

Calcium carbonate precipitation by urease and carbonic anhydrase positive bacteria

Üreaz ve karbonik anhidraz pozitif bakteriler tarafından kalsiyum karbonat çökeltimi

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Abstract

In present study, CaCO₃ precipitation was examined in two ureolytic bacteria. *Bacillus aerius* U2 and *Sporosarcina pasteurii* ATCC 6453 were used as test organisms. The determination of urease and carbonic anhydrase enzyme activities were also determined. For further confirmation of the calcium carbonate mineral type produced by bacteria, XRD, SEM and EDX analysis were done. Strain U2 produced calcite and vaterite. In *S. pasteurii* ATCC 6453, only vaterite was found. The enzyme activity studies showed that both urease and carbonic anhydrase activities was 2-50-fold higher in *S. pasteurii* ATCC 6453 than *B. aerius* U2. Although, *S. pasteurii* ATCC 6453 was better option for microbial calcium carbonate precipitation (MCP) at higher temperature, by *B. aerius* U2 at lower temperature (<30 °C) is made possible to employ in the most geotechnical applications.

Keywords: Precipitation, Calcium carbonate, Carbonic anhydrase, Urease, Ureolytic bacteria.

Öz

Bu çalışmada, iki üreolitik bakteride CaCO₃ çökeltimi araştırılmıştır. Test organizması olarak *Bacillus aerius* U2 ve *Sporosarcina pasteurii* ATCC 6453 türleri kullanılmıştır. Bunların yanında, bakterilerin üreaz ve karbonik anhidraz enzim aktiviteleri de tespit edilmiştir. Bakteriler tarafından üretilen kalsiyum karbonatın mineral tipi konformasyonu için XRD, SEM ve EDX analizleri gerçekleştirilmiştir. *B. aerius* U2 suşu kalsit ve vaterit üretmiştir. *S. pasteurii* ATCC 6453 suşunda sadece vaterit bulunmuştur. Enzim aktivitesi çalışmalarının sonuçlarına göre *S. pasteurii* ATCC 6453, *B. aerius* U2'den 2-50 kat daha fazla aktivite göstermiştir. Yüksek sıcaklıklarda kalsiyum karbonat çökeltimi için *S. pasteurii* ATCC 6453 türü tercih edilebilirken, daha geniş iklim bölgelerini kapsayan düşük sıcaklıklarda (<30 °C) *B. aerius* U2 türünün kullanım potansiyeli bulunmaktadır.

Anahtar kelimeler: Çökeltim, Kalsiyum karbonat, Karbonik anhidraz, Üreaz, Üreolitik bakteriler.

1 Introduction

CaCO₃ precipitation by ureolytic bacteria is one of the important scientific issues due to its importance in engineering. Previous studies in the scientific community have reported that many environmental problems could be solved with ureolytic bacteria [1]-[3]. The formation of microbial calcium carbonate starts with urea hydrolysis catalyzed by the urease enzyme. When ureolytic bacteria are found in calcium-rich media, the reaction between calcium and carbonate ions occurs due to the high pH caused by urea hydrolysis. As a result of this reaction, calcium carbonate crystals precipitate in the environment. The biological, chemical, and physical factors such as pH, temperature, urease enzyme activity and calcium ions can affect the bacterial precipitation [4]-[8]. Also, the bacterium type is an important because the type of mineral, size and biochemical structure of calcium carbonate varies as according to the species of bacteria. In order to determine the effect of bacterial strains, numerous researchers have intensified on this subject [9]-[16]. In this study, we aimed to compare and analyze two bacterial calcium carbonate precipitation by *Bacillus aerius* U2 and *Sporosarcina pasteurii* ATCC 6453. Moreover, the role of

urease and carbonic anhydrase enzymes in this process was determined for the first time. The structures of bacterial calcium carbonate were analyzed by XRD, SEM, DTA and TGA throughout in this study.

2 Material and methods

2.1 Materials

Bacillus aerius U2 and *Sporosarcina pasteurii* ATCC 6453 were used as organisms. *B. aerius* U2 was isolated in our previous study [17]. The bacteria were obtained from Pamukkale University, Department of Biology, Bacteriology Laboratory Denizli, Turkey.

2.2 Calcium carbonate precipitation

For screening precipitation, Calcium Precipitation Medium (CPM), containing 3.0 g/L Nutrient Broth (Difco), 25 mM CaCl₂, 25 mM NaHCO₃ and 333 mM urea, was used. The experimental parameters for mineralization were given in Table 1. The amount of precipitated CaCO₃ was measured by EDTA titrimetric method [18] and calculated by the formula of

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$[CaCO_3=(V1.M.1000)/V2]$, V1: consumed EDTA, M: 1 mL EDTA=0.96 mg CaCO₃ V2: sample amount (mL)].

