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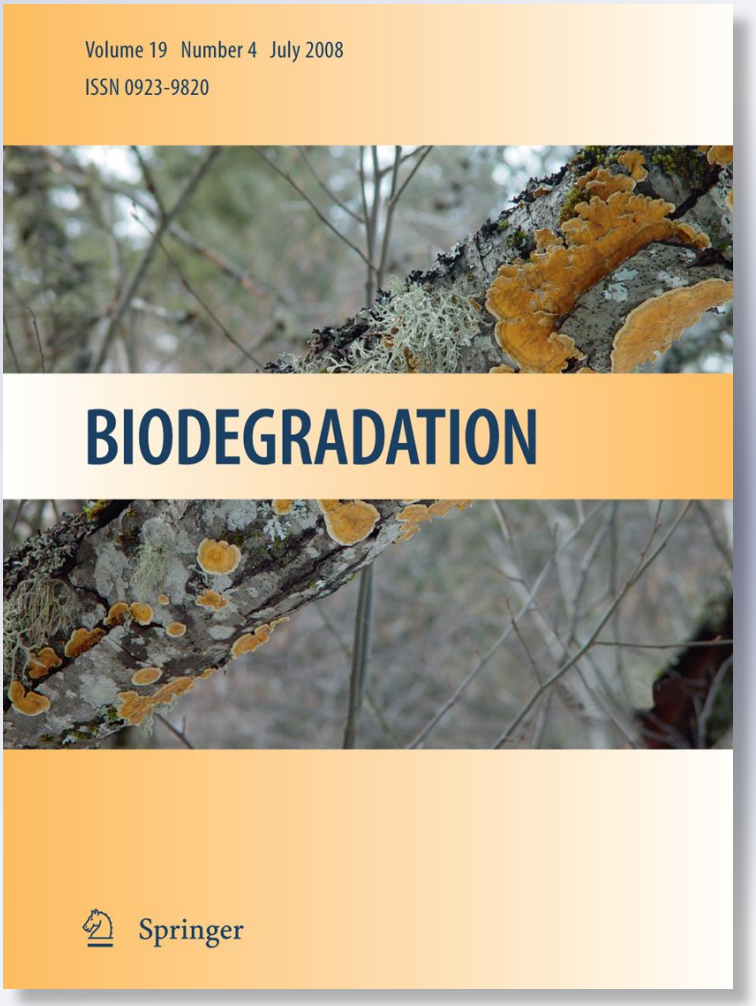

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ORIGINAL PAPER

Biological characterization of lead-enhanced exopolysaccharide produced by a lead resistant *Enterobacter cloacae* strain P2B

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Abstract A lead resistant bacterial strain isolated from effluent of lead battery manufacturing company of Goa, India has been identified as *Enterobacter cloacae* strain P2B based on morphological, biochemical characters, FAME profile and 16S rDNA sequence data. This bacterial strain could resist lead nitrate up to 1.6 mM. Significant increase in exopolysaccharide (EPS) production was observed as the production increased from 28 to 108 mg/L dry weight when exposed to 1.6 mM lead nitrate in Tris buffered minimal medium. Fourier-transformed infrared spectroscopy of this EPS revealed presence of several functional groups involved in metal binding viz. carboxyl, hydroxyl and amide groups along with glucuronic acid. Gas chromatography coupled with mass spectrometry analysis of alditol-acetate derivatives of acid hydrolysed EPS produced in presence of 1.6 mM lead nitrate demonstrated presence of several neutral sugars such as rhamnose, arabinose, xylose, mannose, galactose and glucose, which contribute to lead binding hydroxyl groups. Scanning electron microscope coupled with energy dispersive X-ray spectrometric analysis of this lead resistant strain exposed to 1.6 mM lead nitrate interestingly revealed mucous EPS surrounding bacterial cells which sequestered 17 % lead (as weight %) extracellularly and protected the bacterial cells from toxic effects of lead. This lead resistant strain also showed multidrug resistance. Thus these results significantly contribute to better understanding of structure, function and environmental application of lead-enhanced EPSs produced by bacteria. This lead-enhanced biopolymer can play a very important role in bioremediation of several heavy metals including lead.

