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Biomineralization of $CaCO_3$ by *Bacillus* sp. 8WNM for Application as Bio-Cement

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Abstract

Globally, cement is the most often used building material, but its traditional manufacture comes with significant environmental impacts along with restrictions on cost and quality. Calcium carbonate ($CaCO_3$) is a commonly used substance in this context. To overcome these obstacles, biological building materials are becoming sustainable technology. Recently, the production of $CaCO_3$ by bacteria has garnered attention due to its environmentally friendly and health-conscious approach. In this study, *Bacillus sp.* 8WNM was isolated from Wadi El Natrun lake, Egypt and evaluated for its ability to precipitate $CaCO_3$ through biomineralization using different calcium compounds, including calcium nitrate ($Ca(NO_3)_2$), calcium acetate ($Ca(CH_3COO)_2$), and calcium chloride ($CaCl_2$). The precipitated $CaCO_3$ was characterized using Fourier Transform Infrared Spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM), and energy-dispersive X-ray (EDX) analyses. Among the tested sources, calcium acetate was the most effective, yielding the highest amount of precipitate. XRD analysis confirmed that the obtained $CaCO_3$ exhibited a single-phase structure, while FTIR analysis identified peaks corresponding to $CaCO_3$. The precipitated particles were mainly cubic, consisting of Ca (15.38±1.34%), C (23.21±1.08%), and O (61.41±3.84%). Optimal conditions for $CaCO_3$ precipitation were achieved with a calcium source concentration of 2.5 g/L, an inoculum size of 1.5 mL, and an incubation period of 8 days, resulting in a production yield of 0.236 g/100 mL. The promising results obtained by precipitating $CaCO_3$ using *Bacillus* sp. 8WNM recommend its usage in the bioconstruction materials production that are both financially sustainable and socially and environmentally beneficial.

Keywords *Bacillus sp*. Biomineralization, $CaCO_3$ precipitation, optimization, calcite characterization

1. Introduction

Building construction is an ancient practice that has been started since human civilization. As early as 3000 BC, a mixture of straw and clay were used by the Sumerians as bio-composite materials [1]. Today, the global construction industry is rapidly expanding, driven by the development of nations. However, this growth presents significant environmental challenges [2]. Cement, a material widely favored for its durability and affordability, plays a major role in these issues. Its increased consumption has severely impacted the global environment, with cement production alone contributing emissions up to 3.4% of total global CO_2 and 8–10% of global anthropogenic CO_2 [3]. Additionally, a study by Zhao et al. [4] revealed that in 2019, China produced approximately 2,300 million tons of waste from construction and demolition, making it the world's largest producer of construction waste.

Beyond environmental impact, the cracks formation within the infrastructure are another significant disadvantage of cement-based construction. Cracking is a widespread issue in concrete-based buildings, leading to engineering properties reduction and strength of the material, particularly in surface layers [5]. Environmental factors like temperature changes, external stresses, exposure to substances caused corrosion, and the size of the pores in the materials can all lead to the development of cracks. These elements have a detrimental impact on concrete's mechanical qualities, such as its permeability, tensile strength, and compressive strength. As a result, the building becomes damaged, which results in corrosion of the reinforcement and additional maintenance expenses [6, 7, 8]. These findings reveal the substantial contribution of construction sectors to environmental pollution, highlighting the urgent need to shift towards more environmentally friendly infrastructure materials.

In 1990, a U.S. research group led by Prof. Sookie Bang, along with Gavin McIntyre and Eben Bayer, introduced the concept of using microbes in the construction industry. One notable application of this concept is the use of bacteria to self-heal cracks in concrete, either directly or indirectly [6, 9, 10]. Microorganisms are particularly well-suited for this purpose due to their unique characteristics, including metabolic processes, cell structure, rapid reproduction, growth rates, spore formation, and metabolite production. These attributes enable a wide range of microbial applications [6]. Much recent work has been on the use of microorganisms to enhance the mechanical characteristics of cement, namely via a process called microbially induced calcium carbonate precipitation (MICP). This process enhances the strength of cement by encouraging bacterial mineralization [11].

Microorganisms can precipitate calcium carbonate through various processes, such as photosynthesis, ammonification, denitrification, carbonic anhydrase production, and urease enzyme activity. Among these, urease enzyme activity is widely studied the most, as it efficiently generates carbonate compared to other methods [12]. The selection of bacteria for this process depends on several factors, including spore formation, tolerance to harsh environments, high alkalinity, nutrient survival, growth conditions, and resistance to mechanical

stress [13]. Therefore, the chosen microbes must be capable of withstanding high alkalinity and low oxygen levels and effectively precipitating calcite in mixing with healing agents. The calcium carbonate they produce further increases the environment's alkalinity [5]. Notably, during the calcium carbonate precipitation process, the negatively charged surface of bacterial cells acts as a nucleation center, where positively charged calcium ions accumulate, triggering the nucleation process [14].

Based on research, the most commonly used genus for bacteria-based crack healing in concrete is Bacillus [6, 15, 16]. Bacillus bacteria are known for their spore-forming ability, which allows them to endure extreme conditions. These bacteria have thick cell walls and compact round shape measured between 0.8 and 1 μm in size [6]. Remarkably, they can survive for extended periods, with some studies suggesting a lifespan of around 50 years [8], and others indicating survival for hundreds of years [17]. Their resilience enables them to tolerate various environmental conditions, including exposure to chemicals, ultraviolet radiation, and high mechanical stresses, making them ideal candidates for self-healing applications in construction [5].

