Original paper

The relationship between monosaccharide composition of extracellular polysaccharide and activities of related enzymes in Nostoc flagelliforme under different culture conditions

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Abstract

The relationship between monosaccharide composition of Nostoc flagelliforme extracellular polysaccharide (EPS) and activities of EPS synthesis enzymes under various carbon sources, nitrogen sources and light culture condition was investigated. Culture conditions showed significant influences on both monosaccharide composition and related enzyme activities. Under both carbon and nitrogen sources conditions, mannose mole percentage was increased with the increase of initial mole ratio of C/N and positively related to fructose-1, 6-bisphosphate activity, and glucuronic acid and galactose mole percentages were positively correlated with UDP-glucose dehydrogenase, while arabinose and rhamnose mole percentages were negatively associated with UDP-glucose pyrophosphorylase. Different correlation between monosaccharide composition and enzymes activity from carbon and nitrogen sources conditions was found under light condition. These findings will be helpful to establish a novel fermentation process aimed to produce the N. flagelliforme EPS with desired monosaccharide composition.

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1. Introduction

Cyanobacteria are capable of fixing carbon dioxide (CO2) used to produce biomass, synthesize and secrete extracellular polysaccharide. The cyanobacterial extracellular polysaccharide can be divided in two main forms: the ones bound the cell surface as capsules polysaccharide (CPS fraction) and the others released into the surrounding environment (EPS fraction) (Pereira et al., 2009; Rehm, 2010). The EPS can be easily recovered from liquid cultures and their many physicochemical properties show advantages over the polysaccharides from plants or microalgae (De Philippis, Sili, Paperi, & Vincenzini, 2001; Sutherland, 1998). Due to EPS potential applications in the food, pharmaceutical and environment industries, interest towards polysaccharides has significantly increased in recent years (De Philippis et al., 1998; De Philippis and Vincenzini, 2006; De Philippis et al., 2011).

Generally, physicochemical properties of polysaccharides are highly dependent on their composition and structure (Rehm, 2010; Sutherland, 1998). Therefore many previous studies have concentrated on monosaccharide composition and structure analysis of cyanobacteria EPS (Brüll et al., 2000; Huang, Liu, Paulsen, & Klaveness, 1998; Hu, Liu, Paulsen, Petersen, & Klaveness, 2003; Jensen et al., 2013; Nicolaus et al., 1999; Singh & Das, 2011). However, there is a lack of systematic investigation regarding the mechanisms involved in the regulation of monosaccharide biosynthesis of cyanobacteria EPS. Based on the studies performed in bacterial, fungal and microalgae species, results revealed that EPS biosynthetic mechanisms are very complex, but the pathways and necessary enzymes (such as UDP-glucose pyrophosphorylase and UDP-glucose dehydrogenase) of typical EPS biosynthesis involved in the activation of the monosaccharides and conversion into sugar nucleotides are relatively conserved (Degeest & de Vuyst, 2000; Escalante, Wacher-Rodarte, García-Garibay, & Farrés, 1998; Grobben, Smith, Sikkema, & de Bont, 1996; Horecker, 1966; Looijesteijn, Boels, Kleerebezem, & Hugenholz, 1999; Li, Xu, Xu,
N. flagelliforme is a species of edible terrestrial filamentous cyanobacteria, distributed throughout arid and semi-arid areas (Qian, Zhu, & Chen, 1989; Takenaka et al., 1998). Several studies have demonstrated that N. flagelliforme EPS possess the properties of antivirus, antioxidant, and anti-tumor (Hayashi et al., 2008; Kanekiyo et al., 2005; Kanekiyo, Hayashi, Takenaka, Lee, & Hayashi, 2007). N. flagelliforme can convert light into chemical energy as a group of photosynthetic microorganisms (Raines, 2003). It has efficient carbon concentration mechanisms (CCM) which functions to transport and accumulate inorganic carbon actively (Ci: HCO$_3^-$, and CO$_2$) (Badger & Price, 2003; Ogawa, 1994; Price, Badger, Woodger, & Long, 2008), and can either utilize a combined nitrogen source or fix atmospheric nitrogen (Garcia-Fernandez & Diez, 2004; Pereira et al., 2009). Many investigators have reported the light condition, carbon and nitrogen sources are the important factors mainly affecting energy availability and C/N control the production of the cyanobacteria EPS and their monosaccharide composition (Ge, Xia, Zhou, & Hu, 2014; Huang et al., 1998; Otero & Vincenzini, 2003). Our previous studies have systematically studied the influences of light, carbon and nitrogen sources on cell growth, EPS production and monosaccharide composition of N. flagelliforme (Ding, Jia, Han, Yuan, & Tan, 2013; Han, Sun, Wu et al., 2014; Han, Sun, Jia et al., 2014; Yu, Jia, & Dai, 2010). Yet there is little knowledge about the correlation between relevant enzymes activities in N. flagelliforme EPS biosynthesis and their monosaccharide composition under these different culture conditions.

