

## Characterization of Marine Bacterial Carbonic anhydrase and their CO<sub>2</sub> Sequestration Abilities based on a Soil Microcosm

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### Abstract

The novel technology of biological carbon sequestration using microbial enzymes have numerous advantages over conventional sequestration strategies. In the present study, extracellular carbonic anhydrase (CA) producing bacteria were isolated from water samples in the Arabian sea, India. A potential isolate, *Bacillus safensis* isolate AS-75 was identified based on 16S rDNA sequence analysis. The culture conditions suitable for CA production were 32 °C incubation temperature with 4% NaCl and 10 mM Zn supplementation. Experimental optimization of culture conditions enhanced enzyme activity to 265 U mL<sup>-1</sup>. CA specific gene was characterized and based on the analysis, the CA of *B. safensis* isolate AS-75 was a Leucine (11.3%) with  $\alpha$ -helices as the dominant component in its secondary structure. Based on soil microcosm studies, carbonic anhydrase could sequester CO<sub>2</sub> by 95.4  $\pm$  0.11% in sterilized soil with enzyme (SSE) microcosm. Hence, the application of enzyme was found to be more effective in removing CO<sub>2</sub>.

**Key words:** Carbonic anhydrase; CO<sub>2</sub> sequestration; microcosm; enzyme optimization; kinetics

## Introduction

Tremendous increasing emission of greenhouse gases (GHG) and its deleterious effects on the climate change are the most discussed issue around the globe. Among the various GHGs, carbon dioxide occupies the first position in the anthroposphere and has detrimental effects on the ecosystem<sup>[1]</sup>. Reducing the levels of atmospheric carbon dioxide and increasing the environmental awareness are the main solution for global warming<sup>[2]</sup>. Several ongoing researches were started to combat the atmospheric CO<sub>2</sub> levels by various methods like CO<sub>2</sub> capturing and storage technologies. Among these, one of the promising carbon dioxide sequestrations is by microbial extracellular enzyme, carbonic anhydrase. The biotechnological sequestration of CO<sub>2</sub> using carbonic anhydrase has more advantages like eco-friendliness and improved rate of catalysis. Carbonic anhydrase helps the conversion of CO<sub>2</sub> into carbonate in an increased reaction rate manifold with the high substrate specificity<sup>[3, 4]</sup>.

Carbonic anhydrase (E.C 4.2.1.1) (CA), an enzyme widely distributed in organisms, is involved in the basic metabolic processes such as photosynthesis, respiration, ion transport, etc. The metallo-enzyme containing a zinc core facilitates the interconversion of CO<sub>2</sub> to bicarbonates and vice-versa. During the hydration process, CO<sub>2</sub> enters the active site where it undergoes nucleophilic attack by zinc ion and is converted into HCO<sub>3</sub><sup>-</sup>, which subsequently displaced by a water molecule<sup>[5]</sup>. The role of CA in various physiological processes and its distribution in various systems are well known<sup>[6-9]</sup>. CA is also involved in the biomineralization process in the marine environment. This potential of CA enzymes is recently getting attention due to their contribution in the process of carbon dioxide sequestration. Microorganisms have the ability to form calcite by reacting with atmospheric CO<sub>2</sub>, thereby helping in the carbon sequestration process. The actual role of bacteria in the precipitation remains unclear, however, a matter of debate. Some authors believe this precipitation to be an unwanted and accidental by-product of the metabolism<sup>[10]</sup>, while others think that it is a specific process with ecological benefits for the precipitating organisms<sup>[11, 12]</sup>.

Generally, CA enzymes are with high catalytic activity but possess low stability. Hence, mostly CA enzymes are used in the immobilized state to enhance its stability for various applications<sup>[13- 15]</sup>. Immobilization can be one of the best approaches to reuse the enzyme and it would also establish cost competitive route for commercialization of process<sup>[16, 17]</sup>. CA has been immobilized by adsorption method on Sepharose 4B<sup>[18]</sup> on polyurethane and on steel matrix coated with silica by covalent bonding<sup>[19, 20]</sup>.

Hence, bacteria having CA activity in desired level could be used for the sequestration process. This paper encompasses on the characterization of CA gene and evaluating the ability of carbonic anhydrase in CO<sub>2</sub> sequestration in a soil microcosm.

## **Materials And Methods**

### **Sample site description**

Water and sediment samples were collected from five transects during a multidisciplinary oceanographic cruise onboard *FORV Sagar Sampada* (Cruise No. 369) in the Arabian sea during December 2017.

### **Screening of bacterial strains producing carbonic anhydrase**

Water samples were obtained using CTD Rosette Niskin sampler and samples were plated directly onto Zobell Marine agar plates and incubated at 37 °C. Total plate count was calculated for each sample and the values were expressed as CFU mL<sup>-1</sup>. Carbonic anhydrase-positive strains were screened using Zobell Marine broth (ZMB) supplemented with 3 mM para- Nitro phenylacetate (p-NPA) [21] and the broth would turn yellow for carbonic anhydrase producing strains when incubated at 37 °C.

