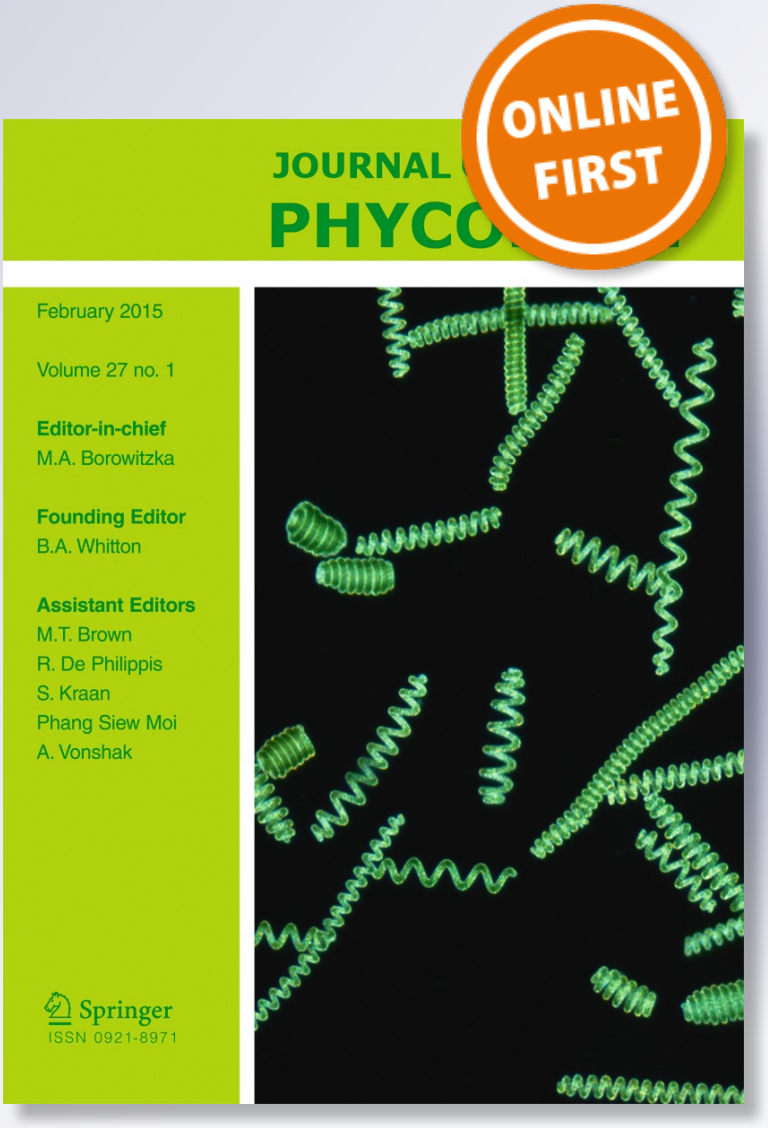
Assessment of alternative sources of seaweed polysaccharides in Argentina: potentials of the agarophyte Gelidium crinale *(Hare ex Turner) Gaillon (Rhodophyta, Gelidiales)*

M. Emilia Croce, Marcelo A. Villar & Elisa R. Parodi

Journal of Applied Phycology

ISSN 0921-8971

J Appl Phycol
DOI 10.1007/s10811-015-0537-1



Springer

J Appl Phycol
DOI 10.1007/s10811-015-0537-1

5TH CONGRESS OF THE INTERNATIONAL SOCIETY FOR APPLIED PHYCOLOGY

Assessment of alternative sources of seaweed polysaccharides in Argentina: potentials of the agarophyte *Gelidium crinale* (Hare ex Turner) Gaillon (Rhodophyta, Gelidiales)

M. Emilia Croce · Marcelo A. Villar · Elisa R. Parodi

Received: 28 July 2014 / Revised and accepted: 26 January 2015

Abstract The phycocolloids industry in Argentina has been based on the importation of non-native species and the exploitation of natural populations of *Gracilaria*, which are currently declining. This study was carried out to evaluate the potential of *Gelidium crinale* as an unexploited source of agar in this country. The yield and composition of polysaccharides extracted from *G. crinale*, as well as the growth potential of this species were studied. In vitro cultures of apical sections were carried out; relative growth rates (RGR) and data were analyzed through repeated measurements. Explants rapidly duplicated their initial size, producing numerous proliferations and achieving structural complexity. RGR of explants, primary proliferations, and secondary proliferations were high and similar. Polysaccharide extraction was done under three conditions: acid pre-extraction treatment+pressure boiled (AP), water pre-extraction treatment+pressure boiled (WP), and water pre-extraction treatment+boiled (WB). Polysaccharides were characterized through infrared spectroscopy, X-ray fluorescence, thermogravimetric, and elemental analysis. Polysaccharides obtained corresponded to the agar-type, and showed high similarity to commercial agar samples, with low sulfate and methyl contents. Higher yields were obtained with pressure extractions; however, acid treatment did not increase the amount of extracted biopolymers. Their degradation occurred in the range 250–400 °C. Polysaccharides obtained under AP conditions had a degradation curve similar to food-grade agar-agar, whereas degradation curves of polysaccharides obtained without acid treatment were similar to biotechnology-grade agar-agar. According to the results, *G. crinale* from Anegada Bay produces a high-quality agar to be considered for the Argentinean agar industry; however, micropropagation techniques would be indispensable to increase its bioavailability.

Keywords Agar · Apices · FT-IR · In vitro culture · Relative growth rate · Thermogravimetry

M. E. Croce · M. A. Villar
Planta Piloto de Ingeniería Química (PLAPIQUI), CONICET-Bahía Blanca, Camino Carrindanga 7.5 km, B8000FTN Bahía Blanca, Argentina

M. E. Croce (✉) · E. R. Parodi
Laboratorio de Ficología Aplicada, Instituto Argentino de Oceanografía (IADO), CONICET-Bahía Blanca, Camino Carrindanga 7.5 km, B8000FTN Bahía Blanca, Argentina
e-mail: ecroce@criba.edu.ar

E. R. Parodi
Laboratorio de Ecología Acuática, Departamento de Biología, Bioquímica y Farmacia. Universidad Nacional del Sur, San Juan 670, B8000ICN Bahía Blanca, Argentina

Introduction

Gelidium species have been traditionally used in the phycocolloids industry for agar-agar production (Lahaye 2001; McHugh 1991). *Gelidium pacificum* Okamura, *Gelidium sesquipedale* (Clemente) Thuret, *Gelidium latifolium* Bornet ex Hauck, *Gelidium rex* Santelices & I.A. Abbott, *Gelidium pusillum* (Stackhouse) Le Jolis, and *Gelidium amansii* (J.V. Lamouroux) J.V. Lamouroux are the most used species (Zemke-White and Ohno 1999). Bio-products currently obtained from *Gelidium* species do not only include the traditional phycocolloids but also novel ones such as bioactive compounds and fibers (e.g., Akakabe and Kajiwara 2008; De la Coba et al. 2009; Seo et al. 2010; Sun et al. 2007).

Despite the fact that *Gelidium* is preferred for bacteriological and pharmaceutical-grade agar, its commercial cultivation is not well developed (Bixler and Porse 2011). *Gelidium*

Published online: 14 February 2015

species grow slowly in culture (Friedlander 2008) and therefore the exploitation is based on the harvesting of natural populations (Melo 1998). *Gelidium* populations have been exploited in Spain, Portugal, Morocco, Chile, South Africa, South Korea, Japan, and Mexico (Melo 1998; Titlyanov and Titlyanova 2010). The perspectives of this group of algae for biotechnology applications are encouraging, and the potential of cultivation of this genus is high (Boulus et al. 2007), but several issues need to be addressed in order to achieve profitable results, especially in developing countries which have outdated technology.

The phycocolloids industry in Argentina is based on the importation of non-native species as well as the exploitation of natural populations. In the 1990s, *Gracilaria verrucosa* (Hudson) Papenfuss, *Gigartina skottsbergii* Setchell & N.L. Gardner and *Macrocystis pyirifera* (L.) C. Agardh were the most important species regarded as raw material for agar, carragenan, and alginate production, respectively (Piriz and Casas 1996). During this decade, Argentina contributed about 10 % of the agarophyte species exploited worldwide (McHugh 1991). However, the current state of the Argentinean phycocolloids industry is discouraging, since native populations of *Gracilaria* are decreasing, presumably because of inappropriate management (Boraso de Zaixso et al. 2006). In this context, the selection of alternative species becomes essential in order to reinforce the availability of this resource, as well as integrating local economies. *Gelidium* populations from Argentina have not been taken into account for the phycocolloids industry (McHugh 1991; Muñoz et al. 2011; Piriz and Casas 1996). Therefore, this study was conducted in order to evaluate their potentials.

