Int J Pharm Bio Sci 2012 Oct; 3(4): (B) 819 - 829

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

ESTIMATION OF HEAVY METAL TOLERANCE AND ANTIBIOTIC SUSCEPTIBILITY OF *Bacillus cereus* ISOLATED FROM MUNICIPAL SOLID WASTE

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ABSTRACT

With the intension to screen heavy metal tolerance and antibiotic susceptibility, a bacterium was isolated and characterized from municipal solid waste dumping yard (Dhapa), Kolkata, India. On the basis of biochemical and 16S rDNA sequence profile, it was identified as *Bacillus cereus*. Tolerance was observed against heavy metals like Cd^{2+}, Co^{2+}, Ni^{2+}, Cr^{3+}, Pb^{2+} , Hg^{2+} by this bacterium and was found that the bacterium was most tolerable to Cd^{2+} and Cr^{3+} compared to other four metals. Study of susceptibility of this bacterium was determined by minimum inhibitory concentration (MIC) value of different antibiotics such as chloramphanicol, carbenicillin, tetracycline, erythromycin, ciprofloxacin, norfloxacin and maximum inhibition was observed by chloramphanicol and norfloxacin. A plasmid was isolated from the selected stain with MW of 45 kb. The plasmid curing experiment revealed that this plasmid was very essential for metal resistance power of the selected isolate.

KEYWORDS: ***Bacillus cereus*, Heavy Metal, Antibiotic resistance, MIC, Plasmid Curing.**

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INTRODUCTION

Heavy metals are often defined as a group of metals whose atomic density is greater than 5 g/cm^3 [1]. Heavy metals are stable and persistent environmental contaminants since they cannot be degraded or destroyed. Some heavy metals are essential which are required by the organisms as micro nutrients and are known as "trace elements". Many agricultural and industrial practices led to environmental pollution by heavy metals. Heavy metals contamination in soil comes from smelters, mining, power stations and the application of pesticides containing metal, fertilizer and sewage sludges. Heavy metals contamination can have significant effects on indigenous microbial populations, microbial activity and nitrogen fixation process in rhizoid. Heavy metals can damage the cell membranes, alter enzymes specificity, disrupt cellular functions and damage the structure of the DNA. Several metal resistance mechanisms have been identified: exclusion by permeability barrier, intra and extra cellular sequestration, active transport, efflux pumps, enzymatic detoxification, and reduction in the sensitivity of the cellular targets to metal ions [2].

Laboratory investigations have shown that many bacteria which are resistant to the effects of high concentrations of heavy metals are concomitantly resistant to several antibiotics [3]. This organism possesses plasmid and as the plasmid is the source of different resistance factor to many antibiotics and heavy metals. [4] In this study, a metal tolerant bacterium was isolated from municipal damp soil, it was identified and its ability to tolerance against different concentration of heavy metals and antibiotics were evaluated. The relationship between heavy metal tolerance and antibiotic resistance properties of isolated bacteria with its plasmid content was also correlated.

MATERIALS AND METHODS

(i) Sample collection Soil samples were collected from the municipal waste dumping ground Kolkata Municipal Corporation, Dhapa, Kolkata. The sample was collected in sterilized plastic bags and transported to the laboratory of Microbiology at the Department of Pharmaceutical Technology, Jadavpur University. The container was maintained at a temperature of 4ºC to ensure minimal biological activity. Processing of the samples for the isolation of bacteria was carried out within 24 hrs of sample collection.

