An attempt in mitigating global warming through carbonic anhydrase mediated carbon sequestration

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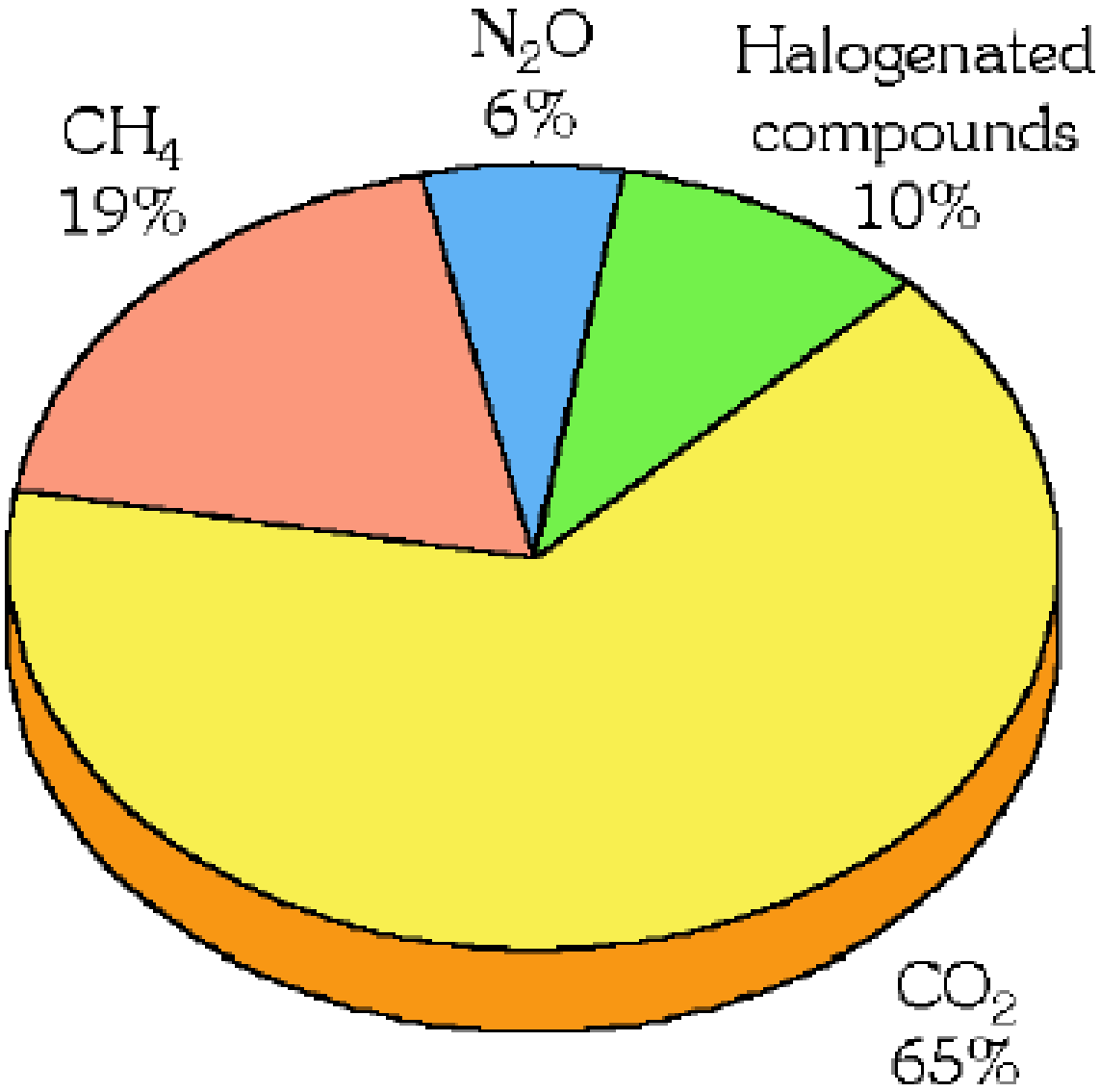
GLOBAL WARMING

Increase in the average temperature of Earth's near-surface air and oceans

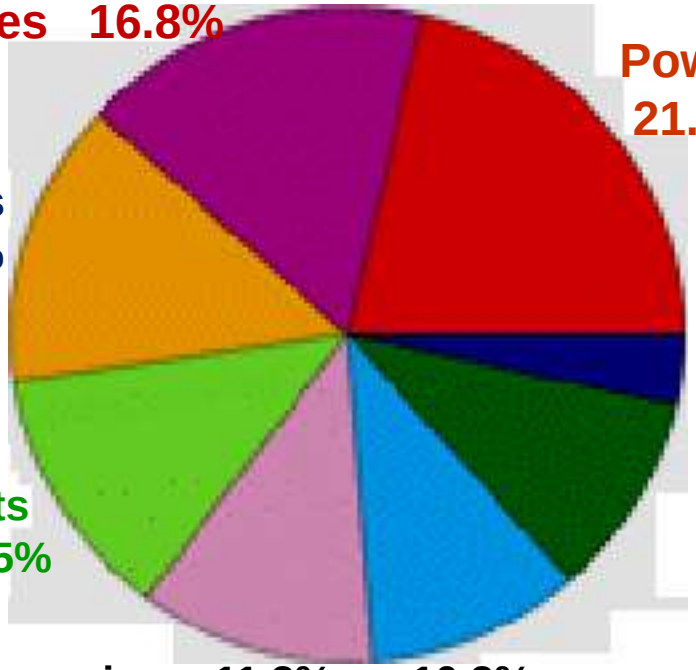
Global surface temperature has increased by 0.74 ± 0.18 °C in 20th century

This may rise further to 1 – 6.4 °C in 21st century, if not checked.

CONTRIBUTION TO GLOBAL WARMING



Annual Greenhouse Gas Emissions



Annual Release of CO_2 into the atmosphere: 22 billion tonnes

Global Warming:The Current Scenario

- In the past 100 years, global temperatures are the warmest at present.
- Atmospheric CO_2 has increased by 31% from pre-industrial levels.
- Ice is disappearing from the Arctic Ocean and Greenland.
- If the Antarctic and the Arctic ice melts, sea levels would rise by almost 11 meters.

Global Warming: The Consequences

Some anticipated effects include:

- Sea level rise of 110 to 770 mm by 2100
- Repercussions to agriculture
- Possible slowing of the thermo-haline circulation
- Reductions in the ozone layer
- Increased intensity and frequency of hurricanes and extreme weather events
- Lowering of ocean pH
- The spread of diseases such as malaria and dengue fever
- Mass extinction events
- Physiological effects involving reduction in the pH value of the blood serum (acidosis)
- Reduction in rains

Combating Global Warming

- Reduction of energy use (per person)
- Shifting from carbon-based fossil fuels to alternative energy sources
- Carbon capture and storage; Geo-engineering including carbon sequestration
- Population control

Mineralization of CO_2

CO_2 reacts with available metal oxides, which in turn produces stable carbonates. This process occurs naturally over many years and is responsible for a large amount of surface limestones.

$$CO_{2\,(g)} \leftrightarrow CO_{2\,(aq)} \quad (1)$$

$$CO_{2\,(aq)} + H_2O \leftrightarrow H_2CO_3 \quad (2a)$$

$$H_2CO_3 \leftrightarrow H^+ + HCO_3^- \quad (2b)$$

$$HCO_3^- + OH^- \leftrightarrow CO_3^{-2} + H_2O \quad (3)$$

$$Ca^{+2} + CO_3^{-2} \leftrightarrow CaCO_3 \downarrow \quad (4)$$

Advantage of the process

- Mineral carbonation is thermodynamically favourable and occurs naturally
- Raw materials such as mineral silicates and industrial wastes rich in MgO and CaO are abundant
- Produced carbonates are stable
- The process can be made economical by utilizing carbonates

Uses of mineral carbonates

- Can be used for synthesis of industrially valuable and useful by-products such as chemicals, cements and construction materials, white pigment in paints, a therapeutic source in antacids and calcium supplements, and tableting excipient as well as remediation of waste feed stocks

- Mineralization process parameters can be optimized to produce high purity valuable metals, silica and carbonate mineral powders

The conventional carbonation pathways are, however, very slow under ambient temperature and pressure.

Carbon sequestration

Carbon sequestration or CCS (carbon capture and storage) can be defined as the capture and secure storage of carbon that would otherwise be emitted to or remain in the atmosphere

Methods of carbon sequestration

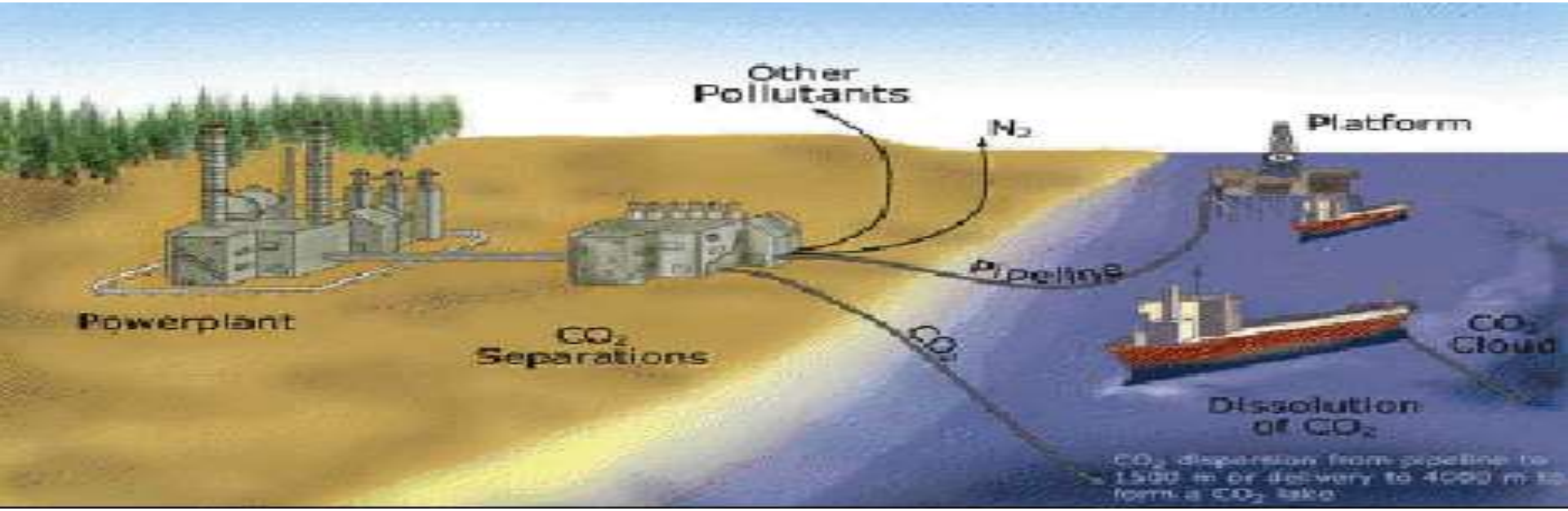
1. Terrestrial sequestration in plants and soil

2. Geological sequestration

- Underground structures eg. Unminable coal seam
- CO_2 is sometimes injected into declining oil fields to increase oil recovery
- CO_2 can also be sequestered in deep saline aquifers where it displaces brine and some of it would get partially dissolved

3. Ocean sequestration

Carbon sequestration by direct injection into the deep ocean involves the capture, separation, concentration, transport, and injection of CO_2 from land or tankers



Drawbacks associated with ocean and geological storage of carbon dioxide

- Future risk of leakage from the site of injection and could cause local ecological damage.
- Separation, concentration and transportation increases the cost of the process

Carbon sequestration using biological systems

Algal cultivation

Heterotrophic microbes

Carbonic anhydrase

Carbonic anhydrases (CA) are one of the fastest known (K_{cat} ranging from 10^5 to 10^7 s^{-1}) and ubiquitously present zinc containing metalloenzymes that catalyzes the interconversion of CO_2 and water to bicarbonate and protons.

$$\mathbf{CO_2 + H_2O \leftrightarrow HCO_3^- + H^+}$$

CA can speed up the process of calcification by catalyzing the rate-determining step (step 2a) in the conversion of CO_2 to $CaCO_3$.

Mechanism of action of CA

$$\mathbf{E\text{-}Zn^{2+}\text{-}OH^- + CO_2 \leftrightarrow Zn^{2+}\text{-}HCO_3^-} \qquad \mathbf{(2a)}$$

$$\mathbf{Zn^{2+}\text{-}HCO_3^- \leftrightarrow E\text{-}Zn^{2+}\text{-}H_2O + HCO_3^-} \qquad \mathbf{(3b)}$$

$$\mathbf{E\text{-}Zn^{2+}\text{-}H_2O \leftrightarrow H^+ \text{-}E\text{-}Zn^{2+}\text{-}OH^-} \qquad \mathbf{(2c)}$$

$$\mathbf{H^+ \text{-}E\text{-}Zn^{2+}\text{-}OH^- + B \leftrightarrow E\text{-}Zn^{2+}\text{-}OH^- + B\text{-}H^+} \qquad \mathbf{(2d)}$$

Types of carbonic anhydrases (CA)

There are at least six distinct CA families (α, β, γ, δ, ζ and η).

These families have no significant amino acid sequence similarity and are an excellent example of convergent evolution.

Desirable characteristics for an ideal CA to be useful for CO_2 mineralization

- **Thermostability**
- **Alkalistability**

Carbonic anhydrase assay methods

Wilbur Anderson assay

Its an electrometric assay in which the time required (in seconds) for a saturated CO_2 solution to drop the pH of 0.02 M Tris·HCl buffer from 8.3 to 7.3 at 0°C is determined. The time without enzyme is recorded at T_0; with enzyme, T.
(pH meter: Metrohm, Switzerland with biotrode electrode)

WA Unit =T_0 –T/T

1 WA unit is defined as the amount of enzyme that causes the pH of a 20 mM Tris buffer to drop from pH 8.3 to 7.3 per minute at 0 °C.

