The Effect of the Presence of Urea and Different Concentrations of HEPES Buffer on Microbial-Induced Calcium Precipitation (MICP) by *Bacillus subtilis.*

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Introduction

Over the past several decades, atmospheric carbon dioxide (CO_2) concentrations have risen significantly, driven mainly by large-scale anthropogenic emissions (Nunes, 2023), where cement production is one of the largest sources; about 8% of CO_2 emitted into the atmosphere is contributed by calcination, high temperature treatment of limestone to produce clinker, the primary ingredient in cement (Nunes, 2023). The production of concrete is expected to reach 5.5 billion tons by 2050 (Isaac et al., 2024).

To address this environmental challenge, innovative carbon capture and sequestration (CCS) technologies are being actively explored (Nunes, 2023). CCS is a climate mitigation technology that prevents CO_2 from being released into the atmosphere (Nunes, 2023). Microbially Induced Calcite Precipitation (MICP) is a CCS process that utilizes the metabolic activity of microorganisms to form solid calcium carbonate ($CaCO_3$), which sequesters atmospheric carbon into a mineral form (Cappa et al., 2025). Cement production leaves industrial waste that is rich in calcium compounds, containing calcium ion (Ca^{2+}), such as calcium nitrate ($Ca(NO_3)_2$) or calcium oxide (CaO), so MICP can offset a portion of the calcination emissions by sequestering atmospheric CO_2, reducing both waste and CO_2 release (Qian et al., 2022). The specific chemical form of the calcium source is not critical, since MICP fundamentally depends on the availability of Ca^{2+} ions (Qian et al., 2022). MICP ensures permanent sequestration, since CO_2 stored as $CaCO_3$ remains stable over geological timescales (Cappa et al., 2025). It also requires less energy than chemical CO_2 sequestration methods because microbial processes operate under ambient conditions, unlike the high temperature required for chemical methods (Qian et al., 2022).

Bacillus subtilis is a well-established model organism for studies of MICP (Hoffmann et al., 2021). It produces carbonic anhydrase (CA) in the presence of CO_2 and urease in the presence of urea. These enzymes enhance and supplement the MICP capability of *B. subtilis* (Qian et al., 2022 & Cappa et al., 2025). CA accelerates the hydration of CO_2, the crucial first step in mineralization (Qian et al., 2022; see Equation 1).

$$CO_2\,(g) \leftrightarrow CO_2\,(aq) + H_2O\,(l) \leftrightarrow H_2CO_3\,(aq) \qquad \text{Equation 1}$$

The alkaline environment developed by urease pushes the equilibrium from carbonic acid (H_2CO_3) to carbonate ions (CO_3^{2-}) (Equations 2 and 3).

$$H_2CO_3\,(aq) \leftrightarrow H^+\,(aq) + HCO_3^-\,(aq) \qquad \text{Equation 2}$$

$$HCO_3^-\,(aq) + OH^-\,(aq) \leftrightarrow CO_3^{2-}\,(aq) + H_2O\,(l) \qquad \text{Equation 3}$$

The byproducts of cement production can generate the primary cation Ca^{2+} required for CO_2 mineralization (Equation 4).

$$Ca(NO_3)_2\,(s) \rightarrow Ca^{2+}\,(aq) + 2\,NO_3^-\,(aq) \qquad \text{Equation 4}$$

By combining CO_3^{2-} and Ca^{2+}, $CaCO_3$ is produced, and atmospheric CO_2 is trapped in a stable solid form for a long period of time (Equation 5).

$$Ca^{2+}\,(aq) + CO_3^{2-}\,(aq) \leftrightarrow CaCO_3\,(s) \qquad \text{Equation 5}$$

This will contribute to lowering the concentration of CO_2 in the atmosphere. This study investigated the use of an industrial calcium byproduct as a sustainable, low-cost calcium source for biomineralization mediated by *B. subtilis* through media preparation, bacterial culture, and filtration. The project focused on how the presence of urea and different concentrations of HEPES buffer (0 mM, 50 mM, and 100 mM) affect the MICP efficiency of *B. subtilis.*

Methods

Media Preparation

Four sets of media were prepared: with urea, without urea, with urea & 50 mM HEPES buffer, and with urea & 100 mM HEPES buffer. Three replicates were tested for each condition. The medium was prepared in 500 mL Erlenmeyer flasks using the components and quantities listed in Tables 1 and 2. After gently swirling to fully dissolve all the compounds, all flasks were covered with aluminum foil and autoclaved at 121 °C for 20 minutes at 15 PSI.

