Isolation and Identification of heavy metal resistant bacterial strains from Chavara industrial area, Kollam (Dt) Kerala

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Abstract

The occurrence of toxic heavy metals in the environment is geogenic or anthropogenic. In mitigating heavy metal polluted environments several treatment strategies and methods are available such as chemical precipitation, filtration, ion exchange, electrochemical treatment, membrane technologies, adsorption on activated carbon, evaporation, phytoremediation etc. This study involved isolation, identification and molecular characterization of heavy metal and drug resistant bacterial strains from the sediment samples of Vattakayal backwaters, near chavara industrial area. Morphological and biochemical tests were carried out for the identification of seven heavy metal tolerant bacterial isolates. Based on the analysis the isolates were identified as *Bacillus cereus, Enterobacter aerogens, Pseudomonas putida, Bacillus licheniformis, Klebsiella pneumonia, Alcaligens faecalis,* and *Citrobacter kosari.* Sequences were matched with previously published bacterial 16S rDNA sequence in the NCBI database using ADVACED BLAST. Antibiotic resistant study showed that 57.14% of the isolates were resistant to at least one antibiotic tested. Phylogenetic tree was constructed by using MEGA4 software and the evolutionary history was inferred by neighbour-joining method. It was found that the strain K1 classified in the branch of Bacillus *cereus* and K4 in the branch of *Bacillus licheniformis*.

Key words: Heavy metal, *Bacillus cereus, Bacillus licheniformis,* Minimal Inhibitory Concentration

Introduction

Heavy metal pollution is a most important apprehension, as it leads to toxicity, risk to human survival and disrupts ecological balance (WHO, 2007). The release of untreated industrial wastewater into the environment has become a foremost concern in the developing countries and is viewed as one of the most important environmental issues. With rapid development of various industries such as mining and smelting of metals, surface finishing industry, energy and fuel production, fertilizer and pesticide industry, metallurgy, iron and steel, electroplating, electrolysis, electro-osmosis, leather industry, photography, electric appliance manufacturing, metal surface treating, aerospace and atomic energy installation, knowingly or unknowingly discharged the metal contaminated pollutants to the surroundings causing serious threat to human beings and other life forms (Volesky, 1990) and hence potent environmental stressors. The microorganisms living in the heavy metal polluted environment are more resistant to metals than the microbe from non-contaminated area (Bahig *et al*., 2008). Das *et al*. (2006) reported that the microorganisms living in the marine extreme environmental conditions have potential tolerance to heavy metals.

The objective of present study was to (a) Isolate and evaluate the metal resistance of bacterial strains from the sediment samples of Vattakayal backwaters. (b) Evaluate the antibiotic sensitivity of the bacterial isolates. (c) Identify and characterise the metal resistant bacterial strains.

Materials and Methods

Sample collection

Surface sediment samples from Vattakayal backwaters (Station 6) were collected using Peterson grab and transported on ice to the laboratory and processed within 5-7 hours of sample collection.

Isolation of heavy metal tolerant bacterial strains from the sediments

Isolation and enumeration of bacteria were carried out by standard serial dilution plate technique (Ansari and Malik, 2007). After incubation, morphologically distinct colonies were picked and purified. Pure cultures of bacterial colonies were prepared and persevered at 4^{o}C as slant cultures for further analysis.

Preparation of metal stock solution

The stock solutions of chromium [Cr(VI)], copper [Cu(II)], Zinc [Zn(II)], cadmium [(Cd(II)] and lead [Pb(II)] were prepared in deionized water and sterilized by filter membrane (0.22 µm) and stored at 4°C. The salts used were potassium dichromate ($K_2Cr_2O_7$), copper (II) sulphate pentahydrate ($CuSO_45H_2O$), zinc sulphate hexahydrate ($ZnSO_46H_2O$), Cadmium nitrate tetra hydrate (Cd $(NO_3)_24H_2O$) and lead nitrate $(PbNO_3)_2$.

Determination of Minimal Inhibitory Concentration (MIC)

Resistance of the bacterial isolates to varying concentrations of heavy metals such as lead, zinc, chromium, and cadmium were determined by agar dilution method (Luli *et al*. 1983). Fresh overnight cultures of the isolates grown in peptone water were aseptically inoculated into nutrient agar plates, which were supplemented with increasing concentration of the aforesaid metals individually (5µg/ml to 500µg/ml). The plates were incubated at room temperature and observed for bacterial growth. The lowest concentration of heavy metals at which no growth occurred when compared with the control plates was considered as the Minimal Inhibitory Concentration (MIC).