CHEMIST'S PRAYER

Lord I fall upon my knees
And pray that all my syntheses
May no longer be inferior
To those conducted by bacteria

OBJECTIVES

1. Selection of a potent carbonic anhydrase (CA) producing bacterial strain
2. Optimization of native CA production
3. Purification and characterization of native CA
4. Cloning, purification and characterization of CA produced heterologously in *E. coli* and *Pichia pastoris*
5. Application of CA in biomimetic carbon sequestration
6. Immobilization of CA and its utility in carbon sequestration

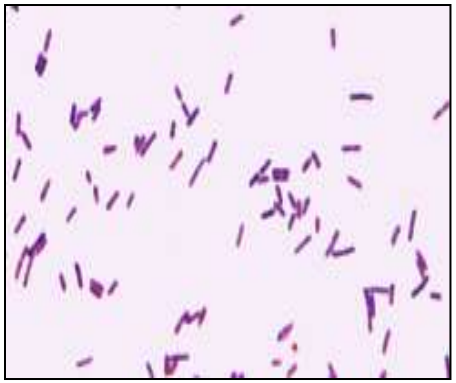
Screening of a potent carbonic anhydrase producing strain*

Strain	Location	U/ml	U/g dry biomass (gdbm)	SEA (U/mg)
***Bacillus halodurans* (TSLV1)**	Extracellular	-	-	-
	Intracellular		6,300 ± 430	4.3
***Geobacillus thermoleovorans* (NP33)**	Extracellular	-		
	Intracellular		-	
***G. thermodenitrificans* (C360)**	Extracellular	-		
	Intracellular		-	
Sporosarcina pasteurii	Extracellular	0.33		0.043
	Intracellular		26 ± 2.0	0.63

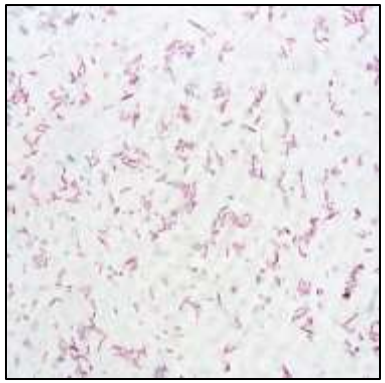
***By Wilbur-Anderson assay**

Identification of the selected bacterium

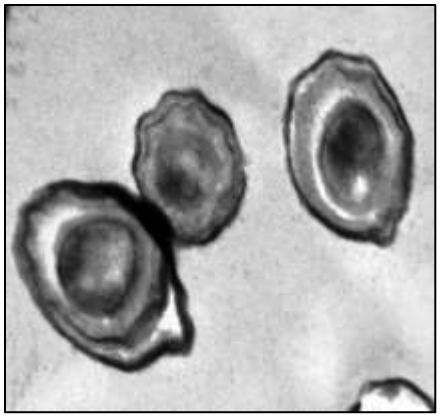
Bacillus halodurans (TSLVI)



Gram's staining



Endospore staining

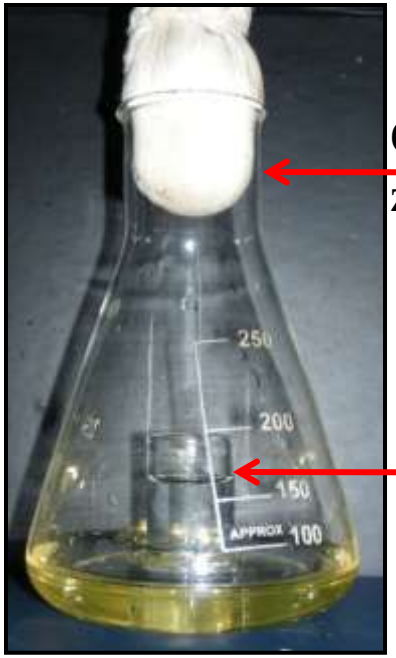


Endospore under TEM

Bacillus halodurans is a rod-shaped, Gram positive, motile and spore forming bacterium isolated from the alkaline sediments of Lonar Lake.

Effect of elevated levels of CO_2 on growth and CA production by *Bacillus halodurans*

CO_2 concentration (%)	U/ gdbm
0	6,344 ± 320
0.03	6,456 ± 385
5.0	5,822 ± 452
10.0	5,004 ± 765



Cotton plug containing zeolite

5M KOH

Optimization of carbonic anhydrase production by *B. halodurans* TSLV1

Enzyme preparation

Cells harvested and washed with saline and resuspended in 10 mL, 20 mM Tris buffer pH 8.3

↓

Sonication at 50% amplitude (3s pulse on 3s off)

↓

Cell debris removed by centrifugation.

↓

Supernatant served as crude enzyme

Optimal variables for carbonic anhydrase production by *B. halodurans*

Culture variables that significantly affected carbonic anhydrase production identified by one-variable-at-a-time approach

Component	%
Starch	0.5
Peptone	0.5
KH_2PO_4	0.1
$MgSO_4$	0.05
pH	8.5
Temperature (°C)	45.0
Agitation	200 rpm
Inoculum age	8 h
Inoculum size	2(%)1.5x10^6 cfu ml^{-1}

Approach	CA production (U / gdbm)	Fold increase in production	Specific activity (U/mg protein)
Unoptimized medium	6, 300 ± 580	1	4.3
Optimized medium (One-variable-at-a-time approach)	25, 000 ± 800	3.97	12

Optimization of CA production by Statistical methods

S.no.	Starch (%)	$MgSO_4$ (%)	Inoculum size (%)	Predicted values	Observed values
1	2.5	0.12	2.49	30,278.8	31,120±985
2	2.13	0.10	3.71	29,573.5	28,203±1,024
3	2.92	0.08	2.99	34,353.2	35,920±1,105
4	3.34	0.07	3.54	30,672.9	29,728±1,000

Approach	CA production (U / gdbm)	Fold increase in production
Unoptimized medium	6,300 ± 350	1
Optimized medium (Statistical approach)	35,920 ± 1105	5.7

Component	%
Starch	2.5
Peptone	0.5
KH_2PO_4	0.1
$MgSO_4$	0.1
pH	8.5
Temperature (°C)	45.0
Agitation	200 rpm
Inoculum age	8 h
Inoculum size	3(%)

Optimized culture variables

Purification of CA from *B. halodurans* TSLV1

Steps involved in the purification process

Crude lysate

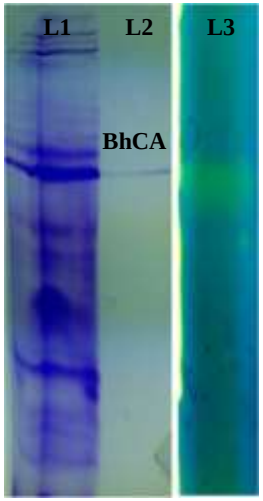
Acetone precipitation(0-50%, 50-70%)

Anion exchange using (Hicapto™)

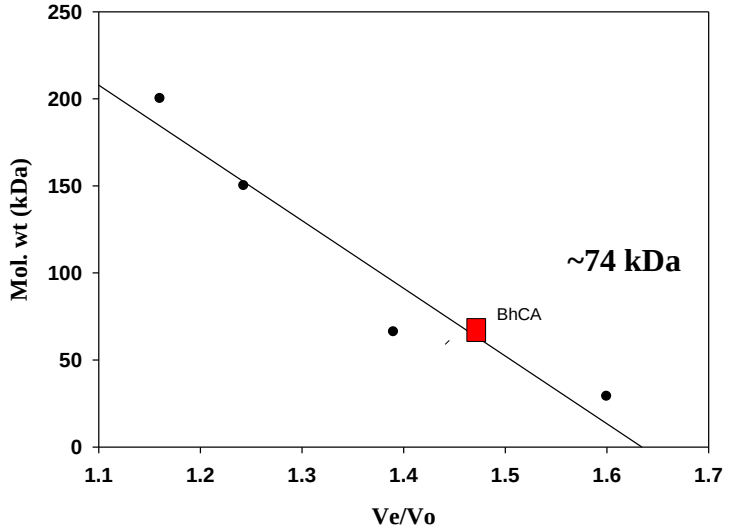
pAMBS affinity chromatography

Purification profile

Purification step	Activity (WA U/mL)	Total activity (WA U)	Protein (mg/mL)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude protein	329.0	21155.0	7.6	43.29	100	1.0
Acetone precipitation	2311	12666	27.18	85	59.8	1.97
Anion exchange using (Hicapto Q™)	4798.0	9076.1	2.5	1912.2	42.9	44.17
pAMBS affinity chromatography	625.88	8136.44	0.05	3,425	38.46	79.11

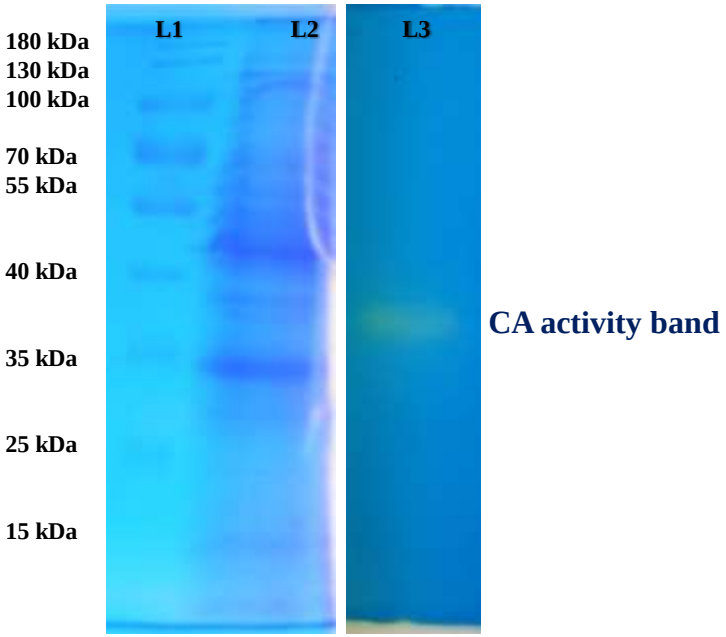


. Figure Native PAGE of purified BhCA along with zymogram. *Lane 1*: Crude lysate, *Lane 2:* Purified BhCA, *Lane 3:* Zymogram of BhCA



Molecular weight markers used with purified BhCA (1) β-Amylase (200); (2) Alcohol dehydrogenase (150); (3) Albumin (66); (4) Carbonic anhydrase (29kDa); (5) Cytochrome C (12.4kDa).

Zymogram of crude lysate of *B. halodurans*

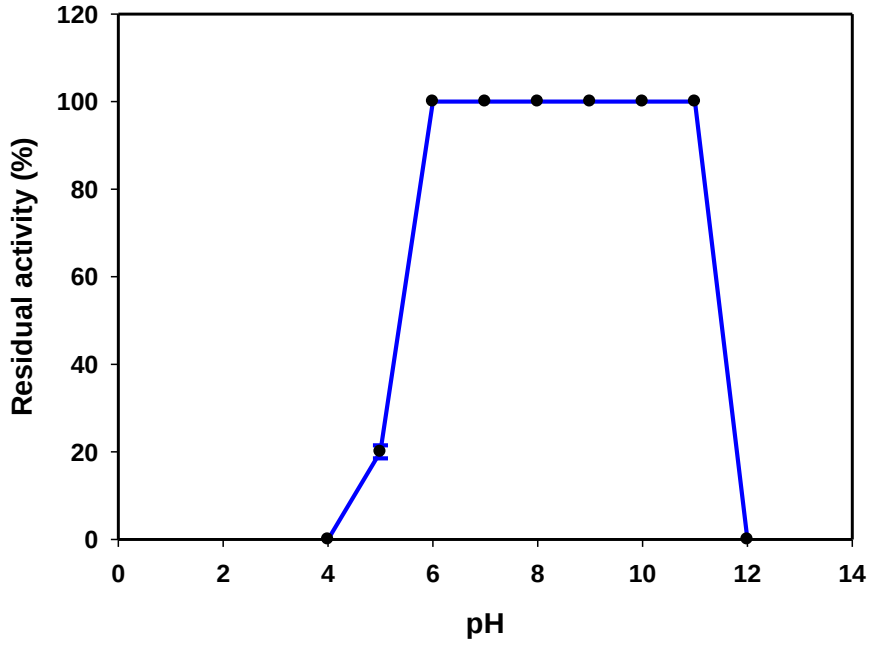


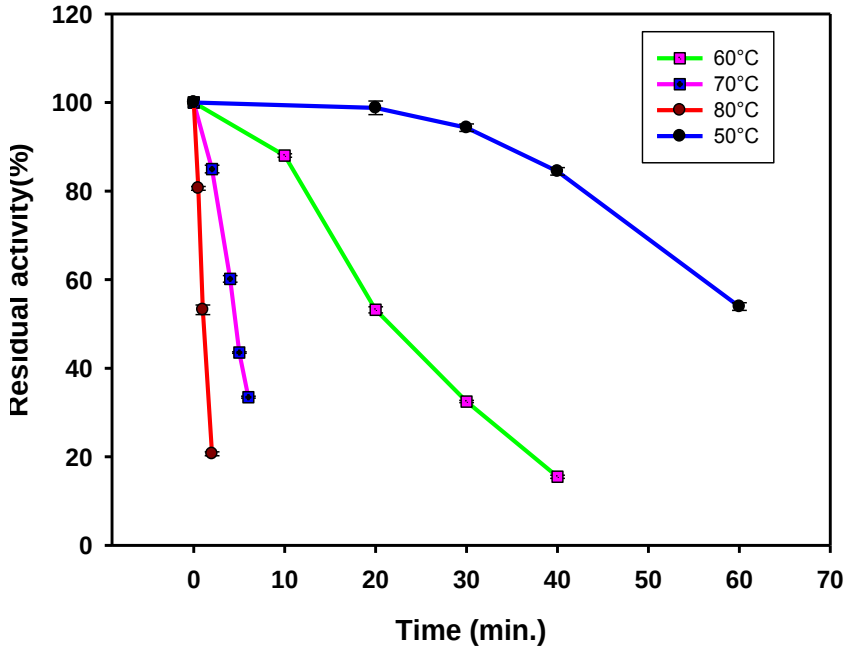
SDS PAGE of crude lysate of *B. halodurans*: L1: Protein molecular weight marker, L2: Crude lysate, L3: Zymogram of CA activity

Environ. Sci. Pollut. Res. (2016): 23: 15236 – 249.

Characterization of BhCA

Effect of different pH and temperature on the stability of BhC





Effect of different pH on BhCA stability:
BhCA is stable in pH 6.0-11.0 for 24 h retaining 100 % activity

Effect of different temperatures on BhCA stability:
$T_{1/2}$ is 65 ± 1, 25 ± 1, 4.7 ± 0.5 and 1.2 ± 0.2 min at 50, 60, 70 and 80° respectively

Effect of CA specific inhibitors on the activity of BhCA

Inhibitor	AZA	EZA	MZA	SA	BSA	SNA	AS
IC_{50} (µM)	0.22	0.33	1.03	8580	4.58	76.2	168.36

***AZA- Acetazolamide**
EZA- Ethazolamide
MZA- Methazolamide
SA- Sulfamic acid
BSA- Benzenesulfonamide
SNA- Sulfanilamide
SA- Sulfamic acid

Effect of different metal ions and anions on enzyme activity

Metal ions	Concentration	Residual activity (%)
Mg^{2+}	1 mM	100±0.30
	5 mM	100±1.04
Zn^{2+}	500 µM	100±1.00
	1 mM	55.47±1.21
Hg^{2+}	500 µM	31.83±0.31
	1 mM	23.37±0.26
	5 mM	0±0.5
Co^{2+}	1 mM	40.43±0.27
	5 mM	27.41±0.46
Cu^{2+}	500 µM	44.60±1.19
	1 mM	36.66±0.38
	5 mM	0±1
Mn^{2+}	1 mM	99.57±1.24
	5 mM	100.±0.95
Ca^{2+}	1 mM	99.43±1.30
	5 mM	100.08±0.73
Ni^{2+}	1 mM	22.65±0.30
	5 mM	18.97±0.14
Fe^{3+}	1 mM	21.36±0.27
	5 mM	0±0.5
Fe^{2+}	1 mM	27.72±1.17
	5 mM	0.64±1.24
Al^{3+}	1 mM	100 ±0.82
	5 mM	100.01±0.61
Ag^{2+}	1 mM	100±0.27
	5 mM	100.±0.61
Sn^{2+}	1 mM	124.71±3.91
	5 mM	148.16±1.28
Pb^{2+}	1 mM	100±0.27
	5 mM	68.92±1.79
Ba^{2+}	1 mM	100.92±1.50
	5 mM	100±0
NH_4^+	1 mM	99.5±1.25
	5 Mm	100±0.5
Na^+	1 M	100.87 ±0.29
	2 M	73.27 ±1.64

