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Biosorption of lead ions by exopolysaccharide producing *Azotobacter* sp.

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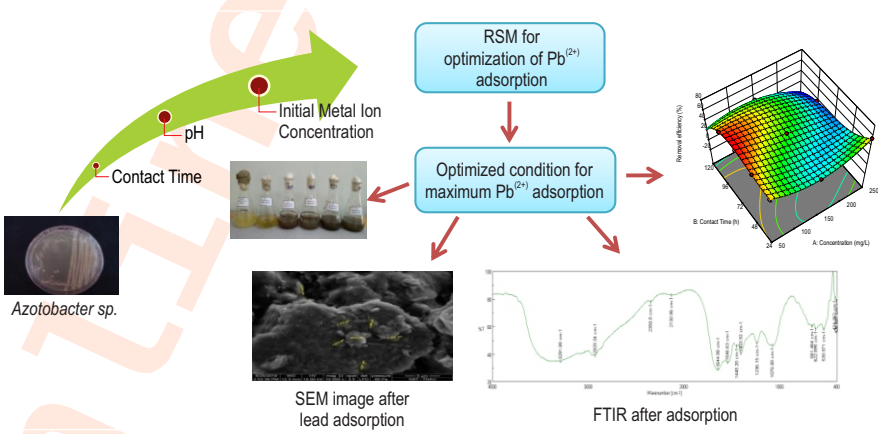
Abstract

Aim: Removal of lead from wastewater using *Azotobacter* species and optimisation of various parameters to maximise the adsorption of lead by response surface methodology as a tool.

Methodology: The bacterial isolate UBI-7 recovered from sewage water irrigated soil was examined for its biosorption potential towards lead. The lead removal efficiency of *Azotobacter salinestris* was studied with respect to metal concentration (50-250 mg l^{-1}), contact time (24-120 hrs), and pH (4-8).Using response surface methodology, these factors were optimized and R^2 value obtained was 0.9710 for lead ions, which indicates the validity of the model.Observation with Fourier Transform Infrared (FTIR), Scanning Electron Microscope imaging (SEM) and Energy Dispersive X-ray Spectroscopic analysis (EDX) were carried out to confirm lead biosorption by *Azotobacter salinestris*.



Results: The lead tolerant bacterium isolated from sewage water irrigated soil (UBI-7) was recognized as *Azotobacter salinestris* by 16S rRNA based gene sequence analysis. The highest removal percentage of Pb (61.54) was 50 mg l^{-1} in 72 hrs equilibration period. Interaction effect between different levels of Pb and different contact time of the solution were found to be significant. Lead biosorption by the organism was confirmed by the changes in stretching intensities of functional groups as well as appearance of strong OH stretching at 3291.69 cm^{-1}. Images obtained from Scanning Electron Microscope and Energy Dispersive X-ray Spectroscopic studies of the bacteria (UBI-7) before and after biosorption clearly indicated lead adsorption.

Interpretation: Current study proves that the functional groups of *Azotobacter salinestris* are involved in lead biosorption from aqueous solution which was confirmed through FTIR.EDX analysis also elucidated the lead absorption by the bacterial cells. Hence, this could be effectively utilized for decontamination of lead from the polluted environment.

Key words: *Azotobacter salinestris,* Biosorption, Lead, Response surface methodology

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Introduction

A rapid urbanization, industrial development and agricultural practices generate enormous toxic pollutants including heavy metals (Manoj *et al.,* 2020; Zhang *et al.,* 2018). Accumulation of heavy metals in the soil, plants and aquatic ecosystem successively amplify the biomagnification process in the food chain (Kaur *et al.,* 2018). Among the heavy metals, lead is one of the hazardous metal causing toxicity to environment and humans (Hu *et al.,* 2020). The main source for lead contamination in water are discharge of wastewater from processing industries, i.e., electroplating, pigment, paint, metal finishing, basic steel work and electric batteries (Ansari *et al.,* 2011). The desirable limit of Pb^{2+} concentration in drinking water is 0.01 mg l^{-1} as described by World Health Organization (WHO, 2017), while Indian standards (IS: 10500, 1991) have specified the permissible limit of 0.05 mg l^{-1} (Kumar and Puri, 2012). Owing to inappropriate treatment technologies and discharge methods, the lead concentration in water has exceeded the permissible limit (Dai *et al.,* 2019), which accumulates in the body causing severe damage to the central nervous system, kidney, bone marrow and liver (Tsoi *et al.,* 2016). Hence, there is an urgent need to develop environmental safety methods to detoxify lead contamination. The conventional methods of lead remediation from the environment can be achieved by ion exchange, chemical oxidation and precipitation, complexation, electrochemical treatment and membrane filtration techniques. Compared to these conventional methods, biosorption is gaining attention due to its economic benefits as well as eco-friendly nature (Belogolova *et al.,* 2020).

Several studies have explored that microbial cells can be used for removal as well as recovery of metal ions (Busi *et al.,* 2016; Mandal *et al.,* 2016), due to small size, fast breeding, resilience to diverse environmental conditions and high metal absorption efficiency. Bacterial detoxification of metals achieved through chelation and surface adsorption mechanism and live cells along with their products are the best candidates for removing soluble and particulate forms of metals from the environment. Gabr *et al.* (2009) reported that hydroxyl, carboxyl, phosphate and amino groups present in the cell wall participate in binding lead ions by living and non-living bacterial cells. Fomina and Geoffrey (2014) demonstrated that the presence of functional groups in the outer layer act as a barrier for metals ions by the mechanism of binding. A wide range of bacterial genera particularly *Bacillus, Pseudomonas, Paenibacillus, Azotobacter, Shewanella* and *Sinorhizobium* have been recorded for metal adsorption properties by several mechanisms such as ion exchange, surface precipitation and complexation.

