Bulletin of Romanian Chemical Engineering Society, Vol. 4, No. 2, 2017 ISSN 2360-4697

EXTRACTION OF BIOMOLECULES FROM A BROWN SEAWEED FROM THE ROMANIAN BLACK SEA SHORE (*CYSTOSEIRA BARBATA*)

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Abstract

Cystoseira barbata, the main brown seaweed species at the Romanian Black Sea shore, contains a number of valuable biomolecules. We focus on the extraction of alginate, fucans and phlorotannins. Alginate is already a natural biomolecule with a well-established market,which is supposed to grow in the near future, as a thickening and gelling agent. Furthermore, fucans have recently been shown to be biologically active. Phlorotannins are also worth investigating as they are polymers of phloroglucinol, a phenolic compound which has a good antioxidant activity. In this paper, alginate, fucans and phlorotannins are extracted in a sequential manner from Cystoseira barbata. Identification and quantification of the biomolecules is carried out by specific methods. The purpose of this paper is to prove the concept of biorefinery applied for a brown seaweed species at the Romanian Black Sea shore.

Key words: *CystoseiraBarbata*, alginate, fucans, phlorotannins

1. Introduction

Seaweed is a source of valuable products with unique properties. In Romania it remains untapped as it is removed from beaches as a waste when it

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accumulates. All three types of seaweed or phyla are represented on the Romanian Black Sea shore: Chlorophyta (green), Rhodophyta (red) and Phaeophyta (brown) [1].Biomass produced at the Romanian Black Sea shore consists mainly of red and green seaweed with values reported between 5 and 25 kg/m^2[2]. The only brown seaweed (kelp) species which still exists is *Cystoseirabarbata*. Even if now biomass is at a low level, values of 4300 t were reported in 1972. Nowadays, the collapse of the *C. barbata* population is largely related to climatic and anthropic factors [1].

Brown seaweed is a well-known source of valuable biomolecules which includes alginate (an acidic polysaccharide), fucoxanthin (a carotenoid pigment) and fucans (sulfated polysaccharides). Apart from these classes, we also find phlorotannins which are polyphenolic compounds with a high solubility in water[3,4]. All of these compounds have been proven to be useful either for their rheological properties (alginate) or for their various biological activities (antioxidant, cytostatic, antiviral etc.) [5-7].

Alginate is one of the most biomaterials with a whole range of applications in food industry and even n textile industry[8]. It is a natural copolymer formed from two uronic acids, β-D-mannuronic acid (M) and α-L-guluronic acid (G) which are linked β-(1,4)[9,10].Together with other water-soluble polysaccharides extracted from seaweed, it formsthe group which is generically called phycocolloids[11]. These biomolecules possess a wide range of possible applications in the food industry and cosmetics, especially as gelling and thickening agents[12].Other important phycocolloids are agar and carrageenan (red algae), fucans (sulfated polysaccharides from brown algae) and ulvans (sulfated polysaccharides from green algae)[11]. Thegross market value for phycocolloids (which also includes alginate) has been estimated at 1 billion US$ per year with an annual global production of 100 thousand tons and it is expected to grow [13].Other uses applied for seaweed include raw material for biofuel production[14-16], polysaccharide based supercapacitors[17] and others.

The main goal of our work is to show that several valuable biomolecules can be extracted in series, following an extraction scheme which resembles that of a biorefinery concept. At this point, we are only focusing on polyphenols, fucans and alginate. Other compounds such as pigments and proteins could also prove interesting of our purpose. Apart from this, we expect that the fibers which are left after the extraction series to be rich in fermentable cellulose which could valorized as biogas or bioethanol [18]. Suchextraction models applied to seaweed have been proposed in literature before[19] and follow a bio-refineryconcept.

2. Materials and Methods

Raw material

C. barbata seaweed were collected from the Black Sea in the city of Mangalia (Latitude: N 43° 49' 9.9048"; Longitude: E 28° 35' 22.5655"). They were then washed with tap water to remove seawater and other impurities and dried in the sun. The resulted dried material was then milled and sieved. *C.barbata*algal powder with particles smaller than 500 µm was used.

