Botanica Marina
Vol. XXIV, pp. 441–444, 1981

Chemical Quality and Production of Agars Extracted from *Gracilaria tikvahiae* Grown in Different Nitrogen Enrichment Conditions*

K. T. Bird, M. D. Hanisak and J. Ryther[1]

Harbor Branch Foundation, RR 1, Box 196, Ft. Pierce, Florida, 33620, USA

[1] *Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, 02543, USA*

(Received November 18, 1980/June 16, 1981)

Abstract

Increasing concentrations of nitrogen fertilizer led to increased growth and internal nitrogen content of *Gracilaria tikvahiae* and decreased yields of agar. The thallus nitrogen content was highly correlated with percent protein and protein:carbohydrate ratios, and percent agar was highly correlated with percent carbohydrate. Agars extracted from thalli grown under nitrogen enriched conditions had higher melting temperatures and greater gel strengths than did agars extracted from less enriched thalli. The data suggest that the increased gel strengths found in these experiments may be due to the greater molecular size of the agar polymers, as indicated by the increased melting temperatures associated with greater gel strengths.

Introduction

The strong interest in phycocolloid production by members of the red algae has resulted in several investigations of physical factors which affect agar and carregeenan content (Neish and Shacklock 1971, DeBoer 1979, Simpson *et al.* 1978). Phycocolloids such as agar are highly complex polysaccharides composed of galactropyranose moieties and their various derivative forms. Variations in the relationships of different subunits to each other account for much of the commercial properties of agar such as melting and gelling temperature, and gel strength (Guiseley and Renn 1977). Gel strength and gelling and melting temperatures have been found to vary seasonally in agars extracted from species of *Gracilaria*; however, no cause and effect relationships were evident (Hoyle 1978, Thomas and Krishanmurthy 1976). The purpose of this investigation was to examine if various levels of nitrogen fertilization would have any effect on the production of agar by *Gracilaria tikvahiae*, and more importantly, whether agar would vary in its chemical quality when extracted from plants grown under different fertilization regimes.

Material and Methods

Thalli of the alga, *Gracilaria tikvahiae* McLachlan, ORCA clone (Ryther *et al.* 1979) were grown in heavily aerated suspension cultures similar to those previously described (LaPointe and Ryther 1978). Samples of the thalli were taken from an ongoing experiment that was investigating the relationship of nitrogen and growth of this clone (Hanisak, unpublished data). This experiment consisted of two series, F and G, each of which had eight treatments of different forms and concentrations of nitrogen nutrients. The cultures sampled had been under cultivation for at least four months prior to use in this phycocolloid study. Series F was sampled in December 1979, and Series G in January 1980. Water temperatures for the week prior to sampling ranged from 11 to 29.5 C for Series F and 7 to 26.5 C for Series G, with average midday temperatures of 18 and 17 C, respectively. Nitrogen was added weekly in several forms, as NO_3^-, NH_4^+, or liquid digestor residue (mostly in the form of NH_4^+), from *Gracilaria* seaweed digestion. Concentrations of the nitrogen additions ranged from no addition to 1500 μM N X 1^{-1}, and are detailed in Table I. Stocking density of the thalli was 2.6 kg wet wt. X m^{-2} in all the treatments. Harvested weed was washed, dried at 70 C, and subsequently ground to a powder. Carbon and nitrogen were determined using a Perkin Elmer Model 240 elemental analyzer, and protein and carbohydrate determined by procedures outlined in Dawes *et al.* (1974).