Stimulators:
Sn^{2+}, SO_4^{2-}

No observable effect of SO_3^{2-}

Anions	Concentration	Residual activity(%)
SO_4^-	1 M	167.8 ±2.76
	0.125 M	100 ±2.44
SO_3^{2-}	1.0 M	100.66 ±0.86
	1.25 M	100 ±0
NO_3^-	0.5 M	100 ±2.08
	1 M	83.01 ±1.66
	1.5 M	75.12 ±2.64
HCO_3^-	0.1 M	100 ±0
	0.75 M	30.25±1.42
CO_3^{2-}	0.01 M	100 ±0.12
	0.05 M	75.63 ±0.66
	0.1 M	60.05 ±1.08
Cl^-	0.5 M	100.27 ±1.71
	1 M	100.8 ±1.53
I^-	1 mM	100.6 ±1.78
	5 mM	99.8 ±1.29
F-	1 mM	100±1.5
	5 mM	100±1.0
Br-	1 mM	100±0.5
	5 mM	100±0.76

Effect of different modulators (inhibitors, ionic and non ionic detergents) on enzyme activity

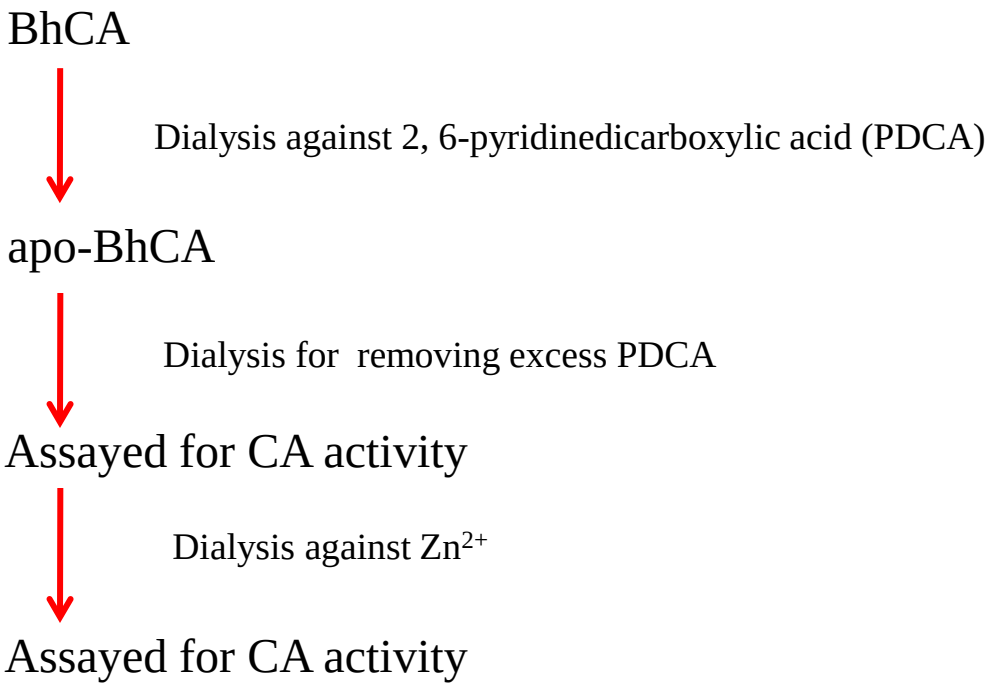
Modulator	Concentration	Residual activity (%)
WRK	1 Mm	96 ±3.7
	5 mM	0 ±0.55
NBS	1 mM	6.0±1.21
	5 mM	0 ±0.53
NEM	1 mM	95.3 ±2.57
	5 mM	0 ±0.41
DEPC	1 mM	76±1.5
	5 mM	0.85±1
PMSF	1 mM	36.06±1.42
	5 mM	27.44±1.76
DTT	1 mM	93.8 ±2.93
	5 mM	43.2 ±3.89
IAA	1 mM	50.3 ±2.36
	5 mM	0 ±0.63
β-ME	1 mM	100±1.02
	5 mM	80.88
TRITON X100	0.1%	84.5 ±1.54
	0.2%	83.22 ±1.5
TWEEN 80	0.1%	100.2 ±1.67
	0.2%	101.5 ±2.07
SDS	1%	99.24
	5%	86.22
EDTA	50 mM	100 ±0.2
	1 M	100.58±0.66

No observable effect of EDTA

DTT- dithiothreitol
β-ME - β-mercaptoethanol
WRK - Woodward's reagent K
IAA - Iodo acetamide
PMSF- phenyl methyl sulfonyl fluoride
NBS - N-bromosuccinimide
NEM - N-ethylmaleimide
DEPC - Diethylpyrocarbonate
EDTA –ethylenediaminetetraacetic

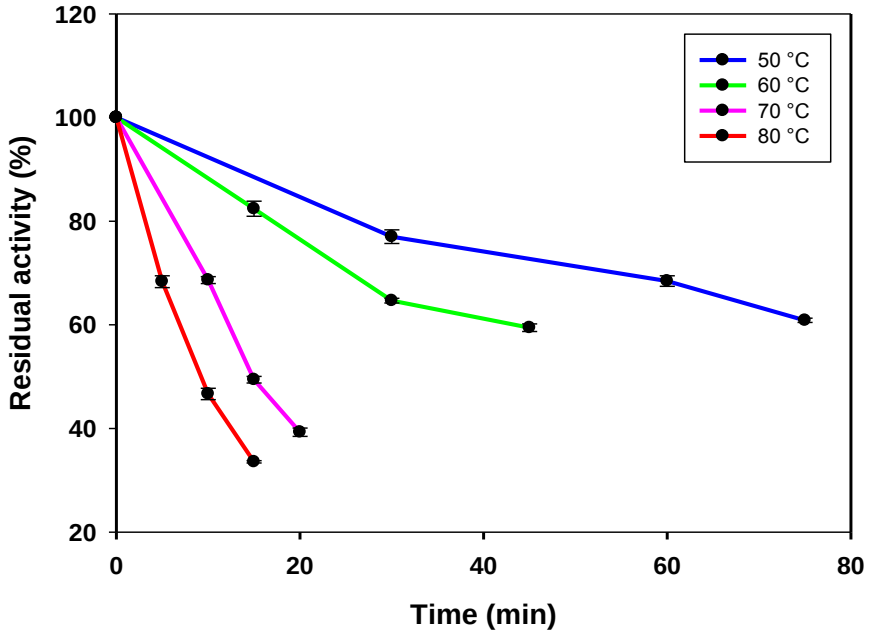
BhCA is a zinc containing metalloenzyme

2, 6-pyridinedicarboxylic acid (PDCA) – specific chelator of zinc ion



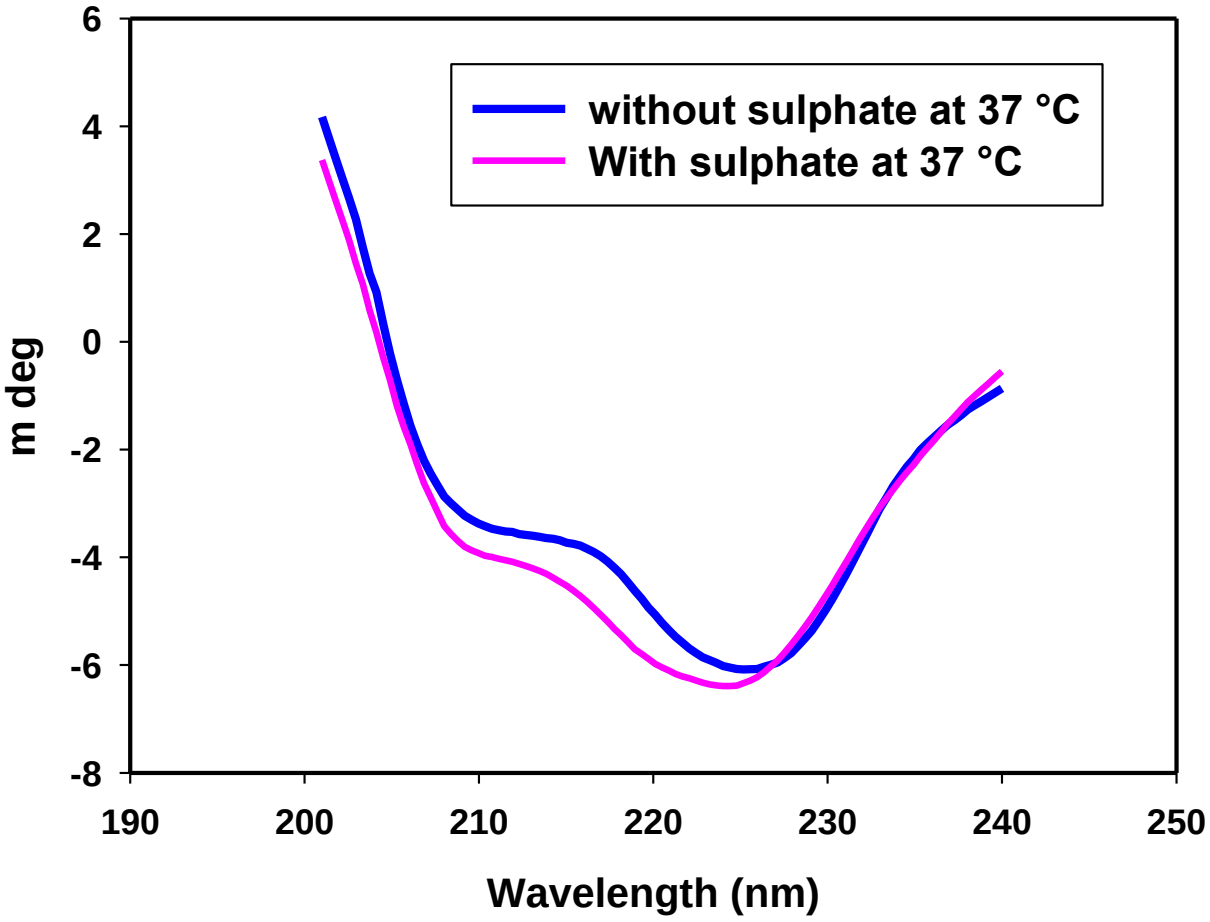
BhCA lost activity upon dialysis against PDCA , and the activity was restored upon dialysis against Zn^{2+} confirmed BhCA to be a zinc metalloenzyme.

Temperature stability of BhCA in presence of sulphate

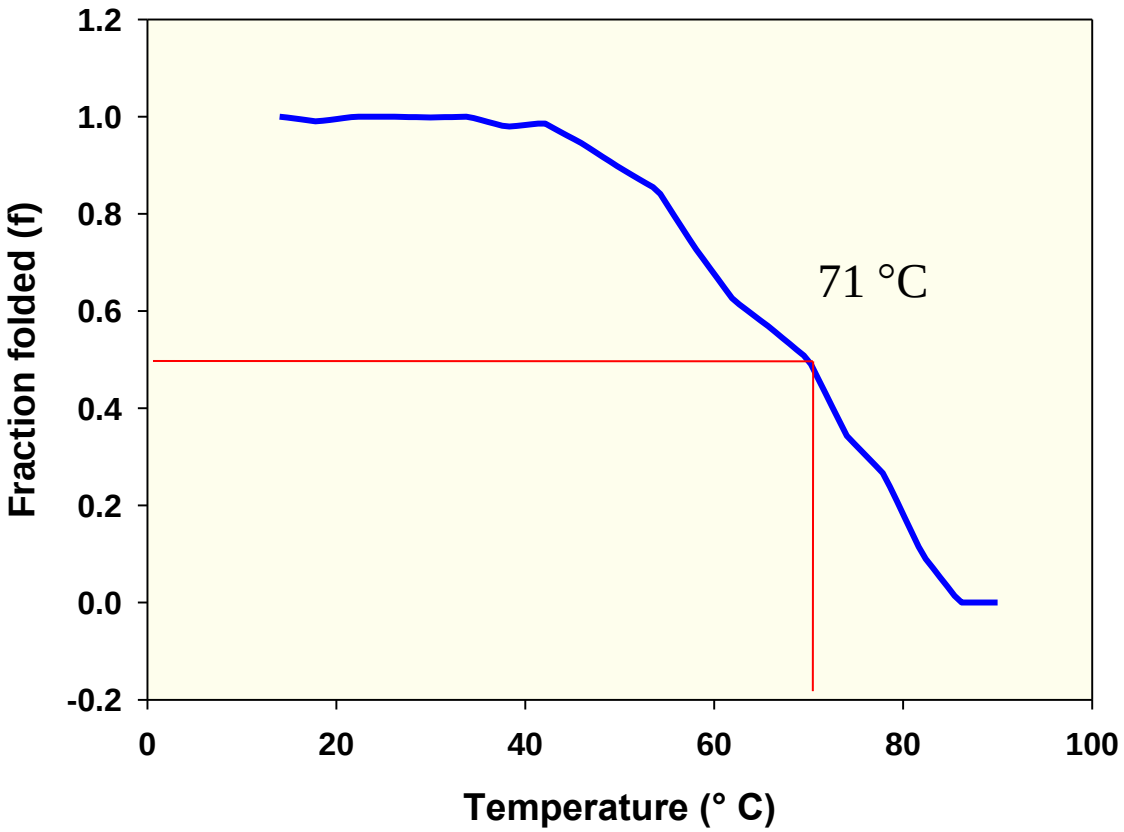


Temperature °C	$T_{1/2}$ min
50	95 ± 1.0
60	54 ± 0.5
70	15± 1.0
80	19 ± 1.0

Far-UV CD spectra of BhCA in presence and absence of sulphate



Melting Temperature (Tm) of BhCA



Thermal unfolding curve for BhCA

Shelf life of BhCA

	Residual activity (%)	
Duration	4 °C	Room temperature
6 months	100	100
12 months	100	100
18 months	100	94
24 months	100	85

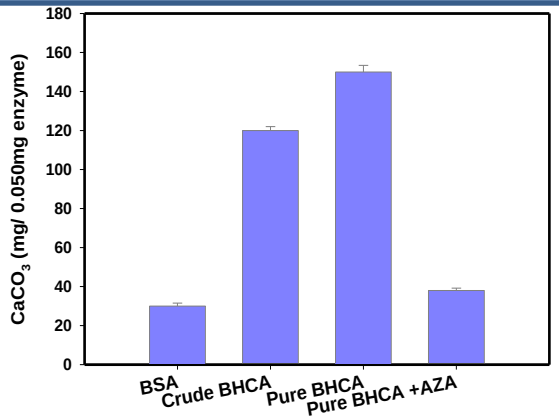
Application of BhCA in biomineralization of CO_2

Turbidometric experiment to study the effect of CA enzymes on acceleration of $CaCO_3$ precipitation

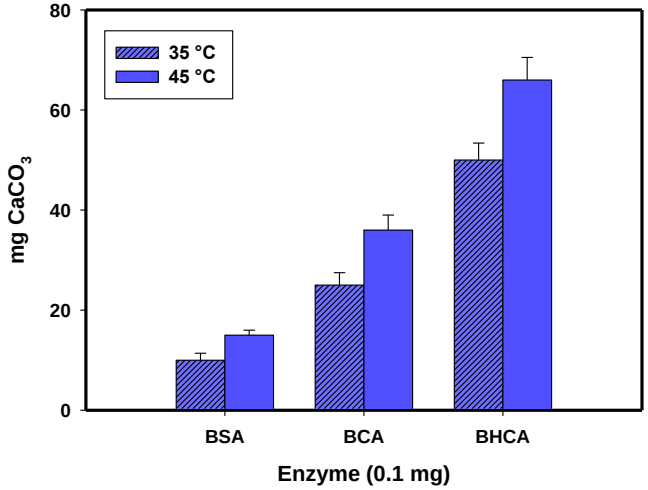
Sample	BSA	BhCA	BCA
Time (s)	130 ± 2.5	8 ± 0.5	38 ± 2.0

