J. Phycol. **39,** 617–623 (2003)

CULTURE OF THE TERRESTRIAL CYANOBACTERIUM, *NOSTOC FLAGELLIFORME* (CYANOPHYCEAE), UNDER AQUATIC CONDITIONS[1]

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Both colonies and free-living cells of the terrestrial cyanobacterium, *Nostoc flagelliforme* (Berk. & Curtis) Bornet & Flahault, were cultured under aquatic conditions to develop the techniques for the cultivation and restoration of this endangered resource. The colonial filaments disintegrated with their sheaths ruptured in about 2 days without any desiccating treatments. Periodic desiccation played an important role in preventing the alga from decomposing, with greater delays to sheath rupture with a higher frequency of exposure to air. The bacterial numbers in the culture treated with seven periods of desiccation per day were about 50% less compared with the cultures without the desiccation treatment. When bacteria in the culture were controlled, the colonial filaments did not disintegrate and maintained the integrity of their sheath for about 20 days even without the desiccation treatments, indicating the importance of desiccation for *N. flagelliforme* to prevent them from being disintegrated by bacteria. On the other hand, when free-living cells obtained from crushed colonial filaments were cultured in liquid medium, they developed into single filaments with sheaths, within which multiple filaments were formed later on as a colony. Such colonial filaments were developed at 15, 25, and 30° C at either 20 or 60 μmol photons·m^{-2}·s^{-1}; colonies did not develop at 180 μmol photons·m^{-2}·s^{-1}, though this light level resulted in the most rapid growth of the cells. Conditions of 60 μmol photons·m^{-2}·s^{-1} and 25° C appeared to result in the best colonial development and faster growth of the sheath-held colonies of *N. flagelliforme* when cultured indoor under aquatic conditions.

Key index words: **blue-green algae; colonial filament; cyanobacteria; desiccation; *Nostoc flagelliforme*; sheath disintegration; single filament**

Abbreviation: **PFD, photon flux density**

The terrestrial cyanobacterium, *Nostoc flagelliforme,* as previously described (Gao 1998), is an economically significant, terrestrial, macroscopic, filamentous species that is distributed on arid or semiarid steppes of northwestern parts of China (also in Algeria, Czechoslovakia, France, Mexico, Mongolia, Morocco, Russia, Somalia, and the United States). It has been used as a herbal ingredient in China and in Japan and as a food delicacy by the Chinese for hundreds of years. Increased market demands that are completely dependent on collection from natural populations and overexploitation are lessening the *N. flagelliforme* resources and seriously damaging vegetation in its growing areas with deterioration of the environment, causing sandstorms of increasing scale (You 2000), the effects of which have even been recognized in Japan (Zhang and Lu 1999). Therefore, *N. flagelliforme* should be cultivated to meet its market demand and to conserve the endangered resource and the environment.

A number of studies have been performed on the ecology, physiology, biochemistry, and culture of *Nostoc* species (Dodds et al. 1995, Gao 1998, Potts 2000). Studies showed that *N. flagelliforme,* after rehydration, required light and potassium to reactivate photosynthesis (Gao et al. 1998a, Qiu and Gao 1999), showed unaffected photosynthetic efficiency until after 50% of water loss (Gao et al. 1998b), and demanded higher CO_2 concentrations to maintain positive net photosynthesis when highly desiccated (water loss >70%) (Qiu and Gao 2001). When submerged, this alga can use bicarbonate as a photosynthetic inorganic carbon source (Gao and Zou 2001). The bicarbonate transport in cyanobacteria is thought to involve an Na^+-dependent process (Bhaya et al. 2000).

Methods of cultivating this alga have been examined both in laboratory and in field. When the alga collected from nature was cultured in an aqueous medium, it grew little and then disintegrated (Zhu et al. 1982, Qian et al. 1989); when exposed to air with enriched CO_2 and periodic watering, it grew by 31% in 15 days (Gao and Yu 2000). The daily net photosynthetic production varied by 132–1280 μmol CO_2·g (dry wt)$^{-1}$, or 0.64%–6.14% (dry wt.) when *N. flagelliforme* was cultured in air with varied levels of CO_2 and water supply (Qiu and Gao 2002). However, field observation recognized only 6% elongation over a year (Dai 1992). These studies presented important data in estimating the growth of *N. flagelliforme* but have not led to any successful cultivation.

