Bioprospecting Ureolytic Rock Bacteria for Calcium Carbonate Precipitation Inducer

Idris, Idris
Research Center for Applied Microbiology, National Research and Innovation Agency

Rustandi, Billyanto
Department of Biology, Faculty of Technobiology, Atma Jaya University

Tri Ratna Sulistiyani
Research Center for Biosystematics and Evolution, National Research and Innovation Agency

Rahmat, Ali
Research Center for Limnology, National Research and Innovation Agency

他

https://doi.org/10.5109/6622882

出版情報：Evergreen. 9 (4), pp.962-972, 2022-12. 九州大学グリーンテクノロジー研究教育センター
バージョン：

Bioprospecting Ureolytic Rock Bacteria for Calcium Carbonate Precipitation Inducer

Idris Idris[1*], Billyanto Rustandi[2], Tri Ratna Sulistiyani[3], Ali Rahmat[4], I Made Sudiana[1]

[1]Research Center for Applied Microbiology, National Research and Innovation Agency, Indonesia
[2]Department of Biology, Faculty of Technobiology, Atma Jaya University Yogyakarta, Indonesia
[3]Research Center for Biosystematics and Evolution, National Research and Innovation Agency, Indonesia
[4]Research Center for Limnology, National Research and Innovation Agency, Bogor, Indonesia

*Author to whom correspondence should be addressed
E-mail: idri002@brin.go.id

(Received September 26, 2022; Revised December 5, 2022; accepted December 7, 2022).

Abstract: The application of calcium carbonate precipitation-inducing bacteria in the past two decades has become an alternative in green technology development, particularly in construction as self healing agent of concrete and in the waste treatment as contaminants remover (e.g., radioactive pollutants and heavy metals). This study aimed to obtain potential bacterial isolates from rock samples that can induce calcium carbonate precipitation and characterize the precipitate produced. This study began with the isolation of bacteria from rock samples taken from an arid area (Malaka, East Nusa Tenggara) using Nutrient Broth-urea-$CaCl_2$ media. Colonies showing the formation of calcium carbonate precipitation were then purified and selected for the ureolytic activity assay using Christensen's urea agar. Bacterial isolates with high ureolytic activity were selected for further characterization of their ability to produce calcium carbonate precipitation. Five bacterial isolates with the best precipitation ability were obtained. Each isolate had a different ability to induce calcium carbonate precipitation, and the resulting crystal morphology was also different. Isolate M 2.6 was the best bacterial isolate capable of inducing the highest calcium carbonate precipitation, which was 2.6 g/L. This isolate was later identified as *Mesobacillus campisalis*. The calcium carbonate precipitate produced by the five selected isolates ranged from 1.4 g/L to 2.6 g/L. The Field Emission Scanning Electron Microscopy (FESEM)-Energy Dispersive Spectroscopy (EDS) characterization revealed that the precipitate resulting from the bacterial isolates was calcium carbonate. This was indicated by the mass percent value, which was dominated by three main elements, namely O, Ca, and C, with a mass ratio of approximately matching $CaCO_3$.

Keywords: bio-mineralization, crystal morphology, limestone, mineral precipitation, self-healing concrete

1. Introduction

During the past two decades, the bacterial-induced calcium carbonate precipitation has become a promising technology for various applications due its environmentally friendly[1)]. Calcium carbonate precipitation-inducing bacteria can be used in the development of self-healing concrete technology as a self-healing agent[2,3)]. Bacteria-induced calcium carbonate precipitation mixed into the concrete can improve the physical and mechanical properties of the concrete structure and automatically repair it when cracks occur[4,5,6)]. Nowadays, there is a necessity to develop construction technology that produce less carbon dioxide emission as well as improve building durability because concrete have been known as one of major contributors in carbon dioxide emission to the atmosphere[7,8)]. Therefore, by lowering the demand for cement consumption, this approach might be an option for developing construction technology that creates less carbon dioxide. Furthermore, this group of bacteria was not only used in concrete self-healing technology but also used in the process of removing contaminants such as radioactive pollutants[9)], heavy metals from liquid waste and groundwater[10,11,12)]. In addition, these bacteria also can be used in disasters, namely to mitigate soil erosion by increasing the compactness of the soil structure[13)]. The utilization of biological technologies have beneficial in low environmental impact as well as low cost, hence support the green technology application and sustainable

development[14,15].

Precipitation of calcium carbonate by microorganisms is a natural phenomenon that occurs in nature[16]. This process was known as biomineralization. Biomineralization is the process of mineral precipitation due to changes in environmental chemistry caused by the activity of microorganisms. Mineral precipitation by microorganisms can take place both intracellular and extracellular[17]. There are more than 60 types of minerals that can be formed through the biomineralization process[18].

Microbially induced calcium carbonate precipitation naturally occurs through a number of cellular biochemical activities[19]. Some of its formation mechanisms include photosynthesis reactions, urea hydrolysis, sulfate reduction, anaerobic sulfide oxidation, and the formation of biofilms and extracellular polymer compounds[1,17]. Among these mechanisms, urea hydrolysis was the most commonly used method in the study of calcium carbonate precipitation by microorganisms, especially bacteria. Bacteria posed faster metabolic activity prompting changes in pH to become more alkaline than other metabolic activities. In addition, the characteristic of the calcium carbonate produced by bacteria was more homogeneous than other microorganisms[20].

Calcium carbonate precipitation through urea hydrolysis takes place in the presence of urease activity. Hydrolysis of urea by urease will produce carbamates and ammonia. The carbamates then spontaneously hydrolyze to form bicarbonate and ammonia. After that, bicarbonate and ammonia will establish an equilibrium in water to form carbonate, ammonium, and hydroxide ions. The accumulation of ammonium ions will increase the pH of the environment and will push the reaction equilibrium towards the formation of carbonate ions. Precipitation will occur when carbonate ions bind to calcium ions attached to the surface of bacterial cells. Bacterial cells play a role in the initial precipitation process by providing a starting place for the development of crystal nuclei (nucleation). Negative charges on bacterial cell surfaces will attract positive charges of calcium ions[17,18,19].

The application of bacterial-induced calcium carbonate precipitation still encounter a number of limitation, particularly in harsh environmental conditions. Consequently, choosing appropriate bacterial strains is one of the crucial steps in the actual application of this approach. Due to their great ureolytic activity, *Sporosarcina pasteurii* strains have been the subject of the majority of research on ureolytic-based bacterial-induced calcium carbonate precipitation.[21] Therefore, the research to find other potential strain of bacteria-induced carbonate precipitation is important to to offer bioresources that can meet the application requirement.

This study aimed to obtain potential bacteria that induce calcium carbonate precipitation through the mechanism of urea hydrolysis. Bacteria were isolated from rocks collected from an arid area in the Malaka District, Atambua Regency, East Nusa Tenggara, thereby potential to obtain a unique character of bacterial strains. We found five potential bacteria and three of them have been not reported anywhere. This study might be a good starting of studying microbially-induced calcium carbonate precipitation in a tropical region country, particularly Indonesia.

