Research Article

Isolation and 16S Ribotyping of Heavy Metal Tolerant Bacterial Strain from Leather Industry Effluent

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Abstract | The release of untreated tannery wastewater poses serious environmental and health risks due to the presence of high concentrations of heavy metals and toxic organic compounds in the wastewater. This study aimed to isolate and molecular identification of bacterial strain from tannery effluents collected from Sialkot, Pakistan. Bacteria were isolated and cultured by using serial dilution on Luria Broth agar. Genomic DNA was extracted through the phenol chloroform method PCR amplification and Sanger sequencing of the 16S rRNA gene were performed. Physicochemical analysis of the effluent revealed higher values of pH (09±1), BOD (Biochemical Oxygen Demand) and (107.89±2 mg/L), COD (Chemical Oxygen Demand) (166.43±2 mg/L) and EC (208.17±2 µS/cm). Heavy metal profiling indicated chromium (27mg/L±1), chloride (17.5mg/L±2), Iron (4.7 mg/L±2), Zinc (1.54mg/L±2), Nickle (0.455mg/L±2) and copper (0.3225mg/L±1). Morphological analysis revealed that bacteria are Gram-positive *Bacillus* species. BLAST analysis of obtained sequence showed 91% similarity to *Bacillus tequilensis*. Subsequently submitted to NCBI under the accession number *PQ141696*. The detection of heavy metal–resistant *Bacillus tequilensis* indicates its potential for absorbing toxic metals including Cr, Cu, Pb, Ni, Fe, and Zn. These results help in understanding native bacterial communities in industrial wastewater and may support the development of eco-friendly treatment approaches in the future.

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Introduction

Water is an indispensable resource that serves as the foundation for all life on Earth and is vital for the existence of all living organisms, ranging from minuscule microbes to massive mammals (Kılıç, 2020). The problem switches when water is being contaminated through different sources. The major contribution of contaminants are industries that release untreated waste water to water bodies and these contaminants become part of food chain. On the other hand, the establishment of industries are essential

for the economic and infrastructural development of countries (Rahman and Bakar, 2019) yet the environmental challenges they pose, particularly in wastewater management, cannot be overlooked. The release of untreated tannery wastewater poses serious environmental and health risks due to the presence of high concentrations of heavy metals and toxic organic compounds in the wastewater (Humayra, 2023).

To meet the challenge of cost and sustainability, industries are adopting the biological methods, such as bioremediation or constructed wetlands, which use natural processes to degrade pollutants more efficiently (Ali, 2022). The origin of harmful metal pollution in rivers is ascribed to companies that release their waste without carrying out recycling activities (Ilyas *et al*., 2019). The tanning industry, which deals with the processing of animal skin, is identified as one of the most harmful industries to the environment (Rivela *et al*., 2004). The leather processing industry uses a substantial quantity of water. It is well known that most leather manufacturers use compounds that contain chromium (Cr), making this industry highly polluted. Chromium (Cr) is a toxic heavy metal that has the potential to cause carcinogenic effects (Ahmed *et al*., 2021). Tanning waste is highly polluted with many substances such as salt, lime sludge, sulfides, and acids. If the tanning process did not conserve the collagen or protein fibers, then the leather would deteriorate in the closet because it prevents them from being biodegradable (Khalid *et al*., 2021).

In contrast to other materials used in fashion, the environmental damage caused by leather is substantial, and it is a major ecological issue. Eutrophication is the consequence of too much plant life in water bodies, which is caused by waste materials, making the water body have less oxygen, thereby asphyxiating organisms. The generation of hypoxic zones, commonly known as dead zones, is a major environmental issue linked to industrial pollution. Such pollution is often associated with activities like cattle rearing and related industrial processing, which contribute to greenhouse gas emissions, land use pressure, and deforestation (Mammadova *et al*., 2020; Amde, 2017).

Exposure to toxic industrial chemicals, particularly chromium, can cause serious health issues including skin irritation and increased cancer risk among exposed workers (Junaid *et al*., 2017; Koki *et al*., 2017). In addition, Hexavalent chromium can enter the ecosystem, leading to major contamination of soil and water (Srivastava *et al*., 2021). heavy metal–contaminated wastewater negatively affects plant systems by reducing seed germination, root growth, and biomass production, while also disturbing normal metabolic activities (Malik *et al*., 2003; Sarker *et al*., 2013).

tannery and chromium-rich wastewaters are most frequently dominated by chromium- and heavy metal–resistant bacteria**,** especially *Bacillus spp.*, *Pseudomonas spp.*, *Klebsiella spp.*, and *Enterobacter spp.*, which are widely reported for their survival and bioremediation potential in such environments (Hossain and Anwar, 2016; Ashraf *et al*., 2018; Kabir *et al*., 2018).

