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Production and properties of agar from the invasive marine alga, *Gracilaria vermiculophylla* (Gracilariales, Rhodophyta)

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Abstract The utilization potential, in terms of agar production, of the invasive alga, *Gracilaria vermiculophylla*, collected at Ria de Aveiro, northwestern Portugal was investigated. The agar yield ranged from 15% to 33%, with pre-extraction treatment with alkali generally increasing the yield. The gel quality (gel strength and apparent Young's modulus) was best (>600 g cm^{-2} and >1,000 kPa, respectively) when alkali treatment with 6% NaOH for 3.5 h was performed. At these pretreatment conditions, the effect of extraction time was also investigated and highest yield and best gel quality were obtained with a 2 h extraction time. By employing these extraction conditions, *G. vermiculophylla* can be a source of industrial food-grade agar. The structure of agar from *G. vermiculophylla* was determined through chemical techniques and FTIR and NMR spectrometry. It is mainly composed of alternating 3-linked D-galactose and 4-linked 3,6-anhydro-L-galactose, with methyl substitution occurring at 16–19 mol% of C-6 in 3-linked units and 2–3 mol% of C-2 in 4-linked units. A minor sulfation on C-4 of 3-linked units was also detected; while precursor units (6-sulfated 4-linked galactosyl moieties) were found in the native extract.

Keywords Agar · Alkali treatment · Extraction · Gel strength · *Gracilaria vermiculophylla* · Invasive alga

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Introduction

An increasing number of marine macroalgae has been reported to be invasive and threaten the ecological balance of coastal ecosystems (Schaffelke et al. 2006; Schaffelke and Hewitt 2007; Williams and Smith 2007). Several management and eradication strategies have been proposed and/or implemented to mitigate impacts of invasive seaweeds (Anderson 2007). One viable strategy is the mechanical removal (harvesting) of the invasive alga and such would yield a tremendous biomass that can be utilized for various applications.

The agarophyte *Gracilaria vermiculophylla* (Ohmi) Papenfuss was first observed in 1996 at the Brittany coast of France (an expansion beyond its original range in East Asia) and thereafter found in several European

locations from Sweden to southern Portugal (Rueness 2005). Its introduction to European waters was speculated to emanate from the aquaculture of Japanese oysters (Rueness and Beaupoil 2003 as cited in Rueness 2005), since it was discovered near an oyster farm (Mollet et al. 1998). Like its type locality in Japan (Terada and Yamamoto 2002), new populations in Europe are concentrated in estuarine environment as entangled mats which are loosely lying on mud (Rueness 2005). We are currently conducting intensive studies on the potential utilization of a newly established population of the alga in Ria de Aveiro, northwestern Portugal. We report here on the production and physico-chemical properties of agar, a commercially valuable biopolymer, from harvested biomass of the invasive alga. To the best of our knowledge, *G. vermiculophylla* is the first agar-producing marine alga which goes invasive.

Agar is a biopolymer synthesized by several genera of marine red macroalgae (Rhodophyta). It is built on a disaccharide-repeating unit of 3-linked D-galactose and 4-linked 3,6-anhydro-L-galactose (3,6-AG) residues, with possible occurrence of sulfate, methoxyl, and/or pyruvate substituents at various positions in the polysaccharide chain (Rees 1969). The type, pattern, and degree of substitution, as well as the molecular weight, determine the gelling properties of agar. The wide range of gel properties makes agar suitable for a diversity of uses in medical, pharmaceutical, industrial, and food applications; this is in contrast to the current situation where only agar that produces strong gels are commercially desired.

To improve the gel properties of the agar extract from *G. vermiculophylla*, we studied the effect of several extraction conditions on gel quality and identify best conditions during processing of the alga. The extraction of agar from agar-bearing seaweeds may involve a pretreatment with alkali in which ‘precursor’, L-galactose-6-sulfate, moieties in the agar backbone are converted to 3,6-AG (Rees 1972), with corresponding increase in gel strength of the extract. For this purpose, two aspects of the pretreatment (alkali concentration and pretreatment duration) were studied in terms of agar yield and physico-chemical properties. Using pretreatment conditions that produce the best gel quality, the effect of extraction duration on agar yield and quality was then investigated and the optimum level was estimated. A detailed physico-chemical characterization of the agar extracted using optimum conditions was then conducted, with subsequent comparison to the ‘native’, non-alkali-treated extract.

The information presented here can be of vital use in the production of valuable product from the invasive seaweed, as its harvesting is advocated as control measure to curb further population expansion and negative ecological impacts.

