Original Article

Isolation and Characterization of a New *Bacillus licheniformis* Strain for Bioleaching Heavy Metals

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Abstract

Introduction: Increased usage and improper management of electronic wastes results in immense environmental pollution. Although conventional techniques are well known for removing heavy metals from the environment, their high cost and severe environmental consequences indicates urgent requirement of cost-effective methods of heavy metals uptake. Bioaccumulation can be considered as an alternative to the traditional methods in terms of their cost effectiveness and maximum recovery of the metal ions.

Materials and Methods: This study deals with the isolation of heavy metals tolerant Gram-positive bacterial strain, Bacillus licheniformis JAJ3 and its application in bioaccumulation of copper, lead and nickel and bioleaching of heavy metals from electronic waste. For this purpose, 16S rRNA sequencing was performed to identify the bacterial strain. The accumulation study was carried out in liquid medium and analyzed using atomic absorption spectroscopy (AAS). Bioleaching activity was checked using one-step procedure. For bioleaching studies of heavy metals, printed circuit boards (PCBs) were used as a source of electronic wastes. Scanning electron microscopy and energy dispersive spectroscopy were used to record the changes before and after experimental procedures.

Results: The organism was able to accumulate 98.6% copper, 64.6% lead and 57.3% nickel. The bioaccumulation reaction followed pseudo second order kinetics model (R^2 value 0.92, 0.92, 0.99 for copper, lead and nickel bioaccumulation respectively). Efficient bioleaching activity was shown by the strain.

Conclusions: The experimental analyses confirmed that the strain is efficient in bioleaching heavy metals from electronic wastes and thus can be used in the management of electronic wastes.

Keywords: Atomic Absorption Spectroscopy; Bacteria; Bioaccumulation; Electronic Wastes; Kinetics; Pollution

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Introduction

Improper handling and management of electronic wastes (e-wastes) affects the environment to a large extent. Disposal of e-wastes directly to the soil releases pollutants in the soil which reaches the ground water further contaminating the water and land.[1] After disposal of e-wastes in the environment, it leads to pollution due to metal corrosion, leaching of heavy metals and sediment re-suspension in soil and water. E-wastes contains different hazardous heavy metals, like copper, lead, cadmium, nickel, lithium, mercury, chromium and zinc.[2] Although heavy metals are one of the most important constituents of earth's crust, excessive amount of heavy metals causes environmental contamination and thus affects human, animals and plants. Presence of heavy metals in living beings affects various cell organelles and enzymes responsible for metabolism, detoxification and damage repairing. The interaction of metal ions with DNA and proteins results in constitutional changes of the DNA and proteins leading to carcinogenesis or cell death.[3]

Remediation of heavy metals using conventional physicochemical methods, such as chemical precipitation, extraction, ion-exchange, membrane filtration, reverse osmosis and electrochemical process are well known. These methods, however, are inefficient in remediation of heavy metals and result in the release of toxic secondary sludge which is one of the major disadvantages of traditional techniques for heavy metal removal.[4] Application of living microbes in recovery of heavy metals from contaminated water or soils, also called bioremediation has been implemented in many cases. Bioremediation can be claimed to be a better choice than conventional techniques as it is cost effective with no hazardous sludge. Bioremediation involves several techniques which are biosorption, bioaccumulation, biotransformation and biomineralization.[5] Biosorption of heavy metals, like copper, chromium, nickel, cadmium and lead using various bacterial strains are reported by several researchers. Some of the bacterial strains used as biosorbents are *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, *Bacillus thuringiensis* and *Thiobacillus ferrooxidans*.[6] Biosorption of heavy metals have also been implemented using fungal strains,

like *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus terreus*, *Penicillium spinulosum*, *Penicillium notatum*, *Penicillium chrysogenum* and also *Rhizopus arrhizus*.[7,8] However, not much articles have reported the bioaccumulation of heavy metals and bioleaching of metal ions from electronic wastes.

In the present study, bacterial isolate has been isolated from soil samples collected from river banks. The isolated strain was identified using 16S rRNA sequencing and checked for its heavy metals tolerance capacity. The bioaccumulation ability of the strain was studied thoroughly along with its kinetics parameters. The application of the strain in recovery of heavy metals from printed circuit boards (PCBs) was assessed.

Materials and Methods
Chemicals Used
All the chemicals were of analytical grade purchased from Himedia. The solvents and other chemicals used in the study were of high purity.

