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Efficacy of Exopolysaccharide (EPS) Producing Chromium Resistant Bacteria in the Removal of Chromium from Wastewater

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ABSTRACT

The contamination of heavy metals has caused major health risks, which has particularly led to toxicological manifestations, causing ailments and diseases like irritation of the skin and lungs that can cause nausea and vomiting. Heavy metals; therefore, impart hazardous effects on overall human health due to their potential to accumulate in the living tissues through the food chain mechanism. Conventional remediation strategies have become a challenge to resolve the rising issues of heavy metals. Among different heavy metals, chromium gained much attention due to its prevalence in different oxidation states; however, Cr (III) (less toxic) and Cr (VI) (toxic), are the most prevalent ones. Hexavalent chromium can be converted by some bacteria to the less insoluble and less toxic Cr (III). The current study was conducted to isolate chromium-resistant microbes from the tannery and dye industries, and their potential was evaluated for the reduction of their toxic form. A total number of 13 isolated chromium-resistant bacteria were screened for the production of EPS and out of 13 isolates, 6 were found positive. The effect of temperature (25°C, 30°C, 35°C, 40°C, and 45°C), pH (5, 6, 7, 8, and 9) and time period (24 hours, 48 hours, and 72 hours) on the exopolysaccharides production was examined. It was found that optimum temperature was 35°C, pH was 8, and the time period was 72 hours, respectively, both for the growth and chromium reduction potential. These conditions seemed to be optimum given that the bacteria (alkaliphiles) were isolated from a slightly alkaline environment, which have the ability to grow in a slightly alkaline environment and temperature ranging from 34°C to 36°C. The average chromium reduction potential of EPS-producing bacteria was 91%, and the average growth of these bacteria was 1.0192, respectively. Significant positive correlation was observed between the number of EPS and chromium reduction of EPS-producing chromium-resistant bacterial isolate.

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Keywords: bioremediation, exopolysaccharides, hexavalent chromium, non-exopolysaccharides, wastewater

1. INTRODUCTION

Heavy metals found naturally in the Earth's crust refer to any metallic chemical or toxic element having a relatively high density. Even at low doses, the majority of them are harmful. Due to their widespread industrial use, free heavy metals concentration in terrestrial environments and aquatic systems are increasing, which have widespread industrial applications (pharmaceuticals, insecticides, plastics, rubbers, tanneries, organic compounds, and wood products). Furthermore, the non-biodegradable nature of heavy metals ensures their long-term existence in the environment [1]. Among all, some prominent heavy metals include arsenic (As), mercury (Hg), cadmium (Cd), chromium (Cr), and lead (Pb). The chromium ion is a chemical element that is found in nature. Chromium is a class-one hazardous substance that can exist in a variety of oxidation states because they are the most stable oxidation states in nature. Only the trivalent chromium Cr (III) and hexavalent chromium Cr (VI), which have nine valence states ranging from 2 to + 6 [2], are significant for the environment. In the environment, Cr (VI) species are highly water-soluble and mobile than Cr (III) species, which are less soluble and less mobile and are classified as an extremely hazardous, cancer-causing, mutation-causing and teratogenic for animals, including humans. Despite this, Cr (III) is supposed to be a vital trace element for glucose, lipids, and amino-acids metabolism, as well as a general nutritional supplement [3]. Coagulation, chemical precipitation, electro-dialysis, evaporative recovery, floatation, flocculation, ion exchange, nanofiltration, reverse osmosis, and ultrafiltration are a few of the most used heavy metals ion treatment processes [4]. There are many methods for removing heavy metals from wastewater, including membrane filtration, adsorption, and ion exchange. Adsorption is one of the best suitable methods for cleaning contaminated water. Moreover, the adsorption method offers various benefits like accessibility, affordability, and environmental friendliness [5].

Sometimes they may cause metal ion removal to be inefficient and unexpected. As a result, it is critical to develop effective, efficient, cost-effective, and environmentally acceptable solutions for reducing heavy metal ion concentrations in the environment down to safe levels. Chromium (VI) is one of the most prevalent harmful wastes, it must be treated before

removal. Commonly utilized therapy includes biological, pharmacological, and physical therapies. Additionally, bioremediation is thought to be the best solution, using soil bacteria [6].

Currently, microorganism-based chromium remediation is considered the finest and cost-effective approach for eliminating Cr pollution. Resistance to Cr has been identified in *Staphylococcus aureus*, *Pediococcus pentosaceus*, and several *Klebsiella* species. =It was found that some chromium-resistant bacteria have the ability to reduce chromium because of reductase enzymes. Hexavalent chromium Cr (VI) is reduced to trivalent chromium Cr (III) by chromate reductases found in chromium-resistant bacteria [7].

Leather production and environmental conservation go hand in hand. the tanning process produces enormous volumes of effluent that may be polluted with a number of substances, due to the several steps involved in soaking and wringing dry raw hides. The leather industry generates a variety of chromium-based pollutants, as chromium-based tanning is widely used due to chromium's versatility. Unwanted wastes that pose a major environmental risk include chromium sludge, chrome-tanned leather shavings (CTLSs), and chrome leather trims [8].

