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Neutral sugars determination in *Chlorella*: Use of a one-step dilute sulfuric acid hydrolysis with reduced sample size followed by HPAEC analysis

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ABSTRACT

The accurate determination of the chemical composition of microalgae is essential when evaluating their potential as feedstock for the production of biofuels and biochemicals. The usual methodology for the determination of total microalgal carbohydrate content combines a two-step acid hydrolysis of the microalgal biomass with an analysis of the released monosaccharides using phenol-sulfuric acid, but the latter is a hazardous procedure. Alternatively, the sugar monomers that are present in the acid hydrolysate can be identified using high performance liquid chromatography (HPLC), whose accuracy may be compromised by compounds derived from the complex cell composition of the microalgae. Aiming to further the extant analytical procedures for determination of the neutral sugar content in microalgae of the *Chlorella* genus, the present study evaluated a one-step dilute sulfuric acid hydrolysis followed by the analysis of the released monosaccharides using high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). The proposed HPAEC-PAD method proved to be specific, sensitive, accurate, precise and robust. The one-step dilute sulfuric acid hydrolysis process showed an acceptable correlation with the widely used two-step technique for the determination of total neutral sugars in *Chlorella*, despite the use of a sixfold smaller sample size. The method was also successfully applied to *Mychonastes homosphaera*, another green microalga of the phylum Chlorophyta, indicating its applicability for broader use beyond the *Chlorella* genus. Furthermore, the combination of both methods is proposed as a useful tool to evaluate recalcitrant glucose moieties in microalgal biomass.

1. Introduction

The ability of some microalgae to accumulate a large amount of lipids has drawn attention from the energy sector to this potential feedstock [1]. Some species are also capable of accumulating a large amount of intracellular starch in addition to the presence of structural polysaccharides in their cell walls. As a result, microalgal-derived glucose features highly in the energy research agenda as well.

Within this context, accurate characterization of the chemical composition of microalgae is essential for an evaluation of their potential to serve as feedstock for biofuels and biochemical production. However, the existence of thousands of microalgae species with different cellular structures and chemical compositions has hindered the development of standard analytical procedures for the characterization of microalgal biomass [2].

The determination of the composition of microalgal carbohydrates requires a preliminary acid hydrolysis to depolymerize the intracellular starch and structural polysaccharides into their monomers, which are then further quantified. A widely used acid hydrolysis method published by the National Renewable Energy Laboratory (NREL) is based on a two-step hydrolysis using H_2SO_4 [3]. This method is reliable, but it is time- and sample-consuming, and it is a multi-step procedure, which increases the chance of experimental errors. Northcote et al. [4] described another method based on a one-step acid hydrolysis with dilute H_2SO_4 that uses smaller biomass samples. Although this method seems to offer improvements when compared to the NREL procedure, to the best of our knowledge, it has not previously been optimized or validated.

Following microalgal acid hydrolysis, the total carbohydrate content of the hydrolysate can be determined using the phenol-sulfuric acid colorimetric method [5]. Although it is simple and fast, this technique involves the use of hazardous chemicals. Furthermore, the accuracy of its data is highly dependent on the choice of the sugar used as a standard, because different sugars provide different conversion coefficients

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[5]. This method also results in a high deviation [6] and can be affected by interference from several algae components [2].

The sugar content of the microalgal acid hydrolysate can be more accurately determined by liquid chromatography. This analytical technique is more costly and time-consuming, but it provides data on the composition of the individual monomeric sugars in the acid hydrolysate. However, the resolution and selectiveness of high performance liquid chromatography (HPLC) techniques do not meet the analytical requirements of complex monosaccharides mixtures, such as the ones found in the acid hydrolysate of microalgae, and may be affected by interference from compounds derived from the complex microalgal chemical composition [7]. The use of high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) is therefore a valuable option, as this liquid chromatography technique is more selective and better indicated for microalgal sample analysis [7].

Therefore, the present study aimed to contribute to the development of a standard method for determination of carbohydrates by verifying the efficiency of the method proposed by Northcote et al. [4], using three Chlorophyta microalgae with different starch contents. In conjunction with this method, an HPAEC-PAD methodology was evaluated for the analysis of neutral sugars in microalgal samples.

2. Materials and methods

2.1. Microalgae

Two species of microalgae belonging to the *Chlorella* genus and one species belonging to the *Mychonastes* genus were used in this study. A dried commercial *Chlorella* sp. strain (Lot 1404181102, Fuqing King Dnarmsa, Fuqing City, China) was selected for its low starch content. *Chlorella sorokiniana* (UTEX1663) was selected as the microalga with an intermediate starch content; it was cultivated in 500-mL Erlenmeyer flasks containing 200 mL of Bold's Basal Medium in an orbital shaker (New Brunswick Scientific Innova 44R, Eppendorf AG, Hamburg, Germany) at 30 °C and 175 rpm under daylight white fluorescent light with a 12-hour photoperiod and a photosynthetic active radiation of 100 μmol/m^2/s (average irradiance). After 30 days of growth, cells were collected by centrifugation at 8000 x *g* for 5 min, freeze-dried (Christ Delta 1-24 LSC, Christ, Osterode am Harz, Germany) at a pressure of ≤1.65 mbar and a shelf temperature of 25 °C for 24 h, and thereafter stored in a freezer. The microalga *Mychonastes homosphaera* (formerly *Chlorella homosphaera* Skuja) (LEAF0708) was chosen for its high starch content and was cultivated and harvested as described elsewhere [8].

