Enzymatic Saccharification of Dilute Acid Pretreated Saline Microalgae, Nannochloropsis Oculata

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Abstract—In this study the saccharification potential of a marine microalgae, N. oculata, was evaluated by employing conventional techniques of hydrolysis and saccharification often used for lignocellulosic biomass. N. oculata was first hydrolyzed using dilute acids viz 5\% (v/v) sulfuric acid, and 5\% (v/v) and 2\% (v/v) phosphoric acid at 160 °C followed by enzymatic saccharification using commercial cellulases, EI or EII. Neither dilute acid hydrolysis nor enzymatic saccharification alone released any sugars. However, hydrolysates after acid hydrolysis were readily saccharified on addition of enzymes EI or EII. The extent of saccharification ranged between 8 and 100\% in all experiments. Sulfuric acid hydrolysis produced furfurals whereas no side products were detected after phosphoric acid hydrolysis. Maximum sugar yield using EI was 345 g sugars/kg ash free dry matter (afdm) within 4 hours whereas EII yielded 360 g sugars/kg afdm within 12 hours. Twice of the nominal enzyme loading facilitated 35\% more sugar release and half the nominal enzyme loading yielded 64\% less sugars. It was concluded that conventional dilute phosphoric acid hydrolysis followed by enzymatic saccharification using commercially available enzymes could be efficient for saccharification of marine microalgae.

Keywords—Biofuels, Nannochloropsis oculata, cellulase, saccharification, microalgae, acid hydrolysis

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Abstract—In this study the saccharification potential of a marine microalgal, *N. oculata*, was evaluated by employing conventional techniques of hydrolysis and saccharification often used for lignocellulosic biomass. *N. oculata* was first hydrolyzed using dilute acids viz. 5% (v/v) sulfuric acid, and 5% (v/v) and 2% (v/v) phosphoric acid at 160°C followed by enzymatic saccharification using commercial cellulases, EI or EII. Neither dilute acid hydrolysis nor enzymatic saccharification alone released any sugars. However, hydrolysates after acid hydrolysis were readily saccharified on addition of enzymes EI or EII. The extent of saccharification ranged between 8 and 100% in all experiments. Sulfuric acid hydrolysis produced furfurals whereas no side products were detected after phosphoric acid hydrolysis. Maximum sugar yield using EI was 345 g sugars/kg ash free dry matter (afdm) within 4 hours whereas EII yielded 360 g sugars/kg afdm within 12 hours. Twice of the nominal enzyme loading facilitated 35% more sugar release and half the nominal enzyme loading yielded 64% less sugars. It was concluded that conventional dilute phosphoric acid hydrolysis followed by enzymatic saccharification using commercially available enzymes could be efficient for saccharification of marine microalgae.

Keywords—Biofuels, *Nannochloropsis oculata*, cellulase, saccharification, microalgae, acid hydrolysis

I. INTRODUCTION

Research efforts are being devoted to biofuels (aspects related to both feedstock and fuel production) due to emerging concerns about global warming and climate change. Even though ethanol is being produced successfully at commercial scale using corn starch or sugarcane juice as feedstock, this approach is not sustainable as it diverts resources from food and feed production to fuel production. The other option is to produce ethanol from agricultural residues or energy crops (terrestrial and aquatic) grown in non-agricultural, marginal lands and wastewater. A considerable amount of work has been done on the simultaneous saccharification and fermentation (SSF) of macro algae or aquatic biomass like spirogyra, sea lettuce, invasive algal feedstock, etc. for ethanol production [1, 2]. These studies promise high yields of ethanol from macroalgae owing to high composition of carbohydrates [1]. Compared to macroalgae, microalgae usually have higher growth rates and lower nutrient demands for growth [3]. Therefore, microalgae could be a preferred biomass for fuel production and they can also be cultivated to be rich in lipids and carbohydrates [4]. Utilization of algae for ethanol requires saccharification of the carbohydrate content followed by fermentation. With terrestrial biomass, saccharification is accomplished by a pretreatment process like acid hydrolysis, steam explosion or heat treatment followed by enzymatic hydrolysis [5]. There are only a handful of studies dealing with enzymatic saccharification of freshwater microalgae as listed in Table 1. These studies utilize commercial and bacterial enzymes for saccharification. From commercial enzyme saccharification literature, only one study demonstrates acid hydrolysis pretreatment (carried out at 121°C with dilute sulfuric acid) before enzymatic saccharification of mixed undefined microalgae culture [6]. Rest of the studies involving both commercial and bacterial enzymes are subjected to direct enzymatic hydrolysis without any pretreatment and uses sulfuric acid to bring down the pH as shown in Table 1.

