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Study on Genomics And Cadmium Resistance Potentiality of *Enterobacter* Isolated From Narcotic Containing Agent.

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ABSTRACT

About seven bacteria were isolated on the basis of their cadmium (Cd^{2+}) resistant properties from narcotic containing beedi samples. Among them only 42.86% isolates shown growth at 1000 mg/L and where NaCd1 was observed with maximum relative growth of 86.89% while compared with control. A high significant difference was seen among these bacterial isolates and their Cd^{2+} concentrations at $P < 0.001$. Primarily, NaCd1 was identified as *Enterobacter* through cultural, morphological, biochemical and physiological tests. The optimum growth was seen at initial pH of 6.0 while incubating at 35ºC and at 2% (w/v) NaCl concentrations. 16S rRNA gene sequencing revealed the NaCd1 as *Enterobacter cloacae* NaCd1 (GenBank Accession No. KT336353). Furthermore, this fact was verified at secondary structural level through structure prediction in Mfold web server. Cadmium resistance potentiality was also observed experimentally. It was found that about 29% Cd^{2+} was removed from the medium incubated for 24 h and from sonicated cells of bacteria about 91.38% Cd^{2+} was recovered. This supports the fact clearly NaCd1 holds promise for effective, economical and eco-friendly metal bioremediation technology for industrial exploitation and pollution free environment and may replace the commercial ion exchange resins that have been used conventionally for metal removal.

Keywords: Beedi, bioremediation, cadmium, Mfold, secondary structure, tobacco

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INTRODUCTION

Pollution is always claimed to be a major problem for the human life as well as the earth and its environment. Release of many toxic metals including mercury, cadmium, chromium, zinc, lead from industrial activities has become a serious source of concern. Metallic pollutants are not easily degraded during composting and can enter into the environment to cause severe hazard for both biotic and abiotic ecosystems. During the last two decades microbial processes are gaining extensive attention in bioremediation and bioleaching of heavy metals [24]. Many recent studies have been well documented on significance of bacteria to remove heavy metal ions from the environment [3]. Heavy metals in soil put adverse effects on agricultural production by interfering food quality, safety, marketability, crop growth and environmental health [2].

Cadmium (Cd^{2+}) accumulation in crop plants, such as tobacco (*Nicotiana tabacum* L.), can lead to human exposure to this carcinogenic metal. Smoking is the most common method of consuming tobacco, and tobacco is the most common substrate smoked. About 2-8% nicotine is present in the dry mass of the tobacco leaves [10]. Bacteria found in tobacco products are saprophytic bacteria which may cause opportunistic infection. Other than microbial infections tobacco practice can also lead to systemic disorders which can lead to many acute and chronic respiratory and pulmonary disorders. Cadmium concentrations in the tobacco samples ranged from 0 - 6.78 µg/g confirmed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and a significant difference in Cd^{2+} concentration and interaction were found among types and in all countries.

Cadmium and cadmium compounds are highly toxic. Cadmium concentration contributes a large number of health conditions, including the major killer diseases like heart disease, diabetes and cancer. Concentration of cadmium in liver, kidney and various other organs are highly toxic than that of lead or mercury. It is toxic at level one tenth that of lead, mercury, aluminium or nickel [14]. Cadmium is produced in different industrial activities like galvanizing, pigments, stabilizers, thermoplastics, batteries and alloys [23]. According to WHO's recommendation cadmium limit in drinking water is 0.005 mg/L.

Studies revealed that the microorganisms isolated from tobacco had been used either for identification or for the nicotine degradation [7, 15, 20]. Heavy metal tolerance test of these isolates was given very less importance. Therefore keeping the above problems in mind, the present study focused on isolation and identification of the bacteria present in tobacco which are resistant to cadmium and their bioaccumulation potential.

