

**ISOLATION AND CHARACTERIZATION OF ALKALI-
TOLERANT BACTERIAL ISOLATES FROM INDUSTRIAL SOIL
FOR POTENTIAL SELF-HEALING PAINT APPLICATIONS**

A Thesis Submitted

In Partial Fulfillment of the Requirements

for the Degree of

**MASTERS OF TECHNOLOGY
in
INDUSTRIAL BIOTECHNOLOGY
by**

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I, Shriya Agrawal (Roll No. 2K24/IBT/09), a student of Master of Technology (Industrial Biotechnology), hereby declare that the Project Dissertation titled **“Isolation and Characterization of alkali-tolerant bacterial isolates from industrial soil for potential self-healing paint applications”**, submitted to the Department of Biotechnology, Delhi Technological University, Delhi, in partial fulfilment of the requirements for the award of the degree of Master of Technology, is original and has not been copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma, Associateship, Fellowship, or any other similar title or recognition.

Place: Delhi

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A handwritten signature in blue ink, appearing to read 'Shriya'.

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CERTIFICATE

I hereby certify that the Project Dissertation titled **“Isolation and Characterization of alkali-tolerant bacterial isolates from industrial soil for potential self-healing paint applications”**, submitted by Shriya Agrawal, Roll No. 2K24/IBT/09, Department of Biotechnology, Delhi Technological University, Delhi, in partial fulfilment of the requirements for the award of the degree of Master of Technology, is a record of the project work carried out by the student under my supervision. To the best of my knowledge, this work has not been submitted, in part or full, for any degree or diploma to this University or elsewhere.

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ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisor, **Dr. Asmita Das**, for her invaluable guidance, support, and encouragement throughout this project. Her expert advice and insightful feedback were instrumental in shaping the direction and outcome of this research.

I also extend my gratitude to the Department of Biotechnology at Delhi Technological University for providing all the essential facilities and resources. Their support was crucial for the experimental work and overall progress of this study.

Lastly, I extend my appreciation to my colleagues and family for their constant support and motivation. Without them, this journey would not have been possible.

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ABSTRACT

The development of micro-cracks in protective paint coatings leads to a decrease in the durability and lifespan of structures due to penetration of moisture, oxygen and other corrosive agents. Traditional repair methods generally require regular maintenance and use of artificial chemical-based sealants. This can be sustainably tackled through autonomous self-healing coatings. Urease-mediated Microbially Induced Calcium Carbonate Precipitation (MICP), a novel bio-engineering method, has recently gained popularity, mainly due to its ability to exhibit calcite precipitation upon moisture ingress, thereby effectively closing cracks without requiring any external assistance.

The current study focuses on the isolation and characterization of alkali-tolerant indigenous bacterial strains and test their potential for self-healing paint applications. Soil samples collected from different industrial sites were serially diluted and cultured to obtain bacterial colonies. Three morphologically different bacterial isolates were purified and characterized under different alkaline conditions and concentrations of urea and calcium chloride. Growth behaviour was analysed at pH 8, 8.5 and 9 as well as with 1%, 2% and 3% concentrations of urea and calcium chloride to determine isolates that can survive under MICP conditions. Among the various isolates obtained, Isolate 2 exhibited better growth as compared to other isolates in the presence of alkaline and nutrient-stressed conditions. Gram staining indicated that Isolate 2 was gram positive while Isolate 1 and 3 were gram negative. Morphology and growth pattern of Isolate 2 was also similar to that of *Sporosarcina pasteurii*, the model organism for MICP based studies.

The outcome of the study imply that the industrial soils could be a potential candidate to identify alkali tolerant bacterial strains for future bio-based self-healing coatings. Additional research on urease activity analysis, precipitation of calcium carbonate and paint compatibility assessment are needed to be done to confirm their practical applicability.

Keywords: Microbially Induced Calcium Carbonate Precipitation (MICP), self-healing paint, alkali-tolerant bacteria, urease activity, biomineralization, sustainable coatings.

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LIST OF ABBREVIATIONS

CaCl ₂	Calcium Chloride
CaCO ₃	Calcium Carbonate
CFU	Colony Forming Unit
LB	Luria–Bertani
MICP	Microbially Induced Calcium Carbonate Precipitation
mL	Millilitre
NCIM	National Collection of Industrial Microorganisms
OD	Optical Density
OD ₆₀₀	Optical Density at 600 nm
pH	Potential of Hydrogen
rpm	Revolutions Per Minute
<i>S. pasteurii</i>	<i>Sporosarcina pasteurii</i>
UV	Ultraviolet
VOCs	Volatile Organic Compounds
w/v	Weight per Volume
°C	Degree Celsius

CHAPTER 1

INTRODUCTION

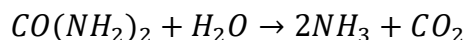
1.1 Microbially Induced Calcium Carbonate Precipitation (MICP) and Self-Healing Paints

The durability of coatings that are used for protection is one of the parameters that affects the lifespan and maintenance of buildings and infrastructure. Coatings that have been applied to concrete, metal and any other building materials will be subject to the effects of the environment, UV rays, water, temperature variations and mechanical friction. This results in the formation of tiny cracks on the coating, causing corrosion of the material below the coating[1]. The cracks lead to ingress of moisture, air, chlorides and chemicals into the structure[2].

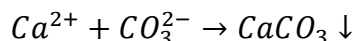
Traditional methods of repairing crack include repainting, use of polymer sealants, and chemical fillers, but these methods are temporary in nature and need frequent manual intervention. Also, most synthetic paints have volatile organic compounds that lead to environmental pollution and harm human health [3]. Hence, due to increasing concerns about sustainable construction and maintenance, self-healing materials have become popular among researchers. These types of materials can repair any damage without the need for manual intervention.

Among various forms of self-healing, biological self-healing using microorganisms is one area that has attracted significant research interest recently. In MICP, or Microbially induced Calcium Carbonate precipitation, calcite is precipitated in the presence of specific microorganisms [4]. Ureolytic MICP is a type of MICP in which bacteria produce carbonates by hydrolyzing urea, and these carbonates react with the calcium ions to form calcium carbonates. The formed crystals of calcium carbonate accumulate in the cracks and pores of concrete, making it less permeable and strong [5].

The overall ureolytic reaction involved in MICP can be represented as:



followed by calcium carbonate precipitation:



A number of genera, such as *Bacillus*, *Sporosarcina*, and *Pseudomonas*, have been examined for MICP applications [6]. Amongst these, *Sporosarcina pasteurii* is the most researched genus owing to its strong urease activity, alkaliphilic character, and ability to precipitate efficiently calcium carbonate crystals [7]. The bacterium has promising applications in self-healing concrete, soil stabilization, and bio cementation investigations.

While considerable advancements have been made in the area of MICP-based self-healing concrete, much less research efforts have been devoted to the application of MICP technology in paint and coatings. Paints represent an extremely difficult microhabitat for bacteria due to highly alkaline medium, absence of nutrients, risk of desiccation, and use of antimicrobials and solvents in formulations [8]. Hence, the choice of bacterial strains with the ability to withstand adverse conditions poses a great challenge in designing bio-self-healing paints.

Cement and construction industry soils are characterized by unique ecological conditions characterized by high alkalinity and calcium content [9]. It seems logical to assume that microorganisms inhabiting these ecological niches can be isolated and used for MICP purposes due to natural adaptation mechanisms allowing them to withstand harsh environmental factors.

The current study has been conducted with the objective of screening alkali-tolerant bacteria obtained from industrial soils that can be utilized in developing self-healing paints. The major aim of this study has been the evaluation of bacteria for growth in varying levels of alkalinity and also with differing levels of urea and calcium chloride. Characterization and identification of bacteria through morphological tests and Gram stains have been carried out as well. The results of this study could lead to the

development of environmentally friendly and bioactive coatings that could enhance the durability and life span of building materials.

