Isolation and characterization of Carbonic Anhydrase producing bacterial isolates for CO_2 conversion

Submitted in fulfilment of the requirements for the degree of

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IN

BIOTECHNOLOGY

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DECLARATION BY STUDENTS

I hereby declare that the project work entitled "Isolation and characterization of Carbonic Anhydrase producing bacterial isolates for CO_2 conversion" submitted to the Department of Biotechnology and bioinformatics, Jaypee University of Information Technology Solan (H.P), is a bonafide record of original work done by me. The work was carried out under the supervision of Dr. Ashok Kumar Nadda.

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This is to certify that the above statement made by the student is true to the best of my knowledge.

SUPERVISOR'S CERTIFICATE

This is to certify that the work titled "Isolation and characterization of Carbonic Anhydrase producing bacterial isolates for CO_2 conversion"by Ishika Bhatti during the end semester in May 2022 in fulfilment for the award of degree of Master of Science in Biotechnology of Jaypee University of Information Technology, Solan has been carried out under my supervision. This work is a bonafide record of her original work. It has not been submitted elsewhere for any other degree/diploma.

_____

Signature of Supervisor

Name of supervisor: Dr Ashok Kumar Nadda
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Date : _____

Ishika Bhatti

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Abstract

Rapid industrialization and burning of fossil fuels is one of the major causes of global warning which has led to the deterioration of a healthy environment. Microbial carbonic anhydrase has been employed for the reduction of CO_2 level in the environment. Soil sample was used in this study in order to search for CA-producing bacteria. In the bacterial cultures, eight isolates were chosen after screening for CA activity. It was found that Isolate no. 5 had the highest concentration of CA activity (1.58 U/ml) out of all the bacteria tested. Several production parameters were optimized for CA production. Incubation temperature 20°C, media nutrient broth, and inoculum volume 0.25%v/v, pH 7 was optimized. The crude enzyme was examined for the conversion of CO_2 into calcium carbonate ($CaCO_3$). Crude CA was shown to have a CO_2 conversion. Bacterial CA precipitation of calcite can be exploited for the sequestration of carbon, can be used in a variety of applications such as paper industry, cements, and construction materials.

Keywords: Carbonic anhydrase, calcium carbonate, sequestration

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Chapter 1: Introduction

Human activity causes global warming by releasing greenhouse gases into the atmosphere. Temperature rise has shown disturbing effects on the global climate, including glacier melting, changes in precipitation patterns, and a decrease in agricultural output(Bose & Satyanarayana, 2017a). In order to save the environment from the potentially catastrophic effects of global warming, a large number of governmental and non-governmental organisations advocate for the shift away from fossil fuel power and toward clean energy sources. (T. Sharma & Kumar, 2021). It is widely acknowledged that the main contributor to the observed rising in the amount of CO_2 levels in the environment is anthropogenic for the emission of CO_2 caused by the continuous increase in the consumption of fossil fuels(W. Zhang et al., 2014). Rapid urbanization and industrial intensification have disrupted the aquatic life as well as the environment, threatening microorganisms. Some microbes are adapted to convert complex harmful materials entering the soil into non-harmful components, thereby preserving soil fertility. As a result, it is vital to screen various enzymes and microbes causing the mitigation of harmful compounds and the regeneration of microbes for normal life(Kumar et al., 2019). Climate change has been linked to both natural factors and human activities. As a result of climate change, the scope of environmental health issues has augmented from household irritants to global scale(Mulenga & Siziya, 2019). To curb this problem, Carbon capture and storage (CCS) with the involvement of microbes provides with the solution to this issue. Through the development of carbon concentrating mechanisms, some bacteria play a critical part in fixing increasing levels of CO_2 into better quality products such as calcium carbonate. A prominent technology in this regard is capturing and storage of carbon and its usage which allow capturing of CO_2 from flue gases and other sources.(Bose & Satyanarayana, 2017b). CO_2 capture is accomplished through the use of chemicals, materials with large pores. Bacteria, algae, and cyanobacteria are among the living organisms that help to reduce CO_2 levels by using their carboxylating enzyme. Due to rapid growth of microbes, they can help in the fixation of CO_2 faster than plants. CA generating microorganisms are advantageous for CO_2 capture and its utilization due to its higher turnover and economic feasibility. (T. Sharma et al., 2020).

Carbonic anhydrases, known as zinc metalloenzymes possessing Zn^{2+} in their active sites. CA is bifurcated into six classes α, β, γ, δ, ζ, η. These are without particular sequence similarity(Bose & Satyanarayana, 2017a).Out of these, archaea, bacteria, fungi, and algae are dominated by beta class. CA is mainly a 30-kDa monomeric protein with no phosphate groups

or disulfide linkages.(T. Sharma et al., 2020). The reversible hydration of CO_2 is catalysed by carbonic anhydrases(del Prete et al., 2020). It catalyses the Hydration and dehydration of carbon dioxide is catalysed by CA. Carbonic Anhydrase is one of the fastest biocatalysts present till date and it has millions of reactions per second turnover rates, and if used properly, they have the potential to make a significant contribution to CO_2 emissions reduction(Hou et al., 2019). These days, CO_2 sequester technologies are considered as part of the global effort to curb the expected disaster. The carbon dioxide sequestration into bicarbonates that was catalysed by an enzyme carbonic anhydrase is among the most potent biological approaches for sequestering carbon dioxide.(Ramanan et al., 2009). Carbonic anhydrase has been found to be prevalent in different organisms included in Archaea and Bacteria categories, shows that enzyme plays a crucial role in prokaryotic life. Carbonic anhydrase is known for having three major classes (α, β, γ) and they are the primarily known to have biological catalytic function (Smith & Ferry, 2000). The monomer interface of CA has three active sites linked with zinc. In a tetrahedral geometry, the zinc is connected to three histidines as ligands, and it is also putatively coordinated to two or three water molecules. CA has been found in various cellular constituents that handle different metabolic and physiological functions. Inorganic carbon transport efficiency is considered to be improved by extracellular CA. (A. Sharma et al., 2009). CO_2 levels can be reduced using the biocatalytic characteristics of carbonic anhydrase (CA). The enzyme carbonic anhydrase (CA) catalyses the exchange of CO_2 and HCO_3^- in solution. Even without CA, CO_2 and HCO_3^- conversion occurs, but the process is very slow. CA is thought to be needed to make sure that CO_2 and HCO_3^- are always available for different metabolic pathways in an organism.(DiMario et al., 2018). Specifically, CO_2 when reacted with alkaline minerals, it is converted to mineral carbonates. Mineral carbonates, like $CaCO_3$ or $MgCO_3$, are stable forms of carbon from a thermodynamic point of view. CO_2 can be stored for a long time after being turned into carbonates. (Chang et al., 2017). Carbonate and silica are some biominerals which are produced by some organisms. Biominerals are often used to make parts of an organism's structure, like shells and skeletons. When the conditions are favourable, they can form in water. Through a process called "biomineralization," CO_2 can be converted into biominerals like calcium carbonate. Natural CO_2 hydration is a slow process, so living things have a special enzyme that speeds up hydration so they can use CO_2 directly.(Kim et al., 2012). Several investigations have shown that when CA activity increased, so did the quantity of soluble calcium, showing that the solubility of calcium as well as ions was enhanced by CA in carbonate rock. CA could therefore be utilised in the recovery and utilisation of CO_2 in the atmosphere, in weathering, and in the creation of carbonate rock.(Z.

