Offprint Botanica Marina Vol. 40, 1997, pp. 63–69

Acetic Acid Pretreatment in Agar Extraction of Philippine *Gelidiella acerosa* (Forsskaal) Feldmann *et* Hamel (Rhodophyta, Gelidiales)

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Application of different pre-extraction treatments and extraction methods were used to isolate agar from *Gelidiella acerosa*. Acetic acid pretreatment entailed soaking the sample in 0.5% acetic acid for 1 hour at 16–20 °C. Alkali pretreatment entailed treatment with 1 N NaOH at 90 °C for 1 hour and neutralization in weak acid for another hour at 16–20 °C. Native agar was extracted directly from air dried samples. One hour extraction using steam pressure at 15–20 PSI and boiling at 100 °C in a water bath were applied respectively. Comparative analysis showed that the acetic acid pretreated and autoclaved sample gave the highest agar yield (29.8 ± 2.41%) and gel strength (676 ± 4 g cm^{-2}) among the extraction methods applied. Other physico-chemical properties of acid-modified agar were measured. Relative viscosity of a 1.0% solution at 65 °C ranged from 5–70 cps. A melting temperature of 90–98 °C is comparable to that of the agars from most Gelidiales, while a gelling temperature of 42–47 °C is relatively high which is suspected to be associated with a number of chemical variables masking or altering the basic structural unit of agar. Temperature hysteresis (difference between gelling and melting temperatures) at 48–50 °C was comparable to that of Difco Bacto agar tested at 50 °C.

Introduction

Gelidiella acerosa (Forsskaal) Feldmann *et* Hamel, a red seaweed, is one of the main sources of raw material for the manufacture of agar in the Philippines (Trono and Ganzon-Fortes 1985, Hurtado-Ponce and Umezaki 1988). In the world market data of raw material for agar production, *G. acerosa* is probably included in the *Gelidium* seaweed harvest as it is often misidentified (McHugh 1991, Armisen 1995). Moreover, there is limited information available in the primary literature on the characteristic profile of the agar from *G. acerosa*.

The different methods, modification of procedures and innovations in extracting phycocolloids from various species of red seaweeds (notably *Gracilaria*, *Pterocladia*, *Gelidium*, and *Gelidiella*) have produced immense variability in the yield and quality of agar (in Table I only the methods applied to *Gelidiella acerosa* are listed). These were done primarily to improve agar yield and quality.

The traditional method of extraction by boiling was modified by using steam pressure to hasten the extraction process (Meer 1980). Other modifications include preliminary bleaching of the raw material using NaOCl, CaO, $CaCO_3$ or CaOH and sun drying before boiling (Rodulfo *et al.* 1985, Nuqui 1987, Hurtado-Ponce 1992 a). Treatment with alkali prior to extraction of *Gracilaria* species has been documented in Japan as early as 1938 (Armisen 1995). This process leads to sulfate hydrolysis and the resulting agar has a significantly higher gel strength (Hong *et al.* 1969). Subsequent experimentation using different combinations of alkali at different concentrations, temperature and duration of treatment were applied to determine the best treatment combination for a certain agarophyte (Guven and Guler 1979, Minghou 1990, Hurtado-Ponce 1992 b). Alkali treatment applied to the species of Gelidiales, *Pterocladia* and *Gelidium* (Lemus *et al.* 1991), showed an improved gel quality compared to their native (untreated) agars. Further pretreatment modifications included a combination of alkali-acid (Rodulfo *et al.* 1985, Anzaldo 1987, Cancino *et al.* 1987, Christeller and Laing 1989, Shafeei *et al.* 1994) or acid-alkali (Gopal 1979, Shafeei *et al.* 1994) treatments. Another modification employed cooking the agarophyte in an acidic medium with the addition of HCl or H_2SO_4 (Thivy 1959, Desai 1967, Guiseley 1968, Okazaki 1971, Anglo *et al.* 1973, Nuqui 1987) or in alkali medium with the addition of $CaCO_3$ or NaOH (Semesi and Mshigeni 1977, Santos and Doty 1978, Hoyle 1978 a, b). With the continuing pursuit to establish an appropriate pre-extraction treatment and extraction methodology, innovations have included the steam explosion as pretreatment in agar extraction from *Gracilaria dura* (C. Ag.) J. Agardh (Murano *et al.* 1993), acid treatment of the polymeric residue from exhaustive aqueous extract of *Gelidium purpurascens* Gardner (Whyte and Englar 1981) and a water-ethanol extraction procedure in *Gracilaria* species (Lahaye *et al.* 1986, Lahaye and Yaphe 1989, Lignell and Pedersen 1989, Chiles *et al.* 1989). Furthermore, the application of different purification processes (Whyte *et*