Cell walls of both Gram-positive and Gram-negative bacteria carry negative charge, which bind to toxic metal cations and its membrane regulates movement of metals. Gram-positive bacteria possess a carboxyl group in the cell wall which are directly involved in metal binding process whereas phosphate groups presented in Gram-negative bacteria contribute significantly to metal binding on cell surface. *Azotobacter* sp. is a Gram-negative free-living nitrogen fixer which has tremendous application in bioremediation of heavy metals due to its exopolysaccharide production mediated biosorption capacity (Rasulov *et al.*, 2013). For example, *Azotobacter spinoculates* in soils reported to promote adsorption of lead on the surface of bacterial cell in chelated form (Belogolova *et al*., 2020) and sixty four isolates of *Azotobacter* sp. obtained from contaminated soils revealed high resistance to herbicide, antibiotics and heavy metals including Pb^{2+} (Aly *et al*., 2014). Similarly, *Azotobacter vinelandii* reported for bioremediation of Zn^{2+} (El-barbary and El-Badry, 2019) and cyanide degradation (Manogaran *et al.*, 2019). Interestingly, *Azotobacter chroococcum* exhibited a maximum exopolysaccharide production under cadmium, chromium, lead and nickel stress mediating metal immobilization (Rizvi *et al.*, 2019). In addition, Rizvi and Khan (2019) observed the level of exopolysaccharides secretion in *Azotobacter chroococcum* CAZ3 increased even under high lead concentration which increased metal chelating efficiency of the bacterium. Moreover, work in this direction is less due to lack of information about the chemical changes during the adsorption process and cellular damage, metal uptake and localization after lead adsorption certainly a way to prove the detoxification mechanism. Keeping in view the above, the present study focused towards isolation of *Azotobacter* sp. from contaminated sites, optimizing various factors for maximum biosorption using response surface methodology in order to detoxify lead from the aqueous solution.

Materials and Methods

Twelve bacterial strains were isolated from treated sewage irrigated soil, near Ukkadam, Coimbatore. All the isolates were purified and named as Ukkadam Sewage Isolate No. 1 to 12, *i.e.,* UBI 1-12. All the isolates were tested in lactose media supplemented with different concentrations of lead 500 ppm and were observed for growth as well as exopolysaccharide production on solid media surface. The isolates were selected based on their distinct appearance in the solid media and lead tolerance as well. Organisms producing colonies with watery surface can be detected macroscopically and hence, were selected as exopolysaccharide producers, which was confirmed with water soluble dye (aniline blue WS).Only three isolates (UBI-3, UBI-7 and UBI-11) showed growth and EPS production on solid media even at high concentration (500 ppm).

The isolates were grown in a broth spiked with 150 ppm lead and cells were harvested by centrifugation. After centrifugation, two volumes of ice cold isopropanol was added and stored at 4°C. Precipitated contents were collected by centrifugation and pellets were dried at 105°C and weighed. Among the three isolates UBI-7 produced higher amount of polysaccharide (4.3 g l^{-1}) and also belonged to Generally Recognized as Safe GRAS group, and was selected for further studies. Morphological, cultural and biochemical characterization was conducted to identify the organism. The result obtained was compared with the Bergey's Manual of Determinative

Bacteriology (Holt *et al.,* 1994) for identifying the culture. Based on the morphological, cultural and biochemical characteristics, the isolate UBI-7 was tentatively identified as *Azotobacter* sp. For authentic identification of strain, a molecular study was carried out by constructing a Phylogenetic Tree. DNA was purified with Insta Gene matrix DNA purification kit. Colonies were selected, picked up and suspended in 0.5 ml of sterilized saline in an eppendorf tube and centrifuged at 10,000 rpm for 10 min. The pellet alone after centrifugation was suspended in 0.5 ml of Insta Gene Matrix (Bio-Rad, USA), incubated at 56°C for 30 min and then heated to 100°C for 10 min. After heating, the supernatant was used for running PCR and purified PCR products of approximately 1,500 bp were sequenced by using primers (27 F 5'AGAGTTTGATCMTGGCTCAG3' and 149 2R 5'TACGGYTACCTTGTTACGACTT) (Weidner *et al.,* 1996).

Sequencing was performed by Sanger's method using Emerald Amp GT PCR Master mix sequencing kit (Applied BioSystems, USA). The sequenced products were resolved on an automated DNA sequencing system (Applied Biosystems model 3730 XL) and subjected to BLAST analysis. From the GenBank, similar type of strain sequence and other phylogenetic related sequences were selected and aligned with multiple sequences. The aligned sequences were subjected to Phylogenetic tree (neighbour joining) construction using MEGA 7 (Kumar *et al.,* 2016). Batch experiment was done to determine the biosorption of lead by UBI-7. The metal adsorbed by the test organisms were analysed by Atomic Absorption Spectrophotometer (Sethuraman and Balasubramanian, 2010) and percent adsorption of metal was calculated by following formula:

$$\text{Adsorption percentage (\%)} = \frac{C_i - C_f}{C_i} \times 100$$

Where, C_i and C_f are initial and final concentration

Optimization using RSM: Response surface methodology was performed to identify the interaction among complex media components and their contribution to biomass production. Design expert 11.0 software (Stat-Ease, Inc., Minneapolis, trial version) was used in the optimization process with the help of Central Composite Design (CCD). The effects of three independent variables, metal concentration (50-250 mg l^{-1}), time of contact (24-120 hr) and incubation pH (4-8) on Pb removal (%) by the isolate UBI-7 was investigated by means of a design used in the RSM for building a second order model for the variables. The effects of three factors were optimized and the response influenced was analyzed using the graphs. Multiple regression analysis of the results was carried out with Stat-EaseInc. Design Expert ver.11 software and the empirical second-order polynomial model was used to explain the behavior of the system. Statistical analyses were done as per Gomez and Gomez (1984).

