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Cultivation of *Nostoc flagelliforme* on Solid Medium

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Abstract - In order to construct an artificial cultivation of *Nostoc flagelliforme* on solid medium, we attempted to assess the viability of approaches, which utilized either BG-11 agar or sand medium using both sterile and non-sterile algal segments. In the trial in which the BG-11 agar medium was inoculated with the non-sterile algal segments, the algae exhibited the rapid growth in the initial 4 days of cultivation. However, after 4 days of cultivation, the growth rate of the algae slowed, and the algal growth was completely stopped by 7 days of cultivation. When the BG-11 medium was inoculated with the sterile algal segments, the algae exhibited the rapid growth for a longer period of 8 days, reaching a length of 24.9 mm. The growth rate during this period was measured to be 24.5%. After the 8 days of cultivation, the algal growth rate began to slow and had almost stopped by the 13 days of cultivation. On the other hand, when the sterile algal segments were inoculated onto a sand plate, the algal segments decomposed, reaching total decomposition after 11 days of cultivation. By way of contrast, the desiccation treatment samples continued to grow for 14 days of cultivation. After 14 days of cultivation, the algae achieved a length of 26.1 mm, with a growth rate of 30.6%. Our results indicate that periodic desiccation may constitute an effective strategy for the prevention of algal decomposition.

Key words : *Nostoc flagelliforme*, cyanobacterium, blue green algae, epiphytic microorganisms

INTRODUCTION

Nostoc flagelliforme (Berk. & Curtis) (Bornet & Flahault) is an edible terrestrial cyanobacterium (blue-green algae), which is normally found in desert or semi-desert regions, including Algeria, China, Czechoslovakia, France, Mexico, Mongolia, Morocco, Russia, Somalia, and the USA (Li 1991). *N. flagelliforme* is a pioneer in desert soil, and performs crucial functions in the improvement of soil and the environment (Tang *et al.* 2000). *N. flagelliforme* is filamentous, and normally not branched, cylindrical, or lamellate. False divergent branching, however, has occasionally been observed in this species. *N. flagelliforme* is known to assume a variety of morphologies, according to its habitat. For example, samples of this species of algae, which grow in soil with a relatively high moisture content, tend to assume a flat shape (Shi *et al.* 1992). One end of each *N. flagelliforme* filament is normally attached to lumps of soil, stones or plants, and appears to be adhesive. *N. flagelliforme* contains canthaxanhin, in addition to the other compounds normally found in Nostoc species, which include echinenone, myxoxanthophyll, beta-carotenoid, allophycocyanin, phycocyanin and chlorophyll. It also

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contains 20~30% of protein, with 19 amino acids, accounting for 39% of total amino acids by weight, about 56~57% of carbohydrates, 1.8% of Ca, 3.3% of N, and 0.1% of P (Lu *et al.* 1990). Moreover, *N. flagelliforme* is called 'Facai' (hair vegetable) by the Chinese, ostensibly due to its hair-like appearance. *N. flagelliforme* has long been consumed in China, and is also prized by overseas Chinese, by virtue of its food and herbal values, as well as its spiritual image. In China, *N. flagelliforme* has long been collected and traded. However, this valuable resource is becoming less and less available as the market demand increases as the result of economic growth in China and elsewhere. However, land exploitation has severely reduced the area in which *N. flagelliforme* can naturally grow, further exacerbating the potential shortage. In Ningxia province, the area in which this species could be found was approximately 3.1×10^6 ha in the 1960s; however, by the 1980s, it had decreased to about 1.7×10^6 ha (Dai 1992) In July of 2000, the Chinese government forbade the collection and trade of *N. flagelliforme*, in order to protect the remaining *N. flagelliforme* sources, as well as its natural environment. In order to preserve this natural resource and still fulfill market demand for *N. flagelliforme*, a host of researchers have reported that *N. flagelliforme* tends to decompose after a given period of cultivation under artificial conditions (Wang *et al.* 1989; Wang *et al.* 1992; Zhu *et al.* 2002; Li *et al.* 2003). Although a great deal of research has been conducted in order to establish a viable set of cultivation conditions for *N. flagelliforme* on agar and sand media, *N. flagelliforme* has yet to be successfully cultured (Qian *et al.* 1989; Huang *et al* 2001; Li *et al.* 2001).

In this study, in order to construct an artificial cultivation of *Nostoc flagelliforme* on solid medium, we attempted to inoculate BG-11 agar and sand medium using both non-sterile and sterile algal segments. The primary objective of this study was to determine the reasons underlying the decomposition of algae in such cultivation, in order to develop a cultivation technique, which would allow the successful cultivation of this terrestrial cyanobacterium.