Materials and methods

Samples of *Gracilaria vermiculophylla* were collected at Ria de Aveiro, Portugal (40°38′2.5″N, 8°40′32.5″W) in January 2008 during low tide. During this time, the live seaweeds were found lying on the intertidal mudflat and were unattached to any hard substrate. The samples were washed with freshwater, cleaned of extraneous matter (other seaweeds and invertebrates—mollusks and crustaceans) and dried in an oven at 60°C. Four-gram dried samples were prepared, each considered as an experimental unit in the succeeding experiments. Samples were extracted within 2 months to prevent any possible agar degradation upon prolonged seaweed storage (Romero et al. 2008).

A voucher sample of the alga was deposited at the Herbarium PO, Oporto University, Portugal, under accession number PO 4012A.

Alkali pretreatment and agar extraction

Dried sample, 4 g, was soaked in a 200-mL alkali solution (0, 2, 4, 6, and 8% sodium hydroxide) at 85°C for 0.5, 1.5, 2.5, and 3.5 h. It is noteworthy that the sample used in the extraction was the whole dried thalli and no grinding was conducted prior to alkali pretreatment. With such, the effect of initial algal material particle size on agar extraction and how particle size is affected by alkali treatment were not assessed in this study.

The alkali solution was then discarded and the algal material was washed thoroughly with freshwater. It is then soaked in 200 mL 0.5% acetic acid for 1 h at room temperature. Acetic acid solution was discarded and seaweed washed again with freshwater. Seaweed was extracted with 200 mL distilled water at 85°C for 2 h. The mixture was homogenized in a blender and filtered using filter cloth. Agar was recovered through freeze–thawing method, after which recovered agar was washed and dehydrated with ethanol (96%) then oven dried at 60°C to yield the agar extract.

For each combination of alkali concentration and pretreatment duration, three replicate extractions were conducted.

Effect of extraction time

By using the alkali pretreatment conditions which yielded agar with the best gel quality (i.e., 6% NaOH and 3.5 h pretreatment duration), the effect of extraction time on agar yield and gel quality was investigated. After alkali pretreatment and acetic acid neutralization, seaweed was extracted with 200 mL distilled water at 85°C for 1.5, 2.5, and 3 h. The rest of the extraction process proceeded as described in the “Alkali pretreatment and agar extraction” section. The

extraction employing the same alkali pretreatment conditions as done in "Alkali pretreatment and agar extraction" section is considered as one level of extraction time (2 h) in the experimental design.

Agar gel characterization

A 1.5% (*w*/*w*) agar solution, in distilled water, was prepared by boiling under stirring. Approximately 15 g of the hot solution was transferred to a cylindrical container (30 mm diameter), covered with aluminum foil, and allowed to set at room temperature for 20 h. Gel depth was approximately 21–22 mm.

A texture analyzer (Stable Micro Systems model TA-XT2, Surrey, England) was used to determine the gel strength and apparent Young's modulus of the agar preparations. It is equipped with a cylindrical probe whose diameter is 10 mm and penetrates at a rate of 0.2 mm s^{-1}. Gel strength is defined as the pressure required to break the gel surface and is determined by assessing the stress for which a discontinuity (local maximum) is seen in the stress–strain curve. Apparent Young's modulus, E, was calculated from the stress–strain curve as described in Hilliou et al. (2006a) without correction for sample buoyancy (Oakenfull et al. 1989). Young's modulus is reported as 'apparent' since penetration test was employed in the determination (sensu Gregson et al. 1999), as opposed to the 'real' Young's modulus which is obtained from uniaxial extension test.

The melting and gelling temperatures of 1.5% agar sample were determined through dynamic rheological measurements in a CSL Rheometer (Carri-Med Ltd., Surrey, England). Parallel plate geometry was used with a crosshatched acrylic geometry (4 cm diameter, 2 mm gap) to avoid slippage. An agar gel slab (ca. 3 mm thick) was loaded on the Peltier plate (pre-heated to 50°C) and the temperature was ramped to 95°C to allow the gel between the plate and geometry to melt. Excess sample was removed and its periphery was coated with paraffin oil to minimize evaporation. A cooling scan (2.5°C min^{-1}) to 20°C was then performed, followed by a heating scan (2.5°C min^{-1}) to 95°C. The storage (G') and loss (G'') moduli were monitored at 6.28 rad s^{-1} with 0.1% strain and the temperatures at which crossover of the moduli occurred ($G' = G''$ or tan $\delta=1$) during the cooling and heating scans were regarded as the gelling and melting temperatures, respectively. Typical curves for these rheological tests are presented in Villanueva et al. (2009).

Chemical characterization

The sulfate content of the agar sample was measured turbidimetrically after sulfate hydrolysis in 1 N HCl at boiling temperature and subsequent precipitation of liberated sulfate with barium ion (Jackson and McCandless 1978). Sodium sulfate served as the standard. The 3,6-anhydrogalactose content (3,6-AG) was determined colorimetrically using the resorcinol–acetal method of Yaphe and Arsenault (1965), with fructose as standard.

