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Research article

Biomineralization of a calcifying ureolytic bacterium *Microbacterium* sp. GM-1

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ABSTRACT

Background: Biomineralization is a significant process performed by living organisms in which minerals are produced through the hardening of biological tissues. Herein, we focus on calcium carbonate precipitation, as part of biomineralization, to be used in applications for environmental protection, material technology, and other fields. A strain GM-1, *Microbacterium* sp. GM-1, isolated from active sludge, was investigated for its ability to produce urease and induce calcium carbonate precipitation in a metabolic process.
Results: It was discovered that *Microbacterium* sp. GM-1 resisted high concentrations of urea up to 60 g/L. In order to optimize the calcification process of *Microbacterium* sp. GM-1, the concentrations of Ni^{2+} and urea, pH value, and culture time were analyzed through orthogonal tests. The favored calcite precipitation culture conditions were as follows: the concentration of Ni^{2+} and urea were 50 μM and 60 g/L, respectively, pH of 10, and culture time of 96 h. Using X-ray diffraction analysis, the calcium carbonate polymorphs produced by *Microbacterium* sp. GM-1 were proven to be mainly calcite.
Conclusions: The results of this research provide evidence that *Microbacterium* sp. GM-1 can biologically induce calcification and suggest that strain GM-1 may play a potential role in the synthesis of new biominerals and in bioremediation or biorecovery.

1. Introduction

Diagenetic microorganisms in nature interact with the surrounding environment through enzymatic action. Nearly two-thirds of the main product of such microorganisms is calcium-bearing minerals or calcite minerals that have the vital role of cementation. Loose rock fragments in nature are cemented into hard rock formation through geological processes over long periods of time. In the process of biomineralization, bacteria induce certain sedimentation and organisms create a microenvironment in their vicinity with conditions that prompt the precipitation of extracellular mineral phases, such as $CaCO_3$ [1,2,3]. Calcium carbonate precipitation, which is performed by various bacterial genera in biomineralization, has been observed in numerous natural environments [1,3,4,5,6,7,8]. These bacteria hydrolyze urea to synthesize urease and produce dissolved ammonium, inorganic carbon, and CO_2. The ammonia released into the surrounding environment subsequently increases pH, leading to the accumulation of insoluble $CaCO_3$ in a calcium rich environment [9,10,11]. In this process, microorganisms provide nucleation sites for calcium carbonate precipitation.

Calcium carbonate precipitation in nature occurs in a wide range of environments [12,13,14,15,16,17]. In biomineralization, the polymorphs, morphology, and bulk density of calcium carbonate precipitation are affected by external conditions, such as environmental pH, temperature, reactant concentration, and precipitation time. However, natural environments lack careful and proper control for precipitation, and the types of precipitates and the conditions under which the precipitates are formed are often more variable, and less predictable [18,19]. Further, calcium carbonate precipitation induced by bacteria has been previously studied for its use in environmental remediation [20], CO_2 sequestration [21], and cementation of materials [22]. Ureolytic bacteria present promising characteristics for the induction of calcium carbonate precipitation should be studied under laboratory conditions for further application in bioremediation technologies. For instance, Ramachandran et al. reported that *Bacillus pasteurii* has certain influence on cementing composites [1]. For the remediation of damaged structural formations, Gollapudi et al. [23] employed selective microbial plugging in which metabolic activities induced calcium carbonate precipitation calcite [23].

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Ghosh et al. [5] discovered a bacterium that leached silica and affected the growth of new silicate phases where a concentration of 105 cells/mL optimized the microstructure of cementing aggregates. Additionally, Elbeik et al. [24] investigated the positive effect of calcite precipitation induced by bacteria as a protective film to prevent corrosion on mild steel [24].

In the present study, a urease synthesis bacterial strain GM-1 was isolated from active sludge and characterized by 16S rRNA gene sequence analysis, and the calcium carbonate polymorphs produced by the strain GM-1 were analyzed using X-ray diffraction. In addition, the biological calcification induced by the strain GM-1 under different conditions was determined. The results suggest that *Microbacterium* sp. GM-1 has potential application in bioremediation of contaminated environments and material technology.

