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ISOLATION AND IDENTIFICATION OF CALCITE FORMING BACTERIA FROM SOIL AND LIMESTONE COLLECTED IN BA RIA VUNG TAU PROVINCE

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ABSTRACT

Calcite-forming by bacteria has been reported in various geological environments including limestone caves, and soil. There are four natural processes by which calcite is formed: carbonic anhydrase (CA), sulfate reduction, nitrate reduction, and urea hydrolysis. The study aims to identify calcite-forming bacteria occurring in limestone areas of Ba Ria Vung Tau province. 128 bacterial strains were obtained on B4 agar. Nine strains (BRVT1, BRVT2, BRVT3, BRVT4, BRVT5, BRVT6, BRVT7, BRVT8, and BRVT9) were selected by seemingly determining high calcite production and urease activity. Among the bacterial isolates, the BRVT1 strain produced is 11.8 g/L calcite precipitate; BRVT2, the BRVT5 strains are 11.4 g/L, and the BRVT4 strain is 12.8 g/L. The 16S rRNA gene sequencing identified isolates as BRVT1 strain is *Bacillus tequilensis*, BRVT2 and BRVT5 strain are *Bacillus cereus* and BRVT4 strain is *Bacillus subtilis*. Further, the microstructure of calcite precipitated was inspected through scanning electron microscopy (SEM), and X-ray diffraction (XRD).

Keywords: 16S rRNA, Bacillus, Ba Ria Vung Tau, SEM-EDX.

1. INTRODUCTION

Calcite is the carbonate mineral and the most stable form of calcium carbonate ($CaCO_3$). Other forms are the minerals aragonite and vaterite. Calcite is a common component in sedimentary rocks, such as limestone, which is largely made up of the shells of dead marine species. Approximately 10 % of sedimentary rock is limestone. Calcite is the main mineral in metamorphic marble. It also occurs in mineral veins in hot springs deposits, and it is often found in caves as stalactites and stalagmites. Calcite can also be found in volcanic rocks, rocks of mantle origin such as carbonatite, and kimberlite, but rarely in peridotite. Calcite, a common form of calcium carbonate, is often the primary component of the shells of marine organisms such as plankton (e.g., lime spines and worms) and red algae. Microbially induced calcite precipitation (MICP) involves the formation of calcium carbonate from a supersaturated solution, driven by the presence of microbial cells and their associated biochemical activities [1]. During the MICP process, microorganisms secrete one or more metabolic products, such as carbonate ions (CO_3^{2-}), which react with environmental ions like calcium (Ca^{2+}), leading to the precipitation of minerals. The formation of calcium carbonate has been proposed to occur through various mechanisms, including photosynthesis, urea hydrolysis, sulfate reduction, anaerobic sulfide oxidation, biofilm activity, and the involvement of extracellular polymeric substances (EPS) [2, 3]. However, the most widely used method for precipitating calcium

carbonate by bacteria is through urea hydrolysis [4]. The precipitation process of calcium carbonate ($CaCO_3$) in microbial-induced calcium carbonate precipitation (MICP) is a simple and highly controllable mechanism capable of generating substantial amounts of $CaCO_3$ within a short time frame [5]. The successful and efficient precipitation of calcium carbonate relies on a combination of biological and chemical factors, including: (1) pH, (2) concentrations of dissolved inorganic carbon (DIC), (3) calcium concentrations, and (4) the availability of nucleation sites. The first three factors primarily affect the concentration of carbonate ions (CO_3^{2-}), determining the saturation state. In contrast, the availability of nucleation sites plays a critical role in ensuring stable and continuous calcium carbonate formation [6]. In the biomineralization process, bacteria act as nucleation sites, facilitating the precipitation of calcium carbonate ($CaCO_3$) in association with bacterial cells. Various parameters significantly influence either the ureolytic activity or the formation of $CaCO_3$ crystals. Bacterial cell surfaces contain negatively charged functional groups that attract and bind divalent cations, such as Ca^{2+} and Mg^{2+}, under neutral pH conditions, making these surfaces ideal for calcite nucleation. However, Ca^{2+} ions bind more readily to the negatively charged bacterial surfaces than Mg^{2+} ions due to their higher ionic selectivity [7]. Subsequently, the bound cations (metal ions) react with anions, such as carbonate, to produce insoluble calcium carbonate ($CaCO_3$). Bacterial cells play a crucial role in this process by providing nucleation sites (heterogeneous nucleation) and influencing the specific mineral types that form. The microbial-induced calcium carbonate precipitation (MICP) process is a highly effective and environmentally friendly technology with diverse applications, including heavy metal and radionuclide remediation, bioconsolidation, biocement production, CO_2 sequestration, and other environmental solutions.

