

Understanding the importance of endosporulation methods for generating endospores that can resist harsh conditions and produce calcite in bio self-healing of concrete

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Abstract. Vegetative cells used for the concrete bio self-healing process often face threatening environmental conditions such as extreme temperature, pH, salinity, shear stress, and starvation during the hardening process and the service life of the concrete. These conditions can eventually lead to cell death. Since endospores are likely to remain dormant for prolonged periods and can survive, germinate, and grow under inhospitable conditions, they are a suitable bacterial phenotype to introduce into concrete for microbial-inducing calcite precipitation. This study investigated how different endosporulation methods affect the endosporulation ratio (*i.e.*, the fraction of vegetative cells that are converted to endospores during endosporulation), as well as the germination ratio (*i.e.*, the fraction of endospores that are converted to vegetative cells following germination) and the microbial-induced calcite precipitation (MICP) performance of germinated endospores after facing harsh conditions of concrete, specifically, freeze and thaw cycling. Results from this study show that thermal shock followed by cell incubation in alkaline conditions leads to increased sporulation and germination ratios. It was also observed that freeze and thaw cycling had negligible effects on calcite production by endospores, while exposure of vegetative cells to these harsh conditions led to not only less biomass and calcite production but also to a lower mass of calcite produced per mass of cells, as determined by thermogravimetric analysis (TGA). The results from this study provide key insights into improving methods for endosporulation and germination to effectively use them for bio self-healing applications in concrete.

1 Introduction

Bio self-healing agents (referred to hereafter as bio-agents) that can perform microbial-induced calcite precipitation (MICP) to seal/heal cracks in concrete can face life-threatening conditions during the mixing of mortar and throughout the service life of concrete [1], therefore, the long-term durability of bio-agents in concrete is critical for bio self-healing of concrete. Due to the vulnerability of vegetative cells against harsh conditions and also the resistivity of inactive dormant endospores to inhospitable environmental conditions, endospores-forming bacteria (e.g., *Bacillus* and *Clostridium* species) can be considered ideal bio-agents for self-healing of concretes [2, 3, 4, 5].

One endospore-forming species widely used for biomineralization purposes is *Lysinibacillus sphaericus* (formerly known as *B. sphaericus*) [6, 7]. *L. sphaericus* is alkaliphilic, produces tightly packed layers of calcite [7], and also has high rates of urea decomposition (K_{urea} 10.8 h⁻¹ OD₆₀₀⁻¹ [8]) and calcite production (3.4 g/l⁻¹ h⁻¹ [9]). Vegetative cells of *L. sphaericus*, as a consequence of the lack of vital living conditions (such as carbon and nutrient deficiencies), can be converted to endospores through asymmetric division named endosporulation [5]. However, it is shown that the quality of endospores

(including the water content, the volume of the endospores, and the sporulation and germination ratios) is affected by environmental conditions [2, 10] (such as temperature, pH, or different concentrations of oxygen, nutrients, salinity (mono and divalent cations), amino acid, and carbon sources) [2, 5, 10, 11] suggesting that endosporulation methods could impact bio self-healing applications in concrete.

Further studies investigating different endosporulation methods are required to assess the important parameters that enhance the MICP performance of germinated endospores. Since literature shows that thermal shock is able to produce the endospores of *L. sphaericus* [2], thus, in this study, the effect of changing pH, the concentration of nutrients, along with the time of heating during thermal shock (the different ways of producing endospores – endosporulation methods) on endosporulation ratio is investigated, then the MICP performance of endospores (with higher endosporulation ratio) was compared with vegetative cells, and finally, the impact of applying freeze and thaw cycles (FTC) on the MICP performance of germinated endospores and vegetative cells was investigated.

2 Material and Methods

2.1 Endospore formation methods

Lysinibacillus sphaericus ATCC 1380 (Strain MB284) was incubated into the culture medium including yeast extract and urea (each 20 g/l) and after 24 hours vegetative cells were collected while they were in their exponential growth phase ($OD_{600} < 1$). Vegetative cells then were washed three times with 1 molar Phosphate Buffer Solution (PBS) and inoculated into the Minimal Salt Media (MSM), Table 1 shows the ingredients of the MSM. After that, cells were put into a boiling water bath (for 30 minutes-heating time) and immediately into an ice bath (for 30 minutes-cooling time) which is named thermal shock (0.5). For further investigation, the heating time duration of 1 hour was also used instead of 30 minutes, which is named thermal shock (1). Thereafter, the cells were incubated into MSM with 3 different pH (5, 7, and 9) for 24 hours in a shaker incubator (30 °C and 120 rpm). Finally, they were washed three times with 2 °C De-ionized water (DI water) then harvested, and incubated into the MSM, with pH 7. For investigating the effects of the concentration of nutrients on the endospore formation ratio, PBS and DI water were also used instead of MSM.

