

Applying the strategy of light environment control to improve the biomass and polysaccharide production of *Nostoc flagelliforme*

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Abstract The strategy of light environment control was applied to improve biomass and extracellular polysaccharides (EPS) production of the cyanobacterium *Nostoc flagelliforme* by adjusting multiple wavelengths (red 660 nm, blue 460 nm, green 520 nm) and light intensity assisted by nitrogen source optimization. A mixed wavelength with low light intensity was more suitable for cell growth. Wavelength shift approach, i.e., manipulation of light wavelength at appropriate culture stages, increased both biomass and EPS production, and optimum shift time was at 9 days. The effects of four nitrogen sources under different light conditions were subsequently evaluated, and urea showed the best performance. The optimized wavelength shift approach (9-day illumination with white light followed by 9-day culture with mixed wavelengths of red/blue/green = 12:5:5) with urea as nitrogen source improved the biomass from 0.72 ± 0.02 to 1.20 ± 0.02 g L⁻¹ (i.e., by 66 %) and EPS production from 27.31 ± 1.00 to 86.65 ± 2.56 mg L⁻¹ (i.e., by 217.3 %). These results provide information on novel culture strategies for microalgal biotechnology by applying light environment control.

Keywords Light environment control · Biomass · Extracellular polysaccharides · Cyanobacterium · *Nostoc flagelliforme* · Light wavelength

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Introduction

Microalgae as potential sources of high-value products have attracted considerable attention, because their biomass and/or extracts are used for various applications such as food and feed (e.g., pigments, polyunsaturated fatty acids, antioxidants) and biofuels (e.g., oils for biodiesel, carbohydrate for bioethanol and biohydrogen) (Spolaore et al. 2006; Chisti 2007; Saeid et al. 2013). Among these products, extracellular polysaccharides (EPS) have wide applications in the food, pharmaceutical, and cosmetic industries because of their rheological properties and biological activities (Parikh and Madamwar 2006; Han et al. 2014a). There are more than 100 cyanobacterial strains belonging to 20 different genera which have been investigated with regard to the production and the release of EPS into the culture medium. The EPS derived from cyanobacteria also often shows advantages over other EPS extracted from plants or microalgae (Pereira et al. 2009).

Nostoc flagelliforme is an edible terrestrial filamentous cyanobacterium with great food and herbal values found in arid and semi-arid areas. It has been used as food in China for more than 2000 years, and contains 20–23 % protein with 19 amino acids (8 of which are essential for human health), about 56–57 % carbohydrates, with its herbal values were recognized more than 400 years ago (Gao 1998). The hot water extract of *N. flagelliforme* has been shown to possess antitumor activity and this may be attributable to nostoflan. Nostoflan, a type of acidic EPS, was confirmed to have outstanding antiviral activity on a variety of enveloped viruses (e.g., HSV-1, human cytomegalovirus, and influenza A virus) (Kanekiyo et al. 2005). Besides that, we previously demonstrated that the EPS of *N. flagelliforme* had high intrinsic viscosity, good emulsification activity, and excellent flocculation capability, indicating it as a very promising candidate for numerous industrial applications (Han et al. 2014a). However, the yield

of biomass and EPS was too low to meet increasing market demand, strongly limiting the potential for biotechnological applications.

Changes in light quality and light intensity have been found to have significant influence on the growth of cyanobacteria and EPS production (Otero and Vincenzini 2003; Mishra et al. 2012; Yang et al. 2012). Therefore, in order to improve biomass and EPS production, it is crucial to develop an efficient process to maximize the utilization of light energy. Consequently, the strategy of light environment control was proposed, which involves the manual adjustment of light quality, light intensity, light cycle, and other environmental factors (Ravelonandro et al. 2008; Sforza et al. 2012). *Nostoc flagelliforme* has chlorophyll *a*, as well as phycobiliproteins, that complement chlorophyll *a* absorbance, which can greatly increase the photosynthetic ability of cells based on Emerson and Brody's theory (You and Barnett 2004). Therefore, the light wavelengths that can be absorbed by both chlorophyll *a* and the phycobiliproteins have much higher photosynthetic efficiency than those could be absorbed by only one of these pigments. In our previous work, investigating the effects of different wavelengths of light on *N. flagelliforme* culture, it was found that blue and red light could promote biomass accumulation and EPS production (Han et al. 2014b). Subsequent metabolomic analysis revealed the strong dependence of EPS production on light spectra and the involved metabolic mechanism of light wavelengths influencing polysaccharide biosynthesis (Han et al. 2015). Based on our previous findings, the strategy of light environment control by adjustment of mixed wavelength and light intensity was proposed to improve biomass and EPS production of *N. flagelliforme*. The effects of the multiple wavelengths and different wavelength ratios on growth and EPS production were investigated.

Materials and methods

Strain and culture conditions

The *Nostoc flagelliforme* cells (TCCC11757) cultivated in BG-11 medium (Liu et al. 2005) were obtained from the Tianjin Key Lab of Industrial Microbiology (Tianjin, China). Inocula were prepared regularly (bi-weekly) in 500-mL Erlenmeyer flasks at 25 °C under cool-white fluorescent light at 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Before all the experiments the cells were first incubated in a dark room for 3 days in order to reduce stored compounds and to avoid the influence of white light from preculture. After that, the cells were cultivated in 500-mL Erlenmeyer flasks containing 200 mL BG-11 medium. Continuous illumination was supplied under mixed wavelength, and light intensity set as 20, 40, and 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$,

respectively. The evaluated mixing ratios were red/blue = 1:1, 7:15, 15:7 and red/blue/green = 12:5:5, 15:5:2. A schematic diagram of the experimental setup is illustrated in Supplementary Fig. S1. Each light-emitting diode (LED) light (Shenzhen Federal Heavy Secco Electronic Co. Ltd., China) was composed of 22 colored bulbs which emitted at specific wavelengths. Red bulbs (660 nm), blue bulbs (460 nm), and green bulbs (520 nm) with half-band width of 5 nm were used. The wattage and voltage of each colored bulb was 1 W and 220 V. Red and blue lights consisted of 22 red and blue bulbs, respectively. The ratio of red/blue and/or red/blue/green is represented by the number of the red, blue, and green bulbs. The light intensity was measured with a quantum sensor connected to LightScout Dual solar quantum light meter (Spectrum Technologies, USA) and controlled by adjusting the distance between the light source and the Erlenmeyer flasks. The flasks were placed in airconditioned environment at 25 ± 1 °C. Cells grown under cool-white fluorescent lights were treated as control.