This study aimed to develop the techniques for the cultivation of this economic but endangered cyanobacterium. First, the algal filaments (colonies) were directly cultivated under aquatic conditions and effects of desiccation during the culture estimated. The methods of producing the "seeds" (free-living cells or developed fil-

[1] Received 29 January 2002. Accepted 19 February 2003.
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aments) and the process of formation of *N. flagelliforme* colonies from unicells were then investigated.

MATERIALS AND METHODS

Materials and treatments of samples. Nostoc flagelliforme Born et Flash was collected in 1999 from Guyuan prefecture of Ning Xia Province and stored dry at room temperature before use in experiments. Colonial filaments were selected from the alga mats and rinsed with distilled water to avoid further contamination before culture under bacteria-exposed or controlled conditions. The surfaces of selected filaments for the bacteria-controlled culture were sterilized by submerging them in 75% alcohol for 30–60 s and then rinsing five to seven times with aseptic water (Chen and Cai 1988). This treatment reduced the number of bacteria in the initial phase of culture by 95%–97% compared with the control treatment. Physiological activity of the colonial filaments was reactivated by maintaining them in BG-11 medium (Stanier et al. 1971) at 25° C and a photon flux density (PFD) of 40 μmol photons·m^{-2}·s^{-1} for 9–10 h (Gao et al. 1998a). The cultures were aerated directly with ambient air or indirectly through a filter (0.2 μm pore size, Cole-Parmer, Vernon Hills, IL, USA) for the bacteria-exposed and control treatments, respectively.

Culture of natural colonial filaments. The colonial filaments were cultured at 25° C and 140 μmol photons·m^{-2}·s^{-1} (14:10-h light:dark cycle) in HGZ medium (Song and Liu 1996), which has soil broth in addition to the otherr components of BG-11 and, and gave rise to better growth in preparative experiments. Five colonial filaments (7–10 cm long, 0.1 cm wide) were placed in 400 mL of aerated medium in a 500-mL flask maintained in a plant growth chamber (EF7, Conviron, Winnipeg, Manitoba, Canada). During culture, filaments were taken out of the media and desiccated at frequencies varying from zero to seven times for 30 min each time or at varied time intervals of 0–3.5 h for one exposure per day. There were five duplicates for each exposure. The desiccating treatments were carried out on a super-purifier clean bench (4 Foot Purifier Clean Bench, Labconco, Kansas City, MO, USA) at 30° C and 15 μmol photons·m^{-2}·s^{-1}. The wet samples lost 90%–95% or 93%–97% of their water after each 30-min or 3.5-h exposure, respectively. The time when the sheath of the filament was ruptured was defined as disintegration time; sheath rupture was examined microscopically (BX 50, Olympus, Tokyo, Japan). The number of bacteria in cultures with different treatments was then determined according to Zu et al. (1993). Culture medium (0.2 mL) was striped on a brewis-peptone-agar plate with a sterilized glass rod. Bacterial colonies on each plate were counted after 24 h incubation at 37° C. Triplicate plates were made for each treatment.

Culture of free-living cells. Free-living cells were obtained from colonial filaments of *N. flagelliforme.* A colonial filament, after sterilization and physiological reactivation as described as above, was sandwiched between two sterilized slides, crushed slightly on the clean bench, and washed into sterilized HGZ medium. Unicell or trichomes were separated from the supernatant and transferred into 300 mL HGZ medium and cultured at 15, 25, and 30° C at three levels, respectively, of PFD, 20, 60, and 180 μmol photons·m^{-2}·s^{-1} (14:10-h light:dark cycle). Trip-

