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Heavy Metal Tolerance by Bacteria Isolated from Rajpardi Lignite Coal Mine, Gujarat

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Abstract

Heavy metal pollution is considered one of the most common and harmful pollution known to cause severity in the case of living beings residing. The present study was conducted to determine the heavy metal tolerance of bacteria isolated from the Rajpardi lignite mine, Gujarat, India, and characterize them. Eight bacteria were isolated and cultured in Nutrient Agar Medium. Morphological and biochemical characterization tests revealed the presence of 3 *Enterobacter* species, 2 *Klebsiella* species, 2 *Bacillus* species and 1 *Streptobacillus* species. Following this, the heavy metal tolerance assay was performed against five different metal salts – Hg, Ni, Al, Pb and Cu by dissolving the metal salts in various broth culture tubes and determining the growth turbidity in UV-Vis Spectrophotometer at an absorbance of 600 nm. All the isolates displayed the highest tolerance against the heavy metal, aluminium, with maximum tolerance shown by bacterial isolates marked LCB3 and LCB9. Thus, the Rajpardi lignite mine can be considered a new source with potential candidates for bioremediation of an environment contaminated with aluminium.

Keywords: Bacteria • Heavy Metals • Lignite • Tolerance

Introduction

The soil in different parts of the world is a habitat to a diverse group of microbial communities, adapted to varying temperatures, pressure, moisture content, and heat. Various bacterial species report growing in extreme conditions, ranging from ice-cold temperatures like the Himalayas (psychrophiles) to soil in extreme heat conditions like volcanoes (Thiolava). Due to their adaptation to such severe conditions, these bacteria are categorized as extremophiles. These places are prone to various changes which may be natural or may have interference by human beings. Heavy metal pollution is a common problem seen in almost every industrial site and is associated with causing damage to the flora and the fauna of the surrounding area. Heavy metals pollute the environment and harm the biological functioning of an organism's growth. These pollutants may cause neurotoxicity, DNA damage, carcinogenesis, cell damage, protein misfolding, and conformational changes [1]. Heavy metals - Mercury and Lead pose a high ecological risk to the environment, followed by metals like copper, nickel, and zinc [2]. The toxicity of the heavy metals contaminates the microbiota and is associated with causing problems in plant development. They are associated with reduced seed germination and inhibit root development in plants [3]. In any living being, accidental consumption of heavy metals increases their concentration in the body by bioaccumulation. It further increases the heavy metal concentration at a subsequent trophic level. This increase is known as biomagnification. Different approaches to phytoremediation techniques against toxic heavy metals have been put forward, including removal of the heavy metals using various plant processes like phytovolatilization, phytodegradation, and phytoextraction. It causes metal immobilization and metal detoxification [4]. However, these bacteria have well equipped themselves with the surrounding environment in a way that they can replenish the soil for them to sustain life by secreting various enzymes and compounds [5]. The physiochemical and enzymatic nature of these organisms that help them thrive in such adverse conditions is of utmost importance in research, promoting the use of natural techniques for treating polluted sites and biodegradation. Lignite, referred to as brown coal, is usually a product in the second stage of the coal formation process, formed by compression of the peat. Extreme pressure and temperature of the soil report to harbour very few bacterial species. Lignite is the lowest quality coal and is considered to undergo processing by the microbiota already present. Mining activities are associated with causing a high amount of heavy metal pollution that causes prominent distress to the local microbiota present in the soil and causes an ecological imbalance. With time, the bacteria adapt to the environment and develop natural remediation strategies to replenish the soil.

