Carbon Bio-sequestration by anhydrase enzyme extracted from spinach

(*Spinacia oleracea)*

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Abstract

Demand for sustainable and new technologies striving for sequestration of greenhouse gases, particularly carbon dioxide, is an area of considerable focus. In recent years there has been an increased interest in using an enzyme biocatalyst, Carbonic Anhydrase (CA) for this purpose. CA is an ubiquitous metalloenzyme that catalyzes the reversible hydration of CO_2 in aqueous biological systems. In this study, Carbonic Anhydrase was extracted and partially purified from a plant source which is spinach leaves. The extracted enzyme was immobilized on different materials for increased stability, recyclability and cost-effectiveness. Immobilization of Carbonic Anhydrase was done on alginate beads, chitosan beads and chitosan film. After immobilization the activities for alginate beads, chitosan beads and chitosan film were found to be 23.37, 20.96 and 17.58 U/mg respectively. Optimum pH for alginate beads and free enzyme was 8, while for chitosan beads and film it was 8.5 and 7.5 respectively. The optimum temperature for free enzyme was 30°C, while for alginate beads, chitosan beads and chitosan film it was 40°C, 35°C and 35°C respectively. For both free and immobilized enzyme, calcium carbonate precipitation was approximately same per unit of enzyme activity. The recyclability of immobilized enzyme was tested up till four cycles. The immobilized enzyme showed better stability than the free enzyme. Alginate beads, chitosan beads and chitosan film retained 76.59, 80.75 and 83% of their activities over a period of 4 weeks. It was concluded that carbonic anhydrase obtained from plant source can be used for CO_2 sequestration purposes. On immobilization, the enzyme has better storage stability, recyclability and can be used in industrial process.

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Last but not the least, I thank God for everything.

Abbreviations

CA - Carbonic Anhydrase

BCA – Bovine Carbonic Anhydrase

BSA – Bovine Serum Albumin

GHG – Greenhouse gases

hCA – Human Carbonic Anhydrase

RBCs – Red blood cells

GI tract – Gastrointestinal tract

HFM – Hollow fibre membrane

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Chapter 1 Introduction

The escalation of greenhouse gas (GHG) levels in atmosphere in the last five decades is believed to be the principal cause of global warming. Among other greenhouse gases, CO_2 is the biggest contributor in respect of its sum display in the atmosphere adding to 60% of global warming effects. The total quantity of carbon in the atmosphere is fixed and is distributed between the lithosphere, biosphere and atmosphere. But since the introduction of industrialization the concentration of CO_2 is increasing rapidly in the atmosphere (IPCC, 2005). IPCC panel also predicts that by the year 2100 the atmospheric CO_2 levels might reach up to 570 ppmv as compared to that of 377 ppmv in 2004, which in turn will lead to a rise in mean global temperature and increase in mean sea level of 38 m.

To reduce the CO_2 emission in the atmosphere there are three alternatives available i.e., reducing CO_2 emissions in the atmosphere, reducing energy intensity and improve sequestration of CO_2. To improve CO_2 sequestration, attempts are being made to escalate CO_2 fixation by developing new approaches to capture and sequester CO_2 and to avoid its discharge into the atmosphere such as ocean sequestration, mineral carbonation, geological sequestration, etc. CO_2 sequestration is a solution to mitigate environment impact and allows us to use fossil energy until renewable energy technologies mature.

CO_2 sequestration methods such as adsorption on membranes, cryogenic systems and use of chemical solvents is highly expensive (Abu-Khader 2006), corrosive and solvent loss occurs (Amornvadee Veawab et al. 1999). CO_2 sequestration to mineral carbonates is an environment friendly, steady, safe and long term sequestration method, but it is a slow process (R Ramanan et al. 2009). Recently an enzyme based CO_2 capturing technology has been described by researchers which mimics naturally occurring CO_2 reactions in living organisms (Frommer 2010).

Carbonic Anhydrase is a zinc metalloenzyme which is found in all living organisms such as plants, animals and prokaryotes (C. Boone et al. 2013). CA catalyzes the conversion of CO_2 to bicarbonates and vice versa (Rishiram Ramanan et al. 2009a).

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \text{.....(i)}$$

CA makes the fastest rapid mass transfer of CO_2 from gaseous phase (Bhattacharya et al. 2003). It can catalyze the hydration of CO_2 at a rate of 10^4-10^6 s^{-1} as compared to that of 6.2 x 10^{-3} s^{-1} (Bhattacharya et al. 2003).

Most of the industrial processes that seek to employ CA for CO_2 capturing and sequestration are quite harsh by biological standards which means that these processes involve extreme change in pH and temperatures (Gonz et al. 2014). The use of free enzyme in solution also has its disadvantages such as non-reusability, low enzyme stability, decreased recovery from the reaction environment (Vinoba et al. 2012) and deactivation of enzyme in strong acid or alkali solutions.

Immobilization of enzyme can improve the stability and reusability of enzyme, provide operational flexibility and would pave a way for cost competitive route for commercialization of the process (Ekrem Ozdemir 2009a). As compared to free enzymes, immobilized enzymes are resistant to environmental changes and more robust (Oviya et al. 2013). Entrapment in matrices, adsorption on solid support, cross-linking with polymers and covalent bonding are some of the methods that have been used for enzyme immobilization. A variation of support materials such as, silica, alginate, chitosan, glass, polyurethane foam have been investigated for CA immobilization (Ekrem Ozdemir 2009b), (Prabhu et al. 2011), (Vinoba et al. 2012).

Chitosan and alginate are inert materials which have been used for immobilization of various enzymes. Chitosan is obtained from deacetylation of chitin. It is user friendly, non-toxic and has protein affinity (B Krajewska 2004). Alginate is a polysaccharide of marine brown algae and a cheap alternative for enzyme entrapment (J. Sharma et al. 2010). Chitosan and alginate

have both been used for immobilization of CA extracted from bacteria, microalgae and commercially available Bovine Carbonic Anhydrase but the effect of immobilization on CA extracted from plants has not been studied yet. The present study thus aims at extraction and partial purification of CA from *Spinacia oleracea* (spinach) and its immobilization on chitosan and alginate to assess its CO_2 sequestration potential.

The specific objectives of this study were:

I. To extract Carbonic Anhydrase from spinach and partially purify it by ammonium sulphate precipitation.
II. Immobilization of Carbonic Anhydrase on alginate beads, chitosan beads and chitosan membrane.
III. Determine the effect of temperature and pH on free and immobilized enzyme.
IV. Assess the CO_2 sequestration potential of free and immobilized enzyme.
V. Study the reusability and stability of free and immobilized enzyme.

Chapter 2 Literature Review

2.1.Climate Change

One of the major challenges the world faces today is climate change. It has been proven unequivocally that climate change is happening at a very fast pace. Climate change is defined as the change in weather patterns which lasts for an extended time period. Climate scientists confirm that the main cause of climate change is global warming. Sometimes climate change and global warming are used interchangeably but they are two different phenomena. On one hand climate change is a global phenomenon engendered primarily by the heat entrapping gases on the earth's surface, while on the other hand global warming results from increasing temperatures across the globe since the industrial revolution. Climate change comprises of increased temperatures because of global warming along with rising sea levels, extreme weather, melting of ice glaciers etc. Global warming is caused by increased greenhouse gases in the earth's atmosphere. It results by the atmospheric entrapment of heat being radiated from the surface of the earth (Pachauri et al. 2014).

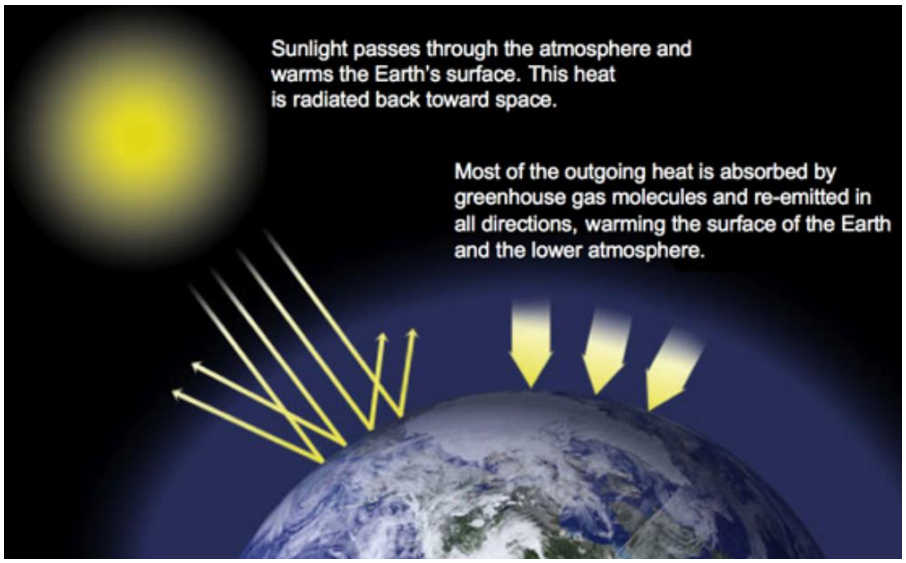

Figure 2.1: Greenhouse gases acting as thermal blanket around the Earth. (Courtesy: NASA Earth Observatory, January 20, 2018).

2.1.1. Greenhouse Effect

The heat from the Sun is one of the reasons for the Earth to be inhabitable by all living organisms including humans. Solar rays falling on earth are reflected into space, however a

tiny portion of these rays are trapped by the layer of gases around the Earth which makes up our atmosphere as shown in figure 1. These gases are called greenhouse gases are some of them are as follows:

→ Water Vapour

→ Methane

→ Nitrous Oxide

→ Chlorofluorocarbons

→ Carbon Dioxide

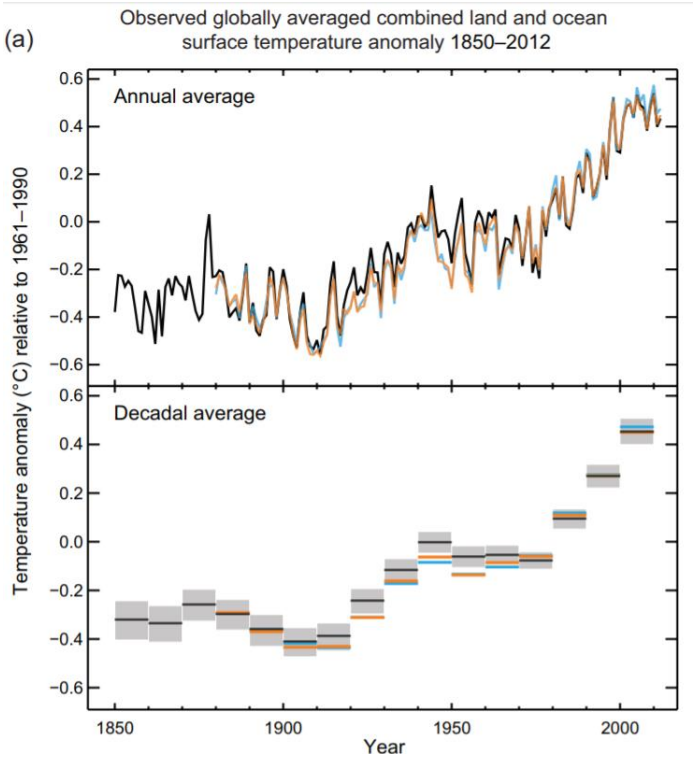

Figure 2.2: Mean annual and decadal change in temperature between 1850-2012 (IPCC, 2013).

The presence of these greenhouse gases help maintains average temperature of earth's surface at 15°C which might be -18°C in the absence of these gases (greenhouse wiki). The average temperatures are expected to rise further in the next century (Dhanwantri et al. 2014).

Since 1850, Earth's temperature was rapidly increasing every decade as shown in figure 2. It can be attributed to climate change and global warming. To take an action against these climatic conditions, it is important to understand the carbon cycle.

2.1.2. Carbon Cycle

On Earth, carbon is an abundant element and the backbone of every form of life on the earth including the oceans, plants, soil, fossils and atmosphere. The flow of carbon from one reservoir to another is known as the carbon cycle. Carbon can be released into the atmosphere by activities such as cutting down trees and burning fossil fuels. If there is a change in any of the cycles, then carbon is shifted from one reservoir to another. Any change that increases the level of carbon in atmosphere is promoting the chances of Earth getting warmer.

The carbon cycle helps maintain a balance which prevents the carbon from entering the atmosphere. The carbon cycle has been divided into two subsystems i.e., the slow carbon cycle and the fast carbon cycle (Riebeek 2011).

The Slow Carbon Cycle: In this, it takes millions of years for the carbon to change forms among oil, oceans, rocks and atmosphere with the aid of tectonic activity and chemical reactions. Every year, on an average of 10-100 million metric tonnes of carbon shifts through the slow carbon cycle.

The Fast Carbon Cycle: The time taken by carbon to go through a fast carbon cycle is calculated in a lifespan. It is the movement of carbon through biosphere. About 100-1000 million metric tons of carbon moves through a fast carbon cycle in a year.

The fast and slow carbon cycles keep a balanced carbon concentration in ocean, plants, land and atmosphere. Today, the carbon cycle is being disturbed by anthropogenic activities. Cutting of dense forests exposes the soil which in turns releases carbon dioxide from decay plants into atmosphere. Without human intervention, carbon would be released by volcanoes into

atmosphere slowly over millions of years (Reichle et al. 1999). This process is accelerated when oil, coal, natural gases are burnt for our daily use.

since 1950, carbon dioxide levels are increasing drastically as shown in figure 2.3. Approximately 30 billion tonnes of CO_2 is generated each year currently (Sheppard 2018).

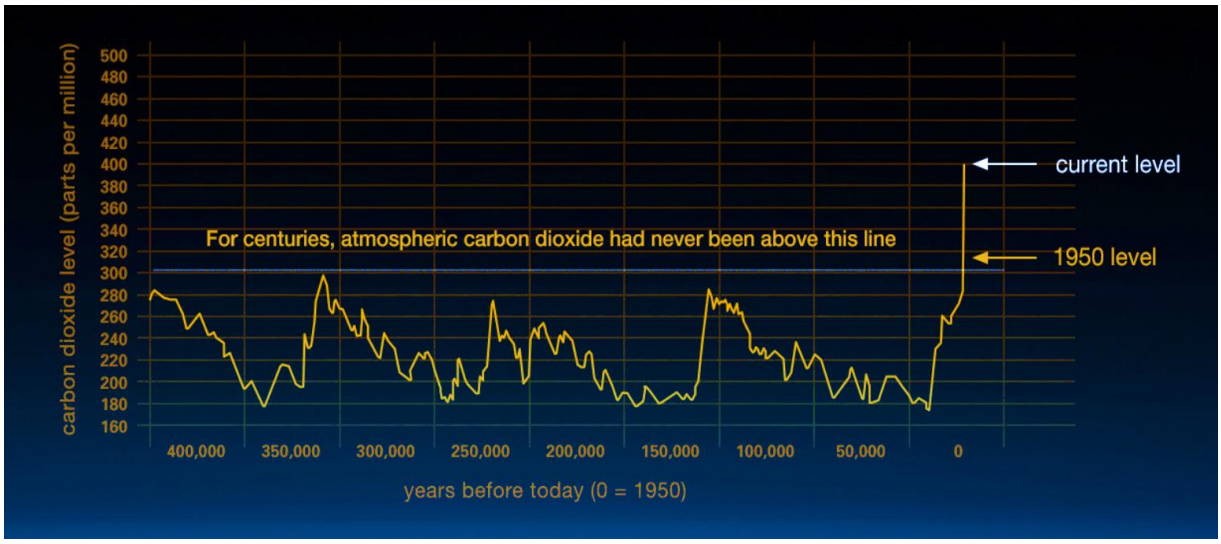

Figure 2.3: Increasing atmospheric CO2 since the Industrial Revolution (Courtesy: NASA Earth Observatory, January 26, 2018).

2.1.3. Mitigating Carbon Dioxide

Overabundance of carbon dioxide in the atmosphere warms up the planet, it also makes the water in oceans acidic which is dangerous for marine life (Riebeek 2011).

Increasing levels of carbon dioxide have substantial impact on earth's climate. Hence, it is crucial to find ways to tackle this problem of growing atmospheric CO_2 emissions. There are ways by which atmospheric CO_2 levels can be alleviated such as (i) developing alternatives for C-based fuels; (ii) capturing and long-term storage of carbon dioxide; (iii) reducing emissions of CO_2 into the atmosphere (Mirjafari et al. 2007).

2.2. Carbon Dioxide Sequestration Methods

Sequestration is a method used for keeping the levels of carbon below the threshold level to maintain a balanced carbon dioxide pool in the atmosphere. It helps in storing the carbon in a form that does not cause global warming (Yamasaki 2003). Many researchers have tried to

classify various sequestration strategies to capture carbon in different forms based on the methods, carbon sinks, organisms etc. A very broad classification is done based on the non-biological and biological methods (Nogia et al. 2016).

2.2.1. Non-Biological Methods

Sequestration done with the help of physical and chemical processes without involving any living organisms comes under this category. This includes chemical, geological and oceanic sequestration.

