ISOLATION AND CHARACTERIZATION OF CHROMIUM-TOLERANT BACTERIA FROM POULTRY EXCRETA

By

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Certification

It is hereby certified that student bearing Roll No. ASH2305MS112M; Session: MS2022-23 has carried out the research work entitled “Isolation and Characterization of Chromium-tolerant bacteria from poultry excreta” for the partial fulfillment of the requirements for the degree of Master of Science in Microbiology, Noakhali Science &Technology University, Bangladesh, under my academic supervision in the Leeuwenhoek, Pasteur Laboratory, Department of Microbiology, Noakhali Science & Technology University.

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Abstract

Chromium (Cr) contamination represents a critical environmental challenge in Bangladesh, largely driven by the integration of tannery byproducts into poultry feed. This study aimed to isolate and characterize chromium-tolerant bacteria from poultry excreta to assess their potential for environmental bioremediation.

Initial screening was conducted on Luria-Bertani (LB) agar enriched with 100 mg/L of potassium dichromate ($K_2Cr_2O_7$). Five high-tolerance strains (ID-01, ID-09, ID-10, ID-11, and ID-14) were selected based on their growth performance across a concentration range of 100–500 mg/L over a 34-h period. The Cr (VI) reduction efficiency of these isolates was subsequently assayed using the 1,5-diphenylcarbazide colorimetric method. Among the isolates, ID-14 exhibited the highest reduction potential, reducing 98.17% of Cr(VI) at 100 mg/L, though efficiency decreased to 48.12% at 500 mg/L concentration.

Biochemical characterization and antibiotic susceptibility testing (Kirby–Bauer method) assessed phenotypic traits and potential resistance co-selection, revealing multidrug-resistant phenotypes, notably in isolate ID-14 which exhibited the most extensive resistance profile, including Ampicillin, Levofloxacin, Ceftazidime & Oxacillin etc. Molecular identification of three top-performing isolates was achieved through 16S rRNA gene sequencing, which revealed that ID-11 matched with *Proteus mirabilis*, ID-10 with *Staphylococcus xylosus* & ID-09 with *Staphylococcus cohnii*. However, the most tolerant strain, ID-14, underwent Whole Genome Sequencing (WGS) to map the genetic architecture of its resistance determinants. WGS revealed that the isolate was *Staphylococcus ureilyticus*. Genomic analysis identified multiple metal resistance determinants, including chromium resistance genes such as *chrA* and *cysk*, which encode chromium efflux and reductive mechanisms, respectively. In addition, genomic mapping uncovered diverse antibiotic resistance genes (ARGs), including the fluoroquinolone resistance genes *norA*, *norB*, beta-lactam antibiotic resistance genes *blaZ, fmtA*, macrolide resistance genes *mphC* & *msrA* and multidrug resistance genes *emrB* & *sdrM*.

Overall, these results identify potential indigenous bacterial candidates capable of mitigating heavy metal pollution and provide essential baseline genomic data for bioremediation strategies.

Keywords: Chromium-tolerant bacteria, Isolation and characterization, poultry excreta, antibiotic resistance, whole genome sequencing.

Table Of Contents

List of Tables

List of Figures

Abbreviations

LB	Luria- Bertani
AST	Antimicrobial Susceptibility Test
NB	Nutrient Broth
MIU	Motility Indole Urease
DPC	Diphenyl-carbazide
TSI	Triple Sugar Iron
e.g.	For example (from Latin *exempli gratia*)
WGS	Whole Genome Sequencing
TSB	Tryptic Soy Broth
Icddr,b	International Center for Diarrheal Disease Research, Bangladesh
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
rRNA	Ribosomal Ribonucleic Acid
OD	Optical Density
etc.	And so on (from Latin *et cetera*)
PGP	Plant Growth-Promoting
CFU	Colony-Forming Unit
MIC	Minimum Inhibitory Concentration
CLSI	Clinical and Laboratory Standards Institute
ChrA	Chromate transporter A (gene/protein)

Chapter 1: Introduction and Review of Literature

1.1 Introduction

Heavy metal pollution has turned into a serious environmental issue in Bangladesh as well as worldwide, where chromium (Cr) is identified as one of the most toxic and persistent pollutants. Despite occurring naturally at trace levels, the large-scale industrial practices such as mining, electroplating, tanning, dyeing, and wood preservation have widely spread chromium contamination in soils and aquatic bodies (1,2). In Bangladesh, chromium accumulation is a serious concern, especially in areas with tannery and textile industries, due to a high level of industrialization in a short time and its association with inadequate waste treatment. The issue is of particular concern as chromium occurs both in highly soluble and mobile and toxic hexavalent [Cr(VI)] and less toxic and even essential for human at trace level trivalent [Cr(III)] forms. Cr(VI) is a strong human carcinogen because of its mutagenic and teratogenic acitvity, and also represents a long-term risk to public health and to the environment (2).

Traditional approaches for the treatment of heavy metals (chemical precipitation and ion-exchange) are expensive and are not practical for implementation on an extensive scale in resource-poor countries such as Bangladesh. The organic materials are chemically treated and are then transformed into material that can be easily disposed of or recycled (3). Consequently, biological methods are becoming more and more interesting, especially employing micro-organisms which are resistant to and able to deactivate heavy metals. Microorganisms have special machinist to resist metal toxicity by biosorption, bioaccumulation, enzymatic reduction and efflux systems. For chromium Cr, some bacteria species are able to transform toxic Cr(VI) into the less harmful Cr(III) by enzymatic reduction and hence reducing its bioavailability and environmental burden(3,4). The strains of *Bacillus*, *Pseudomonas*, *Azotobacter*, have been reported to tolerate the high level of chromium and help in the detoxification of chromium and hence these may be manageable for the bioremediation of chromium (5).

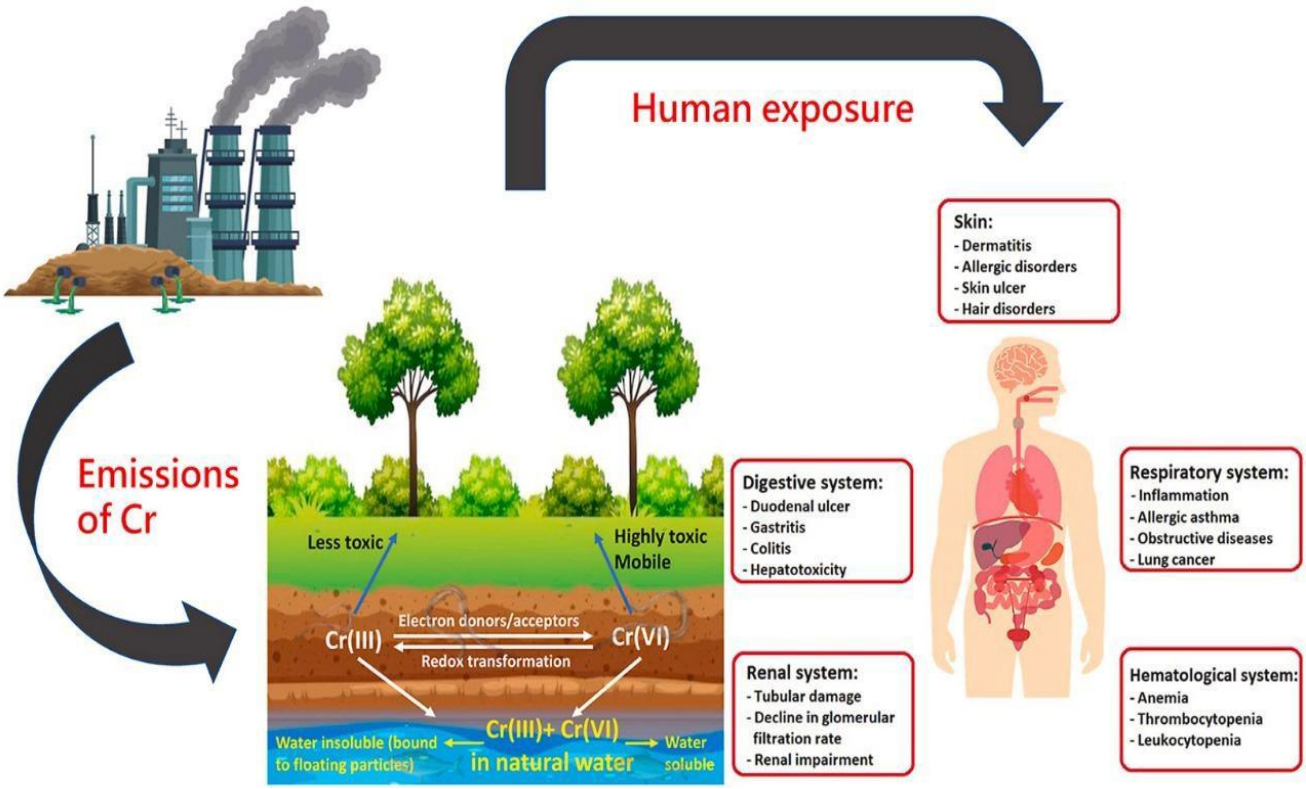


Figure 1.1: Biogeochemical behavior and toxicology of chromium in the soil-water-human nexus.

[This image shows the natural and health effects of chromium emissions. It shows how Cr (III) and Cr (VI) move via soil and water, highlighting their toxic effects on human health, including skin, digestive, respiratory, renal, and hematological systems] (37).

Poultry industry in Bangladesh is significant for food production and rural economics. Nevertheless, poultry production has been linked with heavy metal accumulation and spread. Contamination of chromium in Poultry production could be directly through the feed ingredients and water or indirectly from the former use of tannery wastes in poultry feed which was a source of protein at a low cost (6). Many studies have found high levels of chromium in poultry feed and finally deposit in chicken body, organ and excreta. As a result, poultry litter (feces + bedding) becomes one of the major sources of chromium contamination in agricultural soils as organic fertilizer (7). When chickens eat the Cr-laden feed, the metal will accumulate in their bodies and may ultimately be excreted in their manure. For this reason, poultry litter (feces mixed with bedding material) in such infected environments can have high levels of chromium

(7). For example, the high Cr contents found in the poultry feed samples of the surveyed farms by a study is very alarming and indicates that poultry excreta can represent a burden of heavy metals (8). The fact that Cr was found in the poultry excreta, in addition to the risk of polluting the soil (pure or when litter is applied as manure), raises the possibility of selecting the microflora toward microorganisms capable of tolerate or even proliferate in such a high-metal environment. Basically, in the zone of heavy metal inputs, chicken droppings are a niche habitat for chromium- tolerant bacteria, indicating the cooperation between agriculture and environmental pollution. Apart from issues related to food safety and pollution liability, the occurrence of chromium in poultry waste contributes selective pressure promoting the survival and the growth of chromium-tolerant bacterial communities (7).

The presence of chromium-tolerant bacteria in poultry waste not only is an indication of environmental contamination but also provides information of biotechnological interest. These heavy metal-resistant microorganisms can be used as biological agents to remediate heavy metal polluted sites (9). As they possess chromate reductase enzymes and corresponding genetic determinants, which enable them to reduce or immobilize chromium indicating their ecological importance. Indeed, recent studies conducted in Bangladesh identified various chromium-resistant bacteria from poultry waste, e.g. *Bacillus* and *Brevibacillus* species isolated from chicken excreta, showed tolerance to 1000 ppm Cr(VI) in culture (9). All the isolates from that study had the chr gene, which is responsible for the detoxification of hexavalent chromium. Metal-resistant bacteria such as these could potentially be used for purposes of bioremediation to help remove or detoxify Cr in contaminated materials. Meanwhile, continued heavy metal presence in environments, such as on poultry farms, carry public health implications — heavy metal stress can co-select for antibiotic resistance in bacteria, so the bacteria with chromium resistance also may be carrying around antibiotic resistance genes (7). Actually, poultry farms have been identified as potential sources for multidrug-resistant bacteria in Bangladesh (also locally in Noakhali), and long-term exposure to metals such as Cr is one of the factors that might favor the co-resistance process. These two concerns emphasize the need to characterize those metal-resistant bacterial populations that occur in agricultural environments (10).

The Noakhali region of Bangladesh, being a coastal region and experiencing a rise in poultry industry, has not been extensively studied for heavy metal pollution along with its microbiological effect. In light of data on chromium contamination of poultry feed and litter in other areas of the country, it is important to determine whether such contamination and an

adapted microbial flora occurs in Noakhali as well. Isolating and characterizing chromium-tolerant bacterial strains from poultry excreta in this area will determine how much contamination chromium introduction leads to and which biological changes result from it. Such information is precious for several causes: holding a biological mirror of Cr pollution of Noakhali environment, contributing to risk management studies for agricultural application of poultry manure, and contribution to work towards bio-based polluting mitigation systems using native bacteria. Hence, the study aimed at isolation, and characterization of chromium tolerant bacteria from poultry excreta at Noakhali region. Our study will simply fill this gap and enhance the knowledge of the microbial ecology affected by heavy metal exposure in livestock waste and the application of these hardy bacteria in bioremediation of chromium pollution. In the long run, this study will assist in filling the knowledge gap on heavy metal–microbe interactions in a region in Bangladesh where sustainable management of agro-wastes and environmental health have recently assumed higher priorities.

1.2 Review of Literature

Heavy metals are defined as naturally occurring elements with high atomic mass and density five times greater than water that are toxic even at low levels. Heavy metals are not biodegradable, unlike organic contaminants as they remain in the environments for several years and even decades, and they bioaccumulate in animals, plants, and human. Worldwide, industrialization, mining, high intensity farming and the dumping of urban wastes have stepped up the rate at which heavy metals are being discharged into the biosphere. Their persistence has earned them the dubious distinction as one of the most important pollutants of the 21st century (11).

The most common typical heavy metal contaminants are chromium (Cr), cadmium (Cd), lead (Pb), mercury (Hg), arsenic (As) and the like. Heavy metals, in contrast to organic contaminants are non-biodegradable and accumulate and remain in the soil, water, and living organisms (12). Industrial and agricultural activities, such as metal smelting, tanning and textile production, mining, inorganic waste dumping and pesticide and fertilizer applications containing metal compounds, are the prime sources of heavy metal pollution. Due to the fast industrialized and modern farming, these inorganic pollutants are more being driven into the air, water and soil at the global level. Heavy metals can interfere with biological processes when they are present above trace levels: some are carcinogenic, (arsenic), some are highly poisonous (lead, mercury), and others are essential micronutrients (iron, zinc, copper, manganese) which are toxic in larger amounts.

Heavy metals tend to bioaccumulate there – to concentrate in tissues over time, and move up the food chain through living organisms (12). A side effect of this bio-accumulation is that it will cause chronic toxicity in wildlife and humans, producing effects from growth inhibition in plants to organ damage and cancer in humans. Heavy metal pollution has been identified as an important environmental issue worldwide and it is characterized by stringent monitoring and treatment (13).

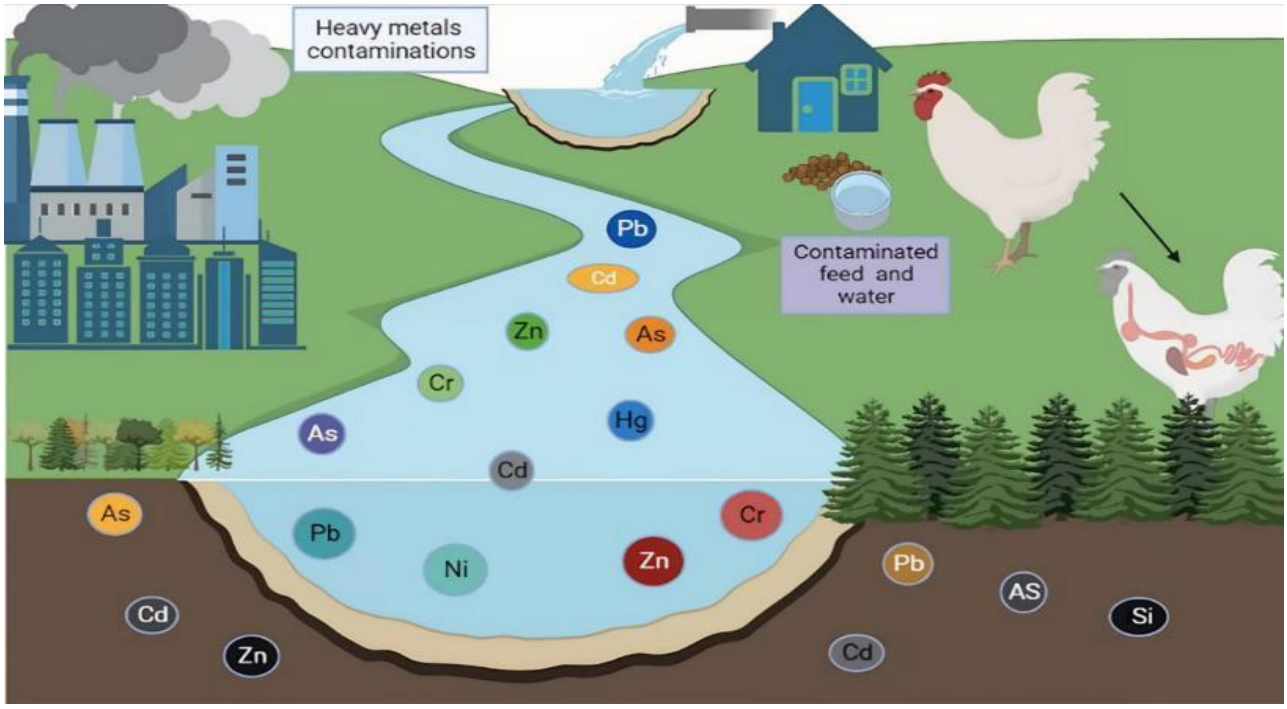


Figure 1.2: Heavy metal contamination in poultry.

[This figure depicts the contamination of water, soil and feed with heavy metals like As, Pb, Cd, Zn and others. It highlights their pathway from pollution sources to chicken exposure, affecting their health] (Biorender).

1.3 Chromium Contamination in Bangladesh

The heavy metal chromium (Cr) is one of the priority toxic pollutants and it is widely used in industries such as metallurgy, electroplating, leather tanning, and pigment. Chromium can occur in various valence states, and Cr (III) and Cr (VI) are the most stable forms in nature. Cr (III) is an essential micro-nutrient at very low concentration, whereas Cr (VI) is a potent toxin, mutagen and carcinogen (14). Cr (VI) compounds are soluble in water, mobile in soil, and readily absorbed into biological systems, potentially representing a significant environmental and public health threat (15). Leather tanneries are one of the anthropogenic sources that are responsible for pollution of the environment with heavy metal including chromium in Bangladesh. Tanneries, located in the Hazaribagh region of the city of Dhaka, have for decades notoriously discharged untreated waste water including hexavalent chromium (Cr(VI)) into the environment. During their height, an estimated 150-200 tanneries in Hazaribagh used to discharge 22,000 cubic litres

of hazardous waste per day (including carcinogenic Cr(VI)) into the Buriganga River, Dhaka's key waterway. That uncontrolled pollution landed Hazaribagh on the World's Top 10 Toxic Hot Spots, with the population plagued with higher incidence of skin diseases and lung ailments due to exposure (16).

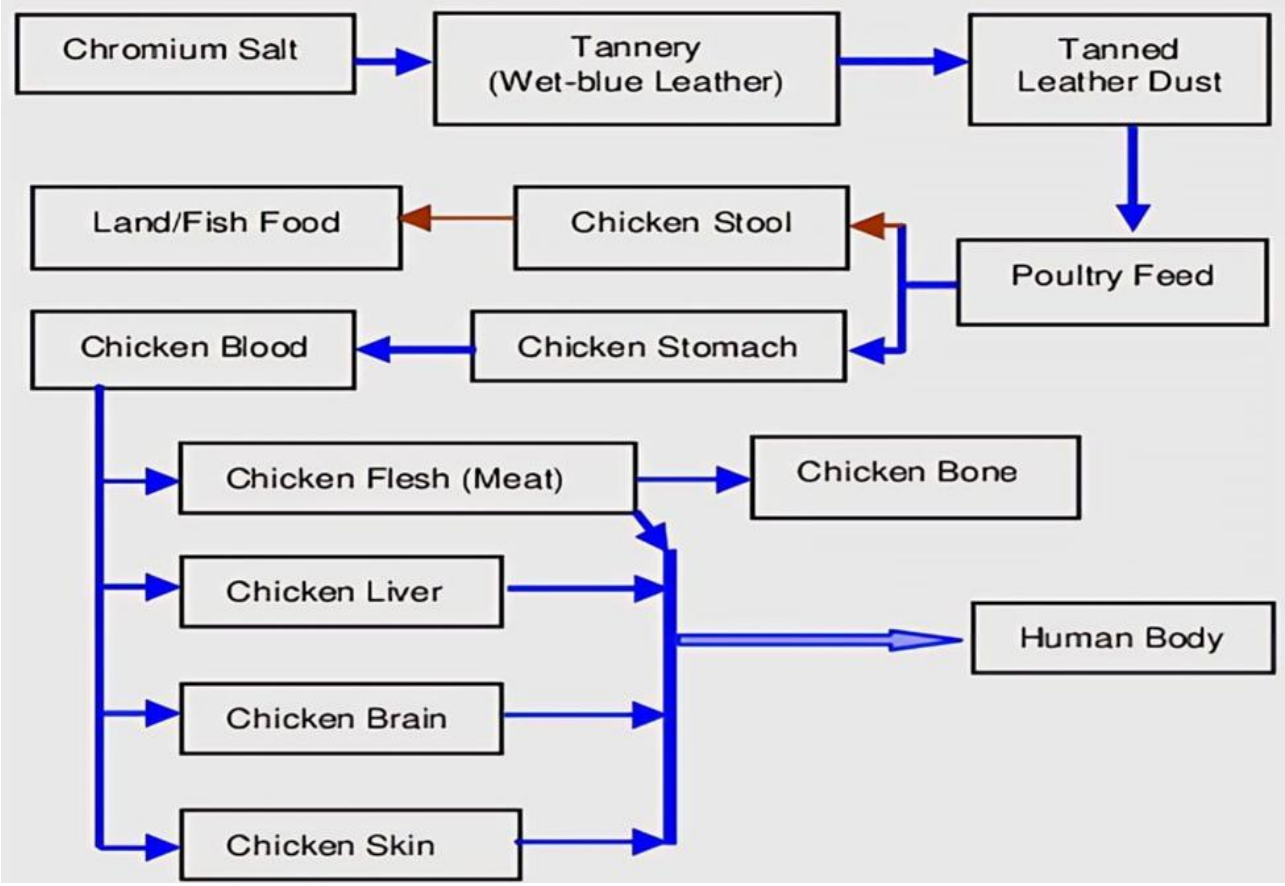


Figure 1.3: Flow Chart of the Transport Mechanism of Chromium from Tannery Wastes to Human Body.

[This flowchart depicts the pathway of chromium exposure, from chromium salts in tannery and leather dust, to poultry feed, chicken tissues, and ultimately to human consumption through chicken meat and by-products] (19).

In addition to those tanneries in Dhaka, chromium and a number of other heavy metals have been found at many media of the environment throughout the country. For example, heavy metals have been detected in agricultural soils and irrigation water in the vicinity of industrial areas and waste dump sites. In a region such as Noakhali in particular – a region with little industry – fears of heavy metal pollution have arisen from more diffuse forms of contamination. Heavy metals pollution was reported to be present in the Meghna River estuarine region of Noakhali coast by previous studies (17). Furthermore, heavy metals can get into the food chain from tainted animal feeding stuffs. A on national survey on poultry feed in Bangladesh revealed alarming levels of chromium, cadmium, and lead in commercial feeds. Accordingly, animals fattened with such feeds can store chromium in their tissues.

In another study, the concentration of chromium in broiler chicken meat available in local markets was higher than what is considered safe for human consumption and the estimated cancer risk over a lifetime due to dietary chromium was also above acceptable levels (18). These results reveal that chromium pollution is not confined to known industrial hotspots but can spread to rural and coastal regions though water, soil and feed, with known implications for environmental and human health (17). Monitoring in locations such as Noakhali is extremely important because of limited baseline information concerning chromium in this area While there is a need to clean up known polluted areas in Bangladesh (e.g. former tannery sites), care should also be taken to monitor in locations where no or little work has been done with respect to heavy metals.

1.4 Environmental Impact of Chromium Pollution

Environmental contamination by chromium is often accompanied with severe ecological and long-term consequences. The most significant oxidation states are Cr(VI) and Cr(III), both with marked differences. Cr(VI) (commonly as chromate or dichromate) is so soluble and mobile and toxic, whereas Cr(III) has a strong tendency to form insoluble compounds that may be adsorbed on to soil particles (20). In the soil, hexavalent chromium is a serious threat to the health of the soil. It changes soil microbial community- high Cr(VI) concentration is not liveable for many useful microbes. It has been reported that Cr(VI) exposure results in soil bacterial biomass and

enzyme activities decrease, and consequently organic matter decomposition becomes slower (Nirola et al. Contaminated soil may be degraded in its fertility and growth of plants. Cr(VI) in soil could be also absorbed by plants to some degree, resulting in phytotoxic effects, including the low germination, growth depression, leaf chlorosis etc. Chromium is not harmless to aquatic ecosystems either. Cr(VI) is easily soluble in water and can disseminate in a surface run-off or leaching manner, thereby affecting rivers and groundwater aquifers (21). Aquatic organisms experience not only lethal, but also sublethal toxicity by chromium, i.e. fish and invertebrates exposed to Cr(VI) may suffer from oxidative stress, gill and liver damage, stunted growth and lower reproductive output. Chromium can bioaccumulate up the aquatic food web, so predators (including people who eat fish) get tiny, concentrated doses. There is also evidence that constant Cr release can perturb the complete biology ecology of aquatic systems, causing biodiversity to disappear.

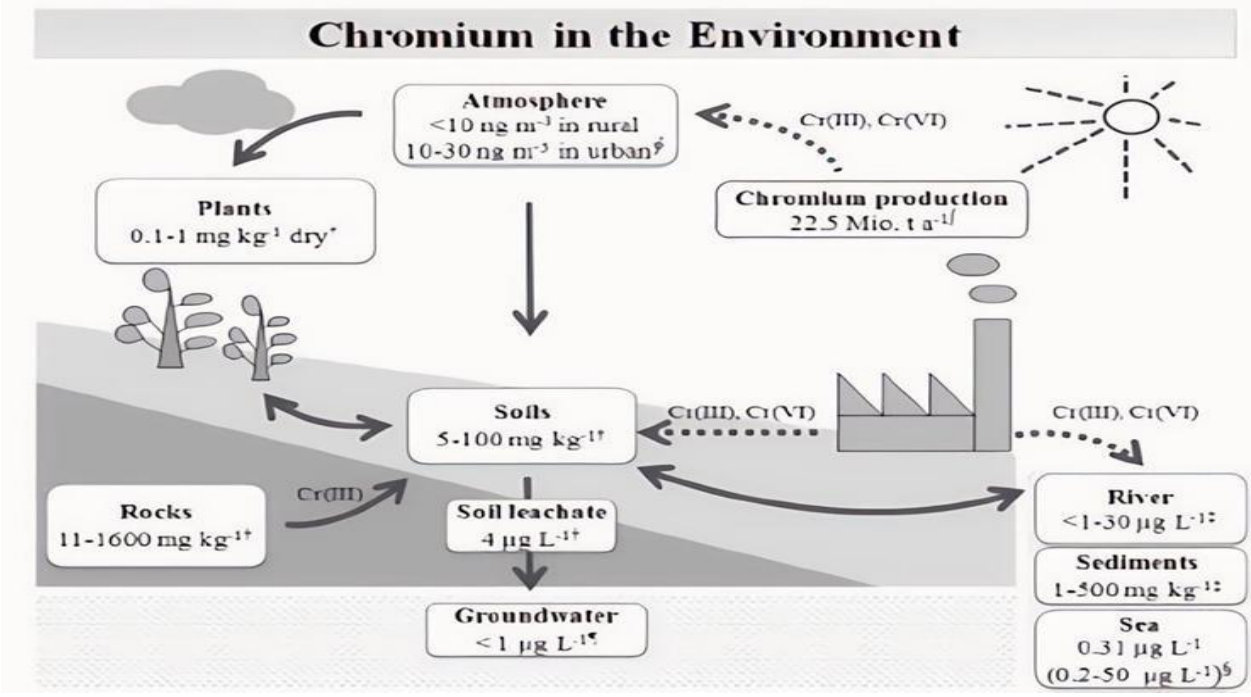


Figure 1.4: General distribution of chromium in the Environment.

[This diagram illustrates the environmental distribution of chromium, showing its presence in the atmosphere, plants, soil, groundwater, rivers, and sediments, alongside chromium production and various chemical forms (Cr(III) and Cr(VI)) (36)].

In Bangladesh, the ecological cost of chromium contamination has been widely publicized close to the former Hazaribagh tannery area. Downstream of Hazaribagh, a stretch of the Buriganga River went biologically dead – and harbors no (or very few) fish or other animal life – because of the high quantities of chromium and other industrial waste in the river (22). River and floodplain sediments contained chromium at concentrations well in excess of natural background levels, and nearby agricultural fields that were irrigated with water contaminated with chromium had chromium added to the soil and crops. There is also a public health dimension to environmental chromium: if water, such as drinking water or water used for irrigation, or crops are contaminated with chromium, then human exposure may be possible (see discussion in section following). In general, chromium pollution causes complex environmental problems, i.e., habitat destruction, wildlife poisoning and health concerns, and thus requires a comprehensive remediation and prevention approach.

1.5 Chromium Toxicity & Health Effects

The health impact of chromium depends upon its chemical form, with trivalent chromium (Cr(III)) being a required trace element in glucose and lipid metabolism, whereas hexavalent chromium (Cr (VI)) is a carcinogen. The International Agency on Cancer Research lists Cr(VI) as a Group 1 carcinogen, that specifically causes lung cancer when inhaled (25,26). Cr(VI) toxicity results from the fact that it can penetrate cells by the sulfate transport system, as chromate ions are similar to sulfate ions. Intracellular reduction of Cr(VI) to Cr (III) intracellularly (by cellular reducing agents like ascorbate and glutathione) occurs via Cr(V) and Cr(IV) intermediate species. This generates ROS and reactive intermediates of chromium, causing oxidative stress, DNA strand breaks, and DNA-protein adducts (27).

