In-Vitro Evaluation of Heavy Metal Tolerance and Biosorptive Potential of Two Native Strains of Bacillus Cereus Against Nickel and Cobalt

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1. Abstract

Heavy metal contamination now a day is one of the major global environmental concerns and industrial effluent is commonly used for irrigation. Increasing industrial rate in the modern world is responsible for increase in concentration of heavy metals. Present study was designed to isolate and identify some indigenous heavy metal tolerant bacteria from textile effluents. Two isolates were screened out showing maximum tolerable concentration and multi metal resistance to Ni and Co and were named as AMIC2 and AMIC3. Molecular characterization confirmed AMIC2 as (Bacillus cerus, accession number LT838345) and AMIC3 as (B. cerus, accession number LT838346). Biosorptive potential was accessed using Inductively Coupled Plasma-Optical Emission Spectroscopy and it was found that AMIC2 reduced Ni (48.4%, 49%) and Co (70.6%, 73.6%) after 24 and 48 hours respectively while AMIC3 reduced Ni (50.6%, 51.8%) and Co (71.8%, 73.2%) after 24 and 48 hours respectively. Fourier transform infrared spectroscopy was used to analyze the functional groups and overall nature of chemical bonds in isolates while Scanning Electron Microscope was performed to detect outer morphological changes in the bacteria in response to metal stress. Overall results suggested that bacteria possessed significant bioremediation potential and could be used in the development of bioremediation agents.

2. Introduction

In general, "Heavy Metal" is a broad term, which is used for the group of metals and metalloids having atomic density greater than 4000 kg m-3 or 5 times more than water. Heavy metal contamination now a day is one of the major global environmental concerns and the main sources of heavy metal contamination are either natural or anthropogenic. Depending on soil pH and their specification these heavy metals can become mobile in soils and in this way, a small part of the total mass can leach to aquifer or can become bioavailable to living organisms (Hookoom, 2013) [17]. Industrial wastewater is commonly used for irrigation in most of the developing third world countries (Bouwer et al., 2002) [9]. Without proper treatment, release of heavy metals in effluent poses a menace to public health because of its persistence, biomagnifications and accumulation in food chain (Issazadeh, 2014) [18]. As the number of industries is being increased day by day in the modern world, with this the concentration of heavy metals is also being increased. Cadmium, chromium, mercury, lead, nickel, cobalt and copper are mainly found in the industrial wastewater (Smrithi, 2012) [42].

Several studies have been conducted to elaborate the effects of these heavy metals on living organisms including animals, plants and human (Chisti, 2004) [11]. In recent years, the use of microbes for removal of heavy metals has achieved great attention. Various microorganisms such as bacteria (Shuttlework et al., 1993) [40], yeast (Salinas et al., 2000) [34], fungi (Anoop et al. 1999) [5], algae (Ahuja et al., 1999) [5], and plants (Chen et al., 1996) [10] have been reported to tolerate and remove heavy metals from aqueous solutions. Bioremediation is a potential cost-effective solution for the remediation of heavy metal contaminated envi-

ronment (Congeevaram et al., 2007) [12]. The advantages of using microbes for bio-remediation include natural occurrence, cheap production, easy availability to treat large volumes of wastewater due to rapid kinetics and high selectivity in terms of removal and recovery of specific metals (Genter, 1996) [15]. Indigenous organisms have the ability to adopt themselves according to the prevailing environments and could flourish under these conditions (Haq, 2000) [16]. Variety of mechanisms has been developed by some microorganisms to deal with high concentrations of heavy metals and usually is specific to one or a few metals (Piddock, 2006). Most microorganisms, posses the efflux of metal ions outside the cell, and genes for tolerance mechanisms has been found on both chromosomes and plasmids (Hookoom, 2013) [17]. Some bacteria can use mechanisms of tolerance and detoxification of heavy metals and still produce chelating agents that bound metals and reduce their toxicity (Kavamura and Esposito, 2010) [20]. Many living bacteria have been reported to reduce or to transform toxic contaminants into their less toxic forms (Solecka et al., 2012) [43].

Faisalabad is the 3rd biggest city in Pakistan after Karachi and Lahore. It is the 2nd biggest city in the province of Punjab after Lahore, and a major industrial center. The city is also known as the "Manchester of Pakistan" (Jaffrelot, 2002). The surrounding countryside, irrigated by the lower Chenab River, produces cotton, wheat, sugarcane, vegetables and fruits. Due to the heavy industrialization different types of waste is being produced by the different industries. The textile zone is playing a vital role in the export of the country but at the same time a lot of environmental pollution is being produced by this zone. It is one of the main polluters in industrial sectors in the compass of degree and the chemical composition of the discharged runoff. Inept dyeing processes often result in considerable residuals of dyes. The presence of these dyes in effluent is considered to be very problematic because of the persistent and recalcitrant nature (Yasar et al., 2013) [50]. Therefore, it is need of the time to analyze these wastes for the isolation and characterization of some indigenous strains of heavy metal tolerant (HMT) bacteria and to explore their potential in bioremediation of common heavy metals founds in such effluents. Keeping in view the above, present study was conducted for the isolation, identification and molecular characterization of indigenous HMT bacterial strains from textile effluent and to evaluate their biosorptive potential against heavy metals.