* Harbor Branch Contribution No. 238
* Woods Hole Oceanographic Contribution No. 4737

0006-8055/81/0024-0441$02.00

Agar was extracted from triplicate 1 g samples of dried material in 50 ml H_2O. After autoclaving for two hours at 121 C, the material was filtered through Celite and a 10 μ filter. The agar was allowed to gel, and was then purified through several repeated freeze-thaw cycles before drying it at 70 C. Yields were corrected for recovery efficiency, determined by recovery of known gravimetric standards of Bacto-Agar. Melting and dynamic gelling temperatures of duplicate 1.5 percent agar gels were determined using procedures described by Craigie and Leigh (1978). Gel strengths of triplicate 1.5 percent agar gels aged 24 h at room temperature were determined with a gelometer similar to that described by Goring (1956). The plunger had a surface area of 1 cm^2 and a descent rate of .18 cm s^{-1}. The percent 3.6 anhydrogalactose (3.6 A, G) was determined by the technique of Yaphe and Arsenault (1965), and the percent sulfate (SO_4^-) by the procedure of Jones and Lethan (1954), after hydrolysis of agar samples in 1N HCl at 120 C for three hours. For purposes of comparison, agar quality determinations were also made with Difco Bacto-Agar, Lot number 660673.

Results

All forms of added nitrogen caused increased growth over the treatments with no nitrogen additions (Tab. I). In Series F, which was fertilized only with NO_3^-, the thallus nitrogen levels varied from 1.3 to 3.2 percent. The Series G treatments, which received nitrogen in the forms of NO_3^-, NH_4^+, and digestor residue, exhibited a much wider range of percent thallus nitrogen. In both series, the percent agar was significantly inversely correlated with thallus nitrogen, although the correlation was much greater in Series G (Tab. II). Series G also had a much wider range of percent agar (Tab. I). In both series, the agar yields were inversely correlated with protein and protein: carbohydrate ratios, and positively correlated with percent carbohydrate (Tab. II). Within the respective series, these correlations were generally greater than those of percent nitrogen to percent agar. Percent protein and protein: carbohydrate ratios were positively correlated to percent thallus nitrogen, and the conversion of percent thallus nitrogen to protein ranged from 2.2 to 3.6.

Increasing thallus nitrogen content was also accompanied by increasing melting temperatures of agars extracted from these thalli (Tab. I). Gel strengths of agars rose with increasing thallus nitrogen levels, although there was considerable differences in the range of gel strengths from agars derived from Series G compared to Series F. Both agar gel strength and melting temperature were significantly correlated to the thallus N levels (Tab. II). The melting and gelling temperatures and gel strength of Difco Bacto-Agar were similar to other values reported (Guisely and Renn 1977), Yaphe and Duckworth 1972).

In Series G, the 3,6 AG was inversely correlated to gel strength while SO_4^- was positively correlated (Tab. III). The agar gel strengths from Series F showed no correlation with 3,6 AG and a significant correlation with SO_4^-. Both 3,6 AG and SO_4^- showed only small ranges

Tab. I. Effect of N fertilization (as NO_3^-, NH_4^+, or liquid digestor residue, "D") on internal thallus N content, C:N ratios, protein, carbohydrate, protein: carbohydrate ratios, growth (as g dry wt × m^{-2} × d^{-1}), agar, and five indices of agar quality, melting and gelling temperature, gel strength, percent 3,6 anhydrogalactose, and percent SO_4^- in the agars.