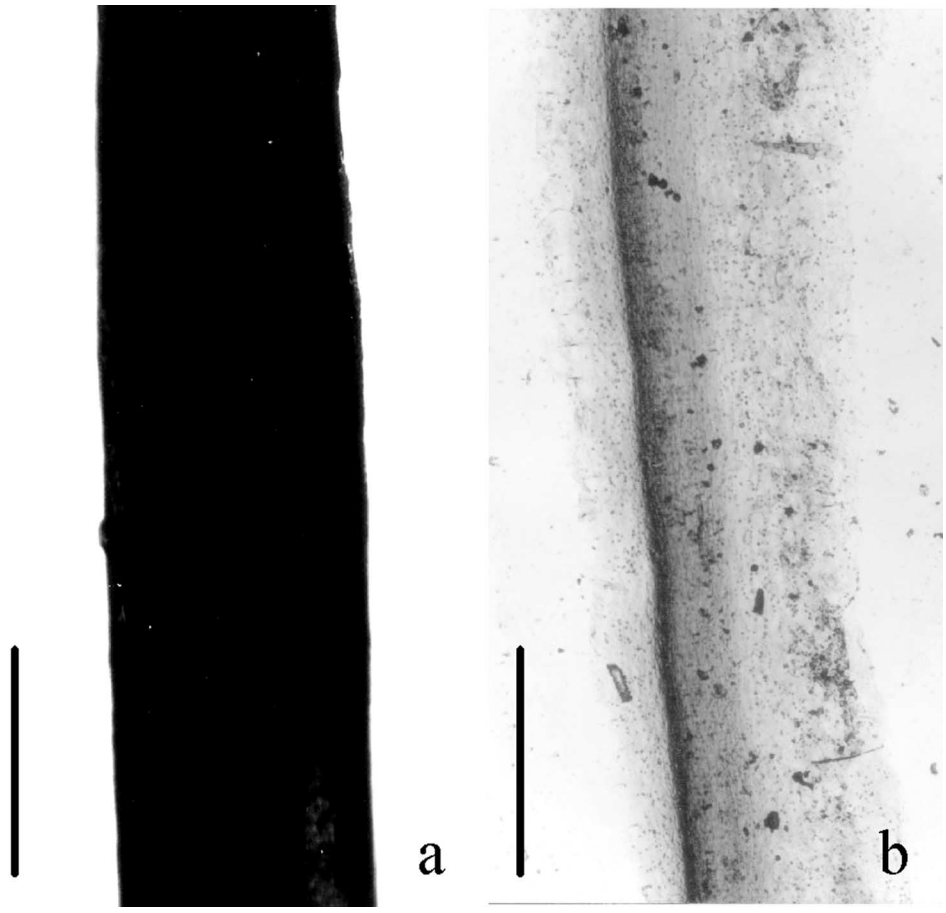

FIG. 1. Sheath integrity (a) and sheath rupture (b, disintegration) in *Nostoc flagelliforme.*

