

**ISOLATION AND FUNCTIONAL
EXPLORATION OF POTENTIAL
CHROMIUM-UTILIZING BACTERIA FOR
BIOREMEDIATION AND
BIOMINERALIZATION APPLICATIONS:
INSIGHTS INTO HEXVALENT CHROMIUM
REDUCTION IN CEMENT INDUSTRY SOIL**

**Thesis Submitted
in Partial Fulfillment of the Requirements for the
Degree of**

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in
INDUSTRIAL BIOTECHNOLOGY**

**by
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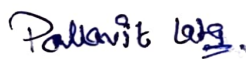
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I Pallavit Lata hereby certify that the work which is being presented in the thesis entitled **“Isolation and Functional Exploration of Potential Chromium-Utilizing Bacteria for Bioremediation and Biomineralization Applications: Insights into Hexavalent Chromium Reduction in Cement Industry Soil”** in partial fulfillment of the requirements for the award of the Degree of Master of Technology, submitted in the Department of Biotechnology, Delhi Technological University is an authentic record of my own work carried out during the period from January 2026 to May 2026 under the supervision of Dr. Asmita Das.

The matter presented in the thesis has not been submitted by me for the award of any other degree of this or any other Institute.


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Isolation and Functional Exploration of Potential Chromium-Utilizing Bacteria for Bioremediation and Biomineralization Applications: Insights into Hexavalent Chromium Reduction in Cement Industry Soil

Pallavit Lata

ABSTRACT

Hexavalent chromium [Cr (VI)] pollution is a serious environmental problem linked to many industrial processes, especially cement production, tanning, electroplating, pigment production and metal processing. The trivalent chromium [Cr (III)] that occurs naturally in raw materials can be converted to more harmful Cr(VI) in the high-temperature clinker production process used in the cement industry. Consequently, cement dust, slurry, contaminated soil and wastewater may be significant sources of chromium pollution. Cr(VI) is highly soluble and mobile, persists in the environment, is carcinogenic, and highly impacts soil microbial diversity, plant growth, aquatic ecosystems, groundwater quality and human health. Of the currently available treatment techniques, conventional physicochemical techniques have several drawbacks, such as expensive treatment cost, incomplete detoxification and production of secondary pollutants, which makes sustainable biological techniques required.

This study aimed to explore the microbial chromium bioremediation and biomineralization from the cement industry-associated environments in an integrated way. The soil collected from the cement industry was used for isolating and functional screening of bacteria that can withstand the stress of heavy metals. The isolates were tested on chromium-amended media to assess the tolerance and adaptation of these isolates to Chromium. Microbial detoxification of hexavalent chromium is important because bacteria that resist chromium may transform the chromium to the less mobile and less toxic trivalent chromium using various mechanisms, including enzymatic reduction, biosorption, efflux-mediated resistance, and extracellular sequestration and precipitation-associated immobilization.

Further study of the urease-associated biomineralization potential of the chromium-adapted isolates was conducted in the presence of varying concentrations of urea. The urease-producing bacteria convert urea to carbonate ions and ammonia, raising the pH in the area and favouring calcium carbonate precipitation. The microbially induced calcium carbonate precipitation process (MICP) can be used in crack repair, surface protection, coatings, paints and self-healing cementitious materials. A functional basis for the identification of the isolates that may be useful for both chromium bioremediation and biomineralization-based material systems is obtained by the combined screening of chromium tolerance and urease activity. Overall, the research contributes to the development of sustainable microbial treatments for environments contaminated with chromium in industries and self-healing building materials.

Keywords: Hexavalent chromium, cement industry soil, chromium-tolerant bacteria, microbial reduction, bioremediation, biomineralization, urease activity, microbially induced calcium carbonate precipitation, self-healing materials, heavy metal detoxification.

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LIST OF ABBREVIATIONS

Cr	Chromium
Cr (III)	Trivalent Chromium
Cr (IV)	Tetravalent Chromium
Cr(V)	Pentavalent Chromium
Cr (VI)	Hexavalent Chromium
MICP	Microbially Induced Calcium Carbonate Precipitation
EPS	Extracellular Polymeric Substances
ROS	Reactive Oxygen Species
NADH	Nicotinamide Adenine Dinucleotide, Reduced Form
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Reduced Form
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
PH	Potential of Hydrogen
SEM	Scanning Electron Microscopy
EDS	Energy Dispersive X-ray Spectroscopy
XRD	X-ray Diffraction
FTIR	Fourier Transform Infrared Spectroscopy
16s rRNA	16S Ribosomal Ribonucleic Acid
°C	Degree Celsius
rpm	Revolutions Per Litre

CHAPTER 1

INTRODUCTION

1.1 Chromium Contamination in Cement Industry-Associated Environments

One of the main environmental issues linked to industrial growth is chromium contamination, especially in regions impacted by the manufacture of cement, tanning, electroplating, pigments, textiles, and metals[1]. Because of its resilience, fluctuating oxidation states, toxicity, and capacity to contaminate soil, wastewater, and groundwater, chromium is particularly significant among the various heavy metals discharged into the environment. Chromium occurs in two major oxidation states in the environment, namely trivalent chromium [Cr (III)] and hexavalent chromium [Cr (VI)]. The biological consequences and environmental behaviour of these two types are very different. In contrast to Cr (VI), which is highly soluble, mobile, persistent, and toxic to living systems, Cr (III) is somewhat less soluble, mobile, and toxic[2], [3]

The strong oxidizing properties of Cr (VI) and its capacity to enter cells through anionic transport systems are the primary causes of its toxicological significance. Cr (VI) may undergo reduction through unstable intermediate states once within the cell, producing reactive oxygen species and harming the cell. This process can have mutagenic, genotoxic, and carcinogenic impacts on proteins, lipids, nucleic acids, and membrane-associated structures[2], [4]. Additionally, soil fertility, microbial diversity, plant development, groundwater quality, and the wider ecological balance of contaminated environments are all impacted by chromium contamination[5]. Therefore, remediation techniques for chromium pollution must be efficient, long-lasting, and compatible with the environment.

Environments associated with the cement industry are a major source of chromium pollution. Chromium is found in cement raw materials naturally, primarily in the form of Cr (III). However, some Cr (III) may be oxidized into Cr (VI) during the high-temperature clinker production process. Hexavalent chromium Cr (VI) may therefore be stored in cement dust, slurry, sludge, wastewater, and adjacent contaminated soil. Because Cr (VI) can remain mobile in aqueous systems and may penetrate nearby soil and groundwater, its presence in cement-impacted soil and wastewater from the cement industry is concerning for the environment. Cement industry-affected areas have complicated environments for chromium exposure since such pollution is not restricted to the liquid effluent phase but may also be responsible for solid residues and dust particles.

When untreated or inadequately treated industrial effluent is discharged into open spaces or aquatic bodies, the environmental danger of Cr (VI) increases. Cr (VI) can travel long distances and survive for extended periods of time in aquatic conditions. Chromium can interact with clay particles, organic matter, microbial biomass, and

mineral surfaces in soil, but its mobility is dependent on pH, redox conditions, organic content, and the presence of reducing agents. Because of its toxicological relevance, the allowable content of chromium in drinking water is often kept at extremely low levels. The chromium-related water quality discussion published by [6] states that the possible carcinogenic risk of chromium in drinking water makes it a significant public health concern.

Native microbial populations are under selective pressure when chromium is present in industrial soil for a long period of time. While resistant or adaptable organisms might face and gain dominance, sensitive microbes may be suppressed. For the purpose of isolating chromium-tolerant microorganisms, these contaminated locations thus offer a helpful biological niche. These organisms are significant because they may have fundamental mechanisms for immobilization, biosorption, reduction, or resistance to chromium. Therefore, the isolation of bacteria from soil contaminated with chromium in the cement industry is important for both studying microbial adaptability under metal stress and identifying possible strains for applications based on bioremediation and biomineralization[7].

1.2 Microbial Chromium Detoxification and Bioremediation

conventional physicochemical techniques for chromium removal, such as Chemical precipitation, ion exchange, adsorption, electrochemical treatment, membrane filtration, and coagulation-flocculation[8]. These techniques may lower the concentration of chromium, but their practical use can often be limited by high operating costs, the need for chemicals, energy, partial detoxification, and the creation of secondary sludge. For long-term cleanup, these drawbacks make conventional treatment less appealing, especially when chromium is present in low quantities or when substantial amounts of industrial effluent need to be treated.

Bioremediation is a sustainable and environmentally favourable option for treating environments contaminated with chromium. The foundation of microbial bioremediation is the capacity of bacteria, fungi, and microbial consortia to use their inherent metabolic activities to change, immobilize, accumulate, or detoxify contaminants. The microbial reduction of Cr (VI) to Cr (III) is important in the case of chromium due to the low solubility, mobility and toxicity of Cr (III) than Cr (VI). Therefore, chromium bioavailability and environmental toxicity are decreased when Cr (VI) is converted to Cr (III)[2], [4].