The Fourier transform infrared (FTIR) spectra of selected native (pretreated with 0% NaOH for 3.5 h) and alkali-treated (pretreated with 6% NaOH for 3.5 h) agar samples were recorded on films using a Bomem MB-series FTIR spectrometer (ABB Bomem Inc., Quebec). Films, ca. 0.003 mm thick, were prepared by drying in an oven (60°C) about 3.2 mL of 0.5% agar solution contained in a 4-cm diameter plastic vessel. Each spectrum is the average of eight scans acquired at 2 cm^{-1} resolution. Sigma agar (A-7002, Lot 043K0052, Sigma-Aldrich Co., St. Louis, MO) served as reference. The absorbance ratio of peaks attributed to 3,6-AG (ca. 935 cm^{-1}) and total sulfate (ca. 1250 cm^{-1}; sensu Hilliou et al. 2006b), hereby denoted as $A_{935}/A_{1,250}$, was determined as semi-quantitative measure for the chemical conversion elicited by the alkali pretreatment. Peak base lines and heights were determined in transmittance mode and calculated peak heights were converted to absorbance (see Rochas et al. 1986 for details).

Nuclear magnetic resonance (NMR) spectroscopic measurements on the same extracts subjected to FTIR analysis were carried out non-spinning at 80°C on a 500-MHz Varian VNMRS spectrometer (Varian, Inc., USA), using a 5 mm HCX triple probe equipped with a z gradient coil. Agar was dissolved in D_2O to a final concentration of 10–15 mg mL^{-1} and 3-(trimethylsilyl)propionic-2,2,3,3-*d*4 acid sodium salt (TSP) was added as an internal chemical shift reference (δ_H=−0.017 ppm; δ_C=−0.18 ppm; sensu van de Velde et al. 2004). Proton and phase-sensitive heteronuclear multiple quantum coherence (HMQC) spectra were acquired using the standard Varian pulse sequences. The degrees of methylation at C6 of 3-linked D-galactose and at C2 of 4-linked 3,6-anhydro-L-galactose units were estimated from the ^{1}H NMR spectral data by the ratio between 1/3 of area of the peaks at ~3.43 and ~3.52 ppm, respectively, and the peak area of H1 of the 4-linked residues [3,6-anhydro-L-galactose (5.13–5.15 ppm)+L-galactose-6-sulfate (5.28 ppm)].

The weight-average molecular weights (M) of agar preparations from "Effect of extraction time" section were determined from their intrinsic viscosities ([η], in mL g^{-1}) using the Mark–Houwink equation (Rochas and Lahaye 1989):

$$[\eta] = 0.07M^{0.72}$$

Intrinsic viscosities of samples in 0.75 M NaSCN were measured using a Cannon-Fenske viscometer (size 50;

Comecta S.A., Barcelona) at 35.0±0.1°C. Agar concentrations between 0.1 and 0.2% (*w*/*v*), whose efflux times are about 1.2 to 2.0 times that of the solvent were used in the extrapolation to zero concentration in a Huggins' plot (Morris and Ross-Murphy 1981).

Statistical analysis

A two-way analysis of variance (ANOVA) was used to determine the main and interactive effects of alkali concentration and pretreatment duration on agar yield, gel, and chemical properties. On the other hand, significant differences in properties of agar extracted with different extraction times were detected by one-way ANOVA. Duncan's multiple range test (DMRT) was carried out as post hoc procedure. To find out significant difference in A_{935}/A_{1250} and molecular weight of native and alkali-modified agars, *t*-test was employed. Pearson's correlation analysis was used to determine the relationship between two agar characteristics.

Results and discussion

Alkali concentration and pretreatment duration significantly influenced the yield and physico-chemical properties of agar from *G. vermiculophylla*, with the former variable exerting greater influence as reflected by higher *F* values (Table 1). The interaction between the two variables was also detected to be significant in all agar parameters investigated (Table 1), implying a significant influence of one to the performance of the other. Low agar yields (ca. 15%) were obtained at 0% NaOH at different pretreatment durations; whereas at higher alkali concentrations, the yields were between 24% and 33% (Fig. 1). In extractions where pretreatment involve no alkali (i.e., 0%), the resultant extracts produced weak gels, which upon freezing and subsequent thawing do not yield firm polymeric networks which are difficult to recover even upon partial dehydration with ethanol. We suspect agar to have leached to the thaw water during the recovery process, hence the diminished yield. Furthermore, since the sample treated with 0% alkali concentration was subjected to similar pretreatment conditions (temperature and duration) as those treated with alkali, agar could have leached out from the seaweed during the pretreatment and removed when the pretreated seaweed was washed. It is noteworthy that without alkali in the pretreatment solution, the algal thallus is relatively softer than those treated with alkali after the pretreatment step. Alkali could have a stiffening effect on the thallus, thereby preventing leaching out of agar from the seaweed during the pretreatment. On the contrary, native (0% alkali concentration) agar yields from other three *Gracilaria* spp. were higher than the alkali-treated counterparts (Freile-Pelegrín and Murano 2005). Generally, the yield decreased with longer pretreatment (Table 2). This suggests that agar leached out to the alkali pretreatment solution, as previously observed in the extraction of agar from *Gracilaria eucheumoides* (Villanueva et al. 1997).

