DISSERTATION

BIOCONVERSION OF LIPID-EXTRACTED ALGAL BIOMASS INTO ETHANOL

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ABSTRACT

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Energy security, high atmospheric greenhouse gas levels, and issues associated with fossil fuel extraction are among the incentives for developing alternative and renewable energy resources. Biofuels, produced from a wide range of feedstocks, have the potential to reduce greenhouse gas emissions. In particular, the use of microalgae as a feedstock has received a high level of interest in recent years.

Microalgal biofuels are promising replacement for fossil fuels and have the potential to displace petroleum-based fuels while decrease greenhouse gas emissions. The primary focus of research and development toward algal biofuels has been on the production of biodiesel or renewable diesel from the lipid fraction, with use of the non-lipid biomass fraction for production of biogas, electricity, animal feed, or fertilizer.

Since the non-lipid fraction, consisting of mainly carbohydrates and proteins, comprises approximately half of the algal biomass, our approach is biological conversion of the lipid-extracted algal biomass (LEAB) into fuels. We used LEAB from *Nannochloropsis salina*, and ethanol was the model product. The first step in conversion of LEAB to ethanol was deconstruction of the cell wall into fermentable substrates by using different acids or enzymes. Sugar release yields and rates were compared for different treatments. One-step sulfuric acid hydrolysis had the highest yield of released sugars, while the one-step hydrochloric acid treatment had the highest sugar release rate. Enzymatic hydrolysis produced acceptable sugar release rates and yields but enzymes designed for algal biomass deconstruction are still needed. Proteins were deconstructed using a commercially available protease.

The hydrolysate, containing the released sugars, peptides, and amino acids, was used as a fermentation medium with no added nutrients. Three ethanologenic microorganisms were used for fermentation: two strains of *Saccharomyces cerevisiae* (JAY270 and ATCC 26603) and *Zymomonas mobilis* ATCC 10988. Ethanol yields and productivities were compared. Among the studied microorganisms, JAY270 had the highest ethanol yield while *Z. mobilis* had the lowest yield for most of

the studied conditions. A protease treatment improved the biomass and ethanol yields of JAY270 by providing more carbon and nitrogen.

To increase ethanol productivity, a continuous fermentation approach was adapted. Continuous stirred tank reactors have increased productivity over batch systems due to lower idle time. The downtime associated with batch fermentation is the time it takes for empting, cleaning, and filling the reactor. Productivity in the continuous fermentation was limited by the growth characteristics of the microorganism since at high flow rates, with washout occurring below a critical residence time. To overcome the washout problem, the use of an immobilized cell reactor was explored. The performance (ethanol productivity) of free and immobilized cells was compared using an enzymatic hydrolysate of LEAB. Higher ethanol productivities were observed for the continuous immobilized cell reactor compared to the stirred tank reactor.

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1 Introduction

1.1 Overview

In this dissertation research, the focus was on ethanol fermentation from lipid-extracted algae biomass (LEAB). LEAB contains considerable amounts of carbohydrates and proteins that can be used as energy source by microorganism. LEAB was deconstructed to fermentable substrates and the hydrolysate was used for ethanol fermentation. The biomass was hydrolyzed with no pretreatment step. An advantage of using LEAB over other types of biomass is lack of lignin, which makes the hydrolysis simpler with no need for a pretreatment step. Algae may contain algaenan, which is a biopolymer recalcitrant to most treatments. Different hydrolysis methods including acid or enzyme treatment were compared based on their resulting sugar release yields and rates. The inhibitors generated by different hydrolysis methods were quantified and their effect on the growth of a yeast strain was studied. The resulting hydrolysates with no added nutrients were fermented with either *Saccharomyces cerevisiae* or *Zymomonas mobilis*. The ethanol production for these two microorganisms was compared in batch fermentations based on ethanol yields. The advantage of this study is using the hydrolysate with no added nutrients. Most of the studies that used LEAB for ethanol fermentation, added other nutrients to the hydrolysate. Our approach was to keep the operating costs minimal by minimal adaptation of the LEAB hydrolysate. Batch fermentation is limited by the ethanol tolerance of the microorganism and by the downtime associated with a batch process. To increase ethanol productivity, process intensification strategies were adapted. Two strategies, immobilized cell reactor and continuous fermentation, were tested. Continuous fermentation of LEAB enzymatic hydrolysate with yeast cells resulted in higher ethanol productivity compared to batch fermentation. The continuous ethanol fermentation in an immobilized yeast cell reactor was compared to a continuous stirred tank reactor. To our knowledge, this is the first study that used LEAB hydrolysate in continuous fermentation. Bioconversion of LEAB has advantages over thermochemical conversions. Biological conversion allows the user to produce a specific product, as well as the option to recover residual proteins for animal feed and other uses. In this study, bioconversion of LEAB to ethanol was evaluated. Ethanol was the model product, but the results can be generalized to other fuels and chemicals. Ethanol was selected as the model product since it can be used as a drop-in

fuel or as a chemical platform for production of bio-based chemicals. Many chemicals that are produced from oil can be produced from ethanol including ethylene, acetaldehyde, and ethyl acetate [1]. The remaining LEAB after ethanol fermentation still contains nutrients and can be recycled back to the algal cultivation pond, be used as fertilizer, or get used for production of more fuels via anaerobic digestion or hydrothermal processing. Our proposed process has the advantage of being capable of producing several value-added products.

1.2 Research goal

The goal of this project was to develop a biochemical process for conversion of lipid-extracted algae biomass to ethanol. Our hypothesis was that the remaining algal biomass after lipid extraction contains considerable amount of carbohydrates and proteins, which can be used as energy sources by microorganisms to produce valuable compounds. The model product was ethanol, but other fuels or value-added chemicals can be produced by selecting a different microorganism for the fermentation step. To accomplish this goal, the following tasks were completed:

1) Deconstruction of LEAB to fermentable substrates using hydrolyzing agents such as acid or enzyme. To achieve this objective, different acids or enzymes were tested and optimized conditions such as concentration of acid or enzyme, temperature, biomass concentration, and reaction time were found.
2) Identification of inhibitory compounds generated during hydrolysis. Some of the hydrolysis conditions generated compounds that were inhibitory to fermenting microorganisms. All hydrolysates were screened for the presence of common fermentation inhibitors.
3) Fermentation of the resulting hydrolysates with different ethanologenic microorganisms with no additional nutrients. Selected ethanologenic microorganisms were tested and compared based on their growth and ethanol yield.
4) Process intensification by continuous fermentation. Ethanol productivities of continuous fermentation of LEAB hydrolysate in a chemostat was compared to an immobilized cell reactor.

2 Background and Literature Review

2.1 Motivation for renewable energy

About 88% of world's energy needs are obtained from fossil fuels [2] and the challenge that the whole world is facing is to meet the mobility and chemical needs of all the nations. Dependence on crude oil is increasing the concerns over national energy security and price stability [3]. Fossil fuel dependence not only affects the economy but also has environmental and political impacts. Diminishing fossil fuel resources and increasing greenhouse gas emissions are among incentives for developing alternative energy sources [3, 4].

United States spends about $1 billion per day on importing oil from volatile regions of the world, which results in serious geopolitical concerns [5]. It is estimated that U.S. had spent $8 trillion on protecting oil cargoes in the Straits of Hormuz (Persian Gulf) since 1976 despite the fact that only 10% of the oil passing through the Straits is actually destined for the U.S. [6]. These are only a few examples of the financial burden on the U.S. economy caused by foreign oil. The trade deficit of U.S. in 2012 only, was $291 billion. This number is shrinking by fostering policies with respect to better fuel economy, increased oil production, and expanded use of renewable fuels. If renewables can displace imported oil then a large portion of this money will be invested here in U.S. and will have a great impact on the economy and job creation [7] .

Some of the environmental issues associated with using fossil fuels are greenhouse gas emissions, air pollution, and acid rain [4]. Biofuels have a net life-cycle reduction in greenhouse gas emissions (GHG) compared to petroleum-based fuels. The GHG impact of a biofuel depends on the energy used for the growth and harvest of the feedstock plus the energy used to produce the fuel. Technologies used for advanced biofuels have the potential to reduce GHGs by 70% to more than 100%, relative to conventional gasoline [7].

Political and geopolitical challenges associated with importing oil from volatile regions of the world are of concern. The oil crisis in 1973 and subsequent rise in fuel prices changed the approach in political circles [8]. Fossil fuel supplies are limited and we probably run out of these resources in a couple hundred years. When the production of petroleum reaches its maximum level, the main concern will be future

energy supply [5]. The solution to this problem is finding renewable sources of energy including solar, wind, and bioenergy [7].

The aforementioned reasons summarize why as a nation we need renewable sources of energy. We need to diversify energy resources and reduce the nation's dependence on imported oil. All sources of renewable and clean energy including solar, wind, and bioenergy are needed.

2.2 Bioenergy

The term bioenergy refers to the energy derived from biological materials such as biomass. The major form of bioenergy is biofuels, which are being used for transportation purposes [9]. Biofuels are categorized depending on the type of biomass used for their production.

Biomass is the only renewable energy source that can replace the whole barrel of petroleum [7], while other resources such as wind or solar do not have this potential. Biomass resources for production of bioenergy and/or biofuels are but not limited to lignocellulosic biomass, municipal solid waste, and algae. Biomass used for energy production is called feedstock. Using biomass as the renewable source for production of fuels has several benefits including stimulation of the economy, improvement of the U.S. trade balance, mitigation of climate impact, increasing energy security, boosting U.S. technology leadership, and enhancing sustainability [7].

Biomass is the only carbon rich material source that can replace fossil fuels and chemicals. Carbohydrates, lignin, triglycerides, and proteins are the chemical structures within biomass that are of significance for a biorefinery. The average composition of synthesized biomass in the world is 75% carbohydrates. This proves why the focus of research and development should be on efficient access to carbohydrates, and their subsequent conversion to final products [8].

First generation biofuels are primarily produced from food resources [10, 11], and compete with land and water usage for production of food or fiber [9]. The advantage of the first generation is their conversion technology, which is economical and environmentally friendly [10]. The products from the first generation of biofuels are biodiesel, corn ethanol, and sugar alcohol.

The second generation of biofuels can be produced from plant waste biomass including agricultural and forest residue, which does not compete with food resources. The main issue with this generation is

developing an economical biomass conversion technology. This is especially true for lignocellulosic biomass since lignin is hard to hydrolyze. Some of the common products of second generation biofuels are bio-oil, lignocellulosic ethanol, butanol, and mixed alcohols [10].

Currently, most of the biofuels, mainly the first generation, are obtained from food sources such as corn grain and sugar cane. This affects the price for food and is the reason why the energy and agricultural markets are closely affected by one another [12]. Food security especially with more than one billion people suffering from lack of dietary energy is becoming more serious. The production of food has also been adversely affected by the greenhouse gas (GHG) emissions. The second and third generations of biofuels are promising since they do not compete with human food.

Biofuels produced from microalgae are classified as third generation and are of interest due to their unique characteristics. When compared with terrestrial plants, the advantages of using microalgae as a potential source of fuels are that there is no requirement for soil fertility and, for marine algae, there is minimal need for fresh water [13]. Other characteristics of microalgae compared to terrestrial plants are higher growth rate of algae, higher productivity per unit land area, lower requirements of fresh water [14]. Another interesting potential of microalgae as biofuel feedstock is the ability to utilize nutrients such as nitrogen and phosphorus from wastewater sources and sequester carbon dioxide from power plants' flue gases [15]. Microalgae grown for biofuels production do not compete with human food; and can actually be used as animal food since it is rich in proteins, vitamins, and other nutrients [12]. Microalgal biomass can be used for human nutrition as supplements or nutraceuticals in the forms of tablets, capsules, and liquids or can be incorporated into snacks and beverages [16]. Algal biomass can be used as food colorant such as astaxanthin. The major market for astaxanthin is the pigmentation agent in aquaculture, primarily in salmon [17]. Fuel production is only one application of the algal biomass [18, 19], while wastewater treatment [20], production of a metabolite such as human nutrients, animal feed, or recombinant proteins are among the others [2]. All these characteristics make the algal biorefinery a promising replacement for petroleum-based refineries.

2.3 The biorefinery concept and replacing the whole barrel

The biorefinery concept is used to describe the production of biochemicals and biofuels in an integrated process from biomass [19]. In a biorefinery setting, different products are produced and recovered by a set of jointly applied technological processes[8]. In other words, the concept of a biorefinery holds a wide range of technologies that can separate biomass resources into their building blocks which can be converted to value-added products, biofuels, and chemicals [8]. A biorefinery facility can produce transportation biofuels, power, and chemicals from biomass. The most common biofuels produced in the world today are bioethanol, biodiesel, and biogas. Some of the common commercially available bio-based products are adhesives, cleaning compounds, detergents, hydraulic fluids, lubricants, paints and coatings, polymers, solvents, and sorbents [8]. Biorefinery can improve the process economics and resolves the issues associated with waste management since it turns a waste stream to value-added products or energy.

Any system should meet seven requisites to be considered a biorefinery [8]. These are:

1. Biomass refining: raw materials are upgraded and refined. A biorefinery separates all the biomass components to be processed for production of a high concentration of a pure chemical such as ethanol or a high concentration of molecules having similar functions such as Fischer–Tropsch fuels.
2. Combustion of residues: the whole feedstock cannot be combusted in a biorefinery system since the whole purpose of a biorefinery is to increase the value of different components of biomass and only the leftovers from other conversion processes can be sent to the combustion unit.
3. Vale added chemicals/materials: production of at least one value chemical besides animal food or fertilizers is necessary.
4. Fuel or energy products: production of at least one biofuel besides heat and electricity is required.
5. Fossil fuel replacement: a biorefinery should be capable of replacing fossil fuel based products including chemicals and energy carriers.
6. Energy self-sufficiency: the energy for biomass conversion should be supplied internally in the form of heat and electricity from the combustion of residues.

7. Waste minimization: all forms of waste production should be minimized. One way is to use the waste produced in a downstream process and send it to the upstream process of another plant [8].

The percent of products being produced from a barrel of oil are as follows: diesel 24%, jet fuel 8%, gasoline 42%, and other products or chemicals 25% (Figure 2-1) [7]. For example, the cellulosic ethanol currently can only displace 42% of a barrel used for production of light-duty gasoline [7]. More research and development are needed on a range of technologies to displace the other 58% of the barrel. The oil refinery uses raw materials such as petroleum and produces consumer goods, while the role of the biorefinery is to convert raw materials originating from a renewable source into the same final consumer goods.

2.4 Microalgal biorefinery

There are many different feedstocks or biomass resources available for production of biofuels and biochemicals [3]. The choice of feedstock is highly dependent on availability and price. For instance, the feedstock for commercial ethanol production in Brazil is sugar cane while in U.S. is cornstarch. Commercial ethanol production from cornstarch is not yet cost effective so other feedstock options such as lignocellulosic biomass or algae are viable replacements [21]. Algae biomass is a good candidate as a feedstock for biofuel production due to its unique characteristics. The focus of the algal biofuel industry has been on the lipids for biofuel production, but large-scale production of algal biofuels is not yet economical. The key to large-scale production of algal biofuels is adapting a biorefinery approach.

High-value molecules other than lipids can be produced using microalgae. One of the applications for algal biomass is fuel production [18, 19], and other applications include wastewater treatment [20], production of a metabolite such as human nutrients, animal feed, or recombinant proteins [2].

A single product strategy is not economical for the algal biofuel industry and a biorefinery approach needs to be adapted. For this reason, most algae companies are adapting a biorefinery approach by having several products, including but not limited to nutraceuticals, animal feed, and bioplastics. Cellana, a Hawaiian algae company, invented a cultivation system called ALDUO, which is a series of photobioreactors coupled with open ponds enabling economic and continuous production of diverse strains of microalgae. Cellana has recently added human and animal health supplements to their product

line. This includes the high-value oils for human nutrition such as DHA and EPA (omega-3 fatty acids) and high-protein algal biomass to replace fishmeal for farmed fish and soymeal for livestock feed [22]. One of the potential applications for LEAB is animal food including cattle and fish [23]. The fishmeal price is almost four times the cattle meal on a per ton basis. The problem with using LEAB as animal feed is that market will be saturated quickly. A recent study has shown that fish cannot utilize the non-starch polysaccharides as energy source since they lack necessary enzymes such as β-glucanases or β-xylanases [24]. Presence of the non-starch polysaccharides in the diet interferes with feed utilization and affects the performance of the fish. Addition of enzymes that degrade the non-starch polysaccharides in the fish meal can mitigate the adverse effect of such polysaccharides [25]. This will not be an issue for green algae since they store their carbohydrates as starch.

The next example is an algae company established in 2010 called Algix. Their focus has been on bioplastic. They co-produce fresh fish and algae biomass in sustainable fish farms, which results in low-cost production of fresh food and bio-based feedstock for the renewable plastics industry. Algix's bioplastic technology blends aquatic feedstocks with commercial polymers to reduce cost and dependence on fossil-fuel and food-based feedstocks [26]. One of the most successful algae companies is Solazyme. Its success relies in the fact that the company has different product lines ranging from high-end personal care products to food and fuels. Their algal flour and protein is intended for human food replacements and additives. One of their famous products is a friction inhibitor, used for horizontal oil drilling, called Encapso. Unlike traditional lubricants, Encapso is composed of micro-sized cells containing pure, custom-engineered lubricating oil [27]. Algenol, another algae company, is using engineered cyanobacteria to produce biomass, ethanol, and biochemicals. Algenol's ethanol fraction goes to fuel and bioplastic production while the biomass is used for production of green crude, diesel, gasoline, and jet fuel. Algenol is going towards biochemicals such as isopropanol, propanol, and isoprene [28].

