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RESEARCH PAPER

Effect of Different Calcium Salts on Calcium Carbonates Formation Induced by Halophilic *Bacillus oceanisediminis* CB1

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Abstract: Biomineralization through the biomimetic CO_2 sequestration process has been gaining attraction in recent years due to the formation of carbonates widely used as raw material in various industrial processes. The deposition and dissolution of calcium carbonate can be affected by physiochemical factors, such as the type of calcium salt. However, most studies have focused on calcium chloride ($CaCl_2$). In the present study, A potent bacterial carbonic anhydrase (CA) producer, *Bacillus oceanisediminis* CB1, was screened on CA activity from mangrove plant *Avicennia marina,* collected from Ghogha, Bhavnagar, India (21.68°N 72.28°E). We premeditated deposition experiments to determine the effects of different calcium salts on calcium carbonate deposition in *Bacillus oceanisediminis* CB1 colonies. The results demonstrated the calcite formation observed in calcium salt-supplemented nutrient agar, calcium chloride, and calcium acetate. Merely uniform distribution and peripheral distribution of calcite particles found in calcium acetate and calcium chloride supplemented into nutrient agar, respectively. Calcite formation was confirmed by staining with Alizarin Red S dye followed by SEM-EDX. This study will provide a vital reference for designing and applying microbial-induced carbonate precipitation using different calcium salts.

Keywords: Carbonic anhydrase (CA); Mineral Carbonation (MC); Carbon sequestration; Mangrove-associated bacteria

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INTRODUCTION

The amount of various Greenhouse Gases (GHGs) is progressively increasing after industrialization and fossil fuel use. CO_2 occupies the first position, and its concentration has reached 410 ppm due to various anthropogenic activities, and CO_2 concentration will reach 600 ppm by 2050 and 700 ppm by 2100 [1,2]. Current physical and chemical methods fail to provide universal, efficient CO_2 sequestration solutions due to the higher cost of operation, low efficiency, hazardous operational process, restriction of plant geography, community acceptance, and safety issues [3]. There is an urgent need to sequester CO_2 using novel and innovative approaches to strengthen the sustainable economy.

Mineral carbonation (MC) is emerging as a long-term safer CO_2 sequestration and storage technology [4]. MC is characterized as an

accelerated weathering of silicate rocks in which metal oxide-bearing materials react with CO_2 to form insoluble carbonates. Additionally, insoluble metal carbonates have been stable and environmentally benign for millions of years. Therefore, MC seems to be a feasible and viable technology [5]. MC also offers various benefits, including wide resource availability, lack of post-storage monitoring, easy public acceptance, favorable chemistry, and marketable product formation. Moreover, the potential of CO_2 sequestration through MC accounts for more than 10000 Gt C, which is sufficient for the next hundred years [5-7]. However, the slow reaction rate and other physiochemical factors hamper the technology's success, which needs to be addressed for commercializing the technology.

The input of carbonic anhydrase (EC 4.2.2.1) (a zinc metalloenzyme biocatalyst) catalyzes the reversible hydration of CO_2 ($CO_2 + H_2O \Leftrightarrow H^+ + HCO_3^-$), which is responsible for speeding up the MC. In search of suitable CA, numerous microbial CAs from different habitats were screened and engineered for carbonate production to sequester CO_2 [4,8-11]. Kupriyanova *et al*., (2007) demonstrated the role of inorganic carbon in a $CaCO_3$ deposition in the mineralization process by *Microcoleus chthonoplastes* which significantly enhanced the formation of $CaCO_3$ and acted as an intimation for the growth of mineralization for CO_2 sequestration [12]. Subsequently, the formation of calcium carbonate ($CaCO_3$) is widely studied among various types of carbonates. Later on, the formation of $CaCO_3$ or other carbonates in bacteria was demonstrated using various sources of calcium or magnesium by carbonic anhydrase (CA). Subsequent research has been carried out to optimize $CaCO_3$ production [4,13]. However, calcium concentration, carbonate concentration, pH of the environment, and nucleation sites influence the formation of $CaCO_3$ through bacterial CA. CA-mediated $CaCO_3$ formation also depends on other physiochemical parameters such as temperature and pressure. However, limited success has been achieved in integrating CA in current CO_2 capture technologies [4,9,11]. Among them, CA-mediated carbonate formation using CO_2 from power station fuel gas seems more promising than another CO_2 capture system for achieving significant CO_2 sequestration [14,15].

