










## Article

# Isolation and Characterization of Indigenous Ureolytic Bacteria from Mindanao, Philippines: Prospects for Microbially Induced Carbonate Precipitation (MICP)

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**Abstract:** Microbially induced carbonate precipitation (MICP), a widespread phenomenon in nature, is gaining attention as a low-carbon alternative to ordinary Portland cement (OPC) in geotechnical engineering and the construction industry for sustainable development. In the Philippines, however, very few works have been conducted to isolate and identify indigenous, urease-producing (ureolytic) bacteria suitable for MICP. In this study, we isolated seven, ureolytic and potentially useful bacteria for MICP from marine sediments in Iligan City. DNA barcoding using 16s rDNA identified six of them as *Pseudomonas stutzeri*, *Pseudomonas pseudoalcaligenes*, *Bacillus paralicheniformis*, *Bacillus altitudinis*, *Bacillus aryabhatai*, and *Stutzerimonas stutzeri* but the seventh was not identified since it was a bacterial consortium. Bio-cementation assay experiments showed negligible precipitation in the control (without bacteria) at pH 7, 8, and 9. However, precipitates were formed in all seven bacterial isolates, especially between pH 7 and 8 (0.7–4 g). Among the six identified bacterial species, more extensive precipitation (2.3–4 g) and higher final pH were observed in *S. stutzeri*, and *B. aryabhatai*, which indicate better urease production and decomposition, higher CO<sub>2</sub> generation, and more favorable CaCO<sub>3</sub> formation. Characterization of the precipitates by scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDS) and attenuated total reflectance Fourier

transform spectroscopy (ATR-FTIR) confirmed the formation of three carbonate minerals: calcite, aragonite, and vaterite. Based on these results, all six identified indigenous, ureolytic bacterial species from Iligan City are suitable for MICP provided that the pH is controlled between 7 and 8. To the best of our knowledge, this is the first report of the urease-producing ability and potential for MICP of *P. stutzeri*, *P. pseudoalcaligenes*, *S. stutzeri*, and *B. aryabhatai*.

**Keywords:** calcium carbonate; microbially induced carbonate precipitation (MICP); ureolytic bacteria

## 1. Introduction

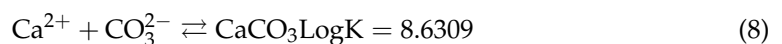
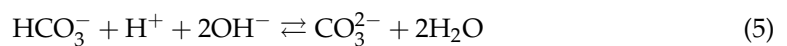
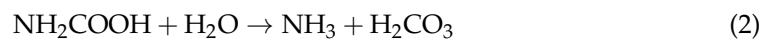
Microbially induced carbonate precipitation (MICP) is a bio-mineralization technique that uses metabolic pathways of naturally occurring bacteria to promote calcium carbonate ( $\text{CaCO}_3$ ) precipitation [1,2]. When precipitated between loose soil particles,  $\text{CaCO}_3$  acts as a binder that holds particles together, improving soil mechanical properties like strength and stiffness [3,4]. Qian et al. [5], for example, treated a sand column via injection of MICP solution and obtained an unconfined compressive strength (UCS) of about 2 MPa. Similarly high UCS values of between 1.76 and 2.04 MPa were reported by Zhao et al. [1] after submerging a premixed bacteria–sand matrix in a solution containing nutrients and cementing agents. Another application of MICP is in the removal of heavy metals from contaminated soils, wastes, and groundwater via adsorption and coprecipitation. Kang et al. [6], for instance, removed >99% of cadmium (Cd) as  $\text{CdCO}_3$  by MICP after 48 h of treatment in column experiments.

The presence of carbonate minerals like calcite and dolomite has also been shown to limit the leaching and transport of lead (Pb), copper (Cu), zinc (Zn), and arsenic (As) in tunnel-excavated rocks [7], contaminated soils and sediments [8], mine tailings [9], and industrial residues [10]. More recently, MICP has been receiving a lot of attention as a low-carbon alternative for ordinary Portland cement (OPC) in geotechnical engineering, and for its ability to impart “self-healing”—a property whereby small cracks are “healed” with time by filling them with carbonate minerals—in concrete and mortar [11,12]. The production and use of cement are major contributors to carbon dioxide ( $\text{CO}_2$ ) emissions [13], so shifting to the use of  $\text{CaCO}_3$  from MICP as a cementing agent for strengthening weak soils or other materials could reduce OPC use and limit its associated greenhouse gas emissions [14]. Another advantage of using MICP instead of OPC to stabilize weak soils is the circumneutral pH (6–8) of the former, which is more conducive for plant nutrient uptake and growth of most soil microorganisms [15].

There are four major metabolic processes for MICP: (i) urea hydrolysis, (ii) denitrification, (iii) iron salt reduction, and (iv) sulfate reduction [16,17]. Among them, urea hydrolysis is the most widely used not only because of its simple and easy-to-control reaction process but also because of its significant production of  $\text{CaCO}_3$  in a relatively short period [18,19]. Zaghloul et al. [20], for example, isolated marine bacteria identified as *Staphylococcus epidermidis* and reported high  $\text{CaCO}_3$  precipitation (~13 g/L) via urea hydrolysis. MICP via urea hydrolysis uses urease-producing (ureolytic) bacteria that produce urease as part of their metabolism [19]. Urease subsequently catalyzes the decomposition of urea ( $\text{CO}(\text{NH}_2)_2$ ) to carbamic acid ( $\text{NH}_2\text{COOH}$ ) and ammonia ( $\text{NH}_3$ ) (Equation (1)). Carbamic acid is unstable in water, rapidly decomposing into  $\text{NH}_3$  and  $\text{CO}_2$  (Equation (2)), enhancing  $\text{CaCO}_3$  precipitation by increasing the pH and solute activities of bicarbonate ( $\text{HCO}_3^-$ ) or carbonate ( $\text{CO}_3^{2-}$ ) in the solution (Equations (3)–(8)).

MICP via urease hydrolysis is a relatively straightforward technique that is dependent on two overarching factors: (i) microbial growth and urease secretion, and (ii) urease stability and inhibition. Microbial growth and enzyme secretion further affect the extent of  $\text{CaCO}_3$  formation depending on various environmental factors including the species of bacteria, bacterial abundance, urea concentration, calcium source, temperature, dissolved oxygen, and pH [21–23]. The pH of the medium, for example, plays an important role

by inducing morphological changes, which promotes microorganism growth, enzyme secretion, and stability of microbes [23]. Similarly, dissolved oxygen was highlighted by Skorupa et al. [24] as another important parameter because it could limit the growth and proliferation of commonly used ureolytic bacterial species like *Sporosarcina pasteurii* under saline and anaerobic conditions. Meanwhile, the urease catalytic reaction (Equation (1)) is influenced by not only the pH [25], temperature [25], and the system but also the presence of inhibitors like amides and esters of phosphoric acid, thiols, hydroxamic acids, phosphinic and thiophosphinic acids, boric and boronic acids, phosphate, heavy metal ions, bismuth compounds, quinones, and fluoride [26].



