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ISOLATION AND IDENTIFICATION OF CHROMIUM-TOLERANT BACTERIA FROM POULTRY LITTER IN DHAKA, BANGLADESH

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Tannery waste, known for its high chromium content, often contaminates chicken feed, accumulating chromium in chickens and their excreted waste. This study aimed to isolate chromium-tolerant bacteria from chicken litter and evaluate their ability to withstand various chromium concentrations. Four bacterial isolates—CM3, CM4, CM5, and CM6 were presumptively identified as *Pseudomonas* sp., *Bacillus* sp., *Staphylococcus* sp., and *Bacillus* sp., respectively. The isolate CM4 showed the highest tolerance, growing at 800 mg/L concentrations. A subsequent experiment focused on CM4, where growth was measured by absorbance at 600 nm across chromium concentrations from 0 mg/L to 3200 mg/L. The results indicated a significant decrease in growth with increasing chromium levels, with near-complete inhibition at the highest concentrations. This study highlights the potential of chromium-tolerant bacteria from poultry litter for bioremediation, particularly in environments contaminated with this heavy metal. These findings contribute to ongoing efforts to mitigate chromium pollution through microbial interventions.

Keywords: Chromium, Heavy metal, Tolerance, Isolates, Chicken litter

INTRODUCTION

Chromium (Cr), a non-essential and highly toxic element for plants and microorganisms, is relatively rare in nature (1). Industrial activities such as mining, metal plating, wood preservation, ink production, dye and pigment manufacturing, glass and textile manufacturing, and corrosion inhibitors in cooling water, significantly contribute to Cr pollution. These activities release effluents and solid wastes containing varying levels of chromium, posing severe health risks to the environment and living organisms. Cr's mobility in the soil, influenced by weathering and biochemical processes, further impacts plant growth and metabolic functions in living organisms (1, 2).

Traditionally, chemical methods are employed for Cr remediation. However, rapid industrialization and urbanization have escalated the consumption of heavy metals, including Cr. While Cr is considered a micronutrient in trace amounts, its higher concentrations present significant challenges. The environmental behavior of Cr is largely determined by its oxidation state. Trivalent chromium [Cr (III)] is less toxic, less soluble at neutral pH, and unable to cross cell membranes. In contrast, hexavalent chromium [Cr (VI)] is highly soluble, more accessible, and poses greater toxicity (3). Cr (VI) is classified as a Group A human carcinogen due to its mutagenic (4), carcinogenic (5), and teratogenic (6) effects on humans, animals (7), and plants (8).

In response to these challenges, bioaccumulation and microbe-based technologies have emerged as viable alternatives. Microorganisms and microbial products have demonstrated efficacy in removing soluble and particulate forms of metals, particularly from diluted solutions (9).

Due to improper disposal and recycling practices, tannery waste is notorious for its high Cr content and often finds its way into animal feed, including chicken feed. This contamination leads to Cr accumulation in chickens' bodies, as they ingest feed laced with this heavy metal (10, 11). Consequently, the Cr is excreted through their excreted wastes (12), resulting in chicken litter that might contain significant levels of Cr. This environment may foster the growth and proliferation of Cr-tolerant bacteria. As chickens are continuously exposed to Cr through their diet, their digestive systems may become a breeding ground for microorganisms that can withstand high levels of this toxic element. Therefore, chicken litter might serve as a rich source for isolating Cr-tolerant bacteria, which can be crucial for bioremediation efforts to mitigate Cr pollution. The primary objective of this study was to isolate Cr-tolerant bacteria from chicken litter and assess their ability to tolerate various concentrations of Cr in nutrient agar media.

MATERIALS AND METHODS

Sampling period & location: Chicken litter was obtained from a nearby chicken-selling van in the Siddeshwari area, Dhaka, Bangladesh in February 2023. Chicken fecal matter was collected in sterile Falcon tubes and promptly transported to the laboratory for immediate processing.

Sample collection: Sterile falcon tubes were prepared and labeled for the collection of chicken litter. A sterile spatula was used to collect the chicken litter. Approximately, 10-15 grams of chicken litter were collected from various spots within the van to ensure a representative sample. The collected chicken litter was immediately transferred into the pre-labeled sterile falcon tubes. The tubes were sealed tightly to prevent any leakage or contamination. The sealed falcon

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tubes were placed in a cooler with ice packs to maintain a low temperature during transportation. The samples were then promptly transported to the laboratory to minimize any potential changes in the sample composition. The samples were stored at an appropriate temperature until further analysis.