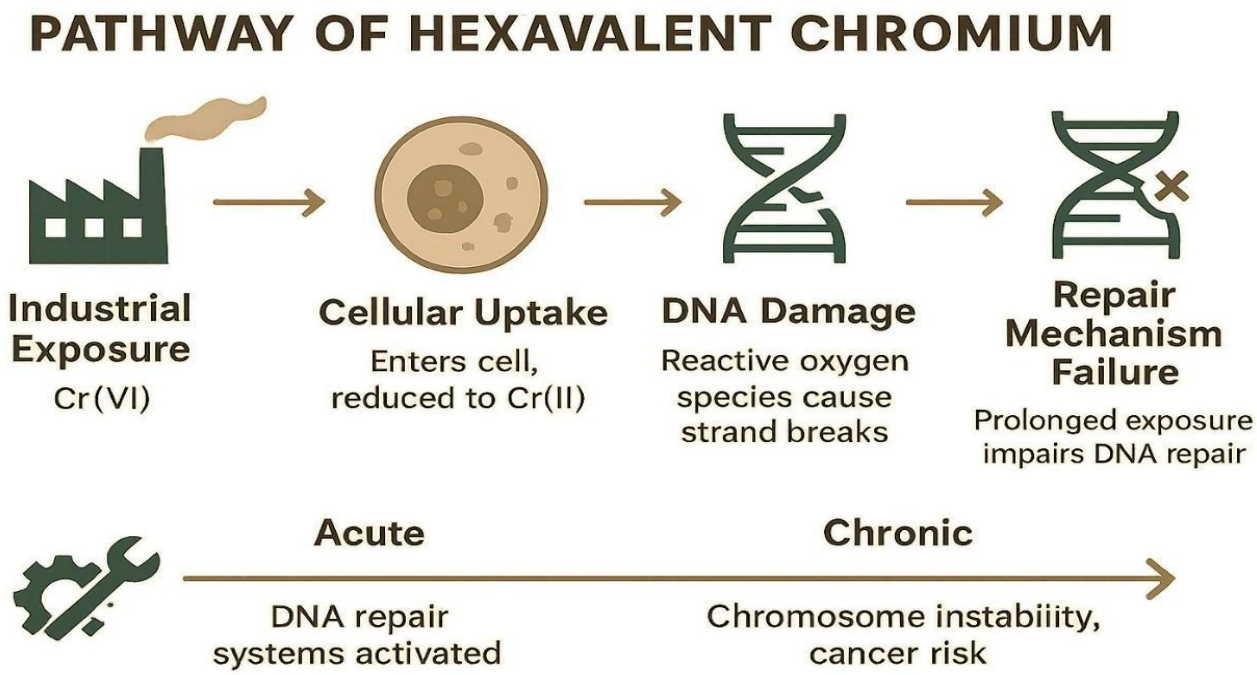


Figure 1.5: Pathway of hexavalent chromium toxicity.

[This diagram provides outline of the pathway of hexavalent chromium (Cr(VI)) exposure, from industrial sources to cellular uptake, DNA damage, and failure of repair mechanisms, leading to acute and chronic health effects such as cancer risk] (28).

The predominantly affected site in occupational settings is the respiratory tract. Inhalation of dusts containing Cr(VI) is associated with chronic irritation of the airways, ulcerations of the mucous nasal membranes – (chrome holes), asthma, and increased rate of bronchitis and pneumonia. Prolonged exposure results in lung cancer and numerous epidemiological studies consistently demonstrate an increased risk for cancer in exposed workers (29). It characteristically presents with severe contact dermatitis, allergic reactions, and characteristic ulcers. Sensitization can be initiated in exposure by 4-25 ppm. Description Chrome ulcers are painless, scabby sores that do not heal well. The prevalence of chromium sensitivity among cement workers is estimated at 4-5 % (30,31).

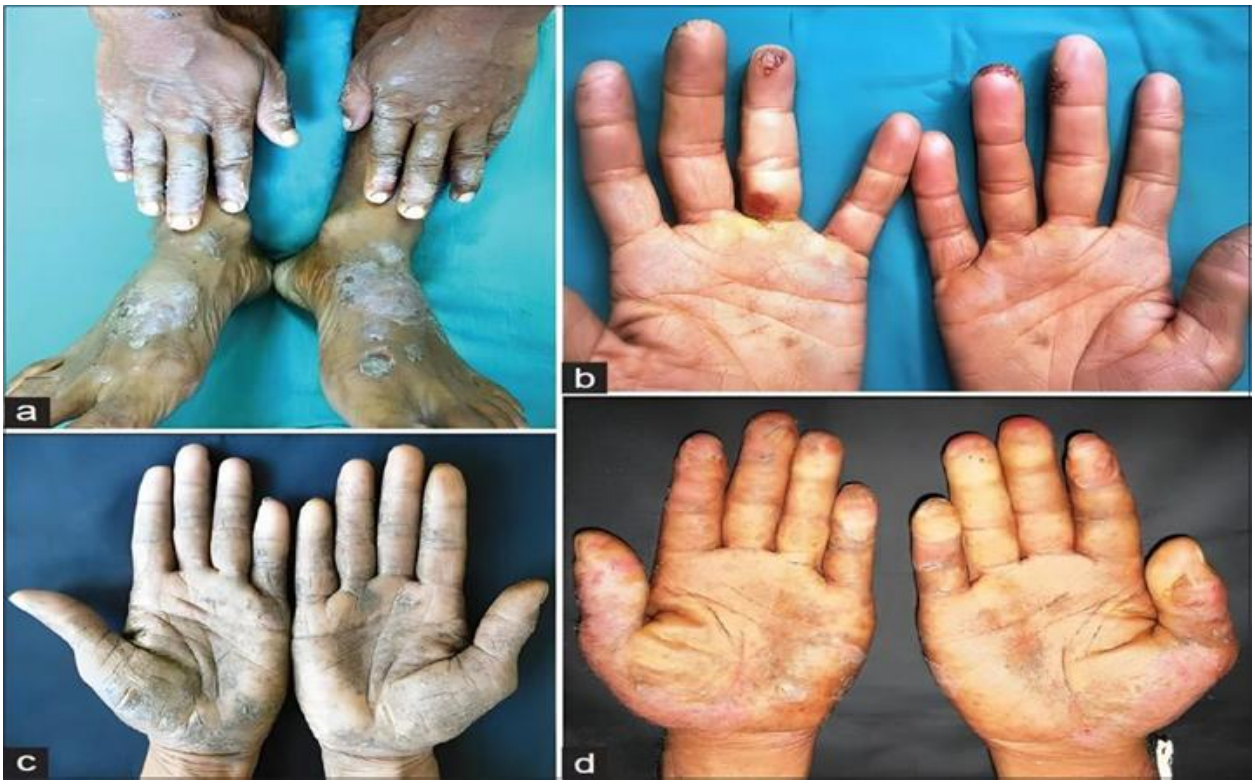


Figure 1.6: Hands and feet showing severe occupational contact dermatitis with ulcers and scaling consistent with chromium-induced skin toxicity.

[This image shows the effects of chromium exposure on the skin, including lesions and discoloration. Panels (a) and (c) show hand and foot damage, while (b) and (d) highlight severe skin reactions on the hands] (38).

Systemic toxicity affects multiple organ systems. Gastrointestinal ingestion causes severe irritation, ulceration, and potential carcinogenesis. Animal studies show temporary microcytic anemia and hematological alterations. Hepatotoxicity and nephrotoxicity of hepatorenal poisoning causes multi-organ acute and chronic effects (32).

Reproductive and developmental effects have also been observed in maternal transplacental transfer of chromium during pregnancy, which results in decreased fetal weight and viability and abnormalities in structure. Cr (VI) exposure interrupts the female reproductive functions by elongating the estrous cycle and decreasing fertility indices (33,34). Conversely, Cr (III) is also 1,000-fold less toxic due to its poor cellular uptake and impermeability through the barrier. Cr (III) is able to complex transferrin and enter cells via receptor mediated endocytosis in a manner that is not toxic, in contrast to being harmful. There has however been some in vivo reduction of the intracellular Cr (VI) to Cr (III) (35).

Regulatory consequences and remediation options are based on the dramatic toxicological differences among oxidation states. Current interventions focus on minimizing Cr(VI) exposure through engineering controls and medical monitoring. Emphasis on environmental remediation addresses reduction or immobilization of Cr(VI) and oxidations state as the overriding toxicological determinant. Such a thorough understanding of the mechanism of chromium toxicology is critical for worker safety and to ensure environmental protection.

1.6 Microbial Tolerance to Heavy Metals

Tolerance to heavy metals is an important adaptive mechanism allowing bacteria to live and grow in heavy metal polluted environments, such as environment contaminated with chromium. Multiple elaborate resistance systems establish the heavy metal tolerance in the bacteria, operating at different levels of the cells (including efflux systems, biosorption, enzymatic detoxification, and the intracellular sequestration). These processes play a crucial role in the knowledge of bacterial resilience in metal-contaminated food products like the excreta of poultry, whose excreta are contaminated with a number of different heavy metals (40,41).

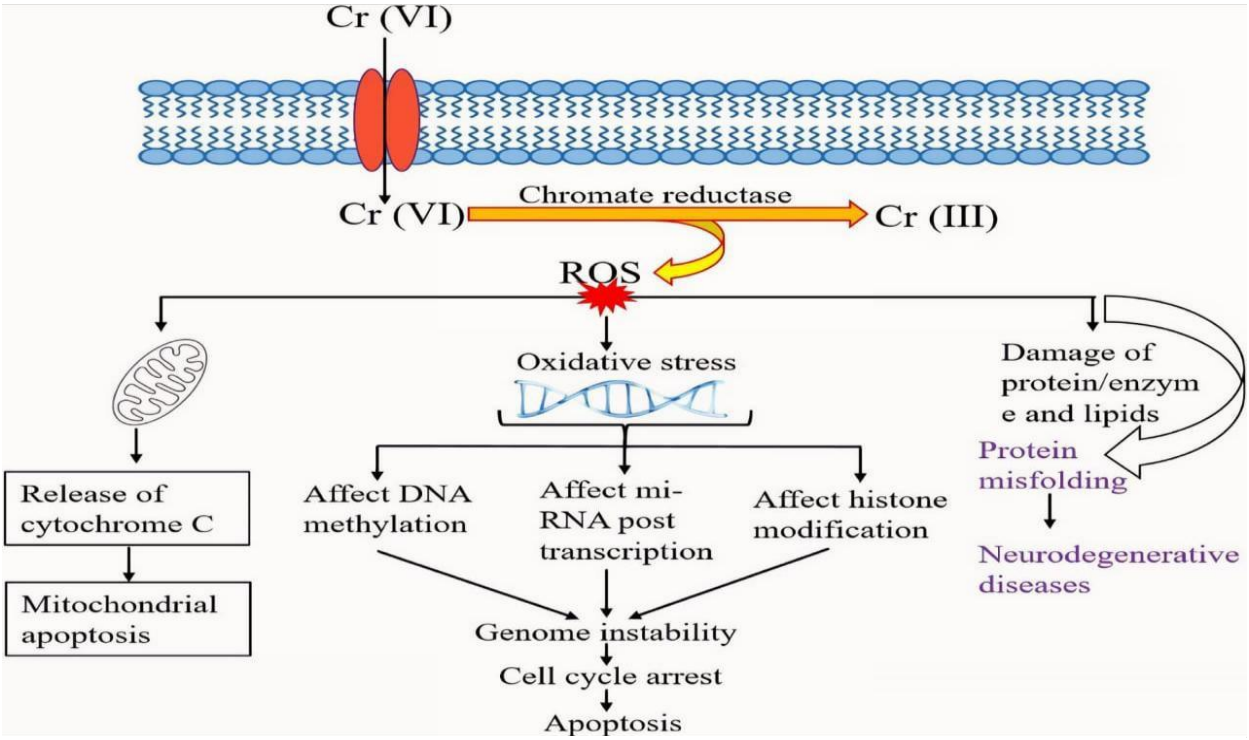


Figure 1.7: The generation of ROS caused by Cr (VI) and their involvement in the destruction of cell organelles.

[This diagram illustrates the toxic pathway of Cr (VI) exposure, showing its reduction to Cr (III), resulting oxidative stress, DNA damage, mitochondrial apoptosis, and potential neurodegenerative diseases due to protein misfolding] (39).

The main means of Bacterial defense against the toxicity of heavy metals is the efflux systems. These transport proteins excrete the toxic metal out of the cell bringing the intracellular metals at levels that are not lethal. The most representative systems are the ATP-binding cassette (ABC) super-family of pumps, resistance-nodulation-cell division (RND) family of transporters, and P type ATPase (42). Among others there has been considerable research on ChrA efflux pump, whose protection runs up to 200mM of Cr(VI) in different bacteria.

The metal sequestration occurs through metal bio sorption mechanisms involving a passive and active process. Active bioaccumulation: This can be achieved through metabolism-based intracellular storage as the metals are transformed into non bioavailable forms by binding with metallo-chaperones and metallo-thioneins (MTs). It has been observed that due to the high efficiency of bioaccumulation, strains of bacteria like *Bacillus megaterium*, *Pseudomonas aeruginosa* and Bacillus cereus can remove up to 91 98.5 percent of various metals that can be considered heavy such as lead, mercury and chromium among others (44). .

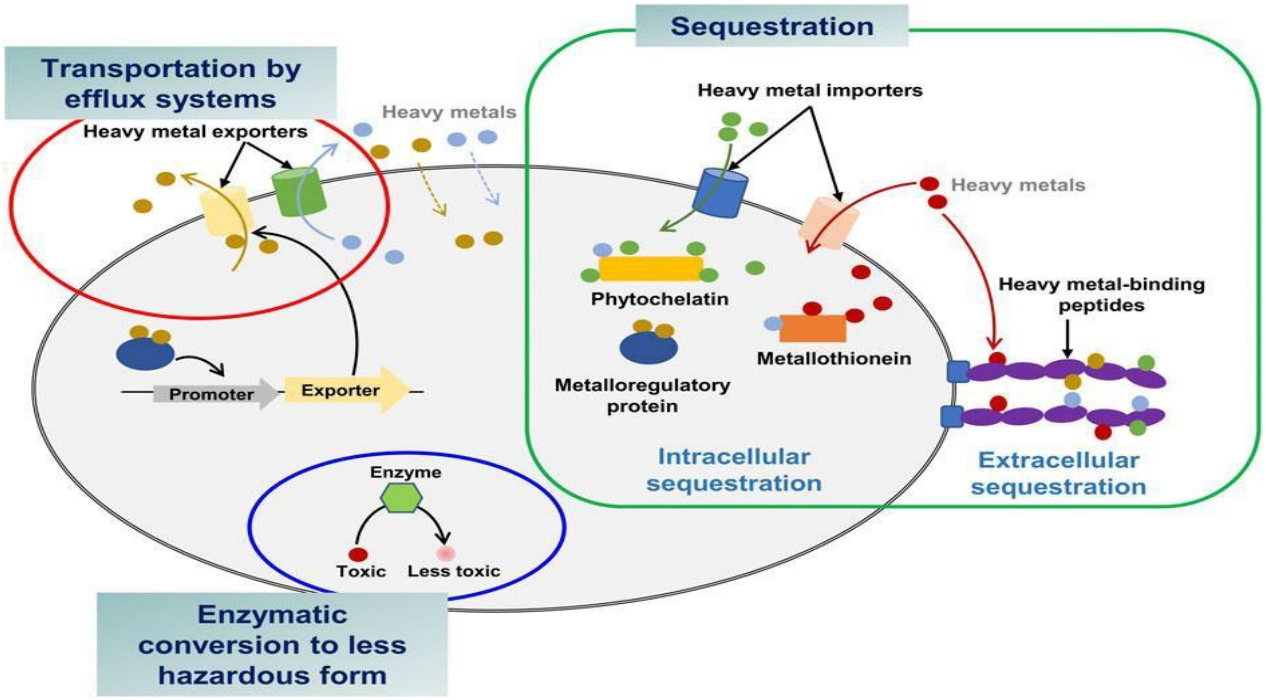


Figure 1.8: Microbial mechanisms of heavy metal tolerance, including efflux, sequestration, and Enzymatic conversion.

[The figure illustrates mechanisms for heavy metal detoxification, including transportation by efflux systems, sequestration (intracellular and extracellular), and enzymatic conversion to less toxic forms, through various proteins and peptides] (46).

The other tolerance mechanism is EPS production. Metal-resistant bacteria produce extracellular polymeric substances (EPS) or films with polysaccharides, proteins and nucleic acids covering the cell. EPS have large quantities of functional groups such as hydroxyl and carboxyl which facilitate bonding between the EPS and heavy metal cations by ion exchange, complexing and precipitation reactions. Studies indicate that *Pseudomonas aeruginosa* and *Azotobacter chroococcum* grow up to produce 1306.7 and 1660 0g mL 1 EPS respectively under exposure to lead stress effectively to chelate metal ions into their non-existence and reduce their bioavailability (45).

The process of enzymatic detoxification is the transformation of toxic metal species to less toxic form. In case of chromium tolerance, Cr(VI) which is very toxic is reduced by bacteria reductase like ChrR and YieF to less toxic Cr(III). This can be a detoxification reaction that is frequently coupled with efflux transport, so that transported out of the cell is a reduced form of the metal. The bacteria also turn on their reactive oxygen species (ROS) detoxification and DNA repair/iron homeostasis enzymes as a heavy metal countermeasure (43).

The overlap between resistance systems differs by a wide margin in different bacterial species of the heavy metal-resistant strains. Gram-positive bacteria are known to be more tolerant to chromium, whereas, mercury tolerance is seen more among Gram-negative bacteria. Such tolerance mechanisms are frequently coded both by chromosomal of plasmid DNA, and facilitate horizontal transfer of resistance traits between bacteria. This has resulted in the evolution of highly complex tolerance systems to resist such environments and bacteria capable of colonization and growth in highly polluted locations are of potential bioremediation and natural biomonitoring interest.

1.7 Chromium-Tolerant Bacteria: A Global Perspective

Several isolates of bacteria that can tolerant and capable of transforming chromium have been found in various different landscapes all over the world. Tannery effluent and waste has provided rich source of such bacteria. Indeed, researchers in India and Pakistan, to take two examples, have retrieved highly Cr-resistant strains in tannery effluents and sludge (47). Such isolates commonly reside within well-known stress-tolerant genera, such as Bacillus, Pseudomonas, Arthrobacter and the Acinetobacter. Bacillus and Pseudomonas bacteria species able to tolerate chromium are frequently found in soil contaminated with chromium (e.g. mining sites or around chromit ore processing plants).

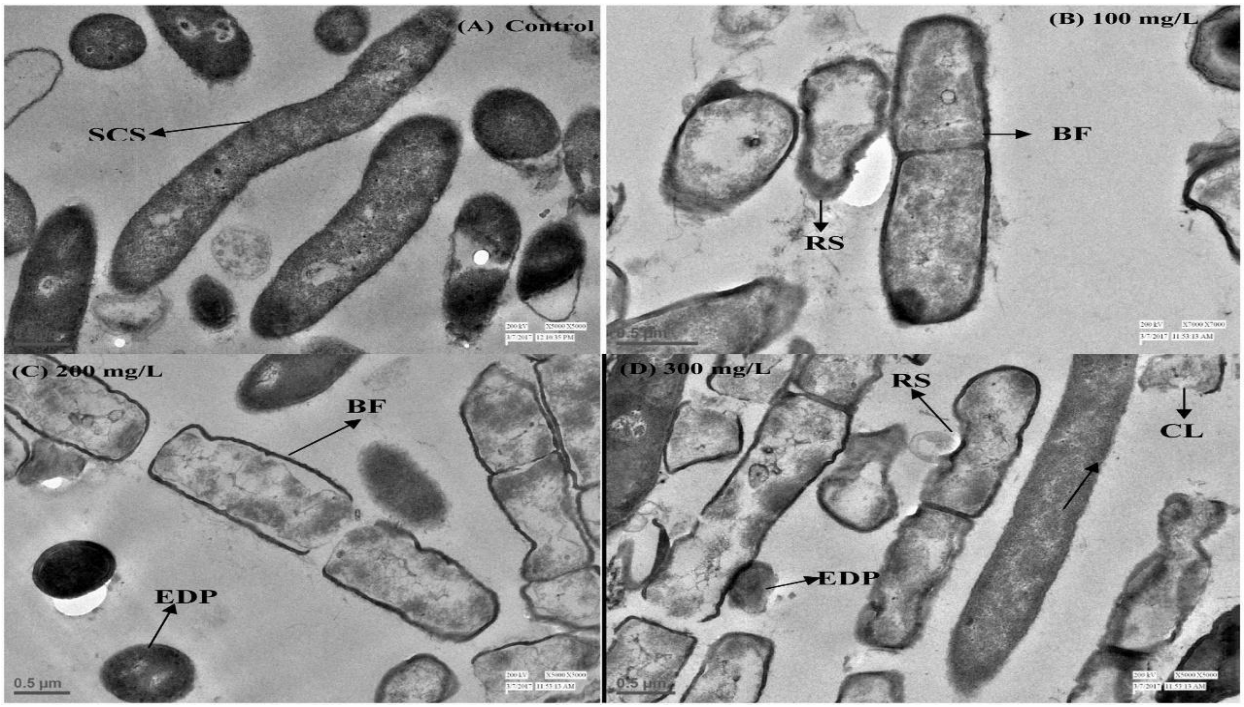


Figure 1.9: Transmission electron microscopy (TEM) images of cross sectioned *B. subtilis* MNU16 cells.

((A) control (without treatment). (B) B. subtilis MNU16 cells at 100 mg/L of Cr(VI) (C) B. subtilis MNU16 cells at 200 mg/L of Cr(VI) and (D) at 300 mg/L of Cr(VI) concentration. Arrows signifies SCS, smooth cell surface; RF, rough surface; BF, binary fission; EDP, electron dense precipitates; CL, cell lysis) (51).

Akin to this, Pseudomonas in some settings has been demonstrated to have a mind-boggling ability to withstand, and cope with, chromium, some using it as an alternate essentially vital oxygen

acceptor in anaerobic respiration (48). Other interesting Cr(VI)-reducing bacteria reported around the world include *Shewanella oneidensis* (another metal-reducer that has been extensively studied; this organism is capable of utilizing Cr(VI) as its respiratory terminal-electron-acceptor), Ochrobactrum species (e.g. an *Ochrobactrum sp.* isolated in a chemical industry site could tolerate Cr(VI) in concentrations up to ~300 ppm, and their reductive enzymes can metabolize Cr(VI)), and *Stenotrophomonas maltophilia*, we even found chromium-resistant bacteria in extreme locations, including the high altitude soils and deep sea sediments implying microbial tolerance to heavy metal stress especially metal components like potassium dichromate (K2Cr2O7) (49, 50).

Overall, the worldwide representation demonstrates that the bacteria resistant to chromium are phylogenetically varied and distributed on the worldwide level at any pertinent areas of chromium pollution. One shared feature of these bacteria is that they contain chromate resistance gene clusters (chr operons), have high minimum inhibitory concentrations of Cr(VI) (usually hundreds to thousands of mg/L), and/or reduce or immobilize chromium.

Figure 1.10: Reduction of hexavalent chromium using *Bacillus safensis* isolated from an abandoned mine.

[The figure illustrates the optimization of Cr (VI) reduction conditions using immobilized *Bacillus safensis* CCMM B629 cells. It shows the processes of biosorption and bioreduction, along with the underlying cellular mechanism] (52).

This international body of knowledge can be applied to Bangladesh and to such regions as Noakhali; exchanging local isolates with better-known strains in other countries allows researchers to determine the usefulness and originality of locally discovered chromium-tolerant bacteria. It also accentuates the fact that the bioremediation cases in other locations have been successful, in some cases in using Bacillus, Pseudomonas, or other genera and that this can be applied in similar contexts of ensuring that such an outcome can be applied in the environs of Bangladesh.

1.8 Poultry Excreta as a Reservoir of Microorganisms

The Poultry excreta contains a rich microbiome of several hundreds of bacterial species, including some heavy metal-resistant strains. These include the gastrointestinal tracts of poultry, which contain about 600-900 bacterial species, with subsequent excretion into the environment and addition to the bacterial environmental pool (53). Studies in Bangladesh have highlighted this problem- a 2025 study of poultry farms throughout the country reported a high prevalence of both resistance to heavy metals and to antibiotics in litter bacteria. According to the findings, poultry environments “represent important reservoirs of bacteria that are dual resistant to heavy metals and antibiotics” (54).

Bacteria resistant to chromium found in chicken waste implies the presence of chromium in either the feeding or water. As an example, Khan et al. (2024) detected chromium-resistant bacteria in the feces of poultry in Bangladesh, verifying chromium contamination of the flock. In these isolates, they were able to grow on chromium-augmented media, which indicates that poultry litter can house such microbes who can survive and even transform toxic metals (55).

The presence of metal-resistant bacteria in poultry waste could therefore be used as an alarm call of contamination and the growth of metal- (and often antibiotic-) resistant bacteria in manure poses health risks should these bacteria contaminate the manure workers or the food chain. On the bright side, certain bacteria that are resistant to chromium can be used in bioremediation to

clean-up waste materials or polluted soil areas like industrial waste areas or mining areas (54).

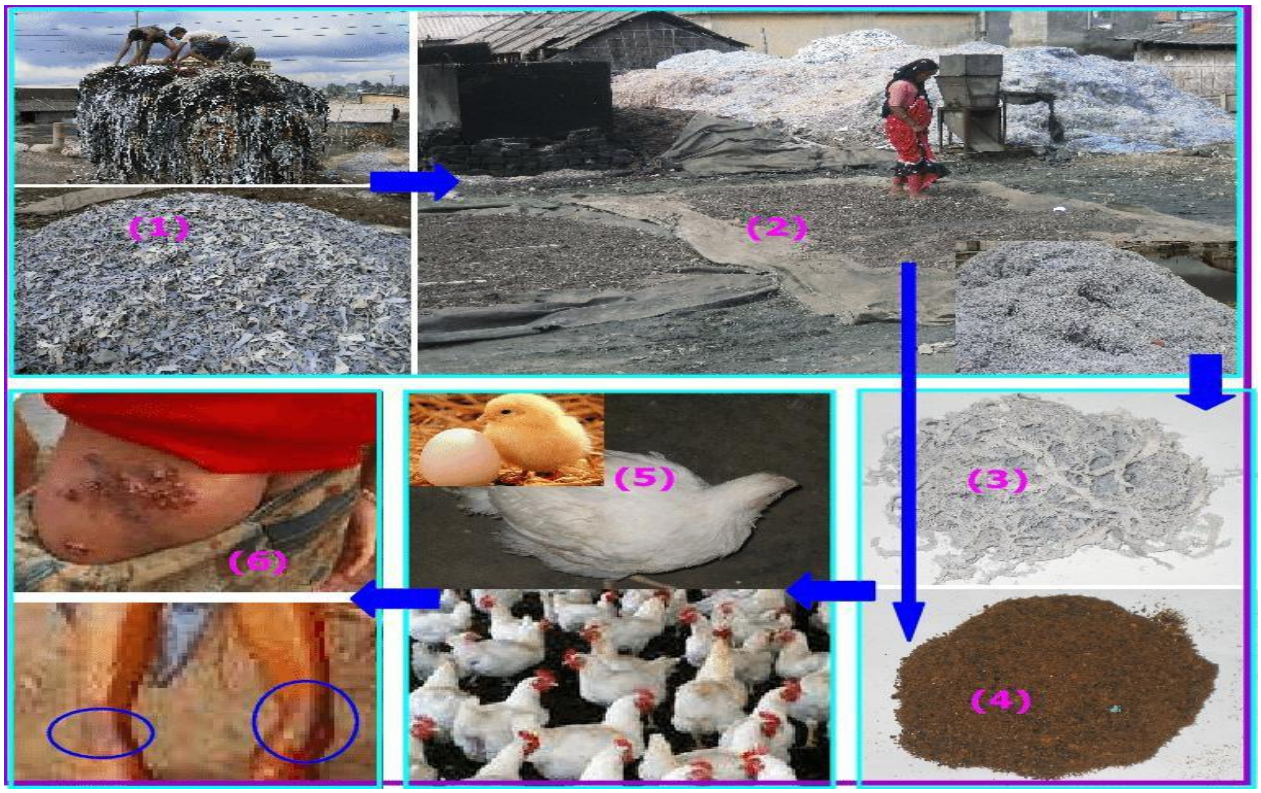


Figure 1.11: Transport Mechanism of Chromium from Tannery to Human Body through Poultry.

[The figure illustrates a process involving the recycling of waste materials (1) into a form of poultry feed (4), highlighting stages of shredding (2), treatment (3), and the potential effects on chickens (5) and human health (6)] (62).

Contamination of chromium in the poultry systems is mainly through the use of tannery solid wastes, especially the dry blue shaving dust and low chromes wet-blue scraps, in poultry foods. Tannery wastes are particularly rich in proteins, and wet blue shaving dust can even contain up to 29,854 mg/kg or chromium, and low-chrome wet-blue scraps can even reach 14,902 mg/kg of chromium. Therefore, the end products poultry feeds produced with such substances may have chromium supported between 3.02 and 618.3 mg/kg which would bioaccumulate in the tissues of the poultry and result in the excretion of waste products (56). The selective pressure by chromium exposure favors the growth of chromium-tolerant communities of bacteria in poultry excreta. Success has been achieved in isolating chromium-resistant bacteria in the poultry feces where isolates have been shown to exhibit resistance towards chromium at concentrations up to 1000 ppm. These bacteria have amazing adaptation patterns, such as the ability to harbor the

genes of chromate reductases, which provides their chromium-reducing functionality. The most prevalent chromium tolerant genera identified are *Bacillus altituidinis, Brevibacillus parabrevis, Pseudomonas spp,* and *Staphylococcus spp*. (57,58).

Biofilm production represents an important survival strategy in chromium-tolerant bacteria in poultry excreta whereby about 67.8 percent of the chromium-resistant isolates exhibit the ability to form a biofilm. This biofilm creation makes bacteria less susceptible to the environmental stresses and adds to the horizontal transfer of genes that may promote the rapid transmission of the resistance mechanism. The duality between the presence of heavy metal tolerance and antibiotic resistance genes in these bacterial populations poses further worries when it pertains to environmental and human health (59,60).

The impact of chromium-tolerant bacteria in poultry excreta is not constrained to the individual farms, since the microorganisms are persistent in the soil and water channels after application of the manure. The microbial communities in poultry-added soil exhibit compositional alteration, where a higher abundance of metal resistance taxa is observed, and the possibility of horizontal gene transfer of the resistance gene. It is vital to comprehend these microbial reservoirs to define a powerful remediation method and control the environmental consequences of chromium pollution in agrarian schemes (59,61).

1.9 Chromium Reduction Microbiology

A common detoxification strategy by many chromium-resistant bacteria is to enzymatically reduce noxious hexavalent chromium (Cr(VI)) to the less toxic trivalent form, Cr(III). The process (ref. bacterial chromate reduction) is of importance due to direct detoxification of the pollutant: Cr(III) is much less toxic and soluble than Cr(VI). Although not every Cr-resistant strain is able to reduce chromate, all known Cr(VI) reducers have intrinsic chromate resistance to survive the metal (63). The degradation process is catalyzed by chromate reductase enzymes (chiefly NAD(P)H-dependent oxidoreductases) which donate electrons to Cr(VI) in a process of reduction (to Cr(III) (64). Various types of these reductases have been described - such as ChrR, YieF, NemA, LpDH - and they may be either in the cytosol or membrane-bound. These enzymes may be coded either in the chromosome or plasmids (65). Resistance determinants borne on

plasmids are frequently encountered; an example is the plasmid-mediated ChrA energy-dependent chromate efflux pump that actively exports chromate ions and reduces the intracellular Cr(VI) concentration (66). Other-ways in which the Cr(VI) reductions by microbes can be carried out include different cell-competencies in the microbe. Some bacteria use extracellular chromate-reducing enzymes that are either secreted as membrane-bound soluble reductase complexes or as secreted reductive agents, and bacterial exposure to Cr (VI) in the cytoplasm is reduced. (Indeed, an inducible chromate reductase in *Bacillus methylotrophicus* was also discovered to be extracellularly released to the medium) More usually, Cr(VI) that gains access to the cell is reduced in the cytoplasm by enzymes driven by NAD(P)H (63).

