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# Preparation of galactans from *Gracilaria debilis* and *Gracilaria salicornia* (Gracilariales, Rhodophyta) of Indian waters

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**Abstract** Superior quality non-methylated and low-sulphated galactans were extracted from two Indian agarophytes namely *Gracilaria debilis* and *G. salicornia* growing naturally along the west coast of India, using an eco-friendly method developed in our laboratory. The galactans were characterised by FT-IR, $^{13}$C NMR, GC-MS, ICP, GPC and rheological measurements. *G. debilis* produced exclusively non-methylated galactan exhibiting the greatest gel strength of 650±25 g cm$^{-2}$ and lowest sulphate of 0.21±0.06%. On the contrary, *G. salicornia* polysaccharide was composed of non-methylated galactose (major) and mannose (minor), having gel strength of 510±25 g cm$^{-2}$ and sulphate of 0.45±0.06%. Very low heavy metal contents were determined in both the galactan samples, which may thus be potentially useful in food and biological applications.

**Keywords** *Gracilaria debilis* · *Gracilaria salicornia* · Galactans · $^{13}$C NMR · GC-MS

## Introduction

*Gracilaria* species are well recognised as a source of linear galactan polysaccharide agar, consisting of the repeating units of (1→3) linked β-D galactopyranosyl and (1→4) linked α-L-3,6-anhydro galactopyranosyl (Fig. 1). *Gracilaria* species have been widely studied and reported in the literature and have played an important role in the business of agar production (Critchley 1993; Armisen 1995). *Gracilaria debilis* (Forsskål) Børgesen and *Gracilaria salicornia* (C. Agardh) Dawson occur naturally at the west coast of India (Oza and Zaidi 2001). Agars, the galactan polymer, are widely used in industries for their excellent gelling or thickening abilities (Selby and Whistler 1993). Many *Gracilaria* spp, e.g. *Gracilaria edulis*, *G. crassa*, *G. foliifera*, *G. corticata*, *G. millardetii*, and *G. fergusonii* occurring in Indian waters, have been reported as the source of agars (Kappanna and Rao 1963). The extraction of low-gel-strength agars from different *Gracilaria* spp of Indian waters has been reported (Siddhanta et al. 1997; Kaliaperumal and Uthirasivan 2001; Meena and Siddhanta 2006). The preparations of superior quality agars and agarose have been reported recently from different Indian agarophytes including *Gracilaria* spp. (Meena et al. 2006; 2007; 2008; Prasad et al. 2007). Krishnamurthy (1989) has reported standing biomass of *G. debilis* to be 1.35 t (corresponding dry biomass was 0.11 t); however, no such data are available for *G. salicornia* from Indian waters. Cultivation of commercially important seaweeds including many agarophytes in Indian waters is being done to ensure uninterrupted availability of the seaweed biomass (Siddhanta et al. 2005). Duckworth et al. (1971) reported alkali-treated methylated agar having gel strength 335 g cm$^{-2}$ (in 2.0% gel) and 0.8% sulphate from *G. debilis* from Barbados. Preparation of low-gel-strength (210 g cm$^{-2}$) agar from *G. salicornia* has been reported in the literature (Oyieke 1993; 1994; Calumpong et al. 1999). Seasonal biomass variation and extraction of low-gel-strength agar from *G. salicornia and G. debilis* from Tanzania, including application in culture of microorganisms have been

