A Review of Isolation and Characterization of Heavy Metal and Antibiotic Co-resistant Bacteria from Tannery Effluent

By

SUJOY SARKER

Student ID : 16136007

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Bachelor of Science in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

Student's Full Name & Signature:

Sujoy Sarker
Student ID: 16136007

Approval

The thesis/project titled "**A Review of Isolation and Characterization of Heavy Metal and Antibiotic Co-resistant Bacteria from Tannery Effluent**" submitted by SUJOY SARKER (16136007) of Spring, 2016 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on "**Date to be given"**

Examining Committee:

Supervisor:
(Member)

_____

Romana Siddique
Senior Lecturer, Department of Mathematics and Natural Sciences
BRAC University

Program Coordinator:
(Member)

_____

Iftekhar Bin Naser, PhD
Assistant Professor, Department of Mathematics and Natural Sciences
BRAC University

Departmental Head:
(Chair)

_____

A F M Yusuf Haider, PhD
Professor and Chairperson, Department of Mathematics and Natural Sciences
BRAC University

Dedicated to

Mai (Grandmother) and Dadu (Grandfather)

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Sujoy Sarker
ID- 16136007

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A review of isolation and characterization of heavy metal and antibiotic co-resistant bacteria from tannery effluent

Abstract:

The use of heavy metals in the tannery industry for leather processing and discharge of untreated effluents from this industry in the environment is a leading challenge to solve. Hence, micro-organism has a great potential to grow in the presence of toxic substances and it is a novel, cost-effective and eco-friendly approach to remove these heavy metals (Cr, Pb, Cu, Zn) from these contaminated sites and safeguard our environment. Toxic substances are hard to degrade, and microbes develop detoxifying mechanisms to counteract these substances' toxic effects. This article reviews the isolation of the heavy metal resistant bacteria and their ability to co-resistant against the antibiotic. *Escherichia coli*, *Staphylococcus sp.*, *Klebsiella sp.*, *Pseudomonas sp.* was identified to resist toxic substances to a minimum inhibitory concentration. In some cases, the same isolates showed co-resistance to multiple heavy metals. All the isolates were also resistant to several antibiotics. My study highlights the organisms' physiological color change in response to any stimuli like heavy metals as the media is incorporated with the heavy metals. Besides, interaction with different toxic compounds is still unclear if these patterns of color change are simply the consequences of heavy metals or any underlying factor or enzyme is responsible for the color transformation.

Keywords: Heavy metal resistant bacteria, Bioremediation, Tannery Effluent, Antibiotic resistance, Co-resistance, color change

Introduction:

In developing countries for the past few years, socioeconomic development has created an industrial revolution that releases a high amount of pollution from the industrial area. Among the industries, the tannery industry has been discharging many effluents into the river without proper treatment and handling. Though the organic compounds can be cleaned by a natural process, whereas inorganic compounds such as heavy metal are tough to destruct as a result can be persisted in the environment for a long time. Metals, metalloids, lanthanides and actinides are heavy metals. It has a greater atomic density, which is greater than 5 gcm^{-3} (Nies, 1999).

Not all the industry use the same kind of heavy metal for their activities and different industry releases different metals in the environment such as the high concentration of chromium is released from the tannery industries and Lead (Pb) and Zn present in higher amount in refinery and fertilizer industry (Gabarrón, Faz, & Acosta, 2017). As mentioned above, every industry uses many toxic components and discharge the leftover effluent into the environment.(Moten & Rehman, 1998). When this heavy metal exposes to a human continually, it causes diabetes, gangrene, keratosis, cancer, and cardiovascular disorder (Zahra, 2017). Different heavy metals have different effects on the human body, such as exposure to Lead and mercury, causing autoimmune disease. Kidney failure, joint pain, neurological dysfunction also are caused by it. Even cadmium can imbalance the calcium level in our body. Endocrine system, damage fragile bones, and lungs are also affected by cadmium exposure (Ayangbenro & Babalola, 2017). Heavy metals are most toxic in their ion forms e.g, Cr^{6+}, Pb^{2+}, Cd^{2+}, As^{3+}, Hg^{2+}. And they enter into the human body and bind to carbohydrates, proteins, and lipids to form an extremely stable toxic condition with a lethal effect and hard to release from the body (Valdman, Erijman, Pessoa, & Leite, 2001).

