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Impact of air entraining admixtures on biogenic calcium carbonate precipitation and bacterial viability

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ABSTRACT

The applications of self-healing in cement-based materials via biomineralization processes are developing quickly. The main challenge is to find a microorganism that can tolerate the restricted environment of cement paste matrix (*i.e.* very high pH, lack of oxygen and nutrients, small pore size etc.). The focus of this work was to determine the possible use of an ammonium salt-based air-entraining admixture (AEA) as a protection method to improve the survival of incorporated *Sporosarcina pasteurii* cells in cement-based mortar. Bacterial cells were directly added to the mortar mix with and without nutrients. Nutrients should be provided to keep the microorganisms viable even at early ages (*i.e.* 7 days). Surface charge of the bacterial cells and *in vitro* biogenic calcium carbonate ($CaCO_3$) precipitation were not affected by the incorporation of AEA. However, introducing AEA did not influence the viability in mortar samples, which might be attributed to the type and chemistry of AEA used.

1. Introduction

Recent research in the field of concrete materials suggested that it might be possible to develop a smart cement-based material that is capable of remediating cracks by activating microbial induced calcium carbonate precipitation (MICP) within the cracked regions [1–3]. MICP is a bio-chemical process in which microorganisms induce mineral precipitation [4].

The use of MICP for civil engineering applications is becoming substantially popular. Recent studies showed that MICP can be used to bind non-cohesive sand particles and improve their properties under shear [5,6]. MICP has also been used in cement-based materials to remediate microcracks, improve mechanical performance and reduce porosity [7–10]. The main challenge for the MICP applications in cement-based materials is to find a microorganism that can tolerate these highly alkaline conditions [11], survive the mixing process, and remain viable with limited access to nutrients [12]. In particular, alkaliphilic and endospore forming microorganisms can tolerate the stresses induced within the cement-based materials. As an early approach Ghosh and Mondal [13] used *Shewanella* species by suspending the cells in the water prior to mortar mixing. It was found that the incorporation of these cells decreased the pore sizes and improved the compressive strength of mortar. Jonkers et al. [14] introduced *Bacillus pseudofirmus* and *Bacillus cohnii* endospores in mortar by simply suspending them in mixing water. These endospores were found to be viable up to 4 months, however incorporation of these endospores reduced the compressive strength of mortar [11]. Similar behavior was observed by Ersan et al. [15] and the authors suggested that the strength decrease due to the incorporation of endospores could be explained by the degradation of proteins by high pH of the cement paste matrix, which might induce the formation of air bubbles. Then, concerns regarding the use of the endospores within the restrictive and high pH environment of cement-based materials have led researchers to propose encapsulation for the endospores. The encapsulation methods consist of immobilizing the bacterial endospores in a protective covering, such as inorganic lightweight porous aggregates (LWAs) [2], polymeric membrane [16,17], microcapsules [8] and hydrogels [18]. Wiktor and Jonkers used lightweight inorganic expanded clay particles to encapsulate *Bacillus alkalinitriculus* endospores and their nutrient source, calcium lactate [2]. With this approach, the researchers replaced a portion of aggregates with LWAs and could extend the viability of the bacteria; however, incorporation of LWAs decreased the compressive strength of the material, which was expected.

Wang et al. [18] developed a biocompatible hydrogel encapsulation

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for *Bacillus sphaericus* endospores to induce self-healing in cement-based mortars. It has been shown that these hydrogels were able to keep the endospores viable within the cement paste matrix and provide self-healing in cracks as large as 0.5 mm within 7 days. Wang et al. [19] also conducted a series of tests to determine the self-healing ability of *B. sphaericus* endospores embedded in micro-silica gel and polyurethane membranes when they were introduced through glass tubes embedded in mortar. The results showed that polyurethane membranes showed a higher self-healing efficiency compared to silica gels in terms of strength recovery and reduction in permeability [19].