Table 1. The experimental parameters of calcium carbonate precipitation.

	<i>B. aerius</i>	<i>S. pasteurii</i>
pH _{initial}	5.5	6.5
pH _{final}	8.64	8.20
Nutrient broth (g/L)	3	3
NaHCO ₃ (mM)	25	25
Urea (mM)	333	333
Temperature (°C)	20	30
CaCl ₂ (mM)	25	25
Inoculation rate (%)	10	10
Days	14	7
Amount of CaCO ₃ (mg/mL)	1319.84	2594.50

2.3 Urease enzyme activity

Urease enzyme activity was measured by phenol-hypochlorite method slightly modified in our lab [19]. Briefly, ureolytic bacteria were incubated in calcium precipitation media (CPM) and Luria Bertani-Miller (LB) supplemented with urea. Then, the enzyme crude extract from *B. aerius* U2 strain and *S. pasteurii* ATCC 6453 were obtained by ultrasonicator. The bacterial enzyme crude extract was added to reaction mixture (phosphate buffer, 0.1 M, pH 7.4 and 50 mM urea) and all incubated at 37 °C for 30 min. At the end of the incubation, 50 µl reaction mixture was added to 500 µl of phenol-nitroprusside solution. For color formation, 500 µl of alkali hypochlorite solution was added and incubated at 37 °C for 20 min. This mixture measured by spectrophotometry at 630 nm. Enzyme activity was determined by standard curve generated with (NH₄)₂SO₄. One unit of urease activity is the amount of enzyme needed to hydrolyze 1 µmol of urea per min at 37°C.

2.4 Carbonic anhydrase enzyme activity

Carbonic anhydrase activity was determined according to method by Armstrong et al. [20]. Some optimization and modifications was done in our lab. Briefly, 100 µl of the crude enzyme prepared by ultrasonicator was added to 900 µl of the mixture consisting of phosphate buffer (100 mM, pH 7.2) and *p*-nitrophenyl acetate (3 mM). Absorbance was measured at 412 nm at least 5 min. Enzyme activity was determined by standard curve prepared with *p*-nitrophenol. One unit of enzyme activity was expressed as 1 µmol of *p*nitrophenyl acetate hydrolysed per minute.

2.5 Minerological analysis (XRD, SEM and TGA)

The XRD analysis of precipitated CaCO₃ from bacteria was done at İstanbul University. TGA analysis was performed in a Perkin Elmer SII-Diamond. The samples were heated from 0 to 1000 °C at a heating rate of 10 °C/min. For SEM analysis, the samples were coated with gold and palladium and analyzed by FESEM. The analyses of TGA and SEM were done at Pamukkale University, Denizli, Turkey.

2.6 Statistical analyses

The Minitab statistical software package was used for statistical analyses. Each data was given as means including their Standard Error of Means (SEM). Student *t*-test was used to compare the data sets and *p*<0.05 was chosen for level of statistical significance.

3 Results and discussion

3.1 Calcium carbonate precipitation

Screening the organisms and parameters of growth for the precipitation of CaCO₃ is laborious and time consuming. However, it is not suspected that bacterial precipitation is both variable according to both bacteria types and growth parameters. In this study, *B. aerius* U2 and *S. pasteurii* ATCC 6453 of their precipitation abilities were compared. Parameters of urea concentration, different initial pH, temperature, inoculation rate and incubation times were tested in our study (data not shown) and the best conditions of both bacteria for precipitation were presented in Table 1. Although both bacteria were capable of precipitating calcium carbonate, the amount of precipitated calcium carbonate was different. For example, the results indicate that highest CaCO₃ mineralization for *B. aerius* U2 was calculated as 1319.84 mg/ml in 14 days. On the other hand, this value was found as 2594.50 mg/ml in 7 days for ATCC 6453 strain. In contrast to *B. aerius* U2, *S. pasteurii* ATCC 6453 accumulated more carbonate in less time. When compared *B. aerius* U2 and *S. pasteurii* ATCC 6453, lower calcium carbonate precipitation was observed in *B. aerius* U2. Our urease activity results were parallel to that precipitation result also. But, this strain could precipitate calcium carbonate properly. It was well established that, urease activity in bacteria varies from strain to strain. For example, urease activities of *B. megaterium* were different from *S. pasteurii* [21]. Also these results were parallel to our results. On the other hand, there are no correlation between urease activity and bio-calcification [22]. Besides these, *Bacillus aerius* U2 achieved maximum calcium carbonate precipitate at the end of a longer incubation (14 days) compared to *S. pasteurii* ATCC 6453 (7 days). *S. pasteurii* ATCC 6453 precipitated more calcium carbonate than *B. aerius* U2 in a shorter time. This was due to higher urease activity. Undoubtedly, the incubation time was a significant advantage. But, the ability of precipitation of U2 at lower temperature was also an advantage of this bacterium. It is well established that, one of the important factor affecting the growth and activity of bacteria is temperature. Studies on bacterial calcium carbonate precipitation in the literature have shown that low temperature is not effective in this process [23],[24]. It was determined that *B. aerius* U2 effectively precipitated calcium carbonate at low temperature (20 °C). Perhaps, *B. aerius* U2 can be used to improve problematic soils by changing mechanical properties of these soils such as uniaxial compressive strength, young's modulus etc. [17].