Preparation of the Aqueous Solution of Heavy Metals
Stock solutions of Cu^{2+}, Pb^{2+} and Ni^{2+} were prepared individually using $CuSO_4.5H_2O$, $PbNO_3$ and $NiSO_4.6H_2O$ respectively.

Collection and Pretreatment of Soil Sample
The soil sample was collected from the river banks of Varanasi, Uttar Pradesh, India (25.3176° N, 82.9739° E). The sample site was chosen based on the exposure of metal contaminants on the river banks. Soil was acquired from the surface layer (0-20 cm) of the site. The collected sample was dried at room temperature, ground and sieved to remove large particles.

Isolation of Heavy Metal Tolerant Bacteria
The soil sample was subjected to serial dilution and spread plate technique for isolation of metal tolerant bacteria. To do so, 0.1 mL of serially diluted soil sample was spread on nutrient agar medium and incubated at 37°C for 24-48 hours. The obtained bacterial isolates obtained were then inoculated in nutrient broth and agar plate supplemented with 100 mg/L of Cu^{2+}, Pb^{2+} and Ni^{2+}. Bacteria which were able to grow in the presence of heavy metals were selected for further studies. Pure cultures were maintained on nutrient agar slants and were stored at 4°C.

Morphological, Biochemical and Taxonomic Characterization
Gram staining was performed to analyze the Gram nature (positive or negative) and the shape of the isolate. Physiological characteristics were studied by performing the biochemical tests, such as, IMViC test (Indole, methyl red, Voges Proskauer and Citrate Utilization), triple sugar iron, nitrate reductase and gelatin hydrolysis test.[9]

Molecular characterization and identification of the strain was carried out by 16S rRNA gene sequence analysis. The bacterial DNA was isolated, purified and used for performing polymerase chain reaction in a final volume of 25 μL volume which contained 5 μL template DNA, 1.5 μL each of forward and reverse primers, 5 μL of deionized water and 12 μL of Taq master mix (Taq DNA polymerase, 0.4 mM dNTPs, 3.2 mM

$MgCl_2$ and 0.02% bromophenol blue). Gene amplification was conducted in three steps: denaturation, annealing and extension. The initial denaturation carried out at 94°C for 3 minutes was followed by another denaturation step at 94°C for 30 seconds. Annealing was performed for 30 minutes at 60°C. Finally, the extension step was accomplished at 72°C for 10 minutes completing one cycle. The cycle was repeated for 35 times. The amplification product was then sequenced with primers 27F and 1492 R. Post amplification, the purification and sequencing of the fragments were obtained using ABI 3130 Genetic Analyzer.[10] The sequence obtained after 16S rRNA gene sequencing was submitted to GenBank National Center for Biotechnology Information database. The ClustalW was used to perform the multiple alignments of the sequences.[11] The phylogenesis analysis was studied using MEGA 7.0 software. For this purpose, the phylogenetic tree was prepared according to neighbor-joining method.

Heavy Metals Tolerance Capacity of the Bacterial Strain
About 1000 ppm stock solution of the heavy metals was prepared for determining the heavy metals tolerance capacity of the isolate. For this purpose, 10-150 ppm of Cu^{2+}, Pb^{2+} and Ni^{2+} were used. The tolerance capacity was detected based on the growth of the bacterial strain. Also, 1 mL of bacterial culture was inoculated in 100 mL of nutrient broth spiked with heavy metals. Optical density was measured at 600 nm to determine the growth of the strain at regular time interval. The concentration of heavy metals was increased until the highest minimum inhibitory concentration was detected.[12]

Growth Kinetics
The growth curve of the isolated bacterial strain was examined in nutrient broth. The strain was inoculated in nutrient broth and incubated for 24 hours. About, 1 mL of the culture was diluted to 10^{-1} after incubation and inoculated in medium to record growth pattern. The growth was checked by measuring the optical density at 600 nm.[13]

Bioaccumulation of Heavy Metals Using the Bacterial Strain
The bioaccumulation experiment of heavy metals using the isolated bacterial strain was conducted in liquid medium. The nutrient broth used for the study was amended with 100 ppm of individual heavy metals and inoculated with 1 mL of bacterial culture. The experimental setup was incubated at room temperature at 120 rpm. Nutrient medium with heavy metals in absence of the organism was incubated in the same conditions and was used as control setup. The percentages of removal of heavy metals from the liquid medium by the bacterial strain were analyzed using atomic absorption spectroscopy (AAS) (Varian Spectra A240).[14] The percentage of bioaccumulation efficiency was calculated using the below formula:

$$\% \ Bioaccumulation \ Efficiency = \frac{C_i - C_f}{C_i} \times 100$$

Where, C_i and C_f signifies the initial and final concentration of heavy metals (in ppm or mg/L).