With a proper microbial selection and nutrient medium, 2% of the initial bacterial inoculum remained viable up to 11 months after mortar mixing without any protective material [20]. The inoculated *S. pasteurii* cells were able to precipitate $CaCO_3$ within the cement paste and were able to improve the microstructure of the 7-day old mortar samples after internal microcracks were induced [21]. However, to extend the period of application and seal the surface cracks the number of viable cells remaining in the mix becomes more critical. Thus, it is important to develop a protection method to increase the survival percentage of the vegetative bacterial cells against the restrictive environment and increase the viable cell retention for longer periods. One of the possible actions to improve the cell viability is to increase the volume of available space for the microorganisms so the stress due to space limitation can be released. Use of AEA in the mixes can provide uniformly distributed air voids, which can enable more voids for the microorganisms to survive.

Previously, Ersan et al. [15] have tested the performance of air entrainment (BASF MasterAir 100) as a protection method for microorganisms embedded in cement paste matrix. The study has only focused on the effects of the AEA protection (1% w/w of cement) in terms of its influence on compressive strength and setting of mortar samples. Use of AEA as a protection method decreased the compressive strength of mortar and further investigation on microorganism viability was not conducted. Stuckrath et al. [22] showed that AEA did not yield any change in the performance of bacterial self-healing when the cells were introduced as spores in LWAs.

The objective of this study is to evaluate the possible use of an ammonium salt-based AEA to improve the survival percentage of the microorganisms in alkaline environment of cement paste. This study reveals the effects of a commercially available ammonium salt-based AEA on biomineralization and the viability of the *S. pasteurii* cells embedded in mortar. Here, we examined the survival of the microorganisms within the cement paste matrix with and without incorporation of nutrients, the morphology of the *in-vitro* biogenic $CaCO_3$ precipitates, and the impact of vegetative cell culture addition on $CaCO_3$ content *within* the cement-based material.

2. Material and methods

2.1. Microorganism growth

Leibniz Institute- German Collection of Microorganisms and Cell Cultures: *S. pasteurii* (DSMZ 33) was grown in Urea-Yeast Extract (UYE) medium composed of tris base (15.8 g), urea (10 g) and yeast extract (20 g) per liter of distilled (DI) water (pH 9). Twelve grams of agar per liter was added to the media when solid medium was required. *S. pasteurii* cells were inoculated in 600 mL of UYE and incubated aerobically with shaking conditions (180 rpm) at 30 °C. Sample aliquots were taken from these media periodically and plated on agar plates. Samples for viable plate counts were serially diluted (10^0–10^{-7}); and the cell concentration was obtained by viable plate counts and represented as colony forming units (CFU/mL). Bacterial growth curves were developed in terms of CFU/mL *vs.* time.

It is known that *S. pasteurii* cells can induce mineral precipitation not only through urea hydrolysis but also by acting as a nucleation site due to their negative surface charge. Thus, it is crucial to determine the influence of nutrients and AEA on surface charge of bacterial cells. To measure the surface charge of *S. pasteurii*, cells were grown in UYE medium until a concentration of 2×10^8 CFU/mL was reached. Then, cells were harvested by centrifugation at 6300 g for 15 min, washed by sterile DI water and resuspended in 4 different media: DI water, DI water + AEA, fresh UYE medium and fresh UYE medium + AEA. BASF MasterAir 200 was used as AEA (2.22 g/L of nutrient medium). These cells were incubated in these media aerobically with shaking conditions at 30 °C for 24 h. Then, the cells were collected by centrifuging, washed and resuspended in sterile 20 mM Tris buffer at pH 9 for testing. A Malvern Zetasizer Nano ZS (Malvern, Worcestershire, United Kingdom) was used to determine the influence of AEA and UYE nutrient medium on zeta potential of the cells. Triplicates of measures were taken from triplicates of samples.

