1   **IMPROVED PERFORMANCE OF LESS PURIFIED CELLULOSIC FILMS**
2   **OBTAINED FROM AGAR WASTE BIOMASS**

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13

14   **Abstract**

15   The residues generated after the extraction of agar from *Gelidium sesquipedale* by
16   means of a hot-water treatment, with (NaOH+HW residue) and without (HW residue)
17   an alkali pre-treatment have been valorized to produce high performance cellulosic
18   films. Both residues were mainly composed of structural carbohydrates (in particular,
19   agar), ashes and lipids. The residual agar could only be completely removed by
20   applying a two-step process based on bleaching and alkaline treatments.

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22   The application of the alkaline pre-treatment for the extraction of agar did not
23   significantly affect the properties of the films produced from the extracted fractions,
24   hence making the HW residue more sustainable and economically viable. The agar
25   remaining in the less purified fractions had a positive effect on the performance of the
26   films, improving their transparency, mechanical properties and water vapour barrier,
27   outperforming benchmark biopolymers; in addition, these materials presented
28   antioxidant capacity inhibiting the degradation of β -carotene.

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36   **Keywords:** seaweed; *Gelidium*; valorization; cellulose; agar; biopolymers; packaging.

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38   **1. Introduction**

39   The severe environmental issues associated to the use of synthetic plastics are currently
40   one of the hot topics in the society. Synthetic plastics are petroleum-based, hence
41   consuming large amounts of fossil fuels for their production; moreover, they are not
42   biodegradable and, thus, after disposal they can accumulate in natural ecosystems up
43   to several thousands of years. Given the large amount of plastics consumed nowadays,
44   with almost 350 million tons produced worldwide in 2017 (*Plastics - the Facts 2018*,
45   2018), it is necessary to find a long-term solution to overcome these issues. The
46   replacement of conventional plastics by biodegradable polymers obtained from
47   renewable natural resources (i.e. biopolymers) is envisaged as the most sustainable and
48   long-term solution. However, the properties of biopolymers are still far from those of
49   benchmark synthetic polymers (especially in terms of barrier and mechanical
50   performance) and their production costs are too high to compete in the market.
51   Furthermore, the raw materials typically used for the production of biopolymers
52   originate from land crops, whose primary use is the food sector. As an alternative,
53   marine resources such as seaweeds and aquatic plants, given their abundance and
54   interesting composition, are also being explored as a source for the production of
55   biopolymers (Benito-González, López-Rubio, Gavara, & Martínez-Sanz, 2019a; Cian,
56   Salgado, Drago, González, & Mauri, 2014; Khalil et al., 2017; Martínez-Sanz, Erboz,
57   Fontes, & López-Rubio, 2018).

58

59   Amongst the several biopolymers which are being currently explored for their
60   utilization as packaging materials, cellulose is one of the most promising. Although
61   cellulose has been typically extracted from land crops, it can also be obtained from

62 aquatic plants and seaweeds. Interestingly, in line with circular economy policies,
63 several recent works have reported on the valorization of low-cost aquatic biomass
64 such as residues directly derived from seaweed/aquatic plants (Benito-González et al.,
65 2019a; de Oliveira et al., 2019; Fontes-Candia, Erboz, Martínez-Abad, López-Rubio,
66 & Martínez-Sanz, 2019) and from industrial processes making use of algal biomass
67 (Chen, Lee, Juan, & Phang, 2016; El Achaby, Kassab, Aboulkas, Gaillard, & Barakat,
68 2018; Singh, Gaikwad, Park, & Lee, 2017) for the extraction of cellulosic materials.
69 Cellulose is typically isolated from the raw biomass by applying a sequential extraction
70 protocol to remove other cell wall components such as lignin, non-cellulosic
71 carbohydrates and other minor compounds (proteins, waxes, pigments, lipids, etc.) (Lu
72 & Hsieh, 2012; Oksman, Etang, Mathew, & Jonoobi, 2011; J. X. Sun, Sun, Zhao, &
73 Sun, 2004). While most of the available studies in the literature focus on the
74 purification of cellulose, recent work has demonstrated that the presence of other
75 components such as non-cellulosic carbohydrates and/or lipidic compounds in less
76 purified fractions may enhance the mechanical and barrier properties of cellulosic
77 films (Benito-González et al., 2019a). Thus, applying simplified extraction protocols
78 may not only be beneficial from an economical and environmental perspective, but
79 also in terms of material performance.

80

81 Macroalgae are classified as brown (*Phaeophyta*), green (*Chlorophyta*), or red
82 (*Rhodophyta*) seaweeds based on the composition of their photosynthetic pigments,
83 although the algal polysaccharides present in the cell walls are highly indicative for
84 the main algal taxa. Red seaweeds and, in particular, seaweed from the *Gelidium*
85 genera, have complex composite cell walls rich in agar and cellulose amongst other

86 polysaccharides (Jeong, Choi, Lee, & Oh, 2012; Park et al., 2011). *Gelidium*
87 *sesquipedale* is the most commonly used seaweed for the industrial extraction of agar
88 in the Mediterranean area due to the relatively high abundance of this species and the
89 high quality of the agar extracted (Carmona, Vergara, Lahaye, & Niell, 1998; Mouradi-
90 Givernaud, Hassani, Givernaud, Lemoine, & Benharbet, 1999). The industrial process
91 for the extraction of agar involves the application of hot-water treatments, which are
92 usually preceded by alkaline pre-treatments to eliminate other components present in
93 the raw seaweed such as proteins and minerals (Martínez-Sanz et al., 2019a; Martínez-
94 Sanz et al., 2019b). However, previous studies have reported on the production of less
95 purified agar-based extracts with reduced costs, increased yields and interesting
96 antioxidant properties by suppressing the alkaline pre-treatment step (Martínez-Sanz
97 et al., 2019a; Martínez-Sanz et al., 2019b). The agar extraction yields previously
98 reported for the simplified heat-based extraction with and without the alkali pre-
99 treatment are within the range of 10-12% and 2-3%, respectively (Martínez-Sanz et
100 al., 2019a). Thus, a large amount of residue is generated after the extraction process,
101 which is currently underutilized, being mostly discarded or, as other seaweed biomass
102 sources, used as soil fertilizer (Haslam & Hopkins, 1996; Tuhy, Samoraj, Michalak,
103 & Chojnacka, 2014). However, this residue is known to be rich in polysaccharides
104 (with up to 55% of sugars) and other interesting compounds such as proteins and
105 minerals (Ennouali, Ouhssine, Ouhssine, & Elyachioui, 2006). Therefore, the
106 exploitation of this residue for the production of cellulosic materials would be an
107 interesting approach for adding value to this waste stream.