Sensitivity to antibiotics

Antibiotic sensitivity of heavy metal resistant isolates was determined by disc diffusion method (Bauer *et al*., 1966). Antibiotic impregnated discs (6 mm diameter, HiMedia) were placed on Muller - Hinton agar plates swabbed evenly with individual isolates and incubated at 37^{o}C for 24 hrs. The antibiotic diffuses out of the disk to form the gradient; the test organism starts to divide, grow and progresses towards a critical mass of cells. The so-called inhibition zone edge is formed at the critical time where a particular concentration of the antibiotic is just able to inhibit the organism before it reaches an overwhelming cell mass or critical mass (Acar and Goldstein, 1996). The diameter of the inhibition zones around the discs was measured in mm. Antibiotic resistance was determined by comparing the diameter of inhibition zone around each antibiotic disk with zone size interpretive chart supplied by Hi-media laboratories, Bombay. The concentrations of the antibiotic present in the disc were ampicillin (10 µg), tetracycline (25 µg), chloramphenicol (25 µg), penicillin (1 µg), Tetracyline (25 µg), streptomycin (10µg) and Sulphatriad (300 µg) respectively (Raja *et al*., 2006).

Multiple Antibiotic Resistance (MAR) indexing of the isolates

The MAR index when applied to a single isolate is defined as a/b where ‘a’ represents the number of antibiotics to which the isolate was resistant and ‘b’ represents the total number of antibiotics to which the isolate was exposed. Isolates with a MAR index value higher than 0.2 is considered to have originated from high-risk source of contamination such as human, commercial poultry farms, swine and dairy cattle where antibiotics are often used.

Identification and characterization of heavy metal tolerant bacterial strains

Morphological

The morphological characteristics of the bacterial colonies including shape, margin, elevation and pigment production were visually observed during isolation. Cell shape of the bacterial strains were examined under microscope (1000x magnification) after Gram staining. Motility of the isolates was tested by semisolid agar.

Biochemical characteristics

Bacterial isolates which are maintained as pure culture on Nutrient Agar were characterized and identified by Biochemical tests of Oxidase, Catalase, Indole, Methyl red, Voges Proakauer, Citrate, Urease, Nitrate reduction, D-Glucose gas production, D- Glucose acid production, Lactose, Mannitol, Sucrose, Phenylalanine Deaminase, H_2S from LIA, Litmus milk reaction, Gelatine, Starch, Lipid etc. as per Bergey's Manual of Determinative Bacteriology: 9th edition (Holt *et al*., 1994) and 8th edition (Buchanan and Gibbons, 1974).

Molecular characterisation

Genomic DNA isolation from culture cells

Genomic DNA was isolated from the bacterial isolates. The pure bacterial colony was inoculated in Luria Bertani broth. After incubation 1ml of the culture was transferred to a sterile micro centrifuge tube and the cells were harvested by centrifugation (12000 rpm) for 10 minutes at room temperature. Supernatant was discarded and the pellet was resuspended in 1 ml of 0.85% (w/v) NaCl solution and centrifuged as above. Discarded the supernatant and added 600µl lysis buffer along with 7 µl of proteinase-K. Vortexed the mixture and incubated at 65ºC for 1 hour. Added equal volume of chloroform: isoamyl alcohol (24:1) with gentle mixing by inverting the tubes for 2-5 minutes. Centrifuged the samples for 15 minutes (12000 rpm) at room temperature. The aqueous phase was collected in another micro centrifuge tube without disturbing the interface and lower phase. Repeated the steps of chloroform: isoamyl alcohol extraction. Again the aqueous phase was collected and added 50µl volume of 3M Sodium Acetate (pH 5.2) followed by equal quantities of ice cold isopropanol, so that the DNA gets precipitated and centrifuged it again at room temperature for 5 minutes at 12000 rpm. The supernatant was discarded and rinsed the pellet twice with 70% ethanol, followed by maintaining the tubes for 1hour in vacuum desiccators. The desiccated DNA samples were completely resuspended in 50µl of DNA dissolving buffer (TE buffer) and stored at -20ºC. The Ultraviolet (UV) absorbance was checked at 260 and 280 nm for determination of DNA concentration and purity. Concentration of DNA was estimated using the formula: Concentration of DNA (mg/ml) = OD 260 ×50 × Dilution factor.

