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Superior quality agar from *Gracilaria* species (Gracilariales, Rhodophyta) collected from the Gulf of Mannar, India

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Abstract *Gracilaria edulis*, *G. crassa*, *G. foliifera*, and *G. corticata* are naturally occurring agarophytes of Indian waters. These agarophytes were evaluated for their agar contents using an improved process recently reported by us (US Patent 2005/0267296A1). The effect of different concentrations of NaOH in the alkali treatment was studied for optimizing the extraction conditions. These *Gracilaria* species of Indian waters produced agars, both native and alkali treated, with different properties confirming the heterogeneity of the agar polymers in this genera, as one would expect. Among these, *G. edulis* and *G. crassa* produced agar polymers having high gel strengths of 490± 8.16 and 800±15.4 g cm^{-2}, respectively, with 8% NaOH treatment as opposed the low gel strength agars that have been reported in the literature to date.

Keywords *Gracilaria* spp. · Agar · Alkali treatment · Rheology

Introduction

The agarophyte *Gracilaria* has been widely studied and reported in the literature (Critchley 1993). Species is not the only factor of variance in the yield and quality of agars (Cote and Hanisak 1986). Environmental factors, such as seasonal variations (Lahaye and Yaphe 1988) and extraction methods (Craigie and Leigh 1978; Armisen and Galatas 1987; Lemus et al. 1991) have been reported to influence the properties of agar as well.

Six species of *Gracilaria* (*G. edulis*, *G. crassa*, *G. foliifera*, *G. corticata*, *G. millardetii*, and *G. fergusonii*) occurring in Indian waters have been reported to be potential sources of agar (Kappanna and Rao 1963). Among these, *Gracilaria corticata, Gracilaria crassa*, and *Gracilaria edulis* are the most common ones and were therefore selected for this study. Many authors have reported extraction of agar from different *Gracilaria* spp. of Indian waters (Siddhanta et al. 1997, 2005; Kaliaperumal and Uthirasivan 2001). Numerous reports are also present in the literature for alkali treatment up to 10% NaOH at 90°C up to 3 h (Rebello et al.1997; Villanueva and Montaño 1999; Freile-Pelegrin and Murano 2005; Praiboon et al. 2006). Sugar-reactive agars have been reported from some Indian agarophytes including *G. edulis* and *G. crassa* in our earlier work (Meena et al. 2006), but there are no reports on the optimization of alkali concentrations for *G. edulis*, *G. crassa*, *G. foliifera*, and *G. corticata*. In this study, we have done systematic studies and optimization of the concentration of alkali for obtaining the best quality agars from these seaweeds. No reports are available on agars of these seaweeds with gel strengths in excess of 250 g cm^{-2}.

In this communication, we report the preparation of superior quality agars having high gel strengths after alkali modification from *G. edulis* and *G. crassa* collected from the Gulf of Mannar. The results of this investigation would be useful in bioprospecting of agarophytes, as well as in the commercial exploitation of the seaweeds mentioned.

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Experimental

Materials

Samples of four *Gracilaria* spp., *G. edulis* (S. Gmelin) P. Silva, *G. crassa* Harvey ex J. Agardh, *G. foliifera* (Forsskal) Børgesen, and *G. corticata* (J. Agardh) J. Agardh, were collected from the natural stocks of the Gulf of Mannar Tamil Nadu, India (8. 46–9.14°N and 78.90–79.14°E). Harvested plants were shade dried, packed in the gunny bags, and transported to our laboratory by road transport. The dry seaweed samples containing 7–10% moisture were stored as received in plastic bags. Before extraction of agar, the seaweed was washed thoroughly with tap water to remove the epiphytes and extraneous impurities. All the sample specimens were submitted to the Central Salt and Marine Chemicals Research Institute, Bhavnagar, Herbarium (AS0416905, AL0201908, AL0204301 and AL0204109) after identification. Difco Bacto agar (0140-01; Detroit, MI, USA) was used as the reference material; it has the following specifications: gelling temperature 35°C, sulphate content 1.77%, and gel strength 600 g cm^{-2} (in 1.5% at 20°C).

