Conversion of lipid-extracted *Nannochloropsis salina* biomass into fermentable sugars

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Abstract

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 °C for 5 hours, while the maximum sugar release rate was obtained with 10% hydrochloric acid under the same conditions. 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.

Keywords: Lipid-extracted algal biomass, *Nannochloropsis salina*, hydrolysis, carbohydrate

1. Introduction

Concerns over high atmospheric greenhouse gas levels and issues associated with fossil fuel extraction are among the incentives for developing alternative energy resources [1]. Biofuels, produced from a wide range of feedstocks, have the potential to reduce greenhouse gas emissions [2-4]. In particular, the use of microalgae as a feedstock has received a high level of interest in recent years [5, 6].

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 [2, 6, 7]. Compared with terrestrial plants, the advantages of using microalgae as a potential source of fuels include the ability to use non-fertile land [8], 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 [6].

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 [9, 10].

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 [11-14] and fertilizers [12-14]. In addition, recycling of the nutrients contained in LEAB for subsequent algal cultivations has also been proposed [15].

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 [16] and to bio-oils by slow pyrolysis [14] and hydrothermal liquefaction [14]. LEAB has also been converted to hydrogen [11] and lactic acid [17] by fermentation. To facilitate fermentation, several methods have been used for conversion of biomass to fermentable substrates, including alkaline and thermo-alkaline conditions [11], acidic conditions [17], and sonication [18]. Little has been published regarding bioconversion of LEAB with no prior treatment [16], or adding it as a sole carbon source to the fermentation medium [16].

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 [19], and may change with growth conditions [20]. Sugars and other small molecules are readily available for fermentation, and starch [21] 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 [20, 22], cultivation phase [20], growth conditions [22, 23], and lipid extraction process [11].

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 [24]. 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 [24, 25].

2. Materials & Methods

2.1. Reagents

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

2.2. 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 in a column, and the extracted biomass was allowed to drain. When drainage ceased, residual hexane was removed from the LEAB with either nitrogen or carbon dioxide gas in a downflow mode. The amount of inert gas used varied by test. No hexane odor was detected when biomass arrived. The LEAB was received as dry biomass and was stored at 4 °C until use.

2.3. 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), 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.

2.4. 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 °C; 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.

2.5. Enzymatic hydrolysis

Commercially available enzymes (pectinase) and enzyme mixtures (Accellerase 1500, Accellerase XC, and 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 [26], 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 °C. 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 °C, 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 °C 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.

2.6. 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 [27]. 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 [28] 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 [27] 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 1). The MBTH method was performed according to the published protocol [29]. 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 [30].

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 μ m 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 [31]: 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.

2.7. 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 [32].

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 2.2 and 2.4, 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 °C on a multiple position stirrer (Corning, USA) and samples for OD measurements and HPLC analysis were taken at 0, 24, and 48 h.

2.8. 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 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 °C, 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.

2.9. 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. Results

3.1. Treatment severity

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

$$CSF = \log \left(t \cdot \exp \frac{(T_H - 100)}{14.75} \right) + |pH - 7| \quad (1)$$

where t is reaction time (min), T_H is the reaction temperature ($^{\circ}\text{C}$), and 100 is the reference temperature ($^{\circ}\text{C}$) [33]. The combined severity factors for different treatments are shown in Table 1. The reaction time used for calculating CSF values for enzymatic hydrolysis and one-step acid hydrolysis was 300 min. The CSF value for the two-step acid hydrolysis was calculated as the sum of the CSF values for the two steps. The duration of the first step was 60 min and the second step was 65 min.

3.2. 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

provided the optimal conditions and revealed interactions between studied factors. The experimental design and the levels of studied factors including the response for the two-step optimization are reported in Supplementary Materials, Table S-1.

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 (Supplementary Materials, Figure S-1). ANOVA results for the optimization are presented in Table S-2. The optimal response (sugar yield) is predicted to occur when the second step is performed with 4% H_2SO_4 at 112 °C for 65 min. Using these conditions, the total sugar release yield and rate for the two-step hydrolysis were determined (Table 1). The conditions used for this hydrolysis were the optimized conditions and the analysis was performed with the MBTH assay. The CSF value for the two-step acid hydrolysis was the highest of all of the methods.

