Journal of Basic Microbiology
Environment · Health · Techniques

Research Paper

Calcium carbonate precipitation by strain *Bacillus licheniformis* AK01, newly isolated from loamy soil: a promising alternative for sealing cement-based materials

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The relevant experiments were designed to determine the ability of indigenous bacterial strains isolated from limestone caves, mineral springs, and loamy soils to induce calcium carbonate precipitation. Among all isolates examined in this study, an efficient carbonate-precipitating soil bacterium was selected from among the isolates and identified by 16S rRNA gene sequences as *Bacillus licheniformis* AK01. The ureolytic isolate was able to grow well on alkaline carbonate-precipitation medium and precipitate calcium carbonate more than 1 g L^{-1}. Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD) analyses, and scanning electron microscopy (SEM)/energy-dispersive X-ray spectroscopy (EDX) examinations were performed in order to confirm the presence of calcium carbonate in the precipitate and to determine which polymorphs were present. The selected isolate was determined to be an appropriate candidate for application in a surface treatment of cement-based material to improve the properties of the mortar. Biodeposition of a layer of calcite on the surface of cement specimens resulted in filling in pore spaces. This could be an alternative method to improve the durability of the mortar. The kind of bacterial culture and medium composition had a profound impact on the resultant $CaCO_3$ crystal morphology.

Keywords: *Bacillus licheniformis* AK01 / Calcite precipitation / Mortar / Surface treatment

Received: July 12, 2013; accepted: September 26, 2013

DOI 10.1002/jobm.201300560

Introduction

Recently, bacterially induced calcium carbonate precipitation has been proposed as an environmentally friendly method to protect decayed ornamental stone. The method relies on the bacterially induced formation of a compatible calcium carbonate precipitate on limestone. Unlike that from lime-water treatment, the carbonate cement formed under bacterial influence appears to be highly coherent [1]. In addition, this technique has been explored for the improvement of the durability of cementitious materials [2, 3]. Microbial carbonate precipitation (MCP) occurs as a byproduct of common microbial metabolic processes, such as photosynthesis [4], urea hydrolysis [5], and sulfate reduction [6]. Microorganisms possess net negative cell surface charge, which acts as a scavenger of divalent cations, including Ca^{2+} and Mg^{2+}, by binding them onto their cell surfaces, thereby making microorganisms ideal crystal nucleation sites an example of MCP [5, 7]. Another benefit of MCP is its ability to sequestrate atmospheric CO_2 through calcium carbonate formation [8]. Calcium carbonate-biodeposition technologies have already been used for consolidation of sand columns [9], for repair of limestone monuments [10] and to a smaller extent for remediation of cracks in concrete [2].

Surface treatment is playing an increasingly important role for the protection of construction materials from

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harmful effects resulting from access of water and other harmful substances. In this paper, we report for the first time, the isolation and identification of an indigenous strain of *Bacillus licheniformis* with significant capability of $CaCO_3$ production that may be appropriate for application to concrete as a healing agent. This was accomplished by (1) testing bacterial isolates for their ability to precipitate $CaCO_3$, (2) measuring urease activity of our $CaCO_3$-precipitating isolates, (3) identifying the $CaCO_3$-polymorphs precipitated by our bacterial isolates, and (4) evaluating surface treating of mortar specimens with our isolates for calcite precipitation in existing pores (mortar healing process).

Materials and methods

Sample collection and bacteria isolation

A variety of viscous samples from surface scrapings and sediment was used as the initial inoculums for enrichment. Twenty samples were collected from different locations including solutional cave (Roodafshan limestone cave, Damavand region, Iran), mineral springs (Damavand region, Iran), and loamy soils (Loot desert, Shahdad region, Iran). One gram of each sample was introduced separately into 9 ml of sterile 0.9% saline solution and inoculated in B4 medium at 30 °C for enrichment and mineral precipitation. The samples from the enrichments were spread on B4 agar with sample dilutions ranging from 10^{-1} to 10^{-6} and incubated at 32 °C for 1 week. Several individual colonies were selected and purified by re-streaking on B4 agar. Of these purified isolates, 11 were chosen for further studies based on their ability to produce mineral precipitates in B4 broth medium. *Sporosarcina pasteurii* DSM-33 (previously known as *Bacillus pasteurii*) was used as control bacterium. *Selection of a bacterial strain, exhibiting optimal precipitate accumulation, urease activity, sporulation, and resistance to alkaline pH*. Precipitate production by each of the bacterial isolates was determined in weight per liter after 7 days in B4 broth after 7 days and expressed as precipitation weight per liter. For the determinations, the calcium carbonate precipitate was separated from the medium by filtration through Whatman No. 1 filter paper. The filter paper with the deposited calcium carbonate was dried for 2 days at 37 °C and weighed. The urease activities of the isolates were qualitatively tested by streaking the purified bacterial cultures on urease test agar (BBL, Becton Dickinson and Company, Sparks, MD). A change in color following incubation for 5 days at 30 °C was recorded as a urease-positive reaction. In continuation of studies on the calcite-precipitating bacteria and their properties, spore formation by isolates was investigated in B4 glucose-free medium in addition to DSM medium. Bacterial isolates were cultured in alkaline medium and their native ability to grow over a wide pH range (pH 7–11) and examined further as a necessary parameter for selection examined. The pH of the media was adjusted with 1 M sodium hydroxide (NaOH).

