Optimization of Indigenous Alkalophilic Bacteria of Sambhar Lake on B4 Medium

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Abstract
This study explores the isolation and characterization of alkalophilic bacterial strains from the hypersaline Sambhar Lake, with a focus on their potential for mineral precipitation, specifically calcium carbonate. Soil samples were collected from the lake and analyzed for physicochemical properties, revealing an alkaline pH (9) and high salinity, conditions conducive to the growth of halophilic bacteria. Bacteria were grown on 10% NaCl nutrient agar and subsequently cultured on specialized B4 medium for mineral precipitation. Five distinct bacterial strains were isolated, identified through 16S rRNA sequencing as Halomonas salifodinae UJSL1, Oceanobacillus kimchii UJSL2, Halobacillus dabanensis UJSL3, Halomonas mongoliensis UJSL4 and Halomonas campaniensis UJSL5.

All strains demonstrated urease activity and produced significant calcium carbonate precipitates. Halomonas salifodinae UJSL1 and Halomonas mongoliensis UJSL4 were the most effective in producing large, well-formed crystals as confirmed by SEM analysis. The findings suggest that these strains hold potential for applications in biocementation, carbon sequestration and environmental remediation. The study contributes valuable insights into the use of halophilic bacteria for sustainable and eco-friendly biomineralization processes.

Keywords: Sambhar lake, Calcium Carbonate, Biomineralization, MICP, Alkalophilic bacteria, B4 medium.

Introduction

The interaction between microorganisms and minerals is a fundamental aspect of biogeochemical cycles, influencing soil formation, nutrient cycling and the overall health of ecosystems[8,15]. Among the various microbial processes, bacteria-induced mineral precipitation (BIMP) has gained significant attention due to its potential applications in environmental remediation, construction materials and biogeotechnical engineering[10]. BIMP refers to the process by which bacteria facilitate the precipitation of minerals through metabolic activities, leading to the formation of stable mineral deposits.

Calcium carbonate ($CaCO_3$) precipitation is one of the most studied forms of BIMP, primarily because it plays a crucial role in carbon sequestration and has diverse applications in industries ranging from agriculture to construction. The ability of certain bacteria to induce $CaCO_3$ precipitation is largely attributed to their metabolic processes such as ureolysis, which elevates the pH of their environment and promotes supersaturation of calcium ions. This phenomenon not only contributes to the formation of mineral structures but also enhances the structural integrity of soils and sediments[1,7,11].

B4 medium has emerged as a preferred growth medium for studying bacterial mineral precipitation due to its optimal composition that supports the growth of various calcifying bacteria[2,22,26]. This medium provides essential nutrients while maintaining a favorable pH conducive to mineral formation. The unique characteristics of B4 medium allow researchers to investigate the mechanisms underlying mineral precipitation and to assess the performance of different bacterial strains in inducing biomineralization. This research aims to explore the optimization of bacterial strains on B4 medium for mineral precipitation, focusing on their capabilities, mechanisms involved and potential applications. Understanding how different bacterial species interact with B4 medium can enhance knowledge of microbial processes that contribute to mineral formation. This insight can be leveraged for innovative solutions in environmental management and engineering.

Review of Literature

An ancient bacterial trait, microbially induced calcite precipitation (MICP), has recently garnered significant attention for its biotechnological applications. This process, a byproduct of bacterial metabolism, involves the presence of nucleation sites on bacterial cell surfaces or extracellular substances, along with an increase in pH and other chemical changes in the extracellular environment. Despite its potential, the molecular mechanisms of MICP and their interactions remain not fully understood, hindering the ability of synthetic biologists and biotechnologists to exploit bacterial biomineralization to its full potential. Since *Bacillus subtilis* does not inherently exhibit noticeable MICP activity, a bottom-up approach was employed in this study to biomineralize it.

