Removal of Chromium and Lead from water through biomineralization

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Dissertation

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Masters of Science

In

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By

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July, 2023

DECLARATION

I hereby declare that work presented in dissertation entitled "**Removal of Chromium and Lead from water through biomineralization**" in partial fulfilment of the requirement for the degree of Masters in Science in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab, is an authentic record of my own work during the period of six months from January 2023 to July 2023, under the supervision of Dr. M. Sudhakara Reddy, Professor, Head of Department, Department of Biotechnology, Thapar Institute of Engineering and Technology. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Dhruv Poddar

Date - 17th July 2023

Place - Patiala

CERTIFICATE

This is to certify that the thesis entitled “Removal of Chromium and Lead from water through biomineralization” submitted by Dhruv Poddar in partial fulfilment of the requirement for the degree of Masters in Science in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab, is a record of student’s own work carried out by her. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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Dhruv Poddar

CONTENTS

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LIST OF ABBREVATIONS

%	Percentage
U/ml	Units per ml
pH	Potential of hydrogen
MICCP	Microbially induced calcium carbonate precipitation
mg/L	Milligrams per Liter
Cr	Chromium
Pb	Lead
AAS	Atomic absorption spectroscopy
BIM	Biologically induced mineralization
BCM	Biologically controlled mineralization
Ca^{2+}	Calcium ions
CO_3^{2-}	Carbonate ions
hrs	Hours
^0C	Degree Celsius
OD	Optical density
nm	Nanometre
SEM	Scanning Electron Microscopy
EDS	Energy Disruptive Spectroscopy

IC	Inhibitory concentration
Conc	Concentration
EDTA	Ethylene diamine tetra acetic acid
Fig	Figure

ABSTRACT

Heavy metal contaminants present in the environment can enter the human body through the food chain and water contamination. The accumulation of heavy metals can lead to various health problems such as problems in the functioning of the brain, lungs, kidneys, liver, and other vital organs of the human body, which can lead to cancer, cardiovascular and neurological diseases, liver damage, and central nervous system disorders and other related diseases. In the present study, heavy metals such as Chromium (Cr) and Lead (Pb) were removed through biomineralization by using *Bacillus* sp. CT5. Biomineralization is the process by which organisms secrete inorganic minerals in the form of carbonate. *Bacillus* sp. CT5 can hydrolyze urea by producing urease enzyme. The isolate has high urease production that is 670.71 U/ml. It can survive a temperature range of 25-55° C and can also grow in a pH range of 6-11. Heavy metal removal was tested at different concentrations of Cr (1.36 µM,1.7 µM, 2.04 µM) which was 71.95%, 87.24% and 87.80% respectively. Pb samples were also tested for assessing the removal. The concentrations at which test was performed were 1.27 µM, 1.59 µM, 1.91 µM and removal efficiency was 99.57%, 99.69% and 99.08% respectively. Through biomineralization, heavy metal gets captured in the lattice sites of crystals of calcium carbonate by the formation of heavy metal calcium carbonate complex. The production of a heavy metal-calcium carbonate complex was also demonstrated by the FESEM and EDS data, indicating that the heavy metals were effectively immobilized by microbially induced calcium carbonate precipitation. This study shows biomineralization is an effective technique to remove heavy metals from contaminated sources.

1 INTRODUCTION

1.1 Heavy metals

Heavy metals are metallic compounds that generally have relatively high densities, atomic weights and specific gravity. Even at low quantities, heavy metals are harmful to the human body. Heavy metals are lustrous, dense, malleable, ductile and conductive. Although heavy metals are important for biological processes but large quantitative levels can cause bio-accumulation and biomagnification. Examples of heavy metals are Pb, Hg, Cr, Ni, Co, As, Cd, Fe and Zn (Mood *et al*., 2021).

1.2 Sources of heavy metal pollution

Anthropogenic activities including metal mining, metal leaching, insecticide, pesticide, and fertiliser usage all contribute to the dispersion of heavy metals. Natural processes including volcanic eruptions, soil erosion, geological weathering, metal corrosion, and metal evaporation from water and soil can also cause heavy metal contamination. Humans exposed to Pb exposure are generally battery workers, paint manufacturers etc (Mood *et al*., 2021).

1.3 Hazardous effects of heavy metals

Exposure to heavy metals such as Pb, Hg, Cr, Ni, Co, As, Cd, Zn and TI is harmful to human health and can cause complications such as gastrointestinal and kidney dysfunction, nervous system problems, vascular damage, skin lesions, birth defects, cancer and immune system dysfunction. Pb is a heavy metal that is extremely harmful to humans and may be absorbed via skin. Pb exposure can result in ailments of the nervous system, lungs, heart, and kidneys. Pb poisoning can lead to anaemia in the human body. Cr is a heavy metal present in the earth`s crust and sea water. Cr exposure can lead to various hazardous diseases. Industries such as metallurgical, refractory, and chemical industries release a large amount of Cr in the form of waste in air, water and soil which causes various health issues and diseases in animals, humans, and aquatic life. Cr exposure can occur through the consumption of Cr contaminated food and water. The bioaccumulation of heavy metals like Cr can cause several illnesses and conditions, including renal, cutaneous, neurological, and gastrointestinal problems. Various cancers, including those of the lung, throat, testicles, bladder, kidney, thyroid, and bone, can also develop as a result of Cr bioaccumulation. (Mood *et al*., 2021).

1.4 Removal of heavy metals by biomineralization

Many hazardous effects of the bioaccumulation of heavy metals can be seen in the human body. Various diseases and disorders can occur due to heavy metal accumulation, so to prevent this bioaccumulation, the removal of heavy metal is necessary to prevent such diseases and disorders. Removal of heavy metals from water or liquids is comparatively tougher than any other form. Heavy metals from liquid are difficult to remove as compared to any other pollutant, as other pollutants can be removed by chemical methods and biological waste water treatment (Knox *et al*., 2000). Microorganisms can break down toxic compounds into lesser toxic compounds and can also immobilize them by using biomineralization or transformation abilities of the microorganisms itself (Ayangberno and Babalola, 2017; Cheng *et al*., 2016). Under severe circumstances, such as media that have been contaminated with heavy metals, microorganisms may be the best option for heavy metal detoxification (Maity et al., 2019; Qian and Zhan, 2016). Heavy metals can be transformed by microorganisms by modifying their physical and chemical properties (Liu et al., 2021). Biomineralization is a process by which microorganisms form inorganic crystals through the natural metabolism of the organism itself (Niedermeier *et al*., 2018). Microbially induced calcium carbonate precipitation (MICCP), which has been the subject of much research, has previously demonstrated to be a suitable and efficient technique for removing heavy metal pollution. (Mitchell and Ferris, 2005; Achal *et al*., 2011, 2012, 2013; Kang *et al*., 2014; Kumari *et al*., 2014; He *et al*., 2019; Kim *et al*., 2021).

For the majority of heavy metals, calcium carbonate is one of the best host matrix (Callagon et al., 2014). Biologically induced mineralization (BIM) and biologically controlled mineralization (BCM) are the two primary categories of biomineralization (Li *et al*., 2014; Zheng *et al*., 2021). Biomineralization is known to form more than 60 minerals such as carbonates, phosphates, sulfides/sulfates, arsenates, silicates, chlorides, fluorides, oxides, hydroxides, and ferromanganese oxides (Schütze *et al*., 2013; Tayang and Songachan 2021; Wu *et al*., 2021). Ureolytic bacteria can form urease enzyme that break down urea in aqueous solutions to carbonate ions which explains rise in the pH of the solution and the formation of carbonate. A effecient method for the bioremediation of heavy metals is microbially induced calcium carbonate precipitation (MICCP). Therefore, the current work suggests employing MICCP in conjunction with ureolytic bacteria to bioremediate heavy metals (Pb and Cr) in aqueous solution. By producing urease enzyme activity and calcium carbonate precipitation, the Bacillus sp. CT5 bacterial strain removes heavy metals from the environment.. Biomineralization potential of ***Bacillus*** **sp.** CT5 bacteria for removal of Pb and Cr was tested by using the technique Atomic Absorption Spectroscopy (AAS). The biomineralized product formed at the end was analyzed by SEM and EDS for verification of the results.

1.5 Research Gap

There are several traditional bioremediation methods. Among the most effective bio-based decontamination techniques are bioaccumulation, bioleaching, bio coagulation, biosorption, and bio flocculation. The main disadvantage of these methods is the potential for harmful heavy metals that have been adsorbed to return to the bioremediation site (Collins and Stotzky,1989). In order to trap heavy metals away from the environment, the biomineralization method functions as a beneficial alternative and appropriate strategy. It has an advantage over other standard bioremediation procedures (Knox 2002). Even though biomineralization-based material creation is now receiving a lot of interest, only a few microorganisms have been completely exploited for their potential. Therefore, using MICCP for heavy metal bioremediation has received relatively little attention and the use of *Bacillus* sp. CT5 for the purpose is seldom noticeable, we have concentrated on it in this study.

2. OBJECTIVES

1. Biomineralization efficacy of *Bacillus* sp. CT5
2. Physiological characterization of *Bacillus* sp. CT5 under metal stress
3. Removal of heavy metal, Cr and Pb from the aqueous environment through biomineralization

3. REVIEW OF LITERATURE

3.1 Heavy metals

Metal complexes, which often have high atomic weights, specific gravities and relative densities are known as heavy metals. Due to increased industrialization, urbanisation, and some risky farming practises, more and more heavy metals are being released into the environment, which has a negative impact on the ecosystem by poisoning the soil and water (Ojuederie and Babalola 2017). Over 20 million hectares of land and more than 5 million individual plots of soil have been discovered to be contaminated by heavy metals or metalloids such Cd, Cr, Hg, As, Pb, Co, Cu, Ni, Zn, and Se (Liu *et al*., 2018). Agricultural practices may further enhance the accumulation levels of Cd, Pb, and Zn in soils close to mining regions in Oman, China, Australia, and the United Kingdom, where Cu and Cd in soils around copper mining areas typically surpass moderate to heavy contamination levels (Chen *et al*., 2022). Heavy metals from the environment can enter the body of a person through the food chain and accumulate over time, compromising the operation of the brain, lungs, kidney, liver, blood components, etc. Cancer, heart and mental illnesses, liver damage, central nervous system abnormalities, and other ailments connected to it might occur from heavy metal exposure. (Jaishankar *et al*., 2014; Tayang and Songachan 2021). For the health of people and all life, heavy metal pollution is a severe issue. Heavy metal disposal is a byproduct of several processes, including chemical production, painting and coating, mining, extractive metallurgy, nuclear energy, and other sectors. The wildlife and vegetation in lakes and streams are negatively impacted by those metals (A. Sayari *et al*. 2005). Pb is a heavy metal that may be absorbed via human skin and is a particularly hazardous heavy metal for humans. Pb exposure can result in problems in the nervous system, lungs, heart, and kidneys. Human anaemia can result from Pb poisoning. Cr is a heavy metal found in both seawater and the crust of the planet. Numerous illnesses can be caused by Cr. Large amounts of Cr are released into the air, water, and soil by human activities including metallurgy, refractory, and chemical production, which affect the health of humans, animals, and aquatic life. Consuming tainted Cr-containing food and drink can demonstrate Cr exposure. Cr bioaccumulation has been linked to several illnesses and conditions, including renal, cutaneous, neurological, and gastrointestinal issues. Cr bioaccumulation can result in the development of several cancers, including those of the lungs, throat, testicles, bladder, kidney, thyroid, bone, etc. (Mood *et al*., 2021).