Zhang et al., 2011). Temperature and enzyme concentration were shown to improve the rate of CO2 hydration in CA, which might be used to sequester CO2.CAs provide a wide range of physiological functions, involving pH regulation, gastric acid generation, and CO_2 transport via tissues towards lungs.(Boone et al., 2013a). In organs like kidney, brain, CA is often involved in regulation of pH and transport of ion. (Ceyhun et al., 2011). This enzyme has a high turnover rate and thus plays a crucial role in photosynthetic microbe's ability to capture and utilize CO_2 from the atmosphere.(Prabhu et al., 2011). Enzyme storage, reusability as well as its stability are the most significant drawbacks. The only way to overcome these constraints is to immobilise enzymes on a suitable matrix ameliorate. Immobilization of enzyme on a solid surface remains appealing because it reduces process expenditure and often improves biocatalyst robustness. Immobilized enzymes are comparatively more stable than native enzymes in terms of pH, ionic strength and temperature. (A. Sharma, Sharma, et al., 2018). Many enzymes can be easily denatured or inactivated when exposed to organic solvents. The advancement of protein engineering and a variety of physical and chemical techniques, such as immobilisation, modification, and trapping, made it possible to stabilise enzymes in organic solvents.(Kumar et al., 2016). CA enzymes, in general, have less stability but increased catalytic activity. As a result, CA enzymes are immobilised to improve their stability for future. Thus, Immobilization is considered as one of the most effective methods for reusing enzymes, as well as establishing a cost-effective path for commercialization of the process(Jaya et al., 2019).

Chapter 2: Review of Literature

CA Structural Characteristics

Carbonic anhydrases are metalloenzymes that are found in almost all living things. They are coded by eight gene families that are not related to each other by evolution: i.e. (α, β, γ, δ, ζ, η, θ-, and ι-CAs)(Iraninasab et al., 2022). The α -class is mostly found in mammalian species, which has 11 different forms, some of which have been linked to diseases. So far, the catalytic mechanism of these monomeric enzymes has been the most studied of the three classes. Researchers have depicted an active site consisting of three histidine ligands. The active site of the β -class, found in algal and bacterial species comprise of one histidine and two cysteine ligands. These enzymes are also polymeric, which means they can exist as dimers, tetramers and hexamers. This enzyme's active site is made up of three histidine ligands, like the active sites of γ -class enzymes, but they belong to a different monomer(Amata et al., 2011). CA uses several types of inorganic carbon, such as bicarbonate, which are involved in the calcium precipitation process that occurs in nature. CAs have been identified in every phylum and class of living things. The currently accepted system of classifying CAs divides them into three core classes: α, β and γ. These classes do not share a significant amount of fundamental sequence identity and are thought to be evolutionarily distinct from one another.

Carbonic anhydrase ha zinc in its active site is kept in place by three histidine side chains. The CA active site has a unique pocket for CO_2 that puts it proximal hydroxide group linked by zinc. This results in the OH^- ions attacking Carbon dioxide and forming a bicarbonate molecule. So, CA speeds up the hydration of CO_2 to make bicarbonate and gives off a proton(Mondal et al., 2016). These enzymes use a two-step process to convert carbon dioxide and water into bicarbonate ions and a proton by reversible hydration. Initially, the nucleophilic attack of a Zn^{2+}-bound hydroxide ion on CO_2 produces HCO_3^-, which is then displaced by a water molecule from the active site. The rate-limiting second step transfer the proton bound water molecule consisting Zn^{2+} to the acceptor of proton or active site residue to restore the catalytically active Zn^{2+} bound hydroxide ion. CA speeds up the interconversion of CO_2 and carbonate form(Alterio et al., 2012). In the presence of Ca^{+2}ions, carbonate ion precipitates to form calcium carbonate. In order to transform CO_2 into solid carbonates for carbon sequestration, extracellular CA increases $CaCO_3$ synthesis at higher pH. $CaCO_3$ precipitation increases as carbonic anhydrase activity rises, and vice versa(Sundaram & Thakur, 2018).

Carbonic anhydrase (CA) is a vital enzyme in the capturing of CO_2 to calcium carbonate (Sundaram & Thakur, 2018). In CCM, CA outside of the cell binds inorganic carbon to the surface of the cell. CA accumulates HCO_3^- in cyanobacteria. Intermediate of HCO_3^-- pool that is very concentrated. CA changes HCO_3^- into CO_2 so that it can be used in the metabolic cycles. In the Calvin cycle, CO_2 is fixed. Starch and fatty acids are made by the cell as a way to store energy. These molecules can be taken apart to make products with added value(Sundaram & Thakur, 2018).

A climatic crisis will be avoided if we use microbes to repair CO_2. (T. Sharma et al., 2020). Technologies for carbon capture and storage (CCS) offer a way to reduce CO_2 emissions from burning fossil fuels, steel industries and refineries, and transporting this CO_2 to storage facilities or trapping them in place.(Chang et al., 2017). CO_2 can be turned into H_2 with the help of renewable energy, CO_2 can be hydrogenated via H_2O electrolysis. Methanol is one of the best products that can come from hydrogenation. Alkali hydroxide-based method was used to capture CO_2 and convert it to methanol. Methanol is produced by converting CO_2 to formate, formaldehyde, and alcohol dehydrogenase, which are all enzymes. Formaldehyde dehydrogenase is responsible for converting formate into formaldehyde, and ADH is the enzyme responsible for converting formaldehyde into methanol. Alcohol dehydrogenase is the enzyme responsible for converting formaldehyde into methanol (Zezzi Do Valle Gomes et al., 2021). Hydrogen was added to a solution of ethylene glycol, and the bicarbonate and formate salts were converted to huge volumes of methanol. An ethylene glycol solution of the base was utilized to extract CO_2 effectively in an integrated one-pot system.CO_2 was then hydrogenated to CH_3OH at temperatures between 100 and 140 °C using Ru-PNP catalysts. Distillation is a simple way to separate the methanol produced.(Sen et al., 2020). In a report about CO_2 hydrogenation, it was said that a high-pressure study up to 412 bars over Cu/Al_2O_3 catalysts in a fixed bed reactor showed high CO_2 conversion and high selectivity to methanol(Bansode & Urakawa, 2014). As a liquid fuel for internal combustion engines and direct methanol fuel cells, CH_3OH is the most intriguing CO_2 hydrogenation result since it may be used immediately. At low temperatures, metal-based homogeneous catalysts that have been built utilising rational catalyst design methodologies can be used to create CH_3OH (Methanol). Carbon dioxide (CO2) is collected and converted to CH3OH at temperatures between 125 and 165 degrees Celsius using pentaethylenehexamine (PEHA) and Ru-PNP complex. Its high boiling point, high basicity, and high nitrogen concentration make polyamines ideal for CO_2 capture. It was found in the research that 79 percent CH_3OH production was produced. Formate was formed from

6% of the CO_2 that was captured during the process. 15% of bicarbonate/carbonate mixture was unreacted(Kothandaraman et al., 2016).

SLC-0111 a derivative of ureido substituted benzenesulfonamide has the capability to inhibit CA isoforms IX and XII. SLC-0111 is presently being tested in Phase I or Phase II clinical trials to treat metastatic solid hypoxic tumours. SLC 0111 demonstrated remarkable anticancer or antimetastatic activity in animal models while maintaining a favourable pharmacokinetic profile(Lou et al., 2011). Pseudomonas aeruginosa, widespread pathogenic environmental bacterium which can infect animals, including humans. It can cause a serious inflammation of lungs, heart, urinary tract, and ulcers, especially in immunocompromised or otherwise susceptible patients with HIV or cancer(Richard et al., 1994). This adaptation is especially advantageous for the bacteria when they infect the lungs, which contain 200-fold higher CO_2 concentrations than the atmosphere (400 ppm), the solubility of CO_2 in water at 298 K is 55mM. P. aeruginosa is susceptible to the majority of existing antibiotics,19 -CAs offer a new target for the development of antibiotics. Lotlikar et al. have found three genes in P. aeruginosa PAO1 that encode three β-CAs (psCA1-3)(Aggarwal et al., 2015). Biocatalyst γ-Carbonic anhydrase which is produced by Aeribacillus pallidus TSHB1 polyextremophile bacteria, has the following conditions such as 32 kDa subunit is the molecular mass, the enzyme is alkali stable and relatively thermostable with $T_{1/2}$ values of 40±1, 15±1, and 8±0.5 min at 60, 70, and 80 °C, respectively. It is stable in the pH range of 8.0 to 11.0(Bose & Satyanarayana, 2017b). According to the study by (Li et al., 2017), CO_2 absorption of CA immobilised onto a polyester polymer increased from 75 mg/min to 197 mg/min. Immobilization of CA enhanced its CO_2 absorption efficiency, pH stability, and thermal stability, according to this study. In other study the comparison of free CA with immobilized enzyme was done and it was found that the immobilised CA on the high porous carbon foam kept 40% of the initial activity after 1 h at 70 and 90 Degrees Celsius, but the free CA dropped fast at both temperatures, with just 8% of the initial activity remaining after 1 h at 70°C(Wu et al., 2020).