Agar gel strength and apparent Young's modulus responded similarly to the different pretreatment conditions (Fig. 1, Table 2). Either of these two gel parameters has been regarded as the metric for agar gel quality (e.g., Mrani et al. 1995; Norziah et al. 2006; Prasad et al. 2007; Li et al. 2008). Lowest gel qualities were obtained at 0% alkali concentration in all pretreatment durations (regarded as native extracts). The gel strengths of native extracts were between 55 and 115 g cm^{-2}, similar to the native extracts from the same alga (*G. vermiculophylla*) collected from Mexico (Arvizu-Higuera et al. 2008; Orduña-Rojas et al. 2008) and from France (Mollet et al. 1998). The gel quality increases with increasing alkali concentration up to 6%, at which the highest value (679 g cm^{-2}) was obtained, and then the quality decreases at the highest concentration investigated. From this observation, 6% is regarded as the optimum alkali concentration in the pretreatment. Working on the several *Gracilaria* spp., Meena et al. (2008) found 8% as the optimum concentration to obtain high quality agar, with higher concentrations not producing any further increment in the gel strength. Similarly, González-Leija

Table 1 Two-way ANOVA on the effect of alkali concentration (A) and pretreatment duration (B) on properties of agar from *Gracilaria vermiculophylla*

Agar property	NaOH concentration (A)		Pretreatment duration (B)		A × B	
	F	*p*	*F*	*p*	*F*	*p*
Agar yield	118.86	0.0001	8.18	0.0002	3.12	0.0034
Gel strength	73.64	0.0001	31.68	0.0001	10.56	0.0001
Apparent Young's modulus	58.60	0.0001	17.14	0.0001	3.51	0.0014
Gelling temperature	138.51	0.0001	8.58	0.0002	7.59	0.0001
Melting temperature	31.48	0.0001	8.16	0.0002	4.11	0.0003
Sulfate content	27.30	0.0001	3.72	0.0189	2.79	0.0074
3,6-Anhydrogalactose content	138.37	0.0001	5.38	0.0033	3.66	0.0010

The two factors and their interaction significantly affected all agar properties ($p<0.05$). *df*: A=4; B=3; A×B=12

