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Actinomycetes as a Possible Source of Bioremediation of Heavy Metal Cadmium from Contaminated Soil

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Abstract

Annually, a significant volume of chemicals, encompassing fertilizers and pesticides, is administered to agricultural soils. Using pesticides and fertilizers, agricultural practices contribute to heavy metal Cadmium (Cd), Copper (Cu), Lead (Pb) and Zinc (Zn) pollution. Heavy metals and pesticides are high at the peak of ecological contaminants, presence of this has introduced grave risks to the health of the population and agronomics. Among heavy metals, cadmium (Cd) is recognized for its high mobility in various environmental settings. Cd has a deleterious effect on plant phenotypic, cytotoxicity (e.g., lowering chlorophyll concentration and limiting photosynthetic effectiveness), and metabolic activities (e.g., chlorosis and necrosis). Microbial bioremediation by using microorganisms is one of the secure, pure, cost operative and eco-friendly technology for decontaminating polluting sites as compared to physical and chemical techniques. Among microbes, Actinobacteria hold a paramount position, serving as key players in numerous biological processes, utilize toxins as carbon source and turn into high concentrations of pesticides, chemical complexes and heavy metals into commercially viable antibiotics, enzymes, proteins, and plant growth promoting hormones. This study is an effort to explore the potent cadmium resistance actinomycetes to reduce cadmium levels to enhance degradation. For this purpose, 53 actinomycetes strains were tested for heavy metal resistance and tolerance to Cadmium against different concentrations. After secondary screening Four potent isolate have the potential to grow at 1000 mg/L concentration of Cadmium in the medium. When they are able to grow on heavy metal containing media it could be beneficial for reduction and elimination of toxic metals from contaminated environment. When it comes to achieving a suitable level of metal tolerance, this potent powerful actinomycetes strain *Streptomyces pactum* have been identified to be promising.

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Introduction

Over the recent past, the prompt escalation of heavy metals as contaminants poses a growing threat to the environment and through the accumulation in soil and subsequent uptake by plants, heavy metals are generating devastation in the environment, particularly in the agricultural sector.[1] This might be caused by the prolonged use of Phosphate fertilizers, the utilization of sewage sludge, industrial waste, and improper irrigation of agricultural regions.[2] Aluminum (Al), lead (Pb), cadmium (Cd), and silver (Ag) are examples of heavy metals that are poisonous to living things and have no biological use.[3] Majority Cd particles are absorbed by plant roots in accordance with portability, bioavailability, and concentration. Cd accumulates in the roots, shoots, and edible sections of plants after entering plant cells by a variety of carriers, including Calcium (Ca) channels.[4] Additionally, the buildup of Cd in plants has harmful consequences, such as the repression of some activities (mineral transport, photosynthetic apparatus, and nutrient intake). Additionally, it may prevent the entry of Fe into plant shoots. In recent decades, the crucial need for heavy metal pollution removal has expanded. Frequently utilized methodologies for the elimination of toxic metals from the surroundings encompass chemical precipitation, reverse osmosis, ion exchange, ultrafiltration, and electrodialysis. Unfortunately, they are frequently ineffective and quite pricey. They may also release poisonous chemicals, which are undesirable and unprofitable5. Therefore, there is a major need for the discovery of low-cost, exceedingly successful, and selective substitutes that can reduce heavy metal toxicity to levels that are appropriate to the environment. Thus, bioremediation is an appealing and effective cleansing method to get rid of toxic waste from a contaminated environment.[6] Environmental heavy metals are treated using bioremediation, which employs a variety of microbes containing bacteria, yeast, fungi, algae, and higher plants as its major treatment agents. The most applicable among these is bacteria as it is both inexpensive and environmentally beneficial. It may be done on-site, costs less to treat, has less impact on the environment, and does not result in secondary pollution.[7] The most significant groupings of microorganisms, the actinobacteria, are in charge of the bioremediation of hazardous heavy metals as well as the destruction and transformation of organic and metal substrates (Cd, Cr, Cu, Pb, Hg and Zn). Streptomycetes, a genus of actinobacteria, play a vital job in the extraction of metals from polluted water and soil. This capability is credited to their metabolic diversity, specific growth characteristics, spore-forming capacity, mycelia formation, and the comparatively quickly colonization of substrates. Because they produce a variety of metal ion chelators, like siderophores, that gives safety from the detrimental impact of heavy metals or selective absorption for particular metabolic activities, actinomycetes have the capacity to survive in environments polluted by metals.[8] To ascertain the indigenous bacterial strains' resistance to cadmium, the current study seeks to extract and identify them from heavy metal-contaminated soil. The traits of the strains' physiology and biochemistry were used to define them.