16S rDNA gene amplification

The bacterial 16S rDNA fragment was amplified from the extracted genomic DNA by using 16S rDNA universal primer, 8F (5'-AGAGTTTGATCMTGG-3') and reverse primer 1492r (5'-ACCTTGTTACGACTT-3'). PCR was performed in a final reaction volume of 25 µl in 200 µl capacity thin wall PCR tube. PCR tubes containing the mixture were tapped gently and spinned briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. The reaction mixture contain deionized water - 16.5µl, Taq buffer without MgCl(10 X) - 2.5µl, MgCl22(15 mM)- 1.0µl and dNTPs mix (10 mM each) - 1.5µl. After the initial denaturation of 5 min at 95ºC there were 29 cycles consists of denaturation at 94ºC for 30 seconds, annealing at 58ºC for 30 seconds, extension at 72ºC for 45 seconds and final extension at 72ºC for 10 min. PCR was carried out in ABI3730xl Genetic Analyzer (Applied Biosystems, USA) . The PCR product were analysed by 1.5% (w/v) agarose gel electrophoresis in 1 X TBE (Tris-Borate-EDTA; electrophoresis buffer) with Bromophenol blue loading dye. Viewed the gels on UV transilluminator and photograph of the gel was taken. The PCR product was sequenced by ABI3730xl Genetic Analyzer (Applied Biosystems, USA). Sequences were matched with previously published bacterial 16S rDNA sequence in the NCBI database using ADVACED BLAST (www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al*., 1997).

Phylogenetic identification

A phylogenetic tree was constructed using the neighbour-joining distance method with the MEGA4 software (Tamura *et al*., 2007) and the reliability of the bootstrap consensus inferred from 1000 replicates. Some reference sequence from the Gene Bank of most closely related to that of Bacillus were used in generating phylogenetic tree (Yang *et al*., 2009).

Results and Discussion

Isolation of heavy metal tolerant bacterial strains from the sediments

The bacterial strains were isolated from the heavy metal contaminated sediments from station 6 (**Plate I**) where the industrial wastewater reaches the estuary. The microbes capable of tolerating individually 50µg/ml of metals like

chromium, copper, zinc, cadmium and lead were isolated. Totally 7 strains (K1, K2, K3,K4, K5, K6 and K7) were isolated based on their difference in colony morphology in the preliminary screening.

Heavy metal resistance patterns of bacterial isolates

In the present work a total of seven metal resistant bacterial strains were isolated from the lake sediments. Most of the isolates showed resistance to one or more heavy metals selected, however, the patterns of tolerance among the seven cultures varied (Table 1). Metal resistance studies of the bacterial isolates showed that about 57% of the isolates showed comparatively high tolerance (≥300µg/ml) to lead, zinc and copper. Resistance to cadmium and chromium was in between 95µg/ml - 300µg/ml and 100µg/ml -250µg/ml respectively. Sampling environments that contain elevated concentrations of heavy metals are a potential source of heavy metal tolerant bacteria. Many researchers reported that indigenous organisms isolated from heavy metal contaminated sites had tolerance to heavy metals toxicity (Andreazza *et al*., 2011).

Among the heavy metals Zn, Cu and Pb were found to be less toxic, whereas Cd and Cr were highly toxic to all strains in the order of resistance Pb>Cu>Zn>Cd>Cr indicating the adaptation of the organism to a stressed environment. Similar observation has earlier been reported (Raja *et al*., 2006). The metal resistance of *Ralstonia* in lake sediments and industrial biotopes has previously been studied (Yong *et al*., 2008). Duan and Min (2004) isolated a strain DKC1 of *Ralstonia eutropha* from a long-term cadmium treated soil sample and found that it had high resistance to cadmium, could grow well on solid medium supplemented with 3 mM cadmium. The plasmids are frequently present in heavy metal resistant bacteria than in common bacteria (Zolgharnein *et al*., 2007). In the present study it was found that the isolates K1 and K4 showed high tolerance to heavy metals (Table. 1)

Table 1 Heavy metal resistance patterns of bacterial strains isolated from Vattakayal Lake Sediments.

