Influence of concrete-related environmental stressors on biomineralizing bacterium used in self-healing concrete

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Abstract

Biogenic calcium carbonate precipitation has been identified as a way to improve durability and remediate cracks in cement-based materials. However, conditions that occur in cement-based materials (e.g., elevated temperature, nutrient depletion, and high pH) might impede microbial-induced calcium carbonate precipitation (MICCP) in these systems. Thus, the effects of heat, nutrient depletion, and pH treatments, all designed to mimic conditions in fresh cement paste, on the ureolytic bacterium *Sporosarcina pasteurii* were examined; specifically, impacts to bacterial viability, ability to hydrolyze urea, and surface charge were assessed. Viability and urea hydrolysis were most impacted by exposure to extreme temperature (55 °C) and extreme pH (13.6), but the impact was greatly reduced with exposure to milder temperature (45 °C) and pH (12.9) conditions. The zeta potential of *S. pasteurii* cells, which was used to approximate surface charge, was minimally affected by all tested conditions; the negative surface charge of *S. pasteurii* cells suggested that the cells might serve as heterogeneous nucleation sites for calcium carbonate precipitation. This was supported by the observation of substantially higher calcite concentrations in bacterial pastes containing viable cells or cells inactivated by autoclaving as compared to cell-free pastes. These new findings provide important insight as to how the conditions within cement paste could influence the viability of microorganisms and MICCP within cement-based materials. Overall, this study shows the importance of bacteria, whether viable or not, as potential heterogeneous nucleation sites for MICCP in cement-based materials.

Keywords: Biomineralization, *Sporosarcina pasteurii*, Zeta Potential, Urea Hydrolysis, Calcium Carbonate

1. INTRODUCTION

Biomineralization, the process by which organisms stimulate the formation of inorganic minerals, has been used to improve the properties of porous construction materials [13-15, 39]; biomineralization can increase the cohesion and stiffness of soil [15, 51], reduce the permeability of portland cement concrete [1, 2, 14], and remediate cracks in concrete [2, 9, 47]. One way that biomineralization is commonly achieved is through ureolytic bacteria; these bacteria possess the urease enzyme, which catalyzes the hydrolysis of urea and triggers a series of chemical reactions that results in microbial-induced calcium carbonate precipitation (MICCP). Urea hydrolysis produces ammonia and carbon dioxide, causing the precipitation of calcium carbonate in the presence of dissolved calcium [35, 41, 44]. The availability of dissolved calcium is critical for MICCP, making the calcium-rich pore solutions intrinsic to cement-based systems [11, 19] chemically well-suited for this process.

