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Evaluation of morphological variation and biomass growth of *Nostoc commune* under laboratory conditions

Yi Diao[1,2] and Zujun Yang[1]*

[1]School of Life Science and Technology, University of Electronic Science and Technology of China, Chengdu-610 054, Sichuan, People's Republic of China

[2]College of Biological and Chemical Engineering, Sichuan Province Key Laboratory of Characteristic Biological Resources of Dry and Hot River Valley, Panzhihua University, Panzhihua 617000, Sichuan, People's Republic of China

*Corresponding Author E-mail: yangzujun@uestc.edu.cn

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Abstract

Nostoc commune is a blue green alga used for health food and herbal medicine due to its nutritional values and antioxidant properties. However, wild type *N. commune* has been decreasing in quantity as a result of ever-growing market demand and environmental pollution. Therefore, artificial culture of *N. commune* is important as it can bring great social and economic benefits. In this article, *N. commune* was cultured in BG11 medium, under which condition morphological variation and biomass growth of *N. commune* were investigated. Results indicated that concentration, fresh weight and dry weight of the colony increased fastest at 40 rpm from the Ist day to 14th day and the fresh and dry weight increased as the culturing time was prolonged, and reached 27.22 g l^{-1} and 0.88 g l^{-1} respectively on 56th day. Aggregated cell mass formed on 4th day and it expanded to asteriated colonies on 10th day. Single microcolonies formed on the 21st day had diameters 200-250 µm. Macrocolonies obtained after 28 days had diameters of 5 mm on 42nd day. Discoid colonies were formed as macrocolonies ruptured on 49th day and the diameter reached 15 mm on 56th day. Results of the present study can promote large-scale industrial production of *N. commune*.

Key words

Biomass, Discoid colony, Macrocolony, Morphology, *Nostoc commune*

Introduction

Nostoc is one of the five genera of family *Nostocaceae* and the name *Nostoc* was used in Europe about 500 years ago (Potts, 1997). As a terrestrial cyanobacterium that also got the name of "Dimuer" in China, *Nostoc commune* Vauch. has been used as dietary supplement and herbal medicine for past 2000 years (Hu 2006; Li *et al.,* 2003).

Earlier studies have described the morphological structure of filament, trichome, vegetative cell, heterocyst, spherical colony and discoid colony of *Nostoc* sp. (Hu, 2006; Briones *et al.*, 2007; Yan *et al.,* 2010). Thalli of *N. commune* is packed with a capsule outside which is filled with winding algae filament and such filament is a colony of trichomes embedded in a sheath. The vegetative cells are about 4.5-6 µm in length and 5 µm in width. Heterocysts occur in the middle or at the end of the filaments which are about 7 µm in diameter and are bigger than the vegetative cells (Hu, 2006).

Moreover, recent studies have shown that *N. commune* contain rich proteins, amino acids, fatty acids, polysaccharides, flavonoids, vitamins and many kinds of minerals (Diao *et al.,* 2012; Li *et al.,* 2003), and these compounds possess anti-tumor, anti-viral, anti-bacterial and anti-inflammatory effects (Kanekiyo *et al.,* 2005; Tamaru *et al.,* 2005). *N. commune* has edible and medicinal value, which contributes to its popularity in China. Besides, it also promotes the growth of crops.

However, wild *N. commune* has been rapidly diminishing in quantity because of increasing market demand and environmental pollution. Additionally, as large amounts of sediment contained in wild colonies can't be cleaned up, the edibility of *N. commune* is influenced. Therefore, *N. commune*

needs to be cultivated under controlled environmental conditions so that the resources can be protected and exploited.

Deng *et al.* (2004) cultivated, *N. commune* in an outdoor pond system and collected 3-4mm colonies but discoid colonies of *N. commune* have not yet been cultured successfully. Therefore, no study has been conducted to evaluate morphological changes and biomass growth of *N. commune* with the extension of incubation time under artificial conditions. This study showed *N. commune* could be cultured, and macrocolonies and discoid colonies were successfully attained under artificial conditions, which made us a unique opportunity to study the variation of morphology and biomass in order to establish an effective methodology for mass cultivation of *N. commune*.

