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Plasmid Mediated Tolerance and Removal of Heavy Metals by *Enterobacter* sp

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Abstract: Problem statement: The role of plasmid in the heavy metal resistance and accumulation by endophytic bacteria was investigated. **Approach:** The experimental results showed that high level plasmid mediated Cd^{2+} and Zn^{2+} resistance in this strain is due to decreased Cd^{2+} and/or Zn^{2+} uptake/accumulation by resistance strain. **Results:** Based on the fact that subsequent plasmid curing experiments demonstrated that the ability to grow in presence of $Cd^{2+}$and Zn^{2+} was encoded by the 98 kb plasmid, whereas the ability to grow in presence of Pb^{2+} appeared to be encoded by the chromosome. The Cd^{2+} and Zn^{2+} removal capacity of the respective metal resistant strain (pBN4) were about 36 and 45 µg g^{-1} DW respectively, while the removal capacity of the both metal by sensitive variant showed a significant high Cd^{2+} and Zn^{2+} removal capacity of 153 and 228 µg g^{-1} DW respectively. **Conclusion:** The isolated endophytic *Enterobacter* was not only tolerant to heavy metals, but also bound considerable amount of heavy metals from the growth medium. The biosorbed order of the metals by parental strain and its cured derivatives strain based on the cell dry weight was found to be in the order of Pb^{2+}> Zn^{2+}>Cd^{2+}.

Key words: Heavy metals, plasmid, *Enterobacter* sp., endophytic bacteria

INTRODUCTION

Contamination of the aquatic environment by toxic metal ions is a serious pollution problem[2,7,17]. Unlike organic pollutants, chemical or biological processes can not degrade toxic metal ions. To remediate the aquatic environment, the toxic metal ions should be concentrated in a form that can be extracted conveniently, possible for reuse or at least for proper disposal. Natural resources including plants and microorganisms are extensively explored to combat metal ion pollution.

Endophytic bacteria are described as non-pathogenic bacteria found within the interior tissues of healthy or symptomless plants[27]. These bacteria are found in most, if not all, plant species, span a wide range of bacterial phyla and are known to have plant growth promoting and pathogen control activities[8,9,25]. Recent research suggests that these beneficial impacts may, in the case of plant growing at contaminated sites, extent to the degradation of xenobiotic compounds[1,6,30,32].

Although endogenous bacterial endophytes capable of degrading hydrocarbons are likely to be widespread, to date only a few studies have assessed the heavy metal removal potential inherent to endophytic communities of heavy metal accumulator plant in phytoremediation. So, Soltan and Rashed[28] found that bacterial strains isolated from root and stem tissues of different heavy metal accumulator plant growing at heavy metal contaminated sites were able to accumulate metals. The distribution of these bacteria was spatially limited both within and between the individual accumulator cultivars. Water hyacinth (*Eichhornia crassipes*) is one of the plant species that attracted considerable attention because of its ability to grow in heavily polluted water together with its capacity for metal ion accumulation[3,4,13,14, 28,33] though other studies have clearly manifested that hyacinths are more efficient in the phytoremediation of heavy metals[16,24,29]. We therefore hypothesized that *Eichhornia crassipes* tissues could be a potential habitat for metal-resistant microorganisms. Because certain microorganisms could enhance the growth and remediation potential of plants[1,3] and because heavy metal-resistant microorganisms could be sources of genes for engineering plants for heavy metal resistance and remediation, our objective of the current study was to identify and characterize heavy metal resistant bacteria in the plant tissues of *Eichhornia crassipes*. Accordingly the aim of this work was to study the tolerance and the uptake of different heavy metals by endophytic bacteria isolated from heavy metal accumulator plant water hyacinth and to investigate the role of endogenous plasmid in metal resistant and removal potential by endophytic bacteria.

MATERIALS AND METHODS

Bacterial strains: The bacterial strains and plasmids used in this study are listed in Table 1. *Enterobacter* sp. BN4 was originally isolated from surface sterilized leaves of *Eichhornia crassipes* growing on irrigation canals, Egypt[5]. The endophytic character of this strain was previously confirmed[5] and identified as *Enterobacter* sp. based on the 16S rRNA gene sequence analysis. The sequences were deposited at Gen Bank (*Enterobacter* sp. Accession no. AM40578).

