

## Article

# Characterization of a Novel CaCO<sub>3</sub>-Forming Alkali-Tolerant *Rhodococcus erythreus* S26 as a Filling Agent for Repairing Concrete Cracks

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**Abstract:** Biom mineralization, a well-known natural phenomenon associated with various microbial species, is being studied to protect and strengthen building materials such as concrete. We characterized *Rhodococcus erythreus* S26, a novel urease-producing bacterium exhibiting CaCO<sub>3</sub>-forming activity, and investigated its ability in repairing concrete cracks for the development of environment-friendly sealants. Strain S26 grown in solid medium formed spherical and polygonal CaCO<sub>3</sub> crystals. The S26 cells grown in a urea-containing liquid medium caused culture fluid alkalization and increased CaCO<sub>3</sub> levels, indicating that ureolysis was responsible for CaCO<sub>3</sub> formation. Urease activity and CaCO<sub>3</sub> formation increased with incubation time, reaching a maximum of 2054 U/min/mL and 3.83 g/L, respectively, at day four. The maximum CaCO<sub>3</sub> formation was achieved when calcium lactate was used as the calcium source, followed by calcium gluconate. Although cell growth was observed after the induction period at pH 10.5, strain S26 could grow at a wide range of pH 4–10.5, showing its high alkali tolerance. FESEM showed rhombohedral crystals of 20–60 μm in size. EDX analysis indicated the presence of calcium, carbon, and oxygen in the crystals. XRD confirmed these crystals as CaCO<sub>3</sub> containing calcite and vaterite. Furthermore, *R. erythreus* S26 successfully repaired the artificially induced large cracks of 0.4–0.6 mm width.

**Keywords:** calcium carbonate; cementitious materials; concrete; repair; ureolysis



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## 1. Introduction

Calcium carbonate (CaCO<sub>3</sub>) formation is a representative example of bacterial mineralization occurring because of the involvement of various bacterial genera in a wide range of natural environments; this process is known as bacterially induced CaCO<sub>3</sub> precipitation (BICP) [1]. Recent studies on BICP cover a variety of fields with important implications for geobiotechnology, civil engineering, and the environment, such as biocement, concrete reinforcement, soil stabilization, carbon dioxide sequestration, wastewater treatment, heavy metal bioremediation, and oil recovery [2,3]. The most promising among them is the use of BICP to repair damaged concrete structures and historically important stone cultural heritages [4].

Various processes, such as physicochemical weathering, biological corrosion, ground subsidence, and human activity, cause cracks in concrete and stone structures. If not treated properly and quickly, cracks will expand further, eventually requiring costly repairs. Currently, resin mortar and epoxy are used to repair cracks, but they react with nitrogen oxides to create ozone, a highly toxic air pollutant which is harmful to humans and the environment [5]. Therefore, from the viewpoint of eco-friendliness and cost reduction, new cementitious materials for repairing structural cracks such as BICP, which is known as the

self-healing technique, have gained attention. Self-healing materials refer to substances that can restore themselves to their original state. In particular, bacterially induced self-healing is described as the process of autonomously sealing cracks generated on the surface of concrete structures by chemical changes (i.e.,  $\text{CaCO}_3$  formation) owing to the metabolic activity of bacteria [5,6].