They are significantly toxic, even in very small amounts, and can cause diseases in humans and animals because they cause irreversible changes in the central nervous system. Other than being carcinogenic and mutagenic, Cr (VI) can induce liver damage, lung congestion, and skin irritation, which can result in ulcers. Additionally, chromium accumulation may also cause birth defects and a decrease in reproductive health [9].

Chromium (Cr+6) is one of the most dangerous heavy metals utilized in current industries including plastic, pigment, wood preservation, steel, leather tanning, cement, paint, dyeing, and fertilizer. These are some of the well-known toxin substances that can cause cancer [10]. Exposure to high concentrations of chromium can result in multiple disorders (epigastria, nausea, vomiting, severe diarrhea, and hemorrhage). For drinking water, the maximum chromium tolerance level is 0.05 mg/L.

2. MATERIALS AND METHODS

2.1 Sample Collection

Surface soil and wastewater samples were gathered from two industrial sites (tanneries and dye factories) in Kasur, Lahore, Pakistan. Using a sterile spatula, soil samples were extracted from clean plastic bags, while wastewater samples were retrieved from tannery industrial effluents. All samples were brought to Lahore Garrison University's biology lab, where they were stored at 4°C for later use.

2.2 Physiochemical Characteristics of Samples

For physiochemical characterization, 2 mL of each sample (for the soil sample, 0.2 g of soil was mixed with 2 mL of distilled water) was taken in a microfuge tube. The pH of samples was observed using pH strips and temperature was measured using a digital thermometer.

3. ISOLATION OF CHROMIUM RESISTANT ISOLATES

3.1 Isolation and Purification

In a sterilized test tube, 1 g of soil and 9 mL of autoclaved distilled water were mixed in a test tube. The prepared samples were added to the agar by using the spread plate technique. A total of 100 microliters from each soil and water sample were then extracted by using a micropipette before being plated on LB-agar plates containing 600 g/ml chromium, spread with a sterile L-shaped glass spreader, and incubated at 37^{o}C for 24 hours. (McGrath and Smith, 1990). To obtain pure cultures, isolates were purified. Isolated colonies were streaked on LB-agar plates supplemented with chromium at 600 µl/ml at 37°C and incubated for 24 hours by using the quadrant streak method [11].

3.2 Microscopic Characterization

For microscopic characterization, gram staining was performed to distinguish between Gram- positive and Gram-negative bacteria. Smears were made/created, air dried, and then heated. The dry slides were stained for 1 minute with crystal violet and then rinsed with distilled water drop by drop until the stain was entirely removed. After washing, slides were flooded with Gram-iodine solution (a mordant) for 1 minute, followed by thorough washing. Ethanol consistency of 95% was used for 30 seconds as a decolonizing agent. After using the Counterstain Safranin for 1 minute,

the area was thoroughly cleaned and washed. Moreover, the slides were dried using blotting paper, which were later examined under a light microscope (100X), and microscopic characters/details were noted [12].

3.3 Biochemical Characterization

Biochemical assays were carried out to confirm the isolates [13]. For this purpose, various tests (Catalase, Urease, Oxidase, DNase Test, Indole, MR, VP, and Starch Agar Test) were performed for the confirmation of bacterial isolates, and results were noted for the identification of bacteria.

3.4 Physiological Characterization

Chromium-resistant isolates were inoculated into prepared Luria broth and incubated at different temperatures (25°- 45°C), different pH levels (5 - 9), and different time periods (24 - 72 hours) at chromium concentration (600 µl/mL) to measure their optical density at 600 nm with a spectrophotometer to determine their influence on the growth of isolates. The results were recorded for all cultures individually. Three replicates were set up for each bacterial isolate and treated in the same manner [13].

3.5 Screening of EPS producing Chromium Resistant Bacteria

The purified chromium-resistant bacteria were screened for EPS production in LB agar containing chromium (600µl/ml). To isolate chromium-resistant EPS-producing bacteria, plates were inoculated with pure bacterial isolates and cultured at 37 °C for 24 hours [14].

3.6 Quantification of EPS producing Chromium Resistant Bacteria

To extract EPS, LB-broth was prepared in a 50ml flask, autoclaved, and inoculated with bacterial isolates. Flasks were then incubated for 24 hours at 37°C. The bacterial culture was then transferred into falcon tubes and centrifuged for 20 minutes at 10,000 rpm [15]. The supernatant was transferred to another falcon tube with cold ethanol and the falcon tubes were incubated at 4^{o}C for 20 minutes. Following the appropriate incubation period, falcon tubes were centrifuged at 10,000 rpm for 15 minutes, the supernatant was discarded, and the weight of the dried pellet was measured.