2.2. Characterization of in-vitro $CaCO_3$ precipitation

To induce *in-vitro* $CaCO_3$ precipitation *via* MICP, the microorganisms require carbonate ($[CO_3]^{-2}$) and calcium ($[Ca]^{+2}$). In terms of reaction mechanisms, 1 mol of urea added in nutrient medium produces 1 mol of $[CO_3]^{-2}$, which can react with 1 mol of $[Ca]^{+2}$ to form 1 mol $CaCO_3$. Even though, the $[CO_3]^{-2}$ was hydrolyzed through urea decomposition, to obtain *in-vitro* biogenic $CaCO_3$ precipitation, external $[Ca]^{+2}$ source was added as Calcium nitrate tetra hydrate- $Ca(NO_3)_2 \cdot 4H_2O$ (28 g/L of nutrient medium) To induce precipitation, *S. pasteurii* cells were incubated in UYE medium and once the cells reach their exponential growth phase (see Section 2.1), $[Ca^{+2}]$ source was added to media. After 24 h of incubation at 30 °C under shaking conditions, precipitates were collected by centrifuging at 6300*g* for 15 min. To investigate the impacts of air entrainment on biogenic $CaCO_3$ precipitation, after 7 h of incubation at 30 °C, BASF MasterAir 200 AEA (2.22 g/L of nutrient medium) was added and the incubation process was continued for another 17 h, then precipitates were collected by centrifuging. Collected biogenic precipitates including the bacterial cells were gold coated and processed by JEOL Scanning Electron Microscope (SEM) (Freising, Germany). The accelerating voltage was kept at 5 kV while the working distance was held at 9–12 mm at various magnifications. To determine the crystal structure of biogenic $CaCO_3$ precipitated, a qualitative X-ray diffraction (XRD) analysis was conducted with BRUKER D8 Advance X-ray Diffractometer (Karlsruhe, Germany). In general, collected precipitates were kept in a drying chamber at 40 °C for 24 h prior to testing. Then, the samples were placed and compacted into a sample holder and analysis was conducted at angles from 10 to 90° 2θ at a step size of 0.02° 2θ. Control samples were prepared by adding $[Ca^{+2}]$ source to fresh UYE medium with and without AEA.

2.3. Determining the number of viable cell retention in mortar

To investigate whether the AEA will improve the survival of *S. pasteurii* cells *within* portland cement mortar. The standard triplicate replicate Most Probable Number (MPN) method was employed to quantify *S. pasteurii* concentrations in the bacterial culture used to prepare all mortar mixes and the remaining viable *S. pasteurii* in hardened mortar samples.

Viable *S. pasteurii* were enumerated *via* MPN analysis in the bacterial culture, at 7 and 28 days after mixing the inoculum into mortar samples. Mortar beams (40 mm × 40 mm × 160 mm) were made with a water to cement ratio (w/c) of 0.45 and a sand to cement ratio of 3 by using ordinary portland cement CEM I 52.5 N. Four different kinds of mortar mixes were prepared to test the effects of AEA on viability of *S. pastuerii* cells. Table 1 summarizes the mixes prepared for the composition of mortars for each series. BASF MasterAir 200 was used as an AEA by 0.2% weight of cement (suggested maximum amount defined by the manufacturer).

For the hardened mortar samples, the viability testing was done

Table 1
Composition of each mortar series for viability testing.

Series	Cement (g)	Sand (g)	Water (g)	UYE medium (g)	Bacterial cells (cells/g of mortar mix)	AEA(g)
R	450	1350	202.5	–	–	–
N	450	1350	–	202.5	–	–
RB	450	1350	202.5	–	4×10^6	–
RB + AEA	450	1350	202.5	–	4×10^6	0.9
NB	450	1350	–	202.5	4×10^6	–
NB + AEA	450	1350	–	202.5	4×10^6	0.9

UYE medium: Urea-Yeast Extract (pH 9); AEA: BASF MasterAir 200 Air Entraining Admixture; "–": not added to the mix. R: Neat paste/mortar; N: Nutrient paste/mortar without bacteria; RB: Bacterial paste/mortar without nutrients and AEA; RB + AEA: Bacterial paste/mortar with AEA and without nutrients NB: Bacterial paste/mortar with nutrients and without AEA; NB + AEA: Bacterial paste/mortar with nutrients and AEA.

with crushed powdered samples. The samples were removed from their curing environment and then ground into a powder using a sterile mortar and pestle. Approximately 60 g of the powdered sample were suspended in approximately 250 mL of fresh UYE medium at pH 9 and the resulting suspension was sonicated in a water bath sonicator at low frequency at 120 W for 10 min. The suspension was allowed to settle for 10 min, and the supernatant was transferred into a sterile tube and vortexed for 1 min. Triplicate serial dilutions were prepared with UYE medium (pH 9) in test tubes. The tubes were incubated at 30 °C for 3 days, at which time the optical density at 600 nm (OD_{600}) was measured. Cell concentrations were estimated from triplicate MPN values by statistical analyses [23].

