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NOTE

Calcite Production by *Bacillus amyloliquefaciens* CMB01

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The bio-mediated production of calcite crystals by calcinogenic bacteria has great applicable value for the restoration of deteriorated calcareous monuments, because of its high purity and coherency. An investigation of the conditions for calcite production by an alkalophilic *Bacillus amyloliquefaciens* CMB01 strain was made. Optimal calcite precipitation occurred when the bacterium was cultured at pH 8.0 and 30°C, and in B4 medium that consisted of 0.4% yeast extract, 0.5% glucose, and 1.5% calcium acetate. Calcium ion of the bacterially induced calcite was analyzed by an inductively coupled plasma (ICP) spectrophotometer. Optical and scanning electron microscopy (SEM) of the calcite revealed a typical rombohedral polycrystalline structure.

Key words: an alkalophilic *Bacillus amyloliquefaciens,* calcite production, calcinogenic bacteria, ICP spectrophotometer, rombohedral polycrystalline structure

Biologically induced mineralization (BIM) occurs through chemical reactions between specific ions or compounds resulted by metabolic activities of an organism in certain environmental conditions (Lowenstam, 1981; Addadi and Weiner, 1997; Bazylinski and Moskowitz, 1997; Douglas and Beveridge, 1998). Carbonatogenesis, bacterially induced precipitation of calcium carbonate (synonym of calcite), is a good example of BIM (Little *et al.*, 1997). Although the bacterial contribution to carbonatogenesis has been suspected for nearly a century, it has remained controversial until not long ago when metabolic studies in bacterial carbonate precipitation and evaluation of bacterial carbonate productivity were reported (MaCallum and Guhathakarta, 1970; Bouquet *et al.*, 1973; Novitsky, 1981). Chemoorganotrophic microorganisms making their living through their catabolic process of organic matter are the major contributors of carbon dioxide in nature. And these microorgamisms are also highly involved in the bio-precipitation of carbonate minerals, which are formed through crystal formation between calcium ion and carbon dioxide (Douglas and Beveridge, 1998).

In recent decades, the calcareous stone statuaries and historic monuments around world have faced accelerated weathering and deterioration because of increasing atmospheric pollution and biodeterioration (Crispim *et al.*, 2003; Ortego-Morales *et al.*, 2003; see references in Rodriguez-Navarro *et al.*, 2003). Consequently, conservation methods are greatly needed for the protection and consolidation of stones before extensive granular disintegration led to irreversible damage. Among the many protection and consolidation treatments available that mainly use inorganic and organic materials, none of them has proven to be quite satisfactory (Pèrez *et al.*, 1995; Cardino *et al.*, 2001).

It has been known that calcite (calcium carbonate) has values of technical and industrial applications for the preservation and restoration of calcareous stone statuaries and historic monuments. One of the applications of calcite is the restorative treatment of porosity and weakening of the superficial structural strength of calcareous stone attributed to calcite leaching. Thus, calcite is needed in high purity and good coherency for better restoration. However, it is a very laborious and expensive process to obtain the highly pure and coherent calcite from natural sources, such as shell crust. Thus, bacterially induced carbonate precipitation has received attention as an environment-friendly method of protecting decayed ornamental carbonate stone, as mentioned in the reports on calcinogenic bacteria, *i.e. Micrococcus* sp., *Bacillus subtilis*, *Bacillus pasteurii*, *Deleya halophila*, *Halomonas eurihalina*, and *Myxococcus xanthus* (Rivadeneyra *et al.*, 1991; 1996; 1998; Tiano *et al.*, 1999; Castanier *et al.*, 2000; Rodrigues-Navarro *et al.*, 2003). Applications of bacterial carbonetogenesis to regenerate the carbonate buildings and historic monuments were practiced by spraying the calcinogenic bacterial suspension

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grown in a suitable medium to the surface of the porous objects followed by feeding the same medium at certain intervals (daily or every two days) for calcite precipitation *in situ* (Le Métayer-Levrel *et al.*, 1999; Castanier *et al.*, 2000).