The findings show that MICP can be induced by heterologous urease production through localized increases in extracellular pH. Furthermore, urease accessory genes responsible for urea and nickel absorption can be co-expressed to enhance this effect, depending on the surrounding conditions. The generation of exopolysaccharide appears to be the key driver of biofilm-promoting environments which can significantly accelerate MICP. The protein component of the biofilm matrix was found to be non-essential. Attempts to modify *Bacillus subtilis'* cell surface charge had minimal impact, suggesting that this organism may naturally possess a highly negatively charged cell surface, making it inherently prone to MICP.

This research highlights the genetic components that could be utilized to develop improved or novel biomineralization processes, as well as the molecular mechanisms that govern MICP in a commercially valuable organism. The study illustrates how genetic factors and environmental chemical composition interact to influence the rate, spatial distribution and crystal formation of MICP. Together, these results lay the foundation for the rational design of application-specific synthetic precipitating strains in future work[10].

Sambhar Lake, a hypersaline habitat located at 27°58′N 75°55′E in Rajasthan, India, is a major source of salt production in the country. A total of fifty-nine moderate halophiles were isolated from the surface lake water, soil and shore sediments, which were then categorized into twenty-two distinct isolates based on their shape, colony characteristics and staining properties. Fourier Transform Infrared (FTIR) spectroscopy confirmed that these isolates were eubacteria, with a characteristic C=O stretching in ester functional groups. Further analysis revealed that some Halomonas isolates shared similar characteristics, suggesting potential phylogenetic relationships.

Phylogenetic analysis, based on FASTA sequences obtained using universal bacterial primers, identified predominantly Gram-positive genera, such as *Alkalibacillus, Amphibacillus, Marinococcus, Piscibacillus, Planococcus, Salinicoccus, Staphylococcus and Virgibacillus*, along with two Gram-negative strains of *Halomonas*. The genus *Amphibacillus* was reported in Sambhar Lake. Despite being moderately halophilic, several isolates showed remarkable salt tolerance, growing in up to 25% salt concentration. All isolates were mesophilic, thriving within a temperature range of 18-42°C, which aligns with the local climate conditions. The hydrolytic activity of these isolates was assessed, revealing eighteen as protease producers, thirteen as lipase producers and ten as cellulase producers.

Additionally, the fatty acid methyl ester (FAME) analysis identified the dominance of C10:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0 and C18:1 fatty acid, with the presence of C16:0 and C18:1 suggesting possible antimicrobial properties of these bacterial strain[23]. In biomineralization and polymorph development, amorphous calcium carbonate (ACC) serves as an important precursor to crystalline calcium carbonates. This study demonstrates that ACC is produced by various bacterial strains isolated from a cave in Hungary and that ACC is protected from crystallization by their extracellular polymeric substance (EPS). The results show that ACC produced by bacteria can form in a humid environment at ambient temperature and remains stable for at least six months, in contrast to ACC produced in a laboratory, which must be stored in a desiccator and kept below 10°C to prevent crystallization.

The ACC-protecting extracellular matrix (EPS) consists of lipids, proteins, carbohydrates and nucleic acids, with a significant presence of components involving long-chain fatty acids. It is proposed that ACC is enclosed in a micella-like structure within the EPS to prevent water intrusion. Unprotected ACC grains crystallize into calcite when the protective layer breaks down and water infiltrates the lysing bacterial cells. Based on the findings, it appears that bacteria can produce ACC and the amount of ACC relative to calcite fluctuates by up to 20% as the colony ages. Given the diverse range of bacteria present in cave sediments within temperate zones, it is likely that ACC is widespread in these environments, with its presence linked to bacterial activity, which, in turn, influences the geochemical signals observed in speleothems[6].

Researchers have explored bacterial calcium-carbonate precipitation (BCP) for various applications including consolidation, cementing and environmental cleanup. The primary aim of the BCP technique is to isolate and identify effective calcifying bacteria. This study investigated various bacteria isolated from rhizospheric soil for their ability to precipitate calcium carbonate in both solid and liquid environments. Research on different cultures revealed that most of the bacteria responsible for calcium carbonate precipitation belong to the Actinobacteria, Gammaproteobacteria and Alphaproteobacteria families. For sand biocementation tests, both pure and mixed cultures of selected strains were used.

