Bacterium isolated from coffee waste pulp biosorps lead: Investigation of EPS mediated mechanism

Shiny Martis B, Aparna K Mohan, Sanjana Chiplunkar, Sandhya Kamath, Louella Concepta Goveas*, C Vaman Rao*

Department of Biotechnology Engineering, NMAM Institute of Technology, Nitte 574110, Karnataka, India

A R T I C L E   I N F O

A B S T R A C T

Kleibsiella pneumoniae Kpn555, isolated from coffee waste pulp showed high level of tolerance to lead with a minimum inhibitory concentration of 900 mg/L. On its growth in nutrient broth supplemented with lead, brown clumps were visualised at the bottom of the flask. On scanning and transmission electron microscopic studies the brown clumps were corroborated to be bacterial cells with lead biosorbed on the cell surface and accumulated inside the cytoplasm. Biochemical and FT-IR analysis of the extracellular polymeric substance produced on exposure to lead revealed its chemical nature as glycolipid with protein moieties. Purified EPS (100 mg/L) could remove 50% of lead from aqueous solution (200 mg/L). Isolation of plasmid from *Klebsiella pneumoniae* Kpn555 revealed the presence of a plasmid of size 30–40 kb. This capability of the bacteria was proven to be plasmid mediated as the *Escherichia coli* DH5α cells transformed with the plasmid of *Klebsiella pneumoniae* Kpn555 also could tolerate 900 mg/L of lead and form brown clumps. This study shows that these bacteria, aided by EPS could serve as an effective agent for the removal of lead from contaminated water environmental samples.

1. Introduction

Contamination of heavy metals is a major concern because of their toxicity and threat to living forms and environment (Mishra et al., 2019). Due to global industrialization and nuclear processes, large amounts of heavy metals have been released into the biosphere (Awual, 2019; Pavithra et al., 2020). Among the different heavy metals like copper, cadmium, arsenic, mercury etc., pollution by lead is worldwide public problem, because it causes serious health hazards in children like permanent brain damage, learning disabilities, hearing losses and behavioural abnormalities whereas in adults, it is known to cause hypertension, heart diseases and detrimental effects on reproductive systems (Awual and Hasan, 2019, 2014). Half-life of lead in blood is about 1 month whereas it takes about 20–30 years to reach half of its initial concentration in the skeleton (Awual, 2019).

The removal of heavy metals from the environment is being done by physico-chemical methods such as mechanical screening, hydrodynamic classification, gravity concentration, adsorption, ion exchange, membrane filtration, electro dialysis, reverse osmosis, ultra-filtration and photo catalysis (Akhtar et al., 2020). Among these methods removal of metals by use of different adsorbents is most commonly used attributing to its ease of use, simple procedure and eco-friendly nature (Awual et al., 2020). Several adsorbents such as activated char-

coal, activated carbon, nanocomposites, nanomaterials and biomaterials are extensively used in removal of heavy metals (Renu et al., 2017). Among various biomaterials used, microbial biomass can utilized in bioadsorption of heavy metals from solutions as they are abundantly available and cost-effective when used in bulk amounts, stability over a wide pH and temperature and minimization of waste created by the detoxification process (Aryal and Liakopoulou-Kyriakides, 2015; Kumar et al., 2018).

In the presence of metal ions, microorganisms such as bacteria, fungi and algae employ different detoxification processes such as oxidation/reduction of metal ions, formation of complex/precipitates, bioaccumulation/biosorption (Elahi et al., 2020). The adsorption of heavy metals on to bacterial cells is facilitated by attachment of heavy metals to functional groups present on extracellular polymeric substance (EPS) secreted by bacteria on exposure to heavy metal stress (Mahapatra et al., 2020). The adsorbed heavy metals are then accumulated intracellularly, post which they are converted to lesser toxic oxidised state via enzyme-dependent metabolism (Sengupta et al., 2021). Although, there are numerous distinct reports describing heavy metal adsorption by bacterial EPS and accumulation of heavy metals into bacterial cytoplasm (Sharma and Shukla, 2021; Cavallero et al., 2020; Huang et al., 2018; Dobrowolski et al., 2017), there are limited papers describing EPS mediated bacterial biosorption and accumulation.

