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Use of bacterial cell walls to improve the mechanical performance of concrete

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ABSTRACT

This research presents the role of bacterial cell walls of *Bacillus subtilis* as a concrete admixture to improve the mechanical performance of concrete. The bacterial cell walls are known to mediate microbially induced carbonate precipitation, a process in which $CaCO_3$ is formed from Ca^{2+} ions and dissolved CO_2. Consistent with such knowledge, incorporation of bacterial cell walls increased carbonation of $Ca(OH)_2$ and formation of $CaCO_3$ in concrete. Furthermore, the bacterial cell walls significantly increased compressive strengths of concrete by 15% while also decreased porosity at 28 days of curing. Assay for $CaCO_3$ precipitation *in vitro* indicated that bacterial cell walls, but not dead cells, accelerated carbonation of Ca^{2+} ions in $Ca(OH)_2$ solution. Since $CaCO_3$ formed can fill up the void, decrease the porosity and increase the compressive strength in concrete, bacterial cell walls could act as a promising concrete admixture with benefits in enhancing mechanical performance and improving other carbonation-related properties.

1. Introduction

Microbially induced carbonate precipitation (MICP) is a natural process that has shaped the earth from ancient time. In this process, calcium carbonate minerals are formed from calcium and carbonate ions. Because calcium carbonate minerals are a homogenous material compatible with concrete and stone and is environmentally friendly, MICP has been studied as a method for stone crack repair [1]. The technology based on *Bacillus cereus*-induced $CaCO_3$ formation has been commercialized for repairing cracked surface of ornamental stones [1,2]. Also by incorporating live bacteria in concrete, MICP has been shown to improve mechanical properties [3–5] and self-healing of concrete [6]. Despite the promises of using live bacteria to induce $CaCO_3$ mineralization in concrete, many drawbacks have been encountered. The effects of bacteria on the mechanical strength of concrete are contradictory in various reports and organic nutrients added to the concrete sometimes had negative effects on mechanical strength [3,7–9]. Also bacteria were not viable in concrete beyond 7 days of curing because pores in concrete during curing shrank to size too small for bacteria to survive [9].

Bacterial cell walls, which comprise the cell surface, are known to be central to MICP [1]. Bacterial cell walls are negatively charged under environment of neutral or alkaline pH, attract the calcium ions in the extracellular environment to react with the carbonate ions and form calcium carbonate minerals on the cell surface, which serve as nucleation centers for further mineralization [10]. Currently, despite the central role of cell walls in MICP, there is no research studying the effects of directly applying cells walls in concrete. One recent research applying cell walls of *Bacillus subtilis* on cracked stone surface showed increased formation of $CaCO_3$ crystal which significantly decreased water permeability of the stone [2]. Direct application of cell walls in concrete may bypass the complications and viability issue of using live bacteria. In addition, cell walls have been shown to be more effective than the live bacteria in binding Ca^{2+} ions and forming $CaCO_3$ crystal since live bacterial cells actively pump H^+ ions across cell membrane to compete with Ca^{2+} ions for binding to the negatively charged cell walls [11,12]. As a result, mutant *B. subtilis* with defective etfA gene that generated excessive amount of H^+ ions could not form $CaCO_3$ minerals on the cell surface [13]. Here, we report the effects of bacterial cell walls on the mechanical properties of cement mortar and quantification of the amount of $CaCO_3$ formation in cement mortar. The effects of cell walls, dead cells and cell wall components in accelerating $CaCO_3$ formation in $Ca(OH)_2$ solutions were also studied. Taken together, our research demonstrated the positive effects of bacterial cells walls on the physicochemical properties of concrete, which indicates that bacterial cell wall may be a novel biological admixture for improving the performance of concrete.

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2. Materials and methods

2.1. Strains and preparation of dead cells and bacterial cell walls

B. subtilis strain 168 (ATCC, Manassas, VA), a well studied calcinogenic model bacterium [2], was selected for this study. *B. subtilis* 168 cells were cultured in nutrient broth (Thermo Fisher Scientific, Hanover Park, IL) at 37 °C. As controls, Gram positive *Micrococcus luteus* and negative bacteria *Escherichia coli* were cultured in Luria broth (Thermo Fisher Scientific). To prepare dead cells, cells were collected during the exponential growth phase, autoclaved at 120 °C for 30 min, centrifuged and re-suspended in physiological saline solution (8.5 g/l NaCl) to a concentration of 10^8 cells/ml before being stored at −20 °C. Bacterial cell walls were prepared as described by Mastromei et al. [2]. Briefly, frozen *B. subtilis*, *M. luteus* or *E. coli* cells were put in a mortar and ground for 15 min with an equal volume of alumina. Grinding with alumina has been determined to be an effective way to disrupt cells and isolate the bacterial cell walls [2]. Disrupted cells were collected by adding physiological saline solution. The suspension was centrifuged at 2500 rpm using Eppendorf 5804R fixed angle centrifuge rotor at 4 °C for 20 min, to remove alumina and unbroken cells. The supernatant will then be centrifuged at 10,000 rpm using the same rotor at 4 °C for 20 min, to separate the two fractions: supernatant (cytosol) and pellet (bacterial cell walls). Pellet was re-suspended in physiological saline solution to a concentration of 0.1 mg wet weight/ml and was stored at −20 °C until further analyses.