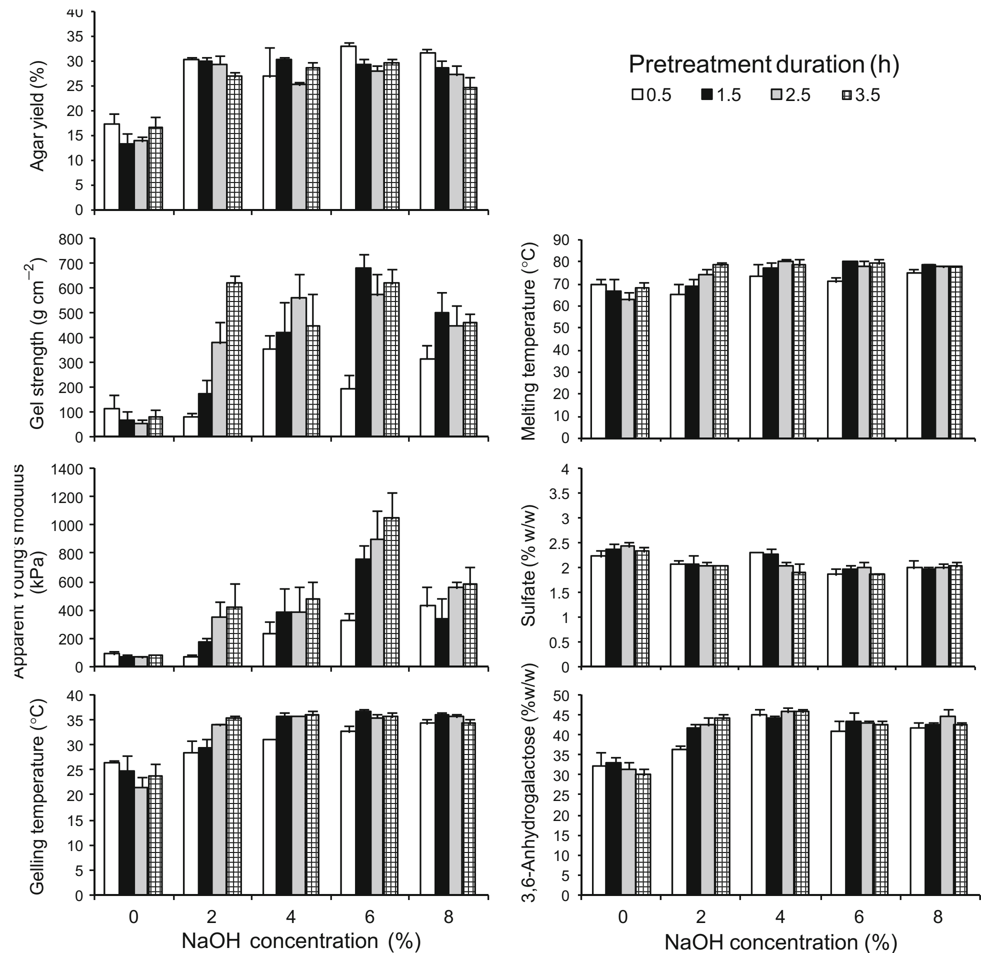

Fig. 1 Yield and physico-chemical properties (mean ± s.d., n=3) of agars extracted from *Gracilaria vermiculophylla* pretreated with different alkali concentrations for different durations. Differences among NaOH concentrations and pretreatment durations are presented in Table 2

et al. (2008) observed that the gel strength of agar extracted from *Gracilariopsis lemaneiformis* did not show further improvement at alkali concentration beyond 5%. Arvizu-Higuera et al. (2008) used 7% in their investigation of the effect of alkali treatment time on agar quality of *G. vermiculophylla*, as adopted from a previous work of Freile-Pelegrín and Robledo (1997). Despite the convergence of optimum NaOH concentrations in the extraction of high quality agar at the range of 5–8%, we found that alkali concentration has a significant interaction with the alkali pretreatment duration (Table 1). This implies that even at sub-optimal alkali concentrations, agar of high quality can be produced by manipulating the pretreatment duration, e.g., involving longer pretreatment. This is exemplified by the gel strength response to different pretreatment durations at 2% NaOH, producing agar with 613 g cm^{-2} gel strength at 3.5 h (Fig. 1).

The gel strength increases with longer duration of pretreatment (Table 2). This is in contrast to the results obtained from the same alga where an inverse relationship was obtained (Arvizu-Higuera et al. 2008). The longer the pretreatment duration ensures longer contact time between the alkali in solution and the agar polymer, thereby increasing the efficiency of conversion of precursor to 3,6-AG. Unless significant degradation of the agar polymer occurs with longer pretreatment, agar gel strength is expected to increase with longer pretreatment up to a certain optimum duration, beyond which the gel strength value plateaus. We observed no deterioration of gel quality with pretreatment up to 3.5 h and from the DMRT results (Table 2), 3.5 h was chosen as the pretreatment duration to produce agar of best quality.

The gelling and melting temperatures of agar extracts were lowest at pretreatment with 0% alkali concentration (21.6–26.4°C and 62.7–70.0°C, respectively) and increased with increasing alkali concentration up to 4% (31.0–35.8°C and 73.6–80.4°C, respectively), then remained constant with higher concentrations (Fig. 1). Both temperatures are significantly lower in agars extracted with the shortest pretreatment duration (0.5 h) compared to those pretreated longer (Table 2).

Alkali concentration, pretreatment duration and their interaction significantly affect the chemical properties of the agar extracts (sulfate and 3,6-AG contents, Table 1). Native agars (pretreated with 0% alkali) showed significantly higher sulfate and lower 3,6-AG contents than their alkali-

Table 2 Duncan's multiple range test (DMRT) groupings of different alkali concentrations and pretreatment times on properties of agar extracted from *Gracilaria vermiculophylla*

Agar property	Alkali concentration (%)					Pretreatment time (h)			
	0	2	4	6	8	0.5	1.5	2.5	3.5
Agar yield	C	AB	B	A	B	a	b	c	bc
Gel strength	D	C	B	A	B	c	b	ab	a
Apparent Young's modulus	E	D	C	A	B	c	b	a	a
Gelling temperature	C	B	A	A	A	b	a	a	a
Melting temperature	C	B	A	A	A	b	a	a	a
Sulfate content	A	BC	B	D	CD	a	a	a	b
3,6-Anhydrogalactose content	D	C	A	B	B	b	a	a	a

Treatments with the same letter (case specific, depending on factor) are not statistically different ($p>0.05$). 'A' and 'a' represent the highest values, 'E' and 'c' being the lowest. See Fig. 1 for data

treated counterparts (pretreated with 2–8% alkali; Fig. 1). Similar observation was obtained for *Gracilaria crassissima*, but not for *G. cervicornis* and *G. blodgettii* (Freile-Pelegrín and Murano 2005). Variability in the chemical response to alkali pretreatment may be due to interspecific differences in alkali-labile sulfate or 'precursor' concentration on their respective native agar. The native agar from *G. vermiculophylla* in this study has a sulfate content of 2.2–2.4% (*w/w*) and that of alkali-treated agar (obtained through the optimum pretreatment conditions, 6% alkali and 3.5 h duration) is 1.9%. Hence, the concentration of the 'precursor' sulfate in the native agar can be estimated to ca. 0.4%.

