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Article

# The Invasive Seaweed *Agarophyton vermiculophyllum* from Oualidia Lagoon (Northwestern Moroccan Atlantic Coast) as a Source of Agar: Yield, Chemical Characteristics, and Rheological Properties

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**Abstract:** Agar is a hydrophilic biopolymer extracted from red seaweed. This phycocolloid consists of two components: agarose and agaropectin. In the present work, agar extracted from the invasive red seaweed *Agarophyton vermiculophyllum* was characterized using physical, chemical, and spectroscopic analyses to investigate the effect of alkaline pretreatment on agar properties. Two extraction conditions, native and alkali-pretreated agars, were comparatively studied. The native yield ( $28.4 \pm 0.9\%$ ) was higher than that of the alkaline-pretreated agar ( $20.4 \pm 0.8\%$ ). The alkali-pretreated agar showed higher gel strength ( $763.8 \pm 57.0 \text{ g cm}^{-2}$ ), gelling ( $36.5 \pm 0.9 \text{ }^\circ\text{C}$ ), and melting ( $83.2 \pm 0.6 \text{ }^\circ\text{C}$ ) temperatures and increased 3,6-anhydrogalactose ( $26.2 \pm 1.9\%$ ) and decreased sulfate contents ( $6.2 \pm 0.8\%$ ) compared with native agar (gel strength:  $204.8 \pm 17.10 \text{ g cm}^{-2}$ , gelling temperature:  $29.5 \pm 0.9 \text{ }^\circ\text{C}$ , melting temperature:  $73.4 \pm 0.7 \text{ }^\circ\text{C}$ , 3,6-AG content:  $13.8 \pm 0.7\%$ , sulfate content:  $10.5 \pm 0.5\%$ ). The alkaline pretreatment improved the agar's gelling properties and significantly influenced its chemical properties. In view of the obtained results, *A. vermiculophyllum* might potentially be thought of as a viable source for the agar industry in Morocco, serving as a local source of agar.

**Keywords:** agar properties; alkaline pretreatment; <sup>13</sup>C NMR; extraction; FTIR; invasive seaweed; North African lagoon



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## 1. Introduction

Agar-agar is a gel-forming substance derived from red macroalgae (Rhodophyceae). This phycocolloid is communally used as a gelling, thickening, and stabilizing food additive [1]. A complex mixture of polysaccharides such as agar and carrageenan can be extracted from red seaweed [2]. Agar consists of two main components, agarose and agaropectin. Agarose contributes high-strength gelling properties to agar, while agaropectin is related to weak gelling properties. Agar is a linear polysaccharide consisting of repetitive units of D-galactose and 3,6-anhydro-L-galactose, linked by alternating  $\alpha$ -(1 → 3) and  $\beta$ -(1 → 4) glycosidic bonds [3].

Agar presents various characteristics such as gelling, stabilizing, absorbing, and lubrication with different applications in the food, medicine, and biotechnological industries. Agar is the first phycocolloid used in the formulation of human food, accounting for 80%

of its consumption [3]. The utilization of agar in food applications is based on its characteristics, such as a hard texture, the heat tolerance of gels, the easy process of gelation, and stability in acidic environments [2]. Furthermore, agar is being researched as an alternative raw material for medicinal applications because of the strong gels it forms. It has been used in the development of injectable and phase-changeable composite hydrogels for treating cancers with chemotherapy and photothermal therapy [4].

Between 2000 and 2018, the amount of seaweed produced worldwide increased by more than two times, from 10.6 to 32.4 million tons [5]. The agar industry has shown a remarkable 7% yearly growth when referring to carrageenan and alginate. Worldwide industrial agar sales reached 14,500 t with a value of USD 246 million in 2015 [6]. Over the past ten years, the market demand has rapidly increased. Now, the agar business processes nearly 91% of all processed biomass using just *Gracilaria* as an agar source [7]. The value of *Gracilaria* is increasing along with its demand because of the high cost of production deficiency in the genus *Gelidium* and its wild populations. Thus, most of the agarophytes produced in the world contain *Gracilaria* [8]. There has been rising interest in the development of sustainable seaweed aquaculture practices that have the potential to increase the production capacity of seaweed polysaccharides. The overall aim is to improve the production capacity of these biomaterials, making them more available for wider use and enhancing their sustainability. Commercially available agar is primarily extracted from *Gelidium* and/or *Gracilaria/Gracilariopsis*. High-quality agar is traditionally made from *Gelidium* species, while *Gracilaria/Gracilariopsis* species result in agar with weaker gel properties. *Gracilaria/Gracilariopsis* are usually grown in Asian countries, producing about 98% of the world's supply [9]. *Gracilaria* is a genus with a large number of species, many of which have been used for a variety of purposes. The red seaweed *Agarophyton vermiculophyllum* (Ohmi) Gurgel, J.N.Norris et Fredericq, 2018 (previously named *Gracilaria vermiculophylla* (Ohmi) Papenfuss, 1967), originally from the northwestern Pacific, however, has virtually spread to almost all temperate estuaries in the Northern Hemisphere over the last 100 years. According to the Algaebase website, *A. vermiculophyllum* is currently accepted as a synonym of *Gracilariopsis vermiculophylla* Ohmi, 1956, and it is considered the basionym.