Calcite precipitation in solution occurs via the overall equilibrium reaction of $Ca^{+2}+CO_3^{-2} \Leftrightarrow CaCO_3$. Because the production of CO_3^{-2} from bicarbonate (HCO_3^{-1}) in water is strongly pH dependent, an increase in CO_3^{-2} concentration occurs under alkaline conditions. There fore, calcium carbonate precipitation readily occurs in alkaline environments abundant of the calcium (Ca^{+2}) and carbonate (CO_3^{-2}) ions (Little *et al.*, 1997; Stocks-Fischer *et al.*, 1999; Rodrigues-Navarro *et al.*, 2003).

Recently, a report on *Bacillus amyloliquefaciens* CMB01, an alkalophilc *Bacillus* producing a potent protease (Ban *et al.*, 2003) was made. This is a mesophilic (25~45°C) and slightly alkalophilic bacterium (pH 7~9), whose optimal growth temperature and pH are 35~40°C and pH 8, respectively. The alkalophilic and heterotrophic nature of this bacterial strain led me to investigate conditions necessary for calcite production.

One ml of a culture of *B. amyloliquefaciens* CMB01 grown overnight in Luria-Bertani (LB, 1% tryptone, 0.5% yeast extract, 0.1% NaCl) broth at 30°C with 150 rpm was seeded to 100 ml of the following media; LB acetate medium (LBA, 1% tryptone, 0.5% yeast extract, 0.05% NaCl, 1% calcium acetate), B4 medium (0.5% yeast extract, 0.5% glucose, 1 % calcium acetate), MM medium (0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.1% glucose, 0.01% $MgSO_4 \cdot 7H_2O$, 1% calcium acetate) and MM-G medium (0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.01% $MgSO_4 \cdot 7H_2O$, 1% calcium acetate). The medium was adjusted at pH 8. The culturing of bacteria was carried out at 30°C for 5 days with continuous aeration at 150 rpm. Bacterial growth was recorded with an optical density at 600 nm (OD_{600}). Separation of the bacterial cell and calcite in the culture broth was made by filtering through a 0.45 μm membrane pore; the calcite was retained on the membrane and the bacterial cell in the filtrate.

Calcite production of the CMB01 strain in different media was expressed as either calcite dry weight (mg)/cell dry mass (mg) or calcite (mg)/OD_{600}. As shown in Table 1, B4 medium gave the highest productivity of calcite versus growth, and the next productive was the MM medium. The B4 medium employed in this study contained 1.0% calcium acetate, whereas the B4 medium used by others in similar studies contained 0.25% calcium acetate (Rivevadeneyra *et al.*, 1991; Tiano *et al.*, 1999). The pH effect on calcite production of the CMB01 strain was investigated by employing the B4 media prepared at pH ranging from 7 to 10. As summarized in Table 2, calcite production was increased at pH 8~9, with a two-fold higher calcite production at pH 8 compared with those at either pH 7 or 10. This observation was conceivable without any doubts since the bacteria grow optimally at pH 8 (Ban *et al.*, 2003) and calcite precipitation in water favors alkaline conditions (Little *et al.*, 1997; Stocks-Fischer *et al.*, 1999). When effect of temperature on the calcite production of CMB01 strain was studied with the B4 medium (pH 8), bacterial culture grown at 30°C yielded slightly but significantly more calcite (6.4 mg/ml of culture) compared with those at 25 and 37°C (5.4 mg and 5.9 mg of calcite /ml of culture, respectively). Since the CMB01 strain is a soil isolate originated from ambient environments, it was not surprising that the calcite production was not heavily temperature-dependent within the range of 25~37°C. An availability of calcium and carbonate ions in the calcinogenic environment is a major determining factor for calcite precipitation, excluding an alkaline environment, and therefore, the calcite production of the CMB01 strain was studied with the B4 medium containing different concentration of calcium acetate. As shown in Fig. 1, calcite production was maximized at 1.5% calcium acetate concentration. Using an ICP spectrophotometer (JY 38 Plus, France. This work was carried out at Center for Research Instruments and Experimental Facilities, Chungbuk National University), a quantitative assay of calcium ion of the bacterially induced calcite showed that about 90% of calcium ion occurred in the calcite sample (*i.e.* 733 ppm Ca^{+2} in 812 μg calcite dissolved in HCl). When the calcite was observed using an optical microscope after a simple staining with methylene blue, a typical crystalline structure was seen (Fig. 2A). Also its texture was observed through scanning electron microscopy (SEM) (Leo 1530, Leo Electron Microscope Ltd, Germany) after

