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BIOREMEDIATION OF LEAD, NICKEL AND COPPER BY METAL RESISTANT *BACILLUS LICHENIFORMIS* ISOLATED FROM MINING SITE: OPTIMIZATION OF OPERATING PARAMETERS UNDER LABORATORY CONDITIONS

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ABSTRACT

Biosorption has been considered as effective process of bioremediation for removal of heavy metals in an ecosystem. In this study, metal resistant bacterial strains were isolated from the soil sample rich in heavy metals like Lead (Pb), Nickel (Ni) and Copper (Cu). Isolates were evaluated for their metal resistant and degradation capacity for applicability in heavy metal removal from the soil. Based on this, one efficient gram positive strain has been characterized by 16S rDNA sequencing and identified as *B. licheniformis.* The effect of metals concentration of biomass and metal sorption efficiency was determined. The optimization study showed that, optimum pH for isolate is 6.0 for Pb, 6.5 for Cu and 7.0 for Ni absorption; optimum media components are 1% glucose, 100 ml of minimal salt solution and 1 g of nitrogen source per liter. The change on surface morphology of cell wall was studied by Transmission Electron Microscopy (TEM). Changes in Chemical functional groups on the surface of bacteria with metal absorption were studied by, Fourier Transform Infrared (FTIR) spectroscopy and crystalline nature by X-ray Diffraction (XRD). The bioreactor was designed and operated at optimized conditions with three different flow rates of medium and result showed the maximum sorption of Pb 86 ± 5% at 192 h, Ni 77 ± 8% at 240 h and Cu 80 ± 6% at 216 h.

KEYWORDS: Bioremediation, *Bacillus Licheniformis*, Metal Resistant, Soil Reactor, Heavy Metal, Biosorption

INTRODUCTION

Heavy metals are those which have densities higher than 5 g cm^{-3}, among these some metals (Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni and Zn) are essential and serves as micronutrients while some other metals (Ag, Al, Cd, Au, Pb, and Hg) are non-essential and have no biological role (Bruins et al.2000). Nonessential metals are potentially toxic as well as carcinogenic to living organisms, whereas essential metals like copper or even though serving as the nutrients they are uncertain at the upper limit of the acceptable range of oral intake (more than 2 - 3 mg day^{-1}) in adult human (IPCS 1998). These toxic heavy metals mobilized and released into the environment by the result of modernized human activities like industries, municipal sewage and mining activities (Lloyd and Lovely 2001). Once the metallic species released into the environment they tend to persist indefinitely, circulating and accumulating in the domestic area, further reaches up to the food chain, thus causing a serious threat to the aquatic and terrestrial organisms as well as human health.

Both the removal and or recovery of heavy metals from contaminated ecosystem are essential and it's a challenging task in the protection of the environment and human health.

There are several chemicals, physical and biological methods were identified to treat heavy metal contaminated area such as adsorption (Olgun et al. 2009), bio sorption (Davis et al.2003), coagulation (Stephenson andDuff1996), chemical oxidation (Mohan et al. 2001), membrane filtration (Sojka et al. 2010), and anaerobic treatment (You et al. 2010). Among these methods, absorption has been considered as effective process because of its efficiency and ability of biological materials to bind heavy metals (King et al. 2007). Several reports revealed that the bacterial community was used for bio sorption of metals due to their characteristic structure of cell wall; especially Gram-positive bacteria have a greater ability to bind metals than gram-negative bacteria (Matyar et al. 2008, Jaafar et al. 2015). This is because of their characteristic cell wall structure formed with teichoic acids and their phosphate groups and carboxyl groups which formed by peptidoglycan layers were main key components for the uptake of metals (Da Costa 1999, Beveridge 1989, McLean et al. 2002, Gadd 2009).

The bio sorption efficacies of bacterial strains obtained from batch studies were providing fundamental information, but these systems were not likely to be employed in industrial application. Fixed-bed system or column studies were more useful for treatment of heavy metal contaminated areas (Colaket al. 2011). In our previous work (Akshata et al. 2014)we studied the applicability of microbial culture in the crude mass for heavy metals (Cr, Pb, Ni and Cu) removal of mining residues and simulated soil residue to develop a bioremediation process. The present study describes the isolation and characterization of heavy metal resistant gram positive bacterial strain from mining soil. And evaluation of its potential use as metal sorbents for the removal of heavy metals in batch and continuous flow column systems was studied in detail.

MATERIALS AND METHODS

Soil sample collection and characterization was carried out as described in our previous work (Akshathaet al. 2014). The chemicals and reagents used were of analytical grade. Nutrient Broth media (NB) and mineral media were procured from Hi-media, Bangalore

Preparation of Metal Contaminated Soil

The soil was contaminated artificially in the laboratory by the addition of respective metal sulfates of Lead, Copper and Nickel separately. A stock solution was prepared with the respective salts by dissolving individually in distilled water to achieve concentration 10,000 mg L^{-1} and stored at 4^0C. Working standards were prepared by adding the desired quantity from stock solutions into the soil sample individually and samples were kept for 3 days on a rotary shaker at room temperature, finally stored at 4^0C.

Isolation of Metal Resistant Bacteria

Wet soil sample (10 g) was homogenized at 3000 RPM for 1 min with 90 ml of sterile-filtered, cold saline solution diluted 1:20 in a sterile warring blender. 0.1 ml samples (1:10 dilution) were spread on plates containing nutrient agar medium containing 10% soil extract; this was spread evenly on the agar surface by L shaped glass rod. The plates were then wrapped in cling film (to retain moisture) and incubated at 35°C for 48 h. A number of morphologically different colonies were randomly selected and counted under a light microscope (Motic BA-210). The colonies were picked from the nutrient agar plate and sub-cultured on nutrient agar medium.

