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Biomimetic Sequestration of CO_2 Using Carbonic Anhydrase from Calcite Encrust Forming Marine Actinomycetes

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ABSTRACT

Background: CO_2 in the environment has been a burning issue and aggravated the threats of global warming. Various strategies are being used to decrease the level of releasing CO_2. Biosequestration is a one of the available nontoxic, robust and eco-friendly approaches but still less evaluated. This study reports the role of microbial Carbonic Anhydrase (CA) in the formation of calcite by utilizing CO_2. **Methods:** The marine *Nocardiopsis lucentensis* was studied for intracellular microbial CA. Isolate grown optimally at 2.5% CO_2 saturated environment and procedure the calcium carbonate encrust in the presence of $CaCl_2$. **Results:** The enzyme was found to catalyze the reaction in a wide range of pH and temperature with an optimum at 7.0 pH and 25°C. The 50 mM NaCl, KCl and $MgCl_2$ were found to support the enzyme activity and 50 mM $ZnCl_2$ increases activity 1.5 fold. CA was able to withstand against sulphanilamide inhibitor and stable over prolonged incubation at 4 and 37°C. **Conclusion:** Calcite formation was evaluated with and without enzyme using marine water as a source of calcium ions. The result of SEM and EDX indicated the formation of larger flower-shaped particles compare to the small cubic particles formed without enzyme. The results suggest that the robustness of enzyme and suitability in the CO_2 capture reactor.

Key words: Calcite formation, carbon dioxide, carbonic anhydrase, biosequestration, marine actinomycetes

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INTRODUCTION

CO_2 is most abundant greenhouse gas in Earth's atmosphere which plays a crucial role in the global warming effect. Currently, CO_2 concentration has increased at to the alarming rate that creates the need of the environmental amelioration either by decreasing the CO_2 emission in the atmosphere or capturing it into benign products using suitable technology for safe future existence[1]. Carbon sequestration is the process of capturing CO_2 and depositing it for long term where it will not enter into the atmosphere[2]. A great initiation is taken by the Inter-Academy Panel on International Issues (IAP) to reduce the 80% of CO_2 in the atmosphere by 2050. Various methods have investigated the solutions of CO_2 capture and that is why the CO_2 solutions hold the broadest portfolio of patents covering the use of carbonic anhydrase for CO_2 capture. CO_2 is naturally captured from the atmosphere through biological, chemical or physical processes. Biological approach is more useful and eco-friendly to capture and mitigate the CO_2. Various biological methods, including the peat bogs, reforestation, wetland restoration and ocean related techniques have been used. Recently, researchers have developed a new biosequestration method using a biocatalyst for the hydration process of CO_2[3]. The cost of carbon sequestration varies depending on the various steps of the process, including separation, compression, transportation and injection of CO_2. Biomimetic CO_2 sequestration does not require any CO_2 transport and produce an eco-friendly end product