Biochemical and molecular identification of the selected isolate

Biochemical identification of the most potent calcium carbonate-producing isolate was performed using standard biochemical tests [11]. Further identification of the isolate was carried out using 16S rRNA gene sequencing. Extraction of bacterial genomic DNA was carried out using the Kit (Roche Applied Science, Germany), according to the manufacturer's instructions. The extracted DNA samples were used as template in the polymerase chain reaction (PCR), using the 27F 5-AGAGTTTGATCCTGGCTCAG-3, 1492R 5-GGTTACCTTGTTACGACTT-3, 785F 5-GGATTAGATACCCTGGTA-3, and 805R 5-GACTACCAGGGTATCTAATC-3. The reaction was carried out in a 25 μl volume containing 1× PCR buffer, 1.5 mM $MgSO_4$, 200 μM each dNTP, 1 μM of each primer, 1 U of pfu (Fermentas) DNA polymerase and 1 ng of template DNA. PCR amplification was performed as follows: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The amplified product was sequenced using an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA). The resulting sequence was aligned with the available almost complete sequence of typed strains of genus *Bacillus* and then with corresponding sequences of representative.

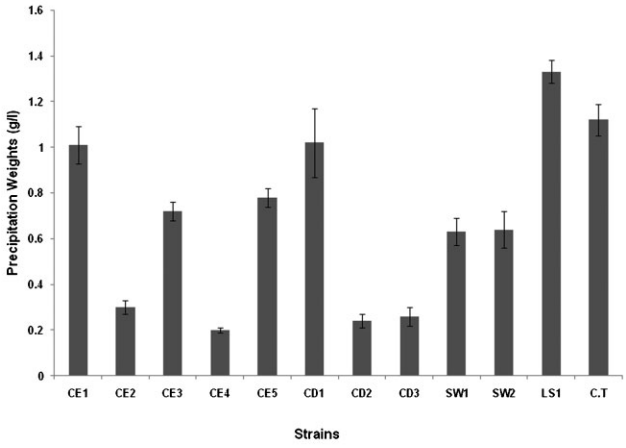

Figure 1. Comparison weight of calcium carbonate precipitation by different strains after 7 days.

Characterization of precipitate by Fourier-transform-infrared (FTIR), X-ray diffraction (XRD), and scanning electron microscopy (SEM)

Calcium carbonate precipitate by different bacterial isolates was analyzed using FTIR) spectroscopy (Perkin-Elmer Spectrometer, FTIR GX 2000). The precipitates were prepared as described above and thoroughly washed in distilled water three times by repeated centrifugation and stored in a vacuum oven for drying. The dried precipitate samples were ground up and powdered with anhydrous potassium bromide (KBr) to give a fine mixture. The spectra were in the range of 400–4000 cm^{-1} with 8 cm^{-1} resolution. The resulting spectra were the average of 16 scans. In order to confirm the presence of calcium carbonate in the precipitate and to identify its polymorphs, XRD analysis was carried out. XRD-spectra were obtained using Equinox 3000 with a Cu anode (40 kV and 30 mA) scanning from 3 to 60° 2θ. The calcite samples were identified by comparing the X-ray profile of the samples with standards established by the International Center for Diffraction data. Morphological analysis of deposited $CaCO_3$ crystals formed in cultures of the bacterial isolate was performed by SEM. To generate the $CaCO_3$ crystals, the bacterial isolate was grown in B4 broth medium for 3 days at 30 °C. After fixation and ethanol dehydration, samples were dried by the critical-point method. The samples were then coated with gold and visualized using a LEO 1455 SEM instrument. Further evidence of the carbonate deposits as calcite crystals was provided with energy-dispersive X-ray spectroscopy (EDX) analysis.

