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Influence of alkali treatment on agar from *Gracilaria cornea* from Yucatán, México

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Abstract

The effect of alkali treatments on the yield, rheological and chemical properties of agar from *Gracilaria cornea* growing along the Yucatán coast were studied in order to evaluate its potential for industrial use in an attractive economic standpoint. Alkali treatment was carried out with NaOH concentrations of 0.5%, 1%, 3% and 5% in a water bath at 80, 85 and 90 °C. Agar yield, gel strength, gelling and melting temperatures, sulphate, 3,6-anhydrogalactose and ash content were determined. The different combinations of NaOH concentration and treatment temperature strongly influenced agar characteristics. There was a variation in the agar content for all NaOH treatments and temperature combinations, ranging between 14.5% to 22.1%. Although the yields obtained for 0.5% NaOH at all temperatures and 1% NaOH at 80 and 85 °C were higher than those required by the industry, the physical and chemical characteristics of the agar were similar to those obtained for native agar from the same species. The gel strengths, sulphate content and gelation hysteresis obtained with agar from the 1% NaOH treatment at 90 °C are in the range required by the food industry. Treatments with 3% and 5% NaOH at all temperatures improved significantly the agar quality giving higher gel strengths (974–1758 g cm^{-2}) than those reported for other *Gracilaria* species.

Introduction

Gracilaria is currently the major agar source worldwide (Critchley, 1993) and numerous studies have been done on the agar yield and quality for species from different geographical areas (Aponte-Díaz & Lemus, 1989; Matsuhiro & Urzúa, 1990; Luhan, 1992; Yenigül, 1993). The genus *Gracilaria* is of particular interest because of the large quantities of several species available in temperate and tropical regions, and for the amenability of its species for mariculture. A recent study on *Gracilaria cornea* from the Yucatán coast has pointed out the economic importance of this tropical species in relation to its agar characteristics (Freile-Pelegrín & Robledo, 1997). It is well known that the quantity and quality of the colloid varies not only among species (Cote & Hanisak, 1986), but they are also influenced by environmental factors (Craigie & Wen, 1984), seasonal variations (Lahaye & Yaphe, 1988) and extraction methods (Craigie & Leigh, 1978; Armisén & Galatas, 1987; Lemus et al., 1991). The yield and the physical properties of agar such as gel strength, melting and gelling temperatures as well as chemical properties, determine its value to the industry.

Generally, *Gracilaria* species produce agars with low quality due to high sulphate concentrations and therefore they are called 'agaroids' (Craigie, 1990). However, the gel properties of many *Gracilaria* agars can be improved by alkali treatment, which converts L-galactose-6-sulphate to 3,6-anhydro-L-galactose (Duckworth et al., 1971), which is responsible for the enhancement of the gel forming ability. This treatment must be adapted to each species of *Gracilaria*, and, in particular, variables like temperature and alkali concentration must be adjusted to obtain as much desulphation as possible, while still avoiding the yield losses that this process can cause (Armisén & Galatas, 1987). One of the problems in the industrial produc-

tion of agars from *Gracilaria* is that alkali treatment produces considerable pollution in the outflows which then must be neutralized, thereby raising production costs (Pickering et al., 1993; Armisén, 1995).

Although native agar extracted from *G. cornea* from Yucatán was recognized as an agaroid, alkali treatment induced a dramatic increase in its quality characteristics. No studies have been reported on the optimum alkali treatment for this alga. The present study was conducted to determine the effect of varying the temperature and alkali concentration on agar yield, rheological and chemical properties in order to obtain an industrial quality agar that would be attractive from an economic standpoint.

Materials and methods

Gracilaria cornea J. Agardh entire plants were collected by diving between 1–3 m depth at Dzilam de Bravo (21°23′N – 88°57′W) during May (1995). Harvested plants were transported to the laboratory, washed thoroughly with tap water and submerged overnight in 10% formaldehyde to prevent enzymatic hydrolysis, sun dried and stored in sealed plastic bags until agar extraction. The algal material collected was free from visible contaminants and was considered as 'pure seaweed'.

