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Isolation and Characterization of Bioremediation Potent Microorganisms from Spectrophotometrically Analysed Heavy Metal (Cr and Cd)-Rich Tannery Effluent.

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ABSTRACT

Microorganisms can become tolerant and highly efficient in degrading toxic heavy metals that cause environmental pollution. This study focuses on the isolation of hexavalent Cr and Cd (II)-resistant bacteria collected from tannery effluents. The isolation was conducted by spread plate method on Agar plates supplemented with their respective heavy metal salts ($K_2Cr_2O_7$ and $CdCl_2$). A total of ten isolates were screened of which, two were subjected to 16sRNA sequencing on the basis of the degree of their resistance to heavy metals. The bacteria, identified from the isolates, were *Flavobacterium psychrophilum* and *Flavobacterium sp. ARSA-103*, which can be used for bioremediation of metal-contaminated effluents.

Keywords: Bioremediation, Heavy Metals, 16sRNA, Atomic absorption spectrophotometry (AAS).

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INTRODUCTION

Uncontrolled urbanization and industrialisation has caused a serious pollution problem due to the disposal of sewage and industrial effluents without proper treatment [1]. Tanning is one of the oldest industries and the treatment and disposal of wastes need to be addressed for maintaining a clean environment [2]. A variety of chemicals are used in the tanning industries, including CaO [lime], NaCl, Na_2CO_3, NH_4Cl, H_2SO_4, tannins and dyes. The dyes used are mainly mineral tanning agents and contribute to pollution. All tanneries need a large amount of water for processing that is supplied by ground water. The discharged effluents from the processing units are stored in large lagoons and pollution occurs as the dissolved salts percolate into the surrounding soil. Thus, the ground water sources are exploited to their fullest potential and polluted to a great extent. Therefore, severe pollution results from a cluster of tanneries in close proximity.

Accumulation of heavy metals [for example, Cu, Cd, Zn, Pb, Ni, Hg and Cr] in soil and water bodies can occur in concentrations that are toxic to plants, animals, humans and aquatic life [3]. Heavy metals present in the wastes are difficult to remove from the environment. Heavy metals have antimicrobial properties using which they can inhibit biodegradation activities [4]. Hence, these heavy metals are non-degradable. The heavy metals interact with enzymes by disrupting their active sites leading to disorder of metabolic activities. Every metal has its unique bio functions and bio toxicities; for example, Cu can enhance the growth of microbes at low concentrations, but suppress their growth at high concentrations. On the other hand, Cd suppresses microbial growth at low concentrations [5]. The presence of non-biodegradable heavy metals in the effluent leads to their existence in the food chain [1]. The permissible limits for Cd and Cr as given by Occupational Safety and Health Administration are 15 µg/l and 500 µg/l, respectively [1].

Microbes play an important role in the bio-geochemical recycling of heavy metals and in cleaning up or remediation of metal-contaminated environments [5]. Microorganisms have adopted a variety of mechanisms for adapting to toxic heavy metals [6,7]. The evaluation of resistance to metals is a complex process. Aquatic microbes become resistant to metals as a result of contamination with effluents. The significant increase of Multiple Metal Resistant [MMR] bacteria is observed in various aquatic systems [8,9]. Human diseases caused by such bacteria could be difficult to treat with drugs [8-11]. The resistance development may be due to nonspecific mechanism involving gene regulation of plasmids and chromosomes, which may be transferable to other microbes due to the presence of a resistance [R]-factor [12]. To survive in metal-stressed conditions, bacteria have used various types of mechanisms to tolerate heavy metal ions. These mechanisms include the efflux of metal ions outside the cell and reduction of the heavy metal ions in a less toxic state. The objective of this study is to isolate and characterize microorganisms from tannery waste water which have heavy metal resistant potential, so we can use those microorganisms in treatment of heavy metals by metabolizing the heavy metals into simpler forms which are not harmful for living beings [6,7]. In this research, the microorganisms were isolated and screened from two tanneries sample which were located in Jalandhar, a city in the north western Indian province, Punjab. It was expected that the isolated and screened microorganisms would demonstrate their ability to survive in adverse conditions and convert the heavy metals into less harmful products.

