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Calcium carbonate precipitation induced by ureolytic bacteria *Bacillus licheniformis*

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ABSTRACT

In this study, we report a novel approach for preparing precipitated calcium carbonate for the first time by *Bacillus licheniformis* using different types of media containing urea as a source of urease enzyme and for the first time using particles of pure calcium. The bacteria used in this study produced urease which catalyzes the hydrolysis of urea ($CO(NH_2)_2$) into ammonium (NH_4) and carbonate (CO_3^{2-}) leading to the precipitation of calcium carbonate. Calcium carbonate precipitation was experimentally studied by spontaneous precipitation at various pH (8–12), temperatures (30, 35, 40, 45, 50 °C), and at different concentrations of urease enzyme (1, 5, 10 mg mL^{-1}). The XRD results showed a mixture of calcite and vaterite. The morphology of calcium carbonate particles prepared was studied under SEM after using it as a bioconsolidation of degraded fresco wall paintings.

1. Introduction

Microbial metabolic activities often contribute to selective cementation by producing intra- or extra-cellular relatively insoluble organic and inorganic compounds. Some bacteria produce glycocalyx on the cell wall which remains in nature after death (MacLoad et al., 1988), other one can accumulate inorganic compounds like phosphate, carbonate, and silicate (Stocks-Fischer et al., 1999).

Bacterially induced mineralization has recently emerged as a method for protection and consolidating decayed ornamental stone (Fujita et al., 2000; Hammes et al., 2003; Dick et al., 2006; Jimenez-Lopez et al., 2007; Okwadha and Li, 2010; Chahal et al., 2013), in addition to protective surface layer and waterproofing properties.

In the patented Calcite bioconcept process, a layer of selected bacteria is sprayed on the surface together with specific nutrients and calcium ions. Over the time, this layer is supplied repeatedly with the nutrient medium then the layer of calcite is precipitated (De Muynck et al., 2010).

The precipitation of calcium carbonate is mainly related to four factors: the concentration of dissolved inorganic carbon, the pH, the concentration of calcium ions, and the presence of nucleation sites. The first three factors are provided by the metabolism of the bacteria while the cell wall of the bacteria will act as a nucleation site. In addition to many several parameters like temperature, and urease enzyme may have an impact as well (Hammes and Verstrate, 2002; Rodriguez-Navarro et al., 2007).

The bacteria used in this study produce urease which catalyzes the hydrolysis of urea ($CO(NH_2)_2$) into ammonium (NH_4) and carbonate (CO_3^{2-}). First, 1 mol of urea is hydrolyzed intracellularly to 1 mol of carbomate and 1 mol of ammonia (Eq. (1)). Then the carbomate is hydrolyzed to form ammonia in addition to carbonic acid (Eq. (2)).

$$CO(NH_2)_2 + H_2O \rightarrow NH_2COOH + NH_3 \quad (1)$$

$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \quad (2)$$

These products are then transformed into bicarbonate (1 mol) and ammonium and hydroxyl ions (2 mol) (Eqs. (3) and (4)). The last two reactions increase the pH value which shifts the bicarbonate equilibrium resulting in the formation of carbonate ions (Eq. (5)) (Ganapathy, 2008; Tittelboom et al., 2010).

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+ (pka = 6.37) \quad (3)$$

$$2NH_3 + H_2O \leftrightarrow 2NH_4 + 2OH^- \quad (4)$$

$$H_2CO_3^- + H^+ + 2HCO_4^+ + 2OH^- \leftrightarrow CO_3^{2-} + 2NH_4^+ + 2H_2O \quad (5)$$

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Precipitation of calcium carbonate phase occurs when a sufficient super saturation is reached with respect to this phase (Eq. (6))

$$CO_3{}^{-2} + Ca^{+2-} \leftrightarrow CaCO_3 (K_{so} = 3.8 \times 10^{-9}) \quad (6)$$

Sometimes the cell wall of the bacterium has a negative charge, the bacterium draws cations as Ca^{2+} to deposit on its cell surface, the Ca^{2+} reacts with the $CO_3{}^{2-}$ ions and precipitate $CaCO_3$ on the cell wall surface which lead to the death of bacteria. (Eqs. (7) and (8)) (Tabler et al., 2011).

$$Ca^{2+} + Cell \rightarrow Cell\text{–}Ca^{2+} \quad (7)$$

$$Cell\text{–}Ca^{2+} + CO_3{}^{2-} \rightarrow Cell\text{–}CaCO_3 \quad (8)$$

In addition, $CaCO_3$ precipitation requires sufficient calcium and carbonate ions so that the ion activity product (IAP) exceeds the solubility constant (K_{so}) (Eqs. (9) and (10)). From the comparison of the IAP with the K_{so} the saturation state (Ω) of the system can be defined; if $\Omega > 1$ the system is oversaturated and precipitation is likely (Morse, 1983):

$$Ca^{2+} + CO_3{}^{2-} \leftrightarrow CaCO_3 \quad (9)$$

$$\Omega = a(Ca^{2+})a(CO_3{}^{2-})/K_{so} \quad \text{with} K_{so\,\text{calcite},25^\circ} = 4.8 \times 10^{-9} \quad (10)$$

The newly formed calcium carbonate creates a coherent carbonate cement of 10:50 μm that coat the treated stones. Also this cement is rooted down to a depth of 1 mm while the porosity of the stone remains unaltered (Rodriguez-Navarro et al., 2003).

