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Isolation and Identification of Chromium Resistant Bacteria from a Polluted Soil

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ABSTRACT: Tannery effluents from the textile industry contain a variety of organic chemicals used in manufacture, including dyes, chrome dyes, and other chemicals. These can result in large-scale liquid waste discharges that contain heavy metals, including hexavalent chromium. Chromium-resistant bacteria were isolated from polluted soil in order to address these heavy metals biologically as environmental contaminants. Using the atomic absorption spectrophotometer AAS, the total amount of chromium in the soil sample was determined to be 0.0863 mg/l. Under ideal circumstances, the effects of pH and different chromium concentrations on the bacteria's ability to bio transform were also examined for a duration of 72–96 hours. Pseudomonas spp., a gram-negative rod-shaped bacterium, was isolated from the soil using a combination of microscopic identification (gram staining) and other biochemical testing. The greatest biotransformation capability was found to be 90.65% and 90.99% at pH 6 and 7, respectively. The results of this study demonstrate that Pseudomonas species are capable of withstanding hexavalent chromium.

Keywords: *tannery effluents, bio-transformation, hexavalent, chromium, resistant bacteria, atomic absorption spectrophotometer (AAS), gram-negative bacteria*

Introduction

The industrial revolution has led to environmental contamination. It has grown to be a significant issue for several centuries and is among the world's biggest issues. It is growing daily as a result of industrialization. Many human activities, like the widespread, large-scale use of chemicals, have increased dramatically in order to support and meet the demands of an overpopulation (Hazarika, 2016). Large liquid waste discharges comprising hexavalent chromium, salts of zinc, sulfates, copper, sodium, potassium, and other organic chemicals can result from the manufacturing of dyes, chrome dyes, and other chemicals in tanning companies. Due to their toxicity, including mutagenicity and carcinogenicity. Life in an ecosystem depends on their treatment before release into the environment (Deepali, 2011).

The United States Environmental Protection Agency (USEPA) states that chromium is an elemental chemical that is considered an environmental pollutant and poses a risk to human health. It is a naturally occurring heavy metal and transition element in the periodic table in soil, rocks, vegetation, animals, gasses, and volcanic ash. While chromium can exist in various oxidation states, trivalent and hexavalent are the most stable. While hexavalent chromium is very hazardous to biological systems, trivalent chromium is essential for the metabolism of proteins, lipids, and glucose in mammals. Because of its high solubility and permeability through the biological barrier, it is easily disseminated through the soil and water (Arshad et al., 2019). It is required in a trace amount as it is an essential micronutrient, but above the permissible level, it is more toxic, carcinogenic, and mutagenic to the plant and animal system. It is therefore recommended that the hexavalent chromium concentration should not exceed 0.005 mg/L in drinking water. Their compound can cause nasal irritation, breathing problems, allergic reactions, skin rashes, reproductive problems, anemia, and occasionally intestinal and stomach ulcers, cancer, gastrointestinal tract tumors, and lung cancer (Deepali, 2011). If the concentration of chromium is higher than 2 ppm, it can inhibit plant growth and seed germination. Additionally, in fruits, vegetables, and trees growing next to a

discharging factory, necrosis resulting in changes in the structure of the chloroplast causes reduced photosynthesis (Arshad et al., 2019). Once Cr (VI) entered into cells, spontaneous reactions occur with the intracellular reductant as ascorbate and glutathione, generating the short-lived intermediates Cr(V) and/or Cr (IV), free radicals and the end-product Cr (III). In the cytoplasm, Cr (V) is oxidized to Cr (VI) and the process produces a reactive oxygen species, referred to as ROS, that easily combines with DNA–protein complexes. On the other hand, Cr (IV) interferes with the normal physiological functions of the cells by binding to the cellular organelles. It is known that Cr (VI) species and hydroxyl radicals cause DNA lesions in vivo (SHANKER et al., 2005). The intermediates that originated from the action of Cr (VI) are harmful to cell organelles, proteins and nucleic acids. Cr (VI) is known to have several dangerous effects on biological systems as it has mutagenic, carcinogenic and teratogenic effects. Moreover, Cr (VI) can induce oxidative stress in cells, damaging their DNA (Sylvia, *et al.,* 2005).