2.2. Preparation of cement mortar specimens

Cement mortar specimens (2 × 2 × 2 in) were prepared using type I ordinary Portland cement and sand (110 μm average grain size) sieved by No. 8 USA standard sieve with cement/sand ratio of 1:2 (volume ratio) and W/C ratio 0.68 (volume ratio). Cement mortar specimens were poured according to ASTM C-31 and cured at room temperature in water for 7 or 28 days. Bacterial reagents of various concentrations (3.3×10^{-3}, 3.3×10^{-1} and 3.3×10^{1} mg/ml for live and dead *B. subtilis* cells; and 3.3×10^{-1} and 3.3 mg/ml for cell walls of *B. subtilis*) were incorporated into the specimens as part of the mixing water to determine their effects on compressive strength, porosity and $CaCO_3$ formation. To provide nutrients for the bacteria, the liquid medium was included when live bacteria were incorporated. Thus, to be consistent, all other bacterial reagents were incorporated together with the liquid medium. The control specimens are amended with the liquid medium only (without the bacterial reagents). The liquid medium (urea-$CaCl_2$) contained the following components per 1000 ml of water: 3 g nutrient broth (Oxoid, Thermo Fisher Scientific), 20 g urea, 10 g ammonium chloride, and 2.12 g sodium bicarbonate (pH = 8) and calcium chloride was added to a final concentration of 25.2 mM. To ensure that components of the liquid medium did not significantly affect physicochemical properties of cement mortar specimens, experiments were performed using specimens amended with individual constituents of the urea-$CaCl_2$ liquid medium. All experiments were performed at least three times.

2.3. Compressive strength and porosity of cement mortar specimens

Standard compressive strengths of the concrete specimens with or without the bacterial cell walls, dead, or live cells were measured following ASTM C109. To investigate the effect on pore structures of concrete, the total void volume and porosity are measured using gas displacement pycnometry (Micromeritics Accu-Pyc 1340) [14]. The measured void volume and porosity were directly related to concrete permeability and provided an indicator of the durability and resistance of a particular concrete specimen against the ingress of aggressive media in the gaseous or liquid state.

2.4. Quantification of $CaCO_3$ mineralization in concrete

$CaCO_3$ mineralization in ground cement mortar specimens was visualized using scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDX; Hitachi S-5500) and quantified using X-ray diffraction (XRD; Rigaku Ultima IV). For SEM/EDX, $CaCO_3$ precipitate formation was directly visualized on the SEM images and identified by passing the secondary electron beams through the specimens in EDX. The accelerating voltage was set at 30 kV and the emission current was set at 20 μA for image acquisition and element analysis using EDX. For XRD, 0.15 g sample was randomly taken from each ground specimen and scanned ranging from 2.0° to 44° at 2.0°/min with the step width of 0.02°. X-ray spectra were then retrieved and analyzed using the PDXL software (Rigaku), from which identity and concentrations of $CaCO_3$ were determined.

2.5. In vitro $CaCO_3$ precipitation assay

The *in vitro* $CaCO_3$ precipitation assay was as described by Addali and Weiner [15] and Mastromei et al. [2] with modifications. Briefly, for each bacterial reagent sample, 10-fold serial dilutions using physiological saline solution was prepared starting from no dilution to at least the 7th serial 10-fold dilution to determine the best dilution for $CaCO_3$ precipitation. Then 0.5 ml of each diluted sample will be added to 1.5 ml of 7.5 mM $CaCl_2$ solution or 0.94 mM $Ca(OH)_2$ solution in six-well cell culture plates. CO_2 gas was introduced to the solutions upon releasing from slow degradation of ammonium carbonate powder and the reaction system was placed in a sealed desiccator at room temperature. Images of precipitate formation in solutions were acquired with a GelDoc™ Molecular Imager System using the Quantity One software (Bio-Rad, Hercules, CA). Samples showing precipitate with amount comparable to that of the controls (without addition of bacterial reagents) were considered negative. Samples to be tested include cell walls from the Gram positive (*B. subtilis* or *M. luteus*) or negative bacteria (*E. coli*), the respective dead cells of these bacteria, and pure peptidoglycan from the *B. subtilis* (Sigma–Aldrich, St. Louis, MO) for an maximum observation period of 14 days.

3. Results

3.1. Compressive strength and porosity of cement mortar amended with cell walls of B. subtilis

Cell walls, dead, or live cells of *B. subtilis* were added to cement mortar specimens and the effects were compared with control specimens without adding any bacterial reagents. At day 28 of curing, cell walls of *B. subtilis* amended at 3.3 mg/ml (10×) and 0.33 mg/ml (1×) concentration caused significant increase of compressive strength by 15.6% and 14.8%, respectively, in comparison with the control specimens (Fig. 1). In contrast, dead cells amended at 3.3 mg/ml concentration did not significantly affect the compressive strength at either the early (day 7) or late stage (day 28) of curing process . Dead cells added at the concentration of 33.3 mg/ml (100×) even significantly decreased the compressive strength at day 28 but not at day 7 (Fig. 2). When live cells were added to concrete at 0.33 mg/ml (1×) or 33.3 mg/ml (100×) concentration, compressive strength was slightly decreased at day 28, although not significantly different than the control (Fig. 3).

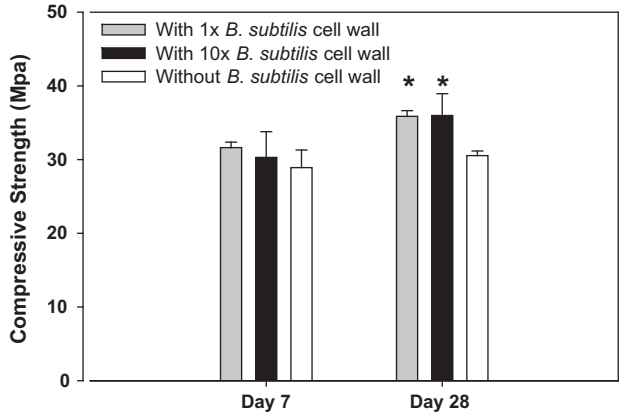

Fig. 1. Compressive strength at day 7 and 28 curing for cement mortar specimens amended with cell walls of *B. subtilis* at 0.33 mg/ml (1×) or 3.3 mg/ml (10×) concentration (cement:sand = 2:3 (volume), W/C = 1:1 (volume), specimens 2 × 2 × 2 in). All experiments were performed at least three times and the results are shown as means ± standard deviation. Star (*) indicates significant difference from the control specimens (without *B. subtilis* cell wall) with 95% confidence interval.