MATERIALS AND METHODS

Collection of samples and metal solution preparation

Prior to the experiments, few pieces of beedi were collected from the local market of Bhubaneswar. Stock solution of Cd^{2+} (1000 mg/L) was prepared from its chloride salt i.e. $CdCl_2$. The glassware used for this purpose, were leached in 2N HNO_3 (nitric acid) and rinsed several times with distilled water before use to avoid any metal contamination. Stock solution was prepared in distilled water and slightly acidified with HNO_3 (2 to 3 drops of concentrated HNO_3) to prevent precipitation, and were sterilized at 121°C for 15 min. These solutions were tested in Atomic Absorption Spectrophotometer (AAS) and kept at 4°C for not more than one month.

Isolation of cadmium resistant bacteria

Isolation of Cd^{2+} resistant bacteria was performed in two major steps i.e. direct and indirect. Initially, the tobacco samples were weighed to 1 g and crushed using mortar and pestle to fine powder of tobacco and then inoculated in peptone water by gentle vortex and incubated for 8 h for enrichment of the culture [21].

In direct method, 1 mL of the above sample was transferred aseptically to 9 mL Luria-Bertani (LB) broth containing 50 mg/L cadmium chloride solution and mixed well by agitation. It was then incubated at 37°C for 24 - 48 h. About 200 µl of the suspension was inoculated onto series of sterile LB agar plates and incubated at 37°C for 24 - 48 h.

The indirect method was based on serial dilution technique [19] in which 1 mL of the above mentioned sample prepared in peptone water was added to a tube containing 9 mL of sterile 0.75% normal saline (10^{-1}) and mixed well. Like this dilution was repeated till 10^{-6}. After that about 200 µl of each dilution was inoculated on to sterile LB agar plates containing 50 mg/L $CdCl_2$ solution, and spread thoroughly with sterile glass spreader and incubated aerobically at 37°C for 24 - 48 h with control devoid of cadmium chloride and incubated along with the other plates.

After observation of any bacterial growth on LB agar in both the above cases, it was re-cultured in normal LB agar for pure culture and then transferred to LB broth containing 40% glycerol in 2 mL Eppendorf tubes, and stored at - 20°C.

Cadmium tolerance study of isolates

Different concentrations of heavy metal were prepared as follows: 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 (mg/L) with final volume of 10 mL in LB broth. Then the metal concentration was taken in separate test tubes and inoculated with 1 mL of 24 h old culture of bacterial isolates. A control was taken consisted of metal deficient medium inoculated with the respective isolates. The tubes were incubated at 37°C for 24 h and examined for turbidity. After confirmed it was streaked on LB agar plate for their growth and results were noted. From the above qualitative screening the most potent isolate showing maximum tolerance was selected for further study.

Partial characterization of potent cadmium resistant bacteria

The potent isolate was partially characterized on the basis of their cultural, morphological, biochemical characterization. The sugar utilization tests were also conducted using seven sugars i.e. cellobiose, fructose, galactose, lactose, mannose, raffinose and xylose. Different physiological tests were done using LB agar plates with their respective parameters and also by using spectro-photometric analysis at 600 nm. The parameters investigated were pH, temperature and NaCl concentrations. Besides, the isolate was checked for the UV and antibiotic resistance tests. The investigated antibiotics were Ampicillin, Bacitracin, Chloramphenicol, Ciproflaxacin, Erythromycin, Fusidic acid, Gentamicin, Kanamycin, Penicillin-G, Streptomycin and Tetracycline. The degradation capability of the isolate towards various substrates was also conducted using various substrates viz. starch, pectin, urea, casein and gelatin.

Molecular identification of potent cadmium resistance bacterial isolate

Molecular identification of the potent cadmium resistance bacterial isolate was carried out by sequencing of 16S rRNA gene and submitted to Gen Bank of NCBI web server. The amplification and sequencing of 16S rRNA gene was carried out at Xcelris Genomics, India. Raw sequence analysis and computation of consensus sequence were performed using Bio-Edit software (7.0.5.3). Homologous sequence identification of the isolate was done by BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in NCBI web server. Multiple sequence alignment and construction of phylogenetic tree was performed using MEGA 6.0 (Molecular Genetics Evolutionary Analysis) package [22]. The resultant tree topology was evaluated by bootstrap analysis of UPGMA data sets based on 3000 re-samplings.

