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Enhancing Ancient Concrete Durability through Urease-Mediated Calcite Precipitation by *Staphylococcus Succinus*: Implications for Sustainable Preservation in Archaeological Sites

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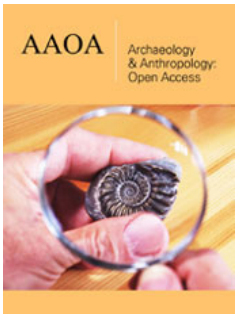

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Abstract

Concrete is a crucial material in construction, but its susceptibility to cracking remains a persistent issue. Microbially Induced Calcite Precipitation (MICP) presents an innovative, eco-friendly approach by harnessing microbes to produce calcite that fills cracks and strengthens concrete. This study aimed to isolate urease-producing bacterial strains with significant calcite-forming potential and optimize conditions for MICP to improve concrete performance. Soil sample was collected from Pakistan, to identify *Staphylococcus succinus* (T1 strain) as a high calcite producer through its ureolytic activity. The methodology involved isolating bacteria, screening for urease production, and assessing calcite precipitation under different environmental conditions such as salt and alkali levels. The T1 strain showed an impressive calcite precipitation rate of 37.90% at pH 8, coupled with excellent salt and alkali resistance. When added to concrete, the T1 strain enhanced compressive strength from 5.0885MPa to 12.3275MPa and split tensile strength from 3.323MPa to 6.514MPa. X-ray diffraction confirmed the formation of well-defined calcite crystals, supporting effective biomineralization. These findings highlight the potential of *Staphylococcus succinus* (T1 strain) to improve concrete durability and offer a sustainable, self-healing solution via MICP.

Keywords: MICP; *Staphylococcus succinus*; Calcite precipitation; Concrete durability; Biomineralization

Highlights

a) *Staphylococcus succinus* demonstrated high calcite precipitation at pH 8.

b) Significant increase in concrete compressive strength with T1 strain treatment.

c) XRD analysis confirmed well-formed calcite crystals in treated concrete.

d) T1 strain showed robust salt and alkali tolerance in varying environmental conditions.

e) Urease activity of *Staphylococcus succinus* promoted effective biomineralization.

Introduction

Concrete is a fundamental material in construction, but it presents notable environmental concerns, such as substantial CO_2 emissions and significant waste production [1]. Cement manufacturing alone contributes roughly 7% of global anthropogenic CO_2 emissions, primarily due to the energy-intensive processes involved in producing clinker, the key component of cement [2]. Additionally, traditional concrete structures degrade over time, particularly due to the formation of microcracks, leading to expensive repair costs [3]. A large portion of construction budgets in some areas is spent on infrastructure maintenance, highlighting the long-term financial burden of conventional concrete [4]. Bioconcrete, a more sustainable alternative, integrates bacterial cells and additives that facilitate self-healing by promoting

calcium carbonate ($CaCO_3$) precipitation through microbial activity [5]. This process effectively fills cracks in the concrete, reducing the need for repairs and extending the material's lifespan. Moreover, the microbial carbonation process accelerates CO_2 sequestration, as bacteria in bioconcrete absorb CO_2 and convert it into stable $CaCO_3$, strengthening the concrete while decreasing the environmental impact of its production [6].

By enhancing both the durability and sustainability of concrete, bioconcrete presents a promising solution for reducing greenhouse gas emissions, prolonging the service life of infrastructure, and lessening the environmental footprint of construction. Reinforced concrete is prone to degradation caused by various physical and chemical factors, leading to cracks that weaken its structural integrity [7]. These cracks may result from temperature changes, freezing and thawing cycles, or the infiltration of water and gases [8]. As microcracks form, they can expand, allowing moisture and salts to reach the steel reinforcement, causing corrosion and eventual structural failure. To mitigate this, self-healing concrete, which incorporates microorganisms capable of precipitating calcium carbonate ($CaCO_3$), has become an area of interest [9]. Bacteria like *Bacillus pseudofirmus* and *Sporosarcina pasteurii* remain dormant in concrete until cracks develop, activating the bacteria to precipitate $CaCO_3$ and seal the crack [10]. The self-healing effect can be further enhanced by embedding nutrients in microcapsules, extending the concrete's healing capability [11]. Despite challenges, such as the limited duration of self-healing, these innovations offer promising approaches for improving concrete durability and extending its service life.