Keywords Exopolysaccharide · Lead · Resistance · Bacteria · Bioremediation

Introduction

Environmental contamination of water bodies due to release of toxic heavy metals such as lead, mercury and cadmium poses serious threat to natural biota including humans (Nies 1999; Fernandes and Beiras 2001; Dirilgen 2011). Lead is well known to inhibit biosynthesis of heme, causes serious neurodegenerative diseases, interferes with kidney function and possesses carcinogenic properties (Fowler 1998; Tong et al. 2000; Watt et al. 2000; Lam et al. 2007). Heavy metals are toxic to natural biota including microorganisms since they inhibit enzyme activity, damage DNA and disrupt membrane permeability (Nies 1999;

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Asmub et al. 2000; Hartwig et al. 2002) but some natural bacterial isolates have potential to colonise sites heavily contaminated with toxic metals by employing various resistance mechanisms which include efflux system, sequestration, reduction, oxidation, bioaccumulation and precipitation as phosphates and sulphides (Nies 1999; Roane 1999; van Hullebusch et al. 2003; Pal and Paul 2008; Taghavi et al. 2009; Sinha and Khare 2011).

Bioavailability of metals is an important factor for metal toxicity since soluble metals can readily penetrate cell membranes (Roane 1999). Therefore metal immobilisation strategies applied by microbes to counteract toxic effect of heavy metals which includes precipitation, intracellular accumulation and extracellular exclusion in biopolymers (Roane 1999; van Hullebusch et al. 2003; Pal and Paul 2008). Biopolymers secreted by bacterial cells are known as exopolysaccharides (EPSs), which consist of macromolecules such as polysaccharides, proteins, nucleic acids, humic substances, lipids and other non polymeric constituents of low molecular weight (Bramhachari and Dubey 2006; Bramhachari et al. 2007). Microbial polymers differ extensively both in specificity and metal-binding characteristics depending on their physicochemical properties therefore chemical composition of the EPS is helpful in order to understand the nature of the metal ion–EPS interactions and its involvement in metal resistance mechanism (Arias et al. 2003; Bhaskar and Bhosle 2006; Morillo et al. 2006). Bacterial EPSs are chemically diverse and are mostly acidic heteropolysaccharides with ionizable functional groups such as hydroxyl, carboxyl, amide, sulphate and phosphoryl which interestingly exhibit very high affinity to heavy metals (Iyer et al. 2004; Bhaskar and Bhosle 2006; Bramhachari et al. 2007; Pal and Paul 2008). Bacterial EPS play a key role in initial attachment of cells to different substrata, cell-to-cell aggregation, protection against desiccation and resistance to harmful exogenous materials (Decho 1990; Iyer et al. 2004; Pal and Paul 2008). Various microbial biopolymers have been shown to have potential to bind heavy metals with differential degree of specificity and affinity (Bhaskar and Bhosle 2006; Pal and Paul 2008). They are high molecular weight polyanionic polymers which bind metals by electrostatic interaction between metal cation and negatively charged components of EPS resulting in metal immobilisation within exopolymeric matrix (van Hullebusch et al. 2003). Enzymatic activities in bacterial EPS assist degradation of organic recalcitrants and transformation of heavy metals along with their subsequent precipitation in the polymeric mass (van Hullebusch et al. 2003; Pal and Paul 2008).

Bioremediation processes are cost effective and are highly efficient as compared to physicochemical methods for removal of heavy metal therefore over last several decades attention has been focused towards exploiting microbes for heavy metal bioremediation. Therefore, understanding the mechanism by which bacteria sequester toxic heavy metals to protect itself from their toxic effects on physiological processes is crucial to the development of microbial processes for concentration, removal and recovery of metals from industrial effluents and solid wastes. Bioremediation of heavy metals involves their biosorption to either biomass or isolated biopolymers. The present communication deals with identification and biological characterization of lead resistant bacteria with reference to EPS and its metal-binding characteristics to explore EPS mediated lead resistance.

Materials and methods

Screening of EPS producing lead resistant bacterial isolate

EPS producing lead resistant bacterial strain was isolated from effluent of lead battery manufacturing plant of Goa, India. Serially diluted effluent sample was spread plated on PYT80 agar supplemented with 0.1 mM lead nitrate and plates were incubated at room temperature (Naik and Dubey 2011). The mucoidal bacterial colonies were selected and further inoculated on PYT80 agar plates amended with different levels of lead nitrate. The bacterial colony growing at highest lead nitrate concentration and showing mucoid exopolymer production was selected for further characterization and designated as strain P2B.