The experiments of optimizing mixed wavelengths and light intensity and nitrogen source were run for 16 days, and samples were taken every 4 days for determining the biomass and EPS content. For analyzing the elemental composition, CO₂ fixation rate and photosynthetic pigment content, samples were taken on 16th day of culture. The experiments of wavelength shift optimization were carried out for 18 days, and samples were taken on the 18th day and analyzed for biomass and EPS content. The experiments of wavelength shift approach with urea as nitrogen source were performed for 18 days, and samples were taken every 3 days to measure the biomass and EPS content.

The measurement of cell growth, EPS production, and cellular elemental composition

The growth increment of the *N. flagelliforme* cells was measured with dry mass (DW) method. Twenty milliliter of the culture medium was centrifuged in a 50-mL centrifuge tube at 4000 \times g for 15 min. The supernatant was removed for determining the EPS production, and the tube (preweighed) with the pellet was washed twice with distilled water and then dried at 80 °C until the weight was constant. The supernatant was dialyzed with distilled water for 24 h to remove salts and other small molecules, and then, the EPS content was determined by a modified phenol-sulfuric acid method using glucose as standard (Dubois et al. 1956). The dry cells were finely homogenized using a mortar and pestle, and a portion (<10 mg) was transferred into pure tin cups. A Flash 1112 Series elemental analyzer (Thermo Finnigan, USA) was used to measure the elemental composition of cells according to the previously reported method with slight modification (McNamara et al. 2013).

The measurement of photosynthetic pigment

To determine pigment content, 5 mL of sample was taken and centrifuged at $4000\times g$ for 10 min. The supernatant was discarded, and then, the cells were resuspended in 5 mL of methanol in a closed centrifuge tube and stored at 4 °C for 12 h in the dark. Then, the cells were centrifuged at $10,000\times g$ for 10 min, supernatant was collected and absorbance was measured at 470 and 665 nm, respectively. The content of chlorophyll *a* and carotenoid (mg L^{-1}) were calculated using the equations of Wellburn (1994):

$$\begin{aligned}\text{Chlorophyll } a(\text{mg L}^{-1}) &= 13.43 \times A_{665} \times V_{\text{methanol}}/V_{\text{cell}} \\ \text{Carotenoid}(\text{mg L}^{-1}) &= (1000 \times A_{470} - 44.76 \times A_{665})/221 \times V_{\text{methanol}}/V_{\text{cell}}\end{aligned}$$

Measurement of CO₂ fixation rate and photosynthetic efficiency

The CO₂ fixation rate was calculated from the carbon content of the biomass and the specific growth rate (μ) as described by Yun et al. (1997) as follows:

$$\begin{aligned}R_{\text{CO}_2} &= \mu \times C_c \times \text{dry weight} \times (M_{\text{CO}_2}/M_C) \\ \mu &= (\ln x_2 - \ln x_1) / (t_2 - t_1)\end{aligned}$$

where R_{CO_2} and μ are the CO₂ fixation rate and specific growth rate, respectively, C_c , M_{CO_2} , and M_C are the carbon content of the biomass (%), molecular weights of CO₂, and elemental carbon, respectively; x_2 is the cell concentration at experimental time t_2 ; and x_1 is the cell concentration at time t_1 .

The photosynthetic efficiency was calculated according to the previously reported method (Hase et al. 2000).

Statistical analysis

To ensure reproducibility of results, each experiment was performed with three replications and the values are expressed as the mean \pm standard deviation. The data were analyzed by analysis of variance (ANOVA) or independent-samples *t* test using the SPSS statistical software (version 20.0). The significance level was set at $p < 0.05$.

Results

Effects of mixed wavelength and light intensity on the biomass accumulation

As shown in Fig. 1a, *N. flagelliforme* was sensitive to the changes of light environment and significant difference in cell growth was observed. Under white light, the growth of *N. flagelliforme* was greatly increased from 20 to 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ($F = 2.379$, $p < 0.001$), but there was no

obvious difference when light intensity increased from 40 to 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ($F = 0.263$, $p = 0.358$). For the mixed LED light wavelength treatments, the biomass of red/blue = 1:1, 7:15, and 15:7 treatments decreased with the increase of light intensity. However, with the increase of light intensity, the biomass of red/blue/green = 12:5:5 treatment remained basically unchanged, and the biomass of red/blue/green = 15:5:2 treatment was firstly increased from 20 to 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, but then remained basically constant from 40 to 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. After 16 days of culture, the highest biomass was achieved at treatment of red/blue = 7:15 with the value of $0.78 \pm 0.02 \text{ g L}^{-1}$, which was increased by 8.33 % compared with control group at 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Effects of mixed wavelength and light intensity on the EPS production

The time courses of EPS production under different light conditions are presented in Fig. 1b. Irrespective of the mixed wavelengths and light intensities, the production of EPS increased with increasing cultivation time. After 16 days of culture, EPS production was increased in the order of red/blue = 15:7, red/blue/green = 12:5:5, red/blue = 7:15, red/blue/green = 15:5:2, red/blue = 1:1, and white light-grown cells, which reached 46.57 ± 0.98 , 45.99 ± 3.25 , 37.36 ± 2.52 , 35.80 ± 1.75 , 31.40 ± 0.80 , and $27.31 \pm 1.00 \text{ mg L}^{-1}$, respectively. The EPS production of red/blue = 15:7 and red/blue/green = 12:5:5 grown cells were 1.71 and 1.68 times that of the white-light grown cells, respectively.