In the study, the effects of culture conditions including different light conditions, concentrations of carbon sources and nitrogen sources on EPS monosaccharide composition and activities of EPS biosynthesis enzymes were investigated. The correlation between monosaccharide compositions and related enzymes was subsequently analyzed, for improved understanding EPS synthesis process in N. flagelliforme and attempting to obtain N. flagelliforme EPS with desired composition in the future.

2. Materials and methods

2.1. Strain and culture conditions

The N. flagelliforme cells (TCCC11757) utilized in liquid suspension cultures were obtained from the Tianjin Key Lab of Industrial Microbiology (Tianjin, China). The cells were cultured in BG-11 medium in 500 mL shake-flask containing 200 mL medium at 25 °C under continuous white fluorescent light at a photon flux density of 60 mol photon/(m$^2$·s) for 16 days. For light condition, the same photon flux density of monochromatic red (660 nm) and blue (460 nm) were used instead of the white fluorescent light as the treatment group. The half-band widths are 5 nm for each monochromatic light, according to manufacturer’s instruction (Shenzhen federal heavy secco electronic Co. LTD., China). The other two culture conditions including carbon sources and nitrogen sources were evaluated. For carbon source condition, initial concentrations of NaHCO$_3$ were set at 0 g/L, 1.26 g/L and 2.94 g/L, respectively, with fixed NaNO$_3$ concentration at 1.50 g/L. For nitrogen source condition, initial concentrations of NaNO$_3$ were set at 0 g/L, 1.50 g/L and 4.50 g/L respectively, with no exogenous addition of NaHCO$_3$.

2.2. Extraction and purification of EPS

The N. flagelliforme culture was centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was added with ethanol to 80% (v/v) and kept overnight at 4 °C. Crude EPS was collected by centrifuging and subsequent freeze-drying. The crude EPS would then be redissolved in deionized water and further purified according to the previously described (Han, Sun, Jia et al., 2014). In briefly, the redissolved solution was dealt with DEAE-650M cellulose anion exchange column (φ2.6 cm × 60 cm) (TOSOH Corporation, Japan) and then processed with a Sephadex G100 gel chromatographic column (φ1.6 cm × 80 cm, Pharmacia Corporation, Sweden). These two column were eluted with 0–1.0 mol/L NaCl solution at a flow rate of 1.0 mL/min and distilled water at a flow rate of 0.5 mL/min, respectively. The fractions of carbohydrates were monitored via phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), and carbohydrate-positive fractions were collected and lyophilized to get the purified EPS.