2.2.1.1. Mineral Carbonation (Chemical)

In this method the atmospheric carbon is converted to a stable compound. Carbon dioxide reacts to various metal oxides like calcium oxide, iron oxide, magnesium oxide etc to form their respective carbonates (Oelkers et al. 2008). The carbonates are stable and can help in avoiding the carbon to liberate into the atmosphere which eventually reduce global warming effects. These carbonates can be stored very easily for a longer time (Rattan Lal 2008). An example of natural mineral carbonation is the weathering of rocks. In this, CO_2 present in rainwater reacts with minerals present in rocks.

$$CO_2(g) + MO \leftrightarrow MCO_3 + heat \text{ ……..(ii)}$$

Such kind of reactions occur in nature but can be replicated in an industrial setting. O'Connor *et al.* described the industrial process of mineral carbonation (O'Connor et al. 2001).

2.2.1.2. Geological Sequestration

Geological sequestration is the process of capturing, transportation and injection of CO_2 into deep geological bed like oil wells, gas aquifers, coal mines, stable rocks (Klara et al. 2003). It depends on many factors which are yet to be understood properly.

An example of geological sequestration is Sleipner project, Norway. CO_2 separated from natural gas in pumped into aquifer below sea level (Sipilä et al. 2008). CO_2 from industry is also being stored in saline aquifers where CO_2 reacts with salts present in the aquifer to form

carbonates (Rattan Lal 2008). Countries like Canada, Australia, Algeria have started small demonstration projects.

2.2.1.3. Oceanic sequestration

Oceanic sequestration involves the direct injection of CO_2 into the deep ocean beds. The gaseous CO_2 forms carbonic acid which then dissociates into hydrogen ion and bicarbonate ion according to the equation given below (IPCC 2005).

$$CO_2(g) + H_2O(l) \leftrightarrow H_2CO_3(aq) \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \text{.....(iii)}$$

This process must be stable, and the CO_2 should be injected at maximum possible depths so that its undisturbed and does not leak into the environment. Since CO_2 is lighter than water, the process must be carried out approximately 3000 meters below the ocean level which is considered stable for CO_2 injection. It prevents carbon dioxide from escaping. (O'Connor et al. 2001).

2.2.1.4. Reforming of methane

Dry reforming is a process to produce synthesis gas (mixture of carbon monoxide and hydrogen) by reacting CO_2 with hydrocarbons such as methane. CO_2 acts as an oxidising agent for the oxidation of methane. The most reduced form (CH_4) is combined with the most oxidized form of carbon (Amin et al. 2018). This process was introduced by Fischer and Tropsch in 1928.

Reforming is of two types, namely steam and dry reforming. The are endothermic in nature so they require higher temperatures to achieve higher conversions of methane.

$$CH_4 + H_2O \rightarrow CO + 3H_2 \text{.....(iv)}$$

$$CO_2 + CH_4 \rightarrow 2H_2 + 2CO \text{.....(v)}$$

2.2.2. Biological methods

Biological sequestration involves the use of living organisms or naturally occurring biological processes to capture and storage of CO_2. Some of the biological ways to sequester carbon are forestation, ocean fertilization, soil sequestration, phyto-sequestration, biocatalyst.

2.2.2.1. Forestation

Forests act as a major carbon pool in which there is continuous exchange of CO_2 with the environment. About 19% of carbon is stored in plants at a global level. Forests can be considered as a carbon source if it releases more carbon into the atmosphere than it holds. Usually, carbon from forests is released by burning of trees or by decaying when they die.

Trees take up the CO_2 from the atmosphere and use it for photosynthesis. They also store the CO_2 in the form of wood (Dhanwantri et al. 2014). Harvested timber, plant woody debris and wooden chips are all sequestering the forest carbon. Hence, afforestation is a feasible way to sequester carbon (Lamb et al. 2005).

2.2.2.1. Ocean fertilization

Ocean nutrition is a way to enrich the upper ocean with the help of nutrients to elevate marine food production and to reduce the levels of CO_2 from the atmosphere (Matear et al. 2004). The ocean removes about 30% of carbon from the atmosphere (Battle et al. 2000). Phytoplankton need macronutrients (nitrate and phosphate) and micronutrients (iron and zinc) to survive and grow. Addition of such nutrients to ocean stimulates the growth of phytoplankton, which in turn consume carbon dioxide through photosynthesis (Ingall et al. 2013).

2.2.2.2. Soil sequestration

A small amount of carbon dioxide that is converted to organic material in plants with the help of photosynthesis is transferred to soil from the roots. As a result, carbon is stored in both organic and inorganic form in the soil (Jansson et al. 2010b). Soil sequestration refers to enhancing the concentration of organic carbon and inorganic carbon content of soil. Soil's

efficiency to sequester carbon depends on rainfall, soil texture, content of clay, moisture content, climate, mineralogy (Metting et al. 2001).

To increase the soil organic carbon levels, the carbon is restored with the help of humification of the soil surface (0.5-1 m depth). Soil organic carbon content is lost by erosion, leaching and mineralization (Rattan Lal 2008). Ways to increase organic carbon content are no-tilling farming (Paustian et al. 2000), nutrient management (Metting et al. 2001) and wood burial (Zeng 2008).

2.2.2.3. Phyto-sequestration

Plants act as potential carbon sink for carbon sequestration. Phyto-sequestration and soil carbon sequestration are more or less corelated terms (Nogia et al. 2016). A huge amount of carbon is stored in plants through photosynthesis. Storing carbon in living biomass, converting the biomass to composites and fibre cement materials are short term alternatives. Long term carbon sequestration is achieved when the biomass containing carbon is transferred to the underground organic and inorganic soil carbon pool. Few ways to achieve long term sequestration are biochar, phytoliths, photo-assimilation of carbon dioxide etc (Jansson et al. 2010a).

2.2.2.1. Biocatalysts

Biocatalysts have the capability to effectively transform carbon dioxide into reduced forms. Enzymes are a promising alternative to catalyze carbon fixation steps. In carbon assimilation pathways, they transform the feedstock into metabolites which are further used for the production of chemicals and wide range of fuels (Alissandratos et al. 2015).

Carbonic anhydrase is one such biocatalyst that has been used for carbon capturing. It is widely being used in industries in the process of conversion and storage of atmospheric carbon dioxide.

Table 2.1: *Sequestration methods advantages and disadvantages.*

Sequestration Methods	Advantages	Disadvantages	Reference
Non-Biological methods			
Mineral carbonation	Long term storage	Potential air, water and soil pollution of surrounding areas.	(Mazzotti et al. 2007)
Geological	Helps in methane and oil recovery	Risk of CO_2 leakage from storage location and high storage cost.	(IPCC 2005), (Sipilä et al. 2008), (Kovscek 2004)
Oceanic	Minimum leakage of stored carbon dioxide	Organisms show reduced rates of growth, calcification, reproduction, mobility and circulatory oxygen supply. Increased mortality over time.	(Lampitt et al. 2008), (IPCC 2005)
Conversion to methanol	Produces a value-added product	Kinetic limitations low	(Barton et al. 2008)
Biological methods			
Forestation	Natural process	Needs improved management strategies	(Larjavaara 2008)
Ocean fertilization	Phytoplanktonic CO_2 fixation is enhanced	Effects the ocean biota	(Lampitt et al. 2008), (Nogia et al. 2016)
Soil sequestration	Decreases soil erosion, help conserve water, increase plant productivity	Soil saturation	(Oren et al. 2001), (R Lal 2004)
Phyto-sequestration	Cost effective, large carbon sink capacity	Plantation efficiency should be increased, deforestation should be reduced	(Jansson et al. 2010c)
Biocatalyst	Long term storage and cost effective	Denatures under harsh conditions	(Nogia et al. 2016)

2.3.Carbonic Anhydrase (CA)

Carbonic anhydrase or carbonic dehydratases belongs to a family of enzymes that catalyzes the hydration of carbon dioxide to bicarbonate and protons and vice versa. This reversible

hydration of CO_2 is fundamental to multiple biological processes such as respiration and photosynthesis. It was discovered in 1933 due to the biomedical applications of its inhibitors (Supuran 2013). It is an ubiquitous enzyme that is found in all kingdoms of life (C. D. Boone et al. 2013).

$$CO_2 + H_2O \xleftrightarrow{carbonic\ anhydrase} H_2CO_3 \leftrightarrow H^+ + HCO_3^- \text{.....(vi)}$$

They are classified as metalloenzymes as most of them contains zinc ion at their active site (Del Prete et al. 2014). Iron, cadmium and cobalt have been demonstrated as metal cofactors for carbonic anhydrase (MacAuley et al. 2009), (Park et al. 2008), (Yee et al. 1996). In human CAII, Zn^{2+} ion is located at the centre of the molecule occupied by three amino acid residues (H94, H96 and H119) as shown in figure 2.4 (Goodsell 2004).

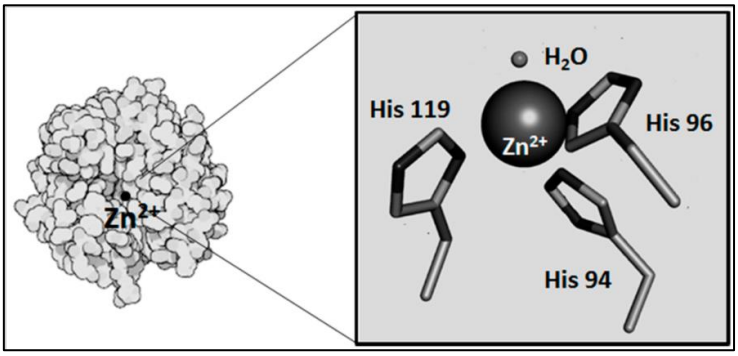

Figure 2.4: Human CAII; metal binding site with zinc ion and amino acid residues (Lionetto et al. 2016).

2.3.1. Mechanism of action

Most carbonic anhydrases have zinc ions at their active site and have similar catalytic activity (Madhumati Mondal, Saumyakanti Khanra. O.N. Towari, K. Gayen 2016). The reversible hydration by all carbonic anhydrase isoenzymes occurs through a process called metal hydroxide mechanism. Carbonic anhydrases have three key amino acid residues (ligands) which differ according to class as shown in figure 2.5. The zinc prosthetic group is coordinated by histidine side chains at three positions and the fourth position is occupied by a water

molecule. The active site contains a pocket for carbon dioxide through which the hydroxide group attached to zinc in brought closer. The electron rich hydroxide ion then attacks the carbon dioxide which results in the formation of a bicarbonate molecule (equation v) (C. Boone et al. 2013). The OH^- bound to Zn ion reacts with Carbon dioxide molecule yielding a Zn bound HCO_3^- ion, which is then replaced by water molecule (equation vi). The HCO_3^- ion in solution can gain H^+ to form $H_2CO_3^-$ (equation vii).

$$E - Zn^{2+} - OH^- + CO_2 \leftrightarrow E - Zn^{2+} - HCO_3^- \text{ .....(vii)}$$

$$E - Zn^{2+} - HCO_3^- + H_2O \leftrightarrow HCO_3^- + E - Zn^{2+} - H_2O \text{....(viii)}$$

$$HCO_3^- + H^+ \leftrightarrow H_2CO_3^- \leftrightarrow 2H^+ + CO_3^- \text{.....(ix)}$$

CA class	Lig
α CA	His, His, His
β CA	His, Cys, Cys
γ CA	His, His, His
δ CA	His, His, His
ζ CA	His, Cys, Cys
η CA	His, His, Gly

Figure 2.5: Amino acid residues according to different carbonic anhydrase classes (Lionetto et al. 2016).

Role of zinc is to facilitate the H_2o to create a proton and a nucleophilic OH^- ion. The nucleophilic water attacks the carbonyl group of CO_2 to convert it to HCO_3^-. This is obtained because of the +2 charge on the zinc ion, which attracts the oxygen of H_2O and deprotonates the water molecule, thus converting it into a better nucleophile so that the OH^- can attack the CO2.

2.3.2. Types of CA

There are five distinct Carbonic Anhydrase families based on conserved nucleic acid sequences in gene sequences of CAs. These enzymes are all called carbonic anhydrase because they

catalyse the same reaction although they have different organization of active center and don't have any structural homology (Rudenko et al. 2015). They use the same catalytic mechanism with different metal atoms at their active sites.

2.3.1.1. α-CA:

α-CA are found in vertebrates (Hewett-Emmett 2000), algae (Fukuzawa et al. 1990), bacteria (Soltes-Rak et al. 1997) (Soltes-Rak et al. 1997), ascomycetes (Elleuche et al. 2010). At least 16 isoforms of carbonic anhydrase has been discovered in mammals (Supuran 2008a). The first ever discovered and purified α-CA was from *Neisseria sicca* in 1972 (Adler et al. 1972). CAs have not been detected in archae (Kumar et al. 2014).

α-CAs are monomers of molecular mass ranging from 29 kDa to 35 kDa (Shazia Faridi Satyanarayana T. 2015). Human CAs structure comprise of a tertiary fold with a 10 stranded β-sheet, Zn is located at the active site coordinated with three histidine residues and a water molecule.

2.3.1.2. β-CA:

It was first discovered in 1939 in plants, but it was not until 50 years later that it was reported to be not homologous to CAs from animals (Rudenko et al. 2015). In 2000 the first crystal structure of β-CA was reported (Mitsuhashi et al. 2000). β-CA are generally found in microalgae(Eriksson et al. 1996), eubacteria, archaebacteria (Smith et al. 2000), higher plants (Elleuche et al. 2010).

β-CAs are dimers, tetramers, hexamers and octamers (Tripp et al. 2001) with molecular mass ranging between approximately 45 kDa to 200 kDa (Shazia Faridi Satyanarayana T. 2015). A dimer is considered as the basic building block of this class of CAs. Zinc is the metal ion at active sites of all the β-CA which is coordinated by three residues i.e., two cystines (Cys32 & Cys90) and one histidine (His87) and a water molecule/hydroxide ion as shown in figure 2.6 (Di Fiore et al. 2015).

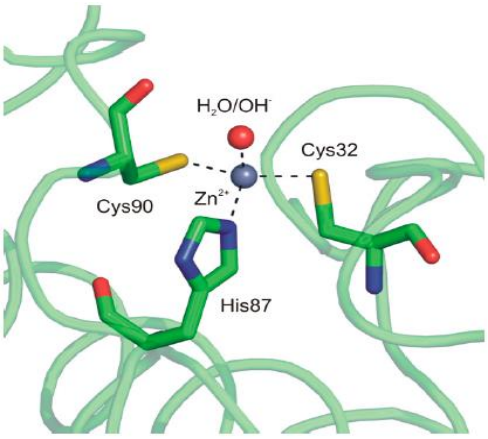

Figure 2.6: Active site representation of β-CA.

2.3.1.3. γ-CA:

γ-CAs are said to have evolved 3-4.5 billion years ago and are one of the most ancient form of carbonic anhydrase. They are found in diatom (Roberts et al. 1997), bacteria, green algae, Archaebacteria (Alber et al. 1994) and higher plants. They have both zinc and cobalt at their active site (Roberts et al. 1997).

They are said to have a homotrimeric structure having a monomeric subunit of molecular mass 20 kDa (Shazia Faridi Satyanarayana T. 2015). The homotrimer has a left-handed parallel beta helix. The active site has the ligand coordinated with three histidine residues (His81, His117, His122) and a water molecule/hydroxide ion (R. R. Yadav et al. 2014).

2.3.1.4. δ-CA:

δ-CAs are found in diatoms. In 1997 it was discovered and purified from *Thalassiosira weissflogii* having a molecular mass of approximately 34 kDa (Roberts et al. 1997). They were structurally very similar to α and γ carbonic anhydrases except for the amino acid sequences (Kupriyanova et al. 2017). δ-CAs have similar active site structure as that of α-family (Rudenko et al. 2015).

2.3.1.5. ζ-CA:

ζ-CAs are mostly found in marine diatoms (Lane et al. 2000). They have cadmium at their catalytic center instead of Zinc which is due to lack of Zinc ions in sea water (Kupriyanova et al. 2017).

2.3.1.6. η-CA:

η-CAs are a relatively new member of carbonic anhydrase family. It has been found in *plasmodium*.

2.3.3. Sources of Carbonic Anhydrase

Carbonic anhydrase is an ubiquitous enzyme. It is found in all organisms including prokaryotes and eukaryotes (Wong 2014). Its availability varies from species to species and many of them have been found to express genes of more than one family.

The first carbonic anhydrase was discovered and purified from human red blood cells in 1933 (The Late U Meldrum et al. 1933) and so far 15 human α-CA isoforms have been discovered having different tissue localization, catalytic activity and cellular distribution in forms of cytosolic, membrane bound and mitochondrial (Aggarwal et al. 2013) as shown in table 2.2. Three of these isoforms are acatalytic and lack Zn^{2+} active site and are known as CA related proteins.

Photosynthetic carbon capture function of CAs have been studied in cyanobacteria and algae. Bacteria and cyanobacteria have been found to have all classes of CAs. Some of the bacteria possessing this enzyme are *Rhodospirillum rubrum, Acetobacter woodii* (Gill et al. 1984)*, Neisseria gonorrhoeae* (Yeates et al. 2008)*, Helicobacter pylori* (Marcus et al. 2005)*, Citrobacter Freundii* (Rishiram Ramanan et al. 2009b)*, Bacillus subtilis , Pseudomonas fragi, Micrococcus lylae,* and *Micrococcus luteus* (A. Sharma et al. 2009). CA obtained from *Methanobacterium thermoautotrophicum* is found to be active at high temperatures (up to 75°C) (Yeates et al. 2008).