Microalgal biomass production has several proposed steps such as cultivation, cell harvest, lipid or product extraction, and downstream processing and conversion technologies. Each step has its own challenges and requires more research and development to be improved and become economically feasible. Large-scale production of microalgae biofuels has not yet been economically feasible and significant improvements in all the proposed steps are still needed. Some of the areas for improvements

are species selection, genetic manipulation of strains to increase lipid accumulation, design of bioreactors, pest management strategies, and finding efficient harvesting techniques, and efficient extraction methods.

2.4.1 Algal cultivation

Selection of the strain of interest depends on the final product(s) and environmental conditions in which the strain is grown. Once the strain of interest has been selected, the first step in a biorefinery is cultivation. Depending on the application of the product, the alga can be cultivated in open ponds or in closed photobioreactors. If the final product is fuel then the economics suggest using open ponds but if the final product is nutraceutical then it is logical to grow the alga in a more controlled environment such as photobioreactors.

The two main algae cultivation techniques are open ponds and closed photobioreactors. While both are costly at this time, the economics of cultivation in closed photobioreactors is especially unattractive [29]. Based on the life cycle analysis done by Resurreccion et al., open ponds have lower energy consumption and greenhouse gas emissions than photo bioreactors, for example 32% less energy use for construction and operation [29]. Photobioreactors have different configurations including vertical-column, flat-plate, and tubular photobioreactors [30]. Mass transfer limitation is the major hurdle in practical application of algal mass culture. More research is still required to improve photobioreactors technologies and perhaps this is one of the major issues that needs to be addressed for mass cultivation of algal biomass [30].

One challenge associated with large-scale production of algal biomass is stable cultivation to maintain the elite strain of algae and pest management. Pest management requires a cheap monitoring technique to identify weedy algae and bacteria long before they become prominent in cultures of elite strains [31].

Fulbright et al. developed PCR-based tools to monitor contaminants (weedy algae) in algal cultures. They found out that qPCR was 10^4 times more sensitive for detecting weeds than flow cytometry. Contamination is a common phenomenon and early detection is necessary for decision making during culture selection for sub culturing or scale-up [31].

McNamee et al. developed a multiplex microarray for the detection of five groups of harmful algal and cyanobacterial toxins found in marine, brackish, and freshwater environments. The feasibility of this system as a rapid, easy to use, and highly sensitive screening tool has been investigated [32].

Not all the bacteria in the algal culture are harmful and some actually improve the growth. Suminto et al. inoculated a growth promoting marine bacterium with three different species of marine microalgae. They showed that the bacterium significantly increased the specific growth rate of one microalga and caused the stationary phase to last longer, while the bacterium did not have any effect on the growth rates of the other two algae but kept their high cell densities in the stationary phase longer. The bacterium was the dominant species in the bacterial flora (> 45%) [33]. De-Bashan et al. co-immobilized freshwater microalgae with the microalgae-growth-promoting bacterium *Azospirillum brasilense* in alginate beads and observed significant changes in microalgal population size, cell size, cell cytology, pigment, and lipid content in comparison with the control (microalgae immobilized in alginate without the bacterium) [34]. It has also been known that algae can acquire vitamin B12 through a symbiotic relationship with bacteria [35]. These studies suggest that maybe co culturing with a growth promoting bacteria is a way to promote the growth of the elite strain of the alga.

A continuous cultivation process needs an efficient monitoring technique before the culture crashes. If the algal culture does not crash and the elite strain is maintained, the next step is cell harvest.

2.4.2 Algal harvest

It is necessary to harvest the culture (to separate the algal cells from the culture medium) when the molecule of interest is reserved inside the cell [36]. Algal harvesting is one of the most energy intensive steps in the algal biorefinery and represents 20–30% of total production costs [36]. The concentration of an algal culture at the point of harvest is usually about 5 g/L, which is a dilute culture in terms of harvesting. The main challenge for harvesting is the dilute concentration of the culture ranging between 0.02% and 0.05% solids [36]. This is one of the reasons for high cost of harvesting, the other may be the negative charge that algal cells carry [37]. Some of the factors affecting the efficiency of the harvesting are cell concentration, pH, and ionic strength [36].

Some of the common harvesting strategies used in the industry are centrifugation, gravity sedimentation, filtration, flocculation, and flotation [36-38]. Usually, microalgal harvesting is a two-step process. In the first step, the biomass is separated from the suspension by flocculation followed by flotation or gravity sedimentation. The first step concentrates the cells into slurry with about 2-7% solid concentration. The slurry is still dilute for downstream processing and needs further concentrating. In the second step or the thickening phase, the slurry gets concentrated up to 95–99% by means of filtration, centrifugation, or thermal processes [36].

Milledge et al. reviewed algal harvesting techniques for biofuel production. They compared the advantages and disadvantages of the common harvesting techniques. They concluded that sedimentation and flocculation have the lowest energy input for microalgal harvesting. There is not one method or combination of methods suited to all microalgae and the degree of concentration will vary with the method [39].

Weschler et al. compared energy demand for the algal biomass production and concluded that the choice of harvesting technology affects the energy demand of other phases. Total energy demand for biomass production depends on final concentration [40].

Feasible algal biofuel production is limited by the lack of cost-effective and low energy means of algal biomass harvesting. For this reason, finding novel harvesting techniques with low energy requirements is essential [36].

2.4.3 Algal lipids

Algal biomass is cultured for the production of target molecules including lipids, pigments, and proteins. So far, the focus of the algal biofuel industry has been on the lipids. Many species of microalgae are capable of accumulating high levels (>50% w/w) of lipids, which can be extracted and converted to biodiesel, green diesel, or green jet fuel [14, 41, 42].

Lipid productivity is a key factor in choosing the right species for biodiesel production [43]. There are techniques available that can increase the lipid production. Courchesne et al. reviewed the progress, challenges, and future perspectives of lipid overproduction using microalgae by different approaches, including the biochemical engineering, genetic engineering, and the emerging transcription factor

engineering approaches [44] . Sharma et al. reviewed some of the most common techniques used in the literature for algal lipid induction. These common techniques are nutrient starvation, temperature and light stress, salinity and pH change, and genetic engineering [45]. Simionato et al. have shown that the triacylglycerol accumulation increased by 38% when nitrogen was removed from the media of Nannochloropsis genus [46]. Dunahay et al. genetically transformed two species of diatoms to manipulate the lipid accumulation in the transformed species [47]. Rodolfi et al. screened thirty species of microalgae for their biomass productivity and lipid content. Four strains (two marines and fresh water) that were robust and had relatively high lipid content were selected for growth in outdoor photo bioreactors under nitrogen deprivation. Both marine strains one of which was *Nannochloropsis* sp. had final lipid contents of about 60%. Once *Nannochloropsis* sp. was grown outdoor in nutrient sufficient and deficient conditions, the lipid productivity increased from 117 mg/L/d in nutrient sufficient media to 204 mg/L/d for the deficient case [48]. Efforts to increase the lipid accumulation are either genetic modifications or environmental factors [49].

Reviewed research summarizes the efforts to increase the lipid productivity to overcome the obstacles for large-scale production of algal biofuels. The two key barriers to commercialization of the algal biofuels are the high cost of algal biomass production and the low yield of target molecule such as lipids [50]. Production of valuable bioproducts alongside fuels is a way to increase the value of algal biomass. A possible solution to economical production of algal biofuels may be a biorefinery approach analogous to oil refineries.

2.4.4 Product (lipid) extraction

Once the algal cells are harvested, the next step is the extraction of the target molecule. Target molecules are not usually secreted out of the cell so cell wall disruption is required to extract the molecule of interest. Lipids are the molecules of interest for the biofuel industry but other molecules like pigments and proteins are of interest.

Lipid extraction techniques can be categorized as mechanical cell disruption to release the lipids contained in the cells or chemical extraction of the lipids by solvents [38]. A good extraction method extracts desirable lipid fractions (neutral lipids) and avoids the non-lipid fraction such as pigments [38].

Pragya et al. reviewed some of the technologies for algal harvesting and oil extraction. Usually a pretreatment step, which acts as cell disruption method, is needed prior to the actual lipid extraction [38].

Lee et al. studied and compared a couple different cell disruption methods, including autoclaving, bead-beating, microwaves, sonication, and a 10% sodium chloride solution. After pretreatment of cells, total lipids were extracted with a solvent extraction technique. They observed different lipid extraction efficiency among different species of algae. Among the pretreatments studied, microwave oven method had the highest lipid efficiency [51].The two bottlenecks in lipid-extraction are the need to use dry algae and the use of expensive solvents. An ideal lipid extraction technique can use wet biomass and hence saves a lot of energy [38].

The lipid extraction is an area that still needs more research and development. Mercer et al. reviewed some of the developments in the algal lipid extraction techniques[52] . They reported that most common extraction technique being used is solvent extraction coupled with mechanical disruption techniques. Most recent development on lipid extraction is promising non-solvent methods including the use of pulse electric field, enzymes, microwaves, ultrasonic energy and mechanical disruption. Yet the effect of these methods on the chemical stability of compounds prone to oxidation needs to be investigated. Some of the new extraction techniques need to be tested at pilot scales [52]. Another criterion for selection of the extraction method is the application of the final product, for example, a solvent extraction is not suitable for food applications.

2.4.5 Conversion technologies

Once the target molecule(s) has been extracted, the remaining of the cells has still some value and can further be processed and converted to fuels or chemicals. The two major pathways for conversion of any type of biomass to biofuels and bioproducts are biochemical and thermochemical conversion technologies [3]. The selection of the conversion technology depends on the biomass composition. The major difference between these two platforms is the catalyst used for conversion.

Thermochemical processes use heat and/or physical catalysts to convert biomass to an intermediate product, followed by a chemical transformation to fuels and chemicals [3]. Hydrothermal liquefaction (HTL) and slow pyrolysis are categorized as thermochemical conversion [53]. HTL has advantages such

as using the wet biomass and the benefit of using all the fractions in the biomass for fuel production. The highest value of bio oil yield based on dry ash-free biomass was 78.3%. As a result of the HTL process, the algal biomass will separate into four phases: biocrude oil, aqueous products, solid residue and gaseous products. The nutrients remained in the wastewater can be recycled back to the algae pond for the growth of next generation biomass. Same approach is true for the carbon dioxide recycle. Algal biomass consists of carbon, hydrogen, oxygen, nitrogen, phosphorus, sulfur, potassium, sodium, but only the carbon and hydrogen fraction can be turned into hydrocarbon liquid fuels. The other elements have to be removed from the biocrude oil to meet the fuel standards [54]; in other words, the biocrude needs to be upgraded.

Slow pyrolysis uses dry biomass, unlike HTL, and the drying step will significantly increase the operating cost. Pyrolysis decomposes biomass in the absence of oxygen and presence of thermal energy. The products of pyrolysis are renewable oil, gas, and char. The bio-oils can be used for direct combustion, or can be upgraded into liquid fuels and bio-chemicals. The key nutrients of biomass such as N, P, and K remain in the resulted biochar from pyrolysis. For this reason, the biochar has the potential to be used as an agricultural fertilizer. The biochar contains carbon, which can improve the quality and productivity of soil, while capture the carbon before it is released to the atmosphere [53].

The ideal algal biorefinery in the case of thermochemical conversion is comprised of these four main stages: microalgae growth and harvesting, fuel production, algae residue processing, and nutrients recovery and recycling. The drawback of this process would be upgrading of the bio oil, which can be done by the conventional methods used for petroleum upgrading. The other problem is losing valuable protein fractions when doing denitrogenation. To use those valuable proteins, one approach is to extract the proteins before the HTL process and add value to the process at the same time the cost for upgrading the fuel is lowered [55]. While thermochemical methods have potential, biological conversion allows the user to produce a specific product, as well as the option to recover residual proteins for animal feed and other uses.

Biochemical conversion relies on biomass transformation through intermediates like sugars, while thermochemical route is based on biomass reduction to building blocks such as H_2 and CO. The biochemical platform uses biocatalysts, heat, and chemicals for conversion of the biomass to a sugar

stream, which is then turned into a fermentation product and byproducts like heat and power. Biochemical conversions include anaerobic digestion, alcoholic fermentation, and bio-photolysis [2].

One major hurdle in conversion of any type of biomass into its constituent building blocks is the deconstruction step due to biomass recalcitrance. In the deconstruction step, the biomass is usually pretreated to decrease the biomass recalcitrance and make the cellulose more susceptible to hydrolysis [3]. A suitable pretreatment process includes disruption of hydrogen bonds in cellulose, breakage of the cross-link between hemicellulose and lignin, increase in the porosity and surface area of cellulose for subsequent hydrolysis treatments [24]. Some of the properties of an ideal pretreatment are production of a digestible pretreated solid, no degradation of pentoses, no inhibition of the subsequent fermentation, minimum size reduction of biomass feedstock, reasonable reactor size and cost, production of no solid-waste residues, simple process, and finally effective at low moisture content [3]. Pretreatment can be chemical, physical, physico- chemical, and biological or a combination of the aforementioned processes. During hydrolysis, the carbohydrate macromolecules are broken down to their monosaccharides. The biological route usually begins with a pretreatment step as is often performed prior to hydrolysis of the biomass to increase the availability of complex carbohydrate molecules to hydrolysis. Algal biomass, including LEAB, may not require a pretreatment step since most algal species do not have lignin in their cell wall structure.

The research described in this dissertation is based on a biochemical conversion of LEAB since lipids are not the only target for the production of biofuels. Carbohydrates and to the lesser degree proteins are of interest for biofuels production [23]. The biochemical composition of microalgal biomass varies among species[56], and may change with growth conditions [57]. On average, the algal biomass cultivated for the biofuel industry has about 50% w/w lipids, 20% carbohydrates, and 30% proteins. If only 10% of U.S. annual diesel usage were to be replaced with algal derived biofuels, then approximately 55 billion kg/yr of dry algal biomass has to be produced. Assuming 50% of dry weight of microalgae biomass is lipids, then 27.5 billion kg/yr of LEAB will be generated (Figure 2-2). The LEAB contains high levels of carbohydrate and protein, which can be used by microorganisms to produce value-added chemicals or fuels via fermentation.

Ethanol was selected here as the model product but the results can be generalized to other fermentation products. Ethanol has the potential to be used as a drop-in fuel in the current gasoline infrastructure up to a 10-15% blend or be used as a platform to produce other biochemicals [1]. Currently, ethanol is being used as a blend with gasoline to improve the octane number of the fuel, and reduce the greenhouse gas emissions [21]. Many chemicals that are produced from oil can be produced from ethanol including ethylene, acetaldehyde, and ethyl acetate [1]. LEAB can also be used to produce various products such as hydrogen, methane, bio-oil, plastics, fertilizers, animal feed, nutrients, electricity, and sorbents [23].

The conceptual process flow diagram for our approach is shown in Figure 2-3. The first step is deconstruction of LEAB to its building blocks followed by fermentation to ethanol using selected microorganisms. An alternate process developed by researchers at the National Renewable Energy Laboratory is shown in Figure 2-4 [58]. In the alternate approach, algae biomass is treated with acid to break open the cell wall and release lipids. The organic phase containing lipids is then separated from the aqueous phase. The aqueous phase containing sugars is sent to fermentation [58].

Another approach proposed is the production of glucose from unextracted algae, which is then separated from the algae solids by filtration. The liquid fraction containing the glucose can be fed to a fermenter for the production of microbial oil using oleaginous yeast, whereas the solid algae residue can be sent off for lipid extraction. The oil production from the yeast could ultimately be integrated with the algae oil in the existing downstream process [59].

Each of the proposed processes for production of fuels and chemicals has its own advantages and disadvantages and only a complete techno-economic and life cycle analysis can determine which one is the feasible one. Additional technology advancement in the key areas are still needed and more R&D is needed to commercialize any of these technologies [3].

2.5 Literature review on applications of residual algal biomass

One of the most promising feedstocks for biofuel production is algal biomass. Microalgae can convert carbon dioxide to potential biofuels and high-value biomolecules in a reaction driven by sunlight [54, 60]. Compared to other sources of feedstock such as terrestrial plants, algae biomass has some unique

characteristics including the ability to use non-fertile land [13], higher growth rates, higher productivity per unit land area, lower requirements for fresh water, and the fact that microalgal cultivations would not divert food supplies [14]. Food versus fuel is a challenging issue in the modern society [12]. Algae biomass has received a lot of attention for biofuel production due to these characteristics.

Different types of renewable biofuels, including methane, biodiesel, and biohydrogen, can be produced from microalgae [60]. The focus of the algal biofuel industry has been on the lipid portion of the algae biomass because this hydrocarbon mixture can readily be converted to biodiesel or renewable diesel [61]. Biodiesel derived from oil crops and animal fat is a carbon neutral renewable alternative to petroleum fuels, but cannot meet the demand for transport fuels. Microalgae is the only source of renewable biodiesel that can meet the demand for fuel production due to their high oil productivity [60]. Algae biomass on average accumulates lipids over 60% of its dry weight [62].

Large-scale production of algal biofuels is not economically feasible yet. One reason is the focus of the industry on one product and in particular lipids. Algal biofuel production is capital intensive and the risks associated with its production are high and somewhat unknown. Recent advances in systems biology, genetic engineering, bioreactor design, and biorefining present opportunities to develop this process in a sustainable and economical way [63].

