Title:

New insights into the role of pH and aeration in the bacterial production of calcium carbonate ($CaCO_3$)

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Abstract

Over recent years the implementation of microbially-produced calcium carbonate ($CaCO_3$) in different industrial and environmental applications has become an alternative for conventional approaches to induce $CaCO_3$ precipitation. However, there are many factors affecting the biomineralization of $CaCO_3$, which may restrict its application. In this study we investigated the effects of pH and aeration as the main two influential parameters on bacterial precipitation of $CaCO_3$. The results showed that the aeration had a significant effect on bacterial growth and its rise from 0.5 SLPM to 4.5 SLPM could produce 4.2 times higher $CaCO_3$ precipitation. The increase of pH to 12 resulted in 6.3 fold increase in $CaCO_3$ precipitation as compared to uncontrolled-pH fermentation. Morphological characterization showed that the pH is an effective parameter on $CaCO_3$ morphology. Calcite was found to be the predominant precipitate during aeration controlled fermentations, while vaterite was mainly produced at lower pH (up to 10) over controlled-pH fermentations. Further increase in pH resulted in a morphological transition, and vaterite transformed to calcite at the pH ranges between 10 and 12.

Introduction

It is widely known that microorganisms contribute to the generation of a wide diversity of minerals such as carbonates, phosphates, sulphides and silicates. Among all bioprecipitate minerals, the production of $CaCO_3$ has drawn much attention due to its role in environmental and industrial applications. The bioprecipitation of $CaCO_3$ can be achieved through biologically controlled mineralization (BCM) and biologically induced mineralization (BIM). BCM is a strictly regulated process and is independent of environmental conditions, while BIM usually occurred in an open environment and the minerals are precipitated as the interaction of microbial metabolic activities and environment [1].

There are different hypotheses for bacterial production of carbonate. The first hypothesis is ionic exchange through the bacterial cell membrane [2]. In this approach which is considered as an active precipitation, microbial $CaCO_3$ precipitation is induced by successful attachment of bacterial cell walls and positively charged Ca^{2+} ions. The production of extracellular polymeric substances (EPS) is assumed as another hypothesis in regard to $CaCO_3$ precipitation through the trapping of Ca^{2+} [3] or due to the production of particular proteins that may influence the precipitation [4]. The last hypothesis is carbonate mineralization through a passive approach as a by-product of microbial metabolism involving either autotrophic or heterotrophic pathway [5]. In autotrophic pathway, the conversion of carbon dioxide to carbonate by microorganism during non-methylotrophic methanogenesis (by methanogenic archaea), oxygenic photosynthesis (by Cyanobacteria) and anoxygenic photosynthesis (by purple bacteria) leads to induce $CaCO_3$ precipitation when a Ca^{2+} source presents in the surrounding. The production of carbonate minerals can also be achieved through the heterotrophic growth of bacteria species including *Bacillus*, *Arthrobacter*, and *Rhodococcus* on organic acid salts such as acetate, lactate, citrate, succinate, oxalate, malate and glyoxylate [6]. In general, the sulphur cycle and the nitrogen cycle are the most common mechanisms of producing $CaCO_3$ through heterotrophic pathway. The sulfur cycle occurs by dissimilatory reduction of sulfate, while urea or uric acid degradation (ureolysis), ammonification of amino acids and dissimilatory nitrate reduction contribute to the production of carbonate in nitrogen cycle. These three nitrogen cycle approaches induce the production of carbonate and bicarbonate ions, and therefore, $CaCO_3$ is produced if the Ca^{2+} ions are present in the

surrounding. On the other hand, if the Ca^{2+} ions are not available in the environment, the bacterial activity may lead to zeolite formation [7].

The precipitation of carbonate minerals by microorganisms broadly occurs in different geological environments such as fresh and marine water [8], soil [9], cave [10, 11] and hot spring [12]. Apart from the role of biomineralization in nature, a large number of applications involving the microbially induced $CaCO_3$ precipitation with important scientific and technological implications have been reported. The main applications of $CaCO_3$ biomineralization are protection of limestone monuments [13], removal of Ca^{2+} ions from wastewaters [14], surface treatment of construction materials [15, 16], improvement of brick properties [17, 18], removal of contaminants and heavy metals from groundwater [19], plugging the pores of oil reservoirs [20], strengthening of sand columns [21], soil consolidation [22, 23], atmospheric CO_2 sequestration [24], and filling concrete cracks [25-27]. Over recent years there has been an increasing interest in the study of the bio self-healing concrete to address the concrete issues. Concrete is one of the most common building materials used in the world; however, it is prone to crack formation, which affects the concrete's integrity, durability and serviceability. A biological approach has the potential to overcome the issues arising from the current techniques to fill the cracks by producing the most compatible material ($CaCO_3$) with concrete. The bacterial production of $CaCO_3$ relies on many parameters, which can affect the bio self-healing performance. It has been reported that bacterial production of $CaCO_3$ is affected by the concentration of dissolved inorganic carbon and calcium, pH, nucleation site and Hartree energy (E_h) [28, 29]. Concrete has a pH of ~12 and exposes many environmental conditions which may inhibit the bacterial metabolism. Therefore, the ability of bacteria to tolerate a high pH and also the capability of $CaCO_3$ precipitation in such conditions are the main challenges to design a sustainable bio-concrete. Although the survivability of bacteria in a relatively harsh environment has been reported [30], the capability of producing minerals in such environments has remained a matter of debate. The availability of oxygen is another significant parameter affecting the biomineralization of $CaCO_3$. In general, aerobic organisms utilize oxygen to facilitate efficient growth leading to produce bio-product under the certain condition. However, the possibility of toxin formation, inhibitory effect on metabolism and respiration in microorganisms may increase if a high supply of oxygen (more than its critical level) presents in the medium. The oxygen toxicity potentially occurs at the exponential growth phase rather than the stationary phase. Considering the robustness of bacterial growth at the exponential growth phase, and its significant role in biomineralization of $CaCO_3$, it is vital to provide a condition to enhance bacterial growth without elaborating a considerable amount of toxin.

The fermentation is a complex dynamic process that may be affected by many factors, including aeration and alkalinity levels. Therefore, the main aims of the present study were to investigate (i) the effect of aeration on bacterial growth and $CaCO_3$ production, and (ii) the performance of bacteria to induce $CaCO_3$ precipitation at the different ranges of pH.

Materials and methods

Chemicals

Calcium chloride anhydrous, urea and yeast extract were obtained from Sigma-Aldrich (St. Louis, MO, USA). Glucose and Bacto™ Peptone were purchased from Becton Dickinson (Becton Dickinson, NJ, USA). Sodium

hydroxide, sodium chloride and hydrochloric acid (36 %) were obtained from a domestic supplier. Active silicone antifoam agent and nutrient agar plates were purchased from Fort Richard Laboratories (Auckland, New Zealand).