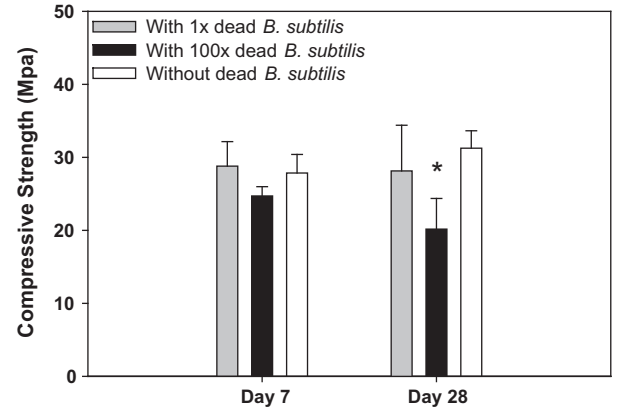

Fig. 2. Compressive strength at day 7 and 28 curing for cement mortar specimens amended with dead cells of *B. subtilis* of 0.33 mg/ml (1×) or 33.3 mg/ml (100×) concentration (cement:sand = 2:3 (volume), W/C = 1:1 (volume), specimens 2 × 2 × 2 in). All experiments were performed at least three times and the results are shown as means ± standard deviation. Star (*) indicates significant difference from the control specimens without dead cells with 95% confidence interval.

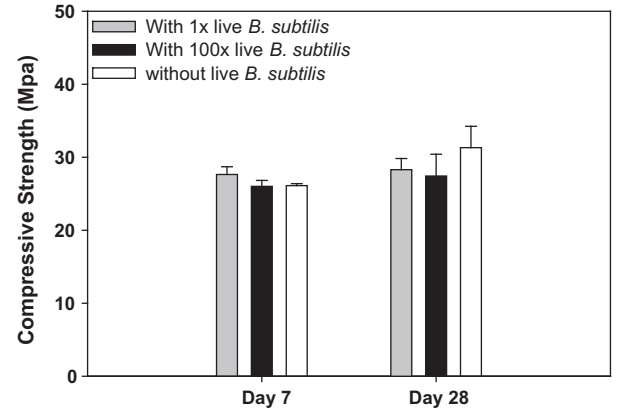

Fig. 3. Compressive strength at day 7 and 28 curing for cement mortar specimens amended with live *B. subtilis* cells of 0.33 mg/ml (1×) or 33.3 mg/ml (100×) concentration (cement:sand = 2:3 (volume), W/C = 1:1 (volume), specimens 2 × 2 × 2 in). All experiments were performed at least three times and the results are shown as means ± standard deviation.

Overall, the dead and live cells of *B. subtilis* had no positive effects on the compressive strengths of the cement mortar specimens while the cell wall of *B. subtilis* increased the compressive strength.

In the above experiments, liquid media (urea-$CaCl_2$) were added to the cement mortar specimens whenever live *B. subtilis* cells were added, similar to previous reports [9]. When the effects of cell walls of *B. subtilis*, or dead *B. subtilis* cells were studied, to be consistent with studies using live *B. subtilis* cells, liquid media were also added. One issue for such experimental design is that the liquid media (urea-$CaCl_2$) may interfere with the effect of the bacterial reagents. Thus the compressive strength of concrete specimens amended only with the liquid media or individual components of the liquid media was measured. Compared to the control specimens amended with only water, components of the media (calcium chloride, sodium bicarbonate, ammonium chloride, nutrient broth, and urea) or the liquid media at the concentration levels used in the study of bacterial reagents had no statistically significant effect on the compressive strengths (Fig. 4).

Having found cell walls to be the only bacterial reagent that had a positive effect on the compressive strength and had the potential to solve the issues of concrete incorporating live bacteria [3,7–9], we performed further study on the effects of bacterial cell walls on concrete porosity using gas displacement pycnometry. Our results indicated that porosity decreased in the presence of cell walls of *B. subtilis* significantly by 2.47% and 1.64% on average at day 7 and 28 of curing, respectively (Fig. 5).

3.2. In vitro $CaCO_3$ precipitation in the presence of cell walls of *B. subtilis*

Having found the dramatic effects of bacterial cell walls in increasing compressive strength and decreasing porosity of concrete, we then performed an *in vitro* $CaCO_3$ precipitation assay to determine whether bacterial cell walls can directly modify $CaCO_3$ precipitation in solution. $CaCl_2$ (7.5 mM) and $Ca(OH)_2$ (0.94 mM) solutions with the addition of cells walls or dead cells of *B. subtilis*

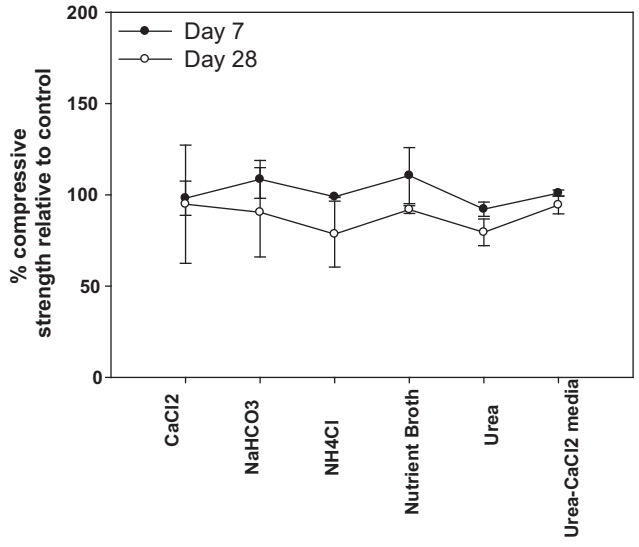

Fig. 4. Effects of individual components of urea-$CaCl_2$ liquid media on compressive strength (cement:sand = 2:3 (volume), W/C = 1:1 (volume), specimens 2 × 2 × 2 in). The *Y* axis is expressed as the percentage of compressive strengths of the concrete specimens incorporate with individual components of the of urea-$CaCl_2$ culture media (indicated on the *X* axis) relative to that of control concrete specimens incorporated with water. All experiments were performed at least three times and the results are shown as means ± standard deviation.