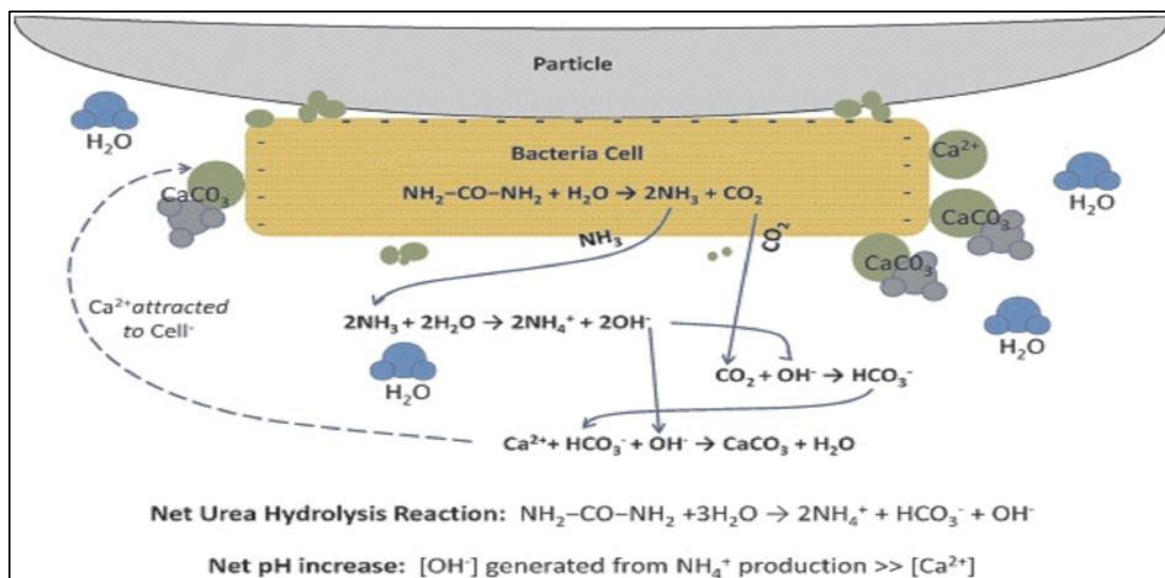


Figure 1.1: Bacteria serving as nucleation site for CaCO_3 precipitation in the sand particles (Source: DeJong et al., 2010).

1.2 Objectives of this study

The present study was carried out to isolate and characterize alkali-tolerant bacterial strains from industrial soil for their potential application in bacterial self-healing paint systems. Industrial soils represent chemically stressed environments that may harbour microorganisms capable of surviving under alkaline conditions and contributing to biomineralization processes such as microbially induced calcium carbonate precipitation (MICP). The study focused on evaluating the suitability of these bacterial isolates for future applications in sustainable self-healing coatings. The objectives of this study are:

1.2.1 To isolate bacterial strains from industrial soil samples using standard microbiological techniques.

1.2.2 To obtain pure bacterial cultures and perform preliminary morphological characterization of the isolates.

1.2.3 To evaluate the growth behaviour of bacterial isolates under varying concentrations of urea and calcium chloride.

1.2.4 To assess the survivability of bacterial isolates under alkaline pH conditions relevant to paint systems.

1.2.5 To compare the characteristics of indigenous isolates with the reference strain *Sporosarcina pasteurii*.

1.2.6 To identify potential alkali-tolerant bacterial candidates for future self-healing paint applications.

CHAPTER 2

LITERATURE REVIEW

2.1 Self-Healing Materials

Self-healing materials represent a recent innovation within the realm of intelligent materials that possess the capacity to self-repair without human interference or minimal intervention. They have been drawn from the natural biological systems that automatically heal themselves from injuries. Within the construction and protective coating industry, there is a growing interest in self-healing systems since they help to enhance the durability of buildings, decrease the frequency of maintenance, and increase the lifespan [10].

Manual crack-sealing, painting over cracks, and replacing broken materials are some of the conventional practices used in coating repairs and cement structures. Unlike these manual methods, the self-healing system involves restoring the material automatically after a crack develops, hence increasing sustainability [11].

Different self-healing concepts have been adopted such as the capsule-based technology, vascular network system, polymers as self-healing agent, and biological self-healing technique. Microbial self-healing represents a relatively new environment-friendly method of self-healing since it enables microbes to precipitate mineral compounds through natural processes [12].

Table 2.1: comparison between conventional paints and self-healing paints

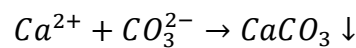
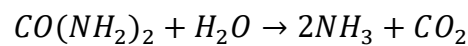
PARAMETER	CONVENTIONAL PAINT	SELF-HEALING PAINT
CRACK REPAIR	Manual	Autonomous
DURABILITY	Moderate	Improved
MAINTENANCE	Frequent	Reduced
SUSTAINABILITY	Lower	Higher

2.2 Microbially Induced Calcium Carbonate Precipitation (MICP)

Microbially Induced Calcium Carbonate Precipitation (MICP) is a biomineralisation process in which the metabolic activities of microorganisms induce the formation of calcium carbonate crystals [13]. This technique has become increasingly popular in recent times due to its usage in repairing cracks, bio-cementing, and the stabilization of soils and building materials.

The biomineralization processes involve the use of different metabolic routes for inducing calcium carbonate precipitation including; photosynthesis, sulfate reduction, denitrification, and ureolysis. One of the most efficient and widely studied process is urease-mediated MICP where urease producing bacteria degrade urea into ammonia and carbon dioxide causing a rise in the concentration of carbonate and increase in pH [14]. In the presence of calcium ions, calcium carbonate precipitation occurs in the form of crystals and accumulate within pores and cracks.

The process can be represented by the following reactions:



Calcium carbonate produced through MICP occurs in three polymorphs; calcite, aragonite, and vaterite. Among these, calcite is the stable form and plays a critical role in the formation of cracks [15].

MICP technology is environmentally friendly since it uses natural biogenic processes and reduces dependence on the repair material. However, some operational challenges remain to be solved, such as the optimisation of bacterial survivability, nutrient availability and environmental compatibility [16].

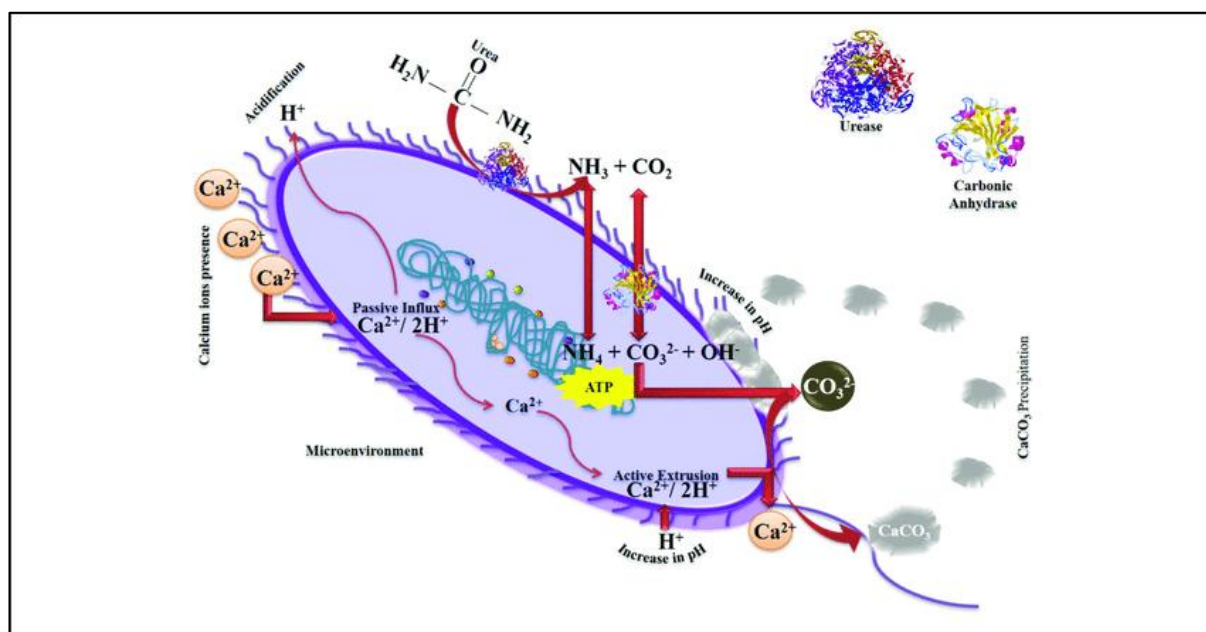


Figure 2.1: Schematic diagram of MICP formation mediated by urease and carbonic anhydrase.

(Source: Castro-Alonso MJ et al., 2019)

2.3 Role of Ureolytic Bacteria in Self-healing Materials

The ureolytic bacteria are essential for MICP because they can secrete enzymes called ureases responsible for hydrolysing urea. Various bacterial strains such as *Bacillus*, *Sporosarcina*, *Pseudomonas* and *Lysinibacillus* are researched for calcium carbonate precipitation application [17].

Among these microorganisms, spore-forming bacteria are considered to be very efficient for use in self-healing structures because spores are able to survive in extreme environmental conditions, such as drying, alkali environment, nutrition scarcity and temperature variation [18]. As soon as the dormant spores are activated by contact with water and nutrients, the biomineralization process starts after crack formation.

The effectiveness of calcium carbonate precipitation is influenced by different factors such as urease activity, calcium ion concentration, pH value, temperature, and nutrients. In fact, bacteria react to changes in their environmental conditions and affect biomineralization [19].