Table 2.2: Isoforms of α-CA found in humans.

Isoform	Tissue Location	Cellular location	Reference
hCA I	GI tract, RBCs	Cytosol	(Supuran et al. 2004)
hCA II	GI tract, RBCs, eyes, kidneys, lungs, brain	Cytosol	(Supuran et al. 2003)
hCA III	Skeletal muscles	Cytosol	(Supuran 2008b)
hCA IV	Kidney, endothelium	Extracellular Membrane bound	(Supuran et al. 2004)
hCA VA	liver	Mitochondria	(Nishimori et al. 2005)
hCA VB	Skeletal muscles, hearts, pancreas, spinal cord, kidneys, GI tract	mitochondria	(Nishimori et al. 2005)
hCA VI	Mammary and salivary glands	Milk/saliva (secretory)	(Nishimori et al. 2007)
hCA VII	Central nervous system	Cytosol	(Vullo et al. n.d.)
hCA-RP VIII	Central nervous system	Cytosol	(Supuran et al. 2004), (Supuran et al. 2003)
hCA IX	Tumours, GI mucosa	Cell membrane associated	(Thiry et al. n.d.)
hCA-RP X	Central nervous system	Cytosol	(Supuran 2008b)
hCA-RP XI	Central nervous system	Cytosol	(Supuran et al. 2003), (Supuran et al. 2004)
hCA XII	Renal, eye, intestinal, tumours, kidneys	Transmembrane	(Whittington et al. 2001)
hCA XIII	Reproductive tract, gut, lungs, brain, kidneys	Cytosol	(Lehtonen et al. 2003)
hCA XIV	Kidneys, brain, liver	transmembrane	(Whittingtons et al. 2004)

As of the moment algae to stand out when to be the most effective of carbon fixing microorganisms. Both micro and macro-algae have been utilized for carbon fixation because of their capability to uptake and utilize carbon at a fast speed, ability to produce secondary products like lubricants and biofuels (Wong 2014). Recently, algal transgenics has become an

area of interest for many researchers. Diatoms like *Phaeodactylum tricornutum* and green algae like *Chlamydomonas reinhardtii* and *Volvox carteri* have been used as model organisms for advanced genetic tools (Walker et al. 2005). Some algae have been used for carbon concentrating mechanisms by growing them in controlled photobioreactors using *Scenedesmus abundans* and *Chlorella pyrenoidosa* (Kargupta et al. 2015). *Scenedesmus obliquus* has been identified as temperature and high CO_2 tolerant microalgae which can sequester carbon form flue gas (de Morais et al. 2007).

Plant carbonic anhydrase was first extracted from leaf cytoplasm by Neish (Neish 1939). Plants have been found to have three types of CA: α, β, γ (Moroney et al. 2001). Plants having all three types of Carbonic Anhydrase are shown in table 2.3. The carbonic anhydrase is present in chloroplast of plants which carry out photosynthesis. Any change in the activity of Carbonic anhydrase directly affects CO_2 fixation and rate of photosynthesis (Ganai 2017). In higher plants, carbonic anhydrases show variations in their distribution as they have different number of genes present in each family. For example, Arabidopsis Thaliana has 19 genes (Initiative 2000), on the other had rice also have similar number of genes (Yuan et al. 2005). In higher plants, isoforms of α-CA, β-CA and δ-CA have the same catalytic mechanism despite their structural difference (S Lindskog 1997).

In C3 plants, the enzyme in leaves constitute 1 to 2% concentration of the total soluble protein (Okabe et al. 1984). About 95% of the total CA is found in chloroplast (Tsuzuki et al. 1985) and the other 5% is present in mesophyll cells. Carbonic anhydrase activity varies in different plants. CA had been diversely compartmentalized among tissues, organs and cellular organelles and his has been exhibited by CAs different physiological roles. CA helps to raise the concentration of CO_2 in the chloroplast which plays a significant role as a substrate by the RuBisCO enzyme, therefore increasing carbon fixation rate.

Table 2.3: List of plants having Carbonic Anhydrase.

Plant	Type of Carbonic Anhydrase	Reference
Physcomitella patens	α, β, γ	(Rathnam et al. 1975)
Gossypium hirsutum (cotton)	β	(Chang 1978)
Arabidopsis thaliana	α, β, γ	(Tsuzuki et al. 1985)
***Lactuca sativa* (lettuce)**	β	(Walk and Metzner 1975)
***Spinacia oleracea* (spinach)**	β	(pocker and Ng 1973)
***Petroselinum crispum* (parsley)**	β	(Tobin 1970)
***Lycopersicon lycopersicum* (tomato)**	β	(Kositsin and khalidova 1974)
Ananas comosus	α, β, γ	(Ming et al. 2015)
Flaveria pringlei	β	(Tetu et al. 2007)
Neurachne munroi	β	(Clayton et al 2016)
Neurachne alopecuroidea	β	(clayton et al. 2016)

In C4 plants, Carbonic anhydrase provides continuous supply of HCO^{3-} at the site of carbon fixation (Rathnam et al. 1975). Carbonic anhydrase also helps in the diffusion of CO_2 through plasma membrane and the chloroplast in plants.

2.3.4. Factors affecting Carbonic Anhydrase enzyme

2.3.4.1. Effect of temperature on Carbonic Anhydrase enzyme

Temperature plays a very important role in the biological activity of any enzyme. After a specific temperature any enzyme can be denatured very easily resulting in the loss of activity. Sarraf et al. studied the temperature dependence of the activity and structure of the enzyme bovine carbonic anhydrase (Sarraf, et al. 2004). Firstly, they noted that the amino-acid

sequences of bovine and human carbonic anhydrase are almost 87 percent identical. It has been shown that there is a decrease in the amount of β-structures and amount of random coil, from 25 to 40°C. But from 40 to 52°C, the amount of helix is slightly decreased and there is an increase in the percentage of β-structures. They reported that the initial decrease may be due to the lower stability of β-structures comparing to helices and enzyme became looser at higher temperatures. The reason of this could be either amino acid may be liberated from their relevant hydrogen bond in protein structure or the establishment of β-structures may become more favorable.

Thermal behaviour of bovine carbonic anhydrase was also carried out by Lavecchia and Zugaro (Lavecchia and Zugaro 1991). They heated the enzyme solution from 40°C to 70°C and measured the activity of carbonic anhydrase. They noted that carbonic anhydrase was active under 60°C, but it lost its activity between 60-65°C. They explained irreversible denaturation as the structural deformation of carbonic anhydrase and it's caused unfolding.

2.3.4.2. Effect of pH on Carbonic Anhydrase activity

An enzyme's activity or the rate of chemical reaction is affected by the structure of the enzyme. When the pH of an aqueous solution changes it leads to changes in the shape of enzyme. The change in pH may also lead to a change in the shape and charge of the substrate as well. This change in structural shapes of the substrate and enzyme can be reversible if the change in pH is within narrow range. But if the change in pH is significant then the substrate and enzyme may go through denaturation. In which case they will not identify each other and there will be no reaction.

pH is the concentration of hydrogen ions in a solution and any slight increase or decrease in pH changes the concentration of the solution. Theses ions are responsible for change in structure of the enzyme, due to breakage of existing bond or formation of new bonds.

2.3.5. Applications of CA

Applications of carbonic anhydrases used in industries are many. Some of them are as follows.

Artificial Lungs:

Respiratory failure affects thousands of patients all over the world. Because of this the patient has to either go through a lung transplant surgery or put on mechanical ventilators. But these solutions are short term as they put pressure on lung tissues, causing damage in the long run (Kaar et al. 2007). An artificial lung is a device capable of replacing mechanical ventilators. They are capable of assisting in respiration without the involvement of lungs. Current models are good for gas exchange but can only be used for patients in intensive care units and are not implantable because of their large size. Hollow fibre membranes have been used to make artificial lungs and lot of work has been done in this area (Kaar et al. 2007). The main issue with artificial lungs as of now is the transfer of CO_2 across the HFM. One was to increase CO_2 removal rate is by immobilizing CA on the membrane. CA treated HFM shows 75% higher rate of CO_2 removal rate than untreated HFM. These findings indicate possibility of smaller artificial lungs being engineered and used (Kimmel et al. 2013).

Biosensors:

Chemical media containing trace number of similar molecules is hard to quantify. To achieve such specificity and sensitivity biosensors can be used. Human carbonic anhydrase (HCA-II) has strong affinity towards zinc, which has been used to quantify trace amounts of zinc in sea water and waste water (Sven Lindskog et al. 1964). The biosensor would work along the sea bed and give out fluorescence signal at the surface upon binding of inhibitor, upon binding of zinc at the active site of CA (Thompson et al. 1993).

CO_2 sequestration for confined spaces:

High levels of CO_2 have negative effects on human beings such as problem in breathing, impaired judgement and even death in extreme cases. Controlling the amount of CO_2 gas in confined spaces is very important. Initially it was NASA who developed CA based CO_2 capturing technology for submarines and spacecrafts. CA was added to thin aqueous buffered films and compressed between porous membranes. The concentration of CO_2 in confined spaces is relatively low as compared to industrial fumes. The CO_2 dissolves in the enzyme containing buffer, then diffuses across the membrane and is removed on the back with vacuum (Pierre 2012).

Drug Delivery:

For treatment of analgesic overdose, CA employed CO_2 responsive cationic hydrogels in antidote delivery has been used. Alternate medicines are effective but have side effects that can lead up to death due to increased CO_2 levels and decreased O_2 levels. The CA treatment involves antidote delivery system that responds to high CO_2 levels. A cationic hydrogen based on DMAEMA polymer has been used for this purpose (Satav et al. 2010).

Blood substitutes:

For surgeries and trauma injuries, a continuous supply of blood is needed. Since, natural blood in limited in supply, there has been progress in development of blood substitutes which consist of 4–5 cross-linked stroma-free hemoglobin (polySFHb) molecules (Ge et al. 2002). These substitutes are found to have few advantages like they can be easily sterilized, stored for long time and contains no blood antigens.

Carbon dioxide sequestration :

Mirjafari et al. (2007) studied the effect of bovine carbonic anhydrase on the hydration of carbon dioxide, and its precipitation in the form of $CaCO_3$. In their study, enzyme solution

prepared in phosphate buffer at different concentrations and then this solution mixed with a solution carbon dioxide saturated water. They reported that the rate of hydration reaction increased with both the temperature and enzyme concentration. They also showed that calcium carbonate precipitation was increased with the help of carbonic anhydrase, but the concentration of the enzyme did not have any effect on the precipitation. They indicated that temperature increase caused increase in calcium carbonate formation and the enzyme activity was not influenced by the pH.

Bond et al. (2007) aimed to develop an enzymatic CO_2 scrubber to reduce CO_2 emissions from fossil fuel burning power plants. They used bovine carbonic anhydrase as a catalyst to improve the rate of CO_2 hydration (Bond, et al. 2007). Investigation of the effect of other chemical species (NOx, SOx) on bovine carbonic anhydrase activity was also done. NOx and SOx is very important in the flue gases. According to their report, high concentrations of NOx (>0.05M) and SOx (>0.005M) inhibited the enzyme activity.

Polymeric membranes have been also used for CO_2 separation from flue gas. These membranes have selective layer that is non-porous film that transport gases by diffusion mechanism. Some types of polymeric membranes are polyarylene ethers, polyarylates, polyethylene oxide, poly carbonates, polymethacrylates which are used for CO_2 separation (Yang et al. 2008), (S. J. Metz et al. 2004).

Ren et al. (2012) used block co-polymers to prepare polymeric membrane. Poly (amide-6-b-ethylene oxide) was used to prepare multilayer polyetherimide (PEI)/polydimethylsilicone (PDMS)/PEBA1657/PDMS composite membranes. They balanced hard and soft blocks to provide good CO_2 separation performance without loss of its permeability (Ren et al. 2012).

Liu et al. (2005) used a type of hollow fibre membrane (hollow fibre poly (ether block amide) (PEBA)/polysulfone (PSf) composite membranes for CO_2/N_2 separation. They showed that

CO_2 permeability increases with increase in gas pressure, whereas N_2 permeability was independent of pressure applied (Liu et al. 2004).

Liu et al. (2005) studied the effect of cations in the produced water from the Permian and San Juan Basins to enzymatic CO_2 scrubber. They used Bovine carbonic anhydrase to accelerate CO_2 hydration by them. It was reported that precipitation of calcium carbonate occurred much faster in the presence of enzyme. They also investigated the effect of temperature on precipitation time. They have shown that the increases in temperature accelerated the precipitation for both enzymatic and control reactions (Liu, et al. 2005).

2.4. Immobilization of CA

Considerable research has been focused on stabilizing CA or binding CA to solid support. There are many advantages of immobilizing CA over free CA enzyme, including the improvement of enzyme stability and operational lifetime, enzyme recover and reuse, easy separation of products and flexibility in reactor design. There are mainly two types of immobilizations i.e., physical and chemical immobilization. Physical immobilization involves adsorption and entrapment while chemical immobilization covalent attachment or cross-linking to a water insoluble support.

Physical adsorption uses weak hydrogen bonds and Van Der Waals interaction for binding of the enzyme to the substrate. It is a simple and cheap method and the integrity of the structure of enzyme is retained mostly by this method. One of the disadvantages of adsorption is the desorption of enzyme from the solid support (Brena et al. 2013). In case of cross-linking and covalent binding the enzyme stability is higher, and the disadvantage is that there are high chances of loss of activity of enzyme. Entrapment method has wide applicability but there are high chances of leaching/leakage of enzyme from the support.

Ray (1997) purified and immobilized Human erythrocyte carbonic anhydrase in polyacrylamide gel. As compared to the soluble enzyme, the immobilized enzyme was considerably more resistant to heat and sulphanilamide action (Ray, 1977).

Carbonic anhydrase has been reported to be immobilized on chitosan based activated alumina–carbon composite beads. Synthesized adsorbent has been characterized by BET, FTIR, XRD and SEM. The optimized condition under which the highest enzyme activity was obtained is pH 9.5 and temperature 45°C at concentration of 1 mg/5 ml (Vinoba et al. 2012).

Bond et al. proposed that bacterial overexpression was the suitable way for the carbonic anhydrase production and they proposed three different supports for carbonic anhydrase immobilization (acrylamide, alginate, and chitosan-alginate) (Bond, et al. 2001). They noted that alginate and chitosan-alginate support were better because these were easy to produce, non-toxic, cheap, biodegradable and environment friendly.

CA immobilized on chitosan beads has also been performed (Wanjari et al. 2011). It has been concluded as well that storage stability of immobilized CA is up to 20 days at -20°C, which is higher than free CA.

There is also report carbonic anhydrase immobilized on surfactant-modified silylated chitosan (SMSC) as support. In this study, silylated chitosan material was treated with surfactant-like hexadecyltrimethylammonium bromide (HDTMABr) to increase the surface area of chitosan by forming mesh network that allows more CA to be embedded. The optimum temperature and pH value were reported to be 35°C and 7, respectively (R. Yadav et al. 2010).

Other researches show that bovine carbonic anhydrase have also been immobilized on different types of support for biomimetic CO_2 sequestration. One of the applications is the immobilization of BCA within polyurethane (PU) foam. For estimating activity, tris buffer containing 10% acetonitrile has been used due to the limit of p-NPA solubility. Stability test was performed, and the result showed that immobilized CA maintains stability and the same

activity after seven washings. The immobilized CA retains the same activity for 45 days stored in tris-buffer at room temperature (E Ozdemir 2009).

Thermal stability of bovine CA immobilized within polyurethane (PU) foam has been done as well (Vinoba et al. 2012). CO_2 gas generated when forming the foams plays the role of creating large pores in the crosslinked polymeric sponge-like material. TGA analysis showed that PU foam is thermally stable at the temperature of 280°C. The optimum temperature for immobilized CA activity was 45°C and 98% stability was obtained at less than 50°C. A decrease in the activity was seen from 50°C and until there was completely no activity at 60°C.

Rationale:

- The raw material (waste from supermarkets) is readily available. This vegetable waste usually ends up at landfills as they are not fit for human consumption anymore. So, it can be used to extract the enzyme (CA) which can further be used for CO_2 sequestration purposes.
- In addition to that not much study has been reported using plant-based enzyme for carbon capture and storage purposes.

Chapter 3
Materials and methods

3.1. Equipment

i. **pH meter:** For this study Sartorius PB-11 basic meter and Ph probe was used.

ii. **Centrifuge**: Thermo Fisher Sorvall RT1 centrifuge was used.

iii. **Ultrasonic bath:** For this study Thermo Fisher Scientific ultrasonic bath (5.7L) was used.

iv. **Magnetic Stirrer:** For this study Fisher Scientific Isotemp® was used.