Microorganisms and growth medium

Bacillus licheniformis ATCC 9789 and *Bacillus sphaericus* NZRM 4381 as the most $CaCO_3$ producing isolates were selected from the previous study [31]. The sterilized growth medium used for bacterial rehydration was composed of 0.5 % (w/v) Bacto™ peptone, 0.5 % (w/v) glucose and 0.05 % (w/v) yeast extract. The medium was then incubated overnight with shaking of 120 rpm at 37 °C. One milliliter of revived bacterial culture was spread on nutrient broth agar plate and incubated at 37 °C for 24 h. The harvested cells were suspended in a solution of sodium chloride (0.9 % w/v). To obtain a pure solution of spores, it was then kept in water bath at 80 °C for 10 min [32]. Subsequently, to remove the cell debris, the spore suspension was centrifuged at 3000 rpm for 12 min.

Colony counting and growth monitoring

The growth rate of the bacteria was calculated by measuring the absorbance at a wavelength of 600 nm (Shimadzu, UV-1700, Kyoto, Japan) during the fermentation process. The optimum fermentation medium for $CaCO_3$ fermentation studies, was composed of urea, calcium chloride and yeast extract [31]. Direct measurement of microbial biomass was also performed by using a nutrient agar plate at the end of the fermentation process. One milliliter aliquot of the medium was taken from the fermentor, appropriately diluted, then spread on the nutrient agar plate and incubated at 37 °C for 24 h to determine the cell numbers. The average was used for counting and the results were expressed as colony forming units per milliliter (CFU/mL).

Bioreactor setup

A 3-L laboratory-scale fermentor (BioFlo/CelliGen 115, New Brunswick Scientific Co., MA, USA) was employed for an aerobic fermentation study. AFS Biocommand (New Brunswick Scientific) was used for controlling the bioprocess and data acquisition. The bioreactor was equipped with a top-driven stirrer and two six-blade Rushton impellers were placed onto the agitation drive shaft (i.e., diameter 52 mm, width 16 mm, length 18 mm). To ensure gas bubble dispersion and provide a high rate of oxygen transfer, four stainless steel internal baffles (i.e., width 13 mm, length 140 mm) were positioned inside the vessel. The maximum adjustable levels of stirrer and aeration were 1200 rpm and 5 standard liter per minute (SLPM), respectively.

A standard two-point calibration method (pH 7 and 12 buffer standards) was performed to calibrate the pH electrode. The pH of the medium was kept constant with a measurement error of 0.01 by automatic addition of base (2M NaOH) or acid (2M HCL). Dissolved oxygen (DO) was measured by DO probe, which was already calibrated using a standard two-point calibration method (0 % and 100 %). The 0 % calibration was conducted by introducing nitrogen into the fermentor at a rate of 1 SLPM until the reading stabilized. The span calibration (100 % air saturated condition) was achieved by the provision of 50 rpm agitation speed and vigorous sparge air into the bioreactor for 30 min. The DO values were recorded according to these calibration set points. The foam level was controlled during batch fermentation by addition of silicon-based defoamer agent into the vessel. To detect

the formation of foam, a level probe, which is sensitive to wetness, was used. The diluted sterilized antifoam was pumped upon the formation of foam (wet contact) to alleviate the biofilm formation.

The external sparger tube attached to the controller was sterilized with the circulation of sodium hypochlorite solution at 1 % for 1 h. The tube was subsequently rinsed with sterilized distilled water to eliminate the chlorine residues. Airflow was then introduced into the vessel through a 6 mm ring sparger and dispersed 10 mm below the impeller. To avoid batch spoilage and environmental contamination, filter-sterilized air (0.2 µm) sparged into the bottom of the vessel.

Design of the batch experiments

The fermentation medium was prepared as previously described; the optimal concentrations were as follows: 40 g/L calcium chloride, 65 g/L urea and 2 g/L yeast extract [31]. The bioreactor was sterilized using an autoclave at 121 °C for 20 min, cooled and then inoculated according to the optimum condition (4.5 % v/v of each isolates). During 180 h of continuous fermentation, samples were taken from the fermentor at 12-h intervals to determine the concentration of soluble Ca^{2+} ions and bacterial growth rate. In the present study, all the experiments were operated under batch fellow condition. The bioreactor was thoroughly cleaned to avoid the clogging of the sparger and attachment of bioproduct to the pH, DO and foam probes at the end of each experiment.

In the first stage of the investigation, the batch fermentations were carried out at various aeration rates and a constant mixing speed of 150 rpm at 35 °C to investigate the effect of aeration on the microbial production of $CaCO_3$. It has been shown that the availability of a high level of CO_2 inhibits the bacterial metabolic activity and subsequently it leads to a decrease in the yields. Therefore, to minimize the inhibitory effects, and also increase the availability of oxygen in the media, the maximum aeration rate was set to 4.5 SLPM. The increase of airflow rate resulted in a massive foam formation during experiment runs. Hence, a sterilized solution of antifoam agent was pumped into the vessel to alleviate the foam formation and provide a better oxygen transfer in the system. In the second stage, the batch fermentations were performed at different levels of pHs with a constant air flow rate of 0.5 SLPM to explore the influence of alkalinity on bacterial growth and $CaCO_3$ precipitation. The pH tended to decrease over experimental runs, and it was stabilized by addition of base to the bioreactor.

Calcium carbonate extraction

During 180 h of fermentation, samples were taken from the fermentor at 12 h intervals to determine the concentration produced of $CaCO_3$. The concentration of soluble Ca^{2+} ions in the filtered medium (0.45 µm) was measured using a benchtop photometer (Palintest, The UK). Calcium chloride solution was used to make a standard curve for measuring the Ca^{2+} ion's concentration and the standard error of measurement was 0.2 mg/L. The fermentation run with no microbial inoculation was used as a negative control. At the end of fermentation, the precipitated $CaCO_3$ crystals were extracted by passing the fermented medium through a 0.2-µm membrane filter paper (Advantec, Tokyo, Japan). To remove impurities the precipitates were washed three times with distilled water, oven dried overnight at 70 °C and kept at a desiccator for crystal characterization.

Statistical analysis

An analysis of variance (ANOVA) was performed using Statistica package version 12 (Tulsa, USA) at 0.05 probability level ($P < 0.05$) to determine whether the variables, including aeration and pH, were statistically significant on the yield. Consequently, the t-test was conducted to compare the yield for each pair of group.

