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Colony development and physiological characterization of the edible blue-green alga, *Nostoc sphaeroides* (Nostocaceae, Cyanophyta)

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Abstract

The edible blue-green alga, *Nostoc sphaeroides* Kützing, is able to form microcolonies and spherical macrocolonies. It has been used as a potent herbal medicine and dietary supplement for centuries because of its nutraceutical and pharmacological benefits. However, limited information is available on the development of the spherical macrocolonies and the environmental factors that affect their structure. This report described the morphogenesis of *N. sphaeroides* from single trichomes to macrocolonies. During the process, most structural features of macrocolonies of various sizes were dense maculas, rings, the compact core and the formation of liquid core; and the filaments within the macrocolonies showed different lengths and arrays depending on the sizes of macrocolonies. Meanwhile temperature and light intensity also strongly affected the internal structure of macrocolonies. As microcolonies further increased in size to form 30 mm macrocolonies, the colonies differentiated into distinct outer, middle and inner layers. The filaments of the outer layer showed higher maximum photosynthetic rates, higher light saturation point, and higher photosynthetic efficiency than those of the inner layer; whereas the filaments of the inner layer had a higher content of chlorophyll *a* and phycobiliproteins than those of the outer layer. The results obtained in this study were important for the mass cultivation of *N. sphaeroides* as a nutraceutical product.

Keywords: Macrocolony; Morphogenesis; *Nostoc sphaeroides*; Photosynthesis

1. Introduction

Nostoc sphaeroides Kützing is an edible blue-green alga that forms spherical macrocolonies, and has been used as a potent herbal medicine and dietary supplement by the Chinese and other indigenous populations in Asia, Europe, and South America for centuries [1–3]. Historically, the effects of *N. sphaeroides* were described as anti-inflammatory, assisting digestion, control of hypertension, immune-boosting, and general improvement of overall well-being [3,4]. Recent studies have suggested that *N. sphaeroides* contains a broad range of compounds that have anti-microbial, anti-viral, anti-tumor, and anti-cancer activities, and it has become an important crop plant with economic potential because of its nutritional and pharmaceutical value [3,5–7]. The market demand for high quality *N. sphaeroides* has increased drastically during the last decade. However, this fast growing market demand has been met with an inadequate supply *of N. sphaeroides* due to its limited production [4].

The spherical macrocolonies of *N. sphaeroides* are collected from its natural habitats, i.e. water-filled rice paddies in some mountain areas in China during winter and spring

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with a long tradition [4,8]. This method of production resulted in an unreliable supply of this product that varied greatly in quality and quantity depending on the season and climate conditions [2,4]. In order to establish a reliable source of *N. sphaeroides*, mass cultivation of this edible *Nostoc* under controlled environmental conditions is needed. However, this approach has failed due to the lack of a clear understanding of the key factors controlling colony morphogenesis and a failure to develop a suitable photobioreactor for large-scale production.

Our previous report showed that *N. sphaeroides* could be cultured under laboratory conditions [9]. In order to establish a reliable methodology for mass cultivation of *N. sphaeroides*, we conducted detailed studies on the physiological factors that affected the formation of *N. sphaeroides* macrocolonies. The structural changes of the macrocolonies during the morphogenesis process of *N. sphaeroides* were also closely monitored. This report demonstrated that the formation of *N. sphaeroides* macrocolonies was strictly controlled by light and temperature conditions.

2. Materials and methods

2.1. Organism and culture conditions

Nostoc sphaeroides was isolated from a rice field in Hefeng County, Hubei Province, China (latitude 29°38′N; longitude 110°38′E) in May 2003. Macrocolonies and trichomes of *N. sphaeroides* were cultured in BG-11_0 growth medium [10]. Cultures were maintained at 25 °C under continuous illumination of 50 μM photons $m^{-2} s^{-1}$.

2.2. Preparation and culture of free trichomes

Trichomes of *N. sphaeroides* were obtained from dispersed colonies using a sterilized glass homogenizer. The homogenate was centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. Free trichomes were resuspended in 10 ml of sterilized BG-11_0 growth medium and transferred into a 500-ml Erlenmeyer flask containing 300 ml BG-11_0 growth medium. The suspension was incubated under a low light intensity of 50 μM photons $m^{-2} s^{-1}$ and 25 °C for four weeks. During this period, free trichomes underwent transformation to form macrocolonies ranging in size from 0.1 to 0.5 mm in diameter.

Microcolonies were defined as colonies with a diameter less than 0.2 mm, and macrocolonies were greater than 0.2 mm.

2.3. Culture of macrocolonies

Colonies with a diameter of 0.1–0.5 mm were used as inocula. The BG-11_0 growth medium was renewed once every 10 days. All the cultures were carried out at continuous illumination of 20, 200, or 500 μM photons $m^{-2} s^{-1}$. The incubation temperatures were 15, 29 °C, or room temperature. The ambient air was aerated. Triplicate cultures were made for each treatment. Throughout the cultivation, macrocolonies of different sizes were selected for structure analysis.

