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Alkaliphilic *Bacillus* species show potential application in concrete crack repair by virtue of rapid spore production and germination then extracellular calcite formation.

T.K. Sharma[1], M. Alazhari[2], A. Heath[2], K. Paine[2] and R.M. Cooper[1]

1 Department of Biology and Biochemistry; 2 BRE Centre for Innovative Construction Materials, University of Bath

BA2 7AY, United Kingdom

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Correspondence

Richard Cooper, Department of Biology and Biochemistry, University of Bath, BA2 7AY, United Kingdom.

Abstract

Aims: Characterisation of alkaliphilic *Bacillus* species for spore production and germination and calcite formation as a prelude to investigate their potential in micro-crack remediation in concrete.

Methods and Results: Conditions, extent and timing of endospore production was determined by dark field light microscopy; germination induction and kinetics was assessed by combining reduction in optical density with formation of refractile bodies by phase contrast microscopy. *B. pseudofirmus* was selected from several species as the most suitable isolate. Levels and timing of calcium carbonate precipitated *in vitro* by *B. pseudofirmus* was evaluated by atomic absorption spectroscopy and structural identity confirmed as calcite and aragonite by Raman spectroscopy and FITR. The isolate produced copious spores that germinated rapidly in the presence of germinants L-alanine, inosine and NaCl. Bacterial cells produced $CaCO_3$ crystals in micro-cracks and the resulting occlusion markedly restricted water ingress.

Conclusions: By virtue of rapid spore production and germination, calcium carbonate formation *in vitro and in situ*, leading to sealing of micro-cracks, *B. pseudofirmus* shows clear potential for remediation of concrete on a commercial scale.

Significance and Impact of Study: Microbial sealing of micro-cracks should become a practicable and sustainable means of increasing concrete durability.

Keywords

Bacillus sporulation, endospore germination, survivability of spores, micro-crack healing, concrete durability.

Introduction

Metabolic activities in microbes yield insoluble organic and inorganic materials, intra- or extra-cellularly (Lappin-Scott *et al.* 1988). The processes that lead to production by living organisms of inorganic material such as phosphorites, carbonates, silicates and iron and manganese oxides in the form of shells, skeletons and teeth are termed bio-mineralization (Beveridge *et al.* 1983; Ghiorse 1984). Biocalcification involves precipitation of calcium carbonate polymorphs and occurs commonly in soil, fresh water and marine environments (Shirakawa *et al.* 2011).

Research into and potential application of bio-calcification has included restoration of limestone on historic monuments and ornamental stone (Rodriguez-Navarro et al. 2003), soil stabilization and microbiologically enhanced crack repair (De Muynk et al. 2010). More generally, $CaCO_3$ precipitation induced by bacterial activity can increase stability of structures in civil engineering (Sarmast *et al.* 2014). The methods mimic what has been occurring naturally, since many carbonate rocks have been cemented over eons by microbe-induced calcium carbonate precipitation (Rodriguez-Navarro et al. 2003).

The most widely accepted hypotheses related to $CaCO_3$ precipitation are based on accumulation of unused calcium ions in the extracellular medium (Rodriguez-Navarro et al. 2003; Shirakawa *et al.* 2011). There are at least two pathways for microbial precipitation of $CaCO_3$: passive and active. Ureolytic bacteria degrade urea to yield CO_2 and NH_3 then accumulation of NH_3 in the vicinity increases pH resulting in passive deposition of $CaCO_3$ (Castanier *et al.* 1999). Active $CaCO_3$ precipitation is a result of ion exchange through the cell membrane and activation of Ca^{2+} and/or Mg^{2+} ion pumps, possibly combined with carbonate ion production (Le Métayer-Levrel *et al.* 1999). In some microorganisms a series

of bioprocesses like photosynthesis, ammoxification, denitrification, sulfate reduction and anaerobic sulfide oxidation induce extracellular precipitation of carbonate (Baskar *et al.* 2009). Calcite, vaterite and aragonite are three polymorphs of calcium carbonate precipitated by bacteria (Wei *et al.* 2015).

The strength and relatively low cost of concrete explain it as the most widely used construction component world-wide (Jonkers *et al.* 2010). However, the low tensile strength of concrete and its susceptibility to cracking often compromise the structural integrity. Although crack widths smaller than 0.2 mm do not impose a structural threat, ingress of chlorides, sulphates and acids result in corrosion of steel reinforcement, or expansion of the hardened cement paste, and this can lead to catastrophic structural damage (Edvardsen 1999; Reinhardt and Jooss 2003). Enormous expenditure is required for maintenance and repair of cracks. It is estimated that the United States spends 4 billion dollars annually on concrete highway bridges as an outcome of reinforcement corrosion (Koch *et al.* 2001).

In order to create a sustainable and cost effective alternative, microbiologically induced calcite precipitation is being studied for application in healing of cracks in concrete (van Tittelboom *et al.* 2010). Bacteria in the form of *Bacillus* species endospores can be added to the concrete mix (naked or encapsulated) while casting but they must withstand extreme alkaline pH values, dehydrating condition within concrete, and survive high mechanical compressive forces during concrete curing (Jonkers et al 2010). Previous investigations on sealing of cracks with bacterially precipitated $CaCO_3$ have involved *B. cohnii*, *B. pasteurii* (syn, *Sporosarcin*a), *B. sphaericus and B. alkalinitrilicus* (Bang *et al.* 2010; Jonkers *et al.* 2010, van Tittelboom et al. 2010; Wiktor and Jonkers, 2011). Calcium carbonate precipitation on application of immobilized *S. pasteurii* cells to the cracks enhanced concrete performance (Bang et al. 2010) and *Shewanella* species have also been shown to achieve crack remediation (Ghosh *et al.* 2005), but these species do not produce endospores.

Application of *Myxococcus xanthus* to restore surfaces of ornamental stones induces new carbonate-crystal grains that are more durable than the original limestone (Rodriguez-Navarro *et al*, 2003). These various studies have typically taken an engineering perspective and often neglected optimization and understanding of the basic microbiology and calcite formation required for the potentially important application of these refractile, crystal-inducing, bacterial strains.

In this work we have avoided use of ureolytic bacteria, because oxidation of urea to nitrous acid and then nitric acid can lead to severe corrosion of building materials (Allan 1999). We describe, often for the first time in the context of bacteria used in concrete remediation, selection from three alkaline-tolerant *Bacillus* spp., evaluation of speed and extent of endospore formation, triggers for spore germination, ability of spores to survive in concrete, and the kinetics, amounts and structural confirmation of calcite production. These features are prerequisites to function, in combination with suitable nutrients and precursors, to create a product effective in crack-healing. The findings are highly encouraging because spore production could be readily commercialized, spores survive the formation of mature concrete and bacterial cells are efficient producers of calcite not only *in vitro* but *in situ*.