(ii) Isolation and Identification of Heavy Metal Resistant Bacteria :

Heavy metals incorporated media were used for the selective isolation of heavy metals resistant bacteria. Basal media nutrient agar (NA) incorporated with salts of heavy metals like Cd^{2+}, Cr^{6+}, Ni^{2+}, Co^{2+} were prepared separately. The concentration of each heavy metal was maintained as follows:

$CdCl_2$: 0.25, 0.5, 0.75, 1.0, 1.25, 1.5mg/ml

$K_2Cr_2O_7$: 0.25, 0.5, 0.75, 1.0, 1.25, 1.5mg/ml

$NiSO_4$: 0.25, 0.5, 0.75, 1.0, 1.25, 1.5mg/ml

$Pb(CH_3COO)_2$: 0.25, 0.5, 0.75, 1.0, 1.25, 1.5mg/ml

$Hg(CH_3COO)_2$: 0.25, 0.5, 0.75, 1.0, 1.25, 1.5mg/ml

$Co(NO_3)_2$: 0.1, 0.2, 0.3,0.35, 0.4, 0.5mg/ml

After the incubation period (24-48 hrs.) the plates were observed for any kind of growth on the media. The isolated and distinct colonies on these selective media were sub cultured repeatedly on the same media for

purification. The pure culture was identified on the basis of their morphology and biochemical characteristics [5].

(iii) Study of Colonial Morphology

Isolated colonies of purified bacterial strain grown on solidified agar plates were observed and data was recorded regarding the form,

elevation, margin and optical feature of the colonies [6].

(iv) Study of Motility Character

The test can be used to check for the ability of bacteria to migrate away from a line of inoculation to physical features like flagella. Craigie's method is used to perform this test. The semi solid nutrient agar medium (contains 0.2%-0.5% of agar) test tube is inoculated with the test organism into the central glass tube and incubated at the relevant temperature for 18-24 hrs.

(v) Study of Cellular Morphological Characteristics

For determining the shape and Gram character, bacterial film was stained by Gram's Method. The slide was examined under the microscope (oil immersion, 100 X). Other staining procedures were conducted for detection of endospore, capsule as per standard protocol.

(vi) Biochemical Characterization

Biochemical characterizations were studied to detect the presence of enzymes namely gelatinase, oxidase, catalase, urase, nitrate reductase, casein hydrolase and amylase. Other tests also were performed included indole test, methyl red and Voges Proskauer test, citrate utilization test, H_2S production test.

(vii) Physiological Characterization

To select the heavy metal tolerance strains, it is necessary to standardize the cultural and physiological conditions of the selected organisms. Among the physico- chemical conditions, temperature, pH and salt (NaCl) concentration were optimized .

pH Profile

pH is a limiting factor, which governs bacterial growth. To determine the pH optima, nutrient broth medium meant for growth of the isolates was adjusted to different pH ranging from 6.0-11.0 and was seeded with 0.1ml inoculum. Post overnight growth at 37°C under shaking condition was measured in terms of OD at 600 nm using colorimeter.

Temperature Profile

For determination of optimum temperature, 0.1ml inoculation was provided into nutrient broth medium and overnight incubation was done at different temperatures like 30, 37, 40 and 50°C. The growth was measured in terms of OD at 600 nm through colorimeter.

Salt (NaCl) Concentration Profile

To study the optimum salt concentration on the bacterial Growth, different concentration of NaCl ranging from 0.5% to 10% were used in nutrient broth. The growth was measured in terms of OD at 600 nm using colorimeter.

(viii) Molecular Characterization

The molecular characterization was done on the basis of 16S rDNA sequence analysis. This analysis was performed by Xcelris Labs Ltd. (Sydney House, Ahmedabad, India). DNA was isolated and evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of

1438 bp rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.The sequence

obtained was submitted to NCBI GenBank.

(ix) Maximum Tolerance of Heavy Metals

The metals used in the study and detailed procedure to determine the tolerance property, in terms of Maximum Tolerable Concentration (MTC) was as per the protocol reported by Schmidt and Schlegel, 1994.The Maximum Tolerable Concentration (MTC) of heavy metal was designated as the highest concentration of heavy metal that allows growth after 48 hrs.[7] Maximum Tolerable Concentration (MTC) of the isolated strain against different metal salts are given in Table 4.

