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Whey as an Alternative Nutrient Medium for Growth of *Sporosarcina pasteurii* and Its Effect on CaCO₃ Polymorphism and Fly Ash Bioconsolidation

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Abstract: Whey in large quantities can cause environmental problems when discarded, because it reduces dissolved oxygen and aquatic life. Nonetheless, it could be used as an easily available and economical alternative to reduce culture medium costs in microbially induced calcium carbonate precipitation (MICP). In this work, a native *Sporosarcina pasteurii* was isolated and then cultured by using different proportions of whey (W) in nutrient broth (NB). The solids were characterized by XRD, FT-IR, TGA, and SEM. The potential applications in bioconsolidation were also studied. Whey concentration was directly related to CaCO₃ production. Higher whey concentrations reduced calcium carbonate purity to nearly 80%. All experiments showed calcite and vaterite fractions, where a whey increment in the media increased calcite content and decreased vaterite content, causing a decrease in crystal size. MICP improved compressive strength (CS) in sand and fly ash. The best CS results were obtained by fly ash treated with 25 W-75 NB (37.2 kPa) and sand with 75 W-25 NB (32.1 kPa). Whey changed crystal polymorphism in biogenic CaCO₃ production. Material bioconsolidation depends on the CaCO₃ polymorph, thus fly ash was effectively bioconsolidated by crystallization of vaterite and sand by crystallization of calcite.

Keywords: biogenic CaCO₃; polymorphism; ureolytic bacteria; whey



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1. Introduction

Calcium carbonate precipitation (MICP) indicates the mineral formation from an oversaturated solution, mediated by the metabolism of microorganisms [1]. In MICP, cells secrete metabolic products (CO₃²⁻) that react with the ions (Ca²⁺) present in the environment with subsequent mineral precipitation. Such substances act as cementing materials and are commonly known as “biocement” [2]. The pathways reported to calcium carbonate formation include photosynthesis [3], urea hydrolysis [4–6], anaerobic sulfide oxidation [7], and by extracellular polymeric substances [3,8].

Urea hydrolysis is the most extensive method of biogenic CaCO₃ production because urease is present in some microorganisms [9–11]. One of the objectives of MICP technology is to isolate and select bacterial strains with increased urease activity, higher stability, and greater tolerance to high ammonium concentrations [12]. There are many ureolytic microorganisms, such as the genus *Bacillus*, that have a higher urease content [13].

Sporosarcina pasteurii is a Gram-positive bacterium used in MICP because of its high enzyme content (which reaches almost 1% by dry weight), and its zero pathogenicity [9,14,15]. This bacterium grows in different kinds of soils and can resist extreme conditions of pH and temperature. For *S. pasteurii* growth, some authors used nutrient broth [4,16,17], yeast extract [18–20], heart brain infusion broth [21], peptone [22], or meat extract [22], all of which were supplemented with urea. However, cheaper substitutes should be sought, due to the necessity of large-scale production. Consequently, it is necessary to implement the use of available, inexpensive agro-industrial waste to reduce culture medium costs [23].

Some alternatives to replacing nutrients and achieving a level of urease activity are torula yeast (*Cyberlindnera jadinii*), brewery waste yeast, Vegemite[®], and acetate (which lowered production costs by 95%) [19]. Additionally, media such as fermented maize liquor [24] and lentils [12] have been used.

Whey is the watery part of milk that results from cheese coagulation in industrial processes. It has a significant carbohydrate, protein and mineral content [25]. When a large amount of whey is discarded as effluent it causes an environmental problem [26] and reduces dissolved oxygen and aquatic life as consequence [27].

There are few reports in the literature on MICP that include the use of whey. Achal et al. [28] studied microbial concrete production using lactose mother liquor (LML) as a different nutrient for bacteria. They added only 10% of this by-product to a yeast extract and nutrient broth medium. The compressive strength of the cement mortar was increased by *S. pasteurii* in all the media used compared to the control (sand with cement 3:1 % *w/w*). No significant difference in bacterial growth, urease production, and mortar compressive strength with LML was found, suggesting that this is another source of typical media. Cuzman et al. [29] tried different nutrients for bacteria such as lactose mother liquor, buttermilk, yeasts, liquid and dried yeasts from breweries, liquid bakery yeast, and Vegemite[®]. They reported that whey and buttermilk waste were potential nutrients for biotechnological applications with *S. pasteurii*. They did not study CaCO₃ crystals or their possible applications. Grabiec et al. [30] studied CaCO₃ biodeposition with *S. pasteurii* in the modification of recycled concrete aggregate, and whey was used as an alternative culture medium. They showed that CaCO₃ biodeposition led to the reduction in aggregate water absorption.

These previous works did not study whey incorporation in different quantities, calcium carbonate production or the type of crystal produced. This paper shows the results of using whey as an inexpensive nutrient source for ureolytic bacteria and its effect on the polymorphism of CaCO₃ precipitate. Additionally, compressive strength changes were analyzed using different material types and whey addition percentages.

2. Materials and Methods

2.1. Bacterial Isolation

Bacteria were isolated from agricultural soils of Sotaquirá and Nobsa (Boyacá, Colombia), following a previously established protocol [31]. Urease activity was determined by the conductivity method [19]. The bacterium with higher urease activity was molecularly identified by amplification of the 16S ribosomal gene [32].

2.2. Culture Media

To increase urease activity and calcium carbonate production, experiments with three types of media were performed: (1) yeast extract (10 g/L), ammonium sulfate (AS, 20 g/L), sodium acetate (SA, 8.2 g/L), and urea (U, 60 g/L); (2) tryptone (10 g/L), AS (20 g/L), SA (8.2 g/L), and U (60 g/L); and (3) nutrient broth (NB, 13 g/L) and U (30 g/L) [33]. Tests were carried out for 24 h at 30 °C and 200 rpm. Then, CaCl₂ solution (30 g/L) was used to precipitate calcium carbonate at pH 7.2. Urease activity, biomass and CaCO₃ production were recorded. Experiments were done by triplicate. Urease activity was determined by the conductivity method [19]. Biomass was measured at 600 nm using a spectrophotometer

Bio Whittaker ELX 80834 (BioTek Instruments Inc., Winooski, VT, USA). Calcium carbonate production was measured by weight difference.