Pearson's correlation analysis revealed that all gel properties are correlated positively with 3,6-AG, but negatively with sulfate content (Table 3). In *G. dura*, 3, 6-AG was also found to be correlated positively to gel strength, however no significant correlation between sulfate content and gel strength existed (Marinho-Soriano and Bourret 2005).

Agar extracted with 0.5 h pretreatment duration has significantly lower 3,6-AG content than those subjected to longer pretreatments (Table 2), corroborating earlier findings of Villanueva et al. (1997). On the other hand, longer times of pretreatment produced agar with lower 3,6-AG content (Arvizu-Higuera et al. 2008).

The properties of alkali-treated agar extracted from *G. vermiculophylla* with different extraction times and ANOVA results are presented in Table 4. Agar yield was significantly affected by extraction time, with 2 h producing the highest yield. Amylase treatment, to eliminate contaminating starch, was not performed during agar extraction; hence, the reported yield for agar could be an overestimate. It was reported that starch accounts for 2.7% of the agar yield in *G. vermiculophylla* similarly processed without amylase treatment (Mollet et al. 1998). Another potential contaminant in the extract is protein; however, nitrogen (measure for protein) levels in the extracts were not determined. Low nitrogen contents, ca. 3–4%, were reported in agar extracts from several *Gracilaria* species (Marinho-Soriano and Bourret 2003, 2005).

Gel quality (gel strength and apparent Young's modulus) also showed significant differences amongst treatments, with shorter extraction times (1.5 and 2 h) producing better gel strengths (Table 4). Despite similar gel strengths, agar produced with 2-h extraction time exhibits significantly higher apparent Young's modulus than agar from a 1.5-h extraction time (Table 4). The explanation underpinning this observation is elusive at this point. From here, we consider 2 h as the optimum extraction time. Gelling and melting temperatures and agar chemical properties, including molecular weight, were not significantly affected by the

Table 3 Pearson's correlation coefficient matrix of gel and chemical properties of agar extracted from *Gracilaria vermiculophylla*

Agar property	Apparent Young's modulus	Gelling temperature	Melting temperature	Sulfate content	3,6-Anhydrogalactose content
Gel strength	0.82	0.88	0.95	−0.60	0.80
Apparent Young's modulus	–	0.77	0.79	−0.70	0.64
Gelling temperature		–	0.94	−0.78	0.92
Melting temperature			–	−0.66	0.84
Sulfate content				–	−0.66

Three replicates in each treatment (see "Materials and methods" section) were averaged to represent a data point in the correlation analysis. Total number of data points: 23; $df=21$. All pairs of agar properties are significantly correlated ($p<0.01$)

Table 4 Yield and physico-chemical properties (mean ± s.d., n=3) of agars extracted from *Gracilaria vermiculophylla* employing different extraction times, including ANOVA results on the effect of extraction time (df=3)

Agar property	Extraction time (h)				F	p
	1.5	2	2.5	3		
Agar yield (%)	24.7±1.7[b]	29.4±0.9[a]	24.5±0.5[b]	26.5±1.3[b]	10.94	0.0033*
Gel strength (g cm^{-2})	636±149[a]	614±59[a]	412±41[b]	394±84[b]	5.76	0.0214*
Apparent Young's modulus (kPa)	767±66[b]	1040±194[a]	712±69[b]	740±98[b]	4.89	0.0323*
Gelling temperature (°C)	36.1±0.2[a]	35.4±1.0[a]	36.2±0.9[a]	35.5±0.9[a]	0.93	0.4699
Melting temperature (°C)	79.7±1.5[a]	79.1±2.3[a]	78.5±0.5[a]	76.2±2.5[a]	2.00	0.1934
Sulfate content (% *w*/*w*)	2.00±0.02[a]	1.86±0.02[a]	1.89±0.10[a]	1.92±0.25[a]	0.63	0.6183
3,6-Anhydrogalactose content (% *w*/*w*)	43.4±0.7[a]	42.5±0.9[a]	43.1±1.0[a]	44.2±0.8[a]	2.11	0.1771
Molecular weight (kDa)	57±17[a]	60±16[a]	54±19[a]	44±14[a]	0.55	0.6610

Means with the same letters do not differ significantly (DMRT, p>0.05)

*p<0.05 (significant)

extraction time. The molecular weights of agars (44–60 kDa) extracted with different extraction times are within the range of molecular weights for most *Gracilaria* agars reported in literature so far (24–124 kDa; Rochas and Lahaye 1989; Romero et al. 2008; but see Rodríguez et al. 2009 wherein high molecular weight (ca. 540 kDa) agar was obtained from *G. gracilis*), and are lower than in *Gelidium* (75–122 kDa; Rochas and Lahaye 1989; Freile-Pelegrín et al. 2007; Pereira-Pacheco et al. 2007) and *Hydropuntia* (342–372 kDa; Pereira-Pacheco et al. 2007) agars.