2.4 *Sporosarcina pasteurii* as a Model Organism

Sporosarcina pasteurii is the most intensively investigated urease-producing bacterium applied in various MICP-based processes. The bacterium is Gram-positive, alkaline-tolerant, non-pathogenic, and produces a high level of urease enzyme and thus highly efficient in calcium carbonate precipitation [20].

The microorganism has found a wide range of applications in investigations dealing with self-healing concrete, crack healing, soil stabilization, and bio-cementation. It is known to be able to tolerate alkaline conditions and deposit stable crystals of calcite and therefore is used as a reference organism in investigations assessing the biomineralizing capability of native bacterial isolates [21].

Earlier investigations have shown that *S. pasteurii* is an efficient producer of calcium carbonate under different environmental conditions and efficiently decreases crack permeability in cement-based matrices [22]. Due to its use in MICP-based applications, *S. pasteurii* is often used as a model bacterium in investigations concerning biological self-healing processes.

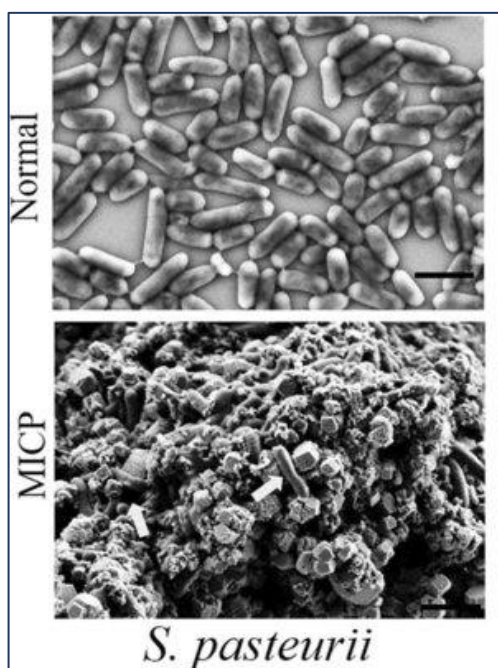


Fig 2.2. SEM images of *S. pasteurii* under normal and MICP conditions.

(Source: Liang Ma et al., 2020)

2.5 Self-Healing Paints and Bacteria Survival Difficulties

Self-healing paints represent innovative coating formulations that restore micro-cracks through self-repair after their emergence. Using bacteria within coating systems represents an environmentally friendly strategy compared to other technologies employed for coating repairs [23].

Although self-healing paint coatings using bacteria represent an attractive prospect, sustaining their viability within paint formulations presents substantial difficulties. Paints are complex chemical systems consisting of numerous substances such as pigments, binders, solvents, additives, and preservatives that can negatively impact bacteria survival [24]. Thus, for the successful development of self-healing paints, it is important to select appropriate microorganisms that will withstand harsh physicochemical conditions.

2.5.1 Physicochemical Constraints on Paints

The pH of architecture paints is between 8 and 9 due to the presence of alkaline substances and compounds that contain calcium. High pH values are generally hostile to many bacterial species but some urease-producing and alkaliphilic bacteria are relatively resistant and can survive in this environment [25].

There are a number of other paint properties that determine survival of the bacteria. For example, some paints include antimicrobial agents that reduce the viability of bacterial cells after long-term storage [26]. Solvent based paints tend to dehydrate bacterial cells by altering osmotic equilibrium. Water-based acrylic paints are found to be relatively more accommodating than solvent-based paints due to their low toxicity and environmental friendliness [27].

Moisture and nutritional requirements of bacteria affect their survival in paints. Bacterial spores become active only when there is sufficient water in the surrounding medium. The entry of water into paints is facilitated by cracking [28]. Thus, the interaction between bacterial cells and coatings is crucial in designing self-healing paints.

2.5.2 Effect of Environmental Parameters on Bacterial Activity

The surrounding environment significantly affects the bacterial growth, urease activity, and calcium carbonate deposition. Parameters such as pH, concentration of calcium ions, temperature, and nutrients determine the performance of the MICP process [29].

Among these factors, alkaline pH becomes especially critical because the deposition of carbonates increases when the conditions are alkaline. Several researchers have shown that the maximum activity of ureolytic bacteria is seen at a pH level ranging from 8 to 9.5, which favors bacterial metabolism and calcite deposition [30].

Moreover, urea and calcium chloride concentration can influence the bacterial growth and biomineralization efficiency. The former acts as a substrate in hydrolysis catalyzed by the urease enzyme, while the latter provides the necessary calcium ions for calcite formation. However, very high concentrations can negatively impact bacteria due to osmotic pressure [31].

In addition, temperature significantly influences the metabolism and enzymes' activity. The majority of ureolytic bacteria used for MICP purposes grow at 30°C to 37°C. The metabolic activity slows down under low temperatures, while high temperatures may affect the stability of urease enzyme and bacteria viability [32].

2.5.3 Bacterial Survival and Growth Behaviour in Coating Matrices

Bacterial survival and activity within matrix coatings are vital parameters for ensuring the efficiency of self-healing coatings. In most bacterial self-healing systems, spore-forming bacteria are used since their spores can survive in adverse environments, including limited nutrient availability, alkaline conditions, and dehydration [33].

Upon the formation of micro-cracks followed by the entry of moisture into the coating layer, bacteria become activated and resume metabolic functions, which initiate the ureolysis process and result in the deposition of calcium carbonate at the site of damage [34].

It has been previously demonstrated that Gram-positive bacteria with spores show higher resistance to alkaline conditions than non-spore-forming bacteria due to having

resistant spore forms and strong cell walls [35]. Evaluating the growth of bacteria under various conditions can be considered one of the key steps in the selection of microorganisms for self-healing purposes.

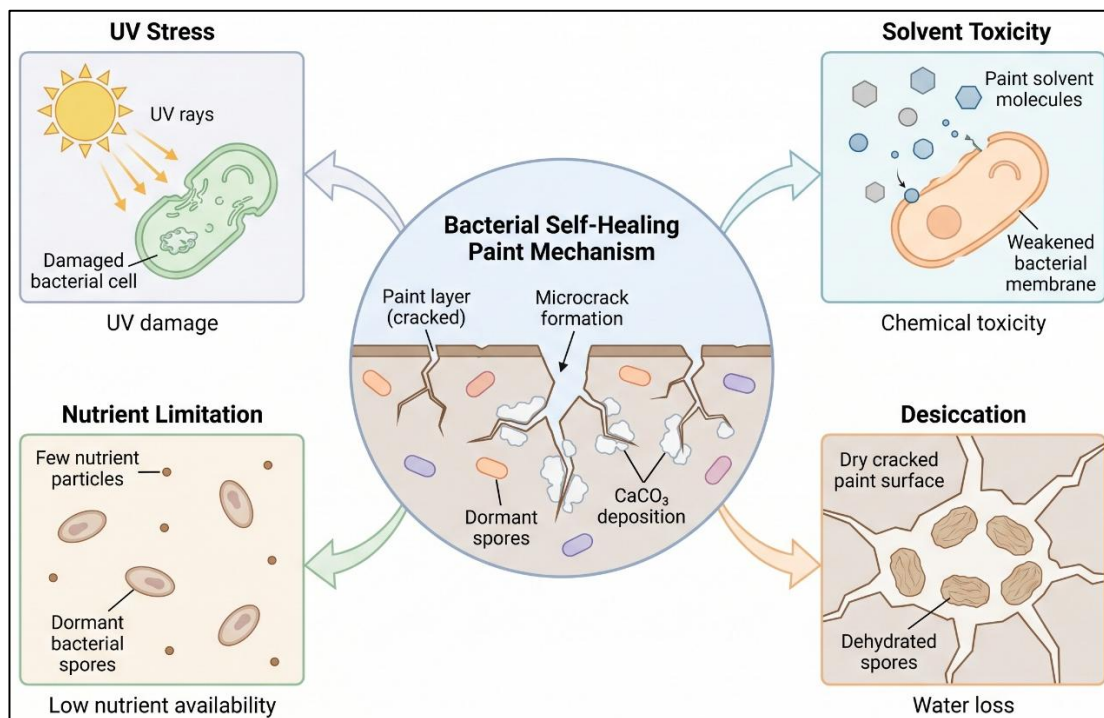


Figure 2.3: Challenges associated with bacterial self-healing paints. (Created with: Biorender)

2.6 Significance of Indigenous Alkali Resistant Bacterial Isolates

Indigenous bacteria from industrial surroundings have been gaining significant research attention lately due to their adaptation to stressful environment conditions. In such case, the natural selection favours bacteria with higher survivability in adverse soil composition, which consists of high pH, variable nutrients, and minerals [36].

