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Kosakonia radicincitans as a Multiple Heavy Metal Bioremediating Bacteria – A Study

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A B S T R A C T

Industrialization and urbanization have intensified the pollution of soil and aquatic ecosystems with multiple heavy metals and co-contaminants, including antibiotics. Microbes in such stressful environments develop unique strategies to survive in such stressful environments and evolve with resistance to multiple metals. Using these organisms to bioremediate multiple metals simultaneously will be a sustainable and cost-effective approach. The present study investigated the bioremediation capabilities of multiple metal-resistant bacteria from sediments at Mahim Creek. A mucoid colony forming bacteria resistant to Cd, Cr and Pb was selected and characterized morphologically and biochemically. Molecular identification through 16srRNA sequencing revealed the organism to be *Kosakonia radicincitans*. MIC (minimum inhibitory concentration) of the organism was found to be 70 ppm for Cr, 100 ppm for Cd and 800 ppm for Pb. Study of the growth pattern of the bacteria was carried out in the presence and absence of the heavy metals. Its bioremediation capability was explored using Atomic Absorption Spectroscopic analysis and was found to have higher reduction efficiency for Cr and Pb than Cd. The organism also exhibited resistance to multiple antibiotics and showed an MAR (Multiple Antibiotic Resistance) index of 0.685.

***Key words*:** Multiple metal resistance, *Kosakonia radicincitans*, MIC, Growth pattern, Bioremediation, AAS, MAR

Heavy metal pollutants are of major concern around the world due to exponential population growth, global urbanization and industrialization. Anthropogenic activities have increased the levels of heavy metals in soil to dangerous levels. The United States Environmental Protection Agency lists Cu, Ni, Cd, Zn, Cr, Se, Ag, Th, Be, As and Pb as the most hazardous heavy metals [1-2]. The high-water solubility of these heavy metals, their recalcitrance to degradation leads to their persistence in the aquatic sediments and soils. This further cause their bioaccumulation and biomagnifications in the terrestrial and marine ecosystems thus affecting the soil and aquatic microbial diversities [3-6]. In the ATDSR's list of 275 hazardous chemicals for human health, Cr ranks 17th, Cd ranks 7th and Pb ranks 2nd [7].

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Cd has an extremely high biological half-life and is classified as group 1 carcinogen to humans [8]. It is a non-essential element and is toxic even at low concentrations of 0.001–0.1 mg L^{-1}. It accumulates in the human body through the food chain, has no benefit to the ecosystem but only harmful effects have been reported [9-10] Smoking of cigarettes leads to Cd poisoning in humans since smokers inhale approximately 10% of the 0.5mcg to 2.0mcg Cd content from one cigarette [11].

Cr exists in several oxidation states, ranging from Cr^{2+} to Cr^{6+}, of which Cr^{3+} and Cr^{+6} are the most stable forms frequently encountered in the environment [12-13]. Cr^{3+} is essential and necessary in trace amounts for natural lipid and protein metabolism and is a co-factor for insulin action however Cr^{6+} is associated with a variety of diseases and pathologies [14]. The high-water solubility and mobility of hexavalent chromium, and its ease of reduction, make it 100–1000 times more toxic than trivalent chromium [15-16]. USEPA has designated hexavalent chromium as a priority pollutant because of its high affinity for oxygen [17]. The International Agency for Research on Cancer (IARC) in its report in 2018 classified Cr^{6+} as group I occupational carcinogen [14], [18]. Cr through food chain leads to bioaccumulation in human body and causes various dermal, renal, neurological, and GI diseases and several cancers including lungs, larynx, bladder, kidneys, testicles, bone, and thyroid [19].

Pb is another non-essential toxic heavy metal detrimental to organisms even at toxic levels. In addition to being a mutagen

and teratogen, it also affects the kidneys, bone marrow, digestive system, cardiovascular system, reproductive system, nervous system, and immune system. Due to the short half-life of Pb in blood and soft tissues (30 – 40 days) and its long excretory half-life in bone (upto 16 years) Pb is difficult to eliminate from living bodies once absorbed [20-21].

