Adaptive Responses and Molecular Characterization of Chromium-Resistant Bacteria from Industrially Polluted Environments of Bangladesh

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Humbly-The Author

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Certification

This is to certify that **Noimul Hasan Siddiquee**, ID No. MUH2305MS109M completed his research under my supervision titled "**Adaptive Responses and Molecular Characterization of Chromium-Resistant Bacteria from Industrially Polluted Environments of Bangladesh.**" This is to confirm that the submitted work is original and acceptable for credit towards the Master of Science in Microbiology degree at Noakhali Science & Technology University.

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Abstract

Background: Industrial effluents released from tannery, dyeing and battery industry contribute largely to heavy metal pollution in both water and soil environments of Bangladesh. Chromium is one of the most problematic metals as it remains in the environment for long time and shows high toxicity, and its mobility increases under acidic conditions, making it risky for ecosystem and human health. Microorganisms subjected to such conditions frequently evolve adaptation methods that facilitate survival under metal stress, rendering them viable instruments for bioremediation. The goal of this study was to find bacteria that can live in or break down heavy metals and then make them more tolerant by passing them through higher and higher levels of heavy metals over and over again.

Methods: Heavy metal degrading and tolerant bacteria were isolated from both water and soil samples collected from different industrial polluted areas of Savar, Dhaka. At first, the bacterial species were identified through selective media culture and common biochemical tests, and finally PCR analysis with species specific primers was used to confirm the identification. Potassium dichromate ($K_2Cr_2O_7$) was used to test tolerance to chromium, the most prevalent heavy metal, in broth dilution and then plate counting. Adaptive evolution was prompted by successive exposure to progressively elevated chromium concentrations.

Stress-driven morphological changes were examined by Gram staining, microscopy, and colony morphology was assessed visually on MacConkey agar.

Results: Water samples showed a wide pH variation (1.01–7.50), with several sites being highly acidic and non-compliant with environmental standards. A total of 32 bacterial isolates were recovered, with *Pseudomonas*, *Klebsiella*, *Escherichia coli*, *Aeromonas*, and *Acinetobacter* as dominant genera. PCR confirmed most of the biochemically identified isolates. Among them, the survival of *E. coli* and *Klebsiella spp.* in chromium salt was recorded as high as 1.5 mM. After successive adaptation with increasing Chromium concentration, the MIC increased to 1.75 mM and 1.5 mM for *E. coli* and *Klebsiella,* respectively. Colony morphology changed significantly in *Klebsiella* under chromium stress, while *E. coli* showed no visible colony-level change. Adapted cells exhibited reduced cell size, irregular morphology, altered cellular arrangement, whitish-centered colony on the MacConkey plate

Conclusion: The study demonstrates that industrial pollution in Savar has created chemically harsh environments that select for chromium-tolerant and adaptively evolving bacteria. The microbial plasticity under chromium stress, highlighting the potential of heavy-metal-tolerant bacterial isolates for efficient bioremediation applications through selecting improved metal-degrading strains.

Keywords: Chromium, heavy metal tolerance, bacterial adaptation, MIC, industrial pollution, Savar, Bangladesh.

Chapter 1

1.1 Introduction

Industrial activities and improper waste disposal are major sources of heavy metal contamination, which endangers the health and environment of humans and other animals [1]. The worldwide increase of heavy metal (HM) contamination has become a serious matter of concern, because it affects not only human beings but also plant life, and as a result the ecosystem is disturbed. Different reports have shown that heavy metals are responsible for delayed growth in children, neurological problems, and also long-term chronic diseases [2]. On the other hand, the behavior of heavy metals in bacterial populations is not simple and shows complex nature. Metals like cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), and zinc (Zn) are needed only in very small quantity for normal metabolic activities and for maintaining enzyme stability in bacteria. However, when these essential metals are present in higher amount, especially at micromolar level, they become toxic; for instance bacteria can tolerate copper in the range of about 2–8 mM and zinc around 0.5–5 mM, but metals such as cadmium and mercury show toxic effect even at comparatively lower high concentrations [3].

Bioremediation approaches often face different difficulties because of the toxic nature and long-term persistence of heavy metals in the environment. Unlike organic pollutants, heavy metals cannot be easily biodegraded or destroyed by heat, and therefore the options for their removal or neutralization are very limited [4]. Although heavy metal pollution can be lethal, some bacterial groups have gradually developed defense mechanisms that help them to survive and even grow under such contaminated conditions. Since bacteria are present almost everywhere and are involved in most biological processes, they require special consideration in discussions related to heavy metal bioremediation. Heavy metals are generally toxic to microbial cells at high concentrations; however, certain bacterial species such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, and some others have shown the ability to tolerate and detoxify different heavy metals [5], [6], [7]. These heavy metal resistant bacteria, when applied together with microbial biotechnology, may provide long-term and effective solutions for the bioremediation of heavy metal polluted sites, while also reducing the environmental damage caused by conventional treatment methods.

Even though microbes can't break down heavy metals in the environment, they can change them into less harmful oxidation states or organic forms. As a result of changes in their oxidation states, metals become less poisonous, more soluble, more readily precipitated, and more vaporized [8]. Bioaccumulation refers to the possibility that microbes may employ metabolic energy to absorb heavy metals. The genetic, physiological, and environmental factors primarily influence the bioaccumulation of heavy metals by microbes [9]. The negative charge on the surface of some microbes' cells allows them to adsorb heavy metal ions, and these microbes can then employ these metals and metalloids as electron donors or acceptors to generate energy [10].

This study set out to isolate, water quality assessment by pH measurement, using biochemical and PCR-based identification, evaluation of heavy metal tolerance, adaptation to higher salt concentration, and assessment of stress-driven morphological changes of gram-negative bacteria collected from water and soil samples taken from an industrial region in Dhaka, Bangladesh. This also annotates the genomic and translational divergence between native (environmental sample isolates) and heavy metal-adapted isolates using the whole genome sequencing method.

1.2 Aims and Objectives of the Research

1. To isolate and identify heavy-metal-tolerant bacteria using cultural, biochemical, and PCR-based molecular techniques.
2. To determine the chromium tolerance capacity of selected bacterial isolates through minimum inhibitory concentration (MIC) assays using potassium dichromate ($K_2Cr_2O_7$).
3. To adapt selected bacterial strains to higher chromium concentrations through successive passage under metal stress.
4. To evaluate stress-induced morphological changes in native and adapted strains using Gram staining.

1.3 Literature review

1.3.1 Heavy Metal Pollution in Industrial Aquatic and Soil Environments

Heavy metal pollution from rapidly expanding industrial activities has become one of the most persistent threats to aquatic and soil environments, particularly around tannery and dyeing clusters in developing countries such as Bangladesh [11], [12]. In such areas, poorly treated or even untreated industrial effluents containing chromium (Cr), lead (Pb), and cadmium (Cd) are regularly

discharged into nearby rivers and surrounding floodplain soils. After entering the environment, these metals tend to accumulate over time, undergo different transformations, and finally create long-term ecological as well as human health impacts [12], [13].

Tanneries mostly depend on chromium salts for leather processing, whereas industries such as dyeing, pigment production, battery manufacturing, and electroplating act as major sources of Pb and Cd within industrial zones [14], [11]. Due to inadequate effluent treatment facilities and also because of malfunctioning or bypassing of central effluent treatment plants, large amounts of metal-containing wastewater are often released directly into surface water bodies, drainage channels, and low-lying waste disposal ponds [15], [16]. Once these metals are released, they strongly attach with suspended particles and organic matter, which helps their deposition in riverbed sediments and in nearby agricultural soils that are irrigated using contaminated water [12], [17]. With time, this process causes gradual enrichment of Cr, Pb, Cd, and other trace metals in sediments and topsoil, and in many cases their concentrations exceed the recommended international guideline values for safe irrigation practices and crop production [13], [18].

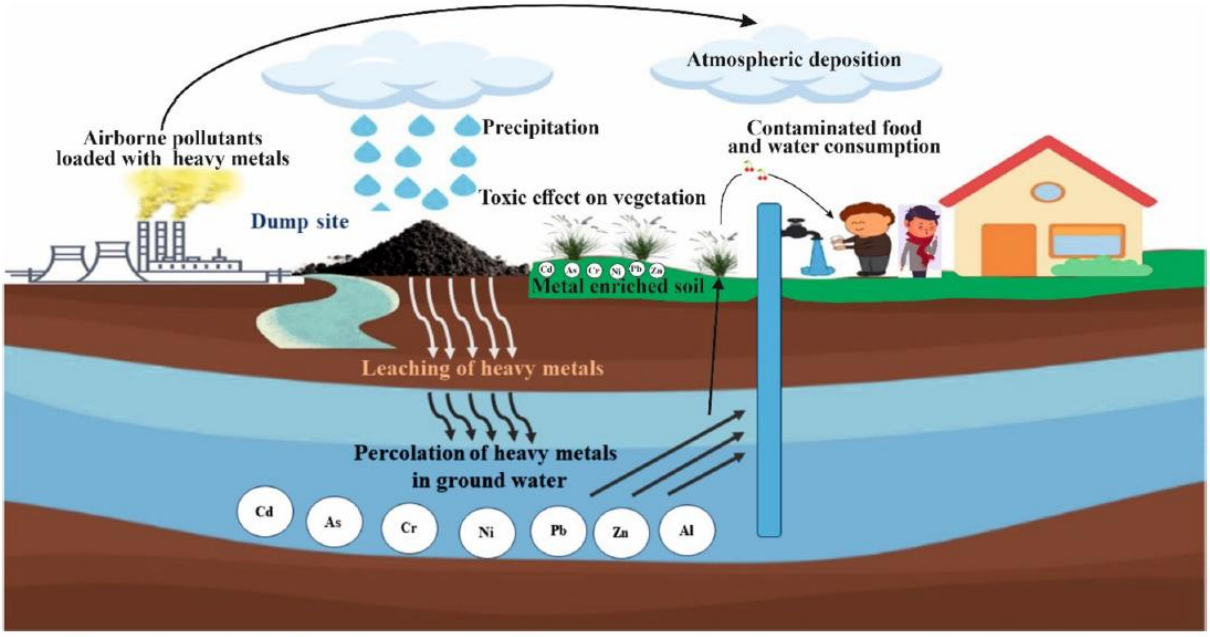

Figure 2.1. Overview of industrial sources of heavy metals and their movement into water bodies and surrounding land ecosystems, causing environmental contamination. [19].

The move of the Hazaribagh tanneries to the Savar Tannery Industrial Estate in Bangladesh was meant to cut down on pollution in the Buriganga River. Instead, it has moved and concentrated the heavy metal burden onto the Dhaleshwari River system [16], [20]. Studies indicate that the estate discharges tens of thousands of cubic meters of predominantly untreated wastewater daily, with high amounts of chromium, lead, cadmium, and other metals found in river water, sediments, surrounding soils, and river-irrigated vegetables [11], [14], [21], [22], [23]. Research in the Savar area has shown that Cr is the most common contaminant and is often the main health risk for communities that use river water, soil, and crops. Pb and Cd also add to the overall health risks for people and the environment [12], [13]. These patterns of contamination show that the Dhaleshwari floodplain is a key place where metals move through the food web, harming fish, cattle, and eventually people living in the area [14], [17].