Materials and Methods

Collection and Analysis of Soil Sample

The soil specimen was obtained from agricultural site in Jamnagar district, Gujarat with a latitude and longitude of the area, 22°18'11" N, 70°18'57" E. Soil sample was taken from the surface to a depth of 13-15 cm. Soil samples were gathered using zippered bags for the sampling process. Stone fragments and plant remains were manually removed and cleaned. A 2 mm brass sieve was used to filter soil sample and air dried. Soil analysis was performed at Envitro Soil Laboratory, Rajkot, which includes soil pH, Electrical Conductivity, Total Organic Carbon, Nitrogen, Phosphorous, Potash, Cation Exchange Capacity, Moisture and heavy metals such as Zinc (Zn), Cadmium (Cd) and Copper (Cu).

Enrichment of Soil Sample

By using a sterile spatula, sample was deposited in sterile Ziplock bags. Soil sample was kept at 4° C and brought to the lab for processing right away. For the enrichment of actinomycetes, the sample was treated to a chemical treatment with $CaCO_3$.[9]

Isolation of Actinomycetes

One gram of soil was mixed with 9 ml of autoclaved N-saline, which repeatedly diluted to dilutions of 10^{-3}, 10^{-4}, and 10^{-5}. On Starch-Casein Agar (SC) medium, 0.1 mL of each dilution were dispersed. Petri dishes were kept in a chamber for culturing at 28°C for 7–12 days after sub-culturing isolated actinobacteria.[10]

Primary Screening of Metal Tolerant Actinomycetes by Plate Assay Method

By adding 100 mg/L of CdCl2 to sterilized SC medium, the isolated actinomycetes colonies were tested for heavy metal resistance to cadmium. The isolated actinomycetes colonies were subjected to a purification process and cultured at 28° C for 10-12 days on SC agar medium. The greatest concentration of heavy metal that allows for development after maximum concentration (7-12 days) that may be tolerated. Isolated actinomycetes colonies that had grown on SC agar supplemented with cadmium were then subjected to increasing levels of cadmium (100, 500, 1000, and 1500 mg/L). When complete inhibition of growth was observed on SC agar with metal supplementation, the testing for microorganism tended tolerance came to an end.[11]

Secondary Screening of Metal Tolerant Actinomycetes by Agar Well Diffusion Method

Test was conducted in SC agar medium plates treated with $CdCl_2$ at concentrations of 100, 500, 1000 and 1500 mg/L. In this instance, wells were created by aseptically removing strips of agar from the center of Petri dishes and then filling them with 500 µL of 100, 500, 1000 and 1500 mg/L $CdCl_2$ solutions. Isolates were inoculated by streaking perpendicularly to the wells. Petri dishes were cultured at 28°C for 10-12 days. The ratio of metal tolerance exhibited by the actinomycetes strain was estimated by determining the proportion in between the length of the observed growth (in millimeters) and the total length of the inoculated streak.

% of growth=(complete inoculated length of streak-inhibited length×100)/(complete inoculated length)

Consequently, the inhibition exerted by the metal heightened with the greater distance of the growing colony from the well's edge. The proximity of microbial growth to the well served as the qualitative measure of metal resistance.[12]

Spore Optimization

Spore optimization was carried out by adding a drop of tween 80 reagent mixed with 5 ml distilled water on a slant. The solution was mixed homogenously and allowed it to settle. 1 ml of supernatant was taken and serially diluted to dilution of 10^{-5} on SCA media supplemented with Cd and without Cd. By using a spread plate approach, the viability of the spores was assessed. After the petri dishes were cultured for 11-13 days at 28°C, colony forming units (CFUs) were counted. Spore counts were performed and compared with control.[13] High spore forming actinomycetes strain was selected for molecular identification. Isolates were proceeded further for their spore counting by using this formula,

