BIODIVERSITAS
Volume 25, Number 5, May 2024
Pages: 2139-2147

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d250532

Micromorphology characterization of crystal calcium carbonate and exopolysaccharides quantification carbonatogenic bacterial LTP4-d isolated from historical painting of Maros-Pangkep karst area, Indonesia

NUR AFIFAH ZHAFIRAH[1], NUR HAEDAR[2,♥], SLAMET SENTOSA[2], FUAD GANI[2]

[1]Graduated School, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Hasanuddin. Jl. Perintis Kemerdekaan Km. 10, Makassar 90245, South Sulawesi, Indonesia. Tel.: +62-411-586016, Fax.: +62-411-588551, ♥email: nurhaedar@unhas.ac.id

[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Hasanuddin. Jl. Perintis Kemerdekaan Km. 10, Makassar 90245, South Sulawesi, Indonesia

Manuscript received: 20 February 2024. Revision accepted: 20 May 2024.

Abstract. *Zhafirah NA, Haedar N, Sentosa S, Gani F. 2024. Micromorphology characterization of crystal calcium carbonate and exopolysaccharides quantification carbonatogenic bacterial LTP4-d isolated from historical painting of Maros-Pangkep karst area, Indonesia. Biodiversitas 25: 2139-2147.* Indonesia possesses the second-largest limestone/karst region globally, trailing only China. Among Indonesia's limestone region, the Maros-Pangkep karst area in South Sulawesi stands out, as it has prehistoric rock art dating back at least 45.5 thousand years. However, over time, those paintings began to deteriorate due to many causes, one of which was overlayering of the deposition of calcium carbonate precipitates covering the surface of the paintings. This can occur due to the presence of microorganisms with the ability to precipitate calcium carbonate on the surface of those paintings. Microorganisms that have the ability to precipitate calcium carbonate are usually known as carbonatogenic bacteria or ureolytic bacteria. This study aimed to isolate the carbonatogenic bacteria and then assess how effectively it precipitates calcium carbonate, also to determine the production of EPS and characteristic micromorfology of $CaCO_3$ produced by carbonatogenic bacteria isolated from historical painting of Maros-Pangkep karst, Indonesia. Results showed that sixty bacterial isolates were successfully isolated from prehistoric paintings in Maros-Pangkep karst, of which 24 isolates were confirmed as carbonatogenic bacteria. The highest amount of $CaCO_3$ precipitate was recorded from LTP4-d isolates, which were able to precipitate 37.62±0.12 $mgmL^{-1}$ calcium carbonate. The highest amounts of ammonia and EPS production were also from LTP4-d. The SEM results showed that $CaCO_3$ precipitate from LTP4-d has a type of vaterite with element contents including C 0.32%; O 15.24%; Ca 84.53%. This study presents novel findings on the capability of carbonatogenic bacteria from prehistoric paintings in Maros-Pangkep karst to induce calcium carbonate precipitation, which can be utilized as a reference for repairing and preserving prehistoric paintings found in the Maros-Pangkep karst area.

Keywords: Calcium carbonate, carbonatogenic bacteria, karst, Maros-Pangkep

INTRODUCTION

Indonesia possesses the second-largest limestone/karst region globally, trailing only China. Among Indonesia's limestone regions, the Maros-Pangkep karst area in South Sulawesi stands out. Since May 24, 2023, UNESCO has designated the Maros-Pangkep karst area as part of the UNESCO Global Geopark list and recognized it as one of the top 10 geopark sites in Indonesia. The karst landscape of the Maros-Pangkep karst in South Sulawesi, Indonesia, obtained prehistoric rock art dating back at least 39.9 thousand years ago (Aubert et al. 2014; Brumm et al. 2021). Over 240 caves and rock shelters have been identified, containing a unique collection of rock art, which is distinguished by its red or mulberry-colored pigments, consists of hand stencils, depictions of animals, and combinations of human and animal figures. However, over time, those paintings have begun to deteriorate due to weathering processes, making it challenging to recognize or identify them. The damage to these paintings was also marked by fading colors or turning black due to the growth of microbes on the painting surfaces. Additionally, this deterioration can occur due to overlayering or the deposition of calcium carbonate precipitates covering the surface of the paintings. This can occur due to the presence of microorganism with the ability to precipitate calcium carbonate on the surface of those paintings.

Microorganisms that have the ability to precipitate calcium carbonate are usually known as carbonatogenic bacteria or ureolytic bacteria. The deposition of calcium carbonate can occur due to the presence of urease enzyme in bacteria that cause a biological mineralization process (Rajasekar et al. 2021). Urease enzyme was used by bacteria to be responsible or catalyze the hydrolysis of urea into ammonia and carbon dioxide, and also to produce calcium carbonate when carbonate (CO_2^{2-}) results from the process of hydrolysis of the urea and binds with calcium (Ca^{2+}). The presence of Ca^{2+} ions can influence the formation of calcium carbonate ($CaCO_3$), with higher levels of Ca^{2+} promoting calcite formation and vice versa (Phang et al. 2018). The electronegativity of bacterial cell walls facilitates the adsorption of cations, including calcium ions, thereby facilitating the deposition of $CaCO_3$ on the cell wall (Gat et al. 2014). Ureolytic bacteria are found in various environments, including limestone formations, sediments, cave formations, and marine sediments (c).

Numerous bacteria are recognized for their role in encouraging extracellular Calcium Carbonate Precipitation (CCP) (Wei et al. 2015; Dhami et al. 2018; Idris et al. 2022). The calcium carbonate precipitation process is greatly influenced by the presence of EPS (Exopolysaccharides/ Extracellular Polymeric Substances). This EPS, comprising polysaccharides, proteins, nucleic acids, and lipids, acts as sites for CCP nucleation due to their anionic chemical properties, which attract nearby metal ions such as calcium (Ca^{2+}). Microbial EPS aids in calcium carbonate precipitation by capturing calcium (Ca^{2+}) ions in the nearby surroundings via the negatively charged functional groups present in EPS. Consequently, this leads to the formation of calcium carbonate precipitates (Omoregie et al. 2016).

