Exopolysaccharide production in *Anabaena* sp. PCC 7120 under different CaCl_2 regimes

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Abstract Influence of various levels of CaCl_2 (0, 1, 10 and 100 mM) on exopolysaccharide production has been investigated in the cyanobacterium *Anabaena* 7120. At the concentration of 1 mM CaCl_2 , growth was found to be stimulatory while 100 mM was sub lethal for the cyanobacterial cells. Estimation of EPS content revealed that EPS production depends on the concentration of calcium ions in the immediate environment with maximum being at 10 mM CaCl_2 . A possible involvement of *alr2882* gene in the process of EPS production was also revealed through qRT-PCR. Further, FTIR-spectra marked the presence of aliphatic alkyl-group, primary amine-group, and polysaccharides along with shift in major absorption peaks suggesting that calcium levels in the external environment regulate the composition of EPS produced by *Anabaena* 7120. Thus, both quantity and composition of EPS is affected under different calcium chloride concentrations presenting possibilities of EPS with novel unexplored features that may offer biotechnological applications.

Keywords *Anabaena* 7120 · Extracellular polymeric substances · Fourier transform infra red spectroscopy · Quantitative reverse transcriptase-polymerase chain reaction · *alr2882* gene · Scanning electron microscopy

Abbreviations

EPS	Extracellular polymeric substances
FTIR	Fourier transform infra red spectrometry
SEM	Scanning electron microscopy
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction

Introduction

Cyanobacteria are ubiquitous photoautotrophic gram negative prokaryotes with the ability to synthesize high-molecular weight hydrated polysaccharide (EPS) layers on their surface which may remain attached to the cell (capsular) or released into the immediate environment (extracellular) (Micheletti et al. 2008; Singh et al. 2014). EPS are complex mixture mainly made up of polysaccharides, proteins, nucleic acids and lipids with various functional groups such as carboxylic, phosphoric, amino and hydroxyl groups (Liu and Fang 2002; Ozturk et al. 2010). These extracellular polymeric substances are believed to have protective role against desiccation, antibiotic, penetration of toxic metals, phagocytosis, phage attack and to produce biofilms (Cammarota and Sant'Anna 1998; Kanmani et al. 2011; Kazy et al. 2002; Sutherland 2001). Cyanobacterial EPS are comparatively better suited basically due to higher growth rate, reproducible physiochemical properties, and economical costs compared to EPS of plant and algal origin. They are of great biotechnological interest and their versatility and novelty presents promising potential to replace the traditional polysaccharides in industries related to textiles, detergents, adhesives, oil recovery, waste water treatment, dreading, brewing, cosmetology, pharmacology and food additives (Quintelas and Tavares 2001; Ruas-Madiedo et al. 2002). Among the various abiotic factors

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affecting survival of microorganisms, salinity is the foremost. Abundance of sodium and calcium salts particularly lead to a process termed as secondary salinization. Since calcium is an important macronutrient affecting various processes in higher as well as lower prokaryotes (Rocha and Vothknecht 2012; Pandey 2008) and fewer studies have been done taking into consideration its role in various metabolic pathways, there seems to be enough evidences to look for its role in extracellular polymeric substances biosynthesis. Considering biotechnological applications of EPS and the possibility of production of novel EPS under salinity particularly resulting from differing calcium levels in the environment, the present study focuses at evaluating the effect of calcium salt concentrations on EPS production, growth behaviour of *Anabaena* sp. PCC 7120 and nature of produced EPS. EPS assembly and export in cyanobacteria occur basically by three modes *wzy*, *ABC*, and *synthase* dependent. *ExoD* is involved in EPS production, however, exact role and/or relationship with the main pathways is still ambiguous (Pereira et al. 2013). Here, expression analysis of *alr2882* encoding *ExoD* protein in *Anabaena* sp. PCC 7120 has been studied to ascertain its involvement in assembly and export of EPS, moreover chemical characterization of EPS has been done by analyzing the functional groups by FTIR in cyanobacterium *Anabaena* sp. PCC 7120.