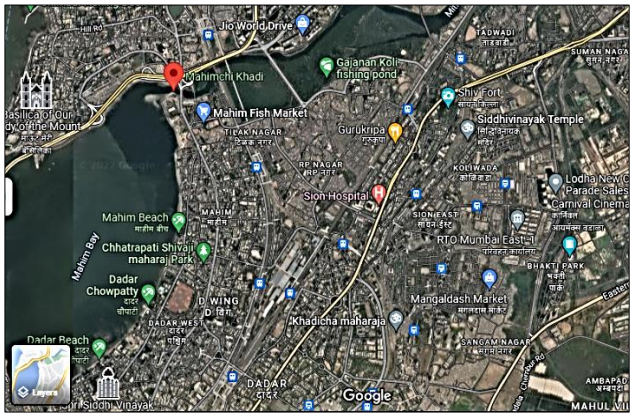
Recent years have seen a great deal of attention devoted to bioremediation of these heavy metals using microorganisms, which has scientific novelty and potential industrial applications [22]. The presence of heavy metals in the niche of the microorganisms pushes it in an imperative situation to develop a resistance or tolerant mechanism against them and the nutritional versatility of the microorganisms can be **e**xploited for the removal of heavy metals from polluted soils [23]. Heavy metal pollutants can also induce antibiotic resistance in metal resistant which poses a serious health threat to humans. This metal driven co-selection of heavy metal and antibiotic resistance can be attributed to the underlying mechanisms of co-selection or cross resistance [24-26].

Coastal areas in India are subjected to anthropogenic pressure and encroachment for human settlements due to the growth of population [27]. Mahim creek in Mumbai has become a major dump site for the wastes generated by residential complexes and small-scale industries around. Mumbai's famous Mithi river notoriously polluted with heavy metal beyond the permissible limits drains off into Mahim creek [28-29]. Micro-organisms in such environment evolve with unique multiple metal resistance as an adaptive strategy to cope with the stress. Such ability of the bacteria can be exploited for an eco-friendly and efficient approach for the simultaneous removal of pollutants [30]. The present study aims at isolating one such bacterium capable of remediating Cd, Cr and Pb, studying the effect of these metals on its growth pattern and its efficacies were assessed and compared using techniques such as atomic absorption spectroscopy.

MATERIALS AND METHODS

Sample collection

Sediment samples were collected around Mahim Creek (19.05048No, 72.83771Eo) (Fig 1) also known as *Mahim chi khadi* in the regional language in sterile containers and immediately taken to lab and kept at 4 °C till further processing.



Isolation of heavy metal resistant bacteria

A preliminary screening for microorganisms resistant to heavy metals present in the sediment sample was done by enriching 1gm of the sample in 50ml of sterile minimal media [Solution A – (KH_2PO_4 – 0.3 grams, Na_2HPO_4 – 0.6gms, NH_4Cl – 0.2 grams, NaCl – 0.5 grams, D/W -80 ml) and solution B – (Glucose 0.8 grams, $MgSO_4.7H_2O$ – 0.01 grams, D/W – 20 ml, pH – 7.2)] supplemented with 10ppm of Cd at RT for 72 hours in shaking conditions at 100 rpm. Serial dilutions of the enriched culture was performed followed by plating onto sterile minimal agar plates supplemented with 10ppm cadmium. The plates were kept at RT for 24 – 48 hours to obtain well isolated colonies [31].

Screening for multiple metal resistance

The isolates obtained in the preliminary step were further streaked on sterile minimal agar plates incorporated with 10 ppm of Cr and 10ppm of Pb separately and incubated at RT for 24 – 48 hours [15]. The organism with the capability to grow in the presence of all the three heavy metals (Cd, Cr and Pb) was considered for further studies.

Morphological and biochemical characterization

Colony morphology of the isolate on sterile minimal media incorporated with Cd, Cr and Pb separately were studied. Tests like indole, methyl red, Voges-Proskauer, citrate, catalase, triple sugar iron (TSI), starch and carbohydrate (glucose, lactose, mannitol, sucrose) utilization was done to study its biochemical characteristics.

Molecular identification of the organism

The isolate was identified by 16srRNA sequencing followed by NCBI BLAST. A phylogenetic relationship with the first ten BLAST results from NCBI Gene Bank was constructed using UPGMA method. The phylogenetic tree was constructed for evolutionary analysis using MEGAX software. The sequences were then submitted at the NCBI gene Bank for accession numbers.

