WJPMR, 2026, 12(8), 304-311

Research Article

WORLD JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.wjpmr.com

Impact Factor: 7.533

ISSN (O): 2455-3301

ISSN (P): 3051-2557

Coden USA: WJPMBB

COMPARATIVE SCREENING OF HEAVY METAL-TOLERANT MICROORGANISMS FOR SUSTAINABLE ENVIRONMENTAL BIOREMEDIATION

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DOI: https://doi.org/10.5281/zenodo.21699032

How to cite this Article: Kartiki Kale, Piyusha Anupkumar Das, Dr. Sandhya Mulchandani* (2026). Comparative Screening Of Heavy Metal-Tolerant Microorganisms For Sustainable Environmental Bioremediation. World Journal of Pharmaceutical and Medical Research, 12(8), 304-311.

Article Received on 05/07/2026 **Article Revised on 25/07/2026** **Article Published on 01/08/2026**

ABSTRACT

Heavy metal contamination is a major environmental concern due to its toxic effects on living organisms and long-term persistence in the environment. The present study evaluated the tolerance and antimicrobial activity of four heavy metals-cadmium chloride, potassium dichromate, copper sulphate, and lead nitrate—against different microbial isolates using agar ditch method, agar cup method, microdilution assay, and Diphenyl Carbazide assay. The antimicrobial activity was determined by measuring the zone of inhibition at different concentrations, while MIC and MBC values were assessed using microtitre plate techniques. The results showed that all heavy metals exhibited concentration-dependent antimicrobial activity. Among the tested compounds, cadmium chloride demonstrated the highest and most consistent antimicrobial effect, followed by potassium dichromate and copper sulphate, whereas lead nitrate showed the least activity. Certain isolates, particularly S-2 and S-8, exhibited high tolerance toward multiple heavy metals. In the chromium reduction study, isolate S-2 (*Bacillus subtilis*) showed nearly 75% reduction of Cr (VI) within 24 hours, indicating strong bioremediation potential. Overall, the findings suggest that heavy metal-tolerant microorganisms can serve as promising candidates for sustainable and eco-friendly bioremediation applications.

KEYWORDS: Heavy metal, antimicrobial, MIC, microtiter, Diphenyl Carbazide.

INTRODUCTION

Environmental pollution caused by heavy metals has become one of the most serious global issues because of rapid industrialization, urbanization, mining activities, electroplating industries, textile manufacturing, agricultural runoff and improper disposal of industrial waste. The continuous discharge of heavy metals such as chromium, cadmium, copper, and lead into soil and aquatic systems leads to the long-term environmental pollution. Heavy metals are non-biodegradable unlike organic pollutants and persist in the environment for a long time and bioaccumulate in living organisms through the food chain. Their accumulation has a negative impact not only on the ecological balance, but also poses serious threats to human health and biodiversity.

At low concentrations the toxic effects of heavy metals on biological systems are well known. They can interact with proteins, nucleic acids and enzymes, interfering with cell metabolism and disrupting crucial biochemical pathways. Heavy metals exposure can lead to oxidative stress, membrane damage, inhibition of enzyme activity and production of reactive oxygen species (ROS). Chromium is one of the common heavy metals and exists mainly in two stable oxidation states, trivalent chromium [Cr (III)] and hexavalent chromium [Cr (VI)]. Cr (VI) is highly toxic, mutagenic and carcinogenic. Another toxic metal is cadmium, which can induce severe toxicity by impairing microbial respiration and enzymatic activities. Lead and copper are also toxic to microorganisms and other living systems at high concentrations.

Microorganisms that live in places with a lot of metals in the environment often find ways to deal with it and survive. They do this by using systems that help get rid of the bad metals or by storing them inside where they will not cause harm. Some microorganisms can even change the metals into forms that are not as bad for them.

This helps them live in places that would be toxic to living things. These microorganisms that can handle metals are very important for cleaning up the environment. They do this in a way that's good for the earth and does not cost a lot of money. This approach is called bioremediation. It uses microorganisms to get rid of or clean up things in the environment. Some kinds of bacteria like Bacillus, Pseudomonas, Acinetobacter and Enterobacter are really good at dealing with metals and helping to clean up the environment. Microorganisms are very important, for bioremediation because they can help remove metals from the environment. Bioremediation is a way to clean up the environment because it uses microorganisms to do the job.

In addition to their impact on the environment heavy metals also have properties. Heavy metals are toxic to cells mainly because they cause proteins to unwind disrupt cell membranes stop enzyme systems from working and cause oxidative stress. These properties have been studied a lot for their potential to fight microorganisms. However, some microorganisms are more or less susceptible to metals depending on the type of metal its concentration and the microorganism's resistance mechanisms. So, it is important to study how microorganisms interact with metals to understand microbial resistance and to find potential microbial candidates, for cleaning up heavy metal pollution.