The formation of calcium carbonate precipitates has positive effects on karst cave rocks, one of which is to strengthen the structure of karst rocks and repair cracks in them. In addition to the positive impact of the presence of calcium carbonate precipitates in karst caves, there are also negative effects that are detrimental to the prehistoric cave paintings found within these karst caves. The presence of calcium carbonate precipitates can form overlayering on the surface of prehistoric paintings and cause damage to these prehistoric paintings. This study aimed to isolate the carbonatogenic bacteria and then assess how effectively they precipitate calcium carbonate, and to determine the production of EPS and ammonia and characteristic micro-morphology of CaCO3 produced by carbonatogenic bacteria isolated from historical printing of Maros-Pangkep karst, Indonesia.

MATERIALS AND METHODS

Study area and sampling sites

Study area

The sampling locations for the isolates of carbonoclastic bacteria causing damage to prehistoric paintings in this study were conducted in five different caves. Among the five cave sampling locations, there are three locations within the Maros District area in South Sulawesi, Indonesia namely Leang Pettae Cave (4° 58’ 43.2” S 119° 40’ 34.2” E), Leang Timpuseng Cave (4° 59’ 54” S 119° 40’ 34.2” E), and Leang Sumpang Bita Cave (4° 56’ 54” S 119° 38’ 57” E), while the other two caves, Parewe Cave (4° 47’ 40.1” S 119° 31’ 7.25” E) and Bulu Sipong Cave (4° 48’ 23.81” S 119° 36’ 36.9” E), are located in the Pangkajene and Kepulauan (Pangkep) District in South Sulawesi (Figure 1).

Sample collection

The surface of the cave wall samples was gently swabed using a cotton swab that has been moistened with sterilized aquadest and was stored in sample bottles that had been filled with NaCl 0.9% to maintain its cellular metabolism. The samples were then stored in a coolbox and transported to the laboratory for further analysis.

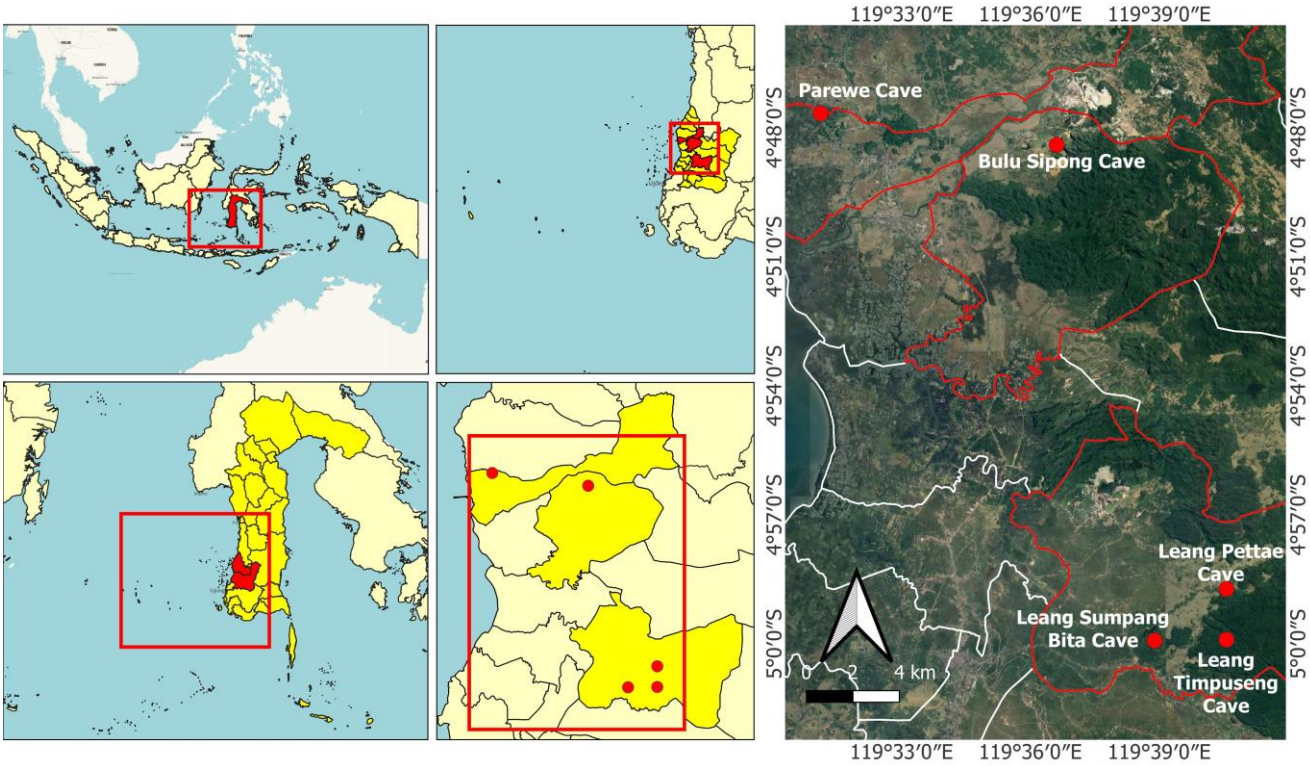

Figure 1. Research sampling at the karst area of Maros-Pangkep, South Sulawesi, Indonesia

Isolation of carbonatogenic bacterial strain

The samples were then cultured on Nutrient Agar media by streaking the surface of the media using a cotton swab with a quadrant streaking technique. Screening of isolates ability to precipitate $CaCO_3$ was conducted through the utilization of Calcium Carbonate Precipitation Agar made with mixing 20 g urea, 2.12 g $NaHCO_3$, 4.14 g $CaCl_2$, 10 g NH_4Cl, 3 Nutrient Broth medium 18 g Agar in 1000 mL destilled water and was sterilized with an autoclave at a pressure of 1 atm and a temperature of 121°C for 15 minutes as described by Zulaika et al. (2021). The growth of carbonatogenic bacteria was characterized by the presence of calcium carbonate crystals around the colonies when observed under a stereo microscope.