2. Materials and methods

2.1. Bacterial strains and culture conditions.

Strain GM-1 isolated from active sludge was cultured with nutrient broth (NB) medium. To investigate the urease activity, strain GM-1 was cultured in a urease selective medium containing (L^{-1}) 1.0 g peptone, 5.0 g NaCl, 2.0 g KH_2PO_4, 2.0 g urea, and 0.1 g glucose with 0.2% phenol red (w/v) as the indicator. Urease selective medium without urea was employed as the control medium, and solid medium was produced by the addition of 1.5% agar. Unless otherwise stated, strain GM-1 was incubated in the medium at 30°C aerobically.

2.2. Bacteria identification

The purity of strain GM-1 was investigated by 16S rRNA gene sequence analysis, and the extraction of genomic DNA was performed with a TIANamp Bacteria DNA Kit (Tiangen). 16S rRNA gene was amplified using bacterial universal primers 27F (5′-AGAGTTTGATC CTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The conditions for PCR were as follows: 5 min of denaturation at 94°C, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min. PCR products were visualized by 0.8% agarose gel (w/v) electrophoresis and purified with a TIANgel Midi Purification Kit (Tiangen). Then, the purified DNA fragments were ligated into pMD19-T vector (TaKaRa) and sequenced by BGI Corp (Beijing). The plasmids were purified by a TIANpure Mini Plasmid Kit (Tiangen), and the 16S rRNA gene sequences were assembled using SeqmanII5.0 (DNASTAR). The partial 16S rRNA gene sequence was analyzed with the BLASTN program online (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Urea hydrolysis of GM-1

For preparation of the inoculum, a single colony of strain GM-1 was inoculated into NB medium and incubated on a rotary shaker (120 rpm) at 30°C for 24 h aerobically. To investigate the urea hydrolysis activity, the cells were inoculated in both urease selective plates and control plates using the streak plate method. Color variation was observed after 2 d of incubation.

2.4. Microbial growth detection

Strain GM-1 was inoculated in NB medium and amended with or without urea, then incubated in an orbital shaker (120 rpm) at 30°C. The growth of cells was identified by optical density at 600 nm (OD_{600}) using a microplate reader (Epoch, Bio-Tek). Cell growth was monitored in culture samples withdrawn at 2 h or 4 h intervals for up to 96 h. Culture samples (200 μL) were removed from the medium to determine OD_{600} each time. Growth calculations were performed at triplicates of each time period.

2.5. Urease activity

Urease activity has proven to be influential on the process of calcium carbonate precipitation induced by urea-hydrolyzing bacteria [25,26]. A conductivity method was performed to examine the urease activity. Urease hydrolyzes non-ionic urea into ionic ammonium and bicarbonate ions, which leads to a proportional increase in the conductivity of the medium. A single colony of strain GM-1 was inoculated into NB medium and incubated in an

