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ORIGINAL ARTICLE

Characterization of carbonic anhydrase from diversified genus for biomimetic carbon-dioxide sequestration

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Abstract Diversified group of bacteria were screened for carbonic anhydrase (CA) activity. Significant CA activity was found in crude enzyme extracts of *Enterobacter* and *Aeromonas* isolates while minimal or negligible CA activity was observed in case of *Shigella* and *Klebsiella* spp. Optimization and characterization study of potent CA producing isolates revealed that the maximum enzyme activity of 3.86 EU/ml was observed in *E. taylorae* and the optimum pH range for enzyme stability was found to be 7.5–9.0 along with an optimum temperature range of 35–50 °C. The molecular mass of CA was 29-kDa indicating α-type with periplasmic and cytosolic location. Present investigation for the first time reports CA in diversified genus and optimized parameters for enhanced production of CA in *Enterobacter* sp. & *Aeromonas* sp. from fresh water bodies that inturn lay down grounds for exploitation of CA from *E. taylorae* as an efficient catalyst for CO_2 sequestration within a bioreactor.

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Introduction

Carbon-dioxide (CO_2) is known to be responsible for an estimated 60% of the global warming from the green house gases generated by human activities [1]. Phenomenal rise in CO_2 concentration have led to disastrous consequences viz. temperature rise, precipitation change, droughts and floods etc. The concern for the rapidly degrading environment confers to the linkage between environmental issues and resource conservation for sustainable development of technologies mitigating dynamics of global ecosystem.

Conversion of CO_2 to carbonates offers the possibility of safe, stable and environmentally benign product for long-term carbon sequestration, as massive carbonate mineral reservoirs have existed for millions of years. Precipitation of calcium carbonate from aqueous solution occurs at suitable super saturation of carbonate ions, a process requiring the formation of bicarbonate ions. Formation of carbonate at high pH posses both economic and environmental concerns, thus a process that operates at mildly basic pH values would be desirable. The rate-controlling step in the fixation of gaseous CO_2 into carbonate ions is the hydration of CO_2. A novel biomimetic approach to carbon sequestration using Carbonic anhydrase (CA; EC 4.2.1.1) provides a viable means to accelerate CO_2-hydration reaction and has been found to be feasible for fixing large quantities of CO_2 into calcium carbonate in presence of suitable cations at moderate pH values *in vitro*. The biomimetic method provides a holistic approach, which requires a robust

Carbonic anhydrase that can perform optimally at high pH and temperature, being minimally inhibited by other gases and ions [2].

Although ubiquitous in highly evolved organisms from the eukarya domain, the enzyme carbonic anhydrase has received scant attention in prokaryotes from the bacteria and archaea domains [3] and has been purified from only five species since it was first identified in *N. sicca* [4,5,6,7,8]. Recent evidences suggest that CA is widespread in metabolically diverse species from bacteria and archaea domain indicating that the enzyme has a more extensive and fundamental role in the prokaryotic physiology than previously recognized [3].

Buzolyova and Somov [9] have reported the presence of CA in *Y. pseudotuberculosis* and *L. monocytogenes,* the pathogens isolated from soil and water. The presence and importance of CA has been demonstrated in *E. coli*, *Salmonella* sp. & *H. pylori* that are well-established pathogens [10]. Such studies provide the impetus to search for potential CA in other members of Enterobacteriaceae and related Aeromonadaceae.

Thus, the aim of this study was to screen bacterial isolates for the presence of Carbonic anhydrase among the members of Aeromonadaceae and Enterobacteriaceae, determining the location of the enzyme in the cell, optimization of growth conditions for maximum enzyme production and characterization of the intracellular enzyme, that would help in establishing CA as an efficient biochemical marker for carbon sequestration and environmental amelioration in the current global warming scenario linked with elevated CO_2 concentration.

Materials and methods

Microorganisms: A total of 40 bacterial isolates belonging to genus *Aeromonas* sp., *Enterobacter* sp., *Shigella* sp. and *Klebsiella* sp. with 10 isolates each, were obtained from Bacterial Germplasm Collection Center (BGCC), Bacteriology laboratory, Dept. of Biological Sciences, Rani Durgavati University, Jabalpur (M. P.), India.

