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Exploring the Indigenous Microbes from Deliming Effluent to reduce heavy metals for greener approach

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Abstract

Bioremediation is an affordable, green technique that makes dealing with heavy metal toxicity simple. From the deliming effluent, five isolates were found. With respect to the heavy metal levels up to 5000 ppm, the MICs for each isolate varied. *Bacillus cereus* B66 OR740559, *Bacillus amyloliquefaciens* BAB-807 OR740568 and *Bacillus rugosus* SPB7 OR740574 were the high resistance isolates that 16S rRNA was able to identify. Using Atomic Absorption Spectroscopy (AAS), the reduction capacity was evaluated. Maximum reduction of *Bacillus cereus* B66 showed maximum reduction at 37℃ pH 9 Pb 92%, Cu 92%. *Bacillus amyloliquefaciens* BAB-807 was seen at 37°C and pH 9 Pb 90%, Cu 90%; at 45°C and pH 9 Pb, Cu, 92%, 93% during 48 hours. However, in 48 hours, 93% of the Pb and Cu were reduced at 55°C in pH 9. The three most important variables were temperature, pH, and incubation time ($p < 0.0001$). Among top three isolates, finding indicates that, for the longest possible exposure duration, *Bacillus cereus* B66 & *Bacillus amyloliquefaciens* BAB-807 reduction is most effective at temperatures of 37°C, 45°C,55°C with an increase in pH. These findings could serve as a foundation for the development of bioremediation techniques for the treatment of tannery effluent.

Keywords: Deliming effluent**,** bioremediation, heavy metals, atomic absorption spectrometry

Introduction

The tannery industry produces enormous quantities of contaminants[1], and the effluents it produces include poisonous pollutants to the flora and wildlife and highly resistant[2]. The elements known as heavy metals have a specific density greater than 4 g cm^{-3}, and certain of these elements are necessary for the basic physiological activities of living things at low concentrations[3], [4].Organisms require specific concentrations of Calcium, Sodium, Magnesium, Potassium, Copper, Iron, Zinc, and Chromium[5] to survive, while high concentrations of these elements are poisonous. The World Health Organisation has defined the permissible heavy metal concentration in water for consumption, and the Food and Agriculture Organisation has outlined the maximum permissible limit of toxic heavy metals in irrigation water [6]. Heavy metals are released into the environment through natural and anthropogenic activities, and they have well-defined toxic effects[7]. Because heavy metals are non-degrading and bioaccumulating, government agencies have imposed rules and legislation to limit their use and discharge into the environment[8].[9]

The procedures include soaking to eliminate impurities and conservation salt, liming/unhairing to remove epidermis and hair, flesh removal to remove this portion of the hide, deliming to remove lime added to the lime/unhairing and to lower pH, bating to prepare collagen fibres prior to tanning, and cleaning the hides using enzymes to remove impurities from the prior procedures(Gallego-Molinaetal.2013; EylemKilic,2014). Within the same drum, deliming and bating procedures are performed. Consequently, effluent from both processes deliming and bating can be attributed to the drum's discharge. Between one and four m3/t of effluent are discharged into the environment by a typical tannery that treats livestock[10]. To remove the lime from the pelt, deliming effluent is an essential unit step in the tannery technique. Neutralising the lime is the process at hand(Eylem Kilic 2015;

Sivakumar et al. 2015). The deliming phase eliminates the surplus liming chemicals employed in the previous unhairing technique by using acids and/or acidic salts. Because the pH needs to be decreased gradually, ammonium salts are often utilised for this purpose. During the deliming process, ammonium salts such $(NH4)_2SO_4$ and NH_4Cl are commonly employed to counterbalance the alkali (Na_2S, $Ca(OH)_2$, and NaOH)(Lei et al. 2020; Zeng et al., 2021) that were introduced to the pelt in the preceding liming step. 2000–4000 mg/L of NH_3–N are left in the deliming effluent after deliming, accounting for 60%–70% of the total NH_3–N in the mixed tannery effluent, which also contains tanning, pickling, soaking, liming, and post-tanning effluents[15]. Since a few decades ago, bioremediation has attracted a lot of attention since it requires few or no chemicals, is inexpensive, produces no solid sludge byproducts, and uses environmentally favourable operating procedures[16], [17]. Using live or dead biomass, bioremediation is the process of transforming, degrading, and detoxifying contaminants to an innoxious state and then mineralizing them into elements like carbon dioxide, nitrogen, and water. The ideal option for bioremediation is bacteria due to their omnipotence, abundance, diversity, tiny size, and special ability to survive and reproduce in both regulated and unregulated conditions while being robust to the environment[18].

Objectives

The objective of this study is to identify the substantial bacteria from deliming effluent that may mitigate the toxicity of heavy metals (Pb, Cr, Cu, Ni, and Cd) in the effluent from tanneries. Although deliming effluent has a large variety of bacteria that can be separated and exploited to improve the treatment of mixed effluent processes, it has a wide pH range. Isolating native microbes from deliming effluent is one of the objectives. Other goals include characterising heavy metal resistance, identifying those bacteria up to phylogenetic level, screening the isolates to find the isolates that can reduce heavy metals, and profiling them as fundamental studies to ensure that they are the foundation for their ability to resist so that the microbes can be used for breakdown in bioremediation framework.

Materials And Methods

Materials

Filter membranes having a pore size of 0.22µm were used to sterilise the solutions of heavy metal salts of copper, lead, and cadmium ($Cu(NO_3)_2$, $Pb(NO_3)_2$, and $Cd(NO_3)_2$). To conduct further tests, heavy metals were created for stock solutions[19][20]

Salt Used for In Vitro Investigation

Chemical name: Copper Nitrate

Chemical formula: $Cu(NO_3)_2$

Molecular weight: 187.56

Atomic weight: 63.546

Chemical name: Lead Nitrate

Chemical formula: $Pb(NO_3)_2$

Molecular weight: 331.2

Atomic weight: 207.2

Chemical name: Cadmium Nitrate

Chemical formula: $Cd(NO_3)_2$)

Molecular weight: 236.42

Atomic weight: 112.40

Sample Collection and Isolation of Bacteria

A tannery sector at the Central Leather Research Institute (CLRI), Chennai, provided samples of deliming effluent for use in bioremediation investigations. After being collected in sterile bottles, the sample were quickly taken to the lab and kept at 4℃ for further assessment. The nutrient agar was sterilised for 15 minutes at 121°C. Separately, 100µl of the serial dilution were spread out on spread and pour plates from 10^{-3} to 10^{-7} and were incubated for 24 hours at 35°C. Following incubation, the plates were examined to determine different types of colonies[21], [22].

Physiochemical properties

Physical and chemical characteristics such as colour, odour, turbidity, pH, total suspended particles, total dissolved salts, chemical oxygen demand (COD), biological oxygen demand (BOD), and chromium were examined in the gathered tannery effluent[23].

Heavy metal Tolerance

Isolates were streaked on nutrient agar plates supplemented with different metals (Cu, Cd, Pb) at increasing concentrations ranging from 50 ppm to 5000 ppm [24]to perform selective screening. For 72 hours, the isolates were cultured at 35℃. Plating was done in triplicates [25], [26].

