Exopolysaccharide Production From *Nostoc* sp. Under Different Nutritional Conditions

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Abstract

In the last decades, biotechnological applications have led to the optimization of the cultural conditions for the production of exopolysaccharides (EPSs) by cyanobacteria. *Nostoc* is a genus of cyanobacteria found in various environments that forms colonies composed of filaments of moniliform cells in a gelatinous sheath. The effect of some important nutritional and growth beside iron nanoparticles on production by *Nostoc* sp. was studied. Optimization of the cultural conditions was carried out using Central Composite Design (CCD), and it was achieved at pH 8.39, 1% of NaCl and 0.8 $g.l^{-1}$ nitrate based in modified BG0 medium and more than 12 $g.l^{-1}$ of EPS was obtained by phenol-sulphuric acid method. The present research investigated the possibility of inducing EPS production in the presence of Fe_3O_4 nanoparticles from optimized microalga cultures. This study revealed that *Nostoc* sp. treatment with 10 $mg.l^{-1}$ Fe_3O_4 NPs induced maximal EPS production (4 $\mu g.ml^{-1}$).

Keywords: Central Composite Design, Exopolysacchride, Nanoparticle, *Nostoc* sp., Optimization.

Introduction

Microalgae are photosynthetic microorganisms that can convert CO_2 from the atmosphere into organic carbon. There are eukaryotic microalgae such as green microalgae, red microalgae, diatoms and dinoflagellates or prokaryotic cyanobacteria red microalgae. Microalgae are a source of numerous compounds that can be used in many branches of industry. Synthesis of such compounds in microalgal cells can be amplified under stress conditions. Human activity, development of industry and natural Earth processes lead to release of numerous metals such as Fe^{2+} Zn^{+}, Cu^{2+}, Cd^{+} and metallic nanoparticles (Fe_3O_4, Ag^{+}, TiO_2) that can act as inhibitor modulators for microalga growth and metabolism. Thus, exposure of in microalga to various metals can be one of the methods to induce cell stress therefore it synthesis of target products (Krystian et al., 2015). One of these products is exopolysaccharides (EPS), which are excreted by cyanobacteria upon exposure to stress condition. EPSs are high-molecular-weight polysaccha-

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rides which are mainly composed of carbohydrates secreted into media (Chengcheng et al., 2017). These products can be easily isolated and purified from the supernatants of fermentation culture with high yields (Chi and Zhao, 2003). In recent years, increasing attention has been focused on the EPSs of microbial origin, which have different chemical structures and physiological functions based on strain and environmental condition. Among microbes, some strains of cyanobacteria (blue-green microalgae) and green algae are good producers of EPSs (Dhanesh et al., 2018). Cyanobacterial EPSs have been proposed to operate in the protection against environmental stress (Jittawuttipoka et al., 2013) and play important roles in biological systems, including the production of fish meal, organic fertilizers, natural pigments and antioxidants (Freitas, 2017) as well as biofilm formation, bioremediation of heavy metals, protection against desiccation and UV, and establishment of symbiosis (Pereira, 2015). In the last decades, extensive efforts have been done continuously to isolate, identify, characterize and functionalize novel and valuable EPSs from different species for different purposes and applications (Chengcheng et al., 2017). So far, over 70 cyanobacterial strains have been shown to yield good EPSs (De Philippis and Vincenzini, 1998). It has been reported that EPS productivities shown by some cyanobacteria are very low in comparison with heterotrophic microorganisms (Zhao and Chi, 2003). For optimal EPSs production, it is necessary to increase the growth rate and EPSs productivity per cell. Moreover, the responses of cyanobacteria which is completely strain- dependent based on different culture conditions, made the optimization of EPS production even more difficult (Pereira et al., 2015). Several researches indicated EPSs production are affected both by nutritional and environmental parameters. Some of the important factors are the presence of metal ions, the availability of carbon substrates and the balance between carbon and other limiting nutrients (Dante et al., 2016). In some researches nutrient deprivation enhanced EPS synthesis, thus, it was proposed as a stimulatory method to maximize the productivity (Federico and Roberto, 2015). Nitrate deficiency has been shown to stimulate polysaccharide synthesis in some microalgae (Arad et al., 1992). The effect of salinity on EPS synthesis is still controversial, and the most probable effects are species-specific (Federico and Roberto, 2015). Further studies need to clarify the factors that activate the biosynthetic machinery of the EPSs. Additionally iron-based nanoparticles (NPs) are increasingly produced and used in many fields, particularly in environmental remediation and biomedicine. Due to the fact that iron-based NPs are increasingly produced and used in many fields, thus, they could easily enter the aqueous environment and may induce toxic effect to the ecosystem. As a result, more and more attention has been paid to the potential impact of Fe-based NPs, especially on aquatic organisms (Mahmoudi et al., 2011; Stefaniuk et al., 2016) and considering some recent studies that have reported that microalgae synthetize some organic compounds such as EPSs in higher amounts as a protective response against metals. So, aim