Herein, this study aim is to evaluate the potentiality of bacterial isolates from alkaline soils as effective self-healing agents in concrete through identifying and characterizing bacteria capable of inducing mineral precipitation ($CaCO_3$). The investigation will include isolating and screening bacterial strains, identifying the most promising isolate using morphological and molecular techniques, and characterizing the self-healing products at a morphological and chemical level.

2. Material and methods

2.1. Sample collection

Wadi El-Natrun, a depression in the Sahara Desert about 80 km northwest of Cairo, Egypt, is where the samples were taken. Seven large, hypersaline, alkaline lakes in this valley are solar-heated and mostly fed by underground seepage from the Nile River and occasional winter precipitation. Notably, the lakes, ranging in depth from 0.5 to 2 meters, are known for their extreme conditions, including high alkalinity, elevated salt concentrations, and high temperatures due to intense solar heating.

2.2.Bacterial isolation

To isolate bacteria, samples were serially diluted from 10^{-1} to 10^{-6}. Subsequently, 1g of soil sample was mixed with 10 mL of 0.8% sterile saline solution and shaken at 150 rpm for one hour at 30°C. Then, 0.5 mL of an appropriate dilution was spread on a medium plate, containing enrichment medium with the following components (g/L): 10 g glucose, 5 g peptone, 5 g yeast extract, 30 g NaCl, 0.1 g $MgSO_4$, 0.5 g KCl, and 20 g agar in PH 9 [18]. For 48 h, each flask was incubated at 37°C on a rotary shaker spinning at 100 rpm. Following enrichment, the organisms were separated and cultured for 24 h at 37°C on nutrient agar medium plates [19]. Then, well-isolated and morphologically unique colonies were transferred to the respective medium slants and kept as stock cultures.

2.3. Growth of pure bacterial isolates in B4 broth media containing calcium acetate as calcium source

Pure bacterial isolates were inoculated in B4 medium with using calcium acetate ($Ca(CH_3COO)_2$), a common admixture in engineering materials, as a calcium ion source. The medium composition per liter included 5 g of dextrose, 4 g of yeast extract, and 2.5 g of calcium acetate [20]. The pH was initially adjusted to 7.0, and a non-inoculated flask was used as a control. For seven days incubation, the cultures were rotated at 150 rpm and 30°C. Then, PH value, amount of precipitate, and turbidity were measured for each flask, and based on these measurements, we have chosen certain bacterial isolates for successive tests.

2.4. Selected bacterial isolates growth in different calcium sources

Different calcium compounds, including calcium nitrate ($Ca(NO_3)_2$), calcium acetate ($Ca(CH_3COO)_2$), and calcium chloride ($CaCl_2$), were used as calcium ion sources. The B4 medium was modified by substituting calcium acetate with the other calcium compounds. For each experiment, which was carried out in triplicate, the medium's pH was adjusted to 7.0. The control group consisted of non-inoculated flasks. For seven days incubation, cultures were shaken at 150 rpm and maintained at 30°C. Samples were collected every 12 to 24 h to quantify the growth of the bacteria using the plate count method.

Precipitates were formed in bacterial cultures and abiotic control flasks after incubation for 7 days. Pre-weighed Whatman No. 1 filter paper (Merck, Germany) was used to filter the precipitates. After twice washing the solid samples by sterile water to get rid of any attached cells, they were dried in a hot air oven set at 45°C until completely dry. Filter paper containing precipitates and empty filter paper were recorded in order to calculate the weight of the precipitates.

2.5. Morphological and cultural characteristics

2.5.1. Gram stain and microscopic examination

Gram staining and microscopic examination were performed on four selected isolates [21]. Smears that had been air-dried and heat-fixed were exposed to crystal violet stain for one minute and then kindly rinsed with tap water. The slides were decolorized until the runoff was clear and then counterstained with safranin for 30 sec. After a final rinse with tap water, the slides were blotted dry and examined under a bright-field microscope at 100x magnification using oil immersion.

2.5.2. Sporulation(Spore-Forming Ability)

The spore-forming ability of the four isolates was assessed by inoculating each into slants containing sterilized B4 medium broth [22]. After a 5-day incubation at 30°C, the slants were boiled at 80°C for 10 min in a water bath. Following that, Samples were transferred to new sterilized B4 medium slants and incubated for an additional 2 days to observe spore formation.

2.6. Optimization of $CaCO_3$ precipitation

2.6.1. Effect of different inoculum size

The inoculum size effect of the chosen bacterial isolate on calcite precipitation was investigated by adding

varying amounts of a 24-hour-old culture (0.5, 1, 1.5, 2, 2.5, and 3 mL) to 100 mL of B4 broth in 250 mL Erlenmeyer flasks. For 48 h in 37°C, the cultures were incubated. Following incubation, precipitates were gathered by filtering. They were air-dried and weighed after being twice cleaned with sterile water to remove any adhering cells.

2.6.2. Effect of different calcium acetate concentration

Calcium acetate in different concentrations (0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 g/L) were tested for their impact on calcite precipitation. Each concentration was prepared in B4 medium, and 100 mL of each was inoculated with the chosen bacterial isolate in 250 mL flasks. Following that, the cultures were incubated for 48 h at 37°C. The precipitates were harvested after incubation, washed twice by sterile water, air-dried, and weighed.