Biodeposition treatment using mixed cultures proved successful and Scanning electron microscopy (SEM) and Energy dispersive X-ray spectroscopy (EDS) analyses of the biotreated materials confirmed the biological nature of the cementation process. X-ray diffraction (XRD) testing showed that all the calcifying bacteria used in sand biocementation produced calcium carbonate, predominantly as calcite. To identify promising candidates for BCP applications, Biolog® EcoPlate was used as a useful tool to help focus sample location selection. Additionally, this research is the first to employ ImageJ software to screen strains for high calcium carbonate production[4].

This study explores the use of alkaliphilic bacteria, isolated from Sambhar Salt Lake in Rajasthan, in the development of self-healing concrete through Microbially Induced Calcium Carbonate Precipitation (MICP). The bacteria strains used in the study including *Halomonas salifodinae* UJSL1, *Oceanobacillus kimchii* UJSL2, *Halobacillus dabanensis* UJSL3, *Halomonas mongoliensis* UJSL4 and *Halomonas campaniensis* UJSL5, were incorporated into concrete, with M20 concrete as the control sample. The results revealed substantial improvements in mechanical properties such as increased compressive and tensile strength along with reduced water absorption.

The bacterial consortium, in particular, showed the best performance, leading to the most significant enhancements due to the MICP process. This process effectively sealed cracks and pores in the concrete. The approach offers several advantages, including a sustainable and cost-effective solution to enhance the durability and longevity of concrete structures, especially in challenging environmental conditions in India. The study demonstrates the potential of utilizing microbial resources to innovate construction materials, contributing to lower maintenance costs and reduced environmental impact while improving structural resilience. The successful use of MICP in self-healing concrete with a bacterial consortium presents a promising pathway for future research and development in eco-friendly building materials[20]. The pioneering studies were conducted by Boquet and colleagues using B4 precipitation medium. This medium has become the preferred choice for researching mineral precipitation *in vitro* with bacterial strains. Numerous studies have used this medium to demonstrate that some environmental isolates can precipitate minerals, while others cannot. Data on mineral precipitation for environmental isolates cultured on B4 were analyzed to determine whether pH and buffer conditions significantly influenced the results. In this study, the potential for calcium carbonate precipitation was assessed by growing 49 strains on B4 plates, collected from various natural habitats in Puerto Rico.

The findings revealed a strong correlation between the colonies' acidity on B4 plates and the absence of calcium carbonate precipitation. By adjusting the pH of the B4 medium to 8.2, calcium carbonate precipitation was reinstated. The role of the medium's buffering capacity in calcium carbonate precipitation was highlighted; acid-base titrations of B4 components showed that yeast extract had insufficient buffering capacity within the pH range of 6.5-7.5. This pH range aligns with the pre-inoculation pH of B4 plates, which was 6.87 (±0.05).

The rapid pH changes, even from minor shifts in the H+/OH- balance during microbial growth and precipitation in B4, may explain its efficient precipitation properties. In conclusion, environmental strains produced crystals on B4 medium, with 90% of those crystals having an amorphous matrix[16]. "

Material and Methods

Soil Sample Collection: Soil samples were collected from Sambhar Lake, a hypersaline environment, using sterile tools[5,18,24]. Samples were stored in sterile containers and transported to the laboratory under controlled conditions to prevent contamination.

Physicochemical Analysis: Physicochemical analysis of the soil samples was conducted to determine parameters such as pH, salinity and mineral composition. The pH of the samples was measured using a pH meter and was found to be 9, indicating an alkaline environment. Salinity was assessed using a salinometer, confirming high salt concentrations typical of hypersaline ecosystems.