Materials and Methods

Culture of *N. Commune*: Fresh *N. commune* samples were collected from NanBu county in Sichuan province of China (latitude 31°40' N, longitude 106°24'E) in 2012. After being rinsed and dried, the collected samples were stored in dry conditions at room temperature for two months before being used for experiments. Five-gram dry samples were soaked in distilled water for 48 hrs. The samples were then sterilized with 70% alcohol (v/v) for 30 sec in order to eliminate surface microorganisms. Then the samples were rinsed five times with sterile water.

The disinfected samples were homogenized in order to obtain the trichomes of *N. commune*, and homogenate was filtered with an 80 micron mesh sieve to obtain free trichomes. The free trichomes were further diluted to a concentration of 1×10^6 CFU ml^{-1} using sterilized BG11 medium. The BG11 medium consisted of macronutrients: 15.0 g l^{-1} $NaNO_3$, 0.04 g l^{-1} K_2HPO_4, 0.075 g l^{-1} $MgSO_4.7H_2O$, 0.036 g l^{-1} $CaCl_2.7H_2O$, 0.001 g l^{-1} $Na_2EDTA.2H_2O$, 0.02 g l^{-1} Na_2CO_3, 0.006 g l^{-1} $(COH)(COOH(CH_2COOH)_2.H_2O$, 0.006 ammonium ferric citrate and micronutrients : 2.86 g l^{-1} H_3BO_3, 0.22g l^{-1} $ZnSO_4.7H_2O$, 0.0179 g l^{-1} $CuSO_4.5H_2O$, 1.860 g l^{-1} $MnCl_2$, 0.390 g l^{-1} $Na_2MoO_4.2H_2O$, 0.049 g l^{-1} $CO(NO_3)_2.6H_2O$.

Two ml free trichomes samples were inoculated into 500 ml conical flasks containing 250 ml BG11 growth medium. They were cultured at 25ºC under continuous illumination of white fluorescent lights for 16 hrs at a mean photon flux density (PFD) of 50 µmol photons·m^{-2}·s^{-1}, and in continuous darkness for 8 hrs every day at the surface of conical flasks, on a KYC 100B-rocking incubator (Shanghai Fuma Test Equipment Company) (Su *et al.,* 2008; Deng *et al.,* 2008). During culturing period, BG11 growth medium was renewed once in every 7 days.

Growth of biomass: Test samples were cultured on the rocking incubator at speeds of 0, 20, 40, 60 and 80 rpm. Growing biomass was estimated by measuring the biomass concentration of *N. commune* according to Su *et al.,* 2008 with minor modifications. The suspensions were inspected from first day to 14th day, and the absorbance was measured at 750 nm to estimate the growth of biomass. Fresh and dry colonies were weighed after 14 days.

In order to investigate the growth of biomass in BG11 growth medium, *N. commune* was inoculated in conical flasks with sterilized BG11 medium at 25ºC at the speed of 40 rpm for eight weeks. The BG11 medium was renewed with fresh sterilized medium and the biomass was determined every week. Dry colonies were weighed every week after the culture solutions were centrifuged at the speed of 8000 rpm for 10 min, the supernatants were discarded, and fresh colonies were heated at 105℃until constant weight was obtained.

Morphological observation : Filaments, microcolonies, macrocolonies and discoid colonies were obtained during different culture times. Thin cross-sections of microcolonies were hand-sliced with a razor blade by hand-sliced method. Filaments suspension and thin cross-sections of microcolonies were observed through a Leica GZ6 anatomic microscope equipped with a Nikon 4500 camera. Diameters of macrocolonies and discoid colonies were detected by vernier caliper and photographed using a Nikon 4500 camera. Morphologies of microcolonies, macrocolonies and discoid colonies were detected every seven days, and 50 separate colonies were chosen. Microcolonies were spherical with diameter less than 0.3 mm, while macrocolonies had diameters are greater than 0.3 mm (Deng *et al.,* 2008b).

The mean and standard deviations were calculated for absorbance, fresh weight and dry weight of *N. commune* cultivated at different shaking speeds. One-way analyses of variance (ANOVA) were employed to test for significant differences where necessary.