In this way, bacteria found in a natural habitat would be likely to have better survivability in alkaline conditions associated with self-healing materials than those grown in a lab. Hence, they could be applied as a source of bacteria for coating and self-healing material technologies.

2.6.1 Industrial Soil as a Potential Source of Alkali-Resistant Bacteria

Industrial soil is characterized by rich bacterial diversity and contains a large number of bacteria that have adapted to live in an alkaline and chemically-stressful environment. Such soil can be considered a resource for alkaline and biomineralizing bacteria [37].

Their isolation could be done through the process of serial dilutions and plates, which allows the selection of bacteria with characteristic morphology. Preliminary alkaline screenings can provide useful data on isolates survival [38].

The utilization of indigenous isolates could provide advantages like environmental adaptation, enhanced stress tolerance, and lesser dependence on microbial cultures from outside sources. It can also aid in developing self-healing systems that are efficient and sustainable both economically and environmentally.

2.6.2 Importance of Preliminary Characterization of Bacterial Isolates

Preliminary characterization of bacterial isolates plays an integral role in selecting isolates that will be subjected to further biomineralization processes. Morphology, in terms of colour, elevation, texture, and margin of colonies, gives information about bacterial diversity [39].

In most cases, Gram stain procedure is carried out for the purpose of distinguishing Gram positive and Gram-negative organisms depending on the structure of their cell walls. Gram positive isolates are preferred over their counterparts for harsh environments since their thick peptidoglycan layer increases resistance [40].

Testing the isolates at different pH levels as well as various concentrations of calcium chloride and urea can also help in assessing the adaptability of bacterial isolates towards the MICP process. These preliminary studies are an important basis for determining potential bacterial candidates for subsequent self-healing paint applications.

2.7 Current Challenges and Future Perspectives in Self-Healing Paint Technology

Despite the promising prospects that have been observed, there are still a number of challenges that prevent the application of self-healing coating technologies in larger scales. The first of the obstacles is the preservation of viability and metabolism of bacteria used in such applications, which can be impaired by the presence of chemicals, paint preservatives, lack of nutrients and harsh environment [41].

One of the other limitations concerns the control of calcium carbonate deposition in the cracks formed on the paint surface, since too much precipitation might result in unfavorable changes in the characteristics of the coating, whereas too little would mean insufficient repair [42].

In order to overcome the limitations, different techniques for bacterial immobilization are studied, including encapsulation using silica gel, lightweight aggregates and polymeric microcapsules [43]. This should enable controlled activation of bacteria inside paint coating when needed.

It is evident that advances in the field of biomineralization technology and microbial biotechnology open up a lot of perspectives regarding the future of self-healing coatings. Additional research on improving survivability of bacteria, compatibility with commercially available paints and durability may contribute towards the development of effective biologically active coatings.

2.8 Research Gap

Though considerable progress has been achieved within the field of microbial self-healing concrete, relatively few studies have been conducted concerning self-healing paints. Most of the studies conducted till now involve laboratory strains such as *Sporosarcina pasteurii*, whereas there is very little research conducted about using indigenous alkali tolerant bacteria for self-healing purposes.

Further, little work has been done to evaluate bacterial survival in paint related alkalinity along with various levels of Calcium and nutrient concentration. Limited

information is available about the preliminary selection of industry soil isolates for self-healing paints.

Hence, this paper aims at identifying and studying the preliminary characteristics of alkali tolerant indigenous bacteria capable of surviving in conditions suitable for MICP for the purpose of self-healing paints.

CHAPTER 3

METHODOLOGY

3.1 Experimental Workflow

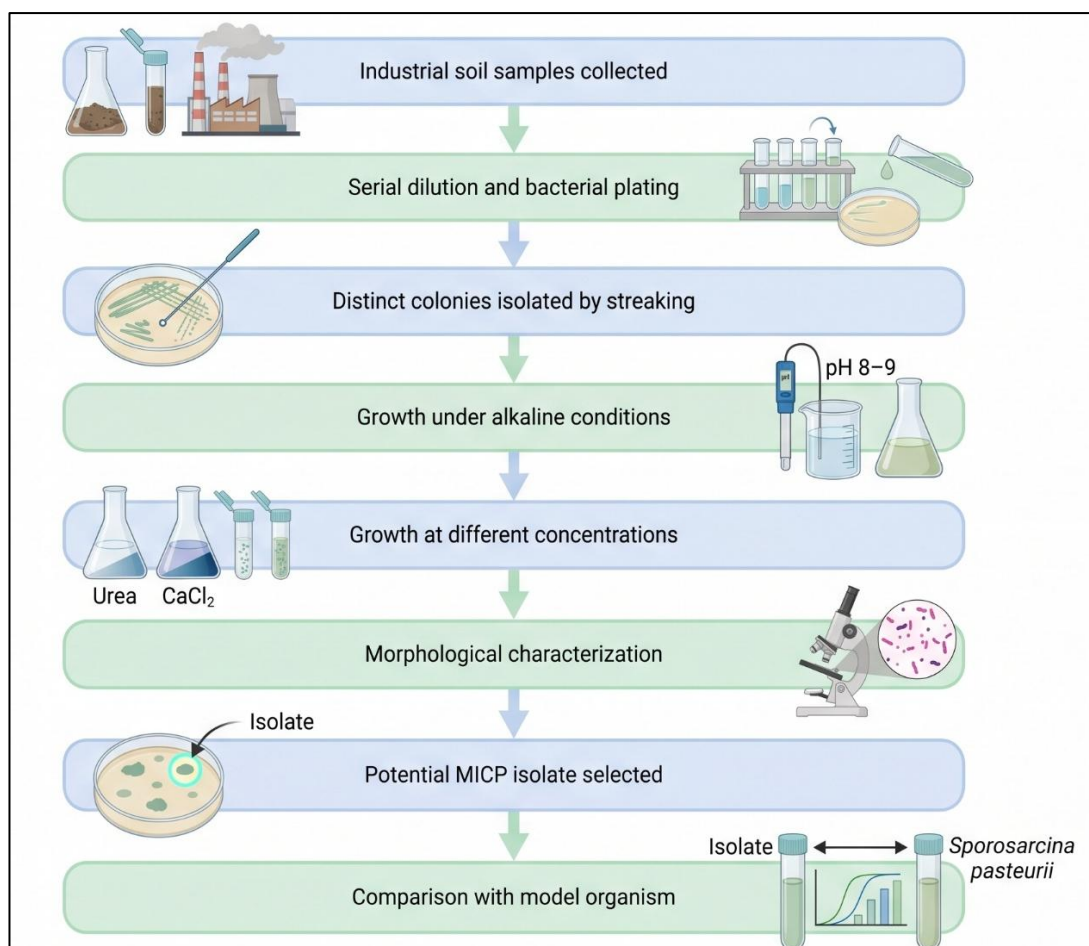


Figure 3.1: Overall experimental workflow for isolation, screening and application of alkali-tolerant bacteria for MICP-based self-healing paints. (Created with: Biorender)

3.2 Soil sampling

Soil samples were collected from four different industrial sites suspected to have microbial populations adapted to chemically stressed and alkaline environmental conditions. Soil samples were aseptically collected using sterile spatulas and were placed into sterile sample collection containers.

The collected samples were correctly labelled and transported to the laboratory for further microbiological analysis. Collection and handling procedures were designed to minimise external contamination. Samples were kept under appropriate laboratory conditions prior to processing.

The industrial soil was chosen as the source of microorganisms because it was expected that microbes that can survive in this environment would be more tolerant to the alkaline conditions and mineral-rich environments found in self-healing paint systems.

