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Research Article

Assessing Indigenous Ureolytic Bacteria Isolated from Gua Damai Limestone for Microbially Induced Calcite Precipitation (MICP)

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ABSTRACT

Low porosity and non-aggregated soil are significant global concerns, presenting substantial environmental hazards. This study determined the capacity of native ureolytic bacteria found in limestone to stabilise soil through the process of microbially induced calcite precipitation (MICP). Six pure bacterial isolates obtained from limestone in Gua Damai, Batu Caves, Selangor were qualitatively assessed for urease production. The isolate S4C4, identified as *Bacillus tropicus* strain NTF4, demonstrated the highest urease activity at 821.654 U mL^{-1}. This isolate precipitated 37.15 ± 9 mg mL^{-1} of $CaCO_3$ after 96 hours of incubation and XRD analysis confirmed the biocementation of organic soils treated by *B. tropicus* strain NTF4, primarily forming calcite and vaterites. Significant calcite polymorph presence in soil samples is attributed to a longer treatment duration which promotes crystal development and stability. Harnessing indigenous limestone ureolytic bacteria with high urease activity presents a promising avenue for green soil bio-stabilisation. This approach potentially unlocks sustainable and scalable applications of microbial-induced calcite precipitation (MICP) in large-scale geo-engineering projects.

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INTRODUCTION

Surface erosion, fluvial erosion, erosion along riverbanks, and mass movement erosion, which is essentially the force of gravity moving a loose combination of soil and rock particles downslope, are examples of the geological processes that cause excessive soil removal and soil deterioration (Yusof et al. 2022). Soil erosion occurs under typical conditions that have existed for millions of years, leading to the creation of new soils (Ahmad et al. 2020). Heavy rains, erosive conditions, poor land management techniques, and construction methods are frequently the causes of landslides (Paramananthan et al. 2021), with excessive grazing, deforestation, and unsuitable farming methods the main causes of increased soil erosion (Retallack 2021). The application of geotechnical techniques, such as chemical grouting like polyurethane, lignosulfonates, and acrylamides, to control or reduce the risk of soil structure collapse is not only expensive but also poses a hazard to human health and the environment (Van Paassen et al. 2010) and thus, many countries have banned their use. Consequently, attention has focused on the search for more passive and environmentally friendly geotechnical techniques for soil structure based on common soil microbes found in soil (DeJong et al. 2015).

The biomineralisation process, also known as microbially induced calcium carbonate precipitation (MICP), is an environmentally friendly technique that has emerged in the fields of geotechnical and civil engineering. It serves as a viable and sustainable alternative for ground improvement, surpassing existing remediation technologies in enhancing the soil's mechanical properties (Naveed et al. 2020). MICP occurs naturally as a byproduct of microbial metabolic activity via a variety of mechanisms including urea hydrolysis, photosynthesis, sulphate and nitrate reduction, and other biochemical processes facilitated by microbes that raise the carbonate saturation state (Leeprasert et al. 2022). The most extensively used method is calcium carbonate ($CaCO_3$) precipitation by urease-producing bacteria through urea hydrolysis (Castro-Alonso et al. 2019; Naveed et al. 2020). $CaCO_3$ formation between the soil particles can help cement the gaps leading to improved geochemical characteristics, particularly the friction angle, cohesion, bending strength, and stiffness (Jiang et al. 2019).

Numerous microorganisms demonstrate urease activity such as *Sporosarcina pasteurii*, a non-pathogenic, endospore-producing bacterium, which thrives in harsh conditions with a basic pH of 9.0 (Chen et al. 2022). However, it is crucial to isolate and study other potential urease-producing bacteria, such as the genera *Bacillus*, *Geobacillus*, *Lysinibacillus*, *Pararhodobactor*, and *Psychrobacillus*, to produce bioresources suitable for a variety of climates and conditions (Yoshida et al. 2010; Ghosh et al. 2019). Suitable bacteria are typically present in challenging environments, especially those with high alkalinity, nutritional scarcity, and intense shearing forces. MICP is only possible if the bacteria survive (Leeprasert et al. 2022) and depends on pH, the amount of dissolved inorganic carbon and calcium, as well as the presence of nucleation sites (Erdmann & Strieth 2022) essential for consistent and continuous calcium carbonate production. Significant calcium precipitation must support the expression of high ureolytic activity by these bacteria for MICP applications (Erdmann & Strieth 2022).