Metal resistance capacity

Each isolate (D1, D2, D3, D4, D5, D8A, D8B, D8C, D9A, D10A, D10B, D12A, D12B) was grown independently on NA agar plates supplemented with 5000 ppm of Cd, Cu, Pb for 24 hours at 35℃ following this, the ability of the isolates to withstand different heavy metals was evaluated[27].

Identification of Isolated bacteria

Based on the characteristics of the colonies, such as colour, texture, form, elevation, gram staining, and biochemical assays, pure cultures of the isolates were identified. The isolates' identities were verified using molecular techniques. Bergey's Manual of Systemic Bacteriology states that the isolates' genus-level identification was provisionally determined[23].

Molecular Characterization

Genomic DNA from bacterial culture was extracted using DNA extraction kits for the isolate. Before starting a PCR reaction, the material was frozen at -4 °C and the extraction process was completed in accordance with the kit manual's recommendations. Three sets of denaturation, each lasting five minutes at 95°C, one minute at 95°C, one minute of annealing, two minutes at 72°C for primer extension, and ten minutes at 72°C for final extension comprised the amplification process. A 1% agarose gel pre-stained with ethidium bromide and a side marker was used to evaluate the size of the DNA based on the PCR result. After being submerged in the gel for approximately half an hour, the 14-well comb was removed. The comb was removed from the gel tray and placed inside the buffer container once the gel had set. The gel was able to sink to a depth of about two to five millimetres after adding 0.5 TBE to the tank. The 16S rRNA gene was amplified by PCR using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), and the resultant PCR products were sequenced. The 16S rRNA sample that had been amplified was forwarded for sequencing. [27], [28].

Phylogenetic Analysis

The sequences were located by using the BLAST programme on the NCBI database. The first ten sequences were chosen and aligned using the multiple sequence alignment software Clustal W, according to their maximum identity score. A phylogenetic tree and distance matrix were made using MEGA 10 software. The NCBI was contacted and asked for the accession numbers after the sequences were submitted to GenBank[29].

Effects of heavy metals on the growth of microbes

The ideal growing conditions were established in relation to the concentrations of three heavy metals. The isolates were cultivated individually in NB broth medium enriched with various heavy metals 100 ppm at 35℃ in a rotary shaker at 150 rpm and pH 7.0. A UV spectrophotometer was used to measure the optical density (OD) at k = 600

nm. The impact of the concentration of heavy metals on their growth was evaluated for each 2 hours of incubation [27].

Bio reduction Assay

Optimization of pH, temperature, and incubation time

A rotatory shaker set at 150 rpm was used to cultivate the bacterial isolate in a flask using NB broth medium. Every conical flask received 100 ppm of sterilised heavy metals (Cu, Cd, and Pb) after the optical density reached 0.6. The flasks were then incubated at 35°C, 45°C, and 55°C for 24, 48, and 72h. The culture was centrifuged for 10 minutes at 8000 rpm after incubation. Following their separation and mixing, the supernatants yielded twice as much concentrated HNO_3.Using a hotplate stirrer, mixtures were heated to 100°C until the volume of the acid-digested mixture returned to its initial level. The extract was collected into a flask, diluted, and filtered through paper using a Whatman filter. This extract underwent analysis using Atomic Absorption Spectrometry, and the reduction capacity percent result was compared to the control. Every parameter was used in this experiment[19], [30].

Statistical Analysis

In every instance, measurements were made in triplicate while monitoring and evaluating the development of bacteria that had been exposed to varying concentrations of heavy metals. The 2010 version of Microsoft Excel was used to record the data and compute the means and standard deviations. The statistical significance of the observed changes was verified using the student's t-test. Statistical analysis was done based on the percentage of deterioration at various temperatures, pH values, and incubation times. Utilising GraphPad Prism and Microsoft Excel, all the data was analysed. Tukey's Test analysis in conjunction with Two-way ANOVA was used to make between group comparisons. At $P<0.0001$the data demonstrated significance[31], [32].

RESULTS AND DISCUSSION

Characteristics of deliming effluent

The physiochemical properties were measured using conventional methods. The deliming effluent had a pH of mildly acidic and was light grey in hue. In deliming, the total dissolved salts were 4352 mg/L. Chemical oxygen demand, or 40995 mg/L, was high in comparison to BOD and COD **(Table 1)**. Electrical conductivity is a numerical expression that represents a water sample's ability to transport an electric current[33]. The total concentrations of the ionised compounds dissolved in water, as well as the temperature at which the measurement is conducted, define the number[31].Analyses were conducted on heavy metals such as copper, lead, cadmium. The ability of a water sample to carry an electric current is measured in terms of electrical conductivity[34]. The current investigation indicates that the tannery effluent's high conductivity may have been caused by salts and other inorganic compounds with good conductivity. Moreover, total suspended solids cause turbidity[35], which hinders light from entering the aquatic system and has an impact on photosynthetic activity. The results of the current study indicated a high organic load in tannery effluents due to the high BOD levels (6830 mg/L). Salt concentrations in deliming are low because several acids and acid salts are employed to reduce the amount of surplus liming agents required in the unhairing process[10], [36]. A high COD level (40995 mg/L) was found in this study. This suggests that there is no way for aquatic life to exist in wastewater. Raj et al. found that the deliming effluent from Chennai had higher COD levels[37]. Poole et al. state that microbial oxygen demand, which is represented in an increase in BOD, is what causes the depletion of dissolved oxygen[25]. This study also showed that deliming effluent has been extremely polluted, thus it is imperative to implement technology that may effectively minimise or degrade the mixed tannery effluent[28].

Table 1 Physiochemical parameters of deliming effluent

Parameters	**Unit**	**Results**
Color	-	light grey

pH@25℃	-	6.71
Electrical Conductivity@25℃	µs/cm	6800
Potassium	mg/L	69
Calcium	mg/L	994
Magnesium	mg/L	184
Sodium	mg/L	401
Total Dissolved Salts	mg/L	4352
Manganese	mg/L	0.01
Biochemical Oxygen Demand (BOD) 3 days @27 ℃	mg/L	6830
Chemical Oxygen Method (COD)	mg/L	40995

Isolation of Bacteria

The Central Leather Research Institute (CLRI) collected deliming wastewater in the tannery sector in latitude 13.0086 and longitude 80.2447. After being serially diluted on nutrient agar media, the samples' respective concentrations were found to be 2218 CFU/ml. After colony forming units (CFU) were directly plated, they were incubated for 24 hours at 35°C. From 10^{-1} to 10^{-7}, colonies were cultivated on spread and pour plates. Five bacterial colonies in all were selected and streaked independently.

Primary screening of heavy metal resistant bacteria

Growth was monitored for three days while thirteen different bacterial isolates, D1, D2, D3, D4, D5, D8A, D8B, D8C, D9A, D10A, D10B, D12A D12B were grown independently on nutrient agar plates enriched with different metals Cu, Cd, Pb at increasing concentrations of 50 ppm to 1000 ppm at 35°C. With respect to different metals, these thirteen isolates demonstrated good resistance. For each heavy metal, all isolates exhibited dense growth. Plates were observed for three days.

