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Algal Production Studies

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HNEI
Hawai'i Natural Energy Institute
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Month 2 Deliverable.

A. Identifying low-cost or waste organic carbon and organic acids from industrial processes.

B. Results of laboratory testing on existing microalgal strains and their affinity for using waste organic acids and carbon.

HAWAI'I BioEnergy

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Month 2 Deliverable.

A. Identifying low-cost or waste organic carbon and organic acids from industrial processes.

Introduction

Hawai'i BioEnergy (HBE) is exploring the production of mixotrophic microalgae as a biofuel crop. A mixotrophic system has the potential for faster growth and higher densities compared to a typical photosynthetic growth system. This is because algae in a mixotrophic growth system can continue to grow without light via heterotrophic processes. This allows microalgae to be grown at greater depth resulting in greater productivity per area and capital basis.

Cost modeling performed by HBE has determined that the costs of the organic carbon (OC) and the conversion rate of OC to lipids are largely the critical drivers of a financially viable mixotrophic growth system. Simply, the prevailing market costs of sugar are too expensive for a commercial mixotrophic system. For example, at HBE's current lipid concentration and the rate at which glucose is converted to biomass, the cost of glucose is $20 per gallon of oil produced. Therefore, it is extremely important to explore alternative sources of OC in order to lower these input costs.

In addition to glucose, literature has shown that mixotrophic microalgae are capable of metabolizing organic acids and other organic carbons via their heterotrophic processes. Largely developed technologies that produce bio-based products, food, fuel, and energy often produce a waste stream that may contain valuable organic acids and other organic carbons. These waste sources can be leveraged to improve the efficiencies of these biorefineries, turning waste into added biomass, and can be suitable alternatives to glucose, which is extraordinarily expensive, costing $575 per ton. This report will summarize the waste streams containing organic acids and other carbon-based compounds that could be a substitute to industrial-grade glucose.

Known Organic Carbons Used by Microalgae

First, to be an adequate substitute for glucose, the OC must be able to be metabolized by mixotrophic microalgae. The following table is a list of OC's that have been shown to be capable of sustaining micro algal growth. As previously mentioned, glucose is normally the preferred OC for mixotrophic algae. This may be because glucose is the simplest sugar, is the primary product of photosynthesis and has a very high-energy content. However, other OC's specifically, other monosaccharides and acetic acid or acetate (salt of acetic acid) have also been shown to support high amounts of growth of microalgae. Acetic acid, like glucose is one of the simplest forms of its respected group, in this case carboxylic acids. Acetic acid/acetate is a product of natural fermentation and therefore is normally present in the environment. This may be why mixotrophic capable algae are frequently able to metabolize acetic acid or more often acetate. Acetate is acetic acid without an H+, and is the normal form in the environment when the pH is above 5.5.

Organic Carbon	Market Cost per Ton	Microalgae affinity to OC based on literature	Source

Glucose	$575	Very High	Perez-Garcia et. al (2010) and others.
Sucrose	$540 (raw)	Very low to low	Samejima and Meyers (1958) and Martinez et. al (1987)
Fructose	$450	Medium	Martinez et. al (1987)
Galactose	$540	Medium	Martinez et. al (1987)
Acetic Acid	$450	Medium to high	Martinez et. al (1987), Perez-Garcia et. al (2010)
Lactic Acid	$500	Very Low to low	Samejima and Meyers (1958)
Glycerol	$500-750 (food grade)	Low to medium	O'Grady and Morgan, 2011
Sodium Acetate	$1420	Medium to high	Samejima and Meyers (1958)
Sodium Formate	$500-800	Low	Samejima and Meyers (1958)
Levoglucosan	$3000	High	Bennett et al. (2009)

Table 1. Current market costs of OC and their reported affinity for growth by microalgae.

Waste Organic Carbons from Biofuel and Bioenergy Production

HBE and its partners are exploring the thermochemical conversion of raw biomass via either pyrolysis or gasification as part of an integrated bio-refinery. An integrated bio-refinery accepts numerous types of feedstocks and produces various products in order to maximize its sustainability both, economically and environmentally. Currently, feedstocks would come from 20,000 acres of a mixture of eucalyptus and sorghum.

Gasification and F-T Process

The gasification process heats biomass to over 800°C in an oxygen-limited environment, in order to prevent combustion, and produces a mixture of carbon monoxide and hydrogen known as syngas. The syngas is then converted to diesel or other types of liquid fuel via the Fischer-Tropsch (F-T) process. The F-T process takes the syngas through a number of chemical reactions to reach its final liquid stage. It is during these chemical reactions that a waste stream rich in OC is produced. According to Majone et al. (2010) this waste stream has a very high chemical oxygen demand (COD) of around 28 mg/l. The large majority, around 10%, of the wastewater is short and long chain alcohols, mainly methanol and ethanol. Around 3% of the wastewater is from organic acids, mainly acetic acid and propanoic acid (Majone et al. 2010).

Pyrolysis

Flash pyrolysis is a technology capable of converting cellulosic residues like eucalyptus or sorghum biomass to a bio-oil through rapid heating in an oxygen free environment. The conversion of biomass to pyrolysis oil can be as high as 70% w/w (Mahfud et al. 2008). This resulting bio-oil has high amounts of water, is acidic and varies in composition depending on feedstock input (Rasrendra and Heeres, 2010). It therefore requires further processing to be converted into higher-value liquid fuels like diesel, gasoline, and jetfuel. A study by Sipila et al. (1998) looked at the composition of the pyrolysis oil from three different feedstocks: wheat straw, Scots pine and a pine-maple mixture. Table 2 below shows the pH and the percentage of some of the main components.

	Wheat Straw Oil	Scots Pine Oil	Maple-Pine Oil
pH of oil	3.7	2.6	2.8
Water %	19.9	11.1	23.2
Total Acids %	10.8	7.3	6.42
Acetic acid %	7.41	3.45	4.59
Formic acid %	1.85	2.47	1.10

Table 2. Composition of three different pyrolysis oils given as percent weight.

There are a number of ways to extract the organic acids from the pyrolysis oil. Mahfud et al. (2008) gives examples of catalytic distillation and membrane separation. A recent report by Rasrendra and Heeres (2010) dealt specifically with extraction of acetic acid from pyrolysis oil. They focused on a continuous reactive extraction system using long-chain tertiary amines. Using this technology, they consistently were able to extract over 70% of the acetic acid from pyrolysis oil in a continuous process.

A study done by Rasrendra and Heeres (2010) looked at the potential different waste products from pyrolysis oil and showed that it also contained carbohydrates. They reported that the two largest components of pyrolysis oil were levoglucosan (10-30 % wt.) and glycolaldehyde (5-15 % wt.). Levoglucosan ($C_6H_{10}O_5$) is a six-carbon ring structure commonly found following pyrolysis of starch and cellulose. It is routinely used as a chemical tracer in atmospheric chemistry studies because it is so commonly associated with thermochemical decomposition of organic material.

Glycolaldehyde ($HOCH_2$-CH=O) is the smallest possible molecule that contains both an aldehyde group and a hydroxyl group. It therefore is also therefore the simplest sugar with 2 carbons. Even though it is a sugar it actually inhibits fermentation and photosynthesis. In photosynthesis, glycoaldehyde inhibits CO2 fixation and stops the calvin cycle. Glycolaldehyde also is responsible for disrupting fermentation and has been identified as a key inhibitor of bioethanol fermentation.

Acetic Acid, Formic Acid, Levoglucosan, and Algal Growth

Acetic acid and formic acid are the two main organic acids produced in pyrolysis. Numerous studies have shown acetic acid or sodium acetate (a salt of acetic acid) as an organic carbon source in microalgae growth systems. Richmond (2007) discussed using acetic acid as the carbon source of a mixotrophic open outdoor culture of microalgae. Acetic acid was used as it led to lower bacterial loads than sugars. The system fed acetic acid automatically when the pH reached 7.0 until it declined to 6.5. They reported that it took between 2 and 4 grams of acetic acid to produce 1g of algae. Another study by Liang et al., (2009) reported algal densities of 0.9 g/l with 31% lipids using a 1% sodium acetate addition. Using 1% glucose they achieved 1.2 g/l and 23% lipids.

Few studies examine the use of formic acid as a mixotrophic algal feed. One study by Samejima and Myers (1958) fed sodium formate (salt of formic acid) to two types of green algae. They reported no significant differences between cultures fed formate and those not receiving anything. However, because pyrolysis oil would produce large amounts of formic acid after upgrading it is important to look for strains that may be able to use it. Formic acid is the simplest of the carboxylic acids.

A study by Bennett et al. (2009) showed that levoglucosan from pyrolysis bio-oil could be extracted from phased separation and then converted to glucose via acid hydrolysis. They then fermented the glucose to ethanol showing a route for cellulose to be converted to ethanol. A study by Luyen et al. (2007) showed that adding levoglucosan to microalgae cultures increased cell density. The fed 10mM of levoglucosan to 8 different microalgae species and all showed over a 100% increase in cell density compared to the control.

Biodiesel

The production of biodiesel from lipids like vegetable oils is rapidly increasing. Vegetable oil, which is a triglyceride, is converted into biodiesel through a multi-step process. Ultimately, the glycerol must be separated from the triglyceride to isolate the long chain fatty acids that make up the backbone of biodiesel. This process is known as transesterification. Glycerol makes up about 10% by weight of triglycerides or for every 9kg of biodiesel, 1kg of crude glycerol is produced (Centi et al., 2009). In 2006, the US produced 150 million gallons of biodiesel, which created over 50,000 tons of crude glycerol. However, without further processing crude glycerol from transesterification has a very low value because of impurities. These impurities make up about 20% of the crude glycerol and consist mainly of water and salts (NaCl, KCl, etc.) (Centi et al., 2009).

Glycerol, which is used in the food industry as a solvent and sweetener and in cosmetics and soap production requires a very pure or “food-grade” quality. Purification of the crude glycerol from transesterfication is costly and is usually out of the range of small to medium biodiesel producers lacking the economies of scale (Pachauria and He, 2006). As biodiesel production has increased, crude glycerol has become a large waste stream with a limited market.

Glycerol and Algal Growth

A potential use of crude glycerol would be a carbon source for heterotrophic or fermentative growth of algae, fungi or bacteria. Pachauri and He (2006) showed glycerol could be fermented by microorganisms

to produce a number of high-value products including hydrogen and succinic acid. Grady and Morgan (2011) grew Chlorella protothecoides, a microalga, successfully in the dark using reagent-grade (high quality) and crude-glycerol as the OC source. The results showed no difference between the sources of glycerol indicating that crude glycerol could be used in algal production systems. Cultures grown on glycerol had a specific growth rate and final lipid yield of 0.1/h and 0.31 g lipid/g substrate, respectively. The values were similar to those observed on pure glucose, 0.096/h and 0.24 g lipid/g substrate.

Waste Streams from Agriculture and Food Processing

Because of the high costs of industrial grade OC (See Table 1), it is important to explore alternative sources of sugars that could lower the OC costs. There are many possible sources within the agricultural and food processing industries. Below, are those HBE has identified that either have an existing large local supply or could be made possible relatively easily.

Sugar Production

Sugarcane processing to granulated sugar produces a by-product of molasses. The composition of molasses is approximately 48% sugar, 24% water and 16% ash. Remaining major constituents include nitrogen and potassium and trace amount of vitamins and metals. The sugars in molasses are mainly sucrose, however, glucose and fructose are also present. A ton of molasses ($140) cost around 75% less than a ton of industrial glucose ($565). A ton of molasses is only around 0.5 ton of sugar. Therefore, a ton of sugar sourced from molasses would cost $280 or about 50% that of raw sugar or glucose.

Molasses and Algal Growth

The properties of molasses, including its high content of available sugars and other growth nutrients makes for a potential high-quality OC feed for mixotrophic algal systems. Yan et al., (2011) studied heterotrophic growth of *Chlorella,* a microalga, using molasses and the decreased production costs of using molasses.

Results of a molasses feeding study by Yan et al., (2011) confirmed that the algae were in fact capable of growing heterotrophically on crude-molasses. However, growth rates and densities were much higher after the molasses underwent enzymatic hydrolysis. Enzymatic hydrolysis converts sucrose to glucose, which is the preferred sugar for the alga.

Results showed that adding 17 g/l of molasses produced 8.8 g/l of algae without hydrolysis and 16.9 g/l of algae with molasses that underwent hydrolysis. The latter, nearly a 1:2 conversion of sugar (molasses ~50% sugar) to biomass is considered extremely efficient. The same study also reported high yields of algae (79 g/l) using a growth media consisting of only molasses in a 5-liter closed fermentor maintaining 30 g/l of molasses. These results confirmed another hypothesis that the molasses had enough nutrients to support algal growth.

The paper reported cost of $3.21, $1.65 and $ 2.21 per liter of oil produced from algae growing on glucose and nutrients, molasses and nutrients and only molasses, respectively. These costs included capitol costs, labor and all other costs associated with the production system. The enzyme responsible for converting sucrose to glucose, β-invertase, costs approximately $300 per kilogram. According to the

study, the use of the enzyme raises the price of a liter of algae oil by approximately $0.31. However, they report that using molasses instead of glucose decreased the costs of a liter of algae oil by approximately $0.90, resulting in a net saving of approximately $0.60 per liter.

It should be noted that reviewing the metrics given in the paper of a 1:2 conversion of molasses to biomass, a lipid content of 45% and a molasses cost of $140 per ton gives a cost of roughly $1.14 of molasses per gallon of oil or $0.30 per liter of oil. These numbers agree with the reported costs Yan et al. (2011) paper and illustrate the optimal metrics of oil content and conversion rate of OC to biomass required for a mixotrophic system to make economic sense.

Sorghum Production

Sweet sorghum is a C_4 crop with high biomass productivity and is being considered as a bioenergy crop for Hawai'i. Sorghum has a sucrose-rich juice that can removed following harvesting. HBE is considering a biorefinery plant utilizing sorghum as a base feedstock. Following harvesting, sorghum would be juiced, dried, and then converted into a liquid fuel. The sorghum juice could then be used as a feed in a mixotrophic microalgae system.

Sorghum Juice and Algal Growth

Gao et al., (2010) studied feeding of sorghum juice to the microalgae, *Chlorella*. They performed hydrolysis on the sorghum juice prior to feeding, as sorghum juice is largely composed of sucrose. Results show that hydrolyzed sorghum juice at a rate of 10 g/l (glucose) produced approximately 5.2 g/l of algae in 120 hours, roughly a 2:1 sugar to biomass conversion efficiency. The same rate of pure glucose produced approximately 3.9 g/l of algae in 120 hours in the study, a 2.5:1 conversion efficiency.

Although the yields of algae using sweet sorghum are less than molasses, it is difficult to compare the two because the results are from two different studies with likely two different species of microalgae. A single study using the same growing method for each OC would be needed to compare different OC's

HBE would ideally have approximately 5,000 acres of sweet sorghum in production, which would generate roughly 45,000 ton of sugar per year.

Coffee Pulp: A possible waste source of sucrose

The process of removing the pulp and skin from a coffee cherry in order to get the coffee bean presents some opportunities for obtaining a cheap local OC source. Around 40% of the total coffee harvest is pulp and mucilage. The composition of coffee pulp and mucilage has a sugar content of approximately 13% (mainly as sucrose)as well as considerable amounts of nitrogen and other nutrients (von Enden and Calvert, 2003). The pulp is normally allowed to dry out, where it begins to ferment and break down, before it is composted.

Hawaii's annual production of 6 million pounds of green coffee bean, produces nearly 7500 tons of coffee pulp. Assuming a 13% sugar content, this represents nearly 1000 tons ($540,000 value) of available sucrose from a waste source. Currently there is little industry or demand for the coffee pulp, although recently, a Hawai'i company has begun marketing an energy drink manufactured from the spent coffee pulp.

In order to extract the sugar from the coffee pulp some sort of processing would need to take place. Likely, pressing the pulp will extract a decent portion of the sugar. However, further processing via enzymatic or mechanical breakdown will likely increase the sugar extraction efficiency but would come with added costs.

Dairy: A source of lactose and lactic acid

Lactose is a disaccharide of galactose and glucose. Cow's milk is around 8% lactose. Cheese production produces a waste liquid after the milk has been curdled known as whey. Whey, when dried is up to 70% lactose. Whey has many commercial uses including nutritional supplements such as whey protein. Wang and Peng (2008) grew Chlorella in the dark on lactose, glucose, sucrose and fructose. Of all the sugars, lactose produced the least amount of algal biomass and resulted in the highest residual sugar reading following the growout. Algal density using 40g/l lactose was just 1.42 g/l, a 28:1 conversion efficiency. For comparison, glucose at 40g/l produced algal densities of 10.7 g/l, about a 4:1 conversion efficiency. This paper along with others has indicated algae have very low affinity for lactose. However, whey can be converted to glucose and galactose via an enzyme. By converting it to simple sugars a mixotrophic algal feed could easily be created.

Silage production for dairy animal feed presents another opportunity for carbon rich waste sources. Silage is a preservation method for harvested grass that allows for long-term storage. It is preserved via natural fermentation that results in an acid-stable food source. However, through both the silage making process and storage of silage, a runoff of silage effluent can be created. This effluent, if not managed properly can be a major pollutant as it has a very high biological oxygen demand.

According to USDA, silage effluent should be diluted by dairy wastewater and used in irrigation or be diverted into an open top liquid manure tank and allowed to evaporate. Silage effluent contains high amounts of lactic acid, water-soluble carbohydrates (simple sugars like glucose and fructose), minerals, and nitrogen.

Other Sugar Crops

Other crops related to Hawai'i with potential for fermentation or heterotrophic microalgae production includes many fruit crops. Juice from crops like papaya, mango, pineapple, etc. all have high amounts of sucrose and could likely supply heterotrophic microalgae with an OC source, especially if the juice is hydrolyzed to glucose. Although these fruits are expensive, it may be possible to obtain rotten, bruised and broken fruit as a cheap OC source.

Estimated Production in Hawaii

Organic Carbon (OC)	Volume: Hawaii	Estimated Cost	Pre-processing	Conversion Rate (g OC per g algal)
From Agricultural and Food Processing				
Molasses	55,000 tons	$140/ton	Sterilization and enzymatic	0.5 to 1.5

	sugar /yr		hydrolysis	
Coffee Pulp	1400 tons sugar /yr	Low	Juicing, sterilization, and enzymatic hydrolysis	1.0 to 2.5*
Sorghum Juice	45,000 tons sugar /yr**	Low	Juice extraction, sterilization and enzymatic hydrolysis	1.0 to 2.5
From Biofuel and Bioenergy production and processes				
Organic Acids Mainly as Acetic Acid	7500 tons /yr**	Low	Reactive extraction	2.0 to 4.0
Levoglucosan	8750 ton /yr**	Low	Phased separation. Acid hydrolysis to glucose also possible.	TBD
Glycerol	150,000+ gallons	Low	Separation process to remove water and salts.	4.0+

*Estimate
** Based upon envisioned HBE biorefinery.

Conversion Processes

Literature cites particular strains capable of utilizing waste and low-cost OC available in Hawaii. Although, microalgae strains likely prefer glucose over these low-cost OC, their use could still result in a greater areal productivity relative to a photosynthetic culture and at a lower cost.

However, if a local strain capable of using a particular low-cost OC is not found, a chemical conversion of the waste OC may be necessary. For example, initial work by HBE has found that acetic acid needs to be converted to acetate to maximize productivity. Another example is sucrose. HBE has currently not identified an algal strain able to grow on sucrose. However, if sucrose is converted to glucose either through an enzymatic or chemical transformation then current algal strains would be sufficient.

Conclusions

This reported has pointed out numerous potential sources of OC that are less costly than industrial glucose because they are often by-products. Many have been reported within the literature to be metabolized by microalgae. Additionally, many of these are currently available or are anticipated to be available locally. Assuming reported conversion efficiencies and utilizing these local, low-cost carbons sources could produce over 96,000 tons of algal biomass each year. This amount of biomass at 25% oil content could produce approximately 5.3 million gallons of bio-oil suitable for further refining for biofuels.

Molasses is the largest single organic carbon source identified in this report and the most expensive. If you remove molasses, and just focus on the cheaper OC, the mixotrophic algal system could potentially produce 2.5 million gallons of oil per year.

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Month 2 Deliverable.

B. Results of laboratory testing on existing microalgal strains and their affinity for using waste organic acids and carbon.

Strain Testing for Affinity to Waste Organic Acids and Carbon.

Initial work has screened a number of algal strains found on Oahu for their capability to grow using acetic acid/acetate (AA) and other organic carbons. Early results show three strains had a significant increase in growth rate when acetic acid was present in complete dark (in **bold**). Two of these strains are capable of growing on glucose too (in **bold**). Currently only one strain has been tested in a mixotrophic system, where both light and AA are applied. Growth rate significantly increased more than the additive effect of the photosynthetic rate and the heterotrophic rate. This indicates that light may be required to get maximum growth with AA. A similar trend was observed in a mixotrophic glucose growout.

More work is being done to understand and maximize the mixotrophic growth of AA and other organic carbons. The strain "SS" will be the primary focus on these experiments as it has shown to be capable of metabolizing both acetic acid and glucose. This work will be reported on in the Month 12 deliverable.

Additionally, new strains are being isolated from both Hawaii and Maui islands to be tested for heterotrophy. The summary of these strain development efforts will be summarized in Month 9 deliverable.