The current study found that cave-dwelling bacteria are prospective sources of urease with the ability to induce calcite crystals (Komala & Khun 2013; Soon et al. 2014). Only native ureolytic bacteria found in limestone caves can produce energy by fixing carbon dioxide into organic compounds (Tomczyk-Żak & Zielenkiewicz 2015), contributing to their growth and survival. Limestone comprises about 50 % carbonate minerals (Fairbridge et al. 1967), predominantly consisting of calcite and aragonite. Thus, a significant diversity of ureolytic bacteria capable of performing MICP is expected in the

limestone, as well as the potential to isolate novel ureolytic bacteria. However, there are few published studies on the exploitation of microbial diversity in these regions, therefore this study evaluated the MICP activity of indigenous ureolytic bacteria isolated from limestone.

MATERIALS AND METHODS

Isolation of ureolytic bacteria from limestone

Limestone samples were collected from Gua Damai, Batu Caves, Selangor (3° 14'52"N 101°41'14"E), at a depth of 5–10 cm using a mechanical drill, chisels, and mallet. All samples were crushed using a mortar and pestle before being mixed with sterile distilled water. The samples were preserved before completing the appropriate bacterial isolation (Omoregie et al. 2018) using calcium carbonate precipitation (CCP) agar. Cultures were incubated at 28 °C ± 0.5 °C for 7 days before being transferred to CCP Minimal Medium Agar and incubated at 28 °C ± 0.5 °C for 24 hours (Aliyu et al. 2023a). The colonies were screened for urease and preserved as a stock culture. The long-term storage of pure ureolytic bacterial colonies was performed according to the modified methods adapted from Bibi et al. (2018).

Urease activity

The pure isolates were streaked and incubated on Christensen's urea agar (pH 6.8) at 28 ± 0.5 °C for 120 hours and observed every 6 hours (Burbank et al. 2012). The change in colour from orange to pink determined a pH increase indicating urease production. To target potential high-urease producers, only isolates that changed colour within 48 hours were selected for further analysis (Wei et al. 2015). The selected isolates were incubated in urea broth (pH 6.8) and shaken at 180 rpm for 120 hours at 28 ± 0.5 °C (Aliyu et al. 2023a). Urease activity was determined by measuring the amount of ammonia released from urea using the phenol hypochlorite assay method described by Kim and Youn (2016).

Calcium carbonate production

Calcium carbonate production was determined by the acid test whereby 10 % HCl was dropped onto the precipitated $CaCO_3$ and the bubble of carbon dioxide gas released signals the presence of carbonate minerals. modified method adapted from Wei et al. (2015) and described by Aliyu et al. (2023a) was used to assess the calcium carbonate production by the selected isolates.

Isolate identification

Molecular identification of the selected isolates was based on 16S ribosomal RNA sequencing.

Molecular identification of the selected isolates was based on 16S ribosomal RNA sequencing. The entire 16S ribosomal RNA gene was amplified from bacterial DNA using universal primers universal primers 27F (5' - AGAGTTTGATCCTGGCTCAG- 3') and 1492R (5' - GGTTACCTTGTTACGACTT- 3') (Wu et al. 2014). The PCR cycling conditions were set with an initial denaturation at 94 °C for 2 minutes, followed by 25 cycles of denaturation at 98 °C for 10 seconds, annealing at 53 °C for 30 seconds, and extension at 68 °C for 1 minute. The forward and reverse sequencing outcomes are merged and refined to create a comprehensive full-length sequence. The nucleotide Basic Local Alignment Search Tool (BLAST) sequence from the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/nuccore/NR_115714.1; https://www.ncbi.nlm.nih.gov/nuccore/NR_157734.1) was utilised to compare the genes for identification. Neighbour-joining trees were created using the Kimura 2-parameter model using MEGA version 7.0 with bootstraps of 1000

replicates after repeated alignment of genes with their closed taxa using the CLUSTAL W method (Kumar et al. 2016).