The key to commercial production of algal biofuel is a biorefinery approach analogous to oil refineries [64-66]. In a biorefinery, algae biomass is grown for the production of oil and other value added chemicals. In addition to lipids, algae can synthetize bioactive molecules like carotenoids, antioxidants, anti-inflammatory, and other valuable organic molecules [66]. All these molecules can be extracted and converted to final products such as food additives, nutraceuticals, and drugs. In addition to biodiesel, other fuels such as methane, biohydrogen, and bioethanol can be produced from the whole or residual algae biomass.

Biofuel production from algal biomass has been recognized, but their sustainability an economic feasibility is still in doubt. Primary fuels that can be produced from algal biomass are hydrogen, methane, biodiesel, and bioethanol [67]. The biomass remained after the extraction of the primary fuel can be a promising source for production of additional fuels or high-value added products. This residual or spent algal biomass contains proteins, carbohydrates, and minerals [61, 67, 68]. Some of the potential

applications for this residual biomass are animal feed, electricity, fertilizers, removal of heavy metals and dyes from wastewater, and production of biofuels [61, 67]. The potential applications for this residual biomass has only been studied to limited degrees, unlike the whole algal biomass [67]. The possible applications of LEAB depend on its algal species, cultivation, harvest, and lipid extraction, since all of these factors affect the biochemical composition of the LEAB [23, 57, 61, 67, 69, 70]. A summary of applications for residual algae biomass is presented here.

The focus in this research was on the biomass remaining after lipid extraction. This biomass is called lipid-extracted algae biomass (LEAB). One of the main applications of the LEAB is bioenergy including biofuels and electricity. Rashid et al. studied the potential of algae biomass and activated sludge for electricity production in microbial fuel cells (MFC). They evaluated both whole and lipid-extracted algae as substrate for electricity production. Various concentrations (1–5 g/L) of dry whole algae biomass were tested and 5 g/L (5000 mg COD/L) of biomass produced the highest voltage of 0.89 V and power density of 1.78 W/m^2 under 1000 Ω electric resistance. They also evaluated LEAB as substrate for the MFC, but the voltage produced by LEAB was only 0.021 V. They speculated that toxic chemicals remained in LEAB after lipid extraction, inhibited the growth of microbes. They suggested further investigation of chemical toxicity of the lipid extraction method [71].

LEAB accounts for 70% of the whole algae biomass on a dry basis and contains carbohydrates and proteins [67]. LEAB can be used for production of additional fuels such as hydrogen, ethanol, methane, and bio-oil. Production of biogas from LEAB is highly desirable due to its high content of carbohydrates and proteins [67]. Zhu presents a theoretical evaluation of ethanol and biogas production from algal residual biomass. LEAB is a threat to the environment if not disposed of properly. To make the microalgal biodiesel sustainable, LEAB needs to be utilized in a fermentation or anaerobic digestion. It is also critical to recycle the N and P contained in the LEAB. Carbohydrates including the storage (starch) and cell wall components (cellulose) can be converted to ethanol. Proteins, carbohydrates, and lipids can be converted to methane. Their proposed process is a fermentation of carbohydrates to ethanol followed by anaerobic digestion of the leftover biomass to methane. The effluent biomass out of the anaerobic digester contains N and P, which can be recycled and used as substrate for algal cultivation. CO_2 generated during the fermentation and anaerobic digester can be recycled to the algal cultivation pond. They proposed that a

combination of ethanol and methane production from the LEAB can improve the sustainability of algal biofuel industry [72].

Yang et al. studied biogas production from lipid-extracted algae biomass extensively [57, 73-75]. They studied different pretreatment methods to improve lipid-extracted *Scenedesmus* biomass solubilization and anaerobic hydrogen production. Studied methods included thermal, alkaline, and thermo-alkaline pretreatments. The highest hydrogen yield of 45.54 mL/g-volatile solid was observed in the case of thermo-alkaline pretreatment at 100 ℃. This yield was three-fold higher than the yield from untreated LEAB, which proved that thermo-alkaline pretreatment at 100 ℃, is an effective method to improve solubilization and increase the hydrogen production from LEAB [57]. Yang et al. performed batch experiments to convert lipid-extracted *Scenedesmus* biomass pretreated by a thermo-alkaline method into hydrogen. To obtain high hydrogen production, repeated batch cultivation was conducted using the pretreated LEAB as feedstocks under optimal pretreatment condition. The optimal pretreatment conditions for LEAB were NaOH dosage of 8 g/L, pretreatment time of 2.5 h and solid content of 6.7%, which resulted in 160% and 500% improvement in the hydrogen yield and hydrogen production rate, respectively [75]. Conversion of the LEAB to hydrogen serves dual role in renewable energy production and sustainable development of algal biodiesel industry. Yang et al. investigated an anaerobic fermentation process to convert LEAB from *Scenedesmus* into hydrogen. They investigated the effects of initial pH, inoculum pretreatments, inoculum concentrations, and substrate concentrations on hydrogen production from LEAB. The best conditions for hydrogen production from fermentation of LEAB was obtained at 36 g volatile solids /L at the initial pH 6.0–6.5 using the heat-treated anaerobic digested sludge as inoculum [73]. Yang et al. studied hydrogen and methane production from lipid-extracted *Scenedesmus* biomass in a two-stage process. Biogas production and energy efficiency of the two-stage were compared to the traditional one-stage process. In the one-stage process, hydrogen is usually not detected as hydrogen is consumed during methanogenesis to produce methane and carbon dioxide as products. The methane yield for the two-stage process was 22% higher than the one-stage process. The two-stage process was more energy efficient and the efficiency increased by 27%. To enhance the methane production rate and reduce the fermentation time, repeated batch cultivation was a useful method to cultivate the cultures. The downside of the repeated batch cultivation was the decrease in

methane yield by increase in the ammonia levels, which suggests inhibition of methane production by ammonia [74].

Bohutskyi et al. evaluated methane production and nutrient recovery from lipid extracted algae biomass of *Auxenochlorella protothecoides* in a semi-continuous anaerobic digester. The methane production from LEAB reached 50% of the predicted maximum yield. The reason that methane production was limited to 50% of theoretical maximum yield was due to biomass recalcitrance and inhibition effects from the residual solvent in LEAB. Energy recovery from algal biomass was increased by 30%. The remaining nutrients in the LEAB are about 40–60% of N and P, 30–60% of Mg, Ca, and S, and 15–25% of Mn and Fe. These nutrients can be recycled from the effluent of the anaerobic digester back to the algal cultivation system. The recycling can reduce cost of the supplied fertilizers by up to 45%. They proposed further optimization to maximize methane yield and nutrient recovery in addition to elimination of solvent residues [76]. Ehimen et al. studied methane production from lipid-extracted *Chlorella* biomass via anaerobic digestion. The aim of their study was to find out how much energy can be recovered from LEAB via anaerobic digestion and what effects lipid extraction and transesterification have on methane yield. They also investigated the codigestion of glycerol (a byproduct from transesterification step) with LEAB and its effect on the produced methane yield. The maximum energy recovery was about 22 MJ/Kg dry LEAB depending on the preceding lipid extraction or transesterification route. Addition of the glycerol to LEAB in the anaerobic digester enhanced energy yields by about 10 MJ/Kg LEAB. They found out that the type of solvent used for lipid extraction have a major effect on methane yield. Use of chloroform inhibits methane production and a rinse step might be needed before biomass gasification. Since LEAB has low C:N ratios, they proposed codigestion of other energy-rich wastes, such as, forestry residues to improve the methane yield from LEAB [77]. Quinn et al. studied methane production for whole and lipid-extracted *Nannochloropsis salina*. Results showed whole microalgae produced 3 times more methane than LEAB due to removal of energy rich lipids for fuel production. They believed that current life cycle analysis modeling in literature is dramatically overestimating methane production from LEAB [78].

Subhash et al. studied the potential of pretreated LEAB as feedstock for dark fermentative hydrogen production using pretreated acidogenic consortia as biocatalyst. Hydrogen production depends on the pretreatment method for extraction of carbon from feedstock. This study proves the feasibility of

microalgae as potential feedstock for simultaneous production of biodiesel and biohydrogen in a biorefinery platform [79].

Nobre et al. evaluated the potential of *Nannochloropsis* sp. in a biorefinery context. The algae biomass was used for the production of biodiesel, biohydrogen, and carotenoids. The lipid extraction method was CO_2 extraction. The effect of extraction factors including temperature, pressure, and solvent flow rate were evaluated on the extraction yield. The best operational conditions were found to be at 40 °C, 300 bar, and a CO_2 flow rate of 0.62 g/min. The effect of adding a co-solvent like ethanol was studied. Addition of 20% of ethanol improved the lipid extraction efficiency by 37% and 70% of the pigments were recovered. The LEAB was used as feedstock to produce biohydrogen through dark fermentation [66].

Hernandez et al. studied lipid extraction and biogas production from four different algae. They found supercritical CO_2 extraction to be the most efficient method for lipid extraction compared to Soxhlet and Kochert methods. They recovered energy maintained in LEAB by anaerobic digestion. They observed higher methane yield from lipid-extracted algae biomass than the non-lipid extracted biomass due to biodegradation of the biomass with supercritical CO_2 extraction [62].

Other than biogas, ethanol and bio-oil are among the potential fuels that can be produced from LEAB. Harun et al. studied ethanol fermentation from whole and lipid-extracted algae biomass. They added both kinds of biomass to a fermentation medium containing essential nutrients including glucose and compared ethanol concentrations. LEAB was obtained after supercritical extraction of lipids. They observed 60% higher ethanol concentration for LEAB than the whole algae. The supercritical extraction with high temperature and pressure caused the algal cell wall to rupture and release the embedded polysaccharides. Therefore, the extraction process made the carbohydrates available to the yeast and this resulted in higher ethanol concentrations. The cell wall of the whole algae was remained intact since no pretreatment was done on it and that was the reason for lower observed ethanol concentration for the whole algal biomass [80].

Talukder et al. developed an acid hydrolysis method that disrupts cell wall of *Nannochloropsis salina* for lipid extraction and carbohydrate deconstruction. Algae biomass was acid hydrolyzed and subsequently treated with hexane to separate lipid from the hydrolysate. The lipid free hydrolysate was

used for lactic acid production via fermentation. The acid hydrolysis improved the lipid extraction by 75% [81].

Bioethanol has been produced from whole algal biomass, different fractions of the biomass other than LEAB, and also from macroalgal biomass. Kumar et al. studied ethanol production from red seaweed along with agar. After agar extraction from the algal biomass, the leftover pulp contained 62–68% carbohydrates, which was enzymatically hydrolyzed and fermented to ethanol with a yield of 0.43 g/g sugars [82]. Kim et al. studied ethanol production from marine algae biomass treated with acid and commercially available hydrolytic enzymes. Ethanogenic recombinant *Escherichia coli* used for fermentation of both mannitol and glucose with a yield of 0.4 g ethanol per g of carbohydrate. It is worth mentioning that this yield was obtained from *L. japonica* hydrolysate supplemented with LB medium [83]. The addition of extra nutrients in the form of LB medium will increase the cost of ethanol production.

Lipids are not always the focus of the algal biofuels. Miranda et al. studied the influence of the type of bioreactors on growth and sugar accumulation of *Scenedesmus obliquus*. A closed-loop vertical tubular photobioreactor was compared to an open-raceway pond and a bubble column. Depletion of nitrate resulted in an accumulation of sugars for all cultivations. The highest biomass production was achieved in the open raceway, biomass from the pond was hydrolyzed with sulfuric acid in an autoclave, and the hydrolysate was fermented by different yeasts in order to choose the best one. The maximum sugar content was 29% g/g, and the highest ethanol concentration obtained by *Kluyveromyces marxianus* was 11.7 g/L [84].

There are two pathways for bioethanol production from microalgae biomass: direct dark fermentation or yeast fermentation of hydrolyzed biomass. Dark fermentation is the anaerobic production of bioethanol by the microalgae itself through consumption of intracellular starch [84]. Ueno et al. investigated the dark fermentation of marine green alga *Chlorococcum littorale* [85]. Under dark anaerobic conditions, 27% of cellular starch was consumed within 24 h at 25 °C. The maximum ethanol productivity was obtained at 30 °C [85].

Lee et al. studied bioethanol fermentation from *Dunaliella tertiolecta* lipid-extracted biomass. They studied chemical, enzymatic, and chemical-enzymatic saccharification for biomass deconstruction and the resulting hydrolysate was used for fermentation. Enzymatic saccharification did not require additional

pretreatment prior to fermentation with *Saccharomyces cerevisiae*. Bioethanol was produced with 82% yield from the saccharification solution with added yeast extract with a concentration of 12 g/L [86].

Gao et al. investigated component analysis of *Pseudochoricystis ellipsoidea* as a novel biodiesel-producing alga. Results showed that proteins and amino acids are abundant in this alga while carbohydrate content is low. For this reason, they used LEAB from this alga as a nutrient source to replace expensive yeast extract in the lactic acid and ethanol fermentation [87].

Pyrolysis and liquefaction are other pathways of liquid fuel production from lipid-extracted algae biomass. The products of these processes are biochar and bio-oil [67]. Wang et al. studied pyrolysis of lipid-extracted *Chlorella vulgaris* in a fluidized bed reactor at 500 °C for nutrient and energy recovery. Yields of bio-oil and biochar were 53 and 31% (w/w), respectively. For comparison, yields of bio-oil and biochar for pine pyrolysis were 68 and 10% (w/w). The bio-oil and biochar represented 57% and 36% of the energy content of the lipid-extracted algae biomass, respectively. About 94% of the energy content of *C. vulgaris* LEAB was recovered in the form of bio-oil and biochar [88].

Vardon et al. studied bio-oil production from raw and defatted algae biomass via hydrothermal liquefaction (HTL) and slow pyrolysis. HTL is ideal for processing high-moisture biomass, while pyrolysis is suited for the conversion of dry feedstocks. Conversion of raw and defatted *Scenedesmus* via HTL and slow pyrolysis produced bio-oils with similar heating values, heteroatom content, and functionality [53]. Zhu et al. studied LEAB conversion to liquid fuels via HTL. The generated bio-oil was further upgraded via hydrotreating and hydrocracking to produce liquid fuels, mainly alkanes. Cost analysis demonstrated that HTL and upgrading is effective for converting LEAB to liquid fuels. Sensitivity analysis identified LEAB feedstock cost, final products yields, and upgrading equipment cost to be the key factors affecting production cost [89].

The cost associated with bio-oil production from biomass is relatively high and the main challenges are the low yield and poor bio-oil quality. The undesired properties of bio-oil, which limit its application as fuel, are high water content, high viscosity, high ash content, high oxygen content, and high acidity. Some of the common techniques for bio-oil upgrading are hydrotreating, hydrocracking, solvent addition/esterification, and emulsification [90].

Broch et al. evaluated the hydrothermal carbonization of whole and lipid-extracted *Spirulina maxima* feedstocks for production of a solid biofuel (hydrochar) and value-added coproducts in the aqueous phase. Hydrothermal carbonization is effective in creating solid hydrochar from both whole algae and LEAB at lower temperatures as compared to lignocellulosic feedstocks. Lower temperature requirement is due to lack of lignin in algae [91].

Another potential application for lipid-extracted algae biomass is animal feed [67]. Patterson et al. evaluated the nutritional value of whole and lipid-extracted algae biomass. Partial addition of the algal biomass to aquaculture diets was studied. LEAB could only substitute for up to 10% of the protein normally provided by fishmeal. Lipid extraction decreased the amount of protein in the residual biomass. Their results suggested that an addition of more than 10% protein from LEAB results in decreased fish performance [92]. Maisashvili et al. determined the values of whole and lipid-extracted algae for aquaculture using hedonic pricing methods based on their nutrient compositions. They compared their estimated price with the ones in literature. They also confirmed that fully replacing a fishmeal with algae, is impossible since it shows poor growth responses [93]. Vidyashankar et al. studied the compositional and nutritional value of defatted *Scenedesmus dimorphus* as animal feed. They tested LEAB in rats and found out that it was safe in short term, single-dose feeding, and long term repeated-dose feeding. They suggested using LEAB in animal feed up to 10 % (w/w) replacement [94]. Gatrell et al. studied addition of LEAB to chicken diet and its effect on creation of omega-3 (n-3) fatty-acid-enriched chicken product. The algae biomass was *Nannochloropsis oceanica* out of biofuel research. The inclusion of LEAB to the corn–soybean meal diet resulted in a linear increase in total n-3 fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). It was concluded that 8 to 16% of LEAB could be added in diets for broilers to produce a n-3 fatty-acid-enriched chicken meat [95]. Leng et al. studied the effect of feeding lipid-extracted algae biomass to laying hens. Inclusion of 15% LEAB to the diet decreased feed intake and egg production as compared with the control diet, but for 7.5% addition of LEAB an increase in egg albumen weight was observed. In conclusion, addition of 7.5% of LEAB in the corn-soybean meal diet had no adverse effect on their health, egg production, or egg quality [96]. Austic et al. observed that defatted *Staurosira* sp. biomass can be added up to 7.5% to soybean meal in diets of broiler chicks [97]. Ekmay et al. studied the nutritional and metabolic impacts of lipid-extracted *Desmodesmus* sp., protease, and non-

starch polysaccharide degrading enzymes in diets for weanling pigs and broiler chicks. The enzyme addition to LEAB was studied to see if it could improve digestion. Pigs that were fed 10% LEAB for 28 days had growth performance comparable to the control group. Broilers that were fed 15% LEAB had 16% better gain/feed efficiency than the control group over 42 days. Supplemental protease improved digestion in pigs, whereas supplemental non-starch polysaccharide degrading enzymes showed negative effects in broilers. They conclude that pigs and broiler chicks tolerated dietary inclusions of 10 and 15% LEAB, respectively [98]. Kim et al. used iron-rich microalgae to elevate blood hemoglobin concentrations. They studied the effectiveness of a lipid-extracted *Desmodesmus* sp. to elevate blood hemoglobin in weanling pigs. LEAB improved hemoglobin levels of marginally anemic pigs by 22-32% [99].