Several methods help us figure out how well microbes can tolerate metals and how well those metals can kill microbes.

The agar ditch method is good for checking if microbes can tolerate toxic metals. The agar cup or agar well diffusion method is often used to see how well a substance can kill microbes by measuring the area where microbes can't grow.

The broth microdilution assay and microtitre plate techniques are considered ways to find out the lowest amount of a substance that can stop microbes from growing which is called the minimum inhibitory concentration or MIC for short and the lowest amount that can actually kill microbes, which is called the minimum bactericidal concentration or MBC. The Diphenyl Carbazide assay is a colour-based method that measures how much chromium is reduced, especially when toxic Cr (VI) is changed into less toxic Cr (III). We use these methods to understand tolerance and antimicrobial activity of heavy metals. Microbial tolerance and antimicrobial activity of metals are important to study. Heavy metals show activity. The antimicrobial activity of metals is determined by several methods. These methods help in understanding the tolerance. The microbial tolerance, to metals is tested using agar ditch method. The Diphenyl Carbazide assay is used for chromium reduction.

METHODOLOGY

1. Agar ditch method (Bowers and Jeffries, 1995)

This method was used as initial screening of the isolates for tolerance levels of toxicants. The 24hr old culture of the isolates were adjusted to 10^8 cfu/ml using Mc Farland standards or Brown's opacity tubes. A St. Nutrient Agar plate was used and 8cm X 1.5cm ditch was aseptically cut out. The weight quantity of the toxicant was dissolved in alcohol/water and added to 10ml sterile molten Nutrient Agar so as to obtain the final concentration. This concoction was poured in the ditch cautiously to avoid overflow of excess media. Once the agar had solidified the isolates were streaked in such a way that they were perpendicular to the ditch and parallel to each other. The plate was incubated at 30°C for 24hrs. The lesser the growth of towards the ditch is highly tolerant to the toxicants.

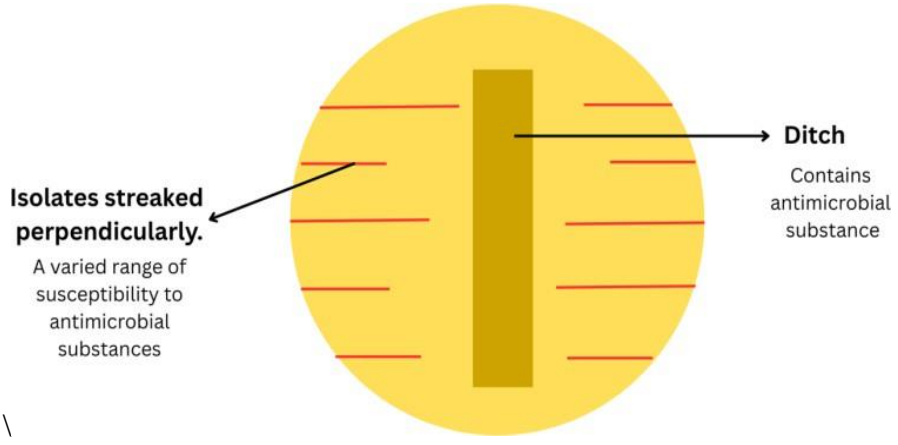

Fig 1: Diagrammatic represent of Agar Ditch Method.

2. Agar cup method (Rose and Miller 1939)

Agar cup method is similar to Kirby-Bauer Disk diffusion assay uses antibiotic disks to study the susceptibility of test organisms by measuring the zone of inhibitions to various array of antibiotics. In agar cup method 15ml of sterile molten Agar is seeded with 1ml of 24hr old culture suspension (10^8 cfu/ml) and poured into a sterile Petri Plate. Once the plate is solidified a sterile 8mm diameter Cork Borer is used to aseptically generate a well that is filled with various concentrations of the

toxicants. The plates are kept upright in the refrigerator for 10mins to facilitate the diffusion followed by incubation at 30°C for 24hrs. The zone of inhibition was measured to determine the toxicant concentration that inhibits growth.