2.3. Effect of Whey on CaCO_3 Crystals and Bioconsolidation Treatment

The influence of different whey concentrations on nutrient broth during 24 h at 30 °C and 200 rpm was assessed by the fermentation of bacterial cultures for 24 h. Table S1 indicates whey composition and its comparison with other studies. The media were labelled as 100 NB, 25 W-75 NB, 50 W-50 NB, 75 W-25 NB, 100 W, where the number indicates the percentage of whey (W) or nutrient broth (NB) used. Then, calcium chloride (30 g/L) was added and mixed for 3 h. The initial and final pH values were 6.5 ± 0.5 and 8.5 ± 0.3 , respectively. The resulting precipitate was collected and centrifuged (10 min at 6000 rpm) and the solid was recuperated and washed three times with distilled water. Next, 20 mL of sodium hypochlorite (10%) was added and allowed to stand for 6 h. Afterward, four water and ethanol washes were done. Finally, the solid was dried at 70 °C for 24 h.

2.4. Characterization of Biogenic CaCO_3 Obtained Using Whey at Different Proportions in Nutrient Broth

The precipitates obtained were evaluated by X-ray diffraction (XRD, Malvern, Westborough, WA, USA,) with $\text{Co K}\alpha$ radiation ($k = 1.789010 \text{ \AA}$) and scanning from 20 to 70° 2 θ (PANalytical X'Pro instrument, Malvern, Westborough, WA, USA). XRD peaks were identified using JCPDS data file. The minerals present in the precipitate were quantified using a Rietveld refinement in the X'Pert HighScore Plus© software with the PDF-4-2012 database and the American Mineralogist Crystal Structure Database [34].

After whey treatments, the Brunauer–Emmett–Teller (BET)-specific surface area, pore volume and size were measured using a physisorption method (ASAP 2020, Micromeritics Instrument Corporation, Norcross, GA, USA). CaCO_3 obtained from different whey treatments was characterized by FT-IR spectra in Thermo Scientific Nicolet iS50 equipment in a range of 3000 to 500 cm^{-1} . Thermogravimetric analysis (TGA) of CaCO_3 obtained was performed using a Setaram Labsys EVO instrument at a heating rate of 10 °C/min in an air flow of 30 mL/min. To study the role of whey addition, calcium carbonate crystals were observed under a scanning electron microscope (SEM, Zeiss, Oberkochen, Germany). The samples were gold-coated with a sputter coating machine (Quorum Q150 R-ES, Zeiss, Oberkochen, Germany) and examined using Carl Zeiss EVO-MA10 equipment (Zeiss, Oberkochen, Germany).

2.5. Tests of Sand and Fly Ash Bioconsolidation

Sand and fly ash were used as materials for bioconsolidation. Material composition is presented in Table S2. Samples were autoclaved at 121 °C for 45 min. They were then placed inside columns and compacted. Plastic columns that were 3 cm in diameter and 10 cm in height were used. Bacterial culture, cementation solution (calcium chloride (30 g/L) and urea solution (30 g/L)) were interspersed using a surface percolation technique. Sand and thermoelectric ashes were treated with separate whey concentrations (two materials, five concentrations and triplicate: 30 samples). Sand and ash controls were packed in columns with sterile materials mixed with media only (without *S. pasteurii*). Treatment was performed by introducing 50 mL of *S. pasteurii* culture and cementation solution (50 mL) into columns every 12 h for 5 days. Afterward, all columns stayed at 20 °C for 28 days before they were taken out of their molds. Finally, all samples were analyzed using a compression testing machine [35]. TEM micrographs of bioconsolidated sand and fly ash were taken using a JEOL JEM 14000 High Resolution Transmission Electron Microscope (JEOL Peabody, MA, USA). Samples were suspended in ethanol and placed on a copper rack before analysis.

2.6. Statistical Analysis

All the experiments were carried out in triplicate. Analysis of variance (ANOVA) was performed, and Tukey's test was applied when statistical significance was found.

3. Results and Discussion

3.1. Bacterial Isolation

Twenty-five bacteria were isolated. Their urease activity (UA) varied between 1.3 and 10.8 mM of urea hydrolyzed/min (Figure 1a). Statistical analysis showed significant differences between evaluated isolates ($F: 1342.3 > F_{crit}: 1.9; P = 2.41 \times 10^{-33}$), and Tukey's test indicated that isolate 9 had the highest urease activity. This isolate was identified as *Sporosarcina pasteurii* of Firmicutes phylum (99% similarity). *S. pasteurii* is a Gram-positive bacterium [13,36,37] and it hydrolyzed 29 mM urea/min [19].

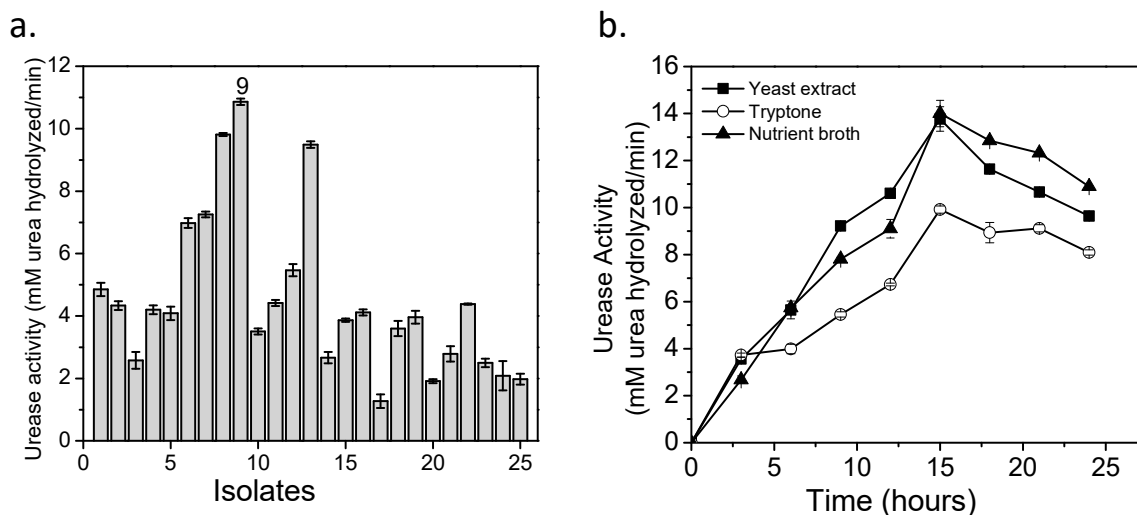


Figure 1. (a) Ureolytic bacteria isolated from different soils of Boyacá. (b) Effect of growth media on urease activity of *S. pasteurii*.