Results and Discussion

Biomass Growth : The absorbance (at 750 nm) of colony concentration had been checked, and shown in Fig. 1, at different shaking speeds from the first day to the fourteenth day (Su *et al.,* 2008). The results showed that the colony concentration increased with the extension of incubation time, and the colonies grew faster at a speed of 40 rpm than at those shaking speeds. The colony concentration (absorbance at 750 nm) increased from 0.086 to 0.698 at 40 rpm. The maximum colony concentration increased correspondingly from 0.353 to 0.698 at different shaking speeds.

Moreover, as illustrated in Table 1, *N. commune* was cultivated at different shaking speeds for 14 days, and fresh weight and dry weight were not directly proportional to the concentration at different shaking speeds. That may be because relative water content of colonies is different at different shaking speeds. Fresh weight and dry weight of *N. commune* at a speed of 40 rpm were significantly higher than those at speeds of 0, 20, 60 and 80 rpm.

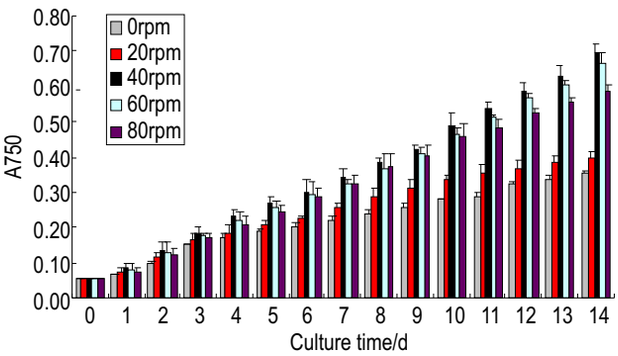

Fig. 1 Absorbance growth at different shaking speeds in BG11 liquid suspension culture of *N. commune*

Table 1 : Fresh weight and dry weight of *N. commune* cultivated at different shaking speeds for 14 days

Shaking speeds (rpm)	Fresh weight (g l^{-1})	Dry weight (g l^{-1})
0	1.269±0.103^{d}	0.0552±0.0098^{d}
20	2.687±0.186^{c}	0.117±0.0112^{c}
40	4.608±0.232^{a}	0.192±0.0134^{a}
60	3.639±0.256^{b}	0.152±0.0142^{b}
80	2.449±0.237^{c}	0.102±0.0121^{c}

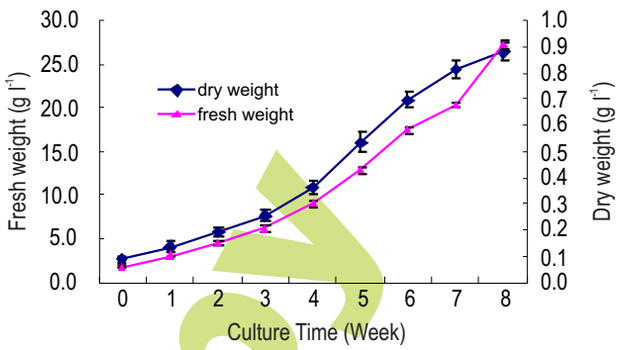

Fig. 2 Fresh weight and dry weight of *N. commune* cultivated in BG11 liquid medium culture at 40 rpm for 8 weeks

It could seem from Fig. 2 that fresh weight and dry weight increased continuously with the extension of culture time. Fresh weight increased from 1.8 g l^{-1} to 27.22 g l^{-1}, and dry weight increased correspondingly from 0.09 g l^{-1} to 0.88 g l^{-1} at 40 rpm for 8 weeks.

Morphologies of *N. commune* was examined from filament to discoid colony during different developmental stages, in order to establish a culture method of *N. commune.* Fresh