Materials and Methods

Bacterial isolates and growth

Three alkalinophilic species *Bacillus pseudofirmus* DSM 8715, *Bacillus cohnii* DSM 6307 and *Bacillus halodurans* 497 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany and stored in 50% (v/v) glycerol at -80°C. They were routinely cultured in buffered Luria Bertani (LB) broth which contained 100

ml l^{-1} Na-sesquicarbonate (Na-sesquicarbonate composition l^{-1}, $NaHCO_3$ 42 g and Na_2CO_3 anhydrous 53 g) to achieve pH 9.5.

Spore preparation, sporulation and germination

Spores were prepared in a sporulation medium and resulting spores were harvested after 48h growth by centrifugation (Jonkers *et al*. 2010). The spore pellet was rinsed thrice with chilled 10mM Tris HCl buffer pH 9 followed by chlorohexidine digluconate treatment (0.3 mg ml^{-1}, 30 min) to kill vegetative cells and further washed thrice with chilled 10mM tris HCl buffer pH 9. For studying spore formation, spore samples were stained (Schaeffer and Fulton 1993).

Spore germination was determined by coupling: (a) a qualitative procedure involving the percentage decrease in OD 600 nm of spore suspensions during germination (Kudo and Horikoshi 1983a; Hornstra *et al***.** 2006) and (b) direct counting of refractile, non-germinated spores by phase-contrast microscopy (x1000) (Powell 1950). Spores were suspended in buffer (Tris HCl, 0.03 mol l^{-1}, pH 9.5) containing germination triggers 10μM inosine and L-alanine, and 0.1 mol l^{-1} NaCl. They were incubated at 30°C in a final volume of 3 ml and adjusted to OD 0.25 before assessments at 15, 30, 45, 60, 90 and 120 min, then overnight. Phase contrast microscopy of freshly harvested spores revealed c. 10% dark and non-refractile vegetative cells, which could be mistaken for germinated spores. Therefore before testing potential stimulants of spore germination, viable vegetative cells were selectively killed with chlorhexidine digluconate. Post treatment, bacterial spores appeared bright and refractile.

Preparation of cement paste specimens and viability of bacterial spores

The survivability of spores in cement paste was studied by adding spores to cement at the same time as the addition of water. Freeze drying involved, washing as described earlier spore pellet thrice with 10mM Tris-HCl pH 9, then chlorohexidine digluconate, then a further

triple wash with Tris-HCl before lyophilisation at -40C in an Edwards Modulyo Freeze Dryer. Dried spores were stored desiccated at 4°C. Fifty mg of freeze-dried spore powder was added to cement (sterilized at 100°C overnight) made to a paste with sterile distilled water, ratio 1:2. This paste was placed in moulds (45 x 45 x 5 mm) and demoulded after 24 h and cured at room temperature (ca. 20°C) in sterile conditions. The moulds had been first surface sterilized, once with chlorohexidine digluconate then with 70% ethanol. The specimens contained either 1.21×10^6 spores g^{-1} of *B. pseudofirmus*, or 1.44×10^6 spores g^{-1} of *B. cohnii* or 4.4×10^6 spores g^{-1} of *B. halodurans*. Three cement pastes were made for each of the bacterial species. The cement used was a Portland fly ash cement, CEM II/B-V 32.5R, conforming to BS EN 197-1. All specimens were stored in a sterile container, which was opened under sterile conditions.

Spore viability was estimated by total viable count from cement specimens aged between 1 and 93 days. One gram of each specimen was comminuted using sterile mortars and pestles, thoroughly vortexed to enhance spore extraction, then one ml aliquots were serially diluted (in triplicate) in sterile Tris-HCl buffer pH 9.5. Aliquots of suitable dilution were plated on LB alkaline agar plates at 30°C overnight and total viable count recorded after 18 h.

Selection of an appropriate medium for quantification of $CaCO_3$ formation

One ml of a culture of *B. pseudofirmus* DSM 8715 grown overnight in LB broth at 30°C, 150 rpm was seeded to 100 ml of the following two media: 1/10th strength LB acetate medium (LBA, containing l^{-1} tryptone 1g, yeast extract 0.5 g, NaCl 1g, calcium acetate 2.5 g); B4 medium containing l^{-1} (yeast extract 4 g, glucose 5 g, calcium acetate 2.5 g) All media were adjusted to pH 8 with NaOH.

Quantification of calcite precipitation *in vitro*

Collection and processing of crystals from liquid cultures was the same for subsequent analyses by atomic absorption spectroscopy (AAS), Fourier-transform-infrared spectroscopy FTIR, Raman Spectroscopy, scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX). Crystals were extracted daily until 8 d for AAS and after 8 d for other analytical methods. Cultures were inoculated and incubated as above, then fluids from triplicate cultures were centrifuged (3,800 x g, 10 min) and pellets washed once with 1/10th LB without Ca-acetate and then twice with SDW; the washed pellet was then filtered on nitrocellulose membranes (3µm, 45mm diam.). Crystals from the membrane were collected, washed thrice with SDW then air dried. The dried crystals were dissolved in 0.6 mol l^{-1} HCl (Schäfer *et al*. 2011) added slowly to avoid effervescence, then analysed for elemental Ca^{2+} by atomic absorption spectroscopy (AAS) (Perkin Elmer AAnalyst 100) with an acetylene nitrous oxide flame at wavelength 422.7nm; slit width 0.7nm. All samples standards and blank contained 2000ppm potassium as an ionisation suppressant.

Chemical and physical characterization of bacterial crystalline precipitates

FTIR. The crystals from 8d cultures were subjected to FTIR and all spectra were recorded in the range 4000-600 cm^{-1}, using a PerkinElmer Frontier NIR/IR FT-IR spectrometer with a Pike Technology Miracle ATR sampling accessory. Each spectrum was the average of 5 scans using a spectral resolution of 2 cm^{-1} .The IR spectra were recorded and stored with software PerkinElmer Spectrum (v. 10.03.09). Spectra were matched with a reference spectrum of calcite from the RRUFF database (http://rruff.info/Calcite/R050128) (Lafuente *et al.* 2015).

Raman Spectroscopy. Raman spectra were recorded using a Renishaw inVia confocal Raman Microscope with a 50x objective lens. Measurements were taken using a 785nm laser in high

confocality mode (20µm slit width) with a 1200 l/mm grating. Laser power was 100% using a 10s exposure with 100 accumulations.

