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Generation of enhanced endospores for microbially induced calcium carbonate precipitation (MICCP) via thermal shock for concrete self-healing

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ABSTRACT

Endospores are desirable phenotypes in Microbially-Induced Calcium Carbonate Precipitation (MICCP) for their durability in challenging environments. This study investigates the impact of various endosporulation methods on endospore morphology and their subsequent performance under harsh conditions. Results revealed that the Thermal Shock (TS) method produced larger endospores, possibly due to multi-layer encasement. Notably, endosporulation and germination ratios, besides MICCP effectiveness, achieved via TS-produced endospores surpass that of the Carbon Starvation method, both *in vitro* and within a cement-paste setting under freeze and thaw cycling. This outcome underscores the pivotal role of utilizing TS-produced endospores for concrete self-healing, particularly in cold areas.

1. Introduction

Ensuring the resilience and optimal performance of bacteria utilized in microbial-induced calcium carbonate precipitation (MICCP) for the self-healing of concrete over time poses a formidable challenge. Throughout the phases of concrete casting, hardening, and service life, bacteria are exposed to a spectrum of environmental stresses. These stressors include high pressure, temperature fluctuations, hypersalinity, shear stress, and exposure to highly alkaline conditions (pH ~ 12), compounded by limitations in oxygen, moisture, carbon sources, nutrients, and living space [1–4]. While the vegetative state of most bacteria often struggles to endure such inhospitable conditions [5], certain bacterial genera, notably *Bacillus* and *Clostridium*, have evolved the ability to form dormant endospores. These endospores exhibit the remarkable capacity to remain inactive in the absence of vital elements, such as carbon and nutrient sources, oxygen, and humidity, over an extended period. This adaptability enables them to germinate upon exposure to favorable conditions [6–9].

Nevertheless, the literature highlights subjecting endospores to harsh conditions during the dormant phase also adversely impacts the viability, bacterial growth rate, and enzymatic activities of germinated endospores [2,3,9]. For example, Wang et al. (2017) noted that exposing endospores to alkaline conditions resulted in a delayed initiation of the exponential growth phase [10]. They further observed that reducing the temperature from 28 °C to 10 °C extended the germination time from 12 hours to 6 days and reduced the rate of urea hydrolysis, from 82.5 to 25.4 mM decomposed urea per hour, resulting in diminished MICCP performance and delayed calcium carbonate production. These adverse effects of harsh conditions on bacterial behavior and MICCP performance may be attributed to a reduction in the germination ratio, representing the percentage of endospores that remain viable during the dormant phase and successfully germinate. This reduction indicates the limited survivability rate of endospores under inhospitable conditions.

One of the defense mechanisms employed by endospores against adverse mechanical, physical, and chemical conditions is attributed to the encapsulation of cells with multi-protective layers, ensuring the safeguarding of the core's genetic content [11,12]. These layers, including the outer membrane, cortex, and inner membrane, play a crucial role in reducing cell membrane permeability to toxic chemicals and enhancing adaptability to extreme environmental factors such as high temperatures, toxins, and enzymatic attacks [7,9,13]. Consequently, augmenting the quantity of these additional protective layers

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Table 1
Procedure for running different endosporulation methods.

Endosporulation method	Culture media	pH of culture media	Temperature during inoculation	Incubation time	Sample's name
Vegetative cells	Yeast extract	7	35 °C	2 days	A
NS method	DI water	7	35 °C	2 days	B
CS method	MSM	7	35 °C	2 days	C
		3			D
		9			E
		7	45 °C		F
			2 °C		G
			35 °C	1 month	C^+
TS method	MSM	7	35 °C	2 days	H
		9			I
		7		1 month	H^+

holds the potential to bolster the resilience of endospores, thereby improving their capacity to survive and germinate after exposure to harsh conditions, ultimately enhancing the germination ratio. Given that the morphology of endospores, encompassing the arrangement of protective layers and the core, can be influenced by various parameters such as the type of species and endosporulation method [14–16], it is hypothesized that engineering the endosporulation method may lead to an enhancement in the thickness of protective layers around the core. This, in turn, could potentially amplify the germination ratio and kinetics of calcium carbonate production.

This study was undertaken to investigate the morphological characteristics of *Lysinibacillus sphaericus* MB284 endospores generated through different endosporulation methods under varying environmental conditions. Our primary objective was to pinpoint an endosporulation method capable of yielding larger endospores, potentially with additional protective layers, as well as achieving a higher number of produced endospores. Subsequently, we delved into the survivability of produced endospores when exposed to harsh conditions including FTC, saline and alkaline conditions and using aged endospores. Following this, we examined the behavior of germinated endospores and their MICCP performance, both *in vitro* and within a cement paste environment after exposure to FTC. The contribution of this study is to illuminate the critical role of employing highly tolerant endospores under challenging conditions in the self-healing of concrete leading to the enhancement of the yield of calcium carbonate production.

2. Material and methods

2.1. Vegetative cells preparation

The strain MB284, identified as *Lysinibacillus sphaericus* 13805™ and deposited as *Bacillus lentus* Gibson, was procured from the American Type Culture Collection (ATCC) based on its notable capacity for endospore production and high calcium carbonate production kinetics [5]. Bacterial growth was monitored *in vitro* by measuring the optical density at 600 nm (OD_{600}) using a Thermo Scientific™ GENESYS 20 spectrophotometer in the absence of added calcium ions, as the production of calcium carbonate can interfere with optical measurements.

To obtain vegetative cells, 1 μL of strain MB284 with an OD_{600} of 1 (approximately equivalent to 10^9 cells/mL) was inoculated into 50 mL of culture media containing 20 g/L of yeast extract until the biomass concentration reached $OD_{600} \approx 1$. Subsequently, 1 μL of this culture was transferred to fresh media with the same composition, repeating the process until the cell concentration reached $OD_{600} \approx 1$. Cells were separated from the supernatant by centrifugation at 7830 rpm and 25°C (Eppendorf 5430 R) for 10 minutes. After three washes with phosphate buffer solution (PBS), the vegetative cells were resuspended in 50 mL of PBS for use in various endosporulation methods described in Table 1.

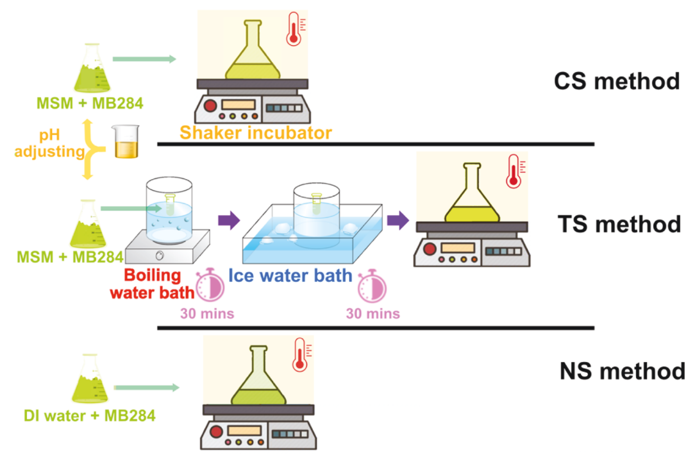

Fig. 1. The schematic representation of various endosporulation methods, including Carbon Starvation (CS), Thermal Shock (TS), and Nutrient Starvation (NS) methods. During endosporulation, the vegetative phenotype of MB284 was utilized and MSM was used as a source of nutrients.

