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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–SO_2–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.

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 *Ascophyllum* [5]. Alginate or alginic acid is a co-polymer of α-L-guluronate (G) and its C5 epimer β-D-mannuronate (M), being arranged as homopolymeric 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) cleave 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.–) cleaves these

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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 alginases 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 Chiloe 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 was 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 alginate, hemicelluloses and pectins from *M. pyrifera* were determined by the acid methanolysis method. 2 ml of methanolysis 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 desiccator at 40 °C below 50 mbar for 20 min. Once samples were completely dry, they were silylated by adding 150 μl of pyridine, 150 μl of hexamethyldisilazane (HMDS) and 70 μl of chlorotrimethylsilane (TMCS), and thoroughly mixed using a vortex. Samples were kept in an oven at 70 °C for 45 min, and then a clear liquid phase was taken to analyze sugar content in a gas chromatograph [12]. 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 acetone 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* was 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 ([DBNH][OAc]) [14] and 1,8-diazabicyclo-[5.4.0]–undec-7-ene–sulfurdioxide–monoethanolamine (DBU–MEA–SO_2–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 McIlvaine buffer (citric acid/disodium hydrogen phosphate [17]) 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 McIlvaine 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.