For all parties involved, managing and treating chromium-contaminated soil presents a problem in this case. Bioremediation utilizing microorganisms is a very expensive technology that may be crucial for plant growth. Certain heavy metals can be readily reduced or built up by some microbes. Because of their enormous surface area-to-volume ratio and ability to interact with nearby metals, these microorganisms are advantageous to the environment since they simultaneously improve other factors of importance, such as the growth of plants. The application of microorganisms to remove environmental pollutants that pose a risk to human health is known as bioremediation. Bioremediation is the biological process of degrading environmental pollutants into less toxic forms using living organisms like bacteria, fungi, or plants. It reduces contaminants to harmless levels under controlled conditions, often utilizing microorganisms that may be native or introduced to the contaminated site (Dahl, *et al.,* 2010). The organisms use metabolic reactions to detoxify pollutants, and when external microorganisms are added to enhance degradation, it is called bioaugmentation. For bioremediation to be effective, microorganisms must enzymatically transform pollutants or use them as carbon sources. The process often involves manipulating environmental conditions to promote microbial activity and accelerate degradation (Dahl, *et al.,* 2010). Bioremediation methods are classified as:

In-situ bioremediation (Treats contaminants at the site without removal) and ex-situ bioremediation (involves removing contaminated material for treatment elsewhere). On-site bioremediation (treatment occurs at a facility near the contamination site). Off-site bioremediation (material is transported to a distant facility for treatment). Techniques like bioaugmentation and biostimulation (adding nutrients or electron donors/acceptors to enhance natural microbial activity) are applied to both in-situ and ex-situ methods for improved results (Microbewiki, 2014). Numerous distinct microorganisms, particularly fungi and bacteria, work in tandem or sequentially to finish the breakdown processes in bioremediation. (Arshad et al., 2019). Microorganisms transform chemicals in ecosystems through various mechanisms. Pollutants can serve as either a carbon and energy source for microbial growth or as terminal electron acceptors. Their ability to degrade and convert toxic molecules highlights their diverse capabilities. (Sylvia, *et al.,*2005). Primary substrate utilization involves an electron acceptor, either aerobic or anaerobic, to facilitate chemical conversions (Harms *et al.,* 2011). Oxygen accelerates the reaction but is not always necessary. This biodegradation process is commonly observed in the breakdown of hydrocarbons and certain pesticides (Sylvia, *et al.,*2005). When the causal transformation of a chemical by the organism uses different substances as its primary source of energy or metabolites (Carbon source), the process is called cometabolism or secondary substrate utilization. This process often involves toxic intermediates and provides no net energy or carbon gain to the organism. The degradation of Trichloroethylene (TCE) is an example, requiring organic growth substrates like propane or butane for enzymatic transformation (Microbewiki, 2014).

Bacteria are widely diverse organisms, and thus make excellent players in biodegradation and bioremediation. There are few universal toxins to bacteria, so there is likely an organism able to break down any given substrate when provided with the right conditions (anaerobic versus aerobic environment, sufficient electron donors or acceptors, etc.). *Pseudomonas putida* is a gram-negative soil bacterium that is involved in the bioremediation of toluene, hexavalent chromium and other product of petroleum refining, in contaminated soils (Sylvia, *et al.,*2005). Dechloromonas aromatica Dechloromonas aromatic – a rod-shaped bacterium that can be used to oxidize aromatics including benzoate, chlorobenzoate, and toluene,

coupling the reaction with the reduction of oxygen, chlorate, or nitrate. *Nitrobacter hamburgensisn, Nitrosomonas europaea* is involved in the nitrification and denitrification of unwanted mineral nitrogen compounds. During nitrification, ammonium is oxidized to nitrite and *Deinococcus radiodurans* is a radiation-resistant extremophile bacterium that is genetically engineered for the bioremediation of solvents and heavy metals e. t. c. (Nordberg H et al., 2024). Current bioremediation applications primarily utilize bacteria, with comparatively few attempts to use fungi. Fungi (Mycoremediation) have fundamentally important roles because they participate in the cycling of elements through decomposition and transformation of organic and inorganic materials. These characteristics can be translated into applications for bioremediation which could break down organic compounds and reduce the risks of metals. In some cases, fungi have an advantage over bacteria not just in metabolic versatility but also in their environmental resilience. They can oxidize a diverse amount of chemicals and survive in harsh environmental conditions such as low moisture and high concentrations of pollutants. Therefore, fungi are potentially an extremely powerful tool in soil bioremediation. The most important fungi used are *Phanerochaete chrysosporium* for *the* degradation of organic pollutants, *Aspergillus oryzae* and so on. These microorganisms can be used for wider applications in treating various environmental pollutants. (Nordberg H et al., 2024). The aim of this research is to isolate and identify the chromium-resistant bacteria from polluted soil. The effect of different concentrations of hexavalent chromium and the effect of pH on the chromium-resistant bacteria will also be studied.

