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Feasibility of posthydrolysis processing of hydrothermal extracts from *Sargassum muticum*

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ABSTRACT

The posthydrolysis of the crude fucoidan extracted during hydrothermal treatment of *Sargassum muticum* was carried out with mineral acid or without added acid and aided by microwave or by ultrasound processing during short periods. The liquors produced under non-isothermal treatments at 150 and 170 °C were subjected to alginate precipitation and the soluble crude fucoidan to posthydrolysis. Acid posthydrolysis caused a steady increase in the monosaccharide content. Ultrasound (US) was the intensified process selected for assisting short term posthydrolysis, which caused depolymerization of crude fucoidan, but did not significantly change the saccharide or sulfate content. The antiradical and cytotoxic properties against lung and colon cancer cells were significantly enhanced by US assisted posthydrolysis.

1. Introduction

Fucoidans are sulfated heteropolysaccharides with low toxicity found in higher amounts in seaweeds than in other sources, and with potential for food, health and cosmetic uses due to their biological properties [1,2]. Fucoidans, composed of variable amounts of fucose, galactose, xylose, uronic acids and sulfate, present a highly diverse structural configuration, molecular weight and sulfation pattern, depending on tissue, growth-stage, geographic location, environmental conditions, season and the extraction process [3].

The biological activity of fucoidan, influenced by both composition and structure [4], can be controlled to fit to desired applications of the final products [5]. However, there is no standardized extraction and purification procedure for fucoidan and most techniques involve the extraction for several hours, usually with water, diluted alkali or acid solutions [3,6]. Extended extraction can be accompanied by undesirable declines in fucose and sulfate content and increased co-extraction of contaminants.

Mild acid hydrolysis is a depolymerizing method for sulfated polysaccharides, showing cleavage specificity influenced by the polysaccharide sulfation pattern. Polysaccharides of high molecular mass present high viscosity, structural heterogeneity and low permeability through the cell membranes; these properties limit their bioavailability and functionality. Depolymerization was proposed to produce low molar mass oligosaccharides with improved physiological activities and novel biological effects. The depolymerization has mainly been conducted by acid, oxidative, enzymatic and radiative hydrolysis [7]. Mild acid hydrolysis is an apparently nonspecific depolymerizing method for sulfated polysaccharides, but shows cleavage specificity influenced by the polysaccharide sulfation pattern [8]. High values of time and temperature could lead to decreased activity, probably due to the partial removal of sulfate groups, formation of sideproducts and degradation of sugars [9]. Preparation of oligosaccharides suitable for analyses was proposed in a process named autohydrolysis, at room temperature for prolonged periods [10]. Thinh et al. [1] also denoted autohydrolysis to acidic polysaccharide hydrolysis under very mild conditions using the $-SO_3H$ groups as the acid source. However, these processes required long times, acidic media or the use of enzymes, which represent an additional cost. Hydrothermal technology was efficient for the simultaneous extraction and depolymerization of fucoidan fractions from *Sargassum muticum*, the molecular weight being highly dependent on the extraction conditions. Maximum fucoidan recovery was attained at 170 °C (5–12 kDa), but the purity was higher at 150 °C (> 80 kDa) [11]. Other authors found that 140 °C was suitable for obtaining low-molecular-weight fucoidan [12] without affecting the degree of sulfation, but also observed partial degradation above 160 °C [13].

Intensified technologies, including microwaves and ultrasound, have also been proposed for the extraction of bioactives from seaweed. The efficient internal microwave heating is achieved by two main mechanisms: dielectric polarization and ionic conduction. The dipole or ion attempts to align with the oscillating electric field and energy is lost

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in this process in the form of heat through molecular friction and dielectric loss [14]. In chemical processes the thermal and kinetic effects are usually the responsible for rate enhancements and altered product distributions [15]. Microwave aided in the enzyme extraction of phlorotannins and antioxidants from brown algae [16], in the acid degradation of polysaccharides, previously extracted with water [17,18], and in hydrothermal processing for controlling modification of the fucoidan polymers [12], maximizing fucoidan yield without compromising purity and structural integrity. Microwave-assisted fucoidan processing in open-vessels was efficient to achieve disruption of the cell wall complex preserving fucoidan structure during controlled modification into more active derivatives requiring short times [5].

The acceleration of chemical and enzymatic reactions caused by ultrasound can be due to cavitation, a phenomenon enhancing the rates and selectivity based on the physical and chemical effects, particularly the extreme reaction conditions in cavitation bubbles. In addition, the generation of free radicals could facilitate polysaccharide hydrolysis. Ultrasound, used for the extraction of bioactives from algae [19–22], has been proposed for polysaccharide degradation in the presence of hydrogen peroxide aqueous solutions to enhance the antithrombotic activity of fucoidans [23].

The present work aims at evaluating the possibility of depolymerizing the *S. muticum* crude fucoidan using intensification processes in a short posthydrolysis stage of the mild acid liquid phase generated during hydrothermal treatments of the whole alga. Neither of these processes requires the external addition of acid. The influence of other variables during sonication on the yields and composition of fucoidans, as well as on their antioxidant and cytotoxic properties, is discussed.

2. Materials and methods

2.1. Materials

Sargassum muticum was collected in Praia da Mourisca (Pontevedra, Spain) in July 2014. Algae were cleaned, washed with tap water, ground and stored in plastic bags at −18 °C until use. The average moisture of the algae was 85.14 ± 0.44% and the proximal composition in dry basis (%) was ash (17.25 ± 0.08), protein (9.80 ± 0.03) and the saccharide mass fraction consisted of glucose (1.00), fucose (0.71), mannose (0.55), rhamnose (0.26), xylose (0.14), galactose (0.10) and sulfate (2.9).

