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Inducible microbial osmotic responses enable enhanced biosorption capability of cyanobacteria

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ABSTRACT

Microorganisms have evolved series of protective or adaptive responses to enhance survivability to fight against stresses in nature and engineered systems. Although the cellular responses and underlying mechanisms have been intensively studied, seldom research explored the possibilities of employing the microbial stress response via an inducible routine for applications of environmental benefits. In this study, we proposed and demonstrated that the biosorption of methylene blue by cyanobacteria could be enhanced via exerting higher osmotic stress, and the enhanced biosorption is attributed to the induced stress responses which elicited the combined effects of exopolysaccharides over-secretion and the up-regulated carbonic anhydrase activity. To our knowledge, this is the first study that employing the inducible physiological responses of microorganisms for enhanced environmental application, and we believe that this research will bring novel insights into the field of applied microbiology and environmental biotechnology.

1. Introduction

Microorganisms live in changing environments with various stresses. These stresses elicit protective or adaptive responses of exposed cells to enhance survivability [1]. Many researchers investigated the genetic and physiological variations in microbes when they were exposed to different environmental stimuli [2,3]. As reported, pH, temperature, antibiotics, nanoparticles and heavy metals could all induce the variation of surface properties including increased secretion of extracellular polymeric substances (EPS), as well as the change of transcriptional profiles of relevant genes [4–8]. Although the cellular stress responses and underlying mechanisms have been intensively studied in various microorganisms in both natural and engineered systems [9], there were no reports to our best knowledge that had taken advantage of the microbial stress response via an inducible routine for environmental benefits.

Environmental biotechnologies, especially biosorption, require sustainable biomaterials with promising performance to remove hazardous pollutants. Previous research demonstrated that the performance of biosorption relies on the cell surface properties to interact with target contaminants [10]. EPS are of grand importance to these specific features [11]. Given the inducing effects of external stress on EPS secretion, we proposed that exerting external stress on microorganisms could enhance its biosorption capacity via inducing microbial stress responses.

Ideal biomaterials for adsorption should possess adequate functional groups and originate from sustainable resources. In this sense, enriched microorganisms are promising candidates, and cyanobacteria have even further advantages. As autotrophs, they directly use CO_2 and solar energy to accumulate biomass. Thus, we chose *Synechococcus elongatus* PCC7942 (*S. elongatus*), a model cyanobacterium with well-characterized physiology and genetics, as an example to investigate whether external stress could induce cellular response and enhance biosorption capability. In addition, *Microcystis aeruginosa*, which is known to be one of the major genuses dominating the algal blooms and is desirable to be removed from aquatic ecosystems, was further tested to confirm the universality of our strategy. NaCl was selected to exert osmotic stress to the cell culture in order to enhance its biosorption capability, since, unlike antibiotics, nanoparticles, or other stressors, NaCl exhibits limited environmental impacts by itself.

In the present study, we demonstrated that it is feasible to exert external stress for enhancing biosorption via inducing the stress responses of cyanobacteria. The adsorption performance of induced cyanobacteria, surface properties as well as the transcriptional analysis of essential genes were investigated. We believe our

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strategy would develop a simple, cheap, and environmental sound method for enhanced biosorption with a novel angle via exploiting the stress responses of microorganisms.

2. Materials and methods

2.1. Strains, culture conditions and sample preparation

S. elongatus was obtained from ATCC (American Type Culture Collection), and the *M. aeruginosa* was obtained from Prof. Li Li's lab (Shandong University). Both of them were cultured in BG11 liquid medium (pH 7.1) at 30 °C with constant light (2000–3000 Lx). *S. elongatus* cells were harvested at stationary growth phase by centrifugation at 10,000*g* for 10 min at 4 °C and washed twice with phosphate buffer saline (PBS, pH 7.2). *M. aeruginosa* cells were harvested at stationary phase by centrifugation at 4,500*g* for 10 min at 4 °C and washed following the same protocol for *S. elongatus*. The pellets were resuspended in PBS and used for subsequent experiments. To distinguish the physical variations caused by osmotic stress from stress responses induced by stress shock for living cells, half of the harvested cells were treated by reacting with 2.5% glutaraldehyde for 15 min at 4 °C and considered as dead cells, to compare with the other half without glutaraldehyde treatment (living cells). Both living cells and dead cells were used for the adsorption experiment under the same conditions.