METHODS AND MATERIALS

Collection of samples from tannery effluent

For bacteriological analyses, the samples were collected from common effluent treatment plants in Jalandhar and transported to the Biotechnology laboratory at Lovely Professional University. The samples were collected in a sterile plastic container. The samples were stored at 4 ^{o}C and were used within 24 hours for isolation of microbes.

Description of Atomic Absorption Spectrometry (AAS)

Samples were placed in air tight containers and sent for AAS located at the Punjab Agricultural University (PAU) in Ludhiana, for the determination of Cd and Cr concentration in the samples. This technique makes use of absorption spectrometry to assess the concentration of an analyte in a sample. It works on the Beer-Lambert law in which the electrons of the atoms in the atomizer can be promoted to higher orbital's (excited state) for a short period of time (nanoseconds) by absorbing a defined quantity of energy (radiation of

a given wavelength). This amount of energy is specific to particular electron transition in a particular element [13].

Isolation of microbes

The bacterial isolates were screened on Nutrient Agar (NA) plates supplemented with 5 mg/l concentration of each metal (Cr and Cd) at one time by the standard spread plate method [14]. Plates were incubated at 37°C for 24 hours and colonies differing in morphological characteristics were selected and used for further studies.

Minimum inhibitory concentration (MIC)

Metal tolerance was evaluated as the MIC of metals such as Cr and Cd. The metal tolerance was determined for different bacterial isolates by the streak plate method. Different concentrations (5 mg/l to 420 mg/l) of each metal were used to check the MIC's for each isolate. Based on the evaluation, MIC was determined at 37°C in every 24 hours. The minimum concentration of heavy metals, at which no growth was observed, was considered as the MIC of bacterial isolates against heavy metals [15].

Identification and characterization of the tannery effluent bacteria

Selected tannery effluent isolates were grown on Nutrient Agar (NA). The shape and colors of the colonies were examined under an optical microscope after Gram staining. Isolates were biochemically analyzed for the activities of Oxidase, Catalase, MR-VP test, Urease test, Gelatin test, Starch hydrolysis test, Indole production and Citrate utilization. The tests were used to identify the isolates according to Bergey's Manual of Determinative bacteriology [16,17]. After identification of isolates they were stored as glycerol stocks in Biotechnology laboratory of Lovely Professional University.

16S rRNA sequencing of isolates

16S rDNA (rRNA) sequencing plays a very important role in the accurate identification of bacterial isolates and the discovery of novel bacteria. It is particularly important in case of bacteria with unusual phenotypic profiles. In this case, the first step of the 16S rDNA sequencing was to separate the gDNA from the isolates. Then the evaluation of the quality was done in 1-2% Agarose Gel. A fragment of 16S rDNA gene was amplified by Polymerase Chain Reaction from the isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel that was continued with purification of PCR amplicon in order to remove the undue containments. The forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 27F and 1492R primers using BDT v3.1 cycle sequencing kit on an ABI 3730xl Genetic Analyser. Consensus sequence of 1468p rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out Basic Local Alignment Search Tool with the database of the National Center for Biotechnology Information Genbank database. Based on the maximum identity score, first ten sequences were selected and aligned using multiple alignment software program. Clustal W. Distance matrix was generated using RDP [18-22].

RESULTS AND DISCUSSION

The AAS analyses of the samples indicated that Sample 1had 0.051 mg/l of Cd, the concentration of Cr being 220.3 mg/l and Sample 2 had 0.002 mg/l of Cd and 26.96 mg/l of Cr which is above the permissible limits for Cd and Cr for both the samples.