*BSA= Bovine serum albumin
BCA= Bovine carbonic anhydrase

Application of BhCA in mineralization based CO_2 sequestration



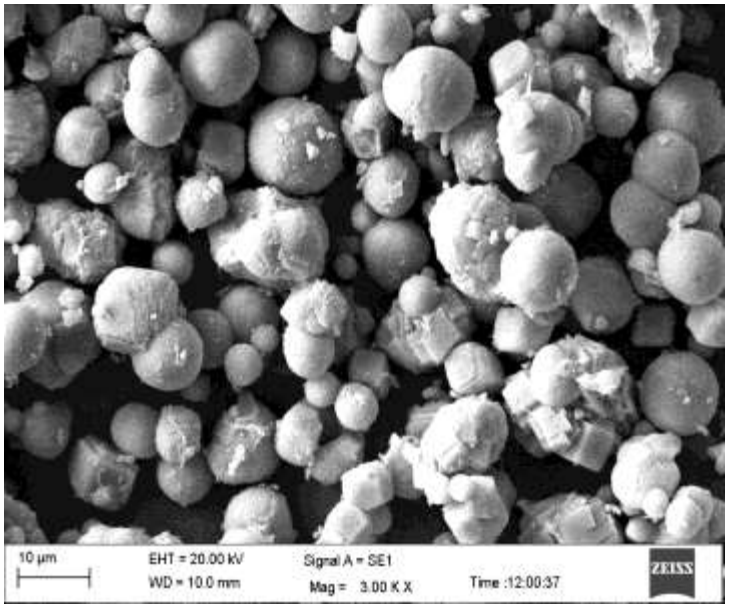
Analysis of carbonate precipitation catalyzed by crude and pure preparations of BhCA. BSA served as a negative control. Specific inhibition of purified BhCA by AZA led to decline in carbonate precipitation

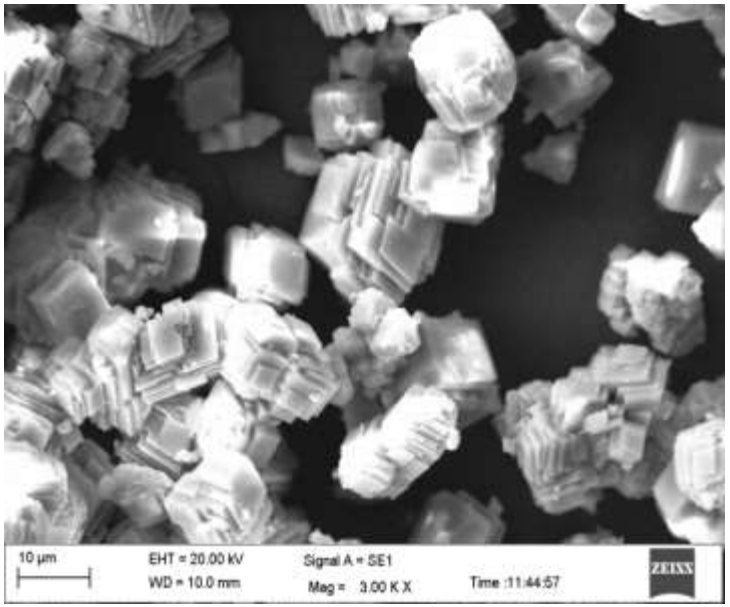


Comparison of sequestration efficiencies of BCA and BhCA at 37 and 45 °C in presence of SO_4^{2-} and NO_3^- in terms of carbonate precipitation. BSA served as a negative control

Mineralization of exhaust gas CO_2 using BhCA and Ca^{2+}

SEM images of $CaCO_3$ precipitate obtained after mineralization of CO_2





(a) Vaterite form of $CaCO_3$ formed in absence of rBhCA; (b) Calcite form of $CaCO_3$ formed in the presence of BhCA

Cloning of *α CA* in *E. coli*

Cloning of α-CA from *B. halodurans*

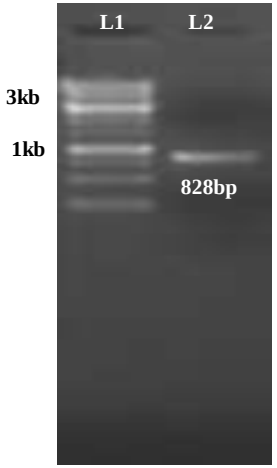
α-CA (828bp)

ATGAAAAAATATTTATGGGGAAAAACGTGTTTAGTTGTATCATTAAGTGTCATGGTGACCGCATGCTCTTCTGCACCTTCCACAGAACCA
GTCGATGAGCCGAGCGAGACACATGAGGAAACGAGCGGTGGCGCACACGAGGTTCATTGGTCTTACACTGGAGACACTGGTCCAGAGC
ATTGGGCAGAGTTGGATTCGGAATATGGTGCTTGCGCTCAAGGAGAAGAGCAGTCACCGATCAACTTAGACAAAGCGGAGGCCGTTGAT
ACCGATACCGAAATCCAAGTTCATTATGAGCCGAGCGCGTTTACGATTAAAAATAATGGTCACACGATTCAAGCAGAGACTACCTCAGA
TGGGAACACGATTGAAATCGATGGAAAAGAATACACACTCGTTCAATTCCACTTCCATATTCCTTCCGAGCATGAAATGGAAGGAAAGA
ATTTAGATATGGAGCTACATTTTGTCCATAAAAATGAAAACGACGAGCTCGCCGTACTCGGGGTCTTAATGAAGGCCGGCGAAGAGAAC
GAAGAGCTAGCGAAGCTATGGTCGAAGCTACCAGCAGAAGAAACAGAAGAAAATATTTCGTTAGATGAGTCAATTGATTTGAACGCGCT
CTTACCAGAAAGCAAAGAAGGATTCCATTACAACGGTTCCTTAACGACGCCTCCTTGCTCAGAAGGGGTAAAGTGGACCGTGCTATCTGA
ACCGATTACTGTTTCACAAGAGCAAATCGACGCGTTTGCTGAGATCTTCCCAGACAATCACCGACCAGTCCAACCTTGGAACGACCGTGA
TGTCTATGACGTGATCACTGAATAG

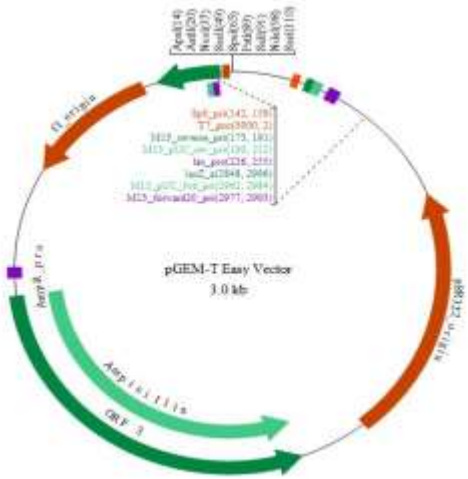
Full length primers:

FP: CCCCCGAATTCATGAAAAAATATTTATGGGGAAAAACGTG

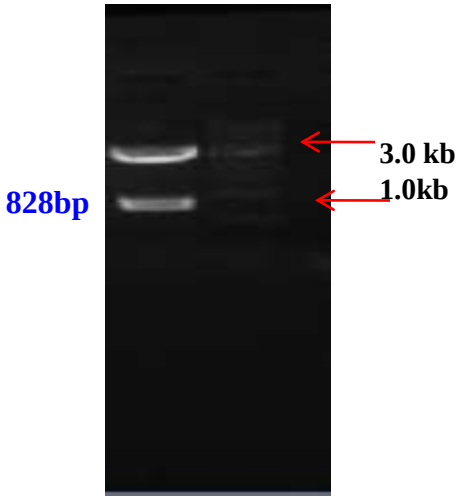
RP: CCCCCGCGGCCGCTTTCAGTGATCACGTCATAGACATCAC



PCR amplification of BhCA



pGEMT-Easy vector



pGEMT fall out

Deduced amino acid sequence of α-CA (275 amino acids)

MKKYLWGKTCLVVSLSVMVTACSSAPSTEPVDEPSETHEETSGGAHEVHWSYTGDTGPEHWAELDSEYGAC

AQGEEQSPINLDKTEAIDTDTEIHVHYEPSSFTIKNNG**H**TIQAETTSDKNTIEIDGKEYTLV**Q**F**H**F**H**IPS**E**HEMEG

KNLDMEL**H**FVHKNENDELAVLGVLMKAGEENEELAQLWSKLPAEETEENISLDESIDLNVLLPESKEGFHYNG

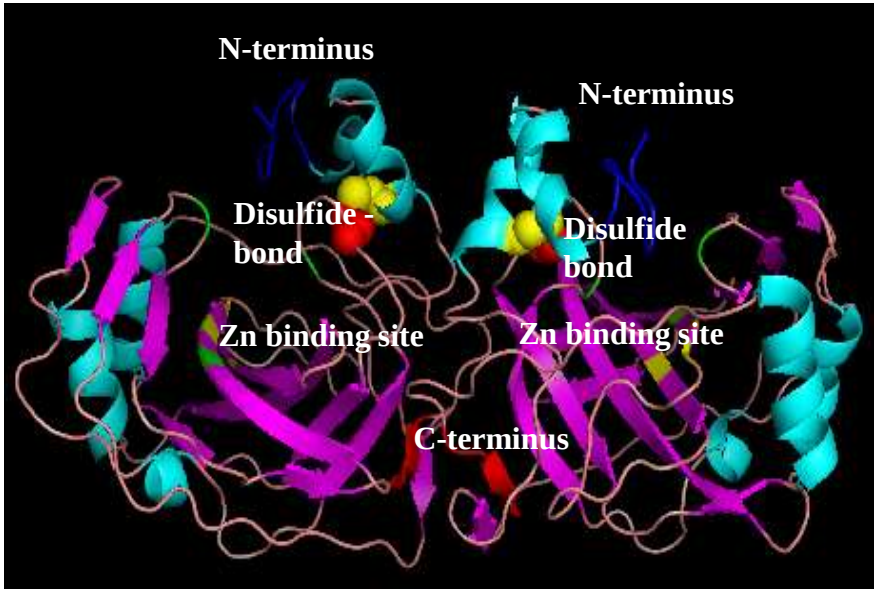
SL**T**TPPCSEGVKWTVLSEPITVSQEQIDAFAEIFPDNHRPVQPWNDRDVYDVITE

Catalytically important amino acids

His 136, His 138, His 155 – involved in zinc binding

Theoretical mol. mass= 31 kDa

Active site : 110-223
Signal peptide : 1-25

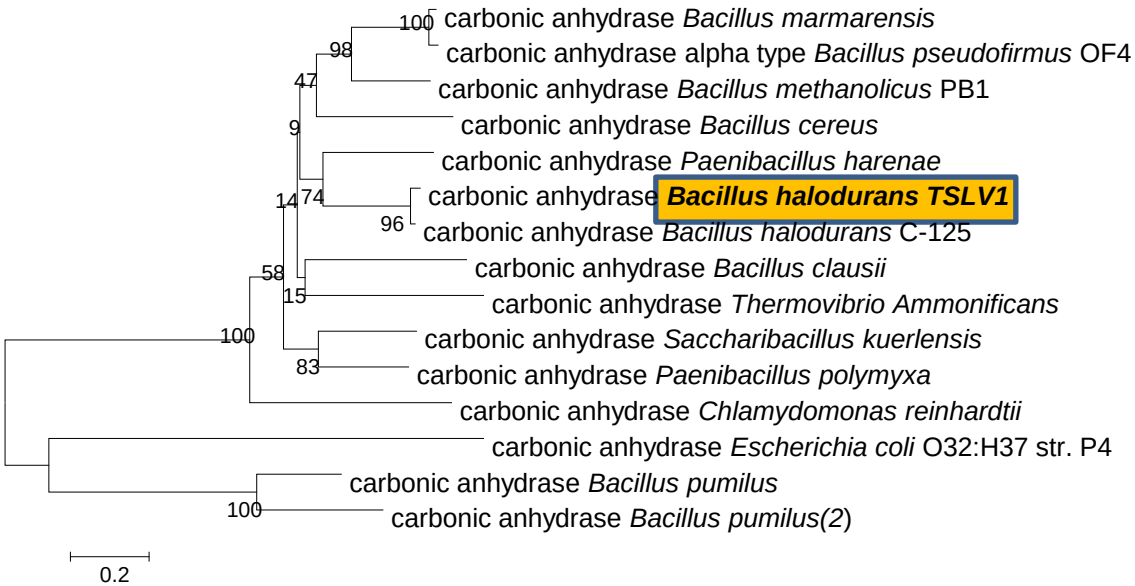


Proposed 3D structure of acidic α-CA from *B. halodurans*. The template α-CA of *Sulfurihydrogenebium azorense* (PDB ID 4x5s.1) shared 43.88% identity with α-CA of *B. halodurans*.

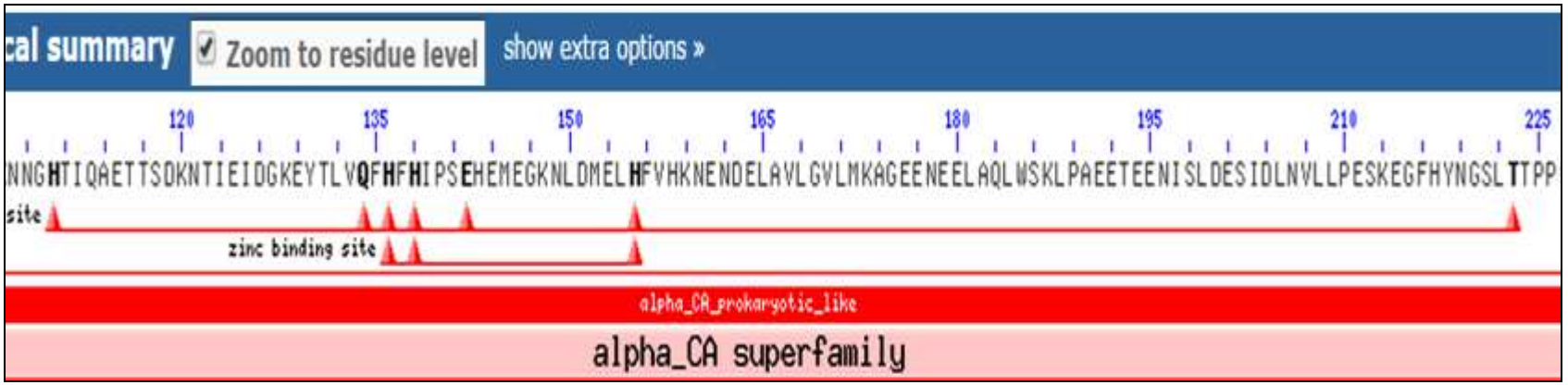
Total number of negatively charged residues (Asp + Glu): 56
Total number of positively charged residues (Arg + Lys): 15

Multiple sequence alignment of α-CA from *B. halodurans* with α-CAs of other microbes