2.2. Osmotic shock and adsorption experiments

Living and dead cells were washed twice with deionized water and resuspended in different concentration of NaCl solution (0, 0.15, 0.25, 0.5 and 0.75 M); they were afterwards shaken for 24 h at 30 °C to enable the osmotic responses. Then, 500 μL of cyanobacterial suspensions were added to 50 mL centrifuge tubes, and 250 μL of 200 mg/L methylene blue (MB) were added as the adsorbates. Finally, the total volume of the solution in each tube was adjusted to 5 mL with deionized water. All tubes were shaken on an orbital shaker at 150 rpm in dark at 25 °C for 3 h to reach the adsorption equilibrium. After filtered by 0.45-μm cellulose acetate membrane, MB in the filtrate was analyzed by measuring the absorbance at 664 nm with a UV-2000 spectrophotometer (UNICO, USA). The performance of adsorption was evaluated by specific adsorption capacity, which is expressed as follow,

$$\text{Specific adsorption} = \frac{q}{q_c}$$

in which q is the adsorbed MB by osmotic stress induced cyanobacteria, mg/g dry weight; q_c is adsorbed MB by control, mg/g dry weight.

2.3. Extraction and characterization of EPS

Following the osmotic shock treatment for *S. elongates* and *M. aeruginosa*, EPS was extracted using a heating procedure (70 °C, 1 h) followed by centrifugation at 10,000 rpm for 15 min [12]. The supernatants were filtrated through a 0.45-μm cellulose acetate membrane and were considered as the EPS solution including exopolysaccharides (PS) and exoproteins (PN). The PS in EPS was measured by anthrone-sulphuric acid method [13], using glucose as the standard, and the PN was determined by the Bradford method [14], using bovine serum albumin as the standard.

2.4. Transcriptional analysis

Transcriptional variations of essential genes in the model cyanobacterium *S. elongatus* were further analyzed using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

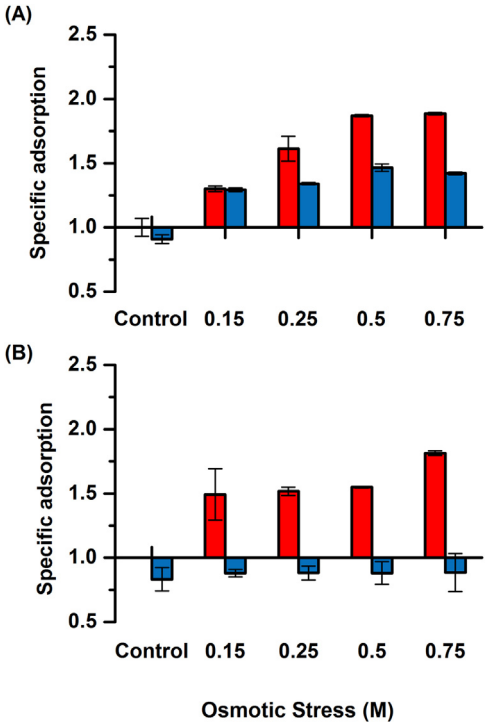

Fig. 1. Specific adsorption of living (red) and dead (blue) *S. elongatus* (A) and *M. aeruginosa* (B) cells with 0, 0.15, 0.25, 0.5 and 0.75 M NaCl shock. The adsorption capacity of living cells for both species exhibited significant increase compared with control, and the results were confirmed by statistical analysis ($p < 0.05$) as significant differences. Error bars represent ±1 SD from the means of at least triplicate measurements. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