Morphological observation of bacterial $CaCO_3$ precipitation

The morphology and the size of precipitated $CaCO_3$ crystals were observed by scanning electron microscopy (SEM, Hitachi S-4700, Tokyo, Japan). Furthermore, the elemental composition of bioprecipitates was analyzed using an energy dispersive X-ray spectroscopy (EDX) equipped with the SEM instrument. The dried bioprecipitates were crushed using a mortar and pestle to obtain a homogeneous powder mixture. The collected bioprecipitates were placed on a sticky carbon tape which was positioned to the aluminum stub and, subsequently, it was platinum coated by a putter coater (Hitachi, E1030) to minimize the image disturbances. Morphological observation and elemental composition study were conducted at 5 KeV and 15 KeV, respectively.

Characterization of bacterial $CaCO_3$ precipitation

The well-crushed bioprecipitates were examined by x-ray diffraction (XRD) for recognition and quantification of $CaCO_3$ morphologies. The powder was back-packed into a sample holder and mounted on Panalytical Empyrean reflectometer (Almelo, The Netherlands). Bioprecipitates were characterized by XRD (CuKα radiation) in a 2θ interval between 15° and 75°. Data were collected for the step size, the voltage and the current of 0.0530°, 45 kV and 40 mA, respectively.

Results

Aeration controlled batch fermentations

In the preliminary study, the variations of cell growth, $CaCO_3$ precipitation, DO and pH were monitored during uncontrolled-pH fermentation. Fig. 1 illustrates the variation of pH, DO, bacterial growth and microbial production of $CaCO_3$ throughout the fermentation process. It was found that the highest concentration of bacterial cell was attained during the first 40 h of fermentation. Interestingly, a correlation was observed between bacterial cell growth and $CaCO_3$ precipitation which more than 60 % of $CaCO_3$ crystals were precipitated over the exponential cell growth (40 h). This indicates the significant impact of cell number as a nucleation site for ionic exchange to induce $CaCO_3$ precipitation. The variations in DO concentration and pH strategy were also monitored during the cultivation time. The concentration of DO decreased sharply and reached its lowest level (15 %) at 30 h. However, an increase in DO concentration was observed from 30 h to 60 h and then it stabilized until the end of fermentation. The same trend for DO and pH profiles was observed during first 70 h of fermentation. pH started to drop suddenly at the exponential phase and reached to the lowest level (pH 7). Afterwards, the pH was increased to the highest level (8.2) and followed a slight decrease for the rest of fermentation period.

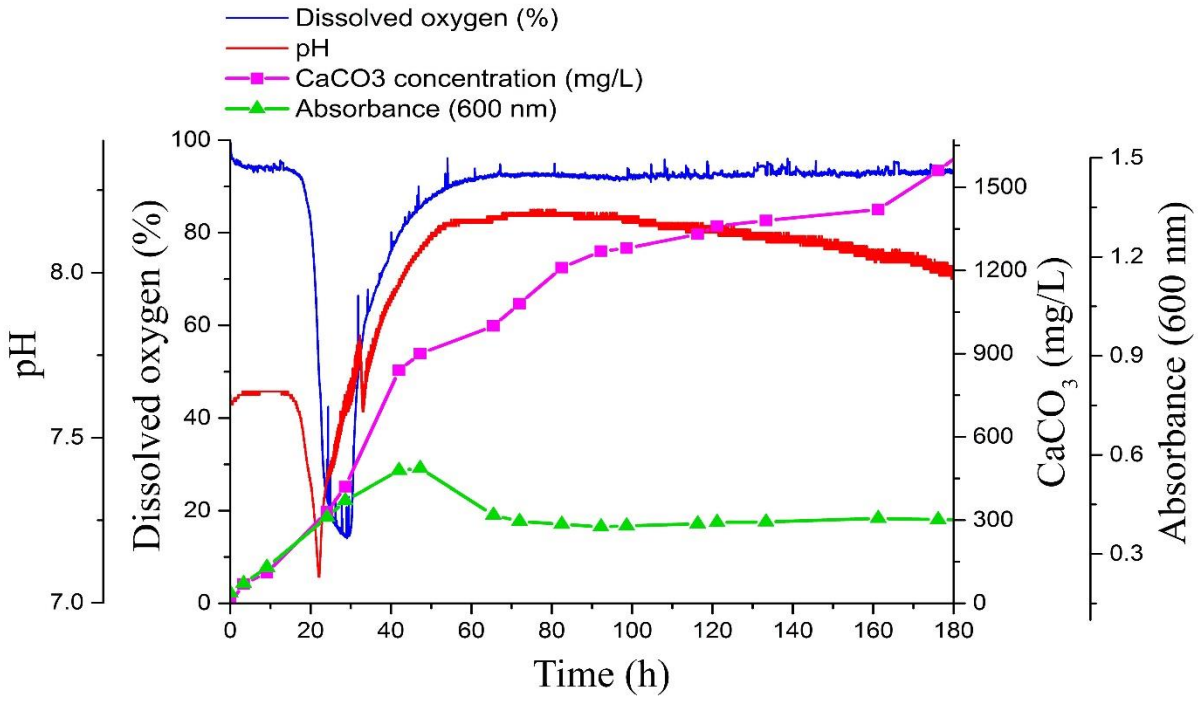

Fig. 1 Variation in bacterial growth, DO, pH and $CaCO_3$ concentration over the course of fermentation

In the next set of experiments, the effect of aeration on the bacterial cell growth and the production of $CaCO_3$ was investigated. As shown in Table 1, fermentation runs were performed at four aeration rates of 0.5, 1.5, 2.5 and 4.5 SLPM under an uncontrolled-pH condition. It was observed that the higher aeration rate was in favor of $CaCO_3$ precipitation. The *p-value* of 0.0029 indicates the addition of oxygen during fermentation had a significant impact on the yield (Table 2). An increase in aeration rate from 0.5 SLPM to 1.5 SLPM resulted in a 45 % increase in the $CaCO_3$ concentration. However, a 3.3-fold increase in the bacterial production of $CaCO_3$ was achieved when the aeration rate reached to 2.5 SLPM. Although the increase of airflow to its upper level (4.5 SLPM) had a positive effect on the $CaCO_3$ concentration (4.3-fold increase), its contribution was not significant as compared to 2.5 SLPM airflow (P>0.05). Fig. 2a shows the concentration of soluble Ca^{2+} during aeration controlled batch fermentations. The results indicated that the majority of insoluble Ca^{2+} was induced during 80 h of fermentation. To validate the results obtained during biosynthesis, the fermentations were repeated in triplicates. The data confirmed that the aeration at 2.5 SLPM was the optimum level for the bacterial production of $CaCO_3$. The possibility of $CaCO_3$ precipitation was also tested in the absence of cells as negative control. In this case, the fermentor was inoculated with no bacteria and the result showed no precipitation took place during the fermentation. The similar profiles for DO were observed for all aeration rates over the course of fermentation. The DO concentration began to decrease at all aeration levels, followed by a gradual increase and reaching a plateau.