Heavy Metal Tolerance

The concentration of the heavy metal-resistant bacteria grown on heavy metal supplemented media is determined by gradually increasing the concentration of the heavy metal[25,38] on the growth media (NA) until the microbes no longer thrive on the plates. The 13 strains were chosen based on their ability to resist 50 ppm to 1000 ppm, and their minimal inhibitory concentration was determined by increasing concentrations on various metals in media ranging at 2000 - 5000 ppm. Some isolates showed dense growth on various heavy metals (Cu, Cd, Pb) at 600,900,1000,2000,3000,4000,5000 ppm. Each day observation was observed **(Fig. 1,2,3)** and compared with control **(Fig.4)**. In this case, D2, D4, D8A, D10B, had moderate growth at 2000, 3000, 4000 and slight growth on 5000 ppm; D5 displayed moderate growth at 1000, 2000, 3000,4000,5000ppm; dense growth was only evident at 600 & 900 ppm. D8B displayed modest growth at 900 – 4000ppm. D9A had dense growth on 600 and 900 ppm and moderate growth on 1000 to 5000 ppm. D9B, D10A displayed moderate growth on a range of metals from 600 to 5,000 ppm. D10A, D10B showed slight growth on 5000ppm. D12B showed moderate growth on 900- 5000 ppm. Among 13 top three isolates, D1, D3, D12A showed robust resistance at 5000 ppm **(Fig.1,2,3,4)** along with dense growth and good tolerance at concentrations of 600, 900, 1000, 2000, 3000,5000 **(Table 2)**. It was discovered that all isolates were able to tolerate, but (D1, D3, D12A) these three-isolate showed strong resistance.

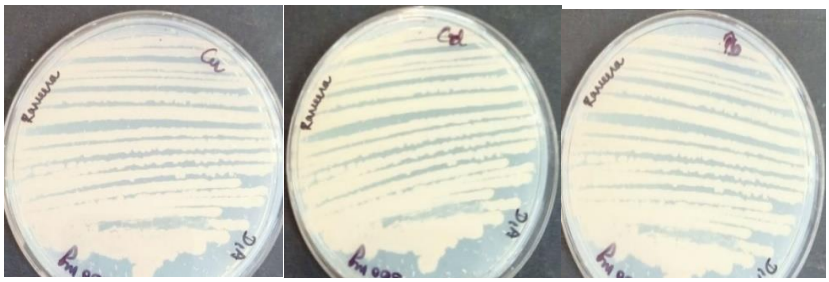


Figure 1. Screening of D1 isolate on Copper,Lead,Cadmium at 5000 ppm

Figure 2. Screening of D3 isolate on Copper,Lead,Cadmium at 5000 ppm

Figure 3. Screening of D12A isolate on Copper,Lead,Cadmium at 5000 ppm

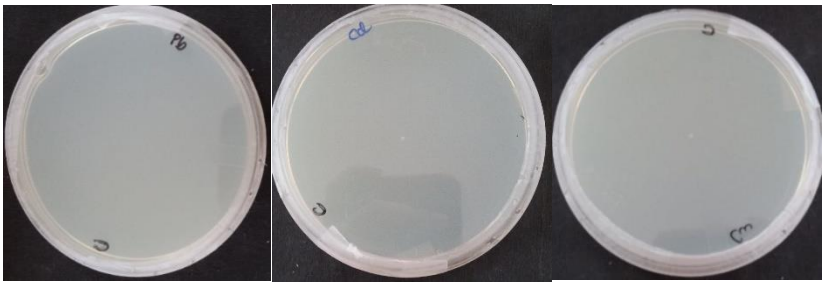


Figure 4. Screening of Control plates on lead resistant at 5000 ppm

Table 2 Minimum inhibitory concentration determination of bacterial isolates

Bacterial Isolates	600 ppm	900 ppm	1000 ppm	2000 ppm	3000 ppm	4000 ppm	5000 ppm
D1	+++	+++	+++	+++	+++	+++	+++
D2	+++	+++	+++	+++	++	++	++

D3	+++	+++	+++	+++	+++	+++	+++
D4	+++	+++	+++	++	++	++	++
D5	+++	+++	++	++	++	++	++
D8A	+++	+++	+++	++	++	++	+
D8B	+++	++	++	++	++	++	+
D9A	+++	+++	++	++	++	++	++
D9B	++	++	++	++	++	++	++
D10A	++	++	++	++	++	++	+
D10B	+++	+++	+++	++	++	++	+
D12A	+++	+++	+++	+++	+++	+++	+++
D12B	+++	++	++	++	++	++	++

***Slight growth (+), Moderate growth (++), Dense growth (+++)**

Identification of strains

Bergey's Manual of Determinative Bacteriology [27], [39]was followed in the identification of the bacterial isolates. The identified morphological, biochemical, and cultural traits were used to characterise the bacterial isolates. The top three isolates (D1, D3, D12A) with possible heavy metal resistance were described **(Table 3)**. Gram staining, colony colour, shape, and texture, as well as catalase and oxidase activities were evaluated. The isolates D1, D3, D12A **(Table 3)** tested positive for Gram-positive bacteria. D1 was found to be white, granular had irregular margin, wavy edge D3 was found to be opaque, white, and had a rough surface with wrinkles around the edges. D12, on the other hand, was found to be creamy white and circular in shape. Both isolates are aerobes, rod-shaped bacteria that form spores. The presence of bubbles suggests that D1, D3 and D12A were catalase positive. When the Oxidase test yields no colour change. D1& D3 were considered negative; when D12 yields a purple tint, it is considered positive.

Table 3 Biochemical Characterization

Bacterial Isolates	**Gram nature**	**Colony colour**	**Shape and Texture**	**Catalase**	**Oxidase**
D1	Gram positive	White	granular, irregular margin, wavy edge	+ ve	-ve
D3	Gram positive	White	Rough, wrinkled, opaque, irregular	+ve	-ve
D12A	Gram positive	Creamy white	Circular	+ve	+ve

Molecular Characterization

The 16S rRNA sequencing was used to amplify the isolate, and phylogenetic tree was created. D1, D3 and D12 strains were found to be *Bacillus cereus* B66 (99.87%), *Bacillus amyloliquefaciens* BAB-807 (100%) and *Bacillus rugosus* SPB7 (99.87%), respectively, with Accession numbers OR740559, OR740568 and OR740574 in the NCBI GenBank database **(Figs. 5,6,7).** The phylogenetic tree was created using MEGA 10 software, and the species belong to the *Bacillaceae* family and the genus is *Bacillus*.