Before and after adsorption of lead, the surface morphology of UBI-7 was characterized using scanning electron microscopy (SEM) and chemical characterization of the surface was analyzed with EDS (energy dispersive X-ray analysis system)(FEI- Quanta 250, Czech Republic). The infrared spectrum of the culture during the experimental study was carried out with absorption spectroscopy (Fourier Transform infrared Spectroscopy, FTIR) to detect active functional groups of bacterial biomass (UBI-7) before and after lead adsorption.

Results and Discussion

Soil samples collected from treated sewage irrigated field was enriched in lead supplemented broth and individual colonies were isolated. From the enrichment, 12 isolates were selected based on their distinct appearance in the solid media and all were observed for lead tolerance level. Organisms which produces colony with watery surface can be detected macroscopically (Suresh Kumar *et al.,* 2007). Colonies which produced watery surface were selected as exopolysaccharide producers, and was confirmed with water soluble dye (aniline blue WS). But there may not be any direct correlation between morphological characteristics on solid medium and ability to produce polysaccharide production in liquid medium. However, some polysaccharides might have formed complexes with aniline dye, which could be used further as a screening tool. The selected isolates showed varying degree of lead tolerance, maximum upto 450 ppm with and without polysaccharide production. Among the three isolates, two isolates, UBI-3 and UBI-11 exhibited tolerance upto 350 ppm and UBI-7 up to 450 ppm. In addition, UBI-7 produced higher amount of polysaccharide (4.3 g l^{-1}) and also belonged to Generally Recognized as Safe GRAS group, hence it was selected for further studies (Table 1).

The selected bacterial isolate UBI-7 was characterized and the morphological, physiological and biochemical results obtained were compared with the standard characteristics described in the Bergey's Manual (Holt *et al.,* 1994) and was tentatively identified as *Azotobacter* sp. The selected isolate UBI-7 was subjected to 16S rRNA gene sequence informatics for authentic identification. The 16S rRNA region of isolated DNA was amplified and the test amplicon of 1500 bp was purified by Gel elution/SAP. A similar search was performed using the BLAST program indicated a close genetic relatedness of UBI-7 isolate with the rRNA sequence of *Azotobacter salinestris* (16S: 68% similarity with the reference strain) in NCBI database. MEGA 7 software was used for phylogenetic tree construction with partial sequence and confirmed that the selected bacterial isolate UBI-7 as *Azotobacter salinestris* (Fig. 1).

The absorption potential of isolates was explored using broth spiked with different concentrations of lead. Various forms of lead salts (lead nitrate, lead sulphate, lead chloride and lead acetate) were examined for its suitability for the adsorption studies. Lead nitrate was found more suitable due to its high water soluble property, when compared to other forms of lead salts. Generally, bacteria have the capacity to uptake metal ions in the range of 1 to 500 mg g^{-1} (Yin *et al.,* 2018). In the present study, even though the selected isolate tolerated upto 450 ppm, the lead

Table 1: Bacterial isolates obtained from the sewage irrigated soil from Ukkadam,Coimbatore

Metal Concentration (ppm)	Bacterial isolates obtained from the sewage irrigated soil																							
	Isolate 1		Isolate 2		Isolate 3		Isolate 4		Isolate 5		Isolate 6		Isolate 7		Isolate 8		Isolate 9		Isolate 10		Isolate 11		Isolate 12	
	G	EPS	G	EPS	G	EPS	G	EPS	G	EPS	G	EPS	G	EPS	G	EPS	G	EPS	G	EPS	G	EPS	G	EPS
10	+++	+	+++	-	+++	+	+++	+	+++	+	+++	+	+++	+	+++	+	+++	+	+++	+	+++	+	+++	-
20	+++	+	+++	-	+++	+	+++	+	+++	+	+++	+	+++	+	+++	+	+++	+	+++	+	+++	+	++	-
30	+++	+	+++	-	+++	+	+++	-	+++	+	++	-	+++	+	++	-	+++	+	+++	+	+++	+	+	-
40	++	-	++	-	+++	+	++	-	+	-	+	-	+++	+	++	-	+++	+	+++	+	+++	+	+	-
50	++	-	++	-	+++	+	++	-	+	-	+	-	+++	+	+	-	++	-	+++	+	+++	+	+	-
100	+	-	+	-	+++	+	++	-	+	-	+	-	+++	+	+	-	++	-	+++	+	+++	+	-	-
150	+	-	+	-	+++	+	+	-	+	-	+	-	+++	+	-	-	+	-	++	+	+++	+	-	-
200	-	-	+	-	++	+	+	-	-	-	-	-	+++	+	-	-	+		++	+	+++	+	-	-
250	-	-	-	-	++	+	-	-	-	-	-	-	++	+	-	-	+	-	+	-	++	+	-	-
300	-	-	-	-	++	+	-	-	-	-	-	-	++	+	-	-	+	-	-	-	++	+	-	-
350	-	-	-	-	+	+	-	-	-	-	-	-	++	+	-	-	-	-	-	-	+	-	-	-
400	-	-	-	-	+	-	-	-	-	-	-	-	++	+	-	-	-	-	-	-	+	-	-	-
450	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-
500	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-

GrowthKey (G) :-: No growth; +: Scanty growth; ++: Moderate growth; +++ : Heavy growth

Exopolysaccharide production by visual observation: + Mucoid or watery surface, Yellowish brown surface; - No slime

Table 2: Lead removal at different time interval with different lead concentration by *Azotobacter salinestris* UBI-7