Bacteria from various habitats are being explored to search for potentially applicable CA for Carbon Capture Utilization and Sequestration (CCUS) [4]. However, inadequate work has been carried out on an exploration of mangroves as a potential CA source. Mangroves are an ecosystem with maximum carbon sequestration potential [16,17]. Further, the carbon sequestration potential of different mangrove plants varies from 10.4 tons/Hector to 71.3 tons/Hector, with *Avicennia marina* having the highest potential (71.3 tons/Hector) [18]). Due to advancements in sequencing technology and metagenomics, it is well known that any plant or organism's functional trait is primarily due to the microbiome it harbors [19]. Bacteria were isolated from the mangrove plant *Avicennia marina* in search of a potential CA producer.

In the present study, potential CA producer was screened based on CA activity. The potent bacteria tested for *in-vitro* formation using two different sources of calcium, and confirmation of calcite formation was carried out by SEM-EDX. The study provides inputs for designing an efficient microbial-induced carbonate precipitation system.

MATERIALS AND METHODS

Chemicals

Sodium chloride (NaCl), Alizarin Red S, calcium acetate ($C_4H_6CaO_4$), and calcium chloride ($CaCl_2$) were purchased from Sisco Research Laboratories Pvt. Ltd.(SRL), India, and were used without any further purification. Nutrient agar was purchased from Hi-Media.

Bacterial strain

Mangrove plant, *Avicennia marina*, and associated soil collected from Ghogha, Bhavnagar, Gujarat, India (21.68°N 72.28°E) used as a source of bacteria. Pooled samples were taken in a sterile plastic bag, transferred to the laboratory, and stored at 4°C until further use. Bacteria were isolated on nutrient agar plates (pH 8 and 9) containing 2.5% NaCl by serial dilution. The potent bacterium was screened based on CA activity [20]. The potent bacterium was identified using 16s rRNA sequencing.

Preparation of deposition system

The potent bacterium's *in-vitro* calcium carbonates formation competence was examined by culturing bacteria on nutrient agar plates supplemented with 25 mM calcium acetate and calcium chloride. The colony was observed under a microscope and scanning

electron microscope (SEM) after 48 hours of incubation.

Characterization

The formation of calcium carbonates is checked by optical microscopy and Scanning Electron Microscopy (SEM). SEM operated at an accelerating voltage of 15.0 kV under vacuum, and images were taken at a magnification of 40K. Confirmation of calcite assured by staining of calcite and Energy-dispersive X-ray spectroscopy (EDX). The colony was observed for calcite crystal formation under a stereomicroscope (5X). Confirmation of calcite was done by flooding a 0.2% w/v of Alizarin Red S dye into a thin section of the colony for 10-15 seconds and washing with distilled water to observe under a stereomicroscope (5X). Subsequently, a thin section of the colony was observed under SEM, and the formation was confirmed through EDX by scanning the colony's thin area for elemental analysis.

RESULTS AND DISCUSSION

A total of 15 different bacteria were isolated from the pooled samples of stem, root, and root-associated soil on a nutrient agar plate supplemented with 2.5% w/v of NaCl. Based on molecular identification, a potent CB1 isolate was found *Bacillus oceanisediminis*. The potent bacterium was selected based on the highest intracellular CA activity and further study.

The isolate *B. oceanisediminis* CB1 was tested for microbial-induced $CaCO_3$ precipitation on nutrient agar augmented with a different calcium salt. The underlying mechanism of $CaCO_3$ formation is well understood in prokaryotes. Bacteria produce HCO_3^- in the presence of CO_2 and H_2O by using the CA and subsequently form $CaCO_3$ using Ca^{2+} and HCO_3^- [21]. Other theories suggest that bacteria's metabolic activity plays a significant role in carbonate production by releasing HCO_3^- and NH_4^+ from the organic matter of medium (such as peptone, yeast extract, and beef extract) in the presence of Ca^{2+} using CA [22]. Several investigators reported calcite formation from a diverse group of bacteria by CA [4]. In the present study, *B. oceanisediminis* CB1 produced $CaCO_3$ in calcium-supplemented nutrient agar using calcium chloride or calcium acetate.

Silva-Castro et al. (2015) reported CaCO3 crystal formation on culture media by bacteria [4,23]. In the present study, different amounts of elemental calcium were reported in different calcium sources (calcium chloride and calcium acetate) supplemented with nutrient agar. The difference in elemental calcium amount indicates the role of calcium salt in the formation of microbial-induced calcite. In contrast, no calcite formation was observed in calcium-devoid nutrient agar, indicating the role of calcium in bio-precipitation processes (Figure 1). The formation of $CaCO_3$ depends on the types of bacterial CA, their efficiency, and CO_2 concentration and source of Calcium.

