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Effects of environmental factors and metal ions on growth of the red alga *Gracilaria chorda* Holmes (Gracilariales, Rhodophyta)

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Abstract

Gracilaria is a potentially valuable source of marine biopolymers such as proteins and polysaccharides. In order to select suitable culture conditions, growth and tolerance of *Gracilaria chorda* Holmes from Shikoku Island in southwest Japan were investigated under variations of temperature (5–30 °C), photon irradiance (20–120 μmol photons m^{-2} s^{-1}), and photoperiod (12:12 h, 14:10 h light:dark regime) in a unialgal culture. *Gracilaria chorda* showed wide tolerances for all factors investigated, which is characteristic of eurythermal species. Maximum growth was observed at 18–24 °C. The optimum photon irradiance for algal growth was 60–120 μmol photons $m^{-2}s^{-1}$. Instead of using ordinary sea salt (NaCl) to prepare artificial seawater, ultra pure salt was adopted. *Gracilaria chorda* grew faster in artificial seawater made with ultra-pure salt than that made with ordinary sea salt, probably because the former medium was clear, while the latter was milky. Effects of some metal ions on the growth were tested with artificial seawater. Iron ions affected algal growth, but cobalt ions did not. This study enables us to determine suitable culture conditions for *G. chorda*. A scaled-up 30 l culture of *G. chorda* under such conditions was successful.

Introduction

The red algal genus *Gracilaria* is harvested and cultured on a commercial scale in many countries because it has considerable economic importance as an agarophyte. The total annual *Gracilaria* production in the world increased to more than 89,000 t, including 50,000 t of cultured production, in 1995. *Gracilaria* plants are also used as sources of traditional seaweed salad in Japan and feed for shellfish (abalone) in many countries. Recently some bioactive substances from *Gracilaria* spp. have been extracted and reported (Kakita et al., 2003).

Seasonality affects agar quality in various *Gracilaria* species (Oza, 1978; Hoyle, 1978, Whyte et al., 1981; Lahaye & Yaphe, 1988; Bird & Ryther, 1990, Luhan, 1992; Yenigul, 1993). For obtaining *Gracilaria* biopolymers of constant quality and quantity, a cultured strain is likely to be more suitable than a wild one.

Environmental factors including temperature, salinity and light play an important role in the growth, reproduction and distribution of marine algae (Gessner, 1970; Gessner & Schramm, 1971; Lüning, 1981; Lobban & Harrison, 1994). Temperature requirements for survival and growth of *Gracilaria* species have been extensively studied (Bird et al., 1979; Laing et al., 1989; Yokoya & Oliveira, 1992). Some *Gracilaria* species require less than 100 μmol photons $m^{-2}s^{-1}$ for optimal growth (Bird et al., 1979; Beer & Levy, 1983), while others require higher irradiance (Lapointe, 1981; Lapointe et al., 1984). However, few data on the effects of such environmental factors on the growth of Japanese *Gracilaria* species in controlled conditions are available (Orsco & Ohno, 1992; Chirapart et al., 1994; Yokoya et al., 1999). The aim of this study is to characterize the physiological responses of *G. chorda* to temperature, irradiance and photoperiod, assessing tolerance

and optimal conditions for growth in unialgal cultures.

Several metals such as iron ions are regarded as essential components for algal growth (Matsunaga et al., 1998). Thus, effects of some metal ions on the growth were also tested in artificial seawater. We therefore tested and selected suitable culture conditions and prepared a scaled-up model of an artificial seawater system for *Gracilaria* cultivation.

Materials and methods

Stock unialgal culture strains

Stock unialgal cultures of the red alga, *G. chorda*, were started from spores released by fertile plants that were harvested in June 1998 from Seto Inland Sea off the coast of Tokushima city, Tokushima Pref., Japan. The establishment of unialgal strains followed the methods of Yamamoto and Sasaki (1987). Stock unialgal cultures were incubated with aeration at 20 °C, in a 14:10 h light:dark regime, in salinity of about 33‰, at photon irradiances of just 40 μmol photons $m^{-2}s^{-1}$ under cool-white lamps to inhibit growth in storage. Provasoli enriched seawater (PES) (Provasoli, 1968) was made using sterilized Yashima surface seawater (Yashima, Kagawa Pref., southwest Japan) without the addition of vitamins, and this medium was used for the stock unialgal culture (Yamamoto & Sasaki, 1987). Medium renewal was carried out bi-weekly.

