Title:

Induced calcium carbonate precipitation using *Bacillus* species

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Abstract

Microbially induced calcium carbonate precipitation is an emerging process for the production of self-healing concrete. This study was aimed to investigate the effects and optimum conditions on calcium carbonate biosynthesis. *B. licheniformis, B. sphaericus,* yeast extract, urea, calcium chloride, and aeration were found to be the most significant factors affecting the biomineralization of calcium carbonate. It was noticed that the morphology of microbial calcium carbonate was mainly affected by the genera of bacteria (cell surface properties), the viscosity of the media, and the type of electron acceptors (Ca^{2+}). The maximum calcium carbonate concentration of 33.78 g/L was achieved at the optimum conditions. This value is the highest concentration reported in the literature.

Introduction

Due to unique mechanical characteristics, concrete is one of the most used materials in the world in which annually a billion tonnes of concrete is produced and consumed. However, concrete tends to crack as it shrinks, and this reduces the concrete lifespan [1]. Although a diverse range of crack treatment techniques are in place, the majority of them are a source of health and environmental risks and, more importantly, they are effective only in the short-term. In recent years a sustainable biotechnological approach has been proposed. Since calcium carbonate is the most compatible substance with concrete compositions, its production through microbial metabolic pathways (biomineralization) has emerged as one of the most promising approaches to overcoming the shortcomings associated with the conventional crack treatment techniques [2-5].

In general, calcium carbonate can be produced through two distinct biomineralization processes, namely biologically controlled mineralization (BCM) and biologically induced mineralization (BIM). In BCM mineral particles are deposited intracellularly in a specific location within or on the cell and the process is independent of environmental conditions [6, 7]. However, BIM usually happens in an open environment as an uncontrolled consequence of metabolic activity [8]. There are many factors affecting BIM including bacteria type, the concentration of dissolved inorganic carbon and calcium, pH, nucleation site, and Hartree energy (E_h) [9, 10]. The production of calcium carbonate through the BIM process can be achieved by heterotrophic pathways (sulfur cycle and nitrogen cycle). The sulfur cycle occurs by dissimilatory reduction of sulfate while oxidizing organic compounds. However, the production of calcium carbonate through nitrogen cycle is achieved by three distinct pathways, namely urea or uric acid degradation (ureolysis), ammonification of amino acids and dissimilatory nitrate reduction. Apart from the aforementioned pathways, the heterotrophic growth of bacteria on organic acid salts, such as acetate, lactate, citrate, succinate, oxalate, malate and glyoxylate leads to induced calcium carbonate precipitation [11]. Since the concrete pH is ~12, the biomineralization pathways must be able to produce calcium carbonate crystals in an alkaline surrounding. The heterotrophic precipitation of calcium carbonate occurs in the alkaline environment and it has relatively higher productivity comparing to other pathways, and therefore they are the most suitable mechanisms in designing a bio self-healing concrete.

To date, several studies have demonstrated the positive influence of microbial compounds on concrete properties. Jonkers et al. [2] investigated the influence of a healing agent on the concrete properties and the crack filling capacity. The precipitation of calcium carbonate due to activation of microbial compound resulted in an

increase in compressive strength and a reduction in pore size of concrete specimens. Likewise, Achal et al. [12] successfully incorporated a microbial healing agent containing *B. sphaericus* in a mortar. It was found that the precipitation of calcium carbonate through ureolysis pathway could fill the cracks and porosities. Their investigation showed that the bio-treated mortar absorbed six times less water than untreated mortar. The purpose of the study performed by Wang et al. [13] was to determine the effect of microbial agent on the crack healing capacity and water permeability. It was found that the crack healing capacity increased from 18–50 % to 48–80 % in the presence of the microbial agent. The precipitation of calcium carbonate through ureolysis pathway also resulted in a tenfold reduction in water permeability. Distribution and the amount of bio-precipitate across the concrete structure are the main criteria to determine the efficiency of the bio self-healing approach. To evaluate the efficiency of bio self-healing concrete, an investigation was performed by Wang et al. [14] to observe the distribution of the bio-precipitates and determine the capacity of crack filling by microbial agent throughout the concrete. The healing ratio of 70–100 % was observed for the crack width ranges 0.05–0.3 mm. Despite a relatively well distribution of precipitates throughout the specimen, the remediation was mostly limited to the crack width less than 0.3 mm.

Until recently the majority of published works have focused on the possibility of calcium carbonate precipitation by various microbial strains and nutrients. Although the microbial self-healing compound has been successfully incorporated in concrete, presence of a healing agent cannot guarantee the filling of the entire cracks, voids and porosities. Since the crack and pore size vary from the micro to macro ranges, the durability of concrete structure will be further increased when the entire cracks and porosities are filled with calcium carbonate. The mineralogy and morphology of precipitates are other important parameters that need to be considered. Calcium carbonate morphologies (calcite, vaterite and aragonite) have different physical properties, such as solubility, density and hardness, that could significantly affect the final concrete bio healing properties.

The objectives of the present study, therefore, are to (i) investigate the effective factors on enhancing the biomineralization of calcium carbonate and (ii) quantify the morphology of the produced calcium carbonate. This investigation opens a new horizon for designing a new protocol to achieve a high-performance bio self-healing concrete.

Materials and methods

Microorganisms and growth medium

Bacillus licheniformis ATCC 9789, *Lysinibacillus sphaericus* ATCC 4525, *Bacillus subtilis* ATCC 6633 and *Bacillus sphaericus* NZRM 4381 were purchased from the NZ culture collection (Porirua, New Zealand). After strains revival, they were cultivated on the optimum growth media containing 0.5 % (w/v) Bacto™ Peptone (Becton Dickinson, New Jersey, USA), 0.5% (w/v) glucose and 0.05% (w/v) yeast extract (Sigma-Aldrich, St. Louis, MO, USA) which were already sterilized by autoclaving at 121 °C for 20 minutes [15]. The cells were scraped from nutrient broth agar plates after two days and the harvested bacteria were suspended in a solution of sodium chloride (0.9 % w/v). The bacteria suspensions were heated in a water bath at 80 °C for 10 min and the spore suspension was then centrifuged at 2000 rpm for 10 min to remove cell debris.

Chemicals

A wide range of chemical compounds, including nutrients and calcium sources, were used for bacterial growth and biomineralization. Calcium chloride anhydrous, calcium lactate pentahydrate, calcium nitrate tetrahydrate, calcium acetate hydrate and urea were purchased from Sigma-Aldrich (St. Louis, MO, USA). BBL™ yeast extract, Bacto™ Peptone were obtained from Becton Dickinson (Becton Dickinson, New Jersey, USA). Sodium chloride and glucose were purchased from a domestic supplier.