3. Meterials & Methods

3.1. Sample Collection & Heavy Metals Analysis

Wastewater samples were collected from the textile effluent. 06 main drains present in and around Faisalabad, Pakistan receiving the textile effluents and surrounding different textile units were selected. From each drain, 05 samples were taken keeping the distance of about 1000 meter between two points (Baby et al., 2014) [7]. After collection, samples were processed for determination of

heavy metals i.e., Cobalt (Co), Chromium (Cr), Nickel (Ni), Lead (Pb) and Zinc (Zn). Samples were digested by following the protocol as previously described by Sinha et al. (2014) [41] and metal analysis was done by using Atomic Absorption Spectrophotometer (AAS) (Hitachi Polarized Zeeman AAS, Z-8200, Japan) following the conditions described in AOAC (1990) [6]. Based on the results of metal analysis, Nickel (Ni) and Cobalt (Co) were selected for further study.

3.2. Isolation of Hmt Bacteria

Tenfold serial dilutions of effluents were prepared in sterile distilled water up to 10-5 as described by Lucious et al. (2013) [23]. Isolation of Ni and Co tolerant bacteria was done through spread plate method as described by Samanta et al. (2012) [35].

3.3. Determination of Maximum Tolerable Concentration (MTC)

MTC of heavy metal was selected as its highest concentration that allowed visible bacterial growth after 48 to 96 hours of incubation. The increasing concentration of both heavy metals (Ni and Co) i.e. (0.5 mM, 1 mM, 1.5 mM, 02 mM, 2.5 mM, 03 mM, 3.5 mM, 04 mM, 4.5 mM, 05 mM, 5.5 mM, 06 mM, 6.5 mM, 07 mM, 7.5 mM, 08 mM, 8.5 mM, 09 mM, 9.5 mM, and 10 mM) were added in pre-sterilized nutrient agar plates for testing the MTCs of bacteria (Vashishth and Khanna, 2015) [46].

3.4. Multi Metal Resistance (MMR)

MMR of bacteria was determined by inoculating them on nutrient agar medium incorporated with Ni, Co and Cr in equal concentration i.e. (1:1:1) as described by Saini and Pant (2016) [33].

3.5. Identification of Bacteria

After 48 hours of incubation, colonies were selected on the basis of morphology, shape and color. All the isolates were purified by repeated streaking on nutrient agar and stored at 4°C for further studies. Identification up to genus level was done on the basis of cultural characteristics, microscopic examination after Gram's staining (shape, arrangement and staining character), and physiological/biochemical characteristics (motility, oxidase reaction, catalase reaction, glucose utilization & fermentation tests and starch hydrolysis). All identification tests were performed following the protocols mentioned in Bergey's Manual of Determinative Bacteriology.

3.6. Molecular Characterization

Ribotyping was done for the molecular characterization of identified HMT bacteria by amplifying 16S rRNA gene. Total genomic DNA was extracted by CTAB method (Wilson, 2001) [49]. Polymerase Chain Reaction (PCR) was used for the amplification of 16S rRNA using 16S rRNA PCR primers, PA (5´-AGAGTTT-GATCCTGGCTCAG-3´, and PH (5´-AAGGAGGTGATCCAGC-CGCA-3´) (Zaheer et al. 2016) [51].

3.7. Phylogenetic Analysis

The 16S rRNA gene from the pure culture sequences from the NCBI database were aligned using Clustal X (Thompson et al., 1997) [45] and the maximum likelihood (ML)-based phylogenetic tree was constructed using MEGA (version 6) (Tamura et al. 2013) [44]. Confidence in the tree topology was evaluated using bootstrap resampling methods (1000 replications), and bootstrap values of at least 50% that demonstrated good support measures were retained.

3.8. Effect of Ni and Co on Bacterial Growth

To observe the effect of Ni and Co separately on bacterial growth, growth curve experiment was conducted in nutrient broth. For this purpose, nutrient broth tubes with Ni (01mM), Co (01mM) and without Ni and Co (control) were prepared. For each bacterial isolate 100 ml medium was taken in one set consisting of 08 test tubes for all three groups (i.e., two with metals and one control), autoclaved and then inoculated with 20 μL of freshly prepared inoculum. These tubes were incubated in shaking incubator at 37ºC at 100 rpm. Then after 0, 4, 8, 12, 16, 20, 24 and 28 hours one tube out of 08 in each group was removed and absorbance was measured at 600 nm. Growth curve was plotted by the readings obtained from the experiment and compared (Shakoori et al., 2010) [38].