Biomineralization, a process where living organisms convert organic materials into inorganic compounds like calcium carbonate ($CaCO_3$), holds great potential for microbial self-healing in concrete [12]. This process leverages microorganisms' metabolic activities to precipitate $CaCO_3$ in various polymorphic forms, such as calcite, vaterite, and aragonite, with calcite being the most stable and suitable for construction purposes [13]. Various bacterial and fungal species, including *Bacillus subtilis*, *B. pasteurii, B. sphaericus, B. cohnii, B. megaterium*, *Staphylococcus succinus*, and fungi like Penicillium and Cladosporium, demonstrate ureolytic activity that facilitates $CaCO_3$ precipitation [14]. Studies indicate that the optimal conditions for microbial calcite precipitation are a pH of 7 and a temperature of 30 °C, with performance declining at higher temperatures [15]. For instance, Bacillus cereus UCP 1615, when combined with calcium lactate and Portland cement, achieved 82% SiO_2 and 18% $CaCO_3$, showing its potential in forming $CaCO_3$ crystals [16]. Furthermore, research by Kim et al. identified *Staphylococcus succinus* and *Sporosarcina pasteurii* as highly efficient in producing calcite under controlled conditions, showcasing the promise of various microbial strains to improve concrete's durability and sustainability.

The purpose of this experiment was to evaluate the potential of *Staphylococcus succinus* in facilitating Microbially Induced Calcite Precipitation (MICP) to enhance concrete durability and sustainability. *Staphylococcus succinus*, a bacterium abundant in soil, was selected for its ability to precipitate calcium carbonate ($CaCO_3$) in response to environmental factors like high pH and calcium availability. The goal was to determine the bacterium's effectiveness in catalyzing calcite formation and its potential to serve as a self-healing agent in concrete, filling microcracks and reducing the need for conventional repairs. This study aimed to explore the innovative application of *S. succinus* in bioconcrete, offering a low-cost, environmentally friendly solution that enhances the longevity of concrete structures while minimizing the carbon footprint of traditional repair methods.

Materials and Methods

Isolation and identification of *staphylococcus succinus*

Soil samples (1 gram each) were collected from Rawalpindi, Punjab, Pakistan, to isolate *Staphylococcus succinus*. The isolation was carried out using a selective medium for urease-producing bacteria, containing phenol red solution (0.2%) at 4mL/L, NaCl (5.0g/L), KH_2PO_4 (2.0g/L), urea (2.0g/L), peptone (1.0g/L), glucose (0.1g/L), and agar (20.0g/L), adjusted to pH 7.0. Colonies exhibiting a fuchsia hue, indicating urease production, were isolated and subcultured on nutrient-agar plates containing peptone (10.0g/L), NaCl (10.0g/L), yeast extract (5.0g/L), and agar (20.0g/L). Purified colonies were inoculated into a seed medium (peptone 5.0g/L, glucose 5.0g/L, beef extract 3.0g/L) and incubated at 37 °C with shaking (180rpm) for 12 hours to promote bacterial growth. For bacterial identification, genomic DNA was extracted using a DNA extraction kit, and the 16S rRNA gene was amplified with universal primers. PCR products were sequenced, and sequence data were compared with public databases to confirm the bacterial strain as *Staphylococcus succinus*. Cultures were preserved at -80 °C with 30% glycerin.

Bacterial growth optimization process

Following isolation, *Staphylococcus succinus* growth was monitored at two intervals: directly after isolation and after 24 hours. Growth was quantified by measuring the Optical Density (OD) at 600nm, with higher OD values corresponding to higher bacterial concentrations. Initial growth measurements were conducted in a nutrient-free medium to determine baseline growth. The medium was subsequently supplemented with urea and $CaCl_2$, and bacterial growth was re-evaluated after 24 hours to optimize the growth conditions.