Identification and maintenance of bacterial isolate

Bacterial strain P2B was grown in PYT80 broth and stored at −20 °C as glycerol stock (20 % v/v) and subcultured once every 2 months. Before its use as inoculums the strain was subcultured on PYT80 agar and incubated at 30 °C for 24 h. Bacterial strain P2B

producing viscous exopolymer in Tris buffered minimal medium (TMM) (Mergeay et al. 1985) containing 0.4 % glucose was tentatively identified using Gram staining, morphological and biochemical characteristics following Bergey's Manual of Systematic Bacteriology (Krieg and Holt 1984), fatty acid methyl ester analysis (Sherlock version 6.0B) and 16S rDNA sequencing. PCR amplification of 16S rDNA was done using primer set 8F (5′-AGAGTTTGATCCT GGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGT TACGACTT-3′) and sequencing was done at Xcelris laboratories, Ahmedabad, Gujarat, India. DNA sequence was compared with GenBank reference database using NCBI-BLAST search (Altchul et al. 1997).

Growth behaviour and lead resistance limit of isolate

Growth behaviour of lead resistant bacterial strain P2B was determined in TMM supplemented with varying concentrations of lead nitrate using 0.4 % glucose as carbon source and 0.5 mM β-glycerol phosphate to avoid precipitation of lead with inorganic phosphate as lead phosphate (Himedia, India). Overnight grown bacterial strain P2B was inoculated in the culture broth, so that final optical density was 0.025 (initial inoculum). Growth conditions were 30 °C, pH 7.2 and constant shaking at 150 rpm. Absorbance was recorded using UV–Vis spectrophotometer (Shimadzu, UV-2450, Japan) after regular time interval at 600 nm. Cross tolerance of the isolate to $HgCl_2$ and $CdCl_2$, was also determined in TMM (Himedia, India).

Antibiotic susceptibility test

Antibiotic susceptibility test was performed following Kirby–Bauer disc diffusion method (Bauer et al. 1966), using Mueller–Hinton agar and antibiotic discs (Himedia, India). Bacterial suspension (0.1 mL) having concentration 5×10^5 cfu/mL was spread plated on Mueller–Hinton agar plates for antibiotic susceptibility test using disc (6 mm) diffusion method.

Purification and characterization of bacterial EPSs

EPS production was monitored in the TMM without (control) and with 1.6 mM lead nitrate in order to study role of EPS in resistance against lead. Purification of EPS was done using modified ice cold ethanol precipitation method (Bramhachari and Dubey 2006). Culture grown in the presence of 1.6 mM lead nitrate and without lead nitrate in TMM (1 L) was harvested separately at 10,000 rpm for 15 min at 4 °C, when culture reached stationary growth phase. Culture supernatant was filtered through 0.22 μ cellulose nitrate filters (Millipore Filters, Bangalore, India). EPS was precipitated from the final filtrate by addition of three volumes of ice cold ethanol and kept at 4 °C overnight. Resulting precipitate was centrifuged and washed with 70–100 % ethanol–water mixture. EPS was dissolved in distilled water and dialysed for 24 h at 4 °C (molecular weight cut off of 13 kDa; Sigma Aldrich, Germany) against distilled water. Bacterial Pellet was resuspended in 300 µL EDTA solution (10 mM EDTA + 1.5 mM NaCl) and heated at 50 °C for 3 min in order to extract cell bound EPS and purified in the same way as extracellular EPS. Extracellular and cell bound EPS was combined, lyophilized and stored at room temperature in sealed bottle until chemical and physical analysis. EPS production was recorded as dry weight of EPS in control and lead exposed conditions. Chemical composition of EPS produced under the stress of 1.6 mM lead nitrate (test) and EPS produced without lead nitrate (control) was analysed by gas chromatography coupled with mass spectrometry (GC–MS), Fourier-transformed infrared (FTIR) and Alcian blue staining methods.

FTIR spectroscopy of EPS

FTIR spectroscopy of lyophilized EPS was done to analyse major functional groups of the purified EPS which were involved in lead binding. Dry grinded EPS (2 mg) was mixed with 200 mg dry KBr, followed by pressing the mixture into 16 mm diameter mould (Bramhachari and Dubey 2006; Bramhachari et al. 2007). FTIR spectrum was recorded in the region 400–4,000 cm^{-1} (SHIMADZU-FTIR 8201PC instrument, Japan).

GC–MS analysis of EPS

GC–MS analysis of EPS was performed to investigate presence of neutral sugars. Acid hydrolysis of lyophilized EPS into monosaccharides and conversion of these sugars into their respective alditol acetates,

which were then analysed by GC–MS (model GC–MS-QP-2010 plus) from Shimadzu, Japan, equipped with a fused silica capillary column coated with CP Sil-88 (25 m id 0.32 mm, ds 0.12; Chrompack, Middleburg, Netherland) and Helium (3 mL/min) was used as a carrier gas (Albersheim et al. 1967; Fox et al. 1988). The following temperature gradient program was used: 75 °C for 2 min followed by an increase from 75 to 175 °C at the rate of 50 °C per min and finally 40 min at 230 °C. The *m*/*z* peaks representing mass to charge ratio characteristic of the monosaccharide sugar fractions were compared with those in the mass spectrum library of the corresponding sugar.