Extraction of native and alkali-treated agar

Different samples of dry *G. edulis, G. crassa, G. foliifera*, and *G. corticata* (20 g each) were soaked in 400 mL water for 1 h at room temperature followed by 2 h at 90°C in a water bath. The soaked seaweed was cooked in an autoclave with distilled water for 1.5 h at 120°C. The cooked seaweed was then homogenized in a grinder mixture, boiled with Celite and charcoal and filtered through a Celite bed under vacuum to obtain the clear extract. The filtrate was held at room temperature for gel formation, and the gelled material was then frozen in the freezer at −15°C for 15 h and thawed to obtain the native agar. Finally the thawed agar was air-dried for 24 h at ambient conditions and then dried in an oven at 50°C for 2 h.

Alkali pre-treatment of the *Gracilaria* species was carried out using 3, 4, 6, 8, 10, and 15% aqueous NaOH solutions following the procedure described by Siddhanta et al. (2005). Different samples of *G. edulis, G. crassa, G. foliifera*, and *G. corticata* (20 g dry each) were soaked in 400 mL tap water for 1 h at room temperature and then treated with 400 mL of various concentrations of aqueous NaOH solutions at 90°C in a water-bath for 2 h. After the alkali treatment, excess was removed by water washing until the washing showed pH in the range of 7–8. The seaweed was then autoclaved with distilled water (1:30 w/v) at 120°C for 1.5 h. Afterwards the alkali-treated agar was obtained by using a similar process as mentioned for the native agar extraction.

Physicochemical analyses

A 1.5% (w/v) solution of agar was prepared in an autoclave at 120°C to minimize the water evaporation. After the formation of gel at room temperature, it was kept at 10°C overnight in a refrigerator. Gel strength was measured at 20°C using a Nikkansui type gel tester (Kiya Seisakusho, Tokyo, Japan). Gelling and melting temperatures were measured as reported by Craigie and Leigh (1978). Metal ion and sulphate content analyses (ICP) were carried out on a Perkin-Elmer ICP-OES Optima 2000DV machine following the method described by Wolnik (1988).

Rheological measurements

Dynamic rheological measurements of sol and gel samples of agars obtained from the four *Gracilaria* spp. and Difco Bacto agar, the reference agar, were carried out on a rheometer (RS1, HAAKE Instruments, Karlsruhe, Germany), as reported earlier (Meena et al. 2007).

Statistical analyses

Analysis of variance (one-way ANOVA test) was carried out by using Microcal Origin, version 6, software (Microcal Software, MA, USA). To carry out the analysis of variance, four replications (n=4) of each parameter in three groups were made. Mean and standard deviation were calculated using Microsoft Excel 2000 software. One-way ANOVA test was conducted for significant differences (when $P<0.01$) between native agar and best quality alkali-treated agars for agar yield, gel strength, and sulphate content from the four *Gracilaria* species.

Results

Physicochemical properties

The effect of NaOH concentration on the yield of agars is presented in Table 1. The mean values of the agar yield for native agar ranged from 16±0.77 to 25±0.76%, with the greatest value (25%) obtained for *G. edulis* and the lowest (16%) for *G. corticata* (Table 1). The yield of agar decreased with the increase in alkali concentration for all the *Gracilaria* species studied. The mean values of the agar yield for alkali-treated agar ranged from 9.5±0.80 to 23±0.89% (Table 1). The greatest (16%) yield for the best quality alkali-treated agar was obtained for *G. edulis* with 8% NaOH pre-treatment and the lowest (9.5%) was obtained for *G. corticata* (Table 1). Significant differences were noted (Table 2) between native agar and best quality

Table 1 Effect of alkali (NaOH) concentration on the physicochemical properties of agar extracted from four Indian *Gracilaria* spp., and one-way ANOVA test for variation in the same treatment group