3.2. Culture Media

Yeast extract and nutrient broth showed similar behavior in UA (Figure 1b). The best value was obtained in nutrient broth, 14.0 mM urea hydrolyzed/min at 15 h. These results are similar to *S. pasteurii* ATCC 11859 (13.7 mM urea hydrolyzed/min) [19] and higher than *S. pasteurii* WJ-2 (3.8 mM urea hydrolyzed/min) [38]. The CaCO_3 yields were: 76%, 86%, and 99% for yeast extract, tryptone, and nutrient broth, respectively. There is a direct relation between UA and CaCO_3 production [19], so it was expected that yeast extract would present the best mineral content. Nevertheless, the better results in nutrient broth confirmed that *S. pasteurii* adapted better to this medium to produce high urease amounts. UA and CaCO_3 production showed statistical differences in ANOVA tests (UA $F: 419.3 > F_{crit}: 5.2$; CaCO_3 $F: 17.1 > F_{crit}: 5.2$).

3.3. Effect of Whey in Nutrient Broth on Biogenic CaCO_3

The effect of whey in nutrient broth on urease activity and CaCO_3 production is shown in Figure 2a,b, respectively. Significant statistical differences were found in the treatments (UA $p_{value}: 2.38 \times 10^{-11}$; CaCO_3 $p_{value}: 1.03 \times 10^{-15}$). The use of pure whey decreased considerably with urease activity, in comparison to the assays containing nutrient broth, due to the high concentration of proteins in whey that are difficult to assimilate by microorganisms [27]. However, calcium carbonate production was similar in whey (100 W) and nutrient broth (100 NB) due to the excess of calcium ions. Although W-NB mixes presented similar urease activity, close to 100, CaCO_3 production depended on protein proportion, where 50 W-50 NB had the lowest precipitate formation.

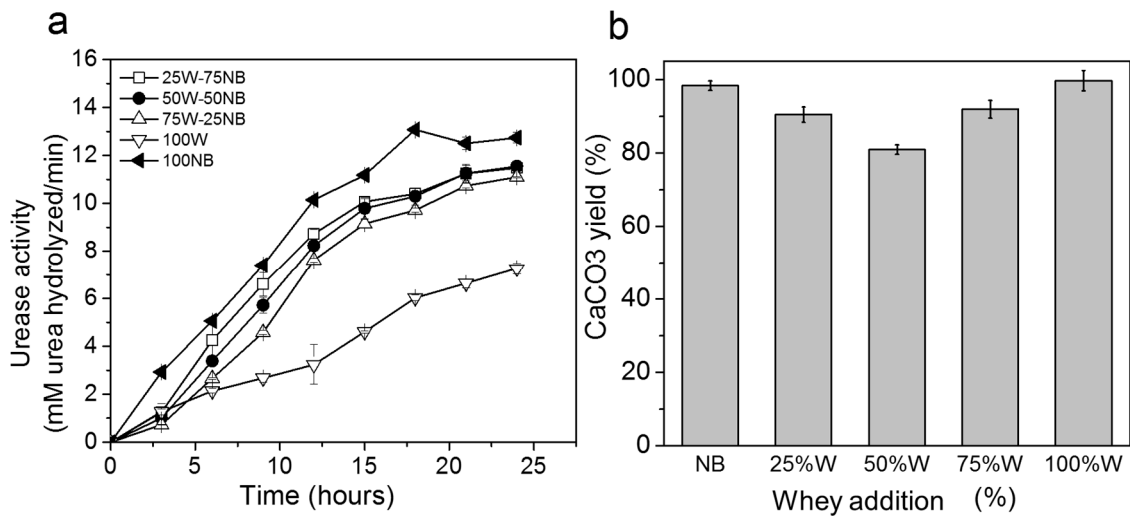


Figure 2. Effect of whey at different proportions in nutrient broth on (a) urease activity, and (b) CaCO₃ yield.

3.4. Characterization of Biogenic CaCO₃ Obtained Using Whey at Different Proportions in Nutrient Broth

It has been reported that the crystal morphology of microbially induced calcium carbonate depends on the microorganism, calcium source, and growth media composition [6,39]. XRD results revealed calcite and vaterite fractions in all experiments (Figure 3).

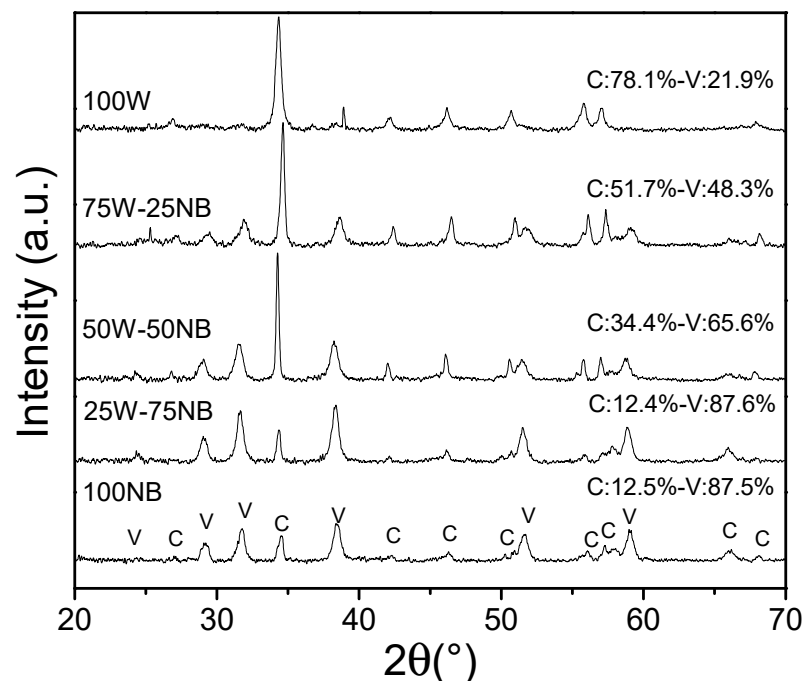


Figure 3. XRD of whey effect on biogenic CaCO₃ production.

From the Rietveld refinement of the XRD patterns, Figure 3 also presents the semi-quantitative ratios of the crystalline phases of both vaterite and calcite for all the precipitates. The weighted residual profile of the refinements (R_{wp}) was below 10% and none presented any variation above 20% with respect to the expected values (R_{exp}). These parameters determined that the refinement models were good to accept the values obtained [40]. In the precipitate obtained with only nutrient broth, *S. pasteurii* produced a high content of

vaterite (87.5%). When whey concentration increased, calcite content increased up to 78.1% for 100 W.