Residual algal biomass can serve as biosorbent to treat wastewater. Adsorption is becoming an alternative to the conventional wastewater treatment. Drawbacks of the conventional methods are high capital cost, low removal efficiency, and large generation of sludge [67]. Mona et al. studied spent algae biomass from a hydrogen bioreactor for biosorption of a textile dye called reactive red 198. Biosorption was mediated by functional groups like hydroxyl, amide, carboxylate, methyl, and methylene groups present on the algal cell surface [100].

Chandra et al. studied the utilization of LEAB as a non-conventional low cost adsorbent. Removal of methylene blue present in liquid phase was evaluated by adsorption with LEAB. The data were fitted to the Langmuir and Freundlich isotherms. This study proved that LEAB could effectively be used as adsorbent for the removal of basic dyes due to the presence of negatively charged functional groups on adsorbent's surface [101].

LEAB is rich in protein and therefore rich in nitrogen content, so it can substitute chemical fertilizers. LEAB contains low carbon to nitrogen ratio, so it is ideal to be utilized as animal feed, fertilizer, or nutrient source for organisms [102]. Maurya et al. used nitrogen rich LEAB of *Chlorella variabilis* and *Lyngbya majuscula* as fertilizer for maize plants. The grain yields for both LEABs were equivalent to that under control condition using chemical fertilizer. It was concluded that LEAB could substitute the chemical nitrogen fertilizer without affecting the yield and quality of the crop. LEAB can reduce the usage of the chemical fertilizers in agriculture industry [102]. Lewis et al. used lipid-extracted *Nannochloropsis salina* as soil amendment for agricultural production. Addition of lipid-extracted algae is a means of increasing

organic carbon. There is a 3% limit to addition of LEAB as soil amendment since soil with excessive nitrate from LEAB addition may cause leaching, runoff, and environmental pollution. Salt sensitive plants may adversely get affected by large LEAB applications [68].

One interesting application of LEAB is serving as nutrient source for lipid production in bacteria or microalgae. Zheng et al. investigated the feasibility of lipid production of *Chlorella* sp. from LEAB and molasses hydrolysate. Five different hydrolysate mixture ratios of LEABs/molasses were tested for cultivation of *Chlorella* sp. and highest lipid productivity of 335 mg/L. day was achieved by using the hydrolysate mixture ratio of LEAB/molasses of 1/4. Results showed that *Chlorella* sp. can utilize mixed sugars and amino acids from LEABs and molasses to accumulate lipids efficiently [103]. In a different study, Zheng et al. treated LEAB enzymatically and used the hydrolysate as a source of nutrients for the cultivation of *Chlorella vulgaris* with/out aeration. LEAB was hydrolyzed into amino acids and sugars by the enzymatic hydrolysis and these compounds were utilized as nitrogen and carbon sources for *C. vulgaris* cultivation under both conditions of aeration. Results showed that aeration favored cell growth and lipid accumulation. Lipid productivity of 116 mg/L. day were observed in the aerated culture [104].

Ma et al. evaluated enzymatic hydrolysates of the lipid-extracted algae biomass as nutritional sources for mixotrophic growth of *Chlorella vulgaris* and lipid production. Both temperature and substrate concentration had a significant effect on cell growth and lipid production. The maximum lipid productivity of 164 mg/L. day were obtained. Results confirmed that LEAB could be utilized by the mixotrophic growth of *C. vulgaris* for microalgal lipid production under the optimal temperature and substrate concentration [105].

A novel process by Trzcinski et al. was introduced to add value to LEAB. They extracted glucose from diatoms before the lipid extraction step, and then glucose in the liquid phase is filtered and separated from the solid biomass. The liquid fraction, containing glucose, is fed to a fermenter for the production of microbial oil using oleaginous yeast. The algae biomass is sent for lipid extraction and the oil produced from yeast will integrate with the algae oil in the downstream process [59].

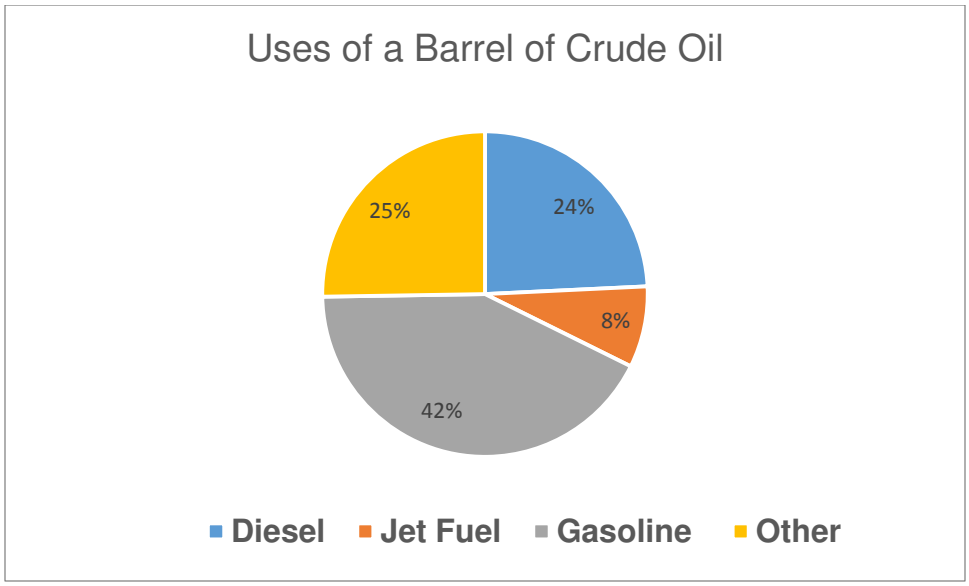

Figure 2-1. Uses of a barrel of crude oil by percentage. Data replotted from “Replacing the whole barrel” [106].

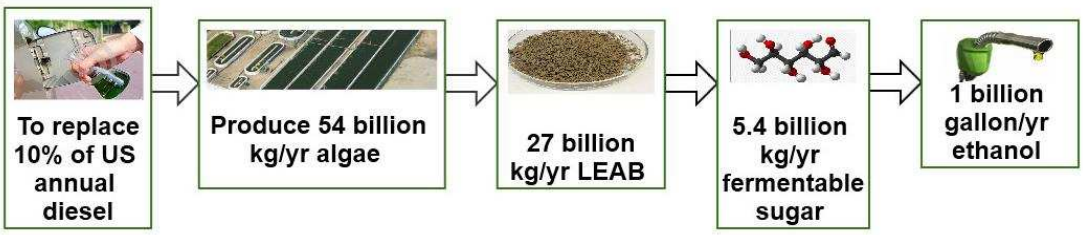

Figure 2-2. Rationale behind using lipid-extracted algal biomass as a feedstock for biofuel production.

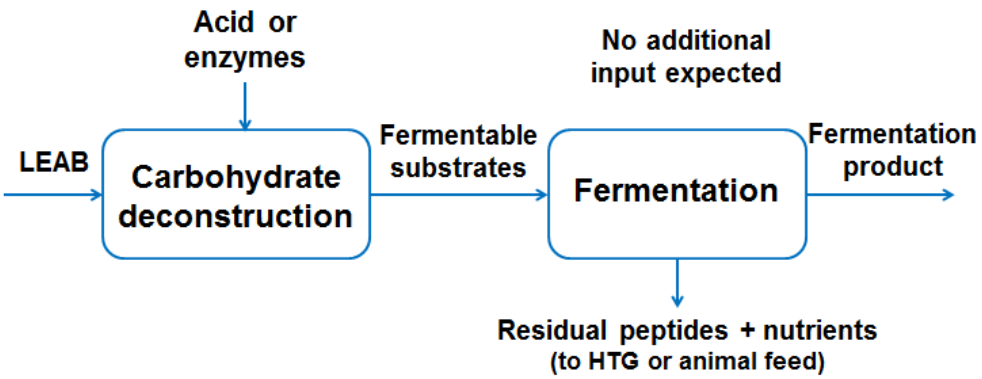

Figure 2-3. Conceptual process flow diagram for bioconversion of LEAB into ethanol, proposed process in this research.

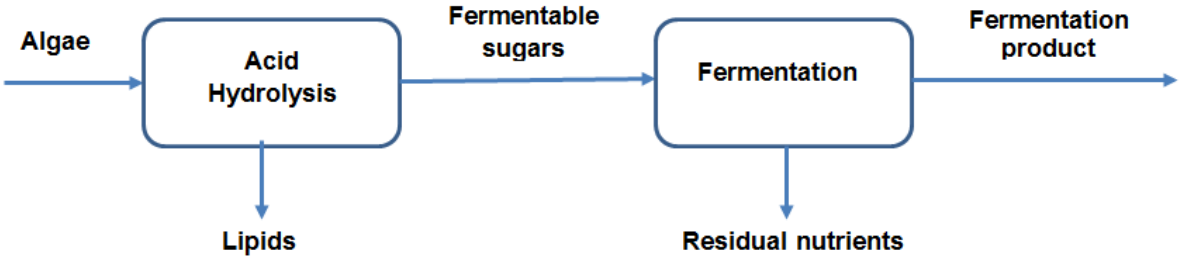

Figure 2-4. Process flow diagram for the proposed process by NREL, an alternative to the process studied in this dissertation research [58].

3 Conversion of Lipid-Extracted *Nannochloropsis salina* Biomass into Fermentable Sugars[1]

3.1 Summary

The primary focus of research and development toward algal biofuels has been the production of fuel from the lipid fraction, with use of the non-lipid biomass for production of biogas, electricity, animal feed, or fertilizer. Since the non-lipid fraction comprises approximately half of the algal biomass, the development of processes to produce additional liquid fuel or higher value products is of interest. We evaluated several hydrolysis methods for the deconstruction of cell wall carbohydrates in residual algal biomass. The hydrolysate, which contains the released sugars, can be used as a fermentation feedstock. For all methods, hydrolysis rates and yields of released sugars were measured. The effects of temperature, acid concentration, and biomass loading on acid hydrolysis were studied. Combined severity factors, an indicator of treatment efficiencies, were evaluated for their correlation to the hydrolysis outcome. An optimal enzyme mixture, which released sugars with an acceptable yield and rate, was found. The ability of the resulting hydrolysates to support the growth of an industrial yeast strain was tested and the levels of common fermentation inhibitors were examined. Of the conditions tested, the highest yield (243.2 mg sugar/g biomass) of released sugar was obtained with a one-step sulfuric acid process with 10% acid concentration at 90 ℃ for 5 hours, while the maximum sugar release rate was obtained with 10% hydrochloric acid under the same conditions. For one-step acid hydrolysis, all the studied factors were statistically significant, while for the two-step process, only acid concentration was significant. This is the first process for conversion of residual algal biomass that does not require pretreatment and that results in a hydrolysate on which yeast can be grown with no added nutrients. Adapting a biorefinery concept by conversion of algal residue to value-added products may improve the process economics.

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3.2 Introduction

Concerns over high atmospheric greenhouse gas levels and issues associated with fossil fuel extraction are among the incentives for developing alternative energy resources[4]. Biofuels, produced from a wide range of feedstocks, have the potential to reduce greenhouse gas emissions [41, 107, 108]. In particular, the use of microalgae as a feedstock has received a high level of interest in recent years [14, 63].

Many species of microalgae are capable of accumulating high levels (> 50% w/w) of lipids, which can be extracted and converted to biodiesel, green diesel, or green jet fuel [14, 41, 42]. Compared with terrestrial plants, the advantages of using microalgae as a potential source of fuels include the ability to use non-fertile land [13], the higher growth rates of microalgae, higher productivity per unit land area, lower requirements for fresh water, and the fact that microalgal cultivations would not divert food supplies [14].

Large-scale production of microalgal biofuels has not yet been shown to be economically feasible, and significant improvements in the proposed processes are still needed. If a biorefinery concept analogous to oil refineries is adapted to produce additional fuels or chemicals from algal biomass, then the process economics could be improved [66, 109].

The focus of the microalgal biofuel industry has been on the lipid portion of the algae biomass because this hydrocarbon mixture can readily be converted to biodiesel or renewable diesel. The biomass remaining after lipid extraction (lipid-extracted algal biomass, LEAB) consists primarily of proteins and carbohydrates. LEAB can be used with minimal additional processing to produce animal feed [53, 57, 67, 110] and fertilizers [53, 67, 110]. In addition, recycling of the nutrients contained in LEAB for subsequent algal cultivations has also been proposed [111].

A few studies have focused on the chemical and biological conversion of LEAB or whole-cell microalgal biomass to fuels and other chemicals. Both intact and lipid-extracted algal biomass have been converted to ethanol by fermentation [80] and to bio-oils by slow pyrolysis [53] and hydrothermal liquefaction [53]. LEAB has also been converted to hydrogen [57] and lactic acid [81] by fermentation. To facilitate fermentation, several methods have been used for conversion of biomass to fermentable substrates, including alkaline and thermo-alkaline conditions [57], acidic conditions [81], and sonication

[112]. Little has been published regarding bioconversion of LEAB with no prior treatment [80], or adding it as a sole carbon source to the fermentation medium [80].

The conversion of intact or lipid-extracted algal biomass to fuels or other chemicals depends on the biomass composition. The biochemical composition of microalgal biomass varies among species [69], and may change with growth conditions [70]. Sugars and other small molecules are readily available for fermentation, and starch [113] can be hydrolyzed with amylase to yield glucose. If a lipid-extraction step is performed, sugars and starches may be separated from the LEAB. The remaining carbohydrates in LEAB are generally complex molecules located in the cell wall. The carbohydrate content and composition of LEAB depend on the species [23, 70], cultivation phase [70], growth conditions [23, 56], and lipid extraction process [57].

While thermochemical methods have potential, biological conversion allows the user to produce a specific product, as well as the option to recover residual proteins for animal feed and other uses. The biological route may begin with a pretreatment step as is often performed prior to hydrolysis of lignocellulosic biomass to increase the availability of complex carbohydrate molecules to hydrolysis. In hydrolysis, those carbohydrates are converted to fermentable sugars. Algal biomass, including LEAB, may not require a pretreatment step.

The goal of the work presented here was to deconstruct the carbohydrates in LEAB into fermentable sugars, producing a hydrolysate with the potential to be used as the source of carbon and nutrients for fermentations by yeast and other microorganisms. Three different methods — two-step sulfuric acid hydrolysis, one-step acid hydrolysis, and enzymatic hydrolysis — were compared on the basis of their rate of hydrolysis and yield of sugar. In addition, evaluation of the presence of fermentation-inhibiting hydrolysis byproducts was conducted. LEAB from *Nannochloropsis salina* was used as a model system; this alga has been considered for large-scale commercial cultivations [114]. Under nitrogen stress, this alga can accumulate oil up to 60% of its biomass on dry weight basis, which makes it an excellent candidate for biofuel production [114, 115].

3.3 Materials & methods

3.3.1 Reagents

All chemicals used were of analytical grade and were purchased from Sigma-Aldrich.

3.3.2 Lipid-extracted algae biomass

Lipid-extracted *Nannochloropsis salina* was provided by Solix Biosystems, Inc. (Fort Collins, CO). Cultivation was in closed photobioreactors and cells were harvested by centrifugation. Whole algal cells were dried and the lipids were extracted with hexane. Residual hexane was removed from the LEAB with nitrogen or carbon dioxide gas. The LEAB was received as dry biomass and was stored at 4 °C until use.

3.3.3 Two-step sulfuric acid hydrolysis

Portions (0.1 g) of LEAB were ground in a standard kitchen blender and further ground with a mortar and pestle. The ground sample was placed into a 4 mL glass vial and mixed with 1 mL of 72% w/w sulfuric acid. The vial was placed into a sonicating bath (Model T1.9C, HealthSonics, IL, USA) for 15 min and then kept in a water bath (Model 210, Napco Heinicke, OR, USA) at 30 °C for 45 min. For the base case of two-step hydrolysis, the sample was removed from the water bath after 1 h, transferred to a 50 mL serum bottle, and diluted to a 4% w/w acid concentration by addition of 26.4 mL of deionized water. The serum bottle was sealed and autoclaved (Steris Cooperation, OH, USA) for 1 h at 121 °C. After completion of the autoclave cycle, samples were cooled at room temperature and then neutralized with 10 M NaOH to pH 7. The amount of NaOH added was recorded to calculate the correct final volume. The residual solid in the hydrolysate was separated by centrifugation at 3000 x g for 10 min. The supernatant, which contained the soluble sugars, was used for sugar and other analyses.

A set of experiments was conducted to optimize the second step of acid hydrolysis. The response surface method with a central composite design was used to design the experiments. The factors that were studied for the optimization of the second step were acid concentration, temperature, and reaction time. For the optimization experiment, the first step of hydrolysis was conducted according to the standard protocol above. The ranges for the factors were: acid concentrations, 1-5% (w/w); temperature, 100-120 °C; and reaction time 60-80 min. Five values of each factor were evaluated. In this design, the

number of blocks was defined as the number of days since not all experiments could be conducted in one day. The combination of the factors that resulted in the maximum sugar yield was designated as the optimal condition. Samples were cooled and neutralized according to the protocol used for the base case.