For further isolation and enumeration of metal resistant bacteria, all the cultures were serially diluted to 2×10^{-6} dilutions and 1 ml of each strain were poured indifferent Nutrient agar plates supplemented with 100 $mgL^{-1}$of metals (Pb, Ni and Cu) individually. The plates were incubated for 2 days at 37^0C (Megharaj et al. 2003).

The experiment repeated in triplicate to isolate metal resistant strain in order to obtain bacterial biomass. The above metal resistant strains were inoculated separately into the nutrient broth medium and incubated for 48 h at 37^0C in a shaker incubator. The biomass is used for the Pb, Ni and Cu degradation ability and optimization study of physical parameters and medium.

Determination of Minimum Bactericidal Concentration (MBC) for Isolates

The MBC of Pb, Ni and Cu at which no colony growth occurred was determined by broth dilution methods (Aleem et al. 2003; Nath et al. 2014). The isolates were inoculated individually into a Nutrient broth medium which containing 10 µg ml^{-1} of the respective metals (Pb, Ni and Cu). There by the metal concentration was gradually increased by 10 µg ml^{-1} each time, until the strains failed to produce the colonies in broth. The last concentration in which isolates failed to grow on the plate was recorded and the same was considered as MBC. An experiment was carried out in triplicate and standard deviation was calculated (Table 1).

Biochemical Characterization of Isolates

Based on the above result six strains were selected which showed resistant and degradation properties of all three metals, and these were carried for further evaluation and species characterization. For characterization the biochemical tests include Grams staining, oxidase test, lactose fermentation test, indole test, citrate test, spore forming, catalase test, mannitol fermentation test and glucose fermentation test (Table 2) and also used specific medium; Brilliance Bacillus Cereus agar (BBC agar) for *Bacillus licheniformis*, streamed and Macconkey agar was used for *Pseudomonas aeruginosa*. *Bacillus lichiniformis*wasshowedhigh resistance to metals (Pb, Ni and Cu) as well as significant sorption of all three metals, the work was further extended to characterize the strain by 16S RDNA sequencing method.

Identification by 16S RDNA Sequencing

Genomic DNA (gDNA) was isolated from bacterial isolates as described by Joseph and David (2001). Briefly, 16S RDNA of Culture was amplified using conventionally purified gDNAby the PCR primers 5'-AGAGTTTGATCATGGCTCAG-3' and 5'-GGTTACCTTGTTACGACTT-3', P3 forward primer and P13 reverse primer respectively, that amplifies approximately 1500 bp length of 16S RDNA gene of bacteria. The primers were commercially synthesized from the Eurofins Genomics (Bangalore). The lyophilized primer was dissolved in nuclease free water according to manufacturer's recommendation to obtain stock primer of 100 µM final concentrations. Using this primer stock, a working premier solution was prepared at a final concentration of 10 µM using nuclease free water.

PCR was performed by 20µL reaction Mixture containing 2.0 µL of DNA, primers at a concentration of 0.6 pmol each, 2.0 mM $MgCl_2$ and dNTPS concentration of 50 µM, as well as 1.25 U of Taqpolymerase and 1X buffer was used. After the initial denaturationfor 4 min at 94°C, there were 32 Cycles consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 45 s and final extension at 72°C for 5 min. PCR products were analyzed by 1% (W/V) agarose gel electrophoresis in 0.5xTBE buffer with ethidium bromide (5 µg ml^{-1}). PCRproducts were then sequenced by In-silico sequence analysis and compared with the National Center for Biotechnology Information (NCBI)

database using the BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the best match of resemblance was selected to identify the bacterium.

Effect of Metal Concentration on Bacterial Growth and Metal Sorption

The selected strain, *B. licheniformis was* tested in a co-relation study between cell growth and reduction of metals. Metal resistant isolate, *B. licheniformis* was inoculated into nutrient broth 100 ml in 250 ml Erlenmeyer flask (pH 7.0) containing different concentrations of Pb, Ni (50 to 1800 mg L^{-1}) and Cu (50 to 2500 mg L^{-1}) separately (Table 1) and incubated for 72 h at 35^{o}Cwith orbital shaking (100 RPM). The inoculum was adjusted to 2 x 10^5 CFU ml^{-1} with saline solution and 2 ml was used for inoculation and the blank was maintained without metals. The cell density was determined by measuring optical density at 600 NM using UV/Vis. Spectrophotometer (Shimadzu, UV-1800) (Kim et al. 2012) and the metals concentration (Pb, Ni and Cu) were estimated by AAS (Atomic Absorption Spectrophotometer).

Optimization of Physical Parameters for Metal Degradation

Optimization study was followed asDhal et al. (2010) with the slight modification, the reduction of metals (Pb, Ni and Cu) with the selected strain (*B. licheniformis*) was carried out under varying physical parameters like pH, temperature and incubation time. While determining optimum pH, the pH ranges were selected 5 to 8 by keeping the temperature (35^0C), individual metals concentration (100 mg L^{-1}) and incubation time (96 h) constant. For determination of optimum incubation time, the varying incubation time was from 24 to 168 h by keeping the pH (7.0) and temperature (35^0C) constant. For the temperature optimization study varied temperature range was considered (20 – 50^0C) with constant pH (7.0) and time (96 h). In the optimization experiments 100 mL of NB medium containing the desired metal concentration with 0.5% glucose taken in a 250 ml Erlenmeyer flask and it was inoculated with 2 ml of inoculums separately. The inoculate of metal resistant strain B. *licheniformis* was adjusted to 2 x 10^5 CFUml^{-1} with saline solution and used 2 ml for all the flasks. At pre-determined time intervals, 1.0 ml aliquots were drawn from the each flask and centrifuged (6000 RPM for 10 min at 10^0C) for respective metal analysis AAS. Growth of the bacteria was monitored at definite time intervals, by measuring the optical density of the cultures at 600 NM (Kim et al. 2012) using UV-visible spectrophotometer (Shimadzu, UV-1800, Japan).