Materials and methods

Organism, growth conditions and experimental set up

Anabaena sp. PCC 7120, heterocyst-forming cyanobacterium, was grown in 1000 mL erlenmeyer flasks under moderate light intensity (3600 lux) provided with cool-white fluorescent lamps in culture room at 28 ± 1 °C in BG11₀ (Rippka et al. 1979) medium, pH 7.4. Cyanobacterial cultures of *Anabaena* sp. PCC 7120 were harvested through centrifugation and inoculated in BG11₀ medium (Supplementary Table 1) supplemented with different levels of calcium chloride (0–100 mM). EPS production has been determined at a wide concentration range (Supplementary Fig. 1) however only four concentrations namely 0, 1, 10 and 100 mM of CaCl₂ have been investigated as these concentrations accounted for significant changes in EPS production. All experiments were repeated thrice to confirm the reproducibility of the results.

Growth behaviour and pigment analysis

Growth was determined in terms of dry weight (Jahnke and Mahlmann 2010) at 20th day. Pigment analysis was done

Table 1 F ratios and levels of significance of multivariate ANOVA test for different parameters of *Anabaena* sp. PCC 7120 for repeated measures of CaCl₂ concentrations (treatments) and their interactions for absorbance at 663 nm and EPS production

Parameters	Treatment
EPS production	0.003023***
Absorbance 663 nm	0.001215***

Level of significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: not significant

by taking absorption spectra scan in the range of 300–800 nm by using Hitachi U 2910.

Isolation and determination of EPS content

Extracellular polymeric substances were extracted by the modified protocol of Cérantola et al. 2000. After 20 days cultivation (late log phase), 50 ml culture volume was centrifuged at $10,000 \times g$ for 10 min at room temperature. Supernatant was discarded, pellets dissolved in double distilled water and boiled at 100 °C for 15 min. Samples were then kept at room temperature for 10 min and 3 µL of 85 % TCA was added. The mixture was centrifuged at $10,000 \times g$ for 30 min. The supernatant containing EPS were taken and equal volume of ethanol was added and kept at 4 °C for overnight. Mixture was then centrifuged at $10,000 \times g$ for 30 min. Precipitate was then washed two times with 96 % ethanol and centrifuged at $10,000 \times g$ for 30 min. Finally, precipitate was dissolved in 1 mL double distilled water and stored at –20 °C. Total carbohydrate contents in extracted EPS samples were determined as per the protocol of Dubois et al. (1956) using glucose as a reference (Torino et al. 2001). EPS contents were calculated from the data obtained with triplicate trials ($n = 3$). One way analysis of variance (ANOVA) was further performed to check the statistical significance of interaction of treatments (0, 1, 10, 100 mM) with parameters namely EPS production and Absorbance at 663 nm (Table 1).

Scanning electron microscopy of *Anabaena* 7120 cells exposed to different concentrations of calcium chloride and the extracted EPS

Scanning electron microscopy was done at 720th day (late log phase) for the cyanobacterium (to check for EPS production) as well as the extracted EPS obtained after its isolation from the cyanobacterium. 0.5 mL of culture was centrifuged at $8000 \times g$ for 10 min. Pellets were washed thrice in Milli-Q (mQ) water (Millipore) and fixed in 2.5 % glutaraldehyde containing alcian blue and lysine (Fassel et al. 1997). Samples were incubated overnight at 4 °C. The pellets obtained after centrifugation at $8000 \times g$ for

30 min were dehydrated with 30, 50, 70, and 100 % ethanol for 15 min each. Further, dehydrated cyanobacterial cells were incubated for 1 h at room temperature in 100 % ethanol. 0.1 % AgNO₃ were added to the pellets and incubated in water bath at 45 °C for 1 h. The resultant pellets obtained after centrifugation were used for SEM analysis. For understanding the nature of extracted EPS, after isolation through the protocol of Cérantola et al. 2000 the samples were straightway subjected to SEM analysis. Fei Quanta 200 microscope fitted with LFD detector at a 20 kV working voltage with a working distance of 10 mm and 5000× magnification was used for SEM microscopy.