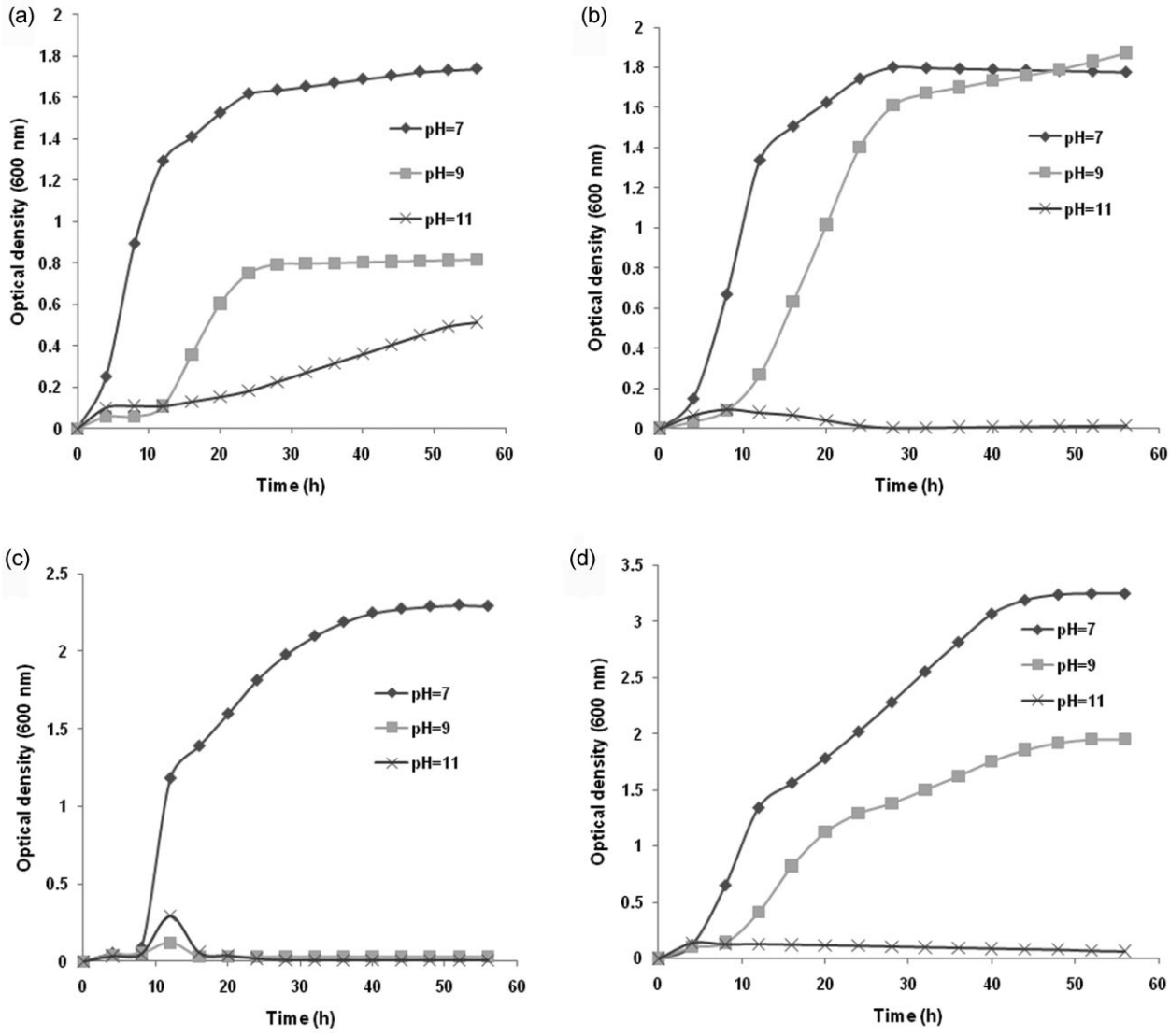

Figure 2. (a) Bacterial growth of C.T strain, (b) LS1 strain, (c) CE1 strain, (d) CD1 strain in alkaline pH.

