NANOPARTICLE MEDIATED WATER SPLITTING IN DARK FOR MICROBIAL GROWTH: PROOF OF CONCEPT

M.Sc. Thesis

2024

For partial fulfilment of the requirements for

the Master of Science in Biotechnology

Submitted to

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Submitted by

Alisha Nepali

Roll No: BT 602/075

TU Regd No: 5-2-37-1316-2014

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Supervisor

Dr. Pramod Aryal

Senior Scientist

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Tribhuvan University
CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Kirtipur, Kathmandu, Nepal

Central Department of Biotechnology
T. U.
Kirtipur-2064

Date: 22 April 2024

Recommendation

This is to certify that the research work entitled **"NANOPARTICLE MEDIATED WATER SPLITTING IN DARK FOR MICROBIAL GROWTH: PROOF OF CONCEPT"** has been carried out by Ms. Alisha Nepali under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is her original findings. I hereby recommend this thesis for final evaluation.

Dr. Pramod Aryal
(Supervisor)
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu
Nepal

Tribhuvan University

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Kirtipur, Kathmandu, Nepal

Date: 22 April 2024

Certificate of Evaluation

This is to certify that the thesis entitled **"NANOPARTICLE MEDIATED WATER SPLITTING IN DARK FOR MICROBIAL GROWTH: PROOF OF CONCEPT"** presented to evaluation committee by Ms. Alisha Nepali is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

Prof. Dr. Krishna Das Manandhar
(Head of Department)
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu
Nepal

Assoc. Prof. Dr. Parash Mani Timilsina
(External Examiner)
Department of Biotechnology
Kathmandu University
Dhulikhel, Kavre
Nepal

Asst. Prof. Dr. Jarina Joshi
(Internal Examiner)
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu
Nepal

Dr. Pramod Aryal
(Supervisor)
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu
Nepal

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Alisha Nepali

Registration No: 5-2-37-1316-2014

ACRONYMS

FTIR	Fourier Transform Infrared Spectroscopy
LB	Luria-Bertini Broth
MR	Methyl Red
M	Molar
MFC	Membrane Fuel Cell
NCF	Nitrogen Carbon Free
NFB	Nitrogen Free Broth
OER	Oxygen Evolving Reaction
PS I	Photosystem I
PS II	Photosystem II
W/V	Weight By Volume
XRD	X-Ray Diffraction
HER	Hydrogen Evolving Reaction
BNF	Biological Nitrogen Fixation

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Abstract

The escalating global population directly correlates with an increased demand for agricultural products. Currently, fossil fuels are the primary energy source due to their accessibility, abundance, and high energy content. By 2050, global energy consumption, which was 17 terawatts (TW) in 2013, is projected to reach 34 terawatts (TW). Fossil fuel combustion is the primary source of CO_2 emissions, releasing approximately 36 billion tonnes annually. These emissions, along with other greenhouse gases like methane and nitrous oxide, exacerbate global warming and climate change. To mitigate high atmospheric CO_2 levels, various policies, technologies, and mechanisms have been proposed. Among these, CO_2 capture and storage (CCS) is a crucial technology. Biological carbon sequestration, particularly the conversion of CO_2 into biofertilizer, is a promising approach. A bacterial consortium, including extracellular electron transfer bacteria such as *Geobacter* spp., diazotrophic *Azotobacter* spp., *Pseudomonas* spp., and diazotrophic autotroph *Azospirillum* spp., was isolated based on Carbon Catabolite Repression (CCR). This consortium can fix atmospheric nitrogen in its reduced form, confirmed by Nessler's test and quantified. Additionally, the consortium can fix atmospheric CO_2 into its reduced form, confirmed by Molisch's test and Anthrone test, and further quantified using the DNS method. Cobalt nanoparticles were synthesized and characterized by FTIR and XRD analysis and employed as a suspension within the consortium to augment the water-splitting mechanism, making electrons and protons available in the media. The introduction of these nanoparticles enhanced the water-splitting mechanism, even in the dark, as confirmed by the optimal growth of the bacterial consortium compared to the consortium without nanoparticles. This technology can be further developed and optimized to address global warming through effective carbon and nitrogen fixation. Additionally, it offers a sustainable alternative to urea in agricultural applications, contributing to more sustainable agricultural practices.

Keywords: *CO2 Capture and Storage (CCS), Biological Carbon Sequestration, Bacterial Consortium, Carbon Catabolite Repression (CCR), Cobalt Nanoparticles, Water-Splitting Mechanism, Microbial Fuel Cell (MFC), Sustainable Agriculture*

Chapter 1
Introduction

1.1 Background

The escalating global population, currently at approximately 7.9 billion, is set to reach nearly 10 billion within the next 50 years. This upward trend directly correlates with an increased demand for agricultural products, presenting a substantial challenge in feeding the growing population. As the world's populace continues to expand, ensuring food sufficiency for the current and future generations becomes an imperative task (Tomlinson, 2013). At present, fossil fuels serve as the primary source of energy due to their accessibility, abundance, and high energy content. By 2050, global energy consumption, which stood at 17 TW in 2013, is projected to double at least, as indicated by (Graham & Hammer 2012; Moniz *et. al*., 2015). Fossil fuel combustion, particularly from coal and petroleum, is the primary source of carbon-dioxide (CO_2) emissions, with approximately 36 billion tonnes released annually. These emissions, coupled with greenhouse gases like CO_2, methane, and nitrous oxide, exacerbate global warming and climate change. Addressing the elevated CO_2 levels in the atmosphere is a significant global challenge, garnering considerable focus from the scientific sector, prompting the search for effective mitigation strategies (Sarkar *et. al*., 2023; Graham & Hammer, 2012).

Various strategies are being globally pursued to mitigate the high atmospheric CO_2 levels, including policies, technologies, and mechanisms. Among these, CO_2 capture and storage (CCS) emerges as a crucial technology addressing the issue. Biological carbon sequestration, particularly the conversion of CO_2 into biomass, holds promise as a sustainable solution for a cleaner environment. Plant photosynthetic process is widely acknowledged as an effective means of sequestering atmospheric CO_2, with certain plant, macrophyte, and algae species identified as efficient CO_2 fixers (Sarkar *et. al*., 2023; Gough, 2008).

In nature, green plants and photosynthetic bacteria play a crucial role in converting CO_2 into oxygen and energy sources through photosynthesis. To transition from fossil fuels to sustainable alternatives, researchers are investigating photovoltaic technologies to harness sunlight for generating the necessary energy, thus facilitating the conversion of recycled CO_2 into fuel materials essential for growth. The photocatalytic reduction of CO_2 with H_2O is a significant reaction, resembling artificial photosynthesis, aiming at carbon storage. Initially conducted using TiO_2 and $SrTiO_3$ as photo-catalysts, the process yielded principal products

like HCOOH and HCHO, with trace amounts of CH_3OH and CH_4. Various semiconductors activated by lamp irradiation, including WO_3, TiO_2, ZnO, CdS, GaP, and SiC, have been employed for photocatalytic CO_2 reduction, albeit with lower efficiency due to thermodynamic and kinetic challenges (Fechete & Vedrine, 2015; Ikeue *et. al*., 2001; INOUE *et. al*., 1979; Navalón *et. al*., 2013). Cobalt catalysts are essential in industrial processes like Fisher-Tropsch synthesis and CO_2 conversion. Electrocatalytic CO_2 reduction offers a promising method for producing important chemicals and fuels using renewable electricity, operating at neutral pH, room temperature with low consumption in energy. Cobalt's outstanding attributes, such as high electrical conductivity, chemical and thermal stability, and strong catalytic performance, make it an ideal candidate for this process. (Usman *et. al*., 2021).

The Calvin-Benson-Bassham cycle is the predominant pathway for CO_2 fixation, pivotal in regulating atmospheric CO_2 levels, operating across various organisms from bacteria to green plants. At its core, RuBisCo, the most abundant protein globally, facilitates the critical step in CO2 reduction. As a multifunctional enzyme, RuBisCo governs both CO2 reduction and ribulose-1,5-bisphosphate oxygenation, linking it to nearly all primary production processes. Given its immense agricultural and environmental importance, RuBisCo is extensively studied (Raven, 2013; Selesi *et. al*., 2005).

We develop the syntrophic growth containing different bacteria and cobalt nanoparticle in a media with free carbon and nitrogen source and without any light forms including sunlight. The reduced nitrogen and carbon sources were further used to make biofertilizer and biohydrogen.

1.2 Hypothesis

1.2.1 Null Hypothesis (H_0):

The nanoparticle based water splitting cannot enhance the microbial growth in dark.

1.2.2 Alternative Hypothesis (H_1):

The nanoparticle based water splitting can enhance the microbial growth in dark.

1.3 Objectives

1.3.1 General objectives

To mediate the water splitting for microbial growth in dark by using the synthesized cobalt nanoparticle.

1.3.2 Specific objectives

- To revive the four different strains of bacteria
- To perform the biochemical tests and grow those particular bacteria in their selective medium
- To detect the presence of reduced carbon source in nitrogen carbon free (NCF) culture media
- To perform single vessel syntrophic growth of those consorts of bacteria
- To develop microbial electrochemical synthesis to augment CO_2 reduction
- To develop water splitting mechanism to augment CO_2 reduction
- To synthesize the cobalt nanoparticles
- To study the role of cobalt nanoparticles in carbon reduction experiments

1.4 Rationale and scope of the study

The significant rise in global energy consumption, driven by population growth and increased lifestyle standards, has predominantly relied on fossil fuels, which contribute to approximately 90% of the world's energy supply. This heavy reliance, notably in transportation and industry, leads to significant emission of greenhouse gases, encompassing carbon dioxide (CO2), nitrous oxide, methane, and fluorinated gases. Recent reports indicate an annual emission of 36 billion

tonnes of CO_2, equivalent to 9.8 billion tonnes of carbon, released into the atmosphere. This highlights the pressing need for measures in addressing environmental impact of fossil fuel usage and reduce greenhouse gas emissions. The increased atmospheric CO_2 levels pose a significant global challenge. Various strategies must be explored to mitigate CO_2 emissions. These include adopting new green technologies that replace fossil fuels with renewable energy sources, employing physical, chemical and biological methods to capture and store CO_2, and converting CO_2 into diverse chemical outputs and fuels. Addressing this issue requires a multifaceted approach in mitigating effects of CO_2 on the environment and combat change in climate effectively (Sarkar *et. al*., 2023).

Chapter 2

Literature Review

2.1 Autoionization of water

Water serves as a versatile solvent in various chemical processes, capable of acting as both an acid and a base. In aqueous solutions, water undergoes self-ionization to form hydroxide (OH^-) and hydronium (H_3O^+) ions, often adopting Eigen- or Zundel-like structures.

$$(2H_2O \rightleftharpoons H_3O^+ + OH^-)$$

The proton in water is integral to its composition, stemming from the autoionization process. This autoionization significantly influences water's properties, facilitating charge transport in biological and electrochemical processes. Research on protons in bulk water reveals their dynamic nature, similar to the hydrogen-bonded network of water molecules (Das *et. al*., 2020; Moqadam *et. al*., 2018).

In liquid water, protons and hydroxide ions exhibit the highest diffusion rates among ions. The speed of proton movement surpasses that of water molecules by a factor of approximately four, whereas hydroxide ions exhibit a mobility roughly 60% slower than that of protons in the overall water environment. More than two centuries ago, Von Grotthuss postulated that the movement of these ions occurs via the conveyance of structural or topological imperfections through successive proton transfer reactions (Bankura & Chandra, 2015).

The intricate network of hydrogen bonds greatly influences the structural and dynamic properties of water. Normally, each water molecule engages in four hydrogen bonds, serving as both a donor and acceptor, thus achieving a coordination number of 4. However, variations from this ideal tetrahedral arrangement, such as under- or over-coordination of water molecules, are considered crucial for dynamic processes like transport within liquid water. These deviations enhance the adaptability and intricate nature of water's behavior (Loparo *et. al*., 2004; Markovitch & Agmon., 2008).