108

109 In this work we aim to valorize the residues generated after the extraction of agar from
110 *Gelidium sesquipedale* seaweed for the extraction of cellulose-based fractions and the
111 production of biopolymeric films. The production of less purified fractions by
112 suppressing some of the purification steps in the extraction process is evaluated, as
113 well as the effect of the remaining impurities on the performance of the biopolymeric
114 films for their use as food packaging materials.

115

116 **2. Materials and methods**

117 **2.1 Materials**

118 **2.1.1 Raw materials**

119 The waste biomass obtained after the extraction of agar from the seaweed *Gelidium*
120 *sesquipedale* was used as the raw material for the production of cellulosic fractions.
121 The raw seaweed was kindly donated by Hispanagar (Burgos, Spain). The extraction
122 of agar was carried out by means of hot water treatments with and without a prior
123 alkaline pre-treatment step with NaOH, as described in a previous work (Martínez-
124 Sanz et al., 2019a).

125

126 **2.1.2 Preparation of cellulosic fractions**

127 A purification procedure previously optimized and applied to aquatic biomass (Benito-
128 González, Martínez-Sanz, & Lopez-Rubio, 2018; Martínez-Sanz et al., 2018), was
129 carried out to sequentially remove cell wall components and obtain different cellulosic
130 fractions from two different biomass waste materials: (i) the residue obtained after the
131 hot water extraction of agar (sample coded as HW) and (ii) the residue obtained after
132 the extraction of agar with an alkaline pre-treatment, followed by hot water extraction

133 (sample coded as HW+NaOH). Figure 1 illustrates the whole extraction process, from
134 the initial agar extraction step to the subsequent purification steps to extract cellulosic
135 fractions.

136 The conventional extraction process consisted of an initial Soxhlet extraction step to
137 remove wax, pigments and oils. Approximately 10 g of ground wet *Gelidium*
138 *sesquipedale* waste biomass were extracted with 800 ml of toluene/ethanol 2:1 (v/v)
139 during 24 h. The resulting material was dried at room temperature overnight.
140 Subsequently, to remove residual pigments and any traces of lignin, the de-waxed
141 material was ground into fine powder and treated for 5h at 70°C with 700 ml of 1.4 %
142 NaClO₂ solution, having the pH adjusted to 3 with acetic acid. After that, the reaction
143 was stopped by quenching with ice, and the excess liquid was decanted. The yellow
144 solid was collected and washed repeatedly with distilled water by vacuum filtration
145 until the filtrate became approximately neutral, hence obtaining the second fraction
146 (referred to as F2 or F2 NaOH, depending on the starting residue). The material was
147 then treated with 400 ml 5% KOH solution for 24 h at room temperature, followed by
148 2h at 90°C, in order to remove the non-cellulosic carbohydrates, yielding pure cellulose
149 (referred to as F3 and F3 NaOH).

150 To optimize the process from an economical and operational perspective, two
151 alternative extraction protocols were also evaluated:

152 (a) Suppressing the initial Soxhlet step: The ground waste biomass was directly
153 subjected to the NaClO₂ treatment, obtaining the fractions designated as F2A and F2A
154 NaOH. Furthermore, the F2A fraction was subsequently treated with KOH as
155 described above, obtaining the fraction labelled as F3A.

156 (b) Suppressing the Soxhlet and bleaching step: The ground seaweed (7 g on dry basis)
 157 was treated with 400 ml 5% KOH solution for 24 h at room temperature, followed by
 158 2h at 90°C, obtaining the F3B fraction.
 159 The obtained fractions were stored in the fridge as partially hydrated materials and a
 160 small amount of each fraction was subjected to freeze-drying for further analyses.

161
 162 **Figure 1.** Flowchart of the different agar extraction protocols applied to the biomass
 163 residues and the subsequent purification steps applied to produce the cellulosic
 164 fractions.

166 2.1.3 Production of cellulosic films

167 Pure lignocellulosic films were produced by adding 0.25 g of cellulosic fractions to 50
168 mL of distilled water and dispersing them by mild sonication, followed by ultra-turrax
169 homogenization until obtaining homogeneous suspensions. These were then vacuum
170 filtered using PTFE filters with 0.2 μm pore size to remove water. The solid material
171 remaining in the filter was then dried at room temperature overnight. The formed films
172 were peeled off the filters and stored at 0%RH.

173

174 **2.2 Chemical analysis of the waste biomass and the extracted fractions**

175 The ash, Klason lignin and structural carbohydrates (designed as holocellulose in the
176 case of higher plants) content in the HW and HW+NaOH residues were determined by
177 means of the standard methods TAPPI T211 om-07, T222 om-06 and ASTM D1104-
178 56, respectively. The protein content in the residues was calculated based on the
179 nitrogen content estimated with Kjeldahl method, multiplied by a factor of 6.25. The
180 lipid content in the residues and the extracted fractions was estimated after performing
181 the Soxhlet extraction of ca. 1 g of material with 800 ml of toluene/ethanol 2:1 (v/v)
182 during 24 h.