2.3. Monosaccharide composition analysis

The monosaccharide compositions of the EPS were determined by gas chromatography–mass spectrometry (GC–MS, Agilent Technologies, CA, USA) as a modified method by previously described (Han, Sun, Wu et al., 2014). In briefly, 2 mg EPS was hydrolyzed with 5 mL of 2 M trifluoroacetic acid (TFA) at 120 °C for 4 h in a sealed glass with N$_2$ protecting. Residual acid was removed by repeated co-distillations with anhydrous methanol. The hydrolyzed product was derivatized for 80 min at 40 °C in 50 μL of 20 mg/mL methoxyamine hydrochloride in pyridine and then added 80 μL N-methyl-N-(trimethylsilyl) trifluoroacetamide for an 80-min treatment at 40 °C. The derivatives were obtained for GC–MS analysis.

The operation was performed in the following conditions: N$_2$ velocity at 1.0 mL/min; injection temperature at 250 °C; detector temperature at 240 °C; initial column temperature programmed at 110 °C for 2 min, then increased to 160 °C at 8 °C/min and then to 230 °C at a rate of 2 °C/min, finally to 250 °C at 5 °C/min and holding for 2 min.

2.4. Activity assays of enzymes involved in EPS synthesis

The enzyme extract method is modified by the method of Looijesteijn et al. (1999) and Tamoi, Murakami, Takeda, and Shigeoka (1998). The N. flagelliforme cells were harvested by centrifugation at 10,000 × g for 10 min at 4 °C, washed twice with cell extract buffer pre-cooled at 4 °C, which was 20 mM phosphate buffer containing 50 mM NaCl, 10 mM MgCl$_2$, and 1 mM dithiothreitol at pH 6.5. The cells pellet resuspended in the extract buffer were disrupted ultrasonically at 20 kHz for 12 min on ice using an ultrasonic processor (SCIENTZ-IIID sonicator, Ningbo Scientz Biotechnology Inc., China). Cell debris was removed by centrifuged at 12,000 × g for 15 min at 4 °C, and the resulting supernatant (cell extract) was used for the enzyme activity assay. The protein content of the cell extract was determined by the method of Bradford (Bradford, 1976).

Activity of EPS synthesis enzymes was measured spectrophotometrically by kinetic assay in a final volume of 250 μL at 24 °C. Reactions were initiated by adding 30 μL cell extract to various reaction mixtures depending on the enzyme, which were listed in Table 1. The enzyme activity was determined by monitoring the formation or disappearance of NAD(P)H by measuring the absorbance at 340 nm (ε$_{340}$ = 6220 M$^{-1}$·cm$^{-1}$), and specific activity were expressed in μmol NAD(P)H (mg protein)$^{-1}$ min$^{-1}$. The blank for each enzyme was the same reaction condition with the same volume of cell extract that had been heated at 100 °C for 5 min. In all
Table 1
The reaction mixtures for the activity detection of *N. flagelliforme* EPS synthesis enzymes.