3.3.4 One-step acid hydrolysis

LEAB hydrolysis was also tested in a one-step method for both hydrochloric and sulfuric acids. Two sets of experiments were performed, each in triplicate. The first was a set of exploratory experiments in which total sugar release was studied as a function of three hydrolysis factors (acid concentration, temperature, and biomass loading) while holding reaction time constant at 24 h. In these experiments, the levels of the factors were: acid concentration, 1 and 10% w/w; temperature, 40, 60, and 90 ℃; and biomass loading, 1 and 10% w/v.

The conditions from these exploratory experiments that yielded the highest sugar concentration were then used in a second set of experiments to evaluate hydrolysis as a function of time.

The amount of LEAB for each experiment was calculated to achieve a desired final total volume and biomass loading. For instance, for a 1% w/v biomass loading and total volume of 10 mL, 0.1 g of LEAB and 9.9 mL of the appropriate acid solution were combined, and the vial was placed in an oven (Model 658, Thermo Fisher Scientific, NJ, USA) at the desired temperature. A separate vial for each time point was used to avoid variations associated with sampling. At different times, the vial contents were cooled at room temperature and then neutralized with 10 M NaOH to pH 7. The volume of NaOH solution was recorded to calculate the exact final volume of neutralized samples. The residual biomass was separated from the hydrolysate by centrifugation at 3000 x g for 10 min. The supernatant was used for sugar and other analyses.

3.3.5 Enzymatic hydrolysis

Commercially available enzymes (pectinase) and enzyme mixtures (Accellerase 1500, Accellerase XC, Accellerase XY) were obtained from Dupont Industrial Biosciences (USA). Accellerase 1500 and Accellerase XC contain multiple enzyme activities, mainly exoglucanase, endoglucanase, hemi-cellulase, and beta-glucosidase. Accellerase XY is an accessory product to supplement whole cellulases with

xylanase activity. All the enzymes were provided as liquid solutions.

Enzymatic hydrolysis was adapted from a published protocol [116], in which a 0.5 g portion of dry, ground LEAB was added to a 20 mL conical flask. To each flask, 5 mL of 0.1 M, pH 4.8 acetate buffer was added. To prevent the growth of organisms during digestion, 0.04 mL of a 10 mg/mL of tetracycline solution in 70% ethanol was added to each flask. Deionized water was added to each flask to bring the total volume to 10 mL after addition of the enzyme suspensions. Prior to the addition of enzymes, the flasks were equilibrated at 50 ℃. In separate tests, the acetate buffer and tetracycline were shown to have no effect on the hydrolysis or the analysis of sugar concentrations.

A similar approach to that taken with the one-step acid hydrolysis was employed, in which a first set of experiments was used to determine the best conditions for enzymatic hydrolysis, and a second set performed to determine the time course of hydrolysis. The goal of the exploratory experiments was to find the concentration of each enzyme in combination with others that resulted in the highest sugar yield from LEAB biomass under specified conditions (50 ℃, biomass loading 1.5% w/v, pH 4.8, 72 h). To account for the sugars released from the LEAB and enzymes prior to hydrolysis, reaction blanks for LEAB only and enzyme only were tested. The LEAB blank contained buffer, water, and the desired amount of substrate. The enzyme blank contained buffer, water, and the corresponding concentration of enzymes. The residual biomass was separated from the supernatant (hydrolysate) by centrifugation at 3000 x g for 10 min. The hydrolysate was used for sugar analysis.

A response surface methodology was used to find the enzyme mixture that resulted in the highest sugar yield. The four factors subject to optimization were the concentrations of Accellerase 1500, Accellerase XC, Accellerase XY, and pectinase. The dosage ranges provided by the enzyme manufacturer were used for setting the lower and upper limits of each factor in the design: Accellerase 1500, 0.05–0.25 mL/g biomass; Accellerase XC, 0.0125–0.125 mL/g biomass; Accellerase XY, 0.005–0.05 mL/g biomass; and pectinase, 0.04–0.153 mL/g biomass. In this experimental design, the number of blocks was defined as the number of weeks since not all experiments could be conducted in one week. The combination of factors that resulted in the maximum sugar yield was designated as the optimal condition.

In the second set of experiments, flasks were incubated at 50 ℃ with shaking at 200 RPM to suspend solids for the period of the experiment. To measure the progress of the reaction in the second set of experiments, 0.5 mL aliquots were removed at intervals and analyzed for sugar concentrations. Sampling continued until the additional release of soluble sugars became negligible. Once samples were removed, they were boiled for 5 min to denature the enzymes, and then centrifuged at 3000 x g for 10 min. The liquid portion was used for sugar analysis with HPLC or colorimetric assays depending on the purpose of the experiment.

3.3.6 Sugar concentration analysis

Depending on the purpose of the experiment, either exploratory or time-course, one of three sugar assays, two colorimetric and one chromatographic, was used.

The goal of the exploratory experiments was to survey a range of different factors and compare the treatment effects while holding reaction time constant. The sugar assay used for this phase was the phenol-sulfuric acid (PSA) assay, which is suitable for detecting monosaccharides and their methyl derivatives, oligosaccharides, and polysaccharides [117]. This method is appropriate for determining the effect of the studied factors on the release of fermentable sugars. The PSA method used was a modification of the Dubois [118] method. Dubois et al. proposed two protocols with different concentrations of phenol solution. In this study, a 5% w/w phenol solution was used. Samples were appropriately diluted so the final absorbance was in the linear range of the spectrophotometer. After addition of the phenol and concentrated sulfuric acid to the samples, the samples are allowed to stand 10 min, then mixed with a vortexer and placed at 30 °C for 30 min. The absorbance was measured at 490 nm. The amount of total sugar was determined by reference to a glucose standard curve.

Factors in the exploratory experiments identified as significant were selected as parameters in the time-course experiments. The method used for quantification of sugars in those experiments was a carbohydrate analysis method based on 3-methyl-2-benzothiazolinone hydrazone (MBTH), which forms a complex with monosaccharide aldehyde groups. The MBTH assay measures free monosaccharides [117] and was used to compare different treatments. Results obtained in the time-course experiments were used for the calculations of sugar release yields and rates (Table 3.1). The MBTH method was performed

according to the published protocol [119]. This method measured the free monosaccharides and the amount of total sugar was determined by a reference to a glucose standard curve. Different monosaccharides have similar responses by MBTH, unlike the PSA assay. The maximum absorbance of all monosaccharides is close to the absorbance wavelength of the MBTH assay and the interfering compounds do not absorb at this wavelength. In contrast, the wavelength used in the PSA assay is where the interfering compounds also absorb [120].

For the optimization experiments related to the two-step sulfuric acid and enzyme treatments, sugar concentrations in the samples were quantified by high pressure liquid chromatography (HPLC). This method is suitable for detection and quantification of individual sugars. Individual sugars were detected and quantified by HPLC. The apparatus used was a Shimadzu Prominence system equipped with a RID-10A refractive index detector and controlled by LCSolution 1.25 software. The carbohydrates were separated on an Aminex HPX-87H (Bio-Rad, Hercules, CA) column with 9 µm particle size and 300 mm x 7.8 mm dimensions. A standard cartridge holder was used to protect the column. The mobile phase was 0.01 N sulfuric acid with a flow rate of 0.6 mL/min, the oven temperature was 65 °C, and the analysis time was 25 min. Limits of detection for common monosaccharides of *N. salina* [30], as determined with standards, are [121]: arabinose 0.2 mg/mL, rhamnose 0.03 mg/mL, fucose 0.04 mg/mL, galactose 0.02 mg/mL, and glucose 0.4 mg/mL.

3.3.7 Growth assays

To evaluate the potential of the acid and enzymatic hydrolysates to support growth, assays were conducted using *Saccharomyces cerevisiae* JAY270, a PE-2 derived diploid. This strain is naturally adapted to the sugar cane fermentation process in Brazil and has a high fermentation efficiency and prolonged persistence [122].

The hydrolysates tested in the growth assays were obtained from the optimized two-step acid method, optimized enzymatic method, and eight of the one-step acid protocols (biomass loading 5% w/v, hydrolysis time 5 h, acid concentration 1 and 10% (w/v), temperature 60 and 90 °C). Both sulfuric acid- and hydrochloric acid-derived hydrolysates were tested. The conditions for the two-step acid hydrolysis and enzymatic hydrolysis are described in Sections 3.2.3 and 3.2.5, respectively. The enzymatic

hydrolysis was carried out for 5 h (biomass loading 5% w/v) and then stopped by boiling for 5 min. For the growth test on the enzymatic hydrolysis, the acetate buffer was replaced with citrate buffer to avoid any complicating effects that may have been posed by the availability of acetate as a carbon source. In separate tests, both citrate and acetate buffers were shown to release the same amount of sugar.

Prior to the assay, an inoculum culture was prepared by growing strain JAY270 for 24 h at 30 °C in a medium composed of 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract. After 24 h, the culture was centrifuged, the supernatant was decanted, and cells were re-suspended in sterile DI water to the original volume. The cells were washed three times to prevent the carry-over of glucose and ethanol. For the growth assay, the pH of the hydrolysates to be tested was adjusted to 6 with 10 M NaOH prior to inoculation with 5% v/v inoculum in a 10 mL serum bottle. The volume of the hydrolysate in each bottle was 5 mL and each bottle had a stir bar for mixing. No other nutrients were added to the hydrolysates. Inoculated serum bottles were placed in an anaerobic chamber (Coy Laboratories, USA) at 30 ℃ on a multiple position stirrer (Corning, USA) and samples for OD measurements and HPLC analysis were taken at 0, 24, and 48 h.

3.3.8 Fermentation inhibitors

During the hydrolysis of biomass, certain chemicals may be generated that are inhibitory to fermentation. The presence of common inhibitory compounds was evaluated using a version of the HPLC technique described in Section 3.2.6. The mobile phase was 0.01 N sulfuric acid with a flow rate of 0.6 mL/min, the oven temperature was 55 ℃, and the analysis time was 50 min. A lower temperature was used here than for sugar analysis because some of the inhibitors are temperature sensitive and may have decomposed at higher temperatures. The presence of the most common inhibitors generated during hydrolysis such as furfural, hydroxymethylfurfural, levulinic acid, formic acid, and acetic acid were examined using standards.

3.3.9 Statistical analysis

Design-Expert version 8 was used for experimental design of response surface methodology (or the optimization experiments). SAS OnDemand for academics was used for statistical analysis of the one-

step acid treatment. The significance of studied factors were compared based on ANOVA results and P-value=0.05.

3.4 Results

3.4.1 Treatment severity

The combined severity factor (CSF) for different acid hydrolysis treatments was calculated as

$$\mathrm{CSF} = \log\left(\mathrm{t}\,.\exp\frac{(T_H - 100)}{14.75}\right) + \left|\,\mathrm{pH} - 7\,\right| \qquad (3.1)$$

where t is reaction time (min), TH is the reaction temperature (℃), and 100 is the reference temperature (℃) [123]. The combined severity factors for different treatments are shown in Table 3.1. The reaction time used for calculating CSF values for enzymatic hydrolysis and one-step acid hydrolysis was 300 min. CSF value for the two-step acid hydrolysis was calculated using the sum of the CSF values for two steps. Each step had different pH, temperature, and reaction time. The duration of the first step was 60 min and the second step 65 min.

3.4.2 Two-step sulfuric acid hydrolysis

The two-step acid hydrolysis process is a common method in the lignocellulosic industry and typically releases the highest amount of sugars. This process was used in this study as a benchmark treatment to estimate the maximum sugar yield from algal biomass. An optimization experiment was conducted to find the conditions for the second step of acid hydrolysis that result in the highest sugar yield from the *N. salina* LEAB. The response surface methodology provides the optimized conditions and reveals any existing interactions between studied factors. The experimental design and the levels of studied factors including the response for the two-step optimization are reported in Table 3.2.

Among the studied factors in the ranges tested, only acid concentration was statistically significant (p <0.0001). The only significant interaction was between temperature and time (Figure 3.1). The ANOVA results for the optimization of the second step of sulfuric acid hydrolysis are presented in Table 3.3. The optimal response (sugar yield) is predicted to occur when the second step is performed with 4% H_2SO_4 at 112 ℃ for 65 min. Using these conditions, the total sugar release yield and rate for the two-step

hydrolysis were determined (Table 3.1). The conditions used for this hydrolysis were the optimized conditions and the analysis was with the MBTH assay. The CSF value for the two-step acid hydrolysis was the highest of all methods.

3.4.3 One-step sulfuric and hydrochloric acid hydrolysis

Since *N. salina* does not contain lignin, milder hydrolysis methods such as one-step acid hydrolysis might be capable of releasing sugars at yields and rates similar to those of the more severe two-step method. Sulfuric and hydrochloric acid were tested. The results of the exploratory experiments for one-step acid hydrolysis revealed that the hydrolysis reaction rate increased with temperature in agreement with the Arrhenius equation (Figure 3.2). Increased biomass loading did not improve the yield with either acid when the concentration of hydrolyzing agent was kept constant (Figure 3.3 and Figure 3.4). In contrast, increased acid concentration resulted in increased sugar yield (Figure 3.3 and Figure 3.4). Based on these results, the conditions chosen for the time-course experiments were: 1 and 10% w/w acid concentration, 60 and 90 °C, and 5% w/w biomass loading.

The time course experiments provided information on the time to obtain maximal sugar release for each condition, as well as estimates of hydrolysis rates under the different conditions. Sugar release rates and yields as well as the CSF values are presented in Table 3.1 for all one-step acid hydrolysis treatments. Results for hydrochloric acid confirmed that the reaction was completed within 5 h (Figure 3.5). Sugar yields and rates were improved by increasing the temperature while keeping the acid concentration constant (Figure 3.5). The same trends were observed for sulfuric acid hydrolysis (Figure 3.6).

3.4.4 Enzymatic hydrolysis

Response surface methodology was used to determine the effect of the enzyme mixture composition on the yield of sugars (Table 3.4 and Figure 3.7). In the enzyme concentration ranges tested, only the pectinase concentration had a statistically significant impact on the sugar yield. None of the interactions between the enzymes was statistically significant. The optimal enzyme mixture was determined to be (per g LEAB): 50 µL Accellerase 1500, 5 µL Accellerase XY, 12.5 µL Accellerase XC, and 153 µL pectinase. A

time course experiment with this optimal enzyme mixture revealed that enzymatic deconstruction of LEAB stopped within 5 h (Figure 3.8). Yield and rate data for enzymatic sugar release are presented in Table 3.1.

3.4.5 Evaluation of inhibitors

Hydrolysates were assayed by HPLC for the presence of common fermentation inhibitors (Table 3.5). The common inhibitor among hydrolysates produced by all treatments is acetic acid, which is also present in the biomass blank treated only with water. In lignocellulosic biomass, acetic acid is liberated by degradation of hemicellulose. Levulinic acid and hydroxymethylfurfural (HMF) are products of the acidic degradation of hexose sugars (glucose, in this case). Levulinic acid and furfural can be further degraded to formic acid. If longer hydrolysis times were tested, formic acid might have been observed. Pentose sugars such as xylose are usually degraded to furfural but furfural was not detected, further supporting the observation of little or no pentose release from the LEAB.

3.4.6 Growth results

A qualitative assessment of the growth of the model yeast, *S. cerevisiae* strain JAY270, on various hydrolysates for 24 h is presented in Table 3.6. No other nutrients were added to the hydrolysates and only the pH was adjusted. Strain JAY270 grew on some but not all of the tested hydrolysates. Lack of growth may have been caused by inhibitors and/or salt generated from neutralization. A detailed chemical analysis of the hydrolysates was not performed, but it was noted that hydrolysates that did not support visible growth contained acetic acid concentrations above 0.25 g/L.

3.5 Discussion

3.5.1 Comparison of sugar analysis methods

The two colorimetric methods used for calculating total sugar concentrations in the hydrolysates were PSA and MBTH. Some of the challenges associated with the PSA method are interference of other compounds present in the hydrolysate, including salts, proteins, and non-carbohydrate compounds such as pigments and lipids [117, 120], insensitivity to some algae-specific monosaccharides [120] , lack of

specificity (e.g., between mono- and disaccharides), and poor detection limit [117]. In this study, the PSA method was used for the exploration phase, while the MBTH method was used for the quantification phase. The PSA method was sufficient to determine the significant factors for different treatments and to quantify the total carbohydrates including the unhydrolyzed polysaccharides.

The MBTH method does not suffer from interferences of salts and proteins and will measure the free monosaccharides released as the product of the hydrolysis treatment [117, 120]. A review on sugar analysis of marine matrices showed that regardless of the hydrolysis method, sugar yields estimated by colorimetric techniques were higher than those estimated by chromatographic methods [117]. This observation may be due to an underestimation of the carbohydrates when using chromatographic techniques or an overestimation when using colorimetric methods due to the presence of similar functional groups (-CH=O) as in sugars [117].

The only monosaccharide that was detected by the HPLC method was glucose and this was in accordance with the findings of Gerken et al. [124] for the related alga, *Nannochloropsis gaditana*. They observed that about 80% of the cell wall mass of *N. gaditana* was composed of carbohydrates, 98–99% of which were formed from glucose monomers.

3.5.2 Acid hydrolysis

Two different acid hydrolysis methods were examined: a standard two-step process and a modified one-step process. The two-step sulfuric acid hydrolysis is a common method for lignocellulosic biomass and is known to generate fermentation inhibitors. Since LEAB has no lignin, our hypothesis was that a milder acid treatment could still release fermentable sugars at an acceptable rate and yield while generating lower levels of fermentation inhibitors. Results for sugar and inhibitor yields (Tables 3.1 and 3.5) supported this hypothesis.