Procedure for MICP evaluation on calcite-activity

To evaluate the potential of *Staphylococcus succinus* for Inducing Calcium Carbonate Precipitation (MICP), a reaction solution was prepared by combining 50mL of water, 1.25g of urea, 2.5g of calcium chloride, and 1g of bacterial suspension in an additional 50mL of water in a 500mL conical flask. A control sample was prepared without calcium chloride. The mixture was incubated at 37 °C, agitated at 100rpm for 6 hours. After incubation, the solid components were separated by centrifugation at 8000rpm for 10 minutes, washed with deionized water, and dried in an oven.

Adopted technique for bacterial growth in alkaline and saline conditions

Staphylococcus succinus was cultured in a seed medium (peptone 5.0g/L, glucose 5.0g/L, and beef extract 3.0 g/L), and the pH was adjusted to values between 7 and 14 with NaOH to test alkaline tolerance. The NaCl concentration was varied between 0% and 5% to assess salt tolerance. Cultures were maintained at 37 °C with shaking at 180rpm, and bacterial growth was monitored by measuring OD600 at 2-hour intervals, determining the optimal conditions for growth in both alkaline and saline environments.

Fiber bio-concrete preparation and testing

Six concrete cylinders (100mm in diameter, 200mm in length) were cast using a mix ratio of 1:2:4:0.6 (C:S:A:W). The mix included 4.6kg of cement, 9.6kg of sand, 19.5kg of aggregates, 2764.6mL of water, and 46g of jute fiber. Three cylinders were prepared without bacteria (control), while three were inoculated with *Staphylococcus succinus* (15mL of bacterial suspension at 10^7CFU/mL), 55g of urea, and 50g of calcium chloride to encourage calcite precipitation. The ingredients were mixed, poured into molds, and allowed to settle overnight. After demolding, the cylinders were cured at room temperature for 28 days recorded to evaluate compressive and tensile strength.

Procedure for analysis of calcium carbonate precipitation

Calcium carbonate precipitates formed by *Staphylococcus succinus* and deposited on crack surfaces were analyzed using X-Ray Diffraction (XRD) with the EXPLORER model. The samples were air-dried, flattened, and placed on XRD. Once cured, the specimens were dried for one day and tested for strength using a Universal Testing Machine (UTM). The maximum load at failure was holders for uniformity. Data were collected using Cu Kα radiation over a 2θ range of 5° to 70°, with a step size of 0.02° and a counting time of 1-2 seconds per step. The diffraction patterns were analyzed using specialized software to determine the mineral composition and structure.

Results

Isolation of urease-producing *staphylococcus succinus*

Staphylococcus succinus was successfully isolated from soil samples taken from Rawalpindi, Punjab, Pakistan, using a specialized medium for detecting urease activity. The strain exhibited a fuchsia color, indicating urease production through the hydrolysis of urea, which altered the medium's pH. The colony was further isolated and purified on nutrient-agar plates, demonstrating consistent growth (Figure 1). Upon examination of its morphological traits, the strain was identified as *Staphylococcus succinus* through 16S rRNA sequencing, and the strain was labeled T1. The bacteria were cultured in a seed medium at 37 °C with shaking for 12 hours, then preserved at -80 °C in a 30% glycerin solution for future use.

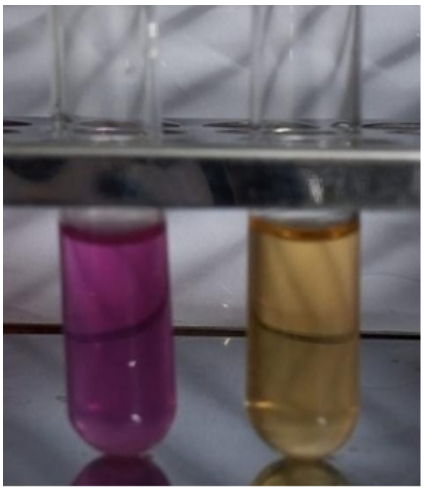

Figure 1: Urease positivity of *staphylococcus succinus*.

