters including municipal, industrial, and agricultural wastewaters have been identified as alternate growth mediums. Presented here is a study of algal growth on three sources of underutilized wastewater, namely produced water from the petroleum and natural gas industry, municipal wastewater from a lagoon-style wastewater treatment facility (Logan City, UT), and water from Great Salt Lake. The algal growth on these and their relative performance as algal nutrient sources against two known media sources was evaluated. Batch growth in 1.2-L reactors using a diatom, *Chaetoceros gracilis*, and a *Chlorella* sp. USU080, on all wastewaters with addition of phosphorus and/or nitrogen was comparable to the two media. Biodiesel productivity was also comparable or remarkably higher in strains grown on wastewater versus media recipes, reaching up to 63% lipid content and 63.8 mg biodiesel L^{-1} day^{-1}. Relative costs for supplying nutrients for each nutrient source is also presented, showing that use of wastewaters is more cost effective than

media recipes and removes the need for a freshwater supply, especially when growth and biodiesel production are comparable.

1. Introduction

Due to an increase in energetic demands in past years and projected for years to come, and with a growing concern of the effect of fossil fuels on global greenhouse gases, the need for alternative, sustainable fuel sources has never been greater. To encourage the development of renewable fuels, the U.S. Federal Government passed the Energy Policy Act of 2005 and the Energy Independence and Security Act of 2007, which mandate that by 2012, all motor vehicle fuel sold in the U.S. must contain 15.2 billion gallons of renewable fuels, 1 billion of which must come from biomass-based diesel, or from feedstock other than corn starch. To help meet this demand for biomass-based diesel production, microalgae have been identified as a potential feedstock due to their capacity to produce high concentrations of lipids (transesterified into fatty acid methyl esters [FAME], or biodiesel) in land areas relatively small compared to food crops such as soybeans and corn, grow at high rates, tolerate land not suitable for agricultural crops, produce value-added products and byproducts, and produce energy in a proposed carbon-neutral scheme (Chisti, 2007, 2008; Hu et al., 2008).

Because microalgae require an aquatic medium for growth, concerns for the availability of sufficient freshwater must be addressed before scaling this process commercially. Algae-based biofuel has been found to require 14 times as much water as corn for the production of the same amount of energy (Clarens et al., 2010). It has also been stated that water availability will be one of the greatest challenges in the twenty-first

century and that lack of water will be one of the key factors in limiting development (WMO, 1997). By the year 2025, it is projected that 3.5 billion, or nearly half, of the human population will live in water-stressed river basin areas (Revenga, 2000). As freshwater supplies are already under strain to sustain human life, it is essential that biofuel production not rely on this limited resource. For these reasons, wastewaters are being considered not only as substitutes for freshwater in microalgal cultivation but also as a source of nutrients, eliminating the need for additional micronutrients.

There is an abundance of wastewater streams globally that could be tapped as a large scale growth medium resource if the microorganism can tolerate the wasteproducts contained therein. Produced water, for example, is a byproduct of the oil and natural gas drilling process contaminated with hydrocarbons, heavy metals, and other salts. An oil well will surface anywhere from 1 to 50 times the volume of produced water than the volume of oil. It is estimated that the United States alone generates 21 billion barrels (882 U.S. gallons) of produced water every year, which is currently disposed of at the high expense of well operators, and consequently, consumers (Clark and Veil, 2009). In addition to wastewater from industrial processes, each city also must handle millions of gallons of municipal waste each day, making it another source of wastewater available. These and countless other wastewater streams carry nutrients and contaminants alike that are currently considered undesirable and therefore underutilized (see Pittman et al., 2011).

It has long been known that algae can remove heavy metals and also remediate N- and P-rich wastewaters, thus preventing downstream eutrophication (Pittman et al., 2011). Many wastewater treatment facilities are utilizing algae for this purpose. Many

experiments have shown that wild algal cultures can grow on municipal (Oswald, 2003; Woertz et al., 2009; Wahlen et al., 2011), and agricultural (Mulbry et al., 2008; Woertz et al., 2009) wastewaters for the purpose of biodiesel production (see also Pittman et al., 2011). The properties of ocean (brackish environmental) water have also been used and investigated as a nutrient source (Takagi et al., 2006; Araujo et al., 2011). However, optimizing high lipid-yielding algal strains on wastewaters to mitigate biodiesel production costs and environmental concerns, and comparing algal performance in growth and biodiesel production against known media recipes has not previously been investigated. In addition, microalgae have never been shown to grow on any produced water source.

Presented here is a comparison of three different wastewaters (industrial, municipal, and brackish environmental water) against two media recipes as water and nutrient sources, and their effects on algal growth and lipid production in two marine strains, *Chaetoceros gracilis*, a diatom, and *Chlorella* sp. USU080, a green alga isolated from the Great Salt Lake.

2. Materials and Methods

2.1 Strains and Culture Conditions

A diatom, *C. gracilis*, and a green alga, USU080 were selected for these studies. *C. gracilis* was obtained from The Culture Collection of Algae at the University of Texas at Austin (UTEX, Austin, TX). USU080 was isolated from a tributary to Great Salt Lake. BLAST analysis of the 18s rRNA gene sequence, obtained by PCR (forward primer GTGCCAAGCAGCCGCGGTAA, reverse primer GGGCATCACAGACCTG),

matched closely with *Chlorella* strains. Stock cultures of each strain were maintained in 250 mL of media in 500 mL baffled flasks, rotating at 140 rpm, and illuminated from overhead by fluorescent lighting (180 μmol photons m^{-2} s^{-1}) on a 14:10 (light:dark) photoperiod. Both strains were maintained on Broad Seawater (BS) media that contained the following components per liter: NaCl (18 g), $NaHCO_3$ (1 g) (none for *C. gracilis* cultures), KCl (0.6), $MgSO_4•7H_2O$ (1.3 g), $CaCl_2•2H_2O$ (100 mg), K_2HPO_4 (250 mg), $Na_2SiO_3•9H_2O$ (70 mg) (none used for USU080 cultures), ferric ammonium citrate (5 mg). 1 mL trace metals were also added per L of media: H_3BO_3 (600 mg L^{-1}), $MnCl•4H_2O$ (250 mg L^{-1}), $ZnCl_2$ (20 mg L^{-1}), $CuCl_2•2H_2O$ (15 mg L^{-1}), $Na_2MoO_4•2H_2O$ (15 mg L^{-1}), $CoCl_2•6H_2O$ (15 mg L^{-1}), $NiCl_2•6H_2O$ (10 mg L^{-1}), V_2O_5 (2 mg L^{-1}), KBr (10 mg L^{-1}).

BS media was prepared according to the composition listed above and in Table 3, and is a modified version of L1 medium (Guillard and Hargraves, 1993). For *C. gracilis*, 70 mg L^{-1} sodium silicate was used and no bicarbonate was added. For USU080, BS was adjusted to 25 mg L^{-1} sodium silicate and 1 g L^{-1} bicarbonate was added. Table 1 also lists the elemental composition of BS, as prepared for USU080 growth.

Composition of Enriched Seawater Artificial Water (ESAW) is also listed in Table 1, according to the published recipe (Berges et al., 2001). Exceptions to this recipe are exclusion of Na_2SeO_3 and substitution for $CoSO_4$ and $MnSO_4$ for $CoCl_2•6H_2O$ and $MnCl_2•4H_2O$, respectively. Sodium nitrate was also adjusted to be equal to BS (300 mg L^{-1} $NaNO_3$).

2.2 Environmental Water Collection and Preparation

Environmental water samples representing municipal wastewater, brackish water, and produced water from the oil and gas industry were collected on site. The municipal wastewater sample was obtained on January 5, 2011, from a lagoon style wastewater treatment facility operated by Logan City (Utah). Brackish water was obtained from a point in the Great Salt Lake where one of its freshwater tributaries mixed with the saline water of the lake on January 19, 2011. Water from each of these two samples was centrifuged to remove suspended solids and was subsequently sterilized by autoclave. Produced water was obtained at a water reinjection site operated by Anadarko Petroleum Corporation near Vernal, Utah. The water reinjected at this site is collected from several wells and thus is a good representative sample of produced water from the Uintah Basin. Produced water was collected in 55-gallon barrels and was stored at room temperature. As needed, water was removed from the barrel, centrifuged to remove residual hydrocarbons, and sterilized by autoclave.

Each environmental water source was supplemented with sodium nitrate as a nitrogen source. For both Great Salt Lake water and produced water 300 mg L^{-1} $NaNO_3$ was added. Great Salt Lake water required additional supplementation with dibasic potassium phosphate to match the N:P ratio of supplemented produced water. Because the phosphate was found to be lower than other waters, only 180 mg L^{-1} sodium nitrate was supplemented so as not to create such a high N:P ratio for algal growth.

2.3 Elemental Analysis of Water

The elemental composition of each environmental water source was determined by ICP analysis prior to each growth (refer to Table 3.1). Elemental analysis of produced water was determined by Utah State University Analytical Laboratories (USUAL) using ICP analysis (Thermo Electron, Marietta, OH) for the following elements: Al, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Si, Sr, and Zn. The nitrate, phosphate, and chloride ion concentrations of produced water were also determined by USUAL (Quikchem 8000, Lachat, Loveland, CO). Utah Veterinary Diagnostics Laboratory (UVDL) determined the concentration of the following elements within both the municipal wastewater and brackish water samples by ICP analysis (ELAN 6000, Perkin Elmer, Waltham, MA): Ag, Al, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Li, K, Mg, Mn, Mo, Na, Ni, P, Pb, Sb, Se, Si, Sn, Sr, Tl, V, and Zn. UVDL also determined the nitrate concentration within these two using the Biospec-1601 (Shimadzu, Columbia, MD).

2.4. Experimental Setup

Experiments were conducted in 1.3-L glass tubes (800 mm x 50 mm diameter) illuminated from the side by fluorescent lighting (approximately 300 μmol photons m^{-2} s^{-1}). Each light bank can accommodate fourteen culture tubes; the inner twelve were used during experiments. Culture vessels were inoculated with a 10% inoculum in logarithmic growth. Each culture vessel was sparged from a single manifold with air supplemented with 1% CO_2. All growth conditions were performed in triplicate. Growth rates were

followed by measuring the optical density at 600 nm using the Cary 50 Bio UV spectrophotometer (Varian, Walnut Creek, CA).

2.5. Harvesting and Dry Weight Determination

Cultures were harvested by centrifugation when the culture entered stationary phase, either when optical density reached a plateau over time or disappearance of the chlorophyll peak in the culture spectrum. The elemental composition of the resulting supernatant from each culture was determined by ICP analysis. The harvested cell pellets were frozen at -80°C then lyophilized (Labconco FreeZone 4.5, Kansas City, MO) to obtain dry algal biomass.

Dry weights were determined by gravimetric analysis. A culture volume of 25 mL was passed through a pre-weighed 47-mm glass fiber filter (Whatman 1822-090) and rinsed with 0.5 mM ammonium formate. The filters were then dried in a 100°C oven, weighed, then heated in a 500°C oven to combust all the filtered organic content (i.e. algae) and leaving only the inorganic ash. To calculate the ash-free dry weight, the ash weight (after 500°C treatment) is subtracted from the total dry weight (after 100°C treatment). The final weight is then multiplied by a conversion factor to give the dry weight in terms of $g\ L^{-1}$.

2.6. Lipid Quantification

Lipid content of each sample was determined using the method described previously (Wahlen et al., 2011). Briefly, lipids contained in algal biomass were converted to fatty acid methyl esters (FAMEs) by direct transesterification which is performed by heating dry algal biomass (100 mg) with acidified methanol (1.8% H_2SO_4,

v/v, 2 mL) in a commercial scientific microwave (Discover S, CEM USA, Matthews, NC) at 90°C for 20 minutes. The FAME was extracted from methanol by the addition of chloroform followed by water to force a phase separation. The chloroform-lipid phase was removed into a 10-mL volumetric flask, and the remaining biomass was washed twice with 3 mL of chloroform to maximize the recovery of FAME. The final volume of the solution was brought up to 10 mL with chloroform and inverted for mixing. Samples of 1 mL were further diluted 1:2 or 1:4 depending on previous lipid content estimation, with addition of 10 μL octacosane as an internal standard. FAME content of each sample was then determined using a gas chromatograph (Model 2010, Shimadzu Scientific, Columbia, MD) equipped with a programmable temperature vaporizer (PTV) injector and a flame ionization detector (FID). Helium was used as the carrier gas and the flow was controlled in constant velocity mode set to a velocity of 50.0 cm sec^{-1}. Sample volumes of 1 μL were injected onto the PTV injector in direct mode. The temperature program for the PTV injector was identical to that used for the column. Analytes were separated using an RTX-Biodiesel column (15m, 0.32 mm ID, and 0.1 μm film thickness) (Restek, Bellefont, PA). The oven temperature was set to 60°C for 1 min and then increased at a rate of 10°C min^{-1} to 370°C for 6 min. FID detector was set to 370°C. The FID detector response was calibrated to FAME using methyl myristate (C14:0), methyl palmitoleate (C16:1), and methyl oleate (C18:1). Methyl ester standards were obtained as pure compounds (Nu-Chek Prep, Inc., Elysian MN) and were diluted with chloroform to obtain concentrations ranging from 0.1 mg mL^{-1} to 1 mg mL^{-1}. Sample and standard peaks were integrated using GCsolution postrun 2.3 (Shimadzu) for total FAME (biodiesel) concentration determination.

3. Results and Discussion

3.1. Elemental Composition of Natural Waters

As shown in Table 1, the three wastewater sources contain some phosphate, a necessary nutrient, salts, and various heavy metals—some essentially found as trace elements in media recipes, such as calcium and iron, and others that could be toxic to growth, such as arsenic.

Much has been said about the N:P molar ratio in marine microalgae media, which, according to the Redfield ratio should approximate 16:1, the same ratio as nutrients found in the deep ocean (Redfield, 1934). It should be noted, however, that many N:P ratios have been published, and that nitrogen limitation can trigger lipid accumulation in algae (Shifrin and Chisholm, 1981; Guschina and Harwood, 2006). Nitrate levels in all wastewaters were found to be below the level of detection prior to their supplementation with sodium nitrate (300 mg L^{-1} in produced water and Great Salt Lake water, 180 mg L^{-1} in municipal wastewater). Produced water contained 6.3 mg L^{-1} total phosphorus, and after nitrate addition had a 17.4:1 N:P ratio, close to the Redfield ratio of 16:1. Phosphate was found in lower concentrations in municipal wastewater (1.79 mg L^{-1}), giving a N:P ratio of 37:1 after nitrate addition. Phosphorus was also found to be very low (0.12 mg L^{-1}) in Great Salt Lake water prior to addition of 6.16 mg L^{-1} potassium phosphate, to equal that of Produced Water. Great Salt Lake water was further supplemented with 300 mg L^{-1} sodium nitrate, also creating a N:P ratio of 17.4:1.

Because marine species have adapted to function in high salt medium, salinity is an important parameter to consider when growing algae. In some diatoms, sodium has

Table 3.1. Nutrient content of three wastewaters and two lab medias.

Element/Ion	Produced Water[a] (mg L^{-1})	Municipal Wastewater[a] (mg L^{-1})	Great Salt Lake[a] (mg L^{-1})	Broad Seawater[a] (mg L^{-1})	Enriched Seawater Artificial Water[a,b] (mg L^{-1})
PO_4^{3-}	1.15			136.3	2.00
NO_3^-	<0.1	<5	<5	218.8	34.1
HCO_3^-	16.55			726.1[c]	126.3
Ag		<0.001	<0.001		
Al	<0.12	0.07	0.089		
As	0.02	0.002	0.003		
B	3.14	0.213	0.563	0.105	4.017
Ba	2.22	0.055	0.055		
Be		<0.001	<0.001		
Ca	502	34.596	41.656	27.26	365.5
Cd	<0.001	<0.001	<0.001		
Cl	15000			11251	16720
Co	<0.005	<0.001	<0.001	0.004	0.0004
Cr	0.008	0.006	0.001		
Cu	<0.008	0.014	0.012	0.006	
Fe	1.055	0.183	0.22	0.875	0.366
K	133.5	11.83	19.929	426.9	342.5
Li		0.019	0.139		
Mg	71.1	27.02	33.499	128.2	1001.6
Mn	0.625	0.012	0.027	0.069	0.133
Mo	<0.15	0.002	0.004	0.006	0.0006
Na	9946	90.871	335.697	7438	8526
Ni	0.003	0.006	0.003	0.0025	0.0004
P	6.285	1.79	0.122	44.49	0.625
Pb	<0.03	0.001	<0.001		
S	350			169.1	80.15
Sb		<0.001	<0.001		
Se	<0.04	0.001	0.002		
Si	36.45	5.422	9.257	2.473[c]	2.226
Sn		0.001	<0.001		
Sr	30.15	0.166	0.201		7.198
Tl		<0.001	<0.001		
V		0.001	0.044	0.0006	
Zn	<0.0058	0.011	0.005	0.0096	0.017
TDS[d]	25900				

[a]Values for Media Recipes are calculated. Wastewater values were determined experimentally.
[b]Berges et al., 2001
[c]Amount listed for USU080. In *C. gracilis*, [HCO_3^-] is 0 mg L^{-1} and [Si] is 6.294 mg L^{-1}.
[d]Total Dissolved Solids, a measurement of ionic strength, or salinity, of the water.

even been found as a symport molecule with silica for cell proliferation (Bhattacharyya and Volcani, 1980). The sodium concentrations found in the wastewaters examined as part of this study varied. Sodium concentrations in produced water (9946 mg L^{-1}) were comparable to BS (7438 mg L^{-1}) and ESAW (8526 mg L^{-1}). Sodium in Great Salt Lake water was significantly lower (336 mg L^{-1}) as this water sample was collected from the shore where a freshwater inlet mixed with the lake water. The municipal wastewater was found to have the lowest sodium concentration of the three waters (91 mg L^{-1}).