RNA was extracted with the RNAprep pure Cell/Bacteria Kit (Tiangen Biotech. Co. Ltd., Beijing, China). Reverse transcription was conducted with the Primescript™ RT reagent kit (Takara Biotech. Co. Ltd., Dalian, China). qRT-PCR was carried out using a Roche LC-480 real-time PCR system. All replicates of each sample were measured at least in triplicates. *rbcL*, *icfA*, *rpoD*, *KpsF* and *cpsB* were selected as targeted genes, and the 16 s rDNA sequence was used as the housekeeping gene. The qRT-PCR results were quantified using $2^{-\Delta\Delta Ct}$ method [15]. All experiments were followed by the protocols from manufacturers. Primer pairs designed for the target genes are listed in Table SI.

3. Results and discussion

3.1. Enhanced biosorption by inducing the osmotic responses of S. elongatus and M. aeruginosa

To study the effect of external salt stress, living and dead cells of two cyanobacterium species were resuspended in different concentrations of NaCl solutions, and MB was selected as a target adsorbate. After reaching the adsorption equilibrium, the specific adsorption of MB was calculated. For *S. elongates* living cells, the specific adsorption of MB increased by 1.30, 1.61, 1.87 and 1.89-fold with the cells that had been treated with 0.15, 0.25, 0.5, 0.75 M NaCl solution, respectively (Fig. 1A and Table SII). However, the increased adsorption of dead cells, which might resulted from the morphological variations, was not as significant as that of living cells. The more significant adsorption enhanced effect of osmotic stress on living cells than dead cells indicating that the increased adsorption was partly resulted from the induced stress responses of living cells, not only the physical change by osmotic stress. We further examinated the strategy on *M. aeruginosa*. Similar results were obtained

(Fig. 1B and Table SII). For living cells, the specific adsorption was increased by 1.49, 1.52, 1.55 and 1.81-fold under 0.15, 0.25, 0.5 and 0.75 M NaCl, respectively. However, we observed severe cell damages when the concentrations of NaCl exceled 0.25 M, and the cells lost the integrity under these high NaCl levels (Fig. S1). Thus, the increased adsorption capability of *M. aeruginosa* induced by higher than 0.25 M NaCl might not resulted from our proposed strategy. At lower osmotic stress (0.15 M), at which the *M. aeruginosa* cells remain intact, we also determined the enhanced adsorption capability induced by osmotic stress (Fig. S1). When we examined the integrity of *S. elongatus* at all tested levels, we found that all cells remained their morphology without distinguishable damage (Fig. S2). Therefore, we concluded that the osmotic stress was able to increase the adsorption performance of both *S. elongatus* and *M. aeruginosa* via inducing physiological changes under certain range of stress levels.

3.2. *Surface characterization of osmotic stress induced* S. elongatus *and* M. aeruginosa

Previous research reported that surface properties play important roles in adsorption [10]. So, the observed effects of osmosis stress on the changes of adsorption capacity may be attributed to the varied surface properties. The EPS contents of the biosorbents that were treated with different NaCl levels were first measured as they are the outer-most layer of the cell which directly interacts with the adsorbates [16,17]. For both living and dead cells of *S. elongatus*, the content of PN did not show obvious changes (Fig. 2B), indicating that PN was not the dominating factor contributing to the varied surface properties. However, the content of PS in *S. elongatus* living cells without NaCl treatment was 5.89 ± 0.12 mg/g dry weight, and it increased in the cells that was treated with NaCl and reached the highest 32.73 ± 1.14 mg/g dry weight in the cells that was treated with 0.25 M NaCl (Fig. 2A). On the other hand, the PS content of dead cells (treated with various concentrations of NaCl) remained similar to the control. This indicated that the variation of PS content was due to the cellular responses, and thus this was only significant in the living cells. Our results were in well agreement with various previous researches. Many researchers have discovered that microorganisms overproduced EPS to protect themselves from environmental stimuli [17–19] and higher amounts of PS production under salt stress were reported in *Synechocystis* sp. [20]. These results corresponded well with the change of FTIR profiles. For the living cells treated with NaCl from 0 (control) to 0.75 M, the differences could be observed in the range corresponding to the functional groups in polysaccharides; the polysaccharide groups band shifted from 1030 to 1050 cm^{-1} for *S. elongates* (Fig. S4). Therefore, the enhanced adsorption of the cells after osmotic shock might be contributed to the varied PS content.