Table I. Yield, physical and chemical properties of agar from *Gelidiella acerosa* resulting from different extraction procedures.

Extraction method	Time (min)	Yield (%)	Strength (g cm^{-2})	Viscosity (cps)	M. temp. (°C)	G. temp. (°C)	SO_4 (%)	3,6-AG (%)	Reference
Untreated and boiled	180	12.6	520[1]	108.9[1]	ND	ND	2.22	32.58	Laserna *et al.* 1981
	360	37.21	493	ND	89.5	38.0	1.64	38.34	Santos and Doty 1983
Untreated and autoclaved	60,60[2]	50–53[3]	367–286[3]	ND	81.0–94.0[3]	44.5–52.5[3]	ND	ND	Thomas *et al.* 1975
	30,20[2]	6.5	ND	ND	87.0[4]	43.5[4]	ND	ND	Diaz-Piferrer and de Perez 1964
	60,30[2]	32.4	251	ND	82.0[4]	42.0[4]	ND	ND	Diaz-Piferrer and de Perez 1964
	60,30[2]	33.3	132	ND	78.0[4]	40.5[4]	ND	ND	Diaz-Piferrer and de Perez 1964
	180	24.0 ± 3.2	33 ± 13	ND	60.7 ± 0.6	35.3 ± 0.6	2.66	ND	Kapraun *et al.* 1994
Bleached and autoclaved	30,20[2]	4.8	ND	ND	84.0[4]	42.0[4]	ND	ND	Diaz-Piferrer and de Perez 1964
	60,30[2]	35.8	326	ND	83.0[4]	42.0[4]	ND	ND	Diaz-Piferrer and de Perez 1964
	60,30[2]	15.4	355	ND	78.5[4]	42.5[4]	ND	ND	Diaz-Piferrer and de Perez 1964
Alkali treated and autoclaved	180	12.7 ± 2.5	189 ± 26	ND	97.6 ± 0.6	46.8 ± 0.3	2.21	ND	Kapraun *et al.* 1994
Alkali treated and boiled with HAc	240	48	300	ND	ND	ND	ND	ND	Pillay 1977
Bleached, HCl treated and autoclaved	30,20[2]	35.4	266	ND	82.5[4]	41.5[4]	ND	ND	Diaz-Piferrer and de Perez 1964
	60,30[2]	44.5	214	ND	81.0[4]	41.0[4]	ND	ND	Diaz-Piferrer and de Perez 1964
HAc treated and autoclaved	**60**	**19.7–34.0[5]**	**360–800[5]**	**5–70[5]**	**90.0–98.0[5]**	**42.0–47.0[5]**	**0.40–2.10[5]**	**39.2–44.2[5]**	**This study**
Difco Bacto agar	–	–	222 ± 18	4 ± 0.3	85.0 ± 1.0	35.0 ± 1.0	1.40 ± 0.4	39.0 ± 4.6	This sutdy

[1] from 2.0% solution in gel strength; from 1.5% solution in viscosity
[2] with reextraction of algal residue
[3] value represent seasonal maxima
[4] from 1.5% solution
[5] minimum and maximum values from 13 sample lots
ND no data

al. 1981, Murano 1991) were also found to improve the agar quality.