3.9. Evaluation of Biosorption Potential

Biosorption potential of indigenously isolated and characterized bacterial strains named as AMIC2 and AMIC3 was determined against two metals i.e., Ni and Co. For this purpose, one set (each containing 02 glass culture bottles) having capacity of 500ml was prepared for each strain supplemented with 200 ml of LB broth with initial metal concentration of 50ppm for both metals. After autoclaving each set was inoculated with 02 ml of 18-hour old bacterial culture having turbidity equal to 0.5McFarland turbidity solution. Culture bottles were kept under constant agitation at 37oC for 24 and 48 hours then centrifuged at 14000 rpm for 05 minutes and supernatants were collected and stored at -20oC for heavy metal analyses. Heavy metals present in solution were measured through Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (Ramyakrishna and Sudhamani, 2016) [32].

3.10. FT-IR Analysis of Bacterial Biomass

FT-IR was used to analyze the functional groups and overall nature of chemical bonds in bacterial strains. Infrared spectra of the control (bacteria grown without metal stress) and tested (bacteria grown with metal stress, Ni or Co) biomass were obtained by grinding 02mg of freeze-dried biomass with 200mg dry potassium bromide (KBr) powder (1:100) ratio in agate mortar. Obtained mixture was pressed to get translucent sample discs using pressure bench press. The FT-IR analysis was performed by using Perkin-Elmer Spectrum Version 10.4.3. The spectral data were collected over the range of 450- 4000 cm-1 (Ramyakrishna and Sudhamani, 2016) [32].

3.11. Scanning Electron Microscopy (SEM)

Outer morphology of the bacterial cells before and after biosorption was examined using SEM (Carl Zeiss Supra 55 Gemin; German Technology, Jena, Germany). Prepared samples were placed on the sample holder (stub) with carbon tape. In order to increase the electron conduction and to improve the quality of micrographs, a conductive layer of gold was made with portable SC7620 'Mini' sputter coater/glow discharge system (Quorum Technologies Ltd, Laughton, UK) (Michalak et al., 2014) [25].

3.12. Statistical Analysis

The data was analyzed by calculating Means ± SE, Analysis of Variance (ANOVA), Regression, co-relation and Z-test was performed by using Minitab software. P value was calculated to see the significance. The results of heavy metal analysis through AAS and physico-chemical analysis of collected effluent samples are shown in (Tables 1 & 2) respectively. From all 30 samples 13 samples were identified possessing Ni tolerant isolates in them but only two samples collected from drains surrounding the textile units located at small industrial estate and main Sargodha Road, Faisalabad Pakistan (SarDP1, SarDP5) shown the presence of isolates which shown MTC of Ni up to 08mM were selected for further studies. Single colonies of bacteria were obtained by repeated streaking and strains were given names (AMIC2 & AMIC3).

4. Results

It is worthy to elaborate that as all 30 samples were collected from 06 locations and from each location 05 points were selected so statistically results are tabulated for 06 locations and their mean and standard error was calculated. The AAS results shown that for all samples Ni, Co, Cr, Pb and Zn was found in the range of 0.168 ± 0.035 to 0.230 ± 0.019 for all samples, Co, 0.128 ± 0.053 to 0.216 ± 0.008, Cr, 0.031 ± 0.020 to 0.098 ± 0.018, Pb, 0.026 ± 0.023 to 0.240 ± 0.160 and Zn, 0.218 ± 0.068 to 0.336 ± 0.016. Out of 30 samples 13 were found to have Ni tolerant bacteria, two samples collected from drains surrounding the textile units located at small industrial estate and main Sargodha Road, Faisalabad Pakistan (SarDP1, SarDP5) shown the presence of isolates which shown MTC of Ni up to 08 mM were selected for further studies. Single colonies of bacteria were obtained by repeated streaking and strains were named as AMIC2 and AMIC3 respectively. MTC of Ni, Co and MMR shown by AMIC2 and AMIC3 is given in Table 1 & 2 respectively. Initial identification of the isolate up to genus level was done by using conventional microbiological techniques as described in Bergey's Manual of Determinative Bacteriology (Table 3). Molecular characterizations of the isolates were done by ribotyping for confirmation of bacterial species. Phylogenetic tree has been shown in (Figure 1). Percentage similarity and GenBank accession number is given in (Table 4). To observe the effect of Ni and Co separately on bacterial growth, growth curve experiment

was conducted in nutrient broth (Figure 2). The biosorption potential of AMIC2 and AMIC3 was determined against Ni and Co. Percentage reduction in metal (Ni & Co) is given in (Table 5).