3.2. Materials

i. ***Spinach:*** Fresh spinach leaves were collected from Superstore and stored at -20°C until used.

ii. ***Enzyme:*** Bovine Carbonic Anhydrase (BCA) was purchased from Sigma-Aldrich®, ≥ 99% purity, specific activity ≥ 2500 W-A units/mg protein, lyophilized powder, and was stored between 2-6°C.

iii. ***Alginate:*** Alginic acid sodium salt from brown algae was obtained from Sigma-Aldrich®, powder, low viscosity and stored at room temperature.

iv. ***Chitosan:*** Chitosan was obtained from Sigma-Aldrich®, medium molecular weight, powder and stored at room temperature.

v. ***Trizma® Base:*** Trizma® base was obtained from Sigma-Aldrich®, ≥ 99.9% purity, crystalline, pH: 10.5-12 and stored at room temperature.

vi. ***Dialysis membrane:*** Dialysis tubing cellulose membrane (76mm) was obtained from Sigma-Aldrich®.

Other Chemicals:

Ammonium Sulphate (≥ 99%), Calcium chloride (≥ 97%) were purchased from Sigma-Aldrich®. Hydrochloric acid and acetic acid were purchased from Fisher Scientific.

CO_2 cylinder: Carbon dioxide (CO_2) cylinder was obtained from Praxair Inc.

3.3. Experimental Procedures

3.3.1. Spinach preparation

Spinach leaves were washed thoroughly with water and the stems were removed and discarded, then the leaves were dried at room temperature for 30 min to removed excess water and then stored at -20°C in a sealed plastic bag.

3.3.2. Enzyme Extraction

Slightly modified procedure of Pocker and Ng (1973) was followed for this step. The procedure was carried out at 4°C. The stored leaves were blended with 20nM Tris-Hcl buffer (pH 8) in a blender. Approximately 1.5 ml of buffer was used for each gram of leaves (Pocker et al. 1973). The suspension was filtered through cheesecloth and the pulp was discarded. This homogeneous mixture was centrifuged at 4000 rpm for 30 min at 4°C. The pellet was discarded, and the supernatant was used to purify carbonic anhydrase enzyme.

3.3.3. Partial purification of enzyme

Enzyme purification was done with the help of ammonium sulphate precipitation. The supernatant obtained from extraction step was used for partial purification of enzyme. The supernatant was brought up to 30% saturation with $(NH_4)_2SO_4$ and stirred for 1 hour at 4°C before centrifuging at 4000 rpm (4°C) for 30 min. The pellets were discarded. To the supernatant more $(NH_4)_2SO_4$ was added to bring the final concentration to 55% and mixed for 1 hour followed by centrifugation at 4000 rpm (4°C) for 30 min. The precipitate was recovered and dissolved in 5ml of 20mM Tris-Hcl buffer (pH 8) and then dialysed against the same buffer at 4°C for 24 hours to remove salts (Marianne K. 1978). The enzyme obtained after dialysis was stored at 4°C for further experiments.

3.3.4. Carbon Dioxide saturated water

Carbon dioxide saturated solution was prepared by passing gaseous CO_2 from a cylinder through 500 ml of deionized water at 0-4°C for 1hour.

3.3.5. Carbonic Anhydrase activity assay

Carbonic Anhydrase activity was assayed by using electrometric method developed by Wilbur and Anderson in 1948. In a glass vial 3ml of 20mM Tris Base buffer (pH 8.3, 25°C) was poured followed by adding 50µl of enzyme solution. pH electrode was placed in the solution while stirring. After the pH reached maximum (pH > 8.5), 2ml of ice-cold CO_2 saturated water was added to the solution. The drop in pH from 8.3 to 6.3 was monitored and the time was recorded for this 2 units pH drop. Chilled distilled water was used in place of enzyme solution for the control (Warrier et al. 2014). Wilbur-Anderson (WA) activity of CA was calculated using the following formula and expressed as WA units per ml of enzyme.

$$\text{Enzyme activity } (U) = \frac{(T_i - T_f)}{(T_f)} \text{ ……..(x)}$$

T_i and T_f signify the time required for 2 units drop in pH in control and in test sample, respectively. T_i stands for time needed for change in pH without the enzyme and T_f stands for time needed for change in pH with enzyme.

3.3.6. Protein estimation

The concentration of protein was assayed according to the method of Lowry with Bovine Serum Albumin (BSA) as the standard protein (Lowry et al. 1951).

Reagents:

- BSA stock solution (1mg/ml)
- Analytical reagents:
    a. 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solution.
    b. 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37% sodium potassium tartarate solution. Analytical reagent was made by mixing 2 ml of (b) with 100 ml of (a).
- Folin-Ciocalteau reagent solution: equal volume of reagent and distilled water was mixed. This reagent is made fresh on the day of use.

For standard plot different dilutions (0.05-1 mg/ml) of BSA solution was prepared by mixing stock BSA solution (1mg/ml) and water. 0.2 ml of protein solution was taken in test tubes and to it 2 ml of the analytical reagent (copper sulphate reagent) was added and mixed. This solution is incubated for 10 mins at room temperature. Then 0.2 ml of Folin-Ciocalteau solution was added to the test tubes and incubated for 30 mins. Water was used as blank for standard plot and Tris-Hcl was used as blank for protein estimation. Absorbance was measured at 660nm.

3.3.7. Preparation of alginate and chitosan beads

3.3.7.1. Alginate beads

4% (w/v) sodium alginate solution was made in distilled water and was stirred for 1 hour. CA enzyme (0.1mg/ml) was added to this solution. It was followed by drop wise extrusion of CA-alginate solution into 2.5% (v/v) $CaCl_2$ solution to form beads. The beads were incubated for 1 hour at 4°C (R. R. Yadav et al. 2012). The beads were then washed with 20mM Tris buffer and kept in the fridge until further use. For control, beads were made without adding the CA enzyme solution.

3.3.7.2. Chitosan Beads

Chitosan solution was prepared by dissolving 2 g chitosan in 100 ml of 1% acetic acid. The solution was stirred at 30°C for 1 hour to obtain a viscous solution (Simsek-Ege et al. 2002). This viscous solution was the degassed for 2 hours followed by adding dropwise in 1M NaOH solution while continuously stirring to form beads. The beads were allowed to stabilize in NaOH solution for overnight. The beads were then washed thoroughly with distilled water to remove excess NaOH.

3.3.7.3. Chitosan Membrane

Membranes were prepared with varying concentration of chitosan (1-2%) and acetic acid (1-2%). Chitosan was dissolved in acetic acid and stirred for an hour at 30°C. Then the chitosan solution was degassed for 1 hour followed by pouring it on glass plates and putting them in

oven at 60°C overnight for drying. After drying the glass plates were immersed in 1M NaOH solution for half an hour and then washed with distilled water to wash off excess NaOH. The sheets were then re-dried at room temperature for 3-4 days (Magalhães et al. 1998).

3.3.8. Enzyme Immobilization

For alginate beads, entrapment method was used for enzyme immobilization. Enzyme was added in the alginate solution and then added dropwise in 2.5% $CaCl_2$ solution to form beads. The beads were incubated for one hour and then washed with Tris-HCl buffer (R. R. Yadav et al. 2012). The immobilized beads were stored in fridge for further experiments.

For chitosan beads and membrane, adsorption was used for enzyme immobilization. The chitosan beads and membrane were incubated with enzyme solution (1mg/ml) for overnight at 4°C with slight stirring. After incubation the beads and membrane were washed with distilled water. The supernatant obtained was used for protein estimation to determine the amount of enzyme immobilized on the substrate.

3.3.9. Thermal and pH stability

The immobilized or free enzymes were kept in Tris-HCl buffer (pH 8.0) at different temperatures (25-60°C) for 1 hour. The enzyme activity was measured to analyse the optimum temperature for both free and immobilized enzymes.

For optimum pH the enzyme activity was measured after incubating the free or immobilized enzyme at various pH ranging from 5.5 to 10 in Tris-HCl buffer for 1 hour at room temperature.

3.3.10. Storage stability

The storage activity of free and immobilized enzyme was determined by storing it for 30 days at 4°C. The enzyme activity was determined every week with the help of enzyme activity assay.

3.3.11. Sequestration of CO_2

1ml of 1M Tris buffer (pH 8) was added to 10ml of CO_2 saturated water and shaken at room temperature. Then to this mixture, 10ml of 2% $CaCl_2$ was added followed by 1ml of enzyme

solution (1mg/ml) and shaken. The precipitate formed was filtered using whatmann filter paper and dried in oven. The amount of precipitate formed was weighed. In case of immobilized enzyme, the enzyme solution was replaced with beads and film.

3.3.12. Recyclability of immobilized enzyme

Immobilized enzyme was used for CO_2 sequestration as mentioned above. The immobilized enzyme was then washed with distilled water and reused again for CO_2 sequestration. This was repeated until no $CaCO_3$ precipitate was obtained in the end.

Chapter 4 Results and discussion

4.1. Extraction and purification of Carbonic anhydrase from spinach

Carbonic Anhydrase was extracted from Spinach and partially purified by ammonium sulphate precipitation method. As mentioned in section 3.3 the spinach was blended in a blender and a homogeneous slurry was obtained, which was then filtered with muslin cloth to obtain the crude extract. The crude extract was then partially purified by adding ammonium sulphate precipitation followed by dialysis as shown in figure 4.1. Commercial CA (Bovine Carbonic Anhydrase) was used to compare the activities of fully and partially purified enzyme. The total activity and specific activity of commercial enzyme, crude extract and the precipitate obtained after partial purification is shown in Table 4.1.

(A) **(B)** **(C)**

Figure 4.1: (A) Crude extract obtained after filtration; **(B)** Precipitate obtained after ammonium sulphate precipitation; **(C)** Dialysis of precipitate.

The specific activity of partially purified enzyme is 621.78 U/mg which is considerably lower than that of commercial enzyme (BCA) 1706.67 U/mg, however, it should be considered that the spinach derived CA was obtained in a simple way and after partial purification it still contained some impurities which explains the differences in activities. It has been reported by Kandel et al. (1977) that partially purified CA from spinach had specific activity of 389 U/mg, specific activity of pecan leaves was reported to be 61.2 U/mg which is significantly less than

what was obtained from spinach in this study. Purification level of the of extracted sample is 1.71 times which indicates that the partial purification step worked, and the sample got purified. Yield is the enzyme activity retained after purification step. The initial enzyme yield is said to be 100%, after purification step it was 188.53 times which indicates that the majority of proteins in the original crude extract was purified.

Table 4.1: Partial purification of CA from spinach leaves.

	Total Protein (mg/ml)	**Total Activity (Units)**	**Specific activity (Units/mg)**	**Yield**	**Purification Level**
Bovine Carbonic Anhydrase (commercial)	5	8533.33	1706.67	-	-
Crude extract	2.573	933.33	362.73	100	1
55% $(NH_4)_2SO_4$ saturation precipitate	2.830	1759.64	621.78	188.53	1.71

4.2. Immobilization on different materials

Immobilization is confinement of enzyme to a support other than the substrates and products. Due to poor regeneration and recovery of enzyme in aqueous solutions, immobilization has drawn a lot of attention. Natural and inorganic polymers have been used for immobilization (Datta et al. 2013). Immobilization techniques have received attention in the past decade as they have several advantages like stability, inertness, physical strength, reusability, ease in separation, more robust and resistant to environmental changes (Lee JF et al. 2015), (Datta et al. 2013). One of the disadvantages of enzyme immobilization is the diffusional limitation of substrate to the enzyme, leaching of enzyme and cost of material (Homaei et al. 2013). For this study alginate and chitosan have been used as immobilization

materials/support. They both are natural polymers. Alginate has been extensively used because of its non-toxic nature and reusability for immobilization as calcium-alginate beads, alginate-xanthan beads for enhanced enzyme activity and chitosan, a derivative of chitin, has several advantages as well such as its easy availability, biodegradability and biocompatibility (Harish Prashanth et al. 2007) (Homaei et al. 2013).

In this study the partially purified enzyme was immobilized on alginate beads, chitosan beads and chitosan film. 4% (w/v) alginate beads, 2% (w/v) chitosan beads were made and immobilized with 0.1 mg/ml of enzyme solution overnight. For chitosan films different concentrations of chitosan (1-2% (w/v)) and acetic acid (1-2% (v/v)) were used for making films and then those films were immobilized with enzyme solution overnight. Table 4.2 and Table 4.3 show the amount of protein that got immobilized on the beads and film and their respective enzyme activities. Entrapment of enzyme was done in alginate beads, it showed specific activity of 23.37 U/g of alginate beads with protein content of 0.00037 g/g of beads which is higher as compared to immobilization of commercial CA on alginate beads which showed specific activity of 26.8 U/g of beads with protein content of 0.0019 g/g of beads (A. Sharma et al. 2011). On chitosan beads the enzyme was immobilized by adsorption method. The beads had specific activity of 20.96 U/g of beads with protein content of 0.039 mg which is better than that has already been reported in literature which shows that chitosan beads were immobilized with CA extracted from *B. Pumilus* by adsorption had specific activity of 2.85 U/mg with protein content of 0.04 mg (Wanjari et al. 2011).

Table 4.2: Total activity, protein content and specific activity of enzyme immobilized on alginate and chitosan beads. For experiments 200 mg of alginate and chitosan beads were taken.

Nature	Total Activity for 200mg beads (U)	Protein content on 200mg of beads(mg)	Specific Activity (U/mg)	Protein content for 1 g of beads (g/g beads)	Total Activity for 1 g of beads (U/g of beads)
Alginate beads	1.74	0.074	23.37	0.000374	8.74
Chitosan beads	0.83	0.039	20.96	0.000199	4.18

For making chitosan films different concentrations of chitosan and acetic acid was used to obtain the optimum concentrations of chitosan and acetic acid because in literature several different concentrations of the same were reported. Two concentrations of chitosan (1 and 2 % (w/v)) were taken along with two concentrations of acetic acid (1 and 2% (v/v)). On increasing the concentration of chitosan, the mixture became very viscous and was heated at 30°C to obtain a homogeneous mixture. Chitosan films with lower chitosan concentration were quite fragile as compared to those made with higher concentration of chitosan (2% (w/v)). Chitosan concentration could not be increased beyond 2% (w/v) as it became very difficult to dissolve even after heating and a very viscous mixture was obtained which was not appropriate for making films. Chitosan film with 2% (w/v) chitosan dissolved in 1% (v/v) acetic acid was the one which showed maximum specific activity (53.88 U/mg) after immobilization of enzyme and hence was selected for further experiments.

Table 4.3: Optimization of chitosan concentration and acetic acid percentage for chitosan film preparation.

Chitosan concentration (g)	Acetic acid (%)	Specific Activity (U/mg)
1	1	30.22
1	2	20.14
2	1	53.88
2	2	22.48

4.2.1. Effect of temperature on free and immobilized enzyme

Temperature is known to have a very significant effect on the activity of enzymes. Most of the enzymes denature at higher temperature which is the reason why enzymes are being immobilized. In industrial processes the temperatures are very high which are not favourable for enzymes. Immobilization of enzymes has been proven to aid in the stability of enzymes at higher temperatures. In this study the effect of temperature on both free and immobilized enzyme was done to see which one is more effective for the enzyme to be used at a higher temperature.

The effect of temperature on free enzyme, alginate beads, chitosan beads and chitosan film were studied by incubating free and immobilized enzyme in enzyme solution (0.1 mg/ml) at temperatures ranging from 25-60°C for an hour and then their activity was measured. Free enzyme showed maximum activity at 30°C as shown in figure 4.2.

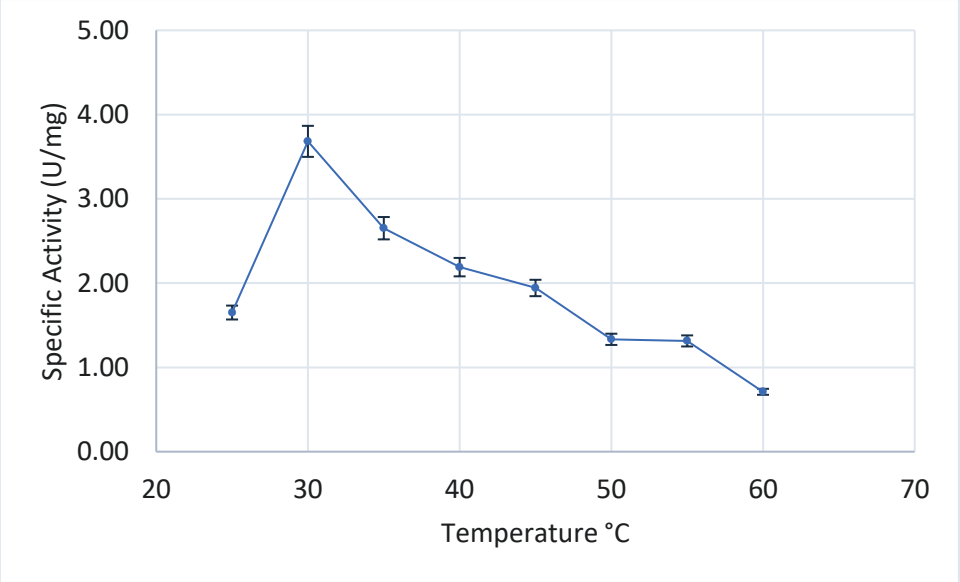

Figure 4.2: Effect of temperature on free enzyme.

As compared to free enzyme the enzyme immobilized on alginate beads is showed in figure 4.3, as the temperature increases the specific activity of the immobilized enzyme increases and reaches maximum at 40°C and then starts decreasing on further increasing the temperature.

