Cement and Concrete Research 98 (2017) 44–49

Contents lists available at ScienceDirect

Cement and Concrete Research

journal homepage: www.elsevier.com/locate/cemconres

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

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ARTICLE INFO

Keywords:
Biomineralization
Air entraining admixture (D)
Calcium carbonate (D)
Cement paste (D)
Thermal analysis (B)

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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http://dx.doi.org/10.1016/j.cemconres.2017.04.005
Received 13 July 2016; Received in revised form 4 January 2017; Accepted 12 April 2017

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