2.4. Thermogravimetric analysis (TGA)

TGA analysis were conducted on cement paste samples as described in Table 1, only sand content was removed. Following the mixing, samples were cast into prismatic molds (2 × 3 × 4 cm). The specimens were initially cured at 100% relative humidity at 21 °C for 24 h. Then the molds were removed, and the samples were further cured by submerging them in UYE medium. The samples were kept in the nutrient media curing until testing occurred. TGA testing was conducted on samples at 1, 7 and 28 days.

At the time of testing, the specimens were removed from the curing solution and a representative sample was taken out from the core of the prism specimens. Then, the samples were pulverized with a pestle and mortar to provide a homogenous form. The powdered sample was ground in the presence of ethanol to stop hydration and preserve the chemical composition of the samples [24]. The samples were kept in a vacuum desiccator for 24 h and then tested in Netzsch STA 449 Jupiter TGA-DTA Analyzer (Germany). The analysis was conducted by heating the pulverized cement paste samples from 40 °C to 1100 °C and mass loss was recorded as a function of time.

3. Results and discussions

3.1. Influence of AEA on zeta-potential of cells and in-vitro $CaCO_3$ precipitation

S. pasteurii is one of the most common microorganisms used for biomineralization applications and it has been assumed that these cells have a negative surface charge in basic environments [3,24,25]. In addition, this species also induces $CaCO_3$ precipitation by serving as nucleation sites, which occur as a result of their negative surface charge that attracts positively charged calcium ions. To determine the possible influence of AEA and nutrients on surface charge of bacterial cells, zeta potential measurements were taken in bacterial cultures grown in UYE medium (pH 9) and then incubated either in DI water or fresh UYE medium with or without incorporating AEA. Then, the cells were again collected by centrifuging, washed and resuspended in sterile Tris buffer at pH 9 for testing. Results of zeta potential measurements are presented in Table 2.

Table 2
Zeta potential for *S. pasteurii* incubated in DI water or UYE medium and resuspended in 20 mM Tris buffer (pH 9); error represents the standard deviation for triplicate sample.

Sample	Incubation pH	Zeta potential (mV)
S. pasteurii in DI water	7.0	− 17.1 ± 3.9
S. pasteurii in DI water + AEA	6.6	− 16.8 ± 3.4
S. pasteurii in UYE medium	9.0	− 46.1 ± 6.7
S. pasteurii in UYE medium + AEA	8.6	− 46.3 ± 6.8

UYE medium: Urea-Yeast Extract (pH 9); AEA: BASF MasterAir 200 Air Entraining Admixture. The measurements were obtained in sterile Tris buffer at pH 9.

S. pasteurii cells incubated in DI water exhibited a substantially lower negative zeta potential as compared to *S. pasteurii* cells incubated in UYE medium. According to Halder et al. [26] possible damages in the cell wall structure can lead to a significant decrease in zeta potential of gram positive cells. Thus, when the bacterial cells were suspended in DI water, the cell wall might be damaged due to osmosis, resulting in a lower zeta potential compared to a system where the cells were suspended in UYE medium. Since cells were suspended in tap water for the mortar mixes, this negative effect might not be observed.

Addition of AEA to cultures resulted with a similar zeta potential compared to their counterpart samples that did not have AEA. Based on zeta potential measurements, it could be concluded that incubation environment had a significant effect on zeta potential of the cells, which might influence the heterogeneous nucleation of $CaCO_3$. This might be due to the difference in alkalinity of these two solutions, such that the absolute zeta potential value of cells were found to be increasing by increasing pH [27].

Fig. 1 shows the SEM images for *in vitro* biogenic $CaCO_3$ precipitation with and without AEA. Precipitates with an approximate size of 5 μm were observed in each solution. The precipitates obtained from cells cultured in UYE medium without AEA (Fig. 1a) exhibited more spheroid vaterite. There was no (or not enough) visible $CaCO_3$ precipitation in control samples, where $Ca(NO_3)_2$ was added to fresh UYE medium with and without AEA. Thus, SEM and XRD analysis could not be conducted on these samples.

