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Accumulation of Exopolysaccharides in Liquid Suspension Culture of *Nostoc flagelliforme* Cells

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Abstract The liquid suspension culture of dissociated *Nostoc flagelliforme* cells was investigated. It was found that the growth rate of *N. flagelliforme* cells and the accumulation of exopolysaccharides (EPS) increased prominently when $NaNO_3$ and KH_2PO_4 were added in the liquid BG-11culture medium though phosphate had little effect on EPS yield for specific mass of cells. *N. flagelliforme* cells grew well at 25 °C and neutral pH, however, a lower or higher temperature and weak alkaline can promote EPS accumulation. With the increase of the light intensity, the growth rate of *N. flagelliforme* cells and the EPS accumulation increase accordingly. When *N. flagelliforme* cells was cultured in BG-11 medium added with 2.5 g L^{-1} of $NaNO_3$ and 0.956 g L^{-1} of KH_2PO_4 at 25 °C with 60 μmol photon m^{-2} s^{-1} of light intensity, 1.05 g L^{-1} cell density and 89.9 mg L^{-1} EPS yield were achieved respectively. Adopting the optimal conditions established in flask culture, the liquid culture of *N. flagelliforme* cells in 20-L photobioreactor for 16 days was conducted and a maximum biomass of 1.32 g L^{-1} was achieved, which was about 17.6-fold of that in the initial inoculation. The yield of EPS was 228.56 mg $L^{-1}$and about 2.23-fold of that in flask culture. Moreover, the polysaccharides' material was released into the culture medium during cell growth. These released polysaccharides (RPSs), which can be easily recovered from the medium, are favorable for industrial applications.

Keywords *Nostoc flagelliforme* cells · Liquid suspension culture · Exopolysaccharides · Culture conditions

The interest in polysaccharides from cyanobacteria is greatly increased recently for their some peculiar advantages over other polysaccharides extracted from plants or marine

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Table 1 Dry cell weight and yield of EPS production of *N. flagelliforme* cells grown for 20 days with different levels of $NaNO_3$ and K_2HPO_4.

Nutrients levels	EPS (mg L^{-1})	DW (g L^{-1})	EPS[a] (mg g^{-1})	Chl contents (mg L^{-1})
$NaNO_3$ (gL^{-1})				
1.0	44±5.3	0.72±0.02	61.8±4.2	5.05±0.02
1.5	73±2.3	0.88±0.03	83.8±2.6	6.16±0.04
2.5	100±7.6	1.13±0.02	94.1±5.3	6.41±0.02
3.5	62±5.0	0.90±0.04	69.3±4.7	6.32±0.03
K_2HPO_4 (gL^{-1})				
0.024	71±5.3	0.87±0.02	81.4±3.3	6.13±0.01
0.238	76±5.3	0.92±0.06	83.3±3.1	6.42±0.03
0.956	97±5.3	1.21±0.02	81.1±4.7	8.43±0.04
1.912	93±5.3	1.14±0.05	82.3±2.6	7.98±0.02

[a] The EPS yield of specific mass; data were expressed as mean±SEM(n=3)

macroalgae [1]. There are more than 100 cyanobacteria strains belonging to 20 different genera which have been investigated with regard to the production and the released of extracellular polysaccharides (EPS) into the culture medium [2]. The EPS play an important role in protecting cells from various stresses [3, 4]. As a new biomaterial, it was found to have a wide range of applications such as improvement of water-holding capacity of soil, removal of heavy metals from wastewater, and being used as food additives [5–7].

Nostoc flagelliforme, which distributes on arid and semi-arid areas, is an edible terrestrial cyanobacteria with great economic value. In China, *N. flagelliforme* is favored by people because of its food and herbal value as well as its pronunciation of "Fa cai", which means "getting rich" in Chinese. In recent years, it was reported that the hot water extract of *N. flagelliforme* has anti-tumor activity and its effective content, nostoflan, is a kind of acidic polysaccharide. Moreover, this acidic polysaccharide was confirmed to have a remarkable anti-virus effect on many kinds of enveloped viruses such as HSV-1, human cytomegalovirus, and influenza A virus [8]. Therefore, *N. flagelliforme* may represent a candidate for the provision of novel polysaccharides that exert a variety of biological effects.

Increased market demands have resulted in the excessive exploitation of *N. flagelliforme*, which, in turn, causes the endangered status of this species and the deterioration of the environment [9]. Therefore, the Chinese government designated *N. flagelliforme* a Category II Protected Plant in 1999. Under the protection regime, picking and trading of wild *N. flagelliforme* are strictly prohibited. As a result, some processes

Table 2 Result of one-way analysis of variance of effects of $NaNO_3$ and KH_2PO_4 on cell dry weight and EPS yield of *N. flagelliforme* cells.