Fourier Transform Infrared Spectroscopy (FT-IR) was used to analyze the functional groups and overall nature of chemical bonds in the isolates. Results of FT-IR spectra for AMIC2 and AMIC3 are given in (Figures 3 & 4) respectively. SEM was performed to check any morphological changes occurred in the outer surface of the bacteria in response to metal (Ni or Co). The results revealed that both metals (Ni & Co) affected the bacterial cell wall. Surface changes can be easily observed when compared to control (bacteria grown without metal stress) as shown in (Figure 5).
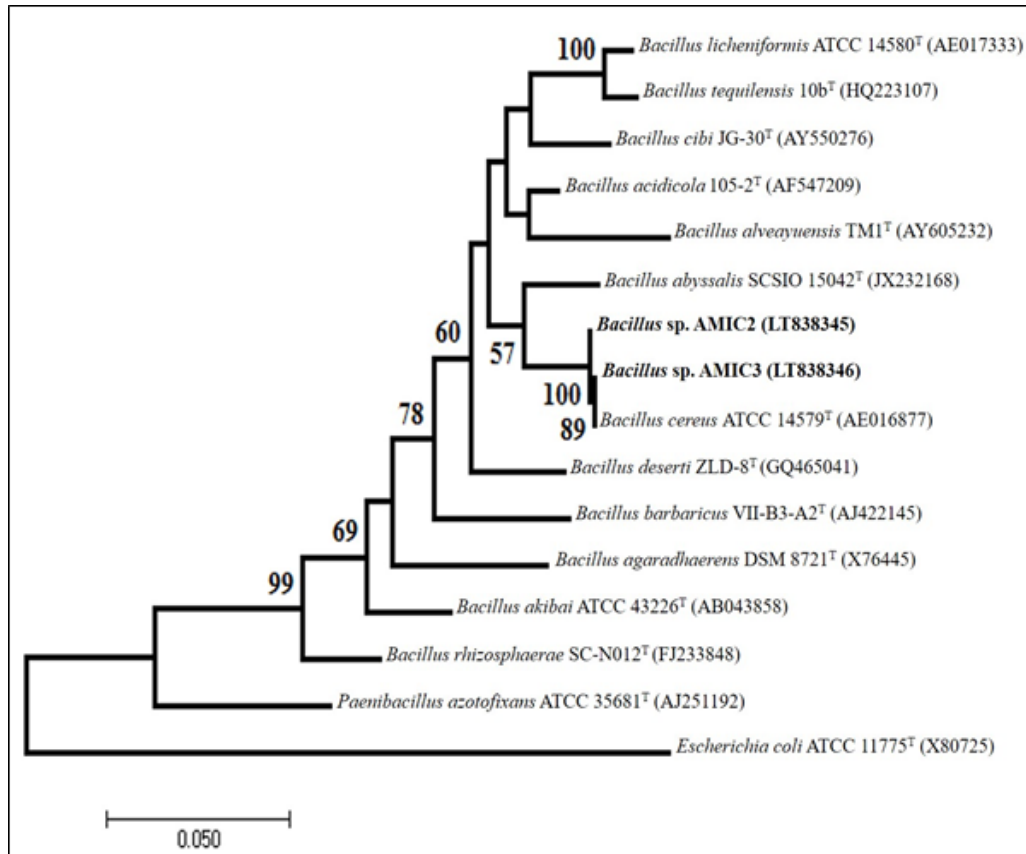

Figure 1: 16S rRNA sequence-based phylogenetic tree of Bacillus cereus isolated from textile effluent constructed by Maximum Likelihood method
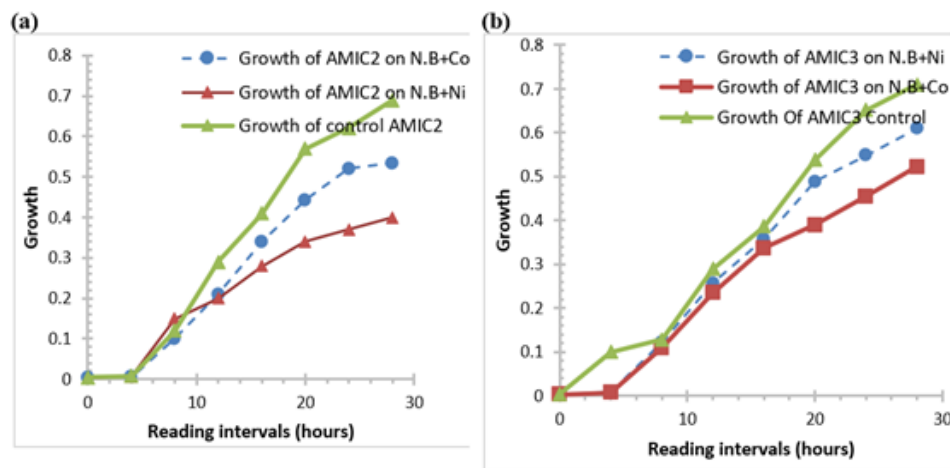