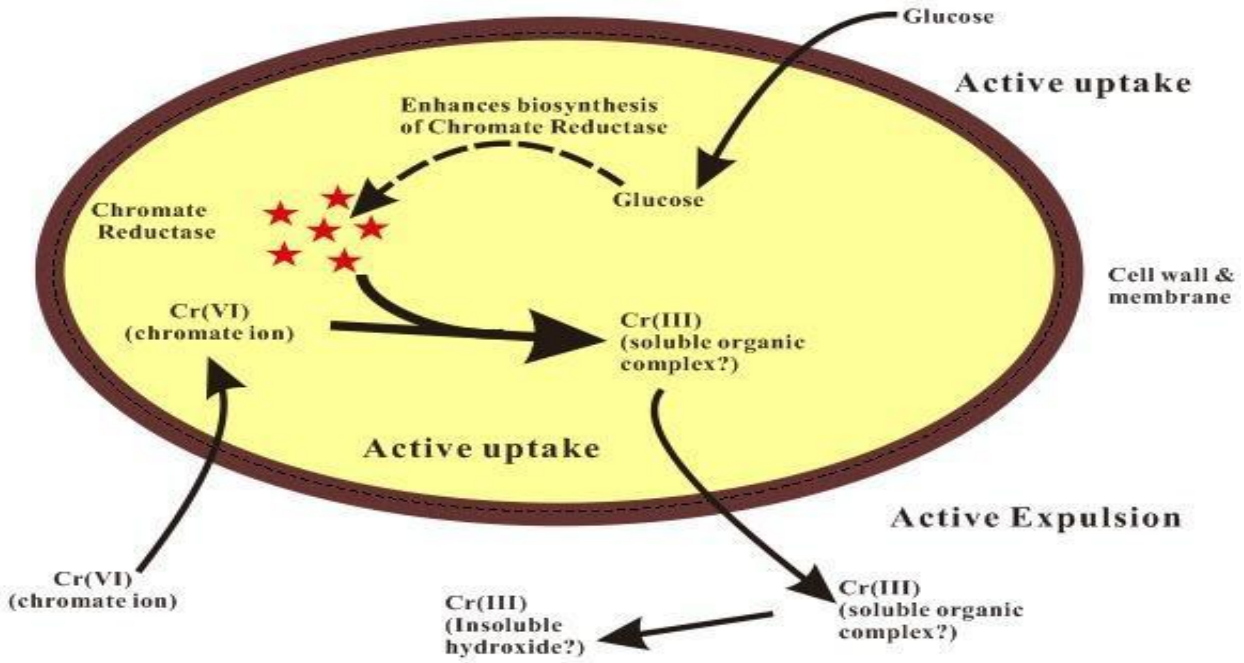


Figure 1.12: Mechanism of bacterial reduction of toxic Cr (VI) to non-toxic Cr (III).

[The figure depicts the cellular process of chromium ion uptake and expulsion, showing the role of chromate reductase in reducing Cr(VI) to Cr(III) and its active transport across the cell membrane] (70).

Chromate reduction exists in two different pathways, enzymatic chromate reduction. In Class I reduction, the enzyme passes on a single electron to Cr(VI) to create a short-lived Cr (V) species that is very susceptible to the O 2 and creates reactive oxygen species (ROS). Class II (Class: semi-tight) chromate reductases (which have no Cr(V) intermediate) produce much less ROS. An example is the major *E. coli* nitroreductase, NfsA, whose mechanism has been characterized,

forming a flavin semiquinone and Cr(V) in reaction with Cr(VI), which redox-cycle to produce large amounts of ROS (67). Similarly, broad-specificity reductases, including a *Vibrio harveyi* flavo-nitroreductase and *E. coli* NemA (flavin reductase) also catalyze the reduction of Cr(VI) to Cr(III), which also gives additional indication of the extended substrate range of chromate reductases. Interestingly, chromate reductase genes are either constitutively expressed or only expressed at the presence of chromate depending on the organism (68).

Table 1.1: Cr (VI) reduction in different microorganisms using reductases with multiple substrate specificity.

Microorganisms	Enzymes	Other substrates	References
Alishewanella sp. WH16-1	Selenite reductase (CsrF)	Selenium (Se)	Xia et al. (2018)
Leucobacter sp.	Dihydrolipoyl dehydrogenase	Dihydrolipoamide	Sarangi and Krishnan (2016)
Caldicellulosiruptor saccharolyticus	Ni-Fe hydrogenase	Hydrogen	Bai et al. (2018)
Vibrio harveyi	Nitroreductase (NfsA)	Nitroaromatic compounds	Kwak et al. (2003)
Staphylococcus aureus LZ-01	NfoR	Flavin and FMN	O'Neill et al. (2020)
Escherichia coli	NemA	Glycerol trinitrate and pentaerythritol tetranitrate	Robins et al. (2013)
Streptomyces violaceoruber strain LZ-26-1	Thioredoxin operon	Thioredoxin	Chen et al. (2014)

Whereas, enzymatic Cr(VI) reduction detoxifies chromate by precipitating Cr(III), the one-electron reduction pathway can produce large quantities of ROS through the Cr(V) intermediate. Therefore, to prevent oxidative damage chrome tolerant bacteria initiate extra defense mechanisms. These include:

1.1.1 **Chromate efflux pumps**: e.g. ChrA transporters that actively extracellularly represent chromate to prevent the build-up of intracellular Cr(VI) (66)

1.1.2 **DNA repair (SOS response):** up-regulation of DNA repair mechanisms in order to repair chromate mediated oxidative damage (63).

1.1.3 **Antioxidant protection:** increased level of antioxidant enzymes (catalase, superoxide dismutase), low molecular (glutathione, vitamins C/E, carotenoids etc.) (63).

The effectiveness of microbial reduction of Cr(VI) may be variable. Other bacteria, such as Pseudomonas and Bacillus strains, come into contact with chromate quickly eliminating 50-80 percent of the chromate in solution within a matter of hours to days. In one research, Bacillus subtilis MNU16 consumed 75 percent of an original 50 mg/L Cr(VI) level in 72 hours to a contention of roughly 13 mg/L (51). The rate and degree by which this reduction occurs is based on factors such as enzymatic capability of the specific organism, the availability of adequate electron donors and environmental factors (pH, temperature, etc.). Bacteria reduction of chromates often works best at slightly acidic to neutral pH and can be suppressed by either excessive concentrations of chromium (VI) or other co-existing contaminating toxic metals.

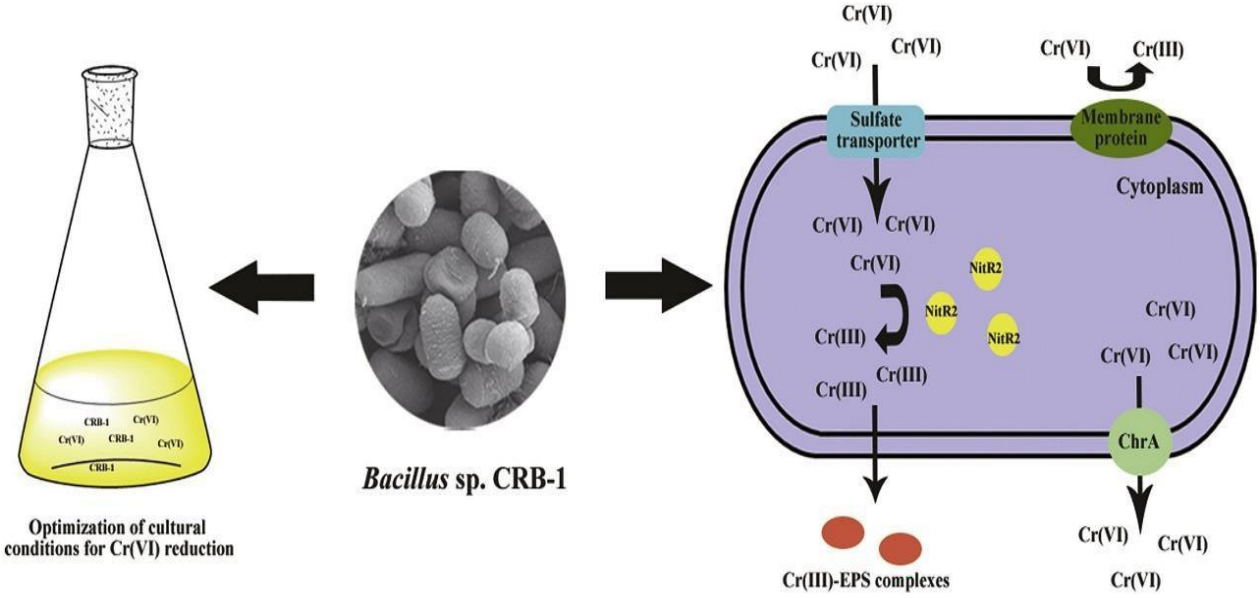


Figure 1.13: Mechanisms of Cr (VI) reduction by Bacillus sp. CRB-1.

[The figure illustrates the optimization of cultural conditions for Cr(VI) reduction using Bacillus sp. CRB-1, showing the uptake, reduction, and expulsion of Cr(VI) and Cr(III) via membrane proteins and transporters] (72).

Interestingly, Cr(VI) also leads to induction of some proteins in some chromate-reducing bacteria. In one example, an Ochrobactrum isolate (strain DM1) was shown to only produce a ~30 Kda protein when exposed to Cr(VI) indicating this formed part of its chromate response and potentially a direct role in reduction/resistance (68). Several chromate reducing bacteria have been shown to possess the gene (chrR) coding chromate reductase as well as additional genes in what is termed as the chr operon which helps the cell defend itself against oxidative damage when reducing the chromate (69).

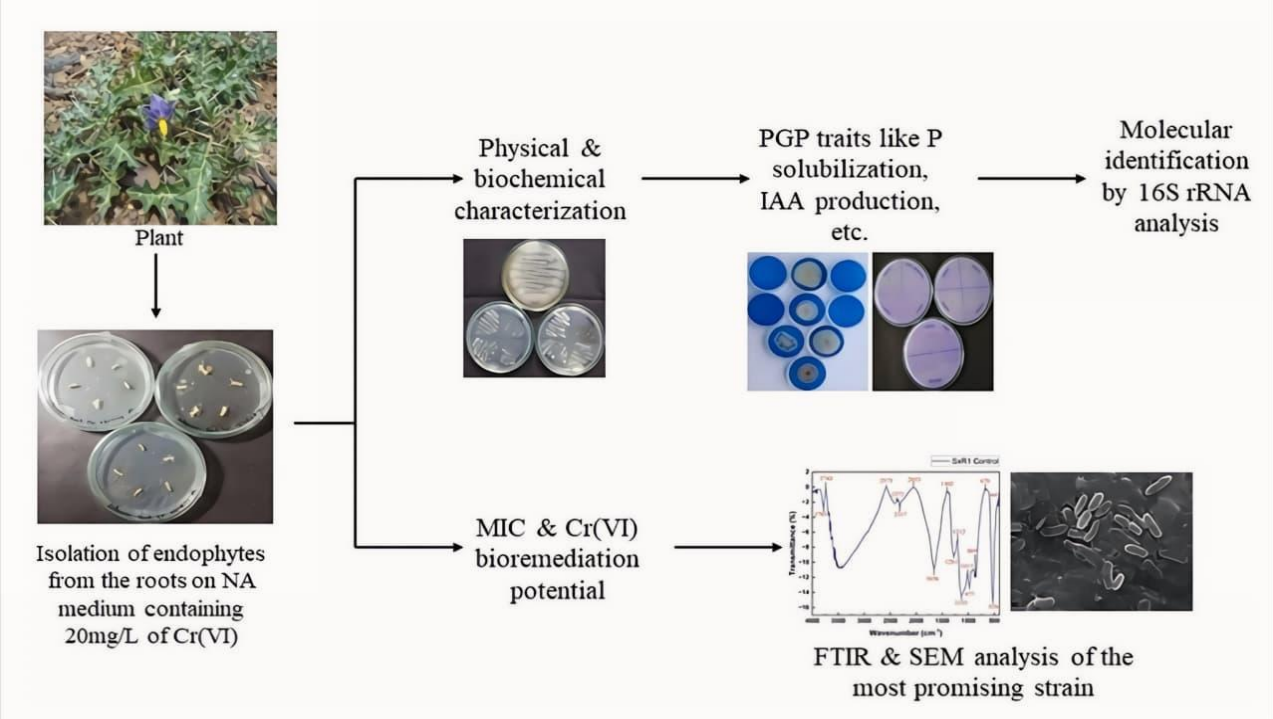


Figure 1.14: Isolation and characterization of hexavalent chromium-tolerant endophytic bacteria inhabiting *Solanum virginicum* L. roots.

[The figure outlines the process of isolating endophytes from plant roots, followed by physical, biochemical, and molecular characterization, including Cr(VI) bioremediation potential, PGP traits, and FTIR & SEM analysis] (73).

In short, the microbiology of redox reactions to chromium entails mechanisms through enzymes and other adaptations of the physiology that transform the Cr (VI) into Cr (III). This microbial transmutation forms the basis of the idea to use bacteria in chromium bioremediation; i.e. to use their innate cleaning capacities in removing contamination.

1.10 Bioremediation Potential Of Chromium-tolerant Bacteria

Hexavalent chromium (Cr) is a highly toxic, mobile and carcinogenic environmental contaminant, that is formed mainly in industrial processes such as tanning industries. Bioremediation using chromium-tolerant bacterial strains provides an environmentally sound and cost-effective way of mitigation this contamination. These microorganisms employ sophisticated mechanisms to detoxify Cr the most noticeable of which include biosorption, active efflux,

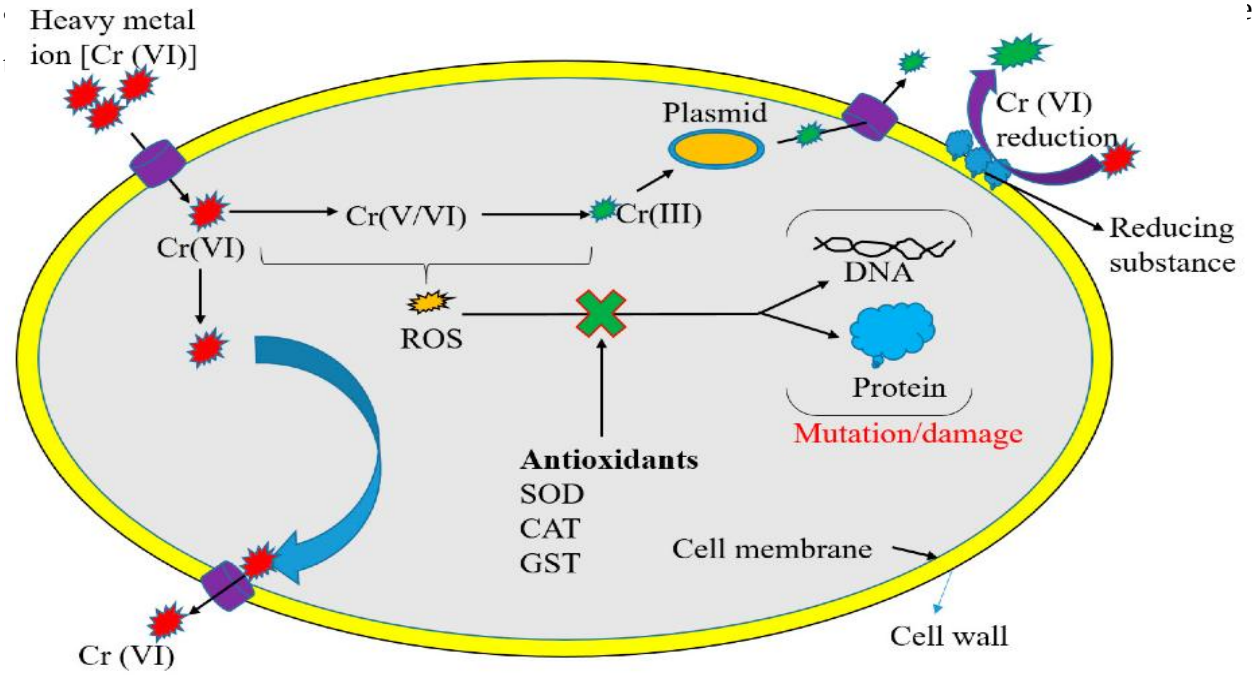


Figure 1.15: Remediation of heavy metals using bacteria and the mechanism of heavy metal reduction in bacterial cells.

[The figure depicts the cellular process of chromium (Cr) reduction, illustrating the conversion of Cr(VI) to Cr(III), ROS generation, antioxidant defense mechanisms, and potential mutations or damage to DNA and proteins] (83).

The focus of the detoxification of chemically diverse intruders (metals, organics) in bacteria is enzymatic reduction, and this is mainly achieved by an array of oxidoreductases. Some characteristic enzymes involved include Chromate reductases (e.g., ChrA, YieF, ChrR, NfsA, NemA), NADH dependent nitroreductases, Fe reductases, Quinone reductases, Hydrogenases and NADH/NADPH dependent flavin reductases. These enzymes catalyze the movement of electrons from electron donors, mainly their reducing agents NAD(P)H to Cr, resulting in its reduction (80,81). The reduction process may be achieved in various localizations in the cell: extracellular, at the cell membrane, and in the cytoplasm of the cell, under both aerobic and anaerobic conditions. For instance, soluble chromate reductases, such as ChrR and YieF have been purified from bacterial strains including ***Pseudomonas putida*** and ***Escherichia coli*** respectively (82).

Numerous genera of bacteria have significant Cr reduction potentialities. Some notable representatives are *Pseudomonas, Bacillus, Enterobacter, Escherichia coli, Shewanella, Acinetobacter, Ochrobactrum* and *Cellulomonas* (78). Specific strains, such as ***Staphylococcus simulans***, have shown significant Cr tolerance and chromate reduction ability (81); however, ***Vogococcus fluvialis*** and certain strains of ***Pseudomonas aeruginosa*** also show efficient chromate detoxification ability (82). The variable enzymatic machinery and adaptability of these microorganisms make them an important place in the case of potential development of effective bioremediation strategies in chromium contaminated environments (40).

1.11 Techniques for Isolation & Characterization of Chromium-tolerant bacteria

The isolation and characterization of strains of chromium tolerant bacteria follow a systematic multi stage protocol. Firstly, samples of the environment are collected at the contaminated areas of chromium. Specimens are enriched in culture supplemented with chromium to selective to the growth of the resistant taxa and with preceding serial dilution and plating procedures which result in the production of individual clonal isolates (84,85). Thereafter, morphological evaluation, enzyme tests, e.g., catalase, oxidase tests, etc.; physiological analysis are administered. Genotypic identification is performed mostly by 16S rRNA gene sequencing (84,86).

Mechanisms responsible for bacterial tolerance and detoxification of chromium are discussed after isolation. One of them is a reduction of the extremely toxic hexavalent chromium Cr (VI) to the less dangerous trivalent state Cr (III) (84). This reduction can occur via both the enzymatic and non-enzymatic pathways, where chromium (VI) detoxification is mediated by chromate reductases (ChrR and YieF) which mediate electron transfer (40,82). Chromium is frequently taken up via sulfate transport channels because of the structural similarity between the ions so that internal reduction can occur.

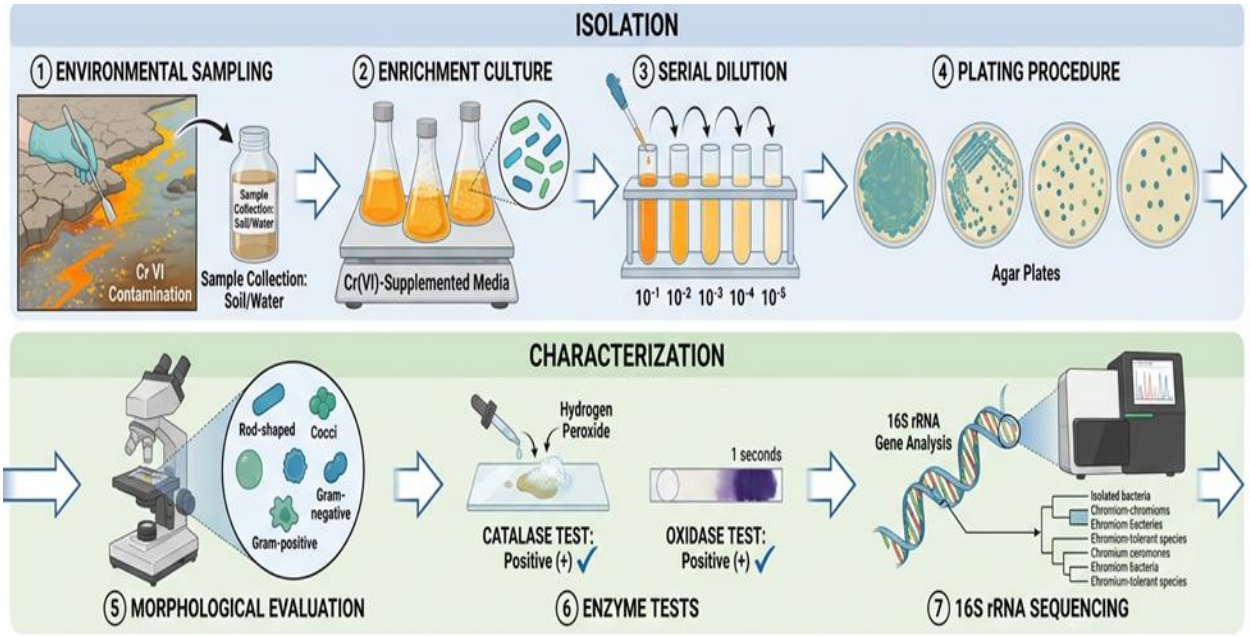


Figure 1.16: Workflow of Isolation & Characterization of Chromium-tolerant bacteria.

[The figure depicts a multi-stage process for isolating and characterizing chromium-tolerant bacteria, including environmental sampling, enrichment culture, serial dilution, plating, morphological evaluation, enzyme tests, and 16S rRNA gene sequencing] (Felo).

Another form of resistance mechanism is an active extrusion of chromate ions out of the cytoplasm through energy-dependent efflux pumps, one of which is the ChrA transporter (43,87). The genes that encode this activity such as chrA and chrB often sit in the operons, such as chrBCAF, and they also play a part in the efflux process (84). Bacteria also may use biosorption where Cr is adsorbed to the cell wall, therefore reducing Cr accumulation within the cell. Other defensive responses include ion transport regulation, DNA repair pathway regulation, and chromium-induced oxidative stress by metabolic adaptations (88,89).

1.12 Challenges in utilizing Chromium-Tolerant Bacteria

Utilization of chromium tolerant bacteria for bioremediation has been one of the interests as a sustainable alternative to the traditional physicochemical methods; however, the major applications and effectiveness of both are limited by several important challenges. A positive issue is the inherent toxicity of Cr (VI), which for even tolerant strains can seriously repress growth, metabolic activity and overall remediation efficiency. Elevated Cr (VI) concentrations may overwhelm cellular defense systems to lower viability reducing the rate of Cr (VI) reduction (90,91) . Furthermore, the product of Cr (VI) reduction, Cr (III), while less toxic and mobile can itself have inhibitory effects on subsequent reduction processes and will compromise cellular functions (90).

There is the heterogeneity and complexity of contaminated environments which is the major problem. The performance of the chromium-tolerant bacteria cultures is significant due to contaminated factors like pH, temperature, nutrient sufficiency, or co-contaminated chemicals into the environment (92). For example, oxygen competes with Cr (VI) as an electron acceptor, hence reducing the anaerobic or facultatively anaerobic reductive capacity of microorganisms (90). In case of complex matrices such as soils, the efficiency of bacterial inoculation is frequently impaired by destruction of microbial vitality, microbial growth inhibition by competition with autochthon microorganisms, and the overall harsh conditions which are common in the polluted sites. Even though successful isolation of Cr (VI)-tolerant strains can be undertaken in such environments, it may not be their best as noted under variable field environment (93,94).

The bioremediation processes are complicated as scaling from the controlled laboratory environment to potential use at large industrial or field scales presents major technological and operating issues. The complex engineering solutions are necessary to maintain optimum conditions through bacteria proliferation including a uniform distribution of inoculator, good mass transfer of zone support, electron donor and substrates, and similarity of environmental conditions in large-scale bioreactors or natural locations (93). Moreover, bioremediation is time-consuming by nature compared to many conventional methods, or it may increase the remediation time. The availability and cost of suitable electron donors and other requisite inducers put the further limits on the scalability of microbial Cr (VI) reduction (71). The urgent

need is on how to solve these scale-up problems by further research and fully exploit the potential of using the microbial chromium bio remediation.

1.13 Research Opportunities and Strategies To understand Enhanced Chromium Tolerance & Bioremediation

Opportunity to carry over laboratory accomplishments in chromium bioremediation to the field scale remediation is an area of continued research. A crucial field of concern entails surmounting problems connected with maintenance of microbial viability and optimum functioning in multifaceted environments matrices (93,95).

Future investigations should include focusing on explaining the interaction between chemical additives such as electron donors and redox mediators and their effect on the Cr (VI) bio-reduction mechanisms and the microbial community structures (71). Advanced omics technologies provide possibilities to screen for new genes and metabolic routes crucial for improved Cr tolerance and detoxification, providing guidelines for their genetic manipulation (71,93).

The most vital interventions to enhance the effectiveness of bioremediation include formation of powerful microbial consortia that are site particular and the use of novel carriers, including bacteria-impregnated biochar, to increase microbial survivability and program (96). In addition, combining biotechnological methods, such as bio-stimulation using different electron donors or the use of microbial fuel cells that would meet both Cr minimization and energy production would also be a viable example of sustainable and cost-effective remediation (97,98). These multifaceted strategies are important to make the microbial-based chromium remediation move forward.

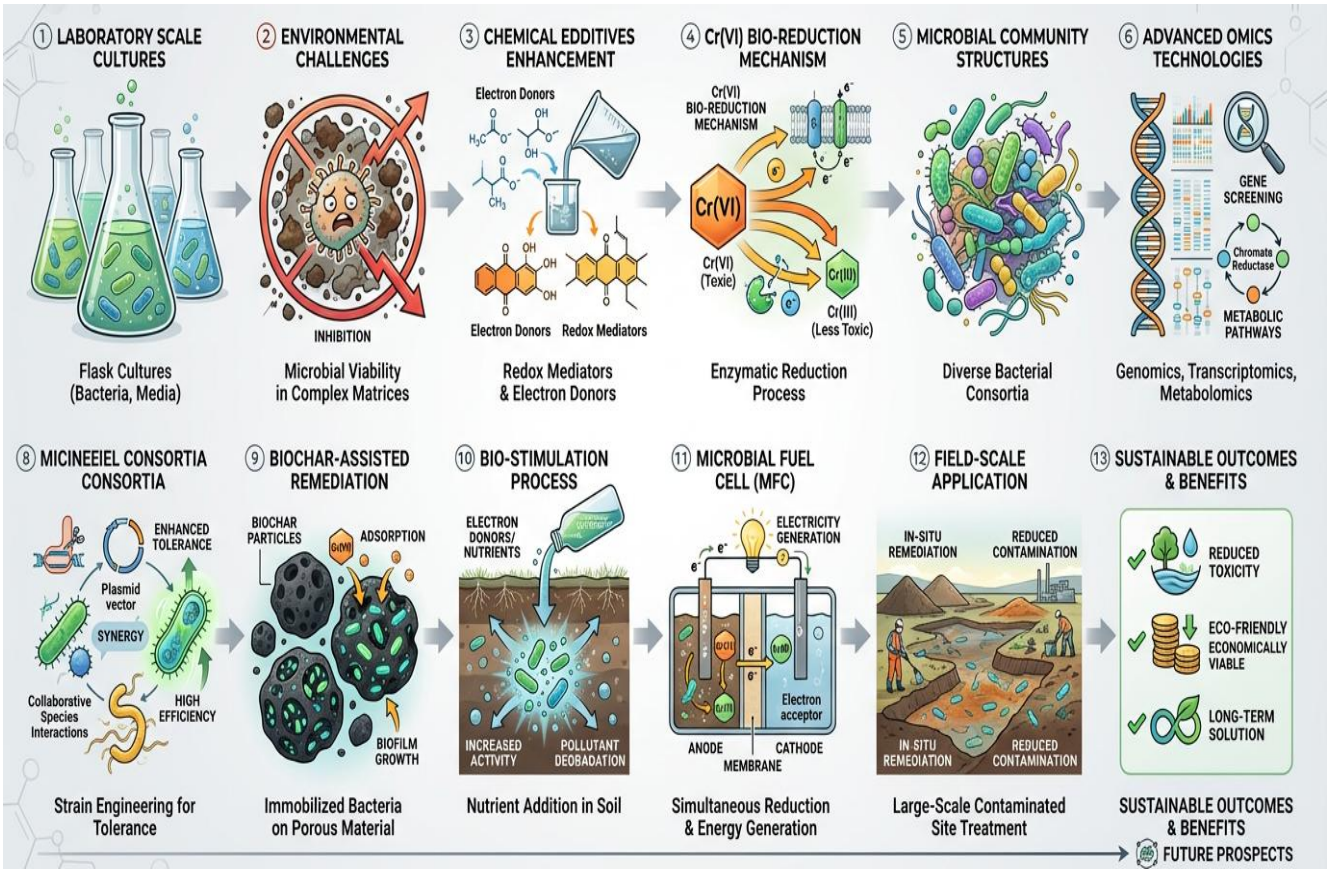


Figure 1.17: Research Opportunities and Strategies for Chromium Bioremediation.

[The infographic outlines strategies and research opportunities in enhancing chromium tolerance and bioremediation, focusing on microbial cultures, chemical enhancements, bio-reduction, advanced technologies, and field-scale applications]. (OREATE)

1.14 Objectives

The specific objectives of this study are:

- To isolate and screen chromium-tolerant bacteria from poultry excreta samples.
- To assay chromium reduction efficiency of the chromium-tolerant isolates.
- To identify & characterize the chromium-tolerant bacterial strains using molecular techniques.
- To explore genetic mechanisms of chromium-tolerance and chromium-reduction efficiency in the most tolerant isolate.
- To evaluate bioremediation potential of the selected isolate.

Chapter 2: Materials and Methods

2.1 Experimental design

This study was performed according to the following experimental design (schematic).

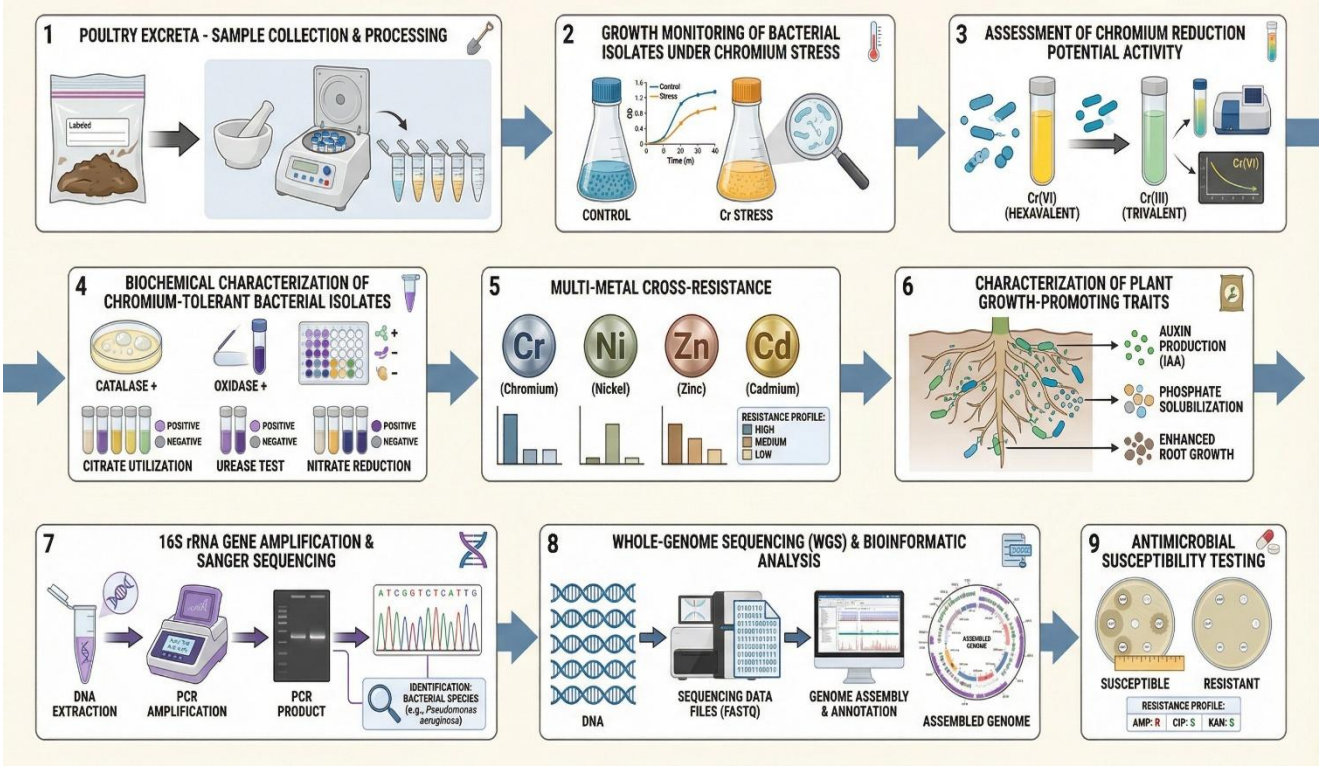


Figure 2.1: Steps for isolating & characterizing Chromium-Tolerant Bacteria.