Table 1. The ingredients of the MSM.

Salts	Concentration	Salts	Concentration
NaNO ₃	1 g/l	ZnSO ₄	5.74 mg/l
K ₂ SO ₄	0.17 g/l	MnSO ₄	3.38 mg/l
MgSO ₄	0.037 g/l	H ₃ BO ₃	1.24 mg/l
CaSO ₄	0.0086 g/l	CoMoO ₄	0.94 mg/l
KH ₂ PO ₄	0.53 g/l	FeSO ₄	0.224 g/l
K ₂ HPO ₄	1.06 g/l	H ₂ SO ₄	0.1 ml/l
KI	1.66 mg/l	CuSO ₄	5 mg/l
DI water	1 liter		

2.2 Cell staining and counting

Immediately after washing cells with DI water, 1 ml of samples were taken and diluted 10 times through serial dilution. Then, 10 µl of the final solution was mixed with 10 µl of PBS and stained (the Schaeffer-Fulton method) on the slides for light microscope investigation (Leitz Diaplan, 020-437.035). For counting the endospores, the number of endospores in one grid (20 µm * 20 µm) was counted and the average values of 10 grids were considered as the number of endospores for each slide.

2.3 Germination of endospores

Cells incubated into the MSM were stored in 2 °C conditions for a month leading to cells completing endospore formation stages. Then, cells were harvested and washed three times with PBS, and incubated into a solution of DI water containing only yeast extract and urea (each 20 g/l) to induce germination. The biomass concentration during the cell growth was measured

based on the optical density at the wavelength of 600 nm (OD_{600}) by the spectrophotometer. The final pH and electric conductivity (EC) of culture media were also measured to evaluate the MICP performance of bacteria. Calcium acetate was used as a source of calcium and added to each sample when the bacteria was in the stationary phase. After adding calcium acetate, the content of each sample (50 ml) was thoroughly well-mixed into a shaker incubator (30 °C and 120 rpm) for 1 hour, then pellets (precipitated solids) were harvested by centrifugation (7830 rpm, 25 °C) for 10 minutes. The pellets (containing biomass & calcite) were dried at 105 °C for 1 hour and then weighed for measuring the total weight of biomass and calcite. Finally, the calcite to biomass ratio of pellets was measured by Thermogravimetric Analysis (TGA) tested in the range of 30 to 900 °C with the ramp rate of 10 °C/minute under nitrogen gas.

2.4 Freeze and thaw cycling and germination ratio

Cells incubated into the MSM were stored at -4°C and 25 °C sequentially (24 hours for each temperature and cycles were repeated 7 times) to stimulate the FTC. Thereafter, cells were incubated into the media containing yeast extract and urea (each 20 g/l), and calcium acetate was added when the bacteria were in the stationary phase. The pour-plate technique was used to count the number of germinated endospores before and after FTC, and the germination ratio was calculated by dividing the number of cells before FTC over the number of cells after FTC.

3 Results and Discussion

3.1 Effect of endospore formation methods on endospore formation ratio

The endospore formation ratio refers to the percentage of vegetative cells that successfully convert to endospores during endospore formation [5]. Since endospores can tolerate harsh conditions, remain dormant, and are able to germinate upon exposure to hospitable conditions, the number of generated endospores at the end of the endospore formation method is an important parameter indicating the efficiency of the endospore formation method. Therefore, microscopic observations were used in this study to quantify the endospore formation ratio for different endospore formation methods.