In one of the articles, it is shown how directed evolution and protein sequence-activity relationships can be used to make new variants of CA that work in some of the toughest conditions that enzymes have to work in. We made a very reliable version of the CA from Desulfovibrio vulgaris that increases CO_2 uptake at temperatures above 100 °C in alkaline

solvents. This is important for effective carbon capture.(Alvizo et al., 2014). Carbon-containing adsorbents made from the leftovers of leather skin have been found to be very good at absorbing CO_2 and to have an interestingly high gas selectivity for CO_2. CO_2 isotherms and the high heat of adsorption show that there are strong CO_2 binding sites, which are related to the amount of nitrogen in the leather materials and the size of the nanoparticulate pores.(Bermúdez et al., 2013). It is possible to capture CO_2 in harmless forms utilising calcium aluminosilicate biomineralization. The reversible hydration of CO_2 by CA produces bicarbonate ions. To make a valuable carbonate, these bicarbonate ions combine with metal ions. Carbonates are stable because they are solid, which means they can last for ages. Metal ion carbonation, or weathering, is a common name for this phenomenon. (Bhagat et al., 2018). CA-bioreactor systems are very efficient at normal pressures and temperatures, which helps keep costs down. Keeping the membranes wet or at least moist will make them more expensive and harder to use in real life.(Boone et al., 2013b). Carbon dioxide (CO_2), has been shown to significantly increase in the growth rates of microalgal species. C4 plants have a greater capacity to fix CO_2 than microalgae. The selection of suitable microalgal strains is determined by their CO_2 fixation and tolerance capacity as well as their lipid potential, both of which are functions of biomass productivity. Microalgae can be cultivated in either open raceway ponds or enclosed photobioreactors. Biological CO_2 fixation also depends mostly on tolerance of particular strains to high temperatures as well as the amount of CO_2, SO_x, and NO_x present in flue gas. After sequestration, the potential uses of microalgal biomass could include the biodiesel production, livestock feed, colorants, and vitamins(Bhola et al., 2014).

When working with enzymes, protein purification techniques like fractionation, concentration, and chromatography helps to study the biological component required for functional, structural and kinetic studies. (A. Sharma, Meena, et al., 2018). The use of CA for CO_2 sequestration was explored, and it was shown that the rate of CO_2 hydration increased in the presence of both enzyme concentration and temperature. (Giri et al., 2018). Because of the activity of intracellular CAs, some eukaryotic algae can precipitate intracellular calcium carbonate. On the other hand, cyanobacteria are only capable of depositing calcium outside of their cells, and the pH plays an essential role in controlling this precipitation. It is probable that the extracellular CAs of cyanobacterial cells will engage in cell mineralization as well as the stabilisation of the pericellular pH. In addition, research has not been done on the cyanobacterial CAs that live in benthic populations.(Kupriyanova et al., 2007).

CA is good for carbon sequestration because it can handle a good amount of CO2, is stable in alkaline environments, and has a high enzyme reaction rate during carbonate conversion. (Giri & Pant, 2019).

Immobilization of CA

CA was immobilised to Sepharose 4B on polyurethane and bound to a steel matrix with silica through covalent bonds. (Jaya et al., 2019). Even though the enzyme has a very fast rate of catalysis, it is not very useful because it is not very stable and can't be used more than once. Both of these issues are frequent in applications involving enzymes. By immobilising CA on various support materials, such as silica, glass, polyurethane foam, and chitosan, a number of these issues have been resolved. This has led to the development of CAs that are more stable at high temperatures and have enhanced enzyme recovery. To produce the material or immobilise the enzyme, however, each of the currently employed techniques requires harsh conditions and a great deal of time, resulting in a low immobilisation efficiency and a significant decrease in enzyme activity.(Jo et al., 2014). Using bioinspired silica—a green way for bovine CA immobilization in bioinspired silica in a single step. Biological silicas are developed under neutral pH and optimum temperature. Offers better stability, high activity, and only takes 5 minutes to make. Diethylenetriamine (DETA) and sodium metasilicate are two examples of bioinspired additives. It was found that the ability of immobilised enzyme to remove CO_2 was about the same as that of free enzyme(Forsyth et al., 2013). Glutaraldehyde cross-linking succeeded by alginate bead encapsulation has been demonstrated in other research to be an effective method for stabilising carbonic anhydrase and accelerating microalgal growth. Floating hydrogel beads constructed from calcium alginate and cross-linked with carbonic anhydrase. This has a number of benefits, which are listed below:

(1) CA effectively captures CO_2 out from the air.

(2) It makes enzymes more stable and extends their lives.

(3) It allows it very easy to recycle enzymes.

For CA Activity Assay, p-NPA Activity Assay was performed. To identify the enzyme in the alginate beads., Coomassie Blue Dyeing was used(Xu et al., 2021). CA was lost during preparation and storage of CA as well as CA-GA beads in the following ways: For the CA beads, the loss of CA in the curing solution was 883%, but for the CA-GA beads, it was 191%.

In the same way, the CA loss in the storage solution was 2.0 0.7% for CA beads and 0.4 0.1% for CA-GA beads. CAGA- beads were less likely to be broken down by the enzymes than CA beads. The free CA can't be recycled through centrifugation or filtration, but the buoyant alginate CA-GA beads can be kept during cultivation or skimmed off the surface of the culture to be used again. This makes it possible to separate them, saves money, and makes them more useful for the rest of their lives(Xu et al., 2021).

Immobilization of carbonic anhydrase on bioactive HFM boosts the diffusional gradient on HFM surface, thereby enabling CO_2 removal (CA). CA is utilised by tissues to increase diffusion within RBC as well as endothelial surfaces of lung capillaries. Using bioinspired method, they developed a bioactive HFM coating immobilised with carbonic anhydrase (CA)(Kaar et al., 2007). On the fibre membrane, hydroxyl groups were deposited as a result of radio frequency flow discharge (RFGD) treatment. Gas permeability tests as well as scanning electron microscopy (SEM revealed that a 30 s exposure time and 25W did not impair the HFM's robustness and gas permeability. Data on gas permeance after activating the coupling of CNBr and CA revealed that CA does not restrict gas diffusion across the membrane. Compared to unmodified devices, these improved CA HFMs increased the CO2 removal rate from PBS by 75% in micro gas exchange devices. Thus, the creation of bioactive hollow fibre membranes has evolved from the need for efficient CO2 removal membranes in respiratory support devices (HFMs)(Arazawa et al., 2012). Using the sol-gel approach, the general structure of bovine carbonic anhydrase II is retained when encased in silica monoliths. Enzyme precipitates in solution at 64°C while it is not in the case of encapsulated enzyme. its unfolding occurs at melting temperature of 51°C. Also, only 77 percent of the encapsulated enzyme is unfolded at 74.0 °C. The glass matrix restricts complete unfolding and refolding and inhibit the movements of the encapsulated protein molecules. Upon encapsulation, several properties of the enzyme are preserved such as overall conformation and K'^{M} value, whereas so me properties are altered for e.g., tendency for unfolding and k'_{cat} value(Badjić & Kostić, 1999).