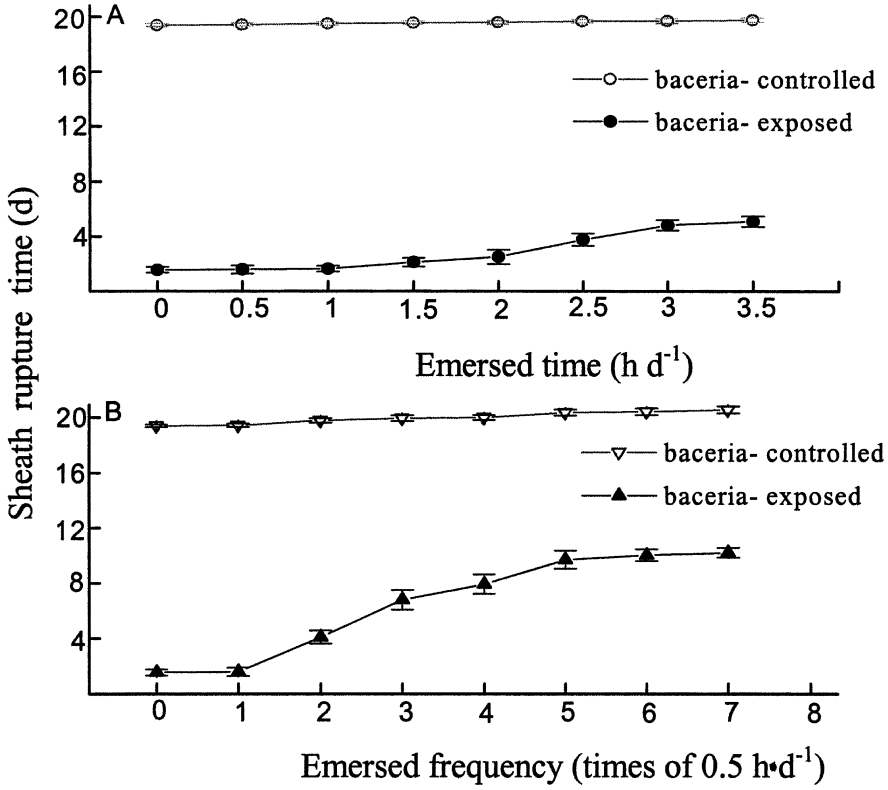

FIG. 2. Effects of desiccation time (A, one exposure per day) and frequency (B, 0.5 h for each exposure) on the disintegration (sheath rupture) of *Nostoc flagelliforme* colonies under bacteria-controlled or -exposed aquatic conditions. Data are means of five samples ± SD.

licate cultures were maintained under each of the nine conditions of light and temperature.

Determination of growth rate. To investigate the growth rate, a single colony or filaments with sheath formed from the unicells were transferred into flasks with 200 mL medium and cultured at 20, 60, or 180 μmol photons·m^{-2}·s^{-1} and 25° C for 13 days. The resulting biomass was collected on a filter paper, dried at 80° C for 12 h, and then cooled in a desiccator before weighing with an electronic balance (Libror Aeu-210, Shimadzu, Kyoto, Japan). Relative growth rate (RGR, %·d^{-1}) was calculated according to the following formula:

$$\mathrm{RGR} = 100 \ln (D_t/D_0)^{-1} \quad (1)$$

where D_0 is initial dry weight and D_t is the dry weight after t number of days. To minimize the measuring error of the small amount of the initial biomass, filaments of 1.23 dry mass in 9 L medium were equally divided into nine parts and collected on filter papers and then dried and weighed.

RESULTS

In the bacteria-exposed cultures, an increased frequency or duration of desiccation postponed disintegration of the *N. flagelliforme* colonies. The sheath of the alga's colonial filaments ruptured (Figs. 1 and 2) in 1–2 days without desiccation (0 times·d^{-1}) in about 10 days with seven continual emersions per day. One treatment of desiccation for 3.5 h per day delayed sheath rupture until 5 days. Seven desiccation treatments for 0.5 h each were equivalent to one treatment for 3.5 h per day in terms of exposure duration but significantly delayed the alga's disintegration ($P < 0.01$, t-test). Thus, desiccation with either treatment appeared to have played an important role in preventing alga disintegration. In bacteria-controlled cultures, the colonial filaments of *N. flagelliforme* disintegrated in about 20 days independent of desiccation frequency and time (Fig. 2).

The number of bacteria in the cultures differed with different treatments (Table 1). More bacteria were formed in the bacteria-exposed cultures compared with bacteria-controlled cultures on the day when the sheath was completely disintegrated. Without desiccation treatment, the number of bacteria colonies in the bacteria-exposed culture was almost five times that in the bacteria-controlled culture ($P < 0.01$, t-test). Sheath rupture time in the former was about 19 days earlier than in the latter, implying a role played by bacteria in the early-phase disintegration of the sheath. In bacteria-controlled cultures (Table 1), when the sheath

TABLE 1. Bacterial abundances in the liquid media of bacteria-exposed or bacteria-controlled cultures of *Nostoc flagelliforme.*

Culture conditions	Desiccation treatments		
	0	7 × 0.5 h·d^{-1}	1 × 3.5 h·d^{-1}
Bacteria exposed	4792 ± 192	2598 ± 160	3111 ± 96.5
Bacteria controlled	1025 ± 163	728 ± 70	826 ± 110

Number of bacterial colonies were estimated on solid agar plates (64 cm^{-2} each) at 37° C after 24 h incubation and then converted to cells per mL in the original suspension. Values are means ± SD ($n = 3$).

was completely ruptured, bacteria in cultures with seven desiccating exposures per day were 46% less compared with nondesiccated cultures ($P < 0.01$, *t*-test), implying a bacteria-inhibiting effect of desiccation.