2.2. Endosporulation methods

Three distinct endosporulation methods were employed in this study, as illustrated in Fig. 1. I) The Nutrient Starvation (NS) method involved inoculating 1 mL of vegetative cells (prepared in Section 2.1.) into 50 mL of distilled water (pH 7) at 35°C for 2 days. II) The Carbon Starvation (CS) method entailed inoculating vegetative cells (1 mL) into 50 mL of Minimal Salts Media (MSM) devoid of carbon sources. The inoculation was performed under varying pH conditions (pH 3, 7, and 9) at temperatures of 2, 35, and 45°C in a shaker incubator for 1 month. The composition of the MSM was detailed in a previous study [5] and further explained in a supplementary file. III) The Thermal Shock (TS) method involved injecting 1 mL of vegetative cells into 50 mL of MSM within sealed falcon tubes. These tubes were then indirectly exposed to a boiling water bath (100°C) for 30 minutes, followed by immediate transfer to an ice water bath for an additional 30 minutes. Throughout these procedures, the tubes remained submerged in both the boiling and ice water baths. Due to the tubes being capped, there was no direct contact between the cells and the water. Instead, heat was transferred via conduction through the tube walls. Subsequently, the pH of the MSM was adjusted to both 7 and 9, and the samples were stored at 35°C for 1 month.

2.3. Number of endospores

To compare the efficacy of different endosporulation methods in converting vegetative cells to endospores, the number of produced endospores serves as a relevant metric. For endospore quantification, 10 μL of the same concentrations of cells inoculated into the MSM or DI water after each endosporulation method were mixed with 10 μL of PBS, stained, and observed under an optical microscope (Leica DM 2700 M, USA). The average number of endospores in one grid (20 μm * 20 μm) from 10 different grids was considered representative of the endospore count [5]. To stain the endospores, The Schaeffer-Fulton method was employed, which involved adding malachite green and safranin to the cells dried on the microscope slides. The gram-staining method was also applied for staining vegetative cells based on adding crystal violet, iodine, and safranin to dried cells.

2.4. Scanning electron microscopy

To scrutinize the generated endospores, scanning electron microscopy (SEM) imaging was conducted using the Apreo 2 S Low Vac – ThermoFisher. The imaging process was executed at 1.00 kV and 0.10 nA, employing an Everhart–Thornley detector (ETD). The reported average length of the 20 endospores produced through both CS and TS methods represents the length of each type of produced endospore. In the process of sample preparation, endospores suspended in culture media underwent initial fixation using glutaraldehyde. Subsequently, gradual dehydration was achieved through a series of ethanol solutions, replacing water with a substance compatible with the SEM vacuum [17]. After ethanol drying, the specimens were affixed to a metal stub using sticky carbon sheets coated with aluminum tape.

2.5. Exposure of endospores to harsh conditions

Stringent conditions were imposed on endospores generated through both CS and TS methods, which were inoculated in 50 mL of MSM. These conditions included exposure to alkaline environments achieved by adjusting the pH to 12, saline conditions induced by the addition of 50 g/L of sodium chloride, and freeze and thaw cycling (FTC) involving 7 cycles of alternating temperatures: −4°C and 25°C, with each temperature phase lasting 24 hours. After enduring these conditions for 2 weeks, the cells were germinated. Furthermore, to assess the durability of the produced endospores before germination, both TS and CS-produced endospores were inoculated in MSM after 100 days as aged endospores, followed by germination. This investigation aimed to evaluate how well the endospores retained their viability and functionality over an extended period, providing insights into the robustness of the produced endospores under aging conditions.

2.6. Germination and growth of bacteria

To assess the germination ratio, two sets of culture media were prepared, comprising MSM and endospores as previously described. One set underwent exposure to various harsh conditions, including FTC, alkaline, and saline conditions (50 g/L NaCl), while the other set was stored at a constant temperature of 35°C under neutral pH. To produce aged endospores, the culture media containing MSM inoculated with endospores were stored in a shaker incubator at 35°C under neutral conditions for 100 days. Following exposure to these conditions, the number of germinated endospores was evaluated on the second day of cell incubation on the agar plates using the pour-plate technique (20 g/L yeast extract and 10 g/L agar) [18]. Quantification of the germinated endospores, as determined by Eq. 1, aimed to elucidate the intricate relationship between environmental stressors and the germination outcome.

$$\text{Germination Ratio} = \frac{\begin{array}{c}\text{Number of germinated endospores}\\ \text{after exposure to harsh conditions}\end{array}}{\begin{array}{c}\text{Number of germinated endospores}\\ \text{before exposure to harsh conditions}\end{array}} \times 100 \tag{1}$$

To evaluate bacterial growth, 1 μL of each culture medium containing MSM inoculated with endospores was introduced into a culture medium comprising 50 mL of MSM, yeast extract, and urea (20 g/L each). The resulting mixture underwent incubation in a shaker incubator, maintained at a constant temperature of 35°C, for 3 days. This incubation duration was selected to facilitate the successful transition of endospores into vegetative cells through the germination process. The controlled environment and nutrient-rich medium supported the optimal conditions for bacterial growth and development during this incubation period.

2.7. MICCP performance measurement

2.7.1. In vitro

To evaluate the *in vitro* performance of MICCP, following the germination of endospores produced in the culture media (as previously described), we directly measured the pH and electrical conductivity (EC) of the culture media. Probes from a pH meter (Scientific Accumet Basic AB15, US) and an EC meter (Mettler Toledo, US) were inserted into the culture media throughout the experiment. To induce calcium carbonate production, we introduced 20 g/L of calcium acetate (serving as the calcium source) into 50 mL of culture media after a 7-day incubation period at 35°C. The resulting mixture underwent agitation in a shaker incubator for 1 hour. Subsequently, we separated the supernatant from the precipitated solid mass (residue) through centrifugation at 7830 rpm and 25°C for 20 minutes. As unreacted yeast extract and calcium acetate remain soluble in the supernatant, we deduced that the solid mass primarily contains produced calcium carbonate, cells, and minor other salts.