Figure 2: (a) Graph showing effect of Ni, Co and without metal (control) on the growth rate of AMIC2 (Bacillus spp.) (b) Graph showing effect of Ni, Co and without metal (control) on the growth rate of AMIC3 (Bacillus spp.)
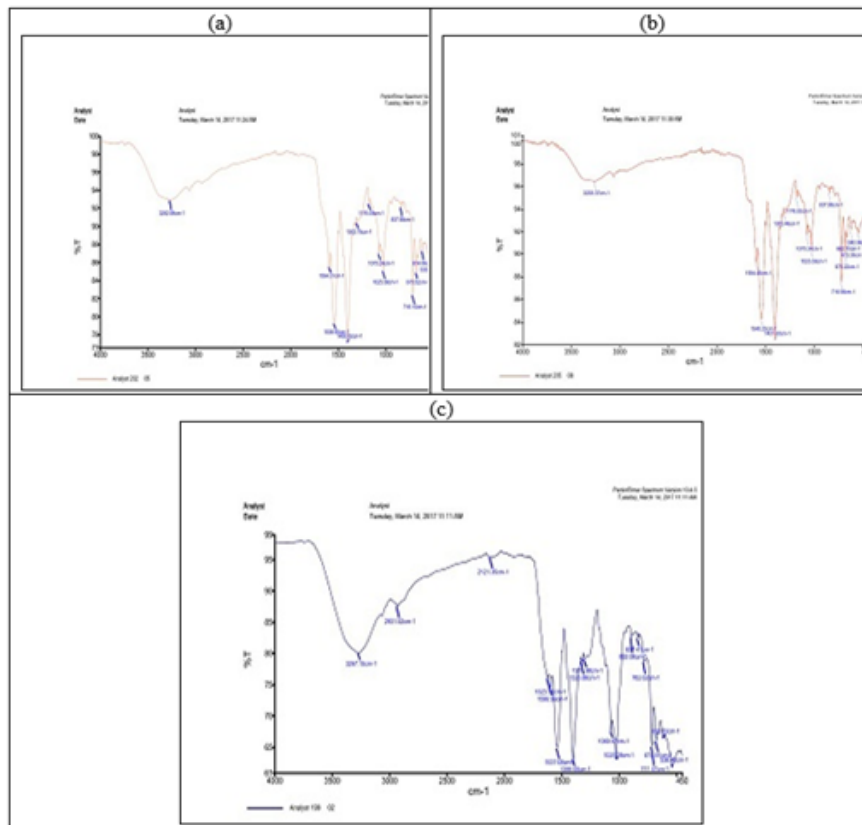
Figure 3: (a) FT-IR spectra of AMIC2 biomass loaded with Ni (b) FT-IR spectra of AMIC1 biomass loaded with Co (c) FT-IR spectra of AMIC1 biomass without metal loading

Table 1: Maximum Tolerable Concentration of Nickel (Ni & Co) and Multi Metal Resistance (Ni, Co and Cr) shown by AMIC2

	Concentration of Ni (mM)																
MTC of Ni shown by AMIC2	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
	Concentration of Co (mM)																
MTC of Co shown by AMIC2	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9
	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
	Concentration of Ni, Co and Cr (mM) at 1:1:1																
MMR shown by AMIC2	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9
	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-

Table 2: Maximum Tolerable Concentration of Nickel (Ni & Co) and Multi Metal Resistance (Ni, Co and Cr) shown by AMIC3

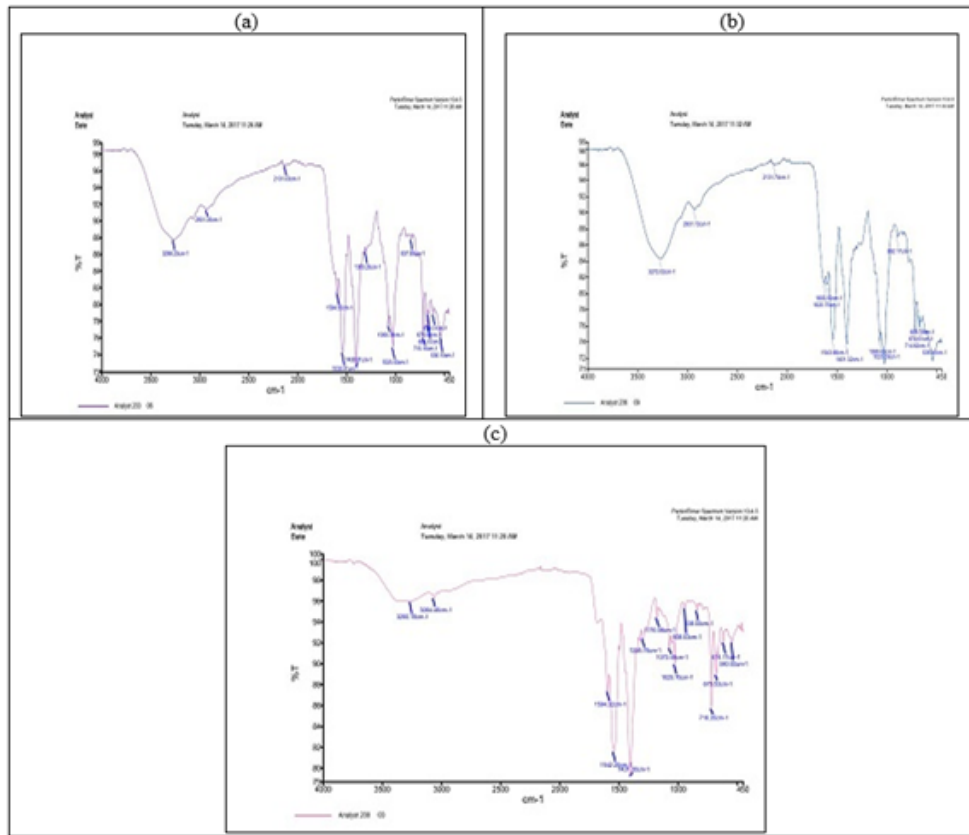|	Concentration of Ni (mM)																
MTC of Ni shown by AMIC3	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
	Concentration of Co (mM)																
MTC of Co shown by AMIC3	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9
	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
	Concentration of Ni, Co and Cr (mM) at 1:1:1																
MMR shown by AMIC3	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9
	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-