2.6.3. Effect of different incubation time intervals

Different incubation times effect on calcite precipitation was studied by adding a 24-hour-old culture to 100 mL of B4 broth in 250 mL flasks. The flasks were incubated for varying intervals at 37°C. After each incubation period, precipitates were harvested, washed with sterile water, air-dried, and weighed.

2.7. calcite characterization

2.7.1. X-ray diffraction (XRD) analysis

X-ray diffraction (XRD) was employed to analyze the crystal structure of powders. The analysis was conducted using a powder X-ray diffractometer (Malvern Panalytical Empyrean, UK) with a CuKα radiation source, operating at 30 kV and 30 mA. The diffraction patterns were recorded over a scan range of 20° to 60°. To further investigate the crystal structure of the precipitates, VESTA (Visualization for Electronic and Structural Analysis) software was utilized. VESTA facilitated 3D visualization of crystal morphologies, structures, and volumetric data. For simulating the powder X-ray diffraction patterns based on the lattice and structural properties, VESTA incorporated RIETAN-FP, a tool for refining crystal structures from diffraction data. The software allowed crystal structures to be represented using five different models: ball-and-stick, space-filling, polyhedral, wireframe, and stick models. These models provided comprehensive insights into the structural and morphological features of the crystals [23].

2.7.2. Fourier Transform Infrared Spectroscopy (FTIR) analysis

FTIR spectra were obtained using a Bruker Vector-22 FT-IR spectrometer, covering the wavenumber range from 4000 cm^{-1} to 400 cm^{-1}. The FTIR spectra were recorded with a resolution of approximately 4-8 cm^{-1}, allowing for detailed analysis of the molecular vibrations and functional groups within the sample.

2.7.3. Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) analyses

A Tescan Vega 3LMH scanning electron microscope (SEM) with a back-scattered electron detector was used to study the sample shape and structure. To determine the precipitate composition, energy dispersive X-ray spectroscopy (EDX) was performed using the same SEM equipped with a Bruker X-ray detector. This allowed for both visualizing

the sample surface and identifying the elements it contained.

3. Results

3.1. Bacterial isolation and screening for $CaCO_3$ precipitation

Twenty bacterial isolates were tested to see if they could produce $CaCO_3$. This capability was assessed based on the amount of precipitate formed, turbidity, and pH (**Table 1**). It is noticeable that the weight of precipitates is directly proportional to the pH and turbidity of the media. Nine isolates, which exhibited the highest $CaCO_3$ precipitates, were further evaluated using various calcium sources to determine the most effective one for $CaCO_3$ precipitation (**Table 2**). Calcium acetate proved to be the most effective source, yielding the highest amount of precipitate. Consequently, four bacterial isolates (4WNM, 8WNM, 11WNM, 15WNM) were selected for subsequent testing.

Table 1: Bacterial isolates screening for $CaCO_3$ precipitation.

Bacterial isolates	PH value	Amount of precipitate (g / 100 mL)	Bacterial growth (O.D) at 600 nm
1WNM	8.52	0.155	2.01
2WNM	8.50	0.096	2.05
3WNM	8.49	0.082	2.03
4WNM	8.63	0.226	2.30
5WNM	8.42	0.067	1.92
6WNM	8.43	0.151	1.56
7WNM	8.52	0.113	2.14
8WNM	8.65	0.238	2.31
9WNM	8.51	0.176	2.12
10WNM	8.39	0.067	1.90
11WNM	8.57	0.207	2.22
12WNM	8.24	0.086	1.97
13WNM	8.30	0.074	1.75
14WNM	8.37	0.113	2.01
15WNM	8.63	0.218	2.10
16WNM	8.54	0.150	2.06
17WNM	8.41	0.052	1.97
18WNM	8.61	0.076	2.01
19WNM	8.52	0.167	2.04
20WNM	8.51	0.056	2.11

Table 2: Effect of different calcium sources on precipitation of $CaCO_3$.

Bacterial isolates	Amount of precipitated $CaCO_3$ (g / 100 mL)		
	Calcium acetate	Calcium chloride	Calcium nitrate
1WNM	.167	.088	.037
4WNM	.203	.093	.042
6WNM	.158	.091	.046
8WNM	.226	.122	.043
9WNM	.184	.041	.021
11WNM	.192	.081	.032
15WNM	.195	.112	.053
16WNM	.163	.039	.026
19WNM	.160	.048	.032

3.2. Morphological and cultural characteristics

The four selected bacterial isolates that showed good precipitation of calcium carbonate were Gram positive and spore – forming (**Figure 1**). Furthermore, their cells were rod-like and arranged in chains or in pairs. On the other hand, they formed mainly wrinkled surface colonies with creamy, off-white to grey-white color. Based on these characteristics, the isolates were categorized as members of Bacillus genus. Subsequently, the strain with the largest single colony (8WNM) was chosen for $CaCO_3$ precipitation and further testing.