2. Materials and Methods

2.1 Rock sampling

Limestone rock samples were taken around the hilly area in the Malaka District, Atambua Regency, East Nusa Tenggara. The sampling coordinates were 90°28'18.2" SL and 124°55'54.3" EL. Stone sampling was carried out aseptically by scraping the surface of the stone using a sterile knife, and the stone chips obtained were inserted into a sterile tube containing 15 mL of a 0.85% (w/v) NaCl solution. Rocks that were difficult to scrape were broken using a sterile hammer. All samples obtained were numbered to facilitate data collection.

2.2 Screening of bacterial-induced calcium carbonate precipitation

Stone fragments that had been stored in a sterile 0. 85% (w/v) NaCl solution were brought to the laboratory and incubated at room temperature (28-30 ℃) with shaking at 104 rpm for 5 days. A total of 50 µL of the culture media from the incubated rock samples were inoculated onto the selection media Nutrient Broth (NB)-urea-$CaCl_2$ using the spreading technique[22]. The composition of the NB-urea-$CaCl_2$ media in one liter was 8 g Nutrien Broth (Himedia, India), 5 g NaCl, 20 g urea, 0.025 M $CaCl_2$, and 15 g agar. The cultures were incubated at 30 ℃ for 7 days. The observation of carbonate precipitation formation by bacterial isolates was carried out every 24 hours using a stereo microscope (Olympus SZ61, Japan). Bacterial isolates that showed carbonate deposition were distinguished based on the morphology of the carbonate crystals formed. Each isolate was assigned a numbering code to facilitate further identification. Each isolate was grown on slanted nutrient agar (NA) media for further characterization

2.3 Urease activity assay of bacterial-induced calcium carbonate precipitation

The urease activity of bacterial-induced calcium carbonate precipitation was confirmed using Christensen's urea agar medium[23]. Each bacterial isolate obtained from the previous stage was inoculated on Christensen's urease solid media and incubated for 48 hours at 30 ℃. Positive urease activity was indicated by a change in the color of the medium from light orange to pink/purple. Negative urease activity was indicated by the color of the medium remaining orange or turning yellow. The bacterial isolates with the highest ureolytic activity were subjected to further analysis.

2.4 Molecular identification of potential bacterial isolates

The molecular identification of bacteria was began with bacterial DNA genome extraction step. The extraction of the bacterial DNA genome was carried out following the method of Packeiser et al.[24]. Cells from single colony on the surface of solid media were removed using a sterile toothpick and suspended in 20 µL of nuclease-free water. The suspension was then vortexed for ten seconds and incubated at 98°C for five minutes. The supernatant was separated from the cell debris by centrifugation, and was used as a DNA template.

The 16S rRNA gene of bacteria was amplified using a universal primer pair, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')[25]. Each tube contains ultrapure water 9.75 µL, GoTaq Green MasterMix (Promega) 2x12.5 µL, primer 27F (10 µM) 0.625 µL, primer 1492R (10 µM) 0.625 µL, DMSO 0.5 µL, and 1 µL template DNA sample with a total volume of 25 µL. Amplification was carried out under Polymerase Chain Reaction (PCR) conditions: predenaturation at 95 ℃ for 90 seconds, followed by 30 cycles consisting of denaturation (95 ℃, 30 seconds), annealing (50 ℃, 30 seconds), elongation (72 ℃, 90 seconds), and final extension at 72 ℃ for five minutes, followed by 4 ℃ for 20 minutes. The amplification products were then seperated through electrophoresis on an agarose gel with a concentration of 1% (w/v). After completion, the gel was immersed in a 5.0 µg/mL ethidium bromide solution for 30 minutes and visualized using a UV transilluminator.

The PCR amplicons were sequenced at Macrogen Korea Laboratory using an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). Sequencing data were processed using the Bioedit program[26]. The 16S rRNA sequence homology was searched on the Eztaxon server. Reference sequences were obtained from the GenBank/DDBJ/EMBL database, which was accessed online via www.ezbiocloud.net[27]. The phylogenetic tree was constructed using the neighbor-joining method[28], which was carried out in MEGA 7.0 software[29] with a bootstrap value of 1000x.

2.5 Observation of calcium carbonate precipitation formation process by potential bacterial isolates

Five potential bacterial isolates obtained from the previous stage were grown on NB-urea-$CaCl_2$ agar media using a sterile loop. The cultures were incubated at 30 C for 5 days. The observation of calcium carbonate precipitate formation was carried out on single colony of each bacteria isolates every 24 hours under a stereo microscope (Olympus SZ61, Japan).

2.6 Production of calcium carbonate precipitation by potential bacterial isolates

Five selected calcium carbonate-precipitating bacteria isolates with high urease activity were further analyzed for their ability to produce calcium carbonate precipitates in liquid medium. The medium used was the same as the initial screening medium without agar addition. Selected bacterial isolates were first subcultured on Nutrient Broth media for 16 hours. A total of 1% (v/v) culture was inoculated into 50 mL of NB-urea-$CaCl_2$ production medium. The cultures were incubated at room temperature for 7 days with shaking at 104 rpm. The calcium carbonate precipitate was separated from the medium using Whatman No.1 filter paper with the help of a vacuum pump. The precipitate obtained was rinsed using distilled water three times. The precipitate was dried in an oven for 24 hours at 60 °C. The amount of calcium carbonate precipitate was weight and expressed in mg/L media.

2.7 Precipitation characterization by FE-SEM and EDS

The calcium carbonate precipitate produced by each bacterial isolate was further characterized to determine its morphology and elemental content. The precipitate morphology was visualized using a Field Emission Scanning Electron Microscope (FE-SEM) (Thermo Scientific Quattro S). Qualitative and quantitative analysis of elemental content on the sample surface was also carried out with this tool because it was equipped with an energy-dispersive X-ray spectroscopy (EDS) detector. This characterization was carried out at Advanced Characterization Laboratories, Cibinong Integrated Laboratory of Bioproducts, National Research and Innovation Agency, Indonesia.

3. Results

3.1 Screening of ureolytic bacterial-induced calcium carbonate precipitation

About 33 isolates of bacteria were obtained at the screening stage using NB-urea-$CaCl_2$ media, which can induce calcium carbonate precipitation. These bacterial isolates came from six different limestone rock samples. The number of bacterial isolates obtained from each rock sample was five to seven. Each isolate was visually distinguished based on the morphological characteristics of the formed calcium carbonate precipitation. The observation results using a stereo microscope showed that there were morphological variations of calcium carbonate precipitation found around the colonies. Generally, calcium carbonate precipitate appeared from the center of the bacterial colony. Its shape like a crystal granule. Some of the morphological forms of calcium carbonate crystals can be seen in Fig. 1.

