Cultivation and screening of microalgal strains for enhanced biomass production using enzymatic treatment

A PROJECT

Submitted in partial fulfillment of the requirement for the award of the degree of

BACHELOR OF TECHNOLOGY

IN BIOTECHNOLOGY

Under the supervision of

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By

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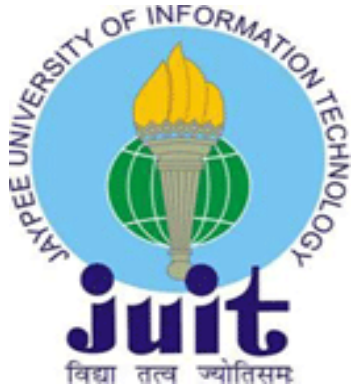
DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS



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CERTIFICATE

This is to certify that the work entitled "**Cultivation and screening of microalgal strains for enhanced biomass production using enzymatic treatment**" pursued by Shagun Sharma (181823) and Kanishk Bhatt (181816) for the partial fulfilment of degree Bachelor of Technology in Biotechnology from Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This part of work has not been submitted partially or wholly to any other University or Institute for the award of any degree or appreciation.

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Sincerely,

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Date: 14th May, 2022

DECLARATION

We hereby declare that the project work entitled 'Cultivation and screening of microalgal samples for enhanced growth using enzymatic treatment', submitted to the Jaypee University of Information Technology, Waknaghat, Solan is a record of original work done by me under the guidance of Dr. Ashok Kumar Nadda, Assistant Professor, Department of Biotechnology and Bioinformatics. This project work is submitted in the partial fulfilment of the requirements of the reward of the degree of Bachelor of Technology in Biotechnology. The results embodied in this thesis have not been submitted to any other university or institute for the award of any degree or diploma.

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Shagun Sharma (181823)

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

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Abstract

The industrial development made over the last six decades, combined with the tremendous growth of the worldwide total, has occurred at a huge environmental expense. From 1960, world GDP per capita had roughly tripled, but carbon dioxide release had almost quadrupled. The previous three centuries have seen almost two-thirds of such a rise. The greatest danger, however, of this growing CO_2 atmospheric concentrations is the "greenhouse effect", which affects the atmosphere's radiation balance to quite an extent. Therefore, lowering CO_2 levels and transitioning to renewable fuels and value products may be a viable alternative for mitigating greenhouse gas emissions. The biological technique, which relies heavily on the metalloenzyme carbonic anhydrase, is one such way for effective CO_2 use. This enzyme is responsible for converting carbon dioxide into bicarbonate ions. Because microalgal biomass uses bicarbonate ions for growth, this entire process may be employed to promote and enhance microalgal biomass in water systems. In relation to existing biomass feedstocks, microalgae have received a lot of interest in recent years and have emerged as an appealing bioenergy source. Knowing the processes of carbon absorption and utilisation is critical for optimizing the regulation of microalgae in converting bicarbonate ions from carbon dioxide plus energy into biomass. Furthermore, the produced microalgae may be harvested in greater quantities by various methods and be utilized in various applications. Production of several value-added goods in industries such as pharmaceuticals, cosmetics, nutraceuticals, and microalgae biorefinery can all benefit from enhanced microalgal biomass. A number of distinct natural, hybrid, plus inorganic compounds have lately been investigated as carbonic anhydrase sequestration carrier substances. Such components are crucial not just for increasing carbonic anhydrase tolerance to harsh conditions but also for enhancing its effectiveness and rate of recuperation from the reactive atmosphere, which lowers the price of using it on a large scale.

Key words: *Carbon dioxide, Microalgae, Metalloenzyme, Carbonic Anhydrase, Immobilization*

Introduction

With rising CO_2 levels in the atmosphere, the greatest threat and fear of the hour is its effect on the atmosphere's energy balance, also known as the "greenhouse effect." As a result, reducing the greenhouse gas effect requires solely reducing CO_2, which can be accomplished by turning it into cleaner resources and vital chemicals. As a solution, CO_2 capturing and processing techniques for value-added products are being researched. CO_2 use has been proposed as a solution and/or supplement to CO_2 geological depository, with the potential to raise the commercial importance of CO_2 collected. For CO_2 collection, various technologies have been developed, the majority of which are dependent on amine-dependent absorption-desorption processes, while some use solid adsorbents to separate CO_2 from flue gases before storage. Such methods not only consume a lot of energy, but they also possess corrosion and operational concerns as a result of the numerous contaminants in the fuel burning or other components where CO_2 is captured *(Global CCS Institute, 2011)*.Biological systems, on the other hand, need less energy but have a smaller range. CO_2 can be converted into organic matter where the source of energy is sunlight. Although this choice is appealing, further testing and execution are needed before it can be enforced. Algae, in fact, are much more competitive and efficient at transforming CO_2 into organic compounds than other terrestrial plants *(Razzak et al., 2017)*. However, when it comes to enzymes, immobilized carbonic anhydrase is quite popular for hydration of CO_2 with a high amount of specificity. Lately many carrier materials for carbonic anhydrase immobilization have been investigated, including a vast variety of organic, hybrid, and inorganic substances. These materials play a vital role not only in increasing carbonic anhydrase resistance to harsh conditions, but also in increasing its performance and recovery rate from the reaction environment, thereby reducing the expense of using it on a large scale *(Wu et al., 2020)*. This is where microalgae come into the picture, since they have high productivity along with other benefits, including the obvious potential to fix CO_2 better (10–50 times) than terrestrial plants, as claimed by many reports *(Yadav et al., 2017; Lam et al., 2012)*. Phototrophic microalgae are yet another potential for biological CO_2 mitigation, as they may thrive on inorganic carbon as a source of food *(Hwang et al., 2014; Markou et al., 2013)*. Microalgal biomass can be utilised to make high-value items like pharmaceuticals and cosmetics, and their lipid products can be used to make biofuels *(Chew et al., 2017)*. When contrasted to terrestrial crops like maize and soybean, microalgae biofuels have high manufacturing outputs per landmass. Algae biomass output is expected to be 2–10 fold that of the finest terrestrial habitats per unit area. Increased photosynthesis efficacy is the fundamental cause for it though. Algae consumes the