*Gracilaria* agar with low agarose content and high sulfate-group concentrations affects quality; one solution is to apply an alkaline treatment with sodium hydroxide [10,11]. The biotic and abiotic conditions, the physiological state of the seaweed, the agar extraction process, and the storage of the seaweed represent some of the variables that influence agar production and gelling qualities [12]. Prior to contemplating commercial applications, it is crucial to optimize alkali concentrations and extraction variables. The agar yield of *Gracilaria* sp. decreases as the NaOH concentration increases, and this variation can range from 10% to 50% depending on the species and season [13]. The abundance of seaweed resources has prompted researchers to explore various methods of utilizing them, including the extraction of a commercially valuable biopolymer and its characterization using diverse techniques. This is the first study carried out in Morocco regarding the extraction of agar from the invasive species *A. vermiculophyllum*. Other studies concerning other species of red seaweed have been conducted along the Moroccan coast dealing with the biochemical composition and seasonal variations in agar from *Gelidium latifolium* and *G. sesquipedale* [14,15]; the seasonal variations in growth and composition of agar from *Gracilaria multipartita* [16]; and agar from *Gracilaria gracilis*, *Gelidium corneum*, and *G. microdon* and its properties [17,18]. The properties of agar derived from the invasive marine seaweed *A. vermiculophyllum*, the effects of the alkaline treatment and extraction times on the agar, the seasonal variations in agar extracted from this red seaweed, and the stability of its colagar have been studied by many authors worldwide [19–22]. The present study hypothesizes that alkaline pretreatment conditions significantly influence the gel quality, yield, and physicochemical and gelling properties of agar from the seaweed *A. vermiculophyllum*.

The research was carried out with the purpose of determining the potential application of the invasive species *A. vermiculophyllum* from Morocco as a commercial agar producer by evaluating the yield and physicochemical, rheological, and spectroscopic characteristics of agar under two types of extraction.

## 2. Materials and Methods

### 2.1. Algal Material

The thalli of *A. vermiculophyllum* were collected in early spring (March) from muddy substrates during low tides at Oualidia Lagoon (northwestern Atlantic coast of Morocco). Samples of *A. vermiculophyllum* were washed and cleaned with fresh tap water to remove sand, debris, and extraneous epiphytes and then cleaned with distilled water. Seaweed specimens were air-dried, sun-dried, and put in an oven at 50 °C until reaching a stable weight and stored for further agar extraction.

### 2.2. Agar Extractions

#### 2.2.1. Native Agar Extraction

The agar extraction was performed according to the technique reported by Arvizu-Higuera et al. [19] and Villanueva et al. [21], with minor adjustments. Dry seaweed material (30 g) was added to distilled water with a ratio of 1:50 for 30 min at room temperature; soaking water was discarded; and then, the agar extraction was performed using hydrated material heated for 2 h at 85 °C in a water bath (Gesellschaft Für Labortechnik mbH, Burgwedel, Germany). The mixture was filtered through layers of cheesecloth. The filtrates were frozen for 24 h and then thawed. The resulting agar was dehydrated with ethanol and dried in an oven at 50 °C until it reached a constant weight.

#### 2.2.2. Alkaline Pretreatment for Agar Extraction

In total, 30 g of the seaweed *A. vermiculophyllum* was soaked for 30 min in 7% NaOH (Sigma-Aldrich, St. Louis, MO, USA). The solution was heated at 85 °C for 30 min. After alkali treatment, the samples were washed completely with tap water and then distilled water, and then, they were neutralized with 0.5% acetic acid (Sigma-Aldrich, St. Louis, MO, USA). The acetic acid solution was eliminated to remove the excess acid, and the seaweed was washed again with tap water and then distilled water. The following steps are similar to native extraction.

For the two agar extraction conditions, extractions were conducted in triplicate.

### 2.3. Spectroscopic Characterization

The Fourier transform infrared (FT-IR) spectra of the selected native, alkali-treated, and commercial agar samples (Bacteriological agar type E, Biokar diagnostics, Beauvais, France) were performed using a Thermo Scientific Nicolet Impact 400D FT-IR Spectrometer (Nicolet Instrument Co., Madison, WI, USA). The FT-IR spectra were scanned in attenuated total reflectance (ATR) mode and were recorded in a wavelength between 4000 and 500  $\text{cm}^{-1}$  with an average of 32 scans at a 4  $\text{cm}^{-1}$  resolution, and eventually, the OMNIC software was used to process the IR spectra (Nicolet, Madison, WI, USA).

Nuclear magnetic resonance ( $^{13}\text{C}$  NMR) spectroscopic measurements of agar samples were carried out at 353 K on an AV II spectrometer (Proton Larmor frequency of 400.33 MHz, Bruker Corporation, Billerica, MA, USA) operating at a frequency of 400 MHz and equipped with pulsed gradient by using a 5 mm Triple Resonance Broadband Inverse probe (Bruker Corporation, Billerica, MA, USA) at a base frequency of 100.62 MHz. During the relaxation delay and mixing period, presaturation was applied. The exponential multiplication apodization functions were performed in one dimension at 0.5 for line broadening before the Fourier transformation.