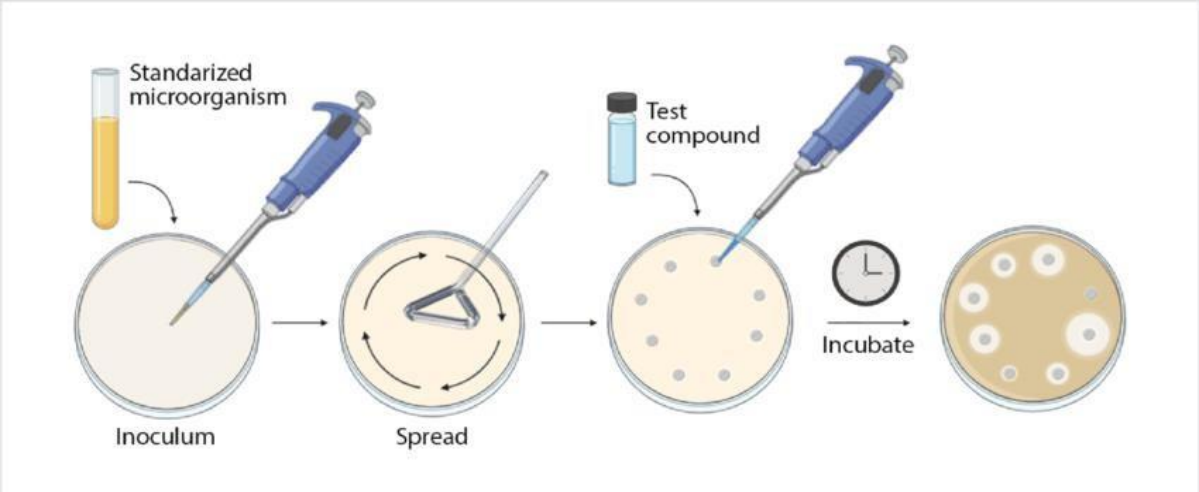

Fig 2: Diagrammatic representation protocol for Agar cup method.

3. Micro Dilution Assay (Lalitha,2005) **and MIC/MBC determination using microtiter plate technique** (Amsterdam,1996)

This is a standardised and high throughput technique used in laboratories to determine the MIC of antimicrobial substances, that probably could be overlooked due to lack of visible growth. It uses a 96 well plate loaded with sample constituents as follows, test organism concentration of 10^6 to 10^5 cfu/ml, various dilution of toxicant and double strength Muller-Hinton Broth. The plate is then incubated at 30°C for 24hrs and using 0.1% of 2,3,5-Triphenyl Tetrazolium Chloride (TTC), a redox indicator to detect viable and non-viable cells colorimetrically by the means of Microtiter Plate Reader. The actual growth values are obtained by subtracting the initial OD readings from the final reading. The same Microtiter plates from the study were used further to determine the MBC values for toxicant.

4. Diphenyl Carbazide Assay

The chromium conversion led bioaccumulation involves conversion mechanism of Cr (VI), an unstable, oxidative and carcinogenic form to Cr (III), a stable form. This reduction can be done by using colorimetric method known as Diphenyl Carbazide Assay.

Selected isolate was cultured overnight in M9 medium containing 10 ppm Chromium, at 30^0C on shaker (100 rpm). The dense culture was centrifuged at 6000 rpm for 10 min at 10°C. The supernatant was discarded and pellet was dissolved in small amount of phosphate buffer to use as inoculum. Later, the culture flasks with 200ml M9 medium including 100 ppm of chromium were inoculated with 10 ppm Chromium tolerant culture prepared in phosphate buffer. The initial culture strength of flask was adjusted. Culture along and an uninoculated control was incubated in a shaker at 100rpm for 48 hrs at 30°C. Growth was monitored at specific time intervals by measuring optical density of the cultures at 540nm. To study the reduction of Cr reduction by isolate, a 2 ml culture sample from the above flask was removed every 4 hours, centrifuged (6000 rpm for 10 min at 10°C). Diphenyl carbazide solution (20µl) was added to 1 ml of the supernatant and allowed to stand for 10 minutes for colour development. The sample was transferred to a quartz cuvette and the absorbance was measured at 540 nm in a UV Spectrophotometer using Distilled Water as a reference. The amount of Cr (VI) in the solution was calculated from the standard graph prepared using a range of 10 to 100µg/ml standard for Cr (VI).

RESULT AND DISCUSSION

1. Agar Ditch Method

According to results obtained all isolates showed growth for all concentrations of chromium. Only S-1 showed no growth on 1.5 mg/ml concentration. All organisms able to tolerate as well as they can degraded the chromium.

For lead nitrate also growth was demonstrated by all isolates for all concentrations of lead. They tolerate to be led and can be degraded lead. Copper S-2, S-5, S-6 and S-8 tolerated all concentrations of copper. It showed growth of these 4 organisms. All the other organisms exhibited the same pattern of sensitivity to copper.

For cadmium S-2, S-5, S-6 and S-8 also showed tolerance to all concentration of cadmium. It showed growth of these 4 organisms. All other organisms showed sensitivity pattern to cadmium. However, S-5 and S-6 presents low growth pattern compared to S-2 and S-8. S-5 and S-6, showed slightly sensitive at 1.25 mg/ml and 1.5 mg/ml. It results that S-2 and S-8 showed high tolerate to all selected 4 heavy metals.