Bacterial strains were isolated from tannery effluent. Ten isolates were screened from initial level of heavy metal supplemented NA medium. Images of the ten isolates are shown in Fig.1. The screening of the isolates was conducted on the basis of their biochemical and morphological properties (Table 1). The ten bacterial isolates were analysed as follows: Isolate 1 was *Neisseria*, Isolate 2 was *Staphylococcus,* Isolate 3 was *Staphylococcus,* Isolate 4 was *Flavobacterium,* Isolate 5 was *Nocardia*, Isolate 6 was *Flavobacterium*, Isolate 7 was *Corneybacterium*, Isolate 8 was *Streptococcus*, Isolate 9 was *Staphylococcus* and Isolate 10 was *Citrobacter.*

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Table 1: Characteristics of bacterial isolates from tannery effluents.

	Is. 1	Is.2	Is.3	Is.4	Is.5	Is.6	Is.7	Is.8	Is.9	Is.10
Indole test	-	-	-	-	+	+	+	+	+	+
Methyl Red Test	+	+	+	+	-	+	+	+	+	+
Vogues Proskauer Test	-	-	-	-	-	-	-	-	-	-
Citrate Utilisation Test	+	+	+	-	-	-	-	-	+	+
Gram Staining	-	+	+	-	+	-	+	+	+	-
Catalase Test	+	+	+	+	+	-	+	-	+	+
Starch Hydrolysis Test	+	-	+	-	-	-	-	+	+	+
Gelatine Hydrolysis Test	+	-	+	-	-	-	+	+	-	+
Urease Test	-	-	-	-	-	-	-	-	-	+

+ symbol depicts that the microorganisms gave a positive result, - symbol depicts a negative result. After performing these Biochemical tests, the isolates were Is.1: *Neisseria,* IS.2: *Staphylococcus,* IS.3: *Staphylococcus,* IS.4: *Flavobacterium,* IS.5: *Nocardia,* IS.6: *Flavobacterium,* IS.7*: Corneybacterium,* IS.8: *Streptococcus,* IS.9*: Staphylococcus,* IS.10: *Citrobacter*.

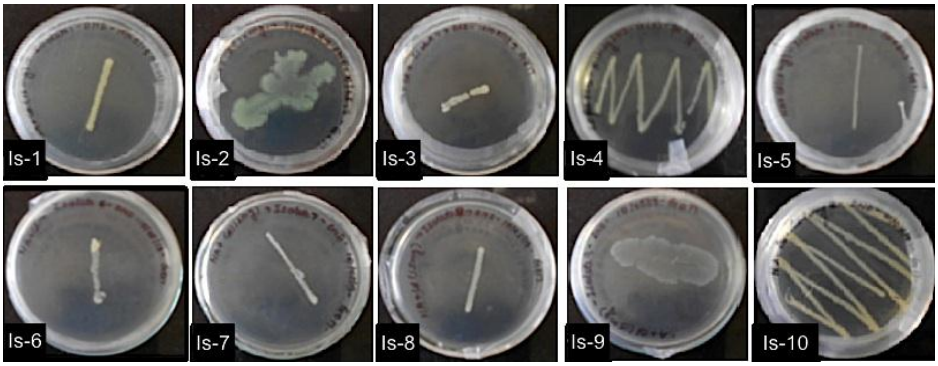

Figure 1: Images of the pure isolates.

The MIC for all isolates was found to vary between 9 to 39 mg/l for Cd, and 214 to 411 mg/l for Cr. It was found that Isolate 4 (*Flavobacterium)* has the highest MIC for Cd, therefore, having high resistance to Cd as seen in Fig.2. The Isolate 6 (*Flavobacterium)* depicted the highest MIC for Cr, hence a high resistance to Cr. It was observed that Cd was more toxic at lower concentrations as compared to Cr. Hence the permissible limits of Cd should be less than that of Cr.