The state of Gujarat in India is a rich source of high-grade lignite that satisfies the fuel needs of the state industries. Different projects under the Gujarat Mineral Development Corporation Ltd. (GMDC) are ongoing in various mines located in the state, namely, Tadkeshwar, Rajpardi, Panandhro, Bhavnagar, Mata No Math, and Umarsar [6]. Rajpardi lignite mine located in the Bharuch district of Gujarat is an open cast mine producing nearly ten thousand tonnes of lignite per year to satisfy and meet the demand for fuel. But due to extensive mining levels down the years in these sites, the associated pollution causing ecological imbalance is of great focus. This mine has never undergone study for the presence of microorganisms. Thus, understanding the microbiota of the mine site could provide an effective strategy for promoting remediation. The presence of heavy metals like copper, nickel and a few others in the groundwater is a threat to the local people residing there. It causes severe diseases. This lignite mine could fulfil the need for a novel source with bacteria highly tolerant to heavy metals and process the way for new bioremediation strategies. So, the current study aims to isolate the bacteria resistant to heavy metals from the lignite coal mine of Rajpardi, located in the Bharuch district of Gujarat. Bacteria, showing tolerance to respective heavy metals, are grown in media containing metals like Hg, Al, Ni, Pb or Cu and are cultured in Nutrient Agar Medium. Characterization of the bacteria is performed using morphological techniques, including gram staining, endospore staining, and motility test. It follows biochemical characterization tests - triple sugar test, catalase test, indole test, MR-VP test, Citrate test, and Starch hydrolysis test (Figure 1).

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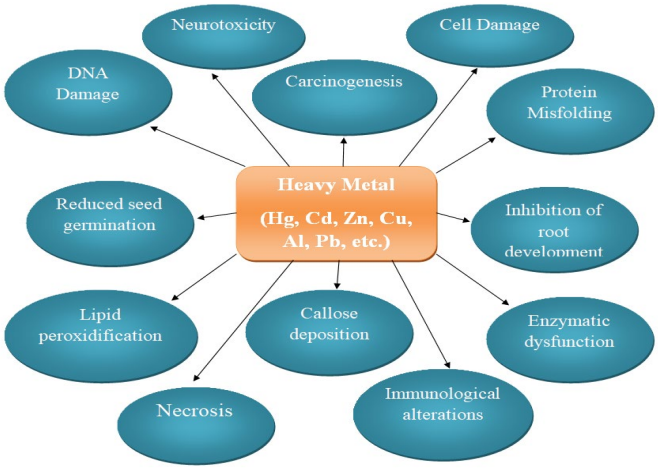

Figure 1. Effect of heavy-metals in living tissue.

Materials and Methods

Surveying and sampling

Rajpardi Lignite mine, Bharuch District, Gujarat: This open cast mine with a lease area of 3849618 square meters is located at the Amod village of Bharuch district in the state of Gujarat. The Road link associated with the mine is the Bharuch-Rajpipla State Highway and the National Highway. Sampling was done by scraping off the topsoil and collecting a lignite rock of nearly 500 grams overseeing Gujarat Mineral Development Corporation Limited (GMDC) [7]. The rock sample is sealed tightly in a polythene bag to get analysed in the lab. A small part of the rock sample is broken into powdered form, and serial dilution is performed.

Microbial analysis

Serial dilution: 0.5 gm of the powdered lignite was weighed and added to 5 mL of distilled water taken in a test tube and shaken to form a suspension. 0.5 mL of this sample was taken and added to another test tube containing 4.5 mL of distilled water, labelled as 10^{-1}. Similarly, sample was diluted to 10^{-2} by transferring 0.5 mL from 10^{-1} to 4.5 mL of distilled water. Similar dilutions were prepared to 10^{-3} [8].

Culturing: Bacterial growth culture was performed by preparing Nutrient agar media plates. With the help of the spread plate method, the coal sample was inoculated. 100 µL from each diluent was spread over the surface of the media.

Subculturing: Bacterial colonies of distinct morphology were picked based on the size and colour of each colony and streaked on the surface of freshly prepared three nutrient agar plates of 20 mL each. These plates were then incubated at 37°C until growth was observed. Following this, pure strains were collected using a pipette tip and inoculated in nutrient broth tubes of 5 mL each. The inoculated broth tubes were incubated at 37°C until growth was observed.

Characterization Tests: The morphological testing of the bacterial isolates was carried out by gram staining, endospore staining, and motility test. The biochemical characterization of the isolates was conducted using the following tests - the catalase, IMViC, TSI and starch hydrolysis tests.

