REMOVAL OF TOXIC METALS IN WASTEWATER THROUGH BIOMINERALIZATION

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Under the guidance of

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CERTIFICATE

Certified that the thesis "**REMOVAL OF TOXIC METALS IN WASTEWATER THROUGH BIOMINERALIZATION**" which is submitted by Miss Manjot Kaur, in the fulfilment of the requirement for the award of the degree of Master of Science in Biotechnology in the Department of Biotechnology (DBT), Thapar Institute of Engineering and Technology, Patiala is a record of the candidate's own independent and original research work carried out by her in our guidance and supervision. The matter embodied in this thesis has not been submitted in part or full to any other institute or university for the award of any degree.

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DECLARATION

It is hereby declared that the work which is being presented in the thesis - REMOVAL OF TOXIC METALS IN WASTEWATER THROUGH BIOMINERALIZATION submitted by me for the award of the degree of Master of Science in the department of Biotechnology, Thapar Institute of Engineering andTechnology, Patiala, Punjab is true and original record of my own independent and original research work carried out under the supervision of **Prof. Dr. M. Sudhakara Reddy,** Professor, Department of Biotechnology. The matter embodied in this thesis has not been submitted in part or full to any other institute or university for the award of any degree in India or Abroad.

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ABSTRACT

Due to anthropogenic activities, heavy metals are one of the most toxic xenobiotics contaminating our soil today. The excessive release of untreated wastewater leads to an increase in salinity in water and soil. Conventional methods are not eco-friendly and more expensive for application on a larger scale. Biomineralization is a natural phenomenon occurring in a wide variety of living organisms which leads to production of minerals. Heavy metals are much more toxic in ionic form and the toxicity is significantly reduced when immobilized by reacting with carbonates, sulphides, silicates etc. due to a considerable decrease in solubility because of precipitation. The present study investigates the role of biomineralization by ureolytic halophilic bacteria to remove heavy metals- cadmium and arsenic through microbially induced calcium carbonate precipitation. Calcium carbonate precipitation by bacteria resulted in a significant decrease in the initial heavy metal concentration and removal through precipitation within 7 days of inoculation. The microstructural analysis of the bio-precipitates using FESEM and EDX revealed immobilization of heavy metal by calcium carbonate.

KEY WORDS- Arsenic, Cadmium, Microbially Induced Calcium Carbonate Precipitation, Biomineralization, Ureolytic, Halophillic.

Chapter 1

INTRODUCTION

As the industry is progressing, heavy metals are becoming one of the most toxic xenobiotics contaminating soil and water today. Heavy metal pollution in soil primarily refers to the accumulation of heavy metals, such as lead, mercury, chromium, cadmium, nickel, and other biologically toxic significant heavy elements in the soil [Abdalla et al., 2012], resulting in quantities that are higher than permissible values. The term heavy metal denotes any metallic chemical element that has high density and is toxic or poisonous at even low concentrations. Heavy metals are generally considered to be those metallic elements whose density exceeds 5 g per cubic cm. Some of the heavy metals are cadmium (Cd), arsenic (As), chromium (Cr), Mercury (Hg), and lead (Pb). Detoxification of heavy metals in water bodies and soil around factories has been an issue for a long time

Many industries such as electroplating, metal finishing and polishing, mining and metallurgy, electronic-circuit production, iron and steel processing, pesticide, insecticide application, and fine-chemical and pharmaceutical production discharge a number of toxic heavy metals into the nature (Eccles, 1999). Many metallic elements are essential for biological systems and should be present in a certain concentration bracket. Rather, they provide the living organisms with important cofactors for metalloproteins and enzymes; therefore, very low quantities can lead to a fall in metabolic reactions. Heavy metals are generally classified as essential or non-essential depending upon their role in different biological functions like maintenance of the cell structure and enzyme catalysis (Bruins et al., 2000). The non-essential heavy metals are those that are not required for cellular activity and are extremely toxic for cells even in micro amounts (Haferburg and Kothe, 2007). At greater concentrations, metals can behave in a harmful way by hindering essential functional groups, replacing other metallic ions, or alteringthe dynamic conformation of biological entities (Collins and Stotzky, 1989). In plants, toxicitydue to heavy metals can lead to reduced biomass in plants, poor seed germination, delayed fruitharvest, lesser nutritional content, and overall lesser root length and shoot length and induce

yellowing of leaves and tissue death (Rai et al., 2021). As the solubility of heavy metal ions is pretty high in the environment, they are often ingested by living beings. As these metals progress through the food chain, large quantities of heavy metals deposit in the system. If these toxic elements are ingested above the allowed standards, they can lead to many diseases and disorders (Babel and Kurniawan, 2004) like cancer, improper working of kidneys and lungs, lesions on the skin, etc. Therefore, we need to execute the appropriate techniques for removing the heavy metals from any polluted wastes or soil.

Many conservative methods used for detoxifying heavy metals are oxidation/reduction, reverse osmosis, membrane filtration, electrochemical methods, and ion exchange. Physical methods such as magnetic separation, electrostatic techniques, physical screening, floatation methods, and separation with regards to density. They possess a lot of downsides, such as less efficiency, high demand for chemicals, higher expenses, by-product formation of poisonous sludge, and dangerous disposal of the substances (Cho et al., 2004; Achal et al., 2012). Conventional physicochemical methods for the decontamination of polluted areas are money intensive and generally do not lessen the pollution problem permanently. These methods are said to be inefficient to remediate the heavy metals to the maximum permitted concentration because of the shortcomings which include sample pre-treatment, labour-intensive and expensive excavation, changes in quality of soil due to treatment with reagents, and feasibility.

Various biological decontamination techniques have been studied for their efficacy on a commercial scale as well as eco-friendliness. Microorganisms are diversely distributed. In extremely unfriendly surroundings also, microorganisms are chosen to be appropriate for the detoxification of heavy metals. It has been documented that various organisms possess the ability to adapt or mutate in order to endure the toxicity of heavy metals. As microorganisms are able to endure and proliferate in unhospitable ecosystems, one certain characteristic of these microbes is that they need to possess an efficient enzyme system and/or mechanism to take in, deposit, detoxify and remediate toxic metals.

Biomineralization is a technique through which organisms synthesize inorganic substances as a virtue of their metabolism. Minerals that are formed through biologically induced mineralization via passive surface-mediated mineralization are metal oxides, metal silicates, sulfates, carbonates, phosphates and sulfides (Dhami et al, 2013). Mineral carbonates are the most predominant out of the above-mentioned. Precipitation through microorganisms is the first step of biomineralization in which the nucleation of the heavy metal ions and the development of mineral crystals takes place (Fig. 1). The bacteria mainly endure the metal

stress by forming inclusions within the cell. A huge diversity of microbes can utilize their cell wall parts or enzymes for the beginning of the precipitation of the nucleation site. The parameters that oversee the efficacy of the precipitation include their ability to proliferate in extremely contaminated regions and the ability to endure heavy metal toxicity. Microbially induced calcium carbonate precipitation (MICCP) via urea hydrolysis is an uncomplicated and extensively used method for carbonate precipitation for a variety of technical applications like sequestration of carbon dioxide, and concrete crack remediation, saving historical buildings from deterioration.

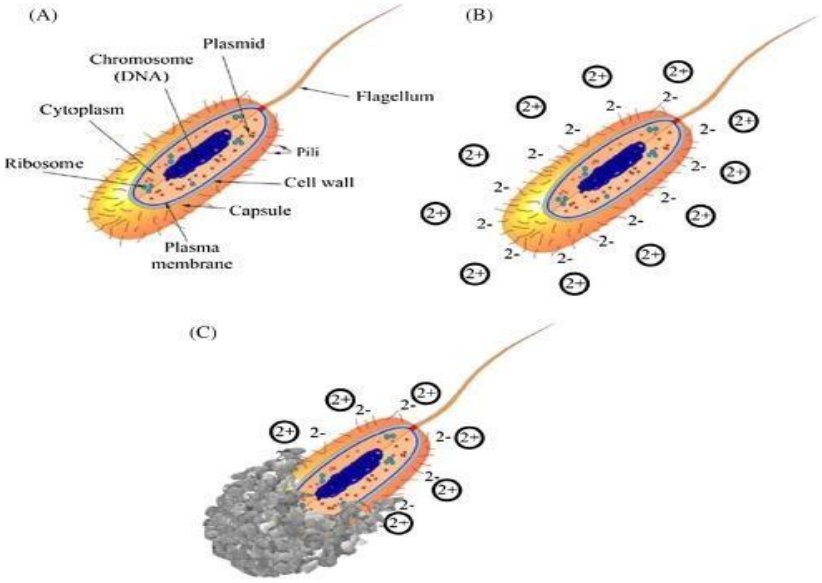


Fig. 1- Extracellular calcium carbonate precipitation by bacterial cell

(Seifan et al. 2016)

Bacteria plays a major role in the process of MICCP by serving as a nucleation site for carbonate crystals. Ureolytic bacteria synthesize the urease enzyme which cleaves urea in aqueous conditions to carbonate ions which leads to an upsurge in pH which results in carbonate formation. Mineralization of heavy metal ions is caused by the reaction of a heavy metal-cell complex after which the ultimate conversion to metal carbonate precipitate takes place. (Hussein et al., 2019). Heavy metals are more toxic in ionic form than the metal carbonate form as the latter is relatively more insoluble and inert. Through precipitation, microorganisms aid in the metal immobilization and lead to chemical changes in metal compounds. Prokaryotic microorganisms, take part in oxidation-reduction reactions and alter the valency of heavy metals, thus altering their activity, which impacts their mobility or toxicity (Gavrilescu, 2004) The insoluble precipitate is formed when heavy metals present in the

contaminated water or soil system react with the species formed during the precipitation. Heavy metals which get surrounded by carbonate in the mineral structures through co-precipitation are generally more stable and less toxic over time (Achal et al., 2013). The process is affected by the microbial population, temperature, pH, and concentration of heavy metals. Microbial bio-remediation proposes a new method for discussing the problem of heavy metal contamination in wastewater and soil, and it has now become an emphasis of new research and development in bioremediation techniques. In the present study the was efficiency of biomineralization was studied on the removal of heavy metals in wastewater.

RESEARCH GAP

Different types of bioremediation techniques such as There are a large number of conventional bioremediation techniques bioaccumulation, bioleaching, bio-coagulation biosorption, and bio-flocculation are classified among the most useful bio-based decontamination methods. The main disadvantage related to these techniques is the potential of discharging of the adsorbed toxic heavy metals back to the site of bioremediation (Collins and Stotzky,1989). Therefore, the biomineralization technique has an edge over the conventional bioremediation methods and serves as an efficient alternative and suitable method to entrap heavy metals away from the natural environment (Knox 2002).

At the moment the advancement of materials based on biomineralization is receiving a great deal of attention, but still very few bacteria have been exploited to their full potential. So, in this study we have focussed on utilization of MICCP for heavy metal bioremediation as that has been relatively less explored and application of halophilic bacteria for the same is almost negligible. With the exponential increase in use of pesticides and fertilizers, salinity of soil is a major agricultural issue.

OBJECTIVES

1. To evaluate the urease enzyme activity and calcium carbonate precipitation of *Bacillus paramycoides* – SL1 under different salt concentrations.
2. Evaluation of urease activity and calcium carbonate precipitation for removal of heavy metals from wastewater.
3. To determine the efficiency of biomineralization potential of *Bacillus paramycoides* – SL1 for removal of heavy metals through Atomic Absorption Spectroscopy (AAS) and microstructural analysis (Field Emission Scanning Electron Microscopy).

Chapter 2

REVIEW OF LITERATURE

2.1 HEAVY METALS

A heavy metal is referred to as any element possessing metal like properties such as ductility, electrical conductivity, high density, stability as cations, specificity to ligands, etc. and having atomic number of more than 20. A more biologically correct organization of metals depending on activities such as ligand forming has been stated by Nieboer and Richardson in 1980. Various metals are important for biotic systems and thus should exist in a particular range of concentration. Actually, they serve as important cofactors for a variety of metalloproteins and enzymes and, therefore, much lower concentrations might lead to deficiency due to a decrease in metabolic reactions. Heavy metals exist naturally on the Earth's crust, and under normal conditions, the concentrations of heavy metals in the soil stay at lower concentrations. Nevertheless, through the past few years, the addition of various heavy metals as a result of human activities into the soils has increased the normal heavy metal load from soil formation, even at a local level (Facchinelli et al., 2001). At greater levels, heavy metals behave in a harmful way by hindering important functional groups, replacing various necessary metal ions, or altering the active state of biomolecules (Collins and Stotzky, 1989). Even though, they are poisonous for both higher-ordered species as well as microorganisms many of these elements are extremely soluble in aqueous systems, well-documented toxins, and oncogenic agents. Nonessential metals can be endured at lower concentrations but hinder metabolic activity at greater concentrations.

2.2 INDUSTRIES EMPLOYING THE USE OF HEAVY METALS

Trace metals and metalloids (Ag, Cd, As, Be, Cu, Cr, Ni, Hg, Sb, Pb, Zn Se, Tl,) are considered to be the primary pollutants (Sparks, 2005); they are formed from various natural sources like rocks and minerals, and human inputs from e.g. farming, metal refineries, energyproduction, electronics, mining and quarrying, sewage release and waste treatment (Landa, 2005; Gilmour and Riedel, 2009). Over the last few decades, the production, as well as releaseof heavy metals, have risen manifold. Compounds of heavy metals are generally utilized in pigments, fertilizers, batteries, or other manufacturing products. Undoubtedly, factories deliver the important provisions of life in modern societies, but these products from industries come hand in hand with unwanted toxic waste that very often is allowed to get discharged into the nature (Milukaite et al, 2010). In Table 1, the permissible limits of various heavy metals and their sources of contamination have been described.

Table 1- Permissible limit of heavy metals in drinking water and their sources of contamination.

Heavy Metal	Permissible limit in drinking water*	Sources of Contamination
Cadmium	3 ug/L	Cadmium producing industries, electroplating and welding, by products from refining of lead, zinc, copper, fertilizer industry, pesticide manufacturers, Cd-Ni batteries, nuclear fission plants.
Arsenic	10 ug/L	Fungicides, pesticides, herbicides, metal smelters, by products of mining activities, chemical wastes
Copper	50 ug/L	Iron and steel industry, wood burning, discharge of mine tailing, disposal of fly ash, municipal and industrial waste disposal
Lead	10 ug/L	Automobile emission, lead smelters, burning of coal and oil, lead arsenate pesticides, smoking, mining and plumbing

Zinc	5000 ug/L	Zinc refineries, galvanizing processes, brass manufacture, metal plating, plumbing.

*BIS 10500-2012 Indian Standards for Drinking water, 2012

2.3 DANGERS OF HEAVY METAL POLLUTION AND METHODS OF HEAVY METAL REMEDIATION

Various heavy metals for example zinc, manganese, copper, iron, and cobalt are needed by the body, (Lane and Morel, 2000) but, they might be harmful when absorbed at higher quantities. Various heavy metals like Pb and Hg do not possess any useful effects on human well-being but rather dangerous to the body if added up in body over a period of time (Chronopoulos et al., 1997). Heavy metals disrupt body's metabolic activities through different ways. Also, they can add up in important body organs like kidney, liver, heart, and brain upsetting regular biological activity (Chronopoulos et al., 1997). When heavy metals are accumulated in the biological systems they disrupt their important activities in body. However, it is a true fact that it is not possible to have an environment entirely free of heavy metals.

The heavy metals can enter the body through many ways such as food, potable water, inhalation etc. Agricultural crops that are irrigated with water polluted by heavy metals also tend to have high quantities of heavy metals in the toxic range (Singh, 2007). Toxic heavy metal waste is polluting soil and water leading to deleterious effects on the health of living beings while metals were discovered and mined to fulfil human needs. Biological systems are unable to destroy heavy metals due to which they can get added up in the body over time to dangerous levels.

The researchers have identified various cancer-causing pathways that involve heavy metal contact (Beyersmann and Hartwig, 2008). Many researchers have examined that cancer causing and mutagenic activity is related to oxidative stress induced by the heavy metals. Also,various oxidation- reduction reactions are carried out by heavy metal ions such as cobalt, chromium, arsenic, nickel etc in the biotic systems. Free radicals are produced as a consequenceof these reactions result in oxidative damage to DNA and protein. Other than causing direct DNA damage, reactive species formed as a consequence of redox reactions involve two other important cancer-causing manifestations in humans. First being the initiation of redox sensitive transcription factors while other revolves around its activity as mitogenic agent. Similarly,

heavy metals interfere with the DNA repair mechanisms leading to carcinogenicity (Genestra, 2008).

Chronic exposure leads to accumulation of heavy metals to such an extent that the body begins to utilize them as a replacement of essential nutrients with leads to an overall imbalance. For example, calcium replaced by lead, cadmium in place of zinc and other important micro nutrients being replaced by aluminium. Furthermore, various metabolic processes of the biological systems are compromised leading to antioxidant imbalance. Likewise, many hormones and enzymes are also disturbed (Mukke and Chinte, 2012). Thus, the need of the hour is to look for long term heavy metal management solutions. Table 2 shows various methods of heavy metal bioremediation, its applications, pros and cons.