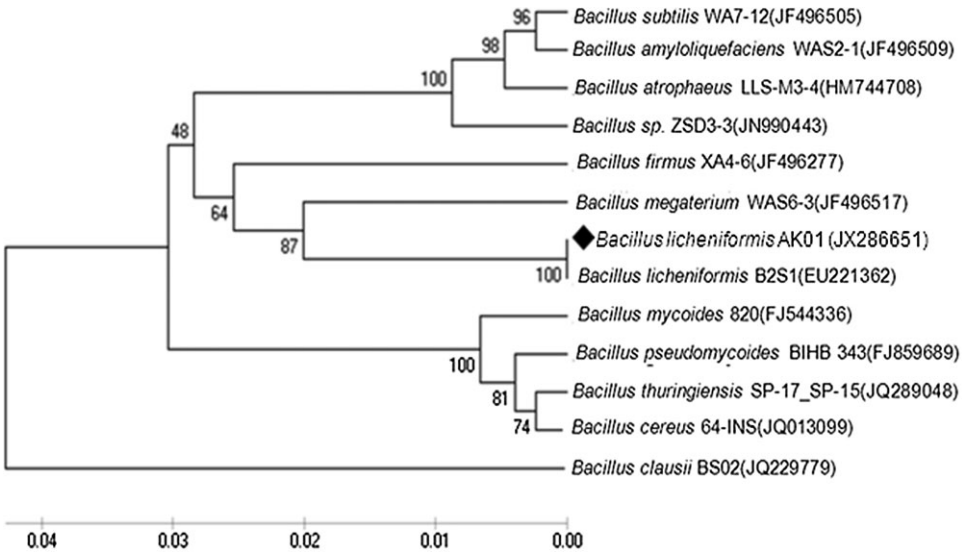

Figure 3. Phylogenetic tree. Numbers at the nodes indicates the bootstrap values on neighbor joining analysis. Bar 0.01 represent sequence divergence.

Surface treatment of cement-based material specimens

To evaluate the calcium carbonate formation by the selected bacterial isolate and the ability of the calcium carbonate to fill pores and microcracks in mortar specimens, mortar cubes aged for 28 days were prepared and cured according to ASTM C109. For this purpose, the selected strain was grown overnight in urea-based nutrient broth, and then mortar specimens were immersed in the culture for 24 h. The mortar samples were then removed from the medium and blotted with a paper towel to remove the excess medium from the surface of the mortar samples. Lastly, samples were submerged in urea-based nutrient broth that also contained calcium chloride for 7 days to let bacteria to produce calcium carbonate precipitates both into the wall pores and cracks on the surfaces. Control specimens were also prepared in a similar way without the bacterial isolate in the urea-based nutrient broth culture medium.

Results

Selection of the best strain on the basis of precipitation weight, urease activity, sporulation, and alkaline resistance

Different bacteria appear to be able to cause the formation of calcium carbonate crystals, whose morphology varied depending on the bacterial strain, substrate, and some other environmental parameters. Bacterially formed calcium carbonate-crystals examined by light microscopy are predominantly single or multiple-needle-shaped. The optimum precipitation rate of the bacterial isolates was determined after 7 days. As shown in Fig. 1, the isolates tentatively designated as CD1 (isolated from cave deposition), CE1, CE3, CE5 (cave stalactites), SW1, SW2 (spring water), LS1 (loamy soil) and C.T (control-*S. pasteurii* DSM-33) formed the most precipitate. Among them, strains LS1, C.T, CE1, and CD1 formed more than 1 g of precipitate per liter. Incorporation of alkalophilic and dormant but viable bacteria in the concrete matrix may contribute to the self-healing of concrete. Screening of sporulating bacterial strains from the collected samples was performed to determine the spore yield of the isolates. Out of all the

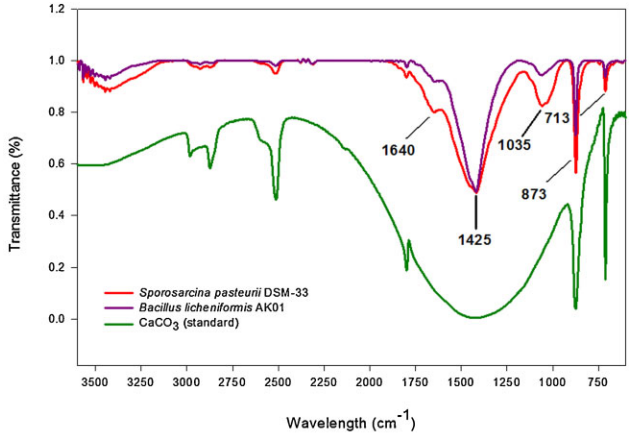

Figure 4. Infrared spectroscopy of *S. pasteurii* DSM-33, *B. licheniformis* AK01 sediment and standard calcium carbonate.