2.4. Light and fluorescence microscopy

Thin cross-sections (0.2–0.5 mm) of macrocolonies cultured under different conditions were obtained by dissection with a razor blade. Eight to ten colonies with the same sizes from each treatment were examined. Cross-sections were observed with a Leica GZ6 anatomic microscope equipped with a Nikon 4500 camera and a Leica microscope DM 5000 B equipped with a digital camera. Fluorescence photographs were observed under ultraviolet (UV) light (340–380 nm excitation filter, 425 nm barrier filter). The cells on the surface of macrocolonies showed red fluorescence under UV light, indicating the presence of chlorophyll and phycobiliproteins. If the center of the macrocolonies lacked the red autofluorescence, but showed a blue autofluorescence, the beginnings of cell degeneration were suggested [11].

2.5. Pigments content measurement

When macrocolonies grown at 200 μM photons $m^{-2} s^{-1}$ and at room temperature reached 30 mm in diameter, three layers could be seen in *N. sphaeroides*: the outer layer, the middle layer and the inner layer. The outer layer was about 0.3 mm in thickness on the surface of the 30 mm colonies [12]. The middle layer was composed of the mucilaginous envelopes ranging from 2 to 6 mm. The center of the 30 mm colonies became liquid and many floccules composed of filaments and mucilage appeared in the liquid inner layer. The outer layer, middle layer and inner layer from 30 mm colonies of *N. sphaeroides* were disrupted into the slurries, respectively, by a glass homogenizer. Triplicate samples were prepared from each treatment group in parallel. Then 5 ml aliquots of the slurries were used for the measurement of dry weight, chlorophyll (Chl) *a* and phycopiliprotein. Chl *a* was extracted with 100% methanol for 30 min at 60 °C in the dark [8]. The content of carotenoid was measured according to Ref. [13]. Phycobiliproteins were extracted in 0.1 M phosphate buffer (pH 7.0). The extract was frozen and thawed several times. The resulting extract was centrifuged and the supernatant was quantified [14].

2.6. Photosynthetic activity measurement

Oxygen evolution of the filaments from the different layers of 30 mm macrocolonies grown at 200 μM photons $m^{-2} s^{-1}$ and at room temperature was measured using a Clarke-type oxygen electrode (Hansatech Ltd., UK) at 25 °C under a series of light intensities. Photosynthetic rates were normalized to Chl *a* content. Photosynthetic

irradiance response (P–I) curves were plotted from $P = P_m \tanh(a \cdot E/P_m) + R_d$ [15].

2.7. Statistical analysis

The mean and standard deviations were calculated for each treatment. One-way analyses of variance (ANOVA) were employed to test for significant differences where necessary.

3. Results

3.1. Developmental stages of *N. sphaeroides*

In order to establish a reliable methodology for the mass cultivation of *N. sphaeroides*, we carried out a detailed study to elucidate the developmental stages of *N. sphaeroides* from trichomes to macrocolonies. Colonies with a diameter of 3 mm were dispersed to obtain trichomes (Fig. 1a), which were then inoculated in a fresh culture medium at a light intensity of 50 μmol photons $m^{-2} s^{-1}$ and 25 °C. The trichome first developed a sheath and became a filament (Fig. 1b) after 2 days of cultivation. As cell division began within the sheath, aggregated cell mass was formed along the filament (Fig. 1c) and expanded into aseriated colonies and finally into spherical colonies (50–200 μm) that were either connected by a heterocyst or separated (Fig. 1d) after 15 days. Colonies with a diameter of 0.1–0.5 mm grown under 200 μM photons $m^{-2} s^{-1}$ at room temperature continuously increased in size (Fig. 1e) as the culture medium was periodically renewed. The largest colony obtained was 33 mm in diameter after 120 days of cultivation (Fig. 1f).

Trichomes or hormogonia released from colonies in any size were able to form new colonies again. The budding from colonies as a method of multiplication was also observed (data not shown). The colony rupture was observed in each stage of the colony development due to nutrition deficiency or unfavorable light intensity and temperature. However, the mechanism controlling the colony rupture is not clear at present. Preventing the colony rupture remains to be a challenge for the mass cultivation of this organism.