For *M. aeruginosa* living cells, both PN and PS showed significant increase within the cells treated with NaCl (0.15 M) compared to those in the control cells (Fig. S3). Interestingly, the PS contents in the *M. aeruginosa* dead cells were very similar to those in the living cells under both conditions (either without or with 0.15 M NaCl treatment), and thus similarly a significant increase of PS content was observed in the NaCl treated dead cells compared to that in the control (dead cells without NaCl treatment). However, the PN contents in the *M. aeruginosa* dead cells were much lower than those in the living cells under same conditions (with or without NaCl treatment) and did not show big difference when compared the NaCl treated cells versus the control.

However, for the *S. elongatus* cells treated with 0.5 and 0.75 M of NaCl, when compared to the cells treated with 0.25 M, the PS content decreased while the adsorption capacity still increased,

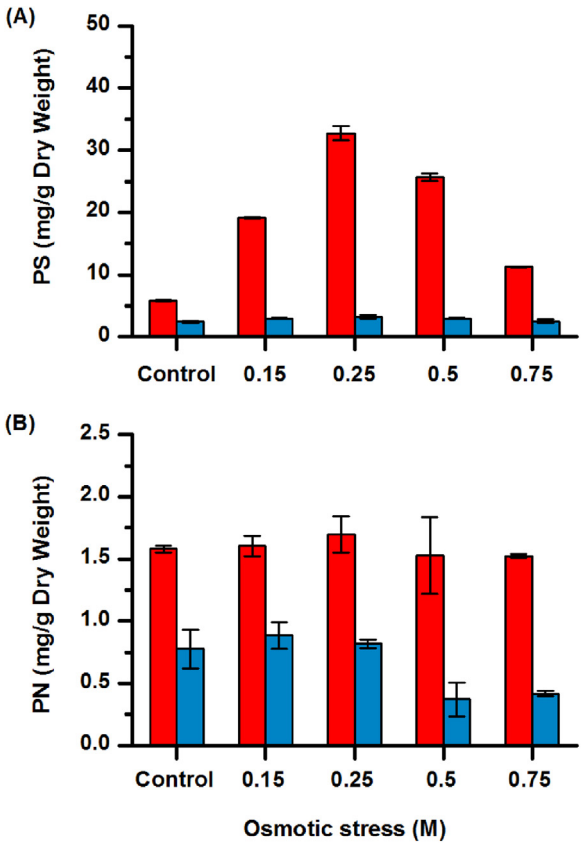

Fig. 2. Exopolysaccharide (PS) (A) and exoproteins (PN) (B) excreted by living (red) and dead (blue) *S. elongatus* cells with 0, 0.15, 0.25, 0.5 and 0.75 M NaCl shock. Error bars represent ±1 SD from the means of at least triplicate measurements. The data of living cells treated with 0.15, 0.25, 0.5 and 0.75 M NaCl show significant difference ($p < 0.05$) compared with control via statistical analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

indicating that PS might not be the only key factor influencing the adsorption in this process.

