

# Modification of heavy metals toxicity by Cyanobacteria *Nostoc* sp. N27P72 and *Nostoc* sp. FB71 in Culture Conditions

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## Research Article

**Keywords:** metal removal capability, extracellular polysaccharides, Gas Chromatography Mass Spectrometry, FT-IR, Nostoc, cyanobacteria

**Posted Date:** April 20th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-414617/v1>

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# Abstract

**Purpose:** Cyanobacteria are ecologically relevant prokaryotes that can be found in environments contaminated with heavy metals. As their photosynthetic machinery imposes high demands for metals, homeostasis of these micronutrients has been extensively considered in cyanobacteria. So far, most studies have focused on treatment of wastewaters using microalgae leads to remarkable reduction of an array of organic and inorganic nutrients, but what takes place in the extracellular environment when cells are exposed to external supplementation with heavy metals remains largely unknown.

**Methods:** Here, *extracellular* polymeric substances (EPS) production in strains *Nostoc* sp. N27P72 and *Nostoc* sp. FB71 isolated from different habitats are reported and compared. Cultures of both strains, supplemented with either glucose, sucrose, lactose or maltose showed that production of EPS and cell dry weight was boosted by maltose supplementation.

**Result:** *Nostoc* sp. N27P72 which was isolated from lime stones was higher, resulting in  $9.1 \pm 0.05$   $\mu\text{g/ml}$  and  $1.01 \pm 0.06$   $\text{g/l}$  in EPS and cell dry. The cell cultures tested for their ability to remove Cu(II), Cr(III) and Ni(II) in media culture containing the maltose and without maltose as control culture. Remarkably, we showed that although these elements can be toxic, supplementing the media culture can effectively sequester their toxic effects by increasing the production of EPSs, carbohydrates and total soluble proteins in comparison to control. The crude EPS showed metal adsorption capacity assuming the order Ni(II) > Cu(II) > Cr(III) from metal-binding experiments. Nickel was preferentially biosorbed with a maximal uptake of  $188.8 \pm 0.14$   $\text{mg (g cell dry wt)}^{-1}$  crude EPS. FT-IR spectroscopy revealed treatment with Ni made changes in the functional groups and glycoside linkages in both strains. Results of Gas Chromatography Mass Spectrometry (GC-MS) to determine the biochemical composition of *Nostoc* sp. N27P72 showed that strong Ni(II) removal capability is suspected to be associated with the high Cyclotrisiloxane and 1,2-Benzenedicarboxylic acid content.

**Conclusion:** The results of these investigates specified that strains *Nostoc* sp. N27P72 is good candidates for the commercial production of EPS and might be utilized in bioremediation field as an alternative to synthetic and abiotic flocculants.

## Introduction

*Nostoc* cyanobacteria species are a large and morphologically diverse group of phototrophic prokaryotes which have found in various habitats. They have has drawn more attention because of the presence of outermost polysaccharidic envelopes, often coupled with the capability to release exocellular polysaccharides (EPS) into the culture medium during cell growth (Mota et al., 2015).

Most of these polymers are characterized by an anionic nature, owing to the presence of uronic acids and/or of other charged groups (Pereira et al., 2013). As a result, these polysaccharides typically keep very high affinity to metallic ions and can be considered very promising as chelating agents for the removal of heavy metals from water (Kumar et al., 2007; De Philippis et al. 2001; Cepoi et al., 2019; Kumaran et al., 2011; Ni et al., 2019).

Many factors influence cell growth and metabolite accumulation in microalgal cell cultures and *extracellular* polymeric substances (EPSs) including nutrients, such as phosphate and nitrogen, temperature, light intensity, aeration rate, and mixotrophic condosion (Otero and Vincenzini 2003); (Helm et al. 2000). Although the presence of EPS is extremely preserved among cyanobacteria, not much is recognized about factors that maximize EPS biosynthesis and *affecting* biosorption *capacity* (Pereira et al. 2009; Klock et al. 2007; De Philippis et al. 2000; Yoshimura et al. 2011; Gupta et al., 2017). Some trace amounts ( $\mu\text{gL}^{-1}$ ) such as copper, cobalt and nickel are essential by cyanobacteria strains as cofactors for the enzymatic activities. Unlike organic contaminants, heavy metals such as, copper and lead are main pollutants of freshwater due to their inherent nature of being persistent, toxic, recalcitrant and non-biodegradable (Micheletti et al., 2008). On the other hand, heavy metal ion concentrations at ppm ( $\text{mgL}^{-1}$ ) level are known to be toxic to the organisms because of irreversible inhibition of many enzymes by the heavy metal ions. Heavy metal uptake capacity of algal biomass has proved to be the highest due to the presence of polymers such as polysaccharides, proteins or lipids on the cell wall surface containing functional groups such as amino, hydroxyl, carboxyl and sulfate, which can act as binding sites for metals (Pereira et al., 2015). A number of articles have been published on phycoremediation investigation and severlal authors have established the fact that treatment of wastewaters using algae, microalgae particularly leads to remarkable reduction of an array of organic and inorganic nutrients, including some of the toxic chemicals (Anjana et al., 2007; Essa and Mostafa., 2012; Farooqui et al., 2017; Principe et al., 2020; Devi and Mehta., 2014). Our previous research found that cell growth and EPS production are highly dependent on the culture conditions. There is no correlation between cell growth and EPS production for cultures being grown in different sources of nitrogen. In contrast, light intensity and cell growth in mixotrophic condition at 150 or 50  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$  have had a highly positive effect on EPS production. In salty grown cultures, thick layers of ASN\_M strain prevent the cells from NaCl stress and hence its growth is maintained without inhibition under the NaCl stress (Nowruzi et al., 2013).

This study was undertaken to assess modification of metal removal capability of two *Nostoc* strains isolated from different habitats, for their ability to remove Cr(III), Cu(II) and Ni(II) from aqueous solutions. We thought that the strain of *Nostoc* sp. N27P72 that is isolated from lime stones of Khuzestan province have many features which include high tolerance to different abiotic stress such as drought and high density of light that make it ideal candidate for the selective removal and concentration of heavy metals, compared to aquatic strain.

The main objective of this work is the use of the mixotrophic conditions to optimize the biosorption controllable factors for the maximum metal removal efficiency of algal biomass. Lead, Nickel and copper were selected because of their contrasting toxicity and essentiality. Furthermore, cell dry weight, carbohydrates content, total soluble proteins, analysis of functional groups by fourier transformed infrared (FT-IR) spectroscopy and Chemical composition of the lyophilized EPS by coupled gas chromatography –mass spectrometry (GC-MS) have been investigated. To the best of our knowledge, modification of culture media by *Nostoc* species has not been reported for the removal of heavy metals and hence this study would be of great importance in increasing the capability of bioremediation of metals from aqueous environment.

## Materials And Methods

## Materials

All materials and reagents were purchased from Sigma-Aldrich unless otherwise specified.

## Cyanobacterial strains

*Nostoc* sp. N27P72 and *Nostoc* sp. FB71 obtained from the Cyanobacteria Culture Collection (CCC) and the ALBORZ Herbarium, at the Science and Research Branch, Islamic Azad University, Tehran.

## Culture conditions

*Nostoc* sp. N27P72 was belong to lime stones of Khuzestan province and *Nostoc* sp. FB71 was isolated from fresh water of Golestan province. *Nostoc* sp. N27P72 was grown at modified Z8IX medium and *Nostoc* sp. FB71 was cultured on liquid media BG11 medium (nitrate free) (Rippka *et al.* 1979) and pH was adjusted to 7.1. Cultures were incubated in a culture chamber at 28° C, and were provided with continuous artificial illumination of approximately 15  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for two weeks (Liu *et al.*, 2014).

the cell cultures used for optimization of metal removal capability were grown in broth (25 ml in 250 ml baffled shake flasks) containing the sugars glucose, maltose, lactose, and sucrose, respectively, as additional carbon source at the concentrations 10 g/l and were grown for 48 h. Samples were taken after 6, 12, 24, and 48 h and were analysed for total produced EPSs, cell dry weight and sugar content. A parallel control experiment was carried out using broth media cultures of Z8IX and BG11 without carbon source supplementation.