SEM and EDX. Morphology and characteristics of the crystals was determined by SEM [JSM 6480LV (JEOL)]: resolution, 3 nm, magnification, 8–300,000, accelerating voltage, 0.3–30 kV. Dried precipitates from 8 d liquid cultures were uniformly spread on aluminium stubs with two-way adherent tabs, air dried then gold-coated by sputtering for 3 min at RT. For EDX specimens were uniformly spread directly on adherent tabs and dried before analysis.

Microbiologically enhanced crack remediation

Bacillus pseudofirmus DSM 8715 was grown in 500 ml of alkaline LB broth (30°C, 150 rpm) for 18-20 h, cells (OD 0.8-0.95) were collected by centrifugation (3,800 x g, 4°C) then washed thrice with 0.03 mol l^{-1} Tris HCl pH 9.5 buffer. A suspension containing $3x10^6$ cells was made up in 10 ml of a designed crack repair medium (CRM) of pH 9.0 containing l^{-1} (calcium acetate 100 g, yeast extract 4 g, glucose 2 g) and was injected once daily for 8 d into micro-cracks (≤ 0.25 mm generated by using a flexural strength machine) of pre-wetted concrete blocks (40x40x160 mm) and incubated in a humid chamber (100% R.H.) at 30°C.

Initial Surface Absorption Test (ISAT)

The ISAT described in BS 1881-208 (British Standards Institution, 1996) was used as measure of the efficiency of the repair material(s) to exclude water. Whilst the test does not measure the bulk permeability of the concrete, it provides a measure of the quality of near surface properties by the rate at which water absorbs into the surface of concrete. This zone is where cracks will manifest and heal, thus most often determines durability of concrete and protection of the steel reinforcement therein. Mortar prisms (40 x 40 x 160 mm) were

prepared in accordance with BS EN 196-1. The cement was a Portland fly ash, CEM II/B-V 32.5 R conforming to BS EN 197-1, and the fine aggregate was standard CEN sand conforming to BS EN 196-1. The water:cement ratio was 1:2 and the aggregate:cement ratio 3:1. In order to avoid cracks penetrating throughout, mortars were reinforced with a fibre polymer mesh placed 15 mm from the base. All mortars were cast in stainless steel moulds, then cured at 95% RH and 20°C for 24 h, followed by immersion in water at 20°C. At 28 d, cracks 0.12 to 0.14 mm maximum width were generated in the centre of the mortar prisms under three-point bending. Controls comprised non-cracked mortars.

Following cracking, three mortars were injected with: (a) water only, (b) crack repair medium only (c) *B. pseudofirmus* in crack repair medium with three control prisms per treatment..

A scaled-down and slightly modified version of the method described in BS 1881-5 was used. The specimens were dried for 72 h at 50°C before testing. A cap of 1600 mm^2 was sealed to the surface and filled with water. The rate at which the water was absorbed into the concrete under a pressure head of 200 mm was measured by movement along a capillary tube attached to the cap. The rate of surface absorption was measured at intervals of 10 min, 30 min, 1 h and 2 h from the start of the test.

Results

Sporulation and sporulation kinetics

Application of endospores would be required in order to survive the hostile conditions of a fresh concrete mix of pH ca. 13. Therefore it was important to study timing, speed and the degree of sporulation in the selected *Bacillus* isolates. Eventually this process could be

required on a commercial scale for use in a self-healing agent. Initially three alkalinophilic *Bacillus* species were chosen and surveyed for spore production *in vitro*.

Sporulation in *B. pseudofirmus* (following growth overnight then inoculation into a sporulation medium) was detectable at 20h and apparently 100% of cells viewed by phase contrast microscopy contained endospores by 26 h, at which stage divisional growth had ceased (Fig. 1 a, b). *Bacillus cohnii* spores were produced rapidly, beginning by 3 h and 98% sporulation was achieved after 6 h (data not shown). *B. halodurans* formed long chains that intertwined causing practical difficulty in microscopic quantification and hence percentage sporulation data could not be recorded. Later this isolate was removed from the survey.

Germination inducers and germination kinetics

For application in self-healing concrete, spores need to germinate rapidly to generate cells that precipitate calcite. Germination inducers have been widely studied in *Bacillus* spp. For example *B. cereus* T most effectively germinates with L-alanine and purine ribosides (Shibata *et al.* 1976). Kudo and Horikoshi (1983) reported germination of alkaliphilic *B. pseudofirmus* 2b-2 required the presence of Na^+ ions along with alanine and inosine.

After 4h at 30°C in the presence of both germinants, 10 µM L-alanine and inosine, ca. 99% (98±0.6) germination occurred in 0.1 mol l^{-1} NaCl, but was lower (82.0±6.2)) in 0.05 mol l^{-1} NaCl and none occurred without NaCl. With NaCl and single inducers, germination was also reduced (inosine only, 55%; alanine only, 36%).

Germination of *B. pseudofirmus* spores increased with combined inducer concentrations ranging from 52% at 2 µM to >99% at 10 µM (Table 1). No germination occurred with NaCl alone in absence of alanine and inosine.

Kinetics of germination was studied using the optimal conditions above (0.1 mol l^{-1} NaCl, 10µM L-alanine and inosine), when germination of *B. pseudofirmus* spores exceeded 99% by

120 min with a corresponding OD reduction of 43.8 % (Fig 2). Evaluation of germination by *B. cohnii* was very difficult because of its small spore size. In any event, germination under these conditions was low and the isolate was not used further.

Survival of spores in cement stone

It was essential to test the survivability of endospores in cement paste during mixing and subsequent hardening, where they would initially experience extreme pH, then compression. The outcome would assist in designing an appropriate way of delivery and predicted longevity in concrete. Estimation of viable spores (CFU g^{-1}) in the aging cement paste specimens was conducted by serial dilution of finely crushed specimens. The non-inoculated cement controls samples did not show any viable growth. In specimens containing *B. pseudofirmus* spores, after 1 and 3 d of curing, ca. 4.4 and 1.4 %, (i.e. 5.3 and 1.7 $x10^4$ spores g^{-1} of the originally incorporated $1.2x10^6$ spores g^{-1}) were recovered as viable colonies (Fig 3). This number declined to 0.58 then 1% between 7 and 28 d when spore survival resulted in 7.1 then $1.4x10^3$ CFU g^{-1}. Beyond 42 d recovery was relatively consistent at ca. 1-4% recovery.

In specimens containing *B. cohnii* spores, after 1 and 3 d of curing, ca. 2.1 and 0.25% of the originally incorporated $1.4x10^6$ spores g^{-1} were recovered as viable colonies; this number declined to 0.15–0.09% between 7 and 28d. Beyond 42 d and up to 93 d recovery again was relatively consistent at ca. 0.52–2.4% (data not shown).