Optical density of *staphylococcus succinus* (T1 Strain)

In the growth optimization experiment, *Staphylococcus succinus* (T1 strain) showed significant growth, as reflected in its Optical Density (OD) at 0 and 24 hours. The OD at 0 hours was 0.015, indicating initial bacterial growth. After 24 hours and nutrient addition, the OD increased to 1.014, signifying substantial growth and suggesting optimal conditions for bacterial proliferation, which could be utilized for further experiments.

Calculation of bacterial-induced precipitation

The Microbial-Induced Calcium Carbonate Precipitation (MICP) potential of *Staphylococcus succinus* (T1 strain) was evaluated by measuring calcite precipitation. The reaction was carried out by incubating the bacterial suspension with urea and calcium chloride, followed by separation, washing, drying, and calcite measurement (Figure 2). At pH 8, the strain precipitated 37.90% calcite, with a weight of 0.296g, demonstrating the strain's efficiency in calcium carbonate precipitation (Figure 3), which can be leveraged in applications such as bioconstruction and environmental sustainability.

Figure 2: Calcite precipitation by T1 without filtration.

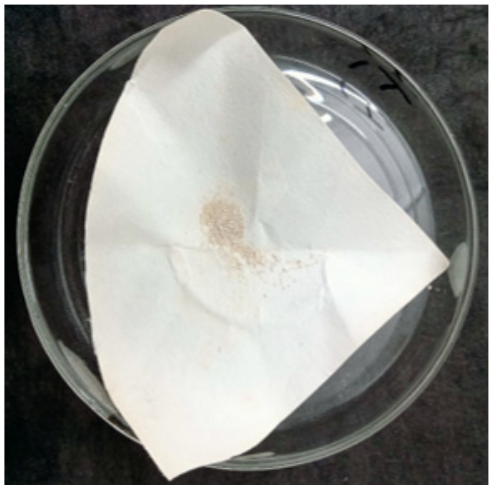

Figure 3: Calcite precipitation by T1 after filtration.

Salt and alkali resistance of *staphylococcus succinus* (T1 strain)

The resistance of *Staphylococcus succinus* (T1 strain) to salt and alkali was tested by evaluating growth at varying NaCl concentrations and pH levels.

a. Salt Tolerance: The strain exhibited optimal growth at 0% NaCl (OD600 of 3.932 at 14 hours) and maintained high growth at 5% NaCl (OD600 of 3.810), demonstrating exceptional salt tolerance (Table 1).

Table 1: Salt tolerance of isolated strain.

Concentration	0hr	2hr	4hr	6hr	8hr	10hr	12hr
0%	2.376	3.678	3.710	3.780	3.785	3.968	3.932
1%	2.426	3.147	3.260	3.592	3.692	3.787	3.797
2%	2.204	3.046	3.246	3.425	3.475	3.738	3.778
3%	2.210	2.772	2.916	3.304	3.354	3.382	3.390
4%	2.840	3.133	3.236	3.570	3.650	3.785	3.810
5%	2.376	3.678	3.710	3.780	3.785	3.968	3.932

b. Alkali Resistance: Growth was steady at pH 7 (OD600 of 0.2917) and pH 8 (OD600 of 0.2315), with peak growth observed at pH 9 (OD600 of 0.203). However, growth decreased beyond pH 9 (OD600 ranging from 0.0490 to 0.0561) (Table 2), indicating that the strain thrives best in neutral to slightly alkaline environments.

Table 2: pH tolerance of isolated strain.

pH	2hrs.	4hrs.	6hrs.	8hrs.	10hrs.	12hrs.	14hrs.
7	0.0796	0.1510	0.1581	0.1663	0.2795	0.2907	0.2917
8	0.1346	0.1714	0.1897	0.2050	0.2305	0.2326	0.2315
9	0.1438	0.1969	0.1979	0.1999	0.2030	0.2030	0.2030
10	0.0296	0.1489	0.1163	0.1469	0.1428	0.1428	0.1428
11	0.0820	0.0820	0.0820	0.0820	0.0820	0.0820	0.0820
12	0.0561	0.0561	0.0561	0.0561	0.0561	0.0561	0.0561
13	0.0561	0.0561	0.0561	0.0561	0.0561	0.0561	0.0561
14	0.0490	0.0490	0.0490	0.0490	0.0490	0.0490	0.0490