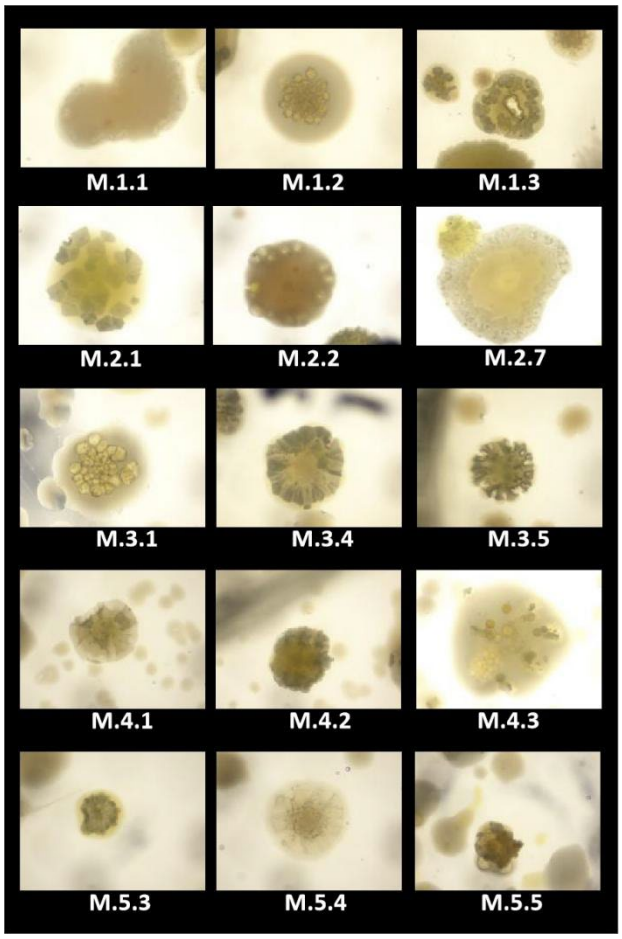

Fig. 1: Crystal morphology diversity of calcium carbonate precipitation induced by different types of bacteria isolated from stone using NB-urea-$CaCl_2$ solid media on the fifth day of incubation.

Fig. 2: Change in color of medium in Christensen's urea agar indicates urease activity. Positive = pink-purple; negative = yellow-orange.

All isolates obtained from the screening stage were then tested for their ureolytic activity qualitatively using Christensen's urea agar media. The assay result showed that only 16 isolates from 33 isolates were positive for ureolytic activity, which was indicated by a change in the color of the medium from orange to pink/ purple (Fig. 2 and Table 1).

Table 1. Urease activity assay of bacterial isolates induced-calcium carbonate precipitation on Christensen's urea solid media.

Sample code	Isolate number	Urease activity
M.1	1	-
	2	-
	3	+
	4	+++
	5	+
M.2	1	-
	2	+
	3	+
	4	+
	5	+
	6	+++
	7	+
M.3	1	+
	2	-
	3	+
	4	+
	5	-
M.4	1	-
	2	-
	3	-
	4	++
	5	++
M.5	1	-
	2	++
	3	-
	4	-
	5	-
	6	-

Note:" - "= urease negative, "+" = urease postive (+ = low, ++ = medium, and +++ = high)

3.2 Molecular identification of potential ureolytic bacteria

Five selected isolates with the best ureolytic activity were further identified to determine their identity. The homology analysis of the 16S rRNA gene sequence using the Eztaxon server can be seen in Table 2. All isolates had a similarity level of above 98% with the strains already in the database. The five isolates were identified as *Bacillus onubensis* (isolate M.1.4), *Mesobacillus campisalis* (isolate M.2.6), *Agrobacterium pusense* (isolate M.4.4), *Staphylococcus wareri* (isolate M.4.5), and *Microbacterium paraoxydans* (isolate M.5.2).

Table 2. The homology analysis of the 16S rRNA gene sequences of bacterial isolates using the EZtaxon server

Bacterial isolate	Top hit taxon	Top hit strain	Similarity
M.1.4	*Bacillus onubensis*	0911MAR22V3	99.93
M.2.6	*Mesobacillus campisalis*	SA2-6	98.93
M.4.4	*Agrobacterium pusense*	LMG 25623	99.70
M.4.5	*Staphylococcus warneri*	ATCC 27836	99.86
M.5.2	*Microbacterium paraoxydans*	NBRC 103076	100.00

3.3 Observation of calcium carbonate precipitate formation process by potential bacterial isolates

Figure 3 exhibited the development of calcium carbonate precipitation of five selected bacterial isolates. It showed that each isolate produced a different morphology of calcium carbonate crystals, particularly in shape and size. Each of isolates had a different period when the calcium carbonate precipitate first emerged. The fastest initiating calcium carbonate precipitation was found on isolates M.2.6 and 2.4.4, which required 48 hours/2 days of incubation. The bacterial isolate that takes the longest time to form calcium carbonate precipitation was M.2.4.5, which takes 96 hours (4 days) incubation.

Precipitation of calcium carbonate by isolate M.1.4 (*Bacillus onubensis*) started with amorphous formation until the second day, began to change into crystal form on the third day, and continued to grow. Whereas in isolate M.2.6 (*Mesobacillus campisalis*), calcium carbonate precipitation was homogeneous and there was a crystal nucleus in the center of the colony which looked darker. As for the appearance of calcium carbonate precipitation on isolate M.4.4 (*Agrobacterium pusense*), calcium carbonate crystals were clearly visible with fairly large size on day 2, different from the other four isolates. However, no significant changes were seen in the days that followed.