Neighbour joining tree for rBhCA

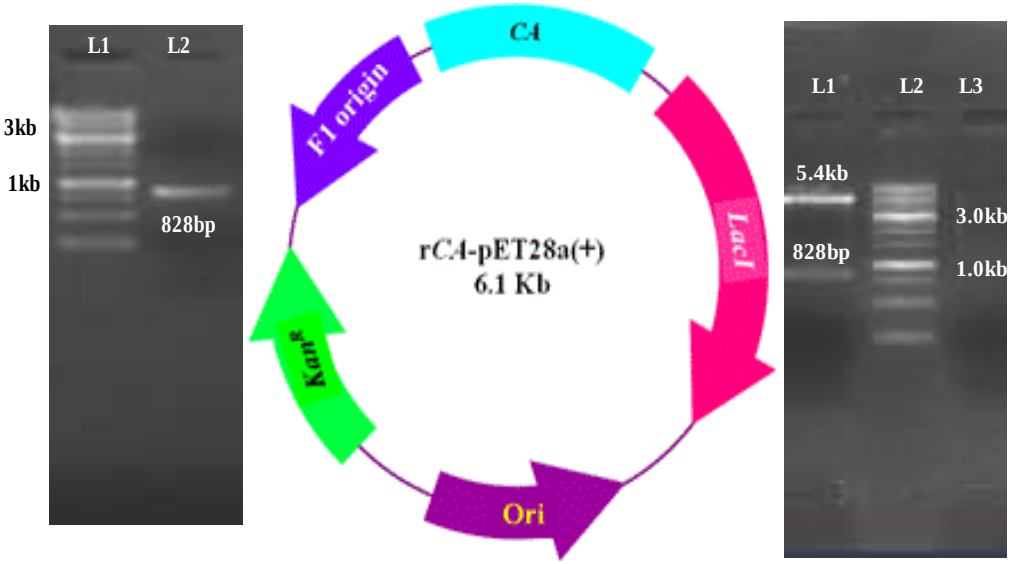


Phylogenetic tree of recombinant α-CA: rBhCA shows highest homology with *Bacillus halodurans* C-125

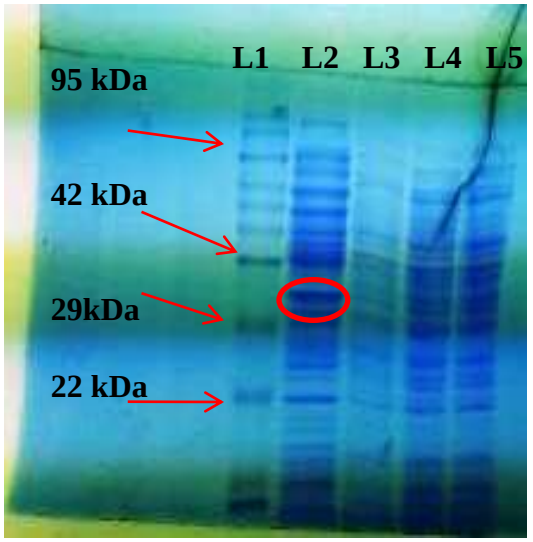


The α-CA encoding gene sequence has been deposited at GenBank database (accession no. KR347171)

Cloning of *BhCA* in pET28a vector & expression analysis after transformation in *E.coli* BL21(DE3)



Construction of the recombinant vector r*BhCA*-pET28a



L1 : Marker, L2: Induced soluble fraction,
L2:Uninduced soluble fraction,
L3: Uninduced inclusion bodies ,
L4:Induced inclusion bodies ,

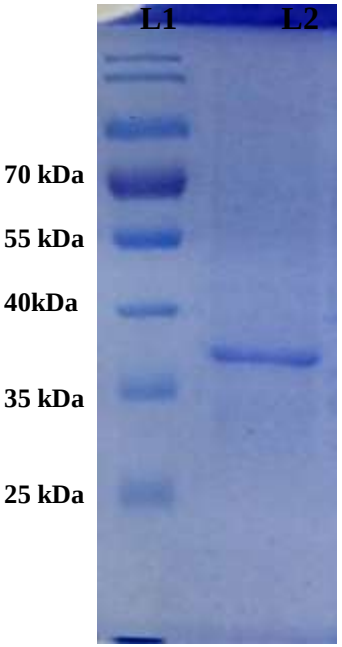
CA production was measured using Wilbur Anderson assay

7,85,000 ± 1000 U/gdbm

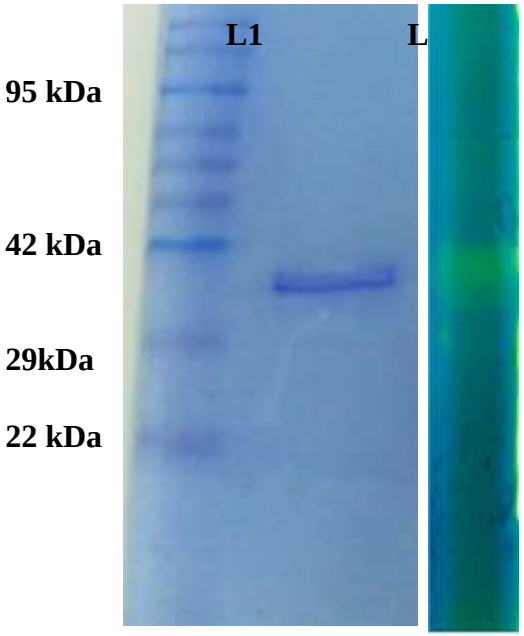
Intern. J. Biol. Macromol. (2017) 31: 3002 -3009

Purification of rBhCA

rBhCA was purified from *E. coli* by using Ni-NTA affinity chromatography. rBhCA was eluted using 300 mM imidazole



L1 : Marker, L2: Purified rBhCA

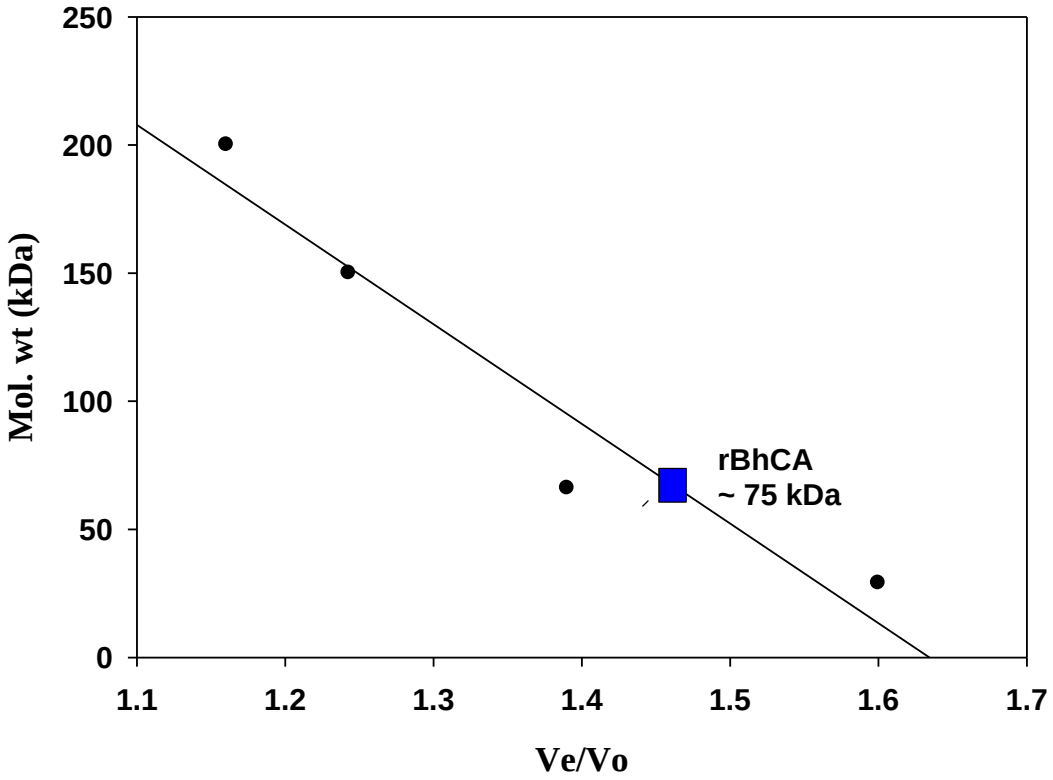


L1 : Marker, L2; Purified rBhCA, L3: Zymogram of CA

	EA (U/ml)	Volume (ml)	Total EA	Protein (mg/ml)	Specific activity (u/mg)	Yield	Fold Purification
Sample loaded	2019.5	5	10,097.5	2.10	961.66	100%	1
Eluate	804.8	8	7,855.6	0.09	8942.2	77.7%	9.2

Characterization of rBhCA

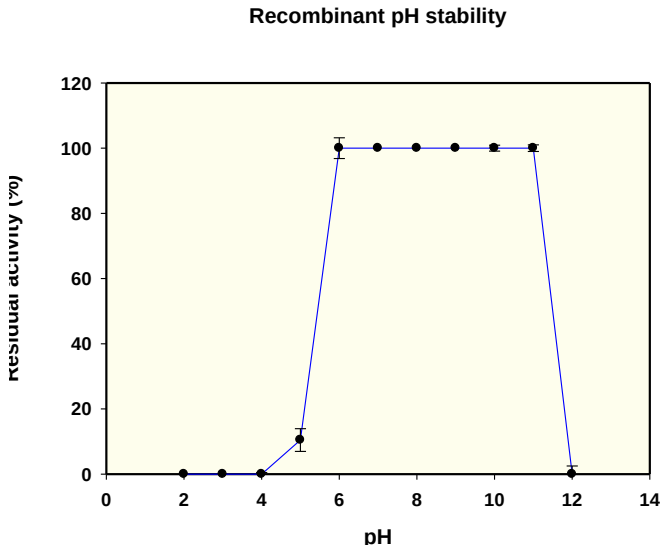
Native molecular weight determination



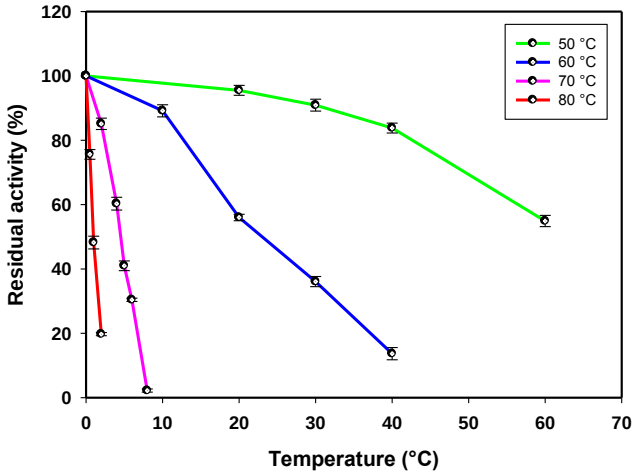
rBhCA mol. wt.~ 75 kDa

Plot of Ve/Vo against molecular weight of proteins on Sephacryl™ S-200 high resolution column (16/60). Molecular weight markers (kDa) used with purified rBhCA. Cytochrome c (12.4kDa), carbonic anhydrase (29kDa), bovine serum albumin (66kDa), yeast alcohol dehydrogenase (150kDa) and sweet potato β-amylase (200kDa)

Effect of different pH and temperature on the stability of rBhCA



Effect of different pH on rBhCA stability



Effect of different temperatures on BhCA stability

$T_{1/2}$ is 64.5 ± 1, 24 ± 1, 4.4 ± 0.5 and 1.0 ± 0.2 min at 50, 60, 70 and 80 °C respectively

Effect of CA specific inhibitors on the activity of rBhCA

Inhibitor	AZA	EZA	MZA	SA	BSA	SNA	AS
IC_{50} (µM)	0.25	0.35	1.0	8610	4.0	76.9	166.5

* Acetazolamide (5-acetamido-1-thia-3, 4-diazole-2-sulphonamide, AAZ), methazolamide (MZA), Ethoxyzolamide (EZA), Sulfanilamide (4-amino benzene sulphonamide, SNA), sulfamic acid (SA), Benzenesulfonamide (BSA) and Ammonium sulfamate (AS)

*IC50 = Half maximal inhibitory concentration

Effect of different metal ions, anions on enzyme activity

Metal ions	Concentration	Residual activity (%)
Mg^{2+}	1 mM	100±0.30
	5 mM	100±1.5
Zn^{2+}	500 µM	100±0
	1 mM	50.5±1.5
Hg^{2+}	500 µM	33.83±1.41
	1 mM	22.08±1.26
	5 mM	0±1.5
Co^{2+}	1 mM	43.44±1.8
	5 mM	25.80±2.6
Cu^{2+}	500 µM	46.60±1.9
	1 mM	34.56±1.58
	5 mM	0±1.6
Mn^{2+}	1 mM	100±0.5
	5 mM	100±1.5
Ca^{2+}	1 mM	100±1.60
	5 mM	100±0
Ni^{2+}	1 mM	20±2.50
	5 mM	15.8±1.54
Fe^{3+}	1 mM	24.36±1.87
	5 mM	0±0.9
Fe^{2+}	1 mM	25.88±2.1
	5 mM	0±.5
Al^{3+}	1 mM	100 ±1
	5 mM	100±1.8
Ag^{2+}	1 mM	100±1
	5 mM	100±2.88
Sn^{2+}	1 mM	126.44±2.55
	5 mM	143.16±3.54
Pb^{2+}	1 mM	100±0
	5 mM	70.06±2.76
Ba^{2+}	1 mM	100±0
	5 mM	100±1.5
NH_4^+	1 mM	100±0.25
	5 mM	100±0.5
Na^+	1 M	1100 ±0.29
	2 M	75.5 ±2.8

Stimulators:
Sn^{2+}, SO_4^{2-}

No effect of SO_3^{2-}

Anions	Concentration	Residual activity(%)
SO_4^-	1 M	170 ±2.5
	0.125 M	100 ±0
SO_3^{2-}	1.0 M	100.±0.5
	1.25 M	100 ±0
NO_3^-	0.5 M	100 **±1.5**
	1 M	85.16 **±1.5**
	1.5 M	77.44**±1.88**
HCO_3^-	0.1 M	100 ±0
	0.75 M	32.55±2.5
CO_3^{2-}	0.01 M	100 ±0
	0.05 M	73.63 ±1.74
	0.1 M	62.54 ±2.45
Cl^-	0.5 M	100 ±1.8
	1 M	100.±1
I^-	1 mM	100.±1.5
	5 mM	99.8 ±1
F-	1 mM	100±1.5
	5 mM	100±1.0
Br-	1 mM	100±0
	5 mM	100±0

Effect of different additives (inhibitors, ionic and non ionic detergents) on enzyme activity

Modulator	Concentration	Residual activity (%)
WRK	1 Mm	92 ±1.5
	5 mM	0 ±0
NBS	1 mM	8.0±1.5
	5 mM	0 ±0.5
NEM	1 mM	97 ±2.6
	5 mM	0 ±0.5
DEPC	1 mM	74±2.0
	5 mM	0.5±1
PMSF	1 mM	38.06±2.6
	5 mM	23.54±0.76
DTT	1 mM	95 ±2.5
	5 mM	45 ±2.8
IAA	1 mM	52.22 ±1.54
	5 mM	0 ±0.5
β-ME	1 mM	100±0
	5 mM	83.4±2.5
TRITON X100	0.1%	87.5 ±2.0
	0.2%	85.22 ±1.8
TWEEN 80	0.1%	100 ±1.5
	0.2%	100. ±0.5
SDS	1%	100±0
	5%	88.5±1.66
EDTA	50 mM	100 ±0.5
	1 M	100±0
2, 6-pyridinedicarboxylic acid	3.34 mM	0

No effect of EDTA

Conserved residues in active site and outside

Trp185, 233
Glu142, 153
Asp117, 204
Cys227
Ser141
H110, H 137, H139, H156

DTT- dithiothreitol
β-ME - β-mercaptoethanol
WRK - Woodward's reagent K
IAA - Iodo acetamide
PMSF- phenyl methyl sulfonyl fluoride
NBS - N-bromosuccinimide
NEM - N-ethylmaleimide
DEPC - Diethylpyrocarbonate
EDTA –ethylenediaminetetraacetic