3.3. *Variations of exopolysaccharide synthesis and essential genes through osmotic shock*

To better elucidate the working mechanism for the enhanced adsorption by the osmosis shock strategy, the transcriptional profiles of *kpsF*, *cpsB*, *rbcL*, *icfA* and *rpoD* in *S. elongatus* were analyzed with qRT-PCR. The *kpsF* and *cpsB* genes are closely related to PS production. In particular, *kpsF* gene is involved in the synthesis of 3-deoxy-D-manno-oct-2-ulosonic acid linker or its activated donor connecting the polysaccharide to a terminal lipid [21], while *cpsB* gene encodes a phosphotyrosine protein phosphatase which is essential for encapsulation and regulation of capsular polysaccharide production [22]. In the cell culture with the osmosis shock using 0.25 M NaCl, the expression level of *KpsF* increased up to 2.47 ± 0.64 folds than the control, but it decreased to 0.55 ± 0.17 fold of the control in the cells treated with 0.75 M NaCl. This result is consistent with the EPS production results (Figs. 2 and 3). The expression level of *cpsB* also decreased to 0.57 ± 0.09 fold in the cells treated with 0.75 M NaCl. This results explained the increased production of PS in the cells with 0.25 M NaCl treatment and the reduced PS secretion from the cells with 0.75 M NaCl treatment.

Furthermore, the photosynthesis-related genes, *rbcL* (encodes the large unit of ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBisCO) and *icfA* (encodes carbonic anhydrase, CA), were investigated. The expression level of *rbcL* decreased to 0.46 ± 0.10 fold in

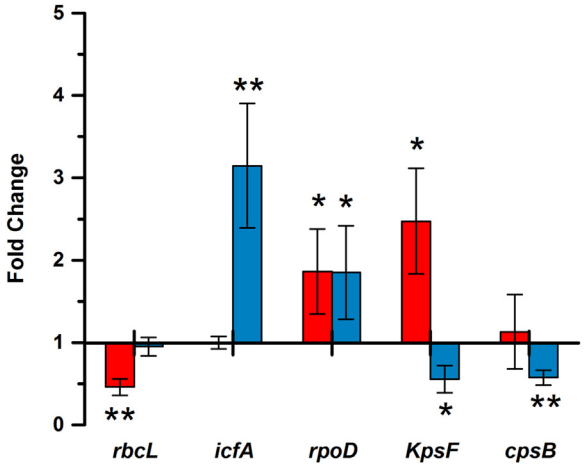

Fig. 3. The transcriptional profiles of *rbcL, icfA, rpoD, kpsF* and *cpsB* of *S. elongatus* treated with 0.25 (red) and 0.75 M (blue) NaCl. The differences between data were evaluated using the Student's *t*-tests with $p < 0.05$ (*) as a significant difference and $p < 0.001$ (**) as highly significant differences. Error bars represent ±1 SD from the means of at least triplicate measurements. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the cells with 0.25 M NaCl treatment (Fig. 3), indicating that the salt stress has adverse effects on the activities of RuBisCO, the core enzyme in Calvin cycle. The CA regulates the carbon concentration mechanism in cyanobacteria, and converts HCO_3^- into CO_2 [23,24]. When HCO_3^- transports through the cell membrane, OH^- will be pumped out [25]. Thus, up-regulated CA activity would result in an increased pH in the microenvironments of cells and higher pH may facilitate the adsorption of MB on cyanobacteria via influencing the functional groups and surface charge of the cells, as well as the chemistry of MB. We observed that the expression level of *icfA* increased up to 3.14 ± 0.76 fold than control in the cells with 0.75 M NaCl treatment (Fig. 3), suggesting that the activity of CA was induced by osmotic stress. Therefore, the enhanced biosorption of the osmotic induced cyanobacteria was resulted from the combined effects of PS overproduction and *icfA* overexpression.