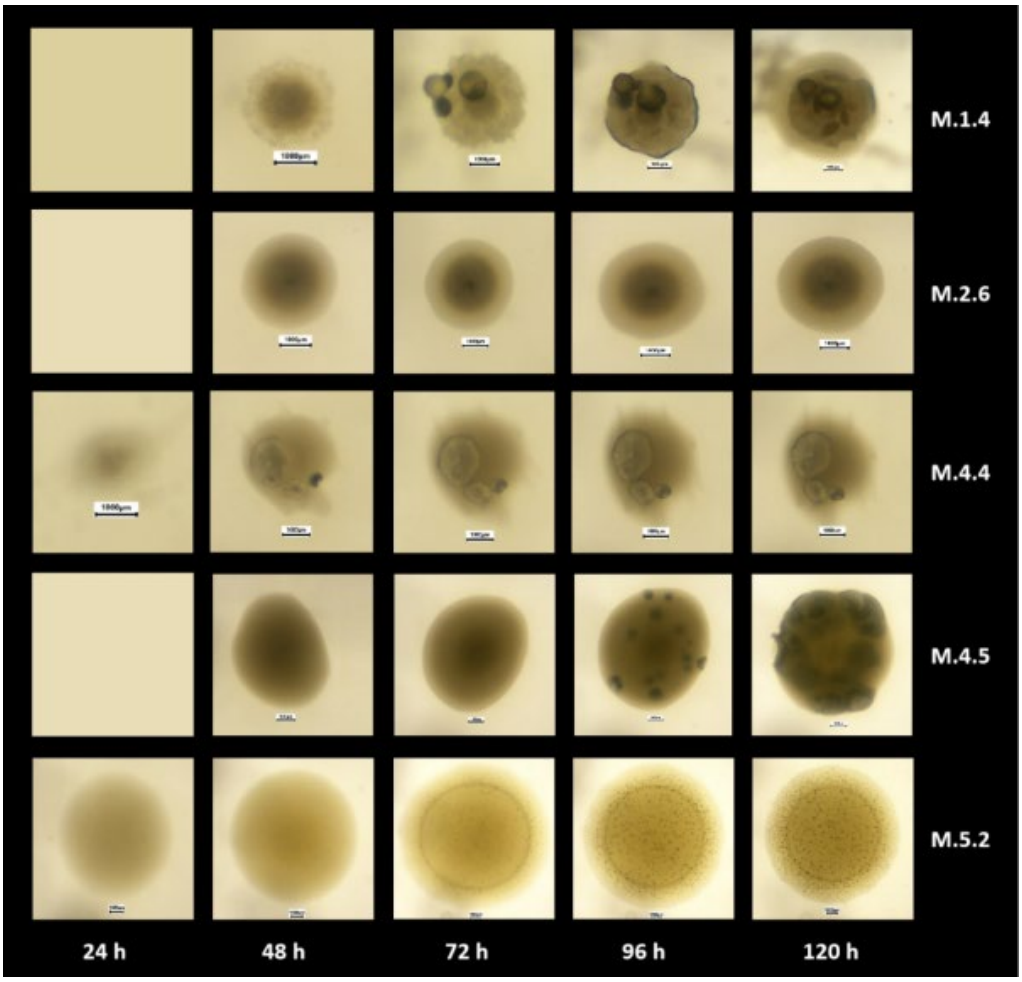

Fig. 3: The development of calcium carbonate precipitation formation by five bacterial isolates with the highest urease activity. Bar size = 1000 μm

The appearance of calcium carbonate precipitation in the form of crystals on isolate M.4.5 (*Staphylococcus wareri*) was only clearly visible on the fourth day and continued to increase in size on the fifth day. On the previous day, it was only seen that there was a slightly darker part in the center of the colony. It was different

again with isolate M.5.2 (*Microbacterium paraoxydans*), which had a homogeneous crystal form, small in size, and spread throughout the colony. Interestingly, the colony also contained calcium carbonate precipitation, which was arranged in a circular, ring-like shape on the inside of the colony.

3.4 Production of calcium carbonate precipitation by potential bacterial isolates

Each isolate had a different ability to induce calcium carbonate precipitation. The calcium carbonate precipitate produced by the five selected isolates ranged from 1.4 g/L to 2.6 g/L. The highest calcium carbonate precipitation was produced by *Mesobacillus campisalis* strain M.2.6, at 2.6 g/L, followed by *Bacillus onubensis* strain M.1.4 at 2.53 g/L (Figure 4a). *Microbacterium paraoxydans* strain M.5.2 had the lowest ability to precipitate calcium carbonate compared to the other four strains, which was only 1.4 g/L.

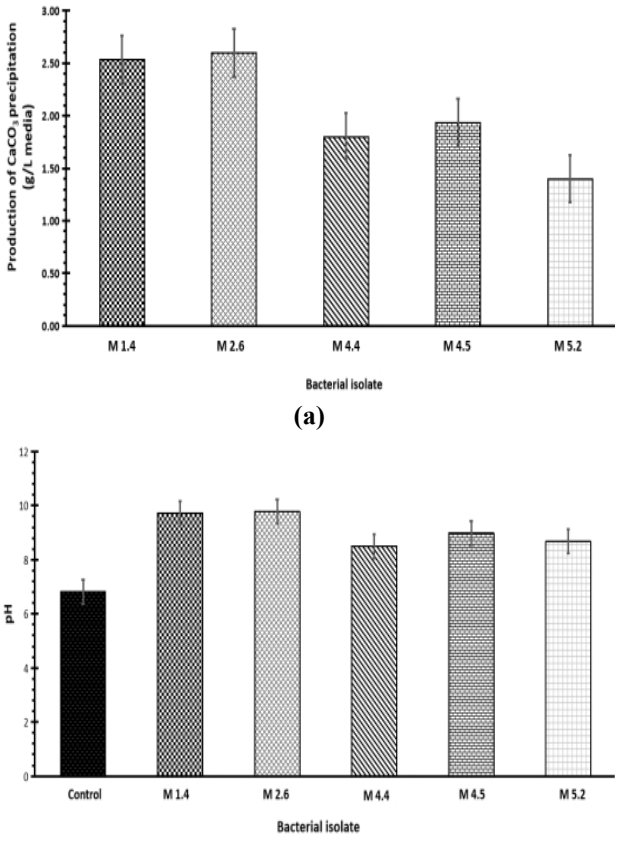

Fig. 4: (a) Production of calcium carbonate precipitate from the five potential isolates after seven days of incubation in NB-urea-CaCl2 liquid media, (b) the final pH of the medium after seven days of incubation.

This study also observed the pH of the media at the end of the incubation period (Figure 4b). All of the pH media of the five potential isolates turn alkaline. The highest pH media was founded on bacterial isolate M. 2.6, reaching a pH of 9.8. There was a positive correlation between the level of urease activity and the level of calcium carbonate precipitation produced. The higher the ureolytic activity of bacteria, the higher the calcium carbonate precipitation produced.

3.5 Precipitate characterization by Field Emission Scanning Electron Microscopy (FE-SEM) - Energy Dispersive Spectroscopy (EDS)

The FE-SEM analysis showed that the morphology of the calcium carbonate produced by each isolate was different in shape and size (Fig. 5) . Calcium carbonate crystals produced by isolates M.1.4, M.2.4, and M.4.4 had a morphology similar to a rhombohedral structure with a flat surface, while the crystal morphology produced by isolates M.4.5 and M.5.2 was more likely to have an irregular shape with spherical and uneven surfaces.

Further characterization of the components contained in the precipitate produced by each isolate can be determined using EDS. The study revealed that three main elements—O, Ca, and C—dominated the mass percent value. All samples obtained from each bacterial isolate showed the same pattern, only varying in the percentage of those three elements.

4. Discussion

Precipitation of calcium carbonate induced by bacteria occurred as a result of cellular biochemical activities. Several cellular biochemical activities have been reported to be responsible for the induced precipitation of calcium carbonate. This study focuses on the precipitation of calcium carbonate as the urea hydrolysis activity. Therefore, urea was used in screening media to select the bacteria that were able to induce calcium carbonate precipitation via urea hydrolysis. The hydrolysis of urea by bacteria promoted the formation of carbonate ions due to the increase in the environment's alkalinity.