Chromium, used as trivalent Cr(III) in tanning, oxidizes to highly mobile, bioavailable, and carcinogenic hexavalent Cr(VI). Lead and cadmium from dyes, pigments, and batteries persist indefinitely, cycling between water, sediment, and biota [17], [24], [25], [26]. In river-soil systems, redox, pH, and organic matter regulate speciation and mobility; however, hazardous forms of Cr, Pb, and Cd are remobilized for plant and benthic uptake. Legacy sediment and soil contamination continue to endanger ecosystems and human health. Cr, Pb, and Cd interfere with metal-enzymes, membranes, DNA/proteins, and produce excess ROS, resulting in oxidative stress, lipid peroxidation, and DNA damage. Long-term exposure causes genotoxicity, cancer, and problems with organs. In aquatic and soil biota, it lowers biodiversity and changes how communities work [27], [28], [29], [30].

1.3.2 Influence of pH on Heavy Metal Mobility and Bioavailability

The pH condition of aquatic and soil environments plays an important role in controlling the solubility, chemical form, adsorption and desorption behavior, and overall bioavailability of heavy metals. Under acidic conditions, the solubility of metals such as Cr, Pb, and Cd increases significantly, which in turn enhances their mobility from sediments into overlying water and also increases their uptake by microorganisms, plants, and aquatic organisms [31], [32]. When the pH drops below 5.5, protons start to compete for binding sites on mineral and soil particle surfaces.

In the case of chromium, low pH conditions help stabilize the highly toxic and mobile Cr(VI) form, whereas at near-neutral pH, microbial as well as abiotic reduction processes convert Cr(VI)

into the less soluble Cr(III) form, highlighting the strong influence of pH on chromium redox behavior. Therefore, regular monitoring of pH in tannery-affected water bodies is important for better assessment of metal toxicity, microbial survival, and the potential efficiency of bioremediation processes, such as Cr(VI) reduction by acid-tolerant microbial consortia [41], [42].

1.3.3 Heavy Metal-Resistant Bacteria in Polluted Environments

Microorganisms that live in environments contaminated with heavy metals often develop different types of resistance mechanisms that allow them to survive under high and continuous metal stress. Heavy metal resistant bacteria have been frequently isolated from industrial effluents, river sediments, and polluted soils, particularly from areas influenced by tannery and dye industries [43], [44]. Several bacterial genera such as *Escherichia*, *Klebsiella*, *Pseudomonas*, *Acinetobacter*, and *Enterobacter* are commonly reported for their resistance to heavy metals, which indicates a widespread adaptive response of bacteria under long-term contamination pressure [44] ,[45].

The resistance of bacteria to heavy metals is controlled by a number of known molecular and physiological mechanisms. These mechanisms include active efflux systems that remove toxic metal ions from inside the cell, enzymatic reduction of highly toxic metal forms into less harmful forms, for example the conversion of Cr(VI) to Cr(III), and also intracellular binding of metals by specific proteins such as metallothioneins. In addition, metals may be adsorbed onto the bacterial cell wall or trapped within biofilm matrices, which helps to reduce their accumulation inside the cytoplasm [46], [47], [48], [49]. The spread of heavy metal resistance traits is further supported by horizontal gene transfer through plasmids and transposons, allowing resistance genes to move quickly among different bacterial populations and increase survival in polluted environments [50], [51].

In chromium contaminated ecosystems, the enzymatic reduction of carcinogenic hexavalent chromium to the less toxic trivalent form is considered a key detoxification process in bacteria. This mechanism not only decreases the solubility and bioavailability of chromium but also explains why chromium resistant bacteria are regarded as potential candidates for bioremediation applications. Many studies near tannery and dye effluent discharge zones have reported bacterial populations that can tolerate millimolar levels of chromium salts along with other heavy metals,

showing strong natural selection for resistant bacterial types in these industrially polluted areas [54], [55].

Moreover, bacterial resistance to heavy metals is often linked with general stress response systems, which helps bacteria to maintain ecological fitness under multiple environmental stresses. The long-term persistence and growth of these bacteria in contaminated sites therefore provide promising biological resources for the sustainable remediation of heavy metal polluted water and soil environments [56], [57], [58].

1.3.4 Heavy Metal Tolerance and Minimum Inhibitory Concentration (MIC) in Bacteria

Metal tolerance assays are commonly used to measure bacterial survival under increasing concentrations of toxic metal salts, usually by broth dilution or agar diffusion techniques. These assays provide important phenotypic information about the resistance capacity of bacteria isolated from heavy-metal-contaminated environments, such as tannery effluents and polluted river sediments. The Minimum Inhibitory Concentration (MIC) refers to the lowest concentration of a metal that is able to completely stop visible bacterial growth after standard incubation conditions, generally 24–48 hours at 37°C. MIC values are widely used as a reproducible and comparative indicator to evaluate resistance among different bacterial isolates, strains, and experimental setups [61], [62].

Potassium dichromate ($K_2Cr_2O_7$) is the most common chemical used to test for chromium toxicity because it is a stable and highly soluble source of hexavalent chromium. This compound is a good example of chromium contamination that comes from chrome-tanning in the leather processing industry. Chromate reductase enzymes quickly break down Cr(VI) inside bacterial cells. This process also leads to the formation of reactive oxygen species (ROS). Cr(VI) usually enters bacterial cells through sulfate and phosphate transport systems, such as ChrB and PitA. The production of ROS creates oxidative stress inside the cell. Because of this stress, proteins, membrane lipids, and DNA become damaged. This damage includes protein carbonylation, lipid peroxidation, and DNA–protein cross-linking. In the final stage, the cell membrane breaks down, and the bacterial cell dies [67], [68], [69].

Studies on bacterial isolates taken from tannery effluents have shown MIC values mostly in the range of 0.5 to 3.0 mM Cr(VI). This is roughly equal to about 26–156 mg/L. These values are commonly reported for *Escherichia coli* and *Klebsiella* species. In comparison, more tolerant bacteria such as *Bacillus subtilis* and *Lysinibacillus* spp. are able to survive at higher potassium dichromate concentrations. These values are usually between 500 and 700 ppm, which corresponds to about 2.7–3.8 mM. These may include chromosomal or plasmid-mediated efflux systems, such as *czc* and *chr* genes. Enzymatic reduction pathways, for example *chrR* and *yieF*, are also involved. In addition, metals can be trapped inside biofilm matrices or managed through changes in cellular metabolism. They are also influenced by horizontal gene transfer and long-term exposure to mixed heavy metals in industrially polluted environments [67], [73].

1.3.5 Microbial Adaptation to Heavy Metal Stress Through Successive Passage

Microbial adaptation to heavy metal stress through successive passage is a well-known process and shows how microorganisms are able to adjust themselves under continuous environmental pressure. Successive passage, which is also called serial subculturing, generally involves repeated growth of microbes in media containing gradually increasing concentrations of heavy metals, and this method is commonly used in laboratory studies to develop metal-tolerant bacterial populations. When bacteria remain exposed to sublethal concentrations of heavy metals for a long time, they slowly undergo different genetic and physiological changes that help them to survive under stressful and unfavorable conditions. These changes can include point mutations, gene amplification, changes in regulatory control systems, and alterations in membrane transport mechanisms that regulate the entry and removal of metal ions. With the progression of several generations, natural selection favors more tolerant bacterial types, and as a result these strains become dominant in the population and show increased resistance to the applied metal stress. [73], [79].

When bacteria are exposed to chromium, chromium-adapted strains regularly display higher minimum inhibitory concentration (MIC) values than their original or native strains. Along with these responses, adaptive modifications may also include greater biofilm development, which protects against metal toxicity, and changes in metabolism that help bacteria use other energy sources or get rid of metals more quickly. These laboratory-adapted strains are useful for figuring out how resistance works and for designing bioremediation methods that work in the real world.

Studying these adaptive mechanisms helps us understand how bacteria deal with pollution from factories and gives us ideas for how to make microbial-based solutions for cleaning up the environment and managing waste in a way that is good for the ecosystem [46], [76].

Chapter 2

2. Methods and Materials

2.1 Sample Collection

Heavy metal-polluted water and soil samples were collected for this study from Savar, Dhaka, where tannery and dye industries are located, which use heavy metals. Both the tannery and dye industries use chromium, lead, and cadmium for the processing of dyes and leather. GPS

coordinates of the sample collection locations are depicted in **Table 2.1** and **Figure 2.1**. A total of 13 water (7 samples) and soil (6 samples) samples were collected from the drain of the tannery effluent, tannery waste dumping station, Dhaleshwari River, and its nearby agricultural field. Water samples were collected into the sterile Falcon tube, and soil samples were collected into the sterile zip-lock bag and transported to the laboratory within 24 hours, maintaining 4°C temperature in the sample transportation box.

Table 2.1. Soil and water sample collection points, along with GPS location.

Sample point name	Longitude	Latitude	Sample type
Apex leather	23°47’12.6’’ N	90°14’41.4’’ E	Soil and water
Tannery drain	23°47’08.5’’ N	90°14’43.2’’ E	Soil and water
Tannery waste dumping station	23°47’12.5’’ N	90°14’47.2’’ E	Soil and water
Dhaleshwari river	23°48’04.2’’ N	90°14’38.6’’ E	Soil and water
Agricultural field near Dhaleshwari River	23°48’04.2’’ N	90°14’38.6’’ E	Soil
Dye industry drain	23°47’32.5’’ N	90°15’21.1’’ E	Water
Pb battery water disposal point	23°43’39.6’’ N	90°21’58.2’’ E	Soil and water

Figure 2.1. Different sample collection points from where soil and water samples were collected.

2.2 pH Measurement of the Water Samples

Measuring the pH of water is essential for ensuring its safety for drinking, protecting aquatic ecosystems, and controlling industrial and agricultural processes. pH levels indicate acidity or alkalinity, which impacts chemical reactions, the effectiveness of disinfectants, and the solubility of toxic metals [104]. The P^H of all the water samples was measured using a pH meter (sensION+ MM340, HACH COMPANY, Loveland, Colorado, U.S.A) to understand the degree of heavy metal pollution and to get an estimation of the availability of aquatic life (**Figure 2.2**).