majority of available energy in cellular division during perfect growth circumstances, providing for quick biomass increase. Many experts believe that using algae biofuels might cut emissions of carbon dioxide from 101,000g CO_2 analogous per million British thermal units (BTU) towards 55,440g *(Rittmann 2008)*. As per the U.S. Environmental Protection Agency Act, biofuel made using microalgae has the ability to satisfy the Environmental Protection Agency's (EPA) Renewable Fuel Standard (RFS 2007) requirements. In addition to biofuel, a microalgal biorefinery might create a variety of by-products such as lipids, protein, and carbohydrates. Again, for generation of biofuels such as green diesel, biodiesel, jet fuel, biogas methane, or green petroleum, converting mechanisms can be included. By providing an optimum growing condition or increasing the build-up of inorganic carbon in microalgae, recent developments in photobioreactor construction *(Fu et al., 2019; Zhou et al., 2017)* and genetic improvement of microalgae have increased their growth rate and allowed CO_2 sequestration. Microalgae are typically small, microscopic species that can be found in both marine and terrestrial ecosystems. As a result, they comprise a distinct range of species which are capable of thriving in a variety of environmental conditions *(Rizwan et al., 2018)*. Other nutrients, such as phosphorus and nitrogen, are consumed by microalgae, enabling them to be used in wastewater treatment *(Carvalho et al., 2011)*. As for its application of wastewater treatment, using microalgae for the removal of nutrients (particularly that of phosphorus and nitrogen) from wastewater has been referred to as green technology since it either reduces or eliminates the chemicals used in wastewater treatment plants. Therefore, both CO_2 capture by photosynthesis and the removal of nutrients from wastewater are the main advantages of this technology. CO_2 collection via combustion products combined with wastewater treatment employing microalgae culture to create biofuel appears to have been a viable substitute to fossil fuels. The entire production cost is greatly decreased by integrating these photoconversion and biochemical stages, making CO_2 collection far more commercially appealing *(Razzak et al., 2017)*.Other than this, silver nanoparticles have also been produced using microalgae. Numerous microalgae populations have been shown to create metallic nanoparticles, with biomolecules and enzymes thought to be essential for its reduction capability. Furthermore, silver nanoparticles might be generated as an intriguing coproduct of microalgae farmed for other uses. In specific, Biomass created via CO_2 biological abatement. Cyanobacteria and green algae were investigated because they are economically important species because of their simplicity of cultivation, vast ecosystem, and adaptability to harsh environments *(Mora-Godínez et al., 2020)*.

The main objective of this thesis is to understand the role of carbonic anhydrase so that carbon dioxide can be utilized and minimized efficiently. The literature review talks about how carbon dioxide can be converted to bicarbonate ions using the metalloenzyme carbonic anhydrase and how

further these produced bicarbonate ions are introduced into a microalgae system where the microalgae absorb the ions and uses them to proliferate. Further in the thesis various experiments have been described which were performed where culture was revived to check for carbonic anhydrase presence, collection of microalgal samples was also carried out from a nearby water source which was then isolated and grown on algal media BG11. The algal sample was then observed under microscope and various observations were made.

Review of Literature

A. Microalgae in CO_2 capture

CO_2 bioconversion based on microalgae is an efficient technology for directly using gaseous CO_2. Inorganic carbon sources like as flue gas or general CO_2 are commonly used in the growth process. Because the conversions happen at normal room temperature, it is an environmentally friendly process. However, optimising physicochemical process parameters and regulating nutritional demands in the cultivation process are critical for achieving higher yields of target products (carbohydrate, lipid). Carbonic anhydrase, an external enzyme, is involved in CO_2 fixation. Bicarbonate and gas CO_2 are both used as carbon sources by microalgae, although bicarbonate is the important carbon species in perhaps the most typical pH range (6.5–10) in microalgae production media. When an industrial exhaust gas stream is fed into microalgae cultures, the CO_2 content is usually greater (0.03–0.05 percent) than in ambient air, resulting in better biomass productivity *(Gonçalves et al., 2016)*. Furthermore, CO_2 contained in media served as a pH buffer, boosting biomass productivity by increasing carbon species availability for absorption. In microalgae, lipid production takes place in the chloroplast. CO_2 from the environment is fixed in chloroplast as an endogenous source of Acetyl-CoA, which is then converted to carbons in the fatty acid chain *(De Bhowmick et al., 2015)*. Chlorella sp., Chlamydomonas reinhardtii, Nannochloropsis sp., Ostreococcus tauri, and Phaeodactylum tricornutum are the most promising microalgae for lipid synthesis and accumulation of triacylglycerol (TAG) *(Zienkiewicz et al., 2016)*.

Furthermore, carbohydrates can take many different forms in microalgae cells. For example, pectin, agar, and alginate in the outer cell, cellulose, hemicellulose in the inner cell, and intracellular products in the inner cell. Photosynthesis allows microalgae to collect monosaccharide glucose. Energy, proteins, lipids, and other carbs are all derived from glucose. When light intensity is too high or a nutrient is lacking (for example, nitrogen stress), glucose synthesis may outpace glucose intake. This much glucose may disrupt the cell's osmotic balance. As a result, the excess glucose was transformed into stored products like as polysaccharides and fat, which will serve as future carbon and energy sources. Glucose is transformed more quickly into carbohydrate first, followed by fat *(Ho et al., 2012)*. When microalgae are grown under ideal growth conditions, they collect mostly

carbohydrate and fats, which might be utilised as a biofuel feedstock. Carbohydrate is typically deposited first, followed by lipid. Operational conditions such as nutrition and light must be appropriately regulated to get a satisfactory microalga - based yield for improving bioenergy production *(Ota et al. 2009)*.

The majority of research on Capturing CO_2 by microalgae has been on reduced CO_2 levels (below 20 percent\). The effects of CO_2 concentrations ranging from 5 to 50% on microalgae growth rate and production of intracellular fatty acids were explored, and it was found that the fatty acid profile and content of microalgae changes greatly. One of the key advantages of employing microalgae to capture CO_2 is that the caught CO_2 does not need to be disposed of. Carbon fixed by microalgae is integrated into carbohydrates and lipids, which may be utilised to make chemicals, meals, or biofuels*(Yue and Chen 2005)*.