Preparation of crude enzyme extract: Cells grown (A_{600nm} = 0.6) in Nutrient broth (pH 7.5) were harvested by centrifugation at 10,000 rpm for 10 min at 25°C. Cell pellets were suspended in 1 ml of Tris: NaCl: EDTA (20 mM, pH 8.0) buffer containing 0.7 mg of lysozyme ml^{-1}, 0.01 mg of RNase-I ml^{-1}, 10 mM Triton-X 100 and incubated at 37°C for 60 min. Cell lysates were centrifuged at 5,000 rpm for 10 min at 4°C. The resulting supernatant was used as crude enzyme extract [11].

Carbonic anhydrase assay: The electrometric method of Wilbur-Anderson [12] was followed with modifications for intracellular CA assay. The samples were assayed at 25°C by adding 100 µl of crude enzyme extract to 3 ml of 20 mM Tris-HCl buffer, pH 8.3. The reaction was initiated by the addition of 2 ml ice cold CO_2 saturated water. The time required for the pH to drop from 8.3–7.3 (t) was measured. The enzyme activity was calculated using the equation, enzyme units (EU) = $(t_c - t) / t$, where t is the time required for the pH change and t_c is the time required for the pH change in control (the crude enzyme extract substituted in reaction mixture by boiled enzyme served as control).

Protein estimation: Protein concentrations were determined by the method of Lowry et al. [13] with Bovine Serum Albumin as standard.

Determination of growth profile and enzyme activity: Erlenmeyer flasks (150 ml) containing 50 ml basal salt solution (BSS), pH 7.5 was sterilized, cooled and inoculated with 2% (v/v) seed culture (A_{600nm} = 0.2) of each isolate separately and incubated at 37°C. Samples were removed at 24 h intervals over a period of 7 days. Growth profile was studied by measuring the absorbance at 600nm and enzyme activity was determined by performing the electrometric assay each time as described earlier. The basal salt solution contained (g/l): Glucose 2.0; K_2HPO_4 7.0; $MgSO_4$ 0.1; KH_2PO_4 3.0; $(NH_4)_2SO_4$ 1.0 ; $FeSO_4$ 0.05 ; $ZnSO_4.7H_2O$ 0.05 $MnSO_4$ 0.05 ; $ZnCl_2$ 0.02 ; $CoCl_2$ 0.04 ; $Na_2MoO_4.2H_2O$ 0.02.

Effect of pH on enzyme production: The effect of pH on enzyme production was determined at different pH values ranging from 7.5 to 9.5 using two different buffers of 0.2M concentration (Tris-HCl for pH 7.5 to 8.5, Glycine–NaOH for pH 9.0 & 9.5). The cells were harvested after 4 days of incubation at 35°C and enzyme activity was determined under standard assay conditions following the electrometric method.

Effect of temperature on enzyme production: The effect of temperature on enzyme production was determined at different temperature range (35°C to 55°C) following four days of incubation in 50 ml BSS medium at optimum pH value of each isolate. The enzyme activity was determined under standard assay conditions following the electrometric method.

Effect of carbon source on enzyme production: Effect of various carbon sources (2% w/v) on CA production was assessed by substituting carbon source: glucose, citrate, lactose or rhamnose individually in BSS medium. The enzyme activity was analyzed after four days of incubation at

optimized pH and temperature for each isolate under standard assay conditions following the electrometric method.

Effect of nitrogen source on enzyme production: Effect of various nitrogen sources (2% w/v) on CA production was assessed by substituting urea, peptone or beef extract individually in BSS medium at optimized pH, temperature and carbon source for each isolate after four days of incubation and the enzyme activity was determined under standard assay conditions following the electrometric method.

Effect of pH on enzyme stability: The stability of the enzyme was monitored by incubating the crude enzyme extract with respective buffers ranging from pH 7.5 to 9.5 as described above. The residual enzyme activity was estimated after 24 h incubation at 35°C under standard assay conditions following the electrometric method.

Effect of temperature on enzyme stability: The thermostability of the enzyme was monitored by incubating crude enzyme extract at a temperature range of 35°C to 55°C for 30 min with respective buffer at optimized pH for each isolate and then the residual enzyme activity was determined under standard assay conditions following the electrometric method.