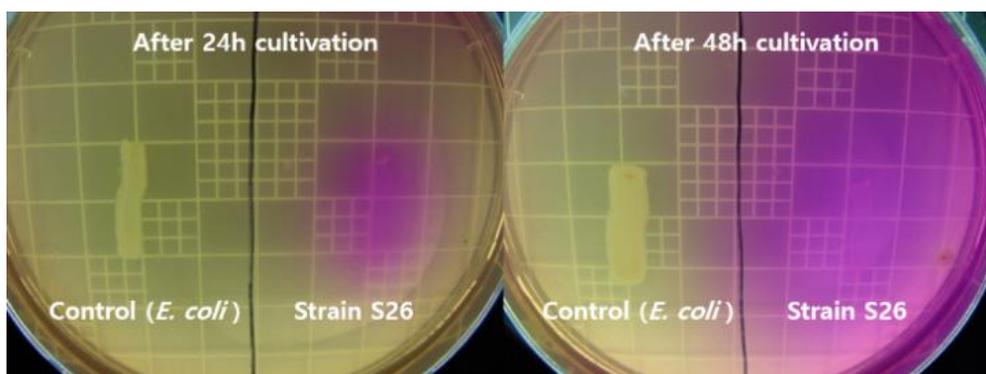
Several processes have been found in which  $\text{CaCO}_3$  is formed by bacteria, including urea hydrolysis, denitrification, sulfate reduction, photosynthesis, and methane oxidation [6,7]. Among them, urea hydrolysis is the most studied and widely applied because of its excellent  $\text{CaCO}_3$  formation efficiency [8,9]. To date, most investigations on BICP by ureolytic bacteria have been conducted using the urease-producing strain of *Sporosarcina pasteurii* [10–12]. This strain can increase the pH by hydrolyzing urea to produce ammonium and bicarbonate ions, thereby accelerating  $\text{CaCO}_3$  formation [8]. However, because concrete is a harsh environment with a high pH, crack repair requires microorganisms that can survive and grow in such an environment. Therefore, the isolation and characterization of other extreme microorganisms are important tasks with regard to securing new biological resources for broader industrial applications.

As part of a project on the isolation of extreme microorganisms, we previously isolated a new strain capable of forming  $\text{CaCO}_3$  from alkaline soil (pH 9) [13]. In this study, the BICP characteristics of the isolated strain and the morphological characteristics of the formed  $\text{CaCO}_3$  were investigated. Furthermore, the rapid crack-repairing ability of this strain was confirmed.

## 2. Results and Discussion

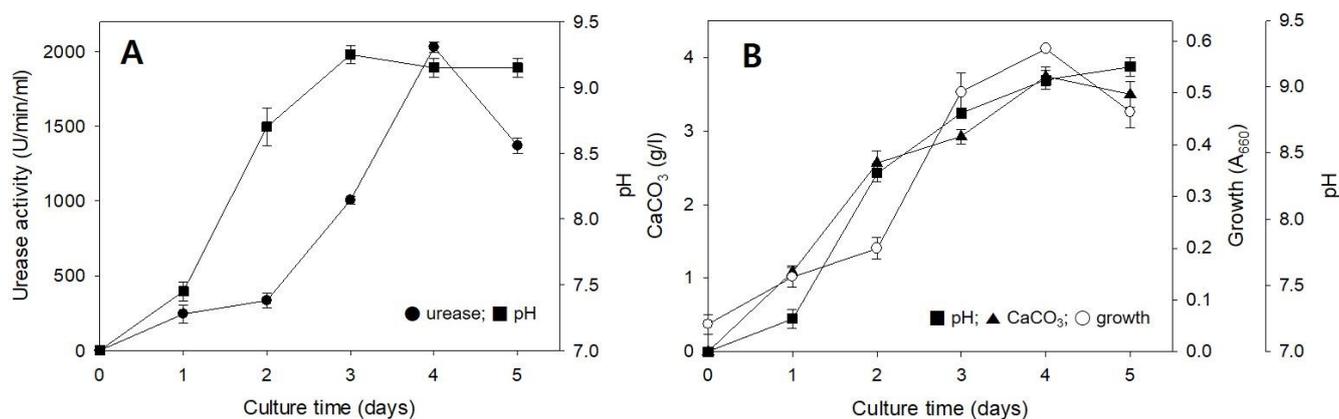
### 2.1. Urease Activities in Solid and Liquid Media

*Rhodococcus erythreus* S26 was inoculated on a urea agar base plate to investigate whether the color change of the medium was caused by a pH increase due to urease activity. As shown in Figure 1, the color of the medium changed from yellow to pink at 24 h, after which the color change of the medium continued to expand, indicating a quick and widespread increase in pH was apparent in strain S26. In contrast, the urease-negative bacterium, *E. coli*, did not change the color of the medium.



**Figure 1.** Photographs showing urease activity of *R. erythreus* S26 and *E. coli* (urease-negative strain) on a urea agar base plate.