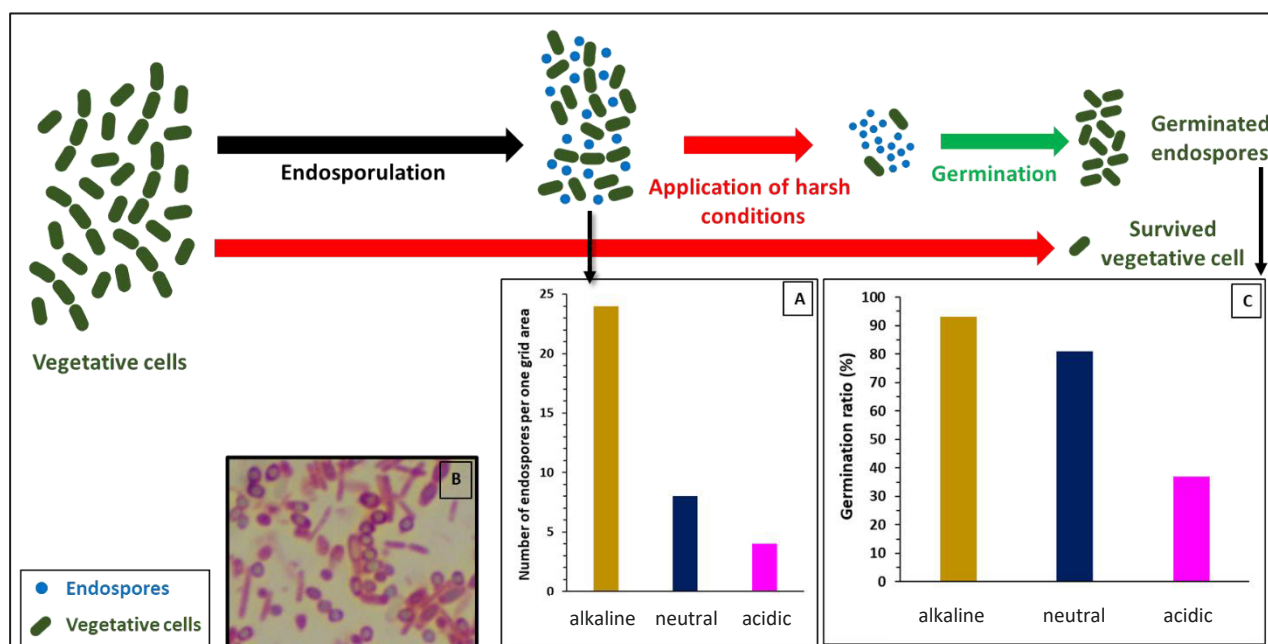


Fig. 1. The schematic of converting vegetative cells to endospores through endosporation, and how applying harsh conditions affects the number of active vegetative cells and endospores. A) The number of endospores were found in one grid area (20 μm * 20 μm) after applying thermal shock (0.5 hour) followed by incubating in different culture media (alkaline, neutral, and acidic conditions). B) The morphology of endospores after thermal shock (1 hour) followed by incubating in alkaline conditions. C) the germination ratio of endospores formed by thermal shock (0.5 hour) followed by incubating in different culture media (alkaline, neutral, and acidic conditions) and after freeze and thaw cycling.

During endosporation, forespores are created inside the body of vegetative cells and converted to endospores, which are released from the vestiges of cells [4]. We hypothesized that the number of endospores that finally are produced is impacted by the endosporation method employed. As Figure 1 A shows, the number of endospores in a certain grid area (400 μm^2) was 24, 8, and 4 for endospores inoculated into the alkaline, neutral, and acidic conditions, respectively. Therefore, we observed that incubating cells at a high pH increased the number of endospores, while low pH had a negative effect on the endospore's generation. Jian et al., [11] also reported that alkaline conditions increased the endosporation rate of *Bacillus cohnii* which are alkalophilic and similar to *L. sphaericus*.

Vegetative cells were also observed along with endospores in all samples which indicates a longer incubation time (more than 24 hours) is required for having a higher endosporation ratio (data was not shown). A longer incubation time allows vegetative cells to pass through several sequential endosporation stages, which are required to eventually form endospores [4]. Comparing Figures 2 A and B indicates that extending the incubation time from 2 days to 1 month had a positive effect on the enhancement of the number of produced endospores both for cells incubated under neutral conditions. Similar results also indicate the positive effect of extending incubation time on the endosporation rate [12].

Also, we found that incubating cells in the media lacking nutrients (including only DI water or PBS) significantly decreased the number of generated endospores and endosporation ratio (Figures 2 C and D). It is hypothesized that forespores need nutrients to

be released from mother cells into the culture media during endosporation [4] and thus it is likely the main reason for the small endosporation ratio of media containing only DI water or only PBS. However, when MSM was used for cell incubation (Figure 2 A), it is observed that the endosporation ratio increased, indicating the media with nutrients leads to having a higher endosporation ratio [11, 13].

