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Overcoming the inhibitory effects of urea to improve the kinetics of microbial-induced calcium carbonate precipitation (MICCP) by *Lysinibacillus sphaericus* strain MB284

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Among different microbial-induced calcium carbonate precipitation (MICCP) mechanisms utilized for biomineralization, ureolysis leads to the greatest yields of calcium carbonate. Unfortunately, it is reported that urea-induced growth inhibition can delay urea hydrolysis but it is not clear how this affects MICCP kinetics. This study investigated the impact of urea addition on the MICCP performance of *Lysinibacillus sphaericus* MB284 not previously grown on urea (thereafter named bio-agents), compared with those previously cultured in urea-rich media (20 g/L) (hereafter named bio-agents$^+$ or bio-agents-plus). While it was discovered that initial urea concentrations exceeding 3 g/L temporarily hindered cell growth and MICCP reactions for bio-agents, employing bio-agents$^+$ accelerated the initiation of bacterial growth by 33% and led to a 1.46-fold increase in the initial yield of calcium carbonate in media containing 20 g/L of urea. The improved tolerance of bio-agents$^+$ to urea is attributed to the presence of pre-produced endogenous urease, which serves to reduce the initial urea concentration, alleviate growth inhibition, and expedite biomineralization. Notably, elevating the initial concentration of bio-agents$^+$ from OD_{600} of 0.01 to 1, housing a higher content of endogenous urease, accelerated the initiation of MICCP reactions and boosted the ultimate yield of biomineralization by 2.6 times while the media was supplemented with 20 g/L of urea. These results elucidate the advantages of employing bio-agents$^+$ with higher initial cell concentrations to successfully mitigate the temporary inhibitory effects of urea on biomineralization kinetics, offering a promising strategy for accelerating the production of calcium carbonate for applications like bio self-healing of concrete.

[**Key words:** Microbial-induced calcium carbonate precipitation mechanisms; Biomineralization kinetics; Urea hydrolysis; Urea-induced growth inhibition; *Lysinibacillus sphaericus* MB 284]

Microbial-induced calcium carbonate precipitation (MICCP) has emerged as a versatile biomineralization technique with diverse applications, including self-healing concrete, environmental remediation, and soil improvement (1–3). Bacteria play a crucial role in MICCP, and various species have been explored for biomineralization purposes, including *Sporosarcina*, *Bacillus*, *Kocuria*, *Penicillium*, *Ralstonia*, *Diaphorobacter*, *Klebsiella*, *Helicobacter*, *Citrobacter*, and *Enterobacter* (4–8). Among these, *Lysinibacillus sphaericus* (formerly known as *Bacillus sphaericus*) stands out as a promising candidate due to its unique properties. Notably, *L. sphaericus* possesses exceptional endospore formation capabilities, allowing it to survive harsh environmental conditions for over 50 years (4,9,10). Additionally, it demonstrates superior urease activity (6.2×10^{-8} h^{-1} mL CFU^{-1}) and calcium carbonate precipitation rate (0.604 h^{-1}) compared to other species (11–16). This enhanced efficiency stems from its ability to synthesize the enzyme urease, which facilitates urea hydrolysis and subsequent carbonate ion production, ultimately leading to calcium carbonate precipitation.

MICCP reactions driven by urea hydrolysis stand out as a particularly efficient biomineralization method due to their rapid reaction kinetics, enabling fast urea hydrolysis and calcium carbonate precipitation compared to other pathways (11,12). While the previously discussed results highlight the promise of urea hydrolysis, some studies have reported potential drawbacks associated with using high initial urea concentrations, particularly affecting *L. sphaericus* strains. Research by Mokhtar et al. (17) observed that increasing initial urea concentration extended the lag phase and decreased bacterial growth, although urea-induced inhibition was not explicitly mentioned. Similarly, Bhat et al. (18) demonstrated that higher urea concentrations reduced urea hydrolysis rates, suggesting an inhibitory effect on bacterial activities.

This study aims to address the knowledge gap regarding the impact of initial urea concentration on biomineralization efficiency. We hypothesized that higher urea concentrations in addition to bacterial growth and urea hydrolysis, negatively affect calcium

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carbonate production kinetics. Therefore, we investigated the relationship between initial urea concentration and its inhibitory effects on *L. sphaericus* strain MB 284 growth (bio-agents) and biomineralization kinetics, considering both short- and long-term effects. Furthermore, we propose a strategy to accelerate calcium carbonate precipitation by mitigating urea inhibitory effects on hydrolyzing urea. This strategy involves employing different initial concentrations, ranging from OD_{600} of 0.01 to 1 of bio-agents^{+} (i.e., bio-agents-plus), cells pre-cultured in media containing 20 g/L of urea. This range aligns with commonly used cell concentrations in MICCP studies, as reported in previous literature (19–21). Utilizing this established range allows for direct comparison of our findings with existing research. Finally, we assess the influence of urea addition and initial cell concentration on calcium carbonate crystal morphology. This study seeks to improve biomineralization kinetics by introducing a potential solution to alleviate urea-induced growth inhibition, for applications in the self-healing of concrete.

MATERIALS AND METHODS

Bacterial preparation The *L. sphaericus* 13805 strain also referred to as MB284, was obtained from the American Type Culture Collection (ATCC). It was cultured in a medium composed of distilled water, 10 g/L of peptone (Research Products International, Mt. Prospect, IL, USA) and beef extract (Sigma–Aldrich, St. Louis, MO, USA), and 5 g/L of sodium chloride to revive the strain. The pH of the culture medium was adjusted to 7 using a phosphate-buffered saline (PBS) solution purchased from VWR (Radnor, PA, USA). Prior to cell inoculation, the culture medium was autoclaved at 121°C for 15 min (ST75935, Harvey SterileMax, Thermo Fisher, Waltham, MA, USA). Subsequently, strain MB284 was incubated in the mentioned culture medium within a shaker incubator at a temperature of 35°C and a speed of 150 rpm (Innova 44, Eppendorf, Enfield, CT, USA) for approximately one day. The growth rate of the bacteria was measured by optical density at a wavelength of 600 nm (OD_{600}) using a spectrophotometer (Thermo Scientific Genesys 20 US, Thermo Fisher).

Once the OD_{600} value of the culture medium reached approximately 1, 25 mL of it was transferred to 50 mL conical polypropylene centrifuge tubes. The sample was centrifuged for 10 min at 7830 rpm, and 25°C (5430 R, Eppendorf) to separate the solid phase containing cells (hereafter called bio-agents) from the supernatant. The bio-agents were subsequently washed three times with a PBS solution and then introduced into the fresh PBS solution. The pour-plate technique was applied to determine the cell concentration. It was observed that MB284 reached a concentration of 10^9 cells/mL when the OD_{600} value reached 1, in line with findings in the existing literature (21).

To induce the production of endogenous urease within the cells, a 3-day incubation period in culture media containing 20 g/L of yeast extract (Sigma–Aldrich) and urea (Alfa Aesar, Heysham, UK) was employed. Subsequently, the culture media was subjected to centrifugation at 7830 rpm for 10 min to facilitate cell harvesting. The harvested cells were then washed three times with PBS solution. The next step involved subjecting cells to sonication (Branson 1800, Emerson, St. Louis, MO, USA) for 30 min, resulting in cell lysis and the release of their contents, which included endogenous urease. To prepare bio-agents^{+}, lysed cells were combined with the same concentration of bio-agents at a volume ratio of 1:5. Fig. S1 visually outlines the distinct steps involved in the preparation of both bio-agents and bio-agents^{+}.