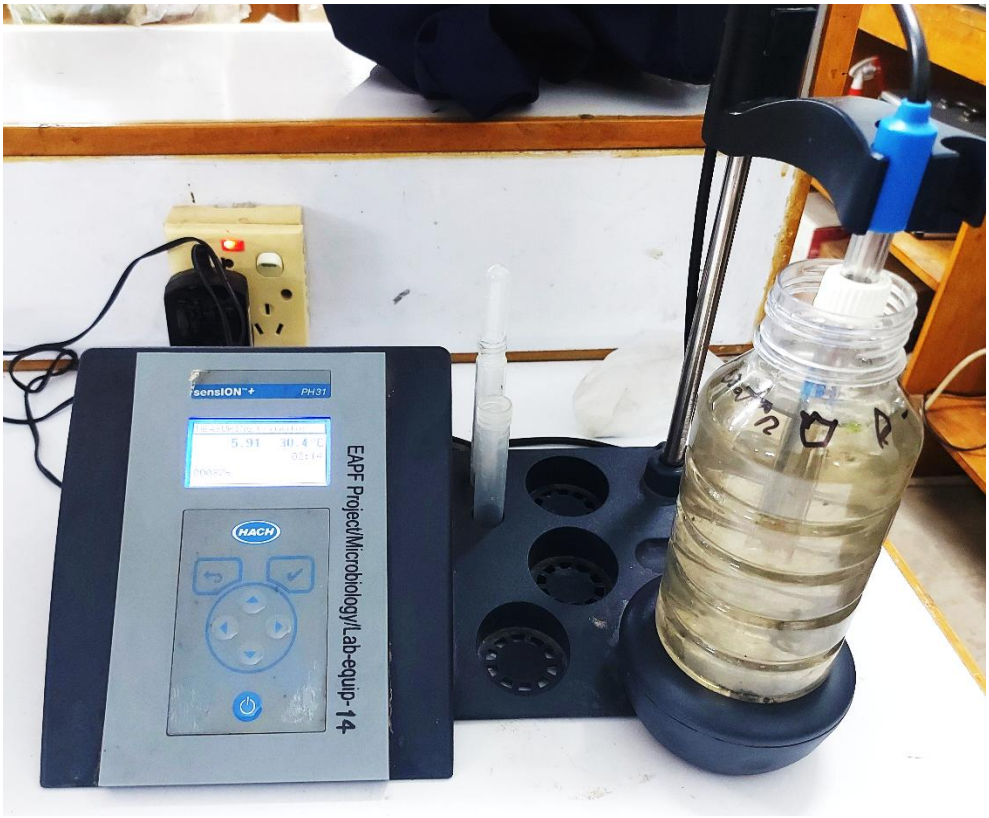

Figure 2.2. PH measurement of the water samples.

2.3 Processing Samples for Further Test

Heavy metal-exposed water and soil samples were processed (serial dilution) for further experiments. For this purpose, from each soil and water sample, 1 gram of soil sample and 1 ml of water sample were added to separate 9 mL of Tryptic Soy Broth (TSB) (HIMEDIA, India) and vortexed for better homogenization, dispersion, and mixture. Then 10-fold serial dilution was conducted for each sample, maintaining aseptic conditions as follows, as **Figure 2.3**.

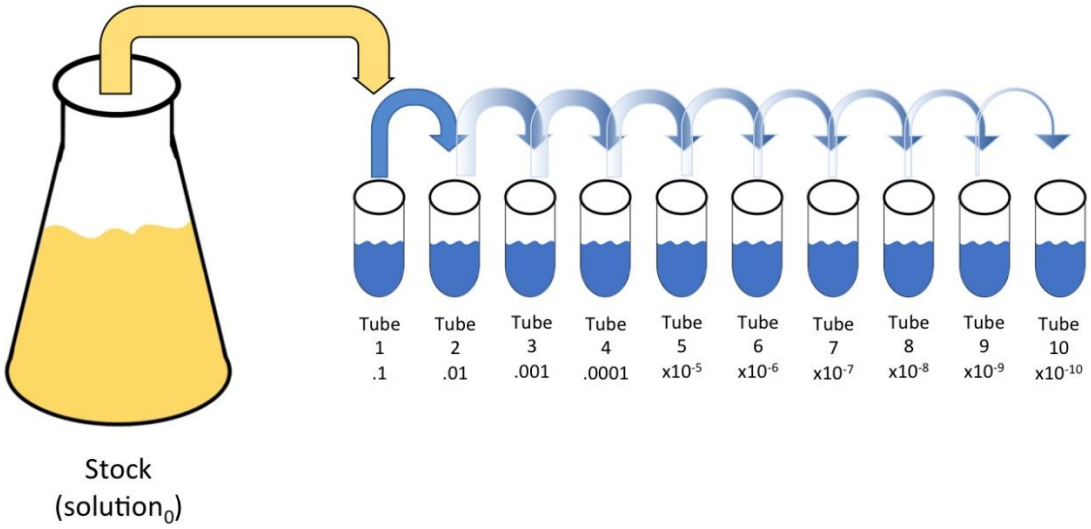

Figure 2.3. 10-Fold serial dilution of the samples to make them suitable for further experiments [105].

2.4 Isolation of Bacteria from Water and Soil Samples

Bacterial isolation is used to separate each type of bacterium from a mixed population in a sample. 10 µL of the sample from each dilution factor for each sample was inoculated into the MacConkey agar (HIMEDIA, India), and the spread plate technique was used to inoculate the samples. Then the inoculated MacConkey Agar plates were incubated for 24 hours at 37°C in an incubator to allow the bacteria contained within the samples to grow. Different types of bacterial colonies were observed and subjected to subculture for a pure culture. Then each isolated bacterium was stocked in a stock medium prepared with 300 mL glycerin and 700 mL TSB for further study.

2.5 Identification of Isolated Bacteria Using Biochemical Test

Biochemical reactions play an important role in identifying bacterial isolates and species [106]. These tests are based on the presence of bacteria-produced enzymes such as catalase, oxidase, urease, gelatinase, and so on. All the isolated bacteria were subjected to biochemical tests to identify their species based on their produced enzyme which show different properties for each bacterium. Triple Sugar Iron (TSI) medium is used to test for bacterial fermentation of three sugars: lactose, sucrose, and glucose; Motility, Indole, and Urea (MIU), which test motility, indole production, and urease activity; citrate; and oxidase test were applied to identify the isolated bacterium from the soil and water samples (**Figure 2.4**). These tests' results, which are frequently

shown by color changes in the media or on filter paper, are then matched to a database or "identification matrix" named Bergey's manual, 9th Edition [107] to verify that the isolates belong to a certain genus and species.

Figure 2.4. Different types of biochemical tests, such as Triple Sugar Iron (TSI), Motility, Indole, and Urea (MIU), citrate, and oxidase, were conducted to identify isolates.

2.6 PCR-Based Identification Confirmation of the Isolates

The most widely used molecular-based method for pathogen detection and quantification is the Polymerase chain reaction (PCR) [108]. This study used seven sets of primers (**Table 2.2**) to identify seven types of bacterial species more accurately over the biochemical test. The primer sets are selected based on our identified bacterial species through biochemical tests. All the isolates identified by biochemical tests were cultivated overnight in Tryptic Soy Broth (TSB) at 37 **°C** for 24 h. Subsequently, bacterial DNA was extracted from these cultures using the boiling DNA method. The PCR reactions were conducted in the following manner: The final volume of each PCR reaction was 25 µL, comprising 12.5 µL of Promega 2X PCR mastermix, 0.6 µL of each forward and reverse primer (10 picoM), 2 µL of the extracted DNA template, and 9.3 µL of nuclease-free water. The nucleotide sequences of the primers are shown in **Table 2.2**.

The PCR technique involved 35 amplification cycles: denaturation at 94 °C for 30 seconds, annealing for 30 seconds, and extension at 72 °C for 1 minute. This was done with a Bio-Rad T100

Thermal Cycler. The PCR products were electrophoresed on a 1.5% agarose gel in 1X Tris-acetate EDTA (TAE) buffer and stained with Ethidium bromide (EtBr). A 1.5% agarose gel was prepared by mixing 0.75g of agarose powder in 50 mL of 1X Tris-acetate EDTA (TAE) buffer, and Ethidium bromide (EtBr) was used as the intercalating agent that inserts itself between the base pairs of double-stranded DNA, making it visible under UV light. AddBio (Korea)100-bp plus DNA ladder was used as a molecular marker, and the Bio-Rad GelDoc EZ Gel imaging device visualised DNA bands under UV light.

Table 2.2. The primer list used for the identification of the isolates, along with the sequence and product length.

Organism Name	Primer Name	Sequence	Product size (bp)	Annealing Temperature	References
Aeromonas spp	*gyrB F*	5′-TCCGGCGGTCTGCACGGCGT-3′	1100	56°C	[109]
	gyrB R	5′-TTGTCCGGGTTGTACTCGTC-3′			
E. coli	*401 F*	5′-TGATTGGCAAAATCTGGCCG-3′	211	56°C	[110], [111]
	611 R	5′-GAAATCGCCCAAATCGCCAT-3′			
Acinetobacter	*Ac696F*	5′-TAYCGYAAAGAYTTGAAAGAAG-3′	397	56°C	[112]
	Ac1093R	5′-CMACACCYTTGTTMCCRTGA-3′			
Pseudomonas spp	*PA-GS-F*	5′-GACGGGTGAGTAATGCCTA-3′	618	57°C	[113]
	PA-GS-R	5′-CACTGGTGTTCCTTCCTATA-3′			
Citrobacter	*ureaseF*	5’-TGAAGCTGAACTACCCGGAATC-3’	454	56°C	[114]
	ureaseR	5’-TGTCCAGGCTCAAAACGTAC-3’			
Klebsiella spp	*wl-5793*	5′-GCTGGCGGTACACGCCAGCCCG-3’	900	65°C	[115]
	wl-5794	5′-AGAAGCATCCTGCTGTGCG-3’			
Enterobacter	*Hsp40-Fw*	5′-GACCTGCGCTACAACATGGAKCT-3’	750	56°C	[116]
	Hsp40-Rv	5′-CCGCGYTCCAAAAGCTTCTTYGAT-3’			

2.7 Evaluation of Heavy Metal Tolerance Among the Isolated Strains

A metal tolerance assay is a method used to determine an organism's ability to withstand high concentrations of toxic metals. Commonly used for bacteria and fungi, these assays measure resistance by observing growth in media with added metals. Two isolates, *Klebsiella* and *E. coli*, were selected for further study based on the Polymerase chain reaction (PCR) based identification confirmation. Among all the confirmed isolates, the selected isolates originating from the Dhaleshwari river water were selected for further study because all the effluent from the drains of different industrial effluents originating from dye and tannery industries fall into the river. For the metal tolerance assay, $K_2Cr_2O_7$ salt was used as the solution. Different salt concentration is used for each isolate: 0.25 mM, 0.50 mM, 0.75 mM, 1 mM, 1.25 mM, 1.50 mM, and 1.75 mM were used for the *E. Coli*, and 0.5 mM, 1 mM, 1.5 mM, 1.75 mM, and 2.0 mM were used for *Klebsiella* (**Figure 2.5**). Tryptic Soy Broth (TSB) medium was used as a solvent to make a solution of different concentrations of $K_2Cr_2O_7$ salt. After inoculation of bacteria in different salt concentrations, the test tubes are allowed to be incubated at 24°C for 24 hours. After incubation, the growth of bacteria was observed by the inoculation of the bacteria from different salt concentrations in MacConkey agar medium using the spread plate method, and the minimum inhibitory concentration of bacteria by salt was identified.

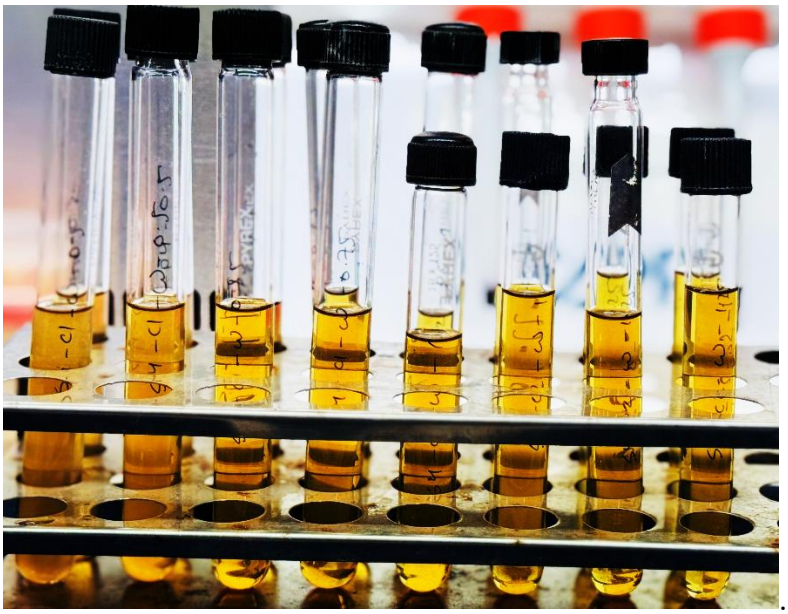

Figure 2.5. Different salt concentrations of $K_2Cr_2O_7$ prepared for MIC testing of *Klebsiella* and *E. coli*.