Temperature, irradiance, and photoperiod

The growth of *G. chorda* was compared among various culture conditions. Variations of temperature (5–30 °C) and irradiance (20–120 μmol photons $m^{-2}s^{-1}$) were tested. Algal growth rates in long-day (14:10 h light:dark) regime were compared with short-day (12:12 h light:dark) regime at three different temperatures (10, 20, and 30 °C). Irradiances were measured with a LI-250 photometer equipped with a LI-193SA spherical quantum sensor (LI-COR, Inc). The controlled conditions were the same as described above for stock unialgal cultures except that irradiance was increased to 60 μmol photons $m^{-2}s^{-1}$ to promote growth.

For each experiment, five replicates of six apical segments (5 mm long and approximately 1.4 mg fresh weight) cut from the stock unialgal culture strains were inoculated into 200 mL conical flasks containing 200 mL of PES. The measurement of total fresh weight of the six apical fragments and the renewal of culture media were carried out weekly in a clean booth. The data on algal growth rate, which were measured from day 14 to day 21 of cultivation, were analyzed by one-way ANOVA (for temperature and irradiance experiments) followed by Tukey's multiple comparison test (Winer et al., 1991) or t-test (for photoperiod experiment). Relative growth rates R were calculated using the formula:

$$R = [\ln(W_t) - \ln(W_0)]t^{-1},$$

where W_0 is the initial fresh weight, W_t is the fresh weight after t days and t is the number of days (Kain, 1987). Growth rate (%) was defined as $R \times 100$.

Artificial seawater

Ordinary sea salt (sodium chloride; NaCl) contains magnesium and other ions as contaminants (Niino et al., 1993). Some adsorbents, such as chelate resins and zeolites, are known to adsorb magnesium ions. Thus, an ultra pure salt (NaCl) was purified from ordinary sea salt by passing a 5.844 % solution of ordinary sea salt through a column of $Na_6Al_6Si_{30}O_{72}{\cdot}24H_2O$-type zeolite (clinoptilolite: Sun-Zeolite Co., Ltd, Akita Pref., Japan) at 27 °C. The solution passed was re-crystallized only once and dried to obtain an ultra pure salt as a white powder. Atomic absorption spectrochemical analysis of the ultra pure salt showed that it contained only about 0.00015 % (w/w) of magnesium ions on average. On the other hand, ordinary sea salt (before adsorption treatment) contained about 0.00753 % (W/W) of magnesium ions.

Artificial seawater solids, Sample A, were made up of 548 g of ultra pure salt (NaCl), 250 g of $MgCl_2{\cdot}6H_2O$, 92.5 g of Na_2SO_4, 35.0 g of $CaCl_2{\cdot}2H_2O$, 15.8 g of KCl, 4.5 g of $NaHCO_3$, 2.25 g of KBr, 0.75 g of H_3BO_3, 0.25 g of $SrCl_2$, 0.13 mg of $FeCl_3{\cdot}6H_2O$, 8.75 mg of glycerophosphate disodium salt pentahydrate ($C_3H_7Na_2O_6P{\cdot}5H_2O$), and 4.0 mg of $NaNO_3$. Artificial seawater solids, Sample B, were a similar composition to Sample A, with the exception of the substitution of ordinary sea salt (NaCl) for ultra pure salt. After mixing of the components mentioned above, several batches of each sample of artificial seawater solids were sealed in laminated bags and stored for 30 days at 20 °C.

After storage for 30 days at 20 °C, 40 g of each sample of artificial seawater solids were dissolved in 1 L of distilled water. The transparency of each

artificial seawater solution was measured as absorbance at 660 nm. The average absorbances of artificial seawater Solution A and B were 0.0005 and 0.0028, respectively ($n = 6$). Artificial seawater Solution A was more transparent than Solution B ($t = -6.139$, $p < 0.001$). The artificial seawater Solution A was named AIST-01.

The growth of *G. chorda* in the artificial seawater Solutions A and B was compared. Each type of artificial seawater solids was dissolved in 25 L of distilled water and these were used as algal culture media. The culture conditions with aeration were set at a temperature of 20 °C, an irradiance of 80 μmol photons $m^{-2}s^{-1}$, a light:dark regime of 14:10 h, in a salinity of about 33‰. Medium renewal was carried out weekly. The data on algal growth rate, which were measured from day 14 to day 21 of cultivation, were analyzed by *t*-test ($n = 5$).