In *Gelidiella acerosa*, the traditional way of preparing dessert gel from this species by the Filipino people entails the use of vinegar or calamansi (*Citrus mitis* Blanco) extract added to the boiling solution of bleached seaweed (Ganzon-Fortes 1994). Based on this traditional extraction protocol, we have conducted the following study to develop an extraction methodology for *G. acerosa* to significantly improve its agar quality. The method adapted involves pretreatment of the seaweed with acetic acid prior to pressure extraction. Comparative extraction methods reported in literature were also used to evaluate the efficiency of our extraction procedure in terms of the resulting agar yield and gel strength.

Materials and Methods

Seaweed samples were collected on a reef flat at Dos Hermanos Is., Bolinao, Pangasinan, Philippines (16°25.71' N, 119°55.87' E). These were cleaned of associated species and foreign material, washed with tap water and air-dried. Native agar was extracted directly from air dried samples. Pre-extraction treatments applied were, weak acid pretreatment, which entail soaking the sample (15 g) in 300 mL of 0.5% acetic acid at 16–20 °C for 1 h, and alkali treatment with 300 mL 1 N NaOH at 90 °C for 1 h, followed by neutralization in 300 mL 0.5% acetic acid at 16–20 °C for another hour. The samples were rinsed thoroughly in tap water after each pretreatment and neutralization. One hour extraction (in triplicate, using aluminum pots containing 300 mL distilled water) was done by means of boiling in a water bath at 100 °C and by steam pressure at 15–20 psi respectively. The 6 different experimental treatments were as follows:

(1) untreated and boiled,
(2) untreated and autoclaved,
(3) alkali treated and boiled,
(4) alkali treated and autoclaved,
(5) acid treated and boiled and
(6) acid treated and autoclaved.

Then 10 g diatomaceous earth was added and the mixture blended to a paste. The resulting slurry was heated to 80 °C in a water bath and filtered in a filter bomb. The filtrate was cooled and frozen overnight. The sample was thawed, washed in tap water several times and the excess water squeezed through a cheesecloth. The agar was dried in an oven at 60 °C. The percentage yield of dried agar was calculated from the dry weight of the original sample (raw material). The agar was powdered for analysis of its gel strength. Gel strength (g cm^{-2}) was measured on a 1.5% agar solution (previously cured overnight at room temperature) (Hurtado-Ponce and Umezaki 1988), using a Gel Tester Model G141-2, Marine Colloids, Inc., Springfield, New Jersey. Only the bottom surface of the gel was tested. Further analyses on the physico-chemical properties of acid modified agar were done. Relative viscosity of a 1.0% solution expressed in centipoise (cps) was measured at 65 °C (Whyte and Englar 1980), using Brookfield Viscometer Model LVF, Brookfield Eng'ng Lab., Inc., Stoughton, Mass. The gelling and melting temperatures were determined from a 1.0% agar stock solution (Whyte and Englar 1980). Percent sulfate content was determined using the turbidometric method of Jackson and McCandless (1978) with Na_2SO_4 as standard. Percent 3,6-anhydrogalactose content was determined using the colorimetric method of Craigie and Leigh (1978) with fructose as standard. All agar analyses were done in triplicate.

The statistical significance of differences in yield and gel strength between pre-extraction treatment, mode of extraction and its interaction were tested using analysis of variance (ANOVA $p = 0.05$), followed by Duncan's multiple range test using a SAS program.

Results

The highest agar yield was obtained from the acetic acid pretreated and autoclaved sample (29.84% ± 2.41) while boiling with the same treatment yielded only one third of the extractable agar (9.76% ± 2.36). In alkali treated samples, high agar yields were extracted from both autoclaved and boiled samples (28.09% ± 2.55 and 27.58% ± 1.71 respectively). Untreated samples gave the lowest agar yield in the autoclaved (9.55% ± 0.49) and boiled (5.94% ± 0.64) extraction methods (Fig. 1). Analysis of variance showed significant differences in pre-extraction treatment ($p < 0.0001$), mode of extraction ($p < 0.0001$) and their interactive effect ($p < 0.0001$).