Silicon is an essential element for the growth of diatoms, as hydrated amorphous silica forms the frustrule, or diatom cell wall (Martin-Jezequel et al., 2000). Silicic acid is a common component of seawater with concentrations ranging from 2-180 μM (0.05-5 mg L^{-1}), averaging 70 μM (2 mg L^{-1}) globally. To reflect the importance of silicon to diatoms, Brzezinski determined the ratio of nitrogen and silicon present in the biomass of a *Chaetoceros* species to be in the range of 1.5-4:1 N:Si (Brzezinski, 1985). Produced water contained the most silicon (36.45 mg L^{-1}) which had a concentration 14 times greater than that of BS, and a N:Si ratio of 2.7:1, within the Brzezinski ratio. Although municipal wastewater had the least amount of silicon (5.42 mg L^{-1}), it still amounted to more than twice that of BS and provides a N:Si ratio of 10.9:1,

Produced water had a higher concentration of many elements such as arsenic, boron (although this was still lower than ESAW), calcium, iron, manganese, sulfur, and strontium. Some of these elements at high enough concentrations have been known to be toxic. One study has shown that arsenate, a species of arsenic, is absorbed into the algal plasmid membrane and causes membrane fluidization at concentrations at 7.5 mg L^{-1} or

higher (Tuan et al., 2008). Though produced water contains only 0.02 mg L^{-1}, it is still ten times as much as was found in either municipal wastewater or Great Salt Lake water.

Municipal wastewater and Great Salt Lake water both had slightly lower concentrations than either media recipe of iron, potassium, and magnesium. All three wastewaters had elevated concentrations of boron, relative to media recipe concentrations. Other elements in wastewaters were within the range of media recipe concentrations. Several of these trace elements have been found to play roles in algal metabolism; for example, iron and zinc have been found to aid in silica transport in diatoms (Martin-Jezequel et al., 2000). Zinc has also been found as a cofactor for utilization of phosphate in phosphate-poor environments in phytoplankton (Arrigo, 2005). Neither elevated nor depressed levels of trace elements in natural waters appeared to affect either growth or biodiesel production.

3.2. Algal Growth Achieved on Wastewaters vs. Media

The goal of these studies is to determine if the bulk of water required for microalgal cultivation could be provided by either underutilized water present naturally in the environment (Great Salt Lake) or wastewater resulting from human activity (produced water and municipal wastewater). As part of this assessment, the growth of two different strains, a diatom (*C. gracilis*) and a green alga (USU080), in the three wastewaters were compared to two laboratory medias. Growth was followed by measuring the optical density (OD) at 600 nm each day. Figure 1 shows the growth of *C. gracilis* in each wastewater as compared with the two media recipes.

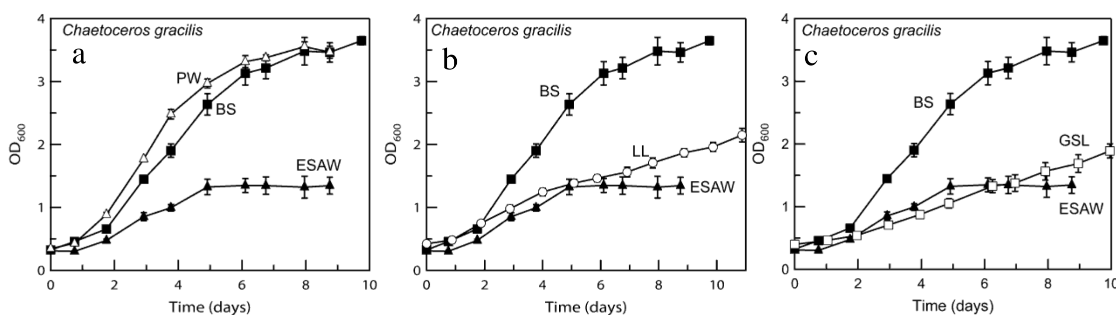

Figure 3.1. Growth of *C. gracilis* on (a) produced water, (b) municipal wastewater, and (c) Great Salt Lake water versus both media waters, measured as OD at 600 nm at various times in the batch process. Water types are signified by ■, Broad Seawater (BS); ▲, Enriched Seawater Artificial Water (ESAW); Δ, Produced Water (PW); ○, municipal wastewater (MW); and □, Great Salt Lake water (GSL).

Growth of *C. gracilis* on produced water (Figure 3.1a) followed closely to that on BS, although on most days growth on produced water measured slightly higher than BS media. ESAW was not an effective media for *C. gracilis* growth as growth on both BS and produced water supported substantially better growth. Growths on municipal water (Figure 3.1b) and Great Salt Lake water (Figure 3.1c) were slow and steady, similar to growth in ESAW media. Due to measurements in the full 300-900 nm daily spectra (data not shown), algal growth in these cultures was determined to have stopped, and were subsequently harvested sooner than ESAW and BS cultures. The slow growth observed in both municipal wastewater and Great Salt Lake water could be due to stress caused by their low salinity.

To further characterize the potential of various underutilized water sources to support microalgal growth, the growth of a green alga, *Chlorella* sp. USU080, was monitored in each wastewater and both lab medias. USU080 was chosen because of the different nutrient requirements that a green alga would have relative to a diatom; most notably their lack of a silicon requirement. The growth of USU080 in each wastewater

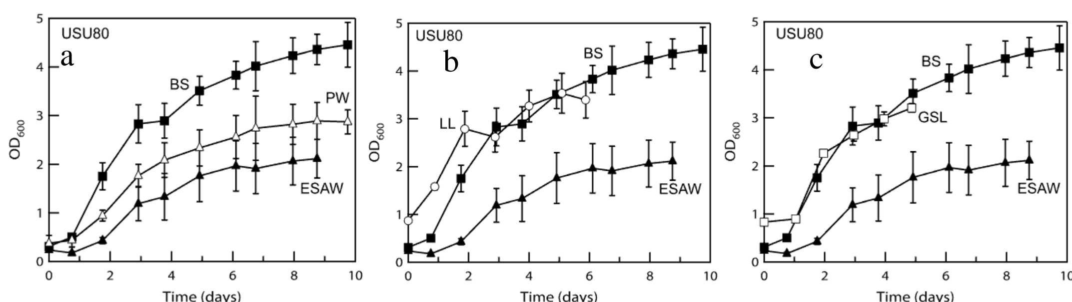

Figure 3.2. Growth of USU080 on (a) produced water, (b) municipal wastewater, and (c) Great Salt Lake water versus both media waters, measured as OD at 600 nm at various times in the batch process. Water types are signified by ■, Broad Seawater (BS); ▲, Enriched Seawater Artificial Water (ESAW); Δ, Produced Water (PW); ○, municipal wastewater (MW); and □, Great Salt Lake water (GSL).

and laboratory media (Figure 3.2) was followed in a similar manner to what was done for *C. gracilis*. Greater variability is generally observed in all growths of USU080 because of the organism's tendency to clump, which at times hinders the aeration in the growth vessel. This is reflected in larger standard deviations than what was obtained for *C. gracilis* OD measurements. Growth curves of USU080 on produced water lie between BS (higher) and ESAW (lower) (Figure 3.2a). A significant difference was observed between the growth curves obtained on BS and produced water, however produced water growth was within error of ESAW growth measurements. Growth of USU080 on municipal water (Figure 3.2b) was comparable to growth on BS with one distinct difference. The growth of USU080 on municipal wastewater began to decline after 6 days while BS culture continued to increase for more than 10 days. The growth of USU080 on Great Salt Lake water (Figure 3.2c) was found to be very similar to the municipal wastewater growths. Like the municipal wastewater growth, OD measurements of USU080 in Great Salt Lake water were nearly identical to BS growths and the culture also began to decline early (5 days) relative to BS (10+ days). Due to

culture stresses (i.e. salinity) that were reflected in the full 300-900nm daily spectra (data not shown), these samples were harvested after 5-6 days.

When considering the viability of using an environmental water source for both the culture medium and the source of nutrients, it is important to verify that the productivity of the algae in these conditions remains as good as in laboratory media. The final biomass yield and biomass productivity of both *C. gracilis* and USU080 on the various nutrient sources is listed in Table 3.2. We used the growth of a diatom and a green alga on two different laboratory medias as a benchmark of microalgae productivity for biofuels. *C. gracilis* achieved its highest biomass productivity (126 mg L^{-1} day^{-1}) when grown in BS and its lowest when cultured in ESAW (76 mg L^{-1} day^{-1}). The productivity achieved on produced water (118 mg L^{-1} day^{-1}) was similar to that of BS, while both the brackish water and the municipal wastewater had intermediate productivity values (88 mg L^{-1} day^{-1} and 82 mg L^{-1} day^{-1}, respectively). In similar fashion the biomass productivity of USU080 was highest among lab medias when grown on BSW (119 mg L day^{-1}) compared with ESAW (104 mg L^{-1} day^{-1}). The biomass productivity rates of USU080 were actually highest in the wastewater sources, with the Great Salt Lake biomass productivity (225 mg L^{-1} day^{-1}) more than twice that of ESAW (104 mg L^{-1} day^{-1}). Growth of USU080 was also strong on both produced water and municipal wastewater (129 mg L^{-1} day^{-1} and 159 mg L^{-1} day^{-1}, respectively). Thus it is evident that growth of both algal strains on these wastewaters is comparable or, in some cases, better than laboratory media sources.

3.3. Lipid Analysis

Nutrient environment plays a critical role in lipid formation in microalgae. In order to understand not just the effect of wastewaters on algal growth but also the lipid production, total lipids from each batch culture were quantified as FAME (biodiesel) (see Table 3.2). Cultures of *C. gracilis* showed greatest lipid quantities in municipal wastewater, producing 570 mg L^{-1} FAME. *C. gracilis* FAME yields from Great Salt Lake growths reached 408 mg L^{-1}, which was similar to that achieved when cultured on BS (422 mg L^{-1}). *C. gracilis* cultured from produced water yielded 191 mg L^{-1}, which although lower than other wastewaters was still greater than the yield of ESAW, at 109

Table 3.2. Biomass yield and productivity, biodiesel yield and productivity, and percent oil content found in *C. gracilis* and USU080 according to nutrient source.

Organism	Nutrient Source	Biomass Yield[b] (mg L^{-1})	Biomass Productivity[c] (mg L^{-1} day^{-1})	FAME Yield[d] (mg L^{-1})	FAME Productivity[e] (mg L^{-1} day^{-1})	% FAME in Biomass[f]
C. gracilis	Broad Seawater	1265 (±133)	126.5 (±2.1)	422 (±94)	37.7 (±10.3)	33.8 (±9.0)
	ESAW[a]	687 (±52)	76.3 (±5.8)	109 (±14)	10.5 (±1.4)	15.9 (±2.5)
	Produced Water	1065 (±42)	118.4 (±4.7)	191 (±22)	19.0 (±2.4)	17.9 (±1.4)
	Great Salt Lake	795 (±194)	87.6 (±5.4)	408 (±141)	44.3 (±8.1)	50.3 (±6.4)
	Municipal Wastewater	897 (±39)	81.6 (±3.5)	570 (±32)	52.9 (±2.9)	63.6 (±1.2)
USU080	Broad Seawater	1388 (±337)	119.1 (±5.3)	443 (±112)	33.3 (±1.4)	31.9 (±2.8)
	ESAW[a]	1096 (±309)	103.7 (±23.0)	361 (±163)	29.1 (±9.5)	31.8 (±6.3)
	Produced Water	1286 (±212)	128.5 (±21.8)	370 (±109)	30.2 (±5.6)	26.3 (±3.8)
	Great Salt Lake	1123 (±24)	224.5 (±4.9)	225 (±22)	45.1 (±4.3)	20.1 (±1.6)
	Municipal Wastewater	953 (±133)	158.9 (±22.2)	383 (±6)	68.8 (±9.3)	40.1 (±1.2)

[a]Enriched Seawater Artificial Water (Berges et al., 2001)
[b]Presented as Volatile Suspended Nonfilterable Solids in mg L^{-1}
[c]Presented as Biomass Yield per number of days grown in the batch cycle in mg L^{-1} day^{-1}
[d]Presented as total mg of all lipids converted to FAME during transesterification per L of batch culture
[e]Presented as FAME Yield per days grown in the batch cycle
[f]Percent of the Biomass found as FAME, as expressed as a percent of mg FAME over mg biomass in each sample

mg L^{-1}. The lipid productivity rates in *C. gracilis* followed the above patterns in FAME ESAW. The percent lipid content (as g FAME per g dry weight) was highest in municipal wastewater, where 63.6% of the dry weight reported as FAME. Growth in Great Salt Lake water also showed high lipid content at 50.3%, much higher than BS at 33.7% lipid. Produced water growths contained only 17.9% lipid, slightly greater than ESAW, which contained 15.9% lipid. Previous growths of *C. gracilis* on produced water under identical conditions have yielded as high as 39% FAME, demonstrating a higher capacity of this water to serve as a growth medium for lipid production.

USU080 cultures yielded greatest lipid content in BS (443 mg L^{-1}), although lipids from municipal wastewater (383 mg L^{-1}) and Produced Water (370 mg L^{-1}) were similar to ESAW (360 mg L^{-1}). USU080 growths on Great Salt Lake water produced the lowest amount of lipid of all USU080 growths (225 mg L^{-1}). Lipid productivity for USU080 was greatest when grown in municipal wastewater, reaching 68.8 mg L^{-1} day^{-1}, which was greatest for both organisms tested. The significantly shorter culture times of both municipal wastewater and Great Salt Lake (45.1 mg L^{-1} day^{-1}) grown USU080 cultures contributed to a higher lipid productivity value. However, in spite of the much shorter growth period, USU080 achieved the highest lipid content when grown on municipal wastewater (40.1% g FAME/g biomass). Lipid contents obtained with the two laboratory medias were both lower with ~32% (g FAME/g biomass), while produced water (26.3%) and GSL (20.1%) grown biomass had the lowest lipid contents. Previous growths of USU080 on produced water under identical conditions reached up to 39% FAME content, showing a greater capacity of this wastewater to foster lipid accumulation. The high lipid productivity achieved on municipal wastewater relative to

BS demonstrates the potential that using an underutilized water source has to provide both the water for growth as well as many of the needed nutrients.

Both strains exhibited high lipid productivity when grown on water obtained from Great Salt Lake and the local municipal wastewater treatment facility. The reason for this high rate of lipid productivity is unknown. It could be attributed to abiotic factors such as osmotic stress caused by the lower salinity of these two samples. *C. gracilis* is a marine species, while USU080 was isolated from Great Salt Lake, and the salinity of the water used for growth was nearly that of freshwater sources. Decreasing saline levels in *C. gracilis* has shown to increase lipid content (Araujo et al., 2011). The municipal wastewater sample obtained had a sodium content of 90 mg L^{-1}, while the sample taken from Great Salt Lake where a freshwater inlet entered the lake had slightly higher sodium content (335 mg L^{-1}). Because of the origin of both of these waters a biological influence could not be ruled out. Although both samples of each water source were autoclaved prior to use, organic carbon would most certainly be present. Some algal species have been shown to grow favorably on organic nitrogen sources (Berman and Chava, 1999) and vitamins (Provasoli and Pintner, 1953), which could be found in these wastewater sources. Further investigation is needed to explain the exact nature of the nutrient stress causing the great accumulation of lipids in these organisms.

3.4. Cost Analysis of Nutrient Sources

The macronutrients nitrogen and phosphorus represent are likely to be the costliest nutrients for microalgal biofuel production. The cost of other “micro” nutrients, however, is not insignificant. Table 3.3 presents the total cost of each nutrient in the two

Table 3.3. Nutrient cost analysis by nutrient source.