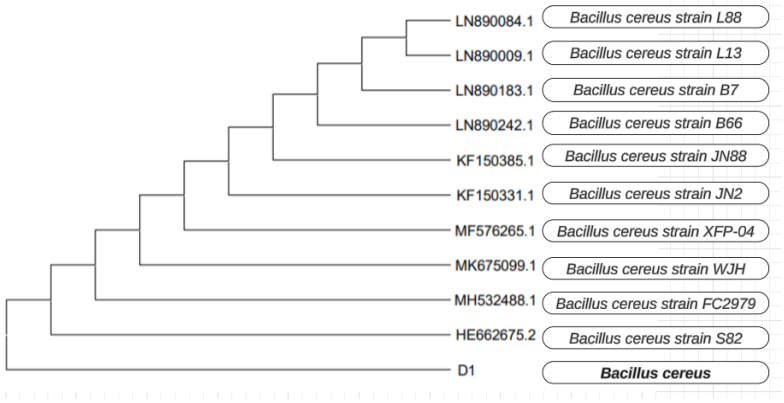


Fig.5 Phylogenetic tree of *Bacillus cereus* B66 (OR740559) and other strains on 16Sr RNA sequences

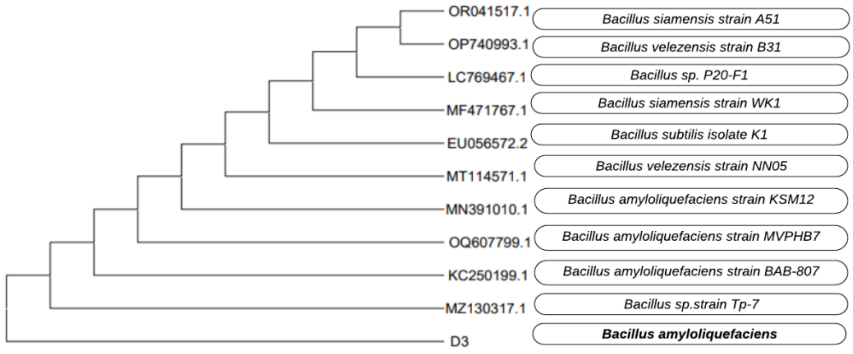


Fig. 6 Phylogenetic tree of *Bacillus amyloliquefaciens* BAB – 807 (OR740568) and other strains on 16S rRNA sequences

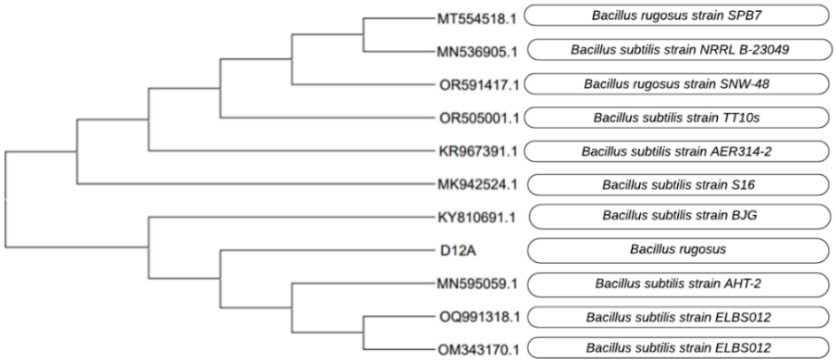


Fig.7 Phylogenetic tree of *Bacillus rugosus* SPB7 (OR740574) and other strains on 16S rRNA sequences

Growth of bacterial isolate on various metals

A growth curve is used to measure the bacterial population of a culture against time. A normal growth curve goes through four phases: lag, exponential, stationary, and death. The "lag phase" is the duration of time required for bacteria to reach a stage at which they may multiply and proliferate quickly[40]. The study looked at the impacts

of several heavy metals that were added at 0.6 OD log phase after being sterilised to a concentration of 100 ppm. A growth curve was used to record D1 isolate's growth in r elation to several heavy metals. For a whole day, the bacterial growth was monitored at 35°C, 45°C, 55°C, with pH 7 as indicated in **(Fig.8,9)**. The promising isolate's resistance to certain heavy metals was assessed. The isolate displayed distinct resistance development patterns to Cu, Pb, Cd. Furthermore, in comparison to other heavy metals at all temperatures and pH ranges, Bacillus species shown susceptibility to Cd and strong resistance to Pb, and Cu. Interestingly, top three isolates exhibited moderate growth at other temperatures and rapid growth at 55◦C and pH 7 **(Fig.10)**. The result of the Cd resistance was phenomenal, however it happened slowly.