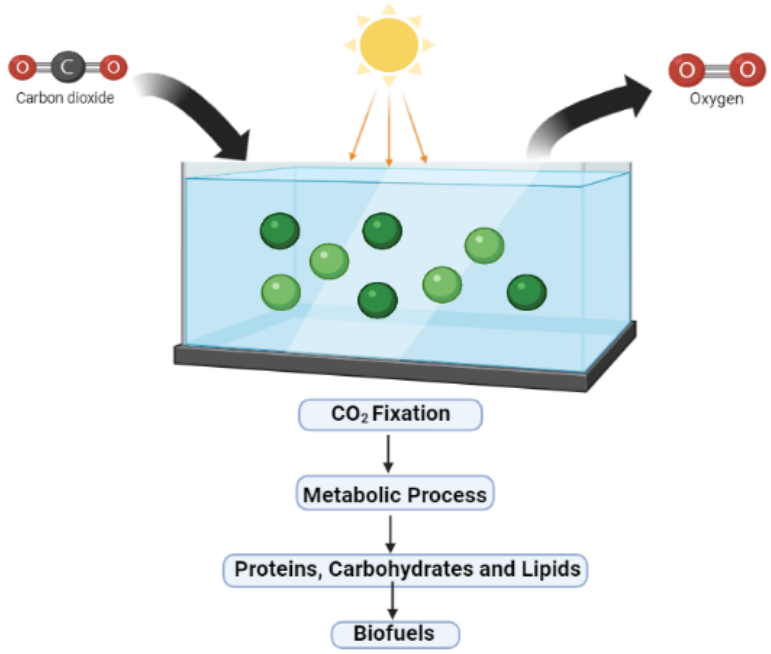

Fig 1: CO_2 Uptake by microalgae and sequestration

B. Carbonic anhydrase and its role in CO2 fixation

The zinc ion in the active site of carbonic anhydrase makes it a metalloenzyme. Carbonic anhydrase catalyses the conversion of CO_2 to bicarbonate and vice versa, a basic physiological process with quick dynamics and a turnover rate of up to 10^6 s^{-1} *(Lindskog et al., 1973; Supuran et al., 2016)*. With its quick kinetics of conversion of CO_2 to bicarbonate under ambient conditions with no external energy input, carbonic anhydrase can become a useful component in the development of efficient CCU technology *(Shanbhag et al., 2016;*

Zhang et al., 2017; Sahoo et al.,2018). Carbonic anhydrase catalyses CO_2 hydration which produces bicarbonate, which can be used as a carbon source for photosynthetic organisms' *(Mondal et al., 2016; Wang et al., 2017)* development as well as the production of formate *(Kim et al., 2017)*, inorganic carbonate *(Srikanth et al., 2018)*, and methanol *(Ji et al., 2015)*. Carbonic anhydrase has been widely investigated in microalgae cultivation, especially in the carbon increasing mechanism *(Swarnalatha et al., 2015)*. Microalgae use bicarbonate as a source of carbon for photosynthesis *(Mokashi et al., 2016)*, and carbonic anhydrase's rapid CO_2 hydrolysis kinetics allow for quick bicarbonate generation from CO_2 *(Schipper et al., 2013)*. Furthermore, applying exogenous carbonic anhydrase to algal growth has been shown to improve biomass growth and/or lipid yield *(Bozzo et al., 2000)*.

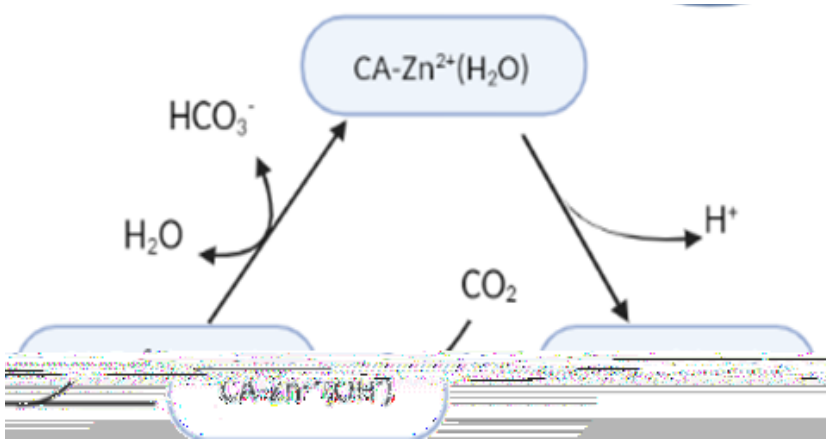

Fig 2: Mechanism of Carbonic Anhydrase

Although the coupling of carbonic anhydrase and microalgae in cultivation has the potential to contribute to the development of an efficient and sustainable biological CCU technologies, the poor stability and re - usability of free carbonic anhydrase typically makes this combination difficult *(Badarau et al., 2006)*.

Nanobiocatalytic techniques that use diverse nanomaterials for both enzyme immobilisation and stabilisation are one of the prospective options to achieve long-term stability and recycling applications of carbonic anhydrase. Because of their huge surface area per unit volume, tunable architectures, and unique physicochemical functions, nanostructured materials such as mesoporous materials, nanoparticles, nanotubes, and nanofibers are attractive candidates for enzyme immobilisation *(Kim et al., 2008)*. A simple and adaptable approach to nano biocatalysis is enzyme precipitate coating (EPC), which consists of

covalent enzyme attachment, enzyme precipitation, and cross-linking processes. Using various nanostructured materials, the EPC technique has been successful in immobilising and stabilising various enzymes such as glucose oxidase *(Kim et al., 2015)*, pyranose oxidase *(Kim et al., 2017)*, acylase *(Lee et al., 2017)*, lipase *(An et al., 2011)*, and carbonic anhydrase *(Hong et al., 2015)* for the prospective success in their practical applications.
One of the approaches for immobilization of CA is nanoflowers. Nanoflower is a newly developed class of nanoparticles having structural similarities to plant blossoms at the nanoscale scale of 100–500 nm *(Zhang et al., 2016; Ye et al., 2016)*. Synthetic approaches like precipitation, ionotropic gelation, and green synthesis are simple, non-toxic, and cost-effective ways to make nanoflowers *(Wang et al., 2014)*.

Cross linked enzyme aggregates are yet another approach for immobilization. It is an easy and economical technique that produces extremely stable biocatalysts and has a high immobilisation yield. The exquisite minimalism, tolerance to organic solvents, and exceptional robustness of CLEAs make them an ideal option *(Cui et al., 2015)*. The technique consists of two stages: (i) precipitating enzyme aggregated clumps in solution using precipitating agents; and (ii) cross-linking enzyme subunits in the aggregates to render them insoluble once the precipitating agent is removed. *(Peirce et al., 2017).* In the influence of the precipitating agent, enzyme precipitation should be as quick as appropriate in comparison to the enzyme denaturation reaction *(Shoevaart et al., 2004)*.