2.2 Sample collection

A total of 10 poultry excreta samples were collected from different areas of Noakhali, Bangladesh at different time intervals & packed in sterile polyethylene (zipper) bags. Those places are:

Table 2.1: List of collection areas of poultry excreta samples.

Sample ID	Collection area	Date of collection
Farm-1	Bismillah Agro Production, NSTU Road, Noakhali	24.04.25
Farm-2	Mrs. Mohammadia Dairy Poultry & Agro Fisheries, Noakhali Mouja, Sadar, Noakhali	27.04.25
Farm-3	Bellal Agro farm, Duck & Hatchery, Noakhali Mouja, Sadar, Noakhali	28.04.25
Farm-4	Afia Poultry Farm, NSTU road.	30.04.25
Farm-5	Tofayel Poultry Farm, Sonapur-Kabirhat road	01.05.25
Farm-6	Rahim Poultry Farm, Kheyaghat, Sonapur, Noakhali.	03.05.25
Farm-7	Sujon Poultry Farm & Hatchery, Sonapur, Noakhali	04.05.25
Farm-8	Mrs. Rajib Poultry Farm, Kheyaghat, Sonapur, Noakhali.	04.05.25
Farm-9	Nurani Poultry Farm, Upazila Halimbazar, Subarnachar, Noakhali.	07.05.25
Farm-10	Nurani Poultry Farm-2, Upazila Halimbazar, Subarnachar, Noakhali.	08.05.25

Figure 2.2: Sample collection area in Noakhali region, Bangladesh.

2.3 Transportation of Samples

The experiments were carried out in the Laboratory of Department of Microbiology, Noakhali Science and Technology University (NSTU). Samples were collected in sterile polyethylene bags and transported to the laboratory under aseptic conditions for further analysis.

Figure 2.3: Transportation of poultry excreta sample.

2.3.1 Sample Processing

The collected samples were processed within six hours after transportation to the laboratory. Serial dilutions of the samples (up to 10^{-8}) were prepared using sterile normal saline solution (9 mL of saline per tube with 1 g of sample). From each dilution, 100 µl was spread onto Luria-Bertani (LB) agar plates supplemented with potassium dichromate ($K_2Cr_2O_7$) at a final concentration of 100 mg/L (74,75). The inoculated plates were incubated at 37°C for 24 hours. Individual colonies showing growth were subsequently subcultured onto fresh LB agar plates containing 100 mg/L $K_2Cr_2O_7$ to obtain pure isolates.

2.3.2 Preparation of $K_2Cr_2O_7$ Solution

Potassium dichromate ($K_2Cr_2O_7$) stock solutions of varying concentrations were prepared to achieve final supplementation concentrations of 100, 200, 300, 400, and 500 mg/L in LB broth. For each target concentration, the appropriate amount of $K_2Cr_2O_7$ powder was accurately weighed and dissolved in 10 ml of sterile distilled water (76,77).

Table 2.2: Preparation details of $K_2Cr_2O_7$ stock solutions.

Target Final [$K_2Cr_2O_7$] in LB Broth (mg/L)	Stock Concentration (mg/L)	Amount of $K_2Cr_2O_7$ per 10 ml stock (mg)
100	5,000	50
200	10,000	100
300	15,000	150
400	20,000	200
500	25,000	250

Each stock solution was prepared separately by dissolving the specified quantity of $K_2Cr_2O_7$ powder into 20 ml sterile distilled water in individual sterile containers. Solutions were mixed thoroughly until the compound was completely dissolved.

After complete dissolution, each potassium dichromate stock solution was sterilized by filtration using sterile 0.45 µm pore-size syringe filters attached to sterile syringes (76, 78). The sterile-filtered solutions were subsequently transferred aseptically into sterile Falcon tubes.

2.3.3 Preparation of Supplemented LB Broth

To prepare the potassium dichromate-supplemented LB broth, 0.5 ml of each filter-sterilized stock solution was aseptically pipetted into individual tubes containing 4.5 ml of sterile LB broth, achieving a total volume of 5.0 ml per tube (76, 78). Tubes were gently mixed by vortexing to ensure complete homogeneity. Using this methodology, final $K_2Cr_2O_7$ concentrations of 100, 200, 300, 400, and 500 mg/L in LB broth were precisely obtained for subsequent experimental use.

2.4 Preparation of Stock Culture

Bacterial isolates were preserved as stock cultures for long-term storage. Tryptic Soy Broth (TSB) was prepared and sterilized by autoclaving, and 750 µL of sterile TSB was dispensed into each stock vial. A loopful of the selected bacterial isolate was inoculated into the broth and

incubated at 37°C for 5–6 h to allow initial growth. Following incubation, 250 µL of sterile 40% glycerol was added to each culture and mixed thoroughly. The vials were labeled with isolate identification codes, vortexed briefly to ensure homogeneity, and stored at –80°C until further use.

2.5 Growth monitoring of Bacterial isolates under Chromium stress

Chromate tolerance of bacterial isolates was evaluated in Luria-Bertani (LB) medium supplemented with potassium dichromate ($K_2Cr_2O_7$). Briefly, 100 µL of each isolate from stock cultures was streaked onto LB agar plates containing 100 mg/L $K_2Cr_2O_7$ and incubated at 37°C for 24 h. Colonies that exhibited growth were selected for further analysis.

For inoculum preparation, a single colony was transferred into 5 mL LB broth and incubated at 37°C for 3–4 h in a shaking incubator. The optical density (OD_{600}) of cultures was adjusted to a uniform level prior to inoculation into 100 mL LB broth supplemented with $K_2Cr_2O_7$ at final concentrations of 100, 200, 300, 400, and 500 mg/L. Control tubes containing LB broth without inoculum served as blanks.

Cultures were incubated at 37°C under shaking conditions, and growth was monitored spectrophotometrically by measuring OD_{600} at 0, 18, 26, and 34 h. Growth profiles were constructed by plotting OD values against each concentration at different time interval, allowing assessment of the tolerance level of individual isolates.

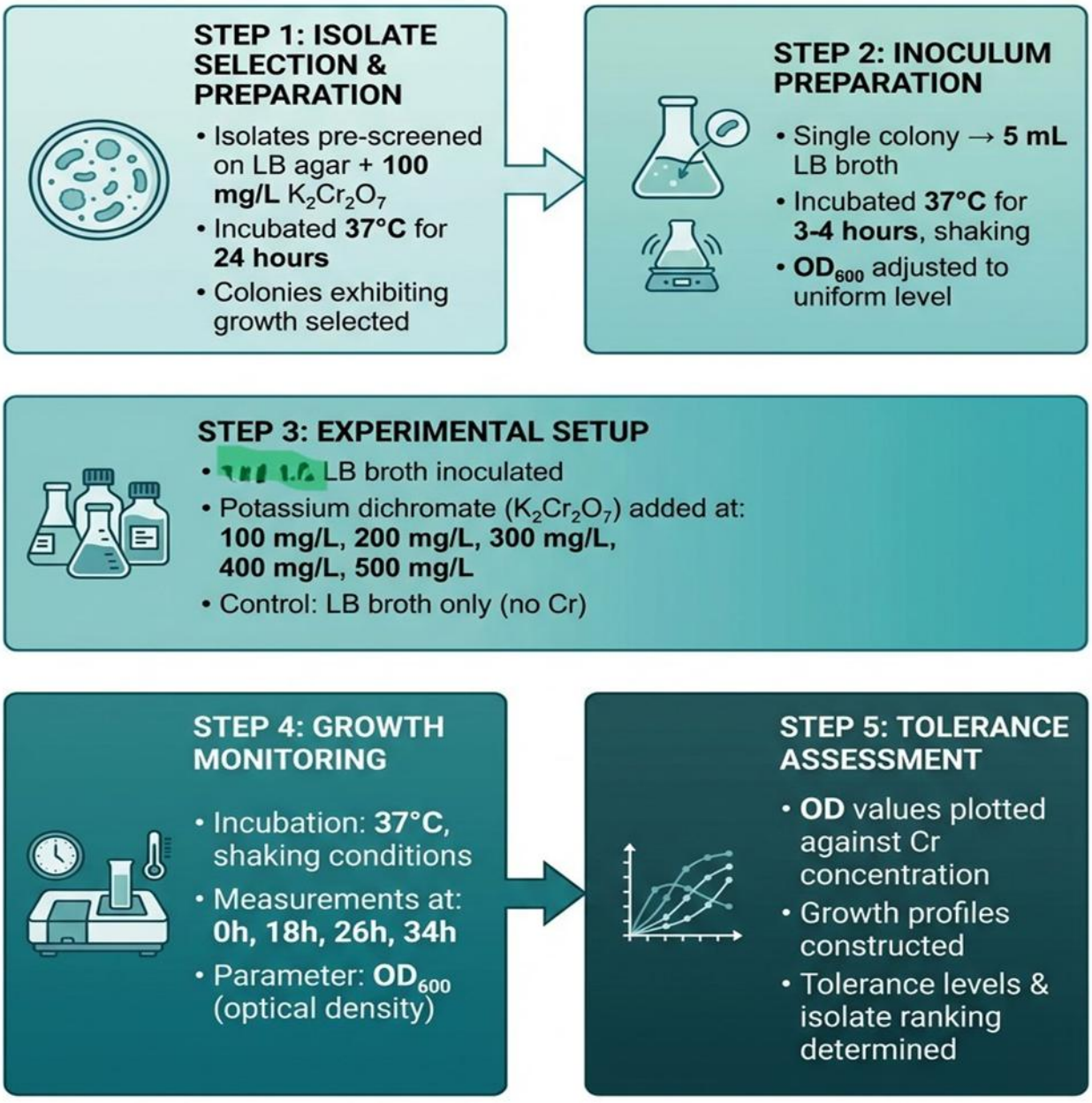


Figure 18: Growth monitoring of Bacterial isolates under Chromium stress.

2.6 Assessment of Chromium Reduction potential Activity

2.6.1 Bacterial Culture Preparation and Chromium Treatment

The top chromium-tolerant isolates were tested separately by inoculating them into Luria-Bertani (LB) broth supplemented with potassium dichromate ($K_2Cr_2O_7$) at final concentrations of 100 and 500 mg/L (79,80). Parallel uninoculated control tubes with equivalent concentrations of $K_2Cr_2O_7$ were also inoculated and developed to establish the baseline levels of hexavalent chromiun [Cr (VI)]. All cultures were incubated at 37°C on an orbital shaker with shaking set to 150 rpm and left for between 24 and 48 h to promote bacterial growth in the presence of chromium stress (81).

2.6.2 Sample Processing and Preparation

Subsequently, after the incubation period, the bacterial cultures and control tubes were centrifuged at 10,000-13,000 rpm for 5-10 min to pellet bacterial cells and then obtain the clear supernatants (80,81). The bacterial pellet was spun down, and the supernatants were collected, being careful of not disturbing the pellet. For high initial readings of A > 1.0, two-fold serial dilutions with sterile distilled water were made in order to fit within the linear range of detection for our spectrophotometer (82,83).

2.6.3 Quantitative Determination of Residual Cr (VI)

The residual Cr (VI) concentration in supernatant samples was determined by the 1,5-diphenylcarbazide (DPC) colorimetric method as previously established (79,82,83). Briefly, 1 mL of processed supernatant (or appropriately diluted supernatant) was transferred to a clean reaction tube and acidified with one drop of dilute phosphoric acid (H_3PO_4) or sulfuric acid (H_2SO_4) at approximately 0.5 M concentration. Subsequently, 2–3 drops of 0.25% DPC reagent (prepared in analytical grade acetone and stored at 4°C in dark conditions) were added to each tube (79,82,83). The reaction mixture was gently mixed and incubated at room temperature for 5–15 minutes to permit complete color development. A reagent blank containing 1 mL distilled water, acid, and DPC was prepared in parallel to zero the spectrophotometer. Absorbance measurements were recorded at 540 nm wavelength, corresponding to the maximum absorption of the purple Cr(VI)-DPC complex (79, 83).

2.6.4 Calculation of Chromium Reduction Efficiency

The percentage of hexavalent chromium reduction was calculated using the formula:
% Of Cr (VI) Reduction = [(A_control – A_sample) / A_control] × 100

where A_control represents the absorbance of the uninoculated control medium at the same chromium concentration and A_sample represents the absorbance of the bacterial culture supernatant (79, 81, 83, 84). For diluted samples, the measured absorbance was corrected by multiplication with the corresponding dilution factor prior to reduction percentage calculation. All experiments were performed in triplicate to ensure reproducibility, and mean reduction values with standard deviations were calculated for each isolate at both chromium concentrations (79, 81, 83, 84).

2.7 Biochemical Characterization of Chromium-Tolerant Bacterial Isolates

The biochemical characterization was performed on all top chromium-tolerant bacterial isolates to determine their metabolic capabilities and enzymatic profiles for species identification. Standard biochemical tests were conducted using 18–24-hour pure cultures grown on LB agar at 35°C ± 2°C, following established protocols. Each test was performed in duplicate and interpreted according to standard microbiological criteria (85).

2.7.1 Gram Staining and Morphological Examination

A heat-fixed bacterial smear made from a pure culture was stained in sequence with crystal violet (60 s), Gram's iodine as the mordant (60 s), and 95% ethanol as the decolorizer (10-15 s) as well as counterstained with safranin (30-45s) (86,87). Stained slides were viewed by oil immersion microscopy (100× magnification) and the morphology of the cells as well as Gram classification was determined. The Gram-positive bacteria were stained purple, whereas the Gram-negative ones were found to be pink.

2.7.2 Catalase Test

The bacterial colonies were transferred to a clean microscope slide and 3% of hydrogen peroxide solution was added dropwise. The formation of immediate bubbles (effervescence) indicated a positive reaction, while the absence of bubbles indicated a negative result (88).

2.7.3 Oxidase Test

Oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride)-impregnated papers or oxidase test strips were moistened with sterile distilled water and bacterial colonies from pure cultures were smeared onto the moistened papers using a sterile wooden stick. Color development was observed within 10-20 seconds. Development of dark purple or blue coloration indicated oxidase-positive result, while no color change indicated oxidase-negative result (89).

2.7.4 Citrate Utilization Test

Simmons citrate agar slants containing sodium citrate as the sole carbon source were lightly streaked with a sterile needle and incubated at 35°C ± 2°C for 18-48 hours. Positive citrate utilization was indicated by visible growth on the slant surface with color change of the medium from forest green to Prussian blue due to alkaline by-products (pH > 7.6). Absence of growth and retention of the original green color indicated a negative result.

2.7.5 Triple Sugar Iron (TSI) Test

TSI agar slants were streaked on the surface and stabbed into the butt to a depth of approximately 0.5 cm using a sterile inoculation needle. Following 18–24-hour incubation at 35°C ± 2°C, results were interpreted based on color changes: yellow butt indicated glucose fermentation; yellow slant and yellow butt indicated lactose and/or sucrose fermentation; red slant with yellow butt indicated glucose fermentation only; black coloration indicated hydrogen sulfide production; and bubble formation or cracks in the agar indicated gas production (90).

2.7.6 Motility-Indole-Urease (MIU) Test

MIU semi-solid medium was stabbed using a sterile needle to two-thirds the depth of the tube with bacterial culture and incubated at 35°C ± 2°C for 24-48 hours. Motility was detected by diffuse growth and turbidity extending away from the stab line, whereas non-motile organisms showed restricted growth along the stab line only. For urease activity detection, pink-red color change of the medium indicated a positive result (pH > 8.0) due to ammonia production, while yellow-orange coloration indicated negative result. Indole production was tested by adding 5

drops of Kovac's reagent to the medium surface; development of a red or pink ring at the surface indicated indole-positive result, while a yellow ring indicated negative result (91).

2.7.7 Methyl Red-Voges Proskauer (MR-VP) Test

MR-VP broth was inoculated with a single bacterial colony and incubated at 35°C ± 2°C for 48 hours. After incubation, the culture was divided into two portions. For the Methyl Red test, 5 drops of methyl red indicator were added to 2.5 ml culture; a distinct red color indicated positive reaction ($pH \leq 4.4$) due to mixed acid fermentation, while yellow color indicated negative result ($pH \geq 6.0$).

For the Voges-Proskauer test, Barritt's reagent A (0.6 ml of 5% α-naphthol in absolute ethanol) and reagent B (0.2 ml of 40% KOH) were successively added to the remaining 2.5 ml culture and shaken vigorously for 30 seconds to expose the medium to atmospheric oxygen. After allowing the tube to stand undisturbed for 30 minutes to 1 hour, development of a red or copper-colored layer at the surface indicated VP-positive result due to acetoin production via the butanediol fermentation pathway, while no color change or yellow coloration indicated negative result (92).

2.7.8 Starch Hydrolysis Test

The bacterial cultures were heavily inoculated onto starch agar plates, which contained 2% soluble starch and incubated at a temperature of 35°C ± 2°C for 24-48 hours. After that, the plates were flooded with Gram's iodine solution and allowed to remain stand for upto 10 minutes. The formation of a clear halo around the bacterial growth indicated a positive result for starch hydrolysis or production of the enzyme amylase, as the bacteria produced small sugars, which are unable to react with iodine (93). Uniform blue-black coloration of the medium including around colonies indicated negative result, demonstrating absence of amylase enzyme production.

Quality Control and Data Recording

All biochemical test results were recorded immediately following the specified incubation period

and interpreted according to standard criteria established by MacFaddin (2000) and Bergey's Manual of Systematic Bacteriology. Biochemical profiles generated from each isolate were compared and compiled for species identification of the chromium-tolerant bacterial strains.

2.8 Antimicrobial Susceptibility Testing of Chromium-Tolerant Bacterial Isolates

The antimicrobial susceptibility patterns of bacterial isolates were determined by a standardized Kirby Bauer disk diffusion method as per Clinical and Laboratory Standards Institute Guidelines. The pure bacterial cultures, stored as stocks, were first revived by inoculating onto fresh plates of Luria-Bertani agar and maintaining 37° temperature conditions for a period of 24 hours. The individual bacterial colonies from the plates were later inoculated into 1.0 mL volumes of Tryptic Soy Broth and incubated at 37°C temperatures for 3-5 hours to obtain actively growing bacterial cultures, which are likely to be in the logarithmic phase of their growth (94,95).

The bacterial inoculums were standardized to a turbidity standard of 0.5 Mc Farland, equivalent to around 1.5 x 10^8 CFU/mL, using a spectrophotometer with a 600 nm wavelength, by adjusting the optical densities between 0.08 and 0.13, and were to be used within 15 minutes once they were prepared. Mueller-Hinton (MH) agar plates were prepared according to manufacturer specifications with a uniform depth of 4 mm and pH adjusted to 7.2-7.4, as this medium is recommended by CLSI for its reproducibility and optimal antimicrobial diffusion properties (96,97). The standardized bacterial suspension was inoculated onto Mueller-Hinton agar plates using sterile cotton swabs through a three-point streaking technique to ensure confluent bacterial growth across the entire agar surface. After allowing the plates to dry for 3-5 minutes at room temperature, commercially prepared antimicrobial-impregnated paper disks (6 mm diameter) were aseptically placed on the agar surface using flame-sterilized forceps cooled in 70% alcohol, individual antibiotic discs were placed onto the agar surface, maintaining a minimum center-to-center distance of 24 mm between adjacent disks to prevent zone overlap. A maximum of 6 antibiotic discs were placed on each 100 mm diameter plate. Each disc was gently pressed onto the agar surface with sterile forceps to ensure complete contact with the medium. Once placed, discs were not moved or repositioned. The inoculated plates were then inverted and incubated at 35°C ± 2°C for 16-18 hours in ambient air (94,98).

Subsequent to incubation, the zones of inhibition around each antimicrobial disc were measured to the nearest millimeter with a ruler, with measurements taken from the bottom side of the plate against a black background with reflected light. The measurement results were interpreted by CLSI M100 performance standards to define each bacterial isolate as either susceptible (S), intermediate (I), or resistant (R) to a given antimicrobial compound (99, 100). The results included measurements of all zones in millimeters and their respective results, either susceptibility, intermediate results, or resistance, for each bacterial isolate tested with each antimicrobial compound. The Multiple Antibiotic Resistance Index was measured if applicable by calculating the number of antibiotics each isolate is resistant to divided by the number of antibiotics tested.

2.9 Multi-Metal Cross-Resistance of Chromium-Tolerant Bacteria

Chromium tolerant bacterial isolates were screened for the cross resistance against cadmium, copper, lead, nickel and silver using a standardized spectrophotometric method based on broth medium. The methodology has been designed so that it is reproducible, accurate and complies with the set protocols for testing resistance to heavy metals for bacteria.

2.9.1. Experimental Setup

The desired chromium-tolerant bacterial isolates were incubated for overnight in Nutrient Broth at 37 degree Celsius with 150 rpm shaking (121). Cadmium (CdCl2.2H2O), copper (CuSO4.5H2O), lead (Pb(NO3)2), nickel (NiCl2.6H2O) and silver (AgNO3) was added to the sterile distilled water to prepare a stock solution (15,000 mg/L) of each of them. These metal stock solutions had filtered (0.22 um syringe filters) to prevent chemical alteration especially for AgNO3 (122).

Five milliliter Nutrient Broth were put in each sterile test tubes prior to autoclave. Then all the NB contained tubes were autoclaved at 121 degree C for 15 minutes. Desired sub-lethal and lethal concentrations of heavy metals were achieved by addition of filter sterilized stock solutions using C1V1=C2V2 formula and adjusted to final volume of 5.0 mL (123). Tube inoculated with certain number of overnight bacterial culture except with negative controls.

2.9.2. Controls and Incubation

The experimental control accounts were as follows: a positive control (broth inoculated but without metals), a negative control (metal contained broth without inoculum), to take into consideration the factors of abiotic turbidity. All bacteria inoculated tubes were incubated at 37 degree Celsius with 150 rpm shaking for total 72 hours (86,121).

2.9.3. Background Checking and Evaluation

Bacterial growth was followed spectrophotometrically measuring the OD at 600 of 0, 18, 36 and 54 hours. Growth curves were constructed and the experiments were replicated. The average reports of the OD600 and standard deviation of the average were calculated to measure the level of the tolerance and get a reproducible result (124).

2.9.4 Assessment of the status of efficiency in metal removal

Following incubation, cultures were vortexed and centrifuged at 10,000 rpm, for 15 min, in order to separate biomass of bacteria and obtain clear supernatants. To neutralize the remaining biological activity and preserve the metal ions so that they could be analyzed afterwards, the supernatants were diluted tenfold with 1% nitric acid (HNO_3) (0.1 mL sample mixed with 0.9 mL of 1% HNO_3) (125).

Bacterial growth was determined spectrophotometrically at 600 nm (OD600) with uninoculated nutrient broth as a blank and is a standard method of determining cell density and growth. Isolates with OD600 value more than 50% of the control were considered to be resistant to respective heavy metal concentration. This evaluation gives insights to the potential of chromium tolerant isolates for bioremediation of multi-metal contaminated environments.

The metal removal efficiency was determined by the following equation:

Percentage (%) of Removal efficiency = [(Absorbance of Control - Absorbance of Sample) / (Absorbance of Control)] x 100

Where, Absorbance of Control is the initial absorbance of uninoculated metal concentration (measured at time zero) and Absorbance of Sample is the final of Absorbance of metal concentration in the bacterial supernatant (without cells) after 72 hours of incubation (125,126).

All experiments were done in duplicate and mean values were used for analysis to ensure reproducibility.

2.10 Characterization of Plant Growth-Promoting Traits in Chromium-Tolerant Bacterial Isolates

The plant growth-promoting traits of chromium-tolerant bacterial isolates were tested qualitatively to check their ability for nitrogen fixation, ammonia production, indole-3-acetic acid production, siderophore production, and phosphate solubilization (127,128).

2.10.1 Nitrogen Fixation Screening

Qualitative screening for nitrogen-fixing bacteria was performed using Yeast Extract Mannitol Agar supplemented with 0.25% (w/v) Congo Red. Isolates were streaked onto YEMA-Congo Red plates and incubated in the dark at 30 degree celsius for 48-72 hours. The observation of characteristic colony morphology, particularly the absence of Congo Red absorption, indicated potential nitrogen-fixing ability (127,129).

2.10.2 Ammonia (NH_3) Production Assay

For ammonia production detection, the bacterial isolates was grown in peptone broth and incubated at 37 ◦C for 48 h. After incubation, 0.5 mL of Nessler's reagent was added to the bacterial suspension. The development of brown to yellow color indicated ammonia production (130).

2.10.3 Indole-3-Acetic Acid Production Test

IAA production was assessed using the Salkowski reagent method. Isolates were cultured in Nutrient Broth containing L-tryptophan for 48-56 hours, typically at 30 degree Celsius with shaking (130) . After centrifugation to obtain a clear supernatant, 1 mL of the supernatant was mixed with 2 mL of Salkowski Reagent (0.1 M FeCl3 in 35% perchloric acid). The mixture was incubated in the dark for 25-30 minutes. A pink to red color change signified IAA presence. An uninoculated medium control was maintained (131).

2.10.4 Siderophore Production Test

Siderophore production was qualitatively determined using a modified ferric chloride (FeCl3) test. From the clear supernatant obtained during the IAA production test, 1 mL supernatant was treated with 2-3 drops of feCl3 solution. A color change, typically to red or purple, indicated siderophore presence. While the Chrome Azurol S assay is a universal method for siderophore detection, the test provides a rapid qualitative indication (132).

2.10.5 Phosphate Solubilization Test

The ability of bacterial isolates to solubilize Phosphate was tested in Pikovskaya's broth media that contained 0.5% tricalcium phosphate P as the sole phosphorus source. The broth tubes were inoculated with the test isolates and incubated at 30^0 C with shaking of 150 rpm for 5-7 days (133). After incubation, 1 ml of culture broths were taken with Eppendorf tube & centrifuged at 10,000 rpm for 10 minutes. The clear supernatants were combined with freshly prepared acidic molybdate-ascorbic acid reagent [(2.5% ammonium molybdate in 5N H_2SO_4 plus ascorbic acid [0.1%)] on a filter paper. The development of blue color is an indication of phosphate solubilization activity of the test isolates (134).

2.11 16S rRNA Gene Amplification and Sanger Sequencing

The sequencing of the 16S rRNA gene of three bacterial test isolates was done in the International Centre of Diarrhoeal Disease Research, Bangladesh (icddr,b) Genome Centre, Dhaka, Bangladesh. Genomic DNA was isolated out of pure cultures using one of the standard commercial DNA extract kits in accordance with their instructions (135).

The amplification of 16S rRNA genes were done with universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The samples of PCR were prepared as instructions; 50 ng of template DNA, 0.5 uM of each primer, 200 µM of dNTPs, 1.5 mM of MgCl2, 1x PCR buffer and 1 U Taq DNA polymerase were placed in each 25 µL PCR reaction (136,137).

The thermal cycling conditions were preliminary denaturation at 95°C of 5 minutes, then in 30 cycles to denaturation at 94º C for 30s, annealing at 55º C for 30s and extension at 72º C of 90s

followed by extension at 72^{o} C for 7 mins (138). PCR products were confirmed on the basis of electrophoresis using a 1.5% agarose gel which was stained with ethidium bromide as well as purified utilizing a commercial PCR cleaning kit. the resulting amplicons were purified and then Sanger sequence was done using ABI 3500 Genetic Analyzer (Applied Biosystems, USA) at icddr,b Genome Centre using the same primers as in the PCR (139,140).

The data of the raw chromatograms was processed and edited with Chromas software (Technelysium Pty Ltd) to obtain consensus sequences (141). Taxonomic identification was done using the BLASTn algorithm and comparing the sequences to the reference sequences in the NCBI GenBank database (142). The sequencing data quality and reproducibility were achieved by performing all the procedures as per the standard operating protocols at icddr,b Genome Centre.

2.12 Protocol of Whole-Genome Sequencing (WGS)

The bacterial isolate was sequenced in its entire genome by the International Centre in Diarrhoeal Disease Research, Bangladesh (icddr,b) Genome Centre in Dhaka, Bangladesh (135). According to the manufacturer's instructions, genomic DNA was purified by using commercially available kits to extract high quality and enough DNA that would be used to sequence it (136). Qubit fluorometric quantification and Nanodrop spectrophotometry were used under the evaluation of DNA concentration and purity, respectively. Again integrity of DNA was confirmed by use of 1% agarose gel electrophoresis (143) .

The sequencing libraries were made by using the Illumina DNA Prep (Nextera DNA Flex) kit according to the standard protocol offered by Illumina Inc. Library quality and concentration were checked before sequencing. Illumina MiSeq Hi-throughput sequencing was used with a paired-end 2x 150 bp sequence, which is ideal in the study of the bacterial whole-genome sequencing as it is highly accurate and comprehensive (144,145).

Raw sequencing reads were assessed on the quality using FastQC and trimmed and filtered on quality with Trimmomatic. Next, high-quality filtered reads were assembled to de novo with SPAdes v3.13.0 assembler which is optimized to assemble bacterial genomes (146,147).

The Prokka was used to annotate the genome and provide insights into protein-coding sequences, rRNA, and tRNA genes, and other leaders. Subsequent analyses of phylogenetic identification, antimicrobial resistance gene profiling and comparative genomics were subsequently used to

define the behavior of the isolates and their genetic lineage (148). All the processes were observed with quality control criteria as required by the icddr,b Genome Centre in order to provide strong and reproducible outcomes of sequencing findings.

Chapter 3: Results

3.1 Cultural characteristics of bacterial isolates

The cultural characteristics of bacterial isolates were studied from poultry excreta samples. A total of 10 poultry excreta samples were collected and inoculated on Luria-Bertani (LB) media plates supplemented with 100 mg/L potassium dichromate. After incubation, colonies with different morphologies appeared on the media plates.

These colonies varied in color and shape, indicating distinct bacterial types. To obtain pure isolates, the colonies were further sub-cultured on fresh LB plates supplemented with 100 mg/L potassium dichromate. Following repeated sub-culturing, well-isolated colonies with stable morphology were obtained.