Figure 4: (a) FT-IR spectra of AMIC2 biomass loaded with Ni (b) FT-IR spectra of AMIC1 biomass loaded with Co (c) FT-IR spectra of AMIC1 biomass without metal loading

Table 3: Morphological and biochemical characteristics of isolated heavy metal tolerant bacterial strains

	Test Name	Isolated heavy metal tolerant bacterial strains	
		AMIC2	AMIC3
Morphological Tests	Cell Morphology	Rod	Rod
	Gram's reaction	+ve	+ve
	Motility	+ve	+ve
	Flagella	+ve	+ve
Colonies characteristics on selective and differential media	Nutrient agar	White to cream colour colonies	White to cream colour colonies
Biochemical tests	Catalase	+	+
	Oxidase	-	-
	Indole	-	-
	VP	+	+
	MR	-	-
	Citrate Utilization	+	+
Carbohydrate fermentation tests	Arabinose	-	-
	Glucose	+	+
	Inositol	-	-
	Lactose	-	-
	Maltose	+	+
	Mannitol	-	-
	Mannose	-	-
	Sucrose	+	+
	Starch	+	+

Table 4: Percentage of maximum similarity and GenBank accession number of HMT bacteria

Sr. No	Strain name	Identified as	Total bases	Homology	Accession No.
1	AMIC2	Bacillus sp.	1433	Bacillus cereus ATCC 14579(Type strain); (AE016877) ;99.86%	LT838345
2	AMIC3	Bacillus sp.	1445	Bacillus cereus ATCC 14579(Type strain); (AE016877); 99.79%	LT838346

Table 5: Comparison of %age reduction in Nickel (Ni) and Cobalt (Co) by AMIC2 and AMIC3

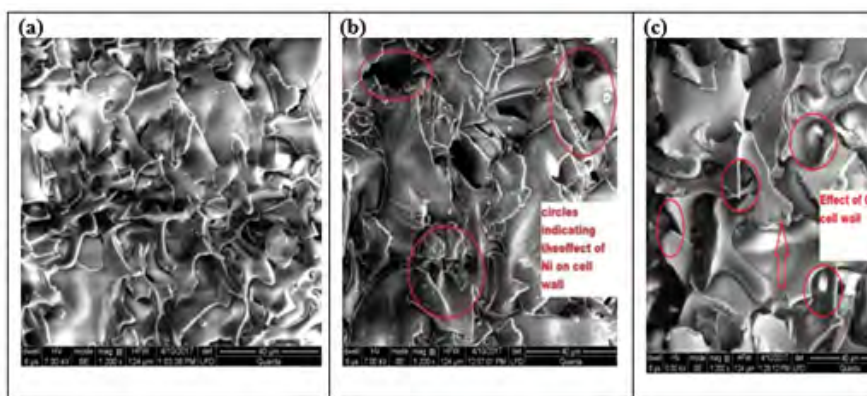| Bacterial strain	Metal	24 hours (S1)			48 hours (S2)			Z- value
		Initial	Final	% age	Initial	Final	% age	S1 vs. S2
AMIC2 (B. cerus)	Ni	50	25. 8	48.4	50	25.5	49	0.20NS
	CO	50	14.7	70.6	50	13.2	73.6	0.45NS
Z-value	Ni vs. CO	2.29*			2.77**			
AMIC3 (B. cerus)	Ni	50	24.7	50.6	50	24.1	51.8	0.20NS
	CO	50	14.1	71.8	50	13.4	73.2	0.23NS
Z- value	Ni vs. CO	2.31*			2.34*			

Figure 5: (a) SEM of Gram +ve Bacteria (AMIC2) grown without metal stress (control) (b) AMIC2 grown with Ni stress (c) AMIC2 grown with Co stress

5. Discussion

Heavy metal tolerant bacteria are assumed to occur; mainly in metal-contaminated sites. Studies showed that presence of metals and other physicochemical parameters play an important role in developing metal tolerance in indigenous bacteria of specific site (Shi et al., 2013) [39]. pH of specific metal contaminated site controls the solubility of metals (Klimek et al., 2012) [22]. The pH values of the effluent samples revealed no significant differences at all localities and ranged from 7.73±0.22 to 8.28±0.33. BOD and COD tests are performed to quantify the relative oxygen reduction effect of waste contaminant (Samudro and Mangkoedihardjo, 2010) [36]. Results showed that the studied effluent samples belonged to highly contaminant wastewaters. So it could be assumed that existing bacteria in a metal stressed environment can develop metal tolerance (Margesin and Schinner, 2001) [24].