Effluents containing heavy metal affect the human body and are hazardous to plants, animals, and aquatic life (Balasubramanian, 2012).To remove heavy metal from industrial effluents, conventional methods are high-cost operations to perform and require technical support to do the job efficiently. The plans include electrochemical treatment, ion exchange, evaporation, chemical precipitation, and reverse osmosis. However, to replace this and make the whole process cost-effective and environmentally friendly, introducing the biological method is an alternative (Srivastava, Chandra, Tripathi, Naraian, & Sahu, 2008). In the biological process, microorganisms are used to remove heavy metals from industrial effluents. Microorganisms

develop a resistance capacity to survive in an environment of a high concentration of heavy metals. It uses waste and converts toxic metals to non-toxic products and some intermediate metabolites (Thassitou & Arvanitoyannis, 2001). Microbes developed some mechanisms to survive in the presence of heavy metals such as metal sorption, extracellular precipitation uptake and accumulation, mineralization and enzymatic oxidation and reduction, efflux of heavy metals from the cells were also stated in many kinds of literature (François et al., 2012; Hrynkiewicz & Baum, 2014; Monteiro, Castro, & Malcata, 2012). Microbes did not develop this mechanism to resist toxic metals from the very beginning. It affects the depletion of many microbes and has led to the development of specific tools that help the microbes grow in this contamination site (Förstner & Wittmann, 2012). Bacteria, yeast, and protozoa are the first microorganisms that are reported to expose initially to heavy metals, present in the industrial effluents (Mergeay, 1991). Isolation and characterization of this heavy metal tolerant microorganisms can be manipulated in order to make the bioremediation process effectively and efficiently in the treatment of wastewater (A. J. O. n. Rajbanshi, 2008). The capacity built up by these organisms to tolerate the toxic compound might have the ability to resist any antimicrobial agents (antibiotic). Antibiotic resistance bacteria is available in hospitals where it is mostly used against bacterial infection. Resistance genes for heavy metal and antibiotic are conferred in the same plasmid of bacteria. So there is a chance of plasmid transformation from one organism to another. Therefore, one bacteria that is resistant to antibiotic might be resistant to heavy metal as well (Chattopadhyay, Grossart, & Manag, 2011).

Aim: In this review paper, numerous literatures have been studied about the heavy metal resistant bacteria and their mode of action towards the toxic metal and their co-resistance towards antibiotics. Several different strategies are discussed about the use of microorganisms in bioremediation from literature reviews. Some insights gained from previous studies, some techniques were conducted, and it is hoped that this study will lead to bioremediation insights.

Research Methodology:

Search Strategy: Several databases were searched for literature reviews. Original articles were studied to gather the information. PubMed, Science Direct, and Google Scholar mostly used for the literature review. In these databases, the search were performed by some keywords, including heavy metal resistant bacteria, tannery effluents, mechanisms of microbial remediation.

Inclusion Criteria: Original scientific research articles that stated microorganisms' use to remove toxic heavy metals from industrial effluent. Even the articles on bacterial co-resistant to the antibiotic were also selected.

Exclusion Criteria: Articles that stated organic compound degradation, phytoremediation, and other biological techniques that did not mention about microorganisms.

Significant Toxicity effects of heavy metals:

The toxicity of heavy metals to microorganisms is its ability to cause some severe effects. The level of impact depends on the availability of heavy metal and the absorption capacity of the organisms (Rasmussen, Sørensen, & Turner, 2000). Several mechanisms have been addressed of this toxicity: destructing ion regulation, breaking fatal enzymatic functions, reacting as redox catalysts in the production of reactive oxygen species (ROS). This addressing causes are directly affecting the formation of Dand as protein (Gauthier, Norwood, Prepas, & Pyle, 2014; Hildebrandt, Regvar, & Bothe, 2007). Heavy metal can change the physiological and biochemical properties of microorganisms.

Chromium presence in two forms, Cr^{6+} and Cr^{3+}. But among them, the first one is the most toxic one compared to the latter one. Cr has a genotoxic effect, which is regarded as the oxidative damage on DNA of the organisms. It also binds to the carboxyl and sulfhydryl groups of enzymes and modifies the DNA polymerase. Even it also takes away the magnesium ions, which is necessary for the action of many enzymes. Chromium alters the cytoskeleton, which is entailed in the loss of the organisms (Cervantes et al., 2001). Cuproenzymes require copper to function as an amine oxidases, cytochrome c oxidases, laccases, methane monooxygenases, multicopper oxidases and tyrosinases. Copper has an alternate state that converts itself to cuprous(1) to cupric (2) oxidation state or vice versa. This property makes copper an ideal candidate for various cellular processes such as energy transduction, iron mobilization, and oxidative stress response. Furthermore, Fenton and Haber-Weis reaction catalyze ROS production through redox reactions in copper, leading to extreme damage of lipids, proteins, DNA, and other cytoplasmic molecules of an organism. When there is an excess amount of copper present in an environment compared to other metals, it binds to the proteins by Irwing-Williams series resulting in protein degradation or disrupting its function (Giner-Lamia, López-Maury, & Florencio, 2014). DNA, the genetic material, can be damaged by aluminum. It