In this study, wild populations of *Gelidium crinale* (Hare ex Turner) Gaillon were evaluated as a source of agar. The aims were (1) to assess the yield of polysaccharides extracted from *G. crinale* under different conditions, (2) to characterize the composition of extracted polysaccharides, and (3) to estimate the growth potential of this species for culture purposes. *G. crinale* is not commonly used as source of agar; however, it is a major component of the oyster reefs located on the north Patagonian coasts, and its morphology, reproduction, and seasonal abundance have been previously examined (Croce and Parodi 2012, 2013). We report for the first time, results of culture trials as well as the preliminary description of polysaccharides obtained from Argentinean *Gelidium* populations.

Materials and methods

Collection and preparation of algal material Several turfs of *G. crinale* were collected during spring from Anegada Bay, Argentina (40° 25′ S; 60° 25′ O). Thalli were rinsed with filtered seawater to remove sediments and epiphytes. A group of thalli were separated for in vitro culture, while the rest were rinsed with tap water, and freeze-dried to be used for polysaccharide extraction.

Polysaccharide extraction and characterization Cell wall polysaccharides were extracted according to traditional methods with some modifications (Craigie and Leigh 1978; FAO 2011; Matsuhiro 1995). Samples were treated, prior to extraction, by soaking the dried algae for 1 h at 16–20 °C in acetic acid 0.5 % or distilled water, both in the proportion of 20 mL g^{-1}alga (Roleda et al. 1997). Polysaccharide extraction was carried out in triplicate, under three different conditions: acid pre-extraction treatment+pressure boiled (AP), distilled water pre-extraction treatment+pressure boiled (WP), and distilled water pre-extraction treatment+boiled (WB). One gram of dried alga was used for each extraction.

Water-treated algae were either boiled at 85 °C±5 °C in a stainless steel vessel under constant stirring, or pressure boiled in a glass baker at 1034–1340 hPa in autoclave, while acid-treated algae were pressure boiled at 1034–1340 hPa in autoclave. Every extraction was made in distilled water in the proportion of 20 mL g^{-1}alga, for 2 h, and repeated using the algal residue for 1 h.

Extracts were filtered through a thermostated funnel using a 225-μm nylon mesh. Water soluble polysaccharides were separated from algal residues by 20 min centrifugation at 2000 rpm, and the supernatants were poured into flat plastic trays. Extracts of each step were combined into the same tray and left to gel at room temperature for 18 h.

After gelification, polysaccharides were obtained by the traditional freeze-thawing procedure. After syneresis, the water was drained. Polysaccharide fraction was frozen for 24 h at −20 °C, freeze-dried for 48 h, and later weighed.

Composition and functional groups of polysaccharides extracted were evaluated using Fourier transform infrared spectroscopy (FT-IR). For each sample, a pellet was prepared by dissolving 1 mg of extract into 99 mg of KBr, which was analyzed in a FT-IR (Nicolet Nexus, USA).

The thermal stability and the decomposition curves of polysaccharides obtained were analyzed through thermogravimetric analysis (TGA) with a TGA Discovery TA (USA), in a dry N_2 atmosphere, at a heating rate of 10 °C min^{-1} in the temperature range of 30 to 900 °C. For chemical characterization of polysaccharides, the elemental composition of lyophilized samples was determined. The content of C and H was quantified using a PerkinElmer elemental analyzer (CHNS/O 2400 Series II). The content of S was determined through X-ray fluorescence spectrometry and used to estimate the content of ester sulfate ($C_6H_8S_2O_{11}$). Oxygen percentage was estimated by difference.

Microscopic morphology of the polysaccharide gels was examined using a scanning electron microscope (SEM). Freeze-dried sections of each polysaccharide were cut with a razor blade, mounted on a sample holder, and coated with gold.

For comparisons, chemical characterization and microscopic evaluation were also performed on commercial agar-agar samples (Soriano® and Britania®), sold as food-grade agar-agar (FA) and biotechnology-grade agar-agar (BA), respectively.

Culture methods Culture assays were carried out using apical meristems, following the methodologies of Collantes and Melo (1995), Titlyanov and Titlyanova (2006), and Titlyanov et al. (2006).

A stock was prepared with several thalli in an aerated aquarium (20 L) with 5 μm filtered seawater and kept in a culture chamber under controlled conditions. The chamber temperature was 7±1 °C, and the photoperiod was 18:6 (light/dark). Photosynthetic active radiation (PAR) was 12 μmol photons m^{-2} s^{-1}.

After 2 weeks in stock, thalli free from epiphytes were examined for growing apices. A total of 74 growing apical sections of 5 mm long were cut and incubated for 1 week in flasks with 100 mL of enriched seawater using modified von Stosch (Grund) medium (Guiry and Cunningham 1984). In order to avoid diatoms growth, 1 mL of germanium dioxide (GeO_2) solution per liter of von Stosch medium was added in each culture chamber (Chapman 1980). Once no diatom was observed in the culture, GeO_2 addition was suspended. Surviving apices were selected for the assay.

Culture was initiated with 27 sections of 2 mm long obtained from cultured apices, from now on called "explants." Culture was carried out in acrylic multiwell plates submerged in an aquarium filled with filtered seawater and enriched with modified von Stosch (Grund) medium. Each explant was located inside a well with 6.5 mL of filtered-enriched seawater. Cultures were aerated through bubbling air (Oliveira et al. 1995).

Culture assays were maintained for 2 months, culture medium of each plate was replaced every week, and the explants were photographed. Several qualitative and quantitative variables were evaluated weekly (Table 1). In order to avoid the growth of microorganisms on their surface, each explant was dragged through solid agar after each measuring stage (Kawai et al. 2005).

Table 1 Qualitative and quantitative variables evaluated in each explant every week of culture

Variables
Explant length: distance between the two ends of the longest axis
No. of primary proliferations (P1): proliferations growing directly on the explant
No. of secondary proliferations (P2): proliferations growing on P1
No. of tertiary proliferations (P3): proliferations growing on P2
No. of quaternary proliferations (P4): proliferations growing on P3
Proliferation length: distance between the base and the apex of each proliferation

Data analyses Yield of extraction was estimated as percentage of algae dried weight, and compared through ANOVA with R software (R Development Core Team 2011). Average comparisons were made through LSD Fisher.

Algal growth was estimated by the increase in length, and was expressed as the relative growth rate (RGR) through the following formula (Brinkhuis 2008):

$$\mathrm{RGR}\ (\%) = \frac{\ln\left(\frac{l_f}{l_0}\right)100}{t} \quad (1)$$

where, RGR is the relative growth rate in percentage, l_0 is the initial length, l_f is the final length, and t is the time between two measurements. Regression lines were fitted and the coefficients of determination (r^2) were obtained. Growth data were analyzed by repeated measure ANOVA with R software (R Development Core Team 2011; Crawley 2007; Kabacoff 2011).

Results

Characterization of polysaccharides Polysaccharide gelation occurred rapidly after pouring the extract into the trays and obtained gels had a light yellowish color.The highest yield was obtained with WP, while the lowest yield was obtained with WB (Table 2). Yields of extraction under pressure-boiled conditions were significantly higher than that obtained without pressure-boiled condition ($p << 0.01$; $df=2$; $F=25.83$); however, no significant differences were found between pressure-boiled conditions ($p=0.09$; $df=6$; $t=2.44$).

Spectra of polysaccharides extracted from *G. crinale* showed high similarity to those corresponding to commercial agar samples (Fig. 1a, b). Characteristic bands of each spectrum are detailed in Table 3.