183

184 **2.3 Fourier Transform Infrared Spectroscopy (FT-IR)**

185 Film samples were analyzed by FT-IR in attenuated total reflectance (ATR) mode
186 using a Thermo Nicolet Nexus (GMI, USA) equipment. The spectra were taken at 4
187 cm^{-1} resolution in a wavelength range between 400-4000 cm^{-1} and averaging a
188 minimum of 32 scans.

189

190 **2.4 ^{13}C CP/MAS Nuclear Magnetic Resonance (NMR) spectroscopy**

191 The solid-state ^{13}C CP/MAS NMR experiments were performed at a ^{13}C frequency of
192 100.63 MHz on a WB-AVIII Bruker spectrometer. The samples were packed in a 4-
193 mm, PSZ (partially-stabilized zirconium oxide) rotor with a perfluorinated polymer
194 (KelF) end cap. The rotor was spun at 10 kHz at the magic angle (54.7°). The 90° pulse
195 width was 2.2 μs and a contact time of 2 ms was used for all samples with a recycle
196 delay of 5 s. The spectral width was 30 kHz, acquisition time 34 ms, time domain
197 points 2k, transform size 8k and line broadening 10 Hz. 20k scans were accumulated
198 for each spectrum. Spectra were referenced to external glycine.

199

200 **2.5 Thermogravimetric analyses (TGA)**

201 Thermogravimetric curves (TG) were recorded with a Setaram TG/DTA92
202 (SETARAM Instrumentation, France). The samples (ca. 10 mg) were heated from 30
203 to 800°C with a heating rate of $10^\circ\text{C}/\text{min}$ under argon atmosphere. Derivative TG
204 curves (DTG) express the weight loss rate as a function of temperature.

205

206 **2.6 Scanning electron microscopy (SEM)**

207 SEM was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating
208 voltage of 10 kV and a working distance of 8-16 mm. Small pieces of the
209 lignocellulosic films were sputtered with a gold–palladium mixture under vacuum
210 before their morphology was examined.

211

212 **2.7 Small Angle X-ray scattering (SAXS) and X-ray diffraction (XRD)**

213 Small angle X-ray scattering (SAXS) experiments were carried out in the Non
214 Crystalline Diffraction beamline, BL-11, at ALBA synchrotron light source

(<http://www.albasynchrotron.es>) in order to characterize the cellulosic films. The experimental setup conditions and data reduction procedures were the same as those described in a previous work (Fabra, Martínez-Sanz, Gómez-Mascaraque, Gavara, & López-Rubio, 2017).

Additionally, XRD measurements of the cellulosic films were carried out on a D5005 Bruker diffractometer. The instrument was equipped with a Cu tube and a secondary monochromator. The configuration of the equipment was θ – 2θ , and the samples were examined over the angular range of 3° – 60° with a step size of 0.02° and a count time of 200 s per step. Peak fitting was carried out using the Igor software package (Wavemetrics, Lake Oswego, Oregon) as described in a previous work (Martínez-Sanz, Lopez-Sanchez, Gidley, & Gilbert, 2015). The crystallinity index was determined by the method reported by Wang et al. (Wang, Ding, & Cheng, 2007).

$$X_c(\%) = \frac{\sum A_{Crystall}}{A_{Total}} \times 100 \quad (4)$$

Where A_{Total} is the sum of the areas under all the diffraction peaks and $\sum A_{Crystall}$ is the sum of the areas corresponding to the three crystalline peaks from cellulose I.

The crystallite size in the direction perpendicular to the cellulose I $_{\beta}$ (200) crystalline plane ($D_{(200)}$) was estimated by employing the well-known Scherrer equation:

$$D_{(200)} = \frac{k \cdot \lambda}{B_{(200)} \cdot \cos \theta} \quad (5)$$

where k is the Scherrer constant (0.94), λ is the X-ray wavelength, $B_{(200)}$ is the full-width at half-maximum of the reflection 200 in radians and 2θ is the corresponding Bragg angle.

237   **2.8 Water vapor permeability (WVP)**

238   Direct permeability to water was determined from the slope of the weight gain versus
239   time curves at 24°C. The films were sandwiched between the aluminum top (open O-
240   ring) and bottom (deposit for the silica) parts of a specifically designed permeability
241   cell with screws. A Viton rubber O-ring was placed between the film and bottom part
242   of the cell to enhance sealability. These permeability cells containing silica were then
243   placed in an equilibrated relative humidity cabinet at 75% RH and 25 °C. The weight
244   gain through a film area of 10 cm² was monitored and plotted as a function of time.
245   Cells with aluminum films (with thickness of ca. 11 µm) were used as control samples
246   to estimate weight gain through the sealing. The tests were done at least in triplicate.

247

248   **2.9 Water uptake**

249   The water uptake of the lignocellulosic films was estimated from sorption experiments
250   at 25°C and 75% RH by means of weight gain using a Precisa Gravimetrics AG
251   SERIES 320XB analytical balance (Dietikon, Switzerland). Square specimens with a
252   total surface area of 6.25 cm² were cut from the films and their initial weight was
253   registered. The assays were carried out at least in triplicate.

254

255   **2.10 Contact angle measurements**

256   Contact angle measurements were carried out at ambient conditions in a Video-Based
257   Contact Angle Meter model OCA 20 (DataPhysics Instruments GmbH, Filderstadt,
258   Germany). Contact angle values were obtained by analyzing the shape of a distilled
259   water drop after it had been placed over the film for 15 s. Image analyses were carried
260   out by SCA20 software.