#### 2.4. Rheological Analyses

The gel strength of the agars was analyzed using a texture analyzer (CT3, AMETEK Brookfield, Middleboro, MA, USA). A 1.5% agar solution was poured into an AMETEK Brookfield gelatin bloom bottle following gel formation at room temperature, and it was subsequently refrigerated at 5 °C overnight to achieve stabilization. Afterward, the gel strength of the agar was assessed at room temperature using a texture analyzer probe (TA10 Cylinder; 12.7 mm diameter, 35 mm length); to measure the load in compression mode, the probe reached a depth of 4 mm during one cycle.

The melting and gelling temperatures of the agars were measured according to Freile-Pelegrin and Robledo's protocol [23], slightly modified. The gelling temperature was recorded by adding hot agar solution and a glass bead (5 mm diameter) to the test tubes. Then, the tubes were tilted up and down until the bead stopped moving, and the gel temperature was directly recorded. The melting temperature was tested using the same tubes by placing them in a water bath and increasing the temperature from 50 to 100 °C at 0.5 °C per min; as the bead dropped into the solution, the melting temperature was measured with a precision digital thermometer.

Hysteresis was calculated as the difference between the melting and gelling temperatures.

#### 2.5. Chemical Properties

The 3,6-anhydro-galactose (3,6-AG) content was calorimetrically measured using the resorcinol–acetal technique [24], using fructose as a standard. The reagent was prepared using resorcinol (Sigma-Aldrich, St. Louis, MO, USA), 1,1-diethoxyethane (Sigma-Aldrich, St. Louis, MO, USA), and concentrated HCl (Scharlau, Sentiment, Spain). The absorbance of 3,6-AG was measured at a wavelength of 555 nm. D-fructose (Sigma-Aldrich, St. Louis, MO, USA) was used as a standard. The 3,6-AG content was calculated and expressed as a percentage (based on dry weight).

The sulfate content was determined following the method of Craigie et al. [25] by hydrolyzing the extracted agar powder in hydrochloric acid HCl (2 N). The dosage was made using a BaCl<sub>2</sub>-gelatin reagent that contained gelatin (Sigma-Aldrich, St. Louis, MO, USA) and barium chloride (Sigma-Aldrich, St. Louis, MO, USA). The sulfate content was estimated using the BaCl<sub>2</sub> turbidimetric method [25], and the turbidity was measured at a wavelength of 550 nm in opposition to the blank. The standard used was K<sub>2</sub>SO<sub>4</sub> (Sigma-Aldrich, St. Louis, MO, USA).

#### 2.6. Data Analysis

A comparison of means was carried out using Student's *t*-test at a significance level of  $p < 0.05$  to find significant differences between the native and alkali-modified agars in order to test whether alkali pretreatment affects agar quality. Pearson's correlation analysis was used to investigate the relationship between agar characteristics for both treatments. Before conducting the above analysis, the data were subjected to a normality assessment using the Kolmogorov–Smirnov test and verification of variance homogeneity via Levene's test. The statistical analysis was performed using the SPSS Statistics software version 26 for Windows.

### 3. Results

#### 3.1. Agar Yield

The methods of extraction used had a direct impact on the agar yield. The agar yield obtained from the invasive species *A. vermiculophyllum* (Table 1) via native extraction ( $28.4 \pm 0.9\%$ , dry weight basis) differed significantly ( $p < 0.000$ ) from that obtained with the alkali pretreatment ( $20.4 \pm 0.8\%$ , dw) (Table 2). The yield and the composition of agars differ depending on species, collection season, environmental factors, extraction process conditions, etc.

**Table 1.** Yield of native and alkali-treated agars from *A. vermiculophyllum* harvested from Morocco compared with *A. vermiculophyllum* collected around the world.

Agar Yield (% dw)	Native Extraction	Alkali Pretreatment	References
<i>A. vermiculophyllum</i> (Morocco)	28.4 ± 0.9	20.4 ± 0.8	Current study
<i>A. vermiculophyllum</i> (Portugal)	18.44	10.8	[20]
<i>A. vermiculophyllum</i> (Portugal)	15	29.4	[21]
<i>A. vermiculophyllum</i> (Mexico)	34.6	15.3	[19]
<i>A. vermiculophyllum</i> (Mexico)	45.7	-	[26]
<i>A. vermiculophyllum</i> (Mexico)	-	11.4 ± 0.4	[27]

(Mean ± SE, n = 3). SE: standard error, dw: dry weight.

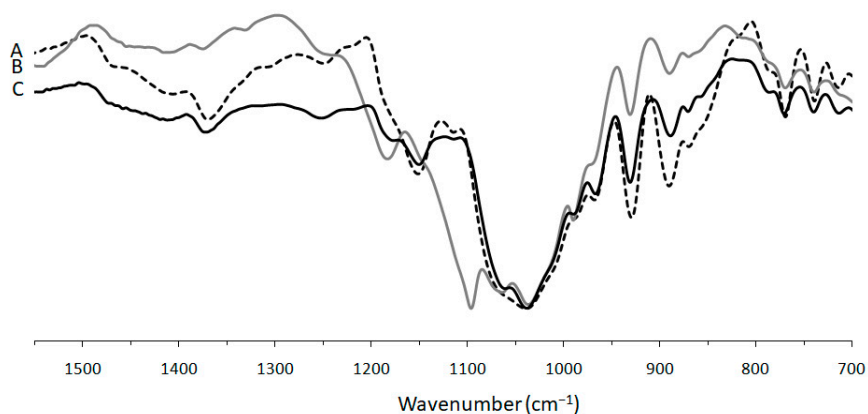
**Table 2.** *t*-test for Equality of Means of agar properties between the alkali and native treatments.