Material and Methods

The materials used for the research work include: Electric Weighing Balance, Hand Shovel, Sample Bag, Petri dishes, Beakers, Measuring Cylinder, pH Meter, Conical Flask, Test Tubes, Digestion Bottles, Retort Stand, 5ml, 2ml, and 10ml Syringes, Micropipette, Funnel, Oven, Atomic Absorption Spectrophotometer (AAS), U–V Visible Spectrophotometer, Cotton, Foil Paper, Hand Gloves, Face Mask, Wire Loop, Bunsen Burner, Autoclave, Incubator, Filter paper, Glass slides, Test tube rake, Compound Light Microscope, Reagent Bottles.

Reagents

The reagents used include Potassium Dichromate, Hydrochloric Acid, Nitric Acid, Sodium Hydroxide, Distilled water, Nutrient Agar and Nutrient broth.

Soil Sampling and Characterization

The soil samples were obtained from three different locations in Gombe Dumping Site, along Bajoga Road, Gombe State. Soil was extracted from 0–15 cm depth (upper surface) and from 15–30 cm depth (subsurface). The sample was kept in a soil sample collection bag while being transported to the laboratory. Physicochemical parameters such as pH were measured by adding soil to water in the ratio of 1:5. The concentration of the chromium was measured initially using an atomic absorption spectrophotometer (AAS) to ascertain the total chromium present in the soil sample.

Growth Media

Nutrient broth and Agar were prepared according to the supplier's instruction and sterilized at 121^0Cfor 15minutes. 100ml of nutrients agar was placed in Petri dishes and allowed to cool.

Isolation of the Chromium Resistant Bacteria

For the isolation of the bacteria, a serial dilution technique from 10^{-1} to 10^{-5} was adopted (Arshad, *et al.,* 2019). Sample of each dilution was streaked on different Nutrient agar plates followed by incubation at 37^0C for 24hours. After 24 hours, three plates marked from 10^{-1}, 10^{-3} and 10^{-5} were picked and out of which 10^{-3} were selected and culture were purified using repeated streaking methods (Arshad, *et al.,* 2019).

Preparation of Chromium Stock Solution

Synthetic solutions were prepared by dissolving 0.283g of potassium dichromate ($K_2C_r2O_7$) in 100ml of distilled water (Yoganand and Umapathy, 2017).

Effect of Chromium Concentrations on Bacterial Growth and Bacterial Resistance to Chromium

The nutrient broth was distributed in three different test tubes and amended with various concentrations of chromium (50, 100, and 150 mg/L). The bacterial culture was quantified in terms of optical density (OD) in the culture medium with the addition of 2ml of Cr (VI) solution to each of the varying concentrations by measuring the absorbance at 600nm against the blank, taken at regular intervals of 24 hours as per standard method. The medium without the addition of metal solution served as a control. The total concentration of the chromium was measured using an atomic absorption spectrophotometer (AAS) (Arshad, *et al.,* 2019).

Effect of Various pH on Chromium Degradation Ability of the Bacteria

The bacterial isolate was inoculated into different test tubes containing the nutrient broth of different pH (starting from 4, 5, 6, 7, 8, 9, 10, and 11) and incubated at 37 ^{o}C for 24, 48, and 78 hours. The initial concentration of chromium used was 100 mg/L (Arshad, *et al.,* 2019).

Identification of the Bacteria

The methods adopted during bacterial identification were Gram Staining and Biochemical tests (Oxidase, Catalase, Urease, Indole, Citrate utilization, and Methyl Red Test).

Results and Discussion

Identification of Chromium Resistance Bacterial Isolate

Macroscopic methods and gram staining techniques were the first approaches used to identify the morphology of the bacteria on the growth media and to differentiate between the gram negative and gram-positive bacteria. As illustrated in figure 4.1, the isolate was found to be gram-negative bacteria with pink-coloured cells and a rod shape. This may be due to the reaction between the safranin dye and the bacterial cell wall. Gram-negative bacteria do not pick up the primary stain (Crystal violet) (Ruiz *et al*., 2017).

