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Induction of Calcium Carbonate by *Bacillus cereus*

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The purpose of this research was to study how the bacteria *Bacillus cereus* (*DCB1*) utilizes calcium ions in a culture medium with carbon dioxide (CO_2) to yield calcium carbonate ($CaCO_3$). The bacteria strain *DCB1* was a dominant strain isolated from dolomitic surfaces in areas of Karst topographies. The experimental method was as follows: a modified beef extract-peptone medium (beef extract 3.0 g, peptone 10 g, NaCl 5.0 g, $CaCl_2$ 2.0 g, glass powder 2.0 g, distilled water 1 L, and a pH between 6.5 and 7.5) was inoculated with *B. cereus* to attempt to induce the synthesis of $CaCO_3$. The sample was then processed by centrifugation every 24 h during the 7-day cultivation period. The pH, carbonic anhydrase (CA) activity, and the concentrations of both HCO_3^- and Ca^{2+} in the supernatant fluid were measured. Subsequently, precipitation in the culture medium was analyzed to confirm, or otherwise, the presence and if present, the formation, of $CaCO_3$. Methods used included X-ray diffraction (XRD), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), and Energy Dispersive Spectroscopy (EDS). Meanwhile, the carbon source in the carbonate was classified by its isotope composition. Results showed that *B. cereus* can improve its pH value in this culture medium; concentrations of HCO_3^- and Ca^{2+} showed a significant decline over the duration of the cultivation period. CA activity reached its maximum during the second day; XRD, SEM, TEM, and isotope analysis all revealed the presence of $CaCO_3$ as a precipitate. Additionally, these results did not occur in an aseptic control group: no detectable level of $CaCO_3$ was produced therein. In conclusion: *B. cereus* can metabolize active materials, such as secretase, by its own growth and metabolism, and can either utilize atmospheric CO_2, or respire, to induce $CaCO_3$ production. Experimental evidence is offered for a concomitant CO_2 reduction and $CaCO_3$ induction by microorganisms.

Keywords: *Bacillus cereus*, biomineralization, CO_2 reduction, calcium carbonate

Introduction

Anthropic activities and industrial production have caused the well-documented greenhouse effect of recent years. Research has successfully shown that carbonate induction by microorganism may be one potential pathway to reduce greenhouse gases (Peng et al. 2010). With metabolism of microorganisms to induce carbonate formation, which fixes atmospheric CO_2 into carbonate minerals or organic substances (Lian et al. 2011; Lian and Hou 2011; Zhang et al. 2011), thus reducing CO_2 concentrations, partially counteracting the greenhouse effect, while offering guidance for future research into the interactions between microorganisms and minerals.

Biomineralization is currently the subject of much attention (Balland-Bolou-Bi and Poszwa 2012; Calvaruso et al. 2006; Lian et al. 2010). Researchers have shown that propagation and metabolism of microorganisms not only accelerates the efflorescence of minerals (Dou and Lian 2009; Lian et al. 2008; Xiao et al. 2012a, 2012b), but also promotes mineral synthesis (Basker et al. 2006; He et al. 2011; Li et al. 2005, 2011). On the one hand, microorganisms can secrete metabolites that can affect biomineralization, e.g., carbonic anhydrase (CA) can accelerate hydration of CO_2, and regulate the concentrations of CO_2 and HCO_3^- (Liu 2001; Zhou et al. 2010). On the other hand, metabolism of microorganisms can change the pH of the surrounding environment, and contribute to the formation and decomposition of carbonates (Cheng et al. 2007; Wang et al. 2011; Yin et al. 1994). This bio-precipitation of carbonates is common in the natural environment: scientists have found many ocean animals, algae, and bacteria that have such a precipitative effect on carbonates (Li et al. 2009); calcium carbonate is one of the important minerals for this biomineralization process and is also among the most common naturally occurring minerals (Xu et al. 2008; Zhang and Xie 2000). Calcium carbonate formation can cause atmospheric CO_2 sedimentation and eliminate CO_2 emissions in water thereby rendering it important to global climate researchers and society as a whole.

Previous research has discovered some microorganisms capable of sedimenting carbonates such as: Li et al. (2011) utilized *B. mucilaginosus*, Hou et al. (2011) utilized *Alternaria* sp., Stocks-Fischer et al. (1999) utilized *B. pasterii*,

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Sancher-Roman et al. (2007) and Wang et al. (2005) utilized a strain of halophilic bacterium, or carbonate-mineralization microbe, respectively to induce successful $CaCO_3$ generation.