3.2 Removal of heavy metals by biomineralization

Heavy metals as they have such harmful effects are necessary to remove from sources. The only way to remediate heavy metals, unlike organic contaminants, is to change them into less hazardous forms or to restrict their mobility, such as by microbial absorption, transformation, mineralization, or immobilization (Ayangbenro and Babalola 2017; Cheng *et al*., 2016). Heavy metals' bioavailability and toxicity are greatly influenced by their environmental forms, with those in the exchangeable state being the most dangerous (Chen *et al*., 2021). Oxidation-reduction techniques, filtration, electrochemical treatment, evaporation, ion exchange, or reverse osmosis are frequently used to remove harmful metals (Radhika *et al* 2006). These techniques have several drawbacks, including a need for significant amounts of energy and chemicals, limited efficiency and frequent production of sludge. Microorganisms can modify the chemical or physical characteristics of heavy metals to change how they are transported and transformed in the environment (Liu *et al*., 2021). Heavy metal pollution in the environment may be remedied using the popular and effective technique of microbial mineralization of heavy metals.

The process by which organisms create extracellular, intercellular, or intracellular crystals is called biomineralization. In Fig. 1, bacterial structure and a conceptual representation of calcium carbonate synthesis are displayed. Mineral precipitation happens when positively charged ions successfully bond with negatively charged microbial cell walls. The crystals produced by this process frequently differ from inorganic mineralization in terms of their appearance and elemental makeup (Niedermeier *et al*., 2018). Indirectly or directly, interactions between heavy metals and microorganisms can influence the development of minerals and change the physical and chemical characteristics of heavy metals and their compounds (Zheng *et al*., 2021). Biologically-induced mineralization (BIM) and biologically-controlled mineralization (BCM) are the two primary categories of biomineralization (Li *et al*., 2014; Zheng *et al*., 2021). BIM is a passive mineralization process in which the organism's metabolic activity modifies the local environment to create conditions favourable for mineral precipitation, whereas BCM is an active mineralization process in which the cellular processes of selective organisms completely regulate the formation, composition, morphology, and location of minerals (Han *et al*., 2020; Krajewska 2018; Li *et al*., 2014; Park and Faivre 2022; Zheng *et al*., 2021). BIM has been utilized in more recent projects as compared to BCM. More than 60 minerals, including carbonates, phosphates, sulfides/sulfates, arsenates, silicates, chlorides, fluorides, oxides, hydroxides, and ferromanganese oxides are known to develop during biomineralization (Schütze *et al*., 2013; Tayang and Songachan 2021; Wu *et al*., 2021).

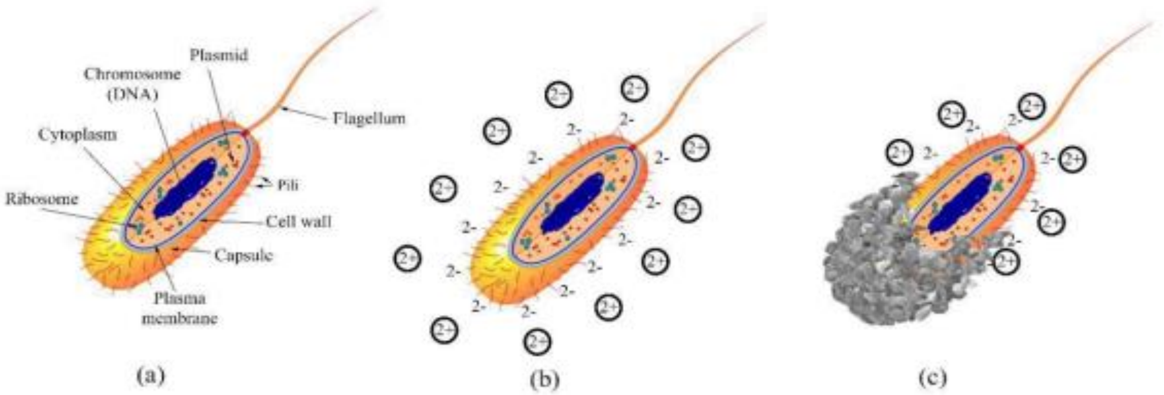


Fig 1. (a) Bacterial cell; (b) Negatively charged cell wall and presence of positive charged ions; (c) Microbial mineralization by binding of ions to cell wall (Seifan et al., 2016)

3.3 Microbially Induced Calcium Carbonate Precipitation (MICCP)

Almost all bacteria have the ability to precipitate $CaCO_3$, according to Boquet *et al*., (1973), whereas Knorre and Krumbein (2000) concluded that MICCP is a byproduct of microbial metabolic activities. Since there is now a lot of interest in the creation of mimic materials based on biomineralization, the major focus of biotechnical engineering research to date has been on microbially induced calcium carbonate precipitation, a component of biomineralization. The carbonate precipitation brought on by microbial metabolic activity is a continuous, pollution-free process.

There have been numerous attempts to use MICCP in different fields, including the removal of heavy metals (Warren *et al*., 2001; Wu *et al*., 2011; Achal *et al*., 2011c, 2012); biodegradation of pollutants (Simon *et al*., 2004; Chaturvedi *et al*., 2006); removal of calcium from waste-water (Hammes *et al*., 2003); bio-remediation (fixation) of metal-contaminated soil and groundwater (Warren *et al*., 2001; Mitchell and Ferris, 2006), atmospheric CO_2 sequestration (Dupraz *et al*., 2009; Ramanan *et al*., 2009; Shaffer, 2010; Mitchell *et al*., 2010; Sharma and Bhattacharya, 2010; Yadav *et al*., 2011), polychlorinated biphenyl removal (Okwadha and Li, 2011). During the MICCP process, Heavy metals can either coprecipitate with Ca^{2+} and CO_3^{2-} during their precipitation and get immobilized in the calcium carbonate lattice, or they can directly precipitate with the produced CO_3^{2-} to form their mineralization products (Khadim *et al*., 2019; Krajewska.2018).

By applying the MICCP phenomenon, heavy metal ions found in the soil, air, and water can be neutralised. Heavy metals with low solubility product constants (Ksp), such as Pb, Cu, Zn and Cd can readily form carbonate minerals with carbonate ions (Khadim *et al*., 2019; Li *et al*., 2013). However, comparable heavy metal ions (such as Pb^{+2}, Cu^{+2}, Zn^{+2}, and Cd^{+2}) occupy the potential sites of certain Ca^{+2} ions in the lattice to create co-precipitation when Ca^{+2} ions engage in MICCP mineralization (Kim and Lee, 2019). Heavy metals and calcium carbonate can both precipitate more quickly as a result of urease-producing bacteria was proved by Li *et al*., (2015). The carbonates are produced by mainly three major metabolic pathways of micro-organisms urea hydrolysis, redox reactions, and photosynthesis.

Microorganisms that generate urease catalyze the breakdown of urea molecules to create carbonate ions. The formation of small carbonate crystals occurs, which are further aggregated into thin sheets, as depicted in Fig. 2. When urease-producing bacteria cause carbonate precipitation, urea is broken down into carbonate ions, and carbonate ions are formed along with metal ions as a result of microbial metabolism (Jalilvand *et al*., 2020). Microorganisms that produce the urease enzyme catalyze the breakdown of urea molecules to create carbonate ions (Seifan and Berenjian, 2019). Small carbonate crystals start to develop and eventually group together to create thin sheets. While the area of carbonate crystals rises, the microbe's surface gaps shrink. (Jose Castro-Alonso *et al*., 2019).

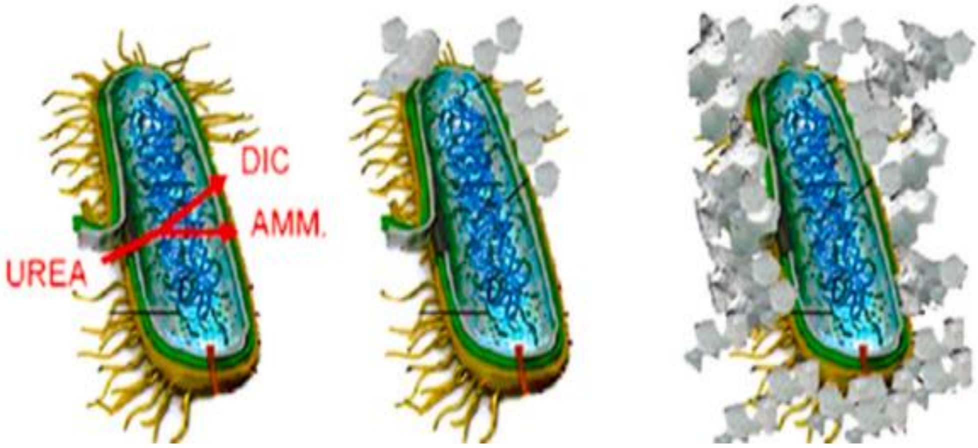


Fig. 2 The process of MICCP by urease producing microorganisms (Modified from Rajasekar et al., 2021; Hammes & Verstraete., 2002).

Fig 3. shows different kinds of metal cations that can alter the morphology of carbonate minerals during MICCP.