Determination of minimum inhibitory concentration (MIC)

The MIC of Cd, Cr and Pb against the isolates were determined by inoculating it in 10ml of sterile minimal media (broth) incorporated with different concentrations of the metals (ranging from 10ppm to 1000ppm), and incubating them at RT for 24 to 72 hours. The growth from the tubes were streaked onto the sterile minimal agar plates with different metal concentrations between (10ppm to 1000ppm) to confirm the minimal inhibitory concentration (MIC) of the isolate. The plates were incubated at RT for 72 hours and the concentration at which isolate failed to grow was considered as its MIC.

Study of the bioremediation potentials of the heavy metals by atomic absorption spectroscopy

The ability of the isolate to reduce Cd, Cr and Pb was studied using Agilent 240FS atomic absorption spectrophotometer. A batch culture method was followed for the study. 1ml of actively growing culture of the isolate (OD adjusted to 0.8 at 600nm) was inoculated into 50ml of sterile minimal medium supplemented with 30ppm and 50ppm of cadmium, 20ppm and 30ppm of chromium and 30ppm, 50ppm, 100ppm and 200ppm of lead and the flasks were incubated at room temperature on a rotary shaker (100 rpm). After 72 hours of incubation, 10ml of the culture was withdrawn aseptically from the respective flasks in a sterile centrifuge tube and centrifuged at 4000 rpm for 25 minutes. The supernatant was considered for further AAS analysis. The cadmium removal rate was calculated using the following formula:

Percentage Metal Absorbed = (HM) i – (HM)f / (HM)i × 100

Where; (HM)i: Initial heavy metal ion concentration (ppm)

(HM)f: Final heavy metal ion concentration (ppm)

Study of the growth pattern of the isolate in the presence and absence of heavy metals

The impact of metal stress on the growth potential of bacteria was observed by assessment of growth curve in the presence and absence of Cr, Cd, Pb separately and in the presence of combination of the metals - Cd & Pb, Cd & Cr and Cd, Cr & Pb. The inoculum for the study was prepared by inoculating 10 ml of sterile minimal medium with the cadmium tolerant isolate and incubated at RT under shaking conditions for 24 hours. For the study of the growth curve, sterile 30ml of minimal medium was inoculated with the actively growing inoculum to get an OD of 0.04 – 0.06 at 600nm and at regular interval of 1 hour and the optical density of the growing culture was recorded. The experimentation was continued until the stationary phase was reached and a graph of time against absorbance at 600nm was plotted to analyze and compare the growth pattern of the isolate under stressed and non-stressed conditions.

Calculation of doubling time 'T' of the 10B

Doubling time or mean generation time, denoted by 'T', is the time it takes for a given population of cells to double. Bacterial culture has millions of cells; therefore, the term mean generation time is used [32]. Doubling time for the isolate 10B under stressed and non-stressed conditions were calculated using Microsoft – EXCEL.

The plot of time versus optical density measurement of the growing culture was scattered. The exponential phase of the growth curve was then converted to log scale and the line of best fit was obtained by selecting the exponential trendline. Then the automated equation in the form of $Y = Ae^{Bx}$ was displayed, where 'A' = the initial amount, 'x' is the period of time over which growth occurs, 'B' is the growth rate per unit of time, 'e' is the base of the system of natural logarithms, and 'Y' is the amount population at time x [33]. The mean generation or doubling time (T) was calculated by the formula doubling time 'Td = Ln (2) / B' where the ln is the natural of value 0.69314 and B value (the growth rate per unit time) provided by EXCEL on the graph.

Antibiotic sensitivity tests

The susceptibility of the isolate to different antibiotics was done by Kirby – Bauer disc diffusion method. Sterile antibiotic discs from Hi Media like Ampicillin (25mcg), Streptomycin (25 mcg), Vancomycin (10 mcg), Kanamycin (5mcg), Erythromycin (10 mcg), Chloramphenicol (25 mcg), Gentamicin (50 mcg), Amoxicillin (25mcg) were used for the tests. Actively growing culture was swabbed onto sterile Mueller Hinton agar plates followed by placing the antibiotic discs on them under aseptic conditions. The plates were then incubated at RT for 24 – 48 hours. After incubation the plates were checked for the presence or absence of zone of inhibition around the discs and the results obtained were interpreted as per the standard Kirby – Bauer chart.