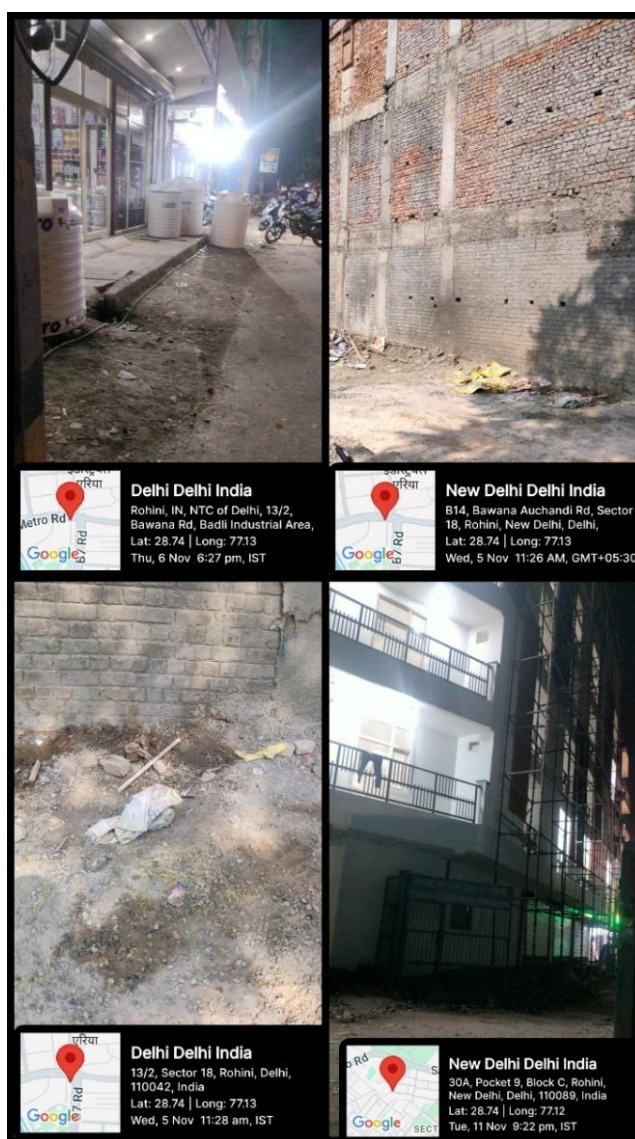


Figure 3.2: Industrial soil sample collection sites used for bacterial isolation.

3.3 Isolation of Bacterial Strains

The isolation of bacterial strains was done through serial dilution and pouring plate method. Around 1g of each soil sample was suspended in distilled water to form the initial suspension.

Serial dilution was done aseptically using sterile dilution blanks. The proper dilutions were chosen for plating to get isolated bacteria strains. Pour plating was done using sterile nutrient agar media.

The inoculated plates were incubated under standard conditions until the growth of the colonies was apparent. Different strains showing varied characteristics in terms of color, size, texture, transparency, elevation, and edge were chosen for purification.

Sub-streaking was done on freshly prepared nutrient agar media to achieve pure bacterial strains. Purified strains were preserved on slants of nutrient agar for use in future experiments.

The isolation procedure made it possible to isolate different strains of bacteria from industrial soils based on their morphological characters.

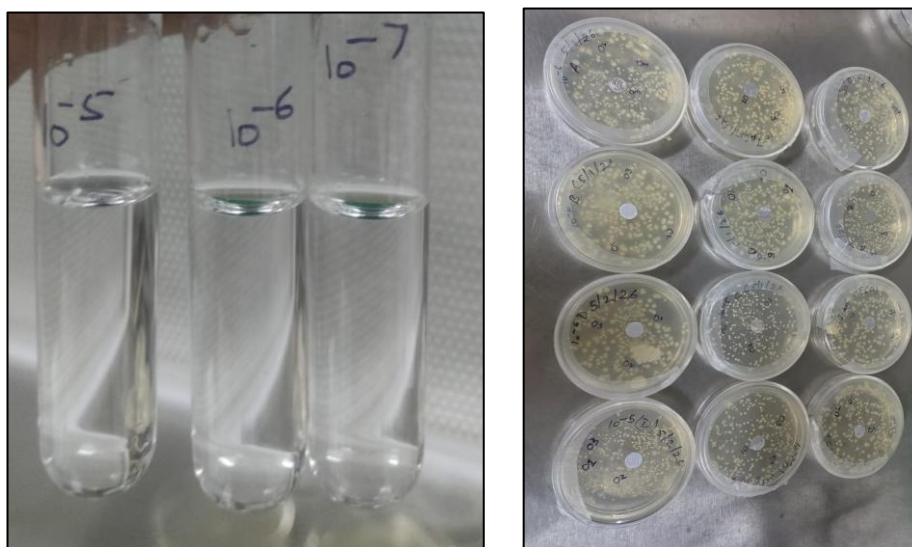


Figure 3.3 Serial dilution and plating procedure performed for isolation of bacterial colonies from industrial soil samples.

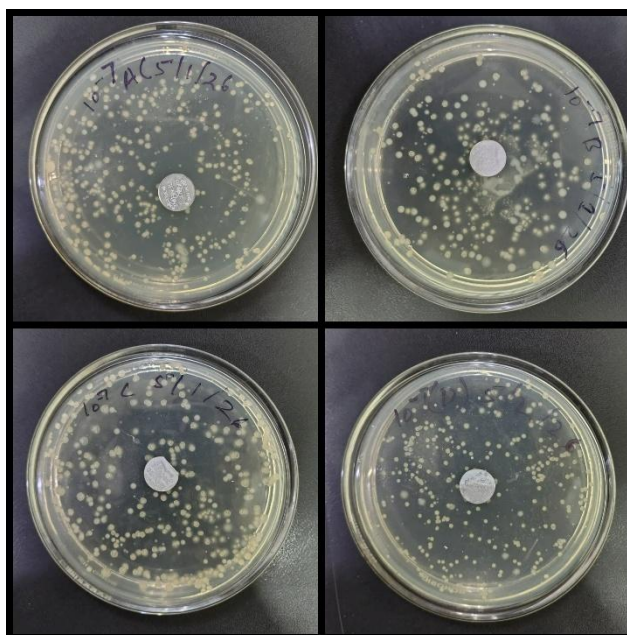


Figure 3.4: Pour plate cultures obtained from industrial soil samples showing distinct bacterial colony growth.

3.4 Preliminary Morphological Characterization of Isolates

Preliminary morphological characterization of the isolated bacteria was carried out based on the colony morphology and microscopic examination. The parameters considered for the purpose included the shape, color, consistency, elevation, transparency, and margin of the colonies.

For microscopic examination, Gram staining method was used. The smears were prepared by suspending bacterial cells in a drop of water on clean slides and staining with crystal violet, Gram's iodine, alcohol decolorization, and safranin.

The stained smears were examined under an oil immersion lens using a compound microscope to ascertain the Gram reaction and cell morphology. From the preliminary observations, certain aspects of bacterial diversity were known.

Gram-positive bacteria were of special significance since ureolytic and spore-forming bacteria involved in biomineralization were Gram positive bacteria that could survive harsh environments.

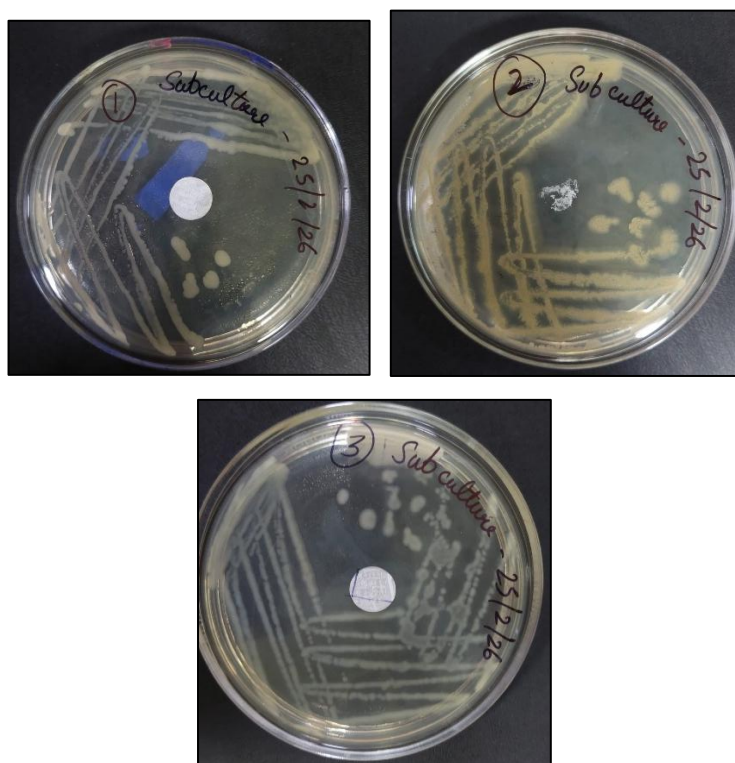


Figure 3.5: Pure bacterial isolates obtained after colony purification.

3.5 Procurement and Maintenance of Reference Strain

Sporosarcina pasteurii, an ureolytic bacteria reference strain, was purchased from the National Collection of Industrial Microorganisms, Pune, India to compare with the native bacterial strains.

The bacterial culture was cultured using microbiological techniques through appropriate media containing nutritional factors. After culturing, the bacteria were maintained under suitable laboratory conditions where the bacteria would grow actively.

Sub-cultures were routinely done to preserve the bacterial viability throughout the experiment period. The reference strain was used as a comparative model since it had known urease activity.