Isolation of Cr-tolerant pure bacterial colony: Nutrient agar (NA) media were prepared according to standard protocols. Media were supplemented with 50 mg/L and 100 mg/L Cr (VI) to observe the growth of bacteria in the presence of chromium. Serial dilutions of the chicken litter samples were performed to obtain different concentrations: 10^3, 10^4, 10^5, 10^6, and 10^7. 0.1 ml of the sample was spread onto the prepared NA media from each dilution. The inoculated plates were incubated at 37°C for 24 hours to allow bacterial colonies to grow. After the incubation period, plates were examined for bacterial growth. Different suitable colonies were selected based on their morphological characteristics. To obtain a pure culture, different bacterial colonies were selected based on their colony morphology. These selected colonies were then transferred individually to separate NA media plates, each containing 100 mg/L of Cr (VI). This process was carried out until pure colonies were isolated.

Identification and Characterization: The identification of the isolates was performed following Bergey's Manual of Determinative Bacteriology (13). Individual bacterial morphology was identified by Gram staining. The selected microbial colonies were morphologically characterized based on their size, shape,
texture, elevation, margin, and pigmentation. Biochemical characterization included starch hydrolysis, TSI, citrate utilization, oxidase, and catalase tests.

Determine the Cr-tolerance of bacteria in nutrient agar media: To detect chromium tolerance, NA media were prepared and supplemented with Cr (VI) at concentrations ranging from 50 to 1600 mg/L. Previously isolated pure bacterial cultures were streaked onto these Cr-supplemented NA plates. The plates were incubated at 37°C for 24 hrs. The Cr tolerance of each bacterial isolate was determined by identifying the highest concentration of Cr (VI) that still supported visible bacterial growth, thereby providing a measure of the isolates' tolerance to Cr (VI).

Determine the Cr-tolerance of bacteria in nutrient broth media: To determine the Cr tolerance of selected bacteria, the nutrient broth (NB) was prepared and supplemented with Cr (VI) at concentrations ranging from 0 to 3200 mg/L. Later, 1% bacterial suspension that corresponds to MacFarland standard 0.5 was inoculated into these Cr-supplemented NB solutions and incubated at 37°C for 24 hrs. After incubation, bacterial growth was assessed by measuring the optical density (OD) at 600 nm using a spectrophotometer. The Cr tolerance of each bacterial isolate was determined by comparing the OD readings across the different chromium concentrations, with higher OD values indicating greater bacterial growth and thus higher tolerance to chromium (VI). This method provided a quantitative evaluation of the tolerance levels of the selected bacteria to varying concentrations of Cr (VI)

RESULTS

After incubating at 37ºC for 24 hours, Total Viable Bacterial Counts (TVBCs) were recorded. For the 50 mg/L Cr concentration, there were 20 colonies from the 10^{-6} dilutions and 17 colonies from the 10^{-7} dilutions. For the 100 mg/L Cr concentration, 9 colonies were observed from the 10^{-6} dilution and 8 colonies from the 10^{-7} dilution (as shown in Table 1).

Table 1: TVBC on NA media containing 50 and 100 mg/L Cr concentrations

Sample	Dilution factor	TVBC (50 mg/L)	TVBC (100 mg/L)
Chicken droppings	10^3	TNTC	TNTC
	10^4	TNTC	TNTC
	10^5	TNTC	TNTC
	10^6	20	9
	10^7	17	8

Single colonies were examined for cultural characteristics after incubation at 37ºC for 24 hours and were labeled as CM3, CM4, CM5, and CM6 (Figure 1).

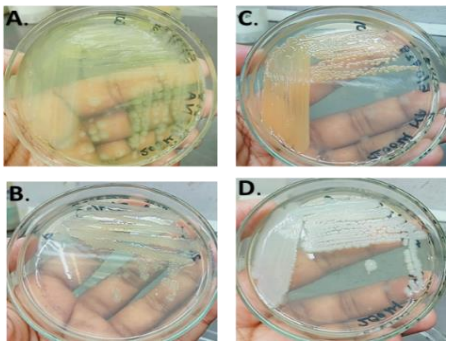

Figure 1: Cultural characteristics of the isolates CM3 (A), CM 4 (B), CM 5 (C) and CM 6 (D) on NA media containing 100 mg/L Cr.