Secondary structure analysis of potent cadmium resistance bacterial isolate

The structural divergence between the bacterial isolate and its close homologous has been studied at secondary structural level of 16S ribosomal RNA. The RNA folding patterns were predicted using Mfold web server (http://www.bioinfo.rpi.edu /application/mfold) for both the 16S rRNA sequences. RNA was folded at a fixed temperature of 37°C and assumed to be constant within the range of temperatures that might occur in vivo or in the laboratory. This enables the server to extrapolate free energies to other temperatures and to fold at these temperatures. RNA sequences were taken as linear; the ionic conditions were fixed at $[Na^{+}]$ = 1 M and $[Mg^{++}M]$ = 0. The predicted secondary structures were compared according to the number of stems, loops [exterior/interior], multiple loops, hairpin loops and bulges to study the conservation at the structural level.

Bio-remediation analysis of the toxic metal using bacterial isolate

The selected potent isolate was subjected for the analysis of Cd^{2+} removal percentage by using AAS by using sonication and chemical digestion protocol. For this, isolate was grown with 1 mg/L of given Cd^{2+} solution as per AAS protocol at a 7.2 pH along with a control and incubated at 35°C for 24 h. At the end of incubation period, the pellet and supernatant were separated by centrifugation at 10,000 rpm for 20 min at room temperature [12]. The supernatant was taken in test tube, washed with 2N HNO_3 (nitric acid) and rinsed several times with distilled water before use to avoid metal contamination if present in it and was analysed for Cd^{2+} content by AAS.

The potent isolate was grown in 100 mL of freshly prepared LB broth (pH 6.0) at 35°C for 24 - 48 h with constant shaking. Stock solution (1000 mg/L) of Cd^{2+} was prepared and adjusted to pH 7.0 using 0.1 M sodium hydroxide and 0.1 M trioxonitrate (V) acid. Cd^{2+} medium (1 mg/L) was made in triplicate set of 100 mL flask and inoculated each with 10 mL of inoculums and a control was kept without inoculum. All flasks were incubated at 35°C for 24 - 48 h. At the end of the incubation period, cells were harvested by centrifugation at 10,000 rpm for 20 min.

Cells were washed thrice with freshly prepared Phosphate buffer saline (PBS) and discarded. About 10 mL of PBS was taken in a 50 mL beaker and cells were dispensed in it. For sonication, amplitude was set to 100, and one full cycle was set in the sonicator setting the temperature at 40°C for 20 min with 45 sec of interval after every 3 - 4 min. Beakers containing the cells were placed under the probe so that the micro tip comes in contact with the cells to carry out the lysis process. After sonication the cells are transferred to sterile test tubes and incubated at 35°C for 24 - 48 h and then analysed for reduction under AAS.

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Statistical analysis

The data recorded during the course of the investigation were subjected to significance testing using a t-test and analysis of variance. Statistical significance was set at $P < 0.05$. Results were denoted as mean ± standard deviation (SD) of triplicate experiments.

RESULTS AND DISCUSSION

Sample collection and isolation of Cadmium resistant bacteria

Tobacco smoking is the practice where tobacco is burnt and the resulting smoke is inhaled which causes various types of diseases and disorders. There are various disorders such as throat and lung cancers in case of practicing tobacco. But microbial diseases from tobacco practice are rarely known. Therefore, microbial presence in tobacco may cause disease in immune-compromised individuals. Microorganisms present in tobacco had been less studied or hardly given any vital importance. The present study is based on the isolation and identification of bacterial community present in tobacco and to check out their efficiency in bioremediation. For this samples of Bidi were collected from local market and isolation was done for observing the incidence of cadmium tolerant bacteria. From the both direct and indirect method, only 7 bacterial isolates were selected from agar plates on the basis of their cultural features and designated as NaCd1 - NaCd7.