<table>
<thead>
<tr>
<th>Detected enzymes</th>
<th>Reaction mixture</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoglucone isomerase (PGI)</td>
<td>50 mM potassium phosphate buffer (pH 6.8), 5 mM MgCl₂, 4U glucose-6-phosphate dehydrogenase, 0.4 mM NADP⁺, 10 mM fructose-6-phosphate</td>
<td>Grobben et al. (1996)</td>
</tr>
<tr>
<td>Fructose-1,6-bisphosphatase (FBPase)</td>
<td>100 mM Tris–HCl buffer (pH 8.0), 0.4 mM NADP⁺, 20 mM MgCl₂, 20 mM DTT, 0.5 U phosphoglucone isomerase, 0.5 U glucose-6-phosphate dehydrogenase, 2 mM fructose-1,6-bisphosphate</td>
<td>Grobben et al. (1996)</td>
</tr>
<tr>
<td>UDP-glucose pyrophosphorylase (UGPase)</td>
<td>50 mM Tris–HCl buffer (pH 7.8), 0.4 mM UDP-glucose, 14 mM MgCl₂, 4U glucose-6-phosphate dehydrogenase, 0.4 mM NADP⁺, 2.1 U phosphoglucomutase, 4 mM inorganic pyrophosphate</td>
<td>Bernstein and Robbins (1965)</td>
</tr>
<tr>
<td>UDP-galactose-4-epimerase (UGE)</td>
<td>50 mM Tris–HCl buffer (pH 8.5), 5 mM MgCl₂, 0.015 U UDP-glucose dehydrogenase, 0.5 mM NAD⁺</td>
<td>Grobben et al. (1996)</td>
</tr>
<tr>
<td>UDP-glucose dehydrogenase (UDGD)</td>
<td>100 mM Tris–HCl buffer (pH 7.5), 5 mM UDP-glucose, 1 mM dithiothreitol, 1 mM MgCl₂, 1 mM NADP⁺</td>
<td>Hung et al. (2007)</td>
</tr>
<tr>
<td>Phosphomannose isomerase (PMI)</td>
<td>50 mM MOPS buffer (pH 7.0), 1 mM CoCl₂, 1 mM NADP⁺, 10 mM mannose-6-phosphate, 4U phosphoglucone isomerase, 4U glucose-6-phosphate dehydrogenase</td>
<td>Shinabarger et al. (1991)</td>
</tr>
<tr>
<td>Phosphofructokinase (PFK)</td>
<td>50 mM Tris–HCl buffer (pH 7.5), 5 mM MgCl₂, 50 mM KCl, 1.25 mM ATP, 0.15 mM NADH, 4.5 U aldolase, 18 U triose-phosphate isomerase, 6.2 U glycerol-3-phosphate dehydrogenase, 5 mM fructose-6-phosphate</td>
<td>Loosjeesteijn et al. (1999)</td>
</tr>
</tbody>
</table>

of the assays, the reaction velocity was linearly proportional to the amount of cell extract. The reaction rate (ΔAbsorbance/min) calculated from the slope of linear plots (R² = 0.95–1.0) from 0 to 3 min or 0-5 min that was normalized to the control. The enzyme activities were calculated using the following equations (Bisswanger, 2004):

\[
\text{Enzyme activity (U)} = \frac{\Delta A_{340}}{\Delta t} \times V_1 \times D / (V_2 \times 0.340 \times d) \tag{2}
\]

where \(\Delta A_{340}/\Delta t\) is the reaction rate, \(V_1\) is the final reaction volume, \(V_2\) is the cell extract volume, \(D\) is the dilution ratio, \(0.340\) is extinction coefficients of NAD(P)H and NADH, \(d\) is colorimetric cup light path.

2.5. Statistical analysis

Experimental data in this study was obtained from three replicates for each treatment, and values were shown as mean ± standard deviation. Statistical significance of correlations between enzyme activities and monosaccharide compositions was based on correlation test. It is performed by correlation coefficient (R²) and significant effects (P < 0.05), which were analyzed based on data obtained under various culture conditions by SPSS statistics vision 2.0.

3. Results

3.1. Effects of culture conditions on monosaccharide composition in *N. flagelliforme* EPS

The monosaccharide composition was analyzed by GC–MS after acid hydrolysis and subsequent trimethylsilyl derivatization. The results showed that *N. flagelliforme* EPS consisted of ten monosaccharides under all culture conditions tested, including aldohexose (glucose, galactose and mannose), ketohexose (fructose), pentose (ribose, xylose and arabinose), deoxysugar (rhamnose and fucose) and uronic acid (glucuronic acid). As shown in Table 2, glucose was the most abundant monosaccharide in EPS under all tested conditions, followed by mannose and galactose. Their mole percentages were obviously influenced by culture conditions. There were no significant differences in the mole percentage of ribose compared with other monosaccharides.

The mole percentage of three monosaccharides (xylose, rhamnose and arabinose) fluctuated obviously under all tested conditions. Under carbon sources of 1.26 g/L and 2.94 g/L NaHCO₃, nitrogen sources of 4.50 g/L NaNO₃ and light condition of red and blue-light, the mole percentage of rhamnose was more than 10%. For carbon sources of 1.26 g/L NaHCO₃ and nitrogen sources of 4.50 g/L NaNO₃, the mole percentage of xylose and arabinose were increased obviously. For other three minor monosaccharides (fucose, fructose and glucuronic acid), the variation of mole percentage were less significant.

