CrossMark

Bioreduction of toxicity influenced by bioactive molecules secreted under metal stress by *Azotobacter chroococcum*

Asfa Rizvi[1] · Bilal Ahmed[1] · Almas Zaidi[1] · Mohd. Saghir Khan[1]

Abstract

Heavy metal pollution destruct soil microbial compositions and functions, plant's performance and subsequently human health. Culturable microbes among many metal abatement strategies are considered inexpensive, viable and environmentally safe. In this study, nitrogen fixing bacterial strain CAZ3 recovered from chilli rhizosphere tolerated 100, 1000 and 1200 µg mL^{-1} of cadmium, chromium and nickel, respectively and was identified as *Azotobacter chroococcum* by 16S rDNA sequence analysis. Under metal stress, cellular morphology of *A. chroococcum* observed under SEM was found distorted and shrinkage of cells was noticed when grown with 50 µg mL^{-1} of Cd (cell size 1.7 µm) and 100 of µg mL^{-1} Ni (cell size 1.3 µm) compared to untreated control (cell size 1.8 µm). In the presence of 100 µg mL^{-1} of Cr, cells became elongated and measured 1.9 µm in size. Location of metals inside the cells was revealed by EDX. A dose dependent growth arrest and consequently the death of *A. chroococcum* cells was revealed under CLSM. *A. chroococcum* CAZ3 secreted 320, 353 and 133 µg EPS mL^{-1} when grown with 100 µg mL^{-1} each of Cd, Cr and Ni, respectively. The EDX revealed the presence of 0.4, 0.07 and 0.24% of Cd, Cr and Ni, respectively within EPS extracted from metal treated cells. Moreover, a dark brown pigment (melanin) secreted by *A. chroococcum* cells under metal pressure displayed tremendous metal chelating activity. The EDX spectra of melanin extracted from metal treated cells of *A. chroococcum* CAZ3 displayed 0.53, 0.22 and 0.12% accumulation of Cd, Cr and Ni, respectively. The FT-IR spectra of EPS and melanin demonstrated stretching vibrations and variations in surface functional groups of bacterial cells. The C-H stretching of CH$_3$ in fatty acids and CH$_2$ groups, stretching of N-H bond of proteins and O-H bond of hydroxyl groups caused the shifting of peaks in the EPS spectra. Similar stretching vibrations were recorded in metal treated melanin which involved CHO, alkyl, carboxylate and alkene groups resulting in significant peak shifts. Nuclear magnetic resonance (NMR) spectrum of EPS extracted from *A. chroococcum* CAZ3 revealed apparent peak signals at 4.717, 9.497, 9.369 and 9.242 ppm. However, ^{1}H NMR peaks were poorly resolved due largely to the impurity/viscosity of the EPS. The entrapment of metals by EPS and melanin was confirmed by EDX. Also, the induction and excretion of variable amounts of metallothioneins (MTs) by *A. chroococcum* under metal pressure was interesting. Conclusively, the present findings establish- (i) cellular damage due to Cd, Cr and Ni and (ii) role of EPS, melanin and MTs in adsorption/complexation and concurrently the removal of heavy metals. Considering these, *A. chroococcum* can be promoted as a promising candidate for supplying N efficiently to plants and protecting plants from metal toxicity while growing under metal stressed environment.

✉ Asfa Rizvi
asfarizvi09@gmail.com

[1] Faculty of Agricultural Sciences, Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh, UP 202002, India

Graphical Abstract
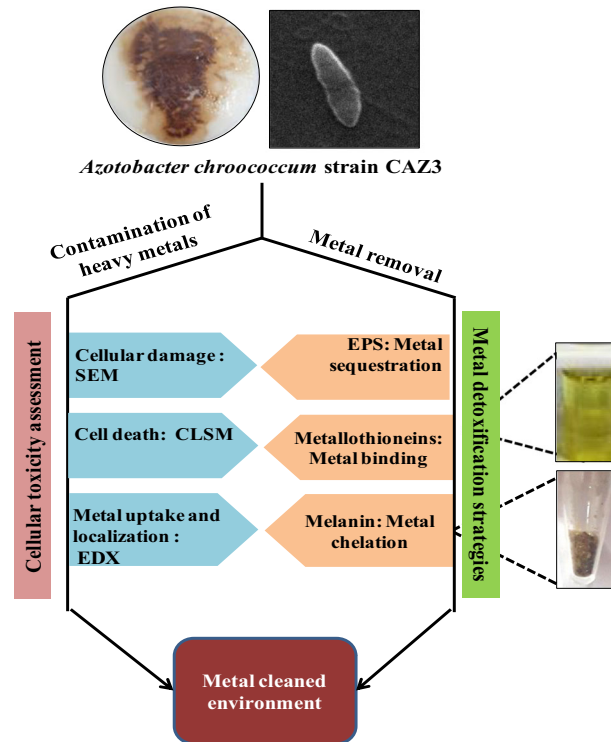
Azotobacter chroococcum strain CAZ3

Introduction

Globally, heavy metal pollution is a major issue which has seriously threatened the sustainability of environment. The toxic heavy metals emerging from sources like, industries, tannery, sewage wastes and other metal discharging industries have been found to adversely affect the density, diversity and physiological activities of soil microbiota (Xie et al. 2016). Among various heavy metals, cadmium, a highly lethal metal is widely used in batteries, for coating, electroplating, PVC stabilizers and as a component in alloy formation in various industries (Sharma et al. 2015). Due to lack of regulatory policy for controlled and safe discharge, cadmium is added to the environment via many anthropogenic activities like combustion of metal ores, burning of wastes and by the use of fossil fuels. However, after it accumulates in soils, Cd can be absorbed by plants leading eventually to the disruption of food chain and indirectly affects human health (Rahimzadeh et al. 2017). Chromium is yet another toxic metal which is used in plating, alloy formation, tanning of animal hides, for inhibiting water corrosion, textile dyes and mordants, pigments, ceramics, refractory bricks, pressure-treated lumber etc. The overuse

and abuse of Cr has however hugely dented human health by acting as carcinogenic and mutagenic element (Oliveira 2012). Nickel which is even though present in very low concentrations in the environment has been found to cause serious human health problems when present beyond threshold concentrations. For instance, Ni can cause- (i) genotoxicity (ii) several types of carcinomas (iii) toxic impacts on the immune system and (iv) damage to other metabolically active tissues (Das et al. 2018).

Some of the distortive/destructive impact of heavy metals on microbes are-(i) alterations in cell surface morphology and growth behaviour (Rizvi and Khan 2017) (ii) cell membrane disruption (Chen et al. 2016) (iii) inhibition of enzyme activity (Alnuaimi et al. 2012) (iv) oxidative phosphorylation leading to lipid peroxidation (Mishra and Mishra 2015) and (v) denaturation of microbial proteins (Ayangbenro and Babalola 2017). However, with the advent of certain state of the art techniques such as SEM (Golding et al. 2016), EDX (Ackerman et al. 2016) and CLSM (Hao et al. 2013), it has now become possible to assess the finer details of metal toxicity to microbes (Gomes and Mergulhão 2017) and plants (Rizvi and Khan 2018) which a few years back were almost impossible. For

example, the SEM reveals the morphological distortions while the EDX provides information on distribution of heavy metals within bacterial cells (Ramya and Thatheyus 2018). Similarly, the CLSM is considered a powerful tool which is used to assess the speciation/composition of metals and hence, the hazardous impact of toxicants on bacterial cells (Hao et al. 2013). However, microorganisms have developed various efficient mechanisms to endure the continuous and prolonged exposure to heavy metals. While surviving under metal stressed environment, metal tolerant microbes transform the toxic heavy metals to less toxic forms (Fashola et al. 2016) and adsorb/desorb (François et al. 2012) metals. Apart from these, the ability of bacteria to- (i) pump out metals and (ii) chelate and trap heavy metals by producing exopolysaccharides (EPS) are some other mechanisms adopted by microbes to detoxify and manage the contaminated sites (Haferburg and Kothe 2010). These features of metal tolerant bacteria are usually exploited to remove toxic metals from metal polluted environments. For example, the EPS among various active biomolecules secreted by a few bacterial species (Ates 2015) provides special protection to the producing organisms while growing under harsh environment (Nocelli et al. 2016). The EPS forms a complex by binding with the ionic forms of heavy metals and makes heavy metals inaccessible to microbes (Zhang et al. 2017). Thus, the toxicity of metals to microbes is restricted. Besides this, the EPS so generated by the bacterial cells also safeguard them from extreme environmental conditions like high temperatures, pH, starvation, and desiccation etc. (Gupta and Diwan 2017). Thus, secretion of EPS could be an adaptive feature which aids the bacterial communities to survive even in the environments heavily contaminated with metals. And hence, the metal chelating ability of the bacterial EPS is being largely employed for bioremediation purposes (Ojuederie and Babalola 2017). This strategy employed by metal tolerant bacteria could be a cost effective approach for metal clean up from contaminated soils.

The synthesis of metallothioneins (MTs) by microbes is yet another important strategy adopted by useful soil microflora to contain/restrain heavy metal toxicity. Metallothioneins (metal binding proteins) comprising of small polypeptide chains with approximately 30% cysteine residues have a strong affinity for metals and therefore, chelates heavy metals like Zn, Ni, Cu, Pb, Cd, Hg etc. through thiolate bonds of cysteine (Diopan et al. 2008). While binding with metals, the MTs provide protection to bacterial cells against any oxidative damage caused due to heavy metal toxicity and metal transportation and regulation (Adam et al. 2014). Since the metallothioneins (MTs) belong to a family of metal-binding proteins, they regulate homeostasis within the cells while simultaneously protecting the cells from deadly effects of metals and combating the stress developed due to the presence of superoxide radicals (Vignesh and Deepe 2017). Metallothioneins also very effectively bind and sequester toxic metals like Cd, Hg etc. Due to their incredible role in substantial mitigation of oxidative stress and protection of bacterial cells from the harsher impacts of metals, MTs have safely emerged as a key component of bacterial cells which allows them to survive actively under metal stressed environments while maintaining the overall regulation of homeostasis within the cells. Also, the metalloregulatory nature of MTs makes them efficient molecules that can attach/attract xenobiotic compounds and thus confer a shield around bacterial cells to thwart metal toxicity (Si and Lang 2018). The production of a brown to black pigment (melanin) by some bacterial strains for example *A. chroococcum* (Banerjee et al. 2014) is yet other mechanism by which the toxicity of metals to microbes could be reduced (Cordero et al. 2017). The melanins have been found to efficiently chelate the metal ions and while doing this, protect bacterial cells from various biochemical (El-Naggar and El-Ewasy 2017) and thermal stresses (Hu et al. 2015) including the heavy metal stress (El-Naggar and El-Ewasy 2017). Melanin pigment due to its ability to absorb a broad spectrum of electromagnetic waves can also provide protection to bacterial cells against ultraviolet radiations. Apart from these, melanin has been reported to safeguard bacterial cells from multiple chemical stresses and high temperatures (Rao et al. 2017). Melanins also possess a remarkable feature of chelating heavy metal ions which can be exploited for detoxification/removal of metal ions from metal contaminated sites (Thaira et al. 2018).

The secretion of EPS, MTs and melanin by metal tolerant bacteria and the ability of these biomolecules to make heavy metals unobtainable for uptake by microbes/plants (microbes assisted phytoremediation) has received little attention as a viable metal cleanup strategy. Considering such a huge gap in this bioremediation area and to test this hypothesis of metal removal strategy, a detailed and systematic study was designed to achieve the following specific objectives (i) to understand how cadmium, chromium and nickel affect bacterial cells employing SEM, EDX and CLSM and (ii) exploring the mechanistic basis of EPS, MTs and melanin in heavy metal removal from derelict environment.

Material and methods

Strain selection, characterization and metal tolerance

The bacterial strain CAZ3 was isolated from rhizosphere of chilli (*Capsicum annum*) grown in heavy metal

contaminated soils. In order to test the ability of this strain to survive under metal stress and hence to determine the tolerance limit, the strain was exposed to 0–2400 µg mL^{-1} each of cadmium, chromium and nickel added to nutrient agar (NA) and nutrient broth (NB) medium. The metal amended plates and tubes were incubated in a bacteriological incubator (York Scientific Industries Pvt. Ltd. India) maintained at 28 ± 2 °C for 48 h. Strain CAZ3 exhibiting maximum tolerance to Cd, Cr and Ni was characterized morphologically and biochemically (Holt et al. 1994). Following this, the strain was identified to species level by 16S rDNA sequence analysis done by Macrogen, Seoul, South Korea. The two primers used were: universal primers, 785 F (5′-GGATTAGATACCCTGGTA-3′) and 907 R (5′-CCGTCAATTCMTTTRAGTTT-3′). Later, strain CAZ3 was identified to species level using BLASTn analysis, the resulting nucleotide sequence was submitted to GenBank sequence database and accession number was assigned. The evolutionary relationship of strain CAZ3 was traced by preparing a phylogenetic tree using MEGA 6.0 software.