Estimation of uronic acid in EPS

Uronic acid content in EPS produced under the stress of 1.6 mM lead nitrate and without lead nitrate was estimated using method described by Ozturk et al. (2010). Glucuronic acid (0–100 μg/mL) was used as standard.

Alcian blue staining of bacterial EPS

Alcian blue staining of EPS was performed to investigate its acidic or basic nature. Alcian blue is cationic dye used to stain acidic polysaccharides. Here bacterial culture grown with 1.6 mM lead nitrate was smeared on slide; air dried and then hydrated with distilled water. Then slide was flooded with 10 μL of 0.1 % alcian blue dye in acetic acid having pH 2.5 for 5 min. Dye was washed with running water; slide was dried and observed under oil immersion microscope (Bhaskar and Bhosle 2005).

SEM–EDS analysis EPS

In order to reveal the role of EPS in lead sequestration, scanning electron microscope coupled with energy dispersive X-ray spectrometric (SEM–EDS) analysis was performed using test bacterial culture grown in TMM supplemented with 1.6 mM lead nitrate. Culture smear was prepared on a glass slide, air dried and then fixed in 3 % glutaraldehyde overnight with 50 mM potassium phosphate buffer. Then glass slide was washed thrice with phosphate buffer and dehydrated in gradually increasing concentrations of ethanol, i.e., 10, 20, 50, 70, 80, 90, 95, and 100 % for 15 min each. Glass slide was then air dried and stored in vacuum chamber prior to SEM–EDS analysis (Naik and Dubey 2011).

Results

Screening and identification of EPS producing lead resistant bacterial isolate

EPS producing lead resistant bacterial strain P2B was gram negative, rod shaped, motile and facultative anaerobe. It showed the presence of enzymes such as catalase, nitrate reductase and gelatinase. Indole, methyl red and urease tests where negative but Voges Proskauer's test was positive and it could also utilise citrate. Based on these biochemical characteristics and as per Bergey's manual of systematic bacteriology, FAME analysis and 16S rDNA sequencing followed by NCBI-BLAST search bacterial strain P2B was identified as *Enterobacter cloacae* (accession no. HQ268733) (Altchul et al. 1997).

Growth behaviour and lead resistance limit of isolate

Enterobacter cloacae strain P2B could resist lead nitrate up to 1.6 mM in TMM (Fig. 1). In addition to lead this strain also showed cross tolerance to $CdCl_2$, and $HgCl_2$ as MIC values for lead nitrate, cadmium chloride, and mercuric chloride were 1.8, 0.3 and 0.05 mM, respectively. This strain also showed resistance to multiple antibiotics such as oleondamycin (15 μg), ampicillin (25 μg), co-trimoxazole (25 μg), mecillinam (33 μg), lincomycin (5 μg), ciphaloridine (30 μg) and chloramphenicol (30 μg).

EPS production

EPS production significantly increased from 28 mg/L without lead nitrate (control) to 108 mg/L with 1.6 mM lead nitrate in TMM.

GC–MS and FTIR analysis of EPS

GC–MS analysis of EPS produced by the test organism in TMM without lead nitrate interestingly revealed presence of neutral sugars such as arabinose 4 %, xylose 9 %, mannose 3 %, galactose 32 % and

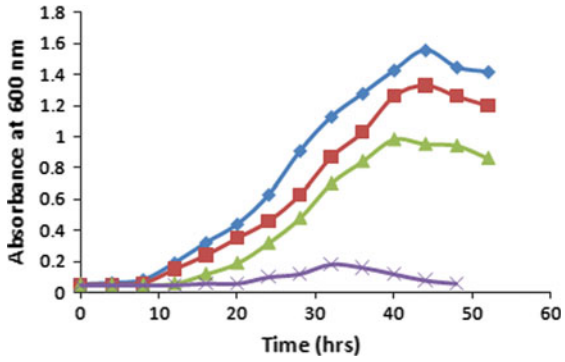


Fig. 1 Growth behaviour of *E. cloacae* strain P2B in TMM amended with different concentrations of lead nitrate. *Diamonds* 0 mM (control), *squares* 1.0 mM, *triangles* 1.6 mM, *times* 1.7 mM