Urease activity screening

Urease screening was carried out to assess the isolate's capacity for urease production using a qualitative urease assay on Christensen Urea Agar. The medium was created by mixing 5 g of NaCl, 1 g of peptone, 1 g of glucose, 2 g of K_2HPO_4, 0.012 g of phenol red and 15 g of agar were dissolved in 900 mL of distilled water and then were sterilized for 15 min in an autoclave at a pressure of 1 atm and a temperature of 121°C for 15 minutes. The media was then cooled to room temperature and then added 20 g of urea nonsterilized in 100 mL of distilled water and was sterilized by filter (Dhami et al. 2018). The bacterial isolates were then streaked onto medium and incubated at 30°C for three days. After 3-days incubation period, a change in color of medium from yellow to cherish pink-purple indicates the positive result. The change in the media's color was caused by the increase of pH value in medium, which indicates the presence ammonium as a result of urea hydrolysis by urease.

$CaCO_3$ precipitation production

$CaCO_3$ precipitation was done by using liquid CCP medium made by mixing 3 g of nutrient broth, 20 g of urea and 25.8 g of $CaCl_2.2H_2O$ with 1000 mL of distilled water. 3 mL of 25%T (The turbidity level of bacterial count in Nutrient Broth media) urease positive bacterial isolates were inoculated into 10 mL liquid CCP medium (Fitri et al. 2023). The liquids were placed on a rotatory shaker at room temperature for 21-days incubation period at 130 rpm. After the incubation period was done, the $CaCO_3$ precipitate was separated by filter paper (Whatman No.42), which had been dried in an oven at 60°C for 3 hours. The $CaCO_3$ precipitate was dried in an oven at 60°C for 4 hours. The weight of the resulting $CaCO_3$ powder was measured using an analytical balance. The calculation of the precipitate weight (Wc) is done by subtracting the combined weight of the precipitate and filter paper (Wfc) from the weight of the filter paper alone (Wf).

Ammonia quantification

Ammonia concentration was measured in a urea-based medium (Utomo et al. 2023). After the bacterial culture was fermented for 21 days in a urea-based medium, it was then filtered using filter paper. 1 mL of the obtained filtrate was taken, 10 mL of distilled water, 4 mL of Na-phenol solution, and 3 mL of NaOCl solution were added to a 50 mL volumetric flask, then shaken until homogeneous and left to stand for 20 minutes. Then, distilled water was added to a volume of 50 mL and shaken again until homogeneous. The ammonia content was measured using a spectrophotometer with a wavelength of 590 nm. This ammonia quantification was conducted three times for repetition. The concentration of the ammonia sample (x) can be calculated by regressing the absorbance values (y) and the constants (a,b) from the equation $y = ax+b$ (Lee et al. 2019).

The preparation of a 10% urea solution was done by weighing 10 g of urea and then added to 100 mL of distilled water. Meanwhile, Na-hypochlorite solution was prepared by taking 281.7 mL of NaOCl and pouring it into 1,000 mL of distilled water. The citrate buffer solution was prepared as follows: 300 g of potassium citrate was weighed and then dissolved in 700 mL of distilled water. Afterward, it was filtered until reached a volume of 1,000 mL, and the pH was measured until it reached 6.7. The sodium-phenol solution was prepared as follows: In the first step, 6.25 g of phenol red was weighed and added to 20 mL of ethanol, then 2 mL of methanol and 18.5 mL of acetone were added, followed by ethanol until reaching a volume of 100 mL and shaken until homogeneous (a). In the second step, 27 g of NaOH was dissolved in 100 mL of distilled water (b). In the third step, solutions (a) and (b) were each taken 20 mL and then added with distilled water until reaching a volume of 100 mL.

The ammonium sulfate stock solution was prepared by weighing 4.717 g of $(NH_4)2SO_4$ and then adding it to 1,000 mL of distilled water. The standard ammonium sulfate solution was prepared by pouring 10 mL of the stock solution into 990 mL of distilled water. The procedure involves preparing 50 mL volumetric flasks, then pipetting the solution for the standard curve in quantities of 0, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, and 20 mL each. The amount was equivalent to 0; 0,2; 0,4; 0,6; 0,8; 1,2; 1,6; 2 ppm NH_3-N/mL. Then, 10 mL of distilled water, 4 mL of sodium phenol, and 3 mL of NaOCl solution are sequentially added and shaken until homogeneous for 20 minutes. After 20 minutes, the volume was adjusted to 50 mL, and the light intensity was measured at a wavelength of 590 nm on a spectrophotometer. A blank solution was prepared as a control for the compound being tested. The blank solution was prepared by dissolving 4 mL of sodium phenol and 3 mL of NaOCl solution in 10 mL of distilled water, then adjusted to a volume of 50 mL and homogenized. The concentration of ammonia was a product of urease activity.

Exopolysaccharides quantification

EPS quantification was conducted using the congo red binding assay according to previous studies (López-Moreno et al. 2014). One milliliter of cell culture from $CaCO_3$ precipitation production medium after 21-days of incubation was mixed with congo red at a final concentration of 3.5 mgL^{-1} and incubated for 30 minutes at 37°C with periodic inversion every 10 minutes. Following centrifugation of the culture at 14.000 rpm for 10 minutes, the OD_{480} of the clear supernatant was recorded. A standard curve for congo red was established by measuring OD_{480} across a concentration range of 0.625 to 80 mgL^{-1}. The fraction of congo red bound to cells was determined by subtracting the remaining congo

red in the supernatant from the congo red present in the medium without cells, using the equation derived from the linear curve (Kim et al. 2015).

Micro-morphology characterization of calcium carbonate precipitate

Micro-morphology characterization in this study was conducted using a scanning electron microscope (SEM) to determining the morphological characteristics of calcium carbonate precipitates obtained from bacterial isolates isolated from prehistoric cave paintings in the Maros Pangkep karst area, South Sulawesi. The calcium carbonate precipitates were plated with platinum before being inspected using FE-SEM. EDS mapping analysis was performed using a NORAN System 7: Silicon Drift Detector (Thermo Fisher Scientific, USA), where the elements on the map were represented by counts.

RESULTS AND DISCUSSION

Sample collection

The sample acquisition was done using a cotton swab and swabbed onto the surface of prehistoric paintings found on the cave wall. The cotton swab was then placed into a tube containing 0.9% NaCl to maintain bacterial metabolism. The samples were then stored in a coolbox and transported to the laboratory for further analysis.