Experimental design and statistical analysis

Plackett-Burman experimental design was used to screen the significant variables affecting the biomineralization process. For this purpose the experimental design was created by MODDE pro software version 11 (Umetrics, Umeå, Sweden). A total of 13 variables at three levels (low, central, high) were selected for screening the most significant factors on calcium carbonate biomineralization as follows: (1) *B. licheniformis* ATCC 9789, (2) *L. sphaericus* ATCC 4525, (3) *B. subtilis* ATCC 6633, (4) *B. sphaericus* NZRM 4381, (5) urea, (6) calcium chloride, (7) calcium lactate, (8) calcium nitrate, (9) calcium acetate, (10) yeast extract, (11) incubation period, (12) temperature, and (13) agitation speed. The statistical importance of each factor was obtained at 0.1 probability level according to the analysis of variance (ANOVA) test and also R^2 was used to evaluate the goodness of fitted model [16].

In order to optimize the microbial calcium carbonate precipitation, the optimum levels of significant factors were determined using response surface methodology (RSM) with a central composite face-centered (CCF) design matrix. A total of 27 experiments runs with three replications at the central point were conducted to determine the optimum levels of the significant variables at three different normalized levels of -1, 0 and 1. In order to predict the production of calcium carbonate, the second-order polynomial regression model was used to fit the experimental data according to the following equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (1)$$

where Y is calcium carbonate concentration (response), β_0 is the constant coefficient, β_i, β_{ii}, and β_{ij} are the coefficients of the linear, quadratic and synergic effects, respectively, and X_i and X_j are the coded values of variables.

Capability of producing calcium carbonate by isolates

To assess the possibility of producing calcium carbonate by isolates, the spores were grown on a B4 medium composed of 2.5 g/L calcium acetate, 4 g/L yeast extract, and 10 g/L glucose [17]. Fifty µL of each isolate was spread on the B4 plates and sealed with parafilm to avoid water evaporation and subsequently they incubated aerobically at 37 °C for two weeks. Autoclaved cell cultures were used as the control sets. Furthermore, a set of B4 medium without calcium acetate was prepared to observe the effect of organic calcium salts on bacterial growth. Individual colonies were taken at different intervals and were washed repeatedly with distilled water and ethanol to observe the formation of crystals.

Calcium carbonate extraction

To extract the produced calcium carbonate, each fermentation medium was passed through vacuum filtration using a 0.2 µm membrane filter paper (Advantec, Tokyo, Japan). The precipitates, subsequently, were washed three times with plenty of distilled water and oven dried overnight at 70 °C. The final pH and absorbance of each medium were just measured prior to filtration by standard pH Meter (Cyberscan 100, Eutech Instruments) and spectrophotometer (Shimadzu, UV-1700, Kyoto, Japan) at 600 nm, respectively.

Morphological observation

The formation of calcium carbonate crystals due to the heterotrophic growth of bacteria on the B4 medium was periodically observed using BX51 polarized microscope (Olympus, Pennsylvania, USA). The precipitates were washed to remove impurities and they were placed onto a glass slide after drying for further observation. Scanning electron microscope (SEM) was performed using Hitachi S-4700 (Tokyo, Japan) to observe the shape and the size of precipitated particles. Moreover, analysis of quantitative elemental composition was performed by energy dispersive x-ray spectroscopy (EDX), which was equipped with a SEM instrument. Prior to mounting the sample into the SEM chamber, the powder was placed on sticky carbon tape attached to the aluminum stub. To prevent image disturbances, specimens were covered with a thin layer of platinum using sputter coater (Hitachi, E1030), and then the samples were mounted into the chamber.

Characterization of microbial calcium carbonate precipitation

X-ray diffraction (XRD) was used as a non-destructive analytical technique to identify and quantify the morphology of precipitated calcium carbonate. The mineralogy of precipitates was examined at room temperature by Panalytical Empyrean reflectometer (Almelo, The Netherland) using the Cu Kα radiation. The precipitated powders were placed into sample holders and exploration range (2θ) was adjusted from 15° to 75°. The step size, the voltage and the current were set to 0.0530°, 45 kV, and 40 mA, respectively.

Quantification approach

Morphological quantification of calcium carbonate was performed by an XRD internal standard method using three sets of calibration curves. Pure calcite was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the pure vaterite and aragonite were synthesized according to the methods presented by Mori et al. [18] and Zhou et al. [19], respectively. Various percentages of calcium carbonate polymorphs and aluminum oxide were mixed, and the calibration curves were constructed based on the maximum peak intensity of polymorphs (Figure 1).

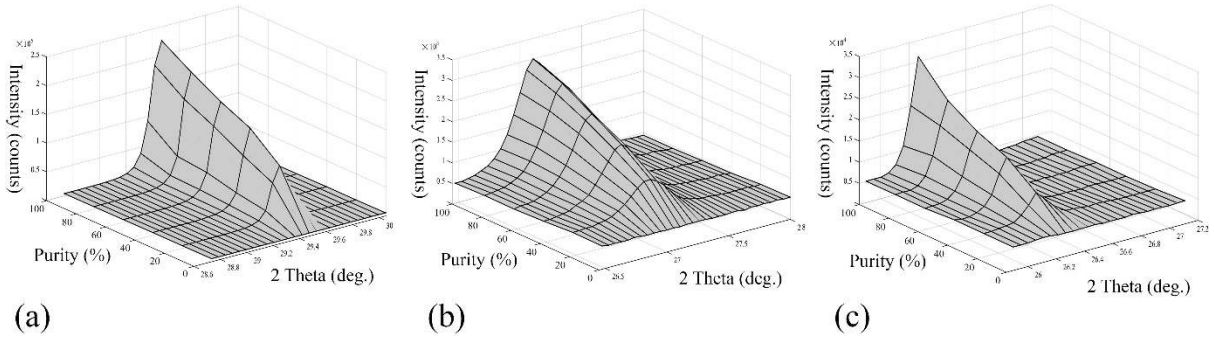

Figure 1 Three-dimensional representation of XRD calibration curves showing the portion of calcium carbonate polymorphs; a calcite (2θ = 29.36°), b vaterite (2θ = 27.11°), and c aragonite (2θ = 26.26°)

Results

Identification of potent calcium carbonate producing bacteria

In the preliminary evaluation, the possibility of microbial calcium carbonate production via selected heterotrophic bacteria was studied. The isolates were tested for calcium carbonate precipitation using B4 solid medium. As shown in Figure S1 (provided in the supplementary material), precipitated crystals at the end of the incubation period possessed strong polarized characteristics. This indicates the crystals were mainly composed of inorganic minerals [20]. No crystallization was observed in the presence of dead cells. This proved that all selected bacteria were capable of producing calcium carbonate. Furthermore, the absence of crystals in B4 media (without calcium acetate addition) confirmed that the presence of organic acid salt is essential for heterotrophic precipitation of calcium carbonate.