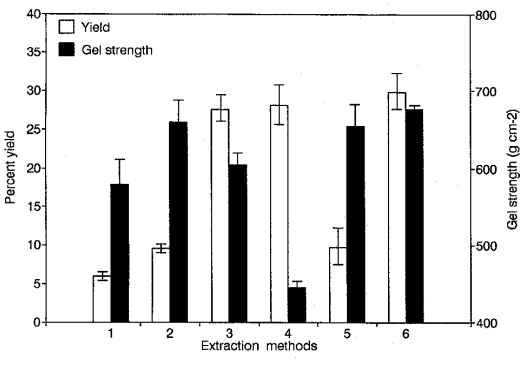

Fig. 1. Yield and gel strength of agar from *Gelidiella acerosa* using different extraction methods (1) untreated and boiled (2) untreated and autoclaved (3) alkali treated and boiled (4) alkali treated and autoclaved (5) acetic acid treated and boiled (6) acetic acid treated and autoclaved. Bars indicate standard deviation.

The highest gel strength was measured on acid modified agar, with a slightly higher gel strength measured from samples isolated by pressured extraction (676 ± 4 g cm^{-2}) compared to boiled extraction (654 ± 28 g cm^{-2}). The gel strength of alkali modified agar was significantly higher in samples obtained by boiling (605 ± 14 g cm^{-2}) compared to autoclaving (445 ± 5 g cm^{-2}). Conversely, the gel strength of native agar was higher in samples extracted by autoclaving (659 ± 27 g cm^{-2}) compared to boiling (579 ± 32 g cm^{-2}) (Fig. 1). Analysis of variance for the gel strength showed significant differences in the mode of extraction ($p < 0.0001$) and its interaction with pretreatment ($p < 0.0001$).

Increasing the pressure extraction time (60, 120, 180 mins) of the acid pretreated samples resulted in an increase in agar yield (22.4% ± 2.8, 24.6% ± 1.7, 28.5% ± 2.3 respectively) but a corresponding decrease in gel strength was observed (497 ± 16.7, 296 ± 56.6, 266 ± 8.5 g cm^{-2} respectively) (Fig. 2). Characteristic profiles of acid modified agar are presented in Table I. Values obtained on similar properties using Difco Bacto agar are also presented for comparison.

Discussion

A pre-extraction treatment using weak acid or alkali was observed to facilitate the extraction process giving higher yields compared to the untreated sample. Moreover, applying steam pressure enabled us to isolate the highest quantity of agar extractable from the raw material. Studies on the extraction of untreated samples have reported that to extract 12.6% agar Laserna *et al.* (1981) boiled their samples for 180 minutes. Santos and Doty (1983) cooked for 360 minutes and extracted 37.21%. Thus boiling required a longer time to obtain a higher agar yield. With pressured extraction (for native agar), Kapraun *et al.* (1994) obtained 24 ± 3.2% after 180 minutes. Higher agar yields were also obtained by Diaz-Piferrer and de Perez (1964) and Thomas *et al.* (1975) at 35.8% and 50–53%, respectively, when after initially pressure extracting for 60 minutes, they repeated the procedure for 30 to 60 more minutes on the same algal residue. However, low agar yields of 4.8–6.5% resulted from bleached and untreated samples when the initial pressure extraction time was only 30 minutes (Diaz-Piferrer and de Perez 1964). Using pre-extraction treatments, Pillay (1977) boiled his sample with acid for 240 minutes after pretreating with alkali and obtained a much higher agar yield of 48%. Kapraun *et al.* (1994), using alkali pretreatment, neutralization and a pressure extraction time of 180 minutes, obtained a low agar yield of 12.7 ± 2.5%. Alkali pretreatment or extraction with alkali has been reported to generally give lower agar yields, a pattern said to be typical for all agarophytes (Lemus *et al.* 1991). This is contrary to our observation where alkali pretreatment and neutralization in weak acid gave high yields (27.58%–28.09%) compared to the untreated samples (5.95%–9.55%). In acetic acid treated material, increasing the pressured extraction time from 60 to 180 minutes was observed to increase the yield by 21%, but a corresponding 47% decrease in gel strength was observed (Fig. 2). Apparently, variation in agar yield is not only affected by extraction time, boiling or applying steam pressure but also by pre-extraction treatment using alkali or acid. Alkali treatment was observed to soften the wiry and tough thalli of *Gelidiella acerosa*. Acidic treatment was reported to facilitate the extraction of the polysaccharides by disrupting crosslinks occurring in the algal structure (Murano 1991).