Table 1. Components in Flasks 1 to 6. Flasks 1 to 3 contain urea (the first condition) and Flasks 4 to 6 do not (the second condition). Table created by Seoyoon Lee using Excel.

Flask #	1-3	4-6
Calcium nitrate (g)	2.1	2.1
LB broth (g)	3.8	3.8
Distilled water (mL)	150	150
Urea (g)	3.0	0

Table 2. Components in Flasks A to F. Flasks A to C contain urea with 50 mM HEPES buffer (the third condition), and Flasks D to F contain urea and 100 mM HEPES buffer (the fourth condition). Table created by Seoyoon Lee using Excel.

Flask #	A-C	D-F
Calcium nitrate (g)	2.1	2.1
LB broth (g)	3.8	3.8
Distilled water (mL)	150	150
Urea (g)	3.0	3.0
HEPES buffer (g)	1.8	3.6

Bacterial Culture

Bacterial culturing procedures were performed under sterile conditions in a biosafety cabinet. *B. subtilis* colonies were transferred from agar plates into 10 mL of autoclaved LB broth in sterile 15 mL tubes. The cultures were incubated in an incubator shaker at 37 °C and 120 rpm for approximately 24 hours or up to one week. Following incubation, bacterial growth was quantified by measuring the optical density at 600 nm (OD_{600}) using a 2802 UV/Vis spectrophotometer blanked with LB broth or Vernier SpectroVis Plus calibrated with water. Each culture was adjusted to achieve $OD_{600} \approx 0.9$. Subsequently, 1 mL of the adjusted culture was inoculated into each experimental flask containing 150 mL of sterile medium. Flasks were sealed with parafilm and incubated at approximately 25 °C and 60-75 rpm for two weeks. After one week of incubation, each flask was supplemented with 10 mL of sterile 10X LB broth (8g of nutrient broth in 100 mL of distilled water) to replenish nutrients.

Precipitate Filtration

After two weeks of culturing, every flask was autoclaved. Subsequently, the autoclaved solutions were centrifuged for approximately 1 minute. The supernatant was discarded, leaving a small volume of liquid in which to resuspend the pallet. The mixture was then poured into a Büchner funnel and filtered using qualitative or quantitative filter paper.

Afterwards, the filter papers were placed onto petri dishes and dried in an incubator oven at 100°C. Samples were dried for at least one day and then massed. The $CaCO_3$ mass was calculated as (filter paper + precipitate) – (filter paper). The $CaCO_3$ yield was normalized by dividing the measured $CaCO_3$ mass by the number of bacterial cells per milliliter in each flask.

Results

Table 3. Total $CaCO_3$ mass produced in each flask and $CaCO_3$ yield normalized per cell. Table created by Seoyoon Lee using Excel.

Flask #	$CaCO_3$ (g)	$CaCO_3$ per cell (g/cell)
1	0.2264	3.14E-10
2	0.0091	1.26E-11
3	0.0137	1.9E-11
4	0.2878	4E-10
5	0.169	2.35E-10
6	0.2352	3.27E-10
A	0.308	3.84E-10
B	0.6672	9.02E-10
C	0.4969	6.71E-10
D	0.145	1.81E-10
E	0.4865	6.06E-10
F	0.5944	7.4E-10

$CaCO_3$, limestone, was expected as the final product of this experiment. All precipitate samples was ivory in color, and they had a powdery texture, similar in appearance to powdered limestone, $CaCO_3$ (Figures 1 & 2).

Qualitative filter paper was used to isolate the solid from Flasks 1 to 4, which may have allowed some precipitate to pass through the filter, resulting in an artificially lower measured mass. Flasks 5 and 6 and Flasks A to F were filtered using quantitative filter paper.

Figure 1. Images of final $CaCO_3$ precipitation on filter papers for Flasks 1 to 6. The top row is Flasks 1 to 3, with urea, from left to right. The bottom row is Flasks 4 to 6, without urea, from left to right. Pictures taken by Seoyoon Lee.

Figure 2. Images of final $CaCO_3$ precipitation on filter papers for Flasks A to F. The top row is Flasks A to C, with urea and 50 mM HEPES buffer, from left to right. The bottom row is Flasks D to F, with urea and 100 mM HEPES buffer, from left to right. Picture taken by Seoyoon Lee.