NaOH (%)	Yield[a] (%)	Gel strength[b] (g cm^{-2})	Sulphate (%)
Gracilaria edulis agar			
0	25±0.76	100±7.30	5.4±0.23
3	23±0.89	135±7.71	3.6±0.15
4	20±0.86	220±8.50	2.7±0.16
6	18±0.83	340±8.56	2.1±0.07
8	16±0.87	490±8.16	1.4±0.07
10	13±0.73	490±7.72	1.3±0.08
15	11±0.77	490±8.34	1.3±0.08
Gracilaria crassa agar			
0	23±0.86	250±15.20	3.2±0.15
3	22±0.73	330±12.65	2.9±0.23
4	21±0.93	420±17.12	2.4±0.15
6	18±0.75	640±16.68	2.0±0.08
8	16±0.86	800±15.40	1.8±0.08
10	15±0.80	800±15.43	1.6±0.07
15	12±0.84	800±16.12	1.5±0.04
Gracilaria foliifera agar			
0	22±0.80	100±8.56	5.7±0.15
3	20±0.85	100±8.06	5.2±0.08
4	18±0.83	100±8.34	4.6±0.15
6	16±0.77	120±8.56	4.1±0.08
8	15±0.73	135±7.63	3.8±0.08
10	14±0.79	135±7.74	3.7±0.07
15	12±0.76	135±8.00	3.7±0.06
Gracilaria corticata agar			
0	16±0.77	100±6.19	6.8±0.23
3	14±0.81	100±8.06	6.0±0.15
4	13±0.77	100±8.85	5.6±0.17
6	12±0.85	100±7.72	4.7±0.08
8	11±0.72	110±6.29	4.2±0.08
10	10±0.85	110±7.93	4.2±0.08
15	9.5 ±0.5	110±8.94	4.2±0.07

All values are calculated as means ± SD of four replicates in three groups. For all values, $P>0.01$ indicates no significant differences in the same group of treatment

[a] The yield was calculated on the basis of bone-dry seaweeds

[b] Gel strength was measured in 1.5% gel at 20°C

alkali-treated agar (with 8% NaOH pre-treatments) yield for the four *Gracilaria* species ($P<0.01$).

The gel strengths of native agars varied significantly among the seaweed species, ranging from 100±6.19 to 250±15.20 g cm^{-2} (Table 1), with the greatest value for gel strength of 250±15.2 g cm^{-2} for *G. crassa* and the lowest 100±6.19 g cm^{-2} for *G. corticata* (Table 1). Generally, the gel strength of the agars increased with increasing concentrations of NaOH (Table 1). Significant increase ($P<0.01$) in the gel strengths to 490±8.16 and 800±15.40 g cm^{-2} with alkali pre-treatment with 8% NaOH was observed for *G. edulis* and *G. crassa*, respectively (Table 1). The gel strengths of the best quality alkali-treated agars were significantly higher ($P<0.01$) than those of the native agars obtained for *G. edulis* and *G. crassa* (Table 2).

The sulphate contents for the alkali-treated agars obtained from the four *Gracilaria* spp. were in the range of 1.3±0.08 to 6±0.15% (Table 1). The lowest sulphate content, 1.4±0.07%, was observed in best quality alkali-treated agar of *G. edulis* (Tables 1 and 2). ANOVA test showed significant differences between native agars and best quality alkali-treated agars (with optimum alkali) in the sulphate contents from the four *Gracilaria* species, $P<0.01$ (Table 2).

The metal ion contents of *G. edulis and G. crassa* agars and those of Difco Bacto agar are presented in Table 3. In case of sodium ion, Difco Bacto agar showed significantly higher values than those of the agars of *G. edulis and G. crassa* studied here (Table 3).

Dynamic rheological properties

The variations in dynamic viscosity of gels of *Gracilaria* spp. and Difco Bacto agars are shown in Fig. 1. The dynamic viscosity of agar gels decreased when the shear rate was increased. These gels showed non-Newtonian (shear thinning) behavior in this experiment (Fig. 1). Agar gel of *G. crassa* showed the least shear thinning and greatest dynamic viscosity under applied shear of the gels studied herein.