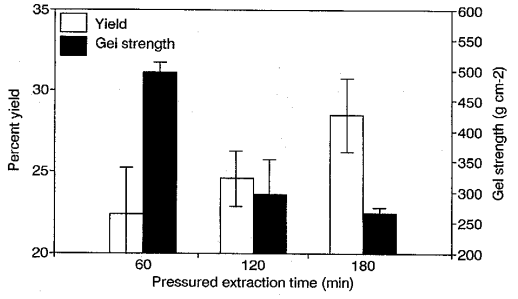

Fig. 2. Effect of extraction time on the yield and gel strength of agar from *Gelidiella acerosa*. Bars indicate standard deviation.

The gel strength of acid modified agar (1.5% agar solution) was significantly higher compared to alkali modified and native agars. Moreover, it gave the highest measured gel strength for *Gelidiella acerosa* agars compared to all reported studies in the literature. The high gel strength of 520 g cm^{-2} previously reported by Laserna *et al.* (1981) is not comparable with our results because their tests were made on a 2% solution. According to Whyte *et al.* (1984) increasing agar concentration from 0.5 to 2.0% will have a corresponding increase in gel strength. We have also observed a corresponding decrease in gel strength with increasing agar extraction time. In *Gracilaria* agars, Hurtado-Ponce (1992 b) observed interactive effects between extraction time and corresponding rheological properties. Thus, extraction time should be carefully monitored in order to get the optimum agar yield and gel quality.

Among the Gelidiales, alkali modified agars from *Gelidium serrulatum* J. Agardh, *G. floridanum* Taylor, and *Pterocladia capillacea* (S. G. Gmelin) Bornet have improved quality in terms of gel strength (relative to the decrease in percent sulfate and increase in 3,6-anhydrogalactose contents) compared to their untreated counterparts (Lemus *et al.* 1991). Likewise, alkali modified agar samples of *Gelidiella acerosa* have significantly higher gel strength (189 g cm^{-2})

than the native samples (33 g cm^{-2}) (Kapraun *et al.* 1994). This observation confirmed our study when boiling was used as the mode of extraction and higher gel strength was measured in alkali modified agar (605 g cm^{-2}) compared to native agar (579 g cm^{-2}). However, when pressure extraction was used, native agar gave a higher gel strength (659 g cm^{-2}) compared to alkali modified agar (445 g cm^{-2}). This observation implied that pressure extraction could have an adverse effect on the structural profile of agar pretreated with alkali.

The characteristic profile of *Gelidiella acerosa* agar (acid modified) in terms of gelling and melting temperature, % sulfate and 3,6-anhydrogalactose (Table I) was comparable to that of the agar from most Gelidiales. The melting temperature, which ranged from 90–98 °C, was similar to that reported for the alkali treated-autoclaved agar (97.6 ± 0.6 °C) of Kapraun *et al.* (1994) and the agars of most Gelidiales (Lemus *et al.* 1991); but was higher than that obtained from other *Gelidiella acerosa* agar samples (Diaz-Piferrer and de Perez 1964, Santos and Doty 1983, native agar of Kapraun *et al.* 1994). The gelling temperature which ranged from 42–47 °C was likewise, comparable to that reported for the treated agar samples of Kapraun *et al.* (1994) (46.8 ± 0.3 °C), but higher than the agars of other Gelidiales (32.5–37.5 °C) (Lemus *et al.* 1991). The presence of sulfate, methoxyl or pyruvate residues in the structural unit of agar has been reported to affect the physical properties of agar gel (Craigie 1990). The increase in gelling temperature is associated with the increase in the methoxyl content of the agarose in the Gracilariaceae (Guiseley 1970) and *Gelidium purpurascens* Gardner (Whyte and Englar 1981). However, in Chilean Gelidiaceae, the highest value in gelling temperature is found in agars with the lowest content of 6-O-methylgalactose (Matsuhiro and Urzua 1991). It is likely that the gel-melting temperature parameter may also be dependent on the molecular weight of the polysaccharide (Selby and Wynne 1973).