Spores/ ml=(No.of colonies×Dilution factor)/(Volume of inoculum)

Morphological and Biochemical Characterization of Actinomycetes Isolates

The morphological and biochemical properties like colony characteristics, Gram staining, Catalase activity, Carbon utilization property, Citrate utilization activity, Starch hydrolysis, Gelatine hydrolysis, Casein hydrolysis, Methyl Red, Voges-Proskauer, Nitrate reduction of strain AMT14, AMT35, AMT37 and AMT52 were performed.[14]

Molecular Identification of Actinomycetes Isolates

The genomic material of highly spore-forming actinomycetes was isolated, and its DNA content was quantified using a 1.0% Agarose Gel, revealing one band of large-molecular-size DNA. A targeted gene segment was then amplified through PCR, yielding a distinct amplicon band upon electrophoresis. Subsequent purification using a column method removed impurities from the PCR product. DNA sequencing of the purified amplicon was performed applying the BDT v3.1 Cycle Sequencing Kit on an ABI 3500xl Genetic Analyzer having primer27 F. The resulting gene sequence underwent BLAST analysis against the NCBI GenBank database. The top ten sequences, based on maximum identity scores, were selected and matched using several alignment programs. Gene sequences were further compared with the GenBank database through the NCBI and BLAST platform. The sequencing methods were implemented by Gene Explore Diagnostics and Research Centre Private Limited in Ahmedabad, Gujarat.[15]

Results

Analysis of Soil Sample

The soil used in this study was contaminated by pesticide. Total Zinc (Zn) and Copper (Cu) contents

demonstrated that it was not contaminated by these metals. On the other hand, it was contaminated by heavy metal Cadmium (Cd). Lower availability of metals in the soil occurs from low pH, which increases the mobility of metals in the soil. Nearly all biological and chemical processes that regulate terrestrial ecosystems are influenced by soil pH. The contents of soil physical characteristics and heavy metals in the investigation area are shown in Table 1 and 2.

Table 1: Physical and Chemical parameters of soil sample

Sr. No.	Tests	Result
1	pH	6.26
2	Electric Conductivity	6.14 mS/Cm^2
3	Total Organic Carbon	17.75 %
4	Nitrogen	0.23 %
5	Phosphorus	0.002 %
6	Potash	0.02 %
7	Moisture	25.97 %
8	Cation Exchange Capacity	17.88 meq

Table 2: Concentration of Heavy metals in soil sample

Sr. No.	Tests	Results (mg/kg)	WHO limits (mg/kg)
1	Zinc (Zn)	7.88	50
2	Cadmium (Cd)	14	0.8
3	Copper (Cu)	0.23	36

The physicochemical analysis of samples contaminated with pesticides has unveiled a substantial concentration of the heavy metal cadmium in the studied site. Moreover, the acidic pH and elevated salinity observed are intricately linked to the utilization of pesticides.

Isolation of Actinobacteria and Screening of Metal Resistant Strains

A sample of soil taken from an agricultural site yielded fifty-three actinobacterial colonies in total. Four actinomycetes strains were resistant to the heavy metal Cadmium out of the total number of actinomycetes strains that were isolated and cultured in starch casein agar with metal supplements. All the four bacterial colonies that were Cadmium resistant were tolerant up to 1000 mg/L, However, at elevated concentrations of metal salts, the growth of these isolates was impeded. None of them were tolerant up to 1500 mg/L of cadmium.

Secondary screening of Actinobacteria by Agar well Diffusion Method

Each individual isolate exhibited a distinct tolerance level compared to others when exposed to the same metal. Out of fifty-three isolates AMT14, AMT35, AMT37 and AMT52 were resistant at 1000mg/L. The total length of the inoculated streak measured 37 mm. The tolerance of cadmium among various actinomycetes isolates was determined based on the length of the growing streak. The growth percentage was computed using the specified formula and was later depicted graphically.