## Determination of cell biomass

Cyanobacteria growth was quantitatively determined by measuring cell dry weight. First cells washed and resuspended in 2 ml of 0.05 M EDTA sodium salt solution (Sardari *et al.*, 2013) and the mixture was shaken at 4 °C for 4 h to remove any capsular EPS. Then cells were harvested and dried in an oven set at 100 °C. The cell dry weight was measured periodically until a constant weight was reached (Nowruzi *et al.*, 2013).

## Isolation of exopolysaccharides

Strains *Nostoc* sp. N27P72 and *Nostoc* sp. FB71 were taken at different times from the cultivations were centrifuged at 4000 rpm for 30 min at 4 °C (SigmaPK). The EPSs were precipitated by adding ethanol and storing overnight at 4 °C. precipitates were harvested and put in a fume hood to evaporate the remaining ethanol. The precipitates then were dissolved in milliQ water and lyophilized (Labconco freeze dry system) to obtain the crude EPSs (Nowruzi *et al.*, 2013).

## Selectivity in the heavy metal removal

The cell cultures were grown in Z8IX and BG11 media culture containing the maltose and without maltose as control culture, were tested for their ability to remove Cu(II), Cr(III) and Ni(II). The cultures (400 ml of cell suspensions in 1000 ml Erlenmeyer flasks) were grown for 10–15 days in an orbital Incubator (Gallenkamp, Loughborough,UK) at  $30 \pm 1^{\circ}\text{C}$  under continuous illumination provided by cool white fluorescent tubes giving

a mean photon flux of  $100 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$  photosynthetic active radiation at the flask surface. Before their use for the experiments, aliquots of the cultures were confined in dialysis tubing and pretreated with 0.1 N HCl and then dialysed against water. Next, the cultures were suspended into metal solutions with continuous stirring. Working solutions of  $10 \text{ mg l}^{-1}$  Cr(III), Cu(II) and Ni(II) were prepared, for each metal, by dilution of  $1000 \text{ mg l}^{-1}$  standard solutions (pH 5.0); the experiments were performed in a thermostat at a temperature of  $25 \pm 1^{\circ}\text{C}$ . For the determination of the kinetics of metal removal, 5 ml samples were withdrawn at known intervals, centrifuged (10 min at  $10\,000 \text{ g}$ ) and filtered through a  $0.45 \mu\text{m}$  membrane. The metal uptake was calculated from the difference between the concentrations of the metals in solution, determined with an Atomic adsorption spectrometer (SpectrAA 10 plus, Varian, CA, USA), at the beginning and end of exposure with the cyanobacterial cell suspensions; Cu(II), Ni(II) and Cr(III) concentrations were determined at 232.0, 359.9 and 324.7 nm respectively.

The amount of metals removed in blanks carried out in parallel without the addition of cyanobacterial cells was subtracted from the experimental values gained in the experiments with the cyanobacterial cultures. All the experiments were performed at least in triplicate and the data are reported as mean values  $\pm$  standard deviation. The metal uptake  $q$ , expressed as mg of metal removed per g of dry biomass, was calculated as:  $q = (C_i - C_f) / m$ . The concentration of the biomass was determined as dry weight ( $\text{g l}^{-1}$ ) by filtering the cell suspension on  $0.45 \mu\text{m}$  filters and by drying the filters at  $100^{\circ}\text{C}$  until constant weight (De Philippis et al., 2003) (Micheletti et al., 2008).

In order to confirm the affection of the metals and to determine the interaction of the cations within the different functional groups of the EPS, metal solution and all incubated EPS were lyophilized after the 24 h dialysis (lyophilized EPS containing maltose and metal solution of Cu(II), Cr(III) and Ni(II)) and total produced EPSs, total soluble proteins, carbohydrate content and chemical composition were measured.

### **Analyzing of carbohydrates content**

5 mg of the sample was mixed with  $2.5 \text{ cm}^3$  of Antrone reagent (0.65 %) in  $\text{H}_2\text{SO}_4$  (65 %). The mixture was incubated for 35 min at  $100^{\circ}\text{C}$ . The absorbance was measured at 620 nm. Carbohydrates content was computed using a calibration curve (Zinicovscaia et al., 2016).

### **Estimation of total soluble proteins**

20 mg of EPS were resuspended in 1 ml of deionized water and sonicated and successively diluted until a homogenous solution was obtained. Protein content was determined according to the Lowry et al. (1951), and a calibration curve was constructed using serum albumin.

### **Analysis of functional groups by FT-IR**

2 mg of lyophilized EPS was grinded with 100 mg dry KBr and pressed into a mold in a uniaxial hydraulic press. FT-IR spectra of the purified EPSs fractions were recorded in the  $4000\text{--}400 \text{ cm}^{-1}$  region using a FT-IR system (Nicolet is5, Ther-moFisher Scientific). The determinations were performed in two independent replicates and are reported as the mean with standard deviations (Mota et al., 2016).

## Chemical composition of the lyophilized EPS

Chemical composition of extracts of *Nostoc* sp. N27P72 was evaluated using a coupled gas chromatography –mass spectrometry (GC-MS). The separation of compounds and their analysis was performed using agilent 7000 series Quadrupole GC-MS system with electron impact ionization. The total GC run time was 32 min and the carrier gas was helium. The initial oven temperature was held at 90<sup>0</sup>C for 1 min and then reached 300<sup>0</sup>C in 13 min, after which it was held at this temperature for 20 min. the injector temperature was 300<sup>0</sup>C.

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns and Fiehn Mass Spectra Libraries. The spectrum of the unspecified component was equaled with the spectrum of the identified components stored in the NIST library. The Name, Molecular weight and Structure of the components of the test materials were ascertained (Sampathkumar and Halith., 2020).

## Statistical analysis

Results of each representative experiment were analyzed by ANOVA, using the statistical software package SPSS version 24 and differences between the groups were detected with Tukey's grouping tests. P(s) values smaller than 0.05 are considered significant. Means and the standard deviation were calculated from the data obtained from three replicates (Nowruzi et al., 2013).

# Results

## Growth and EPS production by the two *Nostoc* strains

The two *Nostoc* strains exhibited a distinct difference in growth behavior. As initial trials with the both strains showed that use of disaccharides (maltose, lactose, and sucrose) as carbon source supplementation generally resulted in higher EPS production than the use of monosaccharides (Glucose), moreover the amounts of total produced EPSs, cell dry weight and residual sugar content was higher in *Nostoc* sp. N27P72 in comparison to *Nostoc* sp. FB71 in all treatments. Consumption of the monosaccharide glucose, and the disaccharides lactose, lactose and maltose was verified (Fig. 1). In all situations, production of EPS was started in the exponential growth phase and was shown to remain in the stationary phase. The consumption rate of glucose, maltose, lactose, and sucrose was nearly uniform in both strains and after 48h all glucose was not consumed and the residual concentration was  $6-6.5 \pm 0.1$  g/l in all treatments. The change in cell concentration was very obvious in both strains in cultures adding maltose and reaching to  $1.01 \pm 0.06$  g/l in *Nostoc* sp. N27P72 and  $0.75 \pm 0.16$  g/l in *Nostoc* sp. FB71 at 48 h. The increase in cell dry weight might be due to consumption of produced EPS, which was reaching a final EPS concentration of  $9.1 \pm 0.05$  µg/ml in *Nostoc* sp. N27P72 and  $7.04 \pm 0.1$  µg/ml in *Nostoc* sp. FB71 (at 48 h). The experiments using cultures without added sugar, showed a slightly lower maximum cell mass ( $0.55 \pm 0.03$  g/l in *Nostoc* sp. N27P72 and  $0.323 \pm 0.05$  in *Nostoc* sp. FB71 after 48 h) and the final EPS concentration was 3.3 µg/ml in *Nostoc* sp. N27P72 and 2.5 µg/ml in *Nostoc* sp. FB71 after 48 h. This indicates that the 10 g/L maltose addition had a small boosting effect on both cell mass and EPS production.