The pH values of the growth media are very important in biomineralization as well as in microbial activity. In addition, studies conducted by many researchers have shown that bacterial calcium carbonate precipitation activity was higher at alkaline pH. [25]-[27]. The initial pH of precipitation in *B. aerius* U2 was 5.5, while *S. pasteurii* ATCC 6453 was 6.5. The final pH values were very closed in both bacteria (8.64 for *B. aerius* U2 and 8.2 for *S. pasteurii* ATCC 6453). In other words, the best precipitation was almost the same alkali conditions. It is well known that urease activity was higher in neutral or alkaline pH. Higher microbial precipitation in alkaline pH was most probably due to higher urease activity in that pH for both bacterial strain.

3.2 X-ray diffraction

According to the results obtained from XRD analysis, amorphous (EPS) phase, calcite and vaterite as calcium carbonate crystals were observed in CaCO_3 produced by *B. aerius* U2. In addition, as the increasing incubation time for *B. aerius* U2 increased the ratio of calcite/vaterite and crystal/amorphous (Figure 1). For ATCC 6453 strain, only vaterite was observed in the XRD analysis of CaCO_3 precipitation medium containing 1000 mM CaCl_2 (14th days) (Figure 2). In other words, calcite was not seen under this condition. There were different forms of microbial CaCO_3 such as calcite, vaterite, aragonite etc. Different bacteria or strains can form different mineral types in different growth conditions [17],[28]-[31]. For example, *Synechococcus leopoliensis* PCC 7942 can form aragonite-like CaCO_3 [31]. *B. megaterium*, *B. cereus*, *B. subtilis* and *B. thuringiensis* can form calcite and vaterite, while *L. fusiformis* produce pure vaterite [32]. This is due to growth conditions and calcium carbonate precipitation parameters.

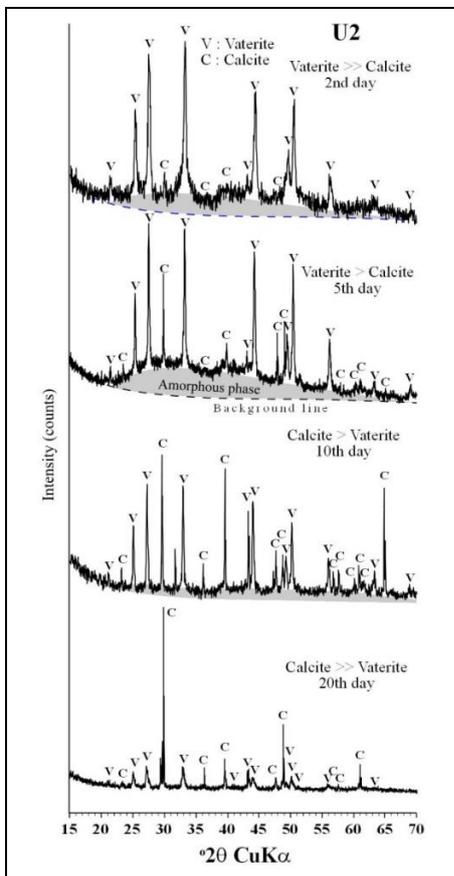


Figure 1. XRD data of precipitated CaCO_3 in *B. aerius*.

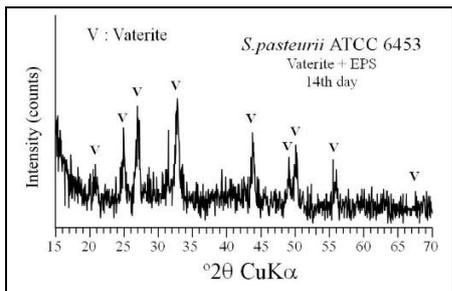


Figure 2. XRD data of precipitated CaCO_3 in *S. pasteurii*.