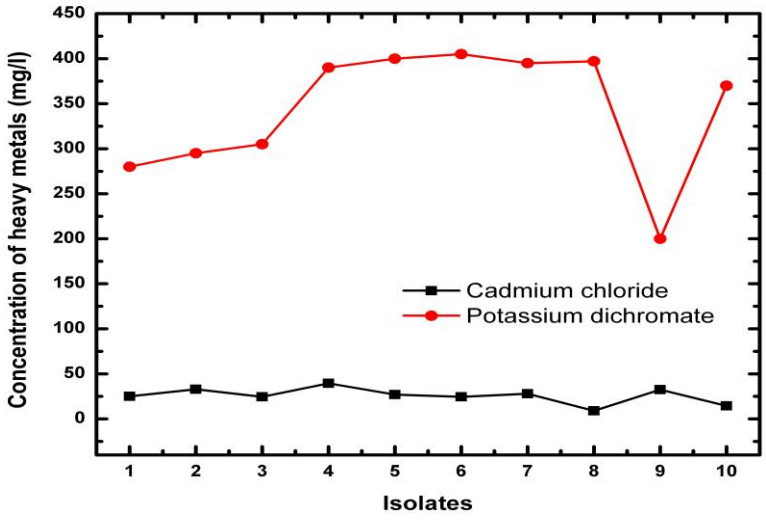

Figure 2: Comparison of the minimum inhibitory concentrations (MIC) of Cr and Cd.

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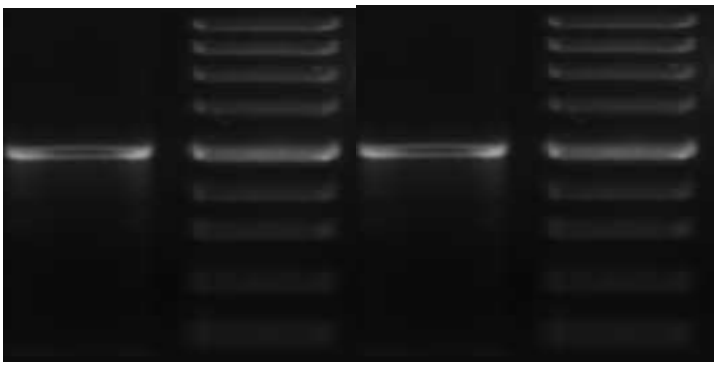
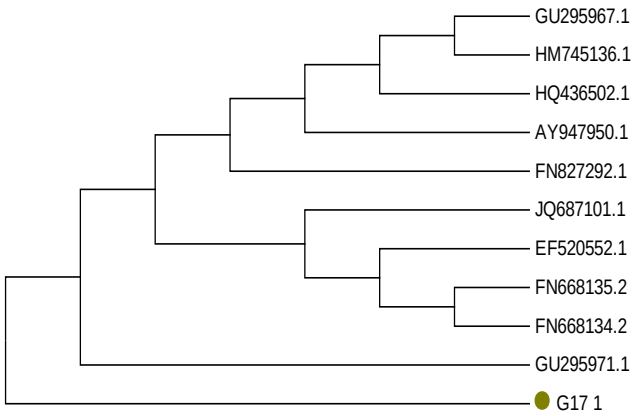
Isolate 4 sent for 16S rRNA was characterised as *Flavobacterium sp. ARSA-103* 16S ribosomal RNA gene (GenBank Accession Number: KJ451477) based on nucleotide homology and phylogenetic analyses (Figs.3a and 4a). A phylogenetic tree or evolutionary tree shows the evolutionary relationships among various biological species based upon similarities and differences in their physical and/or genetic characteristics. The phylogenetic tree shown in Fig.4 shows the relationship of 11 taxa based upon similarities and differences in their physical and/or genetic characteristics. The taxa joined together in the tree are inferred to have derived from a common ancestor. Isolate 6 were characterised as *Flavobacterium psychrophilum strain FLpR 010/09* 16S ribosomal RNA gene (GenBank Accession Number: KJ451476) based on nucleotide homology and phylogenetic analyses (Figs.3b and 4b).