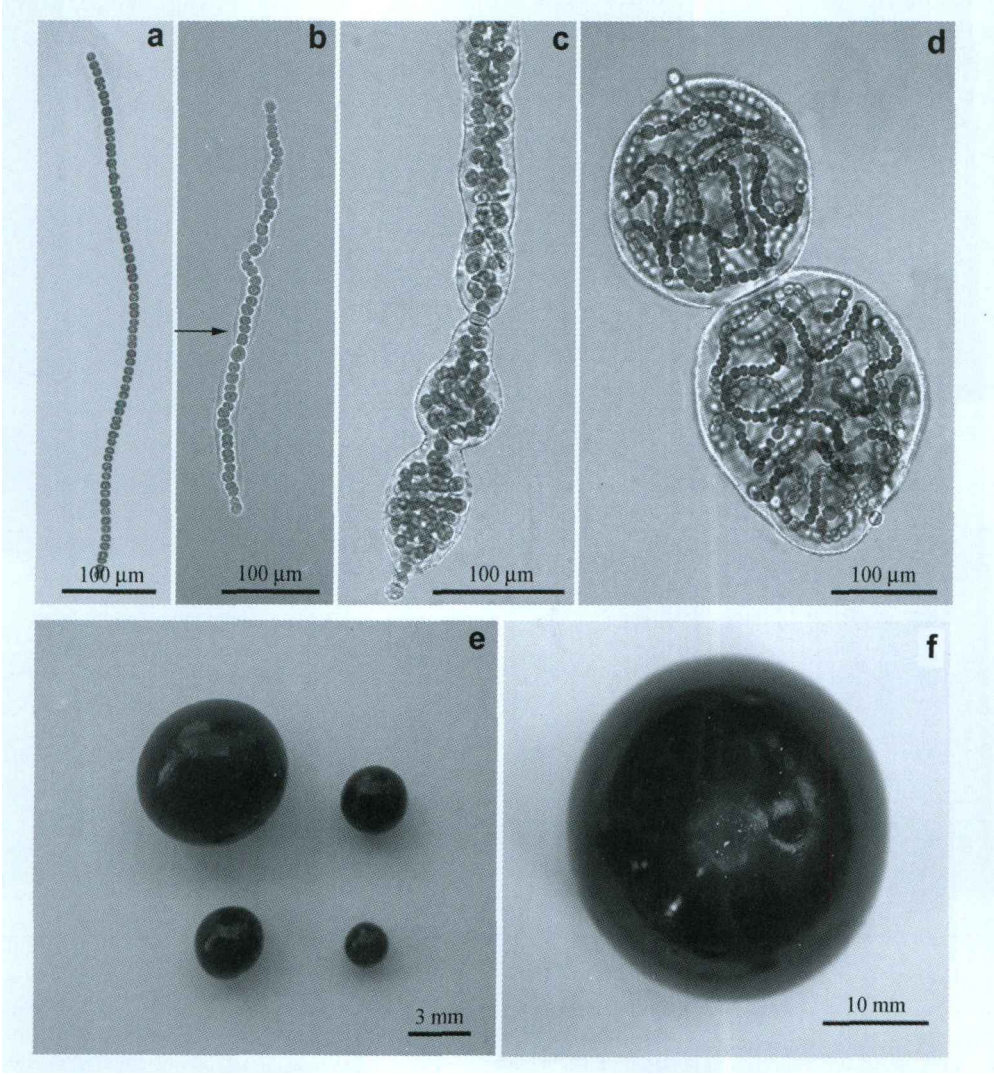

Fig. 1. Developmental stages of *N. sphaeroides*. (a) Trichome; (b) filament; (c) aseriated colonies; (d) microcolonies; (e) macrocolonies of different sizes; (f) ruptured 33 mm colony, one green dot appeared on its surface. Arrow in (b) indicates the sheath. Representatives of at least 10 samples were examined.

3.2. Changes in the internal structure during the development of macrocolonies

As colonies grown under 200 μM photons $m^{-2} s^{-1}$ and at room temperature increased in size to more than 1 mm in diameter, structural differentiation occurred within the colonies and unevenly distributed filaments were observed. Cross-sections of the macrocolonies at each stage were examined to analyze the internal structure. As shown in Fig. 2 1 mm colonies comprised homogeneously distributed filaments that were irregularly contorted in both peripheral region (Fig. 2a and b) and center (Fig. 2e) of the colonies. When macrocolonies grew to 8 mm in diameter, the distribution of the filaments was more concentrated in the peripheral region than in the center (Fig. 2c). This trend became more obvious when the macrocolonies reached the size of 16 mm in diameter (Fig. 2d, g, and h). The filaments were distributed in an ordered manner in the peripheral region, while a much smaller number of filaments remained in the center region. In addition, all filaments were arranged in parallel towards the outside of the macrocolonies.

The array of filaments on the surface of colonies (about 500 μm thick from the surface) was variable as the colonies increased in diameter. In small colonies (e.g. 1–2 mm in diameter) the filaments were contorted and disorderly (Fig. 2a and b); whereas in larger colonies (the diameter greater than 8 mm), the filaments on the surface were arranged in an ordered manner and tended to be located toward the center of the colonies (Fig. 2c), although the interior filaments were still disorderly. Within 16 mm colonies, the interior filaments were much shorter than those in the surface layer. The filaments of the outer layer exceeded 500 μm in length (Fig. 2d), but the filaments of the inner layer were less than 100 μm (Fig. 2g). Such a difference in the filament length was not observed in the smaller colonies (Fig. 2a and e).