3.7 Optimization of EPS Production and Chromium Reduction Potential

Chromium-resistant isolates were inoculated into prepared Luria broth and incubated at different temperatures, different pH levels, and different

time periods at Chromium Concentration (600 µl/mL). The bacterial culture was then transferred into falcon tubes and centrifuged for 20 minutes at 10,000 rpm [16]. The supernatant was transferred to another falcon tube along with double the amount of cold ethanol and the falcon tubes were then incubated at 4^{o}C for 20 minutes. Following the appropriate incubation period, falcon tubes were once more centrifuged at 10,000 rpm for 15 minutes, the supernatant was discarded, and the weight of the dried pellet was measured. Three replicates were set up for each bacterial isolate and treated in the same manner. Deleo and Ehrlich (1994) utilized a spectrophotometer to examine the chromium reduction potential of isolates. The O.D. was measured at 540 nm using 100 µl of supernatant in autoclaved test tubes (containing 1 ml of Di-phenyl-carbazide, and 1 drop of phosphoric acid).

4. RESULTS

4.1 Physiochemical Characteristics of Sample

Water and soil samples were obtained from the tannery and dye industries of Lahore and Kasur. Their physiochemical characteristics (temperature, pH) were observed and recorded individually during and after the sample collection. The temperature was recorded at 34°C for dying and tannery industries. Noticeably, water samples had a pH of 6.

4.2 Isolation and Purification

The bacterial isolates were isolated from the Tannery wastewater and soil by using standard microbiological techniques. For isolation purposes, the collected samples were serially diluted, and subjected to LB agar plates and broth for initial isolation. By using the streak plate method, bacterial isolates were further purified. A total of 13 isolates were obtained from water (n=6) samples collectively, named according to their morphology, and further screened.

All the isolated bacteria were subjected to LB-agar for morphological characterization, each bacterial isolate was observed and recorded according to their colony shape (circular to filamentous), size (pin-point to moderate), pigmentation with entire, lobate, serate, irregular, undulate margins, and elevation types as described in Table 4.3.

4.3 Screening of Chromium-Resistant EPS Producing Bacteria

All of the 13 bacteria that were isolated, grew and tested positive for chromium resistance. Out of 13 chromium-resistant bacteria, only 6 produced EPS and were labelled as TWS-01, TWS-02, TWS-03, TWS-04, DWS-01, and DWS-02 based on their source and site. Chromium-resistant EPS-generating isolates were found microscopically and macroscopically.

4.4 Microscopic Characterization of Chromium Resistant EPS Producing Isolates

Microscopic characters (shape, color, and arrangement) were observed using gram staining. All of the isolated microorganisms were gram-positive in various configurations (chains, clusters, and diplobacilli). Among 6 microscopically identified chromium-resistant EPS- producing isolates, these 6 isolates were isolated from water samples.

4.5 Biochemical Characterization

Biochemical tests (MR, VP, Urease, Catalase, Oxidase, DNase, Indole, and Starch Agar Test) were performed for further validation of isolates. The isolates showed positive results for the Catalase test (bubble formation on slides), Oxidase test (quick color change was observed), Voges Proskauer (formation of brown ring on top), Urease (color change to pink), DNase (green color turn into yellow), and Starch Agar test (color change to orange). All isolated bacteria were negative for the Indole test (no cherry red sing formation) and the Methyl red test.

All bacterial isolates (100%) were positive for the VP, Urease, catalase, oxidase, DNase, and starch Agar Test. Chromium-resistant bacterial isolates (100%) were negative for Indole and MR tests. The biochemical profile of all isolates is described in Table.

4.6 Physiological Characterization

Chromium-resistant isolates development was studied at a number of temperatures (25°C, 35°C, 30°C, 40°C, and 45°C) and pH (5, 6, 7, 8, and 9). After 72 hours of incubation at 35°C, pH 8, and 600 µl/ml chromium concentrations at 600 nm, almost all of the isolates (100%) exhibiting maximum growth .

4.7 Effect of Different Temperature

Bacterial isolates were inoculated in broth tubes with a 600µl/ml concentration of chromium, and incubated at temperatures of 25°C, 30°C, 35°C, 40°C, and 45°C for 24 hours. Following incubation, O.D was recorded at 600 nm by using a spectrophotometer. All of the isolates showed the highest growth at 35°C (with an average growth of 1.0625) and the least growth at 45 °C (with an average growth of 0.5998) suggested that a temperature lower than the requirement rate was not favorable for the growth of bacteria. So, the most suitable temperature for chromium-resistant bacteria was 35 °C (Figure 1).