stabilizes superoxide radicals, which in turn induces the disruption of the DNA. Heavy metals compete with other ions necessary for the enzymatic function and interact with the enzyme to cause the enzyme's configuration change. Thus the enzyme's role has been stopped (Chen et al., 2014). High Cadmium and Lead (Pb) concentration leads to the disruption of the bacteria's cell membrane and damages the DNA. Also, zinc has a stronger affinity to the thiol group of many macro-biomolecules of bacterial cells and thus stop the function of many activities such as the ATP production machinery-glycolysis, the efficiency to tolerate the higher concentration of acid and transmembrane protein translocation (Yazdankhah, Skjerve, & Wasteson, 2018).

Materials and Methods:

Sample Collection:

The water samples were collected from the various industrial areas and used a plastic bottle to collect the water samples from a zone where all the tannery effluents are thrown out without any proper processing. The water sample could be transported to the laboratory at 4^0C freezer box (Abskharon, Hassan, Gad El-Rab, & Shoreit, 2008; Kabir, Fakhruddin, Chowdhury, Pramanik, & Fardous, 2018; Kalaimurugan et al., 2019; Yazdankhah et al., 2018) and in the laboratory, the sample containing bottle were stored in a 4^0C refrigerator.

Isolation of bacterial strain:

The plastic bottle's 1mL water sample was serially diluted from 10^{-1} to $10^{-10,}$ and Nutrient Agar media was prepared for all the serially diluted test tubes. The heavy metal salts (Chromium, Lead, Zinc etc.) were mixed with distilled water to make the stock solution. The 5mg/ml, which is 5ppm, was incorporated into each nutrient agar media plates. 100µl of a water sample from each test tube was added to the NA plate, and platting was done by pour plate technique(Alam, Ahmad, & Malik, 2011). The plates were incubated at 37^0C for at least 48 hours to allow the resistant microbes to grow in the presence of heavy metals (Alagumuthu et al., 2012). The colonies that were able to grow in heavy metals were morphologically selected and purified in a nutrient agar plate (Kalaimurugan et al., 2019).

Preparation of heavy metal aqueous solution:

The stoichiometric amount of heavy metal was taken and liquified in distilled water to prepare the desired heavy metal stock solution. After preparing the stock solution, it was diluted with distilled water to desired concentration by following the equation $M_1V_1=M_2V_2$ and then the final amount was mixed in the solid media.

Minimum inhibitory concentration of heavy metals:

The test isolates were purified in NA plates. The diluted solution of heavy metal were added to the Nutrient agar plates in a triplicates way. The isolate colonies were taken with a loop and streaked on those plates (Abbas, Rafatullah, Ismail, & Lalung, 2014). In this way, the heavy metal concentration was gradually increased to some point like 5ppm, 6ppm, 8ppm, 10 ppm, 12ppm for each NA plates. The lowest concentration where the growth was prevented was the MIC of that specific isolated bacterial (Nath, Deb, & Sharma, 2012). An E.coli DH5-α was used as a control bacteria for comparison to the isolated bacteria. All the plates were incubated at 37^0C for at least 36 hours (Kalaimurugan et al., 2019).

Identification of bacterial strain:

Isolates were grown on nutrient agar media and stored there for further processes. The selected bacteria were identified through bacterial gram staining. It is the first step for finding out whether the bacteria is gram-negative or gram-positive. The following steps were used to identify selected purified bacteria such as oxidase test, urease test, IMViC, nitrate reduction, catalase test, H_2S production, casein hydrolysis, starch hydrolysis, gelatin hydrolysis (Khan, Manchur, Mahmud, & Fatama, 2019). PCR amplified the selected isolates with 16S rDNA gene sequence, and the results were submitted to the GenBank database to find the similarities with any known bacteria. In this way any selected isolates bacteria were identified based on physiological, morphological, biochemical characteristics and 16S rDNA sequence analysis (Abbas et al., 2014).

