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Enhanced biomimetic sequestration of CO_2 into $CaCO_3$ using purified carbonic anhydrase from indigenous bacterial strains

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ABSTRACT

Phenomenal rise in CO_2 concentration have led to disastrous consequences. The present study endeavors the biomimetic sequestration of CO_2 into $CaCO_3$ using biological catalyst; carbonic anhydrase (CA) purified from *Pseudomonas fragi*, *Micrococcus lylae* and *Micrococcus luteus* 2 along with a comparative evaluation of their efficiency against commercial bovine carbonic anhydrase (BCA). At pH range 8.0–9.0 and temperature range 35–45 °C, maximum stability was observed for CA from *M. luteus* 2 followed by *P. fragi* CA, *M. lylae* CA and BCA. *P. fragi* CA demonstrated maximum stability as function of time with respect to pH and temperature. The anionic inhibitors, Cl^-, SO_4^{2-}, NO_3^-, HCO_3 and toxic metal ions viz., lead, arsenic and mercury showed varied inhibitory profile against the four different CAs. The level of inhibition was significantly higher for BCA and *M. luteus* 2 CA compared to *P. fragi* CA and *M. lylae* CA. Calcium estimation was found to be a reliable method for the determination of sequestration efficiency. Indigenous CAs and their consortia exhibited enhanced CO_2 sequestration competence compared to commercial BCA. Sequestration efficiency at 45 °C, under process parameters was found to be maximum for CA consortia (61%) and minimum for commercial BCA (17.8%) indicating its potential application in an onsite scrubber.

1. Introduction

An estimated 60% of the global warming from the green house gases generated by human activities is due to CO_2 [1]. Climate changes due to global warming, pressure of carbon tax and increasing environmental awareness are driving policy makers towards a solution for global warming [2].

Sequestration of CO_2 in the form of a stable, environmentally safe solid carbonate offers obvious appeal for long-term storage of CO_2. The carbonates produced 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 [3]. Precipitation from aqueous solution occurs at suitable supersaturation of calcium and carbonate ions [4]. The hydration of CO_2 to form carbonic acid is the rate-limiting step in the conversion of CO_2 into carbonate ions, which has a forward reaction constant of 6.2×10^{-3} s at 25 °C [5,6]. The enzyme CA catalyses this hydration reaction at or near the diffusion controlled limit [7]. Thus a novel biomimetic approach using carbonic anhydrase has been found to be feasible for fixing large quantities of CO_2 into $CaCO_3$ in presence of suitable cations at modest pH values *in vitro*. This holistic approach requires a robust carbonic anhydrase (CA) functioning at an alkaline pH and high temperature with appreciable tolerance to cations, anions and other inhibitors. Seawater provides a cheap and readily available source of calcium ions and has been considered as a potent supply source for calcium ions [8]. However, to assess the efficacy of carbonic anhydrase in an onsite scrubber the effect of certain metal ions and different anions present in seawater along with SO_4^{2-} and NO_3^- as SOx and NOx in flue gas needs to be evaluated for selection of an efficient carbonic anhydrase.

Carbonic anhydrase is one of the fastest enzyme that catalyses CO_2-hydration reaction with typical reaction rates between 10^4 and 10^6 reactions per second for different forms of this enzyme [9]. Carbonic anhydrase is a zinc metalloenzyme reported to be present in animals, plants and microorganisms [10]. Analysis of amino acid sequences have revealed that all CAs fall into five major classes (α, β, γ, δ and ε), the amino acid sequences of which are not homologous and are supposed to have evolved independently [11]. Although the enzyme has been studied extensively in eukaryotes, it has received scant attention in bacterial and archaeal domain, the enzyme being purified from few prokaryotic species only [12].

Although the biomimetic CO_2 sequestration is still at a research phase, the principle of the process has been successfully validated [3]. This approach holds an overwhelming advantage over other sequestration possibilities such as geological, ocean, terrestrial and

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chemical sequestration. It also provides a point source solution which does not require any CO_2 transport, a benign and ecofriendly end product, along with a quick, efficient and single step process involving one of the fastest enzymes ever known [3,7,13].

The present study aims to evaluate the efficiency of purified CA from *Pseudomonas fragi* (PCA), *Micrococcus luteus* 2 (MTCA), *Micrococcus lylae* (MLCA) and commercial bovine carbonic anhydrase (BCA) from Sigma, under different process parameters for potential application in an on-site scrubber.

2. Materials and methods

Artificial sea water (ASW) and bovine carbonic anhydrase were purchased from Sigma–Aldrich, St Louis, MO, USA. All the buffers used in the experiment were purchased form Hi Media, Mumbai, India. Chloride salts of As^{3+}, Ca^{2+}, Mg^{2+}, Hg^{2+}, Mn^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Pb^{2+}, Fe^{2+}, Ni^{2+}, Se^{2+}, Na^{+}, K^{+}, were used. Sodium salt of $SO_4{}^{2-}$, $NO_3{}^{-}$, $HCO_3{}^{-}$ and potassium salt of F^{-}, Cl^{-}, I^{-}, Br^{-} in the present study used were purchased from Sisco Research Laboratory, Mumbai, India. All other chemicals used were of analytical grade and were purchased from standard sources.