Multiple Antibiotic Resistance (MAR) index of the isolate was calculated using the formula $MAR_{index} = a / b$ where a represents the number of antibiotics to which the isolate was resistant, and b represents the number of antibiotics to which the isolate was exposed [34].

RESULTS AND DISCUSSION

Isolation of metal resistant bacteria and screening for multiple metal resistance

Six well isolated colonies were obtained from the sample enriched in minimal media containing 10ppm Cd by spread plate method which convinced the presence of heavy metal resistant bacteria in the sample. Of the six isolates, the one that showed mucoid colony was selected for further study as it was suspected to produce exopolysaccharide in response to the stress of heavy metal. The mucoid isolate was labelled as 10B.

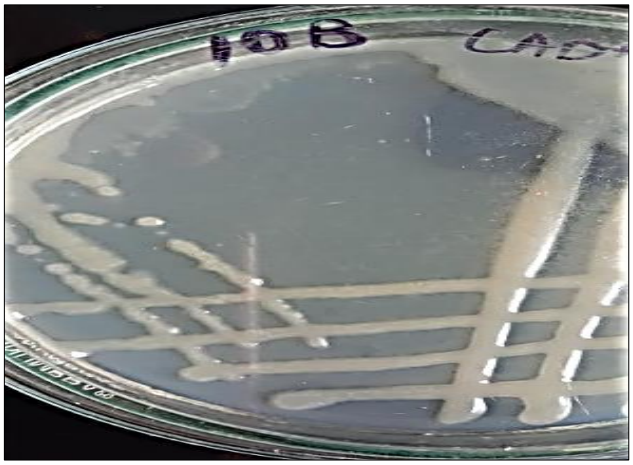


Fig 2 The mucoid isolate 10B on sterile minimal media with 10ppm Cd

Morphological and biochemical characteristics of the isolate

Gram staining of the isolate 10B showed it to be a Gram-negative coccobacillus. The morphological characteristics of the colonies of 10B on sterile minimal agar plate with 10ppm Cd are given in (Table 1). The isolate showed the same morphological characteristics on sterile minimal media with 10ppm Cr and Pb respectively. The organism tested positive for methyl red, catalase and citrate utilization tests and negative for indole, Voges-Proskauer tests. On TSI slant, the organism showed alkaline slant and acidic butt with gas and H_2S production (Table 2). Sucrose, mannitol, and lactose were utilized by the organism without gas production under aerobic conditions, while glucose was utilized with gas production. At anaerobic condition, it utilized sucrose without producing gas, and glucose producing gas (Table 3).

Table 1 Colony characteristics of 10B on sterile minimal agar plate with Cd

Organism	Size	Shape	Colour	Margin	Elevation	Opacity	Consistency	Gram's Nature
10B	Big	Circular	Off-white	Entire	Elevated	Opaque	Mucoid	Gram negative coccobacilli

Table 2 Biochemical characteristics of 10B

Test	Indole	Methyl red	Voges- Proskauer	Urease	Catalase	Citrate	TSI
10B	-	+	-	-	+	+	R/Y/G/-S/B/G/H2S

Key: + (positive), - (negative), R (red), Y (yellow), Gas (gas), S (slant), B (butt)

Molecular identification of the isolates by 16S rRNA method

The partial sequences of the isolate 10B obtained by 16S rRNA were submitted to the NCBI gene database, where an accession number - OM060660 was assigned for the same. The partial sequences obtained by 16S rRNA method were aligned and compared with sequences using NCBI BLAST. The BLAST results showed that the query sequence has 98.97 – 99.92% identity and 100% query coverage with the 16S rRNA

sequences already deposited in the GenBank. To trace the relationship of the isolate 10B with other closely related sequences in the database, a phylogenetic tree (Fig 3) was constructed using cluster algorithm using the first five hits in NCBI nucleotide sequence database and MEGA X software to find the ancestral and evolutionary relationship of the isolate. The tree was generated using the maximum likelihood method with Taimura Nei model and bootstrap replications were set at 1000. The analysis identified the isolate 10B as *Kosakonia radicincitans*.