In many ureolytic species, it has been reported that urease is an enzyme housed within the cytoplasm of cells (22), where it is located intracellularly. Additionally, it has been observed that due to the autolysis of a portion of bacteria containing urease within their cytoplasm, urease is released into the micro-environment, leading to its adsorption onto the surface of intact bacteria (23). Therefore, in this study, sonication was employed to expedite and enhance the cell lysis process of cells that had previously been incubated in urea-rich culture media.

Culture media preparation Prior to investigating the effects of urea and cell concentration on MICCP performance, the culture media composition was optimized. Two types of culture media were employed in this study including defined and undefined culture media. The defined culture media was composed of minimal salt media (MSM) as a nutrient source while 20 g/L of either urea, thiamine (Sigma–Aldrich), ammonium acetate, ethylene diamine tetraacetic acid (EDTA), sucrose (Thermo Fisher), glucose (Aldrich Chemical Company Inc., Milwaukee, WI, USA), and calcium acetate was added as individual carbon source to assess their suitability for MB284 growth. The composition of MSM has been detailed in our previous studies (9,10) and detailed in Table S1 for reference.

For undefined culture media, a commonly used formulation containing 20 g/L either of skimmed milk powder (bioWORLD, Dublin, OH, USA), peptone, yeast, beef, and meat extract (Sigma–Aldrich) was prepared in a 120 mL safety-coated Boston round glass bottle (Qorpak, Clinton, PA, USA) with 50 mL of distilled water. Bio-agents were then inoculated into the autoclaved culture media and stored in a shaker incubator at 150 rpm and 35°C. This study aimed to identify the undefined culture medium that supported optimal bacterial growth and MICCP performance.

Following the selection of the optimal undefined culture media (yeast extract), yeast extract concentration was optimized. Various concentrations (1, 5, 10, 15, and 20 g/L) of the optimal culture medium were then tested to determine the level that yielded the best bacterial growth and biomineralization. Subsequently, the culture media containing the optimal concentration of yeast extract (20 g/L) incubated with bio-agents was used for the main experiments investigating the impact of varying urea concentrations (0, 1, 3, 5, 10, 15, and 20 g/L) on bacterial growth and MICCP performance. Urea was filter-sterilized before addition to the culture media.

Bacterial growth was monitored by measuring OD_{600} after inoculating bio-agents or bio-agents^{+} into the optimized media. It is important to note that OD_{600} measurements were performed in the absence of added external calcium ions to avoid interference from their reaction with soluble carbonate ions in the culture media. All experiments were conducted in triplicate for reproducibility. The steps of experiments are thoroughly demonstrated in Fig. S2.

MICCP performance measurement During MICCP, the micro-environment experiences changes in electric conductivity (EC) and pH, resulting in the production of ammonium and carbonate ions. As part of our experimental approach, pH and EC measurements were conducted by immersing probes from the pH meter (Accumet Basic AB15, Thermo Fisher) and EC meter (Mettler Toledo, Columbus, OH, USA) into the 50 mL of culture media incubated with either bio-agents or bio-agents^{+}. Furthermore, due to the formation of NH_4^+ as another by-product in MICCP reactions, the NH_4^+ concentration in the culture media was quantified using a spectrophotometer (DR 2500, Hach, Loveland, CO, USA).

After the addition of 20 g/L of calcium acetate into 50 mL of culture media containing bacteria to stimulate calcium carbonate production, samples were placed in a shaker incubator at 35°C and 150 rpm for 1 h, followed by centrifugation at 7830 rpm and 20°C for 20 min to terminate the MICCP process. After removing the supernatant from the culture media, the resulting residue (produced solid mass) was subjected to a temperature of 100°C in an oven (Isotemp 550-58 Muffle Furnace, Fisher Scientific) for 24 h to eliminate moisture, transforming it into a dry powder. Subsequently, the dry powder was weighed using an analytical balance (Quintix Semi-Micro Balances, Sartorius, Goettingen, Germany). We assumed that the culture media, urea, and calcium acetate with the examined concentrations (each 20 g/L) were soluble in distilled water. Consequently, any unreacted substances were expected to remain soluble and were subsequently discarded. Therefore, the solid mass that underwent the drying process in the oven primarily consisted of biomass and precipitated salts. Given that calcium ions (Ca^{2+}) exhibit a higher ionic selectivity compared to other commonly present divalent cations in the culture media, such as Mg^{2+} (24), we infer that most of the generated salts are calcium carbonate.

Determination of the calcium carbonate percentage in the solid mass [based on the ratio weight $_{CaCO}$ (g)/weight $_{solid\ mass}$ (g)] was accomplished using thermal gravimetric analysis (TGA Q5000, TA Instruments, New Castle, DE, USA), which was then used to calculate the weight of CaCO3 (Eq. 1). This solid mass was finely ground until the particle size was below 75 μm. About 25 ± 5 mg of the solid mass was placed onto a high-temperature platinum pan and underwent testing between 30 and 900°C, employing a ramp rate of 10°C/min.

$$\text{Weight}_{CaCO_3}(\text{mg}) = \text{Weight}_{\text{solid mass}}(\text{g}) \times \left[\frac{\text{Weight}_{CaCO_3}(\text{g})}{\text{Weight}_{\text{solid mass}}(\text{g})}\right]_{\text{Obtained from TGA test}} \times 10^{-3} \quad (1)$$

To assess the performance of MICCP over time, a series of scarifying samples were prepared, corresponding to the specific conditions of the experiment. These samples were incubated with either bio-agents or bio-agents^{+} and placed in a shaker incubator at 35°C with a constant agitation of 150 rpm. At regular intervals following the incubation of the cells, the EC and pH of each sample were measured. Subsequently, 20 g/L of calcium acetate was introduced into each scarifying sample for calcium carbonate production.

Urease indicator agar plates (UIAPs) were used to detect the urease activity released by bio-agents and bio-agents^{+}. The specific composition of the UIAP is outlined in Table S2. After preparing the UIAP solution, it was autoclaved at 121°C for 15 min. Once the solution's temperature had cooled to 50°C, it was added to 50 mL of a 20 g/L urea solution that had undergone sterile filtration. This amalgam was meticulously mixed and subsequently dispensed into the agar plates.

A 200 μL solution of both bio-agents and bio-agents^{+}, each with a concentration of 10^8 cells/mL (OD_{600} = 0.1), was dispensed into the center of the UIAPs. Control samples included the presence of 1,10-phenanthroline (Chem-Impex International Inc., Wood Dale, IL, USA), a known urea hydrolysis inhibitor (25).

Morphology of calcium carbonate crystals The morphology of the calcium carbonate crystals in the resulting solid mass was analyzed using a Zeiss Supra 50VP field-emission scanning electron microscope (SEM, Zeiss, Jena, Germany) equipped with secondary electron imaging and qualitative energy-dispersive X-ray spectroscopy (EDS) analysis. For sample preparation, we initiated the process by introducing 20 g/L of calcium acetate into 50 mL of culture media containing 20 g/L of yeast extract (with and without 20 g/L of urea) that had been incubated with two initial concentrations of bio-agents^{+} (OD_{600} of 0.01 and 1) for 85 h. The subsequent step involved harvesting the solid mass that resulted from a 1-h

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reaction between the added calcium ions and the produced carbonate ions. This reaction took place in a shaker incubator at a temperature of 35°C. We accomplished the separation of the solid mass from the supernatant through a centrifugation process. Following this, the produced solid mass underwent a 24-h drying period in an oven set at 105°C. Once completely dried, 1 g of the solid mass was meticulously ground to achieve a homogeneous consistency. This ground material was then evenly coated onto carbon-aluminum tape, preparing it for subsequent analysis.

RESULTS AND DISCUSSION

This study aimed to initially optimize the culture media for bacterial growth and MICCP performance. Following the investigation of the short- and long-term effects of varying initial urea concentrations on bacterial growth and MICCP performance, the potential of different bio-agent^{+} concentrations to mitigate growth inhibition observed at high urea concentrations and enhance biomineralization was explored. Finally, the impact of urea addition on the morphology of calcium carbonate crystals was evaluated.