Understanding the link between dissolved inorganic carbon (DIC) concentration and microalgal development is crucial to improving CO_2 fixation efficiency. Microalgae's carbon source is a substantial component of their biomass, and the DIC formed by CO_2 given is their only carbon source *(Kim et al., 2014b).* As a result, *You et al., (2020)* showed CO_2 is quickly converted to bicarbonate (HCO^{3-}) by the CA complexes, resulting in an increase in DIC concentration, leading in fast *C. vulgaris* development. Over the course of a 7-day incubation, the (OD) of *C. vulgaris* was determined every day at 540 nm. *C. vulgaris* grew faster with CA complexes present than in the control group. *C. vulgaris* grew faster than the control group when the CA complex was present. The optical density of *C. vulgaris* 1with the CA complex was nearly 1.6 times greater than that of the control from day 3 to day 5, in the midst of the log phase, indicating fast development. The change in OD was roughly 1.3-fold on day 7 of culture, which was attributed to the oncoming stationary phase. CO_2 (aq), bicarbonate, or carbonate were used to convert the supplied CO_2 to DIC. The temperature,

pH, and quantity of all other salts all have an impact on DIC. *C. vulgaris* transfers DIC intracellularly to be used as a carbon source and grows at different rates depending on the CO_2 concentration. Previous research on the best CO_2 concentration for active *C. vulgaris* growth has found that increasing concentration of CO_2 leads to a higher DIC concentration and promotes lipid formation via increasing acetyl-CoA levels *(Jose & Suraishkumar, 2016)*. Because the concentration of DIC influences *C. vulgaris* growth greatly, increasing the initial DIC concentration is advised to boost *C. vulgaris* growth. The inclusion of NaHCO3 to this approach, on the other hand, may hinder *C. vulgaris* early development. Furthermore, adding more carbon sources creates a constraint since pH changes as concentration increases *(Jinsoo Kim and Ting, 2014; Kim et al., 2014a)*.

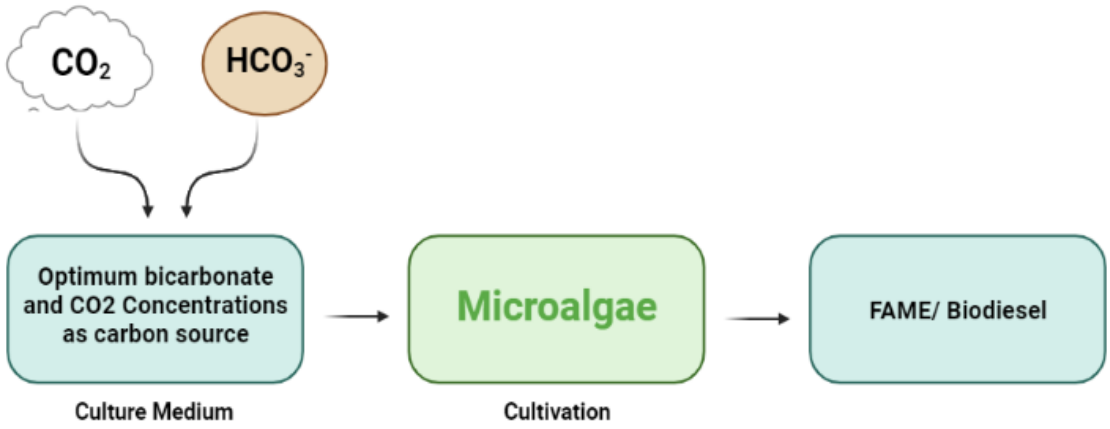

Fig 3: Utilization of $HCO3^-$ by microalgae

C. Value added products from enhanced Microalgal growth

A fairly broad category is high added value compounds. Lipids, proteins, and carbohydrates are used in food and nutraceuticals, while pigments and sterols are used in cosmetics and pharmaceuticals. Because microalgae are among the oldest living forms on the planet, they have a wide range of uses. Throughout long periods of time, they've developed and adapted, resulting in incredible diversity and complexity *(Levasseur et al., 2020)*.

