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Full Length Research Paper

Bacillus mucilaginosus can capture atmospheric CO_2 by carbonic anhydrase

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The study mainly demonstrated whether *Bacillus mucilaginosus* can use atmospheric carbon dioxide (CO_2) through the bacteria excreted carbonic anhydrase (CA) capturing atmospheric CO_2. It had been proved that CA could reversibly catalyze hydration of CO_2. When the bacteria was cultured in the medium containing limestone, Ca^{2+} concentration and CA activities simultaneously increased with the amount of limestone increasing in the medium. Analysis for the bacterial growth kinetics demonstrated Ca^{2+} concentration increased, CA activities enhanced and pH value of broth rose and residual glycerol concentration decreased with prolonging fermentation time. Ca^{2+} concentration of bacterial broth gradually increased with the increasing of amount CA supply and time extension when the bacteria were cultured in the Alexandrov medium containing limestone, in which CA synthesized was extremely low when limestone dissolved into water, CA could elevate Ca^{2+} concentration and pH value, and the bacteria might further elevated the Ca^{2+} concentration and pH value. Therefore, it is suggested that *B. mucilaginosus* can first capture atmospheric CO_2 by CA, then fix atmospheric CO_2 by bacteria metabolism, which complements the content of biology and provide a new strategy for CO_2 sequestration from the air.

Key words: *Bacillus mucilaginosus*, carbonic anhydraseequilibrium point, CO_2 utilization, catalyze.

INTRODUCTION

Before 1936, it was generally assumed that autotrophs were organisms that were capable of synthesizing organic molecules with inorganic material. For example, plants convert carbon dioxide into glucose by photosynthesis using the solar energy. And heterotrophs were incapable of this conversion of inorganic to organic and they obtain their organic compounds by eating or decomposing other organisms. Wood and Werkman (1936) found propionic acid bacteria were able to utilize carbonate or carbon dioxide and to convert it into an organic compound during the propionic acid fermentation with glycerol by propionic acid bacterial. Further, they found that propionic acid bacteria could fix carbon dioxide not only with glycerol as the substrate, but also with mannitol, adonitol, erythritol and rhamnose (Wood and Werkman, 1940). Evans and Slotin (1940, 1941) found that pig liver could fix carbon dioxide (CO_2). Since then, these enzymes which have been found using CO_2 as subsidy carbon sources in heterotrophs involve phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase, phosphoenolpyruvate carboxy-transfer enzyme, pyruvate carboxylase, malic enzyme and isocitrate dehydrogenase (Li et al., 1993). The mechanism of these enzymes in fixing CO_2 into organic molecule by heterotrophys is still not very clear now.

Two key problems of CO_2 fixation are that how CO_2 is captured and where the reducing energy (NADPH or NADH and ATP) is from. In some organism, NADPH can be produced from phosphopentose pathway. NADH and ATP can be produced from EMP pathway and TCA cycle. Wood and Werkman (1936) suggested that succinic acid

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was synthesized by the following pathway: oxaloacetic acid was formed by the addition of CO_2 to pyruvic acid catalyzed by pyruvate carboxylase firstly, then fumaric acid, malic acid and succinic acid were produced sequentially catalyzed by related enzymes.

The carbonic anhydrase (CA), which catalyzes the hydration of CO_2, exists in most living organism including autotroph and heterotroph (Smith and Ferry, 2000; Ilies and Banciu, 2004). Some studies have proved that CA can increase solubility of calcium and magnesium ions from carbonate rocks and the solubility of calcium and the activities of CA were positively correlated (Li et al., 2005, 2007; Nathalie et al., 2009). Based on chemical reactions equilibrium principle and enzyme catalytic properties, enzyme can only increase the reaction rate, shorten the time to reach equilibrium but can not change the reaction equilibrium point, except that the activity of the products was changed. The increase of Ca^{2+} and Mg^{2+} solubility caused by CA indicates that the equilibrium point shifts to the right and bicarbonate may be assimilated by organism. Based on these discussions, the hypothesis has been brought forward that heterotrophys capture CO_2 with CA synthesized by themselves and then convert CO_2 into other organic compounds.