Screening the significant variables on calcium carbonate production

Despite the precipitation of calcium carbonate on B4 media, the effect of key parameters controlling bioprecipitation needs to be considered to maximize the production of calcium carbonate. A higher bacterial cell surface in the fermentation process provides a favorable nucleation site for precipitation of calcium carbonate. Therefore, a liquid state fermentation was chosen to address the limitation of solid state media for bacterial growth, distribution and precipitation. In order to identify the significant factors on biomineralization of calcium carbonate, different concentration of bacteria and nutritional components were grown under various operating conditions. Having a rough estimation of parameter ranges prior to screening study, sets of preliminary experiments were carried out to identify the appropriate level of affecting factors. To determine the concertation of calcium salts, a set of identical media with two concentrations of calcium salts were prepared and incubated at the same conditions. The results disclosed that the production of calcium carbonate was significantly increased in those media containing a lower concentration of calcium salt and the concentration of calcium source exceeded than 40 g/L resulted in a dramatic decline in calcium carbonate precipitation. This finding is in good agreement with results reported in the literature [21, 22]. Thirteen potent variables enhancing the biomineralization of calcium carbonate along with their levels are listed in Table 1.

Table 1 Experimental variables and their level for microbial production of calcium carbonate used in Plackett-Burman design

Variable number	Variable name	Value		
		Low level (-1)	Central Level (0)	High level (+1)
X_1	*Bacillus licheniformis* ATCC 9789 (% v/v)	0	2.5	5
X_2	*Lysinibacillus sphaericus* ATCC 4525 (% v/v)	0	2.5	5
X_3	*Bacillus sphaericus* NZRM 4381 (% v/v)	0	2.5	5
X_4	*Bacillus subtilis* ATCC 6633 (% v/v)	0	2.5	5
X_5	Urea (g/L)	0	32.5	65
X_6	Calcium chloride (g/L)	0	25	40
X_7	Calcium lactate (g/L)	0	25	40
X_8	Calcium nitrate (g/L)	0	25	40
X_9	Calcium acetate (g/L)	0	25	40
X_{10}	Yeast extract (g/L)	0	2	4
X_{11}	Incubation time (hr)	72	204	336
X_{12}	Temperature (°C)	33	39	45
X_{13}	Agitation speed (rpm)	0	70	140

Statistical analysis of variance is displayed in Table 2 to show the effectiveness of various parameters on the production of calcium carbonate. The linear regression coefficient and the adjusted determination coefficient were 0.902 and 0.646, respectively. The goodness of the model was confirmed where the maximum production of calcium carbonate was predicted by only 3 % error. The ANOVA results indicated that only six factors had significant positive effect on calcium carbonate production. Among all isolates, *B. licheniformis* and *B. sphaericus* showed the higher ability to produce calcium carbonate crystals. According to the ANOVA results, yeast extract, calcium chloride and urea showed a greater influence on bioprecipitation of calcium carbonate compared with all the other nutritional compounds. All the operating conditions besides agitation speed were found to be insignificant. Therefore, based on the results, *B. licheniformis*, *B. sphaericus*, yeast extract, calcium chloride, urea and agitation speed were found to be the effective factors on improving the calcium carbonate production.

Table 2 The effects of variables and statistical analysis of Plackett-Burman design matrix

Terms	Coefficient	Standard error	*p-value*
Constant	1.18484	0.203646	0.002118
X_1	0.570689	0.221918	0.049937
X_2	-0.0437929	0.221918	0.851333
X_3	0.532345	0.221918	0.061709
X_4	0.0225073	0.221918	0.923158
X_5	0.0994291	0.221918	0.672867
X_6	0.106424	0.221918	0.651777
X_7	-0.0465857	0.221918	0.842013
X_8	-0.485472	0.221918	0.080337
X_9	-0.439891	0.221918	0.104281
X_{10}	0.767855	0.221918	0.018041
X_{11}	0.0412582	0.221918	0.859817
X_{12}	-0.590383	0.221918	0.044864
X_{13}	0.504381	0.221918	0.072183

X_1 = *B. licheniformis*, X_2= *L. sphaericus*, X_3 = *B. sphaericus*, X_4 = *B. subtilis*, X_5 = Urea, X_6 =, Calcium chloride, X_7 = Calcium lactate, X_8 = Calcium nitrate, X_9 = Calcium acetate, X_{10} = Yeast extract, X_{11} = Incubation time, X_{12} = Temperature, and X_{13} = Agitation speed, R^2=0.902, R^2 (adj.)=0.646

Optimization of microbial calcium carbonate precipitation

In order to optimize the microbial calcium carbonate precipitation, the response surface methodology (RSM) using a central composite face-centered (CCF) design matrix was used to determine the optimum levels of significant variables. For this purpose, a total of 27 experiment runs were carried out, and experimental design with the actual level of variables are shown in Table 3.

To predict the production of calcium carbonate, the experimental results were fitted with a second-order polynomial function. Considering the effective factors, the polynomial regression based model is presented as follows:

$$Y = 7.712 + 1.802X_1 + 1.719X_3 - 5.266X_{10} + 5.817X_{13} - 6.034{X_{10}}^2 + 14.813{X_{13}}^2 - 2.148X_1X_{10} - 5.086X_{10}X_{13} \quad (2)$$

where Y is the response, X_1, X_3, X_{10}, and X_{13} are *B. licheniformis*, *B. sphaericus*, yeast extract and agitation speed, respectively. Analysis of variance (ANOVA) was used to check the adequacy of the model and a R^2 value of 0.885 demonstrated the goodness of the fitted regression model. As it can be seen from Eq. 2, all single and quadratic factors showed a significant effect besides ${X_1}^2$and ${X_3}^2$. However, the only interactive terms of X_1X_{10} and $X_{10}X_{13}$ found to be significant in the production of calcium carbonate.