Metal ions

Fe-free artificial seawater solids, Sample C, was made of the same compounds as Sample A, but lacking $FeCl_3 \cdot 6H_2O$. Forty grams of the artificial seawater solids, Sample C, were dissolved in distilled water, to which was added a predetermined concentration of $FeCl_3 \cdot 6H_2O$ or $CoCl_3 \cdot 6H_2O$ solution, and volume was adjusted to 1 L with distilled water. Various concentrations of $FeCl_3 \cdot 6H_2O$ (0.5, 5, 50 and 500 μg L^{-1}) or $CoCl_3 \cdot 6H_2O$ (0.05, 0.5, 5, and 50 μg L^{-1}) were tested in artificial seawater Batch C. The culture conditions were the same as described above for the salts experiment. Medium renewal was carried out weekly. Data on algal growth rate, which was measured from day 14 to day 21 of cultivation, were analyzed by one-way ANOVA ($n = 3$).

Thirty liter scale cultivation

Artificial seawater Solution A (AIST-01) containing 50 μg L^{-1} of $FeCl_3 \cdot 6H_2O$ was named AIST-01-Fe50. Three 30 L tanks were prepared, each containing 30 L of AIST-01-Fe50 and one algal specimen (fresh weight 1.16 g, 1.30 g, and 1.41 g). Three other 30 L tanks were prepared, each containing 30 L of Yashima surface seawater and one algal specimen (fresh weight 1.10 g, 1.22 g, and 1.37 g). Thirty liter volume unialgal cultivations were maintained simultaneously in three growth chambers, each accommodating two tanks (Koito Seisakusho, Co., Tokyo, Japan, model SNIRI-100) with aeration. The cylindrical tanks used were transparent polycarbonate and had an inner diameter of 350 mm and a height of 460 mm. The culture conditions used were the same as described above for the salts experiment. Medium renewal was carried out weekly. Algal growth rates cultivated in AIST-01-Fe50 were compared with those in surface seawater. The data concerning algal growth rate, which were measured from day 14 to day 21 of cultivation, were analyzed by t-test ($n = 3$).

Results

Temperature had a significant effect on the growth rate of *G. chorda* over three weeks ($n = 5$, $F = 96.662$, $p < 0.001$). The optimum temperature for the growth of *G. chorda* was 18–24 °C. The growth rate of *G. chorda* ranged from 0.13% d^{-1} at 5 °C to 12.3% d^{-1} at 20 °C (Figure 1). The optimum irradiance for the growth of *G. chorda* was 60–120 μmol m^{-2} s^{-1} (Figure 2). Irradiance had a significant effect

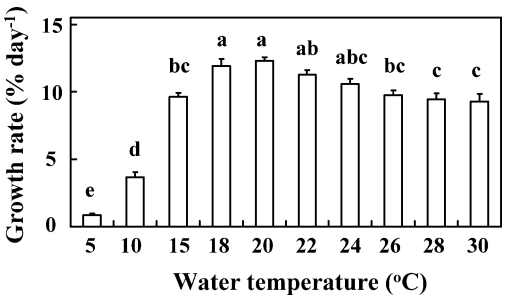

Figure 1. Effect of water temperature on algal growth. Growth rates of *G. chorda* cultivated for three weeks at different temperatures, constant photon irradiance (60 μmol photons m^{-2} s^{-1}) and photoperiod (14:10 h light:dark regime). Each data point is the mean of five replicates (means ± SE). Bars marked with the same letter are not significantly different according to Tukey's multiple comparison test ($p = 0.05$).

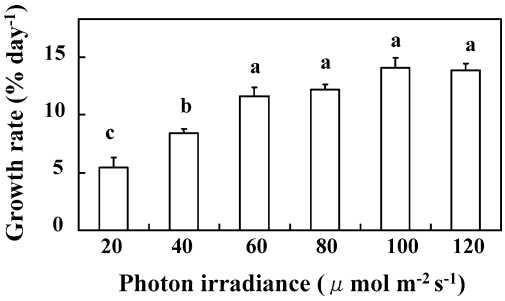

Figure 2. Effect of photon irradiance on algal growth. Growth rates of *G. chorda* cultivated for three weeks at different photon irradiances, constant temperature (20 °C) and photoperiod (14:10 h light:dark regime). Each data point is the mean of five replicates (means ± SE). Bars marked with the same letter are not significantly different according to Tukey's multiple comparison test ($p = 0.05$).