Strain Name	Photosynthetic Daily Growth Rate	Daily Growth Rate (Dark)	Heterotrophic Growth Rate (Dark + AA)	Mixotrophic-Acetic Acid	Mixotrophic-Glucose	Mixotrophic-Sucrose
SS	47%	2.8%	**15.4%**	77%	**250%**	36%
HARC	87%	-7.0%	-16.9%	TBD	97%	66%
HPGB	44%	5.7%	-1.7%	TBD	124%	42%
SS-Chl2	36%	-13.5%	-17.8%	TBD	38%	35%
MG	28%	-17.5%	**6.0%**	TBD	**192%**	29%
WFWP-Scene	46%	-16,6%	-6.4%	TBD	44%	41%
HP-Scene	13%	-12.7%	**8.4%**	TBD	22%	9%

Month 6 Deliverable: Increasing the Lipid Content of Mixotrophic Microalgae

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Hawai'i BioEnergy, LLC

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Introduction

One critical metric for mixotrophic algal production is conversion of organic feeds or organic carbon to lipids. As previously explored and reported, organic feeds are costly and there is a theoretical maximum of the conversion of organic carbon to algae biomass. Therefore, the only way to increase your organic carbon to lipid conversion is to increase the lipid content of the algal biomass. This report details results of attempting to increase the lipid content by adjusting the nutrient levels in the cultures of both phototrophic and mixotrophic cultures.

Materials and Methods

All experiments were conducted in the laboratory in 1L flasks. Flasks were bubbled with air to keep the cultures mixed and supplied with O_2 and CO_2. Cultures were grown autotrophically with just light and mixotrophically with both light and organic carbon. Mixotrophic treatments were fed glucose, acetic acid or lactic acid.

Baseline Lipid Concentrations

Baseline lipid contents were determined for the microalgae grown in both autotrophic and mixotrophic conditions while maintaining ample amounts of all nutrients in the culture.

Increasing Lipid Concentrations

Numerous studies have confirmed that the depletion of nitrogen from the culture media results in increasing lipid concentrations. This is likely because environmental stresses, like nitrogen depletion decrease cell division and slow down the production of cell components without slowing down lipid production. Another theory is that the stress caused by limited N, results in the cell increasing its lipid production as a means of cell survival.

Experimental Design

The research in this report aimed to optimize this nitrogen depletion and subsequent lipid concentration increase by comparing two different ways of depleting nitrogen.

Nitrogen was depleted from the system in one of two ways. First, a limited amount of N was added and the culture was allowed to grow until all the nitrogen had been consumed while phosphate and micronutrients were continually added to maintain adequate levels. This protocol is referred to as the "Limited N Method" throughout this report.

In contrast, cultures were given high levels of nitrogen and all other nutrients until a certain density was achieved. Then the cultures were centrifuged and the high-nitrogen media was separated from the algae. The culture was then re-suspended in new media without nitrogen. This protocol is referred to as "Centrifuge Method" throughout this report.

These methods were varied to include autotrophically grown and mixotrophically grown on glucose, acetic acid, or lactic acid. Lipid concentrations were measured for a baseline, which had no nutrient deprivation stage and at Days 2, 4 and 7 during the nutrient deprivation stage.

Results and Discussion

Baseline Lipid Concentrations

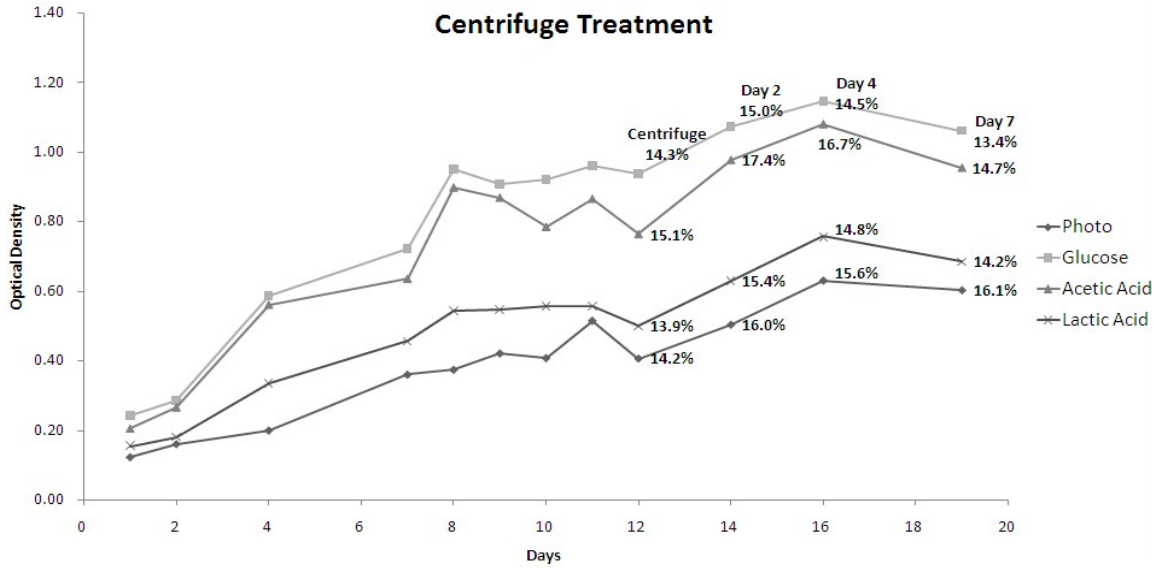
Baseline lipid concentrations for algae were determined by growing in media containing high levels of all nutrients for 14 days. On the 14th day the algae was harvested and analyzed for lipid concentration. Table 1 below is the baseline lipid concentrations as percent biomass. Baseline lipid concentrations ranged from 13.89 to 15.1%. However, large differences in biomass resulted in a large difference in lipid productivities on a daily basis.

Treatment	Lipid Concentration	Protein Concentration	Biomass (mg/L)	Lipid/Liter/Day (mg/L/d)
Photosynthetic	14.23%	38.8%	1108	12.0
Mixotrophic-Glucose	14.26%	36.5%	2692	31.5
Mixotrophic-Acetic Acid	15.10%	29.1%	2487	26.8
Mixotrophic-Lactic Acid	13.89%	40.0%	1776	17.6

Table 1. Baseline Lipid Concentrations of Algae Grown in High Nutrient Media

Effect of Nitrogen Depletion Method

The complete growout measured by optical density with lipid concentrations is given in Figure 1.



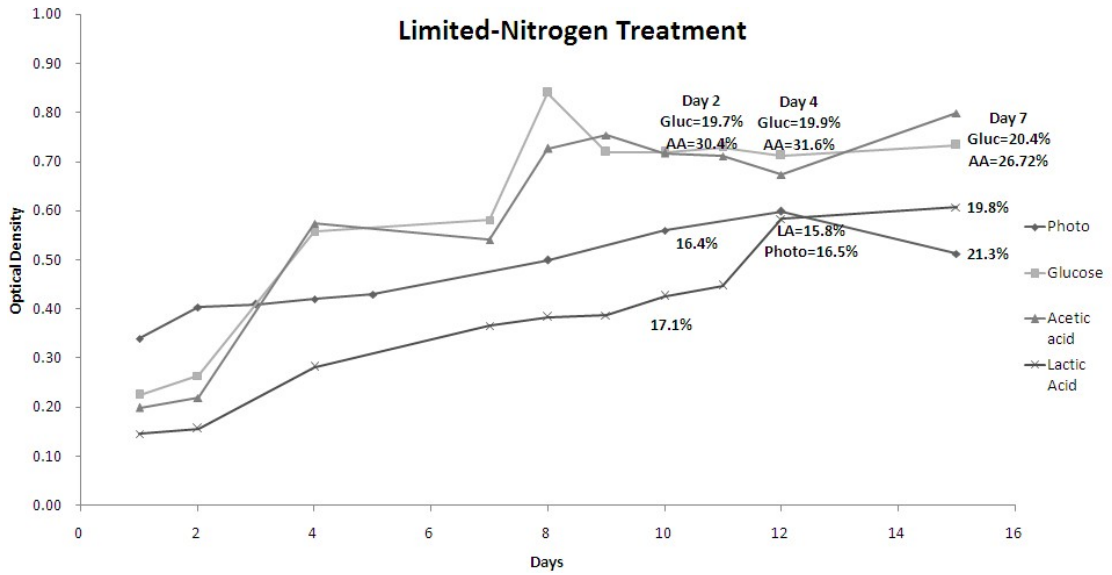


Figure 1. Growouts of both N-Depletion Methods Based on Optical Density with Lipid % at Time Points.

Limited N Method

Regardless of growth method and type of organic carbon feed, all treatments have higher lipid concentrations on day 7 of the nutrient deprivation stage than their counter-part baseline. Day 7 lipid concentrations for the Limited N Method are on average 34% higher than their relative baseline.

All treatments increase their lipid concentrations over time, except the Acetic Acid treatment, which slightly declines on from Day 4 to Day 7. Regardless, there exists a clear trend of increasing lipid concentrations with average growth of concentrations being 0.71% each day.

Treatment	Limited N Method: Lipid			Baseline
	Day 2	Day 4	Day 7	
Photo	16.40%	16.52%	21.32%	14.23%
Glucose	19.76%	19.92%	20.40%	14.26%
Acetic Acid	30.24%	31.56%	26.72%	15.10%
Lactic Acid	17.12%	15.80%	19.80%	13.89%

Table 2. Limited N Method: Lipid Concentrations of Algae Compared to Baseline

Centrifuge Method

Although the Centrifuge Method was in a zero nitrogen environment for as long as the Limited N method, it displayed very little lipid accumulation. Only 2 of the 4 treatments have higher lipid levels

relative to the Baseline at Day 7 of the nutrient deprivation stage. Day 7 lipid concentrations for the Centrifuge Method are on average 1.3% higher than their relative baseline.

Treatment	Centrifuge Method: Lipid			Baseline
	Day 2	Day 4	Day 7	
Photo	16.00%	15.64%	16.08%	14.23%
Glucose	15.00%	14.52%	13.44%	14.26%
Acetic Acid	17.36%	16.72%	14.72%	15.10%
Lactic Acid	15.36%	14.76%	14.24%	13.89%

Table 3. Centrifuge Method: Lipid Concentrations of Algae Compared to Baseline

Over time, the Centrifuge Method results in a decline in lipid concentrations. From Day 2 to Day 7, the lipid concentrations on average experience a negative growth rate of 0.59% each day.

Analysis

Further analysis of nitrogen levels in the culture reveal there is a clear difference between the Limited Method and the Centrifuge Method. (See Figure 2.) The Limited Method had lower nitrogen levels than the Centrifuge Method and had less than 10ppm of nitrogen for three days. Thus, it is likely the Centrifuge Method did not result in a nitrogen deficient environment long enough.

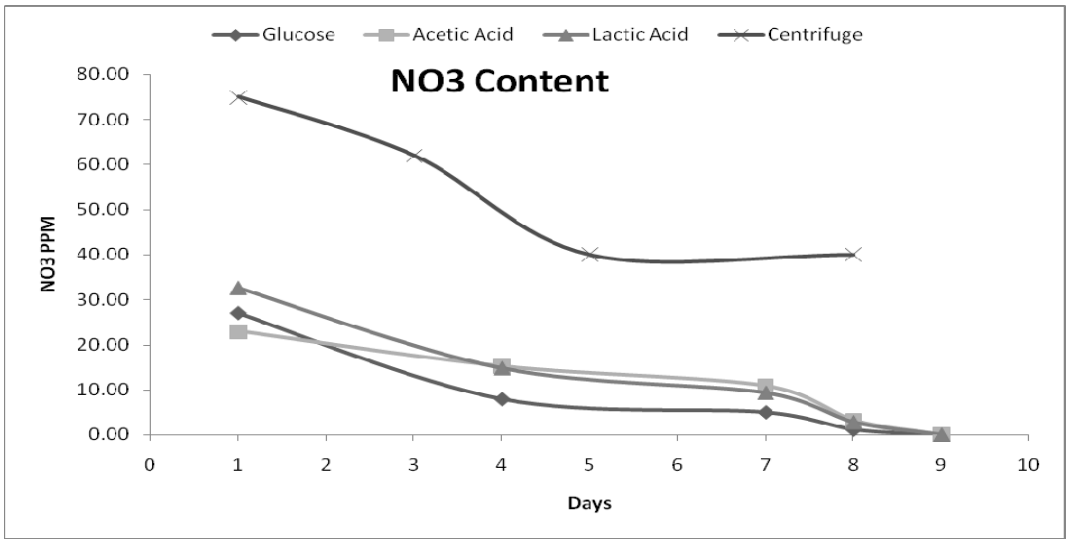


Figure 3. Average Nitrate Levels in Different Media Treatments Prior to Centrifuging or Harvesting

In addition to monitoring nitrogen levels, crude protein levels were also monitored. As seen in Figure 4 there is a clear correlation between lipid levels and protein levels. The lower the protein levels, the higher the lipid levels and vice versa.

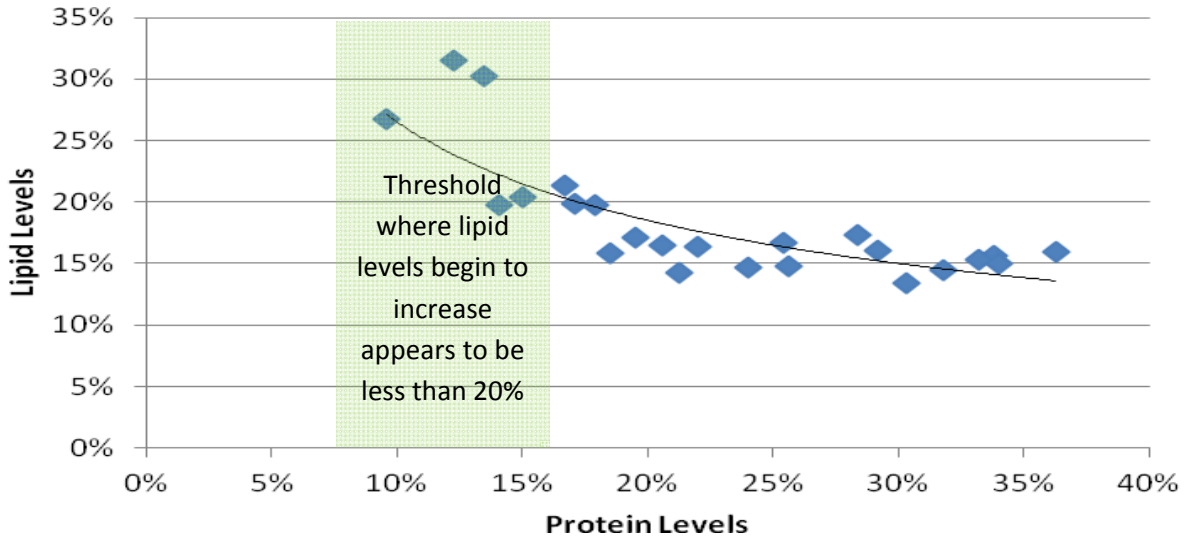


Figure 4. Lipid and Protein Level Correlation

Although the Centrifuge Method does experience protein decreases over the course of the deprivation period, the protein levels were significantly greater, at least ten percentage points higher than its corresponding Limited N treatment. (See Tables 4 and 5) There is perhaps a threshold of protein concentration of less than 20% before lipid accumulation begins to increase. This is reflective in Figure 4, where the slope of the fitted curve is significantly lower for higher protein concentrations than for lower.

Treatment	Limited N Method: Protein			Baseline
	Day 2	Day 4	Day 7	
Photo	22.00%	20.60%	16.70%	38.8%
Glucose	17.90%	17.10%	15.00%	36.5%
Acetic Acid	13.50%	12.30%	9.60%	29.1%
Lactic Acid	19.50%	18.50%	14.10%	40.0%

Table 4. Limited N Method: Protein Concentrations of Algae Compared to Baseline

Treatment	Centrifuge Method: Protein			Baseline
	Day 2	Day 4	Day 7	
Photo	36.30%	33.80%	29.20%	38.8%
Glucose	34.00%	31.80%	30.30%	36.5%
Acetic Acid	28.40%	25.40%	24.00%	29.1%
Lactic Acid	33.20%	25.60%	21.30%	40.0%

Table 5. Limited N Method: Protein Concentrations of Algae Compared to Baseline

It is hypothesized as nitrogen becomes limited, the algal cell is unable to synthesize new protein and internal protein is being consumed to maintain cell health, and likely build lipids. If this is the case, the Centrifuged Method would have needed to be in a nitrogen deprived state longer.

In summary, the Limited N Method was effective at increasing lipids largely because it was more effective at creating a nitrogen deprived environment. Additionally, very close monitoring of nitrogen cycle, from the media to the stored N as protein, would help optimize the accumulation of lipids.

Effect of Organic Feed on Lipid Concentration

Figure 5 below shows the average lipid concentration of each organic feed during the lipid deprivation stage for the Limited N method. The acetic acid feeding has substantially higher lipid levels than the autotrophic treatment and other mixotrophic treatments. (See Figure 5) It had 47% higher lipid levels than the next closest level.

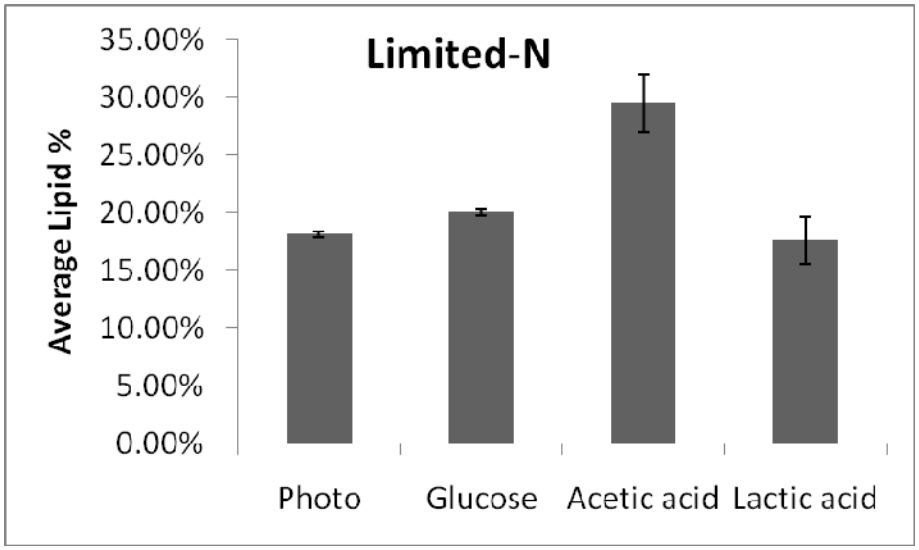


Figure 5. Average Lipid Concentration During Limited N Method with Standard Deviation

Interestingly for the Centrifuge Method, where there was very little nitrogen related stress, the acetic acid treatment also has a higher lipid level than the other treatments. (See Figure 6)

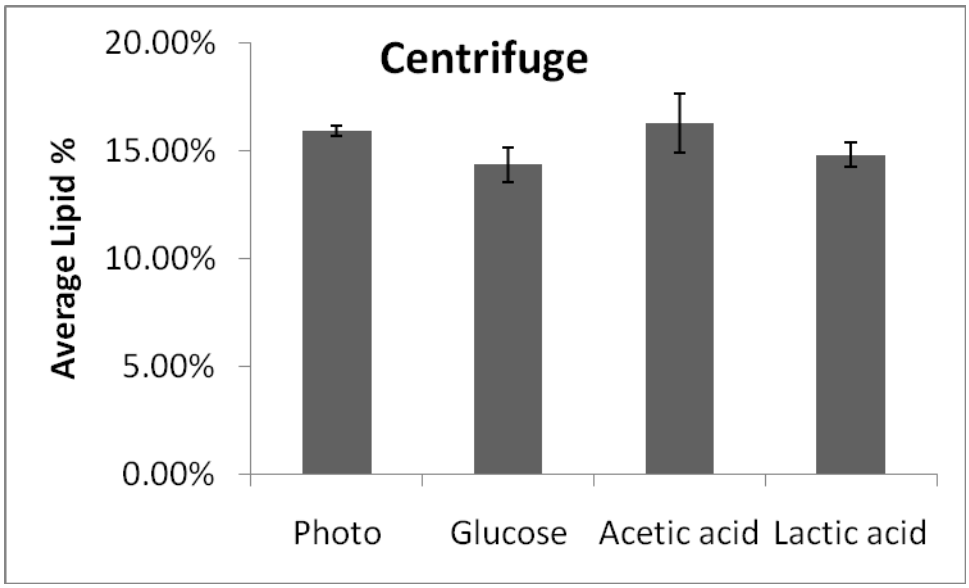


Figure 6. Average Lipid Concentration During Centrifuge Method with Standard Deviation

Analysis

It is clear acetic acid has an impact on lipid levels. In order to confirm this, bacteria populations of both an acetic acid and glucose mixotrophic culture were compared. It was reasoned that higher bacteria levels would increase biomass and "dilute" lipid concentrations. However, bacteria were very similar for both treatments except for a huge spike following the day 2 feeding. This data in conjunction with the N levels in the growout show that acetic acid may directly affect the lipid accumulation mechanism in microalgae.

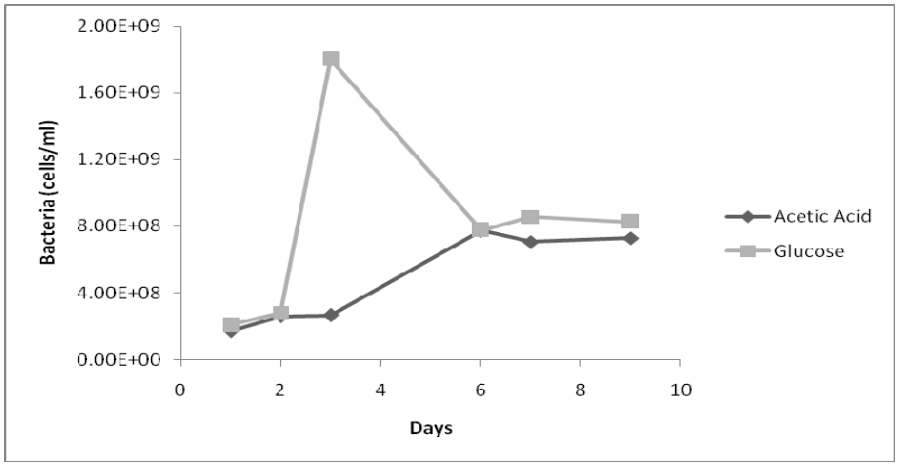


Figure 7. Bacterial Populations of Mixotorphic Cultures

Given that acetic acid is relatively more expensive than glucose it was tested to see if it could be applied as a "finishing" step and quickly and directly impact lipid levels. A culture growing on a glucose media with high rates of N and adding acetic acid in the last 2 days as a "finisher" increased the lipid concentration 14.4% to 18.4%. Although this is less than the concentration of the limited-N Method and acetic acid (29.5%), the culture is able to retain more of its biomass because Nitrogen levels are high and growth remains high.