The ability of detoxification of metals in bacteria can be natural or acquired through plasmids (Marzan et al., 2017). The occurrence of plasmid in metal tolerant strains is often observed in a polluted site rather than an unpolluted site (Lu et al., 2006). Bacterial strains isolated from Yamuna river, New Delhi, India showed antibiotic and heavy metal resistance which was determined as plasmid inherited (Siddiqui et al., 2020).

The present study reports the tolerance, growth kinetics of previously reported heavy metal tolerant *Klebsiella pneumoniae* Kpn555 strain isolated from coffee pulp waste. The investigation of biosorption of lead by the bacteria and its produced EPS was carried out by SEM, TEM and EDS analysis. In order to examine if the heavy metal resistance was genome or plasmid mediated, plasmid isolation and genetic transformation studies were also employed.

2. Materials and methods

2.1. Bacteria and chemicals

The strain used in this study was previously isolated from coffee pulp waste and identified as *Klebsiella pneumoniae* Kpn555 (GenBank accession number KX570899.1) (Mohan et al., 2019).The strain was stored as glycerol stocks at −20 °C and subcultured periodically. All the chemicals used in this study were of analytical grade unless mentioned otherwise.

2.2. Determination of minimum inhibitory concentration (MIC) of lead

Nutrient Broth (NB) (Hi-Media Lab. Ltd., Mumbai, India) was prepared in test tubes and supplemented with different amounts of lead to obtain the desired concentration ranging from 100 – 1500 mg/L. These tubes were inoculated with 1% v/v of 16 h old culture of *Klebsiella pneumoniae* (1×10^6 cells) and incubated for 24 h at 37 °C. The growth of *Klebsiella pneumoniae* Kpn555 in this medium was analysed by measuring the Optical Density (OD) at 600 nm on a UV–visible spectrophotometer (Shimadzu, Japan). MIC was defined as the minimum inhibitory concentration of the heavy metal that showed an OD of 0.1 at 600 nm after 24 h of incubation.

2.3. Growth kinetics and biosorption of lead

NB (100 ml) was supplemented with different concentrations of lead (100 mg/L, 400 mg/L and 800 mg/L) based on MIC results and inoculated with 1%v/v of 16 h old culture (1×10^6 cells). The OD of the broth was measured at 600 nm periodically and the growth kinetics was plotted. The cell free supernatants were analysed for residual lead concentration on an atomic absorption spectrophotometer (AAS – GBS – AVANTA).

2.4. Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy dispersive spectrometry (EDS) analysis

Brown clumps, settled at the bottom of the culture medium were analysed by observing them under SEM (Hitachi S4100, Japan) and TEM. For SEM analysis, the clumps were fixed on the aluminium stubs and coated with a thin layer of gold. For TEM analysis, the brown clumps were first dispersed in water and ultrasonicated for 10 min. A small drop of suspension was placed on a carbon-coated copper grid and dried under an infrared lamp. Transmission electron micrographs were obtained on JEOL-1011 instrument with an accelerating voltage of 100 kV. The composition of clumps was determined by employing EDX analysis in both the cases.

2.5. Extraction and purification of EPS

Klebsiella pneumoniae Kpn555 (2.0%v/v) was cultured in NB supplemented with lead (400 mg/L) for 5 days post which EPS was extracted from the cell free supernatant (Goveas and Sajankila, 2020). The cell free supernatant was acidified to pH 2.0 with 6 M HCl and kept for precipitation overnight at 4 °C. The precipitated EPS were separated by centrifugation at 10,000 rpm for 20 min and resuspended in 0.1 M sodium bicarbonate solution. EPS was purified by extraction thrice with 2:1%v/v chloroform-ethanol mixture at 25 °C.

2.6. Characterization of purified EPS

The qualitative presence of carbohydrate and protein moieties in the purified EPS was performed by Molisch test and Folin-Ciocalteu reagents respectively. Presence of lipids was confirmed by thin layer chromatography using a solvent system of chloroform: methanol: water (70:10:0.5) and subsequent exposure of the plate to iodine vapours (Sims and Larose, 1962). The produced EPS was quantified by estimating the protein, lipid and carbohydrate concentrations by Lowry's method, Phosphovanilin method and Phenol Sulphuric acid method respectively.

In-order to analyse and confirm the chemical composition of purified EPS Fourier transform Infrared Spectroscopy (FT-IR) was performed. Dried EPS was dispersed in potassium bromide (Merck, USA) pellets in the ratio, 1:3 and subjected to an average of 25 scans over the entire range using a FTIR Spectrophotometer (Shimadzu) in the range of 400 – 4000 cm^{-1}.