MATERIAL AND METHOD

1. Collection of samples

N. flagelliforme was collected from the eastern side of the Helan Mountain in Yinchuan, Ningxia, and also in other places, including Qingtongxia, Ningxia, Xining, Qinghai, and Alashanzuoqi, in Inner Mongolia. *N. flagelliforme* was collected from the ground and was stored dry in a disinfected glass bottle. The *N. flagelliforme* cultures were preserved for a period of less than 10 days.

2. Isolation and counting of epiphytic microorganisms

N. flagelliforme was soaked in sterile water for 2 hours, in order to allow the algae to fully absorb the water. The samples were then filtered after grinding. 0.1 mL of the filtrate was inoculated on a medium plate, and epiphytic microorganisms were isolated and counted after 1~3 days of cultivation in an incubator, at 28°C.

3. Treatment and culture of samples

Algae were recollected after fully absorbing the water, and were accurately cut into 20 mm long segments and cultivated according to the following method: The algae were inoculated onto a BG-11 agar medium plate (Wang *et al.* 1992) and positioned on cultivating shelves for cultivation (Treatment a). The algae were immersed in 0.1% $HgCl_2$ solution for 30 seconds after fully absorbing the water, in order to kill any epiphytic microorganisms, then washed 5 times with aseptic water. Surface-disinfected algae were then inoculated onto a BG-11 agar medium plate, and cultivated (Treatment b). The algal samples were immersed in BG-11 medium for 90 minutes beginning at 8:00 everyday, then inoculated onto a sand plate, which contained 1cm of sand instead of agar medium. The algae were kept wet until the next day at 8:00 by spraying with distilled water (Treatment c). The algae were immersed in BG-11 medium for 90 minutes, beginning at 08:00 everyday, then inoculated onto a sand plate (Treatment d). The algae were kept wet until 20:00 by spraying with distilled water, and were then exposed to air for drying until the next day at 08:00. The algae samples were then placed on cultivating shelves for cultivation. Illumination was provided by cool white fluorescent tubes from 08:00 to 20:00 everyday, at a light intensity of 5000 μmol $m^{-2}s$. Cultivation was conducted at 25°C in the light, and 15°C in the dark.

RESULTS AND DISCUSSION

1. Cultivation of non-sterile algae segments on the BG-11 agar medium

When the non-sterile algal segments were inoculated onto the BG-11 agar medium (treatment a), the algae exhibited rapid growth within 4 days of cultivation, and the length of the algae increased by 10.5%. However, after the 4 days of cultivation, the growth rate of the algae slowed, and growth had completely stopped by the 7 days of cultivation (Table 1 and Fig. 1). After 3 days of cultivation, microscopic observation revealed the formation of bacterial micro-colonies, as well as local algal decomposition. This process of disintegration became increasingly obvious after 4 days of cultivation, with marked reductions in the elasticity of the algae and the appearance of a bacterial moss on the medium surrounding the algae. After 5 days of cultivation, the algal decomposition was visible to the naked eye, and the elasticity of the algae had been completely destroyed. On the 8th day of cultivation, the algae were found to be fully decomposed (Fig. 2).

Table 1. Result of cultivation of non-sterile algae segments (n = 100)

Time of cultivation (days)	Growth (%)	State of algae
0		Normal
1	2.0	Normal
2	4.51	Normal
3	8.5	Fairly normal
4	10.5	Elasticity decreased
5	11.5	Decomposed locally
6	12.5	Decomposed
7	13.0	Decomposed badly
8	13.2	Decomposed absolutely

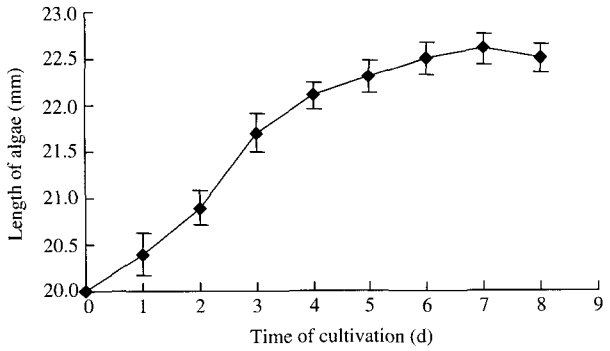

Fig. 1. Result of cultivation of non-sterile algae segments (n = 100).