Media Optimization Studies

The media optimization was carried with optimized physical parameters in a series of experiments by changing one variable media constituent at a time and keeping the other factors constant. Three factors, viz., glucose as carbon source, nitrogen source and M9 Minimal Salt solution (MSS) were chosen to obtain higher percent biosorption of metals (Pb, Ni & Cu). For evaluation of the carbon source, glucose was added as a co-substrate for bio sorption in varying concentrations in the range of 0, 5, 10, 20 and 30 ml of 10% glucose per 100ml. For evaluation of nitrogen sources, NaNO3 was employed in varying concentration range of 0, 0.5, 1.0, 1.5 and 2 g L^{-1}. To evaluate the effect of various inorganic salts, M9 MSS was employed in varying concentration range of 0, 50, 100, 200 and 300 ml L^{-1} by keeping Carbon and nitrogen sources constant. All the experiments were performed in triplicate keeping control without the bacterial isolate (Bhattacharya and Biswas 2014).

Characterization of Sorbed Products on Cells

Transmission Electron Microscope (TEM) is performed by Jaafar et al. (2016) method on JEOL/JEM 2100 microscope with an accelerating voltage of 200 KV and a resolution of 200 NM.

Fourier Transform Infrared Spectroscopy (FT-IR) was used to determine the vibration frequency changes in the functional groups of cellular biomolecules in the metal bio Sorbent isolates. Analysis was carried according to Chen and Yang (2005) with slight modifications. Briefly, two isolates were cultured under optimized conditions in the presence and absence of metals (100 mg L^{-1}) individually. At the exponential stage the cultures were harvested by centrifugation at 3000 RPM for 10 min and washed three times to remove excess metals and 10 µl of the sample was used for infrared spectra of *B. licheniformis* (Both control and metal-loaded) performed in the wave number range of 500-4000 cm^{-1} (Jasco FTIR 4100, Japan).

X-ray Diffraction (XRD) analysis was performed according to Shivashankarappa and Sanjay (2015) by using powder X-Ray Diffractormeter – Rigaku SmartLab 3KM, Japan with Cu-Kα radiation, intensities was recorded from 10° to 80° at 2θ angles. The XRD patterns for dry powder samples of *B. licheniformis* biomass of control and amended with Pb, Ni and Cu (100 mg L^{-1}) was recorded.

Continuous-Flow Soil Reactor Study for Isolate

The effective bioremediation efficacy of the isolate was studied for the heavy metal contaminated soil in lab scale, continuous flow, soil reactor was followed by Jeyasingh and Philip (2005) with the modification in brief, the schematic diagram of the 4 L reactor is shown in the Figure 9. The cylindrical reactor diameter of 19 cm was made up of 3 mm thick acrylic glass sheets with consisted of 2 compartments, the top compartment was of height 20 cm and bottom compartment of height 5 cm in a common diameter which is to collect leachate. The individual metal contaminated (1000 mg kg^{-1}) soil of 3 kg separately was sterilized by autoclaving at 120^{o}C for 30 min, and then enriched with nutrient media (1000 ml). The soil samples were mixed with 50 g dry weight of isolated strains in separately. And the individual soil mixture was loosely packed in to top compartment to the height of 15 cm with 5 cm free boards from the top end of the reactor. The physical parameters were maintained as per the previously determined optimum values (temperature 35^{0}C, pH 6.0, 7.0, and 6.5 for Pb, Ni and Cu respectively, with mental concentration of 1000 mg kg^{-1}, and M9 MSS media with 10 ml of 10% glucose, 100 ml L^{-1} of MSS and 1.5 g $L^{-1}$of Nitrogen source). The reactor was initially incubated for 48 handmineral media was made to flow through the top compartment at different flow rates of 10, 20 and 30 ml min^{-1} in 3 trials. The top of the soil was covered with sterilized cotton to maintain moisture. 24 h after inoculation the 2 g of sample was collected in the interval of 12 h from the sample ports and from bottom compartment leachate was collected. The collected soil samples and leachate was analyzed for heavy metal concentration by AAS to determine the bio sorption efficacy of isolating.

RESULTS AND DISCUSSIONS

Isolation and Identification of Metal Tolerant B. Licheniformis

Soil samples of KGF Iron mine were collected and characterized as described in our earlier work Akshathaet al. (2014). 27 bacterial strains were isolated by serial dilution and sub cultured on Nutrient agar plates. In order to determine their metal degrading properties and tolerance for heavy metals (Pb, Ni and Cu), preliminarily all the 27 strains were maintained in agar plates amended with all three metals (Pb, Ni, & Cu) individually (100 mg L^{-1}).

Among these 27 isolated bacterial strains, 6 strains were shown tolerance as well as metal degradation property for all the three metals. Further, these 6 strains were named (S1, S2, S3, S4, S5 and S6), morphological characterization such as a colony and cell morphology, Gram-reaction, motility, etc. was carried by methods as described by Ghoshet al. (2006). Physiological and biochemical tests were carried out according to the standard protocols described inBergey's Manual of Systematic Bacteriology (Krieget al. 1984).