Alkali treatment

Alkali treatment was carried using 0.5%, 1%, 3% and 5% NaOH concentrations. About 20 g dry weight of *G. cornea* were soaked overnight in 400 mL of the appropriate NaOH solution at room temperature to hydrate the seaweeds. This was followed by 3 h in a water bath at either 80 °C, 85 °C or 90 °C.

Agar extraction

After alkali treatment, the plants were washed with running tap water for 1 h to remove excess NaOH. Samples from light alkali treatment (0.5% and 1% NaOH) were soaked for 2 h in 400 mL of a 0.025% solution of H_3PO_4, while samples from 3% and 5% NaOH were soaked in 400 mL of a 0.025% of H_2SO_4 for the same time. To remove the acid, samples were washed twice with 400 mL of distilled water for 30 min each time. Extraction was carried out by boiling for 1.3 h in 600 mL of distilled water at 6.3–6.5 pH. The extract was ground with a commercial blender, mixed with Celite and finally pressure filtered. The filtrate was allowed to gel at room temperature, frozen overnight and thawed. Finally, the agar was oven dried for 24 h at 60 °C, cooled and weighed to calculate percent agar yield. Extractions and all physical and chemical agar analyses were performed in triplicate.

Physical properties

Dry agar was ground in a Tecator mill and reconstituted in 1.5% w/v solutions to measure the physical properties (gel strength, melting and gelling temperature). Gel strength was measured after gelling overnight at room temperature by measuring the load (g cm^{-2}) causing a cylindrical plunger (1 cm^2 cross-section) to break a standard gel in 20 s (Armisén & Galatas, 1987).

Gelling temperature was measured by adding 10 mL hot agar solution and a glass bead (5 mm diameter) to a test tube (2.3 cm diameter, 6 cm height). The tube was tilted up and down in a water bath at room temperature until the glass bead ceased moving. The gel temperature in the tube was immediately measured by introducing a precision thermometer (0.1 °C divisions) into the agar. Melting temperature of the gel in a test tube (2.3 cm diameter, 16.5 cm height) was measured by placing an iron bead (9 mm diameter) on the gel surface. The test tube was clamped in a water-bath and the temperature raised from 50 to 100 °C at 1 °min^{-1}. The melting point was recorded with a precision thermometer when the bead sank into the solution.

Chemical properties

Percent sulphate was determined in 100 mL Kjeldahl flasks by hydrolyzing 1 g of agar powder (previously dried at 105 °C) in 10 mL concentrated HNO_3 until the complete hydrolysis of the ester sulphate, which was then quantitatively precipitation with barium chloride. The precipitates were collected on ash-free gravimetric filters, dried, ignited and weighed on a precision balance (0.0001 g). The weight of the barium sulphate obtained, multiplied by 0.4116, gave the quantity of sulphate in the agar sample.

The 3,6-anhydro-galactose content (3,6 AG) was determined following the method of Matsuhiro and Zanlungo (1983). An agarose sample kindly provided by R. Armisén (HISPANAGAR) with a known content of 3,6 AG, 50% according to Rochas et al. (1994), was used as the standard.

Ash content

Ash content was obtained by burning 1 g of agar to a constant weight in a porcelain crucible at 600 °C for 3 h in an electric furnace.

Statistical analyses

Data were tested for normality (Kolmogorov-Smirnov) and subjected to the Bartlett's test for homogeneity of group variances using a statistical software package (Stasoft). Pearson's product moment correlation test was used to determine the linear relationship between agar properties. The interaction of the effect of NaOH concentration and temperature on agar properties were assessed by two-way analysis of variance (ANOVA). A post hoc comparison of means by LSD test were done for comparison between treatments.