Next, we subjected the solid mass to a drying process in an oven (Isotemp 550–58 Muffle Furnace, Fisher Scientific) at 100°C for 24 hours, eliminating moisture content and transforming the material into a dry powder. We weighed the samples using an Analytical Balance from Sartorius to determine the quantities of the solid mass. Additionally, we conducted thermogravimetric analysis (TGA) using a TA Q5000 instrument to quantify the amount of calcium carbonate residue. The residue was finely ground to achieve a particle size of less than 75 μm. Finally, approximately 25±5 mg of the solid mass was deposited onto a high-temperature platinum pan, and the test was conducted within a temperature range of 30–900°C, using a ramp rate of 10 °C/min.

$$CaCO_3\ (\text{mg}) = \text{Weight of solid mass (g)} \times \left(\text{Weight ratio of } \frac{CaCO_3}{\text{Solid mass}} (\%)\right)_{\text{Obtained from TGA}} \times 10^{-3} \tag{2}$$

2.7.2. Cement paste

We assessed endospore application in cementitious composites, employing a total of 12 cement paste samples. These samples were formulated with Type I/II ordinary Portland cement, maintaining a water-to-cement ratio of 0.42, following ASTM C150 specifications [19]. The samples were dimensioned at 7.62 × 15.24 cm (diameter × height). Vegetative cells and endospores, generated through both TS and CS methods, were separately introduced into the water mixture to achieve a concentration of 10^9 cells/mL. Subsequently, this mixture was applied to the cement paste samples in triplicate. As references for autogenous carbonation, three samples were prepared with bacterial-free water mixed with cement. All samples were batched and mixed for 5 minutes using a laboratory vacuum mixer. After 24 hours of casting, the samples were de-molded and underwent curing under a moist double-sealed condition at 23°C for 65 days. This curing process, aimed at achieving a high degree of hydration, proves effective in minimizing the autogenous self-healing effect attributed to the hydration of anhydrous cement particles within the samples.

Following the curing process, the cement paste samples underwent FTC, as per the outlined procedure in Section 2.5. Subsequently, controlled tensile loading was applied to induce cracks with a width ranging from 110 to 140 μm in all samples. Each sample's average crack width was measured at ten different points along the crack, resulting in

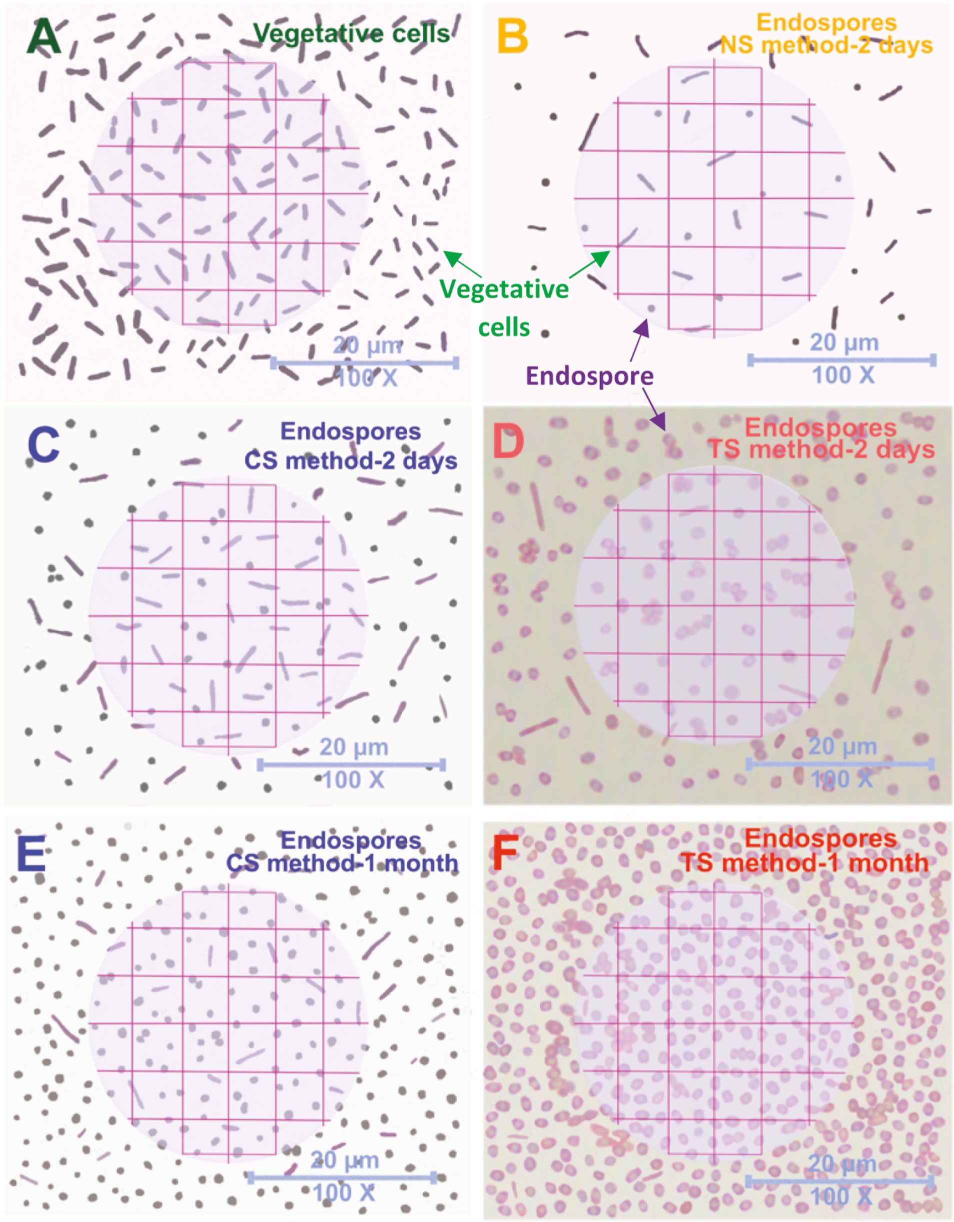

Fig. 2. Optical photomicrograph (X1000 magnification) illustrates the morphology of endospores during different endosporulation methods. A) Cells were inoculated in the MSM containing yeast extract as a carbon source under pH 7 and 35°C after 2 days (sample A). B) Cells were inoculated in DI water under pH 7 and 35°C after 2 days (Nutrient Starvation (NS) method - sample B). C) Cells were inoculated in the MSM under pH 7 and 35°C after 2 days (Carbon Starvation (CS) method - sample C). D) Cells were exposed to the Thermal Shock (TS) method and incubated in MSM at 35°C under pH 7 after 2 days (sample H). E) Cells were inoculated in the MSM under pH 7 and 35°C after 1 month (Carbon Starvation (CS) method - sample C^+). F) Cells were exposed to the Thermal Shock (TS) method and incubated in MSM at 35°C under pH 7 after 1 month (sample H^+). The quality of the images was enhanced using photo editing techniques to eliminate the negative influence of the background on the clarity of stained cells. The images with the effect of the background are provided in the supplementary files.

average crack widths of 134 µm, 128 µm, 121 µm, and 125 µm for cement paste samples containing vegetative cells, CS and TS endospores, and reference samples, respectively. To ensure fairness, the length of the cracks was approximately equal across all samples. The samples then experienced a 23-hour dry period followed by a 1-hour wet period, spanning a total duration of 28 days. During this wet-dry cycle, we immersed the samples in a solution containing yeast extract, urea, and calcium acetate, each at a concentration of 20 g/L. This solution initiated the germination process by providing yeast extract as a carbon source and enhanced the kinetics of MICCP by supplying urea and calcium acetate. We assessed crack healing through MICCP, resulting in a visible white residue within the cracks. To quantify this healing, we employed ImageJ software (version V 1.8.0) to analyze optical microscopy images of the cracked surface. Specifically, we calculated the crack healing percentage by comparing the area of the healed crack to the initial cracked area [20].

$$\text{Crack healing }(\%) = \frac{\text{Healed area of the crack }(\text{mm}^2)}{\text{Initial area of the crack }(\text{mm}^2)} \times 100 \tag{3}$$

3. Results and discussion

3.1. *Impact of endosporulation conditions on the morphology of endospores*

During the process of endosporulation, vegetative cells undergo genetic and physiological transformations, including the development of protein-coating layers around their core [21,22]. However, the impact of altered environmental conditions on endospore morphology and resilience to harsh conditions remains unclear. Our hypothesis proposes that by manipulating environmental factors, cells can augment the protective protein layers, thereby fortifying endospores against extreme conditions. To examine this hypothesis, we exposed vegetative cells to varying levels of the availability of carbon and essential nutrients for bacterial growth, temperature, pH, and incubation duration. Subsequently, we monitored the resultant changes in endospore size and shape.