2.2 Photosynthesis

Chloroplasts, originating from a single ancient photosynthetic cyanobacterium absorbed by a eukaryotic cell over a billion years ago, are fundamental for converting solar energy into essential chemical energy, supporting plant growth and life on Earth. These cellular structures serve as the primary storehouse of chemical energy, housing crucial metabolic processes like photosynthesis, which are indispensable for sustaining life (Timmis *et. al*., 2004; Johnson,

2016). Photosynthesis, a meticulously regulated process, encompasses multiple stages: capturing solar energy, conveying excitation energy, converting it, and transferring electrons from water to NADP+. This sequence generates ATP and initiates enzymatic reactions that assimilate carbon dioxide into carbohydrates. It orchestrates the transformation of sunlight into chemical energy, vital for sustaining life on our planet. (Tanaka & Makino, 2009).

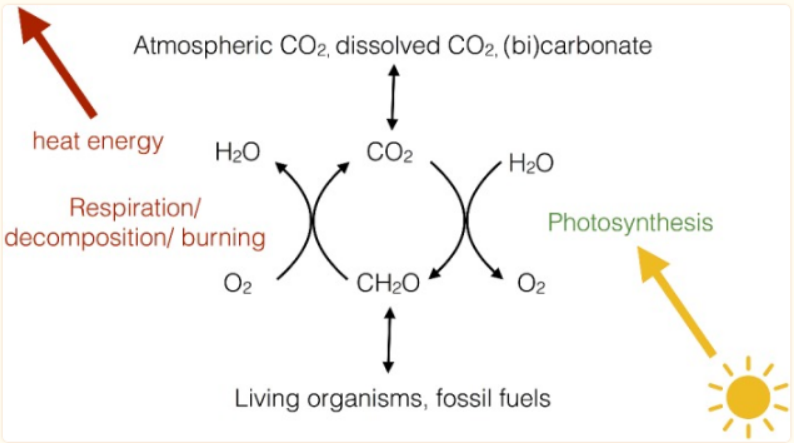

Fig 1: The interconnection among respiration, photosynthesis, and the levels of atmospheric CO_2 and O_2 on a global scale (Johnson, 2016).

Photosynthesis begins with two main stages: the light reactions and the dark reactions. In the light reactions, Photosystem II (PS II), located in the thylakoid membrane, uses light energy to split water molecules, generating oxygen and converting plastoquinone to plastoquinol. Plastoquinol then transfers electrons to cytochrome b6f (cytb6f), which subsequently reduces plastocyanin. This process is crucial for the production of energy-rich molecules in plants. Meanwhile, in the dark reactions, CO_2 fixation occurs via the Calvin-Benson cycle in the stroma. Following this, Photosystem I (PSI) oxidizes plastocyanin, reducing ferredoxin, and subsequently, ferredoxin reduces $NADP^+$ to NADPH through ferredoxin-$NADP^+$ reductase (FNR). The proton gradient created during water splitting and plastoquinol oxidation drives ATP synthesis by ATP synthase, similar to oxidative phosphorylation in mitochondria (Johnson, 2016).

2.3 Types of plants

C3, C4, and CAM represent three distinct carbon fixation pathways in plants. In C3 plants, carbon dioxide enters through stomata and intercellular spaces, where carbonic anhydrase converts it to bicarbonate (HCO_3^-) in the aqueous environment. This enzyme aids in maintaining CO_2 levels for RuBisCo, facilitating the carboxylation of RuBP to PGA in the chloroplast. ATP and NADPH from thylakoid membrane electron transport fuel sugar and

starch production, along with RuBP regeneration in the Calvin-Benson cycle (Price *et. al*. 1994). C_3 plants, constituting 95% of plant species, thrive in cooler, wetter conditions and conduct photosynthesis only with open stomata, leading to higher photorespiration levels. However, their photosynthetic efficiency is generally lower than C_4 plants under standard environmental conditions (Boretti & Florentine, 2019).

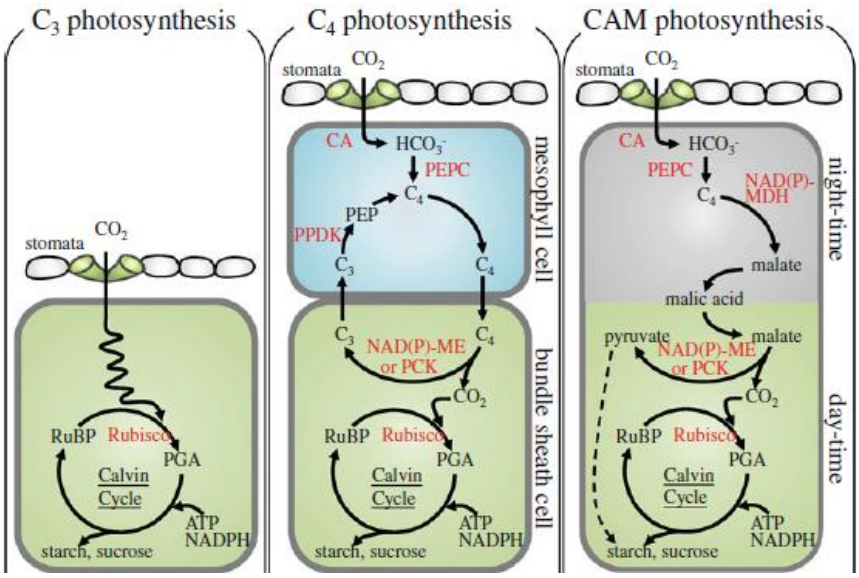

Fig 2: Photosynthesis reaction of C_3, C_4 and CAM (Yamori et. al., 2014).

2.3.1 C_4 plant

C_4 photosynthesis, as explained by Furbank and Hatch (1987) and Jenkins et al. (1989), employs an intricate biochemical process to concentrate carbon dioxide (CO_2) around RuBisCo within bundle sheath cells, exceeding ambient levels. This involves converting CO_2 into bicarbonate and then incorporating it into oxaloacetate (OAA) via phosphoenolpyruvate carboxylase (PEPC) in the cytosol, utilizing phosphoenolpyruvate (PEP). OAA is subsequently either transformed into malate or aspartate, depending on the type of C_4 photosynthesis, which include NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PCK). Malate (or aspartate) is then transported to bundle sheath cells, where it undergoes decarboxylation, releasing CO_2 for RuBisCo fixation, working in conjunction with the Calvin cycle. C_4 plants, making up roughly 5% of plant species, flourish in dry, high-temperature environments, displaying efficient photosynthesis even when stomata are closed.

2.3.2 CAM

CAM photosynthesis employs a unique CO_2 concentrating mechanism, requiring a temporal separation of C_3 and C_4 processes within the same cellular environment. It operates through four phases: nocturnal CO_2 uptake via stomata, followed by CO_2 fixation, malate synthesis, and its storage in vacuoles during phase I; a transition phase at dawn with open stomata for CO_2 uptake (phase II); decarboxylation of malic acid and CO_2 refixation by RuBisCo behind closed stomata (phase III); and another transition phase at dusk with reopened stomata for CO_2 uptake (phase IV). CAM plants, categorized into NAD(P)-ME and PCK types based on malate decarboxylation, capitalize on nocturnal CO_2 uptake when evapotranspiration rates are low, achieving significantly higher water use efficiencies compared to C_4 and C_3 species (Dittrich *et. al*. 1973, 1976). Only a small fraction of plant species employ the CAM photosynthetic pathway.

2.4 RuBisCo

RuBisCo, an essential enzyme in the global carbon cycle, is widely recognized as one of the most prevalent proteins in nature, having originated roughly 3.5 billion years ago. Presently, it accounts for a substantial portion, approximately 95%, of the fixed carbon in the biosphere. Despite its abundance, RuBisCo's efficiency is limited, with only a modest number of CO_2 molecules being fixed per second, typically ranging from two to ten. This inefficiency largely stems from the active sites of RuBisCo being either decarbamylated or obstructed by intrinsic inhibitors, hindering its ability to bind substrates effectively for catalysis (Erb & Zarzycki., 2018). It is a crucial enzyme found in plants, functioning in both carbon fixation within the Calvin cycle and in photorespiration, depending on its interaction with carbon dioxide or oxygen. As a key player in photosynthesis, RuBisCo initiates the process of carbon dioxide conversion into organic compounds, serving as the rate-limiting step in the Calvin cycle (Udenigwe *et. al*., 2017).

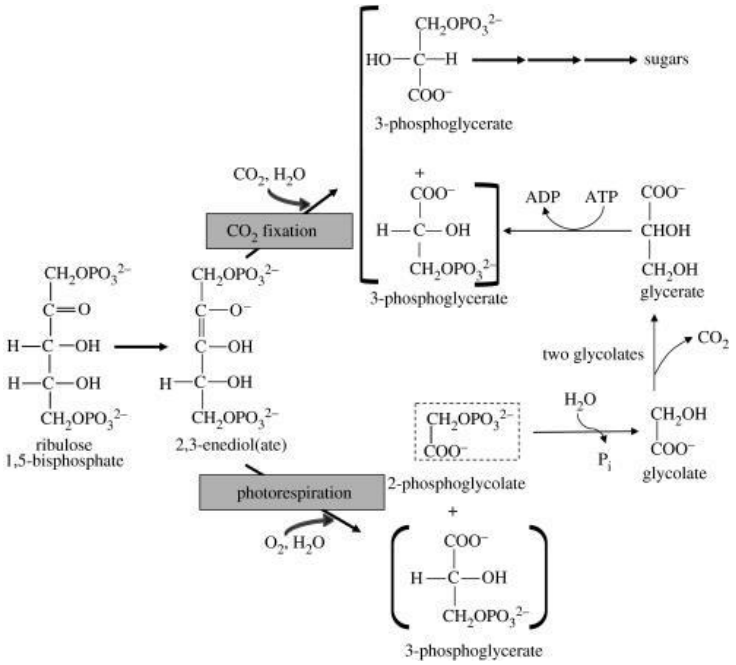

Fig 3: The reaction catalyzed by RuBisCo highlights the challenge aerobic organisms encounter due to the enzyme's fixation of oxygen (BOWES, 1991).

Four different types of RuBisCo exist in nature, labeled as forms I, II, III, and IV. These variations are distinguished by differences in the primary sequence of their approximately 50 kDa polypeptide. However, despite structural diversity, they all share a common building block: the dimer of the large (catalytic) subunit (Tabita, Satagopan, *et. al*., 2008).

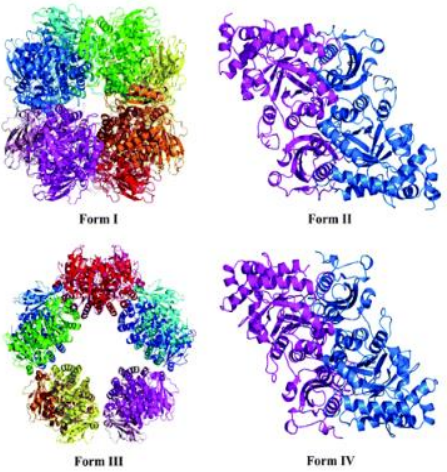

Fig 4: Representative structures of different forms of RuBisCo (Tabita, Hanson, et. al., 2008).

2.5 Carbon-dioxide Fixation

Autotrophic conversion of inorganic carbon to organic matter, pivotal for life's sustenance, initiated biological evolution. Prokaryotes and plants predominantly employ the Calvin-Benson cycle, facilitated by the vital enzyme (RuBisCo), to transform CO_2 into biomass. (Fuchs, 2011).

2.5.1 Aerobic CO2 Fixation Pathway

CBB cycle

The Calvin-Benson-Bassham (CBB) cycle is found in a wide range of organisms, including algae, plants, cyanobacteria, and certain bacterial phyla like Proteobacteria and Firmicutes. It serves as the primary pathway for fixing CO_2 in microorganisms. Important enzymes, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) and phosphoribulokinase (PRK), play crucial roles in this cycle. Additionally, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PRK are involved in RuBisCo regeneration, forming an inactive complex with the regulatory protein CP12 in dark conditions (Santos Correa *et. al*., 2023).