Assessment of disparaging impact on bacterial strain and localization/distribution of metals by SEM and EDX

Cytotoxic damage and morphological changes were determined by growing bacterial cells in NB amended with 50 µg Cd mL^{-1} and 100 µg mL^{-1} each of Cr and Ni. Furthermore, cells were visualized under SEM (model JSM 6510 LV; JEOL, Japan). For SEM analysis, two milli litre cultures of metal treated and untreated cells of strain CAZ3 were centrifuged at 10,000 rpm for 10 min. The cell pellets were washed twice with phosphate buffered saline (PBS) and fixed in primary fixative (2.5% glutaraldehyde and 2% paraformaldehyde) overnight at 4 °C. The pellets were again rinsed with PBS three times for 5 min. each. Following washing, the cell pellets were dehydrated using 30, 50, 70, 90 and 100% ethanol. Then, two micro litre suspensions of the dehydrated pellets were mixed with 8 µL of double distilled water on a glass cover slip and observed under SEM. The localization and percentage of heavy metals inside bacterial tissues was determined by EDX spectroscopy.

Bacterial cell death determined by confocal laser scanning microscopy (CLSM)

Bacterial cells grown in NB medium treated with (25, 50 and 100 µg mL^{-1} each of Cd, Cr and Ni) or without heavy metals (control) were visualized under CLSM (Model LSM-780, Zeiss, Germany) to assess bacterial cell death caused due to heavy metals. For this, bacterial cell pellets were washed three times with PBS for 15 min. and the pellets were dissolved in PBS. A-100 µL of bacterial cell suspension of metal treated and untreated cells was mixed with 10 µL of propidium iodide (PI) prepared in PBS (1 mg mL^{-1}). The bacterial suspension mixed with the fluorescent dye was incubated for 10 min. at room temperature. The resulting suspension was centrifuged (at 5000 rpm for 10 min.) to remove the unbound dye, if any. Furthermore, the cell pellets were re-suspended in 500 µL of PBS and a thin smear was prepared on a glass slide. The samples were then observed for PI stained dead cells with an excitation/emission maxima of 493/636 nm for PI. Samples were prepared in dark to avoid photobleaching of the dye.

Impact of heavy metals on bacterial growth

The influence of heavy metals on growth was determined by culturing a 10 µL of strain CAZ3 into nutrient broth containing 0 to 400 µg mL^{-1} (at a dilution factor of two) of Cd, Cr and Ni. The growth pattern was observed in a 96-well micro titer plate, incubated at 28 ± 2 °C for 24 h. Following incubation, the growth was measured at 620 nm at a 2 h interval and growth curve was constructed.

Heavy metal removal mechanisms adopted by metal tolerant bacterial strain

Extraction of exopolysaccharides (EPS)

The production of EPS by strain CAZ3 under conventional and metal stressed conditions was determined by the method as suggested by Mody et al. (1989). For EPS quantification, the bacterial strain was cultured in 50 mL NB supplemented with 5% glucose (as C source) and treated with 0, 25, 50 and 100 µg mL^{-1} each of Cd, Cr and Ni. The bacterized flasks (100 ml capacity) were incubated at 28 ± 2 °C for 5 days on a rotary shaker incubator (Rivotek) at 120 rpm. The culture broth was spun at 8000 rpm for 30 min. and three volumes of chilled acetone were added to one volume of supernatant to extract the EPS. The extracted EPS was then washed three times with distilled water and acetone and later transferred to a filter paper (No. 42). The filter paper was dried overnight at room temperature and weighed for estimating the amount of EPS released by the bacterial strain.

Characterization of EPS

The structural alteration, metal location and disturbances/variations in functional moieties of dried powdered EPS extracted from metal treated and untreated (control) strain CAZ3 was determined by SEM, EDX, and FTIR respectively. For SEM and EDX, a small fraction of the powdered

EPS was mounted on a stub provided with a double-sided carbon tape. After mounting, the samples were coated with gold coating in a sputter coater prior to visualization under SEM. Following the coating procedure, the EPS samples were visualized under SEM (model JSM 6510 LV; JEOL, Japan) and surface morphology of EPS was recorded. Moreover, the percentage of heavy metals entrapped within the EPS was detected by EDX detector coupled to SEM. The peaks (respective of each metal) appearing in the EDX analysis, gave a clear picture of the elemental composition of EPS extracted from metal loaded and unloaded cells of strain CAZ3. The functional groups of powdered EPS were further assayed by FT-IR spectroscopy. For this, approximately 2.5 mg of powdered EPS was ground and mixed with 75 mg of KBr in a mortar. The powdered material was pressed to prepare translucent discs of the samples. The respective discs were analysed immediately by a spectrometer adjusted in the range of $1000–4000\,cm^{-1}$ with a resolution of $5\,cm^{-1}$ while subtracting the effect of atmospheric H_2O and CO_2. The samples were visualized under FT-IR spectrometer (Thermo Nicolet, Nexus 670) and characteristic spectral peaks corresponding to each metal were recorded.

NMR analysis of EPS To further ascertain the structural composition of EPS, solid state ^1H nuclear magnetic resonance (NMR) was employed. For spectral analysis, D_2O was used as the solvent wherein the dried but crude EPS powder was deuterated with 99% D_2O and NMR spectrum of the sample was recorded at 400.3 MHz for ^1H NMR using Bruker Avance II-500 spectrometer. The chemical shifts in the EPS sample were measured in ppm using tetramethylsilane (TMS) as an internal standard.

Influence of heavy metals on metallothioneins (MTs) secretion by strain CAZ3

The impact of heavy metals on MTs production was assayed by the method of Murthy et al. (2011) with some modifications. The bacterial strain was grown in NB amended with $50\,\mu g\ Cd\ mL^{-1}$ and $100\,\mu g\ mL^{-1}$ each of Cr and Ni for 48 h at $28\pm2\,°C$ on a shaking incubator. A control without heavy metal was also run for comparison. After 48 h, the cultures were centrifuged at 10,000 rpm for 20 min. and the resulting pellets were suspended in lysis buffer comprising of 0.5 M sucrose prepared in 20 mM Tris-HCl buffer (pH = 8.6) and containing 0.01% mercaptoethanol. Sonication of the pellets was done for 2 min. (4 times with 30 sec pulse off) using a Branson Digital Sonifier, Branson Ultrasonics Corporation, Danbury, USA to completely disrupt the bacterial cells. The sonicated sample was centrifuged again at 10,000 rpm for 20 min. and the supernatant was separated from pellet. A-1.05 mL of

cold absolute ethanol (chilled at $-30\,°C$) and $80\,\mu L$ of chloroform were added to one ml of supernatant. The samples were centrifuged under cold conditions at 10,000 rpm for 10 min. with the addition of three volumes of cold ethanol to the supernatant. The suspension was chilled at $-30\,°C$ for 1 h. Centrifugation of the samples was done at 10,000 rpm for 10 min. and the cell pellets were washed with a mixture of ethanol, chloroform and homogenization buffer in the ratio $87:1:12\,\mu L$ and then again centrifuged at 10,000 rpm for 10 min. The pellets were dried to complete evaporation and the dried pellets was re-suspended in $300\,\mu L$ of 5 mM Tris-HCl and 1 mM EDTA (pH = 7). The MTs fraction so obtained was mixed with 4.2 mL of 0.43 mM 5, 5'-dithiobis nitrobenzoic acid (Ellman's reagent) in 0.2 M phosphate buffer (pH = 8) and left at room temperature for 30 min. The concentration of reduced sulfhydryl in the samples was measured at 412 nm and MTs in each sample was quantified by plotting a standard curve of reduced glutathione (GSH). The concentration of metallothionein in the samples was calculated using the standard curve equation of Ana and Garcia-Vazquez (2006).

Extraction and characterization of melanin released under metal stress

Melanin secreted by strain CAZ3 was extracted following the method of Banerjee et al. (2014). For this, 7 days old cultures were grown in NB treated with ($50\,\mu g\ Cd\ mL^{-1}$ and $100\,\mu g\ mL^{-1}$ each of Cr and Ni) or without metals. The bacterial culture was centrifuged at 5000 rpm for 10 min. to obtain cell pellets. The pellets were washed with 5% trichloroacetic acid (TCA) and later with ether-ethanol mixture in a ratio of 1:1 v/v and absolute ether to remove any impurities. The washed pellets were dissolved in 0.05 M sodium carbonate and the mixture was heated in a boiling water bath for 10 min. The material was stored at room temperature for 15 min. The dark brown material so obtained was washed three times with sterile double distilled water and then lyophilized to obtain a dark brown powder which was characterized using SEM, EDX and FTIR techniques.

Measurement of functional group moieties of metal treated and untreated EPS and melanin by FTIR

The destructive impact of heavy metals and stretching vibrations in various functional groups present in EPS and melanin secreted by strain CAZ3 was determined by FT-IR spectrometer (Thermo Nicolet, Nexus 670). For this, dried powder of EPS and melanin (2.5 mg approx.) was blended and ground with KBr (75 mg) in an agate mortar. The translucent discs were prepared by pressing the ground material with the help of 8 tonnes of pressure bench press.

The respective discs were immediately analysed with a spectrophotometer in the range of 1000–4000 cm^{-1} with a resolution of 5 cm^{-1}. The influence of atmospheric H_2O and CO_2 was always subtracted.

Statistical analysis

The results were statistically analyzed by Duncan's multiple range test (DMRT) and the least significant difference (LSD) was calculated using Minitab 17 statistical software at $P \le 0.05$.

Results and discussion

Heavy metal tolerance and bacterial strain identification

Of the total 20 bacterial strains tested for their ability to survive under harsh metal environment, strain CAZ3 exhibited variable growth on NA medium amended with different concentrations of heavy metals. Strain CAZ3 grew well on NA medium treated with 100 $\mu g\ mL^{-1}$ of Cd, 1000 $\mu g\ mL^{-1}$ of Cr and 1200 $\mu g\ mL^{-1}$ of Ni whereas it tolerated 50 $\mu g\ mL^{-1}$ of Cd, 100 $\mu g\ mL^{-1}$ each of Cr and Ni when cultured in liquid medium (Table 1). In general, *A. chroococcum* CAZ3 exhibited greater tolerance to heavy metals when grown on nutrient agar plates as compared to nutrient broth possibly due to the complex/polymeric nature of agar which renders the metals unavailable for uptake by the bacteria as also reported by other workers (Moghannem et al. 2015). In contrast to this, the metals become soluble and hence, are easily available to bacteria when present in liquid medium, thereby resulting in higher levels of toxicity. Similar variation in survivability and metal tolerance ability among bacterial strains for example, *B. cereus* and *B. amyloliquefaciens* has been reported (De Guzman et al. 2016). In this experiment, both bacterial strains tolerated Pb up to a level of 2000 $\mu g\ mL^{-1}$ where as *P. aeruginosa*, *Chryseobacterium* sp., and *B. subtilis* could tolerate 1200 $\mu g\ mL^{-1}$ each of Cd and Ni. In yet another study, Singh and Lal (2015) *Pseudomonas* sp. (P-3) and *Bacillus* sp. (B-2) grew well in liquid medium treated with 350 $\mu g\ mL^{-1}$ of chromium. However, to avert the lethal impact of heavy metals and consequently to survive and multiply under heavy metal pressure, the metal tolerant microbes have evolved many interesting mechanisms such as-(a) complexation of metals within the cell (Ianeva 2009) (b) pumping out of metal ions to the exterior of the cell via efflux pump system (Ma et al. 2016) (c) enzymatic degradation (Marzan et al. 2017) and solubilization of heavy metals (Ayangbenro and Babalola 2017) and (d) transformation of metals from toxic to non toxic state (Chaturvedi

Table 1 Morphological, biochemical and heavy metal tolerance profiling of *A. chroococcum* strain CAZ3

Morphological characteristics	
Colony shape	Irregular with wavy margins
Colony colour	Brownish
Colony morphology	Wrinkled
Pigmentation	Dark brown
Gram reaction	Gram negative
Cell shape	Short rods

Biochemical characteristics	
Indole	+
Methyl red	+
Voges-Proskauer	−
Citrate utilization	+
Nitrate reduction	+
Catalase	−
Oxidase	+
Urease	−
Starch hydrolysis	+
Lipid hydrolysis	+

Minimum inhibitory concentration of heavy metals ($\mu g\ mL^{-1}$)		
	Nutrient agar	Nutrient broth
Cadmium	200	100
Chromium	1200	200
Nickel	1400	200

'+' and '−' indicate positive and negative reactions, respectively

et al. 2015). However, whatever mechanisms they adopt to circumvent metal toxicity, this feature of strain CAZ3 to survive and proliferate in under metal stressed environment is indeed a unique property which could be exploited for various reasons. For instance, with this ability, the bacterial strain will be able to ramify in soils polluted with single or multiple metals and once established, will express their physiological activities which could be beneficial for crops growing in stressed environment. Since this strain was found environmentally interesting, it was further characterized biochemically and molecularly up to species level. Strain CAZ3 displayed Gram negative reaction, and was rod shaped and exhibited dark brown pigmentation (melanin) on maturation. The bacterial strain tested positive for starch and lipid hydrolysis and also displayed a positive reaction for indole, oxidase, and nitrate reduction (Table 1). On the other hand, strain CAZ3 exhibited a negative reaction towards catalase and urease.