Cadmium tolerating capacity of bacterial isolates

Then all the isolates were screened for their Cd^{2+} tolerance capacity. Primarily isolation was done by taking only 50 mg/L cadmium therefore it should be analysed if the isolates can tolerate higher concentrations of cadmium. For this purpose different concentration was taken and results were depicted in Fig. 1. ANOVA test revealed that a highly significant variation of log CFU/mL values was observed among all the isolates at varied cadmium concentrations ($P < 0.001$).

From this it was found that all the isolates showing resistance to Cd^{2+} while differing in their patterns. Luxuriant growths were seen by 100% isolates up to 500 mg/L, while 14.29% showed sensitivity to 600 mg/L of Cd^{2+}. Only 42.86% isolates showed growth at 1000 mg/L. Therefore on the basis of above observations the isolate NaCd1 was selected having maximum Log CFU/mL i.e. 6.36 at 1000 mg/L and considered for further study. In case of NaCd1 a relative growth of 86.89% was observed at 1000 mg/L while compared with the control (without any metal). It confirms that the higher concentrations of cadmium had a little inhibitory to the microbial growth. Castillo-Zacarías and co-workers reported that when the concentrations of metals in the culture media are high enough that leads physiological toxic effect to microorganisms [6]. In this case the metals bind to cellular ligands and displace essential metals from their normal binding sites.

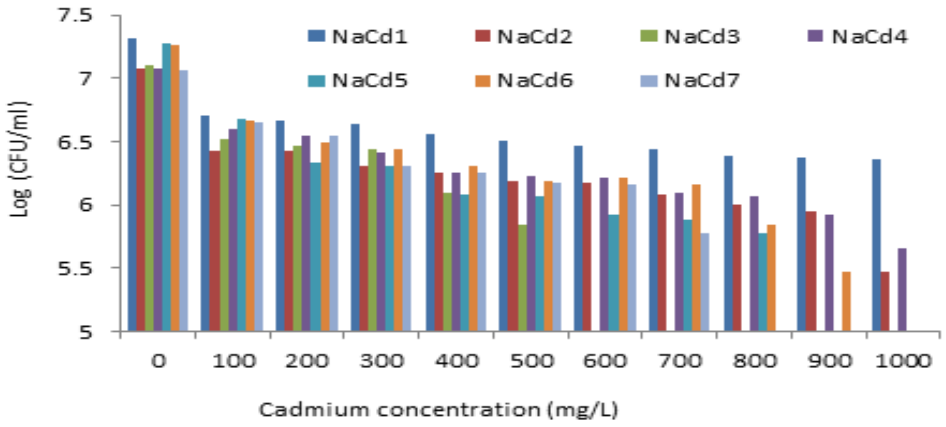

Fig. 1: Heavy metal resistant pattern of the isolates

Partial characterization of NaCd1

From cultural and morphological study all seven isolates except NaCd1 were Gram positive. NaCd1 (LB agar plates) confirmed to be Gram negative, non motile rods, circular, off white, flat elevation, smooth surface and translucent. It showed negative results in indole test, VP and oxidase while positive for MR, citrate, nitrate and catalase test. From sugar fermentation test it was found to be positive for all sugar tested. Similarly, *Klebsiella oxytoca*, *Bacillus* showed positive for catalase, VP, indole and nitrate reduction test [20].

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Responses of the fresh bacterial isolates to toxic metal were very heterogeneous and Foster has reported the resistance to heavy metal in both Gram positive and Gram negative bacteria which also corroborates with our present studies [9]. Mahler and co-workers found that many Gram positive aerobic spore formers were resistant to cadmium and mercury and very few of these strains were antibiotic resistant [17].