From the 10 poultry excreta samples, a total of 25 bacterial isolates were successfully recovered. Their colony characteristics are summarized in Table 4 below.

Table 3.1: Cultural characteristics of bacterial isolates from poultry excreta samples.

Sample ID	Number of Isolates	ID of Isolates	Colony Characteristics
Farm-1 (F1)	3	F1A1	Small, round, light-yellow color.
		F1A2	Translucent white color.
		F1A3	Irregular, Cream color.
Farm-2 (F2)	2	F2B1	Irregular, Yellow-orange color.
		F2B2	Small, circular, Pale-yellow color.
Farm-3 (F3)	3	F3C1	Circular, Off-white color.
		F3C2	Small, Irregular Transparent color.
		F3C3	Circular, Pale-yellow color.
Farm-4 (F4)	2	F4D1	Small, Circular, Cream color.

		F4D2	Small, Circular, Off-white color.
Farm-5 (F5)	2	F5E1	Circular, Bright-yellow color.
		F5E2	Round, Off-white color
Farm-6 (F6)	2	F6F1	Circular, Pale-orange color.
		F6F2	Round, Off-white color.
Farm-7 (F7)	3	F7G1	Small, irregular, Translucent (almost colorless).
		F7G2	Circular, Translucent white color.
		F7G3	Small, round, Translucent white color.
Farm-8 (F8)	3	F8H1	Irregular, Translucent (colorless).
		F8H2	Large, irregular, Cream color.
		F8H3	Small, Translucent white color.
Farm-9 (F9)	2	F9I1	Circular, Cream color.
		F9I2	Irregular, Pale-white color.
Farm-10 (F10)	3	F10J1	Circular, Cream color.
		F10J2	Round, Pale-yellow color.
		F10J3	Circular, Pale-white color.

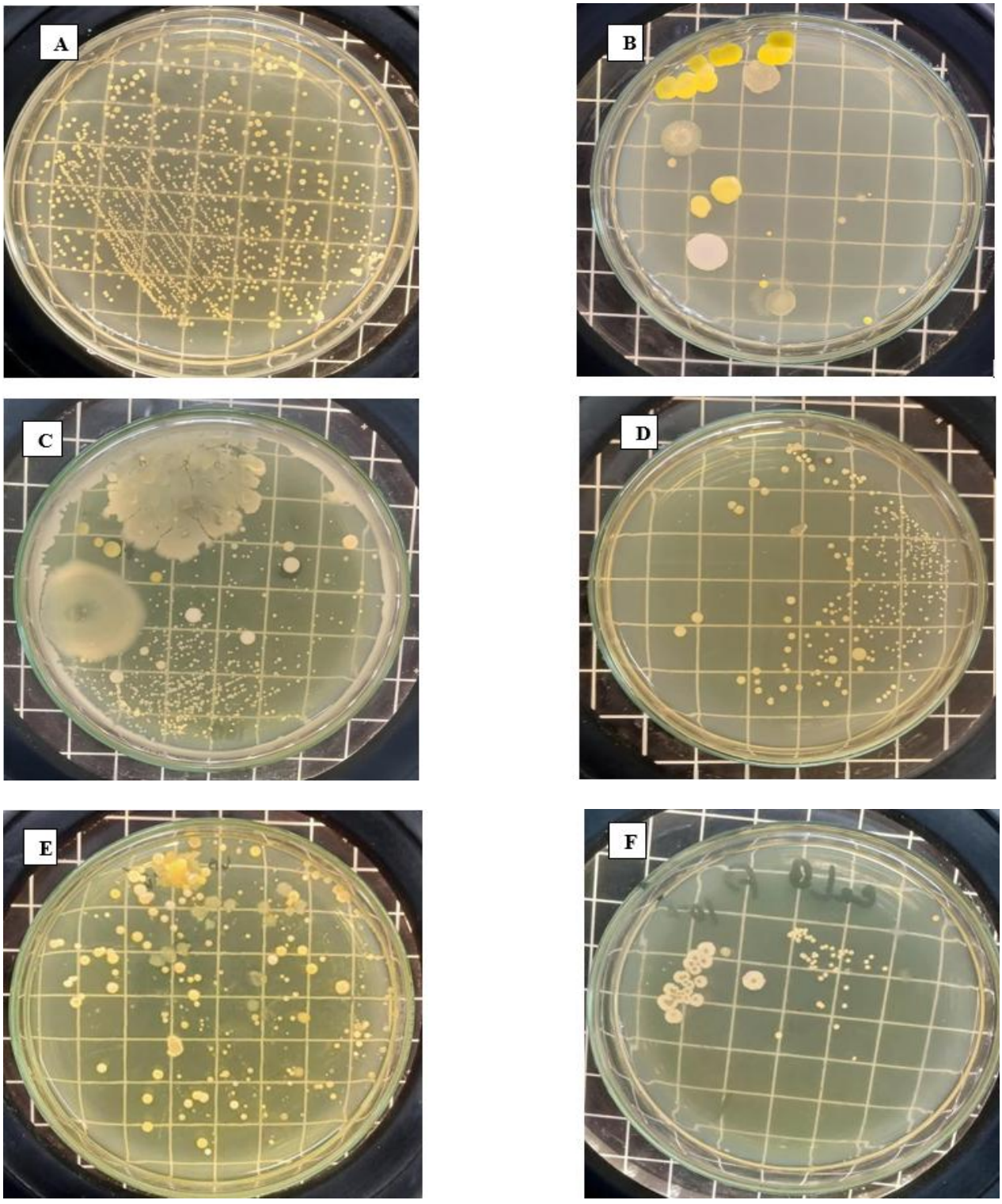
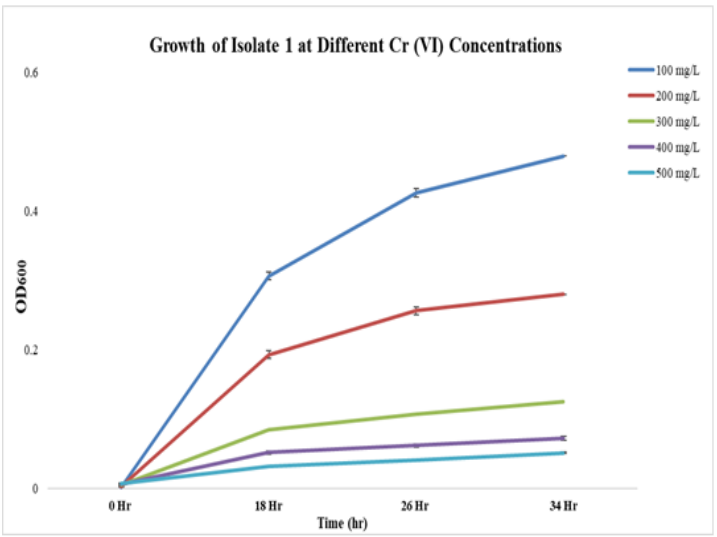


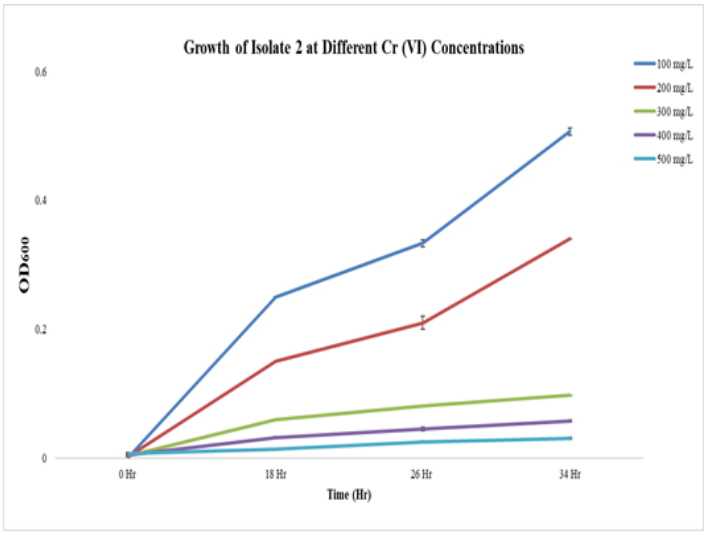
Figure 3.1: Colony morphology on selective media (LB supplemented with 100 mg/L $K_2Cr_2O_7$) of isolates from poultry excreta.

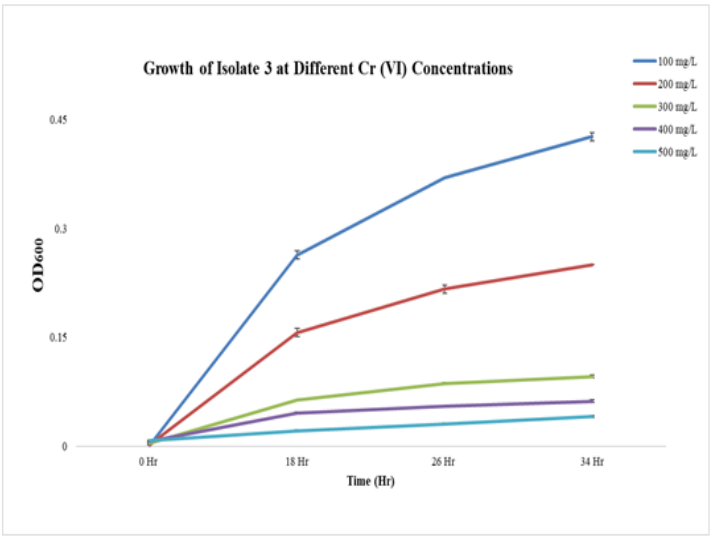
[(A) Numerous small, circular, smooth, convex yellow–cream colonies with entire margins (Micrococcus/Staphylococcus-like). (B) Mixed bacteria with several bright yellow smooth colonies and one large gray-green, fuzzy filamentous colony (mold). (C) Large dry, wrinkled, irregular spreading colony (Bacillus-like) and a circular fuzzy mold-like growth small yellow–cream-colored colonies. (D) Smooth, circular colonies with entire margins and characteristic metallic sheen or opalescent appearance. (E) Heterogeneous mix of colony sizes and textures that includes smooth yellow–cream and several slightly mucoid cream colonies (possible Gram-negative rods). (F) Mixed population showing at least two distinct colony types: (1) small white pinpoint colonies and (2) larger yellowish colonies with irregular margins].

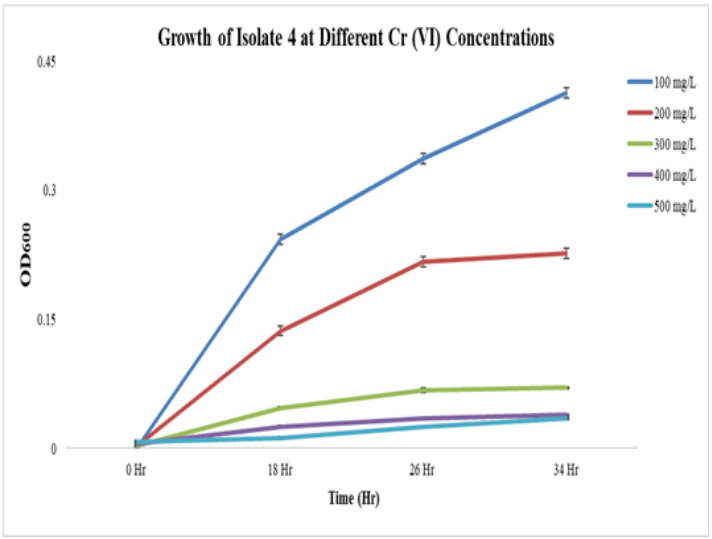
3.2 Determination of Bacterial Growth under Potassium Dichromate Stress

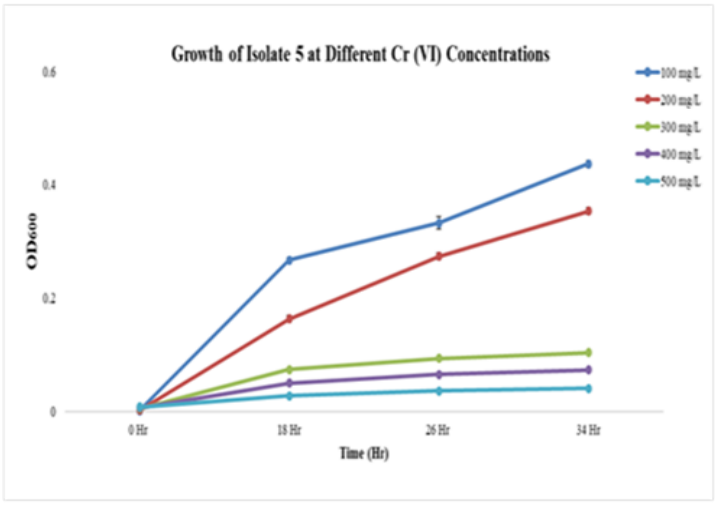
The 25 bacterial isolates were inoculated in Luria-Bertani (LB) broth tubes supplemented with varying concentrations of potassium dichromate (100, 200, 300, 400, and 500 mg/L). After incubation at 370C in different time intervals (18, 26 & 34 hours), optical density (OD) at 600 nm was recorded at defined time intervals (0, 18, 26 & 34 hours) to generate growth curves, thereby assessing the inhibitory effect of potassium dichromate on the bacterial isolates. In this way, OD measurements over time were used to track bacterial growth and evaluate the impact of potassium dichromate on their proliferation. The standard growth curves were constructed for each bacterial isolate to monitor changes in optical density (growth rate) and to assess their tolerance and response under different concentrations of potassium dichromate ($K_2Cr_2O_7$).

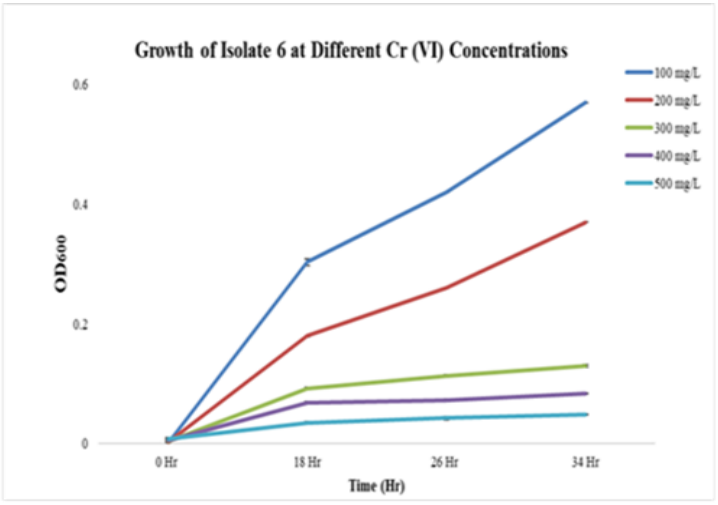

Growth of Isolate 1 at Different Cr (VI) Concentrations
OD600
Time (hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L

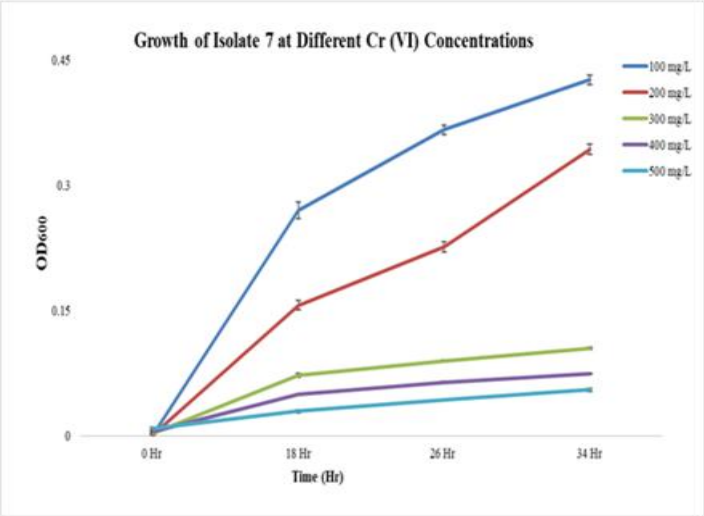

Growth of Isolate 2 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L

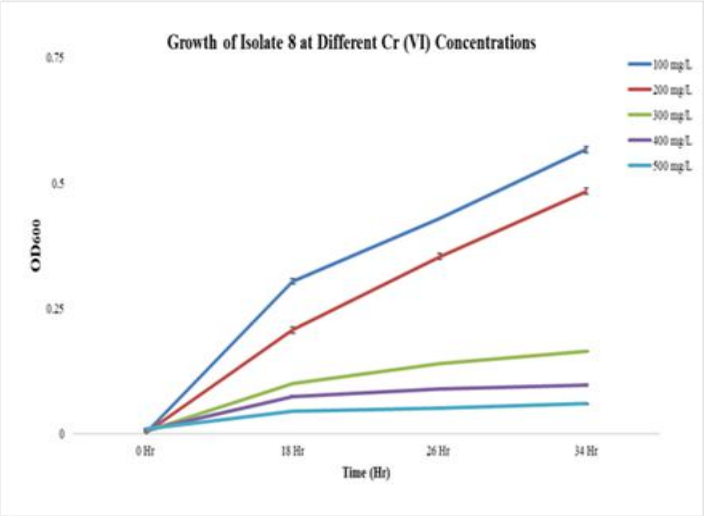

Growth of Isolate 3 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L

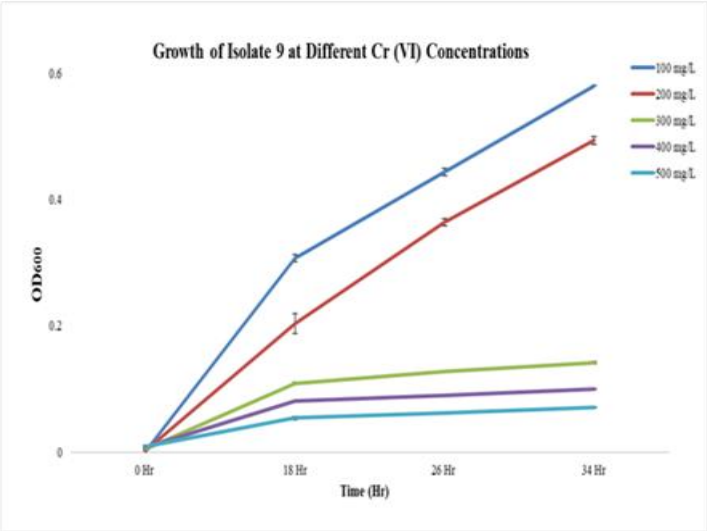

Growth of Isolate 4 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L

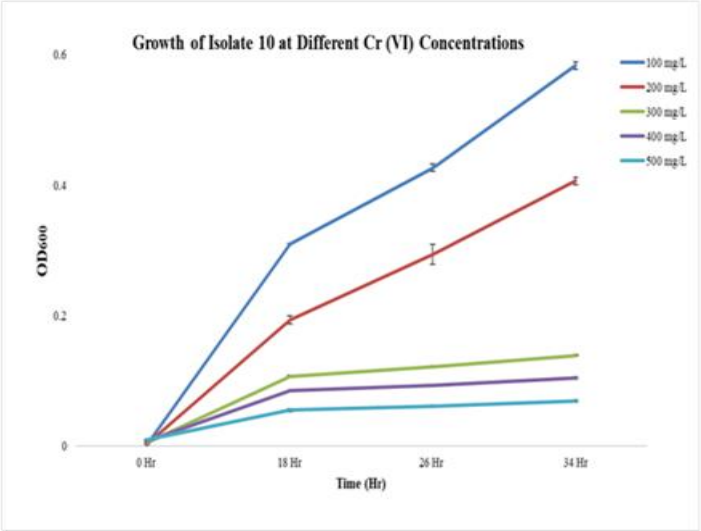

Growth of Isolate 5 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L

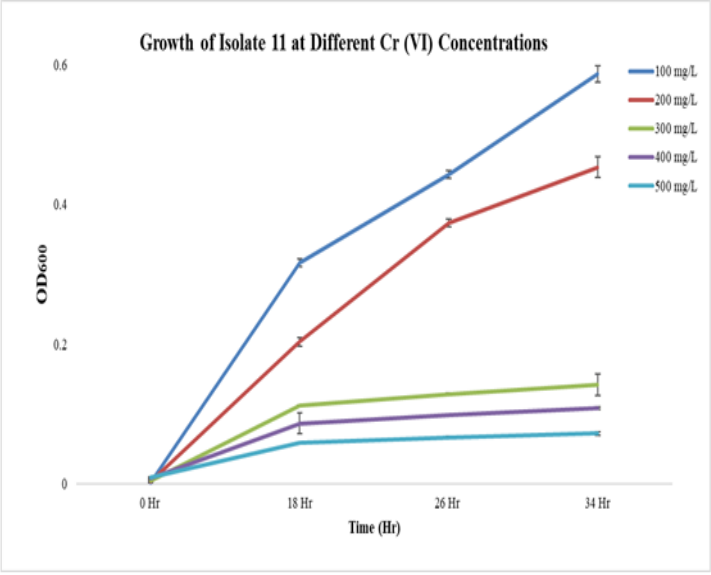

Growth of Isolate 6 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L

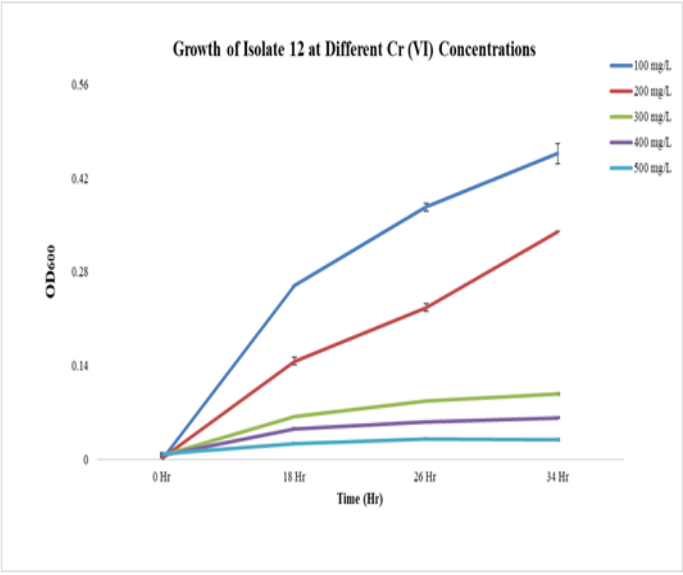

Growth of Isolate 7 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L

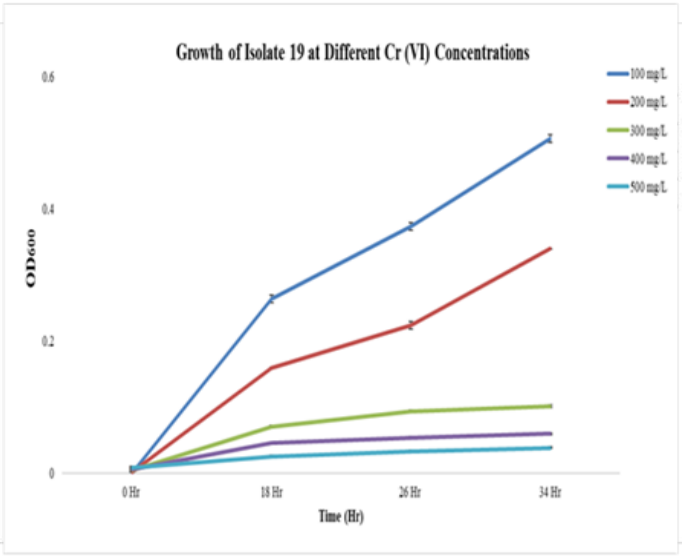

Growth of Isolate 8 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L

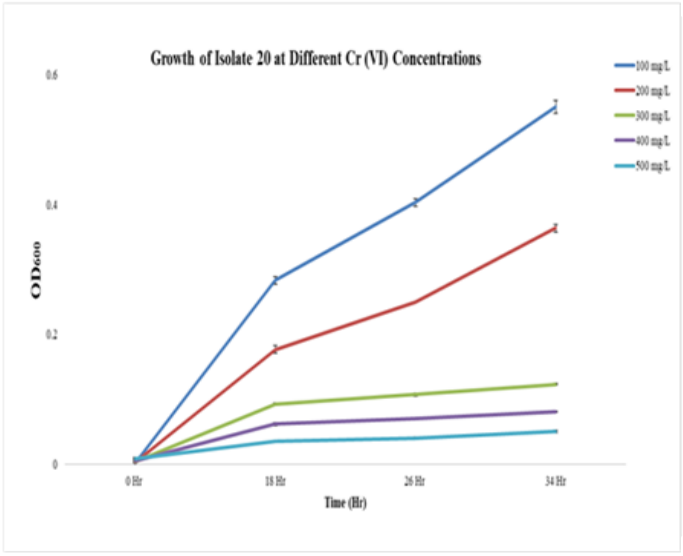

Growth of Isolate 9 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L

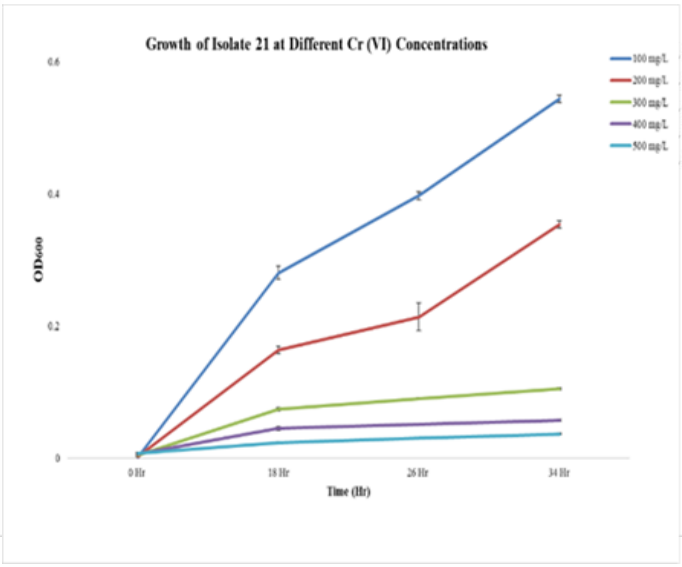

Growth of Isolate 10 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L

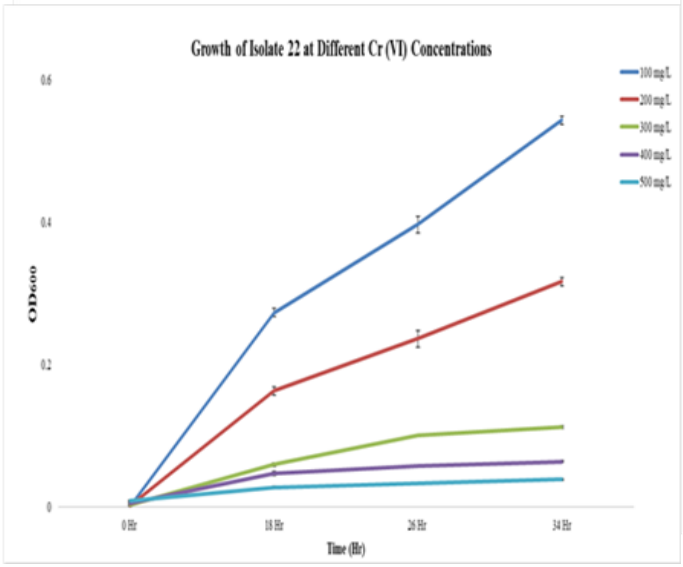

Growth of Isolate 11 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L

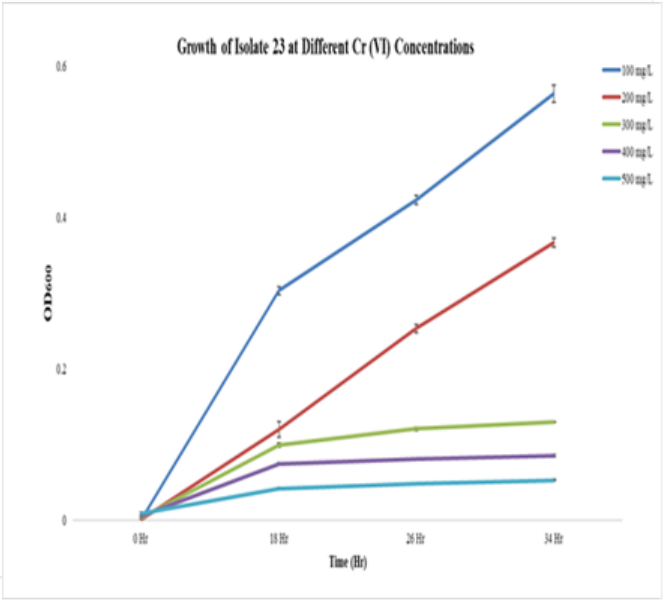

Growth of Isolate 12 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L

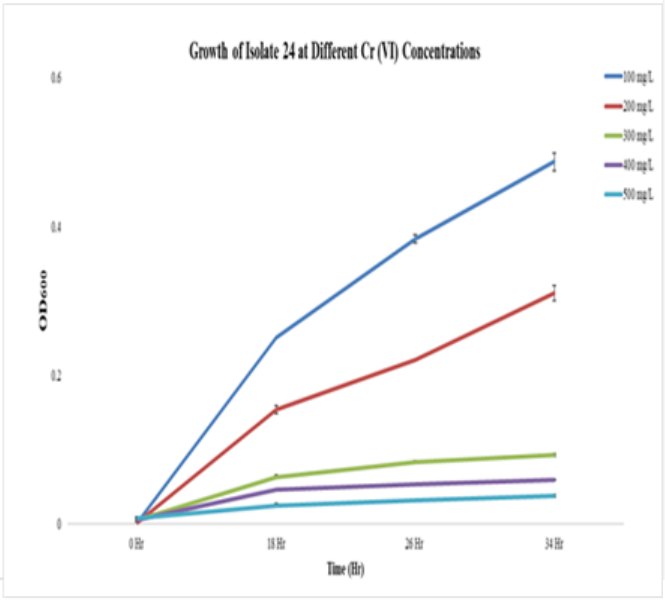

Growth of Isolate 19 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L


Growth of Isolate 20 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L


Growth of Isolate 21 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L


Growth of Isolate 22 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L


Growth of Isolate 23 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L


Growth of Isolate 24 at Different Cr (VI) Concentrations
OD600
Time (Hr)
0 Hr
18 Hr
26 Hr
34 Hr
100 mg/L
200 mg/L
300 mg/L
400 mg/L
500 mg/L

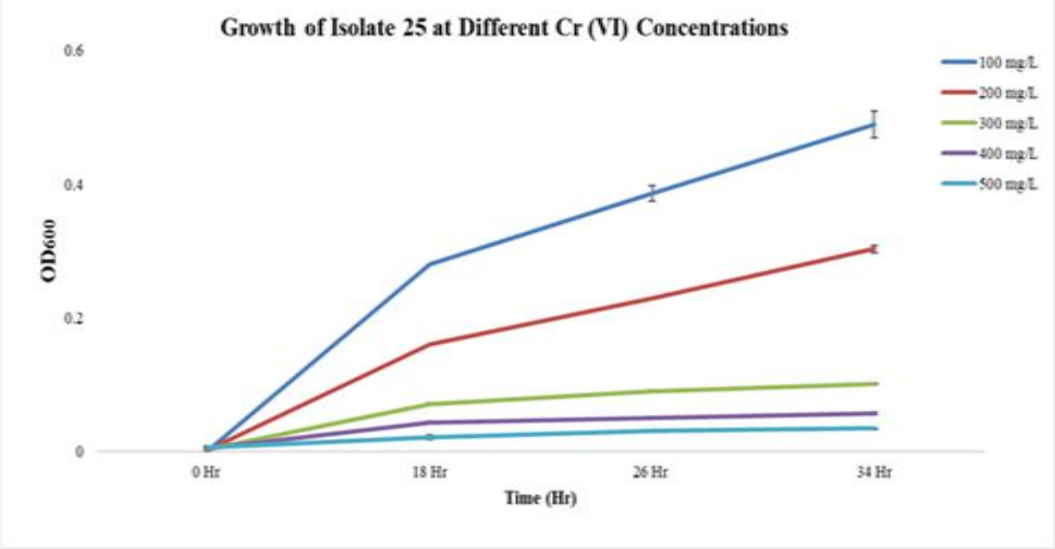


Figure 19: Growth response of bacterial isolates under different concentrations of potassium dichromate.