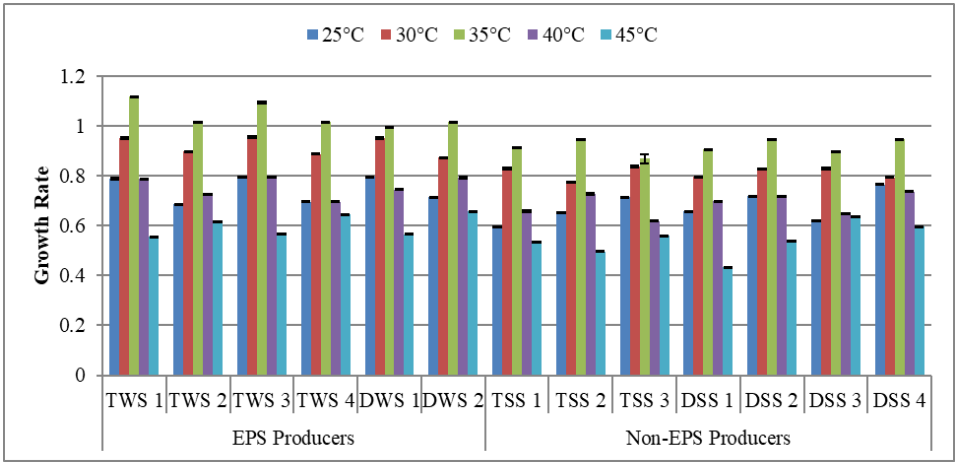

Figure 1. Growth of Chromium Resistant EPS Producing and Non-EPS Producing Isolates at different Temperatures

4.8 Effect of Different pH

To note the cultural growth, the pH of the L-broth was adjusted separately at 5, 6, 7, 8, and 9, and inoculum was given to test tubes. Following the incubation period, the O.D of replicas was measured at 600 nm. At 5 pH maximum growth peaked at 0.6284, and the minimum was noticed at 0.5597. The highest O.D at pH 6 was 0.9125, and 0.8017 was the lowest. 1.2313 was the maximum value at 7 pH whereas 1.0219 is the minimum value. 1.3736 was the maximum value at 8 pH, whereas 1.0812 was the minimum value. Maximum growth at 9 pH was 1.1828, and the minimum was 0.9545. Majority isolates showed maximum growth at pH 8, indicating that chromium-resistant bacteria require a basic environment (pH) followed by neutral (7 pH) for their proper growth (Figure 2).

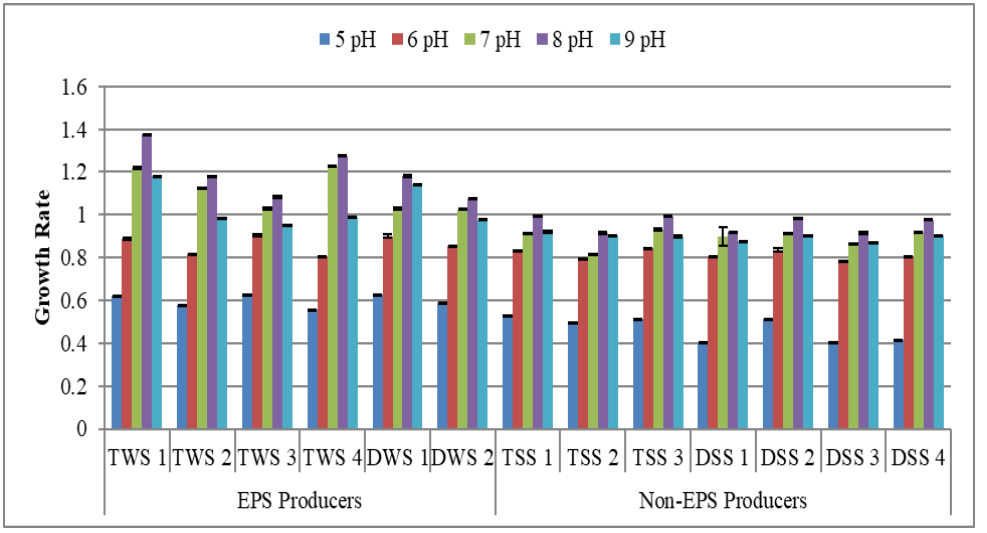

Figure 2. Growth of Chromium Resistant EPS Producing and Non-EPS Producing Isolates at different pH.

4.9 Effect of Different Time Periods

The mean O.D values of replicates with a chromium concentration of 600µg/mL were used to measure growth. The results demonstrated that with 600µg/mL of Cr^{+6}, indicating bacterial growth of selected isolates that was maximum at 72 hours, which also indicated that isolates utilized Cr^{+6} as their nutritive and enhance the ability of bacteria to propagate.