The initial process of calcium carbonate precipitation formation or nucleation starts at the surface of the bacterial cell. Calcium ions are attached to the surface of the outer membrane of bacterial cells, which are generally negatively charged[19,22]. Consequently, calcium carbonate precipitation will be concentrated in the colony when carbonate ions are bound to calcium ions on the surface of the outer membrane of bacteria. Nevertheless, there were also bacterial isolates that induced precipitation scattered outside the colony. A similar phenomenon has also been reported by Reeksting et al., (2020)[20] found in the bacterium *Sporosarcina pasteurii* DSM33. This phenomenon was assumed to correlate with the localized pH change in the medium. Higher urease activity will prompt a rapid upshift in pH medium and promote calcium carbonate precipitation out of bacterial colonies.

It is clear from the ureolytic assay results that not all isolates that caused calcium carbonate precipitation possessed ureolytic activity. Only half of all isolates obtained could hydrolyze urea, as indicated by the change

in media color. The changing in the color of the media from orange to pink/purple occurred as a consequence of the accumulation of ammonium produced from the hydrolysis of urea by bacteria[20].

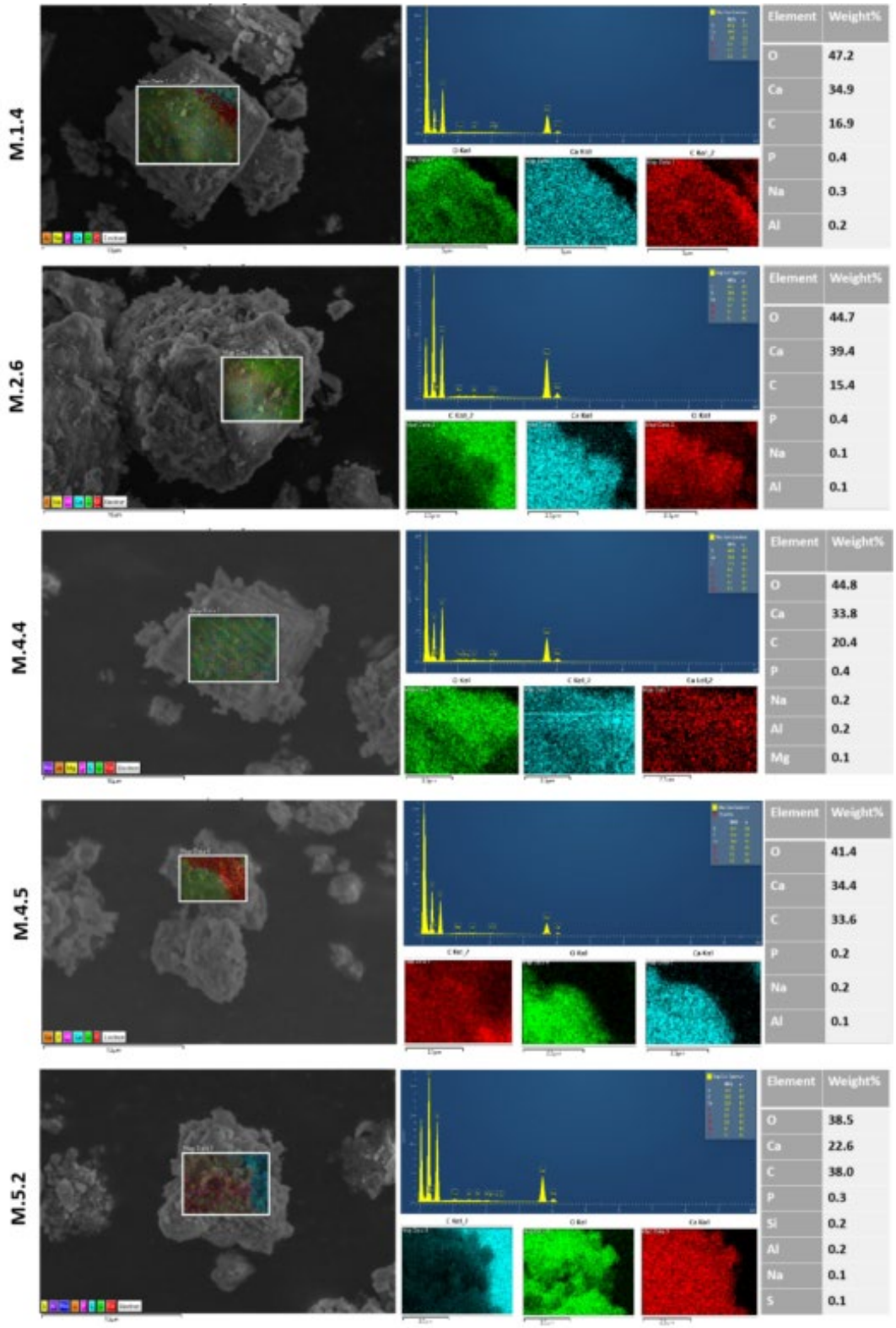

Fig. 5: FE-SEM and EDS analysis of calcium carbonate precipitation produced by each bacterial strain. Left, morphologycal appearance of carbonate precipitation and the area elemental profiling by EDS were indicated by the white box. Center, coresponding EDS spectra showed the peaks of elemental profiling contained in carbonate precipitation. Right, the elemental analysis table showed the amount of each element (weight%).

This finding revealed that urea metabolism is not the only process involved in the calcium carbonate

precipitation by bacteria. Induction of calcium carbonate precipitation by microorganisms can also involve non-ureolytic pathway, for example photosynthesis, as found in the Cyanobacteria group[30].The formation of $CaCO_3$ precipitation through the photosynthesis pathway begins with the capture of CO_2 by cells and will concentrate intracellularly. Then the CO_2-sequestering process will cause the environment in the bacterial cells to become alkaline, thus encouraging the formation of bicarbonate. The presence of calcium around the cells will encourage the formation of $CaCO_3$ precipitation around the cells. $CaCO_3$ precipitation can also be formed from the denitrification process by denitrifying bacteria. Denitrifying bacteria use NO_3^- as an electron acceptor in the oxidation of organic acids to produce CO_2, and then promote the formation of bicarbonate of acid under alkaline conditions. $CaCO_3$ precipitation will form when Ca^{2+} ions are present in the environment[31]. In addition, the sulfate reduction, and extracellular polysaccharide polymer compounds produced by biofilm bacteria can also induce the formation of calcium carbonate precipitation[32,33].

In order to determine the length of time required by each bacterial strain to produce precipitation, the calcium carbonate crystals development was periodically observed for seven days. The observation results showed that the time of precipitation formation varied among bacterial isolates, ranging from 2 to 4 days. Similar observations had been reported by Hammes et al., (2003)[34] on a number of bacterial isolates isolated from various sources, namely soil, pieces of concrete, and mud.The microscopic observation result showed that the precipitation of calcium carbonate formed ranged from 20 hours to 5 days, depending on the bacteria strain. The precipitate formation started with the emergence of amorphous calcium carbonate, indicated by the appearance of a dark area in the center of the colony. Afterward, it was followed by crystal formation and maturation of calcium carbonate crystals[34].