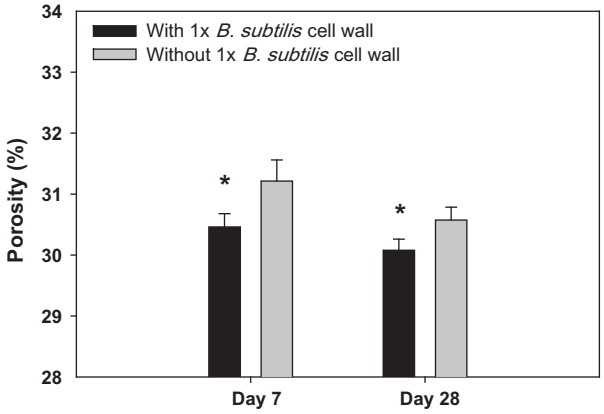

Fig. 5. Porosity of cement mortar specimens amended with or without the cell wall of *B. subtilis* of 0.33 mg/ml (1×) concentration. Star (*) indicates significant difference from the control specimens (without *B. subtilis* cell wall) with 95% confidence interval.

were studied, in comparison to control solutions without addition of bacterial reagents. Our results showed that in $CaCl_2$ solution, $CaCO_3$ precipitant formation was accelerated in the presence of either cell walls or dead cells of *B. subtilis* (Fig. 6a), which was consistent with the previous report [2]. In $Ca(OH)_2$ solutions, however, $CaCO_3$ formation was accelerated only in the presence of cell walls, but not dead cells of *B. subtilis* (Fig. 6b). In the presence of cell walls, the amount of $CaCO_3$ formed in $Ca(OH)_2$ solution reached the maximal level by day 2 whereas the amount in $CaCl_2$ solution reached the maximal level at day 6. Such difference of carbonation rate was not surprising as $Ca(OH)_2$ solution provided an alkaline environment for better dissolution of CO_2 and dissociation of carbonate ions. Given the results of the *in vitro* $CaCO_3$ precipitation assay, it is likely that in hydrated concrete which contains more than 20% free and bound $Ca(OH)_2$, addition of bacterial cell walls may affect rates of carbonation of $Ca(OH)_2$ and alter the development of mechanical properties during curing.

3.3. $CaCO_3$ mineralization in cement mortar amended with cell walls of *B. subtilis*

Having shown that cell walls of *B. subtilis* can accelerate $CaCO_3$ mineralization in aqueous solution (Fig. 6), we then studied whether addition of cell walls of *B. subtilis* can increase $CaCO_3$ mineralization in cement mortar specimens. Upon addition of the cell walls, the amount of $CaCO_3$ formed in ground cement specimens was quantified using X-ray diffraction (XRD) and scanning electron microscope (SEM)/energy dispersive X-ray (EDX). The results indicated that amendment with cell walls increased $CaCO_3$ formation in cement mortar specimens by 10.0 ± 1.2% at day 7 curing, compared with the specimens without cell walls. XRD and SEM results indicated that the $CaCO_3$ formed is of calcite polymorph (Fig. 7a) with rhombohedral crystalline structure (Fig. 7b). EDX point analyses of the SEM specimens showed that the elemental composition was mostly of calcium, carbon and oxygen (Fig. 7c), which was consistent with the mechanism that cell walls of *B. subtilis* induce $CaCO_3$ formation in concrete.