2.1. Source of enzyme

The indigenous purified CA from *P. fragi* (BGCC# 1077), *M. lylae* (BGCC# 1078) and *M. luteus* 2 (BGCC# 1079) were obtained as lyophilized powder from Bacteriology Laboratory, Department of Biological Science, Rani Durgavati University, Jabalpur (MP), India and bovine carbonic anhydrase was purchased from Sigma. A stock solution (1 mg ml^{-1}) of all the four CAs was prepared in distilled water and used for subsequent studies.

2.2. Determination of carbonic anhydrase activity

The method of Wilbur–Anderson [14] was followed with certain modifications. CO_2 of standard grade was introduced at ~1 bar (100 kPa) in 500 ml of Milli Q grade pure water for 1 h at 4 °C. The tube connecting the CO_2 cylinder containing the regulator to apparatus was passed through a rubber cork and the other end was kept closed with the help of a valve. The hole in the cork was immediately sealed after saturation. CO_2-saturated water (3 ml) was immediately added to 2 ml of Tris–HCl buffer (100 mM; pH 8.3) containing 0.5 ml of sample (before the assay test samples were immediately transferred to 4 °C following the ageing experiments with different pH, temperature and metal ions). The reaction vessel was maintained at 4 °C. (The assay was carried at low temperature to slow down the rate of reaction within measurable limits.) The time required for the pH to drop from 8.0 to 7.0 (t) was measured. The time required for the pH change (8.0–7.0) was used as control (t_c) when buffer was substituted for the test sample. The Wilbur–Anderson Units were calculated with equation $t_c - t/t$. The protein content was determined by the method of Lowry et al. [15] and the activity is expressed in Units/ml/mg protein. The pH electrode used in this study is Beetrode electrode with separate reference electrode (Dri-Ref), manufactured by World Precision Instruments Incorporation (WPI). The measurements were carried out in three replicates.

2.3. Determination of pH stability as function of time

The method of Demir et al. [16] was used with certain modifications. The enzyme stability was determined at different pH values ranging from 6.0 to 10.0 using various buffers. The effect of pH on CA stability was determined by incubating 0.02 ml of enzyme (from stock) in 980 μl of citrate–phosphate buffer (pH 6.0–6.5), Tris–HCl buffer (pH 7.0–8.5) and glycine–NaOH buffer (pH 9.0–10.0) at buffer strength of 50 mM. The residual enzyme activity was determined at an interval of 60 min up to a period of 360 min under standard assay conditions.

2.4. Determination of thermostability of CA as function of time

The method of Demir et al. [16] was used with certain modifications. The thermostability was determined at different temperatures ranging from 35 °C to 55 °C by incubating 0.02 ml of enzyme in 980 μl of Tris–HCl buffer pH 8.0. The residual enzyme activity was determined at an interval of 60 min upto a period of 360 min under standard assay conditions.

2.5. Effect of different ions on enzyme stability

The method of Ramanan et al. [17] was used with certain modifications. The effect of different cations (As^{3+}, Ca^{2+}, Mg^{2+}, Hg^{2+}, Mn^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Pb^{2+}, Fe^{2+}, Ni^{2+}, Se^{2+}, Na^{+}, K^{+}) and anions (F^{-}, Cl^{-}, I^{-}, Br^{-}, $SO_4{}^{2-}$, $NO_3{}^{-}$, $HCO_3{}^{-}$) on CA stability was studied individually by incubating 0.02 ml enzyme with 980 μl Tris–HCl buffer (50 mM, pH 8.0) for 1 h at different concentration (0.005–0.2 M) of each ion. The residual enzyme activity was determined at reactants concentration under standard assay conditions.

2.6. Sequestration of CO_2 into calcium carbonate

The CO_2 saturated solution (distilled water and ASW) were prepared at room temperature as described under Section 2.2. CO_2-saturated solution (10 ml) was released from reservoir and mixed with 1 ml of Tris buffer (pH 8.3) in another section connected through a valve. The pH adjusted CO_2 saturated solution was allowed to react individually with purified CA (100 μg from 1 mg ml^{-1} stock) from *P. fragi*, *M. lylae*, *M. luteus* 2 and BCA for 15 min. The bicarbonate solution was released into another vessel through a valve containing 10 ml of $CaCl_2$ solution (at a final concentration of 0.01 mol L^{-1}). Tris buffer pH 9.5 (2 ml, 1 M) was immediately added to the above mixture. The precipitation reaction was carried out by incubating the reaction mixture at 35 °C and 45 °C, respectively for 5 min and analyzing the amount of calcium carbonate formed by drying the sample and weighing the amount of calcium carbonate deposited. The final pH of the solution after precipitation was measured. The same experiment was carried out in absence of enzyme and the amount of calcium carbonate precipitated was determined. The starting time required for visual precipitation was also recorded. The results were expressed in terms of mg $CaCO_3$ formed following control correction.