(x) Study of Antibiotic Sensitivity

To detect the antibiotic susceptibility, the bacterial strain was cultured on Muller-Hinton agar plates [8]. The isolated microbes were assayed against six commercial antibiotics [9] like Chloramphenicol (10µg/disc), Streptomycin (25µg/disc), Tetracycline (10µg/disc), Norfloxacin (10µg/disc), Carbenicillin (25µg/disc), Ciprofloxacin (15µg/disc).The bacterial cultured solution was spread on Muller-Hinton agar plates and antibiotic discs were placed. All the plates were incubated at 37ºC for 24 hrs. On the basis of zone diameter, the isolated strain was classified as resistant or sensitive [9]. Control plates were incubated without antibiotic discs.All the experiments were carried out in triplicate.

(xi) Isolation and Curing of Plasmid DNA

Plasmid was isolated using standard method [10]. The isolated plasmid was characterized by agarose gel electrophoresis [11]. The plasmid curing was carried out according to protocol mentioned by Unaldi et al[12]. The isolated strain was subjected to plasmid curing by chemical agent ethidium bromide (EtBr). Then the culture was subjected to metal tolerance (Cadmium 1.25mg/ml, Cobalt 0.4mg/ml, Nickel 0.75mg/ml, chromium 1.25 mg/ml, Mercury 0.5mg/ml, Lead 0.75mg/ml).

RESULTS

1. Isolation of Heavy Metal Tolerant Bacteria

The heavy metal tolerance property of the isolated strain was confirmed by growing it on heavy metal containing media. One of them was selected for our study and the isolated heavy metal tolerant strain was designated as Strain E.

2. Identification of Isolated Bacterial Strain

Colonial Morphology

Strain E possessed large colony. The colony was circular, convex and margin was entire type. detailed result for colonial morphology has been given in Table 1.

Table 1.
Colonial Morphology of the Isolated Strain E.

Shape	Circular
Size	small
Color	White
Margin	Entire
Elevation	Convex
Surface	Smooth & Shiny
Opacity	Opaque

Cellular Morphology

Cellular morphology such as arrangement, shape and Gram reaction were observed during Gram staining of isolated strain. Cellular shape of the strain was found as rod, whereas cellular arrangement was found in chain as well as scattered form, and the isolated strain was Gram positive. Details for cellular morphology and Gram reaction are given in Table 2.

Table 2.
Cellular Morphology of the Isolated Strain E.

Gram straining	Gram +ve
Shape	Rod
Arrangement	Chain and scattered
Spores	Present
Capsule	Present
Cell motility	Non motile

Biochemical Characteristics

The results of biochemical Characterization experiments are shown in Table3.

Table 3.
Biochemical Characterizations of Isolated Strain E.

Sl no.	Tests	Results
01	**Starch hydrolysis**	**+**
02	**Protein hydrolysis**	**+**
03	**Citrate utilization**	**+**
04	**Urea hydrolysis**	**-**
05	**Indole production**	**-**
06	**Methyl red test**	**+**
07	**Voges Proskauer(VP) test**	**-**
08	**Nitrate reduction**	**+**
09	**Gelatin liquification**	**-**
10	**Catalase**	**-**
11	**Oxidase**	**+**
12	**Triple Sugar Iron Test**	**+**
13	**Sulfide Indole Motility Test**	**+**
14	**Sugar fermentation**	
	Glucose	**Acid + Gas**
	Sucrose	**Acid + Gas**
	Galactose	**Acid + Gas**
	Maltose	**Acid + Gas**
	Mannitol	**Acid + Gas**
	Lactose	**Acid + Gas**

Physiological Characterization

The physiological properties of the isolated strain E was concluded in terms of pH and temperature. The isolate strain E was found to grow within a pH range of 6.0-11.0 with optimum growth at pH 7.0 (Fig. 1). The temperature range found suitable for growth of isolated strain was between 30-40°C, optimum being at 37°C (Fig.2).