Biochemical Test Result

Urease Test

Some bacteria have a urease enzyme. The urease can decompose the urea by hydrolysis to give ammonia and carbon dioxide. Change in the alkaline medium which is shown by the change in colour of the indicator to red-pink indicates a positive result (Ruiz *et al*., 2017). Based on the test and the result found (Table 4.1), there is no colour change which indicates negative results.

Some bacteria deaminate the amino acid tryptophan in order to produce Indole, which is found in peptone water. The test showed a negative result (Table 4.1).

Citrate Utilization

Some groups of bacteria can utilize citrate as their carbon source and ammonia as a source of nitrogen. The citrate is converted to acetoin and Carbon dioxide (Shoaib *et al.,* 2020). The result of this reaction was found to be positive simply because there is a colour production on Simon's citrate agar (Table 4.1).

Oxidase Test

Some aerobic bacteria produce enzyme oxidase as part of their respiratory mechanism. Pseudomonacea and negative Enterobacteriacea families can be differentiated using this test. This enzyme oxidizes a redox dye called TMPPDH, Tetra methyl Paraphenyldiamine Dihydrochloride to deep purple colour (Shoaib *et al.,* 2020). Because of the colour production, this test was also found to be positive (Table 4.1).

Methyl red test

Different Microbes ferment glucose at different pH levels, and produced different end products. Some bacteria have extended pH levels below 4.5. They bacteria glucose to pyruvic acid and then to different type of acids depends on the species of bacteria for which type of acid will be produced (Shoaib *et al.,* 2020). This is indicated by a change in colour of the Methyl red indicator at which is added at the

end of the incubation time. This test was also found to be negative because there is no colour change (Table 4.1).

Effect of Various Chromium Concentrations on Chromium Resistance Bacteria

Pseudomonas spp. shows higher growth at 50mg/l (figure 4.3). However, at elevated Cr (VI) concentrations, the growth of pseudomonas spp. decreased continuously from 50 mg/l to 200 mg/l. It was also reported by some scientists that higher concentrations of Cr (VI) showed an inhibitory effect on the growth of bacteria. This may be due to the toxic effect of hexavalent Cr (Deepali, 2011).

Chromium Biotransformation Observed after 72hrs of Incubation under Various Chromium Concentrations

From the data was observed at neutral pH for up to 72 hrs. The metal removal efficiency increased with an increase in time and significantly increased the percentage degradation by Pseudomonas spp. at 50mg/l and 97.34%, 100mg/l and 90.68%, 150mg/l and 86.46%, and finally 200mg/l and 80.30%. (figure 4.4). *Pseudomonas spp.* have been shown to have high tolerance to Cr (VI) and to be highly effective at reducing it (Arshad et al., 2019). However, higher concentrations of Cr (VI) metal decreased percentage biotransformation, which may be due to lack of availability of the enzyme active sites and more ions are competing for the available binding sites. The reduction in Cr (VI) biotransformation among bacterial cells may be related to the mutagenic and toxic effects of hexavelent Cr (Deepali, 2011).

Effect of Various pH on Chromium Biotransformation Ability of Chromium Resistance Bacteria

According to the literature on bioremediation processes, the pH range of 6-8 was found to be optimal for bacterial growth and biotransformation rate (Mishra et al., 20121). The pH ranges from 4–11 was selected for this research. From above, eleven pH values (4–11) were investigated. As the pH value increased above the optimum level, the biotransformation ability of the pseudomonas spp. Decreased (figure 4.5). Based on the data observed, the optimum pH level for the biosorption ranges from 6–

7. At pH4, the percentage biotransformation was 83.66%, pH5 was 86.60%, pH6 was 90.65%, pH7 was 90.99%, pH8 was 86.82%, pH9 was 86.76%, pH10 was 81.91%, and pH11 was 73.11%. Therefore, the highest percentage was observed at pH 7 and 6. A low percentage is observed because the change in pH of the medium is toxic to the bacterial cells. (AY *et al.,* 2006).

Pseudomonas spp **Growth curves in Nutrient broth at 24, 48 and 72hrs at Varying pH conditions**

It was also observed that changing pH values affect not only the percentage of biotransformation but also the growth of the pseudomonas spp. This may be due to the denaturation of DNA by the hydrolysis of hydrogen bonds holding together the DNA strands at high pH. (figure 4.6). Therefore, changes in pH modify ionization of amino acid functional groups and disrupt hydrogen bonds, which in turn promote changes in protein folding of the molecule, promoting denaturation and destroying activity. (AY *et al.,* 2006).