of this study was to optimize several factors (nitrate, salinity and pH) leading to the maximization of the EPS production from *Nostoc* sp. Then to elucidate the absorption behavior at environmentally relevant concentrations of Fe NPs and to test the hypothesis that suitable dosages of nanoparticles stimulate the growth and EPS production, a series of batch cultivations were carried out at different Fe_3O_4 NPs concentrations.

Materials and Methods

Cyanobacteria culture

Nostoc sp. belonging to cyanobacteria, was obtained form the culture collection of the department of petroleum microbiology, Research Institute (ACECR) using in this study. Following the achievement of axenic cultures, this strain was cultivated in the BG0 medium. *Nostoc* sp. was pre cultured in Erlenmeyer flasks at 30 °C and under continuous light (3000 lux of light intensity) provided by three fluorescent lamps (40 W per lamp). The cultures were aerated using an aquarium pump (Air PUMP AC-9906 model). Samples (3 ml) were taken every two days and chlorophyll a, and carbohydrates in the surnatants were analyzed.

Experimental design by design expert software

The cells in the logarithmic phase of growth were collected from stock cultures and used for incubation in different NaCl salinity (Na^+), pH and NO_3^{-1} concentrations during 6 weeks to investigate the biomass yield and EPS production. Experimental Design Expert 8.0.7.1 was used for experimental design. Level factorials 2^3 with 3 replicates were used to identify the interaction between pH, nitrate and salinity with physiological responses of *Nostoc* sp. In a 2^3 level factorial designs, each factor was varied over 2 levels, +1 and -1, therefore, there was 6 sets of experiments including 2 replicates for factorial design study. Central Composite Design (CCD) was chosen as the experimental design in this study since it is suitable to fit second order models in microbial cultivations (Akin et al., 2008). There were 16 experimental sets with 4 replicates. CCD helps to estimate curvature, and it has 4 center points for 3 factors. Batch experiments were designed with these variables as shown in Table 1.

Fe NPs treatment

For the Fe NPs exposure experiment, Freshly prepared Fe NP, average particle size of 30-50 nm were suspended in deionized water prior to each experiment, and then stirred using an Ultra- homogenizer. The pH was adjusted to 6.8 ± 0.1, which is close to optimal pH condition for algal growth then added to the algal dilutions to achieve the final concentrations 1, 10, 50 and 100 $mg.l^{-1}$. After exposure to Fe NPs, the effects of nano nutrient on the *Nostoc* sp. growth parameters, such as specific growth rate (SGR), chlorophyll a content and amount of EPS were analyzed as follow.

Growth status and SGR determination

Microalga strain growth rate in liquid media was measured (homogenized algal sample by UV-visible spectrophotometer at 750 nm at 2-day intervals (Leganes et al., 1987) then the specific growth rate was calculated as:

$\mu = (\ln N_2 - N_1)/(T_2 - T_1)$.

where N_2, N_1, T_2 and T_1 are max cell count and initial day, final time and initial time respectively.

Table 1. List of the experimental sets, variables (nitrate, pH and salinity) and levels used in The experimental central composite.