Table 2. Outline of different methods used for metal remediation from different environmental sources

Methods of Remediation	Application	Factors Involved	Advantages	Disadvantages	Reference
Physico chemical methods	Magnetic separation electrostatic separation gravity concentration and attrition scrubbing	Particle size distribution, clay content, particulate shape, moisture content, heterogeneity of soil matrix, density between soil matrix and metal contaminants	Easy cheap equipment	Limit clay applicability and efficiency due to high content of calcite, fe & ca, heavy metals.	Dermont et al., 2008 Williford et al, 2000 Fu et al, 2011
Chemical precipitation	Heavy metal removal from inorganic effluent in industry	pH, temperature initial concentration, charge of the ions etc	Simple and easy low cost	Lower solubility of metal sulphides, Emission of toxic hydrogen sulphide during process.	Ku and Jung, 2001
Coagulation & flocculation	Removal of a large number of organic compound and suspended particles in industrial waste water.	Temperature, pH, effluent quality, dosage and type of coagulant	Fast, limited investment.	Production of sludge, application of chemicals, operational costs and transfer of toxic compounds into solid phase	López-maldonado et al, 2014
Electrochemical Treatments	Removal of metals from wastewater streams	Material feed velocity, electrolyte content.	Reduced sludge production, no requirement for chemical use, and ease of operation.	Maximum chemical usage, Huge sludge production, slow metal precipitation, poor settling, the aggregation of metal precipitates, and the long-term environmental impacts of sludge disposal	Mollah, et al, 2001 Aziz et al, 2008

Ion exchange	Most widely used method in water treatment industry r for treating water with low concentration of heavy metals	weight distribution coefficient (Kd) and separation factor (α).	Cost-effective method	Only low concentrated metal solution highly sensitive with the pH	Dizge, N et al, 2009 Hamdaoui, O, 2009
Membrane Filtration	Treatment of inorganic effluent by removing suspended solid, organic compounds and inorganic contaminants such as heavy metals	Size of the particle, solute concentration, pressure, and water flux rate	Lower driving force and a smaller space requirement due to its high packing density	Membrane build-up and clogging. Expensive cleaning, High operational costs	Rether and Schuster, 2003 Petrov, and Nenov, 2004 Trivunac and Stevanovic, 2006
Electrodialysis	Heavy concentrations of heavy metals in wastewater	Flow rate, temperature and voltage at different concentrations	Treatment of highly concentrated wastewater laden with heavy metals to recovery undesirable impurities from water	Membranes replacement and the corrosion process	Chen and Chen, 2003 Kurniawan et al, 2006

2.5 BIOLOGICAL METHODS

Biological methods are environmentally safe, efficient, and budget-friendly techniques. Many bio adsorbents are naturally found. Conventional physico-chemical methods produce harmful by-products that are very difficult to be managed by the factories. Chemical methods prove to be appropriate for inorganic compounds released from factories but the cost of treatment is greater. The employment of biological methods for the removal of xenobiotics from polluted soil or wastewater involves the use of microorganisms by harnessing techniques such as activated sludge treatment, trickling filter, stabilization ponds etc.

2.5.1 ACTIVATED SLUDGE, TRICKLING FILTER AND STABILIZATION PONDS

The activated sludge technique involves the microorganisms degrading the organic material by agitating and providing enough oxygen and removing solids by settling. The sludge from the previous batch is added to the new batch to serve as inoculum and increase the rate of organic degradation (Kurniawan, 2006). A lot of studies involving heavy metal elimination have been conducted on the activated sludge technique in the suspended growth phase.

Trickling filters are made up of layers of stone or plastic media 3 to 10 feet deep to aid the proliferation of microorganisms. Wastewater to be treated is applied by spraying and then allowed to pass through the coarse media and microorganisms degrade the organic substances in aerobic conditions. The water that has passed through the filter gets collected at the bottom then goes through sedimentation (White, 1998).

Stabilization ponds are based on the collective action of microorganisms, sunlight, algae, and oxygen. This is an economical, somewhat inefficient, and a long-term slow process that can be used for different types of effluents.

2.5.2 BIOSORPTION

Adsorption occurs at the surface while absorption is a phenomenon that involves the whole volume of the substance. In adsorption complex formation occurs on the surface of the cell with contaminating heavy metals, from where the heavy metals can be taken into the cell (Danis, 2008). Due to the fact that the surface of the bacterial cells is covered by cell wall and mucous, the heavy metals can be adsorbed and taken inside the cell somewhat easily. A variety of ions in the functional groups present on the cell surface like oxygen, nitrogen, sulfur and phosphorous form complex with metals as coordination compounds. Adsorption is a passive process as it does not involve the requirement of energy while absorption involves the expenditure of energy and occurs solely in living cells (Wang, 2001). Microorganisms can adsorb huge quantities of heavy metal ions quickly. It has been studied that bacillus species

can adsorb more than 60% of its Cu2+ capacity in the first minute of contact and be at equilibrium in about 10 minutes at pH 7.2 (He & Tebo, 1998).

2.5.3 BIOLEACHING

Biomining is a wider term that involves both bio-oxidation [Rahman & Sathasivam, 2015]. and bioleaching, in which movement of positive heavy metal charged particles takes place from insoluble ores generally through biological dissolution or processes related to complex formation [Brunetti, 2012; Volesky, 1995]. Microbes can produce metabolites such as organic acids of low molecular weight which can dissolve heavy metals and minerals containing heavy metals (Chanmugathas and Bollag, 1988). Chanmugathas and Bollag, 1988 proved that microorganisms can efficiently utilize nutrients and energy to release organic acids of low molecular weight and encourage the discharge of Cd in nutritious conditions.

2.5.4 PHYTOREMEDIATION

Various microorganisms, such as mycorrhizal fungi and other living in the soil, can improve the capacity of plants to take in or adsorb heavy metals (Bojórquez and Voltolina, 2016). Intake of cadmium by mycorrhizal plants as compared to that of non-mycorrhizal plants was found to be greater (Joner and Leyval 1997). Mycorrhizal fungi, are able to enhance the surface area of roots due to the presence of mycelia (Trellu et al., 2016). Mycorrhiza that lives within the plants in a symbiotic relationship benefits the host plant to grow resistance to heavy metal ions. If the heavy metal content in the soil reaches harmful levels, the mucus released by the cell wall of fungus can associate with the polyphosphate and low molecular weight organic acid ions in the fungus to chelate the heavy metal ions and lessen the toxicity. Also, the quantity and structure of root exudates modify when the fungi infect the plant roots, thus the oxidation of heavy metals ions is affected in the proximity of the rhizosphere [Niu et al., 2011]. Moreover, plant mycorrhizae generally have a protective mechanism that attaches heavy metals to cell walls and does not allow them to transfer to the plants [Cervantes, 2001]. Also, many studies have shown the efficacy of remediation with the help of plants on soil polluted with various heavy metals, but severe and parched environmental conditions in dry regions restricted its applications (Nieboer, 1980).

2.6 BIOREMEDIATION OF METALS

Bioremediation is the utilization of biotic systems to eliminate various organic and inorganic xenobiotics, in which the most significant organisms being bacteria and fungi for the purpose of reclamation, immobilization of metallic and radioactive pollutants. Microbial Bioremediation involving microbes is a safe and environment friendly method for pollution in

soil. Microorganisms possess a huge potential for breakdown necessary for the detoxification of xenobiotics and then degrade them down into compounds that are comparatively less toxic or immobilize them**.** Metals can be considered different as compared to other pollutants in the sense that they cannot be degraded chemically or biologically in such a manner that their toxicity can be significantly reduced eventually (Knox et al., 2000). Microorganisms do not have the ability to destruct heavy metals; does not matter which path the microorganisms take, the heavy metals are not eliminated (Lovley and Lloyd, 2000). We can say that heavy metals cannot be microbially destructed considering the literal meaning of destruction be any alteration in the elemental nuclear arrangement. The only change in metals that can occur is a change in the oxidation state of organic complexes.

As a result of transformation in oxidation state, the metal may change as one of the following:

(i) Higher water solubility and elimination through leaching,

(ii) Becoming less hazardous,

(iii) Decrease in water solubility in such a way that there is a decrease in heavy metal bioavailability or be eliminated from the site of pollution

(iv) Removal from the contaminated site through volatilization (Garbisu and Alkorta, 1997).

Heavy metal detoxification can be carried out by valence transformation, volatilization or precipitation. Various metals can be reduced with the help of enzymes by microorganisms in biological processes that might not be linked to metal assimilation (Lovley, 1993). Many bacteria can pair the oxidation of lower organic acids, aromatic compounds, alcohols, and hydrogen to cause a reduction of Mn(IV) or Fe(III). Various species of bacteria employ U(VI) as a terminal electron acceptor that can possibly be beneficial for the decontamination of uranium (Lovley, 1993). Sulphate-reducing bacteria are the ones that cause the conversion of sulfate to hydrogen sulfide, which combines with heavy metals to form metal sulphides through precipitation. Metalssulfidess such as cadmium sulphide and zinc sulphide have been described in many bacteria (White et al., 1998 & Iwamoto and Nasu, 2001).

2.7 BIOMINERALIZATION

The process in which minerals are formed by living organisms is called biomineralization. There is a huge percentage of non-soluble carbonate of biotic origin on the surface of the earth which is related to bacteria, algae, fungi, and metazoa (Gadd, 2010). The precipitation of carbonate by microorganisms plays a significant part in coprecipitation of metals and the role of ligation in nature. This naturally occurring process that is observed in numerous geological

situations can be harnessed for a variety of biotechnological applications for example heavy metal decontamination, sequestration of carbon, improved oil recovery and remediation of construction. It is a well-known fact that microorganisms have an array of characteristics that can be utilized to cause differences in speciation of metals, toxic potential, and mobility of metals also they influence the formation of minerals, erosion of minerals or degradation. A large number of minerals of biological origin that we come across are calcium carbonate, oxides, sulphides or silicates (Baeuerlein, 2000; Bazylinski, 2001). Majority of the microbes be it prokaryotes or eukaryotes have symbiotic relation with living organisms on the higher level that can pe a part of the geological phenomena through active interaction (Macalady and Banfield, 2003; Bottjer, 2005; Chorover et al., 2007; Konhauser, 2007; Gleeson et al., 2007; 2008), and majority of these cycles require transformation of metals and minerals.

Microbial precipitation of carbonate takes place as side process in various metabolic activities like photosynthesis (Dupraz et al., 2004), denitrification (Van Paassen et al., 2010), sulfate reduction (Braissant et al., 2007), ammonification (Rodriguez-Navarro et al., 2003), methane oxidation (Reeburgh, 2007) and ureolysis (Fujita et al., 2000). It was reported by Obst et al. in 2009 that the extracellular polymeric substances and cell walls can function as templates for precipitation of carbonate (Obst et al., 2009).

When the biomineralization of calcium carbonate takes place, diverse phases of calcium carbonate are produced as anhydrous polyorphs i.e. calcite, aragonite and vaterite. In hydrated conditions the two crystalline phases are monohydrocalcite ($CaCO_3$.H2O) and ikaite ($CaCO_3$.6H2O). The utmost commonly found calcium carbonate polymorphs are calcite and vaterite.

We can categorize the bacterial mineral formation into two broad categories Biologically controlled mineralization (BCM) and Biologically induced mineralization (BIM). In BCM, the minerals are produced at certain sites inside the cell or on surface under specific conditions of biologically controlled mineralization.

In biologically induced mineralization, due to the metabolic reactions occurring inside the microbes, the minerals are formed extracellularly i.e. outside the cell. In the environment, the process of carbonate precipitation occurs at a very gradual rate lasting many decades therefore if we need to synthesize a huge number of carbonates in shorter duration, then we need to look for microorganisms that have the capacity to influence the conditions for carbonate precipitation in lesser duration. Many species of bacteria have the ability to precipitate the carbonates in high pH surroundings that are rich in calcium ions and many mechanisms as

suggested by Ehrlich, 1996 and Rivadeneyra et al., 2004 can induce bacterial precipitation in the normal surroundings. The process of microbially induced calcium carbonate precipitation works on the dependency of urease enzyme activity. It has been found that a huge diversity of soil microorganisms is involved in this process.

2.8 MECHANISM OF MICCP IN HEAVY METAL BIOREMEDIATION

Heavy metal bioremediation can be brought about by various methods, one such method involves microbial biomass which serves as a heavy metal basin. The heavy metals get entrapped by various methods such as biosorption to the cell walls, extracellular polysaccharides, pigments, deposition within the cells, precipitation of heavy metals around cells. It has been demonstrated by warren et al. in 2001 that cell walls serve as templates for nucleation very efficiently for precipitation of templates. Functional groups with negative charge such as amine, carboxyl or phosphate in the cell wall have the ability to adsorb heavy metal ions (Dittrich and Sibler, 2005; Fein, 2006). Due to this phenomenon, the heavy metal ion concentration rises, and when there is an availability of bicarbonate or carbonate, the oversaturation in relation to carbonates can be attained.

Extracellular polymeric materials have a significant part in microbial calcification as well (Tourney and Ngwenya, 2014), by enhancing precipitation of carbonate (Dupraz et al., 2009). Functional groups such as phosphate, hydroxyl, amine and carboxyl can bind to the metals and are present in the extracellular polymeric substances EPS (Tourney et al., 2008; Dittrich and Sibler, 2010). Microbially induced calcite precipitation (MICP) stemming from biomineralization can be considered as an effective method to decontaminate arsenic from polluted environments with added benefits as compared to current decontamination techniques [Iwamoto, 2001 and white et al, 1998]. This phenomenon is prevalent in most of the ecosystems on earth. There occurs a deposition of minerals when metabolic substances released by the microbes combine with ions or other compounds in the environment.

As a result of biomineralization, calcite is formed that tends to strongly adsorb metalloids on its surface and eventually the ion is incorporated within the crystal lattice (Abdalla, 2012). Significant research has been conducted on biological precipitation of calcite involving the role of ureolytic bacteria (Milukaite, 2010 and Dermont, 2008). Ureolytic bacteria have the tendency to affect the calcium carbonate precipitation by synthesizing the urease enzyme released as a result of metabolic activity of the bacteria. MICP is prevalent in almost every environment of earth and its by-products have the capacity to entrap heavy metals. This can

serve as a great decontamination method for polluted soil. A vast majority of studies for the decontamination of Pb and Sr have been achieved in groundwater through MICP (Smith, 1995). On the outer surface of the bacterial cells, the precipitates of carbonate form due to continuous stratification (Pentecost and Bauld, 1988; Castanier et al., 1999) thus the bacteria can get entrapped inside the growing crystals of carbonate by serving as an initial nucleation site for the crystal to develop (Rivadeneyra et al., 1998; Castanier et al., 1999). When urea is cleaved in the aqueous conditions, ammonia is produced as an unfavourable by product of hydrolysis. Upon further hydrolysis of ammonia, pH rises due to release of OH- and bicarbonates are formed due to dissociation of carbonic acid (Knoll, 2003). Thus, calcium carbonate precipitation is encouraged by this reaction in the presence of Ca^{2+} ions in the solution.

2.9 UREA HYDROLYSIS

A vast proportion of research has been carried out using MICCP are related to ureolysis, mainly in fields of metal decontamination and strength improvement or repair and restoration of civil structures. Urease (urea amidohydrolase; EC3.5.1.5) is quite prevalent in a huge diversity of microbes. As seen in Fig. 2, a mole of urea is converted to a mole each of ammonia and carbonate which upon cleavage in the presence of water produce another mole of ammonia and carbonic acid each. An equilibrium of these products is established in water to form bicarbonate and two moles of ammonium and hydroxyl ions. Due to the presence of hydroxyl ions, the pH rises which leads to a shift in the equilibrium of bicarbonate, as a consequence, carbonate ions are formed. Due to this change, the heavy metals get precipitated in soil or wastewater. Urease activity in microorganisms can be affected by a variety of parameters such as pH (7-8.7), temperature, available urea and by product ammonia concentration, source of carbon, and time of incubation (Hasan, 2000).

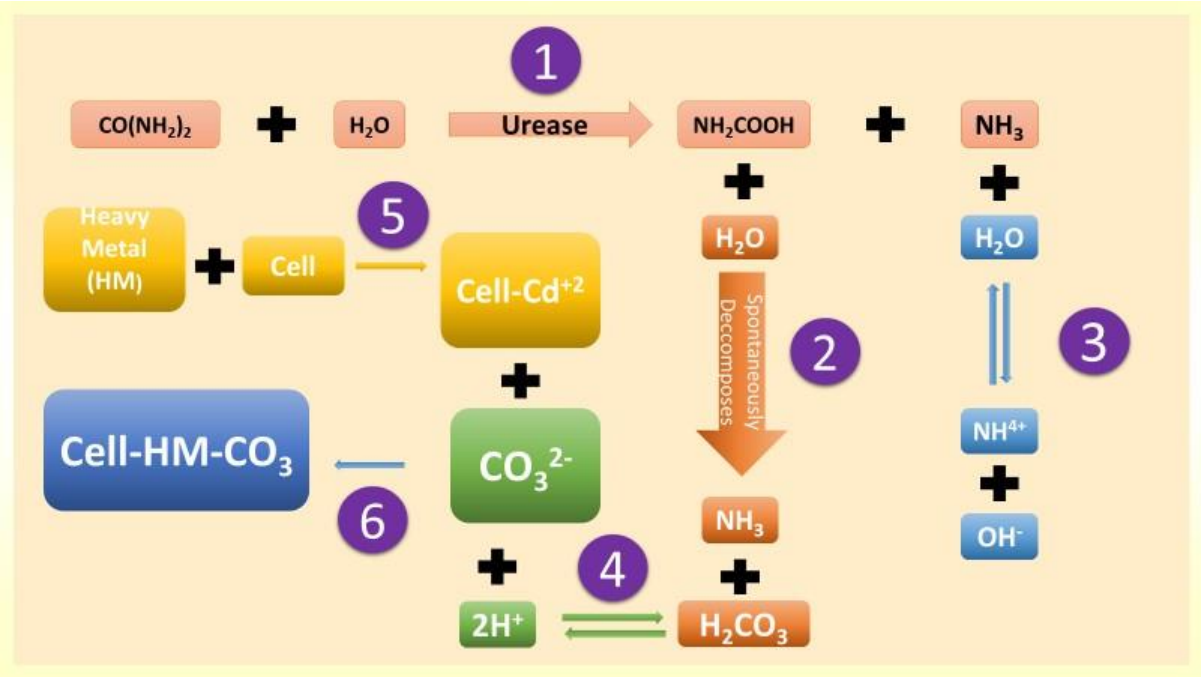


Fig. 2 Reactions involved in formation of heavy metal- calcium carbonate bio-precipitate

2.10 MICCP IN HALOPHILIC ENVIRONMENT

Microbially-induced calcium carbonate precipitation (MICP) has been established as a propitious technique to decontaminate heavy metal/metalloid-polluting sites considering the fact that $CaCO_3$ serves as an excellent host matrix for a large number of metals and metalloids (Callagon et al., 2014). In the past few years the potentials of MICP have been harnessed but comparatively scanty research has been conducted to study the heavy metal decontamination via MICP under salt stress conditions. For example, the waste effluent from factories is an artificial condition is highly saline and various heavy metals ions tend to be present in it (Zhuang et al., 2010). In natural conditions the environments with high salt stress are found in marine regions, salt marshes etc. majority of these environments tend to be at higher danger from heavy metal pollution (Pan and Wang, 2011). A large number of reports suggest that heavy metal contamination is an increasing issue in coastal regions due to sewage from industries and electronic waste (Pan and Wang, 2011).