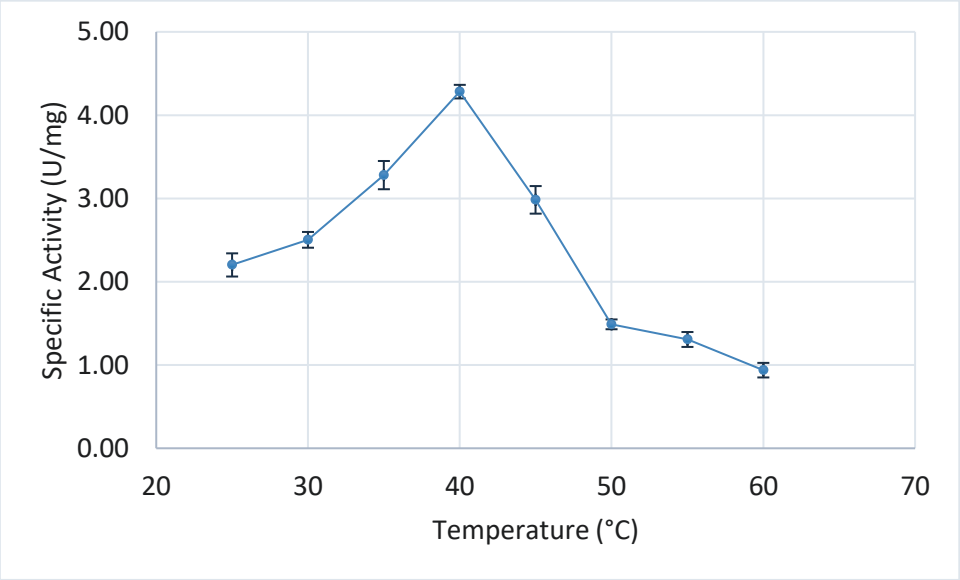

Figure 4.3: Effect of temperature on enzyme immobilized on alginate beads.

Immobilized chitosan beads followed the same pattern as alginate beads. At first as the temperature increased the activity increased as well up till 35°C but then it started decreasing rapidly as the temperature was further increased up to 60°C (figure 4.4). In case of chitosan

film, the enzyme activity was maximum at 35°C which was 5°C higher than free enzyme (figure 4.5).

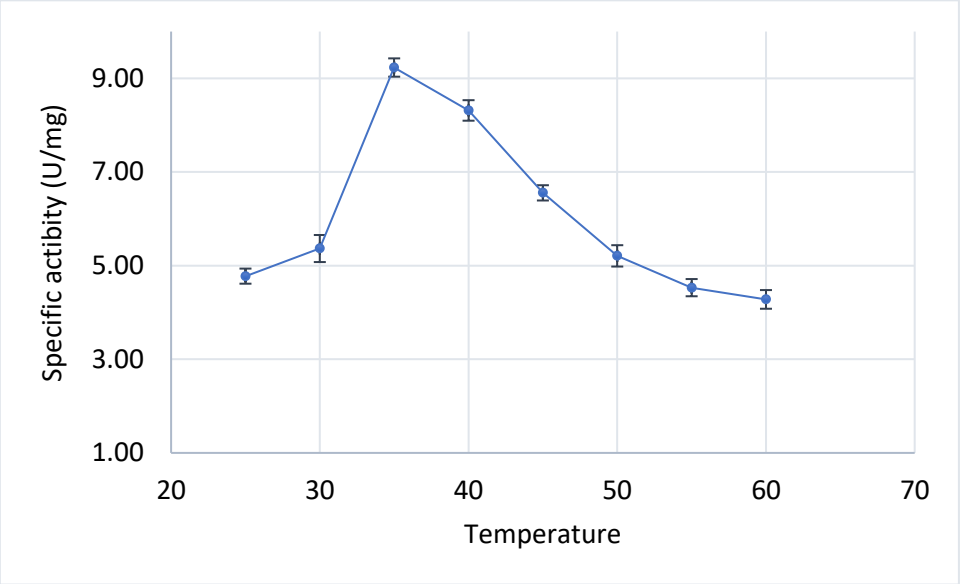

Figure 4.4: Effect of temperature on enzyme immobilized on chitosan beads.

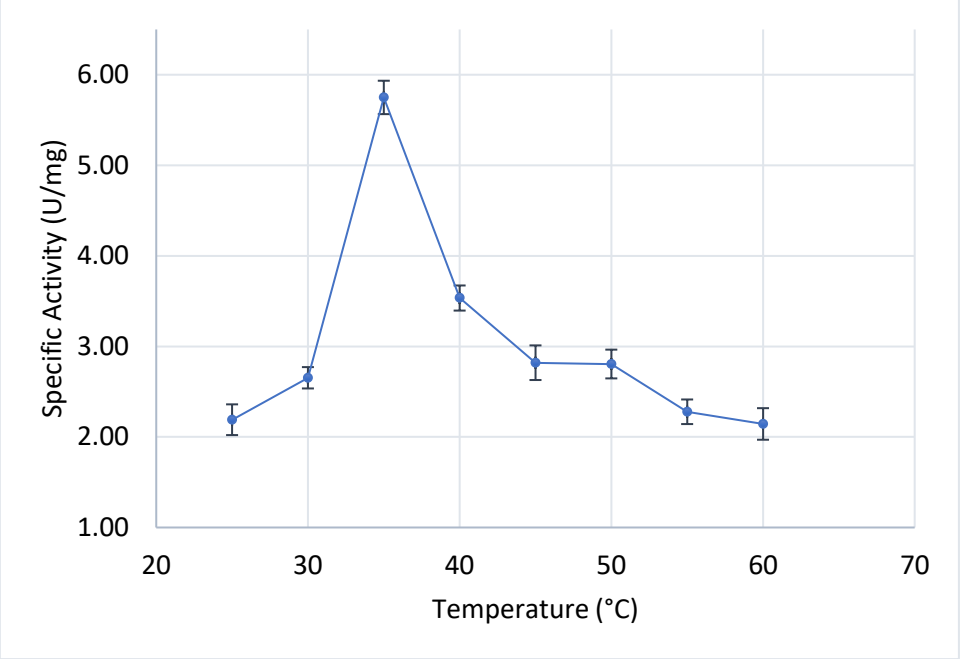

Figure 4.5: Effect of temperature on enzyme immobilized on chitosan film.

Free enzyme showed higher activity at 30°C while alginate beads (40°C), chitosan beads (35°C) and chitosan films (35°C) showed high activity at higher temperatures than free enzyme, this difference in temperatures for free and immobilized enzyme indicates that at

higher temperature the support protect the enzyme from denaturation. It has been reported that that immobilization increases the rigidity of enzyme, which increases the stability towards increasing temperatures compared to free enzymes in solution (Abdel-Naby 1993). The decrease in activity after reaching the optimum activity may be due to denaturation of enzyme at higher temperature which is in concurrence with earlier reported work (R. R. Yadav et al. 2012), (Vinoba et al. 2012). At higher temperatures the protein denatures because of conformational changes and protein unfolding (Vinoba et al. 2012). Thus, it can be concluded that at higher temperatures the immobilized enzymes are more stable than free enzyme.

Alginate beads show highest specific activity at 40°C and chitosan beads at 35°C. In case of alginate beads the enzyme is entrapped in the beads while in case of chitosan the enzyme has been adsorbed on the beads. Enzyme is more stable at higher temperature when immobilized by entrapment than by physical adsorption. In case of physical adsorption, the enzymes are released from the support at higher temperature. These results coincide with those reported by Ohtakara et al, (1988) whose report suggest that immobilization of glucoamylase on chitosan beads showed lesser stability on physical adsorption as compared to that of entrapment or ionic bonding (Skjak-Braek et al. 1989).

4.2.2. Effect of pH on free and immobilized enzyme

One of the most enzyme activity altering parameter in an aqueous medium is pH. Change in pH can alter the shape of protein which can lead to altered protein recognition or the enzyme might lose its activity. pH is a measure of H^+ ions and therefore a good indicator of OH^- ions. The charges on H^+ and OH^- ions interfere with the hydrogen and ionic bond that hold together an enzyme, since they will be repelled or attracted by the charges created by the bonds. This interference causes a change in the shape of the enzyme. Once the shape of enzyme changes the substrate cannot bind to it. pH alterations not only change the shape of

the enzyme but also the charge on the substrate because of which the substrate cannot bind to the active site and cannot undergo catalysis.

For determining the effect of pH on the activity of free and immobilized enzyme, they were incubated for an hour in Tris-HCl buffer prepared at pH ranging from 5.5-10. The specific activity of the free and immobilized enzyme was calculated to obtain the pH at which each of them showed the highest activity.

From figure 4.6 it can be seen that as the pH of the buffer was increased the enzyme activity also increased but after reaching the maximum activity (pH 8) it started decreasing. Alginate beads also showed the same pattern of increase in activity with increase in pH, with highest specific activity at pH 8 followed by a decreasing pattern (figure 4.7). According to literature, for both free enzyme and enzyme immobilized on alginate beads the pH with highest activity has been reported close to 8.5 which coincides with the results in this study (R. R. Yadav et al. 2012).

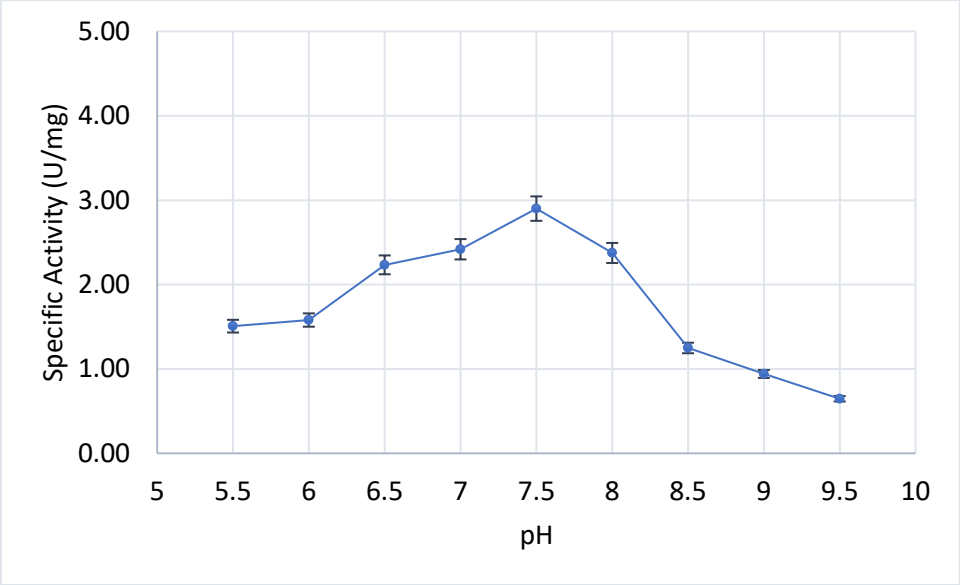

Figure 4.6: Effect of pH on free enzyme.

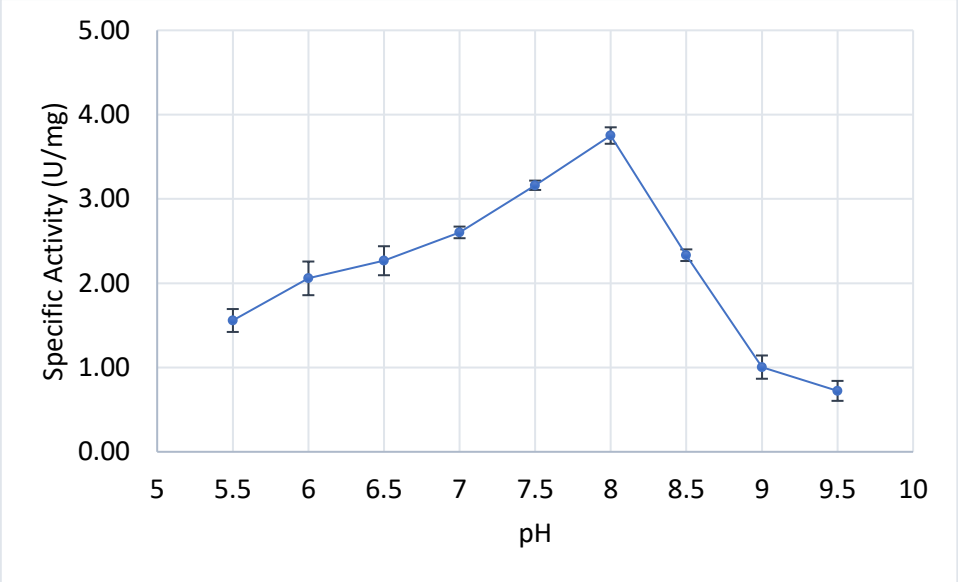

Figure 4.7: Effect of pH on enzyme immobilized on alginate beads.

Chitosan beads showed maximum activity at pH 8.5 and for chitosan film the maximum specific activity was obtained at pH 7.5 (figure 4.8 and 4.9).

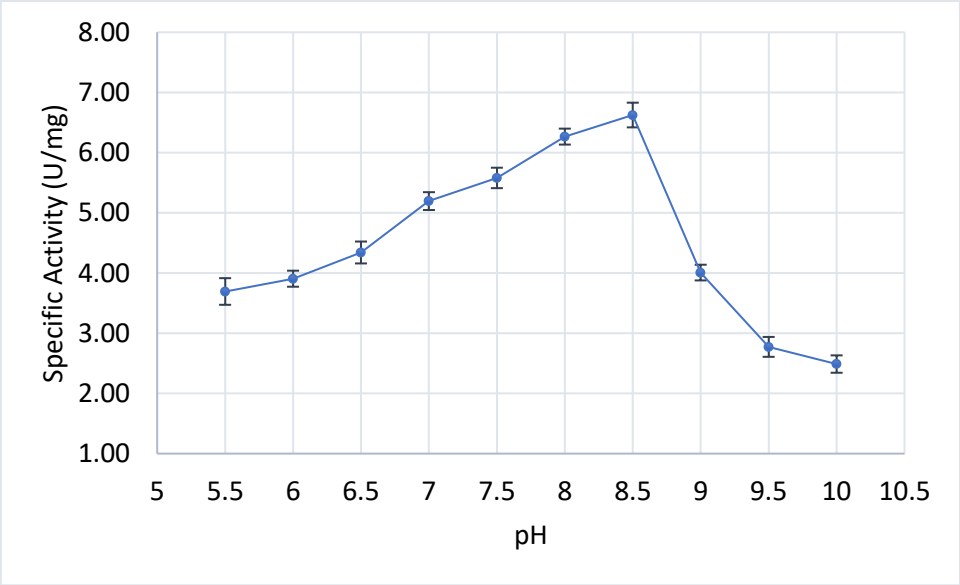

Figure 4.8: Effect of pH on enzyme immobilized on chitosan beads.

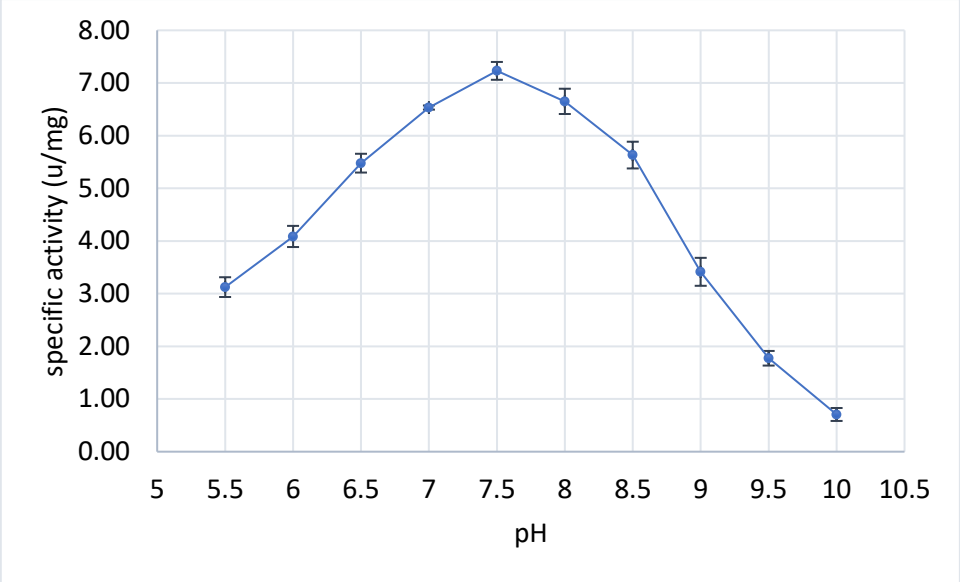

Figure 4.9: Effect of pH on enzyme immobilized on chitosan film.

Out of all the materials used for immobilization Chitosan beads showed the maximum stability of enzyme at pH 8.5 as compared to that of free enzyme at pH 8, alginate beads at pH 8 and chitosan film at pH 7.5. This difference in the optimum pH for chitosan beads and chitosan film is due to the different porosity and adsorption structure (Adarsh et al. 2007), (Ouyang et al. 2014).

4.3.Sequestration of CO_2 by free and immobilized CA

To demonstrate the feasibility of CO_2 sequestration the biomimetic approach using CA from plant domain was done. CA was added to CO_2 saturated water containing calcium chloride solution for enhanced precipitation of carbonate and bicarbonate salts. The immobilized enzymes were used in place of free enzyme in the process to check sequestration efficiency of immobilized enzymes. Table 4.4 shows the $CaCO_3$ precipitate formed after carbonation reaction.