Interest in MICCP to improve the durability of portland cement concrete (primarily by reducing permeability via pore filling) has increased rapidly over the last decade. Portland cement concrete (herein called concrete) consists of sand and gravel embedded in a cement paste matrix. Volumetric changes in the cement paste matrix often lead to the formation of cracks in concrete; these cracks increase the permeability of the concrete and can impact the structural integrity if left unattended. However, in a self-repairing bioconcrete, these cracks could be repaired autogenously through the calcium carbonate precipitation induced by microorganisms [28, 29, 32, 37, 47, 48]. However, one challenge for long-term use of MICCP in concrete is to ensure that the bacteria remain viable during the service life. In bacterial cement paste, where vegetative cells of the bacterium *Sporosarcina pasteurii* (previously *Bacillus pasteurii*) were added to cement paste at a concentration of 2×10^6 most probable number per milliliter (MPN/mL), Basaran [9] reported that 9×10^3 MPN/mL (0.4% of the original inoculum) remained viable after 28 days. Similarly, Jonkers and Schlangen [28] found that 0.12% of *Bacillus cohnii* spores (1.15×10^6 spores/cm³ out of 10^9 spores/cm³ in the original inoculum), 0.01% *Bacillus halodurnas* spores (1.07×10^5 spores/cm³ out of 10^9 spores/cm³ in the original inoculum) and 0.06% *Bacillus pseudofirmus* spores (5.62×10^5 spores/cm³ out of 10^9 spores/cm³ in the original inoculum) survived 10 days after inoculation to cement paste. Understanding the factors contributing to bacterial inactivation (i.e., death) is essential for industrial application of biomineralization in cement-based materials. Bacterial inactivation in cement-based systems might be attributable to sudden environmental changes during the mixing process, including, but not limited to, an increase in temperature due to heat generated by cement hydration, lack of nutrients, and an increase in pH. Previous studies have explored ways to prolong the viability of microorganisms in these systems, such as encapsulating or immobilizing cells [28, 48], but little attention has been given to understanding how vegetative cells are affected by environmental conditions in cement-based systems. Furthermore, the role of inactivated cells and endospores (i.e., bacteria in a dormant state) in MICCP is uncertain. It has been shown that free urease, isolated from bacteria, can induce CaCO₃ precipitation [7]; however, it has not been conclusively determined whether urease activity can persist in microorganisms that have been inactivated. It has also been proposed that inactivated cells might passively aid in calcium carbonate precipitation by serving as nucleation sites for calcium carbonate precipitation [10]. To address these uncertainties and to provide insight regarding the persistence of vegetative *S. pasteurii* in concrete, we investigated the effect of conditions (e.g., heat, nutrient depletion, and high pH) designed to mimic those present in cement-based systems on the viability, urea hydrolysis, and zeta potential of vegetative *S. pasteurii*.

2. MATERIALS & METHODS

2.1. Cement

Texas Lehigh (Buda, TX) portland cement conforming to ASTM C150 Type I and Type II specifications [5] was used for all cement paste mixtures. X-Ray Fluorescence (XRF) was performed using a Bruker S4 EXPLORER to determine the mass composition of oxides (Table 1).

2.2. Microorganism and media

S. pasteurii, a bacterium commonly used in MICCP applications, was selected for this work. This bacterium is alkaliphilic and ureolytic [3, 7, 21, 24, 44, 50]. American Type Culture Collection (ATCC) 6453 *S. pasteurii* was grown in Urea-Yeast Extract (UYE) medium [52]. UYE medium contains 15.75 g/L Tris base, 20 g/L yeast extract, and 10 g/L urea, and the pH was adjusted to 9 with hydrochloric acid. *S. pasteurii* was grown in batch in UYE medium at 30°C with shaking. Absorbance at 600 nm (OD_{600}), which is directly correlated to cell concentration, was monitored during growth using a BIO-TEK Synergy HT spectrophotometer (Winooski, VT, United States) until $OD_{600}=0.8$ was reached. Then, the bacteria were subjected to various treatments designed to mimic the conditions in cement-based systems (Table 2).

2.3. High alkalinity (HA) solution

The HA solution contained 17.94 g/L KOH and 5.24 g/L NaOH [36], and it was used to simulate the high alkalinity environment that is inherent to the pore solution of cement paste. A Thermo Scientific™ Orion™ 720A pH probe (Waltham, MA, United States) was used to measure the pH of the HA solution, with a pH of approximately 13.6. The pH values of fresh cement pastes range from approximately 12.5 to 13.5 [42]; thus, the HA solution represents an extreme pH for a fresh paste. For some experiments, the HA solution was modified to include nutrients (N); HA+N solution consisted of 20 g/L yeast extract and 10 g/L urea in HA solution.

2.4. Cement extract (CE) solution

To serve as a complement to the HA solution, CE solution was prepared by mixing 350 g of cement with 2L of deionized water and continuously stirring the suspension for one hour with a magnetic stirrer [17, 38]. Then, the suspension was vacuum-filtered with Grade 1 qualitative filter paper. The resulting CE solution had a pH of approximately 12.9. The CE solution was used to examine the effects of a more realistic, milder pH as compared to that of the HA solution, and it also provided a more accurate depiction of the chemical composition of an actual cement paste pore solution than does the HA solution. For some experiments, the CE solution was modified to include nutrients; CE+N solution consisted of 20 g/L yeast extract and 10 g/L urea in CE solution.