Table 1 Experimental conditions and results for the aeration controlled batch fermentations

Run	Aeration (SLPM)	Agitation (rpm)	Temperature (°C)	Time (h)	$CaCO_3$ concentration (mg/L)	SD	Cell concentration (CFU/mL)	SD	Yield ($\times 10^{-8}$ mg $CaCO_3$ /cell)
1	0.5	150	35	180	1655.82	359.49	11.9×10^6	4.2×10^5	13.92
2	1.5	150	35	180	3194.40	289.57	21.84×10^6	1.8×10^6	14.63
3	2.5	150	35	180	5473.29	326.87	29.01×10^6	2.8×10^6	18.87
4	4.5	150	35	180	7099.14	336.41	32.00×10^6	1.4×10^6	22.19

SD: Standard deviation

Table 2 Analysis of variance showing the significance of aeration on the bacterial production of $CaCO_3$

Source of Variation	*SS*	*df*	*MS*	*F-value*	*p-value*	*F crit*
Between Groups	7.36448×10^{-15}	3	2.45483×10^{-15}	32.1927	0.0029	6.5914
Within Groups	3.05017×10^{-16}	4	7.62542×10^{-17}			
Total	7.6695×10^{-15}	7				

DF: degree of freedom, *SS:* sum of squares, *MS*: mean sum of squares, *F crit*: F critical value

Controlled-pH batch fermentations

The effect of pH on the bacterial production of $CaCO_3$ and total cell concentration were investigated under controlled-pH batch fermentations. The preliminary testing disclosed that the increase of pH is in favour of microbial $CaCO_3$ production; however, the extent of that requirement is uncertain. In this case, fermentation runs were conducted at pH 9, 10, 11 and 12, and the responses (i.e., yield, $CaCO_3$ and cell concentration) were determined at 180 h of fermentation. The experimental conditions and results for controlled- pH batches are summarized in Table 3.

Table 3 Experimental conditions and results for controlled-pH batch fermentations

Run	pH	Agitation (rpm)	Temperature (°C)	Time (h)	$CaCO_3$ concentration (mg/L)	SD	Cell concentration (CFU/mL)	SD	Yield ($\times 10^{-5}$ mg $CaCO_3$/cell)
1	9	150	35	180	5345.24	316.60	85.00×10^3	4.2×10^3	6.29
2	10	150	35	180	7315.33	347.59	33.15×10^3	1.2×10^3	22.06
3	11	150	35	180	8051.23	375.83	27.20×10^3	2.8×10^3	29.60
4	12	150	35	180	10511.92	324.91	6.80×10^3	5.6×10^2	154.59

SD: Standard deviation

It was found that the bacteria could tolerate in the highest pH; however, the cell viability decreased when the pH increased. The results indicated the viability of bacteria in medium with the pH of 10 decreased by 2.5-fold as compared to pH 9. The least decline in the cell concentration was noticed when the pH of medium increased from 10 to 11. Total cell concentration in the medium with the pH 11 was only 80 % of the counterpart in the pH 10.

The comparison of cell concentration during various pH fermentations exhibited that the maximum drop in cell viability occurred at the pH of 12. A 75 % drop in the cell concentration was observed when the pH increased from 11 to 12. Interestingly, the experimental results showed the selected $CaCO_3$ producing bacteria are able to survive at pH 12 with the final cell concentration of 6.8×10^3 CFU/mL.

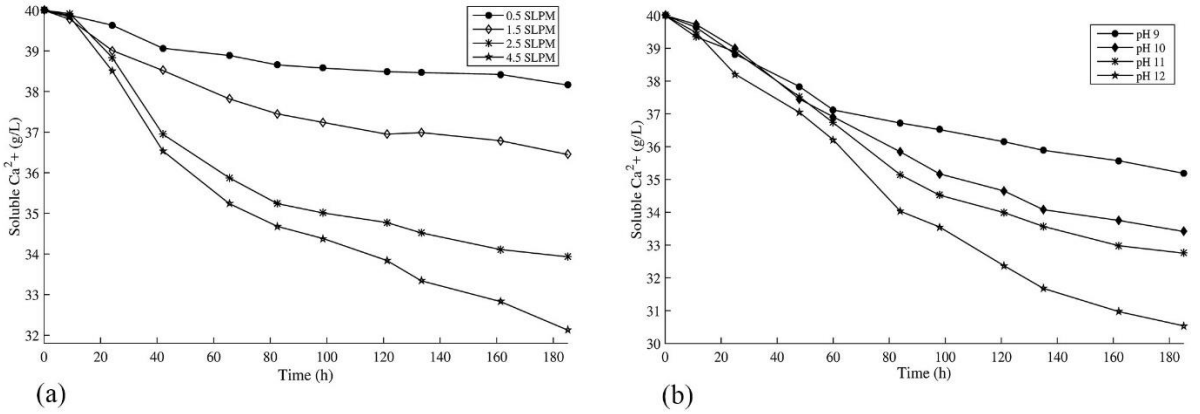

Fig. 2 Soluble Ca^{2+} ion concentration as a function of time at a) aeration controlled fermentations, and b) controlled-pH fermentations

Apart from the viability of bacteria in alkaline conditions, the ability to produce $CaCO_3$ in different pH ranges was investigated. The concentration of precipitated $CaCO_3$ during different pH conditions is displayed in Table 3. The results show that the concentration of $CaCO_3$ increased when pH increased. The maximum concentration of $CaCO_3$ achieved at pH of 12, while the least $CaCO_3$ precipitation obtained in the uncontrolled-pH run. It was found that the bacterial production of $CaCO_3$ in the medium with pH 9 was 3.2-times higher than the uncontrolled-pH. Similar trends were observed when the pH of the medium was altered to 10, 11 and 12. The fermentation experiment under the pH of 10 led to induce 37 % higher $CaCO_3$ precipitation than the pH of 9. However, only 10 % increase in $CaCO_3$ precipitation was achieved when the pH of bioreactor changed from 10 to 11. According to the uncontrolled-pH run, the highest increase in $CaCO_3$ concentration was obtained at pH 12. In this condition a 6.4-fold increase in total induced $CaCO_3$ precipitation was achieved. The concentration of soluble Ca^{2+} during controlled-pH fermentation is shown in Fig. 2b. The results obtained by negative control indicated that no $CaCO_3$ precipitation was induced during the fermentation with no microbial inoculation. Although a decline in the cell concentration was observed when the pH increased, the bacterial production of $CaCO_3$ was increased. The *p-value* of <001 (Table 4) indicated the higher pH value was favourable for the bacterial production of $CaCO_3$.