2. Cultivation of sterile algae segments on the BG-11 agar medium

When the sterile algal segments were inoculated onto the BG-11 agar medium (treatment b), the algae exhibited the rapid growth within 8 days, reaching a length of 24.9 mm, with a growth rate of 24.5% measured during this period. However, this growth rate began to slow after 8 days of cultivation, and growth had almost completely stopped by the 13 days of cultivation (Fig. 3A). The algae maintained their integrity for 14 days of cultivation. These algal segments that had been cultivated for 14 days were then cut into 20 mm pieces, and inoculated onto fresh BG-11 agar medium plates again. The growth patterns of these pieces were similar to those previously reported. After the 8 days of cultivation, the algae reached a length of 24.6 mm, with a

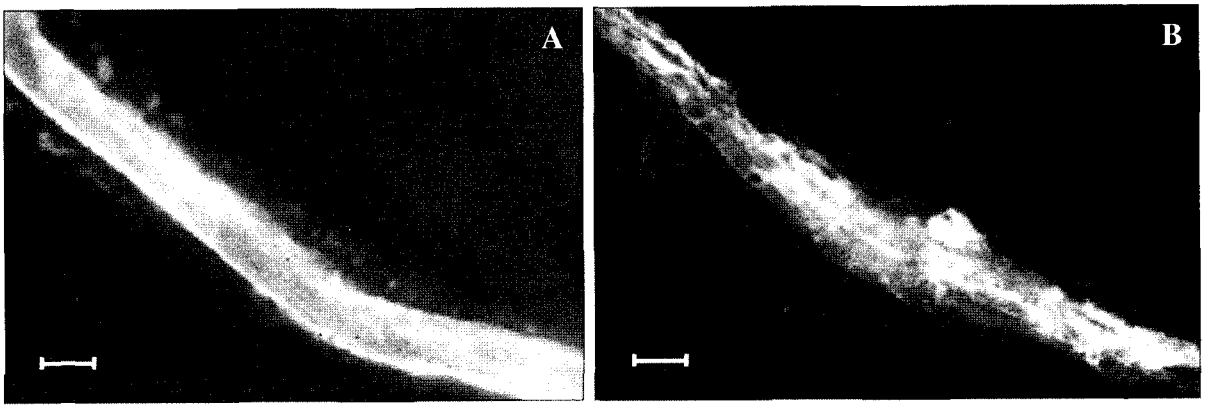

Fig. 2. Photographs of non-sterile segments of algae. A: 4 days of cultivation; B: 8 days of cultivation. Bar = 1mm.

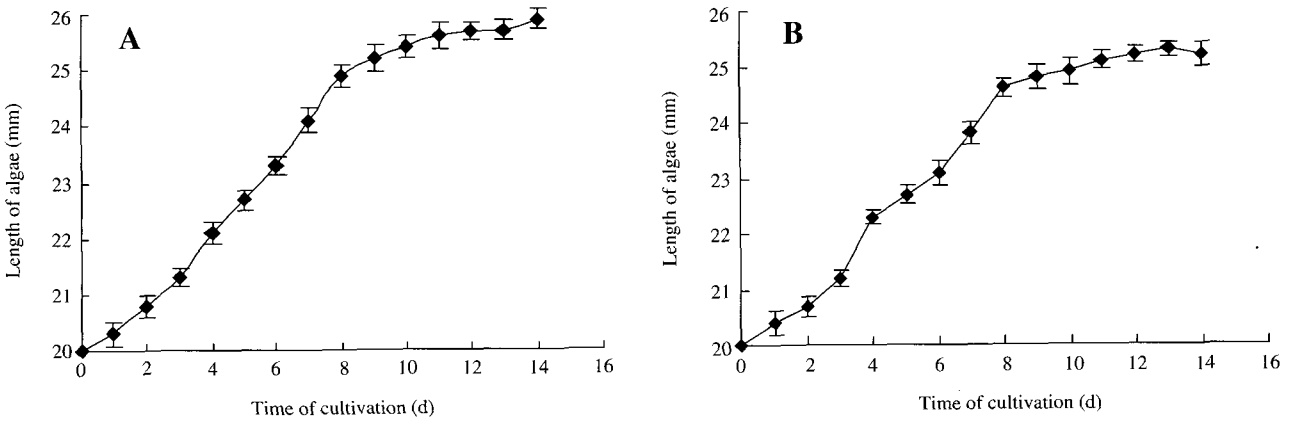

Fig. 3. Result of cultivation of sterile algae segments (n = 100). A: Cut into 20 mm and cultivated. B: Re-cut into 20 mm and cultivated after being cultivated for 14 days.

growth rate of 23.0% (Fig. 3B). This indicates that the observed reduction in the growth of the algae was caused by a shortage of nutriment in the medium. The algae maintained their integrity for 14 days of cultivation, indicating that the reason for the algal decomposition, in this case, was the competitive growth of epiphytic microorganisms. If this is the case, it should be possible to grow algae at normal rates for long periods by eliminating the epiphytic bacteria by surface disinfections.