Run	A: pH	B: Nitrate ($g.l^{-1}$)	C: NaCl Salinity (%)
1	5.99	1.30	1.66
2	6.60	0.60	2.50
3	6.60	0.60	0.81
4	7.50	1.30	0.23
5	8.39	1.99	0.81
6	7.50	2.46	1.66
7	8.39	1.99	2.50
8	7.50	1.30	1.66
9	8.39	0.60	0.81
10	7.50	0.13	1.66
11	6.60	1.99	0.81
12	6.60	1.99	2.50
13	8.39	0.60	2.50
14	9.00	1.30	1.66
15	7.50	1.30	3.08
16	7.50	1.30	1.66

Photosynthetic pigments measurement

Cholophyll a (chl a) was quantified every 7 days by extracting pigments from 1ml of freshly harvested microalga cells, using pure methanol for 24 h at 4 °C. After the removal of the cell debris through centrifugation at 10,000 × g (J2-21M, Bbeckman, Palo Alto , USA) for 5 min , the content of chl a in the supernatant was measured at 665 nm and calculated by the extinction coefficient (Marker et al., 1972). The total pigment was calculated as: chl a ($\mu g.ml^{-1}$) = 13.14 × OD 665.

ExoPolysaccharides content measurement

The cell suspensions were centrifuged at 12000 g for 10 min at 4 °C temperature. The EPSs were calculated by the phenol-sulphuric acid method, using glucose as standard (0-100 $\mu g.ml^{-1}$) (Dubois, 1956).

Data analysis

Design Expert software 8.7.1 used for design the experiments and the results were evaluated by SPSS software to do statics analysis .

Results

Optimization of the growth medium for EPS production

In the first stage, the levels of the factors (concentrations of NO_3^{-1}, pH, NaCl (%) and their interactions on the biomass, and the EPSs were determined through the CCD. In the sec-

ond stage, four different Fe_3O_4 NPs concentration media as 1, 10, 50, 100 mg.l^{-1} were prepared by adding Fe_3O_4 NPs to the optimized cultures tested in the first stage.

Mutual interactive effect of nitrate, pH and NaCl salinity on SGR in Nostoc sp.

In order to analyze the regression equation of the model, three-dimensional surface and 2-D contour plots were obtained by plotting the responses on the Z axis (Fig. 1) against any two variables, while keeping the other variable at zero level. These plots were created to analyze the change in the response surface. Fig. 1a shows the effect of the pH and nitrate concentration on the SGR, while salinity is kept at its respective zero levels. The response surface and contour plots show that higher pH (8-9), lower salinity (1%) and NO_3^{-1} concentrations (1 g.l^{-1}) led to increased SGR.

However SGR decreased when salinity increased, thus, the effect of the interaction between pH and nitrate on SGR is low. The results indicate that the highest SGR (≥45%) is achieved when pH and nitrate concentration are 8:00 and 1.30 g.l^{-1}, respectively. So, at low NaCl salinity, the interaction of high pH and low NO_3 concentrations was significant (Fig. 1a).

Fig. 1b shows the response surface as a function of pH and NaCl salinity, while the nitrate concentration was kept constant. It shows that at 1.29 g^{-1} nitrate. The effect of the interaction between salinity and pH on SGR is more significant and the maximum occurs in the region of 1.29 g^{-1} nitrate and the above 1.29 g^{-1} nitrate, SGR decreases. Generally, the results show that enhancement of the growth observed at nitrate concentration ranging from 9.60 to 1.29 g.l^{-1}. whereas above 1.29 g.l^{-1}, the growth was gradually reduced. The maximum SGR was achieved at 1% NaCl, pH 7.00 to 8.50 and 9-1.29 g.l^{-1}. nitrate concentration. However; it was reduced under the effect of low pH values (Fig. 1c).

Mutual interactive effect of nitrate, pH and NaCl salinity on EPS production in Nostoc sp.

The circular contour plot indicates that the interaction between the corresponding variables is negligible (Figure 1), while the elliptical contour plot indicates significant (Fig. 2). The final response for EPS production in terms of actual factors is presented in Figure 2. The results show that with salinity near 1% when the pH of the medium was changed from 6.00 to 8.40 and the nitrate was on 0.60 to 1.00 g.l^{-1}, the rate of EPS production increased significantly, but with a small increase in salinity (% NaCl) and changing the two variables pH and nitrate, it decreased (Fig. 2a). It is interesting to note that the EPSs secreted by *Nostoc* sp. increased as the concentration of the added nitrate increased from 0.60 to 1.80 g.l^{-1}. However, raising the nitrate concentration above 1.80 g.l^{-1} decreased EPS production (Fig. 2b). Likewise, the EPS secreted by *Nostoc* sp. increased as the concentration of the added NaCl increased from 0.81% to 1.23% (Fig. 2c) and that increment led to EPS productivity reduction, which means 1.23% of added NaCl in the medium was the most suitable for EPS production by *Nostoc* sp. Moreover, the highest EPS concentration at the end of the cultivation reached 12 µg.l^{-1}.