The study addresses the gap in limited characterization of chromium-resistant bacteria from local tannery effluents, particularly their identity and tolerance potential. This is achieved through isolation of native strains followed by molecular identification (e.g., 16S rRNA sequencing) and assessment of heavy metal tolerance under laboratory conditions. The advantage of this work is the identification of indigenous, adapted bacterial strains with potential application in low-cost and eco-friendly bioremediation of chromium-contaminated wastewater.

Aim of the study

This study aimed to isolate and molecular identification of bacterial strain from tannery effluents collected from Sialkot, Pakistan

Materials and Methods

Sample collection and physicochemical analysis

Wastewater samples were collected from multiple leather tanneries located in Sialkot, Pakistan. Samples were aseptically transferred into sterilized screw-capped bottles and transported to the laboratory at Department of Botany, Government College University, and Lahore. Samples were stored at 4 °C until further analysis. Physicochemical parameters including pH, Electrical Conductivity (EC), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), and heavy metal concentrations (Cr, Ca, Fe, Zn, Ni, Cu) were measured using standard protocols (Gana *et al*., 2022). Heavy metal quantification was performed using Atomic Absorption Spectroscopy (AAS).

Bacterial isolation

Serial dilution plating was employed to isolate culture bacteria from the wastewater samples (Ben-David and Davidson, 2014). Nine serial dilutions were prepared using distilled water as a sterile diluent. From each dilution, 100 µL was plated onto Luria-Bertani (LB) agar prepared with the following composition per liter: tryptone (10 g), yeast extract (5 g), NaCl (10 g), and agar (15 g). The autoclaving process at 121°C for 15 minutes with 15 psi pressure was employed for sterilization of the medium (MacWilliams *et al.*, 2006). The plates were cooled before inoculation and incubation at 37°C for 48 hours. Pure culture refers to a culture that contains only one species of bacteria. For isolation of the pure culture, the dominant colony was selected and sub cultured by the streak plate technique (James *et al.*, 2017).

Morphological and Gram Staining Characterization

Bacterial isolates were differentiated based on the composition of their cell walls using Gram staining. Gram staining was performed using the standard Gram staining protocol (Gulzar *et al.*, 2026) Oil immersion was used to examine the slides under a light microscope at 100X magnification (Coico, 2006).

Genomic DNA Extraction/ Agarose Gel Electrophoresis

Modified phenol-chloroform method was employed for the extraction of genomic DNA (Brewster and Paoli, 2013). 1% agarose gel electrophoresis was employed for the assessment of DNA integrity. The UV trans illuminator was employed for visualization of the bands (Armstrong and Schulz, 2015).

PCR Amplification/phylogenetic analysis

PCR amplification of the 16S rRNA gene was performed using universal primers: 27F (5′AGAGTTTGATCCTGGCTCAG-3′) and 1492 R (5′-TACGGYTAC CTTGTTA CG ACTT-3′) (Rahman *et al.*, 2013). Gradient PCR was employed to optimize the annealing temperature for specific amplification (Thomas *et al.*, 2017). The amplicons were validated by gel electrophoresis. Validated PCR products were submitted to Macrogen, Seoul, South Korea.

The obtained sequences were analyzed by NCBI BLAST for taxonomic identification. Sequence alignment and phylogenetic analysis were conducted by MEGA 11 software. The obtained sequence was submitted to GenBank.

Results

Physicochemical analysis of tannery wastewater

All the effluent samples showed high values of key physicochemical parameters. The values are shown in Table 1. The values are higher than the permissible levels, indicating high organic and ionic content in the wastewater.

Table 1: *Physiochemical analysis of waste water.*

Parameters	Standard values	Mean value
BOD (mg L-1)	250	571 ±0.01
COD (mg L-1)	400	570 ±0.05
EC (S m-1)	1000	1566.66 ±0.02
T (°C)	40	51.66 ±0.05

Heavy metal profiling

Some heavy metals were detected in the following descending order based on atomic absorption spectroscopy: Cr > Ca > Fe > Zn > Ni > Cu. Chromium (Cr) has the highest concentration, and this is expected due to its wide application in leather tanning industries. The need for bioremediation methods is evident by the presence of these metals. pH (09±1), EC (208.17±2 µS/cm), COD (166.43±2 mg/L), and BOD (107.89±2 mg/L). Based on Figure 1, the highest concentration was recorded in chromium (27 mg/L±1), chloride (17.5 mg/L±2), iron (4.7 mg/L±2), zinc (1.54 mg/L±2), nickel (0.455 mg/L±2), and copper (0.3225 mg/L±1) respectively as shown in Figure 1.