glucose 49 % out of total sugar content. Whereas percent relative abundance of neutral sugars of EPS produced under the stress of 1.6 mM lead nitrate was recorded as rhamnose 2 %, arabinose 8 %, xylose 15 %, mannose 8 %, galactose 26 % and glucose 37 % out of total sugar content (data not shown). Glucose and galactose were most abundant monosaccharides of the heteropolymers produced under control and lead exposed conditions. Glucuronic acid concentration in EPS was found to be increased from 26 ± 3 (control) to 95 ± 7 µg/mg when exposed to 1.6 mM lead nitrate. The functional group is one of the keys to understand the mechanism of metal binding onto the EPS (van Hullebusch et al. 2003; Pal and Paul 2008). FTIR spectrum of purified EPS produced by *E. cloacae* strain P2B in presence of lead nitrate and without lead nitrate revealed presence of significant difference in absorption peaks of functional groups (Fig. 2a, b) such as broad stretching N–H group at 3,445 was shifted to 3,278 cm^{-1} (hydroxyl group). A broad stretching of C–O–C and C–O between 1,076 and 1,213 cm^{-1} corresponds to the presence of carbohydrates in EPS produced under lead stress whereas control sample showed very weak absorption (1,160–1,250 cm^{-1}). Absorption peak between 1,076 and 1,122 cm^{-1} ascertained the presence of uronic acid, *o*-acetyl ester linkage in the test sample and peaks between 900 and 1,147 cm^{-1} in the control. The absorption peaks of the test sample at 1,662 cm^{-1} (mainly C=O stretch) and 1,529 cm^{-1} (mainly N–H stretch) can be attributed to the amide I and amide II of amide bond due to peptide bond. Carboxyl group at 1,404 cm^{-1} indicates symmetric stretching (van Hullebusch et al. 2003; Iyer et al. 2004; Bhaskar and Bhosle 2006; Pal and Paul 2008; Yin et al. 2011). Whereas in control sample there was significant difference and decrease of intensity and shift in absorption of amide I, II (1,650 and 1,540 cm^{-1}) and carboxyl group (1,461 cm^{-1}). Peak at 804 cm^{-1} ascertains presence of glycosidic linkage in sugars (Bramhachari and Dubey 2006; Bramhachari et al. 2007). It is interesting to note that alcian blue stained the EPS blue and bacterial cells appeared pink confirmed acidic nature of EPS (Supplementary data).