μM N l^{-1}	species of N	% thallus N	C:N	% protein	% carbohydrate	pro:carbo	growth	% agar	melting T (C)	gelling T (C)	gel strength (g cm^{-2})	% 3,6 anhydrogalactose	% SO_4
F Series													
0	–	1.3	23.6	3.4	42.3	.08	0.0	26.3	67.0	38.3	32.5	25.4	2.9
150	NO_3	1.5	19.4	4.8	40.9	.06	5.5	29.3	67.8	40.5	30.0	25.0	2.6
300	NO_3	2.2	13.9	6.5	31.8	.20	10.2	23.6	65.5	39.5	27.5	23.8	2.8
450	NO_3	2.0	14.9	8.4	29.0	.29	11.4	21.7	67.0	41.3	25.0	26.6	3.1
600	NO_3	2.6	12.1	9.1	33.7	.27	13.2	25.3	69.0	41.0	30.0	24.2	2.3
750	NO_3	3.2	8.7	8.8	24.4	.36	9.8	24.0	69.5	41.5	37.5	25.2	3.4
900	NO_3	3.0	9.3	8.8	30.3	.29	10.9	21.0	70.5	41.5	47.5	27.8	3.3
1500	NO_3	3.2	8.9	9.3	24.2	.38	11.1	20.3	71.5	41.5	55.0	24.6	3.7
G Series													
0	–	1.4	21.8	4.6	40.6	.11	3.3	39.6	65.8	41.0	45.0	24.4	0.9
175	D	1.4	22.1	5.1	43.3	.12	4.0	40.1	65.0	39.0	45.0	23.4	1.3
350	D	1.6	16.3	4.5	38.3	.12	9.1	31.6	66.0	41.5	47.5	23.2	1.3
700	D	3.0	9.3	9.1	29.6	.31	10.3	29.1	67.5	42.3	75.0	23.4	1.2
1000	D	3.8	8.0	9.2	24.5	.38	10.2	18.4	74.5	42.0	106.0	20.4	1.9
1400	D	4.9	6.0	10.6	20.1	.53	7.5	23.4	72.0	43.0	110.0	20.8	2.2
1500	NO_3	3.7	8.5	11.1	34.4	.32	8.5	33.9	69.0	43.3	85.0	21.8	1.3
1500	NH_4	5.3	5.3	14.8	21.5	.69	8.8	18.0	72.0	41.3	110.0	20.2	1.2
Difco Bacto-Agar (Lot Number 660673)									88.0	35.5	400.0	33.2	2.2

Tab. II. Correlation coefficient matrix of the percent thallus N, protein, carbohydrate, and agar, and protein:carbohydrate ratios and indices of agar quality: gelling temperature, melting temperature, and gel strength. The G Series data are represented in the bottom correlation matrix, the F Series by the top. Correlation coefficients greater than 0.66 are significant at the 95% level ($p < .05$).

	% N	% pro	% carbo.	pro:carbo.	% agar	melt T	gel T	gel ST.
% N	***	.89	−.88	.92	−.69	.76	.76	.68
% pro	.95	***	−.87	.94	−.73	.63	.88	.43
% carbo.	−.94	−.82	***	−.96	.80	−.53	−.74	−.49
pro:carbo.	.98	.94	−.92	***	−.82	.64	.77	.53
% agar	−.84	−.74	.93	−.85	***	−.45	−.51	−.56
melt T.	.86	−.74	−.90	.81	−.91	***	.69	.88
gel T.	.56	.51	−.58	.44	−.43	.51	***	.43
gel ST.	.97	.89	−.97	.93	−.89	.94	.60	***

Tab. III. Correlation coefficient matrix of five indices of agar quality: gel strength, melting temperature, gelling temperature, percent 3,6 anhydrogalactose, and percent SO_4. The G Series data are represented in the bottom correlation matrix, the F Series by the top. Correlation coefficients greater than 0.66 are significant at the 95% level ($p < .05$).

	gel ST.	melt T.	gel T.	% 3,6 A, G	% SO_4^{--}
gel ST.	***	.88	.43	.23	.76
melt T.	.95	***	.69	.26	.57
gel T.	.60	.52	***	.32	.41
% 3,6 A, G	−.94	−.93	−.42	***	.37
% SO_4	.67	.69	.41	−.66	***

of variations over the ranges of gel strengths (Tab. I). The SO_4^{--} of agars from Series F was generally higher than that of Series G. Melting temperatures of the agars correlated highest with gel strengths in both series, and ranged as much as 7 C between low and high nitrogen thalli. The SO_4^{--} and 3,6 AG of Difco Bacto-Agar were similar to those values reported by Duckworth and Yaphe (1971).

In Series G, there appeared to be differences between the treatments of 1500 μM N × l^{-1} in the forms of NO_3^- and NH_4^+ (Tab. I). The NO_3^- grown thalli showed a lower percent thallus nitrogen, a greater percent carbohydrate, and a greater percentage of agar than did NH_4^+ grown plants. The characteristics of agar extracted from the NH_4^+ grown thalli compared to NO_3^- grown thalli were a higher melting temperature, a lower gelling temperature, and a greater gel strength.