2.8 Adaptation of Selected Isolates to Higher $K_2Cr_2O_7$ Concentration Through Successive Passage

A successive passage experiment involves repeatedly growing a microorganism in a new, specific environment over many generations to observe its evolution [117]. This experiment can test how quickly a population can adapt to a new environmental change. Using a technique called serial subculturing, which involves sequential passage in a growth medium with progressively increasing concentrations of the heavy metal, certain microbial isolates can be adapted to greater concentrations of potassium dichromate ($K_2Cr_2O_7$). This process creates a population with increased resilience by applying constant selection pressure, which only permits the more tolerant individuals to live and procreate. The MIC for each selected bacterium was identified through Metal Tolerance assay. The isolates were adapted to a higher concentration than the MIC concentration through successive passage from a lower concentration to a higher concentration of potassium dichromate ($K_2Cr_2O_7$) solution (**Figure 2.6**). Then, isolates that adapted to the changed, harsh environment are stocked in a stock medium prepared with 300 mL glycerin and 700 mL TSB for further experiments.

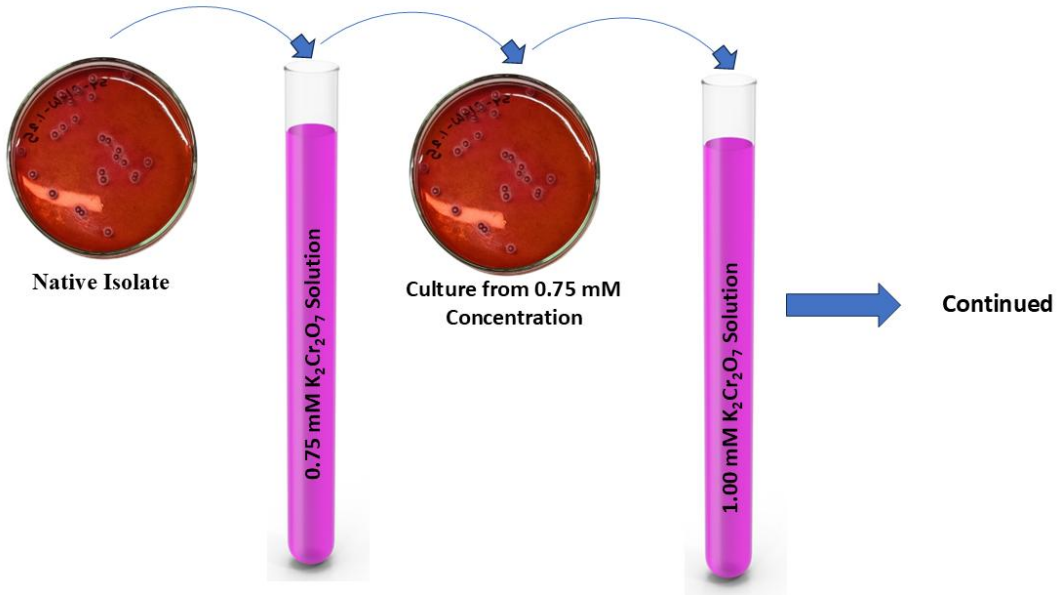

Figure 2.6. Successive passage of the isolates to make them adapted to greater concentrations of potassium dichromate ($K_2Cr_2O_7$) solution.

2.9 Assessment of Stress-Driven Morphological Changes Using Gram Staining

The morphological integrity of the isolates under stress conditions was assessed using standard Gram staining procedures. Bacterial stock from native and stress-exposed types was inoculated in MacConkey agar medium, and then the inoculated MacConkey Agar plates were incubated for 24 hours at 37°C in an incubator. Following standard Gram staining procedure, isolates were subjected to Gram staining, and stained slides were examined under a light microscope using the 100× oil immersion objective. Morphological features—including cell shape, size, and arrangement were recorded. Comparisons between native and stress-exposed cells were made to determine structural alterations induced by the metal stress.

2.10 Effect of Chromium Stress on Colony Morphology of the Isolates

Colony morphology of native and stress-adapted isolates was examined on MacConkey agar to observe any visible phenotypic change under chromium stress. The strains compared in this experiment were native and stress-adapted *Klebsiella* spp. and native and stress-adapted *Escherichia coli*. For chromium exposure, *Klebsiella* was tested at a concentration of 1.5 mM $K_2Cr_2O_7$, while *E. coli* was tested at 1.75 mM $K_2Cr_2O_7$, based on their previously determined tolerance limits.

Chapter 3

3. Results

3.1 pH Measurement of the Water Samples

The pH values of the collected water samples varied widely, reflecting significant differences in their chemical characteristics and potential pollution levels (**Figure 3.1**). The Apex leather industry effluent exhibited an acidic pH of 4.99, whereas the tannery drain water showed an even stronger acidic condition with a pH of 3.94. Extreme acidity was recorded at the lead-acid battery disposal point, where the pH was 1.01, indicating severe acid contamination. The local tannery water also displayed a highly acidic nature with a pH of 3.42, consistent with typical tannery effluent conditions. In contrast, the Dhaleshwari River sample showed a near-neutral pH of 6.10, suggesting comparatively lower levels of acidic pollutants. The dye industry drain presented a slightly acidic to neutral pH of 6.70. Among all samples, the tannery waste dumping station exhibited the highest pH value at 7.50, falling within a mildly alkaline range. Overall, the observed pH distribution highlights substantial variation among industrial discharge sites, with several locations showing pH levels far outside the permissible range for aquatic health and environmental safety.

Comparison with WHO and DoE guidelines showed that 5 out of 7 sampling sites were non-compliant with permissible pH limits (6.5–8.5). WHO states that pH does not have direct health effects but strongly influences water chemistry, metal solubility, and treatment efficiency (https://filerangpur.portal.gov.bd/files/dphe.rangpur.gov.bd/files/06fd444e_18fd_11e7_9461_286ed488c766/WQ%20Standard_Bangladesh.pdf) (**Table 3.1**). The Pb battery disposal point exhibited extreme deviation (pH 1.01), indicating severe acid pollution. Only the dye industry drain and the tannery waste dumping station met compliance standards.

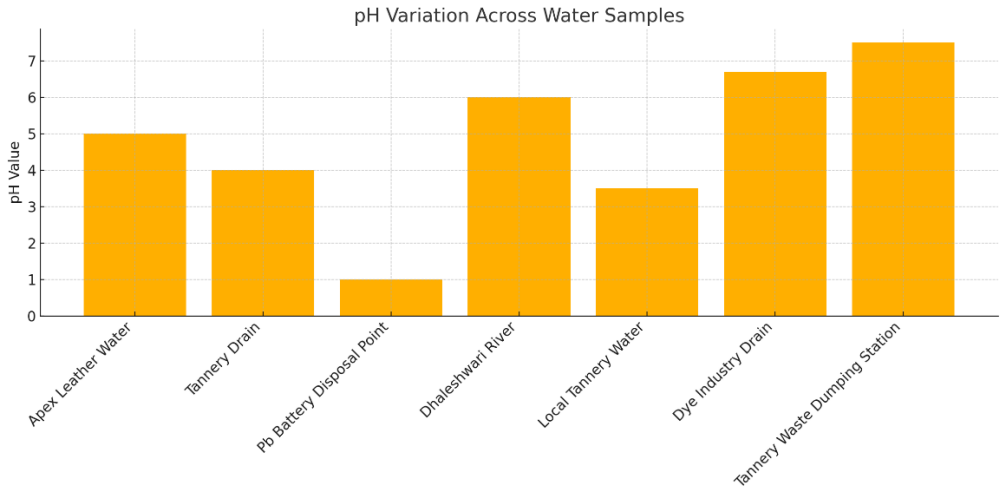

Figure 3.1. pH distribution of water samples from industrial discharge sites and the Dhaleshwari River, indicating severe acidification at the battery disposal and tannery-affected locations.

Table 3.1. Compliance assessment of measured pH values with permissible limits suggested by WHO and the Department of Environment (DoE), Bangladesh.

Sampling Site	pH	WHO/DoE Standard (6.5–8.5)	Compliance
Apex Leather Water	4.99	6.5–8.5	Non-compliant
Tannery Drain	3.94	6.5–8.5	Non-compliant
Pb Battery Disposal Point	1.01	6.5–8.5	Severely Non-compliant
Dhaleshwari River	6.1	6.5–8.5	Marginally Non-compliant
Local Tannery Water	3.42	6.5–8.5	Non-compliant
Dye Industry Drain	6.7	6.5–8.5	Compliant
Tannery Waste Dumping Station	7.5	6.5–8.5	Compliant

3.2 Isolation and Identification of Bacteria Using Biochemical Test

Followed by incubation of the culture plate, a total of 32 bacterial isolates were identified through a series of standard biochemical tests. Based on their biochemical reaction patterns, the isolates were tentatively assigned to seven genera (**Figure 3.2**). The most predominant genus was *Pseudomonas*, which accounted for 9 isolates, indicating its widespread occurrence in the sampled environments. *Klebsiella* spp. represented the second most abundant group with 6 isolates, followed by *Aeromonas* spp. and *Escherichia coli*, each contributing 5 isolates. Additionally, 4 isolates were identified as *Acinetobacter* spp., while *Enterobacter* spp. and *Citrobacter* spp. were

represented by 2 and 1 isolate, respectively. The high representation of *Pseudomonas spp*. (28.1%) suggests strong selective pressure imposed by metal-contaminated environments. Similarly, the occurrence of *Klebsiella* and *Aeromonas* further supports the existence of metal-stressed ecological niches. The comparatively lower abundance of *Citrobacter* and *Enterobacter* indicates possible sensitivity to elevated metal concentrations. The overall biochemical diversity of the isolates reflects a complex and heterogeneous microbial population in the studied water and soil samples.