Kinetics Study

The rate constant during the bioaccumulation reaction using the bacterial strain was examined by applying the experimental results in different kinetic models, such as pseudo first order, pseudo second order, Elovich, intraparticle diffusion and Boyd's Kinetic models. Regression line plotted between ln (q_e-q_t) versus t (pseudo first order kinetics model), and t/q_t versus t (pseudo second order kinetics model), q_t versus Ln t (Elovich Model), q_t versus $t^{0.5}$ (Intraparticle diffusion model) and B_t versus t (Boyd's kinetics model) was evaluated to determine the kinetics parameter followed during uptake of heavy metals by the organism.

The equation denoting the different kinetics models are represented below:

$$\ln(q_e - q_t) = \ln q_e - k_1 t \text{ (Pseudo First Order)}$$

k_1 denotes the pseudo first order rate constant, q_e represents the amount of heavy metals removed at equilibrium (mg/g) and q_t is the amount of heavy metals accumulated at time t.

$$\frac{1}{q_t} = \frac{t}{q_e} + \frac{1}{k_2 q_e^2} \text{ (Pseudo Second Order)}$$

k_2 in the above equation denotes pseudo second order rate constant.

$$q_t = \frac{1}{\beta}\ln(\alpha\beta) + \frac{1}{\beta}\ln t \text{ (Elovich Model)}$$

α represents the accumulation of heavy metals at initial phase (mg/g min) and β denotes the extent of surface coverage with the activation energy while performing chemisorption (g/min).

$$q_t = k_{id} t^{\frac{1}{2}} + C \text{ (Intraparticle diffusion)}$$

Intraparticle diffusion explains the mechanism of diffusion and diffusion coefficient. k_{id} signifies intraparticle diffusion rate constant (mg/g h$^{1/2}$), q_t is the amount of heavy metals uptake after time t and C represents the intercept.

B_t = -0.4977 – ln (1 – F) (Boyd's Kinetic Model)

Boyd's kinetic model is established based on the diffusion by boundary liquid film when the heavy metal uptake is considered as a chemical phenomenon. In the above equation, B_t denotes the mathematical function of F and F signifies the fraction of heavy metals uptake at any point of time t. Therefore, F can be explained as:

$$F = \frac{q_t}{q_e}$$

Where, q_t is the amount of heavy metals accumulated per unit biomass at time t (mg/g) and q_e is the amount of heavy metals accumulated per unit biomass at equilibrium (mg/g).

Bioleaching of Heavy Metals From E-waste using Bacterial Strain

Pieces of PCBs were used for bioleaching heavy metals using the metal tolerant bacterial strain. Two-steps bioleaching process was followed during the experiment. The bacterial strain was inoculated in sterilized Luria Bertani broth and incubated at 30°C for 24 hours. Post incubation, 1% (w/v) of PCB pieces were added to the culture and incubated at 120 rpm. Control was maintained in the absence of bacterial strain.[15]

Results

Isolation of Bacterial Strain From Soil Sample

In the present study, two bacterial strains, designated as BC1 and BC2 were predominantly obtained on the nutrient agar plates. These two strains were screened for heavy metals tolerance in both liquid and agar medium. Among the two strains, BC1 showed flourishing growth in the presence of heavy metals and thus were selected for the further experimental procedures. The pure culture was maintained in nutrient agar slants at 4°C.

Morphological, Biochemical and Taxonomic Identification of the Bacterial Strain

The isolated strain was found to be gram-positive and rod shaped structure. The biochemical analysis of the strain is tabulated in Table 1. The molecular characterization of 16S rRNA gene sequence showed similarity with genus *Bacillus*. The accession ID assigned to the sequence by GenBank was MK113985. The sequence, after CLUSTALW alignment was found to be similar with *Bacillus licheniformis* and thus the strain was named as *Bacillus licheniformis* JAJ3. The phylogenetic tree (Figure 1) was prepared using MEGA 7.