Chemical: Agarose and ethidium bromide were purchased from Sigma (St. Lous, MO, USA), Cadmium Chloride ($CdCl_2.H_2O$), Zinc Chloride ($ZnCl_2$) and lead acetate Pb $(NO3)_2$ were procured from Merck (Darmstadt, Germany). Bacteriological media were purchased from Difco (Difco Laboratories, Detroit, Michigan) and Oxoid (Oxoid Inc., Nepean, Ontario). Chemical reagents used were of the highest grade commercially available

Media and growth condition: Strain BN4 and its derivatives were grown aerobically in Luria-Bertani (LB) medium[26]. For testing the degree of resistance to heavy metal salts, bacterial cells were grown at 30°C in a Tris-buffered Minimal Salts Medium (MSM)[11]. This medium was buffered with 50 mM Tris-HCl (Trizma; Sigma), pH 7, instead of a phosphate buffer to avoid precipitation of insoluble metal phosphates. Phosphorus was added to the medium in the form of sodium β-glycerophosphate. This medium does not complex heavy metal cations. Analytical-grade salts of $CdCl_2.H_2O$, Pb $(NO3)_2$ and $ZnCl_2$ were used to prepare 1.0 M stock solution, which were sterilized by autoclaving and added to MSM media for determination of the minimum inhibitory concentrations (MICs) of the metal ions for each isolate. The range of concentrations used was 0.5-5 mM for lead nitrate, 0.5-5 mM for zinc chloride and 0.1-3 mM for cadmium chloride. Overnight cultures grown in LB Broth were diluted to 10^{-5} cells mL^{-1} then spotted onto LB agar plates or MSM agar plates. Duplicate plates of each isolate were incubated at 28°C for 3 days before growth was scored and MIC (the lowest concentration of metal that completely prevented growth) determined.

Antibiotic resistance: Antibiotic resistance was tested, using Nutrient agar containing tetracycline (20 µg mL^{-1}), kanamycin (100 µg mL^{-1}), ampicillin (100 µg mL^{-1}) or chloramfenicol (25 µg mL^{-1}), which then added aseptically to the medium after autoclaving. Cultures were incubated at 30°C for 7 days.

Table 1: Bacterial strains and plasmids used in this study

Strains or plasmid	Relevant characteristic(s)a	Reference or source
Enterobacter sp.		Accession no. AM40578. This study
BN4	Km^r, Tet^r, Amp^r, Cd^r, Zn^r, Pb^r wild type BN4, pBN4 carrier	
BN4 M	Km^s, Amp^s Cd^s, Zn^s a cured mutant of BN4, plasmidless	This study
BN4T	Km^r, Amp^r, Cd^r, Zn^r transconjugant by mating of BN4 and BN4M , pBN4 carrier	This study
Plasmids pBN4	98 Kb Km^r, Amp^r , Cd^r, Zn^r,, heavy metal resistant plasmid of *Enterobacter* sp BN4	This study

Tet: Tetracycline , Km: Kanamycin, Amp: Ampicillin

Plasmid isolation: Plasmid DNA was isolated from endophytic bacteria essentially by the procedure of Kado and Liu[15]. The isolated plasmid was characterized by agarose gel electrophoresis according to the standard procedure of Sambrook *et al*.[26]. Agarose gel electrophoresis through a horizontal slab gel of 0.8% agarose submersed in TBE (Tris-HCl, Boric acid, EDTA) running buffer at 70 V for 2 h were performed. DNA bands were stained with ethidium bromide for 15 min. and visualized on a UV transilluminator. Molecular weight of the plasmids were determined by using a computer program (DNA size Version 1)[21].

Curing and conjugation: To determine if the resistance genes are encoded by a plasmid or not, Mitomycin C was used to eliminate the plasmid from the strain. Bacterial strain was grown on LB broth containing various concentrations of mitomycin C (5, 10 and 20 µg mL^{-1}) on a rotary shaker at 30°C for 48 h. Appropriate dilutions were spread on LB agar plates. Single colonies were replicated onto nutrient agar supplemented with different heavy metals. Heavy metal sensitive colonies were scored and reported as percent cured. Cured isolates were then reinoculated into MSM with and without metals stress to reevaluate the MIC of each isolate to each metal. For conjugal gene transfer, overnight cultures of donor strain *Enterobacter* sp. BN4 and of the BN4M recipient strain were mixed (1:1) and incubated at 28°C. After overnight growth, the bacteria were suspended in saline (9 g of NaCl L^{-1}), diluted and plated onto selective media and several dilutions were plated on minimal medium agar supplemented with heavy metals.