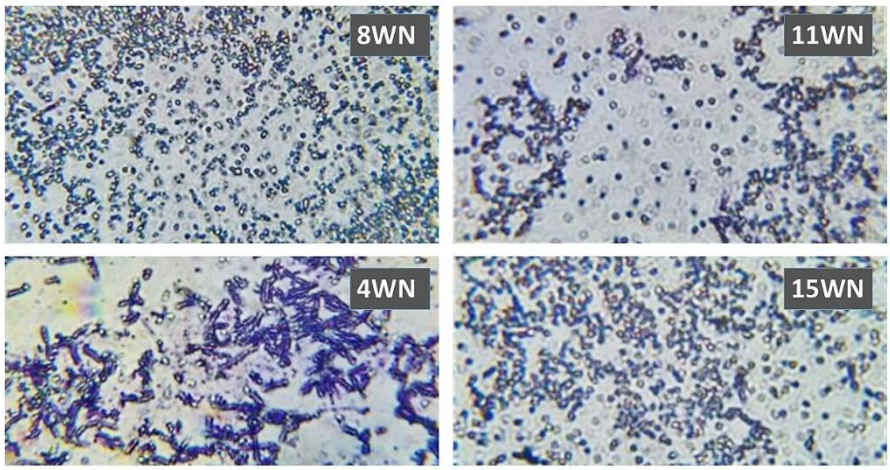

Figure 1: Gram staining and microscopic examination of four selected bacterial isolates.

3.3. Identification of bacterial isolate

The selected bacterial isolate 8WNM was identified as Bacillus sp. 8WNM based on morphological and biochemical characteristics

3.4. Optimization of Precipitation Conditions

To achieve the highest yield of $CaCO_3$ precipitates, the inoculum size, calcium acetate concentration, and incubation time were optimized. The optimal inoculum size for precipitate formation was determined to be 1.5 mL (**Table 3**), while the best incubation time was 8 days (**Table 4**). Additionally, the optimal concentration of calcium acetate for precipitate formation was found to be 2.5 g/L (**Table 5**).

Table 3: The effect of different inoculum size on $CaCO_3$ precipitate formation.

Inoculum size(mL)	Amount of precipitate (g/100mL)	PH
0.5	0.075	8.35
1	0.153	8.48
1.5	0.202	8.60
2	0.173	8.56
2.5	0.194	8.36
3	0.105	8.30

Table 4: The effect of different incubation time intervals on $CaCO_3$ precipitate formation.

Time	Amount of precipitate (g/100mL)	PH
2	0.083	8.23
4	0.135	8.25
6	0.157	8.68
8	0.236	8.70
10	0.172	8.60

Table 5: The effect of different calcium acetate concentrations on $CaCO_3$ precipitate formation.

Calcium concentration (g/l)	Amount of precipitate (g/ 100mL)	PH value
0.5	0.061	8.60
1	0.072	8.658
1.5	0.134	8.78
2	0.176	8.77
2.5	0.204	8.95
3	0.173	8.39
3.5	0.164	8.37
4	0.144	8.35

3.5. XRD analysis

The crystallographic properties of CaCO3 precipitates were confirmed using the XRD technique. As shown in **Figure 2**, the XRD peak patterns of $CaCO_3$ minerals crystalline structure, produced through the Bacillus sp. 8WNM biomineralization activity, were analyzed. According to the ICDD database, these patterns corresponded to $CaCO_3$ in the form of calcite. The experimental XRD spectra matched a single spectral model, suggesting that the obtained $CaCO_3$ has a single phase or structure. The alignment with reference peak revealed that the precipitates displayed the highest peak at 29.5°, which is characteristic of spar calcite.

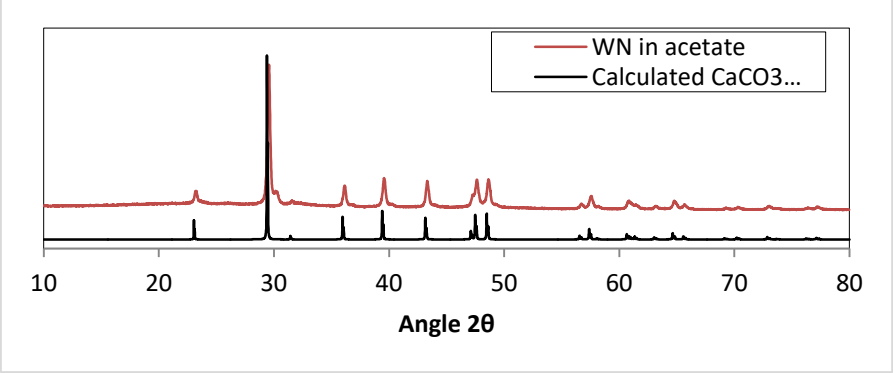

Figure 2: XRD diffractogram of the precipitated $CaCO_3$ produced through the biomineralization activity of Bacillus sp. 8WNM.

3.6. FTIR

FTIR was used to analyze the precipitates, with the results displayed **Figure 3**. Distinct absorption peaks were noticed at 3422 cm^{-1}, 2516 cm^{-1}, 1655 cm^{-1}, 1448 cm^{-1}, 1060 cm^{-1}, 873 cm^{-1}, and 713 cm^{-1}. As confirmed by the infrared characteristic peaks from the control results, these peaks were attributed to $CaCO_3$.