Further, all six strains were eventually chosen for evaluation of their metal resistance performance

Initial heavy metal concentration in the native soil residue was analyzed in AAS method. This analysis showed the presence of heavy metals in high concentration (Akshatha et al. 2014). Among all heavy metals present in the native soil residue, three heavy metals (Pb, Ni and Cu) were selected for the further bioremediation study. These three heavy metals selected were found to be in high concentration in the study site and leads to cause major health and environmental hazards. Spiking of the selected heavy metals was carried out depending on the initial concentration of the heavy metals that were found in the native K.G.F mine soil. The selected heavy metal concentrations were spiked using respective salts for the desired concentration. The heavy metal concentration of copper was spiked up to the concentration of 3000 mg L^{-1}using copper sulphate. The heavy metal concentration of Nickel was spiked up to the final concentration of 1500 mg L^{-1} using Nickel Chloride. The concentration of Lead was spiked up to the final concentration of 2000 mg L^{-1} using Lead Sulphate.

All six bacterial isolates were exhibited high tolerance to all three metals (Cu > Pb > Ni) particular strain S4 exhibited high tolerance to all three metals i.e., copper up to 2166 ± 57.73mg L^{-1} and second highest resistance to lead and followed by Nickel i.e., 1685 ± 115.47mg L-1 and 1246 ± 57.73mg L^{-1} respectively (Table 1). The metal resistant bacterial isolates identified in this study were of three gram positive and three Gram-negatives, these groups that has been often found in metal polluted soils (Piotrowska et al. 2005, Brim, et al. 1999, Kozdro and Van 2000). Based on the morphological and biochemical studies of isolates, the metal-tolerant Gram-negative bacteria in these studies belonged to *Pseudomonas*, *E coli* and *Klebsiella;* whereas Gram +ve strains belongs to *Bacillus*, *Clostridium* and *Micrococcus* (Table 2). The present study suggests that the microorganisms tolerant to metals appear to be the result of exposure to metal contaminated environment, which is fairly consistent with gram positive followed by gram negative. The Isolates were found to be tolerant to different concentrations of all heavy metals, whereas the aboriginal bacterial mass showed very less tolerance, i.e., 433 ± 57.73, 166 ± 57.73 &566 ± 57.73 for Pb, Ni, and Cu respectively, when compared to all isolates (Table 1), this indicates there is need of isolation and evaluation of efficient strains in the contaminated site. Based on this metal tolerance performance the strain S4 was selected for further optimization of physical and chemical parameters to evaluation of metal degrading performance and identified by 16S rDNA method.

The amplified 16S rDNA gene using polymerase chain reaction resulted in a single discrete band of a1.5 kb size in agarose gel. This amplified PCR product wasBLAST searched against the NCBI Genbank database. Based on nucleotide homology, the isolate showed the highest similarity (100.0%) with *B. licheniformis* (Genbank accession number HE993550.1). The identified strain *B. licheniformiswhich* was used in this study was evaluated for metal absorption followed by Zouboulis (2004) from polluted soil for treatment of cadmium and chromium polluted water and wastewater streams and the results showed good preliminary metal removal properties.

Effect of Metal Concentration on Bacterial Growth and Biosorption

The response of metals on the growth of gram positive bacteria (*B licheniformis*) and its metal reduction capacity studies was carried out using nutrient broth medium with lower metal concentration to higher metal concentration. In the nutrient broth media amended with lower metal concentration of 50 mg L^{-1}, no significant negative effects of the metals on the bacterial growth were observed when compared to the control (without metal). Thus, Pb, Ni and Cu at 50 mg L^{-1} had no significant inhibitory effects on the growth responses of mining soil aerobic gram positive bacteria. Simultaneously the absorption of Pb, Ni and Cu at lower concentration had significant effects, 100% of all metals were taken up. However, the rate of biosorption was significantly decreases with increasing metal concentrations. The microbial mass decreased with an increase in the concentration of all the three heavy metals, but final concentration varies with individual metal when compared to the control (without metal), this indicated the toxic effect of the heavy metals on the growth of microorganisms and this is obvious phenomena (Figure 1). This drastic decrease in microbial density caused by increased levels of all three heavy metals was found in this study is in agreement with Kikovic (1997) and Anyanwu et al. (2011).

It has been reported (Rathnayake et al. 2009) that different bacterial strains exhibited different growth rates in the presence of different heavy metals concentration. Ahmad et al. (2005) shown that the toxic effect of the heavy metals was concentration and time dependent for each group of soil organisms. There is no any specific toxicity was observed against these diverse microbial groups (Anyanwu et al. 2011). Thus, in this study, we found that the obtained microbial biomass during the absorption process, represents the different heavy metal has a different final concentration of bacteria. It is, therefore, reasonable to assume that this metal resistant bacteria form a larger part of the total bacterial population at elevated levels of metal contamination of soil when compared to control.

Optimization of Physical Parameters for Metal Degradation

In order to determine optimum pH value for biosorption for isolate, experiment with different range of pH (5.0 – 8.0) with constant temperature and time was conducted. The biosorption capacities for each metal increased with an increase in pH and the optimum biosorption occurred at pH 6 for Pb, pH 6.5 for Cu and at pH 7 for Ni. Oveset al. (2013) has reported the same optimum pH for lead and nickel but pH 6.0 for copper biosorption from gram positive bacteria (*Bacillus thuringiensis*). This different pH values in biosorption of different heavy metals by *B. licheniformis* biomass could be due to the differences in the sensitivity of cell wall molecules to pH. Previous literature reveals that the higher pH value for Ni is may be due to the presence of ligands like carboxyl, phosphate, imidazole and amino groups (Pardo et al.2003) and lower pH value for Cu and Pb may be due to their physiological properties (Sar et al. 1999; Ok et al. 2007) (Figure 2).