Addition of glucose did not stimulate cell growth and EPS production in *Nostoc* sp. N27P72, however it was stimulating in EPS production in *Nostoc* sp. FB71. The consumption rate of glucose during the first 6 h was 1.5 g/l in *Nostoc* sp. N27P72 and 2 g/l in *Nostoc* sp. FB71 but decreased to 0.5 g/l h (6–15 h) and reaching a cell mass of  $0.59 \pm 0.21$  g/l in *Nostoc* sp. N27P72 and 0.25 g/l in *Nostoc* sp. FB71 at 24 h. After 48 h the cell mass finally reached  $0.79 \pm 0.35$  g/l in *Nostoc* sp. N27P72 and 0.38 g/l in *Nostoc* sp. FB71, while production of EPS continued (1.7  $\mu$ g/ml in *Nostoc* sp. N27P72 and 3.9  $\mu$ g/ml during the whole 48 h of cultivation. The cell mass obtained in maltose supplemented cultivations resembled that of the lactose supplementation ( $0.4 \pm 0.28$  g/l in *Nostoc* sp. N27P72 and  $0.5 \pm 0.1$  g/l in *Nostoc* sp. FB71 after 24 h, maintained at 24 h as  $0.94 \pm 0.14$  g/l in *Nostoc* sp. N27P72 and  $0.66 \pm 0.09$  g/l in *Nostoc* sp. FB71. The EPS concentration in this case reached  $6.41 \pm 0.13$   $\mu$ g/ml in *Nostoc* sp. N27P72 and  $5.10 \pm 0.26$   $\mu$ g/ml in *Nostoc* sp. FB71 (after 48 h). Sucrose supplemented cultivations showed that the maximum cell concentration was  $1.15 \pm 0.21$  g/l and the concentration of produced EPS was  $2.01 \pm 0.15$   $\mu$ g/ml in *Nostoc* sp. N27P72 and  $4.32 \pm 0.19$   $\mu$ g/ml in *Nostoc* sp. FB71 after 48 h. The results displayed that while the effects on cell mass were slightly small, production of EPS amplified upon adding of the disaccharides maltose and lactose at stationary and exponential phase.

### Results of optimization of metal removal capability

The time course of specific metal removal ( $q$ ), expressed as mg of metal removed per g of biomass dry weight, by *Nostoc* sp. N27P72 and *Nostoc* sp. FB71 cultivated in media culture containing (10 g/l) maltose, with copper, chromium and nickel in single-metal solutions showed that in single-metal solutions, the kinetics of sorption by the cyanobacterial cultures was always rapid for all the metals tested; the saturation of the metal removal capacity of each strain was achieved within the first 10 min in the metal solution. The metal affinity of the two *Nostoc* strains generally decreased in the order Ni > Cu > Cr. The specific metal uptake ( $q$ ) was very high, in particular for Ni (Fig. 2), which generally were removed in larger amounts than Cu and Cr. Among the strains tested, the highest  $q$  values towards Ni was  $188.8 \pm 0.14$  mg Ni (g cell dry wt)<sup>-1</sup> shown by *Nostoc* sp. N27P72, while the highest values of uptake was  $185.5 \pm 0.24$  by *Nostoc* sp. FB71. In single ion solutions, both *Nostoc* strains tested showed the lowest affinity for Cr namely  $105.65 \pm 0.34$  and  $104.5 \pm 0.1$  mg metal for *Nostoc* sp. N27P72 and *Nostoc* sp. FB71 (g cell dry wt)<sup>-1</sup> respectively.

### Results of optimization of metal removal capability

Results of optimization of metal removal capability showed that adding maltose in culture media in both strains as carbon source supplementation generally resulted in higher EPS production, protein and neutral sugars content in comparison of control. Moreover the amounts of total produced EPSs, protein and neutral sugars content was significantly higher in *Nostoc* sp. N27P72 in comparison to *Nostoc* sp. FB71. Exopolysaccharide concentration in medium containing maltose and metal solution of Ni(II) was 4.87 and 3.10  $\mu$ g/L, while it was 2.72 and 1.8  $\mu$ g/L in control for *Nostoc* sp. N27P72 and *Nostoc* sp. FB71 respectively. Protein concentration in medium containing maltose and metal solution of Ni(II) was 4.87 and 3.10 mg/ml, while it was 0.07 and 0.06 mg/ml in control for *Nostoc* sp. N27P72 and *Nostoc* sp. FB71 respectively. Neutral sugars content in medium containing maltose and metal solution of Ni(II) was 5.64 and 5.16  $\mu$ g/mL, while it was 2.85 and 1.74  $\mu$ g/mL in control for *Nostoc* sp. N27P72 and *Nostoc* sp. FB71 respectively. Nickel removal rate was significantly higher in both strains, it means this metal is more absorbed by polysaccharide envelopes. The reason for removing more nickel according to the results of the diagrams is the higher amount

of EPSs, proteins and neutral sugars content compared to other elements. In fact, More EPS, proteins and neutral sugars content can effectively sequester dissolved metal ions from dilute aqueous solutions (Fig.3).

### Results of analysis of functional groups

Fourier Transformed Infrared (FT-IR) spectroscopy provided useful information about active functional groups that can be used in the determination of polysaccharide composition. So, the FT-IR spectra for both *Nostoc* sp. N27P72 and *Nostoc* sp. FB71 cultivated in media culture containing (10 g/l) maltose, with copper, chromium, and nickel in single-metal solutions were examined. Strong stretching vibrations for OH and weak stretching vibrations for  $\text{NH}_4^+$  at  $3800\text{ cm}^{-1}$  can be detected only in *Nostoc* sp. FB71. The stretching vibration for NH can be identified around  $3779\text{ cm}^{-1}$  only in *Nostoc* sp. FB71. In both samples stretching vibration for OH can be observed at  $3400\text{--}3440\text{ cm}^{-1}$ . The non-symmetric and asymmetric stretching vibrations for CH can be identified at  $2854$  and  $2924\text{ cm}^{-1}$  only in *Nostoc* sp. N27P72. The COOH stretching band ( $1600\text{--}1700\text{ cm}^{-1}$ ) can see in both samples and didn't change after heavy metal treatment. The C-H vibration can be observed at  $1380\text{--}1400\text{ cm}^{-1}$  and a peak at  $1384\text{ cm}^{-1}$  correspondings to bending vibration of  $\text{CH}_2$  can only observe at *Nostoc* sp. N27P72 in presence of Nicle. A peak at about  $1000\text{ cm}^{-1}$  region is associated with C-O polysaccharide and can not observe at *Nostoc* sp. FB71 in presence of Nicle. Furthermore, this peak shift to lower wavenumber at *Nostoc* sp. N27P72 which could be related to polysaccharide conformational changes in both starins. Peaks at  $1040$  and  $1029\text{ cm}^{-1}$  are correlated to polysaccharides skeletal and C-O-C and C-O groups of the anomeric region. Aliphatic esters can be observed at  $1103\text{ cm}^{-1}$  and this peak is removed due to the interaction of Ni, Cr, and Cu to both strains. The  $800\text{--}900\text{ cm}^{-1}$  region depicts several vibrational modes corresponding to the type of glycosidic linkages which is removed after treatments of Cr and Cu with both strains. Peaks in  $840\text{--}860\text{ cm}^{-1}$  region corresponding to  $\alpha$ -glucan and  $890\text{--}910\text{ cm}^{-1}$  corresponding to  $\alpha$  and  $\beta$  glycosidase and as can see these peaks were removed in all samples except Ni treatment at *Nostoc* sp. FB71. Peaks at  $600\text{ cm}^{-1}$  are related to The C-N stretching band ( $600\text{ cm}^{-1}$ ) can see in both sample, but shifted to lower wavenumbers in both strains. Collectively, after treatment of both strains with Ni, Cu, and Cr the FT-IR patterns for both strains changed obviously (Fig 4 and 5).