Bacillus mucilaginosus is a special bacillus species, it can activate some insoluble mineral elements from soil, thus this bacterium has been used as a biological fertilizer (Lian et al., 2000). It has been proved that *B. mucilaginosus* possessed ability of fixing nitrogen and producing little acids. Although the bacteria were extensively applied in agriculture as biological fertilizer, the mechanism of dissolving mineral in soil kept unclear.

To prove the above hypothesis and disclose the mechanism for *B. mucilaginosus* to dissolve mineral element in soil, the correlations between the total amount of Ca^{2+} and the mean CA activity in *B. mucilaginosus* KO_2 culture were analyzed. The paper reported these results and theoretical derivation of our hypothesis.

MATERIALS AND EXPERIMENTAL PROCEDURES

Microbial strain

B. mucilaginosus KO_2 (GenBank database accession number: HM579819) (Mo and Lian, 2011) which is used in the study, was stored at the Environmental Biological Science and Technology Research Center, Institute of Geochemistry, Chinese Academy of Sciences.,

Culture conditions

It is reported that *B mucilaginosus* can grow in the basic medium lacking nitrogen source (Lian et al., 2000), so the basal medium (Alexandrov medium) containing (g/100 ml) sucrose 0.5, Na_2HPO_4 0.2, $MgSO_4 \cdot 7H_2O$ 0.05, $CaCO_3$ 0.01, $FeCl_3 \cdot 6H_2O$ 0.0005 and glass powder 1, was applied. The medium for producing CA was (g/L): glycerol 40, yeast extract 0.7, KNO_3 0.3 and $(NH_4)_2SO_4$ 0.3, $MgSO_4$ 0.05, Na_2MoO_4 0.05, $Na_2B_4O_7 \cdot 10H_2O$ 0.1, 3.5-dinitrosalicylic acid 0.02.

The bacterium was inoculated in 100 ml of basal medium in a 250 ml flask, then the flasks were incubated in a rotary shaker at 150 rpm at 32°C for 4 days for preparing the inoculums. 2% (v/v) inoculums were added aseptically to 250 ml flask containing 100 ml above-stated basal medium. The culture was kept at 32°C for 6 days.

Enzyme preparation

The bacterial was inoculated in 250 ml flask contained 100 ml CA producing medium, then these flasks were incubated in a rotary shaker at 150 rpm, 32°C for 5 days, and then the culture was centrifuged at 4000 x g for 10 min. The supernatant (3000 ml) was precipitated with 80% saturated $(NH_4)_2SO_4$ overnight. The bacterial protein obtained by centrifuge was dissolved in distilled water. The bacterial protein was absorbed by DEAE sepharose column (fast flow). The column was washed with NaCl solution of discontinuous gradient concentrations (0.1, 0.3, 0.5 and 1.5 mol/L) at 2 ml/min. The CA activity was obtained in 0.3 mol/L fraction and was extensively dialyzed (15 kDa) for 16 h in the flowing water. Following dialysis, the dissolved protein in the dialysate was lyophilized and the crude enzyme was obtained and stored at 4°C for further study. All the manipulations were performed at 4°C.

Interaction between CA and limestone

The limestone, containing 54.2% CaO, 1.36% MgO, 0.37% insoluble matter in 1 mol/L HCl, was collected from Caijiaguan, Guiyang city, Guizhou Province, China. After being cleaned and air-dried, the lime stone was ground and particles with diameter less than 0.25 mm were obtained by sieving. To investigate interaction among CA, limestone and *B. mucilaginosus*, six experimental systems were designed (Table 1).

Carbonic anhydrase assay

CA activity was determined by modified colorimetric method according to Verpoorte at al. (1967). The standard assay system was consisted of 1 ml enzyme solution and 1 ml pH6.4 0.2 mol/L phosphate buffer containing 1 mmol/L 4-nitrophenyl acetate (pNPA Sigma-Aldrich, USA) and 0.01 mmol/L diethymalonic acid (Sigma-Aldrich, USA). The reaction was incubated at 35°C for 30 min. The released *p*-nitrophenol was calculated by the increase in absorbance at 400 nm and the standard curve of *p*-nitrophenol. 1 ml enzyme solution was replaced by 1 ml distilled water in blank reaction system. One unit of activity represents the amount of enzyme catalyzing to produce 1 µmol *p*-nitrophenol per min under the assay conditions.