Isolates	Heavy metal concentration (µg/ml)					
	Cr	Cd	Zn	Cu	Pb	Mean
K1	200	300	350	360	450	**332**
K2	150	120	450	300	275	259
K3	100	100	220	290	185	179
K4	250	240	110	380	375	**271**
K5	175	105	230	280	250	208
K6	100	140	350	300	300	238
K7	110	95	340	270	400	243
Mean	155	157.1	292.9	311.49	319.3	

Resistance to antibiotics

A total of seven bacterial strains were isolated from the sediment samples and tested for their sensitivity to 6 common antibiotics (ampicillin, penicillin, sulphatriad, chlorampenicol, streptomycin and tetracycline). 57.14% of the isolates were resistant to at least one antibiotic tested. Resistance to ampicillin (57.14%) and penicillin (57.14%) was noted most frequently. Resistance to sulphatriad (14.28%) and chlorampenicol (14.28%) were relatively low (Table 2, Fig.1 & Plate II). A total of 3 resistant patterns were observed among the isolates and the most frequent (50%) resistant pattern was AMP (ampicillin- penicillin) (Table 3). Out of the 7 bacterial isolates many isolates showed multiple metal and antibiotic resistances. Numerous isolates had acquired multiple drug resistance (MDR). The present study identified the varying multiple antibiotic resistances (MAR) index and resistance patterns of the isolates (Table 3). The result of the present study, in general, agrees with those of other works in which varying frequencies of antibiotic resistance were detected with bacteria isolated from natural environments (Patterson, *et al*., 2007).

Association between metal tolerance and antibiotic resistance

Out of the 7 bacterial isolates many isolates showed multiple metal and antibiotic resistance. The bacterial isolates K1 and K4 showed multiple antibiotic resistance against 3 antibiotics tested and they showed higher heavy metal resistance. The use of antibiotics in medicine and agriculture clearly stimulates the proliferation of antibiotic resistance (Neu, 1992).

Table 2 Antibiotic sensitivity of bacterial strains isolated from Vattakayal lake sediments.

Antibiotic disc	Con.	Isolates						
	(µg/disc)	K1	K2	K3	K4	K5	K6	K7
Ampicillin	10	HR	20 (R)	36(S)	13 (R)	11(R)	21(I)	26(I)
Chloramphenicol	25	22(S)	17 (I)	29 (S)	11 (R)	14 (I)	16 (I)	20 S
Penicillin G	1	HR	19 (R)	30 (S)	HR	11 (R)	22 (I)	26 (I)
Streptomycin	10	26(S)	26 (S)	22 (S)	17 (S)	19 (S)	22 (S)	21 (S)
Sulphatriad	300	HR	30 (S)	31 (S)	30 (S)	20 (S)	30 (S)	30 (S)
Tetracycline	25	21(S)	30 (S)	HS	26 (S)	28 (S)	30 (S)	28 (S)

HR- Highly sensitive; I- Intermediate; S- sensitive; HS – Highly sensitive

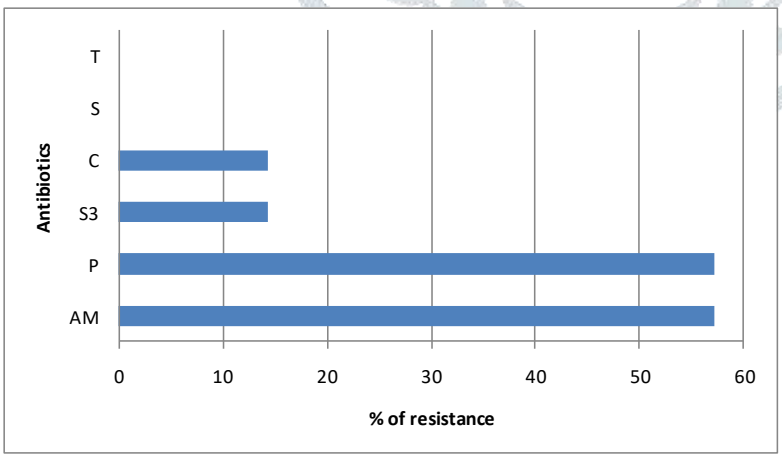
Antibiotic resistance pattern of the selected isolates

Table 3 MAR index and resistance pattern among the bacterial isolates from the sediments of Vattakayal lake.