Effects of mixed wavelength and light intensity on the elemental composition

In order to investigate the effects of mixed wavelengths and light intensities on the cell elemental composition, the percentage of C, H, and N were measured at light intensities of 20 and 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ after 16 days of culture, because these two light intensities had a significant influence on biomass accumulation and EPS production. The percentages of C, H, and N at 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ were slightly higher than those grown under 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Table 1). Among cells grown under various light conditions, the mixed wavelengths and light intensities had no significant effects on C and H ratio ($F = 3.597$, $p = 0.0630$ for C; $F = 4.720$, $p = 0.0631$ for H), but had significant influence on N ratio ($F = 0.08$, $p = 0.016$). The cells under the treatments of red/blue = 7:15 and red/blue/green = 12:5:5 had a higher percentage of N than those grown under other light conditions, which subsequently caused the changes in C-to-N ratio in cells, indicating the differences in cellular components induced by light conditions.

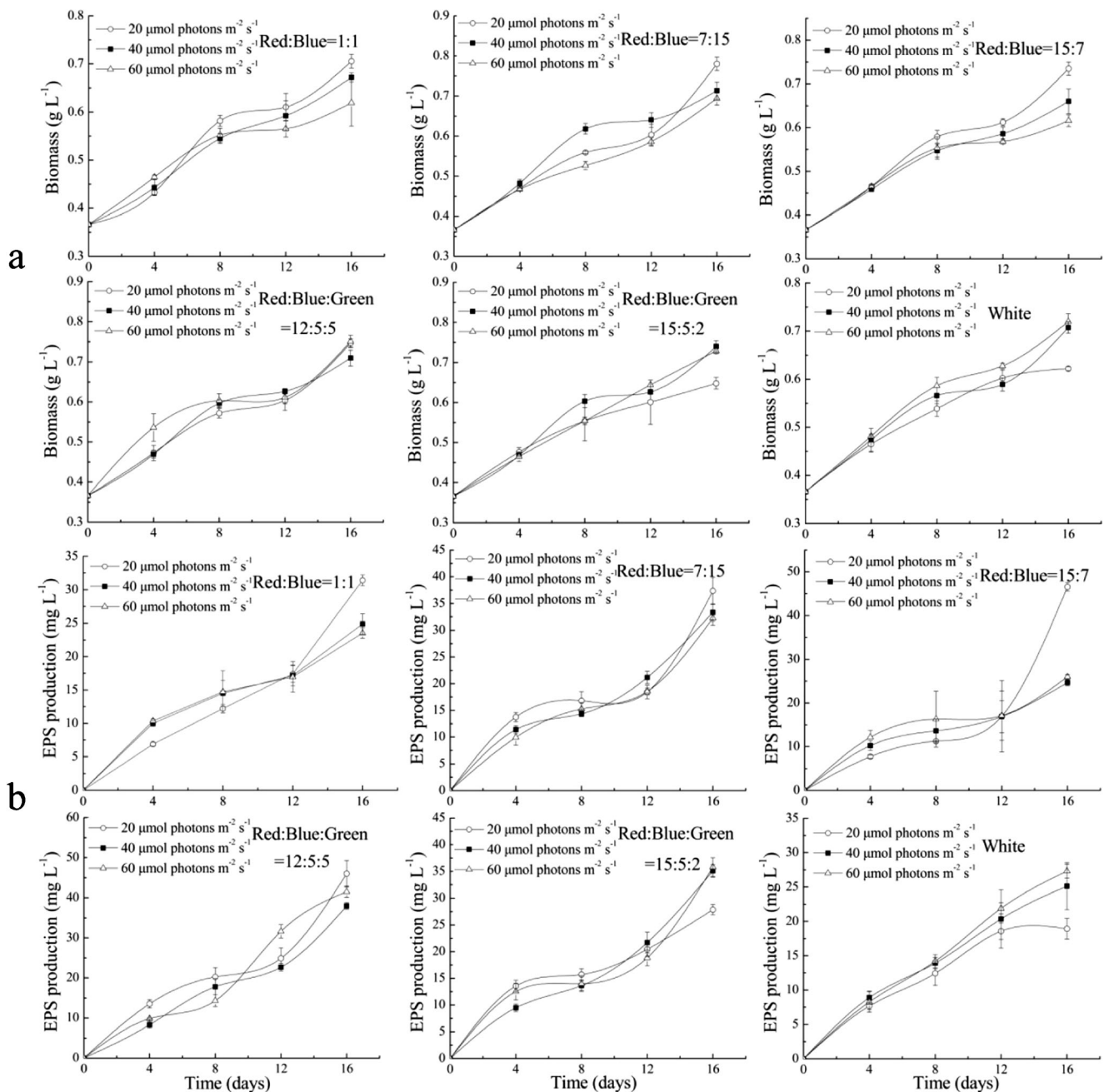


Fig. 1 Effects of mixed wavelengths and light intensity on the biomass accumulation (a) and EPS production (b) of *N. flagelliforme*. Error bars represent \pm SD of the means ($n = 3$)

Effects of mixed wavelength and light intensity on the CO_2 fixation rate and photosynthetic efficiency

Based on the findings of significant effects on the growth of *N. flagelliforme* between 20 and 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, CO_2 fixation rate and photosynthetic efficiency were measured at the two light intensities after 16 days of culture. The results showed that the highest CO_2 fixation rate and photosynthetic efficiency were $45.37 \pm 0.14 \text{ mg CO}_2 \text{ L}^{-1} \text{ day}^{-1}$ and $14.24 \pm 0.14 \%$ in the treatment of red/blue = 7:15 at 20 μmol

photons $\text{m}^{-2} \text{s}^{-1}$ (Fig. 2). Irrespective of the mixed wavelengths, the photosynthetic efficiency at low light intensity was higher than that at high light intensity. The possible reason was that the photosystem II of cells upon exposure to strong light was continually damaged and subsequently reduced the overall photosynthetic efficiency greatly (Murata et al. 2007; Nixon et al. 2010). On the other hand, photosynthetic organisms can reduce oxidative damage by using the excess energy to synthesize protective molecules when the organisms are exposed to saturating light (Li et al. 2009).