In the present study, the saccharification potential of a defined culture of marine microalgae, *N. oculata*, was studied. Saccharification was carried out by using acid hydrolysis pretreatment before enzyme hydrolysis. Acid hydrolysis using different concentrations of phosphoric acid was compared with sulfuric acid. Phosphoric acid was tested because it is milder than sulphuric acid on materials used for construction of off-the-shelf process equipment and produces less inhibitors for fermentation [7]. Commercial cellulase developed for lignocellulosic biomass hydrolysis was tested for saccharification of *N. oculata* and its carbohydrate conversion is studied with different enzyme loading rates. The objectives of this study were to determine an optimal saccharification procedure for *N. oculata* using commercially available enzymes and to compare it with studies done previously with other algal biomass.

II. MATERIALS AND METHODS

A. Feedstock

*N. oculata* culture was cultivated in open raceways at Tyndall Air Force Base (AFB), Florida, at a concentration of 0.8-1 g/L [8]. *N. oculata* was dewatered to a thick slurry of 8% solids. This was accomplished as follows: potassium hydroxide was added to increase pH to 10.8 so as to settle the biomass overnight followed by filtration of settled sludge using a cheese cloth. A one gallon batch of dewatered *N. oculata* was shipped overnight in coolers to Bioprocess Engineering Research Laboratory at University of Florida. On receiving the shipment, *N. oculata* was stored in a chamber at a temperature of 5°C. The batch was well mixed prior to withdrawing samples for saccharification experiments. pH of...
the feedstock was 10.8. The salt content of the algae slurry was 3.5% and no salt removal was done prior to pretreatment or saccharification experiments. The carbohydrate, lipid and protein content of the algae paste was analysed by AFB.

pH of each batch of *N. oculata* for dilute hydrolysis was brought down to 5 by adding dilute acids. 5% (v/v) sulfuric acid pretreatment (referred as 5% H<sub>2</sub>SO<sub>4</sub>) is addition of 5% (v/v) sulfuric acid solution in *N. oculata* till pH reaches 5. Similarly 5% (v/v) phosphoric acid pretreatment (referred as 5% H<sub>3</sub>PO<sub>4</sub>) and 2% (v/v) phosphoric acid pretreatment (referred as 2% H<sub>3</sub>PO<sub>4</sub>) are addition of 5% (v/v) and 2% (v/v) phosphoric acid solution in *N. oculata* respectively.

**B. Ash free dry matter (AFDM) analysis**

The algae paste was analyzed for dry matter, volatile solids and ash content. Dry matter and ash analysis was done by conventional standard method of drying the solids [9]. Total Solids (TS) were determined after drying the wet sample overnight at 105°C. The dried sample was burned at 550°C in a muffle furnace for 2 h to determine the Volatile Solids (VS) content and the AFDM of algae.

**C. Pretreatment**

A Mathis dye beaker apparatus, type number BFA24 manufactured by LabOMAT, Oberhasli, Zurich, was used for acid pretreatment at 160°C and 5 bar pressure. Pretreatment duration was set at 90 minutes for all experiments except for 2% phosphoric acid pretreated samples. For these samples pretreatment duration was 30, 60 and 90 minutes to investigate the effect of duration on saccharification. For acid treatment dilute sulfuric acid or phosphoric acid was added to the sample, this decreased pH of sample to 5. Control pretreatments without acid addition was also carried out.