3-hydroxypropionate cycle

The 3-hydroxypropionate (3HP) cycle, first observed in Chloroflexus aurantiacus, involves the secretion of 3HP during phototrophic growth. It utilizes two bicarbonate (HCO_3) molecules to synthesize glyoxylate through two cycles. The first cycle involves the carboxylation of acetyl-CoA to produce glyoxylate, subsequently converted to succinyl-CoA. The second cycle begins with glyoxylate assimilation and proceeds to regenerate acetyl-CoA. Remarkably, this pathway employs bi- and multifunctional enzymes, enabling 19 steps with only 13 enzymes. Additionally, it operates without oxygen-sensitive steps, functioning under aerobic conditions (Long *et. al*., 2016; Tabita, 2009).

Hydroxypropionate/4-hydroxybutyrate (HP/HB) cycle

The 3-HP/4-HB cycle, identified in Sulfolobales like Metallosphaera and Thaumarchaeota, initiates with acetyl-CoA converting into malonyl-CoA through bicarbonate incorporation. Sequentially, propionyl-CoA is carboxylated to succinyl-CoA via methylmalonyl-CoA with additional bicarbonate. The crucial enzyme, acetyl-CoA/propionyl-CoA carboxylase, produces two acetyl-CoA molecules from one acetyl-CoA, with 3-HP and 4-HB as intermediates. NADPH and ATP serve as cofactors for methylmalonyl-CoA production. In the aerobic HP/HB cycle, an additional half-turn generates succinyl-CoA, further oxidatively converted into oxaloacetate, pyruvate, and PEP (Berg, 2011; Kang *et. al*., 2023).

2.5.2 Anaerobic CO_2 Fixation Pathway

Wood Ljungdahl pathway

Ljungdahl and colleagues (1986) introduced the reductive acetyl-CoA or WL pathway, a pivotal carbon-fixation route found in anaerobic microorganisms. Before the advent of oxygen, these microbes, possibly the earliest autotrophs, utilized inorganic compounds like CO and H2 alongside CO2 for energy. The WLP, one of seven natural carbon fixation pathways, involves converting CO2 into formate and CO, then synthesizing one acetyl-CoA molecule from two CO2 molecules with the aid of NADPH, ATP, and reduced ferredoxin (Kang et al., 2023). Primarily employed by prokaryotes living near thermodynamic limits such as acetogenic bacteria and methanogenic archaea. The pathway not only fixes CO2 but also aids in energy conservation through electrochemical gradient generation. (Berg, 2011).

Reductive TCA cycle

The reductive citric acid cycle (rTCA), initially identified in *Chlorobium limicola* and later detected in various anaerobic or microaerobic bacteria from different taxonomic groups, but not in archaea. Unlike the oxidative citric acid cycle, the rTCA cycle operates in the absence of oxygen, using alternate enzymes to reverse the process. Key cofactors such as thiamine pyrophosphate and succinyl-CoA facilitate carbon fixation, converting acetyl-CoA into pyruvate and ketoglutarate. This cycle yields acetyl-CoA from two CO2 molecules and produces essential intermediates like pyruvate, oxaloacetate, and 2-oxoglutarate crucial for carbon metabolism. (Kitadai *et. al*., 2017).

Dicarboxylate/4-hydroxybutyrate [DC/HB]

The dicarboxylate/4-hydroxybutyrate (DC/HB) cycle was identified in the Archaea species Ignicoccus hospitalis of the order Desulfurococcales (Schipke *et. al*., 1999). This cycle enables the assimilation of CO_2 by converting acetyl-CoA to pyruvate using reduced ferredoxin and bicarbonate to phosphoenolpyruvate via pyruvate synthase and phosphoenolpyruvate carboxylase, respectively. The pathway utilizes cofactors like reduced ferredoxin, ATP, and NAD(P)H for C1 assimilation (Huber *et. al*., 2008). The anaerobic DC/HB cycle employs pyruvate synthase to synthesize pyruvate, which is further utilized to generate other central precursor molecules. Unlike the HP/HB cycle, which relies on NAD(P)H, the DC/HB cycle utilizes ferredoxin/NAD(P)H and is sensitive to oxygen, with the 4-hydroxybutyryl-CoA dehydratase enzyme being inactivated in its presence. Although both cycles produce acetyl-

CoA, they differ primarily in their connection to carbon metabolism, with the DC/HB pathway synthesizing pyruvate from acetyl-CoA via pyruvate synthase (Berg, 2011).

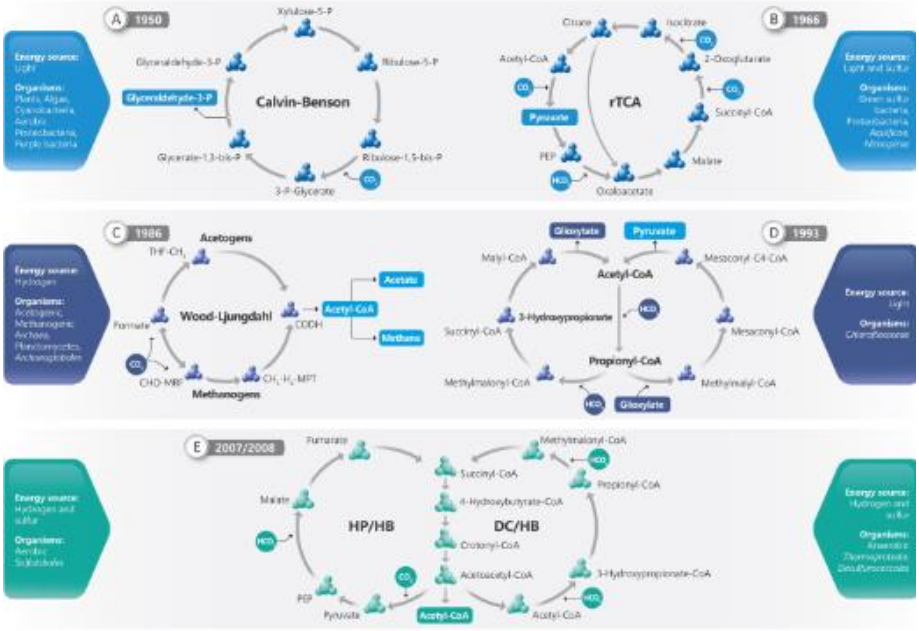

Fig 5: Natural carbon fixation (Santos Correa et. al., 2023).

2.6 Nitrogen fixation

A fertile soil is crucial for optimal plant growth in agriculture, as it facilitates nutrient cycling(Ney *et. al*., 2019) .However, nutrient availability, particularly nitrogen, often limits agricultural productivity (Soumare *et. al*., 2020). Nitrogen fixation is a crucial process that converts atmospheric nitrogen (N2) into ammonia (NH3) under specific oxygen conditions. It contributes about 200 million tons of nitrogen yearly globally, with half of crop field nitrogen attributed to this process. Rhizobia, a group of soil bacteria, form a symbiotic association with legume roots, resulting in nodule formation. Within these nodules, nitrogenase enzymes facilitate the conversion of atmospheric nitrogen into ammonia, vital for sustainable agriculture. (Mahmud *et. al*., 2020). Nitrogenase, a crucial enzyme in nitrogen fixation, comprises: the iron (Fe) protein and the molybdenum-iron (MoFe) protein as two essential metalloproteins. The MoFe protein, forming a tetramer called NifD2K2, weighs about 230 kDa and consists of α and β subunits, NifD and NifK, which share structural similarities, indicating an evolutionary link. It houses two key metalloclusters, the FeMo cofactor for substrate reduction and the P-cluster for electron acceptance from the Fe protein. Structurally, each MoFe protein tetramer contains αβ subunits, each hosting one FeMo cofactor and one P-cluster. Conversely, the Fe protein, NifH2, forms a dimer with two identical subunits, each coordinating a [4Fe:4S] cluster and featuring ATP binding sites at the dimer interface. This

intricate organization underscores the functional interplay within the nitrogenase enzyme system, vital for biological nitrogen fixation (Einsle & Rees, 2020; Poudel *et. al.*, 2018).

2.7 Transcriptional regulation of nitrogenase fixation

In nitrogen-fixing bacteria, the regulation of nitrogen fixation (nif) genes is finely orchestrated in response to diverse environmental signals. Activation occurs under nitrogen scarcity and during symbiotic interactions, while inhibition is triggered by oxygen and intermediate nitrogen levels. Studies on Klebsiella pneumoniae and Rhizobium meliloti have elucidated these regulatory mechanisms (Gussin et al., 1986). In K. pneumoniae, the general nitrogen regulation (ntr) system is pivotal, comprising genes such as ntrA, ntrB, ntrC, glnB, and glnD. Notably, glnD encodes a multifunctional protein with roles in enzyme activity modulation and glutamine sensing. Additionally, NtrB and ntrC form a two-component regulatory system, where NtrB phosphorylates NtrC under nitrogen scarcity, while PII and nitrogen surplus can reverse this process. Phosphorylated NtrC then acts as a transcriptional activator for nifA and related genes involved in nitrogen assimilation (Zhang et al., 2006a).

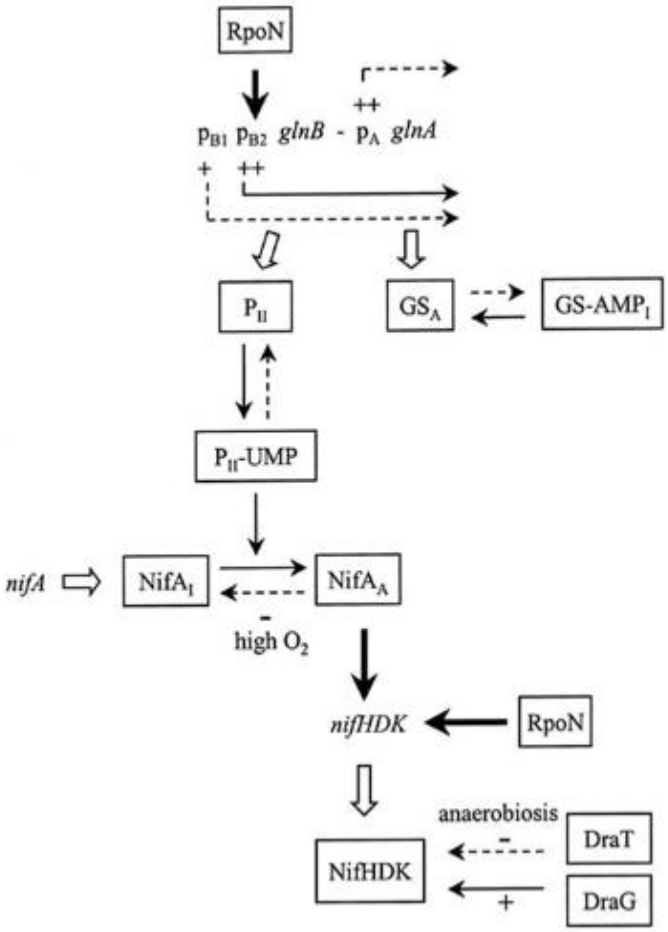

Fig 6: Regulation of Nitrogenase in Azospirillum brasilense: Proposed Model (Steenhoudt & Vanderleyden, 2000).

In Gram-negative nitrogen-fixing bacteria such as Azospirillum and Azotobacter, the expression of nif structural genes depends on two main regulators: NtrA and NifA. Promoters containing σ54 type motifs and upstream activating sequences (UAS) indicative of NifA binding have been identified upstream of various nif genes in A. brasilense and A. vinelandii. Once bound to these sequences, NifA interacts with RNA polymerase, facilitating the formation of an open promoter complex and initiating transcription. NifA proteins from different species share a three-domain structure, comprising a C-terminal domain with a DNA-binding helix-turn-helix motif, a central domain for interaction with RNA polymerase, and a nucleotide-binding site crucial for NifA's activity. While the N-terminal domain of NifA proteins exhibits sequence variation, this structure remains conserved. In A. brasilense, nifA expression is constitutive but diminishes under unfavorable nitrogen fixation conditions. Interestingly, unlike Azotobacter, A. brasilense nifA expression appears unaffected by the regulatory NtrBC proteins, possibly due to the absence of a σ54 consensus sequence in its promoter.