MICP in soil

The bioremediation ability of the indigenous bacteria from limestone was assessed using the modified method of Bibi et al. (2018). Approximately 50 mL of urea medium containing 0.4 g of sterilised farm soil, 20 g L^{-1} of urea, and 3.7 g L^{-1} of $CaCl_2.2H_2O$ were used to inoculate ureolytic bacteria isolates with an initial bacteria concentration of 0.1 at an optical density of 600 nm. The control treatment contained only 0.4 g of sterilised farm soil (black organic soil with a silky texture; pH 4.1 to 4.5) and 3.7 g L^{-1} of $CaCl_2.2H_2O$ in 50 mL of urea media. Both mixtures were inoculated at 28 ± 0.5 °C and consistently shaken at 120 rpm for 30 days. The soil samples were then collected, washed three times with distilled water, and the pellets were oven-dried at 40 ± 0.50 °C for 48 hours. All samples were crushed into evenly distributed particles for X-ray diffraction (XRD) analysis.

X-ray diffraction analysis

The dried treated soil samples were subjected to XRD analysis to confirm calcium carbonate deposition (Dhami et al. 2016) using a Shimadzu 6000 diffractometer to elucidate the crystalline phase composition and differentiate between amorphous and crystalline forms of $CaCO_3$ (calcite, vaterite, or aragonite). The Cu Kα radiation and a Panalytical Empyrean reflectometer were used at an ambient temperature of 28 °C. The exploration range (2θ) was modified at a speed of 2.00 (deg min^{-1}) from 20° to 80° with the Cu anode set at 0.0530°, 30 kV, and 30 mA as the step sizes, voltages, and currents, respectively.

RESULTS AND DISCUSSION

Six ureolytic bacterial isolates were isolated from limestone samples (pH 8.69 to 8.92) (Table 1). Most MICP studies reported that ureolytic bacteria were found in slightly basic environments (Navneet et al. 2011) because they need them to break down urea and make ammonium for the MICP process (Prah et al. 2011). Urea (20 g L^{-1}) was added to the CCP media to prepare a selective medium that only supports the growth of ureolytic bacteria that can tolerate higher urea concentrations (Wei et al. 2015). This modification also produced a distinct and unique odour resembling ammonia gas from the culture plates, therefore, the bacterial activity is responsible for the urea decomposition.

Three isolates changed the agar colour to pink, with isolate S1C5 changing the agar colour in 18 hours, followed by isolates S4C4 and S5C3 (Table 1), indicating the presence of ureolytic bacteria. When urea is hydrolysed by urease, it releases ammonia (NH_3) and carbonic acid (H_2CO_3) which generate hydroxide ions, leading to a pH increase (Mekonnen et al. 2021). Soon et al. (2014) found that ureolytic bacteria thrive within a pH range of 7.5 to 9.5, which aligns with the optimal conditions for MICP. However, ensuring optimal pH conditions is challenging because the solubility of calcium ions at higher pH levels decreases significantly, limiting the bacteria's ability to acquire and utilise these ions (Intarasoontron et al. 2021). The ureolytic bacteria use glucose and peptone in Christensen's urea agar to grow quickly (Dortey et al. 2020). However, only isolates SIC5 and S4 C4 were subjected to further evaluation due to their ability to produce a colour change within less than 48 hours, indicating their high adaptability and potential to establish independent populations (Aliyu et al. 2023a).

Long-term observation revealed that S1C5 and S4C4 achieved the greatest urease activity after 96 hours (Figure 1) and the urease activity of S4C4 exceeds 500 U mL^{-1}, which is comparable to the activity observed in

isolates obtained from cement (Achal et al. 2010) and limestone (Kshetri & Ningthoujam 2016). The exponential rise in urease activity after 24 hours correlated to bacterial population expansion and increased urease enzyme synthesis (Omoregie et al. 2019). Despite a decrease in urease activity in both isolates after 120 hours, possibly due to factors such as insufficient nutrition, metabolic inhibition, cell death, and enzyme breakdown (Jiang et al. 2016), isolate S4C4 maintained urease activity of at least 2.8 % after 96 hours, thus, was selected for further analysis.