3.1.1. Carbon and Nutrient Starvation

Our findings indicated that in the presence of a carbon source (yeast extract), no endospores were observed in vegetative cells (Fig. 2A). This underscores the critical role of carbon scarcity in triggering endosporulation. Endospore-forming bacteria respond to conditions of carbon starvation by detecting reduced accessible energy, initiating a complex quorum-sensing signaling pathway [23]. However, when utilizing the CS method at pH 7 and 35°C, endospores emerged after two days of carbon deprivation (Fig. 2C). Driks' study, which delves into the specific mechanism of endospore initiation in the absence of carbon sources [24], suggests that carbon availability can act as a growth-setting barrier, influencing the initiation of endosporulation in vegetative cells.

Our investigation into the influence of nutrients on endospore production revealed that under conditions of limited carbon source coupled with insufficient nutrients, there is a detrimental impact on the endosporulation process. This results in a notable reduction in endospore production (Fig. 2B), underscoring the pivotal role of nutrient availability in endosporulation. During endosporulation, endospores store essential nutrients within their core and cortex, forming protective layers around the cell. These layers serve to stabilize DNA, protecting it against environmental stress and enhancing long-term survivability [25,26]. Calcium ions play a crucial role by crosslinking the endospore coat proteins, thereby fortifying the protective layers against environmental challenges [27]. Additionally, studies suggest that ions such as Mn^{2+}, Fe^{2+}, Fe^{3+}, and Ca^{2+} act as cofactors during endosporulation. They stimulate the synthesis of enzymes (such as L-arabinose isomerase and dismutase) and facilitate the physiological transition from vegetative cells to endospores [9,28–31].

Vegetative cells were detected in both the CS and NS methods after two days of cell incubation, signifying their incapacity to initiate the endosporulation process and transform into endospores. This inability has a consequential impact on the endosporulation ratio, as discussed later. While carbon depletion served as a signal to halt bacterial growth, modifications in other environmental conditions, such as pH and temperature, potentially intensified the challenges for vegetative cells, making it more challenging to positively stimulate the initiation of the endosporulation process.

3.1.2. pH of Culture Media

Considering that the concentration of hydrogen ions (H^+) influences nutrient absorption, cell membrane permeability, and morphology [32], we investigated to understand how altering the pH of the culture media impacts endospore development during the CS method. Our findings revealed that under acidic conditions (pH=3), vegetative cells displayed low tolerance to acidity, and after the endosporulation process, neither vegetative cells nor endospores were observed (Figure SA). The compromised integrity of cells in acidic environments indicated membrane disruption, protein denaturation, and leakage of cellular components into the culture media. Conversely, alkalinity (pH=9) was found to enhance endospore production, suggesting an accelerated initiation of endospore formation, possibly due to the alkalophilic properties of strain MB284 [5]. Moreover, alkaline conditions can alter cell membrane potential, promoting the formation of a polar septum that partitions cells into distinct compartments, ultimately leading to endospore development [33]. This result aligns with existing literature and demonstrates an enhancement in the endosporulation ratio by elevating the pH of the culture media to 11 [3]. However, the morphology of endospores produced under alkaline conditions was found to be similar to those produced under neutral conditions, indicating the insignificance of elevated pH in changing the size of endospores (data not shown).

3.1.3. Temperature

During the CS method under neutral conditions, we varied the temperature to investigate the impact of extreme temperatures on the morphology of endospores. Notably, we observed that the morphology of endospores remained unaltered after exposing cells to both 45°C and 2°C. Intriguingly, both high and low temperatures were found to stimulate the endosporulation process, leading to an increased production of endospores. Consequently, we posit that strain MB284 perceives extreme temperatures as mild stress, initiating the endospore formation process. This hypothesis is grounded in literature that discusses the role of extreme temperatures in influencing the speed of endosporulation in *Bacillus* species [9].

The TS method was employed to leverage the advantages of extreme temperatures on endosporulation. The results indicated that the TS method not only increased the number of produced endospores but also influenced their morphology by enhancing the average thickness of endospores after two days of cell incubation (Fig. 2D). The underlying mechanism involves the upregulation of heat shock proteins (HSPs) within cells, synthesized in response to abrupt temperature fluctuations [34,35]. These proteins also function as molecular chaperones, ensuring the stability and proper three-dimensional structure of newly formed proteins and repairing damaged proteins caused by heat stress, which play roles in forming layers around the cells [36,37]. Consequently, this orchestrated process leads to the formation of well-structured endospores, complete with multi-layered protective coatings around their cores. It is hypothesized that this cellular adaptation can enhance the resilience of the core cells by safeguarding critical components such as DNA against a variety of stressors [35,38].

Moreover, the examination of the impact of alkaline conditions on the morphology of endospores demonstrated that incubating endospores in culture media with a pH of 9 did not alter the size of endospores produced under neutral conditions. Nevertheless, it increased the quantity of produced endospores (data not shown). Additionally, a parallel endosporulation methodology akin to the TS method was identified in a study conducted by Intarasoontron et al. for MICCP purposes [4]. However, the microscopic images depicting the morphology of the produced endospores were not provided in the study.

3.1.4. Incubation time

Given the intricate nature of the steps involved in endospore production [7,8], the duration of endosporulation can significantly influence the successful conversion of cells into endospores. To delve deeper into this aspect, we extended the inoculation period from 2 days to 1 month, maintaining a temperature of 35°C and a pH of 7. The results illustrated that prolonging the incubation time resulted in an increased production of endospores in both the CS method (Fig. 2E) and the TS method (Fig. 2F). This extension allowed the cells to undergo multiple stages of biochemical transformation, ultimately enhancing the endosporulation ratio [7]. Notably, after one month, a higher quantity of endospores produced through the TS-produced endospores, along with superior size, was observed compared to those produced via the CS method. This underscores the advantages of the TS approach with a one-month incubation time for endospore formation.