Table 4.4: Precipitation catalysed by free and immobilized CA.

Enzyme Nature	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	$CaCO_3$ Precipitate (g)
Free enzyme	8.61	1	172.3	0.089
Alginate Beads	1.74	0.074	23.37	0.018
Chitosan beads	0.83	0.039	20.96	0.009
Chitosan film	0.29	0.017	17.58	0.005

The carbonation capacity of free enzyme was found to be 89 mg as compared to 18mg for alginate beads, 9 mg for chitosan beads and 5 mg for chitosan film. Sharma and Bhattacharya (2010) successfully demonstrated the sequestration of CO_2 to $CaCO_3$ using indigenous CA from *P. fragi*, *M. lylae*, and *M luteus*. In another study, carbon composite beads were used for CA immobilization from *Bacillus Pumilus* showed 19.22 mg precipitate while the free enzyme gave 33.6 mg of precipitate (Prabhu et al. 2011). This shows that carbonate deposition was lower in immobilized enzyme than in case of free enzyme. The lower carbonation rate in immobilized enzyme can be due to lower accessibility of the active site to the substrate. The amount of enzyme immobilized also plays a very important role in carbonation because in case of immobilized enzyme the amount of enzyme that got immobilized on the substrate is very low as compared to free enzyme. Hence, lower enzyme immobilization means lower number of active sites for the substrate to bind and therefore lower carbonation.

Solubility of CO_2 is 3.36 g per 1000 g of water at 0-4°C and 1 atmospheric pressure. Which means 0.0336 g of CO_2 gets dissolved in 10 g of H_2O (CO_2 saturated water). Stoichiometrically, 100 g of $CaCO_3$ has 44 g of CO_2. Theoretically, 0.0076 g of $CaCO_3$ should have been formed from 0.0336 g of CO_2. But, from the actual experiment 0.089 g of $CaCO_3$ was obtained. This increased amount of $CaCO_3$ could be the result of CO_2 that entered the experimental setup while it was opened to add $CaCl_2$ solution and because the setup was not completely sealed.

4.4. Reusability of immobilized enzyme

For industrial applications of enzyme, reusability is an essential parameter since it can reduce the cost of enzyme driven processes. The reusability of CA was evaluated for 4 cycles. The reaction was carried out with in the same manner as CO_2 sequestration but after every cycle the immobilized enzyme was rinsed with Tris-Hcl buffer to neutralize the pH and to remove any excess ions.

Table 4.5: Summary of precipitate of CaCO3 reaction for immobilized enzyme.

	$CaCO_3$ Precipitate (mg)		
Number of cycles	Alginate beads	Chitosan beads	Chitosan film
1	18	9	6
2	12	7	4
3	8	5	3
4	6	3	2.5

Free enzyme could not be reused after one cycle as the enzyme cannot be separated from the solution. The amount of $CaCO_3$ precipitate formed was highest in case of alginate beads (18 mg) than chitosan beads (9 mg) and chitosan film (2.5 mg). The decrease in precipitate formation has been explained because of the leaching effect of enzyme which means that with each cycle the enzyme on the immobilization material is leaching out (Wanjari et al. 2011).

4.5.Storage stability of free and immobilized enzyme

Stability of free and immobilized enzyme was determined by storing them for 4 weeks at 4°C. Samples were taken every week and enzyme activity was assayed.

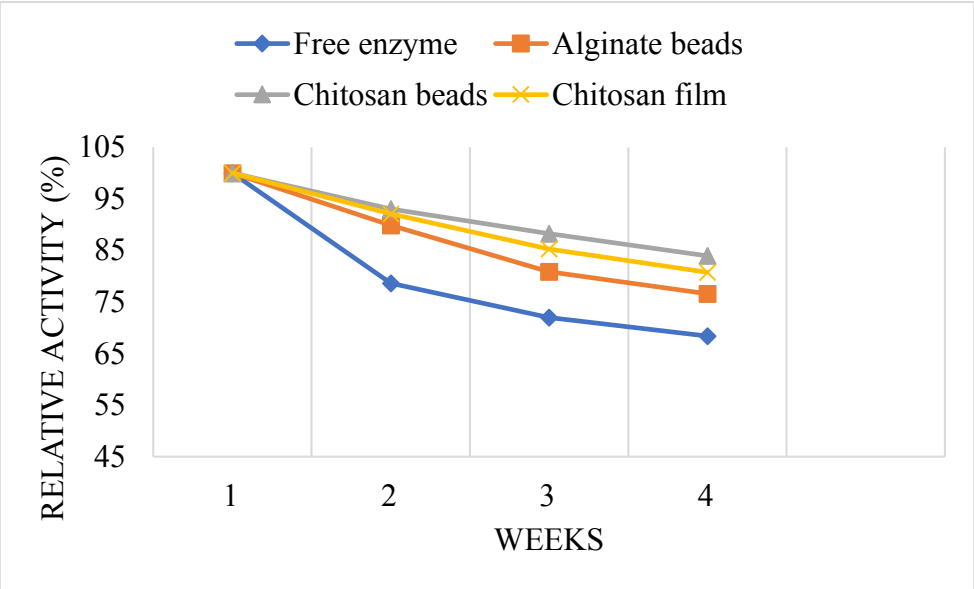

Figure 4.10: Stability of free and immobilized enzyme over a period of 4 weeks.

In case of free enzyme there was a sudden drop in relative activity of the enzyme in the first week, but after that for the rest of the three weeks there was only 20% lost in activity in total. Because of this advantageous shelf life observed, it can be concluded that CA obtained from plants can be used in industries. This observation regarding the good stability of plant CA enzyme coincides with what has been reported in literature (Pocker et al. 1973), (Bednár et al. 2016).

In case of immobilized enzymes, chitosan beads retained about 83% of its original activity after 4 weeks while alginate beads retained 76.5% and chitosan film retained 80.75% of its original activity.

Chapter 5 Conclusion

For this study, the extraction of carbonic anhydrase was done from a plant source namely spinach leaves. The extraction and partial purification of carbonic anhydrase was done successfully with total activity of 1759.64 Units.

The partially purified enzyme was then immobilized on sodium alginate beads, chitosan beads and chitosan film to study the effectiveness of two immobilization techniques on two supports/materials. The immobilization method was used because it is an easy and cheap method to show better reusability and to preserve the stability of enzyme. Optimum temperature and pH for both free and immobilized enzyme was studied. The optimum temperature for immobilized enzyme was better than free enzyme. Free enzyme showed optimum temperature at 30°C, alginate beads had optimum temperature of 40°C while chitosan beads and film had an optimum temperature of 35°C both. The optimum pH for free enzyme was 8 which is lower than that of chitosan beads which is 8.5. Alginate beads and chitosan film showed optimum pH at 8 and 7.5 respectively. Chitosan beads showed higher relative activity in case of optimum temperature and pH.

Transformation of CO_2 to $CaCO_3$ was carried out with the help of both free and immobilized enzymes. Free enzyme produced 89 mg of precipitate per 8.6 U of enzyme activity. Reusability of the immobilized enzymes was performed up till the 4 cycles. Both the free and immobilized enzymes produced relatively same amount of precipitate per unit enzyme. Solubility of CO_2 is 3.36 g per 1000 g of water at 0-4°C and 1 atmospheric pressure. Which means 0.0336 g of CO_2 gets dissolved in 10 g of H_2O (CO_2 saturated water). Theoretically, 0.0076 g of $CaCO_3$ should have been formed from 0.0336 g of CO_2. But, from the actual experiment 0.089 g of $CaCO_3$ was obtained. This increased amount of $CaCO_3$ could be the result of CO_2 that entered the experimental setup while it was opened to add $CaCl_2$ solution and because the setup was not completely sealed.

Stability of free and immobilized was also compared over a period of 4 weeks. The free enzyme showed a relatively better stability than the enzymes extracted from microorganisms. Immobilized enzymes showed better relative stability than free enzyme. Free enzyme retained about 68% of its activity, while chitosan beads retained the maximum relative activity 83% as compared to chitosan film (80.75%) and alginate beads (76.59%). This shows that the immobilized enzyme can be more useful.

Chapter 6 Recommendations for future work

Recommendations for future work include:

Listed below are some are some suggestions for future work with Carbonic Anhydrase:

- Different plant sources can be utilized to check the CO_2 sequestration purposes.
- Cross linking of enzyme can be done during immobilization to prevent desorption or leaching of immobilized enzyme.
- Chemical methods of immobilization can be explored for stability and reuse of enzyme.
- Membrane immobilization of plant-based enzyme can be done for separation of CO_2 from flue gases.

Chapter 7 References

Abdel-Naby, Mohamed A. 1993. “Immobilization OfAspergillus Niger NRC 107 Xylanase and β-Xylosidase, and Properties of the Immobilized Enzymes.” *Applied Biochemistry and Biotechnology* 38 (1–2). Humana Press: 69–81. https://doi.org/10.1007/BF02916413.

Abu-Khader, Mazen M. 2006. “Recent Progress in CO 2 Capture/Sequestration: A Review.” *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects* 28 (14): 1261–79. https://doi.org/10.1080/009083190933825.

Adarsh, and G Madhu. 2007. “A Comparative Study on Metal Adsorption Properties of Different Forms of Chitosan.” *International Journal of Innovative Research in Science, Engineering and Technology (An ISO*. Vol. 3297. www.ijirset.com.

Adler, L, J Brundell, S O Falkbring, and P O Nyman. 1972. “Carbonic Anhydrase from Neisseria Sicca, Strain 6021. I. Bacterial Growth and Purification of the Enzyme.” *Biochimica et Biophysica Acta* 284 (1): 298–310. http://www.ncbi.nlm.nih.gov/pubmed/4627452.

Aggarwal, Mayank, Christopher D. Boone, Bhargav Kondeti, and Robert McKenna. 2013. “Structural Annotation of Human Carbonic Anhydrases.” *Journal of Enzyme Inhibition and Medicinal Chemistry* 28 (2). Taylor & Francis: 267–77. https://doi.org/10.3109/14756366.2012.737323.

Alber, B E, and J G Ferry. 1994. “A Carbonic Anhydrase from the Archaeon Methanosarcina Thermophila.” *Proceedings of the National Academy of Sciences of the United States of America* 91 (15): 6909–13. http://www.ncbi.nlm.nih.gov/pubmed/8041719.

Alissandratos, Apostolos, and Christopher J Easton. 2015. “Biocatalysis for the Application of CO 2 as a Chemical Feedstock.” *Beilstein Journal of Organic Chemistry* 11 (1). Beilstein-Institut: 2370–87. https://doi.org/10.3762/bjoc.11.259.

Amin, Mohamad Hassan, and Suresh K Bhargava. 2018. “A Mini-Review on CO 2 Reforming of Methane.” https://doi.org/10.31031/PPS.2018.02.000532.

Amornvadee Veawab, * and Paitoon Tontiwachwuthikul, and Amit Chakma. 1999. “Corrosion Behavior of Carbon Steel in the CO2 Absorption Process Using Aqueous

Amine Solutions." American Chemical Society . https://doi.org/10.1021/IE9901630.

B Krajewska. 2004. "Application of Chitin-and Chitosan-Based Materials for Enzyme Immobilizations: A Review." *Elsevier*. https://www.sciencedirect.com/science/article/pii/S0141022904001231.

Barton, Emily E, David M Rampulla, and Andrew B Bocarsly. 2008. "Selective Solar-Driven Reduction of CO 2 to Methanol Using a Catalyzed." *J. Am. Chem. Soc* 130: 6342–44. https://doi.org/10.1021/ja0776327.

Battle, M., M.L. Bender, P.P. Tans, J.W.C. White, J.T. Ellis, T. Conway, and R.J. Francey. 2000. "Global Carbon Sinks and Their Variability Inferred from Atmospheric [O.Sub.2] and [[Delta].Sup.13]C." *Science* 287 (5462). American Association for the Advancement of Science: 2467–2467. http://go.galegroup.com/ps/i.do?p=AONE&sw=w&issn=00368075&v=2.1&it=r&id=GALE%7CA61793513&sid=googleScholar&linkaccess=fulltext&authCount=1&u=ocul_lakehead&selfRedirect=true.

Bednár, András, Nándor Nemestóthy, Péter Bakonyi, László Fülöp, Guangyin Zhen, Xueqin Lu, Takuro Kobayashi, Gopalakrishnan Kumar, Kaiqin Xu, and Katalin Bélafi-Bakó. 2016. "Enzymatically-Boosted Ionic Liquid Gas Separation Membranes Using Carbonic Anhydrase of Biomass Origin." *Chemical Engineering Journal* 303 (November). Elsevier: 621–26. https://doi.org/10.1016/J.CEJ.2016.05.148.

Bhattacharya, Sumana, Marc Schiavone, Subhra Chakrabarti, and Sanjoy K. Bhattacharya. 2003. "CO2 Hydration by Immobilized Carbonic Anhydrase." *Biotechnology and Applied Biochemistry* 38 (2). Wiley-Blackwell: 111. https://doi.org/10.1042/BA20030060.

Boone, Christopher D., Sonika Gill, Andrew Habibzadegan, and Robert McKenna. 2013. "Carbonic Anhydrase: An Efficient Enzyme with Possible Global Implications." *International Journal of Chemical Engineering* 2013: 22–27. https://doi.org/10.1155/2013/813931.

Boone, Christopher, Andrew Habibzadegan, Sonika Gill, and Robert McKenna. 2013. "Carbonic Anhydrases and Their Biotechnological Applications." *Biomolecules* 3 (4). Multidisciplinary Digital Publishing Institute: 553–62. https://doi.org/10.3390/biom3030553.

Brena, Beatriz, Paula González-Pombo, and Francisco Batista-Viera. 2013. "Immobilization of Enzymes: A Literature Survey." *Methods in Molecular Biology* 1051. https://doi.org/10.1007/978-1-62703-550-7_2.

Datta, Sumitra, L. Rene Christena, and Yamuna Rani Sriramulu Rajaram. 2013. "Enzyme Immobilization: An Overview on Techniques and Support Materials." *3 Biotech* 3 (1). Springer Berlin Heidelberg: 1–9. https://doi.org/10.1007/s13205-012-0071-7.

Dhanwantri, K, P Sharma, S Mehta, and P Prakash. 2014. "Carbon Sequestration , Its Methods and Significance." In *Environmental Sustainability: Concepts, Principles, Evidences and Innovations*, edited by Prof. (Dr.) Govind Chandra Mishra, 1st ed., 151–57. New delhi: EXCELLENT PUBLISHING HOUSE.

Elleuche, S., and S. Poggeler. 2010. "Carbonic Anhydrases in Fungi." *Microbiology* 156 (1): 23–29. https://doi.org/10.1099/mic.0.032581-0.

Eriksson, M, J Karlsson, Z Ramazanov, P Gardeström, and G Samuelsson. 1996. "Discovery of an Algal Mitochondrial Carbonic Anhydrase: Molecular Cloning and Characterization of a Low-CO2-Induced Polypeptide in Chlamydomonas Reinhardtii." *Proceedings of the National Academy of Sciences of the United States of America* 93 (21): 12031–34. http://www.ncbi.nlm.nih.gov/pubmed/8876257.

Fiore, Anna Di, Vincenzo Alterio, Simona M. Monti, Giuseppina De Simone, and Katia D'Ambrosio. 2015. "Thermostable Carbonic Anhydrases in Biotechnological Applications." *International Journal of Molecular Sciences* 16 (7): 15456–80. https://doi.org/10.3390/ijms160715456.

Frommer, WB. 2010. "CO2mmon Sense." *Science.Sciencemag.Org*. http://science.sciencemag.org/content/327/5963/275.short.

Fukuzawa, H, S Fujiwara, Y Yamamoto, M L Dionisio-Sese, and S Miyachi. 1990. "CDNA Cloning, Sequence, and Expression of Carbonic Anhydrase in Chlamydomonas Reinhardtii: Regulation by Environmental CO2 Concentration." *Proceedings of the National Academy of Sciences of the United States of America* 87 (11): 4383–87. http://www.ncbi.nlm.nih.gov/pubmed/2112252.

Ganai, Bashir Ahmad. 2017. "Carbonic Anhydrase : Mechanism , Structure and Importance in Higher Plants" 7 (August): 17–23.

Ge, J, RM Cowan, C Tu, … ML McGregor - Life Support &, and Undefined 2002. 2002. "Enzyme-Based CO2 Capture for Advanced Life Support." *Ingentaconnect.Com*. https://www.ingentaconnect.com/content/cog/lsbs/2002/00000008/f0020003/art00007.

Gill, Steven R., Paula J. Fedorka-Cray, Rodney K. Tweten, and Bayard P. Sleeper. 1984. "Purification and Properties of the Carbonic Anhydrase of Rhodospirillum Rubrum." *Archives of Microbiology* 138 (2). Springer-Verlag: 113–18. https://doi.org/10.1007/BF00413010.

Gonz, Javier M, and S Zo. 2014. "Carbonic Anhydrases in Industrial Applications" 75 (September 2014). https://doi.org/10.1007/978-94-007-7359-2.