Most importantly, the latter indicates that acetic acid alone and without nutrient deprivation directly acts upon the microalgae and triggers lipid accumulation.

Maximizing Productivity to Maximize Lipid Yield

By limiting the amount of N in the media through gradual depletion, the lipid concentration can be increased over the baseline levels with high levels of N. However, by limiting N slower growth and lower algal biomass levels are to be expected. Limited N cultures had 12% to 40% less biomass compared to cultures receiving ample amounts of nutrients. In Figure 1, this difference in OD can easily be seen. From the fourth day, the limited N treatment begins to underperform against the centrifuge treatment with ample N. This indicated that N is already being limited and beginning to stress the algae. The large increases in optical density following the second and seventh day were from feedings of organic carbon.

To determine the efficacy of each treatment's lipid yield, the biomass and length of growout must be taken account. (See Table 6)

The most productive treatment in terms of maximizing lipids was the centrifuge method grown mixotrophically with acetic acid. This treatment produced over 0.047 g of crude lipid per liter per day of culture. Both the Centrifuge Method with glucose feeding and the Limited N method with acetic acid feeding were similar with 0.043 and 0.046 g per day, respectively. The "finishing" with acetic acid feeding resulted in a lipid productivity of 0.044 g of crude lipid per liter per day.

Method	Biomass at Harvest (mg/l)	Lipid %	Lipids / day (mg/l/d)
Photo-Centrifuge	1656	16.0%	16.6
Photo-Limited-N	1072	21.3%	14.3
Glucose-Centrifuge	3920	14.5%	43.8
Glucose-Limited-N	1800	20.0%	22.5
Acetic Acid-Centrifuge	3020	17.4%	47.7

Acetic Acid-Limited-N	1688	30.2%	46.4
Lactic Acid-Centrifuge	1608	15.4%	22.5
Lactic Acid-Limited-N	1106	17.1%	17.2
Glucose Finished With Acetic Acid	2899	18.4%	44.5

Table 6. Lipid Productivities Based on Nutrient Depletion Method

Conclusions

We were able to increase lipid concentrations by up to 50% by limiting the N in the culture media of a mixotrophic culture compared to supplying high levels of nutrients. Limiting N also decreased algal density in all mixotrophic media. Maximum lipid productivity was achieved by feeding acetic acid in a mixotrophic system regardless of N-depravation method.

Additionally, our results show that that there is a lag time following N-depletion before lipid accumulation takes place. This explains why the Centrifuge Method that only had a maximum of 7 days in an N-depleted media had lower lipid concentrations than the limited N treatment, which an additional week in low N environment. It was suspected that as N levels approached zero and protein levels drop to less than 20%, the cell is stressed and lipid accumulation is triggered.

Utilizing glucose in a mixotrophic system resulted in the highest biomass. However, lipid concentrations did not exceed 20%, which resulted in lower lipid productivities than acetic acid, which reached a 30% lipid concentration. One way to boost the lipid levels was found by adding a quick "finishing" step where acetic acid is fed. This resulted in lipid concentrations increasing from 14.4% to 18.4%, with nearly 3 grams of biomass per liter.

Given the co-product value of protein and the cost of acetic acid relative to glucose, the "finishing" approach may be more commercially viable as it would result in higher product yields with lower input costs.

Appendix 1. Raw Data from N-Depleted Experiment

Treatment	Nitrogen Depletion Method	Harvest Day	Algal Biomass at Harvest (mg/L)	Protein %	Lipid %	Ash%
Photo	Centrifuge	2	1220	36.30%	16.00%	10.82%
Photo	Centrifuge	4	1408	33.80%	15.64%	11.24%
Photo	Centrifuge	7	1656	29.20%	16.08%	1.27%
Photo	Limited N	2	912	22.00%	16.40%	6.38%
Photo	Limited N	4	1016	20.60%	16.52%	6.36%
Photo	Limited N	7	1072	16.70%	21.32%	6.52%
Glucose	Centrifuge	2	2984	34.00%	15.00%	6.75%
Glucose	Centrifuge	4	3920	31.80%	14.52%	7.00%
Glucose	Centrifuge	7	3732	30.30%	13.44%	6.20%
Glucose	Limited N	2	1672	17.90%	19.76%	6.27%
Glucose	Limited N	4	1704	17.10%	19.92%	5.56%
Glucose	Limited N	7	1800	15.00%	20.40%	7.28%
Acetic Acid	Centrifuge	2	3020	28.40%	17.36%	7.63%
Acetic Acid	Centrifuge	4	2764	25.40%	16.72%	7.08%
Acetic Acid	Centrifuge	7	2804	24.00%	14.72%	8.64%
Acetic Acid	Limited N	2	1688	13.50%	30.24%	9.72%
Acetic Acid	Limited N	4	1524	12.30%	31.56%	10.56%
Acetic Acid	Limited N	7	2096	9.60%	26.72%	21.04%
Lactic acid	Centrifuge	2	1608	33.20%	15.36%	7.76%
Lactic acid	Centrifuge	4	1568	25.60%	14.76%	8.41%
Lactic acid	Centrifuge	7	1600	21.30%	14.24%	9.48%
Lactic acid	Limited N	2	1106	19.50%	17.12%	9.73%
Lactic acid	Limited N	4	1116	18.50%	15.80%	10.11%
Lactic acid	Limited N	7	1224	14.10%	19.80%	12.44%

Optimize pH adjustments for large scale

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Introduction

Open mixotrophic algal systems present many challenges in controlling contamination of unwanted bacteria, yeast, algal consumers and other microorganisms. Because of the extremely high growth rate of bacterial populations, they can consume organic carbon and nutrients at a faster rate than the microalgae. This increases production costs and decreases lipid productivity of the system. Further, it can make growing outdoors in an open system unpredictable, making a continuous system substantially more challenging.

Most outdoor, open algal systems are batch systems, resulting in empty ponds and inefficient use of capital. Continuous systems greatly improve productivity and use capital at a greater efficiency. Additionally, most are strictly photosynthetic, resulting in densities or depth being a limiting factor. The ideal system would be a continuous, mixotrophic algal system.

Previous work by Hawai'i BioEnergy, LLC (HBE) tested a number of "extreme environments" in the attempt to find an environment where bacteria were inhibited while algae flourished. The "extreme environments" tested included in the presence of antibiotics, high saline conditions, and extreme pH levels. While all these environments decreased bacterial growth, low pH was the only treatment our mixotrophic algal strain was capable of tolerating. By lowering the pH of the mixotrophic culture with acid, bacterial populations were reduced by upwards of 90% compared to the control.

Although these early experiments were able to identify a possible method for controlling bacteria in an open mixotrophic system, the effect of abrupt pH changes on algal productivity and lipid content had yet to be studied. Therefore, refinement and optimization was needed so that pH manipulations could then be attempted outside in larger-scale ponds.

For this report, laboratory experiments were conducted to ensure that pH adjustments maximized algal productivity while controlling bacteria and then these refinements were applied to large-scale (500 – 1000 liters) outdoor open bio-reactors in an attempt to maximize mixotrophic productivity of lipids in an open system.

Lab Experiments

Effects of Low and High pH on Bacteria and Algae in a Glucose Enriched Environment

Methods

Experiments were conducted to understand how adjusting the pH affected bacterial populations in algal cultures when both glucose and growth nutrients were available. The algae strain used was the same mixotrophic strain that HBE has reported on I the past. It is a green algae of the genus *Chlorella*.

The experiments were conducted in small 200ml flasks that were capped and bubbled with air for mixing. Glucose was added on day one at a rate of one gram per liter (g/l) and nutrients were applied with a concentrated Guilards Recipe for microalgae cultures. The pH adjustments were made using either HCl or NaOH to decrease or increase the pH of the growth environment. The pH was checked daily and was brought back to the targeted pH as needed. Therefore, the culture stayed at or near the targeted pH for the majority of the experiment.

Results

Bacteria

The experiments confirmed that daily adjustment of the pH either up or down was successful in decreasing the bacterial populations (See Figure 1 and Figure 2). Further, the more extreme the pH change the lower bacterial population levels.

Control treatments were not adjusted and allowed to fluctuate naturally. The pH of the controls ranged from 7.4 to 8.2.

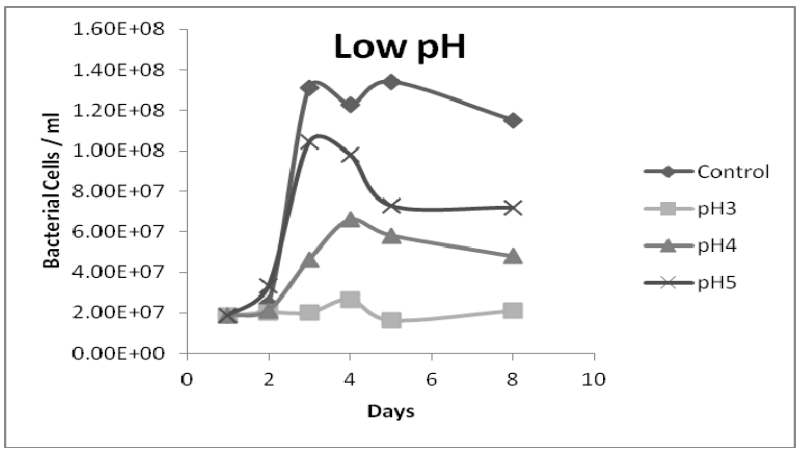


Figure 1. Effect of low pH adjustments on bacterial populations in a mixotrophic algal culture.

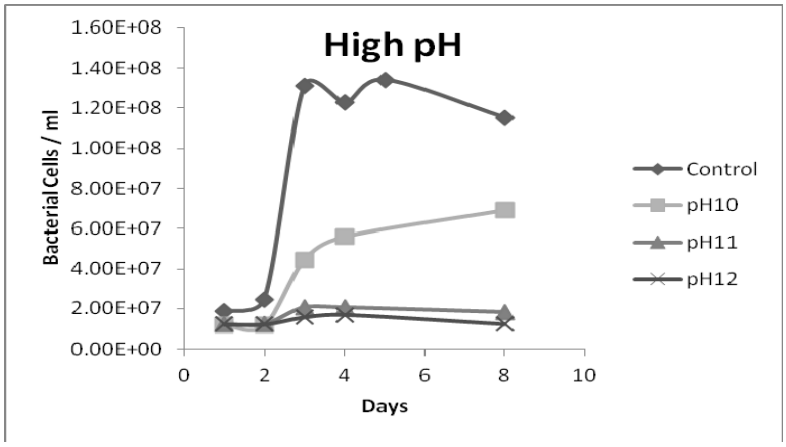


Figure 2. Effect of high pH adjustments on bacterial populations in a mixotrophic algal culture.

Algae

Although the more extreme pH levels resulted in lower bacterial populations, previous initial experiments revealed that microalgal populations would also decrease with more extreme pH levels.

Figure 3 below shows that the microalgae responded well to low pH treatments. The most acidic treatment, pH three, did experience a delay in growth. However, by the end of the growout algal density was only slightly lower than the other treatments. Both treatments with pH levels of four and five finished with slightly higher algal densities than the control, indicating the acid conditions had no negative effect on growth.

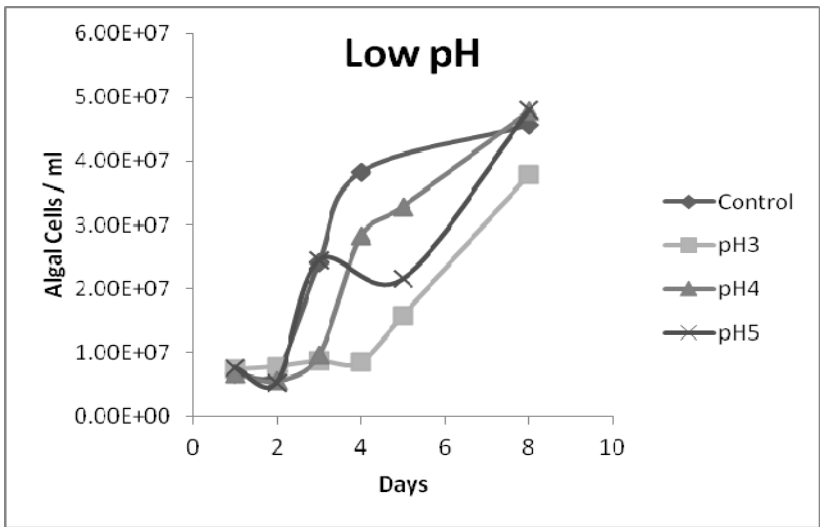


Figure 3. Effect of high pH adjustments on algal populations in a mixotrophic algal culture.

High pH treatments experienced a significantly different response than low pH treatments (See Figure 4). In all treatments where pH was elevated, microalgae performed poorly. Very little growth was measured for the treatment with pH 10, while both the pH 11 and 12 treatments resulted in negative

growth of algae. These results indicate that elevated pH adjustments will not work using our current mixotrophic strain, as it cannot tolerate the high pH conditions.

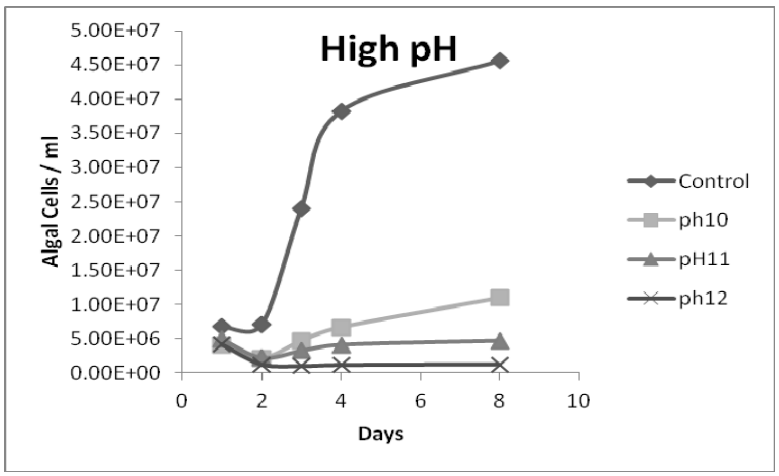


Figure 4. Effect of high pH adjustments on algal populations in a mixotrophic algal culture.

Based on the results of the above work, it is apparent that pH modifications with acid can decrease contamination of bacteria with little to no loss in algae productivity. Lowering a culture to pH 4.0 daily had a higher growth rate and reduced bacteria by approximately 50% compared to the control. We also decreased our bacteria:algae (B:A) ratio from approximately 3.1 to 1.4 by bringing the pH down to four. Higher algal growth rates and lower B:A ratios indicate increased conversion efficiency of glucose to algal biomass.

Effect of Time in Low pH Conditions

Knowing exactly when to lower the pH to maximize the effect of the acidic environment when glucose is added to the system will result in the optimal conversion efficiency. In order to understand how immediately adjusting the pH to 4.0 affects both bacteria and algae, a time-series experiment was conducted.

Methods

Flasks with a stable algae culture were enriched with glucose and were immediately dropped to pH 4.0 with acid. Bacteria and algae counts were collected initially and at 2, 4, 6 and 24 hours following the pH adjustment. The pH of the culture was also monitored.

Results

Results show a significant reduction in both bacteria and algae within two hours of the pH adjustment (See Figure 5). For bacteria, this reduction continued for the complete 24-hour experiment. In contrast, the algae populations stabilized after the initial two hours and sometime after six hours began to grow. This is important as it indicates the time needed to maximize the effect of the low pH environment and

alludes to a feeding strategy where the pH is dropped for at least two hours, in order for the bacterial reduction to begin, prior to introducing organic carbon.

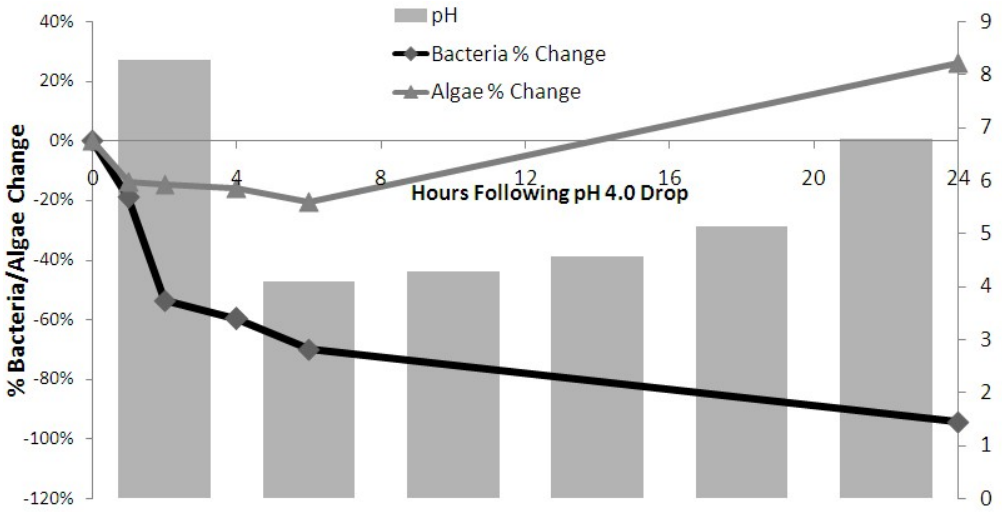


Figure 5. Short term effect of both algae and bacteria following pH 4.0 drop.

Bounce Back Effect

The ability of an algal culture to be lowered to pH 4.0 and spontaneously increase back to its more natural pH range over time is referred to as the "bounce back" effect. This bounce back effect was studied because variable results of pH changes following pH drops were observed in prior experiments. For example, most often a culture lowered to pH 4.0 would return to around pH 7-8 within 24h. However, occasionally a culture would stay at a pH near four and would not return or increase in pH. It is important for the culture to fluctuate pH's and not stay at the same pH indefinitely to prevent bacteria from adapting to the acidic culture conditions.

In order to better understand the relationship of pH following acid manipulations, cultures were measured daily under a variety of conditions. The two main components of an algal culture that may influence the chemistry of the culture include the nutrients and the biomass. Both components were studied to determine if either or both contributed to the bounce-back effect.

Methods

Flasks with different rates of algal biomass and nutrients were lowered to pH 4.0 daily with HCl. The amount of acid (ml) required to lower the pH was recorded. The next day, approximately 24 hours following the previous pH drop, the pH was again measured and brought back to 4.0 if it had risen. This was repeated for five days.

Results

Results showed that the presence or rate of nutrients did not affect the ability of the flask to return or increase in pH following the modification to a pH of four. Instead, flasks stayed at or near pH four

indefinitely after the initial pH modification (data not shown). However, the effect of biomass significantly affected the amount of acid needed to lower the pH and the ability for the culture to bounce back on its own. Cultures with more algal biomass required more acid and bounced back to a higher pH within 24 hours (See Table 1). The results also show that the bounce back effect diminishes after repeated pH manipulations, indicating that as the culture matures and increases in density manual pH raises will need to be performed.

	Day 1		Day 2		Day 3		Day 4		Day 5	
Algal Biomass	pH	HCl Added (ml)	pH	HCl Added (ml)	pH	HCl added (ml)	pH	HCl Added (ml)	pH	HCl Added (ml)
1 g/l	8.7	109	8	58	7.2	15	6.2	8	5.2	3
0.5 g/l	8.1	71	8	59	7.2	15	5.5	6	4.3	1
0.1 g/l	7.1	37	7.7	29	6.7	7	4.9	2	4.0	0
0 g/l	6.5	26	4.7	6	4.2	2	4.0	0	4.0	0

Table 1. Results of the Bounce Back Experiment

Indoor Lab Experiment Conclusions

Indoor lab results showed that it was possible to limit bacterial growth and reduce bacterial populations by adjusting the culture to low pH without affecting the algal growth rate. Therefore, with lower bacterial population a larger percentage of the added organic carbon will be uptaken by the microalgae. Other results previously reported, in Report 3, show that lowering the pH of the culture did not negatively affect the lipid concentration of the microalgae. When these results are combined it is clear that a better conversion efficiency of glucose to lipids can be achieved by manipulating the pH of the growth environment.

Based on the lab results, HBE has determined an optimal feeding and pH adjustment strategy to improve the uptake rate of organic carbon by the microalgae. This strategy includes:

1. The lowering of pH to four to decrease bacteria levels prior to the addition of organic carbon (OC) and ensure a greater rate of OC conversion to alga biomass.

2. Sustained decrease of pH for at least two hours, ensuring the greatest bacteria decrease with minimal harm to microalgae culture.

3. Depending on algal density and culture age, manually increasing the pH following the two hours of reduction through the addition of NaOH.

Application of Strategy to Outdoor Scale Up

Early-Stage Large Scale Outdoor Growouts

Between April and May of 2010 HBE grew a continuous, outdoor, mixotrophic , high density, algal culture. Routine harvests and glucose feedings occurred without any problems. In this system, HBE did attempt some successful pH adjustments to 4.0. However, they had not been optimized.