3.1.5. SEM images of endospores

To scrutinize the morphological changes during endosporulation, in addition to optical microscopy, we employed scanning electron

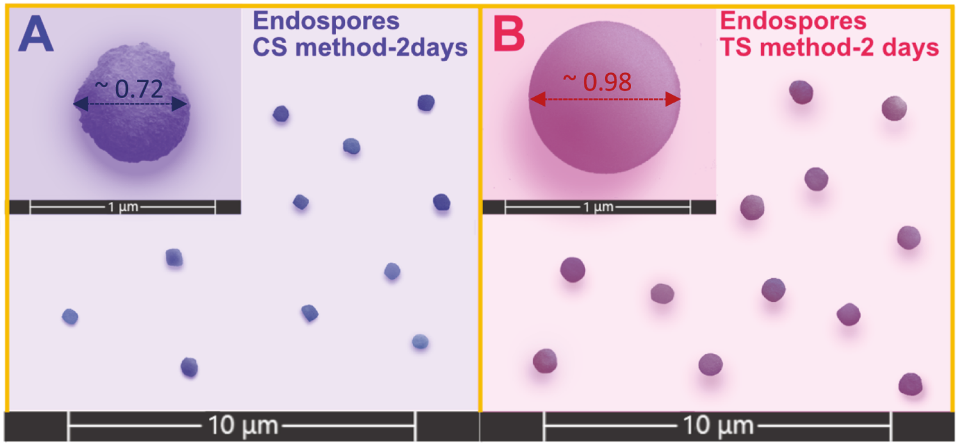

Fig. 3. SEM images depicting endospores generated through the A) Carbon Starvation (CS) and B) Thermal Shock (TS) methods at both 1 and 10 micrometers. For enhanced clarity, endospores were isolated and stained using image techniques. Unaltered images are also provided in the supplementary document.

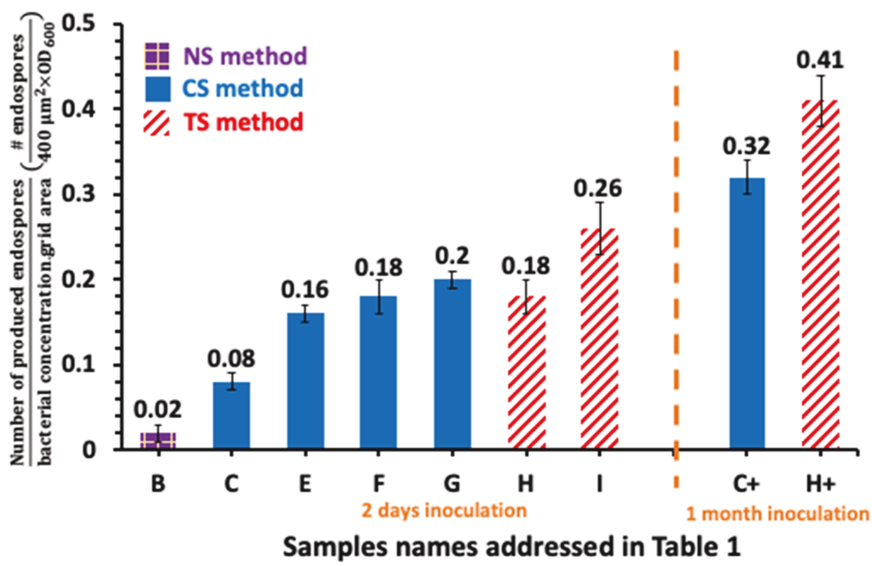

Fig. 4. The number of endospores produced through different endosporulation methods normalized based on the initial OD_{600} of the culture media and the grid area which is shown in Table 1. The error bars indicate the standard deviation of the average of counting 10 grid areas for each endosporulation method.

microscope (SEM) analysis. Our observations revealed that the vegetative cells of strain MB284 had a length of up to 3 μm (data not shown). During endosporulation, the TS method yielded larger endospores (averaging 0.98 μm) compared to the CS method (averaging 0.72 μm). This difference could be attributed to the formation of protein-containing covering layers around the cores in TS-produced endospores. Additionally, we noted that the size variation of TS-produced endospores was lower than that of CS-produced endospores. Furthermore, TS-produced endospores exhibited more rounded angles and smoother surfaces, indicating the influence of the endosporulation method on the morphology and geometry of the produced endospores (Fig. 3). Although it is documented that factors such as strain type, culture media, temperature, humidity, incubation duration, and other stressors like UV irradiation and environmental salinity can impact the size of produced endospores [9,39], the reported size for *Bacillus* species typically ranges up to 1.1 μm [12,39].

3.2. Number of produced endospores

In addition to our qualitative comparative analyses of endospore morphology under various environmental conditions, we quantified the number of endospores resulting from each endosporulation method. This quantity signifies the total number of endospores generated from vegetative cells during the endosporulation process. Assessing the quantity of produced endospores is crucial as a higher number of endospores can potentially lead to an increased number of germinated endospores upon exposure to favorable conditions.

The results demonstrate a congruence between our qualitative observations of endospore morphology and the quantified quantity of endospores (Fig. 4). We noted that the absence of nutrients in the culture media (NS method) adversely affected the production of endospores. Additionally, exposing cells to extreme temperatures (45°C and 2°C) during inoculation resulted in an increased production of endospores. However, acidic conditions (pH=3) inhibited endospore formation, leading to the absence of observed endospores (data not shown).

Conversely, alkaline conditions (pH=9) enhanced endospore production in both the CS and TS methods. Remarkably, under the same conditions, the TS method yielded a higher quantity of endospores than the CS method, suggesting a pivotal role of heat shock proteins in triggering the endosporulation process [40,41]. In conclusion, the TS method appears to facilitate the initiation of endosporulation, priming cells for successful endospore development. Furthermore, extending the

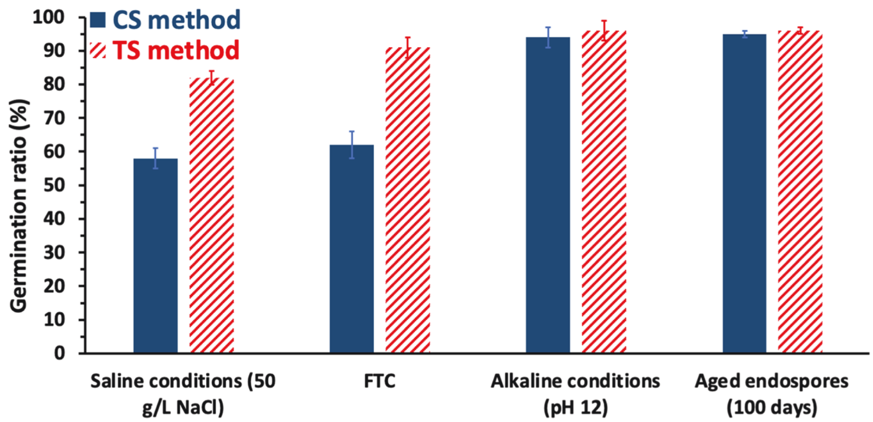

Fig. 5. the impact of exposing various harsh conditions, including saline conditions (50 g/L NaCl), Freeze and thaw cycling (FTC), alkaline conditions (pH 12), and the use of aged endospores (100 days), on the germination ratio of endospores generated through both Carbon Starvation (CS) and Thermal Shock (TS) methods. Error bars are included for each condition, representing triplicate measurements.

length of cell incubation from 2 days to 1 month enhanced the quantity of produced endospores in both the CS and TS methods.