Another critical requirement for MICP is to use indigenous bacteria found locally to avoid environmental problems because of introducing non-indigenous or “alien” microorganisms to a region [27]. To date, this is only the second work in the Philippines to use indigenous bacteria for MICP. The first paper was published by Doctolero and coworkers [28], which used bacteria from the Philippine National Collection of Microorganisms (University of the Philippines Los Banos)—*Bacillus sphaericus* (BIOTECH 1272), *Bacillus subtilis* (BIOTECH 1679), and *Bacillus megaterium* (BIOTECH 1512)—isolated in the past from Laguna in the northern island of Luzon. These authors used these bacterial species to induce “self-healing” properties in oven-cured biochar-containing geopolymers and successfully sealed cracks having sizes between 0.1 and 0.65 mm via MICP. Unfortunately, indigenous bacteria for MICP have yet to be identified and isolated from the southern islands of the Philippines, and this work is the first report of ureolytic bacteria for MICP isolated from Mindanao, Philippines.

In this study, urease-producing marine bacteria in sediments from Iligan City, Mindanao, Philippines were isolated, identified, and grown for MICP. After initial screening, potent urease producers were identified by DNA barcoding and subjected to a biomineralization assay, a test that evaluated the  $\text{CaCO}_3$  production capability of bacterial isolates. The effects of initial pH on biomass and  $\text{CaCO}_3$  formation were also investigated to find the optimum pH for MICP while precipitates generated by the isolates were characterized by the gravimetric acid-washing technique, attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDS).

## 2. Materials and Methods

### 2.1. Sample Collection and Isolation of Ureolytic Bacteria

The bacterial samples used in this study were collected from the coastal areas of Iligan City in Northern Mindanao Island, Philippines (8.2280° N, 124.2452° E). The pH and temperature of seawater in the sampling area were around 7.3–7.5 and 30–32 °C, respectively. Meanwhile, the salinity and dissolved oxygen levels in Iligan Bay have been reported in the range of 34–35 ppt and 5.3–5.7 mg/L, respectively [29]. The isolation of ureolytic bacteria

followed the protocol of Anitha et al. [17]. In brief, coagulated marine sediments were collected using sterile polyethylene bags. After collection, fresh samples were crushed using a sterile mortar and pestle. The bacterial strains were isolated using the serial dilution technique, spread and cultured on nutrient agar prepared using aged seawater (28 g/L, sterilized by autoclaving at 121 °C for 15 min), and incubated at 37 °C for 24 h until visible colonies were obtained. From the nutrient agar, several phenotypically different colonies were collected for purification. The purification procedure of each bacterial isolate under investigation was carried out by repeated agar streak plate method [30].

## 2.2. Screening of Urease Producers

For the screening of urease producers, bacterial isolates were grown in Christensen's agar. The pH of the media was adjusted to 7 and prepared as described by Anitha et al. [17]. Several studies have reported that urea agar base can be used as a quick method to primarily screen for urease-producing bacteria suitable for MICP [31–33]. Urea agar base contains urea and phenol red, which acts as a pH indicator. When urea is hydrolyzed by the bacteria, NH<sub>3</sub> is released and becomes accumulated in the medium, which increases the pH of the environment making it alkaline [34]. Since urea is unstable and breaks during autoclaving, it was sterilized by filtration through a 0.22 µm syringe-driven membrane filter and introduced to the urea agar base medium. Isolates were inoculated and streaked on Christensen's agar plates and were incubated for 24–48 h at 37 °C. Isolates that turned the medium reddish or pinkish color may indicate positive urease production were selected and evaluated in more detail. The hydrolysis of urea by ureolytic bacterial isolates releases NH<sub>3</sub>, resulting in alkaline pH. Phenol red indicator turns pink at alkaline pH values, causing the media to turn pinkish or reddish [17].

## 2.3. Selection of Potent Urease Producer

The urea tolerance test was performed according to Anitha et al. [17], by adding 2% *w/v*, 6% *w/v*, and 10% *w/v* of urea on Christensen's agar plates. These plates were spot inoculated with positive isolates (i.e., urease producer) and incubated at 37 °C. The urease production test was performed via visual observation, and isolates showing rapid development of the pink color on the urea agar plates within 24 h of incubation [35,36] at a high concentration of urea were selected for further evaluation. Morphological characterizations of the bacteria, including colony morphology, gram stain reaction tests and selected biochemical tests, were performed using standard methods.

## 2.4. Genotypic Characterization of Bacterial Isolates

Selected bacterial isolates identified as ureolytic bacteria were sent to the 1st Base Laboratory, Malaysia, for bacterial DNA Barcoding. The bacterial 16S rDNA, full-length 1.5 kb, was amplified using universal primers 27F and 1492R. The total reaction volume of 25 µL contained gDNA purified using 0.3 pmol of each primer, deoxynucleotides triphosphates (dNTPs, 400 µM each), 0.5 U of thermostable DNA polymerase, supplied PCR buffer, and water. The polymerase chain reaction (PCR) was performed as follows: 1 cycle (94 °C for 2 min) for initial denaturation; 25 cycles (98 °C for 10 s; 53 °C for 30 s; 68 °C for 1 min) for denaturation, annealing, and extension. The PCR products were purified by the standard PCR clean-up method and subjected to bidirectional sequencing with universal primers 785F and 907R using BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Bedford, MA, USA).

## 2.5. Microbially Induced Carbonate Precipitation Assay

The identified isolates were subsequently assessed for their microbially induced calcite precipitation (MICP) potential. For this assay, a biomineralization medium was used, consisting of 0.25 M calcium chloride (CaCl<sub>2</sub>) solution, 0.5 M of urea solution, and 0.005 g/mL of nutrient broth per 150 mL consistent with published methods used in previous MICP studies [6,36–40]. CaCl<sub>2</sub> solution was autoclaved and filter-sterilized before mixing to

avoid any contamination while the urea solution was filter-sterilized only to avoid thermal hydrolysis at high temperatures. Two (2) mL of bacterial culture grown in nutrient broth at 30 °C for 24 h was added to 150 mL of the biomineralization media. To investigate the influence of pH on biomass production, biomineralization media for each of the bacterial isolates were prepared at three varying pH levels: pH 7, pH 8, and pH 9. Sterile biomineralization media without bacterial isolates were also used as a control. The media were then incubated in a rotary shaker at 120 rpm and 30 °C for 7 days, and changes in the pH were monitored and recorded every 24 h. The pH was checked aseptically to avoid any potential contamination. After 7 days of incubation, the solution was filtered through a sterile 0.6 µm Whatman® membrane filter (Whatman®, Kent, UK). Each filter paper was put in a separate sterile Petri dish and air-dried at 37 °C for 24 h for subsequent analyses. All incubation experiments were carried out in triplicates to ensure that the differences observed were statistically significant.