However, NtrC plays a vital role in optimizing nitrogen fixation and maximizing nifA expression under nitrogen fixation conditions in A. brasilense, although its effect on the nifA promoter may be indirect. This underscores the complex regulatory mechanisms governing nitrogen fixation in these bacteria, ensuring precise gene expression in response to environmental signals. In the γ-subdivision of proteobacteria, such as A. vinelandii and K. pneumoniae, the gene nifL regulates NifA activity in response to oxygen and fixed nitrogen levels. In A. vinelandii, nifA is often cotranscribed with nifL, but unlike in K. pneumoniae, this transcription is not regulated by NtrBC. NifA's activity is controlled by its inhibitor protein, NifL, which responds to changes in oxygen and fixed nitrogen levels independently. The inhibition of NifA by NifL occurs through distinct mechanisms in response to different environmental cues. This inhibition involves a direct protein-protein interaction between NifL and NifA, resulting in decreased nucleoside triphosphatase activity of NifA when the inhibitory complex forms. Overall, the elaborate regulation of NifA by NifL ensures precise control of nitrogen fixation in response to environmental signals, demonstrating the sophisticated mechanisms bacteria employ to adapt for optimal metabolic efficiency.

2.8 Post translational regulation of nitrogenase activity

In A. brasilense, nitrogenase activity is regulated at the posttranslational level by fixed nitrogen levels and oxygen concentration. Similar to photosynthetic nitrogen-fixing bacteria like

Rhodospirillum rubrum and R. capsulatus, this regulation involves ADP-ribosylation of dinitrogenase reductase. In this process, two enzymes, Dinitrogenase Reductase ADP-ribosyl Transferase (DRAT, encoded by draT) and Dinitrogenase Reductase Activating Glycohydrolase (DRAG, encoded by draG), facilitate a reversible reaction.DRAT transfers an ADP-ribose molecule from NAD to the Arg-101 residue of one subunit of the dinitrogenase reductase homodimer, thereby inactivating the enzyme. Conversely, DRAG removes the ADP-ribose from the modified enzyme, restoring its activity. This intricate posttranslational modification mechanism enables A. brasilense to finely tune nitrogenase activity in response to environmental cues, ensuring efficient nitrogen fixation under varying conditions (Zhang *et. al*., 2006b).

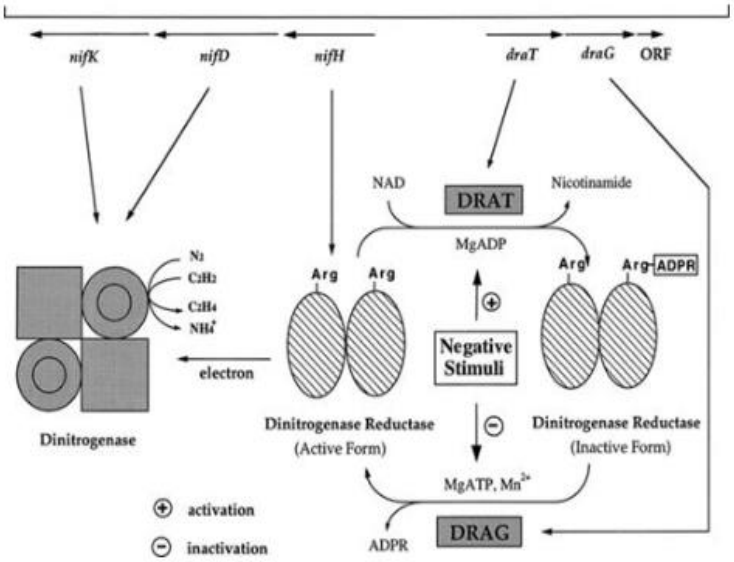

Fig 7: A regulatory mechanism in A. brasilense governs nitrogenase activity by modifying dinitrogenase reductase through ADP-ribosylation. This process, facilitated by the DRAT/DRAG system, entails the addition of ADP-ribose to the Arg-101 residue of dinitrogenase reductase by DRAT during specific conditions, thereby inhibiting its function. Conversely, DRAG removes ADP-ribose from dinitrogenase reductase, restoring its activity in response to changing conditions (Zhang et. al., 2006b).

2.9 Source of electron for nitrogen fixation

Biological nitrogen fixation is a crucial process facilitated by the nitrogenase enzyme in prokaryotes. This enzyme, encoded by Nif gene clusters, converts atmospheric nitrogen (N2) into ammonia (NH3) under normal conditions, demanding ATP, protons, and electrons. The NifH protein and the NifDK complex orchestrate this conversion, with electron carriers like flavodoxins and ferredoxins contributing electrons to NifH's [4Fe-4S] cluster. NifH, in conjunction with the NifDK complex, employs Mg-ATP to transfer electrons, which are subsequently relayed to the [8Fe-7S] cluster in NifDK and eventually to FeMo-co. This cascade of electron transfer culminates in the reduction of N2 to NH3 and H2. Ferredoxins and

flavodoxins, small protein electron carriers, play crucial roles in shuttling electrons from donors to acceptors. Ferredoxins have evolved to selectively associate with specific partner proteins, determined by factors such as binding surface structure and charge, Fd abundance, and reduction potential. This intricate interplay of electron carriers and nitrogenase components facilitates efficient nitrogen fixation in diverse organisms (Bennett *et. al*., 2023).

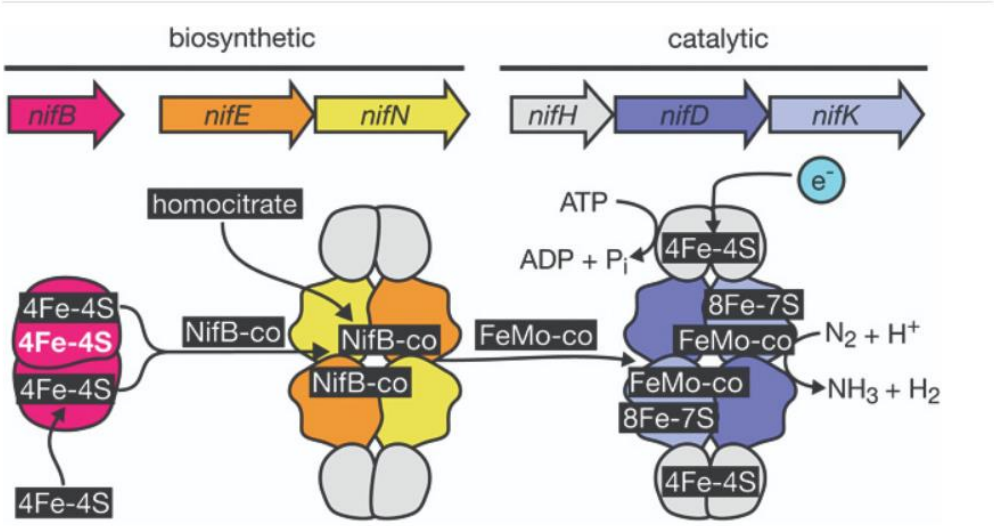

Fig 8: Key Steps in Nitrogen Fixation: MoFe Nitrogenase Catalysis and Essential Gene Functions (Bennett et. al., 2023).

2.10 Genus *Azospirillum*

Azospirilla, a group of Gram-negative bacteria known for nitrogen fixation in the rhizosphere, are acknowledged as beneficial for plant growth. They are found globally, especially in association with grasses and cereals, and include species like *A. brasilense, A. lipoferum, A. amazonense, A. halopraeferens,* and *A. irakense*. These bacteria adjust nitrogen fixation based on environmental cues, thriving in low-oxygen conditions and nitrogen-depleted soils (Zhang *et. al*., 2006b). The genome of *A. amazonense* harbors genes facilitating the conversion of alternative nitrogen sources like nitrate, urea, and dinitrogen into ammonia, which is then assimilated into metabolism via the GS/GOGAT pathway. This conversion process is tightly regulated to minimize energy expenditure, with PII proteins acting as central regulators of nitrogen metabolism.

2.11 Genus *Azotobacter*

Azotobacter bacteria are aerobic, Gram-negative nitrogen-fixing organisms commonly found in soil, forming resilient cysts during adverse conditions. They utilize atmospheric nitrogen to enhance soil nitrogen levels for plant protein synthesis despite sensitivity to acidic pH, high salt, and temperature variations. Employing respiratory protection and mechanisms like

hydrogenase uptake, they safeguard nitrogenase from oxygen (Aasfar *et. al*., 2021). They can withstand harsh environmental conditions by forming resistant structures, making them ideal candidates for biofertilizer development, reducing reliance on chemical fertilizers. When used in consortia with other biofertilizers, *Azotobacter* accelerates their beneficial activities, and certain strains have been identified for their ability to produce antifungal substances, inhibiting phytopathogenic species. *Azotobacter* species express iron-rich nitrogenases and produce siderophore complexes to acquire iron, potentially exhibiting anti-phytopathogenic properties and promoting plant growth. Their utilization of various organic substrates leads to the production of biologically active compounds that stimulate rhizospheric microorganism proliferation. Notably, *Azotobacter* species produce auxin (IAA), a vital phytohormone facilitating root growth, nutrient uptake, and overall plant development (Sumbul *et. al*., 2020).

2.12 Genus *Pseudomonas*

Pseudomonas spp., members of the Pseudomonadaceae family, are diverse and subdivided, with *Pseudomonas fluorescens* and *Pseudomonas putida* being extensively studied for their role in promoting plant growth (Li *et. al*., 2017). Particularly, *Pseudomonas putida* has garnered attention in environmental biotechnology for its proficiency in bio-remediating toxic organic compounds, notably aromatic hydrocarbons. These bacteria are notable for producing secondary metabolites with antimicrobial properties, including 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, hydrogen cyanide, and syringomycin. Many pseudomonads engage with plants, exhibiting biocontrol against plant pathogens and directly fostering plant health and growth as both endophytes and rhizosphere colonizers. Their interactions with plants contribute significantly to enhancing plant disease resistance and overall growth, making them invaluable assets in agricultural practices and environmental management (Silby *et. al*., 2011).

2.13 Genus *Geobacter*

Geobacter sulfurreducens, a Gram-negative δ proteobacterium, thrives in subsurface environments, where it participates in the oxidizing organic compounds like acetate while reducing metals such as Fe(III). Notably, *Geobacter* species, including *G. sulfurreducens*, are instrumental in bioremediating organic and metal contaminants in these environments. Initially considered a strict anaerobe, *G. sulfurreducens* has been found capable of utilizing oxygen as a terminal electron acceptor when present in concentrations of 5% or less (Engel *et. al*., 2020). In the bacterium *G. sulfurreducens*, nitrogen-fixing genes are regulated by several key players, including the sigma factor RpoN and two-component systems like GnfL/GnfM and

GnfK/GnfR. These systems control gene expression, with GnfL/GnfM activating the majority of nitrogen-fixation genes and GnfK/GnfR regulating a subset through transcription anti-termination. During nitrogen-fixation-dependent growth, the phosphorylated form of GnfM predominates, leading to repression of genes like gdhA and activation of nitrogen-fixation gene expression (Ueki & Lovley, 2010a).

2.14 Genus *Bacillus*

Bacillus spp., gram-positive bacteria widely distributed in nature, are commonly found across various environmental niches. Particularly beneficial strains of *Bacillus* form symbiotic relationships with plant roots or rhizospheres, aiding in the development of biofilms that promote plant growth. Utilizing *Bacillus*-based fertilizers enriches soil with nutrients, regulates pathogenic microbial growth, and triggers plant defense mechanisms against pests. These bacteria produce compounds like IAA, gibberellins, cytokinins, and spermidines, helpful in growth, fostering root and shoot cell division and elongation. Additionally, *Bacillus* spp. exhibit iron-chelating properties through siderophore production, facilitating the solubilization of iron from minerals and organic compounds in rhizospheres. Siderophores bind to Fe^{3+} in complex substances, converting it to plant-accessible Fe^{2+} for uptake. *B. subtilis*, a versatile probiotic, shows promise in inhibiting pathogenic bacteria and enhancing nutrient absorption (Radhakrishnan *et. al*., 2017). It is also utilized in industrial applications for the production of various chemicals and vitamins (Su *et. al*., 2020).