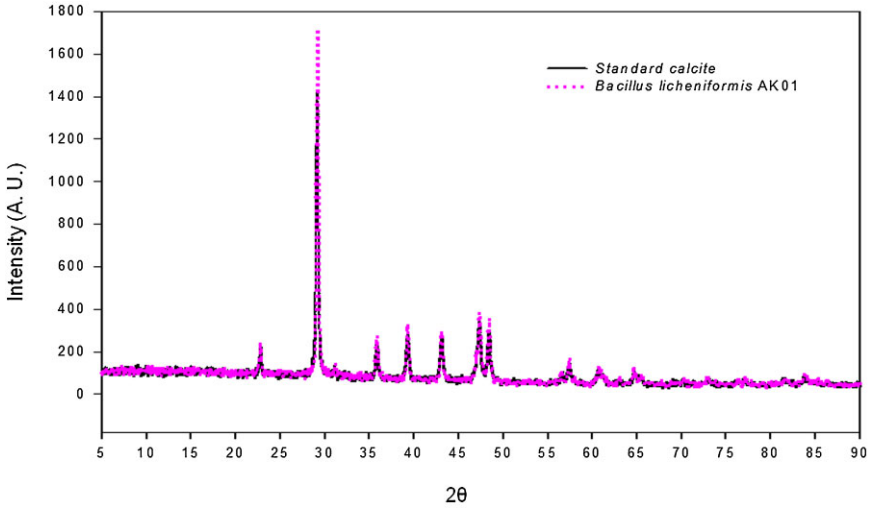

Figure 5. XRD curves of the sediments from strain *B. licheniformis* AK01 and standard calcite.

bacterial isolates, strains LS1 and C.T formed spores after a short incubation in all culture media tested. For growth curve determinations, we cultivated our bacterial strains in B4 medium at pH 7, 9, and 10. As shown in Fig. 2, strain LS1 grew more slowly than the other strains but grew better at alkaline pH. According to Fig. 2B, the optimum pH for growth of strain LS1 was 7. According to Fig. 2a–d, all strains grew best at pH 7. At pH 9, strain LS1 grew best. Carbonate precipitation through alkalinizing metabolisms may play an active role in mineralization. Because urease activity plays a vital role in carbonate calcium precipitation, the isolates were screened by qualitative tube assay. The results showed that strain LS1 was more active than strain C.T. Strains CD1 and CE1 were the least active of the four strains, all of which produced significant amounts of precipitate. Comparing the findings from all four strains, strain LS1 met the requirements of the afore-mentioned factors best.

Identification of selective bacterial strain

Morphological and biochemical characteristics of the selected isolate showed that the selected isolate AK01 was a Gram-positive, long, catalase-negative, spore-forming