### 2.6. Characterization of Precipitates

The yield of biomass/precipitates from the MICP assay was weighed using an analytical balance and those with more than 1 g were further characterized using ATR-FTIR (IR Tracer 100, Shimadzu Corporation, Kyoto, Japan) and SEM-EDS (Hitachi SU3500, Hitachi High-Technologies Corporation, Kyoto, Japan). The mass of CaCO<sub>3</sub> was also determined in representative samples using the gravimetric acid-washing technique [11]. For this method, predetermined amounts of biomass/precipitates were mixed with 100 mL of 5 M HCl and shaken in a thermostat shaker at 100 rpm for 1 h. The residue was then rinsed twice with distilled water, filtered through Whatman no. 2 filter paper, oven-dried, and weighed. The mass difference before and after acid washing was used to calculate the percentage of CaCO<sub>3</sub> in the biomass/precipitates.

### 2.7. Data and Statistical Analysis

The data were expressed as mean and standard deviation of three replicates, and the results were analyzed by one-way analysis of variance (ANOVA) using IBM SPSS V.23 at the significance level of  $p < 0.05$ . The ATR-FTIR spectra were deconvoluted by the Fityk 0.9.8 software using Gaussian peak models [41], while carbonate speciation and precipitation diagrams were created using the Geochemist's Workbench (GWB®, Aqueous Solutions LLC, Champaign, IL, USA) based on the actual solute activity of Ca<sup>2+</sup> used in the experiments.

## 3. Results and Discussion

A total of 36 bacterial isolates were initially identified and screened for their strong urease-producing potential and, among them, 10 isolates were selected based on the urea potency test results. These 10 isolates were further evaluated for their urease-producing ability by culturing them on Christensen's agar plates with 10% urea. Out of the 10 bacterial isolates, 7 samples—coded as S1S6, S1S7, S1S8, S1S19, S2S6, S3S12, and S4S8—exhibited the highest urease activity potential based on the rapid development of the pink color of the urea agar plates within 24 h of incubation in all urea concentrations. Isolates that changed the media color beyond 24 h were excluded in succeeding experiments. The selected bacterial isolates were then initially identified based on colony morphology and biochemical characteristics as summarized in Tables 1 and 2, respectively. Using Bergey's manual [42], comparisons between the biochemical profiles of the isolates were undertaken. Four isolates shared biochemical traits consistent with the *Pseudomonas* genus while the remaining three isolates displayed biochemical attributes aligning closely with characteristic features of the *Bacillus* genus.

**Table 1.** Morphological characteristics of bacterial isolates.

Isolate Code	Gram Stain	Colony Morphology			
		Texture	Size	Color	Form
S1S6	–	smooth	small	white	circular
S1S7	+	smooth	small	white	circular
S1S8	–	smooth	small	white	circular
S1S19	–	smooth	small	white	circular
S2S6	–	smooth	large	colorless	Irregular
S3S12	+	rough	small	yellow	Irregular
S4S8	+	smooth	small	yellow	Circular

Note: “–” means “gram negative staining”; “+” means “gram positive staining”.

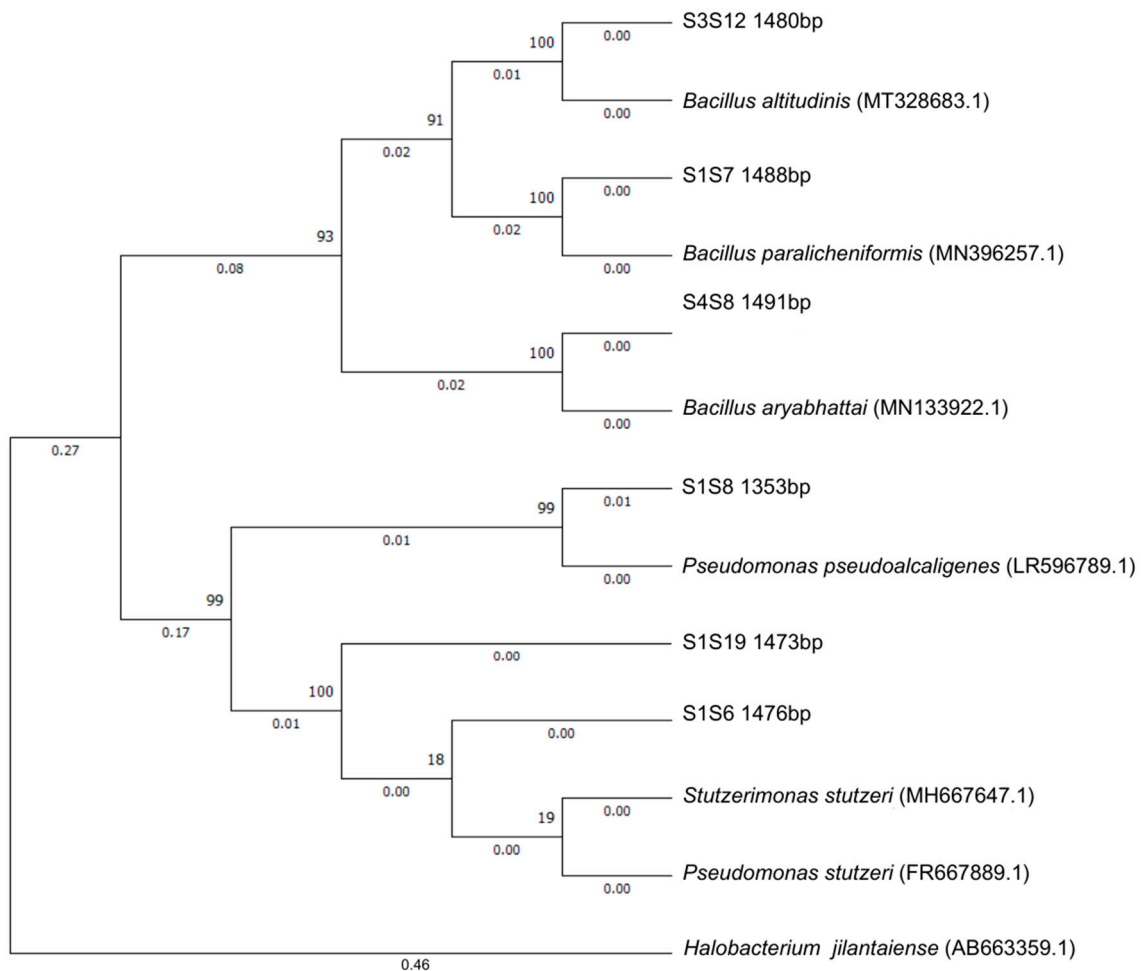
**Table 2.** Biochemical characteristics of bacterial isolates.