Three sharp bands at 3400, 2900, and 1640 cm^{-1}, corresponding to OH, CH_2, and C=O vibrations, respectively, can be observed in the spectrum (Fig. 1a). The substitution by CH_2 groups is denoted by the band at 2900 cm^{-1}, which appeared to be more intense in the spectra of WB samples. In the

Table 2 Average dried weight and yield of extraction of polysaccharides extracted from *Gelidium crinale* under different conditions

Extraction method	Dried weight (g; ±SD)	Yield (%; ±SD)
distilled water+pressure boiled (WP)	0.31 (±0.059)	30.55 (±5.88)
acetic acid+pressure boiled (AP)	0.24 (±0.031)	24.34 (±0.18)
distilled water+boiled (WB)	0.09 (±0.002)	8.62 (±3.14)

SD standard deviation

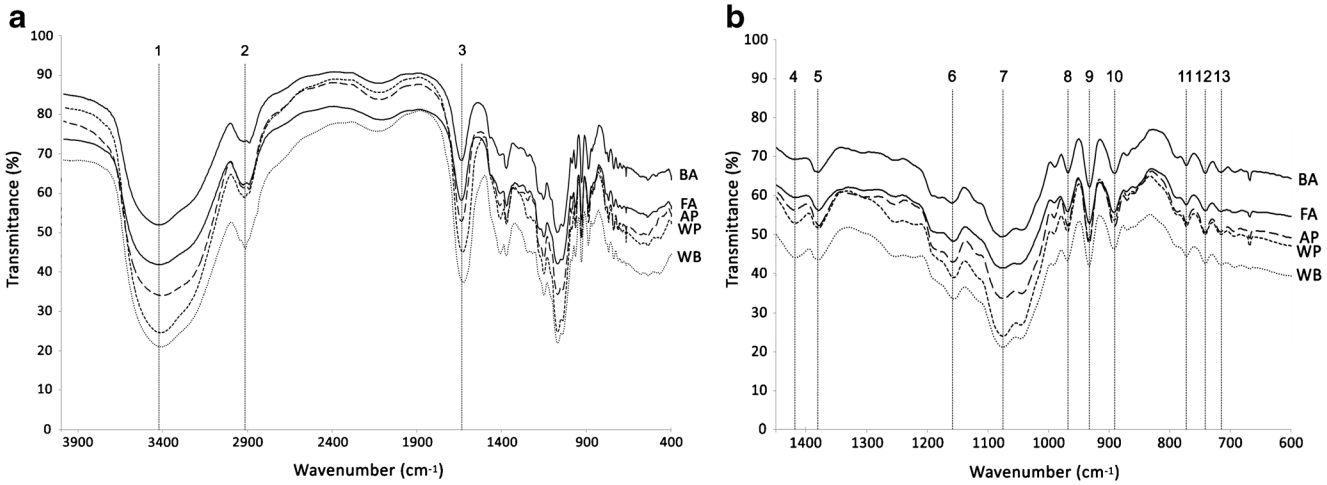


Fig. 1 FT-IR spectra of polysaccharides extracted from *Gelidium crinale*, at different extraction conditions and commercial agars samples. **a** Complete spectrum from 4000 to 400 cm^{-1}. **b** Fingerprint section of the spectrum from 1400 to 600 cm^{-1}. References: *WB* water+boiled, *WP* water+pressure boiled, *AP* acid+pressure boiled, *FA* food-grade agar-agar, *BA* biotechnology-grade agar-agar. *Vertical Broken lines* and *numbers* (*1–7*) denote characteristic bands (Table 3)

fingerprint section of the spectra (Fig. 1b) several bands appeared, but the stronger were between 1000 and 890 cm^{-1} (Fig. 1b). A band at 1400 cm^{-1} was present in the spectra of the extracted polysaccharides, which is associated with the C6 vibration of galactopyranose rings.

All extracted samples, as well as commercial agars, showed some degree of substitution with SO_4^{2-} groups according to the bands at 1370 and 1150 cm^{-1}, respectively. A weak band at 1070 cm^{-1} was present in all spectra with corresponds to the glycosidic linkage C–O–C of 3,6-anhydro-D-galactose. Bands 8, 9, and 10 were the strongest in the spectra of *G. crinale* polysaccharides as well as those of commercial agars. These bands were associated to C–O–C vibration of 3,6 anhydrogalactose. The presence of the 3,6-anhydrogalactose was evident in all samples, but the band had slightly different values in commercial agar samples respect to polysaccharides extracted (Table 3). Band at 891–890 cm^{-1} indicated the presence of unsulfated 3-linked-β-L-galactose, while bands at 770 and 715 cm^{-1} are characteristic of agar-type polysaccharides. However, the later was only present in the spectra of commercial agars and AP samples.

Thermogravimetry curves are represented in Fig. 2. All curves showed an initial weight loss (≈10 %) related to bound water, characteristic of highly hydrophilic compounds such as phycocolloids. After that, polysaccharides extracted from *G. crinale*, as well as commercial agars, exhibited decomposition in the range 250–400 °C up to

Table 3 Characteristic bands of FT-IR spectra of polysaccharides from *Gelidium crinale* extracted under different conditions and commercial agar samples

Wavenumber (cm^{-1})					
Band	Distilled water+ boiled (WB)	Distilled water+pressure boiled (WP)	Acetic acid+pressure boiled (AP)	Food-grade agar-agar (FA)	Biotechnology-grade agar-agar (BA)
1	3417	3422	3407	3426	3425
2	2923	2926	2900	2935	2900
3	1633	1636	1644	1643	1644
4	1415	1414	1417	-	-
5	1376	1376	1375	1376	1377
6	1154	1154	1155	1155	1157
7	1074	1074	1074	1074	1076
8	968	967	967	967	966
9	932	932	932	931	931
10	891	891	890	891	890
11	772	772	772	772	772
12	741	741	741	741	741
13	–	–	716	715	716

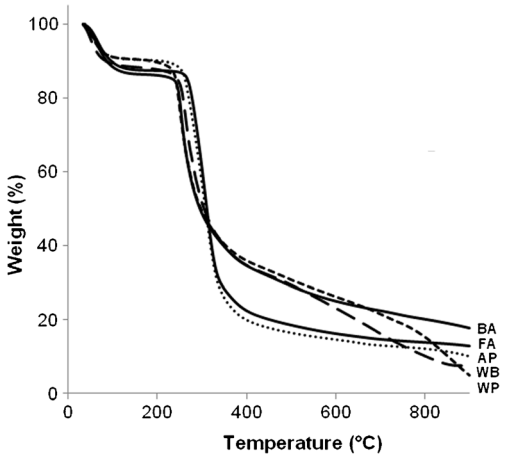


Fig. 2 Thermogravimetric curves of polysaccharides obtained from *Gelidium crinale* and commercial agars samples. References: *WB* water+boiled, *WP* water+pressure boiled, *AP* acid+pressure boiled, *FA* food-grade agar-agar, *BA* biotechnology-grade agar-agar

80 % of weight loss, following the typical degradation of sulfated galactans.

Degradation curves of *G. crinale* polysaccharides followed similar tendencies to those of commercial agars. Degradation of AP polysaccharides was similar to that of FA agar-agar; even when the temperature for the start of degradation was lower. These two degradation curves were the fastest. On the contrary, polysaccharides obtained without acid pre-treatment had similar degradation curves to BA agar-agar.

Curves of WB, WP and BA had the lowest temperature for the start of degradation, between 227 and 235 °C, while AP and FA curves had the highest temperature for the start of degradation, between 250 and 260 °C. Commercial agars and AP polysaccharides were more stable in the temperature range 300–800 °C and continued decomposing over 800 °C. All extracted polysaccharides and commercial agars continued their degradation above 900 °C. The remaining residue at 900 °C was 20 % for BA agar-agar and between 10 and 20 % for the rest of the samples.

The content of C in the polysaccharides extracted was between 38.5 and 41.5 % (Table 4). The highest percentage of C was found in polysaccharides extracted under AP conditions. The content of ester sulfate was between 0.7 and 1.8 %, and the lowest value was found in polysaccharides extracted through AP conditions (Table 4).

Table 4 Content of C, H, O, and ester sulfate of polysaccharides extracted from *Gelidium crinale* under different conditions

Sample	C (%)	H (%)	O (%)	Sulfate ester $C_6H_8S_2O_{11}$ (%)
WB	38.5±0.9	6.6±0.3	54.3±1.7	1.3
WP	38.6±0.3	6.6±0.2	53.0±0.8	1.8
AP	41.5±1.2	7.0±0.5	51.5±1.6	0.7

Microscopic observations Figure 3a–e represent the sectioned surfaces of freeze-dried polysaccharides foams. It can be noticed that pre-extraction treatments with distilled water lead to the formation of a matrix of rather polyhedral "cells" linked by thick walls. These structures were similar to those of commercial agars. On the other hand, the matrix obtained with acid pre-extraction treatment was irregular and more condensed, formed by small and thin lamella. It should be noted that this structure was brittle.