Table 1. Comparison of calcite production by *B. amyloliquifaciens* CMB01 in different media

Medium	Calcite (mg)/Cell mass (mg)[a]	Calcite (mg)/OD_{600}
B4	1.046	5.33
MM	0.807	4.63
LB A	0.343	2.95
MM-G	0.226	1.97

[a]The figure, average of triplicate, represents the dry weights of calcite per dry mass of cells grown in different media (details described in the text)

Table 2. Effect of pH on calcite production of *B. amyloliquifaciens* CMB01 in the B4 medium

pH	Calcite (mg)/Cell mass (mg)[a]	Calcite (mg)/OD_{600}
7.0	0.466	2.42
8.0	0.913	5.83
9.0	0.695	4.16
10.0	0.440	2.24

[a]The figure, an average of triplicate, represents the dry mass of calcite per dry mass of cells grown in different media (details described in the text)

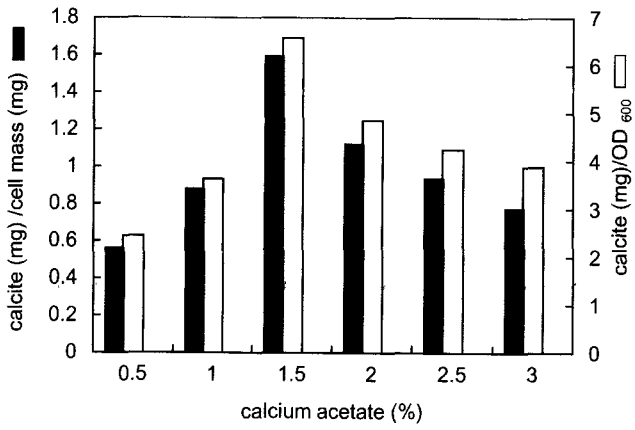

Fig. 1. The effect of calcium acetate concentration on calcite production of *B. amyloquifaciens* CMB01 in B4 medium.

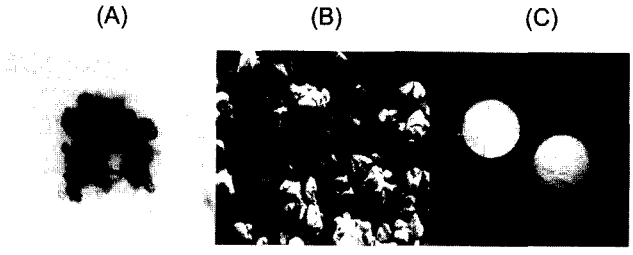

Fig. 2. Optical and scanning electron micrographs of calcite crystals produced by *B. amyloliqufaciens* CMB01 and the calcite stone. A: optical microscopy of calcite after a simple methylene blue staining (400X). B: SEM (a bar is 1 μm). C: the calcite stone made of bacterially induced calcium carbonate (see details in text).

gold coating of the sample (IB: 3 Ion Coater, Eiko Engineering Co., Ltd, Japan). The SEM of the calcite revealed a rombohedral polycrystalline structure as shown in Fig. 2B. Fig. 2C is the mimicked marble made of the bacterially induced calcite by the process of calcination, pressing and sintering. Calcination was carried out for 2 h at 900°C in an electric furnace (SCEF-201, Electric Furnace, Shinseong, Korea), and then the calcinated material mixed with polyvinylalcohol (PVA, 0.3%) was subjected to the press molding (3 ton/m^2, HC-286, Graseby Specac, USA). After 2 h pre-sintering at 500°C to combust the binding material PVA, a real sintering was followed for 3 h at 1,200°C in an electric furnace.

In summary, calcite precipitation by an alkalophilic *Bacillus amyloliquefaciens* CMB01 strain optimally occurred when the cells were cultured at pH 8.0 and 30°C, in a B4 medium consisted of 0.4% yeast extract, 0.5% glucose, and 1.5% calcium acetate. As only a few of the carbonate producing bacteria are applied as a conservation treatment of calcareous ornamental stones (Tiano *et al.*, 1999; Castanier *et al.*, 2000) and regeneration of limestone in buildings and historic patrimony (Le Métayer-Levrel *et al.*, 1999), the alkalophilic *Bacillus amyloliquefaciens* CMB01 would have great values for the conservation of ornamental stones and art work.

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