Table 3 Level of variables examined in optimization using central composite face (CCF) design

Run	Coded levels				Calcite (g/L)	Vaterite (g/L)
	Yeast extract (g/L) (X_{10})	*Bacillus licheniformis* % (v/v) (X_1)	*Bacillus sphaericus* % (v/v) (X_3)	Agitation (rpm) (X_{13})		
1	2 (-1)	3 (-1)	3 (-1)	60 (-1)	0.67	1.21
2	4 (1)	3 (-1)	3 (-1)	60 (-1)	0.12	1.18
3	2 (-1)	5 (1)	3 (-1)	60 (-1)	0.69	3.48
4	4 (1)	5 (1)	3 (-1)	60 (-1)	0.12	1.27
5	2 (-1)	3 (-1)	5 (1)	60 (-1)	1.49	2.09
6	4 (1)	3 (-1)	5 (1)	60 (-1)	0.30	1.08
7	2 (-1)	5 (1)	5 (1)	60 (-1)	0.49	2.09
8	4 (1)	5 (1)	5 (1)	60 (-1)	0.08	1.01
9	2 (-1)	3 (-1)	3 (-1)	100 (1)	0.12	0.56
10	4 (1)	3 (-1)	3 (-1)	100 (1)	0.10	1.26
11	2 (-1)	5 (1)	3 (-1)	100 (1)	11.89	18.08
12	4 (1)	5 (1)	3 (-1)	100 (1)	0.07	1.45
13	2 (-1)	3 (-1)	5 (1)	100 (1)	18.43	11.50
14	4 (1)	3 (-1)	5 (1)	100 (1)	0.08	1.39
15	2 (-1)	5 (1)	5 (1)	100 (1)	8.74	25.04
16	4 (1)	5 (1)	5 (1)	100 (1)	0.07	1.52
17	2 (-1)	4 (0)	4 (0)	80 (0)	0.21	0.53
18	4 (1)	4 (0)	4 (0)	80 (0)	0.14	1.27
19	3 (0)	3 (-1)	4 (0)	80 (0)	0.81	2.80
20	3 (0)	5 (1)	4 (0)	80 (0)	0.23	1.30
21	3 (0)	4 (0)	3 (-1)	80 (0)	0.19	3.33
22	3 (0)	4 (0)	5 (1)	80 (0)	0.30	1.04
23	3 (0)	4 (0)	4 (0)	60 (-1)	2.84	8.21
24	3 (0)	4 (0)	4 (0)	100 (1)	4.95	27.85
25	3 (0)	4 (0)	4 (0)	80 (0)	1.96	8.25
26	3 (0)	4 (0)	4 (0)	80 (0)	2.27	10.56
27	3 (0)	4 (0)	4 (0)	80 (0)	2.70	8.31

Morphological observation

To confirm the production and the crystal shape of calcium carbonate by different microbial strains, SEM was performed. The SEM micrographs of precipitated crystals showed that different morphologies of calcium carbonate can be produced by selected isolates. Figure 2 depicts the calcium carbonate crystals produced in B4 media. The SEM micrographs of colonies revealed that calcite and vaterite were predominant productions of the strains.

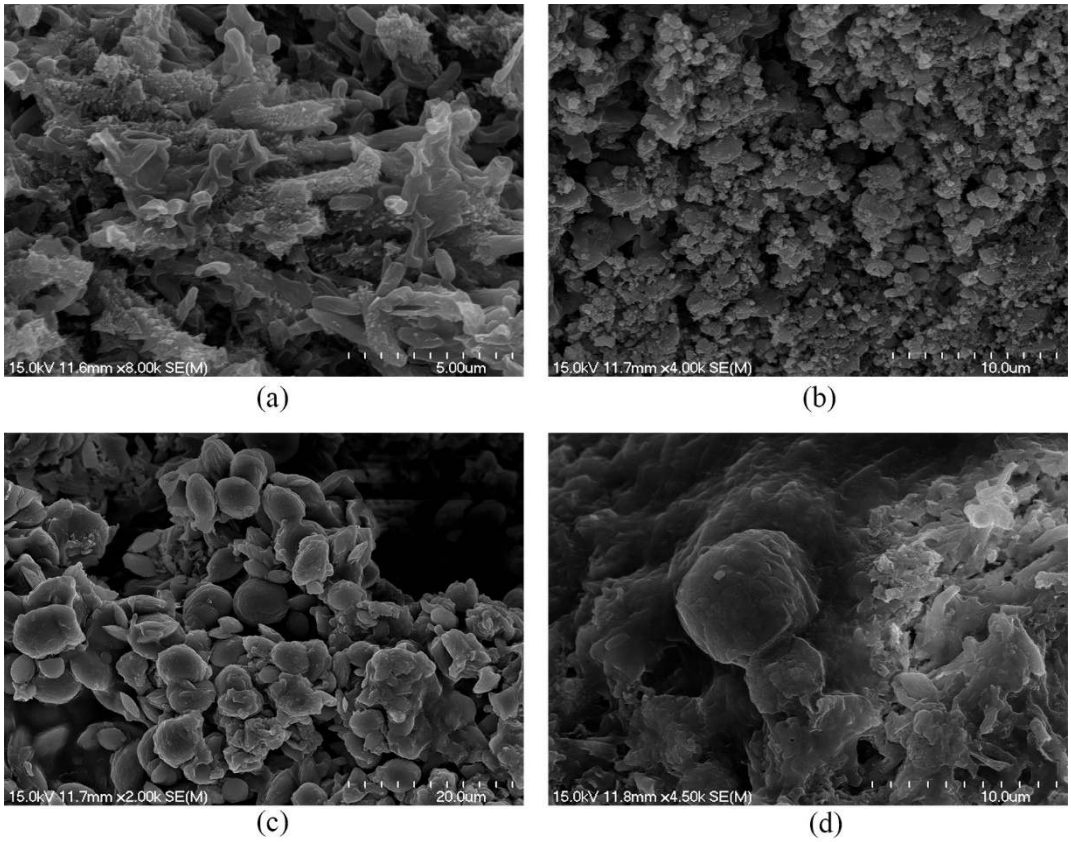

Figure 2 Scanning electron micrographs of calcium carbonate crystals precipitated on B4 media containing microbial strain; a) *B. licheniformis*, b) *L. sphaericus*, c) *B. sphaericus*, and d) *B. subtilis*

Precipitation of calcium carbonate in the screening stage was also studied by SEM analysis. Vaterite and calcite were the main two morphologies in the screening samples. Figure 3a and b show spherical particles predominantly precipitated in the media containing calcium chloride, *B. licheniformis*, *L. sphaericus,* and *B. sphaericus*. Conversely, the micrograph of calcite particles which produced in the media containing calcium lactate, *B. licheniformis,* and *B. subtilis* is depicted in Figure 3c. As expected, a combination of calcite and vaterite were formed in the center points runs which contained all isolates and nutrients (Figure 3d). A comparison between vaterite produced in solid and liquid state fermentation revealed that the crystals precipitated in solid media were smooth while the liquid media produced porous, rough and even broken crystals.

Calcite and vaterite particles can be also distinguished in the optimization samples. Figure 3e presents the SEM micrograph corresponding to the produced crystals in the optimum media. It was noticed that the size of produced crystals in the optimization study was bigger than those precipitated in the screening studies. As shown in Figure

3f, the average vaterite size of 20 µm was observed in the optimized sample which was two times bigger than those produced in the screening stage.