### Result of Chemical composition of the lyophilized EPS by GC-MS

The GC-MS analysis showed cyanobacteria in presence of heave metals change aliphatic compound (2-Ethoxyethanol, 3,3-dimethylhexane, Undecane, Dodecane, 2,6,10-trimethyl  $\boxtimes$  Hydroxylamine, O-decyl  $\boxtimes$  Tetradecane  $\boxtimes$  Nonadecane  $\boxtimes$  Nonadecane  $\boxtimes$  Propionic acid  $\boxtimes$  Dotriacontane  $\boxtimes$  Eicosane  $\boxtimes$  2-Methyldecane) and alkanes compounds (Dotriacontane, Dodecane, 2,6,10-trimethyl, 3,3-dimethylhexane, Eicosane) to rich variety of phytochemical compounds which are effective in heavy metal removal. The active compounds with their retention time (RT), molecular formula, molecular weight, nature of the compound, composition percentage and quality in the hexane extract are presented in Tables 1 to 4.

The total ion chromatograms (TICs) of all samples demonstrated a strong signal, large peak capacity, and reproducible retention time, indicating the reliability of metabolomic analysis. However Cyclotrisiloxane retention time was not in the same range for the Ni(II) and Cu(II) media culture (11.63 to 23.22), while 1,2-Benzenedicarboxylic acid retention time for Ni(II) and Cu(II) media culture was in the range of 23.26 to 25.14.

Moreover Ni(II) and Cu(II) culture revealed a high composition of cyclic and Ester compounds (Cyclotrisiloxane, Bis(2-ethylhexyl) phthalate and 1,2-Benzenedicarboxylic acid), while in Cr(III) cultures there are *aldehydes compounds* (Decanal, 2,3-dimethylbenzaldehyde), *ketones compounds* (6-Bromo-2-hexanone), Ester compounds (2-piperidinone), alcoholic compounds (3,5-Hexadien-2-ol, 2-Hexanol, 1,4-pentanediol), ether compounds (2H-pyran, 3,4-dihydro-6-methyl, 2-Butoxyethanol) and cyclic compounds (Cyclotrisiloxane). The strong Ni(II) removal capability of the *Nostoc* sp. N27P72 was attributed to the abundance of Cyclotrisiloxane (91%) and 1,2-Benzenedicarboxylic acid (91%) characterizes by GC-MS (Fig. 6).

## Discussion

The possible use of exopolysaccharide-producing cyanobacteria for the recovery of valuable metals from industrial wash waters seems to be more promising than most of the other microorganisms (Giner-Lamia et al., 2016; Ni et al., 2019; El Bestawy., 2019; El-Sheekh et al., 2005). The use of cyanobacterial EPS for biotechnological applications depends on the identification of culture parameters that influence the maximum production of the EPS (Chug and Mathur., 2013; Li et al., 2001; Bhunia et al., 2018). Factors such as the amounts of C:N ratio, as well as growth parameters such as light intensity, salinity and temperature have been largely disregarded, and very few exhaustive studies on factors influencing the production of cyanobacterial EPS are available in the literature (Pereira et al., 2009). Though, several elements that can stimulus EPS production, especially pH, dilution rate, growth phase, presence/absence of magnesium, calcium, potassium and heavy metals, as well as the addition of glycoxylate, glucose, citrate, acetate, valerate and EDTA have been sporadically studied (Pereira et al., 2009). Moreover, the responses of cyanobacteria to changes in culture conditions appear to be frequently strain-dependent, making the optimization of EPS production even more difficult (Floutya et al., 2019). It was suggested that some additives, including amino acids, vitamins and precursors, may also play an important role in EPS production during the process of cyanobacteria (Pereira et al., 2011).

In this article, we investigated the effect of adding four different sugars in the culture medium (glucose, maltose, lactose, and sucrose) as carbohydrate source and its effect on the amount of EPS and cell dry weight in two strains of *Nostoc* was investigated. The results of the present investigation indicated that the optimal medium for EPS production by the isolated *Nostoc* sp. N27P72 strain was boosted by maltose supplementation. Previous studies on several bacterial EPS production reported that amino acids and precursor's supplements showed stimulating effect on bacterial growth and EPS synthesis, while others demonstrated that neither additional carbohydrates nor amino acids supplementation affect the EPS level (Maalej et al. 2015). Moreover, among different nature of carbon sources, carbohydrate sugars are the preferred ones for EPS production. In this study, maltose were found to be the most efficient carbon sources.

Unfortunately, large amounts of toxic elements, especially heavy metals which are harmful to human beings, are daily released in nature through human and industrial activities (Tchounwou et al. 2012).

Exopolysaccharides are well suggested as surface-active mixtures for the exclusion of heavy metal pollution. Consequently, to find out microbial EPS with decent metal adsorption selectivity may be very valuable for building up processes aiming to pick up valuable metals from industrial wastewater. Bearing this in mind, we studied supplemented media culture as an additional sugar source exopolysaccharide to evaluation of its efficiency to adsorb various toxic heavy metals (Cu, Ni and Cr) (Mohite et al., 2017). We found that using

maltose as carbon sources was shown to produce a higher amount of EPS, protein and neutral sugars content and it could be a reason for high ability of metal absorbance. This statements has been underlined by Wong and Tam (1984) who stated that algal cells cultivated in the media with very high metal contents also gathered higher metal contents. In spite of this, in few cases, the metal uptake was independent on the external metal concentration, this point was coincided with the conclusion of Wetton et al. (1976). In case of *N. muscorum* grown in wastewater, high concentrations of Cu and Mn did not affect the growth of the microorganism but promote its growth. This may be because of the resistance of the cyanobacterium to Cu and Mn in addition to the occurrence of high content of organic matter, which may detoxify Cu and Mn effects (El-Enany and Issa, 2000).

In fact, the quantity and compactness of different kind of carbohydrates can help to sort the polysaccharidic layer surrounding the cells which can also prevent direct contact between the cells and toxic heavy metals. Actually, it was recently suggested that the high viscosity of the cultures, due to the solubilization in the culture medium of large amounts of a high molecular mass RPS, delayed the free diffusion of copper ions into the media culture (Micheletti et al., 2008). the presence of negatively charged polysaccharidic layers surrounding cyanobacterial cells such as uronic acids, sulphate and ketal-linked pyruvate groups may play an important role in the sequestration of metal cations, and in forming an environment improved in those metals that are crucial for cell growth but are existing at very low concentrations in some environments (Pereira et al., 2009).

We found that strain of *Nostoc* sp. N27P72 that is isolated from lime stones of Khuzestan province have many features which include high amount of EPS and high tolerance to drought and high density of light that make it ideal candidate for the selective removal and concentration of heavy metals, compared to aquatic strain. Actually, in all of the possible systems in which cyanobacteria are involved, the synthesis of EPSs provides a structurally-stable and hydrated microenvironment, as well as a putative physical defensive role against some risky factors, both chemical and physical, representing a boundary between cells and the immediate outer environment, and preserve the cells from toxic heavy metal (Rossi and Philippis., 2015).

The experiments, showed that *Nostoc* sp. N27P72 biomass is characterized by the best efficiency in metal removal, with a  $q_{max}$  (maximum amount of nickel removed per biomass unit) of  $188.8 \pm 0.14$  mg Ni (g cell dry wt)<sup>-1</sup> nickel removed in comparison with the value of  $185.5 \pm 0.24$  mg Ni (g cell dry wt)<sup>-1</sup> of *Nostoc* sp. FB71 biomass. Thus, the metal uptake capacity of EPS under study (especially for nickel) is of great interest regarding other biosorbent efficiencies and the lowest affinity for Cr. Our results contradicted with the result of Chan et al. (1991) who found that the removal of Ni from the mixture of 90% electroplating effluent and 10% raw sewage by two species of *Chlorella* was comparatively low (below 20%). While Kazy et al. (2002) reported that EPS production by a copper-resistant isolate of *Pseudomonas aeruginosa* was considerably higher than its copper sensitive counterpart. Moreover, Ozturk and Aslim., 2008 found Cr(VI) is an important stress factor that increases EPS concentration in cyanobacteria.