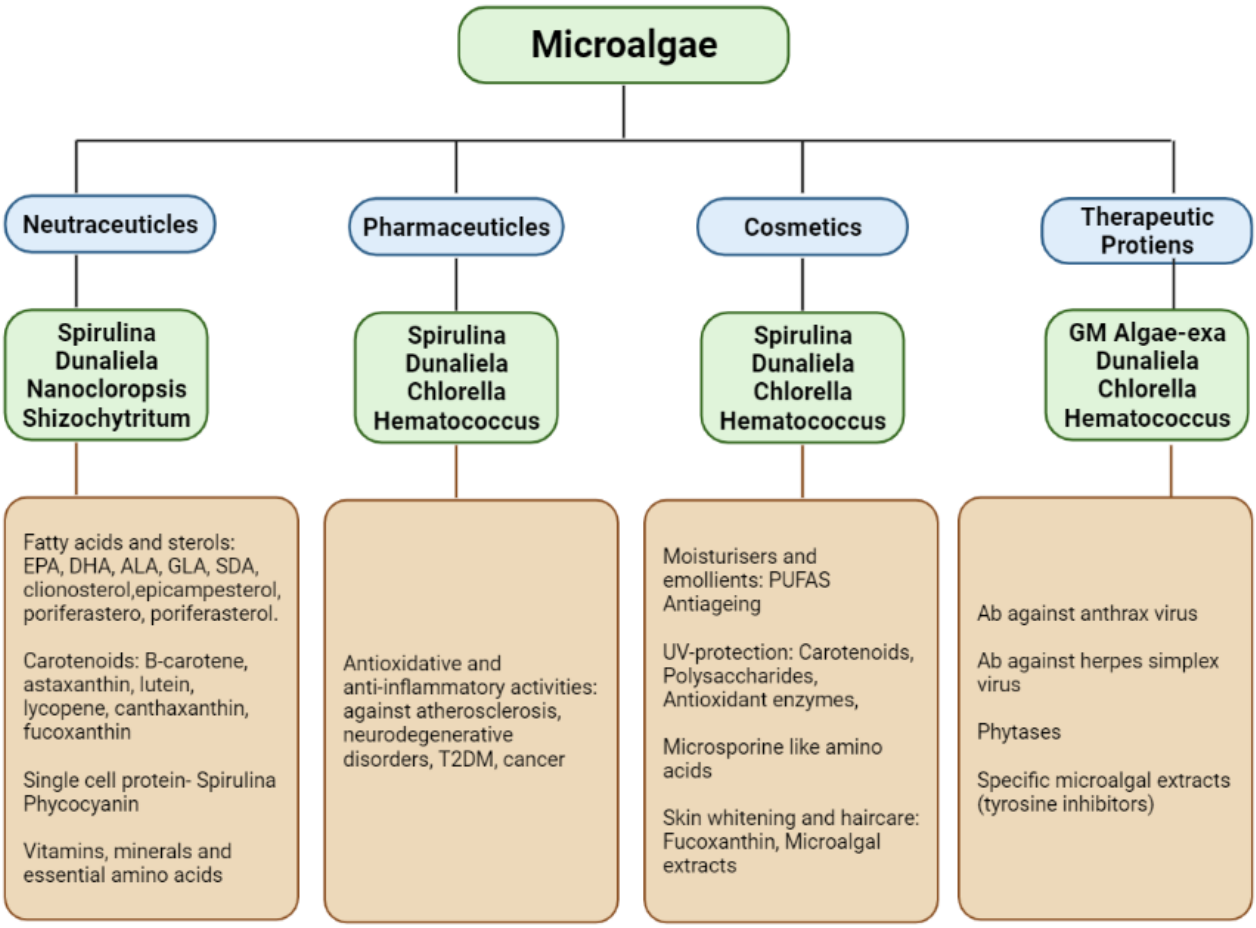

Fig 4: Value added products from microalgae and some applications

Below is the table showing some of the value-added products obtained from microalgae as well as their applications:

Table 1: Value added products from microalgae and their applications

Microalgal Species	**Growth rate (day^{-1})**	**Biomass production ($g\ L^{-1}\ day^{-1}$)**	**Product**	**Applications**
Scenedesmus almeriensis	0.34	0.42	Lutein	Used in Preventing age-related macular degeneration (AMD), cataracts, and the early stages of atherosclerosis, as well as certain

				cancers
Coelastrum species	0.22	0.281	Carotenoids	Bio-flocculation, food supplements, nutraceuticals and cosmetic applications.
Spirulina species.	0.50	1.00.	Fatty acids (palmitic acid and linoleic acid)	Biodiesel production
Chlorella vulgaris	1.38-2.81	0.4	Lipids, Proteins, Carbohydrate, Pigments, Vitamins and Minerals	Biodiesel, antibiotics, Food supplement, animal feed, bio-fertilizers, emulsifiers, food additives, pharmaceuticals, aquaculture feed, Therapeutic, pharmaceutical medicines and cosmetics.
Scenedesmus obtusiusculus	0.3 4	0.52	Carbohydrates, Proteins, Lipids	Biodiesel production, food additives
Desmodesmus sp.	0.96-1.32	0.939	Lipids and proteins	Biodiesel by transesterification and animal feed
Dunaliella sp.	0.25	0.12	Polar lipids, Caretenoids, Glycerol	Nutraceutical, cosmetic, feed applications, food additives.

Haematococcus pluvialis	1.00	0.28	Astaxanthin	Nutraceutical, supplementation in aqua and poultry industries.
Arthrospira Platensis	0.18-0.26	0.122	Natural and probiotic yogurt	Nutritional benefits and properties like water and oil-absorption, as well as the capacity to make and stabilize emulsions and foams.
Chaetocerosm.	1.59 ±0.12	0.243	Sterol	Hypo-cholesterolemia, anti-inflammatory, anticancer, and neurological disorders including Parkinson's disease are some of the positive health benefits.
Galaxauraelongata			Gold nanoparticles	Have antibacterial properties, detects microbial cells and their metabolites, bio-imaging of tumor cells, helps with the study of endocytosis
Desmodesmus abundans	0.357–0.447	0.164-0.183	Silver nanoparticles	Used in antimicrobials, antioxidants, drug delivery, chemical sensing and cosmetics
Nannochloropsis oculata	0.5430	0.856	Pepha-Tight	Has skin tightening properties
Dunaliellasalina	0.26-0.42	0.06-0.19	Pepha-Ctive	Promotes cell growth, enhance the energy metabolism of skin

Conclusion Note

CO_2 sequestration by microalgae is now a viable technology for increasing the synthesis of useful algal biomass while minimizing greenhouse gas emissions. Using a high-value by-product method, microalgal biofuel synthesis may be made more cost-effective. Future advances in microalgae - based biofuel production technology, as well as market factors, might make biofuel production financially viable. Because of its specificity and effectiveness, and the fact that it is ecologically friendly, CA is a key enzyme in this system. The main drawbacks of using free CA in commercial uses on a widespread basis are that it is rigid, expensive, and difficult to recover from reaction conditions, rendering it non-reusable. Carbonic anhydrase immobilization is a solution to this situation.

Enzyme immobilisation techniques also increase enzyme stability and catalytic properties while also speeding up downstream processing. Adsorption, encapsulation, covalent bonding, cross-linking and entrapment are some of the basic immobilisation techniques that underpin all advanced immobilisation techniques. Carbonic anhydrase immobilisation solutions presented in this study included CA CLEA, CA hybrid nanoflowers, CA complexes, and CA EPC all of which enhanced the effectiveness and process of carbonic anhydrase to transform CO_2 to bicarbonate, which was then utilised by microalgae for faster development. Improved microalgae growth, as indicated in the review, might be used to make a number of value-added products, such as cosmetics, pharmaceuticals, nutraceuticals, nanomaterials, and biofuels.

Chapter 3

Materials and Methods

1. **Aim**: Reviving the culture of *Corynebacterium flavescens*

Materials required:

- Nutrient Agar Media
- Isopropanol
- p-nitrophenyl acetate (p-NPA)
- Agar

Theory

Corynebacterium is indeed a Gram-positive bacterium with aerobic metabolism. They are rod-shaped meaning bacilli, and at some stages of life, can be club-shaped, which gave rise to the genus name. All coryneform bacteria contain high G + C concentrations and are just far-off related, making basic phenotypic tests hard to distinguish between them. Alternatively, their cell walls should be examined for the kind of peptidoglycan contained, comprising diamino acid and acyl types, as well as sugars, menaquinones, fatty acids, and polar lipids. Chemotaxonomy is the term for this method of identification, which is time-consuming when dealing with a huge variety of species. Chemotaxonomic approaches have been replaced by molecular techniques in the current taxonomy.