2.15 Water splitting reaction

In natural oxygenic photosynthesis, sunlight is captured by photosynthetic antenna systems and channeled to reaction centers, where it is converted into chemical energy. This process generates oxygen as a byproduct by splitting water molecules (Hou, 2011). This process encompasses oxidation of water to oxygen and the reduction of electron acceptors by Photosystem II (PSII) embedded within thylakoid membrane. Electrons derived from water undergo transportation to Photosystem I (PSI) via electron carrier proteins, culminating in the reduction of NADP+ to NADPH. This linear electron transfer pathway, known as the Z-scheme, is essential for photosynthetic energy conversion (Johnson, 2016).

Photosynthesis presents an opportunity for sustainable energy production, utilizing water as an abundant and non-toxic resource. During optimal situation, clean electricity would drive the electrolysis of water, yielding hydrogen as a fuel, mimicking natural processes. This allows for

the development of artificial systems, such as biological photosynthetic organisms or synthetic catalysts, aimed at efficient large-scale water splitting and hydrogen production, crucial for energy storage in chemical compounds.

Hydrogen, a versatile base chemical, can be converted into various energy-rich materials, such as methane, methanol, or ammonia. The production of molecular hydrogen (H_2) from water involves two catalytic steps: the oxidation of water into oxygen (O_2) and protons, followed by the reduction of protons to H_2. While effective approaches for proton reduction exist, such as biomimetic catalysts derived from hydrogenase research, water oxidation catalysts remain a challenge. First-row transition metal-based catalysts often suffer from stability issues, resulting in low turnover numbers (TON) or turnover frequencies (TOF), and require excessive voltages (overpotential) due to inadequate interfaces with photo-active semiconductors (Cox *et. al*., 2015; Eren & Özkar, 2021).

Sakimoto and colleagues (2016) devised a groundbreaking hybrid system merging *Moorella thermoacetica* (ATCC 39073) with biologically precipitated CdS nanoparticles. CdS, a semiconductor, possesses suitable properties for photosynthesis. *M. thermoacetica* , functioning as both an acetogen and electrotroph, serves as an ideal model to explore hybrid system capabilities. Synthesis of acetic acid by *M. thermoacetica* and CdS involves a two-step, one-pot synthesis. Initially, CdS precipitation is triggered by adding Cd2+ and cysteine (Cys) as the sulfur source. *M. thermoacetica* utilizes photogenerated electrons from illuminated CdS nanoparticles for photosynthesis. Upon photon absorption, CdS generates electron-hole pairs, with the electron contributing to reducing equivalents passed to the Wood-Ljungdahl pathway for acetic acid synthesis from CO_2. Cysteine acts as a quencher for the holes, leading to the formation of oxidized cystine (CySS). This hybrid system demonstrates promising potential for sustainable biotechnological applications (Sakimoto *et. al*., 2016).

Research on the mechanism and cofactors involved in water oxidation at the Oxygen-Evolving Complex (OEC) has garnered significant interest. The potential roles of inorganic carbons (CO_2, H_2CO_3, HCO^{3-}, CO_3^{2-}) at the OEC have been debated, with findings suggesting the involvement of HCO^{3-} ions as mobile proton acceptors. Studies by (Soumare *et. al*., 2020) demonstrated that HCO^{3-} ions aid in transporting protons produced by water oxidation at the OEC within photosystem II, facilitating light-driven O_2 production in the chloroplast's lumen. This insight offers valuable understanding for developing efficient water oxidation catalysts and advancing sustainable hydrogen production technologies.

In (Kanan & Nocera, 2008) developed a catalyst that performed water splitting without the need for excessive driving potential. They used inert indium tin oxide as an electrode in phosphate-buffer containing cobalt (II) ions. Cobalt ions in the presence of chemical oxidants such as $Ru[(bpy)_3]^{3+}$ (bpy, bipyridine) with Eo = 1.26, (where Eo is the standard potential) catalyze the oxidation of water to O_2 in neutral phosphate solutions. They suggested that this catalytic reaction implicates the hydrogen phosphate (HPO_4^{2+}) ions as the proton acceptors as well as are required for formation of heterogenous water oxidation. And it not only forms in situ from earth-abundant materials but also operates in neutral water under ambient condition (Kanan and Nocera, 2008).

In Meyer's research, a mononuclear ruthenium complex has been devised to oxidize water efficiently, crucial for various applications. This complex operates in a solution containing HPO_4^{2-} ions, which serve as proton acceptors. The process involves formation of O-O bond through a mechanism where water, acting as a nucleophile, attacks the highly oxidized Ru=O species. This mechanism proceeds via proton-coupled oxygen atom transfer (Meyer & Huynh, 2003).

2.16 Density functional theory

Density Functional Theory (DFT), a cornerstone in quantum mechanical modeling, finds extensive application across physics, chemistry, and materials science for probing the electronic structure of atoms, molecules, and solids, striking a balance between accuracy and computational efficiency (Kohn & Hohenberg, 1964). Devised by Walter Kohn and Pierre Hohenberg in 1964, with subsequent refinement by Kohn and colleagues (Kohn, 1999), DFT operates on the premise that a system's ground-state energy and other attributes are uniquely determined by its electronic density. Central to DFT is the exchange-correlation functional, which approximates the intricate electron-electron interactions (Hohenberg & Kohn, 1964). Despite its inherent approximations, DFT has enjoyed remarkable success in predicting various material properties, including structural stability, electronic behaviors, and reaction mechanisms, thus significantly propelling the fields of materials science and computational chemistry.

2.17 Microbial Fuel Cells

Microbial fuel cell (MFC) technology has become a focal point in sustainable bioenergy research due to its ability to convert chemical energy into electricity via microbial metabolic

activity. MFCs consist of an anode and cathode separated by a proton exchange membrane (PEM), facilitating electron transfer. In the anode, microbes act as catalysts, breaking down substrates through cellular respiration to produce electrons and protons. These electrons flow through an external circuit, while protons move through the PEM to the cathode, where they combine with oxygen to form water, generating electricity. This process offers advantages such as mild operating conditions, simple setup, and a diverse range of available biocatalyst sources (Wang *et. al.*, 2022).

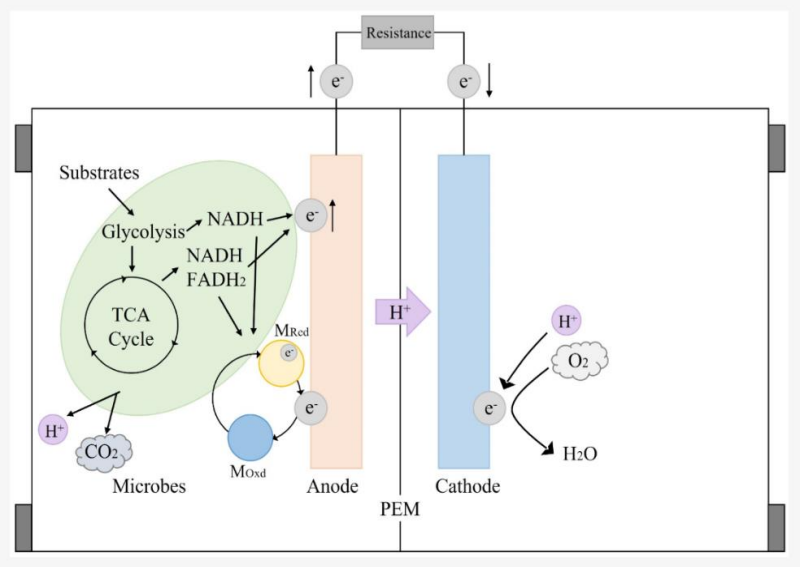

Fig 9: Dual-Chamber Microbial Fuel Cell: Operational Insights (Wang et. al., 2022).

Microbial fuel cells (MFCs) are predominantly applied in conjunction with wastewater treatment, offering a viable solution to address both water pollution and energy shortages. Wastewater often contains organic substances that contribute to water pollution. While conventional aerobic digestion treatments efficiently decompose these pollutants into carbon dioxide, they fail to harness the chemical energy potential within the organic compounds. MFCs, however, utilize these organic pollutants as substrates for microbial metabolic activities, generating electrons in the process. This enables simultaneous organic pollutant degradation and electricity production within the MFC system. Additionally, MFC-based anaerobic digestion technology offers advantages over conventional aerobic methods by reducing energy consumption. This integrated approach showcases the potential of MFCs in providing sustainable solutions for wastewater treatment and energy production (Gul *et. al.*, 2021).

2.17.1 Effect of Anode in MFC

Several microbial species have been identified for their efficient electron transfer to the anode in microbial fuel cells (MFCs). Notable examples include *Rhodoferax ferrireducens*,

Shewanella putrefaciens, *Geobacter metallireducens*, *Geobacter sulfurreducens*, and *Aeromonas hydrophila*. These microbes play a crucial role in MFC operation by directly transferring electrons to the anode. To sustain their metabolic activities, microbes require nutrients, which can be supplied by various waste sources like swine waste, dairy waste, and industrial waste (Malik *et. al*., 2023).

Among these microbes, *G. sulfurreducens* is particularly noteworthy as it belongs to the class of electricigens, which are capable of conserving energy for growth by completely oxidizing organic compounds to carbon dioxide while directly transferring electrons to the anode of the MFC. The oxidation of substrates by microbes at the anode represents the primary source of electron generation in MFC systems. *Geobacter* and *Shewanella* species, known as electrogenic microbes, are commonly employed in MFC applications (Franks & Nevin, 2010).

The study of Geobacteraceae has been significantly advanced by recent breakthroughs, such as the complete sequencing of *G. sulfurreducens*' genome and the establishment of genetic manipulation techniques for this organism. Research involving *G. sulfurreducens* has demonstrated its remarkable ability to fully oxidize electron donors using only an electrode as the electron acceptor. It has been found that this microbe can efficiently transfer electrons to electrodes even without the presence of electron mediators. This electron transfer capability is attributed to a subset of cells that can attach to the electrode and exhibit electron transport rates comparable to those seen with Fe (III) citrate. These discoveries shed light on the dynamics of cell-electrode interactions within the Geobacteraceae family and highlight the potential of these microbes to improve the performance of Microbial Fuel Cells (MFCs) (Bond & Lovley, 2003).

Enhancing microbial fuel cell (MFC) performance hinges significantly on optimizing electron transfer, often achieved through electrode design and material selection (Wang et al., 2022). Effective electrodes should possess traits like robust electrical conduction, corrosion resistance, and stability in harsh environments, while also being biocompatible and offering a high surface area (Wang et al., 2022). Carbon-based materials, prized for their conductivity and stability, dominate electrode choices, including carbon cloth, paper, graphite rods, and granular activated carbon (GGs) due to their microporosity and affordability (Wang et al., 2022). Recent research delves into nanoengineering methods to enhance electron transfer, promising improvements in MFC power density and efficiency (Fadzli et al., 2021). Such advances underscore the pivotal role of electrode design and material innovation in optimizing MFCs for sustainable energy production (Fadzli et al., 2021).

2.17.2 Effect of Cathode in MFC

In microbial fuel cells (MFCs), the anode chamber facilitates the production of protons through microbial processes. These protons then move across a proton exchange membrane to the cathode chamber, completing electrical circuit. At the cathode, electrons are generated and transferred to oxygen, leading to its reduction to water. This continuous reduction process occurs when connection of cathode and anode is facilitated by wire. Several factors, including the type and concentration of electron acceptors, catalyst efficiency, proton presence, and electrode design, impact the efficacy of the cathode reaction (Fadzli *et. al*., 2021).

$$2H_2 \longrightarrow 4H^+ + 4e^-$$

$$O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$$

Oxygen, readily available in the cathode chamber, serves as the primary electron acceptor because of its high oxidation potential and environmentally friendly byproduct production of water. While platinum, a common catalyst due to its ability to accelerate reaction rates by lowering activation energy, its tendency to hinder substrate components and high cost have prompted exploration for alternative catalysts. Najafpour et al. illustrated that addition of a small quantity of potassium permanganate can enhance MFC performance by acting as an oxidizing agent, offering a potentially more efficient and cost-effective alternative to platinum catalysts (Siddiqui *et. al*., 2023).