Interestingly, using optimized culture conditions increases the yield of the *Nostoc* sp. N27P72 EPS, thus improving the chances of its commercial scale production and making it more suitable for special applications, such as the environmental industry. However finding a general pattern for the special effects of metals on EPS synthesis is very hard. It is thought that the effects on EPS synthesis are metal-specific. In

some cases, a shortage of  $Mg^{2+}$  and  $Ca^{2+}$  elicited production, whereas there were no effects in other cases. The increase in EPS synthesis appears to develop the resistance to toxic metals. A study carried out by Ozturk and Aslim [9] showed that *Chroococcus* and *Synechocystis* strains resistant to Cr(VI) created larger EPS amounts compared to the Cr(VI)-sensible isolates. It is promising that a greater EPS concentration inspired by the metal played a role in increasing its immobilization, as suggested by Pereira *et al.* 2013. Though, it was lately reported that in *Cyanothece* sp. CCY 0110, the existence of heavy metals expressively affected its protein profile but did not improve the amount of RPS released by the cells (Mota *et al.* 2016).

Due to the presence of negatively charged groups, primarily carboxyl group they have also been shown to have good sorbent capacity towards positively charged metal ions (Mathlouthi and Koenig, 1987). (Gómez-Ordóñez and Rupérez, 2011) (Ponnuswamy *et al.*, 2013) (Leal *et al.*, 2008). (Mishra and Jha, 2009). Among the parameters that strongly affect the metal uptake capacity of biopolymers is the metal affinity to their functional groups (Delattre *et al.*, 2016). Shuhong *et al.* (2014) reported the implication of O-H, C=O, C-O-C and C=O-C groups of the EPS in the binding of  $Cu^{2+}$ ,  $Pb^{2+}$  and  $Cr^{6+}$  ions.

GC-MS is extensively used method for metabolomics research to date, particularly for enabling the identification and quantification of the metabolites involved in the central pathways of primary metabolism such as amino acids, sugar alcohols, sugars, organic acids and polyamines. In this study, the metabolite profiling analysis in two *Nostoc* species after 24 h exposure to Cu(II), Cr(III) and Ni(II) has been performed using GC-MS analysis. The GC-MS analysis of extract of Ni(II) and Cu(II) *Nostoc* sp. N27P72 revealed cyclic and ester compounds (Cyclotrisiloxane, Bis(2-ethylhexyl) phthalate and 1,2-Benzenedicarboxylic acid), in comparison to control. These compounds in the EPS are recorded as *absorption agent*. Wu *et al* (2006) attributed the cytotoxicity effects of fatty acids to their ability to increase the membrane permeability leading to membrane damage. Mundt *et al* 2003, suggested that fatty acids produced by cyanobacteria as a defense mechanism against other microorganisms might be able to change the permeability of the cell membrane Through interacting with proteins and lipids of the membrane, inhibiting special enzymes or by a layer around the cells. Presence compounds of Benzene derivatives in Ni and Cu cultures may be related to absorption compounds to remove the metal, actually it has been previously been shown that some benzene inhibited  $\beta$ -ketoacyl-acyl carrier protein synthase III, a condensing enzyme that initiates fatty acid biosynthesis in most cyanobacteria, leading to absorption activity of metal. Furthermore, the cytotoxic activity of the pure compound 1, 2- benzene dicarboxylic acid, mono 2- ethylhexyl ester (DMEHE) from marine derived actinomycete *Streptomyces* sp. VITSJK8 was examined against mouse embryonic fibroblast (NIH 3T3) and human keratinocyte (HaCaT) normal cell lines, human hepatocellular liver carcinoma (HepG 2) and human breast adenocarcinoma (MCF-7) cell lines by using MTT assay (Krishnan *et al.*, 2014). 1,2- Benzenedicarboxylic acid, bis(2-methyl propyl) ester was recorded by Alghamdi *et l.*, 2018 as plasticizer and has light and heat stability. The antibacterial properties of olive leaves extract is suspected to be associated with the high cyclotrisiloxane hexamethyl content, which has been tested by Keskin *e al.*, 2012. lastly, as compared to solid wastes created from old approach used for the heavy metal removal, polysaccharides are natural, nontoxic and biodegradable polymers, thus reducing their polluting effects and making them attractive for potential use as metal-absorbent safe materials. In conclusion, the strain *Nostoc* sp. N27P72 may be a suitable candidate for mass production of an ecologically attractive EPS with a potential for use in the bioremediation field.

## Conclusion

From the above described investigational results, it seems obvious that the EPS plays an crucial role in protecting from harmful toxic heavy metal. However, in spite of the large number of studies claiming this role, only a few of them directly investigated the modification of metal removal capability by cyanobacteria under mixotrophic condition. In the present study, the highest EPS production efficiency was found in cultures supplemented by maltose and biomass of *Nostoc* sp. N27P72 possesses a high affinity and a high specific uptake for nickel, comparable with the best performances shown by other microbial biomass, and suggest the possibility to use *Nostoc* sp. N27P72 for the bioremoval of heavy metals from polluted water bodies. The FT-IR spectra showed that treatment of Ni with both strains made obvious changes in functional groups of polysaccharids and linkages. Cyclotrisiloxane and 1,2-Benzenedicarboxylic acid, major constituent of *Nostoc* sp. N27P72 in this study, metal removal capability of *Nostoc* sp. N27P72 extract is suspected to be associated with the high Cyclotrisiloxane and 1,2-Benzenedicarboxylic acid content. Lastly, although the key elements controlling the production of the cyanobacterial EPS have been recognized, inclusive strain-specific studies taking into account the interaction between the variables to know the system reaction to changes, are still missing. This needs a better facts of the genes and metabolic paths complicated in the production of EPS in cyanobacteria.

## Declarations

**Conflict of interest** The authors declare no competing financial interest

**Funding** No funding was received for this work

**Ethics approval** Not applicable

**Availability of data and material** Not applicable

**Code availability** Not applicable

**Informed consent** No human participants were involved in this study

**Consent to participate** Not applicable

**Consent for publication** Not applicable

**Acknowledgements** Not applicable

**Author Contribution** Bahareh Nowruzi supervised and proof-read the manuscript, Elham Ghorbani, Mssomeh NejadAli and Azadeh Hekmat edited and proof-read.

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## Tables

Table 1: Chemical composition of *Nostoc* sp. N27P72 (control) extracts as revealed by gas chromatography mass spectrophotometry (GC-MS).

Area %	Nature of the compound	RT (Mins)	Molecular weight	Molecular Formula	Name of Compound
83%	hydroxyether	8.77	90.12	C <sub>4</sub> H <sub>10</sub> O <sub>2</sub>	2-Ethoxyethanol
96%	Phenolic ester	26.29	278.5	C <sub>17</sub> H <sub>30</sub> OSi	Phenol, 2,4-bis-(1,1-dimethylethyl)
64%	alkane	26.41	212.41	C <sub>15</sub> H <sub>32</sub>	Dodecane, 2,6,10-trimethyl
40%	alkane	27.15	114.23	C <sub>8</sub> H <sub>18</sub>	3,3-dimethylhexane
78%	alkane	28	156.31	C <sub>11</sub> H <sub>24</sub>	Undecane
78%	alkane	29.75	173.2957	C <sub>10</sub> H <sub>23</sub>	Hydroxylamine, O-decyl
59%	alkane	29.95	198.39	C <sub>14</sub> H <sub>30</sub>	Tetradecane
83%	alkane hydrocarbon	30.20	268.5	C <sub>19</sub> H <sub>40</sub>	Nonadecane
35%	Organic acid	30.66	74.08	CH <sub>3</sub> CH <sub>2</sub> CO <sub>2</sub> H	Propionic acid
64%	alkane	30.68	450.8664	C <sub>32</sub> H <sub>66</sub>	Dotriacontane
83%	alkane	31.69	282.5	C <sub>20</sub> H <sub>42</sub>	Eicosane
53%	alkane	31.99	156.31	C <sub>11</sub> H <sub>24</sub>	2-Methyldecane

Table 2: Chemical composition of *Nostoc* sp. N27P72 (Ni(II)) extracts as revealed by gas chromatography mass spectrophotometry (GC-MS).