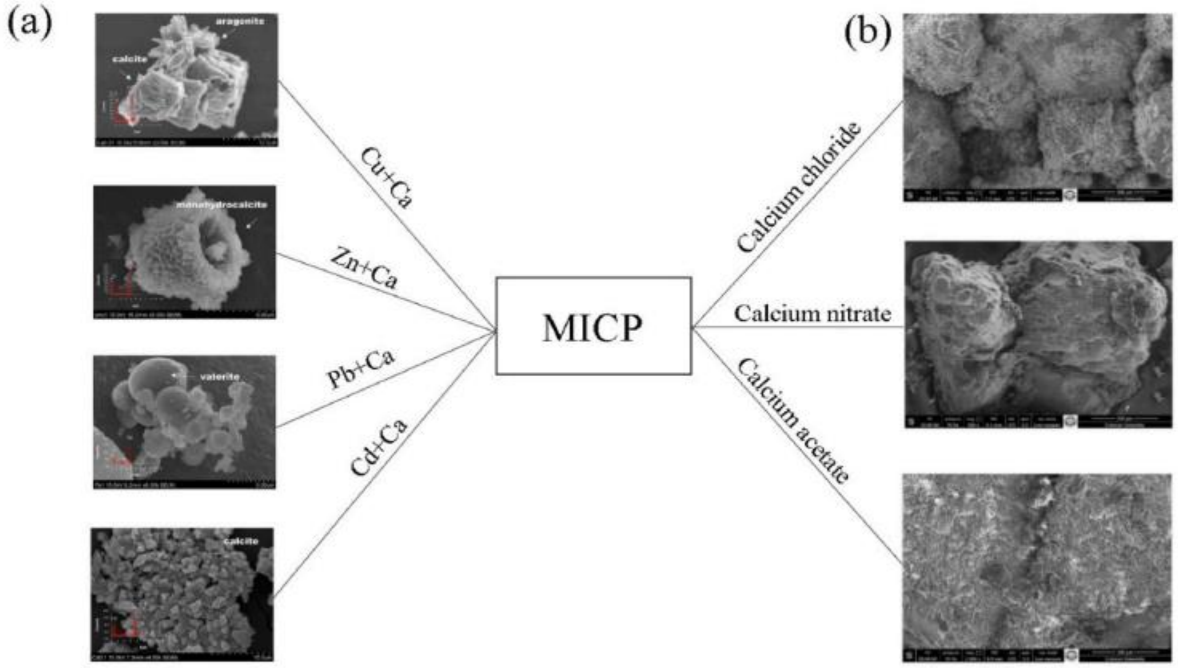


Fig 3. Effects of different ions on mineral morphology of calcium carbonate minerals (Akoguz et al., 2019; Kim et al., 2021; Kumar et al., 2023).

3.4 Optimum conditions for biomineralization

Production of urease enzyme is a major factor affecting biomineralization. The proportion of urea and Ca^{2+} in the medium, the media's pH and ionic strength, the reaction temperature, and the type of bacterial strain all affect the production of the urease enzyme. Biomineralization is significantly influenced by these variables. In the presence of citrate and urea, Sharma et al. (2008) showed a great amount of increase in the synthesis of enzymes.

3.5 Urease enzyme

Urease (UA; EC 3.5.1.5) is a nickel-containing metalloenzyme which is found in a wide range of microorganisms and plants, some of which produce this enzyme in large quantities (Mobley et al., 1989). The first enzyme to crystallise and be discovered to include nickel was urease from Jack bean (Canavalia ensiformis) by (Sumner, 1926). An ideal microbial source of urease for the production of carbonate must be tolerant to high concentrations of urea and calcium. A high level of urease activity that is either constitutively produced (i.e., a consistent amount of enzyme is expressed per cell) or can be reliably activated should also be present in the organism.

The simplest and most controllable method of MICCP, which has the ability to create large amounts of carbonates quickly, is the precipitation of carbonates by urea hydrolysis by ureolytic bacteria. A microbial enzyme called urease hydrolyzes one mole of urea intracellularly into one mole each of ammonia and carbonate, which then spontaneously hydrolyzes into another one mole each of ammonia and carbonic acid. Fig. 4. depicts microbial carbonate precipitation induced by ureolysis.

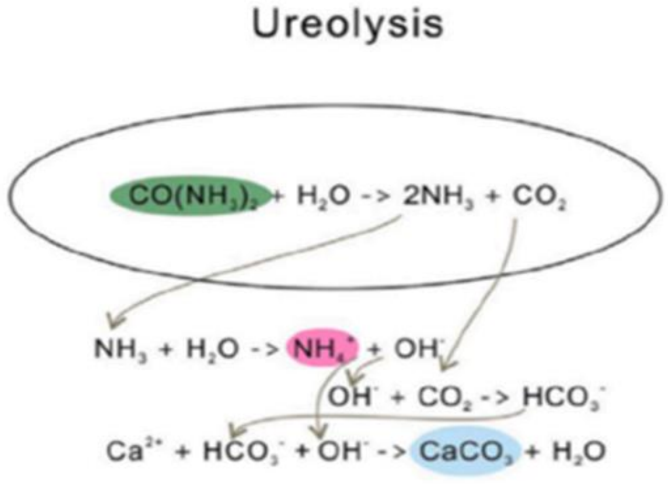


Fig 4. Microbial induced calcium carbonate precipitation by ureolysis mechanism (H. Lin et al. 2023)

3.5 ***Bacillus* sp. CT5**

Using the enrichment culture method, two bacterial strains known as CT2 and CT5 were recovered from samples of very alkaline cement. The bacteria were classified as Bacillus species by various examinations of the 16S rRNA gene sequence and other physiological tests. Both isolates may be categorised as alkalophiles since they were able to thrive in extremely alkaline levels. Both isolates (CT2 and CT5) were Gram positive, rod-shaped, non-motile, and catalase and oxidase positive on an agar plate. No pigments were produced by these isolates. These isolates' morphological and physiological characteristics are similar to those of previously described Bacillus species, which was further supported by 16S rRNA gene analysis. It appears that the genus Bacillus is well suited for long-term survival in harsh conditions. In contrast to CT2, which could only hydrolyse starch, CT5 was able to hydrolyze casein, gelatin, and esculin. It was discovered that both isolates generate sizable levels of urease. It is known that bacteria use urease to hydrolyse urea in order to raise the ambient pH, use it as a supply of nitrogen, and use it as a source of energy. For CT2 and CT5, the urease production was 575.87 U/ml and 670.71 U/ml, respectively. After being incubated for 12–16 hours, the CT2 and CT5 isolates, respectively, showed growth in the pH ranges of 6.5–10 and 6–11, however after being incubated for 72 hours, these isolates were able to survive pH levels of up to 12.6 and 12.9, respectively. When subjected to salinity and temperature tests, CT2 and CT5 were able to survive 0-8% and 0-10% NaCl concentrations and a temperature range of 25-50° C and 25-55° C. The phylogenetic analysis divided CT2 into *B. fusiformis* and *Lysinibacillus sphaericus*, and CT5 into *B. cereus, B. anthracis, and B. mycoides* (Achal *et al*., 2010).

4. MATERIALS AND METHODOLOGY

4.1 Bacterial sample

Bacillus sp. CT5 is a ureolytic bacteria used in the current study for the removal of heavy metals like Pb and Cr from contaminated water. It can withstand a wide range of pH 6–11 and survive in a wide range of temperature 25-55°C. *Bacillus* sp. CT5 was isolated in our lab at TIFAC-CORE, TIET, Patiala (Achal *et al.,*2010). Bacteria was sub-cultured every 10 days on freshly prepared nutrient agar petri plates for the maintenance of the bacteria as shown in Fig. 5.

Fig 5. *Bacillus* sp. CT5 bacteria on nutrient agar plates

Chromium

4.2 Determination of inhibitory concentration (IC_{50}) under chromium stress

- Heavy metal stock solution for Cr was prepared of conc 12 gm/L by adding potassium dichromate ($k_2Cr_2O_7$) from which further working concentrations of metal solutions were prepared.

- Then the stock solution was filter sterilized by passing through a syringe filter with a pore size of 0.22 microns.
- At 37 °C and 120 rpm, the bacterial culture was inoculated into nutrient broth and left to grow overnight.
- 100 ml of nutrient broth was autoclaved in separate flasks.
- Different concentrations of Cr (0.34 µM, 0.68 µM, 1.02 µM, 1.36 µM, 1.7 µM, 2.04 µM, 2.38 µM, 2.72 µM, 3.06 µM, 3.4 µM) were prepared by accordingly adding heavy metal stock solution to every flask.
- The OD_{600} of the overnight culture was taken and adjusted to 0.5 and then 1 ml of culture was added to every flask.
- Then flasks were kept in a shaking incubator at 37°C for 24 hours for incubation at 120 rpm.
- After 24 hours of incubation, 1 ml solution was taken out and OD_{600} was checked with spectrophotometer.
- By using Graph Pad Prism software to display the OD_{600} values obtained for the log of different concentrations, the IC_{50} value for Cr was determined.

4.3 Urease activity under various concentrations of Chromium stress

- Urease activity of the bacterial culture was tested by measuring the amount of ammonia produced due to break down of urea.
- Bacterial cultures were incubated in a shaking incubator at 37°C at 120 rpm for overnight growth.
- 1 ml bacterial culture with $OD_{600} = 0.5$ was re-inoculated into flasks of different concentrations of Cr (0 µM, 0.34 µM, 0.68 µM, 1.02 µM, 1.36 µM, 1.7 µM, 2.04 µM, 2.38 µM, 2.72 µM, 3.06 µM, 3.4 µM).
- All the flasks were incubated at 37°C at 120 rpm for 24 hours.