3.3. Pigment contents and photosynthetic activities in different layers of macrocolonies

To further explore the physiological properties of *N. sphaeroides*, 30 mm macrocolonies grown under 200 μmol photons $m^{-2} s^{-1}$ at room temperature were fractionated into the outer, middle, and inner layers, and the photosynthetic activities and pigment contents of each fraction were analyzed. As shown in Fig. 3, the highest photosynthetic activities measured as oxygen evolution rates were found in the outer layer, while the lowest activities were observed in the inner layer. The maximal net photosynthetic rate (P_m) in the outer layer was about 50% and 400% higher than that in the middle layer

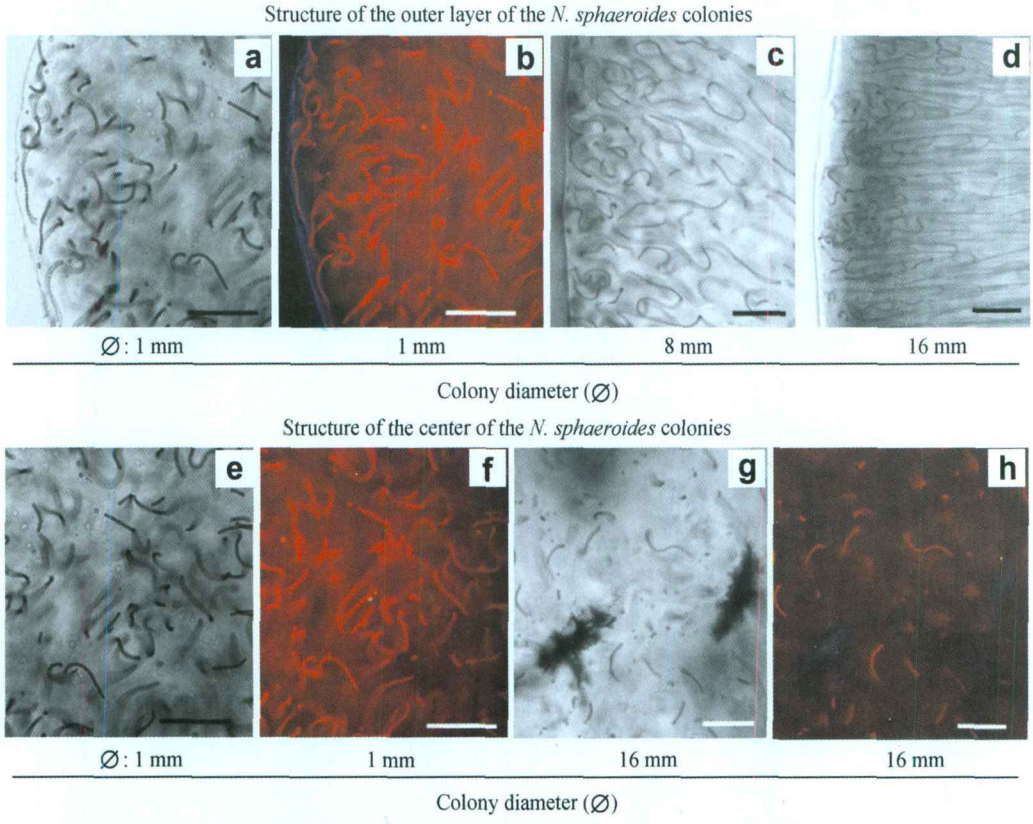

Fig. 2. The structure of the outer layer (top panels) and the center (lower panels) of *N. sphaeroides* colonies grown at 200 μmol photons $m^{-2} s^{-1}$ and at room temperature. (a) Homogeneously distributed filaments within 1 mm colony; (b) autofluorescence of the outer layer shown in (a); (c) outer layer of 8 mm colony; (d) outer layer of 16 mm colony, the distribution of filaments in parallel towards the outside of colony; (e) center of 1 mm colonies; (f) autofluorescence of the center shown in (e); (g) center of 16 mm colonies showing short filaments; (h) autofluorescence of the center shown in (g). Scale bar: 100 μm. Representatives of at least 10 colonies were examined.

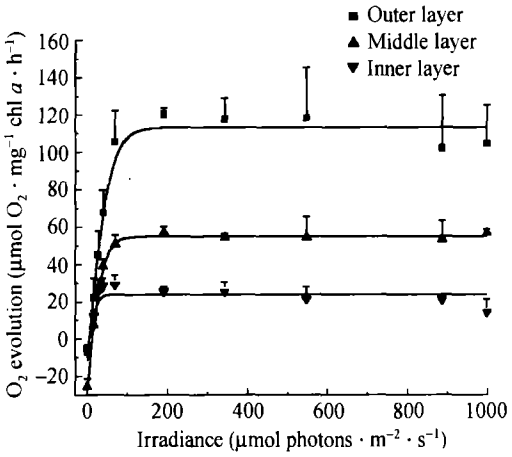

Fig. 3. Light response curve of the filaments in the different layers of 30-mm *N. sphaeroides* colonies ($n = 3$ or 4).