Heavy metal tolerance assay: The heavy metal tolerance assay was conducted as per Singh and Narzary. $HgCl_2$ for Hg^{2+}, $Al_2(SO_4)_3$ for Al^{3+}, $NiCl_2$ for Ni^{2+}, $PbCO_3$ for Pb^{2+}, and $CuSO_4$ for Cu^{2+} were produced as 1 M stock solutions by dissolving the salts in 10 mL distilled water. 1 mM concentration of salt solution taken from stock was added to individual broth culture tubes and incubated at 37°C for 24 hours. The growth turbidity of the culture tubes was determined using UV-Vis spectrophotometer at 600 nm absorbance. Growth was followed by addition of extra 1 mM of the salt solution from the stock. The tolerance assay tested for all the isolates maximum upto a salt concentration of 7 mM. The cultures that showed retardness in growth after a specific salt concentration were discontinued for the particular salt tolerance respective [9-11].

Results

Culturing and subculturing

Lignite coal samples were collected from the Rajpardi Lignite mine, under the supervision of GMDC, and then followed by spreading the soil samples of different diluents on respective nutrient agar plates of 20 mL each. The culture plates were observed for growth after 24 hours of incubation. The Petri plate with samples 10^{-3} did not show any bacterial growth, while the plate with 10^{-1} reported maximum colonies as 3.65×10^{-1} CFU/mL and considered the mother plate. Ten colonies of distinct morphology labelled LCB1 to LCB10 located at different locations on the plate were picked and streaked on the surface of 3 nutrient agar plates for subculturing.

The sub-cultured plates were observed for growth after further 24 hours of incubation. Out of the ten colonies, two colonies did not show any sign of bacterial growth even after 48 hours of incubation. Hence, the remaining eight colonies were considered and transferred to nutrient broth tubes for further studies [12-15].

Characterization tests

The bacterial characterization tests were performed based on studying the morphological and biochemical features of the isolates. Observation under the microscope at 40X and 100X magnification revealed the presence of *Bacilli*. Based on the observations noted from the characterization tests and comparing the results with available data, LCB1, LCB3 and LCB7 were characterized and identified to belong to be the members of the *Enterobacter sp.*, LCB4 and LCB5 to be members of the *Bacillus sp.*, LCB6 and LCB9 to be members of the *Klebsiella sp.* and LCB10 as a *Streptobacillus sp* (Tables 1 and 2).

Heavy metal tolerance assay

The heavy metal tolerance assay was performed by testing the tolerance levels of all the isolates for different heavy metals - Hg^{2+}, Al^{3+}, Ni^{2+}, Pb^{2+} and Cu^{2+} at different concentrations starting from 1 mM taken from the prepared stock solutions. The growth turbidity analyses the tolerance levels of the isolates against the metal salts, determined by measuring the OD values using a UV-Vis spectrophotometer at 600 nm absorbance [16-19].

Table 1. Morphological characterization tests.

S. No.	Bacterial Code	Gram Staining	Endospore Staining	Motility Test
1	LCB1	-	-	-
2	LCB3	-	-	-
3	LCB4	+	-	-
4	LCB5	+	-	-
5	LCB6	-	-	-
6	LCB7	-	-	-
7	LCB9	-	-	-
8	LCB10	+	-	-

Table 2. Biochemical characterization tests.

S. No.	Bacterial Code	Catalase Test	Indole Test	MR	VP	Citrate Utilization Test	TSI agar Test	Starch Hydrolysis
1	LCB1	+	-	-	+	+	A/A	+
2	LCB3	+	-	-	+	+	A/A	+
3	LCB4	+	-	-	+	+	A/A	+
4	LCB5	+	-	-	+	+	A/A	+
5	LCB6	+	-	-	+	+	A/A	-
6	LCB7	+	-	-	+	+	A/A	+
7	LCB9	+	-	-	+	+	A/A	+
8	LCB10	-	-	-	+	+	A/A	-