The measurement result of precipitate production by five potential isolates showed various weights. The highest precipitate production was given by *Mesobacillus campisalis* strain M.2.6. *Mesobacillus campisalis* strain M.2.6 had a higher calcium carbonate precipitation inducing capacity than the *Bacillus megaterium* strain that Dhami et al., (2013)[35] reported, which is 1.87 g/L. These five isolates were still unable to exceed *Sporosarcina pasteurii* (BNCC 337394), which can precipitate 3 g/L calcium carbonate in under 12 hours[36]. However, the composition of the media used was different. Calcium carbonate production was determined not only by the high activity of bacterial urease but also by substrate concentration (urea), $CaCl_2$ concentration, incubation temperature, initial pH of the media, and aeration [18,37]. Beside that, precipitation strategy by separating growth step and inducing precipitation step could increase the production of calcium carbonate precipitation.[38] Therefore, it was necessary to optimize the production of calcium carbonate precipitation for further study.

Based on our knowledge, there have been no reports on the ability of *Bacillus onubensis*, *Mesobacillus campisalis*, and *Agrobacterium pusense* strains to induce calcium carbonate precipitation. Only two strains have been reported, including *Staphylococcus wareri*[39] and *Microbacterium paraoxydans*[40] regarding their ability to induce calcium carbonate. The genus Bacillus has been studied the most in relation to bacteria-induced calcium carbonate precipitation. Because of its high urease activity, ability to survive in harsh environments with the formation of endospores, and capacity to produce extracellular polysaccharide compounds that aid precipitation, the Bacillus genus was well known for its exceptional capacity to induce calcium carbonate precipitation[41]. Nevertheless, several non-ureolytic Bacillus strains were reported to induce calcium carbonate precipitation[42]. However, studies related to non-ureolytic bacteria induced carbonate precipitation are still limited.

Bacillus pasteurii (now known as *Sporosarcina pasturii*) is the most widely used bacterium in the study of calcium carbonate precipitation-inducing bacteria, especially in concrete strength-enhancing applications[41]. Some of the advantages of *S. pasteurii* besides having high urease activity, are more negatively charged cell surfaces, strong cell structures, and low mobility[36]. Numerous studies have been carried out using these bacteria, for example, increasing the compactness of cement and soil[43,44], self-healing concrete[45], and heavy metal bioremediation[46].

Further characterization of the calcium carbonate precipitate obtained from the five potential bacteria strains using FE-SEM showed the surface morphology of the precipitate. There were two types of calcium carbonate precipitate morphology: rhombohedral and spherical. Rhombohedral crystals are characteristic of calcite, which has a more stable crystal form than the spherical form[47]. According to Reeksting et al., (2020)[20], the variation in crystal morphology that each strain of ureolytic bacteria produced was impacted by the ureolytic activity, which had an effect on the precipitation rate. When precipitation occurs rapidly, the crystals formed will be homogeneous, resulting in a rhombohedral crystal. Additionally, it was stated that various mechanisms, such as ureolytic and non-ureolytic bacteria, contributed to variations in crystal shape. In non-ureolytic bacteria, the morphology of the calcium carbonate crystals produced is spherical with a large size due to the slow precipitation process[20].

The EDS analysis offers details on the components present in the precipitate as well as mass percent data for each element. The results of the EDS examination demonstrated that the precipitates generated were, in fact, CaCO3. The mass ratio of approximately equivalent CaCO3 provided evidence of this.

Further study was needed to explore the potential application of these bacteria to develop green technology

deal with various environmental problem whether as a self-healing agent in concrete to develop construction technology for durability building[48,49], heavy metals contaminant remover in wastewater treatment[50,51], or another possible application.

5. Conclusion

Ureolytic bacteria isolated from rock samples were able to induce calcium carbonate precipitation. Five potential bacterial isolates with the best precipitation ability were obtained. The best bacterial isolate induced the highest calcium carbonate precipitation was M.2.6, which was 2.6 g/L. This bacterium was identified as *Mesobacillus campisalis* with a similarity level of 98.93%. The calcium carbonate precipitation produced by the five selected isolates ranged from 1.4 g/L to 2.6 g/L. There was a positive correlation between the level of urease activity and the level of calcium carbonate precipitation produced. Each isolate had a unique ability to induce calcium carbonate precipitation, which resulted in distinct crystal morphology. Bacterial strains M.14, M.2.4, and M.4.4 produced a homogenous rhombohedral-like structure, whereas bacterial strains M.4.5 and M.5.2 produced an inhomogeneous sphere-like structure. The results of further characterization with FE-SEM and EDS showed that the precipitate was CaCO3, which was confirmed by the mass ratio that was close to calcium carbonate and dominated by C, O, and Ca elements.

Acknowledgements

The authors would like to thank the National Research and Innovation Agency for funding support and research facilities. The authors acknowledge the facilities, scientific and technical support from Advanced Characterization Laboratories, Cibinong Integrated Laboratory of Bioproducts, National Research and Innovation Agency through E Layanan Sains.