### **Quantification of CA enzyme**

The bacterial isolates with high carbonic anhydrase activity were selected for further study. The positive isolates were purified on ZMA plate. The isolates were inoculated into 2 mL ZMB and incubated at 37 °C for 24 hrs at its log phase with cell concentration of 0.5 OD McFarland standard. The broth was centrifuged at 10,000 × g for 5 min. The resultant supernatant was used as a crude enzyme for carbonic anhydrase assay [21]. Carbonic anhydrase esterase activity was determined by p-nitro phenylacetate (p-NPA) hydrolysis method [22]. Briefly, the samples were assayed at room temperature by adding 20 µl of the crude enzyme to 700 µl of 15 mM Tris HCl and 300 µl of 3 mM p-NPA. The change in absorbance was measured at 348 nm after 1 min of incubation. The enzyme activity was calculated based on the equation 1.

$$CA \text{ activity (U mL}^{-1}\text{)} = (\Delta A_{348}T - \Delta A_{348}B) \times \frac{1000}{5} \times \text{volume of enzyme} \text{ -equation 1.}$$

### **Molecular identification using 16S rDNA sequencing**

Four bacteria showing consistent and higher CA activity were taken for 16S rDNA sequencing. Genomic DNA was extracted and purified [23]. Polymerase chain reaction (PCR) was performed using 16S rDNA specific primers, 27F: 3'-AGAGTTTGTCTGGCTCA-5' and 1090R: 5'-

GCTCGTTGCGGGACTTAACC-3' <sup>[24]</sup>. Resultant PCR amplicon was purified and sequenced using automated DNA sequencing on ABI 3730xl DNA analyzer (Applied Biosystems, USA). The sequences were analyzed using BLASTN against non-redundant NCBI database to identify the isolate.

### **Optimization for CA production**

One-factor optimization was carried out to understand the influence of various conditions favouring the CA production using the potential bacterial isolates. The effect of pH (3, 5, 7, 9) incubation temperature (25, 35 and 45 °C) Zn concentration (5, 10, 15, and 20 mgL<sup>-1</sup>), NaCl concentration (1-10%), on enzyme production was analyzed. Following 24 hrs of incubation, broth was subjected to centrifugation at 10,000 × g at 4 °C and the resultant supernatant was used as crude enzyme extract for carbonic anhydrase assay.

### **Purification step**

Initial purification of crude enzyme obtained after model validation was performed using ammonium sulphate precipitation method (70%) and the pellet was dialyzed against 250 mM Tris HCl buffer, pH-8.3 at 4 °C for 48 hrs. The enzyme yields and specific activity was calculated. The lysate was applied to affinity column (Sepharose®4B-L-tyrosine-p-aminobenzene sulfonamide) pre-equilibrated with 25.0 mM Tris-HCl/0.1 M Na<sub>2</sub>SO<sub>4</sub> (pH 8.7). After extensive washing with a solution of 25.0 mM Tris-HCl/22.0 mM Na<sub>2</sub>SO<sub>4</sub> (pH 8.7), CA enzyme was eluted with the solution of 1.0 M NaCl/25.0 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 6.3) and 0.1 M NaCH<sub>3</sub>COO/0.5 M NaClO<sub>4</sub> (pH 5.6), respectively <sup>[25]</sup>.

### **Enzyme kinetics**

The enzyme fraction thus obtained was resuspended in Tris Buffer [pH 7.0]. After centrifugation (10,000 × g for 10 min), the precipitate was dissolved in 10 mL Tris buffer (50 mM, pH 8.5) and assayed for CA activity. The soluble protein was estimated according to Lowry et al. <sup>[26]</sup>. Enzymatic assay for carbonic anhydrase was performed according to standard protocols described in the previous section. For studying the kinetics of enzyme, the assay was performed with varying p-nitro phenylacetate concentration (1-5 mM) for carbonic anhydrase. Line weaver Burk (LB) plot was constructed and the Km and Vmax values were calculated from the LB plot.

### **Immobilization on iron nanoparticle**

Fe<sub>2</sub>O<sub>3</sub> nanoparticles (Fe<sub>2</sub>O<sub>3</sub> –Np) synthesized earlier <sup>[27]</sup> was used in the study. The Fe<sub>2</sub>O<sub>3</sub> –Np was spherical with average size of 65 nm. The purified CA fraction was covalently immobilized onto the surface of Fe<sub>2</sub>O<sub>3</sub> –Np <sup>[25]</sup>. Briefly, a stock of purified CA enzyme (100 mg/ml) was prepared in 0.05 M Tris buffer (pH 7.5). An aliquot from this stock was mixed for 30 min with NH<sub>3</sub>·H<sub>2</sub>O pre-

treated Fe<sub>2</sub>O<sub>3</sub>–Np to achieve the homogeneous distribution of CA enzyme. After an hour of incubation at 4 °C, the magnetic Fe<sub>2</sub>O<sub>3</sub>–Np coated with CA were magnetically separated and washed with ethanol and ultrapure water. This was vacuum dried and further used for the experiment. Thermal stability of the immobilized and the free CA was evaluated by incubated at 30, 40 and 50 °C. The present study addresses the environmental engineering application i.e., sequestration in a soil microcosm. We assume that the average or ambient environmental temperature does not exceed 50 °C. After incubation for 1 hr, CA activity was quantified based on the p-NPA assay using a spectrophotometer. Two sets of experiments were performed, one with free-CA enzyme and another with Fe<sub>2</sub>O<sub>3</sub>–Np immobilized CA. The enzyme activity of free-CA and immobilized CA were compared. The enzyme stability was assayed for 1-month period after incubation at 4 °C. One-way ANOVA was carried out using SPSS 11.0 software to determine if there were any significant differences among the specific activity of free and immobilized CA. A level of significance of 0.05 at 5% level was considered.