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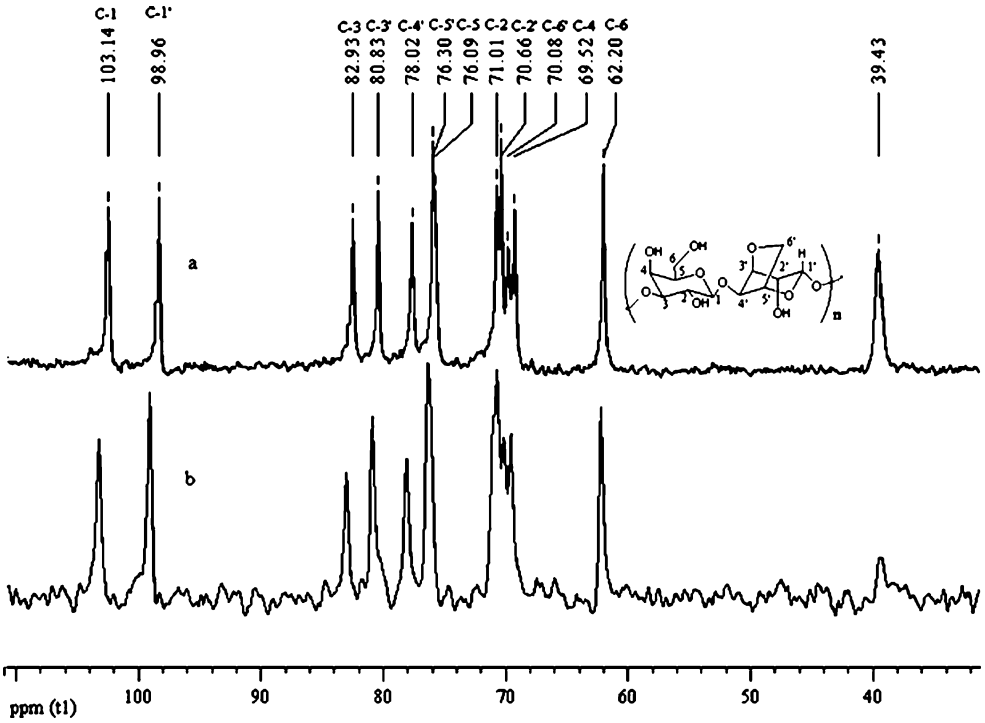


**Fig. 1** $^{13}C$ NMR spectra of alkali-treated galactans of **a** *G. salicornia.* **b** *G. debilis*

reported (Buriyo and Kivaisi 2003; Kivaisi and Buriyo 2007). The prior reports on agars from these two *Gracilaria* species revealed that these are the source of low quality agars. We report herein extraction and physico-chemical characterization of galactans of these two *Gracilaria* species of Indian waters for the first time.

## Materials and methods

*Gracilaria debilis* and *G. salicornia* were collected from the West Coast of India (22.19°N, 68.56°E). Herbarium specimens have been deposited with the CSMCRI Herbarium. Standard sugars Rha, Fuc, Rib, Ara, Xyl, Man, Gal and Glc (AR) were purchased from Aldrich Chem. Co., USA. Other laboratory-grade chemicals were purchased from S. D. Fine Chemicals Ltd., Mumbai, India, and were used without further purification.

*Native galactan extraction* Dry *G. debilis* and *G. salicornia* (20 g each) were soaked in 600 mL water for 1 h at room temperature. The soaked seaweed was cooked in an autoclave with DM water (1:30 *w*/*v*) for 1.5 h at 120°C. The cooked seaweed was then homogenised in a grinder, boiled with Celite and charcoal and filtered through Celite under vacuum to obtain a clear extract. The clear extract was kept at room temperature for gel formation and gelled matter was then frozen at −15°C for 15 h and thawed to produce native agar. Finally, the agar was air-dried for 24 h at ambient conditions and then dried at 50°C for 2 h.

*Extraction of alkali-treated galactan* Alkali pre-treatment was carried out using 10% aqueous NaOH solutions following the procedure described previously for *Gracilaria* species (Meena et al. 2007). Samples of *G. debilis* and *G. salicornia* (20 g dry each) were soaked in 600 mL tap water for 1 h at room temperature and then treated with 600 mL aqueous NaOH at 80°C in a water-bath for 2 h. After the alkali treatment, excess alkali was removed by water washing until the washings 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 similar process as mentioned above for the native agar by freezing–thawing. The alkali (NaOH) used in this study can be recycled and reused for subsequent batches for preparation of alkali-treated agar (cf. Mehta et al. 2008). The percentage yields of dried native and alkali-treated agars were calculated on the basis of dry seaweed with respect to nil moisture content in the seaweed. Native and alkali pretreated extraction processes were repeated three times with the respective seaweeds.