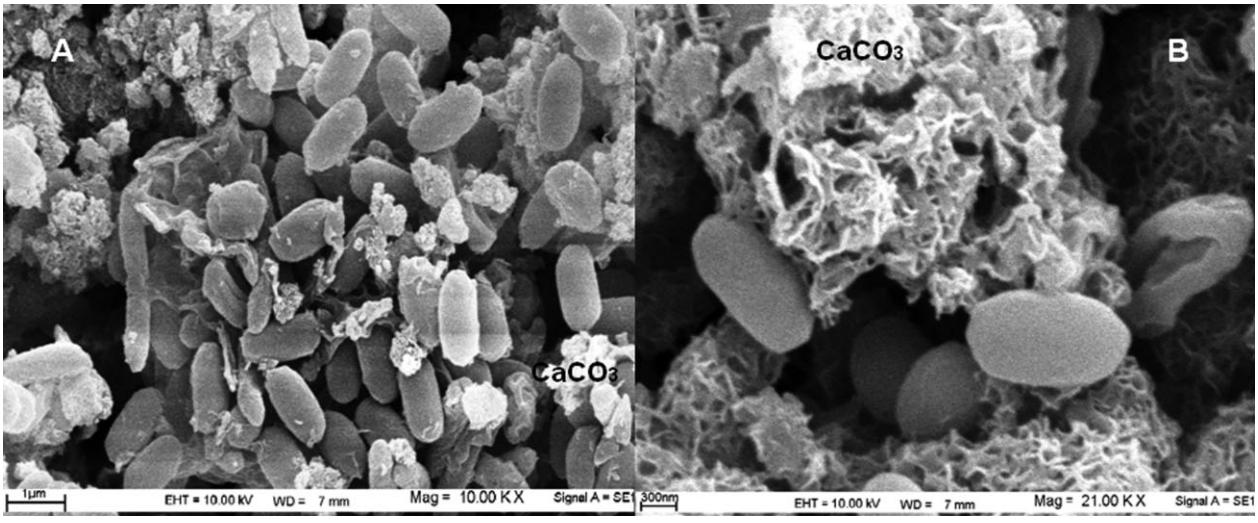

Figure 6. SEM micrograph of microbiologically induced calcite precipitation. As can be seen in (a) and (b), the distinct calcite crystals surrounded by *B. licheniformis* AK01 strain.

rod. Results from 16S rRNA gene sequencing identified our strain as *Bacillus licheniformis* with 100% similarity to *Bacillus licheniformis* strain B2S1 (Fig. 3). We tentatively labeled our strain *Bacillus licheniformis* AK01 and deposited it in GenBank with the accession number JX286651.

Characterization of precipitate as $CaCO_3$ by FTIR and XRD and SEM

We prepared FTIR spectra in the range of 400–4000 cm^{-1} with the dried sediments from liquid cultures of our isolates to determine the nature of the bacterial products.