Results

Agar yield

The agar content for all NaOH treatments and temperature combinations, ranged between 14.5% to 22.1% (Figure 1a). For the treatment with 0.5% NaOH the yield ranged between 14.5 to 17.6%, with the maximum value found at 90 °C and the only significance difference was obtained for this temperature ($p < 0.01$). The treatment with 1% NaOH produced an agar yield between 18.4 and 19.9% with no significance difference among the three temperatures tested. The agar yield ranged from a minimum of 17.6% at 85 °C to a maximum of 22.1% at 80 °C for the treatment with 3% NaOH, with the only significance difference at this temperature ($p < 0.05$). For the treatment with 5% NaOH, the agar yield was lowest at 90 °C (16.6%) and highest at 80 °C (19.4%), with the latter being significantly different from those at 85 and 90 °C ($p < 0.05$).

Physical properties

The variations in gel strength for all NaOH treatments and temperature combinations are shown in Figure 1b. The gel strength varied between 118 and 155 g cm^{-2} for the lowest NaOH concentration with no significance differences among the three temperatures tested. For 1% NaOH concentration the gel strength ranged between a minimum of 173 g cm^{-2} at 80 °C and a maximum of 752 g cm^{-2} at 90 °C, with a highly significant difference ($p < 0.001$). The minimum gel strength of 974 g cm^{-2} found at 80 °C with 3% NaOH rose dramatically to 1647 g cm^{-2} at 85 °C. There was no significant difference between 85 and 90 °C at this concentration. For the highest NaOH concentration (5%) the gel strength ranged between 1694 and 1758 g cm^{-2} with no significance difference among the three temperatures tested.

Chemical properties

In general, sulphate content decreased when NaOH concentration increased (Figure 1c). For the treatments with 0.5% and 1% NaOH sulphate content ranged between 3.33–4.25% and 2.61–3.69%, respectively, decreasing as the temperature increased. The only significant decrease was found at 90 °C for both NaOH concentrations ($p < 0.01$). For the 3% and 5% NaOH treatments, the sulphate varied from 1.54 to 1.90% and 1.53 to 1.75%, respectively. No significance effect of temperature was found at either of these concentrations. There was a clear negative correlation between sulphate content and gel strength for all NaOH concentration at corresponding temperatures ($r = -0.92$, $r = -0.98$, $r = -0.95$; 80, 85 and 90 °C, respectively; $p < 0.001$).

Figure 1d shows the 3,6-anhydro-L-galactose content obtained for the various treatments. The lowest values were obtained at 0.5% and 1% NaOH ranging between 32.6–35.9% and 33.6–36.7%, respectively. At 3% NaOH, the 3,6-anhydro-L-galactose content increased from a minimum of 41.9% at 80 °C to a maximum of 44.6% at 90 °C, with no significance difference between 85 and 90 °C. The 3,6-anhydro-L-galactose content for 5% NaOH treatment ranged between 42.1 and 45.4% with no significance difference among the three temperatures tested. There was a positive correlation between 3,6-anhydro-L-galactose and gel strength for all NaOH concentration at corresponding temperatures ($r = 0.92$, $r = 0.98$, $r = 0.94$; 80, 85 and 90 °C, respectively; $p < 0.001$), and a clear inverse relationship with sulphate content ($r = -0.96$, $p < 0.001$; $r = -0.95$, $p <= 0.001$, $r = -0.87$, $p < 0.005$; 80, 85 and 90 °C, respectively).

The gelling, melting and gelation hysteresis (difference between melting and gelling temperature) are presented in Table 1. Gelling and melting temperatures were low for the 0.5% NaOH treatment (at all temperatures) and 1% NaOH treatment (at 80 and 85 °C). The gelation hysteresis generally increased at higher NaOH concentration and reached values above 50 °C