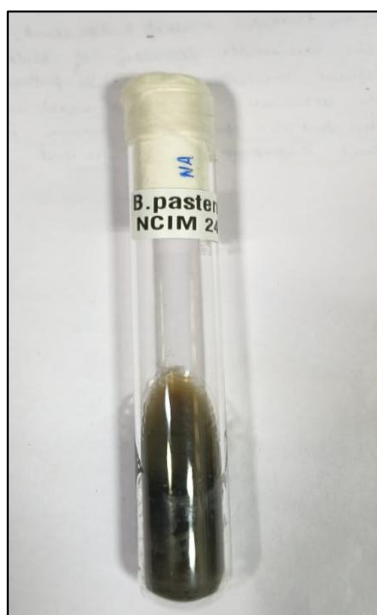


Figure 3.6: Stock of the reference strain: *Sporosarcina pasteurii*.

3.6 Optimization of Urea Concentration for Bacterial Growth

The bacterial isolates as well as *Sporosarcina pasteurii* were subjected to analysis for growth potential in varying urea concentrations. Urea is an important component in the ureolytic process and constitutes an important component in microbial-induced calcium carbonate precipitation (MICP). Hence, optimizing urea concentration is important for determining which bacterial strains are capable of growing under the relevant conditions in self-healing paint systems.

Various concentrations of urea: 1%, 2% and 3% were prepared in the growth medium under sterilized conditions. The bacterial isolates were inoculated individually into the prepared media and incubated in suitable laboratory conditions. Bacterial growth in each case was assessed by visual evaluation of the turbidity and formation of colonies.

It was found that in comparison to low concentrations of urea, high concentrations of urea resulted in relatively reduced growth rates in some bacterial strains. The bacterial isolate identified as Strain 2 displayed growth behaviour similar to the *S. pasteurii* under optimized conditions.

It was found out that some isolates were tolerant to high levels of urea. This implied that the isolates may have a higher potential for applications in urease-based biomineralization processes. This is because the process of ureolysis leads to the formation of carbonate ions which result in the precipitation of calcium carbonate.

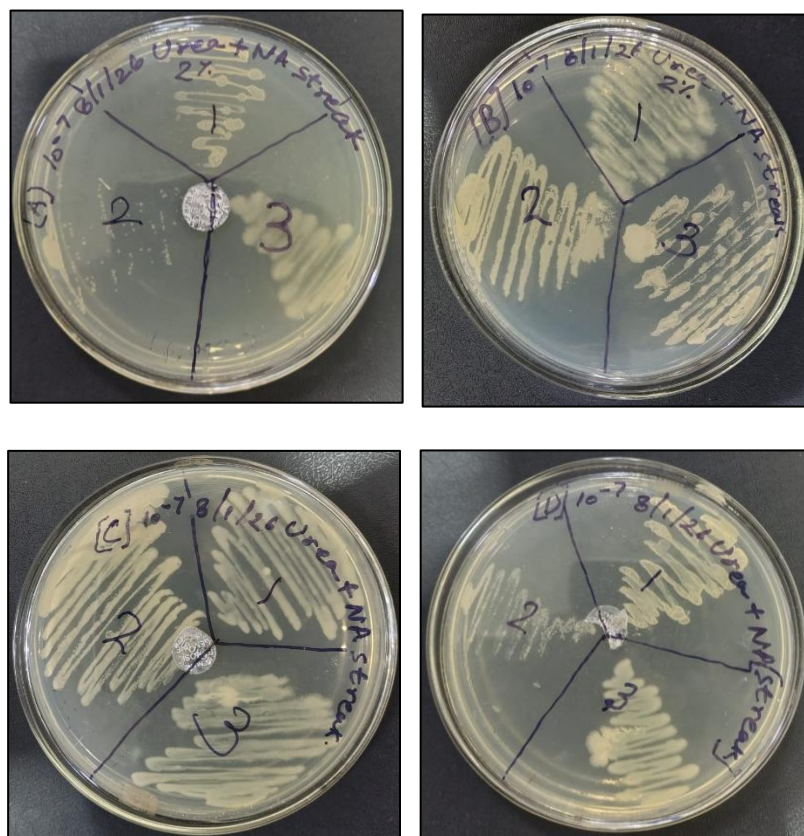


Figure 3.7: Growth response of bacterial isolates under different urea concentrations.

3.7 Optimization of Calcium Chloride Concentration for Bacterial Growth

Calcium chloride optimization experiments were conducted to investigate the capability of bacterial isolates to survive in an environment with a high amount of calcium content due to the process of biomineralization.

Calcium ions serve as important precursors for the formation of calcium carbonate and play a crucial role in the efficacy of self-healing mechanisms due to their interaction with bacteria.

Media with 1%, 2%, and 3% concentrations of calcium chloride were prepared under sterile conditions. Bacterial isolates as well as a control bacterium *Sporosarcina pasteurii* were individually inoculated and then incubated under optimal conditions.

After incubation, the bacterial growth rate was evaluated by the level of turbidity and appearance of colonies in the medium. Some of the isolates had resistance to higher concentrations of calcium chloride compared to others.

One of the indigenous strains (Strain 2) showed similar growth patterns to the control bacterium. This fact indicates possible adaptation of these bacteria to mineral-rich environments and their potential application in future self-healing systems.

The optimization of the concentration of calcium chloride is essential since the high concentration of the calcium ion may lead to the interference of the metabolic processes of bacteria, while low concentration of calcium ions would lower the efficiency of the formation of calcium carbonate.

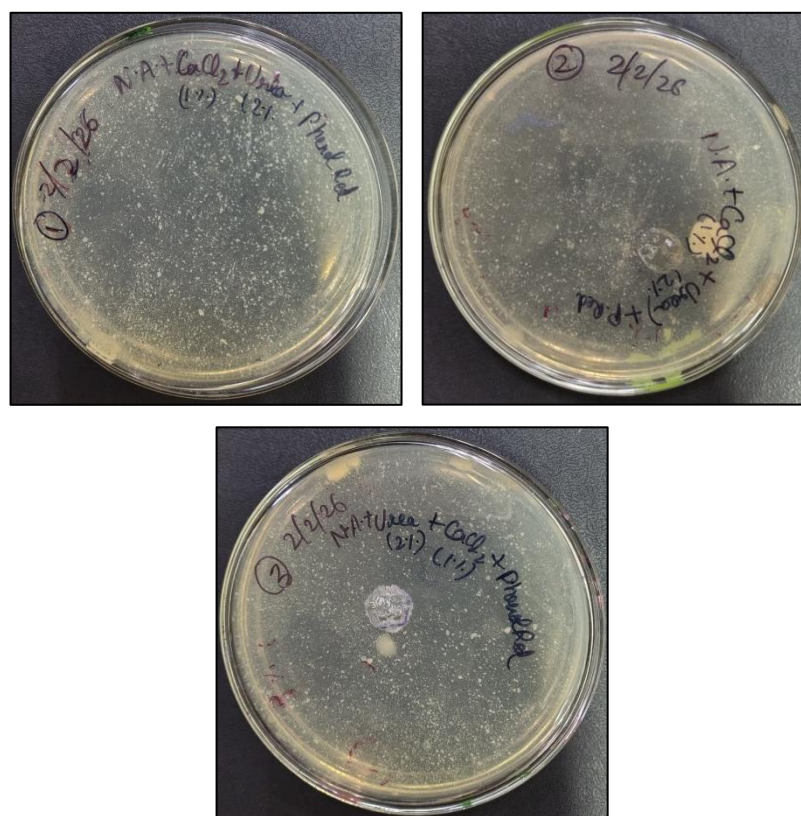


Figure 3.8: Comparative growth of bacterial isolates under varying calcium chloride concentrations.

3.8 Evaluation of Growth Under Alkaline pH Conditions

Since paints have physicochemical properties that make them alkaline, bacteria used for paint self-healing processes should exhibit resistance against high pH values. This calls for a study on growth under such pH conditions, carried out on all the isolated bacteria and *S. pasteurii* strain.

Growth of bacteria under alkaline pH was done by growing them under pH conditions of 8.0, 8.5, and 9.0. pH adjustment was done to the required levels using appropriate laboratory chemicals before sterilization. Subsequent incubation of cultures was done according to standard bacterial growth conditions and results observed.

Various degrees of tolerance under alkaline conditions were recorded among the bacterial isolates. Nevertheless, growth rates increased at pH 9.0 in some of the

isolates. Particularly, Strain 2 indigenous isolate and *S. pasteurii* showed enhanced growth at pH 9.0.

Survival and growth under alkaline pH conditions is an important feature in the use of bacteria within paint self-healing matrix. Resistance against alkalinity can help increase bacterial survival, activity of urease and calcium carbonate precipitation when used to heal cracks in the future.