On the basis of morphological and biochemical properties, strain CAZ3 was presumed as *Azotobacter* which was identified molecularly by 16S rRNA gene sequence analysis
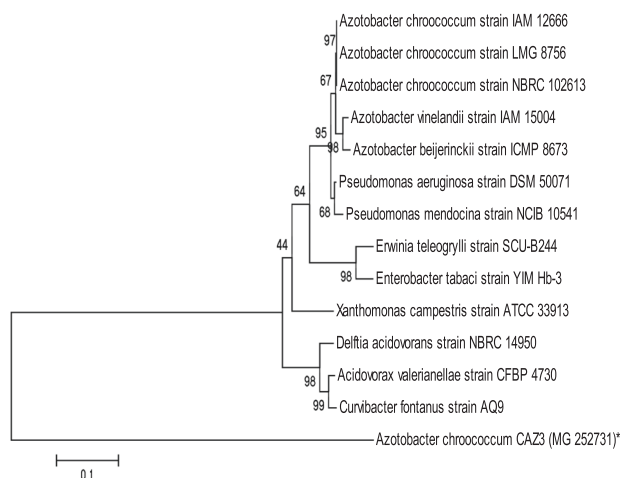
Fig. 1 Phylogenetic analysis based on 16S rRNA gene comparison showing relationship between *Azotobacter chroococcum* CAZ3 and other closely related bacteria. The tree was constructed using Clustal W sequence alignment tool in MEGA 6.0 software

as *Azotobacter chroococcum* (GenBank accession number MG252731) based on percent similarity with other *A. chroococcum* strains already present in the NCBI database (Fig. 1).

Cellular damage to *A. chroococcum* cells revealed under SEM, EDX and CLSM studies

A. chroococcum CAZ3 cells when grown in NB amended with 50 µg mL^{-1} Cd (Fig. 2a) and 100 µg mL^{-1} each of Cr (Fig. 2b) and Ni (Fig. 2c) had distorted cellular morphology when observed under SEM as compared to untreated control cells (Fig. 2d). The metal treated cells displayed shrinkage and elongation and other surface distortions as compared to the untreated bacterial cells whose morphology and size remained unaltered/normal. The average size of cells (1.6 µm) was reduced even though marginally by 6.25% in the presence of 50 µg mL^{-1} Cd, compared to un-treated cells (1.7 µm) yet the distortions were visible. However, among metals, maximum reduction in cell size was observed for Ni which reduced it by 24% (1.3 µm) as compared to control. In contrast, the cells grown in the presence of Cr had elongated cell and the average length of cells was recorded as 1.8 µm. Similar reduction in size of bacterial cells grown with Cd and Ni (Bhagat et al. 2016) and Pb (Girisha 2014) while increase in cell length of bacteria for instance, *Ochrobactrum tritici* 5bvl1 grown under Cr (VI) stress has been reported (Francisco et al. 2010). In yet other study, a significant elongation in the size of Cr treated *E. coli* and *B. subtilis* cells have been revealed under SEM (Samuel et al. 2013). The elongation in bacterial cells following exposure to Cr could possibly be an adaptive mechanism employed by bacteria to protect itself from the

toxic impact of metal. Despite the damage caused by heavy metals, it was interesting to note that the metals were found localized within the cells of *A. chroococcum* CAZ3, as presented in the EDX spectra prepared from bacterial cells after growing them with Cd (Fig. 2e), Cr (Fig. 2f) and Ni (Fig. 2g).

Also, the toxicity of heavy metals to *A. chroococcum* CAZ3 expressed as number of dead cells was clearly visible which however increased progressively with consequent increase in metal concentrations (Fig. 3). In the microimage, the PI stained cells exhibiting red fluorescence indicate the dead cells. Generally, the number of dead cells increased with a progressive increase in metal concentration as presented in Fig. 3a. Conventionally, the untreated control cells did not show any red fluorescence. Mechanistically, the PI dye (a DNA intercalating dye) permeates only through the membranes of dead cells and hence the cells become red in colour. Due to this, the PI dye is considered as an excellent indicator for assessment of metal toxicity. Prior to this study, a similar increase in the number of dead cells of *Burkholderia cepacia* strain PSBB1 with the progressive increase in the concentration of glyphosate was reported by Shahid and Khan (2018). Additionally, the toxic impact of Se causing oxidative stress and DNA damage in *Oreo-chromis mossambicus* has previously been reported (Gobi et al. 2018). Conclusively, even though, *A. chroococcum* CAZ3 tolerated higher concentrations of Cd, Cr and Ni, yet the bacterial membrane was damaged by heavy metals in a dose dependent manner.

After evaluating the growth pattern under CLSM, the inhibitory impact of varying concentrations of Cd, Cr and Ni on *A. chroococcum* CAZ3 were further tested under in vitro conditions. Here also, a variable growth pattern was observed when measured at 620 nm (Fig. 3b). Among metals, Cd in general was found most toxic and inhibited the bacterial growth substantially at 100 ug/ml compared to other metals after 24 h of incubation. The inhibition of bacterial growth exposed to Ni, Cd, and Cr observed under in vitro conditions corroborated the findings recorded by CLSM.

Metal detoxification strategies adopted by *A. chroococcum* CAZ3

Characterization of metal chelating EPS secreted by *A. chroococcum*

Exopolysachharides are polymeric substances synthesized by microorganisms as a protective material to overcome many stressful conditions (Gupta and Diwan 2017). Chief among them are the ionic stresses, antibiotics, heavy metals, and desiccation (Shameer 2016). Due to large surface area, EPS has been found to play tremendous roles in biosorption

Fig. 2 SEM micrographs of *A. chroococcum* CAZ3 grown in the presence of **a** 50 µg mL^{-1} Cd **b** 100 µg mL^{-1} Cr **c** 100 µg mL^{-1} Ni **d** Control. **e–g** represent the EDX spectra of *A. chroococcum* CAZ3 grown with 50 µg mL^{-1} Cd, 100 µg mL^{-1} Cr and 100 µg mL^{-1} Ni, respectively. **h** represents the EDX spectra of untreated control
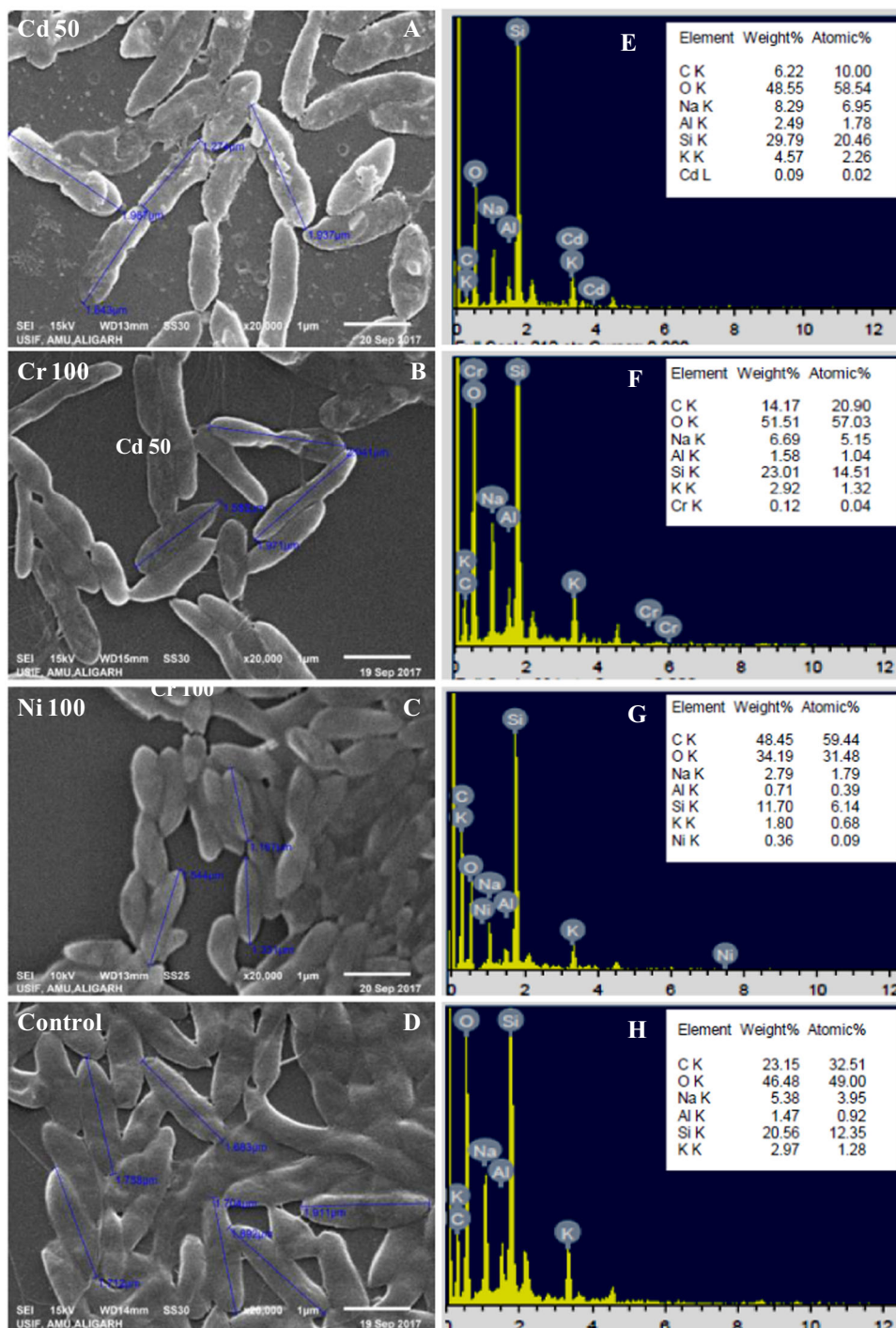
and removal of metals from the contaminated sites (Muthu et al. 2017). Considering this useful and applicable activity of microbes, the production of EPS by *A. chroococcum* CAZ3 while growing under heavy metal stress was assayed. Interestingly, it was observed that *A. chroococcum* CAZ3 survived and maintained the synthesis of EPS even in the presence of metal stress. The strain CAZ3 produced 260 µg mL^{-1} EPS in the presence of 25 µg mL^{-1} Cd which was increased by 23% (320 µg mL^{-1}) when Cd concentration was raised to 100 µg mL^{-1} (Fig. 4). Likewise, at 100 µg Cr mL^{-1}, the concentration of EPS was enhanced by 25% relative to those observed at 25 µg Cr mL^{-1}. Although, the secretion of EPS increased with increasing metal concentrations but Ni at 100 µg mL^{-1} caused a significant drop (77%) in EPS production over control (Fig. 4). In contrast, under conventional growth environment, *A. chroococcum*

CAZ3 secreted maximum amounts (580 µg mL^{-1}) of EPS. In a similar study, *Exiguobacterium* strains UE1 and UE4 under Cr stress have shown maximum release of EPS (Batool et al. 2017). In a follow up study, Abinaya et al. (2018) have confirmed the antioxidant potential, antibiofilm activity and other important biomedical applications of EPS released by *Bacillus licheniformis* Dahb$_1$.

After quantifying the EPS, the powder of EPS was analyzed under SEM. The EPS micrographs of metal treated bacterial cells showed certain polymeric structures while the EDX spectra of the EPS extracted from metal treated cells demonstrated the presence of Cd (Fig. 5a), Cr (Fig. 5b) and Ni (Fig. 5c). The presence of these metals in bacterial cells clearly validated the fact that EPS could chelate heavy metals when compared with control (Fig. 5d).