Heavy Metals Tolerance Capacity of *Bacillus licheniformis* JAJ3

The strain JAJ3 showed luxuriant growth at 120 mg/L of Cu^{2+} and Ni^{2+}. However, in case of Pb^{2+}, the highest growth was recorded at 140 mg/L. Therefore, further experiments for bioaccumulation studies were carried out with 100 mg/L for Cu^{2+} and Ni^{2+} and 120 mg/L for Pb^{2+}.
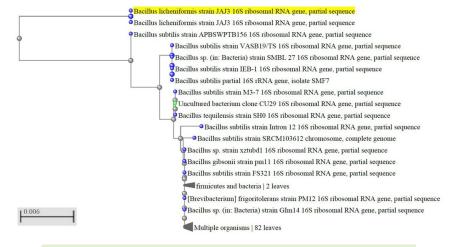
Growth Kinetics

The growth of *Bacillus licheniformis* JAJ3 was examined by analyzing the optical density at 600 nm at regular time

Table 1. Physical and Biochemical Properties of the isolated *Bacillus licheniformis* strain JAJ3

Biochemical Tests	Results Obtained
Gram Characteristic	Gram positive
Shape	Rod-shaped
Indole	Negative
Methyl Red	Negative
Voges-Proskauer	Positive
Citrate utilization	Positive
Carbohydrate fermentation	
• Glucose	Positive
• Lactose	Positive
• Maltose	Positive
• Sucrose	Positive
• Arabinose	Positive

Figure 1. Phylogenetic Tree of *Bacillus licheniformis* JAJ3 Using Neighbour Joining Method.

intervals. The strain JAJ3 was grown in nutrient broth with and without heavy metals. In absence of heavy metals, the strain showed increased growth till 2nd day which remained stable thereafter. However, in case of nutrient medium spiked with heavy metals, the growth of the strain was noticed to increase till 5th day beyond which the growth of the strain was stable (Figure 2).

Bioaccumulation of Heavy Metals Using *Bacillus licheniformis* JAJ3

The bioaccumulation study of Cu^{2+}, Pb^{2+} and Ni^{2+} was carried out with *Bacillus licheniformis* JAJ3 in liquid medium. *Bacillus licheniformis* JAJ3 removed 98.6% Cu^{2+} from the medium within 3 days. In case of Pb^{2+} and Ni^{2+}, 64.6 % and 57.3 % were accumulated by the strain till the 3rd day of incubation beyond which no significant changes were observed (Table 2).

Kinetics Study

The results attained from the bioaccumulation study of heavy metals using *Bacillus licheniformis* JAJ3 were applied to fit into pseudo first order, pseudo second order, Elovich, Intraparticle diffusion and Boyd's kinetics models. The regression plot for the individual models indicated that the bioaccumulation reaction followed pseudo second order kinetics. Complex
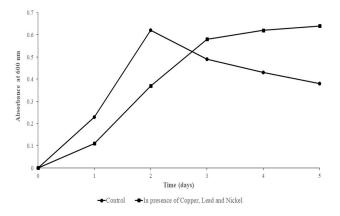
methods, like film diffusion and intraparticle diffusion occurs during interaction of fungal mycelium with heavy metal ions. The linear curve obtained during Boyd's plot did not pass through the origin indicating that the uptake of metal ions is based on film diffusion (Figure 3).

Bioleaching of Heavy Metals from E-waste Using *Bacillus licheniformis* JAJ3

Pieces of PCBs were used as a source of e-waste in this study. The surface of PCB before interaction with *Bacillus licheniformis* JAJ3 was observed to be smooth along with the presence of tiny grooves which showed surface changes after incubation of the pieces with the strain. The bacterial strain used the grooves to attach to the PCB surface and form colonies. With increased time duration, the strain JAJ3 was able to leach out metal ions from the PCB. The SEM image obtained after the interaction of PCB pieces and strain JAJ3 showed cracks on the metal surface. The EDAX spectrum with the elemental weight percentage of heavy metal ions shows the changes of heavy metals concentrations in the PCBs before and after the experiment (Figure 4).

Discussion

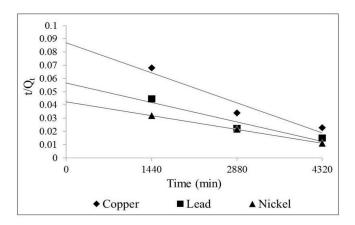The bacterial strain was identified using 16S rRNA sequencing.