[Growth response of bacterial isolates measured as OD_{600} at 0, 18, 26, and 34 hours under different concentrations of potassium dichromate (100, 200, 300, 400 & 500 mg/L)].
From this experimental result, we can interpret that ID 14, ID 11, ID 10, ID 09 & ID 1 has the highest growth response in all tested concentrations among the other 20 test isolates.

3.3 Assessment Of Chromium Reduction Activity

The top four chromium-tolerant bacterial isolates (ID 14, ID 11, ID 10, and ID 09) were evaluated for their hexavalent chromium reduction potential in Luria-Bertani broth supplemented with $K_2Cr_2O_7$ at three different concentrations (100, 300, and 500 mg/L) following 72 hours of incubation at 37°C with 150 rpm shaking. The chromium reduction efficiency was measured by assessing the reduction of Cr (VI) using the diphenyl-carbazide colorimetric method, which is a standard spectrophotometric technique for Cr (VI) quantification.

Table 3.2: Reduction Efficiency at 100 mg/L $K_2Cr_2O_7$ concentration.

Isolates	Media	Concentration of Cr (VI) (mg/L)	Bacterial Supernatant Absorbance (OD)	% of Cr (VI) Reduction	Time (Hour)
ID 14	Luria broth	100	0.023666667	98.17%	72
ID 11	Luria broth	100	0.038	94.71%	72
ID 10	Luria broth	100	0.081	91.92%	72
ID 09	Luria broth	100	0.102333333	87.71%	72

Table 3.3: Reduction Efficiency at 300 mg/L K2Cr2O7 concentration.

Isolates	Media	Concentration of Cr (VI) (mg/L)	Bacterial Supernatant Absorbance (OD)	% of Cr (VI) Reduction	Time (Hour)
ID 14	Luria broth	300	0.431666667	77.43%	72
ID 11	Luria broth	300	0.463333333	74.43%	72
ID 10	Luria broth	300	0.528666667	70.50%	72
ID 09	Luria broth	300	0.754	64.51%	72

Table 3.4: Reduction Efficiency at 500 mg/L $K_2Cr_2O_7$ concentration.

Isolates	Media	Concentration of Cr (VI) (mg/L)	Bacterial Supernatant Absorbance (OD)	% of Cr (VI) Reduction	Time (Hour)
ID 14	Luria broth	500	1.053666667	48.45%	72
ID 11	Luria broth	500	1.161	44.03%	72
ID 10	Luria broth	500	1.284333333	39.93%	72
ID 09	Lb broth	500	1.451	35.68%	72

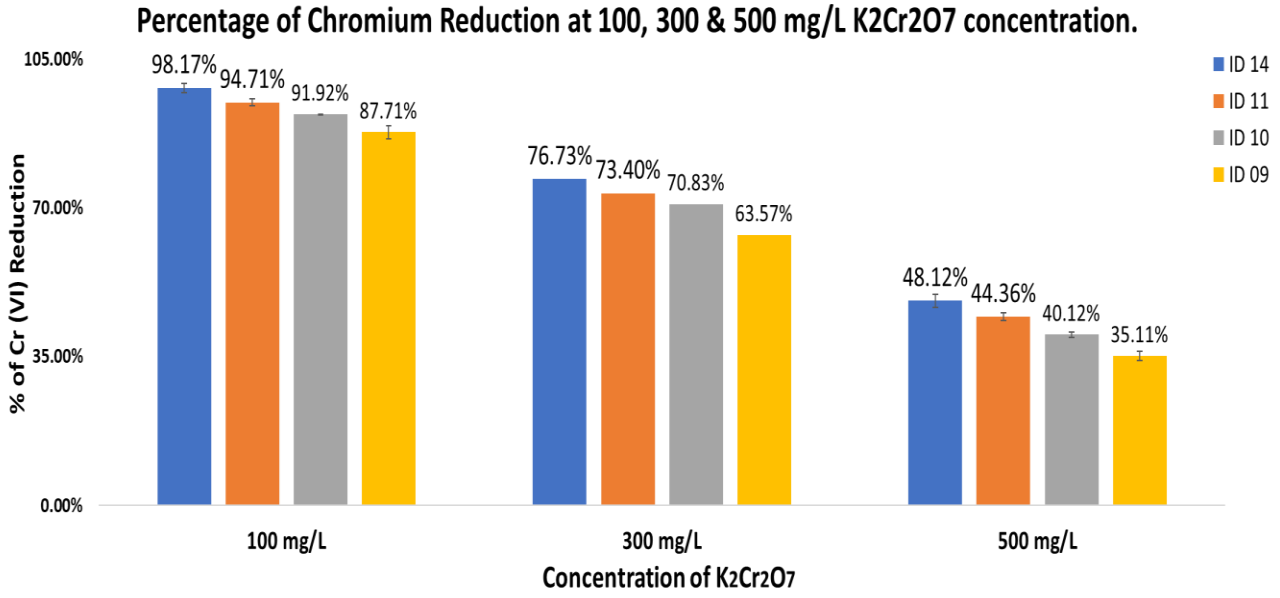


Figure 3.3: Percentage of Chromium Reduction Efficiency at 100, 300 & 500 mg/L K2Cr2O7 concentration.

From this result, we can interpret that all 4 test isolates (ID 14, ID 11, ID 10, ID 09) had reduce hexavalent chromium in Luria broth at 100, 300, and 500 mg/L concentrations over 72 hours. ID 14 achieved highest reduction efficiency: 98.80% (100 mg/L), 77.43% (300 mg/L), and 48.42% (500 mg/L). Efficiency declined with increasing concentration. ID 09 showed lowest reduction capability (88.87%, 64.86%, 35.67% respectively) from other 3 test isolates. All isolates demonstrated chromium-reducing capacity.

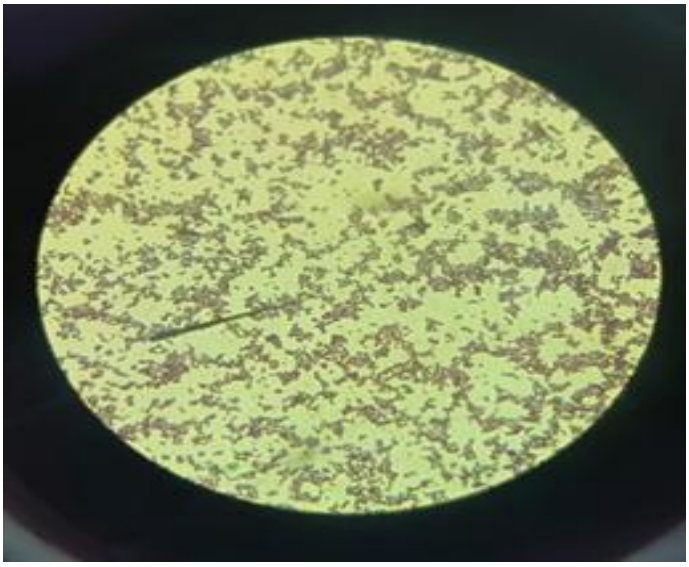
3.4 Biochemical Characterization of Chromium-Tolerant Bacterial Isolates

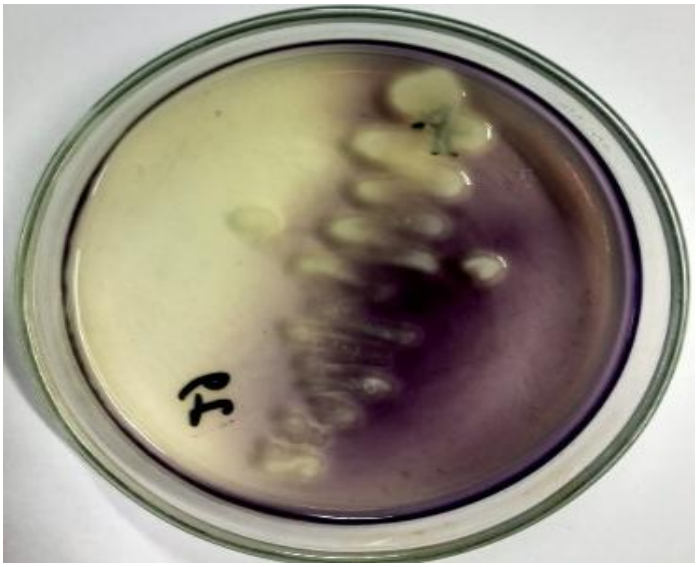
The four chromium-resistant bacterial strains (ID 14, ID 11, ID 10, and ID 09) were characterized with a series of biochemical tests in order to define their phenotypic properties which could be used for preliminary taxonomic identification. The biochemical profile consisted of cell morphology by Gram's stain, motility and metabolic properties by some enzymatic and substrate utilization tests. These included Gram's staining, MIU, TSI, Citrate utilization test, Starch hydrolysis test, Catalase test, Oxidase test and MR- VP tests. Each experiment was carried out in triple to guarantee reproducibility and reliability. The biochemical profiles of the test isolates are presented and each isolate was assigned a presumptive genus-level identification on the basis of the combined biochemical profile.

Table 3.5: Biochemical Test Result.

Isolate Code	Citrate Test	MIU Test	MIU Test	MIU Test	TSI Test	TSI Test	TSI Test	TSI Test	Starch Hydrolysis Test
		Motility	Indole	Urease	Slant	Butt	H_2S	Gas	
ID 14	+	-	-	-	K	A	-	-	+
ID 11	+	+	+	+	K	A	-	+	-
ID 10	-	-	-	-	A	A	-	-	+
ID 09	-	-	-	-	K	A	-	-	-

Isolate Code	Catalase Test	Oxidase Test	MR Test	VP Test	Gram Staining	Presumptive Identification
ID 14	+	-	+	-	+	*Bacillus species*
ID 11	+	-	-	-	-	*Enterobacter spp.*
ID 10	+	+	+	-	+	*Micrococcus species*
ID 09	+	-	-	+	+	*Staphylococcus species*





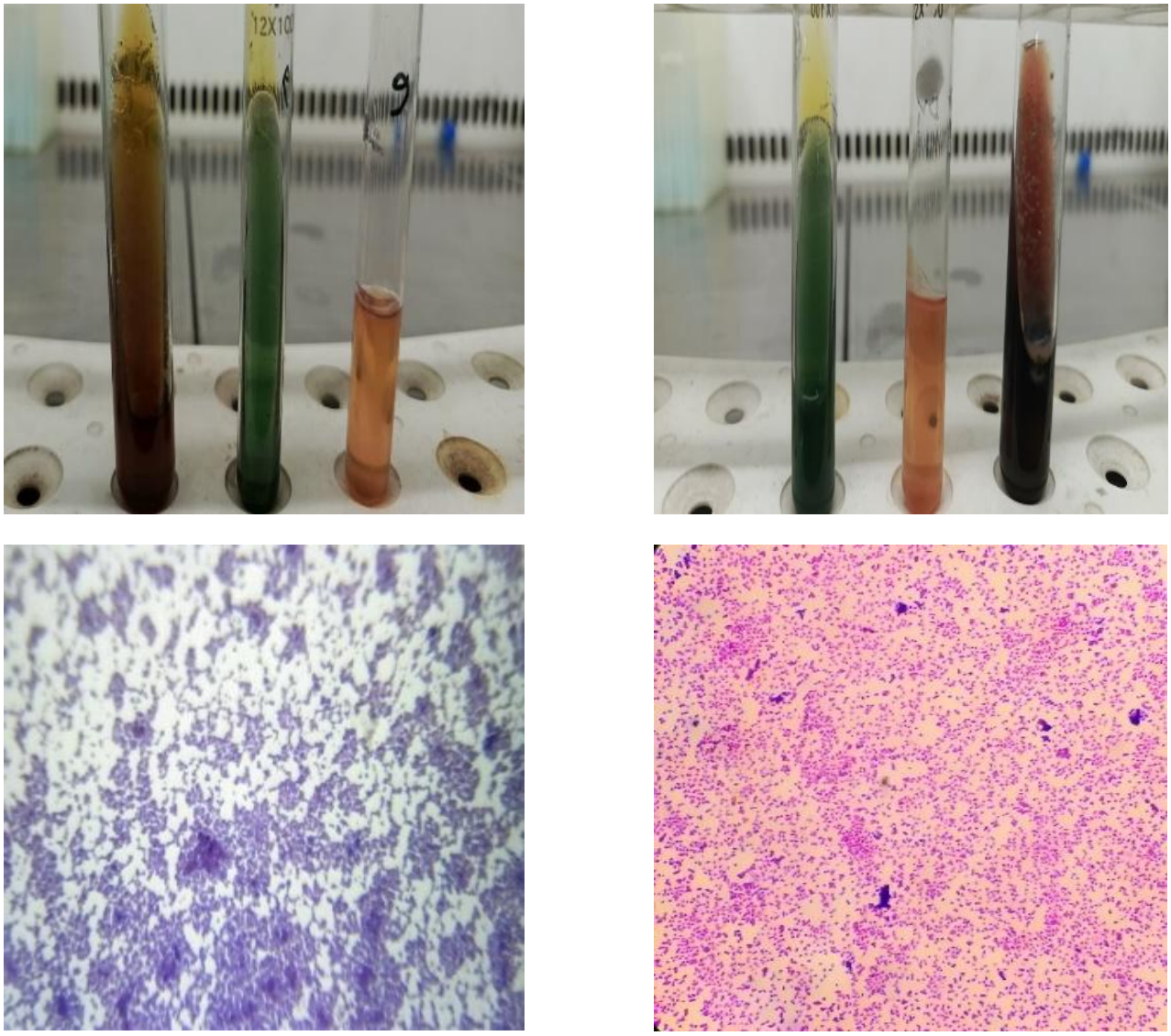


Figure 3.4: Biochemical Tests of Isolates.

On basis of the above test results, 4 test isolates were further biochemically characterized as follows: ID 14—Bacillus species (Gram-positive rod, catalase positive and glucose fermentation); ID 11—Enterobacteriaceae (Gram-negative rod, lactose fermentation. citrate positive); ID 10-Micrococcus species (Gram-positive cocci, catalase positive, limited fermentation); ID 09-Staphylococcus species (Gram-positive cocci, glucose-fermentation). All isolates were catalase positive (antioxidant), oxidase negative, and non-motile. The outcome supports preliminary genus-level identification of the test isolates and suggest diverse bacterial origins of chromium tolerance.

3.5 Growth monitoring of test isolates under different concentrations of other Heavy metals

Four isolates (ID 14, 11, 10, 9) were monitored for the growth characteristics against the presence of Ni, Pb, Ag, Cd and Cu at different concentrations (Ni/Cd: 100-1000 mg/L; Pb: 100-800 mg/L; Ag/Cu: 100-700 mg/L) for 54 hours. Concentration-dependent patterns of inhibition were obtained with optical density. At 100 mg/L, the growth rate of all isolates was progressive (OD increased from baseline 0.002-0.008 to 0.27-0.515). There was moderate inhibition at 500 mg/L, and at the highest concentrations, Ni/Cd at 1000 mg/L (OD 0.0025-0.0135), Pb at 800mg/L (OD 0.001-0.1), and Ag/Cu at 700mg/L (OD 0.0015-0.01) showed metal-specific toxicity levels.

3.5.1 Growth monitoring of test isolates under different $PbNO_3$ concentrations

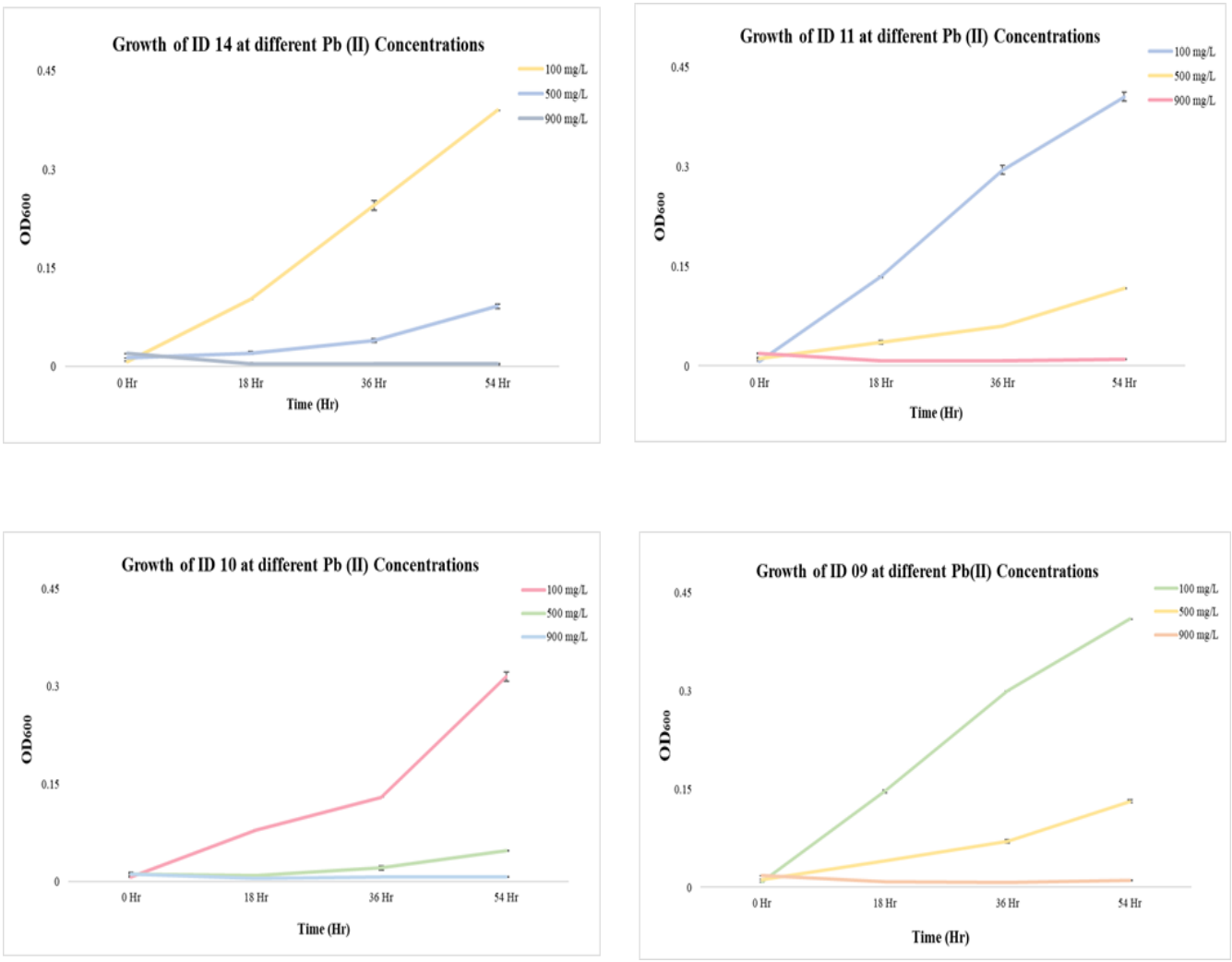


Figure 3.5: Growth response of bacterial isolates under different concentrations of $PbNO_3$.

[Growth response of bacterial isolates measured as OD_{600} at 0, 18, 36, and 54 hour at different concentrations of $PbNO_3$ (100, 500 & 900 mg/L)].

3.5.2 Growth monitoring of test isolates under different $CuSO_4$ concentrations

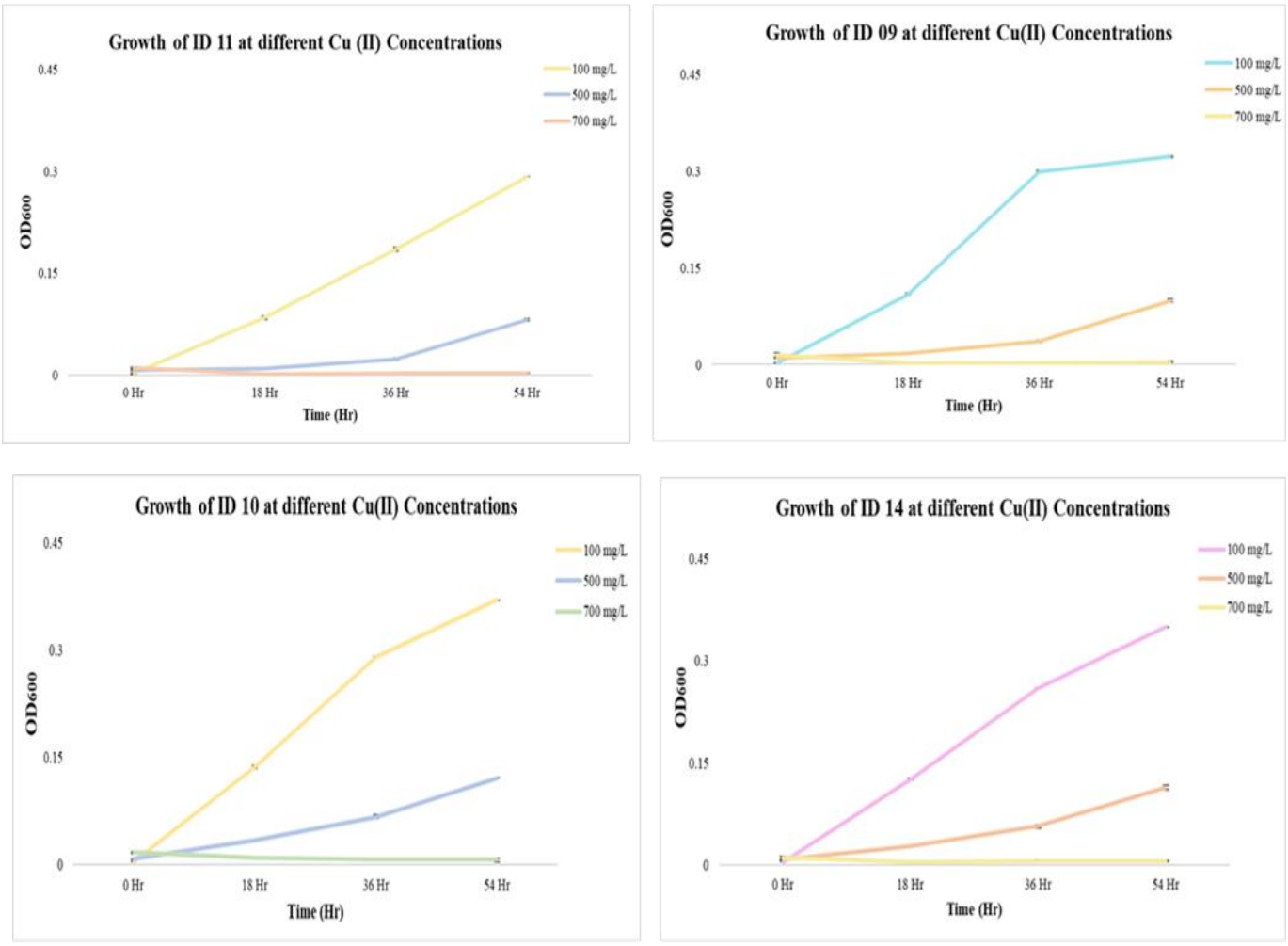


Figure 3.6 : Growth response of bacterial isolates under different concentrations of CuSO4.

[Growth response of bacterial isolates measured as OD_{600} at 0, 18, 36, and 54 hours under different concentrations of CuSO4 (100, 500 & 700 mg/L)].

3.5.3 Growth monitoring of test isolates under different $CdCl_2$ concentrations

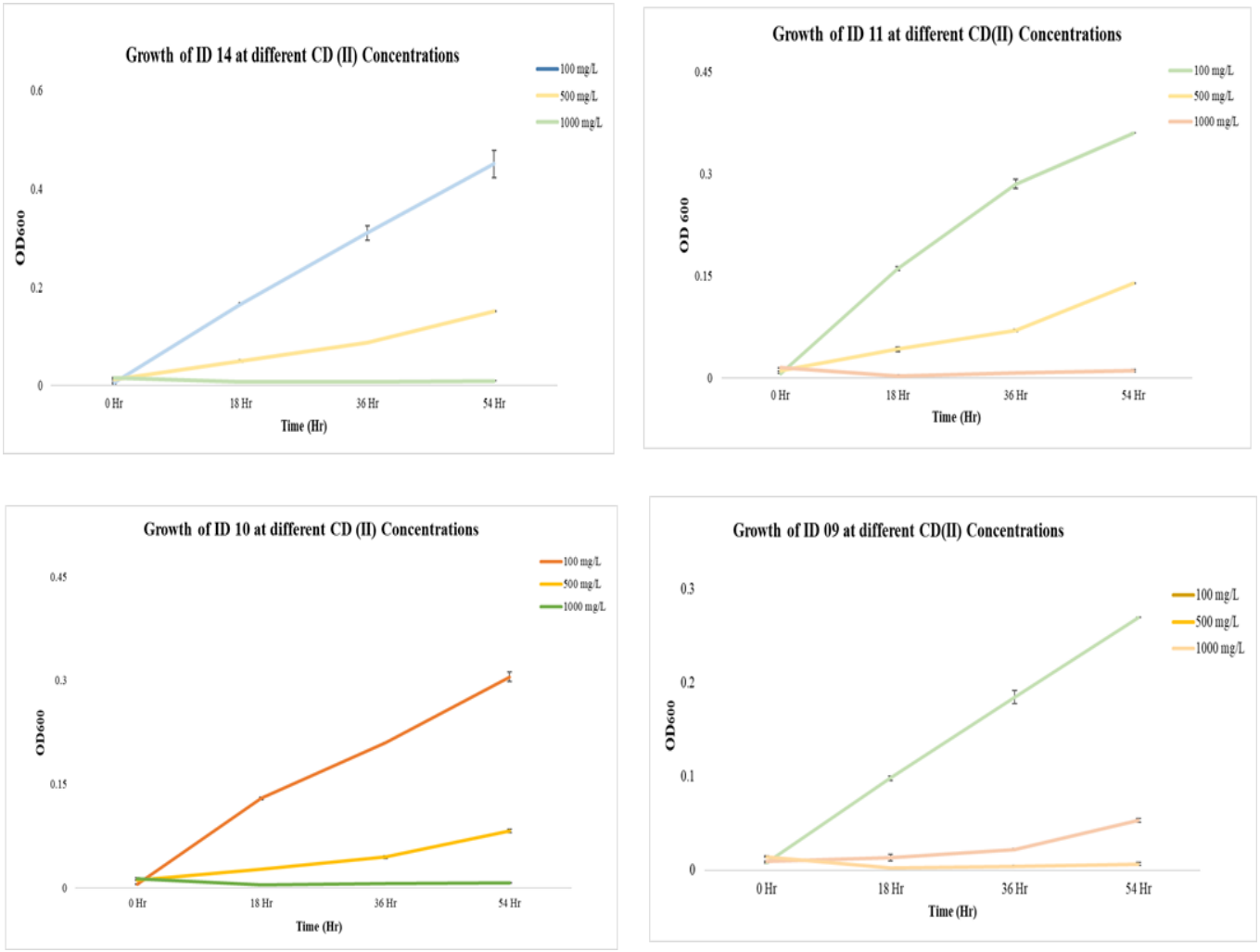


Figure 3.7 : Growth response of bacterial isolates under different $CdCl_2$ concentrations.

[Growth response of bacterial isolates measured as OD_{600} at 0, 18, 36 and 54 hours under different concentrations of $CdCl_2$ (100, 500 & 1000 mg/L)].

3.5.4 Growth monitoring of test isolates under different $AgNO_3$ concentrations

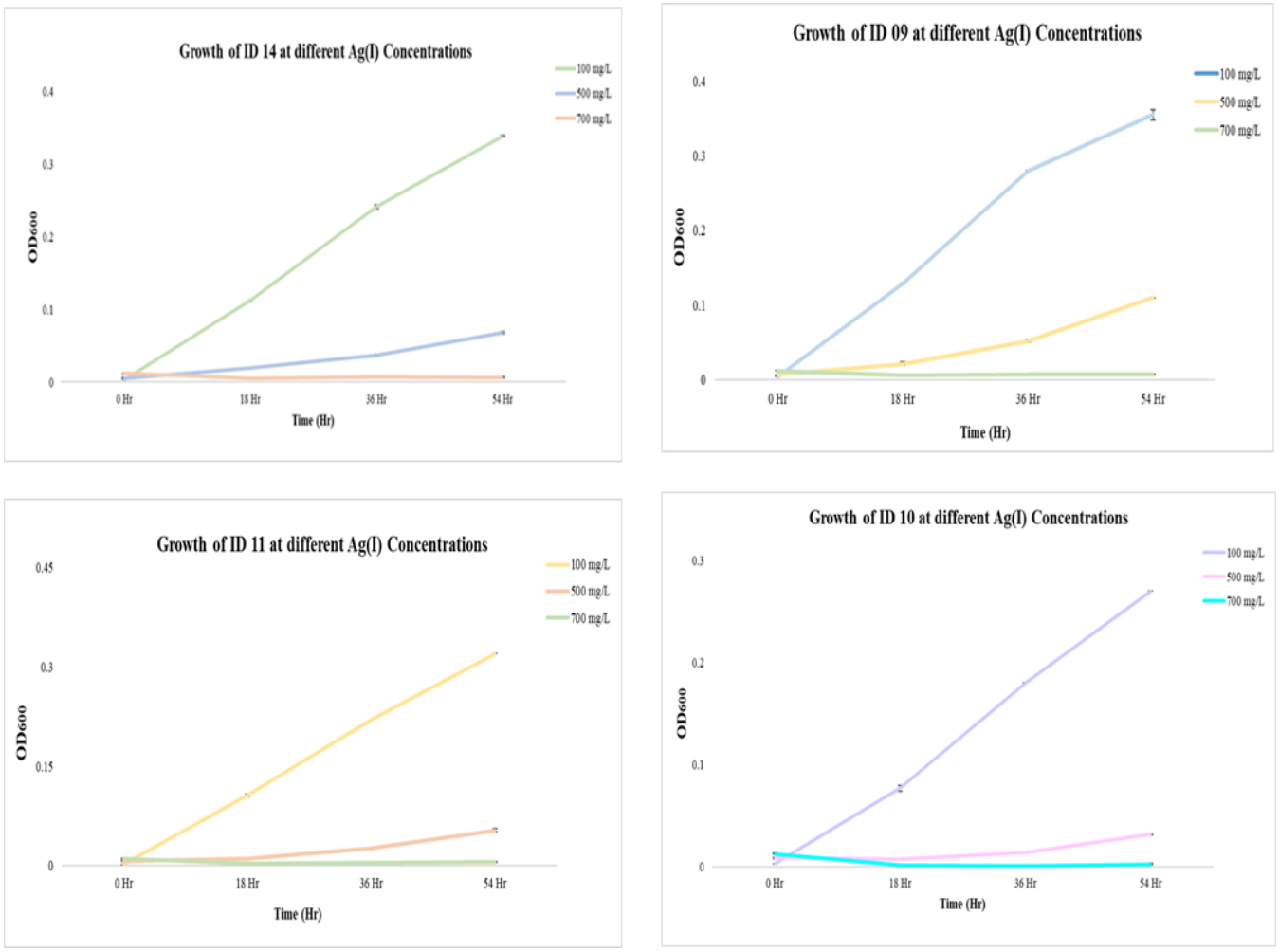


Figure 3.8: Growth response of bacterial isolates under different concentrations of $AgNO_3$.