Extraction

A purified form of alginate was obtained by following a procedure previously presented in literature [20,21]. This procedure is depicted in Fig. 1.

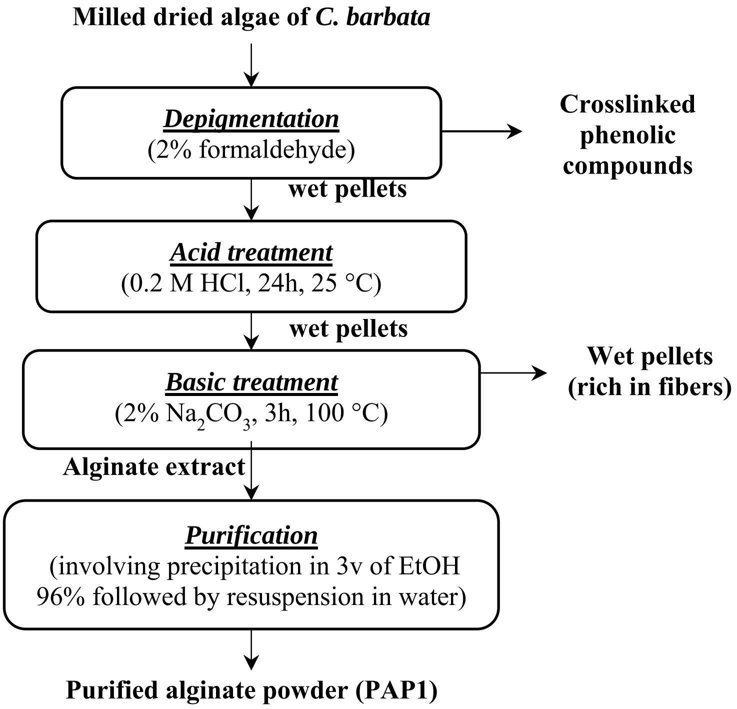

Fig. 1. Alginate extraction (method 1)

25 g of milled dried algae were depigmented with 800 mL of 2% formaldehyde under stirring for 24h at room temperature. The pellets were then removed and washed with distilled water prior to adding them to 800 mL of 0.2 M HCl. This treatment was carried on for 24h, under stirring and at room temperature. The wet pellets were removed and washed with distilled water. Alginate was then extracted by treating the wet pellets with a solution of 2% Na_2CO_3 for 3 hours at 100 °C. The alginate solution was separated from the wet

pellets by filtration (Whitman paper, 25 µm). It was then precipitated with 3 volumes of cooled ethanol (96%). The dried alginate was further purified by dissolution in demineralized water followed by precipitation with another 3 volumes of ethanol. This step was repeated until the pH approached neutral values while the salt equivalent, measured by conductimetry, had a value lower than 1 g/L (Fig. 2). The dried product was further purified by precipitation in cooled acetone followed by dissolution in MilliQ water.