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which is a quick, efficient and single step process ever known for CO_2 sequestration[4,5]. CO_2 solubilization, hydration and carbonate formation readily occurs in an aqueous phase. Hydration of CO_2 is a rapid reaction; however, it can be accelerated using microbial Carbonic Anhydrase (CA) (EC 4.2.1.1) for subsequent fixation into stable mineral carbonates[6]. Carbonate ion produced by CO_2 sequestration provides a for long-term storage of CO_2. Moreover, carbonates are thermodynamically stable, environmentally benign and weakly soluble in water, thus minimizing the concerns over the monitoring requirements and long-term fate of CO_2[5]. Second, the hydration of CO_2 to form carbonic acid is the rate-limiting step which has a forward reaction constant of 6.2×10^{-3} sec at 25°C[7] (it takes only 6.2×10^{-3} sec for a molecule to be converted). So CA catalyzes the hydration reaction at or near the diffusion-controlled limit that the formation of product is much faster than the dissemination of reactants[4]. Carbonic anhydrase producing bacteria were reported from various habitats, including sediments, deep seawater, mangroves soil, karst soil and alkaline soil[8-11]. *Nocardiopsis lucentensis* type strain DSM 44048 was the first time isolated from a salt marsh soil sample near Alicante, Spain in 1993[12]. It was also reported from the Indian subcontinent from different habitats including marine sponge *Dendrilla nigra* and Indian solar salterns[13,14]. However, this is the first report to isolate the bacterium from the seashore water from the India. Data from whole genome sequence indicated the presence of stress-induced protein and antibiotics producing genes suggest the isolate would be the best candidate for the on-site application like bioremediation[15]. CA is a zinc metalloenzyme, ubiquitous and well-studied in eukaryotic organisms. However, it has received scant attention in prokaryotes especially from the bacterial domain, although bacterial CA offers several advantages in CO_2 sequestration[16]. Total three structurally major classes of CA namely α, β and γ are reported in prokaryotes, among them β and γ are widely spread in bacteria while α is rarely found in bacteria. Many individual species contain more than one class of CAs, indicating that carbonic anhydrases have far more extensive and diverse roles in prokaryotes metabolism. Many actinomycetes contain putative carbonic anhydrase genes from more than one class and some even contain genes from all three known classes[17]. Few bacterial CAs from *Enterobacter*, *Aeromonas*, *Shigella*, *Klebsiella*, *Pseudomonas*, *Micrococcus*, *Neisseria* species have been explored for biosequestration of CO_2[18-20]. The biomimetic approach using bacterial CA is necessary for fixing large quantities of CO_2 into calcium carbonate ($CaCO_3$) *in vitro*. This method requires a robust CA which should be functional at an alkaline pH and high temperature with appreciable tolerance to cations, anions and other inhibitors. Moreover, the cost of this enzyme, its catalytic activity, its stability in time and its resistance to certain environmental pollutants are the basic parameters to use and device the enzyme for efficient CO_2 capture reactors[21]. Seawater provides a cheap and readily available source of calcium ions, however, to assess the effectiveness of CA for an on-site use, the effect of certain metal ions and anions present in seawater along with SO_4^{2-} and NO_3^- needs to be evaluated[6]. CA produced by extremophilic bacteria and actinomycetes are relatively more active and stable than CA produce by other bacterial species[22]. Therefore, in the present study, halophilic bacteria-*Nocardiopsis lucentensis* from sea water for the production of CA and its ability to form the mineral crust of carbonate have been explored. *In vitro* evaluation of the CA was performed to optimize various parameters for the maximum enzyme activity and stability to perform CO_2 sequestration.

MATERIALS AND METHODS

Isolation, screening and identification of bacteria: Seawater samples collected from the coastal area of South Gujarat (21°20'0" N, 72°38'0" E), India. For the isolation of the halophilic bacteria, the water sample was inoculated on nutrient agar supplemented with 4% w/v of sodium chloride at pH 7.4. Isolated bacteria were further screened for the production of bacterial carbonic anhydrase[23]. In the presence of esterase, organism develops a yellow color in 3 mM para-nitrophenyl acetate (p-NPA) enriched nutrient agar medium. The isolate was identified by 16S rRNA gene sequence homology.

Bioprospective properties of isolate: The sugar utilization pattern was examined by carbohydrate utilization kit (HiMedia Laboratories Pvt. Limited, India). Moreover, enzyme and antimicrobial activity was detected by agar plates method.

Mass multiplication and carbonic anhydrase activity: The mass multiplication of *N. lucentensis* was done in nutrient broth enriched with 4% w/v NaCl (pH 7.4) at 37°C in shaking condition (120 rpm). Cell mass was collected after 48 h by centrifugation at 10,000 rpm for 10 min at 4°C. The cells were lysed using 20 mM of Glycin-NaOH buffer (pH 9.0) followed by centrifugation at 5,000 rpm at 4°C for 10 min to

obtain the crude extract of CA and stored at -20°C until further analysis. Total protein concentration was determined by the method described by Lowary[24] and the CA activity assay was conducted as described by Adler *et al*.[25]. The Enzyme Activity (EA) was calculated according to following the Eq. 1.

$$EA = \frac{\Delta_T}{X} \quad (1)$$

where, $\Delta_T = (t_T)^{-1}-(t_S)^{-1}$, t_T and t_S, are the time in seconds required for a change in optical density in the presence (t_T) and absence (t_S) of X mg of cell extract; X is mg of protein content of the cell extract.

Growth pattern in the presence of CO_2, $ZnSO_4$ and urea: The pure culture of *N. lucentensis* was inoculated in nutrient broth and incubated at 0.035, 2.5 and 5% CO_2 concentration. After 48 h of incubation, Optical Density (OD) of culture was measured at 660 nm to ensure an increase in cell density. The effects of $ZnSO_4$ and urea on the growth was tested by supplementing nutrient broth with 1 mM of $ZnSO_4$, 0.1% w/v urea and 1 mM of $ZnSO_4$ +0.1% w/v urea and incubated for 48 h. The cell density was measured at 660 nm spectrophotometrically. All the experiments were performed in triplicate.