Bacteria that are resistant to chromium can use a variety of strategies to deal with metal stress. These include chromate reduction, extracellular sequestration, intracellular complexation, efflux-mediated resistance, biosorption on the cell surface, bioaccumulation within the cell, and precipitation-associated immobilization[2], [5], [9].

Generally, biosorption is associated with functional groups on the bacterial cell wall, involving hydroxyl, carboxyl, amino, phosphate and sulfhydryl groups. By interacting with chromium ions, these groups aid in lowering the concentration of free metal in the surrounding medium. Particularly in microbes that form biofilms, extracellular polymeric materials may potentially play a role in metal binding and immobilization.

When chromium enters the microbial cell, intracellular detoxification starts. Chromate reductases and other redox-active enzymes can use electron donors like NADH or NADPH to enzymatically decrease Cr (VI). This mechanism transforms Cr (VI) into Cr (III), which can then precipitate or form stable complexes. To lessen chromium toxicity, microorganisms may also employ intracellular thiol-containing compounds, including glutathione, metallothioneins, and associated metal-binding peptides. These processes enable bacteria to tolerate chromium stress and aid in the detoxification of the environment[2], [9].

Numerous physiological and environmental variables, including pH, temperature, chromium content, dissolved oxygen level, nutritional status, and the availability of electron donors, affect how well microorganisms reduce Cr (VI). Cell surface charge, enzyme activity, and chromium speciation are all impacted by pH. Microbial metabolism and growth rate are influenced by temperature. The level of significance of metal stress is determined by chromium content, and the kind and speed of microbial reduction pathways may be influenced by oxygen availability. Therefore, choosing the right strains is essential to creating effective chromium bioremediation systems. Screening native isolates from contaminated areas is crucial since removal effectiveness can differ greatly amongst microbial species and even strains of the same genus[10], [11]

Several chromium-resistant bacteria have been reported from contaminated soils and industrial effluents. [7]studied the toxicity of Cr (VI) and its decrease by bacteria recovered from soil contaminated by tannery waste, highlighting the significance of native bacteria in chromium detoxification. The ecological significance of microbial adaptation under chromium stress was emphasized by [5], who described Cr (VI)-resistant bacteria isolated from chromium-contaminated soil impacted by tannery activities. The function of native microorganisms in chromium remediation was further confirmed by [12], who showed Reduction of chromate by chromium-resistant bacteria isolated from soils contaminated with dichromate. According to these studies, chromium-contaminated habitats can be important sources of bacteria that have the potential to be used in bioremediation[13].

1.3 Biomineralization Potential of Chromium-Adapted Bacteria

Some microorganisms have the capacity to biomineralize in addition to detoxifying chromium, which has significant uses in material science and environmental biotechnology. The biologically mediated production of minerals by living things or their metabolic processes is referred as biomineralization. Microbially induced calcium carbonate precipitation (MICP) is one of the biomineralization mechanisms that has been studied the most. Bacteria that produce urease hydrolyze urea in MICP to produce carbonate and ammonia ions. The local pH rises when ammonia is released, and carbonate and calcium ions combine to create calcium carbonate precipitates [14]

The potential applications of urease-mediated biomineralization in crack repair, self-healing concrete, cementitious coatings, surface protection, and soil stabilization generated interest. Because they can generate mineral precipitation under the right circumstances and may aid in sealing pores or fissures in damaged materials, urease-positive bacteria are particularly useful in cement-based systems. Urease-aided calcium carbonate mineralization was reviewed by [14], who highlighted its

significance for engineering applications.[15]showed the potential of bacterial mineralization in construction-related applications by demonstrating calcium carbonate mineralization mediated by *Bacillus megaterium* as a biogenic surface treatment for sustainable construction materials.

For conditions associated with the cement industry, the combination of urease activity and chromium tolerance is very crucial. Chromium pollution provides an additional layer of environmental pressure to cement-based systems, which are alkaline and chemically demanding. As a result, bacteria that are meant to be used in cementitious environments contaminated with chromium must be able to withstand metal stress without losing their ability to operate. Chromium-utilizing urease-producing bacteria may have two functions: they may facilitate biomineralization by calcium carbonate precipitation and aid in chromium detoxification through tolerance, reduction, or immobilization.

Thus, chromium-contaminated cement industry soil is a good source for isolation of bacteria with combined bioremediation and biomineralization potential. In the present investigation, the soil from cement industry impacted sites was serially diluted and plated on nutrient agar and minimal media separately, and the distinct colonies were purified and screened on chromium-amended media. Isolates were screened for chromium selectivity by growth under the stress of metal. Further testing was done on the isolates at various chromium concentrations for visible growth, density and development of streak. This screening strategy was useful for distinguishing chromium-sensitive isolates from chromium-tolerant or chromium-adapted bacterial forms.

In addition, urease activity was measured at varying urea levels to identify if chromium-adapted bacteria have biomineralization-related attributes. Using urea concentration as a functional parameter improved the ability to differentiate between those isolates that simply survived the chromium stress and those that might be actively involved in the mineral precipitations resulting from urease activity. Isolates that exhibit both urease and chromium tolerance may be viewed as a more promising strain for dual use in both the chromium bioremediation and self-healing cementitious systems.

The functional classification of the isolates based on their resistance to chromium and urease activity is significant as it gives an application-oriented framework for the selection of useful bacterial strains. These isolates can be classified as chromium-tolerant bacteria, chromium-adapted bacteria, urease-positive bacteria capable of biomineralization and dual-functioning bacteria, which could be of interest in environmental remediation and material-based applications. The use of this approach not only reinforces the relevance of the study but also establishes a connection with future applications in wastewater treatment, contaminated soil remediation, cement-based coatings and self-healing construction materials.

1.4 Rationale and Objectives of the Study

Chromium is increasing in the environment, especially in industrial environments such as cement industry-related ones, where sustainable and biologically compatible approaches to its remediation are needed. Cr (VI) is highly toxic, and Cr (III) is less toxic but persistent and mobile[2], [3]. The biological process is an important detoxification pathway for the bioreduction of Cr (VI) to Cr (III).

Native bacteria can also live in the soil and tolerate chromium stress, while the cement-contaminated soils can also contain native bacteria that can tolerate chromium stress. These bacteria can be studied to see if they can withstand high concentrations of chromium and have the potential to be used in bioremediation.

The current study is based on the integrated concept that the surrounding environments of a cement industry can be contaminated sites that need to be remediated, and as natural reservoirs of chromium-adapted microorganisms. A study was carried out, and a combined approach of bioremediation by chromium and urease-mediated biomineralization was done in order to find bacteria that might be of material-oriented and environmental importance. This is especially important as microorganisms in the cement-associated environment must be tolerant to chemical stress, able to withstand the presence of metals and maintain activity, including urease activity.

The general objective of the present study is to gain information about the environmental relevance of the presence of hexavalent chromium [Cr (VI)] in soil and wastewater from the cement industry and to study the microbial processes in which it is detoxified. The study is targeted on the most important biological processes that play a role in the reduction or immobilization of toxic Cr (VI) in contaminated environments, including biosorption, bioaccumulation, bioreduction, and intracellular sequestration and precipitation-associated immobilization. The study also seeks to isolate bacteria from the soil samples collected from the cement industry, which are contaminated with chromium, using serial dilution and culture-based method and screen the bacterial isolates on the media amended with chromium, and assess their chromium tolerance and adaptation. The urease-associated biomineralization ability of the chromium-adapted isolates is also studied at various concentrations of urea. The isolates have been tentatively classified according to their functional capabilities of chromium tolerance and urease activity for potential use in the bioremediation of chromium and self-healing cementitious systems. Moreover, the study has laid the preliminary groundwork for further quantitative study on the reduction of Cr (VI), molecular identification, urease enzyme estimation and mineral characterization.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Chromium Contamination and Industrial Environmental Concern

One of the main environmental issues caused by rapid industrialization is heavy metal contamination. Heavy metals might remain in soil, sediments, and aquatic systems for extended periods of time because they cannot break down into harmless byproducts as many organic contaminants do. Because of its extensive industrial use, environmental persistence, and toxicity, chromium is one of the heavy metals of particular concern. Industries include the production of cement, tanning, electroplating, pigments, textiles, iron and steel, and other metal-processing processes that discharge chromium into the environment[1]. Soil fertility, groundwater quality, microbial diversity, plant development, and human health can all be impacted by the release of chromium-containing effluents and solid wastes into nearby soil and water systems [2], [4], [9].