[Growth response of bacterial isolates measured as OD_{600} at 0, 18, 36, and 54 hours under different concentrations of $AgNO_3$ (100, 500 & 700 mg/L)].

3.5.5 Growth monitoring of test isolates under different $NiCl_2$ concentrations

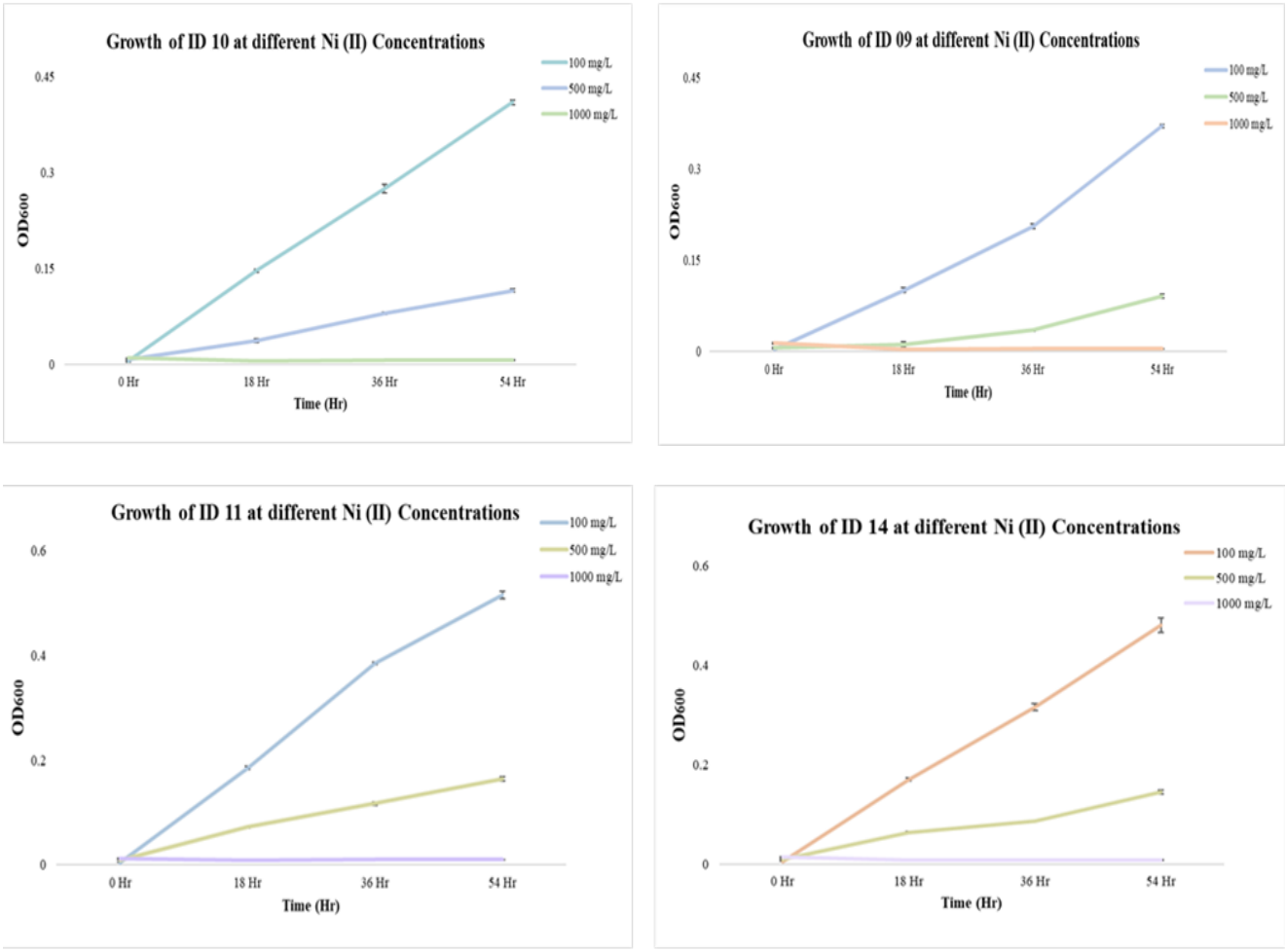


Figure 3.9: Growth response of bacterial isolates under different concentrations of $NiCl_2$.

3.5.6 Assessment Of Metal Removal Efficiency

The removal efficiency of the metal was determined at 100 mg/L, 500 mg/L and higher concentrations (Ni/Cd: up to 1000 mg/L; Pb: up to 800mg/L; Ag/Cu: up to 700 mg/L). At 100 mg/L, variable reduction was observed: isolates (respectively: Ni (33.78-62.14% reduction); Pb (9.08-39.97% reduction); Ag (11.59-48.33% reduction); Cd (21.40-35.02% reduction); Cu (5.24-37.46% reduction)). Efficiency went down precipitously at higher concentrations with most of the isolates at <10% removal at maximum tested concentrations, indicating concentration-dependence limitation in multi-metal bioremediation potential.

3.5.6.1 Reduction Efficiency at 100, 500 & 1000 mg/L $NiCl_2$ concentration

Concentration (mg/L)	100 mg/L		500 mg/L		1000 mg/L	
	Supernatant Absorbance (OD)	Percentage (%) of Ni Reduction	Supernatant Absorbance (OD)	Percentage (%) of Ni Reduction	Supernatant Absorbance (OD)	Percentage (%) of Ni Reduction
Isolate 14	0.108	57.28%	0.4	29.76%	0.83	3.77%
Isolate 11	0.097	62.14%	0.367	36.15%	0.767	6.05%
Isolate 10	0.114	54.77%	0.41	28.28%	0.861	1.60%
Isolate 9	0.168	33.78%	0.53	7.78%	0.95	0.68%

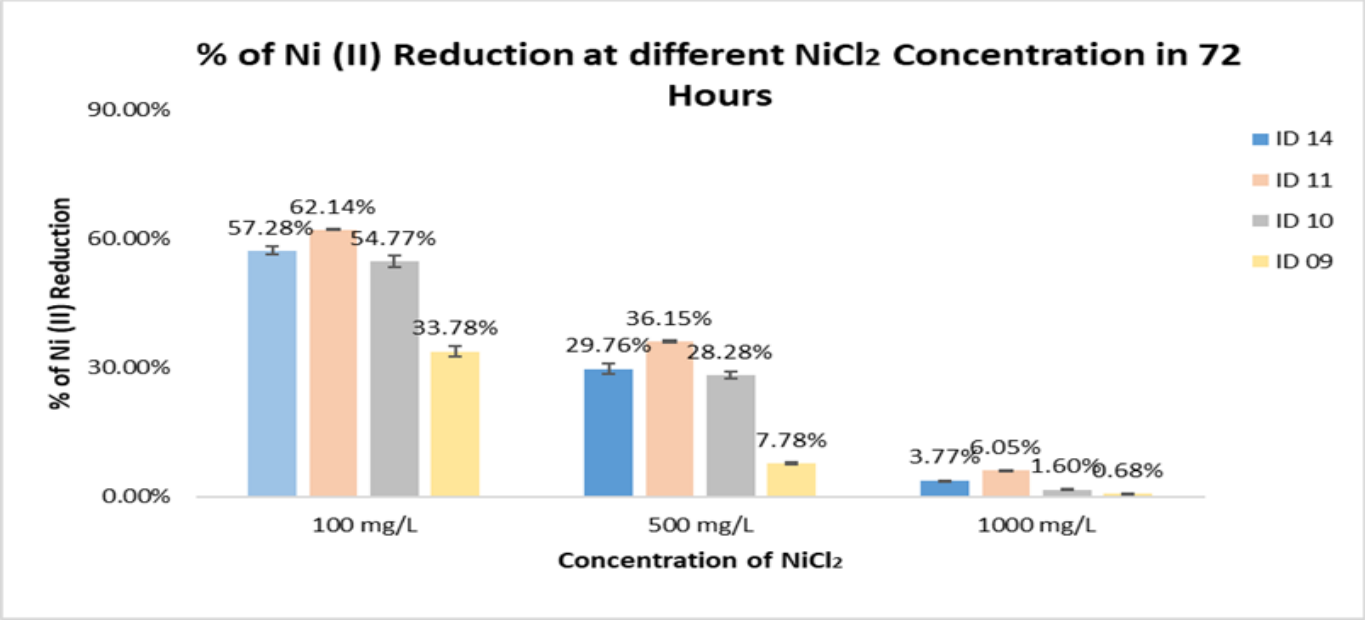


Figure 3.10: Percentage of Ni (II) Reduction Efficiency at 100,500 & 1000 mg/L $NiCl_2$ concentration.

3.5.6.2 Reduction Efficiency at 100, 500 & 800 mg/L $PbNO_3$ concentration

Concentration (mg/L)	100 mg/L		500 mg/L		800 mg/L	
	Supernatant Absorbance (OD)	Percentage (%) of Pb Reduction	Supernatant Absorbance (OD)	Percentage (%) of Pb Reduction	Supernatant Absorbance (OD)	Percentage (%) of Pb Reduction
Isolate 14	0.109	16.90%	0.138	9.08%	0.252	3.43%
Isolate 11	0.09	32.28%	0.153	21.25%	0.275	5.05%
Isolate 10	0.111	14.89%	0.165	7.30%	0.28	1.54%
Isolate 9	0.078	39.97%	0.137	22.42%	0.249	7.62%

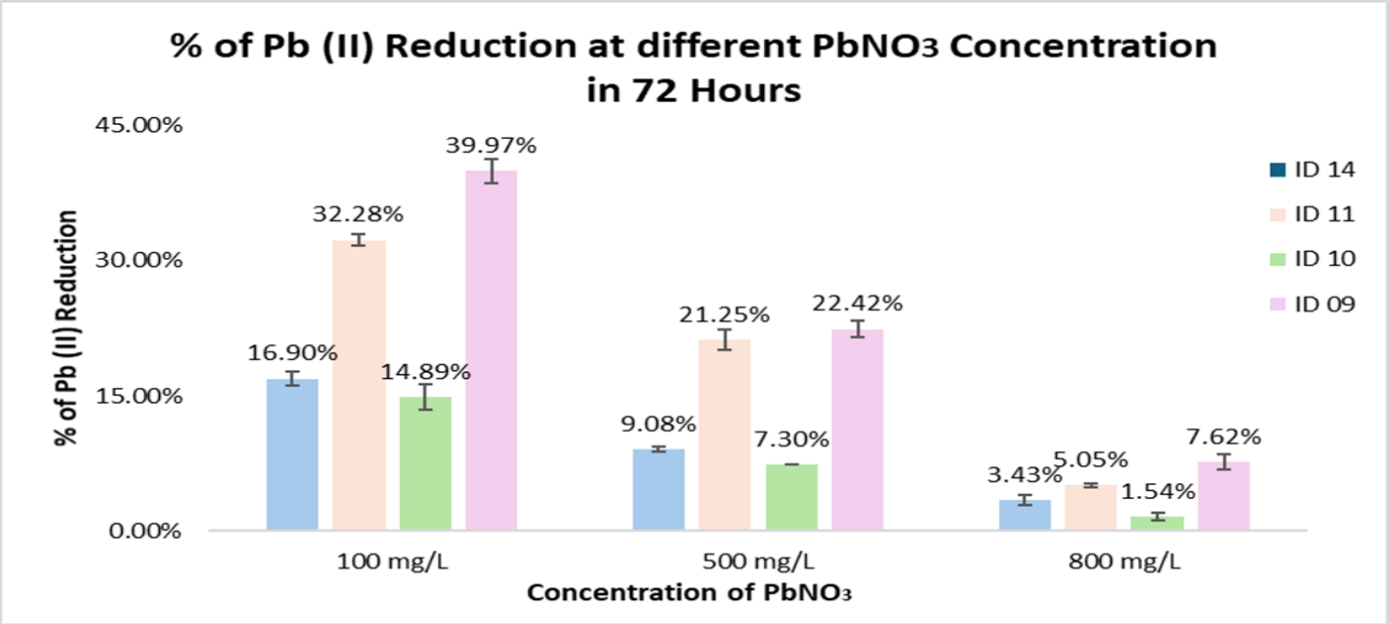


Figure 3.11: Percentage of Pb (II) Reduction Efficiency at 100,500 & 800 mg/L $PbNO_3$ concentration.

3.5.6.3 Reduction Efficiency at 100, 500 & 700 mg/L $AgNO_3$ concentration

Concentration (mg/L)	100 mg/L		500 mg/L		700 mg/L	
	Supernatant Absorbance (OD)	Percentage (%) of Ag Reduction	Supernatant Absorbance (OD)	Percentage (%) of Ag Reduction	Supernatant Absorbance (OD)	Percentage (%) of Ag Reduction
Isolate 14	0.068	46.04%	0.182	17.61%	0.341	5.46%
Isolate 11	0.102	19.52%	0.214	3.48%	0.47	1.22%
Isolate 10	0.114	11.59%	0.21	6.42%	0.434	3.50%
Isolate 9	0.066	48.33%	0.18	19.18%	0.392	7.64%

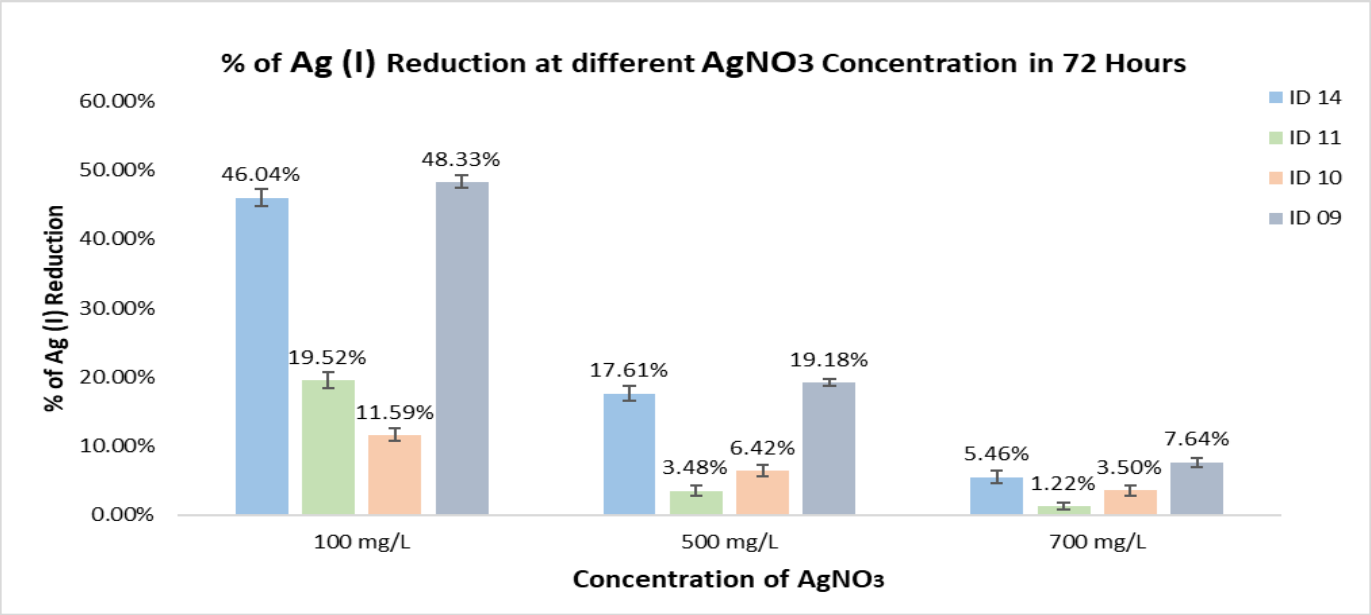


Figure 3.12: Percentage of Ag (I) Reduction Efficiency at 100,500 & 700 mg/L $AgNO_3$ concentration.

3.5.6.4 Reduction Efficiency at 100, 500 & 1000 mg/L $CdCl_2$ concentration

Concentration (mg/L)	100 mg/L		500 mg/L		1000 mg/L	
	Supernatant Absorbance (OD)	Percentage (%) of Cd Reduction	Supernatant Absorbance (OD)	Percentage (%) of Cd Reduction	Supernatant Absorbance (OD)	Percentage (%) of Cd Reduction
Isolate 14	0.102	35.02%	0.162	15.03%	0.33	1.12%
Isolate 11	0.108	31.02%	0.171	10.17%	0.434	0.78%
Isolate 10	0.114	26.13%	0.177	6.60%	0.48	0.41%
Isolate 9	0.125	21.40%	0.185	3.69%	0.569	0.21%

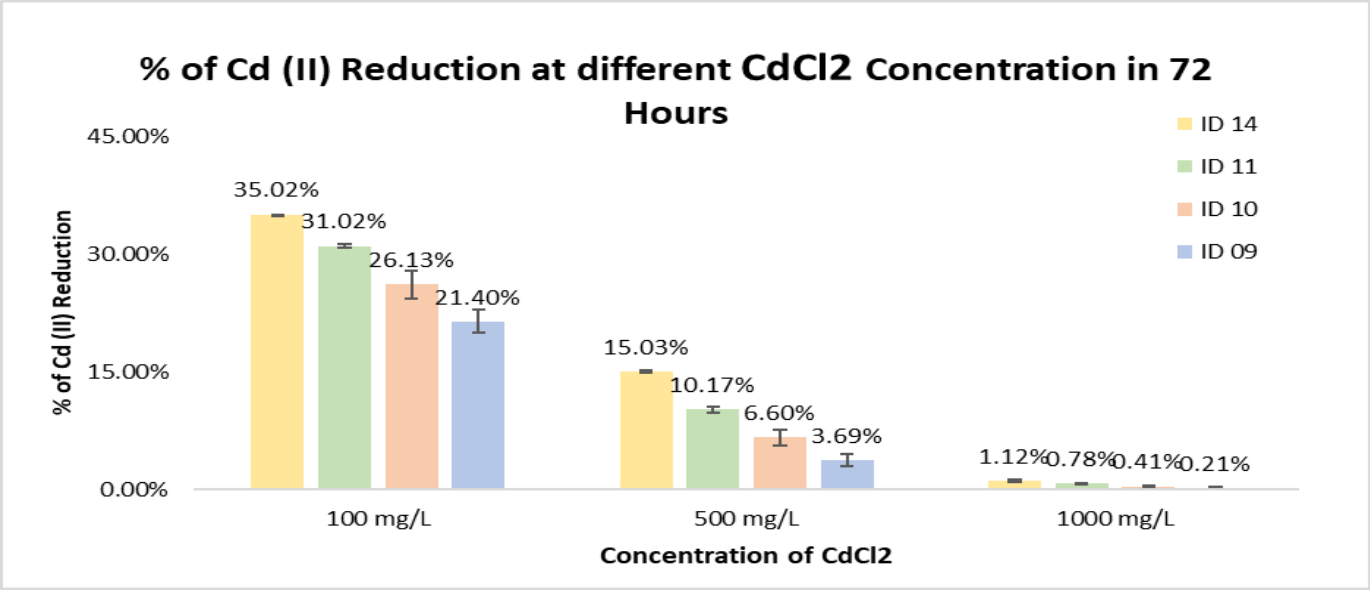


Figure 3.13: Percentage of Cd (II) Reduction Efficiency at 100,500 & 1000 mg/L $CdCl_2$ concentration**.**

3.5.6.5 Reduction Efficiency at 100, 500 & 700 mg/L $CuSO_4$ concentration

Concentration (mg/L)	100 mg/L		500 mg/L		700 mg/L	
	Supernatant Absorbance (OD)	Percentage (%) of Cu Reduction	Supernatant Absorbance (OD)	Percentage (%) of Cu Reduction	Supernatant Absorbance (OD)	Percentage (%) of Cu Reduction
Isolate 14	0.068	32.91%	0.15	13.73%	0.289	5.21%
Isolate 11	0.098	5.24%	0.171	2.90%	0.31	0.94%
Isolate 10	0.065	37.46%	0.143	20.44%	0.278	9.58%
Isolate 9	0.077	24.59%	0.155	10.63%	0.295	3.22%

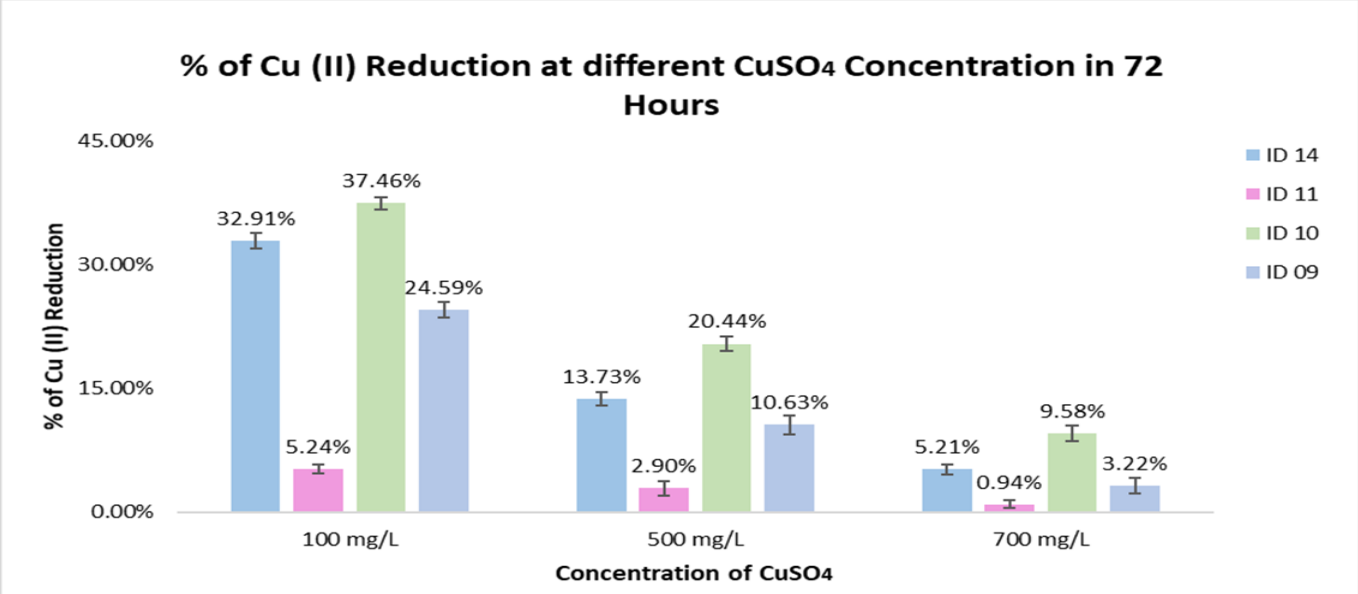


Figure 3.14: Percentage of Cu (II) Reduction Efficiency at 100,500 & 700 mg/L $CuSO_4$ concentration.

3.6 Assessment of multiple PGP traits

The four test isolates (ID 14, ID 11, ID 10, ID 09) were assessed for their plant growth promoting (PGP) characteristics, including phosphate solubilization, ammonia production, nitrogen fixation, indole-3-acetic acid (IAA) production, and siderophore (Fe-chelator). The results of this qualitative evaluation are shown below as negative (−), least active (+), moderately positive (++), and highly active (+++). A description of results by trait is provided below.

3.6.1 Phosphate Solubilization

The potential of solubilizing phosphates greatly differed across isolates. ID 09 showed the greatest solubilization activity followed by ID 14 with moderate solubilization activity. Isolate ID 10 had very little activity and isolate 11 had no phosphate solubilization activity. This means that there are isolate-specific metabolic pathways of mobilization of phosphorus.

3.6.2 Ammonia Production

A total of four isolates were positive in ammonia production and this indicates potential ubiquitous nitrogen cycling. Isolates ID 14 and ID 11 had high production and ID 10 and ID 09 had moderate activity, thus proving their ability to provide bioavailable nitrogen through ammonification processes.

3.6.3 Nitrogen Fixation

Nitrogen fixation ability was only seen in two isolates. ID 10 was moderately nitrogen fixing and ID 14 had minimal activity. In contrast, isolates ID 11 and ID 09 were negative suggesting that they lack the nitrogenase enzyme systems necessary for nitrogen fixation from the atmosphere.

3.6.4 IAA Production

Another characteristic common in all isolates was the production of indole-3-acetic acid (IAA). ID 11 and ID 10 produced most and thus had the greatest potential of stimulating plant growth. The isolates ID 14 and ID 09 showed intermediate IAA production, which supported ubiquitous production ability of auxin.

3.6.5 Siderophore Production

All isolates had a variable but positive siderophore production. ID 11 showed the greatest iron chelating activity followed by ID 14 and ID 10 with moderate production. Isolate ID 09 had the lowest activity which highlights differential strategies for iron acquisition in the chromium-tolerant strains.

Table 3.5: Assessment of multiple PGP traits on Chromium Tolerant isolates.

Isolate ID			**PGP traits**		
	P Solubilization	**NH_3 Production**	**N_2 fixation**	**IAA production**	**Siderophore (Fe-Chelator) Production**
ID 14	++	+++	+	++	++
ID 11	-	+++	-	+++	+++
ID 10	+	++	++	+++	++
ID 09	+++	++	-	++	+

[‘+’=least active ; ‘++’=moderately positive; ‘+++’=most active; ‘-’=negative result]

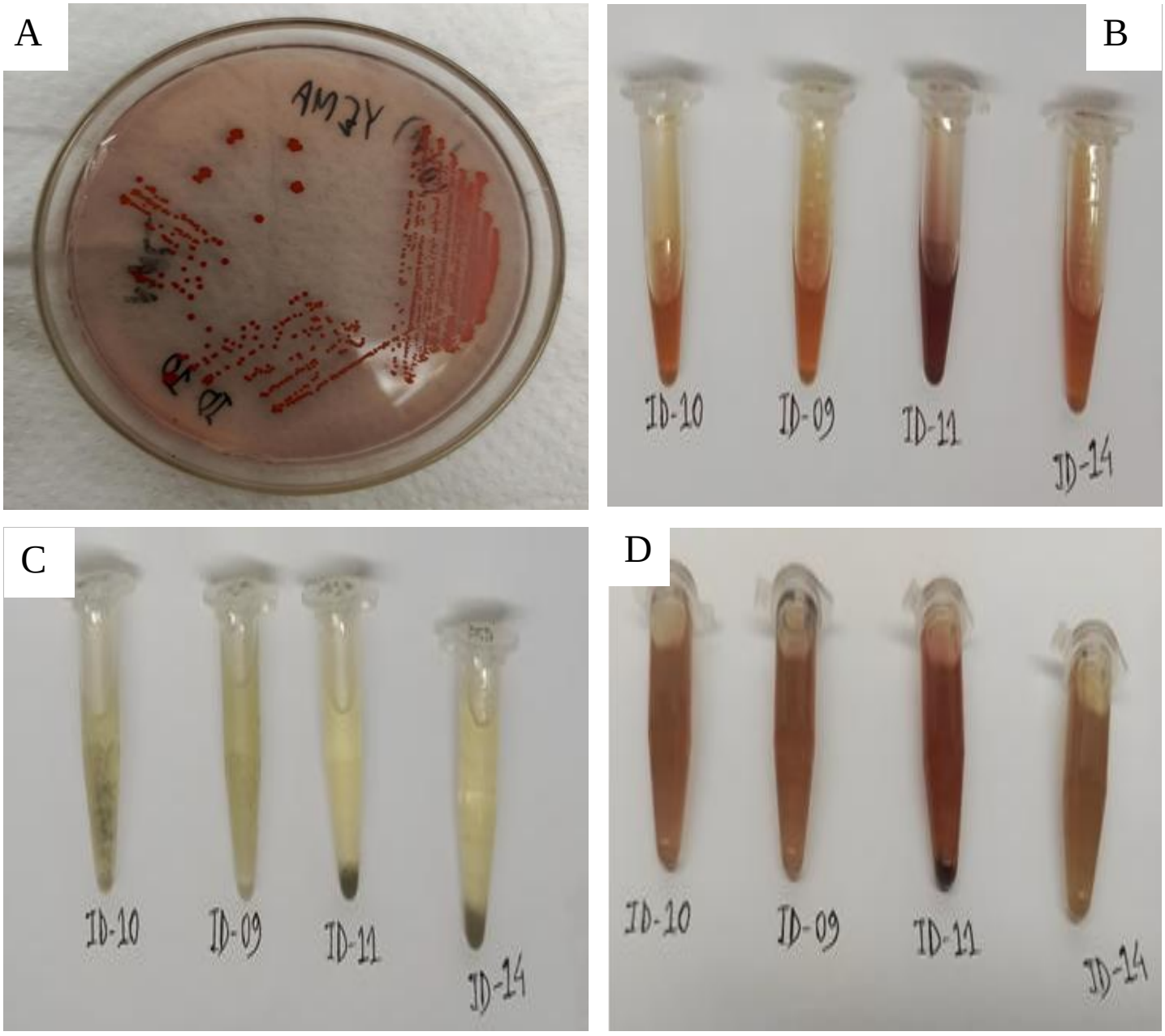


Figure 3.15: Qualitative assessment of multiple PGP traits of the isolated strains.

[(A) Growth of ID-10 on YEMA agar; (B) Siderophore production test result; (C) NH_3 production test results; (D) IAA production test result].

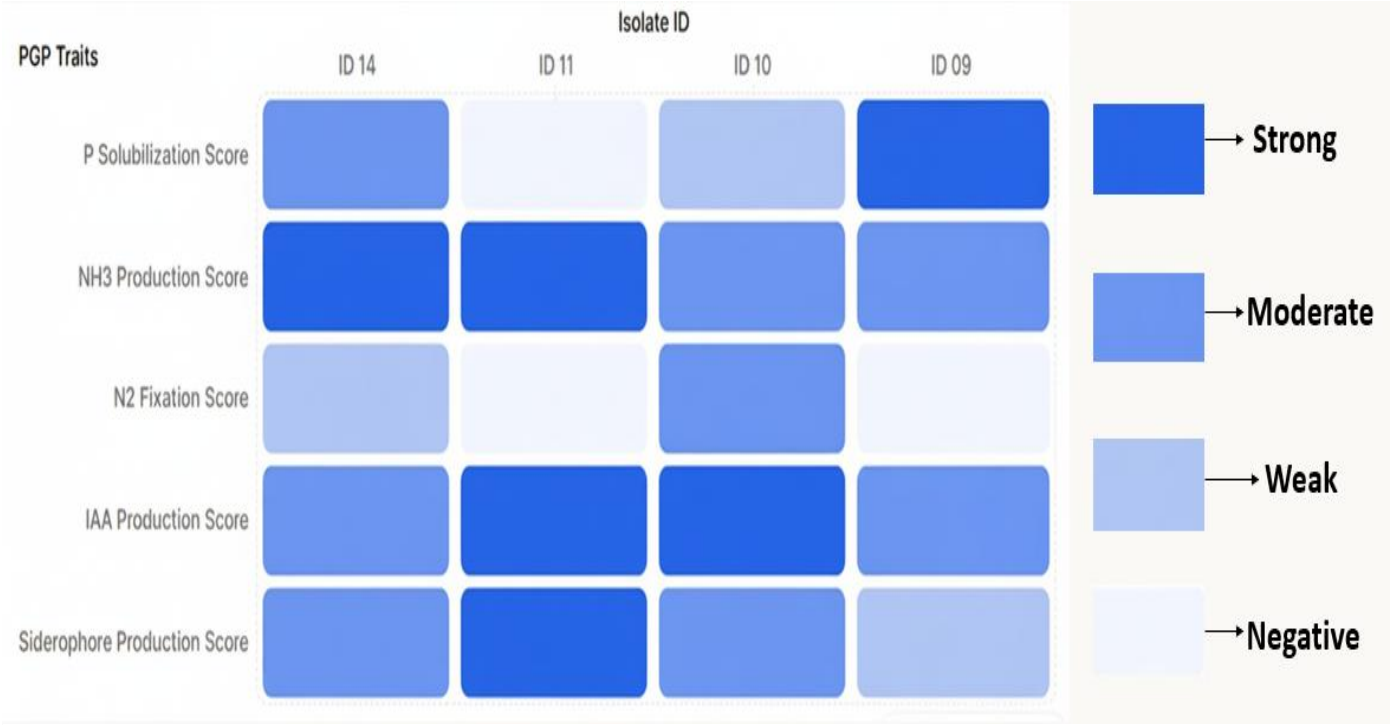


Figure 3.16: Heatmap representation of multiple plant growth-promoting (PGP) traits of chromium-tolerant bacterial isolates.