The EPS extracted from metal treated and untreated cells of *A. chroococcum* CAZ3 was further characterized using FT-IR and various functional groups involved in binding the heavy metal ions to bacterial cell surface were determined. The FT-IR data on metal loaded and unloaded EPS of *A. chroococcum* CAZ3 was recorded in the range of 1000–4000 cm^{-1}. Multiple peaks were obtained in the spectra of metal treated and untreated EPS which indicated diverse functional groups which in turn reflected a complex nature of the extracted EPS. It was interesting to note that some deviations in the peaks corresponding to various functional groups attached to the surface of EPS were recorded when the strain was grown in the presence of 100 µg mL^{-1} each of Cd, Cr and Ni (Fig. 6a). A broad peak appearing in the range of wave number 3281–3428 cm^{-1} could be assigned to N-H stretching of proteins and O-H stretching of hydroxyl groups. The changes in functional group moieties of EPS and shifting/disappearance of peaks due to binding of metal ions have been illustrated in Table 2. Interestingly, the FTIR spectrum of the metal loaded EPS revealed certain modifications. Shifting of peaks was observed at few regions of EPS extracted from Cd, Cr and Ni treated cells of *A. chroococcum* CAZ3 when compared with control. Similar disappearance/shifting of peaks of EPS extracted from numerous bacterial cells grown

Fig. 3 Propidium iodide (PI) staining of metal treated and untreated cells of *A. chroococcum* CAZ3: Number of dead cells increased with increase in metal concentrations. **a** Graphical representation depicting the effect of progressively increasing concentrations of Cd, Cr and Ni on the number of PI stained cells of *A. chroococcum* CAZ3. **b** Growth kinetics of *A. chroococcum* CAZ3 under varying doses of Cd, Cr and Ni compared with untreated control; Error bar shows the mean ± S.D. of three independent replicates
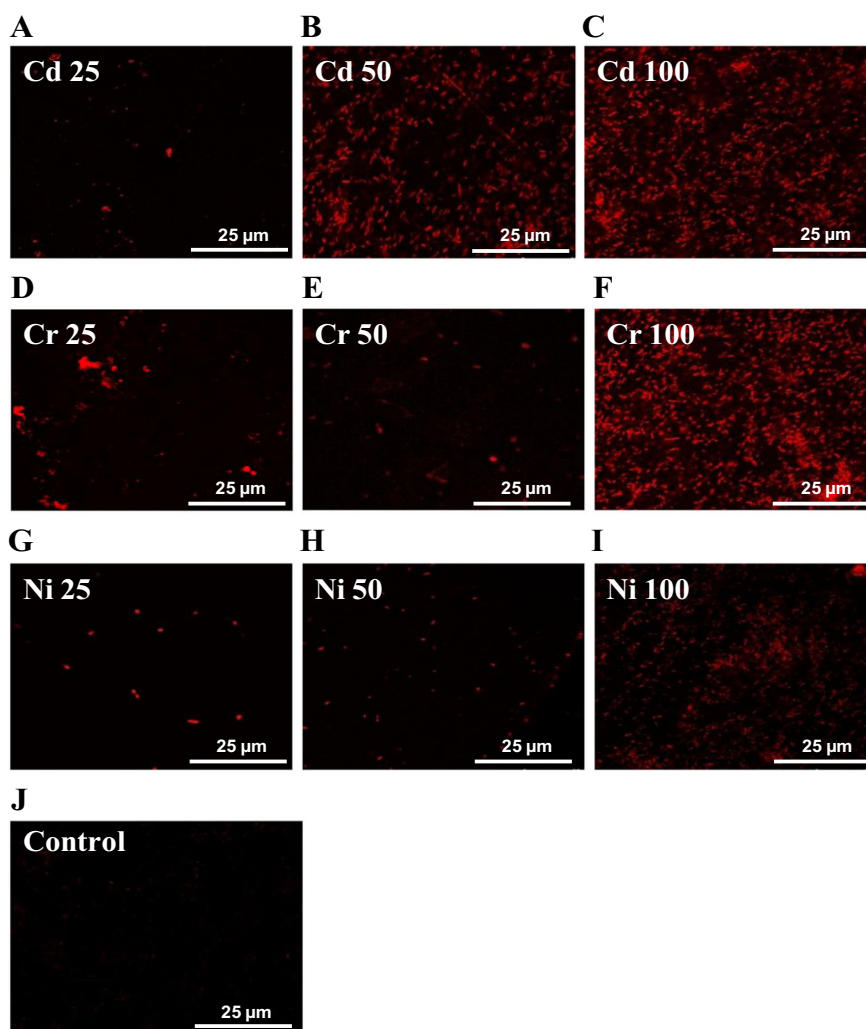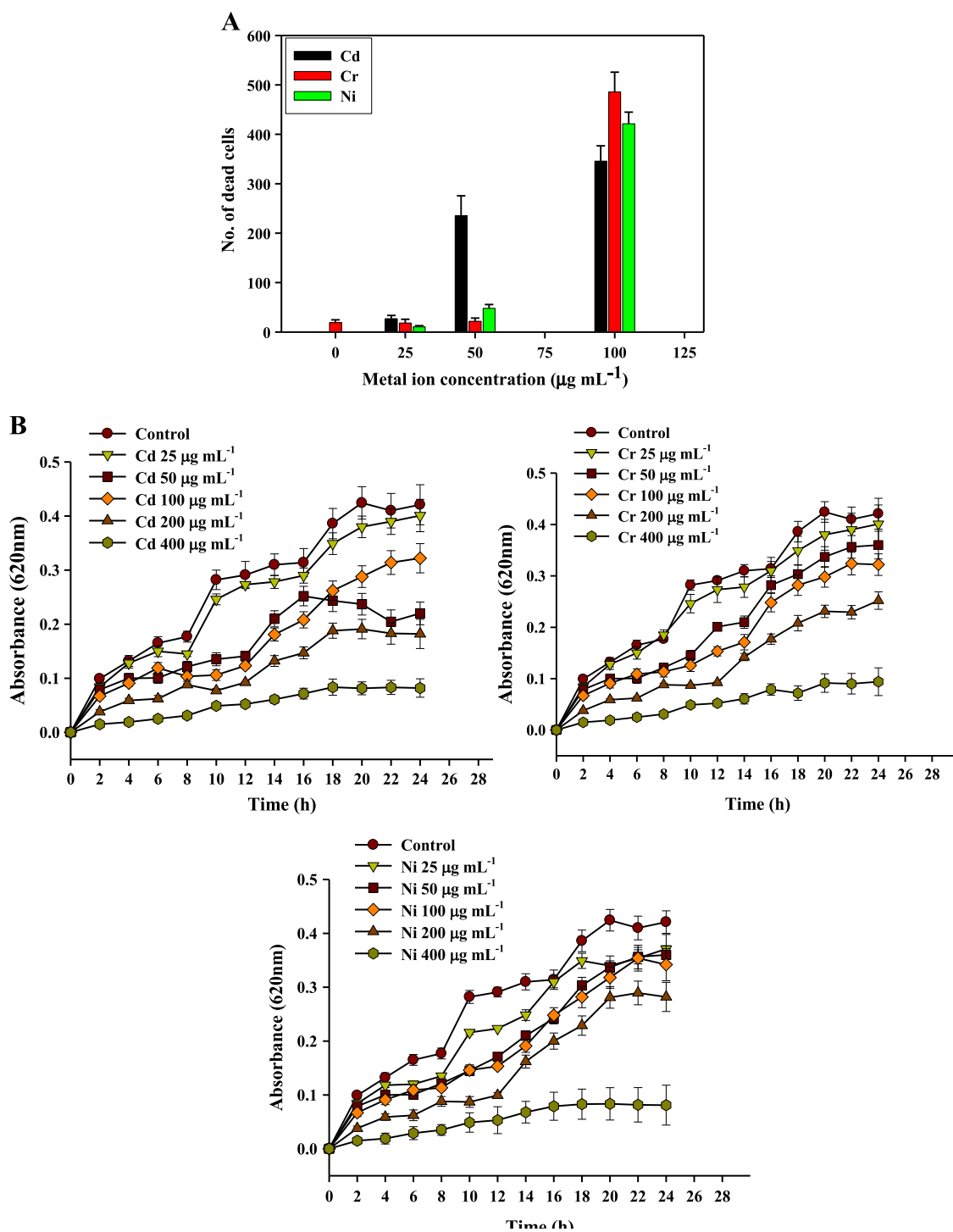
Fig. 3 (Continued)

under metal pressure have been reported (Seneviratne et al. 2016; Batool et al. 2017). The shifting in peaks as compared to control could possibly be due to the change in functional groups present on the surface of metal loaded EPS because of the interactions and binding of metal ions with the bacterial EPS. The production of EPS, thus illustrates a putative mechanism for the elimination of heavy metals from contaminated soils which could eventually serve as a cost effective and eco-friendly biological approach for environmental management.

Since the NMR spectroscopy has widely been used to reveal the structure of EPS (Liang and Wang 2015; Dahech et al. 2013), the EPS extracted from *A. chroococcum* CAZ3 in this study and characterized employing SEM, EDX and FTIR, was further analyzed by NMR in order to better understand the structure of EPS. The ^{1}H NMR spectrum of
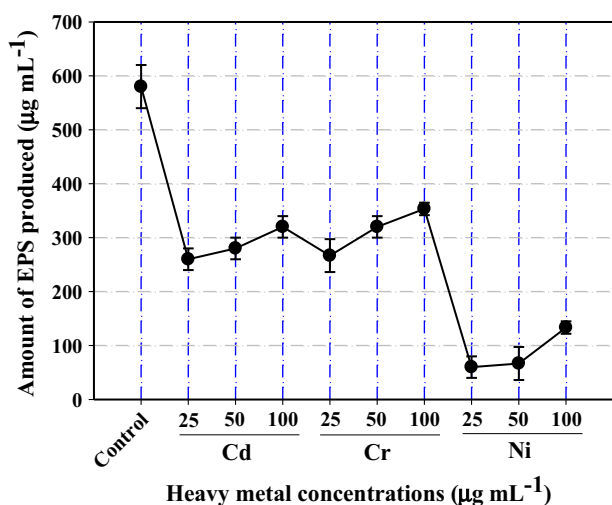
Fig. 4 Quantification of EPS secreted by *A. chroococcum* CAZ3 under control and metal stressed environments

EPS obtained from *A. chroococcum* CAZ3 revealed that the protons appeared at 9.497, 9.369, 9.242 and 4.717 ppm (Fig. 6b). The highest peak signal appearing at 6.438 ppm could represent the peak of solvent D_2O. The NMR peaks were however, poorly resolved due to viscosity/impurity of EPS. The specific characteristic functional groups representing the structure of polysachharides were not detected because the EPS obtained from *A. chroococcum* CAZ3 was not purified in this study. Similar chemical shifts and corresponding functional groups indicating glucan anomeric protons of ^{1}H NMR spectrum of EPS extracted from *P. aeruginosa* B01 has recently been reported (Benit and Roslin 2018).

Metal induced production of metallothionein by *A. chroococcum* CAZ3

Metallothioneins (MTs), a group of low molecular weight proteins (with molecular weight generally ranging from 3500–14000 Da) are rich in cysteine. The thiol group of MTs binds to heavy metals and makes it inaccessible to microbes (Enshaei et al. 2010). While doing this, MTs safeguard the bacterial cells from metal toxicity and oxidative stress that could otherwise have deleterious impact on their survival. Considering the importance of MTs in metal removal, *A. chroococcum* CAZ3 was tested to evaluate its potential to induce MTs production while growing in the presence of Cd (50 µg mL^{-1}), Cr (100 µg mL^{-1}) and Ni (100 µg mL^{-1}). The bacterial cell pellets believed to contain MTs was mixed with Ellman's reagent. For determination of MTs synthesis, the yellow colour so produced was quantified spectrophotometrically in terms of concentration of reduced sulfhydryl in the samples calculated from the standard curve of reduced glutathione. The amount

of MTs in the samples was calculated from the straight line equation (y = mx + c where 'm' is the slope and 'c' is the intercept) of GSH standard curve, wherein the MT content was further calculated by dividing the x-value by 20 since 1 mole of metallothionein is assumed to contain approx. 20 moles of cysteine residues. While comparing the synthesis of MTs both in the presence and absence of heavy metals, bacterial cells grown in metal treated medium showed maximum production of MTs suggesting the inducible role of metals (Table 3). For instance, the untreated cells showed lowest production of metallothioneins (0.4 µ mol) whereas the Cd, Cr and Ni treated cells released 3.6, 0.7 and 2.6 µ moles of MTs, respectively (Table 3). Among metals, Cd was found as the strongest inducible metal and hence, showed maximum production of MTs by *A. chroococcum* CAZ3 compared to Ni and Cr. The production of MTs apart from other active biomolecules under metal stress in general, could therefore, be a possible mechanism which aid the bacterial cells to survive even under metal pressure. In agreement to our findings, similar induction in MTs production by *Bacillus cereus* cells grown in the presence of increasing rates of Pb (Murthy et al. 2011) and *P. aeruginosa* and *P. putida* cells exposed to Cu and Cd have been reported (Enshaei et al. 2010).

