Characterization of Calcium Carbonate Produced by ureolytic bacteria (*Sporocarcina pasteurii* ATCC 6453 and *Bacillus aerius* U2) and Effect of Environmental Conditions on Production of Calcium Carbonate

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Abstract. Microbial carbonate precipitation (MCP) occurs as a byproduct of common microbial metabolic processes by ureolytic bacteria. In this study, the effects of different growth parameters such as urea concentration, temperature, pH and $CaCl_2$ concentration were examined on calcium carbonate mineralization by *Bacillus aerius* U2 and *Sporosarcina pasteurii* ATCC 6453. Mineralogical and textural data show that U2 gave rise to $CaCO_3$ precipitations as amorphous extracellular polymeric substance (EPS) and calcite and vaterite crystals, whereas ATCC 6453 produced EPS and vaterite. For *B. aerius* U2, the initial and final pH levels are 5.5 and 9.28, respectively. The highest $CaCO_3$ mineralization was observed at 20 ^{o}C and 300 mM urea for U2 strain, whereas at 30 °C and 333 mM urea for ATCC 6453. Our results indicate that $CaCl_2$ caused enhanced $CaCO_3$ mineralization. 1000 mM $CaCl_2$ was the most efficient concentration at $CaCO_3$ mineralization in *B. aerius* U2 and *S. pasteurii* ATCC 6453. The obtained data indicate that $CaCl_2$ (1000 mM concentration) caused enrichment for $CaCO_3$ mineralization for *B. aerius* U2 and *S. pasteurii* ATCC 6453. The microbial calcium carbonate precipitation by U2 at lower temperature (<30 ^{o}C) conditions is made possible the method to employ in wider climate zones for geotechnical applications.

Keywords: Ureolytic bacteria, biomineralization, calcium carbonate, vaterite, calcite

1. Introduction

Bacterial calcium carbonate precipitation is very important for environmental and (remediation of heavy metals, wastewater treatment, soil improvement, carbon sequestration, e.g.), remediation of cracked concrete, conservation of monuments, durability of concrete structures (Achal *et al*., 2011; Ferris *et al*., 2003; Fujita *et al*., 2000; Hammes *et al*., 2003; Whiffin *et al*., 2007). When urea hydrolysis occurs in a calcium-rich environment, the calcite precipitates (calcium carbonate) turn into solid crystalline materials (Siddique and Chahal 2011). Ureolytic bacteria related microbial cement formation process begins with urea hydrolysis catalyzed by urease enzyme. Calcium ions in the environment are attracted to the negatively charged ions on the bacterial cell wall by high pH effect. Calcium carbonate precipitates as a result of reaction between carbonate and calcium ions (Bachmeier *et al*. 2002; Wong 2015). Factors affecting this process induced by microorganisms are following: calcium concentration dissolved inorganic carbon concentration, temperature, pH, availability of nucleation zone and urease enzyme activity (Fujita *et al*., 2000; Hammes and Verstraete, 2002; Achal and Pan, 2014; Xu *et al*, 2014; Wong, 2015). On the other hand, the main factor determining the reaction in the microbial calcite precipitation is bacterial strain. Especially, bacterial strains have significant effect on the crystal form, size, morphology and biochemistry of calcium carbonate (Xu *et al*., 2014). For this reason, many researchers have conducted microbial calcium carbonate sedimentation studies with various bacterial strains (Rodriguez-Navarro *et al*. 2003; Bang *et al*. 2001; Rivadeneyra *et al*. 1998; Lee 2003; Cheng and Cord-Ruwisch 2012; Qabany *et al*. 2012; Xu *et al*. 2014; Ivanov and Chu, 2008).

In present study, calcium carbonate precipitation was investigated using *Bacillus aerius* U2 isolated from Israfil River in Denizli (Turkey) and *Sporosarcina pasteurii* ATCC 6453. The effects of different conditions such as initial pH, urea concentration, $CaCl_2$ and temperature were determined. The structure of precipitate produced by bacterial strains was analysed by XRD, SEM, DTA and TGA and the results of mineralization of *B. aerius* U2 was compared with the results of *S. pasteurii* ATCC 6453.

2. Material and Methods

2.1. Bacterial strain and culture

Bacillus aerius U2 was isolated from Israfil River in Denizli (Turkey). The samples were inoculated on Urea Agar containing phenol red. After 2-3 days of incubation, a color change from orange to pink in the medium indicates urea hydrolysis. The bacterial colonies stocked. Gram and

endospore staining were performed to confirm to rod-shaped bacteria.