Bacillus halodurans, after 1 and 3 d of curing was recovered as ca. 0.24% viable colonies of the originally incorporated $4.4x10^6$ spores g^{-1}; this number declined below 0.1% from 7 d onwards (data not shown). Based on the earlier difficulty of assessing spore production microscopically and limited survival in concrete, this isolate was not used further

Optimization of medium for calcite precipitation for calcite quantification

Bacterial isolates for potential use in self-healing concrete must precipitate calcite rapidly and in sufficient quantity in response to crack formation. In order to quantify calcite formation *in vitro* it was essential to design a suitable medium. B4 medium has been used for screening bacteria for calcium carbonate precipitation (Shirakawa *et al*. 2011; Vahabi *et al*. 2015). Although B4 medium supported the greatest growth (Fig 4a), precipitation in controls precluded its use, questioning its value in previous studies.. However, $1/10^{th}$ LBA was selected as it supported good bacterial growth with no precipitation in un-inoculated controls.

In vitro precipitation of calcite in trace amounts was detectable within 48 h of inoculation. The levels of $CaCO_3$ precipitated (Fig 4b) increased up to 6-8 d when yield was mean 2145 to 2330 mg l^{-1}. Although every effort was made to remove bacteria from crystals, inevitably some remained. 165, 171 and 181 cells were detected by CFU in crystals (weights as above) from 6, 7 and 8d respectively. However no elemental Ca^{2+} was detected from equivalent numbers of bacteria, grown in the same medium.

Characterization of precipitate as $CaCO_3$ by FTIR, Raman Spectroscopy, SEM and EDX

FTIR spectra have been extensively used to distinguish crystalline calcium carbonate polymorphs (calcite, aragonite and vaterite) (Ghosh 2001). FTIR spectra (Fig 5a) of crystals from *B. pseudofirmus, B. cohnii and B. halodurans* when analysed in the range of 400–4000 cm^{-1}, showed three major absorption peaks at 1530, 1426, 875, and 712 cm^{-1} peculiar to calcite. In the Raman spectra of Fig. 5b, the most intense Raman bands are observed at low frequencies (50–400 cm^{-1}), which corresponds to the lattice mode vibrations. Calcite has

minor bands at ~282 and 713 cm^{-1}; aragonite has minor bands at ~207 and 704 cm^{-1} (Bischoff *et al.* 1985). In the inset figure of Fig. 5b, a peak at 281 cm^{-1} is observed at frequencies (50–400 cm^{-1}) and peaks at 702 cm^{-1} and 1085 cm^{-1} occur. The FTIR and Raman spectrum reveals the precipitated calcium carbonate as a mixture of calcite and aragonite. The spectra of crystals precipitated by *B. cohnii and B. halodurans* showed similar peaks (data not shown).

SEM analysis (Fig 5c) revealed the crystals of *B. pseudofirmus* from 1/10th LBA medium were uniformly sized, and appeared as multifaceted structures. Energy-dispersive X-ray spectroscopy (Fig.5d) showed that different regions in contained Ca, C, O, and P. (Fig. 5d).

Bacterially induced micro-crack sealing

For proof of concept, a medium (CRM), which provided calcium carbonate precipitation *in situ* (unpublished data), was used. The *in vitro* yields of calcium carbonate by *B. pseudofirmus* cells in this medium were 1403, 3283 and 3870 mg l^{-1} after 3, 6 and 8 d respectively. Levels increased slowly to 4023 mg l^{-1} by 15 d.

After about 8 d, a white precipitate resulted in occlusion of micro-cracks. The precipitated bio-mineral was hard and adhered strongly to the concrete (resisted physical extraction using a scalpel blade). Precipitates were most prominent at the crack surface. SEM images (Fig 6a-c) clearly shows the healing of cracks by *B. pseudofirmus*. The EDX spectrum (Fig 6d) of the precipitated material reveals Ca, C and O. Portland cement contains traces of Si, Al, and S hence these respective peaks are present.

Initial surface absorption test

As is typical of mortars the rate of surface absorption reduced with time as the moisture content of the mortar progressively increases during the test (Fig 7a). The mean initial surface absorption of the non-cracked mortar at 10 min was 0.10 $ml/m^2/s$ and at 2 h was 0.02 $ml/m^2/s$. Typically mortars are considered to have excellent resistance to ingress of water if they have values $\leq$ 0.25 and 0.07 $ml/m^2/s$ at 10 min and 2 h (Concrete Society 1985). These properties were evident in the non-cracked mortars.

The two cracked, control mortars had very high initial water surface absorption values. The water treated control, had initial surface absorptions of 4.1 and 2.4 $ml/m^2/s$ at 10 min and 2 h; whilst the medium control gave even higher values of 8.2 and 6.3 $ml/m^2/s$, respectively. These values reflect minimal resistance to water ingress. Absorption values $\geq$ 0.5 and 0.15 $ml/m^2/s$ are normally regarded as high. In both cases it is likely that water was flowing through the crack without obstruction, rather than ingressing *via* absorption.

Cracks injected with *B. pseudofirmus*, gave in contrast initial surface absorption values of a similar magnitude to that of the non-cracked samples: 0.10 $ml/m^2/s$ at 10 min and 0.03 $ml/m^2/s$ at 2 h. These results clearly show that bacterial activity within the created cracks has resulted in a compound(s) that prevents flow of water through the specimen (Fig 7b). The conferred resistance to water absorption is similar to that of the surrounding intact mortar.

Discussion

It was crucial to determine the timing and quantity of sporulation for experimental purposes and eventually for efficient commercial production. Sporulation in various *Bacillus* species is normally induced by reduced levels of nutrients (Errington 2003), but additionally elemental

Mn^{2+} is required during the active growth phase. Apparently 100% sporulation occurred in *B. pseudofirmus* and *B. cohnii* within 24 h under optimum sporulation conditions. Therefore commercial production of spores could be rapid, reducing production times and cost.

Various amino acids, ribosides, sugars and related compounds can act as germinants, with generally at least one inducer or a combination of inducers necessary for germination. *Bacillus pseudofirmus* germinated rapidly in presence of L-alanine and inosine with 0.1 mol l^{-1} NaCl but there was no germination in absence of NaCl. Germination of *B. cereus* was reported at 10mM inosine and 10 or 100 mM alanine (Shibata *et al.* 1976; Gounina-Allouane *et al.* 2008), whereas optimal concentration of inducers L-alanine and inosine were 0.4 mM for *B. pseudofirmus* No. 2b-2 (Kudo and Horikoshi, 1983). However, for application in self-healing concrete, cost effectiveness is crucial and hence inosine and L-alanine were tested here at lower concentrations than reported elsewhere. Germinants at 10μm, were found to successfully trigger germination.

