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Bio Remediation of CO₂ and Characterization of Carbonic Anhydrase from Mangrove Bacteria

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ABSTRACT

Increasing Carbon Dioxide (CO₂) in the atmosphere has aggravated the threats of global warming and global diversity loss. To decrease CO₂ emission, various physical and chemical based strategies are used to store and sequester CO₂. The biomimetic approach is one of the available harmless, robust and eco-friendly approach but still less evaluated. The present study reports the role of microbial intracellular carbonic anhydrase in the formation of calcite and sequestration of CO₂ by bacteria. Bacterial carbonic anhydrase was screened from *Bacillus altitudinis*, isolated from the mangrove forest of the western coastline of India. The isolate was able to grow optimally at 2.5 % CO₂ saturated environment. The enzyme was found to catalyze the reaction in a wide range of pH and temperature with an optimum at 7.5 pH and 30°C temperature. The enzyme was reported to accelerate the hydration of CO₂ in 50 mM ZnCl₂. Furthermore the enzyme remained active and shown the remarkable activity with sulphanilamide inhibitor. *in vitro* calcite encrust formation activity was tested using growth media supplemented with CaCl₂. Product of sequestration, Calcite, can be used as raw material for building construction.

Key words: Calcite, carbonic anhydrase (CA), biosequestration, carbon sequestration, mangroves

INTRODUCTION

In 20th century anthropogenic activity, industrialization and increasing the population lead to intensification in CO₂ level and leads to the Green House Effect (Yamasaki, 2003). There will be more energy demand in the future and leads to more CO₂ emission. At current stage over 85% of world energy demand is supplied by fossil fuels and are responsible for roughly 40% of total CO₂ emissions (Carapellucci and Milazzo, 2003). According to International Panel on Climate Change (IPCC), by the year 2100, the atmosphere may contain up to 570 ppmv CO₂, causing a rise of mean global temperature of around 1.9°C and an increase in mean sea level of 3.8 m (Stewart and Hessami, 2005). This leads to dramatic changes in the environment and in biodiversity loss through species extinction. To conquer these circumstances either we have to reduce the emission of CO₂ or stabilize CO₂ through carbon sequestration. For later option various physical and chemical methods are used to sequester CO₂ but there is push for the development of technologies to capture and sequester more CO₂ and make it possible to use the product of sequestration as raw material for other industries to make a viable and feasible approach.

Nowadays, policy makers are looking for the development of a novel biomimetic approach for CO₂ sequestration based on the use of an enzyme or biological catalyst. The resulting sequestration system would offer several potential advantages, including a plant-by-plant solution to emission

reduction; no costly CO₂ concentration and transportation steps, safe and stable, environmentally benign product and an environmentally benign process (Liu *et al.*, 2005). Carbonate minerals, such as calcite, aragonite, dolomite and dolomitic limestone, comprise a massive CO₂ reservoir, estimated to contain an amount of carbon equivalent to 150,000*10¹² metric tons of CO₂ (Wright and Colling, 1995). This carbonate minerals offers a geologically proven, safe, long-term repository for CO₂.

Carbonate ion availability play strategic role in carbonation reaction. The hydration of CO₂ to form carbonic acid is the rate-limiting step in the conversion of CO₂ into carbonate in nature but with the help of CA the process will be accelerated. Carbonic Anhydrase (CA) is the fastest enzyme that catalysis CO₂ hydration with typical reaction rate between 10⁴ and 10⁶ reactions sec⁻¹ (Heck *et al.*, 1994). Seawater provides a cheap and readily available source of calcium ions and has been considered as a potent supply source for the formation of CaCO₃ and other mineral carbonate (Bond *et al.*, 2001). However, to assess the effectiveness of carbonic anhydrase for an on-site use, the effect of certain, pH, temperature, metal ions and different anions present in seawater needs to be evaluated. CAs is a zinc metalloenzyme reported to be present in animal, plant and microorganism (Karlsson *et al.*, 1998). However, robust CAs has been reported only from a limited number of microorganisms including bacteria and archea.