Agar Properties	t	<i>p</i> -Value
Agar yield	-10.687	<b>0.000</b>
Melting temperature	17.117	<b>0.000</b>
Gelling temperature	8.890	<b>0.001</b>
Gel strength	16.257	<b>0.000</b>
Sulfate content	-7.491	<b>0.002</b>
3–6 AG content	10.251	<b>0.001</b>

t: *t*-value. The significant values are in bold.

### 3.2. FT-IR Spectroscopy

The Fourier-transformed infrared spectra of the native and alkali-pretreated agars showed similar FTIR spectra (Figure 1) with characteristic peaks compared with the commercial agar (Biokar Diagnostics). The majority of certain agarocolloid-type bands were located between 700 and 1600 cm<sup>-1</sup> [28]. The spectra displayed major bands at 1035 and 1149 cm<sup>-1</sup> corresponding to C-O and C-C stretching vibrations in the pyranose ring typical of all polysaccharides [17,29]; bands at 1241 and 1368 cm<sup>-1</sup> have been recognized in ester-sulfate groups [29,30]. The band located around 715 cm<sup>-1</sup> is attributed to the C-O-C bending mode in glycosidic linkages [28]. The band at 928 cm<sup>-1</sup> is assigned to the C-O vibration of 3,6-AG [18,30]. A minor band at 845 cm<sup>-1</sup> is assigned to the sulfate groups at the C-4 of D-galactose [13,31]. Finally, the band situated at 886 cm<sup>-1</sup> is linked to the C-H bending at the C-1 of β galactopyranosyl [32].