Nutrient Source	Ingredient	Amount (per 1000 L raceway)	Cost ($ per 1000 L)
Broad Seawater	Sodium Chloride	18 kg	73.8272[d]
	Magnesium Sulfate Heptahydrate	1.3 kg	10.1400[d]
	Sodium Bicarbonate	1 kg[a]	5.9194[a,e]
	Potassium Chloride	600 g	8.1432[d]
	Sodium Nitrate	300 g	9.3150[f]
	Potassium Phosphate Dibasic	250 g	5.9669[e]
	Calcium Chloride Dihydrate	100 g	2.2547[d]
	Sodium Silicate Nonahydrate	25 g[a]	0.7040[a,e]
	Ferric Ammonium Citrate	5 g	0.1869[e]
	Boric Acid	600 mg	0.0064[e]
	Manganese Chloride Tetrahydrate	250 mg	0.0295[e]
	Zinc Chloride Anhydrous	20 mg	0.0038[d]
	Copper Chloride Dihydrate	15 mg	0.0007[d]
	Sodium Molybdate Dihydrate	15 mg	0.0028[d]
	Cobalt Chloride Hexahydrate	15 mg	0.0020[e]
	Nickelous Chloride Hexahydrate	10 mg	0.0006[e]
	Potassium Bromide	10 mg	0.0004[e]
	Vanadium Pentoxide Anhydrous	2 mg	0.0003[d]
	Total		116.5151[b]
ESAW[c]	Sodium Chloride	21.2 kg	86.9520[d]
	Magnesium Chloride Hexahydrate	8.4 kg	63.9792[e]
	Sodium Sulfate	3.5 kg	35.1429[e]
	Calcium Chloride Dihydrate	1.3 kg	29.3111[d]
	Potassium Chloride	599 g	8.1297[d]
	Sodium Nitrate	300 g	9.3150[f]
	Sodium Bicarbonate	174 g	1.0300[e]
	Potassium Bromide	86 g	3.6140[e]
	Boric Acid	23 g	0.2453[e]
	Sodium Silicate Nonahydrate	23 g	3.0130[f]
	Strontium Chloride Hexahydrate	22 g	1.7503[e]
	Sodium-EDTA Dihydrate	5.5 g	0.2423[d]
	Sodium Phosphate monobasic	2.9 g	0.0615[e]
	Sodium Fluoride	2.8 g	0.0790[e]
	Iron Chloride Hexahydrate	1.8 g	0.0455[e]
	Manganese Chloride Tetrahydrate	480 mg	0.0566[e]
	Zinc Sulfate Heptahydrate	73 mg	0.0015[e]
	Nickelous Chloride Hexahydrate	1.5 mg	0.0001[e]
	Sodium Molybdate Dihydrate	1.5 mg	0.0003[e]
	Cobalt Chloride Hexahydrate	1.4 mg	0.0002[e]
	Thiamine-HCl	100 mg	0.0052[f]
	Biotin	1 mg	0.0326[e]
	B_{12}	2 mg	0.0049[e]
	Total		243.0121
Produced Water	Sodium Nitrate	300 g	9.3150[f]
Logan Lagoon	Sodium Nitrate	180 g	5.5890[f]
Great Salt Lake	Sodium Nitrate	300 g	9.3150[f]
	Potassium Phosphate Dibasic	19.3 g	0.4606[e]
	Total		9.7756

[a]Amount listed for USU080 growth. *C. gracilis* cultures contained 0 g L^{-1} sodium bicarbonate ($0 L^{-1}) and 70 g L^{-1} sodium silicate ($1.971 per 1000 L)

[b]Total cost of Broad Seawater for USU080 listed. Total cost for *C. gracilis* is $111.8629 per 1000 L

[c]Enriched Seawater Artificial Water (Berges et al., 2001)

[d]Prices quoted from Fisher Scientific, April 12, 2011

[e]Prices quoted from VWR, April 12, 2011

[f]Prices quoted from Sigma Aldrich, April 12, 2011

lab medias and cost of the nutrients added to the three wastewater sources to promote microalgal growth. The cost of preparing BS from laboratory grade chemicals for USU080 growth would be $116.52 per 1000 L raceway ($111.86 for *C. gracilis*). ESAW media is more than twice as expensive to prepare as BS at $243.01 per 1000-L raceway. This is contrasted by the low chemical cost of conditioning produced water to promote vibrant microalgal growth, requiring only the addition of sodium nitrate, at $9.32 per 1000-L raceway. The cost of developing municipal wastewater and Great Salt Lake water into microalgal growth medias was also low. Municipal wastewater required the addition of nitrate ($5.59 per 1000 L) while Great Salt Lake water required the addition of both nitrate and phosphorus ($9.78 per 1000 L). From this analysis, it is shown that the chemical cost of BS is 12.5 times the cost of using produced water and over 20 times more expensive than using municipal wastewater. Purchasing chemicals for ESAW can be 46 times the cost of using a wastewater such as from the municipal wastewater treatment facility.

There are some substitutions which could be made, though the effect on growth has not yet been determined. For example, using urea (Sigma Aldrich, quoted on April 12, 2011) as a nitrogen source instead of sodium nitrate brings the total cost of BS from $116.52 to $107.21 per 1000-L raceway, and similarly reduces the cost of all water sources. Further experimentation would be required to know if such substitutions would decrease biomass or lipid production within the organism.

4. Conclusions

Although nutrient composition of each nutrient source differs, algal growth achieved in all three wastewaters was within the range of media sources in both *C. gracilis* and USU080. Also, lipid production and productivity in wastewaters were also comparable to media sources, with noticeably high lipid productivity in both organisms on municipal wastewater and Great Salt Lake sources. From analyzing the cost of each nutrient source as followed in this experimental procedure, using wastewaters as a nutrient source for algal biodiesel production is much more cost effective (as much as 46 times less the expense) than purchasing chemicals for media, and eliminates the need for freshwater. Replacing media chemicals with wastewaters supplemented only with essential N and P nutrients proves economically and environmentally logical since similar biomass and lipid productivities can be achieved for a lower cost without the need of freshwater.

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CHAPTER 4

OTHER ALGAE EXPERIMENTS

Abstract

Microalgae have been proposed as a possible feedstock for biofuel production, but because of the high monetary and energetic costs of processing algae for biofuel production, attempts must be made to decrease production costs in every step of the process possible. This chapter addresses the importance of using the wastewater resources from oil/gas industry (produced water), municipal wastewater, dairy manufacturing, and potato manufacturing as growth media for microalgae. It was concluded that potato processing water is a suitable medium for certain green algae with addition of nitrate; however, whey permeate does not sustain algal growth nor does produced water sustain cyanobacterial growth. Particular importance of the nutrient management within municipal wastewater is studied so as to understand how the silicate concentrations and salinity of the water can influence the extreme lipid accumulation in diatoms such as *C. gracilis*. The possibility of growing cyanobacteria on produced water for the production of high value products is also discussed. Two cyanobacteria strains were shown to grow on a 1:1 mixture of produced water and laboratory media. Finally, the possibility of growing algae as a possible source of pet food is also discussed.

1. Introduction

In order to make the algal biodiesel process economically and environmentally viable, wastewater is being considered as a nutrient and water source. In July 2008, U.S. oil prices spiked to an all-time high of $147.30 per barrel. The need for research in

alternative fuels caused the passing of the 2007 Energy Independence and Security Act, which mandates a defined increase in the production of biofuels through agricultural ethanol, butanol, or other oil sources. Not just the market price of corn and other agricultural products, but concerns as well are on the rise of the inability to compete farm land for food and fuel. For this and other reasons, microalgae have been proposed as a possible feedstock for cultivation of a biochemical-based fuel product to serve as alternative and sustainable energy (Chisti, 2008). Microalgae grow on non-aerable land receiving lots of sunshine, and as has been previously demonstrated, are capable of using wastewater resources in place of freshwater (Pittman et al., 2011). The whole process of producing oil from microalgae, from the cell to the gas tank, is currently too expensive to commercialize. The technology is not available to provide this form of biodiesel at competitive market prices, especially in the forms of biomass dewatering and drying.

Aside from engineering solutions to the mechanical aspects of the algal biodiesel process, other solutions exist to help mitigate the high cost of biodiesel production. The production of high value products also extractable from algal biomass can help lower overall production costs. Also, increasing the lipid content by understanding the precise role of certain chemical stressors such as salinity and silicate in diatoms, can automatically increase the efficiency of the harvesting and extraction process. Doubling the lipid content in the cell equates to processing half the material to reach the final product. Finding uses for various industrial wastewaters can attract private companies for better disposal of their waste streams.

This chapter includes various experiments and ideas proposed to make the sun-driven process of biodiesel production more cost effective and environmentally sound.

Cyanobacterial high-value products, diatom sodium-silicate-lipid relationships, new industrial wastewaters, dog food, and pharmaceutical bioremediation are topics that are each addressed.

2. Materials and Methods

2.1. Collection and Storage of Wastewaters

Wastewater was also collected from various sites. Produced water was acquired from Anadarko Injection Site near Vernal, Utah, on May 11, 2010, and stored in 55-gallon barrels at the USU Solar Innovations (Outdoor) Facility until use. Aliquots were removed in 10-L jugs as needed and, depending on the blackness of the water, centrifuged for 10 minutes at 8000 rpm for removal of hydrocarbons prior to autoclaving. Samples of produced water were supplemented with sodium nitrate (1-1.5 g L^{-1}) for cyanobacterial growth.

Municipal wastewater was acquired from Logan Lagoon Wastewater Treatment Facility on two occasions: January 5, 2011, and April 29, 2011. Water was centrifuged for debris removal and autoclaved to ensure strain purity following inoculation. Unless otherwise specified, sodium nitrate (300 mg L^{-1}) was supplemented prior to inoculation.

Whey permeate, or Delac, was obtained from Glanbia® Foods in Twin Falls, Idaho on June 3, 2011. Due to the presence of a yellow precipitate (presumably calcium phosphate) which could interfere with light penetration during growth, samples were centrifuged and the subsequent supernatant was additionally filtered (Whatman, Piscataway, NJ). Samples were diluted with distilled water by either two or four times

prior to autoclaving. Sodium nitrate (280 mg L^{-1}, to reach a total of 300 mg L^{-1}) was supplemented after autoclaving.

Four different wastewaters were submitted to our laboratory in July 2011 for research from J.R. Simplot potato processing company in Boise, Idaho: the blancher, serving as essentially a hot water bath for potatoes; influent to the anaerobic digester; effluent to the digester; and water from the clarifier. This water was autoclaved followed by sodium nitrate (300 mg L^{-1}) supplementation.

2.2. Compositional Analysis of Delac and Simplot Wastewater

ICP (ELAN 6000, Perkin Elmer, Waltham, MA) and nitrate (Biospec-1601, Shimadzu, Columbia, MD) analysis were performed on a sample of Delac and each of the four Simplot wastewaters by Utah Veterinary Diagnostics Lab (UVDL). ICP analysis included concentration determination of the following elements: Ag, Al, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Li, K, Mg, Mn, Mo, Na, Ni, P, Pb, Sb, Se, Si, Sn, Sr, Tl, V, and Zn.

Lactose concentration was also determined for Delac sample using a lactose enzyme kit from r-Biopharm AG (Darmstadt, Germany) with the help of Bill McManus of the Nutrition and Food Science Department. Due to the high concentration of lactose, the sample required a dilution of 400:1 dilution.

2.3. Strain Selection and Maintenance Conditions

Synechocystis sp. PCC 6803, *Synechococcus elongatus* PCC 7942, *Chaetoceros gracilis* UTEX LB 2658, *Chaetoceros muelleri* UTEX LB FD 74, BA060-2 (isolated from Great Salt Lake), BA103 (isolated from Great Salt Lake), GA063 (isolated from

Great Salt Lake), *Amphora coffeaformis* UTEX 2039, *Chlorella* sp. USU080 (isolated from Great Salt Lake), and *Neochloris oleoabundans* UTEX 1185, were used for experiments. Each was maintained in 250 mL cultures in 500 mL baffled flasks rotating at 140 rpm with overhead lighting at 140 μmol m^{-2} s^{-1} on a 14:10 light:dark period. Cyanobacteria were maintained using a BG-11 media recipe, which included the following ingredients (per liter): $NaNO_3$ (1.50 g), $MgSO_4 \cdot 7H_2O$ (75 mg), $CaCl_2 \cdot 2H_2O$ (36 mg), citric acid (6 mg), Na_2EDTA (0.104 mg), H_3BO_3 (2.86 mg), $MnCl_2 \cdot 4H_2O$ (1.81 mg), $ZnSO_4 \cdot 7H_2O$ (0.222 mg), $Na_2MoO_4 \cdot 2H_2O$ (0.39 mg), $CuSO_4 \cdot 5H_2O$ (0.079 mg), $Co(NO_3)_2$ (0.0494 mg), ammonium iron citrate (6 mg), $NaHCO_3$ (20 mg), and K_2HPO_4 (30.5 mg). Algae strains were grown on Broad Seawater media, which contains the following nutrients (per liter): NaCl (18 g), KCl (0.6), $MgSO_4 \cdot 7H_2O$ (1.3 g), $CaCl_2 \cdot 2H_2O$ (100 mg), K_2HPO_4 (250 mg), $Na_2SiO_3 \cdot 9H_2O$ (70 mg unless otherwise noted), ferric ammonium citrate (5 mg). Trace metals were also added at 1 mL per L of media: H_3BO_3 (600 mg L^{-1}), $MnCl \cdot 4H_2O$ (250 mg L^{-1}), $ZnCl_2$ (20 mg L^{-1}), $CuCl_2 \cdot 2H_2O$ (15 mg L^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (15 mg L^{-1}), $CoCl_2 \cdot 6H_2O$ (15 mg L^{-1}), $NiCl_2 \cdot 6H_2O$ (10 mg L^{-1}), V_2O_5 (2 mg L^{-1}), KBr (10 mg L^{-1}). The algal strain BA103 was also grown on Broad Seawater with the aforementioned recipe but with addition of $NaHCO_3$ (1 g L^{-1}) and set to pH of 9.0.

For salt and silicate studies with *C. gracilis*, bulk Broad Seawater lacking NaCl and individual bottles of 90 mg, 1 g, 9 g, 18 g, 25 g, and 35 g of NaCl were prepared. Upon autoclaving, 1 L of no-salt media was dissolved into each bottle of salt, creating Broad Seawater at various levels of salinity. Each condition was run in duplicate. The

same method was applied for obtaining municipal wastewater at various salt concentrations (by dissolving in 0, 1, 9, 18, 25, and 35 g NaCl in autoclaved bottles).

2.4. Growth conditions

Experimental cultures were grown in 1.3-L tubes (800 mm x 50 mm diameter) with 1 L media and 110 mL inoculum, unless otherwise indicated for the experiment. Air supplemented with 1% CO_2 was bubbled through tubes for mixing and nutrient delivery. Optical density (UV-2401PC, Shimadzu, Colombia, MD) was monitored daily, with particular observation at 600 nm. Batch growth length was generally dependent on when optical density indicated the start of the stationary phase. Cultures were harvested by spinning at 8000 rpm for 15 minutes. Cell pellet was frozen and lyophilized (Labconco, Free Zone 4.5, Kansas City, MO) and kept frozen until further analysis was needed.

2.5. Total Lipid Extraction

100 mg dried biomass was weighed in a clean, dry microwave vial and mixed with 2 mL acidified methanol (1.8 % v/v H_2SO_4) and stirbar. Each sample was transesterified in a commercial microwave (Discover S, CEM USA, Matthews, NC) at 90°C for 20 minutes. Upon addition of chloroform and mixing with water, a phase separation made it possible to remove excess acid, methanol, and glycerol by removing the upper phase. The FAME in the organic phase was removed and collected in a 10 mL volumetric flask, and remaining biomass was washed twice more with chloroform for maximal lipid recovery. Volume was brought up to 10 mL using chloroform, and upon mixing by inversion into a clean test tube, 1 mL (or appropriate dilution with chloroform) was stored in a GC vial for analysis by gas chromatography.

2.6. Gas Chromatography

Using pure methyl myristate (C 14:0), methyl palmitoleate (C16:1), and methyl oleate (C18:1) (Nu-Chek Prep, Inc., Elysian MN) as standards, six concentrations of FAME mixture were prepared: 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 g L^{-1}. Lipid content, both TAG and FAME, were determined by gas chromatography (Model 2010, Shimadzu Scientific, Columbia, MD) coupled with programmable temperature vaporizer (PTV) and flame ionization detector (FID). The carrier gas used was helium, set at a constant flow rate of 50 cm s^{-1}. A microliter sample was injected into the PTV in direct mode, with programmed temperature set to match the column. The column used to separate analytes was an RTX-Biodiesel column, 15 m long, 0.32 mm ID, and 0.1 μm film thickness (Restek, Bellefont, PA). The oven temperature was set to 60°C for 1 minute, followed by an increase of 10°C min^{-1} to 370°C for 6 minutes. The FID was set to 370 °C. GCsolution postrun 2.3 (Shimadzu) was used for lipid peak integration.

2.7. Carbohydrate Content Determination

Using the phenol-sulfuric acid method (Dubois et al., 1956), 100 mg dry biomass was weighed into a 16 x 100-mm test tube and 5 mL 3M HCl was added. The sample was placed in a boiling water bath for 3 hours, followed by neutralization with sodium carbonate. The resultant mixture was centrifuged, and 0.1 mL of the supernatant was mixed into a fresh test tube and filled to 1 mL with distilled water. Standards containing 0.2, 0.4, 0.6, 0.8, and 1.0 mg mL^{-1} glucose were also filled to 1 mL with distilled water. To each sample and standard, 1 mL of a 5% phenol sample was added, followed by rapid and direct addition of 5 mL 95% sulfuric acid. The test tubes were immediately mixed.

After 20-30 minutes, absorbance by UV-Vis Spectrophotometer (UV-2401PC, Shimadzu, Colombia, MD) was observed at 490 nm. Total carbohydrate concentration was determined from the standard curve using the proper dilutions.

2.8. Dry Mass Analysis

Dry biomass was weighed to report the dry weight as g L^{-1}. Ash content was determined by heating a pre-weighed sample at 500°C for 30 minutes. The weight of the sample remnants after heating represents the ash content of the sample, and is taken as a percent of the original mass.