**D. Enzyme catalyzed saccharification**

Two commercial cellulases used for saccharification experiments in this study were Accellerase Trio (EI) from Genencor and Cellaic CTEch2 (EII) from Novozymes. The nominal loading of EI and EII for optimum hydrolysis was 0.25 ml/g AFDM and 0.05 ml/g AFDM respectively as stipulated by suppliers. Since enzyme activity at saline condition was unknown [10], it was first verified that cellulase activity of both EI and EII was not affected by high salt content. These were verified in separate experiments using cellulose powder as substrate.

Enzyme saccharification experiments were conducted at 50°C in 500 ml glass flasks using different loadings of enzymes EI and EII. It was ensured that 4 g AFDM of algae was used in each experiment. Experiments were done using nominal, half the nominal and twice the nominal dosage of EI and EII. These flasks will be referred to as reaction flasks henceforth. The pH was all reaction mixtures was 5. For thermal pretreated samples without acid addition pH was adjusted to 5 before enzyme reaction. The following treatments were used as controls. For control run I, a batch of algae without pretreatment or enzyme addition was allowed to sit in the reaction flasks at 50°C and pH 5 for 12 hours. Control run II was done with only enzyme additions without acid pretreatment. For control run III algae samples were pretreated at 160°C without acid addition followed by enzyme treatment. Two replications of all control runs and three replications of each experiment were performed.

All glassware were autoclaved at 120°C for 30 minutes to minimize any contamination. The reaction flasks were then kept in an incubator shaker set at 50°C and 350 rpm. pH was maintained between 4.8 to 5 during enzyme saccharification without further addition of acid or base. Samples were withdrawn at 4 hours and 12 hours, centrifuged for 1 min at 14000 rpm and supernatant was filtered with 0.2 µm filter paper and instantly diluted 10 times for 3,5 Dinitrosalicylic acid (DNS) analysis.

DNS method was used for measuring reducing sugars. A calibration curve was made using 0.4, 0.8, 1.2, 1.6 and 2.0 mg/ml glucose concentration saline (3.5%) solutions. 1 ml of diluted samples were heated with 2 ml of DNS reagent at 100°C for 10 minutes. After cooling the sample to room temperature and mixing 2 ml of deionized water, optical density was measured in spectrometer at 580 nm. It was verified that DNS method was not hindered by the presence of salt. Glucose, sucrose, cellobiose, xylose, galactose, arabinose, mannose, fructose, furfural and hydroxymethyl furfural (HMF) concentrations in reaction mixtures was also measured using High Performance Liquid Chromatography (Agilent1200HPLC) [11]. The extent of conversion of carbohydrate in microalgae was reported as grams sugar released per kilogram AFDM of algae. The percentage conversion was calculated as a ratio of grams of sugar released per gram of carbohydrate initially loaded in the reaction mixture multiplied by 100.

**III. RESULTS**

**A. Ash Free Dry Matter (AFDM) and composition of *N. oculata***

Algae samples received contained 7.75% dry matter and 30.45% ash free dry matter (AFDM). That means the AFDM in the dewatered algae sample was 2.36% (w/w). The samples contained on average 25% carbohydrate, 18% lipids and 35% proteins. About 170 ml of dewatered algae was used in each experiment to provide 4 g AFDM and so the amount of carbohydrate initially loaded in the reaction mixture was 1 g.

**B. Control runs for saccharification of *N. oculata***

Control run I, II and III released 1.5 g sugars/ kg AFDM (0.6% carbohydrates conversion), 3.1 g sugars/ kg AFDM (1.2% carbohydrates conversion) and 11.4 g sugars/ kg AFDM (4.6% carbohydrates conversion) respectively. The sugar released after acid hydrolysis was 2.5 g sugars/ kg AFDM (1% carbohydrates conversion). The final sugar results
reported after enzymatic saccharification of hydrolysate would include this initial sugar content. The sugar content of enzyme EI and EII were measured as 0.36 and 0.29 g/ml respectively. The sugars measured after enzymatic hydrolysis were corrected and reported after subtracting the sugars contributed by enzyme.