2.2. Effect of different conditions on mineralization of calcium carbonate by bacteria

For screening precipitation, Calcium Precipitation Medium (CPM) was used. CPM is contained 3.0 g/L Nutrient Broth (Difco), 25 mM $CaCl_2$, 25 mM $NaHCO_3$ and 333 mM urea (Ferris *et al*. 1996; Whiffin *et al*. 2007). The growth parameters such as initial pH (5.0-7.5), temperature (20-42°C), urea (25-350 mM) and $CaCl_2$ (25-1000 mM) concentration on calcium carbonate mineralization by *Bacillus aerius* U2 and *Sporosarcina pasteurii* ATCC 6453 were tested. EDTA titrimetric method was used to determine the amount of calcium carbonate produced by the *Bacillus aerius* U2 and *Sporosarcina pasteurii* ATCC 6453 urease positive bacterium (APHA, 1989). The amount of calcium carbonate calculated by the formula of [$CaCO_3$ = (V1.M.1000)/V2), V_1: consumed EDTA, M: 1 mL EDTA= 0.96 mg $CaCO_3$ V_2: sample amount (mL)].

2.3. X-Ray Diffraction (XRD)

X-ray diffraction (XRD) studies were performed on the Bruker D8 Advance model XRD with Ni filtered CuKá radiation (ë = 1.54056 Å), running conditions of 40 mA, 40 kV, scan-speed 0.005°, time/scan 0.1 sec and 0.2 mm slit using LynxEye detector at Istanbul Technical University, Turkey. Diffraction peaks were plotted as 2θ value and diffracted X-rays were calculated with Bragg's law d = ë / 2 Sinθ.

2.4. Scanning electron microscopy (SEM)

SEM is a useful tool for determining the surface morphology of carbonate products. SEM analysis was carried out to gain insight into the surface morphology of the lyophilized EPS. SEM investigations were performed on gold-coated samples using a Carl Zeiss Supra 40 VP Field Emission Scanning Electron Microscope (FE-SEM) at the Pamukkale University (Denizli, Turkey).

2.5. Differential Thermal Analyses (DTA) and Thermogravimetric Analyses (TGA)

Thermogravimetric analyses (TGA) were carried out on a Perkin Elmer SII-Diamond TG-DTA Instruments thermal analysis system in dinitrogen atmospheres, applying a heating rate of 10 ºC min−1 in a temperature range of 0–1000 ºC at Pamukkale University, Denizli, Turkey.

3. Results and Discussion

Test bacterium was 100% identical to *Bacillus aerius* U2 (GenBanks: KF861609.1, KC713594.1, KF861608.1, KC834069.1 and KF861583.1) (Life Sciences Research and Application Center, Gazi University). In general, the results in Table 1 indicate that the calcium carbonate could be produced by *Bacillus aerius* U2. The highest $CaCO_3$ mineralization was 17073.22 mg/L in 14 days for *B. aerius* U2 and 21828.10 mg/L in 5 days for *S. pasteurii* ATCC 6453.

As known, the pH values of bacterial growth medium are an important for understanding the microbial activity. Especially, pH value plays a key role in the bacterial calcium carbonate mineralization. Also, numerous researchers reported that urease enzyme was active at alkaline pH (Stocks-Fischer *et al*., 1999; Anne *et al*., 2010; Prah *et al*., 2011). In present study, the best value of precipitation was at final pH = 8.17 for *S. pasteurii* ATCC 6453, but *B. aerius* U2 mineralized CaCO3 in more alkaline condition the final pH = 9.28. On the other hand, when the pH increased to 9.28, the urease enzyme of *B. aerius* U2 was more active. Temperature is another significant factor for mineralization. In fact, the temperature is a factor limiting the use of bacteria in industrial applications. However, early studies express that temperature lower than 30 ^{0}C was no not effective on calcium carbonate precipitation (Hammes and Verstrate, 2002; Mitchell and Ferris, 2005; Rodriguez-Navarro *et al*., 2007). In contrary, *B. aerius* U2 continued the calcium carbonate at lower degrees like 20 ^{0}C and it has a remarkable potential to use in the soil strengthening. The calcium carbonate precipitation bonds the soil grains and this process brings higher mechanical characteristics like uniaxial compressive strength, young's modulus etc. (Akyol *et al*., 2016).