2.7. Sequestration of CO_2 into $CaCO_3$ using enzyme consortia

Three indigenous CA (33.3 μg each) were combined together to form an enzyme consortium (100 μg) and compared with 100 μg of BCA for CO_2 sequestration using CO_2-saturated distilled water and ASW. The sequestration study was carried out as described earlier.

2.8. Determining the efficiency of the sequestration process

The sequestration efficiency in each experiment was evaluated by determining the ionic concentration of calcium present before and after carbonate precipitation following the method of Kolthoef et al. [18]. The difference in calcium concentration was considered to be the amount of calcium utilized in formation of calcium carbonate, the same experiment in absence of enzyme was carried out and following control correction the results were expressed in terms of percentage efficiency of the calcium ion utilized.

Table 1
Effect of pH and temperature as function of time on stability of CA from *P. fragi*, *M. lylae*, *M. luteus* 2 and commercial BCA.

	Time (h)	pH									Temperature (°C)				
		6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10	35	40	45	50	55
P. fragi (residual activity %)	0[a]	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	1	75	78	87	99	**100**	**90**	**82**	67	58	**93**	**90**	**87**	80	71
	2	70	75	86	92	**99**	**88**	**80**	65	55	**89**	**86**	**80**	77	68
	3	68	73	86	92	**99**	**85**	**78**	63	50	**80**	**77**	**72**	66	59
	4	60	70	84	90	**94**	**80**	**73**	57	42	**73**	**70**	**63**	58	53
	5	57	68	80	83	**90**	**76**	**69**	54	39	**70**	**63**	**54**	50	44
	6	50	63	75	80	**88**	**72**	**66**	51	37	**65**	**59**	**50**	45	37
M. lylae (residual activity %)	0[a]	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	1	90	96	100	93	**90**	**72**	**65**	50	43	**90**	**88**	**85**	78	69
	2	85	88	90	89	**85**	**70**	**60**	48	40	**85**	**80**	**81**	76	65
	3	83	85	87	88	**80**	**68**	**57**	45	38	**75**	**70**	**67**	60	54
	4	71	73	80	82	**72**	**63**	**53**	40	35	**69**	**63**	**56**	50	44
	5	60	62	73	75	**67**	**59**	**50**	38	30	**60**	**57**	**50**	42	38
	6	55	60	65	69	**63**	**54**	**45**	33	24	**51**	**48**	**40**	38	32
M. luteus 2 (residual activity %)	0[a]	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	1	68	70	77	88	**90**	**100**	**97**	85	80	**97**	**94**	**90**	86	81
	2	65	68	73	83	**87**	**98**	**93**	80	76	**93**	**90**	**88**	83	79
	3	62	65	69	78	**83**	**90**	**88**	75	70	**90**	**86**	**85**	80	76
	4	56	61	67	73	**80**	**88**	**84**	72	68	**87**	**83**	**78**	78	73
	5	52	57	63	70	**76**	**81**	**78**	65	59	**85**	**80**	**75**	73	70
	6	43	40	60	66	**71**	**78**	**73**	62	57	**83**	**77**	**71**	70	68
Commercial BCA (residual activity %)	0[a]	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	1	85	82	95	90	**83**	**79**	**71**	58	50	**90**	**86**	**72**	68	56
	2	80	75	86	82	**78**	**75**	**66**	50	43	**85**	**81**	**67**	61	50
	3	74	69	81	75	**71**	**69**	**61**	45	39	**75**	**72**	**60**	53	41
	4	70	63	73	61	**59**	**55**	**50**	41	33	**69**	**63**	**51**	45	34
	5	65	57	61	56	**51**	**48**	**44**	38	30	**60**	**54**	**43**	39	28
	6	58	51	57	50	**42**	**39**	**36**	33	25	**54**	**48**	**38**	31	23

The values in bold reflect the probable range of process parameters that could significantly affect the enzyme activity in an onsite scrubber.
[a] 100% activity of the four CAs was equal to their specific activity as reported under Section 3.
All the errors are within 5% of SD.

3. Results

The specific activity of CA from *P. fragi* (70.6 U/mg protein), *M. lylae* (66.5 U/mg protein), *M. luteus* 2 (61.0 U/mg protein) and commercial BCA (74.6 U/mg protein) were determined.