*Characterization of galactans* Spectral analyses, e.g. FT-IR and inductively coupled plasma-emission spectrophotometry (ICP) along with the estimations of sulphate contents were carried out as reported earlier (Meena et al. 2007). Determination of 3,6-AG (anhydro galactose) and galactose followed the method of Yaphe (1960). Noise-decoupled $^{13}C$ NMR spectra were recorded on a Bruker Avance-II 500 (Ultra shield) Spectrometer, Switzerland, at 125 MHz. Agar samples were dissolved in $D_2O$ (60 mg $mL^{-1}$) and the

NMR spectra were recorded at 70°C, using DMSO as internal standard (ca. δ 39.5 ppm).

The carbohydrate profiling of the galactan samples was carried out on a Shimadzu GCMS-QP2010 machine, using an SGE BP-225 capillary column and helium as carrier gas, employing temperature programming (160°C to 230°C at 10°C $min^{-1}$). Alditol acetates were prepared following the method of Siddhanta et al. (2001). The average molecular weight (Mw) was determined by gel permeation chromatography (GPC) on a Waters Alliance 2695 machine with RI 2414 detector. Columns (Ultra hydragel 120, Ultra hydragel 500) were eluted with 0.1 M $NaNO_3$ at a flow rate of 0.5 mL $min^{-1}$. Oven and flow cell temperatures were maintained at 45°C for all measurements. Dextrans of different molecular weights, e.g. $4.4\times10^3$, $4.3\times10^4$, $1.96\times10^5$, and $4.01\times10^5$ Da were used as standards for calibration.

Gel strength was measured in 1.5% galactan gel at 20°C unless otherwise stated, on a Nikkansui-type gel tester (Kiya Seisakusho Ltd., Japan). The gelling and melting temperatures of gel samples were recorded as reported earlier (Meena et al., 2007). Apparent viscosity was measured (in 1.5% galactan solution) on a Brookfield viscometer (DV-II+Pro), using SC4-18 spindle at 60 rpm at 80°C. Specific rotations were measured in 0.25% galactan solution at 45°C on a Jasco J-815 CD spectrometer.

Dynamic rheological measurements of sol and gel samples of the alkali-treated galactans were carried out on a rheometer (RS1, HAAKE Instruments, Germany). The plate/plate (35 mm diameter) geometry was selected for dynamic viscosity measurements at 45°C. Oscillation measurements of galactan gel samples in the controlled deformation (CD) mode with a strain value of 0.05 were done at 25°C using the DC50 water circulator. Measurements of $G'$ and $G''$ were performed over 60 min. Subsequently, temperature dependence measurements were carried out immediately after placing the sample on the plate, having covered the exposed part of the sample with silicone oil to minimise losses due to evaporation. All rheological data are means of three replicate measurements.

## Results and discussion

No significant differences were observed in the yields of both the galactans of *Gracilaria* species (Table 1). Yields of alkali-treated galactans were lower than those of the native ones, which may be due to losses caused by the alkali hydrolysis during pre-treatment (cf. Meena et al. 2007; 2008). Native galactans of *G. debilis* and *G. salicornia* had gel strengths of 490±25 and 350±25 g $cm^{-2}$, respectively, whereas respective values in alkali-treated galactans were 650±25 and 510±25 g $cm^{-2}$ (Table 1), as was expected (cf. Duckworth et al. 1971; Oyieke 1993; Meena et al. 2007; 2008). Results of this study are in good agreement with those reported by Duckworth et al. (1971) and Oyieke (1993).

