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Agar Production and Quality from *Gracilaria* sp. Strain G–16: Effects of Environmental Factors

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Abstract

A strain of *Gracilaria* sp., G-16, which produces an agar with high gel strength was grown under controlled culture conditions at 3 different temperatures, 2 light quantum fluxes, 2 salinities, and under N enrichment and starvation. Best productivity occurred at 24 °C, followed by 32 °C, then 15 °C. Higher light quantum fluxes and nitrogen enrichment also contributed to greater productivities. Salinity changes of 17‰ versus 33‰ showed minimal effect on productivity. Slight salinity effects occurred when other culture factors contributed to reduced growth. Agar content was typically greater in plants grown at 17‰ than 33‰, and was generally lower in plants grown at 32 °C than 24 or 15 °C. There was some effect of thallus N levels on agar content. Agar gel strength was greater in the N enrichment treatments, and was also affected by both temperature and salinity. The highest agar gel strength was found in the 32 °C, N enriched, 33‰ treatments. Lower salinities of 17‰ led to reductions in agar gel strength, but more significantly, the N enrichment effect on increased gel strength disappeared.

Introduction

The production of agar by seaweeds shows considerable variability in gel quality and yield both from different algal species and strains (Cote and Hanisak 1986) and due to seasonal fluctuations. For example, Hoyle (1978) found that low agar yields in the winter were associated with the seasonally higher total thallus nitrogen content in two *Gracilaria* species. Agar gel strengths showed different patterns for these two species, even though they coexist. Gel strength did not correlate strongly with any of the measured environmental parameters such as nitrogen, or with biomass abundance. Other authors have noted similar distinct seasonal changes in agar characteristics. A comparison of seasonal changes in gel strength of *Gracilaria tikvahiae* McLachlan agar to that of carrageenan from *Neoagardhiella baileyi* (Kütz.) Wynne *et* Taylor revealed that highest agar gel strengths were found from summer *Gracilaria* samples while highest carrageenan gel strengths were from winter plants (Asare 1980). Agars from *Gracilaria* spp. in California had highest gel strengths from summer collected plants. Gel strength was correlated with biomass availability but inversely correlated with agar yield (Abbott 1980). While these studies document significant seasonal agar quality changes, there is no clarification of how environmental factors affect agar yield and quality. Effects of salinity dilution from rainfall or river run-off into marine habitats might also affect agar quality, but have not been examined.

Some progess has been made by algal culturists. An inverse correlation occured between agar yield and thallus nitrogen content (DeBoer 1979); however, decreases in thallus nitrogen content also led to agars with lower gel strengths, melting and gelling temperatures (Bird *et al.* 1981). This N enrichment effect on gel strength has been reported for other *Gracilaria* spp. (Craigie *et al.* 1984). For the most part, the effects of environmental factors and their potential interactions on agar production and on commercial quality characteristics still remains unknown as there are little data based on *Gracilaria* spp. grown under well defined culture conditions. Therefore, this research ex-

amined the interactive effects of four major environmental factors: light, temperature, salinity and nitrogen nutrients on growth and agar quality of a strain of *Gracilaria* producing an agar with high gel strength (Guerin and Bird 1987). As the intent of the research was to determine how these factors would affect mariculture of this strain to produce commercially acceptable agars, agar extraction procedures incorporated the alkaline pretreatment process.

Material and Methods

Strain G-16 of the red alga, *Gracilaria* sp., was used for these experiments. The alga was grown under controlled culture conditions to examine the effects of interactive environmental factors on agar yield, chemistry, and agar gel behavior. Four interactive environmental factors were examined: light, temperature, nitrogen nutrients, and salinity, in a 3X2X2X2 matrix. The different factors represent a range of environmental conditions: temperatures of 15 °C, 24 °C and 32 °C, salinities of 17 and 33‰, 2 different nitrogen loadings which led to nitrogen deficient and enriched thalli (> 2% of dry wt.), and light intensities at 465 $\mu E\ m^{-2}\ s^{-1}$ (520 $\mu E\ m^{-2}\ s^{-1}$ determined as photosynthetic saturation) and 250 $\mu E\ m^{-2}\ s^{-1}$.