ANOVA and standard error for model surface

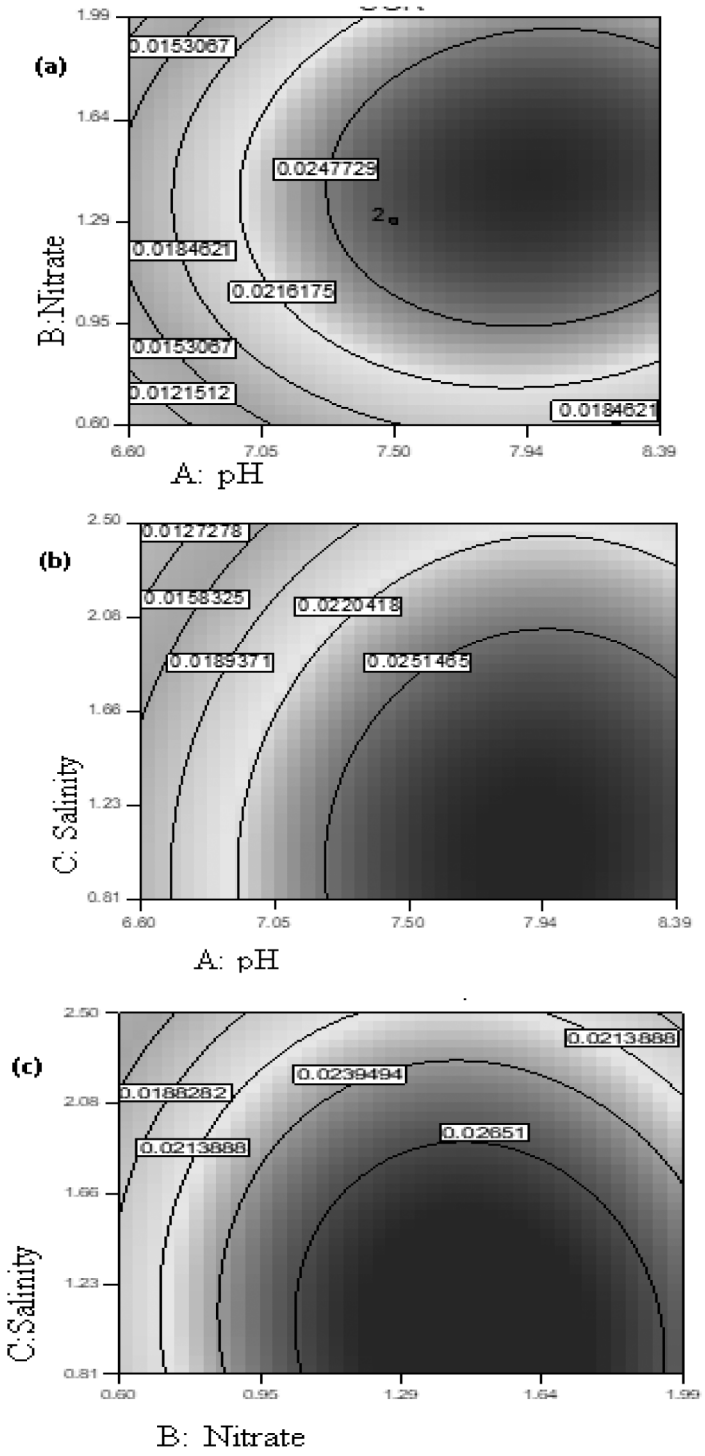

Fig. 1. Response surface and contour plots developed as a function of (SGR): (a) pH and nitrate concentration while the NaCl salinity was kept at 1.66%, (b) pH and NaCl salinity while nitrate concentration was kept at 1.29 $g.l^{-1}$; (c) NaCl salinity and nitrate concentration while pH was 7.50.