3.1. Stability of CA with respect to pH and temperature as function of time

The CA from *P. fragi* was found to retain 88% residual activity at pH 8.0 and 72% residual activity at pH 8.5 and 66% residual activity at pH 9.0 following 6 hr of incubation (Table 1). The enzyme was found to be stable (80%) in the pH range 7.0–9.0 following 3 h of incubation. However, CA from *M. lylae* retained 63%, 54% and 45% residual activity at pH 8.0, 8.5 and 9.0, respectively, following 6 h of incubation. The enzyme exhibited more than 80% stability between pH 7.0 and 8.0, while only 68% and 57% retained at pH 8.5 and 9.0 after 3 h of incubation. The stability profile showed 78% stability for *M. luteus* 2 CA along with 71% and 73% residual activity at pH 8.0 and 9.0, respectively following 6 h of incubation. However, after 3 h of incubation 80% stability was observed between pH 7.5–9.0 for *M. luteus* 2 CA. (Table 1). In contrast only 42%, 39% and 36% stability was observed at pH 8.0, 8.5 and 9.0, respectively for commercial BCA following 6 h of incubation. However, after 3 h incubation at pH 7.0 and 7.5 BCA was found to retain 81% and 75% residual activity, while 79%, 69%, 61%, stability was observed at pH 8.0, 8.5 and 9.0, respectively.

The thermostability profile of CA following 3 h of incubation between 35 °C and 45 °C was in the order; *M. luteus* 2 CA > *P. fragi* CA > *M. lylae* CA > commercial BCA (Table 1). However, after 6 h at a temperature range of 35–45 °C, CA from *P. fragi*, *M. lylae*, *M. luteus* 2 and BCA were found to retain 65–50%, 51–40%, 83–71% and 54–38% residual activity, respectively (Table 1).

The present study indicated that after 3 h of incubation between pH 8.0–9.0 and temperature 35–45 °C, CA from *P. fragi* and *M. luteus* 2 retained 70–90% stability while commercial BCA showed only 60–70% residual activity under similar conditions (Table 1).

3.2. Effect of different ions on enzyme stability as function of time

At highest concentration (0.2 M) of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} ions all four CAs were found to retain more than 75% residual activity (Table 2). However, at 0.2 M concentration of chloride ion CA from *P. fragi*, *M. lylae*, *M. luteus* 2 and BCA exhibited 70%, 71%, 53% and 48% residual enzyme activity, respectively. At 50 mM concentration F^{-}, I^{-}, Br^{-}, showed inhibitory effect on all four CAs. However, the inhibition profile of CAs against these ions provided a distinct mosaic pattern. Correspondingly at 50 mM concentration, Cd^{2+}, Zn^{2+}, Co^{2+} and Fe^{2+}, resulted in 7%, 38%, 28% and 6% increase in enzyme activity in favor of *P. fragi* CA, similar results were obtained for all the CAs involved in the study (Table 2). At 100 mM sulphate ion and 50 mM nitrate ion concentration; CA from *P. fragi* and *M. lylae* retained more than 80% and 90% residual activity. Comparatively BCA and *M. luteus* 2 CA exhibited approximately 65% and 60% residual enzyme activity. At 5 mM concentration of lead, arsenic and mercury; ~70% and ~ 65% of residual activity was retained by CA from *M. lylae* and *P. fragi*, respectively, in contrast more than 50% inhibition of *M. luteus* 2 CA and BCA was observed in presence of lead and mercury (5 mM), while ~30% inhibition was observed in case of arsenic (5 mM). No relevant inhibitory effect was observed at lower (5–50 mM) concentration of manganese and nickel. At 5 mM concentration of selenium, no inhibitory effect on *M. lylae* CA was observed; CA from *P. fragi*, *M. luteus* 2 and BCA retained only 70%, 63% and 65% residual activity, respectively under similar conditions

Table 2
Effect of different ions, metal ions and inhibitors at various concentrations on CO_2-hydration activity of CA from *P. fragi*, *M. lylae*, *M. luteus* 2 and BCA. The enzyme stability is expressed in terms of % residual activity.