### **Characterization of CA gene**

CA gene specific primers were used for amplification of CA gene from the isolate AS-75. The PCR reaction was performed using the primer CA-4 F (5'- AGAGAGCATATGAGCGACTTGACAG-3') and CA-4 R (3'- AGAGAGGGATCCTCAGCAGCAAC -5') and the resultant PCR amplicon was purified and extracted. Nucleotide Sequence from the sequencing chromatogram were translated into a protein sequence using a Translate tool (<http://web.expasy.org>). To examine the physiochemical characteristics of the potential isolate, the Expasy ProtParam tool <sup>[28]</sup> was used and for predicting the secondary structure of protein, the Expasy SOPMA tool <sup>[29]</sup> were used.

### **Homology modelling**

Since the tertiary structure of *Bacillus safensis* carbonic anhydrase was not available in the Protein structural databank (PDB), a model was predicted based on the amino acid sequence obtained after translation of CA gene sequence. SWISS- Model was used for prediction of tertiary structure. 10 templates were searched and models were generated based on the templates. Based on the Z-score value and model quality, the best model predicted was chosen. The quality of built model was evaluated in PROSA-web server (<https://prosa.services.came.sbg.ac.at/prosa.php>). The Ramachandran plot was constructed using RAMPAGE (<http://morded.bioc.cam.ac.uk/~rapper/rampage.php>) to visualize the energetically allowed regions for backbone dihedral angles  $\phi$  against  $\psi$  amino acid residues in enzyme structure.

## **CO<sub>2</sub> sequestration demonstration in a soil microcosm**

A soil microcosm was designed in accordance with Salam et al. [30] with slight modifications for testing the efficacy of potential bacteria and its carbonic anhydrase on CO<sub>2</sub> mitigation. Fresh soil sample (20 g each) was added in each 125 mL reagent bottle and sterilized 3 times to remove any contaminants and to remove normal microbial flora. The sterility of soil sample was confirmed by adding a portion of the sterile sub-sample into nutrient broth media and checked for no visible bacterial growth after 24 hrs of incubation. The sediment sample had  $1.485 \pm 0.51\%$  (mean  $\pm$  S.D) nitrogen,  $1.279 \pm 0.32\%$  carbon and  $0.419 \pm 0.12\%$  hydrogen with a C/N ratio of  $0.861 \pm 0.56$  based on the elemental analysis using CHNS Analyzer (Flash 2000 CHN elemental analyser, Thermo Fisher Scientific, USA). Based on the scanning electron microscope (SEM) (JEOL Model JSM - 6390LV), the sediment particles were large, with pH of 6.3-6.5, average electric conductivity of 1782 -  $\mu$ S/cm and mean salinity of 18 ppt. Six different experimental conditions were used; (i) sterilized soil with AS-75 (SSB = sterilized soil + bacteria AS-75), (ii) unsterilized (native) soil with AS-75 (NSB = native soil + bacteria AS-75), (iii) sterilized soil with enzyme (SSE = sterilized soil + enzyme), (iv) native soil with enzyme (NSE = native soil + enzyme), (v) sterile soil (SS), and (vi) unsterile soil (NS) as control. In the SSB and NSB, 24 hrs old bacterial culture at 0.5 OD (McFarland standard) was used as inoculum (10% v/w). In case of SSE and NSE, partially purified carbonic anhydrase was added (10% v/w). Soil moisture was made uniform using sterile distilled water in case of controls. Unsterile soil microcosm was used to detect the soil respiration from normal microbial flora, whereas in sterile soil microcosm had no organisms for respiration. Approximately, 5% CO<sub>2</sub> gas was purged into the bottles and were sealed airtight. The microcosms were incubated in a water bath at 32 °C for 24 hrs (ambient temperature recorded at the mangrove ecosystem). The concentration of CO<sub>2</sub> in each microcosm was read using a headspace CO<sub>2</sub> analyser (Uniphos Envirotronic Pvt. Ltd, India) and was expressed as % relative reduction. All experiments were carried out in triplicates unless otherwise mentioned and the values were expressed as mean  $\pm$  S.D.