According to different authors, vaterite grains agglomerate into larger particles that transform into calcite. In aqueous solution (culture), the reaction mechanism consists of three phases: (1) a progressive dissolution of small vaterite crystals on the grain's surface, (2) generation of cubic calcite micro-germs (more stable than calcite), and (3) precipitation of cubic calcite providing rhombohedral forms [41]. Furthermore, it is recognized that the nucleation frequency and crystal size are both functions of supersaturation (first) and then the effect of impurities (second). When nucleation takes place in the bacterium cell surface, the supersaturation in basic medium begins with the exclusive formation of vaterite. A higher amount of impurities modifies the crystal growth morphology by interacting with the environment, which changes their size and structure type [42], providing evidence that whey promotes the production of calcite with the lowest crystallite sizes. This fact is probably due to the presence of protein residues in whey, and consequently, this higher impurities content causes preferential precipitation towards calcite. This also reduces the specific surface area (SSA) and increases others such as pore size and pore volume (Table 1). The presence of protein residues was verified with FT-IR and TGA results. Natural calcium carbonate has an SSA from 4 to 10 m²/g [43] and precipitated CaCO₃ from 17 to 25 m²/g [44] and 27 m²/g [45], which is similar to biogenic CaCO₃ obtained in the assay 100 NB.

Table 1. Effect of whey concentration in nutrient broth on crystal size and specific surface area, pore size and pore volume of biogenic CaCO₃.

Treatment	Crystal Size Vaterite (nm) *	Crystal Size Calcite (nm) *	Specific Surface Area (m ² /g)	Pore Volume (cm ³ /g)	Pore Size (nm)	Purity (%) **
100 NB	3.6	3.2	25	0.11	22.5	96
25 W-75 NB	3.6	3.2	23	0.04	8.6	91
50 W-50 NB	3.2	3.6	19	0.05	9.3	83
75 W-25 NB	3.2	3.2	6	0.01	24.8	81
100 W	3.8	2.7	2	0.01	24.8	80

* Crystallite size was determined by Scherrer equation using XRD. ** Purity determined by TGA results.

Infrared transmission spectra of CaCO₃ obtained with different whey additions show strong absorption bands, related to calcite and vaterite crystals. The assignation of these bands has been broadly discussed by Al et al. [46] and Andersen et al. [47], and some of these peaks can be seen in the spectra (Figure S1a, Table S3). The proportion of the band changes with the increase in whey in nutrient broth, confirming the reduction in vaterite content and the increase in calcite phase. The presence of protein residues can be evidenced by the amide bands in Figure S1b.

The incorporation of whey in nutrient broth causes a decrease in CaCO₃ purity, as can be seen in the results of thermogravimetric analysis (Table 1). TGA curves (Figure 4) reveal two water losses between 50 and 100 °C, and 100 and 250 °C. In the first step, bound water goes out, and in the second, structural water is lost. Weight loss in the range of 290 to 480 °C can be due to both loss of water and vaterite transformation to calcite that occurs at 488 °C [48]. Vaterite can transform into cubic calcite under heating conditions and in the absence of oxygen. In the literature, peaks of 481 and 488 °C are attributed to the transformation of vaterite into calcite [41]. This process implies the loss of weight shown in Figure 4. Treatments with high whey concentrations demonstrate weight loss due to the presence of calcite, which contrasts with treatment with 100 NB that has low calcite quantity. The elimination of organic matter can be revealed also in this range of temperature, explaining the highest weight loss. In conclusion, whey addition to nutrient broth decreases calcium carbonate purity, possibly due to the organic matter remaining in the solid, which could be verified with TGA and FT-IR.

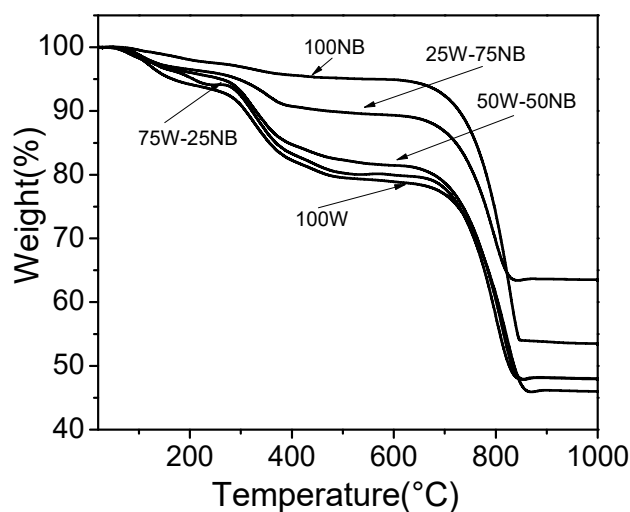


Figure 4. Thermograms of biogenic CaCO_3 using different proportions of whey in nutrient broth.

Figure 5 shows SEM images of biogenic CaCO_3 obtained with nutrient broth (100 NB) and whey (100 W). On the first micrograph (Figure 5a), crystals have a spherical shape, although this could be related to vaterite morphology [49], it is evident by XRD results that the presence of calcite is in minor proportion. This vaterite phase is more soluble and has a lower density compared to calcite, which is why it is not highly recommended for soil bioconsolidation and crack repair [6]. The elongated holes that can be seen on the crystal surface correspond to bacterial cells that act as nucleation sites for crystal growth, which indicates their biological origin [50]. Bacteria were in intimate contact with crystals, demonstrating that cells served as nucleation sites during the MICP process [51]. Figure 5b shows a typical SEM image of CaCO_3 crystals obtained with 100% of whey that preferentially correspond to calcite with a rhombohedral structure [52]. In the present study, *S. pasteurii* and calcium chloride produced vaterite but whey incorporation in the nutrient broth increased calcite content. This confirms that strain, media and calcium type produced a defined crystal phase.