Concrete strength

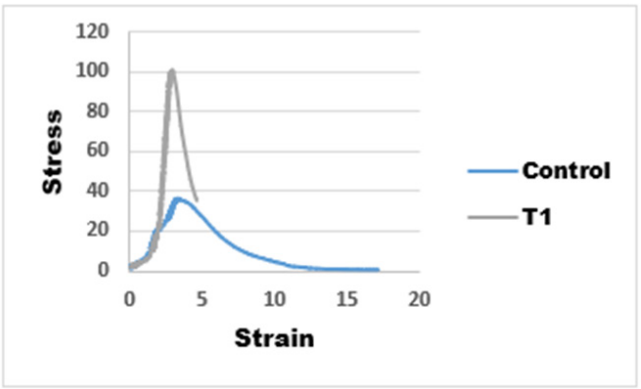

Figure 4: Compressive strength of control and bacterial concrete.

The mechanical properties of the T1-treated concrete showed substantial improvements over the control group. The compressive strength of the control was 5.0885MPa, whereas the T1-treated sample reached 12.3275MPa (Figure 4). Similarly, the split tensile strength of the control was 3.323MPa, while the T1 sample showed a significant increase to 6.514MPa, with a higher load-bearing capacity of 52.81kN compared to 26.94kN in the control (Figure 5). These improvements highlight the potential of *Staphylococcus succinus* in enhancing the mechanical properties of concrete.

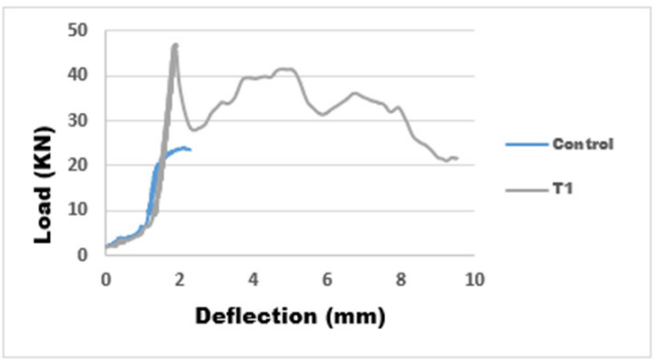

Figure 5: Split tensile of control and bacterial concrete.

XRD analysis

X-Ray Diffraction (XRD) analysis of concrete treated with *Staphylococcus succinus* (T1 strain) revealed key findings regarding calcite precipitation.

a. Peak Analysis: The dominant peak at 29.5° 2θ, characteristic of calcite ($CaCO_3$), confirmed successful calcite precipitation in the concrete (Figure 6). Minor peaks indicated the presence of other calcium carbonate forms but were less prominent than the calcite peak.