Table 3 Carbohydrate utilization of 10B

Organism	Sucrose		Mannitol		Glucose		Lactose	
10B	A	An	A	An	A	An	A	An
	+	+	+	-	+ (G)	+ (G)	+	-

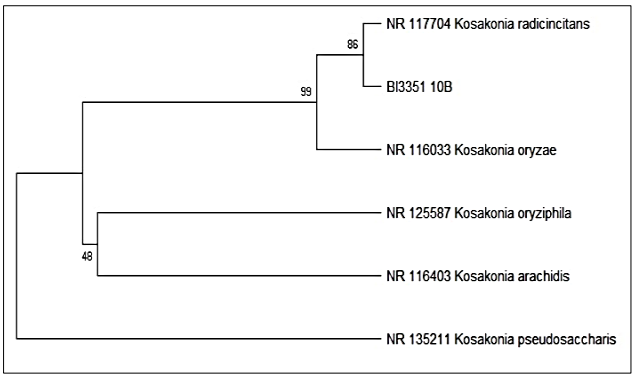


Fig 3 Phylogenetic trees based on 16S rRNA sequencing showing the evolutionary relationship of 10B

Minimum inhibitory concentration (MIC)

MIC of the isolate 10B against Cd, Cr and Pb were studied in the increasing range of 20ppm to 1000 ppm. The isolate 10B was found to be highly tolerant to Pb (800 ppm) followed by Cd (100ppm) and Cr (70ppm).

Study of the bioremediation potentials of the isolate 10B

Using Atomic absorption spectroscopy, the bioremediation capability of the isolate 10B was explored. It was found to reduce 69.23% of 20ppm and 8.5% of 50ppm Cd; 98.38 % of 20ppm and 98.40% of 30ppm Cr and 98.33% of 30ppm, 98.25% of 50ppm, 98.65% of 100ppm and 98.25% of 200ppm of lead, thus, proving it to be a promising tool for bioremediation of sites polluted with multiple metals for a long time.

Growth pattern of the isolate 10B in the presence and absence of heavy metals

A simple and reliable method experimentally bacteria can be grown under stressed and non-stressed conditions and their proliferation can be monitored over a short time scale to understand their response, adaptation and evolution in stressed environments [30]. The growth pattern of 10B in the presence and absence of heavy metals is shown in the (Fig 4a-f). Its generation time 'g' was calculated under the stress of each metal separately and with combination of metals (Cd & Cr, Cd & Pb and Cd, Cr and Pb).