Table 1 Changes of C, H, and N ratio in *N. flagelliforme* grown under mixed wavelength and light intensity (mean ± SE)

Mixed wavelength	Light intensity ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$)	C (%)	H (%)	N (%)	C/N
Red/blue = 1:1	20	41.58 ± 0.06	6.38 ± 0.01	5.76 ± 0.06	7.21 ± 0.07
	60	39.70 ± 0.01	6.35 ± 0.05	4.28 ± 0.02	9.27 ± 0.05
Red/blue = 7:15	20	44.83 ± 0.14	6.79 ± 0.04	9.66 ± 0.31	4.64 ± 0.16
	60	40.06 ± 0.11	6.32 ± 0.02	4.19 ± 0.03	9.56 ± 0.04
Red/blue = 15:7	20	40.68 ± 0.05	6.38 ± 0.05	5.76 ± 0.02	7.07 ± 0.02
	60	38.94 ± 0.02	6.32 ± 0.01	3.99 ± 0.02	9.77 ± 0.01
Red/blue/green = 12:5:5	20	44.68 ± 0.02	6.69 ± 0.03	8.90 ± 0.03	5.02 ± 0.01
	60	43.49 ± 0.06	6.67 ± 0.01	7.53 ± 0.06	5.78 ± 0.03
Red/blue/green = 15:5:2	20	40.32 ± 0.06	6.38 ± 0.02	5.32 ± 0.01	7.58 ± 0.01
	60	39.84 ± 0.08	6.38 ± 0.08	4.78 ± 0.01	8.34 ± 0.01
White	20	45.01 ± 0.13	6.71 ± 0.08	7.97 ± 0.16	5.02 ± 0.07
	60	41.03 ± 0.18	6.38 ± 0.12	5.18 ± 0.05	7.93 ± 0.04

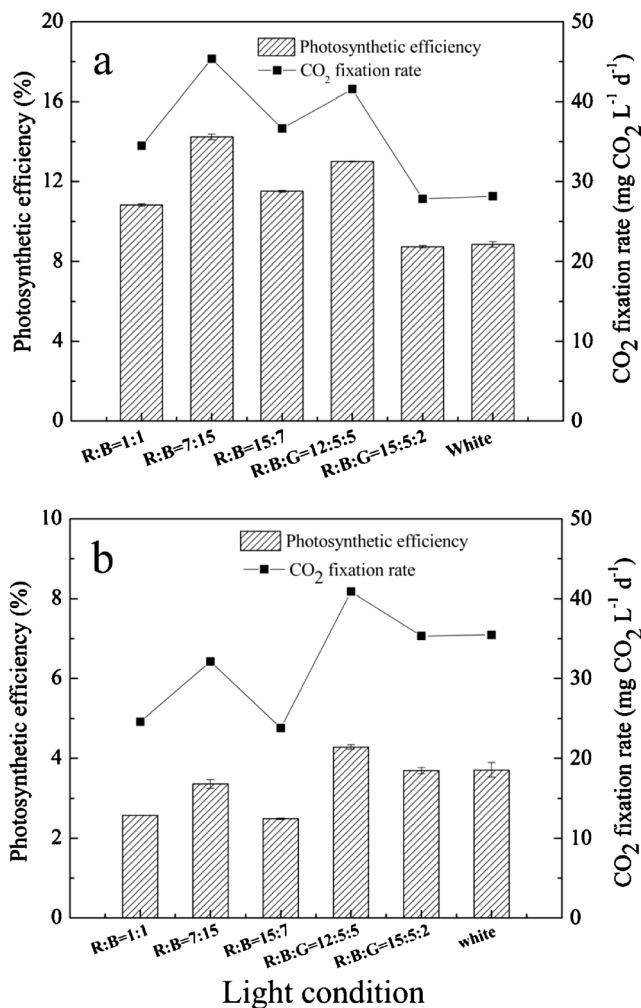


Fig. 2 Effects of mixed wavelengths and light intensity on the CO₂ fixation rate and photosynthetic efficiency after 16 days of culture (a 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, b 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Error bars represent ± SD of the means ($n = 3$)

Evidently, the photosynthetic efficiency was reduced under strong light, which was used for repair of damaged photosystem II and dissipation of excess energy (Sforza et al. 2012).