2. Agar Cup Method

The antimicrobial activity of four heavy metal salts,

cadmium chloride, potassium dichromate, copper sulphate, and lead nitrate, was studied against ten microbial samples (S-1 to S-10) using the agar well diffusion method. We evaluated the effectiveness of each compound by measuring the zone of inhibition at different concentrations.

Among all the metals tested, cadmium chloride showed the strongest antimicrobial effect. As its concentration increased from 0.5 mg/ml to 1.5 mg/ml, the zone of inhibition steadily increased for most samples. Sample S-6 had the highest inhibition (about 4.1 cm), followed by S-10 and S-5. Most samples responded well to cadmium chloride, although a few (S-2, S-8, and S-9) showed lower sensitivity.

Potassium dichromate also showed good antimicrobial activity, but the response varied by sample. Its effect increased with concentration; however, some microbes responded strongly while others did not. Sample S-8 had the highest inhibition (around 3.9 cm), followed by S-9 and S-7. Notably, S-4 showed no inhibition at all, indicating complete resistance.

In comparison, copper sulphate showed moderate antimicrobial activity. The inhibition generally increased with concentration, but the overall effect was less than that of cadmium chloride and potassium dichromate. Sample S-10 had the highest inhibition (about 3.7 cm), followed by S-6 and S-7. Some samples showed only a moderate response, while S-2 and S-8 showed no inhibition, suggesting resistance.

Lead nitrate showed the weakest antimicrobial activity among all the metals tested. There was little to no inhibition at lower concentrations, and only at higher concentrations did some inhibition appear. Samples S-4 and S-5 had the highest response, while S-1, S-2, and S-3 remained mostly unaffected.

Overall, all four metals exhibited better antimicrobial activity as their concentration increased. However, their effectiveness clearly differed. Cadmium chloride was the most effective and consistent, followed by potassium dichromate and copper sulphate, while lead nitrate was the least effective. The differences in how each sample responded suggest that different microbes have varying levels of resistance to these heavy metals.

Graphical Representation of MIC results for heavy metals

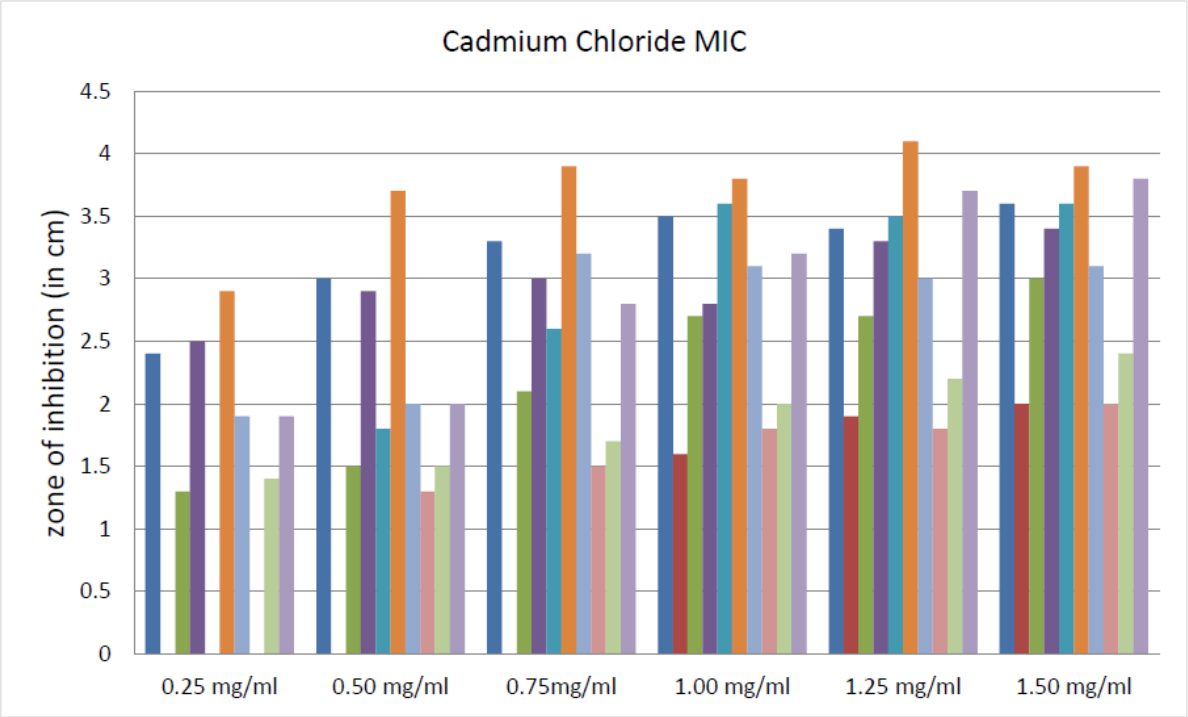

Fig 3: Graphical Representation of MIC results for Cadmium Chloride.