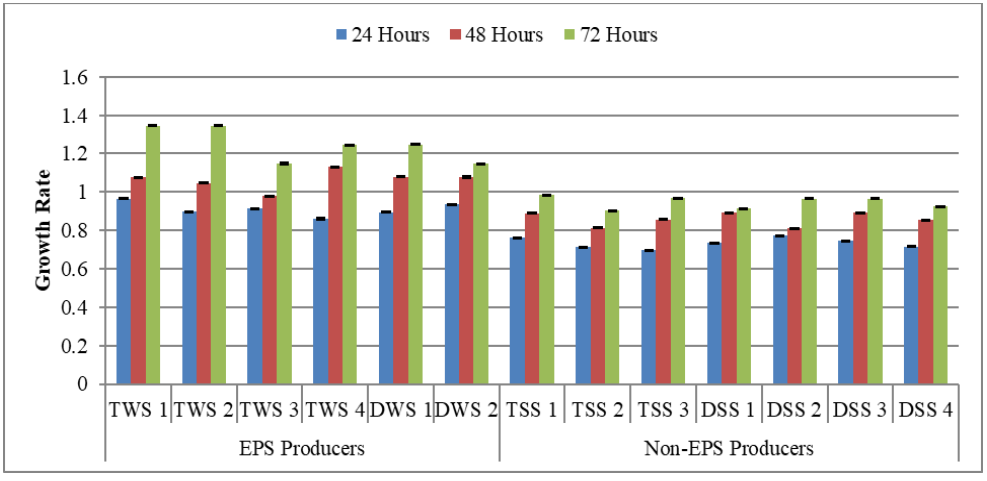

Figure 3. Growth of Chromium Resistant EPS Producing and Non-EPS Producing Isolates at Different Time Periods

5. EPS PRODUCTION

5.1 Effect of Different Temperatures

The selected isolates were placed in L-broth tubes containing 600µl/mL chromium concentration and incubated at different temperature ranges (25°C, 30°C, 35 °C, 40 °C, and 45°C) for 24 hours. Centrifugation was used to count the number of EPS produced by isolates grown in LB Broth. The results showed that Maximum EPS production at 35°C was 1.3411g and the minimum was 1.0421g. The highest EPS production at 30°C was 1.1615g and 1.1128g was the lowest. 0.7722g was the maximum production at 25°C whereas, 0.7019g is the minimum. 0.7824g was the maximum production at 40°C whereas 0.7109 is the minimum value. Maximum production at 45°C was 0.5841g and the minimum was 0.4005g (Figure 4).

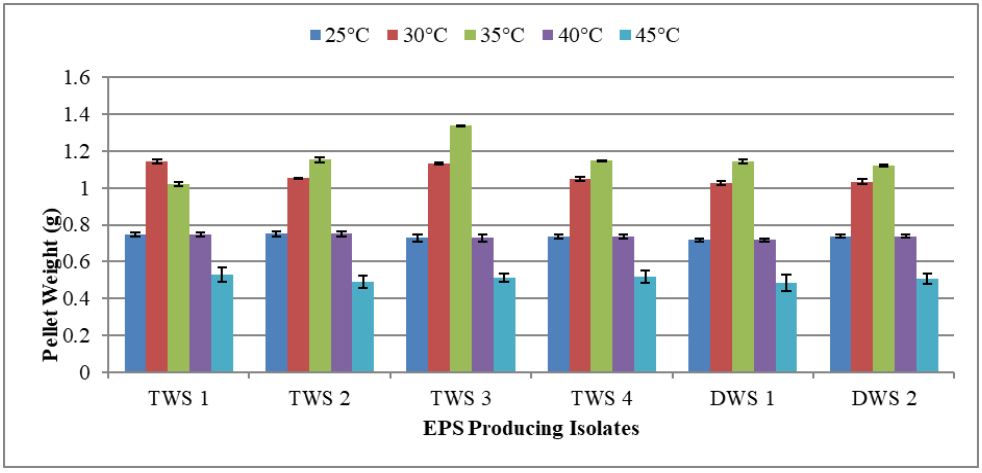

Figure 4. EPS Production at Different Temperatures

5.2 Effect of Different pH

Luria broth was prepared in distilled water and autoclaved in 5 different conical flasks for 15-20 minutes after autoclaving pH was adjusted at (5, 6, 7, 8, and 9) in five flasks separately. Centrifugation was used to count the number of EPS produced by isolates grown in LB Broth. A Maximum production was recorded at 5 pH level (0.1582g) and the minimum was recorded at (0.1017g), respectively. The highest O.D at pH 6 was 1.1611g and 1.0019 was the lowest. 1.6565g was the maximum value at 7pH, whereas 1.3239g is the minimum value. 1.8451g was the maximum value

at 8pH, whereas 1.4561 is the minimum value. Maximum growth at 9 pH was 1.1726 and the minimum was 0.9232 (Figure 5).