Goodsell, D.S. 2004. "Carbonic Anhydrase." *RCSB Protein Data Bank*, January. https://doi.org/10.2210/rcsb_pdb/mom_2004_1.

Harish Prashanth, K.V., and R.N. Tharanathan. 2007. "Chitin/Chitosan: Modifications and Their Unlimited Application Potential—an Overview." *Trends in Food Science & Technology* 18 (3). Elsevier: 117–31. https://doi.org/10.1016/J.TIFS.2006.10.022.

Hewett-Emmett, David. 2000. "Evolution and Distribution of the Carbonic Anhydrase Gene Families." In *The Carbonic Anhydrases*, 29–76. Basel: Birkhäuser Basel. https://doi.org/10.1007/978-3-0348-8446-4_3.

Homaei, Ahmad Abolpour, Reyhaneh Sariri, Fabio Vianello, and Roberto Stevanato. 2013. "Enzyme Immobilization: An Update." *Journal of Chemical Biology* 6 (4). Springer: 185–205. https://doi.org/10.1007/s12154-013-0102-9.

Ingall, Ellery D., Julia M. Diaz, Amelia F. Longo, Michelle Oakes, Lydia Finney, Stefan Vogt, Barry Lai, Patricia L. Yager, Benjamin S. Twining, and Jay A. Brandes. 2013. "Role of Biogenic Silica in the Removal of Iron from the Antarctic Seas." *Nature Communications* 4 (June). https://doi.org/10.1038/ncomms2981.

Initiative, The Arabidopsis Genome. 2000. "Analysis of the Genome Sequence of the Flowering Plant Arabidopsis Thaliana." *Nature* 408 (6814). Nature Publishing Group: 796–815. https://doi.org/10.1038/35048692.

IPCC. 2005. "IPCC Special Report on Carbon Dioxide Capture and Storage, Prepared by Working Group III of the Intergovernmental Panel on Climate Change [Metz, B., O. Davidson, H. C. de Coninck, M. Loos, and L. A. Meyer (Eds.)]." *Cambridge University*

Press, Cambridge, United Kingdom and New York, NY, USA. https://www.ipcc.ch/pdf/special-reports/srccs/srccs_wholereport.pdf.

Jansson, Christer, Stan D. Wullschleger, Udaya C. Kalluri, and Gerald A. Tuskan. 2010a. "Phytosequestration: Carbon Biosequestration by Plants and the Prospects of Genetic Engineering." *BioScience* 60 (9): 685–96. https://doi.org/10.1525/bio.2010.60.9.6.

———. 2010b. "Phytosequestration: Carbon Biosequestration by Plants and the Prospects of Genetic Engineering." *BioScience* 60 (9). Oxford University Press: 685–96. https://doi.org/10.1525/bio.2010.60.9.6.

Jansson, Christer, Stan D Wullschleger, Udaya C Kalluri, and Gerald A Tuskan. 2010c. "Phytosequestration: Carbon Biosequestration by Plants and the Prospects of Genetic Engineering." *BioScience* 60 (685). https://doi.org/10.1525/bio.2010.60.9.6.

Kaar, JL, HI Oh, AJ Russell, WJ Federspiel - Biomaterials, and Undefined 2007. 2007. "Towards Improved Artificial Lungs through Biocatalysis." *Elsevier*. https://www.sciencedirect.com/science/article/pii/S0142961207002360.

Kargupta, Wriju, Anuradda Ganesh, and Suparna Mukherji. 2015. "Estimation of Carbon Dioxide Sequestration Potential of Microalgae Grown in a Batch Photobioreactor." *Bioresource Technology* 180 (March). Elsevier: 370–75. https://doi.org/10.1016/J.BIORTECH.2015.01.017.

Kimmel, J. D., D. T. Arazawa, S.-H. Ye, V. Shankarraman, W. R. Wagner, and W. J. Federspiel. 2013. "Carbonic Anhydrase Immobilized on Hollow Fiber Membranes Using Glutaraldehyde Activated Chitosan for Artificial Lung Applications." *Journal of Materials Science: Materials in Medicine* 24 (11). Springer US: 2611–21. https://doi.org/10.1007/s10856-013-5006-2.

Klara, Scott M, Rameshwar D Srivastava, and Howard G McIlvried. 2003. "Integrated Collaborative Technology Development Program for CO2 Sequestration in Geologic Formations—United States Department of Energy R&D." *Energy Conversion and Management* 44 (17). Pergamon: 2699–2712. https://doi.org/10.1016/S0196-8904(03)00042-6.

Kovscek, Ar. 2004. "Geologic Storage of Carbon Dioxide and Enhanced Oil Recovery. II. Cooptimization of Storage and Recovery."

https://doi.org/10.1016/j.enconman.2004.09.009.

Kumar, R. Siva Sai, and James G. Ferry. 2014. “Prokaryotic Carbonic Anhydrases of Earth’s Environment.” In *Sub-Cellular Biochemistry*, 75:77–87. https://doi.org/10.1007/978-94-007-7359-2_5.

Kupriyanova, E., N. Pronina, and D. Los. 2017. “Carbonic Anhydrase — a Universal Enzyme of the Carbon-Based Life.” *Photosynthetica* 55 (1): 3–19. https://doi.org/10.1007/s11099-017-0685-4.

Lal, R. 2004. “Soil Carbon Sequestration Impacts on Global Climate Change and Food Security.” *Science (New York, N.Y.)* 304 (5677). American Association for the Advancement of Science: 1623–27. https://doi.org/10.1126/science.1097396.

Lal, Rattan. 2008. “Carbon Sequestration.” *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 363 (1492). The Royal Society: 815–30. https://doi.org/10.1098/rstb.2007.2185.

Lamb, David, Peter D Erskine, and John A Parrotta. 2005. “Restoration of Degraded Tropical Forest Landscapes.” *Science (New York, N.Y.)* 310 (5754). American Association for the Advancement of Science: 1628–32. https://doi.org/10.1126/science.1111773.

Lampitt, R.S, E.P Achterberg, T.R Anderson, J.A Hughes, M.D Iglesias-Rodriguez, B.A Kelly-Gerreyn, M Lucas, et al. 2008. “Ocean Fertilization: A Potential Means of Geoengineering?” *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences* 366 (1882): 3919–45. https://doi.org/10.1098/rsta.2008.0139.

Lane, T. W., and F. M. M. Morel. 2000. “A Biological Function for Cadmium in Marine Diatoms.” *Proceedings of the National Academy of Sciences* 97 (9): 4627–31. https://doi.org/10.1073/pnas.090091397.

Larjavaara, Markku. 2008. “A Review on Benefits and Disadvantages of Tree Diversity.” *The Open Forest Science Journal* 1: 24–26. http://www.lerf.eco.br/img/publicacoes/2008_2611 A Review on Benefits and Disadvantages of Tree Diversity.pdf.

Lee JF, Thirumavalavan M, and Lee JF. 2015. “A Short Review on Chitosan Membrane for Biomolecules Immobilization.” *Journal of Molecular and Genetic Medicine* 09 (03).

OMICS International: 1–5. https://doi.org/10.4172/1747-0862.1000178.

Lehtonen, J, B Shen, M Vihinen, … A Casini - Journal of Biological, and Undefined 2003. 2003. "Characterization of CA XIII, a Novel Member of the Carbonic Anhydrase Isozyme Family." *ASBMB*. http://www.jbc.org/content/early/2003/11/04/jbc.M308984200.full.pdf.

Lindskog, S. 1997. "Structure and Mechanism of Carbonic Anhydrase." *Elsevier*. https://www.sciencedirect.com/science/article/pii/S0163725896001982.

Lindskog, Sven, and Per Olof Nyman. 1964. "Metal-Binding Properties of Human Erythrocyte Carbonic Anhydrases." *Biochimica et Biophysica Acta (BBA) - Specialized Section on Enzymological Subjects* 85 (3). Elsevier: 462–74. https://doi.org/10.1016/0926-6569(64)90310-4.

Liu, Li, Amit Chakma, and Xianshe Feng. 2004. "Preparation of Hollow Fiber Poly(Ether Block Amide)/Polysulfone Composite Membranes for Separation of Carbon Dioxide from Nitrogen." *Chemical Engineering Journal* 105 (1–2). Elsevier: 43–51. https://doi.org/10.1016/J.CEJ.2004.08.005.

Lowry, Oliver H, Rose J Randall, and A Lewis. 1951. "The Folin by Oliver." *Readings* 193 (1): 265–75. https://doi.org/10.1016/0304-3894(92)87011-4.

MacAuley, Sheridan R., Sabrina A. Zimmerman, Ethel E. Apolinario, Caryn Evilia, Ya-Ming Hou, James G. Ferry, and Kevin R. Sowers. 2009. "The Archetype γ-Class Carbonic Anhydrase (Cam) Contains Iron When Synthesized in Vivo †." *Biochemistry* 48 (5). American Chemical Society: 817–19. https://doi.org/10.1021/bi802246s.

Madhumati Mondal, Saumyakanti Khanra. O.N. Towari, K. Gayen, G.N. Halder. 2016. "Role of Carbonic Anhydrase on the Way to Biological Carbon Capture through Microalgae-A Mini Review." *Environmental Progress & Sustainable Energy* 35 (6): 1605–15. https://doi.org/10.1002/ep.

Magalhães, Júlia M.C.S., and Adélio A.S.C. Machado. 1998. "Urea Potentiometric Biosensor Based on Urease Immobilized on Chitosan Membranes." *Talanta* 47 (1): 183–91. https://doi.org/10.1016/S0039-9140(98)00066-6.

Marcus, E. A., A. P. Moshfegh, G. Sachs, and D. R. Scott. 2005. "The Periplasmic -Carbonic Anhydrase Activity of Helicobacter Pylori Is Essential for Acid Acclimation." *Journal*

of Bacteriology 187 (2): 729–38. https://doi.org/10.1128/JB.187.2.729-738.2005.

Marianne K., Gornall A. 1978. "Carbonic Anhydrase from Spinach Leaves." *The Journal of Biological Chemistry* 253 (3): 679–85.

Matear, Richard J., and Bronwyn Elliott. 2004. "Enhancement of Oceanic Uptake of Anthropogenic CO $_2$ by Macronutrient Fertilization." *Journal of Geophysical Research* 109 (C4): C04001. https://doi.org/10.1029/2000JC000321.

Mazzotti, Marco, and Switzerland Italy. 2007. "Mineral Carbonation and Industrial Uses of Carbon Dioxide 319 7 Mineral Carbonation and Industrial Uses of Carbon Dioxide." *IPCC Special Report on Carbon Dioxide Capture and Storage*. https://www.ipcc.ch/pdf/special-reports/srccs/srccs_chapter7.pdf.

Metting, F. Blaine, Jeffrey L. Smith, Jeffrey S. Amthor, and R. Cesar Izaurralde. 2001. "Science Needs and New Technology for Increasing Soil Carbon Sequestration." *Climatic Change* 51 (1). Kluwer Academic Publishers: 11–34. https://doi.org/10.1023/A:1017509224801.

Mirjafari, Parissa, Koorosh Asghari, and Nader Mahinpey. 2007. "Investigating the Application of Enzyme Carbonic Anhydrase for CO 2 Sequestration Purposes." *Industrial and Engineering Chemistry Research* 46 (3): 921–26. https://doi.org/10.1021/ie060287u.

Mitsuhashi, S, T Mizushima, E Yamashita, M Yamamoto, T Kumasaka, H Moriyama, T Ueki, S Miyachi, and T Tsukihara. 2000. "X-Ray Structure of Beta-Carbonic Anhydrase from the Red Alga, Porphyridium Purpureum, Reveals a Novel Catalytic Site for CO(2) Hydration." *The Journal of Biological Chemistry* 275 (8): 5521–26. http://www.ncbi.nlm.nih.gov/pubmed/10681531.

Morais, Michele Greque de, and Jorge Alberto Vieira Costa. 2007. "Carbon Dioxide Fixation by Chlorella Kessleri, C. Vulgaris, Scenedesmus Obliquus and Spirulina Sp. Cultivated in Flasks and Vertical Tubular Photobioreactors." *Biotechnology Letters* 29 (9). Springer Netherlands: 1349–52. https://doi.org/10.1007/s10529-007-9394-6.

Moroney, J. V., S. G. Bartlett, and G. Samuelsson. 2001. "Carbonic Anhydrases in Plants and Algae." *Plant, Cell and Environment* 24 (2). Blackwell Science Ltd: 141–53. https://doi.org/10.1111/j.1365-3040.2001.00669.x.

Neish, A C. 1939. “Studies on Chloroplasts: Their Chemical Composition and the Distribution of Certain Metabolites between the Chloroplasts and the Remainder of the Leaf.” *The Biochemical Journal* 33 (3). Portland Press Ltd: 300–308. http://www.ncbi.nlm.nih.gov/pubmed/16746912.

Nishimori, Isao, Tomoko Minakuchi, Saburo Onishi, Daniela Vullo, Andrea Scozzafava, and Claudiu T. Supuran. 2007. “Carbonic Anhydrase Inhibitors. DNA Cloning, Characterization, and Inhibition Studies of the Human Secretory Isoform VI, a New Target for Sulfonamide and Sulfamate Inhibitors.” *Journal of Medicinal Chemistry* 50 (2): 381–88. https://doi.org/10.1021/jm0612057.

Nishimori, Isao, Daniela Vullo, Alessio Innocenti, Andrea Scozzafava, Antonio Mastrolorenzo, and Claudiu T. Supuran. 2005. “Carbonic Anhydrase Inhibitors. The Mitochondrial Isozyme VB as a New Target for Sulfonamide and Sulfamate Inhibitors.” *Journal of Medicinal Chemistry* 48 (24): 7860–66. https://doi.org/10.1021/jm050483n.

Nogia, Panchsheela, Gurpreet Kaur Sidhu, Rajesh Mehrotra, and Sandhya Mehrotra. 2016. “Capturing Atmospheric Carbon: Biological and Nonbiological Methods.” *International Journal of Low-Carbon Technologies* 11 (2): 266–74. https://doi.org/10.1093/ijlct/ctt077.

O’Connor, William K., David C. Dahlin, David N. Nilsen, G.E. Rush, Richard P. Walters, and Paul C. Turner. 2001. “Carbon Dioxide Sequestration by Direct Mineral Carbonation: Results from Recent Studies and Current Status.” National Energy Technology Laboratory, U.S. Department of Energy. https://www.osti.gov/biblio/897125.

Oelkers, E. H., S. R. Gislason, and J. Matter. 2008. “Mineral Carbonation of CO2.” *Elements* 4 (5). GeoScienceWorld: 333–37. https://doi.org/10.2113/gselements.4.5.333.

Okabe, K, SY Yang, M Tsuzuki, S Miyachi - Plant Science Letters, and Undefined 1984. 1984. “Carbonic Anhydrase: Its Content in Spinach Leaves and Its Taxonomic Diversity Studied with Anti-Spinach Leaf Carbonic Anhydrase Antibody.” *Elsevier*. https://www.sciencedirect.com/science/article/pii/030442118490004X.

Oren, Ram, David S. Ellsworth, Kurt H. Johnsen, Nathan Phillips, Brent E. Ewers, Chris Maier, Karina V.R. Schäfer, et al. 2001. “Soil Fertility Limits Carbon Sequestration by Forest Ecosystems in a CO_2-Enriched Atmosphere.” *Nature* 411 (6836). Nature

Publishing Group: 469–72. https://doi.org/10.1038/35078064.

Ouyang, An, and Ji Liang. 2014. “Tailoring the Adsorption Rate of Porous Chitosan and Chitosan-Carbon Nanotube Core-Shell Beads †.” https://doi.org/10.1039/c4ra04131c.

Oviya, M., V. Sukumaran, and Sib Sankar Giri. 2013. “Immobilization and Characterization of Carbonic Anhydrase Purified from E. Coli MO1 and Its Influence on CO2sequestration.” *World Journal of Microbiology and Biotechnology* 29 (10): 1813–20. https://doi.org/10.1007/s11274-013-1343-z.

Ozdemir, E. 2009. “Biomimetic CO2 Sequestration: 1. Immobilization of Carbonic Anhydrase within Polyurethane Foam.” *ACS Publications*. https://pubs.acs.org/doi/abs/10.1021/ef9005725.

Ozdemir, Ekrem. 2009a. “Biomimetic CO $_2$ Sequestration: 1. Immobilization of Carbonic Anhydrase within Polyurethane Foam.” *Energy & Fuels* 23 (11): 5725–30. https://doi.org/10.1021/ef9005725.

———. 2009b. “Biomimetic CO $_2$ Sequestration: 1. Immobilization of Carbonic Anhydrase within Polyurethane Foam.” *Energy & Fuels* 23 (11): 5725–30. https://doi.org/10.1021/ef9005725.

Pachauri, Rajendra K, Leo Meyer, Jean-Pascal Van Ypersele, Sander Brinkman, Line Van Kesteren, Noëmie Leprince-Ringuet, and Fijke Van Boxmeer. 2014. *Climate Change 2014 Synthesis Report The Core Writing Team Core Writing Team Technical Support Unit for the Synthesis Report*. *Russian Federation), Hoesung Lee (Republic of Korea) Scott B. Power (Australia) N.H. Ravindranath (India)*. IPCC. http://www.ipcc.ch/pdf/assessment-report/ar5/syr/SYR_AR5_FINAL_full_wcover.pdf.