RNA extraction and transcript analysis

Total RNA was extracted from the cyanobacterial cells growing under different calcium concentrations (0, 1, 10 and 100 mM) using trizol reagent (Ambion RNA) as per instructions given by the manufacturers' protocol. Primers (*rnpB*: GTGAGGAGAGTGCCACAGAA/TAAGCCGGGTTCTGTTCTCT; *alr2882*: CGGTACAGATGCTTTTGGGT/GACGGGCGATTTTCTCTACA) were designed using Primer 3 software, referring to accessed sequences in the CyanoBase (<http://genome.kazusa.or.jp/cyanobase>) and qRT-PCR of gene *alr2882* was performed. *rnpB* was taken as control due to its constitutive expression. Primer accuracy was also tested using genomic DNA of the cyanobacteria as positive control.

EPS characterization through fourier transform infrared spectroscopy (FTIR)

The chemical characteristics of EPS isolated from cyanobacterial cells grown at different concentrations of CaCl₂ (0, 1, 10 and 100 mM) salts were analyzed using a Fourier transform infrared spectroscopy (PerkinElmer Spectrum Version 10.03.05). All infrared spectra were recorded over the range of 4000–400 cm⁻¹.

Results

Growth behaviour, pigment analysis and EPS production

For EPS determination, late log phase or stationary phase cultures are well suited (Samain et al. 1997; Decho and Lopez 1993; Manca et al. 1996) and accordingly EPS production was determined in each case after its isolation. To understand whether culture condition i.e. growth and the state of pigment levels could affect the EPS production; growth was assessed at the 20th day (late log phase) in terms of dry weight along with a spectrum scan in the range

of 300–800 nm for pigment analysis (Fig. 1a, b). Biomass (gDW) showed an increase by 60 % in case of 1 mM CaCl₂ while it decreased by 40 and 60 % for concentrations 10 mM and 100 mM CaCl₂ respectively. Spectrum scan revealed relatively higher absorption values near 400–500 nm and 660 nm corresponding to phycocyanin and chlorophyll *a* levels respectively. Absorbance values near 400–500 nm and 660 nm were increased by approximately two fold at 1 mM compared to control. Near wavelength 400–500 nm absorbance dropped by 60 and 64 % for concentration of 10 mM and 100 mM of calcium salt compared to control. Likewise, absorbance values decreased by 69 and 84 % at 10 mM and 100 mM of calcium salt concentrations near wavelength 663 nm. Growth in terms of dry weight and spectrum scan both suggest that 1 mM CaCl₂ is optimum, exhibiting high absorbance values while 100 mM is most unsuited for the survival of the *Anabaena* sp. PCC cells. An increase in growth by two fold was observed at 1 mM concentration compared to 0 mM at the same time an inhibition in growth by 60 % at 100 mM CaCl₂ concentration was found. EPS production (Fig. 1a) on the other hand showed different trend, maximum EPS production was found at the inhibitory concentration i.e. 10 mM of the calcium salt. EPS production followed the trend as 10 mM > 0 mM > 1 mM > 100 mM of CaCl₂ salt. EPS production increased by 61 % compared to control (0 mM) at 10 mM CaCl₂. This concentration 10 mM CaCl₂ is thus stimulatory in terms of enhancing EPS production and could be exploited for large scale production of EPS. One way analysis of variance (ANOVA) results also indicated that interaction of treatments (0, 1, 10 and 100 mM CaCl₂) with parameters: EPS production and Absorbance at 663 nm were statistically significant (Table 1).