Biomimetic CO₂ sequestration by CAs is promising approach but still under research and has to be validated before commercial use. The aim of the present research was to investigate *Bacillus altitudinis* for the production of CA and its ability to form the mineral crust *in vitro*. Evaluation of the CA was performed to find out the optimum condition for highest activity and stability of enzyme to carry out CO₂ sequestration.

MATERIALS AND METHODS

Isolation, identification and carbonic anhydrase (CA) activity: *Bacillus altitudinis* isolated from the mangroves of south Gujarat coastline of India and identified by 16S rRNA gene sequencing followed by BLAST at NCBI. *B. altitudinis* was grown on Nutrient Agar (NA) (HiMedia) containing 4% w/v of NaCl (pH 7.4). Potential isolates were screened using para nitrophenyl acetate (pNPA) plate assay for esterase activity (Ramanan *et al.*, 2009). To determine Carbonic Anhydrous (CA) activity, pure culture of *B. altitudinis* was inoculated in nutrient broth containing 4% NaCl and incubated at 37°C under rotatory condition of 120 rpm. Cells were harvested after 48 h and cell mass were collected by centrifugation at 10, 000 rpm for 10 min at 4°C. The cells were lysed using a 20 mM Glycin-NaOH buffer (pH 9.0) followed by centrifugation at 5000 rpm at 4°C for 10 min to obtain the crude extract of CA. Total Protein concentration was determined by the method of Folin-Lowary Classics Lowry *et al.* (1951) and CA activity was estimated as described by Adler *et al.* (1972). Enzyme Activity (EA) (units/mg of protein) was estimated as a specific activity according to following Eq:

$$EA = \frac{(t_r)^{-1} - (t_s)^{-1}}{X} \quad (1)$$

where, t_r and t_s are the times in seconds required for a change in optical density in the presence (t_r) and absence (t_s) of X mg of cell extract. Where, X is mg of protein content of the cell extract.

Effect of CO₂, urea and ZnSO₄ on growth and CA activity: To study capnophilic nature, pure culture of *B. altitudinis* was inoculated in nutrient broth and incubated at three different CO₂ concentration, 0.035, 2.5 and 5%. Similarly, the effects of ZnSO₄ and urea on the growth and CA activity were tested by supplementing nutrient broth with 1 mM ZnSO₄, 0.1%

w/v urea, ZnSO_4 +0.1% w/v urea along with control in four separate flasks. The cell density was measured at 660 nm spectrophotometrically after 48 h to find out growth profile. Also the cells were lysed and CA activity was checked to investigate effect of urea and ZnSO_4 on enzyme production.

Calcite encrust formation by isolate: To investigate the calcite formation, *B. altitudinis* culture was streaked on nutrient agar plates containing 0.75% CaCl_2 as described by Stocks-Fischer *et al.* (1999). Plates were incubated at 37°C for 48 h. After incubation colonies were observed under stereomicroscope to check calcium carbonate precipitates.

Effect of pH, temperature and cations on CA activity: It is necessary to evaluate performance of CA in the presence of salinity (pH), temperature and cations to ensure its feasibility for on site application. The stability of the enzyme was monitored by Demir *et al.* (2001). The stability of pH was investigated at pH 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 using crude enzyme at 37°C. Thermostability of the enzyme was measured by pre-incubating crude enzyme extract for 30 min at 20, 25, 30, 35, 40 and 45°C in buffer (pH 7.5). Enzyme activity was determined under standard assay conditions as described by Adler *et al.* (1972).

The effect of various cations was analyzed using Ramanan *et al.* (2009) method with certain modification. Crude enzyme was incubated for 30 min with monovalent (NaCl and KCl), divalent (MgCl_2 , ZnCl_2 and CaCl_2) and trivalent ions (FeCl_3) at three different concentrations, 50, 100 and 200 mM and its residual activity was measured with control experiment (without ion) using standard assay methods as described by Adler *et al.* (1972).