Chapter 3

MATERIALS and METHODS

Isolation of bacteria

The halophilic bacterial culture was isolated from water sediments collected from Sambhar Lake in Jaipur, Rajasthan, India by our lab at TIFAC-CORE, TIET, Patiala. Sambhar lake is India's largest inland of salinity in lake. It is considered to have rich flora of microorganism that can grow in the presence of high salt concentration (Bhatt et al. 2016).

Preservation and sub-culturing

The halophilic bacterial culture was sub-cultured every 15 days on fresh nutrient agar plates supplemented with 5% sodium chloride for long term maintenance. The bacterial cultures were stored by wrapping the petri plates in parafilm to prevent contamination and drying of plates and refrigerated at 4 °C.

Fig. 3 *Bacillus paramycoides* on nutrient agar plate

Urease activity of culture at varying concentrations of salt stress

- Bacterial culture was analysed for their ureaseactivity by measuring the breakdown of urea and production of ammonia.
- Bacterial culture was grown overnight and O.D. was adjusted using UV spectrophotometer to 0.5.
- The culture with 0.5 OD at 600 nm was reinoculated into 6 different flasks with varying salt concentrations i.e., 0%, 3.5%, 5%, 7.5%, 10%, 15% to find out the maximum

concentration of salt tolerated by the halophilic bacteria and incubated at 37° C in a shaker incubator at 120 rpm.

- The bacterial culture (1 ml) from all flasks was extracted in separate autoclaved eppendorfs and centrifuged at 8000 rpm for 5 minutes.
- The culture filtrate (250 µl) was taken in clean and dried test tubes and added 1 ml of 0.1 M potassiumphosphate buffer (pH 8.0) and 2.5 mL of 0.1 M freshly prepared urea solution.
- The solution was mixed and incubated at 37°C for 5 minutes.
- Add one ml of phenol nitroprusside solution and 1 ml alkaline hypochlorite solution and incubate for 25 minutes at 37°C.
- The optical density was noted at 626 nm a using UV-Vis spectrophotometer.
- The optical density readings were noted at the interval of 24 hours for a period of 7 days.

Calcium carbonate precipitation

- The highest concentration of salt which gave best urease activity was selected.
- Bacterial culture was inoculated in nutrient broth and incubated at 37°C overnight under shaking conditions.
- In 90 ml nutrient broth the Urea and Calcium Chloride was added so the final concentration is 2% Urea and 25 mM Calcium Chloride.
- One ml overnight culture was added to each flask i.e., with salt stress and without salt stress and incubated for 7 days at 37°C.

Preparation of heavy metal stock solutions

- Heavy metal stock solutions were prepared for cadmium and arsenic with concentration of 2 mM each.
- Sodium arsenate heptahydrate was accurately weighed (124.804 mg) and added to 200 ml autoclaved distilled water. It was then passed through a syringe filter of pore size 0.22 microns in order to filter sterilize the stock solution.
- Cadmium chloride (73.328 mg) was added to 200 ml autoclaved distilled water. It was filter sterilized with 0.22-micron filter.

CADMIUM

Determination of Inhibitory Concentration 50 (IC_{50}) value for cadmium under normal and salt stress condition

- To determine the highest concentration of arsenic at which the bacteria are able to grow we need to find the minimum inhibitory concentration of heavy metal for a specific bacterial species.
- Culture was inoculated in nutrient broth and incubated at 37° C overnight under shaking conditions.
- In separate flasks 100 ml nutrient broth was autoclaved in two sets. In one of the sets 5% salt stress was introduced.
- One ml of overnight culture with OD at 600 nm adjusted to 0.5 was added to each of the flasks.
- Heavy metal stock solution was added to the flasks to reach final arsenic concentrations of 0 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM.
- The flasks were then incubated for 24 hours at 37° C under shaking conditions.
- O.D. at 600 nm was noted after 24 hours with the help of spectrophotometer.
- The IC_{50} Value for cadmium under salt stress and normal conditions was found by plotting the OD_{600} values obtained for log of varying concentrations in GraphPad Prism software.

Urease activity under various concentrations of Cadmium stress with and without salt stress

- Bacterial culture was analysed for their urease activity by measuring the breakdown of urea and production of ammonia.
- Bacterial culture was grown overnight at 37° C in a shaker incubator at 120 rpm for 24 hours and O.D. was adjusted using UV-Vis spectrophotometer to 0.5.
- The culture with 0.5 OD at 600 nm was re-inoculated into different flasks with varying cadmium concentrations i.e. 0 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM. and salt stress condition of 5% sodium chloride was introduced to one set of flasks while no salt was added to the second set of flasks incubated at 37° C in a shaker incubator at 120 rpm for 24 hours.
- One ml of bacterial culture from all flasks was extracted in separate autoclaved eppendorfs and centrifuged at 8000 rpm for 5 minutes

- Culture filtrate (250 µl) was taken in clean and dried test tubes and added 1 ml of 0.1 M potassium phosphate buffer (pH 8.0) and 2.5 mL of 0.1 M freshly prepared urea solution and incubated at 37°C for 5 minutes.
- One ml of phenol nitroprusside solution and 1 ml alkaline hypochlorite solution was then added with the help of a pipette and incubated for 25 minutes at 37°C.
- The optical density was noted at 626 nm a using UV-Vis spectrophotometer.
- The optical density readings were noted at the interval of 24 hours for a period of 7 days.

Calcium carbonate precipitation under various concentrations of cadmium and with and without salt stress.

- Bacterial culture was inoculated in nutrient broth and incubated at 37° C overnight under shaking condition.
- In 90 ml nutrient broth Urea and Calcium Chloride was added so that the final Urea concentration is 2% and the concentration of Calcium Chloride is 25 mM in two sets. In one set 5% salt stress was introduced.
- Varying cadmium concentrations i.e. 0 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM were introduced to the flasks.
- One ml overnight culture was added to each flask and incubated for 7 days at 37° C under shaking conditions. The quantification of Calcium carbonate was done using EDTA titration method and the weight of calcium carbonate crystals was taken.

Assessing the removal of cadmium with and without salt stress

- After the completion of 7 days of calcium carbonate precipitation experiment, both sets of flasks i.e. with salt stress and without salt stress were removed from incubation
- The calcium carbonate precipitates were collected in a cellulose membrane filter (Pore size- 0.45 micrometer) using membrane filter apparatus by connecting it to the vacuum pump and the filterate was quantified for the heavy metals using Atomic Absorption Spectroscopy.
- The precipitated powder was collected in the filter paper which was dried using different concentration of ethanol and then left at room temperature for 6 hours.
- After complete drying, the calcium carbonate powder was weighed.

ARSENIC

Inhibitory Concentration 50 (IC_{50}) under various concentrations of Arsenic with and without salt stress

- To determine the highest concentration of cadmium at which the bacteria are able to grow we need to find the minimum inhibitory concentration of heavy metal for a specific bacterial species.
- Culture was inoculated in nutrient broth and incubated at 37° C overnight under shaking conditions.
- In separate flasks 100 ml nutrient broth was autoclaved in two sets. In one of the sets 5 g NaCl was added while in the second set contained no salt stress.
- One ml of overnight culture with OD at 600 nm adjusted to 0.5 was added to each of the flasks.
- Heavy metal stock solution was added to the flasks to reach final arsenic concentrations of 0 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM.
- The flasks were then incubated for 24 hours at 37° C under shaking conditions.
- O.D. at 600 nm was noted after 24 hours with the help of spectrophotometer.

Urease activity under various concentrations of arsenic stress, with and without salt stress.

- Bacterial culture was analysed for their urease activity by measuring the breakdown of urea and production of ammonia.
- Bacterial culture was grown overnight and O.D. was adjusted using UV-Vis spectrophotometer to 0.5.
- The culture with 0.5 OD at 600 nm was re-inoculated into different flasks with varying arsenic concentrations i.e. 0 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM. while one of the sets contained 5% salt stress and incubated at 37° C in a shaker incubator at 120 rpm for 24 hours.
- One ml of bacterial culture from all flasks was extracted in separate autoclaved eppendorfs and centrifuged at 8000 rpm for 5 minutes
- Culture filtrate (250 µl) was taken in clean and dried test tubes and added 1 ml of 0.1 M potassium phosphatebuffer (pH 8.0) and 2.5 mL of 0.1 M freshly prepared urea solution and incubated at 37°C for 5 minutes.

- One ml of phenol nitroprusside solution and 1 ml alkaline hypochlorite solution was then added with the help of a pipette and incubated for 25 minutes at 37°C.
- The optical density was noted at 626 nm a using UV-Vis spectrophotometer.
- The optical density readings were noted at the interval of 24 hours for a period of 7 days.

Calcium carbonate precipitation under various concentrations of arsenic stress

- Bacterial culture was inoculated in nutrient broth and incubated at 37° C overnight under shaking conditions.
- In 90 ml nutrient broth Urea and Calcium Chloride was added so that the final Urea concentration is 2% and the concentration of Calcium Chloride is 25 mM. 5 % salt stress was introduced in one of the sets.
- Varying arsenic concentrations i.e. 0 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM were introduced to the flasks.
- One ml overnight culture was added to each flask and incubated for 7 days at 37° C.

Assessing the removal of Arsenic with and without salt stress

- After the completion of 7 days of calcium carbonate precipitation experiment, both sets of flasks i.e. with salt stress and without salt stress were removed from incubation.
- The calcium carbonate precipitates were collected in a cellulose membrane filter (Pore size- 0.45 micrometer) using membrane filter apparatus by connecting it to the vacuum pump and the filterate was quantified for the heavy metals using Atomic Absorption Spectroscopy.
- The precipitated powder was collected in the filter paper which was dried using different concentration of ethanol and then left at room temperature for 6 hours.
- After complete drying, the calcium carbonate powder was weighed. The heavy metal bioprecipitates thus obtained can be seen in Fig. 3.

Fig. 4 Heavy metal- Calcium Carbonate precipitates obtained after filtration and drying.

SEM and EDS analysis of the Bio-precipitates

The bio-precipitates were analysed by Field Emission Scanning Electron Microscopy (FESEM) to study the topographical information and Energy Dispersive Spectroscopy (EDS) to study the elemental characterization. The samples were coated with a thin layer of gold to increase the conductivity of the sample. Generally, the thickness of coating lies between 20 nm to 30 nm. The imaging was carried out by Carl Zeiss Sigma 500 Field emission microscope and eds was carried by Bruker, QUANTAX 200.

Statistical Analysis

All the experiments were performed in triplicates. The data were analysed by analysis of variance and significant differences among the means were compared by Tukey's test $p < 0.05$. All the analyses were performed using GraphPad Prism 5 software.

Chapter 4

RESULTS

Determination of Inhibitory Concentration 50 (IC_{50}) value for Cadmium and Arsenic under normal conditions and Salt Stress

Inhibitory concentration 50 (IC_{50}) is determined as the concentration of heavy metal required to inhibit the growth of 50% bacterial population. The lower IC_{50} value suggests higher potency of the heavy metal to inhibit the bacterial growth and population. Similarly, a higher IC_{50} value means that the bacteria can tolerate a higher concentration of the heavy metal before showing a significant decline in the growth due to interference of heavy metal with the metabolism.

In fig. 5 (a), the normalized absorbance has been plotted against the increasing cadmium concentrations as a function of logarithm under normal and salt stress conditions. Upon plotting the graph in GraphPad Prism software, the IC_{50} value for cadmium was found out to be 12.84 µM for normal conditions and 9.846 µM under 5% salt stress.

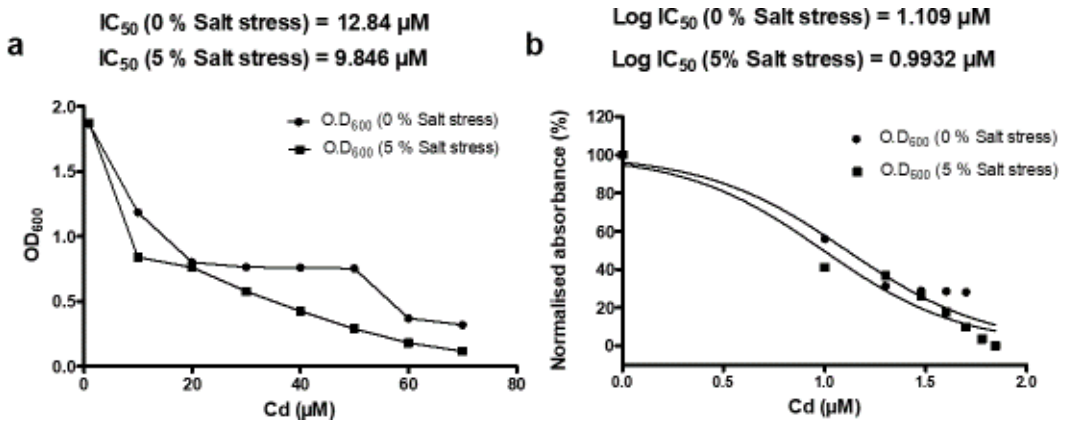


Fig. 5 (a) The effect of increasing cadmium concentrations on OD_{600} under normal and salt stress conditions. (b) Normalized absorbance vs increasing cadmium concentrations

In fig. 6 (a), The arsenic concentrations have been plotted against OD at 600 nm. Fig 6 (b) shows the normalized absorbance been plotted against the increasing arsenic concentrations as a function of logarithm under normal and salt stress conditions. Upon plotting the graph in GraphPad Prism software, the IC_{50} value for arsenic was found out to be 48.54 µM for normal conditions and 62.86 µM under 5% salt stress.

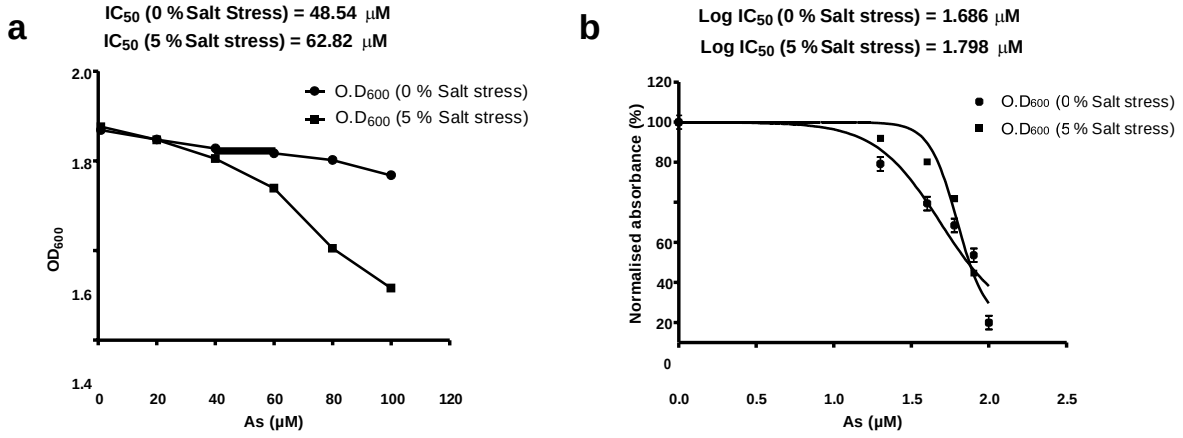


Fig. 6 (a) **The effect of increasing arsenic concentrations on OD_{600} under normal and salt stress conditions. (b) Normalized absorbance vs increasing arsenic concentrations**

Urease activity of the culture at varying salt stress

Urease activity assay is a colorimetric assay in which blue colour is produced whose intensity corresponds to the urease activity. The ammonium carbonate released as a consequence of urea hydrolysis upon action of enzyme urease is taken up by alkaline hypochlorite which is measured by spectrophotometer. The concentration of urease is reported as U/ml. A comparison was done to check the effect on urease activity by changing the salt concentration in the growth media of bacteria. The media was supplemented with 3.5%, 5%, 7.5%, 10% and 15% salt concentration. This was done to find out the highest salt concentration at which the bacteria is able to successfully produce the enzyme urease without compromising on the enzyme yield. As seen in Fig. 6 the maximum urease activity was observed at 5% NaCl concentration after 72 hours of inoculation followed by control i.e., 0% and 3.5 % NaCl concentration. The maximum urease activity at 7.5% NaCl is almost half of urease activity observed in 5 % NaCl. Following the same trend, the urease activity in 10% NaCl concentration is found to be lesser than the urease activity in 7.5% NaCl. Almost negligible urease activity was observed in 15% NaCl concentration. From Fig. 6 we can interpret that the highest salt

concentration at which best urease activity observed is 5%. Therefore, 5% salt stress was introduced throughout the study to replicate highly saline environmental conditions.