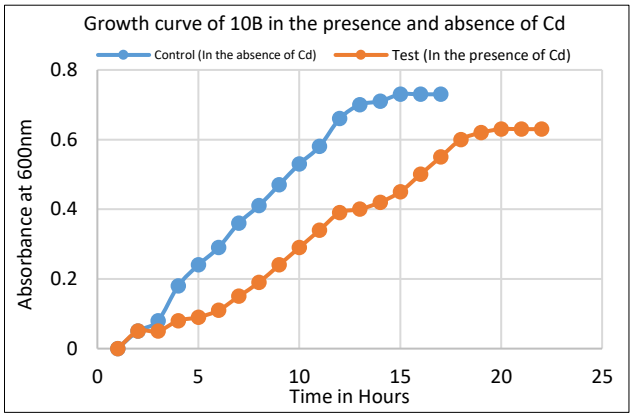


Fig 3a Study of growth under Cd stress

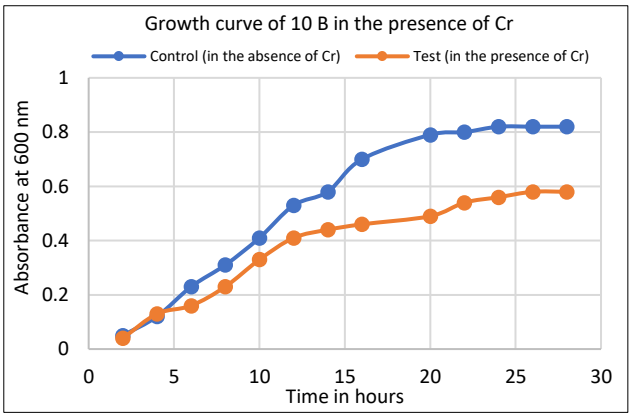


Fig 4b Study of growth under Cr stress

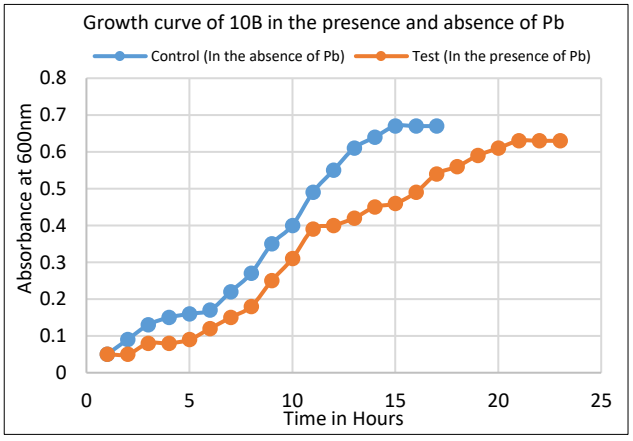


Fig 4c Study of growth under Pb stress

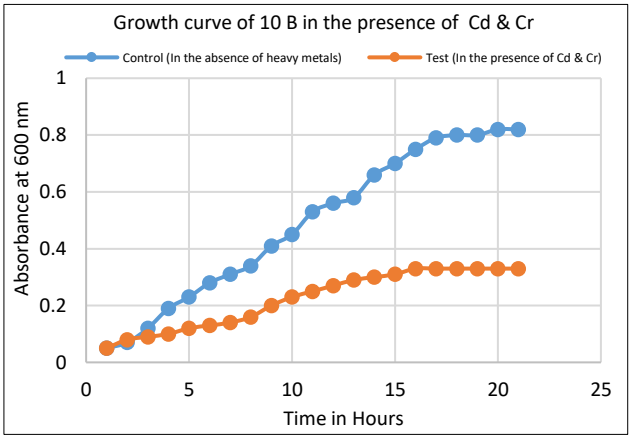


Fig 4d Study of growth under Cd and Cr stress

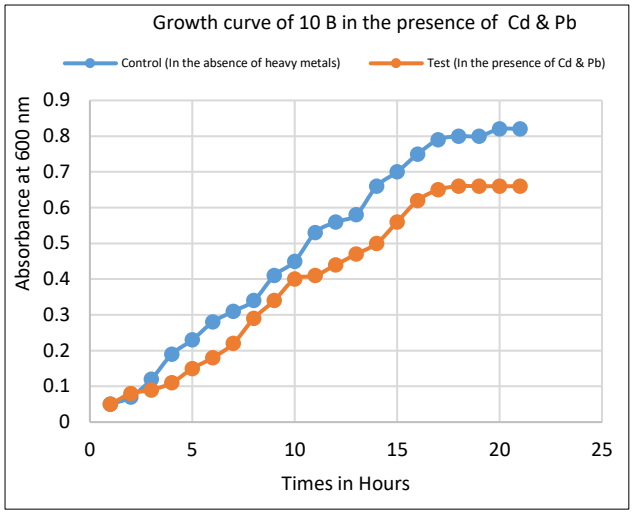


Fig 4e Study of growth under Cd and Pb stress

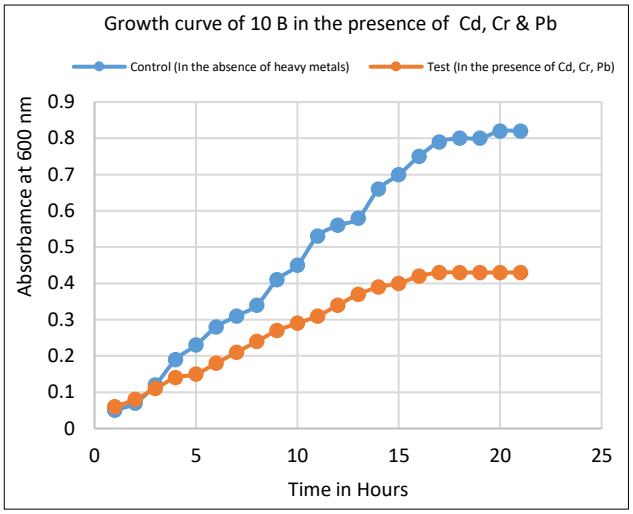


Fig 4f Study of growth under Cd, Cr and Pb stress

Heavy metals affected the growth of the isolate, despite it growing in their presence. An extended lag phase was observed when compared to the control in the absence of heavy metals, is indicative of the organism taking some adaptive measures to deal with the stress. In the presence of Cd (Fig 3a) and Pb (Fig 3c), the organism showed a short lag phase and entered the exponential phase but was observed to have a brief lag again and then moved into the stationary phase. The growth of the isolate in the presence of Cr (Fig 3b), matched that of the control initially. The organism then quickly entered into the log phase but could not prolong in it due to the stress and thus entered the stationary phase. A combination of metals (Cd, Cr, and Pb) had a greater effect on growth showing a long adaptive period and early stationary phase (Fig 3 d, f). In the presence of Cd and Pb very less variation was observed from control and also a brief lag was seen in the middle of the growth before the stationary phase was approached (Fig 3e).

Calculation of doubling time 'T'

The calculated doubling time 'T' for the isolate 10B in the absence (Control) and presence (Test) of the heavy metals using Microsoft – Excel is given in (Table 4). Heavy metals prolonged the doubling time for the isolate. Cr had higher impact on the growth of the bacteria when used separately and with Cd and Pb together. Growth in the presence of combination of Cd and Pb showed the least generation time.

Table 4 Doubling 'T' of 10B in presence of different heavy metals

Name of the metal	Doubling time 'T' for Control (hours)	Doubling time 'T' for Test (hours)