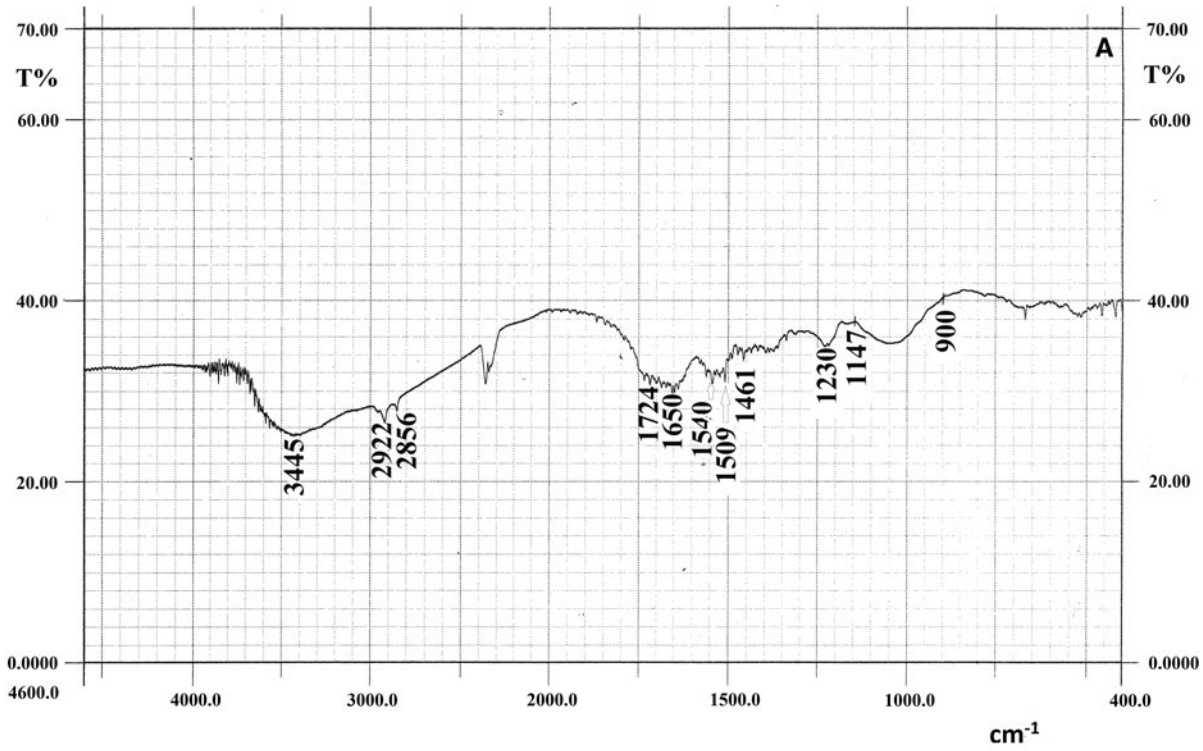
SEM–EDS analysis of EPS

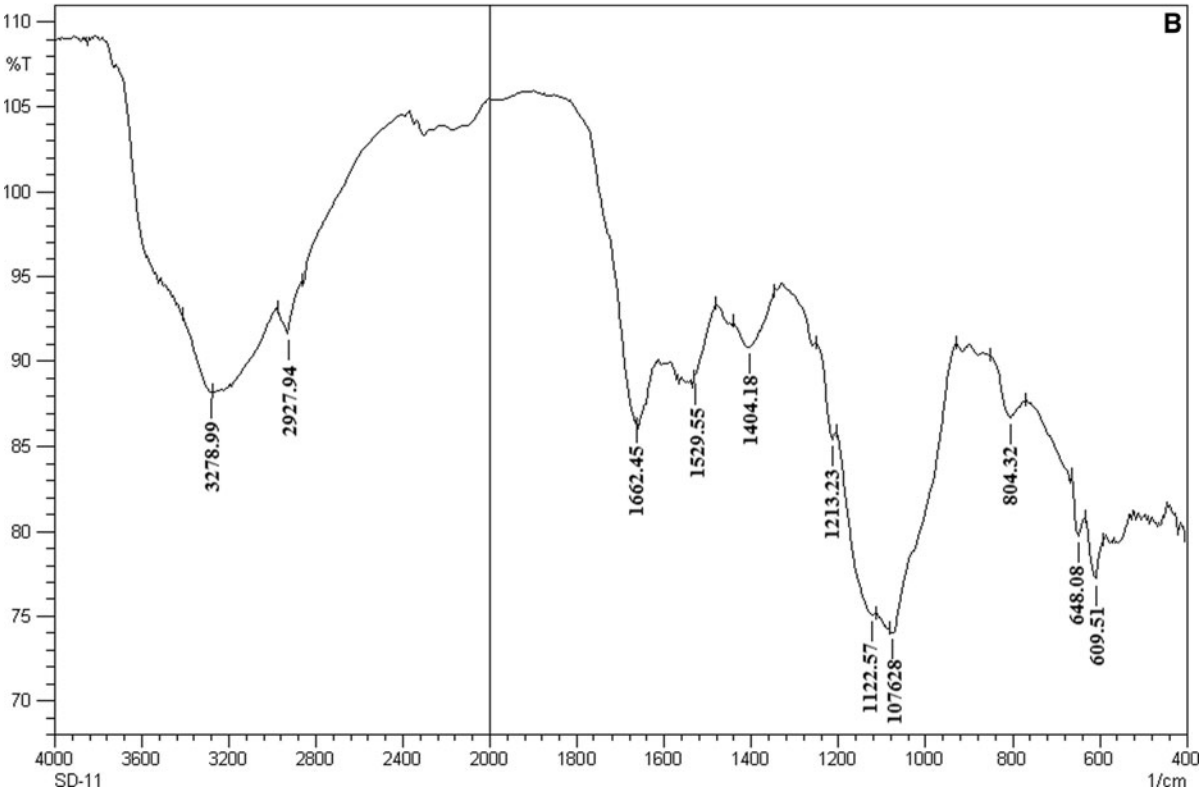
SEM–EDS clearly revealed mucous nature of the EPS surrounding bacterial cells and interestingly confirmed that EPS produced by *E. cloacae* strain P2B in the presence of 1.6 mM lead nitrate sequestered 17 % (as weight %) lead (Fig. 3a, b). SEM images proved that as compared to control, bacterial strain P2B grown under the stress of 1.6 mM lead nitrate produced significantly high amount of EPS (Fig. 3a, b). Also significant alteration in cell morphology as reduction in cell size and shrinkage was evidently observed when cells were exposed to 1.6 mM lead nitrate (Fig. 3a, b).