Table 1. The results of calcium carbonate precipitation for experimental conditions

	***Bacillus aerius* U2**	***Sporosarcina pasteurii* ATCC 6453**
Initial pH	5.5	6.5
Final pH	9.28	8.17
Urea Concentration (mM)	300	333
Temperature (^{o}C)	20	30
Concentration of $CaCl_2$ (mM)	1000	1000
Days	14	7
Amount of $CaCO_3$ (mg/L)	17073.22	21828.10

Calcium sources are a tremendous role in microbial cement formation process. It is known that calcium chloride from calcium sources increases the urease activity and produces more calcium carbonate (Achal and Pan, 2014). In present study, the maximum amount of calcium carbonate was recorded in the medium containing 1000 mM $CaCl_2$ at the end of 14 day by *B. aerius* U2 (17073.22 mg/L). For *S. pasteurii* ATCC 6453, the maximum amount of calcium carbonate was recorded at the end of 7 days in a medium containing 1000 mM $CaCl_2$. The results were in agreement with previous studies (Stocks-Fischer *et al*., 1999; Achal and Pan, 2014). The microbial cement formation process occurs by the formation of ammonium in the presence of urea and calcium and calcium carbonate precipitate in excess calcium. For this reason, urea is required for microbial cement production. When we compared the results with other studies, it was seen that *B. aerius* U2 precipitated calcium carbonate in lower urea concentration (Ferris *et al*., 1996; Fujita *et al*., 2000).

XRD data showed that bacterial calcium carbonate by *B. aerius* U2 are represented by amorphous (EPS) and crystalline (calcite and vaterite) phases. The calcium carbonates from different incubation times were correlated on XRD patterns for U2. The ratio of crystal/amorphous and calcite/vaterite increases together with increasing time (Fig. 1). The maximum amount of calcium carbonate for optimum conditions (7th days, 1000 mM $CaCl_2$) of *S. pasteurii* ATCC 6453 represented by vaterite (Fig. 2). Amorphous (EPS) and crystalline nature of $CaCO_3$ precipitations by *B. aerius* U2 are also confirmed by SEM data (Fig. 3). Amorphous material EPS developed as fine-grained anhedral (amorphous) aggregates. Vaterite and calcite are shown as spherules with 4-10 μm size and euhedral (rhombohedral) crystals, respectively.

Thermal analyses (DTA and TGA) of bacterial calcium carbonates by *B. aerius* U2 indicate two different stages and/or phases (Fig. 4). For DTA curves, the temperatures near 400°C and 700°C represented by exothermic and endothermic peaks, respectively. The exothermic peak indicates a release of energy, related to decomposition of amorphous (EPS) phase; correspond to 10% weight loss for TGA curves. The exothermic peak near 700 °C is related to destruction of calcite and vaterite; correspond to 40% weight loss. The total weight loss values change between 51 and 63%. The maximum weight loss is observed for 2 day incubation time indicating relatively higher amounts of EPS contents as seen XRD pattern (Fig. 1).

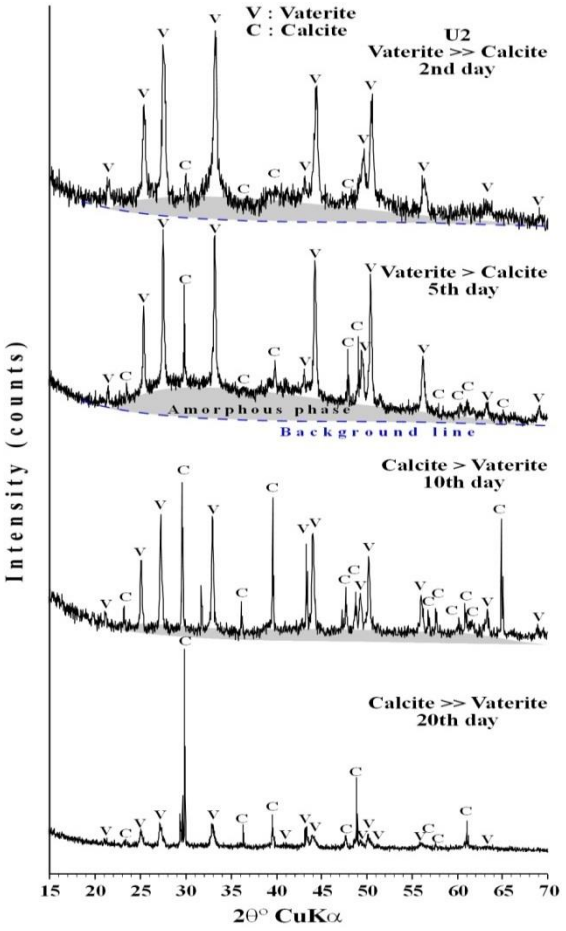

Figure 1. X-ray diffraction patterns of calcium carbonate precipitations by *Bacillus aerius* U2 with respect to the incubation times Gray areas represent amorphous phase (EPS).