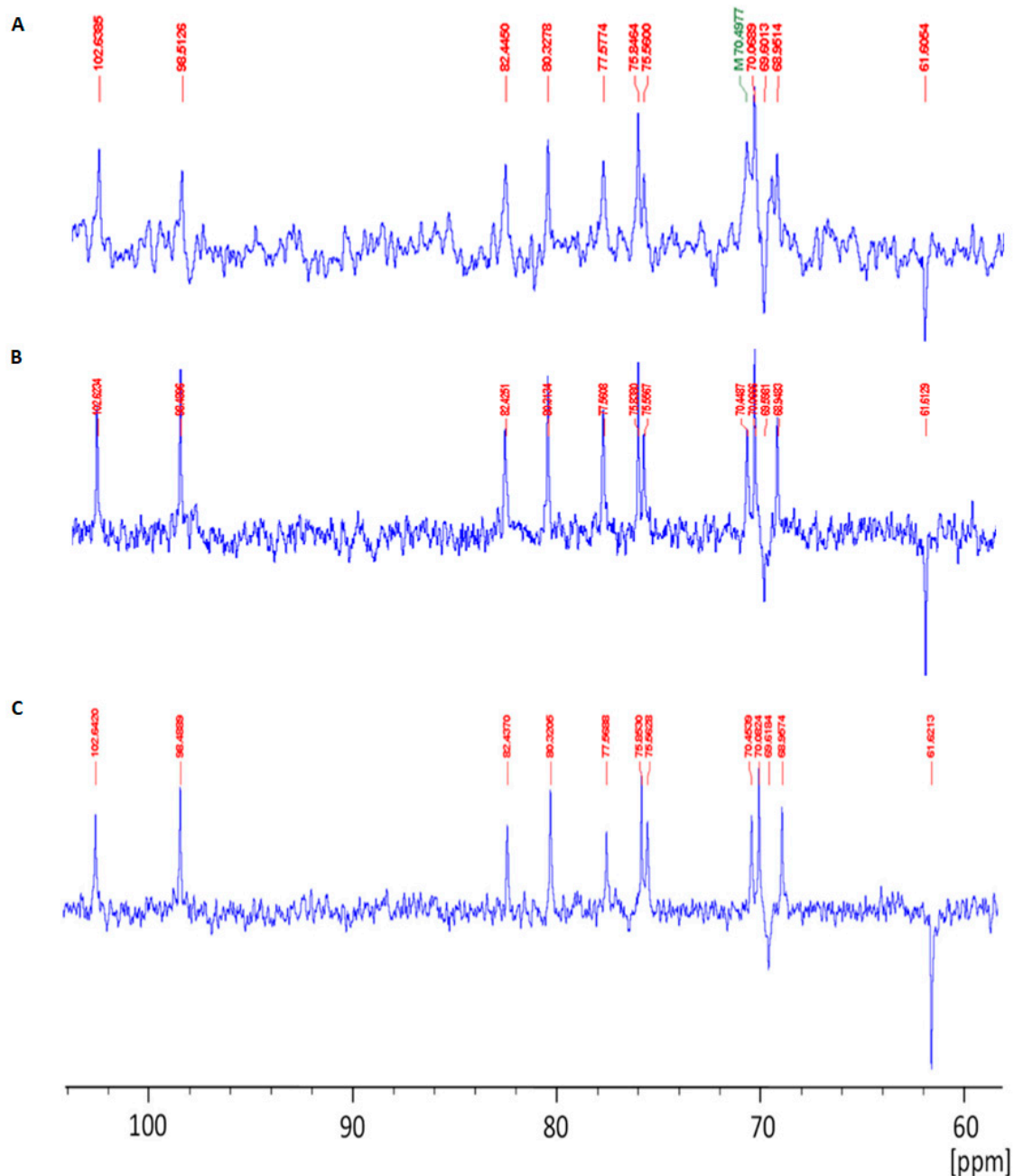


**Figure 1.** FTIR spectra of the agars extracted from *Agarophyton vermiculophyllum*: commercial agar (A), native agar (B), and alkaline pretreated agar (C).

### 3.3. <sup>13</sup>C NMR Spectroscopy

<sup>13</sup>C NMR spectroscopy (nuclear magnetic resonance spectroscopy) is used as an efficient technique to analyze red seaweed polysaccharides. <sup>13</sup>C NMR spectroscopy provides information on the environment of every carbon atom in the molecule. The <sup>13</sup>C NMR spectra of the native and the alkali extractions, as well as the commercial agar, showed 12 major

signals (Figure 2). The signals for 3,6-anhydro- $\alpha$ -L-galactopyranose were identified at 98.5, 80.3, 77.5, 75.8, 70.07, and 69.6 ppm, linked to C1, C3, C4, C5, C6, and C2, sequentially [33,34]. The signal noticed at 102.6 ppm conformed to the C1 of  $\beta$ -D-galactopyranose. In addition, the signals registered at 70.4, 82.4, 68.9, 75.5, and 61.6 ppm corresponded to C2, C3, C4, C5, and C6 and, subsequently, a three-linked  $\beta$ -D-galactopyranosyl unit [33]. The non-appearance of the peak at 59.0 ppm coinciding with the O-methyl group showed a poorly methylated agarose structure.



**Figure 2.**  $^{13}\text{C}$  NMR spectra of agar extracted from *Agarophytonvermiculophyllum*: (A) native agar; (B) agar pretreated with 7% NaOH; (C) commercial agar.

### 3.4. Determination of Gel Properties

The gel strength of the extracted agar revealed a significant difference between both treatments ( $t$ -test,  $p < 0.001$ ). The highest agar gel strength ( $763.8 \pm 57.04 \text{ g cm}^{-2}$ ) was obtained during the alkali pretreatment (Table 3), while the lowest gel strength was recorded

for the native extraction sample ( $204.8 \pm 17.10 \text{ g cm}^{-2}$ ). The gel strength of the tested commercial agar was  $909.8 \pm 187.60$  (Figure 3). The gelling and melting temperatures of the agar gel samples in this study were significantly influenced by both extraction conditions ( $t$ -test,  $p \leq 0.001$ ), with alkaline pretreatment showing the most elevated values ( $36.5 \pm 0.9 \text{ }^\circ\text{C}$  and  $83.2 \pm 0.6 \text{ }^\circ\text{C}$ , respectively), while the lowest values were obtained through the native treatment ( $29.5 \pm 0.9$  and  $73.4 \pm 0.7 \text{ }^\circ\text{C}$ , respectively).