Discussion

In the present investigation we have characterized *E. cloacae* strain P2B which could resist lead nitrate up to 1.6 mM in TMM and produced lead-enhanced EPSs.

The heavy metal-binding capacity of EPSs is usually attributed to the high hydrophilicity of the polymer due to the presence of hydroxyl groups, the presence of ionisable functional groups and the flexible structure of the polymer chains (Iyer et al. 2004; Pal and Paul 2008). Several functional groups were reported to involved in the sorption of Cu(II) and Cd(II) by EPS of *Aspergillus fumigatus* including carboxyl, amide and hydroxyl groups (Yin et al. 2011). Lead resistant *E. cloacae* strain P2B also showed similar results where FTIR-spectrum of EPS produced under the stress of 1.6 mM lead nitrate confirmed the presence of ionizable functional groups able to react with the lead ions such as hydroxyl group at 3,278 cm^{-1}, absorption bands at 1,662 cm^{-1} (mainly





◀ **Fig. 2** FTIR spectrum of purified EPS produced by *E. cloacae* strain P2B **a** in absence of lead nitrate (3,445 cm^{-1} OH^{-}; 1,650 cm^{-1} amide I; 1,540 cm^{-1} amide II; 1,461 cm^{-1} carboxyl group) and **b** in the presence of 1.6 mM lead nitrate (3,278 cm^{-1} hydroxyl; 1,662 cm^{-1} amide I; 1,529 cm^{-1} amide II; 1,404 cm^{-1} carboxyl). Dry grinded EPS (2 mg) was used for FTIR analysis

C=O stretch), 1,529 cm^{-1} (mainly N–H stretch) and carboxyl group at 1,404 cm^{-1} (sym). Absorption band at 804 cm^{-1} clearly ascertained the presence of glycosidic linkage. Absorption peak between 1,076 and 1,122 cm^{-1} ascertained the presence of uronic acid and alcian blue staining further confirmed acidic nature of the EPS. It is well known that uronic acid (glucuronic acid) and acidic functional groups (e.g. carboxyl) of EPS involved in metal sequestration (Bhaskar and Bhosle 2005; Bramhachari and Dubey 2006; Bramhachari et al. 2007; Pal and Paul 2008) and in this study increase in the concentration of glucuronic acid and strong absorption peak of carboxyl group (1,461 cm^{-1}) of test EPS in FTIR spectrum as compared to control EPS revealed involvement of carboxyl group in lead binding to EPS. Pb(II) binding resulted in changes in the absorption frequencies of the various functional groups present in the EPS such as carboxyl, hydroxyl and amide. Increase in intensity and shifts in absorption peaks in the EPS produced under the stress of 1.6 mM lead nitrate as compared to control indicated that the carboxyl, hydroxyl and amine groups of EPS were predominant contributors in lead sequestration and thus protect *E. cloacae* strain P2B from lead toxicity. These results are in agreement with earlier findings (Iyer et al. 2004; Pal and Paul 2008). Also *E. cloacae* strain P2B resist multiple antibiotics along with lead resistance. This result go hand in hand with our earlier reports which showed that metal resistant bacteria also resist multiple antibiotics (Naik and Dubey 2011). Baker-Austin et al. (2006) reported that there is co-selection of antibiotic and metal resistance where co-selection mechanisms include co-resistance (different resistance determinants present on the same genetic element) and cross-resistance (the same genetic determinant responsible for resistance to antibiotics and metals).

GC–MS analysis of EPS revealed that EPS produced under the stress of 1.6 mM of lead nitrate showed different composition of neutral sugars as compared to EPS produced under control condition. Also a strong broad stretching of C–O–C, C–O at 1,076–1,213 cm^{-1} in FTIR spectrum of EPS produced under lead nitrate stress proved presence of sugars in exopolymer as major components which contributes metal-binding hydroxyl group as compared to weak stretching (1,160–1,250 cm^{-1}) in FTIR spectrum of control EPS (Fox et al. 1988; Bramhachari et al. 2007; Pal and Paul 2008). The sequestration of heavy metals by EPS is a metabolism-independent process and mainly depends on interaction between metal cations and negatively charged acidic functional groups of EPS (van Hullebusch et al. 2003; Bhaskar and Bhosle 2006). EPS produced by *E. cloacae* strain P2B in the