Inducibility of heavy metal resistance of strain BN4: A Tris-glucose medium was used to study the inducibility of cadmium, zinc and lead. The pre-cultures were prepared by the inoculation of BN4 in 10 mL of the Tris-glucose medium and left to grow overnight at

30°C on a rotary shaker (100 rpm). For the induction, 0.1 mL of this culture was then added to a 100 mL Erlenmeyer flask containing 10 mL of the Tris-glucose medium with and without heavy metals (e.g. 0.05 mM Cd^{2+}, 0.05 mM Zn^{2+}, or 0.05 mM Pb^{2+}) and incubated at 30 °C on shaker for 18 h. These cultures were then used to inoculate 50 mL media containing sub-inhibitory concentrations of the heavy metals (1 mM Cd^{2+}, 1.5 mM Zn^{2+} or 1.5mM Pb^{2+}) and incubated at 30°C with shaking. The growth of bacteria was monitored periodically by measuring the optical density at 600 nm.

Analysis of Metal removal: Cd^{2+}, Zin^{2+} and/or Pb^{2+} uptake by growing cells was determined at 0.1 mM of tested metals by the parental strain and its cured derivative. The cells were grown in nutrient broth supplemented with desired concentration of metals at 30°C with 150 rpm. After 24 h the bacterial were collected, washed and dried at 55°C for 24 h. After acid digestion (nitric acid: Perchloric acid, 5:3), metal concentration in both washed fluid and pellet were determined using an atomic absorption spectrophotometer (Perkin Elmer analyst 300). Determination of cadmium, zinc and lead was done by using a specific lamp for each metal and at specific wavelengths. All of the metal removal experiments were conducted in triplicate and mean values were used in the analysis of data.

RESULTS

Heavy metal and antibiotic resistance: MIC of various metals for *Enterobacter* sp. BN4 are shown in Table 2. $Zn^{2+}$and Pb^{2+} were less toxic towards *Enterobacter* cells than Cd^{2+}. The order of the metals toxicity to the bacterium was found to be Cd^{2+}> Zn^{2+}>Pb^{2+}. The resistance to antibiotics was also examined for metal tolerant BN4. It was found to be resistance to tetracycline (20 µg mL^{-1}), kanamycin (100 µg mL^{-1}) and ampicillin (100 µg mL^{-1}).

Genetic localization of cadmium, zinc and lead resistance in the endophytic bacteria BN4:

Plasmid content: The plasmid isolated from BN4 was designated as pBN4. The estimated size of the plasmids was approximately 98 Kb (Fig. 1, Lane 1, 3). The location of heavy metal resistance genes on plasmid DNA was determined by plasmid curing and conjugation experiments.

Plasmid curing: To examine the correlation between resistant to cadmium, zinc and lead and the presence of pBN4, mitomycin C was used to cure strain BN4 from its plasmid pBN4. The loss of resistance to Cd^{2+}, Zn^{2+}, or Pb^{2+} in cured strains was used as an indicator of curing (Table 2). Out of three handed *Enterobacter* sp. (BN4) transformants, 200 colonies were cured, showing 66% curing efficiency.

The comparison of resistance before and after curing is shown in Table 2. This was taken as an indication of curing of the plasmids. The results showed that while the BN4 resisted Cd^{2+}, Zn^{2+} and Pb^{2+}, it could not grow on medium containing $Cd^{2+}$and Zn^{2+}after curing. This strain showed a growth on medium containing Pb^{2+} before and after curing. Similarly, the antibiotic resistance of uncured and cured cultures of the isolate was also compared to determine the location of antibiotic resistance genes. The results showed that while the BN4 resisted to Kanamycin and ampicillin, it could not grow on medium containing these antibiotics after curing. To confirm if the resistance genes are encoded by this plasmid, the electrophoretic profile of the plasmid isolated from the original (non-cured) strain was compared with the cured strain. The results of this experiment indicates that each of cadmium and zinc and/or antibiotic-sensitive mutant lost the plasmid pBN4 (Fig. 1; Lane 1). One of these mutants was designated as BN4M.