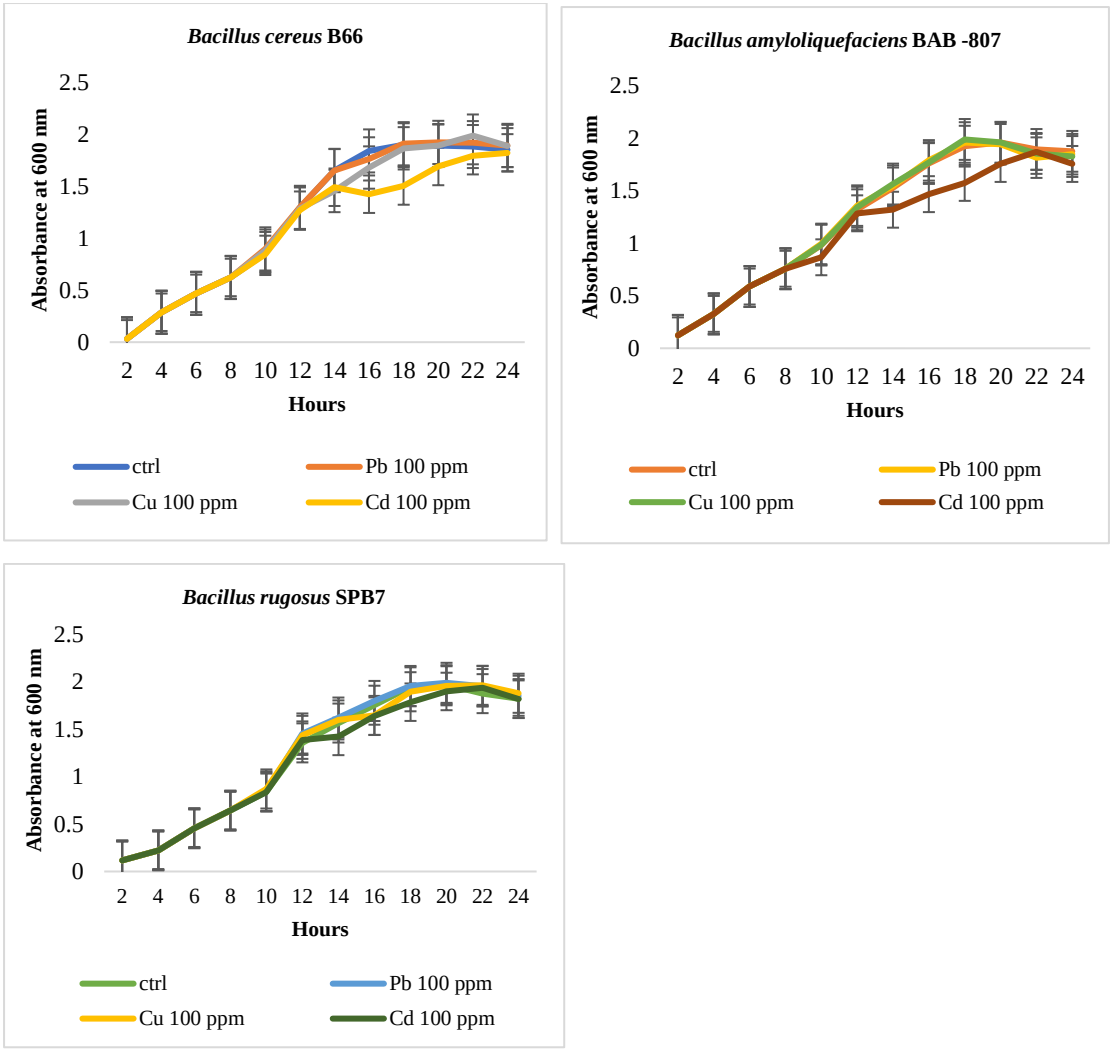


Fig.8 Growth curve of *Bacillus* species at pH 7 and temp 35 °C

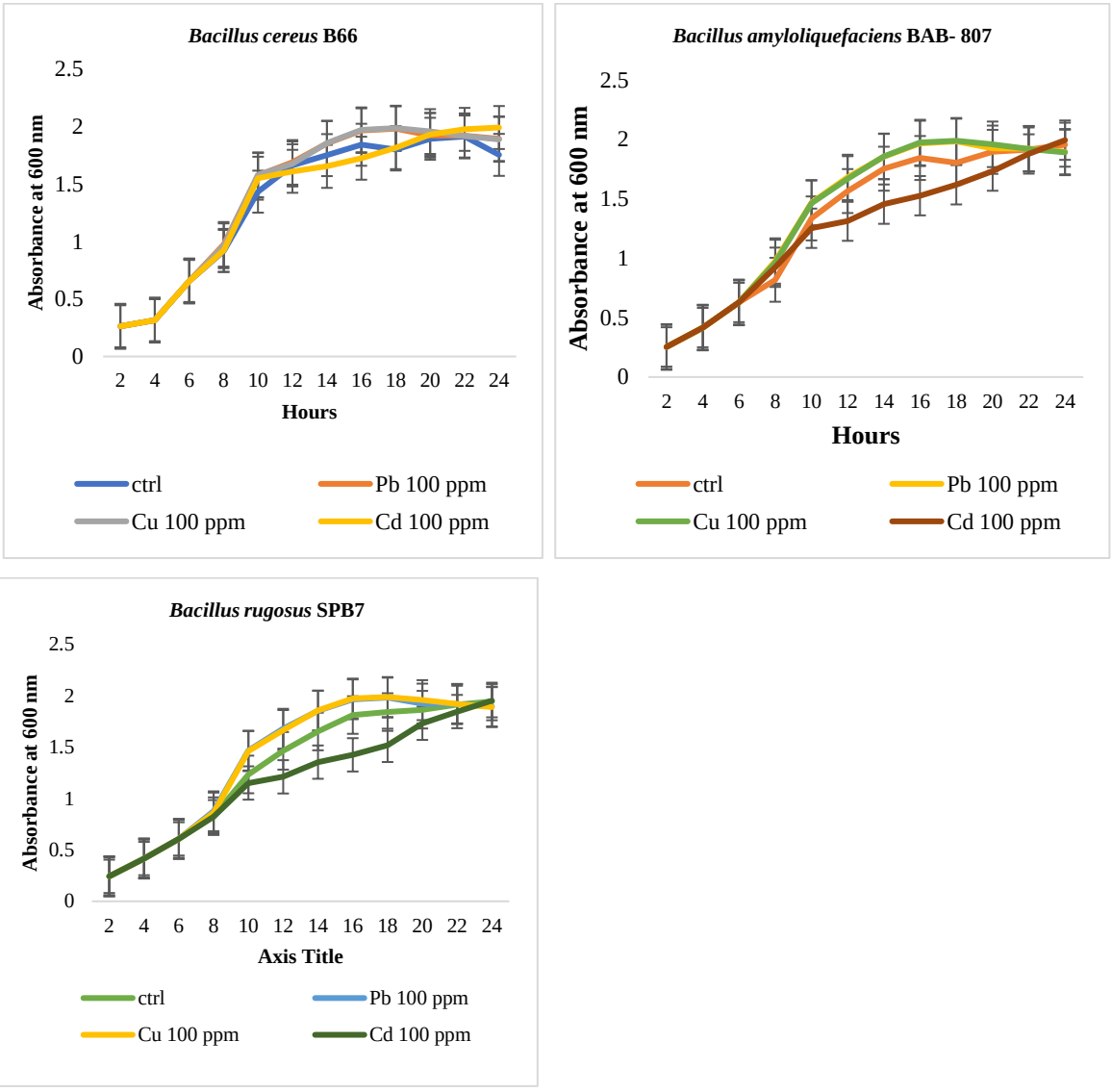
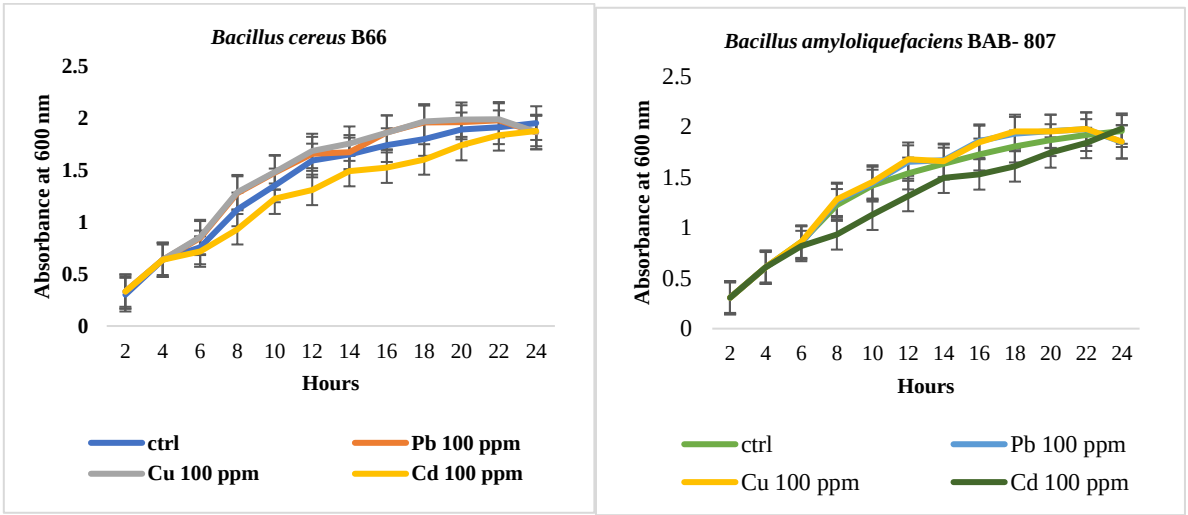