- After 24 hours 1 ml was taken out from every flask and transferred to freshly autoclaved eppendorfs.
- Centrifuged the eppendorfs at 8000 rpm for 10 minutes.
- Culture filtrate (250µl) was taken out from eppendorfs and transferred to clean test tubes.
- Added 2.5 ml of freshly made 0.1 M urea solution and 1 ml of 0.1 M phosphate buffer (pH 0.8).
- For five minutes, test tubes were incubated at 37°C.
- 1 ml of phenol nitroprusside solution was added.
- 1 ml of alkaline hypochlorite solution was added.
- Again, incubated at 37°C for 25 minutes.
- Optical density at 626 nm was noted as shown in Fig. 6.
- Readings were taken after every 24 hours for 7 days.
- pH was also noted for 7 days after every 24 hours

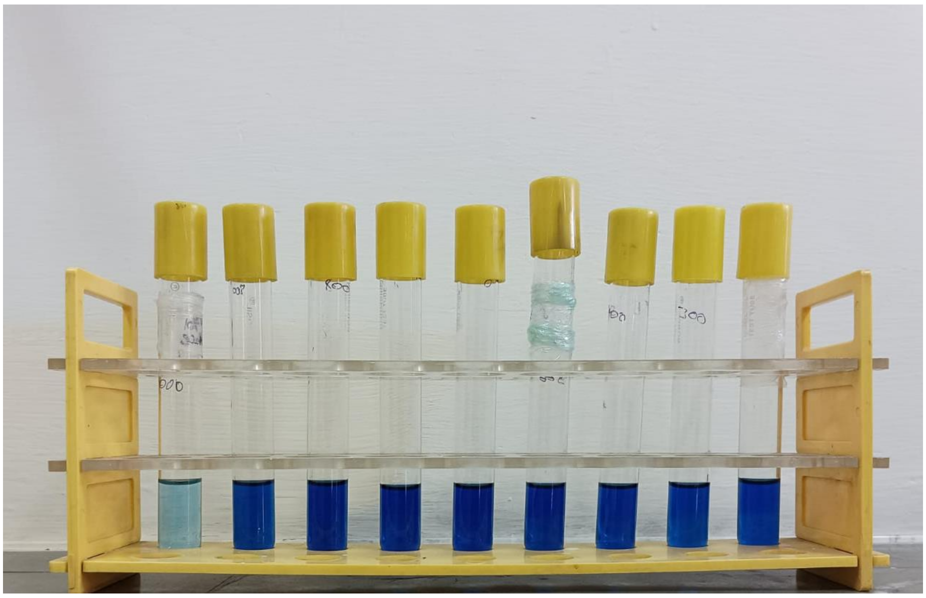


Fig 6. Urease activity test of bacteria under Chromium stress at different concentrations

4.4 Calcium carbonate precipitation under various concentrations of Chromium

- The bacterial culture was inoculated in a freshly autoclaved 100 ml nutrient broth flask and kept for overnight growth at 37°C in a shaking incubator at 120rpm.
- Urea and calcium chloride were added to 100 ml of nutrient broth in separate flasks, resulting in final concentrations of 25 mM calcium chloride and 2% urea.
- The heavy metal stock solution was added to flasks to adjust the final concentration of Cr (0 µM, 0.34 µM, 0.68 µM, 1.02 µM, 1.36 µM, 1.7 µM, 2.04 µM, 2.38 µM, 2.72 µM, 3.06 µM, 3.4 µM).
- One ml of overnight culture is added to every flask after an adjustment of $OD_{600} = 0.5$. After the addition of culture flasks are kept for incubation at 37^0 C, 120 rpm for 7 days in a shaking incubator.
- Quantification of calcium carbonate crystals was done by using the EDTA titration method in which the volume of EDTA used was noted during each titration till the colour of the indicator changed as shown in Fig 7.

Fig 7. Colour change of indicator from pink to blue during start and end point of titration respectively

4.6 Analytical tests

- After 7 days the flasks were removed from the shaking incubator.
- The solution from the flask was centrifuged and the filtrate was transferred to freshly autoclaved flasks and the pellet was collected on a petri plate.
- Further, the filtrate was subjected to Atomic Absorption Spectroscopy (AAS) as test samples for the detection of heavy metals after removal.
- The pellet was collected and drop cast on a copper tape so that it could be examined using a scanning electron microscope (SEM) to determine its topography and an energy disruptive spectrometer (EDS) to determine its elemental makeup.

Lead

4.7 Determination of inhibitory concentration (IC_{50}) under various concentrations of Lead

- Heavy metal stock solution for Pb of conc 12 gm/L was prepared by using Lead nitrate ($(Pb(NO_3))_2$ from which further different concentrations of metal solutions were prepared.
- Then the stock solution was filter sterilized by passing through a syringe filter with the pore size of 0.22 microns.
- The bacterial culture was inoculated in a flask containing 100 ml nutrient broth and was incubated in a shaking incubator overnight at 37°C at 120 rpm.
- 100 ml of nutrient broth was autoclaved in separate flasks.
- By adding the appropriate amount of heavy metal stock solution to each flask, different concentrations of Pb (0 µM, 0.3 µM, 0.6 µM, 0.95 µM, 1.27 µM, 1.59 µM, 1.91 µM, 2.23 µM, 2.55 µM, 2.87 µM, 3.19 µM) were created.
- OD_{600} of overnight culture was adjusted to 0.5 and then 1 ml of inoculum was added to every flask.

- Then flasks were incubated in a shaking incubator at 37^0 C at 120 rpm for 24 hours for incubation.
- After 24 hours of incubation one ml solution was taken out and OD_{600} was checked with spectrophotometer.
- By plotting the OD_{600} values obtained for the log of varying concentrations in Graph Pad Prism software IC_{50} value of lead was calculated.

4.8 Urease activity under various concentrations of lead stress

- The urease activity of bacterial culture was calculated by measuring the amount of ammonia produced.
- The bacterial culture was incubated in a shaking incubator at 37°C at 120 rpm overnight.
- After 24 hours bacterial 1 ml culture was taken out and OD_{600} was adjusted to 0.5 by using spectrophotometer.
- Re-inoculated 1 ml of bacterial culture with an OD_{600} of 0.5 into flasks with various Pb concentrations (0 µM, 0.3 µM, 0.6 µM, 0.95 µM, 1.27 µM, 1.59 µM, 1.91 µM, 2.23 µM, 2.55 µM, 2.87 µM, 3.19 µM).
- All the flasks were incubated in a shaking incubator at 37°C and 120 rpm for 24 hours.
- After 24 hours 1 ml was taken out from every flask and transferred to freshly autoclaved eppendorfs.
- Centrifuged the eppendorfs at 8000 rpm for 5 minutes.
- Culture filtrate (250µl) was taken out from eppendorfs and transferred to clean test tubes.
- Added 2.5 ml of freshly made 0.1 M urea solution and 1 ml of 0.1 M phosphate buffer (pH 0.8).

- For five minutes, test tubes were incubated at 37°C.
- 1 ml of phenol nitroprusside solution was added.
- 1 ml of alkaline hypochlorite solution was added.
- Again, incubated at 37°C for 25 minutes.
- With UV vis spectrophotometer OD at 626 nm was noted as shown in Fig 8.
- Readings were taken after every 24 hours for 7 days.
- pH was also noted for 7 days after every 24 hours.

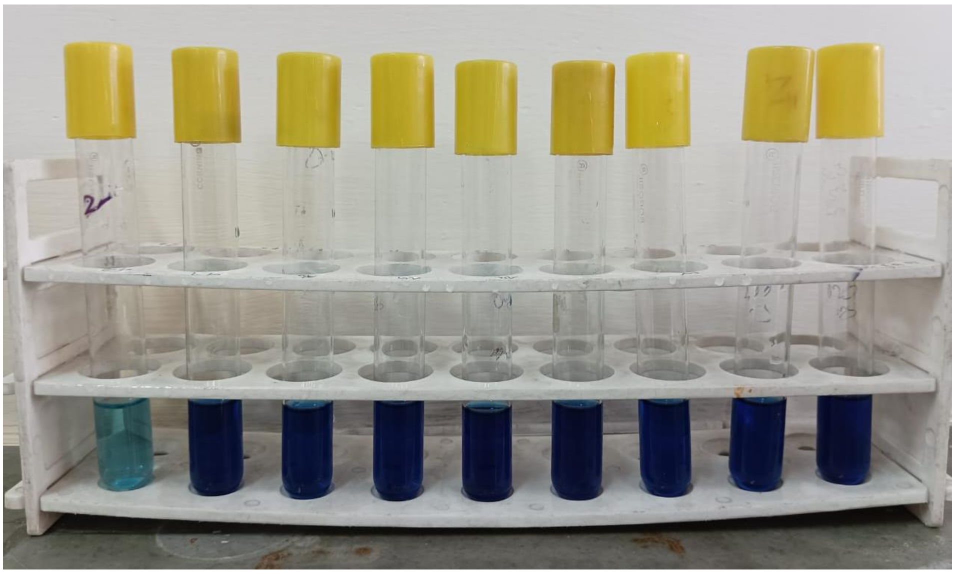


Fig 8. Urease activity of bacteria under lead stress of different concentrations

4.9 Calcium carbonate precipitation under various concentrations of Lead

- Bacterial culture with $OD_{600} = 0.5$ was inoculated in a freshly autoclaved 100 ml nutrient broth flask and incubated for overnight growth at 37°C.
- Urea and calcium chloride were added to 100 ml of nutrient broth in separate flasks, resulting in final concentrations of 25 mM calcium chloride and 2% urea.
- The heavy metal stock solution was added to different flasks to adjust the final concentration of Pb (0 µM, 0.3 µM, 0.6 µM, 0.95 µM, 1.27 µM, 1.59 µM, 1.91 µM, 2.23 µM, 2.55 µM, 2.87 µM, 3.19 µM).

- One ml of overnight culture was added to every flask after adjusting $OD_{600} = 0.5$. After the addition of culture flasks are kept for incubation at 37°C, 120 rpm for 7 days in a shaking incubator.
- Quantification of calcium carbonate crystals was done by using the EDTA titration method (Dhami *et al*., 2016).

4.10 Analytical tests

- After completion of 7 days of the protocol mentioned above flasks were removed from the shaker.
- The solution from the flask was centrifuged and the filtrate was transferred to freshly autoclaved flasks and the pellet was collected on a petri plate.
- Further, the filtrate was deposited to SAI labs for the AAS test (Atomic Absorption Spectroscopy) as test samples for the detection of heavy metals after removal.
- The pellet was collected and drop cast on a copper tape so that it could be examined using a scanning electron microscope (SEM) to determine its topography and an energy disruptive spectrometer (EDS) to determine its elemental makeup.

5. RESULTS

5.1 IC_{50} value determination

The amount of heavy metal required to inhibit the development of 50% of the bacterial population is known as the inhibitory concentration 50 (IC_{50}). The lower IC50 value is a measure of the heavy metal's potency to inhibit bacterial growth. Similar to this, a higher IC50 value means that the bacteria can tolerate a higher concentration of the heavy metal before the heavy metal interferes with the metabolism of the bacteria and causes a discernible decline in growth. IC_{50} value for Pb was calculated to be 1.79 μM. In Fig. 9 (a), The Pb concentrations have been plotted against OD_{600}. Fig 9 (b) shows the normalized absorbance plotted against the increasing Pb concentrations.