Fig. 2 shows the X-ray diffractograms for *in-vitro* biogenic $CaCO_3$ precipitates collected from bacterial cultures with and without AEA. The results of the XRD analysis demonstrated that vaterite was the governing $CaCO_3$ crystal obtained in bacterial culture regardless of the AEA addition; some minor calcite formation was observed with addition of AEA to the medium.

In-vitro biogenic vaterite precipitates according to Oswald's rule, which defined the order of crystallization where the polymorph with the lower solubility precipitates first [28]. Considering the three non-hydrated crystalline polymorphs of $CaCO_3$, the reaction should progress in the order of amorphous calcium carbonate (ACC) to vaterite to aragonite or calcite [4,29]. However, reaction kinetics may be influenced by parameters such as temperature, pH, ion concentration, *etc.* Tai et al. [29] also listed the 2 major influential factors for vaterite morphology as pH and temperature. In addition, according to Mann [4], the crystal shape can also be altered with supersaturation of additives such that irregular shapes of crystals could be observed due to changes in pH and ionic concentration. The AEA used in this study has a pH of 10–11 [30], even though it was basic, it was found that addition of AEA decreases the pH of the media (see Table 2). Although the addition of AEA did not influence the zeta potential of cells, AEA is a surfactant with a negative surface charge that can also attract $[Ca^{+2}]$ in the environment; thus, it might be influential on heterogeneous nucleation of vaterite crystals on bacterial cells. Yet, the AEA studied

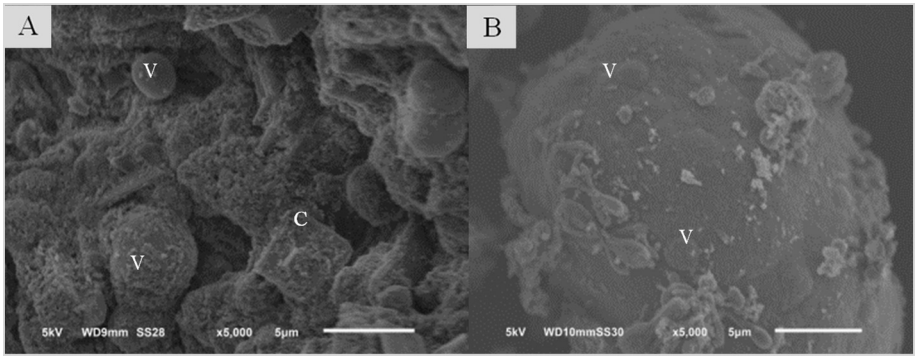

Fig. 1. SEM images of *in-vitro* biogenic $CaCO_3$ induced by (a) *S. pasteurii* grown in UYE medium + $Ca(NO_3)_2$ (b) *S. pasteurii* grown in UYE medium + $Ca(NO_3)_2$ + AEA. C: Calcite; V: Vaterite.

herein does not have a negative effect over *in-vitro* biogenic precipitation, in terms of preventing it or reducing the amount of the precipitate obtained. Thus, the microorganisms were able to trigger nucleation of vaterite crystals even if AEA is present in the specified concentration.

3.2. Impacts of AEA on cell viability in mortar

The initial concentration of viable *S. pasteurii* cells, 4×10^7 cells/mL, in the inoculum prior to mixing with cement (resulting in 4×10^6 cells per g of fresh mortar mix) was determined for all mortar samples (Fig. 3). Since the R and N samples were not inoculated with *S. pasteurii*, the viable bacterial concentrations in those samples were less than the detection limit of the assay (4×10^2 cells/g of hardened mortar).