## **Results And Discussion**

### **Isolation of bacteria with CA enzyme production**

Based on the total plate count, the bacterial abundance ranged from  $1 - 4 \times 10^6$  CFUg<sup>-1</sup>. The maximum bacterial abundance ( $3.34-12.5 \times 10^6$  CFUg<sup>-1</sup>) was recorded at transect T8 of the study and the least at transect T2 ( $1.42-1.6 \times 10^6$  CFUg<sup>-1</sup>). A total of 270 bacterial colonies with different colony morphology were picked from ZMA plate and subjected to further enzyme production analysis. These isolates were screened for carbonic anhydrase activity using ZMB broth. Among 270 bacterial isolates,

there were about 49 isolates showed CA activity ( $63 \pm 0.23$  to  $833 \pm 0.18$  U<sub>mL</sub><sup>-1</sup>), when 1 mL of culture supernatant (crude enzyme) was assayed. CA enzyme is actively involved in many physiological functions of the organisms [8]. Moreover, the present CA is extracellular with high activity. The enzyme could be utilized for the CO<sub>2</sub> capture and over the drawbacks of autotrophic systems used for the same. Being a bacterial derived CA, its cultivation is quite simple than autotrophs which depend on photosynthesis process, photoperiods, nutrient requirements etc. Consider these reason, heterotrophic CA enzyme is important in the capture of CO<sub>2</sub> [31-36]. Li et al., [37] reported an isolate *Psychrobacter* sp. SHUES1 from frozen alkaline samples from Shanghai (China) that produced carbonic anhydrase.

Molecular identification of the bacterial strains was carried out based on 16S rDNA PCR. The obtained sequenced were subjected to nucleotide blast analysis and the isolates were identified as enlisted in Table 1. The isolates were identified *Vibrio parahaemolyticus* (AS-48) [Accession no. MH569862], *Bacillus safensis* (AS-75) [MH569863], *Vibrio rotiferianus* (AS-145) [MH569864] and *Corynebacterium lipophiloflavum* (AS-205) [MH569865]. Several *Bacillus* sp. such as *B. megaterium*, and *B. simplex* were found to produce CA. Achal et al., [38] reported that the *Bacillus* sp. AP6 produced 5.61 EU<sub>mL</sub><sup>-1</sup> CA in the purified fraction.

In the present study, *B. safensis* isolate AS-75 could exhibit a high CA activity compared to the previous reports of *Bacillus schlegelii* (0.0453 U<sub>mL</sub><sup>-1</sup>) isolated from garden soil of Thane, India [39]. However, in general, *Bacilli* sp. are more tolerant to high CO<sub>2</sub> concentrations [40] and their higher carbonic anhydrase production capacity makes them a right candidate for carbon sequestration application [33-35]. Other isolates identified in the study were *Vibrio parahaemolyticus*, *Vibrio rotiferianus* and *Corynebacterium lipophiloflavum*. Though carbonic anhydrase of *Vibrio* sp. has been reported, their CO<sub>2</sub> sequestration ability was not reported. The carbon dioxide and bicarbonate transport mediated by bacterial CAs in the microbial cell has critical role in the bacterial metabolism [41]. Moreover, being opportunistic pathogens, CA is used as a drug target for inhibition of pathogenesis [42-44].

### **One-factor optimization for carbonic anhydrase production**

In this study, only four isolates namely AS-48, AS-75, AS-145, and AS-205 were subjected to optimization studies. At this stage, we used crude enzyme i.e., supernatant of 24 hrs old culture which was at its log phase with cell concentration of 0.5 OD McFarland standard. Higher CA activity (128 U<sub>mL</sub><sup>-1</sup>) was observed at 32 °C compared to the activity (101 U<sub>mL</sub><sup>-1</sup>) at 25 °C for *B. safensis* isolate AS-75 (fig 1a). For other isolates, the CA activity was as low as 5-39 U<sub>mL</sub><sup>-1</sup>. At different NaCl

concentration, *B.safensis* isolate AS-75 produced a maximum activity of 134 UmL<sup>-1</sup> in medium with 4% NaCl (fig 1b). At 10 mM, a maximum CA activity of 192 UmL<sup>-1</sup> was observed for AS-75 (fig 1c). In case of *V. parahaemolyticus* isolate AS-48, CA activity increased with increase in Zn concentration, whereas for *V. rotiferianus* isolate AS-145, *Corynebacterium lipophiloflavum* AS-205, and in AS-75 a decreasing trend was observed beyond 20 mM. Maximum CA activity (265 UmL<sup>-1</sup>) was observed for *B.safensis* isolate AS-75 under alkaline pH (pH 9)(fig 1d). This isolate exhibited no activity at acidic pH of 3 and 160 UmL<sup>-1</sup> at pH 7. However, at pH 3, AS-145 and AS-205 produced CA with an activity of 21 and 7 UmL<sup>-1</sup> respectively. In case of AS-145, pH 7 and in AS-205 alkaline pH did not favour CA production.

Zhang et al.,<sup>[45]</sup> optimized the culture conditions of carbonic anhydrase (CA) production from the strain of *Bacillus mucilaginosus* K02. The CA production increased from  $5.32 \times 10^{-6}$  UL<sup>-1</sup> under the basic medium to 34.53 and  $26.12 \times 10^{-6}$  UL<sup>-1</sup>. The enzyme production in our study was comparable with enzyme production from *Bacillus sp.* (B1)<sup>[36]</sup> and higher than *B. schlegelii*<sup>[39]</sup>, and *B altitudinis*<sup>[46]</sup>.