References

1) T. Zhu, and M. Dittrich, "Carbonate precipitation through microbial activities in natural environment, and their potential in biotechnology: A Review," *Frontiers in Bioengineering and Biotechnology*, **4** (2016). doi: 10.3389/fbioe.2016.00004M.
2) W. Zhang, Q. Zheng, A. Ashour, B. Han, "Self-healing cement concrete composites for resilient infrastructures: A review", *Composites Part B: Engineering*, **189** (), 107892– (2020). doi:10.1016/j.compositesb.2020.107892.
3) C. Qian, T. Zheng, X. Zhang, and Y. Su, "Application of microbial self-healing concrete: Case study," *Construction and Building Materials*, **290**, 123226 (2021). doi:10.1016/j.conbuildmat.2021.123226.
4) H. Chen, C. Qian, and, H. Huang, "Self-healing cementitious materials based on bacteria and nutrients immobilized respectively," *Construction and Building Materials*, **126** 297–303 (2016). doi: 10.1016/j.conbuildmat.2016.09.023.
5) D.A. Golovkina, E.V. Zhurishkina, L.A. Ivanova, A.E. Baranchikov, A.Y. Sokolov, K.S. Bobrov, A.E. Masharsky, N.V. Tsvigun, G.P. Kopitsa, and A.A. Kulminskaya, "Calcifying bacteria flexibility in induction of CaCO3 mineralizations," *Life*, **10** (12) 317 (2020). doi:10.3390/life10120317.
6) J. Feng, B. Chen, W. Sun, and Y. Wang, "Microbial induced calcium carbonate precipitation study using *Bacillus subtilis* with application to self-healing concrete preparation and characterization," *Construction and Building Materials*, **280** 122460. (2021). doi:10.1016/j.conbuildmat.2021.122460.
7) K.T. Zingreu, X. Yang, and M.P. Wan, "Performance analysis of cool roof, green roof and thermal insulation on a concrete flat roof in tropical climate," *Evergreen,* **2** (2) 34-43 (2015). doi:10.5109/1544078.
8) M.I. Sabtu, H. Hishamuddin, S. Nizaroyani, and M.N.A. Rahman, "A review of environmental assessment and carbon management for integrated supply chain models," *Evergreen* **8** (3) 628-641 (2021). doi:10.5109/4491655.
9) A.C. Mitchell, and F.G. Ferris, "The coprecipitation of Sr into calcite precipitates induced by bacterial ureolysis in artificial groundwater: Temperature and kinetic dependence," *Geochimica et Cosmochimica Acta*, **69** (17) 4199–4210 (2005). doi:10.1016/j.gca.2005.03.014.
10) M. Li, X. Cheng, and H. Guo, "Heavy metal removal by biomineralization of urease producing bacteria isolated from soil," *International Biodeterioration & Biodegradation*, **76** 81–85 (2013). doi:10.1016/j.ibiod.2012.06.016.
11) C.H. Kang, J.H. Choi, J. Noh, D.Y. Kwak, S.H. Han, and J.S. So, "Microbially induced calcite precipitation-based sequestration of strontium by *Sporosarcina pasteurii* WJ-2," *Applied Biochemistry and Biotechnology*, **174** (7) 2482–2491 (2014). doi:10.1007/s12010-014-1196-4.
12) Y. Kim, S. Kwon, and Y. Roh, "Effect of divalent cations (Cu, Zn, Pb, Cd, and Sr) on microbially induced calcium carbonate precipitation and mineralogical properties," *Frontiers in Microbiology*, **12** (2021). doi:10.3389/fmicb.2021.646748.
13) H. Raveh-Amit, and M. Tsesarsky, "Biostimulation in Desert Soils for Microbial-Induced Calcite Precipitation," *Applied Sciences*, **10**(8), 2905 (2020). doi:10.3390/app10082905.
14) M. Tanaka, "A comparison study of heap bioleaching sites in chile and Finland for further development of biotechnology for mining," *Evergreen,* **4**(2) 1–7 (2017). doi:10.5109/1929724.
15) A. Rahman, N.B. Prihantini, and Nasruddin, "Biomass production and synthesis of biodiesel from microalgae *Synechococcus* hs-9 (cyanobacteria)

cultivated using bubble column photobioreactors," *Evergreen*, **7** (4) 564–570 (2020). doi:10.5109/4150507.

16) M. Seifan, and A. Berenjian, "Microbially induced calcium carbonate precipitation: a widespread phenomenon in the biological world. Applied Microbiology and Biotechnology," **103** (12) 4693–4708 (2019). doi:10.1007/s00253-019-09861-5.
17) M J. Castro-Alonso, L.E. Montañez-Hernandez, M.A. Sanchez-Muñoz, M.R. Macias Franco, R. Narayanasamy, and N. Balagurusamy, "Microbially induced calcium carbonate precipitation (MICP) and its potential in bioconcrete: Microbiological and molecular concepts," *Frontiers in Materials*, **6** (2019). doi:10.3389/fmats.2019.00126.
18) P. Anbu, C.H. Kang, Y.J. Shin, and J.S. So, "Formations of calcium carbonate minerals by bacteria and its multiple applications," *SpringerPlus*, **5** (1) (2016). doi:10.1186/s40064-016-1869-2.
19) T.D. Hoffmann, B.J. Reeksting, and S. Gebhard, "Bacteria-induced mineral precipitation: a mechanistic review," *Microbiology*, **167** (4) (2021). doi:10.1099/mic.0.001049
20) B.J. Reeksting, T.D. Hoffmann, L. Tan, K. Paine, and S. Gebhard, "In-depth profiling of calcite precipitation by environmental bacteria reveals fundamental mechanistic differences with relevance to application," *Applied and Environmental Microbiology*, **86** (7) (2020). doi:10.1128/aem.02739-19.
21) Y.S. Lee, and W. Park, "Current challenges and future directions for bacterial self-healing concrete," *Applied Microbiology and Biotechnology*, **102** (7) 3059–3070 (2018). doi:10.1007/s00253-018-8830-y.
22) T. Ghosh, S. Bhaduri, C. Montemagno, and A. Kumar, "*Sporosarcina pasteurii* can form nanoscale calcium carbonate crystals on cell surface," *PLOS ONE*, **14** (1) e0210339 (2019). doi:10.1371/journal.pone.0210339.
23) W.B. Christensen, "Urea decomposition as a means of differentiating proteus and paracolon cultures from each other and from Salmonella and Shigella types," *Journal of Bacteriology*, **52** (4) 461–466 (1946). doi:10.1128/jb.52.4.461-466.1946.
24) H. Packeiser, C. Lim, B. Balagurunathan, J. Wu, and H. Zhao, "An extremely simple and effective colony pcr procedure for bacteria, yeasts, and microalgae," *Applied Biochemistry and Biotechnology*, **169** (2) 695–700 (2012). doi:10.1007/s12010-012-0043-8.
25) P. Palaniappan, P.S. Chauhan, V.S. Saravanan, R. Anandham, and T. Sa, "Isolation and characterization of plant growth promoting endophytic bacterial isolates from root nodule of *Lespedeza* sp.," *Biology and Fertility of Soils*, **46**(8) 807–816 (2010). doi:10.1007/s00374-010-0485-5.
26) T.A. Hall, "BioEdit: a user-friendly biological sequence alignment editor and analysis program fro Windows 95/98/NT," *Nucleic Acids Symp Series*, **41** 95–98 (1999).
27) O.S. Kim, Y.J. Cho, K. Lee, S.H. Yoon, M. Kim, H. Na, S.C. Park, Y.S. Jeon, J.H. Lee, H. Yi, S. Won, and J. Chun, "Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species," *International Journal of Systematic and Evolutionary Microbiology*, **62** (Pt_3) 716–721 (2012). doi:10.1099/ijs.0.038075-0.
28) N. Saitou, and M. Nei, "The neighbor-joining method: a new method for reconstructing phylogenetic trees," *Molecular Biology and Evolution*, **4** 406–25 (1987). doi:10.1093/oxfordjournals.molbev.a040454.
29) S. Kumar, G. Stecher, and K. Tamura, "MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets," *Molecular Biology and Evolution*, **33** (7) 1870–1874 (2016). doi:10.1093/molbev/msw054.
30) K. Benzerara, F. Skouri-Panet, J. Li, C. Ferard, M. Gugger, T. Laurent, E. Couradeau, M. Ragon, J. Cosmidis, N. Menguy, I. Margaret-Oliver, R. Tavera, P. Lopez-Garcia, and D. Moreira, "Intracellular Ca-carbonate biomineralization is widespread in cyanobacteria," *Proceedings of the National Academy of Sciences*, **111** (30), 10933–10938 (2014). doi:10.1073/pnas.1403510111.
31) M. Fahimizadeh, P. Pasbakhsh, L.S. Mae, J.B.L. Tan, R.K.S. Raman "Multifunctional, sustainable, and biological non-ureolytic self-healing systems for cement-based materials," *Engineering*, **13** 217-237 (2022), doi: 10.1016/j.eng.2021.11.016
32) L. Baumgartner, R. Reid, C. Dupraz, A. Decho, D. Buckley, J. Spear, K. Przekop, and P. Visscher, "Sulfate reducing bacteria in microbial mats: Changing paradigms, new discoveries," *Sedimentary Geology*, **185** (3–4) 131–145 (2006). doi:10.1016/j.sedgeo.2005.12.008.
33) H.J. Kim, B. Shin, Y.S. Lee, and W. Park, "Modulation of calcium carbonate precipitation by exopolysaccharide in *Bacillus* sp. JH7, *Applied Microbiology and Biotechnology*," **101** (16) 6551–6561 (2017). doi:10.1007/s00253-017-8372-8.
34) F. Hammes, N. Boon, J. de Villiers, W. Verstraete, and S.D. Siciliano, "Strain-specific ureolytic microbial calcium carbonate precipitation," *Applied and Environmental Microbiology*, **69** (8) 4901–4909 (2003). doi:10.1128/aem.69.8.4901-4909.2003.
35) N.K. Dhami, M.S. Reddy, and A. Mukherjee, "Biomineralization of calcium carbonate polymorphs by the bacterial strains isolated from calcareous sites," *Journal of Microbiology and Biotechnology*, **23** (5) 707–714 (2013). doi:10.4014/jmb.1212.11087.
36) L. Ma, A.P. Pang, Y. Luo, X. Lu, and F. Lin, "Beneficial factors for biomineralization by ureolytic bacterium *Sporosarcina pasteurii,*" *Microbial Cell*