Calcite encrust formation by colonies: *N. lucentensis* culture was streaked on nutrient agar plates containing 0.75% $CaCl_2$ as per earlier report[26]. Plates were incubated at 37°C for 48 h and after incubation colonies were observed under stereomicroscope. The calcium carbonate precipitates were visualized as a mineral crust in the peripheral part of the colonies.

pH and temperature stability of enzyme: pH and temperature stability was carried out as described Demir *et al*.[27]. pH stability was tested by measuring enzyme activity after 24 h incubation in 20 mM of phosphate buffer (pH 6.5 and 7.0), 20 mM of Tris-HCl buffer (pH 7.5, 8.0 and 8.5) and 20 mM of glycine-NaOH buffer (pH 9.0). The comparative activity at different pH was calculated by referring enzyme activity at 0 min as 100%. Thermostability of the enzyme was measured by preincubating crude enzyme for 30 min at 20, 25, 30, 35, 40 and 45°C. Residual EA was determined by Adler assay as described above.

Effect of $ZnSO_4$ and urea on enzyme activity: CA is a zinc metalloenzyme; therefore, its activity is directly affected by $ZnSO_4$ and urea. The effects of $ZnSO_4$ and urea were determined by performing experiments in two different systems. The first system contained 3.2 mL of aqueous crude enzyme with 1 mM concentration of $ZnSO_4$ and in second 0.1% w/v urea was added and incubated for 30 min at 37°C and 7.0 pH. After incubation, the residual EA was calculated.

Effect of cations and inhibitor on enzyme activity: For the impact of cations, the enzyme was incubated for 30 min with monovalent (NaCl and KCl), divalent ($MgCl_2$, $ZnCl_2$ and $CaCl_2$) and trivalent ions ($FeCl_3$). Three different concentrations, 50, 100 and 200 mM, of each cation, were used to analyze the effect on CA activity. The residual EA was measured as described in section 2.2 using Adler *et al*.[25] method. The effect of inhibition on the crude enzyme was studied by using sulfanilamide (0.1, 0.5, 1 and 2 mM) as described by Alber and Ferry[28]. The residual EA was determined as described in an earlier section.

Storage stability of enzymes: Stability of the crude preparation of the enzyme was tested at 4°C and 37°C up to three day's incubation. After every 24 h, the residual EA was measured.

Formation of calcite particles: Calcite synthesis was studied by mixing the 1 mL of enzyme (10 Units) with 1 mL of marine water and 1 mL of CO_2 saturated water in a sterile tube and incubated overnight at 37°C. The calcite precipitates were separated by the centrifugation at 10,000 rpm for 10 min and dried at 37°C for 24 h. Similarly, the calcite synthesis was performed without the addition of enzymes and dry precipitates of calcite were collected. Dry powder of calcite was mounted on stubs and examined under scanning electron microscope. For elemental confirmation calcite particles were observed at 3000X magnification with Energy Dispersive X-Ray Spectroscopy (EDX).

RESULTS

Isolation, screening and identification of bacteria: Total 17 isolates were screened for the carbonic anhydrase activity based on the development of yellow color around the colonies from the seawater sample. Yellow color development is due to the hydrolysis reaction of para-nitrophenylacetate (p-NPA) to para-nitrophenol (p-NP) due to esterase activity indicated by isolates (Fig. 1). Further, testing for activity of carbonic anhydrase was carried out by using an intracellular crude extract of cells using standard assay[25].

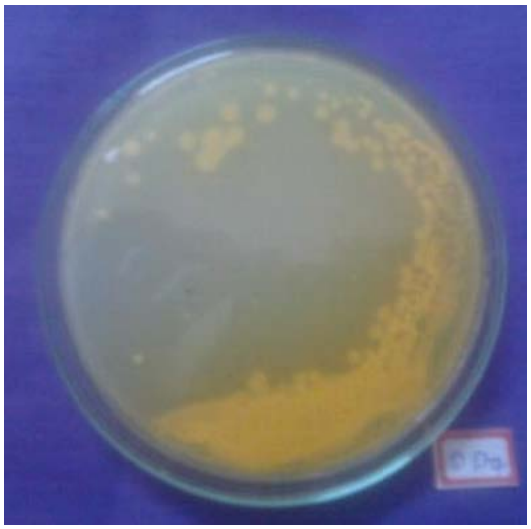

Fig. 1: Plate assay of 48 h old culture showing CA activity with yellow color product p-NP formed around the colonies