The microscopic characteristics of isolates were also examined. Three of the isolates were Gram-positive, while only one was Gram-negative (Table 2).

Table 2: Microscopic examination of the isolates, CM3, CM4, CM5 and CM6.

Isolate No.	Shape	Arrange	Color	Gram reaction
CM3	Rod	Single	Pink	-
CM4	Rod	Single, in pair	Purple	+
CM5	Cocci	Single, cluster	Purple	+
CM6	Rod	Single	Purple	+

Each isolated colony's size, shape, texture, elevation, margin, and pigmentation (CM3, CM4, CM5, and CM6) on NA media were primarily examined (Table 3).

Table 3: Colony morphology of the isolates from poultry litter on NA media containing 100 mg/L chromium concentrations.

Isolate no.	Size	Shape	Texture	Elevation	Margin	Pigmen-tation
CM3	medium	oval	viscous	raised	entire	greenish
CM4	medium	irregular	dry	flat	irregular	off-white
CM5	small	round	moist	flat	entire	yellowish
CM6	small	round	smooth	convex	entire	whitish

The biochemical characteristics of the bacterial isolates, CM3, CM4, CM5, and CM6 were determined through a series of tests as mentioned earlier. The results are summarized in Table 4. Isolates CM4 and CM6 showed positive results for starch hydrolysis, indicating their ability to hydrolyze starch. Isolates CM3 and CM5 tested negative for starch hydrolysis. Isolate CM3 displayed a red slant and a red butt (R/R), indicating no fermentation of the sugars present in the medium. Isolate CM4 and CM5 exhibited a yellow slant and a red butt (Y/R), suggesting glucose fermentation only. Isolate CM6 demonstrated a yellow slant and a yellow

butt (Y/Y), indicating the fermentation of glucose, lactose, and/or sucrose. All the isolates tested negative for gas and H_2S production in the TSI test. Conversely, all the isolates showed positive results for citrate utilization and catalase tests. Isolates CM3, CM4, and CM6 were oxidase positive, and isolate CM5 was oxidase negative. These biochemical tests provided a preliminary identification of the isolates, indicating the diversity of bacterial species.

Table 4: Biochemical test results of the Cr-tolerated isolates.

Isolate no.	Starch hydrolysis	TSI				Citrate	Catalase	Oxidase	Presumptive identification
		Slant	Butt	Gas	H2S				
CM3	-	K	K	-	-	+	+	+	*Pseudomonas* sp.
CM4	+	A	K	-	-	+	+	+	*Bacillus* sp.
CM5	-	A	K	-	-	+	+	-	*Staphylococcus* sp.
CM6	+	A	A	-	-	+	+	+	*Bacillus* sp.

Note: TSI, Triple Sugar Iron; K, alkaline; A, acidic.

Based on the colony morphology, biochemical characteristics and Gram reaction by the Gram staining method. Isolates CM3, CM4, CM5 and CM6 were presumptively identified as *Pseudomonas* sp., *Bacillus* sp., *Staphylococcus* sp. and *Bacillus* sp., respectively (Table 4).

Chromium-tolerant bacteria were isolated from poultry litter, and these isolates were assessed at varying Cr concentrations (50 mg/L to 1600 mg/L). Among the four isolated bacteria, one isolate (CM4) exhibited the highest tolerance to Cr concentration, showing positive growth up to 800 mg/L. Another isolate (CM6) demonstrated positive growth up to 400 mg/L. Additionally, isolates CM3 and CM5 grew at a maximum concentration of approximately 200 mg/L (Table 5).

Table 5: Isolated microbial growth on different Cr concentrations.