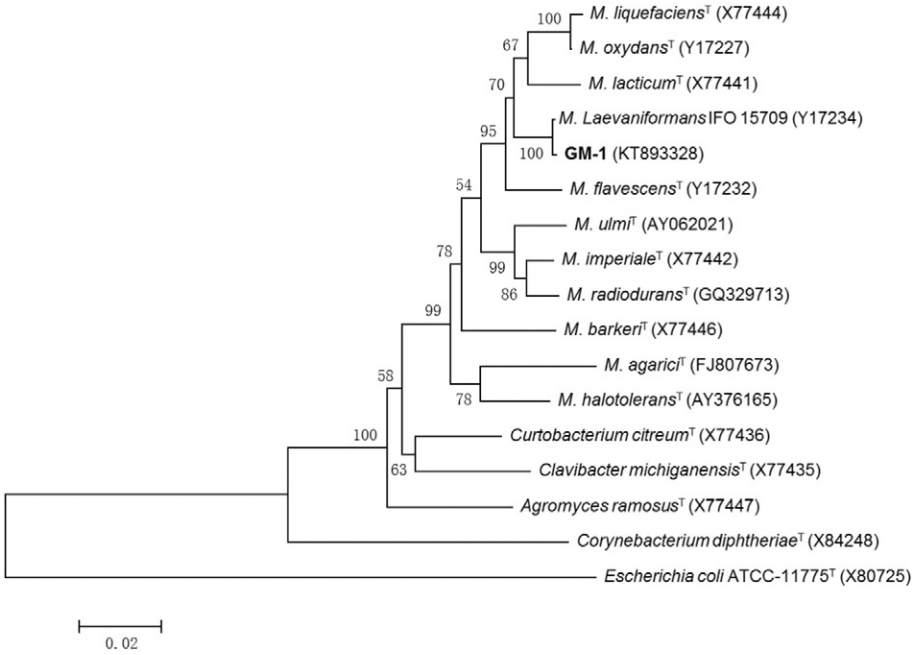

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain GM-1. *Escherichia coli* ATCC-11775^T was used for outgroup rooting. GenBank accession numbers are given in parentheses. Numbers at nodes represent confidence levels from 1000 replicate bootstrap samplings; only values greater than 50% are shown. Bar, 0.02 substitutions per nucleotide position.