($p < 0.001$) and inner layer ($p < 0.001$), respectively (Table 1). The apparent quantum yield (α) and light-saturating (I_k) showed similar trends, indicating declined photosynthetic activities from the outer layer towards the center of the macrocolonies. In contrast, the dark respiration (R_d) and compensation point (I_c) were higher in the middle layer than in the outer layer and inner layers (Table 1).

The three layers of *N. sphaeroides* macrocolonies also showed different pigment contents. The Chl *a* content was about 1.6-fold higher in the inner layer than in the outer layer ($p < 0.005$) (Table 2). At the same time, the contents of carotenoids (expressed as the ratio of carotenoids to chlorophyll–Car/Chl) and phycobiliproteins (phycoerythrin, phycocyanin, allophycocyanin) showed the same trends as observed for the Chl *a* content in the different layers: pigment contents increased significantly from the outer layer towards the center of the macrocolonies ($p < 0.05$). Such a distribution pattern of the pigments was in opposition to that of photosynthetic activity, and might be caused by the availability of light within the colony. In macrocolonies, filaments were mostly concentrated in the outer layer (Fig. 2), which absorbed most of the incoming light. The further toward the center of the colonies was, the less available light was. Therefore, the cells inside the colonies produced more antenna pigments (carotenoids and phycobiliproteins) to cope with the low light condition.

3.4. Effects of light intensity and temperature on the internal structures of N. sphaeroides

Light intensity and temperature played important roles in controlling the morphogenesis process of *N. sphaeroides*. To understand the effect of light intensity on the structure of *N. sphaeroides*, colonies were subjected to a low light intensity of 20 μmol photons m^{-2} s^{-1} and a high light intensity of 500 μM photons m^{-2} s^{-1} at 25 °C. The cross-sections of both the colonies were examined with phase contrast microscopy or autofluorescence after 30 days exposure when colonies increased in size to about 3.4 mm (Fig. 4). The colonies grown under lower light intensity were much softer compared to the colonies grown under higher light intensity. The color of the colonies grown at 500 μM photons m^{-2} s^{-1} was green, while the colonies grown under lower irradiance appeared black-green. Colonies grown under the lower light intensity showed a more homogeneous internal structure (Fig. 4a) compared to the colonies grown under high light intensity (Fig. 4b). Rings were observed in the colonies grown under high light intensity, which were not apparent in colonies grown under low light intensity. In the peripheral region of a colony, denser filaments were found in the colonies grown under high light intensity (Fig. 4d) than in the colonies grown under low light intensity (Fig. 4c). However, in the center of a colony, homogeneously distributed filaments were observed in colonies grown under low light intensity, while only a few filaments were found in the center of the colonies grown under high light intensity. The different distribution patterns of filaments between colonies were confirmed by the autofluorescence observations (Fig. 4e and f). These results indicated that light intensity played a critical role in the structural development of *N. sphaeroides* colonies.

The effect of temperature on colony structure was also examined. Colonies of *N. sphaeroides* were cultivated at 15 or 29 °C under a light intensity of 200 μM photons m^{-2} s^{-1}. After 30 days, cross-sections of the 14 and 3.5 mm colonies were examined and compared. The 3.5 mm macrocolonies grown at 15 °C were more elastic than those grown at 29 °C, the latter colonies contained a large number of dense maculas in the center (Fig. 5b) compared to the colonies grown at 15 °C (Fig. 5a). In addition, the center of the 14 mm colonies became liquid when

Table 1
Photosynthetic parameters of the outer, middle and inner layers of 30 mm macrocolonies of *N. sphaeroides*

Colony section	P_m	α	I_k	R_d	I_c
Outer layer	129.4 ± 12.1^a	2.7 ± 0.3^a	49.6 ± 8.4	11.5 ± 4.0	4.5 ± 2.1
Middle layer	83.2 ± 4.2^b	2.2 ± 0.2^b	37.5 ± 2.2	27.1 ± 2.8	12.2 ± 1.0
Inner layer	30.1 ± 4.2^c	1.9 ± 0.3^b	16.3 ± 3.2	6.2 ± 2.6	3.3 ± 1.5

Maximal net photosynthetic rate, P_m (μmol O_2 mg^{-1} chl a h^{-1}), apparent quantum yield, α (μmol O_2 mg^{-1} Chl a h^{-1})/(μmol photons m^{-2} s^{-1}), light-saturating, I_k (μmol photons m^{-2} s^{-1}), and compensation points, I_c (μmol photons m^{-2} s^{-1}), and dark respiration, R_d (μmol O_2 mg^{-1} Chl a h^{-1}), derived from the P–I curves of the filaments from the different layers of 30-mm *N. sphaeroides* colonies ($n = 3$ or 4). With each column, values with different superscript letters are significantly different at $p = 0.05$ (Tukey's test).