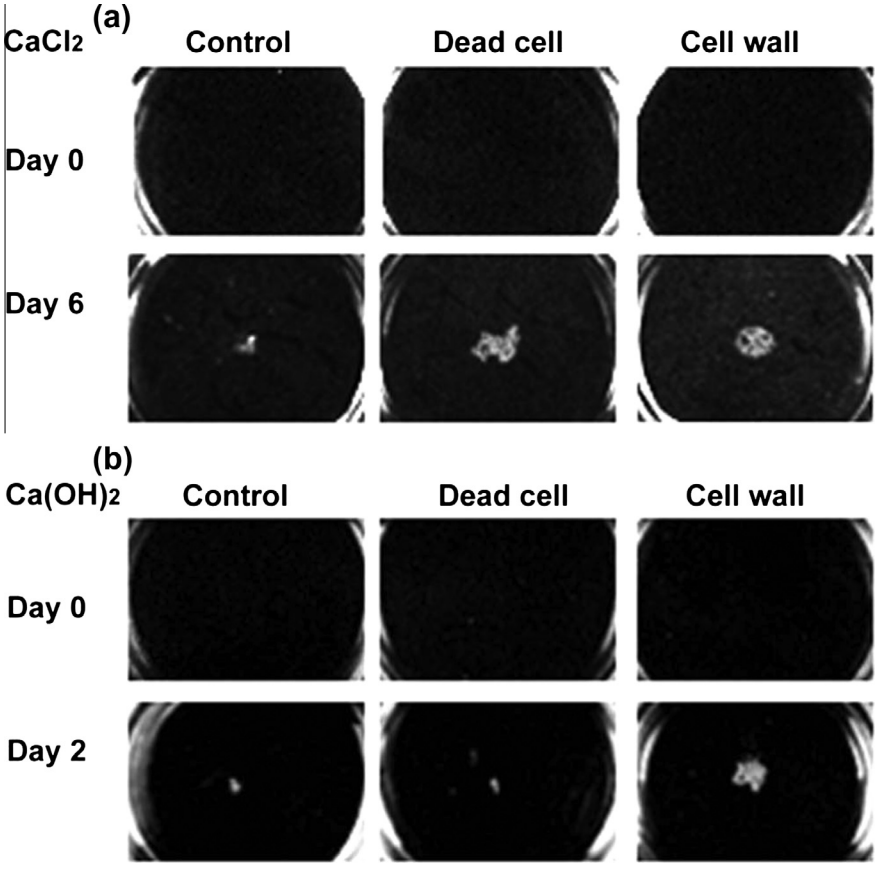

Fig. 6. *In vitro* precipitation of $CaCO_3$ from the (a) $CaCl_2$ (7.5 mM) or (b) $Ca(OH)_2$ (0.94 mM) solution in the presence of the dead cells or cell walls of *Bacillus subtilis*. The results are representative of at least 5 experiments.

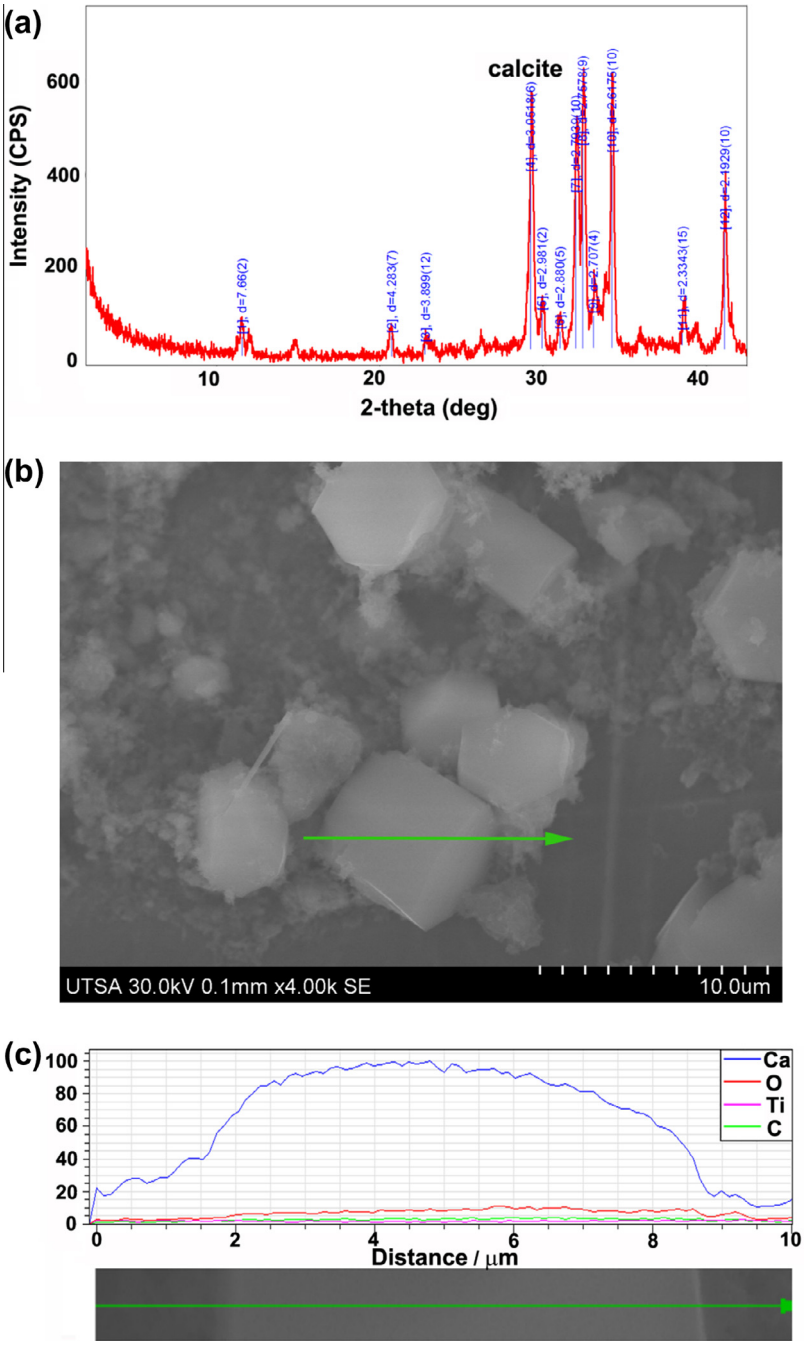

Fig. 7. Measurement of $CaCO_3$ mineralization in ground 7 day-cured cement specimens. (a) Representative X-ray diffraction results at a continuous scanning rate of 2° min^{-1}; (b) $CaCO_3$ crystal formation in the presence of cell walls of *Bacillus subtilis*, as visualized using scanning electron microscopy at 5000× magnification; and (c) energy dispersive X-ray analysis of mineralogical composition of the $CaCO_3$ crystal formed.