Steps in Enzyme Immobilization include the following: (Sheldon & van Pelt, 2013)

1. Selecting a support material whose surface properties specifies the binding relationship for enzymatic bonding.

2. The assessment of parameters throughout the process in order to optimise operational efficiency, to have good yield and activity.

3. Characterizing catalytic properties of derived biocatalyst during process conditions(Bernal et al., 2018).

The chemical alteration of CA boosts stability and improves sequestration, hence enhancing the CO_2 conversion. Immobilization strategies for Immobilization

1. Support-based immobilization
2. Support-free immobilization

1. Support-based immobilization: Consists of adsorption, covalent bonding, and encapsulation.

Adsorption: includes a weak contact between enzyme and support in order to fix enzyme molecules. Preparation and handling are simple, but the enzyme's binding site to the substrate is permanently detached from it, changing its ability to perform its function.

Covalent bonding Despite the fact that covalent bonding permits strong binding of the enzymes and support, this technique demonstrates low enzyme activity. Covalent binding can alter the structure of enzymes and limit enzyme mobility, which can disable biocatalysts.

Encapsulation (entrapment) is an efficient method for encasing enzymes in a polymer membrane to prevent their leaching and deactivation(Cen et al., 2019).

To immobilise enzymes, polymer items like chitosan, agarose, silicon oxide. These are used as suitable supports.

2. Support-free immobilization: Enzymes immobilised on insoluble support materials are stabilised. Support-free immobilisation can be used to reduce their production costs, which rise as their stability and reusability grow.

Cross-linking Compounds involves combining enzyme molecules when required. Enzymes do not require help. These are less expensive and have a greater specific surface area. Cross-linking decreases the chain instability of CLE, which is difficult to manage and has low mechanical strength. Future research should concentrate on improving strength in order to enhance the activity of cross-linked materials(Cen et al., 2019).

Enzyme immobilization can be accomplished through support-based immobilisation and support-free immobilisation, and there is no absolute method or support for enzymes. Each of these has its own advantages and disadvantages(Ren et al., 2021)

Chapter 3: Materials and Methods

Screening of bacteria producing Carbonic Anhydrase

Soil sample was collected from the rock sediments in Waknaghat, H.P. Isolation of bacteria by Serial dilution method. Nutrient Agar plate was inoculated by 1mL dilutions (10^{-4}, 10^{-6}, 10^{-8}) respectively. Incubation was done at 37°C for 24hrs. Streaked plate 8 bacterial isolates on Nutrient agar plates with 3mM p-NPA and incubated at 37°C for 24hrs.

Figure 1: Sample collection from the site

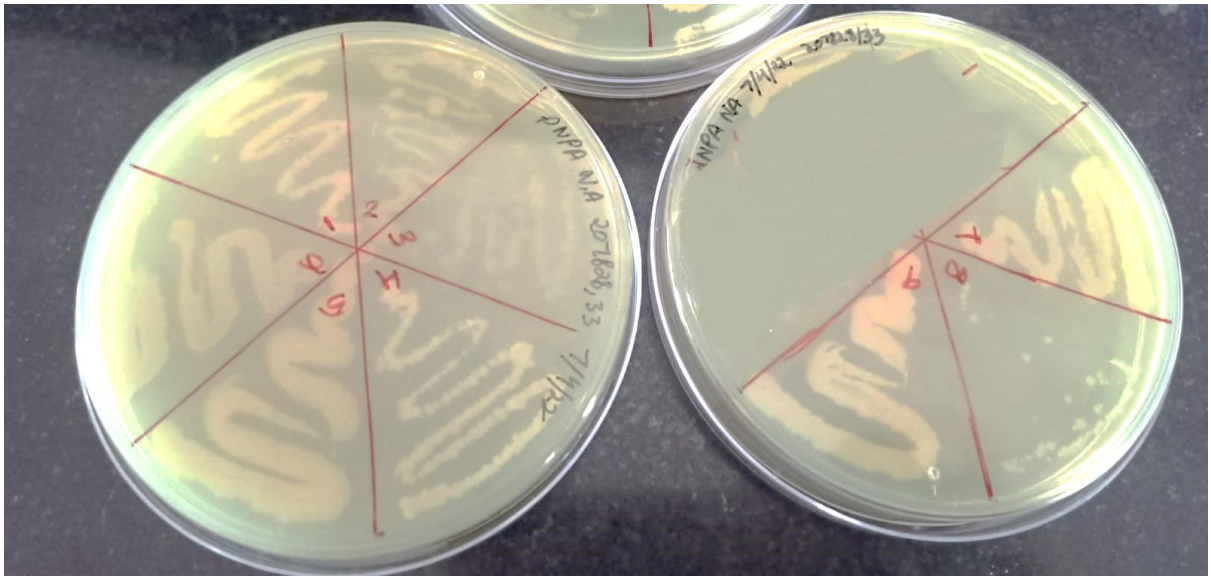


Figure 2: Streaked plate for 8 isolates

p-nitrophenol Standard Curve Preparation

1mg/mL p-nitrophenol stock solution was prepared in isopropanol. 50mM 20mL phosphate buffer was prepared. Different volumes of stock and buffer were added to Eppendorf tubes.200µl of the above samples were loaded to 96 well plate and the OD was taken at 410nm. Standard graph was plotted from the OD reading.

Seed Culture, Production Media Preparation

Preparation of seed culture: Inoculated Loopful of Isolate No. 5 strain from NA streak plate in 50mL nutrient broth and were incubated. Production media preparation: 1% (0.5ml) Seed culture of 8 isolates were inoculated in 50mL Nutrient Broth respectively.

Crude enzyme extraction

50mL of Production Media of 8 isolates respectively were transferred to centrifuge tubes and were centrifuged. Tubes were kept on ice. Collect supernatant in other tubes. Added 5ml phosphate buffer to all the tubes having pellets. Sonicated all tubes for 5 mins with the pulse of 10 seconds in sonicator.

Carbonic Anhydrase- Enzyme assay

For enzyme Assay, 825µl Phosphate buffer was added to Blank, Control, sample tubes. 175µl pNPA was added to control and sample tubes. 25µl enzyme extract was added to sample tubes (8 supernatant, 8 cell lysate tubes). Addition of phosphate buffer and pNPA to the tubes followed by incubation. Reaction mixture was given 5 minutes incubation again in water bath at 37 °C. Absorbance was measured at 410nm. Out of these 8 supernatants, 8 cell lysate tubes, highest enzyme activity of 1.58 U/ml was found in isolate 5 cell lysate(T. Sharma & Kumar, 2021).

Parameter 1: Preparation of Media for Media Optimization

5 different media were prepared for enzyme optimization that is Muller Hinton Broth, Peptone Broth, LB Broth, Basal Salt Media, Nutrient Broth. The composition of other media used were, Basal salt media(g/L) - Na_2HPO_4 2.0, $FeCl_3 \cdot 6H_2O$ 0.005, Sucrose 5.0, $MgSO_4.7H_2O$ 0.5, $CaCO_3$ 0.1; Minimal salt media(g/L) - KH_2PO_4 6.8, $MgSO_4$ 0.2, Na_2HPO_4, 7.8, $ZnCl_2$ 0.02,

$ZnSO_4.7H_2O$ 0.05, $NaNO_3$, 0.085 peptone broth (g/L) - glucose 1.0, beef extract 3.0, NaCl 5.0, peptone 5.0, $CaCO_3$ 6.0(T. Sharma & Kumar, 2021).

The seed culture of isolate No. 5 was prepared and incubated. Production media: 1% (0.5ml) Seed culture of isolate 5 was inoculated in 50mL Muller Hinton Broth, Peptone Broth, LB Broth, Basal Salt Media, Nutrient Broth respectively. 24hrs incubation for 24hrs at 120rpmwas given (T. Sharma & Kumar, 2021).