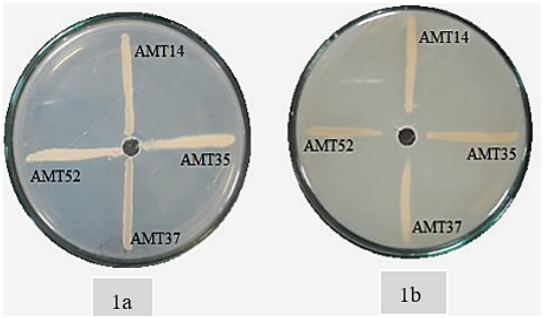


Fig. 1: (1a) Control showing positive result and (1b) Exhibiting growth patterns on SCA plate using Cadmium chloride at the dose 1000mg/L.

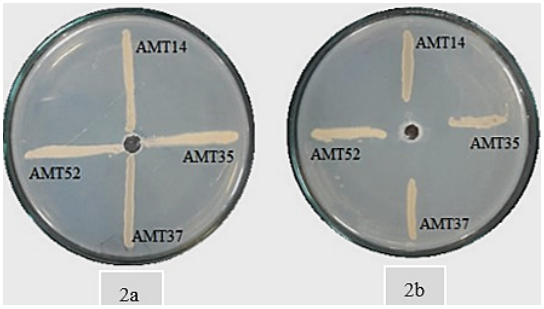


Fig. 2: (2a) Control showing positive result and (2b) Exhibiting growth patterns on SCA plate using Cadmium chloride at the dose 1500mg/L.

Table 3: % of isolates' development on SCA plates

Sr. No.	Actinomycetes colonies	Assess the growth % on agar plates exposed to varying concentrations of $CdCl_2$	
		1000 ppm	1500 ppm
1.	AMT14	84.76	54.05
2.	AMT35	80.13	53.02
3.	AMT37	87.24	86.28
4.	AMT52	78.16	63.92

Note: AMT= Strain number, $CdCl_2$= Cadmium Chloride

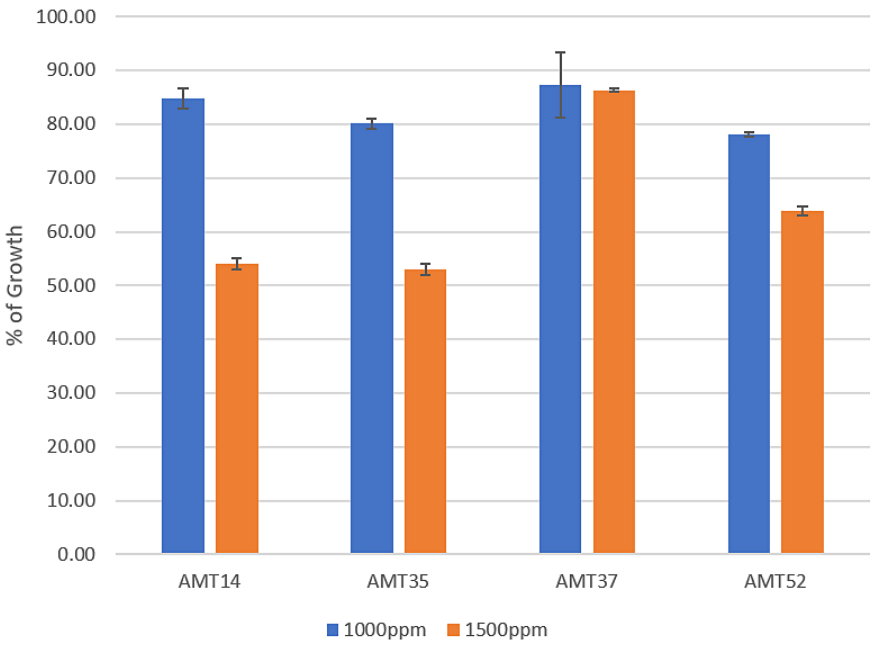


Fig. 3: Graphical presentation of % of growth by all strains at different metal level

Table 4: Highest spore productivity by all bacterial strains

Sr. No.	Name of strain	Dilution	Day of optimization	Control (spores/ml)	Heavy Metal (spores/ml)
1.	AMT14	10^{-5}	6^{th}	28×10^5	21×10^5
2.	AMT35	10^{-5}	5^{th}	46×10^5	8×10^5
3.	AMT37	10^{-5}	6^{th}	15×10^6	8×10^6
4.	AMT52	10^{-5}	5^{th}	39×10^5	23×10^5

