Comparison of different types of pretreatment and enzymatic saccharification of *Macrocystis pyrifera* for the production of biofuel

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**Abstract**

In this work, the brown algae *Macrocystis pyrifera* were pretreated with dilute sulfuric acid, water and three different types of ionic liquids (ILs): 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]), 1,5-diazabicyclo[4.3.0]non-5-ene acetate ([DBNH][OAc]) and 1,8-diazabicyclo[5.4.0]-undec-7-ene-sulfurdioxide–monoethanolamine (DBU–MEA–SO2–SIL), to disassemble the complex polysaccharide structure. After each pretreatment procedure, enzymatic saccharification was performed to release the monosaccharides. The main building blocks of *M. pyrifera* were processed by derivatization via acid methanolysis and subjected to gas chromatographic analysis. It was found that the main constituents were alginate (60.6 wt.%) and cellulose (22.6 wt.%) of total carbohydrate content. The degradation of alginate requires the action of alginate lyase and oligoalginate lyase, which hydrolyze the main chain in a synergistic mechanism releasing uronic acid (unsaturated uronate). Upon saccharification of cellulose, cellulases and β-glucosidase were used allowing the release of glucose. It was found that the best pretreatment strategy for *M. pyrifera* consisted of a pretreatment with 2 vol.% sulfuric acid, followed by saccharification of cellulose with a mixture of cellulases at pH 5.2 for 4 h at 50 °C or by saccharification of alginate with the enzyme lyase/oligoalginate lyase at pH 7.5 for 2 h at 37 °C. The process resulted in a release of 68.4 wt.% of glucose (55.74 ± 0.05 mg glucose/g algae) whereas in the case of alginate 85.8 wt.% of uronic acid (193.7 ± 10.6 mg uronic acid/g algae) was released. To the best of our knowledge this is the first time that saccharification of both cellulose and alginate from brown algae is reported.

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1. Introduction

Algal biomass is considered a good resource of sugars for third-generation biofuel and platform chemicals. In general, algae offer an interesting option to produce sugars for production of bioethanol and platform chemicals unlike other biomass such as corn and bagasse [1]. In addition, algae are characterized for having higher growing rates when compared to terrestrial plants [2]. Earlier pretreatment studies have reported that the high sugar contents of several brown seaweeds can be recovered successfully [3]. An effective pretreatment is necessary to liberate the polysaccharides. Some of the available physical or chemical pretreatment techniques for biomass include acid hydrolysis, steam explosion, alkaline wet oxidation, “green solvents” such as ionic liquids and hot water pretreatment [4]. On the other hand, an enzymatic saccharification process is necessary to liberate the monosaccharides. Therefore, optimal pretreatment and saccharification can increase the amount of fermentable monosaccharides obtained from algae. In the case of brown algae the main polysaccharide is alginate. The saccharification of alginate produces monosaccharides, which can be used to generate biofuels and numerous products of biotechnological interest, such as stabilizers, viscosifiers, and gelling agents for the food, beverage, paper, biomaterials and pharmaceutical industries. Most of the alginate used commercially is obtained from three algae genera: *Macrocystis*, *Laminaria*, and *Asparphyllum* [5]. Alginate or alginic acid is a co-polymer of α-L-guluronate (G) and its C5 epimer β-L-mannuronate (M), being arranged as homopolymetric G blocks, homopolymeric M blocks, alternating GM blocks or random heteropolymeric G/M stretches [6]. The use of enzymes in the saccharification of these polysaccharides is preferred, since enzymatic hydrolysis can be performed under mild conditions, avoiding the accumulation of undesired byproducts and reducing possible environmental contamination. The biodegradation of alginate involving alginate lyase: mannuronate lyase (EC 4.2.2.3) or guluronate lyases (EC 4.2.2.11) cleaves within the chain producing unsaturated uronic acid oligomers with a double bond between C4 and C5 at the nonreducing end. Oligoalginate lyase (EC 4.2.2.2) cleaves these...
oligomers to produce monosaccharides (unsaturated uronate) [7]. Selection of appropriate hydrolytic enzyme and optimal mixture of enzymes are vital to obtain the expected output [8]. These enzymes have been isolated from many sources, including marine algae, marine mollusks, and a wide range of microorganisms, such as bacteria, bacteriophage, marine fungi and viruses [9]. Macrocystis pyrifera, a brown seaweed which grows next to the coasts of Chile and North America, is the largest seaweed on earth, and can reach a length up to 60 m. Underwater, it grows vertically with fronds and bladders around a holdfast, and its foliage forms dense canopies on the sea surface [10]. In addition, M. pyrifera is one of the fastest-growing organisms on Earth. For this reason, we focused our study on M. pyrifera collected from Southern Chile.