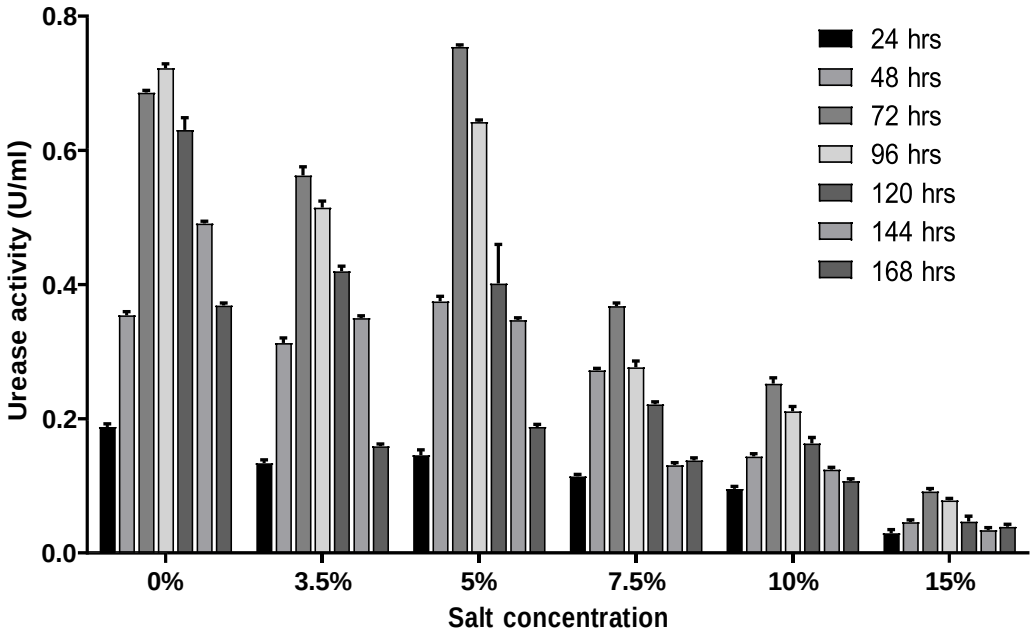


Fig. 6 Change in urease activity over a period of 168 hours supplemented with salt stress of 0% (control), 3.5%, 5%, 7.5%, 10% and 15%. Error bars represent standard deviation (n = 3). The factorial ANOVA results indicating urease activity at different salt concentrations and time period are highly significant (P Values < 0.001).

Urease Activity under various concentrations of Cadmium with and without salt stress

The highest urease activity was reported at 96 hours in cadmium while in arsenic the highest urease activity was observed at 120 hours. The maximum activity of urease enzyme i.e. 722 U/ml at 0 µM as observed in Fig 7. The urease activity at cadmium concentrations of 10 µM, 20 µM, 30 µM and 40 µM was noted to be 616 U/ml, 602 U/ml, 562 U/ml & 525 U/ml. A sharp decrease in the urease activity i.e. 278 U/ml was noted upon reaching the cadmium concentration of 50 µM followed by 60 µM i.e. 172 U/ml. The urease activity at 70 µM showed minimum urease activity of just 58 U/ml.

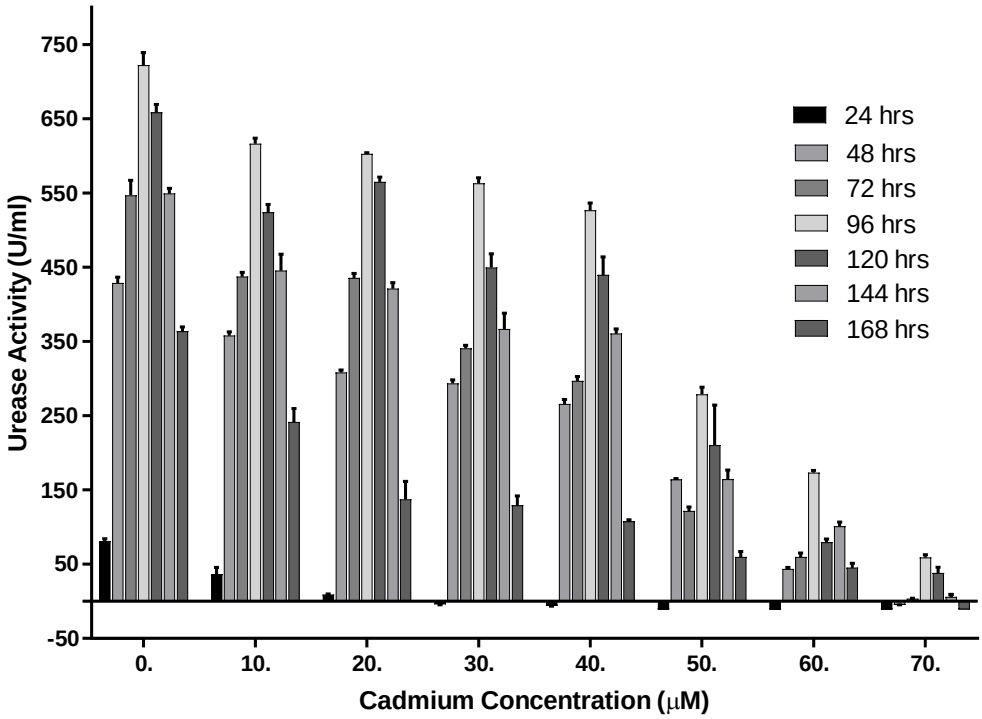


Fig. 7 Change in urease activity over a period of 168 hours (7 days) over varying cadmium concentrations from 0 µM to 70 µM Error bars represent standard deviation (n = 3). The factorial ANOVA results indicating the heavy metal concentration and time period are highly significant (P Values < 0.001).

While under 5% salt stress conditions, at 96 hours, the urease activity was 717 U/ml at 0 µM (Fig 8) which served as control for the experiment. The urease activity at cadmium concentration from 10 µM to 40 µM was 608 U/ml, 547 U/ml, 501 U/ml & 488 U/ml. A significant decrease in urease activity was noted i.e. 236 /ml at cadmium concentration of 50 µM followed by 157 U/ml at 60 µM/. At 70 µM the urease enzyme activity was a meagre 51 U/ml.

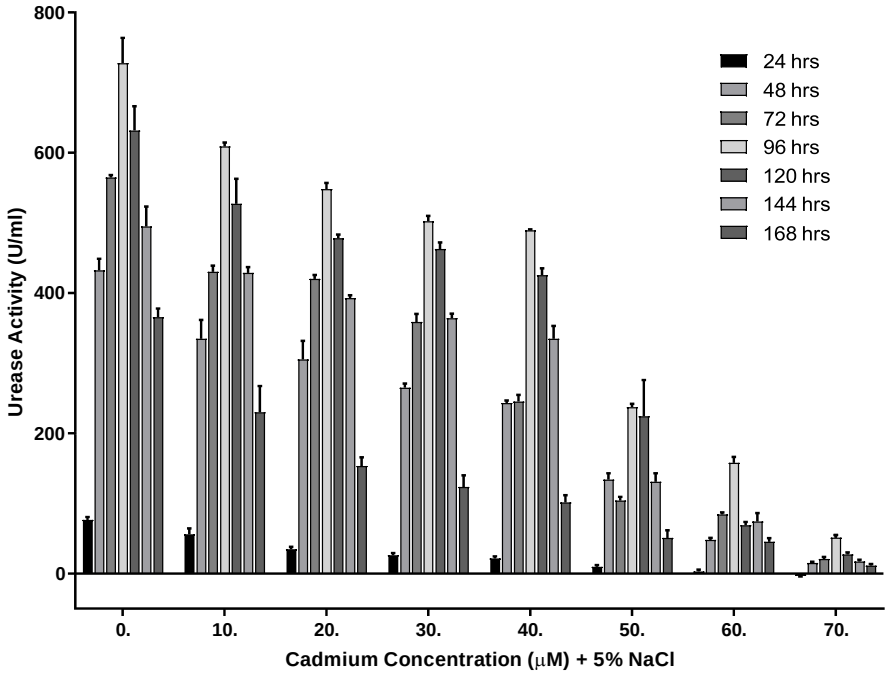


Fig. 8 Change in urease activity over a period of 168 hours (7 days) supplemented with 5% Salt Stress under varying Cadmium concentrations. Error bars represent standard deviation (n = 3). The factorial ANOVA results indicating the heavy metal concentration and time period are highly significant (P Values < 0.001).

Urease activity under various concentrations of Arsenic with and without salt stress

In case of arsenic, the decline in urease activity is not as sharp and distinct as seen in cadmium. The urease activity at arsenic concentrations 0 µM, 20 µM, 40 µM, 60 µM, 80 µM & 100 µM was 855 U/ml, 847 U/ml, 836 U/ml, 824 U/ml, 797 U/ml, 734 U/ml under normal conditions and 816 U/ml, 708 U/ml, 518 U/ml, 435 U/ml, 314 U/ml, 304 U/ml under 5 % salt stress respectively.

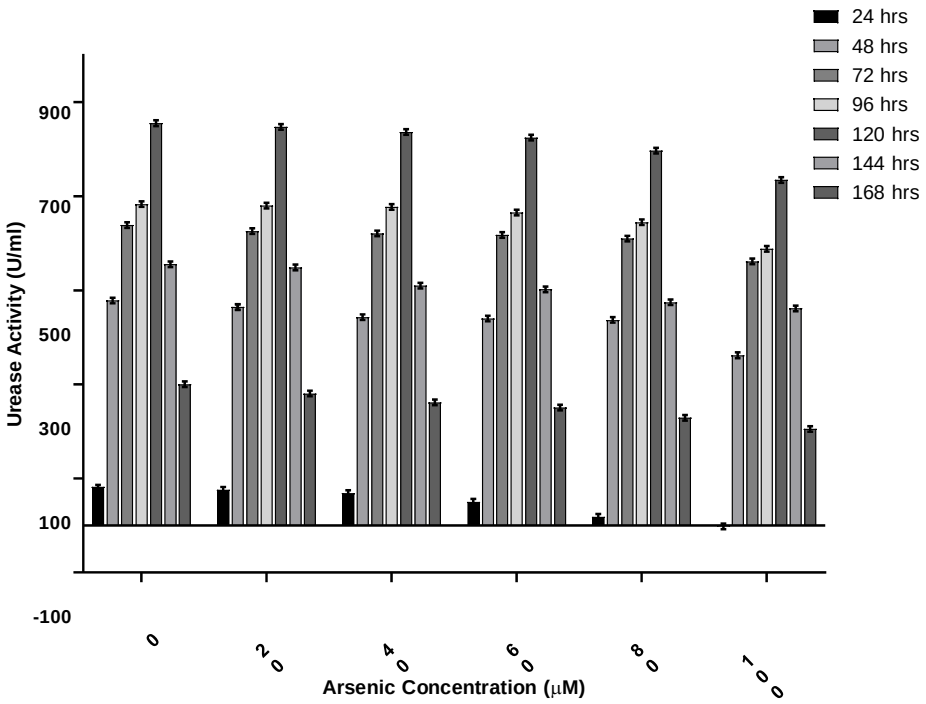


Fig. 9 Change in urease activity over a period of 168 hours (7 days) under varying Arsenic concentrations. Error bars represent standard deviation (n = 3). The factorial ANOVA results indicating the heavy metal concentration and time period are highly significant (P Values < 0.001).

In Fig. 9 and Fig. 10 we can see how the urease activity varies with different concentrations of arsenic through a period of 7 days. Comparing Fig. 9 and 10 we can observe how the salt stress impacts the urease activity. Salt stress of 5% NaCl was introduced to mimic highly saline environments. The salinity in soils is a major issue in industrial waste water and soils surrounding the industrial units that is exposed to the toxic industrial effluent. After 24 hours of inoculation the urease activity is almost negligible and increases fivefoldwithin the next 24 hours. The urease activity is comparatively lesser in the set with salt stress due to an additional osmotic pressure on the bacteria other than the heavy metal stress.

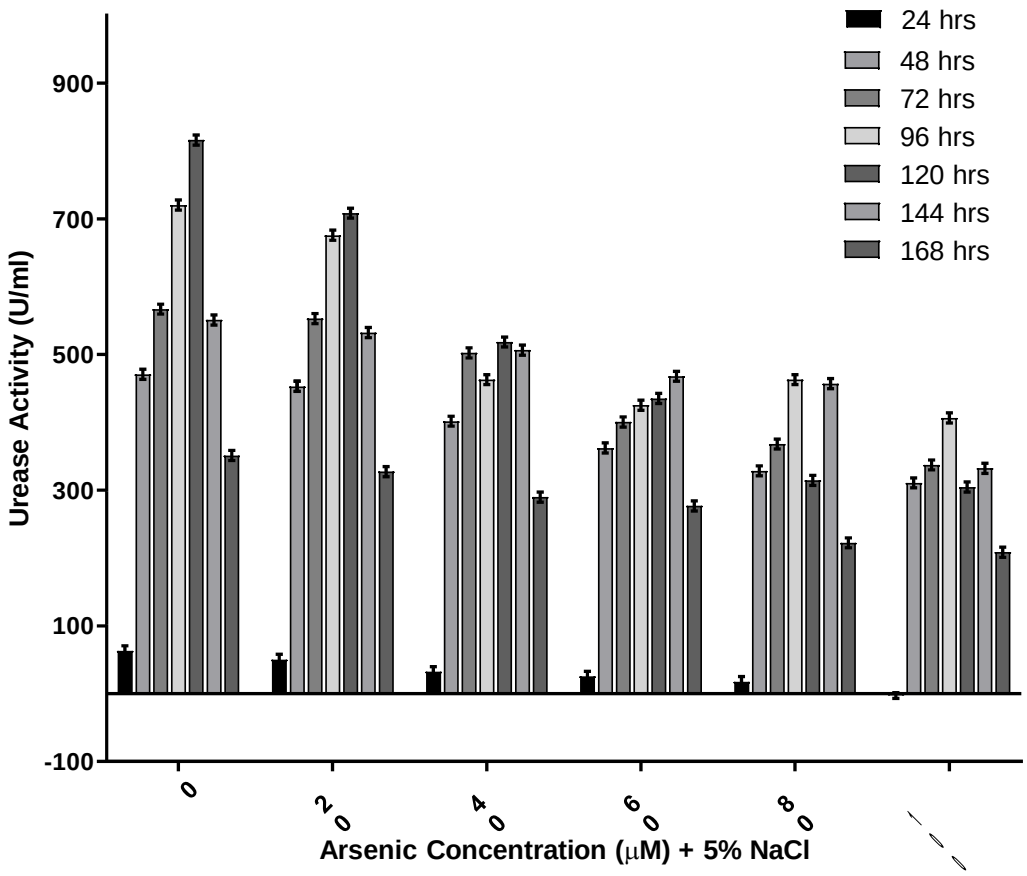


Fig. 10 Change in urease activity over a period of 168 hours (7 days) with 5% Salt Stress under varying Arsenic concentrations from 0 µM to 100 µM. Error bars represent standard deviation (n = 3). The factorial ANOVA results indicating the heavy metal concentration and time period are highly significant (P Values < 0.001).

In Fig. 11 & Fig. 12 it is observed that as the urease activity is increasing, the blue colour becomes more and more prominent since it is a colorimetric assay. The intensity of blue colour is directly proportional to the amount of ammonium ions produced during the reaction occurring in the phenol hypochlorite assay. In Fig. 11 (a) it can be clearly visualised that as the concentration of heavy metal increases from 0 µM to 70 µM (from left to right) there is a proportional decrease in the intensity of blue colour which corresponds to a proportional decrease in the urease enzyme activity as well.

Fig. 11 (b) shows the effect of cadmium concentrations (0 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM) on the urease enzyme activity in the presence of 5% salt stress. As the concentration of cadmium is increasing, a decrease in urease enzyme activity is observed in a similar fashion as in Fig 11 (a). Upon a closer visual comparison, we can also say that the intensity of blue colour (which corresponds to urease enzyme activity) in Fig

11 (a) is slightly more than what can be observed in Fig 11 (b) which lesser urease enzyme activity in the presence of salt stress over the range of same cadmium concentrations at 96 hours.

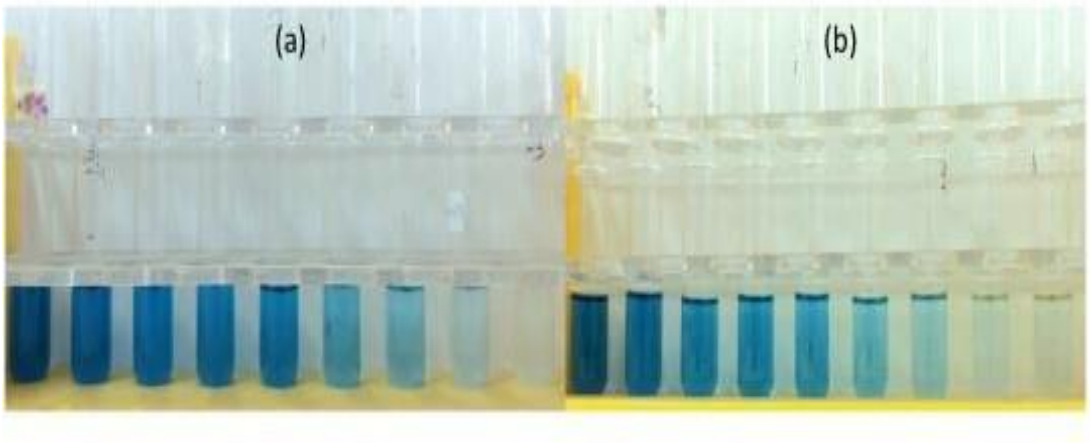


Fig. 11 (a) Urease activity under varying concentrations of cadmium at normal conditions. (b) Urease activity under varying concentrations of cadmium at 5% salt stress conditions

In Fig. 11 (c) it can be seen that as the concentration of heavy metal increases from 0 µM to 100 µM (from left to right) there is a proportional decrease in the intensity of blue colour which corresponds to a proportional decrease in the urease enzyme activity also.