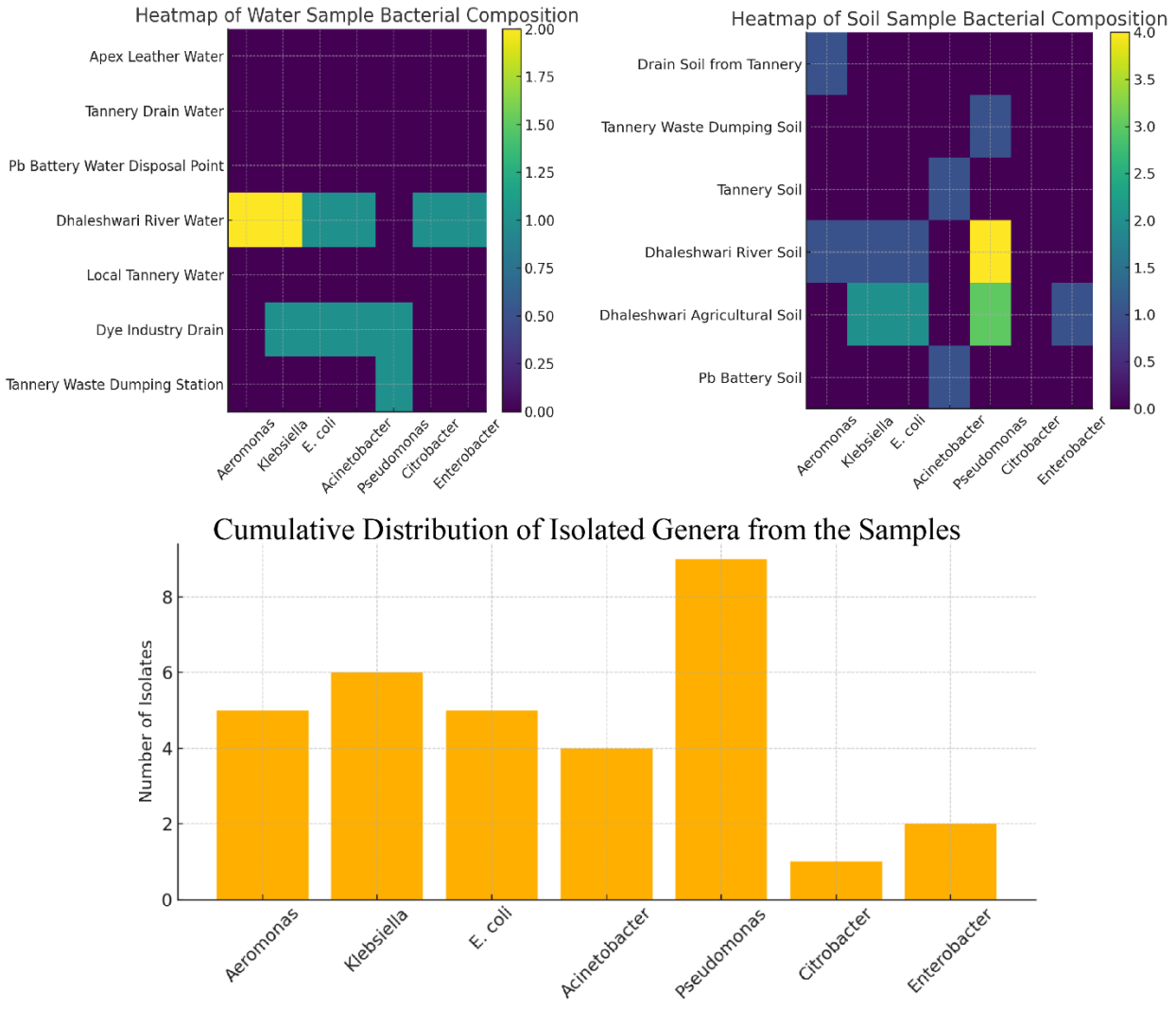

Figure 3.2. Distribution of bacterial genera isolated from industrial and river water and soil samples based on biochemical characterization.

3.3 PCR-Based Identification of the Isolates

PCR-based species identification was performed to validate the biochemical identification and to achieve more accurate confirmation of the selected bacterial isolates (**Figure 3.3** and **Table 3.2**). Among the five biochemically identified *Aeromonas* isolates (ID: S4C4, S4C8, SS1C1, S4C5, SS4C2), all five tested positive, yielding a distinct amplicon of approximately 1100 bp using the *gyrB* primer (**Figure 3.3A**). Out of the six presumptive *Klebsiella* isolates, four isolates (ID: S4C2, S6C3, SS4C3, SS5C7) were PCR-positive, producing bands of approximately 900 bp (**Figure 3.3B**). Similarly, four out of five *Escherichia coli* isolates (ID: S4C1, S6C5, SS4C1, SS5C2) showed positive amplification with a characteristic band size of approximately 211 bp (**Figure 3.3C**). For *Acinetobacter*, two isolates (ID: S6C4, SS3C1) were confirmed positive out of four, displaying PCR products of approximately 397 bp (**Figure 3.3D**). Among the nine presumptive *Pseudomonas* isolates, four isolates (ID: S6C1, SS5C3, SS5C4, SS5C6) were PCR-positive with an expected amplicon size of approximately 618 bp (**Figure 3.3E**). In contrast, no positive PCR amplification was observed for any of the *Enterobacter* isolates, indicating the absence of target gene amplification for this genus under the applied PCR conditions. Overall, the PCR results strongly corroborated the biochemical identification for *Aeromonas*, *Klebsiella*, *E. coli*, *Acinetobacter*, and *Pseudomonas*, while discrepancies observed in a few isolates highlight the importance of molecular confirmation for accurate bacterial identification.

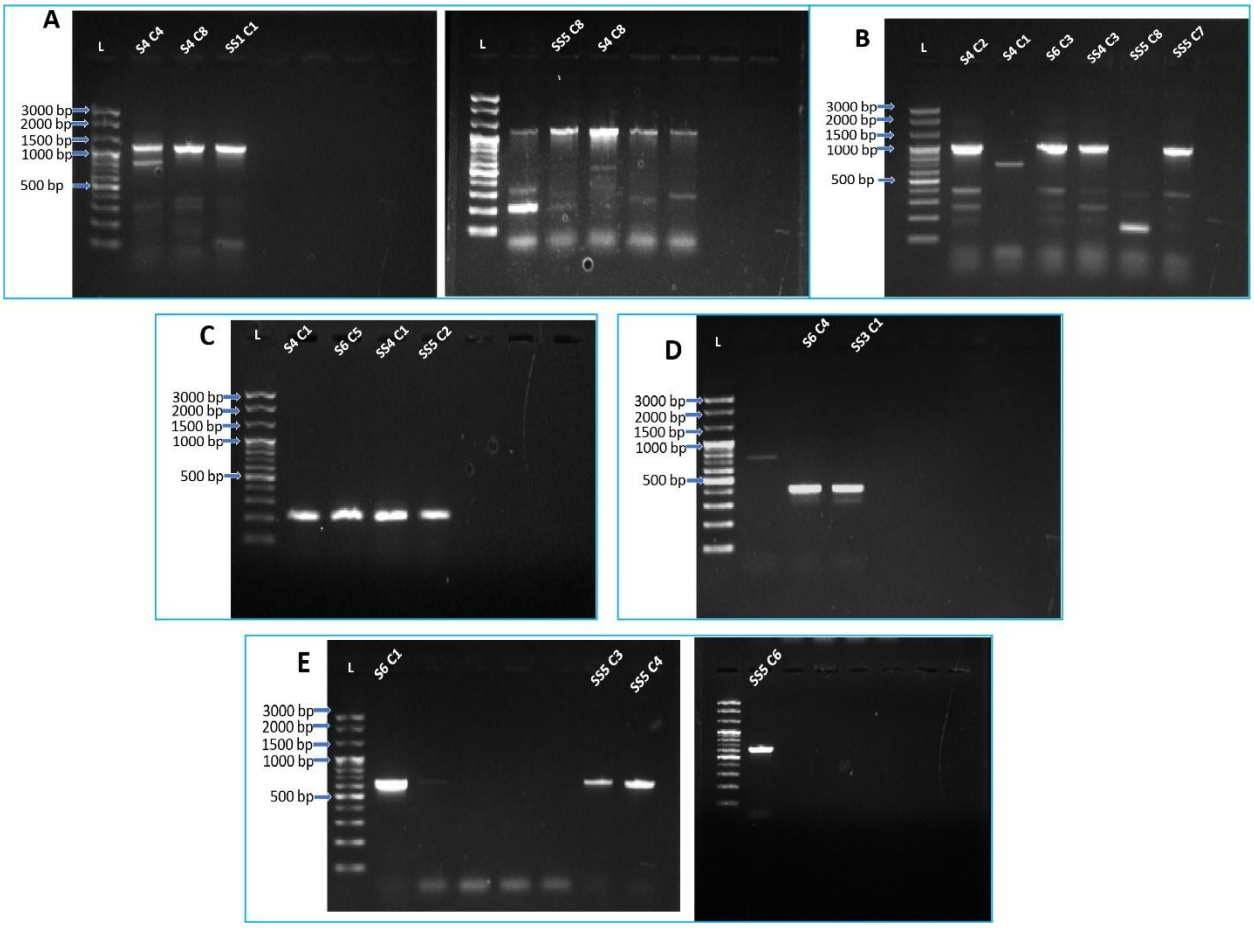

Figure 3.3. PCR amplification result of the species-specific primer to confirm the species of the isolates over the biochemical result. Gel documentation of PCR confirmed species of (A) *Aeromonas,* (B) *Klebsiella,* (C) *E. coli,* (D) *Acinetobacter, and* (E) *Pseudomonas,* respectively.

Table 3.2. PCR confirmation status of biochemically identified bacterial isolates showing the number of PCR-positive isolates, confirmation percentage, and expected amplicon sizes.

Suspected Genus	Biochemically Identified (n)	PCR-Positive (n)	PCR Confirmation (%)	Expected Band Size (bp)
Aeromonas spp.	5	5	100%	1100
Klebsiella spp.	6	4	66.70%	900
Escherichia coli	5	4	80.00%	211
Acinetobacter spp.	4	2	50.00%	397
Pseudomonas spp.	9	4	44.40%	618

Citrobacter spp.	1	0	0%	—
Enterobacter spp.	2	0	0%	—

3.4 Evaluation of Heavy Metal Tolerance Among the Isolated Strains

The chromium tolerance of the PCR-confirmed isolates *Escherichia coli* and *Klebsiella* spp. was evaluated using potassium dichromate ($K_2Cr_2O_7$) at different concentrations. Bacterial growth was assessed after 24 h of incubation by spread-plating on MacConkey agar (**Table 3.3**). For *E. coli*, visible growth was observed from 0.25 to 1.25 mM $K_2Cr_2O_7$. At lower concentrations (0.25–0.75 mM), colony numbers were too numerous to count (TNTC), indicating high growth. At 1.0 mM, growth was markedly reduced (4 colonies), while at 1.25 mM, limited survival was still observed (25 colonies). Complete growth inhibition occurred at 1.5 mM and higher, where no colonies were detected. Therefore, the MIC of $K_2Cr_2O_7$ for *E. coli* was determined to be 1.5 mM. For *Klebsiella* spp., growth was observed up to 1.0 mM. The isolate showed TNTC growth at 0.5 and 1.0 mM, indicating high tolerance at lower concentrations. No growth was detected at 1.5 mM and higher, establishing the MIC of $K_2Cr_2O_7$ for *Klebsiella spp*. as 1.5 mM. Overall, both isolates exhibited the same chromium tolerance. The gradual decline in colony numbers with increasing chromium concentration confirms a dose-dependent inhibitory effect of Cr(VI) on bacterial growth.

Table 3.3. Growth response and minimum inhibitory concentration (MIC) of *E. coli* and *Klebsiella* spp. against potassium dichromate ($K_2Cr_2O_7$) based on colony formation on MacConkey agar.