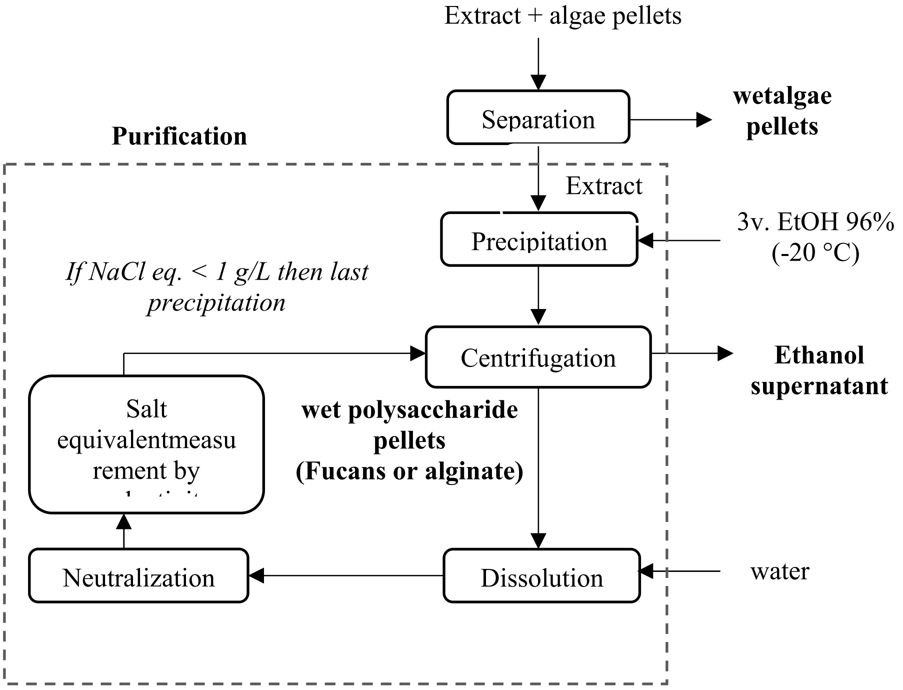

Fig. 2. Purification of polysaccharides

Method 2was adapted from literature [22] and aimed at extracting several groups of biomolecules. Pigments are extracted in 70 % ethanol for 24 h. The pellets are then sequentially extracted under stirring twice in 0.1 M HCl (2 h, 60 °C) to recover the fucans. The pellets are separated by filtration and alginate is extracted with 3 % Na_2CO_3 (2 h, 60 °C) in two steps. Phlorotannins are then removed by washing the wet pellets 4 times with distilled water. The scheme for this extraction scheme is presented in Fig. 3.

In both methods, purification of the polysaccharides followed the scheme presented in Fig. 2 which involves repetitive precipitation in ethanol aimed at removing salt and neutralization. At least after the first precipitation, the solution needs to be neutralized.

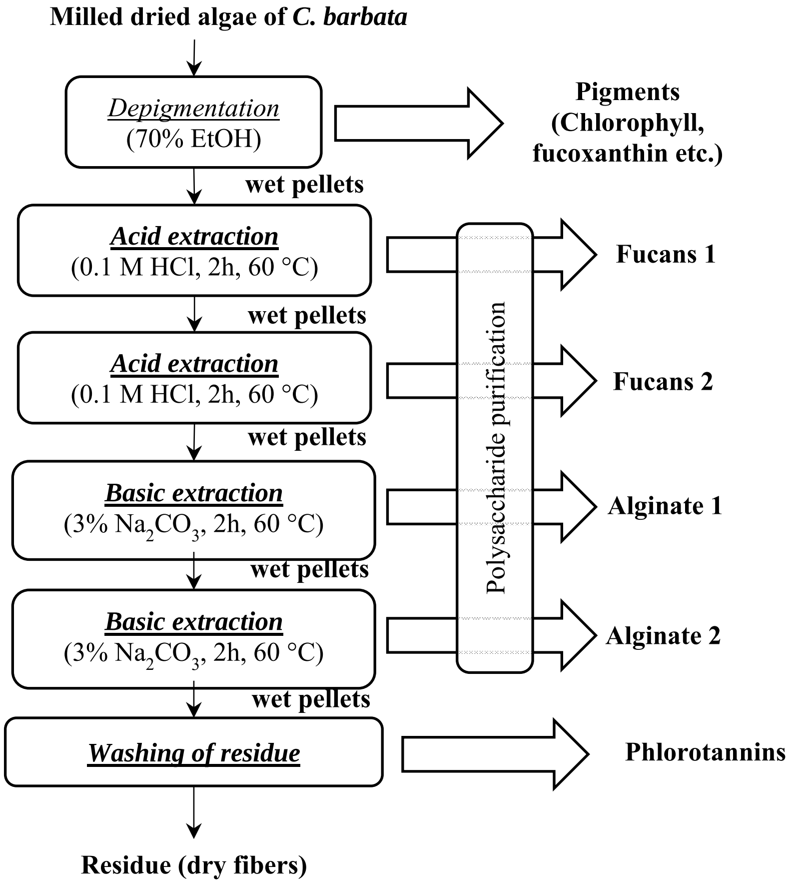

Fig. 3. Sequential extraction scheme (method 2)

Total phenolic content

The method applied for determining the total phenolic content was adapted from literature[23]. 0.5 mL of sample was added to 10 mL of MilliQ water. 0.5 mL of Folin-Ciocalteu reagent is added. 1 mL of Na_2CO_3 (saturated solution) is then added. The sample must be vortexed vigorously. Immediately after, the sample must be placed in the dark and kept there for 1 hour during which it should acquire a blue color. The absorbance at 750 nm is measured. The total phenolic content is expressed as grams of phloroglucinol equivalents (PGE, g/L).