Fig. 11 (d) shows the effect of arsenic concentrations (0 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM) on the urease enzyme activity in the presence of 5% salt stress. As the concentration of arsenic is increasing, a decrease in urease enzyme activity is observed in a similar fashion as in Fig 11 (c). Upon comparison, the intensity of blue colour (which corresponds to urease enzyme activity) in Fig 11 (c) is more than what can be observed in Fig 11 (d) which denotes lesser urease enzyme activity in the presence of salt stress over the range of same arsenic concentrations at 120 hours.

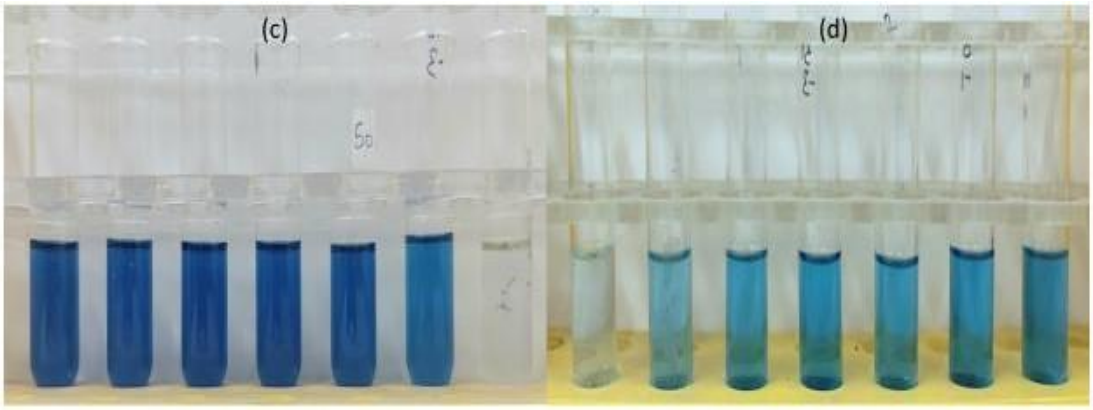


Fig. 12 (c) Urease activity under varying concentrations of arsenic at normal conditions. (d) Urease activity under varying concentrations of arsenic at 5% salt stress conditions

Calcium carbonate precipitation under various concentrations of cadmium with and without salt stress

Calcium carbonate precipitation was observed for a period of seven days from the time of inoculation in nutrient broth in the presence of urea and calcium chloride. The quantification of calcium carbonate precipitation was done after interval of 24 hours using EDTA titration method. In this method the calcium ions present in water form an unstable complex with erichrome black indicator. Ca^{2+} ions are chelated by EDTA upon titration due to which there is a change in colour which is considered as the end point.

As observed in Fig. 13, it is observed that the calcium carbonate precipitation is the highest at normal conditions i.e. without salt stress followed by 3.5% and 5 %. The Calcium carbonate precipitation starts to decrease as the salinity increases. At very high salt concentrations- 10% and 15%, the calcium carbonate precipitation is significantly affected due to extremely high osmotic stress induced by the Na^{+} and Cl^{-} ions upon the bacterial cells that hinders with the normal metabolic functions of the cell.

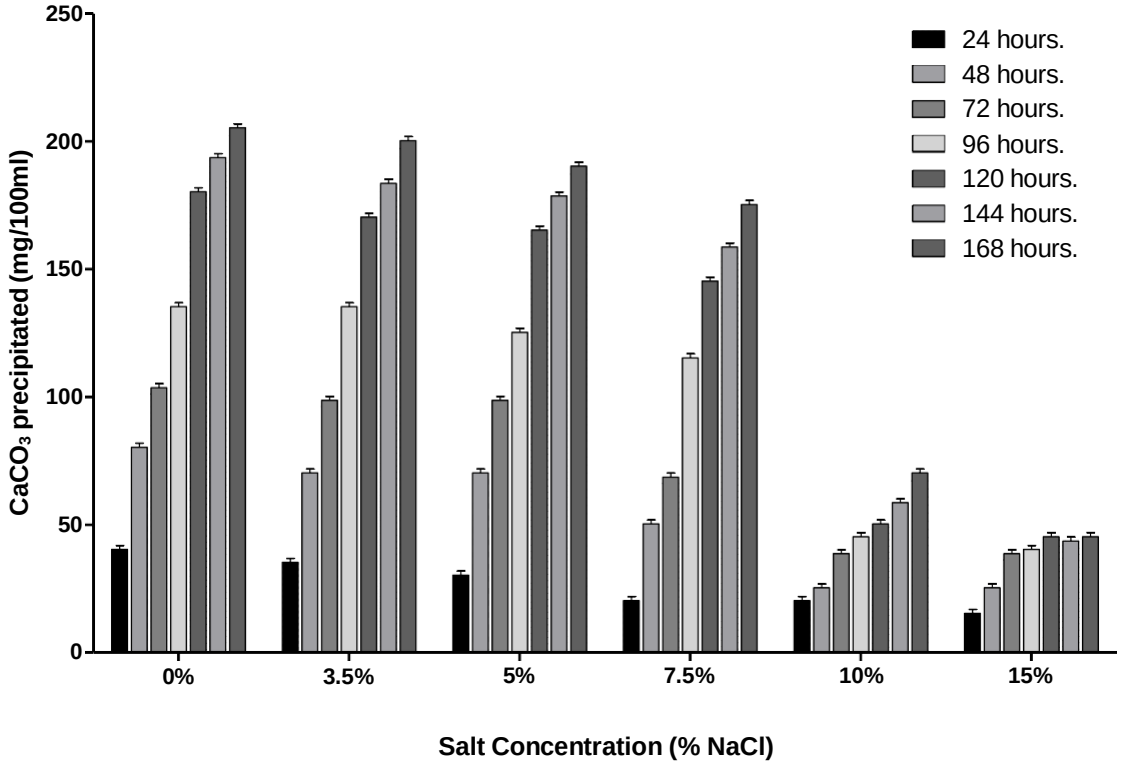


Fig. 13 Change in calcium carbonate precipitation in 100 ml nutrient broth supplemented with different concentrations of salt at time different time period (24-168 hours). Error bars represent standard deviation (n = 3). The factorial ANOVA results indicating the heavy metal concentration and time period are highly significant (P Values < 0.001).

As seen in Fig. 14, on the first day the minimum calcium carbonate was precipitated which increased successively with every 24-hour interval. The increase in calcium carbonate precipitation was observed till the 168th hour which was recorded to be the highest for every concentration of cadmium. Upon completion of 7 days of inoculation, the maximum amount of calcium carbonate precipitated was measured for 0 µM to be 204 mg/100 ml, followed by 189 mg/100 ml (10 µM), 174 mg/100 ml (20 µM), 159 mg/ 100 ml (30 µM), 134 mg/100 ml, (40 µM). Almost a threefold drop was observed in the precipitation of calcium carbonate at cadmium concentration above 50 µM (50 mg/100 ml) followed by 45 mg/ 100 ml in 60 µM concentration of cadmium. At cadmium concentration of 70 µM, lowest calcium carbonate precipitation was recorded i.e. 33mg/100 ml. At 96 hours and 120 hours, a significant increase in the precipitation of calcium carbonate can be seen which can also be attributed to the fact that the urease activity was found to be at its peak at the same number of hours post inoculation.

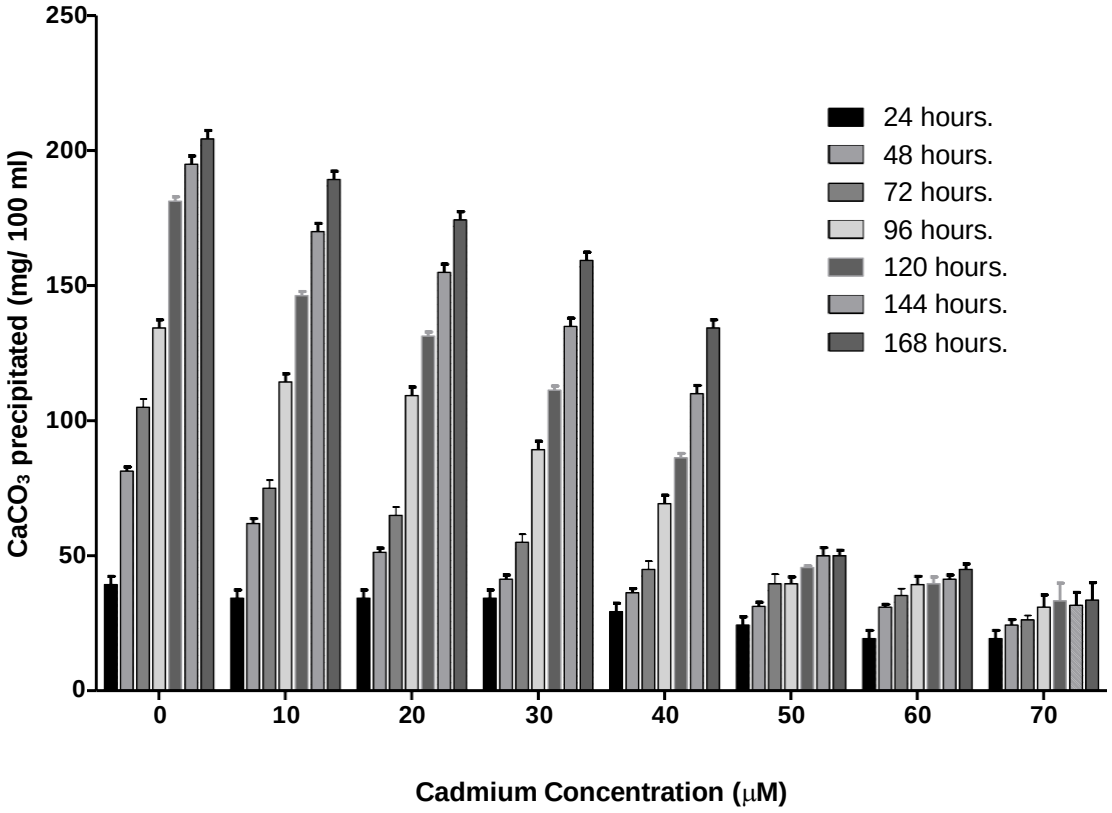


Fig. 14 Change in calcium carbonate precipitation in 100 ml nutrient broth supplemented with different concentrations of cadmium at time different time period (24-168 hours). Error bars represent standard deviation (n = 3). The factorial ANOVA results indicating the heavy metal concentration and time period are highly significant (P Values < 0.001).

In Fig. 15, the increase in calcium carbonate precipitation is observed for a period of 168 hours under the effect of 5% salt stress at cadmium concentrations of 0 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM and 70 µM. The calcium carbonate precipitation was found to be highest in 0 µM cadmium concentration (190 mg/100 ml). It was followed by a constant decrease from 10 µM to 40 µM i.e. 165 mg/100ml (10 µM), 160 mg/100 ml (20 µM), 150 mg/100 ml (30 µM), 125 mg/100 ml (40 µM). The calcium carbonate precipitation was severely impacted at cadmium concentration above 50 µM which was found to be 50 mg/100 ml which was followed by 60 µM i.e. 40 mg/100 ml. Least calcium carbonate precipitation was observed in 70 µM noted as 35 mg/100 ml.

In Fig. 14 & Fig. 15, a comparison can be drawn between the amount of calcium carbonate precipitated in salt stress and normal conditions over a course of 7 days in the presence of cadmium at various concentrations. The trend followed by calcium carbonate precipitation is quite same under 5% salt stress and normal conditions. A significant decrease was observed in both conditions (5% salt stress and normal) above cadmium concentration of 50 µM. It very much corresponds to the urease activity assay results.

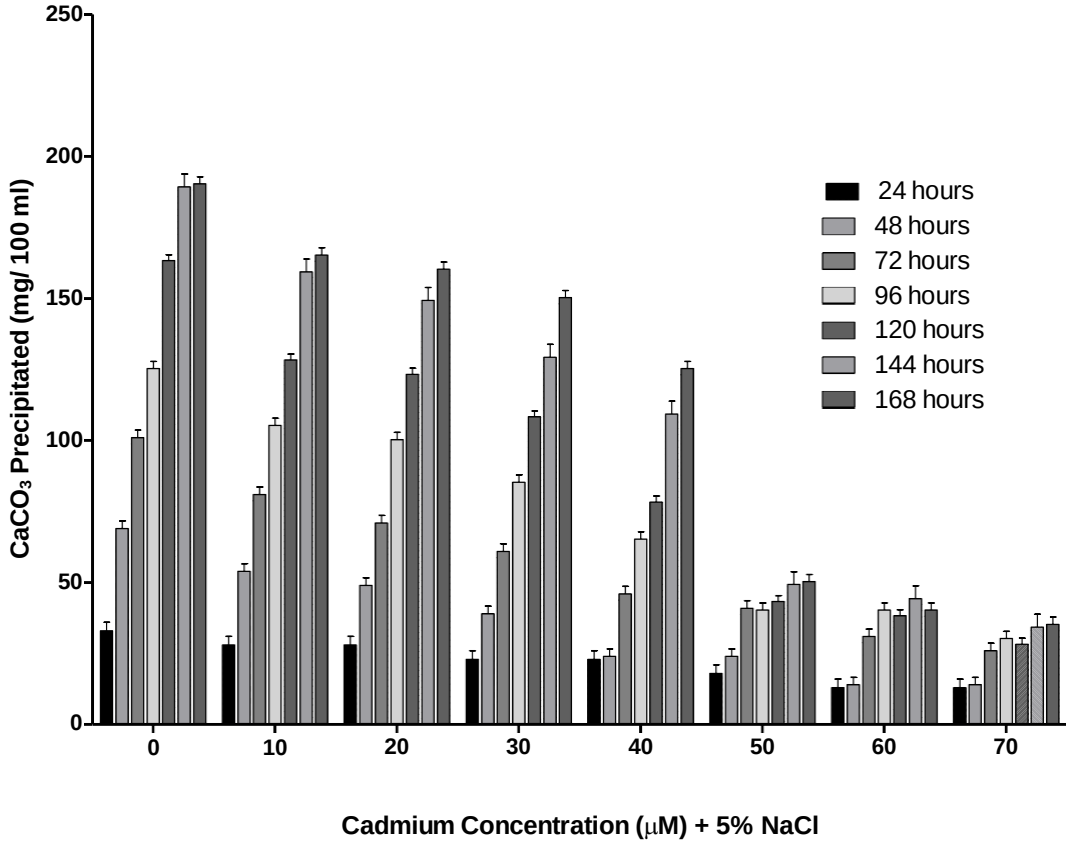


Fig. 15 Change in calcium carbonate precipitation in 100 ml nutrient broth supplemented with different concentrations of cadmium at time different time period (24-168 hours). Error bars represent standard deviation (n = 3). The factorial ANOVA results indicating the heavy metal concentration and time period are highly significant (P Values < 0.001).

The calcium carbonate precipitation under varying arsenic concentrations- 0 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM was calculated for a period of 168 hours (7 days) under normal and 5% salt stress conditions. The culture was inoculated in nutrient broth in the presence of urea and calcium chloride under both salt stress and normal conditions at 0 hour and successive readings of calcium carbonate were taken at an interval of 24 hours after performing the EDTA titration.

In Fig. 16, it is observed that the calcium carbonate precipitation was maximum at the 168-hour interval. The maximum calcium carbonate was precipitated in arsenic concentration of 0 µM (208 mg/100 ml) followed by 20 µM (198 mg/ 100 ml), 40 µM (183 mg/100 ml), 60 µM (178 mg/100 ml), 80 µM (163 mg/100 ml) and lastly, 100 µM (141 mg/100 ml). There was an increase in the calcium carbonate precipitation at the 120-hour interval in all concentrations of arsenic. This is due to the fact that the urease enzyme activity was reported to be the highest at 120-hour interval in case of arsenic.

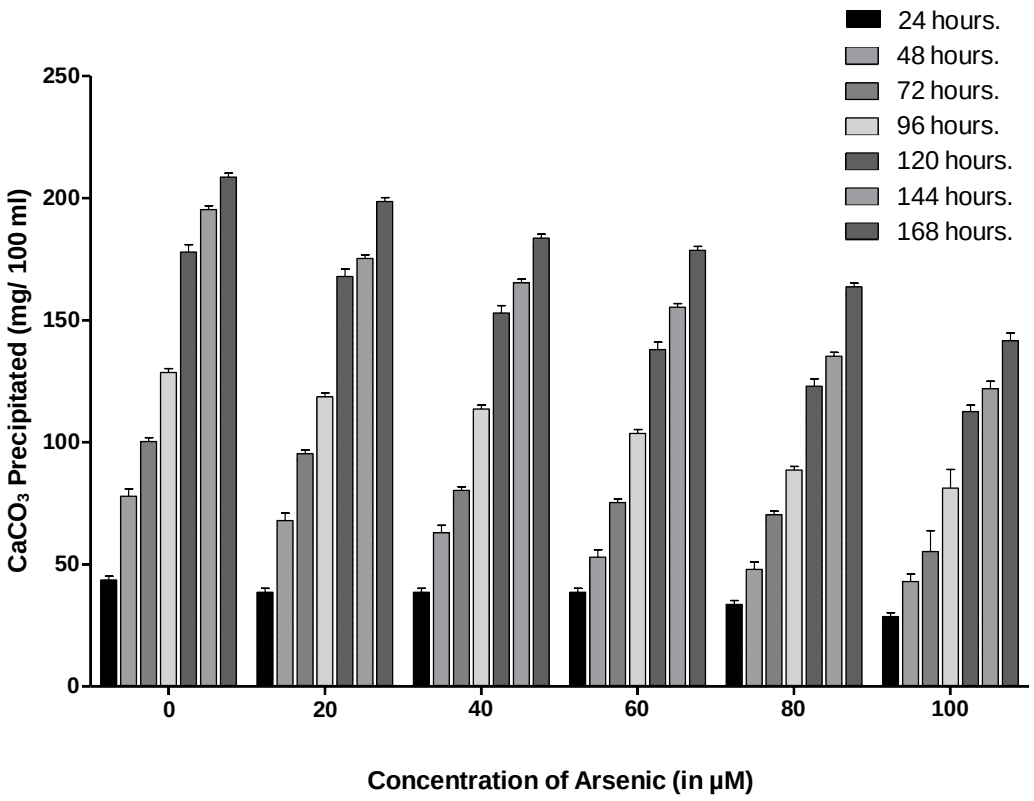


Fig. 16 Change in calcium carbonate precipitation in 100 ml nutrient broth supplemented with different concentrations of arsenic at time different time period (24-168 hours). Error bars represent standard deviation (n = 3). The factorial ANOVA results indicating the heavy metal concentration and time period are highly significant (P Values < 0.001).