Effects of $NaNO_3$ on cell dry weight							$NaNO_3$ on EPS yield					
Source of variance	SS	df	MS	*F* value	*P* value	*F* crit	SS	df	MS	*F* value	*P* value	*F* crit
Between groups	0.2564	3	0.0854	103.6061	9.66E-07	7.5909	4895	3	1631.667	58.7071	8.62E-06	4.066
Within groups	0.0066	8	0.0008				222.3467	8	27.7933			
Total	0.2630	11					5117.347	11				

using *N. flagelliforme* as materials can not be carried out. In order to protect the natural *N. flagelliforme* source and the environment, some methods on cultivating natural colonial filaments have been reported both in laboratory and in field [10–13]. However, because its annual growth rate is less than 6%, this technology was not accepted extensively in industry.

In recent years, there is a growing interest in producing desirable metabolites of plant or alga by utilizing cell culture system. Much works has been done on the cell culture of plant cell [14–16] and microalgal species [17–19]. There are many factors which affect cell growth and metabolite accumulation in cell cultures. These include medium components such as phosphate and nitrogen sources, and culture conditions such as temperature, light intensity, aeration rate, and initial pH [17–20]. Anthocyanin content of *Perilla frurescens* cells differed with different irradiation period [14]. Compared with that in darkness, the growth of callus cells was significantly promoted by continuous red light, and moderately by white light, blue light had nearly no effect on the cell growth [21]. Changes in environmental factors may alter metabolite content in microalgal species. EPA content in *Ellipsoidion sp*. increased with increasing nitrate concentration [22]. Phosphate-starved *Thalassiosira pseudonana* resulted in a decrease of EPA [19]. A preferable growth rate of *N. flagelliforme* cells was reported from the cultivation of free cells isolated from colonial filaments in liquid medium [23–25]. But these studies mainly focus on the growing process of the *N. flagelliforme* cells, the culture condition and the rates of photosynthesis and respiration etc. There is a great lack of knowledge that exists on the relationships between accumulation of exopolysaccharides and the factors that regulate *N. flagelliforme* growth and EPS biosynthesis, thus, this study investigated the effect of nitrate and phosphate concentrations, light intensity, initial pH of culture medium, and temperature on the growth and exopolysaccharides accumulation of *N. flagelliforme* cells in liquid suspension culture condition in order to obtain the optimal conditions for the collection of exopolysaccharides of *N. flagelliforme* cells.

Materials and Methods

Alga Strains and Growth Conditions

The *N. flagelliforme* was collected in the eastern side of the Helan Mountain in Yinchuan, Ningxia of China and stored in dry conditions at room temperature for 36 months before use in experiments. The dissociated (isolated) cells were obtained according to the previous methods we have reported [10, 26]. The cells were cultured in BG-11 medium in 500-ml shake-flask containing 200 ml medium at 25 °C under continuous illumination of 60 μmol photon m^{-2} s^{-1} and grown to its late

K_2HPO_4 on cell dry weight						K_2HPO_4 on EPS yield					
SS	df	MS	*F* value	*P* value	*F* crit	SS	df	MS	*F* value	*P* value	*F* crit
0.2463	3	0.0821	47.5942	1.91E-05	7.5909	1448.25	3	482.75	17.18583	0.0007	7.5909
0.0138	8	0.0017				224.72	8	28.09			
0.2601	11					1672.97	11				

Table 3 Dry cell weight and yield of EPS production of *N. flagelliforme* cells grown for 20 days with different levels of temperature, light intensity, and initial pH of medium.

Cultivation conditions	EPS (mg L^{-1})	DW (g L^{-1})	EPS[a] (mg g^{-1})	Chl contents (mg L^{-1})
Temperature (°C)				
15	37±2.3	0.33±0.03	112±5.3	2.31±0.03
20	43±3.7	0.44±0.05	99±4.7	3.05±0.05
25	73±1.6	1.05±0.06	70±3.2	7.36±0.01
30	67±3.6	0.79±0.03	84±2.9	5.54±0.02
Light intensity (μmol photons m^{-2} s^{-1})				
10	25±2.1	0.27±0.06	93±3.2	1.89±0.01
20	58±3.2	0.59±0.03	98±4.3	4.14±0.02
40	84±5.3	0.88±0.06	95±3.6	6.16±0.05
60	106±3.2	1.14±0.02	93±5.3	7.99±0.03
Initial pH				
5.0	20±5.3	0.41±0.03	49±2.3	2.89±0.02
6.0	37±2.6	0.68±0.02	54±3.3	4.79±0.03
7.0	76±1.8	0.87±0.03	87±2.7	6.11±0.02
8.0	88±3.2	0.84±0.02	104±5.3	5.92±0.03
9.0	106±4.3	0.83±0.05	128±4.3	5.78±0.02