Physiological characterization of NaCd1

Different types of physiological studies were also done by taking both agar plate and broth methods. In case of broth, the observations were taken at 600 nm. From plate assay it was found that NaCd1 can tolerate 25 - 45°C and pH in the range of 4.0 - 10.0 having sodium salt tolerance capacity up to 6% (w/v), which denotes the isolate as mesophilic in nature with slightly halotolerant having wider range of pH tolerance for growth. The above was confirmed in LB broths, which were shown in Fig. 2 - Fig. 4.

NaCd1 showed maximal growth at pH 6.0 and above 50% relative growth was there from pH 6.0 - 12.0. It implies that NaCd1 is an alkalitolerant bacterium as there was only 15.31% relative growth remaining at pH 4.0 (Fig. 2). T-test revealed that there is a significant variation among the population means of initial pH with relative growth percentage ($P < 0.05$) while correlation among them were not significant. This was due to a high drop of relative growth percentage at 4.0 pH.

The optimum growth was found at 35°C having relative growth 100% after that there was a gradual fall of growth and only 17.81% relative growth was left at 60°C (Fig. 3). It denotes NaCd1 as a mesophilic bacterium. T-test revealed that there is a significant variation among the population means of temperature with relative growth percentage ($P < 0.05$) while correlation among them were significant but negative. It means there will be a fall of NaCd1 growth along the increase in temperature. Similar results of significant variation and negative correlation among temperature and pH with the growth of bacteria were found [16].

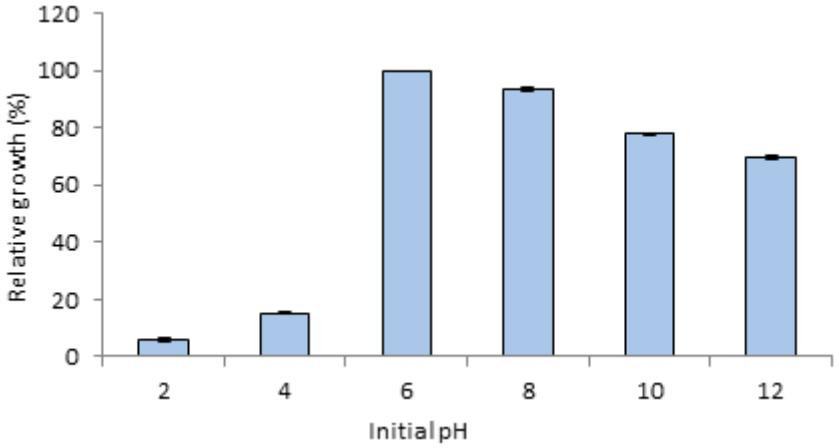

Fig. 2: Effect of pH on growth of NaCd1. The results are the means of 3 independent experiments and the bars correspond to standard deviations ($P < 0.05$).

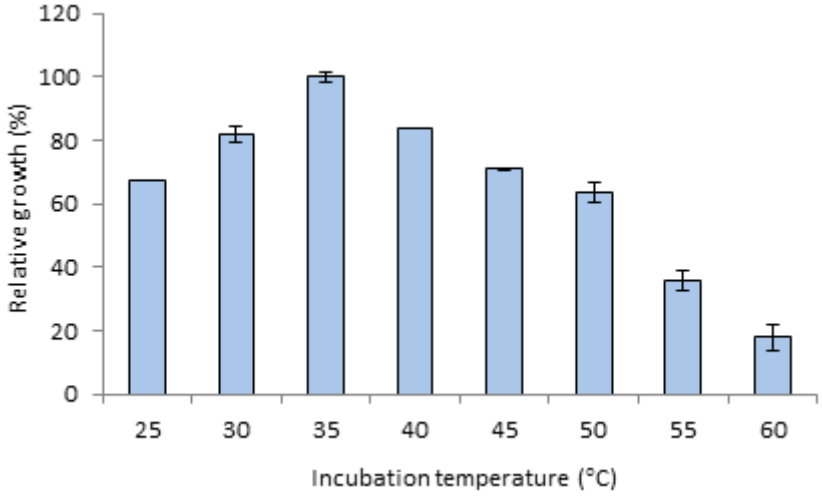

Fig. 3: Effect of temperature on growth of NaCd1. The results are the means of 3 independent experiments and the bars correspond to standard deviations ($P < 0.05$).