The values of apparent viscosity of native polysaccharides of *G. debilis* and *G. salicornia* were 25±1.0 and 35±1.0 cP, respectively, while the corresponding values of the alkali-treated ones were 30±1.0 and 45±1.0 cP (Table 1). The greater viscosity of *G. salicornia* galactans was presumably due to the presence of mannose in the polysaccharides (see GC-MS results below).

No variations were observed in the gelling temperatures (38±0.5°C) of the galactans of both *Gracilaria* species; the melting temperatures, however, varied significantly. The native and alkali-treated galactans of *G. debilis* and *G. salicornia* had melting temperatures 82±0.5°C, 85±0.5°C and 84±0.5°C, 88±0.5°C, respectively (Table 1). Similar variations in other *Gracilaria* species have been reported by Meena et al. (2008). Specific rotation values of alkali-treated galactans of *G. debilis* and *G. salicornia* were $[\alpha]^{45°C}_{589nm}$ −30.09° (c 0.25, $H_2O$) and -18.19° (c 0.25, $H_2O$), respectively. Similar observations have been reported previously (Meena et al. 2007). The corresponding average molecular weights (Mw) of the alkali-treated galactans were $8.25\times10^5$ and $4.48\times10^5$ Da. The same trend was observed in their respective poly dispersity indices, e.g. for 4.23 and 2.38.

Chemical analyses indicated significant variations in 3,6-AG, galactose, and sulphate contents of the galactans obtained from these *Gracilaria* species (Table 1). The 3,6-AG and galactose contents were 28.7±1.0%; 59.2±1.5% and 33.1±1.0%; 55.3±1.5% for native and alkali-treated galactans obtained from *G. debilis*, respectively (Table 1). The 3,6-AG and galactose contents were 23.8±1.0%; 49.6±1.5% and 28.1±1.0%; 53.1±1.5% for native and alkali-treated galactans obtained from *G. salicornia*, respectively (Table 1). The 3,6-AG/Gal ratios for the native and alkali-treated galactans obtained from *G. debilis* were 0.48 and 0.59, respectively, while the respective values for *G. salicornia* were 0.47 and 0.52 (Table 1). Similarly, the sulphate contents were 0.76±0.08% and 3.37±0.12% for native and 0.21±0.06% and 0.45±0.06% for alkali-treated galactans of the above mentioned seaweeds, respectively. It should be pointed out that a combination of lowest sulphate and greatest 3,6-anhydrogalactose contents gives high gel strength as reported earlier (Murano 1995; Siddhanta et al. 1997; Meena et al. 2007; 2008). The high-gel-strength of *G. debilis* galactan in the present study is yet another demonstration of this. Metal ion contents of native and alkali-treated galactans were estimated. Commercially available agar (Fluka Cat. 5038) was used as the reference sample for metal ion comparison. Very low metal ion

**Table 1** Physico-chemical properties of native and alkali-treated galactans extracted from *Gracilaria debilis* and *Gracilaria salicornia* of Indian waters (values are means ± S.D.)

| Seaweed/ agars | Yield[a] (%) | Gel strength[b] (g cm$^{-2}$) | Carbohydrate composition (%) | | | | | Ratio (3,6-AG/ Gal) | Sulphate (%) | Gelling temp. (°C) | Melting temp. (°C) | Apparent viscosity[b,c] (cP) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Ara | Xyl | Man | 3,6-AG | Gal | | | | | |
| *G. debilis* | | | | | | | | | | | | |
| Native | 14.8 (±0.5) | 490 (±25) | ND | ND | ND | 28.7 (±1.0) | 59.2 (±1.5) | 0.48 | 0.76 (±0.08) | 38 (±0.5) | 82 (±0.5) | 25 (±1.0) |
| Alkali-treated | 13.1 (±0.5) | 650 (±25) | ND | ND | ND | 33.1 (±1.0) | 55.3 (±1.5) | 0.59 | 0.21 (±0.06) | 38 (±0.5) | 84 (±0.5) | 30 (±1.0) |
| *G. salicornia* | | | | | | | | | | | | |
| Native | 15.2 (±0.5) | 350 (±25) | 6.09 | 4.49 | 8.42 | 23.8 (±1.0) | 49.6 (±1.5) | 0.47 | 3.37 (±0.12) | 38 (±0.5) | 85 (±0.5) | 35 (±1.0) |
| Alkali-treated | 13.2 (±0.5) | 510 (±25) | ND | ND | 14.0 | 28.1 (±1.0) | 53.1 (±1.5) | 0.52 | 0.45 (0.06) | 38 (±0.5) | 88 (±0.5) | 45 (±1.0) |