The temperature of the medium plays an important role for energy dependent metal sorption by the bacterial cells. Temperature is known to affect the stability of the cell wall molecules, and hence it may cause ionization of chemical moieties present in the cell wall (Congeevaramet al. 2007). In this regard the temperature may simultaneously affect the binding sites on isolated and causing variation in heavy metal removal capacity for the isolate.

Energy-independent mechanisms at the binding sites are may be less likely to be affected by temperature, and

hence the process which responsible for biosorption are largely physiochemical in nature (Bayramogluet al.2003). Biosorption of Pb, Ni and Cu by the isolated bacterial species in this study was appears to be temperature dependent.

In the studied temperature range (20–50^{0}C), the extent of Pb, Ni and Cu (100 mg L^{-1} at pH 7in 96 h) rate of biosorption increased with increase in temperature and at a certain temperature it starts decreased (Table. 3). Interestingly, it was observed that all the metals were taken almost same optimum temperature for reduction, i.e., at 35^{0}C the maximum absorption of 86.33 ± 3.29% for Pb, 74 ± 3.26% for Ni and 77.66 ± 2.49% for Cu in comparison with lower and higher temperatures (Figure 2). Okeke et al. (2008) also reported a similar optimal temperature for growth and Cr (VI) reduction with Bacillus sp. Isolated from soil. The variation of rate of biosorption with respect to temperature is also consistent with the fact that normal growth is observed at ~ 37^{0}C, there is no biosorption is observed during the entire time course in controls (without bacterial cells).

Incubation time is also an important physical parameter of an absorption process of Pb, Ni and Cu, by *B. licheniformis* biomass. The rate of heavy metal biosorption was increasing with increasing time; this may due to high affinity of free metal ion binding sites on bio sorbent but after optimum time the rate of biosorption slowed and reached to equilibrium (Oveset al., 2013). In this study the initially sorption rate was accelerated from 0 h and at certain time moved to equilibrium and that time can be considered as an optimum time required to sorption of heavy metals by *B. licheniformis.* Our experimental data showed the order of biosorption rate was Pb > Ni > Cu (Figure 2). Similarly Gabr et al.(2008) reported the biosorption of Ni > Pb by *Pseudomonas aeruginosa* a gram negative strain, this difference may be due to structural conformation of cell wall molecules.

Media Optimization Studies

As per the previous literature the growth rate of microorganism is dependent on the media compositions, on the other hand, rate of biosorption is very much dependent on the microbial biomass (Puranik and Paknikar 1999; Mapolelo and Torto 2004; Bhattacharya and Biswas 2014). With reference to this the effect of three factors i.e., Carbon source, nitrogen source and MSS of nutrient supplements were made to evaluate the biosorption efficacy of *B. licheniformis.* The experimental design matrix and the result output for the three factors for three metals are presented in Figure 3, 4 and 5.

Effect of glucose: As shown in Figure 3, the growth rate of biomass in the presence of all the three metals increase with increase in glucose concentration and at certain concentration (20 ml of 10% glucose 100 ml^{-1}) gradually decreases due to hypertonic effect. The data show that concentrations of glucose (0-1%) resulted in around 30–40% increase in the metal uptake by the isolate, i.e., at 1% (10 ml of 10%) glucose, the maximum uptake achieved is 88.3±3.5% for Pb, 73±3.6% for Ni and 79±2% for Cu. In correlation to growth rate the sorption was increased with increasing growth, this occurs only up to 10 ml (1%) of glucose thereafter the sorption rate decreases even though increasing in growth up to 20 ml (2%). The trend exhibited by the isolate might suggest that the mechanism of uptake is similar to those of the all the three metals as the uptake decreased by almost 20% following a treatment by 3% glucose. Glucose as a simple alternative carbon source in the media supplement can enhance biodegradation of recalcitrant compounds due to its tendency to improve cell growth (Bhattacharya and Biswas 2014). Also Stoll and Duncan (1996) demonstrated that treatment of yeast cells with glucose resulted in an increase in the removal of Pb, Cu, Cd, Ni and Zn, from electroplating effluents.

MSS: Figure 4 depicts the maximum percentage of heavy metal biosorption with varied concentration of MSS is at 100 ml L^{-1}. The optimum level of absorption of Pb is 81±6%, Ni 95.3 ±2. 5% and Cu is 78.6±4% was observed at 100 ml L^{-1}. It was observed that the growth of the bacterial strain increased with increasing concentrations of MSS and the maximum biomass was obtained up to 200 ml L^{-1} i.e., 2.3, 1.5 and 2.1 OD for Pb, Ni and Cu respectively (Figure 4). And this was probably due to its hypertonic effect of salt solutions.

The maximum percentage of heavy metal sorption achieved by varied concentration of nitrogen is same for all the three metals and it is 1.5 g L^{-1}. The obtained highest sorption was found to be 85±5.0% for Pb, 66±4.5% of Ni and 67± 4.7% for Cu (Figure 5). The concentration of nitrogen in medium varied from 0.0 to 2.0 g $L^{-1}$and there was considerable increase in the biomass as well as sorption of metals simultaneously, the maximum biomass were also obtained at 1.5 g $L^{-1}$of nitrogen.