Crude enzyme extraction- Media

50mL of Production Media of 5 different media were transferred to centrifuge tubes respectively and were centrifuged. Tubes were kept on ice. Discarded Supernatant. Added 5ml phosphate buffer to all the tubes having pellets. Sonicated all tubes for 5 mins with the pulse of 10 seconds in sonicator.

Carbonic Anhydrase Enzyme Assay- Media

For enzyme Assay- 825µl Phosphate buffer was added to Blank, Control, 5 sample tubes, 175µl pNPA was added to control and 5 sample tubes. 5µl enzyme extract was added to 5 sample tubes. Addition of phosphate buffer and pNPA to the tubes followed by 5 minutes incubation. Reaction mixture was given 5 minutes incubation again in water bath at 37 °C. Absorbance was measured at 410nm (T. Sharma & Kumar, 2021).

Parameter 2: Inoculum Size Optimization

Preparation of seed culture: Inoculated Loopful of Isolate No. 5 strain from NA streak plate in 50mL nutrient broth. Incubation at 37 °C for 24hrs at 120rpm.

Production Media Preparation - Inoculum Size Optimization

Production media preparation: 6 falcon tubes consisting 50mL Nutrient Broth were prepared. Seed culture (0.05%, 0.10%, 0.15%, 0.20%, 0.25%, 0.50%) of Isolate No. 5 were inoculated in 6 falcon tubes consisting 50mL Nutrient Broth respectively followed by incubation.

Crude enzyme extraction- Inoculum Size Optimization

Falcon tubes consisting of 50mL Production Media of various inoculum size were centrifuged. Tubes were kept on ice. Discarded supernatant. Added 5ml phosphate buffer to all the tubes consisting pellets. Sonicated all tubes for 5 mins with the pulse of 5 seconds in sonicator. (Kept in Ice) Centrifuged the cell lysate again. Collected the upper phase of the falcon tube in other tube.

Carbonic Anhydrase assay - Inoculum Size Optimization

For enzyme Assay, 825µl Phosphate buffer was added to Blank, Control, sample tubes. 175µl pNPA was added to control and sample tubes. 25µl enzyme extract (6 tubes- cell lysate) was added to sample tubes. Addition of phosphate buffer and pNPA to the tubes followed by 5 minutes incubation. Reaction mixture was given 5 minutes incubation again in water bath at 37 °C. Absorbance was measured at 410nm.

For Bradford's Assay, 80µl Phosphate buffer was added to sample tubes and 100µl was added to control. 1mL was Bradford's reagent was added to control and sample tubes. Addition of 20µl enzyme extract (4 tubes- cell lysate) in the sample tubes. 10 mins incubation was done in room temperature. The tubes were incubated for 10 mins in room temperature. Absorbance was measured at 595nm Bradford 1976.

Parameter 3: Temperature Optimization

Preparation of seed culture: Inoculated Loopful of Isolate No. 5 strain from NA streak plate in 50mL nutrient broth. Incubation at 37 °C for 24hrs at 120rpm.

Production media preparation: 6 Flasks consisting 50mL Nutrient Broth were prepared and autoclaved. 0.5mL Seed culture of isolate no. 5 were inoculated in 6 flasks consisting 50mL Nutrient Broth respectively. The above flasks were incubated at temperature ranging from 20 °C to 70°C respectively for 24hrs at 120rpm.

Crude enzyme extraction- Temperature Optimization: 6 falcon tubes consisting of 50mL Production Media (different temperature conditions) were centrifuged. Tubes were kept on ice. Supernatant was discarded. Added 5ml phosphate buffer to all the tubes consisting pellets. No pellet was seen in the production media optimized at 60 °C, 70°C. Sonicated all 4 tubes for 5 mins with the pulse of 5 seconds in sonicator. (Kept in Ice). Centrifuged the cell lysate again. Collected the upper phase of the falcon tube in other tube.

Enzyme assay – Temperature Optimization: For enzyme Assay, 825µl Phosphate buffer was added to Blank, Control, sample tubes. 175µl pNPA was added to control and sample tubes. 25µl enzyme extract (4 tubes- cell lysate) was added to sample tubes. Addition of phosphate buffer and pNPA to the tubes followed by 5 minutes incubation. Absorbance was measured at 410nm (T. Sharma & Kumar, 2021).

Bradford's Assay- Temperature Optimization: For Bradford's Assay, 80µl Phosphate buffer was added to sample tubes and 100µl was added to control. 1mL was Bradford's reagent was added to control and sample tubes. Addition of 20µl enzyme extract (4 tubes- cell lysate) in the sample tubes. 10 mins incubation was done in room temperature. Absorbance was measured at 595nm Bradford 1976.

Parameter 4: pH Optimization

Preparation of seed culture: Inoculated Loopful of Isolate No. 5 strain from NA streak plate in 50mL nutrient broth. Incubation at 37 °C for 24hrs at 120rpm.

Production media preparation: 6 Flasks consisting 50mL Nutrient Broth were prepared and the pH for the 6 flasks were set at pH 5 to pH 10 and autoclaved. 0.5mL Seed culture of isolate no. 5 were inoculated in 6 flasks consisting 50mL Nutrient Broth respectively. The above flasks were incubated respectively for 24hrs at 120rpm.

Crude enzyme extraction- pH Optimization: 6 falcon tubes consisting of 50mL Production Media (different temperature conditions) were centrifuged. Tubes were kept on ice. Discarded supernatant. Added 5ml phosphate buffer to all the tubes consisting pellets. No pellet was seen in the production media optimized at 60 °C, 70°C. Sonicated all 4 tubes for 5 mins with the pulse of 5 seconds in sonicator. (Kept in Ice). Centrifuged the cell lysate again. Collected the upper phase of the falcon tube in other tube.

Enzyme assay – pH Optimization: For enzyme Assay, 825µl Phosphate buffer was added to Blank, Control, sample tubes. 175µl pNPA was added to control and sample tubes. 25µl enzyme extract (4 tubes- cell lysate) was added to sample tubes. Addition of phosphate buffer and pNPA to the tubes followed by 5 minutes incubation. Reaction mixture was given 5 minutes incubation again. Absorbance was measured at 410nm(T. Sharma & Kumar, 2021).

Bradford's Assay- pH Optimization: For Bradford's Assay, 80µl Phosphate buffer was added to sample tubes and 100µl was added to control. 1mL was Bradford's reagent was added to control and sample tubes. Addition of 20µl enzyme extract (4 tubes- cell lysate) in the sample tubes. 10 mins incubation was done in room temperature. Absorbance was measured at 595nm Bradford 1976.

CO_2 Hydration

Preparation of carbonated water: 500ml distilled water added to reagent bottle. CO_2 is added to the reagent bottle by the CO_2 cylinder. It is done on ice. Distill water pH in the beginning was 9.02. After an hour, pH dropped to 5.2(T. Sharma & Kumar, 2021).

Preparation of reaction mixture (test): 1mL 1M Tris Buffer set at pH 8, 11.5mL of 2% v/v calcium chloride was made by adding 2g calcium chloride in 100mL, 11.5mL CO_2 Saturated water, 1mL (0.5mg/mL) enzyme in phosphate buffer (50mM, pH 7)(T. Sharma & Kumar, 2021).