Isolate	$K_2Cr_2O_7$ Concentration (mM)	Growth (+/–)	Colony Count on MacConkey	Interpretation
E. coli	0.25	+	TNTC	Heavy growth
	0.5	+	TNTC	Heavy growth
	0.75	+	TNTC	Heavy growth
	1	+	4	Strong inhibition
	1.25	+	25	Partial inhibition
	1.5	–	0	MIC
	1.75	–	0	Complete inhibition
Klebsiella spp.	0.5	+	TNTC	Heavy growth
	1	+	TNTC	Heavy growth
	1.5	–	0	MIC
	2	–	0	Complete inhibition

3.5 Adaptation of Selected Isolates to Higher $K_2Cr_2O_7$ Concentration Through Successive Passage

The adaptive response of the selected isolates, *Escherichia coli* and *Klebsiella* spp., to increasing concentrations of potassium dichromate ($K_2Cr_2O_7$) was evaluated through successive passage under progressively elevated chromium stress. The native and stress-adapted strains were compared based on growth response and colony formation on MacConkey agar (**Figures 12 and 13**). Adaptation was achieved after 8 successive passages for *E. coli* and 9 successive passages for *Klebsiella* spp.

3.5.1 Adaptive Response of *E. coli*

The native *E. coli* showed robust growth up to 1.25 mM $K_2Cr_2O_7$, with complete inhibition observed at 1.5 mM and above (**Figure 3.4**). In contrast, the stress-adapted *E. coli* exhibited sustained growth at 1.5 mM, where the native isolate showed no growth, with TNTC colonies observed. Moreover, at 1.75 mM, the adapted strain still survived with 1 colony, whereas the native isolate showed complete inhibition. These findings indicate that the MIC of E. coli increased from 1.5 mM (native) to 1.75 mM (stress-adapted) following successive passage.

3.5.2 Adaptive Response of *Klebsiella* spp.

The native *Klebsiella* spp. showed growth at 1.0 mM, but complete inhibition occurred at 1.5 mM and above (**Figure 3.4**). In contrast, the stress-adapted *Klebsiella* spp. displayed continued growth at 1.5 mM, producing 16 colonies, whereas the native isolate showed zero growth. However, at 2.0 mM, both native and stress-adapted strains exhibited no growth. These results demonstrate that the MIC of *Klebsiella* spp. increased from 1.5 mM (native) to 2.0 mM (stress-adapted) after successive passage.

Overall, both isolates exhibited clear adaptive enhancement of chromium tolerance following repeated exposure to sublethal chromium concentrations. The ability of the stress-adapted strains to survive at concentrations that completely inhibited their native counterparts confirms the effectiveness of successive passage in selecting chromium-tolerant phenotypes.

Table 3.4. Comparative growth pattern and colony formation of native and stress-adapted *E. coli* and *Klebsiella* spp. under increasing concentrations of $K_2Cr_2O_7$ following successive passage.

Isolate	$K_2Cr_2O_7$ (mM)	Native Growth	Adapted Growth	Native Colonies	Adapted Colonies
E. coli	0.75	+	+	TNTC	TNTC
	1	+	+	4	TNTC
	1.25	+	+	25	TNTC
	1.5	–	+	0	TNTC
	1.75	–	+	0	1
Klebsiella spp.	1	+	+	TNTC	TNTC
	1.5	–	+	0	16
	2	–	–	0	0

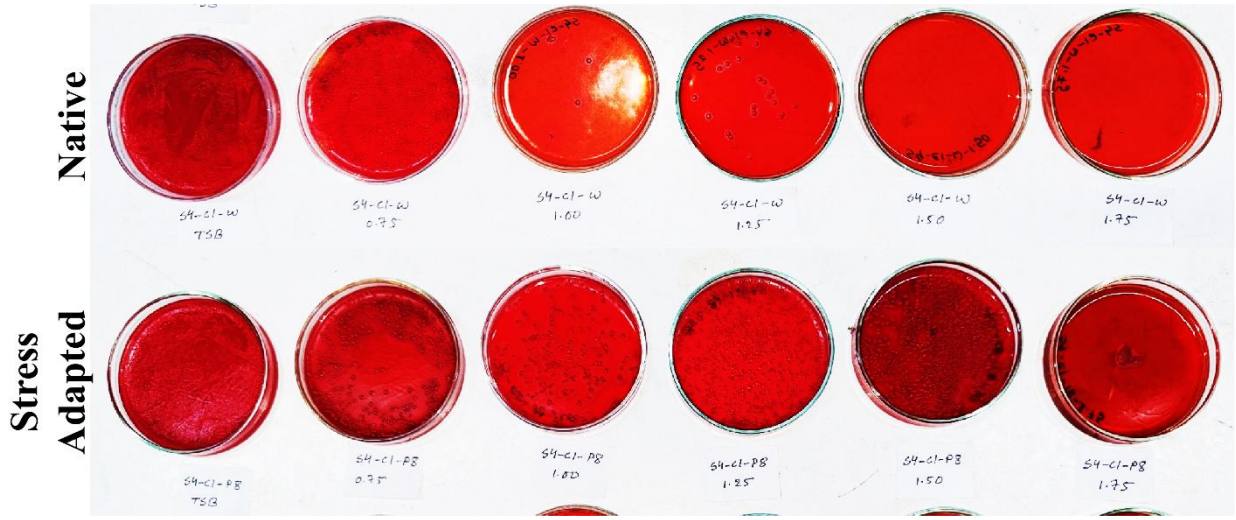

Figure 3.4. Comparative growth of native and stress-adapted *E. coli* on MacConkey agar under increasing concentrations of potassium dichromate ($K_2Cr_2O_7$).

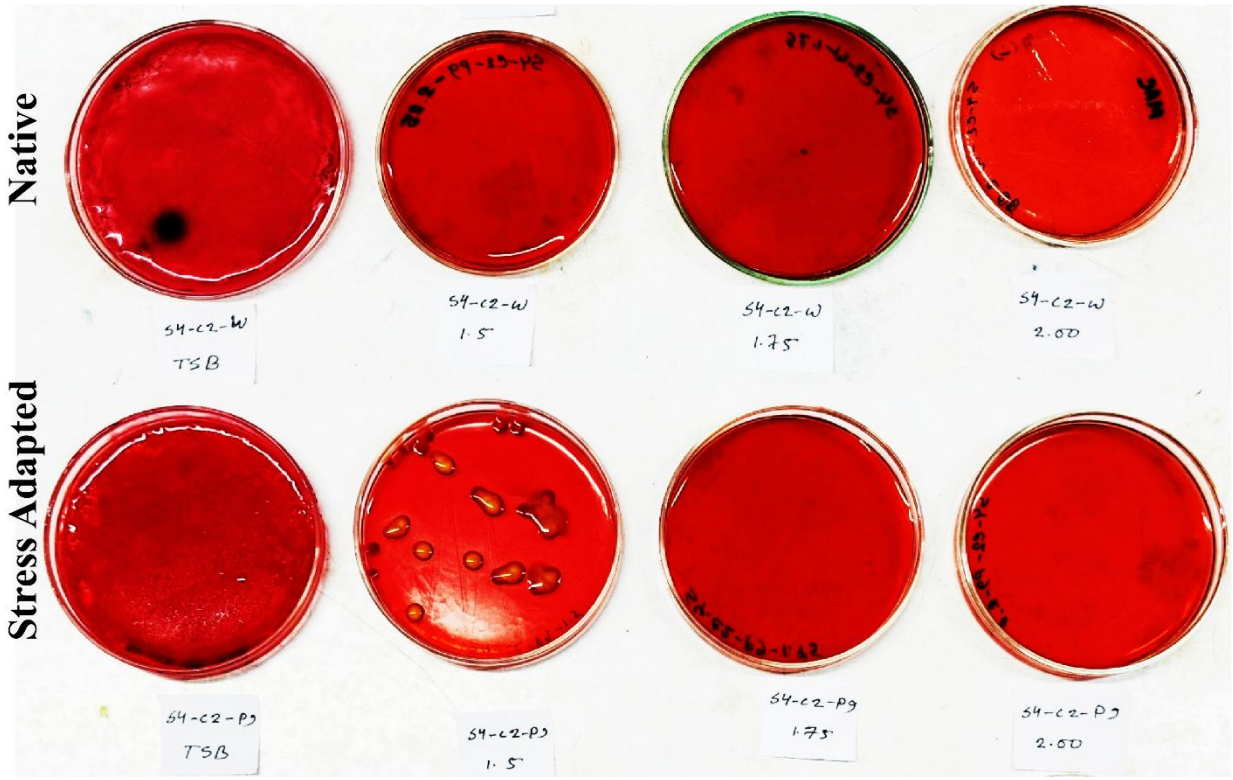

Figure 3.5. Comparative growth of native and stress-adapted *Klebsiella* spp. on MacConkey agar under increasing concentrations of potassium dichromate ($K_2Cr_2O_7$).

3.6 Assessment of Stress-Driven Morphological Changes Using Gram Staining

Stress-induced morphological changes in *E. coli* and *Klebsiella* spp. were evaluated by Gram staining under native and chromium stress–adapted conditions using bright-field microscopy at 100× oil immersion (**Figure 3.6** and **Table 3.5**).

Both native and stress-adapted cells of *E. coli* and *Klebsiella* spp. retained a Gram-negative reaction, appearing pink after counterstaining, indicating that chromium stress did not alter the fundamental cell wall type of either organism.

3.6.1 Morphological Changes in *E. coli*

Native *E. coli* cells (**Figure 3.6A**) appeared as long, uniform rods, predominantly arranged as single cells with well-defined boundaries. In contrast, the stress-adapted *E. coli* cells (**Figure 3.6B**) exhibited pronounced morphological distortion, characterized by shortened, irregular rod-shaped cells, with several cells appearing almost coccoid. Marked changes in cell arrangement were also observed, where adapted cells formed chains, scattered distributions, and aggregated clusters,

unlike the uniform single-cell pattern seen in the native. A clear reduction in cell length was evident in the stress-adapted population, indicating chromium-induced structural remodeling.

3.6.2 Morphological Changes in *Klebsiella* spp.

Native *Klebsiella* spp. (**Figure 3.6C**) displayed long rod-shaped cells, predominantly occurring as single, uniformly stained cells. Upon adaptation to chromium stress (**Figure 3.6D**), the cells became shorter rods and demonstrated a distinct shift in arrangement from single cells to chain formation. A noticeable reduction in cell length was also observed in the adapted *Klebsiella* population, although the overall rod-shaped morphology was still maintained.

Overall, the transition from native to stress-adapted forms in both isolates was associated with cell size reduction, irregular morphology, and altered cellular arrangement, which are classic indicators of metal-induced structural stress and adaptive physiological remodeling.