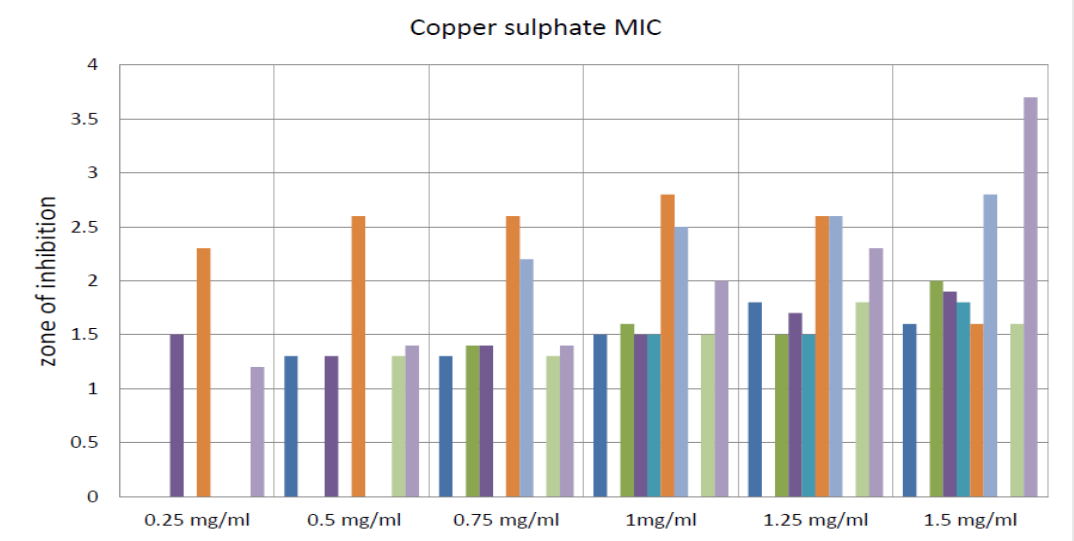

Fig 4: Graphical Representation of MIC results for Copper sulphate.

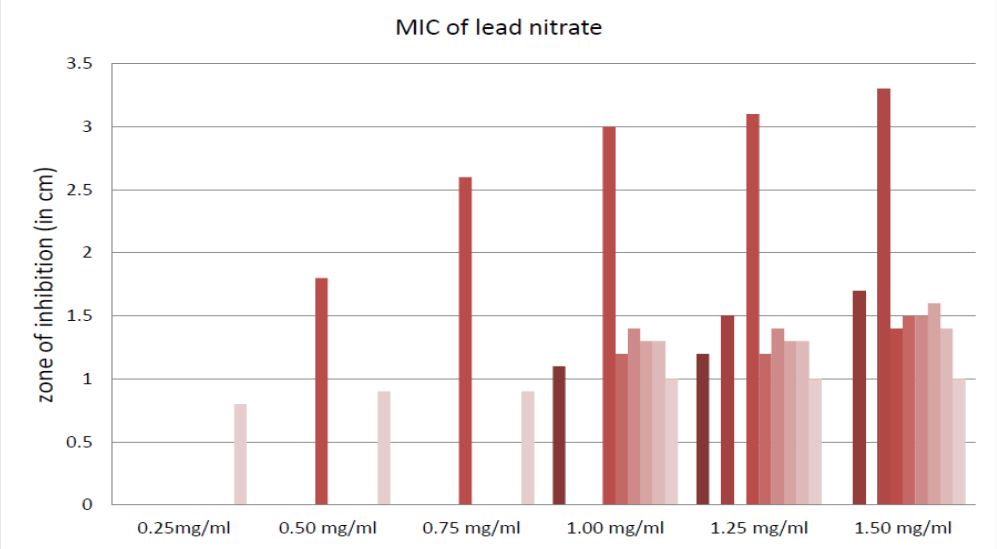

Fig 5: Graphical Representation of MIC results for lead nitrate.