Neutral and Acid Sugars

A colorimetric method is used which also takes into consideration interferences between neutral and acid sugars [24].

Some solutions are prepared beforehand: a resorcinol (6 mg/mL) in MilliQ water, sulfuric acid solution (80%), borax in 97% sulfuric acid (0.12 M) and a solution of 100 mg of m-HBP (m-hydroxybiphenyl) in 1 mL of DMSO (dimethyl sulfoxide).

Neutral sugars assay involves mixing 200 μL of sample, 200 μL of resorcinol solution and 1 mL of 80% sulfuric acid in a screw-cap test tube which is then placed at 90 °C for 30 min. After this, it is left for 30 min in the dark to cool. The yellowish coloration is measured at 450 nm after diluting with 1.4 mL of MilliQ water. Acid sugars assaystarts by adding 200 μL and 1 mL of borax solution to a screw-cap test tube. They are then left for 1h at 90 °C before adding 200 μL of m-HBP. Immediately, the tube is placed for another 2 min at 90 °C. The pink hue of the mixture is then measured at 520 nm.

Values are expressed as glucuronic acid eq. (g/L) for acid sugar content (AcS) and glucose eq. (g/L) for neutral sugar content (NeS). The values are computedusing equations (1) and (2) where α is the specific absorbance of glucose in the neutral sugars assay, β is the specific absorbance of glucuronic acid in the neutral sugars assay and β' is the specific absorbance of glucuronic acid in the acid sugars assay.

$$[AcS] = \frac{Abs_{mHBP}}{\beta'} \tag{1}$$

$$[NeS] = \frac{Abs_{res} - \beta \cdot [AcS]}{\alpha} \tag{2}$$

Dry matter

Dry matter content (DM) was determined by drying a known mass of sample in an aerated oven at 110 °C for 24h.

HPAEC-PAD analysis

Fucose and mannuronic acid content was determined by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) after complete hydrolysis [20,25] of 10 mg of polysaccharide powder in 4.5 mL of 90% formic acid for 6 hours at 100 °C. The samples were then passed through the Dionex ICS-3000 system using an eluent containing 100 mM of NaOH and 100 mM of NaOAc inMilliQ water during 1h at a rate of 1 mL/min [20].

3. Results and Discussion

Mass balance

Table 1 presents the mass balance for dried matter (g), PGE (mg), NeS (g) and AcS (g). It also presents the yields obtained at the end. Thus, we obtain 3.9 g of purified alginate which represents 15.7 % from the initial mass of *C. barbata* powder (<500 µm). Alginate extracted in the second basic treatment could not be recovered. This suggests that one basic extraction is enough to recover most of the alginate [22]. 0.4 % and 0.6 % of the initial mass is recovered as fucans powder in the first and in the second acidic steps. Phlorotannins are mostly recovered at the end when the insoluble algal mass is washed with water. The PGE, NeS and AcS content of the ethanol supernatants is also expressed. This are obtained as described in Fig. 2 regarding the purification of polysaccharides by ethanol precipitation (1 volume of extract + 3 volumes of ethanol 96 %).