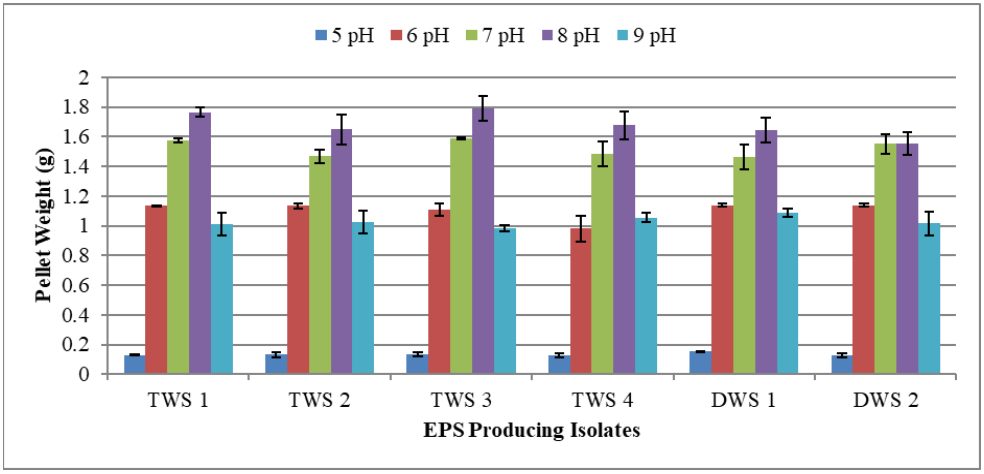

Figure 5. EPS Production at Different pH

5.3 Effect of Different Time Periods

The selected isolates were inoculated in L-broth tubes containing 600µl/mL concentrations of chromium and incubated at different time periods (24 hours, 48 hours, and 72 hours). Centrifugation was used to count the number of EPS produced by isolates grown in LB Broth. The results showed that the maximum EPS production after 24 hours was 1.3367g and the minimum was 1.0437g. The highest EPS production after 48 hours was 1.6568g and 1.3467g was the lowest. 2.1006g was the maximum production after 72 hours whereas 1.4564g was the minimum (Figure 6).

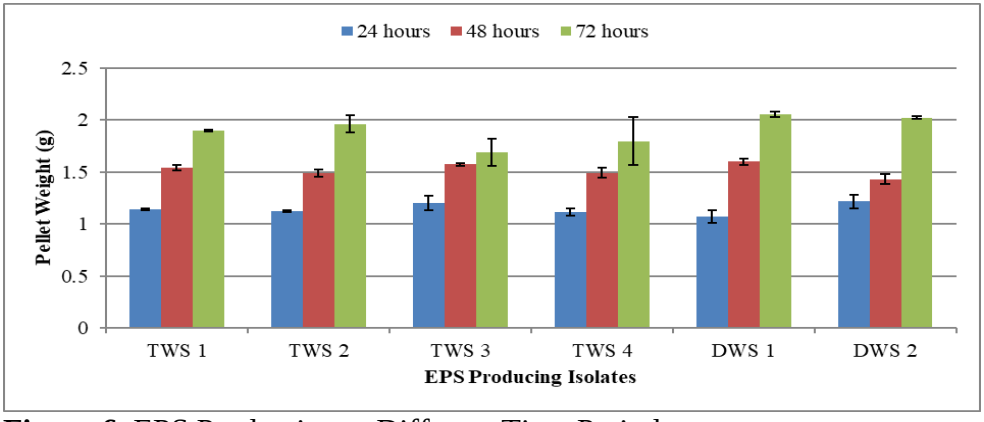

Figure 6. EPS Production at Different Time Periods

6. CHROMIUM REDUCTION POTENTIAL

6.1 Effect of Different Temperatures

Inoculated tubes with chromium (600 micro g/mL conc.) were made according to Deleo and Ehrlich (1994) method and incubated at various temperatures (25°C, 30°C, 35°C, 40°C, and 45°C) to observe chromium reduction. According to the mass spectrophotometer's findings, all of the isolates had the largest reduction percentage (an average of 93%) at 35°C and the lowest (64%) at 30°C (at various temperatures and chromium concentrations). The results showed that temperatures between 30°C and 35°C are more suitable for decreasing chromium at all concentrations. (Figure 7)

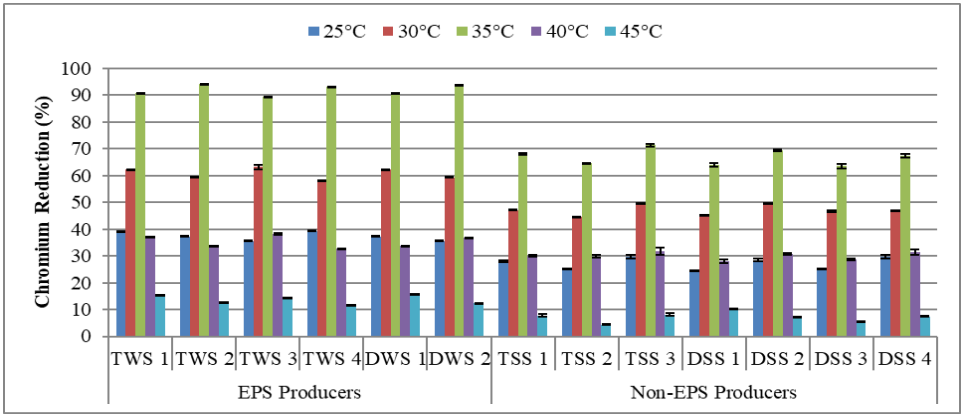

Figure 7. Effect of Varying Temperature on the (Percentage) Ability of EPS and Non-EPS Producing Bacterial Isolates to Reduce Chromium

6.2 Effect of Different pH

To check the chromium reduction at different pH, 5 flasks were prepared in accordance with Deleo and Ehrlich method (1994), pHs (5, 6, 7, 8, and 9) was adjusted separately with the addition of a 600micro g/mL concentration of chromium without cultures. Tubes were equipped with medium, inoculated with isolates, and incubated. The results were recorded using a spectrophotometer (540 nm). The highest percentage of chromium removal from isolates (under various pH conditions and a particular chromium concentration) was recorded at pH 7 (78%) followed by pH 8 (86%). The results indicated that neutral (7) and basic (8) pH are more favorable for bacterial growth even at the specific concentration of heavy metal (Figure 8).