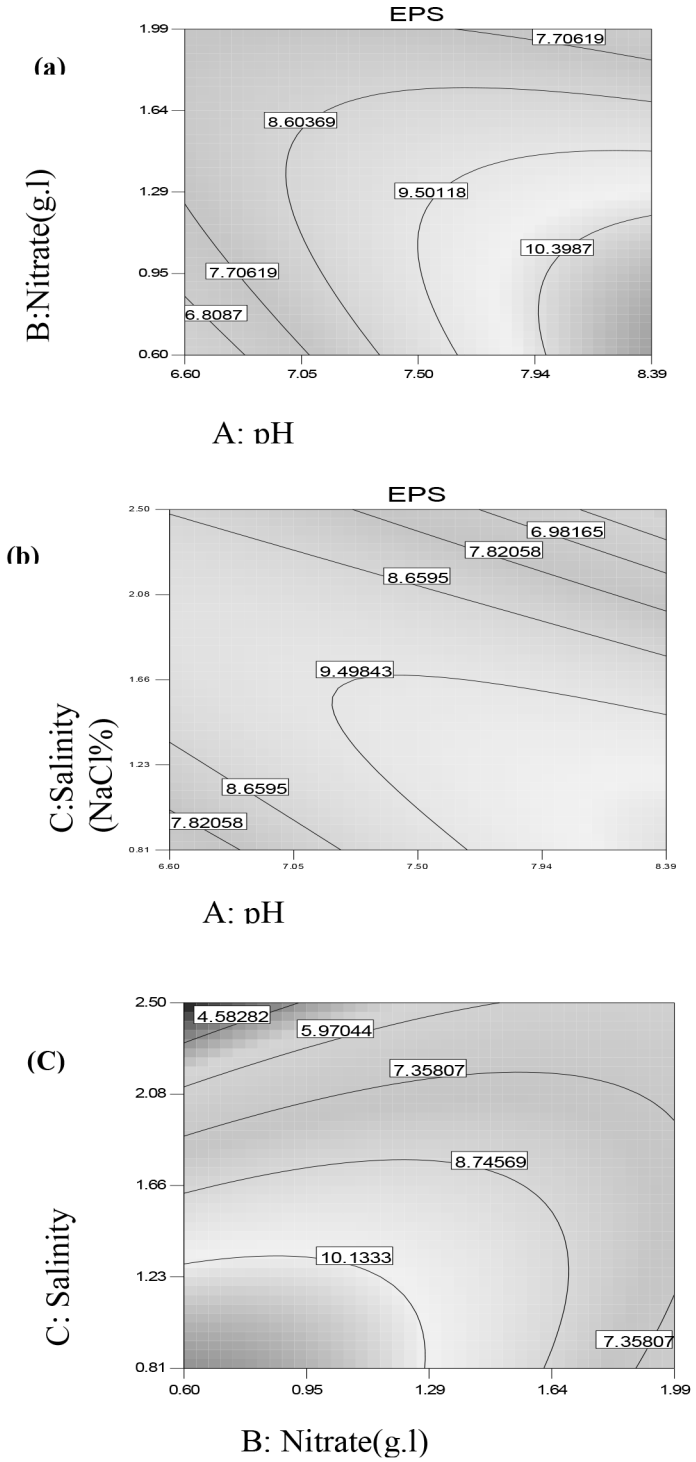

Fig. 2. Response surface and contour plots developed as a function of (EPS): (a) pH and nitrate concentration while the NaCl salinity was kept at 1.02%, (b) pH and salinity while nitrate concentration kept at 1 $g.l^{-1}$, (c) NaCl salinity and nitrate concentration while pH was 7.28.

response

The mathematical relationship of the EPS production for these variables can be approximated by second order polynomial equation. The details of the designed experiments along with the experimental results and predicted values for EPS production efficiency are presented in Table 2. Based on the results, an empirical relationship between the response and variables was attained as shown in the following equation:

EPS ($mg.ml^{-1}$)=9.60-0.15*A+0.92*B-0.73*C-1.53*A*B-1.62*A*C+1.93*B*C-0.46A^2-0.98*B^2-1.21C^2.