Effect of defined culture media on bacterial growth Defined culture media consists of chemical components in which all constituent elements are precisely known and specified. After incubating bio-agents across all tested defined culture media, we observed a lack of growth even after 16 days post-inoculation (data not shown). The inability of bio-agents to thrive in defined culture media suggests a potential deficiency in their capacity to synthesize specific compounds crucial for growth and replication, such as amino acids, lipids, proteins, or cofactors. The inability of MB284 to grow in defined media suggests its auxotrophic nature, meaning it relies on externally supplied compounds for essential cellular processes. While this specific characteristic of MB284 has not been previously investigated, similar auxotrophic behaviors have been documented in other *L. sphaericus* strains, as reported in the literature (26–28).

Effect of undefined culture media on bacterial growth and MICCP performance Undefined nutrient-rich culture media are composed of extracts and digests from yeast, meat, or plants, with the exact chemical composition and quantities of individual components being unknown. Given the auxotrophic characteristics of strain MB284, various undefined culture media, which have been previously employed as carbon sources in the literature (4,29), were utilized in this study. The aim was to investigate the ability of these undefined media to provide the specific components essential for the growth of bio-agents and compare their performance in the MICCP process.

Our findings revealed that there were negligible differences in the duration of the lag phase and the bacterial growth rate (μ) during the logarithmic growth phase among the tested culture media (peptone, yeast, beef, and meat extract), except for skimmed milk powder, which exhibited a notably lower growth rate. The lag phase spanned slightly less than 9 h, and the growth rates measured 1.8, 1.7, 1.7, and 1.6 (OD_{600}/day) for culture media containing beef, yeast, meat extract, and peptone, respectively (Fig. S3A). Comparable results were reported by Zhu et al. (12) and Wang et al. (21), who observed a lag phase of around 8 h and a growth rate of approximately 1.2 (OD_{600}/day) when cultivating *L. sphaericus* in yeast extract.

Furthermore, our experiments demonstrated that the maximum OD_{600} values of cultures grown in culture media contained yeast extract, meat extract, peptone, and beef extract were relatively similar, measuring 1.73, 1.7, 1.61, and 1.54, respectively. These values were approximately three times higher than those obtained from skimmed milk powder. Nonetheless, the final values of OD_{600} for culture media containing yeast and beef extract were slightly higher than that for peptone and meat extract, measuring 1.48, 1.43, 1.23, and 1.15, respectively. Additionally, it was noted that the death phase commenced on the 7th day when using skimmed milk powder, while cultures grown in other nutrient-rich sources remained in the stationary phase for over 40 days indicating that the essential amino acids required for the metabolic processes of bio-agents are naturally present within the undefined nutrient-rich media utilized in this study except for skimmed milk powder. Finally, no bacterial growth was observed in the control samples containing distilled water when they were incubated with bio-agents indicating the role of undefined media in the bacterial growth.

The produced solid mass was quantified after 8 days of cell incubation. The results revealed that when yeast and beef extract components were used in undefined media, a higher amount of biomass and calcium carbonate precipitated compared to cultures with peptone and meat extract (Fig. S3B). Specifically, the solid mass produced from yeast extract, beef extract, peptone, meat extract, and control samples were quantified at 0.36, 0.34, 0.24, 0.21, and 0.023 g, respectively. These measurements were based on the addition of 20 g/L of calcium acetate in 50 mL of each culture medium.

These measurements highlight the marginally superior suitability of yeast and beef extract for fostering the production of carbonate ions during the metabolism of bio-agents, compared to the other examined culture media. The solid mass observed in the control samples can be attributed to the reaction between the calcium ions introduced through calcium acetate and the carbonate ions dissolved in the water within 8 days. Given bio-agents' evident propensity to thrive when nurtured in culture media containing yeast and beef extract, combined with the heightened calcium carbonate production and the noteworthy cost advantage of yeast extract in comparison to other components, these benefits could profoundly influence the selection of the most fitting component – yeast extract for being used in undefined culture media during urea hydrolysis.

Effect of different concentrations of yeast extract on bacterial growth and MICCP performance The choice to employ a yeast extract concentration of 20 g/L was guided by its frequent utilization in previous studies (11,12,15,21,30–32). Nevertheless, we investigated the impact of various yeast extract concentrations (5, 10, 15, and 20 g/L) on bacterial growth. Results showed that increasing the yeast extract concentration positively impacts the slope of the exponential growth phase, along with the maximum and final bacterial concentrations, typically observed after approximately 12 days following cell incubation. This suggests that, within the examined range of yeast concentrations, 20 g/L of yeast extract concentration is the most favorable for improving the kinetics of bacterial growth (Fig. S4A). Furthermore, we observed that an increase in the initial yeast extract concentration resulted in a greater quantity of solid mass production (Fig. S4B).

Boosting the metabolism of bacteria can have a significant impact on increasing the concentration of carbonate ions produced during the MICCP reactions. This enhancement in metabolic activity, in turn, augments the kinetics of calcium carbonate production (2,5,33). Furthermore, it has been documented that bacterial cell surfaces carry a negative charge, enabling them to attract calcium ions and serve as nucleation sites for the growth of calcium carbonate crystals (24). Hence, the positive correlation observed between higher bacterial concentrations, achieved by incubating bio-agents in elevated concentrations of yeast extract, plays a beneficial role in enhancing the yield of calcium carbonate production. The solid mass produced in the control samples can be attributed to a reaction between the calcium ions and carbonate that were dissolved in the yeast extract over 8 days.

It is important to note that as the initial yeast extract concentration increased, there was an upward trend in the amount of

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produced solid mass. This suggests the potential for even greater solid mass production when yeast extract concentrations higher than 20 g/L are used *in vitro* conditions. However, considering the limitations of extrapolating findings from *in vitro* to concrete environments and the potential negative impact on mortar properties at higher concentrations (34), we selected 20 g/L of yeast extract as the culture media for the remainder of the study. This choice allowed for a comparison of our results with existing literature while maintaining practical considerations for potential future applications.

Effect of urea on bacterial growth rate and MICCP performance Based on our findings, it is evident that bio-agents generate bicarbonate ions during the breaking down of the undefined medium. While a specific equation for the biodegradation pathways of undefined culture media remains elusive, Eq. 2 (35) resembles those found in the literature concerning the degradation of organic carbons. This process catalyzes the formation of calcium carbonate through the mechanism of organic carbon oxidation. Additionally, our investigation into bacterial growth and MICCP performance during urea hydrolysis highlights the interplay between urea hydrolysis and organic carbon oxidation mechanisms, with yeast extract utilized as a component in culture media.

$$C_nH_aO_bN_c\ (\text{Undefined culture media}) + (2n - b + c)\ H_2O \rightarrow (n - c)\ CO_2 + c\ NH_4^+ + c\ HCO_3^- \quad (2)$$

Concerning the impact of urea supplementation on the growth rate of bio-agents with an initial OD_{600} of 0.01, two distinct phases in bacterial growth have been revealed (Fig. 1A). Firstly, we observed a temporary growth-inhibited phase, extending for approximately more than 1 day of incubation. Subsequently, the increased growth phase followed the growth-inhibited phase, wherein the biomass concentrations of bio-agents began to rise on the second day. Consequently, urea supplementation enhanced maximum and final bacterial concentrations during the increased growth phase. The preference for supplementing urea to the culture media concerning bacterial concentration during the increased growth phase can be attributed to the alkalophilic nature of bio-agents. As discussed later, we hypothesized that adding urea raised the pH value, creating a favorable environment for bacterial metabolism.