2.5. Treatment conditions

Batch cultures of *S. pasteurii* were grown as described in Section 2.2, and the impact of nine treatment conditions on *S. pasteurii* from those cultures was explored (Table 2). For some treatment conditions, the cells were harvested from UYE medium by centrifugation at 7500 x g for 10 minutes and resuspended in a treatment solution (S, HA, HA+N, CE, CE+N); however, for the remaining treatment conditions, the cells remained in

their original UYE growth medium during treatment (U, HT-45, HT-55, A). Each treatment condition was applied to triplicate 100-mL samples, and all treatments were applied for four hours except for the autoclaving treatment. The untreated (U) sample set served as the control, and it represents live, vegetative *S. pasteurii* cells. The other eight sample sets were treated to examine how conditions designed to mimic those that occur in cement-based systems would impact the viability, urea hydrolysis, and zeta potential of *S. pasteurii*. Additional information about the treatments is provided as follows:

- Heat-treatment (HT) was chosen to elucidate the effect of heat generated by cement hydration on *S. pasteurii*. The maximum allowable temperature in concrete is commonly specified as 57 °C, and the typical temperature rise for concrete using Type I/II cement can range up to 30°C above the placement temperature [22]. Thus, the HT-55 samples were submerged in a water bath at 55 °C to simulate conditions in concrete designed to approach the conventional maximum allowable concrete temperature of 57 °C. HT-45 represents a milder heat-treatment, with those samples being submerged in a water bath at 45°C.
- For the starved (S) sample set, *S. pasteurii* cells were resuspended in distilled water at 23 °C to evaluate the effect of nutrient depletion.
- For the HA samples, *S. pasteurii* cells were resuspended in HA solution (no carbon source and electron donor) at 23 °C to evaluate the effect of high pH coupled with nutrient depletion; by comparison, bacterial cells are typically added to cement paste in their culture medium that contains nutrients. As a result, the degree of nutrient depletion and pH shock in the HA samples might have been more severe than what occurs in bacterial concrete.
- For the HA+N samples, nutrients (N) were added to the HA solution to investigate the effect of high pH on *S. pasteurii* without nutrient depletion; *S. pasteurii* cells were resuspended in HA+N solution at 23 °C.
- The CE and CE+N samples were used to serve as milder complements to the HA and HA+N treatments; *S. pasteurii* cells were resuspended in pre-prepared CE and CE+N solutions at 23 °C.
- For the autoclaved (A) treatment, *S. pasteurii* cells in UYE medium were autoclaved at 121 °C and 100 kPa for 30 minutes to inactivate them. The cells were classified as inactivated based on their subsequent inability to grow in liquid or solid UYE medium.

2.6. Viability

The total number of viable cells was estimated in each treated sample set using the MPN method [46]. Each sample was serially diluted by a factor of 10 (10^0 - 10^{-4} dilutions) in pH 9 UYE medium in 10-mL test tubes. The

dilutions were performed in triplicate for each sample set, and the test tubes were incubated at 30°C with shaking for 96 hours. Then, the number of positive growth responses (indicated by visible turbidity) at each dilution was recorded for each sample set, and this information was used to obtain the MPN index and upper and lower 95% confidence limits from standard tables [46].

2.7. Urea hydrolysis

Urea hydrolysis was examined for the nine sample sets by monitoring ammonia (NH₃) production. *S. pasteurii* was grown in UYE medium to OD₆₀₀=0.8 (Section 2.2), and the cells were treated as described in Section 2.5 and Table 2. Then, cells were harvested from each sample set by centrifugation at 7500 x g for 10 minutes, resuspended in fresh UYE medium, and incubated at 30 °C with shaking. Ammonia concentration was monitored using a Thermo Scientific™ Orion™ High-Performance Ammonia Electrode (Waltham, MA, United States) for 24 hours. The ammonia concentration was measured immediately after resuspension of the cells in fresh UYE medium (t=0), and this value was subtracted from all subsequent measurements to correct for the small amount of NH₃ that was present in the freshly prepared medium.