$CaCO_3$ induction by *B. cereus* has not yet been reported at the time of writing. This kind of germ is a dominant strain found on outcropping carbonate rock surfaces in Karst topographies, where it contributes to both efflorescence and carbonate formation (Tang et al. 2012). To investigate the interaction of microorganisms and carbonate, while learning about the function and ecology of microorganisms in Karst regions, this research provided nutrients and calcium sources for the cultivation of *B. cereus* with a view to checking whether, or not, $CaCO_3$ was thus yielded in the culture. The checks for the presence of $CaCO_3$ included: biochemical index tests, precipitation observation, and XRD phase analysis. Carbon isotope analysis was used to study the carbon source (Piao et al. 2003) and the biological mechanism of $CaCO_3$ formation was analyzed. By doing this, the authors can offer some basic information for the study of the role of carbonate microorganisms in the carbon cycle.

Materials and Methods

Bacterial Strains

B. cereus DCB1 (GenBank serial-number: JN650544) is a dominant strain isolated from dolomite rock surfaces in Guiyang's suburbs.

Bacteria Cultivation and Bio-induction of $CaCO_3$

B. cereus was inoculated and activated in a beef-extract peptone medium at 30°C for 2 to 3 days; then the activated bacteria strains were inoculated into a modified beef-extract peptone medium (beef extract 3.0 g, peptone 10 g, NaCl 5.0 g, $CaCl_2$ 2.0 g, glass powder 2.0 g, distilled water 1 L, and a pH between 6.5 and 7.5) for potential induction of $CaCO_3$ synthesis. In the culture medium, $CaCl_2$ was used as a calcium source: the glass powder had been sieved through a size 60 square aperture mesh and it was intended to act as a nucleation site for the potential synthesis of crystalline $CaCO_3$.

The activated *B. cereus* was inoculated with two loops into 100 mL of the modified medium, which was placed in 250 mL triangular flasks to form two groups: the experimental group (inoculated with *B. cereus*) and the control group (without *B. cereus*). Two parallel protocols were established for two repeat sets of each group. These two groups were then cultivated in a constant temperature oscillation incubator at 30°C and 120 rpm for 7 days.

Measurement of the Culture pH, CA Activity, and HCO_3^- and Ca^{2+} Concentrations

Samples and Measurement of the Culture pH and CA Activity

Sample volumes of 10 mL were taken from the culture medium every 24 h for processing by centrifugation (Anke TGL-16G, China) at 10,000 rpm for 10 min, for the pH, CA, HCO_3^- and Ca^{2+} measurement; and the remainder of the sample were discarded, then the next 10 mL sample was taken (24 h later) from another parallel culture medium. The protocol required the establishment of 16 parallel cultures in one treatment. The pH of the supernatant fluid in the culture was then measured with a pH meter (SevenEasy S20, Shanghai). The test for CA activity was based upon the methods of Zhou et al. (2010) and Pocker and Stone (1967) with minor revisions.

Establishment of standard plot

First, a 1 mM p-nitrophenol titrating solution (0.0139 g p-nitrophenol dissolved in 100 mL of 0.2 M phosphoric acid buffer solution with a pH of 8.0) was used for 3, 2.1, 1.8, 1.5, 1.2, 0.9, 0.6, 0.3, 0.15, and 0 mL respectively, and water added to make up a total volume of 9 mL. Then its absorbance at the 400 nm wavelength was tested and the relevant standard curve obtained. The concentration of p-nitrophenol (mM) was used as the abscissa (x) and the absorbance as the ordinate (y); Equation 1 gives the form of this standard calibration line.

$$y = 5.0499x + 0.0299 (R^2 = 0.9992) \quad (1)$$

Working solution preparation

First, 2.29 g Na_2HPO_4 and 1.9 g NaH_2PO_4 were combined in the 100 mL volumetric flask and the volume metered with deionized water: 0.0181 g of acetic acid p-nitrophenol was dissolved in 1 mL of absolute ethyl alcohol, and combined with 0.156 g diethyl diethylmalonate for dissolution in the phosphoric acid buffer solution.