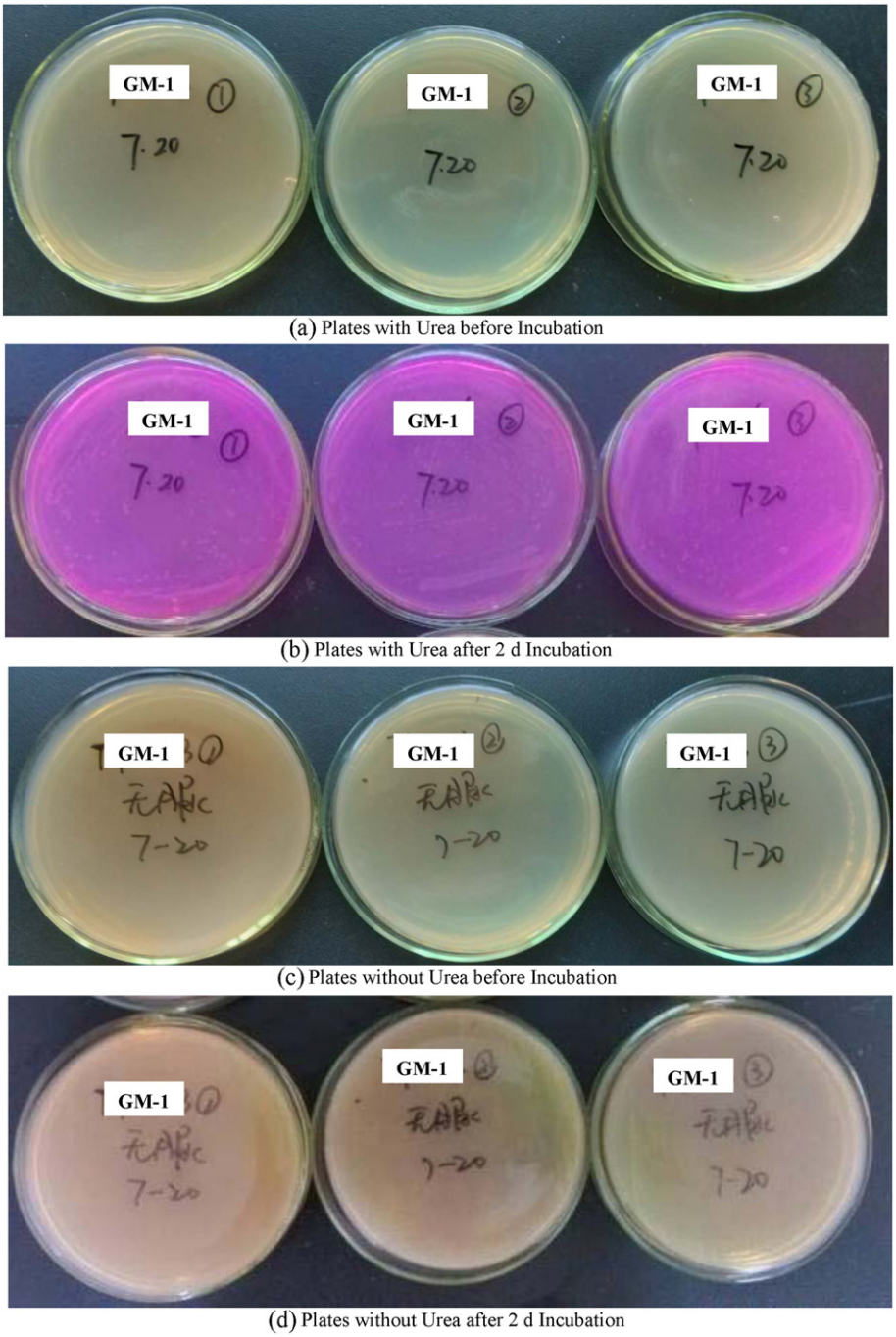

Fig. 2. (a) Plates with urea before incubation. (b) Plates with urea after 2 d incubation. (c) Plates without urea before incubation. (d) Plates without urea after 2 d incubation. The cells of GM-1 were inoculated on urease selective plates with or without urea using streak plate method.

orbital shaker (120 rpm) at 30°C for 96 h. During the process of incubation, 1 mL bacterial suspension was removed from the medium and added into a test tube containing 9 mL of 1.11 M urea (reaction concentration 1 M urea) at 12 h-intervals for 96 h. And the relative conductivity change of the solution (1 mL bacterial suspension + 9 mL of 1.11 M urea) was recorded over 5 min at 30 ± 2°C. One unit (U) of urease activity was defined as the increase in conductivity per min per OD_{600}.

2.6. Urea-resistant experiment

For preparation of the inoculums, a single colony of strain GM-1 was inoculated into NB medium and incubated on a rotary shaker (120 rpm) at 30°C for 24 h. Then the bacterial suspension was spread onto the NB medium supplemented with urea (filter sterilized). The initial concentrations of urea were 20, 40, 60, 80, and 100 g/L, and the conical flasks were incubated on a shaker at 120 rpm at 30°C. The optical density of bacteria at 600 nm in different concentrations of urea indirectly reflected the ability of resistivity to urea. To determine the OD_{600}, 15 incubation experiments (triplicates of each concentration of urea) were prepared.

2.7. Calcium carbonate precipitation

Strain GM-1 was inoculated into modified NB medium containing 20 g/L urea and incubated at 30°C (120 rpm). After incubation for 1, 2, and 3 d, 0.33 M Ca^{2+} was added into the modified NB medium

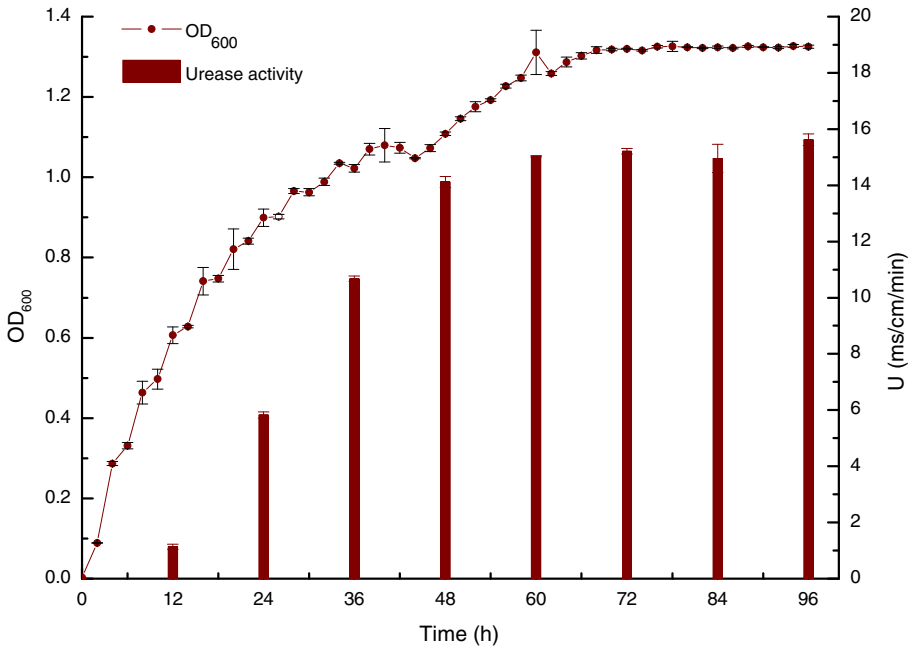

Fig. 3. Growth curve and urease activity of *Microbacterium* sp. GM-1. Overnight cultured cells of GM-1 were inoculated in NB medium and the OD_{600} was detected. One unit (U) of urease activity was defined as the increase of conductivity per min per OD_{600}. Mean values with standard deviations (error bars) from at least three repeats were shown.

with urea mentioned above. The precipitation was collected and analyzed by X-ray diffraction, and samples were evaluated by MDI Jade software. Samples without bacteria inoculation were used as negative controls.