Fig.9 Growth curve of *Bacillus* species at pH 7 and temp 45 ºC



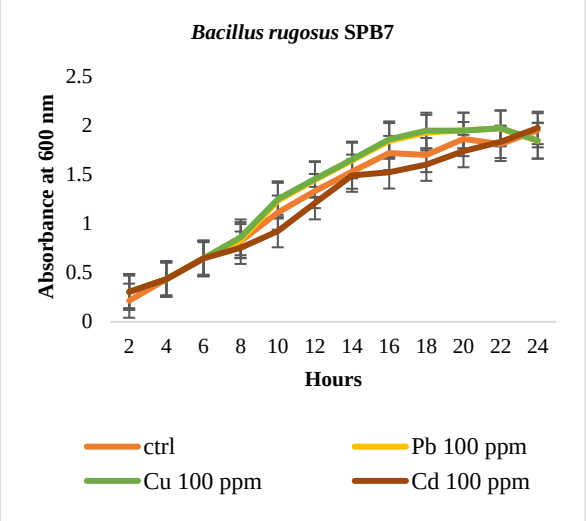


Fig.10 Growth curve of *Bacillus* species at pH 7 and temp 55 ºC

Heavy Metal Reduction capacity

Effect of contact time

Among the factors that affect bioremediation are temperature, pH level, and contact time. This study investigated the circumstances surrounding the isolates' reduction of the heavy metals Cu, Pb, Cd. For 24, 48,72 hours. The isolates' effectiveness at 100 ppm of different heavy metals was investigated at different incubation times at 37℃, pH 7. *Bacillus cereus* B66 was exceptionally effective at reducing Pb, Cu, Cd, as seen in **(Fig. 11A)**. 59% of lead was reduced in 24 hours, 76% in 48 hours, and 95% in 72 hours. In contrast, copper showed reductions of 55% in 24 hours, 79% in 48 hours, and 93% in 72 hours. Cadmium reduction was 43% in 24 hours, 60% in 48 hours, and 83% in 72 hours. By using statistical methods such as the turkey test, *Bacillus cereus* B66 was able to obtain greater efficiency in 72 hours in Pb (95%), Cu (93%), Cd (83%), with ***$p < 0.0001$ when compared to various heavy metals. Here, the rate of deterioration has differed for every metal and has demonstrated a significant time variation in efficiency. Reduction of Pb, Cu, Cd was effective when *Bacillus amyloliquefaciens* BAB-807 **(Fig. 11B)** was used to compare with Lead which demonstrated a 60% drop in 24 hours, a 78% reduction in 48 hours, and a 93% reduction in 72 hours. The reduction in copper was 61% in 24 hours, 78% in 48 hours, and 90% in 72 hours. The reduction in cadmium was 45% in 24 hours, 65% in 48 hours, and 81% in 72 hours. *Bacillus rugosus* SPB7 **(Fig. 11C)** was demonstrated to be effective in lowering Pb, Cu, and Cd. Lead reduction percentages were 58% in 24 hours, 69% in 48 hours, and 91% in 72 hours; in contrast, copper reduction percentages were 63% in 24 hours, 73% in 48 hours, and 90% in 72 hours. Cadmium reduction was 42.30% in 24 hours, 61% in 48 hours, and 80% in 72 hours Previous studies have shown that the bacteria *Bacillus sp*. and *B. amyloliquefaciens* [40], [41] could reduce Cr, which is useful for breaking down heavy metals including lead, cadmium, and zinc that were identified from the sediments of Guilan Bay[42], Iran. The authors reported that after being isolated from contaminated soil, *B. cereus* BUK_BCH_BTE2 and *Bacillus sp*. MNU16[43] showed 92% Pb in 96 hours and 75% Cr in 72 hours.

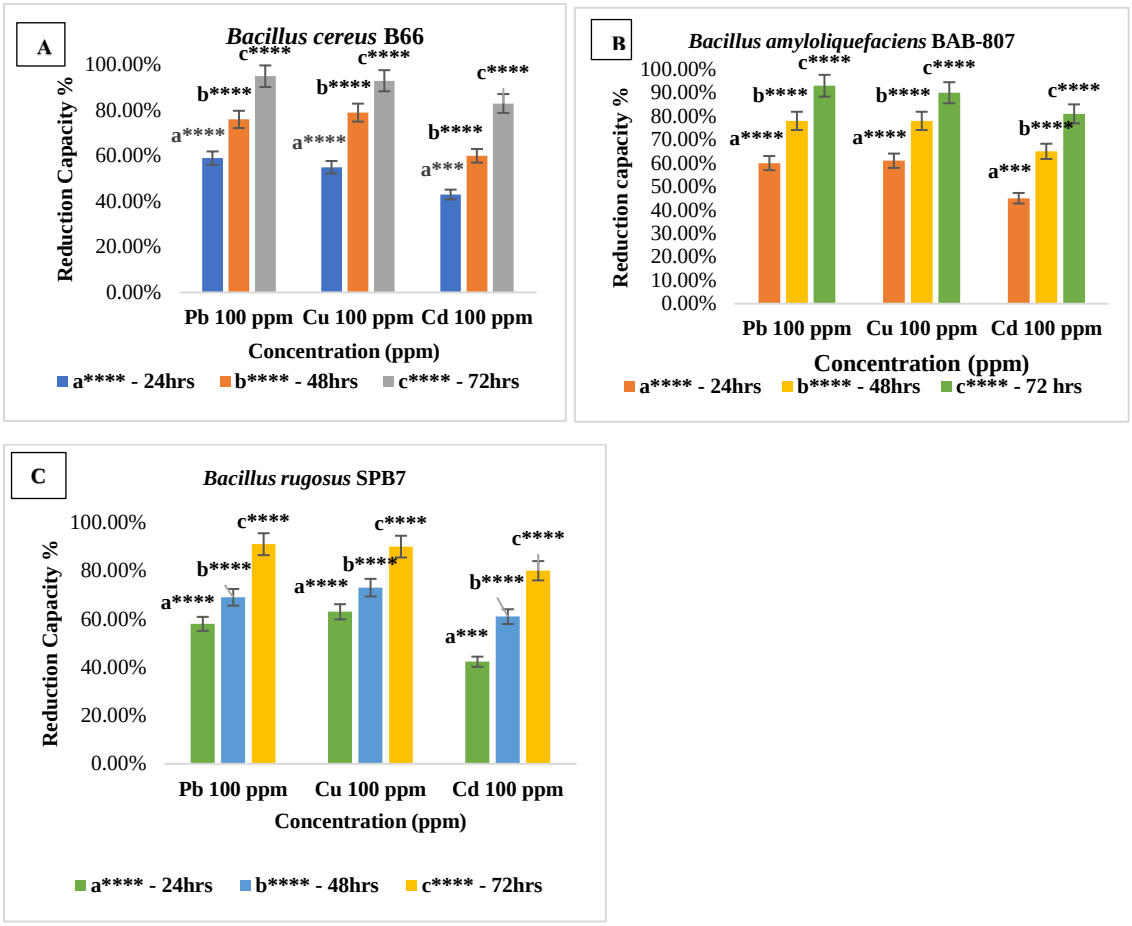


Fig. 11 Values are expressed in mean ± SD (n = 3), statistically significant test for comparison was done by TWO WAY ANOVA followed by Turkey Test. Comparison of Incubation time, Temp 35°C, pH 7, a** - control vs 24 hrs, b****- control vs 48 hrs, c****- control vs 72 h, ***p < 0.001, ****p < 0.0001 and ns – non-significant.**