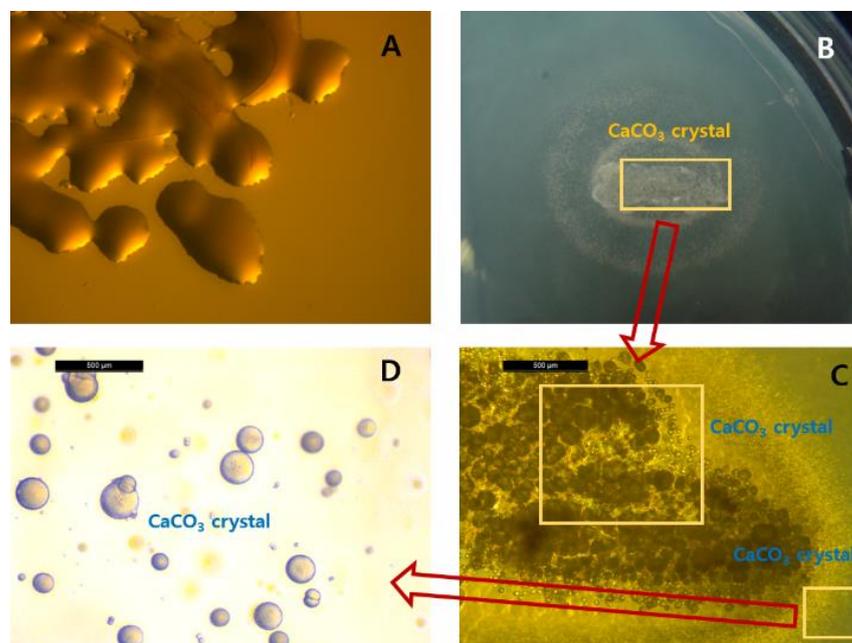
Figure 2A shows the change in urease activity according to the incubation time in NB-urea broth. After urease of 285 U/min/mL was produced at 24 h, it increased proportionally over time, showing maximum activity (2054 U/min/mL) at 4 d. Culture pH increased from 7.0 to 9.1 after 3 d of incubation.



**Figure 2.** Urease activity (A) and CaCO<sub>3</sub> formation (B) of *R. erythreus* S26 in a liquid medium containing CaCl<sub>2</sub>. Error bars ( $\pm$ SDs) are shown when larger than the symbol.

## 2.2. CaCO<sub>3</sub> Formation in Solid and Liquid Media

CaCO<sub>3</sub> formation by strain S26 was investigated using a urea-CaCl<sub>2</sub> plate. As shown in Figure 3, CaCO<sub>3</sub> crystals of various sizes were observed in the colony and the surrounding medium. In particular, glossy crystals were densely distributed covering the colony surface. Most of these crystals were spherical; however, some polygons were also observed. Only spherical crystals were observed in the medium without cell growth around the colonies. No mineral crystals were observed in urease-negative *E. coli*. The shape and size of CaCO<sub>3</sub> crystals depend on the molecular properties of exopolysaccharides secreted by bacterial cells, as well as on the colony form [14]. Furthermore, spherical CaCO<sub>3</sub> crystals, vaterites, are primarily formed in solid medium containing agar [15].



**Figure 3.** Photographs of crystals formed by *R. erythreus* S26 on a urea-CaCl<sub>2</sub> agar plate (A), *E. coli* (urease-negative strain) (B–D), *R. erythreus* S26.

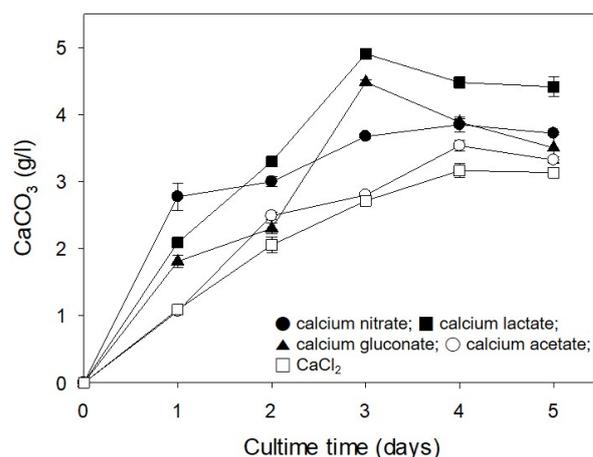
Figure 2B shows typical time courses for pH, cell growth, and CaCO<sub>3</sub> formation in the urea-CaCl<sub>2</sub> broth. The CaCO<sub>3</sub> was formed (3.83 g/L) at 4 d of cultivation when the cell growth reached the late logarithmic growth phase. Cell growth showed the same tendency as observed for CaCO<sub>3</sub> formation. The pH of the culture gradually changed from neutral to alkaline due to urea degradation and showed a pattern similar to that of NB-urea broth.