The free-living unicells obtained from crushed colonial filaments developed into single filaments with sheaths, which later formed multiple filaments (Fig. 3). When cultured at 15° C and 20 μmol photons·m^{-2}·s^{-1}

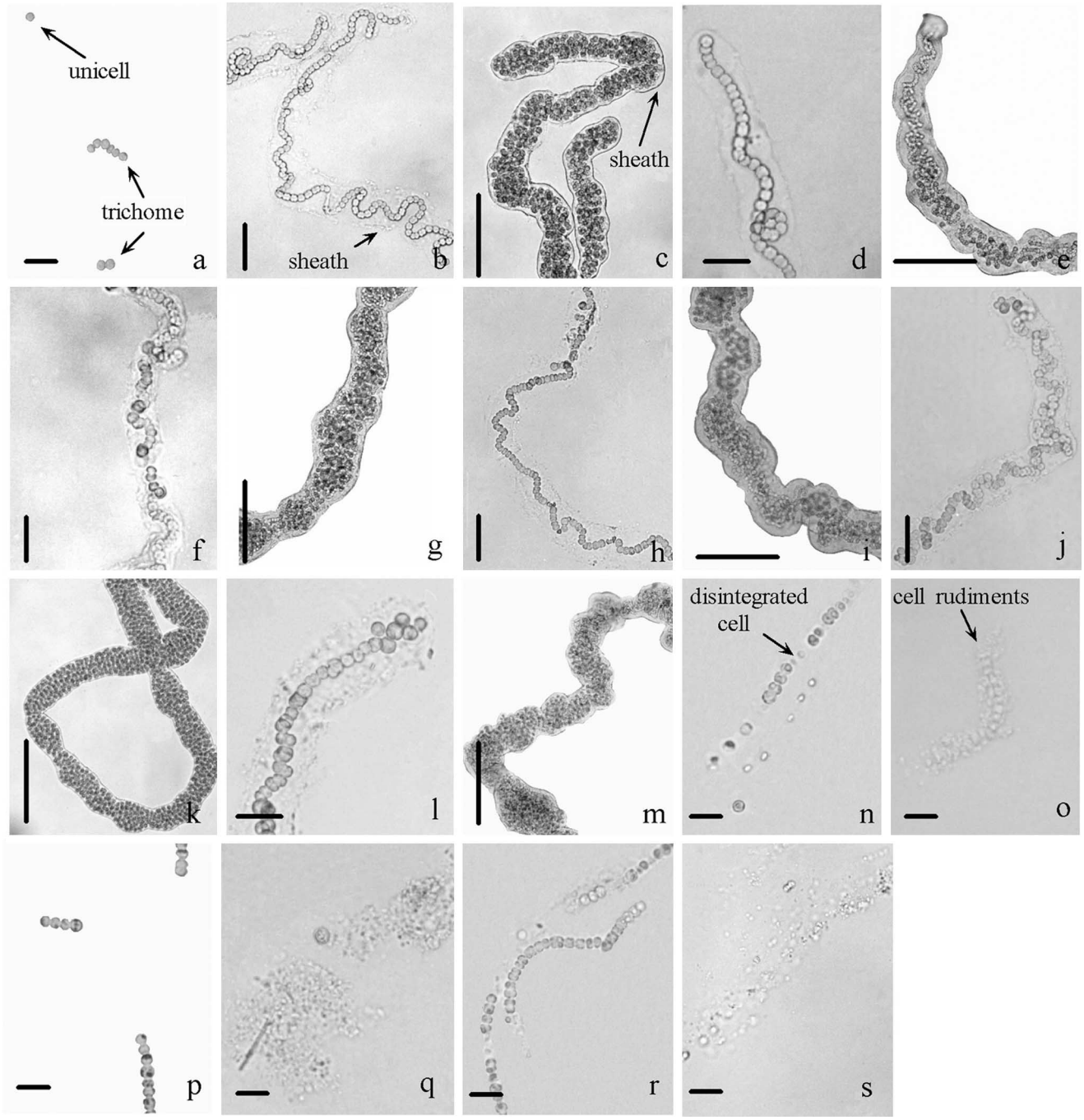

FIG. 3. Developmental process of colonial filaments from free-living cells (a) in *Nostoc flagelliforme* when cultured under aquatic conditions at 15° C, 20 μmol photons·m^{-2}·s^{-1} (b and c); 15° C, 60 μmol photons·m^{-2}·s^{-1} (d and e); 25° C, 20 μmol photons·m^{-2}·s^{-1} (f and g); 25° C, 60 μmol photons·m^{-2}·s^{-1} (h and i); 30° C, 20 μmol photons·m^{-2}·s^{-1} (j and k); 30° C, 60 μmol photons·m^{-2}·s^{-1} (l and m); 15° C, 180 μmol photons·m^{-2}·s^{-1} (n and o); 25° C, 180 μmol photons·m^{-2}·s^{-1} (p and q); and 30° C, 180 μmol photons·m^{-2}·s^{-1} (r and s). (b, d, f, h, j, and l) On day 8, a sheath formed around an elongated filament. (c, e, g, i, k, and m) On day 12 a colonial filament formed with the sheath. (n, p, and r) On day 4 cells disintegrated. (o, q, and s) On day 8 there was further decomposition to rudiments. Bars, 30 μm.

TABLE 2. Formations of sheath and colonial filament under the aquatic conditions at varied levels of light and temperature.

Light (μmol photons·m^{-2}·s^{-1})	Temperature		
	15° C	25° C	30° C
20	sh + cf	sh + cf	sh + cf
60	sh + cf	sh + cf	sh + cf
180	dg	dg	dg

sh, sheath formed; cf, colonial filament formed; dg, disintegrated.