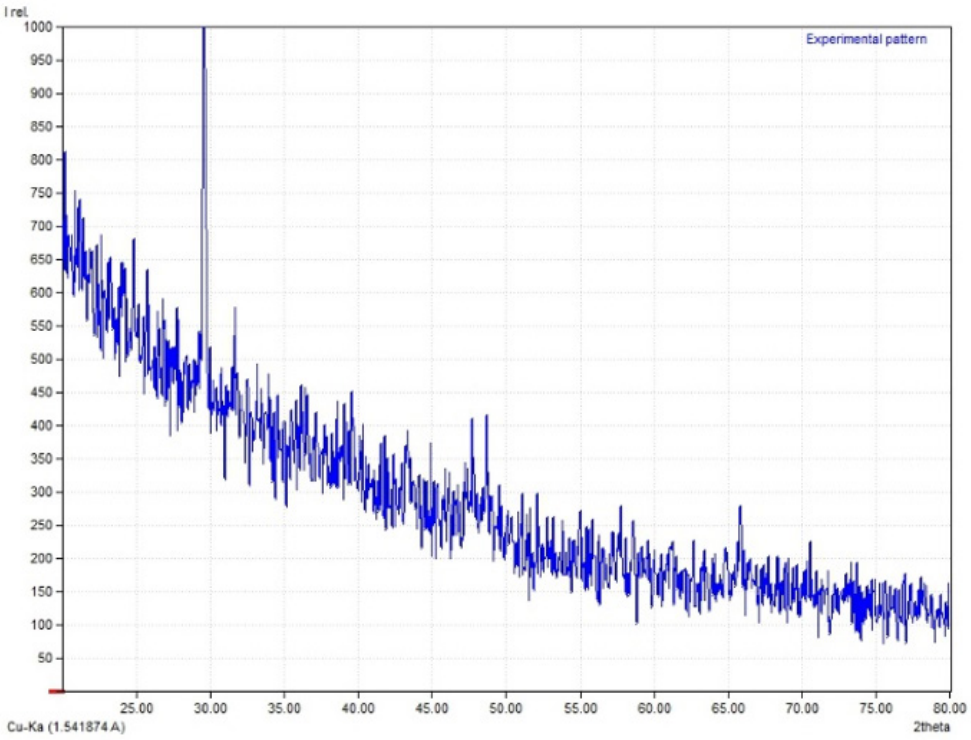

Figure 6: XRD graph of calcite crystals formed by *staphylococcus succinus*.

b. Calcite Quantity: The sharp and intense peaks in the XRD patterns indicated that *Staphylococcus succinus* produced a significant amount of calcite, a result of its high urease activity.

c. Morphology: The XRD analysis suggested that the calcite crystals formed were rhombohedral and well-defined, implying efficient biomineralization and high-quality crystal formation.

d. Noise and Background: The XRD patterns showed minimal background noise, indicating organized crystal growth with fewer amorphous or poorly crystalline phases in the calcite produced by *Staphylococcus succinus*.

Conclusions

The results from the study on *Staphylococcus succinus* (T1 strain) suggest that this bacterial strain demonstrates significant potential for applications in Microbial-Induced Calcium Carbonate Precipitation (MICP), as well as in improving the mechanical properties of concrete.

a. *Staphylococcus succinus* (T1 strain) was successfully isolated from soil samples and identified through urease detection, confirming its ability to produce urease, a crucial enzyme in Microbial-Induced Calcium Carbonate Precipitation (MICP). This urease activity was verified by the fuchsia coloration of the medium, which indicates urea hydrolysis and a pH shift.

b. The strain exhibited significant growth in a bacterial growth optimization experiment, with Optical Density (OD) rising from 0.015 at 0 hours to 1.014 at 24 hours, demonstrating its strong proliferation and suitability for large-scale applications.

c. The T1 strain achieved a 37.90% calcite precipitation rate at pH 8, indicating efficient calcium carbonate biomineralization. This high precipitation rate makes the strain suitable for applications in bioconstruction, where MICP can enhance material durability.

d. *Staphylococcus succinus* showed impressive salt tolerance, with growth sustaining even at 5% NaCl concentration. This resilience to saline conditions suggests its potential for use in marine or coastal environments, where concrete is exposed to high salt concentrations.

e. The strain also demonstrated strong alkali resistance, thriving in slightly alkaline to neutral conditions, making it adaptable for environments with varying pH levels, including those encountered in bioremediation and construction projects.

f. Concrete samples treated with *Staphylococcus succinus* exhibited a significant increase in mechanical properties. The compressive strength of T1-treated samples was 12.3275MPa,

compared to 5.0885MPa in the control, and the split tensile strength increased to 6.514MPa from 3.323MPa in the control. These results suggest that the calcite precipitation induced by the bacterial strain reinforced the concrete, making it stronger and more durable.

g. XRD analysis of treated concrete showed that the T1 strain induced the formation of high-quality calcite, with a dominant peak at 29.5° 2θ characteristic of calcite crystals. The intensity and sharpness of the peaks indicated a uniform and well-defined crystallization process, further supporting the enhanced mechanical properties observed in the concrete samples.

a. Overall, the results suggest that *Staphylococcus succinus* (T1 strain) is highly effective for inducing calcite precipitation, improving concrete strength, and enhancing its resistance to environmental stressors such as salt and alkali. These findings indicate the potential of using this strain for sustainable construction practices, reducing the need for chemical additives and improving the longevity of concrete structures.

Acknowledgement

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