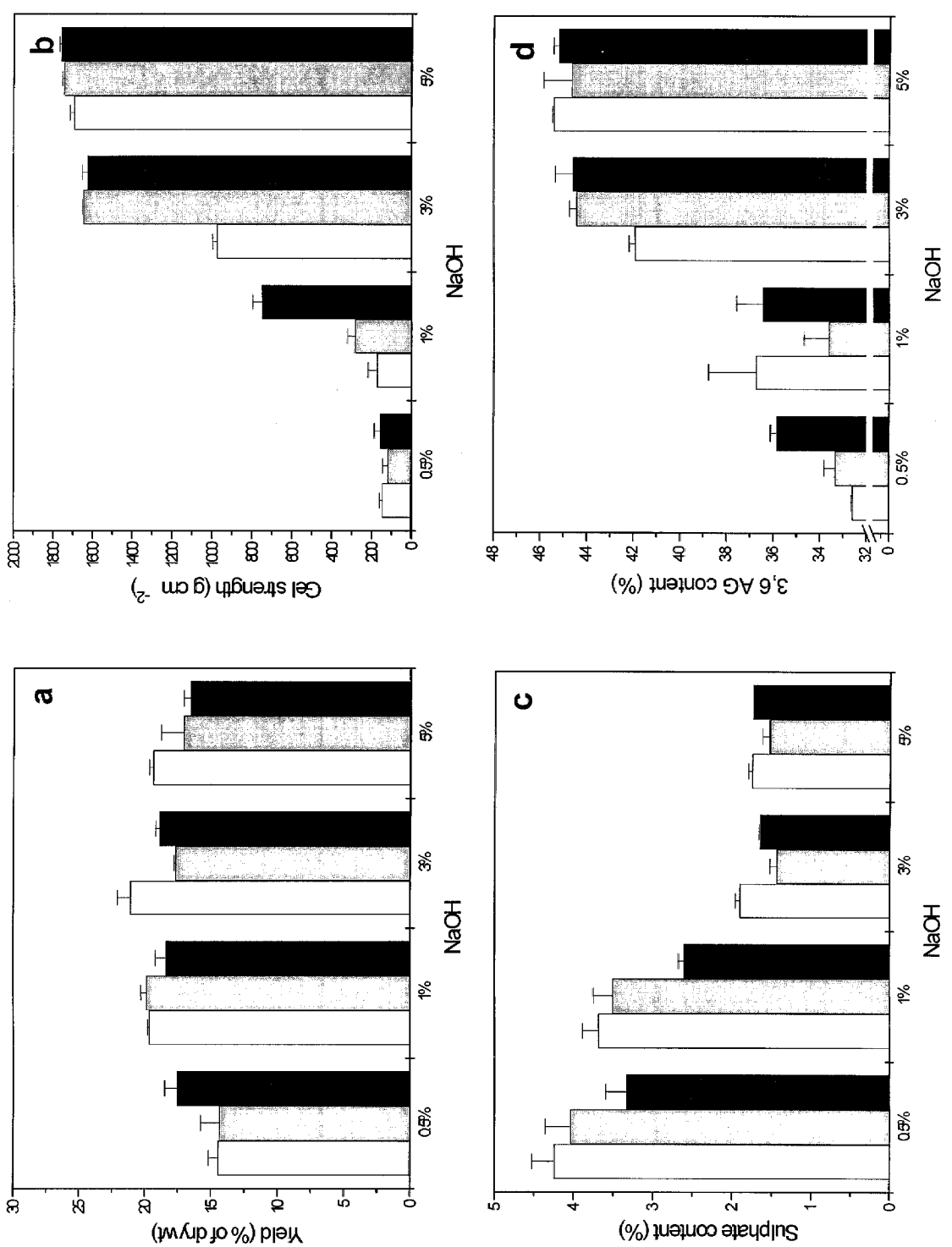

Figure 1. Characteristics of *Gracilaria cornea* agar obtained after different NaOH treatments at three temperatures: 80 °C (white bar), 85 °C (grey bar) and 90 °C (black bar). a. Agar yield. b. Gel strength of 1.5% agar. c. Sulphate content. d. 3,6-Anhydro-L-galactose content. Vertical lines represent standard deviations.