Table 1. Duration for urea agar's colour change for urease activity indication.

Isolates	pH of limestone sample	Hours
S5C1	8.69	Colour unchanged
S5C2	8.69	Colour unchanged
S5C3	8.69	72
S4C4	8.92	24
S1C5	8.78	18
S1C6	8.78	Colour unchanged

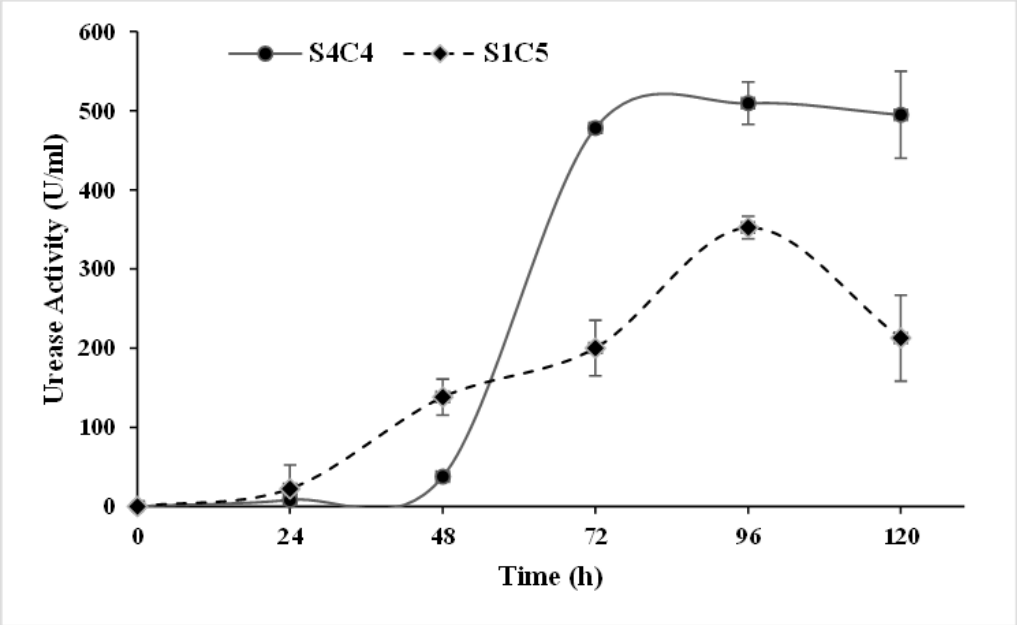
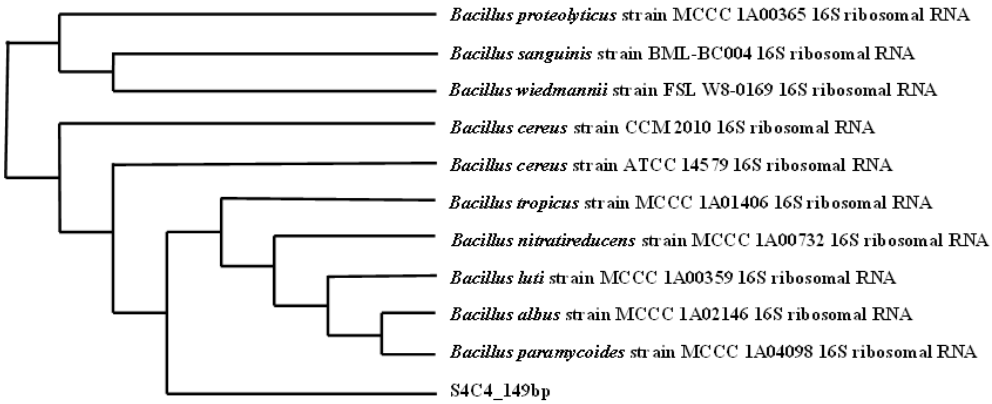


Figure 1. Urease activity of S1C5 and S4C4 in Christensen's urea broth media based on the absorbance of phenol hypochlorite assay at OD626nm.