According to the stoichiometry of the urea hydrolysis reaction, one mole of urea is hydrolyzed to form two moles of NH₃ and one mole of CO₂. UYE medium contains 167 mM urea, so the maximum amount of ammonia that can be produced in each culture is 333 mM. The amount of ammonia present in the medium at t=0 was subtracted from both the ammonia concentration at the time of interest (NH_{3(t=x)}) and the maximum possible ammonia concentration (NH_{3(max)}) in the culture, and the cumulative percent urea consumed was calculated according to Equation 1.

$$\% \text{ urea consumed}_{(t=x)} = \frac{[\text{NH}_3(t=x)] - [\text{NH}_3(t=0)]}{[\text{NH}_3(\text{max})] - [\text{NH}_3(t=0)]} \times 100\% \quad (\text{Equation 1})$$

Urease activity, or the rate of urea hydrolysis, was also calculated for each sample set during the first two hours of the experiment using the ammonia measurements collected at t=0h and t=2h.

2.8. Zeta potential

Zeta potential was measured to estimate the effect of each treatment on the surface potential of the bacterial cells. Zeta potential testing was conducted at 23 °C using a Malvern Zetasizer Nano ZS (Malvern, Worcestershire, United Kingdom). The cells were treated as described in Section 2.5 and Table 2. Aliquots (1 mL) were obtained from each sample set. The aliquots were centrifuged at 4000 x g for 6 minutes to harvest the cells, which were then washed, centrifuged, and resuspended in sterile 20 mM Tris buffer at pH 9. Because CE solution was obtained by filtering a mixture of water and cement through qualitative filter paper as described in Section 2.4, some particulate matter remained in the extracted solution. Thus, the CE and CE+N sample sets were excluded from zeta potential measurements since the presence of the cementitious particulates could affect the results.

Immediately after treatment, zeta potential was measured for triplicate aliquots of the cells resuspended in Tris buffer. Then, additional sample aliquots were stored at room temperature (~23 °C) in either their original UYE medium (for U, HT-45, and HT-55), distilled water (for S), HA solution (for HA), or HA solution + 10 g/L urea + 20 g/L yeast extract (for HA+N) for 28 days. Zeta potential was determined for triplicate 1-mL aliquots obtained from these samples at 3, 7, and 28 days after treatment using the same procedure (cells resuspended in Tris buffer immediately prior to performing zeta potential measurements) to evaluate whether the cells could maintain their surface charge over time. Three replicate cultures were processed for each treatment, where one 1-mL aliquot was taken from each of these cultures at each time point, and zeta potential was analyzed in triplicate for each specimen. Thus, nine data points were obtained for each sample set at each time point.

2.9. X-Ray diffraction (XRD)

2.9.1 Sample preparation and procedure

XRD was conducted using a Siemens Bruker X-ray Diffractometer (Madison, WI, United States) to qualitatively assess the morphology of CaCO₃ in a variety of cement pastes and to quantify CaCO₃ content in each cement paste. A solution-to-cement ratio (s/c) of 0.50 was used for all pastes, and four types of pastes were evaluated:

- Neat paste: prepared with distilled water and cement;
- Nutrient paste: prepared with fresh UYE medium and cement;
- Spent nutrient paste: prepared with spent UYE nutrient medium (obtained by growing *S. pasteurii* in UYE medium to OD₆₀₀=0.8 and then retaining the spent medium after filtering through a 0.2-μm filter to remove cells) and cement;
- Bacterial paste: prepared with untreated (U) and autoclaved (A) sample sets (using *S. pasteurii* cells grown in UYE medium to OD₆₀₀=0.8 and treated according to Table 2) and cement;