In the current study, a correlation coefficient of 0.88 between percentage germination and reduction in optical density confirmed OD as a more rapid and facile technique than microscopy to determine germination, albeit at lower accuracy. For example, ca. 50% reduction in OD corresponded to 100% germination in *B. pseudofirmus* DSM 8715, which was similar to that found for *Bacillus firmus* No.2b-2 (Kudo and Horikoshi, 1983).

Spore survival in cement appears to be low, when compared to the theoretical potential CFU. Detection of viable *B. pseudofirmus* cells long term (42-93d) was only between ca. 1–4 %. Similarly, viability after 9d of *B. cohnii* spores was about 0.5-2.5%. The well documented resilience of *Bacillus* endospores to adverse conditions and the relatively consistent survival over a long time period (93d) in cement might suggest *recovery* of spores from cement matrix is the limiting factor, rather than viability loss due to factors such as initial alkaline pH and consequent compression on curing. In practical terms, provided that the initial inoculum is

high enough, then sufficient numbers of spores could potentially survive and take part in the healing of concrete. In contrast, Jonkers *et al*. (2010) found that viability of spores in cement specimens decreased to negligible levels within 4 months and commented on the likely role of compression when pore sizes in concrete decreased to ca. 1µm.

This is the first critical analysis of quantification and kinetics of bacterial calcite formation. Based on AAS analysis, *B. pseudofirmus* precipitated >2.3 g l^{-1} of $CaCO_3$ after 8 d incubation but production was detectable by 2 d. Amounts produced on a different medium as used in crack-healing exceeded 4 g l^{-1}. Clearly extrapolation of timing and amounts from *in vitro* to *in situ* are currently a matter for speculation. The speed of germination, extent of growth and the duration of conditions suitable for bacterial growth in micro-cracks within concrete are unknown, but will be addressed in future studies. According to weight gain, Rodriguez-Navarro et al. (2003) found most carbonate precipitation occurred in the first 5 to 10 days and Jonkers reported maximal crystal formation after ca. 7 d. Analogously, here we chose 8 d based on our observations of crack occlusion following bacterial treatments.

We obtained a correlation value of 0.99 between dry weight of precipitated, micro-filtered, extracellular material from *B. pseudofirmus* cultures and calculated values of $CaCO_3$ precipitation based on AAS, suggesting that for this isolate at least, AAS could be by-passed for quantification. However, for some species other bacterial products might contribute to dry weight analyses, with extracellular polymeric substances (EPS) an obvious candidate (Aslam *et al*. 2009). Using dry weight determination, *B. pseudofirmus* produced *in vitro* significantly more precipitated carbonate than either of the other two species (data not shown).

Combined with data for sporulation, germination extent and spore survival in concrete, calcite precipitation levels led to the selection of *B. pseudofirmus* as the isolate with greatest potential for use in self-healing concrete.

Crystals were inevitably contaminated with bacteria, so these were analysed for Ca^{2+} content. However there was no elemental Ca^{2+} detected when bacterial cells solubilized in HCl were subjected to AAS. Notably, related *B. subtilis* does not accumulate calcium (Silver and Kralovic 1969). Living cells were found within carbonate crystals by Krumbein (1974), and Morita (1980) detected large numbers of bacteria inside crystals, although viability was not established.

The FTIR spectra of calcite from *B. pseudofirmus, B. cohnii and B. halodurans* showed substantial similarity to standard calcite, with main absorption peaks at 1425 and 873 cm^{-1}, resembling the spectrum of calcite from *B. licheniformis* AK01 (Vahabi *et al*. 2015). However, Raman spectroscopy can discern between potential calcium carbonate polymorphs, calcite, aragonite and vaterite (Dandeu *et al*. 2006) and the combined data from FTIR and Raman indicated a mixture of calcite and aragonite. Calcite predominated over vaterite (a more soluble, rare, metastable polymorph) produced by *B. sphaericus* (DeMuynk et al., 2008a) and by *Myxococcus xanthus* (Rodriguez-Navarrao et al., 2003). All three polymorphs are formed in calcareous deposits of living organisms, with the organic matrix affecting the form in molluscs, but ionic composition greatly influencing the polymorph in solutions (Simkiss (1964). The SEM images of calcium carbonate crystals by *B. pseudofirmus* revealed layers of bioliths (Ferrer *et al*. 1988; Baskar *et al*. 2009); the layered SEM structure of the biomineral could result from the mixture of calcite and aragonite. Different culture media and bacterial isolates can yield variable crystal morphologies (De Muynck et al, 2008a).

Application of *B. pseudofirmus* to micro-cracks resulted in occlusion, by virtue of deposition of strong, adherent material suggested by EDX to be $CaCO_3$. Cracks were effectively sealed based on water absorption tests. The calcium carbonate precipitated remains intact, due to its low solubility at high pH, therefore should provide a permanent as well as effective seal.

Wang *et al*. (2014) found that water permeability was reduced about 10-fold by addition of encapsulated *B. sphaericus***.** A decrease of 65 to 90% in water absorption by mortar and greater resistance to freeze-thaw resulted from deposition of calcium carbonate crystals by surface applied bacteria (De Muynck *et al*. 2008b).

For initial proof of concept, we conducted experiments with unprotected, vegetative cells. Clearly, for eventual practical use, bacteria will have to be encapsulated. Also separate encapsulation of nutrients will be necessary, to include germination inducers and organic source of calcium, as detailed above. Some reports have emphasized the need for protection of bacteria before incorporation into cement mixtures (Bang *et al*. 2001; Jonkers *et al.* 2010; Wang et al. 2014). Encapsulation will not only prevent premature spore germination, but also reduce the bacterial inoculum volumes required and avoid potential damage by nutrients to the properties of concrete (unpublished data).

The germination of endospores in cement stone a realistic situation, which would be dependent on water ingress following micro-crack formation, rupture of nutrient-containing capsules and suitable growth temperatures remains to be assessed. Incorporated oxygen probes might reveal the initial occurrence and even extent of growth. Suitable imaging of crack surfaces should show resulting vegetative cells and associated calcite crystals. Release of nutrients from incorporated capsules could be monitored by basic chemical analysis of one major component (e.g. glucose) from washings made from cracked surfaces. Onset of adverse conditions, such as lowered hydration level, could then trigger a round of sporulation providing potential cells for the next event.