Table 2
Pigment content and carotenoid to chlorophyll ratio (Car/Chl) of the outer, middle and inner layers of 30 mm colonies of *N. sphaeroides*

Colony section	Outer layer	Middle layer	Inner layer
Chl *a* (mg g^{-1} dry weight)	4.2 ± 0.2^b	6.3 ± 0.1^a	6.5 ± 0.2^a
Car/Chl (mg mg^{-1})	0.15 ± 0.02	0.19 ± 0.01	0.26 ± 0.2
Phycoerythrin (mg g^{-1} dry weight)	2.0 ± 0.2^b	2.4 ± 0.2^b	4.2 ± 0.3^a
Phycocyanin (mg g^{-1} dry weight)	3.1 ± 0.4^b	3.7 ± 0.3^b	6.6 ± 0.5^a
Allophycocyanin (mg g^{-1} dry weight)	5.1 ± 0.7^b	5.9 ± 0.6^b	11.2 ± 0.8^a

With each line, values with different superscript letters are significantly different at $p = 0.05$ (Tukey's test). Data are means ± SD ($n = 3$).

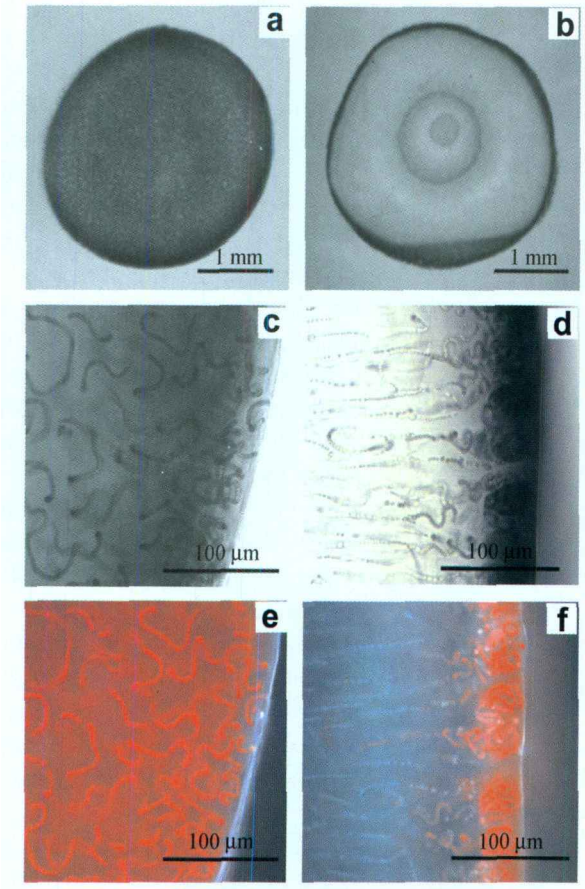

Fig. 4. Effects of light intensity on the structure of 3.4 mm colonies grown at 25 °C. (a) Cross-section of colonies grown under 20 μmol photons m^{-2} s^{-1}, showing a homogeneous internal structure; (b) rings appearing in the cross-section of the colonies grown under 500 μmol photons m^{-2} s^{-1}; (c) formation of filaments within the colonies grown under 20 μmol photons m^{-2} s^{-1}; (d) denser filaments existing within the peripheral region of the colonies grown under 500 μmol photons m^{-2} s^{-1} than that grown under 20 μmol photons m^{-2} s^{-1}; (e) the same filaments as in (c), showing red autofluorescence; (f) the same filaments as in (d), showing that some interior filaments lack red autofluorescence. Representatives of at least 10 colonies were examined.

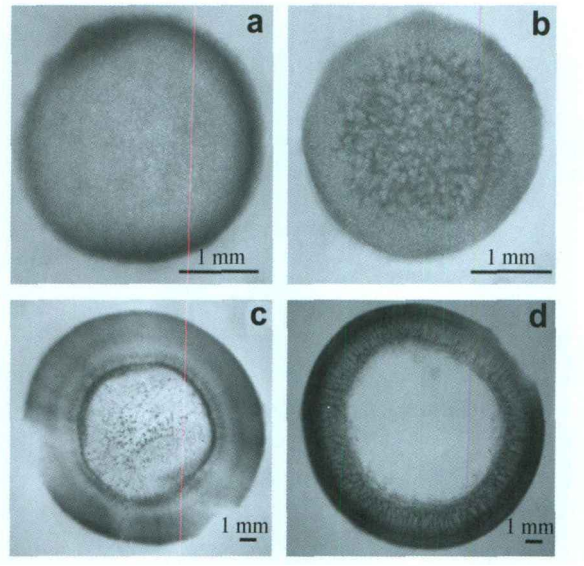

Fig. 5. Effects of temperature on the structure of differently sized colonies grown under 200 μmol photons m^{-2} s^{-1}. (a) Cross-section of 3.5 mm colonies grown at 15 °C; (b) cross-section of 3.5 mm colony grown at 29 °C, showing a large number of dense maculas in the center of the colony; (c) cross-section of 14 mm colony grown at 15 °C, the center of colony remaining gelatinous; (d) cross-section of 14 mm colonies grown at 29 °C, showing that the center of the colony became liquid. Representatives of at least 10 colonies were examined.

grown at 29 °C (Fig. 5d), but remained gelatinous when grown at 15 °C (Fig. 5c).