Diluent preparation

Every 24 h, 10 mL of the culture medium were taken and processed by centrifugation at 10,000 rpm for 10 min, 1 mL of the supernatant fluid was made-up, with water, to a total volume of 5 mL.

The diluent and working solution were mixed together in equal volumetric proportions of 1:1 for 30 min at 35°C, and then rapidly cooled: its absorbance was then tested at the 400 nm wavelength by spectrophotometer (T6 Series - New Century, China).

The enzymatic activity was obtained by virtue of the p-nitrophenol producing amounts according to the standard calibration line. One enzyme activity unit was defined as the enzyme content required for catalysis of 1 μmol p-nitrophenol produced *per* minute.

Measurement of HCO_3^- and Ca^{2+} Concentration

Measurement of HCO_3^- concentration

For the inoculated experimental group and non-inoculated control group, 10 mL of the culture were taken every 24 h and centrifuged at 10,000 rpm for 10 min. Then 3 mL of the supernatant fluid were decanted into 50 mL triangular flasks; meanwhile, 15 mL of cool CO_2-free deionized water, by boiling for 15 min, were added. According to the acidimetry protocols laid down by the People's Republic of China Industry

Standard (covering alkalinity, total alkalinity, bicarbonate, and carbonate), the concentration of HCO_3^- was measured.

Measurement of Ca^{2+} concentration

First, 10 mL of the culture were taken every 24 h from the inoculated and non-inoculated groups, for processing by centrifugation at 10,000 rpm for 10 min. According to protocols laid down by the People's Republic of China EDTA (GB 7476–87 test for calcium ion), the concentration of Ca^{2+} was measured.

XRD Analysis and Observation of $CaCO_3$ Bio-induction

To verify whether or not the $CaCO_3$ arose because of the increase in pH, a culture medium identical to that of the control group was adopted, and its pH adjusted to 9 directly. Precipitates were collected and dried at 65°C together with the precipitates in both the inoculated (experimental) groups and non-inoculated (control) groups that had been cultivated for 7 days. The precipitates were then ground and sieved through a 200 mesh sieve for XRD analysis (D/Max-220, Japan).

Precipitate samples were taken by pipette and examined by TEM (JEM-2000FX, Japan) after 7 days. Meanwhile, the culture medium was filtered and the precipitate samples were fixed by glutaric dialdehyde for 12 h before examination by SEM (S-3400N, Japan) to ascertain their shape.

Carbon Isotope $\delta^{13}C$ Measurement

The glass powder was rinsed in a 5% aqueous HCl solution to avoid potential contamination by $CaCO_3$ resulting in an erroneous carbon isotope measurement. The carbon isotope $\delta^{13}C$ was used to study the carbon source inherent in the $CaCO_3$. The modified beef extract-peptone medium was again adopted; one group of samples containing the medium without $CaCl_2$ was set-up as a comparison (control group). Another group with $CaCl_2$ was set up as the experimental group. Then the inorganic carbon isotope $\delta^{13}C$ in the control group and the resultant solid materials in the experimental group were measured using the seventh day's culture sample.

Inorganic carbon in both the culture (control group) and the solid materials (experimental group) was converted to CO_2 by adding phosphoric acid (Liu et al. 2010). Then the gas was collected and analyzed by gas mass spectrometer (Finnigan MAT 252, Germany) with a view to establishing their carbon isotope $\delta^{13}C$ compositions.

Results and Analysis

Changes to the Culture Medium's pH Over Time

Changing pH values in both the experimental and control groups are shown in Figure 1. Figure 1 shows that the pH in the experimental groups rose over time, indicating that *B. cereus* had an alkalinity-producing effect. Due to its exponential growth in the first three days, the pH increased rapidly, whereas in the control group, the pH showed little change over the same time.

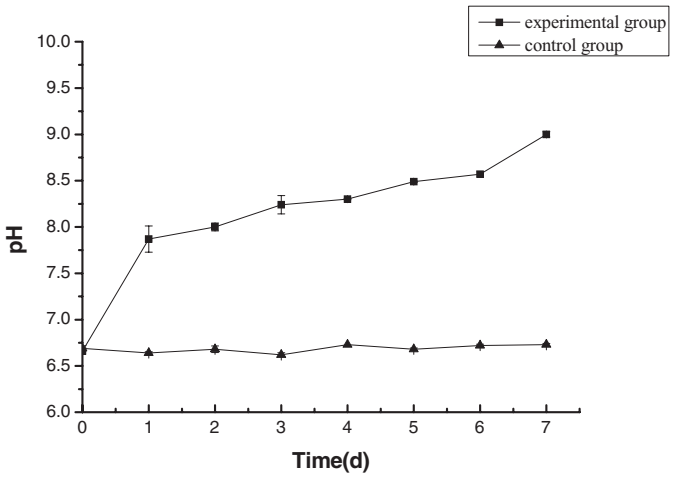

Fig. 1. Changes of pH in experimental and control groups.