3. Results and Discussion

3.1. Pharmaceuticals

During a meeting at which Ned Weinshenker, VP for Strategic Ventures for Economic Development, was present, Liana Etchberger from USU's Vernal Campus presented research done by undergraduate Nicole Glines and faculty member Charley Langley showing the presence of pharmaceutical compounds, particularly estrogens and NSAIDs, in municipal wastewater. These compounds appearing in municipal waste streams is a result of the increase in pharmaceutical drugs, and brings with it troubling downstream environmental effects.

As the research presentation was later mentioned to the Logan biofuels team, ideas were formed of using algae as a means to bioremediate these pharmaceutical compounds. If algae take up harmful compounds from sources like produced water, they may be capable of absorbing harmful drug compounds as well. A simple experiment was

designed to grow on or two strains of algae on the wastewater with drugs, with a control tube of water with no algae treatment. Water analysis done by the Vernal team would indicate whether or not the algae decreased the concentration of the compound of interest. Further contact with Charley Langley to arrange for the acquisition of wastewater for algae growth informed us that the method for pharmaceutical compounds, particularly NSAID, at such low concentrations as found in Vernal municipal wastewater is not currently developed enough for the experiment. No further pursuit of the pharmaceutical bioremediation project followed.

3.2. Cyanobacterial Growth on Produced Water for the Production of Phycocyanobilin

In late January, 2011, a meeting was held with Dr. Jon Takemoto, a professor in the Biology Department, and Dr. Dong Chen, a Biological Engineering professor working with the Synthetic Bio-Manufacturing Center, in which the idea was proposed to grow cyanobacteria, such as *Synechocystis* or *Synechococcus elongatus*, using produced water for the purpose of developing the high-value products phycocyanobilin and biliverdin. These products have clinical uses as antioxidants (McCarty, 2007; Benedetti et al., 2010) valuable in heart disease (McCarty, 2007) and against immunological disorders (McCarty, 2011), and are especially valuable from non-animal sources, since animals create a dozen or so isomers of biliverdin compounds, many of which are ineffective. Cyanobacteria and algae make one isomer of phycocyanobilin, and it is the correct one for clinical use. Both products have competitive market prices, and if they could be formed using mainly produced water resources, they could dramatically reduce the cost of open-heart surgeries and heart transplants.

This collaboration was designed with hopes of combining biodiesel and phycocyanobilin formation in cyanobacteria. Growth of the organism on produced water would make the process more commercially feasible, given the extreme availability of the resource. Because the biodiesel extraction utilizes organic solvents, and phycocyanobilin extraction utilizes water solvents, the two extractions should be able to be performed on the same biomass material.

Growth of the cyanobacteria *Synechocystis* sp. PCC 6803 on produced water had been previously tested, with addition of only 150 mg L^{-1} nitrate. As shown below, growth was successful during the first five days. However, by day seven the culture turned from a dense, lime green color to brown, similar to the color of *Chaetoceros gracilis*, which was growing in the adjacent tube, and the culture was considered contaminated. TAG analysis showed almost no neutral lipid content. The final dry weight as harvested was 0.98 g L^{-1}, which is much lower than typically observed in media growths for this strain. Given the initial success of this strain on produced water, it was decided worth investigating to optimize parameters for larger growths.

Cyanobacteria grow in the laboratory in the BG-11 media, which has a high nitrate concentration (1 g L^{-1}). First, attempts were made to grow both Synechocystis and *Synechococcus elongatus* on produced water with 1 g L^{-1} nitrate supplementation. Within four days, however, *S. elongatus* turned completely white, unable to grow on pure produced water. Synechocystis seemed more resilient to growth on produced water, to some degree. The optical density did eventually double the starting density immediately following inoculation, but after one week it turned the same light orange color that was seen previously.

Next, the two cyanobacteria strains were tested for growth on produced water at various dilutions—if pure produced water was too toxic for growth, perhaps diluting produced water by two or four times with BG-11 media would better match the nutrient environment necessary for successful growth. Over the period of one month, with periodic addition of concentrated sodium nitrate, Synechocystis was able to grow on produced water diluted by half with media from an optical density of 1.18 to 8.53, 8 times the starting density. However, the culture turned a dark, olive green color. Attempts were made to use these cells as inoculum for growth on more concentrated produced water, hoping natural selection would create a culture more robust and resistant to whatever in produced water was inhibiting growth. These attempts all ended in loss of pigment in the culture.

After nearly three months of trying to grow both Synechocystis and *S. elongatus* on various concentrations of produced water, it was determined beyond my scope of research, especially once hearing that our department had not received any funding for the project and that another graduate student in biological engineering, Jonathan Wood, was pursuing the task. It was observed that of the two strains, Synechocystis grew more easily on diluted produced water if given enough nitrate, but that both could achieve some growth if the produced water was diluted enough.

3.3. Growth of Algae on Whey Permeate

In June 2011, the Seefeldt laboratory had an opportunity to discover the possibility of growing algae on a new waste water stream from a cheese manufacturing company in Twin Falls, Idaho, called Glanbia® Foods. This company produces millions

of gallons of waste water per day which they term "Delac," coming from a lactose removal process, that still contains high amounts of lactose and calcium, which currently gets dried down and sold as animal feed. This company is looking for a more profitable use of this waste water resource, possibly for use of algae biodiesel production. The elemental content is shown in the table below.

Table 4.1 shows, for algal growth, low concentrations of nitrate, similar salt concentrations to Broad Seawater and produced water, and extremely high concentrations of calcium and phosphorus. In fact, after just minutes of stirring, the solution would show a bright yellow precipitate, likely calcium phosphate. Iron, aluminum, copper, and

Table 4.1. Compositional analysis of whey permeate.

Element/Ion	Delac (mg L^{-1})
NO_3^-	14.85
Al	0.37
As	0.02
B	1.41
Ba	0.04
Ca	1513.79
Cr	0.12
Cu	0.34
Fe	4.17
K	11636.83
Li	0.09
Mg	524.43
Mn	0.02
Mo	0.12
Na	7188.69
Ni	0.05
P	2959.47
Se	0.05
Si	56.3
Sr	0.48
V	0.02
Zn	0.15
Lactose-monohydrate	172.5 g L^{-1}
Galactose	3.9 g L^{-1}

nickel were also found in higher concentrations than any other investigated wastewater for algal growth.

Upon inoculation with *C. gracilis* and USU080, *C. gracilis* cultures immediately turned red and lost the chlorophyll peak in the optical spectrum. Upon harvesting, a bubblegum pink-colored biomass was revealed in the yellow-orange media. USU080 cultures did not seem to grow very well, either. Optical density barely increased from inoculation, and the resulting culture lost chlorophyll peaks fairly early in the experiment. FAME analysis showed a maximum of 3.5% FAME from growth of *C. gracilis* on Delac diluted 1:3 with water. This is less than the approximated 7-10% lipid content of most presumed inoculum cultures, implying that the organisms lost lipid during the experiment. This concludes that Delac is an unsuitable nutrient and water source for algal growth, and probably for any photosynthetic organism. Its high lactose concentration could make it useful for heterotrophic growth, however.

3.4. Salt/Silicate Diatom Studies

In previous experiments, *C. gracilis* was found to accumulate over 63% of its dry weight as lipid when grown on municipal wastewater from the Logan Lagoon, the highest observed lipid content of all conditions tested. Lipid content was also high in *C. gracilis* when grown on Great Salt Lake water, reaching over 50% FAME content. These values far exceed previously observed lipid content values for this organism. In pursuit of an explanation for the extreme lipid deposition in these diatom cells, it was observed that both of these water sources contained low salt concentrations. Sodium was approximately 100 times lower in municipal wastewater (90 mg L^{-1}) than found in

produced water (9945 mg L^{-1}) or either media recipe (Broad Seawater 7438 mg L^{-1}, Enriched Seawater Artificial Water 8526 mg L^{-1}). Great Salt Lake water also had markedly decreased sodium concentrations (336 mg L^{-1}). Sodium has been identified as an important nutrient in marine diatoms, being used in symport with silicate for uptake into the cell (Bhattacharyya & Volcani, 1980); apparently, decreasing the extracellular salinity can actually decrease the dimensions of the mesopores of biosilica (Vrieling et al., 2007). Thus, silicate uptake, and therefore, growth and proliferation of the cell (Martin-Jezequel et al., 2000), is dependent upon the sodium gradient in the extracellular environment.

Other studies have shown that increasing salinity had detrimental effects on lipid accumulation in the marine algae *Nannochloropsis* (Pal et al., 2011) and *Dunaliella* (Takagi et al., 2006), although significantly lower salinity tends to inhibit growth. It was also found that in *C. gracilis*, increasing sodium chloride from 25 g L^{-1} to 35 g L^{-1} had the effect of lowering the percent lipid content from 60% to 15% (Araujo et al., 2011).

Experiments were designed to discover whether the extreme decrease in salinity in the municipal wastewater and Great Salt Lake water caused the high lipid accumulation in the diatom *C. gracilis*, and to what degree one could control the trigger for lipid accumulation using salt and/or silicate in media. If lipid accumulation could be found similar in both municipal wastewater and Broad Seawater at various salt concentrations, then it would indicate the role of salinity in triggering lipid accumulation in diatoms. Ideally, insights could be gained to the physiological behavior of lipid accumulation in response to its environment such that a continuous culture could be constructed for biofuel production.

The effect of salt concentrations on cultures of municipal wastewater and Broad Seawater. This experiment included twelve cultures of Broad Seawater at six different salt concentrations and twelve cultures of municipal wastewater at the same six salt concentrations, including 0.09, 1, 9, 18, 25, and 35 g L^{-1} salt. The sodium silicate concentration of Broad Seawater was set to equal the silicate concentration found from ICP analysis of municipal wastewater. Figure 4.1 reports the data for both Broad Seawater and municipal water experiments.

As seen in Figure 4.1, low salt concentrations in Broad Seawater increased the lipid content, suggesting that salinity can effect lipid production in at least this diatom species. This trend was not observed in the municipal wastewater samples. Percent lipid content (Figure 4.1b) was reported highest at 65% when 18 g L^{-1} salt was added, compared to 48% at 1 g L^{-1} and 37% at 18 g L^{-1} salt in Broad Seawater. Additionally, lipid accumulation in *C. gracilis* was reported higher in municipal water than at the same salt concentrations in Broad Seawater. This means that although decreasing salinity did cause an increase in lipid production (up to 48% at 1 g L^{-1} from 37% at 18 g L^{-1}) in Broad Seawater, it does not account for the total effect of extreme lipid accumulation in *C.*

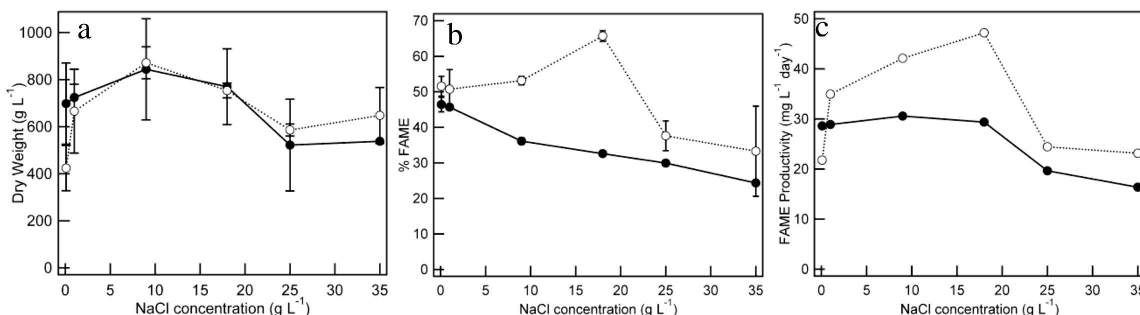

Figure 4.1. Growth of *C. gracilis* on municipal wastewater (○) and Broad Seawater (●) at various salt concentrations. (a) Dry weight measurements, in g L^{-1}, (b) FAME content, as a percent of the dry weight, and (c) FAME Productivity expressed in mg L^{-1} day^{-1}.

gracilis. Something else beyond the salinity inherent to the nature of municipal wastewater, or some combination of factors, is responsible for triggering such high lipid accumulation within these cells.

Also, cell growth (Figure 4.1a) was greater in municipal wastewater than in Broad Seawater, and seemed optimized in both at reduced salt concentrations, between 1 and 9 g L^{-1} salt. Combining biomass and lipid production, FAME productivity (Figure 4.1c) shows that the highest and fastest production of lipids from *C. gracilis* was found in municipal wastewater at 18 g L^{-1} salt, with 47 m L^{-1} day^{-1} FAME being produced.

The cause of extreme lipid accumulation in *C. gracilis* on municipal wastewater did not appear to be caused by the salinity alone, although there was evidence that salinity played a role. Elemental silica was found to be 5.42 mg L^{-1} in municipal wastewater, whereas it is 6.90 mg L^{-1} in Broad Seawater. It was hypothesized that the silicate concentration could influence lipid accumulation in diatoms, and that since literature states that sodium and silicate are used in symport within the cell, an osmotic stress under a specific silicate concentration could trigger excess lipid production in the diatom, eventually in continuous culture settings. This study attempts to find the effect of varying the silicate concentration over a broad range of salt concentrations on lipid production in *C. gracilis*.

Using the previous 2 batches outlined above, two additional batches were set up and run, with a total of 12 tubes of Broad Seawater containing 55 mg L^{-1} sodium silicate (an equivalent of 5.42 mg L^{-1} Si), and twelve tubes of Broad Seawater containing 20 mg L^{-1} sodium silicate (an equivalent of 1.97 mg L^{-1} Si). Results are shown in Figure 4.2 below.

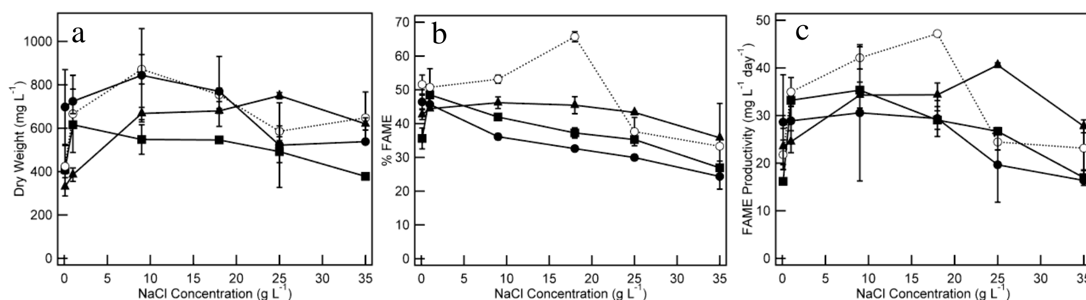

Figure 4.2. Growth of *C. gracilis* on municipal wastewater (○) and Broad Seawater at various salt and sodium silicate concentrations. [Si] = 20 mg L^{-1} (▲), 55 mg L^{-1} (●), and 70 mg L^{-1} (■). (a) Dry weight versus salinity, (b) FAME content as a percent of the dry weight, and (c) FAME productivity expressed as mg FAME L^{-1} day^{-1}.

Analysis of the dry weight data (Figure 4.2a) brings few obvious conclusions. Decreasing the silica concentration below 2 mg L^{-1} decreased the dry weight of cells. However, highest dry weights were optimized not at the highest Si concentration but at the middle, around 5.4 mg L^{-1} Si, in both Broad Seawater and municipal wastewater.

Lipid accumulation (Figure 4.2b) in municipal wastewater cultures was higher than any cultures grown on Broad Seawater. None of the Broad Seawater samples were able to come close to the 65% lipid accumulation seen in municipal water at 18 g L^{-1} NaCl. Cultures grown on Broad Seawater generally accumulated more lipids at lower salt concentrations. However, no general statement could be made as to whether increasing or decreasing the salinity or silica in Broad Seawater had an effect on lipid productivity. Figure 4.2c, showing lipid productivity, has such large deviations and no general patterns from which to draw definite conclusions about salt and silicate balance within this diatom species, except that it is optimized in municipal wastewater with 18 g L^{-1} supplemented salt.

Attempts to repeat the above experiments at random points also brought

inconclusive results. Using municipal wastewater from old stocks and a newly acquired supply, *C. gracilis* was grown with 1 and 18 g L^{-1} sodium chloride added. Results for the lipid content of measured samples are shown below in Figure 4.3 as individual points below the original municipal wastewater data. The repeated experiment gave results with half the lipid content as previously observed, although conditions were identical. Ash content was also run on the dry cells and was found to be quite high for tube experiments (30% for 18 g L^{-1} salt, 15% for cultures without salt added). It remains unknown if the high ash content of these cultures affected other aspects of the data or if this was a normal phenomenon to be expected with repeated growths. What is clear is that the inability to repeat results makes it impossible to gather definite, general results from the data.