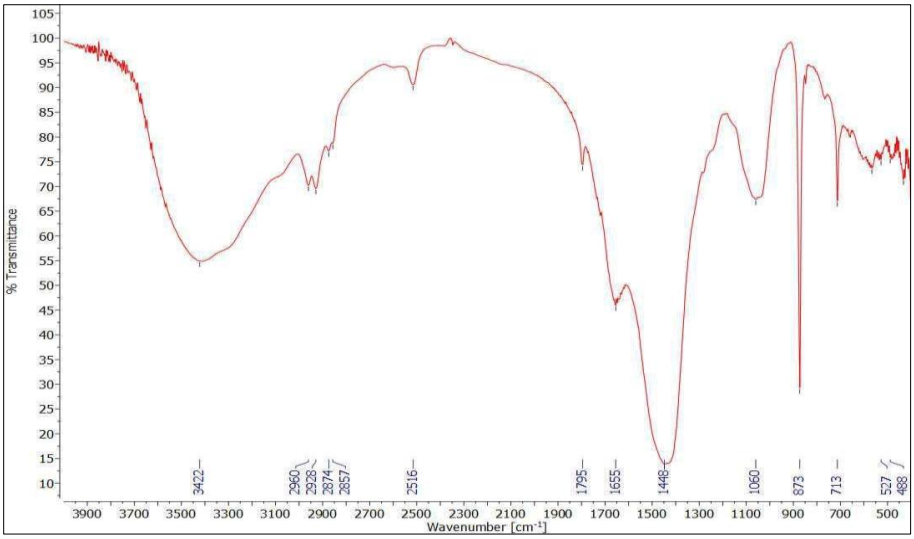

Figure 3: FTIR spectra of the precipitated $CaCO_3$ produced through the biomineralization activity of Bacillus sp. 8WNM.

3.7. SEM and EDX analyses

SEM and EDX analyses were conducted to assess the morphology and purity of the $CaCO_3$ precipitated after 7 days of cultivation. As shown in **Figure 4**, the SEM image clearly displays cubical and tabular-shaped particles, indicating that the crystals were produced through biomineralization by Bacillus sp. 8WNM. The EDX analysis remarked that the primary elements in the biominerals were Ca (15.38±1.34%), C (23.21±1.08%), and O (61.41±3.84%). The elemental ratios closely match those of pure $CaCO_3$, confirming that the precipitated crystals are indeed $CaCO_3$.

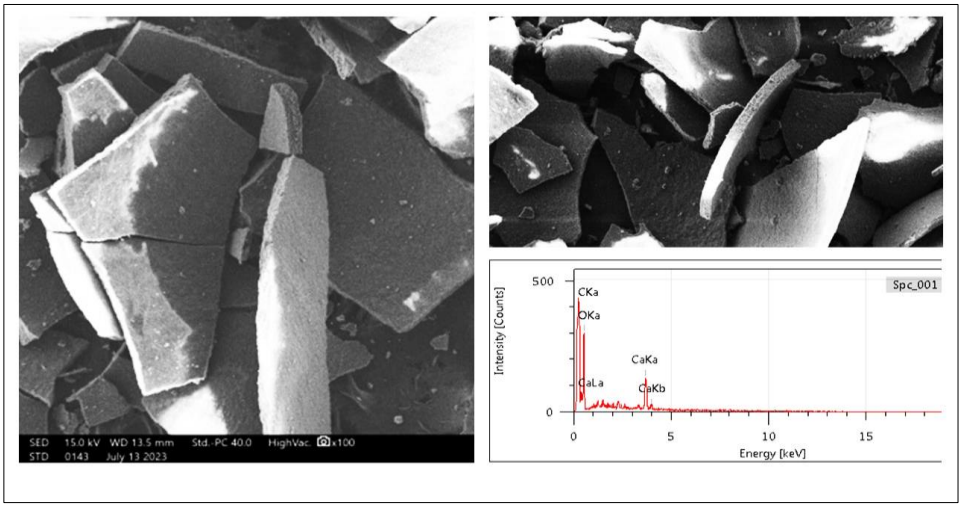

Figure 4: SEM and EDX analyses of the precipitated $CaCO_3$ produced through the biomineralization activity of Bacillus sp. 8WNM.

4. Discussion

The construction industry's heavy reliance on cement is a growing environmental concern, as its production released approximately 2.7 billion tons of CO_2 in 2021 alone, contributing significantly to global pollution. Without addressing this issue, the environmental impact will only escalate [24, 25, 26]. Researchers are increasingly focusing on sustainable alternatives, such as microbe-based materials like biocement, bioblock, and bioconcrete. These materials offer not only a reduction in environmental impact but also superior performance [27, 28]. For example, bacterial concrete enhances compressive strength by 35.15%, tensile strength by 24.32%, and flexural strength by 17.24% compared to conventional concrete, while also significantly lowering water absorption and increasing acid resistance [5]. These advancements highlight the potential for microbial solutions to mitigate the environmental problems associated with traditional cement use, paving the way for more sustainable construction practices.

Microbially induced calcium carbonate precipitation (MICP) has emerged as a result of recent research on the production of biocement by microorganisms in vitro. At moderate temperatures, MICP facilitates the creation of durable and sustainable biocement. In the present work, we have examined the synthesis of biocement by a promising bacterial isolate, *Bacillus sp*. 8WNM.

Numerous microorganisms that produce biocement can be isolated from a variety of environments, including freshwater, marine, and soil [29]. For instance, biocement was made using the Gram-positive *Sporosarcina pasteurii* that was isolated from a marine habitat in Korea. It was discovered that *S. pasteurii* could grow in environments with high calcium concentrations and pH values greater than 8.5 [30]. Furthermore, bacteria isolated from soil have been employed for MICP because they are non-pathogenic, thrive at an ideal pH of 9.0, and can withstand harsh environments [31, 32].

The most often employed strains in MICP are those belonging to the *Bacillus* group. For instance, B. megaterium has been utilized to increase concrete hardness and durability of building material [35], while *S. pasteurii* was employed in concrete remediation, heavy metal contamination and soil enhancement [33, 34]. In extreme environments, *Bacillus sp*. has been shown to stay dormant for up to 200 years [36]. These latent bacterial spores readily absorb moisture from the air and cause the cell to germinate, develop, and form calcite, sealing the gaps in the place. When cracks appear in concrete, the capsules break open, releasing the healing agents [37, 38]. The pH of the surrounding environment rises from 6.5 to 13 as a result of the dissolution of calcium hydroxide that was produced from concrete with accessible moisture [39, 40]. Next, the bacteria that are present get ready to survive in the alkaline environment.