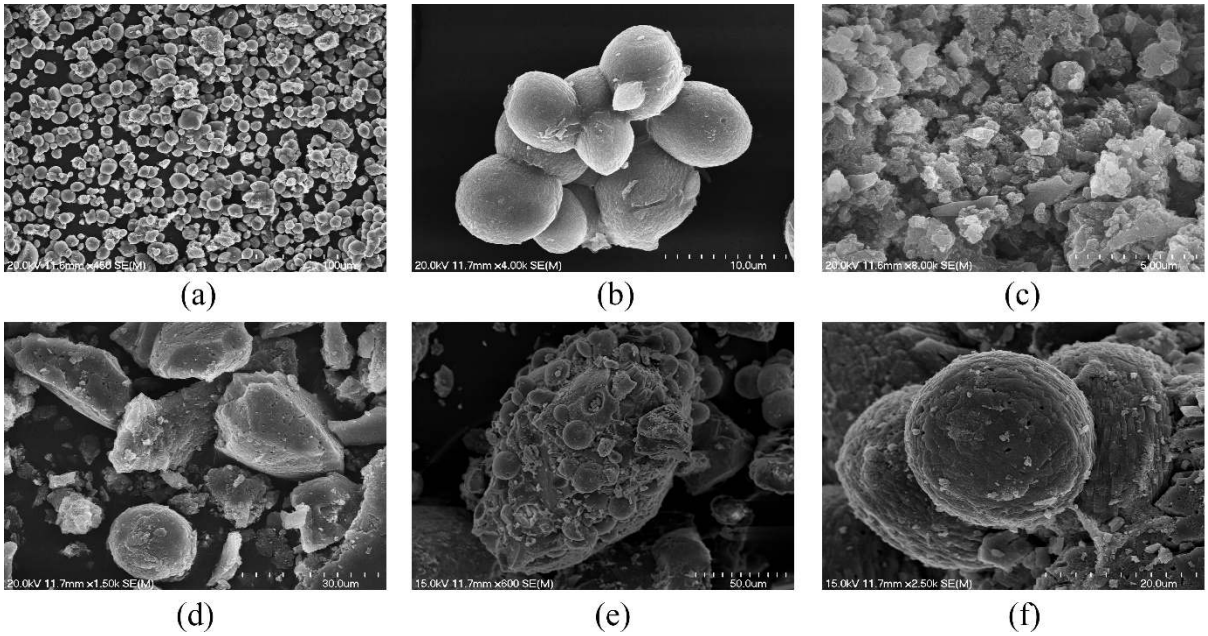

Figure 3 Scanning electron micrographs of calcium carbonate crystals precipitated in liquid media; a-d) vaterite and calcite crystals produced in screening study, e) assemblage of spherical vaterite crystals in optimization study and f) porous structure of vaterite crystals produced in optimization study

Bio-precipitates were further characterized using EDX at 15.0 keV. EDX as an analytical method was employed to detect the elements presented in the newly formed crystals. Elements existing in a sample are detected by atomic number and the amount of them can be determined by the intensity of peaks. To determine the elemental ratio of pure calcium carbonate, EDX was performed for pure calcite and the elemental spectrum is shown in Figure 4a. EDX was also performed for the produced precipitates in the optimized sample to confirm that the precipitated crystals were calcium carbonate (Figure 4b). A high degree of similarity was observed between EDX spectra of the pure calcium carbonate and the optimized sample. The result discloses that calcium, carbon and oxygen are the predominant elements in bio-precipitates. Considering the atomic ratio, it could be concluded that the precipitates were calcium carbonate.

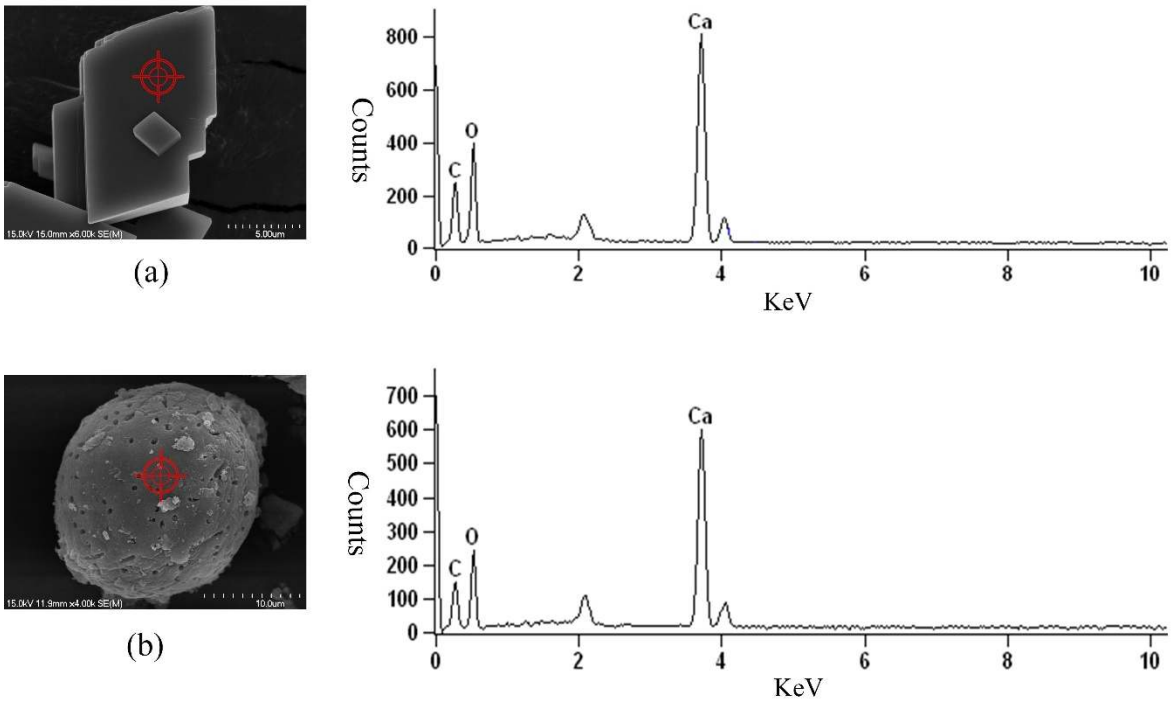

Figure 4 EDX spectra a) pure calcium carbonate and b) precipitated crystal by bacteria in optimization study

Structural and morphological characterization of the produced particles

X-ray diffraction powder (XRD) was performed to analyze the morphology of the produced crystals during the biomineralization of calcium carbonate. The production of two crystals (vaterite and calcite) in B4 media has been supported by XRD examination. As depicted in Figure 5a, XRD spectra confirms the heterotrophic precipitation of calcium carbonate in the B4 media.