These growouts proved that it was possible to maintain a continuous, outdoor, mixotrophic culture in a deep, open system that was repeatedly fed organic carbon. This is significant because there is no published work that has achieved this. However, the costs of glucose were still too expensive at our current conversion rate of sugar to algal biomass and lipids for economic sustainability.

A large goal of the second phase of the ONR work was to look for ways to decrease costs of glucose and other organic carbon (See Report 1) and to attempt to increase our conversion rate of glucose to algal biomass and lipids by decreasing bacteria uptake. The feeding and pH strategies discussed previously are meant to help accomplish this by decreasing the bacterial load.

Initial Outdoor Small Scale Trial

Open, indoor cultures are not as susceptible to environmental contamination as open, outdoor cultures. Thus, open, outdoor cultures were used as a quick way of determining if our feeding and pH strategies would be effective in large scale, open, mixotrophic cultures.

Methods

20-L buckets were used to grow culture for one week and fed glucose three times. Prior to each feeding the pH was lowered to 4.0. The pH was then monitored to assure it bounced back on its own. If it did not begin to rise within 6 hours, it was manually adjusted back to 7.0 with NaOH.

Results

Results overwhelmingly show that strategies developed inside could be successfully applied outdoors. Lowering the pH to 4.0 prior to each feeding reduced bacteria, and resulted in higher algal growth. (See Figure 6). Lowering the pH, decreased bacteria by roughly 75% between day three and day seven where the majority of the glucose feeding occurred. Further, algae density was nearly 100% higher in those days. On Day 3, the B:A ratio was over 40 for the glucose control and was just 4.1 when the pH was regulated.

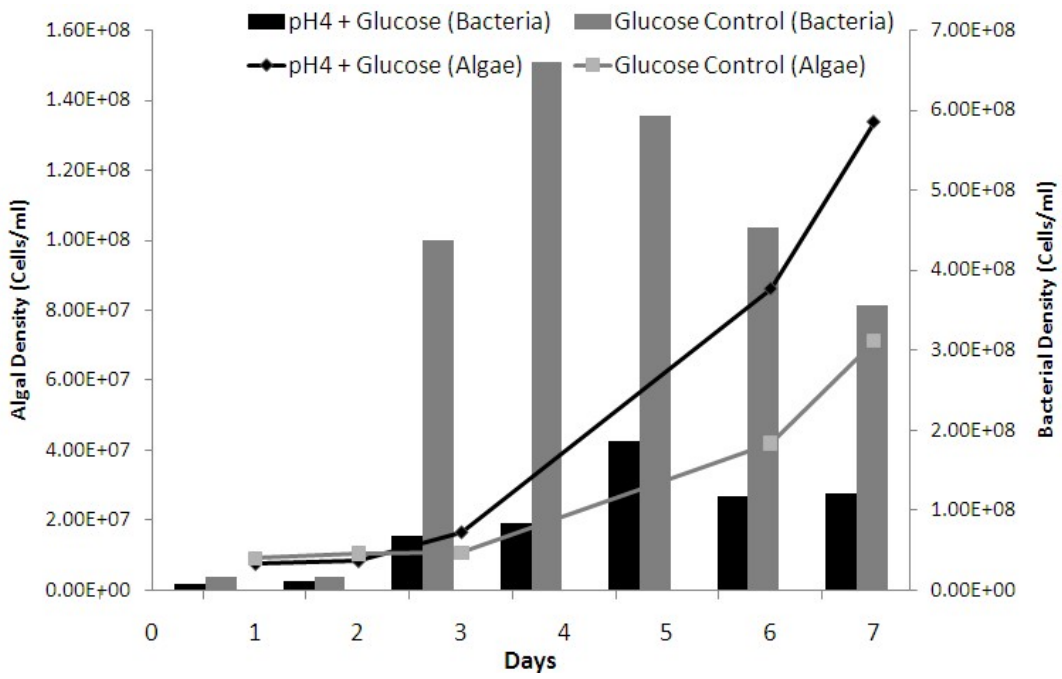


Figure 6. Results of initial outdoor trial to test optimized pH adjustments.

Large-Scale Outdoor Growouts

Based on the results from the indoor and outdoor experiments, HBE attempted to scale-up a growout based on the optimal pH adjustment and feeding strategy outlined above.

Inoculum Production

Inoculum for large outdoor ponds is done through a series of batch cultures starting with small flasks that are continually scaled up to larger vessels. This is done by increasing the vessel ten-fold every time the culture reaches approximately $5x10^7$ cells per ml. Therefore, the culture is diluted down to $5x10^6$ in a vessel 10 times larger than the previous. These densities are chosen because $5x10^6$ is a dilute enough density to allow for high growth while dense enough to effectively compete for nutrients and OC.

Using this method, it takes approximately two to three weeks to produce enough inoculum for a 1000 L pond. The exact method employed for the ponds in this report begin with 10, 1-L flasks started from test tubes in the indoor culture room. These flasks are then brought up to density in about 10 days, combined, and used to inoculate five, 20-L buckets outside. The buckets are then fed to density and used to start a 1000-L pond. The timing can be decreased by increasing the glucose-feeding rate or by increasing the dilution rate (i.e. 1:20 instead of 1:10) but both may compromise the inoculum by increasing bacterial contamination.

First Growout

Two ponds were inoculated in early August 2011 and were pH adjusted and fed glucose the following day. By the third day, Pond 1 had crashed and gone from a light green color to brown and foamy (See

Figures 5 and 6). Pond 2 responded better to the glucose with robust growth for the next 6 days. However, on day 8 it reached its peak density of $1.2x10^7$ cells per ml and then declined from there. As was observed in Pond 1, the color of the culture turned from green to brown in about 24 hours indicating a culture crash.

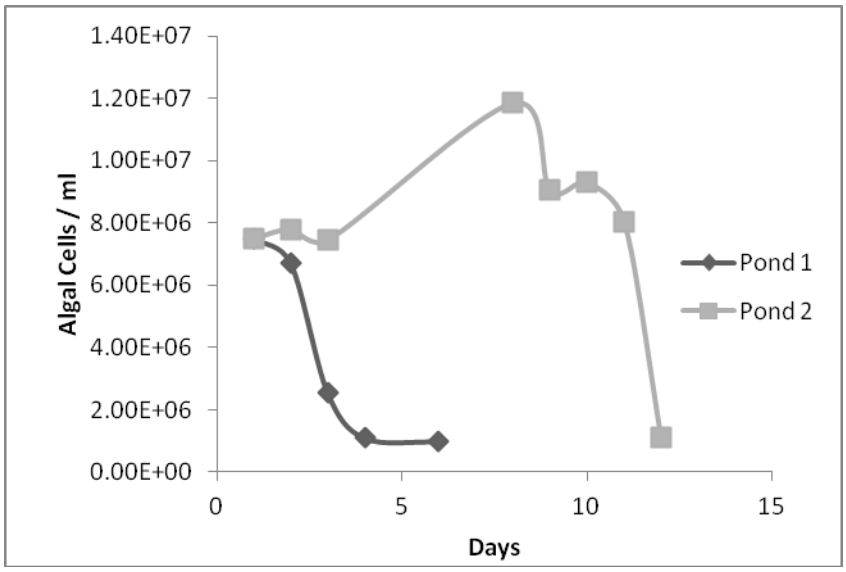


Figure 5. Pond Growouts in August 2011

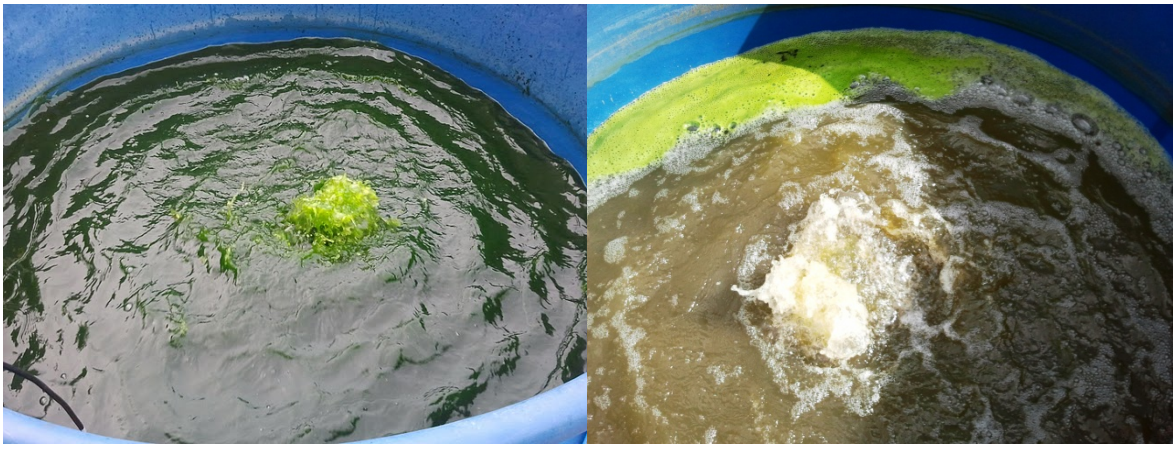


Figure 6. Culture before (left) and after (right) a culture crash.

Following the pond growouts and nearly immediate crashes, HBE reassessed its inoculation and growout practices to attempt to determine what went wrong. One issue noted was the age of the inoculum used for the growout. The inoculum used was taken from a few dedicated "mother" flasks. These mother flasks were maintained by removing 50% of the culture and replacing with nutrified freshwater weekly. However, these mother flasks had been going for over two months prior to the large-scale growout.

Previous work had shown older inoculums is less effective than those more newly grown. It was hypothesized that played a role in the First Growout's crash. .

Second Growout

New inoculum was started in test tubes from the original Chlorella strain that was being kept on agar. HBE stores its culture collection on agar test tubes under low lights in order to preserve the cultures with minimal maintenance or disruption. Single colonies of the Chlorella were picked from the agar, put into test tubes of water, and allowed to acclimate and grow photosynthetically. As the culture acclimated to the new environment, small amounts of glucose were fed in order to begin the scale-up process.

As inoculum was readied for the two ponds, a thorough sterilization of the outdoor ponds was performed. Ponds were drained, cleaned with soap and water, and allowed to completely dry out for 1 week. The ponds were cleaned a second time with diluted bleach water, rinsed and allowed to dry out again. All peripheral plumbing and airline was also sterilized or replaced if possible. By late September, new growouts with fresh inoculum began.

We had more success with the fresh inoculum. Pond 1 went for nearly 20 days, and we conducted five glucose feedings, seven pH adjustments, and two 50% harvests. We were able to reach densities over $4x10^7$ cell per ml, which is nearly 1.3 g of biomass per liter and 14.3% lipids. However, the the significant stability achieved in the first scale-ups in the late Spring was not replicated. Following the second 50% harvest, the pond did not respond to the following glucose feeding and a culture crash was observed overnight (See Figure 7).

The second pond grew very similar to Pond 1 following inoculation. However, it experienced a culture crash following the first 50% harvest on Day 8(See Figure 7).

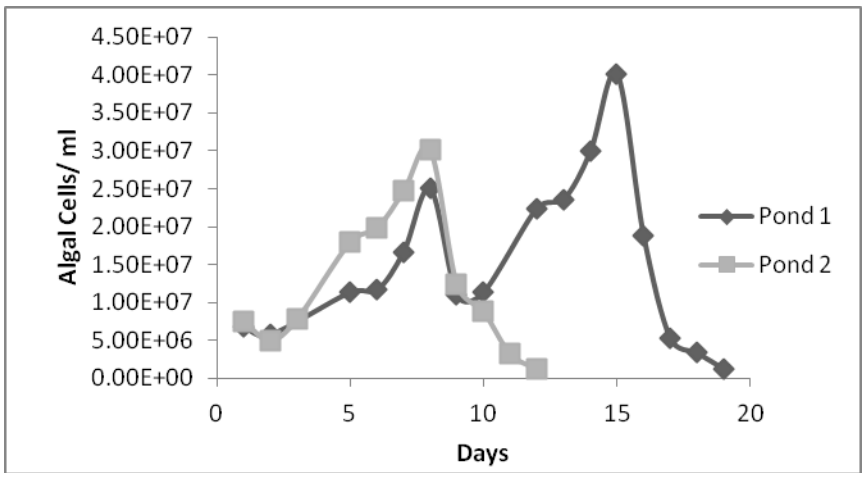


Figure 9. Growth Curves of the second grow-out attempt. Note the 50% harvests of the culture on days 8 and day 15.

The culture crashes in this growout were simillar to Growout 1 where a green culture turned brown in less than 24 hours. Looking at the crashed culture under the microscope, it was apparent that the cells had lysed or burst open as seen by cell fragments and "ghost cells," cells where only the cell wall can be identified. A picture of the crashed culture under the microscope is shown in Figure 10.

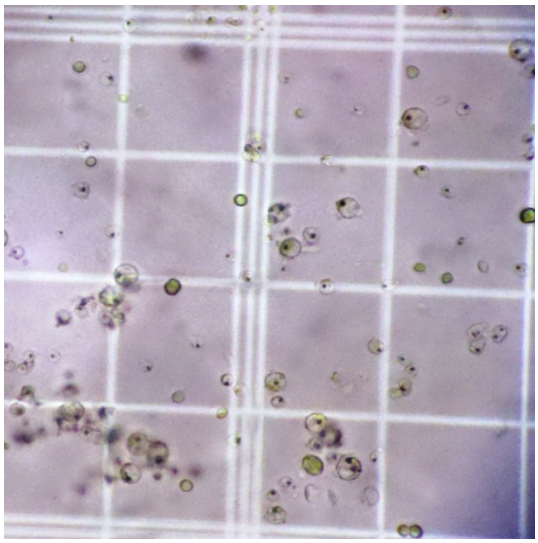


Figure 10. 400x magnification of crashed culture showing lysed cells.

Possible Crash Explanations

Again, following the culture crashes HBE re-assessed and re-evaluated its methods and practices in order to determine practices that may be influencing the crashes. Further, HBE studied the literature for an explanation of the culture crashes. We identified two possible influential factors that could result in a culture crash.

1. Biological stress: According to the literature, many culture crashes can be attributed to viruses or lysing bacteria. If a virus, it could have infected our strain and exist latently or it could be present within the infrastructure or environment of our growth facility.

2. Light stress: Previous work has shown our mixotrophic strain requires light to grow heterotrophically. The large outdoor cultures were commonly crashing at densities where self-shading could greatly decrease light penetration.

Because of the initial success growing outdoor, mixotrophic cultures at a large scale, it was hypothesized that a biological contaminant was being introduced to our cultures. Due to time and resource constraints and difficulty identifying harmful bacteria and virus, focus was placed on determining the source.

Isolating Sources of Contamination

If the biological contaminant was a virus, it could be latent within our seed culture. However, because the crashes do not occur inside, in open cultures, but only outside, it was likely that the contamination was being introduced outside. Further, when we added a small amount of a crashed culture to a healthy indoor flask, we observed a culture crash in the following 48-72 hours. This happened to flasks that were growing both photosynthetically and mixotrophically. This confirmed our suspicion of a biological crash. The possible sources of introduction of a virus to our outdoor ponds include air and water.

Water

Indoor cultures usually are grown in clean autoclaved water to limit bacteria and other contamination while outside tanks and ponds are filled with unfiltered freshwater usually coming from a hose. Hose water, autoclaved water and indoor tap water were all compared outside in buckets.

Results show that buckets got up to approximately $3x10^7$ cells/ml before crashing on the 8th day of the trial (See Figure 11). All treatments performed similarly and water source does not appear to be the cause of the crashes as even sterile autoclaved water crashed. It should be noted that the majority of the crashes observed outside come following a density of approximately $2\text{-}3x10^7$ cells per ml. This is also the density where a photosynthetic culture will usually max out indicating light has become the limiting factor.

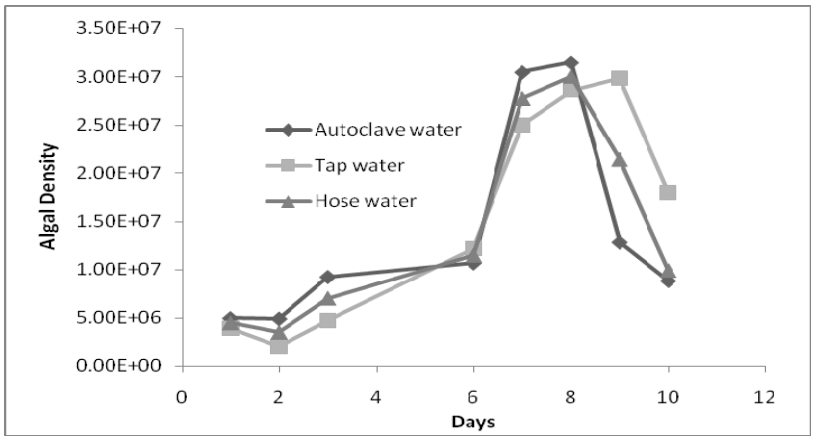


Figure 11. Experiment comparing water source in outdoor buckets.

Outdoor Air Supply

In an attempt to isolate difference from inside and outside we tested the possibility that contamination from our outdoor airline was responsible for the crashes. The airline is responsible for transferring the air used for mixing and aeration from the outdoor air blower. We added a 0.45 µm filter on the airline just before it entered our growing vessel in an attempt to block any biological matter from entering the system via the airline. The filter used will stop most bacteria and larger microorganisms from entering the culture. However, viruses are routinely around 0.20 µm and would likely pass through the filter. Results of this trial resulted in a crash of the vessels both with and without the air filter on day 7 of the growout indicating the filter was not capable of stopping the biological contaminant of concern.

Influence of Light

Previous work indicated that mixotrophic growth required light for robust heterotrophic growth. One major variable that differs from the inside culture room and outside is the amount of light the culture receives. Indoors, cultures always receive 12 hours of light per day, while outdoors the length of light differs over the year due to seasons and also daily due to weather. It was hypothesized that at higher densities, light stress occurs due to self-shading and this in combination of a biological contaminant influences the culture crashes.

In one experiment, buckets were placed both indoors receiving 12 hours of light and outdoors. Buckets placed indoors did not crash and were able to reach $4x10^7$, while those outdoors crashed at

approximately 2x10e^7. Further, the inside buckets remained stable at over 4x10^7 cells per ml for over a week.

To further isolate light variables, we set up flasks indoors and covered them with foil so the culture was in the dark. To some flasks, we added glucose and to others we left alone. None of these flasks indoors in the dark crashed and those that had added glucose grew slightly. Further, we added a small amount of the outside crashed culture to flasks with high and low light. Regardless of light, all flasks crashed. These results indicate that although light is required for photosynthetic growth and greatly enhances heterotrophic growth, it was not a major factor in preventing or causing the crashes.

Location

Based on our experiments it was determined the cause of the crashes was a biological contamination that was being introduced from the environment. Because cleaning and decontamination steps were not successful in eliminating biological contaminants, we attempted a growout in another location, one kilometer away from our existing growing facility.

Inoculum was grown strictly indoors using the buckets referred to in the Influence of Light section above. The pond started slow, but following a feeding and pH drop on day 5, began to grow in log phase (Figure 12). On Day 12, a 50% harvest of the culture was done and the pond was re-filled with freshwater. However, as previously seen, following the harvest the culture crashed turning brown and showing lysed cells under the microscope.

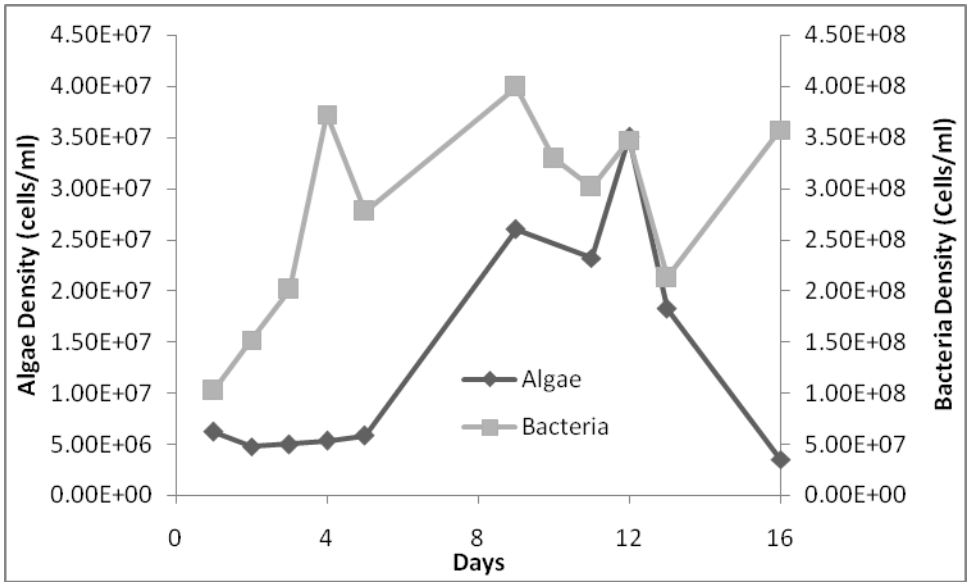


Figure 12. Growth curve of both algae and bacteria in a pond at new outdoor location.

The results of this growout confirm that the virus is not specific to only our original growth facility but exists in other outdoor locations.

Conclusions

Indoors, in our controlled grow room we are able to supply algae with near perfect growing conditions. This includes high amounts of consistent light, constant temperature, sterile water and optimal mixing and gas exchange. When this is combined with the addition of organic carbon, we are able to record some very astounding algal growth rates and keep stable cultures going for very long periods. When the culture was taken outside we consistently achieved densities of around 1 g/l at depths of greater than 12 inches, improving per acre yields. While culture stability was not as great outside as in, we were often able to achieve multiple harvests, again improving per acre yields. However, a long-term (mult-month) culture, necessary for continuous production was not able to be achieved due to an unforeseen, unknown biological contaminant. This introduction represents one of the significant challenges of open mixotrophy, increased culture instability and risk.