In conclusion, decreasing the salinity does seem to have some effect on lipid accumulation in *C. gracilis*. Also, as expected, decreasing the silica concentration generally decreased cell growth. However, the sodium-dependent silica transport system—if one even exists in *C. gracilis*—is an unlikely player in the trigger of extreme

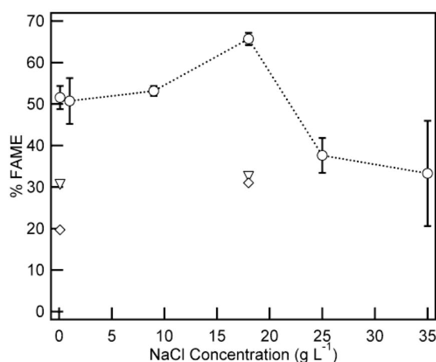

Figure 4.3. Repeatability of growing *C. gracilis* on municipal wastewater with and without salt addition. FAME content is shown as a percent of the dry weight. Old municipal water, ◊; new municipal water,▽; previous experiment, ○)

lipid accumulation. The cause of the high lipid accumulation as seen in growths on municipal wastewater remains inconclusive.

Further studies could be directed at the effect of vitamins in cell growth and lipid accumulation. Some have postulated that the municipal wastewater is rich in vitamins, which would not directly appear in an ICP analysis, but would improve at least algal growth (Provasoli & Pintner, 1953) if not also lipid accumulation.

3.5. Algae for Pet Food

In July 2011, contact was made with representatives from Mars®, Inc. in Tennessee, looking for possibilities of a collaborating project to grow algae for use as pet food. Strains which carried high protein content were desirable, and it was essential that they not contain toxins, particularly microcystins which are normally produced by cyanobacteria. It was also necessary to find a strain of brown algae so as to better blend in with natural canine digestive products. Strains with less offensive odors would be preferred.

Seven strains were identified as potential candidates to meet the above criteria: *Chaetoceros gracilis, Chaetoceros muelleri, Amphora coffeaformis*, BA060-2, BA063, BA103, and GA079. Some cultures grew fast enough to have samples collected twice in the month period. Ash, lipid, and carbohydrate content were determined for each strain and an objective smell rating was assigned for the dried, lyophilized cell mass. Protein content was assumed as the remaining content after the above three were determined, and is presented below in parentheses.

Table 4.2. Results from the growth of six brown algae strains for potential use as pet food. Includes total dry weight of the harvested culture and percent content found as ash, carbohydrates, lipids, and (by subtraction) protein. Also listed is a smell rating on a scale of 1 (weak) to 5 (strong, offensive). GA079 and *C. muelleri* were harvested from two cultures on different days.

Organism	Date harvested	Total dry weight (g L^{-1})	% Ash	% Carb.	% Lipid	(% Protein)	Smell rating (1-5 weak-Strong)
GA079	24-Aug	0.61	23.0%	7.8%	23.5%	(45.6%)	2
GA079	30-Aug	0.5	21.9%	8.6%	23.1%	(46.4%)	1
C.muelleri	17-Aug	1.32	18.1%	30.2%	38.8%	(12.9%)	2
C.muelleri	30-Aug	1.58	20.4%	18.0%	29.5%	(32.1%)	3
BA063	30-Aug	0.93	30.1%	9.6%	11.9%	(48.4%)	4
BA060-2	24-Aug	0.91	10.6%	13.5%	31.9%	(44.0%)	2
BA103	17-Aug	0.93	16.1%	10.7%	46.1%	(27.1%)	4.5
A.coffeaformis	30-Aug	0.76	15.0%	19.6%	20.8%	(44.6%)	2.5

Table 4.2 shows that many strains could be developed for further testing as pet food. Ash content was higher than desired in all strains, with the lowest found in BA060-2 at 10.6%. However, BA060-2 and GA079 were two strains that yielded high protein with a low smell rating.

Work was also done with *C. gracilis* because it had already been grown on a large scale within the laboratory facilities. Using a stock of over 500 g dry material, ash content was tested and found to be over 30%. To decrease the ash content, resuspension in distilled water and centrifugation (after a failed attempt using filter paper) was found to decrease ash content to 10.2%. Lipid content was found to be 29.4%. The remaining sample (approximately 450 g) was sent to Mars®, Inc. for testing at their own facility. A lack of response from Mars®, Inc. has led to an end of interest in this project.

3.6. Algal Growth on J.R. Simplot Wastewater

In September 2011, a meeting was held with Idaho agricultural company J.R. Simplot to determine if a wastewater stream from any point in the potato treatment process could sustain algal growth for biofuel production. ICP and nitrate analysis was performed on the four water sources upon arrival, given in the table below. The phenol-

Table 4.3. Compositional analysis of four wastewaters from Simplot potato processing. Underlined values indicate significant differences in previous tested nutrient sources.

Element/Ion	**Digester Effluent ($mg\ L^{-1}$)**	**Clarifier ($mg\ L^{-1}$)**	**Blancher ($mg\ L^{-1}$)**	**Digester Influent ($mg\ L^{-1}$)**
Nitrate	<5	<5	<5	<5
Ag	<0.001	<0.001	<0.001	<0.001
Al	0.063	0.045	0.235	0.217
As	0.004	0.006	0.006	0.005
B	0.088	0.089	0.141	0.086
Ba	0.029	0.022	0.037	0.042
Be	<0.001	<0.001	<0.001	<0.001
Ca	37.233	32.295	38.305	43.885
Cd	<0.001	<0.001	0.002	0.001
Co	0.001	<0.001	0.001	0.001
Cr	0.004	0.005	0.007	0.008
Cu	0.016	0.033	0.035	0.020
Fe	0.294	0.426	0.252	0.694
K	213.225	233.111	690.843	261.492
Li	0.014	0.013	0.009	0.012
Mg	13.928	12.294	43.319	15.667
Mn	0.061	0.055	0.174	0.108
Mo	0.001	0.003	0.002	0.002
Na	124.353	59.830	132.907	119.480
Ni	0.004	0.003	0.005	0.004
P	30.277	19.056	190.490	34.520
Pb	<0.001	0.001	<0.001	0.001
Sb	<0.001	<0.001	<0.001	<0.001
Se	0.002	0.002	0.005	0.002
Si	15.365	15.793	14.699	18.119
Sn	<0.001	<0.001	<0.001	<0.001
Sr	0.201	0.182	0.172	0.234
Tl	<0.001	<0.001	<0.001	<0.001
V	0.004	0.005	0.005	0.007
Zn	0.031	0.131	0.399	0.095
starch	14.4	59.2	142.1	62.3

sulfuric acid method revealed the starch content of each water source, also given in the table above.

The initial purpose of interest for this water was to be a source of carbon-rich media for heterotrophic organisms such as yeast or bacteria for lipid production. However, the starch content was much lower than expected (data supported by failed attempts to grow high density yeast cultures), and ICP analysis showed a profile better suited for algal growth than yeast growth. Two strains, *C. gracilis* and USU080, were originally selected, and later a third, *N. oleoabundans*, was also tested for growth on the four wastewaters with supplemented sodium nitrate (300 mg L^{-1}), searching for the potential of each strain and wastewater to produce high density lipids.

ICP analysis (Table 4.3) shows acceptable ranges of nutrients in the Digester effluent and the Clarifier, with the exception of slightly elevated levels of zinc than other wastewaters tested. The Digester Influent was also within range of most elements but with slightly elevated levels of aluminum and zinc. The Blancher water was most questionable for algal growth, with higher levels of aluminum, potassium, phosphorus, selenium, and zinc.

C. gracilis failed to grow on any of the Simplot waters (data not shown). USU080 grew acceptably. Because the sodium content was found to be so low, the freshwater green alga *N. oleoabundans* was later tested for growth on the waters and was also successful at growing and producing lipids on three of the four Simplot waters. Lipid and dry weight analysis is shown below in Table 4.4.

As shown below in Table 4.4, it is possible to use Simplot water to produce algal biodiesel using only sodium nitrate as a supplement. USU080 was able to produce the

Table 4.4. Results from growth of USU080 and *N. oleoabundans* growth on Simplot wastewaters. Includes dry weight, FAME and ash content (as a percent of dry weight), days grown, and the FAME and biomass productivity rates, expressed in mg L^{-1} day^{-1}.

	USU080				*N. oleoabundans*		
	Digester Influent	Digester Effluent	Clarifier	Blancher	Digester Influent	Digester Effluent	Clarifier
Dry weight (g L^{-1})	1.54	1.34	1.81	1.16	1.78	1.49	1.00
FAME %	16.1	11.8	19.3	6.6	18.8	17.7	14.5
Ash content %	10.1	13.5	12.0	25.5		4.2	3.3
Days Grown	7	7	7	4	11	6	6
FAME Productivity (mg L^{-1} day^{-1})	35.4	22.7	49.8	19.0	30.4	43.9	24.2

highest biodiesel productivity, at nearly 50 mg L^{-1} day^{-1}, although ash content was high at almost 20%. *N. oleoabundans* also saw high biodiesel productivity at 44 mg L^{-1} day^{-1}, with an ash content of only 4%. The Clarifier water performed best overall in USU080, and the Digester Effluent was best in *N. oleoabundans*, with good performance of the Digester Influent as well in both species. The Blancher water performed the worst, and failed to produce any growth in *N. oleoabundans*. It was concluded that Simplot water was a potential good growth medium for these algal organisms, but not for heterotrophic organisms.

4. Conclusions

Many strategies exist to reduce production costs of algal biodiesel. Additional wastewaters such as whey permeate from the dairy industry and Simplot potato processing industrial wastewater were tested for phototrophic growth in a small selection of microalgal strains. Whey permeate was found to be unsuitable for algal growth, perhaps because of the opacity decreasing light penetration to the culture. Simplot wastewater was found to support growth for two strains of green algae: USU080 and *N. oleoabundans*, with only addition of nitrate. USU080 FAME productivity was seen to

reach nearly 50 mg L^{-1} day^{-1} with 12.0 % ash content when grown on Simplot clarifier water. *N. oleoabundans* reached a FAME productivity rate of 44 mg L^{-1} day^{-1} with 4.2 % ash content when grown on Simplot digester effluent water.

Attempts were made to grow the high value-added product phycocyanobilin using phototrophic cyanobacteria grown on produced water. Initial studies showed successful growth during the first five days of growth followed by a sudden bleaching of color. Attempts to grow two cyanobacterial strains on a mixture of BG-11 media and produced water supplemented with 1 g L^{-1} sodium nitrate, with increasing concentrations of produced water, failed to produce desired growth. Growth was sustained on a 1:1 mixture of produced water and BG-11 media.

Certain strains of brown algae were investigated for their potential to serve as dog food as a possible collaboration with Mars®. An ideal strain would produce cellular content of high protein, high carbohydrate, low fat, and very low ash content, with high biomass productivities and a non-offensive smell. Carbohydrate, lipid, and ash content were determined from each of the six strains tested. Of the six strains tested, BA060-2 showed the most promise with nearly 50% protein and only 10% ash content, with a mild intensity smell rating. However, this premature collaboration has terminated with a loss of contact with Mars® representatives.

Diatom studies were performed to identify a trigger for high lipid accumulation in the species *C. gracilis*. In attempt to find a perfect ratio between sodium and silicate concentrations in solution, difficulties reproducing data led to the termination of studies before reaching any conclusions. Decreasing the salinity in Broad Seawater medium did result in a general increase in lipid accumulation, whereas in municipal wastewater lipid

content was optimized around 18 g L^{-1}. Lipid production at all salt concentrations was higher in municipal wastewater than in Broad Seawater at various tested silicate concentrations, and although data points were not reproducible, it was concluded that another nutrient in the municipal wastewater medium, such as suspected vitamins, causes the high lipid accumulation.

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CHAPTER 5

HETEROTROPHIC GROWTH OF OLEAGINOUS ORGANISMS FOR THE PRODUCTION OF BIODIESEL

Abstract

Oleaginous organisms such as yeast and bacteria have been identified as possible feedstocks for biofuel production because of their ability to accumulate large amounts of their dry weight as oil and grow with much higher densities than microalgae. *Rhodococcus opacus* is an oleaginous bacterial strain and *Cryptococcus curvatus* is an oleaginous yeast strain that have been considered for biodiesel production. Experiments have tracked the uptake of key nutrients such as sugar and nitrogen and the concurrent production of biomass and lipid over the course of several batch processes, including batches where nutrients were spiked part-way through the experiment. Wastewaters, particularly of whey permeate and a local ice cream factory, are considered as a carbon source for the growth of these organisms and described below. Finally, the use of xylose in conjunction with traditional 6-carbon sugars, and eventually wheat straw hydrolysate, were shown to permit the formation of biodiesel.

1. Introduction

While using microalgae as a means of creating renewable energy directly from sunlight and atmospheric CO_2 has many theoretical advantages to other feedstocks for biofuels, the current technology available makes it difficult to offer algal biodiesel at a low enough cost to be competitive with petroleum. Major limitations in harvesting and de-watering techniques arise and drive the cost up, especially when the best large-scale

cell densities reach only 2 g L^{-1}. The monetary and energy expense to remove that much water for biodiesel extraction make it currently impossible to successfully commercialize the process. Until this problem is better addressed, algal biodiesel stands short of other biofuel feedstocks in the search for renewable fuels (Azócar et al., 2010; Samorì et al., 2010).

Other organisms are capable of accumulating lipids in large quantities that could be converted to methyl esters just as algal lipids, and are capable of growing faster and achieve at least ten times the cell density of microalgae. Termed oleaginous microorganisms, or single cell oil, they are capable of accumulating 20% or greater (over 70%) their cell mass as lipid. Additionally, heterotrophic growth is not light or land-limited and can therefore grow in a fermenter of any size any time of the year. The key is finding a cheap, or ideally, waste source of fixed carbon.

While many species and strains of oleaginous organisms exist, our laboratory has accumulated some of these for experimentation. The published findings of just these few select strains includes much about lipid biochemistry; however, to find unique discoveries about these organisms, we began to validate their growth and lipid production capacities as previously stated in the literature. This chapter includes preliminary research done within the bacterial strain *Rhodococcus opacus* PD630 and the yeast strain *Cryptococcus curvatus*, with some implications for applicable growth on waste sources of fixed carbon.

2. Materials and Methods

2.1. Collection and Storage of Waste Carbon Sources

Whey Permeate. Whey permeate, or Delac, was obtained from Glanbia® Foods in Twin Falls, Idaho on June 3, 2011. Due to the presence of a yellow precipitate (presumably calcium phosphate), samples were centrifuged and the subsequent supernatant was additionally filtered (Whatman, Piscataway, NJ). Samples were frozen until use for culture growths.

Blue Bunny® Waste Waters. At the end of November 2011, 2 samples of waste water were provided from the Blue Bunny® ice cream plant near St. George, Utah. One was termed Waste Mix and the other Influent Process. Both samples were divided into 100-mL aliquots and frozen until use for culture growths.

Wheat Straw Hydrolysate. A sample of wheat straw was collected near Pocatello, Idaho on February 4, 2012. Wheat straw was hydrolyzed according to Yu et al. (2011). Briefly, the straw was washed and dried and milled into a mixture of short pieces and dust using a blender. 100 g milled wheat straw was mixed with 1 L of 2% sulfuric acid (20 mL in 1 L) and autoclaved at 121°C for 60 minutes. The solids were removed by subsequent centrifugation and vacuum filtration.

For treatment of non-detoxified hydrolysate, pH was brought up to 5.5 using sodium hydroxide and micronutrients were added in the following quantity per liter: magnesium sulfate heptahydrate (0.4 g), potassium phosphate dibasic (2 g), manganese chloride (3.51 mg), copper (II) sulfate (0.1 mg), and yeast extract (1.5 g). The solution was then sterilized using a 0.2-µm filter. Experiments were run with and without the addition of 0.5 g L^{-1} ammonium sulfate as well.

For treatment of detoxified hydrolysate, the solution was heated to 42°C and the pH was raised to 10.0 using sodium hydroxide. The solution was maintained at 50°C for 30 minutes before lowering the pH to 5.5 using sulfuric acid. Micronutrients were added in the same quantity as described above, and the solution was also filter sterilized using a 0.2-μm filter. Experiments were likewise done with and without the addition of 0.5 g L^{-1} ammonium sulfate.

Carbohydrate content was determined of non-detoxified and detoxified hydrolysate solutions using the phenol sulfuric acid method described below.

2.2. Compositional Analysis of Wastewaters

ICP (ELAN 6000, Perkin Elmer, Waltham, MA) analysis was performed on a sample of Delac and both Blue Bunny® wastewaters upon arrival by Utah Veterinary Diagnostics Lab (UVDL). ICP analysis included concentration determination of the following elements: Ag, Al, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Li, K, Mg, Mn, Mo, Na, Ni, P, Pb, Sb, Se, Si, Sn, Sr, Tl, V, and Zn. Delac water was additionally tested for nitrate concentration (Biospec-1601, Shimadzu, Columbia, MD).

Lactose concentration was also determined for Delac and Blue Bunny® samples using a lactose enzyme kit from r-Biopharm AG (Darmstadt, Germany). Total protein (see below) was also determined in the Blue Bunny® sample by use of the Lowry assay, described below.

Total carbohydrate content of wastewater samples was determined by the phenol sulfuric acid method (Dubois et al., 1956). A predetermined amount of sample (depending on carbohydrate content, dilutions were necessary to fit sample concentration

within standard curve) was added to a clean 16 x 100 mm test tube and filled to 1 mL. Standards of glucose and/or xylose were made containing 0.2, 0.4, 0.6, 0.8, and 1.0 mg mL^{-1}, and filled to 1 mL. To each sample and standard, 1 mL of a 5% phenol solution was added, followed by direct and rapid addition of 5 mL concentrated H_2SO_4. After 20-30 minutes, absorbance at 490 nm was measured, and carbohydrate concentration was determined using the standard curve.