Effect of inhibitors on the enzyme activity: The effect of Azide, Sulfanilamide, Iodide and Nitrate at the reactants concentration of 1.1 X 10^{-4} M, 7.6 X 10^{-6} M, 2.3 X 10^{-2} M and 2.9 X 10^{-5} M respectively in Tris-HCl buffer, pH 8.0 was determined by adding desired concentration of inhibitors in reaction mixture containing crude enzyme extract and incubating it at 37°C for 30 min [5]. The residual enzyme activity was determined under standard assay conditions following the electrometric method. Analogous control experiments without inhibitor in the reaction mixture were carried out.

Localization of enzyme in the cell: Two methods were used to investigate the localization of enzyme in the cell. In the first method periplasmic proteins were preferentially released from bacteria with chloroform [15] while the second method implemented treatment of cells with 0.1M NaOH followed by centrifugation [16]. The second method leaves most of the inner and outer membrane proteins in the pellet and cytoplasmic and periplasmic proteins in the supernatant. The activity of various fractions was determined following the electrometric method.

Determination of molecular weight of Carbonic anhydrase: Molecular weight of Carbonic anhydrase was determined by SDS-PAGE [17] using low range protein molecular weight marker (Bangalore Genei) as standard.

Results and discussions

According to Merlin et al. [18] *E. coli* encoding a β-Class CA is responsible for interconversion of CO_2 & bicarbonate *in vivo*. The demand for bicarbonate was estimated and found to be 10^3 to 10^4 fold greater than would be provided by uncatalyzed intracellular hydration. Therefore enzymatic conversion of CO_2 to bicarbonate is deemed necessary for growth of *E. coli* in aerobic conditions. However, the need for CO_2 in central metabolism cannot be replaced with supplements. The only known supply pathway for bicarbonate is via the hydration of CO_2, the principle function of Carbonic anhydrase.

Out of 40 isolates screened for Carbonic anhydrase activity 19, isolates (9 isolates of *Enterobacter* sp. and all ten isolates from *Aeromonas* sp., showed significant intracellular CA activity while in *Klebsiella* sp. and *Shigella* sp. it was found to be negligible (data not shown). Four isolates i.e. *E. taylorae* (1.887 EU/ml, 0.078 U/mg protein); *E. gergoviae* (2.210 EU/ml, 0.0736 U/mg protein,); *A. hydrophila* (1.878 EU/ml, 0.0626 U/mg protein); and *A. caviae* (1.027 EU/ml, 0.0425 U/mg protein) showed highest CO_2 hydration activity and were further used for optimization and characterization studies.

In the present study, intracellular CA activity of the four isolates was comparable to *H. pylori, P. aeruginosa, S. typhimurium and V. fischeri [10]* however it was less than the activity reported in *A. woodii* [6]. The intracellular enzyme activity was found to be high during the initial phase of growth, however activity decreased when growth was at its maximum followed by slight increase in activity was observed when growth declined. Maximum growth was observed on 5th day for *E. gergoviae*, on 6th day for *A. hydrophila* & *A. caviae* and on 7th day for *E. taylorae* while maximum enzyme activity was observed on 4th day in all the four isolates and minimum enzyme activity was observed on 6th day for *E. gergoviae, E. taylorae* & *A. hydrophila* and on 7th day for *A. caviae* (Fig 1).

A steady enzyme activity profile was observed in case of *E. gergoviae* as compared to the variations in activity profile of other three isolates investigated during the present study. The increase in activity during lag and early log phase could be attributed to the phenomenon of sparking effect, whereby CO_2 is required to meet the biosynthetic demand and is important for organisms to overcome the lag phase [19]. A high load of CO_2 and bicarbonate could result in decreased CA activity during maximum growth. The slight increase in activity as growth retards remain to be investigated.

Maximum CA production in *E. gergoviae* (2.62 EU/ml) & *E. taylorae* (2.96 EU/ml) was observed at pH 8.5.