Based on previous reports (Knowles & Plaxton, 2003; Smith, 1983; Stal & Moezelaa, 1997) and KEGG pathway, a simplified biosynthesis pathway for monosaccharides was proposed (Fig. 1). According to this model, further comprehensive analysis of carbon and nitrogen sources condition (Fig. 2) showed that mole percentage of monosaccharide involved in UDP-sugars synthetic pathway involving glucose, glucuronic acid, xylose, arabinose, galactose and rhamnose was increased when initial mole ratio of C/N was reduced in culture medium composition, while mole percentage of monosaccharide involved in GDP-sugars synthetic pathway including fucose and mannose was obviously decreased. For light condition, mole percentage of monosaccharide of UDP-sugars synthetic pathway was increased, while mole percentage of monosaccharide of other–sugars synthetic pathway involving fructose and ribose was decreased with red and blue light instead of white light.

According to the analysis of the relationship among ten monosaccharides, as shown in Fig. 3, the results showed that glucuronic acid under various culture conditions was closely correlated with galactose. The R² value was 0.6124 under the carbon and nitrogen sources conditions, and for light condition R² value was 0.9473. The comprehensive analysis of the relationship among ten monosaccharides under carbon and nitrogen sources condition showed linear correlation, whereas no correlation was observed under three culture conditions. It implied that there were more similar relationships among monosaccharides under carbon sources and nitrogen sources condition than that of light condition.

3.2. Effects of culture conditions on activity of enzymes involved in *N. flagelliforme* EPS synthesis

Activities of seven enzymes involved in *N. flagelliforme* EPS synthesis were evaluated under various culture conditions (Table 3). FBPase activity was increased with the increase of NaHCO₃ con-
centrations, however, for the rest six enzymes, the activity was the lowest at 1.26 g/L NaHCO₃ and then back up at 2.94 g/L NaHCO₃. Increased concentration of NaNO₃ resulted in reduced enzyme activity except UGDH. For light condition, the activities of PGI and UGDH were the highest under blue-light condition, and the activities of FBPase, UGE, and UGPase was the highest under red-light condition. The PMI activity was significantly increased under both red- and blue-light conditions. Under white-light condition, the activities of enzymes were the lowest except PFK.

### 3.3. Relationship between activities of key enzymes and monosaccharide mole percentages under different culture conditions

The correlation coefficient ($R^2$) between monosaccharide mole percentages and EPS synthesis enzymes was calculated based on results obtained under different culture conditions. For the relationship between enzymes and mole percentages of monosaccharide, it was defined as significant correlation at $R^2 > 0.5$ and $P < 0.05$. For carbon and nitrogen sources conditions, as shown in Fig. 4A, mole percentages of glucuronic acid ($R^2 = 0.9215$, $P < 0.01$) and galactose ($R^2 = 0.5883$, $P < 0.01$) were linked to the activity of UGDH. $R^2$ value was 0.6296 ($P < 0.01$) for mannose and FBPase. Arabinose ($R^2 = 0.7292$, $P < 0.01$) was closely correlated with UGPase. Rhamnose was correlated with the activity of PGI ($R^2 = 0.8211$, $P < 0.01$), PMI ($R^2 = 0.7268$, $P < 0.01$) and UGPase ($R^2 = 0.5146$, $P < 0.01$).

Compared with carbon and nitrogen sources conditions, the trend was opposite under the light condition (Fig. 4B). Mole percentages of glucuronic acid ($R^2 = 0.6004$, $P < 0.05$) was linked to UGDH activity. $R^2$ value was 0.7371 ($P < 0.01$) for mannose and FBPase. Xylose ($R^2 = 0.8143$, $P < 0.01$) was closely correlated with UGPase. Rhamnose was correlated with the activity of PGI ($R^2 = 0.9744$, $P < 0.01$), PMI ($R^2 = 0.7677$, $P < 0.01$).