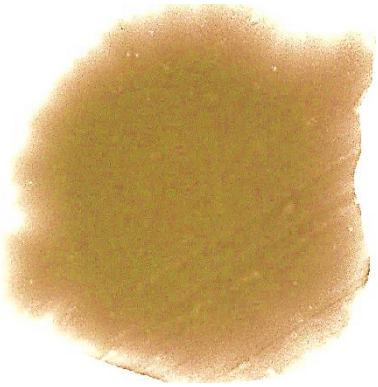

Figure 1. Figure 1. The colony of *B. oceanisediminis* grown on calcium devoid nutrient agar and observed under stereomicroscope (5x). In-vivo calcite formation was not observed by a colony in control experiment.

The confirmation of $CaCO_3$ was carried out using different methods, including staining of $CaCO_3$ crystal through chromogenic dye followed by EDX. In literature, carbonate staining was reported using other stains for petrography, correlation, and Genesis (Gerald 1953). Therefore, a thin section of the bacterial colony was stained by Alizarine Red S dye performed for $CaCO_3$ crystal verification. It is the first report of calcite identification in the bacterial colony through the staining technique. $CaCO_3$ crystal stained with light to dark pink colour indicates that the formed crystal-like structures are polymorphs of calcium carbonate (Figure 2, 3). The distribution of $CaCO_3$ polymorphs was found to vary depending on the source of calcium salt, merely uniform in the bacterial colony in calcium acetate-supplemented nutrient agar (Figure 2) and peripheral distribution in calcium chloride-supplemented nutrient agar (Figure 3) through a stereomicroscope and SEM. The morphology and forms of $CaCO_3$ largely depend on the bacteria's strain [13,24].

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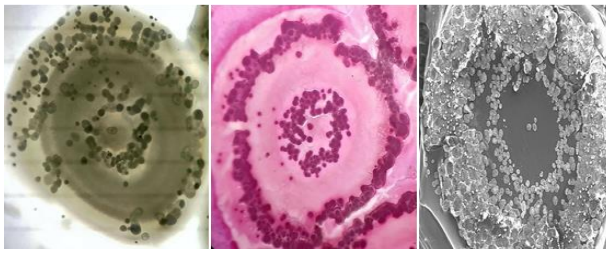

Figure 2. Calcium acetate ($C_4H_6CaO_4$) supplemented nutrient agar colony under stereomicroscope (5x) (Left), staining of $C_4H_6CaO_4$ supplemented nutrient agar colony (5x) (Center), SEM image $C_4H_6CaO_4$ supplemented nutrient agar colony (40K magnification) (Right)

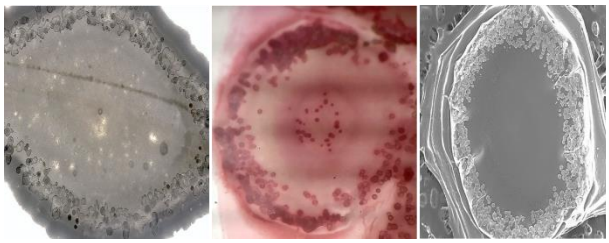

Figure 3. Calcium chloride ($CaCl_2$) supplemented nutrient agar colony under stereomicroscope (5x) (Left), staining of $CaCl_2$ supplemented nutrient agar colony (5x) (Center), SEM image $CaCl_2$ supplemented nutrient agar colony (40K magnification) (Right)

Similarly, the nucleation site may vary depending on the substrate (calcium salt). Additionally, the confirmation of calcite was checked by EDX. Very heightened peak at 3.7 keV suggesting the high concentration of elemental calcium observed in the calcium-supplemented nutrient agar colony. In control (calcium-devoid nutrient agar colony), calcium was absent (Figure 4). In calcium acetate and calcium chloride, the augmented bacterial colony showed nearly 44.91 wt % and 22.64 wt % more elemental calcium than the control group. The difference in the amount of calcium may be due to the different roles of calcium salts on bacterial metabolic activity, resulting in a change in the Ca^{2+} adherence to the bacterial surface, limiting calcium uptake. Moreover, calcium acetate has weak acidity compared to calcium chloride resulting in reduced loss of CO_2 (due to slow CO_2 release) and enhanced utilization of CO_2 by bacteria. In a recent study using *Bacillus cereus*, calcium deposition was observed in the following order, calcium acetate ($C_4H_6CaO_4$) > calcium chloride ($CaCl_2$) > calcium nitrate ($Ca(NO_3)_2$ [25]. Furthermore, the different calcium sources induce crystals with different shapes. The morphological difference in calcium crystal is also strain and species-dependent. Again, Extracellular polymeric substances (EPS) produced by bacteria also play a crucial role in calcium carbonate polymorph formation.