Table 1. Gelling, melting and gelation hysteresis for agar treated with different NaOH concentrations at three temperatures. Data are mean ± standard deviation

Treatment	Gelling T (°C)	Melting T (°C)	Hysteresis T (°C)
0.5% NaOH			
80 °C	35.3 ± 0.2	80.1 ± 0.6	44.8 ± 0.6
85 °C	33.8 ± 1.2	77.2 ± 0.2	43.0 ± 1.0
90 °C	34.8 ± 0.2	77.2 ± 0.7	42.5 ± 0.5
1% NaOH			
80 °C	34.2 ± 0.2	76.2 ± 0.2	42.0 ± 0.5
85 °C	36.0 ± 0.4	81.3 ± 1.4	45.3 ± 1.0
90 °C	39.5 ± 1.1	87.0 ± 1.0	48.2 ± 0.7
3% NaOH			
80 °C	42.2 ± 0.2	91.2 ± 0.2	49.0 ± 0.0
85 °C	42.2 ± 0.2	94.5 ± 0.5	52.2 ± 0.7
90 °C	42.5 ± 0.7	93.7 ± 0.7	51.0 ± 1.5
5% NaOH			
80 °C	43.0 ± 0.7	93.2 ± 0.6	50.2 ± 0.8
85 °C	42.7 ± 0.2	94.7 ± 0.2	52.0 ± 0.2
90 °C	42.5 ± 0.5	96.7 ± 0.2	54.2 ± 0.2

at 3% NaOH and 85 °C. On the other hand, there was a positive correlation between gelation hysteresis and gel strength at all NaOH concentration at corresponding temperatures ($r = 0.90$, $r = 0.97$, $r = 0.95$; 80, 85 and 90 °C, respectively; $p < 0.001$), and an inverse relationship with sulphate content ($r = -0.85$, $r = -0.95$, $r = -0.93$; 80, 85 and 90 °C, respectively; $p < 0.001$).

Ash content

The ash content (data not shown) followed a pattern similar to the sulphate content. The ash content at 0.5% NaOH (4.19–4.84%) was twice that found for 3% (2.39–2.68%) and 5% NaOH (2.69–3.06%). There was a clear positive correlation between the ash and the sulphate content at all NaOH concentrations at corresponding temperatures ($r = 0.97$, $r = 0.91$, $r = 0.95$; 80, 85 and 90 °C, respectively; $p < 0.001$).

Table 2 summarizes the ANOVA on the effects of NaOH concentration, temperature and their interactive effects on agar yield, and physical and chemical properties.

Discussion

Variations in NaOH concentrations and temperatures for the alkali treatment strongly influenced the agar characteristics of *Gracilaria cornea*. However, not all the combinations of alkali concentration and temperatures had a significant effect on agar quality. This was evident for 0.5% NaOH at all temperatures, and for 1% NaOH at 80 and 85 °C. Although the yields obtained for these treatments were higher than those required by the industry (> 8%) (Armisén, 1995), the physical and chemical characteristics of the agar were similar to those obtained for native agar, gel strength between 89–130 g cm^{-2} and sulphate content 4.8–5.5% (Freile-Pelegrín & Robledo, 1997). Thus, the agars obtained with these treatments can be considered as agaroids, due mainly to the low gel strength and high sulphate content.

The agar content found at 1 and 3% NaOH for all temperatures, and 5% NaOH at 80° was similar to those obtained for other tropical species, *G. edulis* (21.8%) from Brazil (Durairatnam, 1987) and *G. cervicornis* (21%) from Venezuela (Aponte-Díaz & Lemus, 1989). Increasing the temperature from 80 °C to 85 or 90 °C at 3% and 5% NaOH concentrations resulted in a yield reduction related to agar diffusion into the water. The slight decrease in the agar yield at 5% NaOH at 80 °C

Table 2. Analysis of variance on the yield and physico-chemical properties of agar from *Gracilaria cornea* treated with different NaOH concentration at three temperatures. A = NaOH concentration; B = temperature; A × B = NaOH concentration and temperature interaction

Sources variation	df	F	p
Agar yield			
A	3	25.12	0.000**
B	2	3.52	0.048*
A × B	6	5.76	0.001**
Gel strength			
A	3	3024.85	0.000**
B	2	194.27	0.000**
A × B	6	77.53	0.000**
Gelation hysteresis			
A	3	104.64	0.000**
B	2	12.79	0.000**
A × B	6	7.91	0.000**
Sulphate content			
A	3	154.66	0.000**
B	2	12.50	0.000**
A × B	6	3.68	0.013*
3,6 AG content			
A	3	175.67	0.000**
B	2	5.52	0.015*
A × B	6	3.13	0.031*

** Highly significant ($p < 0.01$); * Significant ($p < 0.05$)

compared to the yield at 3% NaOH at the same temperature suggests that the former treatment could be producing some degradation of the polysaccharide.