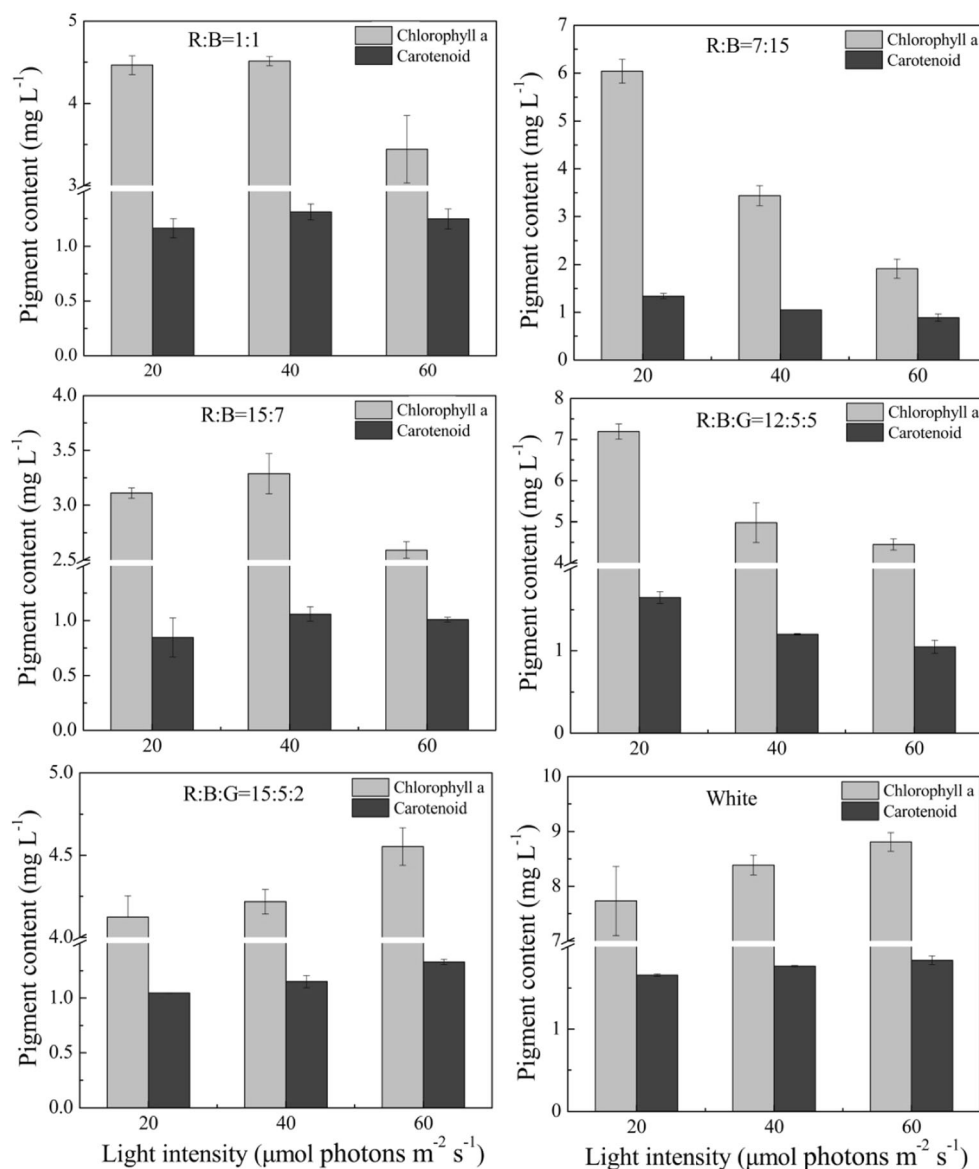
Effects of mixed wavelength and light intensity on the photosynthetic pigment content

The photosynthetic pigment content of *N. flagelliforme* cells grown under different mixed wavelengths and light intensities was measured after 16 days of culture. Notably, the pigment content of *N. flagelliforme* was highly dependent on light intensity, and the chlorophyll *a* contents at treatments of mixed wavelength were all lower than that at white light (Fig. 3). When light intensity was beyond the light saturation point, cells can change the ratio of the light-harvesting pigment in order to maximize the utilization of light energy during the process of photosynthesis (David 2010). The color of the cells cultivated under red/blue = 1:1, 7:15, and 15:7 with intensity of 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ changed from green to yellow. This phenomenon indicated that the photosynthetic pigment composition had changed. Therefore, the ratios of carotenoid to chlorophyll *a* were calculated. This ratio increased with the increase of light intensity except for the treatment of red/blue/green = 12:5:5 and control group (Table 2). The ratios of carotenoid to chlorophyll *a* at treatments of red/blue = 1:1, 7:15, and 15:7 with light intensity of 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ were much higher than in the other treatments.

Effects of wavelength shift approach on biomass production

Compared with white light, the mixed wavelength had no obvious effect on biomass accumulation but a great

Fig. 3 Effects of mixed wavelengths and light intensity on the photosynthetic pigment content of *N. flagelliforme* after 16 days of culture. Error bars represent \pm SD of the means ($n = 3$)



influence on EPS productivity was observed. Thus, to enhance the productivities of biomass and EPS, the

Table 2 Changes of the ratio of carotenoid to chlorophyll a in *N. flagelliforme* cells under different wavelength mixing ratios and light intensities

Wavelength mixing ratios	Light intensity ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$)		
	20	40	60
Red/blue = 1:1	0.26	0.29	0.36
Red/blue = 7:15	0.22	0.31	0.46
Red/blue = 15:7	0.27	0.32	0.39
Red/blue/green = 12:5:5	0.23	0.24	0.24
Red/blue/green = 15:5:2	0.25	0.29	0.29
White	0.21	0.21	0.21

wavelength shift approach, i.e., appropriate manipulation of light wavelength according to different growth stages of *N. flagelliforme*, was proposed. For pinpointing the optimal shift time, the experiments were performed via the shift of light wavelength at different culture stages. The set of experiments was explained in Supplementary Fig. S2, involving ten types of light illumination: continuous red or blue light; white light first and then respective shift to red light, blue light, red/blue = 1:1, 7:15, 15:7 and red/blue/green = 12:5:5, 15:5:2. Light wavelength was shifted to red, blue, red/blue = 1:1, 7:15, 15:7 and red/blue/green = 12:5:5, 15:5:2 after 6, 9, and 12 days of cultivation in white light, respectively.

Under continuous red light, the biomass productivity of *N. flagelliforme* was similar to that under white light, whereas continuous blue light resulted in higher biomass

production (Fig. 4a). As expected, an appropriate switch of wavelength led to the enhancement of biomass production, compared to those under white light. For example, the deployment of white light first and then shift to red light (W6R12, W9R9, W12R6) led to increased cell growth. Among the various wavelength shift approaches, W9F9 displayed the best performance, with the biomass reaching $0.99 \pm 0.02 \text{ g L}^{-1}$, which was an increase of 20.71 % compared with the control group. However, unexpectedly, continuous blue light produced the highest biomass of $1.25 \pm 0.02 \text{ g L}^{-1}$.