3.4. Effects of cell walls, dead cells and peptidoglycan of other bacteria

Bacteria of diverse species are capable of forming $CaCO_3$ crystal in MICP [16,17]. Having shown the effects of cell walls of *B. subtilis* to increase $CaCO_3$ formation in concrete (Fig. 7) and in solution (Fig. 6), we want to know whether cell walls of other bacteria have similar effects and what the effective components of cell walls are. Therefore, cell walls of Gram positive (*B. subtilis* or *M. luteus*), negative bacteria (*E. coli*), the dead cells of these bacteria, or purified peptidoglycan of *B. subtilis*, which is a major biochemical component in cell wall, were compared in the *in vitro* $CaCO_3$ precipitation assay. The cell walls of all Gram positive (*M. luteus*) and negative bacteria (*E. coli*), similarly to the cell walls of *B. subtilis*, accelerated $CaCO_3$ formation in $Ca(OH)_2$ solution. And the dead cells of Gram positive and negative bacteria, similar to the dead cells of *B. subtilis*, did not accelerate $CaCO_3$ formation in $Ca(OH)_2$ solution (Fig. 8a and b). Similarity of results among all bacteria is consistent with the reports that both Gram positive and negative bacteria are capable of causing $CaCO_3$ crystal formation in MICP. This suggests that the cell walls of many bacteria, not only those of *B. subtilis*, may act as bacterial admixtures for modulating concrete properties. Peptidoglycan of *B. subtilis* did not affect $CaCO_3$ formation (Fig. 8c), suggesting that other components but not peptidoglycan in cell walls of *B. subtilis* are responsible for $CaCO_3$ formation.

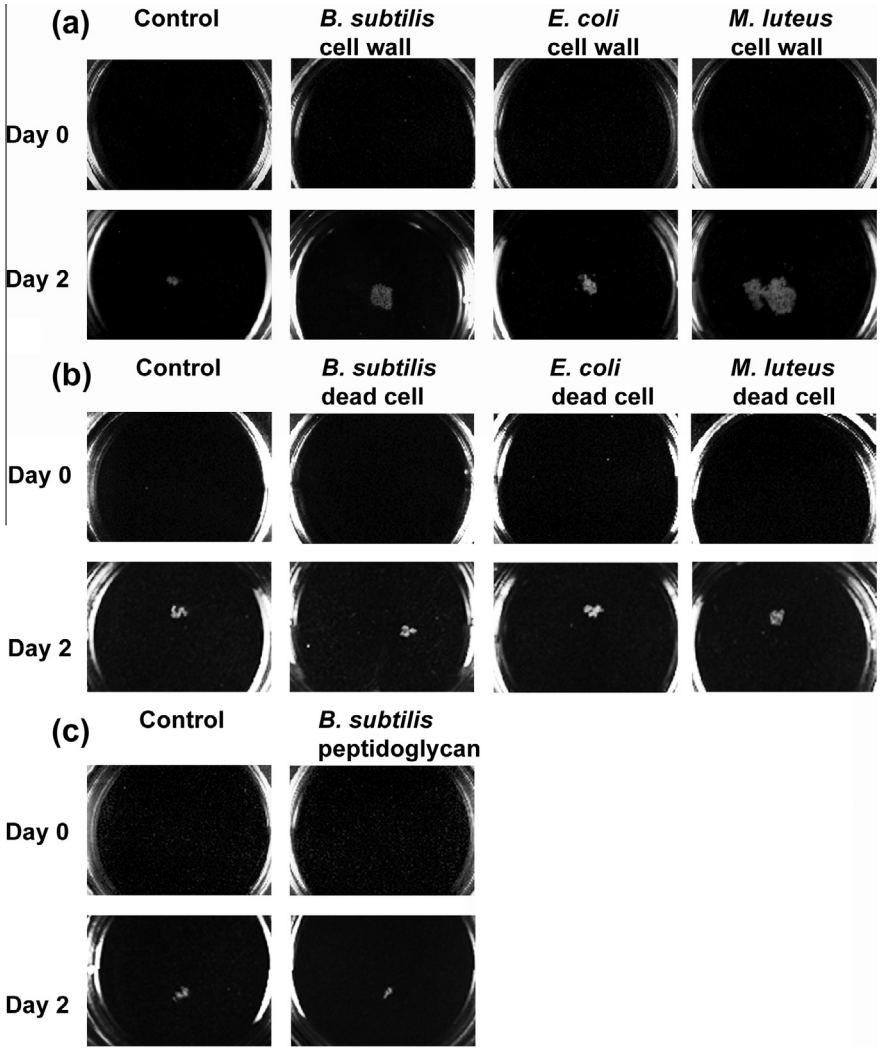

Fig. 8. *In vitro* precipitation of $CaCO_3$ in the $Ca(OH)_2$ solution (0.94 mM) in the presence of (a) cell wall (0.33 mg/l), (b) dead cell (3.3 mg/ml) of various bacteria species or (c) purified peptidoglycan of *Bacillus subtilis* (3 mg/ml). The results are representative of at least 5 experiments.

4. Discussions

4.1. Comparison of the effects of various bacterial reagents as concrete admixtures

Despite the fact that bacterial cell walls play a central role in MICP [1] and bind Ca^{2+} ions better than living bacterial cells [11,12], there is currently no study for the effect of direct incorporation of bacterial cell walls in concrete. Our study indicated that cell walls of *B. subtilis* incorporated in cement mortar specimens caused increase of compressive strength and decrease of porosity (Figs. 1 and 5). Bacterial cell walls increased formation of $CaCO_3$ minerals in cement mortar, as shown in XRD and SEM/EDX analyses (Fig. 7). In addition, bacterial cell walls directly accelerated $Ca(OH)_2$ carbonation and $CaCO_3$ mineralization in aqueous solutions (Fig. 6). Therefore, bacterial cell walls have the potentials to be developed into a concrete admixture with industrial application and thus further research is warranted to fully characterize the actions of bacterial cell walls in concrete.