Initial concentration (ppm)	Time (Hr)				
	24	48	72	96	120
50	52.04±1.17	56.62±0.85	61.54±1.14	56.82±0.88	54.84±0.88
100	42.64±0.84	39.11±0.87	47.35±1.20	43.75±0.59	40.64±0.59
150	26.76±0.06	29.16±0.45	39.61±0.43	34.20±0.58	31.76±0.14
200	25.85±0.29	25.72±0.37	35.82±0.14	31.65±0.67	25.18±0.64
250	16.44±0.41	16.92±0.24	17.51±0.15	16.13±0.24	16.16±0.21
Control	ND	ND	ND	ND	ND

concentrations were fixed between 50 to 250 ppm. The reason being there were no marked differences in growth and colony appearance among the isolates upto 50 ppm. In addition, so far the maximum concentration of lead recorded in sewage irrigated soils in Coimbatore was 184.82 mg kg^{-1} (Pavithrapriya *et al.,* 2015), therefore the maximum spiking concentration was fixed as 250 ppm. The selected isolate UBI-7 is a Gram- negative bacteria and the cell wall of bacteria might have acted as a barrier against Pb ions. Huët *et al.* (2017) demonstrated that the isolates of *Enterococci* and *Bacillus* species also showed polysaccharide production which might have aided in binding of Pb ions. There are several mechanisms by which metals are detoxified, and to understand this phenomenon, cell surface characteristics were studied. Isolate UBI -7 was inoculated into lactose broth, centrifuged at 7000 rpm for 30 min and was further analyzed for structural composition, metal sequestration by SEM with EDX and FTIR technique.

Batch experiment was conducted to study the effect of variables like metal ion concentrations, time of contact and pH on adsorption of lead. Response surface methodology was performed to study the interaction among multiple media components. Optimization of above parameters (metal concentration (50-250 mg l^{-1}), time of contact (24-120 hr) and pH (4-8) was investigated by means of central composite design to assess the Pb removal percent. As far as the effect of metal ion concentration on percent lead removal is concerned, the isolate UBI-7 (*Azotobacter salinestris*) showed decreased removable efficiency with the increasing Pb concentrations, *i.e.,* the highest Pb adsorption was observed at 50 ppm (56.37%) and the lowest was observed at 250 ppm (16.63%) (Fig. 2a).The current study is well correlated with Sethuraman and Balasubramanian (2010), who reported that maximum Cr (VI) removal by *Pseudomonas aeruginosa* increased along with concentration, to certain level and decreased with further increase. Similarly, Shaarani *et al.* (2019) reported that maximum copper removal of 41 % was achieved at low initial metal concentration of 25 mg l^{-1} by *Alcaligenes* sp. and Dai *et al.* (2019) reported *Lactobacillus brevis* increased Pb (II) adsorption from 8.06 to 51.43 mg l^{-1}.

These results are in conformation with the previous reports of Oves *et al.* (2013) in *Bacillus* sp., and Vishan *et al.* (2017) in *Bacillus badius*. Lead ion adsorption percentage depends how metal ion interacts with the binding sites. The maximum metal adsorption at lower Pb concentration may be related to the presence of more adsorption sites on bacterial outer layer. The available sites for electrostatic interaction and ratio between number of binding sites and free lead ions in the solution are gradually decreased with increasing Pb concentration which may be the main reason for reduction in removal percentage at higher Pb concentration (Dai *et al.,* 2019).

The adsorption potential of UBI-7 was increased with increase in contact time from 24 h to 72 h, after that it showed decreasing trend upto 120 h (Table 2).The maximum adsorption of Pb by EPS producing bacteria *Azotobacter salinestris* was 61.54% in 72 hr and the lowest adsorption (16.13%) was observed in 120 hr. In general, the absorption potential varied from 2 - 120 hrs depending on the organism. In the present study, the absorption was maximum at 72 hr, which is in consistent with Chug *et al.* (2016), who reported that highest biosorption of Cr by *Azotobacter beijreinckiiwas* reached at 72 hr (Fig.2a). Contrary to our observation, Kushwaha *et al.* (2012) observed the highest biosorption of lead by *Acinetobacter juniiatv* was reached after increasing the contact time to 120 hr. The effect of pH on the adsorption of lead by UBI-7 (*Azotobacter salinestris*) was determined for pH 4, 5, 6, 7 and 8. As the pH increases (from 4 to 6), the lead removable efficiency of the culture also increased and then decreased upto pH 8 (Fig. 2b). The maximum adsorption of Pb was observed at pH 6 (52.75 %) and minimum at pH 8 (18.83 %). Similarly, Busi *et al.* (2016) reported that an increase in pH increases the lead removal percentage and 81.44 % Pb removal was achieved at pH 10 by bacterial biomass of *Aeromonas hydrophila* Rc1. Vishan *et al.* (2017) reported the optimum pH was found to be 5.0 for maximum biosorption capacity of Pb (II) by *Bacillus badius* AK. In the same way, *Rhodococcus* sp. HX-2 strain attained maximum Pb(II) biosorption at pH=5.0 and decreased beyond (Hu *et al.,* 2020).

The isolate UBI-7 showed maximum absorption at pH 6, which is in line with the findings of Oves *et al.* (2013). The reason for low adsorption of *Azotobacter salinestris* UBI-7 in acidic pH (4 and 5) may be due to competition between metal ions and H_3O^+ions for the adsorption sites (Sofiane and Sofia, 2015). Certain quantity of metal fixed on the surface of the biosorbant material, so saturated. As increasing pH (4 to 6), negatively