Table 4 Analysis of variance showing the significance of pH level on the bacterial production of $CaCO_3$

Source of Variation	*SS*	*df*	*MS*	*F-value*	*p-value*	*F crit*
Between Groups	3.2527×10^{-6}	4	8.1353×10^{-7}	2258	<0.001	5.1921
Within Groups	1.8009×10^{-11}	5	3.6019×10^{-12}			
Total	3.2541×10^{-6}	9				

DF: degree of freedom, *SS:* sum of squares, *MS*: mean sum of squares, *F crit*: F critical value

Morphological analysis of produced $CaCO_3$ crystals

Image analysis was performed using SEM to study the morphological characteristics of produced biominerals. The SEM micrographs of bioprecipitates illustrated the variety of shapes and sizes could be formed when the pH and aeration rate were changed. The SEM micrographs of biominerals produced at different aeration levels are shown in Fig. 3. Fig. 3a and b show the presence of $CaCO_3$ polymorphs during fermentation at airflow rates of 0.5 SLPM and 1.5 SLPM, respectively. An assemblage of bioprecipitates induced at 2.5 SLPM airflow rate is depicted in Fig. 3c. As shown in Fig. 3d, similar morphology was observed for precipitated $CaCO_3$ by selected isolates at the highest aeration rate (4.5 SLPM). Interestingly, Fig. 3e and f depict the imprints of bacterial cells on the surface of the biominerals. This indicates the attachment of Ca^{2+} ions to the negatively charged bacterial cell walls, and then bacterial escaping during biomineralization were accomplished to produce $CaCO_3$ crystals.

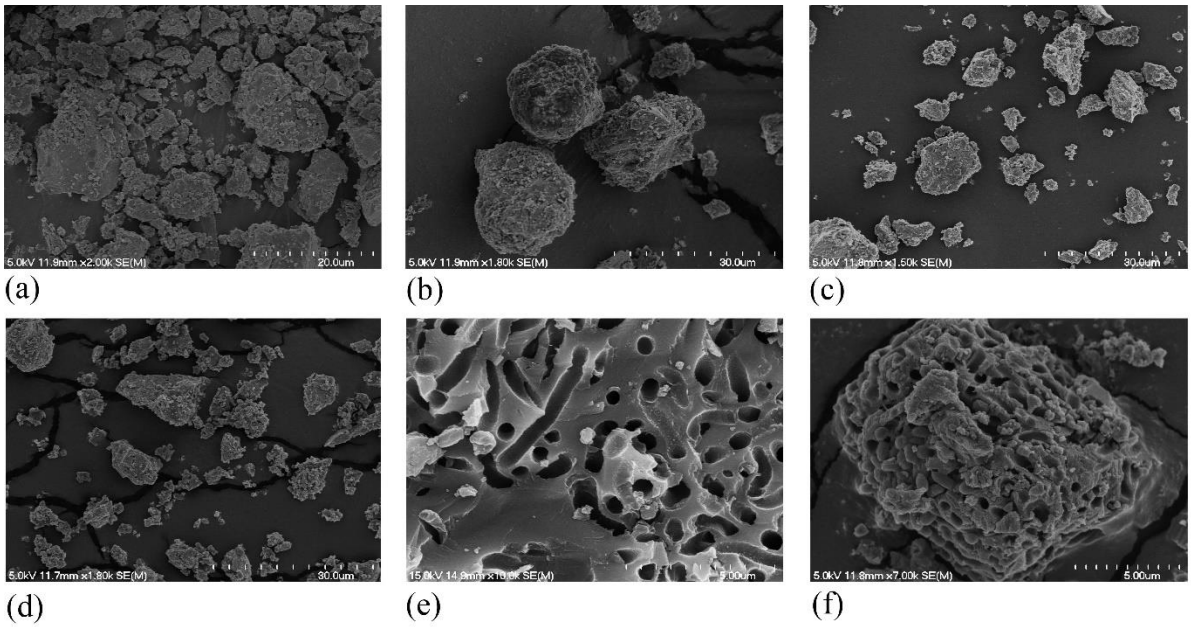

Fig. 3 a-d) Scanning electron micrographs of $CaCO_3$ produced during fermentation at 0.5, 1.5, 2.5 and 4.5 SLPM, and e-f) scanning electron micrographs showing the presence of bacterial imprints on the precipitated crystals at 2.5 and 4.5 SLPM

The morphological observation was also performed for the precipitated biominerals at the controlled-pH fermentation runs. Fig. 4a–d demonstrate the effect of pH on the shape and size of bioprecipitates. The SEM images illustrate the precipitated minerals were mainly between 10 µm and 20 µm. It was noticed that the size of produced $CaCO_3$ crystals decreased with the increase of pH. The average size of biominerals precipitated at pH 9 was two times bigger than those formed at pH 12.