Samples with copper showed growth for the initial concentration. However, 2 mM salt concentration of Cu^{+2} retarded the growth of bacterial isolates, and maximum turbidity at 3 mM concentration for copper was shown by isolates LCB3 and LCB4, after which addition of an extra 1 mM dose of copper reduced the growth of the two isolates. In the case of lead, optical density values for the bacteria LCB4, LCB5, LCB7 and LCB10 decreased at 2 mM salt concentration. The rest of the samples showed growth to a 3 mM salt concentration. A reduction in bacterial growth is observed at a 4 mM salt concentration. The maximum bacterial growth was observed for Aluminium by all the bacterial isolates at 5 mM concentration. The rest of the bacterial isolates showed a reduction in turbidity for the rest of the heavy metals. For Nickel, the isolate LCB4 showed growth only for a 1 mM concentration of salt. Bacterial growth retarded after adding another 1 mM of the metal salt. The rest of the isolates were tolerant to a maximum of 3-4 mM concentration of the metal. High values of optical densities were observed for all the isolates showing their tolerance against the heavy metal, Aluminium, at 7 mM concentration. The culture tubes with the rest of the heavy metals did not exhibit much metal tolerance and were discarded [20-22] (Tables 1-3) (Figures 1-10).

Table 3. Probable genera.

S. No.	Bacterial Code	Probable Genera
1	LCB1	*Enterobacter sp.*
2	LCB3	*Enterobacter sp.*
3	LCB4	*Bacillus sp.*
4	LCB5	*Bacillus sp.*
5	LCB6	*Klebsiella sp.*
6	LCB7	*Enterobacter sp.*
7	LCB9	*Klebsiella sp.*
8	LCB10	*Streptobacillus sp.*

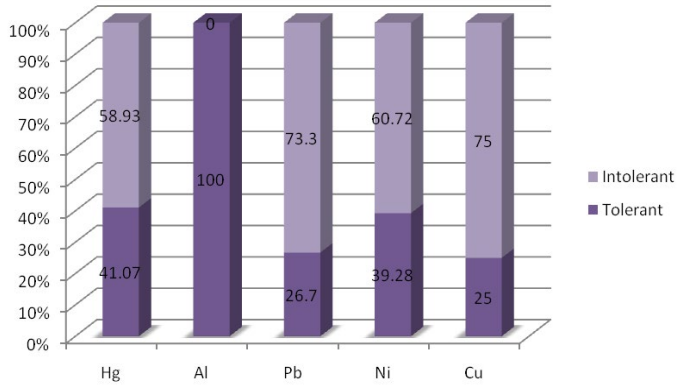

Figure 2. Graph demonstrating the growth tolerance by bacterial isolates against different heavy metals – heavy metal tolerance by bacterial isolates (%) vs. metal intolerant.

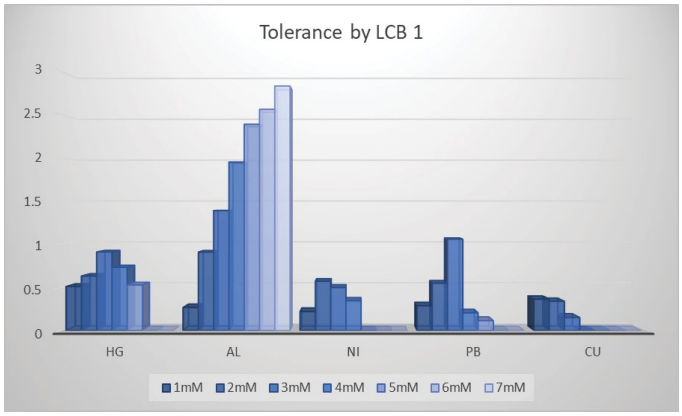

Figure 3. Growth of LCB 1 based on tolerance by different heavy metals.