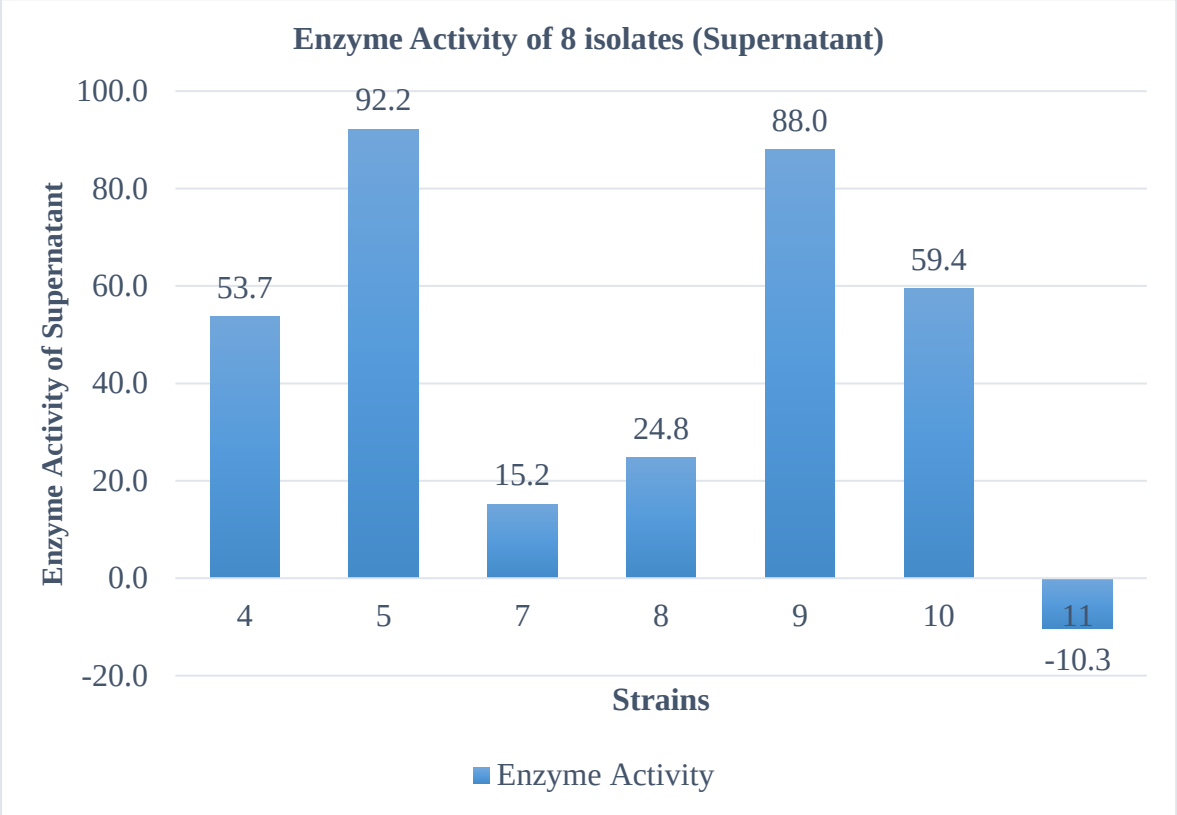
Control: Bovine Serum Albumin (1mg in 1mL phosphate buffer 50mM), 1mL 1M Tris Buffer set at pH 8, 11.5mL of 2% v/v calcium chloride was made by adding 2g calcium chloride in 100mL, 11.5mL CO_2 Saturated water(T. Sharma & Kumar, 2021).

Kept the tube of reaction mixture under room temperature for 24hrs. Centrifuged the tube at 8000rpm for 5 mins. Discarded supernatant and the cell pellet lyophilized for 4 hours in the lyophilizer to remove the water content from the $CaCO_3$ precipitates.

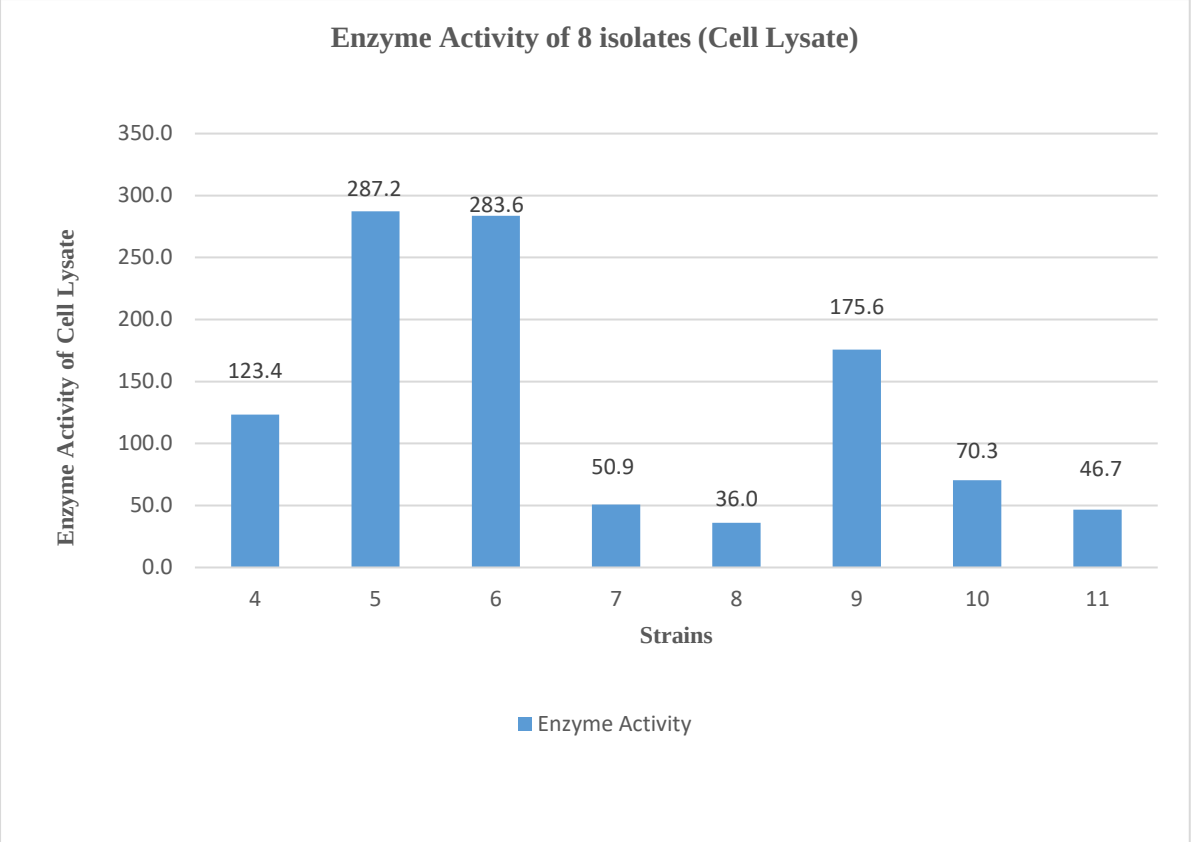
Chapter 4: Results

1. Carbonic Anhydrase Enzyme activity in bacteria

8 isolates were obtained from nutrient agar plates containing 3mM p-NPA. The highest enzyme activity was observed in isolate number 5 for cell lysate. Using Bradford's assay, the protein concentration of the enzyme sample was (7.02 µg/mL). The specific activity was (0.22 U/µg).



Graph 1: Enzyme activity of 8 isolates (Supernatant)



Graph 2: Enzyme activity of 8 isolates (Cell Lysate)

Highest enzyme activity was obtained in cell lysate. Maximum enzyme activity 287.2 µg/min/mL (1.58 U/ml) was seen in Isolate No. 5

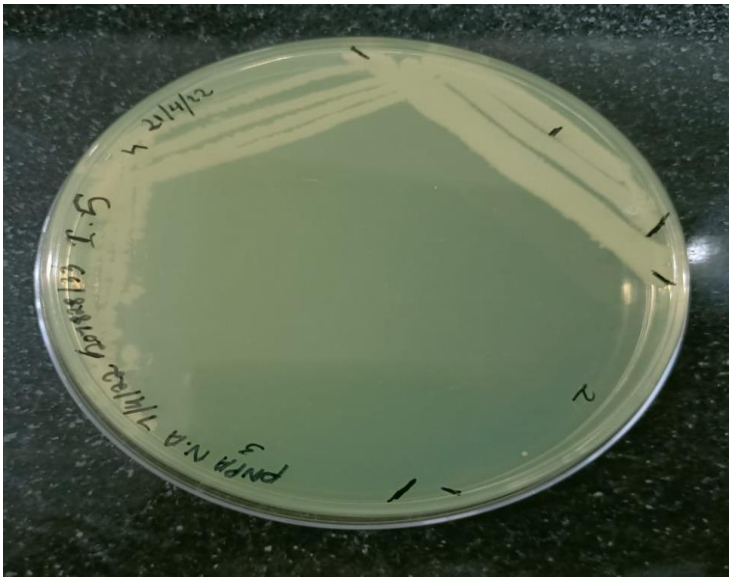


Figure 3: Nutrient Agar Plate for Isolate No. 5

2. Gram staining, Biochemical characterization of the isolated bacteria

Biochemical characterization was done by gram staining, citrate utilization test, urease test.