Determination of antibiotic resistance:

According to Kirby Bauer disc diffusion method, all of the isolates found to be resistant to heavy metals to a specific concentration were examined for antibiotic sensitivity and resistance test. Resistance and sensitivity were measured by checking the inhibition zone and comparing the result with the standard antibiotic disc chart (Nath et al., 2012).

Results and Discussion:

The standardization of the whole process depends on some factors such as culture media employed, the incubation time, the form and concentrations of the heavy metal, as a result the toxicity of the metals may vary in different experiments. That is why there is no fixed method to define the bacterial resistance and tolerance capacity. Some heavy metal resistant bacteria can grow on the nutrient agar media by the presence of any specific heavy metal. Heavy metal and antibiotic resistant bacteria from tannery effluents were isolated by describing procedures. *Staphylococcus sp* was found to be resistant to chromium with MIC value 500µg/ml, which is higher in the presence of chromium. Then *Escherichia coli* showed a MIC value of 200µg/ml to chromium, and Klebsiella sp exhibited a MIC value of 150µg/ml (A. Rajbanshi, 2009). Pseudomonas sp exhibited higher MIC 1400µg/ml for cadmium and 800µg/ml for Lead. Whereas *staphylococcus* showed a higher MIC 1100µg/ml for Lead, and Klebsiella sp has a higher MIC of 1600 µg/ml. MIC of Lead and cadmium for *Bacillus sp* is 1200µg/ml and 1800µg/ml (Nath et al., 2012). The same bacterial strain can be co-resistance to different heavy metals. Such as Cu^{2+} resistant bacteria were found to grow in the presence of cobalt and cadmium with a MIC of 150 µg/ml and 120µg/ml, respectively. This heavy metal resistant bacteria also showed resistance to different antibiotics. It could be possible in a way of plasmid transformation method. Plasmid contains the heavy metal resistance genes and antibiotic resistance genes as well. So this plasmid transfers from one organism to another and it allows others to resistant to several antibiotics too.

SN	Organisms	Sensitive to (antibiotic)	Resistant to (antibiotic)
1	*Staphyloccoccus sp.*	Ciprofloxacin, Vancomycin, Methicillin	Penicillin, tetracycline, erythomycin

2	*E.coli*	Gentamycin, choramphenicol	Ampicillin, cotrimoxazole, Ciprofloxacin, tetracycline
3	*Klebsiella sp*	Gentamycin, cotrimoxazole	Tetracycline, ciprofloxacin, Chloramphenicol,ampicillin
4	citrobacter	Ampicillin, gentamycin, ciprofloxacin	Tertracycline, choramphenicol
5	*Pseudomonas sp.*	Streptomycin	Ampicillin, chloramphenicol, Kanamycin, tetracyclin

Table 1:Antibiotic resistance bacteria isolated from tannery samples (A. Rajbanshi, 2009)

Usage of heavy metal in the tannery, it leads to the emergence of heavy metal resistant bacteria. Besides, almost all heavy metals follow a common mechanism of their toxicity and as a result, bacteria can exhibit co-resistant to multiple heavy metals. But in tannery effluents, some substances induce the bacteria to become resistant towards antibiotics. This phenomenon is still unclear. In tannery wastewater, bacteria is continuously exposed to heavy metal, leading to the development of bacteria's antibiotic resistance capacity. So it might be because of the excessive selective pressure of heavy metal helps the bacteria develop a genetic manipulation of their genes to resist the antibiotic.

My findings:

Water samples were taken from the tannery industrial area, Savar, Bangladesh. Selected isolates were taken from the pour plate technique (incorporated with 1mM any type of heavy metal salt) and streaked on a nutrient agar plate for storage. Heavy metal was incorporated with the NA media or LB agar media. The isolates were streaked on the plate and incubated for a minimum of 36 hours to let them enough time to grow in the presence of heavy metal. However, in some cases, it was noticed that the bacteria changed its color in the presence of heavy metal. It means when bacteria was subcultured on NA media, it was white in color, but in the presence of heavy metal such as potassium dichromate or Lead salt, the bacteria changed its color to yellow or brown. Different isolates of bacteria showed a different color. The lab samples were also used as a control sample and changed its color in the presence of heavy metal. To a specific concentration, lab samples were grown, and it was minimum compared to selective isolates

taken from the tannery's water sample. Some other issues should be mentioned. Such as heavy metal salts need to be incorporated into media (NA) after the media's autoclave. It was noted that heavy metal salts tend to lose their toxicity while autoclaving with the media. So it should be added while pouring the media into the petri dish.