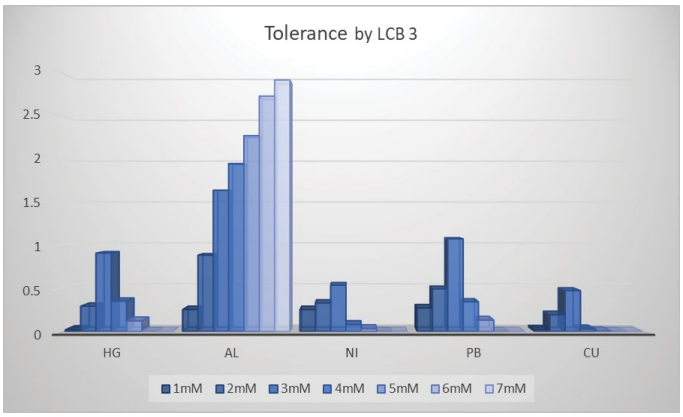

Figure 4. Growth of LCB 3 based on tolerance by different heavy metals.

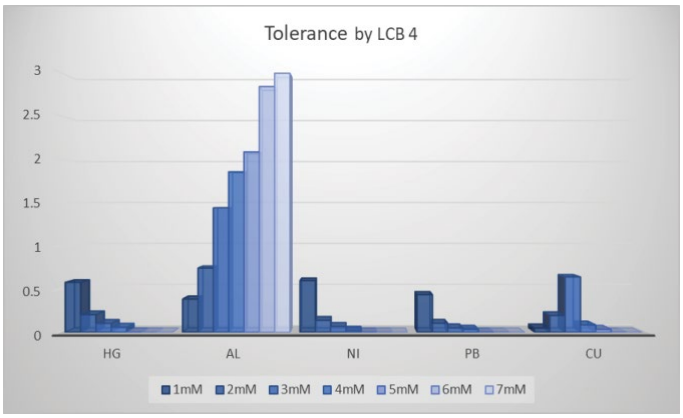

Figure 5. Growth of LCB 4 based on tolerance by different heavy metals.

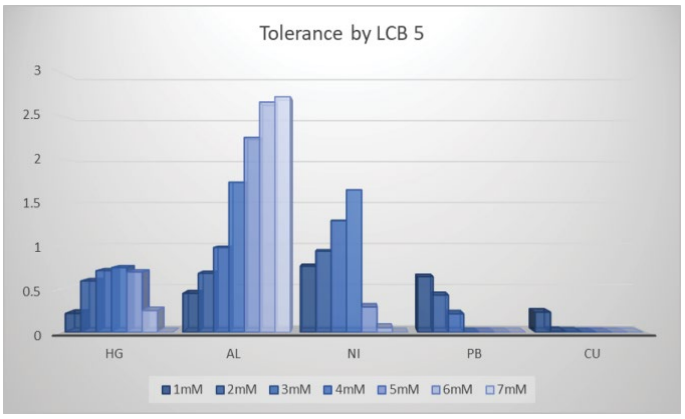

Figure 6. Growth of LCB 5 based on tolerance by different heavy metals.

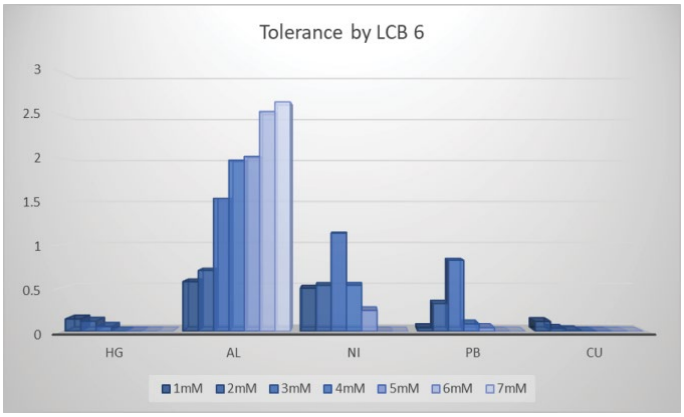

Figure 7. Growth of LCB 6 based on tolerance by different heavy metals.