The oxidation states of chromium are either trivalent chromium [Cr(III)] or hexavalent chromium [Cr(VI)] in natural or industrial environments. These two forms have very different solubility, mobility and toxicity. Cr (III) is less soluble, mobile and toxic in comparison. Whereas Cr (VI) is highly mobile, soluble, persistent and toxic. Cr (VI) usually forms oxyanions - chromate and dichromate, which are readily transported in aqueous systems and could enter the biological cells by anion transport pathways. This makes Cr (VI) more dangerous than Cr (III), especially in the wastewater and groundwater systems[2], [3], [4]

The cement industry-associated environments are important in regard to chromium pollution, as chromium may occur naturally in cement raw materials. A significant amount of Cr (III) can be converted to Cr (VI) during the high-temperature clinker production process. As a result, cement dust, slurry, wastewater, sludge, and soil around the area where the waste is disposed of can be sources of chromium contamination. The contamination is of environmental importance as Cr (VI) can be spread from the industrial discharge point to the surrounding soil and water. Thus, in a site affected by the cement industry, chromium may be present as a multi-compartment pollutant (soil-water contamination) and not as a single-compartment pollutant.

The environmental behaviour of chromium is affected by various physicochemical parameters, including redox potential, pH, organic matter, mineral and microbial activities. In the oxidized state, chromium can exist in the mobile Cr (VI), while in the reduced state, it can transform to Cr (III), which can form insoluble hydroxides or complexes with organic and inorganic ligands. Hence, the reduction of Cr (VI) to Cr (III) is a crucial pathway that can reduce the mobility and environmental risk associated with chromium[2], [4], [9]

Soils with Cr contamination are also selective for the microbial communities. Sensitive organisms can be inhibited, and resistant and adapted organisms can survive. From a

biotechnological point of view, the adaptation is important in contaminated environments, which may be natural sources of chromium-tolerant bacteria. These organisms can possess mechanisms that can be applied in chromium bioremediation, such as biosorption, bioaccumulation, efflux, enzymatic reduction, precipitating, sequestration, and immobilization associated with these precipitates [2], [5], [12]

2.2 Toxicity and Biological Effects of Hexavalent Chromium

The toxicity of Cr (VI) is mainly due to the permeability, oxidizing property and solubility of cell membranes. The chromate ion is structurally analogous to the sulfate and phosphate ions and enters microbial, plant and animal cells via non-specific anion transport systems. The hexavalent chromium [Cr (VI)] can be reduced stepwise to pentavalent chromium [Cr (V)], then tetravalent chromium [Cr (IV)] and finally to trivalent chromium [Cr (III)] upon entry into the cell. This intracellular reduction can result in the formation of reactive oxygen species, leading to oxidative stress and consequent cell damage[2], [4].

Cr reduction can produce reactive oxygen species, which can harm cellular membranes, enzymes, lipids, proteins and DNA. Cr (VI) inhibits growth, disrupts membrane function, alters enzymatic activities and decreases metabolic efficiency in microorganisms. Chromium toxicity can result in inhibition of seed germination, root growth, photosynthesis, chlorophyll content, nutrient uptake and biomass yield in plants. Cr(VI) has been found to cause respiratory toxicity, skin irritation, organ toxicity, genotoxicity and carcinogenic risk in human beings[6], [11], [16]

The Cr (VI) is particularly significant for the environment, because it is capable of being present in the water system and passing through the food chain. The contaminated water can have a direct impact on the aquatic organisms, and contaminated soil can have an impact on agricultural productivity and important microbial processes that serve for nutrient cycling. Decreased diversity of microbes can impact overall soil function, as microbes are important to decomposition, nitrogen cycling, phosphorus solubilization and transformation of organic matter, and loss of these organisms due to Cr may affect overall soil function. The contaminated environments can also be a valuable source for bacteria with bioremediation potential, as the metal stress enriches the microbial population that is resistant to these metals.

The microbial detoxification of chromium is based on the difference in toxicity of Cr (VI) and Cr (III). The biological reduction of Cr(VI) to Cr(III) is considered a preferable detoxification pathway, since Cr(III) is less toxic, less mobile and more prone to precipitation than Cr(VI). Thus, microbial chromium reduction can reduce toxicity as well as the environmental mobility of chromium[2], [4], [7].

2.3 Limitations of Conventional Chromium Treatment Methods

There are several physicochemical techniques used in chromium removal from industrial effluents. They are chemical precipitation, ion exchange, adsorption, coagulation-flocculation, membrane filtration and electrochemical treatment. Chemical precipitation is widely used as soluble chromium species can be transformed into insoluble chromium species and removed from wastewater. This process,

however, can produce considerable amounts of sludge (a waste product) containing metal contaminants, necessitating additional treatment and safe disposal.

Another method being extensively researched for chromium removal is adsorption. Agricultural residues, clay minerals, biochar and activated carbon can be used to remove Cr ions from water-based systems. While adsorption is useful, it can be effective, and its effectiveness relies on pH, adsorbent dosage, contact time, concentration of chromium ion, competing ions, and regeneration capacity of the adsorbent. High removal efficiencies can be obtained by ion exchange and membrane filtration, which could be costly and may require complex maintenance. Electrochemical methods can also be used to remove chromium efficiently, but are generally energy intensive and operating conditions need to be controlled.

The drawbacks of conventional chromium treatment methods are high operating expense, need for chemicals, incomplete removal, production of secondary toxic sludge, and treatment inefficiency in the presence of low levels of metals and process control issues. They do not apply to long-term and large-scale treatment of chromium-contaminated effluents. Hence, biological methods are coming into focus as sustainable solutions, as they are able to operate under mild conditions, and they might also decrease the toxicity of chromium in the environment through natural microbial activities[5], [10], [17]

2.4 Microbial Bioremediation of Chromium

Bioremediation is a technique that involves the use of biological systems to remove, transform, detoxify or immobilize environmental pollutants. The potential of microorganisms to tolerate chromium stress and to reduce or immobilize Cr (VI) is the primary basis of microbial bioremediation in chromium-polluted environments. Biosorption, bioaccumulation, enzymatic reduction, efflux-mediated resistance, extracellular sequestration, immobilization associated with intracellular complexation and precipitation are ways by which microorganisms can detoxify chromium[5], [10], [16], [18] .

The bacteria resistant to chromium were isolated from different contaminated environments, such as soil contaminated with tannery effluents, dichromate-contaminated soil, and industrial effluents. The native bacterial isolates are important in the detoxification of Cr (VI), as reported by[7]. [2]reported that there are several mechanisms of chromium resistance: biosorption, lower uptake, chromate efflux, precipitation and reduction in microorganisms and plants.[5]isolated chromium-resistant bacteria from tannery-polluted chromium-contaminated soil and described these bacteria as chromium-resistant.[12] investigated the ability of chromium-resistant bacteria isolated from dichromate-polluted soils to reduce the chromate and found that the environmental factors pH, temperature and incubation time affect the chromium reduction.

There are two possibilities of microbial Cr (VI) reduction: enzymatic or non-enzymatic. Usually, enzymatic reduction is found to be mediated by chromate reductases and other oxidoreductases that utilize electron donors like NADH or NADPH. The reduction of Cr (VI) can occur either intracellularly or extracellularly or at the cell membrane, depending on the environmental conditions and the

microorganism. The extracellular reducing process is beneficial as it can help to inhibit the entry of Cr (VI) into the cell and thus minimize the intracellular toxicity. Intracellular reduction refers to the cellular detoxification systems that control the reactive intermediates produced during chromium transformation[2], [4].

Biosorption is known as an important passive mechanism for the removal of chromium by microbes. This is the binding of chromium ions to the functional groups on the surface of microbial cells. The carboxyl, hydroxyl, amino, phosphate and sulphhydryl groups in the bacterial cell wall can serve as binding sites for the metal ions. Also, microorganisms may produce extracellular polymeric substances (EPS), which can also bind and immobilize metals. Biofilm-forming microorganisms have proven to be especially valuable in bioremediation, as biofilms create a protective structure that facilitates the survival of the microorganisms in toxic environments and increases metal binding capacity[9].

Bioaccumulation is the process of chromium being absorbed by living organisms. When chromium is in the cytoplasm of the microorganism, it is possible for intracellular molecules like glutathione, metallothioneins, and other thiol-containing molecules to bind and sequester metal ions. These processes decrease the interaction of chromium with sensitive cellular components as well as help the organism to survive the metal stress. Precipitation-related immobilization can happen when Cr (III) precipitates as hydroxides or complexes with extracellular and intracellular ligands, decreasing the bioavailability and mobility of chromium [2], [9].

Factors affecting the efficiency of microbial chromium detoxification are microbial strain, biomass concentration, chromium concentration, pH, temperature, nutrient availability, oxygen level and availability of electron donors. Hence, it is important to select an efficient bacterial strain for efficient bioremediation. Species of the genera *Bacillus* spp., *Pseudomonas* spp. and *Streptomyces* spp. have been frequently reported for their metal tolerance and biosorption ability [4]. High surface binding capacity and metabolic diversity of fungi and microbial consortia can also be a factor in chromium removal.

2.5 Chromium-Tolerant Bacteria from Contaminated Soil

Soil polluted by chromium is a good source for the isolation of soil microorganisms adapted to chromium stress. Exposure of microorganisms to chromium over extended periods results in the selection of resistant microbial populations. These isolates are native and may be better suited for environmental use than non-adapted lab strains that have not been exposed to contaminated field conditions. Isolation of the bacteria resistant to chromium is thus a useful approach to search for possible bioremediation potential bacteria, especially from contaminated soil.