2. MATERIALS AND METHODS

2.1. Isolation of Calcite-Forming Bacteria

Soil and limestone samples from the limestone mountain of Ba Ria Vung Tau province were collected in plastic bags in January 2022. After it will be processed by grinding to a uniform size (< 0.5 mm). Take 25 g of each soil and limestone sample, add into 225 mL of B4 liquid medium with pH 8, and incubate at 35 ^oC, for 24 hours. Samples after proliferation were diluted to concentrations of 10^{-2}, 10^{-3}, and 10^{-4} and inoculated on petri dishes containing B4 agar medium and continued to incubate at 35 ^oC for 48 hours. After 48 hours of culture, single colonies with characteristic morphology appeared and were selected for purification by further inoculating on B4 agar, continuing to subculture several times to obtain a pure line with homogeneous colony form.

2.2. Selection of bacteria capable of rapidly producing calcite

To determine whether the crystals formed by bacteria were calcite, a simple test using diluted hydrochloric acid (HCl) was conducted. The presence of calcite is indicated by its dissolution in HCl, accompanied by the release of tiny gas bubbles of carbon dioxide (CO_2), a result of the reaction with carbonate ions.

Bacterial broth culture samples were inoculated in urea broth and incubated in incubator shaker at 30 ^oC, 200 rpm. A positive test indicated a change in colour from red to hot pink or magenta.

2.3. Identification by morphology - biochemical method

The configuration, margin elevation and colour of the isolate colonies were observed under a light microscope as described by [8]. Gram type determination was conducted

according to the standard staining technique as described by [9]. Biochemical test as such as fermentation test, catalase test, indole test, oxidase test, citrate test as described by [10].

2.4. Identification of selected isolates by 16S rDNA sequencing

16S rDNA of selected strains was amplified by PCR procedure described by Angmo *et al.* (2016). PCR primers 27F (5′- AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) were employed during amplification. Each reaction had a final volume of 25 µL consisting of : 9.5 µL MiliQ water, 12.5 µL My Taq Mix 2x reaction buffer, 1 µL (10 µM) forward primer, 1 µL (10 µM) reverse primer and 1 µL of the template DNA and under the following conditions at 94 °C for 5 min followed by 30 cycles of heating at 94 °C (30 s), primer annealing at 52 °C (30 s) and extension at 72 °C (45 s). The final extension was carried out at 72 °C for 10 min for 1 cycle. Presence of specific PCR products was confirmed by agarose electrophoresis. The DNA sequence of PCR product was carried out by Phu Sa Biochem LTD. Company (https://www.phusabiochem.com/vi/.html). Sequence results were aligned with NCBI database using BLAST algorithm.

2.5. SEM and EDX analyses

Scanning electron microscope (SEM) and an X-ray diffraction (EDS) were used to study the morphological features and elemental composition of the crystals.