2.7. Lead biosorption by purified EPS

Purified EPS (100 mg/L) was added to an aqueous solution of lead (200 mg/L) and incubated at 27 °C at 100 RPM for 7 h. The residual lead concentration was measured every 1 h by atomic absorption spectrophotometry (AAS). The percentage biosorption of lead was calculated as per the following equation

$$\%Biosorption = \frac{Co - Ct}{Co} * 100$$

Where C_o and C_t are the initial lead concentration and lead concentration at time t respectively

The maximum metal uptake was calculated as per the following equation

$$Q = \frac{V(Co - Ce)}{W}$$

Where Q = amount of lead biosorbed (mg/g) after 7 h, V is the total volume of the solution (L), C_i and C_e are the initial and equilibrium lead concentration respectively (mg/L) and W is the weight of EPS added (g).

2.8. Plasmid isolation from Klebsiella pneumoniae Kpn555

Plasmid DNA was isolated using the standard alkaline lysis method (Sambrook et al., 1989). The isolated product was detected by an agarose gel (1%) run. The product was visualized and compared with a standard 10 kb ladder.

2.9. Transformation of the isolated plasmid into Escherichia coli DH5α

Plasmid DNA was isolated from a 50 ml culture in its log phase and was eluted in a TE buffer (10 mM-Tris/HCl/l m-EDTA, pH 8). This isolated plasmid was used to transform 100 μl of *E. coli* DH5α competent cells by calcium chloride method. These transformed strains were tested for their uptake of plasmid after transformation and increased lead tolerance.

2.10. Screening for transformed Escherichia coli DH5α cells and assessment of lead tolerance

The transformed strains were tested for their uptake of plasmid after transformation and increased threshold of lead tolerance. Since *Klebsiella pneumoniae Kpn555* was isolated from coffee pulp waste along with
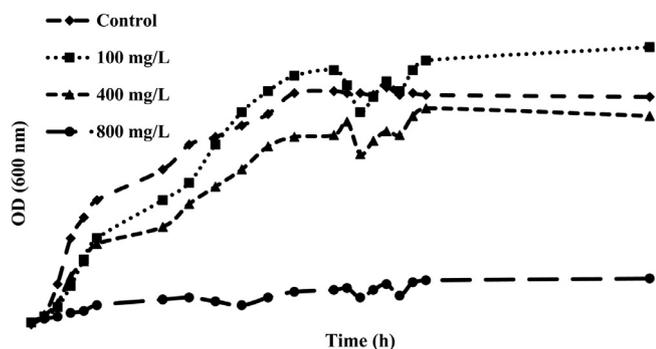
Fig. 1. Difference in growth pattern of *Klebsiella pneumoniae* Kpn555 in the absence and presence of lead.
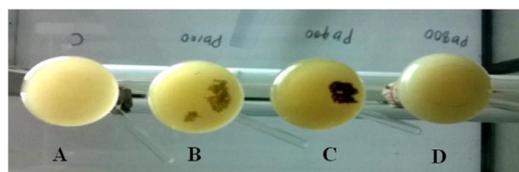
Fig. 2. Brown clumps formed on growth of *Klebsiella pneumoniae* Kpn555 in NB with lead concentration of 100 mg/L (B) and 400 mg/L (C); Clumps absent in NB without lead (A) and in NB with lead concentration of 800 mg/L (D).

Brevibacterium sp. MTCC10313 (Nayak et al., 2012), it also showed tolerance to caffeine (8 g/L). Therefore, the screening for transformation was done by detection of tolerance to caffeine.

The transformed and non-transformed colonies of *E. coli* DH5α were grown in LB medium with different lead concentrations ranging from 100 mg/L to 800 mg/L and their lead tolerance was compared. Bacterial growth was measured by taking OD of bacterial culture at 600 nm.

3. Results and discussion

3.1. Determination of minimum inhibitory concentration (MIC) of lead

The MIC of lead in *Klebsiella pneumoniae* Kpn 555 was found to be 900 mg/L. Beyond this concentration there was complete cessation of the bacterial growth. *Pseudomonas azotoformans* isolated from heavy metal contaminated soil had a MIC of 1000 mg/L of lead (Choińska-Pulit et al., 2018). *Gemella* sp. and *Micrococcus* sp, isolated from tannery effluent could tolerate lead upto 1900 mg/L and 1800 mg/L respectively (Marzan et al., 2017).