Note: AMT= Strain number

Spore Optimization

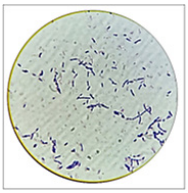
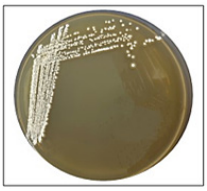
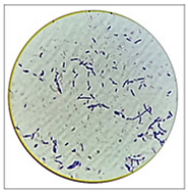
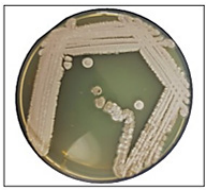
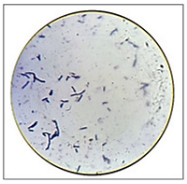
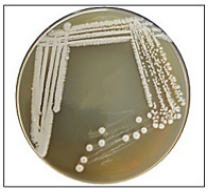
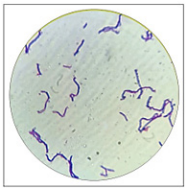
All four actinomycetes isolates were able to grow on SC agar media and able to produce spores. AMT52 having highest spore productivity when compared to control. Thus, it was selected as potent heavy metal tolerant isolates.

Identification of Metal Tolerance Actinomycetes

From soil sample, the isolated actinomycetes were recovered. The morphological traits are still often employed to identify taxa. Microscopic examination of gram-stained slides revealed gram-positive actinomycetes with purple color, rod shape and filamentous structure. Biochemical tests were used to identify the type of bacteria present in isolates.

AMT52 was selected for more detail's studies as it was high spore forming bacterial strain and recognized as *Streptomyces pactum*, a species from the genus *Streptomyces.*

Table 5: Results of Actinomycetes colonies on SCA media and its Gram's staining view under microscope

Strain No.	Isolated Colonies	Gram's stain
AMT14		
AMT35		
AMT37		
AMT52		

Note: AMT= Strain number

Table 6: Colony characteristics of potent actinomycetes

Strain No.	+/-	Shape	Size	Color	Texture	Elevation	Optical Properties
AMT14	+ve	Circular	Small	Chalky White	Rough	Slight Raised	Opaque
AMT35	+ve	Circular	Small	Chalky White	Rough	Slight Raised	Opaque
AMT37	+ve	Circular	Medium	Gray	Smooth	Slight Raised	Opaque
AMT52	+ve	Circular	Small	Gray	Smooth	Slight Raised	Opaque

Note: AMT=Strain number, +ve= Gram positive bacteria and -ve= Gram negative bacteria

Table 7: Results of biochemical tests of potent Actinomycetes

Sr. No.	Tests	AMT14	AMT35	AMT37	AMT52
1.	Catalase	+	+	+	+
2.	Citrate utilization	+	-	+	+
3.	Starch hydrolysis	+	+	+	+
4.	Gelatine hydrolysis	+	-	-	-
5.	Casein hydrolysis	-	-	-	-
6.	Methyl Red	+	-	+	-
7.	Voges Proskauer	+	-	-	+
8.	Nitrate reduction	+	+	+	+