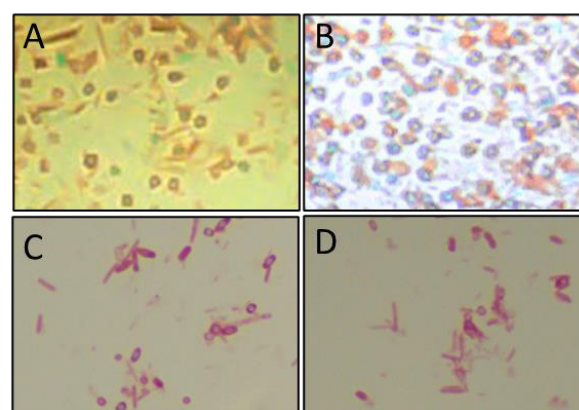


Fig. 2. The stained endospores produced by thermal shock (0.5) followed by incubating in MSM under neutral condition after A) 2-days and B) 1-month; thermal shock (0.5) followed by incubating in C) PBS and D) DI-water under neutral conditions after 2 days.

In addition, we observed that for samples containing cells and media without nutrients (DI water or PBS), the thermal shock could not increase the endosporation ratio, but when cells were incubated into the MSM, the thermal shock enhanced the number of generated endospores (data was not shown). Thus, adding

nutrients to the culture media is more important than applying thermal shock, even though both of these two parameters have prominent effects on the augment of the endosporulation ratio. Lastly, the effect of extending the heating time of thermal shock to two times was tested and it was observed that extending the heating time had a positive effect on the endosporulation ratio (Figure 1 B). It is hypothesized that extending the heating time assists with processes that lead to weakening the integrity of the cell membrane of mother cells so that forespores can be released into the culture media.

3.2 Effect of harsh conditions on germination ratio

Although it is hypothesized that endospores are able to resist harsh environmental conditions more than vegetative cells, the ability of endospores to germinate and grow after exposure to harsh environmental conditions is not well-studied. Germination is a process triggered by the release of soluble factors (such as free water and adenosine triphosphate) from the endospores that stimulate hydrolysis of the endospore's coat, rehydration of the endospores, destabilization of the inner membrane, and digestion of the cytoplasmic membrane [5]. Germination ratio, referred to as the percent of endospores that converts to vegetative cells, is a useful parameter to quantitatively indicate the resistance of endospores against harsh conditions.

It is hypothesized that the endosporulation methods, which directly affect the morphology and quality of endospores, can subsequently affect the germination ratio of endospores that are exposed to harsh conditions. To test this hypothesis, the germination ratio of endospores produced through different endosporulation methods (thermal shock (0.5) followed by incubating under alkaline, neutral, and acidic media) was measured when endospores faced FTC. Figure 1 C shows, the germination ratio of endospores incubated in alkaline, neutral, and acidic conditions was 93%, 81%, and 37%, respectively indicating alkaline conditions had a positive effect on increasing the germination ratio. These results suggest that alkaline conditions provide a more suitable environment for MB284 species to generate more resistant endospores against FTC. A similar observation was reported by Jiang et al. with another alkalophilic endospore-forming species [11].

3.3 Effect of harsh conditions on MICP performance

For calcite production to occur, endospores need to germinate in order to carry out urea hydrolysis and produce carbonate ions. During urea hydrolysis, ammonia is produced by bacteria which increases the pH and electric conductivity (EC) of the environment. Therefore, the bacterial growth rate and also the amount of pH, EC, and produced calcite were measured to characterize MICP performance in this study. The endosporulation method (the thermal shock (0.5) followed by incubating in alkaline MSM) was used to

investigate the effect of harsh FTC on the growth rate and MICP performance of endospores.

Figure 3 shows that the lag phase that is required for vegetative cells to grow before applying FTC is around 6 to 7 hours and for germinated endospores is around 8 to 9 hours. This extra time is hypothesized to be related to the time that endospores need to germinate. However, the maximum biomass concentration of vegetative cells in the stationary phase (1.53 A) was slightly more than that of germinated endospores (1.42 A), which could be due to the existence of some endospores that were either unable to germinate or after germination were not able to grow and multiply. However, FTC had a slightly negative impact on the growth rate and maximum biomass concentration (1.18 A) of germinated endospores compared to endospores that were not exposed to FTC. However, the growth rate of vegetative cells significantly decreased after applying harsh conditions indicating the advantages of using endospores as the bio-agent in the self-healing process of concrete, which under some applications can experience periods of FTC.