Characterization of Sorbed Products on Cells

Figure 3A shows the cells of *B. licheniformis* before interaction with metals while Figure 3B, 3C and 3D shows the bacteria after exposure to Pb, Ni and Cu. Metals were mostly seen on the cell wall, in all the three metal trials, it looks almost like a crust around the cell. The cellular localization of the metals bound by the cells was located mainly within the cell wall. Sinha and Mukherjee et al. (2009) showed that, gram positive; especially Bacillus sp. Cell wall components with phosphate residues i.e polysaccharides, techies and teichuronic acids or phospholipid layers of the membranes can bind more heavy metals. The results of the present study also showed changes in morphology i.e., shrunken, and distorted cell wall in the presence of Cu and depressions in the presence of Pb and Ni and also the size of isolating which exposed to heavy metals, these changes may due to cell surface bounded with metals. Various factors may be responsible for such alterations in cell surface morphology of microbial biomass in the presence of heavy metals (Jaafar et al. 2016).

The patterns of FTIR spectra (500-4000 cm^{-1}) for the native and metal loaded *B. licheniformis* biomass are presented in Figure 6. Based on the principle of FTIR, binding of metal ions by the microbial cells is largely on the functional groups of bacterial cell walls which are made up of macromolecules. The characterization of infrared spectra was based on the literature (Oves et al. 2013;Jeyakumar and Chandrasekaran2013; Giotta et al. 2011; Sar et al. 1999; Chen and Yang et al. 2005) that revealed, peaks originated at wave number around 3300 cm^{-1}, 2350 cm^{-1}, 1650 cm^{-1}, 1200 cm^{-1} and 750 cm^{-1} were believed to be hydroxyl and or amine, alkyl and CHO, C=O of amide groups, COO- and P-O groups respectively. Furthermore, the IR spectral peaks of the metal loaded biomass varied with the unloaded (blank) biomass; similarly the different metals spectral band frequency variance with each other (Figure 6).

The IR spectra revealed a stretching of bands appearing at 3314 cm^{-1} in blank, to 3306, 3299 and 3285 cm^{-1} which was attributed to the interaction of sorbed metals Pb, Ni and Cu respectively with hydroxyl and amide groups. Additionally, stretching of bands observed at 2361 cm^{-1} of blank to 2355, 2348 and 2354 cm^{-1} after biosorption of Pb, Ni and Cu respectively and this could be due to the metal ions were sorbed on the carbon adsorbents similar as reported by Jeyakumar and Chandrasekaran (2013). Likewise, clear shifts from wavenumber 1647 cm^{-1} to 1633 cm^{-1} for Pb added, broadening of peak and stretching to 1639 for Ni added and 1640 cm^{-1} after addition of Cu was due to involvement of C=O groups on the surface of the carbon adsorbents. The peak located at 1222 cm^{-1} in the blank has shifted to 1214 cm^{-1} with the addition of Pb, Ni and Cu which were represented that the metal ion interaction with acetate groups. There is a stretching of bands from 767 cm^{-1} to 768, 780, 774 cm^{-1} with the addition of Pb, Ni and Cu respectively, this

indicates the possibilities of metals interaction with phosphate moiety in the cell wall components. Interestingly, at wave number 659 cm^{-1} inverting the peak intensity and stretching to 674, 667 and 680 cm^{-1} was due to the loading effect of Pb, Ni and Cu respectively, and these broadenings can be explained by the involvement of sulfate ions, the same has been in agreement with Jeyakumarand Chandrasekaran (2013) (Figure 6).

The differences of peak intensity in the metal loaded biomass were substantially lower than the unloaded bacterial biomass. These changes suggest that the stretching of bands occurs to a lesser degree due to the presence of metals and therefore, peak transmittance is consequently reduced. Conclusively, overall variability in spectra following adsorption of the metal ions validated the contribution of functional groups present on cell wall with metal binding.

The powder XRD patterns of isolating grown with the addition of metals (Pb, Ni and Cu) and control without metals in NB media are shown in Figure 6 and represents further insight of sorbet products. It is evident that the sharp peak of the bacterial mass in the absence of metals has disappeared in the XRD patterns, whereas in metals added bacterial cells and several intensity peaks appear, thus indicating the formation of poorly crystalline end product. The sharp peaks of 2θ values at 30 in Pb added, and 22 in Ni and Cu added do not match with control. This is in agreement with the results reported by (Jaafar et al. 2016; Dhal et al. 2010), and this may suggest that Pb, Ni and Cu immobilized on the surface of *B. licheniformis.*

Continuous-Flow Soil Reactor Study

The reactor was constituted and ran first for control (without metals) with mineral media, then all metals separately without bacterial strain aseptically. Further for test runs the reactor received 1000 ml of mineral medium stacking for 48 h then on continuous flow of medium with different flow rates of 10, 20 and 30 ml min^{-1} in three trials further the sample at different sample ports and leachate was collected for every 12 h and then analyzed for metal concentrations (Figure 9).

The performance of reactor with respect to time and flow rate at standardized conditions is presented in Figure 10. Here results depicted that the rate of sorption was depended on both time and flow rate and also showed different rate of sorption for individual metals with respect to time and flow rate. Maximum biosorption of Pb was up to 86 ± 5% at 192 h, 82 ± 8% at 144 h and 80 ± 5% at 120 h in media flow rate of 10, 20 and 30 ml min^{-1} respectively, likewise nickel was sorbed up to 77 ± 8% at 240 h, 74 ± 5% at 192 h and 72 ± 6% at 168 h with medium flow rate of 10, 20 and 30 ml min^{-1} respectively. Similarly maximum sorption of Cu was achieved up to 80 ± 6% at 216 h, 78 ± 8% at 192 h and 78 ± 5% at 144 h with flow rate of 10, 20 and 30 ml min^{-1}. Rest of the metal concentration approximately was found to be at leachate in all the trials and negligible quantity was adsorbed with soil particles.