Effect of Temperature 35°C and pH

One important factor in the bio reduction process is temperature. This experiment serves as an example of how it is done at a steady temperature. For 35°C culture conditions, 100 ppm concentration, pH 5, 150 rpm, and the following reduction%: Pb (64%), Cu (71%), Cd (47%). Pb (76%), Cu (79%), Cd (60%), under growing conditions at pH 7. For 48 hours, the effectiveness of *Bacillus cereus* B66 reduction was evaluated under three distinct pH settings. **(Fig. 12)**. *Bacillus amyloliquefaciens* BAB-807 was cultured at 35°C, pH 5 with the following terms: Pb (51%), Cu (61%), Cd (41%). At pH 7 Pb (78%), Cu (78%), Cd (65%). Pb (90%), Cu (91%), Cd (80%) at pH 9. *Bacillus rugosus* SPB7 showed reduction at pH 5, Pb (61%), Cu (54%), Cd (51%). At, pH 7, Pb (69%), Cu (73%), Cd (61%). At, pH 9 Pb (88%), Cu (89%), Cd (80%). *Bacillus cereus* B66, *Bacillus amyloliquefaciens* BAB-807, *Bacillus rugosus* SPB7 kept at 35°C shows its peak efficiency ****p < 0.0001 when exposed to all heavy metals for 48 hours at all pH ranges.

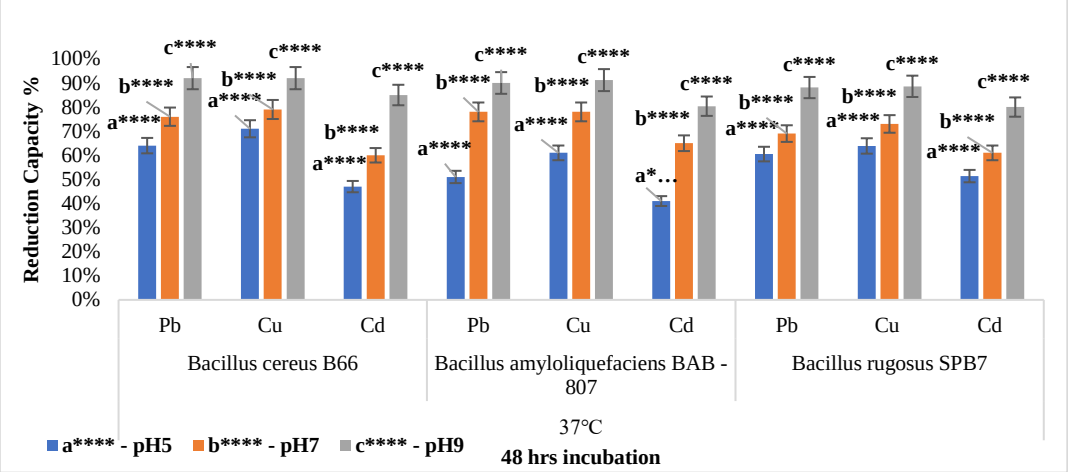


Fig. 12 Values are expressed in mean ± SD (n = 3), statistically significant test for comparison was done by TWO WAY ANOVA followed by Turkey Test. Comparison of Contact time (48hrs), Temp (35°C), pH (5, 7, 9) a** - control vs pH 5, b****- control vs pH 7, c****- control vs pH 9, ***p < 0.001, ****p < 0.0001 and ns – non-significant.**

Effect of Temperature 45°C and pH

The degrading capacity of *Bacillus cereus* B66 was investigated at different pH ranges of 5, 7, and 9 over a 48-hour incubation period at 150 rpm, while the temperature was raised to 45°C. At pH 5, *Bacillus cereus* B66 starts to function at 45°C with (85%) Pb, (89%) Cu, and (79%) Cd. However, at pH 7, it had the highest efficiency with 90% Pb, 91% Cu, and 79% Cd, respectively. Additionally, it is more efficient and demonstrated maximum efficiency with (94%) Pb, (93%) Cu, and (81%) Cd reduction capacity at pH 9 for culture conditions. The efficacy of *Bacillus amyloliquefaciens* BAB-807 and *Bacillus rugosus* SPB7 isolates was examined at 100 ppm of various heavy metals at pH values ranging from 5, 7, 9, and after 48 hours of incubation at 45°C **(Fig. 13)**. According to Sawkat et al., *B. subtilis* and *B. licheniformis* found that, at 125 ppm in 72 hours, the percentages were 85% and 65% in $K_2Cr_2O_7$[21], [44], [45]. Function of *Bacillus amyloliquefaciens* BAB-807 at pH 5 at this temperature 81% for Pb, 82% for Cu, and 73% for Cd. Pb (90%), Cu (90%), and Cd (75%) at pH 7. Pb (92%) Cu (93%), Cd (78%) at pH 9. There has been a noticeable temporal variation in efficiency and a fluctuating rate of deterioration for each metal. It has been found that at pH 6–8, bacterial reduction of Pb, Cu, and Cd is considerable, but at pH 5 or 9, it is minimal[26], [39], [46], [47]The role of *Bacillus rugosus* SPB7 at pH 5 and for Pb (86%), Cu (84%), Cd (73.1%); Pb (90%), Cu (91%), and Cd (75.2%) at pH 7. Pb (92%) Cu (92%) and Cd (78%) at pH 9. Both isolates show their peak efficacy at pH 7.9, ****p < 0.0001, at 45°C.