Growth of explants Explants increased their length during the whole culture period and produced numerous proliferations (Fig. 4). Some explants became epiphytized by microscopic algae. Epiphytes appeared mainly on the older parts of explants.

Explants duplicated their initial size after 4 weeks in culture. Average length of explants significantly increased until the fourth week, after which it remained constant and decreased by the end of the culture (p=0.01; df=8; F=14.2; Table 5; Fig. 5). The maximum absolute length achieved by an explant was 11.25 mm, while the maximum average length was 4.69 mm.

Average RGR of explants was high at the initial week of culture, remained constant during the following weeks and stepwise decreased towards the end (p<<0.01; df=7; F= 15.4; Table 5; Fig. 6). The maximum RGR value achieved by an explant was 11.81 %, while the maximum average RGR was 6.26 %. After 35 days of culture, average RGR of explants became negative. Almost 50 % of explants had negative RGR values by the end of the culture.

The production of proliferations by the explants was abundant and began in the first week of culture (Fig. 7). Primary proliferations appeared on the sectioned parts and on lateral surfaces of explants after seven days of culture, and they were the most abundant of all proliferations (63 %). The percentage of P1 decreased as the production of other types of proliferations increased. Secondary proliferations appeared after 14 days of culture and were less abundant than P1 (32 %). They contributed with low percentage during the first 2 weeks, but their production was increased from the third week until the end of the culture. Tertiary proliferations were the least abundant of all proliferations (5 %) and appeared after 28 days of culture. Only two quaternary proliferations were found during the assay. The maximum average number of proliferations produced by the explants was 16.3 (P1). Proliferation production was maximum in the last 2 weeks of culture (Fig. 7). By the sixth week of culture, almost all explants had reached the 50 % of total proliferation production.

Average length of P1, P2, and P3 showed an increase during the whole culture (Fig. 7). P1 and P2 had similar lengths along the culture, while the length of P3 was always lower (Fig. 5). The increase in length of P1 was lineal, while that of P2 and P3 was exponential (Table 5).

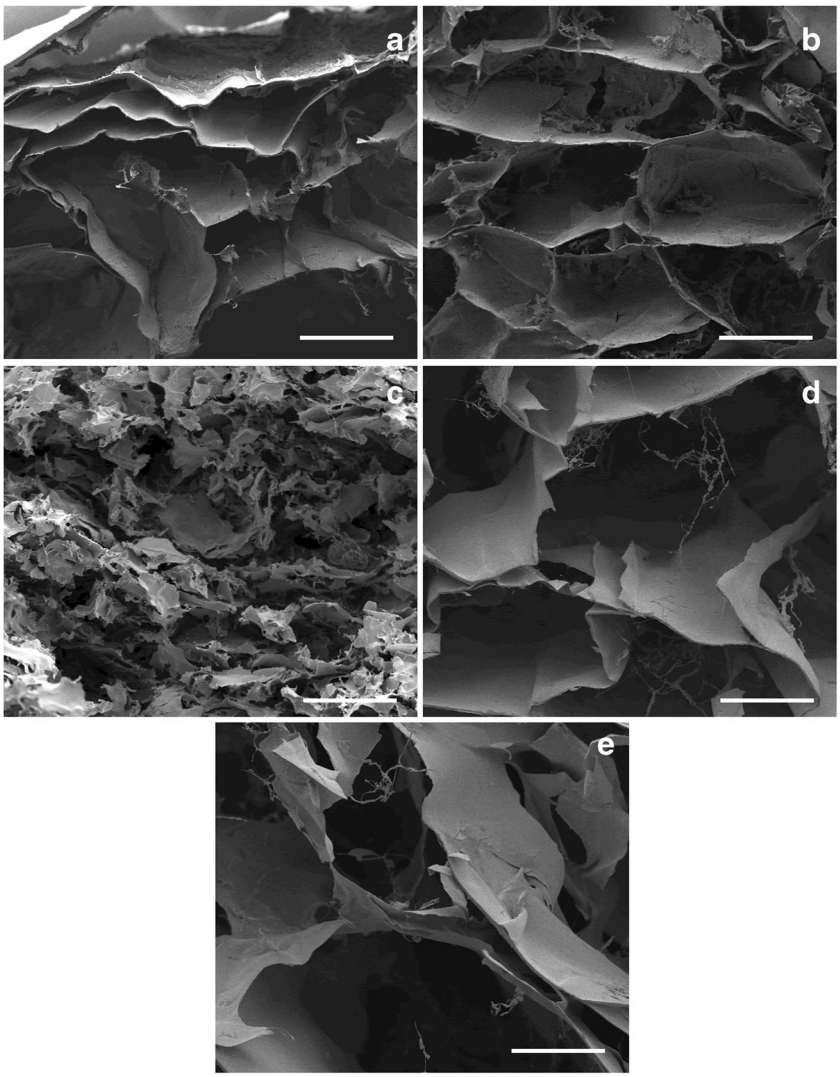


Fig. 3 SEM micrograph of sectioned surfaces of freeze-dried polysaccharides extracted from *Gelidium crinale* and commercial agars samples: **a** distilled water+ boiled, **b** distilled water+pressure boiled, **c** acetic acid+pressure boiled, **d** food-grade agar-agar, **e** biotechnology-grade agar-agar. *Scale*, 200 μm

RGR of P1 and P2 was high during the initial weeks of the culture, and both had a decreasing trend in the following weeks (Fig. 6). On the contrary, RGR of P3 was low during the first week they appeared, increased by the second week and then decreased towards the end of the assay.

RGR of explants, primary and secondary proliferations had a similar trend, with high values at the beginning of the assay and then decreasing at the end of the culture. On the contrary, the behavior of tertiary proliferations RGR was different and was usually lower.

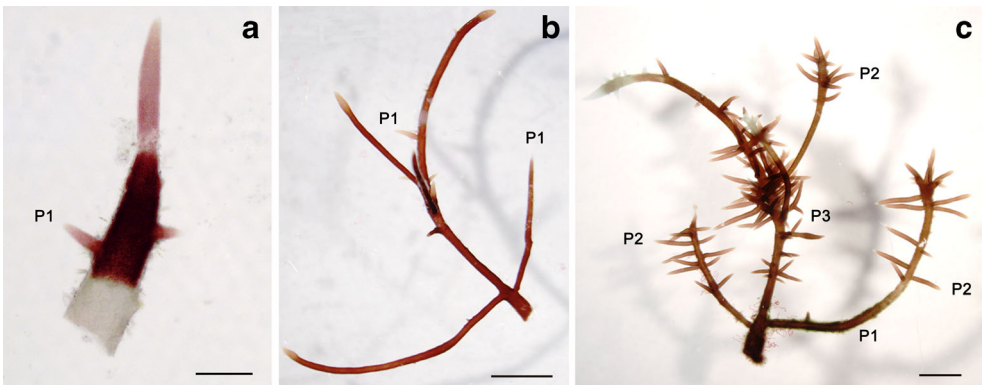


Fig. 4 *Gelidium crinale* explants in vitro culture. Aspect of an explant after **a** 7, **b** 28, and **c** 56 days of culture. *P1* primary proliferations, *P2* secondary proliferations, *P3* tertiary proliferations. *Scale references*: **a** 200 μm, **b** 1.25 mm, and **c** 1 mm

Table 5 Growth equations and significant coefficients of determination (r^2)

Growth curve	Equation	r^2
Explant length	$y=2.23\ x^{0.3}$	0.60
No. of proliferations	$y=1.14\ e^{0.41\ x}$	0.95
Primary proliferations (P1) length	$y=0.4\ x+0.04$	0.97
Secondary proliferations (P2) length	$y=0.44\ e^{0.32\ x}$	0.97
Tertiary proliferations (P3) length	$y=0.17\ e^{0.38\ x}$	0.94
Explant relative growth rate (RGR_E)	$y=-3.57\ \ln(x)+5.5$	0.88
P1 relative growth rate (RGR_{P1})	$y=-5.26\ \ln(x)+9.29$	0.97
P2 relative growth rate (RGR_{P2})	$y=-1.37\ x+8.14$	0.86
P3 relative growth rate (RGR_{P3})	$y=-2.69\ x^2+10.75\ x-4.42$	0.98

Discussion

During the 1990s, Argentina produced around 2 t year^{-1} of dried algae for agar production, being *Gracilaria* the principal raw material for this industry (Zemke-White and Ohno 1999). So far, the extensive meadows of *Gracilaria* have constituted a promising resource in South Patagonian coasts (Boraso de Zaixso 1987). However, the recently reported decline of these natural populations (Boraso de Zaixso et al. 2006) together with the growing demand for natural polymers (Goswami and Naik 2014; Milani and Maleki 2012) leads to the search for alternative species to reinforce agar production in this country. This study evaluated, for the first time, a population of Gelidiales from Argentina as an agar source.