	P. fragi CA				*M. lylae* CA				*M. luteus* 2 CA				BCA			
Ions (M)	0.005	0.05	0.1	0.2	0.005	0.05	0.1	0.2	0.005	0.05	0.1	0.2	0.005	0.05	0.1	0.2
Mg^{2+}	100	92	80	76	100	100	100	93	100	90	82	75	100	94	80	87
Na^{+}	**100**	**100**	**92**	**83**	**100**	**100**	**100**	**100**	**100**	**100**	**85**	**80**	**100**	**100**	**84**	**79**
K^{+}	**100**	**100**	**94**	**86**	**100**	**100**	**100**	**100**	**100**	**94**	**89**	**81**	**100**	**100**	**84**	**79**
Ca^{2+}	**105**	**100**	**88**	**80**	**110**	**110**	**100**	**100**	**100**	**100**	**91**	**84**	**100**	**100**	**90**	**81**
As^{3+}	66	48	31	20	70	65	58	50	63	54	45	36	68	55	36	21
Mn^{2+}	91	85	79	71	100	100	100	100	93	84	80	73	90	84	80	76
Cd^{2+}	**110**	**107**	**100**	**98**	**124**	**121**	**100**	**100**	**108**	**100**	**90**	**84**	**100**	**100**	**90**	**81**
Cu^{2+}	100	100	90	84	100	100	100	100	100	100	94	89	100	100	100	90
Zn^{2+}	**134**	**138**	**100**	**100**	**147**	**140**	**121**	**100**	**110**	**100**	**100**	**100**	**113**	**108**	**100**	**100**
Co^{2+}	**120**	**128**	**100**	**100**	**131**	**129**	**110**	**100**	**100**	**100**	**100**	**91**	**107**	**101**	**100**	**100**
Pb^{2+}	**63**	**40**	**28**	**16**	**71**	**71**	**61**	**54**	**50**	**41**	**32**	**24**	**55**	**33**	**23**	**17**
Fe^{2+}	**107**	**106**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**93**	**88**	**80**	**103**	**100**	**93**	**90**
Ni^{2+}	100	100	87	80	100	100	100	100	100	91	83	75	100	93	89	81
Se^{2+}	70	61	52	40	100	100	91	82	63	51	41	33	65	51	40	28
Hg^{2+}	**68**	**38**	**22**	**14**	**74**	**65**	**57**	**50**	**48**	**37**	**29**	**20**	**58**	**30**	**18**	**12**
F^{-}	93	85	78	65	87	80	66	56	80	73	64	52	84	73	60	55
Cl^{-}	**88**	**80**	**76**	**70**	**90**	**85**	**78**	**71**	**75**	**66**	**59**	**48**	**80**	**70**	**59**	**53**
Br^{-}	84	78	66	54	100	96	84	77	63	54	47	39	77	69	60	51
I^{-}	80	73	61	50	97	92	86	78	72	63	53	46	73	64	55	50
SO_4^{2-}	**100**	**92**	**83**	**71**	**100**	**100**	**92**	**84**	**73**	**68**	**60**	**53**	**100**	**80**	**66**	**57**
NO_3^{-}	**91**	**84**	**70**	**64**	**100**	**94**	**83**	**73**	**69**	**63**	**56**	**48**	**90**	**65**	**60**	**50**
HCO_3^{-}	87	80	70	61	94	88	80	74	73	67	60	54	81	72	61	53

The values in bold reflect the probable range of major ions that could significantly affect the enzyme activity in an onsite scrubber. 100% activity of the four CAs was equal to their specific activity as reported under Section 3.
All the errors were within 5% of SD.

(Table 2). Increase in bicarbonate ion concentration was directly proportional to inhibitory effect irrespective of the type of CA under study (Table 2).

3.3. Sequestration of CO_2 into $CaCO_3$

The successful conversion of CO_2 into $CaCO_3$ in presence of all the CAs has been demonstrated at final pH ~9.2 (Fig. 1). The time taken for the initiation of the carbonation reaction with enzyme was clocked around 15–16 s while for uncatalysed reaction visual precipitation initiated after ~90 s. In presence of CO_2-saturated distilled water, the percentage Ca^{2+} utilization at 35 °C and 45 °C was in the following order; Enzyme consortia CA (58%; 70%) > *M. luteus* 2 CA (52.5%; 63.8%) > *P. fragi* CA (50%; 61.3%) > *M. lylae* CA (43.8%; 61.3%) > BCA (22.5%; 31.3%) (Fig. 2). However, in presence of ASW-based solution containing sulphate and nitrate ions, a modified order was observed; Enzyme consortia CA (50%; 61%) > *P. fragi* CA (38%; 53.3%) > *M. lylae* CA (34.5%; 48.8%) > *M. luteus* 2 CA (31.1%; 44.4%) > BCA (11.11%; 17.8%) (Fig. 3). A positive correlation was observed for the amount of $CaCO_3$ formed with percentage of Ca^{2+} ion utilized (Figs. 2 and 3).

4. Discussion

The precipitation of CO_2 into $CaCO_3$ is deemed to progress at an accelerated rate coupled with generation of an environmentally benign product under economically and environmentally feasible conditions [19]. The principle of biomimetic sequestration has been proved beyond doubt [8]. However, the requirement of a robust carbonic anhydrase functioning optimally under different process parameters is the principal goal. In the present study we have successfully demonstrated the sequestration of CO_2 into $CaCO_3$ using indigenous CA from *P. fragi*, *M. lylae*, *M. luteus* 2 along with commercial BCA. Liu et al. [3] in their study had identified six factors which influence sequestration rate and efficiency in pH range ~8.5. Bond et al. [8] has already used BCA and HCA as model systems to demonstrate biomimetic CO_2 sequestration at a pilot scale. Optimum performance of the biocatalyst at pH range of 8.0–9.0 and temperature range of 35–45 °C is fundamental to the success of the process [20]. In the present study pH and thermostability profile indicated that CA from *M. luteus* 2 and *P. fragi* performed competently compared to *M. lylae* and BCA under objectively defined parameters. CA from bacterial domain exhibiting stability between pH 7.0–8.5 and temperature 35–45 °C has been reported from *P. fragi* and *E. taylorae* [21,22]. However, bovine and human erythrocyte CA having activity in the pH range of 6.5–7.5 and temperature range of 35–40 °C has been reported by Demir et al. [16]. In any approach to CO_2 sequestration, time is an important factor that determines the flow rate and thus cannot be ignored, and the biomimetic approach is no exception. During the study CA from *P. fragi* and *M. luteus* 2 showed remarkable stability compared to BCA at pH range 8.0–9.0 and temperature 35–45 °C. *P. fragi* CA showed highest stability as function of time at different pH and temperature. Thus an enzyme stable under operational parameters for longer duration will be both feasible and economical in terms of sequestration efficiency.