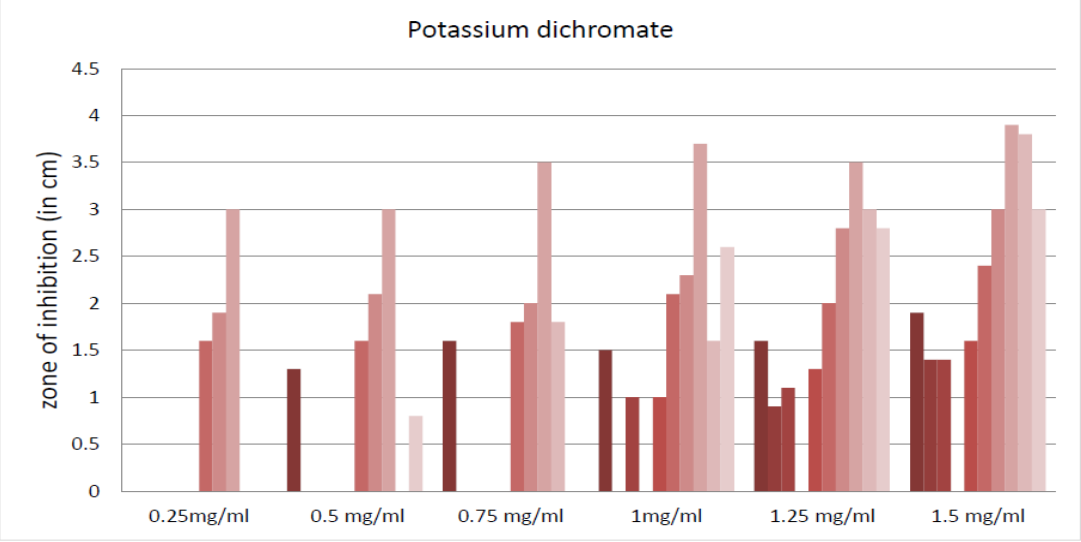

Fig 6: Graphical Representation of MIC results for Potassium dichromate.

3. Micro Dilution Assay

The minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium. The MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by ≥99.9%. The MBC is complementary to the MIC; whereas the MIC test demonstrates the lowest level of antimicrobial agent that inhibits growth, the MBC demonstrates the lowest level of antimicrobial agent that results in microbial death. Antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC. Because the MBC test uses colony-forming units as a proxy measure of bacterial viability, it can be confounded by antibacterial agents which cause aggregation of bacterial cells. The purpose of this study was to evaluate the effects of different heavy metal preparing concentration coated onto 96-well plates to design a method for susceptibility testing. This study was to develop a rapid, simple, and cost-effective broth microdilution method for susceptibility testing of bacteria.

Here in present study for MIC & MBC testing used S-2 isolate against4 heavy metal with different concentration. Reading was taken by microtitre reader at 0 hrs, 24 hrs and after adding 0.1% TTC solution at 540 nm.

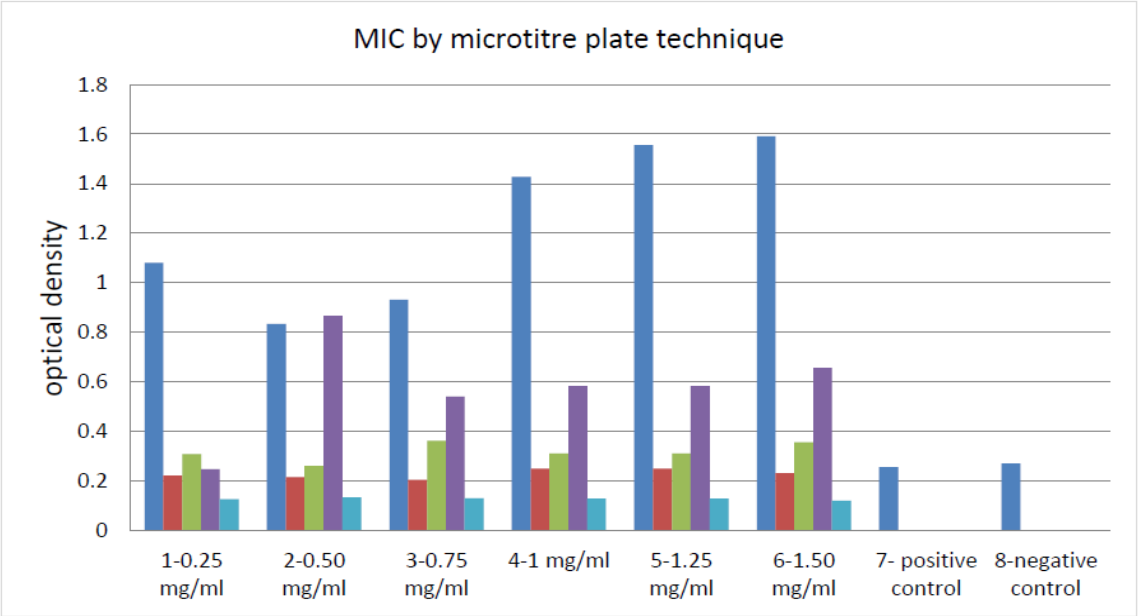

Fig7: Initial readings (at 0 hours).