[a] Yield was calculated on the basis of as received dry seaweeds; and are the mean values of the three replicates

[b] Gel strength and apparent viscosity were measured in 1.5% polysaccharide gel and sol, respectively

[c] Apparent viscosity was measured at 80°C

*ND* not detected

contents were found in the galactans indicating that these can be used as gelling agents of choice, like agar, in food and biological applications. GC-MS analysis and colorimetric determination of 3,6-AG and galactose indicated that native and alkali-treated polysaccharides of *G. debilis* were composed of 3,6-AG and galactose in varying proportions and can be treated as agar polymer (Table 1). The GC-MS data indicated chemical heterogeneity of the native and alkali-treated polysaccharides of *G. salicornia* (Table 1). The native polysaccharide of *G. salicornia* was composed of xylose (4.49%), arabinose (6.09%), mannose (8.42%), 3,6-AG (23.8%) and galactose (49.6%), while the alkali-treated one contained mannose (14.0%), 3,6-AG (28.1%) and galactose (53.1%; Table 1). Indian *G. debilis* produced non-methylated galactan polymer as shown by the single peak of galactitol acetate in the GC-MS, while Duckworth et al. (1971) has reported methylated agar from naturally occurring *G. debilis* from Barbados. FT-IR spectra of the alkali-treated galactans of both *Gracilaria* spp. were recorded. The IR bands were in good agreement with those for an agar sample determined by Christiaen and Bodard (1983). The $^{13}$C-NMR chemical shifts of agars obtained from *G. debilis* and *G. salicornia* were found to be similar to those reported for agar polysaccharide by Usov et al. (1980). The absence of the methyl carbon resonance in both $^{13}$C NMR spectra further confirmed that these agarophytes contained non-methylated polysaccharides (Fig. 1; cf. GC-MS).

The variations in dynamic viscosity of alkali-treated galactans of *G. debilis* and *G. salicornia* galactans were measured. Under high shear rate, strong shear thinning behaviour was observed in both gels. Furthermore, the shear thinning effect was lower in the *G. salicornia* compared to the *G. debilis* galactan gel, indicating a positive correlation with the apparent viscosity values (vide Table 1). The temperature dependence of storage ($G'$) and loss moduli ($G''$) of the alkali-treated galactans were studied. Crossover of $G'$ and $G''$ traces occurred at the gelling point, which confirmed the values obtained by a manual method (Craigie and Leigh 1978). The time-dependent changes of the $G'$ values was also measured. The alkali-treated galactan gels of *G. debilis* exhibited a greater $G'$ value than that of *G. salicornia* agar gel sample. The greater $G'$ value obtained for the alkali-treated galactan of *G. debilis* validated its higher gel strength compared to that of the corresponding *G. salicornia* polysaccharide, which was in good agreement with our previous report (Meena et al. 2006).

In conclusion the alkali-treated polysaccharides of *G. debilis* were composed exclusively of non-methylated galactan having high gel strength and low sulphate, which can be used as agar polymer. On the other hand, *G. salicornia* produced non-methylated galactan with mannose

residues having comparatively lower gel strength and high sulphate; this can be treated as an agaroid polymer. Both these galactans contained low metal ions. These galactan polymers are superior to those reported earlier in the literature from these *Gracilaria* species. These results would also be useful in the bioprospecting work on agarophytes.

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