Bacteria with chromium resistance can be easily recovered by culture-based isolation. This usually includes sampling of contaminated soil, serial dilution, plating on nutrient or minimal medium, selection of morphologically distinct colonies and purification via repeated streaking. Isolates can be purified and then subsequently screened for growth response to metal stress on chromium-amended media. Growth on chromium-containing media is used as an indicator of chromium tolerance and adaptation, a valuable functional indicator. Such screening can be used to select isolates that are able

to tolerate the presence of chromium, and may be further tested for application-appropriate properties.

In this study, the source of soil was the soil of the chromium-contaminated cement industry. Soil was serially diluted and then spread on nutrient agar and minimal media. The morphologically distinct bacterial colonies were picked and purified for further analysis. The isolates were then tested on media containing varying concentrations of chromium supplemented with chromium. The isolates were then tested on chromium-amended media with different concentrations of chromium. Growth, colony density and streak development were used as indicators of chromium tolerance and adaptation. The method can be used to detect bacteria that can survive under the condition of chromium stress and to select isolates that are functionally relevant in the chromium-contaminated site.

A tolerance to chromium is also a desirable characteristic, as bacteria that are resistant to this contaminant could have adaptive abilities that would be beneficial to environmental uses. These can be the mechanisms like decreased chromium uptake, increased efflux, extracellular binding, intracellular sequestration, biosorption and immobilization associated mechanisms. Hence, isolates growing under chromium stress can be referred to as chromium-tolerant or chromium-adapted. The experimental focus on the isolation, tolerance screening against chromium and urease-dependent biomineralization screening offers a solid application background for the assessment of bacteria from contaminated soil from the cement industry.

2.6 Biomineralization and Urease-Producing Bacteria

Biomineralization is a biogenically driven process that can be defined as the influence of living organisms on mineral formation. Biomineralization in microbial systems can take place by metabolic processes changing the local pH, ionic concentration and saturation states. Microbially-induced calcium carbonate precipitation (MICP) is one of the most studied microbial biomineralization processes. Among various applications, MICP has been gaining interest in construction materials, soil stabilization, environmental remediation, and self-healing cementitious systems[14], [19].

Urease-mediated MICP is the most widely investigated precipitation mechanism of microbes. Urease activity of bacteria decomposes urea to ammonium and carbonate ions. The ammonia produces a local pH rise, and the carbonate ions bind with calcium ions to produce precipitates of calcium carbonate. Bacterial cell surfaces are also good nucleation sites because of the presence of negatively charged functional groups that bind calcium ions. When calcium ions are accumulated near the cell surface, the carbonate ions react with these and nucleate the formation of calcium carbonate crystals[14], [19], [20]

[14]discussed the urease-catalysed mineralization of calcium carbonate and its applications in engineering. [15]showed the surface treatment of green building materials via biogenic mineralization of calcium carbonate, assisted by *Bacillus megaterium*. [15]also summarized bacterial biomineralization of calcium carbonates and its engineered applications, which included the role of ureolytic bacteria, biofilms and extracellular polymeric substances (EPS) in the formation of minerals. The present

studies indicate that urease-producing bacteria are capable of precipitating calcium carbonate and can be applied in material protection.

Self-healing cementitious systems have special importance for MICP. Shrinkage, mechanical loading and environmental stress are the reasons why cement-based materials tend to develop microcracks. If there are any cracks allowing the passage of water and harmful ions reduces the ability of the matrix to be durable. Self-healing systems with bacteria are based on microbial calcium carbonate precipitation to seal cracks and permeability. Jonkers et al. (2010) have illustrated the use of bacteria as self-healing materials for the development of sustainable concrete, and they have found that certain alkali-resistant spore-forming bacteria can help to heal the cracks from mineral precipitations.

For application in cementitious environments, bacterial strains must tolerate alkaline pH, limited nutrient availability, desiccation and chemical stress. Soil from the cement industry contaminated with chromium could then be a good source of bacteria to survive in extreme environments. If these bacteria are also urease-positive, they can be applied for biomineralization. In the present study, the urease activity of chromium-adapted isolates was determined at various urea concentrations, which could be used as a point of application for the identification of bacteria with biomineralization associated functional behaviour.

2.7 Microbially Induced Carbonate Precipitation and Heavy Metal Immobilization

Microbially induced carbonate precipitation (MICP) can not only be applied to self-healing construction materials, but also to environmental remediation work. Metal ions can be trapped during the precipitation of calcium carbonate by adsorption to the carbonate surface, co-precipitation in the mineral structure of precipitating carbonate minerals or physical entrapment in the precipitated mineral matrices. This mechanism can be used to deplete the mobility and bioavailability of metals in polluted areas.

Microbially induced carbonate precipitation by ureolytic bacteria for the immobilization of heavy metals was reported by [21], suggesting the possible effect of MICP on the stabilization of metals.[22] gave a review of MICP as a sustainable technique for treatment or entrapment of contaminants and highlighted its importance in heavy metal remediation.[23] mentioned the ureolytic bacteria-induced carbonate precipitation method as a way to immobilize different heavy metals in contaminated environments, suggesting that MICP may have potential in immobilizing them.

The link between chromium tolerance and urease-mediated biomineralization is therefore scientifically important. Chromium-tolerant bacteria may contribute to survival and chromium-associated detoxification processes in contaminated environments, while urease-positive bacteria may promote carbonate precipitation. If both properties are present in the same isolate, the organism may have dual relevance in environmental bioremediation and self-healing material systems. This dual functionality is particularly suitable for cement industry-associated environments, where chromium contamination and cementitious conditions occur together.

2.8 Integrated Potential of Chromium Tolerance and Biomineralization

The integration of chromium tolerance and urease-associated biomineralization provides a functional framework for selecting bacteria with application-oriented potential. Chromium-selective screening helps identify bacteria that can survive under heavy metal stress, while urease screening helps identify bacteria capable of biomineralization-related activity. The combination of both screens allows classification of isolates into chromium-tolerant, chromium-adapted, urease-positive and potential dual-function groups.

This integrated approach is relevant because cement industry-associated environments are chemically complex. Such sites may contain chromium, alkaline residues, mineral particles, cement dust and variable nutrient conditions. Bacteria intended for application in these environments must therefore tolerate metal stress and maintain metabolic functionality. Isolates showing both chromium tolerance and urease activity may be useful for chromium-contaminated soil or wastewater treatment and may also contribute to calcium carbonate precipitation in cementitious materials.

The connection between chromium tolerance and urease-related activity is useful in that it connects environmental survival and biomineralization-related functionality. Growth on chromium-amended media shows the ability of isolates to tolerate heavy metal stress, while urease response shows their ability to participate in the hydrolysis of urea and the generation of carbonate. The present traits favour the selection of multifunctional bacterial isolates from cement industry-associated environments.

CHAPTER 3

METHODOLOGY

3.1 Overview of the Study

This study aimed to isolate and functionally screen bacteria from soil associated with the chromium-contaminated cement industry for possible application in bioremediation and biomineralization. The methodology was culture-dependent, in which contaminated soil was used as the source material, and bacteria that survived under chromium stress were recovered. The experimental study comprised sample collection, sample processing, serial dilution, isolation of bacteria on nutrient agar and minimal media, purification of morphologically distinct colonies, screening of colonies for urease activity at various urea concentrations, growth response of bacteria to varying concentrations of chromium, microscopic observations, characterisation, and functional interpretation of isolates at the colony level.

A selective stress factor was used to isolate the tolerances to chromium-containing conditions, which were chromium. The concentration of urea was used as another functional screening parameter to assess urease-associated biomineralization potential. This simultaneous screening strategy was useful for the classification of isolates based on their ability to tolerate chromium and urease activity, which is a good basis for the identification of bacteria that could potentially be used in chromium bioremediation and self-healing cementitious systems.

The current approach was based on culture-based isolation, chromium tolerance study and functional screening of bacterial isolates obtained from the soil associated with the cement industry by urease activity. This method served as an excellent experimental basis to isolate bacteria that might be useful in chromium-contaminated systems and for use in biomineralization-related applications.

3.2 Sample Collection

Chromium-contaminated soil samples were collected from four cement industry-affected sites. Sampling sites were selected because environments associated with the cement industry may contain residues with chromium, such as cement dust, slurry, sludge and wastewater-affected soil. Soil samples were aseptically collected from contaminated areas using sterile tools to prevent external microbial contamination. The collected soil samples were put in sterile containers and taken to the laboratory for immediate processing.

Care was taken to prevent contamination from non-target surfaces and to maintain aseptic handling during sample collection. The collected soil was a chromium-stressed environmental matrix and thus was considered suitable for isolating bacteria naturally adapted to exposure to heavy metals. Such contaminated sites may provide selective

ecological niches where chromium-tolerant microorganisms can survive and become functionally important for environmental biotechnology.