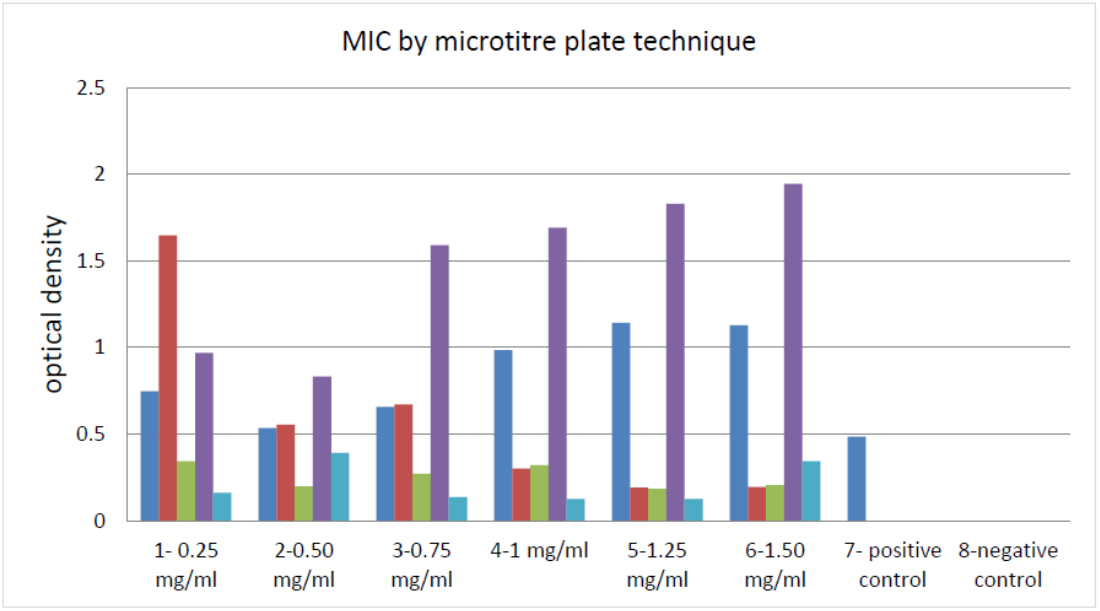

Fig 8: Readings after 24 hours incubation.

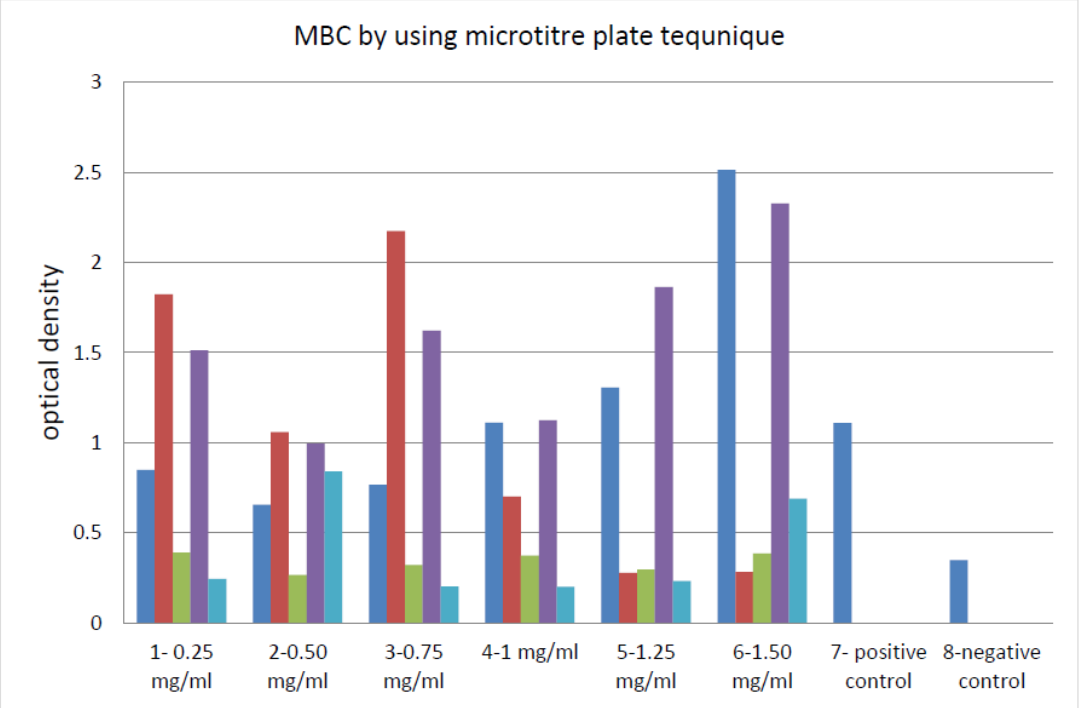

Fig 9: MBC after addition of 0.1% TTC.