Fig. 2: Zone of inhibition surrounding paper disc indicating the antibacterial activity of cell-free medium against lawn growth of *Bacillus megaterium* on nutrient agar plat after 24 h incubation along with the control

Based on the highest enzyme activity, isolate DD7 was screened and identified as *Nocardiopsis lucentensis* using 16S rRNA gene sequence and similarity search in GenBank. The nucleotide sequence of the 16S rDNA is submitted with accession number KJ579952.

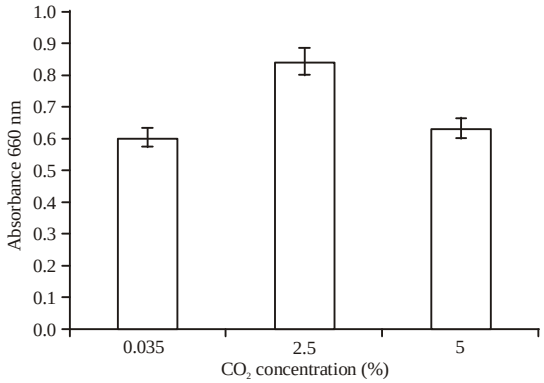

Fig. 3: Growth of isolate in presence of different concentration of CO_2

Bioprospective properties of isolate: Isolate was found to ferment four sugars, including refinance, in-line, sorbitol, mannitol and reported to produce protease, chitinase and cellulase whereas amylase, xylanase and pectinase was not secreted by isolate. Growth inhibition (10 mm) of Gram-positive *Bacillus megaterium* surrounding paper disc, pregnant with 10 μL of cell free extract indicate the production of the antibacterial compound (Fig. 2).

Mass multiplication and carbonic anhydrase activity: *Nocardiopsis lucentensis* have showed maximum growth on nutrient agar at neutral pH at 37°C. After prolonged incubation in liquid culture, isolate produced light pinkish color pigment. *Nocardiopsis lucentensis* is a slow grower and a moderate halophilic actinomycete. The enzyme activity of crude extract was found 12.6 (U mg^{-1} protein).

Growth pattern in the presence of CO_2, $ZnSO_4$ and urea: *Nocardiopsis lucentensis* grows best at 2.5% CO_2 saturation condition than 0.035 and 5% CO_2 (Fig. 3). As demonstrated in the Fig. 3 the growth of isolate was increased in the presence of urea while almost unaffected by the presence of $ZnSO_4$ (Zinc Sulfate). However, the growth remains same in the presence of both urea and $ZnSO_4$ (Fig. 4).

Calcite encrust formation by colonies: The isolated colony on the $CaCl_2$ supplemented nutrient agar plate was observed under stereomicroscope. Large cubic particles were found to aggregate in the peripheral of colonies indicating the calcium carbonate crust (Fig. 5).

Effect of pH and temperature on enzyme stability: The effect of pH on the stability of crude CA was examined in three different buffer systems, including

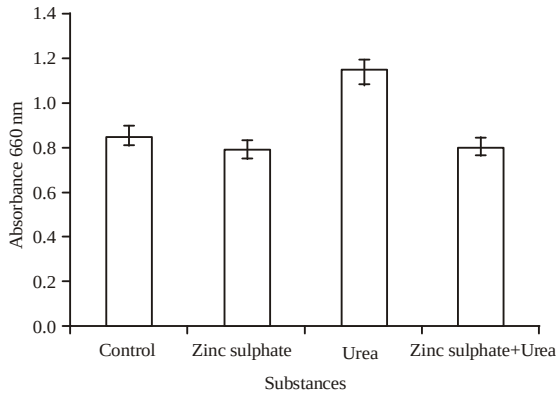

Fig. 4: Effects of zinc sulfate and urea on growth of isolate. Growth was measured as a cell mass turbidity using spectrophotometer