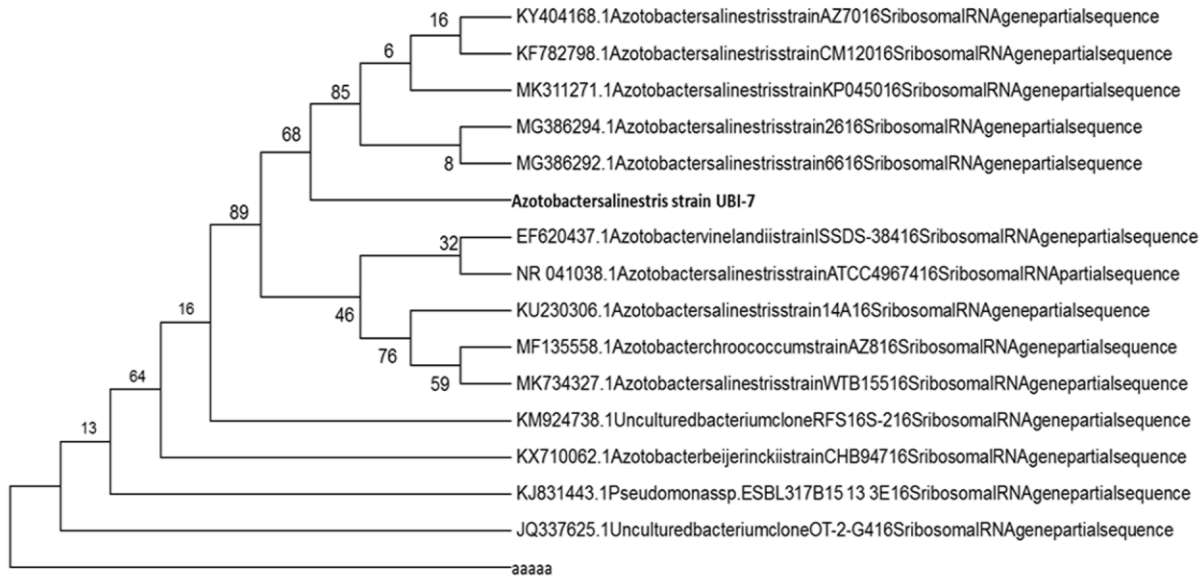

Fig. 1: Phylogenetic tree of UBI-7.

charged functional groups would be exposed in cationic metal might proceeds the adsorption process (Lamelas *et al.,* 2006). While at high pH range (7 to 8), precipitate formation as lead hydroxides by lead ions and hydroxide in the solution might reduce the amount of free lead ions in the solution. This is the reason why unavailability of lead ion for adsorption decreases the removal percentage (Peligro *et al.,* 2016). Therefore, pH dependence of *Azotobacter salinestris* UBI-7 on lead biosorption not only reliant on bacterial surface functional groups but also intracellular metal forms in the aqueous solution (Hu *et al.,* 2020). Similarly, Rasulov *et al.* (2013) reported similar results in Pb and Hg adsorption by *Azotobacter chroococcum* XU1 with the increasing pH from 4-7. Çolak *et al.* (2011) reported that the maximum Pb biosorption by *Bacillus* sp. was obtained at pH 6.0

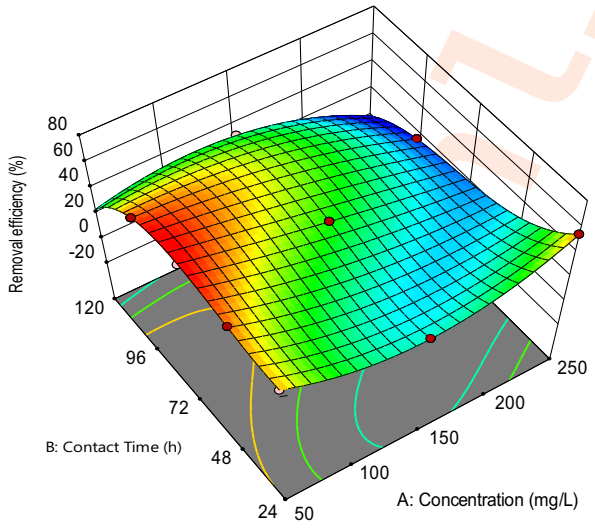

Fig. 2a: Response surface plots for the effect of contact time and initial lead ion concentration (mg l^{-1}) on lead removal (%).

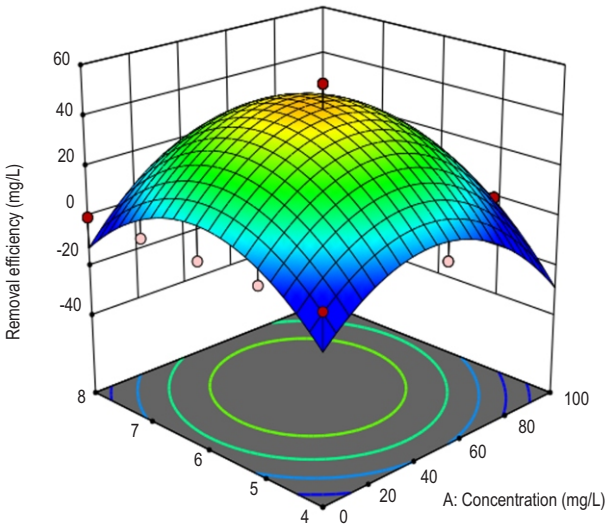

Fig. 2b: Response surface plots for the effect of initial solution pH and initial lead ion concentration (mg l^{-1}) on lead removal (%).

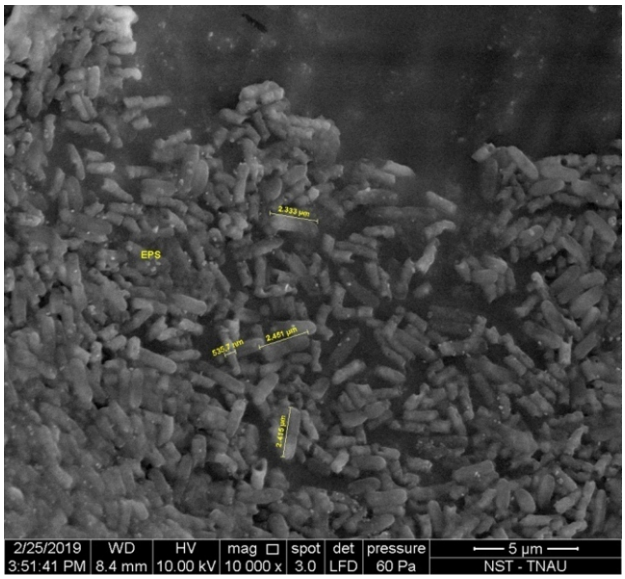

Fig. 3: SEM image of *Azotobacter salinestris* before lead adsorption.