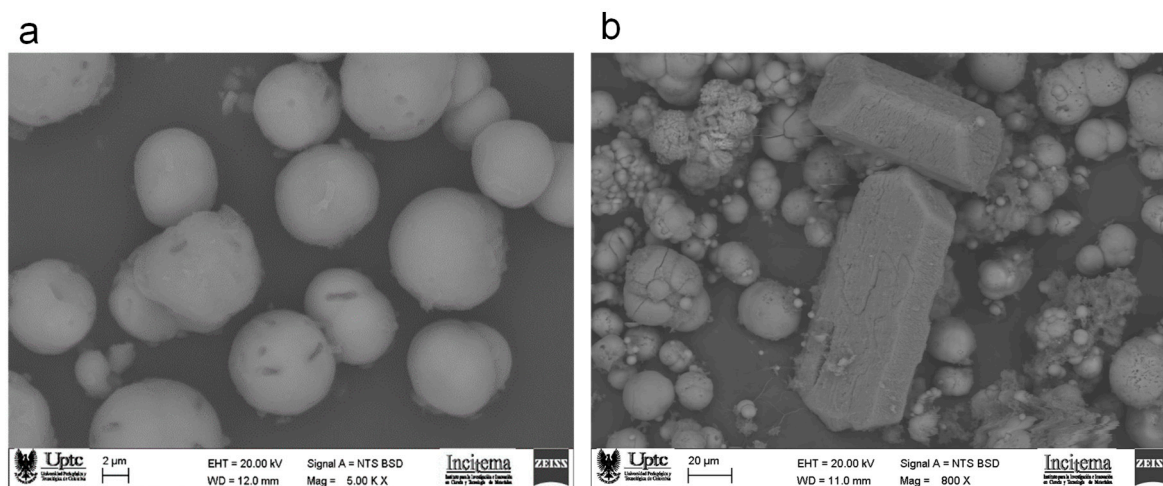


Figure 5. SEM micrographs of biogenic CaCO_3 obtained with (a) nutrient broth (100 NB) shows vaterite crystals, and (b) whey (100 W) with calcite crystals.

3.5. Bioconsolidation of Sand and Fly Ash

The bioconsolidation of sand and fly ash with different BSD concentrations of whey is shown in Figure 6. The results revealed that the highest bioconsolidation degree was

obtained with fly ash and 25 W-75 NB of 37.2 kPa. The increase in compressive strength could have been generated by biogenic CaCO_3 deposition on the *S. pasteurii* surface and within filling material [53]. According to previous characterizations, XRD showed that biogenic CaCO_3 produced by 25 W-75 NB assay had a high vaterite content (87.6%). This type of crystal has a low density and a large specific surface area, which might produce greater adsorption of filled material [54]. After 25% whey, a reduction in the CS trend was observed, probably caused by a vaterite content decrease. Whey has different kinds of components that seal the surface layer of fly ash, which hinders their transport through the column. For this reason, the entire fly ash column compressive strength is lower at high whey concentrations. Fly ash was very well consolidated with vaterite crystals of biogenic CaCO_3 using 25 W-75 NB due to its fine granulometry (Table S2).

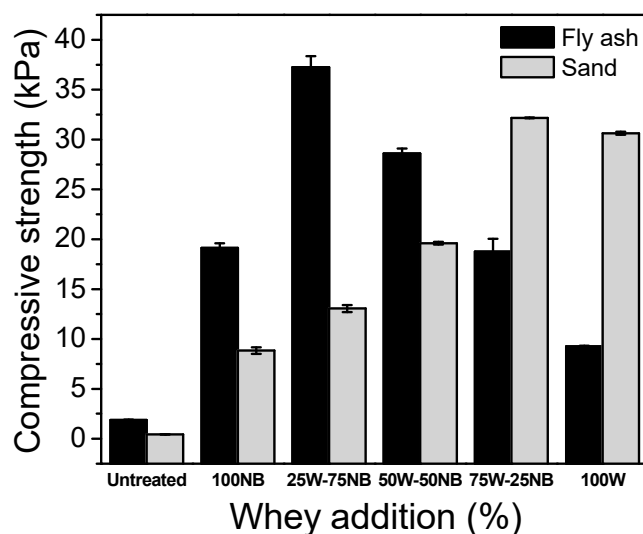


Figure 6. Compressive strength of sand and fly ash treated with different whey additions.

In sand bioconsolidation, compressive strength ranged between 8.8 and 32.2 kPa, and the best results were obtained with 75 W-25 NB and 100 W. Biogenic CaCO_3 produced in 75 W-25 NB had a similar concentration of calcite (51.7%) and vaterite (48.3%), and with this mixture, the maximum compressive strength was obtained. Calcite crystals are rhombohedral, while vaterite crystals are spherical with the same size, which improves the filling of sand pores. A lowest specific surface area and pore volume of calcium carbonate favors a better CS in sand.

Figure 7a shows a TEM micrograph of sand bioconsolidation using 50% of whey. Although sand could not be observed (due to the large particle size), the biogenic calcium carbonate surrounding sand granule junctions was distinguished. Round crystals are the most common, but calcite is also observed. Some elongated black dots that correspond to microbial cells that act as crystal nucleation sites are also noted. Figure 7b shows a fly ash particle mainly surrounded by round crystals, although some straight sides of calcite can also be seen. With these results, it is proven that *S. pasteurii* isolated from agricultural soils could produce calcium carbonate in contact with calcium that is placed in the middle to carry out the bioconsolidation of sand and fly ash.

To reduce the environmental problem due to the release of large amounts of whey in rivers, two alternative uses were proposed. First, the use of whey as a microbial cultivation medium and, second, the potential application as a natural cement using fly ash. Conventionally, the biotechnology process involves the use of expensive media and sterile conditions, and this must be changed by inexpensive and soft production conditions in order to obtain accessible use of *Sporosarcina pasteurii*. It is necessary to find an economical substrate for the growth in bacteria with the production of high urease activity for large-scale production [19]. The cost of 1 L of media (nutrient broth) including

urea was USD 2 in this work and there are other related costs such as water, energy and personnel. Sometimes, inexpensive media are generally subject to low quality control and reproducibility. However, it could be a good choice in order to diminish costs for large biotechnological production. Thus, whey can be an alternative growth media for *Sporosarcina pasteurii* isolated from soils.

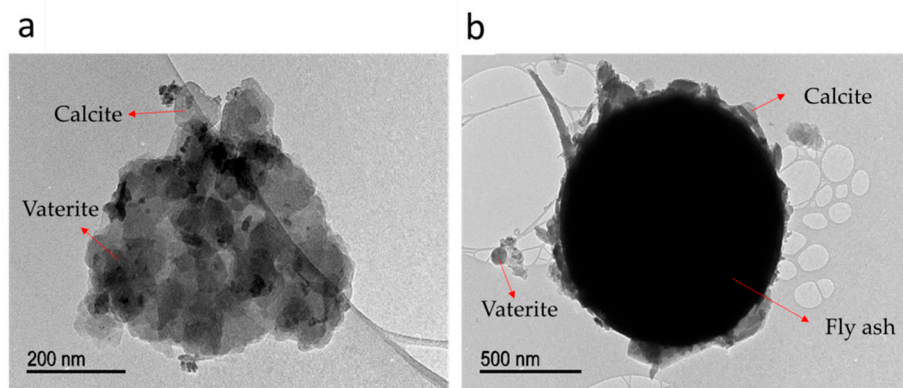


Figure 7. TEM micrographs of bioconsolidation of (a) sand, and (b) fly ash at 50 W-50 NB. These images show vaterite and calcite crystals on these material's surface, such as biocement.