### C. Dilute acid hydrolysis

1) Enzyme EI: As shown in Table 2, enzyme hydrolysis with EI after 5% H$_3$SO$_4$ pretreatment, released 88.6 g sugars/kg AFDM (35.95% carbohydrate conversion) in 4 hours and 29.5 g sugars/kg AFDM (12% carbohydrate conversion) in 4 hours for nominal and half the nominal loading of enzyme respectively. The sugar release dropped to 50.3 sugars/kg AFDM (23.9% carbohydrate conversion) in 4 hours on doubling the enzyme loading. The drop in sugar concentration could be due to contamination in the reaction system. The saccharification reaction was done for 12 hours and maximum sugar release in the reaction setup was attained in 4 hours and maintained thereafter.

With 5% H$_3$PO$_4$ pretreatment, nominal and half the nominal enzyme loading yielded 99.0 g sugars/kg AFDM (40.24% carbohydrate conversion) in 4 hours and 52.1 g sugars/kg AFDM (21.17% carbohydrate conversion) in 12 hours respectively. Double enzyme loading gave higher sugar release of 121.6 g sugars/kg AFDM (49.4% carbohydrate conversion) in 12 hours. 2% H$_3$PO$_4$ pretreatment gave 241.0 g sugars/kg AFDM (97.9% carbohydrate conversion) in 12 hours and 89.3 g sugars/kg AFDM (36.3% carbohydrate conversion) in 12 hours with nominal and half the nominal enzyme loading respectively. Double enzyme loading released 248.0 g sugars/kg AFDM (100% carbohydrate conversion) in 12 hours.

2) Enzyme II: As shown in Table 2, enzyme hydrolysis with EII after 5% H$_2$SO$_4$ pretreatment, released 112.9 g sugars/kg AFDM (45.9% carbohydrate conversion) in 4 hours and 53.8 g sugars/kg AFDM (21.8% carbohydrate conversion) in 12 hours for nominal and half the nominal loading of enzyme respectively. The sugar release increased to 140.7 g sugars/kg AFDM (57.1% carbohydrate conversion) in 12 hours on doubling the enzyme loading. The saccharification reaction was done for 12 hours and maximum sugar release in the reaction setup was attained in 12 hours.

With 5% H$_3$PO$_4$ pretreatment, nominal and half the nominal enzyme loading yielded 153.0 g sugars/kg AFDM (62.2% carbohydrate conversion) in 12 hours and 66.8 g sugars/kg AFDM (27.15% carbohydrate conversion) in 12 hours respectively. Double enzyme loading gave higher sugar release of 195.4 g sugars/kg AFDM (79.3% carbohydrate conversion) in 12 hours. 2% H$_3$PO$_4$ pretreatment released maximum attainable sugars in 4 hours only. Experiments gave 155.0 g sugars/kg AFDM (63% carbohydrate conversion) in 4 hours and 69.5 g sugars/kg AFDM (28.25% carbohydrate conversion) in 4 hours with nominal and half the nominal enzyme loading. Double enzyme loading released 224.0 g sugars/kg AFDM (91% carbohydrate conversion) in 4 hours.

Phosphoric acid pretreatment gave higher sugar release than sulfuric acid pretreatment. 2% loading of phosphoric acid facilitated higher release of sugars than 5% loading. The rate of sugar release was observed to be slower in phosphoric acid than sulfuric acid as maximum sugar release in reaction flask with phosphoric acid was recorded at 12 hours but with sulfuric acid at 4 hours. The difference between sugars released at 12 hours and 4 hours varied from 15% to 89%. Though at 2% phosphoric acid EII gave maximum sugar release at 4 hours. Enzyme EII produced a higher rate of sugar release than EI, irrespective of loading and type of acid. EII showed highest sugar yield among all runs at 2% phosphoric acid. On increasing the enzyme loading, a change between 20-30% in sugar release was observed. Nominal loading could be the optimal loading of commercial enzyme, as it would perform equally well for hydrolysis of microalgae as it is for lignocellulosic biomass.