In addition,  $\text{CaCO}_3$  formation was found to be proportional to urease activity (Figure 2A). From the data shown in Figure 2, the  $\text{CaCO}_3$  formation process can be explained as follows. In the urea- $\text{CaCl}_2$  medium, bacterial urease broke urea into ammonium ions, thereby increasing the pH of the medium. The negatively charged site of the bacterial cell wall was a nucleation site where calcium ions bound, and carbonate ions bound to calcium ions sequentially, forming  $\text{CaCO}_3$  crystals [16].



### 2.3. Influence of Calcium Source on $\text{CaCO}_3$ Formation

The  $\text{CaCO}_3$  formation by microorganisms is greatly influenced by the type of calcium salt [17,18]. Therefore,  $\text{CaCO}_3$  formation by strain S26 was investigated using different calcium sources. In all experimental groups, the amount of  $\text{CaCO}_3$  formed increased in proportion to the incubation time, with the maximum values being observed at 3–4 days.  $\text{CaCO}_3$  formation was in the following order: calcium lactate, calcium gluconate, calcium nitrate,  $\text{CaCl}_2$  (Figure 4).



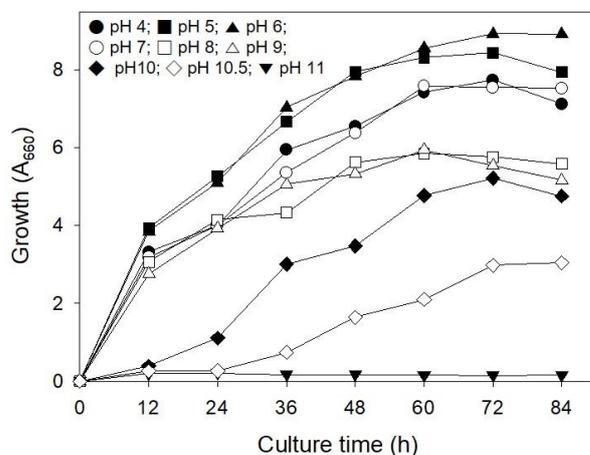
**Figure 4.** Influence of the calcium source on  $\text{CaCO}_3$  formation by *R. erythreus* S26. Error bars ( $\pm$ SDs) are shown when larger than the symbol.

One of the main issues in applying  $\text{CaCO}_3$ -forming microorganisms to concrete crack repair is in exploring the biochemical process of carbonate mineral formation. This process involves converting the soluble calcium source into insoluble  $\text{CaCO}_3$ ; therefore, the influence of the calcium source type is important. Most previous studies have used  $\text{CaCl}_2$  as the calcium source [17–19]. Seifan et al. [20] examined the effects of various calcium sources on  $\text{CaCO}_3$  formation using *Bacillus* species and found that  $\text{CaCl}_2$  was the best source of calcium for the formation of  $\text{CaCO}_3$ . Lee [21] reported that the maximum  $\text{CaCO}_3$  yield by *Bacillus amyloliquefaciens* CMB01 was achieved using calcium acetate. Hence, in the present study, we suggest that the optimal calcium source for  $\text{CaCO}_3$  formation varies depending on the microorganism used.

### 2.4. Influence of pH on Cell Growth

Many microorganisms cannot grow in concrete, which is a highly alkaline environment. To repair cracks in concrete using microorganisms, they must survive and grow in extreme environments [22]. Therefore, the effect of pH on the growth of strain S26 was investigated. As shown in Figure 5, strain S26 showed high growth at pH 4–7, followed by pH 8–10. The cells were able to grow after a lag period of 24 h at pH 10.5, but not at pH 11. Additionally, concrete is strongly alkaline with a pH 11, but the pH decreases to pH 8–10