Cultures were grown in 57 l aquaria. Temperatures were controlled using an heat exchanger system which allowed temperature control for each aquarium. Salinity was modified by dilutions of oceanic seawater to achieve 17‰. Light was provided by high intensity cool white fluorescent lights on a 12 h photoperiod. The lighting was arranged to unidirectionally enter the sides (0.18 m^2) of the aquaria adjacent to the light tubes. Light quanta fluxes was measured with a Biospherical Instrument QSL-100 Quanta meter in the interior of each aquarium, at a central point adjacent to the light exposed side. The aquaria were equipped with longitudinal air line spargers (Bubblewands^R) which were arranged to provide circulation of the algal biomass within the aquaria. The circulation caused the algal thalli to rotate from front to back of the aquaria, in circular vortices. This circulation ensures that all the plant material within the culture tank is uniformly exposed to the same light and culture conditions. Supplemental CO_2 (10%) timed with the light period was also metered into the air lines to reduce C/pH limitations. Checks for pH in the aquaria indicated pH remained between 8.2–8.8. The cultures were provided supplemental nutrients and trace metals to ensure that nitrogen was the limiting growth nutrient (Bird 1984). Nitrogen limitation and enrichment was controlled by adding or deleting NH_4Cl to ensure thallus N content above 2% for N enriched thalli. These cultures were pulse fed nutrients for a 24 h period once a week, prior to weighing and seawater replacement.

Cultures were initiated with 180 g wet weight of growing tips (2–3 cm length) from *Gracilaria* sp. Strain G-16 taken from outdoor cultures. These cultures were at the point of becoming N deficient. Material used for N enriched cultures were pulsed fed with N for 24 h prior to inoculation. The wet weight of each entire aquarium culture was determined weekly. When the culture weights doubled, the culture densities were reduced to the initial density and reinoculated with the new growth tips. After a period of 4 weeks growth in controlled culture, the biomass was harvested, weighed, dried at 60 °C, and prepared for agar extraction. Growth was determined as dry weight productivity using the area of the aquaria sides (0.18 m^2) exposed to the light. Algal material was analysed for percent carbon and nitrogen using a carbon: nitrogen analyzer.

Agar samples from all culture conditions were analysed using techniques described elsewhere (Guerin and Bird 1987). Alkaline pretreatment (1 N NaOH) of the algal biomass was used to improve gel strength. Agar yield was determined using three replicates from each of the experimental treatments. After yield determinations, the replicate agar samples were pooled for the agar chemistry and gel behavior analyses.

Agar gel behavior characteristics consisted of the following analyses (in triplicate): gel strength, melting temperature (Guerin and Bird 1987), and dynamic gelling temperature which was determined as the temperature where a permanent deformation in a liquid agar meniscus is seen when the thermometer is withdrawn. Percent sulfate of agar was determined by the turbidimetric methods described by Jackson and McCandles (1978) and percent 3,6 anydrogalactose of agar carbohydrate by the method of Yaphe and Arsenault (1965). All analyses were compared to a commercial *Gracilaria* agar obtained from TIC Gums, Inc. (New York, N. Y.).

Data from the chemical analyses and gel behavior tests were treated using averaging and variance techniques. Productivity was calculated based on the average of weekly growth measurements with the exception of data from the first week, which consistantly demonstrated prehistory effects. Two sequential replicate experimental runs of 4 weeks were performed. Multifactorial analysis of variance was used to determine effects of single treatments and degrees of interaction between the different treatments on growth, agar yield and gel strength characteristics (Steele and Torrie 1960).

Results

Many of the effects of environmental factors on productivity followed commonly observed patterns. Growth was greater with N fertilization than N starvation in comparative treatments, and with higher light levels. Thallus N levels in N enriched treatments ranged from 2.8 to 5.3%, while those in N starved treatments ranged from 1.0 to 1.8%. Thallus C levels varied only slightly, from 32.4 to 36.9%, and showed no correlation with environmental factors. A temperature of 24 °C led to greater productivity than either 32 or 15 °C. Salinities of 17‰ had little effect on productivity compared to 33‰ within comparative treatments (Table I).

Agar yield was higher in 17‰ treatments than 33‰. Agar yield also appears to be affected by temperature and thallus N. In plants grown at 32 and 24 °C, agar yield was highest in N starved material, while a reciprocal pattern was seen in plants grown at 15 °C, where agar content was higher in N enriched treatments (Table I).