Pastes were prepared by mixing 40 g of cement with 20 g of either distilled water (for neat pastes), fresh UYE medium (for nutrient pastes), spent UYE medium (for spent nutrient pastes), or bacterial cultures (for bacterial pastes) by hand stirring with a spatula for 2 minutes. The paste samples were then poured into 3 x 3 cm cylindrical molds and cured at 100% relative humidity at 25°C for 24 hours. Immediately after curing, the cement paste samples were removed from the molds and crushed with a pestle. A representative aliquot was obtained from the core and pulverized with a mortar and pestle into a powder. The resulting powder samples were passed through a No. 325 sieve such that the particles were finer than 53 μm. The samples were then ground with ethanol to stop hydration [55], and 10% (w/w) zincite was added as an internal standard. The samples were kept in a vacuum desiccator until the time of testing. Analysis was conducted from 25-40° 2θ with 6 seconds of dwell time. The diffractometer was operated at 40 keV and 30 mA, at a step size of 0.02° 2θ. Triplicate paste specimens were prepared for each paste.

2.9.2 Reference Intensity Ratio (RIR) procedure

Calcite was the only polymorph of calcium carbonate that was detected; thus, calcite concentration was quantified in each sample using the RIR method [26]. A ratio was established between the intensities of the primary zincite (internal standard) peak and primary calcite peak by creating samples with known mass ratios of calcite to zincite and plotting the results on a standard curve. Zincite content was maintained at 10% by mass, and calcite content was varied from 2% to 18% by mass. NaCl was used as a filler material, and calcite content was calculated in each paste sample according to the RIR standard curve.

3. RESULTS AND DISCUSSION

3.1. Viability and urea hydrolysis

The concentrations of total viable cells in each sample set, which were evaluated using the MPN method immediately after treatment, are shown in Fig. 1. Most of the treated sample sets contained a lower concentration of viable cells as compared to the untreated sample set. As expected, MPN/mL in the autoclaved sample set (A) was less than the detection limit [46], thus showing that autoclaving successfully inactivated the cells inoculated to that sample set. Of the remaining sample sets, HT-45, HT-55, and HA exhibited the most substantial reductions in total viable cell concentration as compared to the untreated sample set; interestingly, some of the treated sample sets, specifically S, HA+N, CE, and CE+N sample sets, retained a cell concentration more similar to the untreated sample set. As compared to the high alkalinity solution (HA) itself, the addition of nutrients to the high alkalinity solution (HA+N) resulted in a considerable increase in the concentration of viable cells remaining (Fig. 1). These results provide important knowledge about the resilience of *S. pasteurii* cells to different environmental conditions. For instance, in terms of viability, *S. pasteurii* cells were more sensitive to the extreme temperature tested in this study as compared to the tested pH changes. According to the ATCC, the optimum pH of 6453 *S. pasteurii* is 9 [6], but a substantial number of bacteria remained viable in a cement extract solution with a pH of approximately 13.

Monitoring urea consumption provides insight to the potential for MICCP because it is through urea hydrolysis that carbonates are produced. Urea consumption and urease activity, which were calculated from NH_3 measurements as described in Section 2.7, are presented in Fig. 2 and Table 3, respectively. The autoclaved *S. pasteurii* cells (sample set A) did not hydrolyze urea, while the rates of urea hydrolysis by cells in sample sets HT-45, HT-55, S, HA, HA+N, and CE were lower as compared to that of the untreated cells. While urea hydrolysis occurred more slowly in the CE sample set as compared to the untreated sample set (U), the addition of nutrients to the CE solution (CE+ N sample set) completely resolved that issue. As compared to urea hydrolysis by the untreated cells, urea hydrolysis in the first 6 hours of incubation was noticeably reduced in the

HT-55 sample set and was negligible in the HA and HA+N sample sets, despite the fact that the viable cell concentrations in the HA and HA+N sample sets exceeded that in the HT-55 sample set. This shows that cell viability is not the sole predictor of MICCP success in cement-based materials. Taken together, the observations regarding cell viability and urea consumption provide useful information about the potential for bacterial cells to produce carbonates *in situ* in bacterial concrete.