2.3. Strain Selection and Maintenance

Rhodococcus opacus PD630 (DSM 44193) was obtained from the Deutsche Sammlung von Mikroorganismen (Germany), maintained at -80°C as a glycerol stock, and streaked onto agar plates as needed. *R. opacus* growth medium (MIT-nitrate) is given in the following ingredients (per liter), and was combined from media defined in (Kurosawa et al., 2010) and (Chartrain et al., 1998): magnesium sulfate heptahydrate (1 g), calcium chloride dehydrate (15 g), lactose (40 g for inoculum, 120 g for cultures), iron sulfate heptahydrate (0.5 mg), zinc sulfate heptahydrate (0.4 mg), manganese sulfate monohydrate (2 µg), boric acid (15 µg), nickelous chloride hexahydrate (0.01 mg), disodium EDTA (0.25 mg), cobalt chloride hexahydrate (0.05 mg), copper chloride dihydrate (5 µg), sodium molybdate dihydrate (2 mg), ferric ammonium citrate (4.5 mg), potassium phosphate dibasic (3.67 g), potassium phosphate monobasic (1.53 g), and sodium nitrate (1.4 g for inoculum, 9.65 g for cultures).

Cryptococcus curvatus (ATCC 20509) was obtained from the American Type Culture Collection and also maintained at -80°C as glycerol stocks, being streaked onto agar plates as needed. *C. curvatus* growth medium, as cited by Angerbauer et al. (2008),

contained each of the following (per liter): potassium phosphate monobasic (12.5 g), sodium phosphate dibasic (1 g), magnesium sulfate heptahydrate (2.5 g), calcium chloride dehydrate (0.25 g), yeast extract (1.9 g), lactose (40 g), ammonium sulfate (0.5 g), and 0.625 mL/L of the trace metals solution. Trace metals were prepared separately and consisted of the following: ferrous sulfate (16 g L^{-1}), manganese sulfate monohydrate (4 g L^{-1}), aluminum chloride (2.23 g L^{-1}), cobalt chloride hexahydrate (2.92 g L^{-1}), zinc sulfate heptahydrate (0.8 g L^{-1}), sodium molybdate dehydrate (0.8 g L^{-1}), copper chloride dehydrate (0.4 g L^{-1}), boric acid (0.2 g L^{-1}), and potassium iodide (1.6 g L^{-1}) dissolved in 5 N hydrochloric acid.

2.4. R. opacus Growth Conditions

From a single colony from a fresh plate, *R. opacus* was grown in 8 mL MIT-nitrate media in a small culture test tube and placed in a rotating incubator at 30°C. After 2 days, the 8 mL culture was transferred to 50 mL MIT-nitrate media in a 100-mL baffled flask and incubated at 30°C for 2 days. For the batch growth experiment, the inoculum had an optical density (OD) reading of 19.152 at 660 nm. 7.22 mL inoculum was added to each of 8 250 mL baffled flasks with 100 mL MIT-nitrate culture media to give each culture a starting OD_{660} of 0.76. One flask was harvested each day for a total of eight days. Harvested cell pellets were frozen, lyophilized, and kept frozen until further analysis. Supernatants were also collected.

When *R. opacus* was grown on Delac, four flasks of mixed Delac and water were prepared: 25 mL Delac and 75 mL distilled water (25%), 50 mL Delac and 50 mL water (50%), 75 mL Delac and 25 mL water (75%), and 100 mL Delac (100%). Each flask was

supplemented with 3.126 mL of 300 g L^{-1} sodium nitrate, to give a final concentration of 9.65 g L^{-1}. Similar inoculation procedures were followed, with 8 mL inoculum (OD_{660}=20.31) from the 50 mL culture being added to each of four flasks containing a mixture of Delac and water, giving each culture a starting OD_{660} of 1.2. OD was measured daily for a period of 120 hours, at which all cultures were harvested. Cell pellets were frozen, lyophilized, and kept frozen until further analysis. Supernatants were also collected for analysis.

2.5. C. curvatus Growth Conditions

Similar to the experimental conditions just described for *R. opacus*, *C. curvatus* was also grown in an 8-mL culture test tube of Angerbauer media from a single colony streak plate. After overnight growth in an incubator at room temperature to 30°C, 1 mL was added to inoculate 250-mL baffled flasks of 100 mL Angerbauer media. During the first batch experiment, 6 identical 100-mL flasks of the Angerbauer lactose media were inoculated with *Cryptococcus curvatus*, one to be harvested per day. Upon analysis of the data, a second batch of flasks was prepared to be harvested over the course of 10 days, during which all flasks remaining after the fourth day harvest would be spiked with either 40 g L^{-1} lactose and 0.5 g L^{-1} ammonium sulfate, or only 40 g L^{-1} lactose. A third batch growth of a single flask containing 80 g L^{-1} lactose was run until optical density reached a maximum, after 6 days.

Additional experiments of growing *C. curvatus* on Delac as a media source with supplemented ammonium were performed by undergraduate researcher Lynsey Talbot,

whose methods match those described above, supplementing 0.5 g L^{-1} ammonium sulfate and harvesting cultures after 120 hours of growth.

For growths of *C. curvatus* on Blue Bunny® waste mix, 4 100-mL flasks were prepared by dilution with distilled water, just as was Delac, to create the following concentrations of waste mix in water: 25%, 50%, 75%, and 100%. Each flask was supplemented with 0.5 g L^{-1} ammonium sulfate and inoculated with *C. curvatus*. Because of the thick nature of the Blue Bunny® waste mix, OD readings did not follow a great pattern, but were taken daily nonetheless. Cultures were harvested after 120 hours of growth, with the cell pellet frozen and lyophilized, and the supernatant collected.

Experiments involving xylose as a carbon source were done in similar fashion as the above two batch growths, in that 250-mL baffled flasks with 100 mL Angerbauer media containing 40 g L^{-1} of the specified sugar content were inoculated with *C. curvatus* and harvested after 120 hours. OD_{600} was measured daily. The lactose/xylose experiment contained four concentrations of lactose:xylose (1:0, 3:1, 1:1, 0:1). The xylose/glucose experiment contained four different combinations of the glucose:xylose ratio (1:0, 2:1, 1:1, 0:1).

For experiments of *C. curvatus* on wheat straw hydrolysate, 100 mL hydrolysate (detoxified or non-detoxified) was measured into sterile 500-mL baffled flasks. Solutions were tested for growth with and without addition of 0.5 g L^{-1} ammonium sulfate. Cultures were inoculated with fresh *C. curvatus* cells and harvested after 120 hours. OD_{600} was measured daily.

2.6. Dry Mass Analysis

Dry biomass was weighed to report the dry weight as g L^{-1}. Ash content was determined by heating a pre-weighed sample at 500°C for 30 minutes. The weight of the sample remnants after heating represents the ash content of the sample, and is taken as a percent of the original mass.

2.7. FAME Extraction

100 mg dried biomass was weighed in a clean, dry microwave vial and mixed with 2 mL acidified methanol (1.8 % v/v H_2SO_4) and stir bar. Each sample was transesterified in a commercial microwave (Discover S, CEM USA, Matthews, NC) at 90°C for 20 minutes. Upon addition of chloroform and mixing with water, a phase separation made it possible to remove excess acid, methanol, and glycerol by removing the upper phase. The FAME in the organic phase was removed and collected in a 10-mL volumetric flask, and remaining biomass was washed twice more with chloroform for maximal lipid recovery. Volume was brought up to 10 mL using chloroform, and upon mixing by inversion into a clean test tube, 1 mL (or appropriate dilution with chloroform) was stored in a GC vial for analysis by gas chromatography.

2.8. Gas Chromatography

Using pure methyl myristate (C 14:0), methyl palmitoleate (C16:1), and methyl oleate (C18:1) (Nu-Chek Prep, Inc., Elysian MN) as standards, 6 concentrations of FAME mixture were prepared: 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 g L^{-1}. Lipid content, both TAG and FAME, were determined by gas chromatography (Model 2010, Shimadzu Scientific, Columbia, MD) coupled with programmable temperature vaporizer (PTV) and

flame ionization detector (FID). The carrier gas used was helium, set at a constant flow rate of 50 cm s^{-1}. A 1-µL sample was injected into the PTV in direct mode, with programmed temperature set to match the column. The column used to separate analytes was an RTX-Biodiesel column, 15 m long, 0.32 mm ID, and 0.1 µm film thickness (Restek, Bellefont, PA). The oven temperature was set to 60°C for 1 minute, followed by an increase of 10°C min^{-1} to 370°C for six minutes. The FID was set to 370 °C. GCsolution postrun 2.3 (Shimadzu) was used for lipid peak integration.

2.9. Lowry Protein Assay

Total protein content of dry biomass and raw Blue Bunny® waste waters using the Lowry assay, as described in (Peterson, 1977). Briefly, 50 mg dry biomass was mixed in 100 mL distilled water. Samples were sonicated for 3 10-second intervals to reduce sample clumping. Ten milliliters of the cloudy sample was removed into a centrifuge bottle. For liquid samples, 1 mL sample was mixed in 10 mL water in centrifuge bottles. To each sample, 1 mL 0.15% deoxycholate solution was added. After 10 minutes, 1 mL 20% TCA solution was added causing immediate precipitation. The samples were centrifuged for 30-60 minutes at 20000 rpm. Supernatant solution was carefully decanted to remove any excess drop of liquid. The protein pellet was redissolved in 1 mL of a solution (CTC) containing a 1:1:1:1 ratio of the following reagents: 1% cupric sulfate, 2% sodium tartrate, and 1 mg mL^{-1} sodium carbonate; 10% sodium dodecyl sulfate; 0.8 M NaOH; and distilled water. Once the protein pellet had dissolved, it was diluted with 10 mL distilled water. From this solution, 0.2 mL protein solution was removed to a clean test tube and filled to 1 mL. Bovine Serum Albumin

(BSA) standards were also made in test tubes and filled to 1 mL at concentrations of 0.02, 0.04, 0.06, 0.08, 0.1 mg mL^{-1}. To each sample and standard was added 1 mL CTC solution. After 10 minutes, 0.5 mL of 0.33 N Folin-Ciocalteu phenol reagent was added. Absorbances were read at 750 nm after 30 minutes but before 90 minutes. Protein concentrations were calculated using the standard curve and proper dilution factors.

2.10. Total Nitrogen Determination

The total concentration of nitrogen in solution was determined in culture supernatants and raw waste waters by use of QuikChem 8500, Series 2 (Lachat, Loveland, CO).

3. Results and Discussion

3.1. R. opacus Batch Growth

R. opacus has been identified as a unique bacterial strain that is capable of producing and storing significant amounts neutral lipids. Reports in the literature state growth of *R. opacus* at 77 g L^{-1} dry weight and 38% TAG content using glucose and ammonium sulfate as C/N sources (Kurosawa et al., 2010). Another report has demonstrated that neutral lipid production begins at depletion of nitrogen from the environment and continues until the exogenous carbon source depletes (Alvarez et al., 2000), but fails to report growth as dry weight, using optical density instead as a representation of growth. This organism has never been shown to grow on lactose, which is of interest because of the lactose concentration in wastewaters of interest to our laboratory (i.e. Delac and Blue Bunny). Because of the work of lab members, it became

an experiment of interest to understand the nutrient environment and cellular composition of *R. opacus* during a batch growth.

Eight identical 100 mL cultures were started as described above, with one culture harvested a day. Samples were analyzed for cellular dry weight, lipid, and ash content and total nitrogen and lactose concentration of the supernatant media. Results are shown below in Figure 5.1.

As seen above, growth (Figure 5.1a) and lipid (Figure 5.1c) content seemed to reach a peak at day 5, with 32 g L^{-1} dry weight and 44% biodiesel content. There may have been some error in the analysis of nutrients, because it seems unlikely, indeed impossible, that both the exogenous lactose (Figure 5.1b) and nitrogen (Figure 5.1d) concentrations could double after one day of growth, exceeding the amount that was even

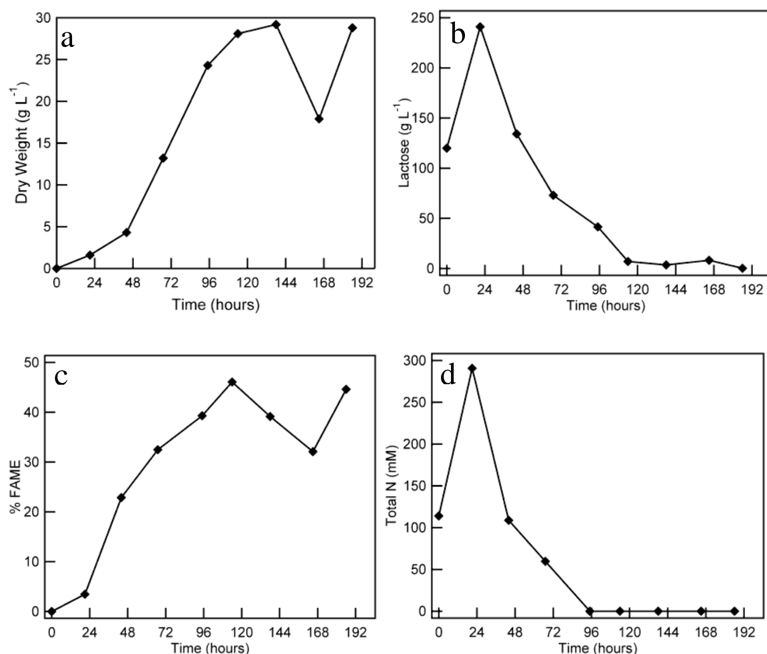

Figure 5.1. Results of batch growth of *R. opacus* in lactose media. (a) Cellular dry weight, (b) lactose concentration of growth medium, (c) FAME content as a percent of the dry weight, and (d) total nitrogen concentration of the grown medium vs. time.

present in the flask to begin with. These anomalies make it difficult to draw definite conclusions from this growth experiment, except that five days, or 120 hours, seems optimal for harvesting maximal dry weight and lipids in the *R. opacus* culture.

3.2. Growth of R. opacus on Delac

Experiments were also done with *R. ocapus* using Delac, the whey permeate wastewater from Glanbia®, supplemented with sodium nitrate (see Table 4.1 for nutrient analysis). Because some nutrients were found to be so high, four different dilutions of Delac were tested: 25%, 50%, 75%, and 100% Delac diluted with distilled water. After five days, cultures were tested for ash and lipid content, dry weight, and remaining nitrogen and lactose in solution. The results are shown in Table 5.1 below.

According to Table 5.1, growth is clearly suppressed at 100% Delac concentration, with minimal utilization of the lactose in solution and high ash content of what cells were recovered. With only 5.4 g L^{-1} dry weight, the 25% Delac concentration produced the greatest lipid of the three tested samples. Other experiments showed that *Cryptococcus curvatus*, an oleaginous yeast organism, was able to grow on Delac at 16 g L^{-1} dry weight and 35% biodiesel content at a 25% Delac dilution supplemented with ammonium sulfate. *R. opacus* is clearly not the best heterotrophic organism for growth

Table 5.1. Results of growth of *R. opacus* on Delac at various dilutions. Includes cellular dry weight, total nitrogen and lactose concentrations remaining in medium at end of growth, and ash and lipid content as a percent of the dry weight.

Flask	**Dry Weight (g L^{-1})**	**[total N] (g L^{-1})**	**[Lactose] (g L^{-1})**	**% Ash**	**% Lipid**
Delac 25%	5.4	0.95	17.9	19.0	16.1
Delac 50%	8.8	1.10	39.8	32.5	5.1
Delac 75%	12.9	1.02	41.8	35.1	2.3
Delac 100%	3.3	1.45	265.8	26.5	

or lipid production on whey permeate.

3.3. Batch Growth of C. curvatus

Among the oleaginous yeast organisms, *C. curvatus* remains a strong competitor for biodiesel production despite the limitation of lacking a working genome. Without genetic manipulation or understanding of its precise metabolic pathways, particularly of lipid metabolism, it has been observed to grow at cell densities of 118 g L^{-1} in a 50-hour fermentation at 25% lipid content (Meesters et al., 1996) and, in our own laboratory, 15 g L^{-1} in 120-hour fermentations at above 70% lipid content. It has been shown to grow on diverse feedstocks, from glucose and sucrose to glycerol (Meesters et al., 1996) and whey permeate (Ykema et al., 1988). Nitrogen limitation seems to be the key trigger for lipid production (Evans and Ratledge, 1984). These characteristics make this workhorse organism an ideal starting place for industrial production of biodiesel (Thiru et al., 2011).

Without genetics, our laboratory needed a better understanding of the effect of the nutrient environment on lipid production in this organism. In a similar fashion as described for *R. opacus*, experiments were designed over a batch process to see the changes in extracellular nutrients and cell composition.