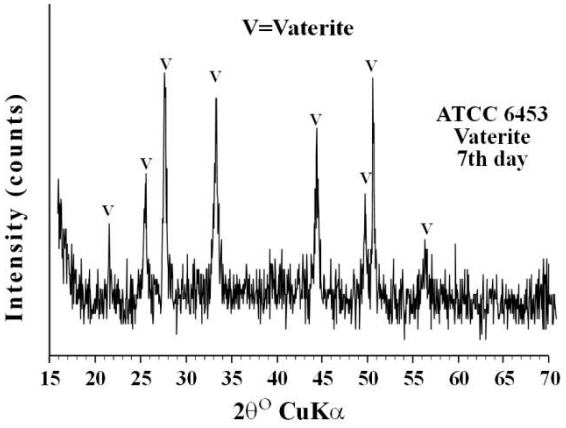

Figure 2. X-ray diffraction patterns of calcium carbonate precipitations by *Sporosarcina pasteurii* ATCC 6453.

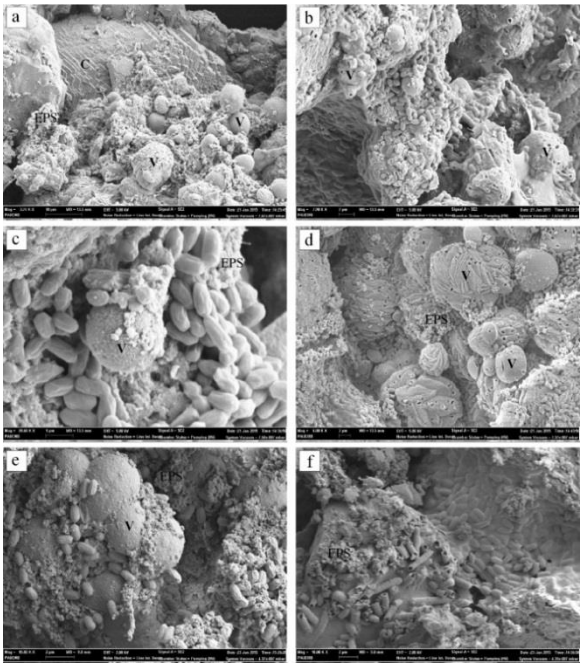

Figure 3. SEM photomicrographs of microbial carbonate formations in *B. aerius* U2. (a) Trigonal calcites (C), spherulitic vaterites (V) and fine-grained polymeric (EPS) formations, (b-e) Ellipsoidal bacteria (1µm) spherulitic vaterites (V) and fine-grained EPS formations, (f) ellipsoidal bacteria and tubular colonies.

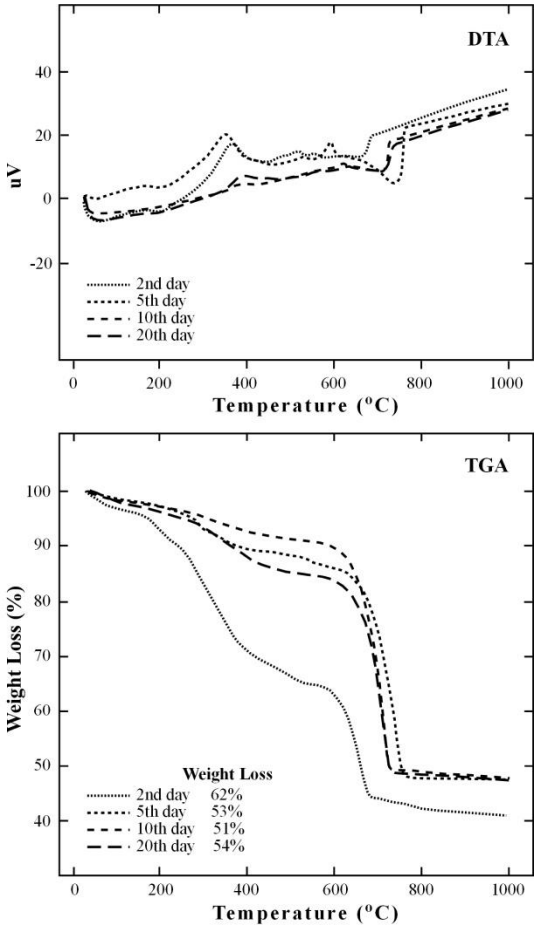

Figure 4. (a) DTA and (b) TGA curves of calcium carbonates depending on the incubation time in *B. aerius* U2.

4. Conclusion

The findings of this study are summarized as follows:

This study is the first information related CaCO3 mineralization of *Bacillus aerius* U2 newly isolated. CaCO3 precipitates of U2 are composed of as amorphous extracellular polymeric substance (EPS) and calcite and vaterite crystals, whereas *S. pasteurii* ATCC 6453 produces EPS and vaterite. The microbial calcium carbonate precipitation by U2 at lower temperature (<30 ^{o}C) conditions is made possible the method to employ in wider climate zones for geotechnical applications.

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