Since the microorganisms were not encapsulated, it was expected to observe a substantial decrease in the bacterial viability, determined by the amount of bacteria that can be cultivated [31]. Even though these cells were known to be alkaliphilic, the restricted environment (*e.g.* lack of oxygen and availability nutrients) led to a significant decrease in cell concentrations even at 7 days. Approximately 3% (5×10^4 cells/g) of the initially inoculated cells (4×10^6 cells/g) remained viable at 7 days when they were incorporated with UYE medium. Only 0.1% (5×10^3 cells/g) of the cells were found viable when these vegetative cells were suspended in tap water without any nutrients at 7 days. At 28 days, the number of viable cells that remained slightly decreased in both NB and RB sample. Achal et al. [32] observed that 3.2×10^4 CFU/mL (0.05% of the initial concentration) of the initial inoculated *Bacillus megaterium* cells (5×10^7 CFU/mL) survived in mortar for 28 days when the unencapsulated microorganisms were introduced into the mix by simply suspending them in a nutrient medium. Even though it is still not clear that the vegetative cells can access the UYE medium within the cement-based matrix, the results suggested that presence of nutrients in the mix increased the viable cell retention compared to the samples that did not include nutrients (see NB *vs.* RB in Fig. 3) even

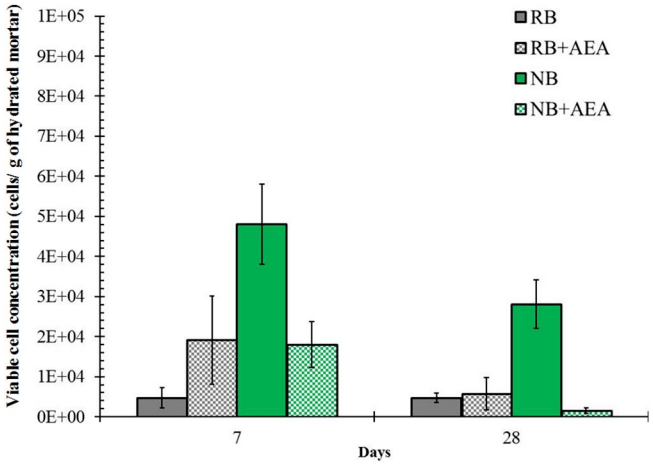

Fig. 3. MPN estimate of viable *S. pasteurii* in *Bacterial mortar* samples over time. The w/c ratio was 0.45. Error bars represents the standard deviation for triplicates of samples ($n = 3$).

28 days after casting.

The addition of 0.2% AEA by cement weight decreased the number of viable cells even at 7-days. In NB + AEA sample, the percentage of viable cells was 0.5% (2×10^4 cells/g) at 7-days, which was lower than NB sample at 7-days (3%). The decrease in viable cell retention due to AEA addition was more severe at 28 days, such that the percentage of viable cells was 0.03% (1×10^3 cells/g) in NB + AEA sample while 0.7% (3×10^4 cells/g) of the initially inoculated cells were found to be viable in NB sample. While addition of AEA in presence of UYE medium decreases the viable cell retention at all ages, incorporation of AEA slightly increased the number of remaining viable cells when the cells were introduced without any nutrients at 7 days. In addition, at 28 days

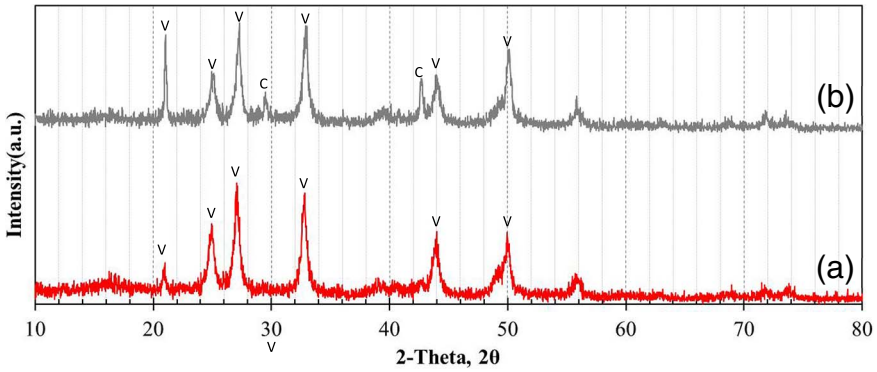

Fig. 2. X-ray diffractograms of *in-vitro* biogenic $CaCO_3$ induced by (a) *S. pasteurii* grown in UYE medium + $Ca(NO_3)_2$ (b) *S. pasteurii* grown in UYE medium + $Ca(NO_3)_2$ + AEA. C: Calcite; V: Vaterite.

the negative impact of AEA on viable cell retention in R + AEA was less compared to its effects on its counterpart N + AEA sample.