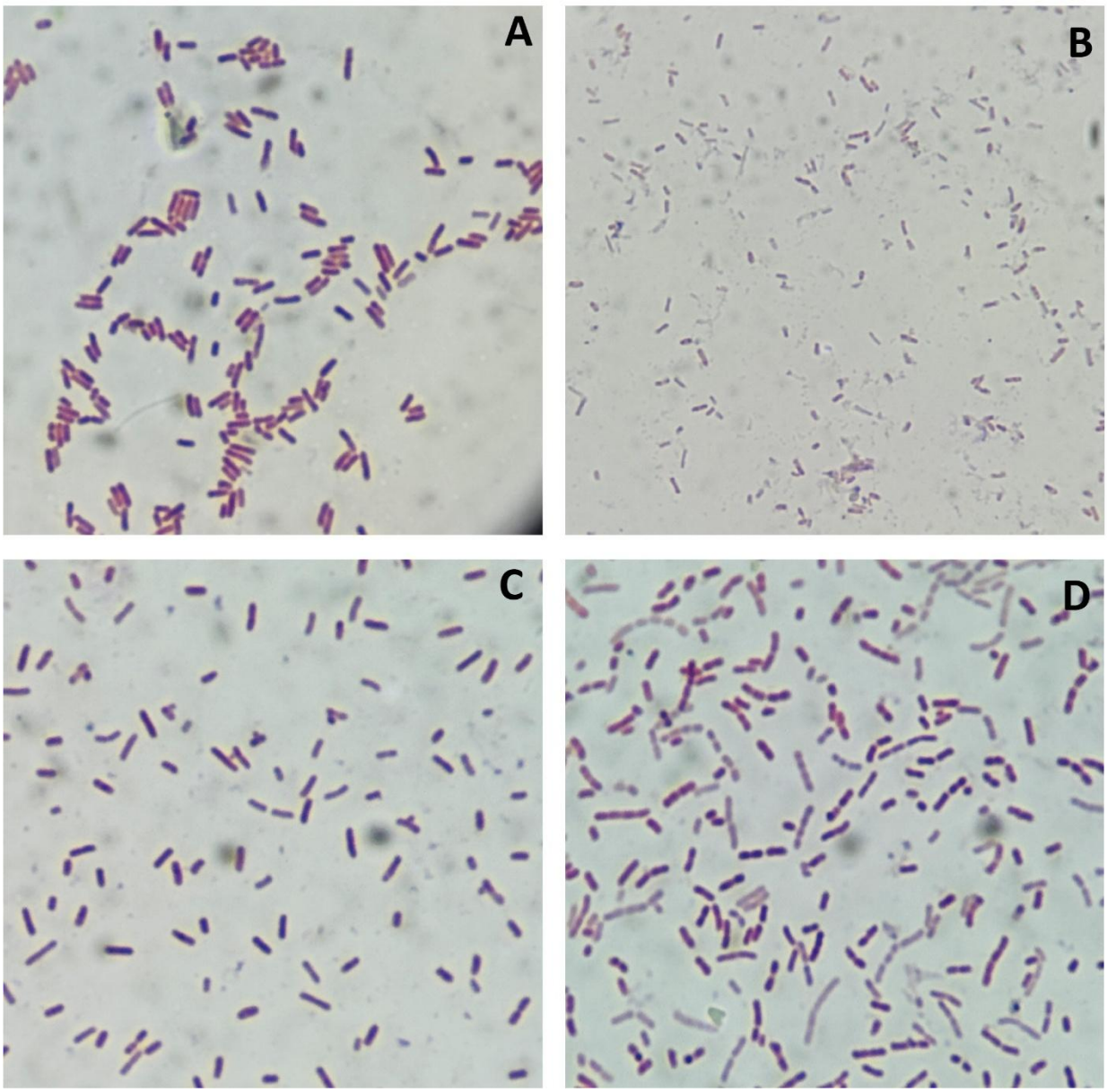

Figure 3.6. Stress-induced morphological changes in *Escherichia coli* and *Klebsiella* spp. under chromium exposure as observed by Gram staining (100× oil immersion).

(A) Native *E. coli* showing long, uniformly stained single rod-shaped cells.

(B) Chromium stress–adapted *E. coli* showing shortened, irregular rods with coccoid-like appearance, aggregation, and chain formation.

(C) Native *Klebsiella* spp. displaying long, single rod-shaped cells. **(D)** Chromium stress–adapted *Klebsiella* spp. showing shortened rod-shaped cells with prominent chain formation. All cells remained Gram-negative under both conditions.

Table 3.5. Comparison of Gram reaction, cell shape, arrangement, and size of native and chromium stress–adapted *E. coli* and *Klebsiella* spp. observed under oil immersion microscopy.

Isolate	Condition	Gram Reaction	Cell Shape	Arrangement	Cell Size
E. coli	Native	Gram –	Long rods	Single rods	Increased length
	Stress-adapted	Gram –	Short, irregular rods (almost coccoid)	Chains, scattered, aggregated	Reduced length
Klebsiella spp.	Native	Gram –	Long rods	Single rods	Increased length
	Stress-adapted	Gram –	Short rods	Chains	Reduced length

3.7 Effect of Chromium Stress on Colony Morphology of the Isolates

Clear differences in colony morphology were observed in *Klebsiella* spp. following chromium adaptation, while *E. coli* did not show any noticeable change **Figure 3.7** and **Table 3.6**.

On MacConkey agar, the native *Klebsiella* (**Figure 3.7A**) produced large, smooth, mucoid, comparatively dry, and deep pink colonies with regular circular margins and raised elevation, indicating strong lactose fermentation. The colonies appeared shiny and well-developed after 24 hours of incubation.

In contrast, the stress-adapted *Klebsiella* (**Figure 3.7B**), grown at 1.5 mM chromium concentration, showed a distinct change in colony appearance. The colonies were smaller in size, less mucoid, and comparatively wet type, with reduced elevation and slightly irregular margins. The pink coloration was noticeably paler than that of the native, suggesting a reduction in lactose fermentation intensity under chromium stress.

For *E. coli*, no visible change in colony morphology was observed between the native and stress-adapted strains at 1.75 mM chromium concentration. Both produced typical pink, smooth, circular colonies with similar size and surface texture on MacConkey agar. This indicates that, under the tested conditions, chromium stress did not cause any colony-level phenotypic variation in *E. coli*.

All observations were consistent across the three replicate plates, confirming that the colony morphology changes observed in *Klebsiella* were reproducible.

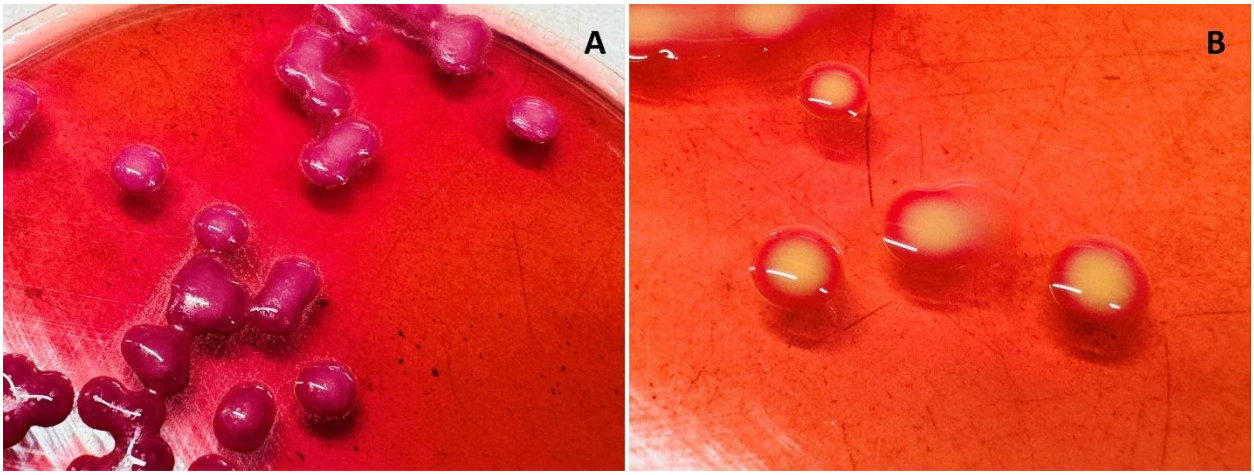

Figure 3.7. Colony morphology of native and stress-adapted *Klebsiella* spp. on MacConkey agar after 24 h incubation at 37 °C.**(A)** Native *Klebsiella* showing large, smooth, mucoid, dark pink lactose-fermenting colonies. **(B)** Stress-adapted *Klebsiella* at 1.5 mM chromium concentration showing smaller, less mucoid, paler colonies with reduced elevation.

Table 3.6. Comparison of colony characteristics of native and stress-adapted *Klebsiella* spp. and *E. coli* grown on MacConkey agar at their respective chromium exposure levels after 24 h incubation at 37 °C under aerobic conditions. Observations were consistent across triplicate plates.

Isolate	Condition	Cr Concentration (mM)	Colony Color	Size	Surface Texture	Margin	Elevation	Lactose Fermentation	Overall Change
Klebsiella spp.	Native	1.5	Deep pink	Large	Smooth, mucoid	Regular, circular	Raised/convex	Strong positive	Typical colony
Klebsiella spp.	Stress-adapted	1.5	Pale pink	Small	Less mucoid, Wet	Slightly irregular	Reduced/flat	Weak positive	Clear change observed

E. coli	Native	1.75	Pink	Medium	Smooth	Regular, circular	Convex	Positive	Typical colony
E. coli	Stress-adapted	1.75	Pink	Medium	Smooth	Regular, circular	Convex	Positive	No visible change

Chapter 4

4.1 Discussion

The results of this study give a general picture of the environmental condition in the chromium-affected industrial area of Savar and also show how the local bacterial communities respond to these conditions. Data collected from different sampling sites clearly indicate chemical stress in the environment. This stress was more obvious at sites related to tannery and battery activities, where the water showed very low pH values. Such acidic conditions are known to increase the mobility and biological effects of chromium. This helps to explain the strong selective pressure acting on microorganisms in these areas. The bacterial isolates obtained from the samples reflect this continuous pressure. Only a small number of bacterial genera were able to survive under these harsh environmental conditions. These findings help to provide background for understanding the physiological changes and adaptive responses discussed in the later parts of the study.

The fact that the pH levels are so different at the several sampling sites shows that industrial waste has caused a lot of chemical damage. The Pb battery disposal point (pH 1.01) and tannery-related sites (pH 3.42–3.94) have very acidic pH values, which means that a lot of acid is being added to the environment. Low pH levels make heavy metals, especially hexavalent chromium, much more soluble and mobile. This makes them last longer in the environment and more harmful to living things [118]. Similar levels of acidity have previously been reported from the Savar–Hazaribagh tannery belt and were strongly associated with elevated chromium levels in surrounding water and sediments [119]. Although the Dhaleshwari River showed near-neutral pH, this likely represents dilution rather than detoxification, as river systems in industrial zones often continue to transport dissolved heavy metals over long distances [120]. Such chemically unstable environments create strong selective pressure on resident microbial communities.

The diversity of bacteria isolated from these contaminated sites—dominated by *Pseudomonas*, *Klebsiella*, *Escherichia coli*, *Aeromonas*, and *Acinetobacter*—is consistent with reports from other chromium-polluted ecosystems. These genera are well known for their ability to tolerate toxic metals through multiple resistance mechanisms, including efflux pumps, enzymatic reduction, metal sequestration, and biofilm formation [121]. Comparable microbial assemblages have been described from tannery- and textile-impacted environments in Bangladesh and neighboring regions [122]. The dominance of *Pseudomonas* and *Klebsiella* in the present study suggests that

prolonged industrial discharge has strongly favored organisms with higher adaptive capacity. In contrast, the relatively low recovery of *Citrobacter* and *Enterobacter* supports earlier findings that chromium resistance is highly genus- and strain-specific and not uniformly distributed among enteric bacteria [123].