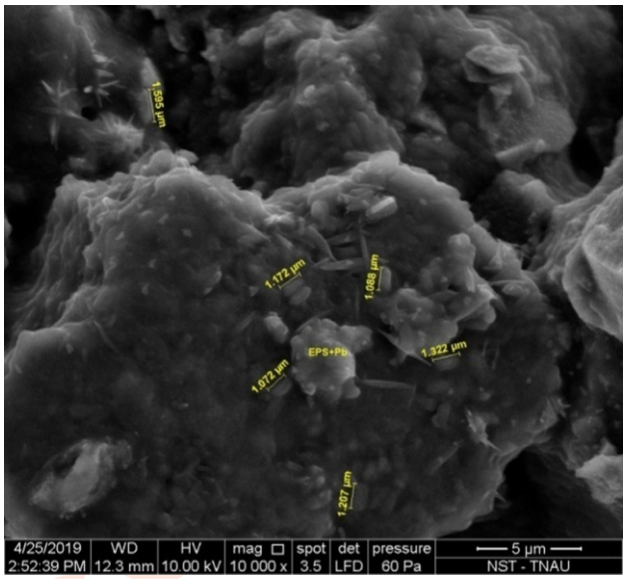

Fig. 4: SEM image of *Azotobacter salinestris* after lead adsorption.

and showed a decreasing trend (pH 8.0). Statistical significance was performed by the analysis of variance (ANOVA). Statistically significant regression was noticed at *F*-value of 1637.84 and p-value <0.0001(>F). The co-efficient value (R^2 =0.9710) pointed that only 0.29 % of the total variable was not explained by the model. The response was well predicted, since R^2 is to closer to 1 which shows the fitness of model as well as highly significant. The value of adjusted determination coefficient (adjusted R^2 = 0.9566) was high, and the predicted R^2 value was 0.9032 depicted that only 10.68 % of the total variations was not included.

This indicating that predicted R^2 is in agreement with the adjusted R^2. At the same time, comparatively low value of the coefficient of variation of 4.59 shows a good precision and reliability of the present study. After the analysis of variances, the regression equation provided the percent lead removal as a function of initial pH, metal ion concentration in the solution as well as the contact time. Multiple regression analysis of the experimental results was done, and the results were fitted with a second-order polynomial equation. The empirical relationship between lead ion removal (*Y*) and independent variables in coded

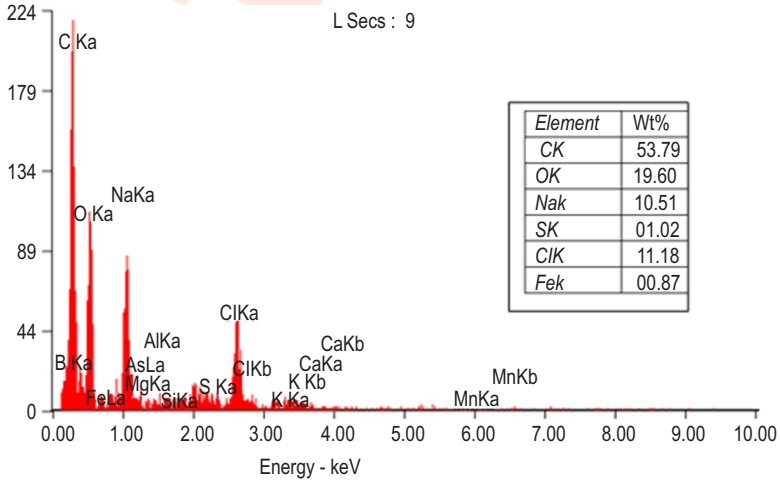

Fig. 5: EDX micrograph of *Azotobacter salinestris* before lead adsorption.

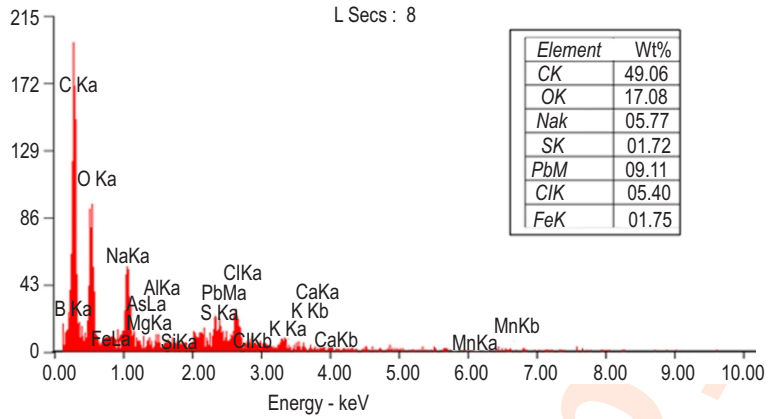

Fig. 6: EDX micrograph of *Azotobacter salinestris* after lead adsorption.

units acquired by the application of Response Surface Methodology are as given below Pb removal (%) (Y)= 39.51–20.53 X_1+20.74 X_2– 6.76 X_1X_2+2.09 X_2-8.78 $X_2$2-21.78 $X_1$2 X_2+15.20 $X_1X_2$2-0.1559 X_3-19.68 $X_3$2 (1), Where X_1 - contact time in hrs; X_2- Pb concentration in mg l^{-1}; X_3 - initial solution pH The cumulative effect of contact time and lead ion concentration on Pb removal at constant biomass dosage and pH is depicted in (Fig. 2a, b). The initial pH exerted stronger influence when compared to initial metal concentration, which could be deduced from the coefficient of factors in equation (1). By setting three factors, the maximum Pb removal was noticed at contact time 72 hr, initial concentration 50 mg l^{-1} as per the software Design Export and lead removal was found to be 61.54 %. In the present study, the experiments were performed at low salt concentrations, which are crucial in final adsorption capacities.