After initial screening, it was observed that 13 out of total 30 effluent samples exhibited bacterial growth on Nutrient agar incorporated with 0.5mM of Ni. After determination of their MTC, it was observed that 02 samples i.e., SarDP2 and SarDP5 found have

some novel bacterial strains which were able to grow on Nutrient agar incorporated with 08mM of Ni. Statistical analyses revealed a highly negative correlation coefficient (r) between the number of isolates and Ni ion concentration for these samples; SarDP2 (r=-0.916x) & SarDP5 (r=-4.90x).

These two samples were then screened for tolerance to Co & Cr and multi metal resistance (MMR) against Ni, Co and Cr. It was evident from the results that bacteria from sample SarDP2 were able to tolerate Co up to 06mM & Cr up to 7.5mM separately and also exhibited MMR to Ni, Co and Cr (1:1:1) up to 5.5mM. Similarly, isolate from SarDP5 was able to tolerate Co up to 6.5mM, Cr up to 07mM and exhibited MMR to Ni, Co and Cr (1:1:1) up to 4.5mM.

These two bacterial strains which were able to tolerate the maximum concentration of heavy metals isolated from effluent samples SarDP2 and SarDP5 were named as AMIC2 and AMIC3 respectively. After Gram's staining of these strains, it was observed that both bacterial strains were Gram +ve rods. After examination of colony characteristics on selective & differential media, biochem-

ical and sugar fermentation tests results, it was confirmed that bacterial strains AMIC2 and AMIC3 isolated from SarDP2 and SarDP5 were Bacillus spp.

The results of present study are in agreement with the work of Abd El Hameed et al. (2015) [2] who performed the similar work by isolating the fungi from phosphatic sources and found a negative correlation between isolates and metal concentrations. Similar study was performed by Selvi et al [37]. (2012) for the isolation and characterization of heavy metal tolerant bacteria from tannery effluents and found that all isolates (Escherichia coli, Bacillus spp., Pseudomonas spp., Flavobacterium spp. and Alcaligenes spp.) exhibited tolerance to heavy metals in the respective order; Pb> Cu> Zn> Cr> Hg. Similarly, Raja et al. (2006) [31] performed a study for the isolation and characterization of metal tolerant Pseudomonas aeruginosa strain and found that isolate showed biosorption potential against all four tested metals (Cd, Cr, Pb and Ni) and the biosorption pattern was found as: Cr (30%) < Cd (50%) < Pb (65%) < Ni (93%).

The results of the present study are also in agreement with the work of Alboghobeish et al. (2014) [4] who isolated Nickel resistant bacteria (NiRB) from wastewater polluted with different industrial sources. Similar study was performed by Ahirwar et al. (2016) [3] for the isolation and characterization of heavy metal resistant bacteria from industrial affected soil and found that bacterial strains identified as Pseudomonas vulgaris, Pseudomonas fluorescence and Bacillus cereus were found to be the most efficient strains in terms of metal resistance.

After the isolation and identification of bacterial strains, growth conditions i.e., pH and temperature were optimized for the isolated and identified heavy metal tolerant bacteria. An optimum growth condition for each strain was determined without and with metal stress. It was found that AMIC2 and AMIC3 (Bacillus spp.) in nutrient broth with and without metal revealed maximum growth in terms of highest OD values at pH 08 and temperature 37oC. Statistical analyses revealed that there was highly significant difference (P<0.01) found in growth pattern of all strains when grown at different temperature and pH and also a highly significant difference (P<0.01) found in growth pattern of all strains when grown without and with metal stress. Then the effect of heavy metals (Ni or Co) was observed on both strains i.e., AMIC2 and AMIC3 (Bacillus spp.). Bacteria were grown without and with metal (Ni or Co) and the growth curve patterns were studied. It was evident from the results that metal ions (Ni or Co) significantly (P<0.05) reduced the rate of growth of both bacterial strains as compared to control group. Similar study was performed by Shakoori et al. (2010) [38] for the isolation and characterization of Cr6+ reducing bacteria. Who found that B. pumilus and Staphylococcus sp. showed optimum growth at temperature 37oC and pH 8 whereas A. faecalis exhibited optimum growth at temperature 37oC and pH 7.