2.18 Proton exchange membrane

Proton exchange membranes (PEMs) are commonly used as dividers in MFCs because of their advantageous traits, such as relatively high cation conductivity and low internal resistance in comparison to other separators. Among these, cation exchange membranes (CEMs) are particularly widespread, specifically crafted for proton transfer within MFCs. Evaluating PEMs largely revolves around their efficacy in enabling proton movement from the anode to the cathode while blocking oxygen, substances, and minerals transfer between two chambers (Rahimnejad *et. al*., 2014).

The membrane's pivotal role in MFC performance hinges on its proton exchange capability. Typically, two types of PEMs are employed: porous proton exchange membranes and nonporous dense membranes. Dense membranes like Nafion primarily serve to separate the anode and cathode compartments, preventing electrolytes and air migration between them (Rahimnejad *et. al*., 2014).

Despite its effectiveness, Nafion's relatively high cost has spurred researchers to seek alternative membrane materials. Various experiments have explored substituting Nafion with alternatives such as sulfonated polyether ether ketone membranes, anion and cation exchange membranes (AEMs and CEMs), ultrafiltration and microfiltration membranes, bipolar membranes, and forward osmosis membranes. These endeavors aim to identify more cost-effective alternatives to Nafion while maintaining or enhancing MFC performance (Siddiqui *et. al*., 2023).

2.19 Mechanism of bio-electron generation

Microbes utilize various organic compounds such as sugars, proteins, and fats as sources of carbon and energy. These compounds undergo metabolic processes, starting with glycolysis, where they are broken down into smaller molecules like acetyl coenzyme A (CoA). The citric acid cycle then oxidizes these molecules, generating electron carriers like NADH and FADH2. These carriers transfer electrons to the cell membrane, where ATP synthase enzymes produce ATP from ADP. This ATP acts as the energy currency of cells, facilitating essential biological processes. In certain environments, bacteria can act as electron acceptors, enabling the production of ATP through respiration-like processes (Singh *et. al*., 2019).

2.20 Mechanisms of proton transfer through the Nafion membrane

Nafion, also referred to as perfluorinated sulfonic acid (PFSA) membrane, is a widely utilized proton exchange membrane (PEM) originally developed by DuPont. It comprises a hydrophobic polytetrafluoroethylene (PTFE) backbone linked to a hydrophilic perfluorinated vinyl ether pendant side chain containing sulfonic acid groups ($-SO_3H$). These sulfonic acid groups, bearing negative charges (SO_3^-), selectively permit the passage of cations while blocking anions. Additionally, they absorb water, ensuring membrane hydration for efficient proton migration. The absorption of water by sulfonic acid groups leads to the formation of ion-cluster domains and water bridges, acting as channels for proton migration, thereby enhancing proton conductivity. The PTFE backbone, characterized by strong C-F bonds between small-sized fluoride atoms, contributes to Nafion's mechanical resilience and chemical stability when swollen with water. Consequently, Nafion exhibits prolonged functionality in fuel cells, lasting over 60,000 hours (Ng *et. al*., 2022).

Proton transfer within proton exchange membranes (PEMs) occurs through surface diffusion, the Grotthuss mechanism (hopping), or vehicular mechanism (diffusion). In surface diffusion,

protons hop between adjacent sulfonic acid groups along the channel or pore wall interface. The Grotthuss mechanism involves protons jumping within the percolation network formed by water molecules, where each water molecule sequentially bonds and releases protons. Meanwhile, vehicular diffusion entails protons migrating alongside water molecules in the form of hydronium ions, driven by concentration gradients and electro-osmosis. Both mechanisms rely on hydration level, with higher water content facilitating faster proton transport. However, extreme temperatures can hinder water diffusion, leading to reduced proton interaction with sulfonic acid groups. Overall, proton conductivity in PEMs depends on temperature, water content, and membrane properties, influenced by all three transport mechanisms (Karimi *et. al*., 2019).

2.21 Syntrophic Consortia

Syntrophy is a symbiotic relationship where multiple organisms collaborate to break down a substrate that none can metabolize individually (Zhang *et. al*., 2023). One prominent example is the partnership between fermentative bacteria and methanogenic archaea, which jointly convert volatile fatty acids (VFAs) into methane. This interaction relies on interspecies electron transfer (IET), particularly involving hydrogen and formate exchange (Kouzuma *et. al*., 2015). In anaerobic environments like digesters, where electron acceptors are scarce, syntrophic partners grow together to overcome energy barriers and share metabolic electrons. Syntrophic communities utilize simple substrates like H_2, formate, methanol, and acetate for electron transfer (Wan *et. al*., 2018). This cooperative relationship is crucial for the complete degradation of complex organic compounds into methane and carbon dioxide in methanogenic ecosystems. Initially, fermentative bacteria hydrolyze polymers into simpler compounds, which are then syntrophically metabolized into methanogenic substrates like H_2, formate, and acetate. Overall, syntrophy plays a vital role in the microbial breakdown of organic matter, facilitating the conversion of natural polymers into methane and CO_2 (McInerney *et. al*., 2009).

2.22 Nanoparticles

When water and carbonate combine with cobalt oxy hydroxide then get reversible reaction of the oxidized from that is removal of 2 electron and 2 proton and final structure of cobalt IV obtained. Simultaneously, there is the formation of the intermediate form and again reduction

form occurs and gets back again into that original form cobalt III structure. Cobalt has its catalytic power towards water splitting.

Electrochemical water splitting involves two key reactions: the hydrogen evolution reaction (HER) at the cathode and the oxygen evolution reaction (OER) at the anode. Cobalt oxides, particularly in their Co3+ state, serve as catalysts rather than mere adsorbents or composites. Cobalt oxyhydroxide, with its layered structure and excellent conductivity, emerges as a

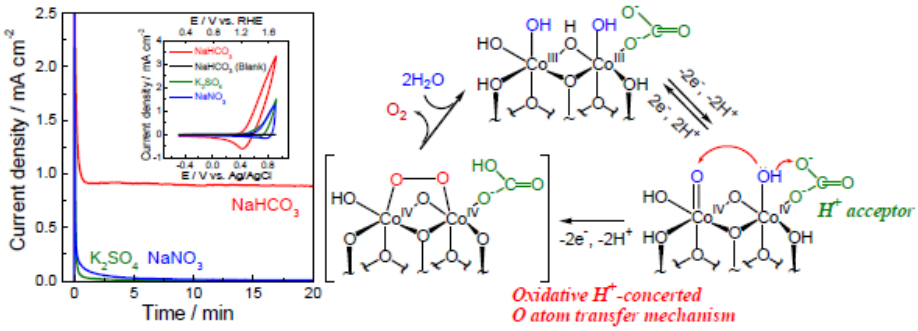

Fig 10: Essential cofactor for carbonate ions for an artificial evolving complex model (Aiso et. al., 2017).

promising electrode material due to its spacious interlayer gaps and well-defined electrochemical capabilities. The formation of CoIV=O species is pivotal for cobalt-based molecular complexes to function as efficient water oxidation catalysts. Moreover, cobalt(II) hexahydrate complexes exhibit potential as electrochemically active species for water oxidation. Alternatively, chemical oxidants combined with dissolved aqua cobalt ions can drive water oxidation, initially producing hydrogen peroxide. In the presence of CoIII, hydrogen peroxide undergoes disproportionation to yield oxygen gas and water. However, it's crucial to note that the kinetics and electrochemical potential of this process differ significantly from those mediated by cobalt catalysts across a pH range of 0 to 14 (Wang et al., 2016).

Chapter 3

Method and Methodology

3.1 Revival of syntrophic bacteria

The four bacteria primarily used in the study which are *Azotobacter*ia spp., Azospirrilium spp., *Geobacter* spp. and *Pseudomonas* spp. were revived from preserved culture by my senior (Pun, 2019).

3.1.1 Protocol for revival of bacteria

First of all, the glycerol stock prepared by Pun, 2019 was inoculated into LB media and incubated at 28°C for 24 hours. Then, the single colonies were sub-cultured into the respective selective media for individual bacteria at 28°C for 24 hours. It was again streaked in LB plates for further use and glycerol stocks of all bacteria were prepared for future use.

3.2 Screening of bacterial isolates on specific media

Screening was done by growing bacteria in nitrogen and carbon free (NCF) media. The isolated scarlet, creamy colonies grown in the nitrogen free basal media (NFB) media were streaked on the NCF media plates and incubated for 3 days at 28°C. The colonies with dew drops appearance were selected and streaked on NCF media plate for pure colony isolation. NCF medium was prepared and acted as specific medium for Azospirrilium spp. Ethylene glycol was added to NCF to make specific media for *Azotobacter* spp. Similarly, *Pseudomonas* spp grew in media containing toulene and *Geobacter* spp grew in media containing aniline (Pun, 2019).

3.3 Morphological characterization of isolates

The isolates of bacteria were further characterized by Gram staining and different biochemical tests.

3.3.1 Gram's staining

A thin and uniform smear of sample was prepared on the microscopic slide by mixing the sample with distilled water and allowed for air dried. Heat fixation was done to fix the smear to the slide. Then, three drops of crystal violet was flooded over the heat fixed smear and allowed to stand for one minute. It was then rinsed with tap water and three drops of Gram's Iodine was flooded and allowed to stand for one minute. Then, rinsing was done by tap water

and decolourizing agent (acid alcohol) was flooded and washed by tap water immediately. After that, counter stain was flooded and allowed to stand for forty five seconds. It was rinsed with tap water again and allowed for air dry. The prepared slide was observed under microscope at 40X and 100X (oil immersion) to observe colour, shape and arrangement of the colonies.

3.3.2 Biochemical characterization of the isolates

Biochemical tests are commonly utilized in the identification of microorganisms, following the flow charts outlined in Bergey's Manual of Determinative Bacteriology. These tests rely on the microorganisms' capacity to utilize specific biomolecules, leading to the production of beneficial organic compounds for their survival. By performing various biochemical tests, distinctions between different microorganisms can be made based on their metabolic abilities.

IMViC test comprises several biochemical assays aimed at assessing specific metabolic activities in bacterial isolates.

The Indole test evaluates an organism's capacity to enzymatically convert tryptophan into indole, indicated by the formation of a red ring when Kovac's reagent is added to the culture after incubation in tryptophan broth.

The Methyl Red-Voges-Proskauer (MR-VP) test assesses acid production from glucose metabolism. The MR test detects stable acid end products, with a positive result indicated by a red color, while the VP test detects acetoin production, marked by a red color after the addition of Barritt's reagents.

Citrate utilization is examined using Simmons citrate agar slants to determine if isolates can utilize citrate as a carbon source, indicated by a change in media color.

Motility and hydrogen sulfide production are assessed using Sulfur-Indole Motility (SIM) agar, where motility is detected by bacterial movement along the line of inoculation, and hydrogen sulfide production is indicated by blackening of the media.

The urease test evaluates an isolate's ability to hydrolyze urea, resulting in an increase in media pH and a color change to pink red due to phenol red indicator.

3.4 Cobalt Nanoparticle (Cobalt-oxyhydroxide) Synthesis

Cobalt nanoparticle was synthesized by chemical method. For this; 100 ml of 0.05 mol/L $Co(NO_3)_2.6H_2O$ (cobalt nitrate hexahydrate) was made and warmed at 45°C. Then, Sodium hydroxide solution (50 ml) of 0.1 mol/L prepared and warmed at 45°C seperately. Then 80 ml

of above cobalt nitrate hexahydrate solution was added dropwise into 50 ml of sodium hydroxide solution. It was allowed to react and see pink precipitate being formed by stirring with magnetic stirrer. After that 25 ml of 0.1M urea solution was added dropwise in a water bath at 90°C with proper stirring (Patil *et. al*, 2016). The precipitate was filtered without disturbing the precipitate. The precipitate was washed several times with deionized water and checked the pH of the solution.