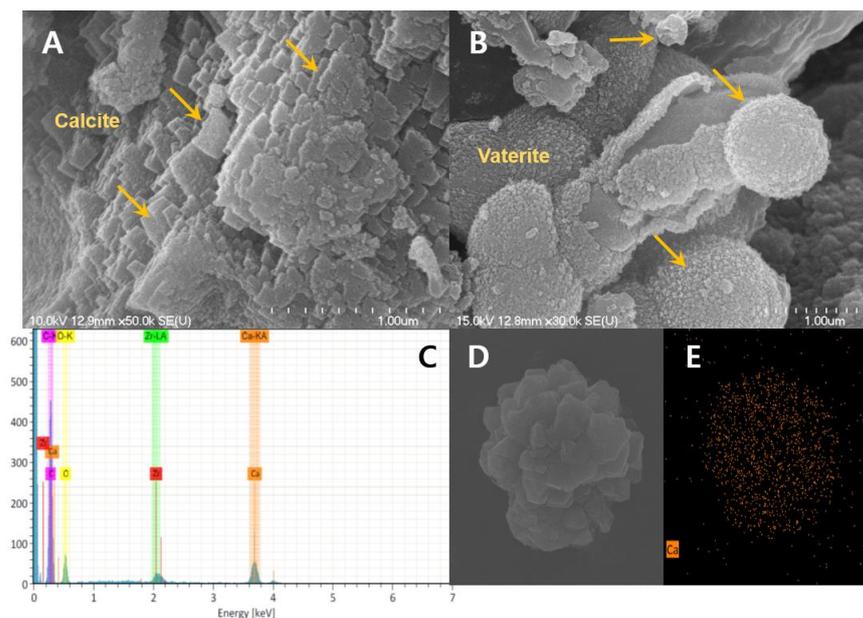
when moisture penetrates inside it through the cracks created [22]. Considering this, strain S26 can form  $\text{CaCO}_3$  after the adaptation period in strongly alkaline concrete and has the potential for in situ applications.



**Figure 5.** Influence of culture pH on cell growth of *R. erythreus* S26. Error bars ( $\pm$ SDs) are shown when larger than the symbol.

### 2.5. Structural Characterization of $\text{CaCO}_3$ Crystals

Morphology of  $\text{CaCO}_3$  formed in the urea-calcium lactate broth was investigated using FESEM. FESEM images showed that the formed  $\text{CaCO}_3$  crystals were mostly rectangular or rhombohedral with an irregular structure, with a size of 0.15–0.25  $\mu\text{m}$  (Figure 6A). A few spherical crystals with particle sizes of 0.35–1.1  $\mu\text{m}$  were also observed (Figure 6B). The elemental composition of the  $\text{CaCO}_3$  crystals was investigated using EDX and elemental mapping. The EDX spectrum showed that the sample contained Ca (25.2 atomic %), C (16.5% atomic %), and O (45.0 atomic %), indicating the presence of  $\text{CaCO}_3$  crystals (Figure 6C). Further elemental mapping confirmed that the crystals consisted of  $\text{CaCO}_3$  (Figure 6D,E).



**Figure 6.** FESEM images (A,B), elemental mapping (C,D), and EDX spectrum (E) of  $\text{CaCO}_3$  crystals formed by *R. erythreus* S26.

As shown in Figure 7, XRD analysis of the crystals showed that two  $\text{CaCO}_3$  polymorphs were formed during the growth in urea-calcium lactate broth; the primary crystal component was rhombohedral calcite, but a small amount of spherical vaterite was also detected, which confirmed the results of FESEM imaging. Thermodynamically, calcite is the most stable  $\text{CaCO}_3$  polymorph and is a major product of many microbial  $\text{CaCO}_3$  formation processes [23]. In contrast, *Proteus mirabilis* [24] and *Delia halophila* [25] form vaterite and aragonite, respectively. Park et al. [26] reported that proteins or minerals produced during metabolism by  $\text{CaCO}_3$ -forming microorganisms were adsorbed on specific crystallographic planes, thereby changing the shape of the crystals. These results suggest that the morphological specificity of the crystals formed is due to differences in the bacterial genera.