**Table 3.** Rheological properties of agars from *A. vermiculophyllum* compared with some important agarophyte species.

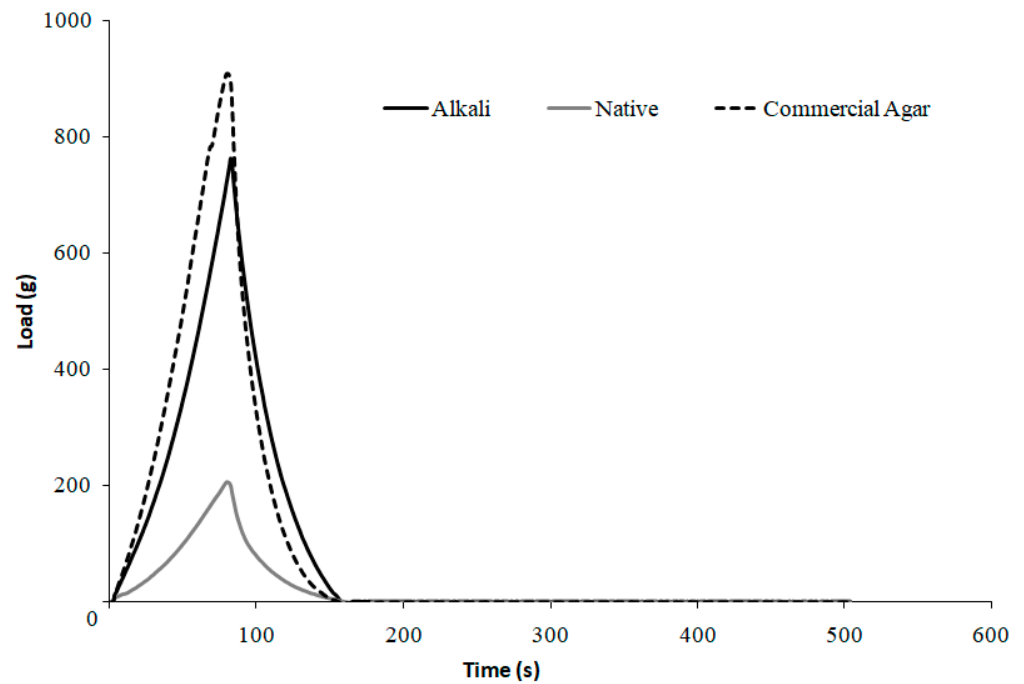
Species	Treatment	Gel Strength (g/cm <sup>2</sup> )	Gelling T° (°C)	Melting T° (°C)	References
<i>A. vermiculophyllum</i> (Morocco)	Native	204.8 ± 17.10	29.5 ± 0.9	73.4 ± 0.7	Current study
	Alkali	763.8 ± 57.04	36.5 ± 0.9	83.2 ± 0.6	Current study
<i>Gracilaria gracilis</i>	Native	105.3 ± 6.0	31.7 ± 0.2	78.5 ± 0.4	[17]
	Alkali	377.3 ± 19.7	35.4 ± 0.3	82.1 ± 0.1	
<i>Gelidium microdon</i>	Native	350.0 ± 17.6	35.1 ± 0.7	85.6 ± 0.2	[18]
	Alkali	489.0 ± 19.4	38.3 ± 0.8	88.3 ± 0.7	
<i>Gelidium corneum</i>	Native	341.0 ± 16.9	36.0 ± 0.9	86.7 ± 0.6	[18]
	Alkali	528.5 ± 11.0	39.2 ± 1.0	89.8 ± 0.1	
<i>Gracilaria fisheri</i>	Native	145.6 ± 34.5	49.25 ± 0.9	72.4 ± 0.1	[35]
	Alkali	228.2 ± 48.1	47.0 ± 0.0	72.3 ± 0.0	
<i>Gracilaria edulis</i>	Native	197.0 ± 72.8	60.2 ± 0.4	92.6 ± 0.3	[35]
	Alkali	239.9 ± 28.3	61.0 ± 1.0	87.6 ± 0.0	
<i>Gracilaria cliftonii</i>	Native	133.0 ± 9	34.7 ± 0.7	85.7 ± 1.2	[36]
<i>Gracilaria veleroae</i>	Alkali	343.5 ± 1.4	43.9 ± 0.3	86.0 ± 0.9	[27]
<i>Gracilaria tenuistipitata</i>	Alkali	624.4–1250.8	28.1–43.2	96.4–99.6	[37]
<i>Gracilaria tenuistipitata</i>	Alkali	482	35.5	103.3	[38]
<i>Gracilaria crassissima</i>	Alkali	1390	50	93	[39]
<i>Gracilaria lemaneiformis</i>	Alkali	1908	41.4 ± 0.5	-	[40]
<i>Gracilaria gracilis</i>	Native	437	31	85	[41]
	Alkali	210 ± 7.2	-	-	[42]
<i>A. vermiculophyllum</i> (Mexico)	Alkali	1064	35.7–39.6	92.4–99.7	[19]
<i>A. vermiculophyllum</i> (Mexico)	Native	85	27.7–36.5	73.9–53.5	[26]
<i>A. vermiculophyllum</i> (Mexico)	Alkali	644.0 ± 49.0	38.3 ± 0.6	99.7 ± 0.3	[27]
<i>A. vermiculophyllum</i>	Alkali	1331	40.7	93.1	[43]
<i>A. vermiculophyllum</i> (Portugal)	Alkali	614	35.4	79.1	[21]
<i>A. vermiculophyllum</i> (Portugal)	Native	247			[20]
	Alkali	348			

### 3.5. Determination of Chemical Properties

The chemical properties of the agar were influenced by the alkaline pretreatment. The 3,6-AG content was negatively linked to the sulfate content. In general, the increase in 3,6-AG content coincided with the decline in sulfate content. There was a significantly negative correlation between the sulfate content and the 3,6-AG content because of the L-galactose-6-sulfate, which was susceptible to converting into 3,6-anhydro-L-galactose under alkali



treatments [26,37,39]. The native agar showed significantly higher sulfate content ( $10.5 \pm 0.5\%$ ) than that of the alkaline pretreatment ( $6.2 \pm 0.8\%$ ) ( $p < 0.01$ ; Table 2). Likewise, the 3,6-AG content differed significantly ( $p < 0.01$ ) between both studied treatment conditions. The alkali-pretreated agar from *A. vermiculophyllum* had the greatest 3,6-AG content ( $26.2 \pm 1.9\%$ ), whereas the native extraction showed the lowest values ( $13.8 \pm 0.7\%$ ) (Table 4).



**Figure 3.** Comparison of texture analysis between the native, alkali-treated, and commercial agars using a texture analyzer.