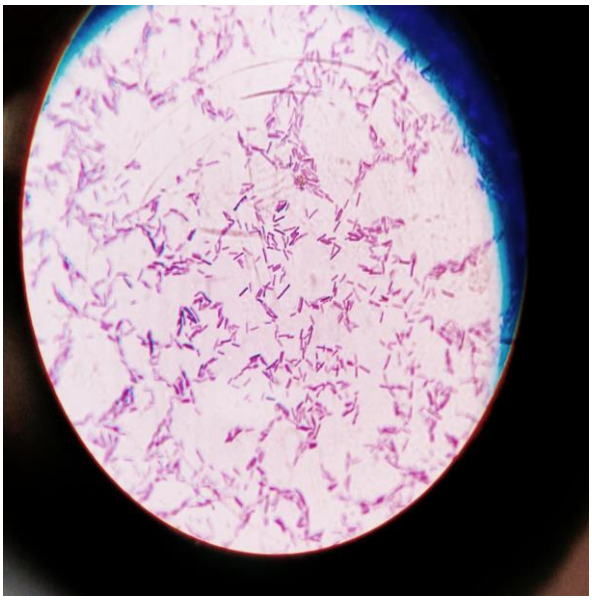


Figure 4: Gram Staining

Microscopic View of Isolate 5

Gram Negative bacteria, Rod shaped Bacilli

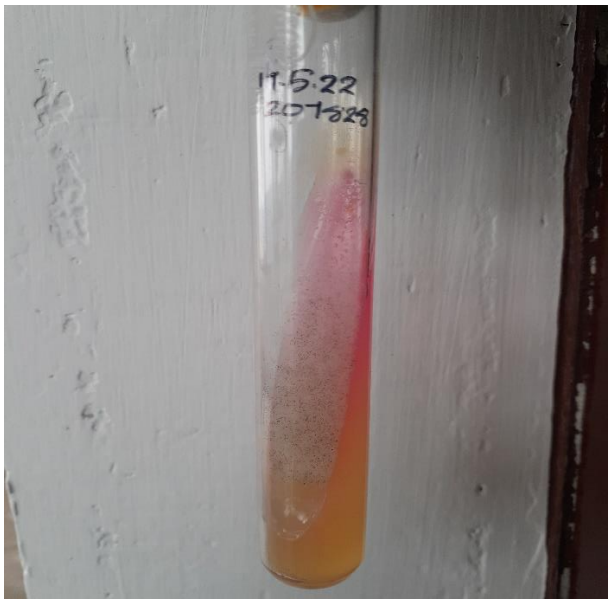
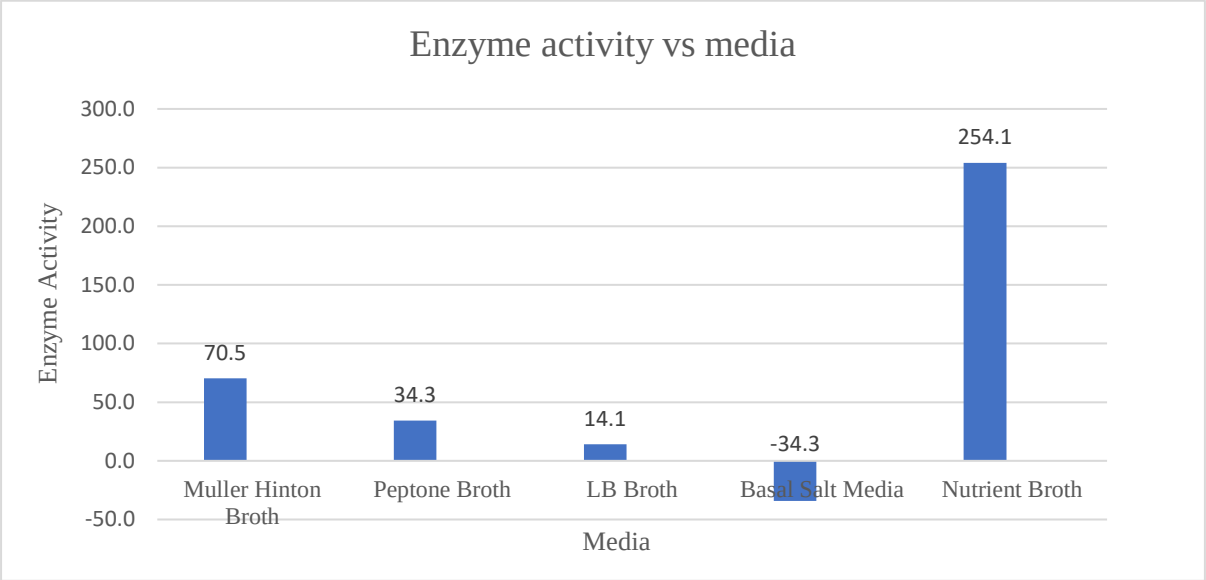


Figure 5: Urease test (observed contamination)

Figure 6: Citrate utilization negative test

3. Production Parameter

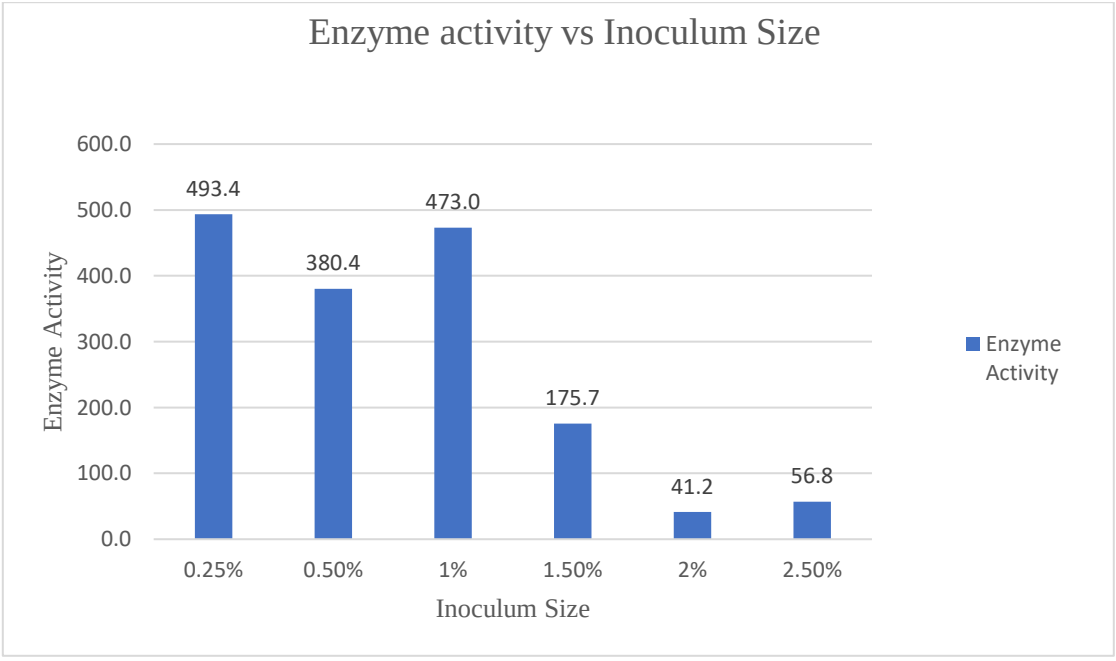
Media Optimization



Graph 3: Enzyme activity vs media

Highest Enzyme Activity 254.1 µg/min/mL (1.4 U/mL) is seen in Nutrient Broth.