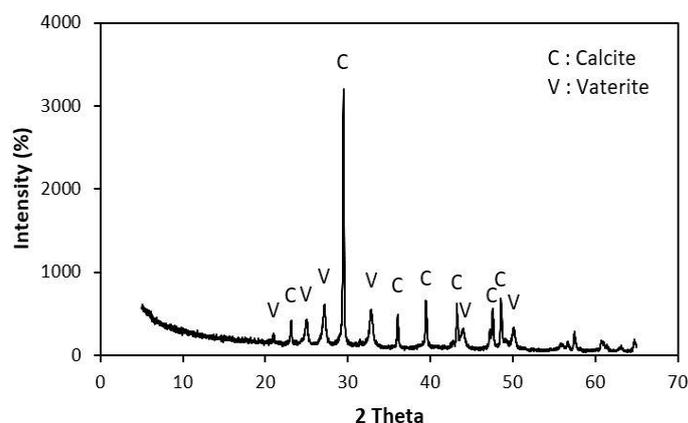
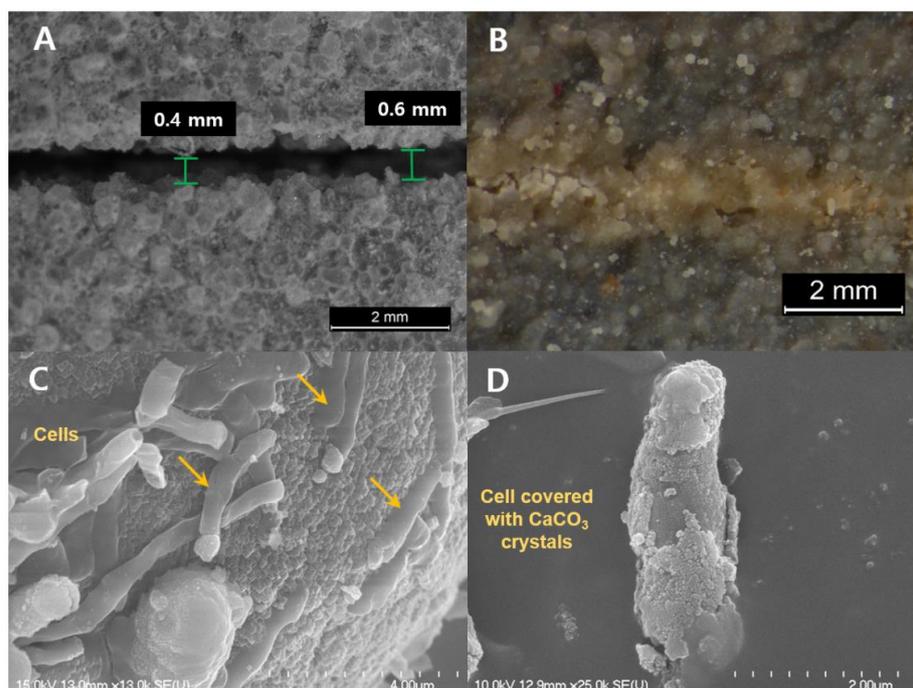


Figure 7. XRD pattern of  $\text{CaCO}_3$  crystals formed by *R. erythreus* S26.

### 2.6. Concrete Crack-Repairing Effect

In order to repair concrete cracks using microbial  $\text{CaCO}_3$  formation, artificial cracks (0.4–0.6 mm) were made and cells were inoculated. As shown in Figure 8A,B,  $\text{CaCO}_3$  precipitates were found in cracks treated with the S26 strain; these cracks were gradually repaired over time and sealed tightly after 6 d. To confirm the microstructure of  $\text{CaCO}_3$  formed in the cracks, the repaired sample was crushed by hand, and then small pieces of  $\text{CaCO}_3$  were observed with FESEM. The formed  $\text{CaCO}_3$  crystal was a compact layered structure composed of polygonal and spherical particles, and rod-shaped cells were observed on its surface (Figure 8C). Some cells appeared to be fossilized by being covered with  $\text{CaCO}_3$  crystals (Figure 8D). De Muynck et al. [27] reported that this dense structure increased the strength of the concrete by giving it stronger adhesion and more tightness. Furthermore, the presence of cells is evidence that strain S26 was directly involved in crack repair, indicating that bacteria act as nucleation sites in the calcium carbonation process. On the other hand, the treatment of a wide crack with  $\text{CaCO}_3$ -forming bacteria does not provide the nucleation site required for  $\text{CaCO}_3$  crystal formation because the cells flow down without attaching to the inner surface of the crack [27]. For example, unlike the results of this study, Park et al. [28] reported that there was no repair effect when *Raoultella ornithinolytica*, *Stenotrophomonas maltophilia*, and *Bacillus thuringiensis* were tested on cracks with widths of 0.22–0.57 mm. Exceptionally, it was reported that *Bacillus sphaericus* LMG25557 completely repaired cracks up to 0.97 mm wide in 21 days [28]. These results indicate that the repairing effect of concrete cracks depend on the microbial species, and strain S26 can be applied to relatively large cracks. Meanwhile, van Titelboom et al. [29] reported that the crack-repairing effect was increased by using resin as a binder that increased the microbial adhesion to cracks with large widths.