### **Purification and enzyme kinetics**

The purified CA enzyme had a specific activity of 245 Umg<sup>-1</sup> with an 8-fold purity and a yield of 74.55%. This was better than the report of Sundaram and Thakur<sup>[47]</sup> who observed a higher specific activity of 481.66 Umg<sup>-1</sup> for CA from *Bacillus sp.* with an yield of 46.61%. CA with specific activity of 600 Umg<sup>-1</sup> from *Halomonas sp.*<sup>[48]</sup> and 385.69 U/mg form *Bacillus sp.*<sup>[47]</sup> were reported previously. Low specific activity of  $5.5 \pm 0.6$  Umg<sup>-1</sup> was also reported for CA derived from recombinant *Pseudomonas fluorescens*<sup>[49]</sup>. The Km (mM) and Vmax ( $\mu\text{mol p-NP mg}^{-1} \text{ protein}^{-1} \text{ min}^{-1}$ ) values of the AS-75 isolate was determined using Lineweaver-Burk plot. A low Km value of 0.073 mM indicated low requirement of the p-NPA for the enzyme to become saturated. Vmax value for the AS-75 was found to be  $522 \mu\text{mol mg}^{-1} \text{ min}^{-1}$  (fig 2). The Vmax value was lower than CA of *Micrococcus lylae* ( $K_m$  2.31 mM;  $V_{\text{max}}$   $769.23 \mu\text{mol mg}^{-1} \text{ min}^{-1}$ ) and *Micrococcus luteus* ( $K_m$  2.0 mM  $V_{\text{max}}$   $1,000 \mu\text{mol mg}^{-1} \text{ min}^{-1}$ )<sup>[31]</sup>. A similar low CA activity was also observed by Söyüt and Beydemir<sup>[50]</sup> in rainbow trout. However, a low Km value indicates that the enzyme requires a small amount of p-NPA to become saturated. Hence, in AS-75, a maximum velocity is reached at a low concentration of p-nitrophenyl acetate.

### **Immobilization of CA on Fe<sub>2</sub>O<sub>3</sub>-Np**

Purified CA fraction was immobilized onto Fe<sub>2</sub>O<sub>3</sub>-Np and the CA activity was evaluated after incubation at different temperatures. An immobilization yield of 87% was observed when immobilized



onto Fe<sub>2</sub>O<sub>3</sub>-Np. There was only slight increase in specific activity of CA in the immobilized state. Statistical analysis demonstrated that there was no significant change in CA activities upon immobilization (P-value, 0.65, > 0.05 at 5% level) (fig 3). The incubation time also had no much influence on the specific activity of CA. Hence, we concluded that the present CA enzyme was quite stable and may be used as such for environmental application. During the environmental engineering applications, enzyme recovery is not considered to be more important than achieving the enzyme functionality. We observed a stability of enzyme upto 1 month in its immobilized state but there was no significant difference in the stability of free and immobilized CA. Though it was claimed that immobilization could improve the stability of CA than in its free state <sup>[51-54]</sup>, in our study, the CA from *B.safensis* AS-75 was quite stable in its free state and hence immobilization was not required. Moreover, the CA was used for CO<sub>2</sub> capture in a soil microcosm and it would be difficult to recovery the enzyme even in an immobilized state from soil during environmental engineering application. Hence, we used the free state CA for the CO<sub>2</sub> capture application. Apart from this, unnecessary or over usage of the immobilized substrate might turn as a pollutant to the environment when used in the field application.

### **Characterization of CA gene**

In this study, since *Bacillus safensis* isolate AS-75 exhibited a high CA activity, the CA gene was sequenced and characterized. The CA gene of AS-75 was amplified by using CA gene specific primers, PCR product obtained was purified and sequenced. The obtained sequence was subjected to NCBI BLAST analysis against non-redundant NCBI database. The obtained sequence had 100% query coverage with 98% similarity with carbonic anhydrase of *Bacillus safensis*. Physicochemical parameters of *Bacillus safensis* CA such as number of amino acid, molecular weight, theoretical isoelectric point, amino acid composition, instability index, aliphatic index, Grand Average of Hydropathicity (GRAVY) were characterized. The *Bacillus safensis* contained 193 amino acids with a molecular weight 21.08 KDa. The pI of the sequence indicated that the carbonic anhydrase protein in *Bacillus safensis* was alkaline in nature, (7.67). The *Bacillus safensis* sequence composed of 23 negatively charged residues and 24 positively charged residues and comparatively high percentage (11.3%) of amino acid present is leucine. *Bacillus safensis* had a carbonic anhydrase protein with an instability index of 43.77 and aliphatic index of 105.03 which positively indicates the thermostability of carbonic anhydrase protein. The GRAVY of carbonic anhydrase protein is negative for *Bacillus safensis* (-0.008). The physicochemical parameters of the CA were predicted using the translated sequence which were comparable with earlier reports <sup>[55-58]</sup>. While characterizing the CA of *Thalassiosira pseudonana*, as an Ala rich protein in which negatively charged amino acid residues

were dominant. Moreover, the enzyme was classified as stable based on instability index but the aliphatic index was lower than in the present study, indicating its low adaptability to high temperatures [59].