Changes in Carbonic Anhydrase Activity with Time

Changes in the medium's CA activity were measured for the inoculated experimental group and the control group: the results are shown in Figure 2. Figure 2 shows that CA activity in the experimental group increased initially, and then decreased: it reached a maximum during the second day and thereafter fluctuated. CA activity in the control group remained practically unchanged. It was supposed that the changing trend of CA activity was related to the growth cycle of the bacteria.

The first 2 days were the exponential growth period during which time the bacteria were vigorous and the CA content concomitantly high. Subsequently, with the onset of the stable growth period, death and weakening bacterial metabolism caused a decrease in CA activity. The control group showed little enzymatic activity. That is thought to be because the distilled water is regarded as a blank while measuring the optical density, and the medium's color made the absorbency increase slightly which led to the false impression of low enzymatic activity. In addition, some bio-material and bacteria may have

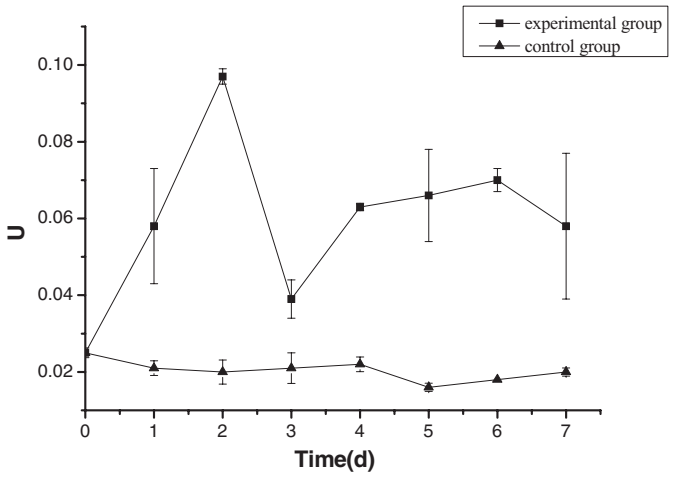

Fig. 2. Changes of carbonic anhydrase activity in experimental and control groups.

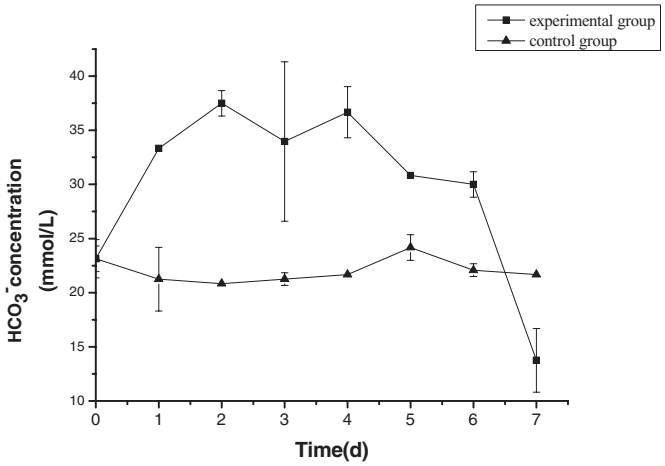

Fig. 3. Changes of bicarbonate ion concentration in experimental and control groups.

been adsorbed on the surface of the precipitates: the resulting CA activity may therefore have been lower than in reality.

Changes of HCO_3^- Concentration in the Medium with Time

The HCO_3^- concentration was measured for the inoculated experimental group and the non-inoculated control group, the results are shown in Figure 3. Figure 3 shows that the concentration of HCO_3^- increased at first and then decreased from the second day. This was because some CO_2 was yielded through bacterial respiration and thus dissolved in the culture medium, producing HCO_3^- thereby increasing the concentration of HCO_3^- in the first two days. With the pH increase, the HCO_3^- was converted into CO_3^{2-}, which then combined with Ca^{2+} in the medium to form $CaCO_3$, accelerating the bicarbonate ion's conversion to CO_3^{2-}. After the fourth day, the bacteria were in their degenerate stage, and the amount of CO_2 yielded by respiration began to decrease, thus the concentration of HCO_3^- declined.