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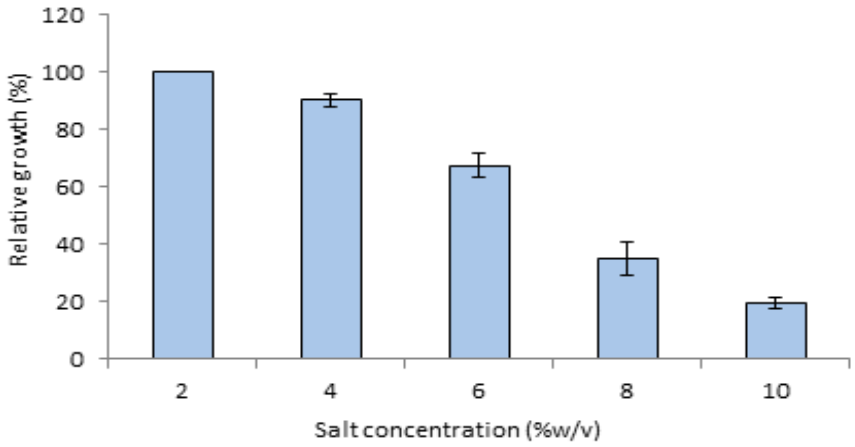

Fig. 4: Effect of salt (NaCl) concentrations on growth of NaCd1. The results are the means of 3 independent experiments and the bars correspond to standard deviations (*P* < 0.001).

Above 50% relative growth was seen up to 6% (w/v) NaCl concentrations and only 19.66% relative growth was seen in case of 10% (w/v) NaCl (Fig. 4). It denotes NaCd1 as a slightly halophilic in nature. T-test revealed that there is a highly significant variation among the population means of varied salt concentrations with relative growth percentage ($P < 0.001$) while correlation among them were significant but negative. It means there will be a fall of NaCd1 growth along the increase in salt concentrations.

Besides, NaCd1 can tolerate UV exposure up to 30 min. NaCd1 was resistant to Ampicillin, Bacitracin, Erythromycin, Fusidic acid, and Penicillin G and rest were susceptible. Many of the *Enterobacter* species have multiple antibiotic resistant that are undetectable in vitro, which makes it difficult to treat in patients [8].

In addition to the above tests the isolate was tested for the production of different enzymes which was confirmed by plate assay method using respective pseudo-selective media. It was able to degrade only protease and negative for amylase, pectinase, and gelatinase.

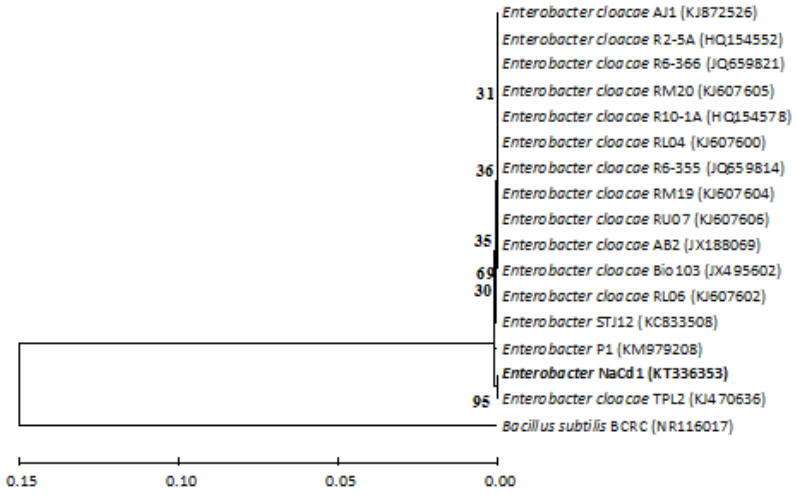

Fig. 5: Phylogenetic relationship of *Enterobacter* NaCd1 with other bacterial isolates. The numbers shown in left side indicates the bootstrap percentages according to which each isolate are clustered together.