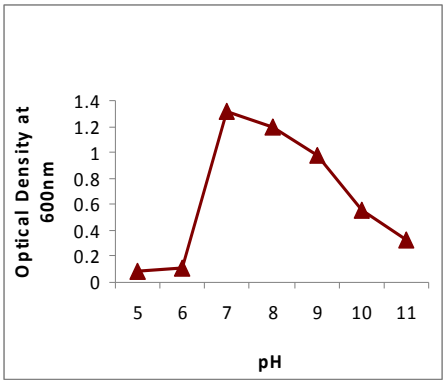

Figure. 1
Graph represents the temperature profile For growth of the isolated strain E

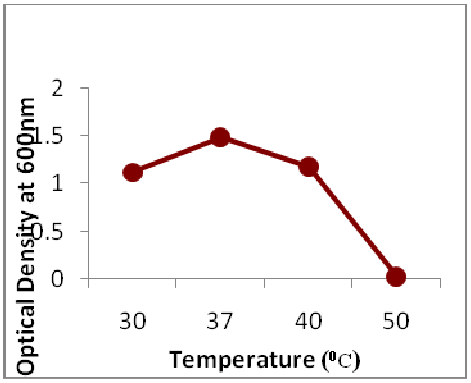

Figure. 2
Graph represents the pH profile for growth of the isolated strain E

The effect of NaCl concentration on growth of the isolated E was also conducted. The optimum growth for isolated strain was found at 0.5% NaCl concentration (Fig.3).

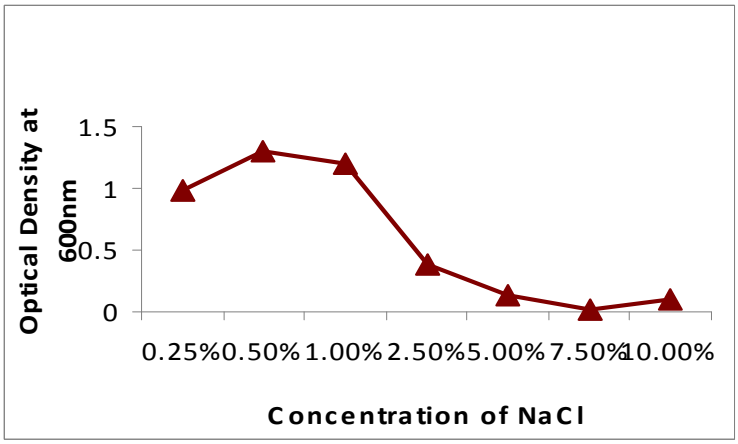

Figure. 3
Graph represents the NaCl concentration profile for growth of the isolated strain E

Molecular Characterization:

N-BLAST search of the 16S rDNA sequence of isolated strain **E** was reported by Xcelris Labs Ltd. (Sydney House, Ahmedabad, India) and reveals that the isolate was found to be *Bacillus cereus* strain IMAUB1022(Gene Bank Accession Number: FJ641009.1) based on nucleotide homology and polygenetic analysis. The sequence obtained was submitted to

GenBank and the bankit no. is 1370734 HM752770.

4. Determination of the Effect of Heavy Metals on Bacterial Growth:

The *Bacillus cereus* exhibited different growth patterns in presence of different heavy metals. The growth curves of strain in presence of different metal concentrations are shown in the Fig 4.

Maximum Tolerable Concentration (MTC) of different heavy metals for the growth of isolated strain were evaluated and depicted in (Table 4) and the pattern of metal tolerance were in the order Cd^{2}> Cr^{3+}> Ni^{2+}> Pb^{2+}>Hg^{2+}>Co^{2+}. The microbial growth decreased with the increase in concentration of heavy metals indicating toxic effect of the heavy metals on the growth of microorganisms [13].