Morphology of *Anabaena* sp. PCC 7120 cells under different calcium chloride levels and the extracted EPS: scanning electron microscopy

Variations in the level of EPS production resulting from exposure to different levels of salinity may lead to a change in surface morphology of the cyanobacterium therefore SEM was carried out to elucidate the impact. SEM of cyanobacterium (Fig. 2) facing different levels of salinity (0, 1, 10, 100 mM CaCl₂) showed a change in morphology (Singh and Mishra 2014, 2016) evidenced by changes in filament length, dimensions of individual vegetative cells, and formation of calcium precipitate i.e. spherical calcium-rich inclusions measuring nearly 70 to 800 nm in diameter were observed at higher calcium levels (10 and 100 mM) (Singh and Mishra 2016). SEM analysis of cyanobacterial samples at 20th day exhibited copious EPS around

Fig. 1 **a** Growth behavior and EPS production under different concentrations of calcium chloride in *Anabaena* sp. PCC 7120. **b** Spectrum scans of pigment under different concentrations of calcium chloride in *Anabaena* sp. PCC 7120

cyanobacterial filaments particularly at 10 mM treatment (Fig. 2). SEM of extracted EPS (Fig. 3) also revealed a difference in nature of EPS produced. Even with respect to extracted EPS the criss cross pattern (surface morphology and topology) was not the same throughout; EPS turns out to be more condensed, rigid forming star shaped aggregations at 0 mM calcium chloride while at other concentrations the EPS seem smooth and forms even meshwork.

Expression analysis of *alr2882* under different levels of calcium chloride

To study change in expression of gene *alr2882* under different calcium regimes qRT-PCR was performed (Fig. 4). Change in the expression of the gene *alr2882* (over or down expression) was analyzed in terms of fold increase/decrease in intensity of bands compared to

Fig. 2 SEM images of the cyanobacterium *Anabaena* sp. PCC 7120 under different levels of calcium chloride as observed on 20th day **a** 0 mM, **b** 1 mM, **c** 10 mM and **d** 100 mM CaCl_2 . Arrow indicates possible EPS matrix

Fig. 3 SEM images of extracted EPS showing different morphology at **a** 0 mM, **b** 1 mM, **c** 10 mM and **d** 100 mM CaCl_2

reference gene *rnpB*. The amplified product of 138 bp showed 1.6 fold up expression of *alr2882* in the cyanobacterium grown at 10 mM CaCl_2 . At 1 and 100 mM CaCl_2 concentration 2.3 and 3 folds decrease in the expression of *alr2882* gene was observed. Thus, this study showed that *alr2882* gene operates in the quantitative production of EPS.

Chemical characterisation of extracted EPS through FTIR

FTIR was done to characterise the EPS on the basis of differences in functional groups. The FTIR analysis of the extracted EPS released under different calcium chloride levels (salinity) has been investigated and summed up in

Fig. 4 qRT-PCR expression analysis of *alr2882* gene encoding protein product ExoD related to assembly and export of EPS in *Anabaena* sp. PCC 7120. **a** Gel image showing differential expression under differing CaCl_2 levels taking *rnpB* as reference gene, **b** bar graph showing change in relative expression of *alr2882* under differing CaCl_2 levels

Fig. 5. FTIR analysis clearly revealed a shift in the major band indicating a change in the structure of the EPS produced therein. Precise differences in the symmetric stretch of the phosphodiester back-bone of nucleic acid ($\sim 1080 \text{ cm}^{-1}$), amide II band of proteins ($\sim 1550 \text{ cm}^{-1}$), and in the area corresponding to the symmetric deformation of CH_3 and CH_2 of proteins and symmetric stretch of carboxylic acids groups ($\sim 1400 \text{ cm}^{-1}$) were observed (Table 2). Further, significant differences were also present in the IR spectra representing calcium ($\sim 2360 \text{ cm}^{-1}$). The absorbance in the mid-IR range is helpful in detecting microbial carboxyl and phosphoric groups involved in mediating bacterial cell interactions with mineral surfaces and with aqueous metal species.