The results indicated that fructose ($R^2 = 0.8725$, $P < 0.01$) and glucose ($R^2 = 0.8082$, $P < 0.01$), galactose ($R^2 = 0.9121$, $P < 0.01$), glucuronic acid ($R^2 = 0.7369$, $P < 0.01$) mole percentage were related to the activity of PGI with different concentrations of NaHCO₃ (Fig. 5A), whereas fructose ($R^2 = 0.8178$, $P < 0.01$) and ribose ($R^2 = 0.7541$, $P < 0.01$), galactose ($R^2 = 0.6624$, $P < 0.01$), glucuronic acid ($R^2 = 0.7757$, $P < 0.01$) were related to it under light condition (Fig. 5B).

### 4. Discussion

Many previously published papers have revealed monosaccharide compositions and mole percentages for EPS from bacterial, fungal and microalgae species (Jing, Mao, Geng, & Xu, 2013; Petry, Furlan, Crepeau, Cerling, & Desmazeaud, 2000; Peng et al., 2015, 2016). Compared with the polymers synthesized by above species, which contain a lower variety of different monomers (usually less than four), cyanobacteria EPS are mainly defined as heteropolysaccharides and 75% of them contain six or more diverse monosaccharides (Huang et al., 1998; Hu et al., 2003; Niclau et al., 1999; Pereira et al., 2009). However, a lack of information regarding the relationship between monosaccharide compositions and activities of related enzymes involved in EPS biosynthesis. Therefore, the effects of culture medium and conditions on monosaccharide composition and the activities of 7 enzymes involved in pathways (EMP pathway and biosynthesis and interconversion of sugar nucleotides) of polysaccharide synthesis of *N. flagelliforme* in a liquid-submerged culture were explored in this work.

The results showed that the monosaccharide components of *N. flagelliforme* EPS varied significantly and were influenced by culture medium and environmental conditions, however, glucose

### Table 2

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Concentration (mM)</th>
<th>Monosaccharide composition (%)</th>
<th>Monosaccharide composition (%)</th>
<th>Monosaccharide composition (%)</th>
<th>Monosaccharide composition (%)</th>
<th>Monosaccharide composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon sources</td>
<td>NaHCO₃</td>
<td>NaNO₃</td>
<td>Rha</td>
<td>Ara</td>
<td>Gal</td>
<td>Glc</td>
</tr>
<tr>
<td>1.26</td>
<td>0.08</td>
<td>0.09</td>
<td>0.10</td>
<td>0.11</td>
<td>0.12</td>
<td>0.13</td>
</tr>
<tr>
<td>2.94</td>
<td>0.08</td>
<td>0.09</td>
<td>0.10</td>
<td>0.11</td>
<td>0.12</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Within a column, different superscript letters a, b indicate significant differences ($P < 0.05$).
Table 3
Effects of carbon sources, nitrogen sources and light condition on activities of EPS synthesis enzymes in N. flagelliforme.

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Enzyme specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PGI</td>
</tr>
<tr>
<td>Carbon sources</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>122.39 ± 14.71*</td>
</tr>
<tr>
<td>NaHCO3 (g/L)</td>
<td>1.26</td>
</tr>
<tr>
<td>2.94</td>
<td>75.52 ± 6.93b</td>
</tr>
<tr>
<td>Nitrogen sources</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>165.84 ± 8.10*</td>
</tr>
<tr>
<td>NaN03 (g/L)</td>
<td>1.50</td>
</tr>
<tr>
<td>4.50</td>
<td>71.82 ± 1.39</td>
</tr>
<tr>
<td>Light condition</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>40.21 ± 2.91*</td>
</tr>
<tr>
<td>Red</td>
<td>88.83 ± 6.93b</td>
</tr>
<tr>
<td>Blue</td>
<td>121.67 ± 8.13c</td>
</tr>
</tbody>
</table>

Within a column, superscript letters a, b, c indicate significant differences (p < 0.05).