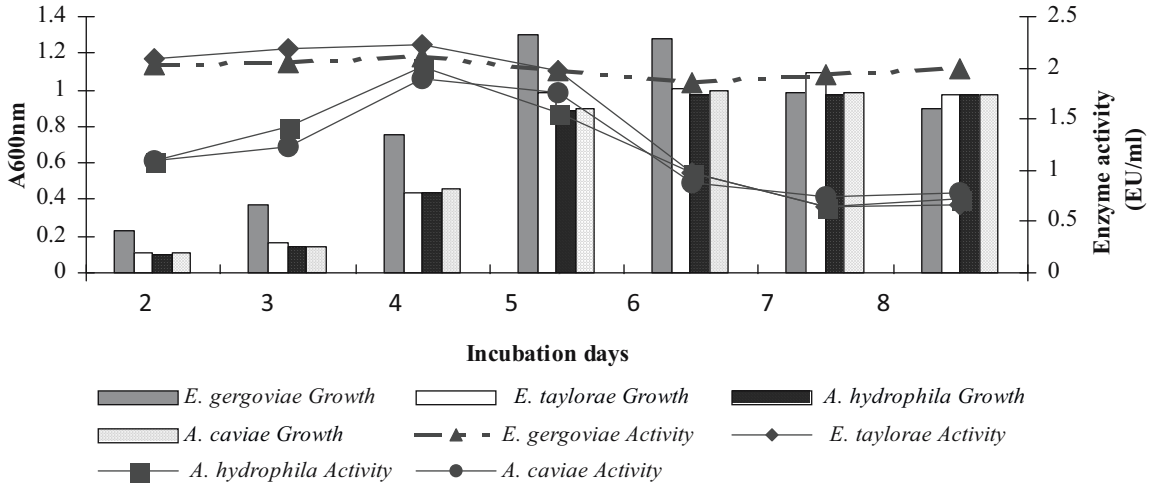


Fig. 1 Effect of incubation days on growth and enzyme activity.

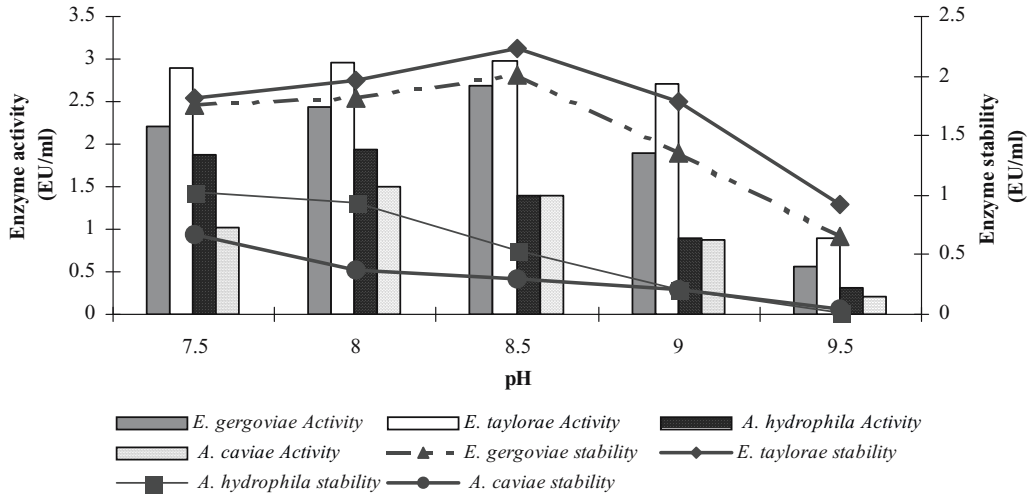


Fig. 2 Effect of pH on enzyme production and stability.

However at pH 8.0 maximum CA production was determined in *A. hydrophila* (1.93 EU/ml) and *A. caviae* (1.51 EU/ml). In *E. taylorae* the stability of the enzyme increased from pH 7.5 to a maximum at 8.5, however 80% and 42% of stability was retained at pH 9.0 & 9.5 respectively. Similar trend was observed for *E. gergoviae*. Maximum enzyme stability was observed for *A. hydrophila* and *A. caviae* at pH 7.5, followed by a progressive decline in stability at higher pH (Fig 2).