CSF can be used as a benchmark to compare efficiencies of treatments and to examine the effect of temperature, reaction time, and pH on biomass conversion [123]. However, using only one equation to predict the conditions that provide the highest yield or rate is an imprecise approach. CSF does not account for other hydrolysis parameters and a significant lack of correlation between sugar yields and CSF values has been reported [123]. The highest CSF value was obtained for the two-step acid

hydrolysis, which correlated with the highest sugar yield (statistically equivalent to the yield for one-step hydrolysis with 10% sulfuric acid at 90 °C) and the highest inhibitor yields (Table 3.1 and 3.5, respectively). For one-step acid hydrolysis, CSF values for hydrochloric acid were higher than sulfuric acid at the same hydrolysis conditions, owing to the higher molarities of hydrochloric acid compared to sulfuric acid at the same mass concentrations. Figure 3.9 shows the correlation of sugar release yields and rates with CSF values. The correlation coefficient for a linear relationship between the sugar yield at 5 h of reaction and CSF is 0.88 while the coefficient between the maximum sugar release rate and CSF is 0.62. The correlation coefficient between total inhibitor yields and the CSF values for the one-step acid hydrolysis was 0.91 (Figure 3.10).

The effects of different factors on acid hydrolysis were studied. Acid concentration was the only significant factor for the two-step process while for the one-step process, acid concentration, type of acid, temperature, and reaction time were all significant. The biomass loading was not a significant factor for the one-step acid hydrolysis.

Recently Talukder et al. [81], reported the hydrolysis of whole *N. salina* biomass with 5% sulfuric acid at 120 °C for 1 h (CSF =9.08), with subsequent lipid extraction using hexane. They reported a total sugar yield of 117 mg/g biomass, which is lower than the yields from the more severe acid treatments reported here and similar to the total sugar yield from enzymatic hydrolysis

The use of an acid hydrolysis process requires neutralization prior to fermentation. In this study, sodium hydroxide was used for neutralization. Other salts such as calcium have lower solubility than sodium, and precipitation of part of the generated salt may occur. Using lignocellulosic hydrolysate, Casey et al. showed that salts can be significant inhibitors of *S. cerevisiae* because of osmotic stress and ion toxicity [125]. Sreekumar et al. observed that calcium ions enhanced the yield and concentration of ethanol for *Zymomonas mobilis* [126]. These studies suggest that selecting certain bases for neutralization may reduce the negative impact of salts on fermentation.

3.5.3 Enzymatic hydrolysis

Enzymatic hydrolysis was achieved by enzymes designed specifically for lignocellulosic biomass. The enzyme mixture included exoglucanase, endoglucanase, hemi-cellulase, beta-glucosidase, xylanase, and

pectinase activities. It is notable that these enzyme mixtures were effective in releasing sugars from the algal biomass used in this study. Although the algal biomass does not contain lignin, the outer cell wall of a related *Nannochloropsis* species is composed of algaenan, which would be expected to block enzyme access [124]. The ability of these non-specific enzymes in achieving about 35% of the maximum sugar yield (Table 3-1) suggests that enzymes penetrated the algaenan layer or that the algaenan layer was disrupted in the extraction process. Using a response surface methodology enabled us to study the interactions among the four enzyme preparations used in this study and to find the optimal enzyme mixture.

The sugar release yield for enzymatic hydrolysis was lower than some of the acid treatments (Table 3.1), which suggests that we still need better enzymes with more specific activities depending on biomass type. Unlike lignocellulosic biomass hydrolysis, which requires about 72 h, enzymatic sugar release from LEAB was completed within 5 h; however some carbohydrates remained undigested. The addition of protease may improve the sugar release from cell wall since some algal cell wall carbohydrates are in the form of glycoproteins [127].

3.5.4 Growth and inhibition of fermenting microorganisms

The fermentation inhibitors generated in the carbohydrate deconstruction methods varied, depending on the biomass composition and severity of the treatment (Table 3.5). The inhibitory compounds are generally grouped into furan derivatives [128], weak acids , and phenolic compounds, while salts from neutralization have also been listed as inhibitors [125].

In this study, the presence of common inhibitors such as furans, sugar degradation products, and weak acids was examined (Table 3.5). Since LEAB has no lignin, phenolic compounds were not expected [129]. Among furans, HMF was detected following some treatments while no furfural was detected (limit of detection 0.14 mg/mL). This result supports the observation that little or no pentose sugar was present to be degraded to furfural.

Inhibitors act through different mechanisms, and different microorganisms have different inhibitor tolerances. Protonated weak acids have different toxicity than their undissociated form, even at the same concentration [130] and thus pH is a crucial factor. The growth experiment in this study was designed to

assess the effect of inhibitors generated as hydrolysis byproducts on the growth of a selected strain of yeast. It was observed that the selected yeast grew on all the hydrolysates (with no added nutrients) except two of treatments, both of which had acetic acid concentrations equal to or greater than 0.25 g/L. The high concentrations of salts from neutralization may also have been an inhibitory factor for those hydrolysates.

3.5.5 Comparison of acid and enzymatic hydrolysis

Acid hydrolysis is still one of the most practical biomass treatment options. Some of its advantages are the availability of mineral acids, effectiveness on almost all kinds of biomass, and high sugar yields and rates. Organic acids such as acetic resulted in much lower yields from LEAB than do the mineral acids tested here (data not shown). Some challenges associated with acid hydrolysis are the requirement for special materials for handling the acid and the need for neutralization. These factors increase process costs, offsetting the cost savings derived from high sugar release rates.

In contrast, enzymatic hydrolysis provides the advantages of milder process conditions (temperature and pH), less inhibitor generation, much lower neutralization requirements, relatively high efficiency, and the better growth of yeast on the enzymatic hydrolysate. However, the high cost of enzyme production is a challenge for enzymatic hydrolysis. It may be possible to overcome this issue by recycling the enzymes or by using them in an immobilized format.

Both types of hydrolysis process suffer from incomplete conversion of cell wall carbohydrates to fermentable sugars. Algal cell walls are composed of fibrillar and amorphous components [131] and the polysaccharide composition of the cell wall varies based on algal taxa [132]. More research is required to find out the effects of cell type, cultivation, harvesting, and lipid extraction techniques on the digestion of cell wall carbohydrates.

3.6 Conclusion

We studied different cell wall carbohydrate deconstruction techniques for lipid-extracted *Nannochloropsis salina*. We developed hydrolysis treatments with no pretreatment step. One-step sulfuric acid hydrolysis had the highest sugar release yield while one-step hydrochloric acid had the highest sugar

release rate. Enzymatic hydrolysis had acceptable sugar release rate and yield, but enzymes specially designed for algal biomass are still needed. Currently, commercially available enzymes for biomass deconstruction are designed for lignocellulosic biomass, which includes lignin, cellulose, and hemicellulose. For production of fermentable substrates from lignocellulosic biomass, the common approach is lignin removal by a pretreatment step followed by a hydrolysis step. In enzymatic hydrolysis or saccharification step, cellulose and hemicellulose are broken down to their constituent components like glucose and xylose. Enzymes designed for lignocellulosic biomass target cellulose and hemicellulose, while not all species of algae are composed of these two biopolymers only. Algal cell walls are composed of fibrillar, matrix, and crystalline polymers. Algal cell walls are typically composed of microfibrillar polysaccharides embedded in matrix polysaccharides and proteoglycans. Cellulose in algal cell wall does not represent the cellulose in plant cell walls. Algal cellulose can be 20 nm in diameter while higher plant cellulose is 3 to 5 nm in diameter [3]. The outer layer of *Nannochloropsis* is composed of algaenan, which is known to block the enzyme access to cellulose inner layer [124]. These are some of the reasons that we need specially designed enzymes for algal biomass deconstruction.

A yeast strain grew on all of the generated hydrolysates except two of the hydrochloric acid treatments. The byproducts of different hydrolysis treatments, which are inhibitors to the fermenting microorganisms were detected and quantified. The LEAB hydrolysate generated by these different treatments can be used as the fermentation media for the production of biofuels or bioproducts.

3.7 Tables and figures

Table 3-1. Summary of carbohydrate deconstruction methods for *N. salina* LEAB. Yield of released sugar refers to the amount released as a result of the hydrolysis process; the LEAB contained 17.3 mg/g sugars prior to treatment. Combined severity factor is defined in Equation 3.1.

Hydrolysis treatment	**Yield of released sugar ±SD (mg sugar/g LEAB)**	**Maximum sugar release rate (mg sugar/g LEAB·h)**	**Combined severity factor**	**Comments**
2-step sulfuric acid	192.3±18.1	96.2	16.8[1]	Heating and significant neutralization required 2 h total reaction time
1-step hydrochloric acid				
1% HCl, 60 °C	15.7±10.8	56.8	7.3	Neutralization required
10% HCl, 60 °C	130.6±14.6	34.5	8.6	5 h reaction time
1% HCl, 90 °C	105.4±25.3	37.5	8.0	BL=5%
10% HCl, 90 °C	164.6±2.1	272.3	9.1	
1-step sulfuric acid				
1% H_2SO_4, 60 °C	15.0±1.9	11.3	6.2	Neutralization required
10% H_2SO_4, 60 °C	49.6±2.8	39.7	7.9	5 h reaction time
1% H_2SO_4, 90 °C	37.4±10.7	43.4	7.9	BL=5%
10% H_2SO_4, 90 °C	225.9±30.7	143.8	9.1	
Optimal enzyme mixture				Mild conditions
50 °C	77.9±3	87.9	3.2[2]	5 h reaction time BL=5%

[1] Calculated based on 65 min for second step.
[2] Only includes pH, temperature, and time effects.

Table 3-2. Experimental design for optimization of the second step of sulfuric acid hydrolysis of LEAB. The studied response is the sugar yield measured by the HPLC method.

Run	Block	Block Type	Factor 1 Acid concentration % (w/w)	Factor 2 Temperature (°C)	Factor 3 Time (min)	Response mg glucose/ g LEAB
1	day1	Center	3	110	70	99
2	day1	Center	3	110	70	81
3	day1	Factorial	4	105	75	116
4	day1	Factorial	2	105	65	41
5	day1	Factorial	4	115	65	120
6	day1	Factorial	2	115	75	70
7	day2	Factorial	4	115	75	71
8	day2	Factorial	2	105	75	49
9	day2	Center	3	110	70	82
10	day2	Factorial	4	105	65	75
11	day2	Factorial	2	115	65	57
12	day2	Center	3	110	70	56
13	day3	Axial	3	100	70	30
14	day3	Axial	1	110	70	14
15	day3	Axial	3	120	70	26
16	day3	Axial	5	110	70	134
17	day3	Axial	3	110	60	98
18	day3	Axial	3	110	80	92
19	day3	Center	3	110	70	87
20	day3	Center	3	110	70	72

Table 3-3. ANOVA results for the optimization of the second step of sulfuric acid hydrolysis. Studied factors included acid concentration, temperature, and reaction time.

Source	**Sum of squares**	**df**	**Mean square**	**F value**	**p-value Prob>F**
Block	1819.29	2	909.65		
Model	16480.90	9	1831.21	12.43	0.0008
A-Acid concentration	10251.56	1	10251.56	69.57	<0.0001
B-Temperature	52.56	1	52.56	0.36	0.5669
C-Time	0.063	1	0.063	$4.242*10^{-4}$	0.9841
AB	171.13	1	171.13	1.16	0.3126
AC	105.13	1	105.13	0.71	0.4228
BC	903.12	1	903.12	6.13	0.0384
A^2	4.17	1	4.17	0.028	0.8706
B^2	3408.17	1	3408.17	23.13	0.0013
C^2	560.67	1	560.67	3.80	0.0869
Residual	1178.81	8	147.35		
Lack of fit	566.31	5	113.26	0.55	0.7367
Pure error	612.50	3	204.17		
Corrected total	19479.00	19			

Table 3-4. ANOVA results for the enzymatic hydrolysis optimization. Studied factors were concentrations of four different enzymes.

Source	Sum of squares	df	Mean square	F value	p-value Prob>F
Block	2566.88	3	855.63		
Model	1740.24	10	174.02	1.01	0.4575
A-Accellerase 1500	134.33	1	134.33	0.78	0.3842
B-Accellerase XC	161.62	1	161.62	0.94	0.3404
C-Accellerase XY	293.49	1	293.49	1.70	0.2013
D-Pectinase	778.62	1	778.62	4.51	0.0412
AB	19.44	1	19.44	0.11	0.7394
AC	182.60	1	182.60	1.06	0.3113
AD	215.54	1	215.54	1.25	0.2719
BC	223.47	1	223.47	1.29	0.2634
BD	40.81	1	40.81	0.24	0.6301
CD	111.22	1	111.22	0.64	0.4280
Residual	5875.98	34	172.82		
Lack of fit	73.15	2	36.57	0.20	0.8184
Pure error	5802.84	32	181.34		
Corrected total	10183.10	47			

Table 3-5. Detected inhibitors and their corresponding yields and concentrations for different LEAB hydrolysates. ND = below detection limit. Y indicates inhibitor yield (mg inhibitor/g LEAB) and C indicates inhibitor concentration (mg inhibitor/mL). Value ranges are ± one standard deviation.

Treatment	Formic acid		Levulinic acid		Acetic acid		HMF		Furfural	
	Y	C	Y	C	Y	C	Y	C	Y	C
HCl 1%, 60 ℃, 5 h	ND	ND	ND	ND	2.75±0.09	0.14±0.00	ND	ND	ND	ND
HCl 10%, 60 ℃, 5 h	ND	ND	1.36±0.05	0.05±0.00	6.51±0.75	0.26±0.03	ND	ND	ND	ND
HCl 1%, 90 ℃, 5 h	ND	ND	0.35±0.07	0.05±0.00	3.97±0.38	0.19±0.02	0.22±0.04	0.03±0.00	ND	ND
HCl 10%, 90 ℃, 5 h	ND	ND	6.39±0.65	0.24±0.02	8.26±1.36	0.32±0.05	0.98±0.14	0.04±0.01	ND	ND
H_2SO_4 1%, 60 ℃, 5 h	ND	ND	ND	ND	2.09±0.21	0.1±0.01	ND	ND	ND	ND
H_2SO_4 10%, 60 ℃, 5 h	ND	ND	ND	ND	4.86±0.21	0.2±0.01	ND	ND	ND	ND
H_2SO_4 1%, 90 ℃, 5 h	ND	ND	ND	ND	4.06±1.01	0.2±0.05	ND	ND	ND	ND
H_2SO_4 10%, 90 ℃, 5 h	ND	ND	2.22±0.12	0.09±0.00	6.15±0.70	0.25±0.03	ND	ND	ND	ND
Two-step sulfuric	ND	ND	14.59±0.53	0.05±0.00	18.18±0.73	0.06±0.00	ND	ND	ND	ND
Biomass blank treated at 60 ℃, 5 h	ND	ND	ND	ND	1.19±0.01	0.06±0.00	ND	ND	ND	ND
Biomass blank treated at 90 ℃, 5 h	ND	ND	ND	ND	1.22±0.01	0.06±0.00	ND	ND	ND	ND
Enzymatic Hydrolysis, 50 ℃, 5 h	ND	ND	ND	ND	2.73±0.90	0.14±0.05	ND	ND	ND	ND

Table 3-6. Growth results for JAY270 on different hydrolysates of *N. salina* LEAB after 24 h. 0 = no visual growth, + = detectable growth, ++ = dense growth.

Treatment	**Growth observed**
HCl 1%, 60 ℃	+
HCl 10%, 60 ℃	0
HCl 1%, 90 ℃	++
HCl 10%, 90 ℃	0
H_2SO_4 1%, 60 ℃	+
H_2SO_4 10%, 60 ℃	+
H_2SO_4 1%, 90 ℃	+
H_2SO_4 10%, 90 ℃	+
Two-step sulfuric acid	+
Optimal enzyme mixture	++

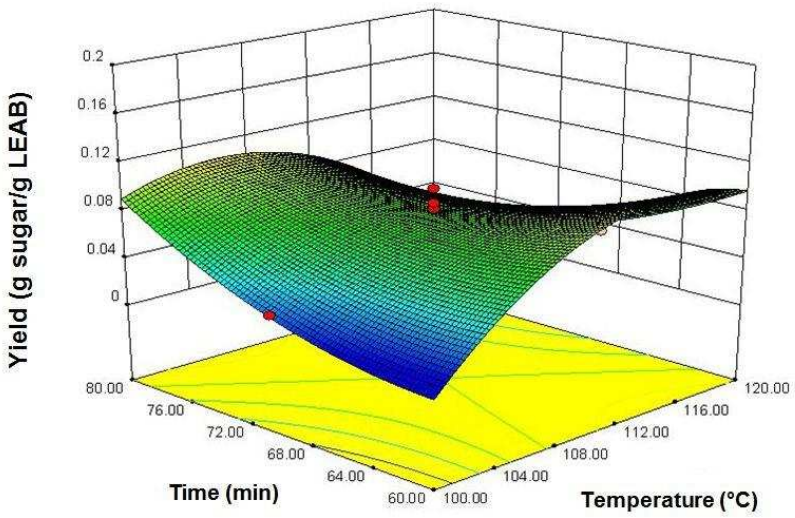


Figure 3-1. Response surface plot for the optimization of the two-step sulfuric acid hydrolysis of LEAB. Plot shows the existing interaction between time and temperature on the released sugar yield. Data have been analyzed by HPLC. Number of biological replicates=3.