Isolation of Halophilic Bacteria: The soil samples were enriched in 10% NaCl nutrient agar (NA) medium to select halophilic bacterial strains[21]. Approximately 1 g of soil sample was suspended in 10 mL sterile saline, serially diluted and spread on the medium. Plates were incubated at 37°C for 48 hours and morphologically distinct colonies were selected for further study.

Figure 1: Sample collection site of Sambhar Lake.

Biochemical Characterization: Isolated bacterial strains underwent biochemical characterization including tests for urease activity, catalase activity and carbohydrate fermentation patterns. These assays helped in identifying metabolic properties relevant to their mineral precipitation potential.

Growth on Specialized B4 Medium: The isolated strains were inoculated into B4 medium optimized for mineral precipitation. The medium composition included 10 g/L glucose, 5 g/L yeast extract, 2.5 g/L calcium chloride ($CaCl_2$) and 2 g/L sodium carbonate (Na_2CO_3) with the pH adjusted to 8. Plates and liquid cultures were incubated at 37°C for 7 days to assess the mineral precipitation tendency of the strains."

Selection of Best-Grown Strains: The best-growing strains on B4 medium, which formed significant mineral deposits, were isolated using the streak plate method to obtain pure cultures. These strains were further analyzed for calcium carbonate precipitation through Scanning Electron Microscopy (SEM) to examine crystal morphology and composition[25].

16S rRNA Sequencing: Pure cultures of the selected strains underwent genomic DNA extraction. Universal primers were used to amplify the 16S rRNA gene, which was subsequently sequenced and analyzed for species identification[19]. The identified strains included:

- *Halomonas salifodinae (UJSL1)*
- *Oceanobacillus kimchii (UJSL2)*
- *Halobacillus dabanensis (UJSL3)*
- *Halomonas mongoliensis (UJSL4)*
- *Halomonas campaniensis (UJSL5)*

The sequences were deposited in the NCBI GenBank database and accession numbers were obtained for each strain.

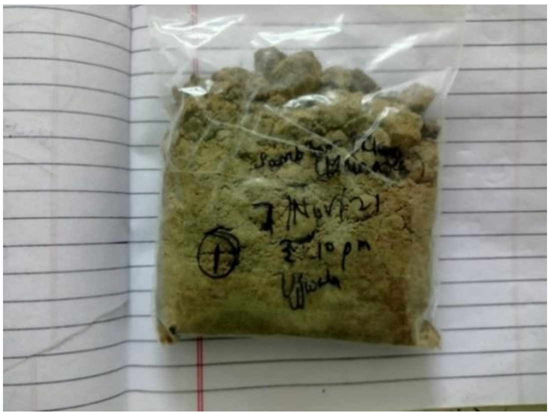

Figure 2: Soil sample collected of site

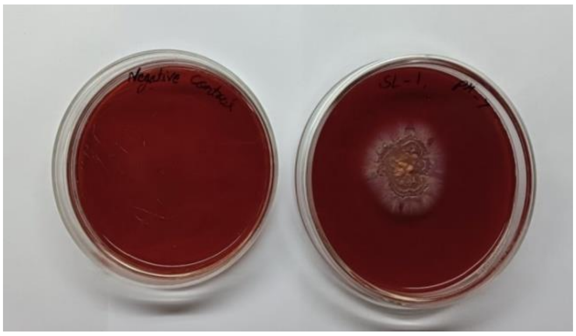

Figure 3: Bacteria cultured in B4 medium.

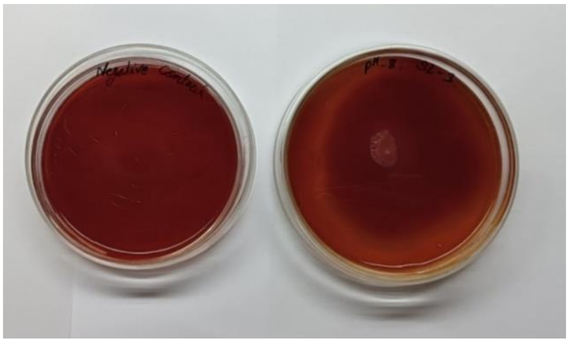

Figure 4: Optimization of bacteria on different pH on B4 medium

Mineral Analysis: The calcium carbonate precipitates formed by the selected strains were collected, washed with distilled water, dried and analyzed. The shape of the mineral deposits was studied using Scanning electron microscopy (SEM)[12,14].