Isolate no.	Concentration (mg/L)					
	50	100	200	400	800	1600
CM3	+++	++	+	-	-	-
CM4	+++	++	+	+	+	-
CM5	+++	++	+	-	-	-
CM6	+++	++	++	+	-	-

The next experiment was conducted solely with the CM4 isolate, as it demonstrated the highest tolerance in the previous experiment. Figure 3 shows the growth of the CM4 isolate measured by absorbance at 600 nm across a range of chromium concentrations from 0 mg/L to 3200 mg/L. The data indicated that the absorbance, and consequently the growth of CM4, decreases as the chromium concentration increases. At 0 mg/L, the absorbance is at its peak (approximately 0.15), indicating robust growth in the absence of chromium. A significant drop in absorbance is observed as the chromium concentration reaches 25 mg/L. Further increases in Cr concentration (50 mg/L to 3200 mg/L) result in very low absorbance values, indicating minimal to no bacterial growth at these higher concentrations. This trend demonstrates the inhibitory effect of Cr on the growth of the CM4 isolate, with the highest concentrations nearly completely inhibiting growth.

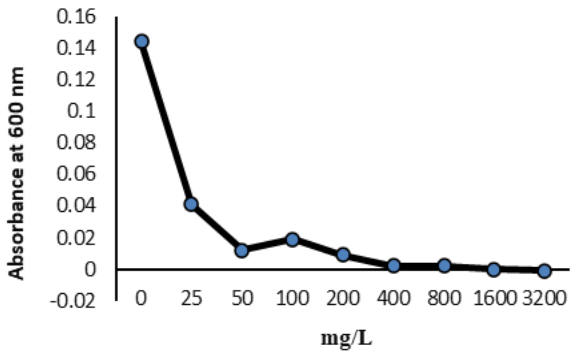

Figure 3: Growth of CM 4 isolates at increasing chromium concentrations. Absorbance was measured at 600 nm.

DISCUSSION

The isolation of Cr-tolerant bacteria from chicken litter underscores the environmental impact of tannery waste disposal and its unintended consequences on animal feed. The presence of Cr-tolerant microorganisms in the chicken litter suggests that chickens, through their diet, serve as a medium for the proliferation of these bacteria. This finding aligns with previous studies that have highlighted the role of contaminated environments in fostering microbial resistance to heavy metals (14, 15).

In this study, four bacterial isolates namely CM3, CM4, CM5, and CM6 were identified, with CM4 exhibiting the highest Cr tolerance, demonstrating growth up to 800 mg/L. This is significant, as previous research has indicated that 100 to 250 mg/L are minimum inhibitory concentrations (MIC) for most bacterial species (16). The ability of CM4 to thrive at such high concentrations suggests a potential for bioremediation applications, particularly in environments with severe Cr contamination.

The observed decrease in bacterial growth with increasing Cr concentrations, as indicated by the absorbance data, highlights the toxic effect of Cr on bacterial cells. Cr, particularly in its hexavalent form (Cr (VI)), is known to disrupt cellular processes by generating reactive oxygen species and interfering with DNA replication and repair mechanisms (17). The significant drop in growth at concentrations as low as 25 mg/L suggests that while CM4 is tolerant, it is not immune to the detrimental effects of Cr, particularly at higher concentrations.

The identification of *Bacillus* sp. among the isolates is particularly noteworthy, as this genus has been widely recognized for its role in bioremediation due to its resilience and ability to form endospores under harsh environmental conditions (18-20). Cr-tolerant *Bacillus* sp. in chicken litter may reinforce the potential of using these bacteria in bioremediation strategies to reduce Cr pollution. These bacteria possess the ability to survive and thrive in environments contaminated with chromium, suggesting their potential for use in mitigating the environmental impact of Cr. Chicken litter, being a nutrient-rich and readily available agricultural waste product, could serve as an effective medium for cultivating Cr-tolerant *Bacillus* strains. The use of these bacteria in bioremediation processes not only contributes to the detoxification of contaminated soils and water but also aligns with sustainable waste management practices, offering a dual benefit in environmental conservation. Further research into the mechanisms of Cr resistance and remediation capacity of these *Bacillus* species will be essential in optimizing their application in real-world pollution control efforts.

CONCLUSION

In conclusion, the isolation and characterization of Cr-tolerant bacteria from chicken litter present promising prospects for bioremediation. The CM4 isolate, in particular, could be further studied and potentially developed into a bioremediation agent to mitigate Cr pollution in contaminated environments. Further research is needed to explore the genetic and biochemical mechanisms underlying the Cr tolerance exhibited by these isolates, which could enhance their application in environmental cleanup efforts.

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