The number of free thiol groups located within a MT protein represents the formation of metal-cysteine clusters responsible for bridging thiolates which eventually results in binding of more metal ions to even smaller number of cysteine residues. In particular, Cd^{2+} ions eventhough have fixed geometry but the bridging ligands generally modify the ratio of bound vs free thiols forming a varied cluster (Rubino 2015; Irvine and Stillman 2017). The metal bound MTs appear as protein chains wrapped around a cluster formed from thiol-metal binding with numerous cysteine residues holding the metal ions via thiolate groups. On the other hand, MTs generally cannot directly bind Cr ions but can eliminate the ROS (developed due to Cr toxicity) by means of cysteine residues, thereby providing protection to the cells from toxic Cr ions (Kimura et al. 2015). This was evident from our findings also where, minimum amounts of MTs were recorded in Cr treated cells as compared to Cd and Ni. Moreover, MT-1 and MT-2 molecules, in some cases, are known to bind chromate ions as well. Interestingly, the apo-MT molecule exhibits higher binding affinity to Cr ions and forms a stable complex once bound to the MT molecule (Dziegiel et al. 2016). In case of Ni, saturation binding of Ni^{2+} ions occurs at seventh position, resulting in the formation of M-7 MT. The binding is assisted by means of thiol groups via displacement of native Cd or Zn ions. Despite this, the exact stoichiometry of Ni binding to MTs is still not clearly understood. A hypothetical model representing the structure of metal bound MT is presented in Scheme 1 where Cd, Cr and Ni ions have been shown to

Fig. 5 SEM micrographs and EDX spectra of EPS secreted by *A. chroococcum* CAZ3 in the presence of **a** 50 µg mL^{-1} Cd **b** 100 µg mL^{-1} Cr **c** 100 µg mL^{-1} Ni and **d** Control. The EDX spectra represent the elemental composition of EPS extracted from metal treated and untreated cells
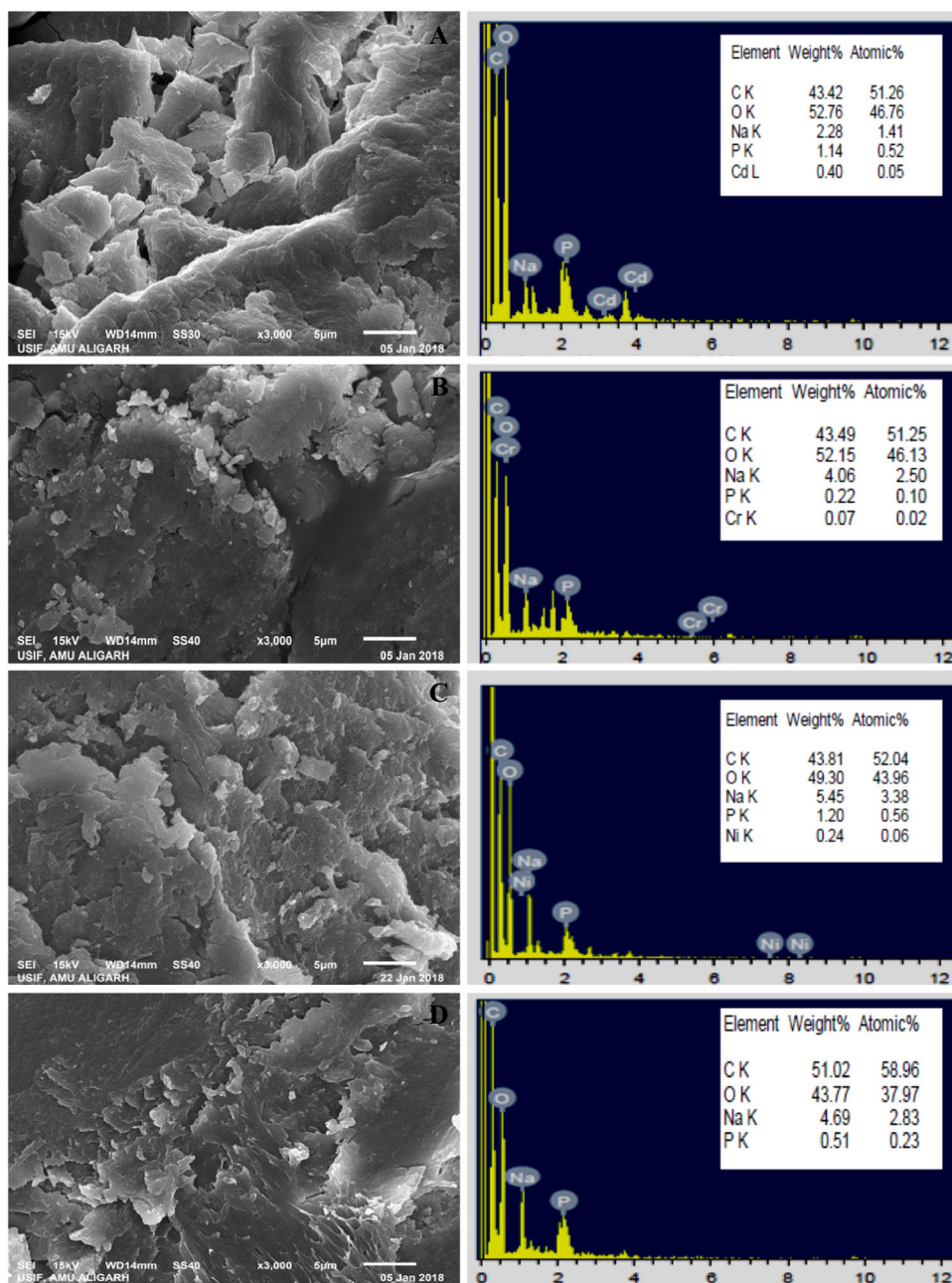
bind at specific sites on the protein chain, resulting in a cluster held together through thiol groups.

Due to their metal binding ability, the MTs could serve efficiently in the abatement of metal toxicity and hence, the bacteria possessing this potential could be exploited for removal of metals (bioremediation) from contaminated environment.

Melanin: importance in metal detoxification

Melanin, a dark brown to black coloured polymeric and amorphous substance, is synthesized by many prokaryotic organisms (Banerjee et al. 2014) including nitrogen fixing organisms (Maru and Gadre 2016). They are known to protect microbial cells from harmful effects of electromagnetic radiations, ROS, high temperatures and heavy metals (Drewnowska et al. 2015) by trapping environmental stressors for example, heavy metals (El-Naggar and El-Ewasy 2017). Taking this into consideration, the melanin synthesized by *A. chroococcum* CAZ3 was extracted and characterized. The SEM micrographs of melanin extracted from metal treated and untreated cells revealed that the pigment was an amorphous substance with no well defined structures. Moreover, the SEM images showed irregular

Fig. 6 a FT-IR spectra of metal loaded and unloaded EPS showing characteristic peaks corresponding to various functional groups involved in binding of heavy metal ions. b ^{1}H NMR spectrum of EPS extracted from the cells of *A. chroococcum* CAZ3
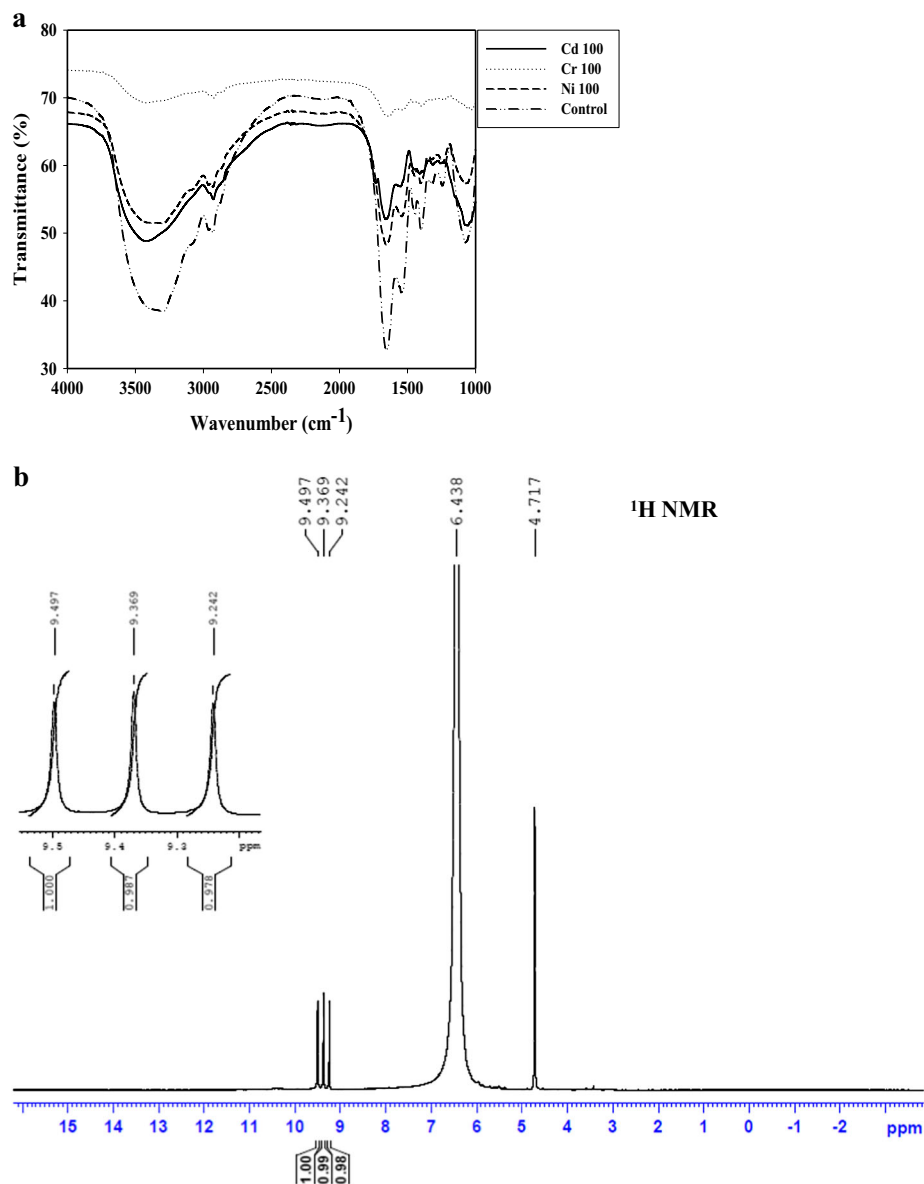
clumping of the aggregates (Fig. 7a–d). In earlier experiments, Tarangini and Mishra (2013) have also confirmed similar features of melanin extracted from *Pseudomonas* sp. The EDX analysis of melanin obtained from untreated cells of *A. chroococcum* CAZ3 revealed the presence of C, O, N and Na (Fig. 7d) as the key elements found in bacterial melanin. Also, the melanin extracted from strain CAZ3 grown with metals displayed the adsorption of Cd (Fig. 7a), Cr (Fig. 7b) and Ni (Fig. 7c) when visualized under EDX spectroscopy. Moreover, it was interesting to note that in addition to C, O, N and Na, the melanin obtained from metal treated *A. chroococcum* cells, also exhibited a fairly good percentage of Cd, Cr and Ni which clearly indicated the metal chelating/binding property of melanin.

The relationship between metal ion concentration and melanin production is however, still not clearly understood. Yet in avian species, it has been found that higher levels of metals like Zn, results in greater synthesis of melanin (Chatelain et al. 2014). This study therefore, has tried to establish a link between metal concentration and melanin based colouration in birds. Likewise, the melanin pigment formation within the feathers of birds influenced by accumulation of Cu has been reported (Edwards et al. 2016). In case of bacterial cells, there are few reports available suggesting the synthesis of melanin by bacteria under heavy metal stress as a protective mechanism and its role in metal ion chelation. However, how the increasing concentration of heavy metals enhances the secretion of melanin is yet to be established. Despite all these conflicting reports, the

Table 2 FT-IR assignments of metal loaded and unloaded EPS extracted from *Azotobacter chroococcum* CAZ3

Peaks in untreated control	Peaks in metal loaded EPS of A. chroococcum CAZ3 (Wave number cm⁻¹)			Assignments/stretching vibrations	Reference
	Cd	Cr	Ni		
3281–3428	3386–3459	3412–3501	3288–3453	N-H stretching of proteins and OH stretching of hydroxyl groups	Maquelin et al. (2002)
3072	–	2933	3078	Asymmetric C-H stretching of –CH₃ in fatty acids	Maquelin et al. (2002)
2933	2923	2856	2970	Asymmetric stretching of C-H of CH₂ group	Maquelin et al. (2002)
1656	1653	1643	1656	Stretching in amide I of β-pleated sheet	Maquelin et al. (2002)
1541	1545	1526	1535	Stretching in amide II group	Movasaghi et al. (2008)
1443	1459	–	1453	CH₂ scissoring mode of acyl chain of lipids	Fukuyama et al. (1999)
1396	1412	1399	1402	Symmetric stretching of C-O in carboxylic group	Lu et al. (2011)
1317	1310	–	1320	Amide III band components of proteins	Movasaghi et al. (2008); Maquelin et al. (2002)
1240	1256	1234	1240	P=O asymmetric stretching of PO₂	Maquelin et al. (2002)
1072	1056	1046	1065	P=O symmetric stretching of PO₂	Maquelin et al. (2002)

'–' indicates disappearance of peaks with respect to control

Table 3 Metallothionein content in untreated and metal treated cells of *A. chroococcum* CAZ3

Treatment	x-value from curve equation	MT content (μ mol) [x-value/20]
Control	7.2[c] ± 0.7	0.4[c] ± 0.03
Cd 50 μg mL⁻¹	71.6[a] ± 6.5	3.6[a] ± 0.6
Cr 100 μg mL⁻¹	12.9[c] ± 2.7	0.7[c] ± 0.1
Ni 100 μg mL⁻¹	51.2[b] ± 4.3	2.6[b] ± 0.2
LSD	8.7	0.4
F-value (treatment)	150.7	150.7