261

262 **2.11 Mechanical properties**

263 Tensile tests were carried out at ambient conditions of typically 24°C and 50% RH on
264 a Mecmesin MultiTest 1-i (1 kN) machine (Virginia, USA) with the Emperor™
265 software. Pre-conditioned rectangular-shaped specimens with initial gauge length of 8
266 cm and 1 cm in width were cut directly from the films. A fixed crosshead rate of 25
267 mm/min was utilized in all cases. The elastic modulus (E), tensile strength (TS), and
268 elongation at break (ϵ_B) were determined from the stress-strain curves, estimated from
269 force–distance data obtained for the different films. At least, two specimens of each
270 film were tensile tested as to obtain statistically meaningful results.

271

272 **2.12 UV and visible transmittance**

273 Spectral transmittance of film samples was recorded on a 8453 Agilent UV–Vis
274 spectrophotometer. A suitable size of rectangle film sample was directly inserted in a
275 quartz cuvette and scanned in the UV-visible range 200–700 nm with an empty cuvette
276 as a reference. UV and visible transmittance factor T_F (transmittance of a film sample
277 per unit thickness) is defined as the following expression:

$$278 \quad T_F = \frac{T_{300}}{x}, \frac{T_{350}}{x}, \frac{T_{450}}{x}$$

279 where T_{300} , T_{350} or T_{450} is the value of transmittance at 300 nm (UVB), 350 nm (UVA)
280 or 450 nm (visible), and x is the film thickness (μm).

281

282 **2.13 β -Carotene-linoleic acid assay**

283 The antioxidant capacity of the cellulosic films was evaluated by the β -carotene-
284 linoleic acid assay, according to (Martins et al., 2013). In brief, 2 mg of β -carotene

was dissolved in 10 mL of chloroform. 2 mL of this solution were placed on a rotary evaporator and the chloroform was evaporated. Then, 50 μ L of linoleic acid and 400 mg of Tween 40 were added and the content of the flask was mixed with stirring. After that, 100 mL of aerated distilled water was transferred to the flask and stirred vigorously. Approximately 25 mg of films, or 0.5 mL of water as a control were transferred to test tubes and then 5 mL of the β -carotene emulsion were added. The samples were incubated in a water bath at 50 °C for 120 min. The absorbance of each sample at 470 nm was measured every 15 minutes using a spectrophotometer. The determinations were carried out in triplicate.

2.14 Statistics

All data have been represented as the average $\pm$ standard deviation. Different letters show significant differences both in tables and graphs ($p \leq 0.05$). Analysis of variance (ANOVA) followed by a Tukey-test were used.

3. Results and discussion

3.1. Characterization of the waste biomass and the extracted cellulosic fractions

The residues generated after the extraction of agar from *Gelidium sesquipedale* seaweed by applying hot-water treatments with (NaOH+HW) and without (HW) an alkali pre-treatment were valorized in this work to evaluate the possibility of extracting cellulose-rich fractions. The composition of the two types of residues was investigated and the results are compiled in Table 1. As observed, both residues were mainly composed of structural carbohydrates (i.e. cellulose and non-cellulosic carbohydrates). Although a detailed carbohydrate composition of *Gelidium sesquipedale* is not

309 available in the existing literature, seaweed from other *Gelidium* species have been
310 reported to contain ca. 15-17% cellulose (Chen et al., 2016; Park et al., 2011). Thus,
311 the remaining carbohydrate must correspond to residual agar and other
312 polysaccharides such as floridean starch, which are less abundant in the raw seaweed
313 (Carmona et al., 1998). As expected, given the small lignin contents typically reported
314 for *Gelidium* seaweed (Chen et al., 2016; Wi, Kim, Mahadevan, Yang, & Bae, 2009),
315 the lignin content in both residues was quite low. The residues also contained
316 significant amounts of ashes and lipids and minor amounts of proteins. These
317 components were more abundant in the HW residue, which is not surprising since they
318 can be digested by the alkali pre-treatment applied to the NaOH+HW residue
319 (Martínez-Sanz et al., 2019a; Martínez-Sanz et al., 2019b).

320

321 After subjecting the residues to the extraction protocols described in section 2.1.2,
322 different cellulosic fractions were obtained, which were expected to present distinct
323 composition and degree of purity (cf. Figure 1). The extraction yields obtained for the
324 F2 fractions were: $12 \pm 2\%$ for F2, $19 \pm 2\%$ for F2A, $27 \pm 1\%$ for F2 NaOH and $27 \pm 5\%$
325 for F2A NaOH. Comparing these values with the structural carbohydrate content in
326 the residues, it is evident that a significant amount of carbohydrates was digested upon
327 the NaClO_2 treatment. The greater extraction yields obtained from the HW+NaOH
328 residue were already anticipated due to its higher content in structural carbohydrates.
329 However, it should be considered that the alkali pre-treatment not only digested the
330 non-carbohydrate components present in the seaweed, but also produced a partial
331 degradation of the carbohydrate fraction. Hence, a significant amount of material was
332 lost and the efficiency of the process was much lower. The agar extraction yields were

ca. 2-3% vs. 10-12% and the amount of residue generated was 32 wt.-% (with respect to the raw seaweed) vs. 62 wt.-% for the extraction with and without the alkali pretreatment, respectively. Since the HW residue would clearly be more interesting from an economical perspective, it was selected to isolate cellulose by means of more complex purification protocols (cf. Figure 1). As expected, lower extraction yields were obtained for the more purified F3 fractions: 8±2% for F3, 9±1% for F3A and 11±1% for F3B. The similar extraction yields of F3A and F3B suggest that most of the compounds removed by NaClO₂ were also digested with KOH.

Table 1. Composition of the agar-extracted *Gelidium sesquipedale* waste biomass.

	Structural carbohydrates (%)	Lignin (%)	Ash (%)	Proteins (%)	Lipids (%)
HW	42.0 ± 3.4 ^a	2.1 ± 0.4 ^a	13.8 ± 0.5 ^a	1.6 ± 0.2	25.0 ± 3.2 ^a
NaOH+HW	59.9 ± 6.9 ^a	4.1 ± 2.1 ^a	7.8 ± 0.7 ^b	< 1%	10.0 ± 1.3 ^b

All the values are provided on a dry basis. Values with different letters are significantly different ($p \leq 0.05$).