MAR Index	Resistance Pattern	No. of isolates showing the pattern	% of occurrence of pattern
0.5	$AMPS_3$	1	25
0.5	AMCP	1	25
0.33	AMP	2	50

(AM- ampicillin, P- penicillin, S_3 – sulphatriad, C - chlorampenicol,)

Fig. 1 Percentage of antibiotic resistance of bacterial isolates from Vattakayal lake sediments.



(AM- ampicillin, P- penicillin, S_3 – sulphatriad, C - chlorampenicol, S - streptomycin and T - tetracycline).

Importantly, a substantial number of reports suggest that metal contamination in natural environments could have an important role in the maintenance and proliferation of antibiotic resistance (Alonso *et al*., 2001). This is of particular concern considering that anthropogenic levels of heavy metals are currently several orders of magnitude greater than levels of antibiotics (Stepanauskas *et al*., 2005). Berg *et al*. (2005) found that soil microbes isolated from a copper-amended field were more resistant to copper and antibiotics than strains isolated from control plots 21 months after copper amendment. Additionally, copper resistant strains were significantly more resistant to ampicillin and sulfonamide than copper sensitive isolates, which strengthened the argument that the traits are co-selected

Identification and characterization of heavy metal tolerant bacterial strains

Morphological and Biochemical characteristics

The morphological and biochemical tests were carried out for the identification of seven heavy metal tolerant bacterial isolates. These tests were performed following the Bergey's Manual of Determinative Bacteriology: 9th edition (Holt, *et al.*, 1994) and 8th edition (Buchanan and Gibbons, 1974. Based on the analysis the isolates were identified as *Bacillus cereus, Enterobacter aerogens, Pseudomonas putida, Bacillus licheniformis, Klebsiella pneumonia, Alcaligens faecalis,* and *Citrobacter kosari* (Table 4).

Molecular characterization of heavy metal tolerant bacterial strains

The Genomic DNA was isolated from the pure culture pellets of isolates K1 and K4 and 16S rDNA fragment was amplified by PCR. The PCR amplicon was purified by column purification in order to remove contaminants (Figs. 2a & b). The sequencing of purified gene segment was done using ABI3730xl Genetic Analyzer (Applied Biosystems, USA) (Figs. 3 a & b). The comparative analysis of the sequences of isolates with already available database using BLAST (Basic Local Alignment Search Tool) showed that the strains were close to the members of genus *Bacillus*. The highest sequence similarity of the sediment bacteria are as follows: K1, *Bacillus cereus* (showed 99% similarity with *Bacillus cereus* Accession No: 346665.1) and isolate K4 (96% similarity with *Bacillus licheniformis* strain CICC 10180, accession No: AY859478.1).The 16S rDNA sequences were submitted in the National Center for Biotechnology Information (NCBI) data bank under the accession numbers JQ863364 (*Bacillus cereus*) and JQ863365 (*Bacillus licheniformis*)

Table 4 Morphological and biochemical characteristics of metal tolerant bacterial isolates from the sediments of Vattakayal backwaters.

Organisms	*Bacillus cereus* (K1)	*Enterobacter aerogens* (K2)	*Pseudomonas Putida* (K3)	*Bacillus licheniformis*(K4)	*Klebsiella pneumonia*(K5)	*Alcaligens faecalis* (K6)	*Citrobacter kosari* (K7)
Morphology	*Abundant,opaque,white,waxy growth*	Abundant, thick, glistening growth	Thin viscous growth	Irregular with lobate margine	White transluscent	Thin viscous growth	Thin abundant growth
Gram staining	**+R**	**-B**	**-B**	**+R**	***-B***	***-B***	***-B***
Motility at 37º C	+	+	+	+	-	-	+
Oxidase	-	-	+	-	-	+	-
Catalase	+	+	+	+	+	+	+
Indole	-	-	-	-	-	-	+
Methyl red	-	-	-	-	-	-	+
Voges Proakauer	+/-	+	-	+	-	-	-
Citrate	-	+	+	+	+	+	+
Urease	-	-	-	-	+	-	+
Nitrate reduction	+	+	+	+	+	-	-
D-Glucose gas production	**A**	+	-	**A**	+	-	+
D-Glucose acid production	-	+	-	+	+	-	+
Lactose	-	+	-	+	+	-	+
Mannitol	-	+	-	**A**	+	-	+
Sucrose	**A**	+	-	+	+	-	+
Phenylalanine Deaminase	+	-	-	-	-	-	-