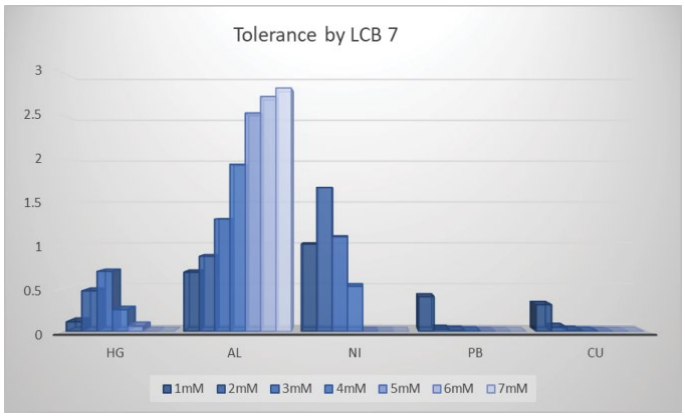

Figure 8. Growth of LCB 7 based on tolerance by different heavy metals.

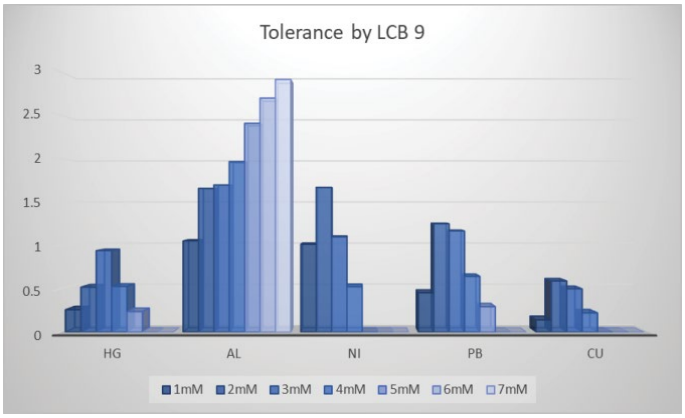

Figure 9. Growth of LCB 9 based on tolerance by different heavy metals.

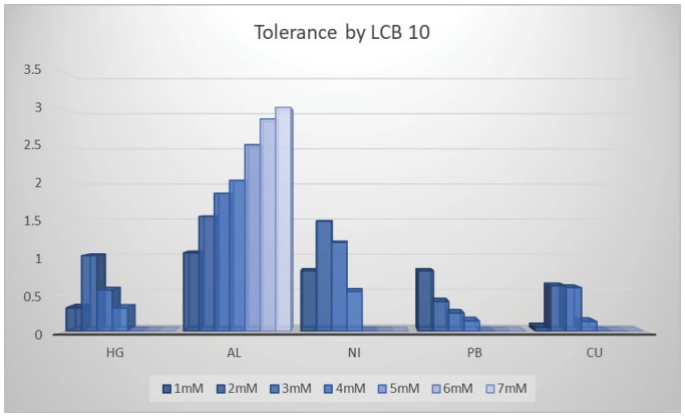

Figure 10. Growth of LCB 10 based on tolerance by different heavy metals.

Discussion

The open-cast lignite mine of Rajpardi in the Bharuch district of Gujarat, under the Gujarat Mineral Development Corporation, is a reservoir containing a vast diversity of bacteria like *Bacillus sp*., *Enterobacter sp*., *Klebsiella sp.,* and many more. The bacteria differ in their morphological and biochemical activities and thus are characterized based on their features exhibited. Aerobes or facultative anaerobes, lignite bacteria digest glucose to generate acetyl methyl carbinol. The microorganisms residing in these mines are most tolerant to the heavy metal Aluminium. The resistance shown by the isolates against this heavy metal promotes their use towards bioremediation of soil contaminated with aluminium. Copper is known for its antimicrobial property, clearly visible from the optical density values noted for the different culture tubes inoculated with the salt-containing Cu^{2+}. Maximum tolerance against copper was observed at a 3 mM concentration of the salt by both the *Bacillus sp.* (LCB3 and LCB4). The rest of the isolates started depleting from the 2 mM concentration of the copper salt. As for the metal salts mercury, nickel, and lead, bacteria showed tolerance for these metals up to 4 mM salt concentration. Later these cultures started to lose their resistance to 5 mM concentration which is noticed from the decreasing optical density values of the culture mixture.