3.2. Growth kinetics and biosorption of lead

The concentration of lead added was chosen on the basis of MIC obtained. Growth was found to decrease in the presence of 800 mg/L of lead. Fluctuation was visualised in growth pattern in the stationary phase, which was absent in the control (Fig 1). It could be presumed that, in the presence of lead, probably some genes of *Klebsiella pneumoniae* Kpn 555 get switched on in the stationary phase, leading to the production of some products that help in combating the stress caused by high concentration of lead thereby reducing its toxic effects on the growth of the bacterium.

Also, brown clumps were visualised in the medium only at 100–400 mg/L (Fig 2). The clumps attached together to form a mat like structure. The mat was reformed even after the clumps were dispersed suggesting that there could be some binding component which caused the agglomeration.

On AAS studies it was observed that the maximum biosorption of lead was observed in the first 15 h after which it remained constant (Fig. 3). This coincided with the irregular fluctuations observed during
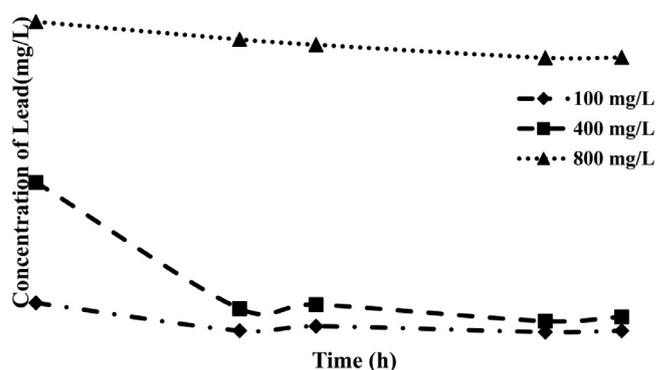
Fig. 3. Decrease in concentration of lead from NB supplemented with lead, on growth of *Klebsiella pneumoniae* Kpn555 for 48 h.

the bacterial stationary phase. This may probably mean that, as the bacterium reaches the stationary phase, the presence of the lead causes a toxic effect on it due to which it releases some amount of lead back into the medium. Once the bacteria adjust to the lead concentration in the stationary phase, it again continues to biosorb lead on its surface. This can be confirmed from the pattern where in, a slight decrease is observed in all the three cases at about 45 h. The agglomerates observed in 400 mg/L of lead supplemented medium were lysed and the lysate was subjected to AAS. The cell lysate showed 367 mg/L lead which confirmed that the bacteria indeed formed agglomerates due to the secretion of some binding material and biosorbed lead out of the medium and deposited it on its surface. *Aspergillus* species isolated from soil samples near the metal plating industry was grown in medium containing different concentrations of lead (0.2–1.5 mM) to determine its resistance to heavy metals and it was observed that the organism was found to accumulate lead particles outside and inside the cell (Pavani et al., 2012).

3.3. Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy dispersive spectrometry (EDS) analysis

On subjecting brown agglomerates to SEM analysis, it was seen that the brown agglomerates were clumps of bacteria with some surface deposition. Deposition was also observed in the interstitial spaces (Fig. 4A). EDS analysis confirmed the presence of lead in these deposits (Fig. 4B). Similar studies on *Bacillus licheniformis* showed a reduction in size of bacterial cells which was attributed to biosorption of lead onto the bacterial cell surface (Girisha, 2014). *Enterobacter* sp. J1 isolated from wastewater treatment plant also showed surface deposition of lead, copper and cadmium ions on the surface of cells as visualised by SEM (Lu et al., 2006).

The agglomerates obtained were subjected to TEM and corresponding EDS analysis. TEM images showed the presence of dark circular deposits in the bacterial cytoplasm which were confirmed by EDS analysis as lead (Fig 5A and 5B). *Klebsiella* sp. 3S1 isolated from wastewater treatment plant showed that lead ions were bio accumulated into the cytoplasm and adsorbed on the cell surface (Muñoz et al., 2015). On growth of *Acinetobacter junii* in medium supplemented with 250 mg/L of lead, isolated from coal mine showed surface deposition and bioaccumulation as visualised by SEM and TEM analysis (Kushwaha et al., 2017).