4. Diphenyl Cabazide Assay

Chromium primarily exists as Cr (III) and Cr (VI), with the latter being more toxic. A popular remediation technique is the conversion of Cr (VI) to Cr (III) followed by the quantitative determination of Cr(III) with the Diphenyl cabazide assay. showed 80 % reduction of Cr(VI) in bioreactors with an initial load of 50 mg/L over 8 h. Further studies identified Acinetobacter sp. as effective in Cr(VI) removal under aerobic conditions. The present study was conducted on S-2 (*Bacillus subtilis*) which removed nearly 75% Cr in 24 h and the maximum reduction in Cr was observed in first 8 h (50%). The reduction rate showed two phases, a fast initial phase and a slower degradation phase, which could be due to saturation of metal binding sites. Different microbes have shown different efficiencies in Cr (VI) reduction, and Bacillus sp. has been able to tolerate and effectively reduce Cr (VI) in different studies.

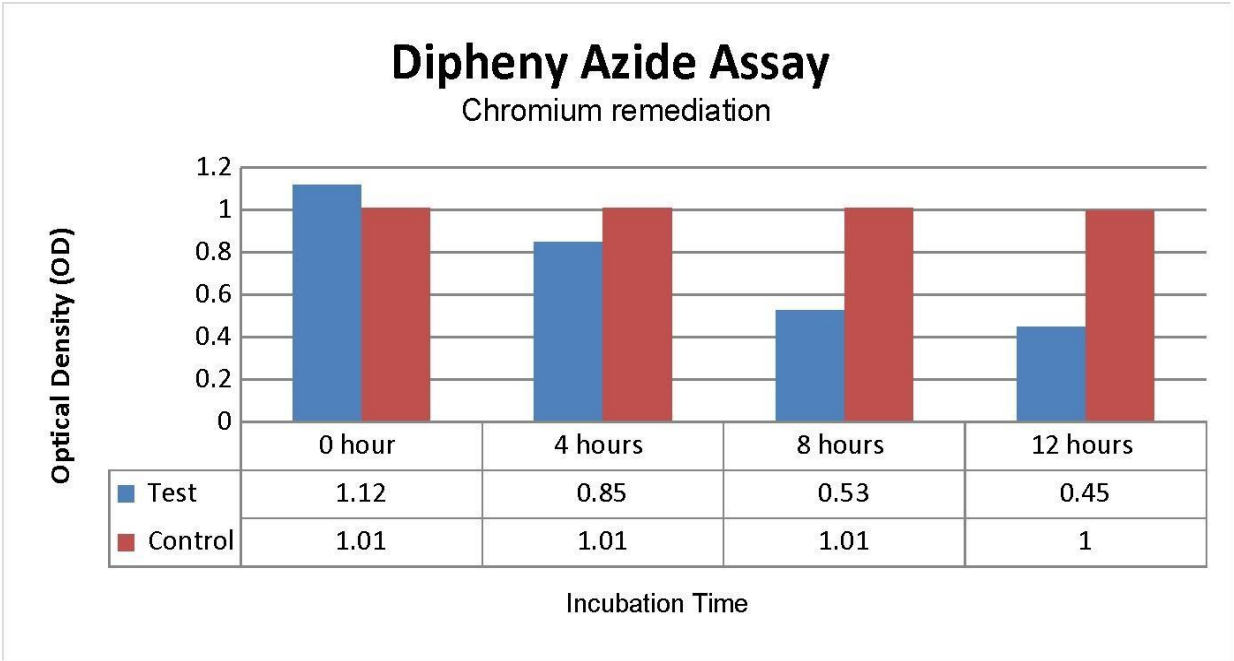

Fig 10: Chromium remediation using Diphenyl Azide Assay Conclusion.

The present study was conducted to evaluate the antimicrobial activity and tolerance of four heavy metals viz. cadmium chloride, potassium dichromate, copper sulphate and lead nitrate against different microbial isolates by agar ditch, agar cup, microdilution and Diphenyl Carbazide assays. All four metals demonstrated concentration-dependent antimicrobial activity with increased inhibition at increased concentrations. The strongest and most consistent antimicrobial effect was observed with cadmium chloride followed by potassium dichromate and copper sulphate while the weakest activity was observed with lead nitrate. The variation in zone of inhibition among the isolates showed the variation in sensitivity and resistance mechanism of microbes against heavy metal stress.

The study also showed that a few of the isolates S-2 and S-8 in particular were highly tolerant to different heavy metals indicating their ability to survive under toxic conditions. In Diphenyl Carbazide assay, isolate S-2 (Bacillus subtilis) showed significant chromium reduction efficiency with about 75% reduction of Cr (VI) in 24 h. The results demonstrate the dual role of microorganisms in heavy metal tolerance and bioremediation. The overall results indicate that some of the microbial isolates can be used in eco-friendly bioremediation of heavy metal contaminated areas.

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