Isolate Code	Oxygen Requirement	Sugar	Motility	H <sub>2</sub> S Production	Citrate	Oxidase Test	Catalase Test	Coagulase Test	Methyl Red	Vogues Proskauer	Growth in MacConkey Agar	Growth in Cetrimide Agar	Initial Identification
S1S6	Obligate aerobes	Absence of carbohydrate fermentation	–	–	+	+	+	–	–	–	+	+	<i>Pseudomonas</i> sp.
S1S7	Facultative Anaerobes	Fermentation of dextrose, lactose, sucrose	–	–	+	–	+	+	–	–	–	–	<i>Bacillus</i> sp.
S1S8	Obligate Aerobes	Dextrose fermentation only	+	–	–	+	+	–	–	–	+	+	<i>Pseudomonas</i> sp.
S1S19	Facultative Anaerobes	Dextrose fermentation only	–	+	–	+	+	–	–	–	+	–	<i>Pseudomonas</i> sp.
S2S6	Obligate Aerobes	Fermentation of dextrose, lactose, sucrose	+	–	+	+	+	–	–	–	+	–	<i>Pseudomonas</i> sp.
S3S12	Facultative Anaerobes	Dextrose fermentation only	+	–	–	–	+	+	–	–	–	–	<i>Bacillus</i> sp.
S4S8	Facultative Anaerobes	Fermentation of dextrose, lactose, sucrose	–	–	–	–	+	+	–	–	–	–	<i>Bacillus</i> sp.

Note: “–” means “absent”; “+” means “present”.

For more detailed identification, the bacterial isolates were subjected to 16s rDNA barcoding analysis, and the results were analyzed using NCBI BLAST to match with reference sequences from the database. The sequence with the highest percent identity value was used as the reference sequence for the bacterial isolates. Based on the NCBI BLAST results (Table 1; Figure 1), two of the isolates were identified as belonging to the genus *Pseudomonas*: S1S6 (*Pseudomonas stutzeri*) and S1S8 (*Pseudomonas pseudoalcaligenes*). Both *Pseudomonas* spp. are well-known as denitrifying bacteria and have been used as biocleaning agents for the removal of nitrate and sulfate salts from wall paintings and heritage structures [36,41,43–45]. To the best of our knowledge, however, this is the first report of their urease-producing behavior and potential for MICP. Meanwhile, three of the bacterial isolates were identified as belonging to the genus *Bacillus*: S1S7 (*Bacillus paralicheniformis*), S3S12 (*Bacillus altitudinis*), and S4S8 (*Bacillus aryabhatai*). *Bacillus paralicheniformis* was recently reported by Hoffman et al. [46] and Rajabi Agereh et al. [47] as a urease-producing bacterium suitable for MICP. Similarly, *Bacillus altitudinis* has been reported to generate carbonate minerals in mangroves and manganese-contaminated environments [48,49]. In contrast, this is only the second report in the literature on the urease-producing ability of *Bacillus aryabhatai* and its suitability for MICP [50]. One bacterial isolate was identified as *Stutzerimonas stutzeri* (S1S19) belonging to the genus *Stutzerimonas*, a recently defined genus that was previously classified as part of the genus *Pseudomonas* [51]. Although this

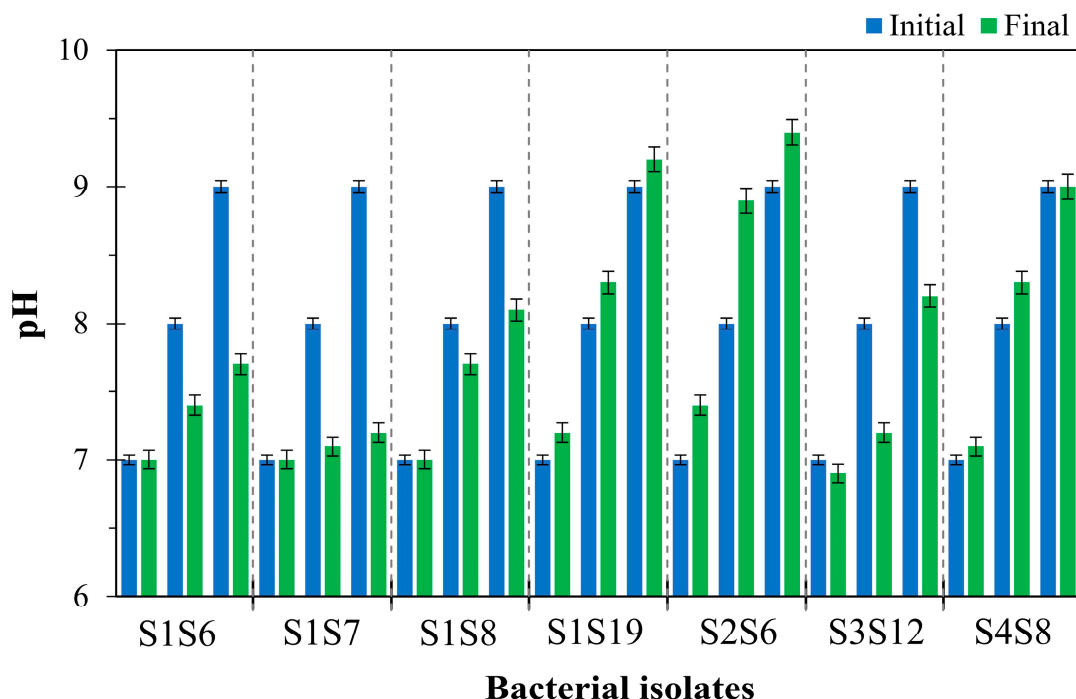
bacterium had previously been isolated from wastewater sludge and seawater and was found capable of degrading residual pharmaceuticals like paracetamol [52,53], this is the first report of its urease-producing capability and potential for MICP. Finally, bacterial isolate S2S6 was not identified due to the presence of multiple microbial types within the submitted culture, warranting further purification. The biochemical identification and DNA barcoding results were complementary except for the microbial consortium, S2S6 isolate.



**Figure 1.** Molecular phylogenetic analysis (Mega v.11.0.10) by maximum likelihood method and Tamura–Nei model with bootstrap analysis based on 1000 replications on partial 16s rDNA sequences from our isolates and reference sequence from NCBI BLAST, illustrating the phylogenetic position of the six isolates and its related taxa.