3.3 Sample Preparation

One gram of chromium-contaminated soil was weighed aseptically and suspended in sterile phosphate buffer. The suspension was mixed well in order to separate the microbial cells from the soil particles and to obtain a uniform suspension. Soil is a heterogeneous matrix consisting of mineral particles, organic matter, microbial biomass and contaminants; thus, proper homogenization was crucial. Homogenization helped in the equal distribution of microbial cells in the suspension and increased the efficiency of the subsequent serial dilutions.

Samples were prepared in a phosphate buffer to ensure a stable suspension medium. The soil suspension after homogenization was used as a stock suspension for serial dilution. Serial dilution is a common microbiological method for stepwise reduction of microbial load and for obtaining well-separated colonies on solid media [24].

3.4 Serial Dilution of Soil Suspension

The prepared soil suspension was diluted in serial dilutions 1000 times up to 10^{-7} dilution level. Aseptically, a known quantity of soil suspension was placed in sterile dilution blanks and thoroughly mixed at each dilution level. The dilution process was taken step by step to decrease the microbial level of the sample.

Proper dilutions were used to plate on solid media. Well-separated bacterial colonies were obtained by using well-diluted suspensions from appropriate dilutions. Using serial dilution helped to obtain morphologically different isolates and also decreased overcrowding on the plates. Care was taken in each dilution step to ensure sterility and consistency.

3.5 Isolation of Bacteria on Nutrient Agar and Minimal Media

Appropriate serial dilutions were made and aliquots of each dilution were spread on nutrient agar and minimal media plates. The general-purpose medium used was nutrient agar, which was used to culture a variety of heterotrophic bacteria that were present in the contaminated soil sample. Organisms were isolated in minimal media that allowed growth in the relatively nutrient-poor environment. The application of nutrient-rich and minimal media helped to grow various types of culturable bacteria from the chromium-contaminated soil.

The inoculated plates were incubated at 37°C for 24–48 hours. Plates were then inspected for growth of bacteria after incubation. The colonies growing on the plates were observed for differences in their morphology such as size, shape, colour, margin, elevation, opacity and texture. Colonies varying in morphology were chosen for purification. Colour, shape, texture, surface area, etc. of the colonies were clearly visible, and subsequently, different bacterial isolates were recovered from the soil sample.

A culture-dependent isolation method was applied to this study due to the aim of the study, which was to isolate viable bacterial isolates to test for chromium tolerance and urease-associated biomineralization.

3.6 Purification of Bacterial Isolates

Colonies were selected and further purified by repeated streaking on new agar plates. Repeated streaking was carried out to obtain single colonies of each bacterial type. The purification process was continued until colonies with uniform morphology were obtained. These were taken to be individual bacterial isolates and the subsequent screening of the bacteria for chromium and urease was carried out after the bacteria were purified.

All the purified isolates were stored on the proper medium of agar for further screening. The purer ones were labelled and preserved for further analysis. Colony morphology of purified isolates was systematically noted and comprised colour, shape, size, elevation, margin, texture and opacity. These observations provided preliminary phenotypic information and aided in the differentiation of isolates in future screening phases.

3.7 Chromium-Selective Screening

Bacterial isolates were purified and then tested on the media containing different concentrations of chromium. Three different concentrations of chromium (0.02%, 0.04% and 0.06%) were used to prepare the medium for the selective culture of chromium. The selection of chromium was to be done with the aim of isolating metal-resistant isolates. The selectively pressure differentiated the isolates that are chromium sensitive from chromium tolerant or chromium adapted isolates using the use of the chromium.

Aseptic inoculation of each purified isolate onto the chromium-amended media was done. For comparison, the normal growth and growth under chromium stress were compared using control plates without chromium. The inoculated plates were incubated at 37°C and were checked for growth after incubation for their appearance. Chromium-amended media were used, and growth was recorded in terms of development of colonies, streaking and growth response.

There was an increase in chromium concentration for the use of assessing the tolerance level. The lowest level of chromium at which an isolate grew was the initial screening level, with isolates that grew at higher levels of chromium considered more adapted to chromium stress. An important selection criterion for the identification of isolates that survived under chromium stress and demonstrated adaptation to the presence of chromium in the growth medium was growth on chromium-containing media.

3.8 Evaluation of Growth Response Under Chromium Stress

The growth response of the selected isolates was determined over the range of the chromium gradient. Isolates were compared by the criteria of their visible growth, colony density and streak development on chromium-amended media. Growth pattern

on chromium-containing plates was compared with the growth pattern on the control to evaluate the stress of chromium.

Those isolates that grew less as the chromium concentration increased were considered moderately tolerant or affected by stress. Isolates that had visible growth throughout the chromium gradient were considered chromium-tolerant or chromium-adapted. Variations in growth response indicated different stress resistance to chromium in isolates. Variations may be related to cellular resistance mechanisms, membrane transport behaviour, metal binding capacity and stress response systems [2], [5].

Growth response assessment was used as a functional screening tool to assess the bacterial isolate under the Cr stress and to select more tolerant ones for further application-oriented assessments.

3.9 Urease Activity Screening at Different Urea Concentrations

The urease-associated biomineralization potential of chromium-adapted isolates was then determined using media with various concentrations of urea. Three different concentrations of urea (0.5%, 1% and 2%) were used for measuring urease activity. The rationale for the use of different urea concentrations was to test whether urease associated activity occurred with different substrate levels when grown with the chromium-tolerant isolates.

Bacteria possess urease, which breaks urea into ammonia and carbonate ions. The ammonia production increases the local pH, and in the presence of calcium ions these can react with carbonate ions, under suitable conditions, to form precipitates of calcium carbonate. This process plays a major role in microbially induced calcium carbonate precipitation and has been investigated for the application of self-healing concrete, cementitious coating and crack repair and protection[14], [19]

The isolates were inoculated in the urea media and incubated at 37°C. The plates were then incubated and then observed for urease response. The activity of urease was recorded at different concentrations of urea and it was noted that there was some variation. The higher the response for the isolates at higher urea levels the higher was urease associated biomineralization potential.

This step was a sort of functional screening method for identification of the chromium adapted isolates with urease-associated biomineralization potential. The urease activity was important because it was an indicator of biomineralization-related processes, as urea hydrolysis by urease will produce carbonate and thus will create favourable conditions for calcium carbonate precipitation.

3.10 Microscopic Examination and Gram Staining

Bacterial isolates were purified by serially diluting them and then cultured on nutrient broth at a later stage, after which they were Gram stained and examined via microscope. The basic cellular morphology, staining behaviour of the isolates were observed by gram staining. The method enabled the differentiation of isolates based on their gram reaction and gave information about the shape and arrangement of the

cells. One of the most popular preliminary staining methods in bacteriology for morphological characterization is Gram staining.

Purified bacterial culture smears were made on clean glass slides. Air-dried, heat-fixed smears were prepared. Then, the staining was done following the conventional Gram staining protocol. The stained slides were examined using the microscope. Shape, organization and staining patterns of the cells were noted. The majority of purified cultures were studied for characteristics including rod type, staining uniformity and pattern of arrangement.

Gram staining and morphological observations gave important phenotypic data, which were useful in the selection of representative isolates for molecular identification.

3.11 Colony Morphology Analysis

Phenotypic characterisation of purified isolates was performed with respect to their colony morphology. Colony size, colour, margin, elevation, opacity, texture and surface appearance were the parameters observed. These characteristics distinguished isolates and served as a visual basis for the selection of morphologically different forms of bacteria.

The morphology of the colonies depends on the species of bacteria, the growth medium, and the growth conditions and physiological state of the bacteria. The functional classification of the isolates was assisted by colony morphology, microscopic observation, chromium tolerance screening and urease response in this study.

3.12 Functional Classification of Isolates

The screening results of both chromium and urea were analysed and used to categorize the bacterial isolates into functional groups. At the screening level, isolates that grew on chromium-amended media were considered to be chromium-tolerant or chromium-adapted. Urease-positive and biomineralization-associated isolates were defined as isolates that demonstrated urease activity. Isolates that were tolerant of chromium and urease were deemed as potential dual-function isolates.

The functional types were: chromium-tolerant, chromium-adapted, urease-positive with biomineralizing activity and isolates that might have dual application for chromium bioremediation and for self-healing cementitious applications. It was important to this classification as it related to the functional ability associated with urease under chromium stress and the survival of the microbes. This dual screening is helpful for the identification of isolates that could be useful in further research for environmental and material applications.

The classification offered an application-oriented classification system to classify isolates based on their chromium tolerance and urease-related functional behaviour.

CHAPTER 4

RESULTS

4.1 Recovery of Bacterial Isolates

The chromium-contaminated cement industry soil sample was serially diluted and spread on the nutrient agar and minimal media. After 24-48 hours of incubation at 37°C, the culture plates showed bacterial colonies. There were visible differences between several colonies in size, colour, opacity, surface texture, elevation and margins.

Morphologically distinct colonies were selected from the primary plates and purified by repeated streaking. After purification, colonies with uniform morphology were obtained and maintained for further screening. The purified isolates were used for chromium tolerance screening, urease activity screening, colony morphology analysis and microscopic examination.