Based on the result, it is shown that among three metals Pb is maximum sorbed in shortest time fallowed by Cu and Ni; comparatively it is similar order of metals sorbed with batch studies in NB medium(without soil). But final quantity of sorption is lesser in reactor studies, this may due to the efficiency of strain in soil is lesser because of metal adsorbed to soil particles was not available to bacterial cells and also some amount of metals leached out along with the mineral medium. Figure 10shows at lower flow rate of medium total percent of sorption is slight more but with increasing flow rate to 20 ml min^{-1} the percent sorption decreasing slightly with shorter duration and at 30 ml min^{-1} the rate of sorption

reaches constant, hence by this we can consider the optimum flow rate as 20 ml min^{-1} for all the three metals. Whereas optimum time to reach maximum sorption to three metals weredifferent I.e., 144 h for Pb and Cu and 192 h for Ni.

CONCLUSIONS

The present study focuses on the ability of bacterial strains isolated from metal contaminated mining site to biosorption of Pb, Ni and Cu. In the native bacterial community *B licheniformis* was selected for batch studies to optimize initial metal concentration, physical parameters and media components. Further the experiment was extended to continuous flow bioreactor with soil was carried out to study the biosorption pattern to Pb, Ni and Cu in mining soil. The growth pattern of *B licheniformis* in the presence of metals is showed Cu > Pb > Ni and the metal sorption capacity was found to be Pb > Cu > Ni. The metal sorption on the surface of bacterial cells was evidenced by TEM, FTIR and XRD studies. The biomass of metal tolerant *B. licheniformis* successfully removed the metals such as Pb, Ni and Cu from soil in a laboratory scale bioreactor. The maximum biosorption occurred for Pb is 86 ± 5% at 192 h, 82 ± 8% at 144 h, and 80 ± 5% at 120 h with flow rate of 10, 20 and 30 ml min-1 respectively similarly for Ni results showed 77 ± 8% at 240 h, 74 ± 5% at 192 h and 72 ± 6% at 168 h with flow rate of 10, 20 and 30 ml min-1 respectively and for Cu it showed 80 ± 6% at 216 h, 78 ± 8% at 192 h and 78 ± 5% at 144 h with flow rate of 10, 20 and 30 ml min-1 respectively. This study validates that the biomass of *B. licheniformis* could be a highly efficient reliable biosorbing agent for effectively removing heavy metals from contaminated soil environment.

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Table 1: Determination of Metal Tolarance Range for Isolates in MBC

Strain No	Minimum Bactericidal Concentration (mg L-1)		
	Lead	Nickel	Copper
Ab	433 ± 57.73	166 ± 57.73	566 ± 57.73
S1	1050 ± 57.73	816 ± 57.73	1266 ± 115.47
S2	833 ± 57.73	633± 115.47	933 ± 57.73
S3	566 ± 57.73	533 ± 57.73	833 ± 115.47
S4	**1685 ± 115.47**	**1246 ± 57.73**	**2166 ± 57.73**
S5	766 ± 57.73	633 ± 57.73	766 ± 57.73
S6	1533 ± 57.73	1066 ± 57.73	1666 ± 57.73

Table 2: Biochemical Characterization of Isolates

Sl. No	Biochemical Test	Test Results					
		S1	S2	S3	S4	S5	S6
1	Gram's staining	+	-	-	+	-	+
2	Starch hydrolysis	×	×	×	+	×	×
3	Oxidase test	×	+	-	×	-	×
4	Lactose fermentation	×	×	+	×	+	×
5	Indole test	×	×	+	×	+	×
6	Citrate test	×	×	-	+	+	×
7	Spore forming	+	×	×	×	×	×
8	Catalase test	×	×	×	×	×	+
9	Mannitol fermentation	×	×	×	×	×	+
10	Glucose fermentation test	×	-	×	×	×	-
11	NaCl (6.5%) Media at 55^0C	×	×	×	+	×	×
12	MacConkey broth media	×	-	×	×	×	×
13	Pseudo F & P agar	×	+	×	×	×	×
14	BBC agar	×	×	×	+	×	×