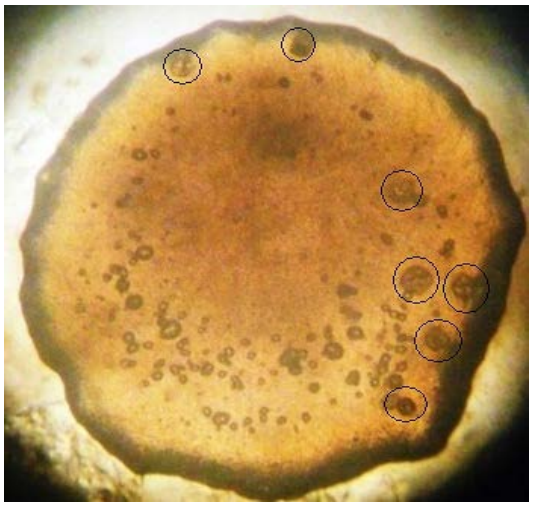

Fig. 5: Mineral crust of calcium carbonate formed is cubic shaped present in peripheral of bacterial colony. A 10X image of 48 h old growth on nutrient agar plates containing $CaCl_2$ captured using stereomicroscope

20 mM of phosphate buffer (pH 6.5 and 7.0), 20 mM of Tris-HCl buffer (pH 7.5, 8.0 and 8.5) and 20 mM of Glycine-NaOH buffer (pH 9.0). The enzyme showed maximum activity at pH 7.0 while at higher pH enzyme decreases its activity to 72 and 50% at pH 7.5 and 8.0, respectively. Similarly, at lower pH Enzyme Activity (EA) was a decline than neutral pH (Fig. 6). Thermostability of the enzyme was measured at various temperatures ranging from 20-45°C. There was a progressive decline in the stability of CA following an increase above 25°C. The enzyme showed maximum activity at 25°C while 93, 87 and 77% activity were

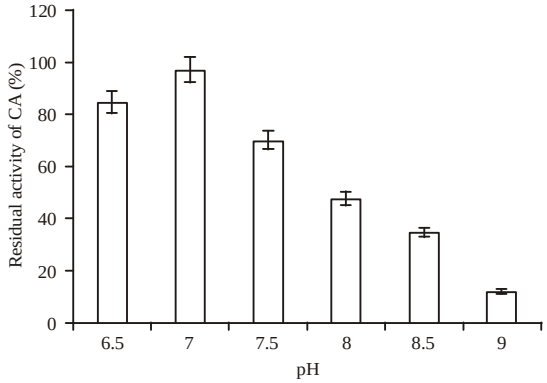

Fig. 6: Effect of pH on stability of crude enzyme. The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 24 h at 37°C temperature. The enzyme activity was measured according to the standard enzyme assay (100% CA activity was equivalent to 12.60 U mg^{-1} protein at pH 7.0)

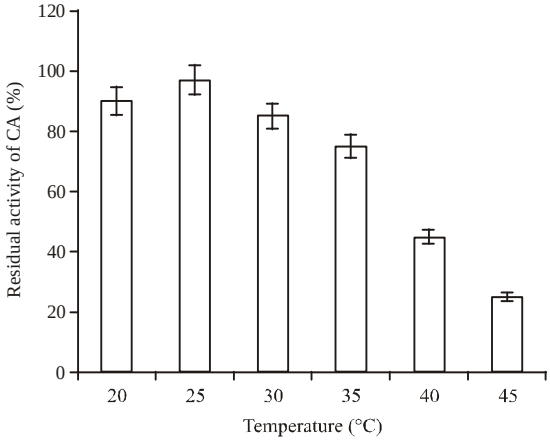

Fig. 7: Effect of temperature on the stability of crude enzyme. The thermostability of the enzyme was determined by incubating the reaction mixture containing the enzyme at different temperatures ranging from 20-45°C after 30 min. The enzyme activity was measured according to the standard enzyme assay (100% CA activity was equivalent to 9.70 U mg^{-1} protein at 30°C temperature)

reported at 20, 30 and 35°C, respectively. There was a drastic decline in the EA at 40 and 45°C that is 46 and 25%, respectively (Fig. 7).

Effect of $ZnSO_4$ and/or urea on enzyme activity: CA activity was stimulated by the addition of 1 mM of $ZnSO_4$ but 0.1% urea suppressed the enzyme activity (Fig. 8).