Isolation and selection of carbonatogenic bacterial strain

Result of isolation showed that a total of 60 bacterial isolates were successfully obtained from the samples. In this research, only 24 isolates had the ability to precipitate calcium carbonate, as indicated by the presence of crystal calcium carbonate around its colonies in the CCP medium. The carbonatogenic isolates obtained from Leang Timpuseng cave were LTP1-a, LTP2-b, LTP3-a and LTP7-d. Isolates SPB3-b, SPB5-a and SPB7-a were obtained from Sumpang Bita cave, while LPE1-a, LPE4-d, LPE6-b, LPE1 were isolates from Leang Pettae cave. From Bulu Sipong cave, five isolates were obtained, namely BSP3-c, BSP6-a, BSP7-a, BSP7-c and BSP7-d. Six isolates, i.e., PRW1-a, PRW2-a, PRW2-c, PRW4-c, PRW5-a and PRW6-a were isolated from Parewe cave. The appearance of calcium carbonate precipitate around the colonies varies in shape and size, as depicted in Figure 2.

Figure 2. Carbonatogenic bacteria in CCP medium

Urease screening

The results of urease screening showed positive results of all isolates. Among the twenty-four isolates that tested positive for urea hydrolysis, two isolates demonstrated weak hydrolysis ability, namely LTP5-a and LTP2-a, as indicated by the absence of color change throughout the media. In contrast, the other twenty-two isolates exhibited high urea hydrolysis ability, characterized by color change across the entire media.

$CaCO_3$ precipitation and collection

The calcium carbonate precipitation test was conducted using NB U/Ca media, which is an enriched medium containing urea as an energy source for bacteria and calcium, and is useful as a material for forming calcium carbonate precipitates. The calcium carbonate precipitation will be visible at the bottom of the medium and appear as white sediment adhering to the erlenmeyer flask after the incubation period. It was found that all 24 isolates of carbonatogenic bacteria were able to precipitate calcium carbonate. Figure 3 shows the calcium carbonate precipitate on the wall of the Erlenmeyer flask produced by the LPE6-d bacteria.

Based on the results obtained, it is evident that the amount of calcium carbonate precipitate produced by each bacterial isolate varies. The highest amount of calcium carbonate precipitate was produced by the bacterial isolate LTP4-d, with a quantity of 37.62±0.12 $mgmL^{-1}$, followed by the bacterial isolate PRW1-a, which produced 30.54±0.17 $mgmL^{-1}$. Meanwhile, LPE1-a bacterial isolate the showed lowest (4.21±0.10 $mgmL^{-1}$) ability to precipitate calcium carbonate (Table 1).

EPS quantification

The results of EPS quantification produced by carbonatogenic bacteria in this study revealed that each bacterium exhibited varying capacities for EPS production. The calculation of EPS value is done with three replicates and taking the average value as the final value entered into the calculation. The highest EPS production capability being observed in the LTP 4-d isolate with the measured EPS resulting being 64.41±0.02 $mgmL^{-1}$ and SPB7-a with the result was 49.83±0.38 $mgmL^{-1}$. Meanwhile, the lowest EPS production being observed from BSP7-c with the result was 2.90±0.03 $mgmL^{-1}$ (Figure 4).

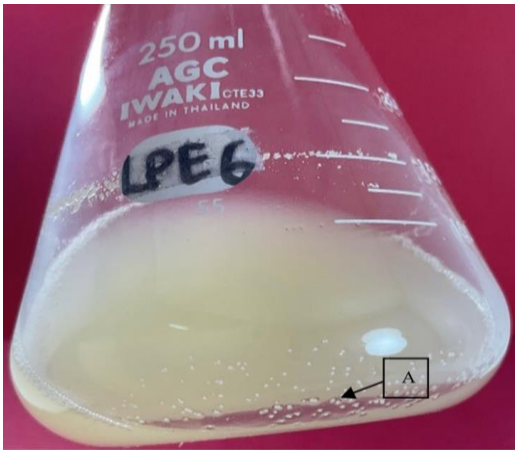

Figure 3. $CaCO_3$ Precipitation in the culture medium NB U/Ca. A: $CaCO_3$ particle

Table 1. Weight of $CaCO_3$ precipitates produced during 21-days of incubation

Isolates	Precipitate weight (mg)			Standard Deviation (SD)	Precipitate weight ($mgmL^{-1}$)
	Precipitate weight and filter paper weight (Wfc)	Filter paper weight (Wf)	Precipitate weight (Wc)		
LTP1-a	1169.3	1087.7	81.60	0.26	8.16±0.26
LTP2-b	1172.7	1021.6	151.10	0.35	15.11±0.35
LTP3-a	911.5	808.3	103.20	0.26	10.32±0.26
LTP4-d	1078.2	702	376.20	0.12	37.62±0.12
LTP7-d	1173	1007	166.00	0.23	16.6±0.23
SBP3-b	1186.6	1087.4	99.20	0.10	9.92±0.10
SPB5-a	1002.3	790	212.30	0.10	21.23±0.10
SPB7-a	1197.5	960.8	236.70	0.12	23.67±0.12
LPE1-a	852	809.9	42.10	0.10	4.12±0.10
LPE5-a	1060.5	783.1	277.40	0.10	27.74±0.10
LPE5-b	1154.5	977.9	176.60	0.25	17.66±0.25
LPE6-a	1159.1	1088.5	70.60	0.82	7.06±0.82
LPE6-d	925.1	810.8	114.30	0.32	11.43±0.32
BSP3-c	1145	1002.3	142.70	0.21	14.27±0.21
BSP6-a	1158.3	984.2	174.10	0.35	17.41±0.35
BSP7-a	860.9	765.5	95.40	0.32	9.54±0.32
BSP7-c	1116.4	1008.2	108.20	0.10	10.82±0.10
BSP7-d	1195	922.1	272.90	0.15	27.29±0.15
PRW1-a	1230.3	924.9	305.40	0.17	30.54±0.17
PRW2-a	888.4	765.5	122.90	0.25	12.29±0.25
PRW2-c	992.3	806.2	186.10	3.87	18.61±3.87
PRW4-c	800.1	508.7	291.40	0.38	29.14±0.38
PRW5-a	1088.6	965.9	122.70	0.21	12.27±0.21
PRW6-a	1101.9	935.1	166.80	0.30	16.68±0.30

Ammonium quantification

After twenty-one-day incubation period, the isolate that produced the highest amount of ammonia was the LTP4-d isolate with a measured ammonia level of 877.80±1.04 ppm, followed by the BSP7-d isolate with a measured ammonia level of 819.71±0.05 ppm, while the isolate with the lowest ammonia content was the LPE6-a isolate with a measured ammonia level of 168.26±0.18 ppm, and the LPE1-a isolate with a level of 174.48±0.09 ppm (Figure 5).