The values from the three replicates for each condition were averaged to obtain a representative mean (Figure 3). Figure 3 shows that the $CaCO_3$ yield in the presence of urea is substantially lower than in the absence of urea. When 50 mM HEPES buffer is added to the urea condition, the MICP efficiency increases significantly relative to both the without-urea and with-urea conditions. The 100 mM treatment also showed an increase but was not statistically significantly different from without urea. Hence, the condition where urea and 50 mM HEPES buffer are present to adjust to the proper pH level result in the best efficiency of MICP among the samples.

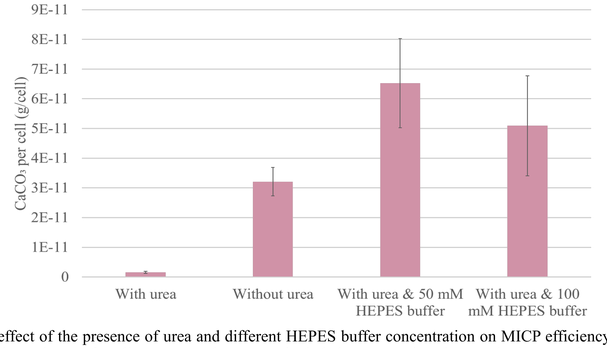

Figure 3. The effect of the presence of urea and different HEPES buffer concentration on MICP efficiency. To reflect the variability within each set of replicates, error bars were included, representing the standard error of the three (or two) measurements. Figure created by Seoyoon Lee using Excel.

Discussion

Among the treatments, the condition where urea and 50 mM HEPES buffer were both present resulted in the best MICP efficiency. Figure 3 shows that the alkaline environment created by urea assisted the ureolytic pathway when buffer was present. When there was not a buffer that could control the pH, the high alkalinity suppressed the bacterial activity, eventually hindering the MICP efficiency.

The results of this study suggest that the presence of urea on its own decreases the MICP efficiency, but in the presence of 50 mM HEPES buffer, the MICP efficiency rises significantly. However, the overlapping error bars for the 50 mM and 100 mM HEPES buffer conditions (Figure 3) suggest there is no significant difference of MICP efficiency between 50 mM and 100 mM conditions. 50 mM HEPES was adequate to adjust the alkalinity created by 333 mM urea and the other components. Comparison of the samples without urea to those with urea & HEPES shows that maintaining a proper alkaline environment to activate urease is more influential for MICP efficiency than accelerating CO_2 hydration by using CA (Figure 3). Overall, the data highlights that the ureolytic pathway is a major contributor to MICP. However, urea by itself appears to create an excessively alkaline environment, which inhibits bacterial activity and ultimately reduces $CaCO_3$ production.

In the work of Hoffmann et al. (2021), to test the optimal condition of MICP for *B. subtilis,* urease and related transport genes were modified. The study assessed MICP efficiency by using colorimetric urease activity assays, precipitation onset time on agar plates, and the number and spatial distribution of visible $CaCO_3$ crystals, whereas this study quantified MICP efficiency by measuring the dry mass of $CaCO_3$ precipitation collected from liquid cultures. By using urea, Hoffmann et al. (2021) suggested that maintaining urease activity and ureolytic pathway is crucial for MICP, which aligns with our result: that ureolysis plays a big role in MICP efficiency when the proper concentration of urea and buffer are used.

This experiment shows how calcium waste made during cement production can be sequestered in a stable form. Since calcination is a major factor that contributes to the increase of atmospheric CO_2 (Nunes, 2023), the results of this study demonstrate a possible pathway to offset a portion of these emissions through MICP, reducing both waste and CO_2 release. Anywhere concrete infrastructure is widespread and cement manufacturing is a significant emitter, this approach could supplement existing carbon-capture strategies with a biologically driven option. These results also demonstrate that significant improvement in mineralization can be achieved through chemical condition management, specifically urea availability and HEPES buffer, without requiring genetically engineered strains, increasing the scalability and regulatory feasibility of industrial adoption.

For future work, MICP efficiency at different CO_2 concentrations can be explored to compare the two enzymes CA and urease. Since Flask 1 was omitted when calculating the average for the ‘with urea’ condition, as a fly had entered the flask and potentially introduced additional bacteria, more replicates should be prepared for future experiments. Further optimization may involve testing a wider range of urea concentrations or different types of buffers. Finally, genetically modified *B. subtilis* can be examined under the same experimental conditions as in this study.

Conclusion

- The presence of urea by itself decreases the MICP efficiency, but in the presence of 50 mM and 100 mM HEPES buffer, the MICP efficiency rises significantly.
- Ureolytic pathway is critical for MICP .

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