The final pH of the isolates either decreased or increased depending on the bacterial species (Figure 2). At pH 7, there were no observed changes in the pH level of all isolates after 168 h. Under pH 8 conditions, four isolates—*P. stutzeri* (S1S6), *P. pseudoalcaligenes* (S1S8), *B. paralicheniformis* (S1S7), and *B. altitudinis* (S3S12)—exhibited a noticeable decrease in pH, while two isolates—*S. stutzeri* (S1S19) and *B. aryabhatai* (S4S8)—showed a slight pH increase. At pH 9, the decrease in pH of the four isolates noted above was more significant, with only a minimal pH increase observed for the remaining two isolates. All bacterial isolates, regardless of the initial pH, generated biomass/precipitates, which were absent in the controls (without bacteria) (Figure 3). More extensive generation of biomass/precipitates was also observed at pH 7 and 8, depending on the bacterial species. The highest amounts of biomass/precipitates were obtained at an initial pH of 8 for *P. stutzeri* (S1S6), *B. paralicheniformis* (S1S7), *P. pseudoalcaligenes* (S1S8), *S. stutzeri*

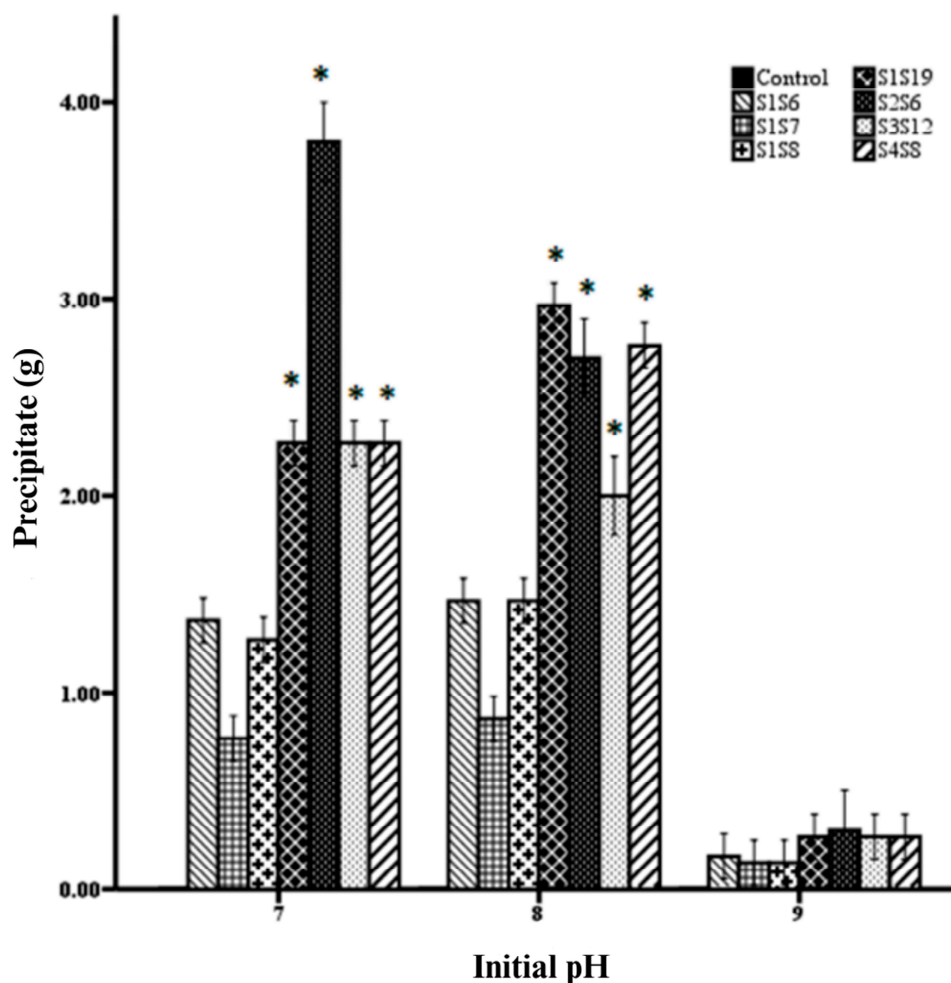
(S1S19), *B. altitudinis* (S3S12), and *B. aryabhatai* (S4S8). At an initial pH of 9, however, the biomass/precipitates produced in all bacterial isolates dramatically decreased (Figure 3).



**Figure 2.** Initial and final pH of bacterial isolates in the biocementation assay experiments (S1S6: *P. stutzeri*; S1S7: *B. paralicheniformis*; S1S8: *P. pseudoalcaligenes*; S1S19: *S. stutzeri*; S2S6: microbial consortium; S3S12: *B. altitudinis*; S4S8: *B. aryabhatai*).

The pH of the system is controlled by bacterial metabolic processes and chemical precipitation of carbonates (Equations (6)–(8)) [29]. The breakdown of urea, and the formation of urease and its by-products by bacteria generates alkalinity ( $\text{OH}^-$ ) via  $\text{NH}_3$  generation and hydrolysis (Equations (1)–(4)), while the formation, dissolution, and speciation of  $\text{CO}_2$ , including carbonate precipitation, lower the pH via proton ( $\text{H}^+$ ) production (Equations (4), (6), and (7)). In addition, the pH of the isolates could be directly influenced by bacteria because of their inherent pH tolerance and buffering ability [52]. It is interesting to note that biomass/precipitates were more abundant in *S. stutzeri* (S1S19), *B. aryabhatai* (S4S8), and microbial consortium S2S6 (Figure 3), which could be attributed to higher bacterial activity, better urease decomposition and  $\text{CO}_2$  generation (Equations (1)–(4)), and enhanced precipitation of carbonate minerals under alkaline conditions (Equations (7) and (8)). As illustrated in Figure 4a, a higher concentration of dissolved  $\text{CO}_2$  (i.e., higher  $\text{CO}_2$  fugacity due to microbial activity (Equation (2))) promoted calcite formation by not only lowering the pH of nucleation from  $\sim 9$  to  $\sim 6.5$  but also enhancing the overall extent of precipitation. In addition, dissolved inorganic carbon starts to speciate at pH 8 to the carbonate ion ( $\text{CO}_3^{2-}$ ), which is more readily precipitated than carbonic acid ( $\text{H}_2\text{CO}_3$ ) and bicarbonate ion ( $\text{HCO}_3^-$ ) as implied by the positive Log K of reaction between  $\text{CO}_3^{2-}$  and  $\text{Ca}^{2+}$  (i.e., more spontaneous process) (Figure 4b; Equations (6)–(8)).