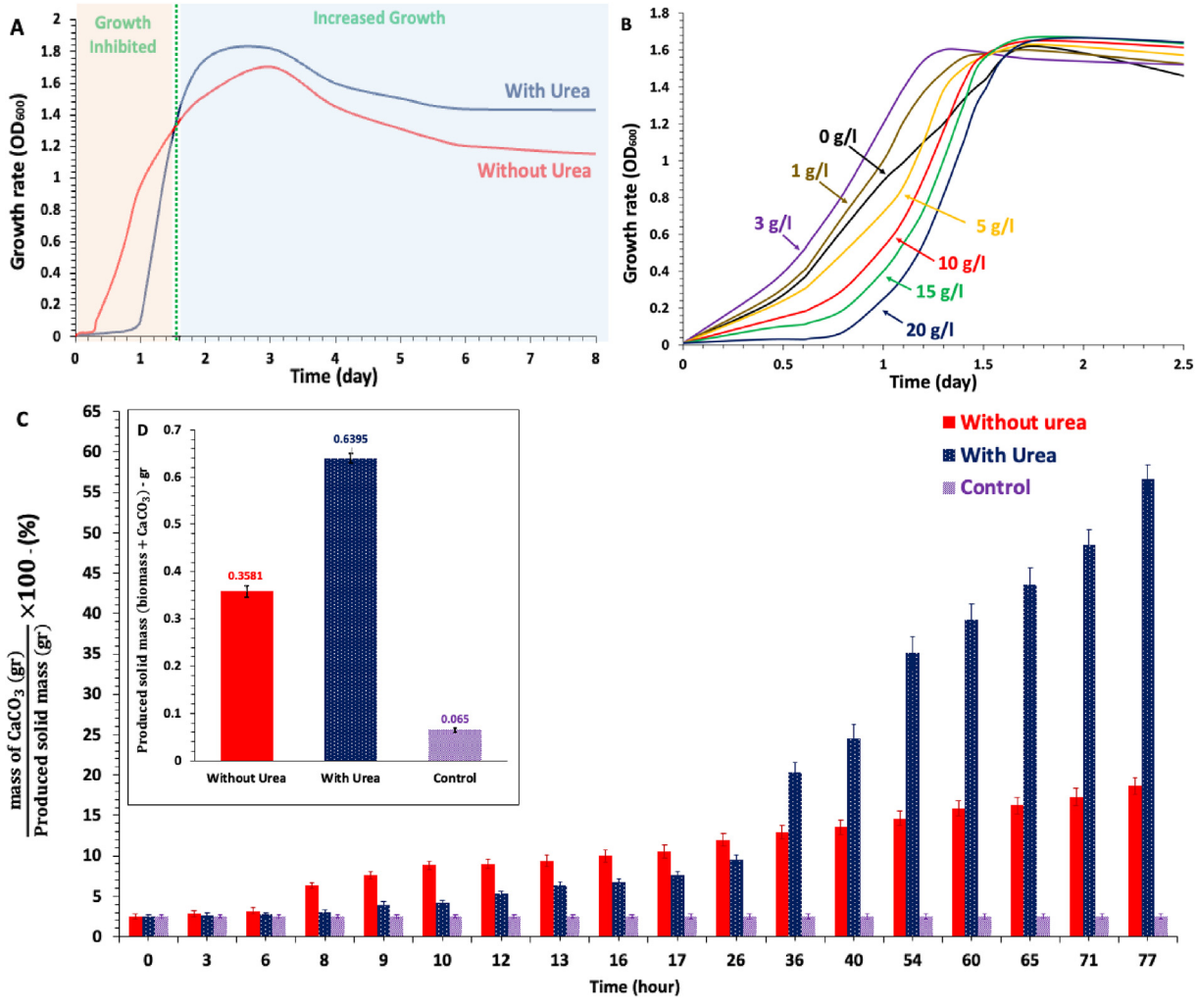

FIG. 1. (A) The growth rate of bio-agents over 8 days to assess the inhibitory effect of urea supplementation on the initiation of cell growth. In all samples, there was a base of 20 g/L yeast extract. However, one group was supplemented with an additional 20 g/L of urea, while the other group did not receive any additional urea. (B) The growth rate of bio-agents over 2.5 days when it was supplemented with 20 g/L of yeast extract and a range of urea concentrations spanning from 0 to 20 g/L. Triplicate measurements of OD_{600} were conducted, and each graph represents the average of these measurements. (C) The ratio of calcium carbonate to the produced solid mass at various time intervals from the incubation of bio-agents in two sets of experimental samples. One set contained 20 g/L of yeast extract, while the second set contained both yeast extract and urea, each at 20 g/L. (D) The produced solid mass in samples containing yeast extract (20 g/L) with and without 20 g/L of urea after 8 days of incubation with bio-agents. In both experiments, calcium acetate was introduced as a calcium source after each time interval to the culture media that had been incubated with bio-agents. The standard variation indicates the degree of proximity among the three replicates for each experiment set. Control samples consisted of 20 g/L of yeast extract without undergoing cell incubation.

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Prompted by the findings indicating the temporary inhibitory effect of urea on bacterial growth, the relationship between varying urea concentrations, ranging from 0 to 20 g/L, and growth inhibition when the initial cell concentration was 0.01 was investigated. The results showed that urea concentrations of 3 g/L and lower do not induce inhibition in bio-agents. However, when initial urea concentrations exceed 3 g/L, bio-agents exhibit reduced growth within the first one and a half days following cell inoculation (Fig. 1B). The observed trend, indicating the extension of the lag phase with higher initial urea concentrations, aligns with the findings of Mokhtar et al. (17), who reported that enhancing the initial urea concentration delayed the initiation of the growth of *L. sphaericus*.

Moreover, the investigation by Bhat et al. (18) revealed that elevated urea concentrations, ranging from 5 to 243 mg/mL, led to a delayed urea hydrolysis rate in the growth of mixed microbial consortia sourced from loamy soils. Additionally, Wang et al. (21) reported a decrease in the urea decomposition ratio by *L. sphaericus* when the initial urea concentration ranged from 200 to 3000 mM. Another study assessing the urease activity of *Thiobacillus thiooxidans*, an autotrophic bacterium, found that higher initial urea concentrations inhibited both cell growth and urease activities (36). Consequently, our experimental results align with existing literature, supporting the inhibitory effect of high urea concentrations on bacterial growth rates. However, our findings suggest that this inhibitory effect is temporary and diminishes after approximately 1 day, contingent on the initial urea concentration.

It is worth noting the correlation between increased urea concentrations (10, 15, and 20 g/L) and higher final biomass concentrations. This observation suggests that while there is an initial inhibitory effect from urea, higher concentrations of urea ultimately lead to greater biomass concentrations. While we observed that bio-agents cannot utilize urea as their sole carbon source, the exact extent to which it participates in co-metabolism remains uncertain. However, the direct connection between the ultimate biomass concentration and the initial urea concentration can be attributed to the higher pH levels induced by elevated urea concentrations, which will be discussed further in this section. It is postulated that this elevated pH environment fosters the growth of strain MB 284, as mentioned earlier.