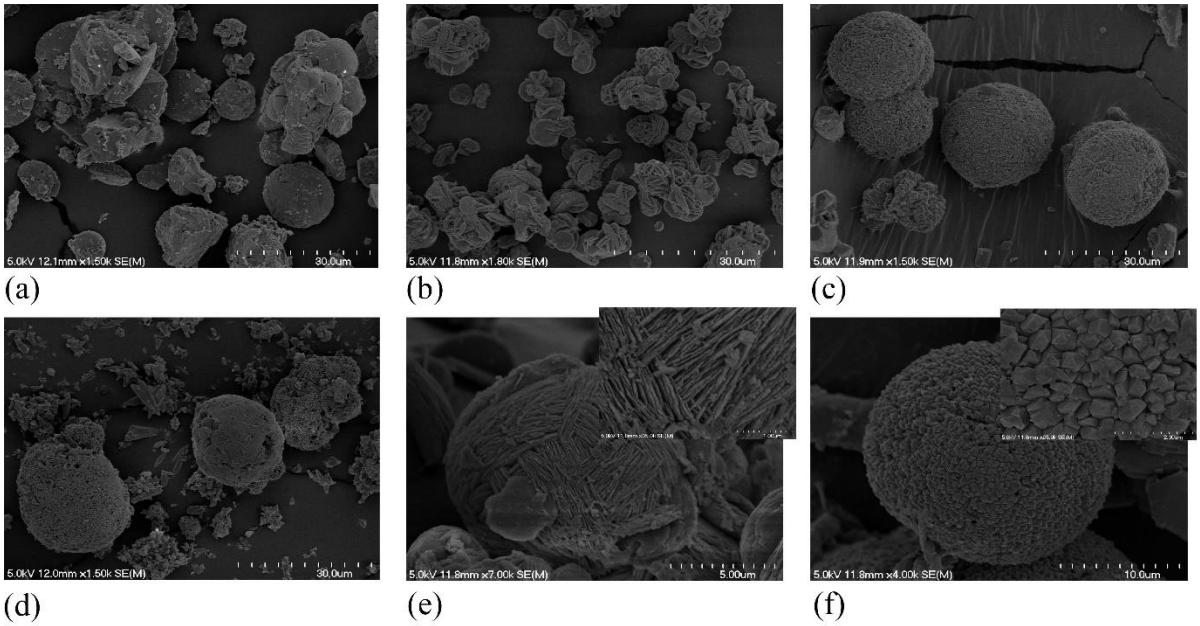

Fig. 4 a-d) Scanning electron micrographs of $CaCO_3$ produced during controlled-pH fermentations at pH 9, 10, 11 and 12, and e-f) scanning electron micrographs showing the surface structure and texture of crystals precipitated at pH 10 and 12

Despite the similarity in the shape of precipitated $CaCO_3$ crystals in controlled-pH batches, different degrees of porosities and crystal surface textures was observed. In general, the bioprecipitates became rough and porous as the pH was increased. A distinct surface morphology was observed during the course of fermentation at a pH of more than 10. Fig. 4 e and f demonstrate the magnified polycrystal surfaces induced at pH 10 and 12, respectively. The fermentation process at pH 10 led to precipitate spherical particles composed of compact layers, while the crystal observation at pH 12 was completely different from those particles observed at a lower pH. Most strikingly, the surface of the bioprecipitates deposited at pH 12 was highly rough and composed of randomly rhombohedral oriented particles with a size of 0.2-0.4 µm.

Elemental composition analysis was conducted using EDX at 15.0 keV to determine the elements presented in the bioprecipitates. The elements were detected and quantified based on the intensity of peaks. The EXD analysis was performed for the deposited crystals during aeration controlled and controlled-pH fermentation runs. As shown in Fig. S1 (provided in the supplementary material), EXD analysis was also conducted for pure $CaCO_3$ to compare with the spectra obtained during fermentations. Fig. 5a, b depicts the EDX spectra for the precipitated crystals during the fermentation at aeration controlled and controlled-pH, respectively. The EDX analysis indicated that calcium, carbon and oxygen were the most abundant elements existing in bioprecipitates. The production of $CaCO_3$ by bacteria was also confirmed by the comparison between EDX spectra obtained from pure $CaCO_3$ and the crystals precipitated in aeration controlled batch fermentation (Fig. 5a). Fig. 5b represents EDX spectra of bacterially induced $CaCO_3$ crystals precipitated in controlled-pH batch fermentation experiments. A high degree of similarity between pure $CaCO_3$ crystals spectrum and those precipitated in different pH media confirmed the biominerals were $CaCO_3$.

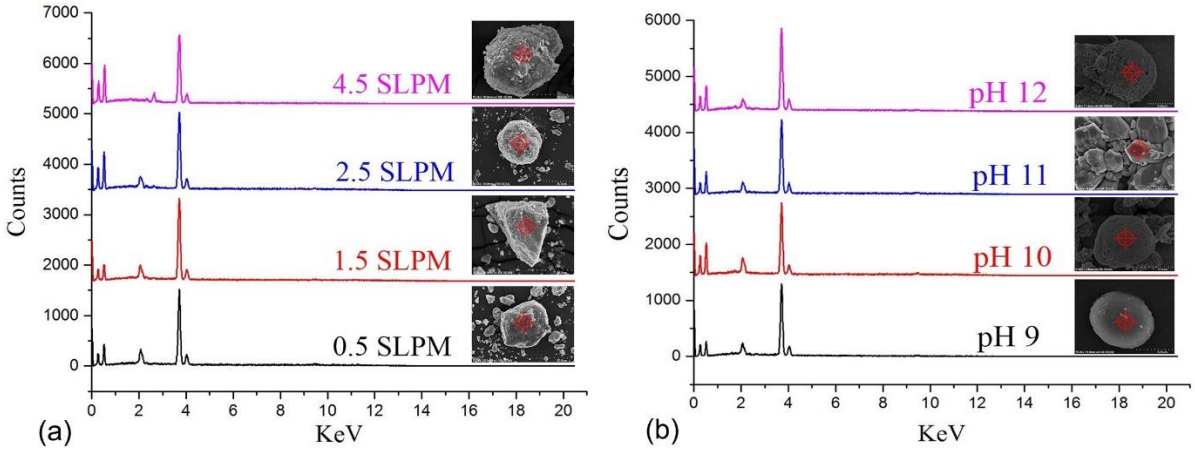

Fig. 5 EDX spectra of precipitates over a) aeration controlled fermentations, and b) controlled-pH fermentations

Structural characterization of produced $CaCO_3$ crystals

In all cases, precipitated biominerals were characterized by XRD to confirm the precipitation of $CaCO_3$ and determine the presence of polymorphs (calcite, vaterite and aragonite) in the bioprecipitates. Fig. 6 demonstrates XRD spectra for induced $CaCO_3$ particles during fermentations. The XRD spectrum for pure calcite is shown in Fig. S2 (provided in the supplementary material).

As shown in Fig. 6a, the most intensive peaks occurred at the angle of 29.3°, indicating the majority of crystals formed during aeration controlled batch fermentations were calcite. As expected, no mineralogical changes were detected following the introduction of different aeration into the fermentor. However, pH was found to be a significant factor to change the morphologies (Fig. 6b). The results indicated that the increase of pH to 10 led to precipitate a higher portion of vaterite, while the further increase of pH (up to 12) resulted in more calcite precipitation. Therefore, it can be concluded that the most transformation of vaterite to calcite occurs at pH 10–11.