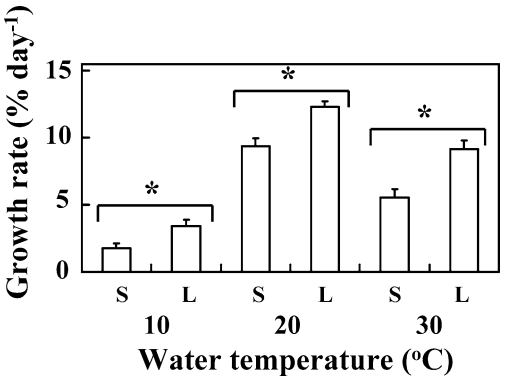

Figure 3. Effect of photoperiod on algal growth. Growth rates of *G. chorda* cultured for three weeks at different photoperiods and temperature (10, 20, or 30 °C) but constant photon irradiance (80 μmol photons $m^{-2}s^{-1}$). Each data point is the mean of five replicates (means ± SE). L: photoperiod is14:10 h light:dark regime. S: photoperiod is12:12 h light:dark regime. *Indicates that growth under a short-day (SD) was statistically different to growth under a long-day (LD) regime.

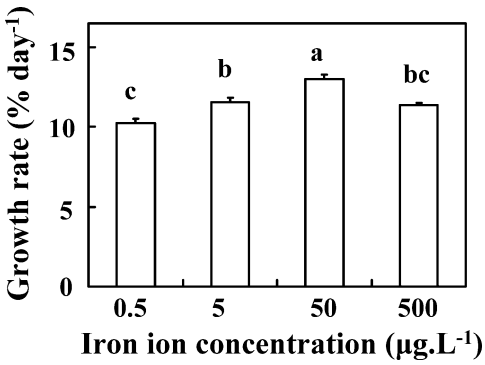

Figure 4. Effect of iron ions on algal growth. Growth rates of *G. chorda* cultivated for three weeks in artificial seawater Solution A containing different iron ion concentrations (0.5, 5, 50, and 500 μg l^{-1}), constant temperature (20 °C), irradiance (80 μmol photons $m^{-2}s^{-1}$) and photoperiod (14:10 h light:dark regime). Each data point is the mean of three replicates (means ± SE). Bars marked with the same letter are not significantly different according to Tukey's multiple comparison test ($p = 0.05$).

on growth rates of *G. chorda* over three weeks ($n = 5$, $F = 25.948$, $p < 0.001$). Growth rates varied from 5.44% d^{-1} at 20 μmol photons $m^{-2}s^{-1}$ to 14.1% d^{-1} at 100 μmol photons $m^{-2}\,s^{-1}$. Photoperiod also had a significant effect on growth rates of *G. chorda* over three weeks (10 °C: $n = 5, t = -2.648, p < 0.05$, 20 °C: $n = 5, t = -4.305, p < 0.005$, 30 °C: $n = 5, t = -4.131, p < 0.005$). Growth rate in a 14:10 h light:dark regime was greater than that in a 12:12 h light:dark regime (Figure 3). From the results of temperature and light experiments, suitable culture conditions were set at a temperature of 20 °C, an irradiance of 80 μmol $m^{-2}s^{-1}$, and a light:dark regime of 14:10 h.

The growth rates of the algae over three weeks of cultivation in artificial seawater A and B were 13.1% d^{-1} and 11.8% d^{-1}, respectively. Differences in the culture media had a significant effect on *G. chorda* growth rate ($n = 5, t = 4.555, p < 0.005$). The average fresh weights of algae cultured in artificial seawater A and B were 62.6 mg, and 52.1 mg, respectively.

$FeCl_3 \cdot 6H_2O$ concentration in artificial seawater A had a significant effect on growth rates of *G. chorda* ($n = 3$, $F = 18.467$, $p < 0.005$). Maximum algal growth was observed in a $FeCl_3 \cdot 6H_2O$ concentration of 50 μg L^{-1} (Figure 4). On the other hand, $CoCl_3 \cdot 6H_2O$ concentration did not affect the growth rate of the alga (data not shown).

On 30 L scale culture, the growth rates of algae over three weeks of cultivation in AIST-01-Fe50 medium and Yashima surface seawater were 9.2% d^{-1} and 1.6% d^{-1}, respectively. The artificial seawater solution (AIST-01-Fe50) accelerated algal growth of *G. chorda* over Yashima surface seawater ($n = 3, t = 29.303, p < 0.001$). After three weeks cultivation, the average fresh weights of algae cultured in AIST-01-Fe50 medium and Yashima surface seawater were 9.37 g/tank and 2.95 g/tank, respectively ($n = 3$).

Discussion

Gracilaria chorda tolerated a wide range of temperature variation, from 5 to 30 °C. The broad temperature tolerance of *G. chorda* is in accordance with observations that *Gracilaria* species from temperate waters tend to be eurythermal (Bird et al., 1979). Maximum growth of *G. chorda* was observed at 18–24 °C, and similar results have been observed in *G. tikvahiae* McLachlan (Bird et al., 1979), *G. chilensis* (Laing et al., 1989 as *G. sordida* Nelson); Yokoya and Oliveira, 1992, *Gracilaria* sp. (chorda-type) (Chirapart et al., 1994), and *G. vermiculophylla* (Yokoya et al., 1999). *Gracilaria chorda* has temperature responses similar to *G. tikvahiae* and *G. vermiculophylla*, growing well in temperatures as high as 30 °C, and tolerating low temperatures without necrosis of the thallus (Bird et al., 1979; Yokoya et al., 1999).