(Fig. 3, b and c), unicells or trichomes (Fig. 3a) became single filaments that elongated with time. Visible particles were congregated along the filament on day 4; these particles became denser on day 8, and a filmy sheath was formed (Fig. 3b). At this point, there was only one filament within the sheath. As the filament elongated and contorted, more and more filaments were produced as a colony within the sheath that became thicker on day 12 (Fig. 3c). Similar developmental processes were observed at 15° C, 60 μmol photons·m^{-2}·s^{-1} (Fig. 3, d and e), at 25° C, 20 μmol photons·m^{-2}·s^{-1} (Fig. 3, f and g), at 25° C, 60 μmol photons·m^{-2}·s^{-1} (Fig. 3, h and i), at 30° C, 20 μmol photons·m^{-2}·s^{-1} (Fig. 3, j and k), and at 30° C, 60 μmol photons·m^{-2}·s^{-1} (Fig. 3, l and m). The colonial sheath was yellowish and the colonial filaments were thicker when light was increased from 20 to 60 μmol photons·m^{-2}·s^{-1}. No morphological changes were associated with the changes in temperature. When cultured at 180 μmol photons·m^{-2}·s^{-1} (Fig. 3, n and o), however, unicells or trichomes did not develop into filaments. On day 4 some cells had disintegrated (Fig. 3n), whereas on day 8 most cells had decomposed to cellular debris (Fig. 3o). This PFD resulted in similar destruction of the cells at 15, 25, and 30° C (Fig. 3, n–s). It appeared that the sheath and colonies were formed at 20 and 60 but not at 180 μmol photons·m^{-2}·s^{-1}, and the formation or decomposition was independent of temperature within the range of 15–30° C (Table 2).