Table 2 shows the prediction quadratic model for response EPS. The results show that B, C, AB, AC, BC, B^2, C^2 are significant model terms, whereas A and A^2 have no significant effect on the model. The results show that the Model F-value of 31.42 implies the model is significant. Values of P-value less than 0.05 indicate that the model terms are significant. The "Lack of Fit F-value" of 0.33 implies the Lack of Fit is not significant relative to the pure error. Hence, it is favorable for the parameter to be not significant. The R-squared value for this model is 0.9680, closer to 1, indicates that the model is reliable for EPS productivity prediction. According to the ANOVA results, regression model presents a high correlation coefficient (R^2 = 0.9792) for the EPS production. The "Predicted R-Squared" of 0.8671

Table 2. ANOVA and standard error for model surface response's EPS ($mg.ml^{-1}$).

EPS Source	Sum Squares	Df	Mean Square	F Value	p-value	
Model	105.72	9	11.75	31.42	0.0002	Significant
A pH	0.32	1	0.32	0.86	0.3898	
B-nitrogen	11.70	1	11.70	31.29	0.0014	
C-salinity	7.35	1	7.35	19.65	0.0044	
AB	18.66	1	18.66	49.90	0.0004	
AC	21.16	1	21.16	56.61	0.0003	
BC	29.95	1	29.95	80.12	0.0001	
A^2	1.94	1	1.94	5.19	0.0630	
B^2	8.93	1	8.93	23.88	0.0027	
C^2	13.64	1	13.64	36.49	0.0009	
Residual	2.24	6	0.37			
Lack of Fit	1.40	5	0.28	0.33	0.8574	not significant
Pure Error	0.85	1	0.85			
Cor Total	107.97	15				

is in reasonable agreement with the "Adj R-Squared" of 0.9481.

Effect of different concentrations of Fe NPs on the growth of Nostoc sp.

Dose-response curves of the iron-based NPs nanoparticle 4 different concentrations on the *Nostoc* sp. growth are shown Fig. 3. Significant statistical differences in the concentration of biomass for different treatments compared to the control were observed for *Nostoc* sp. According to fig 3, enhancement of growth was observed at concentrations ranging from 1 to 100 mg l^{-1}, whereas at 100 mg.ml^{-1} Fe NPs after 4 weeks of cultivation, it significantly decreased in comparison with the growth in the other treatments. However, the biomass obtained in cultivation with 10 mg.ml^{-1} Fe NPs was approximately 125 % higher in comparison with the control after 6 weeks. In other words, the stimulatory effect of 10 mg.ml^{-1} Fe NPs was more pronounced compared to the other treatments. This difference is demonstrated in Fig. 3.

Effect of different concentrations of Fe NPs on EPS production in Nostoc sp.

EPS was measured, under different Fe NPs concentrations during 6 weeks to determine the effect of Fe NPs (Fig. 4). As shown in the Figure 4, in all Fe NPs concentrations, the amount of EPS increased after 4 weeks and maximum amounts of EPS were obtained during the late exponential growth phase. However, the more significant increment of EPS was observed with 3.231 and 3.504 µg.ml^{-1} in the cultivation media containing 10 and 100 mg.ml^{-1} Fe_3O_4 NPs, respectively.

Discussion

To use the cyanobacterial EPS in biotechnology we need to identify factors that influence the synthesis of EPS subsequently, optimize the productivity of the polymer. In the present study, central composite design was applied to *Nostoc* sp. to appraise the incorporate effect of

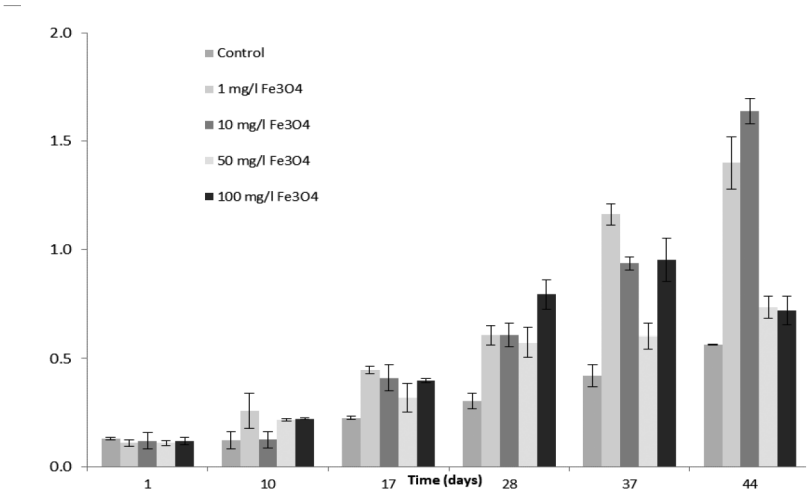

Fig. 3. Effect of different concentrations of Fe NPs on the DW of *Nostoc* sp. during 44 days. values represents ±SD. Those with error bar are significantly different ($p<0.05$).