To better understand the compositional differences between the extracted fractions, they were characterized by means of FT-IR and the obtained spectra are shown in Figure 2. As observed in Figure 2A, the spectra from the extracted F2 fractions were significantly different to those from the residues. In general, all the samples showed a broad band centered at ca. 3300 cm⁻¹, associated with the stretching vibration of hydrogen bonds in OH groups. This band became sharper and more intense in the F2 and F2 NaOH fractions, confirming their more hydrophilic character. The peaks centered at ca. 1730 cm⁻¹ and 1530 cm⁻¹, assigned to the presence of hemicelluloses

(non-cellulosic carbohydrates) and lignin (Seca et al., 2000; X. F. Sun, Xu, Sun, Fowler, & Baird, 2005), were visible in all the F2 fractions, being more intense those extracted from the HW residue. Several bands indicative of the presence of agar were also detected in all the samples. In particular, the bands located at 1260 cm^{-1} (characteristic of sulphate ester groups), 930 cm^{-1} (assigned to the presence of 3,6-anhydro-galactose residue) and 890 cm^{-1} (corresponding to the C-H bending at the anomeric carbon in β -galactopyranosyl residues) (Gómez-Ordóñez & Rupérez, 2011; Pereira, Sousa, Coelho, Amado, & Ribeiro-Claro, 2003) were clearly observed in the residues and the fractions, suggesting that some residual agar remained in the samples. On the other hand, the peaks at 1100 , 1050 and 985 cm^{-1} (corresponding to C-C, C-O, C-H stretching and C-OH bending modes in cellulose (Khiari, Marrakchi, Belgacem, Mauret, & Mhenni, 2011; Oh, Yoo, Shin, & Seo, 2005)) (pointed by arrows in Figure 2A), became more intense in the extracted F2 fractions, confirming a greater concentration of cellulose after bleaching. As expected, most of the bands characteristic of non-cellulosic carbohydrates were absent in the spectra of the F3 fractions (cf. Figure 2B). The spectra from F3 was identical to that previously reported for pure cellulose from other marine resources (Benito-González, López-Rubio, & Martínez-Sanz, 2019b; Chen et al., 2016; Martínez-Sanz et al., 2018), showing cellulose characteristic bands such as those appearing at 1430 , 1300 , 1100 and 1050 cm^{-1} (pointed by arrows in Figure 2B), hence confirming the purification of cellulose. Interestingly, the sample subjected to a single alkaline treatment (F3B) displayed several peaks indicative of the presence of residual agar, confirming that the two-step purification protocol was required to obtain high-purity cellulose.

Figure 2. FT-IR spectra of (A) the agar-extracted *Gelidium sesquipedale* waste biomass and the F2 fractions and (B) the F3 fractions. Arrows point out to cellulose characteristic bands.

383

384 Solid-state ^{13}C CP/MAS NMR characterization was also carried out and the results are
385 show in Figure 3. As observed, the cellulose characteristic peaks had a very similar
386 appearance for all the fractions, suggesting no major structural changes in the cellulose
387 microfibrils. Interestingly, all the F2 fractions and, to a lesser extent, the F3B fraction,
388 displayed additional small peaks located at ca. 70.6 ppm, 80.6 ppm, 99.1 ppm and
389 102.9 ppm (highlighted with arrows in Figure 3). These peaks are known to correspond
390 to the carbons from the β -D-galactopyranose (coded as G1-G6) and 3,6 anhydro- α -L-
391 galactopyranose residues (coded as A1-A6) in agar (Guerrero, Etxabide, Leceta,
392 Peñalba, & De la Caba, 2014), hence confirming the presence of residual agar. This
393 suggests that a two-step purification process combining the bleaching and basic
394 treatments is required to remove all the agar initially present in the residue. It should
395 be noted that small peaks located at ca. 35 ppm, indicative of the presence of lipids in
396 cellulosic samples (Benito-González et al., 2019a; Tuo Wang, Park, Cosgrove, &
397 Hong, 2015), were detected in all the samples in which the Soxhlet treatment was
398 omitted.

399

402 **Figure 3.** ^{13}C CP/MAS NMR patterns of the cellulosic fractions extracted from the
 403 agar-extracted *Gelidium sesquipedale* waste biomass ((A) F2 fractions and (B) F3
 404 fractions). Cellulose characteristic peaks are designated as C1-C6 whereas agar
 405 characteristic peaks (G1, G2, A1, A2 and A3) are pointed by arrows.
 406

407 To determine the effect of the different composition on the thermal stability of the
408 cellulosic fractions, these were analyzed by TGA. As deduced from the derivative
409 thermogravimetric profiles shown in Figure 4, the more purified F3 and F3A fractions
410 showed much sharper degradation peaks. The F2 fractions presented broad peaks with
411 an initial shoulder at 230-260 °C, followed by the main degradation peaks, with
412 maxima at 303°C for F2, 299 °C for F2A, 320 °C for F2 NaOH and 316 °C for F2A
413 NaOH. The initial degradation shoulder was ascribed to the presence of agar
414 (Martínez-Sanz et al., 2019a), while the main degradation peak corresponded to
415 cellulose (Amiralian et al., 2017; Song, Hu, Zhu, Wang, & Chen, 2004). The slightly
416 greater thermal stability from the fractions obtained from the alkali-treated residue
417 might be indicative of a greater cellulose content, which is not surprising given their
418 greater amount of structural carbohydrates in the NaOH+HW residue (cf. Table 1).
419 Further purification of the fractions led to increased thermal stability, with the F3 and
420 F3A fractions showing their maxima in the degradation peaks at 353 °C and 352 °C,
421 respectively. Interestingly, the F3B fraction showed a degradation behaviour similar
422 to that from the F2 fractions, with a lower degradation temperature of 326 °C. This
423 again supports the fact that some residual agar remained in this fraction.