[a] The EPS yield of specific mass; data were expressed as mean±SEM(n=3)

growth phase as inoculums. Prior to inoculation, the inoculums were microscopically examined to make sure that the dissociated cells with capsule are the dominant form.

For the flask culture, each component of the BG-11 medium was sterilized separately by autoclaving at 121 °C for 25 min, after which the cooled solutions were mixed in the 500-ml shake-flasks and 10% (v/v) of the inoculums was inoculated. $NaNO_3$ and K_2HPO_4 were used for the adjustment of N or P levels. And the flask was put on HZQ-Q illuminated electric shaker (Haerbin Dongliang Electronic Technology Company). The cultivation was conducted at given temperature, pH and light intensity with 130 r $min^{-1}$of rotating speed. Each experiment was conducted with three replicates. On this basis, the amplified cultivation was continued in a 20-L turbine-agitated photobioreactor. The reactor was 48 cm high and 26 cm in diameter. Three white fluorescent lights were vertically installed and two six-bladed turbine impellers with the diameter of 9.5 cm were also fixed inside the bioreactor. The vertical distance between the two impellers was 14.0 cm and the lower impeller was positioned 8.0 cm above the reactor bottom. The reactor was aerated through an air filter and a ring sparger with a pore size of 1.0 mm, which was 3.0 cm above the

Table 4 Result of one-way analysis of variance of effects of temperature, pH and light intensity on cell dry weight, and EPS yield of *N. flagelliforme* cells.

Effects of temperature on cell dry weight							Temperature on EPS yield					
Source of variance	SS	df	MS	F	P-value	F crit	SS	df	MS	F	P-value	F crit
Between groups	0.9782	3	0.3261	165.1013	1.56E-07	7.5909	2784.25	3	928.0833	118.8581	5.66E-07	7.5909
Within groups	0.0158	8	0.0019				62.4666	8	7.8083			
Total	0.9940	11					2846.717	11				

reactor bottom and the air flux was 0.8 m^3h^{-1}. The cultivation was carried through at 25 °C and 200 r min^{-1} of stirring speed with continuous illuminated condition.

Extraction of EPS of *N. flagelliforme*

The culture solution was condensed with ultrafiltration. Thereafter 4 times of alcohol (95% *v*/*v*) was added with stirring and deposited for 24 h and then the coarse exopolysaccharides were collected by centrifugation.

Analytical Methods

The growth increment of the *N. flagelliforme* cells was measured with dry mass (DW) method. Polysaccharides in culture solution were determined by means of modified phenol-sulphuric acid method [27]. Nitrate concentration was assayed by salicylic acid method and phosphate ion concentration was determined by molybdenum blue colorimetry. Data were analyzed by one-way analysis of variance (ANOVA).

Results and Discussion

Effects of $NaNO_3$ and KH_2PO_4 on Growth Rate of *N. flagelliforme* Cells and Accumulation of EPS

The cell growth rate and the EPS yield are very important parameters for biotechnological applications of cyanobacteria. Liquid suspension culture of dissociated cells offered an alternative approach to get higher growth rate and EPS yield of *N. flagelliforme.* Although the free cells in liquid suspension culture failed to progress into cyanobacterial thallus with a thick gelatinous sheath like the wild ones, a large amount of EPS secreted into the culture medium, which facilitated the EPS extraction process. Because *N. flagelliforme* cells were only encapsulated with a thin slime layer in the liquid culture, their growth got rid of the confine of gelatinous sheath. Accordingly, nitrate and phosphate accommodation became the key limiting factors for cell growth. Table 1 shows that the cell growth and EPS accumulation changes with nitrate or phosphate concentration in the medium. Data were analyzed by one-way analysis of variance. The results of ANOVA of each factor effects on the cell dry weight or EPS yield were given in Table 2. Elevating nitrate concentration from 1.0 g L^{-1} to 2.5 g L^{-1}or phosphate concentration from 0.024 g L^{-1} to 0.956 g L^{-1} raised the