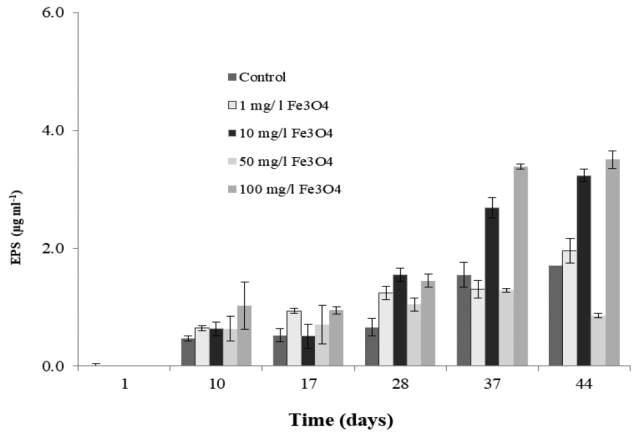

Fig. 4. Effect of different concentrations of Fe NPs on EPS production in *Nostoc* sp. during 44 days.

pH, nitrate concentkration and NaCl salinity. It has been well documented that pH can greatly affect the cell growth of *Nostoc* sp. The results indicated that the cyanobacteria prefer to grow in alkaline pH. Thus, SGR and EPS increased faster, whereas lower pH resulted in a decrease in the a polysaccharide production. These results are in agreement with those suggesting that *Chroococcus* sp. is able to produce high amounts of EPSs a growth medium at pH of 7.5 (Otero and Vincenzini, 2003). Statistical analyses show strong positive correlations in the cell density with parameters like pH, showing that not only nutrients but also pH is considered important in controlling the growth of cyanobacteria (Khosravi Rineh et al., 2011). PH influence on the nutrient solubility, uptake, enzymatic activity and redox reaction. The active oxygen released by cyanobacteria during the photolysis then the organic matter is destroyed and the enzymes get inactive (Delattre et., 2016). The correlation between the source or amount of nitrate and the production of EPSs has been evaluated for several cyanobacteria. During the present study, it was observed that changes nitrate concentration in the medium affected the growth and EPS production and that excess nitrate increased the SGR of *Nostoc* sp. when nitrate concentration was further increased, it decreased. It seems that the optimum nitrate concentration should be fixed at medium. Similar results were reported by Nicolaus et al. (1999). (for *Nostoc minutum*, suggesting that the EPS production was maximal when the NO_3^{-1} concentration was tripled. The high yields of EPS (12 $\mu g.l^{-1}$) in present study were in agreement with the values reported for cyanobacteria by adding nitrate to soluble carbohydrates (Dante, 2016). Nitrate is one of the most essential elements for the synthesis of cell metabolites. The presence of a nitrogen source in the culture medium resulted in an increase in EPS synthesis, perhaps due to the lower energy required for the assimilation of nitrogen compared with the energy needed for nitrogen fixation (Kumar et al., 2007). This was the case only for the non-capsulated strain PCC 7413, in which case much higher total