Table 2 Analysis of variance for significant differences in yield, gel strength, and sulphate content of native and 8% alkali-pretreated agar extracted from *Gracilaria* species

Properties	Agar	Value ± SD	*P*
Gracilaria edulis			
Yield[a] (%)	Native agar	25±0.76	<0.01
	Alkali-treated agar	16±0.87	
Gel strength[b] (g cm^{-2})	Native agar	100±7.30	<0.01
	Alkali-treated agar	490±8.16	
Sulphate (%)	Native agar	5.4±0.23	<0.01
	Alkali-treated agar	1.4±0.07	
Gracilaria crassa			
Yield (%)	Native agar	23±0.86	<0.01
	Alkali-treated agar	16±0.86	
Gel strength (g cm^{-2})	Native agar	250±15.20	<0.01
	Alkali-treated agar	800±15.40	
Sulphate (%)	Native agar	3.2±0.15	<0.01
	Alkali-treated agar	1.8±0.08	
Gracilaria foliifera			
Yield (%)	Native agar	22±0.80	<0.01
	Alkali-treated agar	15±0.73	
Gel strength (g cm^{-2})	Native agar	100±8.56	<0.01
	Alkali-treated agar	135±7.63	
Sulphate (%)	Native agar	5.7±0.15	<0.01
	Alkali-treated agar	3.8±0.08	
Gracilaria corticata			
Yield (%)	Native agar	16±0.77	<0.01
	Alkali-treated agar	11±0.72	
Gel strength (g cm^{-2})	Native agar	100±6.19	>0.01
	Alkali-treated agar	110±6.29	
Sulphate (%)	Native agar	6.8±0.23	<0.01
	Alkali-treated agar	4.2±0.08	

$P<0.01$ indicates significant difference between the native and alkali treated agars

[a] The yield was calculated on the basis of bone dry seaweeds

[b] Gel strength was measured in 1.5% gel at 20°C

The temperature dependence of storage (G′) and loss (G″) moduli of *Gracilaria* spp. and Difco agar gels was studied (Fig. 2). The storage modulus increased with decreasing temperature for all the agar gel samples. Maximum G′ values were observed for *Gracilaria crassa* agar gel indicating more rigidity than those of the other *Gracilaria* spp. (i.e., *G. edulis, G. foliifera and G. corticata*) agar gels studied here. Furthermore, the G′ values of the *G. crassa* agar gel were comparable to those of Difco agar gel, which was used as the reference gel sample.

Table 3 Comparison of metal ion contents (ppm) in best quality alkali-treated agars of *G. edulis* and *G. crassa* with those of Difco Bacto agar

Metal ions	*G. crassa* (alkali-treated agar)	*G. edulis* (alkali-treated agar)	Difco Bacto agar
Ca	≤1,280	≤1,410	1,790
Cd	Nil	Nil	NR
Co	Nil	Nil	<10
Cr	Nil	Nil	NR
Cu	≤0.078	≤1.66	<10
Fe	≤0.32	≤0.53	20
K	≤420.3	≤558.9	1,210
Mg	≤190	≤243	680
Mn	≤0.92	≤1.5	<10
Na	≤1,233	≤1,652	8,370
Ni	≤0.15	≤1.2	NR
Pb	Nil	Nil	<10
B	≤1.30	≤2.2	NR
As	Nil	Nil	NR
Zn	≤3.77	≤3.9	<10

NR Not reported

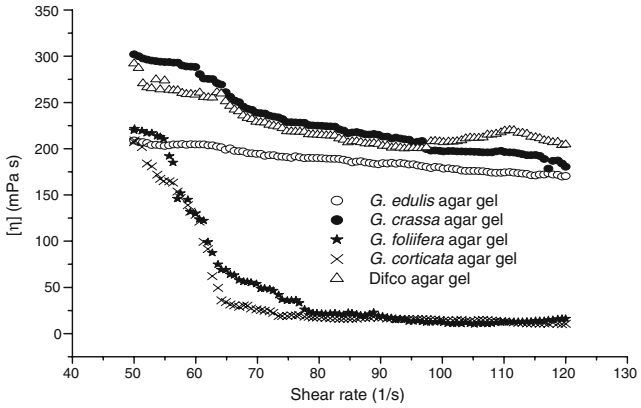

Fig. 1 Dynamic viscosity profiles of alkali-treated agar gel samples extracted from four *Gracilaria* species and Difco bacto agar used as reference agar gel sample

Discussion and conclusions

We reported earlier (Siddhanta et al. 2005) that the gel strength of agar depends on the post-alkali treatment step, wherein the best result was obtained if the pH of the seaweed was maintained in the range 7–8. This prevents degradation of the acid-sensitive galactan polymer backbone present in the cell wall, which contains the 3,6-anhydrogalactose moieties that are responsible for the high gel strength, presumably due to the build-up of local concentrations of acid during neutralization.