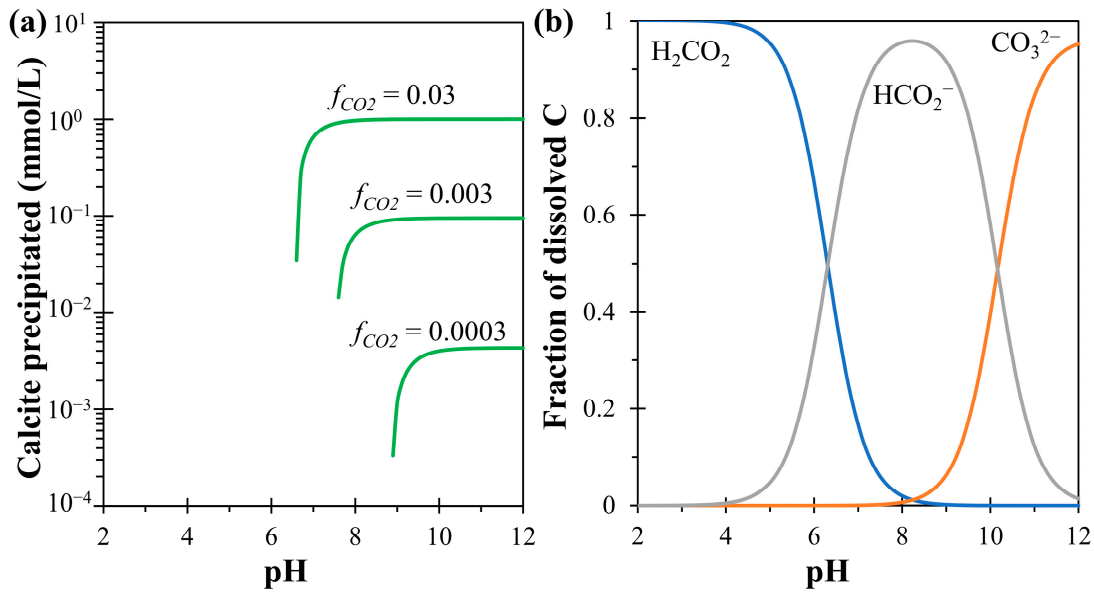




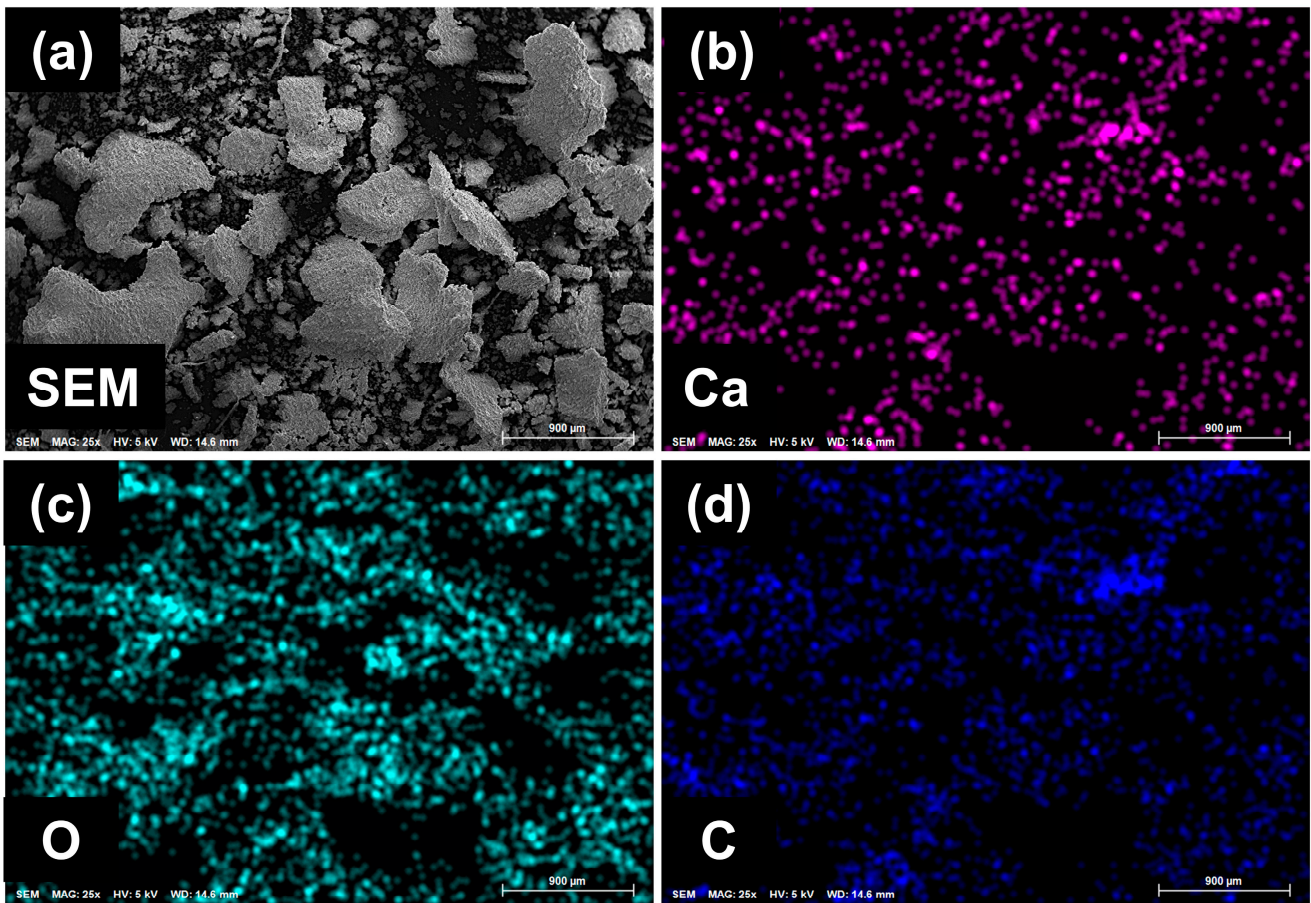
**Figure 3.** Amounts of biomass/precipitates collected from each bacterial isolate as a function of pH (S1S6: *P. stutzeri*; S1S7: *B. paralicheniformis*; S1S8: *P. pseudoalcaligenes*; S1S19: *S. stutzeri*; S2S6: microbial consortium; S3S12: *B. altitudinis*; S4S8: *B. aryabhatai*). Cultivation of the isolated ureolytic bacteria was performed in a medium containing 0.25 M  $\text{CaCl}_2$  and 0.5 M urea in 250 mL flask. Vertical error bars indicate standard deviations, and ANOVA with Tukey's procedure was used to compare the variance between different groups at a level of significance of 0.05 (\*).