and soluble carbohydrates were produced in nitrate-grown culture. Nitrogen deprivation induces high accumulation of EPSs, probably because it is related to the increase in the C: N ratio, thereby promoting the incorporation of carbon into polymers (Kumar, 2007). Several researches have reported that in some cyanobacteria living in extreme environments such high salinity, the environment induces diverse metabolic changes, including an increase in the production of some carbohydrate, whether salinity has an effect on EPS synthesis is still controversial, and the most probable effects are species-specific (Chen, 2006). In this investigation, it was observed that maximum SGR and EPS content was obtained by adding NaCl in intermediate value (1%), which means this range is suitable for tested cyanobacterium activity. EPS production in *Dunaliella salina* increased concomitantly with the increase in salt concentration (NaCl%). Similarly, Qurashi and Sabri (2012) observed high EPS production by halophilic bacteria under NaCl stress. Supplementation of NaCl in cultures of *Lyngbya stagnina* led to a decrease in growth and an increase in EPS production. It is concluded that the presence of NaCl in the nutrient medium is necessary for enhanced EPS production in *Lyngbya stagnina* (Namita, 2013). In some cyanobacteria living in extreme environments environment induces various structural and metabolic changes, including a decrease in respiration and an increase in the production of some carbohydrates, sucrose, which functions as an osmotic solute protecting the membranes against desiccation (Pereira, 2009). However, some exceptions have been reported, suggesting that an increase in NaCl concentration did not affect or even lower the EPS production (Chen et al., 2006). Generally, under salt stress, cyanobacteria also produce larger amounts of EPSs. It has been presumed that the increased discharge of EPS can have a function equivalent to that of the achievement of salt endurance (Chen et al., 2006). As a conclusion, the results obtained by Design Expert show that the highest cell density of cyanobacteria and EPS could be due to the increase in nitrate and pH. Optimal conditions for EPS production by *Nostoc* sp. do not exactly concurrent with those for cell growth. In fact, the results suggested that the EPS in the culture medium could have been synthesized during the late exponential growth phase and progressively released into the culture medium during the stationary growth phase, in which growth is limited by different factors (Pereira et al., 2009). It is recommended that the optimized parameters be replicated in pilot scale photobioreactor to verify the transferability of the results to industrial scale. Since nanoparticles have a number of unique characteristics in terms of physicochemical, reactive, and electrical properties, they have high interaction with organisms as well as potential toxicity (Lanzhou et al., 2012). According to our results, the presence Fe NPs (10 $mg.l^{-1}$) enhances the growth of *Nostoc* sp. compared to the control. The highest biomass accumulation (1.639 $mg.ml^{-1}$) was obtained at 10 $mg.l^{-1}$ of Fe NPs concentration, which reveals that this amount of NPs is more favorable for the microalgae, whereas further increase in the concentration of Fe NPs is toxic, specially during

the second phase of the exponential growth. The results correspond with those reported by Baky et al. (2012), who reported that maximum *Scenedesmus obliquus* biomass yield (2.4 $g.l^{-1}$) is produced at 10 $mg.l^{-1}$ Fe^{3+}. It may be due to the metal ions released from nanoparticles that stimulate the growth of microalgae (Krystian et al., 2015). Another investigation found that the cultures grown at a higher concentration of Fe NPs could not regenerate when inoculated into a fresh medium. So at a high concentration of Fe NPs, the cell wall uptake of nutrition is blocked, but in a normal medium the cell becomes regenerated to uptake nutrition and the cell wall transport gets activated (Yuvakkumar et al., 2011). Besides, in the present study it was detected that Fe NPs inhibit the chl a content in *Nostoc* sp. (data not shown). A similar study of the cultures to which different concentrations of nano molybdenum were added revealed that a low amount of nano molybdenum is favorable for the synthesis of chlorophyll a whereas higher concentrations of NPs inhibited the biosynthesis of chlorophyll a. The inhibitory effect of NPs on the activity of multiple microalga strains was considered to be due to reactive oxygen species (ROS) generation or the physical damage caused by NPs themselves. It could also be due to the metal ions released from NPs, light shading effect, interactions with the composition of media or the synchronic effect of different factors (Krystian et al., 2015). Our experiments revealed the EPS of the treated microalgae(10, 50, 100 $mg.l^{-1}$) was higher than that of the control. Additionaly, a considerable amount of EPS was in the medium at 10 $mg.l^{-1}$ of Fe NPs; hence this concentration is the most suitable dosage in relation to EPS production in the optimized growth conditions as well as developing microalgae production systems. One of the remarkable aspects of microalgae is that it is possible to produce manifold industrially important compounds from metal-exposed microalgae, however, they may also have detrimental effects on the growth. A possible strategy to overcome this problem could be the cultivation of microalgae under non-stressed conditions in order to obtain higher yield biomass, followed by the addition of metals for inducing stress and synthesis of target products in microalgae. Metals at higher concentrations are toxic to microalgae, but at lower concentrations, they can be stimulating for growth. Consequently, more investigations should be focuse on the search for the best EPS producer microalga then optimize suitable factors and doses of NPs in relation to the selected strain.

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