2.6. Statistical analysis

The results were reported as the mean ± standard deviation (S.D.) based on three independent experiments. Statistical analysis was conducted using Minitab v.18 software to perform an analysis of variance (ANOVA). A p-value of < 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Isolation and basic characterization of bacteria

A total of 122 soil and limestone samples were collected from different districts of Ba Ria Vung Tau provine (Table 1). From the results obtained, 128 bacterial strains were obtained on B4 agar. Nine strains (BRVT1, BRVT2, BRVT3, BRVT4, BRVT5, BRVT6, BRVT7, BRVT8, and BRVT9) were selected by seemingly determining high calcite production and urease activity based on the intensity of the pink color produced in the urea broth (Fig. 1)

Table 1. Samples collected in Ba Ria Vung Tau province

Serial Number	Location	Amount	Characterization
1	3B quarry - Hoa An 1 (Dinh mountain)	23	Soil, stone
2	Chau Pha quarry (Dinh mountain)	14	Soil, stone
3	Chau Phan quanry, Phu My (Dinh mountain)	13	Soil, stone
4	Toc Tien quarry (Toc Tien - Thi Vai mountain)	8	Soil, stone
5	Thanh Tam quarry (Toc Tien - Thi Vai mountain)	4	Soil, stone
6	Phu Duc Chinh quarry (Toc Tien - Thi Vai mountain)	5	Soil, stone
7	So quarry (So mountain)	7	Soil, stone
8	Ninh Giao quarry (So mountain)	17	Soil, stone
9	Hung Phong stone production and mining company (Duc Me Long Huong mountain)	10	Soil, stone, grit
10	Hung Phong quarry (Kim Dinh mountain)	8	Soil, stone
11	Truong Phi mountain	6	Soil, stone, grit
12	Ninh Dam mountain	7	Soil, stone

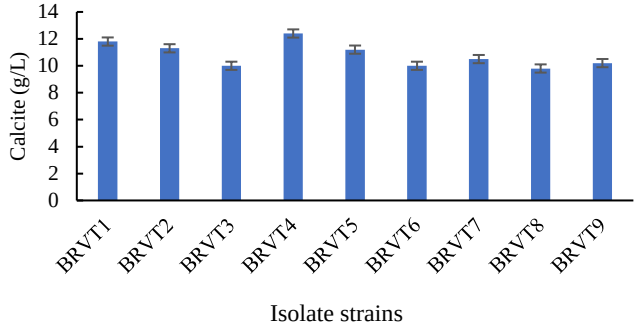

Fig. 1. Calcite production of nine isolated strains

It is essentially difficult to differentiate the species on the basis of colony morphology. Colonies were irregular, raised, opaque colonies and were best observed under reflected light. The colonies of the BRVT1 isolate had a smooth texture, cream white in color, regular shape, and raised elevation. The colonies of the BRVT2 isolate had rough texture, white color, regular shape, and flat elevation. The colonies of the BRVT3 isolate had rough texture, cream color, irregular shape, and flat elevation. The colonies of the BRVT4 isolate had rough texture, white color, circular in shape, and convex elevation. The colonies of the BRVT5 isolate had smooth texture, cream in color, irregular shape, and flat elevation. The colonies of the BRVT6 isolate had smooth texture, white cream color, irregular shape, and raised elevation. The colonies of the BRVT7 isolate had a smooth texture, cream color, circular shape, and convex elevation. The colonies of the BRVT8 isolate had a smooth texture, white color, circular shape, and convex elevation. The colonies of BRVT9 isolate had a rough texture, brown color, irregular shape, and flat elevation as shown in Fig. 2.

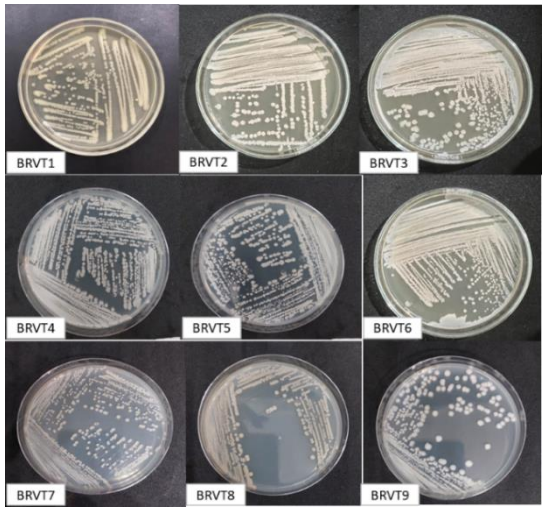

Fig. 2. Morphology of nine isolated strains

3.2. Identification by 16s rRNA sequencing of the bacteria

From the results of calcite precipitation, four strains with the highest results were identified by 16S rRNA sequencing (BRVT1, BRVT2, BRVT4, BRVT5). The sequence analysis of the 16S rDNA gene of the isolates was determined and compared with those of reference *Bacillus* spp strains in the database using NCBI blast (http://www.ncbi.nlm.nih.gov). 16s rDNA sequence