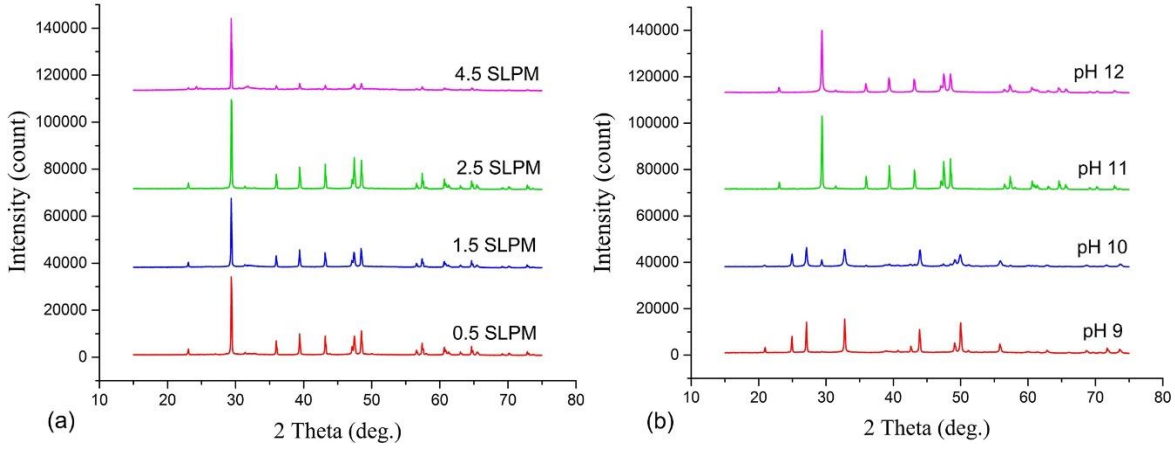

Fig. 6 XRD spectra for a) aeration controlled batch fermentations, and b) controlled-pH batch fermentations

Discussion

Aeration controlled batch fermentations

Aeration and agitation are involved in different extend in overall mass and oxygen transfers in the fermentation process. Aeration provides oxygen to the bacteria and has a significant effect on the bulk mixing of the fermentation fluid, while agitation contributes to the distribution of nutrient and oxygen in the medium. It is known that the metabolic pathway and the metabolic fluxes can be affected by oxygen transfer rate. Some bioprocesses demand a high oxygen transfer rate conditions, while others require controlled oxygen transfer rates in order to regulate oxygen uptake rates [33]. The effect of oxygen on the microbial growth and bio-products has been comprehensively documented. For instance, Zhang et al. [34] reported that the provision of oxygen supply could enhance the bacterial precipitation of $CaCO_3$. The same observation was noticed by Wang et al. [35] and Ramachandran et al. [36]. They examined the possibility of $CaCO_3$ precipitation in different parts of the specimen. The results demonstrated that the precipitates concentrated in those areas where oxygen was sufficiently available. For an efficient bacterial production of $CaCO_3$, it is important to keep the oxygen level at optimum level throughout the fermentation. The result obtained during fermentation showed that the production of $CaCO_3$ can be enhanced if a sufficient amount of oxygen is available in the media. Overall, *p-value* less than 0.05 indicated the significant effect of aeration on the bacterial growth, metabolic activity and $CaCO_3$ precipitation. Data demonstrate the higher aeration rate resulted in an increase in both $CaCO_3$ and cell concentration. High and low DO concentrations have a significant impact on the bacterial growth. Statistical *t*-test analysis showed that there was no significant increase in the yield when the aeration rate increased from 0.5 SLPM to 1.5 SLPM. This might be attributed to the provision of an inadequate supply of oxygen into the fermentor to robust bacterial growth, increase the nucleation sites and bacterial production of $CaCO_3$. Conversely, a significant increase in the microbial yield was obtained when the airflow increased to 2.5 SLPM. In comparison to 0.5 SLPM airflow, higher growth was observed when a 2.5 SLPM aeration was used. Well-distribution and transportation of nutrients to the cells and adequate supply of oxygen for germination might be the main reasons for increasing the yield. Despite the increase of yield at 4.5 SLPM airflow, the statistical analysis displayed no significant improvement in the yield as compared to 2.5 SLPM. Although the concentration of $CaCO_3$ was increased when the aeration increased from 2.5 SLPM to 4.5 SLPM, the number of cells did not substantially enhance. This is attributed to maintaining DO concentration greater than its critical level and the presence of a facultative anaerobic bacterium in the medium. Unlike the other *Bacillus* species that are typically aerobic, *B. licheniformis* is facultative anaerobe, allowing for growth in additional ecological niches. In this stage of the investigation, it was shown that the production of $CaCO_3$ was evidently correlated with the aeration rates. Since the provision of oxygen is required for bacterial germination, metabolism, and consequently $CaCO_3$ precipitation, the utilization of oxygen releasing compounds is suggested to compensate for the lack of oxygen.

Controlled-pH batch fermentations

Another factor affecting the performance of bacteria to induce $CaCO_3$ precipitation is the medium pH. The preliminary study showed a pH variation during the fermentation of $CaCO_3$ which is due to factors including (i) NH_3(g) dissolution, (ii) CO_2(g) dissolution and (iii) acid generation during the bacterial production of $CaCO_3$ [37, 38]. Based on the results, pH dropped from 7.6 to 7.2 at the beginning of fermentation due to increased respiration,

leading to enrichment in CO_2 and, consequently, acidifying the surrounding [39]. However, after 21 h of fermentation, bacteria maintained robust growth in exponential phase which resulted in releasing more NH_4^+ and, subsequently, pH was increased to 8.2 (Eqs 1-3). According to Eq.1 the hydrolysis of one mL of urea by bacteria generates one mole of NH_3 and NH_2COOH. As can be seen from Eq. 2, simultaneously one extra mole of NH_3 is produced from hydrolysis of NH_2COOH. The production of OH^- from the reaction between NH_3 and H_2O results in an increase in pH which favors $CaCO_3$ precipitation (Eq. 3) [40, 41].

$$CO(NH_2)_2 + H_2O \rightarrow NH_2COOH + NH_3 \quad (1)$$

$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \quad (2)$$

$$2NH_3 + 2H_2O \rightarrow 2NH_4^+ + 2OH^- \quad (3)$$

The results indicated that a greater rate of $CaCO_3$ precipitation was induced when the pH was increasing. Since the bacterial production of $CaCO_3$ is used in different environmental conditions, it is important to investigate the effect of pH on the efficiency of biomineralization process. Controlled-pH batch fermentations were performed at different pH levels to identify the effect of alkalinity on the viability and capability of producing $CaCO_3$ crystal by bacteria. The cells are highly sensitive to external pH and the selected bacteria are well adapted to grow in alkaline conditions, enabling them to induce precipitating $CaCO_3$ at high pH levels. The results disclosed that the inoculation of bacteria in a higher pH medium had a negative effect on the cell viability. As the pH was increased, the number of cells decreased and reached to its lowest value at pH of 12, but still remained viable at concrete's pH. This indicates the selected isolates are able to survive in more extreme pHs following a decrease in the number of the cells. The effect of pH on bacterial growth and $CaCO_3$ precipitation has been reported in the literature. Kaur et al. [5] isolated bacteria from calcareous soil (pH 11) and determined the ability of the isolates to induce $CaCO_3$ precipitation. It was found that some of the isolates, including *Bacillus megaterium* and *B. cereu*, could growth in a pH range of 6.5 to 11.5, while the other isolates (*B. thuringiensis*, *B. subtilis* and *Lysinibacillus fusiformis*) were able to survive at a pH range of 6-10. In another investigation, Kim et al. [30] tested different bacterial strains onto Difco nutrient broth with pH of 11 to determine whether they were capable of growing on alkaline condition. The colonies that were able to grow in this condition were considered as possible $CaCO_3$-precipitating isolates. Among the isolates, it was found that only *Sporosarcina sp* and *Bacillus sp* were capable of growing in such alkaline environment. In general, bacterial cells are quickly adapting to environmental conditions at a lower pH by showing a shorter lag phase. However, their viabilities decrease with the increase of the alkalinity. This reduction is attributed to the inhibitory effect of alkalinity, which causes a prolonged lag in growth [42].