The CA of *B.safensis* isolate AS-75 was  $\alpha$ -helix rich based on the secondary structure prediction. They are the first component formed during protein folding and is the most stable element [55, 56]. The carbonic anhydrase secondary protein structure prediction result showed that the protein has 49.22% alpha helix, 16.06% extended strand, 9.33% beta-turn and 25.33% random coil (fig 4 a). Although  $\beta$ -CAs were found to be essential for microbial growth of *Escherichia coli* [60], *Helicobacter pylori* [61], and *Corynebacterium glutamicum* [62], their full physiological role in the biosphere still remains unclear [63]. Certain CA enzymes have a compact structure with a  $\beta$ -sheet core with 5 anti-parallel strands, 4 or more  $\alpha$ -helices and a shallow. They can function in the dimers or larger multi-oligomeric states [5].

Homology model of the carbonic anhydrase of *B safensis* was predicted using SWISS-MODEL server (fig 4b). Based on the template search about 50 templates were suggested. About 10 templates with higher similarity were chosen for model generation. The quality of the predicted model was analyzed using RAMPAGE and PROSA web server. The models predicted using the template 1g5c.3A with highest favoured regions of amino acid residues in Ramachandran Plot and z-score was chosen. The molecular surface of the predicted model was depicted in fig 4c.

### **Carbon dioxide sequestration in soil microcosm**

In the present study 6 different soil microcosm was established to find the efficacy of CA of *Bacillus safensis* AS-75 strain in CO<sub>2</sub> sequestration. The bacterial isolate AS-75 could reduce the headspace CO<sub>2</sub> by  $85 \pm 0.49\%$  in microcosm with sterilized soil with bacteria (SSB) and by  $40 \pm 2.54\%$  in unsterilized soil with bacteria (NSB) (fig 5). In the sterile soil with enzyme (SSE), the effect was due to the carbonic anhydrase alone as no microbial activity existed in this set-up. Interestingly, this could remove about  $88 \pm 0.07\%$  of the CO<sub>2</sub> indicating a highly efficient CO<sub>2</sub> sequestration process. In case of unsterile soil with enzyme, a reduction of  $68 \pm 0.14\%$  of headspace CO<sub>2</sub> was observed.

In the present study, the soil microcosm set up using AS-75 isolate and CA enzyme demonstrated significant reduction in headspace CO<sub>2</sub>. The reduction was  $85 \pm 0.49\%$  in SSB,  $40 \pm 2.54\%$  in NSB, and  $88 \pm 0.07\%$  in SSE. The reduction was higher in experiment setup with carbonic anhydrase alone. The CO<sub>2</sub> emission is contributed by the microbial activity, root respiration, degradation process and heterotrophic respiration of soil fauna [64, 65]. The antagonistic effect of other microbial flora and contribution of normal soil respiration may influence the CO<sub>2</sub> sequestration during

the soil microcosm study especially in NSB. Moreover, some chemicals i.e., tannin like compounds largely found in mangrove plants could interfere with the biological decomposition and might inhibit the microbial enzymes <sup>[66, 67]</sup>. In order to rule out the errors contributed by the above mechanisms, we eliminated the root respiration by removing plant parts and root debris. However, in case of unsterile soil, GHG emission was contributed by the microbial respiration. Apart from this, several abiotic factors like humidity, soil temperature, nutrient availability and pH-value also influence the gas flux in the soil <sup>[68-70]</sup>. We have not used culture as control since we try to demonstrate the CO<sub>2</sub> capture phenomenon in the soil microcosm. The sterile soil will give the environment free of microbes hence there is no other contributors for microbial respiration. A baseline experiment was conducted to exclude the headspace CO<sub>2</sub> conducted with soil and without etc. Further, the chance of simply dissolving in water as bicarbonate – the moisture of soil was made uniform in all microcosm and the during sequestration process mediated by CA, the formation of calcite is a general process. Since there were no prior reports demonstrating the carbon capture in a soil microcosm using CA, this may be, to the best our knowledge, a pioneer attempt to shed light on the process and to show how much reduction of headspace CO<sub>2</sub> could be achieved.

Since, the enzyme as such was introduced into the microcosm, and CA being an enzyme with high reaction speed, the sequestration occurred very efficiently than SSB. When bacteria were introduced into the microcosm, it may take longer time to acclimatize as well as to synthesize extracellular enzyme to achieve significant quantity to perform the sequestration function. To conclude, the potential of our isolate *B. safensis* AS-75 to reduce maximum headspace CO<sub>2</sub> in the microcosm level is highly important.

## **Conclusion**

The efficiency for carbon sequestration by carbonic anhydrase produced by *B. safensis* AS-75 strain in the mangrove soil microcosm experiment setup was high with a reduction of  $88 \pm 0.07\%$  CO<sub>2</sub>. The present soil microcosm study proved to be effective in CO<sub>2</sub> capture in a confined space, and hence, the utilization of this mentioned CA for CO<sub>2</sub> capture in industrial process might be highly efficient. This forms the first attempt to demonstrate the CO<sub>2</sub> capture in soil microcosm and further studies are required to scale up the process. In environmental engineering applications, enzyme recovery is not considered more important than achieving the enzyme functionality. Since free CA enzyme from this study was stable with no significant difference in its activity compared to its immobilized form, we recommend the free-state CA for CO<sub>2</sub> capture process. Future studies will incorporate scale-up studies to evaluate the CO<sub>2</sub> capture efficiency at a high CO<sub>2</sub> saturated environment.