$CaCO_3$ micro-morphology characterization

The utilization of SEM in this research was conducted to determine the morphological characteristics of calcium carbonate precipitates obtained from bacterial isolates originating from prehistoric cave paintings in the Maros Pangkep karst area, South Sulawesi. The surface structure of the calcium carbonate produced by the LTP4-d isolate appears as hexagonal crystals (Figure 6). This appearance of hexagonal morphology structure in the calcium carbonate precipitate indicates that the formed crystal veterite.

Based on theoretical calculations, the expected weight percentage of $CaCO_3$ Ca content was 40%. However, the microanalysis results showed a Ca content of 68.42%, indicating a difference of 28.42%. Similarly, the expected Carbon (C) level was 12%, but the microanalysis revealed a C content of 0.87%, resulting in a difference of 11.13%. Additionally, the anticipated oxygen levels should be 64%, but the direct microanalysis yielded an oxygen content of 30.71%, indicating a difference of 33.29%. Based on the SEM-EDX analysis, it is concluded that the scale is indeed composed of crystalline $CaCO_3$ (Figure 7, Table 2).

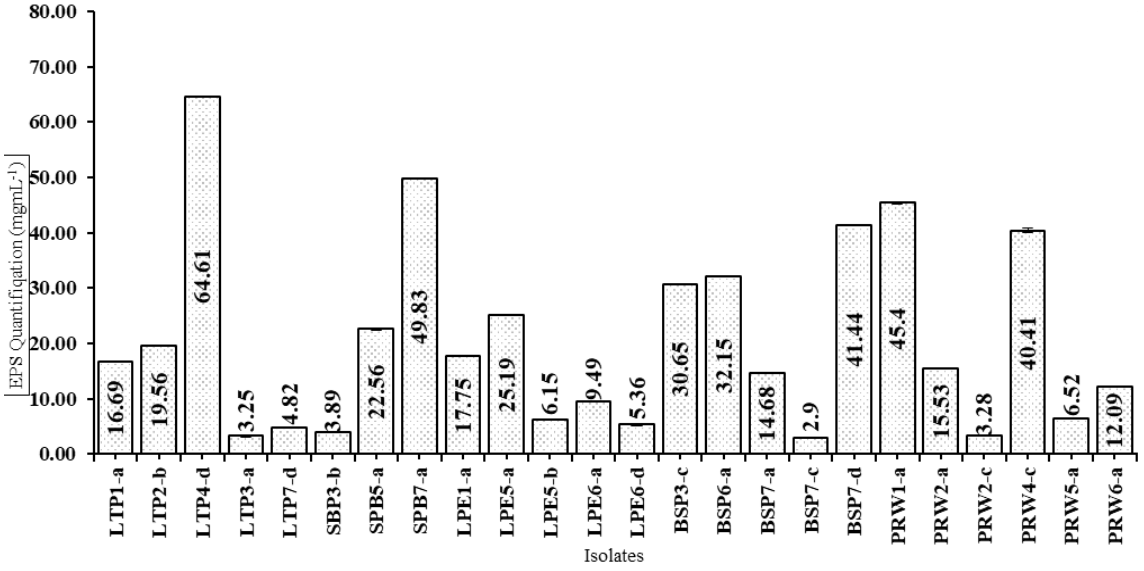

Figure 4. EPS formation of carbonatogenic bacteria

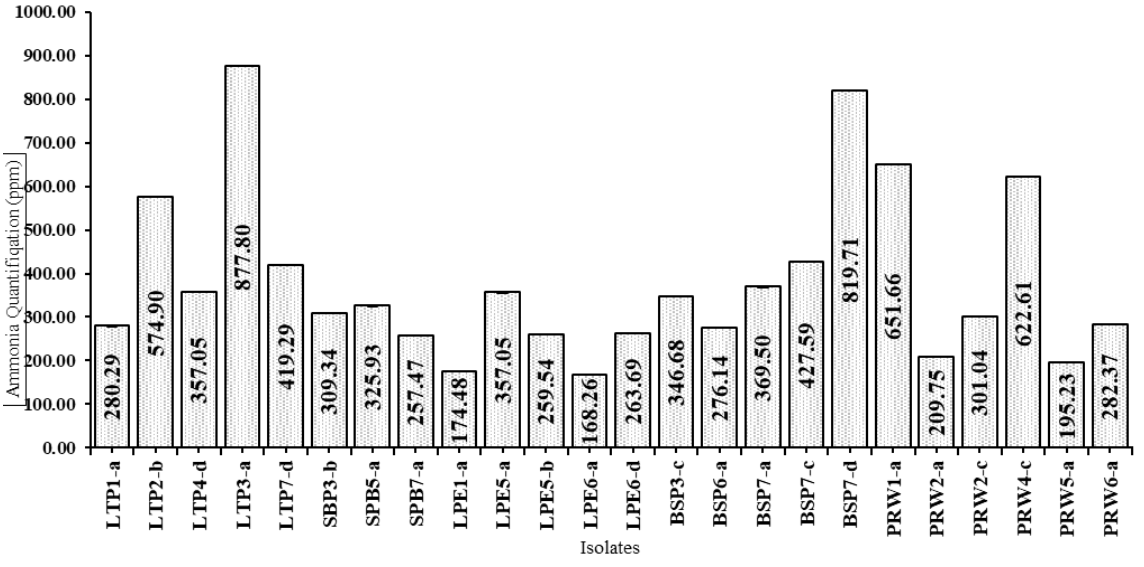

Figure 5. Ammonia quantification of carbonatogenic bacteria

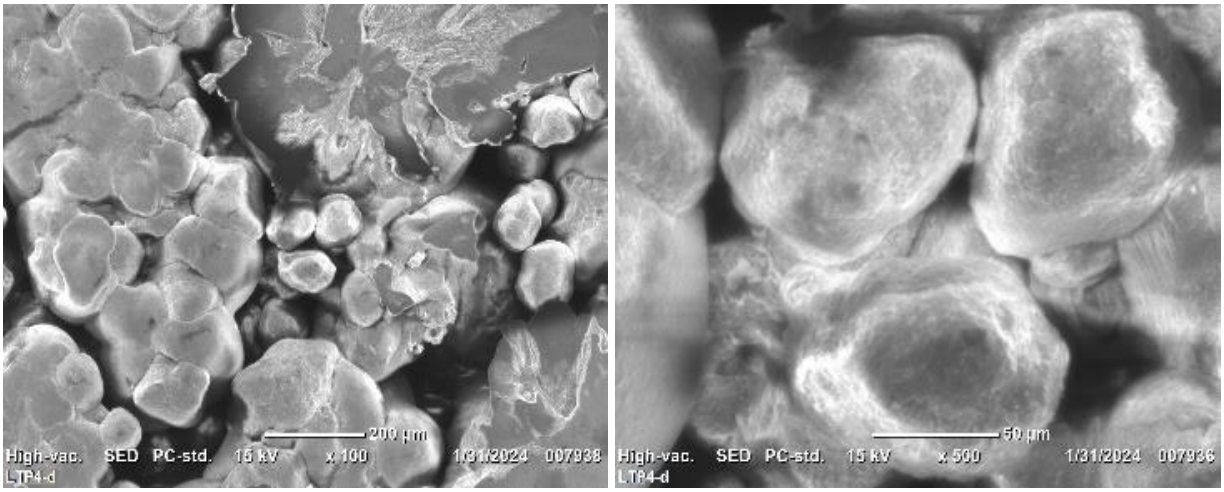

Figure 6. Morphological appearance of $CaCO_3$ precipitation from LTP4-d isolate

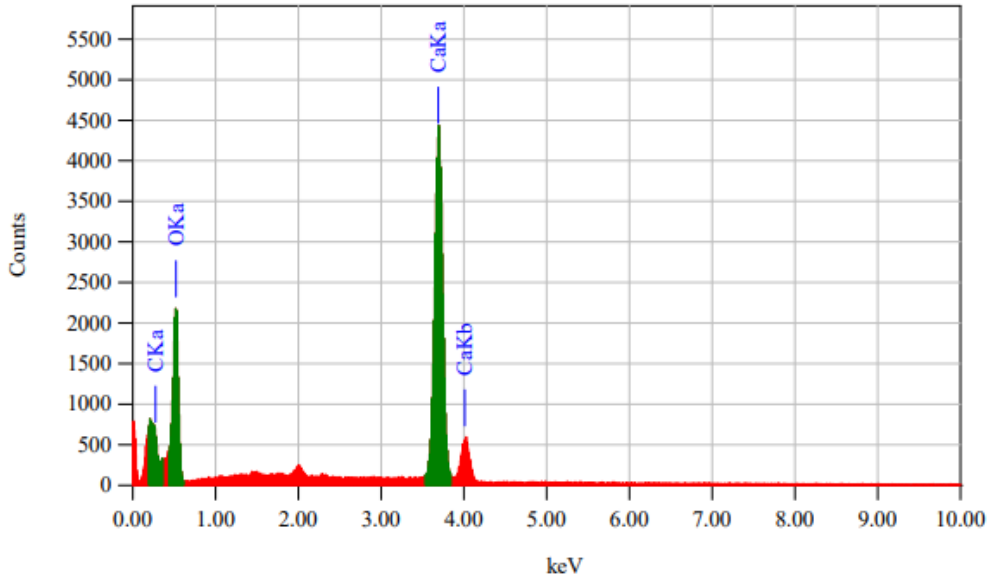

Figure 7. EDS mapping analysis of $CaCO_3$ from LTP4-d isolate

Table 2. The composition of $CaCO_3$ from LTP4-d using Energy-Dispersive X-ray spectroscopy analysis

Elements	Mass%	Atom%
C	0.32	0.87
O	15.14	30.71
Ca	84.53	68.42
Total	100	100

Discussion

Carbonatogenic bacteria is one type of bacteria capable of precipitating calcium carbonate. The precipitation of calcium carbonate by carbonatogenic bacteria contributes to the deterioration of prehistoric paintings on the surface of cave rock walls. The characteristics of carbonatogenic bacteria can be observed from the formation of calcium carbonate precipitate around bacterial colonies when grown on CCP media shown in. The crystal $CaCO_3$ precipitate around the colony was formed due to the enzymatic action of urease, and free carbon ions (CO_3^{2-}) were generated, leading to the binding of free calcium ions (Ca^{2+}) in CCP media, resulting in the formation of $CaCO_3$ crystals (Wei et al. 2015). The formation process of calcium carbonate precipitates is highly influenced by the urease enzyme activity produced by bacterial isolates. The results of this study are consistent with research conducted by Wei et al. (2015), who obtained 20 isolates of calcium carbonate precipitating bacteria from marine sediments showing crystal formation around their colonies after a 7-day incubation period using CCP agar media. The formation process of calcium carbonate precipitates is highly influenced by the urease enzyme activity produced by bacterial isolates.