Since it doesn't have any characteristics that could hurt humans or cause illness, the bacterium *Bacillus sp* is regarded as a harmLess organism [41]. Furthermore, according to Galano et al. [6], it can survive at a pH of 14. Bacillus sp was proved in various studies to be involved in self-healing concrete applications [9, 42, 43]. In the current

study, while *Bacillus sp*. 8WNM was able to grow in the presence of all calcium sources examined in this work, calcium acetate produced the highest growth and pH, and this was in consistent with previous studies; for example, *Lysinibacillus sp.* strain YL, grown on calcium acetate [$Ca(C_2H_3O_2)_2$], precipitated up to 8 gL^{-1} of calcium carbonate ($CaCO_3$) in 7 days, surpassing results with calcium chloride ($CaCl_2$) and calcium nitrate [$Ca(NO_3)_2$] [44]. While, in the case of *Bacillus* sp., the highest precipitate production was approximately 0.9 gL^{-1} using $CaCl_2$ as a calcium source [45]. Consequently, this can be explained by the possibility that calcium acetate [$Ca(C_2H_3O_2)_2$] serves as an additional carbon source, alongside the dextrose and amino acids in B4 medium, while also providing calcium for the precipitation process.

It is noticeable that the pH of media started with a value of 7, and once increasing the counts of viable cells, the pH value increased, inducing an alkaline environment supporting more $CaCO_3$ precipitates, especially in using calcium acetate. A similar pattern was observed in *B. licheniformis* and *Lysinibacillus sp*., where $CaCO_3$ precipitation occurred following an increase in pH, rather than at the beginning of cultivation [44, 46]. This can be explained by the need for cells to neutralize acetic acid ions from calcium acetate by absorbing one proton from each water molecule. Consequently, the naturally low buffered B4 medium became more alkaline, favouring more precipitation of $CaCO_3$. Consequently, calcium acetate [$Ca(C_2H_3O_2)_2$] was the most effective calcium source for $CaCO_3$ production [46].

Optimizing calcium carbonate production involves determining the ideal cellular concentration, incubation time, and calcium acetate levels. High bacterial cell counts (10^6-10^8) have been shown to enhance calcium carbonate precipitation through MICP. Studies with *S. pasteurii* ATCC 11859 confirmed that calcite precipitation efficiency decreases at high calcium concentrations (above 0.5 M) but improves at lower concentrations (0.05 to 0.25 M). Similarly, another study with Proteus vulgaris demonstrated a 100% increase in calcite precipitation at 250 mM calcium concentration [47]. In our study, we found that the optimal conditions for $CaCO_3$ precipitation were achieved with a calcium source concentration of 2.5 g/L, an inoculum size of 1.5 mL, and an incubation period of 8 days. When the calcium concentrations ranged from 0.01 to 0.0625 M, the calcite precipitation efficiency was increased. However, when calcium concentration exceeds 0.0625 M, the calcite precipitation efficiency was reduced.

XRD analysis of the precipitated $CaCO_3$ indicated a single structure corresponding to calcite, consistent with findings from various studies [44, 49]. Similarly, FTIR analysis confirmed the presence of $CaCO_3$, with absorption peaks matching those reported in other research [50]. Broad bands were detected at approximately 3422 cm^{-1} and 1655 cm^{-1}, corresponding to O–H stretching and bending, respectively. The strong band at 1448 cm^{-1} and the one at 873 cm^{-1} were linked to the asymmetric C–O stretching and C–O bending vibrations of calcite [52]. Furthermore, SEM and EDX analyses confirmed the cubical shape and purity of the precipitated $CaCO_3$, aligning

with findings from other studies [44, 53, 54].

5. Conclusion

This study successfully demonstrated the potential of *Bacillus sp*. 8WNM in the biomineralization of calcium carbonate ($CaCO_3$), particularly as a sustainable alternative to traditional cement in construction materials. The isolate showed a strong ability to precipitate $CaCO_3$, especially when calcium acetate was utilized as the calcium source, yielding the highest amount of precipitate under optimized conditions. The $CaCO_3$ produced was confirmed to have a single-phase calcite structure, with the morphological and chemical analyses revealing its high purity and cubical shape. These findings highlight the possibility of using *Bacillus sp*. 8WNM in the production of bio-cement, potentially reducing the environmental impact associated with cement production and usage. It is suggested that further research and development could lead to practical applications in sustainable construction practices.