Area %	RT	Nature of the compound	Molecular weight	Molecular Formula	Name of Compound
49%	11.55	six-membered heterocyclic	82.1	C <sub>5</sub> H <sub>6</sub> O	pyran
90%	12.02	Heterocyclic compound	138.3	H <sub>6</sub> O <sub>3</sub> Si <sub>3</sub>	Cyclotrisiloxane
91%	15.97	Heterocyclic compound	138.3	H <sub>6</sub> O <sub>3</sub> Si <sub>3</sub>	Cyclotrisiloxane
91%	19.62	Heterocyclic compound	84.12	C <sub>5</sub> H <sub>8</sub> O	Cyclotrisiloxane
91%	23.22	Heterocyclic compound	138.3	H <sub>6</sub> O <sub>3</sub> Si <sub>3</sub>	Cyclotrisiloxane
90%	23.51	Quinoline Ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
91%	24.01	Quinoline Ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
91%	24.13	Quinoline Ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
91%	24.35	Quinoline Ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
91%	24.55	Quinoline Ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
91%	24.63	Quinoline Ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
91%	25.04	Quinoline Ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
91%	25.14	Quinoline Ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid

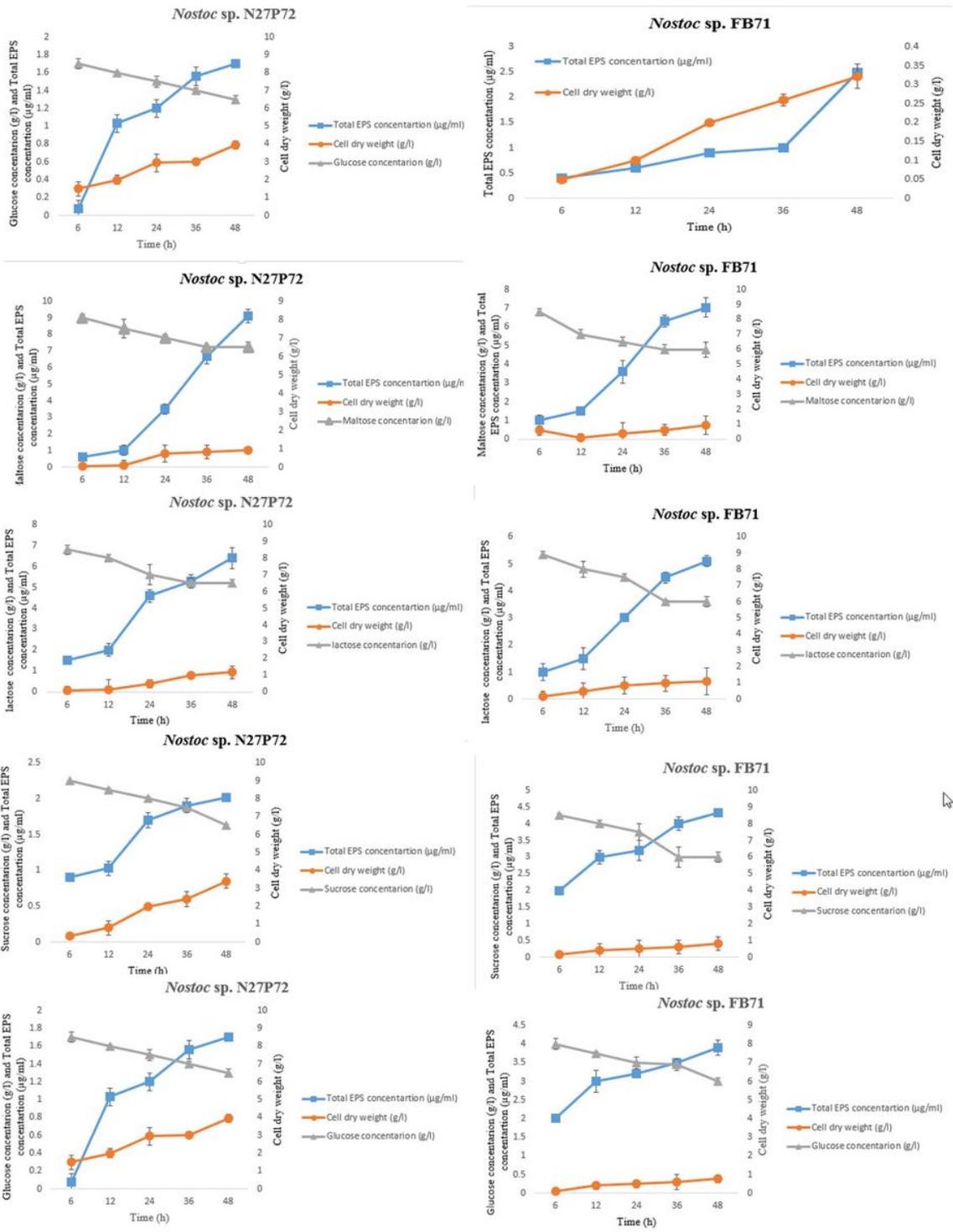
Table 3: Chemical composition of *Nostoc* sp. N27P72 (Cr(III)) extracts as revealed by gas chromatography mass spectrophotometry (GC-MS).

Area %	RT	Molecular weight	Nature of the compound	Molecular Formula	Name of Compound
37%	10.5	327.25	ester	$C_{16}H_{10}F_5NO$	Tranylcypromine, pentafluorobenzoyl
43%	10.85	102.17	Six carbon alcohol	$C_6H_{14}O$	2-Hexanol
64%	11.73	138.3	Heterocyclic compound	$H_6O_3Si_3$	Cyclotrisiloxane
87%	11.83	138.3	Heterocyclic compound	$H_6O_3Si_3$	Cyclotrisiloxane
60%	12.70	98.14	enol ether.	$C_6H_{10}O$	2H-pyran, 3,4-dihydro-6-methyl
72%	13.35	118.17	glycol ether	$C_6H_{14}O_2$	2-Butoxyethanol
40%	15.12	104.15	diol	$C_5H_{12}O_2$	1,4-pentanediol
91%	15.97	482.8	Heterocyclic compound	$C_{23}H_{30}O_4Si_4$	Cyclotetrasiloxane
9%	16.29	96.13	alcohol	$C_6H_{10}O$	3,5-Hexadien-2-ol
9%	18.40	179.05	ketone	$C_6H_{11}BrO$	6-Bromo-2-hexanone
9%	18.55	99.13	lactam	$C_5H_9NO$	2-piperidinone
91%	19.63	230.5	silicone	$H_{10}O_5Si_5$	Cyclopentasiloxane
20.5	20.5	166.17	aldehyde	$(CH_3O)_2C_6H_3CHO$	2,3-dimethylbenzaldehyde
20.64	20.64	118.09	Organic acid	$C_4H_6O_4$	Succinic acid
23.04	23.04	156.26	aldehyde	$C_{10}H_{20}O$	Decanal

Table 4: Chemical composition of *Nostoc* sp. N27P72 (Cu(II)) extracts as revealed by gas chromatography mass spectrophotometry (GC-MS).