3.3 SEM analyses

Figure 3 and 4 present SEM photomicrographs of precipitated CaCO_3 in strains *B. aerius* U2 and *S. pasteurii* ATCC 6453. Amorphous phase (EPS) and the mineral type of the calcium carbonate in *B. aerius* U2 strain and *S. pasteurii* ATCC 6453 strain were confirmed by SEM analysis. EPS as polymeric formations in amorphous structure, spherical vaterite and trigonal structure was shown in photomicrographs (Figure 3). In addition, the SEM-EDX analysis performed on the *S. pasteurii* ATCC 6453 calcium carbonate precipitate determined the mass percentage of the elements contained in the sample (Figure 4). The presence of vaterite and calcite observed in XRD and DTA-TGA analyses in bacterial calcium carbonate precipitates was supported by the results of SEM-EDX analysis.

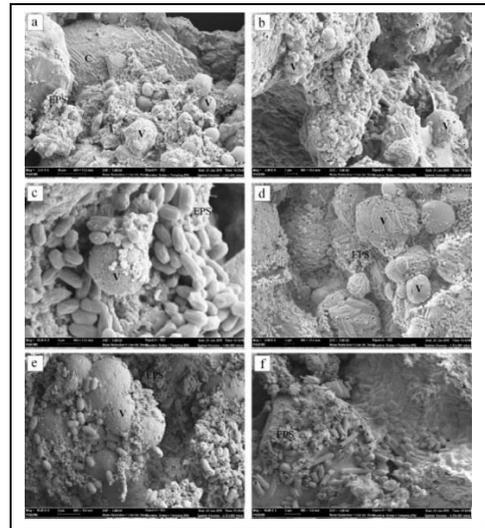


Figure 3. The SEM images of precipitated CaCO_3 in *B. aerius*. (a): Calcite (c): vaterite (V) and amorphous formations (EPS), (b-e): Cells formed bacillus (1µm) vaterite and polymeric formations, (f): Cell colonies.

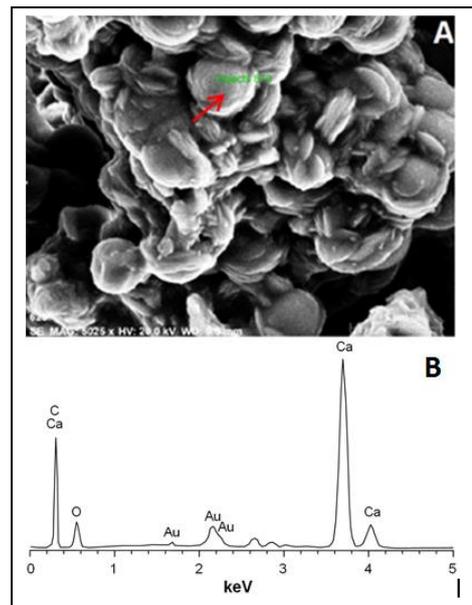


Figure 4. SEM-EDX analysis of precipitated calcium carbonate in *S. pasteurii*. (A): Vaterite formations. (B): Mass % of elements.

3.4 Thermal analyses

Two phases were observed by *B. aerius* U2 as endothermic and exothermic in the thermal analysis of calcium carbonate sediments (Figure 5). The exothermic peak corresponds to an energy release associated with the decomposition of the amorphous (EPS) phase, which corresponds to a 10% weight loss. Calcite and vaterite destruction was observed in thermal analysis as seen exothermic peak close to 700 °C. This caused approximately 40% weight loss. Moreover, this was increased up to 63% at the end of the 48 hour incubation (51-63%). This confirms the XRD analysis results by demonstrating the high amount of EPS as amorphous phase in the bacterial calcium carbonate precipitate (Figure 2).

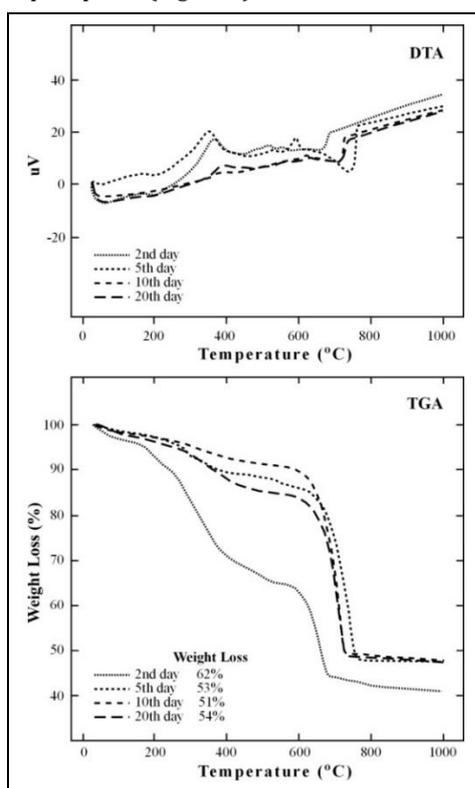


Figure 5. DTA and TGA analysis of precipitated calcium carbonate in *Bacillus aerius*.