Phylogenetic research utilising neighbour joining (unrooted trees) indicated that S4C4 was affiliated with the genus Bacillus, exhibiting 99.9 % similarity to *B. topicus* strain MCCC 1A01406. Thus, isolate S4C4 was known onwards as *Bacillus tropicus* strain NTF4 (Figure 2), a Gram-positive bacterium that produces urease and can generate endospores under conditions of low nutrition or temperature (Thakur et al. 2021; Shen et al. 2022). Previous studies have identified *B. tropicus* as having urease activity in limestone, which is commonly observed in cave materials (Elmanama & Alhour 2013; Omoregie et al. 2019). *Bacillus* sp. possesses cell walls with many layers that are structurally adapted to endure abiotic stress and their urease production can be induced, constitutive, or repressed (Ibarra-Villarreal et al. 2021). Several Bacillus species such as *B. licheniformis*, *B. sphaericus*, *B. megatarium*, *B. thuringiensis*, *B. cereus*, *B. paramycoides*. and *B. pseudomycoides* produce urease and cause calcite precipitation (Helmi et al. 2016; Moravej et al. 2018; Mukherjee et al. 2019; Algaifi et al. 2020; Aliyu et al. 2023b).

Bacillus tropicus strain NTF4 produced up to 37.15 mg mL^{-1} of precipi-



(a)

1 caggatgaac gctggcggcg tgcctaatac atgcaagtcg agcgaatgga ttaagagctt gctcttatga agttagcggc ggacgggtga
91 gtaacacgtg ttaagagctt gctcttatga agttagcggc ggacgggtga gtaacacgtg ggtaacctgc ccataagact gggataactc
181 cgggaaaccg gggggtctaa taccggataa cattttgaac cgcatggttc gaaattgaaa ggcggcttcg gctgtcactt atggatggac
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991 tggtttaatt cgaagcaacg cgaagaacct taccaggtct tgacatcctc tgacaaccc tagagataggg cttctccttc gggagcagag
1081 tgacaggtgg tgcatggttg tcgtcagctc gtgtcgtgag atgttgggtt aagtcccgca acgagcgcaa cccttgatct tagttgccat
1171 cattaagttg ggcactctaa ggtgactgcc ggtgacaaac cggaggaaggt ggggatgac gtcaaatcat catgcccctt atgacctggg
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1441 ccgtcacacc acgagagttt gtaacacccg aagtcggtgg ggtaaccttt ttggagccag ccgcctaagg tgggacagat gattggggtg
1531 aagtcgt

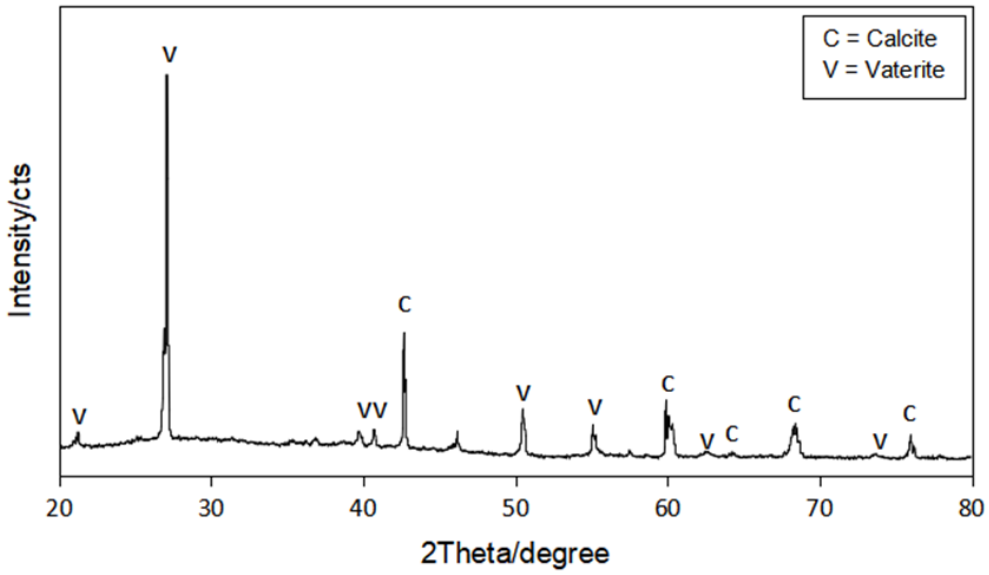
(b)

Figure 2. (a) Phylogenetic Tree – Neighbour Joining (Unrooted Tree) by NCBI Blast Tree Method, as compared to known species for isolate S4C4; (b) S4C4 16S ribosomal RNA, partial sequence.

