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Short Communication

# Induction of calcite precipitation through heightened production of extracellular carbonic anhydrase by $CO_2$ sequestering bacteria

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ABSTRACT

The thermo-alkalotolerant bacterium exhibiting heightened extracellular carbonic anhydrase (CA) activity, survived at 100 mM sodium bicarbonateand 5% gaseous $CO_2$ was identified as *Bacillus* sp. by 16S rRNA sequencing. Extracellular carbonic anhydrase was purified by ammonium sulfate precipitation, gel filtration chromatography and affinity chromatography with a yield of 46.61% and specific activity of 481.66 U/mg. The size of purified carbonic anhydrase was approximately 28 kDa in SDS-PAGE gel filtration and further their role in calcium carbonate production was correlated. The purified enzyme was stable with half-life of 25.36 min at 90 °C and pH 8. $K_M$ and Vmax values of the enzyme were 1.77 mg/mL and 385.69 U/mg respectively. The production of calcite was confirmed by Scanning Electron Microscopy (SEM) analysis, FTIR, and Energy-Dispersive X-ray (EDX) analysis. Carbonic anhydrase and calcite deposition coupled with $CO_2$ fixingbacteria is a significant approach for $CO_2$ sequestration.

## 1. Introduction

The concentration of $CO_2$ in the atmosphere is around 400 parts per million (ppm) and is rising by 2 ppm per year (NOAA, 2017). In the present scenario, carbon capture and storage (CCS) through microbes is an approach to mitigate this problem. Some of the bacteria play very important role in sequestering higher level of $CO_2$ into value added products like calcium carbonate, biofuels and biosurfactants through development of carbon concentrating mechanism (Sundaram and Thakur, 2015b). Carbonic anhydrase (CA) is a major enzyme which plays an important role in carbon concentrating mechanism (CCM) and sequestration of $CO_2$ into calcium carbonate (Kaplan and Reinhold, 1999). CA (EC 4.2.1.1) is a zinc-containing enzyme that catalyzes the reversible dehydration of $HCO_3^-$ to $CO_2$. Among the five distinct classes of CA (α, β, γ, δ, and ε), β-CA is present in cynobacteria and chemolithotrophs while *Bacillus* sp. consists α-CA esterase activity (Ramanan et al., 2009).

The activity of extracellular CA enhanced with increased $HCO_3^-$ and $CO_2$ concentration and has potential role in calcite precipitation by mineralization and nucleation process during the sequestration process (Zhang et al., 2011, Sharma et al., 2009). The use of CA for the conversion of $CO_2$ into bicarbonates increases the rate of the reaction manifolds with high substrate specificity (Bond et al., 2001). Some eukaryotic algae are able to precipitate intracellular calcium carbonate due to the activity of intracellular CAs but some cyanobacteria can deposit calcium only outside the cells, and such precipitation is strictly controlled by pH. It is likely that extracellular CAs of cyanobacteria cells might stabilize the pericellular pH and participate in cell mineralization (Kupriyanova et al., 2007).

Here, we report the identification and partial purification of an extracellular alkalotolerant CA from *Bacillus* sp. This study, describes the optimal culture conditions for increased production of alkaline CA, its characterization and MALDI-TOF spectral analysis. The application of partially purified extracellular CA has been studies for $CO_2$ sequestration and calcite precipitation, showing promising results to develop commercial eco-friendly product.

## 2. Materials and methods

### 2.1. Sampling site and microorganism

The soil sediment was collected for isolation of bacteria capable of carbon dioxide sequestration as reported earlier (Sundaram and Thakur, 2015a,b) and stored in refrigerator at 4 °C until used for further analysis (Thakur, 1995).

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**Table 1**
Partial purification of extracellular CA produced by *Bacillus* sp.

| Purification step | Volume | Total activity (U) | Total protein (mg) | Specific activity (U mg$^{-1}$) | Yield (%) | Fold purification |
|---|---|---|---|---|---|---|
| Crude Extract | 50 | 310 | 170 | 1.82 | 100 | 1 |
| $(NH_4)_2$ $SO_4$ (80%) precipitation | 30 | 280 | 95 | 2.94 | 90.32 | 1.615 |
| PEG dialysis | 20 | 210 | 45 | 4.66 | 67.74 | 2.56 |
| Sefadex G-100 Chromatography | 15 | 190 | 3.5 | 54.28 | 61.29 | 29.824 |
| Affinity Chromatography | 5 | 144.5 | 0.3 | 481.66 | 46.61 | 264.28 |

### 2.2. Bacteria and culture condition

The bacterial strain, *Bacillus* sp., used in this study, was taken from our previous study (Sundaram et al., 2012) and inoculated in minimal salt media (MSM) at 200 rpm; temperature at 37 °C. The growth media was optimized by adding glucose (1% w/v) as additional carbon source and 100 mM $NaHCO_3$ and $CaCl_2$ as CA inducer to enhance the production of $CaCO_3$. CA activity was determined after 48 h of incubation. Cell free culture supernatant was obtained after centrifugation at 10,000 × *g* for 10 min.

### 2.3. Enzyme assay

Carbonic anhydrase enzymatic activity was assayed by the Wilbur-Anderson method (Khalifah, 1971) with slight modification as reported by Srivastava et al. (2015).