Molecular identification of cadmium resistance bacteria NaCd1

The potent cadmium resistant bacteria, NaCd1 was found as Gram-negative rod shape and belongs to the genus *Enterobacter* as revealed from morpho-physiological and biochemical characterization. Further identification of potent bacterial isolate was done by sequencing of 16S rRNA gene and construction of phylogenetic tree. Primarily, the BLAST (Basic Local Alignment Search Tool) hits obtained for 16S rRNA gene sequence of cadmium resistance *Enterobacter* bacterial isolate indicated its closeness with *cloacae* species and is submitted to Gen Bank of NCBI web server (Accession No. KT336353). The evolutionary history was inferred using the UPGMA method in MEGA 6.0 [27]. The analysis involved seventeen nucleotide sequences including *Bacillus subtilis* BCRC (Accession No. NR116017) was taken as an out group. The tree evaluation was done through bootstrap analysis for 3000 replicates. The phylogenetic tree revealed that the potent

cadmium resistance *Enterobacter* NaCd1 clustered with *Enterobacter cloacae* TPL2 (Fig. 5) supported by 95% bootstrap value, suggesting a strong evolutionary closeness between *Enterobacter* NaCd1 and *Enterobacter cloacae* TPL2.

Saha carried out an experiment for the presence of bacteria and fungi at Bangalore, India and found *Klebsiella oxytoca*, *Bacillus* among bacterial species and *Aspergillus sp.* amongst fungi as dominant in tobacco sample [20]. Same findings were also reported [13]. Another report of getting 18 nicotine-degrading bacteria, which could utilize nicotine as the sole carbon and nitrogen sources, were isolated from tobacco plantation soils in Mile, Yunnan Province, China [15]. These bacteria were belonged to *Arthrobacter* spp., *Ochrobactrum* spp., *Sphingobacterium* spp., *Sinorhizobium* sp., *Alcaligenes* sp., *Pseudoxanthomonas* sp. and *Delftia* sp.

Chaudhary reported about 7 bacterial isolates from tobacco and tentatively identified them as *Bacillus alvei, B. circulans, B. cereus* and *Lactobacillus* sp. [7] while few bacteria in genera *Alcaligenes*, *Pseudoxanthomonas*, *Arthrobacter*, *Ochrobactrum*, *Alcaligenes* and *Delftia* were also been isolated from the same [26].

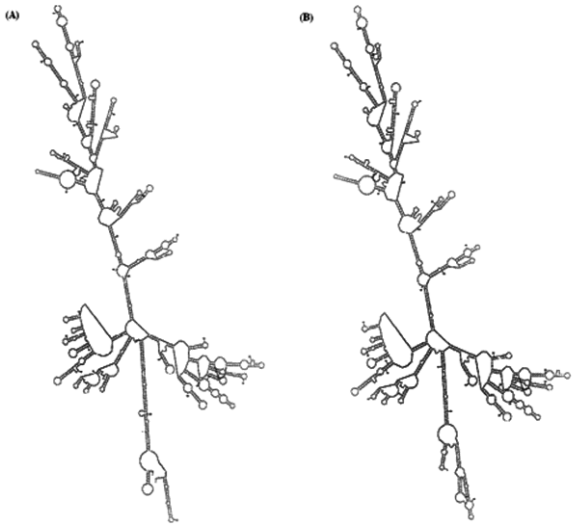

Fig. 6: Comparison of phylogenetic relationship at secondary structural level between (A) *Enterobacter* NaCd1 with (B) *Enterobacter cloacae* TPL2 is depicted here.