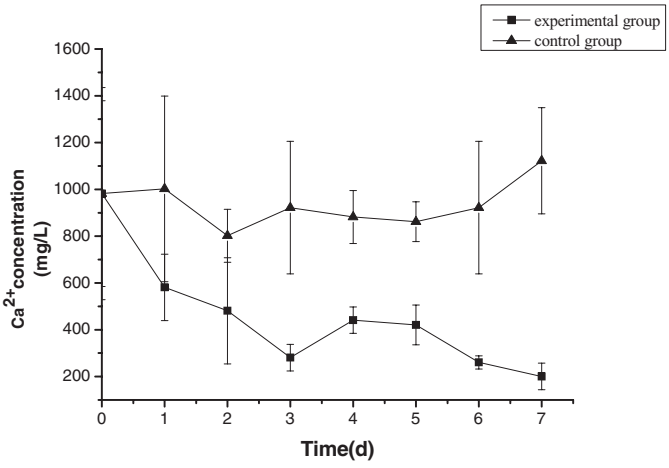

Fig. 4. Concentration of calcium ions in experimental and control groups.

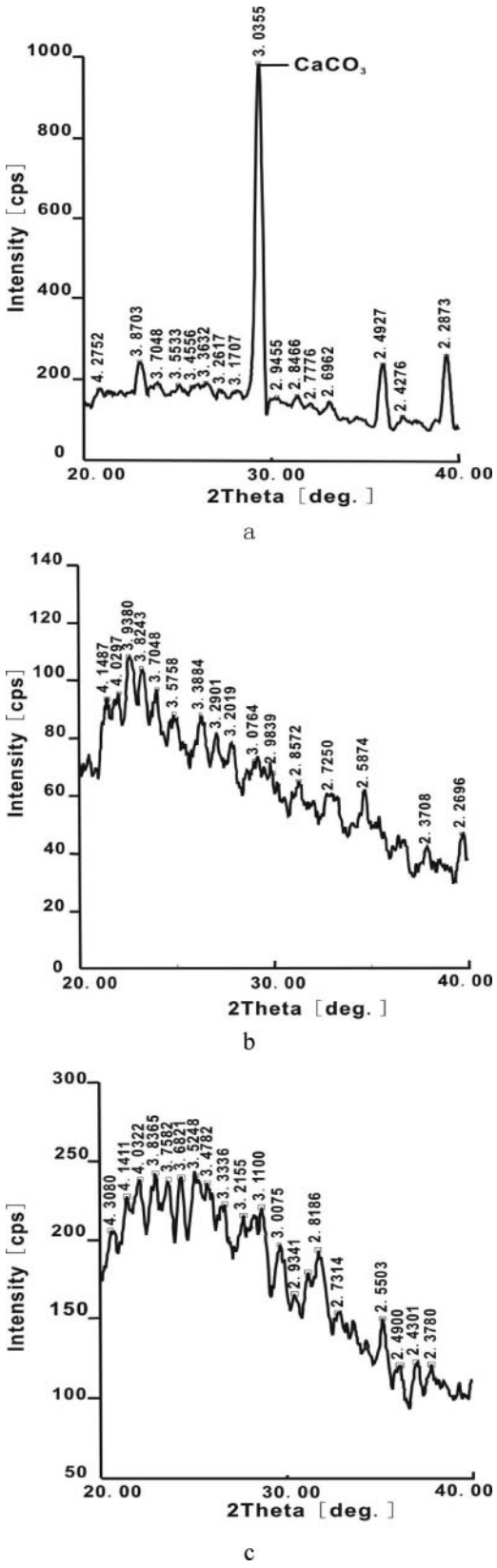

Fig. 5. XRD results; (a) experimental group, (b) control group, (c) pH = 9 control group.