analysis showed that there was a strong similarity (95% - 100%) between our test strains and representative strains in the database of *Bacillus* spp strains, which may indicate that 16s rDNA gene sequence data is helpful for the identification of bacteria at the species level. The results showed that they were closely related to one another as well as to cultured bacteria belonging to the *Bacillus* groups. BLAST results suggested that the closest relatives are *Bacillus tequilensis* (BRVT1), *Bacillus cereus* (BRVT2, BRVT4), and *Bacillus subtilis* (BRVT5). A phylogenetic (neighbor-joining) tree was constructed and is shown in Fig. 3. The local *Bacillus cereus* strain demonstrated high urease activity and has the potential to be a viable and cost-effective solution for bio-self-healing concrete through the bioprocess of MICP [11].

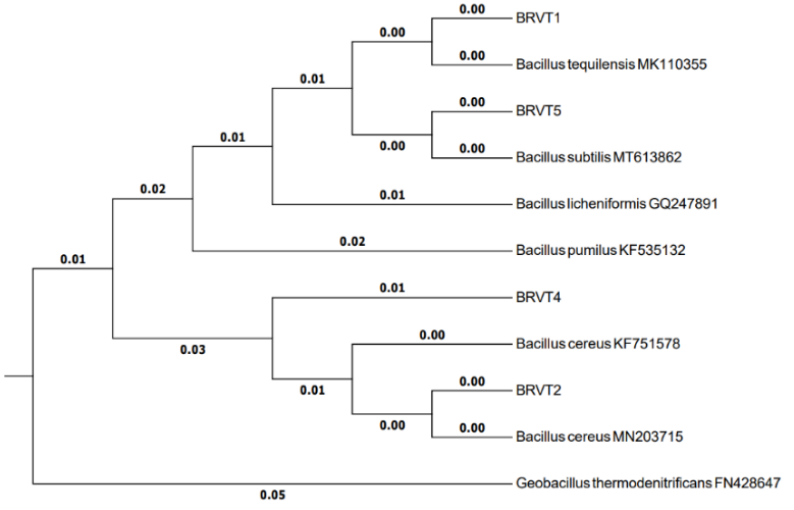

Fig.3. Molecular phylogenetic analysis by maximum likelihood method based on almost-full-length 16S rRNA gene sequences illustrating the phylogenetic position of BRVT1, BRVT2, BRVT4 and BRVT5 and related taxa.

Biochemical tests were conducted to confirm the identification. The biochemical characteristics of these bacteria are given in Table 2.

Table 2. Biochemical characteristics of isolate strains

Tests	BRVT1	BRVT2	BRVT4	BRVT5
Gram	+	+	+	+
Colony	+	+	+	+
Urease	–	–	+	–
Catalase	+	+	+	+
Indol (I)	–	–	–	–
Methyl Red (MR)	+	+	+	+
Mobility	+	+	+	+

(- :negative; +: positive results)

3.3. SEM analysis of precipitated calcite

Calcite precipitated from the bacterial strains was dried and examined under SEM complemented with EDS. Figure 4 represents the crystal morphology of $CaCO_3$. According to it is also endorsed by the literature that bacterial type influences the crystal morphology. Calcite, vaterite and aragonite are three different crystalline polymorphs of $CaCO_3$ existing naturally.