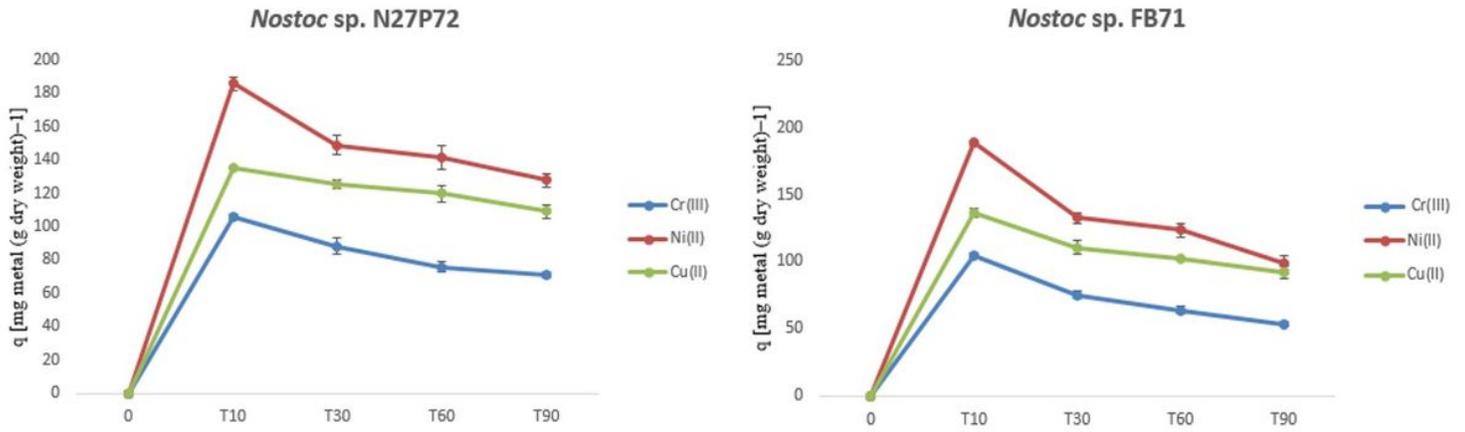
Area %	RT	Nature of the compound	Molecular weight	Molecular Formula	Name of Compound
78%	11.63	Heterocyclic compound	138.3	H <sub>6</sub> O <sub>3</sub> Si <sub>3</sub>	Cyclotrisiloxane
78%	11.71	Heterocyclic compound	138.3	H <sub>6</sub> O <sub>3</sub> Si <sub>3</sub>	Cyclotrisiloxane
90%	15.89	Heterocyclic compound	138.3	H <sub>6</sub> O <sub>3</sub> Si <sub>3</sub>	Cyclotrisiloxane
7%	16.31	methyl ketones	84.12	C <sub>5</sub> H <sub>8</sub> O	4-penten-2-one
90%	19.61	Heterocyclic compound	138.3	H <sub>6</sub> O <sub>3</sub> Si <sub>3</sub>	Cyclotrisiloxane
43%	23.26	mono ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
91%	23.42	mono ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
80%	23.54	diester of phthalic acid	390.6	C <sub>24</sub> H <sub>38</sub> O <sub>4</sub>	Bis(2-ethylhexyl) phthalate
91%	23.81	mono ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
91%	24.03	mono ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
91%	24.15	mono ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
91%	24.57	mono ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
91%	25.05	mono ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid
91%	25.54	mono ester	166.1308	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	1,2-Benzenedicarboxylic acid

## Figures



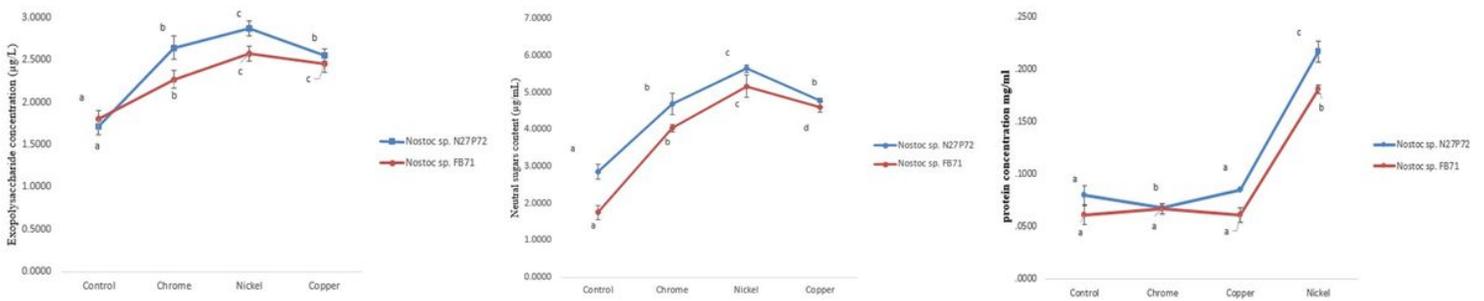
**Figure 1**

Growth profile and EPS production of *Nostoc* sp. N27P72 and *Nostoc* sp. FB71 cultivated in media culture containing (10 g/l) glucose, sucrose, lactose and maltose, separately and media culture without additional sugars as a control. Symbols indicate (●) for cell dry weight, (□) for total EPS concentration, and (Δ) for sugar concentration in the media. Results are the mean of duplicate measurement.



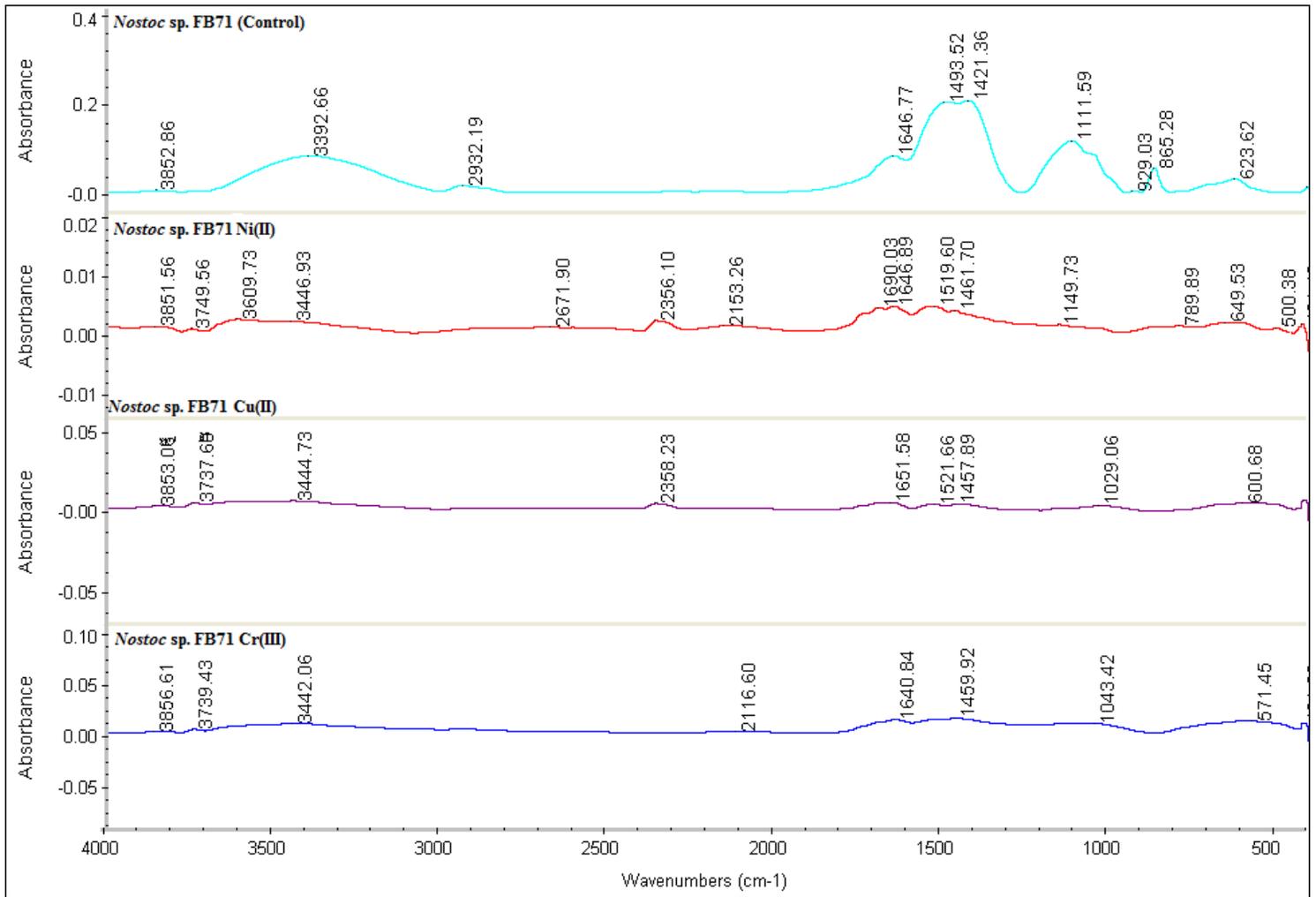
**Figure 2**

Time course of specific metal removal ( $q$ ), expressed as mg of metal removed per g of biomass dry weight, by *Nostoc* sp. N27P72 and *Nostoc* sp. FB71 cultivated in media culture containing (10 g/l) maltose, with copper, chromium and nickel in single-metal solutions. Symbols represent the mean of at least three replicates and bars represent the standard deviation, if larger than the dimensions of the symbols.