Other assay

Ca^{2+} content assay was measured by using a PerkinElmer model AA300 atomic absorption spectrophotometer. Glycerol in broth was determined by refraction analysis on LR35B portable refractometer with glycerol as standard curve (Zhu et al., 2004). Sample pH was determined with a PHS-3C$^+$ pH meter.

RESULTS

Cultured in the medium supplied with more limestone, the bacterium will produce higher CA activities significantly increased. With the amount of limestone increasing from

Table 1. Design and purpose of six test systems. The second test was carried in Braun Biostat Q fermenter (B. Braun, Germany) with equipped with pH and dissolved oxygen electrodes.

System	Methods	Study purpose
1	The bacterial was cultured in the medium for CA synthesis contained different concentration of limestone at 32°C for 6 days	To explore the relation between amount of carbonic anhydrase and dissolved Ca^{2+} concentration
2	The bacterial was cultured in the medium contained 7g/L limestone	To study the change pattern of CA, dissolved Ca^{2+} concentration, glucose consumption and pH of medium
3	The bacterial was cultured in Alexandrov medium contained 7 g/L limestone and added different concentration of CA (0, 20, 40 and 60 μU/ml, respectively) after sterilized at 32°C for 6 days	To analyze the correlation between dissolved Ca^{2+} concentration and CA concentration during bacterium growth
4	0.1 g limestone dissolved into 10 ml pure water	To analyze the correlation among dissolved Ca^{2+} concentration, CA and the bacterial.
5	0.1 g limestone dissolved into 10 ml pure water and 40 μU/ml CA	To analyze the correlation among dissolved Ca^{2+} concentration, CA and the bacterial.
6	0.1 g limestone dissolved into 10 ml pure water, 0.1g fresh the bacterial	
7	0.1 g limestone dissolved into 10 ml pure water, 0.1 g fresh the bacterial and 40 μU/ml CA	

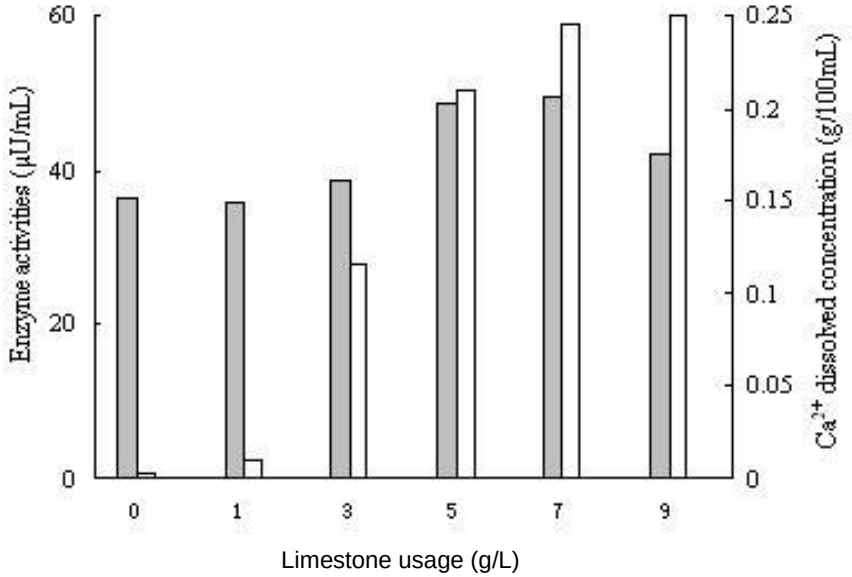

Figure 1. Supplied limestone was beneficial for *B. mucilaginous* K02 to synthesize CA. Enzyme activity (■); Ca^{2+} ion dissolved concentration (□). *B. mucilaginous* KO_2 was cultured at 32°C for 6 days.

1 to 7 g/L, the CA activities and the dissolved Ca^{2+} concentration increased correspondingly (Figure 1). When the amount of limestone was over 7 g/L, the CA activity would reduce and Ca^{2+} dissolved would not increase anymore. Here, it is deduced that some limestone promotes extracellular CA synthesize.