3.4. Characterisation of purified EPS produced by Klebsiella pneumoniae Kpn555

The yield of EPS after purification was found to be 1.23±0.04 g/L. In response to the stress imposed by lead (400 mg/L) on the bacteria, the EPS that was produced by *Klebsiella pneumoniae* Kpn555 was found to exhibit a positive result for Molisch's test while a mild colour change was observed in response to Folin-Ciocalteu test thereby suggesting a lower
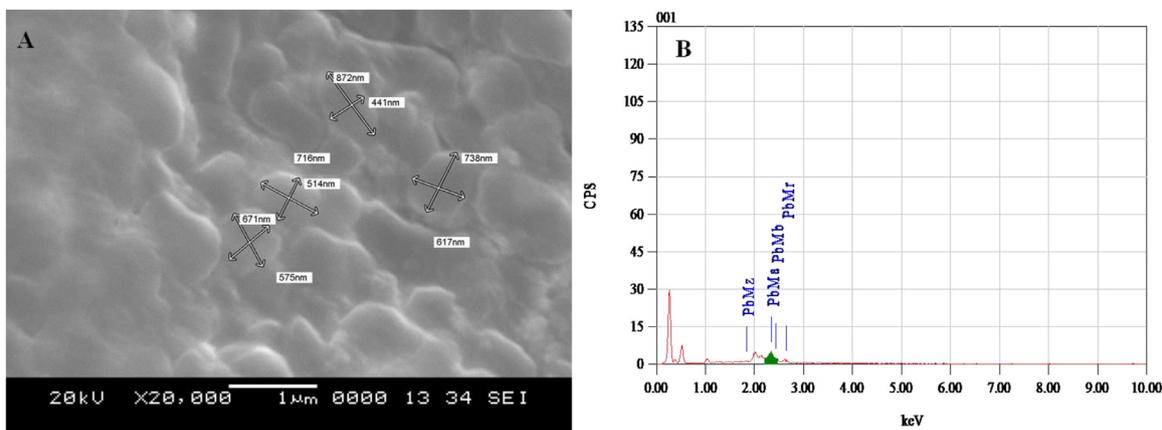
Fig. 4. SEM micrograph of brown clumps showing cells of *Klebsiella pneumoniae* Kpn555 with surface deposition (A); EDS analysis confirming presence of lead on the bacterial cell surface (B).
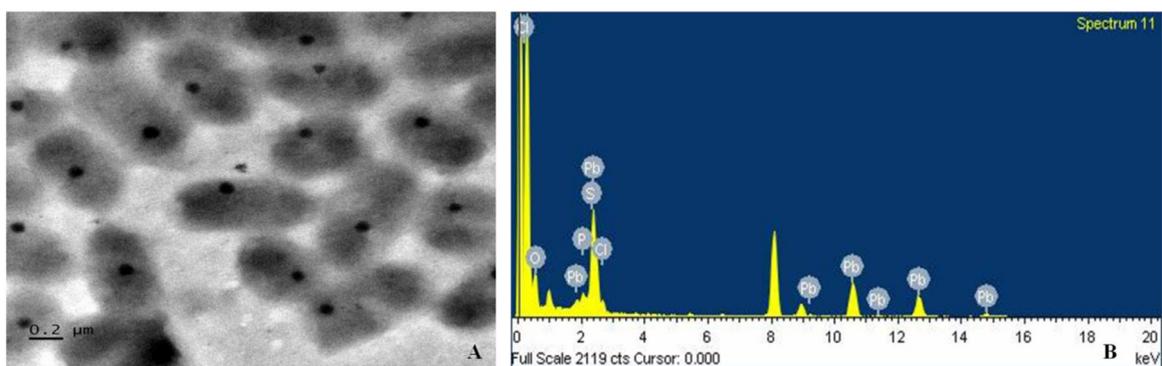
Fig. 5. TEM micrograph of brown clumps showing accumulation of lead as circular deposit in the cytoplasm of *Klebsiella pneumoniae* Kpn555 (A); EDS analysis confirming presence of lead in the circular deposits (B).
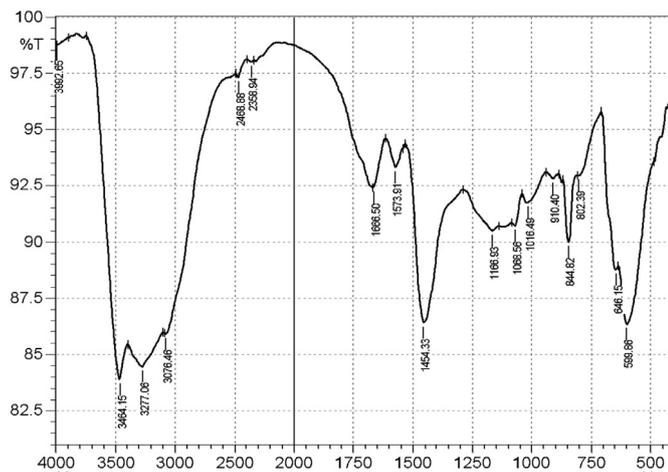
Fig. 6. FTIR spectrum of EPS produced by *Klebsiella pneumoniae* Kpn555.