Secondary structure analysis of cadmium resistance *Enterobacter* NaCd1

To confirm species homology between *Enterobacter* NaCd1and *Enterobacter cloacae* TPL2, secondary structures of both were predicted in Mfold web server [27]. The secondary structures with lowest free energy were selected for divergence analysis. The secondary structural features of rRNA regions, as shown in (Fig. 6), were analyzed based on conserved stems and loops, even in difference in sequence length. Mostly in both cases the structures share common folding pattern in terms of same number of branching with variable loops. The only difference is found at the tail topology of both structures in terms shape, branch length and number of loop which may be due to differences in the nucleotide sequences. Such prediction results support the conservation of rRNA folding patterns between *Enterobacter* NaCd1and *Enterobacter cloacae* TPL2 which implicates a strong homology between them at their species level.

Bio-remediation analysis of toxic metal using *Enterobacter cloacae* NaCd1

The isolate NaCd1 was subjected for the analysis of Cd^{2+} removal percentage by using AAS. After growth the supernatant was subjected for AAS analysis which showed reading of 0.71 mg/L which means there was a removal of 29% Cd^{2+} from the medium from initial Cd^{2+} (1 mg/L) sample, which confirmed that there is a possibility of adsorption of Cd^{2+} by the NaCd1 from the medium. Therefore, the culture pallet was collected and sonicated for the presence of cadmium percentage found within the cells which was found to be removed in the primary test. But after AAS analysis of sonicated cells the reading was 0.265 mg/L, which is only 91.38% of the total removed Cd^{2+}. Therefore, it was confirmed that the NaCd1 have capability to not only tolerate Cd^{2+} but also can uptake it successfully.

The above results corroborates with the work of Castillo-Zacarías and co-workers who isolated phenol-resistant bacteria from industrial polluted effluents in Monterrey, México which were identified as *Klebsiella pneumonia, Enterobacter cloacae* and *Pseudomonas aeruginosa*, with Cd^{2+} removal rate of 23 to 78% by *E. Cloacae*, 23 to 64% by *P. aeruginosa* and 24 to 64% by *K. pneumonia* [6]. Haq and co-workers reported about 86% removal of cadmium (100 mg/l) from medium within 24 h by *E. Cloacae* [11].

Bacillus sp. and *Aeromonas* sp. were shown to be resistant to the toxicity of heavy metals and the persistence of these isolates in the presence of the respective heavy metals may be as a result of the possession of heavy metal resistant plasmids [4, 18]. Kermani and co-workers had reported about cadmium resistant of *Pseudomonas aeruginosa* which was found to tolerate cadmium up to the concentration of 80 mg/L [12]. Similar results are obtained by *Vibrio harvei* [1].

Metal ion binding to the cell surface may be due to covalent bonding, electrostatic interaction, Van-der Waals forces, extracellular precipitation, redox interaction or combination among the processes [5]. The negatively charged groups on the bacterial cell wall adsorb metal cations, which are then retained by mineral nucleation [25]. This above findings can be useful for the bioremediation process by using the *E. Cloacae* NaCd1 isolate of beedi sample.

CONCLUSION

Phenotypic characterization along with 16S rRNA gene sequencing generally considered as a more reliable methods for identification of bacteria than when any of these are used alone. Characterization of *Enterobacter* NaCd1 bacterial isolate from narcotic agent (beedi) shows the potentiality of cadmium resistance. The evidence at sequence homology search, molecular phylogeny and secondary structural folding pattern strongly suggested the identification of species as *cloacae* for *Enterobacter* NaCd1 bacterial isolate. The present investigation opens a wide space for more study on cadmium resistance potentiality of different *Enterobacter* bacterial species. Microbes can significantly affect the distribution of metals in the environment, since they have developed means to use them as their requirements or metabolism. This clearly holds promise for effective, economical and eco-friendly metal bioremediation technology for industrial exploitation and pollution free environment.

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