The majority of *Gelidiales* species have agar yields around 20–30 % (Freile-Pelegrín et al. 1999; Macler and West 1987; Oliveira et al. 1995). The values obtained for *G. crinale* from Patagonia were in the same range and were similar to those reported by Boulus et al. (2007), except for yields obtained under WP conditions which were even higher. Yields of extraction were similar to those reported for commercial species such as *Gelidium latifolium* (Mouradi-Givernaud et al. 1992), *Gracilaria gracilis* (Stackhouse) M. Steentoft, L.M. Irvine & W.F. Farnham (Rodriguez et al. 2009) and other *Gracilaria* species (Macchiavello et al. 1999; Marinho-Soriano 2001; Meena et al. 2008). Specimens of *G. crinale* used in the present study were collected in spring, when average water temperature is 20 °C in Anegada Bay (Álvarez and Ríos 1988; Borges 2006; Croce 2013); therefore higher yields would be expected for specimens collected in winter, according to Boulus et al. (2007).

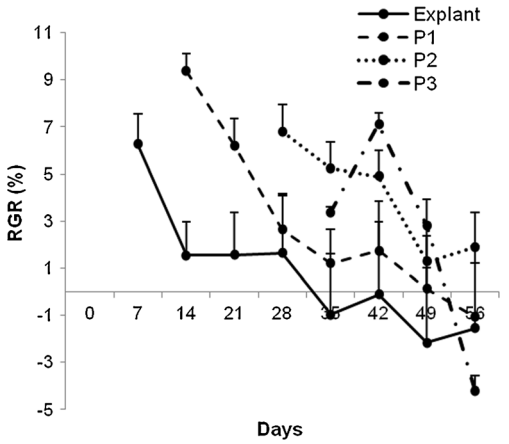


Fig. 6 Relative growth rate (*RGR*) of *Gelidium crinale* explants and proliferations during in vitro culture. *P1* primary proliferations, *P2* secondary proliferations, *P3* tertiary proliferations. *Error bars* denote standard deviations. No. of replicates for explants growth: n=27. No. of replicates for proliferations growth in the successive weeks: P1 (n=65, 65, 59, 32, 33, and 15); P2 (n=20, 20, 20, 18, and 10); P3 (n=5, 5, 4, and 2)

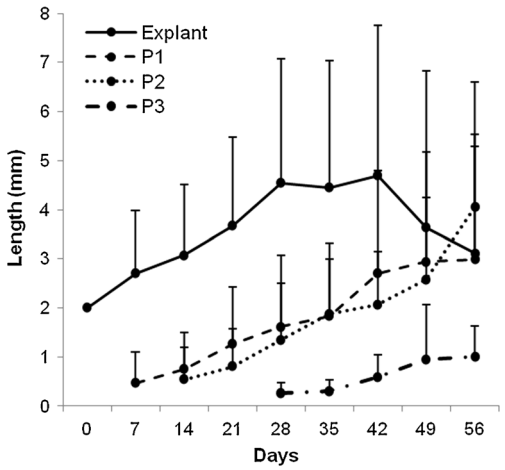


Fig. 5 Average growth length of *Gelidium crinale* explants and proliferations during in vitro culture. *P1* primary proliferations, *P2* secondary proliferations, *P3* tertiary proliferations. *Error bars* denote standard deviations. No. of replicates for explants growth: n=27. No. of replicates for proliferations growth in the successive weeks: P1 (n=65, 65, 65, 59, 32, 33, and 15); P2 (n=20, 20, 20, 20, 18, and 10); P3 (n=5, 5, 5, 4, and 2)

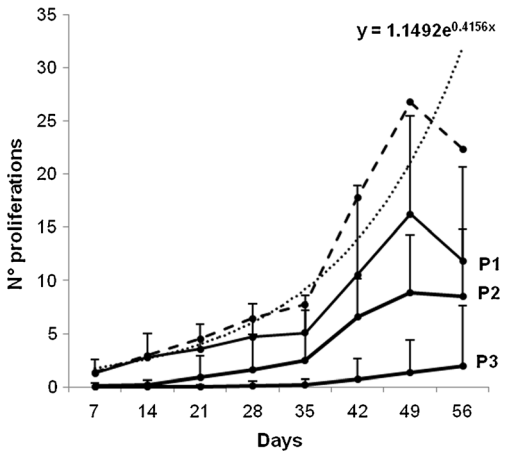


Fig. 7 Average number of proliferations and tendency (*dotted line*) produced by *Gelidium crinale* explants during in vitro culture. *Dashed line* represents the total average. *P1* primary proliferations, *P2* secondary proliferations, *P3* tertiary proliferations. Number of replicates=27

The use of steam pressure during the extraction, significantly increased the amount of polysaccharides extracted, in good agreement with Roleda et al. (1997). As suggested by these authors, extractions with boiling water may need more time to reach yield values similar to those obtained with steam pressure. A positive effect of steam pressure on extraction yield was also found for polysaccharides obtained from *Gelidium spinosum* (S.G. Gmelin) P.C. Silva (Ben Said et al. 2012). On the other hand, the pre-treatment with acetic acid did not significantly enhance the extraction of cell wall polysaccharides. The pre-treatment of the dried algae with acid has been recommended for algae with rigid and resistant thalli such as *Gelidium*, *Gelidiella*, and *Pterocladia*, in order to improve extraction efficiency (Imeson 2010). According to previous studies, acetic acid facilitates the extraction of polysaccharides by disrupting crosslinks in the algal structure (Murano 1991). However, in the present study, yield of extraction obtained under acid+pressure-boiled conditions was comparable with that obtained under water+pressure-boiled conditions, contrary to the results obtained by Roleda et al. (1997) for *Gelidiella acerosa* (Forsskål) Feldmann & G.Hamel. These results reinforce the idea that efficiency of pre-extraction treatments is highly dependent on algal species (Imeson 2010); therefore, these methods should be species-specific.

Sulfated galactans are the main polysaccharides present in red algae, which are responsible for the flexibility of the thalli, hence those species containing high quantity of these polysaccharides are usually rubbery and bendable (Lee 2008). The amount of galactans obtained for this species was relatively high considering that the thalli are inconspicuous and have a wiry consistency (Croce and Parodi 2013).

Infrared spectroscopy has demonstrated to be a non-destructive tool that allows identifying functional groups for the evaluation of seaweed phycocolloids and the comparison of biopolymers obtained by different extraction methods (Gómez-Ordóñez and Rupérez 2011; Kačuráková and Wilson 2001; Matsuhiro and Rivas 1993; Rochas et al. 1986; Sur and Güven 2002). Polysaccharides obtained from *G. crinale* were consistent with those of the "agaran" group according to many distinctive bands of the IR spectra, in addition to their similarity with commercial agars. Previous studies have reported an agaran-like structure of *G. crinale* polysaccharides, composed of two types of α-galactose units but no 3, 6-anhydro-α-galactose residues (Pereira et al. 2005). However, in the present study, the occurrence of 3,6-anhydrogalactose residues was evident through the intense band at 932 cm^{-1} in all samples coincidently with the bands of commercial agar samples at 931 cm^{-1}. It is known that 3,6-anhydrogalactose rings are formed after the loss of sulfates in the C6 position of α-galactose units (McCandless and Craigie 1979). High contents of this residue and therefore low sulfate content are related to stronger gels (Amirsen 1995). This may explain the strong gels that are rapidly formed after pouring the extract into the trays.