424

Figure 4. Derivative thermogravimetric (DTG) curves of the cellulosic fractions extracted from the agar-extracted *Gelidium sesquipedale* waste biomass. (A) F2 fractions and (B) F3 fractions.

3.2. Characterization of cellulosic films from the extracted fractions

The extracted cellulosic fractions were used to generate films from aqueous suspensions. All the fractions presented film forming capacity and Figure 5A shows

434 the visual appearance of the produced films. As observed, all the films were translucent
435 and presented a whitish tonality. Interestingly, the films from the most purified
436 fractions (F3 and F3A) presented an opaquer appearance. This may be due to the more
437 amorphous character of the non-cellulosic components, such as agar, present in the
438 less purified films, which favored the transmission of visible light. As observed in
439 Figure 5B, all the films presented very low transmittance values in the UVA, UVB and
440 visible wavelength regions. Surprisingly, the F3B film presented much higher
441 transmittance values, which could be attributed to a greater content of amorphous agar
442 in this particular fraction.

443

444

Figure 5. (A) Contact transparency from the cellulosic films obtained from the agar-extracted *Gelidium sesquipedale* waste biomass. From top to bottom and left to right: F2, F2A, F2 NaOH, F2A NaOH, F3, F3A and F3B. (B) Transmittance factor of the cellulosic films in the UV-B, UV-A and visible wavelength regions.

The morphology of the films' surface was analyzed by SEM and representative images are shown in Figure 6. The films from the F2 fractions presented a very similar morphology of continuous and rough surfaces, very different to that previously observed in cellulosic films from other aquatic resources, where fibrous material was clearly identified (Benito-González et al., 2019a; Martínez-Sanz et al., 2018), but comparable to that previously observed in agar films (Martínez-Sanz et al., 2019b). This suggests that the residual agar acted as an amorphous matrix in which the remaining components were embedded. The presence of entangled cellulose fibres was clearly observed in the pure cellulose F3 film, whereas the F3A and F3B films

460 presented a more compacted structure where the cellulose fibres seemed to be
461 embedded in an amorphous matrix.

462

463

464 **Figure 6.** SEM images from the surface of the cellulosic films obtained from the
465 agar-extracted *Gelidium sesquipedale* waste biomass. Scale bars correspond to 30
466 μm .

467
468 To evaluate the impact of the non-cellulosic components on the crystallinity of the
469 films, XRD analyses were carried out and the obtained patterns are plotted in Figures
470 7A and 7B. All the films presented a very broad diffraction band within the range of
471 $12\text{-}18^\circ$, followed by a sharp peak located at ca. 22.6° , indicating the presence of
472 crystalline cellulose I. Cellulose crystallinity was calculated from the area of the
473 crystalline peaks and the values are listed in Table 2. As observed, in general the
474 crystallinity increased with the purification degree, being the F3 film the most
475 crystalline. The greater crystallinity of the F2 fractions extracted from the NaOH-
476 treated residue are in agreement with the FT-IR and TGA analyses and confirm that a
477 greater amount of cellulose was present in these samples. The presence of significant
478 amounts of amorphous agar in the F2 and F2A fractions, as well as the F3B fraction,
479 was responsible for their lower crystallinities. The crystallinity value for the F3 film
480 ($X_C \sim 56\%$) is within the range of values previously reported for cellulosic fractions
481 extracted from other agar-rich red seaweed biomass, which vary from 33% (Singh et
482 al., 2017) to 66% (El Achaby et al., 2018). Remarkably, most of the samples showed
483 two narrow peaks located at 21° and 26.7° arising from the presence of mineral
484 components naturally occurring in *Gelidium* seaweed, such as silica (SiO_2) and
485 weddellite ($\text{CaC}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$) (El Achaby et al., 2018; Martínez-Sanz et al., 2019a). The
486 results indicate that none of the applied treatments was able to completely remove
487 these minerals, which remained even in the most purified F3 fraction.

488

489 Small angle X-ray scattering (SAXS) experiments were also carried out and the
 490 obtained scattering patterns are displayed in Figures 7C and 7D. Most of the samples,
 491 except for the F3B film, presented a power-law behavior within the range of 0.002-
 492 $0.006 \text{ \AA}^{-1}$, characterized by exponents of -3.6-3.9, which are indicative of surface
 493 fractal structures (i.e. rough interfaces at the corresponding size range of ca. 105-315
 494 nm). The F3B film showed the appearance of a shoulder-like feature centered at ca.
 495 $0.006 \text{ \AA}^{-1}$ (pointed out by an arrow in Figure 7D), which is similar to that previously
 496 reported for agars extracted from *Gelidium sesquipedale* (Martínez-Sanz et al., *in*
 497 *press*), thus confirming the greater amount of residual agar in this fraction.

498

499

500 **Figure 7.** (A-B) XRD patterns and (C-D) SAXS Kratky plots of the cellulosic films
501 obtained from the agar-extracted *Gelidium sesquipedale* waste biomass.