Results

This research provides a comprehensive analysis of the isolation, characterization and mineral precipitation potential of bacterial strains obtained from Sambhar Lake.

Physicochemical Analysis of Sambhar Lake Soil Samples: The soil samples exhibited high salinity and an alkaline pH of 9, ideal for halophilic and alkaliphilic bacterial growth.

Bacterial Isolation on NaCl-Enriched Media: Several morphologically distinct bacterial colonies were isolated from the 10% NaCl nutrient agar medium. The high salinity supported the growth of halophilic strains adapted to extreme conditions.

Biochemical Characterization of Isolates: The isolated strains exhibited varying biochemical traits, with high urease activity observed in most strains, correlating with their potential for calcium carbonate precipitation.

Mineral Precipitation on B4 Medium: All strains demonstrated mineral precipitation on B4 medium, with visible deposits forming after 7 days of incubation.

SEM Analysis of Mineral Precipitation: SEM analysis revealed significant differences in the morphology of calcium carbonate crystals produced by the strains.

Identification of Bacterial Strains via 16S rRNA Sequencing: The 16S rRNA sequencing confirmed the identification of the isolated strains. Their sequences were submitted to NCBI and accession numbers were obtained.

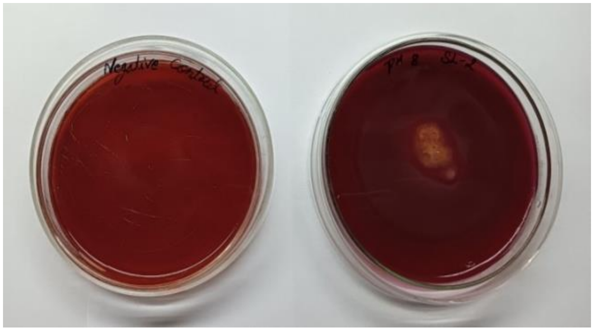

Figure 5: Optimization of bacteria on different temperature on B4 medium.

Discussion

This research highlights the isolation and characterization of halophilic and alkaliphilic bacterial strains from the hypersaline environment of Sambhar Lake and evaluates their mineral precipitation potential. The discussion integrates findings with implications for biomineralization applications. The alkaline pH (9) and high salinity of the Sambhar Lake soil provide a unique ecological niche for halophilic bacteria. Such conditions are conducive to bacteria that thrive in extreme environments and possess specialized mechanisms for survival and metabolic activity.

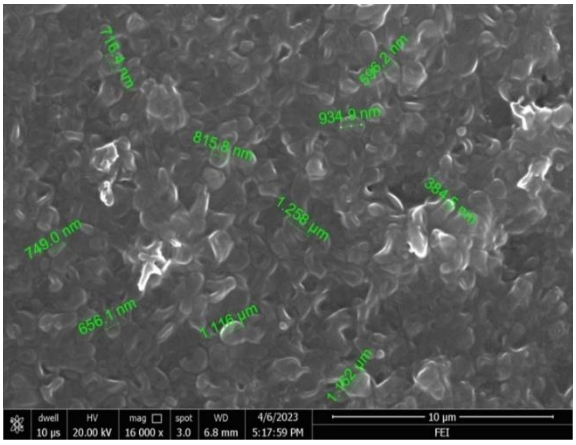

Figure 6: SEM analysis of optimized bacteria

Table 1
Biochemical Characterization of Isolates

Isolate	Urease Activity (U)	Catalase Test	Carbohydrate Fermentation
Halomonas salifodinae UJSL1	45.2 ± 2.1	Positive	Glucose, Lactose
Oceanobacillus kimchii UJSL2	38.5 ± 1.9	Positive	Glucose
Halobacillus dabanensis UJSL3	42.0 ± 2.0	Positive	Glucose, Sucrose
Halomonas mongoliensis UJSL4	46.3 ± 2.3	Positive	Glucose, Lactose, Sucrose
Halomonas campaniensis UJSL5	40.5 ± 2.2	Positive	Glucose, Sucrose