AEA are known to be strong surfactants, which concentrate at the air-water interface and reduce the surface tension encouraging the formation of stable air bubbles [33]. Generally, AEA are composed of a negatively charged hydrophilic head and a hydrophobic tail. The hydrophobic tail is attracted by the air phase, while the hydrophilic head is oriented towards the water phase; and the negative charge on the head induces a repulsive force to prevent the coalescence of the bubbles [33]. Even though it was hypothesized that AEA can create extra pore space to improve the bacterial viability, the results suggested that addition of AEA rather induces a negative impact on the viability. This might be due to the working mechanism of AEA, which are mainly used to stabilize tiny air bubbles in concrete. Hypothetically, it was assumed that the microorganisms were entrapped in entrained air voids, however, due to the presence of hydrophobic tails the water based nutrient medium could not get into the voids. Thus, this mechanism may separate microorganisms from the nutrient. This might be the reason why the decrease in viable cell retention was more severe in NB + AEA compared to the R + AEA samples in which no nutrients were present.

The incorporated cells have been stressed and partially killed due to the restricted environment of cement paste, even if the nutrients were provided (NB sample). However, since there is no urea to decompose in the RB and RB + AEA sample, the cells already suffered from nutrient depletion and a lower survival percentage was observed. Thus, it should be noted that nutrient medium is required to keep the vegetative cells viable even for 7 days. The results obtained suggested that the type and chemistry of AEA used was crucial for bacterial viability, thus due to the working mechanism AEA showed lack of suitable characteristics for protection of bacterial cells, especially when the ureolytic vegetative cells were incorporated with their nutrient media. However, this mechanism is promising for use of endospores rather than vegetative cells. With this approach, AEA can entrap endospores and keep them deactivated by separating the nutrient incorporated in the mixing water from spores. Further studies should be conducted to better understand how the mechanism of AEA impacts the viability and determine the possible use of other types of air-entrainment admixtures as a protection barrier for bacteria in self-healing cement-based materials.

3.3. $CaCO_3$ precipitation within cement-paste

The DTG curve was used to pinpoint the exact temperatures between which the calcium hydroxide (CH) and $CaCO_3$ in cement paste samples (Table 1) decomposed (Fig. 4). Using these temperatures, the weight losses due to CH and $CaCO_3$ decomposition were calculated

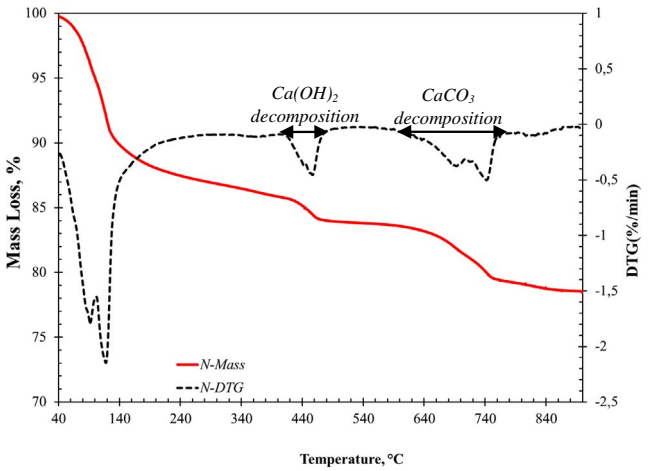

Fig. 4. Representative mass loss (TGA) and DTG curve for 3 day old Bac-UCSL sample to illustrate Ca $(OH)_2$ and $CaCO_3$ content calculation for cement paste samples.

Table 3
Mass percentages of calcium hydroxide (CH) and calcium carbonate ($CaCO_3$) in cement paste samples at 3, 7 and 28 days. A w/c of 0.45 was used.

Series	CH mass %			$CaCO_3$ mass %		
	3	7	28	3	7	28
R	11	12	11	7	7	7
N	7	11	17	7	7	8
RB	6	9	9	7	6	7
RB + AEA	8	8	8	6	6	6
NB	8	12	10	10	10	9
NB + AEA	7	12	8	10	8	9

from the TGA curve. Finally using molecular weights, the weight loss from CH and $CaCO_3$ decomposition was converted to the CH and $CaCO_3$ contents as mass percentages in cement pastes.