Effect of inhibitor: Four different concentrations of sulfanilamide was used to study the effect on CA. Crude enzyme was incubated in 0.1, 0.5, 1 and 2 mM at the 37°C for 30 min (Alber and Ferry, 1994). The residual enzyme activity was determined under standard assay conditions and compared with control (without inhibitor) to find out IC_{50} value.

RESULTS

Isolated thirty marine bacteria were screened for CA activity using plate assay method. Potent isolate NAL-2 was selected based on development of yellow colored product (p-NP) formation around the colonies as shown in Fig. 1. Isolate was tested for maximum CA activity by Adler *et al.* (1972) method using crude extract. Isolate was identified as *Bacillus altitudinis* by decoding the 16s rRNA gene sequence followed by similarity search in GenBank.



Fig. 1: Plate assay of culture exhibiting CA activity with yellow product of p-NPA formed around the colonies

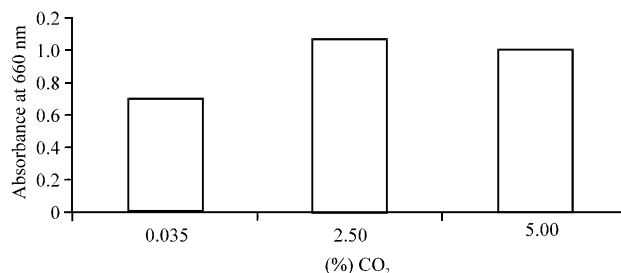


Fig. 2: Growth kinetics in presence of different concentration of CO₂ in incubator

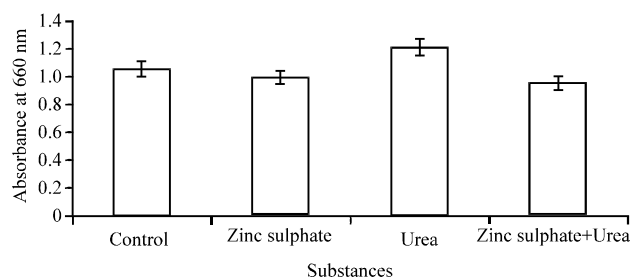


Fig. 3: Effects of zinc sulphate and urea on growth, where, ZnSO₄ = 1 mM and urea = 0.1%

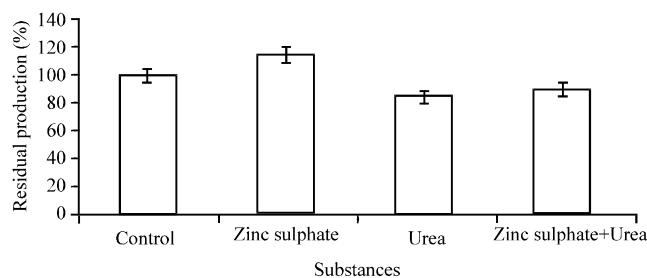


Fig. 4: Effect of zinc sulphate and urea on enzyme production, where, ZnSO₄ = 1 mM and urea = 0.1%

The isolate grew best at 2.5% CO₂ saturation condition suggest the capnophilic nature of bacteria (Fig. 2). Growth of isolates was increased in the presence of urea, while almost unaffected in the presence of ZnSO₄ but growth was remains same in the presence of both urea and ZnSO₄ (Fig. 3). CA production was stimulated by the addition of 1 mM ZnSO₄ but 0.1% urea was found to suppress the activity. However, the combine effects of 1 mM ZnSO₄ with 0.1% urea do not significantly affect the enzyme activity (Fig. 4). Large cubic particles were found to aggregate in colony indicating the calcium carbonate crust, when isolate grown on solid media supplemented with CaCl₂ (Fig. 5). The effect of pH on the stability of crude CA was examined in two different buffer systems, 20 mM Tris-HCl buffer (pH 6.5-8.5) and glycine-NaOH buffer (pH 9.0). The enzyme was found to retain maximum activity at pH 7.5 while more than 72% enzyme activity was retained at pH 8.0 and half of the enzyme activity was found at pH 8.5 after 24 h incubation at 37°C. In more alkaline pH, drastic decrease in the enzyme activity was reported (Fig. 6).