The absence of bands at 1240 cm^{-1} and in the region 800–890 cm^{-1} observed in every spectrum suggests a very low sulfate content of *G. crinale* galactans, a characteristic also associated with agars (Pomin and Mourão 2008). Specifically, the absence of bands at 867 cm^{-1} implicates the lack of sulfate at C6 of L-galactose, reinforcing that the structure of extracted polymers had 3,6-anhydrogalactose rings in its composition (Usov 2011). The fact that this band is lacking in all extracted samples, similarly to commercial agars samples, suggests that the 3,6-anhydrogalactose rings were already formed in the native polysaccharide, therefore, no alkali treatment would be needed to improve this characteristic (Rees 1961). Unsulfated C6 of the native polysaccharide extracted from *G. crinale* were also found by Pereira et al. (2005). Although two bands associated with sulfate groups were present in the spectra (1150 and 1370 cm^{-1}), these bands were very weak and may be related with SO_4^{2-} in C2 and C3 of α units, as it has been reported by Pereira et al. (2005). The band at 890–891 cm^{-1} is characteristic of agar and is associated to unsulfated 3-linked β-D-galactose (Matsuhiro 1996; Sekkal et al. 1993). This band was observed in the spectra of polysaccharides extracted from *G. crinale* as well as in the spectra of commercial agar samples.

The spectroscopic analysis also denoted a low degree of methylation in the polysaccharides obtained, in good agreement with the results previously reported by Murano et al. (1998) for *G. crinale* as well as for other species of the genus (Guerrero et al. 2014). The intensity of the corresponding band was reduced in AP and WP extractions, which resembled the spectra of commercial agars; this suggests that methyl groups can be lost when steam pressure is applied during the extraction. The presence of substitution groups negatively affects the properties and quality of the polysaccharide (Imeson 2010; McHugh 2002; Murano 1995). For example, *Gelidium* is preferred over *Gracilaria* for producing bacteriological and pharmaceutical-grade agar because of the lower sulfate content of their galactans (Bixler and Porse 2011). The low content of substitution groups found in agar extracted from *G. crinale* demonstrates its high quality.

The degradation of *G. crinale* polysaccharides followed the typical pattern of agar and agarose degradation, where pyrolysis starts around 250 °C (Nishinari and Watase 1983; Xia et al. 2014; Zhou et al. 2006). Acid pre-treatment in the extraction gives polysaccharides with a higher temperature for the start of degradation, therefore increasing the thermal stability of the polymer. This treatment also increased the rate of decomposition, resulting in a product similar to commercial agar samples.

The content of C in the extracted polysaccharides was similar to those reported for species of *Gracilaria* and *Gelidium* (Rahelivao et al. 2014; Murano et al. 1993). The content of ester sulfate of agar is usually between 1.5 and 2.5 %, which is

low compared with carrageenans (Imeson 2010). Polysaccharides extracted from *G. crinale* by different treatments had a low content of ester sulfate. On the other hand, values obtained were in the range reported in the literature for *G. crinale* as well as for other *Gelidium* species such as *G. serrulatum*, *G. floridanum*, and *G. pusillum* (Murano et al. 1998). The levels of ester sulphate were typically lower than those reported for *Gracilaria* (Murano 1995; Rebello et al. 1997).

The microscopic analysis allowed the identification of differences in the structural conformation of the polysaccharides obtained as related to the extraction method. The matrix of the polysaccharides extracted with distilled water was typical of agar foams (Xia et al. 2014; Lee et al. 1997), while the structure of the polysaccharides extracted with acid was more compact, irregular and fragile.

G. crinale grew rapidly during in vitro culture and explants were able to increase five times their initial size in only two months. Similarly, Titlyanov et al. (2006) found increases in length of six to nine times the initial size of *Gelidium* sp. The final length reached by the explants was low, but the number of proliferations produced by the explants was highly significant, compared with other *Gelidium* species (Rodríguez 1996). Average RGR of explants was similar to that obtained for *Gelidium sclerophyllum* W.R. Taylor (Rodríguez 1996), *G. amansii* (Shunzo 1971), *Gelidium* spp. (Titlyanov et al. 2006; Titlyanov and Titlyanova 2006), *Gelidium coulteri* Harvey (Macler and West 1987), *G. crinale* (Boulus et al. 2007), and even larger species of *Gelidium* (Fredriksen et al. 1993). Average RGR of explants was higher than that obtained for *Pterocladia* (Santelices 1976), *Gelidium robustum* (N.L. Gardner) Hollenberg & I.A. Abbott (Pacheco-Ruiz and Zertuche-Gonzalez 1995), and *G. sesquipedale* (Carmona et al. 1996; Seoane-Camba 1997); whereas it was lower than that obtained for *Gelidium pulchellum* (Turner) Kützing (Sousa-Pinto et al. 1999) and *G. rex* (Rojas et al. 1996). *G. crinale* also reached maximum RGR values that were higher than other commercial species of *Gelidium* such as *G. pulchellum* (Sousa-Pinto et al. 1999), *G. sclerophyllum* (Rodríguez 1996), *G. sesquipedale* (Carmona et al. 1996; Seoane-Camba 1997), *G. amansii* (Shunzo 1971), and *G. robustum* (Pacheco-Ruiz and Zertuche-Gonzalez 1995).

According to Boulus et al. (2007), the growth rate of this species is positively related to environmental temperatures. The same has been observed for other *Gelidium* species (Macler and West 1987; Macler and Zupan 1991). Several authors have found that growth rate of *Gelidium* species could be positively influenced by high light intensity and high ammonium concentration in water (Boulus et al. 2007; Fredriksen et al. 1993; Macler and West 1987). The values of growth rate obtained in the present study were relatively high considering that light intensity and temperature were set at relatively low values during the culture.

The most important outcome of the culture was the high rate of proliferation production, in good agreement with previous results (Titlyanov and Titlyanova 2006). Similar complex structures reached by cultured algae were observed in species of *Pterocladia* because of radial proliferations (Santelices 1976). Since explants reached a high architectural complexity, the potential of this species for culture through vegetative micropropagation techniques becomes relevant, with important advantages for the commercial exploitation (Macler and Zupan 1991).

Proliferations, especially primary ones, had RGR values higher than RGR of explants, which may reflect an advantage for surviving after excision by rapidly branching, hence increasing the surface available for attachment. This behavior may increase the probability of adhesion to the substratum favoring vegetative reproduction. It is known that *G. crinale* has medullar and cortical rhizines, which are the same components of hapteria (Croce and Parodi 2013); therefore any proliferation produced by the explant should have the potential to develop attachment organs. It has been demonstrated that the production of attachment structures requires external stimuli such as calcareous substrates (Orduña-Rojas and Robledo 2002; Salinas 1991; Santelices and Varela 1994), plantlet freezing-thawing (Titlyanov et al. 2006), and thallus shading (Titlyanov and Titlyanova 2006), although proliferation production showed no polarity and no rhizoids were observed. As suggested by Titlyanov et al. (2006) proliferated explants without rhizoids may be suitable for tank bubbling cultivation instead of plantation.

RGR of explants and primary proliferations decreased after 4 weeks, indicating a stage in the culture where explants should be either re-sectioned or transplanted to a superior culture phase. According to our results, it was evident that sectioned apices from *G. crinale* employed much of the algal resources to the production and elongation of proliferations; therefore it would be more accurate to estimate the growth of this species through volumetric variables.

Epiphytism is common in *Gelidium* cultures (Friedlander 2008) and may damage the growth rate (Boulus et al. 2007). Considering the higher percentage of epiphytes on older parts of the explants compared with proliferations, it would be appropriate to separate the original fragment from the rest of the plantlet at certain point in the culture in order to reduce epiphytism.

Among other applications, recent studies have demonstrated important activity of sulfated galactans from *G. crinale* as anti-inflammatory, analgesic, and anticoagulant (Assreuy et al. 2012; de Sousa et al. 2011; Percot et al. 2009; Pereira et al.,2005). Despite the decrease of the worldwide production of *Gelidium* (Bixler and Porse 2011) and the absence of a successful methodology to achieve a profitable cultivation (Friedlander 2008), the high potential of this species for medicinal and industrial applications denotes the importance of further attempts.

The high quality and yield of agar extracted from *G. crinale* lead us to consider this species as an alternative resource to reinforce agar industry in Argentina, if appropriate culture techniques are developed, which are indispensable to increase its bioavailability. Given the strong association of *G. crinale* with native and exotic mollusks in this habitat (Croce and Parodi 2012, 2013), polyculture initiatives should be considered.

Acknowledgments Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional del Sur, for research funding via grants PGI 24B/145 and PIP 11220100100503 to E. R. Parodi and PIP 11220110100428 to M. A. Villar.