The change in precipitation of calcium carbonate was noted for a period of 168 hours from the time of inoculation. It is observed in Fig. 17 that the calcium carbonate precipitation was maximum at the 168-hour interval. The maximum calcium carbonate was precipitated in arsenic concentration at 5% salt stress was 0 µM (189 mg/100 ml) followed by 20 µM (164 mg/ 100 ml), 40 µM (159 mg/100 ml), 60 µM (149 mg/100 ml), 80 µM (124 mg/100 ml) and lastly, 100 µM (49 mg/100 ml). There was an increase in the calcium carbonate precipitation at the 120-hour interval in all concentrations of arsenic. This is due to the fact that the urease enzyme activity was reported to be the highest at 120-hour interval in case of arsenic.

Upon comparing the results of calcium carbonate precipitation as seen in Fig. 16 and 17 in arsenic under salt stress and normal conditions, overall the same trend was followed in both the conditions. The calcium carbonate precipitation was impacted at 100 µM concentration of arsenic under salt stress as it was found to be lower than the calcium carbonate precipitation at the same concentration without salt stress.

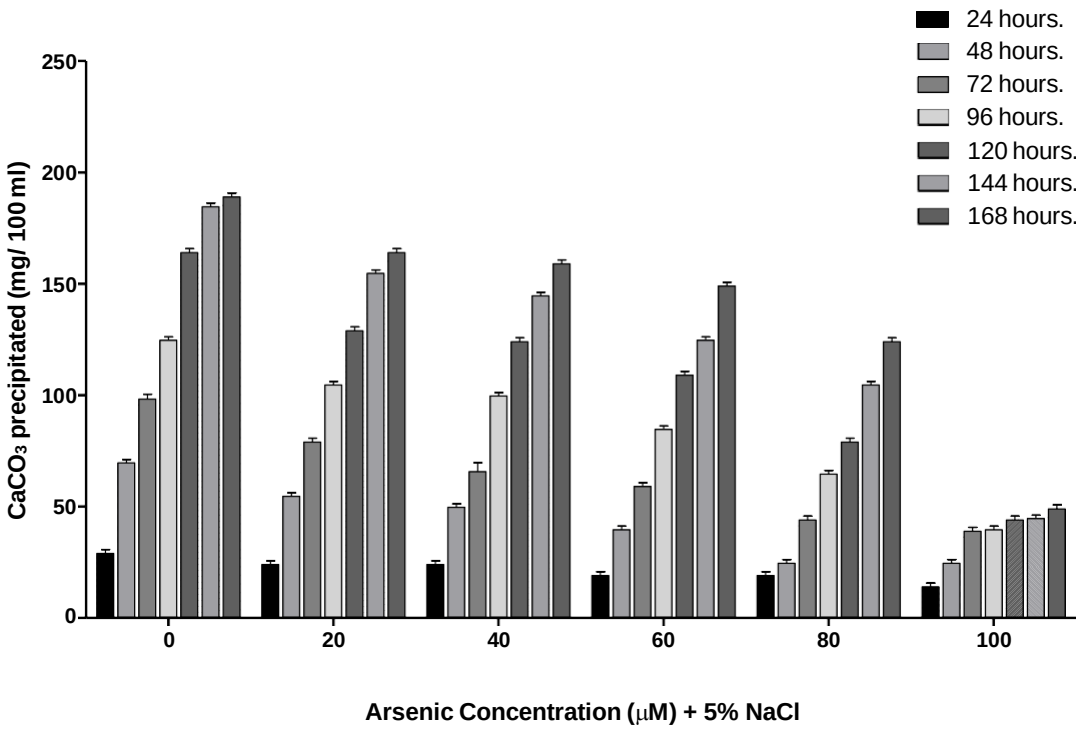


Fig. 17 Change in calcium carbonate precipitation in 100 ml nutrient broth supplemented with different concentrations of arsenic at time different time period (24-168 hours). Error bars represent standard deviation (n = 3). The factorial ANOVA results indicating the heavy metal concentration and time period are highly significant (P Values < 0.001).

pH variation in calcium carbonate precipitation

Due to the ureolytic action of enzyme urease, urea is hydrolysed and as a consequence, ammonium and hydroxyl ions are generated that lead to a rise in pH of the medium. This phenomenon leads to binding of Ca^{2+} ions to the heavy metal present in the solution. The trend observed in the change of pH over time showed that there was a slight reduction in the pH upon addition of urea and calcium chloride for the first 5 hours considering the addition of urea and calcium carbonate to be done at 0 hour. This reduction in pH can be explained considering the dissociation of ions in water upon addition. The pH then increases till 96 hours after which a plateau is observed. The increase in the pH is due to the presence of ammonium ions and also the evolution of carbon dioxide during the ureolysis in similar observations by Achal et al. (2012). In Fig. 18 (a) and Fig. 18 (b), the change in pH for different cadmium concentration is observed for a duration of 0 to 10 hours and 24 to 168 hours and Fig. 18 (c) and Fig. 18 (d) show the change in pH under various cadmium concentrations observed for a duration of 0 to 10 hours followed by 24 to 168 hours.

Similarly, Fig. 18 (e) and (f) show the change in pH over a range of arsenic concentrations for a period of 0 to 10 hours and subsequently, for 24 to 168 hours. Fig. 18 (g) and (h) show the pH for different arsenic concentrations for a period of 0 to 10 hours and for 24 to 168 hours.

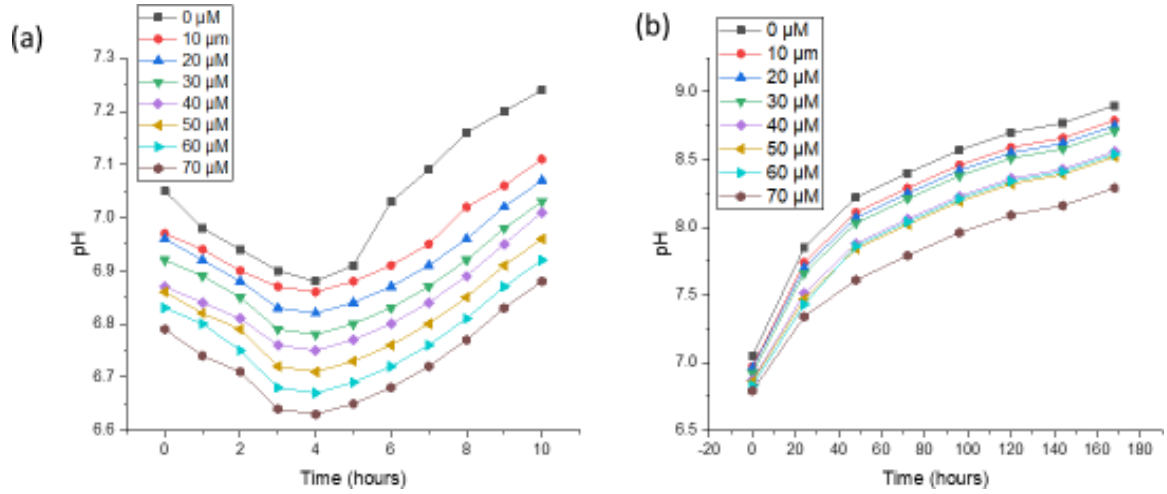
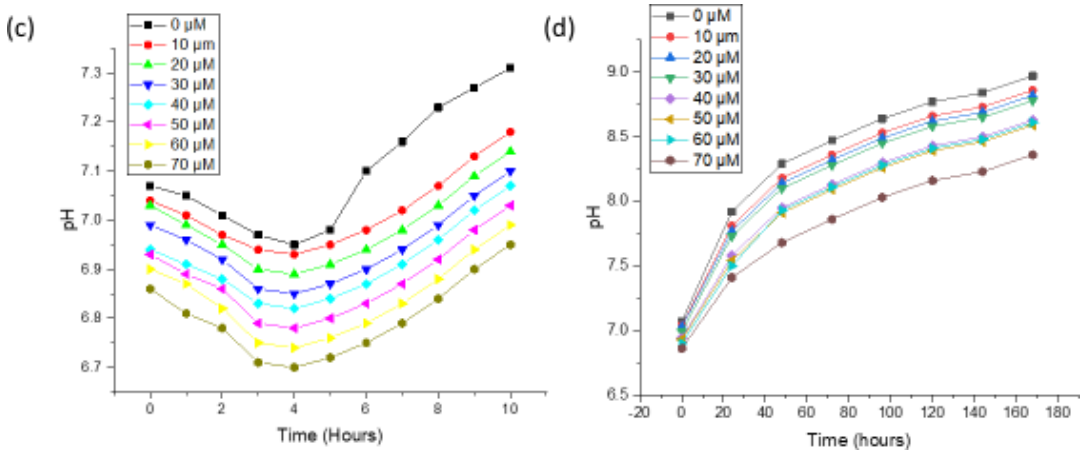


Fig. 18 (a) pH variation in nutrient broth medium supplemented with different concentration of cadmium (0 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM) for initial 10 hours of incubation (b) Subsequent change in pH from 24 to 168 hours post inoculation in nutrient broth medium supplemented with different concentration of cadmium (0 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM).

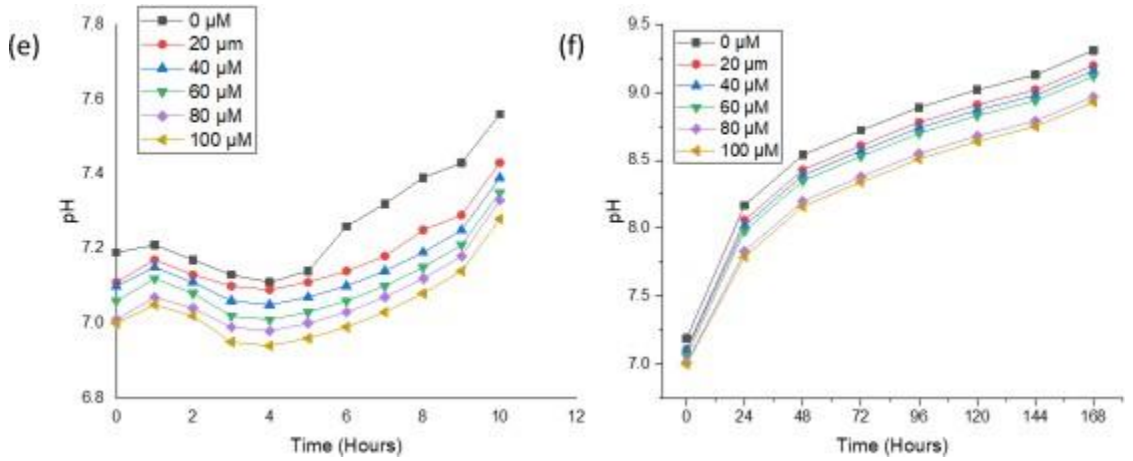
In Fig. 18 (c) the variation in pH is observed for a period of 0 to 10 hours. The concentration of cadmium taken as control was 0 µM. The pH change in control is more abrupt than the cadmium concentrations from 10 µM to 70 µM. In Fig. 18 (d) the pH changed from 7 to 8.5 from day 0 to day 7.



(c) pH variation in nutrient broth medium supplemented with different concentration of cadmium (0 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM) for initial 10 hours

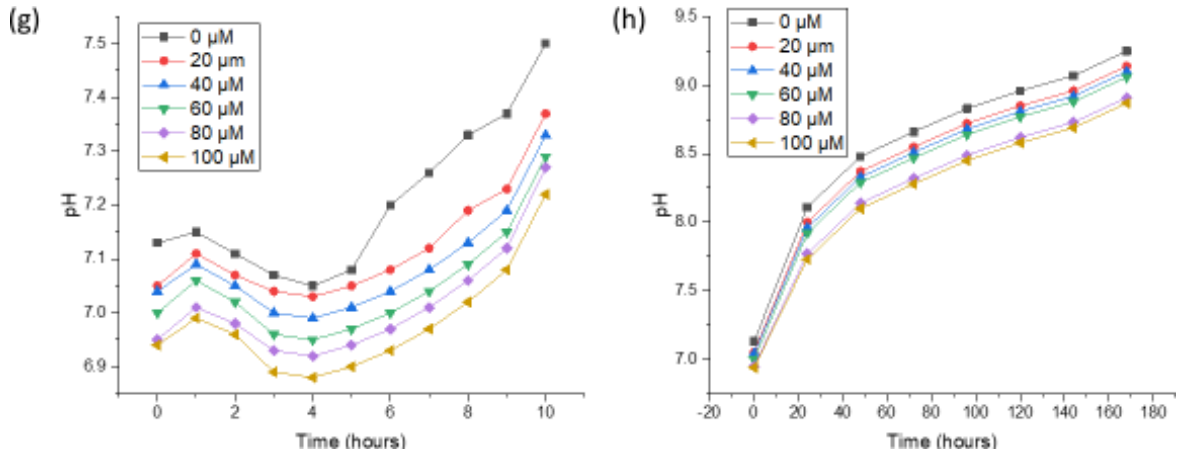
of incubation under 5% salt stress (d) Subsequent change in pH from 24 to 168 hours post inoculation in nutrient broth medium supplemented with different concentration of cadmium (0 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM) and 5% salt stress.

In Fig. 18 (e) a decrease in pH is observed in the first 5 hours which rises from 6 hour onwards for calcium carbonate precipitation under varying concentrations of cadmium at 5% salt stress. Fig 18 (f) shows an extreme change in pH in the first 24 hours which then rises till day 7 from pH 7 to approximately 9.5.



(e) pH variation in nutrient broth medium supplemented with different concentration of arsenic (0 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM) for initial 10 hours of incubation (f) Subsequent change in pH from 24 to 168 hours post inoculation in nutrient broth medium supplemented with different concentration of cadmium (0 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM).

Fig. 18 (g) shows a decrease in pH for the first 5 hours which keeps on increasing. In Fig 10 (h) there is a sharp rise in the pH in the first 24 hours which keeps on rising till day 7.



(e) pH variation in nutrient broth medium supplemented with different concentration of

arsenic (0 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM) for initial 10 hours of incubation at 5% salt stress (f) Subsequent change in pH from 24 to 168 hours post inoculation in nutrient broth medium supplemented with different concentration of cadmium (0 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM) at 5% salt stress.

4.6 Assessing the removal of cadmium and arsenic with and without salt stress

The quantification of heavy metals was done by atomic absorption spectroscopy. In this technique, the sample is made to atomize. The free atoms thus produced absorb UV or visible light and make transitions to higher electronic energy levels. The concentration of the heavy metal is determined from the extent of absorption. The percentage of heavy metal removal was assessed by subtracting amount of heavy metal detected divided by the mount of heavy metal present in the solution initially. Fig. 19 and 20 compare the heavy metal removal by microbially induced calcite precipitation under highly saline and non-saline conditions for cadmium and arsenic respectively. In Fig. 19 it is observed, as the initial concentration of cadmium in the solution is increasing, the percentage of removal by MICCP is also increasing up to a concentration of 40 µM. At 50 µM the percentage of removal decreases sharply. A similar trend is observed in case of arsenic in saline and normal conditions. The percentage of removalof arsenic increases up to 60 µM of arsenic concentration after which a decline in removal efficiency is observed. As seen in Fig. 19 and 20 the maximum concentration of cadmium and arsenic that can be removed with maximum efficiency is 40 µM and 60 µM respectively in normal conditions.