**Figure 8.** Repair of a concrete crack by *R. erythreus* S26-induced  $\text{CaCO}_3$  before (A) and after (B) incubation. B is a photograph showing that the crack was sealed when the cells were cultured for 6 d. (C,D) are FESEM images showing  $\text{CaCO}_3$  crystals and  $\text{CaCO}_3$ -forming cells in the crushed sample after crack repairing.

### 3. Materials and Methods

#### 3.1. Bacterial Strain and Culture Conditions

*R. erythreus* S26 isolated from alkaline soil in Korea was used in this study [13]. It can form  $\text{CaCO}_3$  in a urea- and  $\text{CaCl}_2$ -containing medium. Nutrient broth was used for the preservation and pre-culture of the strain. For pre-culture, a 250 mL Erlenmeyer flask containing 50 mL of nutrient broth was inoculated with a colony from a culture plate, followed by incubation at 30 °C and 200 rpm for 18 h. Unless otherwise noted, 2% (v/v) of the pre-culture was inoculated into the main medium and incubated under the conditions mentioned above.

#### 3.2. Urease Activities in Solid and Liquid Media

The S26 strain was inoculated on a urea agar base plate (0.1% gelatin, 0.1% glucose, 0.5% NaCl, 0.2%  $\text{K}_2\text{HPO}_4$ , 2% urea, and 0.0012% phenol red, pH 7.0) and incubated at 30 °C for 24–48 h. This medium also contained phenol red, which indicates pH change associated with ammonia production due to urea hydrolysis. A color change from orange to pink indicated urea hydrolysis. A negative control was prepared using *Escherichia coli* KCCM 40880, a known non-ureolytic bacterium.

In addition, strain S26 was inoculated into NB-urea broth (0.3% nutrient broth and 2% urea, pH 8.0) and incubated at 30 °C, and time-dependent urease activity was investigated. Urease activity was determined using the method described by Natarajan [30] with some modifications. Briefly, 250  $\mu\text{L}$  of sample was added to a previously mixed 250  $\mu\text{L}$  of 0.1 M potassium phosphate buffer (pH 8.0) and 500  $\mu\text{L}$  of 0.1 M urea solution. The mixture was incubated at 37 °C for 5 min, after which 500  $\mu\text{L}$  of phenol nitroprusside solution was added, followed by 500  $\mu\text{L}$  of alkaline hypochlorite solution. The mixture was then incubated at 37 °C for 25 min, followed by absorbance measurement at 626 nm, using ammonium chloride (0–100  $\mu\text{M}$ ) as the standard. One unit of urease activity is defined as the amount of enzyme hydrolyzing 1  $\mu\text{mol}$  urea/min/mL.

### 3.3. CaCO<sub>3</sub> Formations in Solid and Liquid Media

To investigate the formation of CaCO<sub>3</sub> by strain S26, cells were inoculated on a urea-CaCl<sub>2</sub> plate (0.3% nutrient broth, 2% urea, 0.212% NaHCO<sub>3</sub>, 1% NH<sub>4</sub>Cl, 25 mM CaCl<sub>2</sub>, and 1.5% agar, pH 7) and incubated at 30 °C. CaCO<sub>3</sub> crystals formed around the colonies were observed using a stereoscopic microscope (Laica S6 D, Leica Microsystems, Wetzlar, Germany).

To quantify CaCO<sub>3</sub> formation, cells were inoculated into urea-CaCl<sub>2</sub> broth and incubated at 30 °C and 200 rpm, and the amount of CaCO<sub>3</sub> was measured over time. The culture was centrifuged at 500 × g for 3 min to remove the supernatant, and the precipitated CaCO<sub>3</sub> was collected. CaCO<sub>3</sub> was washed with distilled water to remove residual cells and culture components and was dried at 105 °C for 2 h to measure the dry weight [31].