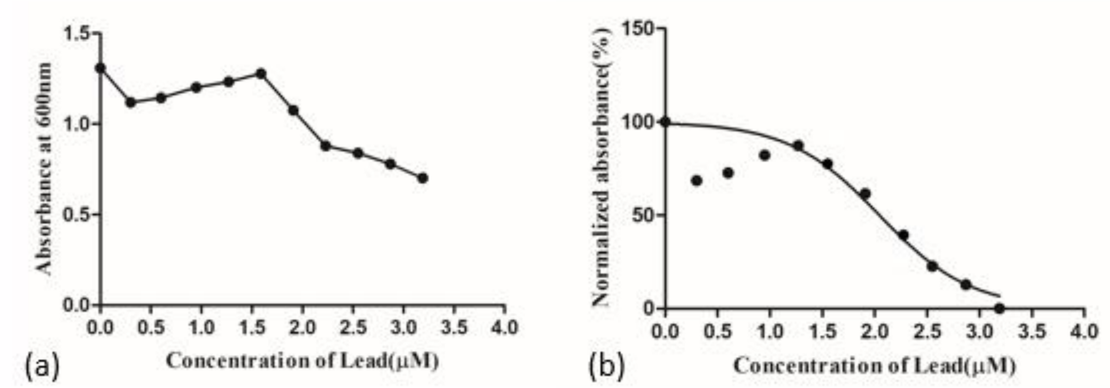


Fig 9.a. The effect of increasing lead concentrations on OD_{600} (b) Normalized absorbance vs increasing lead concentrations

IC_{50} value for Cr was calculated to be 1.64 μM by plotting the normalized absorbance against increasing concentrations of Cr on the graph pad prism. In Fig. 10 (a), The Cr concentrations have been plotted against OD_{600}. Fig 9 (b) shows the normalized absorbance plotted against the increasing Cr concentrations.

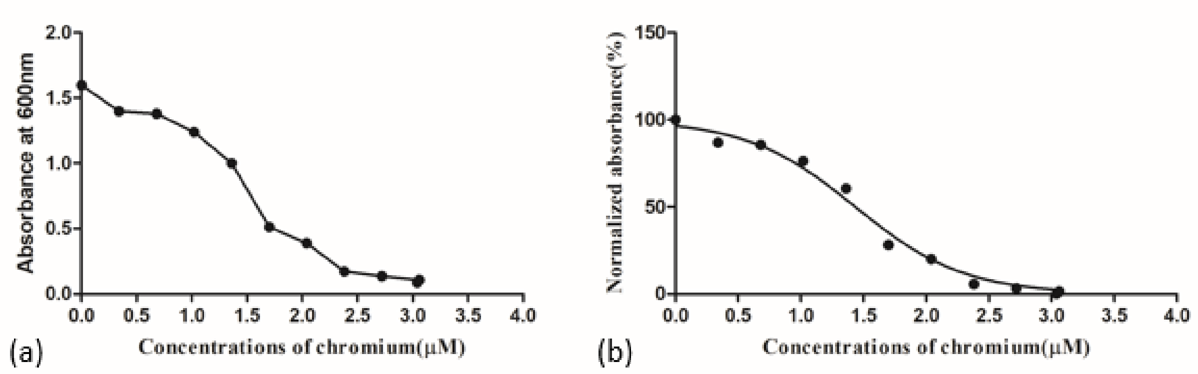


Fig 10.a. The effect of increasing chromium concentrations on OD_{600} (b). Normalized absorbance vs increasing chromium concentrations

5.2 Urease activity of *Bacillus* sp. CT5 under various concentration of Chromium and Lead stress

Urease activity of *Bacillus* sp. CT5 under Cr and Pb stress was tested until a decrease in activity was observed. The quantity of the enzyme hydrolyzing 1 mol of urea per minute is known as one unit of urease, and the larger the amount of hydrolyzed urea, the higher the urease activity. Urease activity was highest spotted in Cr stress to be highest in control (0 μM) concentration at 120 hrs which was calculated to be 641 U/ml. A major rise in urease activity was seen at 120 hrs at each concentration. Urease activity of Cr concentrations 0.34 μM, 0.68 μM, 1.02 μM, 1.36 μM, 1.7 μM, 2.04 μM, 2.38 μM, 2.72 μM, 3.06 μM, 3.4 μM were calculated to be 575 U/ml, 571 U/ml, 569 U/ml, 574 U/ml, 573 U/ml, 571 U/ml, 568 U/ml, 559 U/ml, 546 U/ml and 185 U/ml respectively. A sudden decrease was observed after 3.06 μM as at Conc 3.4 μM was noted to be 185 U/ml. Fig. 11 depicts the change in urease activity under Cr stress at different concentrations, Fig 11. shows highest urease activity at 120 hrs.

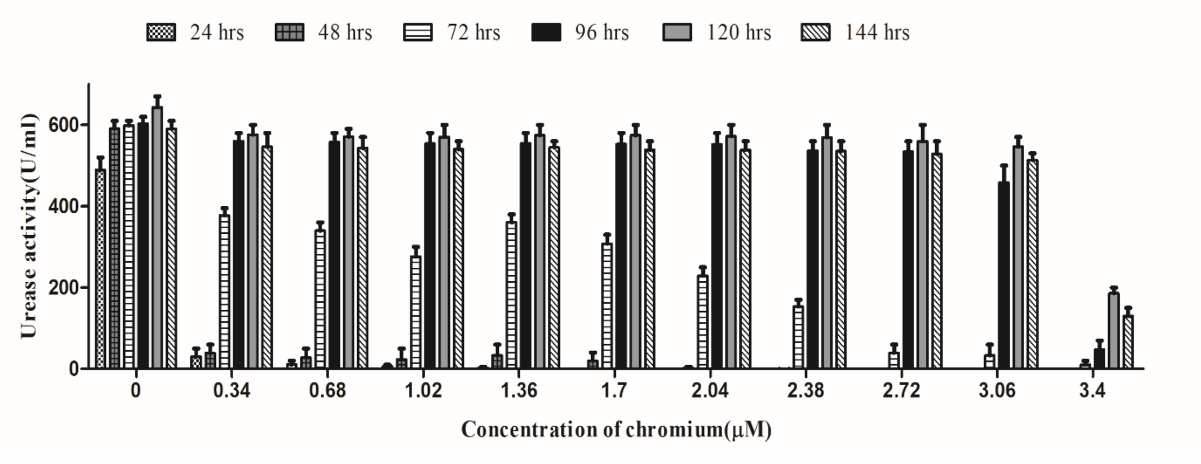


Fig 11. Change in urease activity under various concentrations of chromium stress

Similarly, urease activity under Pb stress was also tested and the highest urease activity was observed in control (0 μM) to be 648 U/ml at 96 hrs. Urease activity at Pb concentrations 0.3 μM, 0.6 μM, 0.95μM, 1.27 μM, 1.59 μM, 1.91 μM, 2.23μM, 2.55 μM, 2.87 μM, 3.19 μM was calculated to be 620 U/ml, 594 U/ml, 591 U/ml, 590 U/ml, 588 U/ml, 587 U/ml, 585 U/ml, 582 U/ml, 90 U/ml and 82 U/ml. There was a sudden decrease in urease activity at 2.87 μM metal concentration as shown in Fig 12.

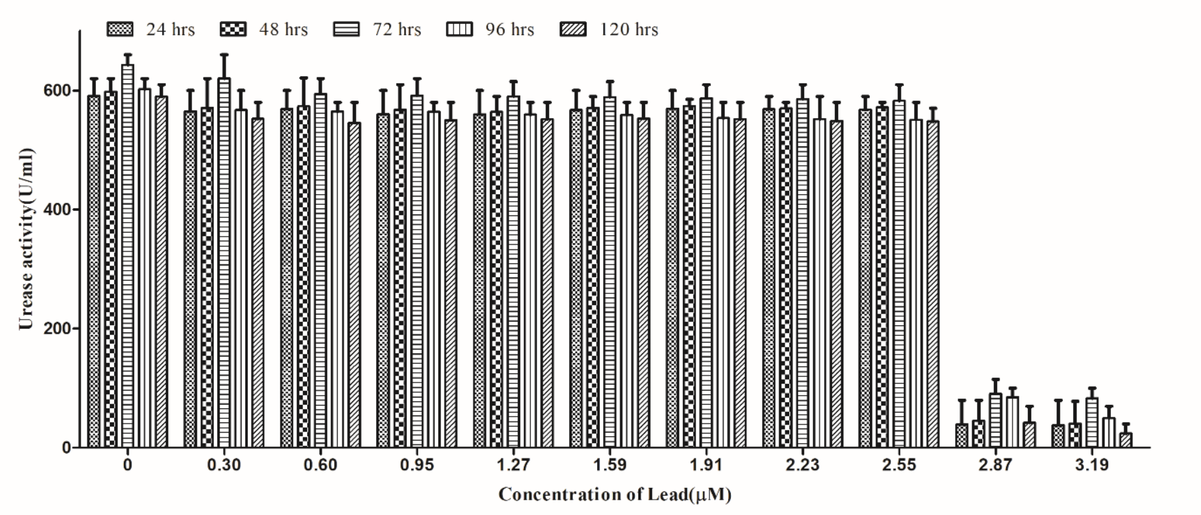


Fig 12. Change in urease activity under various concentrations of lead stress

5.3. Calcium carbonate precipitation under various concentrations of Chromium and Lead

Seven days after the incubation, calcium carbonate precipitation was seen in nutrient broth that had been supplemented with urea and calcium chloride. The EDTA titration method was used for measuring calcium carbonate precipitation. In this procedure, the Ca^{2+} in the water combines with the Erichrome black T indicator to produce a pink coloured unstable compound. After titration, EDTA chelates Ca^{2+} ions, causing a colour shift that is regarded as the end point.

Calcium carbonate precipitation was tested under Cr stress on concentrations 0 µM (control), 0.34 µM, 0.68 µM, 1.02 µM, 1.36 µM, 1.7 µM, 2.04 µM, 2.38 µM, 2.72 µM, 3.06 µM, 3.4 µM and amount of calcium carbonate precipitated was observed to be 182 mg/100ml, 172 mg/100ml, 167 mg/100ml, 150 mg/100ml, 145 mg/100ml, 142 mg/100ml, 133 mg/100ml, 92 mg/100ml, 71 mg/100ml, 64 mg/100ml, 32 mg/100ml respectively. The highest calcium carbonate precipitation was observed 182 mg/100ml in control (0 µM) at 168 hrs. The lowest recorded calcium precipitates were 32 mg/100ml at 3.4 µM Cr concentration as depicted in Fig 13.