The surface morphology of *Azotobacter salinestris* before and after Pb adsorption was studied under Scanning Electron microscope. The SEM images of *Azotobacter salinestris* showed rod shape and size (2µm) on the external surface of bacterial biomass. The microporous structure was observed with 10000 X resolution and the image was taken with a particle size of 5 µm. The surface of *Azotobacter salinestris* was smooth before Pb adsorption (Fig.3), however, after Pb adsorption was found rough surfaced (Fig.4). Several studies have reported modification is shape of *Pseudomonas aeruginosa* strain, (Zolgharnein *et al.,* 2010) and *Klebsiella* sp. (Munoz *et al.,* 2015) after lead adsorption. Jin *et al.* (2016) observed that cells of *Arthobacter* sp. shrunk significantly after lead adsorption. *Azotobacter chroococcum* CAZ3 treated with Cd, Ni and Cr displayed shrinkage of cells under SEM observation (Rizvi *et al.,* 2019). These results are consistent our observation that bacterial cell was rod shaped and cell surface was smooth before adsorption. After adsorption of Pb, the bacterial surface had significant changes like rough, broken and wrinkled and porous. This modification in shape and roughness of bacterial species might be due to adsorption of Pb ions on the surface of *Azotobacter salinestris.* EDX analysis is an appropriate method to analyze metal adsorption on the bacterial biomass, an indirect analyses of the metal ions consequence. The elemental composition of EPS producing *Azotobacter salinestris* before and after Pb adsorption was examined through EDX analyser (Fig. 5, 6).

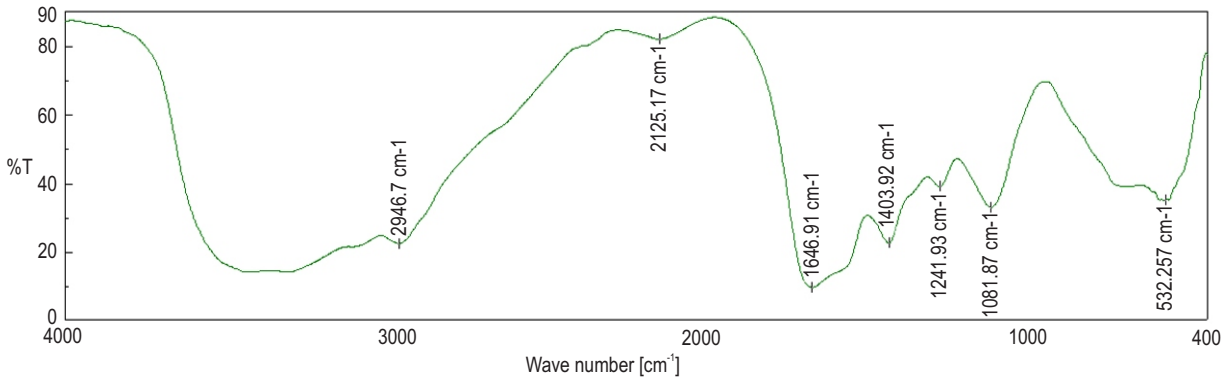

Fig. 7: FTIR analysis of *Azotobacter salinestris* before lead adsorption.

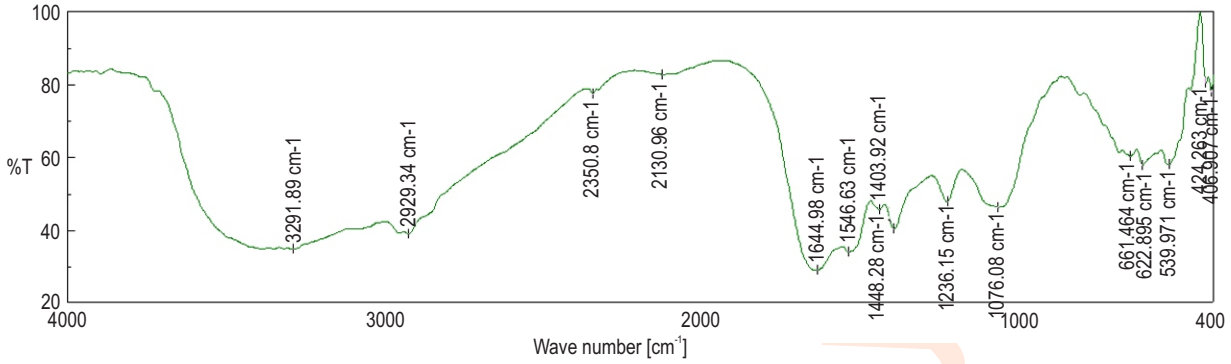

Fig. 8: FTIR analysis of *Azotobacter salinestris* after lead adsorption.

The EDX analysis indicated that the chemical composition of *Azotobacter salinestris* significantly changed after lead adsorption. The EDX spectrum of bacterial biomass in native condition exhibited different peaks of carbon, oxygen, sodium, chloride, calcium and magnesium; whereas the EDX spectra attained after lead adsorption revealed distinct peaks for relevant lead metal as well. The percentage weight of elements carbon (C) and oxygen (O) decreased from 53.79 to 49.06 and 19.60 to 17.085 %, respectively; whereas iron (Fe) and potassium (K) increased from 0.87 to 1.75 % and 1.14 to 2.80 %, respectively after lead uptake. Lead content was found to be 9.11% after lead adsorption, which was not detected before spiking with lead. In the current study, before Pb adsorption, the sodium content was 10.27 %, but after Pb adsorption it was reduced to 5.77 %, which is in accordance with the report of Erkaya *et al.* (2014), who also observed that after adsorption there was a reduced sodium ion in bacterial biomass. Similarly, Qiao *et al.* (2019) reported reduced Na+ concentration in *B. subtilis* X3 biomass after Pb adsorption. This may be due to sodium being the most suitable ion to exchange with lead from aqueous solution and this action of adsorption bridging between lead ions and EPS surface of *Azotobacter salinestris* promotes immobilization of lead ions (Qiao *et al.,* 2019). An increase in the iron content was noted after adsorption on the surface of bacterial biomass might be due to surface alteration through iron impregnation. Muñoz *et al.* (2015) also observed changes in chemical composition of *Klebsiella* sp. obtained by EDX.