proportion of protein in the EPS structure. Furthermore, appearance of a yellow spot on the TLC plate in response to iodine vapours led to the conclusion that the EPS produced is in fact a glycolipid with protein groups. Quantitative analysis revealed that carbohydrates, lipids and proteins were present in the ratio of by 33.76: 57.04:9.2 respectively.

The FTIR spectra of the EPS produced (Fig 6), exhibited a medium absorption band at 3427.51 cm^{-1} and 3464.15 cm^{-1} which implied the presence of O–H groups corresponding to carboxylic acid functional group. The peak observed at 2358.94 cm^{-1} points to the presence of the

C = C functional group. The presence of C = C nitrile group as part of the structure of EPS can also be concluded from the FTIR graph. While the peak observed at wavenumber corresponding to 3076.46 cm^{-1} was assigned to the C–H group of alkenes/ alkenyl functional group, that observed at a wavenumber range of 1015 – 1200 cm^{-1} was assigned to C–O-C/ C–O stretches of sugars. A distinct pattern of 3 peaks observed in the wavenumber range of 500 – 800 cm^{-1} is suggestive of alkyl halide (C-X) groups, wherein X corresponds to the halogen of alkyl halides. The FTIR results were a reaffirmation of the glycolipoproteinaceous nature of the EPS produced.

3.5. Bioremoval of lead by purified EPS

When EPS (100 mg/L) was incubated with lead solution of concentration 200 mg/L, a white precipitate was observed by one h. The aqueous solution was centrifuged to separate white precipitate and the clear supernatant was subjected to AAS. Lead concentration was found to steadily decrease till 5th h of incubation after which it plateaued. Lead concentration of 105 mg/L was observed at the end of 5 h (Fig 7). This observation implies that EPS concentration of 100 mg/L is sufficient for the bioremoval of 50% of total lead added to the solution. The maximum lead binding capacity by purified EPS was 475 mg/g. The mechanism of this action can be explained by the plausible interaction between the alkyl halide groups present in the EPS and the lead ions. A rhamno-lipid EPS isolated from Gram negative bacilli had the ability to biosorps chromium and it was found to bind to 50% of the chromium concentration present in the sample (Karnwal et al., 2014). The maximum lead binding capacity of EPS produced by *Acinetobacter junii* in response to lead stress was observed as 1071 mg/g (Kushwaha et al., 2017). The
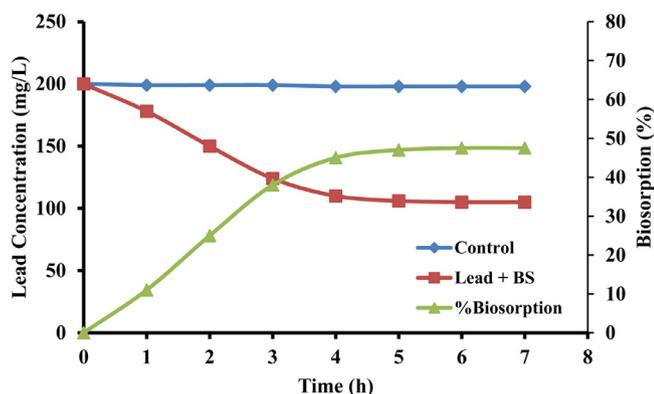
Fig. 7. Bioremoval of the lead by EPS of *Klebsiella pneumoniae* Kpn555.
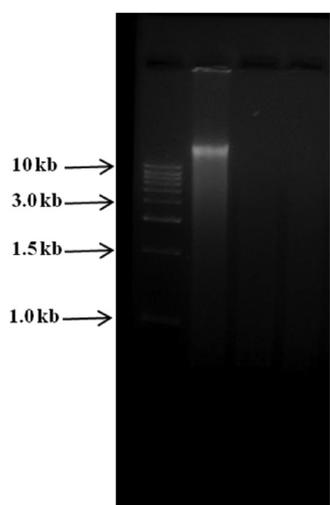
Fig. 8. Plasmid isolated from *Klebsiella pneumoniae* Kpn555 (Lane 1: 10Kb marker, Lane 2: Plasmid).
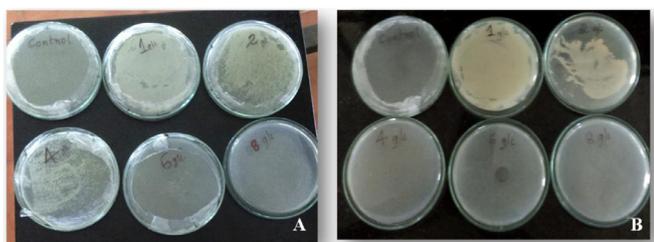
Fig. 9. Growth of transformed *E coli* DH5α colonies till 6 g/L of caffeine (A); Growth of non-transformed *E coli* DH5α colonies till 2 g/L of caffeine (B).