light intensity on cell dry weight

SS	df	MS	F	P-value	F crit
1.2642	3	0.4214	198.3059	7.59E-08	7.5909
0.017	8	0.0021			
1.2812	11				

light intensity on EPS yield

SS	df	MS	F	P-value	F crit
10946.25	3	3648.75	275.4813	2.07E-08	7.5909
105.96	8	13.245			
11052.21	11				

cell dry weight by 56.1% or 37.6% respectively ($P<0.01$). However, when further increasing nitrate concentration to 3.5 g L^{-1} or phosphate concentration to 1.912 g L^{-1}, the cell dry weight of *N. flagelliforme* cells was slightly decreased. In our previous study we reported photosynthetic rate of *N. flagelliforme* cells was increased to a maximum of 148.9 μmol mg Chl^{-1} h^{-1}, when $NaNO_3$ was 2.5 g L^{-1} in culture medium [23]. The data in this work support our previous conclusion. The EPS productions correlate with the cell growth ($P<0.05$). Contradictory results have been reported for different species of cyanobacteria regarding the relationship between nitrogen metabolism and EPS synthesis. Nitrogen starvation has been described to enhance EPS synthesis in both diazotrophic and non-diazotrophic cyanobacteria [17]. On the other hand, growth and EPS production was higher in the presence of combined nitrogen in different *Anabaena* strains [28]. In this work, under 2.5 g L^{-1} of optimal nitrate concentration or 0.956 mg L^{-1} of optimal phosphate concentration, the highest EPS production came up to 106.3 mg L^{-1} or 97.6 mg L^{-1} respectively. The EPS yield of specific mass *N. flagelliforme* cells increased with nitrate concentration ($P<0.05$). However, phosphate concentration has no obvious effect on that.

Effects of Temperature, pH, and Light Intensity on Growth Rate of *N. flagelliforme* Cells and Accumulation of EPS

The impacts of temperature on cell growth and EPS accumulation are presented in Table 3. *N. flagelliforme* cells grew well within the range of 15–30 °C. After 20 days cultivation, the highest cell dry weight of 1.05 g L^{-1} was found at 25 °C and the volumetric yield of EPS also came to the maximum, though the EPS yield for specific mass of *N. flagelliforme* cells at 25 °C was the minimum ($P<0.01$).

The light intensity has marked effect on the growth of *N. flagelliforme* cells in liquid suspension culture (Table 3). Data were analyzed by one-way analysis of variance. The results of ANOVA of each factor effects on the cell dry weight or EPS yield were listed in Table 4. When light intensity increased from 10 μmol photon m^{-2} s^{-1} to 60 μmol photon m^{-2} s^{-1}, the dry cell weight at harvesting time was increased from 0.27 g L^{-1} to 1.14 g L^{-1} accordingly ($P<0.05$). The light intensity has similar effect on EPS production to cell growth rate ($P<0.05$). But the EPS yield of specific mass *N. flagelliforme* cells was unchanged. The cell growth rates and EPS yields at different pH values showed insignificant difference (Table 3). *N. flagelliforme* cells can grow in the wide pH range and grow better near the neural pH condition. However, the alkaline condition is in favor of EPS accumulation ($P<0.01$).

From the above results we knew that *N. flagelliforme* cells in liquid suspension culture could grow in relatively wide ranges of temperature and pH with an optimum growth rate at

Table 4 (continued)

Initial pH on cell dry weight						Initial pH on EPS yield					
SS	df	MS	F	P-value	F crit	SS	df	MS	F	P-value	F crit
0.4446	4	0.1111	111.8859	2.94E-08	5.9943	15423.28	4	3855.821	287.3904	2.84E-10	5.9943
0.0099	10	0.0009				134.1667	10	13.4167			
0.4545	14					15557.45	14				

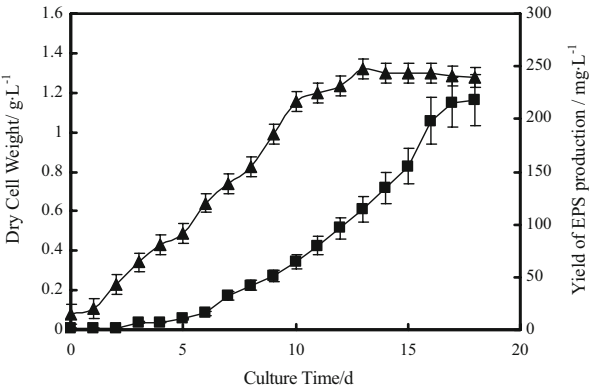

Fig. 1 Time profiles of cell growth and EPS accumulation of *N. flagelliforme* cells in 20 L bioreactor (*filled square* yield of EPS production; *filled triangle* dry cell weight)