H_2S from LIA	-	-	-	-	-	-	-
Litmus milk reaction	**Peptonization**		**Peptonization**	**Peptonization**		**Alkaline**	
Gelatine	+	-	-	+	-	-	-
Starch	+	-	-	+	-	-	-
Lipid	+/-	-	+	+	-	-	-

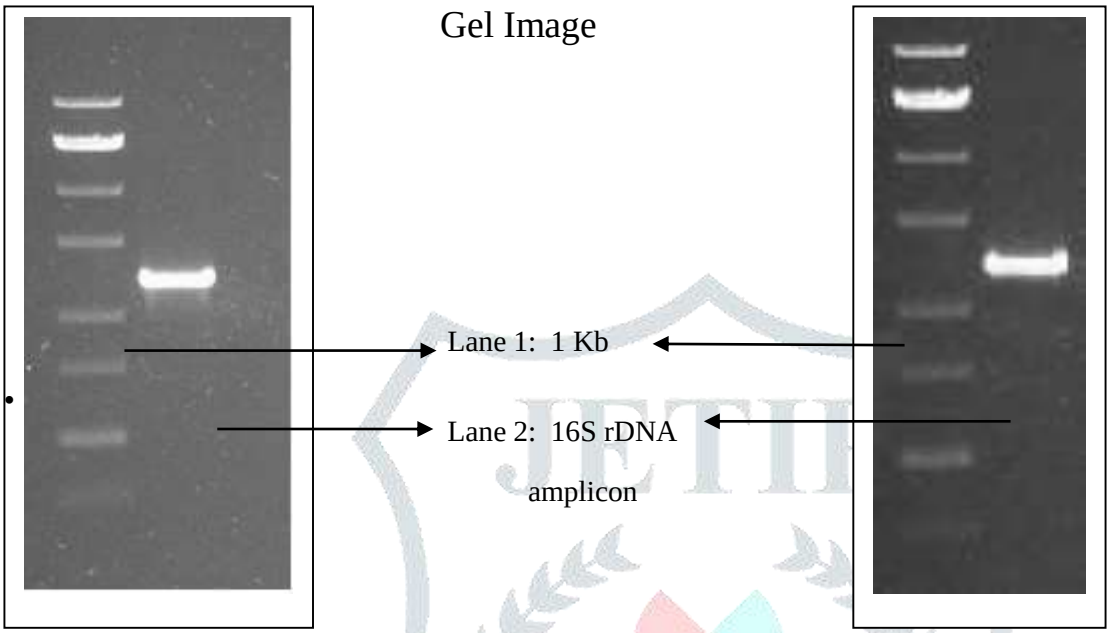


Fig. 2a Gel image for K1

Fig. 5 2b Gel image for K1

Fig. 3a Consensus Sequence Data Culture K1: (877bp)

TGCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGC
ATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGT
GAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGG
AGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTA
GTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGT
CTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAG
GAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGA
CTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATT
AAGCACTCCGGCCTGGGGAGTACGGGCCGCAAGGCTGAAACTCAAAGGAATTTGACGGGGG

Fig. 3b Consensus Sequence Data Culture K4: (458 bp)

TGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTCAGCGGCGGACGGGTGAGTAACACGTGGGT
AACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCAT
GGTTCAATTATAAAAGGTGGCTTTTACCTACCACTTACAAATGGACCCCCGGCGCATTACCTATTTGGGG
AGGTAACGGCTCACCAAGGCAACAATGCTTACCCAACCTGAAAGGTGGATCGGCCACCCTGGAACTGAA
ACACGGCCCAAACTCCTACGGAAGGCACCATTAGGAAATCTTCCGCATGGAACAAAAGTCTGACGAACC