Urease is one of the enzymes known to hydrolyz urea to produce ammonia and carbon dioxide (Kappaun et al. 2018). The amount of ammonia produced by each bacterium

varies. This difference may be caused by the influence of environmental factors or the influence of the bacteria's genetics. Result of urease screening exhibited that all 24 isolates of carbonatogenic bacteria showed positive response. Zulaika et al. (2021) successfully obtained 6 isolates of carbonate-dissolving bacteria from limestone mountains, where all six isolates were positive for urea hydrolysis through testing using Christensen Urea Agar media.

The urease enzyme activity in urea hydrolise process results in carbonate and ammonium, which are by-products of the urea hydrolysis process, while the carbonate or bicarbonate obtained bind with the available calcium in the media, forming calcium carbonate ($CaCO_3$) which the ability of bacteria to precipitate calcium carbonate also varies. This difference can be influenced by the environment. Bacteria accustomed to abundant urea sources in their surroundings enhance their ability in the process of calcium carbonate precipitation. Certain environments are rich in calcium sources including caves, limestone formation or soil (Omoregie et al. 2019). Recent research indicates that urea is plentiful in CCP environments due to contributions from diverse origins such as mammal urine, surface water infiltration, human actions, and agricultural residue (Okyay et al. 2016). The presence of urea in caves or limestone formations can originate from animal urine in the vicinity, secondary metabolites from microbial activities, remnants of microbial biomass, rainwater carrying urea from the soil surface, or the activities of humans and other animals that can leave urea waste in the cave environment. Koning et al. (2022), described that because of these environmental challenges, bacteria employ different routes to generate energy, and the occurrence of MICP (Microbially Induced Calcite Precipitation) relies on four factors: (i) Environmental pH, (ii) dissolved inorganic carbon, (iii) calcium ion concentration, and (iv) the prevalence of nucleation sites which is nucleation site of calcium carbonate is held by the presence of exopolysaccharides.