2.2. Hydrothermal treatment

Ground defrosted *S. muticum* samples (66.67 g) were contacted with water at a liquid:solid ratio of 30:1 (wt) and the suspension was heated up to 150 or 170 °C in a pressurized reactor (Parr Instr., Il., USA). Once the selected temperature was reached, the reactor was cooled and the liquid and solid phases were separated by filtration. The alginate in the liquid phase was precipitated by adding 1% (w/v) $CaCl_2$ (*Acros Organics*), and separated by centrifugation and filtration through cellulose acetate filters (0.45 μm, Sartorius Stedim Biotech) (Fig. 1). The saccharide content of the extracts was 30% of the dry weight, the mass ratio of fucose:galactose:glucose:xylose:mannose being 1.0:0.16:0.15:0.12:0.06 for treatment at 150 °C, and the mass ratio of fucose:galactose:glucose:xylose:mannose:rhamnose being 1.0:0.86:0.56:0.16:0.08:0.05 for treatment at 170 °C.

2.2.1. Acid posthydrolysis of hydrothermal liquors

The alginate-free hydrothermal liquid phase was treated with sulfuric acid (to reach final concentrations of 0.5 and 1%) in an orbital shaker at 175 rpm at room temperature during 1, 2, 3, 4 and 72 h. This treatment was carried out as a control procedure and all experiments were performed in duplicate.

2.2.2. Microwave assisted posthydrolysis of hydrothermal liquors

The alginate-free autohydrolysis liquid phase was placed into a glass flask into the 1.5 L Pyrex extraction vessel and irradiated at selected power and time in an open multimode microwave extractor (NEOS-GR, Milestone Srl, Italy) operating at 2.45 GHz. Temperature was monitored by an external infrared sensor. Experiments were performed in duplicate.

2.2.3. Ultrasound assisted posthydrolysis of hydrothermal liquors

The alginate-free liquid phase from the hydrothermal treatment (50 mL sample) was treated with an ultrasonic water bath (P-Selecta) kept at 25 °C. The conditions used were: power 150 W and 40 kHz for 5–30 min. The study of the influence of the major variables was performed using a probe sonicator (Labsonic P, Sartorius) at 20–60 °C in 100 mL vessels (50 mL sample) at a frequency of 24 kHz and power 400 W, for 5 to 25 min. During these experiments maximal increases in temperature up to 10 °C were observed. Experiments were performed in duplicate and analysis in triplicate.

2.2.4. Analytical methods

Ash content was determined after calcination at 575 °C. Samples of liquors, previously filtered through 0.45 μm membranes, were neutralized with barium carbonate, and diafiltered to remove salts (Spectra/Por Float-A-Lyzer G2 Dialysis Device, MWCO: 100–500 Da, SpectrumLabs). Samples were assayed by HPLC for xylose, mannose and galactose using a 1100 series Hewlett-Packard chromatograph equipped with a RI detector and a 300 × 7.8 mm CARBOsep CHO 682 column (Transgenomic, Glasgow, U.K.) operating at 80 °C. Distilled water was used as the mobile phase at 0.4 mL/min. Glucose, rhamnose, fucose and acetic acid were determined in the same instrument and a 300 × 7.8 mm Aminex HPX-87H column (BioRad, Hercules, CA) operating at 60 °C with 0.003 M H_2SO_4 at 0.6 mL/min as mobile phase. The content of saccharides was determined in duplicate from the concentrations of monosaccharides present in samples subjected to a quantitative posthydrolysis (4% sulfuric acid at 121 °C, 20 min).

The sulfate content of samples was determined following two methods. A solution of ground ashes (25 mg) in 50 mL mq-water was filtered through Whatman N° 44. Samples, diluted in ultrapure water to 100 mg/L, were analyzed in duplicate in an ionic chromatograph Metrohm Advanced model IC-861 with a conductivity detector IC-819 and separation was achieved in a Metrosep A Supp 5-250 (250 × 4 mm) column (32 °C), using 3.2 mM sodium carbonate/1 mM sodium bicarbonate as mobile phase at 0.70 mL/min. The barium chloride-gelatin method was used after hydrolysis of the whole alga with 4% trichloroacetic acid (TCA) (Sigma-Aldrich). $BaCl_2$-gelatin reagent was prepared by mixing 0.5 g gelatine powder (Scharlau) in 100 mL of hot water (60–70 °C), and kept at 4 °C for at least 6 h, before adding 0.5 g $BaCl_2$ (Sigma-Aldrich). Samples of liquors from hydrothermal treatments or distilled water (0.2 mL), TCA (3.8 mL) solution and gelatine-$BaCl_2$ reagent (1 mL) were mixed and maintained 15 min at room temperature. Absorbance was read at 500 nm against a standard curve prepared with K_2SO_4 (Panreac). All determinations were done in triplicate.

High Performance Size Exclusion Chromatography (HPSEC) was used to determine the molar mass distribution of the crude fucoidans using two 300 × 7.8 mm TSKGel G3000PWXL columns in series (Tosoh Bioscience, Stuttgart, Germany), and a 40 × 6 mm PWX-guard column. The mobile phase was distilled water (flow rate, 0.6 mL/min) and a refractive index detector was used. Dextrans (1000–80,000 g/mol) (Fluka, St. Louis, MO, USA) were used as calibration standards. Freeze-dried samples were blended with KBr and dried with an infrared lamp for 30 min. FTIR spectra were recorded at 400–4000 nm at 25 scans/min in a Bruker IFS 28 Equinox equipment with an OPUS-2.52 software for data acquisition System 450-MT2. HPSEC and FTIR analysis were performed in duplicate.

2.2.5. Antiradical and cytotoxic properties

The ABTS radical cation ($ABTS^{\cdot+}$) [2,2-azinobis(3-ethyl-