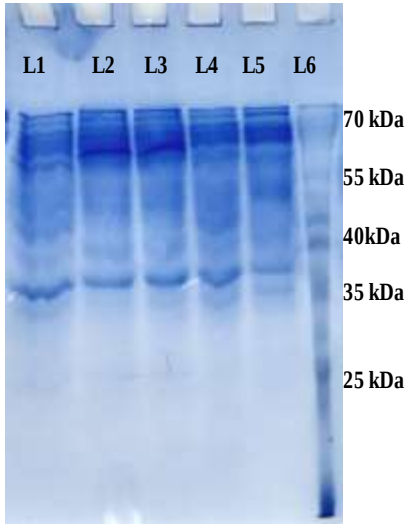
Site directed mutagenesis for confirming the catalytic residues

H_{137} -Y CAForward Primer: CACACTCGTTCAATTC**T**ACTTC**C**ATATTCCTTCCGAG
H_{137} -Y CAReverse Primer: CTCGGAAGGAAT**A**T**G**GAAGT**A**GAATTGAACGAGTGTG

H_{139}-Y CAForward Primer: CACACTCGTTCAATTC**T**ACTTC**C**ATATTCCTTCCGAG
H_{198}-Y CAReverse Primer: CTCGGAAGGAATAT**G**GAA**G**T**A**GAATTGAACGAGTGTG

H_{156}-Y CAForward Primer: AATTTAGATATGGAGCTT**T**ATTTTGTCCATAAGAATG
H_{156}-Y CAReverse Primer: CATTCTTATGGACAAA**A**T**A**AAGCTCCATATCTAAATT

H_{110} -Y CAForward Primer: ACGATTAAAAATAATGGT**G**C**T**ACGATTCAAGCAGAGAC
H_{110}-Y CAReverse Primer: GTCTCTGCTTGAATCGT**A**G**C**ACCATTATTTTTAATCGT

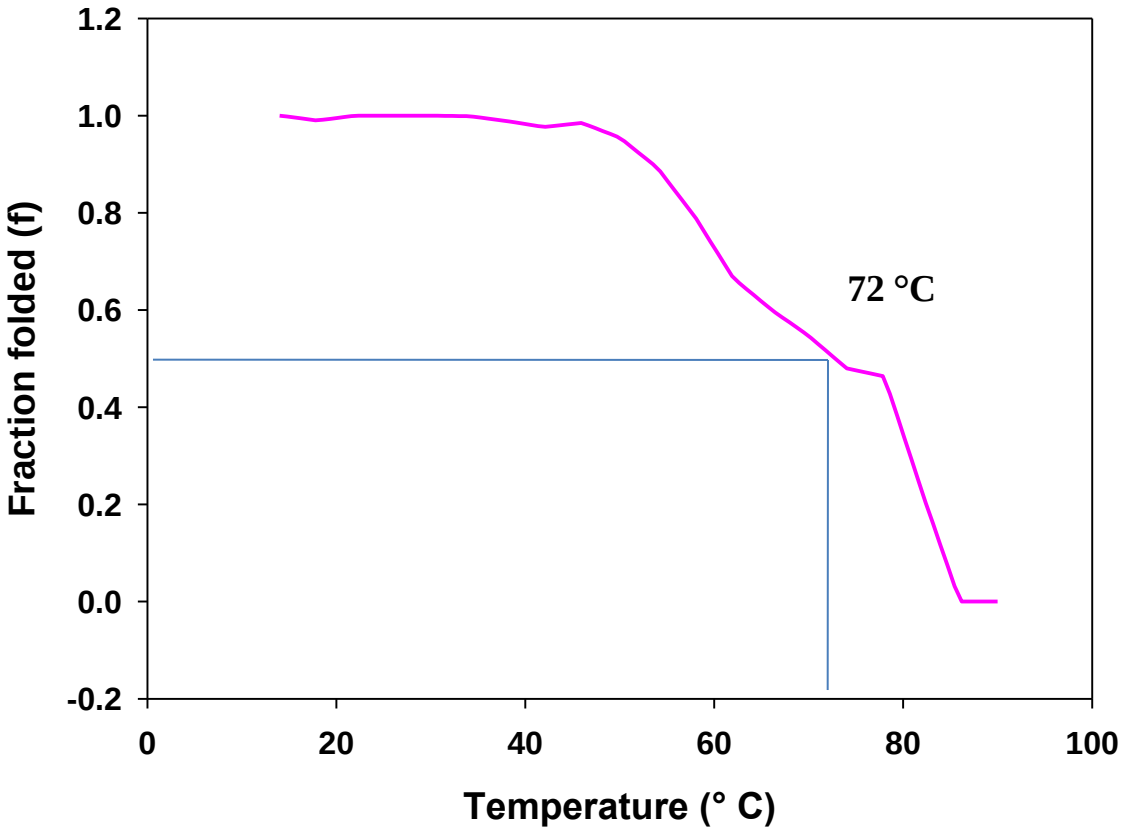


Complete loss of activity occurred with all substitutions

H137Y; H139Y; H156Y (Y=tyrosine)

SDS PAGE showing expression of muteins. L1-L4: Crude lysates of the muteins H110, H137, H139, H156; L2: Uninduced crude lysate; L6- Protein Markers

Melting Temperature (Tm) of rBhCA



Thermal unfolding curve for rBhCA

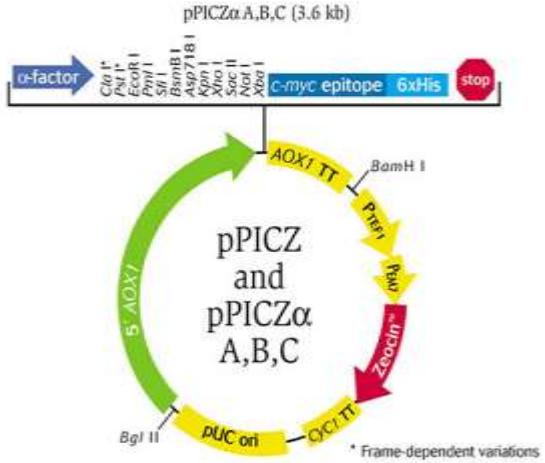
Comparison of wild type and recombinant CA

Properties	Native CA	Recombinant
Production (U/gdbm)	35,000 ± 800	7,85,000 ± 1,105
pH stabilty	6-11	6-11
Thermal stability ($T_{1/2}$ at 50°C)	65 ± 1min	64.5 ± 1 min
Tm	71 °C	72 °C
Mol. mass	~74 kDa	~75 kDa
Specific activity (U/mg protein)	3,425 ± 95	8, 942 ±112

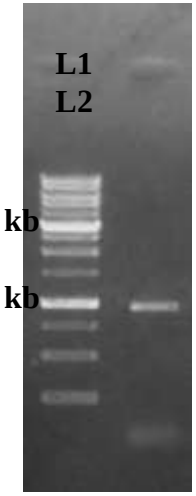
Fold improvement in CA production=22.4

Cloning of *BhCA* in *Pichia pastoris*

Cloning and expression of *BhCA* under AOX1 promoter



Genomic DNA isolation from the *Pichia*-pPICZ- αCA clone



Amplification of αCA from the genome of *Pichia* pPICZαCA construct . L1: αCA (828bp)
L2: DNA Ladder

Linearized pPicZα+ *αCA* constuct

Vector backbone 3.1kb

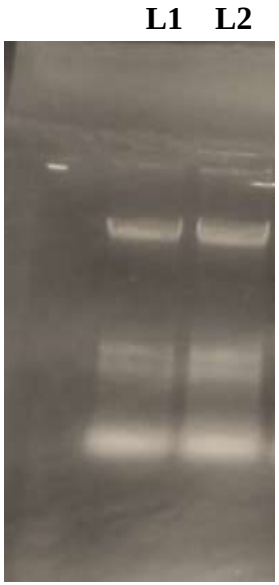
828bp α CA

Clone confirmation by digesting the construct with L1: *EcoRI* ; L3: *EcoRI* and *XbaI*

CA production- 1 U/mL

Cloning and expression of *BhCA* under GAP promoter using pGAPZα vector

Confirmation of pGAPZ- *BhCA* construction by double digestion. L1: αCA fall out after digestion with EcoRI and XbaI, L2: DNA Ladder

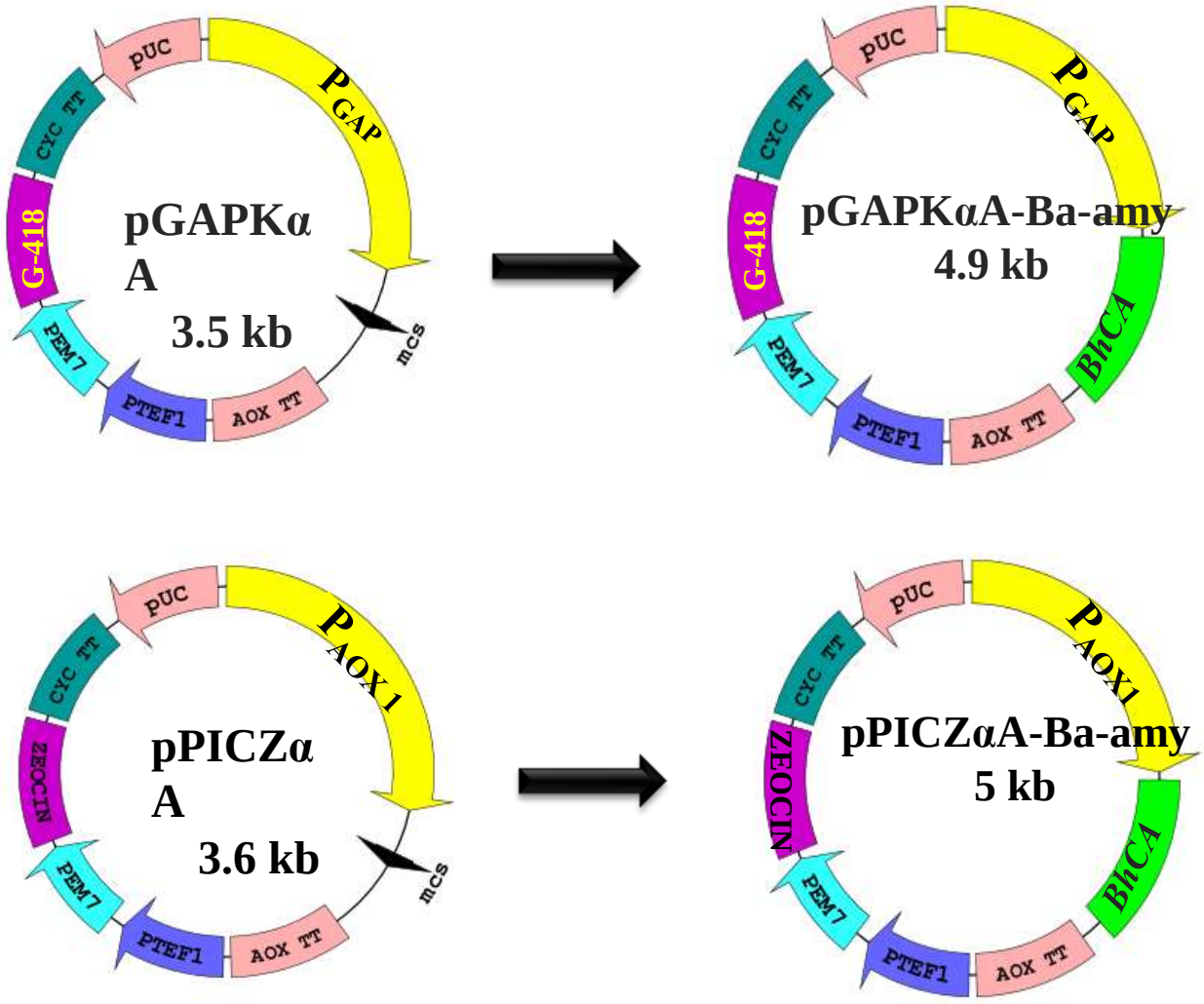


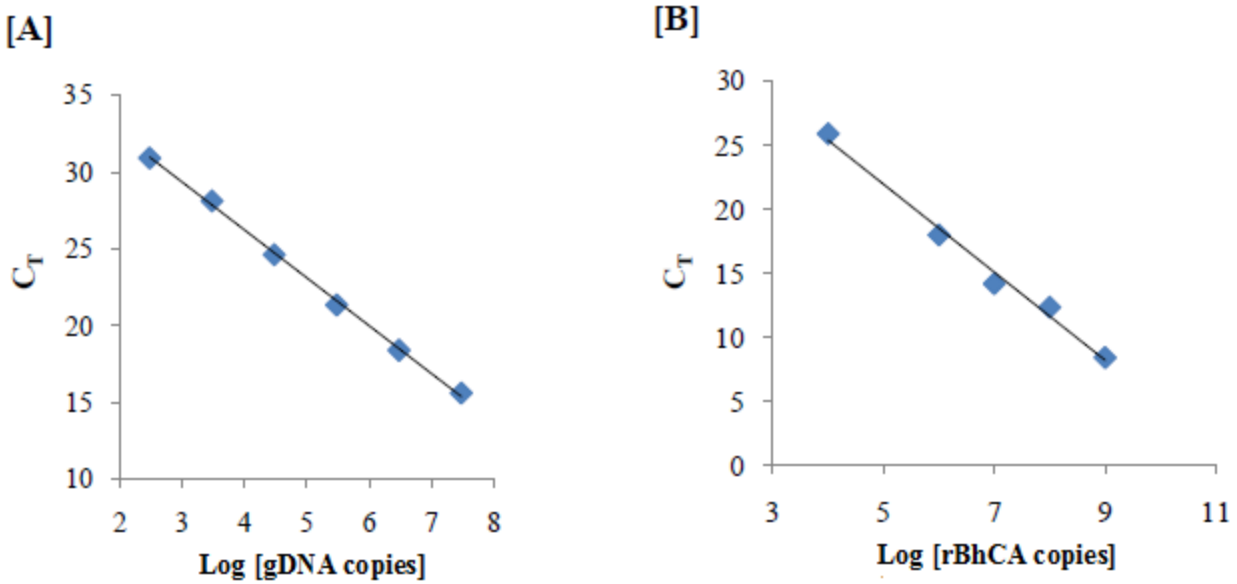
Genomic DNA isolation from the *Pichia*- pGAP Z-*BhCA* clone

Amplification of α-CA from the genome of *Pichia* pGAP Z-*BhCA* clone . L1: BhCA (850bp)
L3: DNA Ladder

After OVAT 25 ± 2 U/mL of rBhCA production was attained.