Extracellular Polymeric Substances (EPS) are one of the metabolic products of bacteria with compositions that vary depending on the bacterial species and environmental conditions. Generally, EPS consists of polysaccharides, proteins, lipids, and various other organic compounds. The production of EPS by microorganisms plays a critical role in the formation, maintenance, and spread of biofilms. EPS facilitates the attachment of biofilms to surfaces, offers mechanical stability, and temporarily immobilizes cells (Kim et al. 2015). The formation of microcolonies surrounded by an EPS matrix is a common feature of all biofilms, particularly in terrestrial environments on rocks, where EPS protects against drying out and exposure to sunlight. Based on the results obtained in the present study were higher compared to the EPS levels obtained in the study by Kim et al. (2015), who isolated carbonatogenic bacteria from concrete, where the highest EPS calculation was found from JH3 isolate with an EPS count of 10.01 mg/mL. The difference in the amount of EPS production can be influenced by various factors. López-Moreno et al. (2014), explained that differences in EPS levels can be caused by varying amounts or qualities of calcium. However, on the other hand, the influence of genetic factors on bacteria also greatly affects EPS formation in bacteria. The higher EPS levels obtained, the greater the strength of the stone or precipitate that can be produced. EPS contributes to the deterioration of stone monuments by stabilizing biofilms, aiding in the capture of particles, increasing surface soiling, leading to the clogging of stone pores, and enhancing the ability of cells to bind cations released from the stones, thus contributing to the weakening of the mineral substrate (Wei et al. 2015). In the process of forming calcium carbonate precipitation, the role of EPS is crucial, where EPS is utilized as nucleation sites in calcium carbonate precipitation, EPS provides substrates as matrices for calcium carbonate deposition and aids in the binding and accumulation of calcium carbonate particles (Bains et al. 2015). Microbial EPS promotes calcium carbonate precipitation by binding available calcium Ca^{2+} ions in the surrounding environment with negatively charged functional groups of EPS, this facilitating the formation of calcium carbonate precipitates (Kim et al. 2015).

In addition to producing EPS, the process of calcium carbonate precipitation also generates ammonia which one mole of urea hydrolyzed will yield one mole of ammonia and one mole of carbonate. Thus, the production of urease enzyme greatly influences the amount of ammonia produced. The higher the production of urease enzyme, the higher the resulting ammonia product. The availability of urea in the media is utilized by bacteria as a carbon source, where bacteria produce urease enzyme to hydrolyze the available urea.

The precipitation of calcium carbonate in bacteria result in various types of calcium carbonate precipitates. The most frequently occurring polymorphs of calcium carbonate, namely calcite and vaterite, are typically brought about by carbonatogenic bacteria through the process of urea hydrolysis (Feng et al. 2022). Additionally, biochemical factors like the amino acid composition within urease significantly influence the crystalline structure of the resulting calcium carbonate precipitate. Research by Sondi and Salopek-Sondi (2005) proposed that the lower concentration of Asp and Flu favors the prevalence of calcite, whereas a higher concentration of these amino acids tends to promote the formation of vaterite. Calculation of the components composing calcium carbonate can also be analyzed using X-Ray Diffraction (XRD) analysis. The purpose of XRD analysis is to determine the crystal structure and composition of materials by measuring the diffraction pattern produced when X-rays are scattered by a sample. Based on the results obtained from picture 5, the type of calcium carbonate crystal from LTP4-d is classified as a stable crystal type, although it has not yet entered the calcite phase, which is the optimal phase in the formation of calcium carbonate crystals.

In conclusion, the isolation results of bacteria from the prehistoric cave paintings of the Maros-Pangkep karst rocks yielded 60 isolates from five caves (Leang Pettae, Leang Timpuseng, Leang Parewe, Gua Sumpang Bita, and Gua Bulu Sipong), among which 24 isolates were classified as carbonatogenic bacteria. The analysis of calcium carbonate precipitates in carbonoclastic bacterial isolates yielded varied results, with the highest obtained from the LTP4-d bacterial isolate at 37.62±0.12 mg, while the lowest was obtained

from the LPE1-a isolate at 4.12±0.10 mg. The analysis of Extracellular Polymeric Substances (EPS) content, the highest produced by carbonoclastic bacteria originating from the prehistoric cave paintings of Maros-Pangkep karst, was found in the LTP4-a isolate with a total EPS content of 64.41±0.02 mgmL^{-1}. Additionally, the analysis of the micro-morphological structure of calcium carbonate precipitates produced by carbonatogenic bacteria from the prehistoric cave paintings of Maros-Pangkep karst revealed the presence of veterite calcium carbonate with elemental compositions of C (0.32%), O (15.14%), and Ca (84.53%). Considering the preservation of prehistoric paintings found in the Maros-Pangkep karst area, it would be beneficial to propose future studies focusing on the following aspects: environmental monitoring, microbial communities, biological conservation strategies, material analysis; cultural heritage management and interdisciplinary approaches. By focusing on these areas in future studies, researchers can contribute to the development of effective conservation strategies that safeguard the invaluable prehistoric paintings found in the Maros-Pangkep karst area for future generations.

ACKNOWLEDGEMENTS

The authors thank the Faculty of Mathematics and Natural Science, Universitas Hasanudin, Makassar, Indonesia, for this research.

REFERENCES

Aubert M, Brumm A, Ramli M, Sutikna T, Saptomo EW, Hakim B, Morwood MJ, van den Bergh GD, Kinsley L, Dosseto A. 2014. Pleistocene cave art from Sulawesi, Indonesia. Nature 514: 223-227. DOI: 10.1038/nature13422.