The objective of this study was to compare different pretreatments before the enzymatic saccharification with a mixture of cellulases and algines in order to enhance the release of monosaccharides. Particular interest in glucose and uronic acid (unsaturated uronate) as renewable chemical precursors and their possible biotechnological application, such as bioethanol production, was the focus of this work. This is the first time where saccharification of cellulose and alginate from M. pyrifera is performed.

2. Materials and methods

2.1. Algae

M. pyrifera was grown in Chiloé Islands 30 km southeast Puerto Montt, Chile. It was harvested in December 2013 and kindly donated by Professor Buschmann, University of Los Lagos. The algae were collected and dried for 48 h at 60 °C. The algae were cut into 10 to 15 mm long chips and 5 to 10 mm wide chips, with cutoff sieve up to 3.5 mm.

2.2. Characterization of algal biomass

Proximate composition of M. pyrifera was performed by Food Analysis Center, University of Los Lagos: Agribusiness Institute, University of La Frontera according to the method described in Reference [11]. Carbohydrates were calculated as 100% – (Humidity + Protein + Ash + Fatty material). The algae were characterized to determine their carbohydrate content using the acid methanolysis and acid hydrolysis methods.

2.2.1. Acid methanolysis

The alginates and hemicelluloses from M. pyrifera were determined by the acid methanolysis method. 2 ml of methanol reagent containing 2 M of HCl in methanol was added to 10 mg of freeze dried algae samples, as well as a calibration solution containing carbohydrates. Tubes were incubated at 100 °C for 3 h. 200 μl of pyridine was added to neutralize the excess of HCl, and 1 ml of internal standard solutions containing 0.1 mg/ml of sorbitol and resorcinol in methanol, respectively, were added to each sample. After mixing, methanol was evaporated at 50 °C under a nitrogen stream and then further dried in a vacuum oven at 40 °C below 50 mbar for 15 min. Once samples were completely dry, they were silylated by adding 80 μl of pyridine, 170 μl of hexamethyldisilazane (HMDS) and 70 μl of chlorotrimethylsilane (TMCS), and thoroughly mixed using a vortex. Samples were incubated at 70 °C for 45 min, and then clear liquid phase was taken to analyze sugar content in gas chromatograph [13]. These analyses were performed in duplicate.

2.2.2. Acid hydrolysis

The cellulose content from M. pyrifera was determined by the acid hydrolysis method. 200 μl of sulfuric acid 72 vol.% was added to each 10 mg algae sample and 10 mg cellulose powder was used as a standard and placed in a vacuum oven until it reached a pressure below 50 mbar. This step was repeated three times. Then the samples were kept under a fume hood for 2 h, whereupon 2 ml of distilled water was added to each sample. 4 h later 6 ml of distilled water was added and the samples were left under a fume hood overnight at room temperature. The next day samples were placed in an autoclave at 125 °C for 90 min, and then left to cool at room temperature. Two droplets of bromocresol green were added to each sample as an indicator, and then barium carbonate was added to neutralize the samples until the liquid phase turned blue. 1 ml of internal standard containing 5 mg/ml of sorbitol in distilled water was added to each sample, and then centrifuged for 10 min. 1 ml of the liquid phase was taken from each sample and transferred to another test tube, where 1 ml of acetic acid was added. Samples were evaporated under a nitrogen gas stream at 60 °C, and then further dried in a vacuum oven at 40 °C below 50 mbar for 15 min. Once samples were completely dried, they were silylated by adding 80 μl of pyridine, 170 μl of hexamethyldisilazane (HMDS) and 70 μl of chlorotrimethylsilane (TMCS), and thoroughly mixed using a vortex. Samples were incubated at 70 °C for 45 min, and then clear liquid phase was taken to analyze sugar content in gas chromatograph [13]. These analyses were performed in duplicate.