When single filaments with sheaths were cultured at 25° C and varied light levels for about 2 weeks, the biomass yield increased with increased PFD from 20 to 180 μmol photons·m^{-2}·s^{-1}; the highest yield at 180 μmol photons·m^{-2}·s^{-1} was 2.6 and 1.7 times those at 20 and 60 μmol photons·m^{-2}·s^{-1}, respectively (Fig. 4). The relative growth rate was also highest at 180 μmol photons·m^{-2}·s^{-1}, being, respectively, 3.0 and 1.5 times those at 20 and 60 μmol photons·m^{-2}·s^{-1}; the differ-

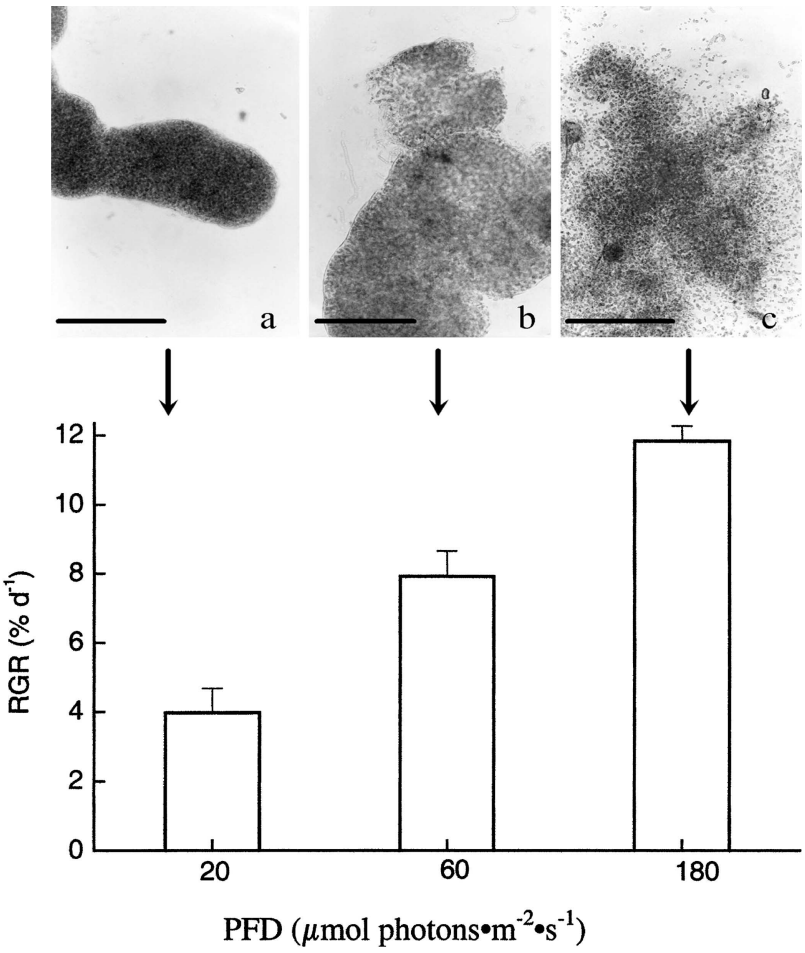

FIG. 4. Relative growth rate (RGR) and morphological changes (a–c) of the laboratory-obtained filaments of *Nostoc flagelliforme* when cultured under aquatic conditions at 20, 60, and 180 μmol photons·m^{-2}·s^{-1} and at 25° C. Bar, 80 μm. Data are the means ± SD (n = 3).

ence was significant ($P < 0.05$, t-test) (Fig. 4). However, the filaments had disintegrated, and no colonies were formed under the highest light level. By contrast, colonial filaments were developed under the other lower light regimes. Conditions of 60 μmol photons·m^{-2}·s^{-1} and 25° C resulted in greater colonial development and faster growth with the ensheathed single filaments of *N. flagelliforme* when cultured indoors.