502

503 The performance properties of the films were also characterized to evaluate their
504 potential as food packaging materials. The mechanical properties were assessed by
505 means of tensile testing and the most representative parameters obtained from the
506 stress-strain curves are summarized in Table 2. In general, the mechanical properties
507 of the films were not largely affected by the purification degree of the fractions.
508 Despite the apparently greater cellulose content in the F2 fractions obtained from the
509 NaOH+HW residue, their films presented lower elastic modulus and tensile strength
510 and higher elongation at break than the films from the HW residue fractions. This can
511 be explained by the better mechanical performance of agar films (Martínez-Sanz et al.,
512 2019b) as compared with cellulose microfibrils. Thus, the greater amount of residual
513 agar in the F2 and F2A fractions led to more rigid and tougher films than F2 NaOH
514 and F2A NaOH. Skipping the Soxhlet treatment gave rise to more ductile films, most
515 likely due to the presence of small amount of plasticizing lipidic compounds. The
516 mechanical properties of the F2 film are superior that those previously reported for
517 holocellulosic fractions extracted from *Posidonia oceanica* biomass ($E \sim 0.8$ GPa and
518 $\epsilon_b \sim 1.1\%$) (Benito-González et al., 2019a) and from *Arundo donax* ($E \sim 1.5$ GPa and
519 $\epsilon_b \sim 1.5\%$) (Martínez-Sanz et al., 2018), again supporting the favorable effect of agar in
520 the *Gelidium*-derived fractions. Although further purification of the F2 fraction was
521 not beneficial for the mechanical performance of the films due to the removal of agar,
522 the properties of the F3A film were improved as compared with the F2A film (probably
523 due to the removal of non-holocellulosic impurities). Interestingly, the F3B film

presented the most optimal mechanical properties, with the highest elastic modulus and tensile strength and a good compromise for the elongation at break. This material presented very promising properties, showing better mechanical performance than cellulosic films extracted from *Arundo donax* ($E \sim 2.3$ GPa and $\epsilon_b \sim 2.4\%$) (Martínez-Sanz et al., 2018) and more ductile behavior than the cellulosic films from *Posidonia oceanica* biomass ($E \sim 4.2$ GPa and $\epsilon_b \sim 2.1\%$) (Benito-González et al., 2019a).

Table 2. Extraction yields, crystallinity, mechanical and water barrier properties of the cellulosic films obtained from the agar-extracted *Gelidium sesquipedale* waste biomass.

	Extraction yield (%)	X _C (%)	E (MPa)	TS (MPa)	ϵ_b (%)	$P_{H_2O} \cdot 10^{-13}$ (Kg·m/s·m ² ·Pa)
F2	12.1 ± 1.9 ^a	36.2	1955.9 ± 453.3 ^b	32.7 ± 17.7 ^a	4.4 ± 1.4 ^c	1.03 ± 0.26 ^a
F2A	18.6 ± 2.1 ^b	33.5	969.0 ± 233.9 ^c	17.3 ± 4.4 ^a	5.0 ± 0.9 ^b	0.69 ± 0.22 ^a
F2 NaOH	27.3 ± 1.3 ^c	42.3	1227.0 ± 447.3 ^c	27.1 ± 6.4 ^a	6.0 ± 0.5 ^b	1.03 ± 0.18 ^a
F2A NaOH	27.3 ± 4.7 ^c	43.0	576.4 ± 124.1 ^d	17.3 ± 3.6 ^a	8.4 ± 0.6 ^a	1.22 ± 0.13 ^a
F3	8.0 ± 1.7 ^a	55.6	1220.2 ± 158.9 ^c	22.9 ± 1.5 ^a	4.5 ± 0.4 ^b	0.99 ± 0.09 ^a
F3A	8.6 ± 0.6 ^a	49.3	1635.4 ± 136.2 ^b	27.1 ± 3.8 ^a	3.8 ± 0.6 ^c	1.11 ± 0.17 ^a
F3B	10.7 ± 0.7 ^a	29.7	2493.4 ± 162.8 ^a	38.4 ± 5.0 ^a	5.0 ± 1.1 ^b	0.83 ± 0.07 ^a

X_C: Crystallinity index determined from the XRD patterns; E: Young's modulus; TS: tensile strength; ϵ_b : elongation at break; P_{H_2O} : water permeability. Values with different letters are significantly different ($p \leq 0.05$).

With regards to the water vapour permeability (WVP) of the films, the results, also compiled in Table 2, evidence no major differences between the samples. As opposed to previous works (Benito-González et al., 2019a; Martínez-Sanz et al., 2018), the removal of non-cellulosic carbohydrates and further purification of cellulose did not have a positive impact on the barrier performance of the films. This might be again

related to the greater barrier capacity of agar films compared to pure cellulose. For instance, values of $0.86 \cdot 10^{-13} \text{ Kg} \cdot \text{m/s} \cdot \text{m}^2 \cdot \text{Pa}$ have been measured for agar films (plasticized with 10% glycerol) (Martínez-Sanz et al., 2019b), while pure cellulosic films from other aquatic biomass resources present WVP values around $1.2\text{-}19 \cdot 10^{-13} \text{ Kg} \cdot \text{m/s} \cdot \text{m}^2 \cdot \text{Pa}$ (Benito-González et al., 2019a; Martínez-Sanz et al., 2018). Accordingly, the two less purified fractions F2A and F3B gave rise to the most optimum films in terms of water vapor barrier, outperforming benchmark biopolymers such as starch (Benito-González et al., 2018) and PLA (Martínez-Sanz, Lopez-Rubio, & Lagaron, 2012).

The water sorption capacity of the films was also evaluated through gravimetric tests (cf. Figure 8A). According to what was expected, the removal of the non-cellulosic carbohydrates (mainly agar) in the F3 and F3A fractions produced a decrease in the amount of water absorbed by the films. This can be directly related to the more amorphous character of agar-rich domains, more accesible to moisture than the impermeable cellulose crystallites. The results indicate that the lower permeability of the agar-containing films is obviously not related to a reduction in the water sorption, but must be a consequence of a hindrance in the diffusion of water molecules through the films, similar to what has been reported for starch films (Benito-González et al., 2019b).

The water affinity of the films' surface was also evaluated through contact angle measurements. The estimated contact angle values (cf. Figure 8B) evidence that the most purified F3 film showed a much more hydrophilic surface (contact angle $\sim 21^\circ$).