2.2.3. Gas chromatography

About 1 μl of a silylated sample was injected via a split injector (260 °C, split ratio 1:15) into a 30 m/0.32 mm i.d. column coated with dimethyl polysiloxane (HP-1, Hewlett Packard), the film thickness being 0.17 μm. The column temperature program was 100 − 4 °C/min–175 °C followed by 175 − 12 °C/min–290 °C. The detector (FID) temperature was 290 °C. Hydrogen was used as a carrier gas.

2.3. Pretreatment

2.3.1. Dilute sulfuric acid

0.5 g of dry M. pyrifera were pretreated with 1.5 ml of sulfuric acid (2 vol.%) or water. Algae and solvent were introduced into glass vials, placed in a thermostated oil bath at 120 °C for 1 h, this temperature was chosen because previous data at 80 °C and 120 °C showed higher yield at 120 °C (data not shown). After incubation, the tubes were removed from the oil bath and the algae were washed with water six times and dried at 37 °C for 3 days.

2.3.2. Ionic liquid

Three different ionic liquids (ILs) were used: 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]) from Sigma, 1,5-Diazabicyclo[4.3.0]non-5-ene acetate ([DBN][OAc]) [14] and 1,8-diazabicyclo[5.4.0]-undec-7-ene-sulfurdioxide—monoethanolamine (DBU–MEA–SO2–SIL) [15].

Algae and solvent were introduced into glass vials, placed in a thermostated oil bath at 120 °C for 1 h. After incubation, the tubes were removed from the oil bath and the algae were washed with water six times and dried at 37 °C for 3 days.

2.4. Enzymatic saccharification

2.4.1. Saccharification with cellulases

For saccharification with cellulases, samples containing 0.1 g of algae were incubated with a commercial cellulase enzyme complex: cellulases from Sigma (Celluclast; 10 FPU/g algae was determined as described by Ghose [16]) and β-glucosidase from Sigma (10 U/g algae; 1 U of activity was defined as the amount of enzyme required to hydrolyze 1 μmol of p-nitrophenyl-[β-D-glucopyranoside per min at pH 4.0 and 37 °C). The optimum pH was determined using Methylcellulose buffer (citric acid/di-sodium hydrogen phosphate) in a pH range of 4.8 to 7.5, incubating for 4 h at 50 °C. The effect of temperature on enzyme activity was analyzed. The samples were incubated for 4 h in Methylcellulose buffer pH 5.2 at 28.5, 37 and 50 °C. The standard condition for saccharification of cellulose was pH 5.2 at 50 °C under stirring (200 rpm) and then centrifuged. The enzymatic saccharification of algae was performed in triplicate. Quantitation of glucose was performed using the Kit RandoxGluc-PAP.
3. Results and discussion

3.1. Composition of algal biomass

Carbohydrate content in *M. pyrifera* was 37 wt.% based on chemical analysis (proximate composition). Carbohydrate content analyzed after derivatization of samples subjected to acid hydrolysis and acid methanolysis methods was 36 wt.%, which confirms the total value reported by chemical analysis (Table 1). The main carbohydrate components in this *M. pyrifera* are alginate (60.6 wt.%), cellulose (22.6 wt.%) and fucoidan (4.6 wt.%), in accordance with the results reported by other authors for these algae [20,21]. The main component among the carbohydrate in *M. pyrifera* was alginate (Table 1). Total amount of glucose contained in *M. pyrifera* was 81.5 mg glucose/g dry algae, whereas total amount of uronic acids, including alginic, galacturonic acid and glucuronic acid, was 225.7 mg uronic acids/g dry algae.