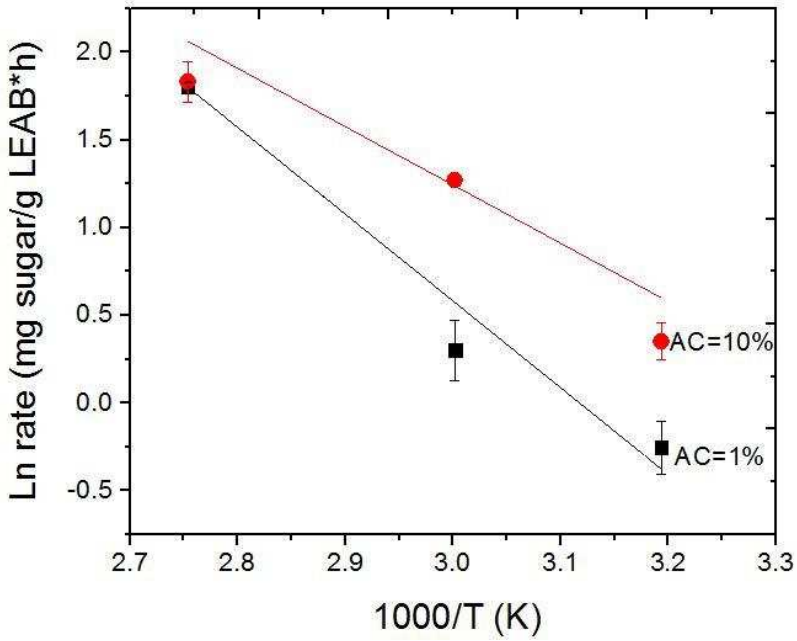


Figure 3-2. Sugar release rate dependence on temperature for one-step sulfuric acid hydrolysis. The reaction time was 24 h and the biomass loading was 1%. Results obtained by MBTH assay. Error bars are ± one standard deviation. Number of biological replicates=3.

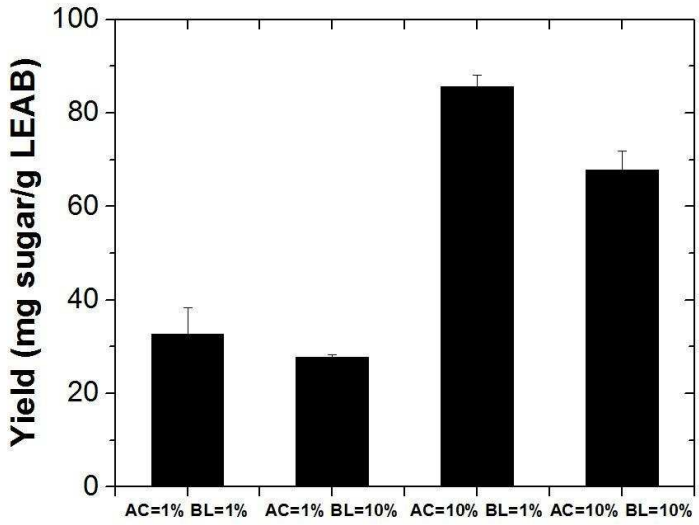


Figure 3-3. Sugar yield dependence on acid concentration (AC) and biomass loading (BL) for one-step sulfuric acid hydrolysis at 60 ℃ and 24 h of reaction. Error bars are ± one standard deviation. Number of biological replicates=3.

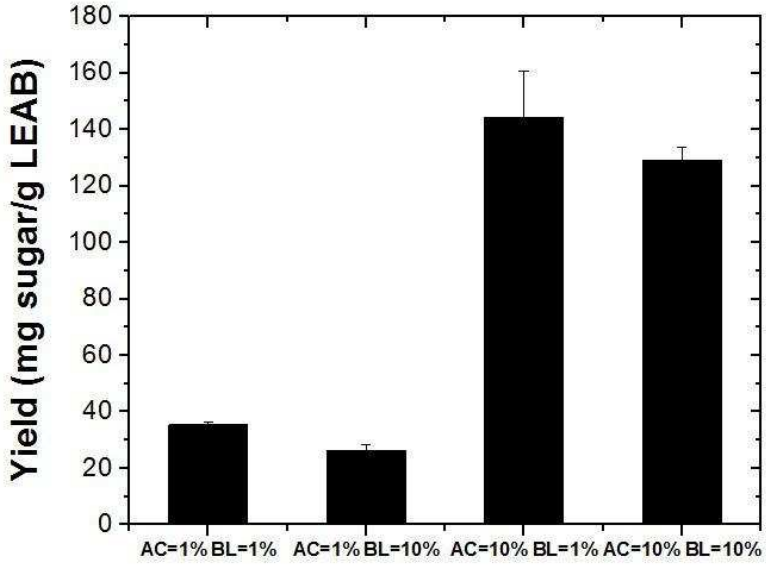


Figure 3-4. Results of sugar yield dependence on acid concentration (AC) and biomass loading (BL) for one-step hydrochloric acid hydrolysis at 60 ℃. The reaction time was 24 h. Error bars are ± one standard deviation. Number of biological replicates=3.

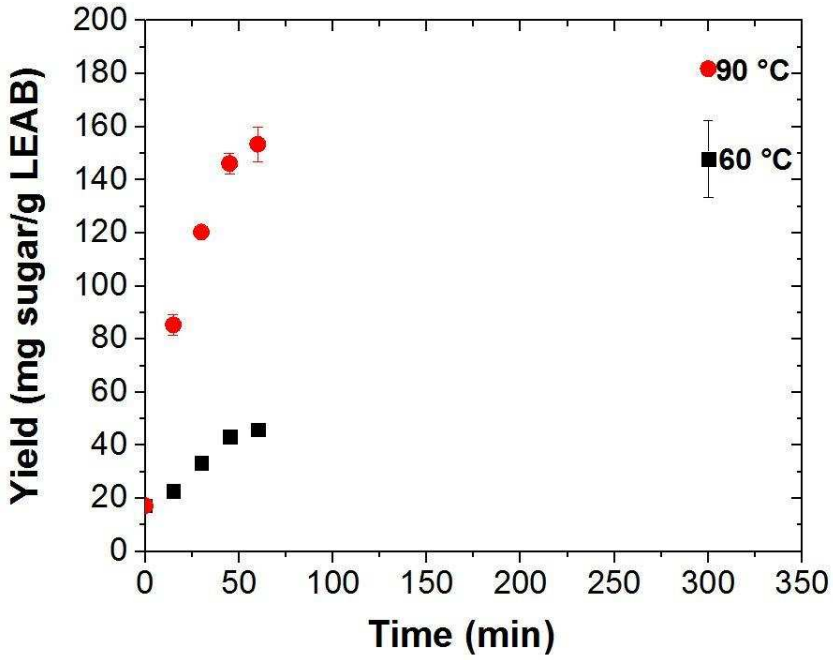


Figure 3-5. Sugar yield time dependence for one-step hydrolysis with hydrochloric acid at 60 ℃ and 90 ℃, 5% biomass loading, and 10% acid concentration. Error bars are ± one standard deviation. Number of biological replicates=3.

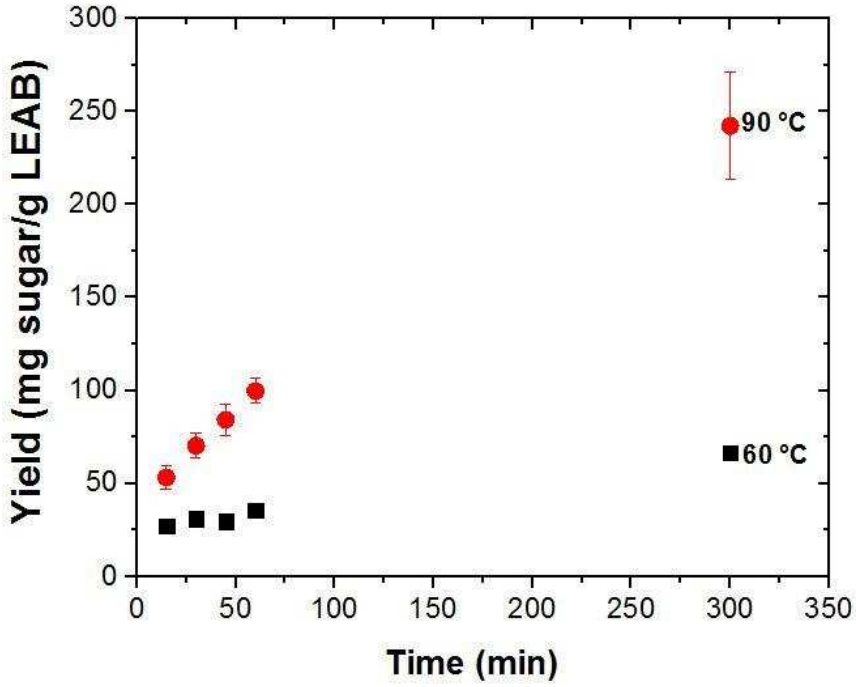
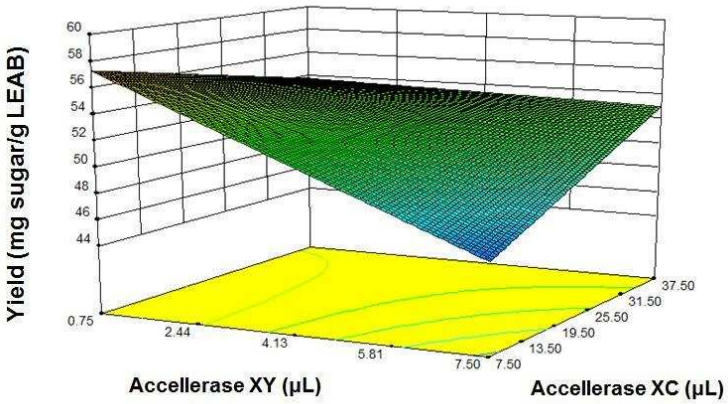


Figure 3-6. Time course of sugar yield for one-step sulfuric acid hydrolysis at 60 and 90 ℃. The biomass loading was 5% and the acid concentration was 10%. Data analyzed by MBTH. Error bars are ± one standard deviation. Number of biological replicates=3.



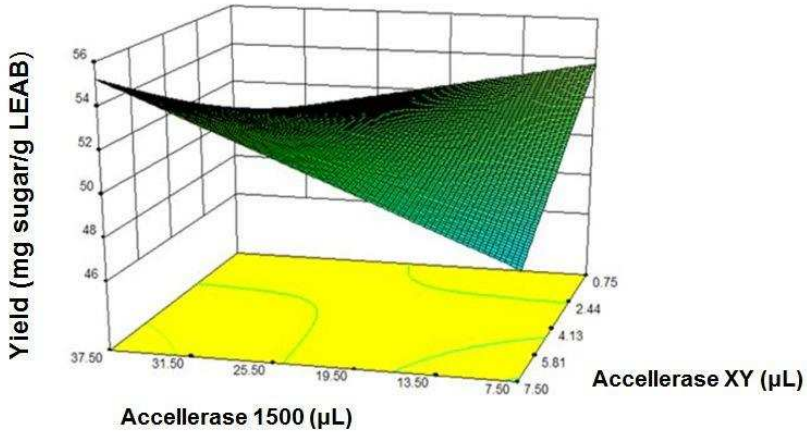


Figure 3-7. Response surface plot for the optimization of enzymatic hydrolysis, hydrolysis conditions was 1.5% biomass loading at 50 ℃. Plot shows the interaction between Accellerase 1500 and Accellerase XY on sugar yield. Number of biological replicates=3.

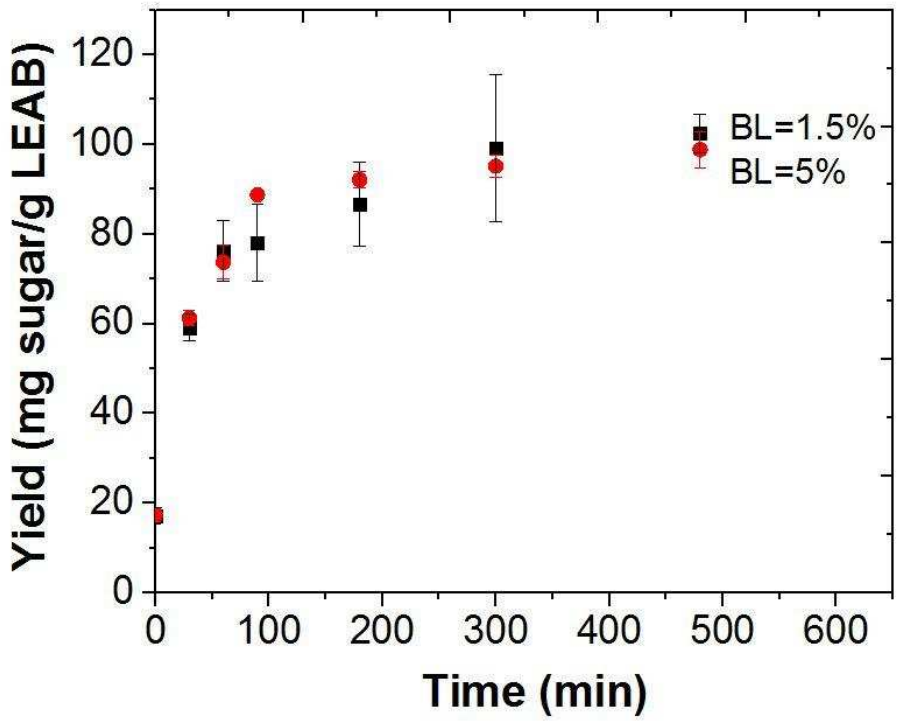


Figure 3-8. Sugar release rate by the optimal enzyme mixture at 50 ℃, pH 4.8, and biomass loading 1.5 and 5%. Error bars are ± one standard deviation. Number of biological replicates =3.

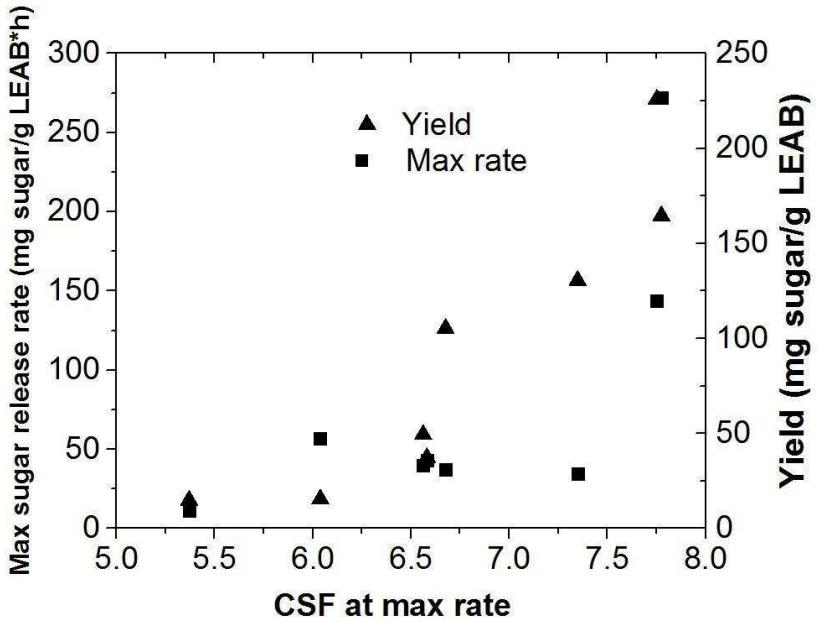


Figure 3-9. Correlation of sugar release yield and maximum sugar release rate with combined severity factor for acid hydrolysis.

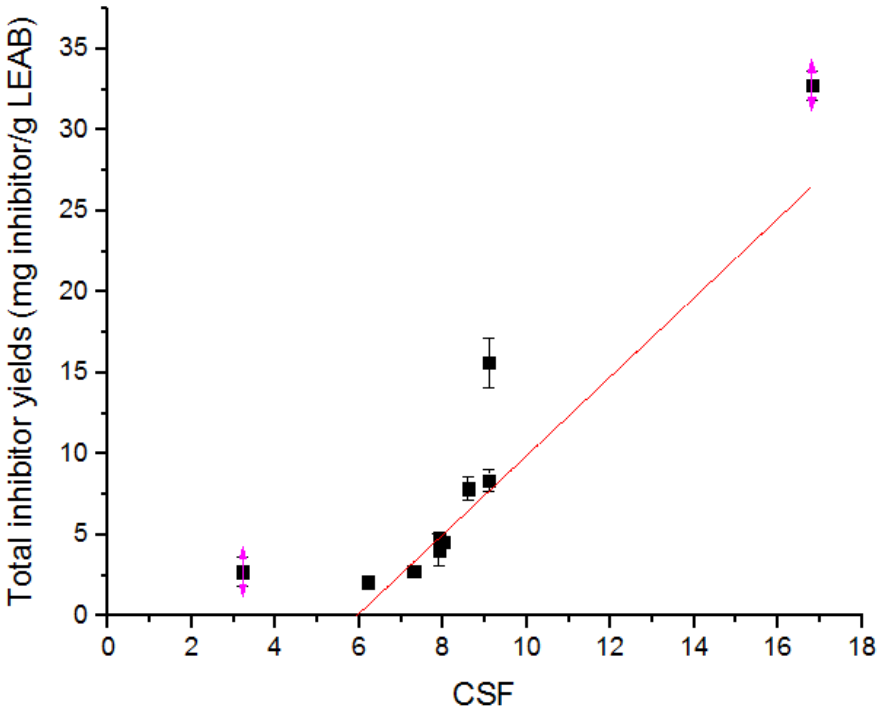


Figure 3-10. Correlation of total inhibitor yield with combined severity factor for one-step acid hydrolysis. Adjusted R-square=0.77. Error bars are ± one standard deviation. Number of biological replicates =3.