Agar quality was strongly affected by the environmental factors. Highest gel strength was found for agars extracted from plants grown at 32 °C, N enriched, 33‰ seawater, followed by plants grown at 24 °C in the same N and salinity conditions. In 33‰ treatments, N enrichment led to higher gel strengths than N starved treatments, a pattern which has been

Table I. Effects of temperature, light, salinity and nitrogen loading on *Gracilaria* sp. Strain G-16 productivity, agar quality, and agar chemistry. ± = (SD)*

Light (465 μE m^{-2} s^{-1})						
Temperature	32 °C		24 °C		15 °C	
Nitrogen Loading	Enriched	Starved	Enriched	Starved	Enriched	Starved
Salinity 33‰						
Productivity	3.6 ± 1.0	2.7 ± 1.2	5.2 ± 0.9	2.9 ± 0.5	2.4 ± 0.8	0.9 ± 0.3
% N	4.5 ± 0.2	1.0 ± 0.5	3.8 ± 0.3	1.1 ± 0.4	4.2 ± 1.0	1.4 ± 0.3
% C	34.8 ± 1.6	34.8 ± 1.5	34.0 ± 3.0	35.0 ± 0.8	34.5 ± 0.7	35.0 ± 1.4
% Agar	19.8 ± 3.4	19.9 ± 4.2	17.8 ± 3.2	23.1 ± 1.9	20.0 ± 4.2	18.8 ± 3.4
Gel Strength	949 ± 174	562 ± 44	808 ± 135	722 ± 218	588 ± 33	693 ± 50
% 3,6 A,G	47 ± 5.5	50 ± 3.3	46 ± 3.3	46 ± 1.4	45 ± 3.8	45 ± 2.3
	50.9 ± 7.8	28.8 ± 12.8	39.2 ± 3.2	32.9 ± 3.2	40.8 ± 4.8	35.9 ± 7.7
Salinity 17‰						
Productivity	4.7 ± 1.5	1.3 ± 0.5	5.4 ± 1.3	3.1 ± 0.7	2.0 ± 0.5	1.4 ± 0.4
% N	3.1 ± 0.2	1.0 ± 0.2	3.4 ± 1.7	1.0 ± 0.6	3.4 ± 0.7	1.4 ± 0.4
% C	36.3 ± 1.1	36.9 ± 1.9	34.9 ± 2.5	36.5 ± 1.0	36.4 ± 1.2	36.3 ± 0.7
% Agar	21.1 ± 3.1	20.4 ± 4.9	20.3 ± 5.1	30.0 ± 6.4	25.6 ± 2.6	27.3 ± 3.2
Gel Strength	638 ± 40	661 ± 57	583 ± 89	639 ± 268	760 ± 114	657 ± 98
Gel T	46 ± 2.3	55 ± 2.6	42 ± 5.1	47 ± 2.6	43 ± 5.7	45 ± 3.3
% 3,6 A,G	39.2 ± 0.9	30.1 ± 3.5	38.1 ± 4.7	37.5 ± 4.7	36.0 ± 3.4	26.6 ± 2.4
Light (250 μE m^{-2} s^{-1})						
Salinity 33‰						
Productivity	3.3 ± 2.0	2.0 ± 0.9	4.1 ± 1.5	3.3 ± 1.3	2.4 ± 1.1	1.0 ± 0.2
% N	5.1 ± 0.9	1.2 ± 0.5	5.3 ± 0.3	1.2 ± 0.2	4.6 ± 0.4	1.8 ± 0.4
% C	34.0 ± 2.1	34.0 ± 1.9	32.4 ± 1.2	34.9 ± 0.7	33.5 ± 1.4	33.7 ± 0.9
% Agar	14.4 ± 3.4	22.5 ± 2.2	18.3 ± 6.9	23.8 ± 6.4	24.4 ± 3.8	18.3 ± 2.3
Gel Strength	965 ± 106	442 ± 13	829 ± 181	594 ± 301	799 ± 228	718 ± 123
Gel T	51 ± 1.5	53 ± 1.0	44 ± 5.3	48 ± 1.7	45 ± 2.9	44 ± 2.8
% 3,6 A,G	36.0 ± 3.4	30.4 ± 3.2	33.8 ± 3.6	34.0 ± 4.5	33.8 ± 1.3	37.4 ± 2.8
Salinity 17‰						
Productivity	3.3 ± 1.2	1.3 ± 0.5	3.5 ± 0.5	2.5 ± 1.5	2.1 ± 0.5	1.2 ± 0.2
% N	4.8 ± 0.3	1.9 ± 0.1	3.8 ± 0.5	0.8 ± 0.5	2.8 ± 0.7	1.4 ± 0.4
% C	36.1 ± 1.5	37.0 ± 0.8	34.9 ± 1.6	35.6 ± 0.9	34.9 ± 2.2	36.4 ± 0.4
% Agar	20.5 ± 2.0	23.8 ± 3.4	29.8 ± 4.3	23.6 ± 1.9	28.9 ± 0.6	23.6 ± 0.3
Gel Strength	790 ± 77	618 ± 124	611 ± 95	672 ± 90	638 ± 144	465 ± 28
Gel T	47 ± 7.7	52 ± 5.5	47 ± 4.3	44 ± 5.7	43 ± 6.8	50 ± 1.8
% 3,6 A,G	34.3 ± 3.9	28.5 ± 4.5	31.3 ± 3.2	30.3 ± 3.2	27.8 ± 2.8	26.8 ± 3.5