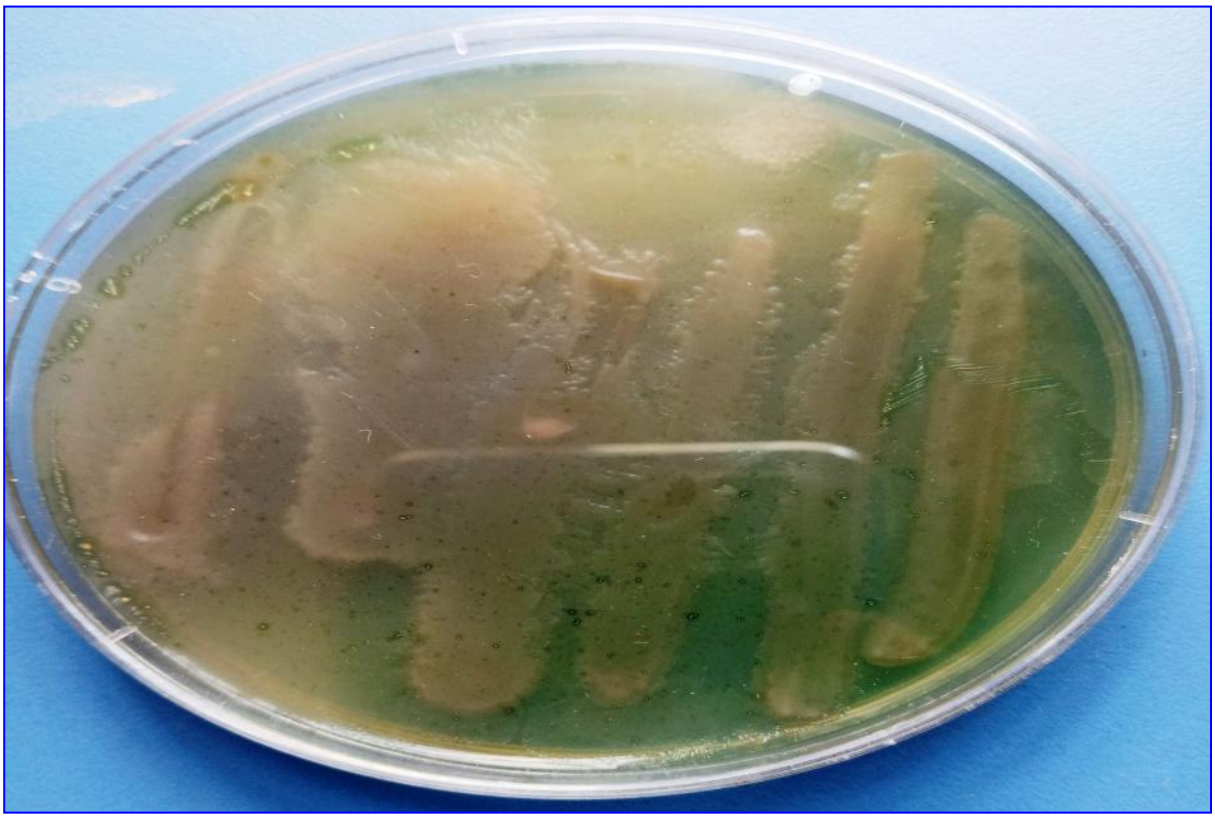


Figure 4.1: Shows the morphology of the bacteria on the Nutrient agar.