Regardless of the pH, the biomass/precipitates produced by the bacterial isolates had flaky morphologies and were predominantly composed of Ca, C, and O based on SEM-EDS observations (Figures 5 and 6). The biomass/precipitates collected from the isolates were further characterized by ATR-FTIR, and the results showed that they were a mixture of bacterial debris, entrained agar, adsorbed water, and calcium carbonates (Figure 7). In *P. stutzeri* (S1S6), *P. pseudoalcaligenes* (S1S8), and *B. altitudinis* (S3S12) at an initial pH of 7, a broad infrared (IR) absorption band was observed centered at  $1645\text{ cm}^{-1}$ , which was assigned to Amide I ( $\nu\text{ C=O}$ ,  $\nu\text{ C-N}$ ) vibrations of proteins and peptides present in bacterial biomolecules [53]. The final pH in these three bacterial isolates decreased below 7, which likely promoted bacterial deaths in the system, a deduction supported by the presence of carbohydrates (i.e., excess food) in the biomass/precipitates as implied by the IR band at  $1020\text{ cm}^{-1}$  and shoulder at  $1110\text{ cm}^{-1}$  [53]. The IR peaks of biomolecules and carbohydrates were not observed in bacterial isolates *B. aryabhatai* (S4S8) and *S. stutzeri* (S1S19), both of which have an initial pH of 8 and final pH of  $\sim 8.5$ . The disappearance of these IR bands was likely because of the more conducive environment for bacterial growth and proliferation, fewer bacterial deaths, and more rapid consumption of available nutrients in solutions like carbohydrates. This deduction is supported by the pH trends and amounts of

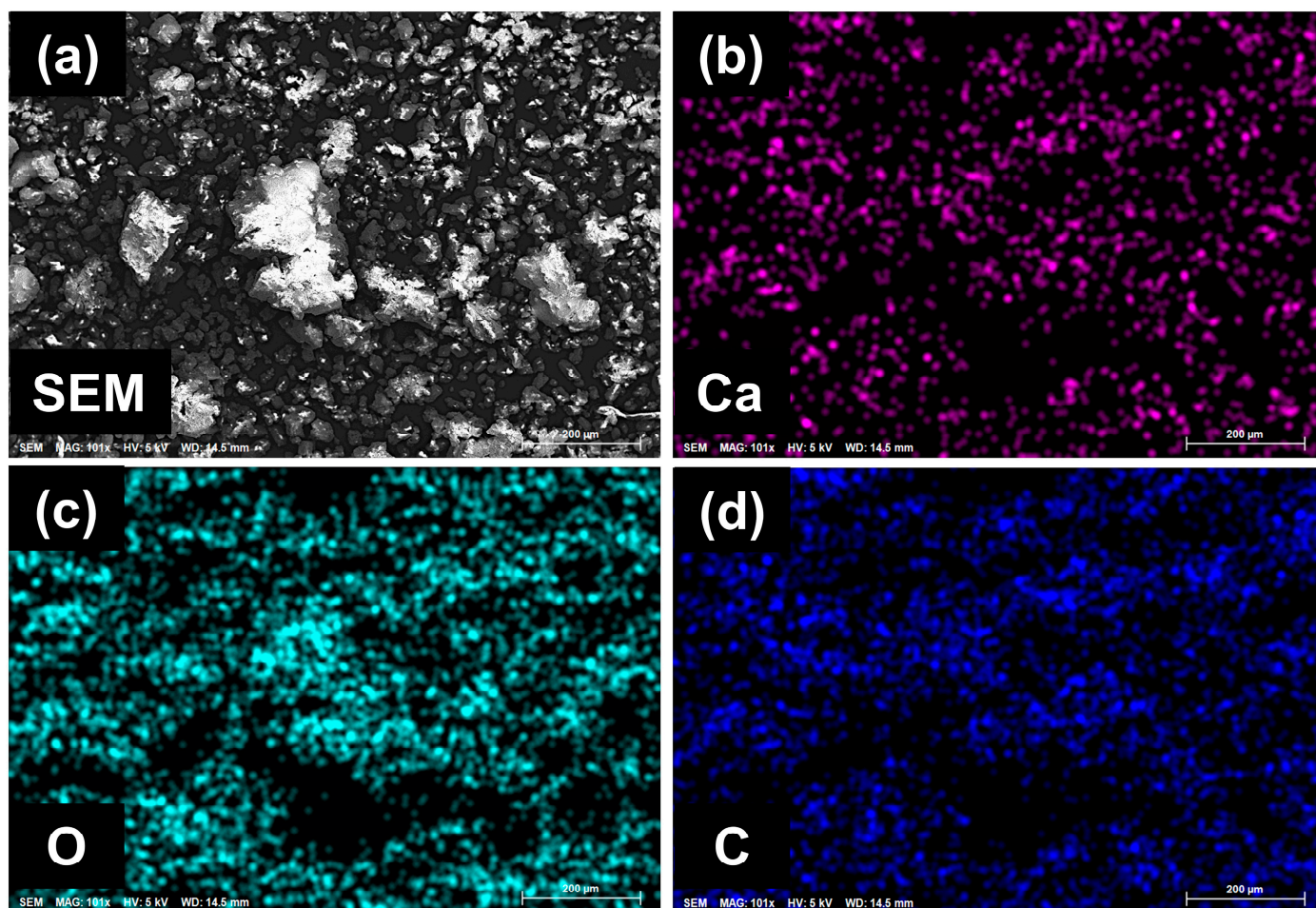
biomass/precipitates noted previously. Meanwhile, the broad IR absorption peak between  $3700$  and  $3000\text{ cm}^{-1}$  could be attributed to adsorbed water, which is to be expected because the biomass/precipitates were only air-dried (Figure 7b) [19].



**Figure 4.** (a) Precipitation of calcite with pH at different  $CO_2$  fugacities ( $f_{CO_2}$ ), and (b) equilibrium speciation diagram of dissolved inorganic carbon equilibrated with atmospheric  $CO_2$ .



**Figure 5.** SEM-EDS observation of biomass/precipitates formed in S1S19 (*S. stutzeri*) at pH 8 (a) and elemental maps of Ca (b), O (c), and C (d).