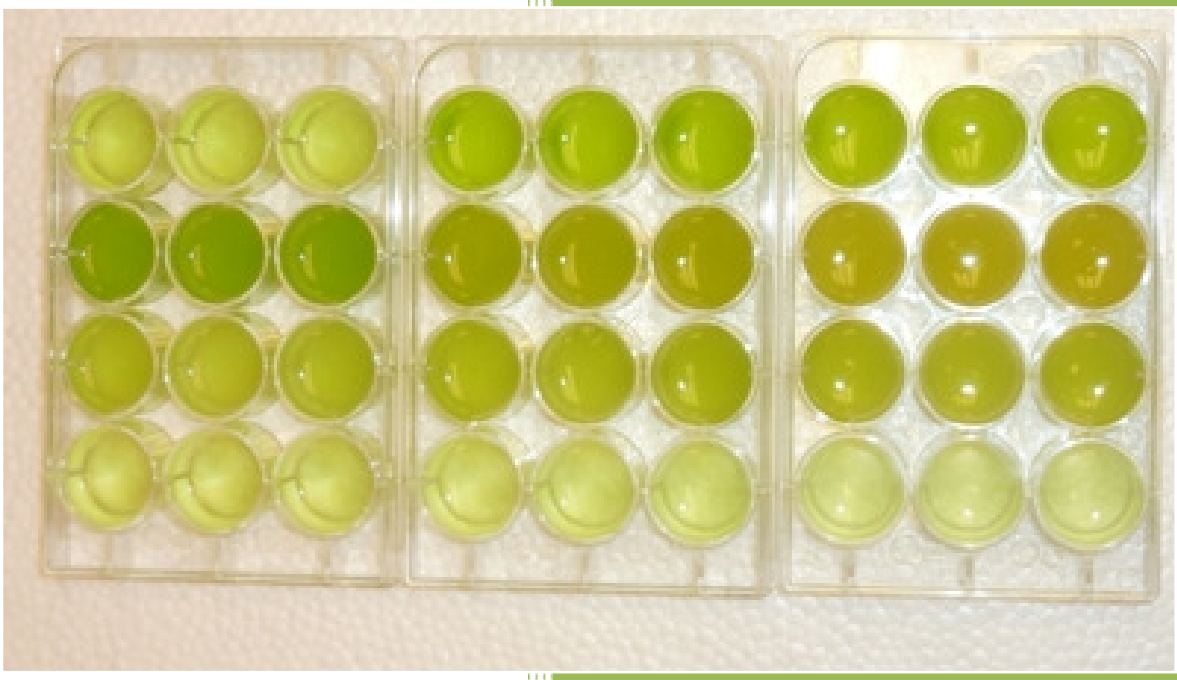
Follow-up Work

The symptoms of the crashes we experienced closely resemble that of an algal virus. We hypothesize that a virus may have infected our strain and been introduced either through environmental sampling conducted or from contamination at our outdoor growing facility. During the past year, there has been continuous construction adjacent to our growing facility. The movement of soils may have aerosolized a virus and transferred it into our culture and growing facility. Further, even though we sterilized our growing facility, some viruses can require numerous years of drying out to be killed.

Further, algal viruses are very small and can be passively diffused into the algal cell. An algal virus infection may remain latent in an algal cell for many generations and as the algae multiply all new cells carry the latent virus. However, when the virus senses stress to the host cell the latent virus may become lytic. This change ruptures the cell, releasing the virus into the environment. In both the first and second growout, crashes were followed by either a harvest or a glucose feeding. This may have caused enough stress to the culture to induce a lytic viral infection. In brown algae, it has been reported that the change from latent to lytic stage of the virus occurs when the algae reaches maturity. In other cases, we too experienced crashes as cultures matured and got to light limiting densities.

In an attempt to determine if we had a virus, we obtained a DNA primer from the typical chlorella phycodnavirus. However, upon extraction we got a negative result inferring we did not have the DNA virus associated with our algae culture. However, we were only able to rule out a specific yet common DNA viruses and it is still likely we have a virus. Further work is needed to confirm if it is in fact a virus. Although this work is expensive and requires special equipment, if time and funding permits, we plan to continue this research.

New Strain Sourcing and Selection



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Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the Office of Naval.

Hawaii BioEnergy, LLC

2/6/2012

Introduction

Hawaii BioEnergy, LLC (HBE) is conducting research funded through the Office of Naval Research (ONR) on mixotrophic microalgae production systems. The general goal of HBE's work is to optimize and increase the productivity of microalgae to maximize land area and minimize capital costs. Previous work has focused on growing algae in deep ponds fed with organic carbon and ways to increase the efficiency of this process. One way to increase the efficiency was to lower the pH of the culture prior to feeding OC. This resulted in lower bacteria levels and increased uptake of OC by the algae. The previous work has all utilized existing HBE algae strains, however these strains were not sourced from areas that were known to contain high amounts of OC or low pH's. It is thought that by identifying land that has a naturally low pH or been in contact with OC, particularly sugars, more efficient strains can be sourced and identified.

Land Identification

Low pH Areas

In Hawaii and other tropical areas with warm temperatures and high rainfall, acidic soils are common. Acidic soils are caused from natural weathering, as base cations like Ca+ and K+ leach out and more stable materials like Fe and Al oxides remain. Further, when agricultural fertilizers like urea are added, soil acidity is enhanced. Pineapple, once a common crop in Hawaii prefers acidic soil in the range of pH 4.5-6.0. Farmers often chemically manipulate the soil to decrease the pH by adding sulfur or iron.

HBE, using GIS soil maps, identified areas with highly weathered soils characterized by high amounts of Fe and Al oxides. These areas where associated with soils of the order Oxisol and Ultisol which are the most weathered types of soil. Appendix 1 contains soil maps of the four main Hawaiian Islands mapped by soil order. Oahu and Kauai contain large amounts of Oxisols and Ultisols becasue they are older and more weathered islands, while Hawaii Island contains no Oxisols or Ultisols. Hawaii Island, where there is soil, is mostly classified as Andisols, are from volcanic ash and are usually slightly acidic to neutral. Eastern Maui in areas around Hana contain more weathered Andisols that have soil pH's classified as highly acidic.

Areas Rich in OC

Soils that may contain high amounts of organic carbon, particularly sugars or carbohydrates will potentially have microalgae associated with them that are capable of growing heterotrophically. Land that has been used to grow sugar cane or land that at one time was used in sugar cane processing may likely contain soil that has absorbed large amounts of sugar at some point.

Again using GIS and historic agricultural maps HBE was able to identify and locate both current and previous land that had been used to grow sugar or was where a sugar mill was located. Appendix 2 contains historic maps of Hawaii Island and Maui Island showing where sugar mills where once located.

Land Access, Permitting and Sampling Procedure

Once sample locations with likely low pH or high OC content were identified, HBE obtained permits and rights access those sites. Because private property access is difficult and contains much liability, HBE focused on its partner lands and public lands.

Partner Lands

Two of Hawaii BioEnergy's partner companies include Grove Farm (GF), a large landowner on Kauai and Kamehameha Schools (KS), a large landowner on Oahu and Hawaii Island. HBE obtained permission to collect soil and water samples on both GF and KS land and worked with their land managers to identify optimal sample locations.

Public Lands

Public lands that are controlled by the Division of State Parks routinely grant access to land by obtaining a Special Use Permit. HBE obtained special use permits for Oahu, Maui and Hawaii Islands and focused on State Park Land that either once was sugar cane (much of East Hawaii Island and some Maui Island) or was located on low pH weathered soils (Windward Oahu).

Sampling Method

Soil Collection

Samples were collected from both soil and freshwater locations. For soil, around 1kg of surface soil was collected from 2-3 locations in the sampling area. The soil was bagged and stored at room temperature for about 24h until it was processed.

Soil Processing

From each soil sample, two 25g sub-samples were taken. The sub samples were mixed with 50ml of tap water and were placed on a shaker for 24h. Following the shaking, the samples were allowed to settle for approximately 5m to allow the majority of the soil particles to fall out. Finally, two 1ml samples of the water were then plated on agar plates containing algal growth nutrients and placed under light for 1 week to allow microalgal colonies to form. Agar plates are routinely used to isolate microorganisms including microalgae.

Water Collection and Processing

Water samples were collected from numerous streams, ponds and pools using 50ml screw-top tubes. Following sample collection samples were stored in a refrigerator for 24h and then plated onto agar plates and incubated for 1 week under light.

Sample Locations

Approximate sample locations are shown overlaid on the soil maps in Appendix 1.

Strain Evaluation and Laboratory Results

Strain Isolation

Methods

Following the plating of samples (described above) isolation of single strains was conducted via streaking of agar plates. From the original sample plate, individual algal colonies were picked off with a sterile inoculating loop and streaked onto a clean agar plate. The new plate was allowed to grow and again a single algal colony was plucked off and re-streaked on a new plate. By repeating this method multiple times, a single species of algae was isolated onto a unique agar plate.

Once isolation was successful, a single colony from the plate was put into a 10ml tube of water and allowed to grow under light. As the algae continued to grow it was scaled up into 200ml flask.

Results

Results of the strain isolation work resulted in 83 algae strains.

Initial Growth Rates

Methods

Daily growth rates for the 83 algal strains were determined for photosynthetic, mixotrophic and heterotrophic growth using 24-well plates and allowed to grow for eight days. Plates were started at optical density (OD) of 0.1 and measured at a wavelength of 750nm. Optical density was read daily and daily growth rate was calculated as a percentage. For mixotrophic growth rates, glucose was added at a rate of 0.5 g/l on the second day. Heterotrophic growth rates were determined as the difference between photosynthetic and mixotrophic growth rates.

Results

The complete list of growth rates and densities reached over the eight-day growout are given in Appendix 3. Table 1 is a summary of the data showing the highest and lowest growth rate as well as the average and median growth rate of the 83 strain collection.

	High	Low	Average	Median
Photosynthetic	136.5% (B-N1)	0.5% (B-C3)	48.90%	44.80%
Mixotrophic	142.7% (B-N1)	-1.3% (M-G1)	55.40%	53.30%
Heterotrophic	49.1% (O-B1)	-21.8% (M-P2)	6.50%	7.60%

Table 1. Summary of 83 strain growout to determine growth rates.

Figure 1 below is a histogram showing the distribution of growth rates both photosynthetic and mixotrophic. Generally, it shows mixotrophy improved growth rates and moved strains to the right of the graph.

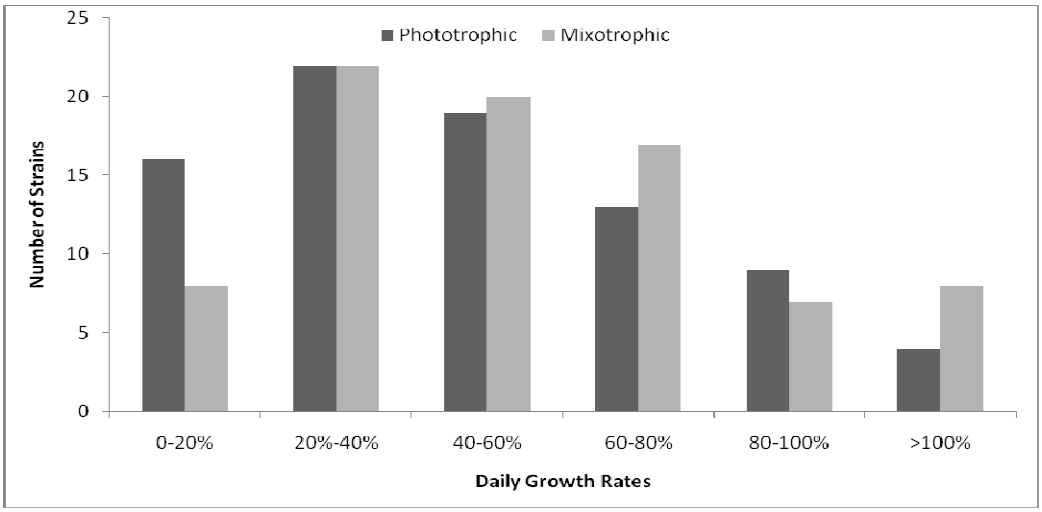


Figure 1. Histogram showing growth rates of heterotrophic and mixotrophic growth rates.

Strain Selection and Further Evaluation

Strains were selected for further evaluation based on total growth rate, maximum OD and most improved growth with glucose addition. Based on the results of the initial 83-strain growout, 23 strains or approximately 28% of the strain collection was selected for further evaluation.

Methods

Selected strains were further analyzed in 200ml and 1000ml flasks. Strains were evaluated to determine their tolerance to saltwater, affinity to a high pH bicarbonate media and low pH environments, and affinity to sucrose and glucose. Further, each of the selected strains was analyzed for crude lipid, protein and ash content grown under non-limiting conditions.

Results

The results of the growouts in selected environments and the lipid and protein contents are provided in Appendix 4.

Saltwater

Results indicate that no strain appears to tolerate salt water or even 50% saltwater. On average, growth rates were approximately 82% less than that of the photosynthetic control in freshwater. However, some strains, particularly M-H3 and K-C1 grew at growth rates around 50% of the control indicating they had somewhat more tolerance of saltwater.

High pH Bicarbonate Media

Of the 23 selected strains, three showed an increase in growth rates with the addition of 10g/l bicarbonate to the growth media. Bicarbonate adds inorganic carbon, a limiting nutrient, to the growth media, however, it also increases the pH to often intolerable levels. Being able to tolerate high pH is a desired characteristic for an algal strain because application of carbon via bicarbonate is often much

more efficient than adding CO_2 gas. Further, maintaining a high pH can greatly reduce contamination of unwanted microorganisms. Strains M-H1, M-H3 and M-K4, all sourced from similar environments on Maui are able to tolerate the high pH and use the carbonate to increase their growth rate. Table 2 below, shows the three strains capable of growing in the bicarbonate media and an average of the other 20 strains as a comparison.

	Bicarbonate Media	Photosynthetic Control	Island
M-H1	118.9%	53.9%	Maui
M-H3	66.3%	58.0%	Maui
M-K4	69.7%	56.5%	Maui
Average of other strains	7.5%	46.0%	

Table 2. Daily growth rates of selected strains grown with and without a bicarbonate media.

Glucose

The effect of glucose was studied on all 83 strains and was duplicated on the 23 selected strains. However, there was some variability between the two growouts. For example, strains that showed some affinity for glucose in the first run did poorly the second run while other strains did better the second run. This is not surprising because we are using OD measurements as a proxy for algal density and OD will also measure fungal or bacterial contaminations, which are common in organic carbon added cultures. However because so many strains are being screened the quicker OD measurement is preferred.

Looking at both Growout 1 and Growout 2 there are seven strains that consistently performed better with glucose added. These strains are given in Table 3. Three of the strains were sourced from soils while the other four came from freshwater streams.

	Photosynthetic Growth Rate	Mixotrophic Growth rate	Island Collected
B-N2	32.9%	52.7%	Hawaii
B-N4	27.1%	46.5%	Hawaii
M-D4	63.4%	74.5%	Maui
M-F2	27.4%	46.8%	Maui
M-F3	89.0%	98.3%	Maui
O-B1	54.3%	83.6%	Oahu
O-C4	70.0%	82.2%	Oahu

Table 3. Strains that repeatedly increased growth under mixotrophic conditions.

Sucrose

Glucose is the preferred organic carbon for mixotrophic microalgae because glucose is a simple mono-saccharide that also is the main product of photosynthesis. In contrast, sucrose is a disaccharide compromised of glucose and fructose bonded via a covalent glycosidic bond. Most mixotrophic algal strains do not have the capability to consume sucrose likely because of the difficulty breaking the bond and getting to the glucose and the fructose. However, there are reports of microalgae being able to grow heterotrophically using sucrose.

Of the strains reported above that showed capability of using glucose, B-N2 and B-N4 also showed similar affinity for sucrose. Both strains increased daily growth by more than 18% with the addition of sucrose. These strains sourced from old sugar cane land, have likely evolved with sucrose present in the soil and acquired the enzymes to break down sucrose to glucose.

Low pH Environment

Previous work by HBE has proven that modification of the pH of a culture can lower bacterial and other contamination in an open, mixotrophic growth environment. Specifically, temporary pH drops with acid to pH 4.0 and manual raises back to normal growing conditions has shown to have no negative effect to our original mixotrophic strain.

The 23 selected strains were grown with and without near daily decreases of pH to 4.0. Density was measured daily via optical density (OD). Results show three strains did significantly better by manipulating the pH with acid while ten strains did significantly worse (See Figure 2). Significance is defined as having more than a 10% growth rate difference. The remaining ten strains showed very little difference with and without the pH4.0 modification.

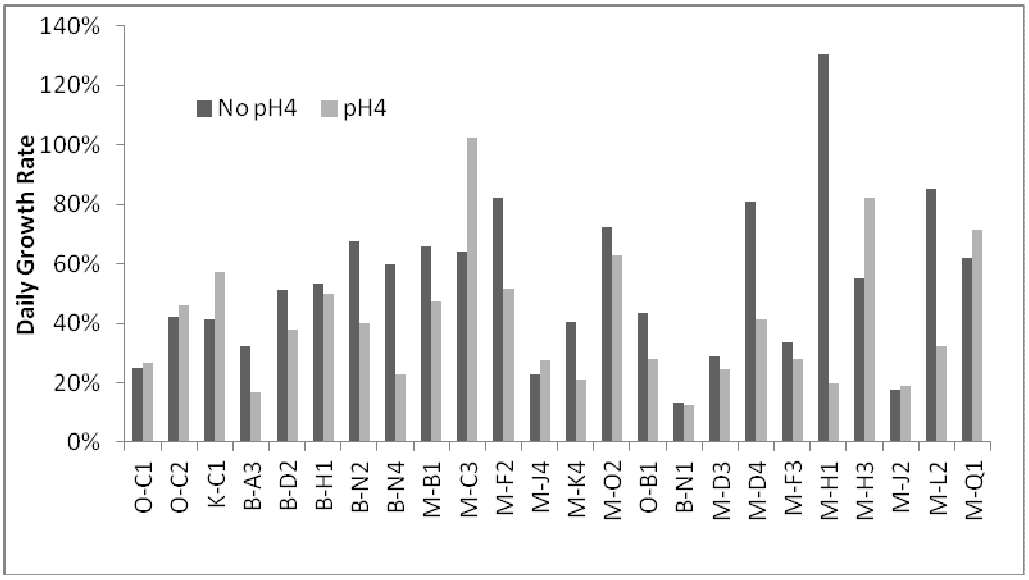


Figure 2. Growth rates with and without pH modifications.

The strains identified above as able to use glucose, only M-F3 did not do significantly worse when a pH4 modification was done to the culture.

Lipid and Protein Content

Baseline crude lipid and protein was determined for each of the 23 selected strains. Baseline numbers are based on growouts in a non-limiting environment. HBE has shown that by "finishing" cultures in a low nitrogen phase, lipid contents can be increased by over 100%. However, because each strain is slightly different on how to maximize lipids baseline numbers allow for better comparison between strains.

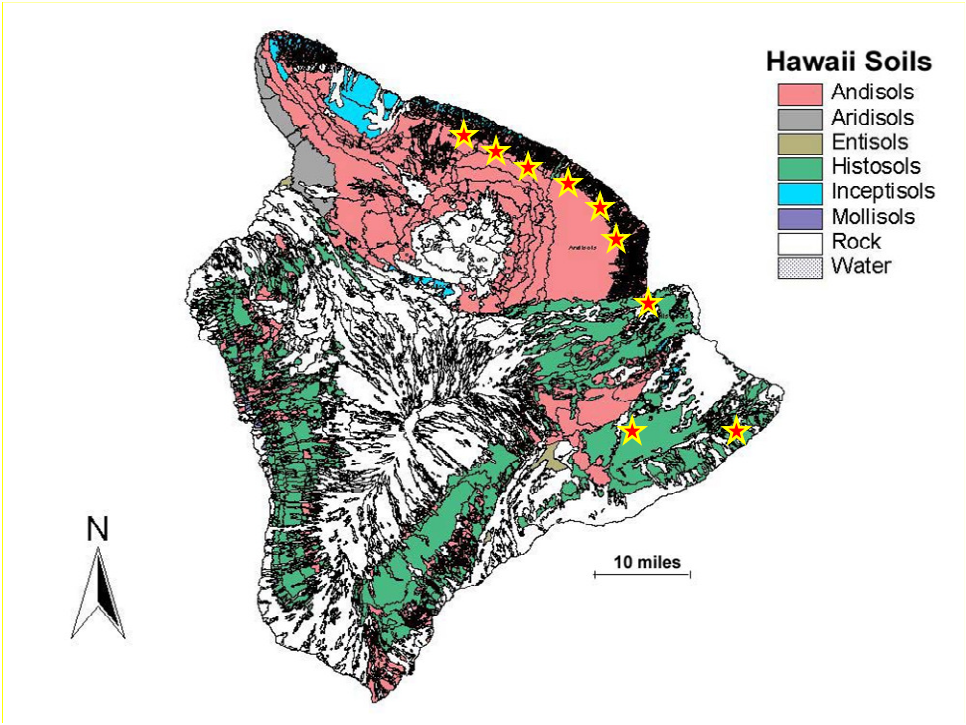
Lipid and protein contents are given in Appendix 3 for the 23 selected strains. Lipid content ranged from 9% to 19% of dry weight and protein contents ranged from 14 to 54% of dry weight. We were unsuccessful in getting enough biomass to do lipid and protein analysis for two strains, B-N2 and O-B1. The strains appear to be macro-algal and would only grow on the surface of the flask.