As shown in Table 3, 30 minutes, 60 minutes and 90 minutes of pretreatment with 2% H$_3$PO$_4$ at 160°C followed by a nominal loading of EII, yields 69 g sugars/kg AFDM (27.8% carbohydrate conversion), 238 g sugars/kg AFDM (95.96% carbohydrate conversion) and 242 g sugars/kg AFDM (97.6% carbohydrate conversion) respectively. This indicates that pretreatment time can be further reduced to 60 minutes to get almost equivalent amount of sugar yield as obtained from 90 minutes pretreatment process.

Trace amount of HMF and cellobiose was produced during saccharification of algae treated with sulfuric acid as compared to undetected levels of these during phosphoric acid pretreatment. In Table 4, concentration of different sugars and by products measured from HPLC analysis is reported and compared to reducing sugars measured from DNS analysis (it should be noted that sucrose is not a reducing sugar). Quantities of undetected sugars are not mentioned in the table.

IV DISCUSSION

*N. oculata* is a unicellular, thick cell walled spherical microalgae. The total carbohydrate composition of *N. oculata* is about 25% of AFDM (or in the dewatered samples used here it was 7.7% of dry matter), out of which 88% is typically polysaccharide. 68.2% of polysaccharide is glucose, rest being fucose, galactose, mannose, rhamnose, ribose and xylose [12]. 35% of AFDM is protein and 18% is lipid. Rest of the composition is amino acids, fatty acids, omega-3, unsaturated alcohols, ascorbic acid. The carbohydrate content can be as high as 26% if grown outdoors [13]. C: N ratio in the *N. oculata* can be controlled by different growth conditions. *N. oculata* has very high productivity and cheaper to grow under saline condition. Hence it is a promising feedstock for commercial biofuel production.

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Algae have a simpler structure as compared to lignocellulosic biomass, but a thick cell wall is responsible for entrapping cellulose and other carbohydrates. The cell wall of chlorophytic phytoplankton (N. oculata) is composed of cellulose fibers distributed within a complex organic matrix[12], which makes cellulose accessibility difficult for enzymes as seen from control run I. A pretreatment is required to break open the cell wall and cellulose becomes accessible to enzyme hydrolysis. Disruption in micro algal cell wall due to acid hydrolysis has been shown in previous studies [14]. Acid hydrolysis could yield 2.5 g sugars/ kg AFDM which equals only 1% of total carbohydrates conversion in N. oculata. As shown in results, N. oculata could not be saccharified with heat, acid or enzyme hydrolysis alone.

In all published studies, algal biomass was subjected to a pretreatment in the form of dewatering, freezing, drying, and in some cases extraction. The carbohydrate conversion varied from 7.5% to 95%. In the present study N. oculata was concentrated only by alkali treatment and was not exposed to any other additional treatment prior to studies conducted here. Some algal biomass like Chlamydomonas reinhardti (microalgae) and spirogyra (algal biomass) can accumulate high starch content via photosynthesis and have simpler cell wall rich in cellulose [15]. They may not require any pretreatment prior to enzymatic hydrolysis. These species result in higher sugar yields and carbohydrate conversion of up to 100% but at the expense of fresh water utilization, high cost and longer growth rates. As shown in Table 1, enzyme hydrolysis of marine micro algae with 53% of carbohydrates could result only in 23.8% carbohydrate conversion in biomass. Another study shows that after ultrasonication pretreatment, enzyme hydrolysis (from fungi derived enzyme) of fresh water species Chlorococcum humicola gives upto 68.2% of carbohydrate conversion[16]. Studies done with micro algal cell wall debris produced after lipid extraction show carbohydrate conversion as low as 7.5% after acid and enzyme hydrolysis [6].