Maximum growth of *G. chorda* occurred at irradiances of 60–120 μmol photons $m^{-2}s^{-1}$, and these responses probably influence its intertidal distribution along the Japanese coast. These irradiances are

higher than those observed for *G. tikvahiae*, which was light-saturated for growth at less than 50 μmol photons $m^{-2}s^{-1}$, and became necrotic at about 65 μmol photons $m^{-2}s^{-1}$ (Bird et al., 1979). On the other hand, optimum growth in higher light levels was reported in *G. foliifera v. angustissima* (Harvey) Taylor (Lapointe, 1981) and *G. chilensis* (Laing et al., 1989, as *G. sordida*). The results of temperature and irradiance experiments show that *G. chorda* is tolerant of wide variations in temperature and irradiance. The result of the photoperiod experiment indicates that *G. chorda* grows well under a long-day photoperiod, similar to that in their natural environment. These findings show that *G. chorda* could be cultivated economically in temperate brackish regions.

In Japan, most salt (NaCl) is purified from seawater. The final step in Japanese salt manufacturing is drying of recrystallized salt slurry at 130 °C. $MgCl_2 \cdot 6H_2O$ (magnesium chloride hexahydrate), which is adsorbed to the surface of NaCl crystals, changes to MgOHCl (basic magnesium chloride: magnesium hydroxide chloride) during heat treatment at temperatures higher than 110 °C. (Niino et al., 1992). After heat treatment, MgOHCl is resolved with moisture in the air, changing to $Mg(OH)_2$ (magnesium hydroxide), and finally to basic magnesium chloride [$Mg_2(OH)_3Cl.4H_2O$ (dimagnesium trihydroxide chloride tetrahydrate)] or [$Mg_3(OH)_5Cl \cdot 4H_2O$ (trimagnesium pentahydroxide chloride tetrahydrate)]. Basic magnesium chloride induces production of $CaCO_3$ (Niino et al., 1993). $CaCO_3$ is insoluble and reduces the transparency of a solution. Artificial seawater containing $CaCO_3$ has disadvantages of low transparency and the promotion of adhesion of insoluble $CaCO_3$ to the algal surface. These two disadvantages inhibit algal growth.

Because the ultra pure salt lacks magnesium ions, the artificial seawater Sample A (ultra pure salt-based) was more transparent than artificial seawater Sample B (ordinary sea salt-based): this may explain the faster growth in A. The high transmission of the seawater prepared with ultra pure salts seems to be effective for acceleration of algal growth. Because low light transmission in the medium results in a wide distribution of irradiation in a culture tank in seawater prepared with non-purified salts, algal growth rates are likely to remain lower than those in seawater prepared with ultra pure salts, even if the light intensity is increased. It may be more effective with large scale tanks.

Commercial scale cultivation of algae in tanks has the advantage of only minor biological contaminants compared with those in the field. Thus, high quality algae are obtained from tank culture. One of the biggest problems in commercial scale culture is that algal growth rate declines when algal density increases. Most commercial scale cultures aim to obtain a lot of cultured algae rapidly. Optimum irradiance and nutrients for algal growth throughout the tank are necessary for rapid growth and high density cultivation. Artificial seawater Solution A (ultra pure salt-based) was more transparent than Solution B (common salt-based). Components of artificial seawater can be manipulated to provide a suitable medium for each algal species. Although we have completed only a laboratory-scale manufacturing process for ultra pure salt, with a yield of 20 kg, the establishment of a large manufacturing process for ultra pure salt would be essential for its application to commercial scale culture. This would enable production of an artificial seawater solution useful for algal cultivation, though some limitations may remain to be solved.

Conclusions

This study enabled us to identify the optimum temperature and light conditions for cultivating *G. chorda*. Using artificial seawater also enabled the study of the effects of Fe and Co ions (micro-nutrients) on algal growth. Artificial seawater is useful for (1) obtaining growth responses to micro-nutrients, (2) maintaining constant quality, (3) growing axenic cultures, (4) cultivating algal strains that are susceptible to microorganisms. A 30 L scale culture of *G. chorda* under such conditions was successful. Ultra pure salt was superior to ordinary sea salt as a component of artificial seawater solids for algal growth.

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