Note: +=Positive and -= Negative

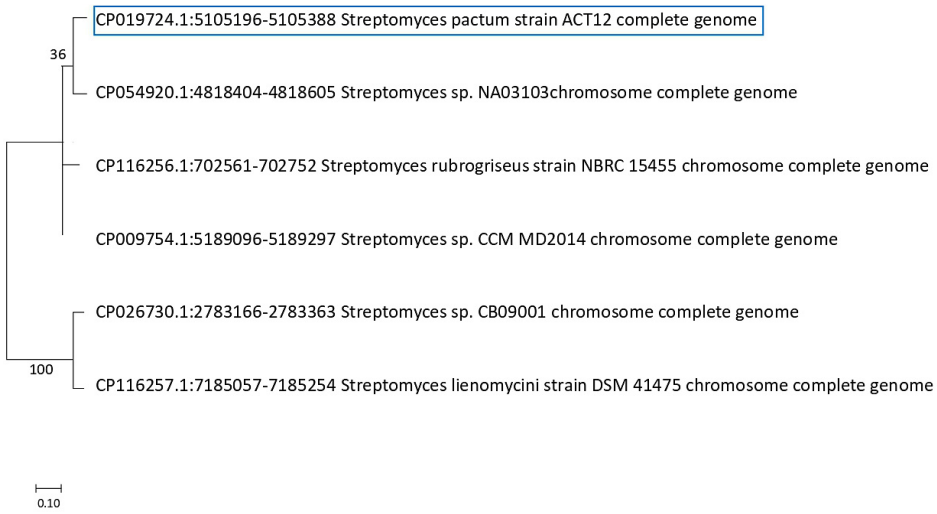


Fig. 4: Molecular Phylogenetic analysis of AMT52

Discussion

The usage of pesticides in agricultural regions has sharply increased in the past several years. Pesticides with composite and triple super phosphate had the highest levels of Cd.[16] Cadmium concentration in the studied region was highest than Zinc and Copper. After the enhancement of a soil sample, fifty-three actinomycetes that were tolerant of heavy metals

were isolated, only four of the isolates expressed this sensitivity. The actinomycetes were selected in the presence of cadmium at concentrations of up to 1000 mg/L. All the isolates were recognized as Streptomyces spp. based on their morphological and biochemical characteristics. Strain AMT52 shown highest spore optimization in a presence of heavy metal cadmium compared to control, which was identified by molecular analysis as *Streptomyces pactum.* Bagot and others isolated actinomycetes from soil contaminated with cadmium and found best growth capacity of actinomycetes in a presence of 10 mg/L cadmium.[17] Hamedi and his colleagues highly identified resistance strains of actinomycetes genera showed resistance to 9.2mM $CdCl_2$ including *Streptomyces, Nonomuraea, Saccharothrix, Streptosporangium* and *Promicromonospora.*[18] Rajivgandhi and others revealed Nocardiopsis dassonvillei as excellent biological source for heavy metal depletion from waste as they reduced heavy metal cadmium contamination up to 85.20%.[19] These studies suggest variability in the potency of bacteria towards heavy metals to which they are resistant. Actinobacterial spore cells produced good metal accumulating results in the current investigation, particularly for cadmium. To improve the bioremediation capacity of these strains, more research should be developed at the molecular level to assess and discover the mechanisms involved in the relationship between metal resistances and accumulation.

Conclusions

Areas contaminated with pesticides are deemed appropriate for isolating microorganisms resistant to heavy metals, serving as valuable reservoirs for potential bioaccumulation processes due to their high contamination levels with metallic elements. The process of heavy metal accumulation can be defined as the gathering of these components within the ecological systems. The restoration of such environments is notably intriguing, particularly through the application of eco-friendly remediation techniques aimed at enhancing their overall quality. We believe that several of the actinobacteria especially *Streptomyces pactum* we have chosen can contribute significantly to the clean-up of contaminated sites. However, additional investigation is required to improve our strains' biological process capabilities and to maximize their ability to eliminate heavy metals from polluted environments.

Authors' Contribution

In the manuscript the first and second author (corresponding author) has contributed in following sectors

- Conception or design of the work- The core idea behind the concept of work and incorporation of the components for the smooth progression of the research work is mentored by Dr.Mousumi Das and performance of the laboratory activity with documentation has completed by Ms.Tasnim Musani
- Data collection- This part of the manuscript was completed by the research worker during her part of work in Ph.D. Idea of compilation and drafting was mentored by Dr. Mousumi Das
- Data analysis and interpretation – This part was contributed by both the authors covering types of representation (selection of Table or graph), selection of images; documentation of the summarized data etc
- Drafting the article–The drafting of the manuscript was performed by Ms.Tasnim Musani
- Critical revision of the article- The analysis and addressing the comments and analysing the details of the manuscript components was guided by Dr. Mousumi Das and implemented by Ms. Tasnim Musani
- Final approval of the version to be published– This part was mutually decided by First and corresponding author with sharing majority of the rights by Corresponding author

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Conflict of Interest

The authors affirm that there are no conflicts of interest pertaining to the research presented in this paper, and they have no financial or personal relationships that could influence the work.

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