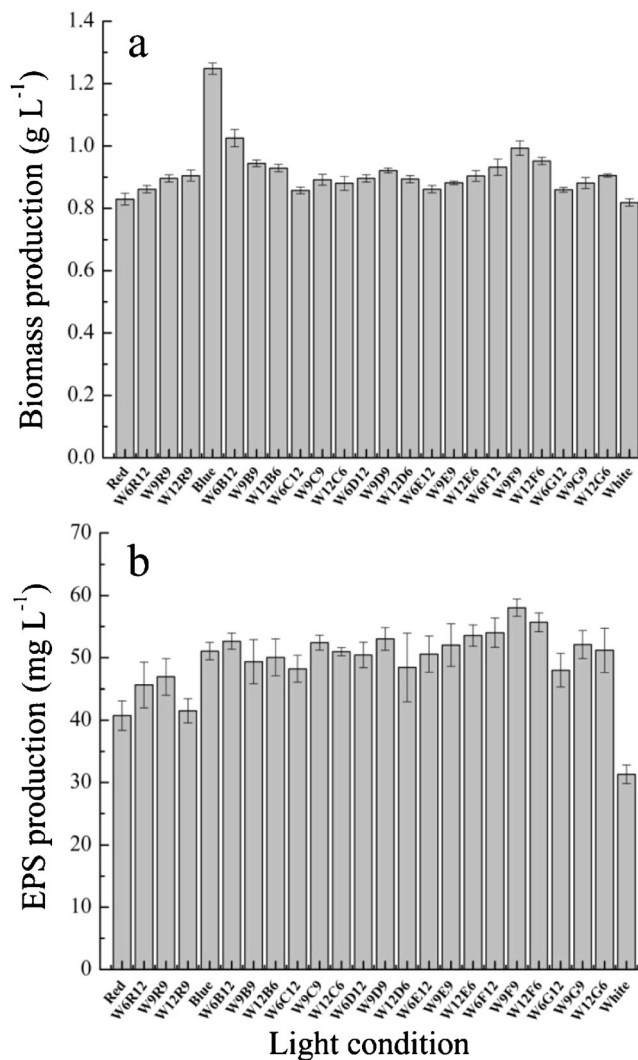


Fig. 4 Effects of wavelength shift approach on the biomass accumulation (a) and EPS production (b) of *N. flagelliforme*. The blue light, red light, red/blue = 1:1, 7:15, 15:7, red/blue/green = 12:5:5, 15:5:2, and white light are represented by B, R, C, D, E, F, G, and W, respectively. The experimental results obtained from wavelength shift are denoted by the form of W6R12 (6-day illumination with white light followed by 12-day culture with red light) or W6B12 (6-day illumination with white light followed by 12-day culture with blue light) and so on. Error bars represent \pm SD of the means ($n = 3$)

Effects of wavelength shift approach on EPS production

The effect of wavelength shift approach on EPS production also was studied. Obviously, EPS production was highly dependent on light conditions (Fig. 4b). It was noteworthy that overall EPS yield could be significantly increased via the switch of wavelength, possibly owing to the efficient growth with the help of sophisticated manipulation of light condition. The highest EPS content was reached at W9F9 with the value of $58.10 \pm 1.40 \text{ mg L}^{-1}$, which was an increase of 85.61 % compared with the control group.

Effects of different nitrogen sources on biomass accumulation

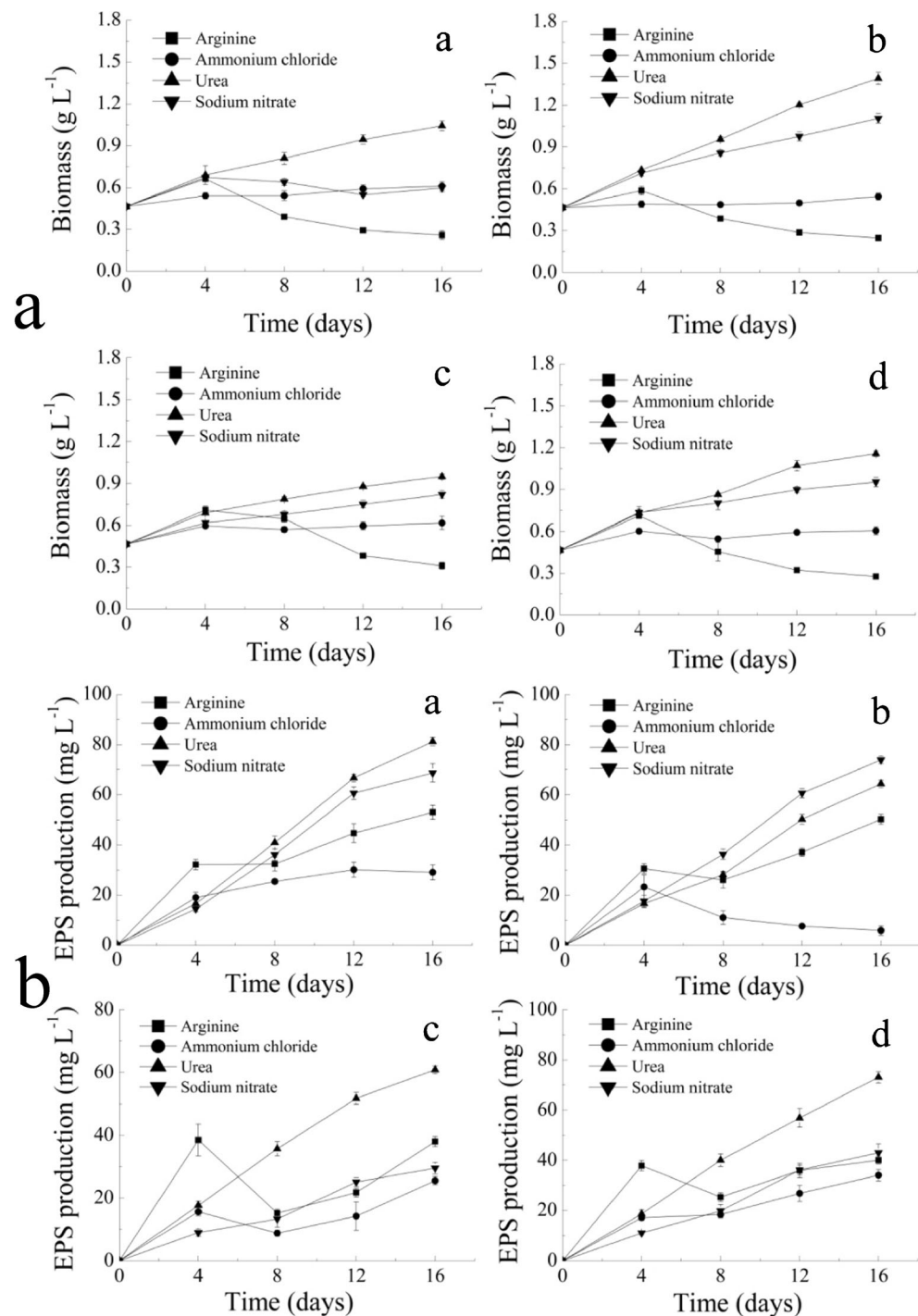
To optimize the nitrogen source for *N. flagelliforme* grown under different light environment, the effects of different nitrogen sources (urea, NH_4Cl , NaNO_3 , and arginine) on biomass and EPS production were investigated based on BG11₀ medium with equal mole basis of nitrogen. After precultivating the cells under white light, they were incubated in BG11₀ medium for a week and then transferred to dark environment for 3 days to fully reduce storage nitrogen sources and avoid the influence of light from preculture. Thereafter, cells were transferred to continuous red or blue light with light intensity of $20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and white light with light intensity of 20 or $60 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