Furthermore, it was observed that urea supplementation consistently lowered the ratio of calcium carbonate within the produced solid mass at all measurement points during the initial 26 h of cell incubation (Fig. 1C). This phenomenon reflects the inhibitory effect of urea on the growth of bio-agents. The inhibition in bacterial growth constrained metabolic processes, resulting in a reduced production of carbonate ions and, consequently, a decrease in calcium carbonate production. However, after 26 h of cell incubation, the composition of calcium carbonate within the solid mass exhibited an increased rate. This shift is attributed to the alleviation of the temporary inhibitory effect of urea on bacterial growth, leading to an enhanced bacterial concentration and the hydrolysis of urea. The hydrolysis process results in an augmented production of carbonate ions (Eqs. 3–7) and, consequently, calcium carbonate (Eq. 8).

$$CO(NH_2)_2 + H_2O \rightarrow NH_2COOH + NH_3 \quad (3)$$

$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \quad (4)$$

$$2NH_3 + 2H_2O \leftrightarrow 2NH_4^+ + 2OH^- \quad (5)$$

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+ \quad (6)$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \quad (7)$$

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3 \quad (8)$$

Moreover, the influence of urea exposure on the yield of the produced solid mass over 8 days of cell incubation was investigated and revealed that urea supplementation enhanced the production of the solid mass from 0.358 g (samples without urea) to 0.640 g (samples with urea) (Fig. 1D). These findings emphasize the ultimately positive impact of urea hydrolysis on the produced solid mass, even in cases where there is an initial delay in the onset of calcium carbonate production.

As NH_4^+ and CO_3^{2-} ions are generated through both organic carbon oxidation and urea hydrolysis mechanisms, resulting in an increase in EC, the impact of urea supplementation on EC changes over time was investigated while culture media were incubated by bio-agents with an initial OD_{600} of 0.01. A delayed trend in the increase of EC values was observed until approximately 10 h of cell incubation in the absence of added urea, corresponding to the lag phase for bacterial growth and metabolism. The addition of urea extended this delayed trend to 26 h. This longer lag phase can be attributed to the inhibition of urea, which delayed bacterial growth and the production of ammonium and carbonate ions. However, following this initial delay, urea supplementation led to an enhanced slope of EC increment from 7.76 mS/cm/day (samples without urea) to 45.88 mS/cm/day. The increase in the EC values in the absence of urea can be attributed to the generation of NH_4^+ and CO_3^{2-}, stemming from the biodegradation of yeast extract during

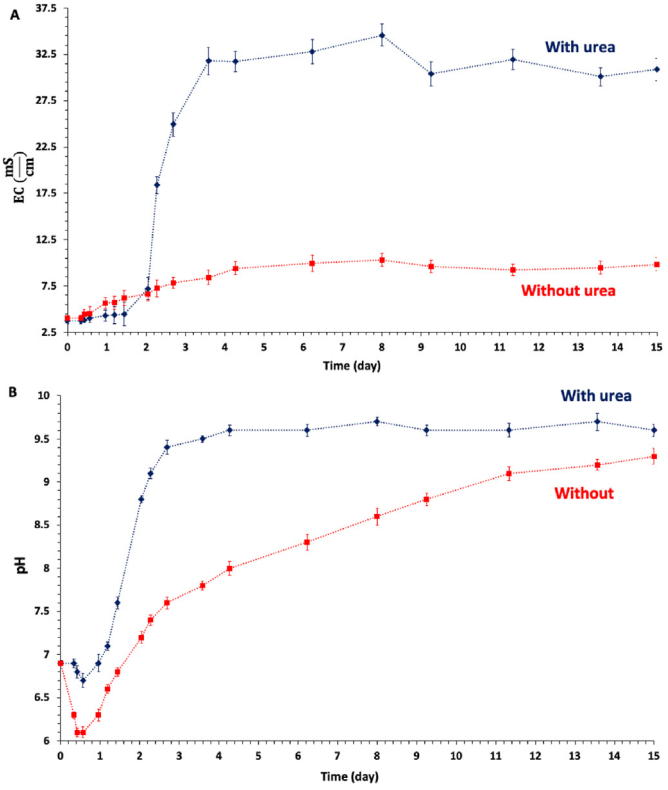

FIG. 2. The change of (A) electric conductivity (EC) and (B) pH of a culture media containing 20 g/L of yeast extract with and without 20 g/L of urea over time. The standard deviation reflects the degree of proximity or dispersion among the three replicates.

the process of organic carbon oxidation (Eq. 2), while the hydrolysis of urea was the reason for the observed surge in the trend of EC changes (Fig. 2A).

pH tracking also revealed a reduction trend ranging from 6.9 to 6.1 within the first 10 h of cell incubation in the urea-free culture media, postulated to be associated with two key factors. First, the decomposition of yeast extract may generate weak organic acids (such as amino acids) and inorganic acids (like sulfuric or sulfurous acid), as represented in Eq. 9. Second, the hydrolysis of yeast extract releases bivalent minerals, such as calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions. These ions interact with carbonic (CO_3^-) and phosphoric ions (PO_4^{3+}), leading to the precipitation of insoluble minerals like $CaCO_3$, $MgCO_3$, and $Ca_3(PO_4)_2$. This process contributes to an increase in the solution's acidity (Fig. 2B).

$$SO_{2(s)} + H_2O \rightarrow H_2SO_{3(eq)} \leftrightarrow H^+ + HSO_3^- \quad (9)$$

However, a relatively minor pH reduction was observed from 6.9 to 6.7 when urea was supplemented to the culture media, postulating that the inclusion of urea had a slight alkalizing effect on the culture media, which may have counteracted the pH reduction caused by the decomposition of yeast extract. This effect helped maintain a more stable pH value over the 26 h of cell incubation. The ability of urea to enhance the dissociation of weak acids also extends to its influence on water ionization, resulting in a decrease in hydrogen ion activity (37). Although urea supplementation delayed the onset of pH increment from 10 h to 26 h of cell incubation, the rate of pH increments significantly increased from 0.22 to 1.2 units per day by adding urea to the culture media.

The upward trend in pH values in the lack of urea can be attributed to the degradation of complex organic nitrogen-rich compounds, such as proteins and peptides found in yeast extract, into simpler nitrogen entities, specifically ammonia (NH_3). Ammonia, functioning as a weak base, can capture protons (H^+), leading to the formation of ammonium (NH_4^+) and hydroxide (OH^-) ions. This chemical transformation contributes to the alkalinity of the culture medium, as represented in Eq. 5. However, the exponential increase in pH coincided with the trend in EC when urea was supplemented, suggesting that urea hydrolysis and subsequent ammonium production were the primary factors driving this significant pH rise. The pH values following urea supplementation remained relatively stable at approximately 9.6 for at least two weeks of the cell incubation period. These results align with those of Wang et al. (21), who reported that the pH of culture media when incubated with *L. sphaericus* reached around 9.2 and remained constant.

To delve deeper into the possible inhibitory effects of urea on bio-agents, we expanded our investigation to include an analysis of the initial rate (slope) of urea decomposition. This was accomplished by measuring the concentration of ammonium, a product of the urea hydrolysis process, across a range of initial urea concentrations, which varied from 0.25 to 24 g/L urea, all within a medium containing 20 g/L yeast extract. The rate of ammonium production was subsequently normalized by the final bacterial concentration, using OD_{600} as the scaling factor (Fig. S5). We observed a trend consistent with the Haldane–Andrews equation and the substrate inhibition model (38). At lower concentrations, heightened degradation rates were noted, indicative of first-order kinetics. However, as the concentration exceeded a specific threshold, approximately 3 g/L, there was a rapid decline in degradation rates.

This observed phenomenon aligns with insights presented by Bhat et al. (18). Their study similarly revealed a parallel trajectory in the urea hydrolysis process conducted by mixed consortia from loamy soil, consistent with the kinetics described by Haldane–Andrews. It illustrated the inhibitory effect of urea on the initial rate of urea hydrolysis. Subsequently, employing data modeling techniques, they established that the substrate inhibition model provided a robust fit, outperforming the Michaelis–Menten model. Our results harmonize with similar observations in the literature, offering compelling evidence for the inhibition of bacterial growth rates and metabolisms by urea.