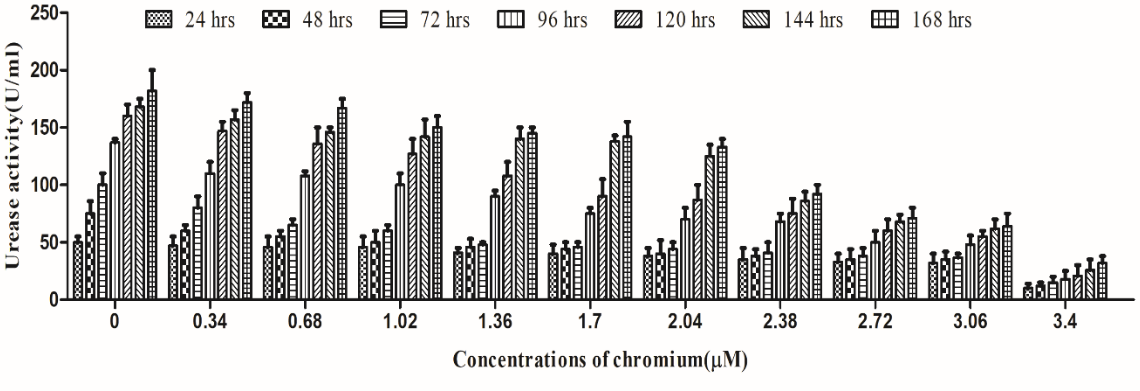


Fig 13. Change in calcium carbonate precipitation at different concentrations of chromium stress till 168 hrs

Pb stress on concentrations of control (0 µM), 0.3 µM, 0.6 µM, 0.95 µM, 1.27 µM, 1.59 µM, 1.91 µM, 2.23 µM, 2.55 µM, 2.87 µM, 3.19 µM was examined for calcium carbonate precipitation. The amount of calcium carbonate precipitated was calculated to be 217 mg/100ml, 198 mg/100ml, 179 mg/100ml, 167 mg/100ml, 157 mg/100ml, 150 mg/100ml, 140 mg/100ml, 90 mg/100ml, 72 mg/100ml, 68 mg/100ml, 60 mg/100ml respectively. The highest calcium carbonate precipitates were observed 217 mg/100ml in control (0 µM) at 168 hrs. The lowest recorded calcium precipitates were 60 mg/100ml at 3.19 µM concentration as shown in Fig 14.

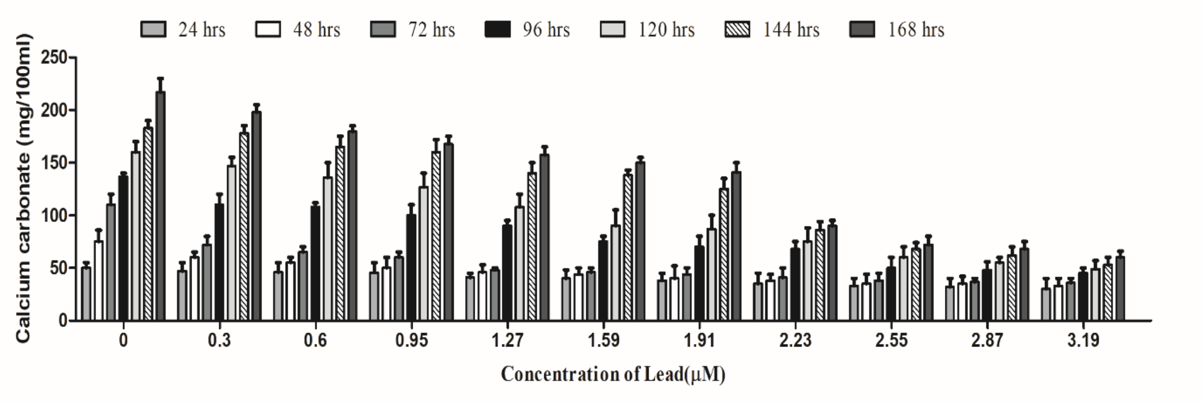


Fig 14. Change in calcium carbonate precipitation at different concentrations of lead stress till 168 hrs

After observing the results of calcium carbonate precipitation under both Cr and Pb stress concentrations selected for AAS test were 1.36 µM, 1.7 µM, 2.04 µM for Cr stress and 1.27 µM, 1.59 µM, 1.91 µM for Pb stress. These concentrations were selected for AAS test as a sudden drop in calcium carbonate precipitation can be observed after 2.04 µM which indicates that heavy metal stress after 2.04 µM is higher due to which hindrance in bacterial functioning increases which intern would lead to less removal of heavy metals in higher concentrations.

5.4. pH variation while calcium carbonate precipitation

Due to the production of urease enzyme by *Bacillus* sp. CT5, urea is hydrolysed which resulted in the production of ammonium ions due to which increase in the pH of the medium can be observed. This phenomenon causes the heavy metal present in the solution to bond to Ca^{2+} ions. Similar research by Achal et al. (2012) demonstrates that the production of carbon dioxide during ureolysis, as well as the presence of ammonium ions, are both responsible for the pH increase. Change in pH was observed till duration of 168 hrs and the pH values at 144 hrs were recorded to be maximum for each concentration. In the case of Cr pH was rising till 144 hrs but after 144 hrs there was negligible change in pH as shown in Fig 15.

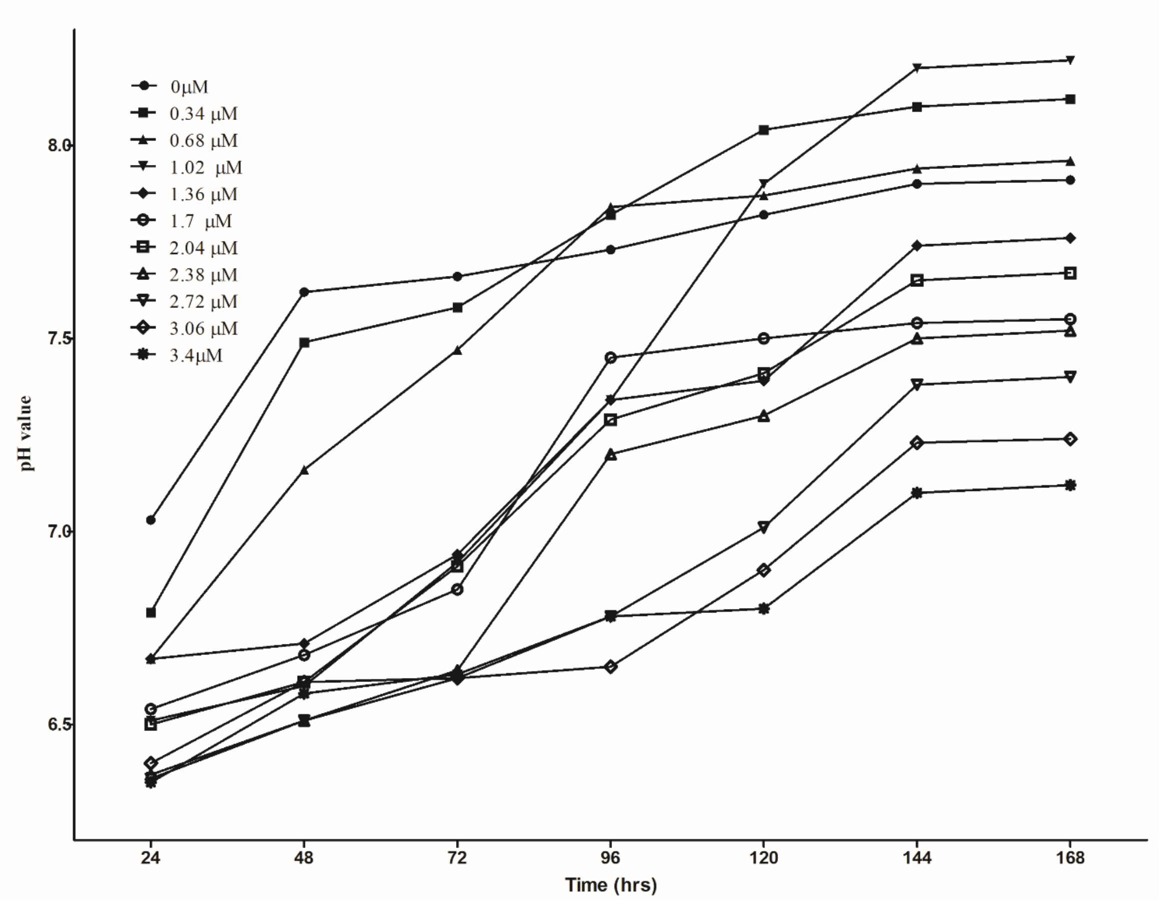


Fig 15. Change in pH from 24 to 168 hours post inoculation in nutrient broth supplemented with different concentration of chromium

pH values were noted for different Pb concentrations till 168 hrs and a major rise was seen till 120 hrs and after 120 hrs the change in pH was noted to be negligible as shown in Fig 16.

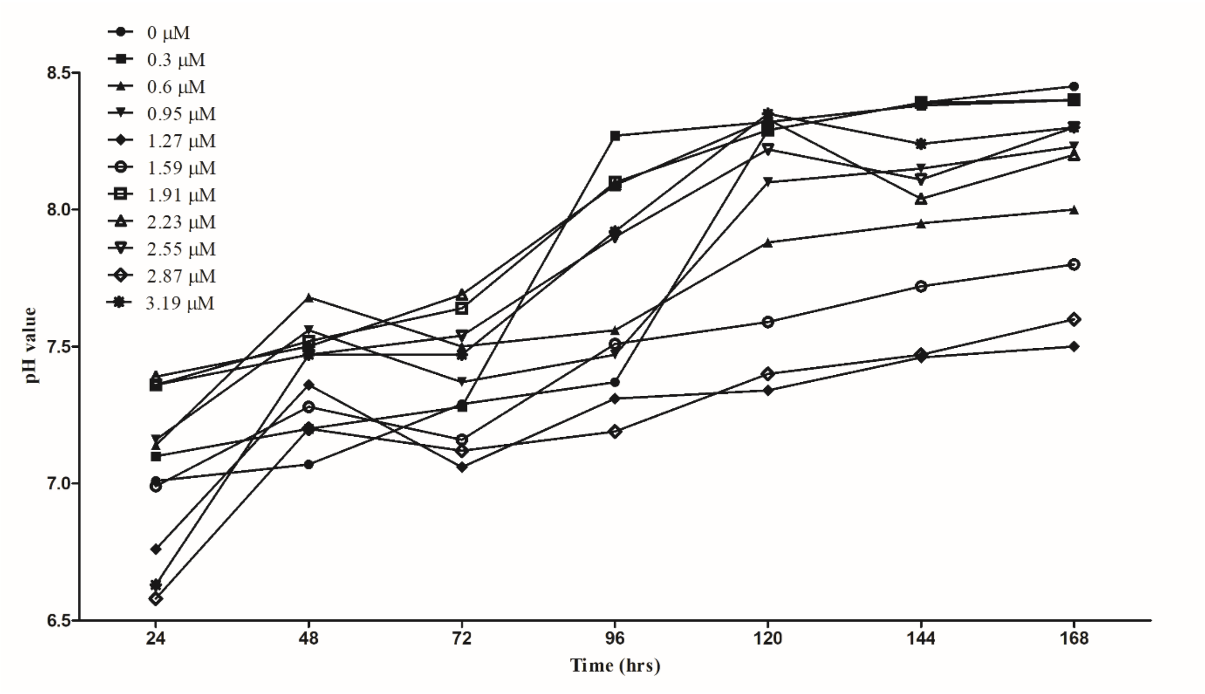


Fig 16. Change in pH from 24 to 168 hours post inoculation in nutrient broth medium supplemented with different concentration of lead