These experiments, as seen in the Figure 5.2, show the relationship between carbon uptake (Figure 5.2b) and cell dry weight (Figure 5.2a), as well as between extracellular nitrogen concentration (Figure 5.2e) and lipid content (Figure 5.2d). In the first batch run, as shown in the dotted line, nitrogen is depleted during the first day, after which is seen a monstrous climb in lipid content within the cell. Also observed is the continuation of cell growth throughout the batch until the carbon source depletes, at day

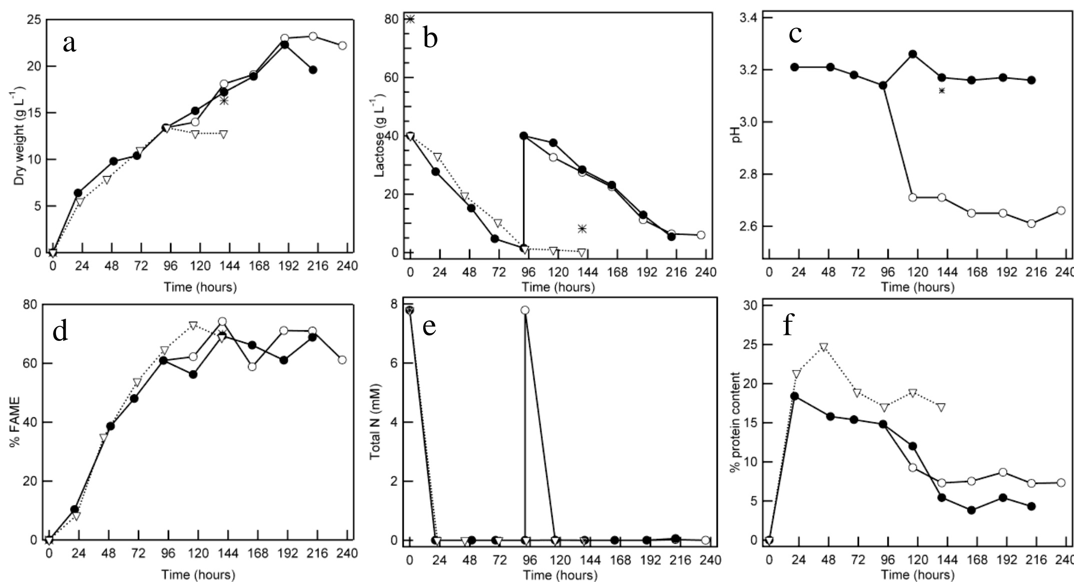

Figure 5.2. Results from the batch growth of *C. curvatus* on lactose media. (a) Cellular dry weight, (b) lactose concentration in the growth medium, (c) pH, (d) cellular FAME content as a percent of the dry weight, (e) total nitrogen concentration of the growth medium, and (f) cellular protein content as a percent of the dry weight, all as functions of time. Symbols are defined as follows: ▽, first batch growth during 6 days; ●, second batch growth during 9 days with a lactose spike on day 4; ○, second batch growth during 10 days with a lactose and nitrogen spike on day 4; and *, single flask containing 80 g L^{-1} lactose grown for 6 days.

four, after which cell dry weight levels off.

The second batch growth follows the same trend until day four, when cultures were spiked with either extra carbon and nitrogen or just carbon. When spiked with ammonium, the cells take up the nitrogen source again within the first day (Figure 5.2e); however, FAME content within the cell does not increase much over the next five days (Figure 5.2d) as it was already at such high levels in the cell. What is noticeable is the slight decrease in lipid content during the day following the spike. It has been shown in literature with *R. opacus*, the presence nitrogen can cause the mobilization of carbon within the cell from storage lipids to other carbon sources (Alvarez et al., 2000); a similar

pathway may be occurring here in *C. curvatus*. With regards to the carbon spike and cell growth, in both cases lactose continues to be depleted and the cell continues to grow (Figure 5.2b). It is interesting to note that there was no large difference in growth in cultures which received a nitrogen spike versus those which received no nitrogen spike. The lactose was removed from solution at the same rate, and growth under both conditions seemed maximized at day eight, or four days after the spike. Comparing this second batch experiment to the first reveals that doubling the amount of carbon source did increase the dry weight of the cells (Figure 5.2a), but only by 50%, not the 100% as might be expected. Also, not all the carbon was utilized in the carbon spike; whether a toxic byproduct or another limiting reagent in the original medium prevented further depletion of the available carbon remains unknown.

The third flask contained 80 g L^{-1} lactose, the same amount as the spiked cultures of the second batch but all at once at the beginning of the growth. Although it is unknown how the available lactose, growth, and lipid content changed during the six days, it was shown that the culture was unable of using up all of the available carbon by the presumed end of the batch growth (Figure 5.2b). High lipid content (Figure 5.2b) was still achieved (68%), but cell growth (Figure 5.2a) was similar to growths with half the amount of carbon (16.3 g L^{-1}). It is possible that the cell required an equal ratio of C:N to produce a higher dry weight as hoped.

3.4. The Capacity of Blue Bunny® Wastewaters to Support Heterotrophic Growth

At the end of November 2011, two samples of waste water were provided from the Blue Bunny® ice cream plant near St. George, Utah. As shown in Table 5.2, the composition was analyzed for both inorganic and organic material to observe the potential of both Waste Mix and Influent Process waste waters for growth of

Table 5.2. Compositional analysis of Blue Bunny wastewaters. Underlined values show large differences in laboratory media concentrations.

Element/Compound (mg L^{-1})	Blue Bunny Waste Mix	Blue Bunny Influent Process
Ag	<0.001	<0.001
Al	2.446	0.092
As	0.034	0.007
B	0.986	0.119
Ba	0.26	0.024
Be	0.003	<0.001
Ca	1282.473	35.556
Cd	0.006	0.001
Co	0.016	0.001
Cr	0.117	0.007
Cu	0.423	0.039
Fe	20.279	0.481
K	2100.593	248.329
Li	0.045	0.018
Mg	187.832	13.389
Mn	0.772	0.064
Mo	0.087	0.004
Na	747.373	66.704
Ni	0.133	0.003
P	1030.245	21.772
Pb	0.005	0.001
Sb	0.004	<0.001
Se	0.169	0.003
Si	26.899	17.328
Sn	0.009	<0.001
Sr	1.655	0.207
Tl	0.003	<0.001
V	0.018	0.006
Zn	5.219	0.16
Lactose (g L^{-1})	41.9	0.86
Total Carb. (g L^{-1})	109	2.6
Protein (g L^{-1})	13.1	0.524

heterotrophic oleaginous organisms. Due to the favorable carbohydrate content of the Waste Mix, four flasks were inoculated with *C. curvatus*, similar to the Delac experiment with *R. opacus*, at the following concentrations (diluted with distilled water): 25%, 50%, 75%, and 100%. They were harvested after five days, the typical time for *C. curvatus* growth. The Influent Process water was considered too carbon-poor to sustain heterotrophic growth; however, the ICP analysis seems favorable toward algal growth, especially freshwater strains. Experiments could be designed in the future for such potential.

According to Table 5.3, as the concentration of Waste Mix increased, the yeast grew less. Following centrifugation, the more concentrated cultures seemed to consist more of chocolate syrup than white yeast cells. Mass was lost in the 100% Waste Mix culture from adhering to the sides of the culture flask during the five days. Otherwise, dry weight increased as waste mix became more concentrated, which leads one to wonder how much of the dry weight was yeast and how much was from the raw Waste Mix. FAME content from the culture followed the opposite trend, increasing in FAME content as the dilution of waste mix increased. This demonstrates that yeast are indeed present in the culture and are responsible for concentrating the lipid in solution upon harvesting. When FAME productivity is considered, it is seen that for every L of raw Waste Mix, a 25% mix with 75% distilled water will produce 7.8 g biodiesel L^{-1} day^{-1}, or 39 g L^{-1}.

Ice cream labels indicate the high fat content of ice cream of any given flavor. When diluted to a proper amount, either to create the ideal C:N ratio or else bring other elements present in the waste mix below toxicity, yeast appear to be able to consume the sugars and the fats in solution. The question can be posed if fermentation is even

Table 5.3. Results from *C. curvatus* growth on Blue Bunny Waste Mix at various dilutions. Includes cellular dry weight, FAME content as a percent of the dry weight, FAME productivity, and the FAME productivity of the raw wastewater when water dilution is accounted for.

	Blue Bunny Waste Mix Dilutions			
	25%	**50%**	**75%**	**100%**
Dry Weight (g L^{-1})	14.3	34.2	45.2	36.2
% FAME	68.5	48.5	40.3	33.3
FAME productivity (g L^{-1} day^{-1})	2.0	3.3	3.6	2.4
Raw Waste Mix productivity (g FAME L^{-1} day^{-1})	7.8	6.6	4.9	2.4

economically worth the effort. Analysis of raw Blue Bunny Waste Mix revealed interesting data. Upon centrifugation, the Waste Mix separates into three layers: solids (47 g L^{-1}), liquids, and a top layer presumed to be fat. This layer is difficult in the centrifuge tubes to separate from the liquid, although in an industrial setting, it is likely that scraping off this top layer could be sufficient. Attempts to lyophilize the top two-layer mixture for transesterification gave poor results, nevertheless yielded 16% FAME content. The solids, in contrast, contained 26% lipid. This means that the Waste Mix, without fermentation, can produce 12.2 g biodiesel L^{-1}. Comparing the 25% Waste Mix dilution fermentation to raw waste mix, one can see that the yeast adds to the 12.2 g lipid L^{-1} already available by converting the sugars within the mix to produce a total of 39 g L^{-1} biodiesel within the solids layer upon centrifugation.

3.5. Use of Mixed Hexose and Pentose Sugars for Yeast Growth

Yeast fermentation, as seen above, hold great potential for biodiesel production, with high cellular densities, high lipid content, short batch times, and its ability to ferment anywhere regardless of sunlight. The comparison of yeast to algae as a biodiesel

feedstock is remarkable. However, the greatest limitation of yeast fermentation as a viable biodiesel feedstock is its need for a carbon source. Conventional fixed carbon sources, such as lactose or sucrose, compete with food stocks and perpetuate the same problem as corn ethanol. Food waste sources are the solution, but there must be a large enough consistent/steady stream of waste products to make appreciable amounts of biodiesel. Blue Bunny® Waste Mix and Glanbia® Delac work well but will not replace our need for other sources of fuel to replace petroleum.

Many researchers are working to make hydrolysate products from biomass that is otherwise not put to human use, or at least underutilized. Wood, straw, grasses, other forestry waste, agricultural residues, and municipal solid waste are all sources of lignocellulose. Wood, for example, is made of primarily cellulose and hemicellulose, with components also of lignin and ash. Cellulose consists of polymers of glucose with β(1,4)-linkages; hemicellulose consists of branched polymers of mixed 5- and 6-carbon sugars, particularly xylose and glucose, but also mannose and galactose (Palmqvist and Hahn-Hägerdal, 2000). Thus, hydrolysis of these polysaccharides would make available a large quantity of available sugars for fermentation. However, it has been observed by many researchers that inhibitors also prevent cell proliferation due to the presence of weak acids from hydrolysis (i.e. acetic, formic, levulinic) (Dunlop, 1948; Ulbricht et al., 1984; Palmqvist and Hahn-Hägerdal, 2000).

Protocols are being developed to hydrolyze lignocellulosic biomass for the production of biofuels, but the next challenge still exists to find, or modify, an organism capable of utilizing not just the common hexoses but pentoses as well. The following study demonstrated the ability of *C. curvatus* to grow on xylose comparative to lactose,

Table 5.4. Results of *C. curvatus* growth on combinations of lactose and xylose. Includes cellular dry weight, percent of the dry weight found converted to FAME, and the amount of sugar remaining in the medium after growth.

	Lactose	**3:1 Lactose:Xylose**	**1:1 Lactose:Xylose**	**Xylose**
Dry weight (g L^{-1})	12.7	10.8	11.3	10.4
% FAME	68.0	61.6	62.1	71.5
FAME (g L^{-1})	8.6	6.7	7.0	7.4
Total Unused sugar (g L^{-1})	1.77	1.24	0.99	2.19

and also its ability to utilize a combination of the sugars in fatty acid synthesis.

Using Angerbauer media and varying the sugar source, cultures containing 40 g L^{-1} xylose, 20 g L^{-1} xylose and 20 g L^{-1} lactose, and 10 g L^{-1} xylose and 30 g L^{-1} lactose were inoculated with *C. curvatus*, with an additional flask with 40 g L^{-1} lactose run as a control, for a 5-day batch growth. Routine analyses provided the data as shown in Table 5.4 above.

Growth on xylose alone saw an 18% decrease compared to the lactose control (Table 5.4). Both cultures with mixtures of lactose and xylose had growth between that of either pure sugar source. Lipid content of all cells grown showed similar results, demonstrating that the carbon source did not disrupt the lipid synthesis pathway. Although there was a slight decrease in lipid content in cells grown with both lactose and xylose, any effect of growing cells on the carbon-source mixture is difficult to confidently state, given the lack of statistical analysis.

A combination of glucose and xylose seems more biologically relevant to hemicellulose hydrolysate products than does lactose and glucose. A recent estimate of corn stalk states that ideal hydrolysis would yield glucose and xylose at a ratio of 2:1 (Zhao et al., 2008). Given this information, the above experiment was repeated by substituting glucose for lactose, and testing the more biologically relevant glucose:xylose

Table 5.5. Results of *C. curvatus* growth on combinations of glucose and xylose. Includes cellular dry weight, percent of the dry weight found converted to FAME, and the amount of sugar remaining in the medium after growth.

	Glucose	2:1 Glucose:Xylose	1:1 Glucose:Xylose	Xylose
Dry Weight (g L^{-1})	13.5	12.9	12.7	12
% FAME	71.43	67.57	69.84	67.76
FAME (g L^{-1})	9.64	8.72	8.87	8.13
Total Unused Sugar (g L^{-1})	1.44	1.50	1.55	1.82

ratios of 2:1 and 1:1, in addition to the pure glucose and pure xylose sugar concentrations for comparison. The results are shown in Table 5.5.

Dry weight of cells was reported highest in glucose growths, with decreasing cellular densities as the xylose content increases, up to an 11% decrease. This is a small difference in growth capacity. Lipid contents of samples were within reasonable experimental error of each other, leading to the conclusion that lipid production occurs evenly independent of the two sugar sources. Considering the starting sugar concentration was 40 g L^{-1}, the concentration of remaining sugar in the media following growth was almost total, with xylose growths leaving the highest of the 4. The greatest amount of lipid was produced in the glucose media; however the glucose/xylose mixtures also gave acceptable yields.

3.6. Use of Wheat Straw Hydrolysate as a Sugar Source for C. curvatus Growth

As stated above, lignocellulosic biomass represents a large amount of underutilized sugars that could potentially be used for the production of energy-dense fuels such as FAME in oleaginous organisms. Protocols are being developed for the hydrolysis of the cellulose and hemicellulose sugar polymers. However, the hydrolysis of hemicellulose and lignin produces toxic byproducts such as acetic acid, furfural,

formic acid, levullinic acid, and phenolic compounds that inhibit microorganism growth (Palmqvist and Hahn-Hägerdal, 2000). Yu et al. (2011) demonstrated the growth of several oleaginous strains on wheat straw hydrolysate, both detoxified and non-detoxified forms, among which was *C. curvatus*. To verify these results within our own laboratory, attempts to repeat the experiment of non-detoxified and detoxified wheat straw hydrolysate as sugar sources within *C. curvatus* were performed.

A total carbohydrate analysis showed similar yield to that published by Yu et al. (2011). Non-detoxified hydrolysate contained slightly higher sugar content, with an estimated 27 g L^{-1} sugar content. Detoxified hydrolysate contained about 26 g L^{-1} sugar. Table 5.6 shows the growth results from both hydrolysates from with and without nitrogen supplementation.

As seen below, the dry weight was found to be similar but slightly higher in non-detoxified and detoxified forms of hydrolysate when external nitrogen was added. However, sufficient growth was also observed without the addition of nitrogen, with 8.9 g L^{-1} dry weight in detoxified hydrolysate and 8.2 g L^{-1} in non-detoxified hydrolysate. Cellular FAME content greatly increased in cultures without supplemental nitrogen, from 24% to 37% in detoxified hydrolysate and 25% to 35% in non-detoxified cultures. Total

Table 5.6. Results from growth of *C. curvatus* on wheat straw hydrolysates. Dry weight is given in g L^{-1}, percent FAME as a percent of dry weight, and FAME amount and productivity rate reported from dry weight, percent FAME, and/or days of batch growth.

	DTH[a]+N[b]	DTH[a]	NDTH[c]+N[b]	NDTH[c]
Dry weight (g L^{-1})	9.4	8.9	9.2	8.2
% FAME	24.5	37.3	24.8	34.8
FAME (g L^{-1})	2.30	3.32	2.28	2.86
FAME Productivity (g L^{-1} day^{-1})	0.46	0.66	0.46	0.57

[a]Detoxified Hydrolysate
[b]Supplemented with 0.5 g L^{-1} ammonium sulfate
[c]Non-detoxified Hydrolysate

FAME content of the culture and productivity rates show that FAME production is highest in cultures without adding nitrogen, and are slightly higher in detoxified cultures. Non-detoxified hydrolysate is still a viable culture medium if economic production costs are more favorable to cut out the detoxification steps.

4. Conclusions

Heterotrophic growth of oleaginous organisms is a promising method of biofuel production. Lipid productivities are magnitudes of order higher than that tested in phototrophic organisms (7.8 g L^{-1} day^{-1} in yeast compared to 0.07 g L^{-1} day^{-1} in algae). Nutrient uptake kinetics and lipid production was monitored in the bacterial organism *R. opacus* and *C. curvatus*. It appears that in *C. curvatus*, dry weight increases throughout the batch until the carbon source is exhausted, and that adding more carbon at the end of the growth can increase cell density to some extent. Also, the lipid content of the cell rapidly increases once the nitrogen has been depleted from the cell. Spiking the culture with nitrogen and carbon near the end of a growth does not seem to effect growth or lipid production.