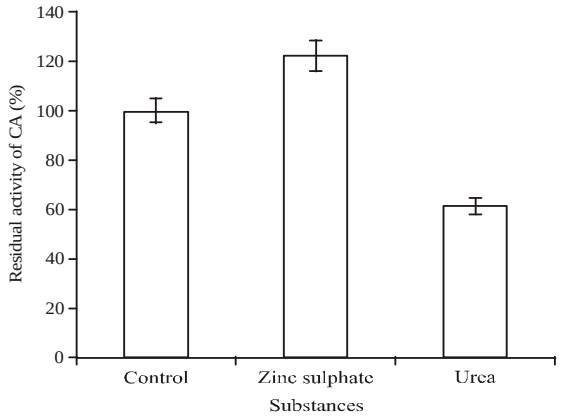

Fig. 8: Effect of zinc sulfate and urea on enzyme activity (100% CA activity of control was equivalent to 10.70 U mg^{-1} protein)

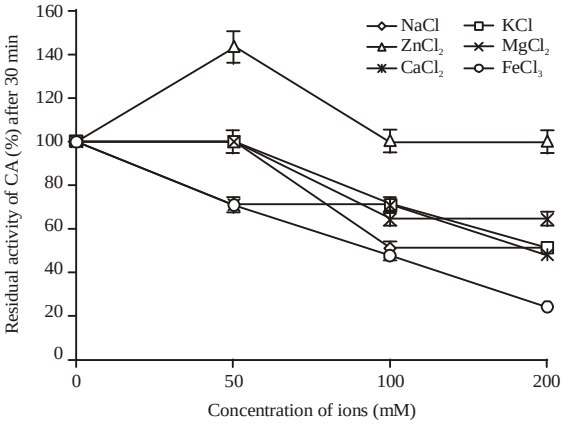

Fig. 9: Effect of metal ions on enzyme activity. Enzyme activity measured in the absence of metals ions was taken as 100% (12.76 U mg^{-1} protein). The remaining CA activity was measured after pre-incubation of enzyme with each concentration of each ion after 30 min at room temperature

Effect of cations and inhibitor on enzyme stability: Three concentrations (i.e., 50, 100 and 200 mM) of each cation were used to test the CA activity after 30 min incubation (Fig. 9). All the cations are essential for the optimum activity of the enzyme. Zn^{+2} was found to stimulate the EA. Except Ca^{+2} and Fe^{+3}, all were reported to support the EA. However, 200 mM concentration of all the cations except Zn^{+2} suppressed the activity due to the inhibition of the enzyme. Activity in the 200 mM of $ZnCl_2$ indicates the requirement of zinc for the catalysis.

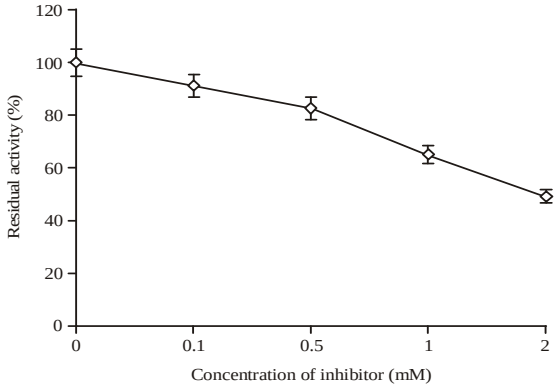

Fig. 10: Effect of inhibitor on enzyme activity. Activity of enzymes in the absence of inhibitor was calculated as 100% (12.76 U mg^{-1} protein)

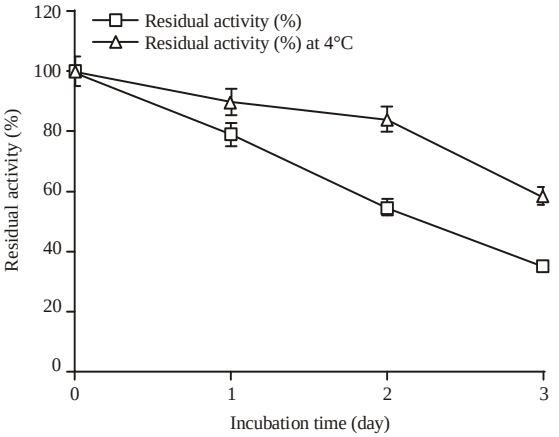

Fig. 11: Storage stability of the enzyme. Activity of 0 day was calculated as 100% (12.76 U mg^{-1} protein) and subsequent day's activity was compared with 0 day

Four different concentrations of sulphanilamide (0.1, 0.5, 1 and 2 mM) were tested and results indicated good stability at each tested concentration of inhibitor. Furthermore, inhibition in the presence of 0.1 mM concentration of inhibitor was negligible (Fig. 10). The result is indicative of greater resistance to the inhibitor that makes this enzyme more suitable for field applications.