In this study, we are reporting a novel approach for preparing precipitated calcium carbonate by *Bacillus licheniformis* using various types of media containing urea as a source of urease enzyme and using particles of pure calcium on Fresco mural paintings, which contain pigments, plasters, and many of mortar layers.

2. Materials and methods

2.1. Bacterial culture conditions

B. licheniformis used in this study (known to be isolated from sewage in the El dakhlah Oasis, New Valley Governorate, Egypt) was purchased from the Microbiological Resource Center (Cairo MIRCEN Center), Ain Shams University, Cairo, Egypt. This strain showed a high urease activity and a continuous formation of dense calcium carbonate crystal in liquid medium.

Five Different liquid cultural media were used as follows:

(a) YE–Ur–$CaCl_2$ consisted of 20 g L^{-1} yeast extract, 20 g L^{-1} urea and 50 g L^{-1} $CaCl_2$ (De Muynck et al., 2010).
(b) Broth media consisted of 2.1 g L^{-1} Nutrient Broth, 1.48 g L^{-1} $NaHCO_3$, 7 g L^{-1} NH_4Cl, 7 g L^{-1} Urea and 25 mM $CaCl_2$ (Ganapathy, 2008).
(c) B4 consisted of Calcium Acetate $Ca(C_2H_3O_2)_2$ 2.5 g L^{-1}, Yeast Extract 4 g L^{-1}, and Glucose $C_6H_{12}O_6$ 10 g L^{-1} (Baskar et al., 2006).
(d) YE–Ur consisted of 20 g L^{-1} Yeast and 20 g L^{-1} Urea (Whiffin, 2004).
(e) YE–Ur–Ca consisted of 20 g L^{-1} Yeast, 20 g L^{-1} Urea and 50 g L^{-1} pure calcium.

Liquid media were sterilized by autoclaving for 20 min at 120 °C, urea was added after autoclaving by means of filtration through a sterile 0.36 μm Millipore filter (Millipore USA). Culture was incubated for 24 h at 30 °C in a shaker at 150 rpm. Culturing was conducted under sterile conditions.

2.2. Crystals formation

B. licheniformis was inoculated into flasks containing 100 mL of each culture medium and incubated at 30 °C. After 72 h, crystals collected from liquid culture were transferred to distilled water and washed out of impurities. The washed crystals were air dried at 37 °C.

2.3. Mural painting samples

A set of samples with 50 × 50 × 20 mm were prepared from (Quartz, Calcite, & Gypsum) (3:1:1) (w:w:w) respectively, then were covered by lime layer (white wash) and were colored by natural pigments. The prepared samples were immersed in the media and incubated in an incubation room at 37 °C for 5 days.

2.4. Factorial experimental design

Factorial experiments were designed based on the important factors that affect bacterial precipitation such as pH, temperature and urease enzyme. For each test, 20 mL of the culture medium were inoculated by 50 μL of *B. licheniformis* suspension and then were incubated for 24 h at different pH value (7, 8, 9, 10, 11, 12) at temperature 35 °C, different temperatures (30, 35, 40, 45, 50 °C) with pH = 8, and at different concentration of urease enzyme (1, 5, 10 mg mL^{-1}) at pH = 8, T = 35 °C.

2.5. Scanning electron microscope (SEM)

The Morphology and Mineralogical composition of the deposited calcium carbonate crystals were investigated using scanning electron microscope (SEM). SEM micrographs were obtained using a Jeol JSM 5600LV Model Philips XL 30 attached with EDX Unit, with accelerating voltage 30 K.V., magnification 10× up to 400,000× and resolution for W. (3.5 nm). Samples surface were first coated with carbon then with gold.

2.6. X-ray diffraction analysis (XRD)

The mineralogical composition of the precipitation calcium carbonate was determined by XRD analysis. The purified crystals were examined by X-ray diffraction (XRD powder diagrams) with Philips PW 1140 and Rigaku-Miniflex Ca 2005 diffractometers equipped with a Ni Filter and a Cu-$K\alpha$ radiation source, and identified according to JCPDS and ASTM, 1974, 1981 criteria. The diffraction peak corresponding to planes 104 (d_0.3 nm) was used to determine approximate mg content of calcite (Goldsmith and Heard, 1961).

3. Results

3.1. Bacterial culture

The results showed that calcium carbonate could be produced by bacterial strains *B. licheniformis* in all culture media. Fig. 1 shows that a higher amount of calcium carbonate was produced and precipitated by the media contain calcium particles. On the other hand, the B4 showed the lowest value of calcium carbonate precipitation.

3.2. Factors affecting $CaCO_3$ precipitation

3.2.1. pH value

The result showed (Fig. 2) that the maximum amount of $CaCO_3$ precipitation is at pH = 8. However, further increase in the alkalinity of the media showed decrease in calcium carbonate precipitation.