Factories, **19** (1) (2020). doi:10.1186/s12934-020-1281-z.

37) A I. Omoregie, G. Khoshdelnezamiha, N. Senian, D.E.L. Ong, and P.M. Nissom, "Experimental optimisation of various cultural conditions on urease activity for isolated *Sporosarcina pasteurii* strains and evaluation of their biocement potentials," *Ecological Engineering*, **109** 65–75 (2017). doi:10.1016/j.ecoleng.2017.09.012.
38) L. Leeprasert, D. Chonudomkul, C. Boonmak, "Biocalcifying potential of ureolytic bacteria isolated from soil for biocementation and material crack repair," *Microorganisms* **10**(5) 963 (2022). doi:10.3390/microorganisms10050963Y.
39) Han, B. Sun, H. Yan, M. Tucker, Y. Zhao, J. Zhou, Y. Zhao, and H. Zhao, "Biomineralization of carbonate minerals induced by the moderate halophile *Staphylococcus Warneri* YXY2," *Crystals*, **10** (2) 58 (2020). doi:10.3390/cryst10020058.
40) F. Jroundi, M.T. Gonzalez-Muñoz, K. Sterflinger, and G. Piñar, "Molecular tools for monitoring the ecological sustainability of a stone bio-consolidation treatment at the royal chapel, Granada," *PLOS ONE*, **10** (7) e0132465 (2015). doi:10.1371/journal.pone.0132465.
41) S.C. Chuo, S.F. Mohamed, S.H. Mohd Setapar, A. Ahmad, M. Jawaid, W.A. Wani, A.A. Yaqoob, and M.N. Mohamad Ibrahim, "Insights into the current trends in the utilization of bacteria for microbially induced calcium carbonate precipitation," *Materials*, **13** (21), 4993 (2020). doi:10.3390/ma13214993.
42) Lee, Y.S., Kim, H.J., Park, W. "INon-ureolytic calcium carbonate precipitation by Lysinibacillus sp. YS11 isolated from the rhizosphere of Miscanthus sacchariflorus," *Jornal of Microbiology,* **55**, 440–447 (2017). doi:10.1007/s12275-017-7086-z
43) Y. Tang, J. Lian, G. Xu, Y. Yan, and H. Xu, "Effect of cementation on calcium carbonate precipitation of loose sand resulting from microbial treatment," *Transactions of Tianjin University*, **23** (6) 547–554 (2017). doi:10.1007/s12209-017-0084-8.
44) K. Wen, Y. Li, S. Liu, C. Bu, and L. Li, "Development of an improved immersing method to enhance microbial induced calcite precipitation treated sandy soil through multiple treatments in low cementation media concentration," *Geotechnical and Geological Engineering*, **37** (2) 1015–1027 (2018). doi:10.1007/s10706-018-0669-6.
45) J. Xu, X. Wang, and B. Wang, "Biochemical process of ureolysis-based microbial $CaCO_3$ precipitation and its application in self-healing concrete," *Applied Microbiology and Biotechnology*, **102** (7) 3121–3132 (2018). doi:10.1007/s00253-018-8779-x.
46) A.J. Mugwar, and M.J. Harbottle, "Toxicity effects on metal sequestration by microbially-induced carbonate precipitation," *Journal of Hazardous Materials*, **314** 237–248 (2016). doi:10.1016/j.jhazmat.2016.04.039.
47) C.-P. Tsai, J.-H. Ye, C.-H. Ko, Y.-R. Lin, "An experimental investigation of microbial-induced carbonate precipitation on mitigating beach erosion," *Sustainability*, **14** 2513 (2022). doi:10.3390/su140525131.
48) H. Nurjaman , S. Suwito, D. Dinariana, G. Suprapto, B. Budiono, M. Fau, "Development of Numerical Model of a High Performance Precast Concrete System Equipped with Base Isolation,"Evergreen **9** (2) 547-555 (2022). doi:10.5109/4794186.
49) A.A. Nasser, N.M. Sorour, M. A. Saafan, R.N. Abbas, "Microbially-Induced-Calcite-Precipitation (MICP): A biotechnological approach to enhance the durability of concrete using Bacillus pasteurii and Bacillus sphaericus," *Heliyon* **8** e09879 (2022). doi:10.1016/j.heliyon.2022.e09879.
50) Y. Masaki, "Characteristics of Industrial Wastewater discharged from Industrialized Provinces and Specific Industrial Sectors in China based on the Official Statistical Reports," *Evergreen,* **3** (2) 59–67 (2016). doi:10.5109/1800873.
51) Torres-Aravena, Á.E.; Duarte-Nass, C.; Azócar, L.; Mella-Herrera, R.; Rivas, M.; Jeison, D. "Can Microbially induced calcite precipitation (MICP) through a ureolytic pathway be successfully applied for removing heavy metals from wastewaters?" Crystals, **8**, 438 (2018). doi:10.3390/cryst8110438