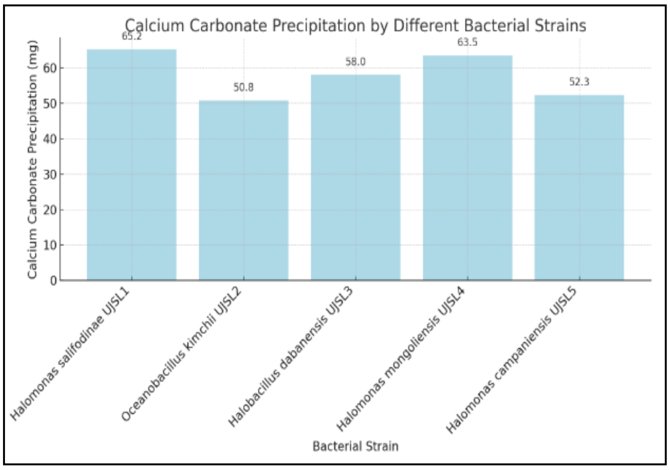

Figure 7: The amount of precipitation (in mg) produced by each strain, showing *Halomonas salifodinae UJSL1* as the most efficient producer, followed by *Halomonas mongoliensis UJSL4*.

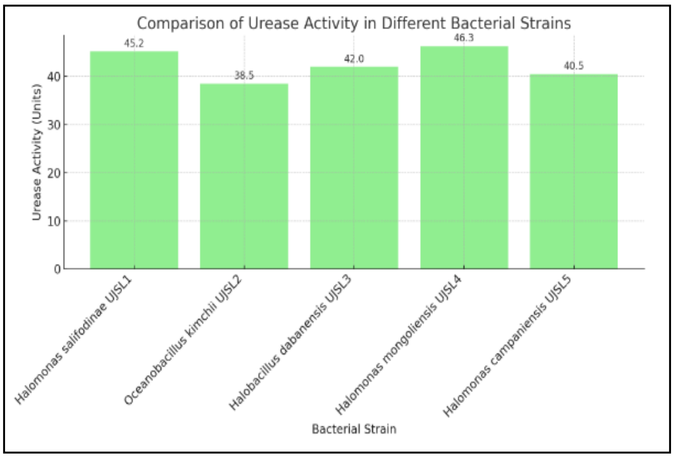

Figure 8: *Halomonas mongoliensis UJSL4* exhibited the highest urease activity, followed by *Halomonas salifodinae UJSL1* and *Halobacillus dabanensis UJSL3*.

Table 2
Showing Mineral Precipitation on B4 Medium

Strain	Calcium Carbonate Precipitate (mg)	Precipitation Appearance
Halomonas salifodinae UJSL1	65.2 ± 2.5	Large, uniform crystals
Oceanobacillus kimchii UJSL2	50.8 ± 2.3	Small, irregular deposits
Halobacillus dabanensis UJSL3	58.0 ± 2.6	Medium-sized, uniform crystals
Halomonas mongoliensis UJSL4	63.5 ± 2.8	Large, well-defined crystals
Halomonas campaniensis UJSL5	52.3 ± 2.4	Small, clustered crystals