6. References

1. M. Dougoud. Mycelium infrastructures for impermanent futures: revitalization of an industrial site through the manufacturing and research of mycelium-based biocomposite materials. 2018.
2. C. Lee, H. Lee, O.B. Kim. Biocement fabrication and design application for a sustainable urban area. Sustainability;10(11) :4079-82. 2018.
3. M.J. Castro-Alonso, et al. Microbially induced calcium carbonate precipitation (MICP) and its potential in bioconcrete: microbiological and molecular concepts. Front Mater;6:126. 2019.
4. X. Zhao, R. Webber, P. Kalutara, W. Browne, J. Pienaar. Construction and demolition waste management in Australia: a mini-review. Waste Manag Res;40. 2021.
5. D. Alemu, W. Demiss, G. Korsa. Bacterial performance in crack healing and its role in creating sustainable construction. Int J Microbiol;2022(1):6907314. 2022.
6. P. Ghodke, S. Mote. The self-healing concrete—a review. Int J Adv Eng Technol;11(1):29-34. 2018.
7. V. Achal, A. Mukherjee. A review of microbial precipitation for sustainable construction. Constr Build Mater;93:1224-35. 2015.
8. C. Jin, R. Yu, Z. Shui. Fungi: a neglected candidate for the application of self-healing concrete. Front Built Environ;4(62). 2018.
9. H. Italia, I. Patel, J. Shah. Experimental study of bacterial self-healing effect on concrete: a review. J Civil Eng Environ Technol;3(1):78-81. 2016.
10. J. Bashir, I. Kathwari, A. Tiwary, K. Singh. Bio concrete—the self-healing concrete. Indian J Sci Technol;9(1):1-5. 2016.
11. S. Joshi, S. Goyal, A. Mukherjee, M.S. Reddy. Microbial healing of cracks in concrete: a review. J Ind Microbiol Biotechnol;44(11):1 511-25. 2017.
12. R. Rautela, S. Rawat. Analysis and optimization of process parameters for in vitro biomineralization of $CaCO_3$ by *Klebsiella pneumoniae*, isolated from a stalactite from the Sahastradhara cave. RSC Adv;10(14):8470-9. 2020.

13. S. Luhar, I. Luhar, F.U.A. Shaikh. A review on the performance evaluation of autonomous self-healing bacterial concrete: mechanisms, strength, durability, and microstructural properties. J Compos Sci;6(1). 2022.
14. M. Kahani, F. Kalantary, M.R. Soudi, L. Pakdel, S. Aghaalizadeh. Optimization of cost-effective culture medium for *Sporosarcina pasteurii* as biocementing agent using response surface methodology: upcycling dairy waste and seawater. J Clean Prod;253:120022. 2020.
15. N. Kumarappan, P.H.C. Sudharsan. A comparative analysis of bacteria-made bio brick and conventional brick. Int J ChemTech Res;11(2):132-7. 2018.
16. A.P. Borse, A. Kannuzia, M.T. Student. Experimental study of microbial concrete. Int J Tech Innov Mod Eng Sci;3(5):49-54. 2017.
17. H.M. Jonkers, E. Schlangen. A two-component bacteria-based self-healing concrete. In CRC Press, Boca Raton, FL, USA. 2009.
18. D.H. Tambekar, V.R. Dhundale. Studies on the physiological and cultural diversity of bacilli characterized from Lonar lake (MS) India. Biosci Discovery;3(1):34-9. 2012.
19. W. Gunkel, G.E. Jones, C.E. ZoBell. Influence of volume of nutrient agar medium on the development of colonies of marine bacteria. Helgoländer wiss Meeresunters;8:85-93. 1961.
20. E. Boquet, A. Boronat, A. Ramos-Cormenzana. Production of calcite (calcium carbonate) crystals by soil bacteria is a general phenomenon. Nature;246(5434):527-9. 1973.
21. B.B. Biswas, P.S. Basu, M.K. Pal. Gram staining and its molecular mechanism. Int Rev Cytol;29:1-27. 1970.
22. C. Ankolekar, T. Rahmati, R.G. Labbé. Detection of toxigenic Bacillus cereus and *Bacillus thuringiensis* spores in US rice. Int J Food Microbiol;128(3):460-6. 2009.
23. P. Siroha, D. Singh, R. Soni, J. Gangwar. Comparative study on crystallographic representation of transition metal oxides polymorphs nanomaterials using VESTA software: case study on Fe_2O_3 and TiO_2. In AIP Conf Proc;2006(1). AIP Publishing. 2018.
24. M.Z. Khaiyum, S. Sarker, G. Kabir. Evaluation of carbon emission factors in the cement industry: an emerging economy context. Sustainability;15(21):15407. 2023.
25. N. Mohamad, K. Muthusamy, R. Embong, A. Kusbiantoro, M.H. Hashim. Environmental impact of cement production and solutions: a review. Mater Today Proc;48:741-6. 2022.
26. M. Soomro, V.W. Tam, A.C.J. Evangelista. Production of cement and its environmental impact. In Recycled Concrete; pp. 11-46. Woodhead Publishing. 2023.
27. P.V. Premalatha, M. Geethanjali, S. Sundararaman, C.S. Murali. An experimental investigation on self-healing concrete using "*Bacillus subtilis*." Mater Today Proc. 2023.
28. Y. Dorfan, Y. Morris, B. Shohat, I. Kolodkin-Gal. Sustainable construction: toward growing biocement with synthetic biology. Res Directions: Biotechnol Design;1. 2023.
29. G. Daniella, R. Zeev, T. Michael. Soil bacteria population dynamics following stimulation for ureolytic microbial-induced $CaCO_3$ precipitation. 2016.