Figure 3.9: Experimental setup for evaluation of bacterial growth under alkaline pH conditions.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of Bacterial Strains from Industrial Soil

Soil samples from four different sites provided various colonies of bacteria after serial dilutions and pouring plates. Differences in their colony appearances such as colour, texture, edges, elevation, and opacity suggest a variety of microorganisms thriving in the industrial environment.

Further subculturing made it possible to isolate pure colonies of bacteria that can be used for experimentation. The chosen bacteria have exhibited improved growth properties in alkaline and stressed environments. Such isolates will thus be studied to enhance urea concentration, calcium chloride concentration, and alkalinity.

The isolation of bacterial strains from industrial soil confirms earlier research, which showed that stressed environments might contain organisms with adaptive traits important for mineralization and self-healing applications [44].

4.2 Morphological Characterization of Isolates

From the preliminary analysis, there were distinct morphological differences observable from the isolated bacterial strains through colony morphology and Gram stain reactions. Some of the bacterial isolates had creamy white coloured, circular and smooth colonies while others had rough edges and different elevations.

Under microscopic observation, one of the indigenous bacterial strain (Strain 2) appeared to belong to Gram-positive bacteria and the other 2 strains (Strain 1 and 3) appeared to be Gram-negative bacteria. Generally, Gram-positive bacteria are known to offer great advantages when it comes to biomineralization processes due to the thickness of the cell wall, stress resistance and alkaline survival [45].

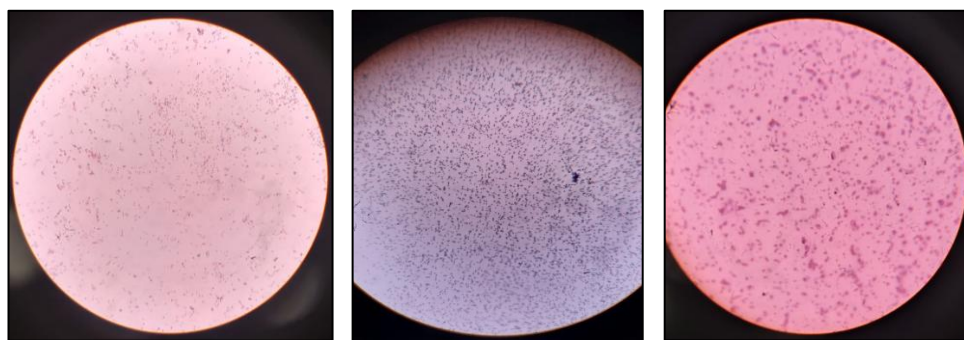


Figure 4.1: Microscopic observations of bacterial isolates obtained through Gram staining (40× magnification).

Among the indigenous strains, one strain (Strain 2) appeared to have colony morphology similar to that of *S. pasteurii*. The similarities between Strain 2 and *S. pasteurii* suggested that both strains might share physiological traits as well.

Table 4.1 : Colony Morphology & Growth Characteristics of bacterial isolates obtained

Feature	Isolate 1	Isolate 2	Isolate 3
Colour	White	Yellowish	White–grey
Texture	Thick, foggy	Thin, stringy/stretchy	Hazy, smooth
Growth pattern	Dense	Thin streaks	Thin streaks
Elevation	Flat	Slightly raised	Flat
Growth at pH 9	Moderate	High	Moderate
Growth in 2% urea + 2% CaCl ₂	Low-Moderate	Highest	Moderate
Gram nature	Gram-negative	Gram-positive	Gram-negative
Cell shape	Rod-shaped	Rod-shaped	Rod-shaped

4.3 Growth Response Under Different Urea Concentrations

The bacterial isolates were investigated for growth response at various concentrations of urea, an essential ingredient for calcium carbonate precipitation by ureases. The study revealed that there was a dependence of bacterial growth on urea concentration used in the medium.

It was observed that bacteria grew at low urea concentration but with increasing urea concentration, growth differences were observed among them. Some isolates did not show good growth at high urea concentrations presumably owing to osmotic pressure and high ammonia levels produced due to ureolysis.

From the indigenous isolates examined, Strain 2 was relatively resistant to urea at high concentrations. In addition, the growth behaviour observed was identical to that of *S. pasteurii*.

Ability to withstand urea concentration is essential for biomineralization as effective ureolysis results in the production of carbonate ions required for biomineralization reactions [46].

Based on these results, it can be stated that industrial soils contain microbes which have adapted to chemically stressful conditions and thus can be used for isolating biomineralization associated bacterial strains.

Table 4.2: Growth Response Under Different Urea Concentrations

Isolate	1%Urea	2% Urea	3% Urea	Observation
Strain 1	Good	Moderate	Reduced	Sensitive at high concentration
Strain 2	Good	Good	Moderate	High urea tolerance
Strain 3	Moderate	Reduced	Poor	Growth inhibited
<i>Sporosarcina pasteurii</i>	Good	Good	Good	Stable growth observed

4.4 Growth Response Under Different Calcium Chloride Concentrations

Optimization of calcium chloride was conducted in order to study the adaptability of the isolates towards different calcium ion concentrations during calcium carbonate precipitation reactions.

Growth patterns of the isolates varied at 1%, 2%, and 3% of calcium chloride concentrations. While moderate concentrations facilitated bacterial growth in most cases, higher concentrations had different effects on some strains growth patterns. Increased levels of calcium ions might affect osmotic balance and metabolism in bacteria, thereby affecting their survival rate.

The native isolate Strain 2 demonstrated relatively consistent growth at different concentrations and exhibited growth patterns like those of *S. pasteurii*. This observation suggests that the isolate might be adapted to thrive in mineral-rich surroundings suitable for biomineralization.

Calcium is an essential element in MICP since the reaction involves calcium ions combining with carbonate ions produced during ureolysis to form calcium carbonate precipitates. Thus, bacterial strains that can thrive in high concentrations of calcium ions have more potential applications in self-healing coatings in the future [47].

Table 4.3: Growth Response Under Different Calcium Chloride Concentrations

Isolate	1% CaCl ₂	2% CaCl ₂	3% CaCl ₂	Observation
Strain 1	Moderate	Good	Reduced	Sensitive at high concentration
Strain 2	Good	Good	Moderate	Consistent growth observed
Strain 3	Moderate	Moderate	Poor	Reduced growth at 3%
<i>Sporosarcina pasteurii</i>	Good	Good	Moderate	Stable growth pattern



Figure 4.2: Comparative growth of bacterial isolates under optimum urea (2%) and calcium chloride (2%) concentrations.

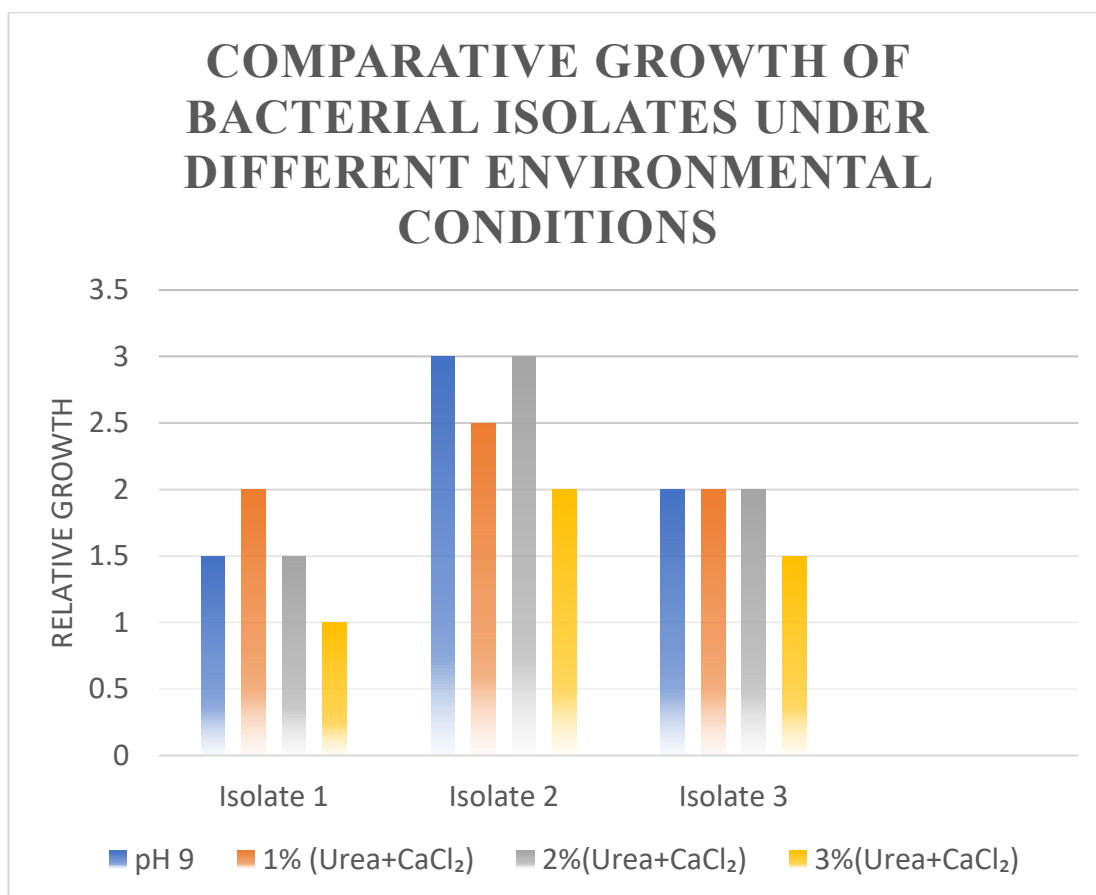


Fig. 4.3: Comparative growth analysis of bacterial isolates under alkaline conditions and varying concentrations of urea and CaCl₂