When cement is added to water, a series of exothermic chemical reactions occurs that eventually results in the stiffening and hardening of the cement paste. The heat that is released from these reactions (the amount of which is governed by the content and type of cementitious materials in the concrete), increases the temperature of the concrete from that of the placement temperature; placement temperature generally ranges between 10 and 32 °C [45, 53]. In this study, an increase in temperature from 45°C to 55°C resulted in a marked decrease in viability (Fig. 1) and initial urea hydrolysis (Fig. 2) by *S. pasteurii*. Thus for bacterial concrete applications, especially where excessive heat generation is a concern (e.g., mass concrete and hot-weather concreting), precautions can be taken to ensure that the temperature of the concrete does not increase too much. Such precautions might include the use of low-heat cements or supplementary cementing materials such as fly ash [8] or the precooling of the mixing water [53].

Furthermore, this study showed that exposure to extreme pH (13.6 in the HA and HA+N sample sets) temporarily halted urea hydrolysis (Fig. 2) by *S. pasteurii* (for several hours) even after the cells were resuspended in pH 9 UYE medium. Fortunately, as compared to pH 13.6, exposure to a milder and more realistic pH for cement paste pore solutions (12.9 in the CE and CE+N sample sets) had far less impact on the viability and urea hydrolysis of *S. pasteurii*. Every microorganism has an optimum pH range and avoiding extreme pH values for the microorganism of interest aids its survival and growth. Thus in concrete applications where the cement paste is inoculated with vegetative microorganisms, cements with high soluble alkali contents and/or high gypsum contents, which tend to increase the pH [34], should be avoided; alternatively, experiments at pH values relevant to the cement paste at hand should be conducted to verify the survival of and induction of calcium carbonate precipitation by the chosen microorganism. Interestingly, the presence of nutrients (urea and yeast extract) provided some protection to the cells exposed to high pH conditions (HA+N and CE+N), resulting in higher viability retention (Fig. 1) as compared to their respective high pH treatments in the absence of nutrients (HA and CE). Thus, inoculating vegetative cells to cement paste in a nutrient medium as compared to standard mixing water could result in greater bacterial viability retention.

3.3 Zeta potential

The zeta potential results in Fig. 3 show that cells in all sample sets were negatively charged. The most negative initial zeta potential reading (-35.05 ± 2.36 mV) was obtained from untreated *S. pasteurii* cells; the least negative initial zeta potential reading (-27.38 ± 1.08 mV) was obtained from cells harvested from the HA+N sample set. Zeta potential changed minimally over time, with zeta potential readings generally not deviating more than ± 5 mV from the initial zeta potential reading for a particular treatment.

Bacterial surface charge could be of substantial importance for MICCP [1, 2, 4, 18]; this surface charge can be approximated using zeta potential [54], which measures the electrokinetic potential of the discontinuous phase in a colloidal dispersion. It has been assumed in several studies [1, 12, 23] that *S. pasteurii* has a net negative surface charge because most bacterial cells possess a negative zeta potential in neutral and basic environments [18, 20, 30, 43, 49]. In the current study, we showed that *S. pasteurii* possesses a robust negative surface charge, as approximated by zeta potential measurements at pH 9 (Fig. 3), both for untreated cells and cells exposed to a variety of treatment conditions (heat-treatment, lack of nutrients, high pH, and autoclaving).

The zeta potential of the cells was reasonably constant for up to 28 days in each sample set, indicating that even stressed or inactivated cells could sustain a negative surface charge over time. It is possible that this negative charge could attract positively charged calcium ions, with the cell surface acting as a site of heterogeneous nucleation for CaCO_3 precipitation [25, 31, 35, 40, 4, 16, 44, 47]. Using scanning electron microscopy on samples from microbial-plugged sand columns, Stocks-Fischer et al. [44] observed that *S. pasteurii* appeared to be embedded in biogenic calcite crystals and suggested that the bacteria acted as nucleation sites for biological precipitation of calcium carbonate. Similarly, Dhimi et al. [16] observed bacterial imprints on the surface of carbonate crystals, again suggesting that the bacteria might serve as nucleation sites. The precipitation of minerals on the cell surface, or epicellular mineralization, is not only a common occurrence, but it is a distinctive feature of biomineralization [33]. Further, the presence of organisms as platforms for precipitation lowers the energy barrier for crystallization, which allows biominerals to form more rapidly and abundantly [27]. Therefore, if epicellular mineralization can occur in bacterial cement paste systems, it seems that all cells, even stressed or dead cells, could passively contribute to calcium carbonate formation by providing sites for heterogeneous nucleation to occur.