Figure 1: Bacteria colonies are visible in the presence of lead (5mM)

Figure 2: SS4 isolates is visible in the presence of K2Cr2O7(3mM)

Figure 3: Klebsiella (lab sample) is visible in the presence of Lead(3mM) and its color changed from white to moderate brown in the presence of lead

Figure 4: E.coli (lab sample) is grown in lead(3mM) corporated media and color changed to moderate brown

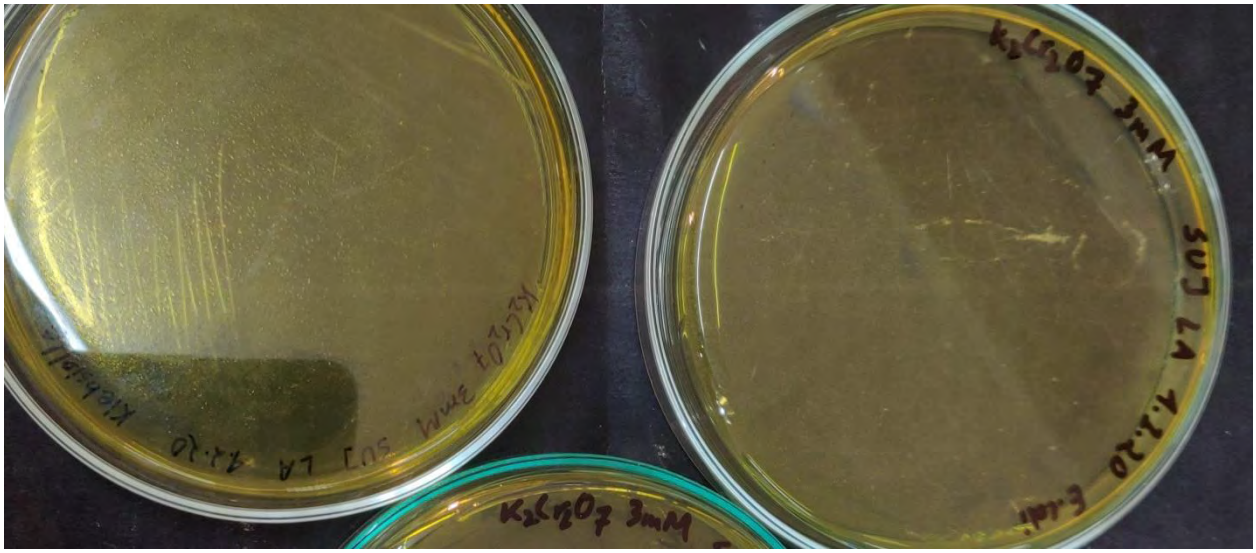

Figure 5: In the presence of K2Cr2O7(3mM), the both lab samples were not grown

In the presence of Lead, the bacteria tend to change its color to brown. It was so visible in the petri dish.

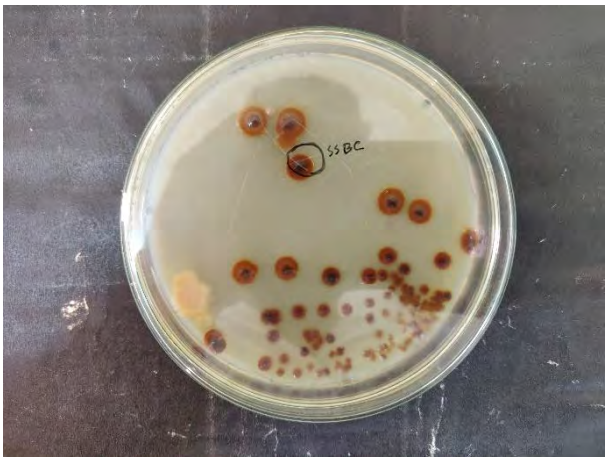

Figure 6: In the presence of Lead (3mM), the isolate SSBC changed its color to brown.