4. Discussion

Although a number of researchers have investigated the intricacies of the life cycle or development of *Nostoc* [3,12,16–20], these studies were mainly focused on the formation of microcolonies, with little attention devoted to the development of macrocolonies [3,21,22]. The lack of information on the development and internal structure of the macrocolonies was partially due to the fact that there was little knowledge about the cultivation of macrocolonies under the controlled laboratory conditions. In our previous work [9] *N. sphaeroides* colonies were successfully cultivated up to 33 mm in diameter under laboratory conditions, providing us a unique opportunity to study the morphogenesis and internal structure of macrocolonies. The current study presented the detailed information on the internal structure of macrocolonies during the developmental stages of *N. sphaeroides* as well as the effects of environmental factors on the internal structure of macrocolonies. Based on the results obtained in this study, we propose the development of the internal structure for the *N. sphaeroides* as illustrated in Fig. 6.

Microcolonies were first developed from trichomes after a series of cell divisions and the formation of sheath (Fig. 1a–d). At this stage, the filaments were homogeneously distributed within a colony (Fig. 2a and e). The

microcolonies continued to increase in size accompanied by the differentiation in their internal structure, e.g. filaments were more concentrated in the peripheral region of a colony (Figs. 2d and g; 4d and f) under certain growth conditions and various ring structures started to appear (Fig. 6c–f). Other structural features of macrocolonies, such as dense maculas, rings, compact core and liquid sphere in the center, developed gradually as the diameter increased. As the colony size further increased, the center became gelatinous or liquid due to the degradation of the filaments. The peripheral region or middle layer of the colonies was firm and solid because of the concentrated filaments (Fig. 6e–g). Under favorable growth conditions, the macrocolonies maintained these structures intact and continued to increase in size. The largest macrocolonies obtained were 33 mm in diameter (Fig. 6g).

The rupture of a colony could take place at any stage of macrocolony development due to unfavorable growth conditions, such as inadequate light intensity, unfavorable temperature, or nutrient deficiency. The rupture usually leads to disintegration of a colony and the release of trichomes or hormogonia, which form new microcolonies. Budding from a colony is occasionally observed as a way of reproduction; however, the exact conditions that precipitate the budding process are not clear.

In large colonies, the diffusion of materials into and away from the center of the macrocolonies is difficult compared to small colonies. Ninety percent of the incoming light is absorbed within 100 μm of the surface of a colony of *Nostoc* [1]. Cells within a larger colony could experience light levels and wavelengths differently from those within a smaller one as a result of the shading and absorption by the