Compared to these published studies, N. oculata gave 100% carbohydrate conversion to sugars after dilute acid hydrolysis followed by saccharification using commercial cellulases. Most common pretreatment technique to improve cellulose digestibility before enzyme hydrolysis is dilute acid hydrolysis. Sulfuric acid is often used in hydrolysis of macro/micro algae, saline crops, starch, cellulosic and woody biomass as it is considered a stronger hydrolyzing agent than phosphoric acid[17]. Sulfuric acid causes dehydration of monosaccharides and the side reactions results in formation of HMF, which could significantly inhibit biological reactions.

Phosphoric acid pretreatment is mild, non-corrosive on process vessels, non-toxic, safe, economic and resulted in no furfural production in microalgae hydrolysis making it a preferable candidate for pretreatment. Cellulobiose indicates the incomplete breakdown of cellulose to simple sugars. HMF is produced due to side reactions in sulfuric acid pretreatment[7].

This explains the lower sugar yield in sulfuric acid pretreatment than phosphoric acid. Phosphoric acid pretreatment studies have been conducted for corn stover biomass. It has achieved only 56 % of hydrolysis as compared to 75 % of hydrolysis from sulfuric acid [18]. Present study compares the performance of dilute acid pretreatment for microalgae with sulfuric acid with phosphoric acid and it indicates microalgae have different behavior for dilute phosphoric pretreatment as compared to lignocellulosic biomass. It was observed phosphoric acid treatment gives equivalent saccharification yield from microalgae as sulfuric acid treatment in lignocellulosic biomass. But our results show that phosphoric acid treatment could give 27% more saccharification in microalgae than sulfuric acid. The sugar released at 4 hours and 12 hours shows an increase of only 15-80%. The short duration for optimum release of fermentable sugars offers the advantages of eliminating contamination, reducing inhibition effects, and making the process economically effective.

5% H₃PO₄ pretreated algae has higher solids loading than 2% H₃PO₄ pretreated algae. Higher substrate loading results in higher viscosity, which in turn increases the content of insoluble materials and thus hinders efficiency of enzyme hydrolysis. This explains higher saccharification resulted from microalgae treated with 2% H₃PO₄ than 5% H₃PO₄.

Cellulases are being commercially produced for specifically breaking down plant cellulose to sugars in lignocellulosic biomass. The hydrolysis studies with commercial enzymes shows higher yields and faster kinetics of sugar release, easy to use and is apt for commercial applications [19]. There are only a handful studies of enzymatic hydrolysis of fresh water algae (macro and micro) from cellulase but none with saline microalgae. This study throws light on feasibility, optimization and possible scale up applications of enzymatic hydrolysis of a defined marine microalgae culture by a commercial enzyme, cellulase. Commercial enzymes having defined optimized working conditions and loading rates for cellulosic biomass was used for enzymatic hydrolysis of microalgae in present study. Enzymes hydrolysis experiments were conducted with nominal, half and double the nominal dosage to optimize the loading rates of cellulase for N. oculata. The results show that half the nominal enzyme loading performs poorer than nominal loading and double the enzyme loading gives only 34% higher sugar release than nominal enzyme loading. Hence concluding it that cellulase performance for N. oculata is similar to that for lignocellulosic biomass.

EI and EII are two commercial enzymes, which targets cellulose, hemicellulose and cellulobiose for degradation. In the recommended dosage, EII enzymes have high concentration and stability, higher conversion yields and more tolerant towards inhibitors. EII had 5 times less recommended loading than EI and works efficiently in the high solids concentration, which ensures higher sugar release per batch of biomass.
100% carbohydrate conversion to sugars was observed when treated with 2% phosphoric acid at 160°C for 60 minutes and subsequently hydrolyzed by commercial enzyme EII at nominal loading without addition of any buffer in the reaction flasks. Increase in sugar release was observed with increased dilution.