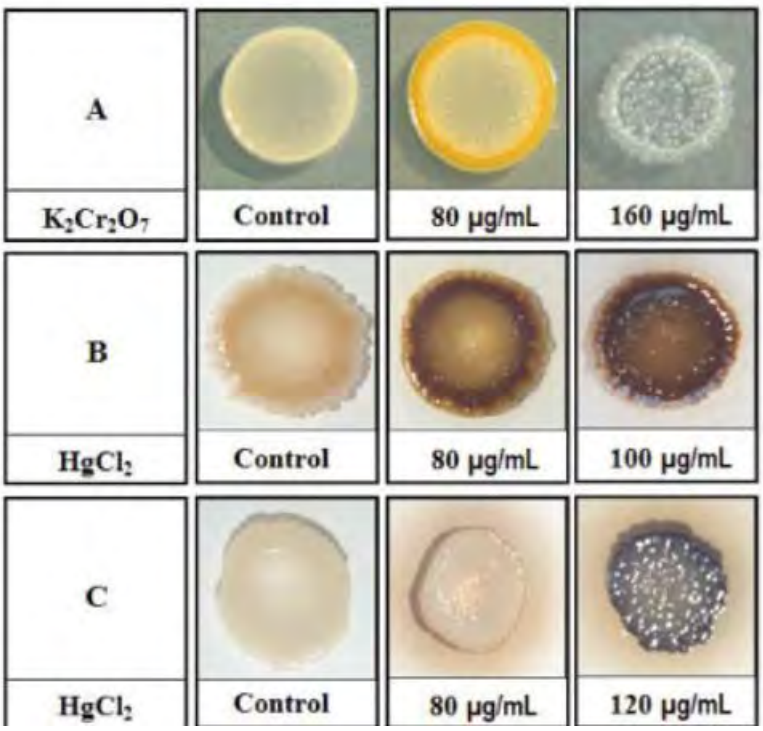

Figure 7: In the presence of heavy metal, the bacterial strain changes its color. (Silva et al., 2012)

A. *S. aureus* strain grown in media without metal and with metal
B. Gram negative rod shaped strain grown in media without metal and with metal
C. Gram positive rod shaped strain grown in media without metal and with metal

This physiological color of bacteria changes due to the presence of metals that were incorporated into solid media. *S. aureus* also showed such as color change, and it turned into yellow pigmentation while going on a media in the presence of a certain amount of $K_2Cr_2O_7$ (Silva et al., 2012), but the growth was suppressed when the concentration increased to 160mg/ml. Such characteristics can be seen in *P.aeruginosa*. One of the strains of this bacteria showed a slightly green without the toxic metal but after adding the heavy metal, the color turned to dark green color and the intensity of the color rose to the highest in the concentration of 160mg/ml of $K_2Cr_2O_7$. In my findings, a colony was turned to brown color in the presence of lead. Whereas in the presence of $HgCl_2$, *Sphingomonas paucimobilis* and *Brevundimonas vesicularis* turned to dark brown from yellow at a concentration of (60-100) mg/mL (Silva et al., 2012). This observation of the changing in color might be due to any chemical modification of metals when it reacts to the bacteria. Moreover, it may not be connected to natural pigmentation induction because the study was done in the absence of light exposure. It is quite

possible regarding Lead that the brown color was observed because the strain may have the ability to reduce the lead and precipitated it. As a result, the bacteria turned to brown. The bacteria might also release some substance that reacts with the heavy metal or bioaccumulation of the metal in a reduced form in the media.

Conclusion:

This review paper stated that the isolates were found in tannery effluents can grow in heavy metal contamination sites. The heavy metal usage in the tannery industry for processing leather and animal skins to make products such as belts and shoes allows the environmental bacteria to be continuously exposed to heavy metal that results in building resistance capacity. Heavy metal resistant bacteria isolated from different sites may not give the exact result. The unique characteristics of each isolate to any particular area may vary from other regions. So MIC value for isolated bacteria from various tannery samples could show dissimilarities. Much more research should be done on this bioremediation process through bacteria as it is cost-effective and offers great potential to remove heavy metals from the environment. Genetic modification of the resistance capacity of the bacteria is a field to work on. Due to the ability to change its colors, bacterial cells can be used as biosensors. Further investigation would be done to determine the exact mechanisms of resistance to heavy metals. It is essential to understand the full extent possibilities of whether this co-resistant bacteria can be moved to the environment and affect people. More research is needed to understand these isolated bacteria's degradation capacity as research has been going on under laboratory conditions. In contrast, the contaminated tannery site is a large area where the environmental condition is a primary factor. Slow degradation capacity may limit the practical use of bacteria in the bioremediation process, and this specific field needs to be focused on. Biotechnology has been regarded as a useful tool for the treatment of hazardous waste from tannery effluents.

Conflict of interest:

No conflict of interest was influenced in this research.

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