Values are mean of three independent replicates; ± indicates standard deviation. Values denoted with different letters are significantly different at $P \leq 0.05$
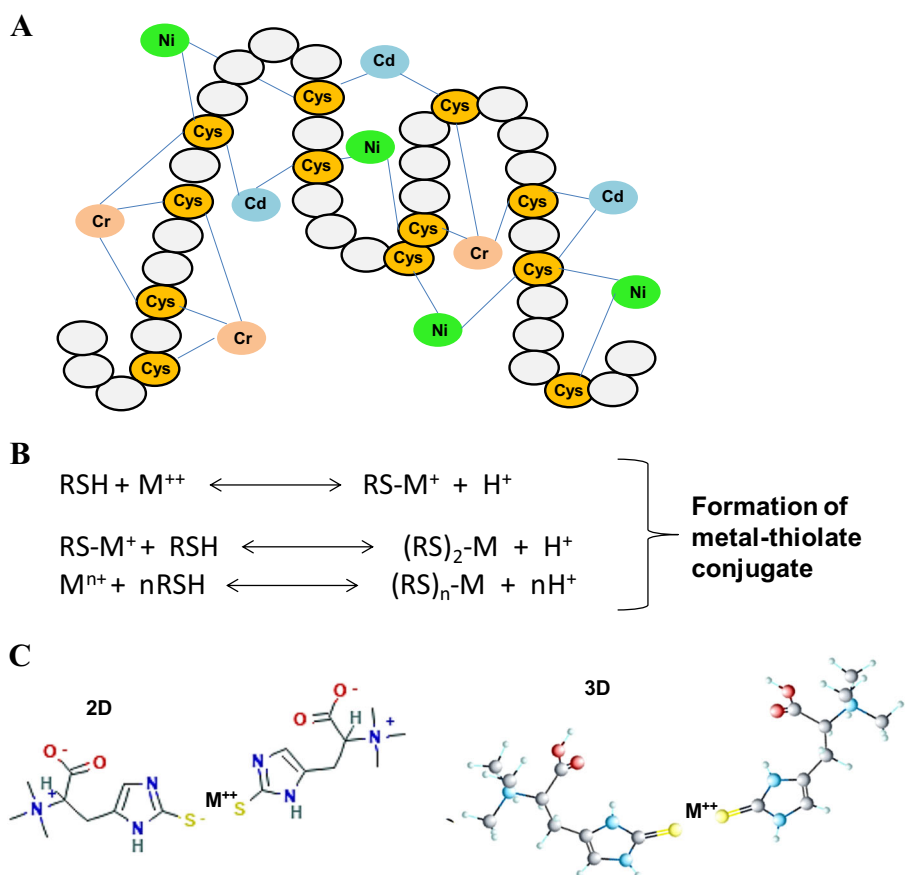
melanin synthesized by *A. chroococcum* CAZ3 in our study was induced largely by highest amounts of Cd, which was followed by Cr and Ni. The release of melanin under heavy metal influence thus suggests that melanin had higher affinity for Cd^{2+} ions as compared to Cr and Ni ions. And hence, greater amounts of melanin were released under Cd pressure.

Also, the peaks of various functional groups obtained in the FT-IR analysis of melanin extracted from metal treated and untreated (control) cells of *A. chroococcum* CAZ3 was investigated in the range of 1000–4000 cm⁻¹ (Fig. 8).

The IR spectra of melanin recovered from metal treated cells exhibited significant deviations corresponding to several functional groups when compared with the peaks in untreated control (Table 4).

In case of melanin obtained from Cd treated cells, a disappearance of peaks at 1399 and 1170 cm⁻¹ was observed in IR spectra. Also, peak disappearance at 1170 cm⁻¹ was also recorded in the presence of Ni. Peak disappearance is indicative of disturbances in the functional groups in the presence of heavy metals. In this regard, a study revealed the stretching vibrations and bending in NH group, C=C stretching and CH₂–CH₃ bending which are characteristic of melanin (Apte et al. 2013). In our studies, a broad peak in the range of 3291–3431 cm⁻¹ was also observed in the presence of Cr. In another similar study, several peaks were detected in the range of 650–1750 cm⁻¹ in *P. aeruginosa* cells corresponding to alterations in carbohydrates, lipids and proteins of the bacterial cells. In addition to this, some other peaks representing hydroxyl groups were also recorded in the synchronous and asynchronous spectra (Durve and Chandra 2014). Overall, the metal chelating property of melanin could play an important role in detoxifying heavy metals from metal enriched soils. And hence, the melanin producing *A. chroococcum* CAZ3 could be a superb choice for removing metals and thus successfully remediating the soils contaminated with various heavy metals.

A

B

$$RSH + M^{++} \longleftrightarrow RS\text{-}M^+ + H^+$$

$$RS\text{-}M^+ + RSH \longleftrightarrow (RS)_2\text{-}M + H^+$$

$$M^{n+} + nRSH \longleftrightarrow (RS)_n\text{-}M + nH^+$$

**Formation of
metal-thiolate
conjugate**

C

2D

3D

Mechanistic basis of melanic plumage/coat coloration

The melanin pigment in general, play important roles in
protection against UV radiations, chemical stresses, ther-
moregulation and camouflage (Côte et al. 2018). Melanin
synthesis is a complex process which involves a series of
reactions catalyzed by various enzyme complexes. Pri-
marily, the process of melanogenesis begins with the oxi-
dation of L-tyrosine catalyzed by tyrosinase (a key regulator
of melanin synthesis) which is encoded by the TYR locus
and is highly conserved among various species. This
enzyme has two main components- (a) tyrosine hydroxylase
and (b) dopa oxidase. Both of these components work in
unison to synthesize L-dopaquinone. This o-quinone so
generated, plays a pivotal role in animal melanogenesis
(Solano 2014) resulting in the formation of either eumelanin
or pheomelanin. The compound L-dopaquinone is highly
reactive and during the synthesis of melanin, reacts with
multiple functional groups including amino, thiol and
hydroxy groups. A well known pathway for melanogenesis
known as the Raper–Mason pathway, involves an intra-
molecular cyclization of L-dopaquinone which gets con-
verted into L-leukodopachrome (L-cyclodopa) through a
nucleophilic reaction where the side chain amino group on
position 6 of the aromatic ring is attacked. This reaction

depends mainly on pH. Similarly, the synthesis of eume-
lanin is also influenced by the pH which however, is
inhibited under acidic environment (Galván and Solano
2016). Post eumelanogenic pathway, L-leukodopachrome
behaves as an unstable indoline exhibiting strong properties
of reduction. It then undergoes a spontaneous redox reaction
along with the precursor L-dopaquinone. The second most
pivotal intermediate formed during melanogenesis, L-
dopachrome, plays a crucial role in eumelanogenesis path-
way (Nasti and Timares 2015). Conclusively, during mel-
anin synthesis, L-Tyrosine serves as the precursor, L-
dopachrome enhances the tyrosinase activity and L-tyrosine
induces the synthesis of melanosome. The product, dopa, so
released, is known to up regulate melanin synthesis and
melanocytes regulate the local and global homeostasis of
melanogenic systems (D'Mello et al. 2016). Once secreted
endogenously, the melanin pigments provide coat coloura-
tion to mammals and birds. For instance, eumelanin imparts
black and grey coat colour while pheomelanin imparts
brown and red coat colour. For example, coat colour in
animals is determined mainly by the ratio of eumelanin and
pheomelanin along the hair and feathers and its spatial
distribution across the body (Lin et al. 2013a).

The production of a specific type of melanin is regulated
by melanocortin-1 receptor (MC1R) which is present within

Fig. 7 SEM micrographs and EDX spectra of melanin pigment isolated from the cells of *A. chroococcum* CAZ3 in the presence of **a** 50 µg mL^{-1} Cd **b** 100 µg mL^{-1} Cr **c** 100 µg mL^{-1} Ni and **d** Control. The EDX spectra displays the key elements present in melanin along with heavy metal ions trapped inside the melanin extracted from *A. chroococcum* CAZ3
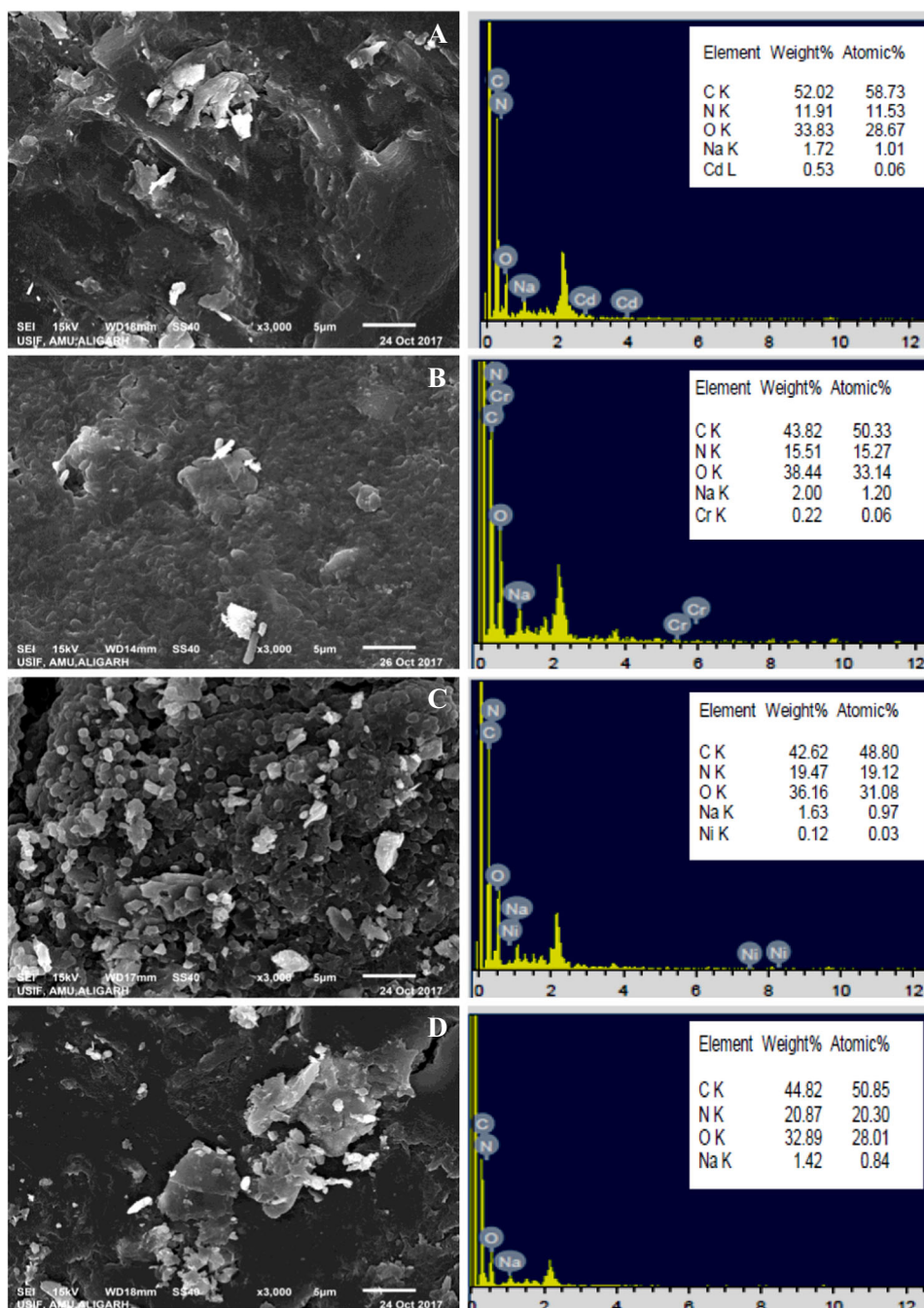

melanocytes and its two ligands- (i) α-melanocyte stimulating hormone (α-MSH) which is secreted from the pituitary glands and (ii) the agouti signalling protein (ASIP) (Chen et al. 2015). The melanocytes produce dark eumelanin when they are activated by α-MSH. The MC1R activity is inhibited when ASIP ligand binds and results in the synthesis of pheomelanin. On the contrary, in many species of birds and mammals, white coat colour is due to the absence of pigment in hair or feathers (Zimova et al. 2018). In case of avian species, the anagen stage of hair/feather growth represents melanogenesis and is restricted within the melanocytes (pigment-producing cells). Melanins get transferred to keratinized cells i.e., keratinocytes of the developing hair or feathers in birds through melanosomes (Chen et al. 2015). Broadly, the entire phenomenon of melanic plumage/coat coloration is genetically controlled involving a series of reactions along with the formation of intermediates and products responsible for melanin synthesis.