3.3. Resilience assessment of germinated endospores

Throughout dormancy, endospores encounter challenging conditions within the concrete that hinder the germination process when favorable conditions arise. Factors such as DNA damage, compromised cell membrane integrity, and disruptions in the regulation of germination-related proteins may impede their transition into vegetative cells [42,43]. To gauge the resilience of the produced endospores against harsh conditions, we examined the influence of subjecting endospores to severe conditions on the germination ratio before and after exposure. Four distinct harsh conditions were considered in this study.

- **Saline conditions:** The salinity of pore solutions in concrete varies based on the salt content of the water and sand used in its production, especially when exposed to seawater and sea sand [44]. To mimic these conditions, a saline culture media containing 50 g/L of NaCl, close to the world's average ocean salinity, was utilized for evaluating the germination ratio.
- **FTC:** FTC is a common cause of creating fractures during the service life of concrete [45]. Consequently, endospores often experience fluctuating high and low temperatures while embedded in concrete.
- **Alkaline conditions:** In alkaline conditions, the pH of concrete pore solution typically ranges between 12.5 and 13.9, influenced by variables such as cement type, water-to-cement ratio, and alkali metal oxides [46]. Interestingly, observations indicate that during urea hydrolysis in alkaline environments, the pH of culture media tends to decrease [10]. Thus, pH 12, which closely aligns with the pH of the concrete micro-environment, was chosen as the initial condition for germination evaluation in the culture media. This selection allows for a representative assessment of bacterial behavior under conditions akin to those encountered during concrete self-healing processes.
- **Endospores' age:** Endospores, before exposure to favorable conditions for germination, may endure extended periods within the concrete. Therefore, the germination ability of endospores was examined after 100 days of storage in carbon-depleted culture media to assess the durability of the produced endospores.

Given the elevated yield of endospore production achieved through the TS method, characterized by a one-month incubation period at 35°C under pH 7 (denoted by sample H^+) and the formation of endospores with multi-layered coverings, we initiated investigations into their tolerance against challenging conditions. As a benchmark, the CS method, commonly utilized for endospore production in the literature for MICCP purposes, also involving a one-month incubation period at 35°C under pH 7 (denoted by sample B^+), was employed for comparative analysis.

The findings indicated that CS-produced endospores maintained 58% of their germination abilities following exposure to saline conditions. In contrast, those generated via the TS method exhibited a significantly higher germination rate of 82%. Likewise, when subjected to FTC, the CS-produced endospores displayed a germination ability of 62%, while the TS-produced endospores demonstrated robustness, boasting a germination rate of 91%. Notably, subjecting endospores to pH 12 and assessing the age of endospores did not significantly impact the germination ratio for either method, indicating that these factors did not act as harsh conditions for the germination of endospores. The resistance of produced endospores to pH 12 can be attributed to the alkalophilic nature of strain MB284. Additionally, the insignificant impact of endospore age on germination can be attributed to the method of endospore preparation, with both sets produced in carbon-free culture media (Fig. 5).

High salinity disrupts the ionic balance, leading to changes in osmotic pressure and causing cell membrane shrinkage. Consequently, cellular contents may leak [47]. Exposure to extreme temperatures can also damage DNA by reducing the cell membrane permeability [48,49]. Additionally, ice crystal formation within cells during FTC can rupture cell membranes, causing physical injury to cellular organelles [50]. The superior germination ratio observed with the TS method may be attributed to the formation of multi-coating layers around the endospore cores. These protective layers reduce cell permeability, providing a shield against osmotic stress, and act as thermal blankets during temperature fluctuations. The superior germination ratio observed with the TS method may be attributed to the formation of multi-coating layers around the endospore cores. These protective layers reduce cell permeability, providing a shield against osmotic stress.

3.4. Impact of salinity on bacterial behavior

We explored the impact of exposing endospores to salinity conditions (50 g/l NaCl) on their growth potential post-germination for both TS and CS-produced endospores. A comparison of the bacterial growth curves of germinated endospores revealed no significant difference when they were not subjected to saline conditions. This suggests that the type of endosporulation method (TS or CS) has no notable effect on the bacterial growth rate under normal conditions (pH 7, 35°C, 0 g/l NaCl).

However, when endospores were exposed to saline conditions, we observed a negative impact on the growth curves of both germinated endospores produced through the TS and CS methods. Particularly,

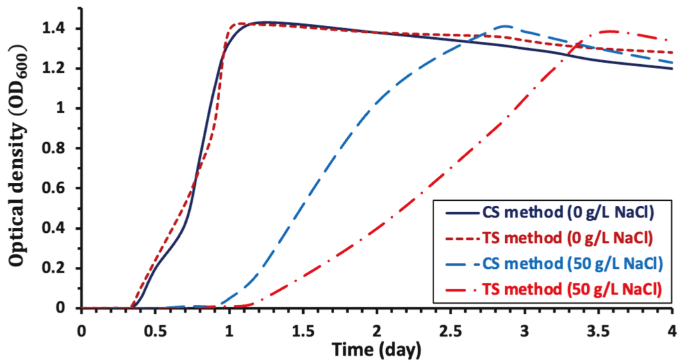

Fig. 6. The growth curves of germinated endospores produced through both TS and CS methods were assessed under saline conditions (50 g/L of NaCl). The growth curves were also examined in the absence of NaCl (0 g/L) to provide a reference for both sets of produced endospores. The presented curves represent the average of three replicates for each condition.

germinated endospores from the TS method exhibited a slightly shorter lag phase than those from the CS method before entering the exponential growth phase, indicating higher adaptability of TS-produced endospores to saline conditions. Furthermore, the slope of the exponential growth phase was significantly steeper for germinated endospores produced through the TS method compared to the CS method (Fig. 6). This observation aligns with the higher germination ratio observed in the TS method. These findings highlight the superior ability of germinated endospores produced through the TS method, as opposed to the CS method, to thrive after exposure to saline conditions.

3.5. Impact of FTC on bacterial behavior and MICCP performance in vitro

To evaluate and compare the bacterial behavior and MICCP performance of post-germinated endospores produced by the TS and CS methods, they underwent FTC. The bacterial behavior and MICCP performance of vegetative cells were also examined and utilized as a reference in this study. The quantity of active cells during MICCP directly influences the yield of calcium carbonate production by enhancing carbonate ion generation and providing more nucleation sites for the formation of calcium carbonate crystals. Our findings indicate that exposure to FTC had a less detrimental effect on the maximum observed concentrations of TS-produced endospores compared to CS-produced endospores after 90 hours of cell incubation. This suggests that TS-produced endospores exhibit a higher capacity to remain alive and metabolically active after exposure to FTC. However, we observed a significant reduction in the maximum cell concentration for vegetative cells, indicating their inability to maintain activity following exposure to FTC (Fig. 7A).