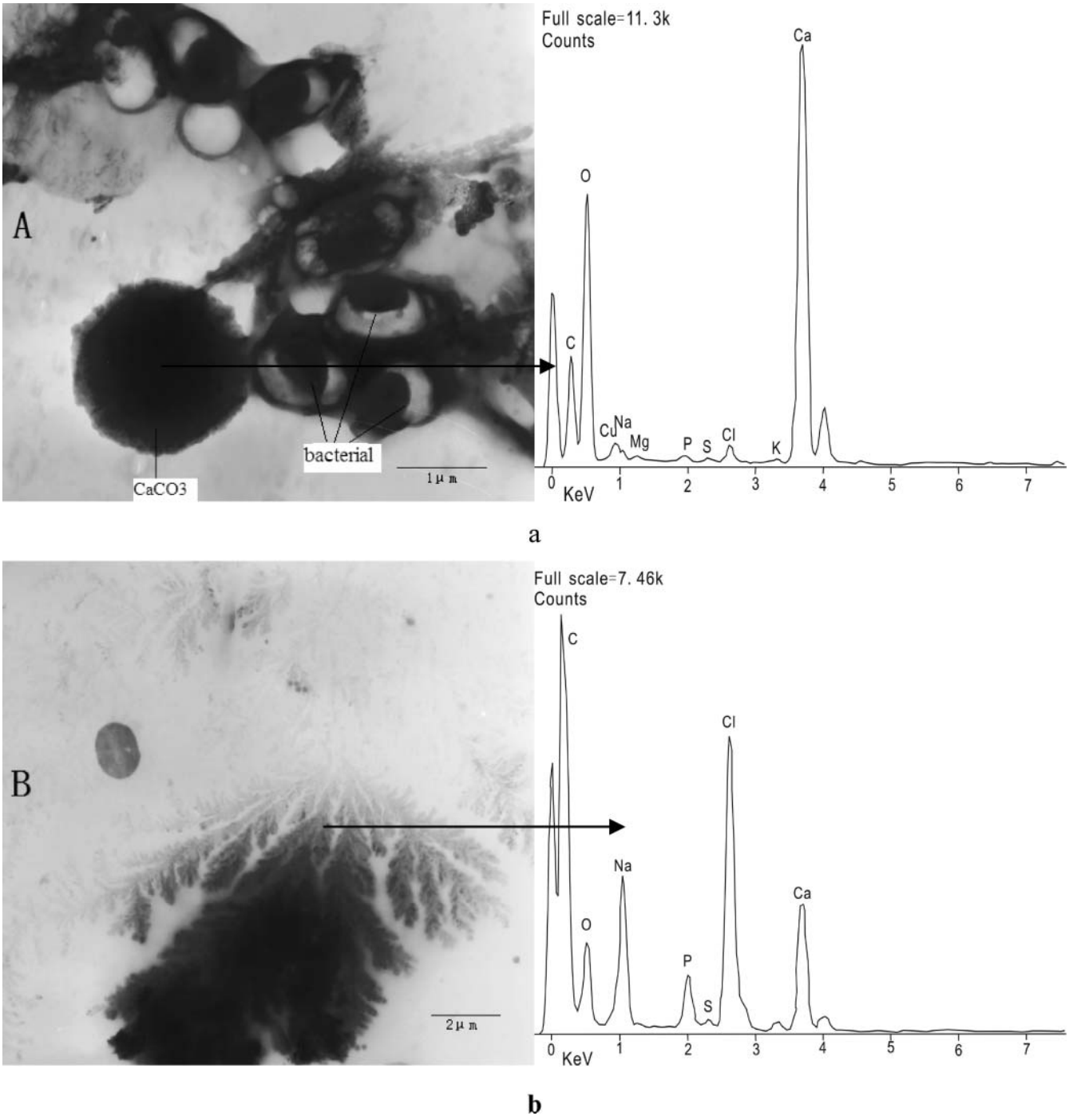

Fig. 6. TEM results and EDS image of the experimental and control groups; (a) experimental group, (b) control group.

The above reactions involve:

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$
$$HCO_3^- \rightleftharpoons CO_3^{2-} + H^+$$
$$CO_3^{2-} + Ca^{2+} \rightleftharpoons CaCO_3$$

The concentration of HCO_3^- in the medium first increased and then decreased; meanwhile, the CA activity had some effect on both CO_2 and HCO_3^- concentrations. However, the concentration of HCO_3^- in the blank control group showed no change; what little fluctuation there may have been caused by atmospheric CO_2.