Bains A, Dhami NK, Mukherjee A, Reddy MS. 2015. Influence of exopolymeric materials on bacterially induced mineralization of carbonates. Appl Biochem Biotechnol 175 (7): 3531-3541. DOI: 10.1007/s12010-015-1524-3.

Brumm A, Oktaviana AA, Burhan B, Hakim B, Lebe R, Zhao J-X, Sulistyarto PH, Ririmasse M, Adhityatama S, Sumantri I, Aubert M. 2021. Anthropology oldest cave art found in sulawesi. Sci Adv 7 (3): eabd4648. DOI: 10.1126/sciadv.abd4648.

Dhami NK, Mukherjee A, Watkin ELJ. 2018. Microbial diversity and mineralogical-mechanical properties of calcitic cave speleothems in natural and in vitro biomineralization conditions. Front Microbiol 9: 40. DOI: 10.3389/ fmicb.2018.00040.

Fitri L, Aulia TB, Fauzi A, Kamil GH. 2023. Characterization and screening of urease activity of ureolytic bacteria from landfills soil in Banda Aceh, Indonesia. Biodiversitas 24 (2): 910-915. DOI: 10.13057/biodiv/d240229.

Feng Z, Yang T, Liang T, Wu Z, Wu T, Zhang J, Yu L. 2022. Biomineralization of calcium carbonate under amino acid carbon dots and its application in bioimaging. Mater Des 217: 110644. DOI: 10.1016/j.matdes.2022.110644.

Gat D, Tsesarsky M, Shamir D, Ronen Z. 2014. Accelerated microbial-induced $CaCO_3$ precipitation in a defined coculture of ureolytic and non-ureolytic bacteria. Biogeosciences 11 (10): 2561-2569. DOI: 10.5194/bg-11-2561-2014.

Idris I, Rustandi B, Sulistiyani TR, Rahmat A, Made Sudiana I. 2022. Bioprospecting ureolytic rock bacteria for calcium carbonate precipitation inducer. Evergreen 9: 962-972. DOI: 10.5109/6622882.

Kappaun K, Piovesan AR, Carlini CR, Ligabue-Braun R. 2018. Ureases: Historical aspects, catalytic, and non-catalytic properties - A review. J Adv Res 13: 3-17. DOI: 10.1016/j.jare.2018.05.010.

Kim HJ, Eom HJ, Park C, Jung J, Shin B, Kim W, Chung N, Choi I-G, Park W. 2015. Calcium carbonate precipitation by *Bacillus* and *sporosarcina* strains isolated from concrete and analysis of the bacterial community of concrete. J Microbiol Biotechnol 26 (3): 540-548. DOI: 10.4014/jmb.1511.11008.

Koning K, McFarlene R, Gosse JT, Lawrence S, Carr L, Horne D, Van Wagoner N, Boddy CN, Cheeptam N. 2022. Biominerlization in cave bacteria-popcorn and soda straw crystal formation, morphologies, and potential metabolic pathways. Front Microbiol 13: 933388. DOI: 10.3389/fmicb.2022.933388.

Lee M, Gomez MG, San Pablo ACM, Kolbus CM, Graddy CMR, DeJong JT, Nelson DC. 2019. Investigating ammonium by-product removal for ureolytic biocementation using meter-scale experiments. Sci Rep 9 (1): 18313. DOI: 10.1038/s41598-019-54666-1.

López-Moreno A, Sepúlveda-Sánchez JD, Mercedes Alonso Guzmán EM, Le Borgne S. 2014. Calcium carbonate precipitation by heterotrophic bacteria isolated from biofilms formed on deteriorated ignimbrite stones: Influence of calcium on EPS production and biofilm formation by these isolates. Biofouling 30 (5): 547-560. DOI: 10.1080/08927014.2014.888715.

Okyay TO, Nguyen HN, Castro SL, Rodrigues DF. 2016. CO_2 sequestration by ureolytic microbial consortia through microbially-induced calcite precipitation. Sci Total Environ 572: 671-680. DOI: 10.1016/j.scitotenv.2016.06.199.

Omoregie AI, Ong DEL, Nissom PM. 2019. Assessing ureolytic bacteria with calcifying abilities isolated from limestone caves for biocalcification. Lett Appl Microbiol 68 (2): 173-181. DOI: 10.1111/lam.13103.

Omoregie AI, Senian N, Ye Li P, Hei NL, Leong DOE, Henry Ginjom IR, Nissom PM. 2016. Screening for urease-producing bacteria from limestone caves of Sarawak. Borneo J Res Sci Technol 6 (1): 37-45. DOI: 10.33736/bjrst.213.2016.

Phang IRK, Chan YS, Wong KS, Lau SY. 2018. Isolation and characterization of urease-producing bacteria from tropical peat. Biocatal Agric Biotechnol 13: 168-175. DOI: 10.1016/j.bcab.2017.12.006.

Rajasekar A, Wilkinson S, Moy CKS. 2021. MICP as a potential sustainable technique to treat or entrap contaminants in the natural environment: A review. Environ Sci Ecotechnol 6: 100096. DOI: 10.1016/j.ese.2021.100096.

Sondi I, Salopek-Sondi B. 2005. Influence of the primary structure of enzymes on the formation of $CaCO_3$ polymorphs: A comparison of plant (*Canavalia ensiformis*) and bacterial (*Bacillus pasteurii*) ureases. Langmuir 21 (19): 8876-8882. DOI: 10.1021/la051129v.

Utomo WP, Wu H, Ng YH. 2023. Quantification methodology of ammonia produced from electrocatalytic and photocatalytic nitrogen/nitrate reduction. Energies 16 (1): 27. DOI: 10.3390/en16010027.

Wei S, Cui H, Jiang Z, Liu H, He H, Fang N. 2015. Biomineralization processes of calcite induced by bacteria isolated from marine sediments. Braz J Microbiol 46: 455-464. DOI: 10.1590/S1517-838246220140533.

Zulaika E, Utomo MAP, Pangestu AS, Alami NH, Shovitri M, Prasetyo EN, Setiawan E, Luqman A, Dwianita Kuswytasari N, Irawan C. 2021. Novel carbonatogenic bacterial strain isolated from limestone quarry in East Java, Indonesia to improve concrete performance. Biodiversitas 22 (9): 3890-3898. DOI: 10.13057/biodiv/d220935.