3.4 XRD

Calcite was the only polymorph of calcium carbonate detected in each of the paste samples, and the mass percent of calcite in each paste sample was determined according to the RIR procedure described in Section 2.9.2 (Table 4). Calcite content was considerably higher in the bacterial pastes as compared to the neat, nutrient, and spent nutrient pastes at 1 day. As compared to neat paste, bacterial paste prepared with untreated bacterial cells and

bacterial paste prepared with bacterial cells inactivated by autoclaving contained 110% and 62% more calcite, respectively (Table 4). The increase in calcite content in the bacterial paste with untreated cells is due to urea hydrolysis by the cells and possibly heterogeneous nucleation. Since the cells in the autoclaved sample set did not hydrolyze urea when resuspended in fresh UYE medium after autoclaving (Section 3.2 and Fig. 2), the increase in calcite content in the bacterial paste with autoclaved cells is due to urea hydrolysis in the culture prior to autoclaving [52] and possibly heterogeneous nucleation. The possible importance of heterogeneous nucleation in bacterial pastes is highlighted by examining the calcite concentration of paste specimens prepared with cell-free, spent nutrient medium. In this study, the cell-free, spent nutrient medium contained the by-products of ureolytic activity (e.g., carbonates) that were likely similar to the levels in the culture used to prepare the bacterial paste with untreated cells, yet the calcite content in the pastes prepared with spent nutrient medium was substantially lower than those in the bacterial pastes; relatedly, the calcite concentration in the spent nutrient paste was more similar to those in the neat and nutrient pastes, even though the spent nutrient paste likely contained a carbonate concentration that was greater than those in the neat and nutrient pastes (Table 4). Taken together, these results suggest that the presence of the negatively charged cells (whether viable or inactivated by autoclaving, Section 3.3 and Fig. 3) might have promoted the formation of calcite by via heterogeneous nucleation in bacterial pastes.

4. CONCLUSIONS

Ureolytic bacteria are commonly used in bacterial concrete studies to induce calcium carbonate precipitation. To elucidate the influence of environmental stressors designed to mimic conditions that these bacteria might encounter in cement paste, the impact of heat, nutrient depletion, and high pH was studied on *S. pasteurii* cells. Of these treatments, extreme temperature (55 °C for 4 hours) and extreme pH (13.6 for 4 hours) caused the largest decline in viable cell concentration and initial rate of ammonia production via urea hydrolysis. By extension, the rate of carbonate production by *S. pasteurii* cells, a critical step in the MICCP process, would be decreased for cells exposed to these treatments. Less extreme treatments of the bacteria with heat (45 °C for 4 hours) and pH (12.9 for 4 hours) yielded greatly reduced impact to viability and urea hydrolysis. Although cell viability and urea hydrolysis were impacted by some of the treatments employed in this study, zeta potential, which was used to approximate surface charge, was minimally affected. Heat treatment, nutrient depletion, and high pH had no discernible effect on the zeta potential of *S. pasteurii* cells as compared to untreated cells. Additionally, the zeta potential of treated and untreated *S. pasteurii* cells remained relatively constant for up to 28 days after treatment, which indicated that these cells could maintain a negative surface charge over time. Thus, the negatively charged *S. pasteurii* cells might serve as heterogeneous nucleation sites for CaCO₃