Ultimately, *rpoD* encodes the sigma factor which regulates the global cellular stress responses in cyanobacteria [26]. The expression level of *rpoD* increased up to 1.86 ± 0.52 and 1.85 ± 0.57 fold compared with the control in the cells with 0.25 and 0.75 M NaCl treatments, respectively (Fig. 3). This results suggested that the cellular stress responses have been actually up-regulated under osmotic shock, and further evidenced our proposed strategy that the enhanced biosorption was induced via the physiological stress responses by exerting NaCl stress.

4. Conclusion

In summary, our study demonstrated that it is feasible to employ cellular stress responses of cyanobacteria induced by osmotic stress for enhanced biosorption. The characterization of the cell surface properties, and the analysis of the transcriptional profiles of some relevant genes explained the enhanced adsorption performance of the cells with our proposed strategy. To our best knowledge, this is the first study that utlizing the inducible physiological responses of microorganism for enhanced environmental application. This simple, cheap and environmental friendly routine can not only be applied to the model microbes with sufficient physiological and genetic information but also the ones lacking such detailed information. Especially for those harmful microorganisms that present in the contaminated ecosystems and need to be removed, such a strategy will add further benefits for harvesting waste materials for environmental benefits. We believe this research will bring novel insights into the field of applied microbiology and environmental technology and will trigger more interesting applications and relevant endeavors.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2017.01.002.

References

[1] K. Poole, Stress responses as determinants of antimicrobial resistance in gram-negative bacteria, Trends Microbiol. 20 (2012) 227–234.
[2] S. Fulda, S. Mikkat, F. Huang, J. Huckauf, K. Marin, B. Norling, M. Hagemann, Proteome analysis of salt stress response in the cyanobacterium Synechocystis sp. strain PCC 6803, Proteomics 6 (2006) 2733–2745.
[3] C.W. Nde, H.-J. Jang, F. Toghrol, W.E. Bentley, Global transcriptomic response of Pseudomonas aeruginosa to Chlorhexidine diacetate, Environ. Sci. Technol. 43 (2009) 8406–8415.
[4] J. Uchiyama, Y. Kanesaki, N. Iwata, R. Asakura, K. Funamizu, R. Tasaki, M. Agatsuma, H. Tahara, A. Matsuhashi, H. Yoshikawa, Genomic analysis of parallel-evolved cyanobacterium Synechocystis sp. PCC 6803 under acid stress, Photosynth. Res. (2015) 1–12.
[5] L.R. Hoffman, D.A. D'Argenio, M.J. MacCoss, Z. Zhang, R.A. Jones, S.I. Miller, Aminoglycoside antibiotics induce bacterial biofilm formation, Nature 436 (2005) 1171–1175.
[6] Y. Yang, P.J.J. Alvarez, Sublethal concentrations of silver nanoparticles stimulate biofilm development, Environ. Sci. Technol. Lett. 2 (2015) 221–226.
[7] V. Tremaroli, M.L. Workentine, A.M. Weljie, H.J. Vogel, H. Ceri, C. Viti, E. Tatti, P. Zhang, A.P. Hynes, R.J. Turner, Metabolomic investigation of the bacterial response to a metal challenge, Appl. Environ. Microbiol. 75 (2009) 719–728.
[8] H.T. Nguyen, H. Razafindralambo, C. Blecker, C. N'Yapo, P. Thonart, F. Delvigne, Stochastic exposure to sub-lethal high temperature enhances exopolysaccharides (EPS) excretion and improves Bifidobacterium bifidum cell survival to freeze–drying, Biochem. Eng. J. 88 (2014) 85–94.
[9] J. Qiao, S. Huang, R. Te, J. Wang, L. Chen, W. Zhang, Integrated proteomic and transcriptomic analysis reveals novel genes and regulatory mechanisms involved in salt stress responses in Synechocystis sp. PCC 6803, Appl. Microbiol. Biotechnol 97 (2013) 8253–8264.
[10] K. Vijayaraghavan, Y.-S. Yun, Bacterial biosorbents and biosorption, Biotechnol. Adv. 26 (2008) 266–291.
[11] Q. Yu, J.B. Fein, Sulfhydryl binding sites within bacterial extracellular polymeric substances, Environ. Sci. Technol. 50 (2016) 5498–5505.
[12] S.S. Adav, D.-J. Lee, Extraction of extracellular polymeric substances from aerobic granule with compact interior structure, J. Hazard. Mater. 154 (2008) 1120–1126.
[13] F.A. Loewus, Improvement in anthrone method for determination of carbohydrates, Anal. Chem. 24 (1952) 219.
[14] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[15] F. Pinto, C.C. Pacheco, D. Ferreira, P. Moradas-Ferreira, P. Tamagnini, Selection of suitable reference genes for RT-qPCR analyses in cyanobacteria, PLoS One 7 (2012) e34983.
[16] S. Zhang, Y. Jiang, C.-S. Chen, J. Spurgin, K.A. Schwehr, A. Quigg, W.-C. Chin, P.H. Santschi, Aggregation, dissolution, and stability of quantum dots in marine environments: importance of extracellular polymeric substances, Environ. Sci. Technol. 46 (2012) 8764–8772.
[17] P.-F. Xia, Q. Li, L.-R. Tan, X.-F. Sun, C. Song, S.-G. Wang, Extracellular polymeric substances protect Escherichia coli from organic solvents, RSC Adv 6 (2016) 59438–59444.
[18] N. Joshi, B.T. Ngwenya, C.E. French, Enhanced resistance to nanoparticle toxicity is conferred by overproduction of extracellular polymeric substances, J. Hazard. Mater. 241–242 (2012) 363–370.
[19] C.-C. Chien, B.-C. Lin, C.-H. Wu, Biofilm formation and heavy metal resistance by an environmental Pseudomonas sp, Biochem. Eng. J. 78 (2013) 132–137.