XRD spectra of the produced crystals in the optimization stage are presented in Figure 5b where the angle of 29.36° and 27.11° represent calcite and vaterite, respectively. Although calcite and vaterite were detected in all samples, this ratio was not consistent across all the samples. However, no aragonite was precipitated in screening and optimization samples. It was noted that the media containing a low concentration of *B. licheniformis* and *B. sphaericus* under a lower level of agitation speed (60 rpm) produced maximum calcite. Whereas, the increase of *B. licheniformis* and agitation speed (100 rpm) led to precipitate the least calcite. This variation was also observed in the screening stage. Figure 5c depicts XRD spectrum for the optimized sample where the lattice planes of 104 and 113 indicate the formation of calcite and vaterite, respectively.

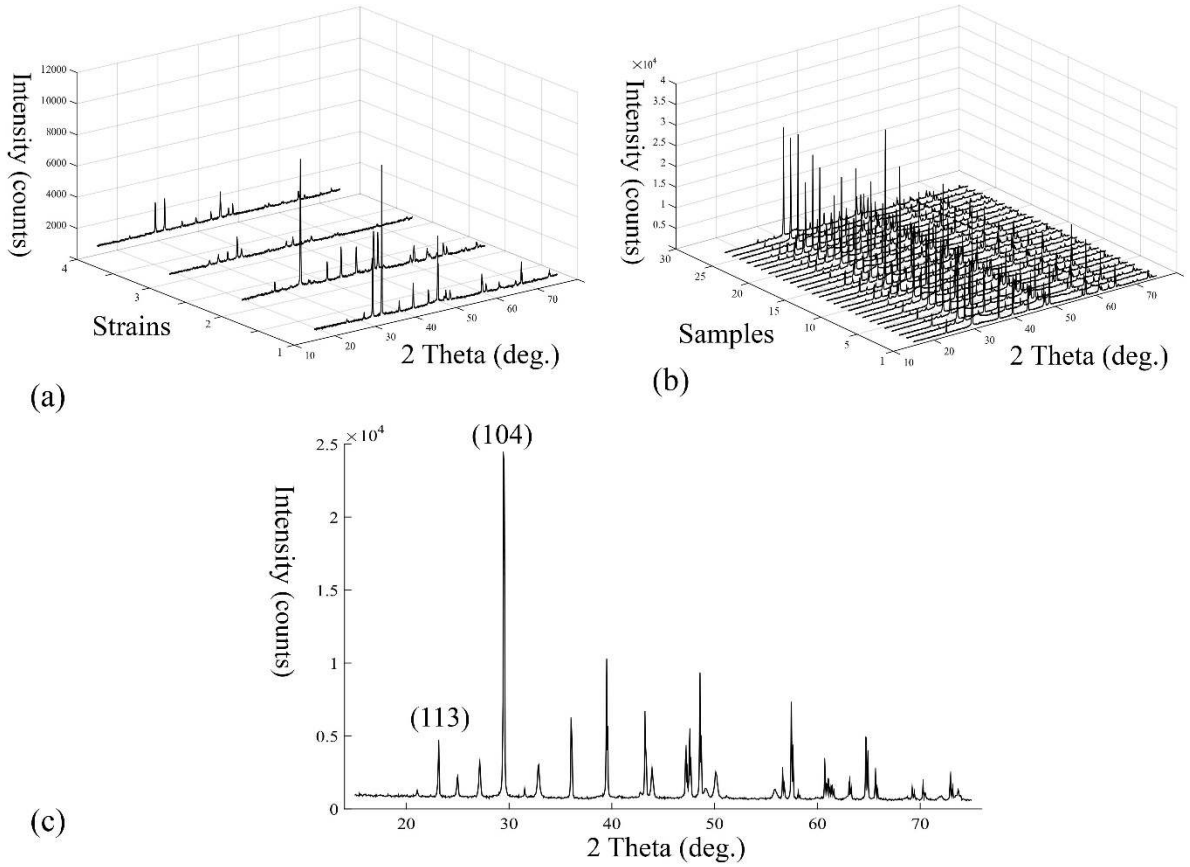

Figure 5 XRD patterns of calcium carbonate precipitated in; a) the B4 media, b) the optimization study, and c) the optimized sample produced by the optimum levels of variables

Morphological quantification

The compatibility, quality and amount of precipitates are the prime factors influencing the performance of bio self-healing concrete. Calcium carbonate is one of the most useful substances for sealing the cracks due to high compatibility with the concrete composition. The quality of a filler is defined as efficient bonding with the concrete and the ability to withstand for a long time. Physical properties of the microbial calcium carbonate precipitation strongly rely on the portion of each polymorph. Calcium carbonate polymorphs have a hardness between 3–3.5 (Mohs scale) and they are poorly water soluble. These properties make the calcium carbonate an efficient long-lasting filler. However, the ability to fill more space is believed to contribute to enhance the efficiency of bioconcrete. Since the density of vaterite is less than calcite, more space can be occupied by vaterite particles. Unlike calcite and aragonite particles which can be precipitated in diverse color (colorless, white, yellow and brown), vaterite particles are usually colorless. Therefore, they can be precipitated in every part of a concrete structure without compromising the appearance of the structure. In this study a morphological quantification was performed to determine the calcium carbonate morphological ratio. The different morphology results in different peaks and intensities. The most intensive peaks occurred at the angle of 29.36° (2θ), 27.11° (2θ), and 26.26° (2θ) for calcite, vaterite and aragonite, respectively. Figure 6 shows the ratio of calcite to vaterite in optimization stage. The maximum percentage of calcite was produced in sample 13 while the maximum percentage of vaterite was

precipitated in sample 10. Considering the weight of bio-precipitates, the maximum amount of calcite and vaterite was 18.43 g/L in sample 13 and 27.85 g/L in sample 24, respectively (see Table 3).