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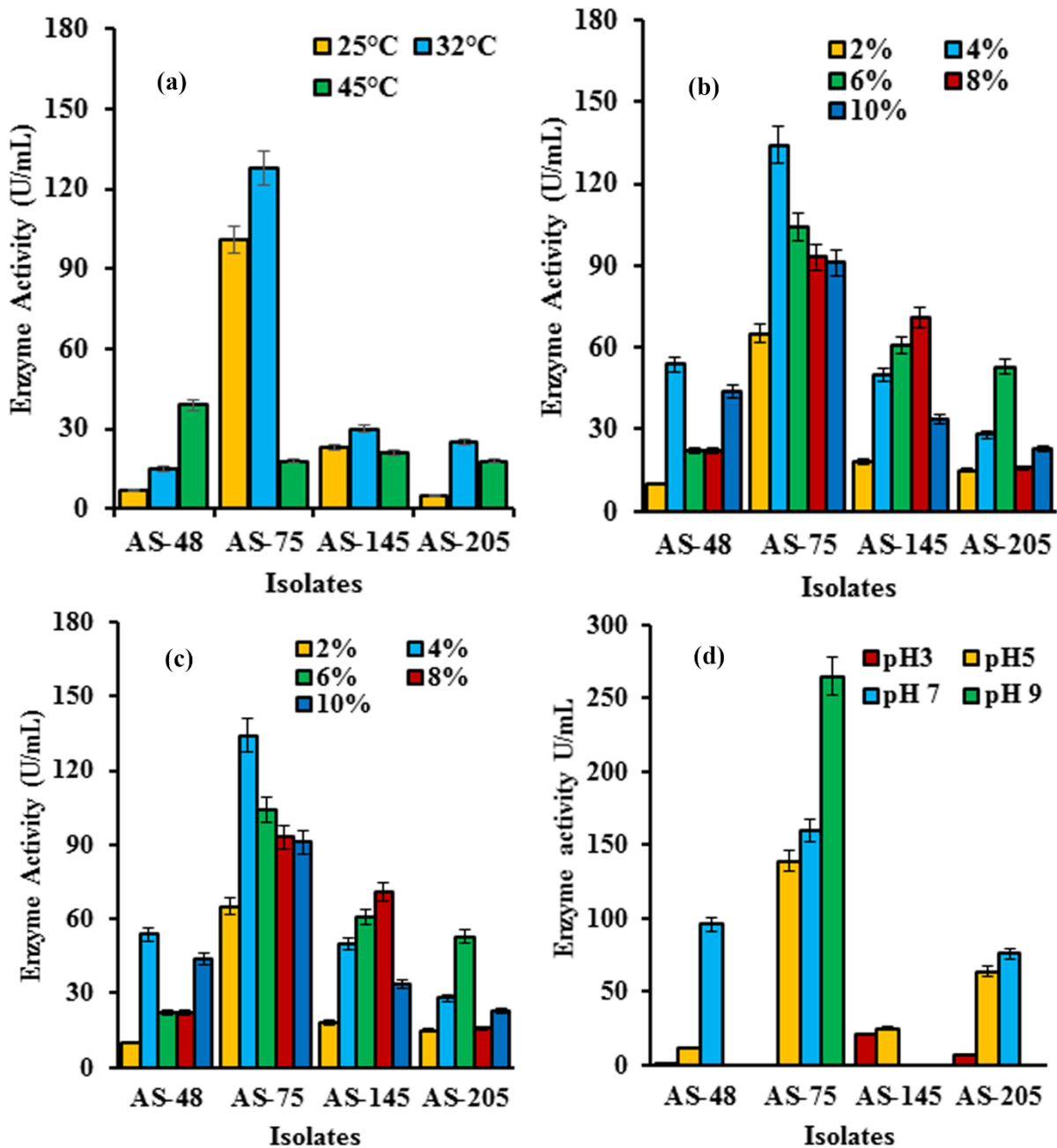
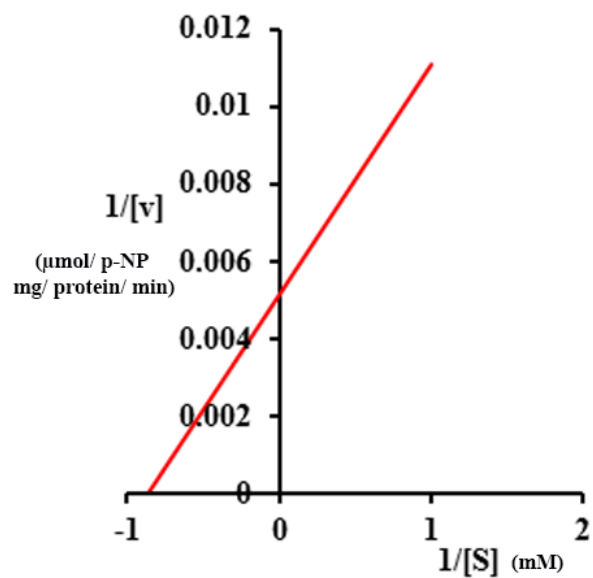
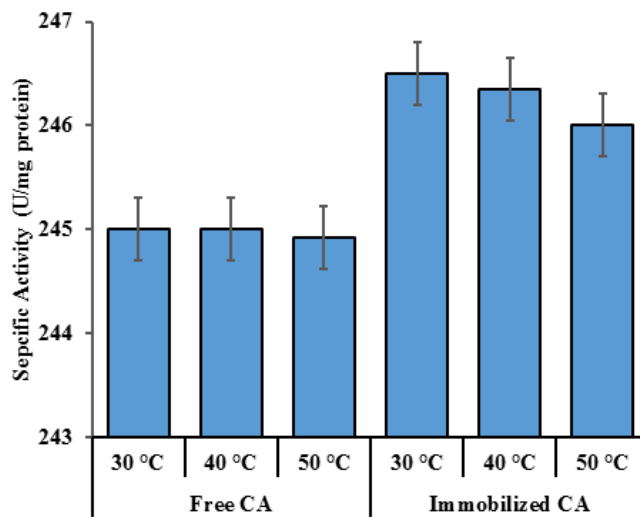