The size of cracks must be a significant factor in terms of the ability of bacterial precipitates to result in effective occlusion. Crystals produced *in vitro* from shaken, liquid cultures, after processing (centrifugation and filtration) were ca. 2.5µm (light microscopic

analysis), yet crack widths 100 times greater were effectively sealed by applying bacterial cells. Crystal size and morphology can be influenced by many factors including nucleation density and localised supersaturation. Presumably various factors contribute to the presentation of crystals, to include incorporation within a bacterial biofilm (Aslam *et al.* 2009). Negatively charged bacterial EPSs are effective at binding cations and act as nucleation sites for mineral deposition. Also bacterial cell wall functional groups such as hydroxyl and phosphate become increasingly negative at high pH with affinity for cations and isolated bacterial membranes will precipitate $CaCO_3$ (see Rodriguez-Navarro et al., 2003). These authors added that *in situ*, bacterial attachment to limestone then formation of carbonate around bacteria ensures strong adhesion of the newly formed cement. Nucleation points within the static environment could create larger crystal accumulations (Rivadeneyra *et al.* 1996**)**. Biomineralized calcite is likely to be more resistant to dissolution than calcite formed by inorganic precipitation as the former is less soluble (Morse, 1983). Crack healing of up to 0.46 mm wide cracks was recorded by Wiktor and Jonkers (2011), but the specimens were submerged in water for 100 days, in contrast to an 8 day period used here.

Based on our studies of sporulation, germination, *in vitro* calcium carbonate precipitation in alkaline conditions and ability of spores to survive within concrete, *B. pseudofirmus* appears to be an appropriate strain to use as a self-healing agent for bio-mineralization in cement-based materials. Ongoing work includes testing *Bacillus* spp. with a wider range of optimal growth temperatures, for applications from temperate to tropical regions. Also we are investigating spore-forming *Bacillus* spp. with neutral pH growth optima to function within aged concrete as the pH reduces towards neutrality as an outcome of carbonation (Pade and Guimaraes, 2007). In order to provide convincing evidence as to applicability and durability, future studies need to be in depth and applied to treated cement stone *in situ*.

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Conflict of Interest

No conflict of interest declared.

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Table 1. Effect of germinant concentrations on *B. pseudofirmus* % spore germination in presence of L-alanine (A) and inosine (I) in NaCl (0.1 mol l^{-1}) after 4h at 30°C. All mean values ±SD are generated from experiments done in triplicate.

Inducer concentrations (0.1mol l^{-1} NaCl)	Germination (mean %)
2µM (A+I)	52.0 ± 1.54
4µM (A+I)	86.3 ± 1.73
6µM (A+I)	89.0 ± 2.49
8µM (A+I)	94.3 ± 0.58
10 µM (A+I)	99.6 ± 1.24

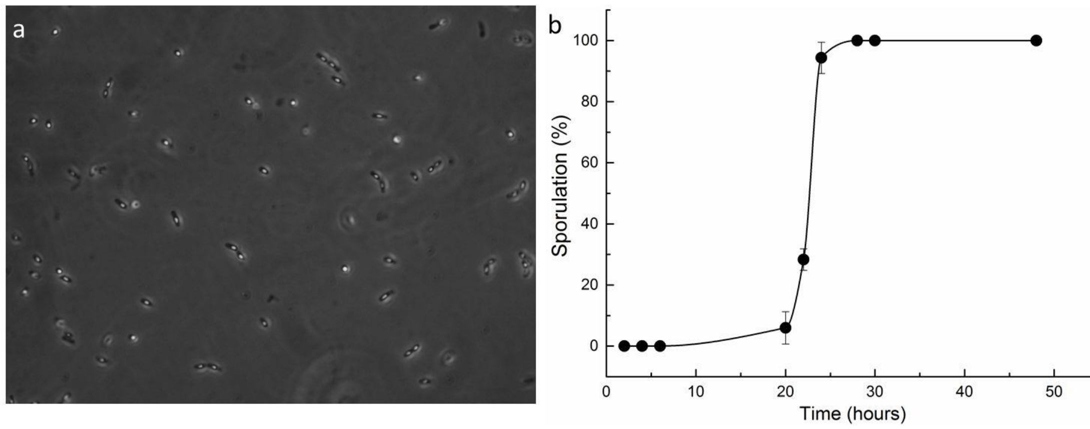

Figure 1 Spore formation and sporulation kinetics in *B. pseudofirmus.* (a) Phase contrast microscopy (b) % sporulation determined with stained spores. Error bars represent SD.

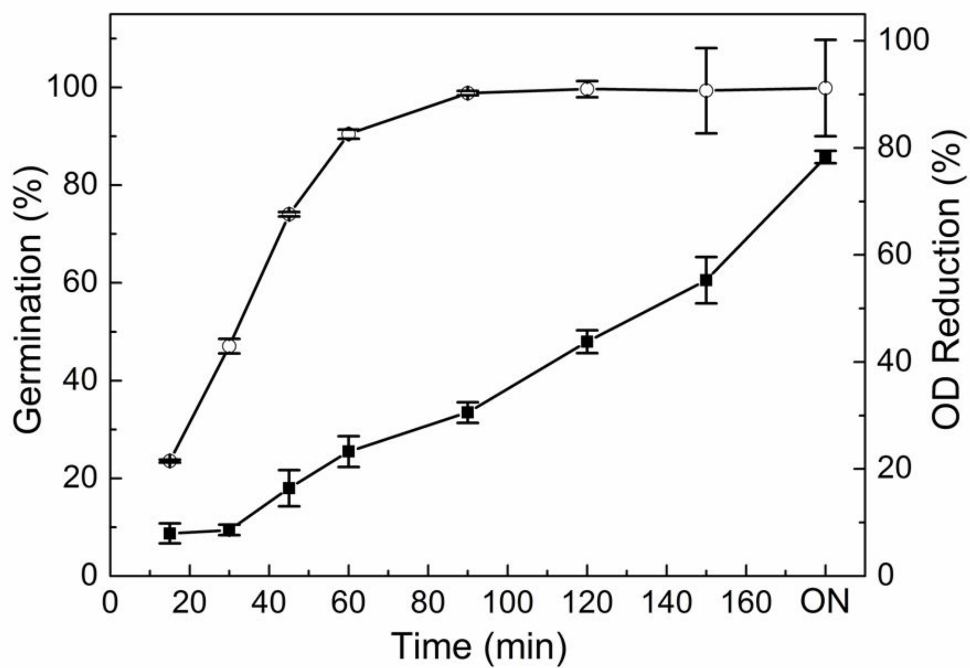

Figure 2 Kinetics of *B. pseudofirmus* spore germination as determined by phase contrast microscopy (%) (—○—) and reduction in OD (%) (—■—) in the presence of alanine (10 μM), inosine (10 μM) and NaCl (0.1 mol l^{-1}) in 30mM Tris HCl (pH 9.5). Error bars show SD.