Effect of utilizing bio-agents$^+$ on expediting urea hydrolysis rate It is observed that urea inhibition temporarily affected bacterial growth, with the initiation of biomineralization observed after about 26 h of *in vitro* cell incubation. However, the duration of the inhibition period during concrete self-healing is uncertain and may potentially be extended. The delay in solid mass production upon crack initiation could contribute to crack propagation and the need for more time to fill void spaces during urea hydrolysis. This may also increase the consumption rate of available carbon and nutrient sources, emphasizing the need to mitigate the inhibitory effect of urea on MICCP reactions in concrete self-healing.

Ureolytic bacteria rely on the secretion of urease, catalyzing the hydrolysis of urea. This enzymatic process expedites the production of ammonium and carbonate ions, playing a pivotal role in the MICCP process (2,12,13,33). Our hypothesis suggests that temporary urea inhibition of bacterial growth and biomineralization, as we observed in previous sections, can be mitigated by employing cells previously cultivated in urea-rich media (bio-agents$^+$) containing endogenous urease. The pre-produced endogenous urease can initiate the conversion of urea, thereby reducing the urea concentration in the environment. This leads to the alleviation of urea inhibition on bacterial growth, accelerates bacterial metabolisms, expedites the carbonate ions production, and enhances the initial yield of calcium carbonate. Consequently, by utilizing bio-agents$^+$, we anticipate observing a quicker initiation of bacterial growth and an enhancement in the kinetics of calcium carbonate production.

The investigation into the rate of urea hydrolysis, conducted by stamping bio-agents and bio-agents$^+$ onto the central region of UIAPs designed to quantify urease release capacity, revealed notable differences. Bio-agents released urease after approximately 32 h, while bio-agents$^+$ exhibited urease release after only around 20 h. The regions affected by urease release, darker areas marked with dotted circles in Fig. 3, clearly indicate this difference. Control experiments involved introducing 1,10-phenanthroline, a urease inhibitor, to the UIAP before stamping it with bio-agents and bio-agents$^+$. In these experiments, no spreading darker area appeared on the surface of UIAP over the 38-h experiment duration, confirming that the observed darker area resulted from urease-mediated urea hydrolysis (data not shown). Therefore, the accelerated urea hydrolysis by bio-agents$^+$ can be reasonably attributed to the presence of endogenous urease, facilitating the conversion of urea to ammonia.

Effect of employing bio-agents$^+$ on bacterial growth and MICCP performance Based on the observation that bio-agents$^+$ displayed a faster initiation of urea hydrolysis, it is postulated that this acceleration in urease production can alleviate the growth inhibition caused by urea, consequently expediting the onset of bacterial growth. Therefore, in this experiment, the bacterial growth rates of bio-agents$^+$ and bio-agents, each with an initial OD_{600} of 0.01, were compared over 3 days in a culture medium containing 20 g/L of yeast extract and urea.

The results revealed that incubating the culture media with bio-agents$^+$ led to a shorter lag phase, occurring at approximately 18 h after cell incubation, in contrast to bio-agents, which exhibited a lag phase of approximately 21 h. This finding suggests a reduction in the inhibitory effect of urea on the bacterial growth rate, thus confirming the potential positive impact of employing bio-agents$^+$ in mitigating urea inhibition. Despite the observed differences in

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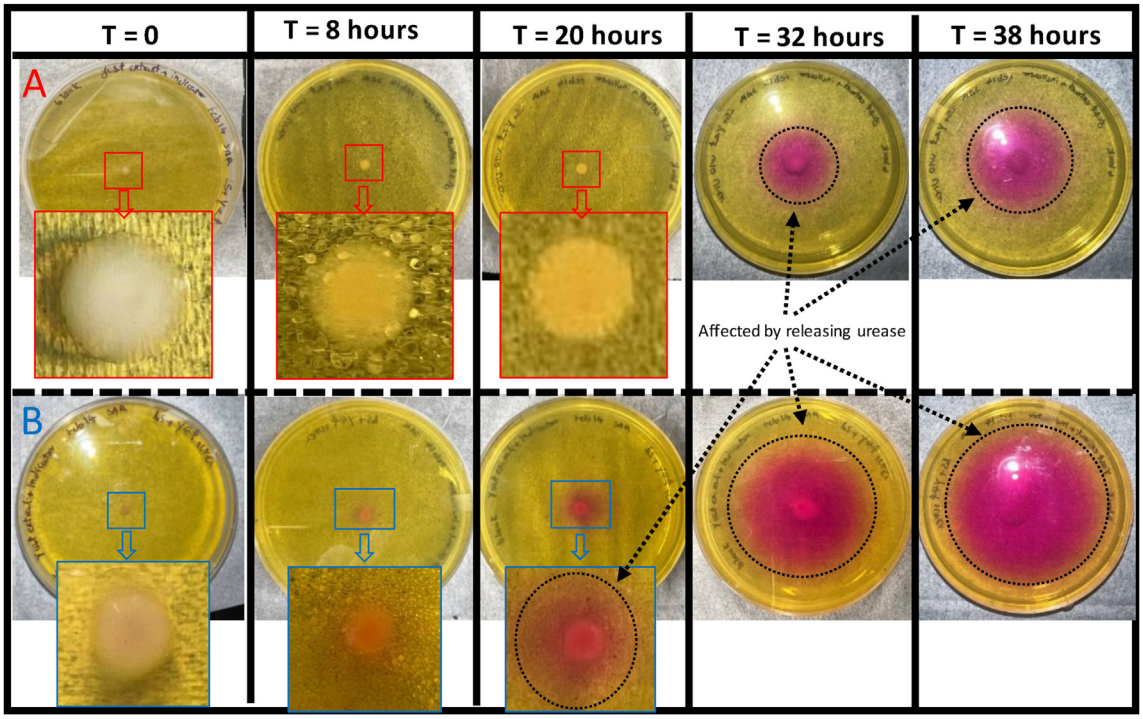

FIG. 3. The urea hydrolysis rate was assessed over a 38-h incubation period using a urease indicator agar plate (UIAP) with (A) bio-agents and (B) bio-agents^{+}. The dotted circles on the agar plate mark the areas where urease was released by the stamped cells. Each experiment was conducted with five replicates, and the time noted at each step represents the average based on observations from these 5 replications. To maintain simplicity, only one sample for each step is shown as a representative of all five replicates.

the lag phase, the final biomass concentration was nearly identical for both groups of cells after 3 days of cell incubation (Fig. 4A).

Following the positive impact of employing bio-agents^{+} on expediting the initiation of bacterial growth, we further compared the yield of calcium carbonate production by bio-agents^{+} and bio-agents over around 3-day periods of cell incubation at various time intervals. As depicted in Fig. 4B, an increase in the initial yield of produced calcium carbonate was observed after 26 h of cell incubation when bio-agents^{+} were incubated in the culture media compared to bio-agents. This suggests potential advantages in incubating culture media with bio-agents^{+} for enhancing the initial yield of calcium carbonate production.

Effect of the initial cell concentrations on bacterial growth and MICCP performance Despite the challenges in comprehending the inhibition pathway that hinders the growth of strain MB284, existing literature emphasizes the importance of the resilience of certain toxic chemicals to the toxic tolerance exhibited by different cell concentrations (39). Therefore, this section aims to investigate the dependency of the growth-inhibitory effect of urea on different bacterial cell concentrations. Considering the superior performance of bio-agents^{+} in overcoming the inhibitory effects of urea, this section explores the incubation of bio-agents^{+} in culture media containing yeast extract and urea (both at 20 g/L) to examine bacterial growth rates and MICCP performances.