Cadmium	3.78149	5.406764
Lead	3.78149	5.839488
Chromium	3.7814	7.894615
Cd, Cr, Pb	3.78149	6.769015
Cd, Pb	3.78419	4.940465
Cd, Cr	3.78419	6.382571

Table 5 Antibiotic susceptibility test of 10B

Name of the antibiotic	Zone of inhibition (mm)	Inference
Ampicillin (25 mcg)	Zero	Resistant
Streptomycin (25 mcg)	24	Sensitive
Vancomycin (10 mcg)	Zero	Resistant
Kanamycin (5 mcg)	12	Resistant
Erythromycin (10 mcg)	13	Resistant
Chloramphenicol (25mcg)	24	Sensitive
Gentamycin (50 mcg)	21	Sensitive
Amoxicillin (25 mcg)	Zero mm	Resistant

Antibiotic sensitivity tests

Heavy metal resistant bacteria also exhibit co-resistance to antibiotics. The heavy metals co-regulate the antibiotic resistant genes and decrease the susceptibility of the organisms to antibiotics [35]. 10B was found to be resistant to Ampicillin, Vancomycin, Kanamycin and Erythromycin but sensitive to Streptomycin, Cloramphenicol and Gentamicin (Table 5). The MAR index was found to be 0.625 for the isolate which is higher than the accepted value of 0.25 suggesting the isolate to be a multiple drug resistant organism [32].

CONCLUSION

This study obtained six different isolates resistant to heavy metal. Among these, one isolate with mucoid colonies was selected for further investigation. It was found to be resistant to Cd, Cr and Pb and identified as *Kosakonia radicincitans* by 16S rRNA sequencing. It exhibited different MIC values with Cd, Cr and Pb. Study of the bioremediation potentials of the organism convinces it to be a promising tool for eco-friendly microbial bioremediation. The response of the

organism to Cd, Cr and Pb was understood by growth curve analysis and their doubling time. The organism was also found to be multiple metal resistant with an MAR index which might be due to co-selection of heavy metal and antibiotic resistance genes after a prolonged exposure to metal pollutants. The mucoid colony of the organism might be due to the synthesis of exopolysaccharie as a defense to against the pollution. Further investigations are needed to prove the organism bioremediation and industrial applications.

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