It is well known in microbially induced calcium carbonate precipitation (MICP) that the crystal phase depends on different parameters such as bacteria strain, urease activity, calcium sources, and media [55–57]. In this work, we established that varying the concentration of whey on nutrient broth could modify the vaterite: calcite ratio by using, a higher amount of whey in the medium, which influences calcium carbonate purity (obtained by TGA results). This control of polymorphism produces a specific type of calcium carbonate phase which has relevant implications and applications. Vaterite has a high surface area as compared with calcite, which indicates that this polymorph could be employed as a catalyst/support in different reactions and for the absorption of heavy metals in wastewaters and contaminated soils. Likewise, these crystals could bring stability to minerals and they can be utilized in different industries, for example as a filler in thermoplastics or as composite materials, among others.

Consequently, the distinct CaCO_3 morphologies affect the production of concrete building blocks by cementing natural fly ash. Our results demonstrate that sand and fly ash bioconsolidation could be employed in the construction industry. This depends on the particle size of the filler, as of the crystal morphology of carbonate obtained. With materials such as fly ash (small grain size), CaCO_3 obtained with low whey concentrations in culture is optimum, while with high grain size materials such as sand, CaCO_3 obtained from high whey quantities should be utilized. In all cases, both treatments increase compressive strength and their high thermal behavior (decomposition temperature above 600 °C) which would be advantageous in their potential use as a cement alternative. From an environmental point of view, this process could be an interesting alternative that will provide sustainability to construction processes. Bioconsolidation with *S. pasteurii* isolated from agricultural soils in Colombia could be an inexpensive alternative to treating these materials. However, before that, it is necessary to develop pilot-scale studies that verify the presented results in large-scale production, especially for the bioconsolidation of sand with 100 W. Future research directions could address the use of these polymorphs obtained by *Sporosarcina pasteurii* isolated from soils in different industries, such as for filler and catalysts.

4. Conclusions

Whey was successfully employed as an economical nutrient source for the growth of *Sporosarcina pasteurii* isolated from agricultural soils in Boyacá (Colombia). To the usual

nutrient broth, various concentrations of whey were added. This generated different CaCO₃ crystallizations. Thus, crystal morphology is influenced by whey incorporation because the production of calcite crystals is directly related to the presence of impurities present in the media. The increase in whey percentage in the culture media promoted calcite production with urea (30 g/L) and calcium chloride (30 g/L) at 200 rpm and 30 °C for 24 h. Bioconsolidation experiments evidenced that *S. pasteurii* increased compressive strength, probably due to the presence of biogenic CaCO₃ on the material. This parameter depended on the different polymorphs formed as a kind of filler. Whey addition in nutrient broth promoted calcite production and this type of crystal is more stable and has a higher density compared to vaterite. This is important in sand bioconsolidation treatments. However, in fly ash columns, vaterite showed the best results because of its high specific surface area and lower density. From a materials perspective, this research opens an interesting double benefit perspective: using a wide surplus of whey to create solid structures (using costless natural ashes as filler material) instead of causing environmental problems.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/ma14102470/s1>, Figure S1: IR Spectra of CaCO₃ produced with different whey treatments, Table S1: Whey composition used as culture media of *S. pasteurii* in comparison with other studies, Table S2: Composition of sand and fly ash used in bioconsolidation, Table S3: Information bands of IR spectra of biogenic CaCO₃.

Author Contributions: Conceptualization, R.L., S.C. and J.J.M.; methodology, G.C., G.R.; validation, G.R., J.J.M.; investigation, J.J.M., A.P.; resources, H.A.R.; data curation, R.L., J.J.M.; writing—original draft preparation, S.C.; writing—review and editing, R.L.; visualization, A.P.; supervision, J.J.M.; project administration, H.A.R.; funding acquisition, R.L., J.J.M. and S.C. All authors have read and agreed to the published version of the manuscript.

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References

1. Bosak, T. *Calcite Precipitation, Microbially Induced*; Encyclopedia of Earth Sciences Series; Reitner, J., Thiel, V., Eds.; Springer: Berlin, Germany, 2011; pp. 223–227.
2. Rong, H.; Qian, C.-X.; Li, L.-Z. Study on microstructure and properties of sandstone cemented by microbe cement. *Constr. Build. Mater.* **2012**, *36*, 687–694. [[CrossRef](#)]
3. Castanier, S.; Le Metayer-Levrel, G.; Perthuisot, J.C. Ca-carbonates precipitation and limestone genesis—The microbiogeologist point of view. *Sediment. Geol.* **1999**, *126*, 9–23. [[CrossRef](#)]
4. Stocks-Fischer, S.; Galinat, J.K.; Bang, S.S. Microbiological precipitation of CaCO₃. *Soil Biol. Biochem.* **1999**, *31*, 1563–1571. [[CrossRef](#)]
5. De Muynck, W.; De Belie, N.; Verstraete, W. Microbial carbonate precipitation in construction materials: A review. *Ecol. Eng.* **2010**, *36*, 118–136. [[CrossRef](#)]
6. Dhami, N.K.; Reddy, M.S.; Mukherjee, A. Biomineralization of calcium carbonates and their engineered applications: A review. *Front. Microbiol.* **2013**, *4*, 314. [[CrossRef](#)] [[PubMed](#)]