Table 1

Mass balance for extraction of biomolecules (method 2)

Name	**Dry weight (g)**	**Dry weight% /25g**	**PGE (mg)**	**NeS (g)**	**AcS (g)**
EtOH 70% extract	1.63	6.5	0.77	0.102	0.037
Acid ethanolsupernatant 1	2.85	11.4	58.37	0.714	0.020
fucans 1 powder (0.1032 g)	***0.10***	***0.4***	3.85	0.043	0.030
Acid ethanolsupernatant 2	2.42	9.7	14.89	0.227	0.020
fucans 2 powder (0.143 g)	***0.14***	***0.6***	5.08	0.029	0.040
Basic ethanolsupernatant 1	2.00	8.0	22.94	0.049	0.009
alginate 1 powder (3.9249 g)	***3.92***	***15.7***	1.51	1.006	2.634
Basic ethanolsupernatant 2	0.96	3.8	14.41	0.029	0.003
alginate 2 powder	N.D.	N.D.	N.D.	N.D.	N.D.
Washing water	2.36	9.4	***415***	0.164	0.119
Dry fibers (5.1 g)	-	**20.4%**			
TOTAL	**21.5**		**537**	**2.365**	**2.912**
percentage of 25 g	**86%**		**2%**	**9%**	**12%**

Phlorotannins

Generally, an important contamination in alginate products arises from pigments and polyphenolic compounds (phlorotannins). Since method 1 (Fig. 1) is aimed at extracting alginate, polyphenols are removed in the first step by crosslinking them with 2% formaldehyde. This ensures that they remain insoluble during the subsequent steps, especially during the basic extraction of alginate.

In the case of method 2, phlorotannins are desirable. To extract the pigments (chlorophyll, fucoxanthin), ethanol 70 % (vol.)[26]. This concentration is also equivalent to the one in the precipitation phase where 1 volume of polysaccharide extract is mixed with 3 volumes of ethanol 96% in order to precipitate the polysaccharides[20]. During the depigmentation step in method 2, polysaccharides are not extracted.

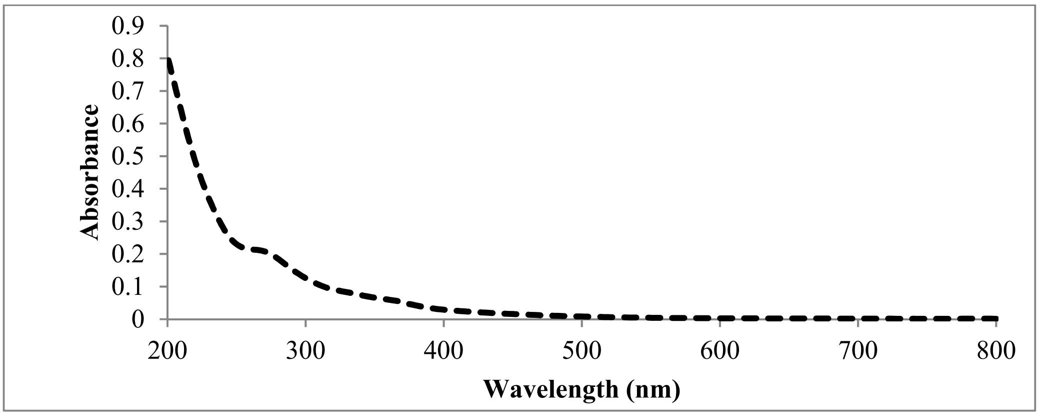

Fig. 4. UV spectra for the washing water rich in phlorotannins

We observe from Table 1 that 77% of the phlorotanninsextracted during method 2 are solubilized in the stream obtained by washing the wet pellets which left after the basic extraction of alginate (Fig. 3). They are identified by the polyphenols assay described in section 2 and by matching its UV spectra (Fig. 4)with one found in literature [3].

Polysaccharides

We identify and quantify the peaks corresponding to fucose (3.02 min - Fuc) and mannuronic acid (23.08 min - ManA) which can be seen in 5 and **Error! Reference source not found.** (totally hydrolyzed alginate obtained via method 1 and method 2). We estimate the concentration of guluronic acid from the ratio of its specific peak (22.7 min - GulA) to that of mannuronic acid assuming that its retention time is close to that of mannuronic acid [27].