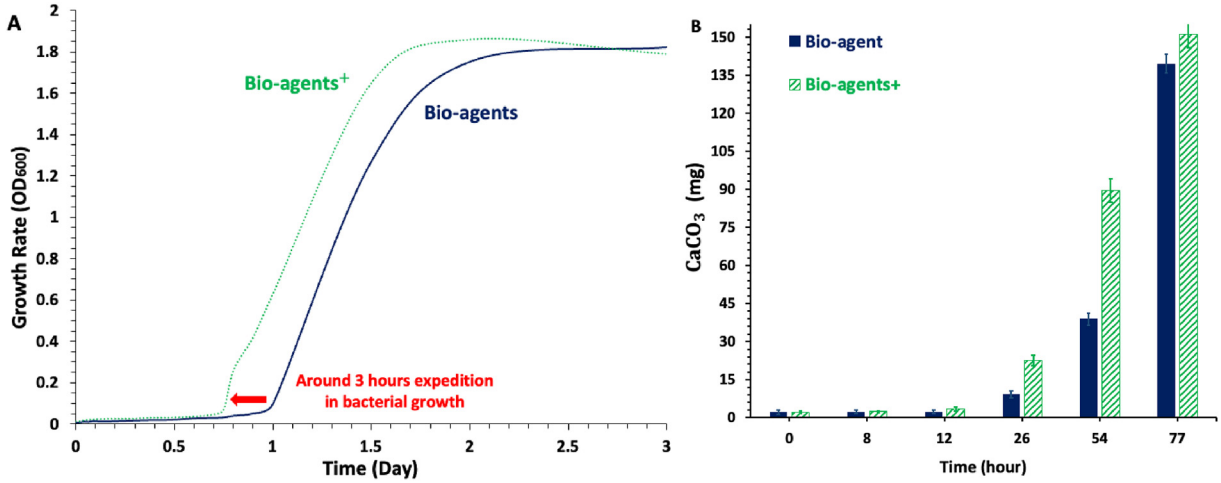

FIG. 4. (A) The growth rate and (B) the quantity of calcium carbonate produced by bio-agents and bio-agents^{+} during incubation in culture media containing urea and yeast extract (20 g/L each) over time. (A) Each graph depicts the average of three replicates. (B) Calcium acetate (20 g/L) was introduced at the end of each interval to samples with 50 mL of culture media. The standard deviation for triplicates is displayed on each bar.

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The comparison of bacterial growth rates at various initial cell concentrations revealed a significant reduction in the duration of the lag phase for bacterial growth as the initial concentration of bio-agents^{+} increased from an OD_{600} of 0.01 to values exceeding 0.1. This observation underscores the close relationship between cell concentrations and the temporary inhibition of urea on bacterial growth, with higher cell concentrations effectively mitigating the temporary growth inhibition induced by elevated urea concentrations (Fig. 5A). The results indicate the enhanced adaptive strategy of the high concentrations of strain MB284 to cope with urea inhibition. Additionally, the higher availability of pre-produced urease housing in elevated concentrations of bio-agents^{+} is expected to reduce urea concentration and alleviate its inhibitory effect on bacterial growth, as evidenced by the decreased length of the lag phase with increasing cell concentrations. Furthermore, a significant disparity in final cell concentrations was noted between samples incubated with higher initial cell concentrations (OD_{600} ranging from 0.1 to 1) and those with lower initial cell concentrations (OD_{600} of 0.01). This difference underscores the crucial role of utilizing higher initial cell concentrations in alleviating urea inhibition and enhancing the final biomass concentration.

Fig. 5B also illustrates that the quantity of produced solid mass, normalized based on the corresponding final OD_{600} values after 85 h of cell incubation, was higher with increased initial OD_{600} values, emphasizing the advantage of incubating a high cell concentration for enhancing biomineralization purposes. These findings are consistent with the literature, highlighting that elevating the initial cell concentrations has the potential to improve both the urea hydrolysis rate and the yield of calcium carbonate (21,36,20,19). The observed enhancement in biomineralization yield for samples incubated with higher initial cell concentrations may be attributed to providing more nucleation sites for carbonate deposition and the overall higher production of urease, mitigating urea inhibition, and amplifying carbonate ion production. Moreover, the use of bio-agents^{+} with more pre-produced urease further promoted the kinetics of urea hydrolysis and carbonate production, ultimately augmenting the rate of calcium carbonate production.

It is noteworthy to emphasize that prior research by Murugan et al. (20) revealed that, while increasing initial cell concentrations enhanced calcium carbonate production, the quantity of calcium carbonate produced remained comparable for samples with an OD_{600} of 0.4 and 0.5. Furthermore, Wen et al. (19) reported no significant difference in the amount of calcium carbonate produced between samples with initial cell concentrations of 0.6 and 1. In contrast, our observations indicate a subtle yet persistent increase in the quantity of produced solid mass, normalized based on OD_{600}, with an elevation in the initial concentration of bio-agents^{+}. We posit that this observed increasing trend in the amount of produced solid mass, across the examined range of OD_{600}, is likely attributed to the higher content of pre-produced urease within bio-agents^{+}, leading to an enhanced production of carbonate ions.

Furthermore, monitoring the EC values of culture media incubated with various initial concentrations of bio-agents^{+} revealed an accelerated surge in EC when the OD_{600} exceeded 0.1, indicating the production of ammonium and carbonate ions (Fig. 5C). Despite a short lag phase in bacterial growth observed in Fig. 5A, EC values rose shortly after incubating the culture media with bio-agents^{+}. The initial increase in EC values can be attributed to the pre-produced endogenous urease within the bio-agents^{+} hydrolyzing urea. Additionally, after the initial increase and subsequent bacterial growth, the rise in EC values can be linked to the decomposition of yeast extract and urea. There is also a direct relationship observed between the rate of EC increment and concentrations of bio-agents^{+}, indicating the enhanced rate of yeast extract decomposition and urea production with increased cell concentrations.