[20] S. Ozturk, B. Aslim, Modification of exopolysaccharide composition and production by three cyanobacterial isolates under salt stress, Environ. Sci. Pollut. Res. 17 (2010) 595–602.
[21] S.B. Pereira, R. Mota, C.P. Vieira, J. Vieira, P. Tamagnini, Phylum-wide analysis of genes/proteins related to the last steps of assembly and export of extracellular polymeric substances (EPS) in cyanobacteria, Sci. Rep. 5 (2015) 14835.
[22] J.K. Morona, R. Morona, D.C. Miller, J.C. Paton, Streptococcus pneumoniae capsule biosynthesis protein CpsB is a novel manganese-dependent phosphotyrosine-protein phosphatase, J. Bacteriol. 184 (2002) 577–583.
[23] H. Fukuzawa, E. Suzuki, Y. Komukai, S. Miyachi, A gene homologous to chloroplast carbonic anhydrase (icfA) is essential to photosynthetic carbon dioxide fixation by Synechococcus PCC7942, Proc. Natl. Acad. Sci. U. S. A 89 (1992) 4437–4441.
[24] T. Nishimura, O. Yamaguchi, N. Takatani, S.-i. Maeda, T. Omata, In vitro and in vivo analyses of the role of the carboxysomal β-type carbonic anhydrase of the cyanobacterium Synechococcus elongatus in carboxylation of ribulose-1, 5-bisphosphate, Photosynth. Res. 121 (2014) 151–157.
[25] L.-R. Tan, P.-F. Xia, X.-F. Sun, N. Guo, C. Song, Q. Li, S.-G. Wang, Ecological insights into low-level antibiotics interfered biofilms of Synechococcus elongatus, RSC Adv. 6 (2016) 78132–78135.
[26] A. Goto-Seki, M. Shirokane, S. Masuda, K. Tanaka, H. Takahashi, Specificity crosstalk among group 1 and group 2 sigma factors in the cyanobacterium Synechococcus sp. PCC7942: in vitro specificity and a phylogenetic analysis, Mol. Microbiol. 34 (1999) 473–484.