5.5. Assessing the removal of Chromium and Lead through AAS

Atomic absorption spectroscopy was used to quantify heavy metals. The sample must atomize in order to use this technique. After absorbing visible or ultraviolet light, the resultant free atoms change to higher electronic energy levels. The degree of absorption is used to calculate the heavy metal's concentration. Over 70 distinct elements may be identified with AAS. By subtracting the quantity of heavy metal detected from the amount of heavy metal initially present in the solution, the percentage of heavy metal removal was calculated. Quintelas (2009) demonstrated that a biofilm of E. coli supported on kaolin can remove Cr 20 % to 100 %. In our research, 1.36 μM, 1.7 μM, 2.04 μM were selected for analysing removal as there was a drop in calcium carbonate production after these particular concentrations and Cr stress increased and percentage removal was calculated to be 71.95%, 87.24% and 87.80% respectively as shown in Fig 17. Jalilvand et al., (2020) reported *S. rhizophila* removed 96.25% of Pb, *V. boronicumulans* removed 95.93% of Pb, *S. pasteurii* eliminated 98.71% of Pb after 72 hours of incubation. In our research removal was calculated to be 99.57%, 99.69% and 99.08% in the following concentrations of Pb (1.27 µM, 1.59 µM, 1.91 µM). 1.27 µM, 1.59 µM, 1.91 µM were selected for analysing the removal of Pb as there was a drop in calcium carbonate production after these concentrations which shows that Pb stress in higher concentrations increased which resulted in hindrance with *Bacillus* sp. CT5 functioning.

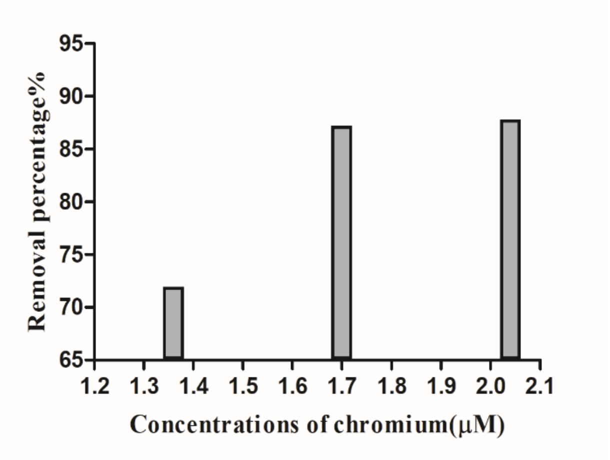


Fig 17. Percentage removal of chromium at different concentrations by AAS

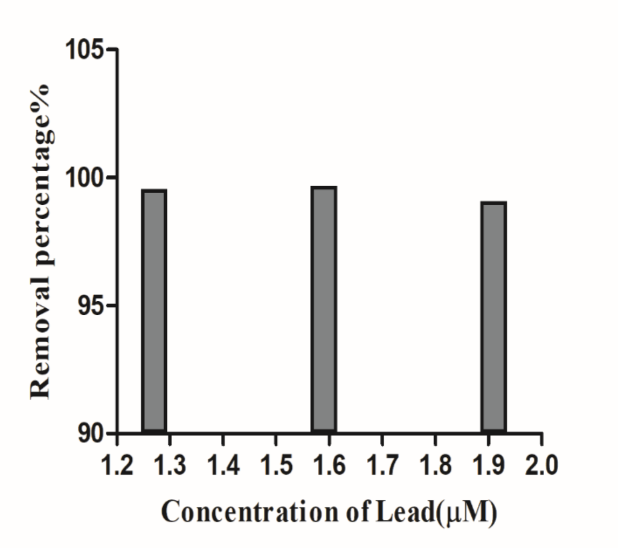


Fig 18. Percentage removal of lead at different concentrations by AAS

5.6. SEM and EDS analysis of the bio-precipitates

Scanning electron microscopy (SEM) was used to examine the morphology of the precipitated calcium carbonate crystal. The calcium carbonate-heavy metal ion combination was shown using FESEM. EDS was performed to determine the percentage of heavy metal content and other elements present in the precipitates obtained. Fig 19. and Fig 21 show heavy metal-calcium carbonate bio-precipitates' SEM pictures containing calcium carbonate crystals and bacterial cells. Calcium carbonate crystals and bacterial cells were labelled as CC and BC respectively as depicted in Fig 19 and Fig 21. Fig 22 and Fig 23 show the elemental makeup of heavy metal-calcium carbonate bio-precipitates along with the EDS graphs.

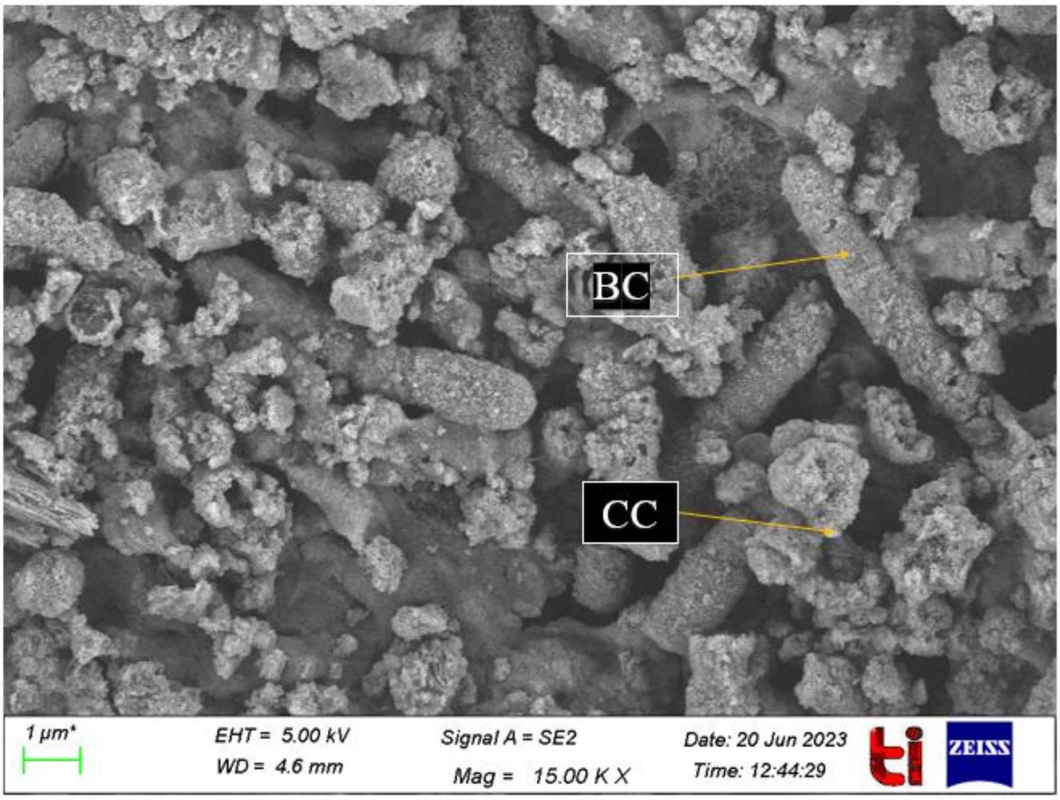
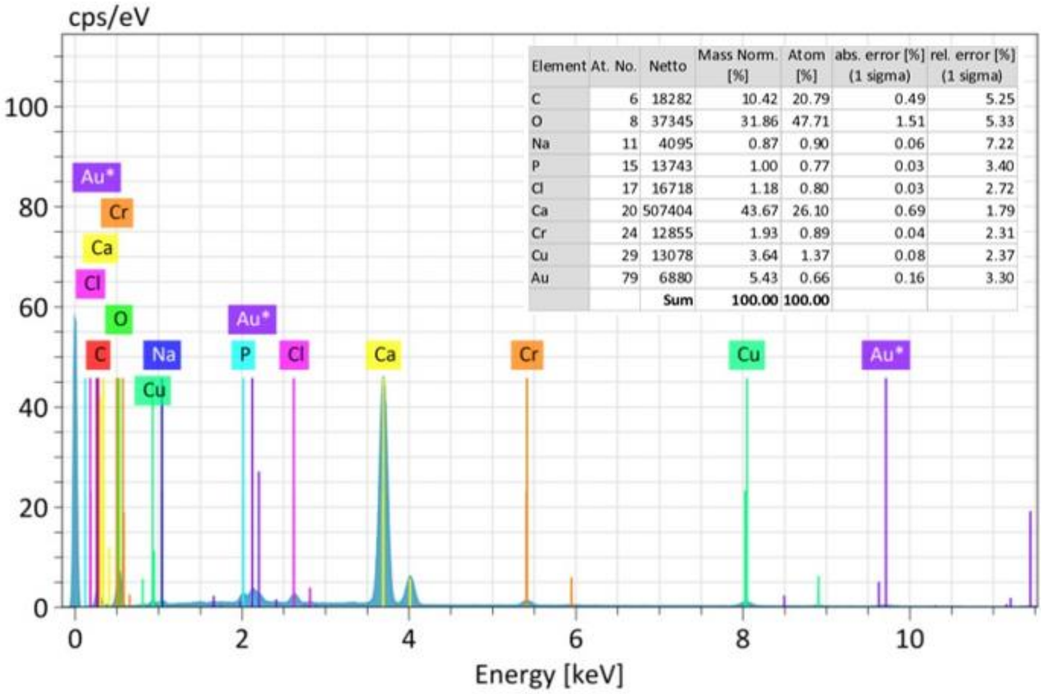