7. Warthmann, R.; Van Lith, Y.; Vasconcelos, C.; McKenzie, J.; Karpoff, A. Bacterially induced dolomite precipitation in anoxic culture experiments. *Geology* **2000**, *28*, 1091–1094. [[CrossRef](#)]
8. Arias, J.L.; Fernández, M.S. Polysaccharides and proteoglycans in calcium carbonate-based biomineralization. *Chem. Rev.* **2008**, *108*, 4475–4482. [[CrossRef](#)]
9. Hammes, F.; Boon, N.; De Villiers, J.; Verstraete, W.; Siciliano, S.D.; Villiers, J. Strain-specific ureolytic microbial calcium carbonate precipitation strain-specific ureolytic microbial calcium carbonate precipitation. *Appl. Environ. Microbiol.* **2003**, *69*, 4901–4909. [[CrossRef](#)]
10. Hammes, F.; Verstraete, W. Key roles of pH and calcium metabolism in microbial carbonate precipitation. *Rev. Environ. Sci. Biotechnol.* **2002**, *1*, 3–7. [[CrossRef](#)]
11. DeJong, J.T.; Mortensen, B.M.; Martinez, B.C.; Nelson, D.C. Bio-mediated soil improvement. *Ecol. Eng.* **2010**, *36*, 197–210. [[CrossRef](#)]
12. Charpe, A.U.; Latkar, M.V.; Chakrabarti, T. Microbially assisted cementation—A biotechnological approach to improve mechanical properties of cement. *Constr. Build. Mater.* **2017**, *135*, 472–476. [[CrossRef](#)]
13. Achal, V.; Mukherjee, A.; Kumari, D.; Zhang, Q. Biomineralization for sustainable construction—A review of processes and applications. *Earth-Sci. Rev.* **2015**, *148*, 1–17. [[CrossRef](#)]
14. Bang, S.S.; Galinat, J.K.; Ramakrishnan, V. Calcite precipitation induced by polyurethane-immobilized *Bacillus pasteurii*. *Enzyme Microb. Technol.* **2001**, *28*, 404–409. [[CrossRef](#)]
15. Mitchell, A.C.; Ferris, F.G. The influence of *Bacillus pasteurii* on the nucleation and growth of calcium carbonate. *Geomicrobiol. J.* **2006**, *23*, 213–226. [[CrossRef](#)]
16. Qian, C.; Wang, R.; Cheng, L.; Wang, J. Theory of microbial carbonate precipitation and its application in restoration of cement-based materials defects. *Chin. J. Chem.* **2010**, *28*, 847–857. [[CrossRef](#)]
17. Ferris, F.G.; Stehmeier, L.G.; Kantzas, A.; Mourits, F.M. Bacteriogenic mineral plugging. *J. Can. Pet. Technol.* **1996**, *35*, 56–61. [[CrossRef](#)]
18. Ramachandran, S.K.; Ramakrishnan, V.; Bang, S.S. Remediation of concrete using micro-organisms. *ACI Mater. J.* **2001**, *98*, 3–9.
19. Wiffin, V. Microbial CaCO₃ Precipitation for the Production of Biocement. Ph.D. Thesis, Murdoch University, St Ives Murdoch, Australia, 2004.
20. Eryürük, K.; Yang, S.; Suzuki, D.; Sakaguchi, I.; Katayama, A. Effects of bentonite and yeast extract as nutrient on decrease in hydraulic conductivity of porous media due to CaCO₃ precipitation induced by *Sporosarcina pasteurii*. *J. Biosci. Bioeng.* **2015**, *120*, 411–418. [[CrossRef](#)]
21. Mitchell, A.C.; Ferris, F.G. The coprecipitation of Sr into calcite precipitates induced by bacterial ureolysis in artificial groundwater: Temperature and kinetic dependence. *Geochim. Cosmochim. Acta* **2005**, *69*, 4199–4210. [[CrossRef](#)]
22. Nosouhian, F.; Mostofinejad, D.; Hasheminejad, H. Influence of biodeposition treatment on concrete durability in a sulphate environment. *Biosyst. Eng.* **2015**, *133*, 141–152. [[CrossRef](#)]
23. Achal, V.; Mukherjee, A. A review of microbial precipitation for sustainable construction. *Constr. Build. Mater.* **2015**, *93*, 1224–1235. [[CrossRef](#)]
24. Achal, V.; Mukherjee, A.; Reddy, M.S. Effect of calcifying bacteria on permeation properties of concrete structures. *J. Ind. Microbiol. Biotechnol.* **2011**, *38*, 1229–1234. [[CrossRef](#)] [[PubMed](#)]
25. Patel, A.K.; Vaisnav, N.; Mathur, A.; Gupta, R.; Tuli, D.K. Whey waste as potential feedstock for biohydrogen production. *Renew. Energy* **2016**, *98*, 221–225. [[CrossRef](#)]
26. Fernandes, M.; Fornari, R.C.; Mazutti, M.A.; De Oliveira, D.; Ferreira, F.; Cichoski, A.J.; Cansian, R.L.; Di Lucio, M.; Treichel, H. Production and characterization of xantham gum by *Xanthomonas campestris* using cheese whey as sole carbon source. *J. Food Eng.* **2009**, *90*, 119–123.
27. Aider, M.; De Halleux, D.; Melnikova, I. Skim acidic milk whey cryoconcentration and assessment of its functional properties: Impact of processing conditions. *Innov. Food Sci. Emerg. Technol.* **2009**, *10*, 334–341. [[CrossRef](#)]
28. Achal, V.; Mukherjee, A.; Basu, P.C.; Reddy, M.S. Lactose mother liquor as an alternative nutrient source for microbial concrete production by *Sporosarcina pasteurii*. *J. Ind. Microbiol. Biotechnol.* **2009**, *36*, 433–438. [[CrossRef](#)]
29. Cuzman, O.A.; Ritcher, K.; Wittig, L.; Tiano, P. Alternative nutrient sources for biotechnological use of *Sporosarcina pasteurii*. *World J. Microbiol. Biotechnol.* **2015**, *31*, 897–906. [[CrossRef](#)]
30. Grabiec, A.M.; Klama, J.; Zawal, D.; Krupa, D. Modification of recycled concrete aggregate by calcium carbonate biodeposition. *Constr. Build. Mater.* **2012**, *34*, 145–150. [[CrossRef](#)]
31. Al-Thawadi, S.; Cord-Ruwisch, R. Calcium carbonate crystals formation by ureolytic bacteria isolated from Australian soil and sludge. *J. Adv. Sci. Eng. Res.* **2012**, *2*, 12–26.
32. Li, H.; Song, Y.; Li, Q.; He, J.; Song, Y. Effective microbial calcite precipitation by a new mutant and precipitating regulation of extracellular urease. *Bioresour. Technol.* **2014**, *167*, 269–275. [[CrossRef](#)]
33. De Muynck, W.; Verbeken, K.; De Belie, N.; Verstraete, W. Influence of urea and calcium dosage on the effectiveness of bacterially induced carbonate precipitation on limestone. *Ecol. Eng.* **2010**, *36*, 99–111. [[CrossRef](#)]
34. Downs, R.T.; Hall-Wallace, M. The American mineralogist crystal structure database. *Am. Mineral.* **2003**, *88*, 247–250. Available online: <https://goo.gl/Lwfd48> (accessed on 20 August 2020).
35. American Society for Testing Materials. *Standard Test Method for Compressive Strength of Hydraulic Cement Mortars*; ASTM C109M; ASTM International: West Conshohocken, PA, USA, 2007.