maximum adsorption capacity of a microporous silica-based conjugate in adsorption of lead ions was observed as 179.82 mg/g (Awual et al., 2019).

3.6. Plasmid isolation from Klebsiella pneumoniae Kpn55 and transformation into E coli DH5α

The plasmid was isolated from *Klebsiella pneumoniae* Kpn55 using alkaline isolation procedure and subjected for agarose gel electrophoresis to determine its size. The size of the plasmid could be approximated as 30–40 Kbp (Fig. 8). This plasmid was used for transforming *E coli* DH5α and the transformed cells showed tolerance upto 6 g/L caffeine whereas the non-transformed cells could tolerate only 2 g/L of caffeine (Fig. 9).

Fig. 10. Absence of brown clumps in NB supplemented with 400 mg/L of lead when competent *E coli* DH5α cells were grown (left); Presence of brown clumps in NB supplemented with 400 mg/L of lead when transformed *E coli* DH5α cells were grown (right).

3.7. Assessment of lead tolerance in transformed colonies

The transformed *E. coli* DH5α colonies were selected from agar plate supplemented with 6.0 g/L caffeine and grown in NB medium supplemented with different concentrations of lead (100, 400 and 800 mg/L). The transformed *E. coli* DH5α cells grew at all concentrations whereas the non-transformed colonies could tolerate only upto 400 mg/L of lead. The transformed colonies also formed agglomerates in NB supplemented with 400 mg/L lead (Fig. 8). This means that, the genetic ability of lead resistance and bioaccumulation of lead by *Klebsiella pneumoniae* Kpn555 is due to the genes present on the plasmid and not the genomic DNA. *Halomonas* BVR 1 isolated from an electronic industry effluent could tolerate lead upto 400 mg/L and heavy metal resistance in this strain was also plasmid mediated (Manasi et al., 2016).

4. Conclusion

The outcome of our study reveals that *Klebsiella pneumoniae* Kpn555, previously isolated from coffee pulp waste showing high resistance to cadmium, lithium and mercury (Mohan et al., 2019), showed tolerance to 900 mg/L lead. SEM and TEM studies revealed the adsorption and bioaccumulation of lead on the bacterial surface and in its cytoplasm. EPS produced in response to lead stress, characterised as glycolipid with protein moieties, adsorbed lead with adsorption capacity of 475 mg/g. This capacity of bacteria was proved to be plasmid mediated which was confirmed by transformation of isolated plasmid into *E coli* DH5α. In addition to utilization of *Klebsiella pneumoniae* for lead removal from polluted sites, transformation of other bacteria with its plasmid could also be employed. Further investigations should be carried out to optimise lead removal by *Klebsiella pneumoniae* Kpn555 and transformed bacteria. These strains could be exploited as a novel bio-sorbent in bioremediation of lead.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

CRediT authorship contribution statement

Shiny Martis B: Methodology, Investigation, Writing – original draft, Writing – review & editing. **Aparna K Mohan:** Methodology, Investigation. **Sanjana Chiplunkar:** Methodology, Investigation. **Sandhya Kamath:** Methodology, Investigation. **Louella Concepta Goveas:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **C Vaman Rao:** Conceptualization, Resources, Validation, Writing – review & editing.

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