The substrate p-nitrophenyl acetate has been utilized in esterase and lipase activity investigations. It acts as the substrate for bacteria to produce carbonic anhydrase.

Methodology

- Weigh 5.6 g of Nutrient Agar Media.
- Add 200 ml of water.
- Add 2% agar (4g) for 5.6 g of Nutrient Agar Media.
- Add 5 ml of p-nitrophenyl acetate into it.
- Keep it for autoclave.
- Pouring is done in Petri plates.

2. Enzyme assay

2.1 **Aim**: Seed culturing for enzyme production and extraction

Material Required

- Falcon Tubes
- Inoculation Loop
- Bacterial Culture Plates
- Nutrient Agar Media

Methodology

- In laminar airflow bacterial culture plates, media, and falcon tubes were taken and in 31 falcon tubes, 5 ml of media was poured.
- Then from 4 unknown bacterial sample plates which were divided into 31 different sections, using an inoculation loop, the colony was picked and in 31 falcon tubes, the different strain was dissolved in them.
- These were then placed in a shaker incubator for 24 hours for proper mixing at 40°C.

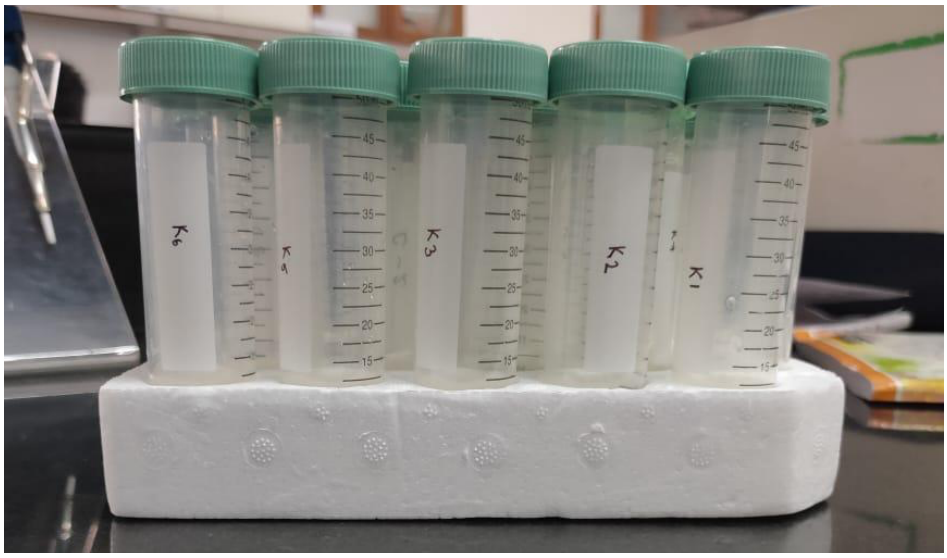

Fig. 8: Falcon tubes containing media and unknown bacterial samples

Fig 9: Falcon tubes placed in shaker for 24-hour period

2.2 **Aim**: Production media preparation and inoculation

- Making Nutrient Broth: Take 8.4g Nutrient Broth and add 300 ml water.
- 10 ml nutrient broth was taken and from the previous culture, 1 ml of culture was taken.
- These were then placed in a shaker incubator at 120 rpm for 24 hours for proper mixing at 40°C.

Fig. 10: Media inoculated with culture placed in the incubator shaker

2.3 **Aim**: To perform enzyme extraction

- These 31 falcon tubes were then centrifuged at 8000 rpm for 10 minutes.
- The supernatant was then transferred to 15ml falcon tubes and was kept at 4°C.
- Falcon carrying pellet was left undisturbed and was kept in ice.

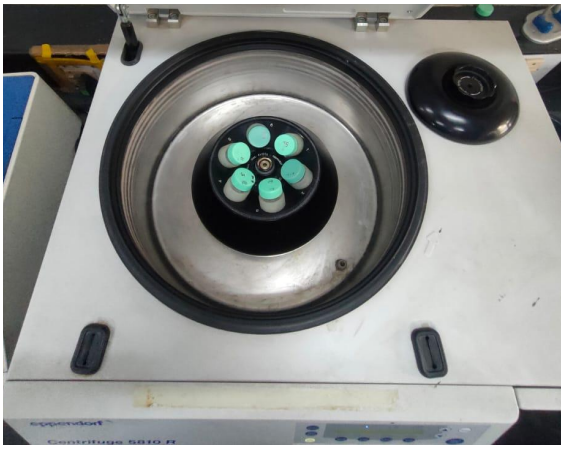

Fig. 11: Centrifugation performed at 8000 rpm

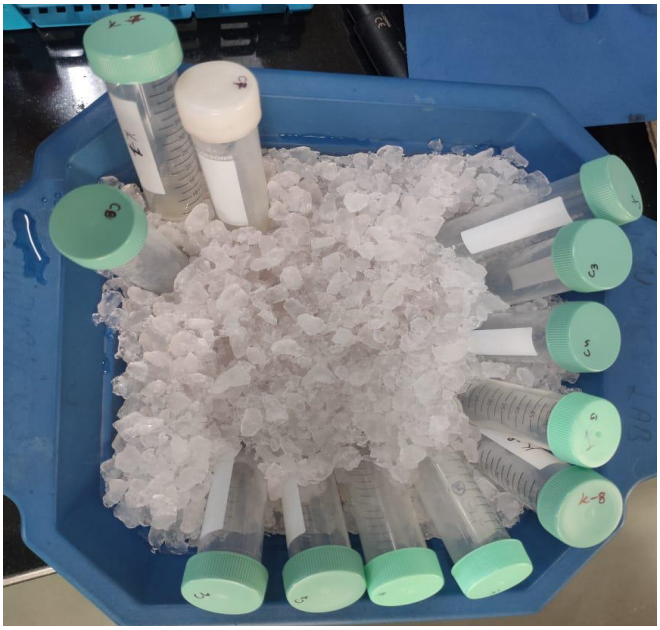

Fig 12: Falcon with pellet kept in ice undisturbed

2.4 **Aim**: To check the presence of the extracellular and intracellular enzymes.

- Next to check the presence and concentration of enzyme extracellularly, p-NPA as a substrate for the enzyme was prepared.
- Tris buffer of pH 8 was prepared.
- 31 vials were taken and 750 µl buffer + 250 µl p-NPA and 20 µl of the enzyme were added to it.
- It was incubated at 30°C for 5 minutes in dark.
- Next OD was taken at 460 nm.
- To check the presence of enzyme intracellularly, sonication was carried out and the same procedure was repeated for supernatant.