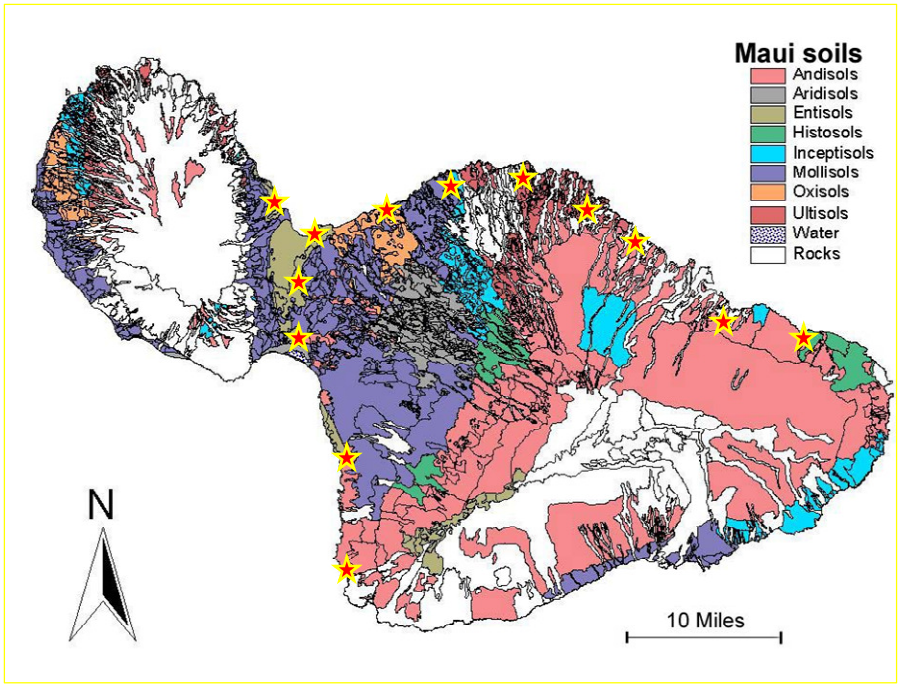
Of the strains identified as able to grow in a high pH bicarbonate media, M-K4 had the highest lipid content of 14.4%. The other two strains were 10.8% (M-H3) and 8.7% (M-H1) lipid per dry weight. Of the strains identified as being able to use glucose, lipid contents ranged from 9.3% to 14.1% with M-D4 being the highest.

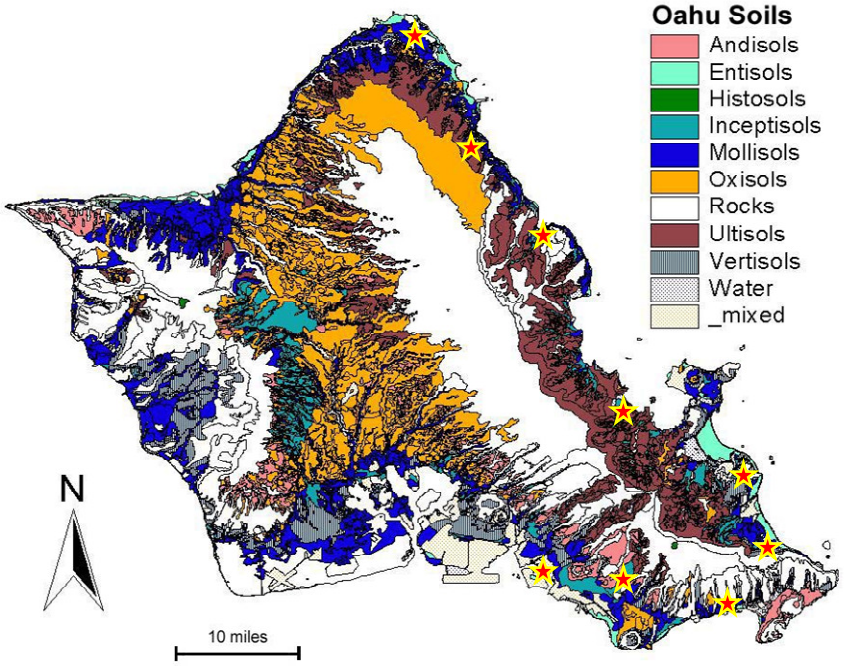
Conclusions

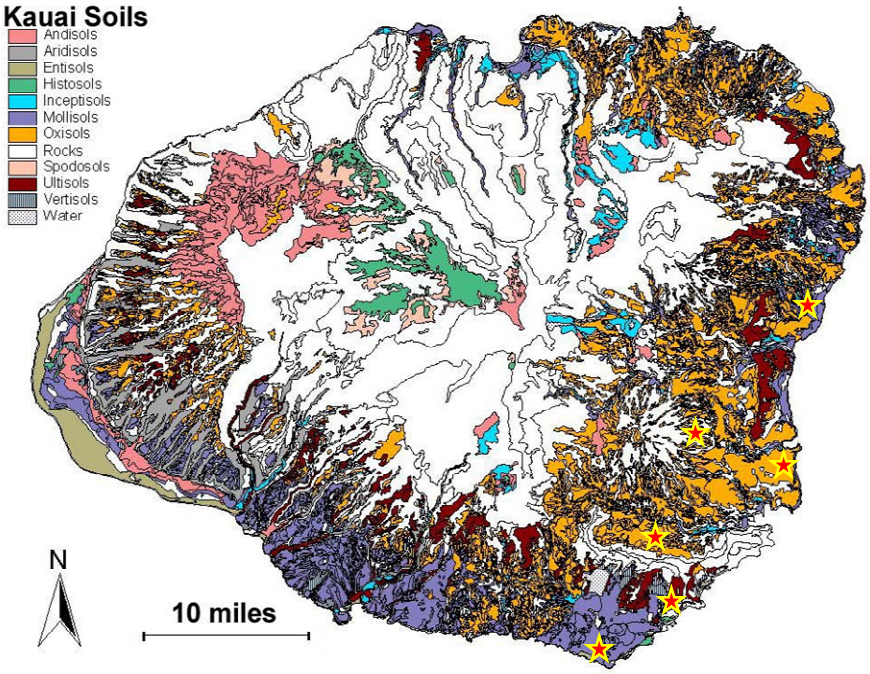
Strain sampling across the four main Hawaiian Islands resulted in 83 new strains. Sample locations were focused on areas where a low pH or presence of organic carbon was suspected. Of the 83 strains isolated, seven have mixotrophic capabilities and will warrant further research to better understand their commercial capabilities. Of the 23 selected strains tested for low pH tolerance, nearly half appear to be able to tolerate pH manipulation without negatively affecting growth rates. However, only one of the mixotrophic capable strains has tolerance for low pH. This data points to the importance of strain sourcing and the matching of strains with production systems.

Appendix 1. Soil maps with algae sample locations.









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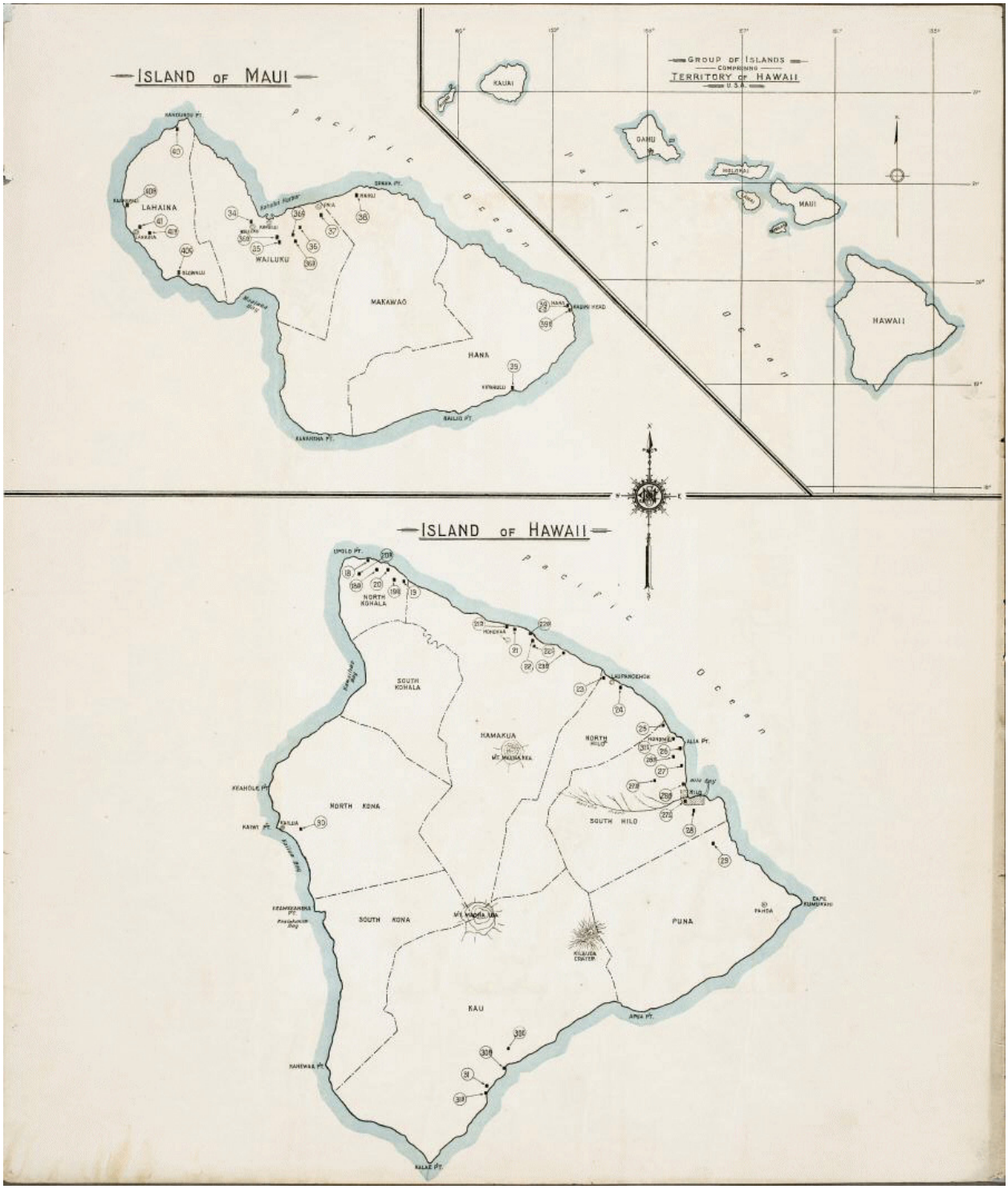
Appendix 2. Sugar mill and canner map. Source: Sanborn Map Company. University of Hawaii.



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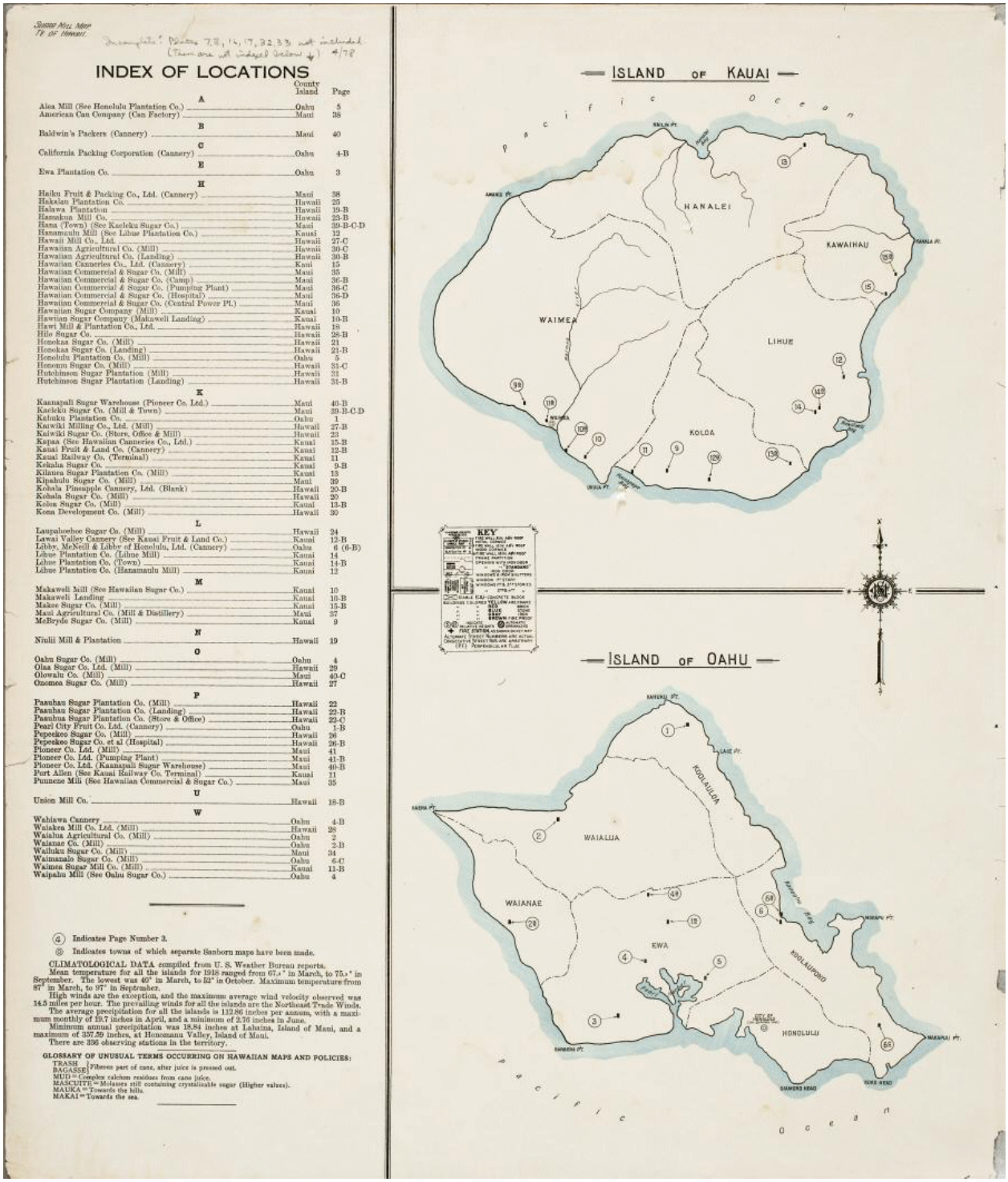

Sugar Mill Map
Tr. OF Hawaii

Incomplete? Pages 7,8,14,17,32,33 not included
(There are 18 indexed below) 4/78

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(4) Indicates Page Number 3.

(3) Indicates towns of which separate Sanborn maps have been made.

CLIMATOLOGICAL DATA compiled from U. S. Weather Bureau reports.

Mean temperature for all the islands for 1918 ranged from 67.5° in March, to 75.5° in September. The lowest was 45° in March, to 52° in October. Maximum temperature from 87° in March, to 97° in September.

High winds are the exception, and the maximum average wind velocity observed was 14.5 miles per hour. The prevailing winds for all the islands are the Northeast Trade Winds.

The average precipitation for all the islands is 132.86 inches per annum, with a maximum monthly of 19.7 inches in April, and a minimum of 2.76 inches in June.

Minimum annual precipitation was 18.84 inches at Lahaina, Island of Maui, and a maximum of 357.59 inches, at Honomanu Valley, Island of Maui.

There are 336 observing stations in the territory.

GLOSSARY OF UNUSUAL TERMS OCCURRING ON HAWAIIAN MAPS AND POLICIES:

TRASH } Fibrous part of cane, after juice is pressed out.
BAGASSE }
MUD = Complex calcium residues from cane juice.
MASCUITE = Molasses still containing crystallizable sugar (Higher values).
MAUKA = Towards the hills.
MAKAI = Towards the sea.

ISLAND OF KAUAI

HANALEI
WAIMEA
KAWAIHAU
LIHUE
KOLOA

KEY

ISLAND OF OAHU

KOOLAULOA
WAIALUA
WAIANAE
EWA
HONOLULU

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Appendix 3. 83 strain data sheet.

Strain Name	Photosynthetic Density on Day 8	Photosynthetic Daily Growth Rate	Mixotrophic Density on Day 8	Mixotrophic Daily Growth Rate	Heterotrophic Daily Growth Rate**
B-A1	0.75	58.9%	0.82	61.7%	2.8%
B-A3	1.23	134.3%	1.17	127.7%	-6.6%
B-B2	0.96	15.8%	0.81	18.2%	2.4%
B-C3	0.10	0.5%	0.12	3.3%	2.8%
B-D1	1.02	99.8%	0.39	86.5%	-13.3%
B-D2	1.21	95.2%	1.15	83.8%	-11.4%
B-E1	1.10	86.1%	1.02	69.1%	-17.0%
B-G4	0.44	27.5%	0.43	33.3%	5.8%
B-H1	1.22	132.4%	1.04	121.0%	-11.4%
B-H3	1.23	57.4%	1.21	69.3%	11.8%
B-I1	0.26	16.2%	0.35	25.7%	9.5%
B-I2	0.50	42.2%	0.58	63.2%	21.0%
B-J1	0.23	14.8%	0.25	17.0%	2.2%
B-J2	0.27	27.2%	0.28	34.2%	7.0%
B-K1	0.56	59.6%	0.48	49.7%	-10.0%
B-K3	0.37	52.4%	0.34	39.1%	-13.4%
B-K4	0.26	18.2%	0.35	27.8%	9.6%
B-L4	0.36	25.1%	0.39	33.1%	8.1%
B-M1	0.96	70.5%	1.02	86.0%	15.5%
B-M2	0.97	48.9%	1.02	51.3%	2.4%
B-M3	1.12	52.7%	1.22	57.6%	4.9%
B-M4	0.95	65.0%	1.05	74.7%	9.7%
B-N1	1.28	136.5%	1.33	142.9%	6.4%
B-N2	0.57	51.8%	0.73	70.0%	18.2%
B-N3	0.35	13.8%	0.46	21.4%	7.7%
B-N4	0.45	38.8%	0.59	54.7%	15.9%
K-A1	0.55	24.1%	0.60	27.6%	3.6%
K-A4	0.82	41.4%	0.94	40.7%	-0.8%
K-B1	0.46	39.2%	0.68	60.2%	21.0%
K-C1	0.78	63.2%	0.83	79.4%	16.2%
K-C2	0.37	19.9%	0.50	26.1%	6.3%
K-C3	0.16	6.1%	0.24	16.1%	10.0%
M-A1	0.38	37.2%	0.43	47.1%	9.9%
M-A2	0.24	21.4%	0.32	34.8%	13.4%
M-A3	0.43	28.0%	0.36	38.0%	10.0%
M-A4	0.47	30.2%	0.70	58.5%	28.3%
M-B1	0.28	6.1%	0.29	24.5%	18.4%
M-C1	0.24	31.2%	0.29	38.4%	7.2%
M-C2	0.53	56.5%	0.61	79.2%	22.7%
M-C3	0.63	99.7%	0.84	126.3%	26.6%
M-C4	0.56	27.2%	0.65	41.6%	14.3%
M-D1	0.30	24.5%	0.38	35.4%	10.8%

M-D2	0.44	12.4%	0.52	14.6%	2.2%
M-D3	0.84	70.6%	0.74	56.4%	-14.2%
M-D4	0.91	46.3%	1.01	54.3%	8.0%
M-E2	0.86	87.5%	0.95	99.9%	12.4%
M-E3	0.15	7.5%	0.13	6.8%	-0.7%
M-F1	0.35	31.7%	0.41	36.8%	5.1%
M-F2	0.44	45.0%	0.64	72.1%	27.1%
M-F3	1.02	105.9%	1.04	114.5%	8.6%
M-F4	0.79	78.0%	0.69	72.6%	-5.4%
M-G1	0.18	12.7%	0.09	-1.3%	-14.0%
M-G2	0.32	10.8%	0.35	23.6%	12.8%
M-G3	0.17	2.2%	0.19	10.3%	8.1%
M-G4	0.21	18.2%	0.31	25.6%	7.5%
M-H1	0.82	55.0%	0.92	49.0%	-6.0%
M-H3	0.69	71.7%	0.93	80.3%	8.6%
M-I1	0.56	51.4%	0.44	42.0%	-9.5%
M-I2	0.40	44.8%	0.52	53.3%	8.6%
M-I3	0.35	39.6%	0.43	53.1%	13.4%
M-I4	0.36	45.6%	0.53	52.2%	6.5%
M-J1	0.34	39.3%	0.30	38.1%	-1.2%
M-J2	0.73	62.5%	0.87	70.1%	7.6%
M-J3	0.16	9.8%	0.20	17.0%	7.2%
M-J4	1.25	79.7%	1.28	85.9%	6.2%
M-K1	0.67	47.0%	0.76	55.6%	8.7%
M-K4	0.99	64.6%	1.00	65.9%	1.3%
M-L2	1.28	75.9%	1.20	72.3%	-3.6%
M-N2	0.28	25.5%	0.40	34.8%	9.3%
M-N4	0.32	23.0%	0.44	33.8%	10.8%
M-O2	1.06	98.0%	0.93	104.9%	6.9%
M-P2	1.09	89.8%	0.99	68.0%	-21.8%
M-P3	1.00	79.1%	0.95	71.6%	-7.5%
M-Q1	1.02	65.9%	0.94	58.7%	-7.2%
M-Q2	0.25	25.5%	0.27	28.3%	2.8%
O-A1	0.48	33.7%	0.66	55.9%	22.2%
O-B1	0.38	26.2%	0.62	75.3%	49.1%
O-B3	0.89	43.6%	1.04	59.3%	15.7%
O-B4	0.99	48.8%	1.05	53.4%	4.6%
O-C1	1.06	83.8%	1.25	101.2%	17.4%
O-C2	0.29	23.2%	0.43	32.5%	9.3%
O-C3	0.90	77.8%	1.06	89.0%	11.1%
O-C4	1.02	98.1%	1.21	116.5%	18.5%

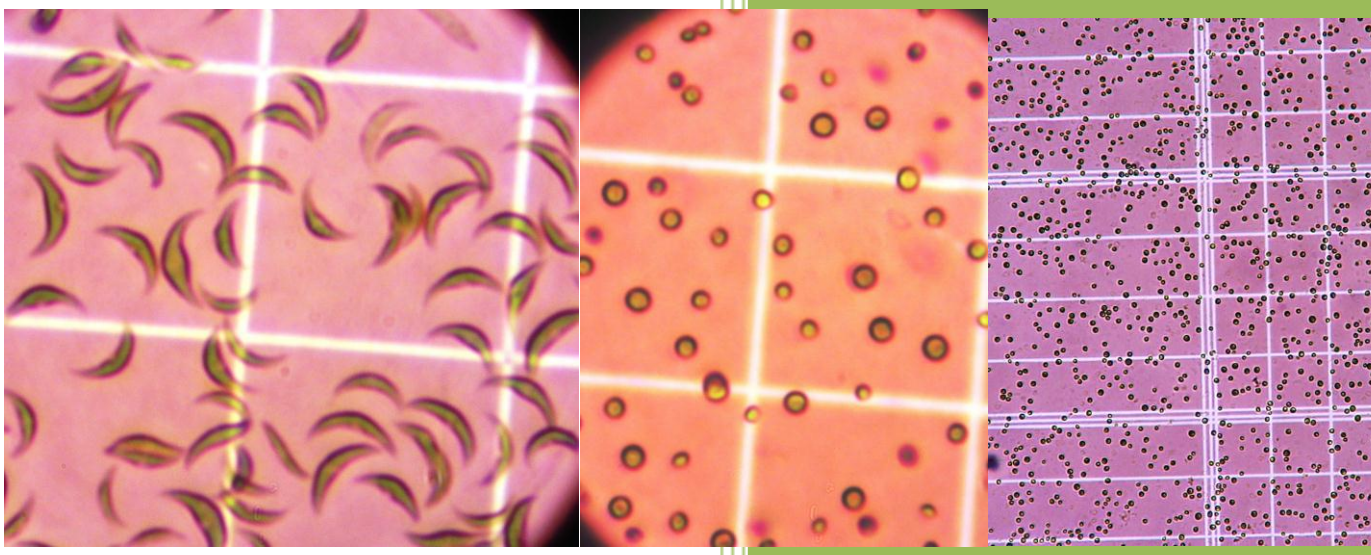
** Heterotrophic growth calculated as the difference between Mixotrophic and photosynthetic.