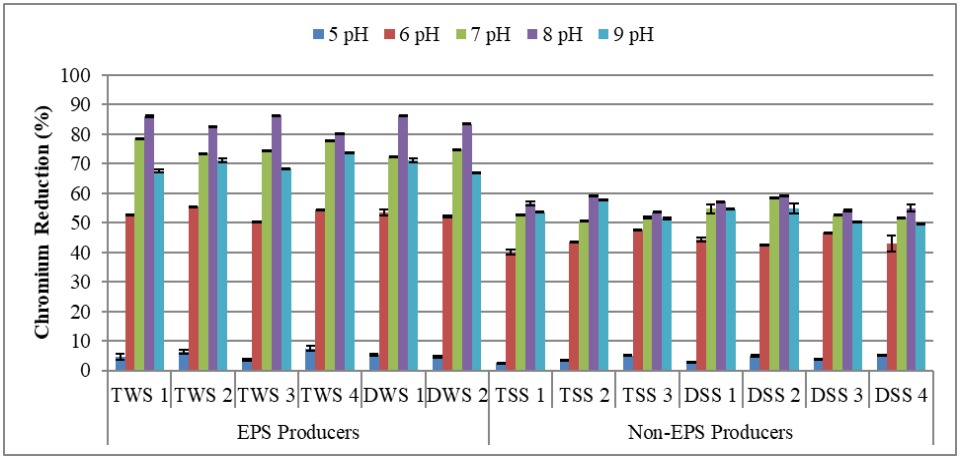

Figure 8. Effect of varying pH on the (Percentage) Ability of EPS and Non-EPS Producing Bacterial Isolates to Reduce Chromium

6.3 Effect of Different Time Periods

The Deleo and Ehrlich (1994) method was used to calculate the percentage of chromium metal removed across different time periods (24 hours, 48 hours, and 72 hours). Chromium's reduction potential was highest at 72 hours, with an average percentage of 93%, followed by 48 hours (83 percent), and 24 hours (65 percent). As time passed, the reduction rate grew several folds, as shown in Figure 4.25. The results indicated that selected EPS-producing isolates reduced more chromium at the maximum time of incubation (Figure 9).

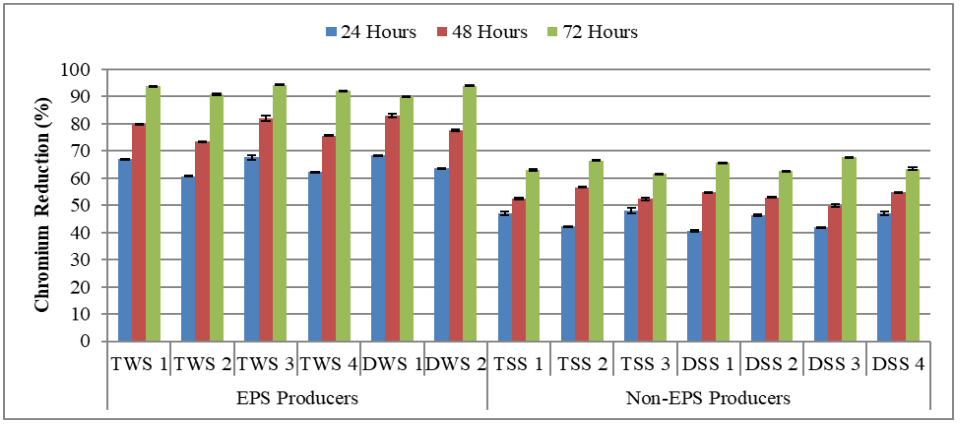

Figure 9. Effect of Varying Time Period on the (Percentage) Ability of EPS and Non-EPS Producing Bacterial Isolates to Reduce Chromium