Ammonia and carbonate ions, both polar substances, are generated during the decomposition of urea and yeast extract. The increase in the amount of EC in the culture media serves as a metric indicating bacterial metabolic activity and the potential for calcium carbonate production resulting from the reaction between carbonate and calcium ions. Our results indicate that the observed reduction in EC was smaller for TS-produced endospores compared to CS-produced endospores after exposure to FTC conditions. However, the corresponding values for vegetative cells significantly decreased (Fig. 7B). In addition to EC, pH was measured as another metric to compare the behavior of cells and MICCP performance. Elevated pH indicates the hydrolysis of urea and the production of ammonia. The results were consistent with the EC changes and indicated that TS-produced endospores experienced a lower pH reduction than CS-produced endospores after exposure to FTC. We also

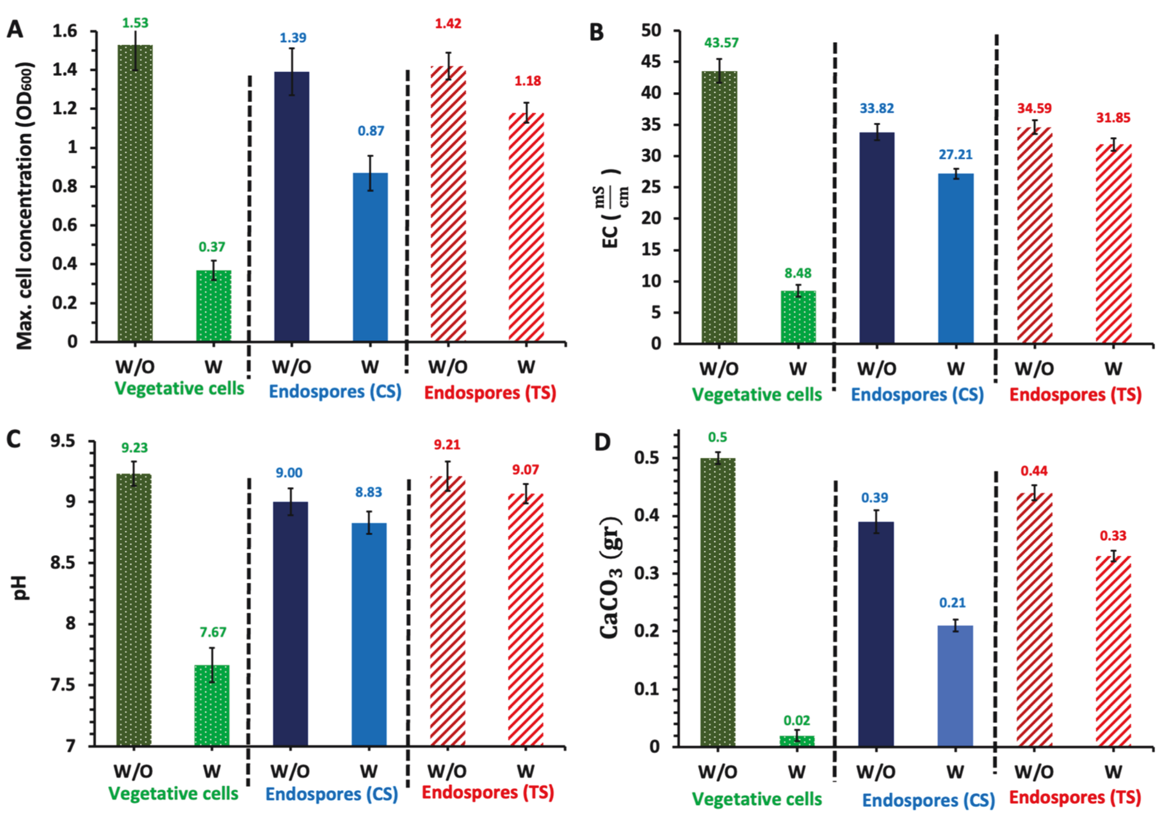

Fig. 7. The effects of exposing Freeze-Thaw Cycling (FTC) on A) maximum obtained cell concentrations, B) Electrical Conductivity (EC), C) pH, and D) produced calcium carbonate of vegetative cells and endospores produced through the Thermal Shock (TS) and Carbon Starvation (CS) methods after 90 hours. "W/O" refers to cells that were not subjected to FTC, while "W" indicates cells that were exposed to FTC. The standard deviation indicates the proximity of results after triplicates.

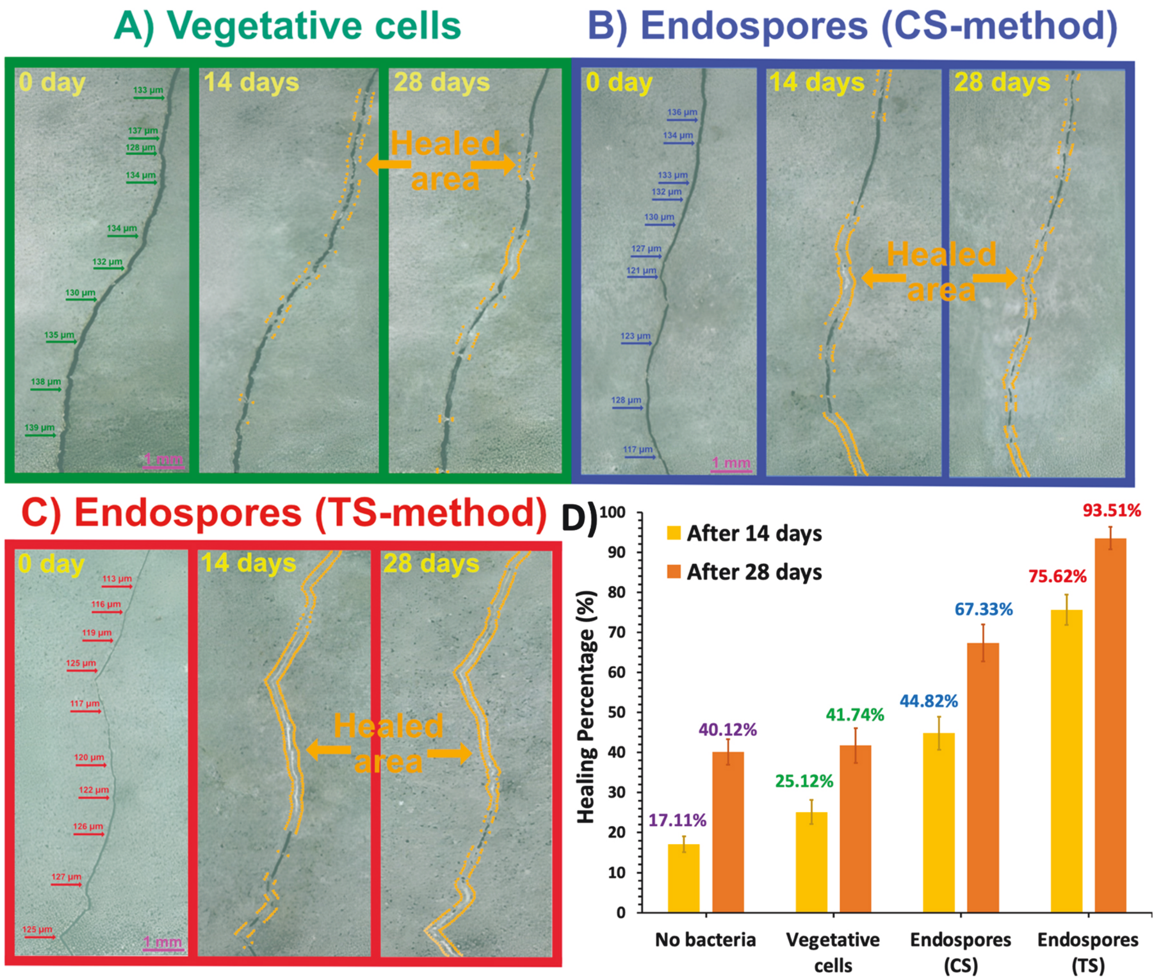

Fig. 8. Crack filling in cement paste specimens following their exposure to FTC including A) vegetative cells and endospores produced through B) CS and C) TS method. The healed areas of filled cracks are bounded by broken orange lines. Each specimen is a representative of three specimens with similar conditions. D) The average of crack filling percentage for different cement paste specimens. The error bars, which represent the data from three replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

observed a significant decrease in the final pH value for vegetative cells (Fig. 7C).