4 Conclusion

Algal biofuels have the potential to replace the petroleum-based fuels, but their commercial scale production is not economically feasible yet. More research and development is still needed in all the proposed steps for algal biofuels production. One promising solution to improve the economics is adopting a biorefinery approach. In a biorefinery setting, value-added chemicals in addition to fuel and energy are produced. The concept of biorefinery holds a wide range of technologies that can separate biomass resources into their building blocks, which can be converted to value-added products, biofuels, and chemicals [8]. A biorefinery facility can produce transportation biofuels, power, and chemicals from biomass. The body of work presented in this dissertation represents a biorefinery strategy in industrial algal biofuel production.

Algal biomass is usually grown for lipid production since it accumulates up to 60% of its dry weight as lipids, which can be extracted and converted to fuels such as biodiesel. The remainder of the biomass consists of carbohydrates and proteins, which can be converted to additional fuels or products. Potential applications for the residual biomass after lipid extraction are electricity, animal feed, fuel, biochar, and fertilizer. Technologies that can produce fuels from residual algal biomass include anaerobic digestion, pyrolysis, hydrothermal liquefaction, and fermentation. Methane and hydrogen can be produced via anaerobic digestion and ethanol through fermentation. A literature review in Chapter 2 of this dissertation covered current research on conversion of the residual algal biomass into different products ranging from food to fuel.

The two main biomass conversion technologies are thermochemical and biochemical. The approach studied in this work was based on a biochemical conversion pathway. In a biochemical approach, biomass is deconstructed to fermentable substrates, such as sugars and amino acids, by hydrolysis. The biomass hydrolysate, contacting fermentable substrates, is then fermented. While thermochemical methods have potential, biological conversion allows the user to produce a specific product, as well as the option to recover residual proteins for animal feed and other uses.

In this study, lipid-extracted algae biomass was converted to ethanol. Ethanol was selected as the model product for validation of the proposed technology. Once the feasibility of our process is confirmed,

products other than ethanol can be produced by selecting a different microorganism. Advantage of ethanol production is that it can be used as a drop-in fuel in the current gasoline infrastructure or as a chemical platform for production of biobased chemicals. Many chemicals that are produced from oil can be produced from ethanol including ethylene, acetaldehyde, and ethyl acetate [1].

The first step in biochemical conversion of LEAB was deconstruction of biomass to fermentable substrates. Deconstruction of lignocellulosic biomass contains a pretreatment step followed by saccharification. Pretreatment is required to separate lignin from cellulose and hemicellulose in the biomass. Once lignin is removed, cellulose and hemicellulose are available to the hydrolyzing agents like enzymes. Unlike lignocellulosic biomass, most algae species do not contain lignin so a pretreatment step is not needed. Some species of algae including *Nannochloropsis gaditana* contain algaenan in their cell wall. Algaenan is a resistant biopolymer and its deconstruction is challenging [124]. Lack of lignin does not necessarily infer easier carbohydrate deconstruction since some algal cell walls may contain algaenan.

We studied several carbohydrate deconstruction methods with no pretreatment step. Some of our studied deconstruction methods were developed by adapting protocols from lignocellulos biomass industry. The challenge for developing saccharification methods specific to the algal industry is the lack of sufficient studies on algal cell wall composition due to diversity in algal species and their cell wall composition. Carbohydrate deconstruction methods that were developed in this research are one-step acid hydrolysis, two-step sulfuric acid hydrolysis, and enzymatic hydrolysis. The two-step sulfuric acid hydrolysis is a common method for hydrolyzing lignocellulosic biomass. This method is severe and it was hypothesized to release the maximum amount of sugar from LEAB. For this reason, two-step sulfuric acid hydrolysis was used as a benchmark. Since algae do not contain lignin, it was hypothesized that milder treatments like one-step acid hydrolysis are capable of releasing sugars at an acceptable sugar rates and yields. One-step acid hydrolysis was developed with milder temperatures and pH compared to two-step acid hydrolysis. Enzymatic hydrolysis with lower temperature and pH than acid treatment was also developed.

An advantage of enzymatic treatment over acid hydrolysis is milder process conditions, which results in lower CAPEX and OPEX. Acid hydrolysis requires higher CAPEX due to material requirements for

handling acid to avoid corrosion. Usually a conservative Ni-Cr-Mo (NCR) metallurgy will be selected. NREL uses 316 stainless steel for their sulfuric acid based pretreatment, but hydrochloric acid presents the added complication of chloride stress cracking. NCR is approximately 3 times as expensive as carbon steel and 1.5 times the cost of stainless. Acid hydrolysis has higher OPEX than enzymatic hydrolysis due to higher temperature and lower pH values and the necessity for neutralization. The major cost associated with enzymatic hydrolysis is the cost of enzymes. Unlike lignocellulosic biomass industry, which requires about 72 h for enzymatic hydrolysis, enzymatic sugar release from LEAB was completed within 5 h; however some carbohydrates remained undigested. The addition of protease may improve the sugar release from cell wall since some algal cell wall carbohydrates are in the form of glycoproteins [127].

Sugar release yields and rates of different treatments were measured experimentally and efficiencies of treatments were compared. If experimental saccharification is not an option, an empirical formula called Combined Severity Factor (CSF) can predict severity of a hydrolysis treatment. CSF can be used as a benchmark to compare efficiencies of treatments and to examine the effect of temperature, reaction time, and pH on biomass conversion [123]. However, using only one equation to predict the conditions that provide the highest yield or rate is an imprecise approach. CSF does not account for other hydrolysis parameters, like the type of catalyst, and a significant lack of correlation between sugar yields and CSF values has been reported [123]. For the tested hydrolysis conditions, we calculated the CSF values. A linear correlation between CSF and maximum sugar release rate and yield existed. By increasing severity of the treatments, higher sugar release yields and rates were observed due to more severe process conditions.

One major issue with acid treatment is generation of salts and byproducts that are inhibitory to fermentation. The inhibitory compounds are generally grouped into furan derivatives[128], weak acids , and phenolic compounds , while salts from neutralization have also been listed as inhibitors [125]. Some of the common inhibitors are furfural, hydroxymethyl furfural (HMF), levulinic acid, formic acid, and acetic acid. LEAB hydrolysates generated by different saccharification methods were screened for the presence of common inhibitors. A linear correlation existed between the calculated CSF values and the measured combined total inhibitor yields. The more severe the hydrolysis treatment gets the more inhibitors are

generated. The challenge of the carbohydrate deconstruction is the existence of a tradeoff between the complete conversion of carbohydrates and production of inhibitors. Since LEAB has no lignin, phenolic compounds were not expected [129]. Among furans, HMF was detected for some treatments while no furfural was detected. No furfural detection supports the observation that little or no pentose sugar was present to be degraded to furfural.

One major roadblock was accurate measurement of the released sugars. Hydrolysis methods were compared based on released sugar yields and rates. Process calculations are all based on sugar measurements so we need accurate methods to quantify sugars. Different individual and total sugar assays were tested and compared. The two colorimetric methods used for calculating total sugar concentrations in the hydrolysates were PSA and MBTH. Some of the challenges associated with the PSA method are interference of other compounds present in the hydrolysate, including salts, proteins, and non-carbohydrate compounds such as pigments and lipids [117, 120], insensitivity to some algae-specific monosaccharides [119], lack of specificity (e.g., between mono- and disaccharides), and poor detection limit [116]. The MBTH method does not suffer from interferences of salts and proteins and will measure the free monosaccharides released as the product of the hydrolysis treatment [117, 120]. A review of sugar analysis of marine matrices showed that regardless of the hydrolysis method, sugar yields estimated by colorimetric techniques were higher than those estimated by chromatographic methods [117]. This observation may be due to an underestimation of the carbohydrates when using chromatographic techniques or an overestimation when using colorimetric methods due to the presence of similar functional groups as in sugars[117]. In this study, chromatography techniques were preferred over the colorimetric assays for individual sugar analysis. Comparison of treatment efficiencies was based on the sugar release yield and rate calculated based on the results of the MBTH assay.

Among the hydrolysis methods that we developed, one-step sulfuric acid hydrolysis had the highest yield of released sugars, while the one-step hydrochloric acid treatment had the highest sugar release rate. The effects of different factors on acid hydrolysis were studied. Acid concentration was the only significant factor for the two-step process, while for the one-step process, the acid concentration, type of acid, temperature, and reaction time were significant. The biomass loading was not a significant factor for the one-step acid hydrolysis. Enzymatic hydrolysis produced acceptable sugar release rates and yields

but enzymes designed for algal biomass deconstruction are still needed. Enzymes designed for lignocellulosic biomass target cellulose and hemicellulose, while not all species of algae are composed of these two biopolymers only. Algal cell walls are composed of fibrillar, matrix, and crystalline polymers. Cellulose in algal cell wall does not represent the cellulose in plant cell walls. Algal cellulose can be 20 nm in diameter while higher plants cellulose is 3 to 5 nm in diameter [3]. The outer layer of *Nannochloropsis* is composed of algaenan, which is known to block the enzyme access to cellulose inner layer [124]. For these reasons, we need enzymes specifically designed for algal biomass deconstruction.

The second step in biochemical conversion of LEAB was ethanol fermentation of the hydrolysate with no added nutrients. To our knowledge, this is the first study that used LEAB hydrolysate as the sole nutrient source for fermentation. No conditioning step was needed, except pH adjustment and sterilization. Different microorganisms were tested for fermentation and their ethanol production was compared.

Reference media were setup to evaluate suitability of hydrolysates with no added nutrients as fermentation media. One of the hurdles to ethanol production from biomass is the production or introduction of inhibitory compounds to the ethanologens due to hydrolysis and/or conditioning steps [61].The hydrolysate has a complex matrix containing unknown chemicals that may be inhibitory to the fermenting microorganisms. To evaluate the effect of nutrients (or carbon to nitrogen ratio) and inhibitors on growth and ethanol production, a reference medium was set up. This reference was a defined medium with the same carbon, nitrogen, and phosphorous concentrations as in the hydrolysate without inhibitors or unknown compounds. The carbon content of the reference medium was calculated from the glucose concentration of the hydrolysates and not the total sugar concentration. The reason was, the majority of the monosaccharides released during the hydrolysis of *N. salina* were glucose [61, 124] and not all monosaccharides could be consumed and converted by the fermenting microorganisms. Yeast available nitrogen concentrations in the hydrolysates were measured to calculate the required ammonium sulfate concentrations for the reference medium. Phosphate ion concentrations in the hydrolysates were measured to calculate the required amounts of potassium sulfate. Required C/N for anaerobic yeast growth producing ethanol is 8.5. C/N ratios in hydrolysates were lower than 8.5, which resulted in less efficient fermentation. Each hydrolysate and its reference medium had the same C/N ratio, but higher

growth was observed on most of the hydrolysates. Higher growth on the hydrolysates than on the reference media was observed for most of the studied conditions. This shows that LEAB hydrolysates do not contain inhibitors to the extent that can negatively affect the growth and the hydrolysates may contain more nutrients (like minerals and minor nutrients) than the reference medium.

Carbohydrates are not the only group of compounds required for optimal growth and most microorganisms need nitrogenous compounds for successful fermentation [133]. Nitrogen limitation is usually the main reason for a sluggish fermentation [134] and therefore providing free amino nitrogen (FAN) will improve the fermentation efficiencies and will shorten fermentation time [133]. Proteins remained in LEAB can serve as nitrogen source to increase biomass production and/or improve ethanol fermentation. Residual proteins and glycoproteins must be hydrolyzed to release FAN, which can be assimilated by microorganisms. FAN consists of di- and tripeptides, and primary amino nitrogen from free amino acids [135]. Depending on the carbohydrate hydrolysis treatment, a separate protein hydrolysis may be needed. If the hydrolysis is an acid treatment, it is thought that it hydrolyses both carbohydrates and proteins but for an enzymatic hydrolysis, a separate enzyme activity is needed to increase the FAN. Protease treatment of the LEAB enzymatic hydrolysis resulted in higher growth for both of the studied yeasts compared to the non-protease treatment; protease treatment resulted in a significant increase in biomass yield of JAY270 compared to the non-protease treatment. This proves the hypothesis that more FAN in the fermentation medium improves the fermentation.

Of the studied microorganisms for ethanol fermentation of LEAB hydrolysates, JAY270 had the highest ethanol yields while *Z. mobilis* had the lowest for most of the studied cases. JAY270 is naturally adapted to sugar cane fermentation in Brazil and is known for its high ethanol and biomass production [122]. Lower ethanol yields for *Z. mobilis* on hydrolysates and their references may be due to lack of an essential nutrient.

One of the common fermentation inhibitors are salts, which are generated by neutralization of hydrolysates [125, 136]. Salts are inhibitory to the fermenting microorganisms through osmotic stress or ion toxicity [125, 137]. Results showed that the base factor, or in other words the generated salt, has a significant effect on the ethanol yields. The highest ethanol yield was observed for the potassium hydroxide. Using lignocellulosic hydrolysate, Casey et al. showed that salts can be significant inhibitors of

S. cerevisiae because of osmotic stress and ion toxicity [125]. Sreekumar et al. observed that calcium ions enhanced the yield and concentration of ethanol for *Zymomonas mobilis* [126]. These studies suggest that selecting certain bases for neutralization may reduce the negative impact of salts on fermentation. In this study, potassium hydroxide used for neutralization resulted in higher ethanol yields compared to sodium hydroxide and ammonium chloride.

It was shown that LEAB hydrolysate could be used with no additional nutrients as a suitable medium for ethanol fermentation. Our results are in accordance with literature results. The highest ethanol yield was observed with JAY270 on the sulfuric acid treatment 0.12 g ethanol/g LEAB. Reported ethanol yield from lipid-extracted *Chlamydomonas reinhardtii* was 0.24 g ethanol/g LEAB [138]. It should be noted that the carbohydrate content of *Chlamydomonas reinhardtii* was about two times higher than our *Nannochloropsis salina*. Ethanol production from *Chlorococum sp* resulted in 0.38 g ethanol/ g LEAB, but this was with added nutrients to the hydrolysate [80]. A yield of 0.14 g ethanol/ g LEAB was reported from *Dunaliella tertiolecta* [86]. A review by Doan et al. reported a range of ethanol yield about 0.011 to 0.52 g ethanol/g dried algae biomass depending on the species of the microalgae [139].

To intensify the process and increase ethanol productivity, a continuous fermentation approach was adapted. The higher cell concentration in the continuous system results in the increased productivity over batch systems. The titer of ethanol production in batch fermentation is limited by the ethanol tolerance of the microorganism. In a continuous process, this problem can be overcome by removal of the ethanol and introduction of fresh feed to the reactor. The downtime associated with batch fermentation is not an issue in the continuous fermentation. Productivity in the continuous fermentation is limited by the growth characteristics of the microorganism since at high flow rates, the dilution rate exceeds the maximum specific growth rate of the microorganism and washing the culture from the reactor happens [140]. Immobilized cell reactor (IMCR) allows dilution rates higher than specific growth rate of the microorganism and has higher density of cells, which allows higher conversions. Advantages of IMCR are dense cell populations, high productivity, and high resistance to toxic chemicals [140]. We studied continuous fermentation and compared the performance of a CSTR to an IMCR for LEAB ethanol fermentation. To our knowledge, this is the first study that uses algal biomass hydrolysate in continuous ethanol fermentation with no added nutrients.

Biological reactions are mostly slow and selection of the most efficient reactor is an important task [140]. This applies to ethanol fermentation from biomass. To decrease the cost of ethanol fermentation and increase ethanol productivity, one strategy is continuous fermentation. Some of the advantages of continuous fermentation over batch are higher conversion rates and faster fermentation rates. Continuous fermentation has higher volumetric efficiency due to increased yeast cell concentration compared to batch systems [141]. The other strategy to increase productivities is process intensification by using immobilized cell column. Immobilizing yeast provides high cell densities, which, in combination with high flow rates, leads to short residence times and increased productivities. Advantages of immobilized cells are longer operating lifetime, lower operating cost, less inhibition (also include ethanol inhibition), higher volumetric productivity, and higher ethanol concentration in the outlet.

In this study, continuous fermentation in a chemostat was compared to the immobilized cell reactor. The highest ethanol productivity for the IMCR was 40 g/ L. h , but only about 40% of glucose gets converted at this flow rate and about 60% of the glucose remains unconverted in the effluent of the reactor. For optimal operation of the IMCR, the system will be operated at flow rates with reasonable ethanol productivities and glucose conversions. The highest ethanol productivity for the CSTR was about 1 g/ L. h. This proves our hypothesis that an IMCR achieves higher productivities than the CSTR. We showed that LEAB hydrolysate can be converted to ethanol in an IMCR with 40 times higher productivities than the CSTR.

In the process configuration described in this dissertation, separate hydrolysis and fermentation (SHF) was used. In SHF, hydrolysis and fermentation are performed in separate reactors and at their optimal process conditions. The capital cost associated with SHF is high due to having separate reactors, but the advantage of SHF is better conversion rates. To lower the cost, one strategy is simultaneous saccharification and fermentation (SSF). In SSF, both hydrolysis and fermentation are performed in one reactor under optimal conditions for fermentation. The disadvantage of SSF is incomplete conversion of biomass to sugars due to lower temperature and pH in the reactor. One recommendation is to compare process economics and LCA of LEAB conversion into ethanol via SHF and SSF. SSF is predicted to have lower water and energy requirements for the reactor, but at the price of lower biomass conversion.

We developed an integrated process for conversion of lipid-extracted algal biomass to ethanol. Each step was carefully studied and necessary optimizations were performed. The challenges associated with each step were addressed. This study proved that LEAB hydrolysate has the potential to be converted into ethanol with no added nutrients.

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