3. Aim: Collection of Algal Sample and preparation of growth media BG11

Material Required

- Falcon tubes
- Spatula
- Various stock solutions and chemicals (Na_2CO_3, $NaNO_3$) for the preparation of BG11
- Algal Sample

Theory

BG11 Broth is an all-purpose media for growing and maintaining most algae (usually blue-green algae). Photoautotrophic algae thrive in this BG11 media. They need light to function. This media is made up of synthetic carbon and nitrogen sources, as well as various inorganic salts. For the production of algae, an illuminance of 2,000 to 3,000 lux is ideal. Subjecting to neon light for a duration of 24 hours a day is ideal for maintaining the algae. A lubricant proof sheet is frequently used to cover incubation flasks. They thrive at temperatures between 20 and 25 degrees Celsius.

Methodology

- Algal sample was collected from a nearby water source in 2 falcon tubes using a spatula.
- Next BG11 was prepared for the growth of the collected algal sample.

Preparation of BG11:

I. Stock-1 (g/l):

- EDTA Na_2H_2O - 0.1
- Ferric Ammonium Citrate- 0.6
- Citric Acid.H_2O- 0.6
- $CaCl_2.2H_2O$- 3.6

II. Stock-2 (g/l)

- $MgSO_4.7H_2O$- 7.5

III. Stock-3 (g/l)

- K_2HPO_4- 3.05

IV. Stock-4 (g/l)

- H_3BO_3- 2.86
- $MnCl_2.4H_2O$- 1.81
- $ZnSO_4.7H_2O$- 0.222
- $CuSO_4.5H_2O$- 0.79
- $CoNO_3$- 0.050
- $NaMoO_4.2H_2O$- 0.391

For the preparation of BG11, the following amount of stock solution and other chemicals were taken:

- Stock 1- 10 ml
- Stock 2- 10 ml
- Stock 3- 10ml
- Stock 4- 1 ml
- Na_2CO_3- 0.02 g/l
- $NaNO_3$- 1.5 g/l

4. Aim: Preparing BG11 plates and streaking

Material Required

- BG11 Media
- Algal sample
- Petri Plates

- Inoculating loop

Methodology

- In LAF the BG11 media was poured into Petri plates.
- These were left to solidify.
- The collected algal sample was taken and using inoculation loop streaking was performed in laminar airflow.

5. Aim: Preparing slides of obtained algal sample and viewing under a microscope.

Material Required

- Algal sample
- Microscope
- Glass Slide
- Coverslip

Methodology

- Obtained algal sample was kept in a flask which was then left undisturbed for 24 hours.
- About 10 μl of the above green floating microalgae was extracted, and placed on a clean glass slide which was then covered with a coverslip.
- The slide was then viewed under the microscope at 100X using emulsion oil.

Results and Discussion

Experiment 1

Observation

Yellow bacterial colonies were observed on the Petri plates.

Fig 5: NA Media

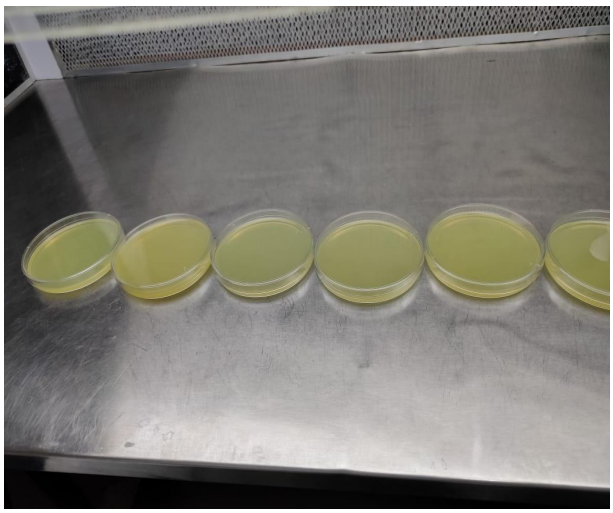

Fig. 6: Pouring Media in LAF

Results

The appearance of yellow colonies indicates carbonic anhydrase presence.

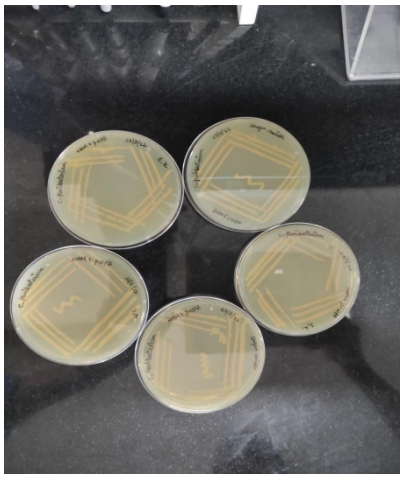

Fig. 7: Results after incubation

Experiment 2

Observation and Results

The enzyme was obtained intracellularly.

Experiment 3

Observations

Fig 13: Collection of algal samples from a nearby water source

Results

- Algal sample was obtained successfully.
- Algal growth media i.e. BG11 was prepared successfully.

Experiment 4

Observations and Results

Algal sample growth was observed on the Petri plates containing BG11 growth media.

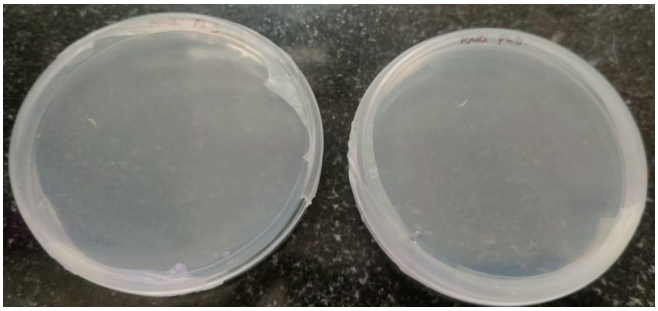

Fig 14: Petri plates containing BG11 media

Experiment 5

Observation and Results

Under microscope various algae were observed on the placed slide.