* Productivity measured as g · m^{-2} d^{-1}, gel strength as g · cm^{-2}, gel T in °C, % 3,6 A,G anhydrogalactose of agar carbohydrate. A commercial *Gracilaria* agar gave gel strengths of 650 ± 50, gel T of 38 °C, and 39.6% 3,6 A,G.

reported previously (Bird *et al.*, 1981). The lower salinity generally led to lower gel strength. More significantly, the effects of N enrichment on gel strength observed at 33‰ were not evident at 17‰. The percent 3,6 anhydrogalactose (of agar carbohydrate) ranged from 26 to 50%. Gel strength was correlated with percent 3,6 anhydrogalactose ($r = 0.74$, significant at $p < 0.05$). Sulfate analyses of the agar indicated levels of 2% or less (usually < 1%), and showed no correlation with gel strength (data not presented). The gel strength of *Gracilaria* sp. G-16 agars was typically higher than a commercial lot of agar, 650 g/cm^2. Agar gelling temperature fell in a range of 43 – 55 °C, and generally showed somewhat higher values for the 32 °C, N starved treatments (Table I). Agar melting temperatures were uniformly high, 99 – 100 °C for all treatments (data not presented).

Multi-factorial analysis of variance was used to examine statistical significance and interactions of treatment effects on productivity, agar yield, and gel strength. Statistical trials were attempted by combining the triplicate measurements of each experiment resulting in six entries for each of the 24 treatments. In these trials, data were bimodally distributed and heterogeneity of variances significant. Transformations of the data normalized the distribution, but homogeneity of variance could only be obtained by using the means within each treatment for the two experiments. Consequently, there were only two entries (1/experiment) for each treatment. In order to combine the two experiments, it was necessary to determine whether there was significant differences between the two experiment runs. Analysis of variance was used to determine whether there were significant differences between the two experiments due their sequential nature. The analysis indicated no significant differences between the two experimental data sets ($p < 0.01$), hence the data from the two experiments could be combined. Combining the two experiments reduced error degrees of freedom, hence the 0.10 level of significance was used.

Productivity was significantly affected by temperature, light and N enrichment as independent factors. Significant interaction occurred for temperature × N enrichment × salinity. Both higher light and N enrichment led to significantly greater productivity. Comparisons of means for temperature effects indicated that productivity at 24 °C > 32 °C > 15 °C. Agar yield was significantly affected by salinity and temperature. Agar yield was significantly higher at 17‰ than 33‰. Comparisons of means for temperature effects indicated that agar yield at (15 °C = 24 °C) > 32 °C. Significant interactions were temperature × N enrichment as well as temperature × N enrichment × light. Gel strength was significantly increased by N enrichment, with the significant interaction being temperature × salinity × N enrichment (Table II).

Table II. Statistical significance of results of multifactorial analyses of variance on the effects of temperature, light, salinity and nitrogen loading on productivity and agar quality of *Gracilaria* sp. Strain G-16. N = 2

Statistical significance		Comparison of means (Duncan's multiple range)
Productivity Transformation: log (x + 1)		
Temperature	$p < .001$	24 °C > 32 °C > 15 °C
Nitrogen	$p < .001$	
Light	$p < .091$	
Temp. × Sal. × Nit.	$P < .078$	
Agar Yield No transformation		
Salinity	$p < .001$	(15 °C = 24 °C) > 32 °C
Temperature	$p < .044$	
Temp. × Nit.	$p < .047$	
Temp. × Nit. × Light	$p < .028$	
Gel Strength Transformation: log x		
Nitrogen	$p < .015$	
Temp. × Nit. × Sal.	$p < .078$	