The composition of C and O, as well as Fe and K were changed from before to after adsorption. It may be due to deposition of lead ions, metal sequestration and precipitation of organic functional groups involved in lead adsorption. Jin *et al.* (2016) observed alteration in the chemical composition of *Arthobacter* sp. after adsorption of lead. The relative abundance of chloride (Cl−) ions was 11.18 % before lead adsorption and it decreased after lead adsorption, indicating loss of chloride ions to 5.41%. Vishan *et al.* (2017) recorded same due to accumulation of lead ions in *Bacillus badius* acquired by EDX. This indicates that the lead binds on the surface of EPS producing bacteria through displacement of chloride (Cl−) by ion exchange mechanism. These alterations in chemical composition of bacterial biomass might be due to lead adsorption on UBI-7 surface which may have taken place due to ion-exchange mechanism. FTIR spectroscopy method was used to differentiate changes in the functional groups present in the surface of *Azotobacter salinestris* before and after adsorption of Pb ions. The FTIR spectrum of *Azotobacter salinestris* showed varied peaks within the interval of 4000-400 cm^{-1}, which showed the complex chemical nature. The FTIR micrographs of *Azotobacter salinestris* before and after absorption of Pb are given in Fig. 7, 8. The FTIR analyses was carried out at 24, 48, 72, 92 and 120 hrs, the maximum adsorption was observed at 72 hr. Therefore, 72 hrs was discussed here.

The band at 2946.7 cm^{-1} was assigned to the vibration of alkane groups in EPS producing *Azotobacter salinestris.* The prominent stretch in the –C-H group at 2929.3 cm^{-1} after lead adsorption might be associated with lead adsorption. The strong intensity of alkene functional group with (=C-H) band was detected at 1646.91 cm^{-1} before absorption, and after Pb absorption it changed to 1644.98 cm^{-1}. The nitro compounds (N-O) at wavelength of 1548.21 cm^{-1} appeared after lead adsorption at 72 hrs and amine groups were disappeared. The functional groups such as carboxyl, amino, phosphate and sulfate groups on the bacterial surface can bind with heavy metals and accumulate on the bacterial surface. The cationic and anionic functional groups present in the exopolysaccharide might have accumulated the metal ion efficiently (Yin *et al.,* 2018). Our results also line with the reports of Singh *et al.* (2010) who reported that the adsorption peaks at 3200-3500 cm^{-1} indicates the involvement of carboxylic acid and amino groups, and also significant changes in the stretching intensities after adsorption. The surface chemical functional groups identified by FTIR are carboxyl, hydroxyl, amino and carbonyl groups may be involved in the biosorption of heavy metals.

The band corresponding to 2125.17 cm^{-1} (-C=C) before absorption changed to 2130.96 cm^{-1} after absorption. Moreover, the band at wavelength 1241.93 cm^{-1} showed that presence of

alkyl halide group before absorption which changed to 1236.15 cm^{-1} after absorption. These changes in band group might be due to interaction lead ions with *Azotobacter* sp. (Liu *et al.,* 2015). After Pb adsorption, *Azotobacter salinestris* exhibited alcohol ring structure (O-H stretch) and alkane (C-H stretch) at the band 3291.69 cm^{-1} and 2929.34 cm^{-1}, respectively (Fig.8). The band corresponding to alkyne (C=C), alkene (C=Cstretch), alkyl halide (C-Cl stretch) were detected at 2130.98 cm^{-1},1236.15 cm^{-1} and 661.464 cm^{-1}, respectively. Similarly, Feng *et al.* (2012) reported that functional groups such as-OH, $–CH_2$,C=O, -N-H,-COO-, -C-N were expressed in biomass of *Lactobacillus plantaram* after lead adsorption. These FTIR results confirmed that adsorption process of lead ions by *Azotobacter salinestris* is mainly attributed to the presence of functional groups on outer biomass (Qiao *et al.,* 2019).

The appearance of strong and broad peak due to OH stretching (3291.69 cm^{-1}) in *Azotobacter salinestris* may be due to Pb adsorption. This is in confirmation with the study of Liu *et al.* (2015) who observed that hydroxyl (-OH) stretching was the dominant metal adsorbing group. Similarly, Chug *et al.* (2016) reported that carboxyl groups are involved in adsorption of Cr (IV) by *Azotobacter beijreinckii.* The presence of negative charge (carboxylic and hydroxyl groups) on the surface of bacterial biomass may be the reason for lead adsorption. The alcohol group was detected in *Azotobacter salinestris* biomass after adsorption of lead which may be due to the stress caused by the metal. Hence, shifting in peaks after lead biosorption relative to control is correspondence with lead binding on bacterial surface by EPS and overloading of metal concentration disturbs EPS functional groups (Rizvi and Khan, 2019). Therefore, from this study it can be concluded that the identified *Azotobacter salinestris* (UBI-7) is a good candidate for remediation of lead contaminated environment. So this isolate can be effectively exploited as a potential organism for the remediation of lead. However, the biosorption potential of *Azotobacter salinestris* was studied with whole biomass. The purification of exopolysaccharide may further strengthen the understanding of metal interaction as well as developing exopolysaccharide as bio sorbent commercially for lead contamination.

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Add-on Information

Authors' contribution: Dr. P. Dhevagi: Objective formulation and overall guidance for the research work, preparation of manuscript; **Ms. S. Priyatharshini:** Laboratory work, Phylogenetic tree construction, RSM analysis, FTIR studies; **A. Ramya:** SEM sample preparation and imaging, EDX analysis; **M. Sudhakaran:** AAS analysis

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