Discussion

The inverse relationship observed between agar yield and thallus nitrogen levels in *Gracilaria tikvahiae* is one which has been observed by other workers for both carrageenan and agar (Neish and Shacklock 1971, DeBoer and Ryther 1977, DeBoer 1979). It should be noted that the range of agar yields from Series G was much greater than those of Series F, even when comparing similar thallus nitrogen levels. This difference might be due to responses of the plants to NO_3^- versus liquid digestor residues (mostly in the form of NH_4^+), or to some other environmental factor such as temperature or water quality.

The response of increasing growth rates and thallus nitrogen content in *Gracilaria tikvahiae* to increasing concentrations of supplied nitrogen have also been noted by DeBoer and Ryther (1977). The high correlations between thallus nitrogen and protein and protein: carbohydrate ratios suggest that these parameters all may be used as indicators of the nitrogen status of algae. It is interesting to note that the conversion factor for percent nitrogen to percent protein was not close to 6.25, a value commonly used. Use of percent thallus nitrogen for conversion to protein probably doesn't sufficiently consider nitrogen in other metabolic forms such as amino acids, RNA, DNA, or internal inorganic nitrogen in the form of NO_3^- or NH_4^+. The close correlation between carbohydrate and agar suggests that determinations of carbohydrate may provide a useful and rapid procedure for estimating agar yields in various algal populations.

The increases in gel strength and melting temperatures with increasing thallus nitrogen indicates that the nutrient regime of the thalli greatly affects the commercial quality of the agar derived from those plants. From these data it would appear that plants grown under moderate to high levels of nitrogen fertilizer produce a better commercial quality of agar in regards to gel strength. There were differences in the range of gel strengths between Series F and G, the latter producing agars with stronger gels. As noted earlier, these differences might be due to either the type of nitrogen fertilizer used or other environmental factors. Hoyle's data (1978) indicate seasonal variations in gel strengths of agars derived from *Gracilaria coronopifolia* and *bursa-pastoris* did not always correspond to seasonal variations in thallus nitrogen levels.

The increases in gel strength found in the experiments cannot be explained by current models of this phenome-

non, which describe gel strength as due to higher levels of 3,6 AG and lower levels of SO_4^{--} (Rees 1972, Yaphe and Duckworth 1972). There was no real correlation of 3,6 AG to gel strength, while SO_4^{--} was positively correlated. The ranges of SO_4^{--} and 3,6 AG were low in comparison to the ranges of gel strengths, even though these values were within the ranges of other *Gracilaria* spp. (Duckworth *et al.* 1971, Whyte and Englar 1980). Series F did have a higher SO_4^{--} and lower range of gel strengths than did Series G, which does conform to the proposed mechanisms of agar gel strength. In both series gel strength correlated highly with melting temperature, an indicator of greater molecular weight and size of the agar polymers (Selby and Wynne 1973). The longer polymers may be more capable of interacting with each other and forming a greater three dimensional lattice between water and the gel helices, hence agars with higher gel strengths. Rees (1972) has described how other workers have found that long chains are capable of gelling, whereas short chains might not gel.

From the management perspective of maximizing agar production and commercial quality, there are still several important questions to be examined. As alkaline treatment is known to increase the quality of poor agars, it will be necessary to evaluate this treatment on agars extracted from plants grown under low and high nitrogen fertilization regimes. Also, it is clear that more attention needs be paid to the effects of different types of nitrogen fertilizer on agar quality, and how other environmental factors interact with nitrogen in affecting agar quality. From this study, it is apparent that more attention should be given to examinations of agar quality indices when doing phycocolloid production studies, as from a commercial point of view, agar quality indices when doing phycocolloid production studies, as from a commercial point of view, agar quality is of prime importance.

Acknowledgements

The authors wish to thank D. Andrews and B. Faulkner for elemental and water quality analyses, and L. Williams and R. Stenberg for assistance during the project. Thanks are also extended to Dr. T. Wang for use of laboratory facilities. This research was supported in part by a Department of Energy Grant, SERI contract No. XR-9-8133-1 and the Harbor Branch Foundation.