References

Akakabe Y, Kajiwara T (2008) Bioactive volatile compounds from marine algae: feeding attractants. J Appl Phycol 20:661–664

Álvarez CHA, Ríos HF (1988) Estudios Litorales en las Bahías de San Blas y San Antonio Oeste. Instituto Tecnológico de Buenos Aires y Universidad Nacional de Mar del Plata, Mar del Plata, Argentina

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

Assreuy AMS, Amorim RMF, Brizeno LAC, Pereira LDP, de Sousa AAS, Aragão GF, Pereira MG (2012) Edematogenic activity of a sulfated galactan from the red marine algae *Gelidium crinale*. Pharm Biol 50:1194–1198

Ben Said R, Romdhane MS, El Abed A, M'Rabet R (2012) Recherche des facteurs les plus influents sur le rendement et la qualité de l'agar-agar de la rhodophycée *Gelidium spinosum* (S.G. Gmelin) P.C. Silva. Afr Sci 8:113–130

Bixler HJ, Porse H (2011) A decade of change in the seaweed hydrocolloids industry. J Appl Phycol 23:321–335

Boraso de Zaixso AL (1987) *Gracilaria verrucosa* in Golfo Nuevo, Chubut, Argentina. Biological parameters and environmental factors. Hydrobiologia 151/152:239–244

Boraso de Zaixso AL, Ciancia M, Cerezo A (2006) Seaweed resources of Argentina. In: Critchley AT, Ohno M, Largo DB (eds.) World seaweed resources-an authoritative reference system. Electronic version 1.0. Eti Information Services Ltd: www.etiis.org.uk. 26 pp

Borges ME (2006) Ecología de las ostras en ambientes del sur bonaerense: cultivo y manejo de sus poblaciones. PhD thesis, Universidad Nacional del Sur, Bahía Blanca, Argentina

Boulus A, Spaneir E, Friedlander M (2007) Effect of outdoor conditions on growth rate and chemical composition of *Gelidium crinale* in culture. J Appl Phycol 19:471–478

Brinkhuis BH (2008) Growth patterns and rates. In: Littler MM, Littler DS (eds) Handbook of phycological methods, ecological field methods: macroalgae. Cambridge University Press, New York, pp 461–477

Carmona R, Vergara JJ, Pérez-Llorens JL, Figueroa FL, Niell FX (1996) Photosynthetic acclimation and biochemical responses of *Gelidium sesquipedale* cultured in chemostats under different qualities of light. Mar Biol 127:25–34

Chapman ARO (1980) Methods for macroscopic marine algae. In: Stein JR (ed) Handbook of Phycological Methods, Culture Methods and Growth Measurements. Cambridge University Press, Cambridge, pp 87–104

Collantes G, Melo CU (1995) Cultivo de tejidos y células en algas marinas. In: Alveal K, Ferrario ME, Oliveira EC, Sar E (eds.) Manual de métodos ficológicos. Universidad de Concepción, Concepción. Pp 457-477

Craigie JS, Leigh C (1978) Carrageenans and agars. In: Hellebust JA, Craigie JS (eds) Handbook of Phycological Methods, Physiological and Biochemical Methods. Cambridge University Press, New York, pp 109–131

Crawley MJ (2007) The R book. John Wiley & Sons, Chichester, UK, 942 pp

Croce ME (2013) Bioecología de la comunidad de macroalgas bentónicas de los arrecifes de ostras en Bahía Anegada (Buenos Aires): potencialidades de explotación y cultivo algal. PhD Thesis, Universidad Nacional del Sur, Bahía Blanca, Argentina

Croce ME, Parodi ER (2012) Seasonal dynamic of macroalgae in intertidal pools formed by beds of *Crassostrea gigas* (Mollusca, Bivalvia) on the north Patagonian Atlantic coast. Bot Mar 55:49–58

Croce ME, Parodi ER (2013) The turf-forming alga *Gelidium crinale* (Florideophyceae, Rhodophyta) on Atlantic Patagonian coasts. Bot Mar 56:131–141

De la Coba F, Aguilera J, Figueroa FL, De Gálvez MV, Herrera E (2009) Antioxidant activity of mycosporine-like amino acids isolated from three red macroalgae and one marine lichen. J Appl Phycol 21:161–169

De Sousa AAS, Benevides NMB, de Freitas PA, Fiúza FP, Queiroz MGR, Morais TMF, Pereira MG, Assreuy AMS (2011) A report of a galactans from marine alga *Gelidium crinale* with in vivo anti-inflammatory and antinociceptive effects. Fundam Clin Pharmacol 27:173–180

FAO (2011) Production, properties and uses of agar. Retrieved from http://www.fao.org/docrep/X5822E/x5822e03.htm on 20 May 2014

Fredriksen S, Rico JM, Rueness J (1993) Comparison of *Gelidium latifolium* (Grev.) Born. et Thur. (Gelidiales, Rhodophyta) isolates from Spain and Norway. J Appl Phycol 5:117–122

Freile-Pelegrín Y, Robledo D, Serviere-Zaragoza E (1999) *Gelidium robustum* agar: quality characteristics from exploited beds and seasonality from an unexploited bed at Southern Baja California, Mexico. Hydrobiologia 398/399:501–507

Friedlander M (2008) Advances in cultivation of Gelidiales. J Appl Phycol 20:451–456

Gómez-Ordóñez E, Rupérez P (2011) FTIR-ATR spectroscopy as a tool for polysaccharide identification in edible brown and red seaweeds. Food Hydrocoll 25:1514–1520

Goswami S, Naik S (2014) Natural gums and its pharmaceutical application. J Sci Innov Res 3:112–121

Guerrero P, Etxabide A, Leceta I, Peñalba M, de la Caba K (2014) Extraction of agar from *Gelidium sesquipedale* (Rhodophyta) and surface characterization of agar based films. Carbohydr Polym 99: 491–498

Guiry M, Cunningham E (1984) Photoperiodic and temperature responses in the reproduction of the north-eastern Atlantic *Gigartina acicularis* (Rhodophyta: Gigartinales). Phycologia 23:357–367

Imeson A (2010) Agar. In: Imeson A (ed) Food stabilizers, thickeners and gelling agents. Wiley, London, p 368

Kabacoff RI (2011) R in action. Data analysis and graphics with R. Manning, New York, USA. pp 447

Kačuráková M, Wilson RH (2001) Developments in mid-infrared FT-IR spectroscopy of selected carbohydrates. Carbohydr Polym 44:291–303

Kawai H, Motomura T, Okuda K (2005) Isolation and purification techniques for macroalgae. In: Andersen RA (ed) Algal culturing techniques. Elsevier Academic Press, London, pp 133–143

Lahaye M (2001) Developments on gelling algal galactans, their structure and physic-chemistry. J Appl Phycol 13:173–184

Lee RE (2008) Phycology. Cambridge University Press, Cambridge, UK, 547 pp

Lee J-P, Lee K-H, Song H-K (1997) Manufacture of biodegradable packaging foams from agar by freeze-drying. J Mater Sci 32:5825–5838

Macchiavello J, Saito R, Garofalo G, Oliveira EC (1999) A comparative analysis of agarans from commercial species of *Gracilaria* (Gracilariales, Rhodophyta) grown in vitro. Hydrobiologia 398/399:397–400

Macler BA, West JA (1987) Life history and physiology of the red alga *Gelidium coulteri*, in unialgal culture. Aquaculture 61:281–293

Macler BA, Zupan JR (1991) Physiological basis for the cultivation of the Gelidiaceae. Hydrobiologia 221:83–90

Marinho-Soriano E (2001) Agar polysaccharides from *Gracilaria* species (Rhodophyta, Gracilariaceae). J Biotechnol 89:81–84

Matsuhiro B (1995) Aislamiento y caracterización de ficocoloides. In: Alveal K, Ferrario ME, Oliveira EC, Sar E (eds.) Manual de métodos ficológicos. Universidad de Concepción, Chile. pp 657–675

Matsuhiro B (1996) Vibrational spectroscopy of seaweed galactans. Hydrobiologia 326/327:481–489

Matsuhiro B, Rivas P (1993) Second-derivative Fourier transform infrared spectra of seaweed galactans. J Appl Phycol 5:45–51

McCandless EL, Craigie JS (1979) Sulfated polysaccharides in red and brown algae. Annu Rev Plant Physiol 30:41–53

McHugh DJ (1991) Worldwide distribution of commercial resources of seaweeds including *Gelidium*. Hydrobiologia 221:19–29