567 The presence of other components in the F3A and F3B fractions led to a marked
 568 increase in the contact angle values ($\sim 92^\circ$ and $\sim 95^\circ$, respectively), which may be
 569 attributed to the presence of agar (with reported contact angles of $78\text{--}114^\circ$ (Martínez-
 570 Sanz et al., 2019b)) and to a lesser amount of free hydroxyl groups in the surface of
 571 the films due to cellulose-agar interactions. Thus, the presence of agar promoted a
 572 more hydrophobic behaviour at the surface of the films, but on the other hand, its more
 573 amorphous character enabled greater amounts of water to be sorbed within the internal
 574 structure of the films.

575

576

577

578 **Figure 8.** (A) Water sorption and (B) calculated contact angle values for the cellulosic
 579 films obtained from the agar-extracted *Gelidium sesquipedale* waste biomass.

580

581 A previous work has shown that non-purified cellulosic fractions extracted from
 582 aquatic biomass may present antioxidant properties (Fontes-Candia et al., 2019). In
 583 order to assess the antioxidant capacity of the produced films, β -carotene bleaching
 584 assays were carried out by soaking the films in β -carotene emulsions and evaluating
 585 their potential to inhibit the degradation of the emulsion when heating. The absorbance
 586 of the β -carotene emulsions at 470 nm as the heating reaction progressed was measured
 587 and the results are shown in Figure S1. The β -carotene emulsion was almost
 588 completely bleached after 2 h, whereas some of the cellulosic films, especially F2A
 589 and F3B, were able to delay the degradation of β -carotene, with the emulsion still
 590 showing a yellow coloration after 2 h. The total inhibition values estimated at the end
 591 of the experiments were $20 \pm 1\%$ for F2, $38 \pm 9\%$ for F2A, $10 \pm 3\%$ for F2 NaOH, 13
 592 $\pm 2\%$ for F2A NaOH, $7 \pm 1\%$ for F3, $12 \pm 1\%$ for F3A and $40 \pm 5\%$ for F3B. Clearly,

the inhibition capacity of the films was negatively correlated with the degree of purity of the fractions. To the best of our knowledge, the antioxidant capacity of cellulosic films has not been studied before. Fontes-Candia et al. reported ca. 33% inhibition for non-purified cellulosic fractions extracted from *Arundo donax* biomass when produced in the form of aerogels (Fontes-Candia et al., 2019), while the films did not show any significant antioxidant capacity. Although antioxidant capacity has been typically linked to the presence of phenolic compounds in plant extracts, some studies have shown that the phenolic content of red seaweeds is not directly correlated to their antioxidant potential (Tello-Ireland, Lemus-Mondaca, Vega-Gálvez, López, & Di Scala, 2011), which in turn may be ascribed to other bioactive components such as sulphated polysaccharides, peptides and aminoacids (Rocha de Souza et al., 2007; Tao Wang et al., 2010; Yuan, Westcott, Hu, & Kitts, 2009). In fact, no significant amounts of phenolic compounds were detected in the fractions by means of the Folin-Ciocalteu colorimetric assay. However, it should be considered that some polyphenols can be bound to cellulose (Liu, Martinez-Sanz, Lopez-Sanchez, Gilbert, & Gidley, 2017) and thus not being detected by standard protocols. Although the UV-Vis spectra from all the films showed the appearance of the absorbance band characteristic from phycoerythrins (i.e. proteins with photosynthetic activity) at 564 nm (Beer & Eshel, 1985), the relative intensity was the same for all the films; therefore, this would not explain the greater antioxidant capacity of F2A and F3B. Consequently, the antioxidant capacity of the less purified fractions must be related to the release of bioactive compounds such as glycosylated polyphenols and minerals, which can be interacting with the agar and/or the cellulose in the films.

617 The results presented in this work indicate that the less purified F3B and F2A fractions
618 would be the optimum choice to develop food packaging materials since both present
619 a good compromise between optimum performance properties (mechanical properties,
620 barrier performance, transparency and thermal stability) and high extraction yields and
621 lower production costs (given the reduced amount of purification steps required for
622 their production).

623

624 **4. Conclusions**

625 Cellulose-based fractions were extracted from the residues generated after the
626 extraction of agar from *Gelidium sesquipedale* seaweed by applying a hot-water
627 treatment, with (NaOH+HW residue) and without (HW residue) an alkali pre-
628 treatment. The residues were mainly composed of structural carbohydrates (in
629 particular, residual agar); however, they also contained significant amounts of ashes
630 and lipids, especially in the case of the HW residue. The residual agar was only
631 removed completely after applying a two-step process based on bleaching and alkaline
632 treatments, producing the most purified cellulosic fractions with yields of ca. 8-9%.

633

634 All the obtained fractions presented film forming capacity and produced materials with
635 good performance. Although the holocellulosic fractions extracted from the
636 NaOH+HW residue seemed to present higher cellulose contents, the properties of the
637 films were very similar to their counterparts obtained from the HW residue. This,
638 together with the greater mass losses produced by the alkaline pre-treatment (lower
639 agar extraction yields and reduced amount of residue), would make the utilization of
640 the HW residue more economically viable.

641

642 The residual agar found in the less purified fractions acted as an amorphous matrix in
643 which cellulose was embedded and was seen to have a positive impact on the
644 performance of the films, improving the transparency, the mechanical properties and
645 the water vapour permeability. In particular, the less purified F2A and F3B films
646 presented superior properties to those reported for benchmark biopolymers such as
647 starch and PLA. Moreover, these films were able to delay and inhibit the degradation
648 of β -carotene to a greater extent than the most purified ones.

649

650 These results evidence that the large waste streams generated after the extraction of
651 agar, currently underutilized by the industry, can be valorized to produce high
652 performance bio-based films based on cellulose and residual agar by means of
653 simplified protocols.

654

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661

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663

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843 **Supplementary Material**

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845 **Figure S1.** Kinetics of β -carotene bleaching for the control emulsion and the
 846 emulsions in contact with the cellulosic films obtained from the agar-extracted
 847 *Gelidium sesquipedale* waste biomass.