Finally, based on the findings observed in this study, the metal-microbes interaction and the roles of EPS, MTs and melanin secreted by metal tolerant *A. chroococcum*
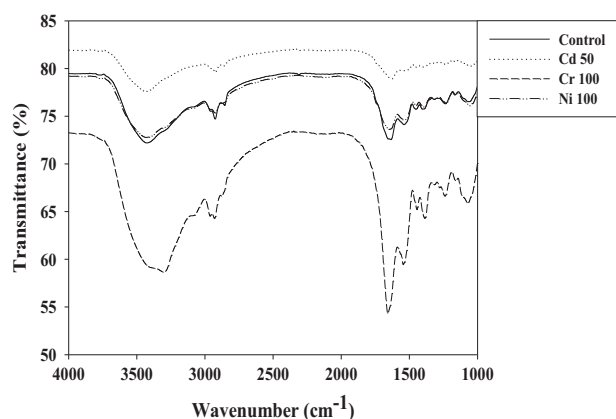
Fig. 8 FT-IR spectra of melanin pigment derived from metal treated and untreated cells of *A. chroococcum* CAZ3 exhibiting characteristic peaks which corresponds to vibrations/ disturbances in various functional groups that participate in metal binding

CAZ3 in metal removal/detoxification is summarized in Fig. 9.

Conclusion

The deleterious impact of heavy metals on *A. chroococcum* strain CAZ3 observed under in vitro studies was further confirmed by microscopic observation under SEM and CLSM. Moreover, despite the toxicity shown by heavy metals, some percentage of metals was found inside the bacterial cells as revealed by EDX. This accumulation of heavy metals confirmed that the bacterial strain could thrive well even under harsh metal stress. The EPS secreted by *A. chroococcum* was found to accumulate significant amounts of Cd, Cr and Ni which was revealed by EDX. The release of EPS by *A. chroococcum* induced by heavy metals and its ability to chelate metals could be a defense strategy adopted by the bacterial strain to protect itself from metal toxicity and consequently to survive, proliferate and perform normal physiological activities in stressed environments. This novel and fascinating trait of *A. chroococcum* is likely to provide new insight in this research area of inexpensive bioremediation technology. The secretion of MTs by viable cells under the influence of heavy metals was yet another interesting feature of *A. chroococcum* CAZ3 which after binding, greatly diminished the metal toxicity. This finding thus, established the protective role of MTs secreted by *A. chroococcum* CAZ3 while growing under metal stressed environment. Among all the test metals, Cd was found as the most efficient inducer of MTs in *A. chroococcum* cells. Also, *A. chroococcum* synthesized a brown pigment melanin which very effectively trapped the heavy metals and hence, reduced its availability and toxicity. The entrapment of heavy metal ions by melanin was visible under EDX

Table 4 FT-IR assignments of melanin extracted from metal treated and untreated cells of *Azotobacter chroococcum* CAZ3

Peaks in untreated control	Peaks in metal treated melanin of *A. chroococcum* CAZ3 (Wave number cm^{-1})			Assignments/stretching vibrations	Reference
	Cd	Cr	Ni		
3425	3425	3291–3431	3431	O–H stretching of hydroxyl groups	Maquelin et al. (2002)
2929	2923	2923	2923	Stretching vibrations of CH$_2$ and CH$_3$ of phospholipids, cholesterol	Huleihel et al. (2002)
2847	2853	2859	2840	Alkyl and CHO group	Oves et al. 2013
1646	1627	1665	1640	Alkenes	Seneviratne et al. (2016)
1532	1520	1545	1538	COO$^-$ of carboxylate groups	Oves et al. (2013)
1456	1405	1443	1449	Amide II	Movasaghi et al. (2008)
1399	–	1380	1393	Symmetric stretching of C–O of carboxylic groups	Lu et al. (2011)
1240	1246	1234	1246	Amino groups	Seneviratne et al. (2016)
1170	–	1164	–	C–O and C–C stretching, C–O–H and C–O–C deformation of carbohydrates	Movasaghi et al. 2008; Maquelin et al. (2002)
1062	1068	1068	1049	C–O or SO and PO stretching vibrations	Aravindhan et al. (2004); Loukidou et al. (2004)

'–' indicates disappearance of peaks with respect to control
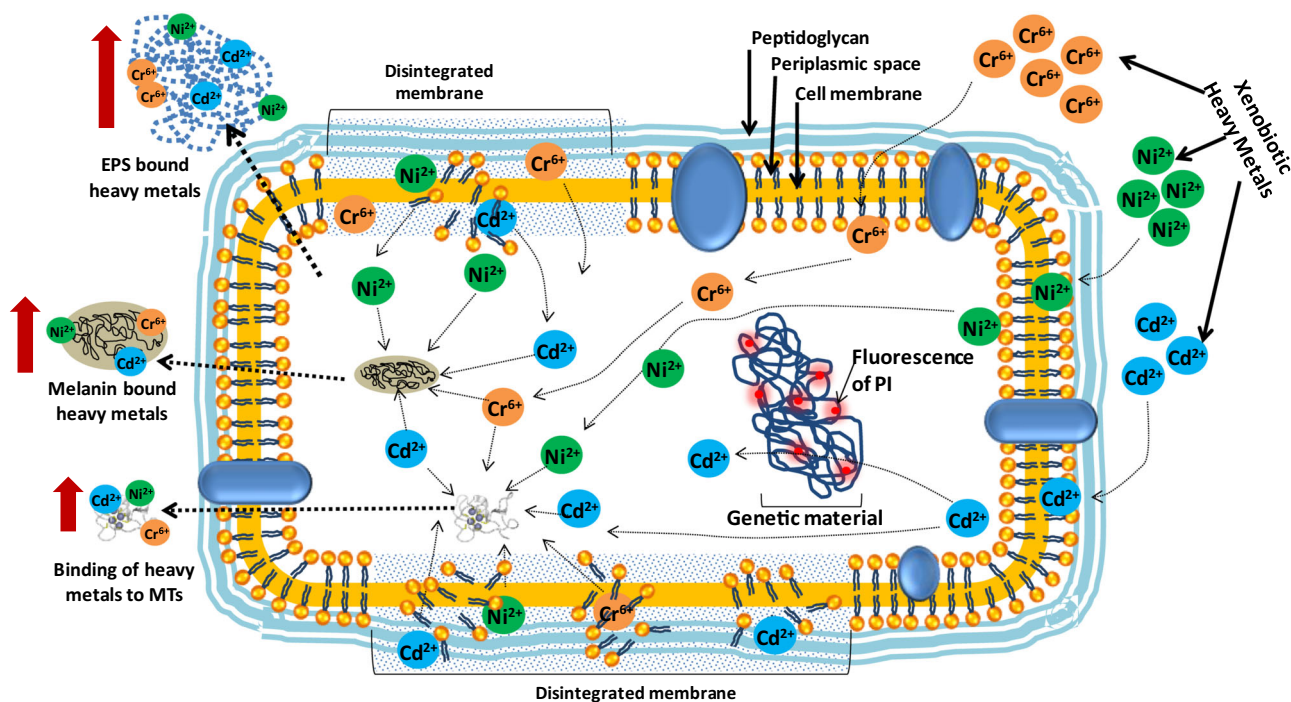
Fig. 9 The schematic representation elucidating the interaction of bacterial cell with the heavy metal ions and their removal/detoxification by active biomolecules (EPS, MTs and melanin) secreted by *A. chroococcum* CAZ3 under metal stress

which further strengthened the fact that bacterial melanin can play significant role in heavy metal removal/detoxification. This melanin related finding is likely to help readers to better understand the metal removal strategy (bioremediation) and to apply melanin producing bacterial strains under contaminated soils. Overall, the microbial management confirmed the potential of nitrogen fixing *A. chroococcum* CAZ3 to detoxify the contaminated environment through secretion of EPS, MTs and melanin. These metal detoxifying properties together with conventional nitrogen fixing ability makes *A. chroococcum* CAZ3 an exciting and most appropriate candidate for heavy metal removal from contaminated soils. Broadly, the application of *A. chroococcum* could be a cost effective and environmentally viable approach in metal cleanup program vis-a-vis, the management of polluted environment.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

Abinaya M, Vaseeharan B, Divya M, Vijayakumar S, Govindarajan M, Alharbi NS, Khaled JM, Al-anbr MN, Benelli G (2018) Structural characterization of *Bacillus licheniformis* Dahb1 exopolysaccharide-antimicrobial potential and larvicidal activity on malaria and Zika virus mosquito vectors. Environ Sci Pollut Res 25:18604–18619

Ackerman CM, Lee S, Chang CJ (2016) Analytical methods for imaging metals in biology: from transition metal metabolism to transition metal signaling. Anal Chem 89:22–41

Adam V, Chudobova D, Tmejova K, Cihalova K, Krizkova S, Guran R, Kominkova M, Zurek M, Kremplova M, Jimenez AM, Konecna M (2014) An effect of cadmium and lead ions on *Escherichia coli* with the cloned gene for metallothionein (MT-3) revealed by electrochemistry. Electrochim Act 140:11–19

Alnuaimi MM, Saeed IA, Ashraf SS (2012) Effect of various heavy metals on the enzymatic activity of *E. coli* alkaline phosphatase. Int J Biotechnol Biochem 8:47–59

Ana RL, Garcia-Vazquez E (2006) A simple assay to quantify metallothionein helps to learn about bioindicators and environmental health. Biochem Mol Biol Edu 34:360–363

Apte M, Girme G, Bankar A, RaviKumar A, Zinjarde S (2013) 3, 4-dihydroxy-L-phenylalanine-derived melanin from *Yarrowia*

lipolytica mediates the synthesis of silver and gold nanostructures. J Nanobiotechnol 11:2

Aravindhan R, Madhan B, Raghava Rao J, Unni Nair B, Ramasami T (2004) Bioaccumulation of chromium from the tannery waste water: an approach for chrome recovery and reuse. Environ Sci Technol 38:300–306

Ates O (2015) Systems biology of microbial exopolysaccharides production. Front Bioengg. Biotechnol 3:200

Ayangbenro AS, Babalola OO (2017) A new strategy for heavy metal polluted environments: a review of microbial biosorbents. J Environ Res Public Health 14:94

Banerjee A, Supakar S, Banerjee R (2014) Melanin from the nitrogen-fixing bacterium *Azotobacter chroococcum*: a spectroscopic characterization. PLoS ONE 9(1):e84574

Batool R, Marghoob U, Kalsoom A (2017) Estimation of exopolysaccharides (EPS) producing ability of Cr (VI) resistant bacterial strains from tannery effluent. J Basic Appl Sci 13:589–596

Benit N, Roslin AS (2018) Isolation and characterization of larvicidal extracellular polysaccharide (EPS) from *Pseudomonas aeruginosa* B01. Int J Curr Microbiol Appl Sci 7:109–120

Bhagat N, Vermani M, Bajwa HS (2016) Characterization of heavy metal (cadmium and nickel) tolerant Gram negative enteric bacteria from polluted Yamuna River, Delhi. Afr J Microbiol Res 10:127–137

Chatelain M, Gasparini J, Jacquin L, Frantz A (2014) The adaptive function of melanin-based plumage coloration to trace metals. Biol Lett 10:20140164

Chaturvedi AD, Pal D, Penta S, Kumar A (2015) Ecotoxic heavy metals transformation by bacteria and fungi in aquatic ecosystem. World J Microbiol Biotechnol 31:1595–1603

Chen Y, Chao Y, Li Y, Lin Q, Bai J, Tang L, Qiu R (2016) Survival strategies of the plant-associated bacterium *Enterobacter* sp. strain EG16 under cadmium stress. Appl Environ Microbiol 82:1734–1744

Chen CF, Foley J, Tang PC, Li A, Jiang TX, Wu P, Widelitz RB, Chuong CM (2015) Development, regeneration, and evolution of feathers. Annu Rev Anim Biosci 3:169–195

Cordero RJ, Vij R, Casadevall A (2017) Microbial melanins for radioprotection and bioremediation. Microb Biotechnol 10:1186–1190

Côte J, Boniface A, Blanchet S, Hendry AP, Gasparini J, Jacquin L (2018) Melanin-based coloration and host–parasite interactions under global change. Proc R Soc B 285(1879):20180285

Dahech I, Fakhfakh J, Damak M, Belghith H, Mejdoub H, Belghith KS (2013) Structural determination and NMR characterization of a bacterial exopolysaccharide. Int J Biol Macromol 2013 59:417–422

Das KK, Reddy RC, Bagoji IB, Das S, Bagali S, Mullur L, Khodnapur JP, Biradar MS (2018) Primary concept of nickel toxicity–an overview. J Basic Clinic Physiol Pharmacol https://doi.org/10.1515/jbcpp-2017-0171

De Guzman MLC, Arcega KSG, Cabigao JMNR, Su GLS (2016) Isolation and identification of heavy metal-tolerant bacteria from an industrial site as a possible source for bioremediation of cadmium, lead, and nickel. AdvEnviron Biol 10:10–16

Diopan V, Shestivska V, Adam V, Macek T, Mackova M, Havel L, Kizek R (2008) Determination of content of metallothionein and low molecular mass stress peptides in transgenic tobacco plants. Plant Cell, Tissue Org Cult 94:291–298

D'Mello SA, Finlay GJ, Baguley BC, Askarian-Amiri ME (2016) Signaling pathways in melanogenesis. Int J Mol Sci 17:1144