**Table 4.** Agar physicochemical properties from *A. vermiculophyllum* and some agarophyte species.

Species	Treatment	Sulfate Content	3,6-AG Content	References
<i>A. vermiculophyllum</i> (Morocco)	Native	$10.5 \pm 0.5$	$13.8 \pm 0.7$	Current study
	Alkali	$6.2 \pm 0.8$	$26.2 \pm 1.9$	
<i>A. vermiculophyllum</i> (Mexico)	Alkali	1.86	42.5	[21]
<i>A. vermiculophyllum</i>	Alkali	-	$38.9 \pm 0.9$	[27]
<i>Gracilaria gracilis</i>	Native	$0.65 \pm 0.0$	$5.6 \pm 0.4$	[17]
<i>Gracilaria edulis</i>	Native	$7.5 \pm 0.1$	$37.0 \pm 2.8$	[35]
	Alkali	$4.9 \pm 0.0$	$43.0 \pm 0.4$	
<i>Gracilaria fisheri</i>	Native	$4.5 \pm 0.1$	$35.1 \pm 0.0$	[35]
	Alkali	$2.8 \pm 0.0$	$38.8 \pm 1.8$	
<i>Gracilaria cliftonii</i>	Native	$5.9 \pm 1.5$	-	[36]
<i>Gracilaria veleroae</i>	Alkali	-	$41.6 \pm 0.3$	[27]
<i>Gracilaria tenuistipitata</i>	Alkali	7.7–3.4	38.9–44.7	[37]
<i>Gracilaria tenuistipitata</i>	Alkali	1.6	-	[38]
<i>Gracilaria crassissima</i>	Alkali	-	43	[39]
<i>Gracilaria lemaneiformis</i>	Alkali	$0.7 \pm 0.0$	$41.3 \pm 0.8$	[40]
<i>Gracilaria gracilis</i>	Native	6.8	-	[41]
<i>Gracilaria salicornia</i>	Native	1.45	19.4	[42]
	Alkali	1.15	20.9	
<i>Gracilaria crassa</i>	Native	2.2	16.9	[42]
	Alkali	1.9	18.2	

#### 4. Discussion

The present study demonstrated that all the properties of the agar extracted from *A.vermiculophyllum* varied between both treatments. Other studies have confirmed the results accomplished using *A. vermiculophyllum*, with a higher agar yield during the native extraction (18%) compared with that attained using the alkaline treatment (10%) on *A. vermiculophyllum* from Portugal [20]. Belattmania et al. [17] reported that the yields of agar extracted from the red seaweed *Gracilaria gracilis* from the southern Moroccan Atlantic coast were  $20.5 \pm 1.3$  and  $15.16 \pm 2.5\%$  dw from the alkaline pretreatment (NaOH) and native extraction, respectively. The decrease in the agar yield was induced by subjecting *Gracilaria* to the alkaline treatment at high temperatures (80–100 °C) before the extraction process, resulting in the degradation of polysaccharides and agar failure due to diffusion during the pretreatment process [2,39]. On the other hand, an opposite effect for alkaline treatments has been demonstrated by other studies. Praiboon et al. [35] reported that agars obtained from pretreated *Gracilaria fisheri* and *G. edulis* using 5% NaOH exhibited a greater yield (34.3–39.6%) in comparison with native agars (10.9–13.3%). Alkaline pretreatment, using either NaOH or KOH within a concentration range of 3–7%, had the potential to enhance the agar yield [38]. In a study conducted by Vuai in 2022 [44], it was observed that different treatments had varying effects on the agar yield. The agar yield from *G. Salicornia*-treated agar was found to be  $21.9 \pm 0.7\%$ , which was higher compared with an untreated agar yield ( $15.8 \pm 0.7$ ). Additionally, agar treated with *G. corticata* resulted in a yield of  $27 \pm 0.7$ , slightly higher than the yield of untreated agar at  $26.2 \pm 1.3\%$ . *G. edulis*-treated agar had a yield of  $17.2 \pm 1.6\%$ .

Concerning gel strength, Orduña-Rojas et al. [26] indicated a gel strength value of  $158.0 \text{ g cm}^{-2}$  for 7% alkali-pretreated agar extracted from *A. vermiculophyllum* (previously called *Gracilaria vermiculophylla*) from Mexico. According to Arvizu-Higuera et al. [19], the alkali-pretreated agar gel strength accounted for  $1064 \text{ g cm}^{-2}$ . The breaking gel strength of alkali-pretreated agar from *G. vermiculophylla* is higher ( $348 \text{ g cm}^{-2}$ ) than that of native agar ( $247 \text{ g cm}^{-2}$ ) [20]. Rodríguez-Montesinos et al. [27] reported a seasonal pattern for agar gel strength from *G. vermiculophylla*, with the lowest value recorded in the summer ( $524 \text{ g cm}^{-2}$ ) and the highest gel strength value in winter ( $793 \text{ g cm}^{-2}$ ). This current study supports findings from previous research conducted on *Gracilaria tenuis-tipitata* from Bangladesh according to Mohibbullah et al. [45]. It was found that the gel strength was lowest ( $132.78 \pm 2.99 \text{ g cm}^{-2}$ ) in native agar without any treatment. However, when the alkaline pretreatment was applied, there was an increase in gel strength ( $201.33 \pm 5.44 \text{ g cm}^{-2}$ ).