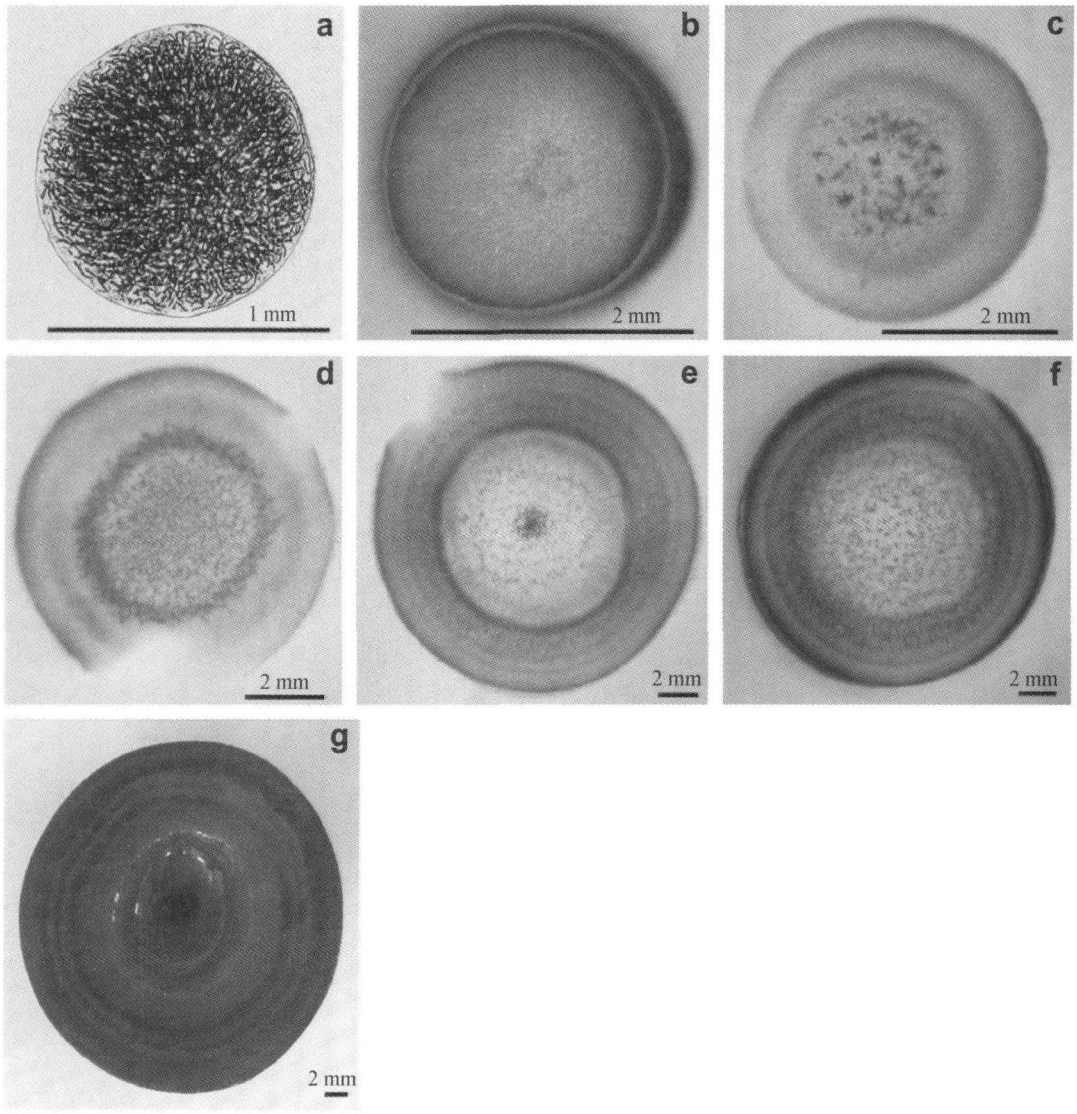

Fig. 6. Cross-sections of colonies with different diameters (cultured at 200 μmol photons m^{-2} s^{-1} and at room temperature) showing a series of structural changes in the cross-section of (a) the 1 mm colony, showing a long sheath homogeneously entangled and enwrapped in a sheath; (b) 2 mm colony, showing dense maculas in the center; (c) 3.4 mm colony, showing one ring; (d) 8 mm colony, showing two rings; (e) 16 mm colony, one core appeared in the center; (f) 16 mm colony, no core appeared in the center; (g) 28 mm colony, the center appeared to be in a liquid state. Representatives of at least 10 colonies were examined.

outer layer [23]. Since *N. sphaeroides* contains a large amount of polysaccharides [5], it is postulated that the lack of light and large amount of polysaccharides in the inner layer of the larger colonies could result in the existence of shorter filaments in the inner layers of the colonies as compared with their outer layer [16].

The internal structure is tightly controlled by light intensity and temperature [24–26]. The present study revealed that the light intensity affected the tortuous level of filaments and the array of filaments in *N. sphaeroides*. Under different growth temperatures, the internal structure of *N. sphaeroides* exhibited a unique appearance (Fig. 5). Macrocolonies incubated at the temperatures above 30 °C were softer and much easier to break down than those incubated at lower temperatures. This is in agreement with previous observations that *N. sphaeroides* is usually found in water-filled paddies in winter and harvested in spring [8]. The annual mean temperature in its natural habitat is about 12.2 °C [10]. During growth, the environmental temperature is low, possibly near or below 0 °C [8]. In our studies, it also appeared that lower temperatures (e.g. 15–23 °C) were advantageous for the formation of steady structures of macrocolonies.

Both irradiance and spectral composition greatly influenced pigment composition [26]. The energy limitation in the inner layer of 30-mm macrocolonies may be partially offset by an increase in the amounts of photosynthetic pigments generated to increase the light-capturing ability per cell [26,27]. Photosynthetic performance in a single macrocolony is regulated differentially in *N. sphaeroides* through its gelatinous thickness. The filaments collected from different layers of macrocolonies showed differences in photosynthetic rates and pigment composition. These results follow the classical view of the response of algae and plants to different light intensities [15,28].

By successfully cultivating *N. sphaeroides* under laboratory conditions, we were able to conduct systematic studies to decipher the developmental stages of *N. sphaeroides* colonies. This study clearly showed the sequential changes in the interior structure of *N. sphaeroides* colonies with the increase of the colony size. The effects of light intensity and temperature on structural development were also demonstrated. More studies are underway to elucidate the physiological and biochemical changes that occur during colony development. This new information about the growth and morphology of *N. sphaeroides* could be potentially used for the scale-up production and increased field cultivation.

Acknowledgements

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