The success of the approach is determined by the availability of calcium ions for $CaCO_3$ precipitation. Seawater has been considered as a readily available and cheap source of calcium ions [8]. However, the effect of ionic strength, salinity, anionic inhibitors, toxic metal ions and other ions on the relative stability of biocatalyst requires in depth analysis before considering the biocatalyst as a potential candidate for an onsite scrubber. In the present study we have used ASW to simulate sea water as cation source. The study indicated that all the four CAs were functionally active in presence of Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} ions; these four ions constitute the bulk of the seawater [8]. Chloride ion exists as the most abundant ionic entity in sea water [8], and is thus believed to have a profound impact on biomimetic CO_2 sequestration. CA form *M. lylae* and *P. fragi* were inhibited upto 30% in presence of 0.2 M chloride ion concentration, while BCA was inhibited upto 52% at the same ionic concentration. In sea water F^{-}, I^{-} and Br^{-}, are present in trace amount [8], at 5 mM concentration of these anions, *M. lylae* CA showed minimum inhibition followed by *P. fragi* CA, BCA and *M. luteus* 2 CA. SOx and NOx are an integrated part of flue gas, their concentration being determined by the quality of coal along with the *modus operandi* of a

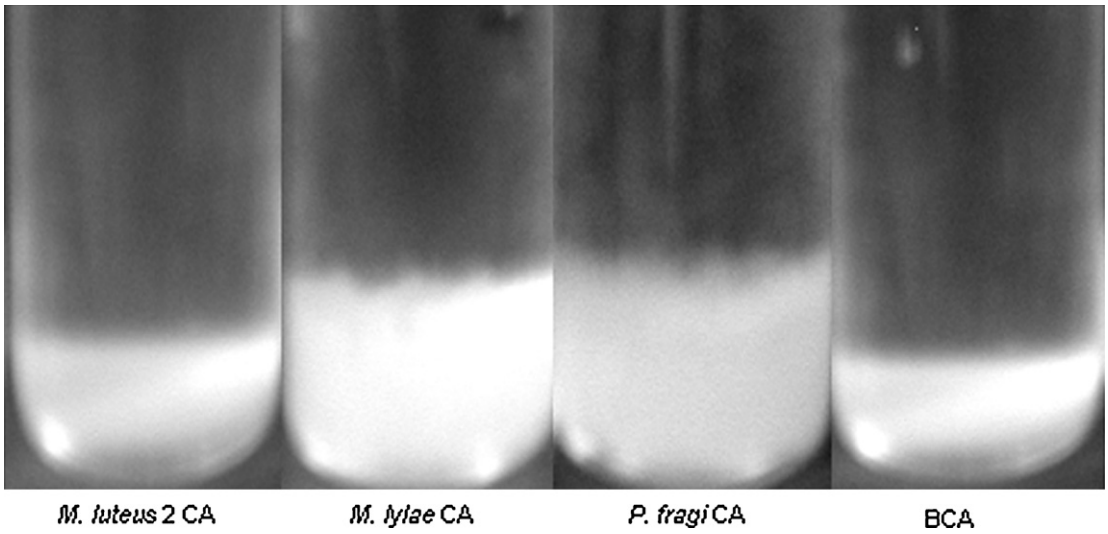

Fig. 1. Precipitation of CO_2 into $CaCO_3$ using indigenous (*M. luteus* 2, *P. fragi*, and *M. lylae*) CA and commercial BCA under similar conditions.

power plant [19]. At different concentration of sulphate and nitrate ions CA from *M. lylae* and *P. fragi* exhibited excellent and consistent stability compared to commercial BCA. The performance of a biocatalyst in the presence of SOx and NOx has important economic implications. The concentration of SOx and NOx can be reduced to certain extent using a flue gas desulphurization (FGD) system; however, considerable economic penalty will be associated with it. Thus a CA with appreciable tolerance to sulphate and nitrate ions will be an automatic choice for selection. In flue gas, lead (29–290 mg/ton), mercury (45–450 mg/ton) and arsenic (350–400 mg/ton) are essentially present in trace amounts [19]. In presence of such toxic heavy metals CA from *M. lylae* and *P. fragi* were inhibited to a lesser extent compared to BCA and *M. luteus* 2 CA. Cd^{2+}, Zn^{2+}, Co^{2+} and Fe^{2+}, were found to have positive impact on CA activity. Zn^{2+}, Cd^{2+} and Fe^{2+} have been reported to increase enzyme activity in extracellular CA from *P. fragi* and other CAs [21,23]. The difference in the response of four CAs to various ions can be attributed to the subtle changes in the amino acid residues present in their active site [24].