At day 3 of testing the CH content that was obtained in cement paste samples including nutrient and bacterial cells (RB, RB + AEA, NB and NB + AEA) was lower as compared to their counterpart control sample (R) (Table 3). Since the induction period ends as a result of the precipitation of CH and calcium silicate hydrate [33], previously this decrease was attributed to the extended induction period and delay in initial set [20]. However, at 7 and 28 days, all NB pastes had similar levels of CH.

Addition of nutrients (N sample), urea-yeast extract, did not yield an increase in the $CaCO_3$ content relative to the control sample (R). Similarly, when the bacterial cells were incorporated without any nutrients (RB), there was no change in the $CaCO_3$ mass percentage compared to the control sample. Also, there was no significant difference in the RB + AEA sample compared to the R and RB sample due to addition of AEA. The $CaCO_3$ content was slightly higher in cement paste samples having both nutrients and bacterial cells (NB and NB + AEA samples) compared to other cement paste samples. Even though this increase was limited, the presence of nutrients and cells triggered a $CaCO_3$ precipitation *within* the restricted environment of the cement paste, particularly at 3 days. The $CaCO_3$ mass percentage in the NB sample stayed constant at 3 and 7 days, while it decreased slightly at 28 days, which suggests the precipitation occurred at really early ages (*i.e.* before 3 days). This might indicate that to induce biogenic $CaCO_3$ precipitation within the cement paste matrix, the viable cell concentration remaining should be at least in the order of 10^4 cells/mL. However, similar studies have shown that the bacterial cell concentration should be at least in the order of 10^6 cells/mL in order to induce biogenic carbonate precipitation [34,35]. On a related note, while the biogenic $CaCO_3$ precipitation was affected by viable cell concentration and presence of cells, it might be also related to the surface charge of the cells. Throughout the literature, *S. pasteurii* cells have been known to induce $CaCO_3$ precipitation by serving as nucleation sites, which occur as a result of their negative surface charge that attracts positively charged calcium ions [3,36]. Even though the incorporation of AEA did not influence the zeta potential of these cells, the zeta potential dropped significantly in absolute value when the cells were suspended in DI water (see Table 2). Overall, these results suggest that maximizing biogenic $CaCO_3$ precipitation at early ages (up to 7 days) requires the presence of vegetative cells and required nutrients, urea and yeast extract.

4. Conclusions

We demonstrated that a fraction of the inoculated vegetative *S. pasteurii* cells could survive in the restrictive environment of cement paste without prior encapsulation and were able to induce $CaCO_3$ precipitation within cement paste. *In-vitro* experiments revealed that morphology of $CaCO_3$ crystals was slightly affected by the addition of AEA, while it does not affect the zeta potential of *S. pasteurii cells*.

However, the zeta potential of cells was affected when the cells were suspended in DI water. The cell viability was highly influenced by incorporation of AEA such that there was a decrease in viable cell concentration with the addition of 0.2% AEA (w/w cement), when the cells were inoculated with their nutrients. However, there was no negative impact of AEA on cell viability when cells were incorporated without their nutrients. This was attributed to the working mechanisms of AEA, where the non-polar ends of AEA act as a barrier between the nutrients and the bacterial cells. However, this mechanism is promising for use of endospores instead of vegetative cells, where the spore cells should be deactivated. TGA results demonstrated a slight increase in $CaCO_3$ content *within* the cement paste matrix, when the viable cell concentration remained above a threshold value, which was related to presence of nutrients. This suggests that biomineralization might be obtained at early ages within the cement paste matrix using a simple procedure that does not require encapsulation of the microorganisms. Special precautions have to be taken for designing self-healing bacterial concrete when AEA use is required. Self-healing ability of the vegetative bacterial cells in presence of AEA should be further investigated. Overall, the results of this study demonstrate the survival of vegetative *S. pasteurii* in mortar, which is promising for the design of a self-healing mortar that can remediate early age shrinkage cracks and microcracks occurring due to excessive loading.

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