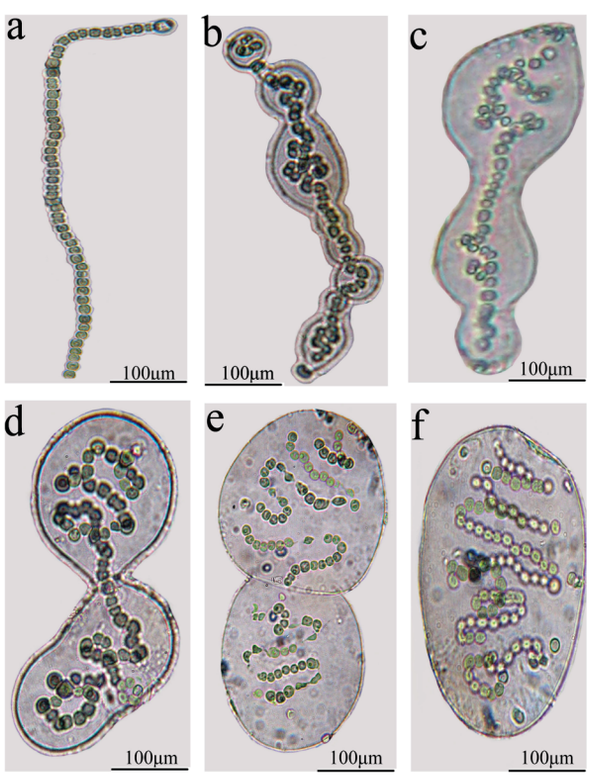

Fig. 3 : Filament, aseriated colonies and microcolonies of *N. commune* (a) filament, (b) aseriated colonies (aggregated cell mass formed), (c) aseriated colonies (the sheath began to invaginate), (d) aseriated colonies (the sheath invaginated continuously), (e) microcolonies (diameters from 100 µm to 150 µm), (f) microcolonies (diameters from 150 µm to 250 µm). Representatives of 50 samples were examined

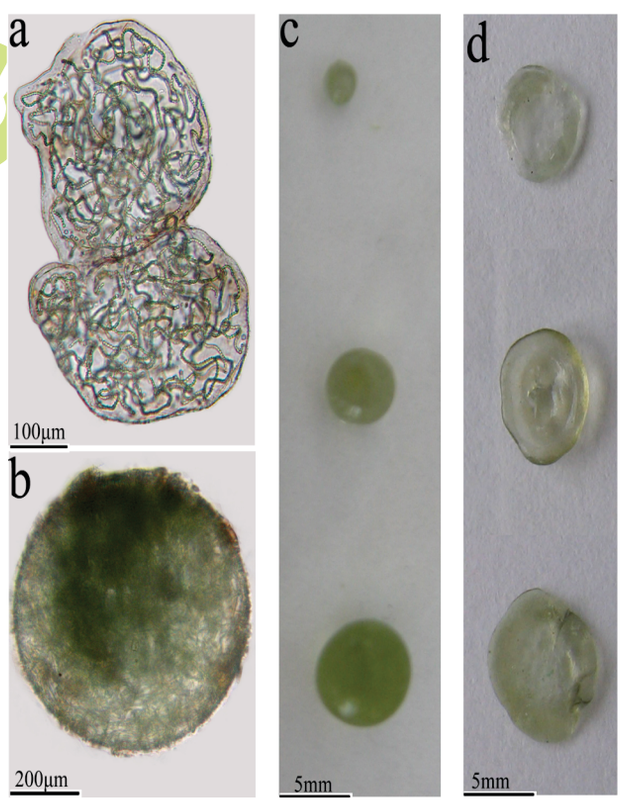

Fig. 4 : Macrocolonies and discoid colonies of *N. commune* (a) macrocolonies (diameters from 0.4 mm to 0.5 mm), (b) macrocolonies (diameters from 0.8 mm to 1.0 mm), (c) macrocolonies (diameters from 1.5 mm to 5 mm), (d) discoid colonies (diameters from 10 mm to 15 mm). Representatives of 50 samples were examined

samples of *N. commune* were ground into homogenates and filtered to obtain free trichomes with an 80 micron mesh sieve. After 3 days a sheath was formed outside the trichome and a filament was produced from trichomes (Fig. 3a). On 4th day of culture the cell began to divide, and an aggregated cell mass was formed along the filament within the sheath (Fig. 3b). In the aggregated cell mass, the sheath invaginated, and cells continued dividing and expanded to aseriated colonies after 10 days (Fig. 3c). The sheath invaginated continuously and the aggregated cell mass continued expanding into spherical colonies (100-200 µm) (Fig. 3d). When invagination of the sheath was nearing the end, diameters of spherical colonies were 150-250 µm after 14 days (Fig. 3e). After 21 days, single microcolonies were formed and their diameters reached 200-250 µm, respectively (Fig. 3f).