2.8. Orthogonal test

According to Benini et al. [27], urease catalyzes the hydrolysis of urea, the final step of organic nitrogen mineralization, using a bimetallic nickel center. Thus, it was essential to probe the effect of Ni^{2+} on the amount of precipitation induced by GM-1. Filter sterilized Ni^{2+} solution was injected into the NB medium containing 2% urea with initial Ni^{2+} concentrations of 0, 100, and 200 μM, then the samples were incubated for 2 d. Moreover, an orthogonal test was carried out to probe the main factors that affect the amount of calcium carbonate precipitation, including concentrations of Ni^{2+} and urea, pH, and cultivation time. Concentrations of Ni^{2+} used in the medium were 0, 50, 100, and 200 μM; urea concentrations used in the medium were 30, 40, 50, and 60 g/L; pH values were 7, 8, 9, and 10; and culture times were 16, 24, 48, and 96 h. All conical flasks were incubated on a shaker at 30°C (120 rpm). After the culture process, 0.50, 0.67, 0.83, and 1.0 M Ca^{2+} were added into culture media with 30, 40, 50, and 60 g/L urea, respectively. Then, complete precipitation of cultures occurred prior to determining concentrations of Ca^{2+} in the supernatant solution of flasks using an atomic absorption spectrometer (AAS).

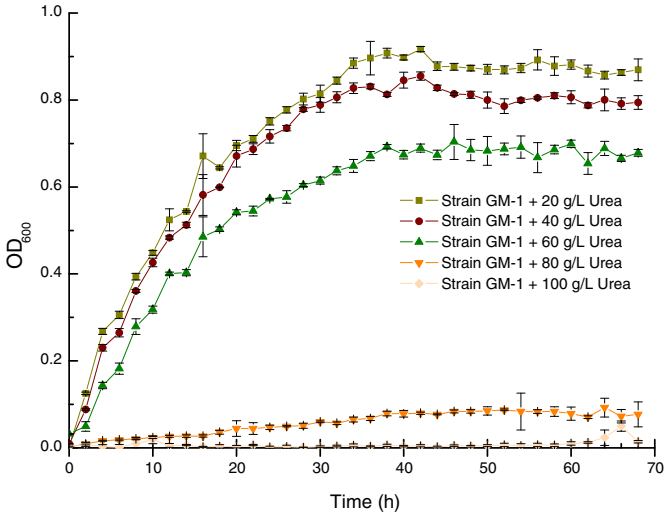

Fig. 4. Effect of different concentrations of urea on the growth of bacteria. Overnight cultured cells of GM-1 were inoculated in NB medium with urea (20 g/L, 40 g/L, 60 g/L, 80 g/L, 100 g/L, respectively). Mean values with standard deviations (error bars) from at least three repeats were shown.

3. Results and discussion

3.1. Isolation and identification of bacteria

The results show that the strain GM-1 belongs to the genus *Microbacterium*, and the phylogenic tree of the strain GM-1 related to *Microbacterium* is shown in Fig. 1. Molecular characterization by 16S rRNA gene sequencing and phylogenetic analysis, based on the neighbor joining method, was used to identify the strain GM-1 as *Microbacterium laevaniformans* with 99% similarities.