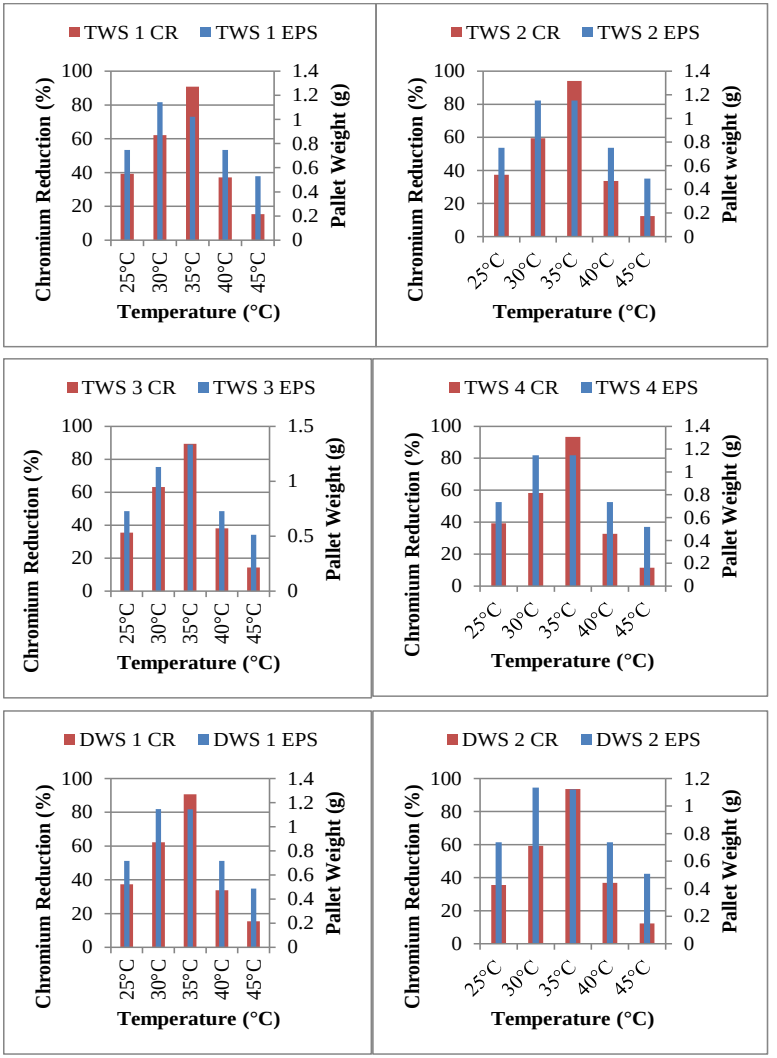

Figure 10. Correlation between the EPS production and Chromium Reduction of Chromium- Resistant Isolates

7. DISCUSSION

Chromium exists in a variety of oxidation states; however, trivalent and hexavalent chromium are the most common. Due to its prevalence and poisonousness, it is a major ecological contaminant found in the environment. Cr (VI) is a powerful skin irritant and carcinogen for living organisms [17, 18], as well as harmful to herbs (plants), aquatic species, and bacteria (Park et al., 2004). Thirteen (13) bacterial isolates were recovered

from the samples and different industries in the current study. Only six isolates (TWS-01, TWS-02, TWS-03, TWS-04, DWS-01, and DWS-02) were found to have EPS-generating chromium reduction potential when tested (CRP). CRP was measured using the 1, 5-diphenyléarbazide technique [9]. To enhance the growth and CRP of each isolate individually, numerous conditions such as varied temperature, pH, and time period were analyzed and optimized. OD was obtained at 600 and 540 nm for assessing cultural growth and reduction potential levels.

The influence of various temperatures, pH, and incubation periods on the growth of chromium-resistant bacteria were also investigated. The maximum growth of chromium-resistant isolates was observed at 35°C with a pH of 8 with chromium (600 µg/ml). A study conducted in Shiraz-Iran showed that the growth of bacteria increased as the time of incubation and temperature increased up to 35°C and then decreased as the temperature increased to 45°C, whereas the ideal pH was found to be 7.0 and 8.0 for bacterial growth. No significant difference in the growth of bacteria was recorded in the media with or without chromium.

There is a strong significant correlation between EPS production and chromium reduction. *Straptobacilli* spp. and *Bacillus* were found to be chromium-resistant. These bacterial isolates were utilized to reduce chromium from the wastewater in different conditions and at several levels (pH, temperature, time periods, and concentration of chromium).

7.1. Conclusion

A reduction in the hazardous effect can be caused by heavy metal contamination and their mobility can help to accumulate the drawbacks of conventional toxic products. Hence, it was observed that increased Cr (VI) elimination and EPS production in the isolated chromium-resistant bacteria, exhibited the remediation of chromium-polluted soil and water. Cr (VI) reduction and their growth is greatly influenced by temperature, pH, and time period, and it was also observed that the optimum temperature was found at 35ºC, pH 8, and 72 hours of incubation. The correlation between the number of EPS and chromium reduction was thoroughly checked, and it was found that there is a strong significant correlation between EPS production and chromium reduction. Chromium reduction by bacterial isolates is a cost-effective and eco-friendly approach. Thereby, the result

indicated that EPS production is an attractive opportunity to remove chromium from wastewater.

CONFLICT OF INTEREST

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

DATA AVAILABILITY STATEMENT

Data availability is not applicable as no new data was created.

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