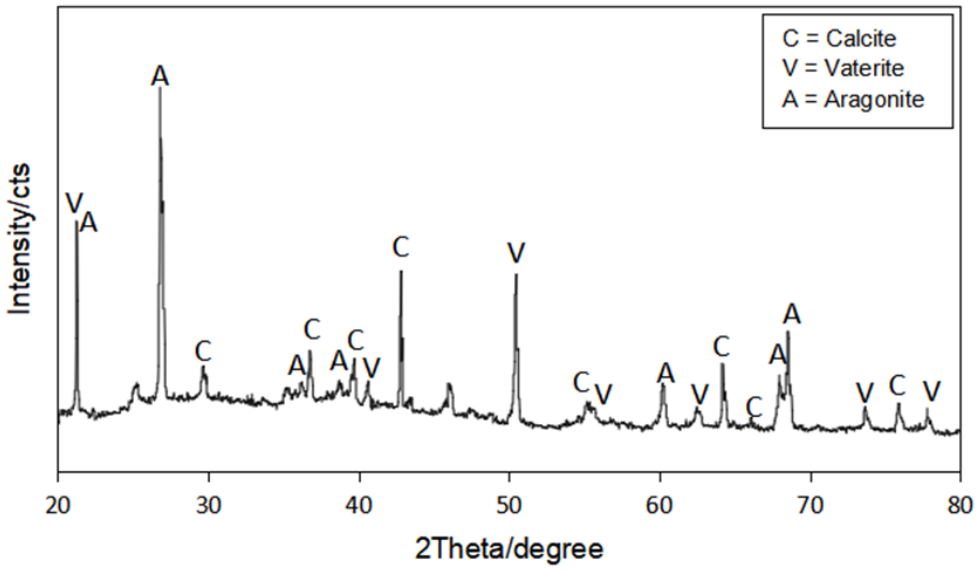
tates containing $CaCO_3$ (Richardson et al. 2014), confirming its capability for MICP. There have been extensive reports that bacterial isolates possess the potential to produce calcite precipitates (Al Qabany et al. 2011) and significantly influence the $CaCO_3$ crystal size, morphology, form, and biochemistry making the selection of bacteria critical for successful solidification (Seifan & Berenjian 2019). Despite *B. tropicus* strain NTF4 $CaCO_3$ precipitation being significantly lower than that of *B. lentus* and *B. diminuta* (Wei et al. 2015), extrinsic factors like media, physicochemicals, and reactant ions can mitigate this effect (Aliyu et al. 2023b). There are differences between species in the amount of $CaCO_3$ precipitation suggesting that there may be differences in how active urease is utilised. Urease catalyses urea hydrolysis liberating carbonate (CO_3^{2-}) ions, so higher urease activity may more CO_3^{2-} to accumulate in the culture medium, which in turn encourages more $CaCO_3$ to precipitate. A steady amount of urease activity in the microenvironment also helps make more dissolved CO_2 in the form of HCO^{3-} or CO_3^{2-} and ammonium ions, which raises the pH (Zaghloul et al. 2021).

The untreated soil sample's XRD 2 Theta diffraction angles reveal vaterite peaks at 21.29°, 27.13°, 40.74°, and 42.67°, along with distinct calcite peaks at 46.19°, 60.35°, and 68.28° (Figure 3), suggesting the formation of $CaCO_3$ crystals in line with Aliyu et al. (2023b) who reported that vaterite and calcite had likely been formed in the soil sample before the test. This is because farm soil is usually urea-rich and has a variety of in situ ureolytic bacteria. Also, sudden changes in temperature, salinity, or pressure can promote vaterite formation (Zhu & Dittrich 2016; Oral & Ercan 2018). According to Reeksting et al. (2020), decreased urease levels can affect the shape of the

crystals and the changes that happen during biomineralisation, and the lack of urea and urease activity could cause vaterite crystals to form with unique properties.