Morphologies of macrocolonies and discoid colonies of *N. commune* are shown in Fig. 4. Colonies with size of 0.4-0.6 mm were obtained after 28 days, as the BG11 medium was periodically renewed every 7 days (Fig. 4a). The diameters of spherical colonies increased to 0.8-1.2 mm after 35 days (Fig. 4b). After 42 days, macrocolonies with the largest diameter of 5 mm were obtained (Fig. 4c), and spherical colonies of various diameters could be obtained as colonies ruptured and released hormogonia and trichomes which could form new colonies again. After spherical colonies reached 5 mm, the spherical macrocolonies ruptured and formed discoid and blue-green colonies, in the middle section of which there was a sizeable dent after 49 days. In discoid colonies, filaments curved and intertwined in the internal thalli, and gum formed outside the colonies. After 56 days, the diameters of discoid colonies reached 15 mm (Fig. 4d).

Although the life cycle and development of *N. commune* has been reported earlier, these studies mainly focused on filaments and microcolonies (Martinez *et al.*, 1985; Komárek *et al.*, 1989; Dodds *et al.*, 1995; Mollenhauer, 1998). Few researches have attempted to study the development of macrocolonies and discoid colonies because there is little knowledge about their cultivation under artificial conditions (Martinez *et al.*, 1986; Crispim and Gaylarde, 2005). In the present study, macrocolonies and discoid colonies of *N. commune* were successfully cultured, and the diameters of macrocolonies reached 5 mm and discoid colonies reached 15 mm, respectively. The successful culture of macrocolonies and discoid colonies created an opportunity to study morphological variation and biomass growth of *N. commune.*

The present results provide detailed information on morphological changes of microcolonies, macrocolonies and discoid colonies during the growth period of *N. commune*. Microcolonies develop from filaments after cells division and aggregated cell mass are formed along the filaments, and the diameter of microcolonies are 200-250 µm after being cultured for 21 days. Macrocolonies can be produced from microcolonies after microcolonies continued to increase in size, and the largest diameter reached 5 mm after 42 days. Macrocolonies can rupture and develop into discoid colonies after being cultured for 49 days. Morphological changes during growth period can be used to estimate the growing state of colonies and to guide the artificial cultivation of *N. commune*.

The low growing rate has been viewed as an obstacle in the artificial culture of *Nostoc* (Gao and Ye, 2003). The current study suggests that biomass concentration, fresh weight and dry weight of *N. commune* could increase with the culture time. And they grew faster at a speed of 40 rpm than at the other speeds, so morphological variation and biomass growth of *N. commune* were studied when colonies were cultured at 40 rpm. The biomass of *N. commune* increased eleven times more at 40, 60 and 80 rpms in 14 days. High growth rate showed that new technique could be used for artificial cultivation of *N. commune.*

At 0 and 20 rpms, secretion of colonies adhered to the surface of colonies and could form an investment (Hill *et al.*, 1994). The investment could prevent colonies from nutrient absorption and gas exchange, and resulted in light attenuation when light passed through the colonies. This may be why the lower colonies' concentrations were obtained at low shaking speeds (Su *et al.*,2008).

At 60 and 80 rpm, the rupture of colonies can take place and exocellular polysaccharidic investment cannot form. The exocellular polysaccharidic investment is a stable morphological structure in *Nostoc* and cyanobacteria (Martin and Wyatt, 1974; Bazzichelli *et al.*, 1989; Philippis *et al.*, 2000). Rupture can take place during the development of microcolonies and macrocolonies due to rotation of rocking incubator and renewing BG11 medium. The rupture can lead to disintegration of colonies and release of filaments and hormogonia. At 60 and 80 rpm, microcolonies cannot grow into macrocolonies due to bursting, and rupture of macrocolonies can lead to the formation of new microcolonies and are unable to form discoid colonies (Prosperi, 1989; Kondratyeva and Kislova, 2001).

Therefore, it is important to control shaking speed and to prevent the colony from rupturing during cultivation of *N. commune.* The mechanism of colonies' burst remains unclear. Therefore, studying and controlling colonies' burst can help microcolonies grow into macrocolonies, and can help macrocolonies grow into discoid colonies (Gao *et al.*, 2003; Deng *et al.*, 2008b).

The results of this study can contribute not only to the protection and exploitation of *N. commune*, but could also provide a standarized technique for the large-scale industrial production of *N. commune*.

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