3.2. Urea hydrolysis of GM-1

The strain GM-1 was incubated in both urease selective medium and the control medium without urea. A remarkable color change from orange (Fig. 2a) to red (Fig. 2b) was observed for plates containing urea only 2 d after incubation, indicating a positive urease result. However, the color of the negative control (plates without urea) remained orange after 2 d (Fig. 2c and Fig. 2d). These results demonstrate that GM-1 is capable of urea hydrolysis, accounting for

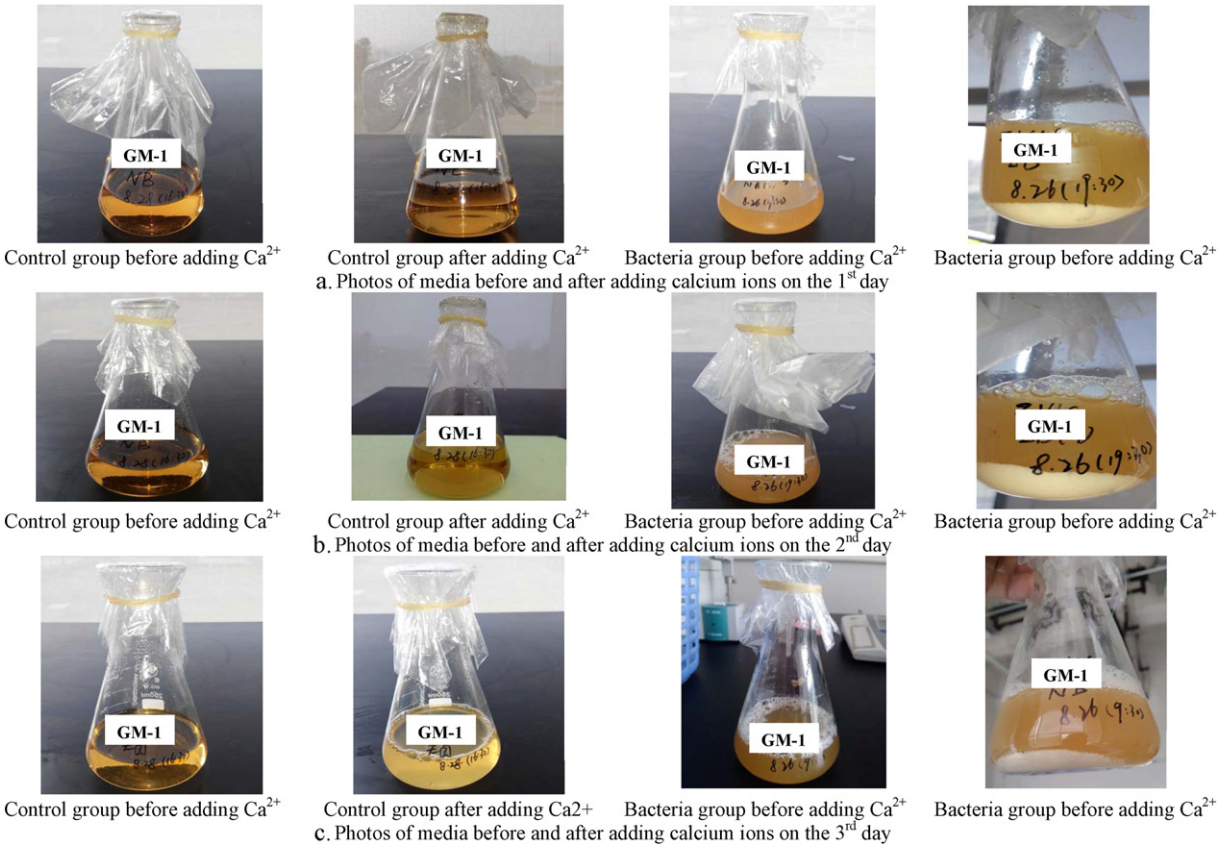

Fig. 5. Calcium carbonate precipitation experiment. (a) 1st day; (b) 2nd day; (c) 3rd day. Strain GM-1 was inoculated into NB medium contained 20 g/L urea and cultured aerobically (120 rpm) at 30°C. Uninoculated medium were the controls. All the cultures were added 0.33 M Ca^{2+} (final concentration).

the generation of CO_3^{2-} and NH_4^+. When NH_4^+ increases the pH value, phenolphthalein becomes more alkaline and changes to a red color.