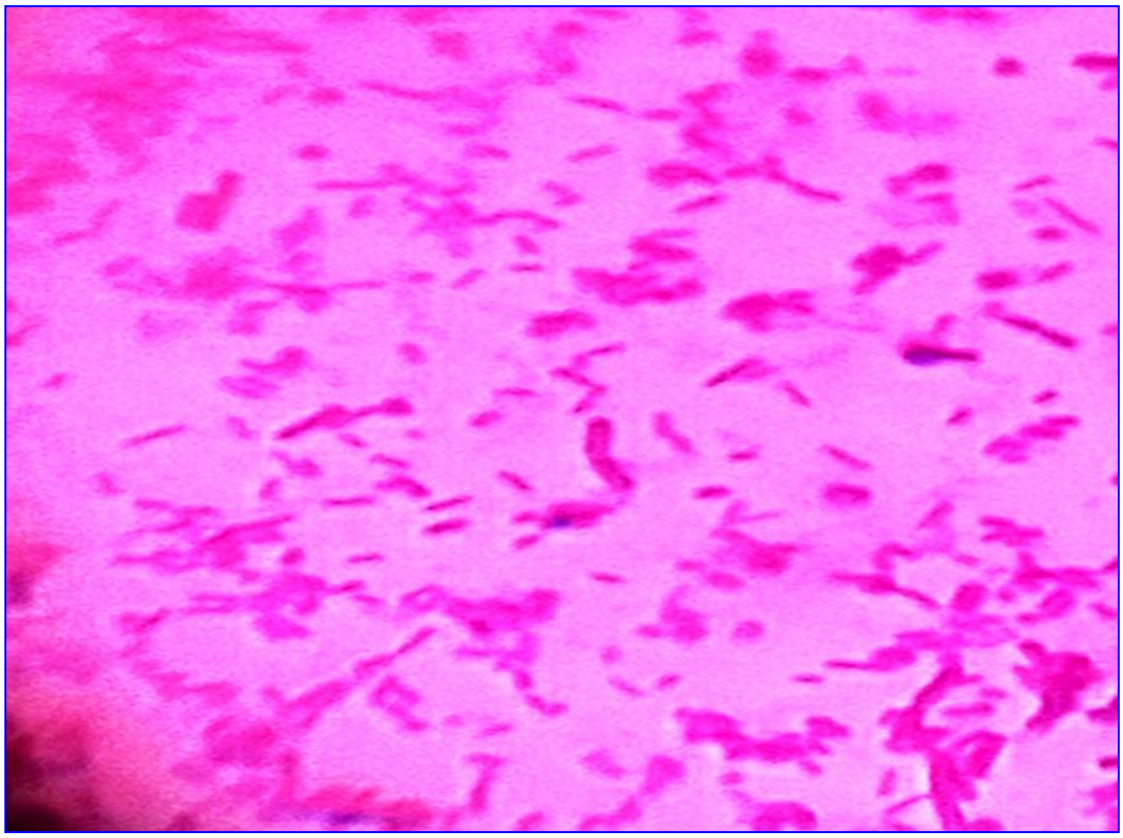
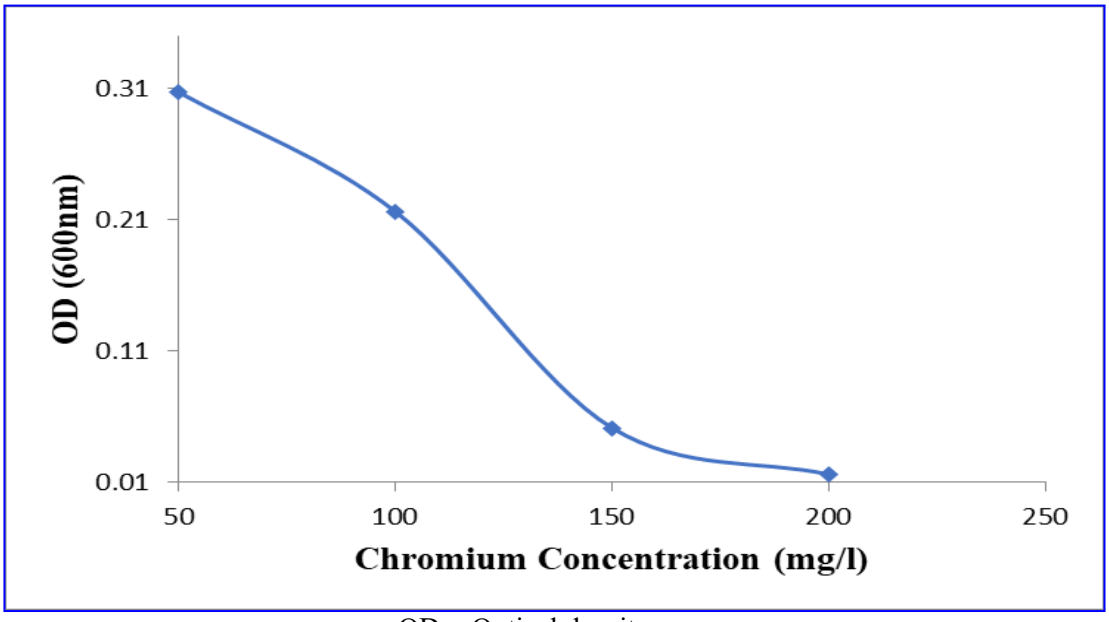


Figure 4.2. Gram stains smear of the isolate observed under a compound light microscope using an oil immersion (100x objective lens).