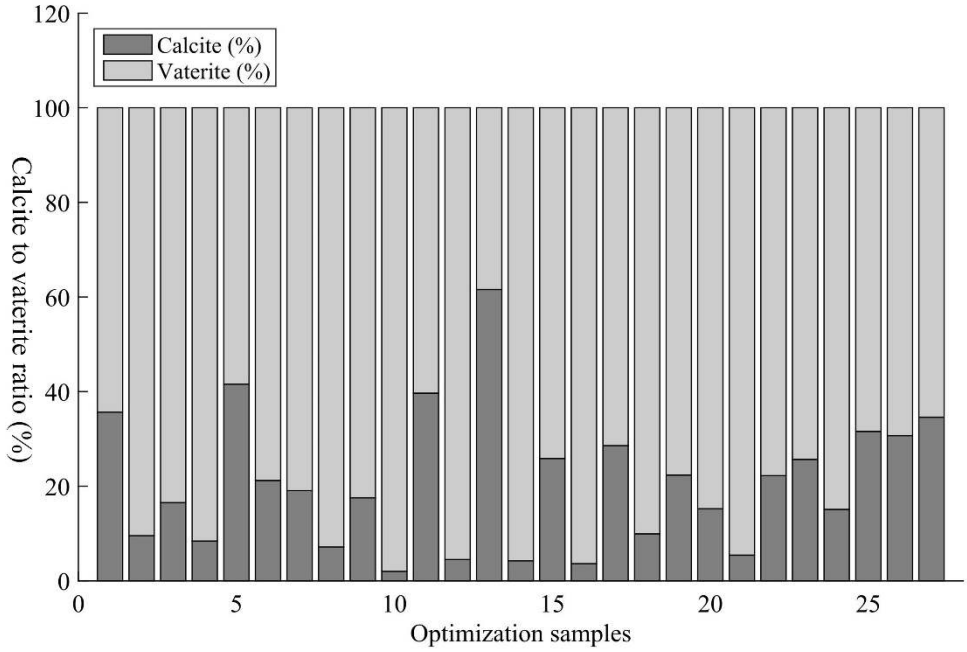

Figure 6 The ratio of calcium carbonate polymorphs (calcite to vaterite) precipitated in optimization study

Validation of model

In order to determine the optimal levels of variables, the regression equation, by remaining inside the region of experimental levels, was solved. The model predicted a 35.47 g/L of calcium carbonate for conditions using 2.0 g/L (yeast extract), 4.5 % (v/v) (*B. licheniformis*), 4.5 % (v/v) (*B. sphaericus*), 40 g/L (calcium chloride), 65 g/L (urea), 100 rpm (agitation speed) at 35 °C. To validate the model, duplicate samples were prepared based on the suggested concentrations. It was noted that the observed and predicted results had a high degree of similarity in the production of calcium carbonate by only 5 % of error.

Discussion

As the bacterial cells serve as nucleation sites for precipitation of calcium carbonate, screening of effective factors on the biomineralization process was performed. All of the isolates were selected from *Bacillus* species because of producing endospores which help bacteria to survive in harsh conditions such as heat, cold and radiations for long periods. The bacteria used in this study are not pathogenic to humans, plants and animals, and therefore there is no foreseeable issue for their application in construction materials. Various concentration of these bacteria were used for the screening step. Although heterotrophic growth of all strains showed that they are capable of producing calcium carbonate in the solid media, the screening results indicated that only *B. licheniformis* and *B. sphaericus* have significant capability for calcium carbonate production (p<0.1). Figure 7 presents the response contour plots to visualize the influence of the effective variables on the production of calcium carbonate. Each surface plot shows the effect of two variables on the response by keeping the other variables at their zero levels. As can be

depicted from Figure 7a, a relatively high concentration of *B. licheniformis* and *B. sphaericus* facilitated the precipitation of calcium carbonate in the media containing a fixed concentration of urea and calcium chloride. The plot also shows that the optimum bacterial concentration was at 4.18 % and 4.21 % (v/v) for *B. licheniformis* and *B. sphaericus*, respectively, to achieve the maximum production of calcium carbonate. Correlation between microbial growth rate and calcium carbonate production (response) are presented in Figure S2 in the Supplementary Material. It shows that an increase in the number of cells provides the higher nucleation sites and, consequently, more calcium carbonate crystals are precipitated.

In the biomineralization process, calcium carbonate is induced when calcium ions accumulate extracellularly in a certain condition. In the screening studies, the effect of four types of calcium source, namely calcium chloride, calcium lactate, calcium nitrate and calcium acetate on biomineralization of calcium carbonate, were investigated. Different concentrations of calcium sources were used in order to evaluate the effectiveness of calcium ions on biomineralization. Based on the analysis of variance results, it can be concluded that calcium chloride is the most preferred calcium source to induce calcium carbonate crystals. Although the presence of calcium source for microbial calcium carbonate precipitation is crucial, the concentration of Ca^{2+} has a great influence on the efficiency of the process. In this study we successfully demonstrated that the presence of low and excessive amounts of Ca^{2+} have an adverse impact on microbial production of calcium carbonate. A high concentration of Ca^{2+} may inhibit the activity of microbial strain and, consequently, the production of calcium carbonate is affected. On the other hand, a few electron acceptors are involved in ionic reaction when a low concentration of Ca^{2+} is used.

Generally, nutritional starvation may contribute to a decrease or cessation of bacterial growth and effective metabolism. Therefore, the presence of appropriate concentrated nutrient is essential to increase the effectiveness of biomineralization. Yeast extract as a nitrogen source was tested due to its availability and high-performance. As shown in Table 2, the presence of yeast extract had a positive influence on the calcium carbonate biosynthesis. However, a high concentration of yeast extract showed an inhibitory effect on the calcium carbonate production. Bacterial cell wall was inhibited when a high concentration of yeast extract was used which prevented electron transportation between existing calcium ions in the media and negatively charged cell walls. It was found that the utilization of yeast extract (more than 3 g/L) dramatically declined the microbial calcium carbonate precipitation. Figure 7b and c demonstrate the interactive effects of yeast extract, *B. licheniformis,* and *B. sphaericus* on the production of calcium carbonate. The response increased with the increase in *B. licheniformis* concentration from 3.6 to 5 % (v/v); however, the production of calcium carbonate decreased as the concentration of yeast extract reached its upper level. The similar trend was observed when *B. sphaericus* and yeast extract were used. Apart from the influence of bacteria and nutritional compounds, operating conditions, such as temperature, agitation speed and incubation period, may have an influence on biomineralization of calcium carbonate which requires further investigation.

Three levels of temperatures (33 °C, 39 °C and 45 °C) were considered to study the effect of temperature on microbial precipitation of calcium carbonate. The screening study revealed that bioprecipitation of calcium carbonate is not significantly affected by the temperature (see Table 2). This indicates that the biomineralization of calcium carbonate is applicable in a wide range of surroundings. Since the concrete structures are built in various environments, this finding demonstrates that the efficiency of a bio self-healing concrete is not affected

by temperature variations. Once a crack forms in the concrete, an urgent action is required to prevent the crack extension and deterioration of the structure. Therefore, the incubation period was another factor which was considered in screening stage. To analyze the effect of incubation period on biomineralization of calcium carbonate, three levels of incubation period were investigated. The screening results indicated that the incubation time is not an efficient factor on the production of calcium carbonate. It was observed that the maximum crystals were precipitated at the beginning of the fermentation process and the rate of calcium carbonate precipitation decreased with the time. Conversely, the ANOVA results showed that agitation speed had a positive effect on bioprecipitation of calcium carbonate among operating conditions. In this study agitation was used to increase the oxygen transfer rate to microbial cells. Various agitation speeds were considered to evaluate their effect on the biomineralization of calcium carbonate. Agitation is not only beneficial for bacterial growth, but also provides more interactions between negatively charged bacteria cells and electron acceptors present in media (Ca^{2+}). The interactive effects of agitation speed, microbial strains and yeast extract on biomineralization of calcium carbonate are depicted in Figure 7d–f. It was found that the increase of these variables besides, yeast extract, increase the production of calcium carbonate. The maximum amount of bio-precipitates can be achieved when the concentration of *B. licheniformis*, *B. sphaericus*, shaking speed and yeast extract are adjusted at 4.21 % (v/v), 4.18 % (v/v), 100 rpm, and 2 g/L, respectively.