Ongoing work focuses on optimization of phosphoric acid loading and duration of thermal hydrolysis for 100% saccharification.

<table>
<thead>
<tr>
<th>Algae</th>
<th>Type</th>
<th>Pretreatment (duration)</th>
<th>Carbohydrate (% DM)</th>
<th>Sugar released (g/kg DM)</th>
<th>Carbohydrate conversion (%)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spirogyra (macro)</td>
<td>Fresh water</td>
<td>Alkali (2 hours)</td>
<td>64.0%</td>
<td>666</td>
<td>104</td>
<td>[1]</td>
</tr>
<tr>
<td>N. oculata (micro)</td>
<td>Salt water</td>
<td>Phosphoric acid (1 hour)</td>
<td>25.0%</td>
<td>248</td>
<td>100*</td>
<td></td>
</tr>
<tr>
<td>Nitzammudini (macro)</td>
<td>Fresh water</td>
<td>Sulfuric acid (1 hour)</td>
<td>41.5%</td>
<td>70.2</td>
<td>16.3</td>
<td>[20]</td>
</tr>
<tr>
<td>Chlorella vulgaris (micro)</td>
<td>Salt water</td>
<td>None</td>
<td>53.0%</td>
<td>126</td>
<td>23.8</td>
<td>[21]</td>
</tr>
<tr>
<td>Dead micro algae (micro)</td>
<td>unknown</td>
<td>Sulfuric acid (1 hour)</td>
<td>1.00%</td>
<td>60.0</td>
<td>7.50</td>
<td>[6]</td>
</tr>
<tr>
<td>Chlorococcum humicola (micro)</td>
<td>Fresh water</td>
<td>Sulfuric acid (0.5 hours)</td>
<td>32.5%</td>
<td>221</td>
<td>68.2</td>
<td>[14]</td>
</tr>
<tr>
<td>Chlamydomonas reinhardtii (micro)</td>
<td>Fresh water</td>
<td>None</td>
<td>60.0%</td>
<td>570.</td>
<td>95.0</td>
<td>[22]</td>
</tr>
</tbody>
</table>

* present study

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Sugar released (in g sugars/ kg AFDM) at different loadings of enzyme during saccharification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme loading</td>
<td>Duration of enzymatic saccharification (hours)</td>
</tr>
<tr>
<td></td>
<td>5% H2SO4</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>0.5X EI</td>
<td>29.5±3</td>
</tr>
<tr>
<td>1X EI</td>
<td>88.6±3</td>
</tr>
<tr>
<td>2X EI</td>
<td>50.3±3</td>
</tr>
<tr>
<td>0.5X EII</td>
<td>45.1±3</td>
</tr>
<tr>
<td>1X EII</td>
<td>112.9±3</td>
</tr>
<tr>
<td>2X EII</td>
<td>111.2±3</td>
</tr>
</tbody>
</table>
| 1X corresponds to nominal loading recommended by enzyme supplier. 0.5X and 2X corresponds to half and double nominal loadings.

TABLE 4
Concentration of sugars and other by products from treatment with nominal EII loading

<table>
<thead>
<tr>
<th>Component</th>
<th>5% H2SO4</th>
<th>5% H3PO4</th>
<th>2% H3PO4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/L</td>
<td>g/L</td>
<td>g/L</td>
</tr>
<tr>
<td>HMF</td>
<td>0.0363</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.6022</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.59</td>
<td>1.86</td>
<td>2.1</td>
</tr>
<tr>
<td>DNS (reducing sugars)</td>
<td>1.75</td>
<td>1.9</td>
<td>2.12</td>
</tr>
</tbody>
</table>

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ABBREVIATIONS

SSF simultaneous saccharification and fermentation
TS total solids
DM dry matter
VS volatile solids
DNS dinitrosalicicyc acid
HMF hydroxymethyl furfural
AFDM ash free dry matter
HPLC high performance liquid chromatography
EI Accellerase Trio
EII Cellic CTech2