Table 2: MICs of heavy metals for *Enterobacter* sp. BN4 and its derivatives in different cultural conditions

Bacterial strains	MIC of Cd^{2+} (mM)		MIC of Zn^{2+} (mM)		MIC of Pb^{2+} (mM)	
	MSM	LB	MSM	LB	MSM	LB
BN4	2.1	3.0	4.0	5.0	4.8	6.0
BN4M	0.3	0.6	0.5	0.7	3.6	5.8
BN4T	2.1	3.0	4.0	5.0	4.8	6.0

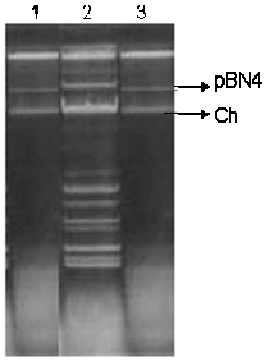

Fig. 1: Plasmid profile of strain BN4 and its derivatives. (Lane 1), wild type strainBN4; (Lane 2), *Shigella flexneri* 49 used as size standard: (Lane 3), BN4T transconjugant obtained by mating of BN4 and BK4M, Ch; chromosomal DNA

Plasmid conjugation: Wild type strain BN4 was used as a donor of pBN4 plasmid to ensure that cadmium and zinc resistance genes are located on this plasmid. The strain BN4M was marked to be rifampin resistance (data not shown) and was used as a recipient strain. All obtained transconjugants were true rifampin resistance. These transconjugants acquired the resistance to Cd^{2+} and Zn^{2+} and contained a plasmid were shown to be identical to pBN4 by agarose gel electrophoresis (Fig. 1; Lane 3). The frequency of plasmid transfer from BN4 to BN4M ranged from 2×10^{-5} per recipient cell.

MIC comparison between wild type strain and its derivatives: Examination of growth of the plasmid bear strain BN4 and its cured derivative on different medium compositions containing varied concentrations of heavy metals showed variable degrees of resistance to one or more of three metals: Cadmium, zinc and lead (Table 2). On rich medium (LB), wild type strain BN4 (pBN4) exhibited high resistance to cadmium and zinc in comparison with the cured strain (Table 2). However, the MICs of Pb^{2+} for the wild type strain and its cured derivative were very close (Table 2). Tris-buffer was not utilized as a carbon or nitrogen sources by the tested strains (data not shown). In the present study, the level of cadmium, zinc and lead resistance in strains BN4 and its cured strain BN4M when grown on Tris-medium amended with glucose as sole carbon and energy source was determined. As reported in Table 2, the MIC of Cd^{2+} for the sensitive cured strain was 0.1 mM, whereas the MICs for wild type strains (BN4) were 2 mM. Furthermore, Strain BN4 exhibited the highest level of resistance to zinc (3 mM), although its resistance level was much higher than that of sensitive strain (Table 2). The results in Table 2 showed that no great difference among the MICs of Pb^{2+} for the tested strains.

Regulation of heavy metal resistance genes in BN4: Whether the Cd^{2+}, Zn^{2+}, or Pb^{2+} resistance property in strain BN4 is inducible or constitutive was determined by examining the growth curves. Figure 2 shows that, the induced and uninduced cells passed a lag period of 6 hr indicating that the pb in strain BN4 is constitutively expressed. In contrast, the cells of this strain pre-grown in the presence of Cd^{2+} or Zn^{2+}, started to grow after a shorter lag phase than the uninduced cells (Fig. 2).

Heavy metal removal by endophytic bacteria BN4 and its cured derivative: The heavy metal-resistant endophytic strain BN4 and its cured derivative BN4M were tested for their ability to remove cadmium, zinc and/or lead from the growth medium.