Appendix 4 Selected 23 Strains Growout data.

Name	High pH Bicarb Media	100% SW	50% SW	Sucrose	Glucose	Photo	Lipid	Crude Protein
B-A3	0.97%	0.93%	9.82%	25.20%	22.32%	24.27%	19.1%	14.4%
B-D2	6.92%	1.50%	9.68%	29.60%	35.38%	35.71%	11.2%	40.7%
B-H1	0.24%	2.30%	2.46%	12.69%	14.94%	14.54%	12.7%	40.0%
B-N1	2.27%	4.73%	3.10%	101.20%	98.52%	91.84%	13.5%	46.8%
B-N2	9.92%	1.74%	6.16%	32.57%	35.36%	14.04%	NA	NA
B-N4	2.93%	5.05%	8.70%	37.33%	38.42%	15.52%	10.9%	54.2%
K-C1	-2.41%	16.87%	39.75%	36.15%	42.66%	42.58%	13.3%	30.6%
M-B1	-1.25%	0.81%	9.43%	31.49%	27.98%	32.94%	9.6%	52.5%
M-C3	6.43%	0.35%	7.54%	24.82%	31.76%	40.46%	10.9%	41.3%
M-D3	8.96%	9.78%	1.65%	77.38%	77.93%	61.87%	12.1%	41.6%
M-D4	25.47%	10.59%	-2.98%	71.13%	94.70%	80.57%	14.1%	39.1%
M-F2	3.71%	5.01%	8.29%	23.51%	21.56%	9.70%	12.9%	39.6%
M-F3	35.83%	13.66%	-0.18%	70.43%	82.06%	71.95%	11.2%	43.4%
M-F4	-2.24%	8.90%	14.29%	46.43%	39.52%	82.31%	14.4%	51.9%
M-H1	118.92%	40.28%	22.50%	77.13%	65.51%	53.94%	8.7%	46.7%
M-H3	66.28%	24.56%	21.50%	84.64%	45.24%	58.03%	10.8%	35.6%
M-J2	9.38%	0.52%	-6.73%	71.35%	67.29%	67.37%	12.7%	49.5%
M-J4	4.53%	2.39%	15.75%	49.70%	45.27%	51.11%	14.2%	37.8%
M-K4	69.69%	20.83%	26.32%	57.70%	58.14%	56.45%	14.4%	36.6%
M-O2	0.68%	0.59%	4.97%	19.46%	22.17%	19.21%	14.9%	48.7%
O-B1	10.83%	15.78%	6.66%	85.52%	91.88%	82.35%	NA	NA
O-C1	10.48%	8.31%	20.73%	41.19%	42.10%	40.43%	9.0%	44.8%
O-C4	16.18%	10.07%	20.65%	45.68%	47.85%	41.93%	9.3%	41.0%

HAWAI'I BioEnergy

Organic Acids as a Feed for Mixotrophic Microalgal Production



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Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the Office of Naval Research.

Hawaii BioEnergy, LLC

July 9, 2012

1.0 Introduction

Mixotrophic and heterotrophic algal systems have the potential for faster growth and higher densities compared to a typical photosynthetic growth system. This is because these systems can continue to grow without light via heterotrophic processes. This allows microalgae to be grown at greater depth resulting in greater productivity per area and capital basis.

Cost modeling performed by Hawaii BioEnergy (HBE) has determined that the costs of the organic carbon (OC), specifically sugar, and the conversion rate of OC to lipids are the critical drivers of a financially viable mixotrophic growth system. Simply, the prevailing market costs of OCs are too expensive. For example, at HBE's current lipid concentration and the rate at which glucose is converted to biomass, the cost of glucose is $20 per gallon of oil produced. Therefore, it is extremely important to explore alternative and waste sources of OC in order to lower these input costs.

In addition to sugar, literature has shown that mixotrophic microalgae are capable of metabolizing organic acids via their heterotrophic processes. However, in some cases chemical alteration or neutralization of the acid must be done to make it a viable algae feed. This report will summarize HBE's work to optimize the use of organic acids on multiple algal strains including modifications of the acid prior to feeding and methods of using acids as a finishing step to quickly increase biomass and lipid contents.

Sources of Organic Acids

Organic acids can be found in waste streams of a number of different industries and processes. In an earlier report we reviewed numerous industrial waste streams that may contain opportunities to capture low-cost organic acids. This included biofuel production and livestock production.

Biofuel Production

Thermochemical conversion processes like pyrolysis, convert cellulosic biomass to a bio-oil. This bio-oil can contain considerable amounts of both acetic acid and formic acid. Table 1 below shows typical yields of both acetic and formic acid in pyrolysis oil and approximate yields from a 40 million gallon pyrolysis facility.

	Pyrolysis Oil (PO)	40M Gallon PO Facility
Total Acids	10.8 %	4.32M gallons
Acetic acid	7.4 %	2.96M gallons
Formic acid	1.9%	0.76M gallons

Table 1. Acid concentrations of pyrolysis oil and approximate acid yields from a 40M gallon PO facility. (Sipila et al., 1998)

2.0 Experimental Work Feeding Organic Acids

Introduction

Numerous mixotrophic and heterotrophic experiments in the past have concluded that glucose is highly preferred by our main algal strains. Preference is defined as getting the most efficient or highest growth response per unit of carbon. For glucose we routinely achieve 1.3 g of algal biomass per gram of carbon added in the form of glucose. The theoretical limit, assuming algae is 40% carbon, is 2.5 g of algal biomass per gram of carbon. However, heterotrophic respiration and bacterial and fungal contaminants will decrease the conversion efficiency.

Methods and Rates

All of the experiments in this report were conducted in laboratory controlled conditions. Controlled conditions ensured optimal performance and minimal contamination therefore the best results and most reliable data.

Because different organic carbons are being added to the cultures and an objective is to compare the results, rates of carbon additions were properly calculated to ensure equivalent amounts of carbon were added. For example, adding 1ml of lactic acid and 1 ml of formic acid to different cultures would result in 35% more carbon being added to the culture receiving lactic acid.

Initial Tests with Acetic Acid

According to the literature (Samejima, 1958, Perez-Garcia et al., 2010), microalgae have the most affinity to acetic acid (AA) relative to other organic acids. Therefore, AA was selected for initial testing and range finding. Results from the initial AA experiments were then applied to the other organic acids.

Acetic acid was applied to algal cultures at different rates to determine if high concentrations and in turn lower pH impacts microalgae growth. Initial rates were based on matching carbon with equivalent glucose amounts. For example, our standard protocol is to add glucose at a rate that matches expected growth. For example we normally add 1 g/l glucose (0.4 g carbon) to an algal culture at 0.1 optical density (around 0.4 g/l DW) and then double that rate (0.8 g carbon) roughly 72 hours later when the culture is roughly 1 g/l of biomass DW. This protocol routinely produces approximately 1.3g of biomass per g of carbon.

Results in Figure 1 show all but the lowest rate of AA actually killed the culture. This was somewhat surprising because at the highest rate, the pH of the culture was only 3.8 after AA was added. This is a pH that has routinely been tolerated by our cultures with pH amendment through an addition of hydrochloric acid (HCl) (See Previous Reports). HCl was used as a means to control bacteria. In contrast to HCl, organic acids are able to penetrate the cell wall via diffusion and lower the inter-cellular pH resulting in cell death (Dibner and Buttin 2002). Because of this process, organic acids are routinely used

for sterilization and in food preservation to inhibit bacterial growth (Dibner and Buttin 2002). At the lowest rate of 0.01 g C, growth rates were not significantly different from the photosynthetic control indicating that there was no effect. This was not surprising as 0.01 g C is a very low rate that would only contribute a negligible amount of heterotrophic biomass.

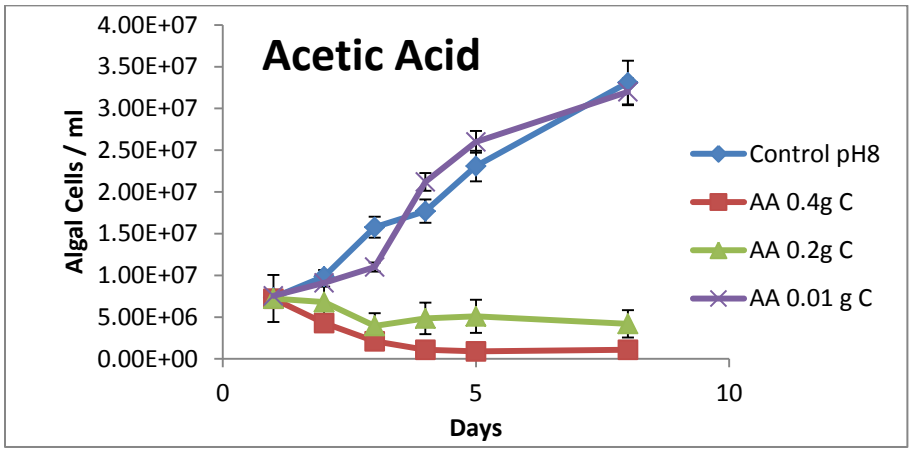

Figure 1. Results of adding acetic acid to algae cultures with a photosynthetic control for reference.

Optimization of Acetic Acid by Chemical Conversion to Improve Uptake

In order to successfully use AA as an alga feed we hypothesized that neutralizing the acid with a base and therefore converting the AA to acetate was required. By doing this we would remove the toxic effect of the organic acid. At a pH above 5.5, the majority of acetic acid is in the form of acetate as shown in Figure 2.

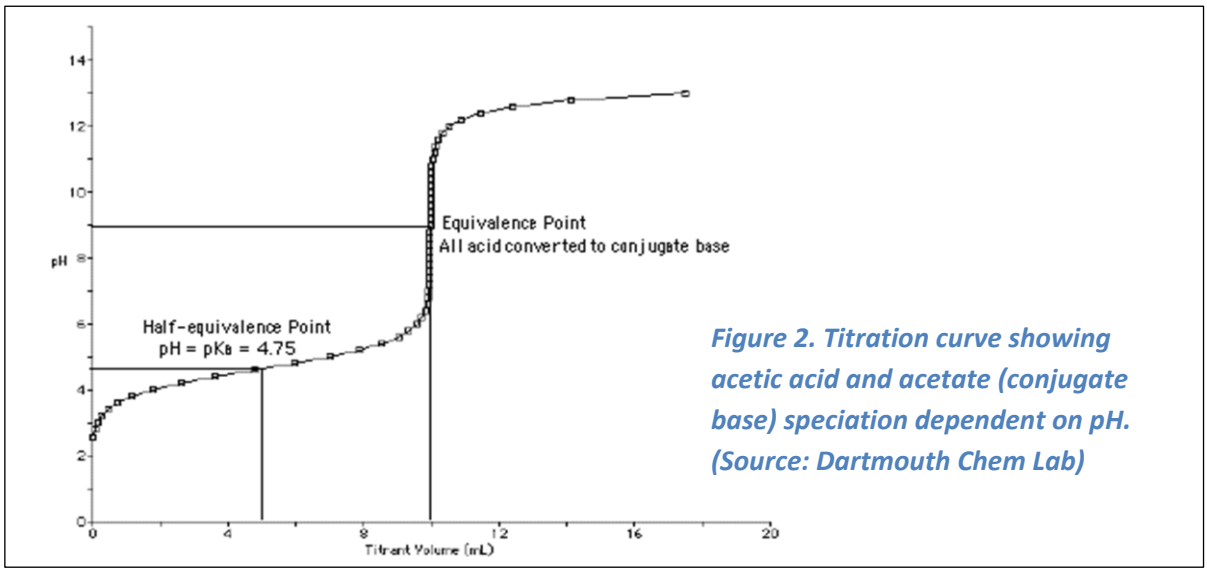

Figure 2. Titration curve showing acetic acid and acetate (conjugate base) speciation dependent on pH. (Source: Dartmouth Chem Lab)

To test our hypothesis we added AA at a rate of 0.4 g C per liter of culture and immediately raised the pH to pH 4.0, 5.0, 6.0, 7.0 and 8.0 with NaOH. Results in Figure 3 show that growth was highest at pH's

above 7.0 indicating that AA must be converted to acetate and be at a neutral pH to optimize the metabolization by the microalgae.

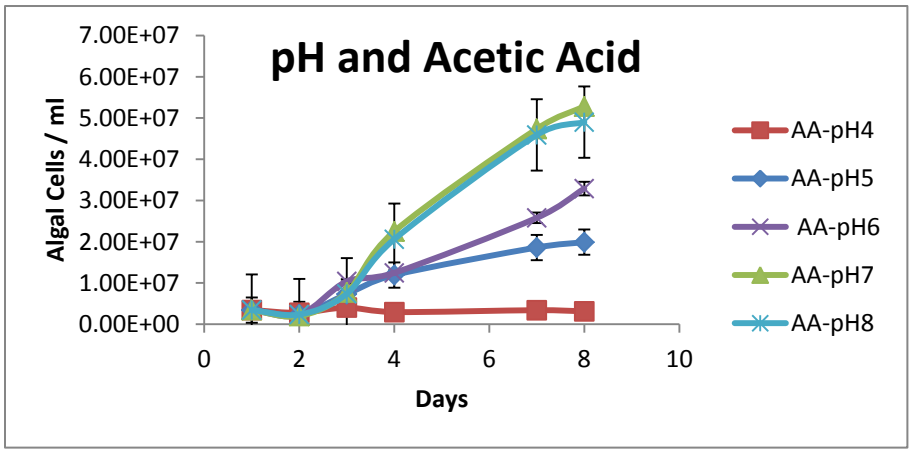

Figure 3. Growth of microalgae with acetic acid (AA) raised to different pH's with NaOH.

Following confirmation that neutralization of AA was required to be an effective heterotrophic feed, we tested different rates via a one-time feeding of AA raised to pH 7.0 with NaOH. The one-time feeding to cultures at 0.4 OD showed that the rate of 0.8g C of AA per liter resulted in the highest growth rate (Figure 4). The highest rate (1.6 g C) resulted in lower growth. This could possibly be attributed to the high amounts of NaOH needed to neutralize the acid which resulted in increased sodium concentrations. A carbon rate of 0.8 g/l is twice what we normally add when feeding glucose, however a similar growth response was observed indicating that glucose is about twice as efficient of a feed as AA. One reason for this may be that glucose has nearly three times more energy (standard enthalpy of combustion) per mole than acetic acid even though both have similar C contents. Therefore, metabolism of glucose should result in more heterotrophic growth as more energy is consumed via uptake.

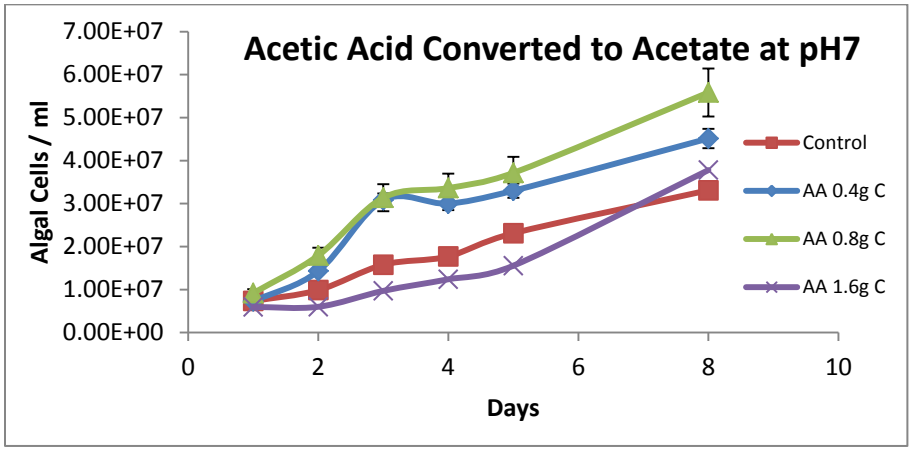

Figure 4. Results of adding neutralized acetic acid (acetate) to algae cultures with a photosynthetic control for reference.

A follow-up experiment was conducted to confirm that complete conversion of AA to acetate was taking place and that adding AA and neutralizing it with NaOH did not have any unknown negative effects. These were both tested by comparing the AA/NaOH treatment with that of adding reagent grade sodium acetate. For both feeds, equal amounts of carbon were added as a means of equalizing the treatments. Results in Figure 5 show the algae growth curves were nearly identical for both treatments indicating that the AA/NaOH does not impact growth.

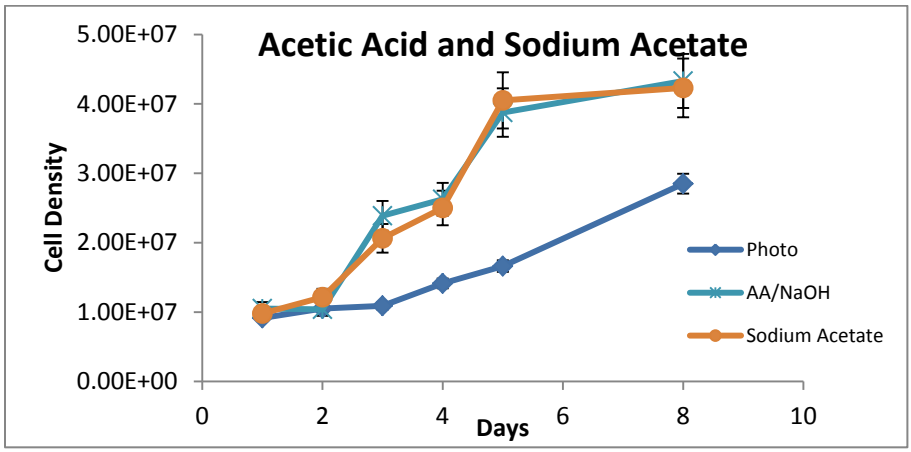

Figure 5. Comparison of acetic acid (neutralized to pH7) and acetate in mixotrophic algae cultures with photosynthetic growth curve for reference.

Optimization of Feeding Based on Rate

In an attempt to optimize feeding we conducted a rate experiment where the same amount of acetic acid (1.44 g of C) was added to cultures but the rate of addition was differed. The exact feeding regime is shown in Table 2. Rates were determined based on expected growth curves and theoretical conversion rates. For example, for AA-2 and AA-3 we fed the most C during days 3 and 4 when we expected the algae to be in a logarithmic growth phase and therefore requiring the most amount of C to continue the high growth rate.

Days	1	2	3	4	5	6	7	8
AA-1	1.44	0	0	0	0	0	0	0
AA-2	0.60	0	0	0.84	0	0	0	0
AA-3	0.19	0	0.87	0	0.38	0	0	0

Table 2. Grams of C (as acetic acid) added on a daily basis.

Results of the experiment show that by adding more frequent, smaller amounts of AA, growth rates were highest. Growth rates increased significantly with increasing rate frequency (Figure 6).