The volume of the solution was made to 20 ml by adding deionized water and warmed at 45°C. To 5 ml of 8 mol/L NaOH, 2ml of synthesized sodium percarbonate was added dropwise while the precipitate was stirred with magnetic stirrer for 18 hours. And then it was dried at 65°C in oven for two days. Finally, the quantity of dried cobalt nanoparticles formed was obtained by measuring in the weighing machine. The synthesized amount of nanoparticle was stored and characterized by FTIR and XRD Preparation of sodium percarbonate.s

Fig 11: Formation of pink precipitate while preparing cobalt nanoparticle

Sodium per carbonate synthesis was done in the laboratory using predefined protocol. Sodium percarbonate was prepared by mixing sodium carbonate (10g) and sodium silicate (0.175g) in 17.5 ml of distilled water. 20 ml of hydrogen peroxide (30%W/V) was added containing 0.325g of magnesium sulphate heptahydrate and slurry of sodium percarbonate was dried in an oven at 60-65°C for 20 minutes (Abdel-bary *et. al*, 1981).

3.5 Determination of biological Nitrogen fixation by using Nessler's reagent

Nitrogen, an essential nutrient for plant growth, is abundant in the Earth's atmosphere but often deficient in tropical soils. Biological nitrogen fixation (BNF) is a vital process that converts

atmospheric nitrogen into a usable form for plants. This involves microbes containing the nitrogenase enzyme, which enhances soil fertility by transforming nitrogen into a form accessible to plants. Accurately measuring the amount of fixed nitrogen is crucial for optimizing symbiotic nitrogen fixation efforts (Shi et al., 2023).

3.5.1 Quantification of atmospheric nitrogen fixation by Nessler's reagent

A loop full of bacteria was inoculated in 10ml NCF media and incubated at 28°C for 48 hours. After 48 hours, production of ammonia by the bacteria was tested by the reaction of incubated bacterial sample with Nessler's reagent. The quantitative reaction of Nessler's reagent with ammonia is:

$2(HgI_2.2KI) + NH_4OH + 3NaOH \rightarrow HgO.Hg(NH_2)I + 3H_2O + 4KI + 3NaI$

The development of brown to yellow color indicated positive test for biological nitrogen.

Quantification was done by centrifuging 1ml culture at 12,000 rpm for 5 minutes and the supernatant was collected for spectrophotometric analysis. For this, 2ml solution containing 1700µl of distilled water, 200 µl cultured supernatant and 100µl Nessler's reagent were mixed and leave for 10 min at room temperature. Finally, absorbance was observed at 435 nm and the amount ammonia production was calculated using standard curve.

Standard curve was plotted by taking the UV absorbance at 435 nm of ten different concentration (0mM-100mM) of ammonium hydroxide (NH_4OH) treating with 100µl Nessler's reagent. Nessler's reagent was mixed properly with the different concentration of ammonium hydroxide in different tube and UV absorbance at 435 nm was noted (Pun, 2019).

3.6 Reduced carbon detection test

The detection of capability of CO_2 reduction by isolated bacteria was also done by culturing bacteria in NCF liquid media. For this, 100µl of over-night culture of was transferred into the 10ml of the NCF broth and incubated at 28°C for 3 days. After three days, growth rate was observed at spectrophotometer at 600nm. Then cell from broth media was separated by centrifugation at 5000 rpm and supernatant was used to detect carbohydrate in the NCF media following different two tests methods.

Molisch's test

This test is a general test for all carbohydrates, in this test, carbohydrates when react with conc. H_2SO_4 get to from furfural and its derivatives. These products react with sulphonated α-naphthol to give a purple complex.

For this test, 1ml of supernatant sample was taken in test tube and one drop of 5% α-naphthol was added then, about 1ml conc. H_2SO_4 was added from the wall of the test tube, then purple color development was observed. Alongside, NCF media and glucose containing NCF media were also taken as negative control and positive control respectively.

Anthrone test

It is also another general test for all carbohydrates. In this test also, carbohydrate gets dehydrated when react with conc. H_2SO_4 to form furfural. This furfural reacts with anthrone to give bluish green colored complex. For this, 1ml of sample was taken and 2ml of anthrone reagent was added. Then it was mixed well and observed color development.

3.6.1 Quantification of Reduced carbon source (RCS) present in culture by DNS test (3,5-dinitrosalicylic acid)

Stock solution of glucose with concentration 1mg/ml was prepared. Then 1ml of glucose with different concentration starting from 1µg/ml to 10µg/ml were prepared along with distilled water as blank. They were treated with 1ml of DNS reagent and left for bathing for 5min at boiling water bath and then cooled them at room temperature. After cooling, 1ml of 40% Rochelle's salt was added then absorbance at 540nm was observed and calibration curve for carbohydrates was obtained.

For this, 1ml sample was taken from the NCF and normal culture in Eppendorf tube and bacterial cell was separated by centrifuging at 5000 rpm for 5 min. obtained supernatant was poured in the clean test tubes and 1ml of DNS reagents was added. Then sample and reagents were mixed well by vertexing for few second, and boiled for 5 min. Boiled sample was cooled down at room temperature, and 1ml of 40% Rochell salt was added. Finally, well mixed sample was subjected to the spectrophotometer and read absorbance at 540nm.

Sumner and colleagues devised the dinitrosalicylic acid reagent for determining reducing sugars. This solution comprises dinitrosalicylic acid, Rochelle salt, phenol, sodium bisulfite, and sodium hydroxide. Rochelle salt is included to prevent oxygen dissolution, phenol enhances color production and counteracts urine-derived phenol, while bisulfite stabilizes color

in the presence of phenol. The alkaline component is essential for glucose's reducing action on dinitrosalicylic acid (Miller, 1959).

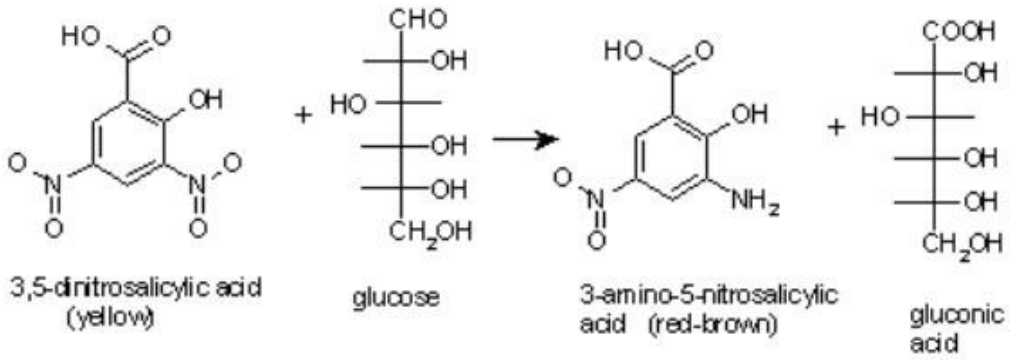

Fig 12: Redox reaction between DNS and reducing sugar

3.7 Microbial Electrochemical synthesis

While syntrophic bacterial growth improves growth rates, it remains insufficient for further implementation. To address this, we developed a technique where external electrons and protons are supplied to the syntrophic culture in a microbial fuel cell (MFC). Prior to setting up the MFC, the proton exchange membrane and graphite electrode were cleaned according to a specific protocol.

Cleaning protocol of Nafian Membrane

- Boil two hours in 3% H_2O_2 at 100°C
- Boil two hours in distilled water at 100°C
- Boil two hours in 0.5M H_2SO_4 at 100°C
- Boil two hours in distilled water at 100°C

Cleaning protocol of graphite electrode

- Ultra-sonicate for 15min in 70% methanol
- Ultra-sonicate for 15min in 70% acetone
- Ultra-sonicate for 15min in distilled water
- Expose in UV for 15min
- Either vaccum dry or overnight oven dry.

All the MFC apparatus were sterilized using 70% ethanol (Pun, 2019)

Then MFC was set up by joining two MFC apparatus and keeping the proton exchange membrane between them. 250ml of Phosphate buffer (pH 7) was prepared and poured in anodic chamber of MFC, along with 250ml of NCF media was also prepared and poured in cathodic

chamber of MFC. Similarly, 250ml of NCF media was prepared in the conical flask for normal culture of bacterial mixture. These were subjected to autoclave for the sterilization. After sterilization, overnight culture of four bacteria were inoculated aseptically (inoculum) diluted 100 times in media) in cathodic chamber MFC and conical flask containing NCF media. The graphite electrodes were used in both chamber and 3 volt electricity was supplied for the dissociation phosphate buffer. This setup was incubated at room temperature for 7 days and daily growth rate was observed by reading spectrophotometer at 600nm.

Chapter 4

Result and discussion

4.1 Revival of syntrophic bacteria

The bacterial isolates belonging to previously mentioned six genera preserved in glycerol stocks were revived in both LB broth and LB agar media. It was further sub-cultured and stored for future uses. The glycerol stock was prepared from the LB broth.

4.2 Culture of bacteria in selective medium

After the revival of bacteria in LB plates and agar, the selective media was prepared for different organisms. NCF media was prepared for the selective growth of Azospirrilium sp. This media would suit the growth of Azospirrilum because of its ability to fix nitrogen and carbon dioxide for its survival. NCF media along with the incorporation of ethylene glycol makes a selective medium for *Azotobacter* sp because of its peculiar ability to tolerate the level of ethylene glycol in the media. Likewise, *Pseudomonas*sp would grow in the NCF media with toluene and *Geobacter* sp would grow in NCF and aniline containing media.

4.3 Biochemical tests

Biochemical tests including Sulphide indole motility test, MR-VP test, Citrate utilization test, Triple sugar iron test, Oxidative and fermentative tests and urease test for different strains of bacteria were done. The results from the test are tabulated below.

Table 1: Results for different biochemical tests

SN	**Test**	***Azospirillum***	***Azotobacter***	***Pseudomonas***	***Geobacter***	***Bacillus***
1	Indole	Positive	Negative	Negative	Negative	Negative
2	MR	Negative	Negative	Negative	Negative	Positive
3	VP	Negative	Negative	Negative	Negative	Negative
4	Citrate	Positive	Positive	positive	Negative	Negative
5	TSIA	R/Y	R/Y	R/Y	R/Y	R/Y
6	O/F test	Oxidative	Oxidative	Oxidative	Oxidative	Oxidative
7	Urease	Positive	Positive	Positive	Positive	Positive

Notes-Y/Y-Yellow/yellow, R/R-Red/red, Y/R-Yellow/red

4.4 Co-culturing of different bacteria in NCF media

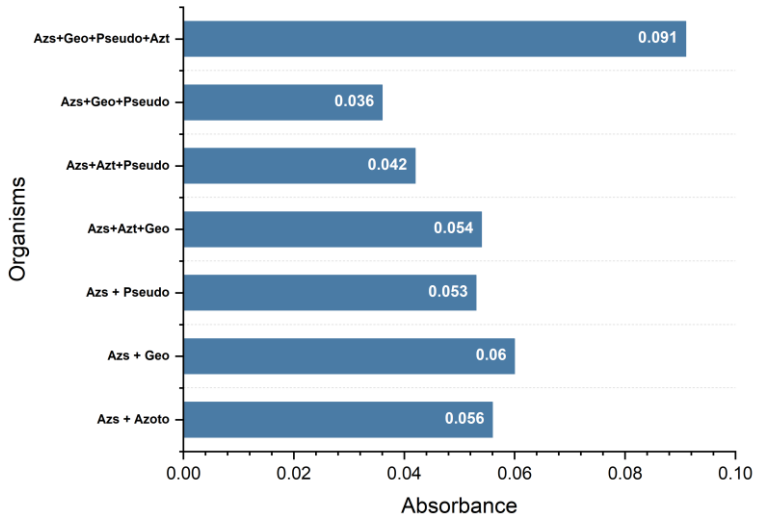

Fig 13: Growth absorbance recorded against the different bacterial population co-cultured.