PCR-based molecular confirmation significantly strengthened the reliability of bacterial identification obtained through biochemical testing. Although biochemical tests remain useful for preliminary screening, heavy metal stress is known to interfere with normal enzyme expression and metabolic activity, leading to atypical biochemical behavior and occasional misidentification [121]. The complete molecular confirmation of all *Aeromonas* isolates and the high confirmation rate of *E. coli* indicate that their diagnostic genetic markers remained stable under environmental stress. Partial confirmation for *Klebsiella*, *Acinetobacter*, and *Pseudomonas* has also been reported in earlier studies from industrial effluent-contaminated sites in Bangladesh [124], emphasizing the necessity of molecular tools for accurate identification of environmental isolates.

Chromium tolerance assays revealed that both *E. coli* and *Klebsiella* spp. exhibited substantial intrinsic resistance, with native MIC values of 1.5 mM $K_2Cr_2O_7$. These MIC values are comparable to those reported for chromium-resistant bacteria isolated from tannery wastewater in India and Bangladesh [54]. The gradual decline in colony number with increasing chromium concentration clearly demonstrates a concentration-dependent toxic effect of Cr(VI). Hexavalent chromium is known to enter bacterial cells via sulfate transport systems and generate reactive oxygen species, which damage DNA, proteins, and cellular membranes [125]. The similar tolerance levels of both organisms suggest that they may share conserved detoxification strategies, including chromate efflux systems, enzymatic reduction of Cr(VI) to the less toxic Cr(III) form, and intracellular metal chelation [123].

One major outcome of this study is the increase in chromium tolerance after repeated exposure to higher metal concentrations. With successive adaptation, the MIC value of *E. coli* increased from 1.5 to 1.75 mM. In the case of *Klebsiella* spp., the MIC increased from 1.5 to 2.0 mM. This gradual change in MIC clearly shows that the bacteria were adapting under chromium stress. The results provide experimental evidence that adaptive evolution occurred during continuous exposure to chromium. Such adaptive responses observed through serial subculturing can be explained by adaptive laboratory evolution, where long-term sublethal stress favors the selection of more

tolerant bacterial types over successive generations [126]. Similar increases in chromium tolerance after repeated exposure have been reported for several bacterial species, including *E. coli* and *Pseudomonas* [127]. These adaptive changes are often associated with enhanced efflux pump activity, upregulation of chromate reductase enzymes, improved oxidative stress defenses, and alterations in membrane permeability [128].

The morphological alterations seen by Gram staining that were caused by stress further confirm the isolates' ability to respond to chromium exposure. Both native and adapted strains of E. coli and Klebsiella spp. retained their Gram-negative characteristics, demonstrating that chromium stress did not modify the essential structure of the outer membrane. However, adapted cells of both organisms showed marked reductions in cell size and changes in cell arrangement. In adapted *E. coli*, the transformation from long rods to short, irregular, almost coccoid forms with aggregation and chain formation suggests severe disturbance of normal cell division and cytoskeletal organization. Similar morphological changes under heavy-metal and oxidative stress have been documented and are believed to represent a survival strategy that reduces the surface-to-volume ratio and limits metal influx [129]. In *Klebsiella* spp., the persistence of rod shape with reduced cell length and chain formation suggests partial disruption of septation processes, possibly involving interference with FtsZ-mediated cytokinesis. Comparable stress-induced chain formation has been reported in other members of the *Enterobacteriaceae* under heavy-metal exposure [45].

In addition to microscopic changes, a distinct alteration in colony morphology was observed in stress-adapted *Klebsiella* spp., whereas *E. coli* showed no visible colony-level change. The transition from large, smooth, mucoid colonies in native *Klebsiella* to smaller, less mucoid, paler colonies in the adapted strain suggests chromium-induced modification of surface polysaccharides and capsule production, or production of any enzyme, which needs further confirmation. Capsule and extracellular polymeric substances are known to play important roles in heavy-metal binding and protection, and changes in their expression under metal stress have been documented in several Gram-negative bacteria [130]. The absence of visible colony alteration in *E. coli* suggests that the adaptive mechanisms in this organism may be more intracellular and metabolic rather than surface-structural, highlighting species-specific strategies of chromium tolerance.

When the tolerance data, adaptive MIC shifts, morphological remodeling, and colony variation are considered together, a clear and consistent pattern of chromium-driven adaptation emerges. The adapted strains not only survived higher chromium concentrations but also exhibited pronounced structural and phenotypic plasticity. This coordinated physiological and morphological response strongly suggests underlying genetic and proteomic reprogramming. Such findings strongly justify the future application of whole-genome sequencing and proteomic analysis, as proposed in the objectives of this study, to identify the exact resistance determinants, regulatory mutations, and stress-response pathways involved in chromium adaptation.

From an applied perspective, the chromium-tolerant and chromium-adapted *E. coli* and *Klebsiella* spp. isolated in this study show promising potential for use in bioremediation of tannery- and battery-impacted environments in Bangladesh. Their ability to withstand high chromium concentrations and to adapt rapidly under selection pressure suggests that they could be exploited in biological treatment systems, such as bioaugmentation in wastewater treatment plants or constructed wetland systems. However, both organisms are also recognized as opportunistic pathogens. Therefore, any environmental application must be preceded by detailed biosafety evaluation, including assessment of virulence factors, antibiotic resistance, and horizontal gene transfer potential, as heavy-metal exposure is known to co-select for antibiotic resistance [45], [131].

Although the present study provides strong phenotypic evidence of chromium tolerance and adaptive evolution, the underlying molecular mechanisms were not directly investigated. Genomic and proteomic approaches will be necessary to determine whether adaptation involved mutations in chromate transporters, oxidative stress regulators, DNA repair systems, or energy metabolism pathways. Nevertheless, this work presents important baseline data on chromium-resistant bacteria from an industrial region of Bangladesh and demonstrates how severe environmental stress can shape microbial survival, structure, and function.

In conclusion, long-term industrial pollution in the Savar region has created strong selective pressure on local microbial populations. Because of this pressure, chromium-tolerant and chromium-adapted bacteria have gradually emerged in this area. The combined analysis of environmental pH, microbial diversity, chromium tolerance levels, adaptive evolution, and morphological as well as colony-level changes gives an overall picture of how microorganisms

respond to chromium stress. These findings provide useful information on microbial metal resistance in Bangladesh. They also support the future development of environmentally sustainable bioremediation strategies for chromium-contaminated sites.

4.2 Limitations and Future Research Directions

Although this study was able to show chromium tolerance, adaptive evolution, and stress-induced changes at both morphological and colony levels in *E. coli* and *Klebsiella* spp., the detailed molecular and biochemical mechanisms behind these changes are still not clearly understood. Many observations remained unresolved in this work, mainly due to limited time and scope of the study. Because of this, deeper insight into these adaptive processes could not be achieved. In future studies, more advanced molecular and structural approaches should be included to better explain these adaptation mechanisms.

Comparative whole-genome sequencing (WGS) of native and chromium-stress-adapted isolates would be highly useful to identify specific genetic mutations associated with enhanced chromium tolerance. Genome-wide comparison could reveal alterations in chromate transporter genes, oxidative stress regulators, DNA repair systems, membrane transport proteins, and energy metabolism pathways that may have contributed to the observed increase in MIC and survival capacity. Such genomic studies are widely used to explain adaptive evolution under long-term environmental stress [126], [132].

In addition to genome-level analysis, RNA sequencing (RNA-Seq) or transcriptomic profiling could be applied to understand how global gene expression patterns change under chromium stress. By comparing transcriptional profiles of native and stress-adapted strains, it would be possible to identify differentially expressed genes involved in chromium reduction, efflux systems, antioxidant defense, and cell wall synthesis. RNA-Seq has become a powerful and widely accepted tool for studying stress-responsive gene regulation in bacteria [133]. In addition to transcriptomic analysis, proteomic analysis for the production of any enzyme. Please highlight on this issue.

The distinct change in colony morphology observed in stress-adapted *Klebsiella* spp. also indicates possible alterations in surface polysaccharides, capsule formation, and metabolic activity. Therefore, metabolomic analysis would be valuable for comparing intracellular and extracellular metabolites between native and adapted strains. Such studies may reveal stress-induced changes

in carbohydrate metabolism, lipid biosynthesis, and extracellular polymeric substance production that may explain the reduced mucoidy and altered colony texture [134], [135].

For more detailed visualization of stress-induced structural changes, advanced imaging techniques such as Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) should be employed. SEM would allow high-resolution examination of surface topology, aggregation, and extracellular matrix formation, while TEM would provide information on internal ultrastructural changes such as membrane disruption, variation in cell wall thickness, and intracellular granule formation. These electron microscopy techniques are widely used for detailed bacterial ultrastructure analysis under stress conditions [133].

In addition, proteomic analysis of native and stress-adapted isolates could help identify chromium-responsive proteins involved in detoxification, stress tolerance, redox balance, and metabolic adaptation. Comparative proteomic profiling would link gene expression to functional protein production, providing direct evidence of molecular mechanisms involved in chromium adaptation [136].

Chapter 5

5. Conclusion

This study gives a general idea about the environmental condition of some selected industrial areas of Savar. It also shows how indigenous bacteria respond to chromium stress in these areas. The pH values measured at different sampling sites were not the same and showed wide variation. At several industrial discharge points, the water was found to be extremely acidic. This strong acidity indicates a serious chemical imbalance in the environment. Such imbalance is mainly caused by long-term disposal of industrial waste. Acidic conditions like these favor the mobilization and bioavailability of toxic heavy metals. Hexavalent chromium is especially affected. As a result, both environmental risk and biological stress increase.

Chromium tolerance assays showed that both E. coli and Klebsiella spp. already had a certain level of resistance to chromium. The native MIC value was 1.5 mM $K_2Cr_2O_7$ for both bacteria. After repeated exposure to gradually increasing chromium concentrations, the level of tolerance increased further in both organisms. The increase in MIC values after adaptation clearly shows that the bacteria responded to chromium stress. This result indicates that the bacteria are not only chromium tolerant but can also adapt over time under continuous metal stress. Such adaptive behavior reflects the flexible nature of microbial survival in polluted environments.

The morphological observations induced by stress provided additional evidence regarding the influence of chromium exposure on the structure of bacterial cells. While the Gram-negative traits of both organisms persisted, significant decreases in cell size and alterations in cell arrangement were noted in the adapted strains. The observed structural changes align with physiological adjustments driven by stress and could enhance survival in toxic environments. Furthermore, alterations in colony morphology were noted in stress-adapted Klebsiella strains, while comparable changes were not observed in E. coli, indicating species-specific surface or metabolic reactions to chromium stress.

Overall, the findings of this study shows that long-term industrial pollution in the Savar area have created strong selective pressure on microbial population. Because of this pressure, chromium-tolerant and adaptively evolving bacteria is able to survive in such polluted environment. Different kind of evidences was collected during this study. This include physicochemical analysis,

microbial identification, metal tolerance assay, adaptive evolution experiment, and morphological observation. Taken together, these results help to explain how bacteria respond to chromium stress in contaminated ecosystems.

The chromium-tolerant and chromium-adapted bacterial isolates identified in this study may have potential application in bioremediation of contaminated site. However, the studied organisms are opportunistic pathogen in nature and may cause health risk. For this reason, any practical use should only be considered after detailed molecular characterization and proper biosafety evaluation is done. This study therefore provide important baseline information regarding chromium resistance in environmental bacteria from Bangladesh and also give a foundation for future molecular as well as applied research.