After the molecular characterization of the isolated strains, biosorption potential of both bacterial strains i.e. AMIC2 and AMIC3 (B. cerus) was determined against two metals i.e. Nickel (Ni) and Cobalt (Co) through Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES). %age reduction in metal concentrations after 24 and 48hours were determined. The results showed that AMIC2 (B. cerus) reduced Nickel (Ni) 48.4 and 49% whereas reduction of Cobalt (Co) was 70.6 and 73.6% after 24 and 48hours. Similarly, AMIC3 (B. cerus) reduced Nickel (Ni) 50.6 and 51.8% whereas reduction of Cobalt (Co) was 71.8% and 73.2% after 24 and 48hours respectively. For statistical analysis Z-test was performed to compare the biosorption potential of each bacterial strain at two different incubation times and to compare the biosorption capacity of each bacterial strain for both metals i.e., Ni and Co at each incubation time. Results of statistical analyses shown that there was no significant difference (P>0.05) in the metal absorption capacity of all the bacterial strains when incubated for 24 hours and 48 hours but there was a significant difference (P<0.05) for biosorption capacity of each bacterial strain for both metals. Results shown that there was a significant difference (P<0.05) in the reduction of Ni and Co for all the strains. It was evident from the results of biosorption experiment that reduction pattern for Ni was found as AMIC3>AMIC2 and pattern for Co was found as AMIC2>AMIC3.

The results of present study are in agreement with the work of Das et al. 2016 [13] who found that Enterobacter sp. and Klebsiella sp. isolated from industrial effluents significantly (P<0.05) reduced Pb. Similar results were reported by Abbas et al. (2014) [1] who found that Pseudomonas sp. M3 isolated from wastewater samples were able to reduce 70% Cd from medium. In another study Abbas et al. (2014) [1] found that Enterobacter sp. and K. pneumonia sp. isolated from industrial effluents significantly (P<0.05) reduced Ar. Similar results were documented by Alboghobeish et al. (2014) [4] who found that K. oxytoca decreased 83mg/l of Ni+2 from the medium after 72 hours. Similarly, Gawali et al. (2014) [14] evaluated the bioremediation potential of heavy metal tolerant bacteria isolated from industrial wastewater. They found that E. coli was able to remove Pb and Cu with removal %age of 45% and 62% respectively. P. aeruginosa was able to remove Cd, Ni and Co with removal %age of 56%, 34% and 53% respectively. While E. acrogens was able remove Cd and Cu with removal %age of 44% and 34% respectively.

FT-IR study was carried out to confirm the difference between functional groups in relation to biosorption of metal (Ni and Co) using metal-loaded (Ni or Co) biomass in comparison to control (bacteria grown in normal conditions). The control sample demonstrated the presence of a number of absorption peaks and reflected the complex nature of the biomass. A change of absorption bands was observed, when we compared the FT-IR spectra of control and metal loaded biomass. After the evaluation of AMIC2 (B. cerus)

spectra it was observed that there was a change in peak at 3500–3200 cm-1 regions in spectrum of Ni and Co and was considered as the binding of Ni and Co with amino and hydroxyl group. Similarly, a change in peak at 2900-3000 cm−1 regions in spectrum of Ni and Co was considered as the binding of Ni and Co with –CH2 groups combined with that of the CH3 groups. A similar change in peak at 1300–1067 cm−1 regions considered as the binding of Ni and Co with carboxyl and phosphate groups. Similarly, the evaluation of AMIC3 (B. cerus) spectra it revealed that there was a change in peak at 3500–3200 cm-1 regions in spectrum of Ni and Co and that was considered as the binding of Ni and Co with amino and hydroxyl group. While a change in peak at 2900-3000 cm−1 regions in spectrum of Ni and Co was considered as the binding of Ni and Co with –CH2 groups combined with that of the CH3 groups. A similar change in peak at 1300–1067 cm−1 regions considered as the binding of Ni and Co with carboxyl and phosphate groups.

The results of the present study are in agreement with Park et al. 2005 who performed a similar study and described that a peak at 3500–3200 cm-1 region is due to the stretching of the N–H bond of amino groups and indicates bonded hydroxyl group. Similarly, Kazy et al. (2006) [21] described that the absorption peaks at 2900–3000 cm−1 are attributed to the asymmetric stretching of C–H bond of the –CH2 groups combined with that of the CH3 groups. Pistorius, 1995 [29] described that the peaks in the range 1300–1067 cm−1 are attributable to the presence of carboxyl and phosphate groups. Pradhan et al., 2007; [28] Volesky, 2007 [48] insisted that mainly functional groups including (hydroxyl, carbonyl, carboxyl, sulfonate, amide, imidazole, phosphonate and phosphodiester) are responsible for the biosorption of metals. Quintelas et al. 2009 [30] performed a similar study and observed that functional groups on the biomass, such as hydroxyl, carboxyl and phosphate groups, would be the main binding sites for biosorption of the studied heavy metals by E. coli. Similar results were documented by Kang et al. (2006) who compared the FT-IR spectra of pristine and chromium loaded biomass and found that P. aeruginosa before and after metal binding indicated that –NH is involved in Cr (VI) biosorption.

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