E. gergoviae (2.62 EU/ml) and *E. taylorae* (2.92 EU/ml) showed maximum enzyme production at 40°C and 45°C respectively however both *A. hydrophila* (1.57 EU/ml) and *A. caviae* (1.96 EU/ml) showed maximum enzyme production at 40°C. In *E. gergoviae* the enzyme showed maximum stability at 35°C and retained 98%, 92% and 86% stability at 40°C, 45°C and 50°C respectively. In *E. taylorae* maximum enzyme stability was observed at at 35°C & 40°C, however 95% and 90% stability was retained at 45°C and 50°C respectively. *A. hydrophila* and *A. caviae* showed maximum enzyme stability at 35°C with a progressive decline in stability at higher temperatures (Fig 3).

Maximum Carbonic anhydrase production from *E. taylorae* was determined at an optimum pH of 8.5 and temperature 45°C. To the best of our knowledge there are no reports on CA enzyme having optimal temperature of 45°C at pH 8.5 in eubacterial domain. However CA isolated from human erythrocyte plasma membrane showed optimum pH of 7.5 [20]. Similar results have been reported for bovine erythrocyte plasma membrane [20]. In contrast, the extra cellular CA of stromatolite forming cyanobacterium, *Microcoleus cathonoplastes* showed two peaks of activity at around pH 10.0 and 7.5 [1]. The optimum temperature for CA obtained from human erythrocyte plasma membrane was 35°C and in other mammalian CA it was found to be

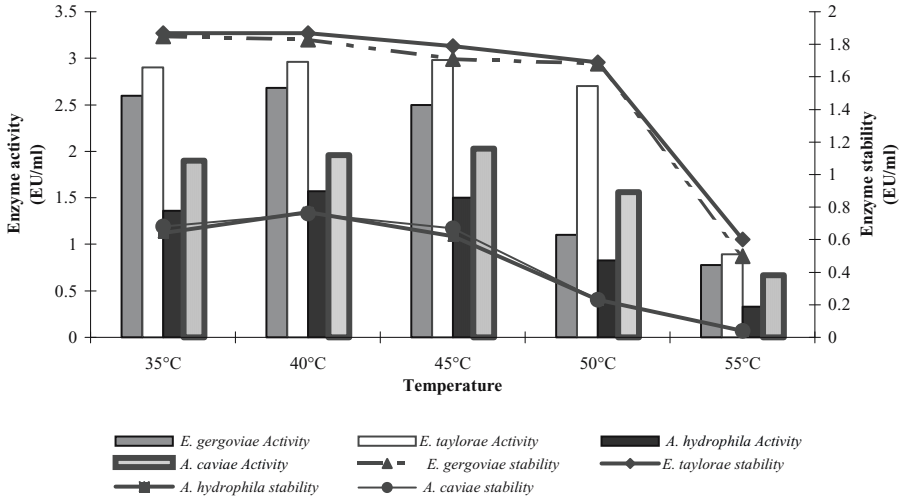


Fig. 3 Effect of temperature on enzyme production and stability.

Table 1. Effect of Carbon sources and Nitrogen sources on the production of Carbonic anhydrase enzyme (EU/ml)

Carbon Source	*E. gergoviae*	*E. taylorae*	*A. hydrophila*	*A. caviae*
Glucose	2.10	2.23	1.92	1.76
Citrate	2.62	3.08	2.05	2.09
Rhamnose	2.08	2.08	1.18	1.21
Lactose	1.36	1.98	1.06	1.06
Nitrogen source				
Beef extract	2.01	1.72	1.15	1.12
Peptone	1.58	1.56	1.09	1.93
Urea	2.68	3.86	2.10	1.15

~37°C [21]. However, the CA from *M. thermoautotrophicum* was found to be active at 75°C [14].

Among all the carbon sources tested citrate was found to maximally increase enzyme production from all the four isolates (*E. gergoviae,* 2.62 EU/ml; *E. taylorae*, 3.08 EU/ml; *A. hydrophila,* 2.05 EU/ml; *A. caviae,* 2.09 EU/ml). Incorporation of urea as nitrogen source in BSS medium resulted an increase in enzyme production from *E. gergoviae* (2.68 EU/ml); *E. taylorae* (3.86 EU/ml) and *A. hydrophila* (2.10 EU/ml) however, maximum enzyme production from *A. caviae* (1.93 EU/ml) was observed when peptone was used as nitrogen source (Table 1).