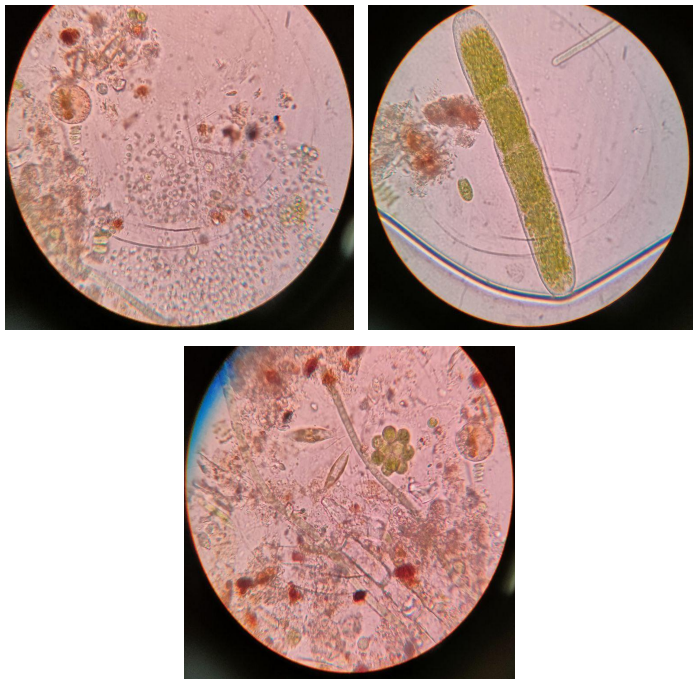

Fig 15: Various algae were observed under microscope at 100X

In the images above following algae were seen:

- Small crescent ones are *Selenastraceae*.
- *Scenedesmus sensu lato*
- *Desmodesmus*
- *Uronema*
- *Chlorella*
- *Chlamydomonas*
- *Diatoms*
- Big crescent shaped are *Closterium*

Discussion

This report contains the work carried out in the lab in alignment with the review of literature. The results obtained were: (i) The appearance of yellow colonies while reviving the culture of *C. flavescens* indicates carbonic anhydrase presence. (ii) The enzyme assay was done and the enzyme was obtained intracellularly, (iii) Algal sample was obtained successfully and algal growth media i.e., BG11 was prepared successfully, (iv) Algal sample growth was observed on the Petri plates containing BG11 growth media, (v) Under microscope various algae were observed on the prepared slides of isolated microalgae.

The media employed in reviving Corynebacterium flavescens was Nutrient agar, whereas in an experiment conducted the media used in growing Corynebacterium was Tinsdale agar, that includes H2S markers L-cysteine and sodium thiosulfate *(Todar, 2008)*. Again, seed culturing media used in the experiment was Nutrient agar media while research publications used Hestrin–Schramm (HS) medium (*Schramm et al., 1957*) and corn steep liquor and molasses medium for bacterial seed culture in several tests (*El-Saied et al., 2008*).

As compared to our culturing of microalgae on BG11 Medium, *Llyod et al., 2021* showed that, microalgae were isolated using BBM, a standard chemically defined medium. It is primarily of ecological, biotechnological, and commercial importance to isolate, cultivate, and identify microalgae native to a given habitat *(Mobin et al., 2002)*. Random sampling was used to collect surface water samples in pre – sterilized jars from publicly available inland water sites (n = 31) in the north, Centre, east and west zones. Pellets were crushed,

washed, and resuspended in sterile Bold basal medium (BBM) broth, which was serially diluted until transparent. The loopful from the past three clear aliquots were microscopically screened for the presence of algae and grown on BBM agar using a spread-plate. For 24 hours, triplicate isolation samples were incubated at 23°C with a tube light source. 2–2.5 percent agar was used to prevent swarming contamination. Isolates were subcultured until they were completely pure. Long-term supplies were kept in 15 percent glycerol-BBM at 20 °C, whereas short-term stockpiles were kept in BBM slants *(Lloyd et al., 2021)*.

Conclusion

The looming impact of global warming and industrialization is increasingly visible in the global landscape. Carbon dioxide, the most significant GreenHouse Gas (GHG), has risen to dangerously high amounts. While co2 capture and storage (CCS) systems have indeed been extensively researched, these are inefficient in respect to cost, and its protracted environmental protection is a problem. Bio-sequestration of carbon dioxide employing microalgal cellular manufacturers, on the far extreme, have been identified as a potential technique of recovering CO2 into biomass through photosynthetic processes that may then be employed to generate biofuels and certain other significant products. Thereby, microalgae has gotten a lot of interest for CO_2 sequestration and biofuel generation since they can collect and transform CO_2 to algae biomass at considerably greater levels than traditional cellulosic biofuels. Biological fixing of CO_2 might just be the very efficient carbon process here on the planet. The majority of previous research on biologically based carbon capture using microalgae has focused on reduced CO_2 levels (<20 percent). There seems to be a few research on the effects of elevated CO_2 concentrations (>20 percent) on microalgae cultivation, CO_2 biological fixation efficiency, and fatty acid profile. Even as the worldwide CO_2 level rises, it is critical to concentrate on this sector. Apart from this, microalgae may be used as cost-effective bioreactors for manufacturing high-value-added new physiologically active chemicals, and they can thrive on low-cost materials and many other value-added products. Furthermore, as the numbers grow, so does the requirement for nutritious foods and medical goods. Algae are a major contender as well as the greatest necessary nutrients due to their rapid development rate and extensive variety of health advantages. The majority of the investigations which have already been published thus far have been carried out on bench size equipment in tightly restricted environments. Several elements, including the availability of sufficient CO_2, micronutrients, and lighting, must be explored and improved in order to use the optimal parameter circumstances in industrial biofuel synthesis.

Chapter 6

Summary

This project report represents our work on the topic “Cultivation and screening of microalgal samples for enhanced growth using enzymatic treatment”. It contains the review of literature for the topic as well as the experiments carried out in the lab related to same. The objectives carried out in the lab were: (i) Reviving the culture of Corynebacterium flavescens, (ii) Enzyme assay, (iii) Collection of Algal Sample and preparation of growth media BG11, (iv) Preparing BG11 plates and streaking, (v) Preparing slides of obtained algal sample and viewing under a microscope. The results were obtained accordingly as mentioned in the above part of the report. The outcomes of the major project are: (i) Understood the characteristics of different microalgal species. (ii) Learned about the mechanism and immobilization strategies for Carbonic Anhydrase, (iii) Understood how microalgae captures and utilizes the carbon dioxide and bicarbonate ions for enhanced growth (iv) Learned about the valu,e-added products that are obtained from different microalgal species,

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