(a)



(b)

Figure 3. XRD spectra of (a) uninoculated soil sample and (b) *B. tropicus* inoculated soil sample after 30 days of incubation period.

All three CaCO3 crystalline polymorphs were precipitated with calcite becoming the main precipitate by *B. tropicus* strain NTF4. The XRD spectra confirmed that *B. tropicus* strain NTF4 precipitated more calcites than the control, with distinct peaks at 29.72°, 42.81°, 55.14°, 66.13°, 68.01°, and 75.93° and several vaterite and aragonite peaks were also detected (Figure 3b). *Bacillus tropicus* strain NTF4also precipitated aragonite, and over time, aragonite will gradually convert into calcite through recrystallisation (Kontoyannis & Vagenas 2000). Aragonite being less stable with a faster dissolution rate is more likely to transform into calcite than vaterite, lowering its concentration in the soil (Chen et al. 2022).

Reeksting et al. (2020) found a correlation between urease activity and the rapid precipitation of vaterite crystals which later transformed into calcite crystals. *Bacillus tropicus* with significant urease activity enhanced the for-

mation of kinetically preferred vaterite crystals (Kakelar et al. 2016). $CaCO_3$ precipitation began with the formation of metastable spherical vaterite crystals before transformational disintegration and dissolution formed calcite crystals (Al-Thawadi & Cord-Ruwisch 2012). Vaterite is rarely found in the environment despite its crucial role as a building block for calcite production (Mwandira et al. 2017). However, the degree of supersaturation in the environment significantly influences the rate of transformation from vaterite to calcite. According to Spanos and Koutsoukos (1998), the rate at which vaterite dissolves and calcite crystallises is similar when the supersaturation ratio is between 1.2 and 1.5. However, the pH greatly influences any changes in the supersaturation conditions (Oral & Ercan 2018).

Studies by Bang et al. (2010) and Achal et al. (2013) reported that calcite was the main form of biomineralisation, whereas Akyol et al. (2017) found that vaterite was the most common form of $CaCO_3$. The dominant calcium carbonate polymorphs in the ureolysis-driven MICP in this study were in line with most previous studies which also identified calcite and vaterite as the most frequently precipitated forms (Al-Thawadi & Cord-Ruwisch 2012; Algaifi et al. 2020). Calcite is generally considered better than vaterite in MICP because it is more stable than at low solution supersaturations (Rajasekar et al. 2021) and the presence of urease enzymes from *B. tropicus* strain NTF4 favours calcite formation over vaterite. Calcite also reigns supreme in MICP applications due to its superior thermodynamic stability (Chang et al. 2017) and ability to offer better cementation with improved mechanical properties (Xu et al. 2024). The interfacial energy during the transformation of amorphous calcium carbonate (ACC) to vaterite is lower than ACC to calcite, which promotes more vaterite formation compared to calcite. However, this alone is not sufficient to offset the advantages of calcite in terms of stability and crystallinity (Yi et al. 2021). Future comprehensive investigations of these factors should be conducted to optimise MICP. Nevertheless, the present study demonstrated dense calcium carbonate formation by *B. tropicus* strain NTF4 on and within soil grains, highlighting their potential as ureolytic bacterial agents for soil stabilisation via MICP.

CONCLUSION

Bacillus tropicus strain NTF4, an indigenous bacterium that produces urease, causes biocementation by causing calcium carbonate to develop and solidify in the soil. Most precipitation occurred within 48 hours and the precipitated $CaCO_3$ consisted predominantly of calcite and vaterite. This indigenous strain exhibited notable resistance to elevated urea levels and demonstrated alkaline tolerance showcasing its intriguing capabilities as an agent for biocementation.

AUTHORS CONTRIBUTION

All authors collaborated to carry out this work. AWAS and NTF performed the study, analysed the data and drafted the manuscript. RG and NAA carried out data validation and draft review. MM designed, oversaw the study, also reviewed the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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