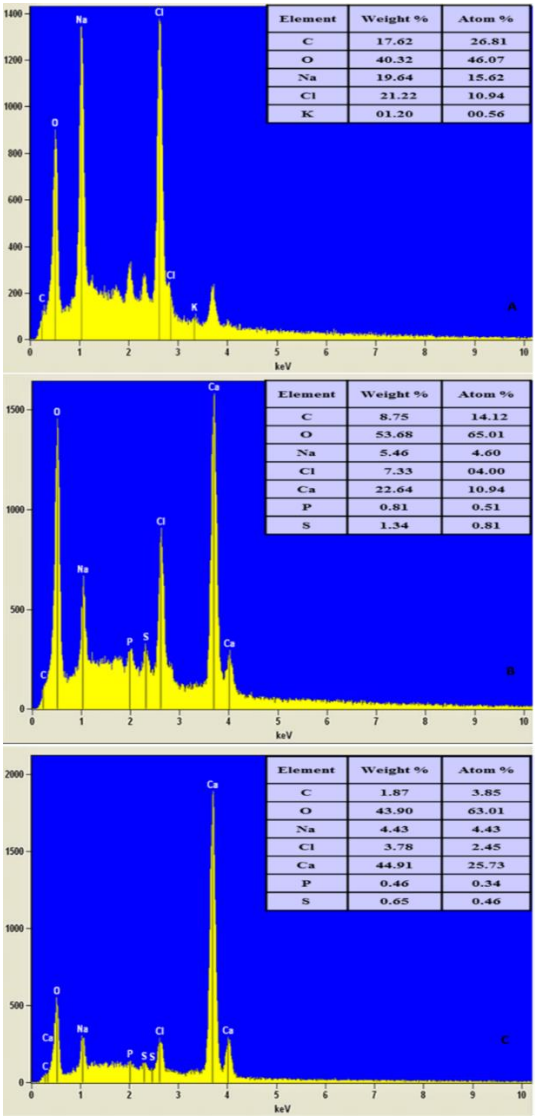

Figure 4. EDX of control colony with Weight % (Top Image), EDX of $CaCl_2$ supplemented nutrient agar colony with Weight % (Middle Image), EDX of $C_4H_6CaO_4$ supplemented nutrient agar colony with Weight % (Bottom Image)

De Muynck et al. (2007) observed the change in the water adsorption capacity of mortar cubes in the presence of *Bacillus sphericus* and $CaCl_2$ or $C_4H_6CaO_4$. Nearly five-time reduction in water adsorption was seen in a blend of *B. sphaericus* and calcium chloride

than control. However, less water adsorption-reduction was noticed in a blend of *B. sphaericus* and $C_4H_6CaO_4$ than in a blend of *B. sphaericus* and $CaCl_2$ [26]. Thus, it may be implicit that in agar plates containing $CaCl_2$, the restriction water that serves as a potential source for HCO_3^- ($CO_2 + H_2O$ ($H+ + HCO_3^-$) restricted calcite formation calcium acetate supplemented agar. Similarly, it may also contribute to the difference in the distribution pattern of calcite in the colony due to more metabolic preference. It concluded that calcium acetate and calcium chloride calcite formation were found, but more versatile calcite formation was observed in calcium acetate-supplemented media. Thus, a different source of Ca^{2+} could affect the physiological activities of microorganisms and mineral deposition.

CONCLUSION

Mangrove forest harbors bacterial diversity capable of calcium deposition. Potent CA producer B. oceanisediminis CB1 was able to precipitate calcium when grown on nutrient agar media supplemented with calcium acetate and calcium chloride. Calcium acetate caused a high calcium deposition than calcium chloride. A further detailed investigation is required to find out the type of calcium polymorph generated by bacteria. However, the proposed research will help to understand and design carbonic anhydrase-mediated microbial-induced carbonate precipitation

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CONFLICT OF INTERESTS

All authors declare to have no conflict of interests

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