N. flagelliforme was sensitive to the changes of nitrogen sources, and significant differences in cell growth were observed (Fig. 5a). Irrespective of light conditions, with arginine as nitrogen source, the biomass increased during the first 4 days, but decreased later. Microscopic examination showed that some cells had disintegrated, which could explain the phenomenon of the culture medium becoming transparent. With increasing culture time, the biomass remained basically unchanged with NH_4Cl as nitrogen source; this might be caused by the inhibitory effect acidification due to H^+ release from NH_4^+ during cultivation (Li et al. 2010). NaNO_3 as nitrogen source promoted the growth of *N. flagelliforme* except when illuminated with red light. Among the four nitrogen sources, urea was the most effective one for the growth of *N. flagelliforme*, especially when illuminated with blue light, and the biomass reached $1.39 \pm 0.04 \text{ g L}^{-1}$, an increase of 19.8 % compared with the control group.

Effects of different nitrogen sources on the EPS production

When illuminated with red or blue light, the EPS productivity of *N. flagelliforme* was decreased with NH_4Cl or arginine as nitrogen source, which might be due to the poor growth (Fig. 5b). Under red light, the highest EPS content of

Fig. 5 Effects of different nitrogen sources on the biomass (a) and EPS production (b) of *N. flagelliforme* under different light environments (a, red light, 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; b, blue light 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; c, white light, 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; d, white light, 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Error bars represent \pm SD of the means ($n = 3$)



N. flagelliforme reached $81.21 \pm 1.72 \text{ mg L}^{-1}$ with urea as nitrogen source, followed by NaNO_3 ($68.72 \pm 3.66 \text{ mg L}^{-1}$). However, under blue light, the highest EPS content of *N. flagelliforme* was reached ($73.90 \pm 1.49 \text{ mg L}^{-1}$) with NaNO_3 as nitrogen source, while the EPS content was only $64.30 \pm 1.51 \text{ mg L}^{-1}$ with urea, which were 1.72 and 0.88 times that of white light ($60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) grown cells, respectively.

Wavelength shift approach with urea as nitrogen source

The growth of *N. flagelliforme* did not show a lag phase and was almost linear when illuminated with white light, which might be due to the rich nutrient and favorable environment (Fig. 6a). When the cells were shifted to the mixed wavelengths, the growth gradually slowed except under red/blue/green = 12:5:5 light (Fig. 6a). The shift of cells to red/blue/

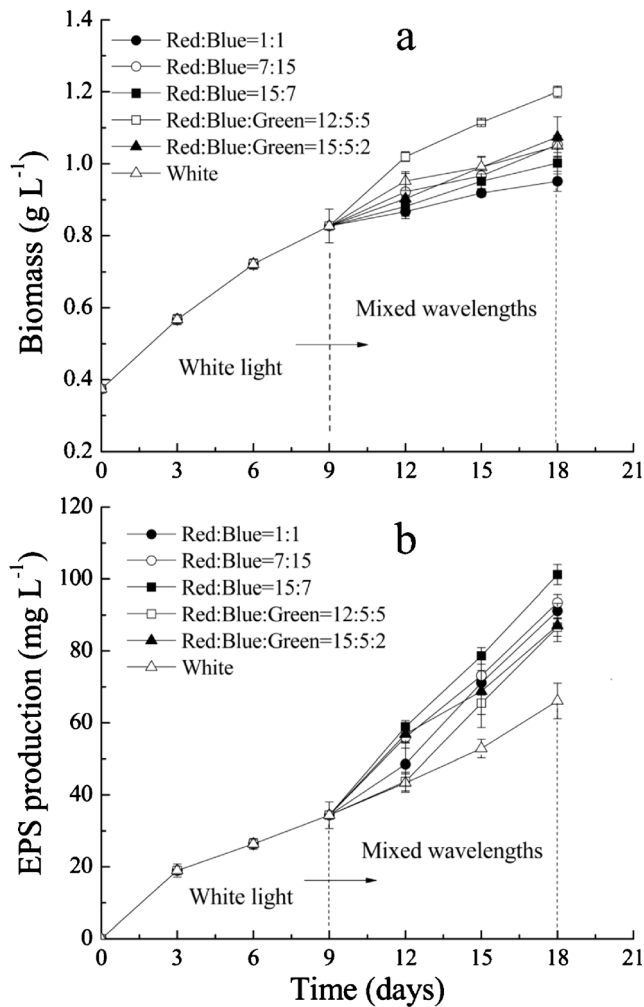


Fig. 6 Effects of wavelength shift approach on biomass (a) and EPS production (b) of *N. flagelliforme* with urea as nitrogen source. Error bars represent \pm SD of the means ($n = 3$)

green = 12:5:5 from white light promoted the biomass reaching a maximum value of $1.20 \pm 0.02 \text{ g L}^{-1}$, an increase of 14.31 % compared with the control group.