30. A.J. Phillips, A.B. Cunningham, R. Gerlach, R. Hiebert, C. Hwang, B.P. Lomans, et al. Fracture sealing with microbially-induced calcium carbonate precipitation: a field study. Environ Sci Technol;50(7):4111-7. 2016.
31. S.S. Bang, J.K. Galinat, V. Ramakrishnan. Calcite precipitation induced by polyurethane-immobilized *Bacillus pasteurii*. Enzyme Microb Technol;28(4-5):404-9. 2001.
32. H. Li, Y. Song, Q. Li, J. He, Y. Song. Effective microbial calcite precipitation by a new mutant and precipitating regulation of extracellular urease. Bioresour Technol;167:269-75. 2014.
33. M. Li, X. Cheng, H. Guo. Heavy metal removal by biomineralization of urease producing bacteria isolated from soil. Int Biodeterior Biodegrad;76:81-5. 2013.
34. A.J. Phillips, R. Gerlach, E. Lauchnor, A.C. Mitchell, A.B. Cunningham, L. Spangler. Engineered applications of ureolytic biomineralization: a review. Biofouling;29(6):715-33. 2013.
35. N.K. Dhami, M.S. Reddy, A. Mukherjee. Synergistic role of bacterial urease and carbonic anhydrase in carbonate mineralization. Appl Biochem Biotechnol;172:2552-61. 2014.
36. S. Morsali, G. Yucel Isildar, Z. Hamed Zar gari, A. Tahni. The application of bacteria as a main factor in self-healing concrete technology. J Building Pathol Rehabil;4:1-6. 2019.
37. S. Mondal, A. Ghosh. Microbial concrete as a sustainable option for infrastructural development in emerging economies. In ASCE India Conference 2017; pp. 413-423. Reston, VA: American Society of Civil Engineers. 2017.
38. A. Magil, D.C. Noriesta, A.M. Pamela, R.P. Alviaradviser. Exploratory research using bacteria (Bacillus subtilis) as a self-healing concrete: a basis for strengthening infrastructure in the Philippine setting. Int J Recent Technol Eng;8(1):125-129. 2019.
39. J. Luo, X. Chen, J. Crump, H. Zhou, D.G. Davies, G. Zhou, ... C. Jin. Interactions of fungi with concrete: significant importance for bio-based self-healing concrete. Constr Build Mater;164:275-285. 2018.
40. R.R. Menon, J. Luo, X. Chen, H. Zhou, Z. Liu, G. Zhou, ... C. Jin. Screening of fungi for potential application of self-healing concrete. Sci Rep;9(1):2075. 2019.
41. T. Pérez-Sánchez, I. Ruiz-Zarzuela, I. Blas, J.L. Balcázar. Probiotics in aquaculture: a current assessment. Rev Aquac;6(3):133–146. 2014.
42. M. Galano, M.W. van den Dungen, T. van Rij, H.E. Abbas. Safety evaluation of food enzymes produced by a safe strain lineage of *Bacillus subtilis*. Regul Toxicol Pharmacol;126:105030. 2021.
43. Y. Tie, Y. Ji, H. Zhang, B. Jing, X. Zeng, P. Yang. Investigation on the mechanical properties of *Bacillus subtilis* self-healing concrete. Heliyon;10(14). 2024.
44. J. Ekprasert, T. Pongtharangkul, P. Chainakun, I. Fongkaew, K. Khanthasombat, R. Kamngam, ... S. Boonlue. Kinetic model of a newly-isolated *Lysinibacillus* sp. strain YL and elastic properties of its biogenic CaCO3 towards biocement application. Biotechnol J;17(1):2100124. 2022.

45. S. Wei, H. Cui, Z. Jiang, H. Liu, H. He, N. Fang. Biomineralization processes of calcite induced by bacteria isolated from marine sediments. Braz J Microbiol;46:455-464. 2015.
46. D.A. Golovkina, E.V. Zhurishkina, L.A. Ivanova, A.E. Baranchikov, A.Y. Sokolov, K.S. Bobrov, ... A.A. Kulminskaya. Calcifying bacteria flexibility in induction of CaCO3 mineralization. Life;10(12):317. 2020.
47. G.D. Okwadha, J. Li. Optimum conditions for microbial carbonate precipitation. hemosphere;81(9):1143-1148. 2010.
48. S.P. Chaparro-Acuña, M.L. Becerra-Jiménez, J.J. Martínez-Zambrano, H.A. Rojas-Sarmiento. Soil bacteria that precipitate calcium carbonate: mechanism and applications of the process. Acta Agronómica; 67(2):277-288. 2018.
49. E.H. Zaghloul, H.A. Ibrahim, D.E.S. El-Badan. Production of biocement with marine bacteria; *Staphylococcus epidermidis* EDH to enhance clay water retention capacity. Egypt J Aquatic Res;47(1):53-59. 2021.
50. X. Zhou, W. Liu, J. Zhang, et al. Biogenic calcium carbonate with hierarchical organic−inorganic composite structure enhancing the removal of Pb(II) from wastewater. 2017.
51. S. Kang, Y. Zhao, W. Wang, et al. Removal of methylene blue from water with montmorillonite nanosheets/chitosan hydrogels as adsorbent. Appl Surf Sci;448:203–211. 2018.
52. M. De Beer, F.J. Doucet, J.P. Maree, L. Liebenberg. Synthesis of high-purity precipitated calcium carbonate during the process of recovery of elemental sulphur from gypsum waste. Waste Manag;46:619–627. 2015.
53. M. Seifan, A. Khajeh Samani, S. Hewitt, A. Berenjian. The effect of cell immobilization by calcium alginate on bacterially induced calcium carbonate precipitation. Fermentation;3(4):57. 2017.
54. R. Rautela, S. Rawat. Analysis and optimization of process parameters for in vitro biomineralization of $CaCO_3$ by *Klebsiella pneumoniae*, isolated from a stalactite from the Sahastradhara cave. RSC Adv;10(14):8470-8479. 2020.