Discussion

The observed environmental effects on productivity of *Gracilaria sp. G-16* agree with previously reported results for similar *Gracilaria* spp., and will not be discussed in great length. Productivity was greater at the higher light intensity and with N enrichment versus starvation, effects well known in algal physiology. The higher productivities at 24 °C followed by 32 °C, then 15 °C have also been observed in outdoor cultivation of this species (LaPointe *et al.* 1984a). The lack of significant salinity effects on productivity agree with previous physiological and growth studies which demonstrate that *Gracilaria* can have broad tolerances to changes in salinity (Dawes *et al.* 1978). Salinity was only a factor when it interacted with other environmental effects (i.e. temperature and N

loading). These salinity effects were noted primarily when the thalli were stressed by the other environmental factors.

Agar yield was affected by salinity, an environmental parameter largely ignored in agar production research. Under long term, lower salinity conditions, agar deposition between cell walls might provide additional structural support for turgid cells. Agar yield was also affected by temperature, especially at higher temperatures where yield was lower. Total acid soluble carbohydrate content has been reported to also decrease with increasing temperature from 15 to 30 °C in *Gracilaria tikvahiae* (La Pointe *et al.* 1984 b). The effects of N starvation on yield were observed primarily in interactions with temperature and light. In general, agar yields were slightly higher in thalli grown under the lower light intensity, especially during N starvation. A small effect of irradiance on agar content of *Gracilaria* has also been reported by Rotem *et al.* (1986). Also, N starvation effects were most noticable at 24 °C, the temperature where productivity was highest. Previous research done in outdoor cultivation systems of N limitation effects on *Gracilaria tikvahiae* agar yield (Bird *et al.* 1981, DeBoer 1979) may reflect conditions suitable for higher productivities. Also, the extraction methods used in these studies may have resulted in floridean starch inclusions with the extracted agars, magnifying the N effects (Yaphe personal communication, Rotem *et al.* 1986). The multiple effects and interactions of environmental factors on agar yield strongly suggest that agar content is linked to both rate of total carbohydrate metabolism as well as partitioning of different kinds of carbohydrates to meet physiological requirements. Nitrogen and light have been shown to play a regulatory role in carbon partitioning into agar and starch fractions in *Gelidium coulteri* Harv. (Macler 1986).

The correlation of nitrogen enrichment with increasing agar gel strength has been reported previously (Bird *et al.* 1981, Craigie *et al.* 1984). It is notable, however, that this N effect on gel strength disappeared at the reduced salinity. There was little apparent difference in gel strengths between comparative treatments at 17‰ differing only in N loading. The higher gel strengths found with N enrichment at 33‰ suggest some role of agar in salinity acclimatization. Since N loading reduces overall carbohydrate content in *Gracilaria* (Bird *et al.* 1982), a more complex agar might be required at higher salinities, ameliorating reductions in osmotic oligosaccharide content. The interactions of temperature with gel strength and the highest gel strength at 32 °C versus 24 °C indicate that this property is independent of algal productivity.

There was also a higher gelling temperature in the nitrogen starved, 32 °C treatments. While this study did not determine methoxylation content of the agars, Craigie *et al.* (1984) have found higher levels of 4-O-methyl-L-galactose in agars extracted from N deficient plants, but lower 6-O-methyl-D-galactose. Differences in these patterns might contribute to the gelling temperature differences found in this study. Future experiments are planned for more detailed analyses of selected agar samples from this report.

The degree to which *Gracilaria* sp. Strain G-16 shows these patterns of productivity, agar content and agar quality in outdoor cultivation systems will be reported elsewhere. These patterns also correlate with those found in Taiwanese pond culture of *Gracilaria*, where highest gel strengths were found in the summer (temperatures greater than 30 °C) under conditions of high light. Neither pond salinity or thallus N content were reported (Wang and Yang 1980). Likewise, similar patterns of high summer gel strengths have been observed for *Gracilaria* spp. in California and Rhode Island (Abbott 1980, Asare 1980). In a study of coexisting *Gracilaria* spp., Hoyle (1978) found *G. bursapastoris* (S. Gmelin) Silva had bimodal patterns of high gel strengths during both summer and winter months in Hawaii, and for *G. coronopifolia* J. Ag., continually through winter and early summer. Since only thallus N and seawater N concentrations were reported, it is uncertain whether the gel strength patterns are due to species specific differences or some unreported environmental factors. Clearly, both more culture work with additional species and increased monitoring in environmental studies will be required to clarify the causes of the observed variability in agar quality.

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