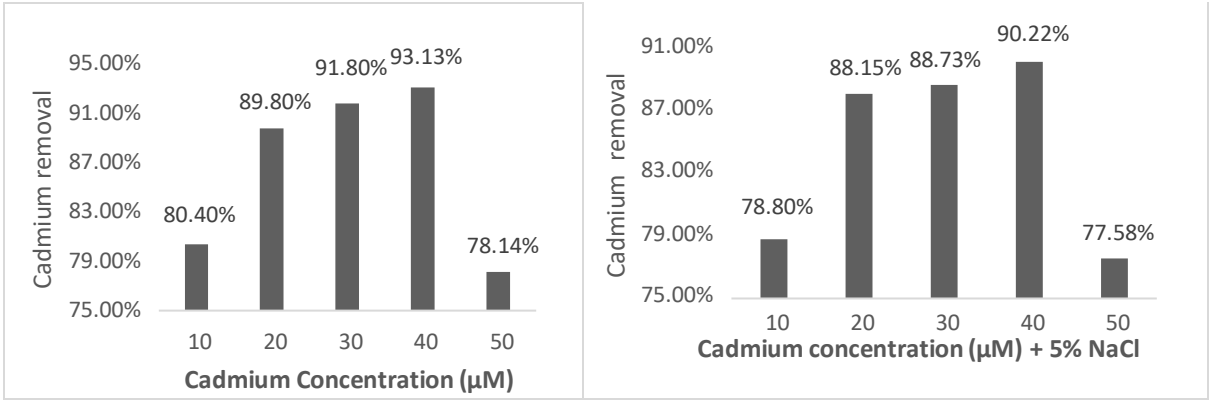


Fig. 19 Graphs showing the removal percentage of cadmium through MICCP under normal and 5% salt stress conditions

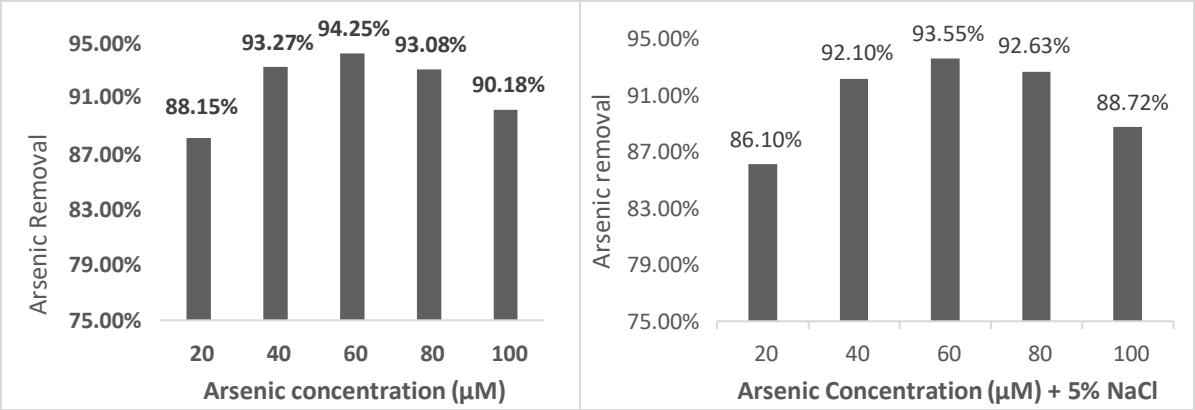


Fig. 20 Graphs showing the removal percentage of arsenic through MICCP under normal and 5% salt stress conditions

4.7 FESEM and EDS analysis of the bio-precipitates

Scanning electron microscopy was performed to study the morphology of the precipitated calcium carbonate crystal. The crystal structure was studied using FE-SEM and EDS was performed to find out the percentage of calcium carbonate and heavy metal content in the precipitate to ensure that the heavy metal ions have been trapped within the calcium carbonate crystal lattice. The SEM images obtained for the heavy metal-calcium carbonate bio-precipitates are shown in Fig. 21. The structure of calcium carbonate crystals with the morphology resembling the calcite polymorph of calcium carbonate was observed labelled as CC in Fig. 21. The EDS graphs along with the elemental composition of heavy metal- calcium carbonate bio-precipitates is shown in Fig. 22 and 23.

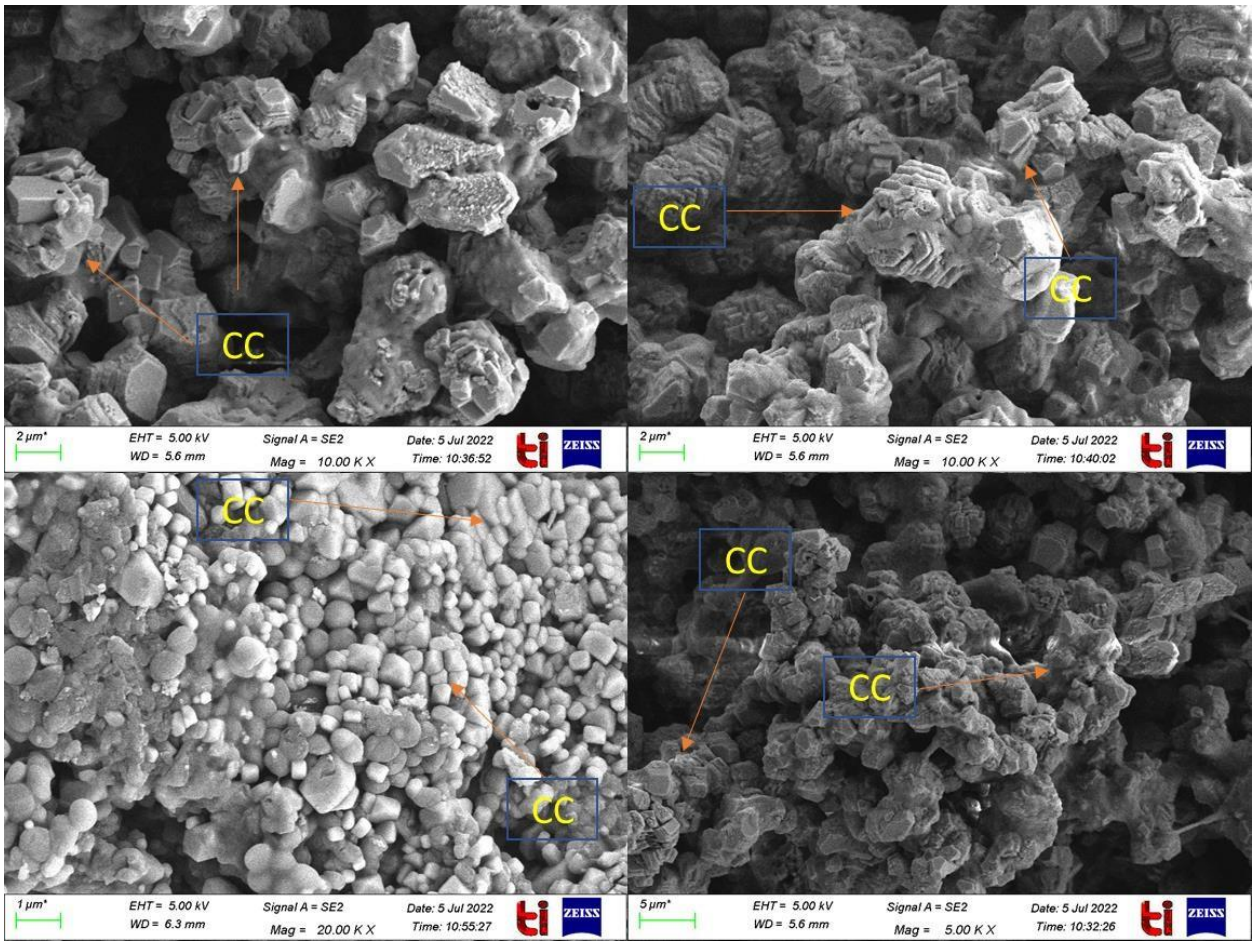


Fig. 21 Microcrsytalline structure analysis through FESEM

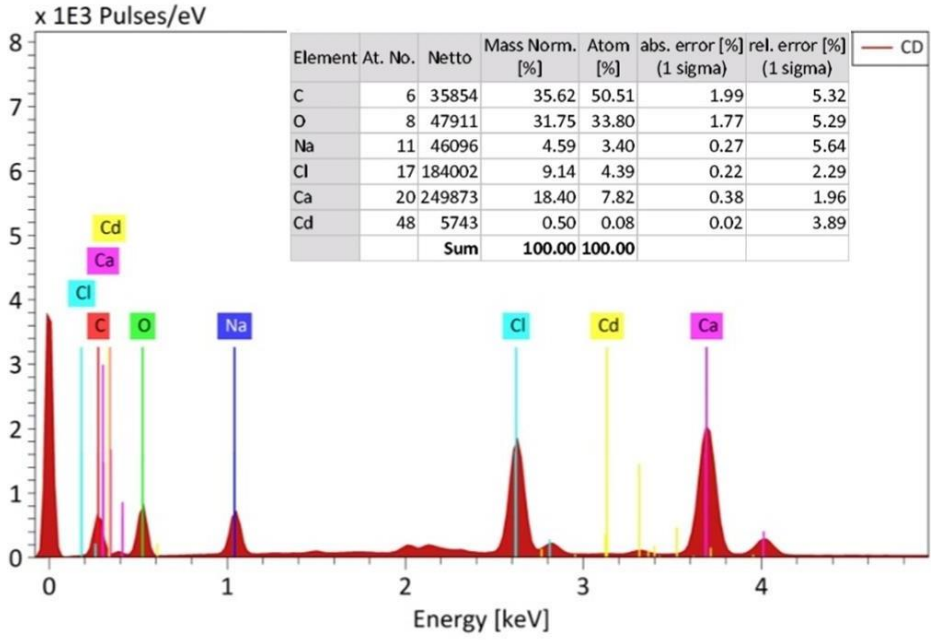
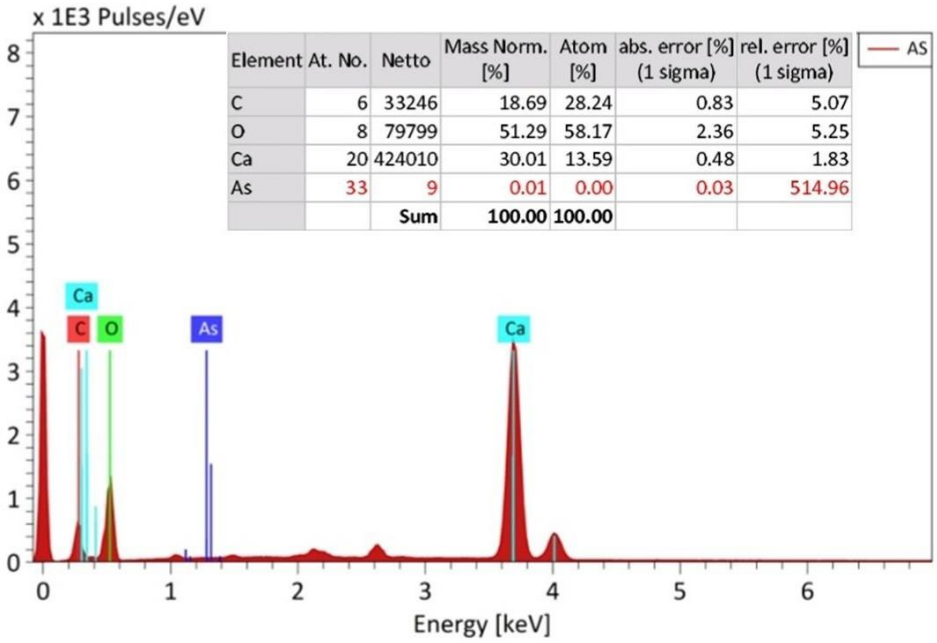


Fig. 22 EDX analysis to determine the elemental composition of cadmium-calcium carbonate bioprecipitates.



Element	At. No.	Netto	Mass Norm. [%]	Atom [%]	abs. error [%] (1 sigma)	rel. error [%] (1 sigma)
C	6	33246	18.69	28.24	0.83	5.07
O	8	79799	51.29	58.17	2.36	5.25
Ca	20	424010	30.01	13.59	0.48	1.83
As	33	9	0.01	0.00	0.03	514.96
		Sum	**100.00**	**100.00**		

Fig. 23 EDX Analysis to determine the elemental composition of Arsenic- calcium carbonate bioprecipitates.

DISCUSSION

Heavy metals are more toxic in ionic form than the metal carbonate form as the latter is relatively more insoluble and inert. Through precipitation, microorganisms aid in the metal immobilization and lead to chemical changes in metal compounds.

It was observed that as the time progresses, the amount of calcium carbonate precipitates also goes up. As the concentration of heavy metals in solution was increased, the precipitation of calcium carbonate decreased because heavy metal ions act as an inhibitor to the normal cellular functions of bacteria. Similar observations have been made by Li et al. (2014). This can be described owing to the fact that the number of free metal binding sites is decreasing and so is the number of viable bacterial cells. Also, the heavy metals interfere with the bacterial DNA repair mechanisms and proliferation.

The initial concentration taken for cadmium was 0 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM and for arsenic 0 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM. The heavy metal removal percentage in cadmium was 93.13% and 90.22% under normal and saline conditions respectively while heavy metal removal percentage of 94.25% and 93.55% was noted for arsenic under normal and saline conditions. No heavy metal removal was observed in control. In a study performed by Kang et al. (2014), almost 99 % of the Cd ions could be removed using MICP in the BPU (beef extract, peptone, urea) media. The ability of *S. ginsengisoli* CR5 to remediate arsenic was first investigated by Achal et al. (2012) which showed that it was able to remediate 96.3% of arsenic at the end of 168 hours. Xanthomonas sp. B13 has been described to remediate 96.9% of arsenic in aqueous media by Aksornchu et. Al. (2008). Nagvenkar and Ramaiah reported 92% removal of arsenic by Enterobacteriaceae at the end of 5 days.

The FESEM and EDX results also reported the formation of heavy metal-calcium carbonate complex which means the immobilization of the heavy metals was successfully achieved through microbially induced calcium carbonate precipitation.

CONCLUSION

The urease activity assay results denoted that the highest salt stress that the bacteria can tolerate without compromising on the urease activity was 5% salt stress. Inhibitory concentration 50 (IC_{50}) was determined as the concentration of heavy metal that can to inhibit the growth of bacterial population by 50%. the IC_{50} value for cadmium was found out to be 12.84 µM for normal conditions and 9.846 µM under 5% salt stress while the IC_{50} value for arsenic was found out to be 48.54 µM for normal conditions and 62.86 µM under 5% salt stress.

The highest urease activity was reported at 96 hours in cadmium while in arsenic the highest urease activity was observed at 120 hours. The urease activity at cadmium concentrations of 10 µM, 20 µM, 30 µM and 40 µM was noted to be 616 U/ml, 602 U/ml, 562 U/ml & 525 U/ml. The urease activity at cadmium concentration under 5% salt stress was 10 µM to 40 µM was 608 U/ml, 547 U/ml, 501 U/ml & 488 U/ml. The urease activity at arsenic concentrations 20 µM, 40 µM, 60 µM, 80 µM & 100 µM was 847 U/ml, 836 U/ml, 824 U/ml, 797 U/ml, 734 U/ml under normal conditions and 708 U/ml, 518 U/ml, 435 U/ml, 314 U/ml, 304 U/ml under 5 % salt stress respectively.

The maximum amount of calcium carbonate precipitated was measured was 189 mg/100 ml (10 µM), 174 mg/100 ml (20 µM), 159 mg/ 100 ml (30 µM), 134 mg/100 ml, (40 µM) under cadmium concentrations. In case of cadmium under 5% salt stress the amount of calcium carbonate precipitated was 10 µM to 40 µM i.e. 165 mg/100ml (10 µM), 160 mg/100 ml (20 µM), 150 mg/100 ml (30 µM), 125 mg/100 ml (40 µM). The maximum calcium carbonate was precipitated in arsenic concentration was 20 µM (198 mg/ 100 ml), 40 µM (183 mg/100 ml), 60 µM (178 mg/100 ml), 80 µM (163 mg/100 ml) and lastly, 100 µM (141 mg/100 ml). The maximum calcium carbonate was precipitated in arsenic concentration at 5% salt stress was 20 µM (164 mg/ 100 ml), 40 µM (159 mg/100 ml), 60 µM (149 mg/100 ml), 80 µM (124 mg/100 ml) and lastly, 100 µM (49 mg/100 ml).

The trend observed in the change of pH over time showed that there was a slight reduction in the pH upon addition of Urea and Calcium Chloride for the first 5 hours considering the addition of urea and calcium carbonate to be done at 0 hour. This reduction in pH can be explained considering the dissociation of ions in water upon addition. The pH then increases till 96 hours after which a plateau is observed.

The heavy metal removal percentage in cadmium was 93.13% and 90.22% under normal and saline conditions respectively while heavy metal removal percentage of 94.25% and 93.55% was noted for arsenic under normal and saline conditions. No heavy metal removal was observed in control. The FESEM and EDX results also showed the formation of heavy metal-calcium carbonate complex which means the immobilization of the heavy metals was achieved successfully through microbially induced calcium carbonate precipitation.

REFERENCES

Abd-Alla, M.H.; Morsy, F.M.; El-Enany, A.W.E.; Ohyama, T. Isolation and characterization of a heavy-metal-resistant isolate of Rhizobium leguminosarum bv. viciae potentially applicable for biosorption of Cd^{2+} and Co^{2+}. *International Biodeterioration & Biodegradation.* 2012, 67, 48–55.

Aziz, H.A., M.N. Adlan, and K.S. Ariffin, Heavy metals (Cd, Pb, Zn, Ni, Cu and Cr (III)) removal from water in Malaysia: Post treatment by high quality limestone. *Bioresource Technology*, 2008. 99(6): p. 1578-1583

Baeuerlein E. 2000 *Biomineralization* Weinheim, Germany: Wiley-VCH;

Bazylinski D. A. 2001; Bacterial mineralization. In *Encyclopedia of Materials: Science and Technology* pp 441–448 Amsterdam: Elsevier.

Beyersmann D, Hartwig A. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. *Arch Toxicol.* 2008; 82: 493– 512.

Bhatt, H. H., Pasricha, R., & Upasani, V. N. (2016). Isolation and characterization of a halophilic cyanobacterium Euhalothece SLVH01 from Sambhar salt lake, India. *International Journal of Current Microbiology and Applied Sciences*, *5*(2), 215-224.

BIS 10500-2012 Indian Standards for Drinking water, 2012

Bojórquez, C.; Voltolina, D. Removal of cadmium and lead by adapted strains of Pseudomonas aeruginosa and Enterobacter cloacae. *Revista internacional de contaminacion ambiental* 2016, 32, 407–412.

Bottjer D. J. 2005; Geobiology and the fossil record: eukaryotic, microbes, and their interactions. *Palaeogeogr Palaeoclimatol Palaeoecol* 219:5–21

Braissant, O., Decho, A. W., Dupraz, C., Glunk, C., Przekop, K. M., and Visscher, P. T. (2007). Exopolymeric substances of sulfate-reducing bacteria: interactions with calcium at alkaline pH and implication for formation of carbonate minerals. *Geobiology* 5, 401–411.