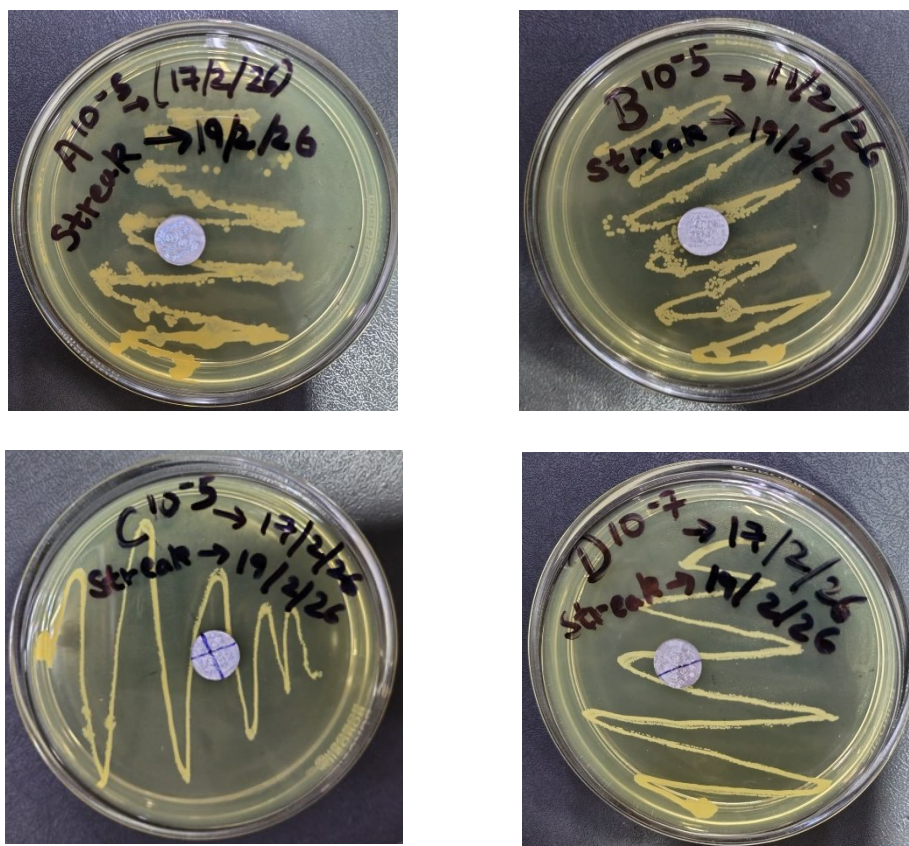


Figure 4.1: Purification of isolates by the streak plate method

4.2 Growth of Isolates on Chromium-Amended Media

Purified bacterial isolates were screened on chromium-amended media containing 0.02%, 0.04% and 0.06% chromium. Growth was observed on chromium-containing plates after incubation. The plates containing chromium showed growth following incubation. Isolates were found to have varying growth responses at various concentrations of chromium.

Several isolates appeared to grow at 0.02% chromium concentration. Selected isolates were found to grow at 0.04% concentration of chromium, but the intensity of the streaks and their density differed. Comparatively, fewer isolates were visible at the 0.06% concentration of chromium. Some isolates continued to grow over the chromium concentration range, and others had decreased growth in the presence of increasing chromium concentrations.

Growth response was evaluated based on three criteria: colony development, streak intensity and growth density (visually). The chromium-amended media, therefore, allowed comparison of isolate growth behaviour under increasing chromium concentration.



Figure 4.2a: Growth of bacterial isolates on chromium-amended media at 0.02% chromium



Figure 4.2b: Growth of bacterial isolates on chromium-amended media at 0.04% chromium



Figure 4.2c: Growth of bacterial isolates on chromium-amended media at 0.06% chromium

4.3 Comparative Growth Response Under Chromium Stress

All the bacterial isolates exhibited growth differentials under chromium stress. Some of the isolates exhibited good and sustained growth at all concentrations of chromium tested, while others saw a decrease in growth at higher levels of chromium. The response of isolates differed in terms of growth density, streak development and visible colony formation.

The growth pattern of the isolates was compared and grouped based on the growth pattern on the media amended with chromium. Isolates showing visible growth at more than one chromium concentration were recorded as chromium-tolerant or chromium-adapted isolates. Isolates showing comparatively stronger growth at higher chromium concentration were selected as more prominent chromium-adapted isolates for further urease activity screening.

4.4 Urease Activity of Chromium-Adapted Isolates

Chromium-adapted isolates were screened for urease activity using media supplemented with different urea concentrations: 0.5%, 1% and 2%. After incubation, a urease response was observed among selected isolates. There was variation in the urease activity of the isolates at the various levels of urea.

Some isolates were weakly urease and had a delayed urease response at the concentration 0.5% urea. Selected isolates exhibited a more distinct urease activity at 1% urea. In some cases, there seems to be a higher urease-associated response at 2% concentration of urea. Some of the isolates were found to be consistently active in all the different concentrations of urea used.

The urease response was observed as changes in the chromogenic urea-containing medium. Variation in response allowed the discrimination of the isolates based on their urease-associated functional activity.

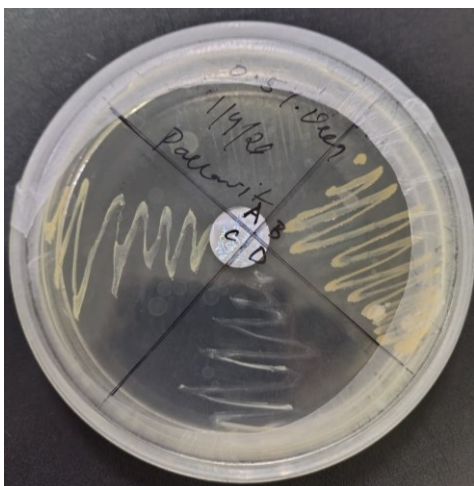


Figure:4.4a: Urease response of chromium- adapted bacterial isolates grown in media with 0.5% urea

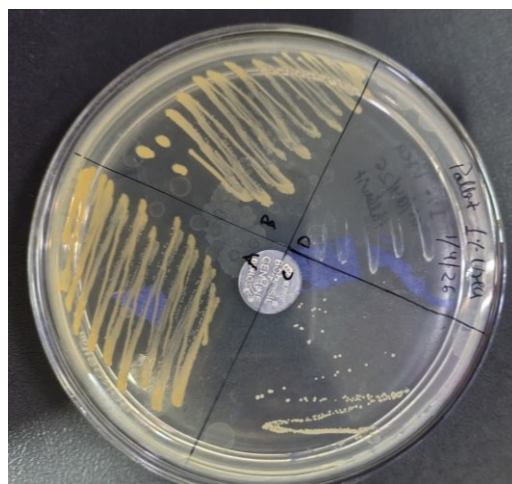


Figure:4.4b: Urease response of chromium-adapted bacterial isolates grown in media with 1% urea

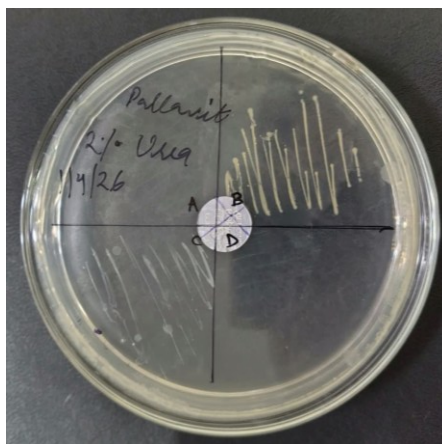


Figure:4.4c: Urease response of chromium-adapted bacterial isolates grown in media with 2% urea

4.5 Urea-Based Differentiation of Isolates

There was variation in the responses of the bacterial isolates to the urea concentration series. A few isolates had a weak urease reaction, and a few isolates had a moderate or strong urease reaction. Isolates with visible urease activity at any of the urea concentrations tested were considered as urease-positive isolates.

On the basis of urease response, the isolates were subdivided as weakly ureolytic, moderately ureolytic and strongly ureolytic. Functionally important isolates were selected for both urease response and chromium tolerance as well.

4.6 Colony Morphology of Purified Isolates

The colony morphology of purified bacterial isolates was analyzed. The colonies of the isolates differed in size, shape, colour, margin, elevation, opacity, and surface texture. Some colonies were smooth and opaque; some had an irregular texture and margin.

Repeated streaking of the purified colonies resulted in colonies with a consistent morphology. Morphology of the colonies was recorded for differentiation and documentation.

4.7 Microscopic Characterization of Purified Isolates

Gram-stained purified isolates were subjected to microscopic examination. The observed bacterial cells were mostly rod-shaped with uniform staining patterns. Clear bacterial morphology was observed in purified cultures in the microscopic images.

The cellular arrangement and staining behaviour were recorded for the purified isolates. Macroscopic observation was complemented by microscopic observation, which gave information at the level of the cells of the isolates.