3.5 Enzymes activities

It is well established that Urease and Carbonic anhydrase enzymes are involved in bacterial calcium carbonate precipitation. These two enzymes have different role in this process. Urease involved in calcium carbonate mineralization by hydrolyzing urea into ammonium and carbonate in the alkaline environment. On the other hand, the carbonic anhydrase catalyzes reversible hydration of CO₂. Urease and carbonic anhydrase activities were determined for the first time in two different medium (LB-UREA and CPM) in both bacteria. Urease enzyme activity of U2 was calculated as 1.52±0.1 µmol/min/mg protein in LB-urea medium and 0.44±0.02 µmol/min/mg protein in CPM. Urease enzyme activity was induced approximately 3-fold in LB-urea medium compared to CPM. *S. pasteurii* ATCC 6453 urease enzyme activities were found 5.36±0.1 and 24.98±5.0 µmol/min/mg protein for LB-urea and CPM, respectively. In contrast to strain U2 urease, strain ATCC 6453 urease activity was higher in CPM (Table 2). Similar to our results many bacterial strains

including *Bacillus* and *Pseudomonas* with high enzyme activities showed high CaCO₃ precipitation [33]. Omoregie et al. [22] reported that urease activities for the bacteria isolated from limestone caves had higher urease activity. In addition, Sun et al. [21] emphasized that the urease activity of *B. megaterium* was lower than that of *S. pasteurii* at high temperatures such as 25 °C and 30 °C. It is well established that urease enzyme activity was regulated by environmental conditions such as nitrogen content. The nitrogen amount of CPM and LB-urea were different. Therefore, enzyme activity was different in two medium. Moreover, type, physiological requirement, and nitrogen demands are different for two bacteria. Therefore, urease enzyme activities may regulate different manner in two bacteria. Although strain U2 showed low urease activity in CPM, CPM also made calcite mineralization [34]. However, future works are needed to solve underlying mechanisms of urease induction in different medium.

In addition to urease, carbonic anhydrase activity was also determined in this study. Carbonic anhydrase enzyme activity is highly effective in calcium carbonate precipitation. This enzyme activity for *B. aerius* U2 was found 30.75±1.76 and 42.30±5.4 nmol/min/mg protein in LB urea and CPM, respectively. 1.38-fold induction was found in two different medium but this change was not found statistically significant. Similarly 1.82 fold difference was found in between CPM and LB-urea medium for ATCC 6453 (61.9±5.7 nmol/min/mg protein for LB urea and 112.9±9.4 nmol/min/mg protein for CPM). These results showed that *S. pasteurii* ATCC 6453 had higher urease and carbonic anhydrase activity than *B. aerius* U2 (Table 2).

Table 2. Urease and carbonic anhydrase enzyme activity.

medium	Bacteria	Urease activity (µmol/min/mg)	Carbonic anhydrase activity (nmol/min/mg)
LB-Urea	<i>B. aerius</i>	1.52±0.1	30.75±1.76
	<i>S. pasteurii</i>	5.36±0.1*	61.9±5.7*
CPM	<i>B. aerius</i>	0.44±0.02	42.30±5.4*
	<i>S. pasteurii</i>	24.98±5.0*	112.9±9.4*

*: Significantly different from the respective *B. aerius* U2 value (p<0.05).

4 Conclusions

In this study, it was examined CaCO₃ precipitation in two ureolytic bacteria and verified mineral types with XRD and SEM for the first time. *S. pasteurii* ATCC 6453 synthesized vaterite crystals. The calcium carbonate precipitates consisted of calcite and vaterite crystals in *B. aerius* U2. Also, strain U2 precipitated calcium carbonate at 20 °C. Bacterial calcium carbonate mineralization is a highly complex metabolic activity of cells. If we want to solve an environmental problem, we must investigate and optimize each organism under its own conditions of urea hydrolysis and bio-mineralization. We thought that *B. aerius* U2 could be used as a potential bacterium in a wide range of climatic zones, mainly in geotechnical applications. And finally, this study was the first document on the urease and carbonic anhydrase activities of both bacteria in LB-UREA and CPM.

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