LCB1 was identified as an *Enterobacter sp*. from the characterization tests that showed the least tolerance against copper. The isolate couldn't even grow at a low concentration of 2 mM. Growth was constantly depleting from 2 mM onwards. Tolerance against the metal Nickel followed copper, with subsequent bacterial growth even at 2 mM concentration of the salt, followed by reduced tolerance from 3 mM salt concentration, showing its least tolerance capacity against the metal. Tolerance levels of the isolate against the two different heavy metals - mercury and lead are nearly similar. They showed growth till 3 mM concentration of the respective salts, despite the tolerance against mercury being more observed from the turbidity of the culture, whereas 4 mM concentration of lead drastically reduced the growth of the bacteria illustrating its least tolerance against the metal. Tolerance against mercury could still be thought of as an option for the bacteria as these bacteria serves the potential to develop resistance against the metal. The tolerance against Aluminium, LCB1 showed increased tolerance against the metal with the subsequent addition of 1 mM of the salt in the broth culture. Nearly consistent growth was observed for the bacteria from base zero to 7 mM concentration of the metal salt, which marks the potential for the bacteria LCB1 for the bioremediation of soil contaminated with aluminium.

The growth turbidity values for LCB3 identified as *Enterobacter sp*. manifested that the bacteria showed similar results in growth against nickel, mercury, and lead. The turbidity of the culture mixture increased significantly up to a concentration of 3 mM, at which point the bacteria lost resistance to the heavy metal salts. LCB3 can be deemed more tolerant to copper than LCB1 since it grew up to a 3 mM concentration of salt, but LCB1 did not. However, the isolate showed remarkable tolerance for aluminium as they attempted to develop to a concentration of 7 mM.

LCB4 and LCB5, both *Bacillus* strains, demonstrated similar resistance to the aluminium metal salt by increasing growth turbidity values. LCB4 is tolerant only at low concentrations of 1 mM against the metals Hg, Ni, and Pb, whereas it is nearly similar to LCB3 in the case of tolerance against Cu. By contrast, LCB5 reported polar opposite results in the case of tolerance against Hg, Ni, and Cu. Both LCB4 and LCB5 showed comparable metal tolerance to copper, except that an extra 1 mM concentration of the salt significantly inhibited the development of LCB4, even though the data for LCB5 indicate that LCB4 has a comparative better tolerance to Pb. Tolerance against the well-known toxic metal Hg up to 4 mM and maintaining the growth turbidity up to 5 mM describes the potential of LCB5 to develop resistance against mercury and could serve as a future candidate for bioremediation of soil contaminated with mercury. Similarly, only LCB5 demonstrated a relatively high tolerance for nickel up to a 4 mM concentration of the salt, a concentration at which no other isolate could survive, indicating its potential for developing a Ni-based bioremediation method.

LCB6 and LCB9, both identified as *Klebsiella* sp., LCB7 (*Enterobacter* sp.), and LCB10 (*Streptobacillus* sp.), did show similar tolerance levels against Al as all the other isolates. LCB6 reports being slightly less tolerant than LCB9 for Hg. Similar tolerance levels are observed for LCB6, LCB7, LCB9, and LCB10 for tolerance against nickel, but LCB9 is the only isolate to show a comparative very high tolerance from the rest of the isolates against lead could grow drastically even at a 2 mM concentration of the salt and nearly maintaining the growth. LCB9 and LCB10 showed comparatively higher tolerance to copper compared to the rest of the isolates. An optimization technique could increase their potential for developing copper resistance, allowing them to be employed as future candidates for copper pollution bioremediation [23].

Conclusion

Every bacterial isolate from the study shows its tolerance against the metal, aluminium illustrating their togetherness for a scope in bioremediation purposes. The modern world seeks molecular techniques for developing

novel strategies against heavy metal bioremediation. That may include bioengineering the mechanisms of the bacteria at the molecular level and then testing them, which may raise ethical issues. The use of indigenous bacteria provides the capacity of the bacteria to strive and does influence the potential of other microbes towards remediation of the polluted environment. These bacteria have already been exposed to the heavy metal present in the soil, slowly developing tolerance to heavy metal, which increases further with time. A long-time of exposure to the metals enhances their tolerance against these metals. As observed from the study, a minute optimization can promote their use for bioremediation of the contaminated environment.

Declaration

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