Furthermore, the effect of wavelength shift approach with urea as nitrogen source on EPS productivity was analyzed. Notably, the EPS productivity was low during the cultivation under white light, but using wavelength shift approach, the EPS productivity was greatly enhanced (Fig. 6b). After 18 days, the highest EPS production of $101.17 \pm 2.82 \text{ mg L}^{-1}$ was achieved with the red/blue = 15:7 treatment and this value was 1.53 times that of white-light-grown cells.

Discussion

Several studies have reported that mixed LED light wavelength treatments were more suitable for microalgal growth than monochrome LED light wavelength treatments (Cheng and Zheng 2014). We have previously reported the effects of

light quality on *N. flagelliforme* EPS production (Han et al. 2014b) and revealed the influence mechanism of EPS biosynthesis upon light quality (Han et al. 2015), indicating that blue and red lights promoted biomass accumulation and EPS production compared with white fluorescent light and other monochromatic light. Although green light is not considered as the optical absorption spectrum of photosynthesis, it has been found to increase the content of chlorophyll and phycobiliprotein, compared to white light (Kim et al. 2015). Therefore, in this study, the effects of mixed wavelength (red 660 nm, blue 460 nm, and green 520 nm) and light intensity on *N. flagelliforme* culture were examined.

The results indicated that mixed wavelengths with relatively low light intensity was more suitable for cell growth and promoted the EPS formation (Fig. 1a). In the present study, the maximum biomass of *N. flagelliforme* was obtained with $20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of red/blue = 7:15, while the highest EPS content was achieved at treatment of red/blue = 15:7 with $20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Since blue and red light can induce photosystem I and enhance photosystem II, they are usually used to improve photosynthetic efficiency, biomass, and EPS production (You and Barnett 2004; Ravelonandro et al. 2008). However, the red and blue light wavelengths should be selectively provided, because only an appropriate mixing ratio of red and blue light wavelengths could yield the highest biomass or EPS, which was consistent with the findings of Kim et al. (2013).

As shown in Table 2, the ratios of carotenoid to chlorophyll *a* at the treatments of red/blue = 1:1, 7:15, and 15:7 with $60 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ were much higher than those in other treatments. The ratio of carotenoid to chlorophyll *a* is usually considered as a marker of cellular oxidative level (Schütüter et al. 1997). Thus, it could be inferred that the growth inhibition under high light intensity might be due to the formation of harmful ROS and oxidative stress as a result of photoinhibition (Powles 1984; Li et al. 2009). This also explained the result that the mixed wavelengths with relatively low light intensity were more suitable for cell growth. However, there was one exception where the content of biomass and EPS were rapidly increased under red/blue = 15:7 with $20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ after 12 days, and this phenomenon needs further investigation.

Compared to continuous white light, the switch of wavelength greatly improved biomass accumulation; however, continuous blue light displayed better performance (Fig. 4a). This might be due to the fact that shorter wavelengths, such as blue light, deliver more energy, which could penetrate deeper through the culture medium, and thus, the cell density increased faster. Ribulose biphosphate carboxylase/oxygenase and carbonic anhydrase are the enzymes that affect the CO_2 fixation rates in microalgae (Beardall and Raven 2016). Atta et al. (2013) have shown in *Chlorella* that the activity of these enzymes in cells grown

under blue light was high, ultimately leading to increased biomass productivity.

Different from biomass production, overall EPS yield could be significantly increased via the switch of wavelengths (Fig. 4b). During the process of shift from white light to red or blue light, the earlier shift resulted in higher EPS production, which was because blue and red lights promoted EPS production compared with white light (Han et al. 2014b). However, when shifting the white light to mixed wavelengths, with the increase of culture time in white light, EPS production first increased and then decreased, except that shifting to red/blue = 15:7 led to a continuous increase in EPS production. Therefore, it was proposed that there should be an optimum time to shift the light wavelength, because it was not effective if applied at either too early or too late. According to the results from wavelength shift approach, the optimum shift time in this study was 9 days. Therefore, appropriate manipulation of light environment could improve biomass as well as EPS production.

Among the four nitrogen sources tested in the study, *N. flagelliforme* grew best with urea under both red and blue light, while under red light, urea promoted EPS production the best, but NaNO₃ was the most suitable one for EPS production under blue light. The results showed that cyanobacteria might have preference for utilization of different nitrogen sources under varied light conditions, which might be a result of the regulation of N assimilation in cyanobacteria being greatly influenced by photosystem I light and photosystem II light (Singh et al. 2009). Therefore, the suitable nitrogen source should be selected based on the actual light condition.

The application of wavelength shift approach plus nitrogen source optimization further enhanced biomass and EPS production. Taken biomass and EPS production into consideration, compared with previous culture conditions, i.e., continuous white light and with NaNO₃ as nitrogen source, the optimized wavelength shift approach (9-day illumination with white light followed by 9-day culture with red/blue/green = 12:5:5) with urea as nitrogen source improved the biomass from 0.72 ± 0.02 to 1.20 ± 0.02 g L⁻¹ and EPS production from 27.31 ± 1.00 to 86.65 ± 2.56 mg L⁻¹, which was increased by 66.67 and 217.28 %, respectively. Appropriate manipulation of light environment could improve biomass as well as EPS production, and the strategy for microalgal cultivation via the manipulation of light environment has been successfully demonstrated in *N. flagelliforme*, which could be easily expanded to other species of cyanobacteria and microalgae. Hopefully, the findings revealed in this work will help microalgal biotechnology and open the way to achieve further improvements.

In conclusion, the biomass and EPS production of *N. flagelliforme* were greatly improved by light environment control. The results demonstrated clearly that manipulation of light environment could significantly increase biomass and

EPS production. The optimized light conditions increased the biomass from initially 0.72 ± 0.02 to 1.20 ± 0.02 g L⁻¹ and EPS production from 27.31 ± 1.00 to 86.65 ± 2.56 mg L⁻¹, respectively. Based on this study, the strategy of light environment control in the cyanobacterial culture is proposed to improve biomass and EPS production.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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