The kinetic analysis of bacterial growth in the medium for CA synthesis containing 7 g/L limestone was shown in Figure 2. The glycerol concentration decreased from initial 37.6 to 6.25 g/L after 120 h of culture (Figure 2); the broth pH slowly rose in the first 48 h, rapidly rose between 48 and 120 h and reached steady-state after 120 h; CA activity slowly increased in the first 48 h, then rapidly increased in the following 72 h, and did not

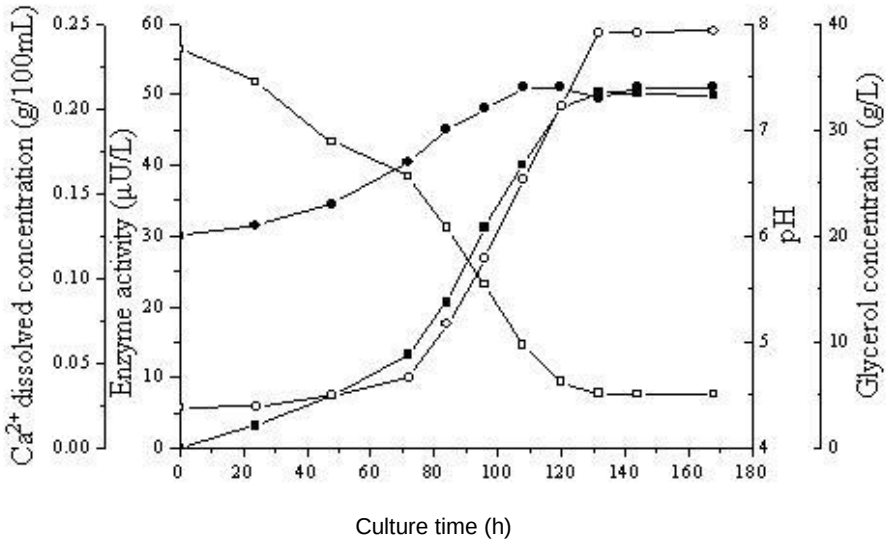

Figure 2. Typical curve of B. mucilaginosu K02 cultured in medium for carbonic anhydrase systhesis supplied with 7 g/L limestone. Ca2+ dissolved concentration (○), CA production (■), glycerol consumption (□) and pH (●) by B. mucilaginosus K02 in a 5-L B. Braun Biostat Q fermenter.

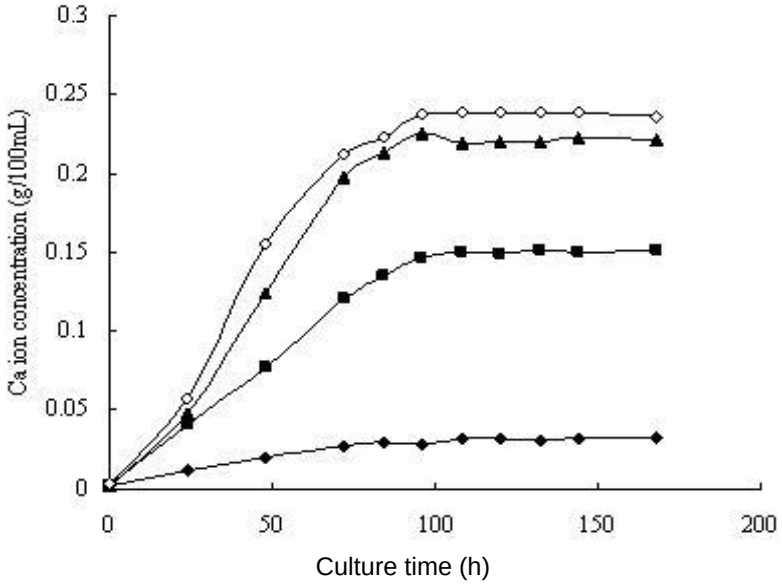

Figure 3. Effect of carbonic anhydrase on Ca released from limestone. Amount of the crude CA production supplied: 0 μU/ml (♦), 20 μU/ml (■), 40 μU/ml (▲) and 60 μU/ml (◊).

markedly increase during the last 48 h; the significant increase of Ca^{2+} concentration was only found from 48 to 120 h. These results demonstrated that the concentration of dissolved Ca^{2+} from limestone was always companied by the increase of CA activities.