The agar yield decreased as the concentration of NaOH increased in all the seaweed species studied. The decrease in yield of alkali-treated agar compared to that of native agar may be attributed to the possible degradation and losses of the polysaccharides in the alkaline liquor (Freile-Pelegrin and Robledo 1997; Siddhanta et al. 2005). It may be noted (Table 3) that the metal ion contents of alkali-treated agars of *G. edulis* and *G. crassa* were lower than those of Difco Bacto agar, indicating the potential utility of these agars in bacteriological applications.

The dynamic viscosity decreased with increasing shear rate, corresponding to a shear-thinning behavior in agar gels as expected, which may be due to the phase or order transfer in the gels during the shear (Fig. 1). The agar gel sample of *G. crassa* showed the least gel thinning and highest dynamic viscosity, indicating more firmness of *G. crassa* agar gel under applied shear rate, presumably due to the high intermolecular association in this gel sample. The storage modulus (G′) and loss modulus (G″) in all gel samples increased with decreasing temperature (Fig. 2a, b), but the increment in G was maximum for the agar gel sample of *G. crassa*, followed by *G. edulis, G. foliifera* and *G. corticata*, indicating a greater elasticity of the *G. crassa* agar gel (cf. Meena et al. 2007).

In this study, all species showed high melting temperatures (84–86°C) and low gelling temperatures (35–40°C), which fall within the range of the United States Pharmacopoeia (USP) standards, indicating their commercial importance (cf. Rebello et al. 1997). The low gelling temperature (35±0.76°C) of *G. crassa* agar gel indicates that it would be more useful for bacteriological and biotechnological applications. The gelling temperatures and sulphate contents of *G. crassa* agar are comparable with those of Difco Bacto agar.

This work dispelled the prevalent myth of poor quality status of certain agarophytes sourced from the Indian waters. In addition, the "sugar reactivity" of the agars of *G. edulis* and *G. crassa* has been reported by Menna et al. (2006). Thus the agarophyte species *G. edulis* and *G. crassa* occurring in Indian waters could be used for producing superior quality agars for commercial exploitation. To our knowledge, this is the first report of such superior quality agars that have been prepared from these two *Gracilaria* species. The results reported herein would be beneficial for bioprospecting of agarophytes.

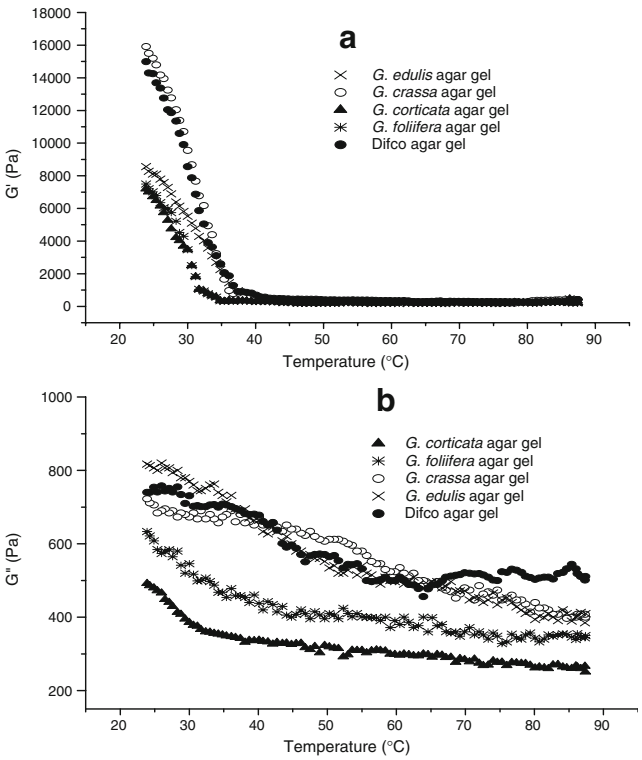

Fig. 2 Temperature dependence of **a** storage (G′) modulus and **b** loss (G″) modulus of best quality alkali-treated agar gel samples extracted from four *Gracilaria* species and Difco agar used as reference agar gel sample

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