2.2. Carbohydrate determination assays

The carbohydrate content was analyzed using the one-step hydrolysis method proposed by Northcote et al. [4]. Briefly, 4 mg of freeze-dried microalgae were weighed in capped centrifuge tubes. Then, 2 mL of 9.1% (w/w) H_2SO_4 were added to the tubes, which were placed in a water bath at 100 °C for 6 h. Test times of 1,2, and 4 h were also evaluated. The hydrolysates were neutralized by the addition of $CaCO_3$ and analyzed using HPAEC-PAD. The total reducing sugars were quantified using the dinitrosalicylic (DNS) method and expressed as glucose-equivalents [9]. The carbohydrate content was also determined using the two-step acid hydrolysis method described by the NREL [3] for comparison. Briefly, 25 mg of freeze-dried microalgae were weighted in capped glass tubes. Then, 250 μL of 72% (w/w) H_2SO_4 was added, and the tubes were placed in a water bath at 30 °C. The tube contents were periodically stirred with a glass rod to ensure total contact between the acid and the sample. After 1 h, 7 mL of deionized water was added, diluting the acid to a 4% (w/w) concentration, and a second hydrolysis step was carried out in an autoclave for 1 h at 121 °C. The hydrolysates were neutralized with the addition of $CaCO_3$ and analyzed by HPAEC. Standard monosaccharides were also subjected to both acid hydrolysis methods as a control for quantifying sugar degradation.

2.3. Acid hydrolysis of standard materials

Commercial polysaccharides were characterized according to the two aforementioned methodologies to verify their hydrolysis efficacy. Microcrystalline cellulose (Avicel®, Fluka, Milwaukee, USA) and commercial corn starch (Maizena®, Unilever, São Paulo, Brazil) were used as representatives of cellulose and starch, respectively. The red macroalga *Kappaphycus alvarezii* (Rhodophyta), after carrageenan extraction to increase its cellulose content, was also characterized by both methods as a standard cellulose-containing material.

2.4. Acid hydrolysis of starch-free microalgae

In order to compare the efficiency of the two methods for the hydrolysis of the structural polysaccharides of the three evaluated species, the microalgae were subjected to enzymatic hydrolysis of their starch content using commercial amylases, according to the Megazyme method [10]. The microalgae were milled in a vibratory mill (Vibratory Micromill Pulverisette 0, Fritsch, Idar-Oberstein, Germany) and then extracted using 80% ethanol at 80–85 °C for 5 min. The extracted microalgae were then hydrolyzed with α-amylase for 6 min at 100 °C, followed by hydrolysis with amyloglucosidase for 30 min at 50 °C. This procedure was repeated twice to ensure complete starch hydrolysis. The resulting materials were thoroughly washed with distilled water until no free glucose was detected in the supernatant. The starch-free microalgae samples were freeze-dried before being subjected to one- and two-step acid hydrolysis.

2.5. High performance anion exchange chromatography (HPAEC) analysis

2.5.1. Reagents and chromatography standards

Standards comprising arabinose, rhamnose, xylose, cellobiose (Sigma-Aldrich, St Louis, USA), galactose, glucose, and mannose (Merck, Darmstadt, Germany) were used. A 50% (w/w) sodium hydroxide solution (NaOH) (Merck, Darmstadt, Germany) was utilized for the mobile phase preparation and the water was purified in a Milli-Q® integral water purification system (Millipore, Billerica, USA).

2.5.2. Equipment

An Ion Chromatography System 5000 (Dionex, Thermo Fisher Scientific, Waltham, USA) was used for method validation. The HPAEC system was equipped with a column oven compartment (Model DC-5), a single gradient Vacuum Degas Pump (Model SP-5), an autosampler with thermostatic cooling (Model AS-AP), a post-column pneumatic controller with a 375 μL reaction coil (Model PC-10), and a pulsed amperometric detector (Model DC-5) consisting of an electrochemical detection cell with a working gold electrode and as Ag/AgCl reference electrode. Data acquisition, analysis, and reporting were performed using Chromeleon® 6.8 software (Dionex, Thermo Scientific, Waltham, USA).

2.5.3. Chromatographic conditions

Experiments were performed with the previously described system using the CarboPac PA1 analytical column (4 × 250 mm with 10 μm particle size; Thermo Fisher Scientific, Waltham, USA), along with the CarboPac PA1 guard column (4 × 50 mm with 10 μm particle size; Thermo Fisher Scientific, Wisconsin, USA). Both columns were used at 15 °C. An aliquot (5 μL) was injected into the columns at a constant flow rate of 1.25 mL/min under the following gradient conditions: ultrapure water from 0 to 42 min, 300 mM NaOH from 42 to 52 min and ultrapure water from 52 to 62 min, along with the post-column addition of 450 mM NaOH with a constant flow rate of 0.4 mL/min throughout the run. The solvent bottles were continuously supplied with nitrogen gas to prevent bicarbonate contamination.