Table4
MTC of Heavy Metals

Isolated Strain	Heavy Metals					
	Cd^{2+}	Cr^{3+}	Ni^{2+}	Co^{2+}	Pb^{2+}	Hg^{2+}
Bacillus sp.	1.25mg/ml	1.25mg/ml	0.75mg/ml	0.4mg/ml	0.75mg/ml	0.5mg/ml

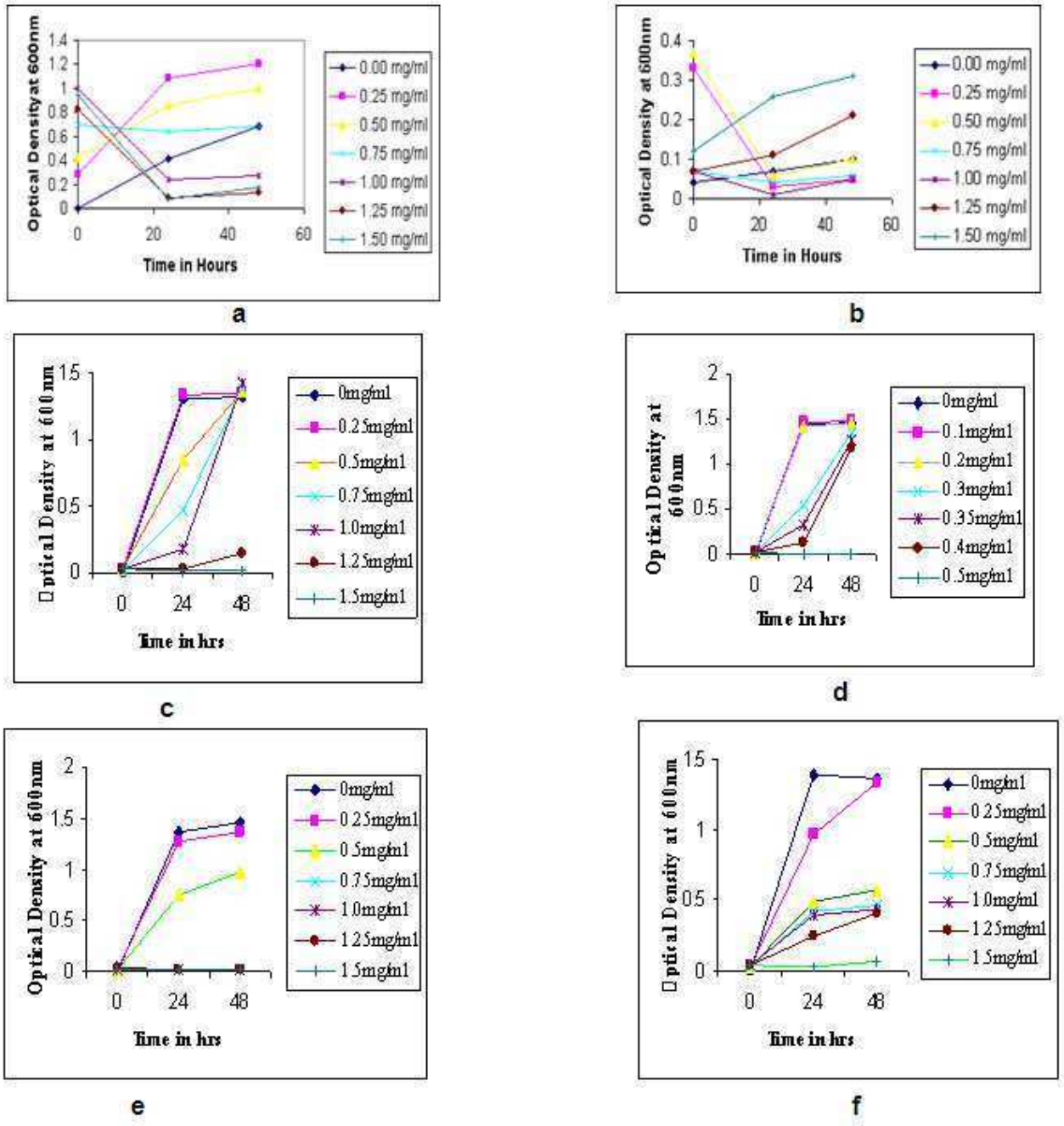

Figure. 4

Growth curves of Strain E in presence of different concentrations of different heavy metals.The X axis depicts time in hours where as Y axis represents bacterial growth presented in terms of OD at 600 nm

a. Growth curve of Strain E in presence of Pb^{2+}.
b. Growth curve of Strain E in presence of Hg^{2+}.
c. Growth curve of Strain E in presence of Cd^{2+}.
d. Growth curve of Strain E in presence of Co^{2+}.
e. Growth curve of Strain E in presence of Ni^{2+}.
f. Growth curve of Strain E in presence of Cr^{3+}.