3.3. 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 (Supplementary Materials, Figure S-2). Increased biomass loading did not improve the yield with either acid when the concentration of hydrolyzing agent was kept constant (Figures 1 and S-3). In contrast, increased acid concentration resulted in increased sugar yield (Figures 1 and S-3). 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 1 for all one-step acid hydrolysis treatments. Results for hydrochloric acid confirmed that the reaction was completed within 5 h. Sugar yields and rates were improved by increasing the temperature while keeping the acid concentration constant (Figure 2). The same trends were observed for sulfuric acid hydrolysis (Supplementary Materials, Figure S-4).

3.4. Enzymatic hydrolysis

Response surface methodology was used to determine the effect of the enzyme mixture composition on the yield of sugars (Supplementary Materials, Table S-3 and Figure S-5). 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 μ L Accellerase 1500, 5 μ L Accellerase XY, 12.5 μ L Accellerase XC, and 153 μ L pectinase. A time-course experiment with this optimal enzyme mixture revealed that enzymatic deconstruction of LEAB stopped within 5 h (Figure 3). Yield and rate data for enzymatic sugar release are presented in Table 1.

3.5. Evaluation of inhibitors

Hydrolysates were assayed by HPLC for the presence of common fermentation inhibitors (Table 3). 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.

3.6. 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 2. No other nutrients were added to the hydrolysates and only the pH was adjusted. Strain JAY270 grew on all of the tested hydrolysates except for the two one-step acid hydrolysis treatments with 10% HCl. 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.

4. Discussion

4.1. 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 [27, 30], insensitivity to some algae-specific monosaccharides [30], lack of specificity (e.g., between mono- and disaccharides), and poor detection limit [27]. 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 [27, 30]. 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 [27]. 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 ($-\text{CH}=\text{O}$) as in sugars [27].

The only monosaccharide that was detected by the HPLC method was glucose and this was in accordance with the findings of Gerken et al. [34] for the related alga, *N. 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.

4.2. 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 1 and 3) 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 [26]. 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 [33]. 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 1 and 3, 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 4 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 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.

Recently Talukder et al. [17], 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 [35]. Sreekumar et al. observed that calcium ions enhanced the yield and concentration of ethanol for *Zymomonas mobilis* [36]. These studies suggest that selecting certain bases for neutralization may reduce the negative impact of salts on fermentation.

4.3. 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 [34]. The ability of these non-specific enzymes in achieving about 35% of the maximum sugar yield (Table 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 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 [37].

4.4. 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). The inhibitory compounds are generally grouped into furan derivatives [38], weak acids, and phenolic compounds, while salts from neutralization have also been listed as inhibitors [35].

In this study, the presence of common inhibitors such as furans, sugar degradation products, and weak acids was examined (Table 3). Since LEAB has no lignin, phenolic compounds were not expected [39]. 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 [40] 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.

4.5. 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 [41] and the polysaccharide composition of the cell wall varies based on algal taxa [42]. 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.

4.6. Conclusions

We studied different cell wall carbohydrate deconstruction techniques for lipid-extracted *Nannochloropsis salina* and developed hydrolysis treatments with no pretreatment step. 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. A yeast strain grew on all of the generated hydrolysates except for those produced by the one-step hydrolysis with 10% hydrochloric acid. Thus, the LEAB hydrolysates generated by several of the treatments studied here can be used as the fermentation media for the production of biofuels and bioproducts.