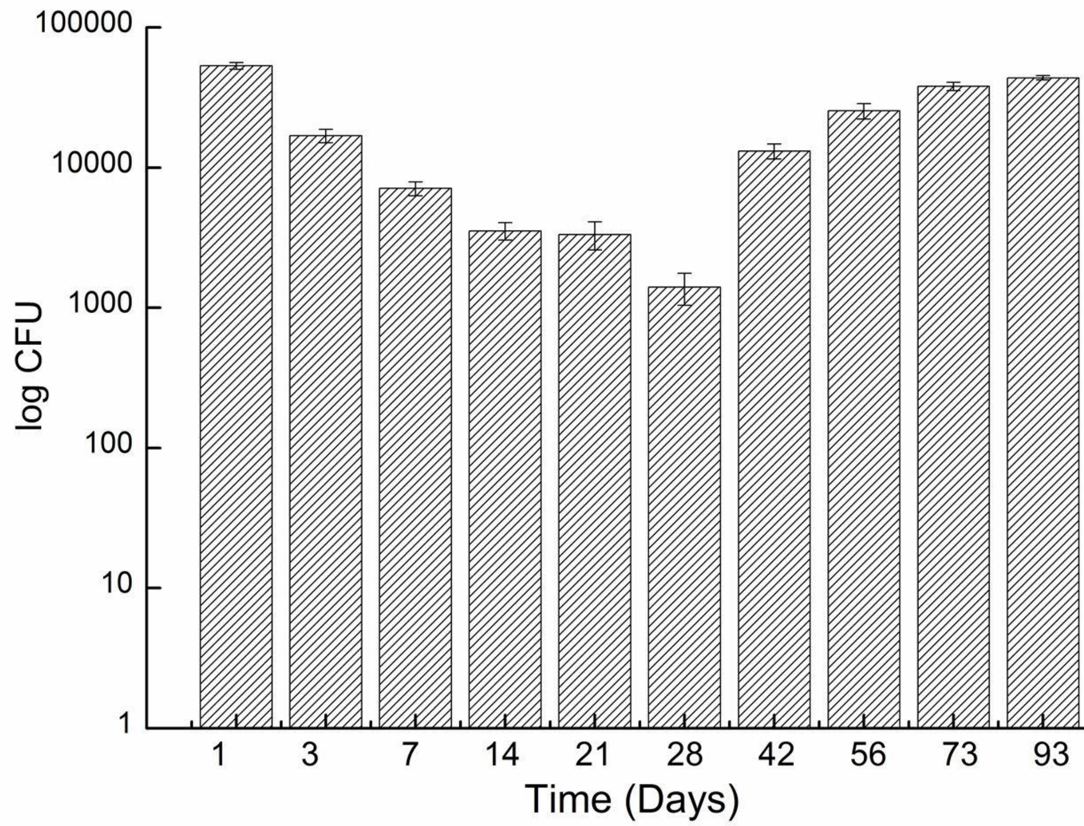

Figure 3 Survivability of *Bacillus pseudofirmus* DSM 8715 spores in cement paste up to 93 d. Error bars show SD.

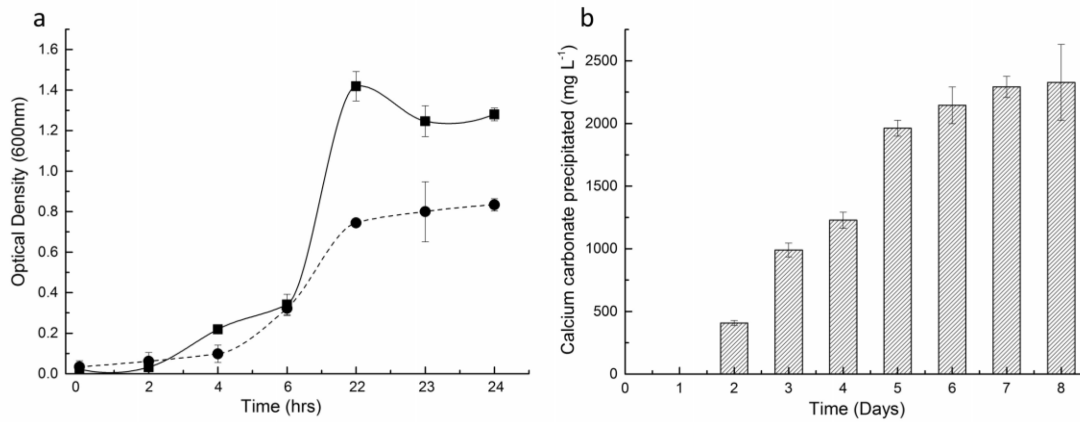

Figure 4 (a) Growth of *Bacillus pseudofirmus* DSM 8715 in two calcite inductive media: (┈┈◆┈┈) LBA (1/10) and (━■━) B4. (b) Quantification by Atomic Absorption Spectroscopy of $CaCO_3$ precipitated during growth of *B. pseudofirmus* DSM 8715. Error bars in both figures show SD.