Fig. 1. Proposed biosynthesis pathways of sugar nucleotides for N. flagelliforme EPS. The solid arrows indicate the central pathways (Calvin cycle, tricarboxylic acid cycle (TCA) and Pentose Phosphate Pathway (PPP)) and the pathways were potentially active in all the strains studied. The dashed arrows and boxes indicate putative pathways and precursors of EPS respectively.

Fig. 2. The effects of culture conditions on monosaccharide mole percentage of UDP-sugars, GDP-sugars and other sugars synthesis pathways. (A) carbon and nitrogen sources. (B) light condition. In Fig. 2A, the initial mole ratio of C/N was calculated based on exogenous addition of NaHCO3 and NaN03.

was always major monosaccharide component. The mole percentages of arabinose showed negative correlation with UGPase under carbon and nitrogen sources conditions, while xylose showed positive correlation with UGPase under light condition. UGPase was involved in the production of UDP-glucose, which was a key precursor to polysaccharide synthesis (Bernstein and Robbins, 1965; Degeest & De Vuyst, 2000; Grobben et al., 1996). There was notable correlation of galactose with glucuronic acid, and they both had positive correlation with UGDH, which was required for the synthesis of the precursor UDP-glucuronic acid (Granja, Popescu, & Marques, 2007). Some literatures have reported the UDP-sugars were closely related to activities of EPS synthesis key enzyme (Grobben et al., 1996; Harding, Raffo, Raimondi, Cleary, & Ielpi, 1993; Looijesteijn et al., 1999; Oka & Jigami, 2006). The results suggested that there might be feedback regulation of arabinose to UGPase. The regulation of galactose and glucuronic acid by UGDH might exist in N. flagelliforme under carbon and nitrogen sources conditions.
For carbon and nitrogen sources conditions, FBPase activity was observably increased with increased initial mole ratio of C/N. The change of monosaccharide percentage involved in GDP-sugars synthetic pathway showed the similar trend, especially mannose. FBPase catalyzes the conversion of fructose-1,6-diphosphate into fructose-6-phosphate, which occurs in two essential steps: one involved in gluconeogenesis and the other in Calvin cycle (Looijesteijn et al., 1999; Tamoi et al., 1998). Thus, it might be inferred that the initial mole ratio of C/N promoted the photosynthetic CO$_2$ fixation, and then significantly increased the percentage of GDP-sugars synthetic pathway, especially mannose, particularly when added the NaHCO$_3$ into the culture medium.

PGI catalyzes the reversible isomerization of glucopyranose-6-phosphate and fructofuranose-6-phosphate playing a key role in glycolysis and gluconeogenesis pathways (Grobben et al., 1996; Peng et al., 2015, 2016). The correlation of mole percentages of monosaccharides with PGI activity suggested that influence patterns between carbon sources and nitrogen sources were different. The results that the activity of PGI was related to mole percentage of fructose and some UDP-sugars synthetic pathway monosaccharides (glucose, galactose, gluconic acid) only when different concentrations of NaHCO$_3$ was added, indicating that carbon sources exerted direct influence. While the variations of nitrogen sources resulting in the different correlation between monosaccharide composition and PGI activity might act in an indirect way by influencing carbohydrate metabolism, which increased the C/N and subsequently promoted the transfer of carbon flux from protein synthesis (Feuillade, Feuillade, & Jolivet, 1982; Huang et al., 1998; Otero & Vincenzini, 2003; Pereira et al., 2009).