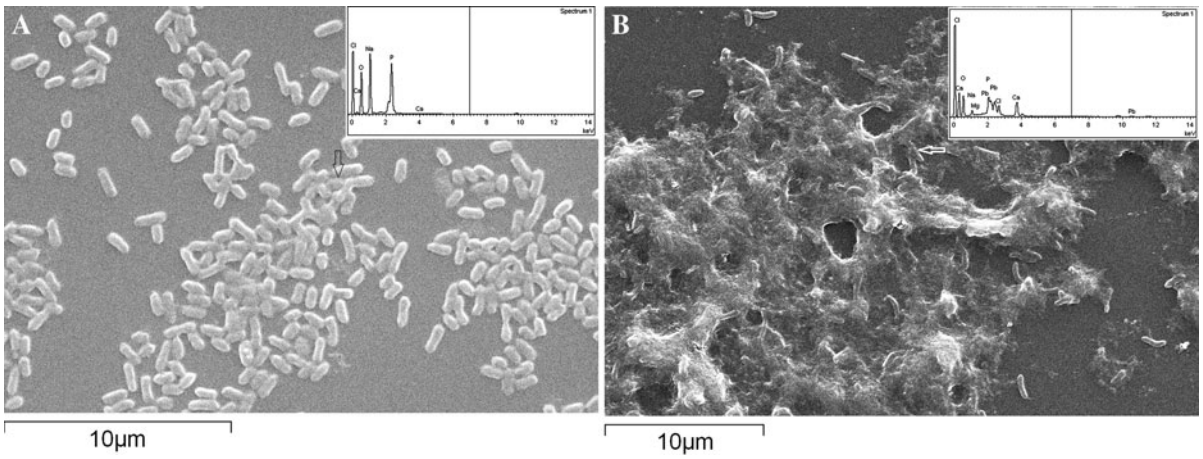


Fig. 3 Photomicrograph of exopolymer surrounding *E. cloacae* strain P2B grown with 1.6 mM lead nitrate (**b**) and *E. cloacae* strain P2B grown without lead nitrate (**a**) using SEM–EDS (SEM—magnification, ×9,000) (*arrows* are pointing to the area considered for EDS analysis)

presence of 1.6 mM lead nitrate contained high amount of sugars with hydroxyl group as revealed by FTIR and GC–MS analysis, enabling it to become hydrated, swell and yield viscous solutions and bind high amount of lead (17 %). Comparing FTIR spectrum of control EPS with EPS produced under lead stress evidently confirmed that EPS produced under lead stress has higher percentage of ionisable functional groups which could bind lead ions than control EPS. Also alteration in cell morphology as shrinkage and increase in EPS production showed by *E. cloacae* strain P2B when exposed to high levels of lead nitrate appeared to be the defensive response of bacterial cells against toxic lead and our earlier report also support this finding (Naik and Dubey 2011).

It was reported that Cr(VI)-resistant *Methylobacterium mesophilium* MU141 exposed to 15- and 35-ppm Cr(VI) concentrations, produced more EPS than its control cells and EPS involved in Cr sequestration (Ozturk et al. 2008). Also in case of *Pseudomonas mendocina* P2d where increase in EPS viscosity was observed in presence of high levels of benzoate. The EPS was apparently formed, therefore, as a protective measure against the toxic effects of benzoate (Royan et al. 1999). In present finding also enhanced synthesis of bacterial EPS from 28 to 108 mg/L dry weight in presence of lead, FTIR spectrum and SEM–EDS analysis demonstrated that EPS of *E. cloacae* strain P2B could sequester 17 % lead (as weight %) due to presence of higher number of ionizable negatively charged functional groups and thus protects bacteria from lead toxicity. Since EPS produced by *E. cloacae* strain P2B which resist 1.6 mM lead nitrate sequester highest amount of heavy metal (lead 17 % by weight) reported in case of *E. cloacae* strains (Iyer et al. 2004; Sinha and Khare 2011) therefore this bacterial strain may serve as potential bioremediative agent (lead biosorbent) in lead contaminated environmental sites.

Conclusion

Our studies have clearly demonstrated that the lead ions could interact with carboxyl, hydroxyl, amide groups and glucuronic acid from different chains of the polyanionic EPS produced by *E. cloacae* strain P2B acting as an electrostatic bridge between them producing finally a mesh of polymer large enough to sequester very high levels of lead. In the present investigation we have demonstrated lead-enhanced EPS production in *E. cloacae* strain P2B sequestering significantly very high amount of lead (17 %) for the first time, as there was no such report so far. Bioremediation technology is simple, cost effective, highly efficient and environmentally sustainable as compared to physico-chemical methods, therefore this lead resistant bacterial strain may serve as a potential biotechnological agent to bioremediate lead contaminated sites.

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