DISCUSSION

The present study showed that when cultured under submerged aquatic conditions, colonial disintegration of *N. flagelliforme* was closely associated with propagation of bacteria, and periodic exposures to desiccation were important to prevent disintegration of *N. flagelliforme* colonies. Increases in both duration and frequency of desiccation were effective, although the latter was more effective in delaying disintegration. Frequent alternation of desiccation and rehydration might have stimulated the production of some antibacterial substances by the alga. Desiccation could also have enhanced the textural elasticity of the colonial sheath in a way that prevents disintegration, because desiccation favors the synthesis of drought-resistance proteins by *Nostoc* species (Scherer and Potts 1989, Zhong et al. 1992); some of these proteins may play a key role in maintaining sheath texture (Hill et al. 1994, Potts 2000). Bacteria may directly or indirectly restrain the growth of algae and even decompose their cells (Middelboe et al. 1995). In the present study, the sheath of *N. flagelliforme* could have become the nutritive base for bacterial growth, leading to faster disintegration when the bacteria were not controlled. In bacteria-controlled or desiccation-treated cultures, disintegration of the colonial filaments of *N. flagelliforme* was significantly delayed, suggesting reduced activities of bacteria during culture. Periodic desiccation appeared to depress or slow down bacterial growth, because drying kills bacteria (Norman 1973). However, the colonial filaments of *N. flagelliforme* were eventually decomposed even in the bacteria-controlled cultures in about 20 days. This phenomenon could be attributed to both suppressed synthesis of compounds required for sheath formation and slow but progressive decomposition by bacteria. Under dehydration stress, some plants accumulate sucrose (Thomas and James 1993); sucrose also occurs in cyanobacteria (Hill et al. 1997) and may be involved in sheath formation by *Nostoc*. The change from the terrestrial to the aquatic environment might have hindered this process in *N. flagelliforme*. In addition, culture under aquatic conditions could also affect the components of its colonial sheath. Bazazichelli et al. (1985, 1986) reported that additional polysaccharides in *N. commune* were composed of fluid and fibrillar components. Variation in the ratio of the components displaces the sheath toward the gel or sol states (Bazzichelli et al. 1985). Rehydration of *N. commune* led to marked changes in the rheological properties of the extracellular polysaccharides close to the cell surface, and these were presumably associated with gel to sol transition (Hill et al. 1997). Such changes presumably occur through the secretion of one or more carbohydrate-modifying enzymes regulated via phosphorylation and dephosphorylation of protein-bound tyrosine (Potts 2000). The gel to sol transition might have occurred in *N. flagelliforme* when cultured under aquatic conditions.

The present study showed that a free-living cell obtained from *N. flagelliforme* colonies could redevelop into a colonial filament with sheath under aquatic conditions at 20 or 60 μmol photons·m^{-2}·s^{-1} and 15, 25, or 30° C. Light rather than temperature was crucial for the formation of filamentous colonies. *Nostoc flagelliforme* experiences dramatic changes in temperature in its natural habitats, with annual air temperature ranges of −29 to +35° C and soil surface temperature from −29 to +66° C (Gao 1998). Temperature changes from 15 to 30° C do not affect morphological development even under aquatic conditions. Cyanobacteria have been found in the field at PFD they are unable to tolerate in culture (Allison et al. 1937). *Nostoc flagelliforme* cells in the present study did not develop into colonial filaments at 180 μmol photons·m^{-2}·s^{-1}, although they grew fastest at this PFD. Photoinhibition did not occur and affect development, because cellular biomass yield was highest at this PFD. However, light-induced substances might not have met the requirements for the colonial formation when PFD was increased to 180 μmol photons·m^{-2}·s^{-1}. Photoinhibition was not recognized in *N. flagelliforme* collected from the wild even at a very high PFD (1140 μmol photons·m^{-2}·s^{-1}) (Qiu and Gao 2001). This indicated that *N. flagelliforme* cells could tolerate high light. It is possible that processes inhibiting the formation of the sheath-held colonies were enhanced or activated at the high PFD.

The colonial filament's sheath formed under aquatic conditions differed from that in the wild, with the former transparent and the latter opaque. Chemical and physical properties of the sheath might be altered when it is formed in liquid culture. The laboratory-induced sheath contracted and became brownish due to desiccation when the colony was exposed to air and swelled when rehydrated, displaying a similar capability of retaining water as that of the wild colony. From a practical point of view, the laboratory-generated colonial filaments of *N. flagelliforme* may be used as "seeds" for mass cultivation and resource restoration, though additional experiments testing their capacity for growth under terrestrial environments are required.

This study was funded by the National Science Foundation of China (No. 30270036), the Chinese Academy of Sciences, and Guangdong Natural Science Foundation. We are grateful to Dr. Hiroyuki Takenaka, MAC Gifu Research Institute, for partially supporting the research.

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