The strong inverse relationship between the gel strength and sulphate content, and the high correlation between the former with 3,6-anhydro-L-galactose content as the NaOH concentration increased has been explained as the elimination of the energetically unstable sulphate ester at C–6 of the L-galactose to increase the 3,6-anhydro-L-galactose content thereby improving the gelling properties (Murano, 1995). The high 3,6-anhydro-L-galactose content obtained in the agar from the 3 and 5% NaOH treatments are similar to those found for *Gracilaria chilensis* agar (43.0%) (Matsuhiro & Urzúa, 1990), the major industrial agar source.

The low gelation hysteresis found for the 0.5% and 1% NaOH treatments would be related to the gelation process. The thermo-reversible transition of agar can be highly perturbed by the presence of charged groups, like sulphate, which can interfere with intermolecular hydrogen bonding (Murano, 1995). This is seen in the inverse relationship between gelation hysteresis and sulphate content of agar from *G. cornea*.

The high positive correlation between ash and sulphate content as the NaOH concentration increased is consistent with the alkaline hydrolysis of sulphate. A maximum ash content of 5% is acceptable for commercial agar, although it is normally maintained between 2.5 and 4% (Armisén & Galatas, 1987). Ash content in the agar extracted from *G. cornea* with 1% NaOH concentration at 90 °C was within this range, reaching values similar to those obtained from *Gelidium* agars for alkali concentrations of 3% and 5% NaOH.

It is impossible to assign a general extraction method valid for any agarophyte to evaluate its yield and at the same time obtain a good quality agar for industrial use. The international food market currently requires a gel strength equal to or greater than 750 g cm^{-2} (1.5% gel) and a sulphate content less than 4%, usually 1.5–2.5% (Armisén, 1995). In our study, the gel strength values, sulphate content and gelation hysteresis obtained with agar from 1% NaOH treatment at 90 °C are in the ranges requires by the food industry. Nevertheless, the agar quality improved significantly for the treatment with 3% and 5% NaOH, with higher gel strength than those reported for other *Gracilaria* species (Yenigül, 1993; Chirapat & Ohno, 1993).

Although the maximum gel strength was found at 5% NaOH treatment, ANOVA showed that there was no significant different between 3% NaOH (except for 80 °C) and 5% NaOH. On the other hand, the slightly decreased agar yield at this last concentration suggests that the optimum alkali treatment is 3% NaOH in a temperature range of 85 to 90 °C. However, if we consider that the Japanese agar industries use NaOH concentrations of 1–2% (Tagawa & Kojima, 1972; cited in Armisén, 1987), the agar obtained from *G. cornea* treated with 1% NaOH at 90 °C produces an agar adequate for industrial purposes.

Quality is usually evaluated in terms of the industrial applications and determines the chemical tailoring of agar preparations (Murano, 1995). In this regard, high quality agar obtained from treatments at 3 and 5% NaOH could be used for specific applications such as bacteriological grade agar or agarose which are usually prepared from *Gelidium* and *Pterocladia*. Never-

theless, other information such as optical clarity and incubation with specific test bacteria are needed to corroborate this particular use.

A relative new grade is the 'sugar reactive' in which the gel strength increases as sugar is added to the agar solution. This effect can be produced by high molecular weight agar of low sulphate content. Commercially, agar possessing this characteristic are derived from *Gracilaria chilensis* (Armisén, 1995). On this regard, *G. cornea* agars modified by the treatments with 3 and 5% of NaOH with the highest gel strength and low sulphate content, could be used as a sugar reactive agar source.

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References

Aponte-Diaz M, Lemus AL (1989) Comparative study of the agar obtained from three species *Gracilaria* feasible for culture in Venezuela. In de Oliveira EC, Kautsky N (eds), Cultivation of Seaweeds in Latin America. USP/IFS, Brazil: 117–119.