Additionally, we explored the impact of exposing cells to FTC on the production of calcium carbonate after 90 hours of cell incubation. The results indicated that the MICCP performance of vegetative cells that were not subjected to FTC was higher than that of endospores. However, following exposure to FTC, the cell concentrations, EC, pH, and the quantity of produced calcium carbonate were lower for vegetative cells than for endospores. The yield of calcium carbonate was higher for TS-produced endospores than for CS-produced endospores (Fig. 7D).

The lower MICCP performance of endospores before exposure to FTC than vegetative cells is attributed to the inability of some percentages of produced endospores to germinate and metabolically become active. However, the susceptibility of vegetative cells to FTC significantly decreased their MICCP performance compared to endospores. Furthermore, the higher MICCP performance of TS-produced endospores than CS-produced endospores can be attributed to the greater resistivity of endospores covered with multi-layers to remain active after exposure to FTC.

3.6. Impact of FTC on surface crack filling rates

The disparity between the conditions within the concrete environment and *in vitro* settings may influence the performance of embedded cells in MICCP reactions. It has been affirmed that the viability and healing efficacy of embedded endospores in concrete were adversely affected after exposure to challenging conditions [51]. Studies indicate that subjecting concrete to FTC can result in structural and material damage, impacting its overall integrity and longevity [52,53]. Alongside the deteriorating impact of FTC on a concrete structure, existing literature highlights a 50% reduction in the viability of endospores within the concrete, with only 5% of bacterial cells ultimately surviving during the concrete curing process following FTC exposure [54]. Subsequently, various endosporulation techniques, such as sugar coating, have been employed in the literature to enhance the survivability of endospores against FTC [55].

In this study, the efficacy of endospores produced through the TS and CS methods in filling surface cracks when cement paste was exposed to FTC was compared. Additionally, vegetative cells were utilized as a reference to assess the role of endosporulation in the crack filling of cement paste. The results demonstrated that following the exposure of hardened cement paste to FTC and subsequent submersion in a solution containing MSM, yeast extract, urea, and calcium acetate, the healing percentage for cracks by TS-produced endospores was 75.62% and 93.51% after 14 and 28 days, respectively. In contrast, for those containing CS-produced endospores, the healing percentage was 44.82% and 67.33% after the same time intervals. These findings indicate the superior performance of TS-produced endospores in withstanding harsh conditions in cement, including mixing, desiccation, and FTC. Moreover, they demonstrate the ability to remain metabolically active after germination, leading to an accelerated rate of crack filling (Fig. 8).

During FTC, the formation of ice crystals occurs both inside and outside of the cells. Intracellular crystals can damage delicate structures, leading to cell death or damage. Additionally, razor-sharp crystals outside of the cell membranes can cause injury to the cells. Moreover, as ice forms in the extracellular space, water from inside the cells rushes out through aquaporins to dilute the higher concentration of solutes, desiccating the cells and leading to cell death, ultimately entering the death phase [56,57]. Therefore, the presence of multi-covering layers around the cells in TS-produced endospores can act as a shield to protect the cell membrane and as a blanket to limit the formation of ice crystals. A comparative study also indicated that the crack repair rate at 28 days for concrete specimens subjected to FTC was smaller than that of specimens not subjected to FTC [58]. Subsequently, expanded perlite was utilized as a carrier to protect endospores from FTC, maintaining the crack repair rate.

Our findings also indicated that the corresponding crack-filling rates for vegetative cells were 25.12% and 41.74% after 14 and 28 days, respectively, which were lower than those observed for cracks filled by endospores produced through both the TS and CS methods. It is assumed that, since the cement paste content is carbon-free, endosporulation was triggered following the mixing of vegetative cells into the mortar. The process of endosporulation occurred by adding vegetative cells into the cement paste and can be compared with the NS method, given the carbon and nutrient-free content of concrete. Since a significant reduction in the number of produced endospores was observed through the NS method, it is concluded that the lower performance in crack filling by vegetative cells may be attributed to the lack of nutrients in the pore solution, leading to a reduction in the number of vegetative cells that could successfully be converted to endospores. This underscores the necessity of adding pre-produced endospores, rather than vegetative cells, into the cement paste as a healing agent. Additionally, we observed healing percentages of 17.11% and 40.12% after 14 and 28 days, respectively, for the bacteria-free cement paste specimens, referred to as autogenous filling, which occurred due to the leftover reagents (Fig. 8). Water entering the cracks during the wet-dry cycles causes un-hydrated cement particles to produce calcium carbonate [4].

4. Conclusion

This study underscores the pivotal role of endosporulation methods in shaping the efficacy of MICCP reactions. Through our investigation into various endosporulation methods, we have elucidated that endospores of strain MB284, when produced via the TS method, exhibit a distinctive multi-layered structure around the core, resulting in altered endospore morphology. Furthermore, our observations reveal that besides expediting endosporulation, the multi-layered structure provides a protective barrier, safeguarding core genetic components against adverse environmental conditions, including FTC, alkaline, and saline environments.

Moreover, our findings highlight the superior performance of TS-produced endospores in enhancing the germination ratio, leading to higher yields of calcium carbonate production and increased surface crack filling rates in both *in vitro* and cement paste conditions, compared to endospores produced through the CS method.

Given the significant enhancement in MICCP efficacy achieved through TS-produced endospores, future research endeavors should focus on exploring the potential of employing these endospores to bolster the mechanical properties, such as compressive and flexural strength, of healed concrete subjected to FTC.

CRediT authorship contribution statement

Seyed Ali Rahmaninezhad: Writing – original draft, Methodology, Data curation, Conceptualization. **Divya Kamireddi:** Writing – review & editing. **Reva M. Street:** Writing – review & editing. **Mohammad Houshmand:** Writing – review & editing, Data curation. **Amir Sadighi:** Writing – review & editing. **Ahmad Raeisi Najafi:** Funding acquisition, Writing – review & editing, Project administration. **Christopher M. Sales:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Yaghoob (Amir) Farnam:** Writing – review & editing, Project administration, Funding acquisition. **Caroline L. Schauer:** Funding acquisition, Writing – review & editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.conbuildmat.2024.135528.

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