McHugh DJ (2002) Prospects for seaweed production in developing countries, FAO Fisheries Circulars. Pp 28

Meena R, Prasad K, Ganesan M, Siddhanta AK (2008) Superior quality agar from *Gracilaria* species (Gracilariales, Rhodophyta) collected from the Gulf of Mannar, India. J Appl Phycol 20:397–402

Melo RA (1998) *Gelidium* commercial exploitation: natural resources and cultivation. J Appl Phycol 10:303–314

Milani J, Maleki G (2012) Hydrocolloids in the food industry. In: Valdez B (ed.) Food industrial processes—methods and equipment. INTE CH. -38

Mouradi-Givernaud A, Givernaud T, Morvan H, Cosson J (1992) Agar from *Gelidium latifolium* (Rhodophyceae, Gelidiales): biochemical composition and seasonal variations. Bot Mar 35:153–159

Muñoz J, Kumar V, Fotedar R (2011) Seaweed culture with special reference to Latin America. In: Fotedar RK, Phillips BF (eds) Recent advances and new species in aquaculture. Wiley-Blackwell, Oxford, pp 252–276

Murano E (1991) Extraction and purification of phycocolloids from agarophytes. In: Reina GG, Pedersen M (eds.) Seaweed cellular biotechnology, physiology and intensive cultivation. Universidad de las Palmas de Gran Canaria, Spain. pp 303–307

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

Murano E, Toffanin R, Halvor Knutsen S, Focher B, Rizzo R, Paoletti S (1993) Evaluation of steam explosion as pretreatment in agar extraction from *Gracilaria dura* (C. Agardh) J. Agardh (Gracilariaceae, Rhodophyta). J Appl Phycol 5:417–424

Murano E, Jellús V, Piras A, Toffanin R (1998) Cell wall polysaccharides from *Gelidium* species: physic-chemical studies using MRI techniques. J Appl Phycol 10:315–322

Nishinari K, Watase M (1983) Effect of alkali pretreatment on the rheological properties of concentrated agar-agar gels. Carbohydr Polym 3:39–52

Oliveira EC, Paula EJ, Plastino E, Petti R (1995) Metodologías para cultivo no axénico de macroalgas marinas *in vitro*. In: Alveal K, Ferrario ME, Oliveira EC, Sar E (eds.) Manual de métodos ficológicos. Universidad de Concepción, Concepción. pp 429–447

Orduña-Rojas J, Robledo D (2002) Studies on the tropical agarophyte *Gracilaria cornea* J. Agardh (Rhodophyta, Gracilariales) from Yucatán, México. II. Biomass assessment and reproductive phenology. Bot Mar 45:459–464

Pacheco-Ruiz I, Zertuche-Gonzalez JA (1995) Effect of water movement on the growth of *Gelidium robustum* (Gardn.) Hollend. and Abb. (Rhodophyta). Cienc Mar 21:59–70

Percot A, Yalcın A, Aysel V, Erdugan H, Dural B, Güven KC (2009) β-Phenylethylamine content in marine algae around Turkish coasts. Bot Mar 52:87–90

Pereira MG, Benevides N, Melo MR, Valente AP, Melo FR, Mourão PA (2005) Structure and anticoagulant activity of a sulfated galactan from the red alga, *Gelidium crinale*. Is there a specific structural requirement for the anticoagulant action? Carbohydr Res 340: 2015–2023

Piriz ML, Casas G (1996) Macroalgas de interés comercial en las costas del sur de Chubut y norte de Santa Cruz. Centro Nacional Patagónico (CONICET), Puerto Madryn, Argentina

Pomin VH, Mourão PAS (2008) Structure, biology, evolution, and medical importance of sulfated fucans and galactans. Glycobiology 18: 1016–1027

R Development Core Team (2011) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/

Rahelivao MP, Andriamanantoanina H, Heyraud A, Rinaudo M (2014) Structure and rheological behaviour of agar extracted from Madagascar sea coast algae. Open Macromol J 7:1–6

Rebello J, Ohno M, Ukeda H, Kusunose H, Sawamura M (1997) 3,6 anhydrogalactose, sulfate and methoxyl contents of commercial agarophytes from different geographical origins. J Appl Phycol 9: 367–370

Rees DA (1961) Estimation of the relative amounts of isomeric sulphate esters in some sulphated polysaccharides. J Chem Soc 5168–5171

Rochas C, Lahaye M, Yaphe W (1986) Sulfate content of carrageenan and agar determined by infrared spectroscopy. Bot Mar 29:335–340

Rodríguez D (1996) Vegetative propagation by fragmentation of *Gelidium sclerophyllum* (Gelidiales, Rhodophyta). Hydrobiologia 326/327:361–365

Rodriguez MC, Matulewicz MC, Noseda MD, Ducatti DRB, Leonardi PI (2009) Agar from *Gracilaria gracilis* (Gracilariales, Rhodophyta) of the Patagonic coast of Argentina—content, structure and physical properties. Bioresour Technol 100:1435–1441

Rojas R, León N, Rojas R (1996) Practical and descriptive techniques for *Gelidium rex* (Gelidiales, Rhodophyta) culture. Hydrobiologia 326/327:367–370

Roleda MY, Montaño NE, Ganzon-Fortes ET, Villanueva RD (1997) Acetic acid pretreatment in agar extraction of Philippine *Gelidiella acerosa* (Forsskaal) Feldmann et Hamel (Rhodophyta, Gelidiales). Bot Mar 40:63–69

Salinas JM (1991) Spray system for re-attachment of *Gelidium sesquipedale* (Clem.) Born. Et Thur. (Gelidiales: Rhodophyta). Hydrobiologia 221:107–117

Santelices B (1976) Nota sobre cultivo masivo de algunas especies de Gelidiales (Rhodophyta). Rev Biol Marina Dep Oceanol Univ Chile 16:27–33

Santelices B, Varela D (1994) Abiotic control of reattachment in *Gelidium chilense* (Montagne) Santelices & Montalva (Gelidiales; Rhodophyta). J Exp Mar Biol Ecol 177:145–155

Sekkal M, Huvenne JP, Legrand P, Sombret B, Mollet JC, Mouradi-Givernaud A, Verdus MC (1993) Direct structural identification of polysaccharides from red algae by FTIR microspectrometry I: localization of agar in *Gracilaria verrucosa* sections. Microchim Acta 112:1–10

Seo Y-B, Lee Y-W, Lee C-H, You H-C (2010) Red algae and their use in papermaking. Bioresour Technol 101:2549–2553

Seoane-Camba JA (1997) *Gelidium sesquipedale* cultivation in Galicia (Spain). Lagascalia 19:179–186

Shunzo S (1971) Mariculture of seaweeds and its problems in Japan. Proceedings of the First US-Japan meeting on aquaculture at Tokyo, Japan

Sousa-Pinto I, Murano E, Coelho S, Felga A, Pereira R (1999) The effect of light on growth and agar content of *Gelidium pulchellum*

(Gelidiaceae, Rhodophyta) in culture. Hydrobiologia 398/399:329–338

Sun J, Wang YJ, Zhu LY, Yang RY (2007) Study on antifungal and antioxidant activities of alcohol extract of *Gelidium amansii*. Food Sci 10:009

Sur M, Güven KC (2002) Infrared studies on *Phyllophora nervosa* agar and comparison with various agars and carrageenans. Turk J Mar Sci 8:143–156

Titlyanov EA, Titlyanova TV (2006) Production of plantlets of the red alga of *Gelidium* genus (Rhodophyta) from thallus fragments. Russ J Mar Biol 32:284–288

Titlyanov EA, Titlyanova TV (2010) Seaweed cultivation: methods and problems. Russ J Mar Biol 36:227–242

Titlyanov EA, Titlyanov TV, Kadel P, Lüning K (2006) Obtaining plantlets from apical meristem of the red alga *Gelidium*. J Appl Phycol 18:167–174

Usov AI (2011) Polysaccharides of the red algae. Adv Carbohydr Chem Biochem 65:115–217

Xia K, Liu X, Zhao J, Zhang X (2014) The physicochemical property characterization of agar acetate. Carbohydr Polym 110:32–37

Zemke-White WL, Ohno M (1999) World seaweed utilization: an end-of-century summary. J Appl Phycol 11:369–376

Zhou J, Zhou M, Caruso RA (2006) Agarose template for the fabrication of macroporous metal oxide structures. Langmuir 22: 3332–3336