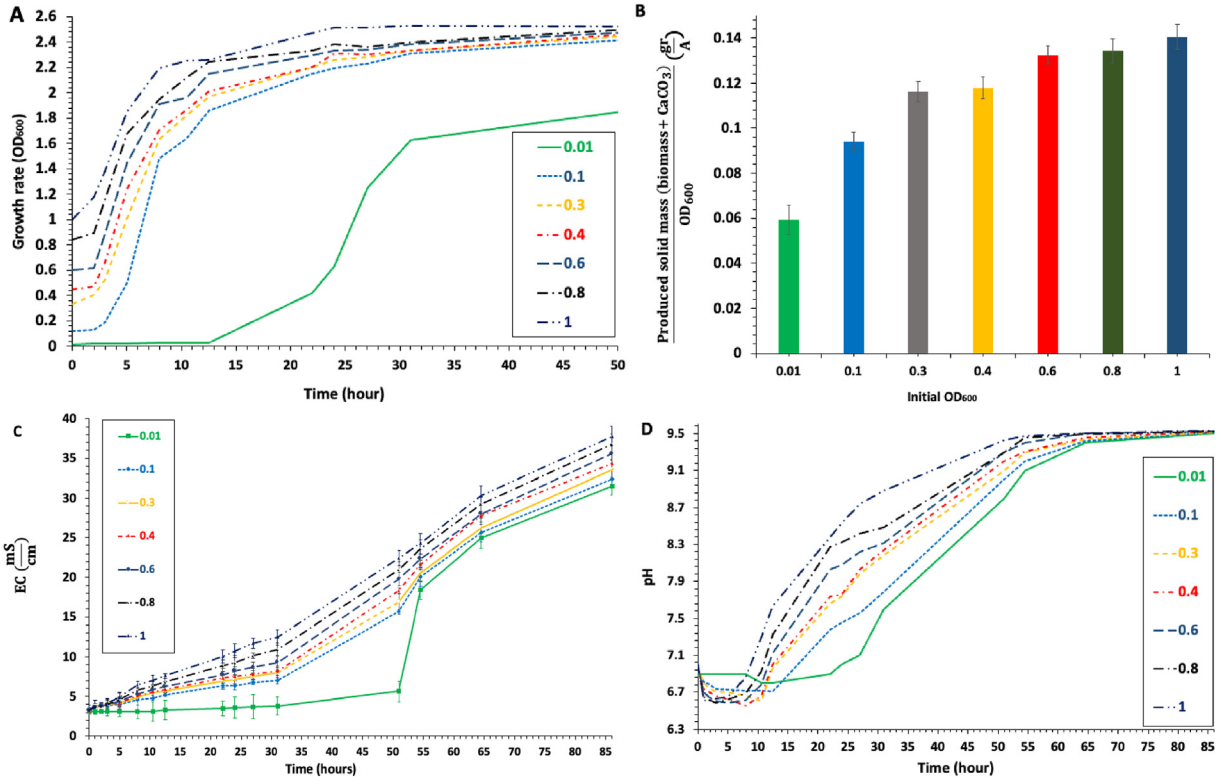

FIG. 5. The impact of incubating culture media with different initial cell concentrations on (A) bacterial growth rate, (C) EC, and (D) pH changes over time. (B) The amount of produced solid mass normalized based on the final OD_{600} after 85 h of cell incubation in different initial cell concentrations. (A, D) Each graph represents the average of three replications. (B, C) The standard deviation, referring to triplication, shows the proximity of data for each data point.

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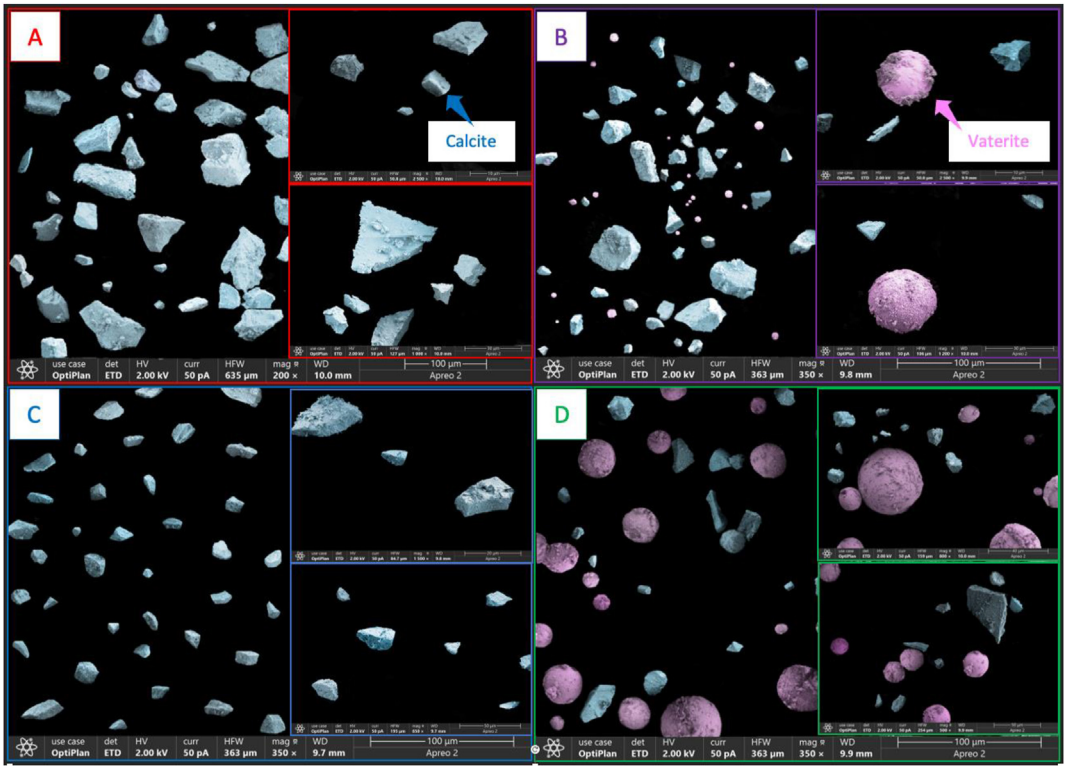

FIG. 6. The SEM images of produced $CaCO_3$ in different experimental conditions. The initial cell concentration was 0.01 (OD_{600}) and the culture media contained 20 g/L of yeast extract, (A) without urea and (B) with 20 g/L of urea. The initial cell concentration was 1 (OD_{600}) and the culture media contained 20 g/L of yeast extract, (C) without urea, and (D) with 20 g/L of urea. To enhance the visibility of the produced crystals, they were isolated, and specific crystals of calcite and vaterite were marked.

An initial pH reduction was observed across the range of bio-agents$^+$ concentrations, attributed to the production of organic and inorganic acids and the consumption of carbonate ions. However, an earlier and more pronounced upward trend in pH values was noticed in samples with higher initial cell concentrations (Fig. 5D). This trend suggests that a high concentration of bio-agents$^+$ can accommodate a greater amount of pre-produced endogenous urease, enabling the conversion of urea to ammonia and then increasing pH. Furthermore, although the pH values were higher for samples incubated with higher cell concentrations, they eventually stabilized at approximately 9.50 $\pm$ 0.02 after around 85 h of cell incubation.

Effect of urea supplementation on morphology of produced crystals of calcium carbonate Exploring the impact of different calcium carbonate crystals on the structural integrity of healed concrete (19), this section delves into the investigation of the role of urea in the formation of diverse calcium carbonate crystals in culture media with varying initial cell concentrations. The findings revealed the presence of calcite crystals irrespective of urea supplementation or initial cell concentrations (Fig. 6). Remarkably, the diversity in the size of calcite crystal formations was more pronounced when urea was introduced to the culture media. Additionally, the quantity of vaterite exhibited a significant increase in urea-supplemented culture media, indicating a delay in the conversion from vaterite (characterized by spherical crystals) to calcite and the subsequent growth of calcite crystals. Vaterite formation occurs shortly after the rapid dehydration process leading to amorphous calcium carbonate (ACC). In contrast, the slower recrystallization (solution–dissolution) process results in the stable formation of calcite (40). Previous studies suggest that certain organic components, such as amino acids, copolymers, and surfactants, can transiently stabilize vaterite (40,41). Considering that urea leads to the production of soluble microbial products (SMPs) like urease, it is hypothesized that urea plays a role in delaying the transformation of vaterite to calcite.

Moreover, an elevation in the quantity of generated crystals was noted with an increase in the initial cell concentration from OD_{600} 0.01 to 1. This observed correlation between the number of crystals and initial cell concentrations aligns with established literature, emphasizing the role of cells as nucleation sites for calcium carbonate formation (20,19). For improved clarity, calcium carbonate crystals were isolated, and specific crystals of calcite and vaterite are highlighted in Fig. 6. To facilitate a thorough comparison, primary images depicting calcium carbonate crystals formed in culture media with an initial OD_{600} of 0.01 without urea (Fig. S6), initial OD_{600} of 0.01 with urea (Fig. S7), initial OD_{600} of 1 without urea (Fig. S8), and initial OD_{600} of 1 with urea (Fig. S9), along with different magnifications, are shown in the supplementary material.

A key limitation of this study lies in ensuring the long-term effectiveness of endogenous urease within bio-agents$^+$. In real-world self-healing applications, these agents may reside in concrete for extended periods and be exposed to harsh environmental conditions like extreme temperatures, high salinity, and low humidity. Therefore, evaluating the impact of these parameters on the resilience of bio-agents$^+$ housing endogenous urease is crucial. Determining the half-life of endogenous urease within these cells is an essential area of future exploration. This will shed light on the specific conditions and duration under which bio-agents$^+$ offer significant advantages over traditional bio-agents for MICCP applications.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2024.03.004.

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