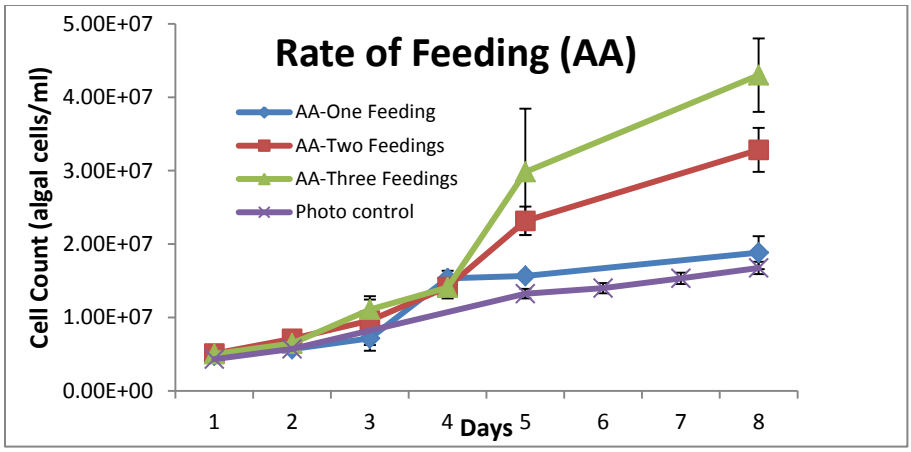

Figure 6. Algal growth rates at different feeding regimes of acetic acid.

Bacteria populations were monitored throughout the experiment. It was hypothesized that more frequent, smaller feedings would keep bacteria levels lower and would contribute to better conversion of AA to algal biomass because bacterial uptake would be less. Results (Figure 7) show that bacteria densities decreased with increasing feeding rate. AA-1 showed an immediate spike in bacteria and then a slow decline as C went limiting. Because the entire feeding of AA was added initially, when algal densities were low, bacteria consumed the majority of the AA and kept algae growth limited. In contrast, AA-2 and AA-3 with multiple, smaller feedings produced lower bacterial densities and higher algal densities. This was because AA was added when algal densities were higher (days 3-5) and more AA was consumed by the algae.

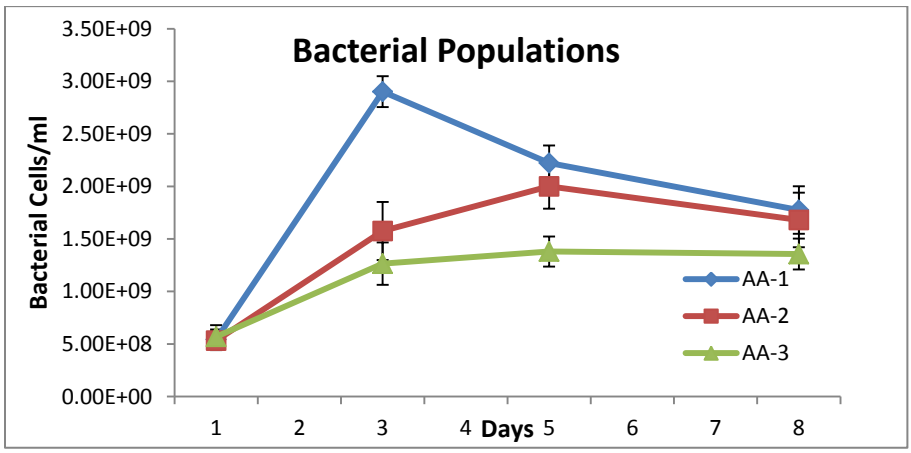

Figure 7. Bacterial densities and different feeding rates.

The same experiment was also performed using glucose as the organic carbon. Results (Figure 8) were similar to that of the AA feeding. Results showed that smaller, more frequent feedings resulted in higher algal densities and more efficient conversion of organic carbon to biomass. Further, results confirmed earlier observations that carbon from glucose results in approximately twice the biomass per unit of C than AA.

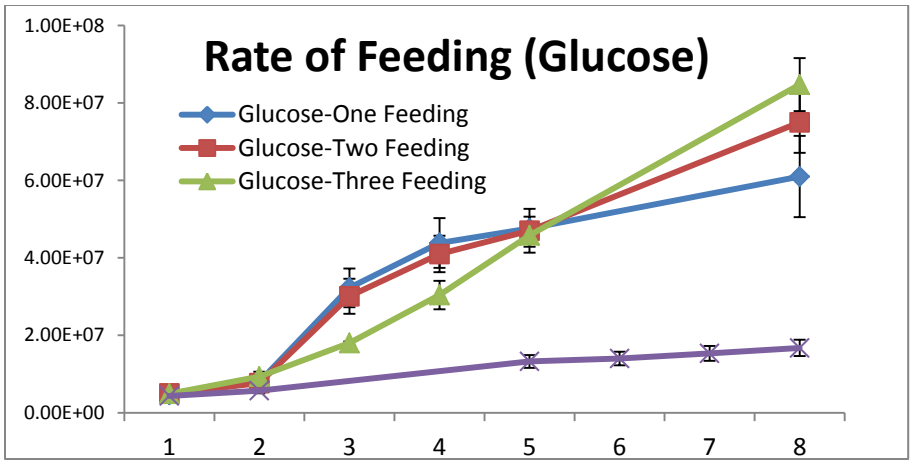

Figure 8. Algae growth rates at different feeding regimes of glucose.

Other Organic Acids

Lactic Acid

Initial tests with lactic acid (LA) confirmed that it had to be neutralized with NaOH in order for it to be successfully added to the algal culture (data not shown). Without neutralization, results were similar to acetic acid where cultures were killed by the organic acid effects.

Once LA was neutralized (converted to lactate) and added to the culture we confirmed that it could be metabolized by the algae in both mixotrophic and heterotrophic growth environments. However, the algae's affinity for lactic acid was found to be less than for acetic acid (Figure 9). Growth curves in Figure 4, show that the algae grown with AA with had significantly higher growth rates and higher densities than those grown with LA. One possible explanation for this is that acetate ($C_2H_3O_2$) is a simpler molecule than lactate ($C_3H_5O_3$) and may be easier to access and consume via enzymatic breakdown.

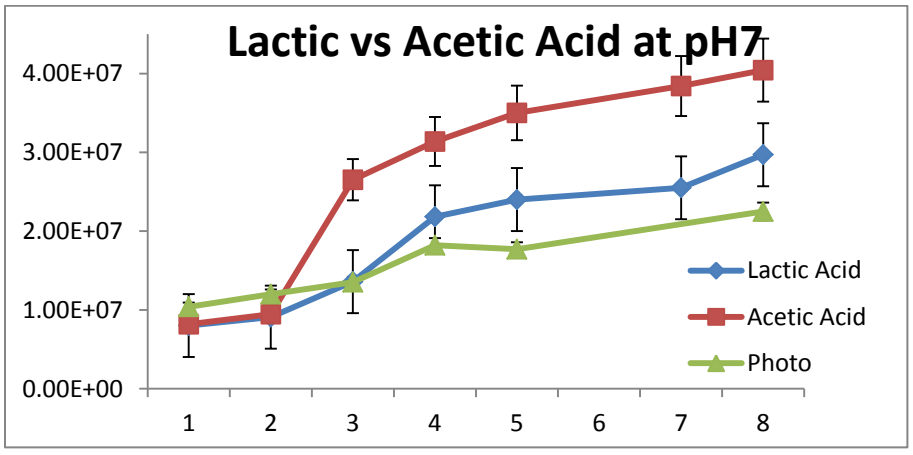

Figure 9. Lactic acid and acetic acid in a mixotrophic growth system with photosynthetic growth curve for reference. Error bars are standard deviation of replicates.

Formic Acid

Unlike lactic acid and acetic acid, microalgae cultures showed no affinity for formic acid even after being neutralized with NaOH and converted to formate. Addition of formate at a 0.4 g carbon/L rate resulted in slower growth than the photosynthetic controls indicating the formate was negatively influencing the culture (Figure 8).

In an attempt to verify the negative effect of formate, we added a combination of glucose and formic acid to microalgal cultures. We hypothesized that by adding glucose, inhibition by formic acid could be reduced because of the strong affinity of the algae for glucose. Results (See Figure 10) show that growth rates were still lower than the photosynthetic control indicating formic acid/formate does inhibit algal growth.

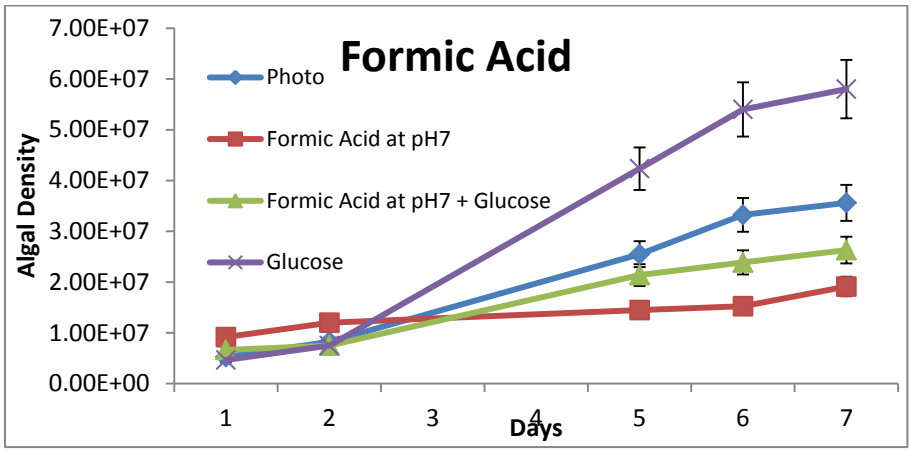

Figure 10. Results of applying formic acid (formate) to algal cultures. Growth curves of photosynthetic and glucose are given for reference.

Conversion Affinity

The conversion affinity for organic carbons from highest to lowest: glucose, acetate (AA), lactate (LA), formate (FA). The data is summarized in Table 3, illustrating how much algal biomass was created from 1g of the specified organic carbon added to the culture using the optimized feeding strategy described above.

Organic Carbon	Algal Biomass per g of C
Glucose	1.33
Acetate	1.04
Lactate	0.84
Formate	0.09

Table 3. Algal affinity of selected organic carbons.

Other Strains

Twenty-six strains of microalgae were screened for their affinity to acetic acid. For all 26 strains, AA and AA neutralized with NaOH to pH7 were added at a rate of 0.4g C /L. No strains had a positive growth

rate with acetic acid that wasn't neutralized with NaOH. However, after neutralization, three strains, B-N1, M-E2 and B-A1 showed increased growth rates with acetic acid. All three strains were also capable of metabolizing glucose and all had a higher affinity for glucose. Of the three, B-A1 showed the least affinity for the acetate, while M-E2 and B-N1 had considerably higher affinity (See Table 4) although less than glucose. The figures given in Table 4 are based on a feeding regime where AA was added at two different times in the growout. It is expected that the affinity would increase if the optimized, smaller more frequent feeding regime was used.

Strain Name	Algal Biomass per g of C (Glucose)	Algal Biomass per g of C (Acetic Acid)
M-E2	1.25	0.99
B-N1	1.79	1.47
B-A1	1.12	0.57

Table 4. Affinity of three different algal strains to organic carbon.

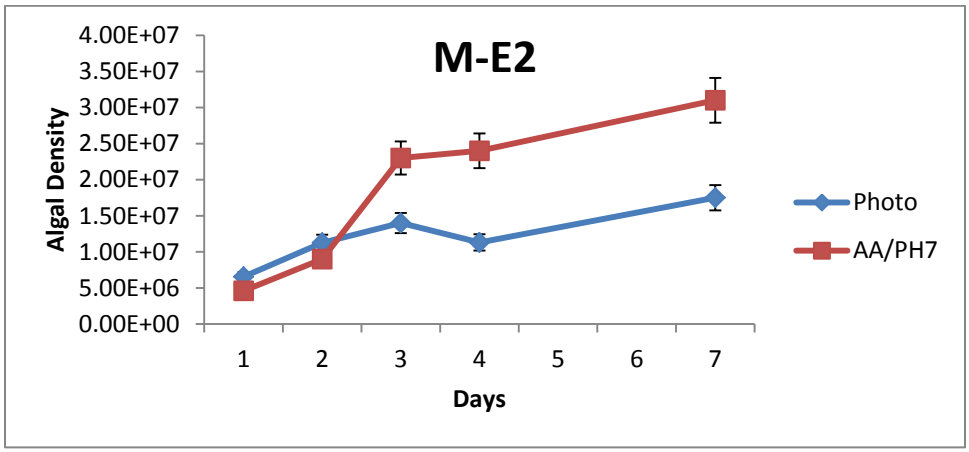

Figure 11. Growth curve of M-E2 with acetic acid at pH7 and a photosynthetic control for reference.

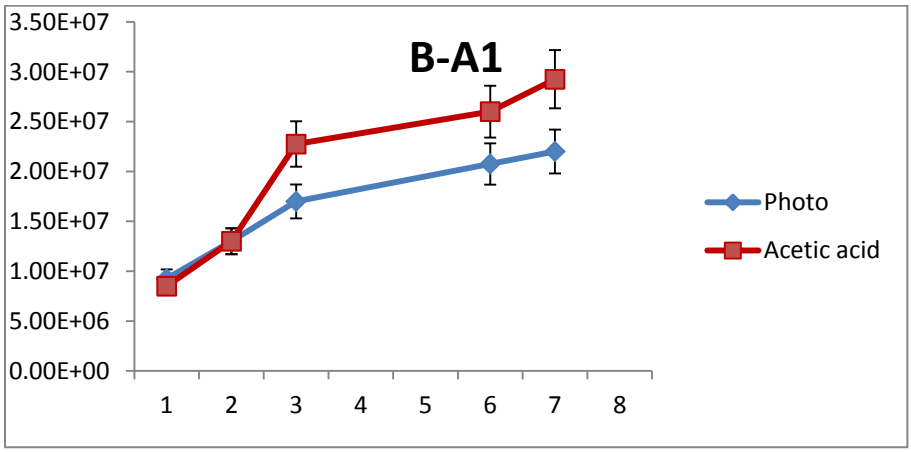

Figure 12. Growth curve of B-A1 with acetic acid at pH7 and a photosynthetic control for reference.

Algal strain B-N1 was further evaluated for affinity to lactic and formic acid both neutralized with NaOH. Growth was significantly higher compared to a photosynthetic control when both lactic acid and acetic acid was added to the culture. Of the two acids, B-N1 had a higher affinity for acetic acid as indicated by the higher growth rate and higher maximum density. Similar to previous results, formic acid resulted in decreased growth rates compared to photosynthetic controls.

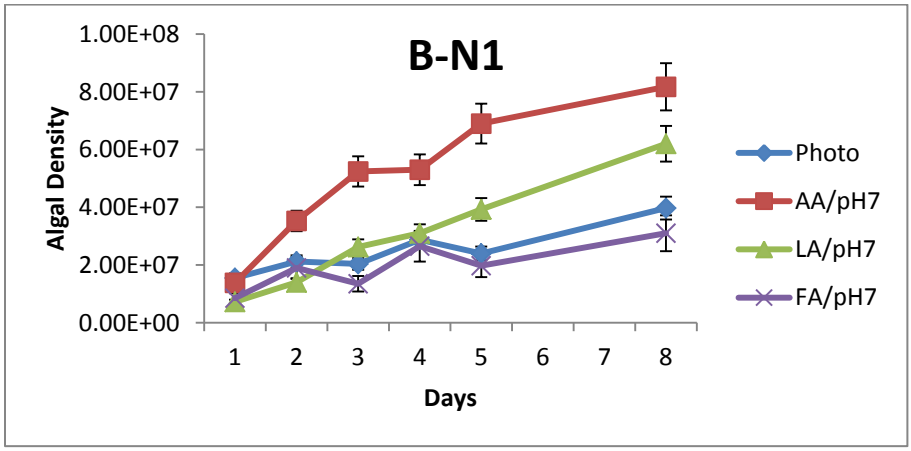

Figure 13. Growth curves of B-N1 grown with a variety of organic acids. A photosynthetic control is given for reference.

Lipid Concentration with Organic Acids

An experiment was conducted to compare lipid concentrations of cultures receiving different types of organic carbon. Results showed that acetic acid (raised to ph7) produced higher lipid concentrations than other treatments (figure 14). These cultures were not starved of nitrogen, which is a common means of increasing lipid concentrations. Similar results were also attained in a previous report that compared different methods of N-starvation as a means of increasing lipids. In that report, acetic acid also had highest lipid concentrations regardless of N-starvation method.

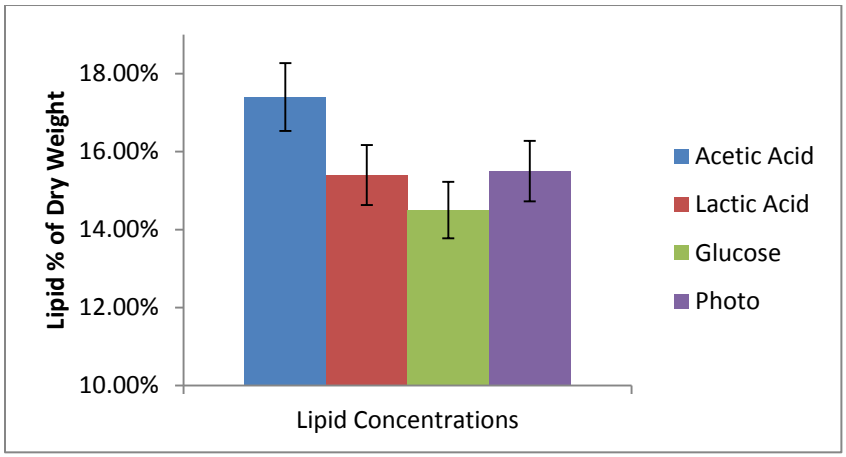

Figure 14. Lipid concentrations of microalgae under different organic carbon additions without nutrient starvations.

A recent study in the journal *Plant and Cellular Physiology* by Fan et al (2012) showed that adding acetate at levels that exceed the capacity of starch synthesis results in oil synthesis even in non-limiting nutrient growth media. They concluded that this method of lipid accumulation is preferred over the nutrient starvation method because algae continue to grow in these conditions. Under N-starvation, growth is severely limited while lipids accumulate.

Conclusions

Organic Acid Feeds

The high commodity cost of sugars for heterotrophic and mixotrophic algal growth systems are highly prohibitive to successful commercial fuel production. However, organic acids may be sourced cheaper if taken from agricultural or processing waste streams. We identified three organic acids, acetic, lactic and formic that all could be potentially sourced cheaply in a waste stream. Two of the organic acids, acetic and lactic were successfully proven to support growth in a heterotrophic environment. However, they had to first be neutralized with NaOH which converted them to their respected conjugate base.

All microalgae that we identified as being capable of using organic acids were able to also metabolize glucose. For all strains, glucose had a higher conversion efficiency than organic acids. However, if the organic acids could be obtained at low costs they would make more economically efficient feeds.

Through a series of optimization experiments we identified an upper feeding limit when using organic acids. A rate of C above 0.8 g/l hindered growth likely because of the large amount of NaOH needed to neutralize the acid. Further, smaller doses of organic carbon kept bacterial populations lower and subsequently increased algal uptake and conversion efficiency. Therefore the optimal feeding regime would consist of daily or every other day feeding where the amount of C added is based on the current density and the projected growth rate. In addition, lipid contents could be expected to be higher when frequent acetate feedings were performed.

Commercial Potential with an Integrated Pyrolysis Oil Refinery

Hawaii BioEnergy is currently evaluating the development of a 40 million gallon pyrolysis refinery. Based upon our work, it is estimated that over 6.8 million kg of algal biomass could be produced from the acetic acid by-product. This mixotrophic algal system could be used to produce additional, high-value, co-products like animal feeds or nutraceuticals. While this remains to be a relatively small amount, these could replace a portion of import needs in Hawaii.

Further Work

Hawaii BioEnergy through Office of Naval Research (ONR) funding, has been conducting R&D of open-system mixotrophic algae work for the last two years. In this time, HBE has isolated and identified over

50 local algae species throughout the State. Additional research was completed to decrease the costs of mixotrophic algae systems. We looked a three specific ways of reducing costs of the mixotrophic system.

1. Increased growth efficiency of bioreactor: In order to maximize capital costs of building ponds, designs that increase growth rates and productivities need to be researched. Through our ONR funding we designed a novel bioreactor that separated photosynthetic and heterotrophic growth areas via a moveable membrane. Therefore, we could theoretically have high percentages of the culture in a photosynthetic environment when densities were low and there were high amounts of light. Then as densities increased and less light became available, more and more of the culture was moved to the lower, dark area where heterotrophic growth took place while the top layer maintained high available light. This bioreactor design maximizes productivity on an areal basis, which is critical in land limited areas like Hawaii.

2. Optimize mixing: A large cost of growing algae in deep ponds is the power required to move the water and keep the algae suspended. Further, sub-optimal mixing can decrease growth rates by allowing microalgae to fall out of suspension. Using computational fluid dynamics, we modeled different mixing systems and instrument placement in order to maximize the mixing efficiency in systems greater than one acre in area. Using these mixing regimes, we then tested them and successfully grew stable mixotrophic cultures in the large scale ponds.

3. Reduce contamination: Contamination has been a re-occurring theme in our open system mixotrophic R&D. Contamination reduces the uptake of organic carbon by the algae and instead it is consumed by faster growing low oil content organisms like bacteria and fungus. Reducing contamination is important because the costs of organic carbon can be very expensive. Through the project we demonstrated that spiking the cultures with HCl to a pH of around four just prior to feeding OC was successful in controlling bacteria. Results showed that doing this pH spike allowed algae to uptake a higher percentage of the OC compared to bacteria.

Heterotrophic and mixotrophic systems offer great benefit because of their potentially high growth rate under light-limiting conditions. The latter enables greater production and harvesting efficiency as greater biomass is produced using less land, water, and energy.

Although we were successful in accomplishing many of our goals through the ONR funding, there is still work that needs to be conducted to fully understand both the economics and the productivity of mixotrophic growth systems. Most importantly, additional work is needed to improve the conversion of organic carbons to biomass in open systems. More work is needed to understand if this is even capable with current algae strains. For example, axenic heterotrophic work in closed fermenters should allow for quantification of maximum conversion efficiencies for each strain. In conjunction, more work needs to be done to better understand how to maximize lipid contents using acetate while keeping the culture growing.

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