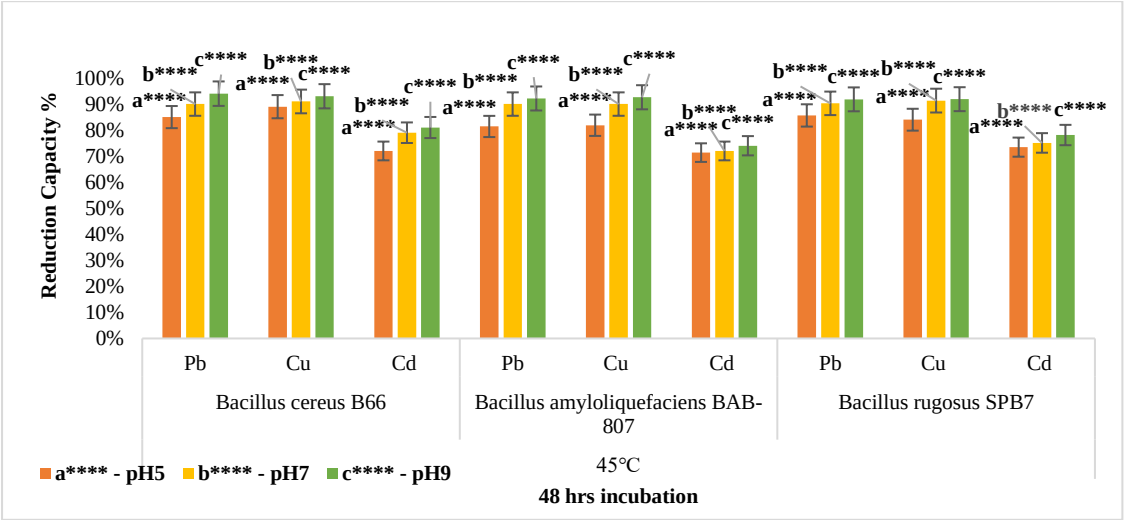


Fig. 13 Values are expressed in mean ± SD (n = 3), statistically significant test for comparison was done by TWO WAY ANOVA followed by Turkey Test. Comparison of Contact time (48hrs), Temp (45°C), pH (5, 7, 9) a** - control vs pH 5, b****- control vs pH 7, c****- control vs pH 9, ***p < 0.001, ****p < 0.0001 and ns – non-significant.**

Effect of Temperature 55°C and pH

The effectiveness of Bacillus cereus B66 was investigated at several pH ranges by raising the temperature to 55°C at the mesophilic optimum range (5,7,9). Under test circumstances with a pH of 5, 55°C revealed 90% Pb, 87% Cu, and 70% Cd in 48 hours. After 48 hours of incubation, the conditions available at pH 7 were determined to be successful for 93% Pb, 93% Cu, and 77% Cd. Maximum reduction was seen at 95% Pb, 95% Cu, and 83% Cd under pH 9 conditions. It has been discovered that at neutral and alkaline pH (7 & 9), the breakdown process is considerably more rapid and efficient when temperature and pH are increased. *Bacillus amyloliquefaciens* BAB-807 and *Bacillus rugosus* SPB7 isolates were tested for their effectiveness at 100 ppm of different heavy metals at pH values between 5, 7, 9 as well as after 48 hours of incubation at 55°C **(Fig. 14)**. (87%) Pb, (87%) Cu, and (71%) Cd were detected in *B. amyloliquefaciens* isolate at pH 5. 90% Pb, 90% Cu, and 75% Cd are present at pH 7. (90%) Pb, (93%), (75%) Cu, and pH 9. At pH 5, *B. rugosus* revealed (85%) Cu, (86%) Pb, and (73%) Cd. Conversely, at pH 7, 90% Pb, 91% Cu, and 76% Cd were present. Pb (92%), Cu (91%), and Cd (76%) at pH 9. When exposed to heavy metals in the medium, the bacteria required a longer incubation duration at 37°C, 45°C, 55°C at pH 7 and pH 9, for the purpose of repair or adaption, as indicated[42], [43], [48], [49], [50], [51].

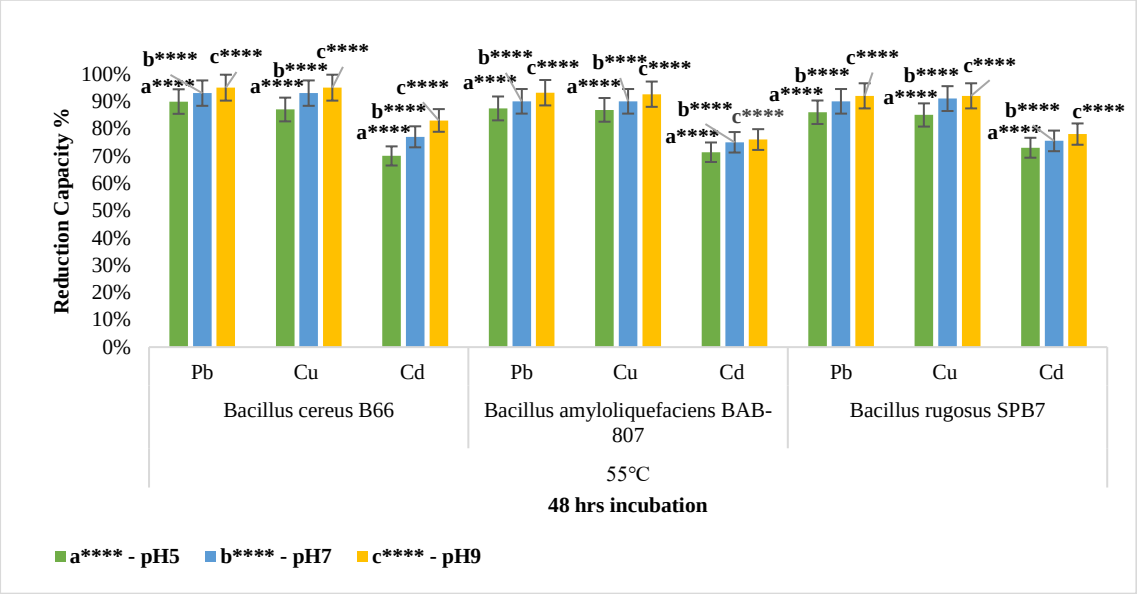


Fig. 14 Values are expressed in mean ± SD (n = 3), statistically significant test for comparison was done by TWO WAY ANOVA followed by Turkey Test. Comparison of Contact time (48hrs), Temp (45°C), pH (5, 7, 9) a** - control vs pH 5, b****- control vs pH 7, c****- control vs pH 9, ***p < 0.001, ****p < 0.0001 and ns – non-significant.**

Conclusion

The current study involved the isolation of five isolates from a novel source deliming effluent, and the potentiality of each isolate to degrade heavy metals was evaluated. To show which isolates would be most suitable for bioremediation of heavy metal pollutants, the isolates were examined for physiochemical parameters, indigenous bacteria, different heavy metal resistance capacity, growth curve analysis of heavy metals, Minimum Inhibitory Concentration, and heavy metal reduction. Based on a variety of biochemical and molecular characterisation combined with phylogenetic tree analysis, we identified *Bacillus cereus* B66, *Bacillus amyloliquefaciens* BAB-807 and *Bacillus rugosus* SPB7. It became evident that a variety of parameters, such as pH, temperature, and

incubation time, had a significant impact on the reduction of each heavy metal. Study indicated that both strains were able to tolerate till 5000 ppm and showed effective and massive reduction capacity at 100 ppm at various pH 5,7,9 various temperature 37℃,45℃,55℃ and incubation period 24,48,72 hrs which is a novel report. According to my research, when compared to top three isolates *Bacillus cereus* B66 & *Bacillus amyloliquefaciens* BAB-807 had substantial potential and it is optimum at temp 37℃, 45℃, 55℃ at pH 5,7,9 without affecting its physical and chemical conditions for bioremediation and to reduce toxicity in mixed tannery effluent.

Conflict Of Interest

The authors declare that there is no conflict of interests regarding the publication of this article

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