Hydrolysis of urea results in accumulation of both bicarbonate and ammonia in the cell, which favors the physiological and regulatory links between urea and bicarbonate metabolism [22]. The incorporation of nickel into the active site of urease is dependent on CO_2 / HCO_3^- metabolism which is in turn regulated by carbonic anhydrase [23]. The fact that *E. gergoviae, E. taylorae and A. hydrophila* are urease positive and showed high CA activity when urea was incorporated into the medium consolidates the role of CO_2 in urease positive organisms.

Use of sulphanilamide and azide inhibited more than 80% enzyme activity whereas nitrate and iodide were found to inhibit more than 50% enzyme activity in all four isolates (Fig. 4). Inhibition due to Sulphanilamide and azide has been reported in all known mammalian Carbonic anhydrases (α-type) [10]. The α–class Carbonic anhydrases is highly sensitive to inhibition by sulphanilamide [24]. However γ–type CA from *M. thermophila* was found to be less sensitive to inhibition by sulphanilamide [5].

The knowledge of location of enzyme in the cell helps in understanding the physiology of the enzyme. Chloroform treatment method was preferred over cell fractionation method, as use of NaOH in extraction resulted in loss of enzyme activity [25]. Enzyme activity was assayed in periplasmic and cytosolic fractions. Significant activity was observed in both the fractions for *E. taylorae* (periplasmic, 1.69 EU/ml; cytosolic, 1.55 EU/ml) and *E. gergoviae* (periplasmic, 1.63 EU/ml; cytosolic, 1.53 EU/ml). Similar results have been reported in *H. pylori* and bacteria from the karst areas [26,27], whereas only the periplasmic fraction revealed a significant activity in *A. hydrophila* (1.56 EU/ml) and *A. caviae* (1.57 EU/ml). Similar results

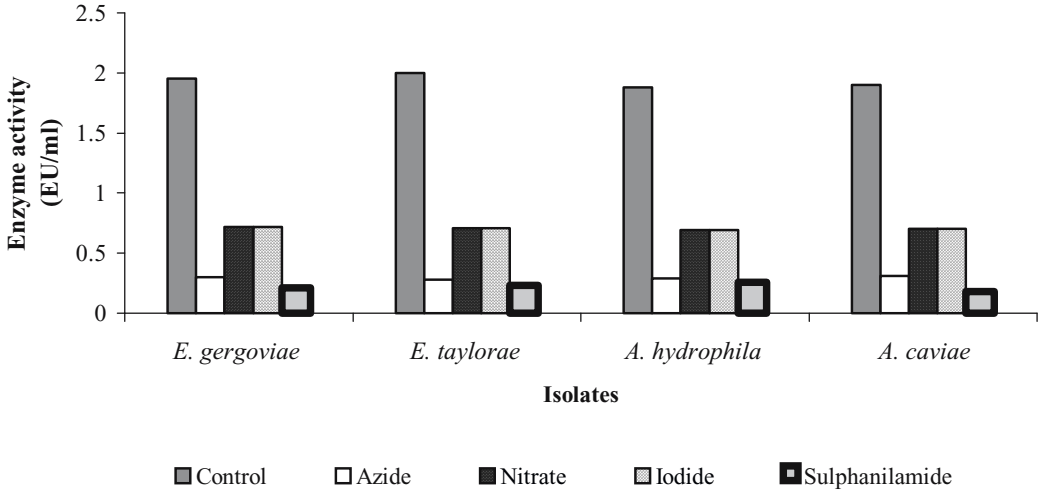
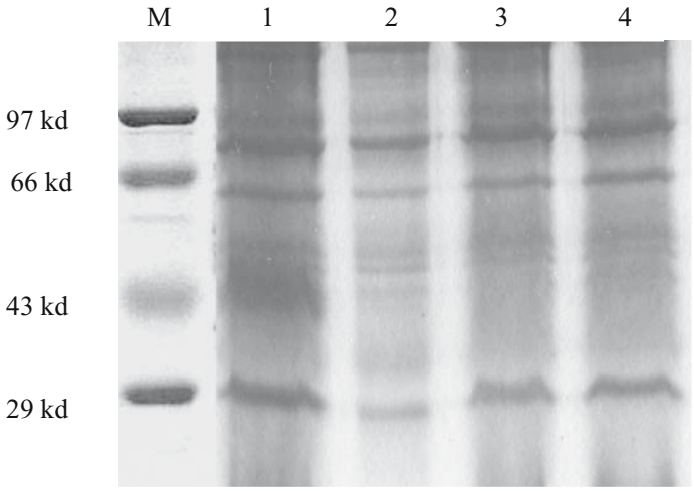