The CO_2 sequestration study demonstrated the feasibility of the biomimetic approach using CA from bacterial domain. The present study indicates that calcium estimation can be used as an effective method for determination of sequestration efficiency. Titrimetry can therefore be considered as a cost effective and readily accessible method for sequestration analysis. In the present study enzyme consortia was found to be most effective for sequestration. The three indigenous CAs performed better compared to commercial BCA at 35 °C and 45 °C and also in presence and absence of ASW-based solution. Modest heating at 45 °C resulted in increased $CaCO_3$ precipitation. This result is justified by a similar observation from Liu et al. [3]; however, it is in stark contrast to the study of Mirjafari et al. [25]. In the present

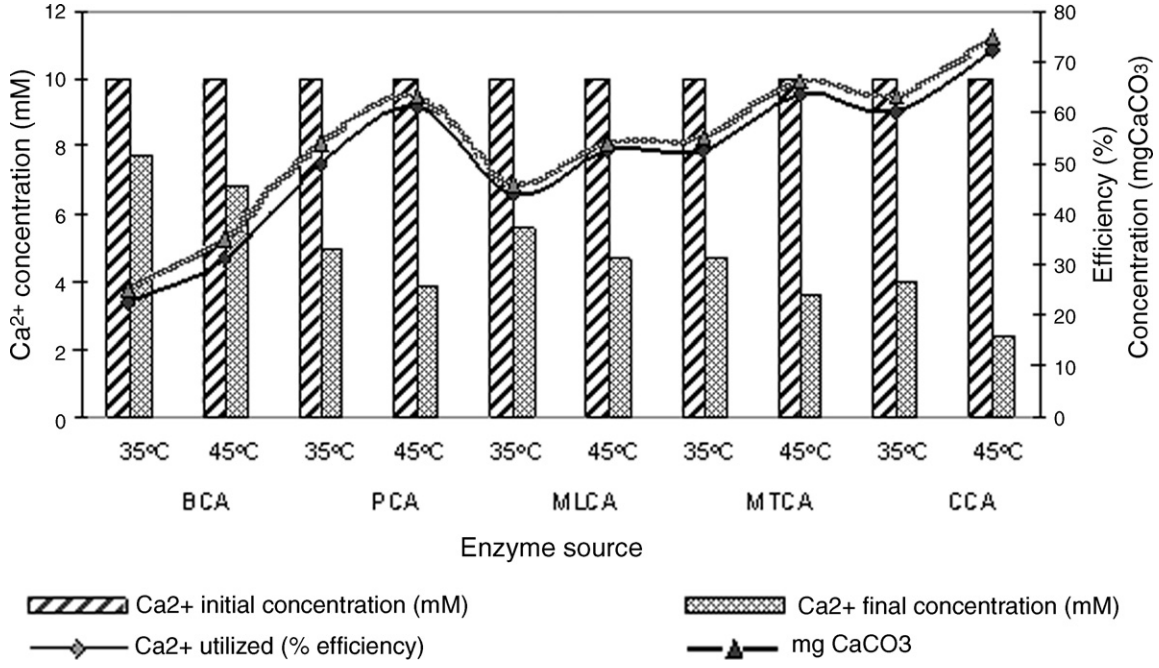

Fig. 2. Determination of CO_2-sequestration efficiency using CO_2 saturated distilled water and $CaCl_2$ solution, at 35 °C and 45 °C. 100 μg of BCA (bovine carbonic anhydrase), PCA (*P. fragi* CA), MLCA (*M. lylae* CA), MTCA (*M. luteus* 2 CA) and 100 CCA μg (enzyme consortia CA) were used in study.