These findings suggested that changing carbon and nitrogen sources conditions to increase initial mole ratio of C/N obviously influenced GDP-sugars synthetic pathway, especially mannose, by enhancing FBPase activity. But the difference between carbon sources and nitrogen sources in the influence on UDP-sugars synthetic pathway was that the addition of NaHCO$_3$ mainly affected the activities of PGI, UGPase and UGDH, whereas nitrogen sources regulated the activities of UGPase and UGDH.

Light conditions significantly influenced the UDP-sugars and other-sugars synthetic pathways, while had little effects on GDP-sugars synthetic pathway. The correlation between monosaccharides and enzyme activity was obviously different from that of carbon and nitrogen sources conditions. It was possibly due to that the monosaccharide composition might not be actually regulated by light (Ge et al., 2014; Otero, & Vincenzini, 2003). This result indicated that regulation of N. flagelliforme EPS synthesis by light condition was a more complex process compared with carbon sources and nitrogen sources. It might be inferred that light condition influence polysaccharide biosynthesis via the reprogrammed carbon metabolism depending on the comprehensive regulation of the key enzymes in EPS synthesis, which increase metabolic flux to UDP-sugars and thereby causing the differences in monosaccharide composition.

In addition, UGE, the Leloir pathway key enzyme catalyzing the interconversion of UDP-galactose and UDP-glucose, seemed not to play an important role in controlling the sugar compositions, which were also found in other studies (Degeest & de Vuyst, 2000; Escalante et al., 1998). PFK and PMI displayed little correlation with monosaccharide components under any tested conditions.

In the reported microbial polysaccharides pathway, rhamnose might also be activated in the form of dTDP-rhamnose (Fig. 1) (Freitas, Alves, & Reis, 2011; Laws, Gu, & Marshall, 2001) besides UDP-rhamnose (Kyoto Encyclopedia of Genes and Genomes, http://www.kegg.jp/kegg/pathway.html), which was the main form discussed in the study. In this paper, some correlation between rhamnose and tested enzymes was found, however it couldn’t clearly infer the activation form of the rhamnose in N. flagelliforme, which needs further investigation. Other nine monosaccharides basically conformed to the proposed biosynthesis pathways according to the results.

The above results also verified that the synthesis of EPS in cyanobacteria should probably follow the pathways proposed in the current study. In this model, CO$_2$ is fixed via the Calvin cycle, and enters the metabolism pathway as 3-phosphoglycerate. Then 3-phosphoglycerate is converted to fructose-6-phosphate, which participates in the EMP pathway (the direction of pyruvate formation), tricarboxylic acid (TCA) cycle and pentose phosphate pathway (PPP). For the biosynthesis of EPS, anabolic pathways of sugar nucleotide synthesis are necessary for providing the activated sugar monomers, and fructose-6-phosphate and glucose-6-phosphate are the important intermediates. Different culture medium and conditions would be related to precursor availability and therefore affected monosaccharide composition of EPS.

The comprehensive analysis of the relationship between monosaccharides and related enzymes showed similar correlation under carbon and nitrogen sources conditions. However, the correlation showed quite different trend under light condition, compared with carbon and nitrogen sources conditions. It might be deduced that similar mechanism was utilized for carbon and nitrogen sources to influence EPS synthesis, while light condition affected the EPS composition in a different mode. The underlying mechanism influencing EPS synthesis needs to be further investigated.

5. Conclusion

The effects of culture conditions on monosaccharide composition of N. flagelliforme EPS and related enzymes were investigated in
Fig. 4. Relationships between monosaccharide mole percentages and activities of EPS synthesis enzymes under (A) carbon and nitrogen sources, and (B) light condition.
the study. Culture conditions showed significant influences on both monosaccharide composition and related enzyme activities. The correlation between monosaccharide composition and enzymes activity was analyzed. The results suggested that the correlation between enzymes and monosaccharide composition was similar under carbon and nitrogen sources culture conditions, whereas which showed significant differences under light condition. The findings would improve the understanding of EPS synthesis process in N. flagelliforme and help obtain N. flagelliforme EPS with desired composition.

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References


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