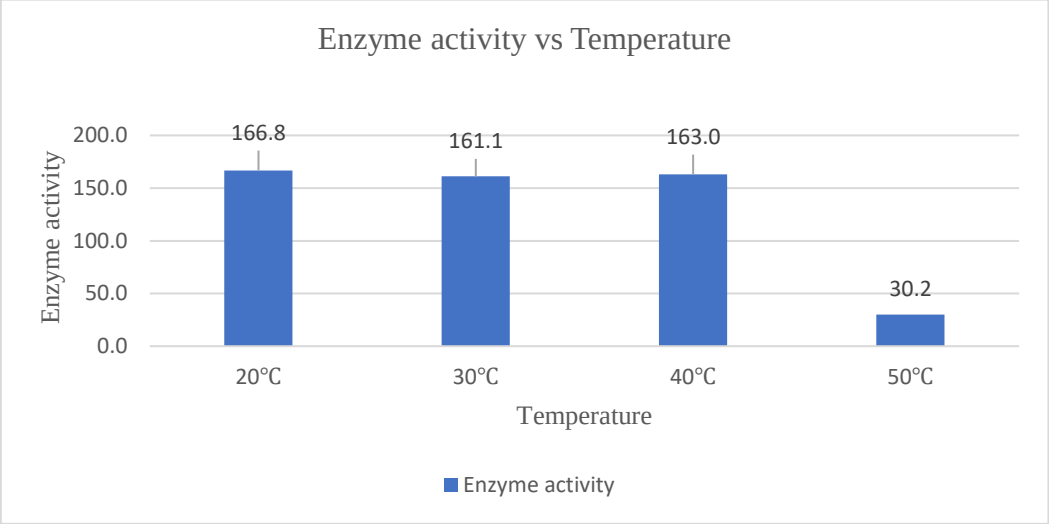
Inoculum size optimization



Graph 4: Enzyme Activity v/s Inoculum size

Highest enzyme activity 493.4 µg/min/mL (3.17 U/mL) was seen in Inoculum size 0.25%.

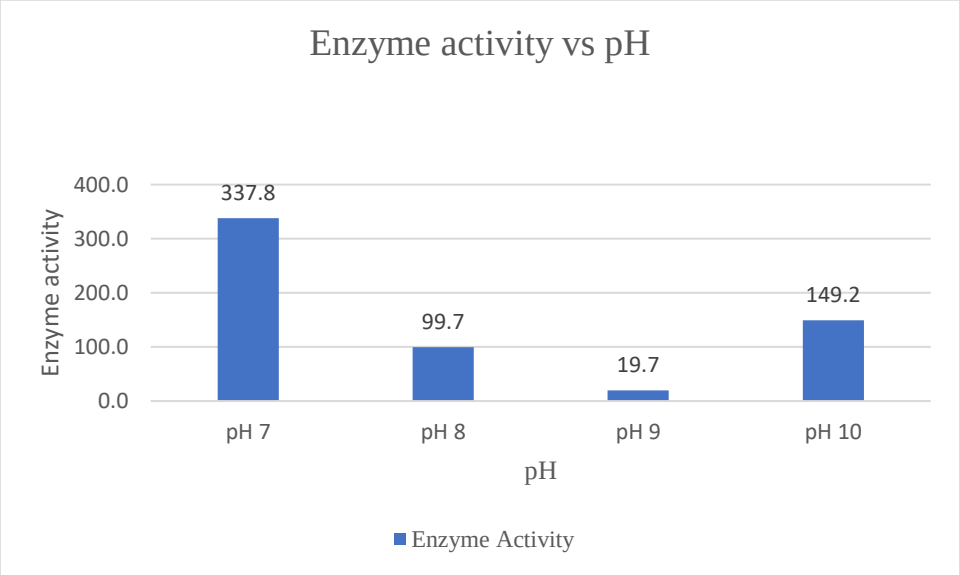
Temperature Optimization



Graph 5: Enzyme activity vs Temperature

Highest enzyme activity 166.8 µg/min/mL (0.92U/mL) was seen at 20°C

pH Optimization



Graph 6: Enzyme activity vs pH

Highest enzyme activity was 337.8 µg/min/mL 1.86 U/mL (pH 7)

4. CO_2 Hydration

6.4mg $CaCO_3$ precipitates were obtained from 20µL of enzyme extract in 50mL of reaction mixture.

Chapter 5: Discussion

8 isolates that were obtained from the soil sample were checked for Carbonic Anhydrase. The highest enzyme activity (1.58 U/ml) was seen in cell lysate of isolate no. 5. The isolate found to be gram positive, rod shaped, bacilli. CA activity detected in bacterial sample states that it possesses extracellular enzyme(s), It has been reported that CA can be produced by a number of different pathogenic bacteria, such as *Yersinia pseudotuberculosis* and *Listeria monocytogenes*, which were isolated from soil and water(Buzolyov 1999). Optimization of production parameters was done by optimizing the media, inoculum size, pH, temperature. Out of 5 media, Nutrient Broth gave the highest enzyme activity and was optimized. (A. Sharma et al., 2009) reported that the maximum CA synthesis from *Pseudomonas fragi* occurred in peptone broth, which is in opposition to the findings that were just presented. At a temperature of 20 degrees Celsius, CA production was found to be at its highest. In addition to this, it was observed that there was a reduction in the CA activity as the temperature continued to rise whereas (T. Sharma & Kumar, 2021) reported highest enzyme activity at 40°C. The volume of inoculum optimized was 0.25% whereas in another paper it was shown that 1.5% inoculum size was optimized for CA production which was *Aeribacillus pallidus*(Bose & Satyanarayana, 2016). The effectiveness of CA in converting CO_2 was evaluated by determining the quantity of $CaCO_3$ that was produced during the experiment. The amount of $CaCO_3$ produced was 6.4 mg $CaCO_3$/mg of protein.

Chapter 6: Conclusion

CO_2 conversion is a challenging topic of research, and no optimum solution has yet been found for large-scale CO_2 conversion. Chemicals, minerals, and biological molecules have all been used in a variety of CO_2 conversion processes. However, each of these CO_2 capture has its own set of constraints, which makes commercial application more complex. In addition to the utilisation of the material and chemicals, one can take advantage of the numerous microorganisms that have potent CA and are able to endure the extreme working conditions of the process. Regarding the topic of biological carbon mitigation, it can be said without a doubt that microbes offer benefits that are beneficial to the environment as well as the economy. For the sequestration process, CA with enhanced catalytic activity is being employed. The enzyme can be put to good use by converting atmospheric carbon dioxide into calcite, which results in the production of a product that is stable, environmentally beneficial, and suitable for long-term storage. Enzymes for industrial usage are frequently derived from microorganisms as it is considered cost effective. The production parameters of Carbonic Anhydrase were optimised. Hydration of CO_2 resulted in the formation of calcium carbonate. Value-added materials, such as CaCO3, can be exploited and used in a variety of applications such as pharmaceuticals, paint and coating materials and paper as well as concrete and other construction materials. Purification and immobilisation of the CA enzyme will improve reaction efficiency in future research. Using bacterial CA precipitation of calcite, carbon can be sequestered and employed in a range of applications, including as paper, cements, and building materials. In order to address the environmental issues raised by CO_2 emissions, the application scope of CA has been broadened. Due to the fact that CA possesses the highest catalytic efficiency for CO_2 hydration.

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