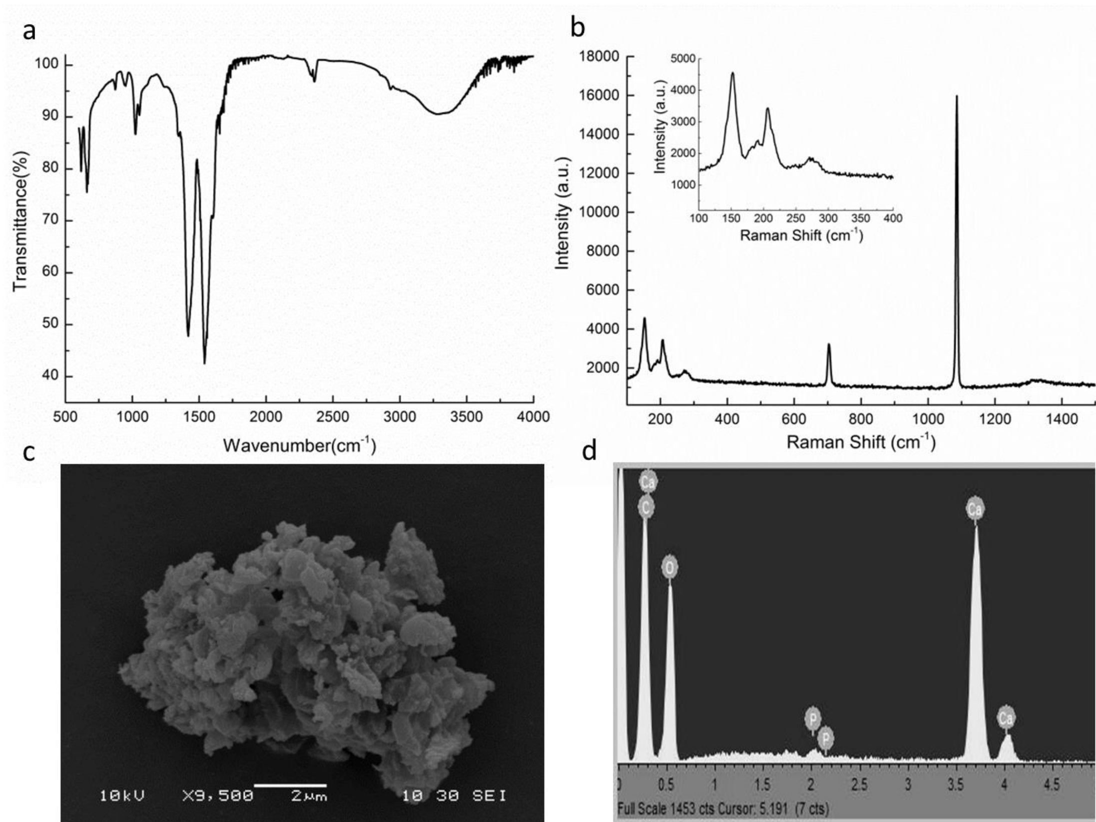

Figure 5 (a) Infrared spectroscopy of *Bacillus pseudofirmus* DSM 8715 precipitate. (b) Raman shift of calcite crystals precipitated by *B. pseudofirmus* DSM 8715. (c) SEM images of crystals precipitated by *B. pseudofirmus* DSM 8715 from liquid LBA culture medium. (d) EDX representative spectrum of calcite crystals.

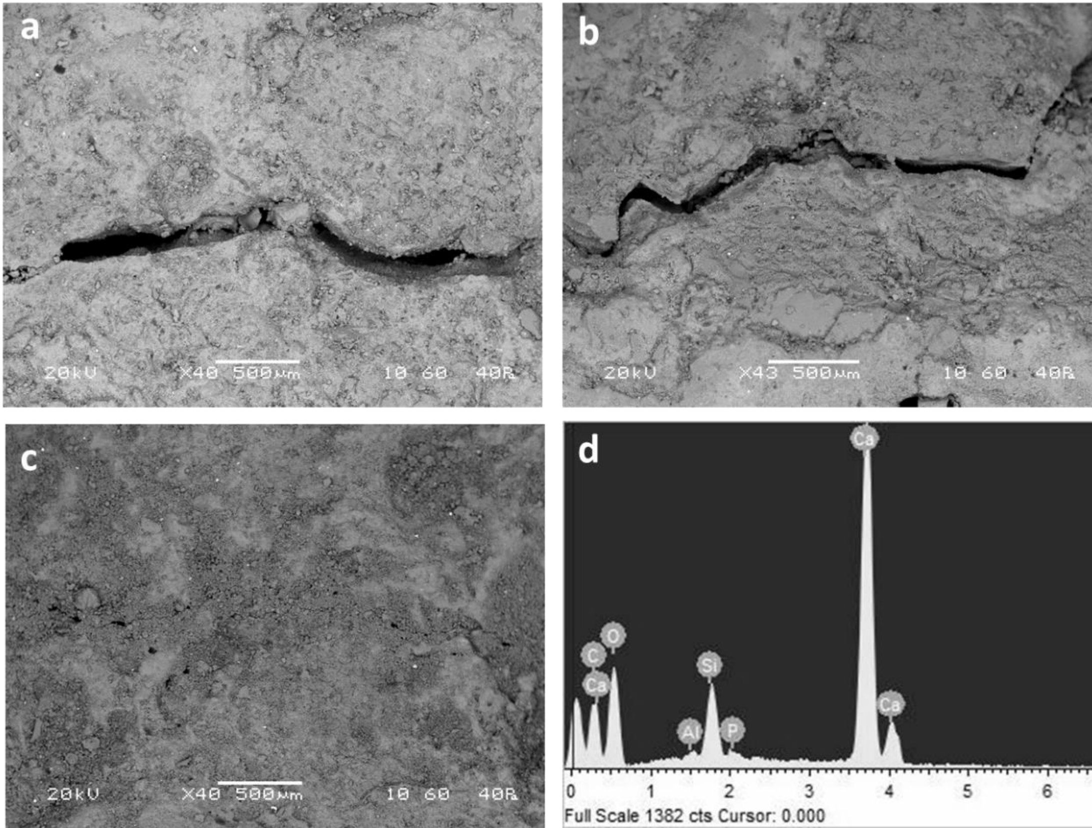

Figure 6 (a) SEM image of a control crack. (b) SEM image of medium impregnated crack. (c) SEM image of bio-deposited material within crack impregnated with bacteria and medium. (d) EDX spectrum of calcite deposited within the crack.

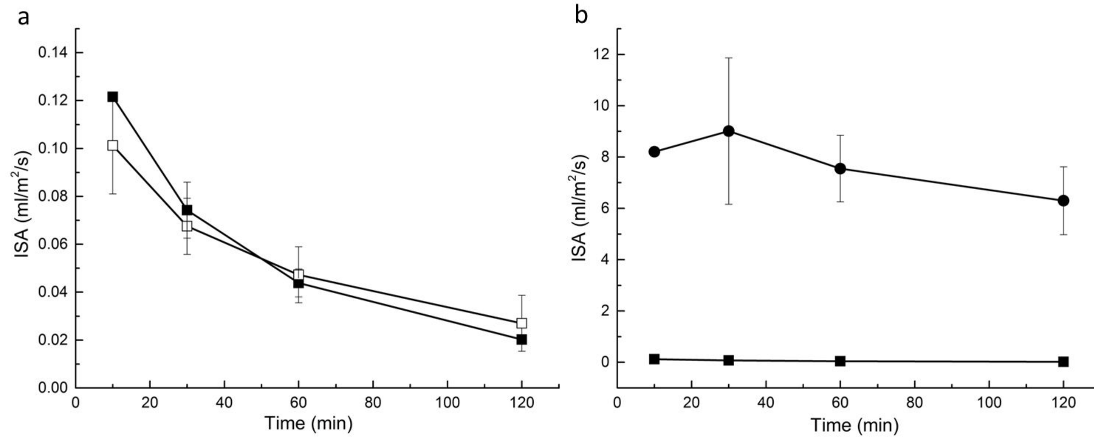

Figure 7 (a) Initial surface absorption test plot of non-cracked cement sample (—■—) and sample containing bacteria in crack repair medium (—□—). (b) ISAT plot of non-cracked cement sample (—■—), sample injected with medium (—●—) and sample injected with medium+bacteria (—□—).

* In figure 7b, the two lower data sets are overlapping and are seen as a single line.