In contrast, our results showed that live bacteria cells incorporated in concrete did not cause increase of compressive strength (Fig. 3). Such results are not unexpected, given the different results from many species of live bacteria incorporated in concrete [3–5,9]. The thermophilic and anaerobic bacteria *Shewanella* sp., but not *E. coli*, caused formation of $CaCO_3$ minerals and significant increase of compressive strength of concrete [3,5]. Bacteria including *Arthrobacter crystallopoietes*, *Sporosarcina soli*, *Bacillus massiliensis*, and *Lysinibacillus fusiformis* caused $CaCO_3$ mineral formation; however, only *A. crystallopoietes* increased compressive strength significantly [4]. Soil bacteria *Bacillus cohnii* caused $CaCO_3$ mineral formation but significantly decreased compressive strength in concrete [9]. Our results revealed the effect of *B. subtilis* on compressive strength of concrete. The different results might be due to differences of bacteria species and experimental conditions.

Contrary to the effects by cell walls of *B. subtilis*, the dead cells of *B. subtilis* had no positive effect on the compressive strengths (Fig. 2). In fact, decrease of compressive strength by dead cells of *B. subtilis* of high concentration at 28 days of curing was observed (Fig. 2). Such results are consistent with the results of *in vitro* $CaCO_3$ precipitation assay that dead cells of *B. subtilis* (Fig. 6) and other Gram positive and negative bacteria (Fig. 8) did not cause acceleration of $CaCO_3$ formation in $Ca(OH)_2$ solutions. One explanation for the differences between bacterial cell walls and dead cells is that the components of bacterial cells such as proteins and polysaccharides may carry negative charges under the basic pH in $Ca(OH)_2$ solutions, which interfere with the effect of negatively charged cell walls as nucleation centers of further $CaCO_3$ formation.

When the effects of cell walls, dead and live cells of *B. subtilis* were compared, the liquid medium (urea-$CaCl_2$) was incorporated in all cement mortar specimens. Liquid media incorporated in cement mortar specimens may possibly interfere with the effects of the bacterial reagents, as indicated by previous studies using media to mix bacteria with concrete [3,5,9]. Ghosh et al. found that addition of media had no effects on compressive strength [3,5]. Jonkers et al. observed that addition of calcium acetate, peptone, or yeast extract, which are components of the bacteria culture media, caused decrease of compressive strength of concrete, while addition of calcium lactate caused a slight increase of compressive strength, compared to the control adding water [9]. Therefore, we studied the effects of liquid media and found that addition of the liquid media and the individual components including calcium chloride, sodium bicarbonate, ammonium chloride, nutrient broth, and urea, were statistically indistinguishable from addition of water for compressive strength at 7 or 28 days of curing (Fig. 4). The reason for such results may be that the amounts of media or the components used in concrete were too small to cause significant effects. For example, calcium chloride is known to affect compressive strength development at day 7 (moderately increased) and 28 (decreased) of curing as an accelerating admixture [18,19]. However, such effects require a concentration between 0.5% and 2%. In our experiments, calcium chloride of concentration 25.2 mM, which corresponds to 0.28%, was used, which was too low to affect compressive strength significantly. Taken together, the cell culture media used in our experiments should not be a confounding factor for the action of the bacteria reagents under investigation.

4.2. Mechanisms of the effects of bacterial cell walls on compressive strength of concrete

Our *in vitro* $CaCO_3$ precipitation results showed that the bacterial cell walls are capable of accelerating $CaCO_3$ formation in $Ca(OH)_2$ solutions. The XRD and SEM/EDX results indicated that bacterial cell walls increased $CaCO_3$ formation in cement mortar. Thus we have good evidence to believe that bacterial cell walls accelerate concrete carbonation, consistent with the established role of cell walls in MICP. A number of studies for concrete carbonation have reported that formation of $CaCO_3$ in concrete can increase the mechanical strength of concrete [20–22]. Thus, the increase of compressive strength and decrease of porosity by cell walls of *B. subtilis* may be caused by acceleration of calcium carbonate mineralization in concrete. A number of potential mechanisms are proposed below. First, by converting $Ca(OH)_2$, which is the most important source of Ca^{2+} in concrete but plays a weak structural role, into solid $CaCO_3$ with strong structural role, $CaCO_3$ formed can increase the compressive strength. Second, $CaCO_3$ can fill the void in concrete, decrease porosity, and improve the particle packing efficiency, thereby making concrete dense and increasing the compressive strength as described in the particle packing model [23]. Third, prior research has shown that $CaCO_3$ directly added in cement paste became nucleation centers for C–S–H formation during cement hydration, and accelerated cement hydration [24,25]. Thus another possible mechanism for the increase of compressive strength and decrease of porosity is that $CaCO_3$ mineralization induced by cell walls creates nucleation centers for C–S–H formation which accelerates further C–S–H formation. Fourth, it is possible that bacterial cell walls, via the negative charges carried, may directly act as nucleation center for concrete hydration, similar to the report for the role of negatively charged C–S–H particle as nucleation center in concrete hydration [26]. Despite explanatory power of these potential mechanisms, exactly how acceleration of calcium carbonate mineralization caused increase of compressive strength and decrease of porosity in our study is not known and $CaCO_3$ formation may potentially lead to opposite outcomes, depending on the study context. In the literature, $CaCO_3$ formation could lead to increase [27,28], decrease [21,29], as well as both decrease and increase of porosity (in different parts of the specimen) [30]. As Johannesson and Utgenannt put it, "The calcium carbonate formed has a very low solubility and will therefore contribute to a clogging of the concrete pore network. However, the volume expansion involved in (carbonation) reactions will cause microcracks in the carbonated zone. Therefore, it is difficult to predict the change of transport properties for gases, such as CO_2 and O_2, due to carbonation [31]. Likewise, $CaCO_3$ formation has been reported to have positive effects on the compressive strength of concrete [20–22], as well as have negative effects when uneven expansion of the concrete on the surface versus the inside cause crack formation and decrease of compressive strength [32]. To determine the exact mechanisms, further studies are needed by using more elaborate tools such as neutron diffraction [33] and thermogravimetric analyses [34] to monitor the physicochemical changes during concrete curing in the presence of the cell walls.