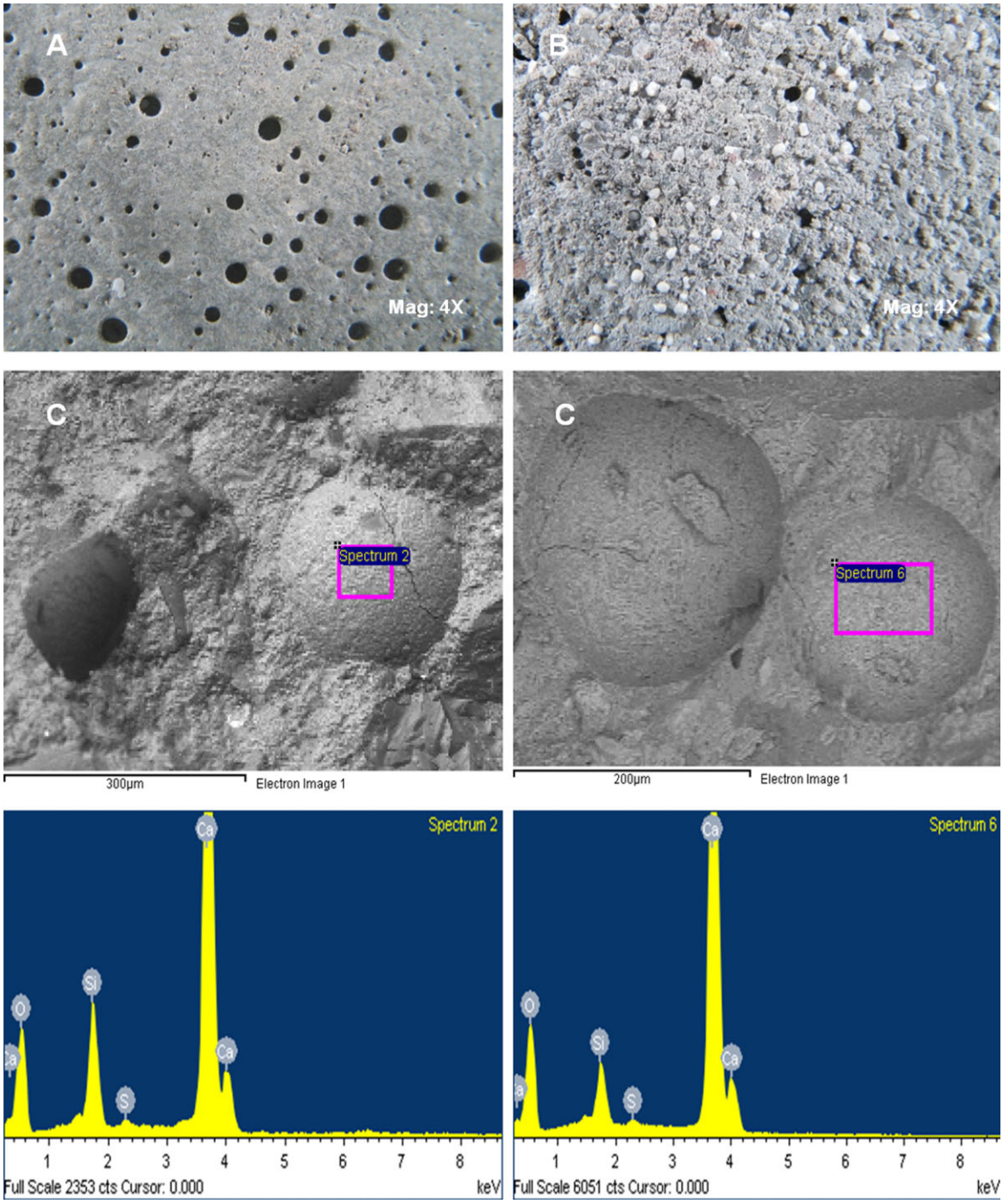

Figure 7. Surface treatment of mortar specimen, (a) control mortar specimen without any formation of biodeposition on its surface, (b) formation of white crystals on the surface of mortar specimen that plug tiny pores using *B. licheniformis* AK01, and (c) EDX spectra of calcite crystals formed into the wall pores and cracks on the mortar surfaces.

The spectra are shown in Fig. 4. The spectrum from the AK01 sediment showed substantial similarity to that of standard calcium carbonate with main absorption peaks at 1425, 873, and 713 cm^{-1}. The absorption at 1640 cm^{-1} in the $CaCO_3$ spectrum is attributable to water molecules because $CaCO_3$ is very hygroscopic. Three major absorption peaks were around 1430, 875, and 712 cm^{-1} and also the largest one had been absorbed in the range of 1430 cm^{-1} and two other peaks were sharp that were attributable to the vibration of carbon-oxygen double bond (CO) in the carbonate ion (Fig. 4). XRD spectra of the precipitates formed by the bacteria are shown in Fig. 5. In the diffraction profile an intense peak at 2θ angle with a value of around 30° shows the $CaCO_3$ calcite structure. The presence of calcite crystals in the AK01 precipitate was shown by the significant similarity of their spectrum to that of the calcite standard (Fig. 5). The SEM micrographs of microbiologically induced calcite precipitate are shown in Fig. 6a and b. Deposition of calcium carbonate appeared at the cell surface of *B. licheniformis* AK01 (Fig. 6b). The deposition seemed to be uniform over the entire surface. In addition, small particles of calcite surrounding the AK01 cells are seen (Fig. 6b).

Surface treatment of specimens

Figure 7b clearly shows that *B. licheniformis* AK01 deposited white calcite crystals on the mortar specimen, which filled the pores and cracks. No such crystals appeared on a mortar specimen that was incubated in the absence of the bacterial strain. We confirmed in a further study that B. licheniformis AK01 has the ability to improve the surface of cement-based materials and may thereby decrease the surface porosity to make it resistant to aggressive water. Thus, this strain is an appropriate candidate for biomineralization in cement-based material as a healing agent. The results obtained from EDX analysis demonstrated that crystal spheres formed into the wall pores and cracks on the mortar surfaces were predominantly composed of calcium (48.13%; Fig. 7c).

Discussion

In this research, different indigenous bacterial strains were compared as to their ability to precipitate calcium carbonate. Our results indicate that *Bacillus licheniformis* AK01 is the most effective candidate for calcium carbonate precipitation. It precipitated 1.33 g of calcium carbonate per liter in 7 days, which was the largest amount precipitated by any of the candidates tested. This was 18% more calcium carbonate than *S. pasteurii* DSM-33 precipitated. The characterization of AK01 precipitate indicated that it was calcite. The AK01 strain had the highest level of urease activity and as an alkalophilic strain could grow at pH $\geq$ 9. It was able to tolerate the harsh fresh mortar environment as well. Based on our results, we may safely conclude that AK01 may be an appropriate strain for biomineralization in cement-based materials. However, this research does not define alternative eco-friendly application of isolated strain as a self-healing agent for improving concrete properties. Further studies are underway to be able to answer to these urgent questions in details.

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