Fig. 4 Effect of inhibitors on enzyme activity.



Lane 1. M- Low range molecular weight marker (Bangalore Genei), Lane 2. *E. gergoviae,* Lane 3. *E. taylorae,* Lane 4. *A. hydrophila*, Lane 5. *A. caviae*

Fig. 5 Molecular weight of Carbonic anhydrase using SDS-PAGE.

have been reported in *N. gonorrhoea*, *R. palustris* and *C. reinhardtii* [25,28,29]. Periplasmic location of the enzyme is significant in maintenance and regulation of pH and ionic concentration. Both cytosolic and periplasmic CA plays an important role in interconversion of carbon dioxide and bicarbonate, along with ion transport. The subunit molecular weight determined by SDS-PAGE showed the presence of ~29kDa protein in all the four isolates (Fig. 5).

Similar results have been reported for α–CA in *H. pylori*, *R. palustris* and *C. reinhardtii* [26,28,29]. On the basis of more than 80% inhibition with sulphanilamide and a molecular weight in the region of ~29 kDa, it can be predicted that the CA from all the four isolates evaluated during the present study is of α-type, however confirmation has to be done through immunoblotting.

Present investigation for the first time reports the screening, optimization and characterization of CA in diversified genus. Screening of large number of isolates for carbonic anhydrase activity, followed by optimization and characterization of four selected isolates revealed *E. taylorae* as the most potent strain for CA production, with a total activity of 3.86 EU/ml, functioning optimally at pH 8.5 & 45°C and stable at alkaline pH (7.5–9.0) and high temperature range (35–50°C). Overexpression and purification of this enzyme can help in finding a potent CA that would be beneficial in

the long-term goal of development of an efficient bioreactor for carbon-dioxide sequestration.