3.3. Growth curve of bacterium GM-1

Fig. 3 displays the growth curve and urease activity of strain GM-1 in NB medium, which roughly shows that GM-1 grew rapidly. The exponential growth phase occurred between 2 and 40 h, and the growing pace of bacteria decreased between 40 to 96 h. The metabolism was active when cells were in the exponential growth phase, which contributed to urease synthesis and calcium carbonate precipitation. The optical incubation time was 24 h, as can be observed through the growth curve of GM-1. Urease activity increased quickly during the exponential growth phase and became constant at 15 ms/cm/min (conductivity per min per OD_{600}), which corresponds to the microbial growth tendency.

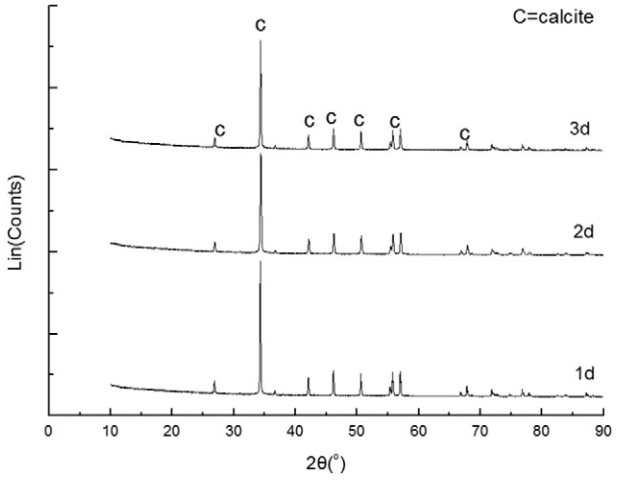

Fig. 6. XRD Analysis of white precipitate, obtained from bacteria groups. XRD spectra were obtained using a Cu anode and scanning from 10° to 90° 2θ. MDI Jade software was used to evaluate the samples.

3.4. Urea-resistant experiment

Urea is an indispensable element and the primary source of CO_3^{2-} in biomineralization. The concentration of urea, to some extent, affects the concentration of CO_3^{2-} and, therefore, the amount of calcium carbonate precipitation. Because high concentrations of urea inhibit the growth of bacteria, it is necessary to explore and determine the urea-resistance capacity of GM-1.

It can be observed in Fig. 4 that the urea concentration directly affects the growth of GM-1. When urea concentrations were 20, 40, and 60 g/L, the maximum optical densities were determined to be 0.7, 0.8, and 0.9, and cell growth of GM-1 was inhibited by 80 and 100 g/L urea. These results indicate that GM-1 can resist high concentrations of urea up to 60 g/L. Establishing a relationship between the optical density of bacteria and the concentration of urea may allow the

Table 1
Effect of different concentrations of Ni^{2+} on the amount of $CaCO_3$ induced by bacteria.

Ni^{2+} (μM)	Initial Ca^{2+} (g)	Ca^{2+} in supernatant (g)	$CaCO_3$(g)
0	1.333	0.853 ± 0.011	1.200 ± 0.028
100	1.333	0.662 ± 0.019	1.678 ± 0.048
200	1.333	0.804 ± 0.022	1.323 ± 0.055

Table 2
$L_{16}(4^5)$ Orthogonal test design.

Ni^{2+} (μM)	Urea (g/L)	pH	Time (h)
50	30	7	16
100	40	8	24
150	50	9	48
200	60	10	96

optimization of culture conditions when the concentration of urea is controlled between 20 and 60 g/L.

3.5. Experiment of calcium carbonate precipitation

In order to understand the interaction between the production of carbonate ions in the presence of ammonium and added calcium, a series of calcium carbonate precipitation experiments were carried out with incubation for 1, 2, and 3 d (Fig. 5). Uninoculated controls were used as a comparison for calcium carbonate precipitation during the experiments. In bacteria groups, some white precipitate was produced after the addition of Ca^{2+}, which also occurred for d 1, 2, and 3; however, there were no significant changes in control groups for these days. All of the precipitate was collected for XRD analysis in order to understand its composition, and the results reveal that calcium carbonate is the main composition of the precipitate (Fig. 6).