Park, H, PJ McGinn, and FMM Morel. 2008. “Expression of Cadmium Carbonic Anhydrase of Diatoms in Seawater.” *Aquatic Microbial Ecology* 51 (2): 183–93. https://doi.org/10.3354/ame01192.

Paustian, K., J. Six, E.T. Elliott, and H. W. Hunt. 2000. “Management Options for Reducing CO2 Emissions from Agricultural Soils.” *Biogeochemistry* 48 (1). Kluwer Academic Publishers: 147–63. https://doi.org/10.1023/A:1006271331703.

Pierre, Alain C. 2012. “Enzymatic Carbon Dioxide Capture.” *ISRN Chemical Engineering* 2012: 1–22. https://doi.org/10.5402/2012/753687.

Pocker, Y., and Joan S.Y. Ng. 1973. “Plant Carbonic Anhydrase. Properties and Carbon Dioxide Hydration Kinetics.” *Biochemistry* 12 (25): 5127–34. https://doi.org/10.1021/bi00749a016.

Prabhu, Chandan, Aarti Valechha, Snehal Wanjari, Nitin Labhsetwar, Swati Kotwal, T. Satyanarayanan, and Sadhana Rayalu. 2011. “Carbon Composite Beads for Immobilization of Carbonic Anhydrase.” *Journal of Molecular Catalysis B: Enzymatic* 71 (1–2): 71–78. https://doi.org/10.1016/j.molcatb.2011.03.011.

Prete, Sonia Del, Viviana De Luca, Andrea Scozzafava, Vincenzo Carginale, Claudiu T. Supuran, and Clemente Capasso. 2014. “Biochemical Properties of a New α-Carbonic Anhydrase from the Human Pathogenic Bacterium, Vibrio Cholerae.” *Journal of Enzyme Inhibition and Medicinal Chemistry* 29 (1): 23–27. https://doi.org/10.3109/14756366.2012.747197.

Ramanan, R, and K Kannan. 2009. “Bio-Sequestration of Carbon Dioxide Using Carbonic Anhydrase Enzyme Purified from Citrobacter Freundii.” *Springer*. https://idp.springer.com/authorize/casa?redirect_uri=https://link.springer.com/article/10.1007/s11274-009-9975-8&casa_token=ABweLeni3wgAAAAA:JdZrJ3ptdNEcVpAZwRTCb8n9W2ZBiWRi-DxXj4uR2_hZRbYkowS1DiEf6xDA2xFnR4WG2GUcRXvctqxR.

Ramanan, Rishiram, Krishnamurthi Kannan, Nadimuthu Vinayagamoorthy, Kunga Mohan Ramkumar, Saravana Devi Sivanesan, and Tapan Chakrabarti. 2009a. “Purification and Characterization of a Novel Plant-Type Carbonic Anhydrase from Bacillus Subtilis.” *Biotechnology and Bioprocess Engineering* 14 (1): 32–37. https://doi.org/10.1007/s12257-008-0099-z.

———. 2009b. “Purification and Characterization of a Novel Plant-Type Carbonic Anhydrase from Bacillus Subtilis.” *Biotechnology and Bioprocess Engineering* 14 (1). The Korean Society for Biotechnology and Bioengineering: 32–37. https://doi.org/10.1007/s12257-008-0099-z.

Rathnam, CKM, VSR Das - Zeitschrift für Pflanzenphysiologie, and Undefined 1975. 1975. “Aspartate-Type C-4 Photosynthetic Carbon Metabolism in Leaves of Eleusine CoracanaGaertn.” *Elsevier*. https://www.sciencedirect.com/science/article/pii/S0044328X75801486.

Reichle, Dave, John Houghton, Sally Benson, John Clarke, Roger Dahlman, Doe George Hendrey, Howard Herzog, et al. 1999. “Carbon Sequestration DRAFT (February 1999).” https://www.netl.doe.gov/publications/press/1999/seqrpt.pdf.

Ren, Xiaoling, Jizhong Ren, Hui Li, Shichao Feng, and Maicun Deng. 2012. “Poly (Amide-6-b-Ethylene Oxide) Multilayer Composite Membrane for Carbon Dioxide Separation.” *International Journal of Greenhouse Gas Control* 8 (May). Elsevier: 111–20. https://doi.org/10.1016/J.IJGGC.2012.01.017.

Riebeek, Holli. 2011. “The Carbon Cycle : Feature Articles,” June. NASA Earth Observatory. https://earthobservatory.nasa.gov/Features/CarbonCycle/.

Roberts, Samantha B., Todd W. Lane, and Francois M. M. Morel. 1997. “CARBONIC ANHYDRASE IN THE MARINE DIATOM THALASSIOSIRA WEISSFLOGII (BACILLARIOPHYCEAE)1.” *Journal of Phycology* 33 (5). Blackwell Publishing Ltd: 845–50. https://doi.org/10.1111/j.0022-3646.1997.00845.x.

Rudenko, N. N., L. K. Ignatova, T. P. Fedorchuk, and B. N. Ivanov. 2015. “Carbonic Anhydrases in Photosynthetic Cells of Higher Plants.” *Biochemistry (Moscow)* 80 (6): 674–87. https://doi.org/10.1134/S0006297915060048.

S. J. Metz, and M. H. V. Mulder, and M. Wessling*. 2004. “Gas-Permeation Properties of Poly(Ethylene Oxide) Poly(Butylene Terephthalate) Block Copolymers.” American Chemical Society . https://doi.org/10.1021/MA049847W.

Satav, Sunita S., Shreedhar Bhat, and S. Thayumanavan. 2010. “Feedback Regulated Drug Delivery Vehicles: Carbon Dioxide Responsive Cationic Hydrogels for Antidote Release.” *Biomacromolecules* 11 (7): 1735–40. https://doi.org/10.1021/bm1005454.

Sharma, Anjana, Abhishek Bhattacharya, and Ankita Shrivastava. 2011. “Biomimetic CO2sequestration Using Purified Carbonic Anhydrase from Indigenous Bacterial Strains Immobilized on Biopolymeric Materials.” *Enzyme and Microbial Technology* 48 (4–5): 416–26. https://doi.org/10.1016/j.enzmictec.2011.02.001.

Sharma, Anjana, Abhishek Bhattacharya, and Shyamlata Singh. 2009. “Purification and Characterization of an Extracellular Carbonic Anhydrase from Pseudomonas Fragi.” *Process Biochemistry* 44 (11). Elsevier: 1293–97. https://doi.org/10.1016/J.PROCBIO.2009.07.022.

Sharma, J, R Mahajan, and VK Gupta. 2010. “Comparison and Suitability of Gel Matrix for Entrapping Higher Content of Enzymes for Commercial Applications.” *Indian Journal of Pharmaceutical Sciences* 72 (2). OMICS International: 223. https://doi.org/10.4103/0250-474X.65010.

Shazia Faridi Satyanarayana T. 2015. “Applicability of Carbonic Anhydrases in Mitigating Global Warming and Development of Useful Products from CO2.” *Climate Change and Environmental Sustainability* 3(2) (December): 77–92.

Sheppard, Michael. 2018. “Carbon Capture and Sequestration.” *Society of Petroleum Engineers*. http://www.spe.org/industry/docs/carbonsequestration.pdf.

Simsek-Ege, Fatma Arzum, Gillian M. Bond, and John Stringer. 2002. “Matrix Molecular Weight Cut-off for Encapsulation of Carbonic Anhydrase in Polyelectrolyte Beads.” *Journal of Biomaterials Science, Polymer Edition* 13 (11): 1175–87. https://doi.org/10.1163/156856202320892948.

Sipilä, J., S. Teir, and R. Zevenhoven. 2008. “Carbon Dioxide Sequestration by Mineral Carbonation Literature Review Update 2005–2007.”

Skjak-Braek, Gudmund., Thorleif. Anthonsen, Paul A. Sandford, and Norway) International Conference on Chitin and Chitosan (4th : 1988 : Trondheim. 1989. *Chitin and Chitosan : Sources, Chemistry, Biochemistry, Physical Properties, and Applications*. London ; Elsevier Applied Science,. https://searchworks.stanford.edu/view/1351905.

Smith, K S, and J G Ferry. 2000. “Prokaryotic Carbonic Anhydrases.” *FEMS Microbiology Reviews* 24 (4): 335–66. http://www.ncbi.nlm.nih.gov/pubmed/10978542.

Soltes-Rak, E, M E Mulligan, and J R Coleman. 1997. “Identification and Characterization of a Gene Encoding a Vertebrate-Type Carbonic Anhydrase in Cyanobacteria.” *Journal of Bacteriology* 179 (3). American Society for Microbiology (ASM): 769–74. http://www.ncbi.nlm.nih.gov/pubmed/9006032.

Supuran, Claudiu T. 2008a. “Carbonic Anhydrases: Novel Therapeutic Applications for Inhibitors and Activators.” *Nature Reviews Drug Discovery* 7 (2): 168–81. https://doi.org/10.1038/nrd2467.

———. 2008b. “Carbonic Anhydrases: Novel Therapeutic Applications for Inhibitors and Activators.” *Nature Reviews Drug Discovery* 7 (2). Nature Publishing Group: 168–81.

https://doi.org/10.1038/nrd2467.

———. 2013. “Carbonic Anhydrases: From Biomedical Applications of the Inhibitors and Activators to Biotechnological Use for CO2capture.” *Journal of Enzyme Inhibition and Medicinal Chemistry* 28 (2): 229–30. https://doi.org/10.3109/14756366.2013.761876.

Supuran, Claudiu T., Andrea Scozzafava, and Angela Casini. 2003. “Carbonic Anhydrase Inhibitors.” *Medicinal Research Reviews* 23 (2). Wiley-Blackwell: 146–89. https://doi.org/10.1002/med.10025.

Supuran, Claudiu T., Andrea Scozzafava, and Janet. Conway. 2004. *Carbonic Anhydrase : Its Inhibitors and Activators*. CRC Press.

The Late U Meldrum, BY N, and F J W Roughton. 1933. “CARBONIC ANHYDRASE. ITS PREPARATION AND PROPERTIES.” *The Physiological and Biochemical Laboratories, Cambridge.* https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1394121/pdf/jphysiol01616-0010.pdf.

Thiry, A, JM Dogne, B Masereel, CT Supuran - Trends in pharmacological, and undefined 2006. n.d. “Targeting Tumor-Associated Carbonic Anhydrase IX in Cancer Therapy.” *Elsevier*. Accessed August 2, 2018. https://www.sciencedirect.com/science/article/pii/S0165614706002203.

Thompson, Richard B., and Eric R. Jones. 1993. “Enzyme-Based Fiber Optic Zinc Biosensor.” *Analytical Chemistry* 65 (6). American Chemical Society: 730–34. https://doi.org/10.1021/ac00054a013.

Tripp, Brian C., Kerry Smith, and James G. Ferry. 2001. “Carbonic Anhydrase: New Insights for an Ancient Enzyme.” *Journal of Biological Chemistry* 276 (52): 48615–18. https://doi.org/10.1074/jbc.R100045200.

Tsuzuki, M, S Miyachi, GE Edwards - Plant and cell Physiology, and Undefined 1985. 1985. “Localization of Carbonic Anhydrase in Mesophyll Cells of Terrestrial C3 Plants in Relation to CO2 Assimilation.” *Academic.Oup.Com*. https://academic.oup.com/pcp/article-abstract/26/5/881/1856766.

Vinoba, Mari, Margandan Bhagiyalakshmi, Soon Kwan Jeong, Yeo I I Yoon, and Sung Chan Nam. 2012. “Immobilization of Carbonic Anhydrase on Spherical SBA-15 for Hydration and Sequestration of CO 2.” *Colloids and Surfaces B:*

Biointerfaces 90 (1): 91–96. https://doi.org/10.1016/j.colsurfb.2011.10.001.

Vullo, D, J Voipio, A Innocenti, C Rivera, … H Ranki - Bioorganic & medicinal, and undefined 2005. n.d. "Carbonic Anhydrase Inhibitors. Inhibition of the Human Cytosolic Isozyme VII with Aromatic and Heterocyclic Sulfonamides." *Elsevier*. Accessed August 2, 2018. https://www.sciencedirect.com/science/article/pii/S0960894X04015136.

Walker, Tara L., Chris Collet, and Saul Purton. 2005. "ALGAL TRANSGENICS IN THE GENOMIC ERA1." *Journal of Phycology* 41 (6). Blackwell Science Inc: 1077–93. https://doi.org/10.1111/j.1529-8817.2005.00133.x.

Wanjari, Snehal, Chandan Prabhu, Renu Yadav, T. Satyanarayana, Nitin Labhsetwar, and Sadhana Rayalu. 2011. "Immobilization of Carbonic Anhydrase on Chitosan Beads for Enhanced Carbonation Reaction." *Process Biochemistry* 46 (4). Elsevier Ltd: 1010–18. https://doi.org/10.1016/j.procbio.2011.01.023.

Warrier, Rekha R, Suriyanarayanan Lalitha, and Chellapan Savitha. 2014. "A Modified Assay of Carbonic Anhydrase Activity in Tree Species." *BBR - Biochemistry and Biotechnology Reports* 3 (1): 48. https://doi.org/10.5433/2316-5200.2014v3n1p48.

Whittington, A, A Waheed, and Baebara U. 2001. "Crystal Structure of the Dimeric Extracellular Domain of Human Carbonic Anhydrase XII, a Bitopic Membrane Protein Overexpressed in Certain Cancer Tumor Cells." *National Acad Sciences*. http://www.pnas.org/content/98/17/9545.short.

Whittingtons, DA, JH Grubb, A Waheed, … GN Shah - Journal of Biological, and Undefined 2004. 2004. "Expression, Assay, and Structure of the Extracellular Domain of Murine Carbonic Anhydrase XIV IMPLICATIONS FOR SELECTIVE INHIBITION OF MEMBRANE." *ASBMB*. http://www.jbc.org/content/279/8/7223.short.

Wong, Tuck Seng. 2014. "Carbon Dioxide Capture and Utilization Using Biological Systems: Opportunities and Challenges." *Journal of Bioprocessing & Biotechniques* 04 (03). https://doi.org/10.4172/2155-9821.1000155.

Yadav, Raju R., Kannan Krishnamurthi, Sandeep N. Mudliar, S. Saravana Devi, Pravin K. Naoghare, Amit Bafana, and Tapan Chakrabarti. 2014. "Carbonic Anhydrase Mediated Carbon Dioxide Sequestration: Promises, Challenges and Future Prospects." *Journal of Basic Microbiology* 54 (6): 472–81. https://doi.org/10.1002/jobm.201300849.

Yadav, Raju R., Sandeep N. Mudliar, Ajam Y. Shekh, Abhay B. Fulke, Sivanesan Saravana Devi, Kannan Krishnamurthi, Asha Juwarkar, and Tapan Chakrabarti. 2012. "Immobilization of Carbonic Anhydrase in Alginate and Its Influence on Transformation of CO2to Calcite." *Process Biochemistry* 47 (4): 585–90. https://doi.org/10.1016/j.procbio.2011.12.017.

Yadav, Renu, Snehal Wanjari, Chandan Prabhu, Vivek Kumar, Nitin Labhsetwar, T. Satyanarayanan, Swati Kotwal, and Sadhana Rayalu. 2010. "Immobilized Carbonic Anhydrase for the Biomimetic Carbonation Reaction." *Energy & Fuels* 24 (11). American Chemical Society: 6198–6207. https://doi.org/10.1021/ef100750y.

Yamasaki, Akihiro. 2003. "An Overview of CO2 Mitigation Options for Global Warming-Emphasizing CO2 Sequestration Options." *JOURNAL OF CHEMICAL ENGINEERING OF JAPAN* 36 (4): 361–75. https://doi.org/10.1252/jcej.36.361.

Yang, Hongqun, Zhenghe Xu, Maohong Fan, Rajender Gupta, Rachid B Slimane, Alan E Bland, and Ian Wright. 2008. "Progress in Carbon Dioxide Separation and Capture: A Review." *Journal of Environmental Sciences* 20 (1). Elsevier: 14–27. https://doi.org/10.1016/S1001-0742(08)60002-9.

Yeates, Todd O., Cheryl A. Kerfeld, Sabine Heinhorst, Gordon C. Cannon, and Jessup M. Shively. 2008. "Protein-Based Organelles in Bacteria: Carboxysomes and Related Microcompartments." *Nature Reviews Microbiology* 6 (9). Nature Publishing Group: 681–91. https://doi.org/10.1038/nrmicro1913.

Yee, Donald, and François M. M. Morel. 1996. "In Vivo Substitution of Zinc by Cobalt in Carbonic Anhydrase of a Marine Diatom." *Limnology and Oceanography* 41 (3): 573–77. https://doi.org/10.4319/lo.1996.41.3.0573.

Yuan, Q, S Ouyang, A Wang, W Zhu, … R Maiti - Plant, and Undefined 2005. 2005. "The Institute for Genomic Research Osa1 Rice Genome Annotation Database." *Am Soc Plant Biol*. http://www.plantphysiol.org/content/138/1/18.short.

Zeng, Ning. 2008. "Carbon Sequestration via Wood Burial." *Carbon Balance and Management* 3 (1). Nature Publishing Group: 1. https://doi.org/10.1186/1750-0680-3-1.