<table>
<thead>
<tr>
<th>Carbohydrate content (mg/g of dry algae) in <em>M. pyrifera</em></th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate**  **</td>
<td>218.9 ± 27.4</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Fucose</td>
<td>16.7 ± 0.9</td>
</tr>
<tr>
<td>Fructose</td>
<td>ND ± ND</td>
</tr>
<tr>
<td>Galactose</td>
<td>10.7 ± 1.6</td>
</tr>
<tr>
<td>Galacturonic acid</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Glucose*</td>
<td>81.5 ± 10.0</td>
</tr>
<tr>
<td>Glucose (cellulosic)**</td>
<td>76.3 ± 11.3</td>
</tr>
<tr>
<td>Glucose (non-cellulosic)</td>
<td>5.2 ± 1.3</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>6.6 ± 1.3</td>
</tr>
<tr>
<td>Mannitol</td>
<td>16.0 ± 1.6</td>
</tr>
<tr>
<td>Mannose</td>
<td>6.0 ± 0.9</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Xylose</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>361.3 ± 23.8</td>
</tr>
</tbody>
</table>

3.2. Enzymatic saccharification of algal biomasses

Glucose and uronic acid present in *M. pyrifera* are of particular interest as renewable chemical precursors focused this study on enzymatic saccharification with cellulases and algines. First, the algae were pretreated with water for 1 h at 120 °C and then, to determine the optimum conditions in the enzymatic saccharification process, experiments were carried out using cellulases and algines.

3.2.1. Kinetic properties

3.2.1.1. Determination of optimum pH. *M. pyrifera* pretreated with water was incubated with cellulases and β-glucosidase in McIlvaine buffer at different pH. The optimum pH for the enzymes was 5.2 (Fig. 1A) shows the glucose liberated after saccharification for 4 h. The results indicate that the activity of celloses decreased at high pH (Fig. 1A). When the algae were incubated with alginase lyase and oligoalginololate lyase in McIlvaine buffer at a different pH the optimum pH of 7.5 was obtained (Fig. 1B) shows the uronic acids liberated after saccharification for 2 h. The activity of alginic lyase and oligoalginololate lyase were clearly decreased by low pH (pH 5.2). Other alkaline alginates lyases have been characterized [22–24] and oligoalginololate lyase [25,26] but the optimum pH of the mixture of alginic lyase and oligoalginololate lyase was important to determine.

3.2.1.2. The effect of temperature on enzyme activity. *M. pyrifera* pretreated with water was incubated with cellulases and β-glucosidase in McIlvaine buffer pH 5.2. The best temperature condition for these enzymes was 37 °C. Alginate lyase and oligoalginololate lyase was chosen to simulate the one used in the saccharification/fermentation process [27]. The temperatures of 28 and 50 °C were chosen in order to compare the effect of the other enzyme's optimal temperature. In the current study, an enzymatic saccharification strategy has been established and optimized for the production of glucose and uronic acid from brown algae *M. pyrifera*. The enzymatic saccharification was performed separately because the cellulases have better activity with different pH and temperature conditions from the alkagines.

3.3. Pretreatment and enzymatic saccharification under optimal conditions

The brown algae *M. pyrifera* were pretreated with dilute sulfuric acid, water and three different ionic liquids (ILs): [EMIM][OAc], [DBNH][OAc] and DBU–MEA–SO2–SIL, in order to open the complex polysaccharides; after that, enzymatic saccharification was performed to obtain the monosaccharides.

The main purpose of using ionic liquids to treat algae is their known ability to de-crystallize cellulose in forestry biomass, as well as disrupt the lignin and hemicellulose matrix. The aim of using IL for treating algae is to spread the use of these solvents studying the possible improvement of the enzymatic digestibility of IL treated algae. Particularly, brown algae are known to possess high content of carbohydrates, principally alginic and cellulose, which enzymatic digestibility may be improved for using such treatments.