Fig 19. FESEM image of calcium carbonate crystals in chromium sample



Element	At. No.	Netto	Mass Norm. [%]	Atom [%]	abs. error [%] (1 sigma)	rel. error [%] (1 sigma)
C	6	18282	10.42	20.79	0.49	5.25
O	8	37345	31.86	47.71	1.51	5.33
Na	11	4095	0.87	0.90	0.06	7.22
P	15	13743	1.00	0.77	0.03	3.40
Cl	17	16718	1.18	0.80	0.03	2.72
Ca	20	507404	43.67	26.10	0.69	1.79
Cr	24	12855	1.93	0.89	0.04	2.31
Cu	29	13078	3.64	1.37	0.08	2.37
Au	79	6880	5.43	0.66	0.16	3.30
		Sum	**100.00**	**100.00**		

Fig. 20 EDS analysis to determine the elemental composition of chromium-calcium carbonate bio-precipitates

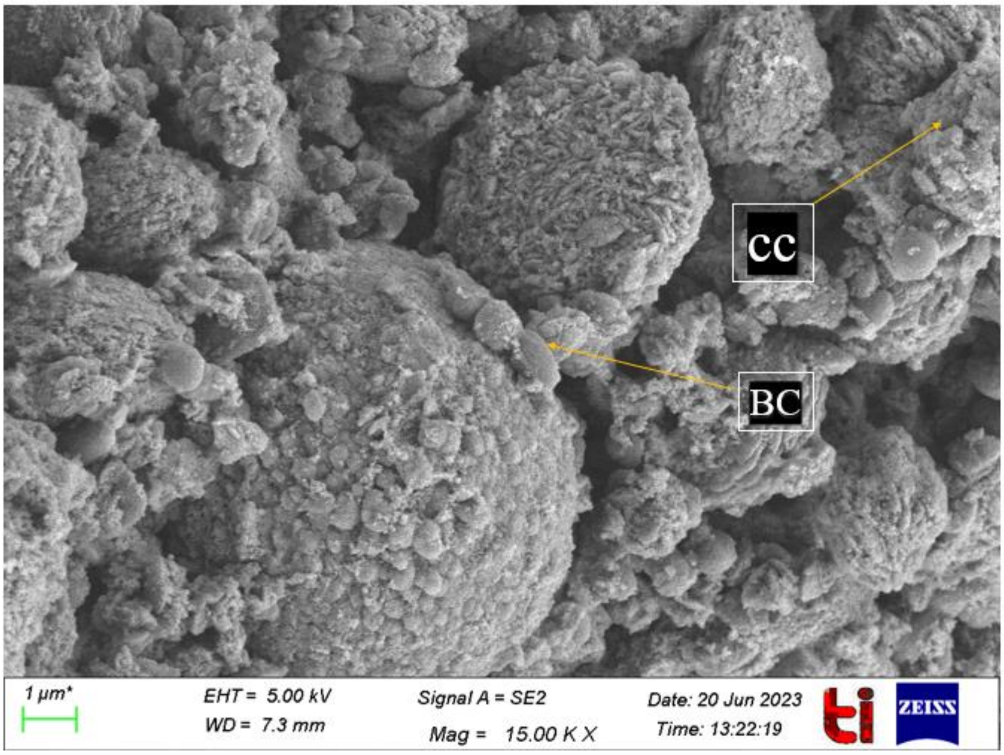
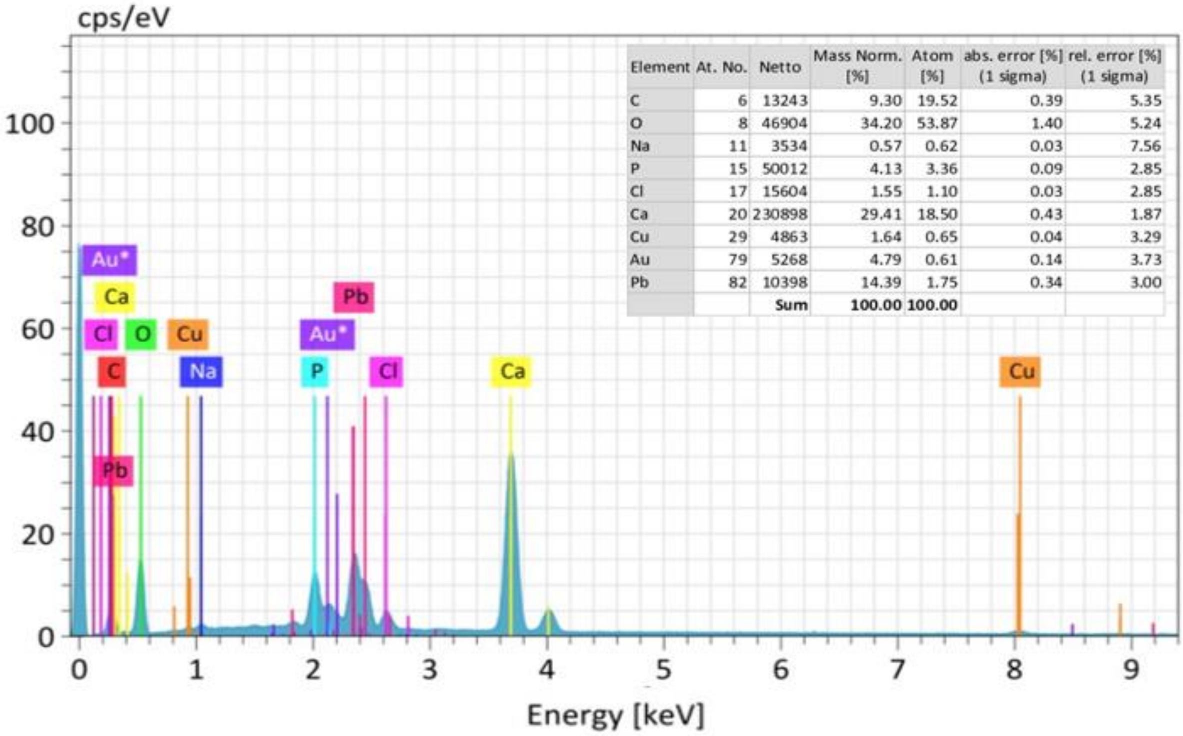


Fig 21. FESEM image of calcium carbonate crystals in lead sample



Element	At. No.	Netto	Mass Norm. [%]	Atom [%]	abs. error [%] (1 sigma)	rel. error [%] (1 sigma)
C	6	13243	9.30	19.52	0.39	5.35
O	8	46904	34.20	53.87	1.40	5.24
Na	11	3534	0.57	0.62	0.03	7.56
P	15	50012	4.13	3.36	0.09	2.85
Cl	17	15604	1.55	1.10	0.03	2.85
Ca	20	230898	29.41	18.50	0.43	1.87
Cu	29	4863	1.64	0.65	0.04	3.29
Au	79	5268	4.79	0.61	0.14	3.73
Pb	82	10398	14.39	1.75	0.34	3.00
		Sum	**100.00**	**100.00**		

Fig. 22 EDS analysis to determine the elemental composition of lead-calcium carbonate bio-precipitates

6. CONCLUSIONS

IC_{50} values for Pb and Cr were calculated to be 1.79 µM and 1.64 µM respectively.

Urease activity was highest observed in Cr stress to be highest in control (0 µM) at 120 hrs which was calculated to be 641 U/ml. Urease activity was observed increasing after 72 hrs in each concentration but the major rise in urease activity was observed at 120 hrs at every concentration. Urease activity under Pb stress was also tested and the highest urease activity was observed in control (0 µM) to be 648 U/ml at 96 hrs. There was a sudden decrease in urease activity after 2.55 µM as at 2.87 µM urease activity was recorded to be 90 U/ml and at 3.19 µM it was recorded to be 82 U/ml respectively.

Calcium carbonate precipitation under Cr stress was observed to be 182 mg/100ml, 172 mg/100ml, 167 mg/100ml, 150 mg/100ml, 145 mg/100ml, 142 mg/100ml, 133 mg/100ml, 92 mg/100ml, 71 mg/100ml, 64 mg/100ml, 32 mg/100ml for concentrations 0 µM (control), 0.34 µM, 0.68 µM, 1.02 µM, 1.36 µM, 1.7 µM, 2.04 µM, 2.38 µM, 2.72 µM, 3.06 µM, 3.4 µM respectively. The highest calcium carbonate was observed 182 mg/100ml in control (0 µM) at 168 hrs. The lowest recorded calcium precipitates were 32 mg/100ml at 3.4 µM concentration. A sudden drop in calcium carbonate precipitation after a concentration 2.04 µM states that hindrance occurred in the functioning of *Bacillus* sp. CT5 as the heavy metal stress increased with increasing concentration of Cr.

Calcium carbonate precipitation in case of Pb was observed to be 217 mg/100ml, 198 mg/100ml, 179 mg/100ml, 167 mg/100ml, 157 mg/100ml, 150 mg/100ml, 140 mg/100ml, 90 mg/100ml, 72 mg/100ml, 68 mg/100ml, 60 mg/100ml for concentrations 0 µM (control), 0.3 µM, 0.6 µM, 0.95 µM, 1.27 µM, 1.59 µM, 1.91 µM, 2.23 µM, 2.55 µM, 2.87 µM, 3.19 µM respectively. The highest calcium carbonate was observed 217 mg/100 ml in control (0 µM) at 168 hrs. The lowest recorded calcium precipitates were 60 mg/100 ml at 3.19 µM concentration. A sudden drop in calcium carbonate precipitation after 2.55 µM states that hindrance occurred in the functioning of *Bacillus* sp. CT5 as the heavy metal stress increased with increasing concentration of Pb.

pH was noted till 168 hrs and the pH values at 144 hrs were recorded to be maximum at every concentration in the case of Cr pH was rising till 144 hrs but after 144 hrs there was a negligible rise in pH values were observed for different lead concentrations till 168 hrs and major rise was seen till 120 hrs and after 120 hrs the rise in pH was observed to be negligible. A rise in pH can be understood as *Bacillus* sp. CT5 is a urease producing bacteria and due to break down of urea and release of ammonium ions due to hydrolysis of urea results in the increase in pH.

Atomic absorption spectroscopy was used for the quantification of the removal of heavy metals. 1.36 µM, 1.7 µM, 2.04 µM were selected for the AAS test as there was a drop in calcium carbonate production after these concentrations which shows increased Cr stress that leads to hindrance in the functioning of *Bacillus* sp. CT5 and percentage removal was calculated to be 71.95%, 87.24% and 87.80% respectively. Similarly, Pb samples were also tested for assessing the removal. The concentration at which the test was performed were 1.27 µM, 1.59 µM, 1.91 µM and removal were calculated to be 99.57%, 99.69% and 99.08% respectively.

The FESEM and EDS data also showed the formation of a heavy metal-calcium carbonate complex, showing that the heavy metals were successfully immobilized by biomineralization.

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