precipitation. This is supported by quantitative XRD results that showed that bacterial cement paste prepared with untreated bacterial cultures contained substantially more calcite than did cell-free pastes; paste prepared with untreated bacterial cultures contained 110%, 72%, and 96% more calcite than did neat cement paste, cement paste prepared with nutrient medium, and cement paste prepared with spent nutrient medium, respectively. Furthermore, *S. pasteurii* inactivated by autoclaving had no remaining viability nor ureolytic activity, but they maintained a similar negative surface charge to untreated cells; bacterial cement paste prepared with this autoclaved cell culture resulted in a substantial increase in calcite content as compared to that in cell-free pastes; paste prepared with the autoclaved culture contained 62%, 33%, and 51% more calcite than did neat cement paste, cement paste prepared with nutrient medium, and cement paste prepared with spent nutrient medium, respectively. Overall, this study suggests the potential importance of bacteria, whether viable or not, as heterogeneous nucleation sites for MICCP in cement-based materials.

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472 **Tables:**

473 **Table 1** Type I/II portland cement oxide composition; LOI: Loss on ignition

Oxides	% (w/w) composition
CaO	63.63
SiO ₂	20.14
Al ₂ O ₃	5.42
Fe ₂ O ₃	2.47
MgO	1.32
SO ₃	3.09
Na ₂ O	0.17
K ₂ O	0.95
LOI	2.82

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Table 2 Summary of treatments conducted to simulate environmental conditions in concrete and characterization analyses conducted to evaluate the effects of the various treatments

Sample Name	Treatment applied to <i>S. pasteurii</i>	Post-treatment characterization analyses			
		Viability	Urea hydrolysis	Zeta potential	XRD
U	None	x	x	x	x
HT-45	Heat-treated: 45 °C, 4 hrs	x	x	x	-
HT-55	Heat-treated: 55 °C, 4 hrs	x	x	x	-
S	Starved (resuspended in distilled water), 4 hrs	x	x	x ^b	-
HA	Resuspended in HA, 4 hrs	x	x	x ^b	-
HA+N	Resuspended in HA+N, 4 hrs	x	x	x ^b	-
CE	Resuspended in CE, 4 hrs	x	x	-	-
CE+N	Resuspended in CE+N, 4 hrs	x	x	-	-
A	Autoclaved	x	x	x	x

“x” indicates that the specific analysis was conducted for that particular sample treatment.

“-” indicates that the specific analysis was not conducted for that particular sample treatment.

“^b” indicates that the treatment was applied for longer than the typical 4 hrs (i.e., up to 28 days for zeta potential analysis).

Table 3: Urease activity for the first two hours of urea hydrolysis experiments; sample sets HA, HA+N, and A did not exhibit any urease activity during this time. Error represents the standard deviation for triplicate samples.

Sample set	Urease activity (mM/min) from t=0 to t=2h
U	1.166 ± 0.053
HT-45	0.920 ± 0.064
HT-55	0.164 ± 0.005

S	0.706 ± 0.036
CE	0.585 ± 0.033
CE+N	1.343 ± 0.021

Table 4 Mass % calcite (w/w) at 1 day; error represents the standard deviation of triplicate samples; Neat: Neat paste, Nutrient: Nutrient paste; Spent Nutrient: Spent Nutrient paste; Bacterial-U: Bacterial paste mixed with untreated cells from U sample set; Bacterial-A: Bacterial paste mixed with autoclaved cells from A sample set

Paste sample	% Calcite
Neat	7.68 ± 0.77
Nutrient	9.35 ± 1.23
Spent Nutrient	8.21 ± 0.51
Bacterial-U	16.11 ± 1.08
Bacterial-A	12.42 ± 1.67

Figures:

Fig. 1 Total viable cell concentration in each sample set estimated by Most Probable Number (MPN); error bars represent the upper and lower 95% confidence limits

Fig. 2 Calculated urea consumption in UYE medium at 30°C with shaking by *S. pasteurii* for each sample set; error bars represent the standard deviation of triplicate cultures

Fig. 3 Zeta potential of *S. pasteurii* (in pH 9 Tris buffer at 23°C) at 0, 3, 7, and 28 days after treatment; error bars represent the standard deviation for triplicate measurements of triplicate cultures