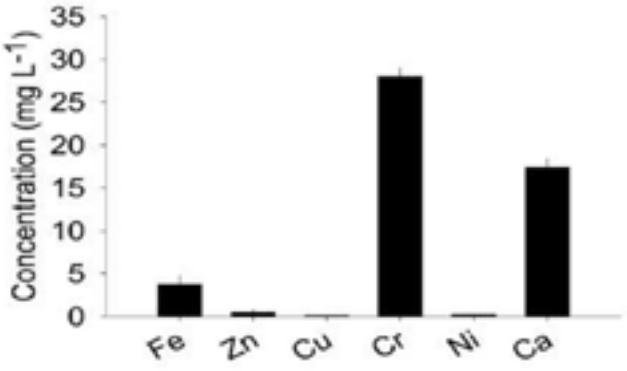


Figure 1: *Heavy metals concentration in Tannery waste water by using Atomic absorption spectrophotometer (AAS).*

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Bacterial isolation and morphological characterization

Serial dilution and plating on LB agar resulted in the growth of distinct bacterial colonies. Dominant colonies were selected and purified using the streak plate method. Gram staining and subsequent microscopic observation revealed rod shaped Gram-positive bacteria as shown in Figure 2. Presumptive genus was *Bacillus*.

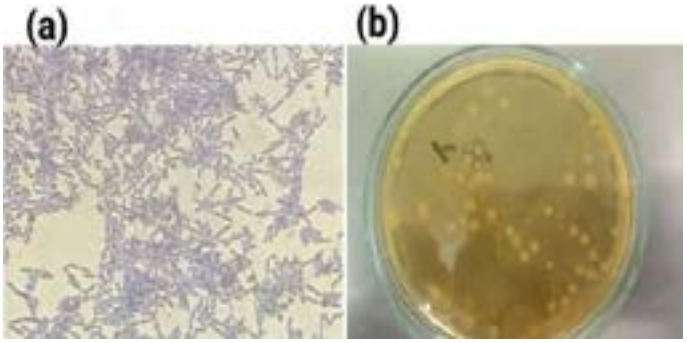


Figure 2: *Identification and characterization of bacteria (a) Gram-positive Bacillus tequilensis species observe under Microscopic at 10X (b) growth of bacteria on nutrient agar media*

BLAST Analysis for molecular identification via 16s rrna gene sequence

Genomic DNA was successfully extracted using the phenol-chloroform method and verified through 1% agarose gel electrophoresis as shown in Figure 3a. PCR amplification of the 16S rRNA gene using universal primers (27F and 1492R) yielded a clear band of expected size 1348 base pairs. Figure 3a and 3b. NCBI BLAST was used to identify the bacterial strain through obtained gene sequence. Results showed 91% sequence similarity with *Bacillus tequilensis*.

Gene sequence was deposited in NCBI GenBank under the accession number PQ141696.

Phylogenetic analysis

The phylogenetic tree was constructed using the 16S rRNA gene sequence in MEGA 11 software by the Maximum Likelihood method. The resulting tree is shown in Figure 4.

Discussion

The tannery industry in Sialkot, Pakistan, is a major source of environmental pollution due to the discharge of untreated wastewater containing high concentrations of hazardous materials and heavy metals. In this study, microbiological and molecular techniques were used to isolate and identify bacterial strains from tannery effluent to understand their diversity, adaptability, and potential role in bioremediation.

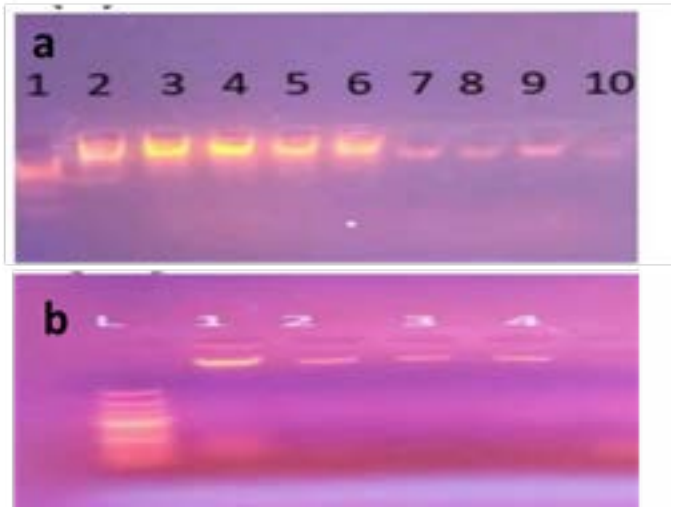


Figure 3: *16S rRNA gene amplification from bacterial isolates (a) DNA bands seen on gel electrophoresis (b) polymerase chain reaction (PCR) amplification results*



Figure 4: *Maximum likelihood phylogenetic analysis of the query isolate based on the Tamura–Nei model using MEGA 11*

Gram staining confirmed the presence of Gram-positive *Bacillus* species in the isolated samples. These findings are consistent with previous studies (Saha *et al.*, 2015) which reported that *Bacillus* species are commonly associated with tannery effluents due to their ability to survive in heavy metal-rich environments. Several *Bacillus* species such as *Bacillus subtilis*,

Bacillus brevis, *Bacillus pumilus*, *Bacillus alcalophilus*,

Bacillus badius, *Bacillus firmus*, and *Bacillus lentus* have also been documented in similar studies (Hauwa *et al*., 2023).