To study the effect of chromium-on-chromium resistance bacteria, the experiment was carried out using $k_2Cr_2O_7$as a source of chromium 50mg/l, 100mg/l, 150mg/l and 200mg/l.



OD = Optical density

Figure 4.3: Growth of the bacterial isolate *(Pseudomonas spp)* in Nutrient broth observed after 48hrs of incubation under various chromium concentrations

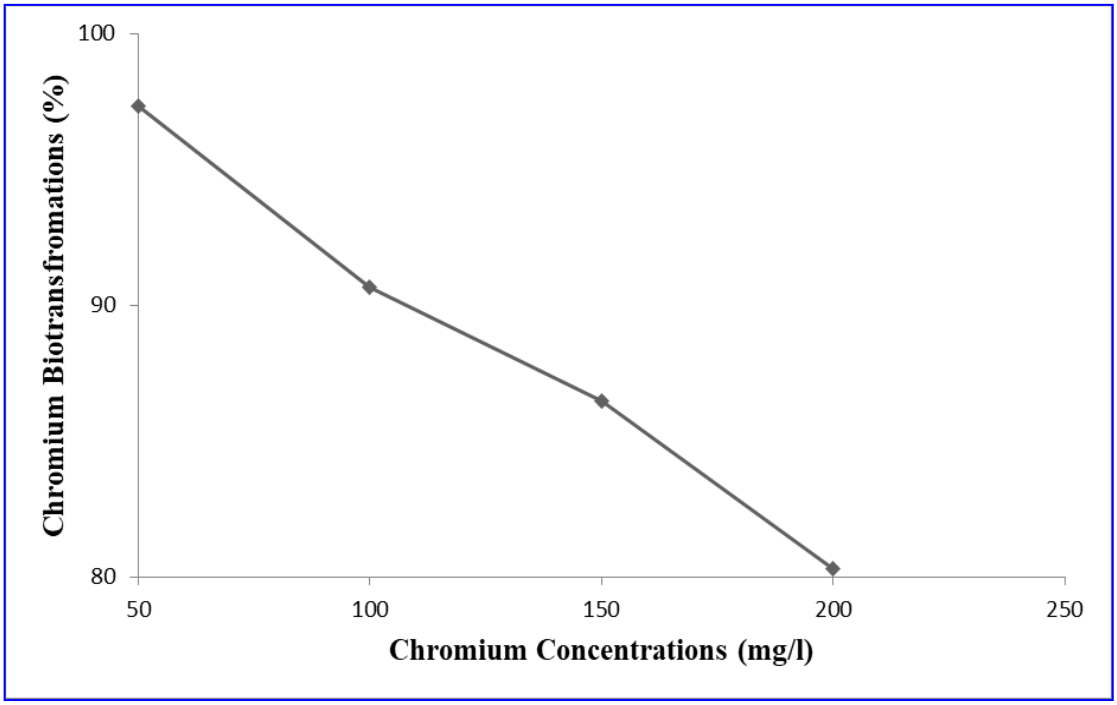


Figure 4.4 Chromium biotransformation observed after 72hrs of incubation under various chromium concentrations