Fig. 3 shows the glucose liberated after pretreatment and saccharification with cellulases. The result shows that the best experimental conditions found for *M. pyrifera* was the pretreatment with 2 vol.% sulfuric acid and saccharification with cellulases mix liberated 55.74 ± 0.05 mg glucose/g dry algae corresponding to 8.4% wt.% of the total amount of glucose. In the enzymatic hydrolysis, there is no great difference in glucose liberation after 4 h when the pretreatment was water or ionic liquids (data not shown). When liquid ion prepretreatments were used, 46.7–37.3% less glucose was released in the enzymatic hydrolysis in...
comparison with dilute sulfuric acid (Fig. 3). These results may be explained because the dilute sulfuric acid has low viscosity compared to ionic liquids. Acid pretreatment destroys the algal cell wall by breaking hydrogen bonds and the low viscosity of dilute sulfuric acid favors diffusion of the solvent through the carbohydrate matrix in algae.

Fig. 4 shows the uronic acid released after pretreatment and saccharification with alginases. The result shows that the best experimental conditions found for M. pyrifera was the pretreatment with 2 vol.% sulfuric acid. Subsequent saccharification with alginase mix liberated 193.7 ± 10.6 mg uronic acid/g dry algae corresponding to 85.8 wt.% of the total amount of uronic acids present in the fresh algae. The pretreatment listed in order of performance of alginate conversion are dilute sulfuric acid > DBU–MEA–SO₂–SIL > [DBNH][OAc] > [EMIM][OAc]. These results may be explained because sulfuric acid has high hydrolytic power, able to cleave hydrogen bonds within the carbohydrate matrix in the algae. In Table S1, supplementary data, compares the yield of uronic acid in the saccharification step compared to solvent pH measured at a nominal 100 g/l concentration in water, where the higher the pH is the better the uronic acid yield obtained. This correlation agrees with the usual industrial conditions at which alginate is extracted, where sodium carbonate at pH 10 is usually used for alginate extraction from brown algae [28,29].

The solvency power of certain ionic liquids is associated with their ability to disrupt the Van der Waals and hydrogen bond interactions within the biomass matrix [3]. Deniaud-Bouët et al. [30] indicated that cellulose is embedded within the alginate network, with only few covalent bonds between these two networks. Alginate is more exposed in the cell wall of brown algae, so for this reason liberation of uronic acids after pretreatment with ionic liquids could be easier compared to cellulose conversion.

[EMIM][OAc] is a well-known IL for its ability to dissolve cellulose and is used for biomass fractionation [31–33]. It has a melting point reported by commercial suppliers around 30 °C, and pH 5.4 at 100 g/l concentration in water, being rather acidic. In terms of its viscosity, it is very variable within a wide range of temperatures as reported by Radhi et al. [34]. They reported a value of viscosity of 2.86 Pa·s at 30 °C and...
0.33 Pa·s at 90 °C, compared to 0.30 mPa·s which is the viscosity of water at 90 °C.

[DBNH][OAc] is prepared in-situ by mixing DBN and acetic acid, forming a solid salt at room temperature. According to thermogravimetric analyses, it has a melting point around 61 °C and decomposition temperature around 176 °C. [DBNH][OAc] has a pH 9.5 at 100 g/l concentration in water, being rather basic. [DBNH][OAc] has been used to prepare strong cellulose by dry jet-wet spinning of previously dissolved cellulose in the IL and also acetylation of xylans [35,36].

DBU–MEA–SO₂–SIL as shown in Supplementary Fig. S1, is prepared by bubbling SO₂ into a mixture containing DBU and monoethanolamine, forming a highly viscous ionic liquid at room temperature. This ionic liquid corresponds to a new family of IL called switchable ionic liquid, where the concept is triggering ionic properties by bubbling an acid gas in the mixture of an organic an organic super base and an alcohol. Ionic liquids containing glycerol as the alcohol (DBU–glycerol–SO₂) have been fully characterized by different methods [14]. DBU–MEA–SO₂–SIL has a pH 11 at 100 g/l concentration in water, being rather...
Table 2: Liberation of glucose from brown algae.