To further prove the correlation between dissolved Ca^{2+} and CA activities, *B. mucilaginosus* KO_2 was cultured in the basal medium containing 7 g/L limestone in which the activities of the CA synthesized by the bacterium was very low. During the culture, the broth was sampled and Ca^{2+} concentration was determined. After the bacterium was inoculated to the medium, different amount of aseptic CA crude enzyme was synchronously added to the medium (Figure 3). The Ca^{2+} concentration of broth was

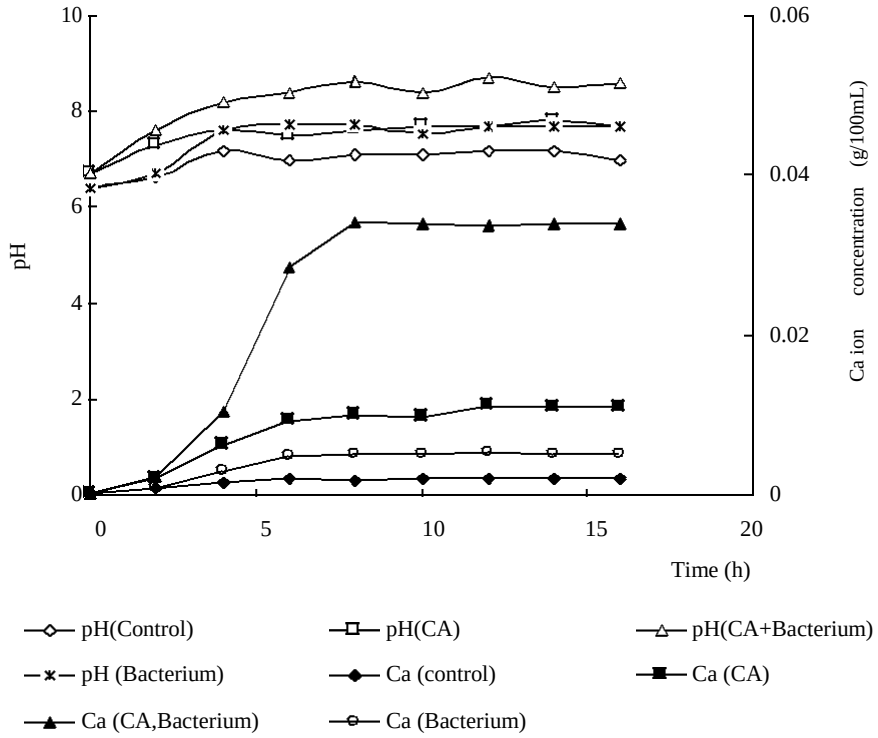

Figure 4. Effect of CA and B. mucilaginosu KO2 on Ca released from limestone. The pH and Ca2+ dissolved in water (◊ and ♦, respectively), pH and Ca2+ dissolved in water and crude CA (□ and ■, respectively), pH and Ca2+ dissolved in water (and) and bacterial, and pH and Ca2+ dissolved in water, crude CA and bacterial.(Δ and ▲, respectively).

all the time very low and less than 0.033 g/100 ml if CA was not added. If slight carbonic anhydrase was supplied, Ca^{2+} concentration would increase significantly. Moreover, Ca^{2+} concentration gradually increased with increasing the amount of CA supplied. As a whole, no matter whether CA was added or not, and no matter how much CA was added, the Ca^{2+} concentration rapidly increased from 0 to 72 h and after 72 h, the Ca^{2+} concentration hardly changed. It is reasonable to believe that CA enhanced the solubility of the limestone and increased Ca^{2+} concentration in the broth, which were supported by some previous studies (Li et al., 2005, 2007; Nathalie et al., 2009; Lindskog et al., 1971; Atkin et al., 1972; Fridlyand and Kaler, 1987).

The Ca^{2+} concentrations in control system (pure distilled water) were always lower than 0.0021 mg/100 ml (Figure 4). If only the strain was added to the pure water, Ca^{2+} concentration and pH were different between control system and the water + bacterium system after 2 h of cultivation and the difference gradually increased with time prolonging. If only the crude enzyme was added to the pure water, Ca^{2+} concentration was significant higher than the control system and over 4 times higher after 6 h (Figure 4) and pH was also significant higher than control system. If the strain and enzyme were added to the pure water at the same time, Ca^{2+} concentration would further increase and reached 0.339 mg/100 ml at 10 h, and was 3 and 6 times higher than that in enzyme solution and bacteria solution, respectively (Figure 4). If the bacteria and CA simultaneously added, the pH showed the same tendency, and reached 8.4 at 10 h and not only markedly higher one of the control system (7.1), but also higher one of enzyme solution (7.7) and bacteria solution (7.54), respectively (Figure 4). These results further proved above results from test 1-3.