Storage stability of enzymes: The crude enzyme was kept at two different temperatures 4 and 37°C. Residual EA was measured at every 24 h up to 3 days. Prolong incubation at 4 and 37°C cause the loss of activity. The enzyme was more stable in cold condition and has nearly 84% activity after two days when stored at 4°C (Fig. 11).

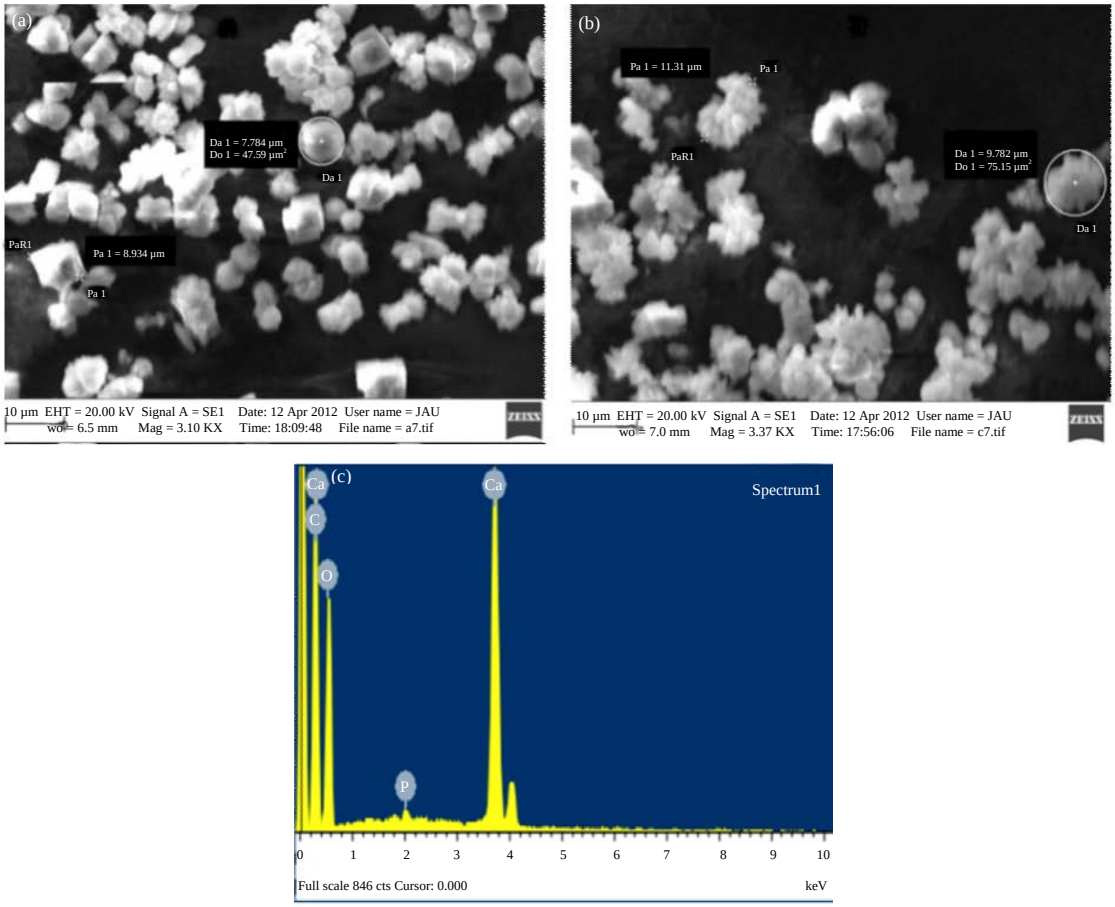

Fig. 12(a-c): EM and EDX analysis of calcite (a) SEM image of cube shaped heterogeneous particle formation without enzyme, (b) SEM image of flower shaped homogeneous particle formation using enzyme and (c) EDX profile of calcite powder indicating the very high pick at 3.7 keV with satellite peak at 4.0 keV

Analysis of calcite particles by SEM and EDX: There were obvious differences in the size and morphology of $CaCO_3$ crystals formed after enzymatic process. The $CaCO_3$ crystals formed by enzyme were rough-surfaced and flower shaped, whereas, smooth-surface cubic crystals of $CaCO_3$ displayed in the control reaction (Fig. 12a, b). EDX analysis showed that majority of elements of crystals were Ca, C and O which strongly support the SEM analysis and also confirms the formation of polymorph of calcium carbonate ($CaCO_3$) formation containing (Fig. 12c).