The sulfate content varied from 0.4% to 2.1%, this was low compared to the other studies (1.39–2.66%) but there were differences in the methodology used in determining this characteristic. Conversely, our agar sample also registered a high gel strength (range = 360–800 g cm^{-2}). This corroborated similar findings from other agarophytes that the degree of sulfation is negatively correlated with gel strength (McCandless and Craigie 1979, Lemus *et al.* 1991, Armisen 1995, Murano 1995). On the other hand, Kapraun *et al.* (1994) characterized the agar from their Philippine samples of *G. acerosa* as having a high sulfate content (2.21–2.66%) and low molecular weight. The corresponding very low gel strength values (33–189 g cm^{-2}) that they obtained are attributed to seasonal minima. Biologically, the L-galactose 6-sulfate residue is synthesized in algae as a precursor of the 3,6-anhydrogalactose and it is enzymatically converted to the anhydro form by sulphohydrolases (Wong and Craigie 1978). However, since the enzymatic activity of the sulphohydrolases in *Gracilaria* seems to be lower than that of *Gelidium*, a higher number of unfinished 6-sulphated molecules is found in the agar extracts of *Gracilaria* (Murano 1995). Alkaline hydrolysis is usually applied as an extraction pretreatment to convert the L-galactose 6-sulfate into the 3,6-anhydro form. With the high 3,6-AG content of our *Gelidiella acerosa* agar (ranged from 39.2% to 44.2%), even without alkali modification, it was stipulated that a galactan with stable chemical conformation structure was synthesized by *G. acerosa* comparable to that from *Gelidium*.

We now assess the quality of the agar from *Gelidiella acerosa* for its possible industrial use. Industrial agars for the international market are of different types or grades and each has its own specifications. Food grade agar requires a gel strength greater than 750 g cm^{-2} at 1.5%, whereas, bacteriological agar requires a softer gel strength (i.e., 222 g cm^{-2}, measured from the Difco Bacto agar) and is not normally accepted for food use (Armisen 1995). Moreover, the microbiological application of agar depends on the required high temperature hysteresis – difference between the gelling and melting temperatures (Meer 1980, McHugh 1991). The gelling and melting temperatures specifications for bacteriological agar according to the American Society of Microbiology Specification (Meer 1980) are 33–38 °C and 80 ± 5 °C, respectively, giving a temperature hysteresis of 42–52 °C. Therefore, the agar that we have isolated from *G. acerosa* which has a temperature hysteresis of 48.0–50.3 °C can pass for bacteriological grade agar.

The interactive effect of acetic acid pretreatment and steam pressure used in agar extraction from *G. acerosa* has produced by high yield with a corresponding high gel strength compared to the other methods tested. The method developed is relatively simple and requires a shorter extraction time. However, further purification of the extracted agar is recommended to produce a higher quality that will pass the specifications for both food and bacteriological grades. The effect of acid treatment on the structural chemistry of *Gelidiella* agar to give in a higher quality is not yet known. Further chemical analyses involving enzymatic degradation, ^{13}C-NMR and IR spectroscopic studies are needed to elucidate its chemical structure.

Acknowledgements

We thank the Philippine Council for Aquatic and Marine Research and Development for financial support, and the National Committee on Marine Sciences of UNESCO National Commission of the Philippines for the scholarship grant to Michael Y. Ro-

leda. We offer our sincerest thanks to Nell Oca, Edith Boo, Edmund Taylo and Emily Eugenio for helping in the collection of samples; to Cynthia Pagba, Rowena Grace Rumbaoa and Benjamin Que for technical assistance; and to the two anonymous referees who made helpful criticisms of an earlier manuscript. This is contribution no. 265 from the Marine Science Institute, University of the Philippines.

Accepted 22 November 1996

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