Representative native and alkali-treated agars possess almost similar FTIR spectra, which are also in close resemblance with that of a reference agar from Sigma (spectra not shown). Bands diagnostic of total sulfate (1,250 cm^{-1}) and 3,6-AG (935 cm^{-1}) were resolved in all the spectra. The $A_{935}/A_{1,250}$ of native agar (6.41±0.67, mean ± s.d.) is significantly lower (t-test, p=0.0033) than that of alkali-treated agar (9.83±0.65). This indicates that the relative concentration of 3,6-AG to total sulfate increases upon alkali treatment, corroborating results of the chemical analyses. A minor peak is resolved at 845 cm^{-1} for both extracts, indicating partial sulfation at 4-position of D-galactose moieties (Matsuhiro and Rivas 1993). Sulfate at this position is persistent even after alkali treatment (alkali-stable) and, hence, it accounts for the sulfate content (ca., 1.9%, from chemical analysis) of agar extracted employing optimum alkali pretreatment conditions. Minor substitution of this sulfate ester, alongside with small amounts of sulfate at 2-position of 3,6-AG, was also detected in agar from the same alga collected in France (Mollet et al. 1998). Several *Gracilaria* agars were found to possess this sulfation pattern and exhibit gelation behavior influenced by alkali metal ions (Murano et al. 1995, 1996; Villanueva and Montaño 1999). No investigation on the effect of alkali metal ion on the gel properties of the agar extracts from *G. vermiculophylla* was conducted in this study; however, if an effect is demonstrated it can offer an additional property and potential use for the polymer. The presence of alkali-labile sulfate ester at 6-position in L-galactose units was not detected in the FTIR analysis of the native agar, i.e., no discernible peak at 820 cm^{-1}. The low level of the alkali-labile sulfate (ca. 0.4% as calculated from the chemical analysis, see above) may be beyond the detection limit of this spectroscopic analysis.

The HMQC spectra of the native and alkali-treated agars reveal that the major component of both extract is an alternating 3-linked D-galactose and 4-linked 3,6-anhydro-L-galactose (Fig. 2). The carbon chemical shifts reported here are about 2.1 to 2.3 ppm more downfield than those from previous reports (e.g., Usov et al. 1980; Lahaye et al. 1989; Falshaw et al. 1999). This discrepancy, as also observed by Pereira et al. (2003), is traced to the difference in the reported chemical shift of the internal standard (dimethyl sulfoxide) used in previous studies to what was recently found out when measured in polar solvent (van de Velde et al. 2004).

Aside from the basic agar repeating unit, considerable level of methyl substitution was found at C-6 of the 3-linked units, occurring at 16.5 and 19.1 mol% in native and alkali-treated agars, respectively. Comparable level of this methylation was found from agar of the same alga collected in France in which 14.6% was determined through monosaccharide analysis using gas chromatography (Mollet et al. 1998). Another methyl substitution detected was at C-2 of 4-linked units, occurring minimally at 2–3 mol% of these units. This minor substitution was detected from the ^{1}H NMR spectra of both extracts through the presence of a peak at 3.52 ppm (barely discernible in topmost spectra in Fig. 2). A cross-peak for this substituent