25 °C and pH of 7.0. Some works have reported the effects of temperature on the microalgar cell culture. Optimal temperature for cell growth and metabolite accumulation is dependent on the species cultured, and even for the same species temperatures which are suitable for cell growth and metabolite production may be different [29]. In this study, *N. flagelliforme* cells in liquid suspension culture accumulated more EPS at either lower or higher growth temperatures than at 25 °C (Table 3) ($P<0.01$), which coincided with the view of Bi et al. [25]. The similar phenomenon occurred in suspension cultures of *Perilla frutescens* cells [29]. The mechanism of effects of temperature on cell growth or metabolite production needs further studying.

There is a common link between the respiratory and photosynthetic electrons transport chains in cyanobacteria. Light intensity not only regulates photosynthetic electron transport rate but also the respiratory electron transport rate, further, it can alter the immobilization rate of CO_2, the respiration rate and the energy level of algae cells. The previous work showed that the light period and light wavelength both have important effects on cell growth or metabolite production [14, 21]. However, whole light period beneficial to cell growth and EPS accumulation [30]. *N. flagelliforme* cells can trap more light energy as the energy source and assimilate CO_2 as the carbon source in growth when

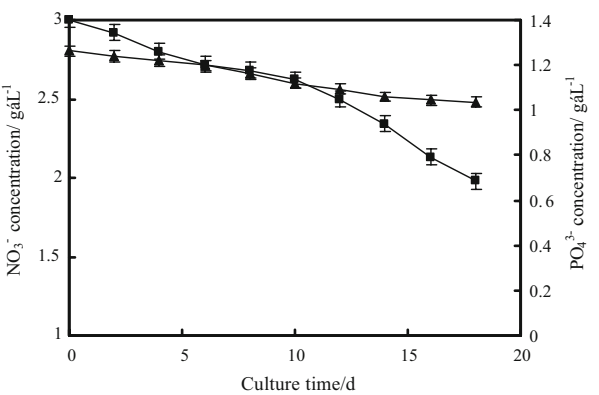

Fig. 2 Time profiles of $NO_3^-$and PO_4^{3-}concentration in the medium of 20 L photobioreactor (*filled square* NO_3^- concentration; *filled triangle* PO_4^{3-} concentration)

the light intensity is raised. Therefore, increasing the light intensity can increase the cell growth and EPS accumulation. A similar result was previously reported for other species in this genus [17, 31].

In general, cells can only grow within a certain pH range, and metabolite formation is also often affected by pH [20]. For example, *Ganoderma lucidum* can grow well at initial pH value within the range of 3.5–7.0. At an initial pH of 6.5, a maximum in biomass of 17.3 g L^{-1} by dry weight (DW) was obtained, as well as a maximal specific production of ganoderic acid of 1.20 mg 100 mg^{-1} DW and total production of 207.9 g L^{-1}. The analogous results were obtained in this paper. *N. flagelliforme* cells can grow within the initial pH value varied from 5.0 to 9.0. The weak alkaline condition facilitated EPS accumulation, which was in accordance with ecological characteristics of *N. flagelliforme* in nature.

The time profile of cell growth and EPS accumulation of *N. flagelliforme* cells in a 20-L photobioreactor is shown in Fig. 1 and the nutritional salts consumption in liquid suspension culture are shown in Fig. 2. It could be seen that in the whole culture phase the nutrient salt concentration decreased. The *N. flagelliforme* cells in photobioreactor showed a linear growth without lag phase and came to stable phase after 14 days. The highest cell concentration came up to 1.32 g L^{-1}, which was 17.6-fold of initial inoculation amount. The EPS mainly accumulated after ninth day of cultivation. At the end of cultivation, the EPS yield reached 228.56 mg L^{-1}.

Improving the cell growth rate and EPS accumulation is a key problem for the cultivation of microalgae. Liquid suspension culture in closed photobioreactor provided a feasible solution for this problem and makes the large-scale cultivation *N. flagelliforme* cells applicable. In this study, *N. flagelliforme* cells grew well in liquid suspension culture of 20-L photobioreactor. Based on our present study, there is 1.32 g L^{-1} of biomass and 228.56 mg L^{-1} of EPS were achieved for 16 days cultivation of *N. flagelliforme* cells in 20-L photobioreactor.

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