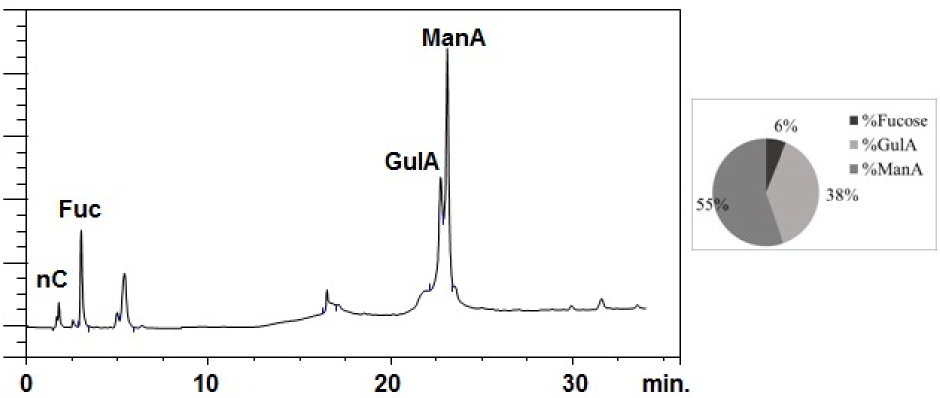

Fig. 5. HPAEC chromatogram for totally hydrolyzed alginate (Method 1)

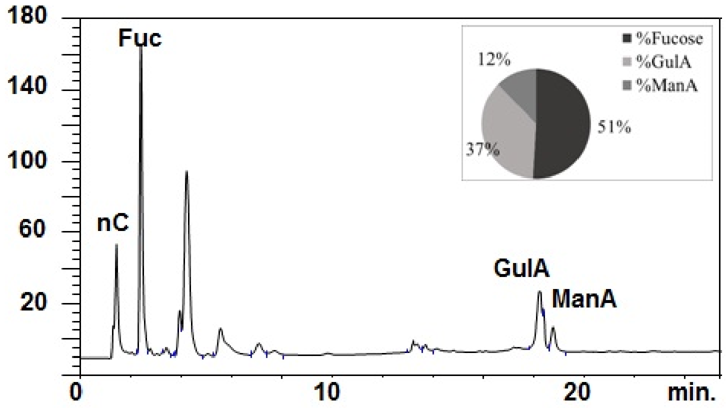

Fig. 6. HPAEC chromatogram for totally hydrolyzedfucans (Method 2)

We notice that in the alginate obtained by method 1 (Fig. 5)we have a low impurity of 6% fucose, related more likely to fucans which were not extracted in the acidic step. However, in the fucans powder purified from the first acidic extract, we observe that there is a large uronic acids impurity which could be due to the partial hydrolysis of alginate which could arise in the acidic medium.

An important characteristic of alginate is the ratio between mannuronate and guluronate (M/G). It is well known that this feature influences the thickening and gelling properties of the alginate [28,29]. By looking at the chromatogram in Fig. 5 we estimate that the M/G value for *C. barbata* recovered from the Romanian Black Sea shore is 1.44. This value needs to be confirmed by NMR [30].

4. Conclusions

Two extraction methods were adapted from literature in order to extract alginate, fucans and phlorotannins from *Cystoseirabarbata* seaweed recovered from the Black Sea.

The first extraction method yielded a purified alginate for which we determined an M/G ratio of 1.44. The hydrolyzed product fed to the HPAEC-PAD instrument contained a 6% fucose impurity associated to fucans.

The second extraction methodwasapplied for the sequential extraction of phlorotannins,fucansand alginate. Using this method, 1%of the initial mass of *C. barbata* is extracted as fucans, while 15.7% as alginate.Most phlorotannins (77% of PGE (mg) found in all streams) are recovered by washing the insoluble material following the basic extraction of alginate. In total, 537 mg of phlorotannins (measured in phloroglucinol equivalents) are extracted from 25 g of dried milled seaweed.

For future studies, our preoccupation will be to test various purification methods and propose new applications for the extracted alginate, fucans and phlorotannins.

Acknowledgements

Financial support from Romanian Society of Chemical Engineering for attending the 20th RICCCE Conference is gratefully acknowledged by Bogdan Trică.

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