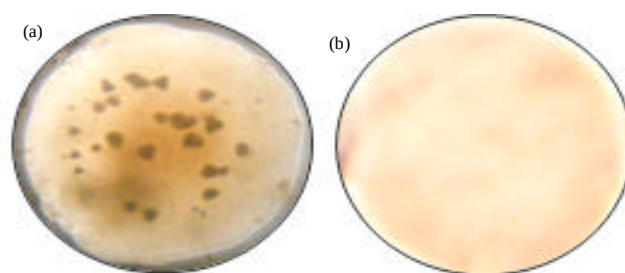


Fig. 5 (a-b): (a) Colony showing mineral crust of calcium carbonate when CaCl_2 supplement in media and (b) In absences of CaCl_2 in media. A 10X image of 48 h old colony captured using stereomicroscope

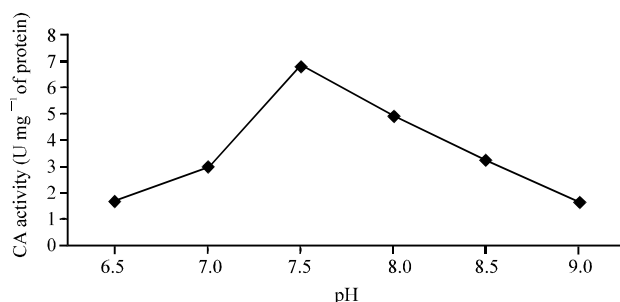


Fig. 6: Effect of pH on enzymes activity

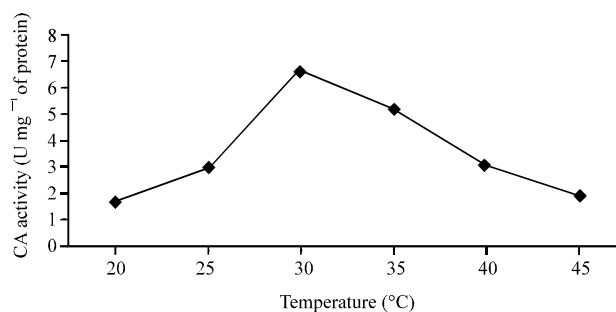


Fig. 7: Effect of temperature on enzyme activity

Thermostability of the enzyme was measured at various temperatures ranging from 20-45°C for 30 min in 20 mM Tris-HCl buffer (pH 7.5). There was a progressive decline in the stability of CA following an increase in temperature ranging from 30-45°C. The enzyme showed 100% activity at 30°C while 80% activity at 35°C. While there was a drastic decline in the residual activity at 40 and 45°C (Fig. 7).

Specific concentration of each cations was used to test the CA activity after 30 min incubation (Fig. 8). All the cations are essential for the optimum activity of the enzyme. Zn^{+2} was found to stimulate the enzyme activity. Similar results found for Na^{+2} and Ca^{+2} at lower concentration. However, 200 mM concentration of all the cations expects Zn^{+2} suppressed the activity due to the inhibition of the enzyme. Activity in the 200 mM ZnCl_2 indicates the requirement of zinc for the catalysis.

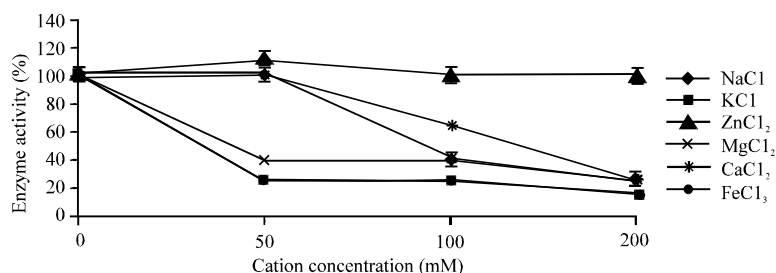


Fig. 8: Effect of metal ion on enzyme activity

Table 1: Effect of sulphanilamide (inhibitor) on enzyme activity

Concentration of sulphanilamide (mM)	Residual activity (%)	IC ₅₀ value
0	100	2.0 mM
0.1	93.7	
0.5	85.75	
1	67.1	
2	52.3	