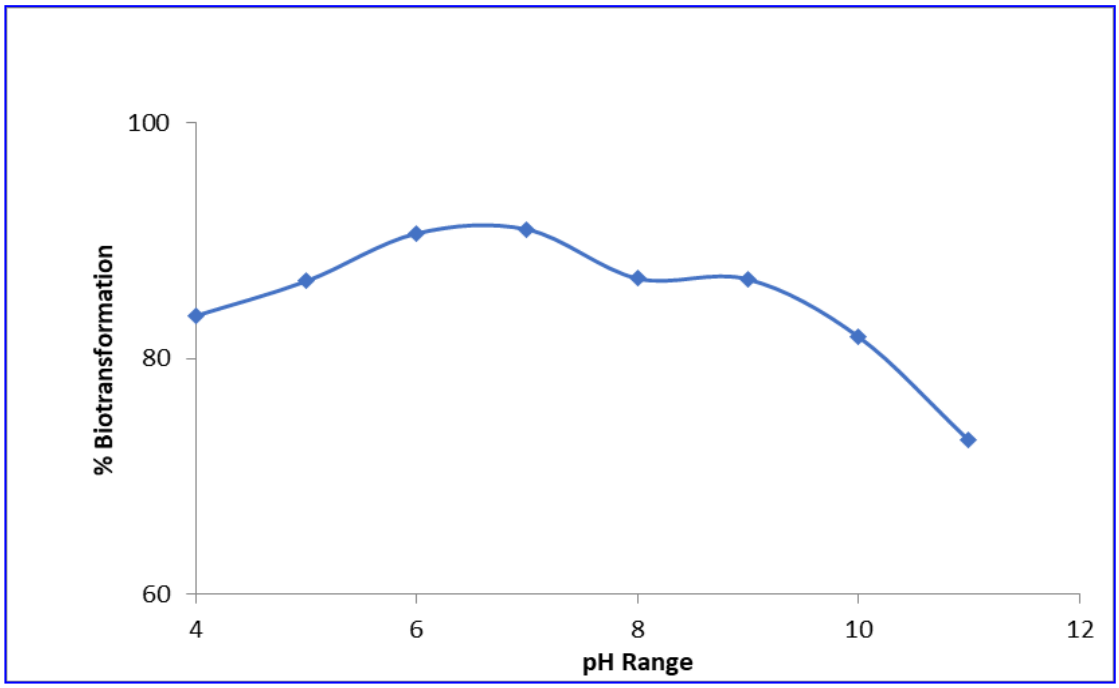


Figure 4.5: Effect of pH on Biotransformation ability of the *Pseudomonas spp*

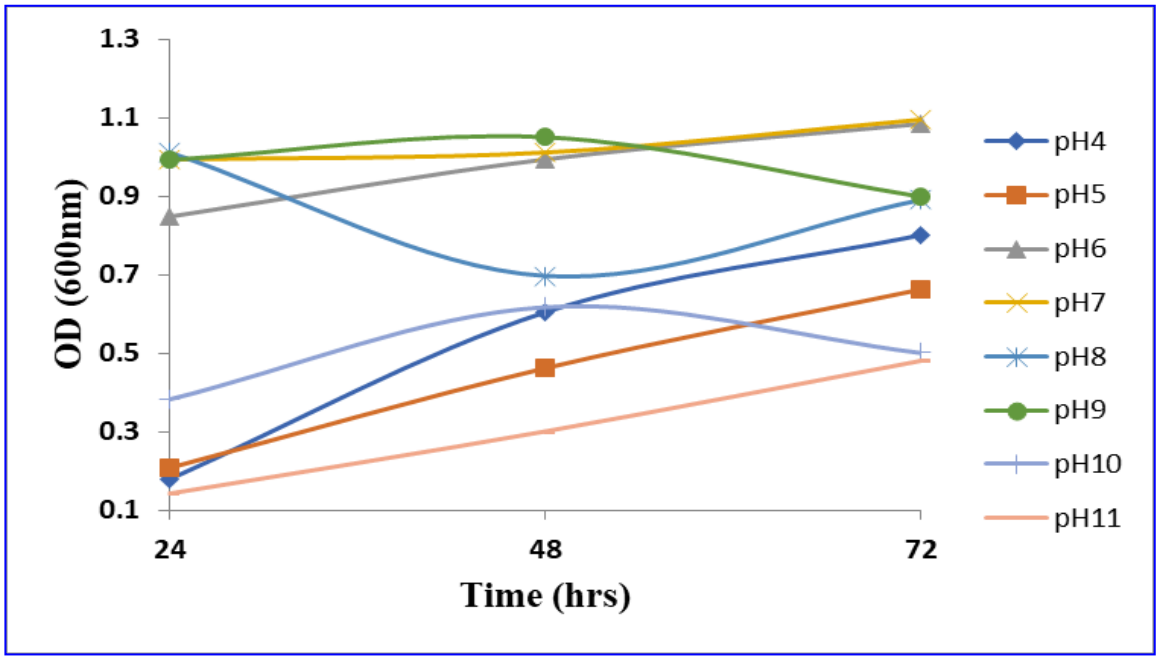


Figure 4.6: *Pseudomonas spp* Growth curves in Nutrient broth at 24, 48 and 72hrs at varying pH conditions.

Conclusion and Recommendations

It can be concluded from the present study that pseudomonas spp. (gram negative bacilli) was isolated as the chromium resistance bacteria and have a great potential to remove hexavalent chromium from the aqueous solution of chromium and soil. The above findings in the present study show that the isolated Pseudomonas spp was found to be a good biosorbent and can be used for the removal of heavy metals from industrial wastes. Further study for the application of this technology is needed with specific attention. The technology, when upgraded, will be a benefit to industrialists in tackling the pollution problem of mines and waste water.

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