36. Tournay, J.; Ngwenya, B.T. Bacterial extracellular polymeric substances (EPS) mediate CaCO₃ morphology and polymorphism. *Chem. Geol.* **2009**, *262*, 138–146. [[CrossRef](#)]
37. Tiano, P.; Biagiotti, L.; Mastromei, G. Bacterial bio-mediated calcite precipitation for monumental stones conservation: Methods of evaluation. *J. Microbiol. Methods* **1999**, *36*, 139–145. [[CrossRef](#)]
38. Kang, C.H.; So, J.S. Heavy metal and antibiotic resistance of ureolytic bacteria and their immobilization of heavy metals. *Ecol. Eng.* **2016**, *97*, 304–312. [[CrossRef](#)]
39. Abo-El-Enein, S.A.; Ali, A.H.; Talkhan, F.N.; Abdel-Gawwad, H.A. Utilization of microbial induced calcite precipitation for sand consolidation and mortar crack remediation. *HBRC J.* **2012**, *8*, 185–192. [[CrossRef](#)]
40. Mccusker, L.B.; Von Dreele, R.B.; Cox, D.E.; Loue, D.; Scardi, P. Rietveld refinement guidelines. *J. Appl. Crystallogr.* **1999**, *32*, 36–50. [[CrossRef](#)]
41. Wolf, G.; Königsberger, E.; Schmidt, H.; Königsberger, C.; Gamsjäger, H. Thermodynamic aspects of the vaterite calcite phase transition. *J. Therm. Anal. Calorim.* **2000**, *60*, 463–472. [[CrossRef](#)]
42. Aquilano, D.; Otálora, F.; Pastero, L.; García-Ruiz, J.M. Three study cases of growth morphology in minerals: Halite, calcite and gypsum. *Prog. Cryst. Growth Charact. Mater.* **2016**, *62*, 227–251. [[CrossRef](#)]
43. Tegethoff, F.W.; Rohleder, J.; Kroker, E. *Calcium Carbonate: From the Cretaceous Period Into the 21st Century*; Springer: Basel, Switzerland, 2001.
44. Preisig, D.; Haid, D.; Varum, F.J.; Bravo, R.; Alles, R.; Huwyler, J.; Puchkov, M. Drug loading into porous calcium carbonate microparticles by solvent evaporation. *Eur. J. Pharm. Biopharm.* **2014**, *87*, 548–558. [[CrossRef](#)]
45. Bang, J.H.; Jang, Y.N.; Kim, W.; Song, K.S.; Jeon, C.W.; Chae, S.C.; Lee, S.; Park, S.; Lee, M. Specific surface area and particle size of calcium carbonate precipitated by carbon dioxide microbubbles. *Chem. Eng. J.* **2012**, *198–199*, 254–260. [[CrossRef](#)]
46. Al, M.M.; Rashid, I.S.; Qinna, N.A.; Jaber, A.M.; Badwan, A.A. Calcium Carbonate. In *Profiles of Drug Substances, Excipients and Related Methodology*, 1st ed.; Elsevier Inc.: Amsterdam, The Netherlands, 2016; Volume 41, pp. 31–132.
47. Andersen, F.A.; Brečević, L. Infrared spectra of amorphous and crystalline calcium carbonate. *Acta Chem. Scan.* **1991**, *45*, 1018–1024. [[CrossRef](#)]
48. Aouad, S.; Saab, E.; Nassrallah-Aboukaïs, N.; Abi-Aad, E.; Aboukaïs, A. The use of vaterite as a trap for metallic ions in water study of copper/vaterite and manganese/vaterite systems. *Leban. Sci. J.* **2009**, *10*, 101–111.
49. Jimenez, C.; Jroundi, F.; Rodríguez, M.; Arias, J.M.; González-Muñoz, M.T. Biomineralization induced by *Myxobacteria*. *Commun. Curr. Res. Educ. Top. Trends. Appl. Microbiol.* **2007**, *1*, 143–154. [[CrossRef](#)]
50. De Muynck, W.; Debrouwer, D.; De Belie, N.; Verstraete, W. Bacterial carbonate precipitation improves the durability of cementitious materials. *Cem. Concr. Res.* **2008**, *38*, 1005–1014. [[CrossRef](#)]
51. Ghosh, T.; Bhaduri, S.; Montemagno, C.; Kumar, A. *Sporosarcina pasteurii* can form nanoscale calcium carbonate crystals on cell surface. *PLoS ONE* **2019**, *14*, 1–15. [[CrossRef](#)]
52. Choi, H.; Choi, H.; Inoue, M.; Sengoku, R. Control of the polymorphism of calcium carbonate produced by self-healing in the cracked part of cementitious materials. *Appl. Sci.* **2017**, *7*, 546. [[CrossRef](#)]
53. Achal, V.; Pan, X.; Özyurt, N. Improved strength and durability of fly ash-amended concrete by microbial calcite precipitation. *Ecol. Eng.* **2011**, *37*, 554–559. [[CrossRef](#)]
54. Udrea, I.; Capat, C.; Olaru, E.A.; Isopescu, R.; Mihai, M.; Mateescu, C.D.; Bradu, C. Vaterite synthesis via gas-liquid route under controlled pH conditions. *Ind. Eng. Chem. Res.* **2012**, *51*, 8185–8193. [[CrossRef](#)]
55. Anbu, P.; Kang, C.; Shin, Y.; So, J. Formations of calcium carbonate minerals by bacteria and its multiple applications. *SpringerPlus* **2016**, *5*, 250. [[CrossRef](#)]
56. Park, S.J.; Park, Y.M.; Chun, W.Y.; Kim, W.J.; Ghim, S.Y. Calcite-forming bacteria for compressive strength improvement in mortar. *J. Microbiol. Biotechnol.* **2010**, *20*, 782–788.
57. Chaparro, S.P.; Becerra, M.L.; Martínez, J.J.; Rojas, H.A. Soil bacteria that precipitate calcium carbonate: Mechanism and applications of the process. *Act. Agron.* **2018**, *67*, 277–288. [[CrossRef](#)]