Changes of Ca^{2+} Concentration in the Medium with Time

The concentration of Ca^{2+} in the medium was tested in both the inoculated experimental group and the non-inoculated

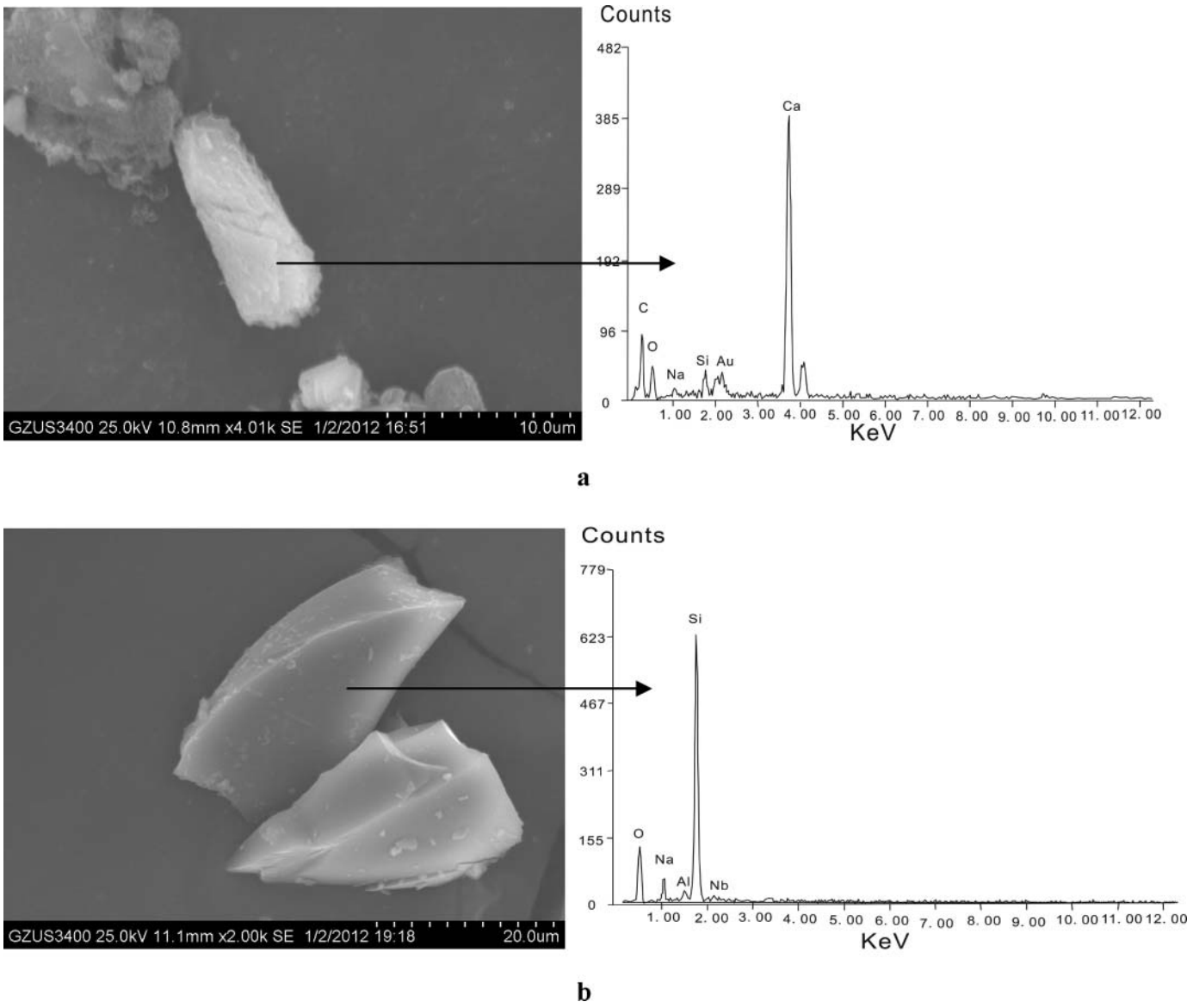

Fig. 7. SEM results and EDS image of the experimental and control groups; (a) experimental group, (b) control group.

control group: the results are shown in Figure 4. Figure 4 shows that the concentration of Ca^{2+} in the experimental group declined over time; the control group showed some fluctuation but there was no significant decreasing trend. This was because the bacterial growth absorbed Ca^{2+} and some Ca^{2+} was transformed into $CaCO_3$ in the experimental group. Although the fluctuation in the concentration of Ca^{2+} in the control group may have been because some Ca^{2+} adhered to the glass powder and became commixed therewith. The rise in the sixth and seventh days may have been caused by water evaporation from the medium.