3. Epiphytic microorganisms in different growing areas

We isolated epiphytic microorganisms from different samples. These microorganisms were identified as bacteria, belonging to the *Bacillus* sp. Fungi were not detected on the surfaces of the experimental samples. The numbers of bacteria, which grew on the samples, were found to vary considerably, according to the areas in which they grew. The numbers of bacteria growing on the sample collected from the eastern side of Helan Mountain were far higher than those observed on the samples collected from the other three areas (Fig. 4). The eastern side of Helan Mountain has fairly good rainfall, especially compared to the rainfall rates of the other three areas. This suggests that water is the principal factor influencing the growth of epiphytic bacteria.

N. flagelliforme grows in desert and semi-desert areas, in which the annual rainfall is about 50 ~ 300 mm, whereas

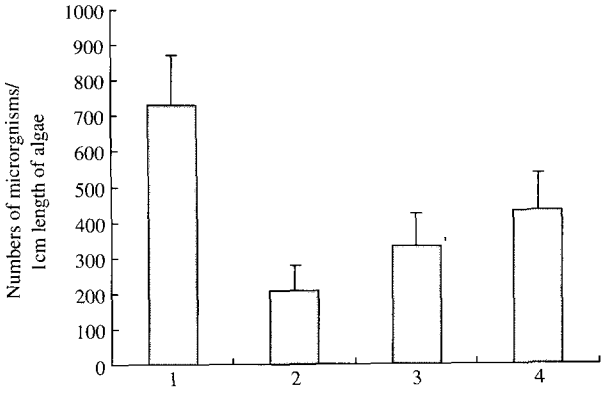

Fig. 4. Numbers of epiphytic microorganisms in different growing areas. The length of algae was measured after algae absorbed water fully (n = 100). 1: Helan mountain, 2: Qingtongxia, 3: Alashanzuoqi, 4: Xining.

the annual evaporation is 10 to 20 times the amount of rainfall (Wang and Liang, 1989). In its natural habitats, the water which fuels the growth of *N. flagelliforme* originates from brief rainfalls, and the dew that forms at night. Due to the rapidity with which it loses water after absorbing it (Gao. 1998), *N. flagelliforme* is subjected to dry conditions most of the time, and is therefore naturally not susceptible to the predations of epiphytic bacteria, which require abundant water. However, in artificial cultures of *N. flagelliforme*, the water provided tends to be sufficiently abundant to meet the needs of algal growth. As a result, epiphytic bacteria are also able to reproduce abundantly during the cultivation process, resulting in algal decomposition.

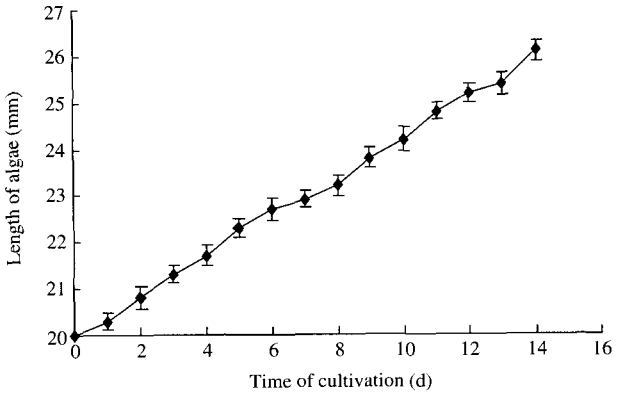

Fig. 5. Result of cultivation of algae segments on sand plate (n = 100).

4. Cultivation of algae segments on sand medium

On the sand plate, the algal segments maintained in a wet state (treatment c) completely decomposed after only 11 days of cultivation. However, the desiccation treatment samples (treatment d) maintained integrity and growth rates for 14 days of cultivation. At the end of 14 days, the algae in this group reached a length of 26.1 mm, with a growth rate of 30.6% (Fig. 5). This result indicates that periodic desiccation constitutes an effective method for the prevention of algal decomposition. Due to the complexity inherent to surface disinfection, as well as the effects induced by disinfector "hangover", surface disinfection is inappropriate for artificial *N. flagelliforme* cultures.

CONCLUSION

In order to preserve this valuable natural resource and still fulfill market demand for *N. flagelliforme*, new methods for artificial cultivation are clearly necessary. Therefore, we attempted to determine the reasons underlying the decomposition of the algae, in pursuit of a viable cultivation technique for the artificial cultivation of this terrestrial cyanobacterium. In order to cultivate any such edible cyanobacterium, the growth and spread of epiphytic bacteria must be controlled. Surface disinfections and periodic desiccation are both fairly effective methods for the control of epiphytic bacterial growth. However, due to the inherent complexity of operation involved with surface disinfection, as well as the deleterious effects associated with disinfector "hangover", surface disinfection is not a suitable technique in the artificial cultivation of *N. flagelliforme*. In large-scale field cultivation of *N. flagelliforme*, the algae should be watered and water absorption should be maintained during the daytime, in order to stimulate growth when the temperature and solar radiation conditions are suitable, and kept dry at night, in order to control the growth of epiphytic bacteria.

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