DISCUSSION

There are two pathways by which CA increase Ca^{2+} concentration in the broth. One pathway is that CA facilitates CO_2 releasing, partly relieving the CO_2 inhibition on the bacterial metabolism, accelerating the metabolism of bacteria and synthesizing excessive organic acid, which expedited limestone dissolving and Ca^{2+} releasing through replacement reaction. The acidity of organic acid was stronger than that of H_2CO_3, and the medium pH value will be reduced. In fact, the kinetic analysis of the bacterial grown in the optimum medium supplied with 0.7 g limestone showed that pH value would not reduce but increase with

the growth time extension (Figure 2). Therefore, the increase of calcium ion concentration in broth can not be explained by excessive organic acids excreted by bacterium but contributed to CA. CA elevates Ca^{2+} concentration in broth by another pathway. That is CA catalyzes the reversible hydration of CO_2, accelerating HCO_3^- and H^+ ion formation (Sharma et al., 2009; Liu and Dreybrodt, 1997).

$$CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$$

It has been reported in many literatures that microorganisms participate in weathering and extracellular CA activity from microorganism was positively correlated with the total amount of Ca^{2+} in leachates of soil (Li et al., 2005, 2007; Nathalie et al., 2009; Liu, 2001) CA catalyzed H^+ reacts with $CaCO_3$.

$$CaCO_3 + H^+ \Leftrightarrow Ca^{2+} + HCO_3^-$$

According to characteristics of enzyme reaction, the increase in enzyme amount can speed up the reaction and shorten the time to reach equilibrium, but it does not change the reaction equilibrium point. The fact that Ca^{2+} concentration increase and extracellular CA activity in the culture were positive correlated demonstrated that CA could capture atmospheric CO_2 and was beneficial to the Ca^{2+} dissolved from limestone. That Ca^{2+} concentration dissolved from limestone further rose after *B. mucilaginosus* KO_2 was added to the react system accounted for that the balance point of CO_2 hydration reaction move towards the right after bacterium were supplied.

Provided that other conditions (such as temperature and pressure, etc.) remain unchanged, the reaction equilibrium shifting to the right can be achieved by increasing substrate (limestone) concentration or decreasing product (HCO_3^- or Ca^{2+}) concentration. It was clear that only the products decreasing, but not substrate increasing can facilitate equilibrium shifting to the right. In the broth, only bacteria could consume HCO_3^- or Ca^{2+} (products). If Ca^{2+} consumed by the bacterium caused the reaction equilibrium to shift to the right, Ca^{2+} concentration should decrease. In fact, Ca^{2+} concentration in the reaction solution increased but not decreased, which explained that consuming Ca^{2+} by *B. mucilaginosus* KO_2 could not contribute to the increase of Ca^{2+} concentration. Therefore, the unique way that bacterium led to increase Ca^{2+} concentration was that the bacterium had consumed HCO_3^-. Bacterium utilizing HCO_3^- contributed to the concentration of HCO_3^- reducing and the reaction equilibrium shifting to the right, namely the side product, Ca^{2+} increasing.

In order to prove the pathway, the test of limestone dissolving in water also validated the deduction. CA could increase the solubility of limestone and elevate culture pH and the bacterium could further increase the solubility of limestone and elevate the culture pH by altering the reaction equilibrium point (Figure 4). The only pathway that the bacterium alters the reaction equilibrium point is that bacteria consume HCO_3^-, which is the production of CO_2 hydration reaction. Based on these deductive reasoning, the hypothesis was suggested that *B. mucilaginosus* KO_2 captured atmospheric CO_2 through hydration of CO_2, and then HCO_3^- was utilized by bacterium and finally converted into the bacterial metabolism. The result and hypothesis obtained from the study possess profound significance for using limestone, controlling CO_2 in the air and reducing greenhouse gas emissions. Certainly, some questions like how bicarbonate is metabolized by bacteria *in vivo*, whether bicarbonate reductase exists in bacteria and where the reducing power, NADH or NADPH, comes from need further intensively study.

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