These organisms are especially useful for biofuel production where a stream of waste carbon is available. Whey permeate from Glanbia Foods, ice cream waste from Blue Bunny®, and lignocellulosic biomass hydrolysate were investigated as sources of waste nutrients for oleaginous biomass growth.

Both *R. opacus* and *C. curvatus* were shown to grow on whey permeate, termed "Delac," which contains over 170 g L^{-1} lactose. In *R. opacus*, growth was best achieved when Delac was diluted with three times its volume of water, at 25% of its original

concentration. Dry weight reached 5.4 g L^{-1} with 16% lipid content and 19% ash. *C. curvatus* showed much more promise as a strain capable of growing on this sugar-rich medium, reaching 16 g L^{-1} dry weight and 35% lipid content. Both organisms required addition of a nitrogen source for growth.

Blue Bunny waste mix solids were isolated and found to contain 12.2 g L^{-1} FAME prior to inoculation of any microorganism. However, diluting the sample to various amounts revealed the ability of *C. curvatus* to increase the FAME concentration by using the available sugars (109 g L^{-1} in the raw mixture) and converting them to lipid. When the waste mix was diluted to 25% its original concentration, FAME concentration reached 39 g per liter of raw waste mix, or 7.8 g FAME per raw liter per day. It was determined that Blue Bunny® waste mix is an excellent substrate for *C. curvatus* in the production of biofuels, requiring only addition of a nitrogen source.

Biologically relevant mixtures of glucose and xylose as found in various lignocellulosic biomass were tested as possible sugar sources in the yeast *C. curvatus*. It was determined that growth could be achieved on either sugar source alone, or mixtures of the two. Although growth was slightly lower (11% decrease) on xylose medium compared to glucose medium, differences were small and cellular FAME content seemed unchanged. It was determined that both sugar sources were acceptable sources of sugar in *C. curvatus* growth.

Finally, wheat straw was hydrolyzed according to previously determined protocols and tested for capacity to support growth of *C. curvatus*. This process produces a culture that was found to contain 25-29 g L^{-1} sugar for heterotrophic growth. It was shown that this process can produce a cellular dry weight of 8.9 g L^{-1} with 37% FAME in

cultures using detoxified hydrolysate, and 8.2 g L^{-1} dry weight with 35% FAME in cultures using non-detoxified hydrolysate. Lipid productivities were seen highest in detoxified hydrolysate at 0.66 g L^{-1} day^{-1}.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Driven by a need for alternative, sustainable fuel sources without placing additional burdens on the already taxed freshwater resources, various wastewaters have been investigated as a source of growth media in the production of biofuel from oleaginous organisms.

Produced water is one such wastewater of extreme abundance (an estimated 20 billion barrels per year in the U.S.) currently being disposed of at great cost (Clark and Veil, 2009). With some preliminary efforts to remove hydrocarbons, several strains of microalgae were shown to successfully grow with addition of nitrate and phosphate nutrients. Among these strains are two that consistently reach higher biomass and lipid productivities than other strains tested: *Chaetoceros gracillis*, a brown diatom; and USU080, a green *Chlorella* species isolated from the Great Salt Lake. USU080 was shown to successfully grow in a scaled-up experimental 220-L raceway. Nutrient addition was optimized within *C. gracilis*. While phosphate addition did improve growth and lipid content, it was not found necessary if production costs were desired to be maintained low. Nitrate addition was optimized during several experiments, finally showing that supplementation with 300 mg L^{-1} was ideal for lipid productivity.

Remediation was also explored as a means to improve marketability of produced water as a growth medium, since microalgae have been used as decades for wastewater treatment. Concentrations of contaminants were observed before and after algal growth, and after extensive analysis several conclusions were made. Water quality standards vary

greatly across the country, and so it becomes difficult to standardize a single biofuels process to utilize the nutrients from produced water and end up with a water source that can then be disposed of as any wastewater. Microalgae are successful at removing nutrients like phosphate and nitrate, as well as magnesium, iron, and calcium. However, the high salinity of produced water brings the Total Dissolved Solids (TDS) orders of magnitude higher than permitted at any location in the state of Utah, and microalgae have no promise to uptake large amounts of salt. The salinity alone makes it difficult to advertise remediation efforts at all within the wastewater biofuels project.

Aside from produced water, two other wastewaters, namely municipal wastewater from a nearby treatment facility and environmental brackish water taken near a fresh-water inlet of the Great Salt Lake were tested for its capacity to serve as growth media for microalgal growth in the production of biodiesel. These three wastewaters were tested for their capacity to grow both *C. gracilis* and USU080 species and produce lipids as compared to two laboratory-based media recipes. It was shown that all three wastewaters had comparable growth to that of the media recipes, with similar or vastly higher lipid productivities in wastewaters. To demonstrate the economic impact of using a wastewater to replace the purchased chemicals of laboratory medias, the chemical cost of each water source and media was determined. It was found that using a laboratory media can cost up to 24 times the cost of a wastewater, with comparable or better growth and lipid productivities.

Additional efforts to increase the efficiency of the algae to biodiesel project were also made. Phycocyanobilin was initially explored as a high value added product of cyanobacterial growth. Brown algae strains were tested for their capacity to serve as pet

food. Attempts to understand the relationship between silicate and sodium concentrations in diatoms were made to better identify the trigger of extreme cellular lipid accumulation. Initial efforts were unsuccessful, although it was found that adding salt to municipal wastewater can induce the cell to store over 65% of its dry weight as lipid. Other wastewaters were investigated for their ability to support algal growth. Whey permeate was determined unsuitable for algal growth; however, potato processing wastewaters obtained from Simplot Foods showed great promise to support growth of USU080 and *Neochloris oleoabundans* strains.

Non-phototrophic organisms also hold great potential to serve as sources of biological oils that can be used as biodiesel. Heterotrophs such as yeast and bacteria have many advantages as feedstocks for biodiesel that currently limit microalgae, such as high cellular densities, known genomes, faster growth rates, and no land- or light-limitations. These organisms do require fixed carbon sources to grow, and therefore it becomes necessary to utilize streams of waste carbon in the production process. Nutrient uptake and cellular composition was observed throughout the batch lives of bacterial strain *Rhodococcus opacus* PD630 and yeast strain *Cryptococcus curvatus*. In the yeast strain, it was found that dry weight increases until the carbon source depletes from solution, and lipid accumulation seems to increase as soon as nitrogen is taken up from the cells. Whey permeate, a wastewater resource from dairy manufacturing with high lactose concentrations, was found to grow *C. curvatus* well with only addition of ammonium sulfate; *R. opacus* grew somewhat only when the wastewater was diluted to 25% strength. Blue Bunny® Waste Mix, a wastewater resource from an ice cream plant containing many sugars and fats, was also found to support growth of *C. curvatus*, with

up to 7.8 g biodiesel being formed from every liter of waste mix per day when the waste mix is diluted to 25% strength. *C. curvatus* was able to grow on mixtures of glucose and xylose, the two major components of lignocellulosic biomass. It was also shown to grow on wheat straw hydrolysate without the addition of an external nitrogen source. Heterotrophs, particularly the yeast organism *C. curvatus*, hold great promise as a feedstock for biodiesel when sources of waste carbon are considered.

Phototrophic microalgae and heterotrophic bacteria and yeast all hold the capacity to utilize wastewaters for the production of biodiesel. Because of limitations on freshwater resources, it will be necessary to incorporate technologies and biological strain selection that include the replacement of wastewater for freshwater for large-scale production of biodiesel. The vast technological development of over a century is taking the idea of Rudolph Diesel's peanut oil diesel engine in 1900 to the potential of powering thousands of vehicles with a sustainable biological fuel from wastewater. Diesel's prophetic statement is becoming a reality: "motor-power can still be produced from the heat of the sun…even when all our natural stores of solid and liquid fuels are exhausted" (Nitske & Wilson, 1965).

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APPENDICES

APPENDIX A. PERMISSION TO USE

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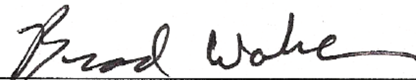
Signed  ______________________________

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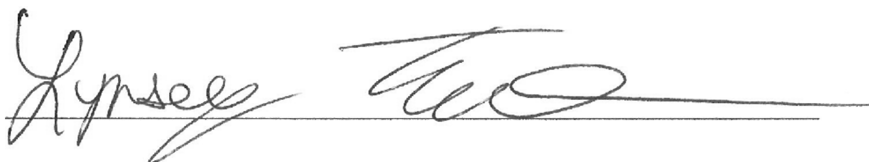
Data from growths of *Cryptococcus curvatus* grown on Delac media.

Signed 

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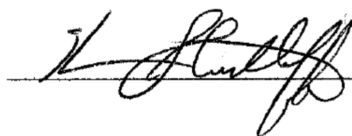
Signed 

APPENDIX B. Developed Protocols

Protocol for Total Carbohydrate Determination (Phenol Sulfuric Acid Method)
Valerie Godfrey December 2011

Prepare solutions:

1. 5% Phenol: 5 g phenol in 100 mL distilled water.
2. Standard Glucose (1 g/L): 100 mg glucose in 100 mL water.
3. Working Standard Glucose (100 mg/L): 10 mL of standard glucose solution diluted to 100 mL.

For dry biomass, the following presteps are necessary:

a. Weigh out 100 mg biomass in a test tube.
b. Add 5 mL of 3 M HCl.
c. Place test tube(s) in a boiling water bath for 3 hours. Stir occasionally.
d. (I find it most helpful to empty contents into clean 200-250 mL beaker for this step:) Neutralize solution by adding sodium carbonate until effervescence stops.
e. Fill volume to 100 mL.
f. Remove 3-5 mL into a clean test tube and centrifuge for 2 minutes.

For all samples (liquid or pre-treated and now suspended dry material):

1. Prepare standard solutions:
    a. Pipette 0.2, 0.4, 0.6, 0.8, and 1.0 mL working glucose stock into separate test tubes. Fill to 1 mL.
2. Pipette 0.1 mL sample solution into new test tube. Fill to 1 mL. (Note: some samples will need to be diluted to varying degrees so as to fit within standard curve)
3. Set a blank with 1 mL water.
4. Add 1 mL 5% phenol solution to each tube.
5. In the fume hood and with gloves/goggles/coat, add 5 mL 96% sulfuric acid to each tube. (I find that the 5 mL pipette works well, or the serological 10 mL pipette. Addition should be rapid and directly into solution.) Tubes will be hot to touch. Use only tube rack, not Styrofoam holder.
6. Vortex carefully (use appropriate vortex speed for test tube).
7. After 30 minutes, read absorbance at 490 nm.
8. Calculate the amount of carbohydrate using the standard curve.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. Colorimetric method for determination of sugars and related substances, 1956. Anal. Chem., 28, 350-356.

Protocol for Total Protein Determination (Lowry Method)

Valerie Godfrey December 2011

Prepare solutions:

1. CTC (copper-tartrate-carbonate): 100 mg cupric sulfate and 200 mg sodium tartrate dissolved in 50 mL distilled water. Dissolve 10 g sodium carbonate in 50 mL distilled water. Slowly mix the two solutions together. Stable for 2 months at room temperature.
2. 10% SDS (sodium dodecyl sulfate): 10 g in 100 mL distilled water.
3. 0.8 M NaOH: 8 g in 250 mL distilled water.
4. 0.15% DOC (deoxycholate): 150 mg in 100 mL distilled water.
5. 20% TCA (trichloroacetic acid): 10 mg in 50 mL distilled water.
6. Reagent A: Mix equal volumes of stock CTC, NaOH, SDS, and distilled water (25 mL each works well for many samples at a time). Keeps for 2 weeks at room temperature. Solution may form white-light blue crystals which will dissolve in mild heat or is also fine to use if mixed thoroughly before using.
7. Reagent B: One volume Folin-Ciocalteu phenol reagent (2 N) mixed with 5 volumes distilled water. Stable at room temperature in amber storage bottle.
8. BSA (bovine serum albumin): Stored in main lab freezer as 10 mg/mL microcentrifuge tube stocks. Completely thaw one stock and pipette out 50 µL into a new 1.5 mL microcentrifuge tube . Dilute to 1 mL with distilled water (0.5 mg/mL). Prepare a working stock by removing 600 µL into a test tube and diluting to 3 mL (0.1 mg/mL).

Procedure:

a. Weigh out 50 mg biomass in a 150 mL Erlenmeyer flask (works well in batches of 6 samples).
b. Add 100 mL of distilled water.
c. Sonicate each solution for 10 seconds until chunks of biomass are dissolved (up to 30 seconds total, algae biomass takes longer than yeast).
d. Remove 10 mL biomass solution into xxx centrifuge tubes. (If samples are already in solution, remove sufficient volume to contain 1-50 mg protein)
e. Add 1 mL 0.15% DOC solution. Mix and wait 10 minutes (cutting this time down seems to lead to poor cell pellet formation).
f. Add 1 mL 20% TCA solution and mix. Protein will immediately begin to precipitate.
g. Tare bottles for centrifugation using distilled water. Using the SS-34 rotor, spin for at least 30 minutes at 20,000 g. (I prefer using the old Sorvall centrifuge because it takes much longer to slow down and disturbs the cell pellet less; also, less people like to use it and when I'm running multiple batches I can generally keep the rotor between cycles.)
h. Take a beaker for waste (100 mL plastic beaker sufficient) to the centrifuge room. Immediately decant solution from pellet. If protein pellet dislodges into solution, re-centrifuge. (It is typical to see translucent "floaties" in supernatant, this is okay). Wipe away any drops of supernatant from inverted tube with a Kimwipe.

i. To protein pellet in centrifuge bottles, add 1 mL Reagent A to dissolve protein. Stir with a metal spatula to aid if necessary.
j. Add 10 mL distilled water.
k. Remove 0.2 mL dissolved protein solution to a clean test tube. Fill to 1 mL.
l. Prepare BSA standards (best done during centrifugation process): Remove 50, 100, 250, 500, 750, and 1000 μL working BSA stock (0.1 mg/mL) into six clean test tubes and fill each to 1 mL.
m. Prepare blank solution of 1 mL distilled water in clean test tube.
n. To all samples, standards, and blank, add 1 mL Reagent A. Mix and wait 10 minutes.
o. Add 0.5 mL Reagent B. Mix.
p. After 30 minutes (before 90 minutes), read absorbance at 750 nm. Color loss is 1-2% per hour following.
q. Calculate protein content using standard curve.

O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall. J. Biol. Chem, **193** (1951), pp. 265–275.

Peterson, G.L., 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83, 346-356.

Chemical Principle:
TCA is known to precipitate all proteins in solution (the acid disrupts the surface charges of the protein folding which causes proteins to aggregate and precipitate from solution). Deoxycholate aids this process lysing the cell membrane and recovering membrane proteins as well. Complete resolubilization of the protein pellet free of other interfering substances (i.e. lipids) is necessary for accurate reading. Interfering substances will cause a lower protein yield. The phosphomolybdic tungstic mixed acid in the phenol reagent reacts with mainly tyrosine residues to cause a blue color with an absorbance maximum at 750 nm.

Note: Some algae papers hydrolyze biomass in 1 M NaOH for up to 24 hours before TCA precipitation. It has been cited that such conditions causes oxidation of lipids which react with the Lowry assay.

Process for preparation of wheat straw hydrolysate (Yu et al., 2011).
Valerie Godfrey February 2012

1. Wash straw and dry completely
2. Blend straw to powder
3. Acid pre-treatment: Using 20mL H_2SO_4 per L distilled water (2% v/v), stir in 100 g milled straw per L dilute acid
4. Autoclave (121°C for 60 min.)
5. Centrifuge
6. Vacuum filter (store at 4°C until further use)
7. Using NaOH, bring the pH up to 5.5
8. Add micronutrients of following concentrations, readjusting pH to 5.5 as needed:
    a. 0.4 g L^{-1} $MgSO_4 \cdot 7H_2O$
    b. 2 g L^{-1} KH_2PO_4
    c. 3.51 mg L^{-1} $MnCl_2 \cdot 4H_2O$
    d. 0.1 mg L^{-1} $CuSO_4 \cdot 5H_2O$
    e. 1.5 g L^{-1} yeast extract
9. Filter through a 0.22 µm membrane

Additional detoxification steps

1. Wash straw and dry completely
2. Blend straw to powder
3. Stir in 100 g milled straw to 1 L dilute sulfuric acid (20 mL H_2SO_4 in 1 L).
4. Autoclave (121°C for 60 min.)
5. Centrifuge
6. Vacuum filter (store at 4°C until further use)
7. Heat to 42°C while stirring
8. Add NaOH until pH reached 10.0. Maintain temperature at 50°C on a heated stir plate for 30 minutes.
9. Reacidify with sulfuric acid to pH 5.5
10. Add micronutrients listed in step 8 above, readjusting pH as needed.
11. Filter through a 0.22 µm membrane

Yu, X., Zheng, Y., Dorgan, K.M., Chen, S., 2011. Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. Bioresour. Technol. 102, 6134-6140.