We also performed *in vitro* $CaCO_3$ precipitation assay (Fig. 8) to study the cell walls of Gram positive bacteria such as *B. subtilis* and *M. luteus* and Gram negative bacteria such as *E. coli*, dead cells of these bacteria species, as well as, pure peptidoglycan from *B. subtilis*. These bacterial reagents have different structures and biochemical compositions. Gram positive bacteria contain a thick cell wall (20–80 nm thickness) and an inner cell membrane. The cell walls of Gram positive bacteria contain more than 50% peptidoglycan as well as covalently linked polymers including teichoic acids and proteins [35]. Gram negative bacteria contain a thin cell wall (10 nm) sandwiched between an inner and outer cell membrane. The cell walls of Gram negative bacteria contain 10–20% peptidoglycan as well as lipid and lipid protein but no teichoic acids [36]. Besides cell walls and membrane, the biochemical components of the main cell body include proteins, DNA, polysaccharides. Our results showed that cell walls, but not dead cells, of *B. subtilis* and other Gram positive and negative bacteria, caused acceleration of $CaCO_3$ formation in $Ca(OH)_2$ solutions. One explanation is that the components of dead bacteria cells such as proteins and polysaccharides may carry negative charges under basic pH in the $Ca(OH)_2$ solution, which interfere with the effect of negatively charged cell walls as nucleation center for $CaCO_3$ formation. The results that the cell walls of Gram positive and negative bacteria behaved similarly in the *in vitro* $CaCO_3$ precipitation assay suggest that cell walls of these bacteria may also be potential concrete admixtures. We also found that pure peptidoglycan had no effects on $CaCO_3$ precipitation in $Ca(OH)_2$ solution (Fig. 8b), which ruled out the possibility that peptidoglycan is the component in bacterial cell wall responsible for accelerating $Ca(OH)_2$ carbonation. Together with the results that the cell walls of Gram positive and negative behaved similarly in the *in vitro* $CaCO_3$ precipitation assay, this suggests that yet-to-be-identified common components of Gram positive and negative bacterial cell walls, such as proteins, is responsible for $CaCO_3$ precipitation. Another issue to consider is the size distribution of the cell walls. Our current experiments did not directly address this issue. The raw preparation of cell walls used in this study, which was prepared as described in [2], is reported to be a mixture of cell wall and cell membrane, which are composed of the following biochemical components, polysaccharides, lipid, and proteins [2]. The purified peptidoglycan (Fig. 8c), which was addressed in this study as a major polysaccharide component of cell walls, was purchased from Sigma–Aldrich but had no accompanying molecular weight information. In the literature, the molecular weight of peptidoglycan from a similar Gram positive *Streptococcus* sp. is at least 5×10^7 Dalton [37]. In order to gain insight into the effective components, it is necessary to perform further biochemical characterization of

cell walls in the future and the size distribution of the components should be studied using methods such as gel permeation chromatograph [38].

4.3. Potentials of bacterial cell walls as a concrete admixture

Our results indicated that the bacterial cell walls can be used as promising biological admixtures to improve the performance of concrete. Use of biologically derived admixtures in the construction industry is not uncommon, for example, the polysaccharide-based Welan gum and Diutan gum purified from bacteria [39]. Here we discuss the potential consequences of using bacterial cell walls as a concrete admixture. Such cell wall concrete admixture can improve mechanical properties and transport properties of concrete, as our result indicated. In addition, cell walls may have potential influence on those concrete properties related to carbonation including autogenous crack healing of concrete [40], re-absorption of CO_2 by concrete [41], and alkalinity reduction in the concrete matrix [18]. First, autogenous crack healing of concrete refers to the ability of concrete to heal the cracks by itself and is accepted to be mainly caused by precipitation of $CaCO_3$ in the crack during concrete carbonation [42]. Currently autogenous repair has a limitation on the width of crack it can repair (less than 0.2 mm) [40,43]. A recent research has shown that incorporation of live bacteria in concrete can repair cracks up to 0.46 mm [6]. Bacterial cell walls may have similar effects in overcoming the limitation. Second, during useful life of concrete (typically 70 years) less than 40% of CO_2 emitted during manufacturing of cement is re-absorbed [44,45]. The fact that bacterial cell walls may accelerate the re-absorption of CO_2 may have huge environmental impact as current manufacturing of concrete contributes 5% to the annual anthropogenic CO_2 release [46]. Third, bacterial cell walls may reduce the alkalinity of concrete by accelerating concrete carbonation. This has the possible consequence of loss of protective layer on steel rebar surface and corrosion of steel reinforcement [47]. Therefore, the effect of cell walls on reducing the alkalinity in concrete should be investigated by carefully monitoring the pH of concrete matrix [48].

5. Conclusion

In an effort to determine the effect of bacterial cell walls on physicochemical properties of concrete, our results showed that bacterial cell walls of *B. subtilis* significantly increased compressive strength and decreased porosity. In contrast, live and dead cells of *B. subtilis* and components of the urea-$CaCl_2$ media did not increase compressive strength. Consistent with the results in concrete, cell walls of Gram positive bacteria (*B. subtilis* and *M. luteus*) and negative bacteria (*E. coli*), but not dead cells of these bacteria, accelerated $CaCO_3$ formation in $Ca(OH)_2$ solution. Therefore, bacterial cell walls may accelerate carbonation of concrete and alter carbonation-related physicochemical properties of concrete and thus have the potential to be an admixture of concrete with benefit in enhancing not only mechanical performance, but also other carbonation-related concrete properties.

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