Drewnowska JM, Zambrzycka M, Kalska-Szostko B, Fiedoruk K, Swiecicka I (2015) Melanin-like pigment synthesis by soil Bacillus weihenstephanensis isolates from Northeastern Poland. PLoS ONE 10:e0125428

Durve A, Chandra N (2014) FT-IR analysis of bacterial biomass in response to heavy metal stress. Int J Biotechnol Photon 112:386–391

Dziegiel P, Pula B, Kobierzycki C, Stasiolek M, Podhorska-Okolow M (2016) Metallothioneins: structure andfunctions. In: Sutovsky P, Clascá F, Kmiec Z, Korf HW, Singh B, Timmermans JP, Schmeisser MJ (Eds) Metallothioneins in Normal and Cancer Cells, Advances in Anatomy, Embryology and Cell Biology pp 3–20. Springer, Switzerland

Edwards NP, vanVeelen A, Anné J, Manning PL, Bergmann U, Sellers WI, Wogelius RA (2016) Elemental characterisation of melanin in feathers via synchrotron X-ray imaging and absorption spectroscopy. Sci Rep 6:34002

El-Naggar NE, El-Ewasy SM (2017) Bioproduction, characterization, anticancer and antioxidant activities of extracellular melanin pigment produced by newly isolated microbial cell factories *Streptomyces glaucescens* NEAE-H. Sci Rep 7:42129

Enshaei M, Khanafari A, Sepahey AA (2010) Metallothionein induction in two species of *Pseudomonas* exposed to cadmium and copper contamination. Iran J Environ Health Sci Engg 7:287

Fashola MO, Ngole-Jeme VM, Babalola OO (2016) Heavy metal pollution from gold mines: Environmental effects and bacterial strategies for resistance. Int J Environ Res Public Health 13:1047

Francisco R, Moreno A, Morais PV (2010) Different physiological responses to chromate and dichromate in the chromium resistant and reducing strain *Ochrobactrum tritici* 5bvl1. Biometals 23:713–725

François F, Lombard C, Guigner JM, Soreau P, Brian-Jaisson F, Martino G, Vandervennet M, Garcia D, Molinier AL, Pignol D, Peduzzi J (2012) Isolation and characterization of environmental bacteria capable of extracellular biosorption of mercury. Appl Environ Microbiol 78:1097–1106

Fukuyama Y, Yoshida S, Yanagisawa S, Shimizu M (1999) A study on the differences between oral squamous cell carcinomas and normal oral mucosas measured by Fourier transform infrared spectroscopy. Biospectroscopy 5:117–126

Galván I, Solano F (2016) Bird integumentary melanins: biosynthesis, forms, function and evolution. Int J Mol Sci 17:520

Girisha ST (2014) Lead Bioremediation with respect to mining and industrial effluents. Int Res J Environ Sci 3:58–61

Gobi N, Vaseeharan B, Rekha R, Vijayakumar S, Faggio C (2018) Bioaccumulation, cytotoxicity and oxidative stress of the acute exposure of selenium in *Oreochromis mossambicus*. Ecotoxicol Environ Saf 162:147–159

Golding CG, Lamboo LL, Beniac DR, Booth TF (2016) The scanning electron microscope in microbiology and diagnosis of infectious disease. Sci Rep 6:26516

Gomes LC, Mergulhão FJ (2017) SEM analysis of surface impact on biofilm antibiotic treatment. Scanning https://doi.org/10.1155/2017/2960194

Gupta P, Diwan B (2017) Bacterial exopolysaccharide mediated heavy metal removal: A review on biosynthesis, mechanism and remediation strategies. Biotechnol Rep 13:58–71

Haferburg G, Kothe E (2010) Metallomics: lessons for metalliferous soil remediation. Appl Microbiol Biotechnol 87:1271–1280

Hao L, Li J, Kappler A, Obst M (2013) Mapping of heavy metal ion sorption to cell-extracellular polymeric substance-mineral aggregates by using metal-selective fluorescent probes and confocal laser scanning microscopy. Appl Environ Microbiol 79:6524–6534

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Gram negative aerobic/microaerophilic rodsand cocci. In: Holt JG (Ed) Bergey's Manual of Determinative Bacteriology. 9th edn. Williams & Wilkins, Lippincott, pp 93–168

Hu WL, Dai DH, Huang GR, Zhang ZD (2015) Isolation and characterization of extracellular melanin produced by *Chroogomphus rutilus* D447. Am J Food Technol 10:68–77

Huleihel M, Salman A, Erukhimovich V, Ramesh J, Hammody Z, Mordechai S (2002) Novel optical method for study of viral carcinogenesis in vitro. J Biochem Biophys Methods 50:111–121

Ianeva OD (2009) Mechanisms of bacteria resistance to heavy metals. Mikrobiolohichnyi Zh (Kiev, Ukr: 1993) 71:54–65

Irvine GW, Stillman MJ (2017) Residue modification and mass spectrometry for the investigation of structural and metalation properties of metallothionein and cysteine-rich proteins. Int J Mol Sci 18:913

Kimura T, Onodera A, Okumura F, Nakanishi T, Itoh N (2015) Chromium (VI)-induced transformation is enhanced by Zn deficiency in BALB/c 3T3 cells. J Toxicol Sci 40:383–387

Lin SJ, Foley J, Jiang TX, Yeh CY, Wu P, Foley A, Yen CM, Huang YC, Cheng HC, Chen CF, Reeder B (2013a) Topology of feather melanocyte progenitor niche allows complex pigment patterns to emerge. Science 340:1442–1445

Liang TW, Wang SL (2015) Recent advances in exopolysaccharides from *Paenibacillus* spp.: production, isolation, structure, and bioactivities. Mar Drugs 13:1847–1863

Loukidou MX, Zouboulis AI, Karapantsios TD, Matis KA (2004) Equilibrium and kinetic modeling of chromium (VI) biosorption by *Aeromonas caviae*. Colloid Surf A Physicochem Eng Asp 242:93–104

Lu X, Wang J, Al-Qadiri HM, Ross CF, Powers JR, Tang J, Rasco BA (2011) Determination of total phenolic content and antioxidant capacity of onion (*Allium cepa*) and shallot (*Allium oschaninii*) using infrared spectroscopy. Food Chem 12:637–644

Ma Y, Oliveira RS, Freitas H, Zhang C (2016) Biochemical and molecular mechanisms of plant-microbe-metal interactions: Relevance for phytoremediation. Front Plant Sci 7:918

Maru V, Gadre S (2016) Melanin pigment production studies from *Azotobacter vinelandii*. Int J Adv Lif Sci 9:44–49

Marzan LW, Hossain M, Mina SA, Akter Y, Chowdhury AMA (2017) Isolation and biochemical characterization of heavy-metal resistant bacteria from tannery effluent in Chittagong city, Bangladesh: Bioremediation viewpoint. Egypt J Aquat Res 43:65–74

Maquelin K, Kirschner C, Choo-Smith LP, van den Braak N, Endtz HP, Naumann D, Puppels GJ (2002) Identification of medically relevant microorganisms by vibrational spectroscopy. J Microb Methods 51:255–271

Mishra A, Mishra KP (2015) Bacterial response as determinant of oxidative stress by heavy metals and antibiotic. J Innov Pharm Biol Sci 2:229–239

Mody BR, Bindra MO, Modi VV (1989) Extracellular polysaccharides of cowpea rhizobia: compositional and functional studies. Arch Microbiol 1:2–5

Moghannem SA, Refaat BM, El-Sherbinya GM, El-Sayeda MH, Elsehemyb IA, Kalaba MH (2015) Characterization of heavy metal and antibiotic-resistant bacteria isolated from polluted localities in Egypt. Egypt Pharm J 14:158–165

Movasaghi Z, Rehman S, Rehman DI (2008) Fourier transform infrared (FTIR) spectroscopy of biological tissues. Appl Spectros Rev 43:134–179

Murthy S, Bali G, Sarangi SK (2011) Effect of lead on metallothionein concentration in lead resistant bacteria *Bacillus cereus* isolated from industrial effluent. Afr J Biotechnol 10:15966–15972

Muthu M, Wu HF, Gopal J, Sivanesan I, Chun S (2017) Exploiting microbial polysaccharides for biosorption of trace elements in aqueous environments-scope for expansion via nanomaterial intervention. Polymers 9:721

Nasti TH, Timares L (2015) MC 1R, Eumelanin and Pheomelanin: Their role in determining the susceptibility to skin cancer. Photochem Photobiol 91:188–200

Nocelli N, Bogino PC, Banchio E, Giordano W (2016) Roles of extracellular polysaccharides and biofilm formation in heavy metal resistance of rhizobia. Materials 9:418

Ojuederie OB, Babalola OO (2017) Microbial and plant-assisted bioremediation of heavy metal polluted environments: A review. Int J Environ Res Public Health 14:1504

Oliveira H (2012) Chromium as an environmental pollutant: insights on induced plant toxicity. J Botany https://doi.org/10.1155/2012/375843

Oves M, Khan MS, Zaidi A (2013) Biosorption of heavy metals by *Bacillus thuringiensis* strain OSM29 originating from industrial effluent contaminated north Indian soil. Saudi J Biol Sci 20:121–129

Rahimzadeh MR, Rahimzadeh MR, Kazemi S, Moghadamnia AA (2017) Cadmium toxicity and treatment: an update. Casp J Int Med 8:135

Ramya D, Thatheyus AJ (2018) Microscopic investigations on the biosorption of heavy metals by bacterial cells: a review. Sci Int 6:11–17

Rao N, Prabhu M, Xiao M, Li WJ (2017) Fungal and bacterial pigments: Secondary metabolites with wide applications. Front Microbiol 8:1113

Rizvi A, Khan MS (2018) Heavy metal induced oxidative damage and root morphology alterations of maize (*Zea mays* L.) plants and stress mitigation by metal tolerant nitrogen fixing *Azotobacter chroococcum*. Ecotoxicol Environ Saf 157:9–20

Rizvi A, Khan MS (2017) Cellular damage, plant growth promoting activity and chromium reducing ability of metal tolerant *Pseudomonas aeruginosa* CPSB1 recovered from metal polluted chilli (*Capsicum annuum*) rhizosphere. Acta Sci Agric 1:36–46

Rubino FM (2015) Toxicity of glutathione-binding metals: a review of targets and mechanisms. Toxics 3:20–62

Samuel J, Paul ML, Ravishankar H, Mathur A, Saha DP, Natarajan C, Mukherjee A (2013) The differential stress response of adapted chromite mine isolates *Bacillus subtilis* and *Escherichia coli* and its impact on bioremediation potential. Biodegradation 24:829–842

Seneviratne M, Gunaratne S, Bandara T, Weerasundara L, Rajakaruna N, Seneviratne G, Vithanage M (2016) Plant growth promotion by *Bradyrhizobium japonicum* under heavy metal stress. South Afr J Bot 105:19–24

Shahid M, Khan MS (2018) Glyphosate induced toxicity to chickpea plants and stress alleviation by herbicide tolerant phosphate solubilizing *Burkholderia cepacia* PSBB1 carrying multifarious plant growth promoting activities. 3Biotech 8:131

Shameer S (2016) Biosorption of lead, copper and cadmium using the extracellular polysaccharides (EPS) of *Bacillus* sp., from solar salterns. 3Biotech 6:194

Sharma H, Rawal N, Mathew BB (2015) The characteristics, toxicity and effects of cadmium. Int J Nanotech Nanosci 3:1–9

Si M, Lang J (2018) The roles of metallothioneins in carcinogenesis. J Hematol Oncol 11:107

Singh Y, Lal N (2015) Investigations on the heavy metal resistant bacterial isolates in vitro from industrial effluents. World J Pharm Pharm Sci 4:343–350

Solano F (2014) Melanins: skin pigments and much more-types, structural models, biological functions, and formation routes. New J Sci 1:1–28

Tarangini K, Mishra S (2013) Production, characterization and analysis of melanin from isolated marine Pseudomonas sp. using vegetable waste Res J Engg Sci 2:40–46

Thaira H, Raval K, Manirethan V, Balakrishnan RM (2018) Melanin nano-pigments for heavy metal remediation from water. Sep Sci Technol 2:1–10

Vignesh KS, Deepe Jr GS (2017) Metallothioneins: emerging modulators in immunity and infection. Int J Mol Sci 18:2197

Xie Y, Fan J, Zhu W, Amombo E, Lou Y, Chen L, Fu J (2016) Effect of heavy metals pollution on soil microbial diversity and bermudagrass genetic variation. Front Plant Sci 7:755

Zhang Z, Cai R, Zhang W, Fu Y, Jiao N (2017) A novel exopolysaccharide with metal adsorption capacity produced by a marine bacterium *Alteromonas* sp. JL2810. Mar Drugs 15:175

Zimova M, Hackländer K, Good JM, Melo-Ferreira J, Alves PC, Mills LS (2018) Function and underlying mechanisms of seasonal colour moulting in mammals and birds: what keeps them changing in a warming world? Biol Rev 93:1478–1498