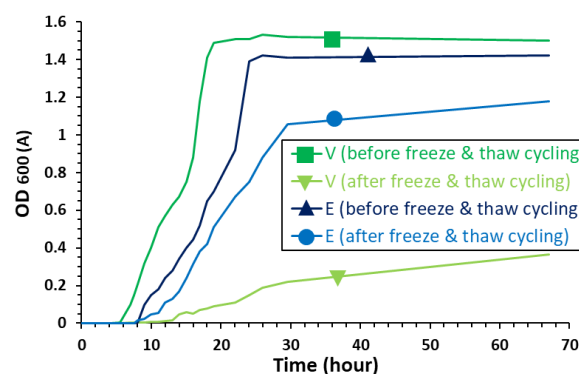


Fig. 3. The impact of freeze and thaw cycling on the growth rate of vegetative cells (V) and germinated

Since urea is a nonpolar component, its biodegradation to ammonia and carbonate ions increases the EC of the culture media [6]. As Figure 4 A shows, although the culture media incubated with endospores had lower EC than that incubated with vegetative cells, FTC had a significant negative effect on the amount of EC of vegetative cells. The production of ammonia enhances the pH of the culture media and since alkaline conditions decrease the solubility of calcite in water, it increases the calcite precipitation rate and is favorable for the bio self-healing process. The pH of culture media incubated with vegetative cells and endospores was nearly similar and applying FTC did not have a significant effect on the final pH of the culture media incubated with endospores (Figure 4 B), however, vegetative cells that faced FTC were not able to increase the pH.

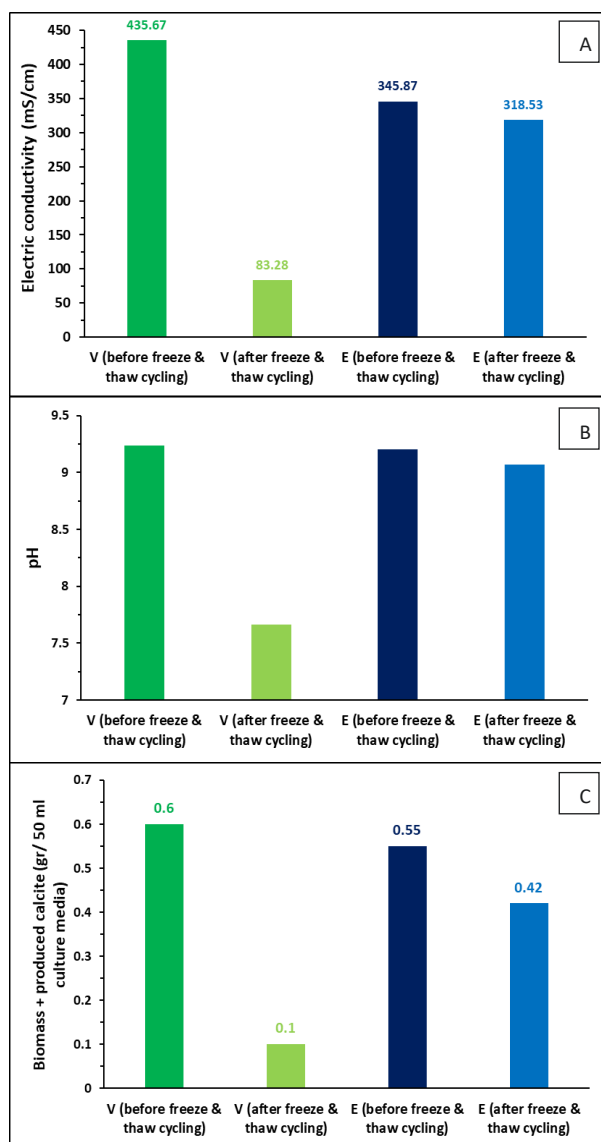


Fig. 4. The impact of FTC on the final amount of A) EC, B) pH, and C) combined biomass & calcite produced by vegetative cells (V) and germinated endospores (E).

The addition of calcium acetate to culture media containing reduced organic carbon and nutrient sources leads to strain MB284 metabolically producing carbonate ions that drive calcite precipitation. Through

that metabolism, we expect the bacteria to grow and generate biomass. Since carbon sources and nutrients (yeast extract and urea) and excess calcium acetate are soluble in the culture media, it is expected that biomass and calcite (generated solid phase) can be separated from the culture media via centrifugation. Figure 4 C compares the final amount of combined biomass and calcite produced by vegetative cells and germinated endospores before and after applying FTC. The final amount of combined biomass and calcite produced by germinated endospores before applying FTC was slightly lower than what vegetative cells produced. However, comparing the performance of germinated endospores and vegetative cells after applying FTC indicates that vegetative cells cannot tolerate harsh conditions as well as endospores, so had a lower growth rate and calcite production. It is hypothesized that FTC causes damage to cellular membranes of vegetative cells and other cellular structures (e.g., proteins and cell walls), leading to these lower rates of bacterial growth and calcite production.