References

Craigie, J. S. and C. Leigh. 1978. Carrageenans and agars. *In*: (J. A. Hellebust and J. S. Craigie, eds.) *Handbook of Phycological Methods. Physiological and Biochemical Methods.* Cambridge University Press. pp. 109–132.

Dawes, C. J., J. M. Lawrence, D. P. Cheney, and A. C. Mathieson. 1974. Ecological studies of floridian *Eucheuma* (Rhodophyta, Gigartinales). III. Seasonal variation of carrageenan, total carbohydrate, protein, and lipid. *Bull. Mar. Sci. 24*: 286–299.

DeBoer, J. A. 1979. Effects of nitrogen enrichment on growth rates and phycocolloid content in *Gracilaria foliifera* and *Neogardhiella baileyi* (Florideophyceae). *Proc. Int. Seaweed Symp. 9*: 263–273. Science Press, Princeton.

DeBoer, J. A. and J. H. Ryther. 1977. Potential yields from a waste recycling algal mariculture system. *In*: (R. W. Krauss, ed.) *The Marine Plant Biomass of the Pacific Northwest.* Oregon State Univ. Press. pp. 231–249.

Duckworth, M., K. C. Hong, and W. Yaphe. 1971. The agar polysaccharides of *Gracilaria* species. *Carbohyd. Res. 18*: 1–9.

Goring, D. A. I. 1956. A semimico gelometer. *Can. J. Techol. 34*: 53–59.

Guisely, K. B. and D. W. Renn. 1977. Agarose: purification, properties, and biomedical applications. FMC Corporation, Rockland, Maine. 34 pp.

Hoyle, M. D. 1978. Agar studies in two *Gracilaria* species (*G. bursapastoris* (Gmelin) Silva and *G. cornopifolia* J. Ag.) from Hawaii. II. Seasonal aspects. *Bot. Mar. 21*: 347–352.

LaPointe, B. E. and J. H. Ryther. 1978. Some aspects of the growth and yield of *Gracilaria tikvahiae* in culture. *Aquaculture 15*: 185–193.

Jones, A. S. and D. S. Lethan. 1954. A submicromethod for the estimation of sulfur. *Chem. Ind.* (London): 622–663.

Neish, A. C. and P. F. Shacklock. 1971. Greenhouse experiments on the propagation of strain T4 of Irish moss. *Atlantic reg. tech. report serial no. 14*: 25 pp.

Rees, D. A. 1972. Shapely polysaccharides. *Biochem. J. 126*: 257–273.

Ryther, J. H., L. D. Williams, M. D. Hanisak, R. W. Stenberg, and T. A. DeBusk. 1979. Biomass production by marine and freshwater plants. *3rd Annual Biomass Energy Conference Proceedings.* Solar Energy Research Institute, Golden, Colorado. pp. 13–24.

Selby, H. H. and W. H. Wynne. 1973. Agar. *In*: (R. L. Whistler, ed.) *Industrial Gums, Polysaccharides, and Their Derivatives.* Academic Press, N. Y. pp. 29–48.

Simpson, F. J., A. C. Neish, P. F. Shacklock, and D. R. Robson. 1978. The cultivation of *Chondrus crispus*. Effect of pH on growth and production of carrageenan. *Bot. Mar. 21*: 229–235.

Thomas, P. C. and V. Krishnamurthy. 1976. Agar from cultivated *Gracilaria edulis* (Gmel.) Silva. *Bot. Mar. 19*: 115–117.

Whyte, J. N. C. and J. R. Englar. 1980. Chemical composition and quality of agars in the morphotypes of *Gracilaria* from British Columbia. *Bot. Mar. 23*: 277–283.

Yaphe, W. and G. P. Arenault. 1965. Improved resorcinol reagent for the determination of fructose, and of 3,6 anhydrogalactos in polysaccharides. *Anal. Biochem. 13*: 143–148.

Yaphe, W. and M. Duckworth. 1972. The relationship between structures and biological properties of agars. *Proc. Int. Seaweed Symp. 7*: 15–22. Univ. of Tokyo Press, Tokyo.