5. Antibiotic Susceptibility Test

In the present study, ***Bacillus cereus*** exhibited sensitive to most of the tested antibiotics. Results are given in Table 5.

Table 5
Result for Antibiotic Sensitivity

Sl No.	Antibiotics	Result
1	Cholramphenicol (10 µg/disc),	Sensitive
2	Carbenicillin (25 µg/disc),	Sensitive
3	Tertacycline (10 µg/disc),	Sensitive
4	Norfloxacin (10 µg/disc),	Sensitive
5	Ciprofloxacin (15 µg/disc),	Sensitive
6	Erythromycin (30 µg/disc),	Sensitive

6. Plasmid Profile and Curing

The plasmid DNA was successfully isolated from the strain *Bacillus cereus.* Plasmid profile of the isolated strain exhibits a single band indicating the presence of a plasmid (Fig.5).

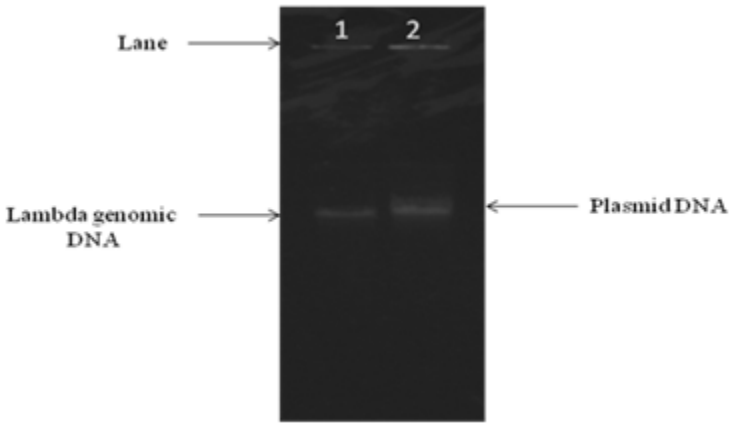

Figure. 5
Plasmid profile of the isolated strain E subjected to electrophoresis on 1% agarose gel.

Lane 1: Lambda genomic DNA(48Kb)
Lane 2: Plasmid DNA of *Bacillus cereus.*

Plasmid curing is carried out to confirm whether the genes for resistance are encoded by genomic DNA or plasmid DNA. After plasmid curing from the isolated strain, *Bacillus cereus* lost its metal resistance ability to certain metals represented earlier (Fig. 4).The above result indicates that the genes for heavy metal resistance of the isolated strain may be reside on plasmid DNA.

DISCUSSION

Now days, metal resistance among bacterial population is becoming a major global concern. The investigation highlights the presence of metal tolerant microbial population in the Kolkata Municipal Corporation waste dumping yard, Kolkata.

Since survival of a strain depends on salt concentration, temperature and pH profile, it was clear that this strain has ability to survive in adverse condition and in stressed environment tolerance mechanism is developed [14].

Multiple resistance isolates exhibits resistance towards group of antibiotics [15] but increase in heavy metal concentration leads to decrease in antibiotic resistance [16]. *Bacillus cereus* exhibited high metal tolerance but sensitive to various antibiotics. It was confirmed from the study heavy metal tolerance was mediated by plasmid [17].

CONCLUSION

The heavy metal tolerant soil bacterium indicates that soil is polluted with heavy metals. Mainly Cadmium and chromium tolerance revealed that study locations of kolkata are contaminated by these metals. Heavy metal remediation and extracting rare metals can be done using *Bacillus cereus* as it resist a wide range of heavy metals. The present investigation has widened the scope for research and development of metal tolerance from bacterial origin.

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