XRD Analysis

XRD measuring the precipitate from the experimental, control, and pH = 9 group, respectively, are shown in Figure 5. Figure 5(a) shows that the experimental group inoculated with *B. cereus* had a significant $CaCO_3$ peak indicating that the principal mineral present was calcite; Figure 5(b) shows that the control group had no $CaCO_3$ peak: substances present were mainly noncrystalline; Figure 5(c) shows no $CaCO_3$ peak. As evidenced above, $CaCO_3$ particles were yielded when *B. cereus* was cultivated for 7 days, whereas the noninoculated control group yielded no detectable $CaCO_3$. This led the authors to suggest that the presence of $CaCO_3$ particles was not only related to the rising pH, but also interrelated to microorganism induction.

Observation and Analysis of the $CaCO_3$ Particles

TEM observation of the precipitate samples in both experimental and control groups was conducted and results shown in Figure 6. Figure 6 shows that bacterial and spherulitic $CaCO_3$ particles were found in the inoculated experimental group.

The particles' morphology, with evidence from EDS analysis, suggested that the $CaCO_3$ was organic in origin: the hollow spaces around the bacteria resulted from differential contractile forces between the bacteria and the surrounding culture medium because of the heating effect of the incident high-energy electrical beam. In the control group, the above phenomenon could not be observed, and the leaf-shaped materials were mainly inorganic crystalline salts. The near-spherical materials seen were mainly organic compositions in the medium and Na, Ca, and S-based substances.

The results of SEM observations are shown in Figure 7. Figure 7 shows that some crystals of granular $CaCO_3$ were yielded in the experimental group, and their surface morphologies were irregular: EDS showed the necessary evidence that this was $CaCO_3$. In the control group, only larger particles of glass powder could be seen.

No precipitation was found in the group of samples whose pH had been directly adjusted to 9.0 implying that pH may not be the main agent affecting $CaCO_3$ formation. In summary, the experimental and control groups were distinctly different in terms of bacteria shape, and the presence of $CaCO_3$ particles was thought to be related to the added bacteria.

As is well-known, microbial growth progresses by using the carbon source from the medium and will thus induce carbon isotope fractionation (Lian et al. 2006; 2008). A control group without $CaCl_2$ was established to find the fractionation data for inorganic carbon isotopes in the medium that originated from microbial respiration: these data were then compared with the isotope data for the $CaCO_3$ formed in the group with $CaCl_2$ (experimental group) and evidence as to whether or not the carbon source of $CaCO_3$ arose from microbial respiration or organic carbon bio-degradation was thus produced. The carbon isotope $\delta^{13}C$ value of that $CaCO_3$ in the experimental group was -10.86 ‰ (compared with PDB), compare that to the inorganic carbon isotope $\delta^{13}C$ value in the medium of the control group with -9.256 ‰ (compared with PDB) and this indicated that formation of the $CaCO_3$ particles was related to biological agents. This result clearly indicated that the strain DCB 1 metabolized organics to CO_2 and then reacted with Ca^{2+} in the culture to yield solid $CaCO_3$.

In addition, the growth and reproduction of microorganisms can secrete a variety of extracellular products, such as polysaccharides, enzymes, etc. (Du et al. 2008; Hu et al. 2011). These substances for calcium carbonate induction may also exert an as yet uncertain influence which the authors recommend as an avenue for further research.

Conclusions

The beef extract-peptone medium containing $CaCl_2$ that was inoculated with *B. cereus* can induce $CaCO_3$ crystal production. *B. cereus* has properties yielding alkalinity-production and CA, which can regulate the pH of the culture medium and in turn, the concentration of HCO_3^--induced $CaCO_3$ production.

The rock microorganism that can induce $CaCO_3$ by their metabolism is one carbon-sink which has, as yet, been ignored by related researchers (Lian and Hou 2011; Lian et al. 2011). Due to the soil and water being rich in calcium ions in Karst regions, the potential for soil microorganisms to reduce CO_2 and induce $CaCO_3$ formation awaits realization and indeed further exploration.

Acknowledgments

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