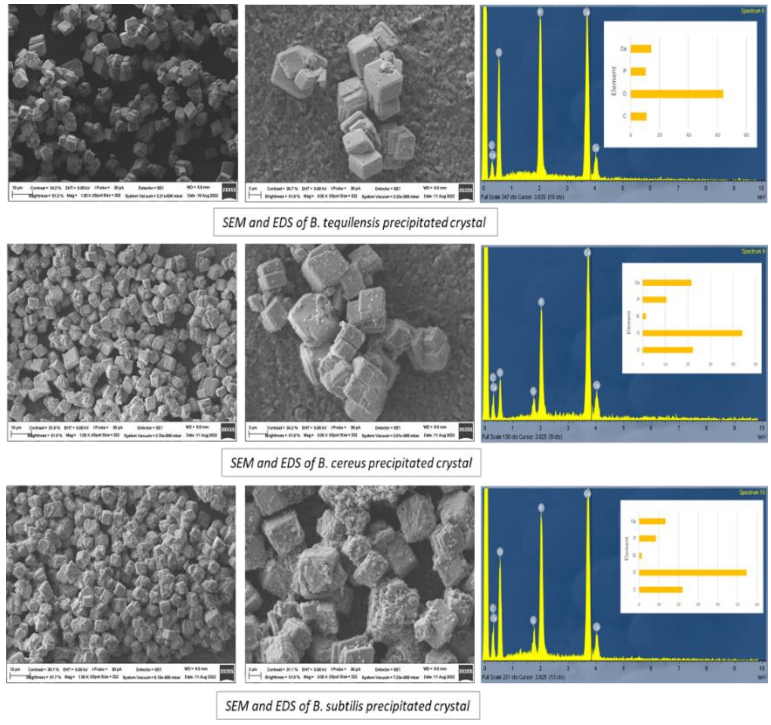

Fig. 4. SEM and EDS analysis of precipitated calcite

You should have a conclusion that $CaCO_3$ from 3 isolates belonging to 1 type is calcite and compare this calcite with that from other *Bacillus* spp. of another author.

4. CONCLUSIONS

The results of this research confirmed the occurrence of microbially induced calcium carbonate precipitation. In the study, new strains of *Bacillus tequilensis*, *Bacillus cereus*, and *Bacillus subtilis* were isolated from soil and limestone samples collected from different districts of Ba Ria Vung Tau province, and characterized based on their distinctive physiological, and morphological characteristics and the sequences analysis of the 16S rDNA gene of the isolate strains.

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TÓM TẮT

PHÂN LẬP VÀ KHẢ NĂNG VI KHUẨN TẠO TỦA CALCITE ($CACO_3$) THU THẬP TẠI TỈNH BÀ RỊA VŨNG TÀU

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Canxit hình thành bởi vi khuẩn đã được báo cáo trong các môi trường địa chất khác nhau bao gồm các hang động đá vôi và đất. Có bốn quá trình tự nhiên mà canxit được hình thành: carbonic anhydrase, khử sunfat, khử nitrat và thủy phân urê. Mục đích nghiên cứu là xác định vi khuẩn tạo canxit xuất hiện ở vùng núi đá vôi tỉnh Bà Rịa Vũng Tàu. Thu được 128 chủng vi khuẩn trên môi trường thạch B4. Chín chủng (BRVT1, BRVT2, BRVT3, BRVT4, BRVT5, BRVT6, BRVT7, BRVT8 và BRVT9) đã được chọn bằng cách xác định hoạt tính urease và sản xuất canxit cao. Trong số các chủng vi khuẩn phân lập được, chủng BRVT1 tạo kết tủa canxit là 11,8 g/L; chủng BRVT2, BRVT5 là 11,4 g/L và chủng BRVT4 là 12,8 g/L. Giải trình tự gen 16S rRNA đã xác định được chủng BRVT1 là *Bacillus tequilensis*, chủng BRVT2 và BRVT5 là *Bacillus cereus* và chủng BRVT4 là *Bacillus subtilis*. Ngoài ra, vi cấu trúc của canxit kết tủa được kiểm tra thông qua kính hiển vi điện tử quét (SEM), nhiễu xạ tia X (XRD).

Từ khóa: 16S rRNA, Bacillus, Ba Ria Vung Tau, SEM-EDX.