Armisén R (1995) World-wide use and importance of *Gracilaria*. J. appl. Phycol. 7: 231–243.

Armisén R, Galatas F (1987) Production, properties and uses of agar. In McHugh DJ (ed.), Production and Utilization of Products from Commercial Seaweeds. FAO Fish. Tech. Pap. 288: 1–57.

Craigie JS (1990) Cell walls. In Cole KM, Sheath RG (eds), Biology of Red Algae. Cambridge Univ. Press, New York: 221–257.

Craigie JS, Leigh C (1978) Carrageenans and agars. In Hellebust JA, Craigie JS (eds), Handbook of Physiological Methods. Cambridge Univ. Press, Cambridge: 109–131.

Craigie JS, Wen ZC (1984) Effects of temperature and tissue age on gel strength and composition of agar from *Gracilaria tikvahiae* (Rhodophyceae). Can. J. Bot. 62: 1665–1670.

Chirapat A, Ohno M (1993) Seasonal variation in the physical properties of agar and biomass of *Gracilaria* sp. (*chorda* type) from Tosa Bay, southern Japan. Hydrobiologia 260/261: 541–547.

Critchley AT (1993) *Gracilaria* (Rhodophyta, Gracilariales): An economically important agarophyte. In Ohno M, Critchley AT (eds), Seaweed Cultivation and Marine Ranching. JICA, Japan: 89–112.

Cote GL, Hanisak MD (1986) Production and properties of native agars from *Gracilaria tikvahiae* and other red algae. Bot. mar. 29: 359–366.

Duckworth M, Yaphe W (1971) The structure of agar. Part 1: Fractionation of a complex mixture of polysaccharides. Carbohydr. Res. 16: 189–197.

Durairatnam M (1987) Studies of the yield of agar, gel strength and quality of agar of *Gracilaria edulis* (Gmel.) Silva from Brazil. Hydrobiologia 151/152: 509–512.

Freile-Pelegrín Y, Robledo D (1997) Effects of season on the agar content and chemical characteristics of *Gracilaria cornea* from Yucatán, México. Bot. mar. 40: 285–290.

Lahaye M, Yaphe W (1988) Effects of seasons on the chemical structure and gel strength of *Gracilaria pseudoverrucosa* agar (Gracilariaceae, Rhodophyta). Carbohydr. Polymers 8: 285–301.

Lemus A, Bird K, Kapraun DF, Koehn F (1991) Agar yield, quality and standing crop biomass of *Gelidium serrulatum*, *Gelidium floridanum* and *Pterocladia capillacea* in Venezuela. Food Hydrocolloids 5: 469–479.

Luhan Ma RJ (1992) Agar yield and gel strength of *Gracilaria heteroclada* collected from Iloilo, Central Philippines. Bot. mar. 35: 169–172.

Matsuhiro B, Urzúa C (1990) Agars from *Gracilaria chilensis* (Gracilariales). J. appl. Phycol. 2: 273–279.

Matsuhiro B, Zanlungo A (1983) Colorimetric determination of 3,6-anhydrogalactose in polysaccharides from red seaweeds. Carbohydr. Res. 118: 276–279.

Murano E (1995) Chemical structure and quality of agars from *Gracilaria*. J. appl. Phycol. 7: 245–254.

Pickering TD, Sladden VH, Furneaux RH, Hemmingson JA, Redfearn P (1993) Comparison of growth rate in culture, dry matter content, agar content and agar quality of two New Zealand red seaweeds, *Gracilaria chilensis* Bird, McLahlan *et* Oliveira and *Gracilaria truncata* Kraft. J. appl. Phycol. 5: 85–91.

Rochas C, Brûlet A, Guenet JM (1994) Thermoreversible gelation of agarose in water/dimethyl sulfoxide mixtures. Macromolecules 27(14): 3830–3835.

Yenigül M (1993) Seasonal changes in the chemical and gelling characteristics of agar from *Gracilaria verrucosa* collected in Turkey. Hydrobiologia 260/261: 627–631.