Strategy for construction of *pGAPKαA-BhCA* construct





qPCR standard curves. [A] qPCR standard curve for *GAP* gene; [B] qPCR standard curve for rBhCA

Number of copies of *BhCA* gene in the recombinant: 2

Recombinant BhCA production: 48 U ml^{-1}

Environ . Sci. Pollut. Res. (2018). 25: 6838-6849

Purification of rBhCA from *Pichia pastoris*

Crude protein

↓

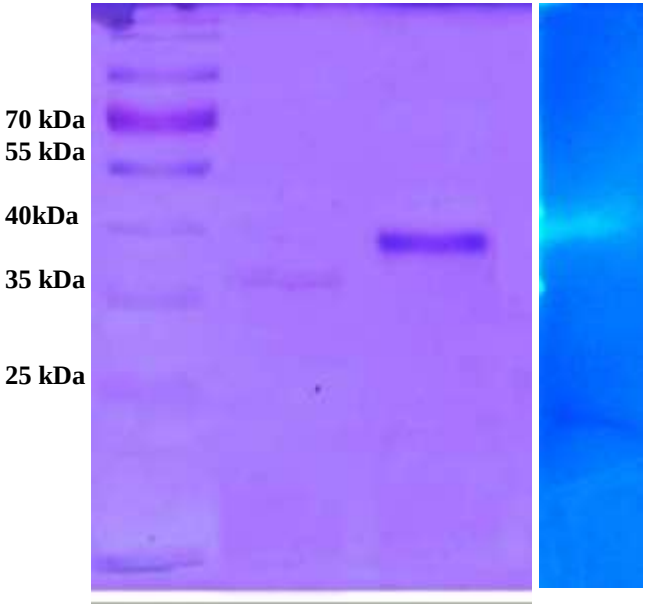
Ultrafiltration

↓

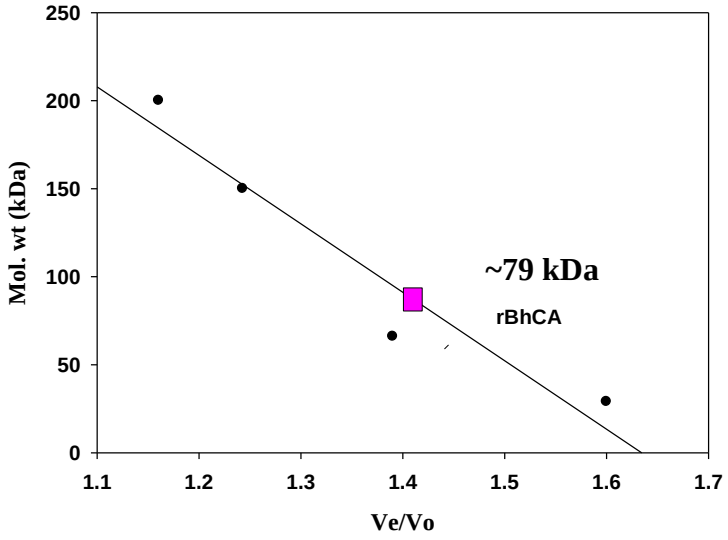
Acetone precipitation

↓

pAMBS affinity chromatography

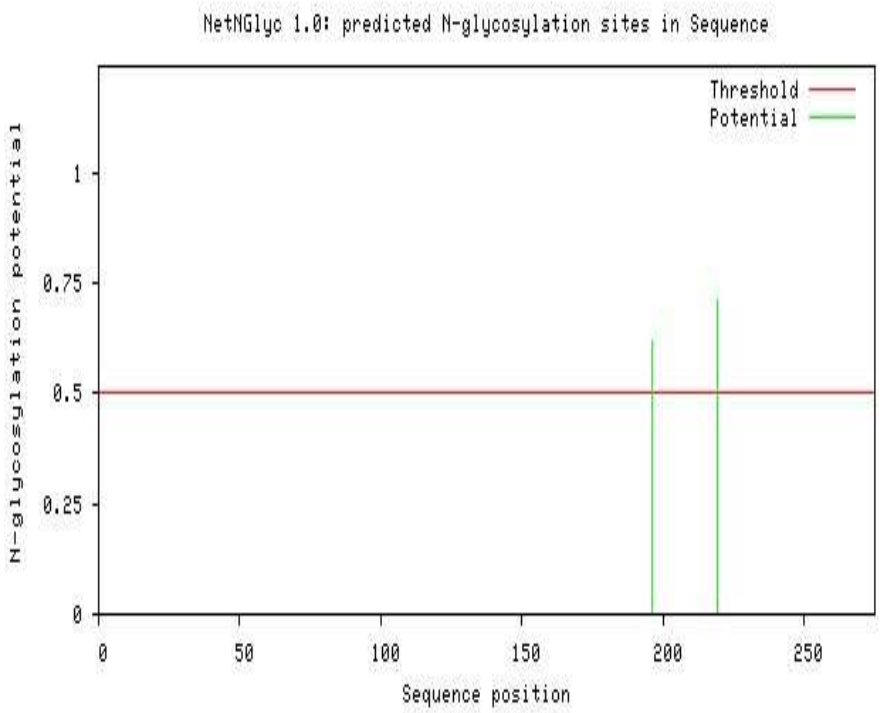


L1 : Marker, L2: Purified rBhCA *from E. coli*, L3, Purified rBhCA from *Pichia*



Plot of Ve/Vo against molecular weight of proteins on Sephacryl™ S-200 high resolution column (16/60). Molecular weight markers (kDa) used with purified rBhCA. Cytochrome c (12.4kDa), carbonic anhydrase (29kDa), bovine serum albumin (66kDa), yeast alcohol dehydrogenase (150kDa) and sweet potato β-amylase (200kDa)

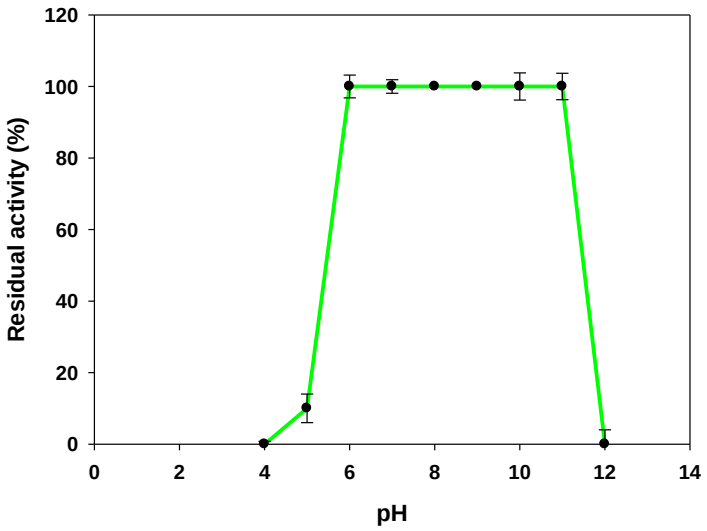
In silico analysis of glycosylation sites using NetNGlyc 1.0 server



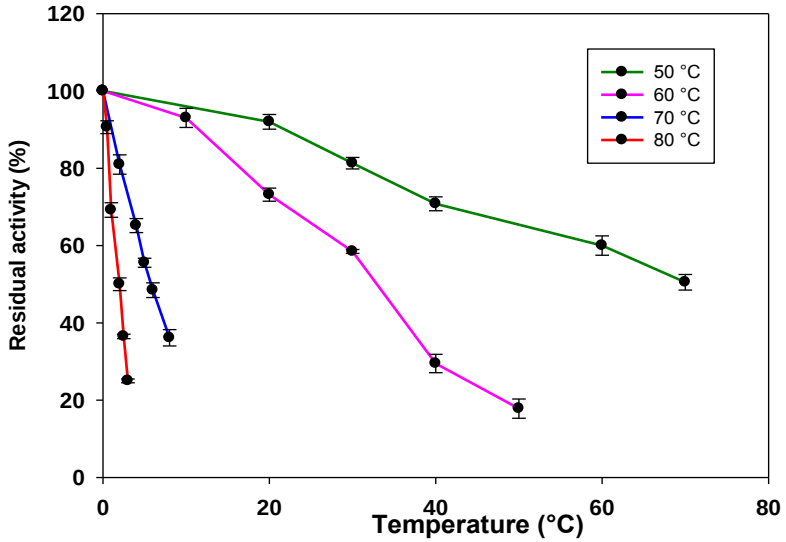
N-glycosylation sites predicted by NetGlyc 1.0 Server

3D model of rBhCA showing distribution of the N-glycosylated residues (green spheres) O-glycosylated residues (magenta spheres)

Characterization of rBhCA expressed in *P. pastoris*



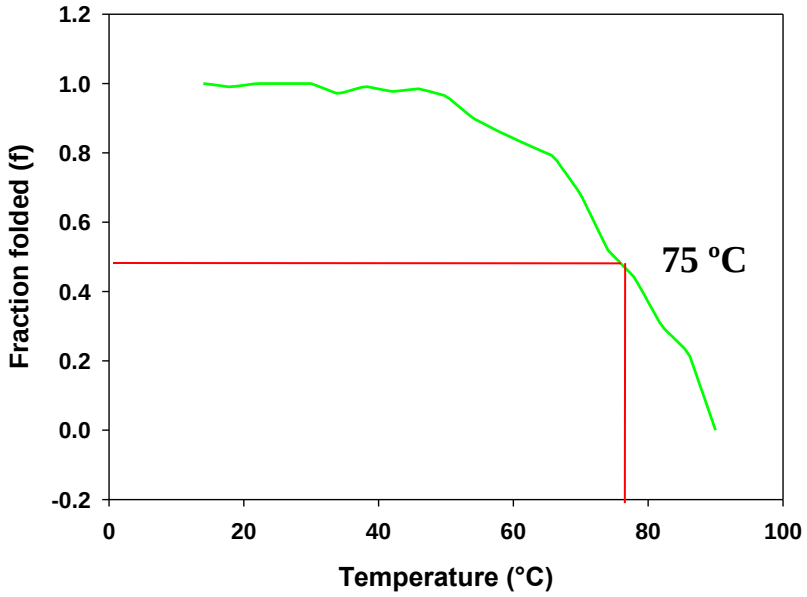
Effect of different pH values on rBhCA stability



Effect of different temperatures on pichBhCA stability

$T_{1/2}$ is 72 ± 1.1, 32 ± 1, 7.0 ± 0.5 and 2.0 ± 0.15 min at 50, 60, 70 and 80° respectively

Melting Temperature (Tm) of rBhCA



Thermal unfolding curve for pichBhCA

Comparison of rBhCA expressed in *E. coli* and *Pichia*

Properties	rBhCA in *E. coli*	rBhCA in *Pichia*
Production (UL^{-1})	2,53,231 ± 2,875	48, 000 ± 200
pH stabilty	6-11	6-11
Thermal stability ($T_{1/2}$ at 50°C)	64.5 ± 1 min	72 ± 1 min
Tm	72 °C	75 °C
Mol. Wt.	~ 75 kDa	~ 79 kDa

BhCA as virtual peroxidase

Disadvantages of natural heme based peroxidases

- Rapid inactivation
- yield aldehyde side products
- show low enantioselectivity

rBhCA as peroxidase

rBhCA

↓ (Dialysis against PDCA in Tris Buffer pH 5.5, O/N, RT)

Apocarbonic anhydrase

↓ (Dialysis against $MnCl_2$, pH 6.95)

Mn-substituted CA

↓

Check peroxidase activity

25 U of peroxidase / mg protein

Immobilization of rBhCA

Immobilization of CA on montmorillonite K10 by physical adsorption

Montmorillonite K 10 + deionized water

↓ vigorously stirred for 6 h

Filtered, dried at 120 °C for 12 h and calcined at 350 °C for 12 h.

↓

Mixed with equal volumes Tris buffer solution (pH 8.3)and enzyme solution

↓

Shaken for 1 h in a water bath shaker at room temperature.

↓

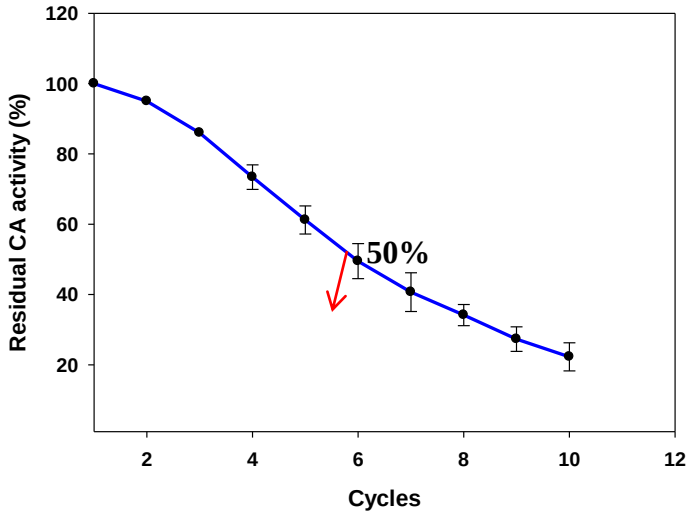
Centrifuged for 1 h

↓

Washed several times

↓

Enzyme assay



CA immobilization on montmorillonite

Immobilization of CA on montmorillonite K10by covalent attachment

Calcined montmorillonite K 10 +10% (v/v) solution of 3-amino propyl triethoxy silane in acetone

↓ vigorously stirred for 3 h at RT

Filtered, washed several times with acetone until the washings became colourless ,dried at 80 °C for 12 h

↓

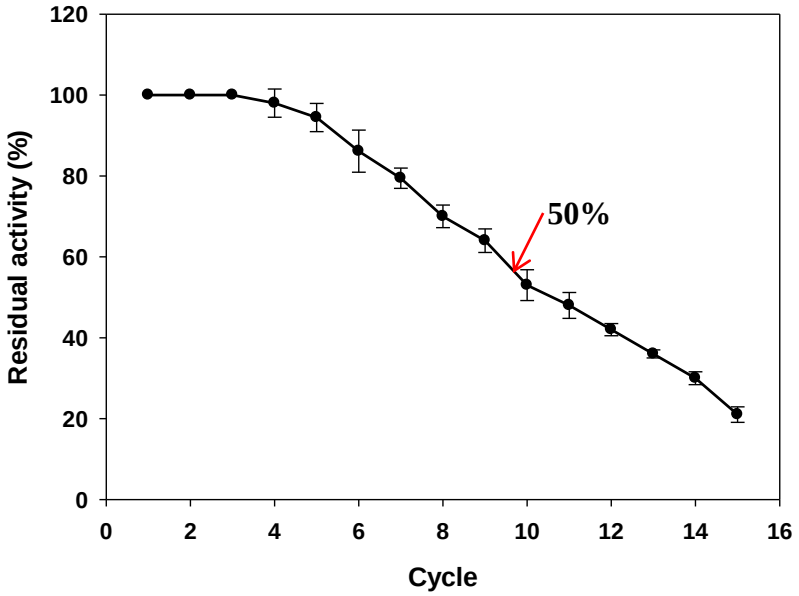
Silanized clay +10% aqueous solution (v/v) of glutaraldehyde and stirred vigorously for 3 h

↓

Filtered, washed free of excess glutaraldehyde and dried at 60 °C for 12 h

↓

This functionalized clay was used for CA immobilization



Immobilization of CA on magnetized aniline nanofibers

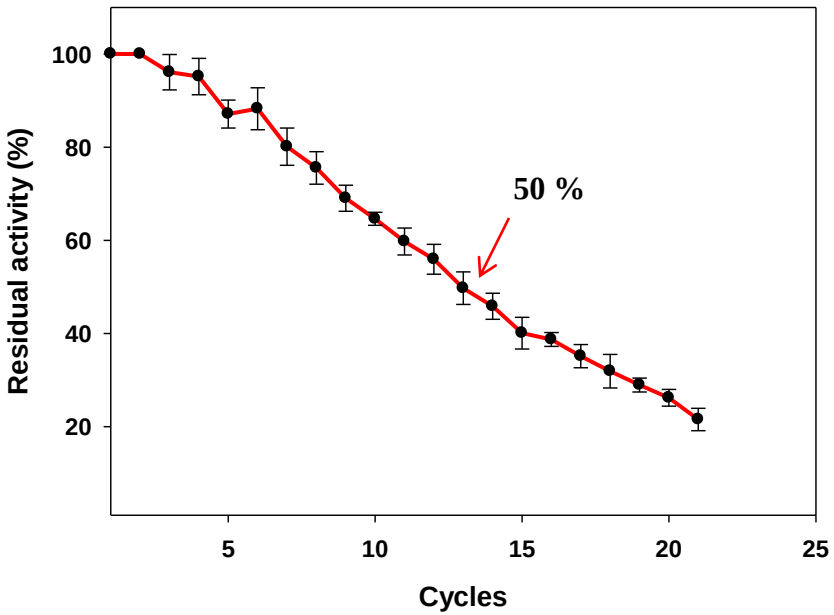
Magnetite iron oxide nanoparticles were prepared by coprecipitation of Fe^{2+} and Fe^{3+} with NH_4OH using the method described by Mahdavi et al. 2013.