Four different concentrations of inhibitor, sulphanilamide (0.1, 0.5, 1 and 2 mM), were tested and result indicated good stability at each tested concentration of inhibitor. In the presence of 0.1 mM concentration of inhibitor, negligible inhibition was found even IC₅₀ value was also found 2 mM (Table 1). The result is indicative of greater resistance against the inhibitors so enzyme from isolates is suitable at field scale also.

DISCUSSION

Carbonic anhydrase is a widespread enzyme in prokaryotes (Smith *et al.*, 1999). Still there is no much data available on capability of marine capnophiles for CO₂ sequestration (Nafi *et al.*, 1990). p-NPA plat assay is a very specific method and can be devised for the high throughput screening of CA. Bacterial growth at high CO₂ concentration in alkaline condition at elevated temperature is a remarkable property making it potent strain fit for the encrust formation in harsh condition. Mineral crust formation by colony is much related to the earlier reports (Dick *et al.*, 2006). Due to urease activity of isolate, it is able to grow luxuriantly in the presence of urea and thus increase alkalinity, leading to calcite precipitation (Chen *et al.*, 2008). Stability of enzymes in a wide range of pH and temperature was significant and concurred with earlier reports (Sharma and Bhattacharya, 2010; Sharma *et al.*, 2008). Due to the stability in alkaline and saline condition, enzymes are compatible in the carbon mineralization reaction including the reaction of $\text{CaCl}_2 + \text{CO}_2 + 2\text{NaOH} \rightarrow \text{CaCO}_3 + 2\text{NaCl} + \text{H}_2\text{O}$. Stability of enzyme at higher concentration of suphanilamide suggest impending on site application for CO₂ sequestration. The stability of enzymes and proteins *in vitro* at various pH, temperature, inhibitor and cations concentration remains a critical issue in biotechnology.

Being a metalloenzyme group, CA activity is increased in the presence of Zinc. Apart from zinc metal ions Ca⁺² were found to enhance enzyme activity. This result is very similar to the earlier report (Tripp *et al.*, 2004). These ions constitute the bulk of the seawater (Bond *et al.*, 2001) and is thus believed to have a profound impact onbiomimetic CO₂ sequestration. Activity in the presence of MgCl₂ indicates that the enzyme is suitable for the reaction of carbon mineralization like $\text{Mg}_2\text{SiO}_4 + 2\text{CO}_2 \rightarrow 2\text{MgCO}_3 + \text{SiO}_2$.

In the bacterial culture medium, Ca^{+2} is not likely utilized by bacterial metabolic processes; it just accumulates outside the cell (Silver *et al.*, 1975). As a result of enzymatic reversible hydration of CO_2 produces the HCO_3^- which is transformed into CO_3^{+2} or HCO_3^- around the cell, commencing the growth of CaCO_3 crystals around the cell. There are wider implications for natural carbonate precipitation, since bacteria are ubiquitous in nature and CA is widespread in prokaryotes and certain eukaryotes (Smith *et al.*, 1999). 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

This is the first study to provide perspective on biomimetic CO_2 sequestration using CA from mangroves isolated microorganism. 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 compatible for the carbon mineralization. It has been known that microorganisms play an important role in promoting calcite precipitation. Positive Influence of CO_2 , Zinc and urea were evaluated for enzyme production and cell growth. Study of bacterial CA is one of the important biomimetic approaches for CO_2 sequestration. Calcite precipitation by bacterial CA leads to the formation of calcite particles that can be used as a supplementary material in the construction of the building.

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