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Tables

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

Hydrolysis treatment	Yield of released sugar $\pm$ SD (mg sugar/g LEAB)	Maximum sugar release rate (mg sugar/g LEAB·h)	Combined severity factor	Comments
2-step sulfuric acid	192.3 $\pm$ 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 $\pm$ 10.8	56.8	7.3	Neutralization required
10% HCl, 60 °C	130.6 $\pm$ 14.6	34.5	8.6	5 h reaction time
1% HCl, 90 °C	105.4 $\pm$ 25.3	37.5	8.0	BL=5%
10% HCl, 90 °C	164.6 $\pm$ 2.1	272.3	9.1	
1-step sulfuric acid				
1% H ₂ SO ₄ , 60 °C	15.0 $\pm$ 1.9	11.3	6.2	Neutralization required 5 h reaction time

10% H ₂ SO ₄ , 60 °C	49.6±2.8	39.7	7.9	BL=5%
1% H ₂ SO ₄ , 90 °C	37.4±10.7	43.4	7.9	
10% H ₂ SO 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 2. 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 °C	+
HCl 10%, 60 °C	0
HCl 1%, 90 °C	++
HCl 10%, 90 °C	0
H ₂ SO ₄ 1%, 60 °C	+
H ₂ SO ₄ 10%, 60 °C	+
H ₂ SO ₄ 1%, 90 °C	+
H ₂ SO ₄ 10%, 90 °C	+
Two-step sulfuric acid	+
Optimal enzyme mixture	++

Table 3. 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 $\pm$ 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 °C, 5 h	N D	N D	ND	ND	2.75 $\pm$ 0.09	0.14 $\pm$ 0.00	ND	ND	N D	N D
HCl 10%, 60 °C, 5 h	N D	N D	1.36 $\pm$ 0.05	0.05 $\pm$ 0.00	6.51 $\pm$ 0.75	0.26 $\pm$ 0.03	ND	ND	N D	N D
HCl 1%, 90 °C, 5 h	N D	N D	0.35 $\pm$ 0.07	0.05 $\pm$ 0.00	3.97 $\pm$ 0.38	0.19 $\pm$ 0.02	0.22 $\pm$ 0.04	0.03 $\pm$ 0.00	N D	N D
HCl 10%, 90 °C, 5 h	N D	N D	6.39 $\pm$ 0.65	0.24 $\pm$ 0.02	8.26 $\pm$ 1.36	0.32 $\pm$ 0.05	0.98 $\pm$ 0.14	0.04 $\pm$ 0.01	N D	N D
H ₂ SO ₄ 1%, 60 °C, 5 h	N D	N D	ND	ND	2.09 $\pm$ 0.21	0.1 $\pm$ 0.01	ND	ND	N D	N D
H ₂ SO ₄ 10%, 60	N D	N D	ND	ND	4.86 $\pm$ 0.21	0.2 $\pm$ 0.01	ND	ND	N D	N D

°C, 5 h										
H ₂ SO ₄ 1%, 90 °C, 5 h	N D	N D	ND	ND	4.06±1.01	0.2±0.05	ND	ND	N D	N D
H ₂ SO ₄ 10%, 90 °C, 5 h	N D	N D	2.22±0.12	0.09±0.0 0	6.15±0.70	0.25±0.0 3	ND	ND	N D	N D
Two-step sulfuric	N D	N D	14.59±0.5 3	0.05±0.0 0	18.18±0.7 3	0.06±0.0 0	ND	ND	N D	N D
Biomass blank treated at 60 °C, 5 h	N D	N D	ND	ND	1.19±0.01	0.06±0.0 0	ND	ND	N D	N D
Biomass blank treated at 90 °C, 5 h	N D	N D	ND	ND	1.22±0.01	0.06±0.0 0	ND	ND	N D	N D
Enzymati c Hydrolysi s, 50 °C, 5 h	N D	N D	ND	ND	2.73±0.90	0.14±0.0 5	ND	ND	N D	N D

Figure captions

Figure 1. Effects on sugar yield on acid concentration (AC) and biomass loading (BL) for one-step sulfuric acid hydrolysis at 60 °C and 24 h of reaction. Error bars indicate +/- one standard deviation.

Figure 2. Sugar yield as a function of time for one-step hydrolysis with hydrochloric acid at 60 °C and 90 °C, 5% biomass loading, and 10% acid concentration. Error bars indicate +/- one standard deviation.

Figure 3. Sugar release rate by the optimal enzyme mixture at 50 °C, pH 4.8, with 1.5% (■) and 5% (●) biomass loadings. Error bars indicate +/- one standard deviation.

Figure 4. Correlation of sugar release yield and maximum sugar release rate with the combined severity factor for one-step acid hydrolysis.

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