Fig 1. Effect of various conditions on the carbonic anhydrase production using bacterial potential isolates from Arabian Sea. (Crude enzyme was used for the enzyme assay)

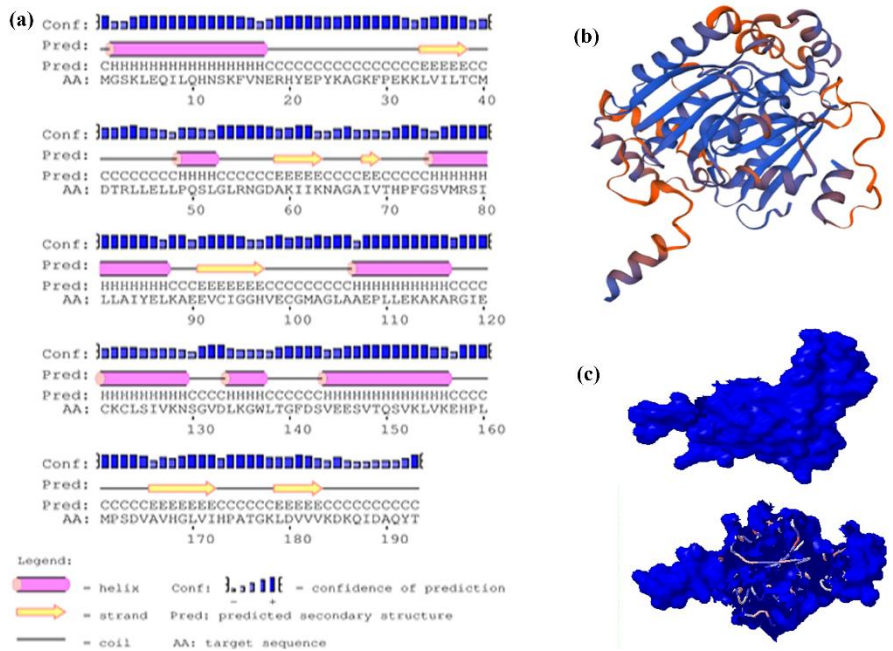
a) Effect of incubation temperature (25, 32 and 45°C) b) NaCl Concentration (2-10%) c) Zn concentrations (1-10%) and d) pH (3-9)



**Fig 2.** Double reciprocal plot for determination of carbonic anhydrase (CA) activity of *Bacillus safensis* isolate AS-75 (Purified enzyme fraction used)

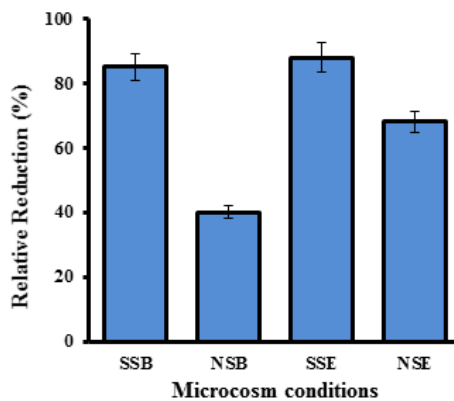


**Fig 3.** Specific activity of carbonic anhydrase in free and immobilized state at different incubation temperatures.



**Fig 4.** Predicted secondary and tertiary structure of *Bacillus safensis* isolate AS-75 carbonic anhydrase

- a) Secondary structure predicted using PSIPRED
- b) Ribbon structure of homology model predicted
- c) Molecular surface with anterior and posterior view



**Fig 5.** Microcosm experiment for evaluating the headspace CO<sub>2</sub> reduction using the *Bacillus safensis* isolate AS-75 and the carbonic anhydrase derived from the isolate. Relative reduction is the expressed as mean  $\pm$  S.D of triplicate experiment.

**Table 1:** Molecular identification of potential bacterial isolates using 16S rRNA ribotyping

<b>Isolates</b>	<b>Identified organism</b>	<b>Identity</b>	<b>Accession numbers</b>
<b>AS-48</b>	<i>Vibrio parahaemolyticus</i>	99%	MH569862
<b>AS-75</b>	<i>Bacillus safensis</i>	100%	MH569863
<b>AS-145</b>	<i>Vibrio rotiferianus</i>	99%	MH569864
<b>AS-205</b>	<i>Corynebacterium lipophiloflavum</i>	100%	MH569865