3.6. Optimization of the culture conditions

Table 1 shows the effect of Ni^{2+} on the amount of $CaCO_3$ induced by GM-1, which follows the sequence: 1.200 ± 0.028 g (0 μM), 1.678 ± 0.048 g (100 μM), and 1.323 ± 0.055 g (200 μM). This suggests that the presence of Ni^{2+} promoted the precipitation of calcium carbonate to some extent, but excessively high concentrations of Ni^{2+} reduced the amount of calcium carbonate. As suggested by Benini et al. [27], urease catalyzes the hydrolysis of urea, the final step of organic nitrogen mineralization, using a bimetallic nickel center.

An orthogonal test was designed based on the results listed in Table 2, and Table 3 shows the intuitive analysis and results of this test. Table 4 shows the variance analysis of the orthogonal test, where Equation 1, Equation 2, and Equation 3 were used to calculate the precipitation rate of Ca^{2+}.

Table 3
The intuitive analysis and result of the orthogonal test.

	Ni^{2+} (μM)	Urea (g/L)	pH	Time (h)	Precipitation rate (%)
1	50	30	7	16	70.751
2	50	40	8	24	75.088
3	50	50	9	48	77.594
4	50	60	10	96	99.330
5	100	30	8	48	68.484
6	100	40	7	96	75.269
7	100	50	10	16	79.021
8	100	60	9	24	81.499
9	150	30	9	96	68.688
10	150	40	10	48	74.718
11	150	50	7	24	78.814
12	150	60	8	16	81.369
13	200	30	10	24	69.613
14	200	40	9	16	76.174
15	200	50	8	96	78.231
16	200	60	7	48	81.093
K_{j1}	322.763	277.536	305.927	307.315	
K_{j2}	304.273	301.249	303.172	305.014	
K_{j3}	303.589	313.660	303.955	301.889	
K_{j4}	305.111	343.291	322.682	321.518	
k_{j1}	80.691	69.384	76.482	76.829	
k_{j2}	76.068	75.312	75.793	76.254	
k_{j3}	75.897	78.415	75.989	75.472	
k_{j4}	76.278	85.823	80.671	80.380	
R	4.794	16.439	4.878	4.908	

Table 4
Variance analysis of the orthogonal test.

Source	Type III sum of squares	df	Mean square	F[a]	Sig[b]
Ni^{2+}	64.045	3	21.348	0.994	0.502
Urea	561.816	3	187.292	8.723	0.054
pH	64.010	3	21.337	0.994	0.502
Time	56.496	3	18.832	0.877	0.542
Error	64.465	3	21.472		
Total	96,251.139	16			

[a] F: value of F test.
[b] Sig.: significant difference.

$$M_0 = \frac{M_u \times 40}{60} \quad \text{[Equation 1]}$$

$$M_p = M_0 - M_s \quad \text{[Equation 2]}$$

$$P(\%) = \frac{M_p}{M_0} \times 100\% \quad \text{[Equation 3]}$$

where M_u is the quantity of urea added into the medium (g); M_0 is the initial quantity of Ca^{2+} (g); M_s is the quantity of Ca^{2+} in culture supernatant (g); M_p is the quantity of Ca^{2+} in $CaCO_3$ (g); and P is the precipitation rate.

As shown in Table 3, the influence of four factors on the amount of calcium carbonate induced by GM-1 follows the sequence: urea concentration > culture time > pH value > Ni^{2+} concentration. The optimal culture conditions were: 50 μM Ni^{2+}, 60 g/L urea, pH 10, and culture time of 96 h in which the precipitation rate reached 99.33%. Further, Table 4 reveals that the concentration of urea plays the most important role in the induction of calcium carbonate precipitation.

4. Conclusions

The present study demonstrates that *Microbacterium* sp. GM-1, a Gram-Positive isolate of active sludge, induces urea hydrolyzation and calcium carbonate precipitation. The strain GM-1 resisted concentrations of urea up to 60 g/L and simultaneously produced urease, and when GM-1 was cultured with both urea and Ca^{2+}, calcium carbonate precipitation occurred. Optimized calcite precipitation culture conditions were determined to be: concentrations of 50 μM Ni^{2+} and 60 g/L urea, pH value of 10, and culture time of 96 h. We further discovered that calcite was the dominant calcium carbonate form of the calcium carbonate polymorphs produced by GM-1. The results of this research suggest that *Microbacterium* sp. GM-1 is a potential candidate for synthesis of new biominerals and has potential application in environmental bioremediation and biorecovery.

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