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References

1. Kupriyanova E, Villarejo A, Markelova A, Gerasimenko L, Zavarzin G, Samuelsson G, Los DA & Pronina N (2007) Extracellular Carbonic anhydrases of stromatolite forming cyanobacterium *Microcoleus cathonoplastes.* Microbiology 153:1149–156
2. Bond GM, Stranger J, Branchvold DK, Sinsek FA, Medina NG & Egeland G (1999) Development of integrated system for biomimetic CO_2 Sequestration using the enzyme carbonic anhydrase. Energy & Fuels 15:309–31
3. Smith KS & Ferry JG (2000) Prokaryotic Carbonic anhydrases. FEMS Microbiol Rev 24:335–366
4. Adler L, Brundell J, Falkbring SO & Nyman PO (1972) Carbonic anhydrase from *Nisseria sicca* strain 6021. Bacterial Growth and Purification of enzyme. Biochemica et Biophysica Acta 284:313–323
5. Alber BE & Ferry JG (1994) A carbonic anhydrase from the archaeon *Methanosarcina thermophila.* Proc Natl Acad Sci USA 91:6909–6913
6. Braus-Stromeyer S, Schnappauf G, Braus GH, Gobner A & Drake HL (1997) Carbonic anhydrase in *Acetobacterium woodii.* J Bacteriol 179:7197–7200
7. Gill SR, Fedorka PJ, Tweten RK & Sleeper BP (1984) Purification and Properties of Carbonic anhydrase of *Rhodospirullum rubrum*. Arch Microbiol 138:113–118
8. Yagawa Y, Shiraiwa Y & Miyachi S (1984) Carbonic anhydrase from the blue green algae (cyanobacterium) *Anabaena variabilis*. Plant Cell Physiol 25:775–783
9. Buzolyova LS & Somov G (1999) Autotrophic assimilation of CO_2 and C_1 compounds by pathogenic bacteria. Biochem (Moscow) 64 (10):1357–1361
10. Smith KS, Zakubzick C, Whittam TS & Ferry JG (1999) Carbonic anhydrase is an ancient enzyme wide spread in prokaryotes. Proc Natl Acad Sci USA 96:15184–15189
11. Dean CR & Ward OP (1992) The use of EDTA or polymixin with lysozyme for the recovery of intracellular products from *Eschericia coli.* Biotechnology Techniques 6 (2):133–138
12. Wilbur KM & Anderson NG (1948) Electrometric and Colorimetric determination of Carbonic anhydrase. J Biolchem 176:147–154
13. Lowry OH, Rosenbrough NJ, Forr AL & Randall RJ (1951) Protein measurement with Folin Phenol Reagent. J Biolchem 193:265–275
14. Smith KS & Ferry JG (1999) A plant type (β- Class) carbonic anhydrase in the thermophilic methanoarchaeon *Methanobacterium thermoautotrophicum*. J Bacteriol 181: 6247–6253
15. Ames G, Prody FL & Kustu S (1984) Simple, rapid and quantitative release of periplasmic proteins by choloroform. J Bacteriol 160:1181–1183
16. Russel M & Model P (1982) Filamentous phage precoat is an integral membrane protein: analysis by a new method of membrane preparation. Cell 28:177–184
17. Laemmli UK (1970) Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature (London) 227:680–685
18. Merlin C, Masters M, Mcteer S & Corlson A (2003) Why is carbonic anhydrase essential to *E. coli*. J Bacteriol 185(21): 6415–6424
19. Charles HP & Roberts (1968) Carbon dioxide as a growth factor for mutants of *E. coli*. J Gen Microbiol 51: 211–224
20. Demir N, Demir Y & Coskum P (2001) Purification and characterization of carbonic anhydrase from human erythrocyte plasma membrane. Turk J Med Sci 31:477–482
21. Demir Y, Demir N, Nadarglu H & Bakan E (2000) Purification and characterization of carbonic anhydrase from bovine erythrocyte plasma membrane. Biochem & Biotechnol 30 (1): 49–59
22. Stahler MF, Ganter L, Katherin L, Manfred K & Stephen B (2005) Mutational analysis of *Helicobacter pylori* carbonic anhydrases. FEMS Immunol Medical Microbiol 44: 183–189
23. Park IS & Hausinger RP (1995) Requirement of carbon dioxide for *in vitro* assembly of urease nickel metallocenter. Sci 267:1156–1158
24. Earnhardt JN, Qian MC, Lakkis MM, Bergenhem NC, Laipis PY, Tashian RE & Silverman DM (1998) The catalytic properties of marine carbonic anhydrase VII. Biochem 37: 10837–10845
25. Chirica LC, Elleby B, Jonsson BH & Lindskog S (1997) The complete sequence, expression in *E. coli*, purification and some properties of carbonic anhydrase from *Nisseria gonorrhoeae*. Eur J Biochem 244:755–760
26. Chirica L, Peteresson C, Hurtig M, Jonsson BH, Boren T & Lindskog S (2002) Expression and localization of α and β Carbonic anhydrase in *Helicobacter pylori*. Biochemica and Biophysica Acta 160:192–199
27. Li W, Yu LJ, Wu Y, Jia LP & Yuan DX (2007) Enhancement of calcium release from limestone by microbial extracellular carbonic anhydrase. Bioresource Tech 98:950–953
28. Pushkas LG, Inui M, Zahan K & Yukawa H (2000) A periplasmic α- type carbonic anhydrase from *Rhodopseudomonas palustris* is essential for bicarbonate uptake. Microbiology 146:2957–2966
29. Rawat M & Moroney JV (1995) The regulation of carbonic anhydrase and ribulose 1,5 biphosphate carboxylase / Oxygenase activase by light and CO_2 in *Chlamydomonas reinhardtii*. Plant Physiol 109:937–944