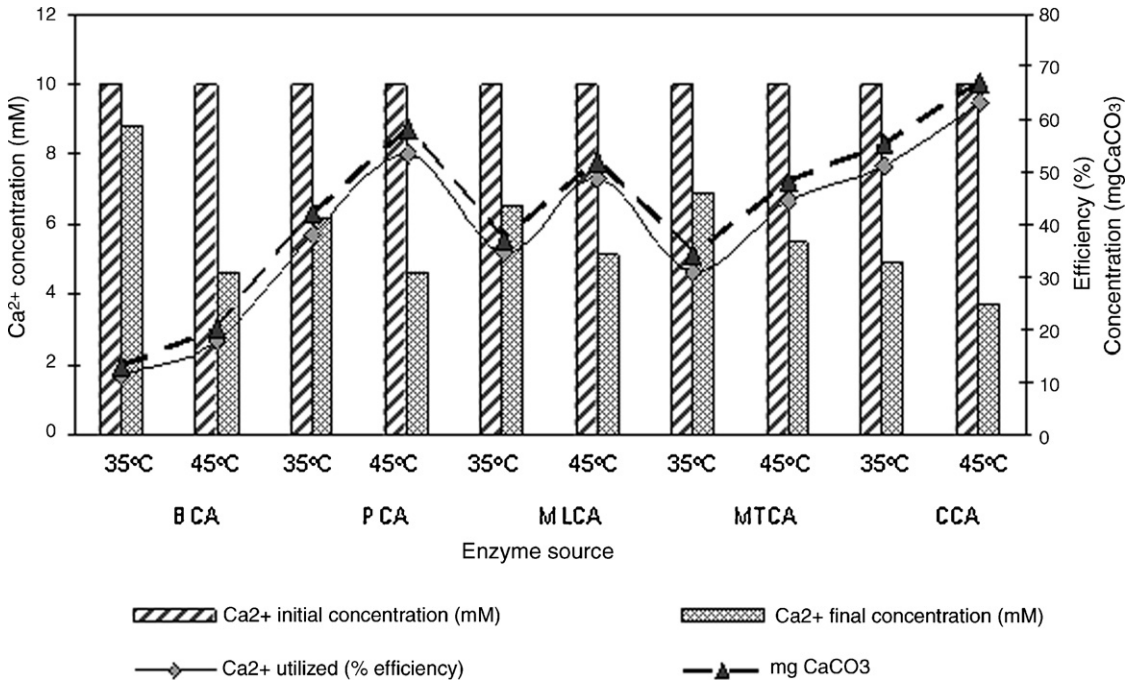

Fig. 3. Determination of CO_2-sequestration efficiency using CO_2 saturated ASW, nitrate and sulphate ions and $CaCl_2$ solution, at 35 °C and 45 °C. 100 μg of BCA (bovine carbonic anhydrase), PCA (*P. fragi* CA), MLCA (*M. lylae* CA), MTCA (*M. luteus* 2 CA) and 100 CCA μg (enzyme consortia CA) were used in study.

study the enhanced sequestration efficiency achieved by consortia CA (61.0%), *P. fragi* CA (53.3%), *M. lylae* CA (48.8%) and *M. luteus* CA (44.4%) compared to commercial BCA (17.8%) under process parameters simulating an onsite scrubber, substantiating the importance of robust CA from bacterial domain for biomimetic CO_2 sequestration. The present study also provides vital information regarding different physical and chemical factors that will influence the calcium carbonate precipitation by carbonic anhydrase in an onsite scrubber. At 45 °C, using ASW-based solution; the sequestration efficiency of *M. luteus* 2 CA decreased by 19.4% followed by BCA (13.5%), consortia CA (7.95%) and *M. lylae* CA (3.75%). This data corroborates to the inhibition profile of CAs for different ions. The significantly reduced sequestration efficiency of BCA and *M. luteus* 2 CA is reflected by their low CA activity in presence of different ions. Interestingly between pH 8.0–8.5 and temperature 45 °C, *M. luteus* 2 CA showed maximum stability compared to other CAs, a result consistent with its sequestration efficiency in absence of ionic inhibitors. Thus, ascertaining the key role of physical factors for accomplishment of biomimetic CO_2-sequestration.

In the present study successful calcium carbonate precipitation was achieved at pH ~ 9.2. The sequestration process has to be carried out near ambient conditions, as recourse to caustic conditions will not be environmentally favorable. Thus the whole experimental setup was adjusted in the pH range 7.5–9.5, in order to strike proper balance with respect to enzyme activity, precipitation of calcium carbonate and environmentally amenable conditions. A much higher rate of $CaCO_3$ precipitation was observed in presence of enzyme catalysed reaction compared to uncatalysed reaction. Similar reports have been documented by Favre et al. [26] and Mirjafari et al. [25]. Liu et al. [3] have reported successful calcium carbonate formation using different brines at pH 8.5–8.7. Ramanan et al. [17] studied the carbonation reaction using tris buffer at pH 8.3. Formation of calcium carbonate using tris buffer at various pH has also been demonstrated by Favre et al. [26].

The most interesting phenomena observed were the capability of enzyme consortia to sequester maximum calcium carbonate under all process parameters. This result is of substantial significance as for the first time application of an amalgamation of indigenous CAs with varying properties has been successfully used to demonstrate enhanced biomimetic sequestration of CO_2 into $CaCO_3$ compared to commercial bovine CA under process parameters simulating an on-site scrubber.

5. Conclusion

The study for the first time provides a comparative analysis of CAs from indigenous bacterial strains and commercial BCA with the perspective of biomimetic CO_2 sequestration. It also proves the importance of different physicochemical factors and the central role they play in a successful carbonation reaction. The study primarily demonstrates that the enzyme consortia from indigenous bacterial strains can provide a better alternative to the existing source of biological catalyst in lieu with designing of a more efficient onsite scrubber.

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