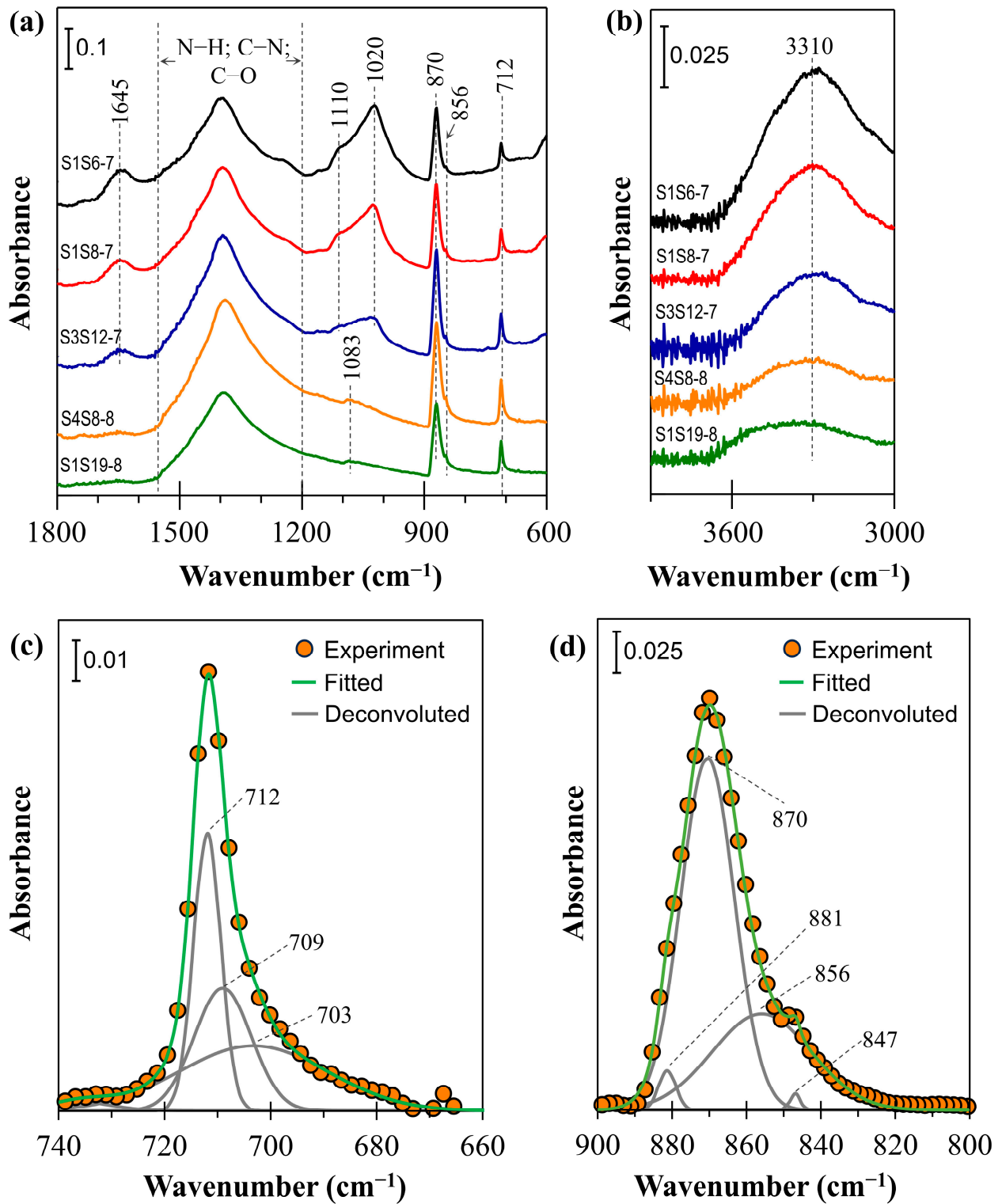


**Figure 6.** SEM-EDS observation of biomass/precipitates formed in S1S8 (*P. pseudoalcaligenes*) at pH 9 (a) and elemental maps of Ca (b), O (c), and C (d).

The ATR-FTIR spectra also detected the presence of three carbonate minerals—calcite, aragonite, and vaterite—in all five isolates (Figure 7a). The IR absorption bands at 870 and 712  $\text{cm}^{-1}$  are assigned to the out-of-plane and in-plane carbonate deformation vibrations in calcite, respectively [54,55]. More IR bands of carbonate minerals were revealed by deconvoluting the complex peaks around 740–660  $\text{cm}^{-1}$  and 900–800  $\text{cm}^{-1}$  (Figure 7c,d). The IR bands at 1083, 856, 847, 709, and 703  $\text{cm}^{-1}$  could be attributed to carbonate stretching, bending, and deformation vibrations in aragonite while those at 881  $\text{cm}^{-1}$  are assigned to the out-of-plane carbonate bending vibration in vaterite [54,55]. Finally, the  $\text{CaCO}_3$  production of bacterial isolates were estimated using the gravimetric acid-washing technique, and the results suggest around 75% to 95%  $\text{CaCO}_3$  abundance in the generated biomass/precipitates (Table 3). Based on these results, all 6 bacterial isolates—*P. stutzeri*, *P. pseudoalcaligenes*, *B. paralicheniformis*, *B. altitudinis*, *B. aryabhatai*, and *S. stutzeri*—are promising for MICP provided that the pH is controlled between 7 and 8.

**Table 3.** Percentage of carbonates in biomass/precipitates from the biomineralization assay.

Sample	Mass of Precipitate (mg)	Mass of Carbonate (mg)	% Carbonate
S1S19	105.7	97.1	91
S3S12	214.5	203.7	95
S4S8	98.8	74.7	75.6



**Figure 7.** ATR-FTIR spectra of biomass/precipitates in bacterial isolates at 1600–600 cm<sup>-1</sup> (a) and 3800–3000 cm<sup>-1</sup> (b), and deconvoluted peaks at 740–660 cm<sup>-1</sup> in S4S8 at initial pH 8 (c), and 740–660 cm<sup>-1</sup> in S4S8 at initial pH 8 (d). Note that S1S6-7 is *P. stutzeri* at initial pH 7, S1S8-7 is *P. pseudoalcaligenes* at initial pH 7, S3S12-7: *B. altitudinis* at initial pH 7; S4S8-8 is *B. aryabhatai* at initial pH 8, and S1S19-8 is *S. stutzeri* at initial pH 8.

#### 4. Conclusions

This study isolated indigenous, ureolytic bacteria from Iligan City, Philippines, identified them using DNA barcoding, and elucidated their potential for MICP using the biomineralization assay, including the characterization of biomass/precipitates by the gravimetric acid-washing technique, ATR-FTIR and SEM-EDS. The findings of this study are summarized as follows:

1. Seven (7) bacterial isolates with strong potential for urease production were identified out of the 36 initially screened isolates based on the urea tolerance test.
2. Among the seven isolates with strong urease production potential, two were identified from the genus *Pseudomonas* (*P. stutzeri* and *P. pseudoalcaligenes*), three from the genus *Bacillus* (*B. paralicheniformis*, *B. altitudinis*, and *B. aryabhatai*), one from genus *Stutzerimonas* (*S. stutzeri*), and one remained as a microbial consortium (S2S6), which needs further studies for isolation and identification of bacterial species.
3. The six identified bacterial isolates performed better at pH 8 than under more alkaline conditions (pH 9).
4. The observed high carbonate precipitation in *S. stutzeri* and *B. aryabhatai* could be attributed to their more alkaline final pH, implying better urease formation and decomposition, CO<sub>2</sub> generation, and CaCO<sub>3</sub> precipitation under these conditions.
5. Three carbonate minerals—calcite, aragonite, and vaterite—were identified in the precipitates by SEM-EDS and ATR-FTIR.

The findings of this study contribute valuable insights into the utilization of indigenous, urease-producing bacteria isolated from the southern island of the Philippines for MICP as a sustainable technology in the local construction and geotechnical engineering sectors, including efforts for environmental rehabilitation and carbon capture, storage, and sequestration.

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