Although viability of bacteria in an alkaline environment is critical, the capability of producing $CaCO_3$ by bacteria in such a harsh condition is another important parameter to be considered. In contrast to viability, a higher pH found to be significant in the production of $CaCO_3$. The higher precipitation of $CaCO_3$ at the higher level of pH might be due to the production of more enzyme which facilitates the generation of CO_3^{2-} and, consequently, more $CaCO_3$ crystals are induced. It has been stated that urease enzyme activities depend on pH value [43, 44]. More urease released in the medium results in more production of CO_3^{2-}. Carbonate tends to dissolve in the medium at low pH levels rather than precipitate [45]. Therefore, $CaCO_3$ precipitation is increased as the pH level in the medium is increased. In general, the soluble Ca^{2+} ion concentration showed a decreasing trend indicating the precipitation of $CaCO_3$ during time (Fig. 2 b). The same trend was observed for the concentration of soluble

Ca^{2+} ion when the pH increased. However, the rate of $CaCO_3$ precipitation was decreased with the increase in fermentation time. This might be due to the reduction in the number of viable cells in the medium.

Despite the decline in the cell viability, the ANOVA results indicated the increase of pH was effective to increase the yield (mg/CFU). The same trend was also found where the optimum medium showed the highest urease activity and $CaCO_3$ precipitation, but lower growth rate [44]. The comparison was made between each pair of batch results and it was concluded that the pH 12 has the most significant influence on the yield. The controlled-pH fermentation experiments disclosed that the selected isolates were alkali tolerance and their ability to induce $CaCO_3$ enhanced with the increase of pH.

Morphological observation and crystal characterization

In general, physical and chemical characteristics of $CaCO_3$ precipitates, such as crystal size, specific surface area, morphology, purity and brightness, are the main criteria that are usually considered to be used in different industrial applications. Physical properties of $CaCO_3$, including density, solubility and hardness, largely depends on the percentage of each polymorph (i.e. calcite, vaterite and aragonite). Calcite and aragonite are the two most dominant $CaCO_3$ polymorphs precipitated in nature, while vaterite is the other anhydrous $CaCO_3$ polymorph that rarely occurs in nature. Morphological analysis using SEM is a common technique to evaluate the crystal properties and perform elemental chemical analysis. Different saturation levels during mineralization leads to precipitate various $CaCO_3$ crystalline polymorphs. Furthermore, bacteria metabolic activities, cell surface characteristics, fermentation medium compositions and the concentration of extracellular polymeric substances (EPS) are attributed to the production of different morphologies.

The fermentative $CaCO_3$ crystals induced in the aeration controlled batches displayed different morphologies from those formed in the controlled-pH batches. Most precipitated crystals in aeration controlled fermentation runs were a combination of egg-shaped or irregular shape, while the produced biominerals in controlled-pH runs were predominately regular spherical or egg-shaped at a lower alkalinity condition. The consistent XRD spectra during aeration controlled fermentations also showed that the aeration had no effect on the $CaCO_3$ morphology transformation. The formation of calcite attributed to the presence of EPS in the medium which inhibits vaterite precipitation and this can be used to biosynthesis a high-ordered $CaCO_3$ morphologies.

$CaCO_3$ polymorphism and morphology can also be affected by abiotic factors. It has been reported that the pH not only facilitates the formation of $CaCO_3$, but it also has an influence on the morphology of $CaCO_3$ [46, 47]. Unlike the biominerals precipitated during aeration controlled batches, vaterite particles were found the predominant polymorphs at pH 9–10. This indicates that the pH had a regulatory effect on the morphology of the precipitated $CaCO_3$ crystals. The increase in pH from neutral to alkaline (up to pH 10) was assumed to inhibit the transformation of vaterite to calcite. However, the release of dissolved organic carbon (DOC) from the EPS at a high pH complexes Ca^{2+} ions in solution, leading to change the saturation level in the bulk solution and facilitating the transformation of vaterite to calcite [48]. Interestingly, a transition was found in the surface structure of precipitated crystals during controlled-pH fermentation runs. In general, the crystals formed at a higher alkaline condition appeared more poorly ordered with rougher crystal faces. The decrease of the bacterial cell and organic macromolecules somewhat contributed to changing the surface structure from smooth to rough. Furthermore, the

polycrystals' formation at high pH condition may arise from the quick adsorption of Ca^{2+} ions at different interfaces over biosynthesis of $CaCO_3$. The same crystal structure for the spherical vaterite particles was previously observed during lysozyme mediated $CaCO_3$ mineralization [49].

In this study we have clearly shown the effect of aeration and pH on the bacterial growth and $CaCO_3$ precipitation. We found that $CaCO_3$ precipitation is correlated with the growth of bacteria at aeration controlled fermentations, while the efficiency of $CaCO_3$ production is linked to the alkalinity at controlled-pH fermentations. The results indicated that aeration had significant effect on both bacterial growth and biosynthesis of $CaCO_3$, while the exceeding amount of oxygen was not effective on improving the yield. Despite the decline in cell viability, the production of $CaCO_3$ was enhanced with the increase of pH. Overall, the results indicated that oxygen starvation and low alkalinity were the main reasons leading a decrease in the yield and possibly bio self-healing concrete performance. SEM, EDX and XRD were used to visualize and characterize the $CaCO_3$ crystals produced over the course of fermentation. The result disclosed that aeration had no effect on the $CaCO_3$ morphology. In contrast to aeration controlled batches, morphological transition occurred when different levels of pH were used. The increase of pH resulted in changing the surface structure of bioprecipitates from smooth single crystals to rough oriented polycrystals.

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Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

This study does not contain any studies with human participants or animals performed by any of the authors.

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