### 3.4. Influence of Calcium Source on CaCO<sub>3</sub> Formation

Urea-CaCl<sub>2</sub> broth was used as a basic medium to study the effect of various calcium salts on the formation of CaCO<sub>3</sub> by strain S26. Calcium sources (calcium acetate, calcium gluconate, calcium lactate, calcium nitrate, and CaCl<sub>2</sub>) at 25 mM were separately added to the medium. Cultivation was performed at 30 °C and 200 rpm, and CaCO<sub>3</sub> formation was measured at each incubation time.

### 3.5. Influence of pH on Cell Growth

To investigate the pH tolerance of strain S26, cells were inoculated into tryptic soy broth adjusted to pH 4–11, respectively, and incubated at 30 °C and 200 rpm. Cell growth was measured at 660 nm in a time-dependent manner.

### 3.6. Crystal Analysis

The morphology of the CaCO<sub>3</sub> crystals was studied using field emission scanning electron microscopy (FESEM) (Carl Zeiss, SUPRA 40VP, Oberkochen, Germany). Prior to analysis, the dried CaCO<sub>3</sub> crystals were mounted on specimen stubs with double adhesive tape, sputter-coated with platinum, and examined at 40 kV. Elemental analysis was then conducted using an energy dispersive X-ray (EDX) detector connected to the FESEM. X-ray diffraction (XRD) analysis of the CaCO<sub>3</sub> crystals was performed using a Panalytical diffractometer (Empyrean series 2, Almelo, Netherlands) at 40 kV and 30 mA using CuK $\alpha$  radiation ( $\lambda = 1.54 \text{ \AA}$ ). Data were collected in the 2 $\theta$  range of 20°–80° at a scan rate of 10°/min.

### 3.7. Repairing Test of Concrete Crack

A concrete paste was prepared by mixing cement (ordinary Portland cement type 1), sand, and water at a ratio of 6:3:4 (*w/w*), and the pH of the finished concrete paste was 10.6. Artificial cracks were induced by casting concrete paste on a Petri dish, inserting a 150  $\mu\text{m}$  thick polyester film up to a depth of 5 mm, and placing it at room temperature for 1 d. After removing the film, the cracked concrete was cured for 20 d in tap water.

The crack repair effect of strain S26 was investigated using a cured concrete specimen. Strain S26 was cultured in nutrient broth (30 °C, 200 rpm, 24 h), and then centrifuged at 12,000 × g for 5 min to collect the cells. The cell suspension (100  $\mu\text{L}$ ) diluted to A<sub>660</sub> = 1 in urea-calcium lactate medium was injected into the crack every 12 h (repeated for 3 d), and the crack repair was observed using the stereoscopic microscope while incubating at 30 °C.

## 4. Conclusions

In the present study, we described an environment-friendly and efficient microbiological method for sealing cracks using *R. erythreus* S26 as a concrete healing agent. This method does not require the use of hazardous chemicals to repair concrete cracks. Strain S26 was able to produce the urease required for CaCO<sub>3</sub> formation. When calcium lactate was supplied as a calcium source, the CaCO<sub>3</sub> formation was the highest, and the formed CaCO<sub>3</sub> crystals were a mixture of calcite and vaterite. In addition, strain S26 was able

to grow in a strongly alkaline environment with a pH of 10.5. These results imply that *R. erythreus* S26 is not only useful for in situ repair of concrete cracks, but also to improve the compressive strength of mortar. To explore these possibilities, detailed studies on the reinforcement and durability of concrete are currently being conducted. In addition, research on the development of an economical medium capable of maximizing CaCO<sub>3</sub> formation for the efficient repair of wider cracks within a shorter time should be carried out.

**Author Contributions:** Conceptualization, H.-J.S.; investigation, S.C., S.P. and M.P.; methodology, S.C. and S.P.; project administration, Y.K.; visualization, M.P. and Y.K.; supervision, H.-J.S.; writing—original draft preparation, K.M.L. and O.-M.L.; writing—review and editing, K.M.L. and H.-J.S. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The data presented in this study are available in the article.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Sample Availability:** Samples of the compounds are not available from the authors.

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