Table 3
Showing SEM Analysis of Mineral Precipitation

Strain	Crystal Morphology	Crystal Size (µm)
Halomonas salifodinae UJSL1	Well-formed calcite crystals	6.5 ± 0.5
Oceanobacillus kimchii UJSL2	Irregular calcite structures	4.2 ± 0.4
Halobacillus dabanensis UJSL3	Uniform calcite crystals	5.0 ± 0.6
Halomonas mongoliensis UJSL4	Large, distinct calcite	6.3 ± 0.4
Halomonas campaniensis UJSL5	Clustered calcite crystals	4.8 ± 0.3

Table 4
Identification of Bacterial Strains via 16S rRNA Sequencing

Isolate	Identified Species	NCBI Accession Number
UJSL1	*Halomonas salifodinae*	PP837498
UJSL2	*Oceanobacillus kimchii*	PP837499
UJSL3	*Halobacillus dabanensis*	PP837500
UJSL4	*Halomonas mongoliensis*	PP837501
UJSL5	*Halomonas campaniensis*	PP837502

The physicochemical analysis aligns with previous studies emphasizing the biodiversity and resilience of microorganisms in hypersaline ecosystems.

The successful growth of bacteria on 10% NaCl nutrient agar medium underscores their halophilic nature. Biochemical characterization further revealed urease activity, a key enzymatic function associated with calcium carbonate precipitation. Urease facilitates urea hydrolysis, increasing carbonate ions in the medium, which precipitate calcium carbonate in the presence of calcium ions. The study demonstrates the efficacy of B4 medium in promoting mineral precipitation. After 7 days of incubation, all isolates exhibited calcium carbonate deposits, with *Halomonas salifodinae* UJSL1 and *Halomonas mongoliensis* UJSL4 producing the highest quantities. The controlled conditions provided by B4 medium facilitated optimal mineralization, showcasing the metabolic versatility of these isolates.

Scanning electron microscopy (SEM) provided detailed insights into the morphology of calcium carbonate crystals. Strains like *Halomonas salifodinae* UJSL1 produced large, uniform calcite crystals whereas others such as *Oceanobacillus kimchii* UJSL2 formed irregular structures. These variations reflect strain-specific differences in metabolic pathways, ion availability and extracellular polymeric substances influencing crystal nucleation and growth. The identification of strains via 16S rRNA sequencing affirmed their classification within halophilic and alkaliphilic genera such as *Halomonas* and *Oceanobacillus*. These genera are known for their ecological significance and industrial potential, particularly in environments where biomineralization and bioremediation processes are essential.

The submission of sequences to NCBI enhances genomic databases, paving the way for future comparative studies. The ability of these strains to precipitate calcium carbonate holds promise for several applications: The production of calcite by bacterial isolates can be utilized in bio-cementation processes for sustainable construction materials. The mineralization process contributes to carbon sequestration by locking atmospheric CO_2 into stable calcium carbonate. The strains can be deployed in saline or alkaline environments to remediate heavy metal contamination through biomineralization.

Conclusion

This study successfully isolated and characterized halophilic bacterial strains from the hypersaline Sambhar Lake and demonstrated their potential for biomineralization through calcium carbonate precipitation. The physicochemical properties of the lake's soil provided a favorable niche for these bacteria which were identified as *Halomonas salifodinae UJSL1*, *Oceanobacillus kimchii* UJSL2, *Halobacillus dabanensis UJSL3*, *Halomonas mongoliensis UJSL4* and *Halomonas campaniensis UJSL5* through 16S rRNA sequencing. The strains exhibited significant urease activity and mineral precipitation capabilities on B4 medium, with *Halomonas salifodinae UJSL1* and

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Halomonas mongoliensis UJSL4 emerging as the most efficient producers of calcium carbonate.

SEM analysis confirmed the morphology of precipitated calcite, showcasing the strains' ability to produce stable, well-defined crystals. These findings highlight the potential of these isolates for applications in biocementation, carbon sequestration and environmental remediation. Further studies should focus on optimizing their performance for large-scale industrial applications to explore their role in natural ecosystems. This research lays the foundation for leveraging halophilic bacteria in sustainable and eco-friendly technologies.

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