3.8 16s rRNA sequencing

After DNA purification and cycle sequencing, samples were run on an ABI 3130 Genetic Analyzer (Applied Biosystems, USA). The resulting chromatogram files were processed using Sequence Analysis 5.2, and contiguous sequences were assembled with DNA Baser Assembler. The consensus sequences were compared with those in the GenBank nucleotide database using BLAST via the NCBI website.

3.7.1 Data analysis of the Isolate ID 11

Table 3.6: Sequence similarity analysis of ID-11 by NCBI BLAST.

SI.	Description	Max Score	Total Score	Query Cover	E. value	Percent Identity	Accession Number
1	*Proteus mirabilis* strain PM2021_60 16S ribosomal RNA gene, partial sequence	1969	1969	100%	0	99.98%	OP393090.1
2	*Proteus mirabilis* strain WRMOE Egy3 16S ribosomal RNA gene, partial sequence	1731	1731	98%	0	99.65%	OR362780.1
3	Bacterium NLAE-zl-H140 16S ribosomal RNA gene, partial sequence	1716	1716	98%	0	98.28%	JX006369.1
4	*Proteus mirabilis* strain QHD 16S ribosomal RNA gene, partial sequence	1711	1711	98%	0	96.18%	KJ699115.1

3.7.2 Data analysis of the Isolate ID 10

Table 3.7: Sequence similarity analysis of ID-10 by NCBI BLAST.

SI.	Description	Max Score	Total Score	Query Cover	E. value	Percent Identity	Accession Number
1	*Staphylococcus xylosus* strain F3CCA1 16S ribosomal RNA gene, partial sequence	1275	1275	100%	0	99.87%	MZ974954.1
2	*Staphylococcus xylosus* strain Huaian_57_2 16S ribosomal RNA gene, partial sequence	1237	1237	100%	0	99.67%	MN252052.1
3	*Staphylococcus saprophyticus* strain CMST-TC-ASG3 16S ribosomal RNA gene, partial sequence	1168	1168	100%	4.00E-179	98.57%	PQ107655.1
4	*Staphylococcus sp.* strain NM10 16S ribosomal RNA gene, partial sequence	1109	1109	100%	0	97.02%	OR592268.1

3.7.3 Data analysis of the Isolate ID 09

Table 3.8: Sequence similarity analysis of ID-09 by NCBI BLAST.

SI.	Description	Max Score	Total Score	Query Cover	E. value	Percent Identity	Accession Number
1	*Staphylococcus cohnii* strain BMBLM7_2 16S ribosomal RNA gene, partial sequence	2673	2673	100%	0	99.99%	MG996846.1
2	*Staphylococcus cohnii* strain Tr15B 16S ribosomal RNA gene, partial sequence	2647	2647	100%	0	99.66%	MH210861.1
3	Bacterium strain S288 16S ribosomal RNA gene, partial sequence	2641	2641	99%	0	99.79%	MZ461828.1
4	*Staphylococcus ureilyticus* strain HBU7259 16S ribosomal RNA gene, partial sequence	2636	2636	99%	0	99.72%	MW365210.1

3.9 WGS Data analysis

3.9.1 Genomic Characterization of Isolate ID-14

The aforementioned experimental research was further validated through comprehensive genome sequencing and analyses. Sequencing of the genomic DNA revealed that the complete genome of Isolate ID14 was assembled into 86 contigs, totaling 2,704,251 bp with an average GC content of 32.6%. A total of 2684 genes were predicted, including 836 hypothetical proteins & 72 RNA genes. Additional genome features are presented. The circular view of the genome from PROKSEE online software (v1.0.0a6) is also presented, highlighting the GC distribution and GC-skew of the genome. The analysis revealed that the isolated organism is *Staphylococcus ureilyticus*

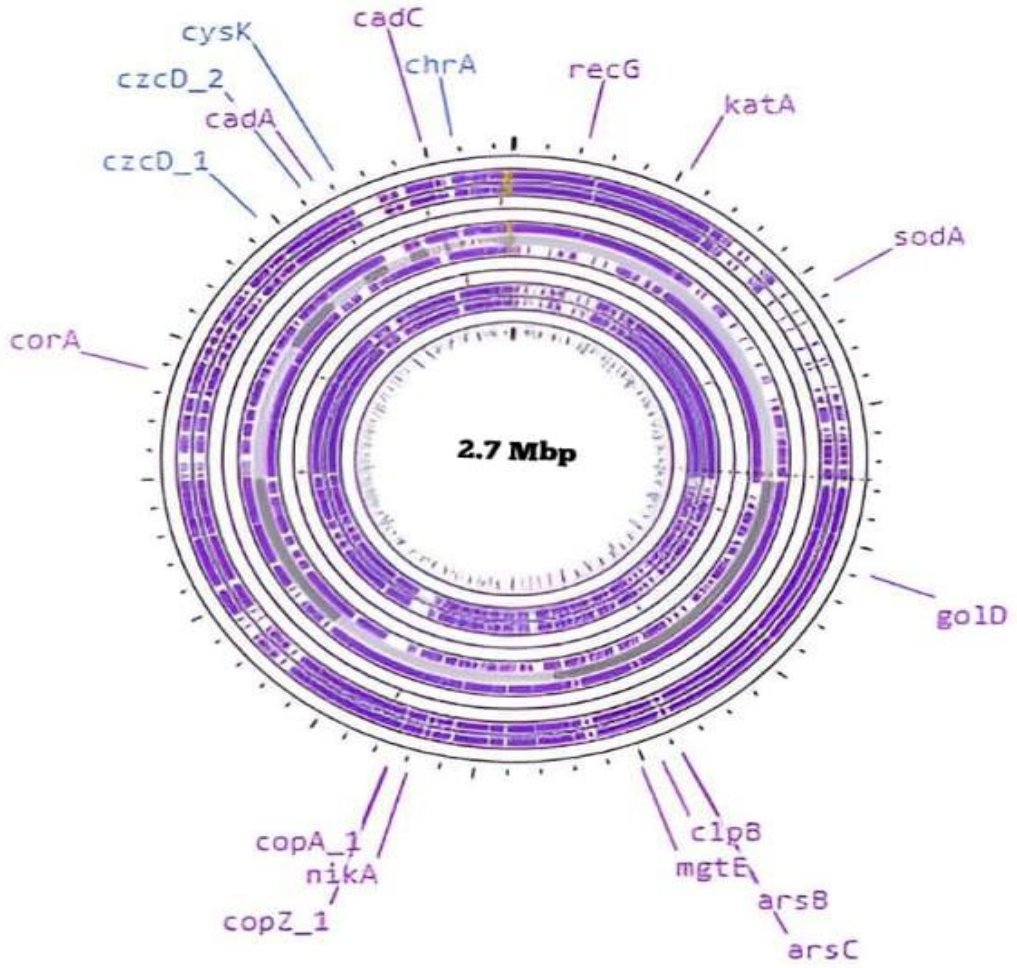


Figure 3.17: Genomic Map of Heavy Metal Resistance Genes in a Bacterial genome.

[This figure displays a circular map of the 2.7 Mbp bacterial genome, highlighting key heavy metal resistance genes such as cadA, copA, and arsC, along with their locations].

3.9.2 Annotation of Heavy metal associated genes

We already observed the ability of ID-14 to grow in the presence of various heavy metals, including chromium, cadmium, nikel, zinc & lead. To further support these findings, the annotated genome of ID-14 was analyzed (via RAST, Patric & Prokka) for the presence of heavy metal tolerance genes. The results revealed abundant genes associated with resistance to chromium, cadmium, cobalt, and zinc.

Table 3.9: Metal-Specific Resistance & Transport Genes.

Target Metal	Gene Name	Function
Arsenic	*arsB*	Pumps toxic arsenite out of the cell (Efflux).
	arsC	Chemically converts arsenate to arsenite for removal (Detoxification).
Cadmium / Zinc / Cobalt	*cadA*	Efflux pump that removes toxic cadmium, zinc, and cobalt.
	cadC	Regulatory protein that detects cadmium levels and activates cadA.
	czcD (1, 2, 3)	Membrane transporter that ejects cobalt, zinc, and cadmium ions.
Chromium	*chrA*	Pumps toxic chromate ions out of the cell (Efflux).
Copper	*copA_1*	Efflux pump that removes excess copper to prevent toxicity.
	copZ_1	Copper chaperone that safely delivers copper ions to the efflux pump.
Gold	*golD*	Likely binds or transports gold ions to confer resistance.
Iron	*fecD*	Transports iron (dicitrate) into the cell to prevent deficiency.
	fur	Regulates iron uptake genes to prevent iron overload and toxicity.
Magnesium (Affects Ni/Co)	*corA*	Primary magnesium transporter; its regulation limits toxic cobalt/nickel influx.
	mgtE	Magnesium transporter that competitively blocks uptake of toxic metals.
Molybdenum	*modA*	Transports molybdenum, an essential trace element, into the cell.
Nickel	*nikA, nikD*	Components of a specific transport system to import nickel safely.

Table 3.10: Metal Non-specific Resistance genes.

Target / Function	Gene Name	Role in Metal Resistance
Oxidative Stress	*katA, katE_1*	Neutralizes hydrogen peroxide produced during metal stress.
Oxidative Stress Protein Repair	*sodA*	Neutralizes superoxide radicals generated by heavy metals.
	trxA, trxB	Repairs proteins damaged by oxidation (thioredoxin system).
	pnrB	Reduces toxic compounds; often reduces metals like Chromate(VI) to less toxic forms.
	cysK	Produces cysteine (thiol), which captures and neutralizes heavy metals.
	clpB, dnaK	Chaperones that fix or recycle proteins unfolded by metal

		toxicity.
DNA Repair	**recG**	Repairs DNA strand breaks caused by metal-induced reactive oxygen.

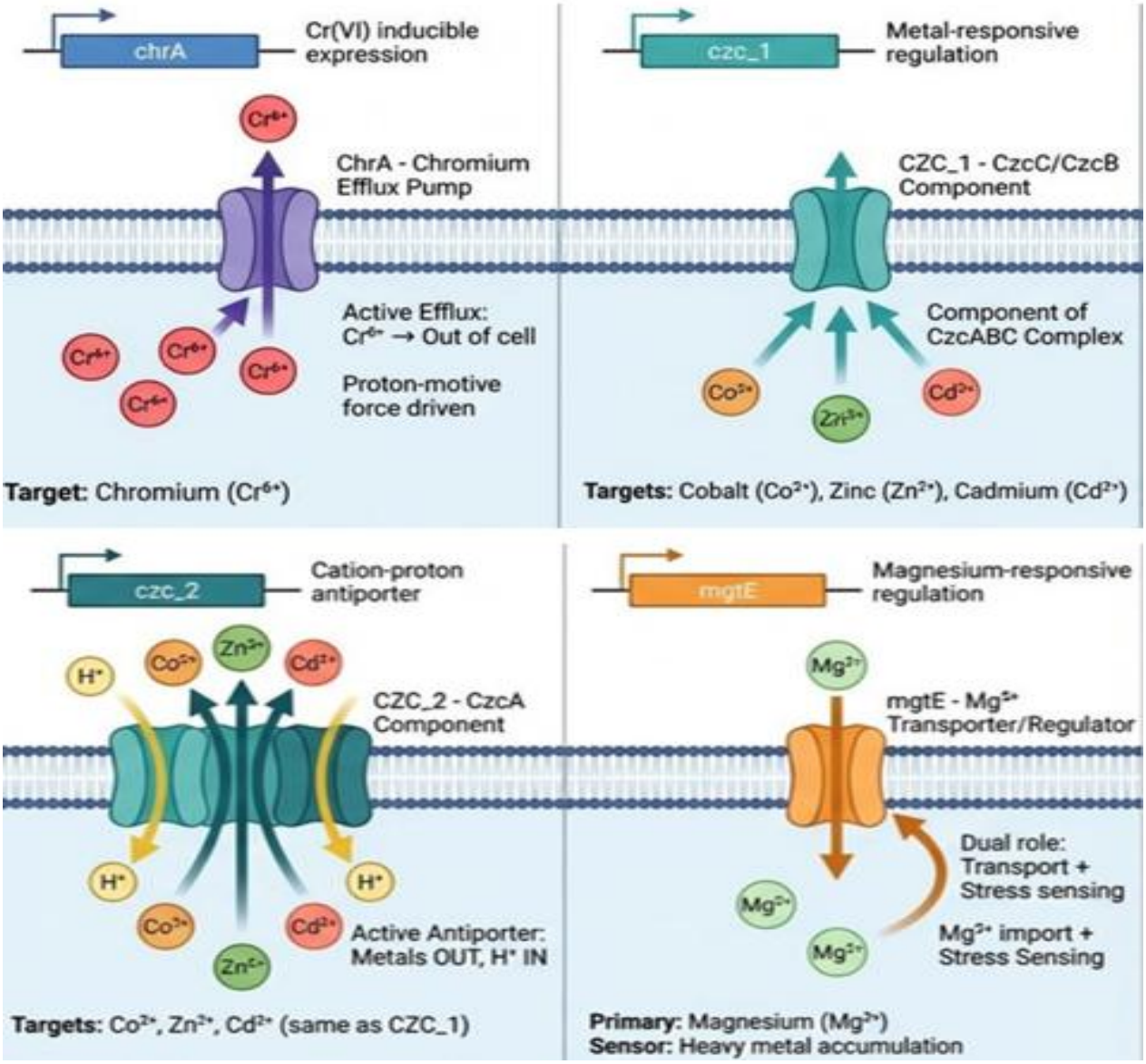


Figure 3.18: Mechanisms of Heavy Metal Transport and Regulation in Bacterial membranes**.**

[This figure illustrates the mechanisms of heavy metal transport in bacterial cell membranes, featuring the roles of ChrA, Czc, and MgtE transporters. Each component is responsible for expelling or regulating ions such as 98 | P a g e chromium (Cr), cobalt (Co), zinc (Zn), cadmium (Cd), and magnesium (Mg). ChrA actively pumps chromium out of the cell in response to Cr(VI) exposure, while CzcC/CzcB complexes help regulate cobalt, zinc, and cadmium levels. MgtE, which acts as

both a transporter and stress sensor, plays a dual role in magnesium homeostasis and heavy metal stress response] (88, 103).

3.9.3 Annotation of Antibiotic resistance associated genes

Like heavy metal resistance associated genes, some antibiotic resistance genes are also annoted for Isolate ID-14.

Table 3.11: Genes related to antibiotic resistance in the ID14 genome.

	Beta-Lactam Resistance (Penicillins, Methicillin, Cephalosporins)
Gene Name	**Function in Antibiotic Resistance**
blaZ	Produces beta-lactamase enzymes that break open the ring structure of penicillin, rendering it ineffective.
mecA	Encodes an alternative penicillin-binding protein (PBP2a) that allows cell wall building to continue despite the presence of methicillin.
femB / femX	Essential auxiliary factors that stabilize the cell wall, allowing the high-level expression of methicillin resistance.
fmtA	Modifies cell wall structures (teichoic acids) to reduce the binding and effectiveness of beta-lactam antibiotics.
lytR	Regulates cell wall turnover and autolysis, contributing to tolerance against cell-wall attacking antibiotics.

	Macrolide, Lincosamide & Streptogramin (MLS) Resistance
Gene Name	**Function in Antibiotic Resistance**
mphC	Enzymatically inactivates macrolide antibiotics (like erythromycin) by adding a phosphate group to them.
msrA	Acts as an efflux pump to actively expel macrolide and streptogramin B antibiotics from the cell.
lnuA	Enzymatically inactivates lincosamide antibiotics (like lincomycin) via nucleotidylation.
salE	Likely confers resistance to lincosamides or streptogramins, protecting the bacterial ribosome.
	Glycopeptide Resistance (Vancomycin)
Gene Name	**Function in Antibiotic Resistance**
vanT / vanY	Enzymes that modify cell wall precursors (peptidoglycan termini) so that vancomycin cannot bind to them.
	Fluoroquinolone Resistance (Ciprofloxacin, etc.)

Gene Name	Function in Antibiotic Resistance
norA / norC / norG	Major facilitator efflux pumps that rapidly eject fluoroquinolones from the cell before they can act.
norB (_1, _3)	Pumps fluoroquinolones and other toxins out of the cell; often active when norA is not.
sdrM	A multidrug efflux pump that transports norfloxacin and other compounds out of the bacterial cytoplasm.

	Fluoroquinolone Resistance (Ciprofloxacin, etc.)
Gene Name	**Function in Antibiotic Resistance**
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norB (_1, _3)	Pumps fluoroquinolones and other toxins out of the cell; often active when norA is not.
sdrM	A multidrug efflux pump that transports norfloxacin and other compounds out of the bacterial cytoplasm.

	Multidrug Efflux & General Defense
Gene Name	**Function in Antibiotic Resistance**
qacC_1 / qacJ	Efflux pumps that remove quaternary ammonium compounds (antiseptics) and some intercalating dyes.
emrB	A multidrug efflux pump that extrudes various hydrophobic antibiotics and toxins.
sepA	A small multidrug resistance (SMR) pump that removes antiseptics and toxic dyes from the cell.
yknY	Part of a transporter system that protects the cell from host antimicrobial peptides and some drugs.
ydfJ	A putative multidrug transporter likely involved in pumping out toxic substances or drugs.
recA	Triggers the SOS response, which repairs DNA damage caused by antibiotics and facilitates the acquisition of new resistance mutations.

3.7 Antimicrobial Susceptibility Testing

The AST has been done for the four test isolates to check their effectivity or sensitivity against different antibiotics. These organisms show different ranges of zone inhibition in MHA agar plates after incubation. By measuring the diameter of ZOI (mm), we can interpret their sensitivity against different antibiotics.

Table 3.11: The AST of isolates against AMP, IPM, AZM, C, Ak & CIP.

Isolate ID	**ID 14**	**ID 11**	**ID 10**	**ID 09**
Antimicrobial Agents				
AMP	R	S	R	R
IPM	S	S	S	S
AZM	S	I	S	R
C	I	S	S	S
Ak	I	S	S	S
CIP	I	R	S	S

Table 3.12: The AST of isolates against COT, LE, CAZ, LZ & F.

Isolate ID	**ID 14**	**ID 11**	**ID 10**	**ID 09**
Antimicrobial Agents				
COT	I	S	S	S
LE	R	I	S	R
CAZ	R	R	R	R
LZ	S	R	I	S
F	S	I	S	S

Table 3.13: The AST of isolates against OX, RD, DA, TE, CN.

Isolate ID	**ID 14**	**ID 11**	**ID 10**	**ID 09**
Antimicrobial Agents				
OX	R	R	R	R
RD	S	R	S	I
DA	I	R	R	R
TE	R	S	I	I
CN	I	S	S	S

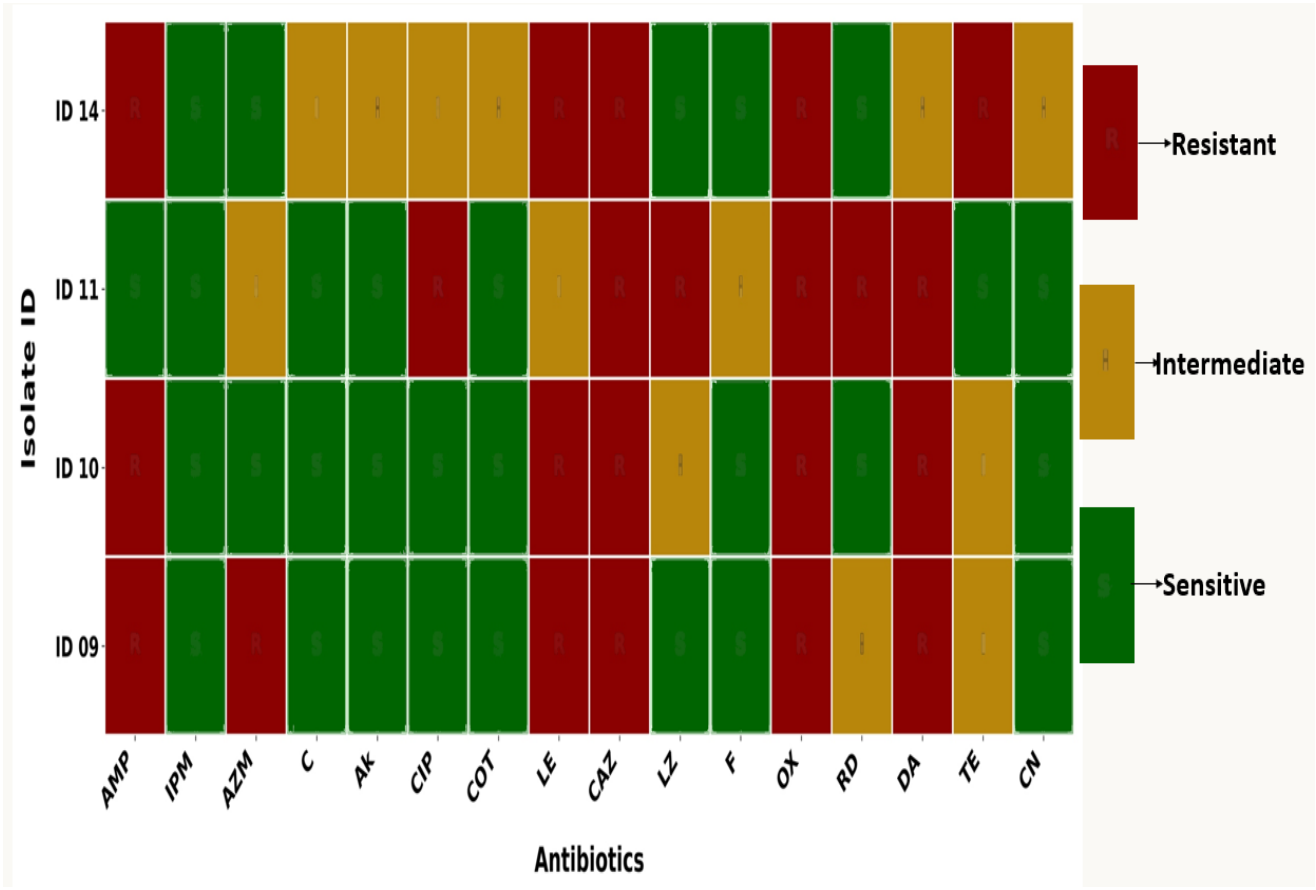

Figure 3.19: Antibiotic susceptibility profile of chromium-tolerant bacterial isolates.

[Color coding: Green = Sensitive (S), Yellow = Intermediate (I), Red = Resistant (R)].

Chapter 4: Discussion

Isolation of 25 chromium-tolerant bacteria of poultry excreta in Noakhali, Bangladesh fills a knowledge gap with regard to characterizing heavy metal-microbe interactions in farming settings. While chromium contamination is known as a major environmental problem in Bangladesh, concerning microbiological investigation about chromium resistant bacteria in agricultural areas, there is a paucity of studies. This study shows that diffuse agriculture pathways, as opposed to localized industrial sources, are important agents of heavy metal distribution and localized selective pressure of specialized microbial communities.

Five isolates, namely ID-14, ID-11, ID-10, ID-09 and ID-01 exhibited amazing tolerance and showed growth at 500 mg/L potassium dichromate (of the order of 200mg/L Cr(VI)) as does internationally reported chromium-resistant isolates. This reduction in optical density, attributed to an increased concentration of chromium, indicates the existence of a metabolic price to active resistance mechanisms, and that there is a number of complementary tolerance mechanisms such efflux pumps, cell surface remodeling and intracellular sequestration. Most importantly, the isolates actively lower toxic chromium hexavalent [Cr(VI)] to less toxic chromium trivalent chromium [Cr(III)], via enzymatic processes, a reaction obligatory to bioremediation processes because Cr(III) separates and accumulates in neutral to alkaline conditions.

The biochemical characterization showed Gram-positive bacteria which were mostly heterotrophic, which indicates that multi-strain consortia strategies can be better than monocultures in terms of large-scale remediation. Moreover isolates showed cross-tolerance to lead, silver, copper, cadmium and nickel at up to 1000 mg/L, and this result is evidence of non-specific resistance mechanisms that it is useful in polymetal-contaminated sites. One of the concerns proved to be that there was resistance to multiple antibiotic classes across several strains, which meant that heavy metal and antibiotic resistance determinants were co-selected, a danger to the population as these can be transferred horizontally.

Whole-genome sequencing of the isolate ID-14 exhibited chromosomal and plasmid-borne chromium resistance genes, such as chromate reductase, metal efflux pump and other complementary acquisition pathways. It is important to note that most of the isolates had plant growth promoting properties such as phosphate solubilization, nitrogen fixation, and the production of siderophores, which allowed them to be used in combined bioremediation methods that used phytoremediation in place of bioaugmentation. The occurrence of chromium-tolerant bacteria in Noakhali, a coastal agricultural area with no known heavy industry, indicates widespread chromium contamination via diffuse pathways, and probably from

chromium-contaminated poultry feed from by-products of tanneries. It demonstrates that the level of heavy metal pollution is much broader than so-called industrial hotspots, as it involves the poultry farming systems of the entire country as potential sources and enhancers of heavy metal and antibiotic resistance determinants, and has far-reaching food safety and environmental health outcomes.

Chapter 5: Conclusion

This initial total survey identified 25 morphologically and biochemically unique chromium-tolerant bacterial strains of poultry excreta at Noakhali, Bangladesh with long-term chromium contamination demonstrating selective pressures on the stable populations of specialized bacterial communities with remarkable heavy metal tolerance and enzyme detoxification properties. Five isolates (ID-14, ID-11, ID-10, ID-09 and ID-01) showed outstanding level of tolerance by maintaining growth in 500 mg/L potassium dichromate (approx. 200 mg/L Cr(VI)), similar to chromium-resistant reference strains from other countries.

Most importantly, the mechanism of chromium tolerance within such isolates is actively enzyme reduction and not passive. Isolates reduce toxic hexavalent chromium [Cr(VI)] to less toxic trivalent chromium [Cr(III)], which can give a real bioremediation capability in the form of detoxification and immobilization. The domination of Gram-positive bacterium with Bacillus-like species is consistent with the existing data on the planet and so forms phenotypically and genetically close populations within the agricultural systems of South Asia.

These isolates exhibit cross-tolerance to various heavy metals (lead, silver, copper, cadmium, and nickel) up to 1000 mg/L of concentration and so non-specific mechanisms of general metal resistance and not chromium-specific mechanisms. This versatility phenotype increases the practical applicability in the environmental polymetal-contaminated settings. Notably, isolates show plant growth-promoting properties such as phosphate solubilization, nitrogen fixation and siderophore production, which have not been reported systematically with chromium toxicity, allowing to integrate bioremediation by combining phytoremediation and bioaugmentation.

Genome sequencing of isolate ID-14 and 16S rRNA sequencing of three other high-performing isolates revealed the presence of genetic determinants that encode chromate reductase and metal efflux pumps and both chromosomal and plasmid-based resistance genes that ensure phenotypic stability. The existence of chromium tolerant bacteria in Noakhali, which was long outside known industrial contamination areas suggests that chromium pollution extends much beyond known hotspots such as the Hazaribagh tanneries to being diffused through diffuse agricultural pathways involving tannery by-products in commercial poultry feed.

Nevertheless, incomitent identity of antibiotic-resistant chromium-tolerant bacteria poses serious threats to the general health of the population by the possibility of horizontal transfer of genes to the pathogenic

bacteria. The potential application of genetic engineering to the environment in the future must possess stringent risk assessment, safety measures as well as the deletion of antibiotic resistance genes should be considered prior to the use. This research provides a scientific basis for sustainable and economically feasible bioremediation approaches under the context of careful risk management for preserving the agricultural productivity, environmental quality, and public health in Bangladesh and other similar regions of the use.

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APPENDICES

Appendix A: Media Composition

1. Luria-Bertani (Miller) Broth (pH 7.0)

Component	Concentration
Tryptone	10.0 g
Beef extract	5.0 g
Sodium chloride	10.0 g
Distilled water	1000 ml

2. Tryptic Soy Broth (TSB) (pH 7.3 ± 0.2 at 25°C)

Component	Concentration
Tryptone (Pancreatic digest of casein)	17.0 g
Papaic digest of soybean meal	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	2.5 g
Glucose (dextrose)	2.5 g
Distilled water	1000 ml

3. Glycerol Stock Solution (40%)

Component	Concentration
Glycerol	40 ml
LB broth	60 ml

4. Cr^{6+} Stock Solution

Component	Concentration
Potassium Dichromate ($K_2Cr_2O_7$)	Varies (in mg)
Distilled water	20 ml (each)

Appendix B: Equipments

Equipment	Specification	Use
Orbital Shaker Incubator	ST30, NUVE, Turkey	Incubation and shaking
Incubator	Incucell, MMM Group, Germany	Incubation of culture
Laminar Airflow	Class II BSC, Esco, Singapore	Aseptic environment
Analytical Balance	KD-UBED-600, Fuzhou Lucky Star Co., Ltd., China	Weight measurement
Magnetic Stirrer	MSH-20A, Wisd (Daihan Scientific), South Korea	Proper mixing of the solution
Colony Counter	1803006, ISOLAB, Germany	Count colonies on culture plates.
Autoclave	WiseClave, Wisd (Daihan Scientific), South korea	Sterilization
Low-temperature-Freezer (4°C)	WFA-2D4-0501, Walton, Bangladesh	Preservation of culture
AC (Automatic Voltage Regulator)	MDR-5000VA,KEBO Electric Co., Ltd., China	Electricity supply
Deep Freezer (-80°C)	Lexicon II ULT Freezer, Esco, Singapore.	To preserve chemicals, media and samples
Distilled Water Plant	BioBase incorporation, China	Preparation of distilled water
Spectrophotometer	BioBase incorporation, China	Quantitative studies
Vortex Machine	VM-1000, Digisystem Laboratory Instruments, Taiwan	Shaking

Microwave Oven	Sheldon Manufacturing (SHEL LAB), United States	To heat and liquefy if necessary
Water Bath	DSB-1000E, Digisystem Laboratory Instruments, Taiwan	To ensure warming condition and avoid solidification
Gel Electrophoresis Unit	Bio-RAD,USA	To observe banding pattern of DNA and RNA