Calcite is consistent with the texture of concrete and also its adhesive properties enhance the strength of bio self-healed concrete. Thus, it is important cracks be filled mostly with calcite instead of biomass. Therefore, although Figure 4 C indicates the total amount of biomass and calcite produced by germinated endospores and vegetative cells, it is important to determine the portion of calcite in the precipitated solid. As Figure 5 illustrates, the solid precipitated by vegetative cells and germinated endospores before facing FTC contained 83% calcite, 17% biomass, and 80% calcite, 20% biomass, respectively which is similar to the endospores after facing FTC (79% calcite and 20% biomass), while the majority of solid produced by vegetative cells after FTC (81%) was biomass and only 19% was calcite.

After FTC, the ratio of calcite to biomass did not change for germinated endospores indicating FTC did not affect the amount of calcite produced by each germinated endospore, while a majority of vegetative cells lost their calcite production abilities. After FTC, a large portion of vegetative cells became dead and could not grow to consequently produce carbonate ions to react with calcium ions, therefore having lower calcite to biomass ratio.

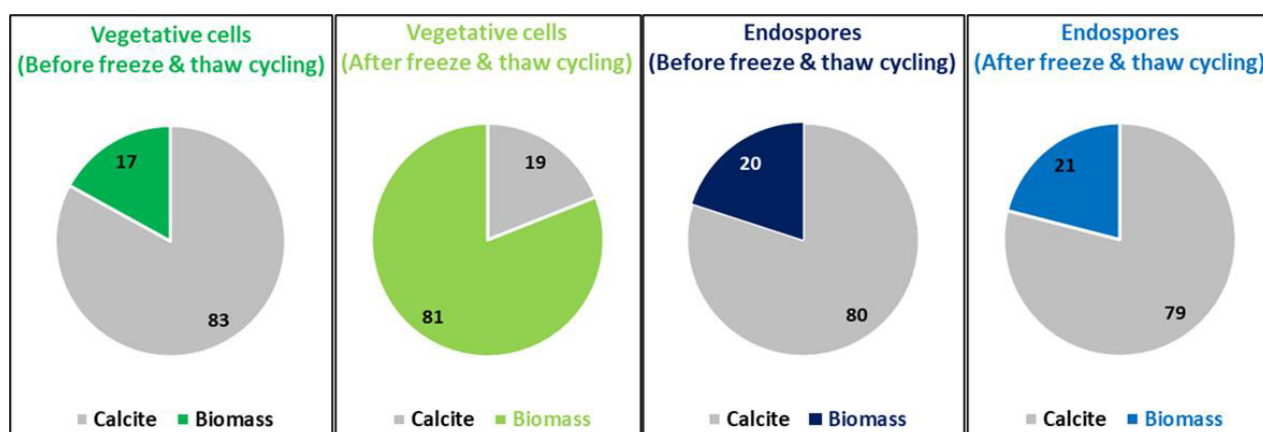


Fig. 5. The calcite (C) biomass (B) ratio for vegetative cells and germinated endospores before and after facing FTC

4 Conclusion

In this study, it was shown that the physiology of endospores can be affected by different endosporeulation methods. In particular, it was found that the presence of nutrients in the culture media and extending the heating period during thermal shock increased the endosporeulation ratio. Furthermore, it was shown that endospores produced via thermal shock and incubated under alkaline conditions achieved the best germination ratio. In addition, it was observed that exposure to FTC, which is a harsh condition commonly experienced by concrete, had a minimal effect on the growth rate and MICP performance of germinated endospores, while it severely decreased the growth rate and the MICP performance of vegetative cells. On a per biomass basis, germinated endospores produce more calcite than vegetative cells after being exposed to FTC.

Altogether, this study not only confirms the advantages of using endospores over vegetative cells as bio-agents in concrete bio self-healing but also demonstrates that the endosporeulation method used to create endospores can have a significant impact on the survivability of bio-agents and the MICP performance of germinated endospores that may face harsh and inhospitable conditions of concrete. Since experiments in this study were only conducted in aqueous solution within test tubes, it is suggested that future experiments investigate the effect of FTC on the survivability and MICP performance of endospores in the concrete specimen.

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