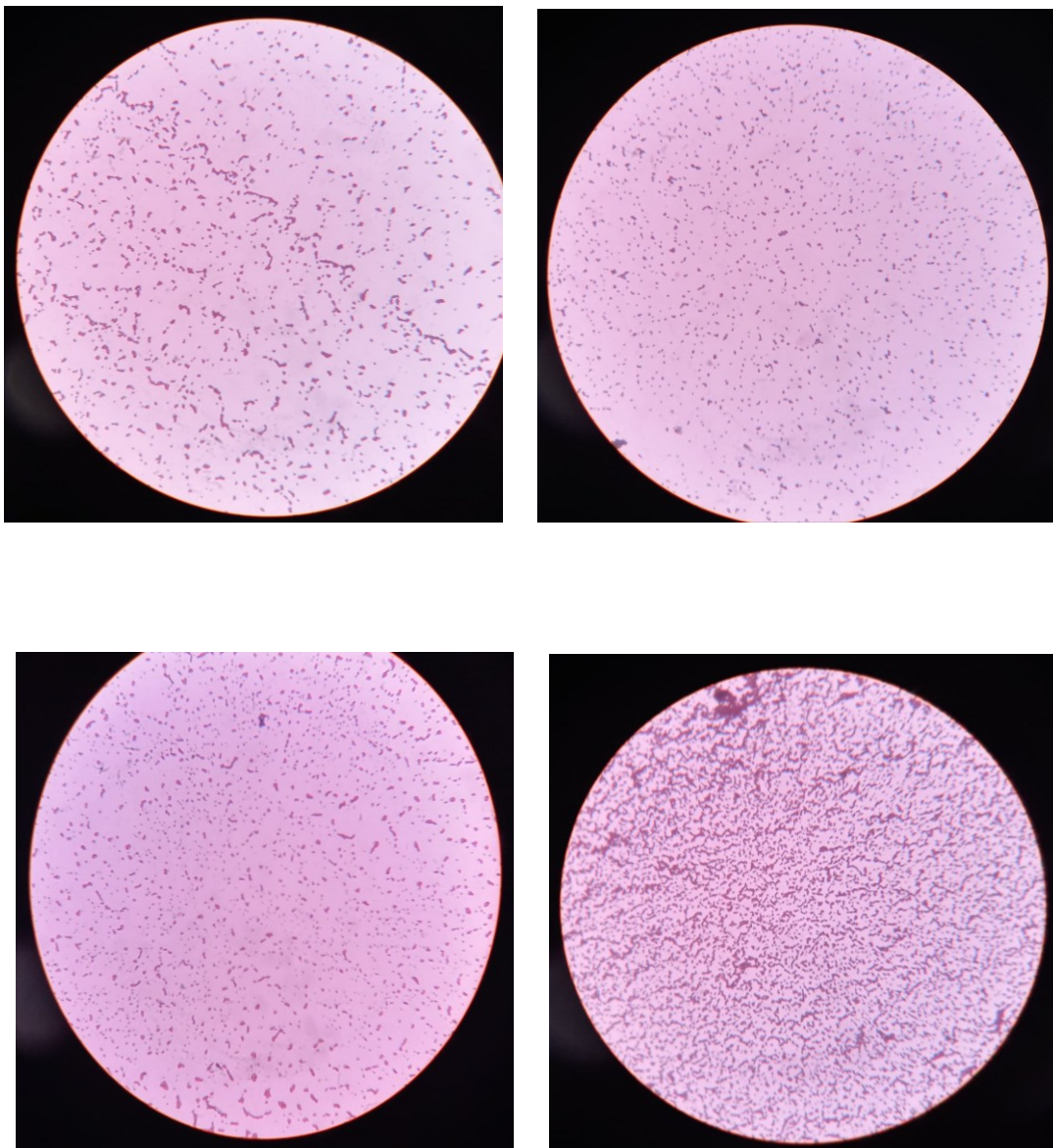


Figure 4.7. Pure bacterial isolates of the adapted bacteria were Gram-stained and examined under a microscope

4.8 Functional Classification of Isolates

The bacterial isolates were grouped according to two major screening parameters, viz., growth response on chromium-amended media and urease activity on urea-amended media. Thus, the isolates showed different functional behaviours in these observations. Some isolates tolerated chromium stress but had weak urease activity, while others showed chromium tolerance with moderate or strong urease activity. A further set of isolates showed both a clear urease-associated response and chromium tolerance, indicative of their possible dual functional relevance. Therefore, classification was done based on the visible growth in the presence of chromium and urease response at different concentrations of urea, which helped in identifying isolates of potential importance for bioremediation and biomineralization based applications.

Table 4.8: Bacterial isolates are functionally categorized according to urease activity and chromium tolerance

Functional category	Chromium growth response	Urease reaction	Potential application
Chromium-tolerant with weakly urease activity	Growth on chromium-amended media	Weak or limited urease response	Chromium tolerance study
Chromium-tolerant with moderate urease activity	Growth maintained at selected chromium concentrations	Moderate urease response	Bioremediation and biomineralization screening
Chromium-tolerant with strong urease activity	Growth maintained across the chromium gradient	Strong urease response	Dual-function significance
Potential dual-function isolate	Chromium tolerance/adaptation	Clear urease-associated activity	Remediation and self-healing of the cracks

CHAPTER 5

DISCUSSION

Contamination of chromium in cement industry-related environments is a major environmental problem due to the presence of heavy metals and alkaline and mineral residues of the industry in these environments. Soil from the cement industry can contain chromium particulates, cement dust, wastewater-associated contaminants and slurry residues. In such systems, microorganisms are subjected to stresses in the environment, and only those that are adapted to the contaminated environment will survive. From the present study, conclusions can be drawn that chromium-contaminated soil from the cement industry can harbour a native bacterial population adapted to the stress induced by the industrial activities.

The significance of isolating morphologically distinct colonies from contaminated soil is that chromium contamination can have a strong effect on the diversity and survival of the microbes. Hexavalent chromium [Cr (VI)] is known for its high solubility, mobility and oxidative toxicity. These can be transported into microbial cells via anion transport systems and, upon intracellular transformation, produce reactive oxygen species (ROS), which have the potential to affect proteins, nucleic acids, membrane systems and enzyme activity, among others [2], [4]. So, the fact that viable colonies could be found in the chromium-contaminated soil suggests that the recovered bacteria had physiological characteristics that enabled them to survive under chromium-stressful conditions.

Chromium-amended media (0.02%, 0.04% and 0.06% chromium) enabled the evaluation of the behaviour of bacterial growth under increasing heavy metal stress. This variation in growth response observed between isolates suggests that there is some variation in the tolerance ability of isolates for chromium. There was a significant reduction in growth at higher concentrations of chromium for some isolates and visible growth at all concentrations for others. Variation of this kind is a common feature in environments contaminated with metals, as they possess different mechanisms of physiological adaptation, metal-binding capacity, efflux capacity and stress response mechanism [5], [7].

Those isolates able to grow on chromium-amended media can be considered chromium-tolerant or chromium-adapted. Tolerance of bacteria against chromium is an important property that is required for bacteria to be used in environments contaminated with chromium. The resistance of surviving bacteria to chromium may involve biosorption, reduced uptake, efflux-mediated resistance, extracellular binding or immobilization through precipitation [2], [5], [9]. These mechanisms could decrease the harmful effects of chromium on the cell and promote microbial survival in a contaminated environment.

The results also support the importance of the use of native isolates from a contaminated environment. The actual physicochemical conditions of the site,

including chromium stress, alkaline residues, mineral particles and variable nutrient availability, are imposed on indigenous bacteria from the chromium-contaminated cement industry soil. The natural selection of the conditions might also yield bacteria that are better adapted for environmental use than those found in other environments. Other previous studies have also shown that the soils contaminated with chromium may harbour resistant bacteria, which may have relevance for chromium detoxification and bioremediation [5], [7], [12].

The study was extended by screening for urease activity as a second functional dimension. Urease-producing bacteria hydrolyse urea to ammonium and carbonate ions. Ammonia increases the local pH, and carbonate ions can react with calcium ions to form calcium carbonate. The process is named as microbially induced calcium carbonate precipitation (MICP) and is important in the applications related to biomineralization, like in the case of crack repair, self-healing concrete, cementitious coating, protection of surfaces and soil stabilization[14], [15].

The different urease response observed in the three different concentrations of urease (0.5%, 1% and 2%) suggests that the chromium-adapted isolates had varied functional behaviours associated with urease. There was a weak or delayed response for some isolates at lower urea concentrations and a strong response at higher urea concentrations. This difference could be due to the difference in the production of urease enzyme, the capacity of utilization of substrate, metabolic activity and tolerance to alkaline conditions caused by urea. Thus, it is suggested that the different levels of urea concentration allowed the identification of isolates showing greater biomineralization-associated potential.