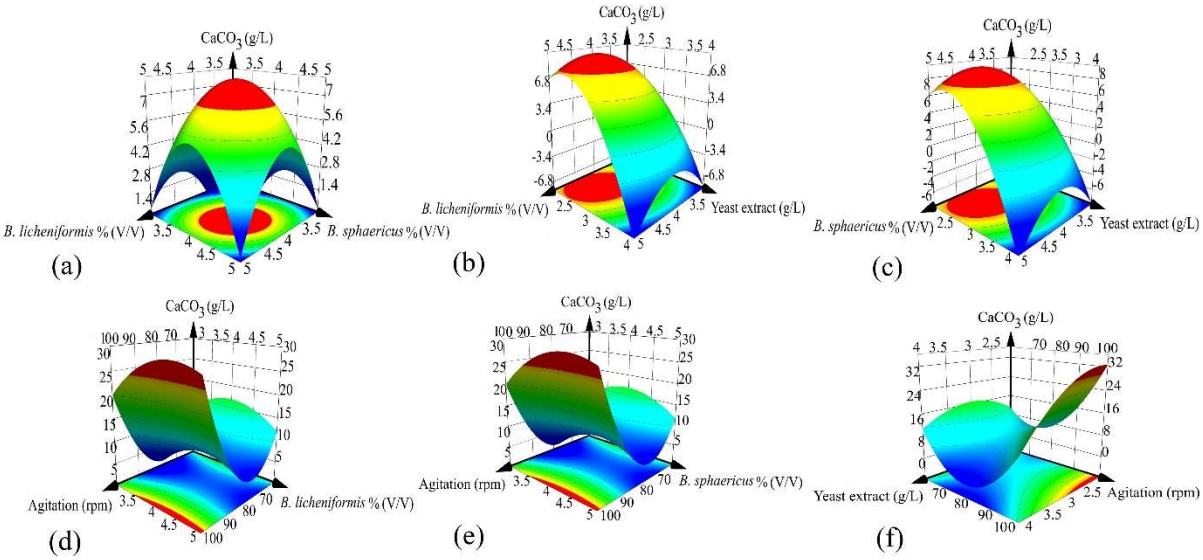

Figure 7 Three-dimensional response surface plots for calcium carbonate production showing the interactive effects of a) *B. licheniformis* and *B. sphaericus*, b) *B. licheniformis* and yeast extract, c) *B. sphaericus* and yeast extract, d) agitation speed and *B. licheniformis*, e) agitation speed and *B. sphaericus*, f) yeast extract and agitation speed

Calcium carbonate properties, including particle size, its distribution, morphology, specific surface area, brightness and chemical purity, have a strong impact on its application in various industries [23]. Among these factors morphological aspect is one of the most significant characteristics. The diversity of calcium carbonate mineralization and various saturation levels result in the production of different polymorphs (calcite, vaterite and aragonite). The reason for producing various polymorphs through biomineralization of calcium carbonate is not well understood. However, factors, such as bacteria surface wall properties, bacteria metabolic activities,

extracellular polymeric substance (EPS) content and the composition of media, may have an influence on the morphology and the size of produced crystals.

Bacterial cell wall provides a nucleation site, allowing the positive ions to attach to a negatively charged bacterial cell surface to form minerals. The bacterial cell surface differences are mainly due to the amount of peptidoglycan, the amidation level of free carboxyl and the availability of mycolic and teichoic acids. For instance, the absence of mycolic acids in *Arthrobacter* sp. causes a hydrophilic cell wall, whereas the present or production of mycolic acids in *Rhodococcus* sp. results in hydrophobic cell wall and, consequently, it is likely to influence cell surface charge [24, 25]. The composition of medium and concentration of EPS also affect the formation of various morphologies. It was reported that the abundance of EPS and the type of amino acids in the medium have a certain influence on the mineralogy of precipitates [26]. It should be pointed out that the crystal size may be affected by EPS and the composition of media. This study indicated that the type of electron acceptor also had an effective influence on morphology. It was found that calcite particles were mainly produced when bacteria utilized organic acid (calcium lactate), whereas vaterite crystals predominantly precipitated when calcium chloride was used as an electron acceptor. Apart from these the viscosity of the medium also showed an impact on production of different morphologies. It was noted that the probability of calcite formation in a natural environment improves as the viscosity of the medium increases [27]. The precipitation of crystals revealed that the likelihood of producing vaterite by isolates increased when the water activity increased. This study showed that operating conditions and nutritional substances, such as yeast extract and urea, had no influence on the morphology; the only parameters affecting the microbially produced calcium carbonate morphology were the genera of bacteria (cell surface properties), the viscosity of the media and the type of electron acceptor (Ca^{2+}).

The effectiveness of a bio self-healing concrete relies on various factors, including the amount of bio-precipitates and the possibility of activation in diverse environments at a short period of time. The utilization of suitable microbial compounds at their optimum levels can significantly enhance the efficiency of bio self-healing concrete by filling the entire cracks and porosities. Various parameters, including microbial strains, media compositions and operating conditions, were investigated to determine the effective parameters on biomineralization of calcium carbonate. The results indicated that *B. licheniformis*, *B. sphaericus,* yeast extract, urea, calcium chloride and agitation speed had a significant influence on biomineralization efficiency. However, it was found that temperature and incubation time were not significant factors on calcium carbonate biosynthesis. It was noticed that calcite and vaterite particles were predominantly produced by *B. licheniformis* and *B. sphaericus*. To determine the influential parameters on calcium carbonate morphologies, a novel morphological quantification using XRD was performed. The study demonstrated that the bacterial cell surface properties, the viscosity of the medium and the type of electron acceptor (Ca^{2+}) were the effective factors on the morphology of bio-precipitates. Since a self-healing concrete reduces inspection and maintenance costs, it can be expected that the bio-concrete could make its way to the market in the early future.

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Conflict of interest

The authors declare that they have no competing interests.

Ethics

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