ACCCCCCCGGGATTGATGAAGGTTTTCGAATCGAAAAACTCTGTTGTTAGGAAAAAACAATTACCGTTC
AATTAGGGGGGTACCTTGACGGTACTTAACCAAAAAGCAACGG

Phylogeny

Phylogenetic tree was constructed by using MEGA4 software and the evolutionary history was inferred by neighbour-joining method (NJ) (Fig. 5.4) by using published sequence from Gene Bank. The phylogenetic relationship was illustrated based on the alignments of 16S rDNA from 8 different Bacillus species namely, *B. cereus*, *B. thuringiensis*, *B. subtilis*, *B. anthracis*, *B. licheniformis*, *B. olivae*, *B. aerius*, and *B. sonorensis* and the strains K1 and K4. The bootstrap consensus was inferred from 1000 replicates. It was found that the strain K1 classified in the branch of Bacillus *cereus* and K4 in the branch of *Bacillus licheniformis* where the bootstrap value is 100.

Phylogenetic tree

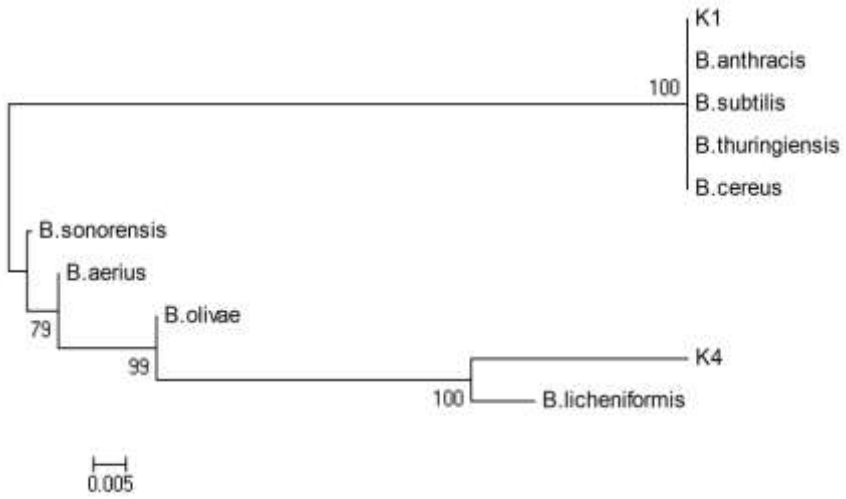


Plate I

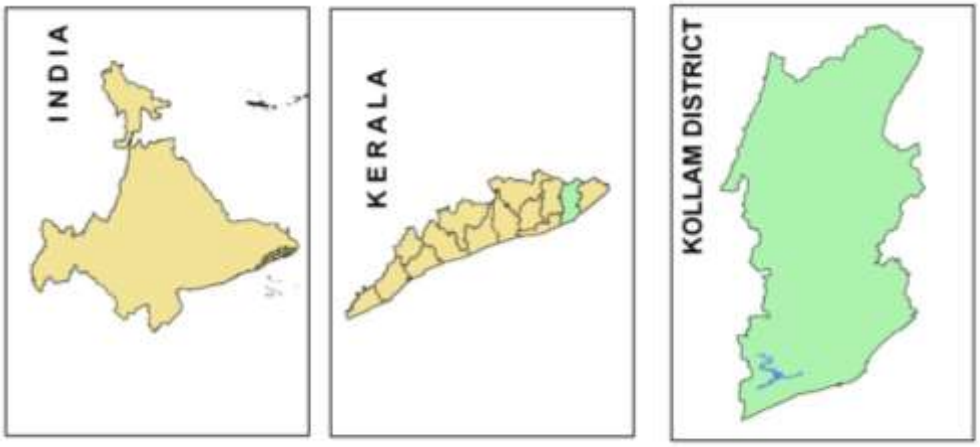


Plate II

Isolates

K1: *Bacillus cereus*
K2: *Entrobacter aerogens*
K3: *Pseudomonas putida*
K4: *Bacillus licheniformis*
K5: *Klebsiella pneumonia*
K6: *Alcaligens faecalis*
K7: *Citrobacter kosari*

Antibiotics

Ampicillin 10 µg
Chloramphenicol 25µg
Penicillin G 1 µg
Streptomycin 10 µg
Sulphatrad 300µg
Tetracycline25 µg

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