<table>
<thead>
<tr>
<th>Brown algae</th>
<th>Conditions</th>
<th>Glucose yield</th>
<th>Uronic acid yield</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Macrocystis pyrifera</em></td>
<td>Pretreatment: 2 vol.% H₂SO₄ at 120 °C for 1 h</td>
<td>68.4 wt.%</td>
<td>85.8 wt.%</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Saccharification: 10 U cellulases and 10 U β-glucosidase, 3 U alginate lyase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 U oligoalginate lyase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Saccharina japonica</em></td>
<td>Pretreatment: 0.06 vol.% H₂SO₄ at 170 °C for 15 min</td>
<td>84.0 wt.%</td>
<td>Not determined</td>
<td>[41,42]</td>
</tr>
<tr>
<td></td>
<td>Saccharification: 15 FPU cellulase and 70 U β-glucosidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Laminaria japonica</em></td>
<td>Pretreatment: 1 wt.% H₂SO₄ at 120 °C for 30 min</td>
<td>Not determined</td>
<td>28.08 wt.%</td>
<td>[43]</td>
</tr>
<tr>
<td></td>
<td>Saccharification: alginate lyase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Nizamuddin zanardini</em></td>
<td>Pretreatment: 7 wt.% H₂SO₄ at 120 °C for 45 min</td>
<td>70.2 wt.%</td>
<td>Not determined</td>
<td>[44]</td>
</tr>
<tr>
<td></td>
<td>Saccharification: 15 FPU cellulase and 45 U β-glucosidase</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

basic. High ability of lignin solubility have been observed in birch biomass, leaving mainly cellulose and hemicelluloses in the undissolved fraction [37].

In this work, when saccharification was performed without pretreatment, low liberation of glucose (6.4 wt.%) and uronic acid (18.3 wt.%) was observed (Figs. 3 and 4). Consequently, these results confirm the need of pretreatment for *M. pyrifera* biomass to achieve alginate hydrolysis during saccharification, with an increase in glucose liberation up to 4.68 fold compared to saccharification of algae when no treatment is performed.

Maximum sugar recovery during acid pretreatment has been achieved by combining pretreatment time; temperature and acid concentration [38,39]. Elevated temperatures caused decomposition of glucan to formic acid via glucose and resulted in formation of degradation products. In some cases, degraded products in pretreated hydrolysate are removed prior to saccharification, fermentation to avoid process inhibition. Activated charcoal was used to remove hydroxymethyl furfural after subjecting *Kappaphycus alvarezii* to dilute acid pretreatment [40]. In this paper, the algae were washed for removing possible degraded products before enzymatic saccharification.

In this study, the liberation of glucose and uronic acid during this treatment of *M. pyrifera* with cellulases and alginate liberated high amounts of glucose and uronic acid: 68.4 wt.% (55.74 mg glucose/g dry algae) and 85.8 wt.% (193.7 mg uronic acid/g dry algae), respectively. Table 2 shows glucose yield from different brown algae using pretreatment with H₂SO₄ and enzymatic saccharification with cellulases. In addition, uronic acid (unsaturated uronate) is the most abundant component in brown algae and is necessary to run the pathway from the sugars to the liberation of glucose and uronic acid for a biotechnological application such as third generation ethanol production.

4. Conclusion

This study shows that the main components in *M. pyrifera* are alginate, and cellulose and the best experimental conditions found for this brown algae was the pretreatment with 2 vol.% sulfuric acid for the liberation of glucose and uronic acid after enzymatic saccharification. Additionally, the use of “green solvents” with the ionic liquids DBU–MEA–SO₃–SiL and [DBNH][OAc] as pretreatment were relatively efficient solvents for the liberation of uronic acid in the saccharification step.

Optimum conditions for enzymatic saccharification using a mixture of cellulases and alginases were determined. Saccharification with cellulases was optimal at pH 5.2 and 50 °C for 4 h and for alginases at pH 7.5 and 37 °C for 2 h.

In conclusion, the pretreatment with 2 vol.% H₂SO₄ for 1 h and saccharification with cellulases and alginases liberated 68.4 wt.% of glucose and 85.8 wt.% of uronic acid of the maximum monosaccharide content in *M. pyrifera*.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2015.11.023.

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References