Bacillus tequilensis is a Gram-positive, spore-forming bacterium initially found in fermented agave, later identified in contaminated soil. It thrives in harsh environments, including those with heavy metals, and produces industrially important enzymes. Research indicates its potential in bioremediation (Gómez-Gil *et al*., 2008; Singh *et al*., 2019). These traits suggest that the identified strain might survive in tannery effluent and could aid in detoxification and environmental sustainability.

The universal primers (27F and 1492R) targeting the 16S rRNA gene were found to be effective for PCR amplification. These results are in agreement with (Sujatha *et al*., 2012) who successfully identified *Bacillus thuringiensis* from tannery effluent using the same primer set.

Heavy metal analysis using atomic absorption spectroscopy revealed the presence of Cr, Fe, Ni, Cd, Zn, and Mg in wastewater samples. Among these, chromium was found at the highest concentration. The presence of heavy metals in wastewater poses serious risks to human health and agricultural productivity. Chromium, widely used in leather tanning, is a known carcinogen and has been reported to inhibit seed germination and reduce crop yield (Khatun *et al*., 2024).

Several studies have also reported elevated levels of Cr, Cd, Pb, Zn, Cu, Fe, and Ni in tannery effluents, highlighting the need for effective treatment strategies.

Bioremediation is considered a cost-effective and eco-friendly approach for treating such contaminated wastewater. Various bacterial strains, including *Citrobacter freundi*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Bacillus subtilis*, *Lactococcus lactis*, *Bacillus megaterium*, *B. carboniphilus*, and *B. licheniformis*, have shown potential for heavy metal bioabsorption (Wróbel *et al*., 2023).

As stated by (Rameshkumar *et al*., 2017) the present study supports that *Bacillus tequilensis* isolated from tannery effluent possesses physiological and genetic characteristics suitable for bioremediation. The successful amplification and sequencing of the 16S rRNA gene further confirm its molecular identity and adaptability. With increasing industrialization, contamination of freshwater resources is expected to rise, making it essential to explore microbial-based remediation strategies (Kumar *et al*., 2025).

Molecular identification through phylogenetic analysis provide taxonomic classification and promotes the discovery of novel strains for potential applications in environmental biotechnology, industrial microbiology, and agriculture. Understanding the microbial diversity of tannery effluents may ultimately support the development of microbial consortia for bioremediation, contributing to environmental restoration and public health protection.

Conclusions and Recommendations

The isolation and molecular identification of a bacterial strain from tannery effluent samples collected from Sialkot, Pakistan, were effectively demonstrated in this study. Physicochemical analysis confirmed the toxic nature of the wastewater with elevated BOD, COD, EC, and heavy metals such as Cr, Ni, Cu, and Zn. The isolated *Bacillus tequilensis* demonstrated strong adaptability to heavy metal–contaminated environments and potential for bioremediation. Molecular identification confirmed its taxonomic identity. The use of indigenous metal resistant bacteria offers a sustainable and cost-effective approach for wastewater treatment. Future studies should aim at identifying the functional genes responsible for metal resistance and investigating the potential use of these strains in designed bioreactors or microbial communities for wastewater treatment.

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Novelty Statement

This study reports the isolation and 16S rRNA-based molecular identification of a native heavy metal–tolerant bacterial strain from tannery effluent in Sialkot, Pakistan. The identified strain, Bacil-lus tequilensis, demonstrated tolerance to multiple heavy metals, indicating its potential

for biore-mediation of industrial wastewater. These findings provide baseline information for the future development of sustainable and eco-friendly wastewater treatment strategies.

Authors' Contributions

This work was carried out through the collective efforts of all authors involved.
Muhammad Umer Farooq Awan and Wasim Abbas: supervised and conceptualize the study.
Annum Gul: performed formal analyses and investigation.
Muhammad Khizar Sultan: performed data curation.
Ali Hasnain, Ammara Ahsan, Muhammad Shoib, Ramna Gulzar, Samra Rafiq, Maryam Iqbal, Awais Riaz, Adeel Ahmad and Aqsa Ahmad: Reviewed and edited the manuscript.
All authors reviewed and approved the final manuscript.

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Data availability
All data analyzed during this study are included in this article.
Declarations

Generative AI and AI-assisted technology statement
The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Ethical approval
Ethical approval was waived because the study did not involve human participants or animal experiments.

Conflict of interests
The authors declared no conflicts of interest related to this article.

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