DISCUSSION

Carbonic anhydrase is a widespread enzyme in prokaryotes but significant research has not been conducted using marine actinomycetes for sequestration of CO_2. *Nocardiopsis lucentensis* is a capnophile that grow best at high CO_2 concentration. Very few capnophiles are evaluated for sequestration of CO_2 using carbonic anhydrase[29]. Bacterial growth at high CO_2 concentration in alkaline condition and elevated temperature is a remarkable property. These properties make it a potent strain to form encrust in harsh condition. Non-ribosomal peptide called Lucentamycins is well reported from the *Nocardiopsis lucentensis* strains[30]. The result also indicated the production of antibacterial compounds which gives it an advantage to survive the competition of the indigenous bacterial population. This bacterium grows luxuriously in the presence of urea by hydrolysis using urease. The urea hydrolysis reaction increases alkalinity of the environment, leading to $CaCO_3$ precipitation[31]. Due to the stability in alkaline conditions, the enzyme is compatible with the carbon mineralization reaction, including the reaction of $CaCl_2 + CO_2 + 2NaOH \rightarrow CaCO_3 + 2\,NaCl + H_2O$. CA was reported to resist in 2 mM concentration of

inhibitor that make this enzyme more suitable for on-site application in biosequestration.

In vitro stability of enzymes and proteins remains a critical issue in biotechnology. Both storage and operational stabilities affect the usefulness of enzyme-based reactions. Storage stability or self-life of an enzyme refers to retaining catalytic abilities in the period between production and eventual use. Operational stability describes the persistence of EA during a process, i.e., under usage conditions[32]. Greater storage stability of the crude enzyme indicate its long self-life which is beneficial for application. At large scale applications, the use of crude enzyme is preferred over purified preparation to avoid the cost of purification and makes the processes economically more viable[33].

Zinc is a well-known activator of CA as it is a zinc metalloenzyme and a similar result was reported in the present investigation. Apart from zinc metal ions Na^+, K^+ and Mg^{+2} were found to enhance enzyme activity. These ions are abundantly present in the seawater. Chloride ion exists as the amplest ionic entity in sea water and thus believed to have a profound impact on biomimetic CO_2 sequestration[3,6]. Activity in the presence of $MgCl_2$ indicates that the enzyme is suitable for the reaction of carbon mineralization like $Mg_2SiO_4 + 2CO_2 \rightarrow 2MgCO_3 + SiO_2$.

In the bacterial culture medium, Ca^{+2} is not utilized by bacterial metabolic processes, it just accumulates outside the cell[34]. As a result of enzymatic reversible hydration of CO_2, HCO^{-3} produces which transform into CO_2^{-3} or HCO^{-3} around the cell, commencing the growth of $CaCO_3$ crystals around the cell. Compared to the experimental system without the enzyme, the precipitation rate of Ca^{2+} was significantly faster in the presence of the enzyme. Moreover, SEM analysis indicated that there were bacterial inputs in the synthesis of calcite polymorph crystals in the experimental system and it was confirmed by EDX analysis. Thus, the enzyme might serve as nucleation sites for calcite precipitation. Bacterial CA produced microscopic flower shaped calcite particles that are totally concurred with the finding of Li *et al*.[10]. At industrial scale, usage of CA is mainly for carbon sequestration and calcite production; both are evaluated in the study very successfully. There are wider implications for natural carbonate precipitation, since bacteria are ubiquitous in nature and CA is widespread in prokaryotes and certain eukaryotes[35]. Thus, the contribution of bacterial CA to the carbon cycle needs to be examined while studying the role of microorganisms in the carbon cycle.

CONCLUSION

To provide perspective on biomimetic CO_2 sequestration using CA from marine actinomycetes is the first study to our knowledge. The study proves the importance of various physicochemical factors for the growth of bacteria and the central role they play in the successful encrust formation. The study also demonstrates the robust nature of microbial CA and compatibility for the carbon mineralization. It has been known that microorganisms play a significant role in promoting calcite precipitation. The positive influence of CO_2, zinc and urea were evaluated for bacterial growth and enzyme production. Furthermore, calcite precipitation by bacterial CA leads to the formation of calcite polymorphs that can be used as a supplementary material for the construction of the building.

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