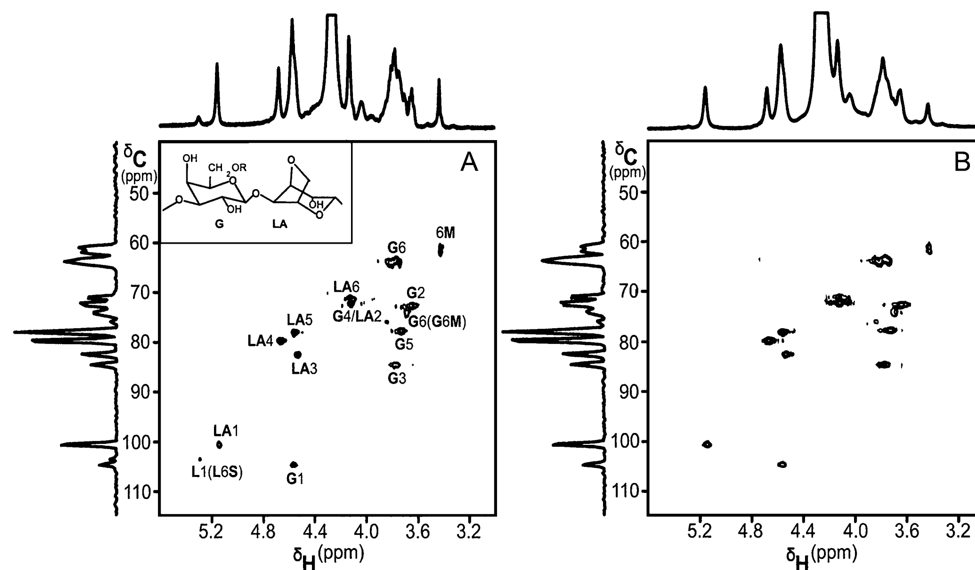

Fig. 2 HMQC spectra with ^{1}H experiment (*top*) and ^{13}C projection (*left*) of native (**a**) and alkali-treated (**b**) agar from *Gracilaria vermiculophylla*. Inset in panel **a** is the proposed structure of the major polysaccharide from the alga. *R* could either be H (81–84 mol%) or CH_3 (16–19 mol%). Other minor components resolved in individual spectra are presented in the text. Assignments were done with reference to Usov (1984), Usov et al. (1980), and Lahaye and Rochas (1991), with 2.1–2.3 ppm difference across carbon chemical shifts (as discussed in the text). Chemical nomenclature of residues follows the shorthand notation system described in Knutsen et al. (1994). *G* 3-linked α-D-galactose, *LA* 4-linked 3,6-anhydro-α-L-galactose, *L* 4-linked α-L-galactose, *M O*-methyl, *S* ester sulfate. Numerical components represent the carbon number in each residue

is absent in the HMQC spectra, implying a concentration which is below the detection limit of the analysis. On the other hand, the 6-*O*-methyl substitution is resolved at around 3.42, 61.6 ppm (^{13}C, ^{1}H) in both extracts. Additional carbon resonances which are resolved due to this methylation include those of G6 (where the methyl ether is attached, 73.9 ppm) and G5 (adjacent to the methylated G6, 75.7 ppm; Lahaye et al. 1989; Lahaye and Rochas 1991). For the native agar, a minor cross-peak in the HMQC spectrum at 103.25, 5.28 ppm is assigned to L1 of a 6-sulfated 4-linked galactose unit, the precursor residue. Interestingly, this signal is absent in the spectrum of the alkali-treated extract, suggesting the alkali-labile nature of sulfate ester at L6. Signals attributable to G4S were not resolved in the NMR analysis, contrary to what has been revealed in FTIR analysis.

The molecular weights of the native and alkali-treated agar samples (same as those subjected to FTIR analysis) do not differ significantly (*t*-test, $p=0.586$; 54.0±9.3 and 60.3±15.5 kDa, respectively). This shows that alkali treatment at optimum conditions (6% NaOH for 3.5 h) does not lead to depolymerization of agar. While in carrageenan, alkali treatment with >1% KOH for 3 to 5 h caused lowering in viscosity, implying depolymerization (Freile-Pelegrín et al. 2006).

Agar from the invasive alga *G. vermiculophylla* exhibits a wide range of gel properties dependent on the alkali pretreatment conditions employed in the extraction. With gel strengths ranging from 55 to 679 g cm^{-2}, it can either be for traditional domestic use (<600 g cm^{-2}) or as industrial food-grade type (≥600 g cm^{-2}; Armisen 1995). The same alga, collected in Mexico, was found to possess a maximum gel strength of 1064 g cm^{-2} after alkali pretreatment (Arvizu-Higuera et al. 2008), while a soft gel (195 g cm^{-2}) was produced by agar from a sample collected in France even after alkali treatment (Mollet et al. 1998). The variation in gel quality among the *G. vermiculophylla* agar extracts from these studies could be due the different alkali pretreatment procedures employed. Lower concentration of alkali (2.4% NaOH) was used in the extraction of agar from the algal sample collected in France—hence, lower gel quality; compared to that employed for samples collected in Mexico (7%). In the present study, a range of alkali concentration (2–8%) was employed for the algal samples collected in Portugal and, consequently, the agar extracts exhibit a wide range of gel quality (from low grade for traditional domestic use to high grade for industrial use). Furthermore, the variability in agar gel quality among samples in these studies may suggest that geographical origin (possibly local environmental conditions where the seaweeds grow) could partly influence the quality of agar from the alga (c.f. Rebello et al. 1997). As this invasive alga has become more and more widespread throughout the world, this proposition can be investigated more thoroughly.

Another factor that could possibly influence the quality of agar from the alga is the season of collection. All throughout the collection months representing distinct

seasons, very poor gelling properties of native extracts were found from the alga harvested in Mexico (Orduña-Rojas et al. 2008). With such seasonal data pertaining to the poorly gelling native extract, a recommendation for an optimal season to harvest during commercial exploitation of the bioresource is not possible. In future work in this line, however, we suggest extracting agar from seasonal samples with the employment of alkali treatment. A seasonal pattern may emerge for the alkali-treated, commercially viable product, besides industrial processing for agar production by and large involves alkali treatment.

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