Figure 2. Growth Pattern of *Bacillus licheniformis* JAJ3 in Presence and Absence of Heavy Metals.

Figure 3. The Suitable Kinetics Model Followed for the Bioaccumulation Study of (a) Cu^{2+}, (b) Pb^{2+} and (c) Ni^{2+}.

Table 2. Kinetic Parameters Obtained During Bioaccumulation of Heavy Metals by *Bacillus licheniformis* JAJ3

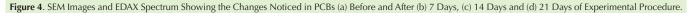| Heavy Metals	Kinetic Models										
	Pseudo-First Order			Pseudo-Second Order			Intraparticle Diffusion		Elovich Model		
	q_e (mg/g)	k (min^{-1})	R^2	q_e (mg/g)	k (g/mg min)	R^2	K_{id} (mg/g h$^{0.5}$)	R^2	α (mg/g min)	β (g/mg)	R^2
Copper	19.31	0.57	0.86	44.05	0.005	0.92	48.67	0.86	2.6E6	0.018	0.86
Lead	75.89	0.62	0.6	68.03	0.003	0.92	44.36	0.84	3E29	0.29	0.84
Nickel	1.26	0.19	0.8	96.15	0.002	0.99	47.84	0.79	2.1E16	0.08	0.79

The obtained consensus sequence was used for determining the similarity of the strain with other strains. Phylogenetic tree was prepared using molecular evolutionary genetics analysis (MEGA). The MEGA estimates an appropriate phylogenetic tree to analyze the molecular evolution of any organism, which helps in understanding their taxonomical background.[16,17] The heavy metals served as carbon and energy source for the strain JAJ3 which helped in the survival of the bacterial strain for appended time duration.[18] Bioaccumulation is a phenomenon of aggregation of heavy metals within the microbial cell through membrane transport systems.[19] The accumulation efficacy of the strain JAJ3 was analyzed using AAS. The strain proved to be much efficient in the accumulation of metal ions. In a previous report, consortium of certain bacterial strains, such as *Pseudomonas* sp., *Alcaligenes faecalis*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* could accumulate 92% of Cu^{2+} within incubation of 13-15 days.[20] *Bacillus cereus*, *Bacillus sphaericus* and *Bacillus subtilis* are few strains from the genus of *Bacillus* with reports on Cu^{2+} accumulation.[21] The effect of *Bacillus* sp. in removing lead from aqueous solution has been reported by Tunali et al.[22] The obtained bioaccumulation experimental results, appropriately fit into pseudo second order model which explained that the heavy metal uptake occurred through chemisorption.[23] During the bioleaching study, the control PCB piece was observed to be smooth with the presence of small grooves on its surface. After interaction of 7 days, the bacterial strain was able to attach to the surface of the PCB piece and colonize on the surface followed by cracking the metal piece and creating bigger crevices with increased duration in time. In the control EDAX spectrum, the presence of aluminum, nickel and lead were observed along with other certain trace elements. After the experimental process, lead and nickel were completely removed which was confirmed by the disappearance of the peaks in the EDAX spectrum. In earlier reports, iron oxidizing bacteria were reported to be capable of leaching copper from PCBs. *Leptospirillum ferriphilum* and *Sulfobacillus thermosulfidooxidans* extracted copper from PCB. The interaction of bacterial strains with the electronic wastes decreased the toxicity of the wastes and thus reduced the negative impact of the wastes on the environment.[24]

Conclusions

The current study dealt with the isolation of *Bacillus licheniformis* JAJ3 from soil sample collected around the river bank in Varanasi, India. Based on the preliminary studies, it was concluded that the strain JAJ3 could tolerate copper, lead and nickel. Bioaccumulation study revealed high efficiency of *Bacillus licheniformis* JAJ3 to accumulate heavy metals. The bioaccumulation reaction followed the pseudo second order kinetics model. Bioleaching activity of the strain JAJ3 was investigated using PCB pieces. The results confirmed that the strain was able to leach out metal ions from PCB pieces. Thus, the strain can be considered as a candidate in the management of mentioned contamination.

Figure 4. SEM Images and EDAX Spectrum Showing the Changes Noticed in PCBs (a) Before and After (b) 7 Days, (c) 14 Days and (d) 21 Days of Experimental Procedure.

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