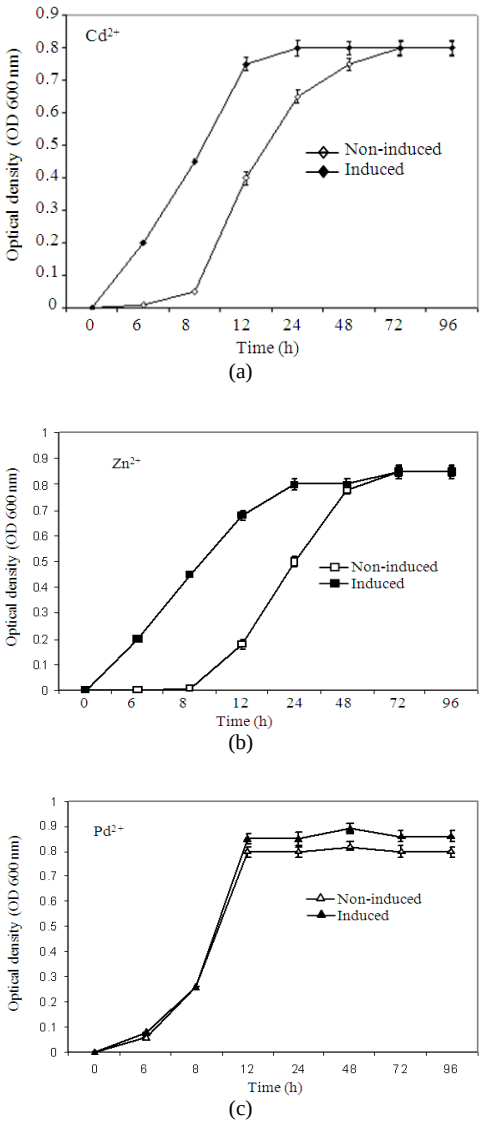

Fig. 2: Growth of *Enterobacter* sp. strain BN4 in presence of 1 mM $CdCl_2$, 1.5 mM $ZnCl_2$ and 1.5 Pb $(NO_3)_2$. Cells were induced by overnight growth in presence of 50 µM Cd, Zn, or Pb

The parental strain and its cured derivative were grown in the presence of 0.1 mM of each metal. Metals which loosely bound with cells (in washing) and accumulated metal in the cells were determined. Curing of plasmid in the parental strain resulted in substantial increase in Cd^{2+} and/or Zn^{2+}-uptake (both accumulated and loosely bound Zn^{2+} or Cd^{2+}) (Fig. 3). However, the accumulated and loosely bound Pb^{2+} uptake were the same in the parental strain and its cured derivative (Fig. 3).

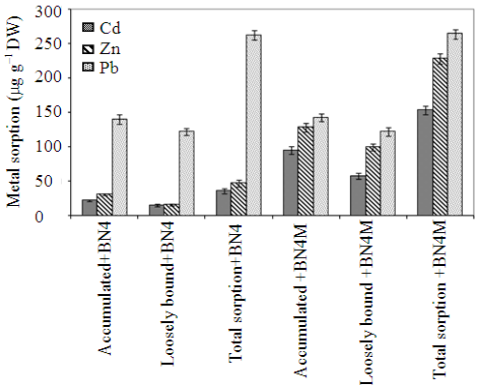

Fig. 3: Cadmium, zinc and lead uptake as accumulated, loosely bound and total metal sorption by metal resistant strain BN4 and its cured derivative BN4M

DISCUSSION

The differences between MIC of either wild type strain or its derivatives on rich medium or Tris medium could be explained by those mentioned previously by Mergeay *et al*.[19] They demonstrated that the interference of high phosphate content in usual minimal medium with metals effect, can lead to overestimation of the MIC[19]. In comparison to previous studies on the metal tolerance of endophytic *Enterobacter* sp.[27], the endophytic bacterium isolated in the present study exhibited more resistance (in Tris medium and at pH 7) to Cd^{2+}, Zn^{2+} and Pb^{2+}. A direct comparison of these MIC estimates with those reported by other authors is not conclusive. These discrepancies in the MIC results could be resulting in differences in the metal-binding capacities of the media used. However, the MIC estimated for *Enterobacter* sp. BN4 for Cd^{2+}, Zn^{2+} and/or Pb^{2+} in Tris medium as well as the MIC of the same metals for BN4 in rich medium, were at levels regarded as those typical for metal resistant species[18-20]. Multiple antibiotic resistance shown by isolate BN4 might be associated with heavy metals resistance. In many studies, the association between metal tolerance and antibiotic resistance have been reported[27]. It has been suggested that under environmental conditions of metal stress, metal and antibiotic resistant microorganisms will adapt faster by the spread of R-factors than by mutation and natural selection[32].

Enterobacter BN4 was able to grow at high concentrations of Cd^{2+}, Zn^{2+} and Pb^{2+} in liquid medium, which might be important for the capacity of this bacterium to survive in different source of pollution with elevated heavy metal levels. It was found that the toxicity of Cd^{2+} was higher than that of Zn^{2+} and Pb^{2+}, that consistent with the results in solid media in the present study and many other reports[27]. This difference in toxicity could be explained by the conditions of the isolation and selectivity of microbial culture techniques adopted in each study, particularly with respect to the nature and specificity of growth media[18-20]. These results also indicating that cadmium and zinc resistance in strain BN4 are inducible expressed whereas, lead is constitutively expressed. Similar observations have been reported by others[11,12] in which Cd^{2+} and Zn^{2+} resistance genes were induced in *Pseudomonas putida*.