Concerning gelling and melting temperatures, other studies on the invasive seaweed *G. vermiculophylla* [26] have reported different gelling and melting temperatures during alkaline pretreatment (7%), which were 41.8 and 81.4 °C, respectively. The melting temperatures for an alkali pretreatment time of 0.5 h and a 2 h extraction time for alkali-treated agar were, respectively, 94.2 and 98.1 °C. However, the gelling and melting temperatures for the native extraction were 30.5 and 22.3 °C, respectively [19]. Agar from integrated, multitrophic, aquacultured *G. vermiculophylla* from Ria de Aveiro (northwestern Portugal) showed  $40.7 \pm 0.2$  °C as the gelling temperature and  $93.1 \pm 0.5$  °C as the melting temperature [43]. Some works have indicated that applying an alkaline pretreatment prior to agar extraction increases the agar's gelling and melting temperatures. For example, Villanueva et al. [21] mentioned that the gelling and melting temperatures of *G. vermiculophylla* agar exhibit the lowest values (21.6–26.4 and 62.7–70.0 °C, respectively), yet pretreatment with NaOH concentrations of up to 4% lead to improvements in both gelling and melting temperatures (31.0–35.8 and 73.6–80.4 °C, respectively). Mohibbullah et al. [45] reported that the melting temperatures of water- and NaOH-pretreated agars were  $85.93 \pm 0.34$  °C and  $86.00 \pm 0.25$  °C, respectively, and the gelling temperatures were recorded as  $37.67 \pm 0.22$  °C, and  $37.97 \pm 0.12$  °C.

Regarding the chemical characteristics, Orduña-Rojas et al. [26] indicated that minimal 3,6-AG content in *G. vermiculophylla* was found in native agars (6.7%), while 7% NaOH-

pretreated samples showed the greatest 3,6-AG content (33.8%). According to Yarnpakdee et al. [38], the 3,6-AG content for agars with NaOH pretreatments increased with an increasing alkaline concentration of up to 5%. The employment of an alkaline treatment before agar extraction was able to cleave the sulfate ester bond at the C-6 of L-galactose with the simultaneous formation of 3,6-AG (Figure 4), resulting in the enhancement of the gel-forming characteristics [46]. The gel strength enhancement after alkaline treatments is related to the elimination of a less energetically stable axial sulfate ester positioned at the C-6 of the L-galactopyranose unit, resulting in the production of more stable 3,6-anhydro-L-galactose [2]. Agar with 3,6-AG content produces a strong gel and, conversely, agar with low 3,6-AG content produces a weak gel [11,47,48]. A comparison of the mean (*t*-test) between the nature of the treatment and the chemical properties (sulfate and 3,6-AG content) has shown a significance level of  $p < 0.01$ , which means the relationship is highly significant; therefore, it is likely that there is a relationship between the two variables, which shows that the treatment affects the sulfate and 3,6-AG contents (Table 4). Pearson's correlation analysis (test of significance, two-tailed) was used to determine the correlation between the studied parameters previously cited. Pearson's correlation analysis of the native extraction showed a strong positive correlation ( $r = 1.000$ ,  $p = 0.000$ ) between the agar yield and the gelling temperature. The melting temperature also correlated positively with the 3-6 AG content ( $r = 1.000$ ,  $p = 0.013$ ), which is in agreement with the study by Villanueva et al. [21]. The agar yield did not show any significant correlation with either gel strength or sulfate content, which is in accordance with the study by Yousefi et al. [49], who worked on *Gracilaria corticata* from the Persian Gulf. In the same context, Wang et al. [37] confirmed the presence of a positive correlation between 3,6-anhydrogalactose content and the melting temperatures of agar from *Gracilaria tenuistipitata*. However, no significant correlations were obtained among any of the agar properties for the alkaline pretreatment condition. In contrast with these observations, according to Marinho-Soriano and Bourret [50], all gel properties are significantly positively correlated with 3,6-AG content and negatively with sulfate content. Concerning the species *Gracilaria dura*, the 3,6-AG content correlated positively with gel strength, but there was no significant correlation between sulfate content and gel strength. In the report of Orduña-Rojas et al. [26], who worked on the two agarophyte species *A. vermiculophyllum* and *Gracilariopsis longissima*, the sulfate content showed a negative correlation with gel strength ( $r = -0.95$ ) for *G. longissima*. Also, the sulfate content for *A. vermiculophyllum* did not show a statistically significant difference compared with the low-alkali treatments, but it did show the opposite with the high-alkali treatments. The 3,6-AG content revealed a significant correlation with gel strength for *A. vermiculophyllum* ( $r = 0.91$ ) and *G. longissima* ( $r = 0.89$ ). This result was not in agreement with our obtained results.



higher gel strength, which is considered a crucial parameter for agar applications. It was observed that the alkali pretreatment had a positive effect on gel strength, leading to an improvement in its value, increased gelling and melting temperatures with a rise in 3,6-AG content, and a reduction in sulfate levels. Overall, the satisfying yield, as well as the physical and chemical characteristics of the extracted agar, makes this invasive species a possible candidate for commercial purposes as a local agar source. Furthermore, this study provides new possibilities for future research, such as the optimization of extraction conditions and the seasonality and improvement of seaweed storage techniques.

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