### 2.4. Purification and characterization of extracellular enzyme

The extracellular protein purification was carried out by 80% $(NH_4)_2SO_4$, PEG (Poly ethylene glycerol) dialysis and Sephadex G-100 column chromatography at 4 °C. The fractions having CA activity were pooled and applied to Sepharose-4B-L tyrosine-Sulphanilamide affinity column chromatography equilibrated with 25 mMTris-Cl (pH 7.0). Protein bands excised from SDS-PAGE were further prepared for MALDI-TOF/MS analysis (Matrix assisted laser desorption ionization time-of-flight mass spectrometry) as earlier discussed by Mishra and Thakur (2011).

The kinetic constants of the purified carbonic anhydrase were determined using *p*-NPA (*para*-nitrophenylacetate) as substrate under optimal assay conditions through Michaelis-Menten equation and the apparent $K_M$ and Vmax calculated. The effect of pH (4–10), temperature (50 °C–90 °C) and half life of the enzyme were also carried out as discussed earlier (Mishra and Thakur, 2011). The inhibitory effect of various chemicals (cyanate, trithiocarbonate, acetazolamide, $CuSO_4$, EDTA, Sulfamide, phenyl boronic acid, halide and bromide) was determined correspond to CA activity.

### 2.5. Production and characterization of $CaCO_3$

$CaCO_3$ precipitation was carried out after hydration of $CO_2$ in an enrichment culture. $CO_2$, (1% and 5%) and 100 mM $NaHCO_3$ added in MSM followed by addition of 0.1 M $CaCl_2$ at 150 rpm and 30 °C for 21 days. The amount of $CaCO_3$ was determined after filtration (Whatman No. 1 filter paper having 90 mm in diameter) and air dried at 37 °C for 48 h. The pH of medium was adjusted to 8 with 1 M sodium hydroxide (NaOH). $CaCO_3$ particle size, morphology and structure were measured by SEM, EDX, FT-IR as earlier discussed by Srivastava et al. (2015).

### 2.6. Statistical analysis

All experimental data were expressed as means ± standard deviation of three replicates and analyzed with sigma plot 9 and 11 statistical package (Systat Software, San Jose, CA).

## 3. Results and discussion

### 3.1. Enrichment, isolation and characterization of bacterium

The thermo-alkalo tolerant *Bacillus* sp. (gene bank accession number JX473586) was shown to sequester $CO_2$ chemolithotrophicallyin minimal salt medium (MSM) through continuous culture in chemostat. This bacterium was selected on the basis of highest extracellular CA production. It is worth noting that very few bacteria have been reported for producing extracellular CA i.e. *Bacillus* sp. GLRT102Ca, *Bacillus cereus* and *Pseudomonas fragi* (Sharma et al., 2009).

### 3.2. Carbonic anhydrase purification and MALDI-TOF-MS analysis

Using batch culture of the *Bacillus* sp., ~7 U/mL extracellular CA has been obtained in 48 h. The crude cell extract is brought to 80% saturation in $(NH_4)_2SO_4$ followed by PEG (poly ethylene glycol) dialysis, Sephadex-G100 gel filtration and affinity chromatography. This protocol afforded 264.28-fold purification of carbonic anhydrase from the culture filtrate with a yield of 46.61% and specific activity of 481.66 U/mg (Table 1). The purified carbonic anhydrase produced single band on an SDS PAGE gel at molecular mass of approximately 28 kDa. Most of the studies had reported the molecular weight of the carbonic anhydrase in the range 26–90 kDa (Pierre, 2012). The purified band of bacterial CA was further studied for MALDI-TOF/MS analysis and the sequence coverage of the peptide against the *Bacillus* carbonic anhydrase reached 32.5%. In MALDI-TOF-MS analysis of extracellular CA purified from *Bacillus* sp. six peptides were found to correspond exactly to internal sequence of carbonic anhydrase belonging to *Bacillus* sp.

### 3.3. Characterization of carbonic anhydrase

The kinetic constants of the purified carbonic anhydrase were determined using PNPA as substrate under optimal assay conditions through Michaelis-Menten equation and the apparent $K_M$ and Vmax values of the enzyme were 1.77 mg/mL and 385.69 U/mg respectively. In the present work, *para*-nitrophenylacetate (*p*-NPA) was used as substrate for kinetic studies and the enzyme showed typical Michaelis-Menten kinetics. The $K_M$ value for the CA is low which shows that the CA has good affinity with substrate. The extracellular CA activity was found maximum at pH 8 and it became inactive (20% activity) at pH 4 and 58% active at pH 10. The optimum temperature for CA activity was 60 °C; however the CA was active over broad range of temperature (50 °C–90 °C). The half life of the enzyme are 56.80 min, 51.00 min, 54.94 min, 36.86 min and 25.36 min at 50 °C, 60 °C, 70 °C, 80 °C and 90 °C, respectively. Thus enzyme exhibited high activity and good stability under alkaline conditions, making it a potent tolerant in environmental condition (Mishra and Thakur, 2011).

Some inorganic anions like cyanate and trithiocarbonate inhibits CA at 20 mM concentration could inhibit up to 51% and 59% of the enzyme activity respectively (Fig. 1). In case of organic solvents, there was complete inhibition by acetazolamide at 50% v/v concentration (Fig. 2). Chemicals like $CuSO_4$, EDTA, sulfamide and phenyl boronic acid, halide, bromide were less effective in inhibiting the enzyme