Bruins M. R., Kapil S., Oehme F. W. (2000). Microbial resistance to metals in the environment. *Ecotoxicol. Environ. Saf.* 45 198–207. 10.1006/eesa.1999.1860

Brunetti, G.; Farrag, K.; Soler-Rovira, P.; Ferrara, M.; Nigro, F.; Senesi, N. The effect of compost and Bacillus licheniformis on the phytoextraction of Cr, Cu, Pb and Zn by three Brassicaceae species from contaminated soils in the Apulia region, Southern Italy. *Geoderma* 2012, 170, 322–330.

Callagon, E., Fenter, P., Nagy, K.L., Sturchio, N.C., 2014. Incorporation of Pb at the calcite (104)-water interface. *Environ. Sci. Technol.* 48, 9263–9269.

Castanier, S., Le Metayer-Levrel, G., and Perthuisot, J. P. (1999). Ca-carbonates precipitation and limestone genesis—the microbiogeologist point of view. *Sediment. Geol.* 126, 9–23.

Castanier, S., Le Metayer-Levrel, G., and Perthuisot, J. P. (2000). "Bacterial roles in the precipitation of carbonate minerals," in *Microbial Sediments*, eds R. E. Riding and S. M. Awramik (Heidelberg: Springer-Verlag), 32–39.

Cervantes, C.; Campos-García, J.; Devars, S.; Gutiérrez-Corona, F.; Loza-Tavera, H.; Torres-Guzmán, J.C.; Moreno-Sánchez, R. Interactions of chromium with microorganisms and plants. *FEMS Microbiol. Rev.* 2001, 25, 335–347.

Chanmugathas, P.; Bollag, J.M. A column study of the biological mobilization and speciation of cadmium in soil. *Arch. Environ. Contam. Toxicol*. 1988, 17, 229–237.

CHEN, L. and Q. CHEN, Industrial application of UF membrane in the pretreatment for RO system [J]. *Membrane Science and Technology*, 2003. 4: p. 009.

Chorover J., Kretzschmar R., Garcia-Pichel F., Sparks D. L. 2007; Soil biogeochemical processes within the critical zone. *Elements* 3:321–326

Chronopoulos J, Haidouti C, Chronopoulou-Sereli A, Massas I. Variations in plant and soil lead and cadmium content in urban parks in Athens, Greece. *Sci Total Environ*. 1997; 196: 91– 98.

Collins, Y.E., Stotzky, G., Factors affecting the toxicity of heavy metals to microbes. In: ed. T.J. Beveridge and R.J. Doyle. Metal ions and bacteria, Wiley, Toronto, Canada, 1989, pp. 31-90

Crittenden, J.C., et al., Understanding and Improving Process Performance of Advanced Oxidation Processes (AOPs).

Danis, U.; Nuhoglu, A.; Demirbas, A. Ferrous ion-oxidizing in Thiobacillus ferrooxidans batch cultures: Influence of pH, temperature and initial concentration of Fe2+. *Fresenius Environ. Bull.* 2008, 17, 371–377.

Dermont, G., et al., Metal-contaminated soils: remediation practices and treatment technologies. *Practice Periodical of Hazardous, Toxic, and Radioactive Waste Management,* 2008. 12(3): p. 188-209.

Dittrich, M., and Sibler, S. (2010). Calcium carbonate precipitation by cyanobacterial polysaccharides. *Geol. Soc. Spec. Publ.* 336, 51–63.

Dizge, N., B. Keskinler, and H. Barlas, Sorption of Ni (II) ions from aqueous solution by Lewatit cation-exchange resin. *Journal of hazardous materials,* 2009. 167(1): p. 915-926.

Dupraz, C., Visscher, P. T., Baumgartner, L. K., and Reid, R. P. (2004). Microbe-mineral interactions: early carbonate precipitation in a hypersaline lake (Eleuthera Island, Bahamas). *Sedimentology* 51, 745–765.

Dupraz, S., Ménez, B., Gouze, P., Leprovost, R., Bénézeth, P., Pokrovsky, O. S., et al. (2009b). Experimental approach of CO_2 biomineralization in deep saline aquifers. *Chem. Geol.* 265, 54–62.

Ehrlich, H. L. (1996). How microbes influence mineral growth and dissolution. *Chem. Geol.* 132, 5–9.

Fein, J. B., Daughney, C. J., Yee, N., and Davis, T. A. (1997). A chemical equilibrium model for metal adsorption onto bacterial surfaces. *Geochim. Cosmochim. Acta* 61, 3319–3328.

Fu, F. and Q. Wang, Removal of heavy metal ions from wastewaters: a review. *Journal of Environmental Management,* 2011. 92(3): p. 407-418

Fujita, Y., Ferris, F. G., Lawson, R. D., Colwell, F. S., and Smith, R. W. (2000). Subscribed content calcium carbonate precipitation by ureolytic subsurface bacteria. *Geomicrobiol*. J. 17, 305–318.

G.S. Nagvenkar, N. Ramaiah, Arsenite tolerance and biotransformation potential in estuarine bacteria, Ecotoxicology 19 (2010) 604–613

Gadd G. M. 2008a; Bacterial and fungal geomicrobiology: a problem with communities. *Geobiology* 6:278–284

Gadd, G. M. (2010). Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology* 156, 609–643.

Garbisu, C., Alkorta, I., Bioremediation: principles and future. *J Clean Technol. Environ. Toxicol. Occup. Med.,* 1997, 6, 1-16

Gavrilescu, M. Removal of Heavy Metals from the Environment by Biosorption. *Eng. Life Sci.* 2004, 4, 219–232.

Genestra M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cell Signal*. 2007; 19: 1807– 1819.

Gilmour C., Riedel G. 2009; Biogeochemistry of trace metals and metalloids. In *Encyclopedia of Inland Waters* pp 7–15 Edited by Likens G. E. Amsterdam: Elsevier

Gleeson D., McDermott F., Clipson N. 2007; Understanding microbially active biogeochemical environments. *Adv Appl Microbiol* 62:81–104

H. Eccles. Treatment of metal-contaminated wastes: why select a biological process *Trends Biotechnol*., 17 (1999), pp. 462-465

Haferburg G., Kothe E. (2007). Microbes and metals: interactions in the environment. *J. Basic Microbiol.* 47 453–467. 10.1002/jobm.200700275

Hamdaoui, O., Removal of copper (II) from aqueous phase by Purolite C100-MB cation exchange resin in fixed bed columns: Modeling. *Journal of hazardous materials,* 2009. 161(2): p. 737-746

Hasan, H. (2000). Ureolytic microorganisms and soil fertility: a review. *Commun. Soil Sci. Plant Anal.* 31, 2565–2589.

Hattab, S., Boussetta, H., and Banni, M. (2014). Influence of nitrate fertilization on Cd uptake and oxidative stress parameters in alfalfa plants cultivated in presence of Cd. *J. Soil Sci. Plant Nutr.* 14, 89–99.

He, L.M.; Tebo, B.M. Surface charge properties of and Cu(II) Adsorption by Spores of the Marine Bacillus sp. Strain SG-1. Appl. Environ. Microbiol. 1998, 64, 1123–1129.

Hussein J.Khadim Saad H.Ammar Shahlaa E.Ebrahim .Biomineralization based remediation of cadmium and nickel contaminated wastewater by ureolytic bacteria isolated from barn horses soil

Iwamoto, T., Nasu, M., Current bioremediation practice and perspective. *J. Biosci. Bioengin.*, 2001, 92(1), 1-8.

Järup, L., and Åkesson, A. (2009). Current status of cadmium as an environmental health problem. *Toxicol. Appl. Pharmacol.* 238, 201–208. doi: 10.1016/j.taap.2009.04.020

Joner, E.J.; Leyval, C. Uptake of 109Cd by roots and hyphae of a Glomus mosseae/Trifolium subterraneum mycorrhiza from soil amended with high and low concentrations of cadmium. New Phytol. 1997, 135, 353–360.

Knoll, A. H. (2003). Biomineralization and evolutionary history. *Rev. Mineral. Geochem.* 54, 329–356.

Knox, A.S., Seaman, J., Adriano, D.C., Pierzynski, G., Chemophytostabilization of metals in contaminated soils. In: ed. D. L: Wise, D. J. Trantolo, E. J. Cichon, H. I. Inyang, and U. Stottmeister. *Bioremediation of contaminated soils.* Marcel Dekker, Inc., New York, 2002, pp. 811-836

Konhauser K. 2007 *Introduction to Geomicrobiology* Oxford: Blackwell;

Ku, Y. and I.-L. Jung, Photocatalytic reduction of Cr (VI) in aqueous solutions by UV irradiation with the presence of titanium dioxide. Water Research, 2001. 35(1): p. 135-142.

Kurniawan, T.A., et al., Physico–chemical treatment techniques for wastewater laden with heavy metals. Chemical engineering journal, 2006. 118(1): p. 83-98.

Landa E. R. 2005; Microbial biogeochemistry of uranium mill tailings. *Adv Appl Microbiol* 57:113–130

Lane TW, Morel FM. A biological function for cadmium in marine diatoms. *Proc Natl Acad Sci*. 2000; 97: 4627– 4631.

López-Maldonado, E., et al., Coagulation– flocculation mechanisms in wastewater treatment plants through zeta potential measurements. *Journal of hazardous materials*, 2014. 279: p. 1-10.

Lovley, D.R., Dissimilatory metal reduction. *Annu. Rev. Microbiol*., 1993, 47, 263-290.

Lovley, D.R., Lloyd, J.R., Microbes with a mettle for bioremediation. Nat. Biotechnol., 2000, 18, 600-601. Miller, R.R., Phytoremediation. Ground-Water Remediation Technologies Analysis Center (GWRTAC)- Technology Overview Report TO-96-03, GWRTAC-O-Series, 1996, p. 11.

Macalady J., Banfield J. F. 2003; Molecular geomicrobiology: genes and geochemical cycling. *Earth Planet Sci Lett* 209:1–17

Milukaite A., Sakalys J., Kvietkus K., Vosyliene M.Z., Kazlauskiene N., Karlaviciene N. Physico-chemical and ecotoxicological characterizations of urban storm water runoff. Pol. J. Environ. Stud. 19, 1279, 2010

Mollah, M.Y.A., et al., Electrocoagulation (EC)—science and applications. Journal of hazardous materials, 2001. 84(1): p. 29-41.

Mukke V, Chinte D. Impact of heavy metal induced alterations in Lipase activity of fresh water crab, Barytelphusa guerini. *J Chem Pharm Res*. 2012; 4: 2763– 2766.

Naeem, A., Saifullah, Ghafoor, A., and Farooq, M. (2015). Suppression of cadmium concentration in wheat grains by silicon is related to its application rate and cadmium accumulating abilities of cultivars. *J. Sci. Food Agric.* 95, 2467–2472.

Nieboer, E., Richardson, D.H.S., The replacement of the non-descript term “heavy metals” by a biologically and chemically significant classification of metals ions. Env. Pollution (B), 1980, 1, 3-26.

Niu, Z.X.; Sun, L.N.; Sun, T.H. The Bioadsorption of Cadmium and Lead by Bacteria in Root Exudates Culture. J. Soil Contam. 2011, 20, 877–891.

Obst, M., Dynes, J., Lawrence, J., Swerhone, G., Benzerara, K., Karunakaran, C., et al. (2009). Precipitation of amorphous CaCO3 (aragonite-like) by cyanobacteria: a STXM study of the influence of EPS on the nucleation process. *Geochim. Cosmochim*. Acta 73, 4180–4198.

P. Aksornchu, P. Prasertsan, V. Sobhon, Isolation of arsenic-tolerant bacteria from arsenic-contaminated soil, Songklanakarin *J. Sci. Technol.* 30 (2008) 95–102

Pan, K., Wang, W., 2011. Trace metal contamination in estuarine and coastal environments in China. *Sci. Total Environ*. 421–422, 3–16.

Pentecost, A., and Bauld, J. (1988). Nucleation of calcite on the sheaths of cyanobacteria using a simple diffusion cell. *Geomicrobiol. J*. 6, 129–135.

Petrov, S. and V. Nenov, Removal and recovery of copper from wastewater by a complexation-ultrafiltration process. Desalination, 2004. 162: p. 201-209.

Rahman, M.S.; Sathasivam, K.V. Heavy Metal Adsorption onto Kappaphycus sp. from Aqueous Solutions: The Use of Error Functions for Validation of Isotherm and Kinetics Models. *Biomed. Res. Int.* 2015, 2015, 126298.

Rai G. K., Bhat B. A., Mushtaq M., Tariq L., Rai P. K., Basu U., et al. (2021). Insights into decontamination of soils by phytoremediation: a detailed account on heavy metal toxicity and mitigation strategies. *Physiol. Plant* 173 287–304. 10.1111/ppl.13433

Rascio, N., and Navarri-Izzo, F. (2011). Heavy metal hyperaccumulating plants: how and why do they do it? And what makes them so interesting. *Plant Sci.* 180, 169–181.

Raskin, I., Kumar, P.B.A.N., Dushenkov, S., Salt, D.E., Bioconcentration of heavy metals by plants. *Curr. Opin. Biotechnol*., 1994, 5, 285-290

Reeburgh, W. S. (2007). Oceanic methane biogeochemistry. Chem. Rev. 107, 486–513.

Rether, A. and M. Schuster, Selective separation and recovery of heavy metal ions using water-soluble N-benzoylthiourea modified PAMAM polymers. *Reactive and Functional Polymers*, 2003. 57(1): p. 13-21.

Rivadeneyra, M. A., Parraga, J., Delgado, R., Ramos-Cormenzana, A., and Delgado, G. (2004). Biomineralization of carbonates by *Halobacillus trueperi* in solid and liquid media with different salinities. *FEMS Microbiol. Ecol*. 48, 39–46.

Rodriguez-Navarro, C., and Doehne, E. (1999). Salt weathering: influence of evaporation rate, supersaturation and crystallization pattern. *Earth Surface Process. Landforms* 24, 191–209.

Rodriguez-Navarro, C., C. Jimenez-Lopez, A. Rodriguez-Navarro, M. T Gonzalez-Muñoz, and M. Rodríguez-Gallego. 2007. Bacterially mediated mineralization of vaterite. *Geochim. Cosmochim. Acta* 71: 1197-1213

Rodriguez-Navarro, C., Rodriguez-Gallego, M., Ben Chekroun, K., and GonzalezMunoz, M. T. (2003). Conservation of ornamental stone by Myxococcus xanthus-induced carbonate biomineralization. *Appl. Environ. Microbiol.* 69, 2182–2193.

S. Babel, T.A. Kurniawan. Cr (VI) removal from synthetic wastewater using coconut shell charcoal and commercial activated carbon modified with oxidizing agents and/or chitosan *Chemosphere*, 54 (7) (2004), pp. 951-967

Seifan M, Samani AK, Berenjian A. Bioconcrete: next generation of self-healing concrete. *Appl Microbiol Biotechnol* 2016;100:2591602.

Singh MR. 2007. Impurities-Heavy Metals: IR prespective

Smith, L., Contaminants and remedial options at selected metal-contaminated sites. Technical resource report, 1995, Battelle, Columbus, OH (United States).

Sparks D. L. 2005; Toxic metals in the environment: the role of surfaces. *Elements* 1:193–196

Tourney, J., Ngwenya, B. T., Mosselmans, J. F., Tetley, L., and Cowie, G. L. (2008). The effect of extracellular polymers (EPS) on the proton adsorption characteristics of the thermophile *Bacillus licheniformis* S-86. *Chem. Geol.* 247, 1–15.

Tourney, J., Ngwenya, B. T., Mosselmans, J. F., Tetley, L., and Cowie, G. L. (2008). The effect of extracellular polymers (EPS) on the proton adsorption characteristics of the thermophile *Bacillus licheniformis* S-86. *Chem. Geol.* 247, 1–15.

Trellu, C.; Mousset, E.; Pechaud, Y.; Huguenot, D.; van Hullebusch, E.D.; Esposito, G.; Oturan, M.A. Removal of hydrophobic organic pollutants from soil washing/flushing solutions: A critical review. *J. Hazard. Mater.* 2016, 306, 149–174.

Trivunac, K. and S. Stevanovic, Removal of heavy metal ions from water by complexationassisted ultrafiltration. *Chemosphere,* 2006. 64(3): p. 486-491.

Van Paassen, L. A., Daza, C. M., Staal, M., Sorokin, D. Y., Van Der Zon, W., and Van Loosdrecht, M. C. (2010). Potential soil reinforcement by biological denitrification. *Ecol. Eng*. 36, 168–175.

Volesky, B.; Holan, Z.R. Biosorption of heavy metals. Biotechnol. Prog. 1995, 11, 235–250.

Wang, Y.; Guo, J.; Liu, R. Biosorption of heavy metals by bacteria isolated from activated sludge. *Appl. Biochem. Biotechnol*. 2001, 91–93, 171–184.

Warren, L. A., Maurice, P. A., Parmar, N., and Ferris, F. G. (2001). Microbially mediated calcium carbonate precipitation: implications for interpreting calcite precipitation and for solid-phase capture of inorganic contaminants. *Geomicrobiol. J.* 18, 93–115.

White, C., Sharman, A.K., Gadd, G.M., An integrated microbial process for the bioremediation of soil contaminated with toxic metals. *Nat. Biotechnol.,* 1998, 16, 572-575.

Williford, C., R.M. Bricka, and I. Iskandar, Physical separation of metal-contaminated soils 2000: CRC Press LLC, Boca Raton.

Zhuang, X., Han, Z., Bai, Z., Zhuang, G., Shim, H., 2010. Progress in decontamination by halophilic microorganisms in saline wastewater and soil. *Environ. Pollut*. 158 (5), 1119–1126.

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