4.5 Evaluation of Growth Under Alkaline pH

Evaluation of bacterial growth under alkaline pH was performed in order to find out the compatibility of the bacterial isolates with regard to incorporation in paint systems. Considering the presence of an alkaline environment in paint systems, survival of bacterial isolates in high pH condition becomes a crucial factor for future applications in self-healing paint systems.

The bacterial isolates were grown at pH 8.0, 8.5, and 9.0. Differences were noticed in growth patterns of isolates at different pH values. While certain bacterial isolates

showed low growth rate at a low alkaline environment, some showed comparatively higher growth rates at pH 9.0.

Strain 2 showed growth patterns that are more desirable for an alkaline environment and the growth pattern of Strain 2 was similar to the control organism *Sporosarcina pasteurii*. Survival under increased pH could possibly mean adaptation of the bacteria to industrial alkaline environment.

Results from this study are consistent with the previous research which suggests better survivability of alkaliphilic and alkali tolerant bacteria in biomineralization environment [48]. Such factors become highly relevant with respect to sustainability of bacteria and urease enzymes in future paint system applications.

Table 4.4: Alkali tolerance of bacterial isolates under different pH conditions.

Isolate	Growth at pH 8	Growth at pH 9	Growth at pH 10	Overall Tolerance
Strain-1	++	++	+	Moderate
Strain-2	+++	+++	++	High
Strain-3	++	+	+	Moderate

4.6 Comparative Analysis with *Sporosarcina pasteurii*

The native bacterial isolates from industrial soil were compared with *S. pasteurii* according to colony morphology and growth properties under optimized experimental conditions.

Of all the bacterial isolates, Strain 2 exhibited similarity in several characteristics compared with *S. pasteurii*. Common features include growth properties, alkalinity resistance, and variation in substrate concentrations.

Molecular identification was out of the scope of this research; nevertheless, similar physiological characteristics suggest the possibility of biomineralization properties of the native bacterium. The results indicate that industrial soil environments are potential

sources of bacteria that can adapt to the conditions necessary for self-healing coating application.

Comparison with a known ureolytic bacterium helped confirm the evaluation of the native bacterial isolate as well as the prospects for further exploration of the bacteria in MICP-related applications.

CHAPTER 5

CONCLUSION

This research work was carried out with the purpose of isolating and characterizing alkali-tolerant bacteria from industrial soil as prospective bacterial cultures for self-healing paint applications. The use of industrial soil from various sources resulted in successful isolation of diverse morphologically distinct bacterial strains that could survive in chemically stressed environmental settings.

The serial dilution and pour plate method allowed the isolation and purification of various bacterial colonies. Preliminary identification showed that the predominant majority of bacteria were of Gram-positive type since these bacteria were often characterized by stress resistance and biomineralization properties.

These bacteria were studied for their growth ability under different concentrations of urea and calcium chloride, two key substrates that participate in microbially-induced calcium carbonate precipitation (MICP). Some bacteria showed better growth behavior at high substrate concentrations, demonstrating adaptation to a mineral environment.

Finally, survival abilities of bacterial strains were tested under alkaline pH condition. It was found that some selected strains could maintain good growth activity at certain pH levels relevant to paints. As for morphological and growth behavior, Strain 2 was similar to *S. pasteurii*.

Conclusions drawn from this experiment indicate that soil samples taken from industrial areas have considerable potential in providing alkali-resistant bacterial strains that could possibly be used in future self-healing paints based on biomineralization. Even though it is not within the context of this experiment to examine the bacteria's physiology in detail, the above observations would lay the basis for future experiments regarding the use of bacterial self-healing paints.

Additionally, the experiments carried out show the need to optimize environmental and nutritional conditions for ensuring viability of the bacteria within the artificial

environment provided by the paints. It should be mentioned that the experiments add value to an ongoing trend towards development of sustainable self-healing coating materials.

5.1 Future Scope

This current study gives an overview of the identification of bacterial strains that have the potential to survive in alkaline and stressful chemical environments for use in self-healing paint technology. Nevertheless, various issues need further elaboration for practical implementation and technology improvement.

Future research may involve biochemical and molecular identification of the isolated bacteria through advanced analytical methods such as 16s rRNA sequencing. Molecular identification will not only help validate the taxonomical identity of the indigenous bacteria but will give more information about their biomineralization potential.

Additional research may entail quantitative measurement of urease activity and calcium carbonate precipitation ability under optimum conditions. This process will help understand the true biomineralization capacity of the identified bacteria.

The encapsulation of bacterial spores into real paint formulations is yet another critical field that needs investigation. The process of encapsulation will help in improving the survivability of bacteria in harsh paint environments.

Future research will also consist of:

- a) tests on crack healing efficiency,
- b) tests on paint film durability,
- c) microscopic observation of the deposit of calcium carbonate,
- d) and evaluation of the stability of bacteria films under stressful environmental conditions.

Comparative experiments using native microorganisms as well as typical strains of ureolytic bacteria can also assist in selecting suitable strains for sustainable self-healing coatings.

The application of microbiological principles to materials science and coatings technology could play an important role in achieving sustainable development.

5.2 Limitations

In the current study, there was an emphasis on the isolation and initial characterization of bacteria strains tolerant to alkaline conditions obtained from industrial soil. However, in-depth molecular and biochemical characterization of the isolates was not done within the context of the current study.

Quantification of urease activity and determination of calcium carbonate precipitation by the bacteria were not included in the present study. While some growth optimization experiments were done using varying concentrations of urea, calcium chloride, and alkalinity levels, there was no detailed biomineralization efficiency analysis in the study.

Direct integration of isolated bacteria into paint substrates to evaluate their self-healing properties was also not included in the current study. These considerations may be pursued in the future studies to provide evidence of self-healing paint with bacteria.

In spite of the limitations, the current study provided key insights on the ability of indigenous bacterial isolates to survive under future self-healing coating conditions.

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APPENDICES

APPENDIX A: List of Publications

1. A paper entitled “**Enhancing Paint Durability Using *Sporosarcina pasteurii*: A Critical Analysis of Urease-Mediated Calcium Carbonate Precipitation for Autonomous Crack Healing**” was presented at the **9th International Conference on Advances in Biosciences and Biotechnology (ICABB-2026)** organised by Jaypee Institute of Information Technology during January 28–30, 2026.
2. A paper entitled “**Isolation of Alkali-Tolerant Bacteria from Industrial Soils and Their Preliminary Screening for MICP-Based Self-Healing Paints**” was presented at the **International Conference on Emerging Trends in Health, Environment and Sustainability (ETHES-2026)** organised by Gurugram University during March 21–22, 2026.










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GURUGRAM UNIVERSITY, GURUGRAM
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DEPARTMENT OF BIOSCIENCES
organizes
International Conference on
Emerging Trends in Health, Environment and Sustainability (ETHES – 2026)
Health and Environment: Challenges, Innovation and Sustainable Solutions

Certificate of Participation

This is to certify that Mr./Ms. **Shriya**
 from **Department of Biotechnology, Delhi Technological University, New Delhi, India**
 has participated as a delegate and presented **Poster** presentation on
 the topic **Isolation of Alkali-Tolerant Bacteria From Industrial Soils and Their Preliminary Screening For**
Micp-Based Self-Healing Paints in the *International Conference on*
Emerging Trends in Health, Environment and Sustainability (ETHES-2026) on the theme
"Health and Environment: Challenges, Innovations and Sustainable Solutions"
 on **21st - 22nd March 2026** held at Gurugram University, Gurugram.
 We appreciate their active participation and valuable contribution towards the success of the conference.



Prof. Dhirender Kaushik
Convener



Prof. Rani Devi
Convener



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