Discussion

Extracellular polymeric substances of cyanobacterial origin offer much advantage over other groups of microorganisms. Processes and environmental variables may regulate the structure and composition of extracellular polymeric

substances (Pereira et al. 2009). Thus of many known factors affecting EPS; salinity is one of it. Salinity is basically due to prevalence of Na^+ and Ca^{2+} salts. Since calcium affects the structure and integrity of cell membrane; its variation in the immediate environment may also result in affecting EPS; their structure and production (Naeem et al. 2009). The present investigation is an attempt to correlate EPS production and CaCl_2 tolerance in *Anabaena* sp. PCC 7120. Further, attempts have also been made to draw a link between CaCl_2 levels and the nature and the composition of EPS produced in each case and the possible involvement of *alr2882* gene in EPS production.

EPS synthesis has been reported to be a major end product of photosynthesis (Moore and Tisher 1965). The results in the present study also suggest that EPS production is altered by the state of the cell culture particularly growth (Ramos et al. 2001; Noghabi et al. 2007) and photosynthetic pigments. Growth in terms of dry weight and absorbance lead to conclusion that 1 mM CaCl_2 is optimum while 100 mM is most unfavourable for the growth and survival of the *Anabaena* sp. PCC cells. High EPS production at the inhibitory concentration i.e. 10 mM of the calcium salt is in accordance to results obtained by other workers where it was shown that different environmental stresses increased production of EPS (Fernandes and Tomé 1989; Ozturk and Aslim 2010; Priester et al. 2006; Sutherland 2001). Higher EPS production at 10 mM Ca^{2+} in the studied cyanobacterium is very much similar to marine *Pseudoalteromonas* sp. isolate, where calcium (10 mM) caused major changes in the proteome and enhanced the amount of EPS and surface-associated biomass (Patrauchan et al. 2005). Most likely similar conditions must be operating resulting in enhanced EPS formation at 10 mM CaCl_2 concentration. Since EPS production is an energy expensive process hence at the sub lethal concentration of 100 mM calcium chloride EPS levels were low (Cunningham and Munns 1984). Also, these results indicated a regulatory role by calcium where either its presence or absence affected EPS production (López-Moreno et al. 2014).

EPS release is believed to protect microbial cells from desiccation and other environmental stresses enhancing their survival (Hill et al. 1997; Kazy et al. 2002; Mezhdoud et al. 2014). In the present investigation, SEM analysis revealed that there were significant alterations in morphology of the cyanobacterium and in the nature of EPS produced. Similar observations related to SEM analysis of EPS have been reported by a number of workers (Jia et al. 2011; Kończak et al. 2014; Ozturk et al. 2010). The reason behind differential level of EPS production under different regimes of calcium might be due to the fact that gram negative cyanobacterial cell wall possess acidic functional groups on its surface which become more negatively

Fig. 5 FTIR transmission spectra of EPS produced by *Anabaena* sp. PCC 7120 under different levels of calcium chloride **a** 0 mM, **b** 1 mM, **c** 10 mM and **d** 100 mM showing shift in absorption patterns

charged with increase in pH displaying an increased affinity for cations such as Ca^{2+} (Rodriguez-Navarro et al. 2012). Similar observations relating to cyanobacterial EPS production along with partial characterization has also been made by other workers as well (Filali et al. 1993; Hill et al. 1994).

EPS synthesis is a complex process involving a number of genes in cyanobacteria (Pereira et al. 2009) till date three basic assembly and export pathways have been deduced first is *wzy* dependent, second is ABC transporter dependent and last is ATP-synthase dependent. ExoD is involved in EPS production, however its exact role and/or

Fig. 5 continued

Table 2 Wave numbers (cm^{-1}) of dominant peaks obtained from FT-IR transmission spectra