Metal-tolerant bacteria have evolved various resistance and detoxification mechanisms[10,19,20]. The resistance mechanisms are chromosomally encoded or, more often, plasmids of different size and showing conjugative capabilities are carriers of metal-resistance genes[31]. Curing of the plasmid in the isolated strain was done by using mitomycin C in order to determine the presence of metal ion resistance genes on plasmid or chromosomal DNA. This study also revealed the high efficiency of mitomycin C in curing the plasmid. Cured and uncured cultures were compared for their resistance against metal ions and antibiotics. The comparison clearly showed the tolerance to Zn^{2+} and Cd^{2+} appeared to be associated with plasmid, as confirmed by the conjugation data. However, Lead resistance gene was shown to be present on the chromosomal DNA rather than the plasmid DNA as the cured and uncured cultures remained similar in Pb^{2+} resistance. Previously, Piotrowska-Seget *et al*.[20] reported that resistance to toxic metals (Zn^{2+} and Cd^{2+}) have been found in bacteria from clinical and environmental origins and genetics determinant of resistance are frequently located on plasmids or transposons. In contrast, Roan[22] and Roan and Kellog[23] did not find plasmids in Pb^{2+}-resistance isolates collected from contaminated soils. Similar report was presented by Haq *et al*.[10], for loss of plasmid linked drug resistance after treatment curing agent. Here I found that curing with mitomycin C resulted in loss ampicillin and kanamycin resistance in BN4 strain. The location of resistance genes for antibiotics, metal resistance on plasmid of *Enterobacter* sp. BN4 was confirmed by conjugation of BN4M mutant and cured BN4 , whereby the transconjugants showed all characteristic of wild type strain BN4.

Wild type BN4 and its cured derivatives isolated in this study show different efficiencies in removal of Cd^{2+}, Zn^{2+} and Pb^{2+} from the medium. They accumulated a significant amount of these metals within 24 h. The accumulation fraction of Cd^{2+} and/or Zn^{2+} was more relative to loosely bound one either in parental or cured derivative. A comparison of parental strains and its cured derivative exhibited that with the loss of plasmid, Cd^{2+} and Zn^{2+}-sorption (accumulated and loosely bound) of bacterial strains increased 4 and 6 fold, respectively (Fig. 3). These results reflected that high level plasmid mediated Cd^{2+} and Zn^{2+} resistance in

this strain is due to decreased Cd^{2+} and/or Zn^{2+} uptake/accumulation by resistance strain.

Plasmid-determined reduced accumulation of Cd^{2+} have been described in *Pseudomonas putida*[12]. Roan and Kellogg[23] observed that different strains of Cd-resistant bacterial isolates varied in their resistance level due to potentially varied mechanism of resistance. Significant reduction of soluble Cd^{2+} was observed during growth of plasmid-bearing *Bacillus* strain H9 and *Pseudomonas* H1. Similarly, three strains of bacteria isolated from industrial effluents (*Enterobacter cloacae* and *Klebsiella* spp.) were resistant to high concentrations of Cd^{2+}, Pb^{2+} and Cr^{2+} in the growth media and could remove approximately 85% Cd^{2+} during growth[10]. Further, the Cd^{2+}, Zn^{2+} and Pb^{2+} removal efficiency was growth-phase dependent. This result implicated the advantage of plasmid-encoded Cd^{2+} and Zn^{2+} resistance genes, allowing for further genetic manipulation and enhanced efficiency.

CONCLUSION

In view of the results of metal accumulation experiments, it was concluded that endophytic isolate was not only tolerant to heavy metals, but also bound considerable amount of heavy metals from the growth medium. The biosorbed order of the metals by parental strain and or its cured derivatives strain based on the cell dry weight was found to be in the order of Pb^{2+}> Zn^{2+}>Cd^{2+}.

Conjugation frequency of Cd^{2+} and Zn^{2+} resistance genes suggests that horizontal gene transfer may be an important factor for the development of metal resistance microorganisms in the environment and may be linked to the distribution of these resistances in nature.

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