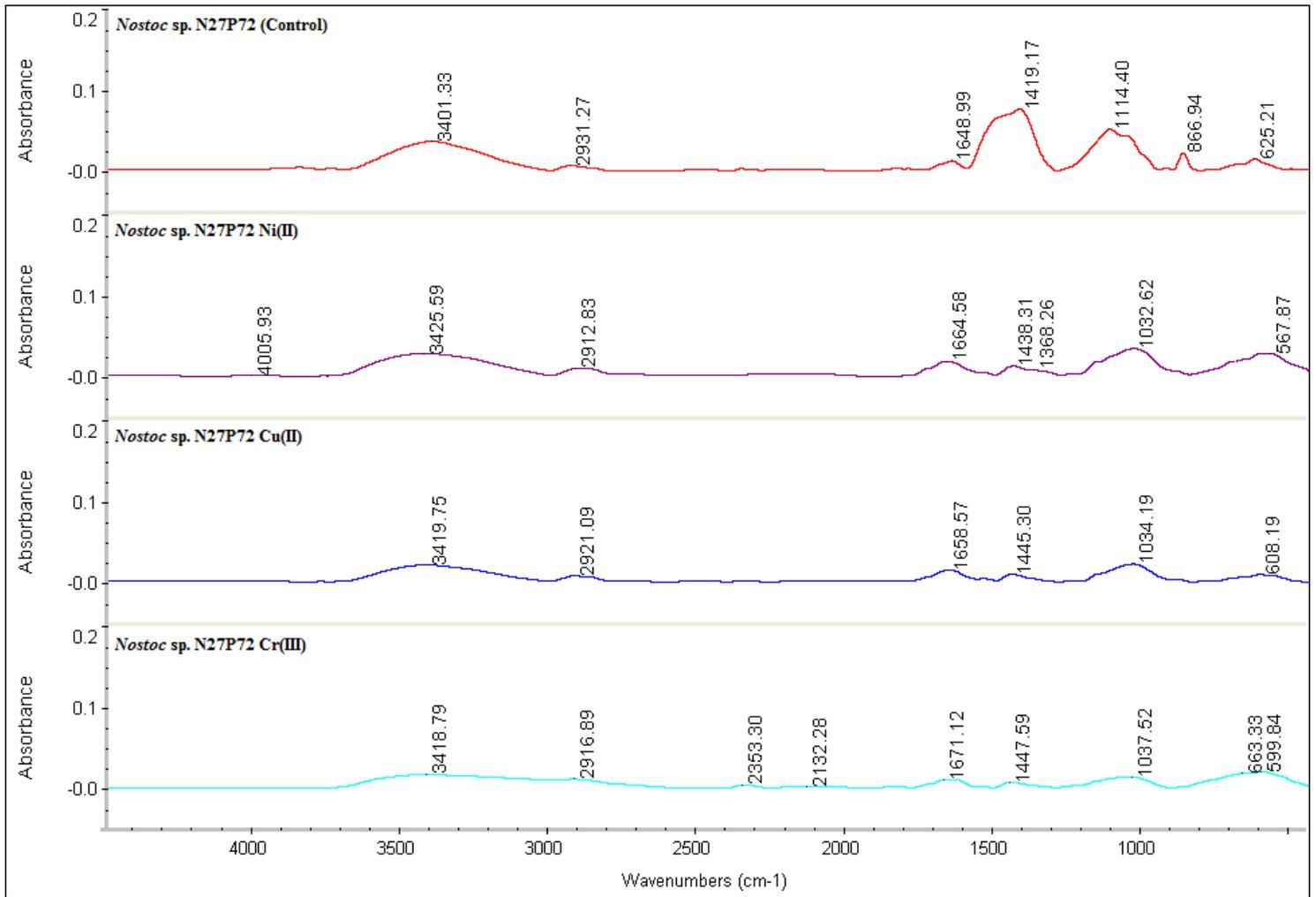
**Figure 3**

Comparison of total produced EPSs (a), Neutral sugar content (b) and total soluble proteins (c) of *Nostoc* sp. N27P72 and *Nostoc* sp. FB71 in lyophilized EPS containing maltose and metal solution of Cu(II), Cr(III) and Ni(II). All the values are mean of at least three replicates  $\pm$  standard deviation.



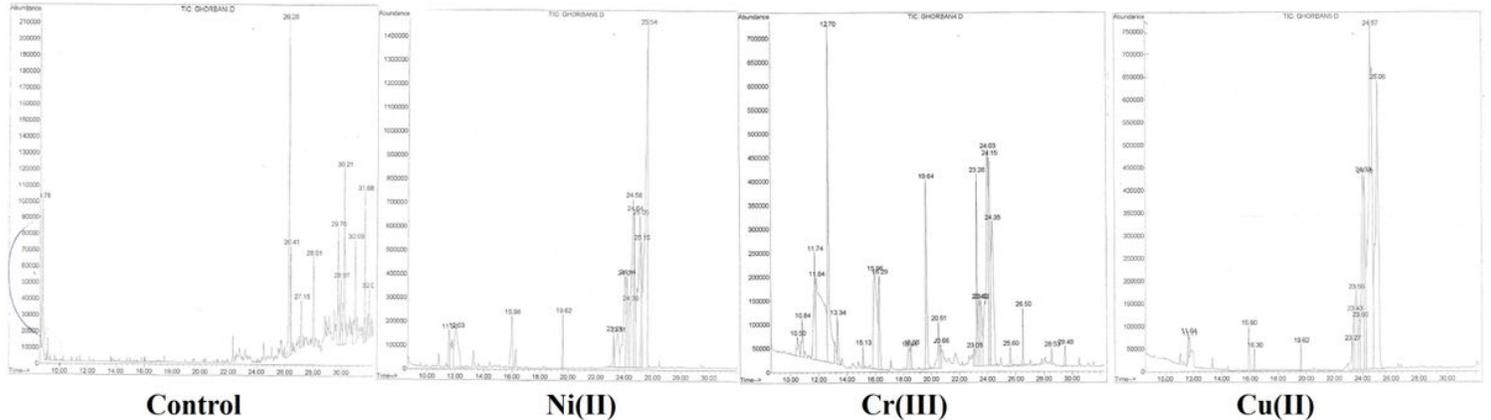
**Figure 4**

Fourier transform infrared (FTIR) spectra of EPS from *Nostoc sp. FB71* against control or exposure to a solution containing 400 ml of cell suspensions in 1000 ml Erlenmeyer flasks of Cu(II), Cr(III) and Ni(II).



**Figure 5**

Fourier transform infrared (FTIR) spectra of EPS from *Nostoc sp. N27P72* against control or exposure to a solution containing 400 ml of cell suspensions in 1000 ml Erlenmeyer flasks of Cu(II), Cr(III) and Ni(II).



**Figure 6**

GC-MS chromatogram of the extract of *Nostoc sp. N27P72*, in media culture containing the maltose and without maltose as control culture, were tested for their ability to remove Cu(II), Cr(III) and Ni(II).