0.1% APS + 5 ml of an aniline monomer solution in 1 M HCl

↓ Rapid mixing

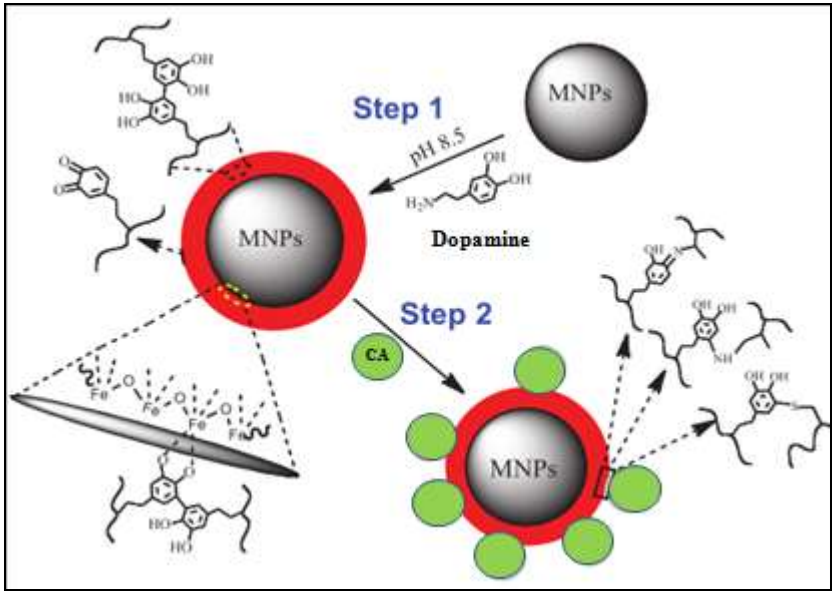
↓ + 10% (w/v) of iron oxide (nanoparticle)

↓ Wash to remove remaining HCl

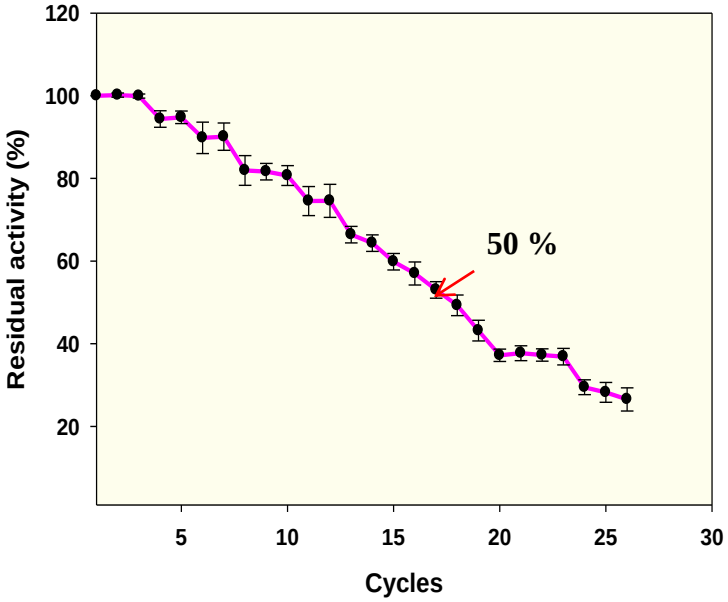


Reusability of CA immobilized on MNPs

Immobilization of CA on dopamine coated iron MNPs

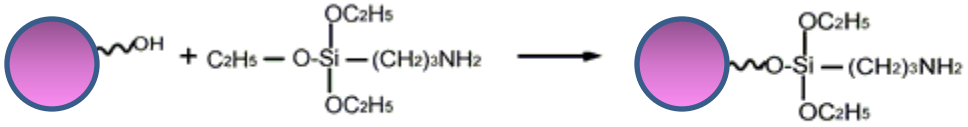


Surface modification of MNPs with polydopamine

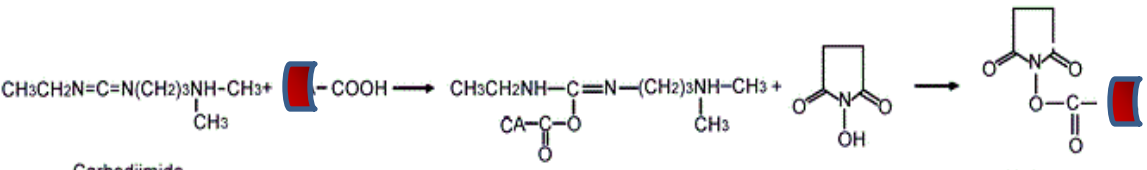


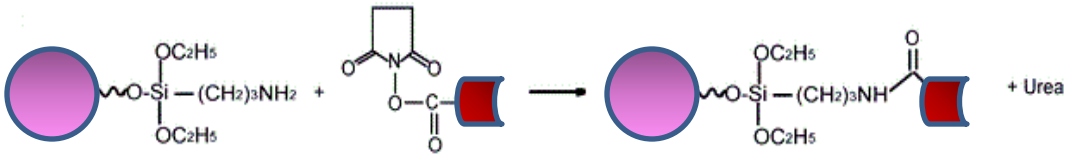
Reusability of CA immobilized on dopamine coated MNPs

Immobilization of CA on silanized iron MNPs

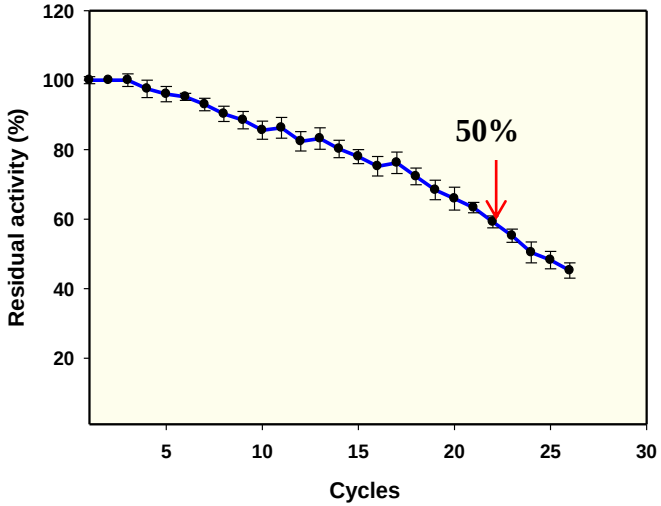


Silanization of MNPs with APTES





EDC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide) activation of carboxyl groups of CA



Reusability of CA immobilized on silanized iron MNPs

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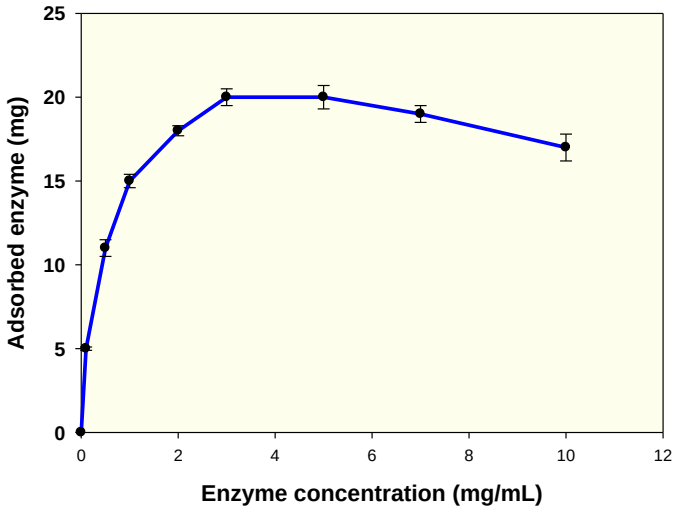
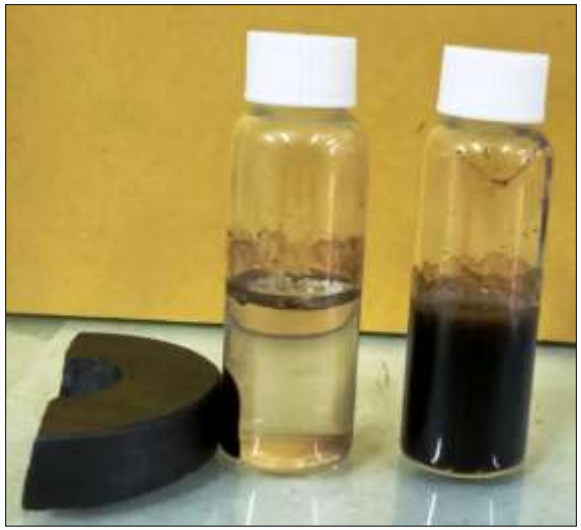
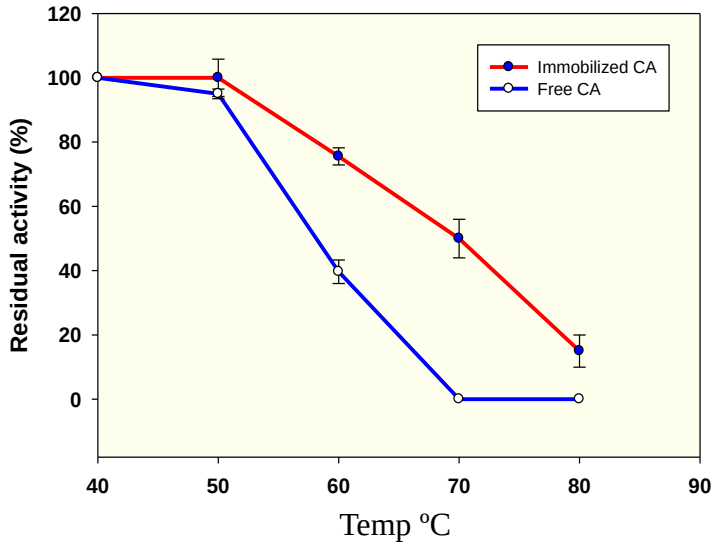
Effect of enzyme concentration on immobilization of CA on Si-MNPs

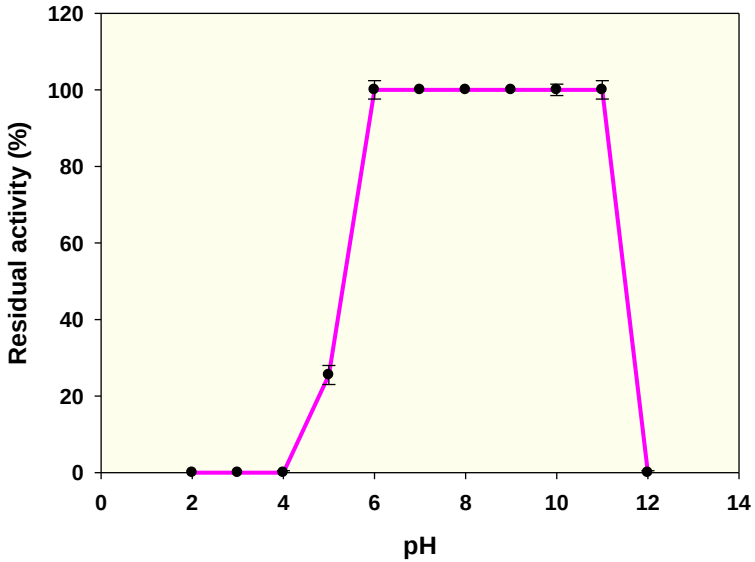


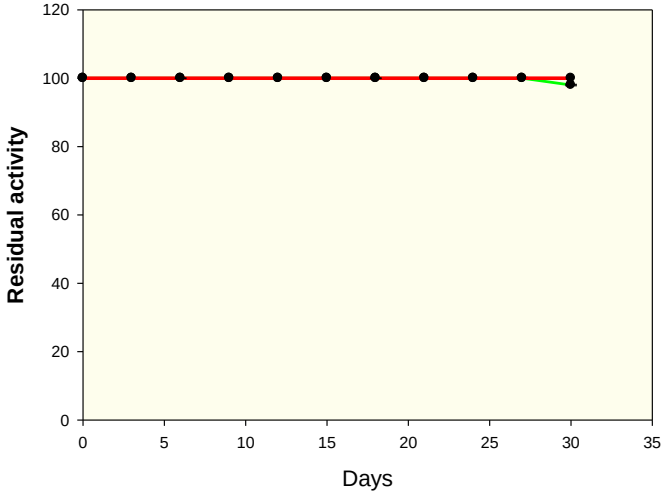


CA-Si-MNPs aqueous suspension after and before magnetic separation

Characterization of CA immobilized on iron MNPs







Effect of anions and metal ions on the immobilized rBhCA

Anion	Concentration	Residual activity (%)
SO_4^{-}	1.0 M	100±0
	1.25M	172 ±3.0
SO_3^{2-}	1.0 M	100.±0.5
	1.25M	100 ±0
NO_3^{-}	0.5 M	100 ±1.5
	1.0 M	85.1 ±1.5
	1.5 M	77.4±1.8
Pb^{2+}	1.0 mM	100±0
	5.0 mM	85.0±3.0
Hg^{2+}	500 µM	20±2.0
	1.0 mM	0±2.8

Conclusions

Conclusions

- *B. halodurans* produces alkalistable and moderately thermostable intracellular α-CA (BhCA) which is tolerant to SOx and NOx present in flue gas.

- The gene encoding BhCA was cloned and heterologously expressed in *E. coli* and *P. pastoris*. Recombinant BhCA displays similar characteristics like the native CA

- Site directed mutagenesis confirmed the identity of catalytically important amino acid residues (H110, H 137 and H 139, H156) of BhCA.

- Application of BhCA in mineralizing CO_2 from flue gas has been confirmed.

- rBhCA has been successfully immobilized on iron MNPs.

PUBLICATIONS

- **S. Faridi, T. Satyanarayana**, Novel alkalistable α-carbonic anhydrase from the polyextremophilic bacterium *Bacillus halodurans:* characteristics and applicability in flue gas CO_2 sequestration, Environmental Science and Pollution Research (2016) 23: 15236-15249 [DOI 10.1007/s11356-016-6642-0].

- **S. Faridi, H. Bose and T. Satyanarayana,** Characteristics of recombinant α-carbonic anhydrase of *Bacilllus halodurans* TSLV1. International Journal of Biological Macromolecules (2016). 89: 659-668 [DOI : 10.1016/j.ijbiomac.2016.05.026].

- **S. Faridi, T. Satyanarayana**, Thermo-alkali-stable α-carbonic anhydrase of *Bacillus halodurans*: Heterologous expression in *Pichia pastoris* and applicability in carbon sequestration, , Environmental Science and Pollution Research (2017) 25: 6838 – 6849 (DOI: 10.1007/s11356-017-0820-6).

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Dr. Shazia Faridi


Thank You