Significantly, the ability to tolerate chromium and urease activity occurs together for some applications in the cement industry. The cementitious environments are generally alkaline and chemically stressful, and chromium additionally brings a stress factor from heavy metals. Bacteria that are chosen for these types of applications, then, should be able to withstand the chromium stress and retain functional activity under the cement conditions. Isolates with both urease response and chromium tolerance may be useful as they link two key functional traits, heavy metal tolerance and activity linked to biomineralization.

Results are presented in a functional classification (chromium-tolerant, weakly ureolytic, moderately ureolytic, strongly ureolytic and possible dual-function groups), which gives an application-oriented description of the results. This classification can aid in the identification of isolates that might have relevance for systems contaminated with chromium, biomineralization systems, or both. Isolates with good growth under chromium stress conditions could be important in chromium-related environmental applications, while isolates with a higher urease activity could be important in biomineralisation-related cementitious applications. If an isolate possesses both the traits, it is a very useful group as they possess both metal tolerance and urease-associated functional ability.

The colony morphology and microscopic examination further confirmed the separation of purified colonies. These differences in colony size, colour, opacity, margin, elevation and texture reflect differences among the culturable bacterial population recovered from the contaminated soil. The microscopic examination of Gram staining revealed that the bacterial cells were mostly rod-shaped and had

uniform staining. Observations were made at phenotypic and cellular levels, and this information was useful in documenting the purified isolates. Gram staining and colony morphology are two useful initial characterization techniques that are usually used for microbiological studies before additional biochemical and molecular analysis is performed[25].

The results obtained in the present work have been similar to those of the previous studies conducted on chromium-resistant microorganisms from the contaminated environment.[7]reported bacteria that are resistant to chromium in tannery waste soil, and [5]reported chromium-resistant bacteria in chromium-contaminated soil affected by tannery activities. Additionally, researchers [12]found bacteria that tolerate chromate in soils contaminated with dichromate. The findings of these studies are consistent with a contaminated environment being able to increase bacterial quantities that are tolerant to heavy metals. This study broadens the knowledge to cement industry-associated chromium contaminated soil, which is of concern because cement manufacturing may be a source of contamination of the soil with chromium-containing dust, slurry and wastewater.

There is also a correlation with previous studies on urease-producing bacteria and calcium carbonate precipitation, as a component of biomineralization. [19]showed that *Bacillus megaterium* can be used as a biogenic surface treatment for green building materials by its calcium carbonate mineralization process. [15]and [26] conducted reviews on bacterial calcium carbonate biomineralization and the mineralization of engineered systems, respectively. The urease response of the chromium-adapted isolates in the present study is in accordance with the relevance of these bacteria in cement based biomineralization applications. In MICP, metal ions can be immobilized through adsorption to the carbonate surfaces, co-precipitation, or inclusion in carbonate mineral matrices. [21], [22], [23] have discussed the importance of MICP for the immobilization of contaminants and remediation of heavy metals. As a result, the urease-positive chromium-adapted bacteria can be useful in the self-healing cementitious systems or in the immobilization-based remediation of contaminated environments.

The present study is focused on the importance of the integration of Chromium tolerance screening with Urease-associated screening. A large number of microbial studies are dedicated to the study of tolerance to heavy metals or to the study of the biomineralization, while this study is dedicated to both. This is an ideal product for use in cement industry-related environments where chromium is also found. The combined screening strategy will give a scientific basis for the selection of multifunctional bacteria that could be applied in waste management, environmental biotechnology and material biotechnology.

The environmental properties of possibly chromium-adapted bacteria can contribute to the sustainable use of sites contaminated with chromium. The traditional methods of treatment for chromium could have high operational costs, including the use of chemicals, sludge formation, and process complexity. Biological methods with modified microorganisms are an alternative and environmentally friendly approach to biosorption, immobilization, bioaccumulation and transformation processes[11], [16]. Isolates obtained in the work are therefore helpful as biological candidates for exploring microbial approaches in the environments where chromium is contaminated in the cement industry.

A material science perspective of urease-positive isolates is bio-based, self-healing systems. Microcracks are frequently formed in cementitious materials subjected to shrinkage and mechanical load, and environmental (weather) exposures. Such cracks enhance permeability, which can let water and ions in and decrease the durability. Precipitation of calcium carbonate via urease activity can be used to seal cracks and create protective mineral coatings on materials, which can increase their performance [14], [15]. Hence, the recovery of urease-positive chromium-adapted isolates links chromium-contaminated environmental biotechnology to sustainable construction material applications.

Overall, the present study will show that it is a good source of bacteria tolerant to chromium and with potential for urease-related biomineralization in soil contaminated with chromium from the cement industry. The findings confirm that the naturally occurring bacteria can be used as multifunctional bioremediation and biomineralization agents from contaminated environments.

CHAPTER 6

CONCLUSION AND FUTURE SCOPE

Hexavalent chromium [Cr (VI)] is an important concern in the environment because of chromium contamination in cement manufacturing environments, and it is persistent, mobile and toxic. Wastewater and soil discharged from the cement industry can be a great reservoir of chromium pollution due to the presence of chromium in raw materials, leading to their being released into the environmental systems as dust, slurry, sludge and effluent generation. Hexavalent chromium is a highly toxic compound that is mobile and soluble, therefore in environments affected by industry, there is an ecological risk to its presence, and it is considered a subject of sustainable biological methods and utilization for chromium-contaminated sites.

In the present study, chromium-contaminated soil from the cement industry was found to be an excellent source of culturable bacterial isolates that are tolerant to heavy metals. Morphologically different viable colonies of bacteria were recovered from the contaminated soil, which suggested the presence of microbial populations in the soil that were native adapted to industrial-stress conditions. This isolation and purification of bacterial colonies was helpful as a basis for functional screening and further characterization of the chromium-adapted isolates.

Chromium-selective screening showed that selected isolates of bacteria were able to grow on chromium-amended media with different chromium concentrations. Different growth response in 0.02%, 0.04% and 0.06% chromium was observed, suggesting that various responses to chromium tolerance and adaptation among isolates on growth responses. A few isolates grew visibly under the increasing levels of chromium, suggesting their ability to survive under chromium-enriched conditions. Bacteria from metal-contaminated locations might have developed tolerance to heavy elements like chromium, as they may exhibit mechanisms like biosorption, bioaccumulation, efflux-mediated resistance, and processes involving the sequestration and immobilization of heavy elements, which apply to chromium bioremediation[18][2], [5], [9].

In addition, urease activity further showed that there were chromium-adapted isolates that had functional potential associated with biomineralization. Urease-positive bacteria are important because they convert urea to ammonia, which produces carbonate ions and alkaline conditions that facilitate the formation of a calcium carbonate. This phenomenon is closely related to MICP, and it is important for areas in self-healing cementitious materials, crack repair, coating systems and surface protection [14], [19]. The larger urease-associated activity and the differentiation of bacteria based on their response in the different urea concentrations aided in the identification of the biomineralization potential of the isolates.

The combined measures of the chromium tolerance and urease activity screened gave the framework to classify the isolates based on the application. The tolerant, adapted and chromium urease-positive strains could be regarded as potential bioagent candidates for the integrated bioremediation and biomineralization-based applications.

This dual functional value is especially interesting to the cement industry-related environment, where chromium pollution and cementitious conditions are linked. The research consequently links environmental biotechnology to material-based applications to search for environmental bacteria with the potential to cope with chromium stress and/or be involved in biomineralization-related processes.

The purified bacterial isolates were differentiated and characterized using the Colony morphology and microscopic examination. The differences in size and pigmentation observed in the various colonies, margins, texture and opacity of the recovered culturable bacterial colony population suggested diversities. The morphology of bacteria was evaluated by a microscopic study, and representative bacteria were selected for further taxonomic and functional evaluation. Results of these observations supported the use of a culture-based approach for screening and served as phenotypic validation of separate forms of bacteria retrieved from the chromium-contaminated soil from the cement industry.

In the overall conclusion, it is summarized that the chromium-contaminated soil from the cement industry has a great potential to isolate bacterial isolates that are chromium-resistant and urease-positive for use in the field of ecological purposes.

The screening procedure adopted here was successfully able to select bacterial isolates that may be useful for chromium bioremediation and urease associated biomineralization. The results contribute to creating a microbial solution for the reduction of heavy metals like chromium in polluted industrial sites and might be regarded as suitable for the application of the Cr-adapted bacteria in self-healing cementitious systems. Add to this the chromium tolerance and the functional screening of the biomineralization- and the study is of considerable value for the development of microbial technologies in industrial biotechnology, environmental cleanup, and sustainable construction materials.

The study scope involves the evaluation of chromium contamination as an environmental problem, microbial mechanisms for detoxifying Cr (VI), isolation of bacteria from the soil contaminated by the cement industry and screening of the bacteria under Cr (VI) stress and evaluation of their biomineralization potential for urease. The results obtained in the study will serve as a preliminary basis for future molecular identification, quantitative chromium reduction assay and quantification of urease and mineral characterization by advanced techniques like 16S rRNA sequencing, SEM-EDS and XRD.

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