CaCl ₂ concentration (mM)	Functional groups				
	OH	CH ₂	C=O	C–O	C–O–C
0 mM	3388.30	2926.32	1650.97	1459.30	1076.01
1 mM	3416.00	2925.80	1646.50	1455.00	1079.18
10 mM	3412.81	2923.90	1645.09	1421.80	1071.03
100 mM	3430.61	2923.37	1634.84	1426.00	1029.20

relationship with the main pathways is still unclear (Pereira et al. 2013) (Supplementary Fig. 2). In this lieu, expression of *alr2882* gene (<http://genome.kazusa.or.jp/cyanobase>) operating in last steps of assembly and export of EPS was monitored in the cyanobacterium *Anabaena* sp. PCC 7120. *alr2882* (genome.microbedb.jp/cyanobase/*Anabaena*/genes/*alr2882*) encodes exopolysaccharide synthesis protein ExoD (MW 22.23 kDa). The nucleotide length for *alr2882* is 633 (initial position 3516116 and terminal position 3516748) having a translated peptide of 210 aa (Pfam id PF06055) (Supplementary Fig. 3). At 10 mM CaCl₂ the expression of *alr2882* was maximal. Expression level fell down respectively by two and three fold under 1 mM and 100 mM CaCl₂ concentration. The results thus indicate that *alr2882* operate in regulation of EPS biosynthesis in *Anabaena* sp. PCC 7120. Although the exact mechanism of EPS assembly in *Anabaena* sp. PCC 7120 still remains elusive, involvement of *alr2882* (ExoD) is confirmed through qRT-PCR.

FTIR is a very good technique that could be used to understand the composition of biological products on the basis of differences in functional groups (Ozturk et al. 2010; Srivastava et al. 2015). As evidenced from the FTIR spectra of isolated EPS obtained from cyanobacteria growing under different calcium salt regimes; the spectra shows different absorption peaks when level of calcium in the external medium changes. Out of four dominant peaks observed, the broadest peak (3276 cm^{-1}) represents the O–H bond in

water (Peng et al. 2003). A significant difference was also revealed in the area representing calcium ($\sim 2360\text{ cm}^{-1}$) (Chen et al. 2015). Peak near this wavelength showed variation with the level of calcium in which they were grown. This is a clue to the possibility that calcium plays role in the biosynthesis of EPS. The maximum absorbance observed around 1045 cm^{-1} can be attributed to the C–O bond in polysaccharides (Yee et al. 2004). Presence of strong bands $3420\text{--}3468\text{ cm}^{-1}$ indicates the existence of –OH groups of glucose moieties. These bands are also overlapped with –NH groups (Ozturk et al. 2010). Strong absorption peak around 1550 cm^{-1} represent CONH– group of Amide II in proteins. This absorption was strongest at 0 mM followed by 10 mM and least at 1 mM. The $1000\text{--}1125\text{ cm}^{-1}$ range characterises the presence of uronic acid, O-acetyl ester linkage bond (Ozturk et al. 2010). Sharp absorption was found for EPS from 0 mM and 10 mM CaCl₂ concentration in this range pinpoints the abundance of uronic acid residues in the extracted EPS. Severe absorption at 1151 (due to symmetrical and asymmetrical C–O–S vibration) at 0 and 10 mM treatment confirm the presence of sulphate groups as reported for other cyanobacterial exopolymers (Hussein et al. 2015). Occurrence of numerous bands in control (0 mM) fewer than 1000 cm^{-1} is possibly due to several visible bands and/or presence of probable linkages between monosaccharides. Thus, external calcium concentration in the medium has the potential to change the composition of the EPS released by the cyanobacterium.

Conclusion

Considering the immense importance and economic applicability of EPS; the present investigation basically outlining EPS production, its chemical characterization (FTIR) as well as transcript analysis manages well to correlate EPS production from the cyanobacterium *Anabaena* sp. PCC 7120 to changes in extracellular levels of calcium chloride. Changes in calcium concentration may be used as a factor to regulate EPS production at mass scale also. New and novel EPS may even be reported in near future using salinity as a controlling factor. More of transcriptional level studies need to be done so as to get a clearer picture of EPS synthesis and regulation at molecular level. Further, structural as well as rheological studies on the extracted EPS would bring additional information for applications in biotechnology related industries.

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Compliance with ethical standards

Conflict of interest All the authors declare that they don't have any conflict of interest.

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