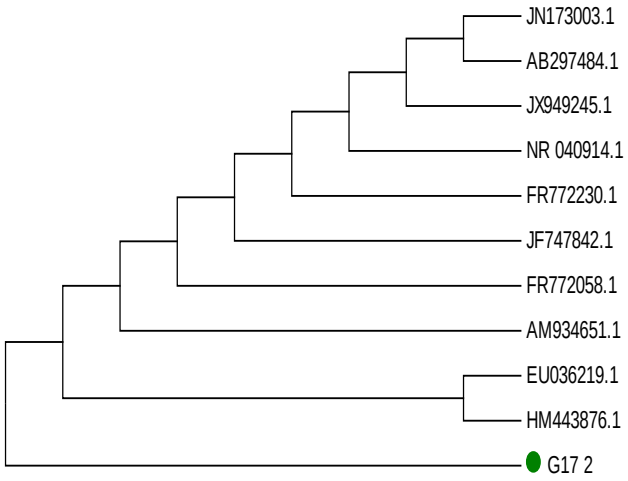


(a) (b)

Figure 3: Gel images of 16SrDNA amplicon of (a) Isolate 4 and (b) Isolate 6; Lane 1: 16S rDNA amplicon band; Lane 2: DNA marker.



(a)



(b)

Figure 4: Phylogenetic trees of (a) Isolate 4 and (b) Isolate 6.

It was noticeable that different bacterial strains such as Isolate 1 (*Neisseria)*, Isolate 2 (*Staphylococcus),* Isolate 3 (*Staphylococcus),* Isolate 4 (*Flavobacterium),* Isolate 5 (*Nocardia)*, Isolate 6 (*Flavobacterium)*, Isolate 7 (*Corneybacterium)*, Isolate 8 (*Streptococcus)*, Isolate 9 (*Staphylococcus)* and Isolate 10 (*Citrobacter)* are showing tolerance to two heavy metals viz. Cd and Cr. The tolerance of the isolates to heavy metals is neither lost nor altered when the isolates were stored in NA at 4 ^{o}C. The MIC determined for the two heavy metals varied between 5mg/l to 420 mg/l depending on the metal and bacterial isolate. This study showed a high incidence of metal resistance for the bacterial isolates. Many bacterial species isolated from industrial zones were shown to develop resistance to heavy metals [1]. The increase in the MIC of metals among the bacterial population in any system could be a sign of risk to the ecosystem and all living species.

In most of the studies, metal resistance has been reported to hold an association with antibiotic resistance [23]. In the current study, it is believed that the isolates that were resistant to heavy metals have some properties of antibiotic resistance. Due the presence of metals, metal and antibiotic resistance in microorganisms helps them to survive longer by the spread of resistant factors rather than by mutation and natural selection [24]. Isolates had different resistant factors against heavy metals; for example, a low concentration of Cd was able to inhibit the growth of microorganisms whereas Cr inhibited growth only at higher concentrations. Hence, Cd is more toxic than Cr at lower concentrations (Fig.2). From 16S rRNA sequence analyses, Isolate 4 was found to be *Flavobacterium sp. ARSA-103* and Isolate 6 was *Flavobacterium psychrophilum strain FLpR 010/09.*

This study revealed the capacity of the bacterial strains viz. *Flavobacterium psychrophilum strain FLpR 010/09* and *Flavobacterium sp. ARSA-103* to tolerate and grow at different concentrations of heavy metals (Cd and Cr). According to Joseph and Walter [25] *Flavobacterium psychrophilum* which is a fish pathogen is resistant to heavy metals and can grow efficiently in the heavy metal stressed environment. Therefore it can be used to remediate heavy metals so as to make the environment heavy metal pollution free.

CONCLUSIONS

The heavy metal resistant microorganisms depicted in this work are pathogenic in nature that would cause diseases in human beings. Therefore, industries involving effluent treatment and discharge should take proper precautions while working with effluent containing these microorganisms and prevent mixture of the effluent discharge with the ground water. The microorganisms that were exposed and, thereafter, survived in the highly enriched heavy metals environment are expected to adapt to this stress by developing various resistance mechanisms. These mechanisms, on the other hand, could be utilized for the treatment and removal of heavy metals from tannery effluent [26]. According to the experimental results, this study identified and evaluated bacteria that could be used to remediate heavy metal contaminated wastewater and sewage. Further studies can be done to investigate the type of chemicals or proteins secreted by the microorganisms in order to gain resistance against heavy metals.

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