Note: + Result positive, - Result negative, × not applicable

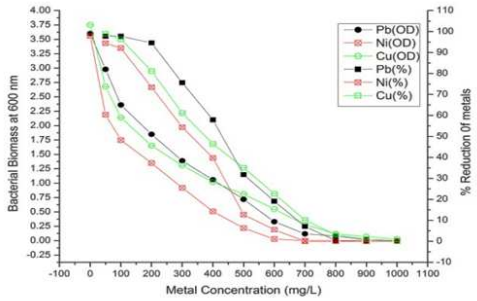

Figure 1: Effect of Metal Concentration on Bacterial Growth and Biosorption

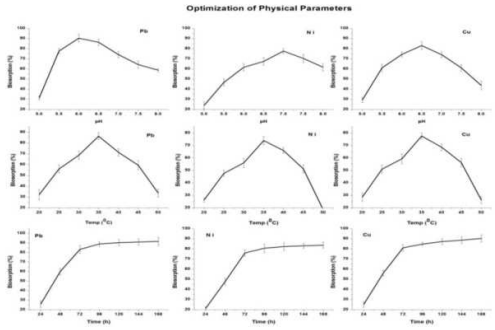

Figure 2: Optimization of Physical Parameters for Metal Degradation

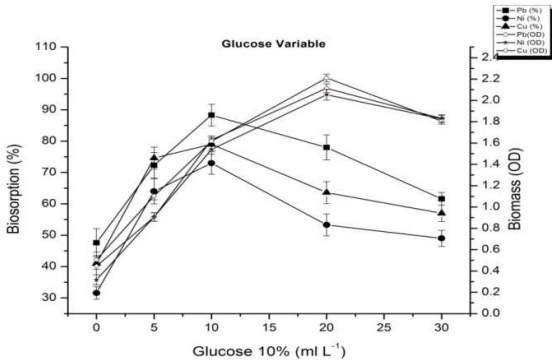

Figure 3: Optimation of Carbon Source with Respect to Growth and Biosorption

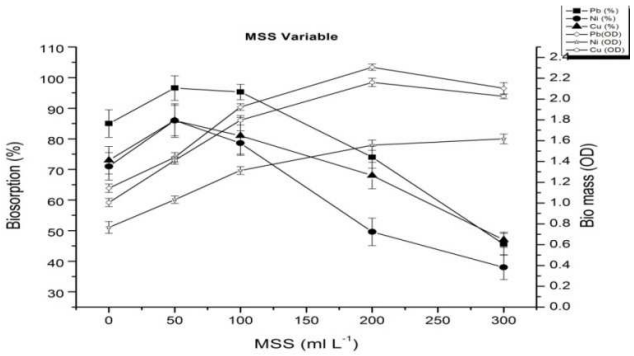

Figure 4: Optimation of MSS with Respect to Growth and Biosorption

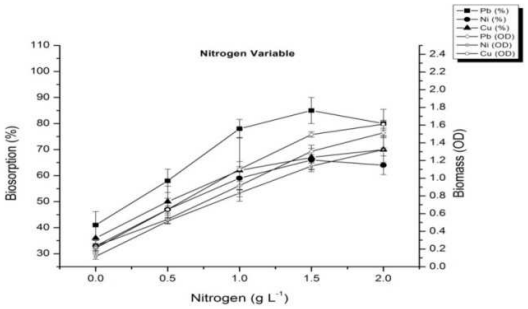

Figure 5: Optimation of Nitrogen Source with Respect to Growth and Biosorption

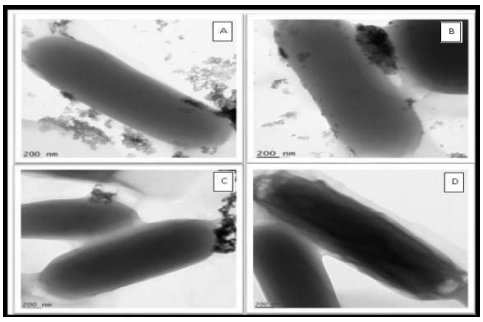

Figure 6: TEM Analysis of the Bacterial Cells Grown for 72 h without (A) or with Pb (B), Ni (C) and Cu (D) Showingelectron Dense Grains Distributed on the Cell at 200 nm

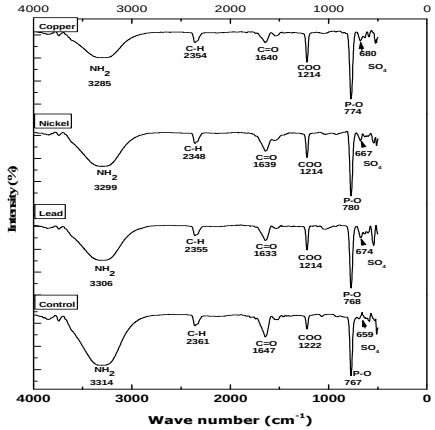

Figure 7: FTIR Spectra of *b. Licheniformis* Massbefore and after Biosorption of Metals

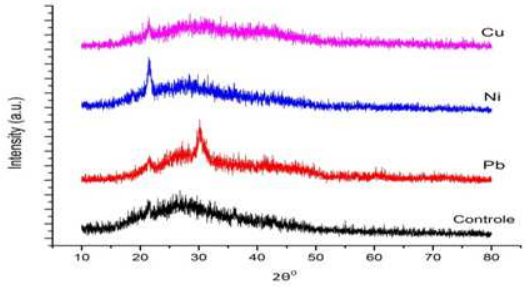

Figure 8: X-Ray Diffraction Spectra of *b. Licheniformis* Massbefore and after Biosorption of Metals

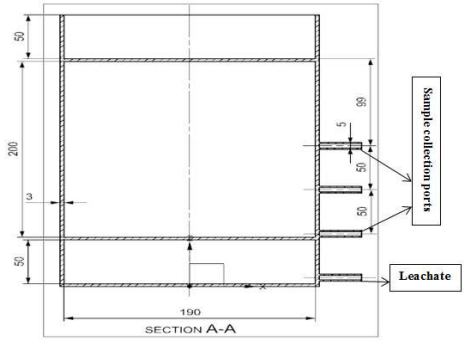

Figure 9: Schematic Outline of Laboratory-Scale Continuous Flow Soil Reactor

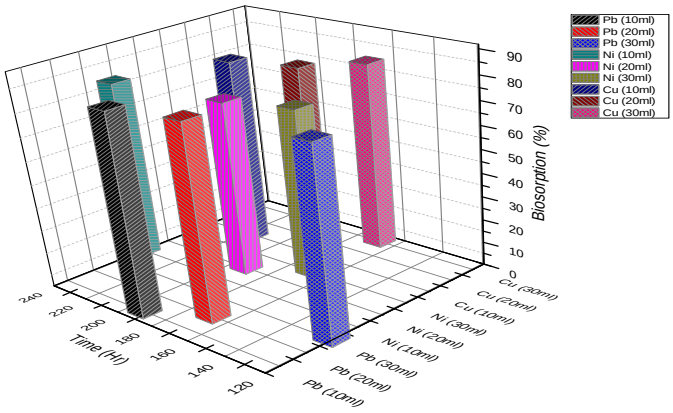

Figure 10: Biosorption of Pb, Ni and Cu in the Soil With Respect to Time and Flow Rate in Reactor Under Optimum Conditions