Syntrophic growth of different bacterial mixture such as *Azospirillium* + *Geobacter* (Azos + Geo), *Azospirillium* + *Geobacter* + *Pseudomonas* (Azos + Geo + Pse), *Azospirillium* + *Azotobacter* + *Pseudomonas* (Azos + Azo + Pse) and *Azospirillium* + *Geobacter* + *Azotobacter* + *Pseudomonas* (Azos + Geo + Azo + Pse) were designed for reducing surplus amount of carbon into reduced carbon in a nitrogen carbon free media (NCF). As single or mono culture condition limit its potential. The single vessel culture or syntrophic culture consists of autotrophic like *Azospirillum* spp. which help in fixing carbon dioxide into reduce carbon with the help of RuBisCo, along with *Pseudomonas* spp., which help in fixing atmospheric nitrogen into ammonia, *Azotobacter* spp., which are fast respiring and helps in creating anaerobic or anoxic condition, this condition is pivotal for proper functioning of enzyme RuBisCo. Similarly, *Geobacter* spp are capable of donating as well as accepting electrons which helps in carbon fixation through Calvin cycle. The *Geobacter* spp. which are capable of accepting or donating electron this helps in converting inorganic carbon into reduced carbon.

Atmospheric CO2 and N2 dissolved in the media were taken as the carbon and nitrogen source, where electron and proton generated from splitting of water helps in CO2 and N2 fixation, following varied biochemical reactions.

The growth or population density of various bacterial mixtures was assessed using a spectrophotometer. Population density, which indicates the number of bacteria in the culture, was estimated by measuring the turbidity of the culture, usually expressed as OD (optical

density) at a wavelength of 600 nm. This measurement provides a convenient way to track the growth of bacteria in the mixtures over time.

Microbes, which thrive in nutrient-poor environments, likely don't follow typical growth patterns observed in lab cultures, with abundant nutrients. They metabolize slowly and have extended generation times, making it unlikely for them to display the typical growth stages seen in batch and continuous cultures.

Bacteria accumulate reserves during periods of nutrient abundance, storing them in various forms such as metabolite pools, compounds, granules, and elemental inclusions. These reserves serve as a crucial resource during periods of nutrient scarcity, determining the bacteria's ability to sustain themselves.

Off different bacterial mixture the one with four different bacteria shows the higher growth than the other bacterial mixture. As the media was devoid of nitrogen and carbon it is presume that atmospheric carbon dioxide and nitrogen was dissolved into the media. These dissolved carbon dioxide is converted into reduced carbon are used for energy generation. The genus *Geobacter* which has the capacity to donate electron as well as to accept electron, bacteria extract the electron from the metal which are present in the media. These electron play vital role in splitting water and thus helps in the carbon fixation through calvin cycle in the carbon reducing bacteria.

Azotobacter has the ability to grow rapidly and fix large amounts of nitrogen quickly by nitrogen fixation and convert atmospheric nitrogen into ammonia, which in turn is taken up and utilized by the plants (Prajapati *et. al*., 1970). To protect the nitrogenase enzyme it respire rapidly by consuming oxygen thus increase the carbon dioxide reduction in the enzyme RuBisCo.

Hydrogen released during the process of nitrogen fixation by the *Pseudomonas* spp., are metabolized by the hydrogenase enzyme into proton and electron, are used during carbon dioxide reduction.

From the above result we can conclude that in syntrophic culture containing different bacteria help each other, attach to each other, and find interesting possibilities to exchange electrons, nutrients or other compounds, and in turn form highly efficient cooperative metabolic processes different bacterial mixture. Syntrophy manifests across various scales of metabolic collaboration, from single cells to entire microbial communities. Microorganisms seek and

attach to each other to facilitate the exchange of electrons, nutrients, and other compounds, fostering highly efficient cooperative metabolic processes (Morris *et. al*., 2013).

4.5 DFT analysis

4.5.1 Molecular Electrostatic Potential

The analysis of electron density distribution around the molecule was conducted using molecular electrostatic potential (MEP). MEP serves as a gauge of the strength of nearby charges, including nuclei and electrons, at specific locations. By utilizing colors, MEP illustrates the shape and dimensions of the molecule. The electrostatic potential values are represented by distinct colors: blue denotes positive regions, indicating nucleophilic sites for nucleophilic attacks, while red indicates negative regions, representing electrophilic sites for electrophilic attacks. Green marks areas with zero potential within the molecule. On surface mapping, MEP intensifies in the following sequence: red, orange, yellow, green, and blue.

4.5.2 Frontier Molecular Orbital Analysis

Molecular orbitals provide a visual representation of how electrons are distributed in three-dimensional space. The energy difference (Eg) between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) is a crucial factor in quantum chemistry. This gap, known as the stability index, influences the chemical reactivity and kinetic stability of molecules.

$$Eg = E_{LUMO} - E_{HOMO}$$ (Hema *et.al.*, 2020)

Specifically, the energy of the HOMO (EHOMO) is linked to the ionization potential, while the energy of the LUMO (ELUMO) is associated with electron affinity. Understanding this energy gap is essential for predicting the electron transport properties of molecules. (Padmaja et.al., 2009).

The HOMO region of the molecules indicates the region with electron filled in its orbital whereas the LUMO region indicated the orbital with unoccupancy of the electron in its orbital. Therefore, the HOMO region of the molecule involves in the electron transferring during the process of reaction and LUMO region of the molecules involves in the electron receiving during the chemical or biochemical process of reaction.

The red and green mesh around HOMO and LUMO map of these molecules indicates the electron density around each phenomenon. The red mesh indicates the region with high

probability of electron density or distribution whereas the green region indicates the lower probability of electron density or activity.

4.5.3 MEP, HOMO and LUMO analysis of CO_2, CO_3, H_2O, Urea, Cobalt oxyhydroxide

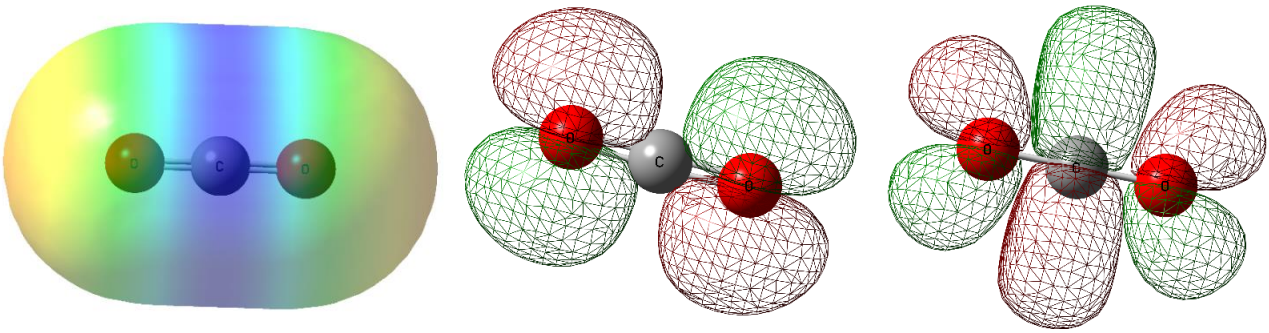

Fig 14: MEP, HOMO and LUMO graphical analysis of carbon dioxide.

Carbon-dioxide consists of two oxygen atom bonded to carbon with double bonds. From the figure we can observe the two opposite oxygen atom are shown in red color, thereby representing negative electrostatic potential. This is because of the presence of C=O. therefore, this red region represent the area of high electron density, thereby able to form bond with nucleophilic species. While, the area around the carbon is shown in blue color, thereby representing positive electrostatic potential, such that it has ability to receive electrons from electron rich species. The green region area represents zero potential area. Therefore, based on this MEP analysis we can say, electron density is distributed in higher concentration to oxygen atoms than carbon atom.

The highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) provides and information on how a reaction takes place. In this molecule of CO2, the HOMO housing high energy electrons, drives internal excitation, while LUMO awaits incoming electrons, therefore, acting either as an acceptor or donor.

During an internal excitation, electron jumps from this HOMO region i.e., from oxygen atom to the LUMO region. Similarly, when electron rich molecules comes closer to this LUMO region, it accepts electron and involve in reaction. Whereas, any electron loving compounds

come closer to this region, electron is transferred to LUMO from HOMO region is shared, thereby, facilitating in chemical reaction.

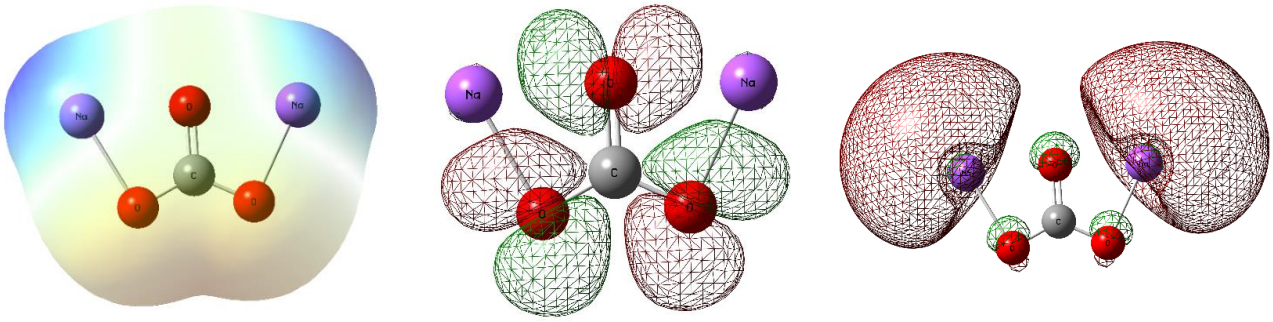

Fig 15: MEP, HOMO and LUMO graphical analysis of carbonate.

Carbonate on the other hand consists of three oxygen atom bonded to carbon atom via one double and two single bonds. On comparing MEP between CO_2 and CO_3^{2-}, distribution of electron density is different between three of the oxygen atoms. Thereby, suggesting carbonate is more susceptible to attack or involving in the reaction.

The HOMO region here is concentrated on the oxygen atoms. These oxygen atoms will have the highest energy occupied orbitals in the molecules. Therefore, HOMO helps in the internal

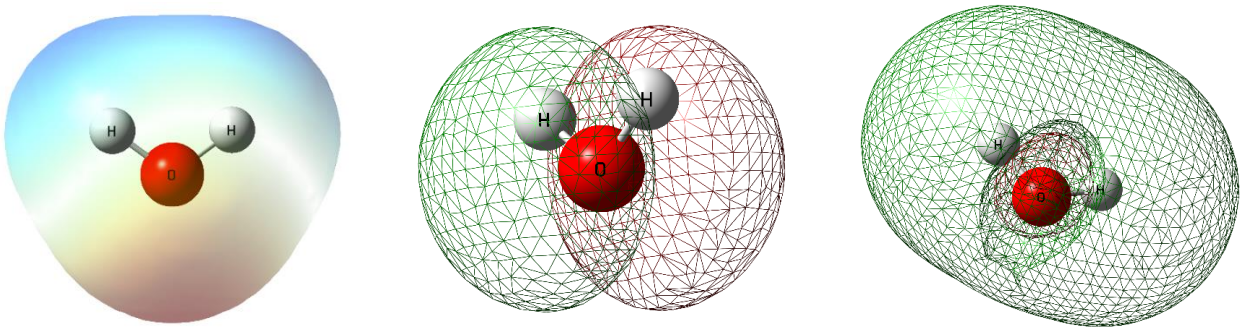

Fig 16: MEP, HOMO and LUMO graphical analysis of water.

excitation of the molecules, transferring electrons to LUMO region of the carbonate. With this HOMO and LUMO region, the chemical reactivity can be facilitated.

Water consists of two hydrogen atom linked to oxygen atoms. The oxygen atom here represents negative electrostatic potential, with high electron density such that it has the capability to donate shared pair of electron and forming bond. While, blue region around hydrogen atom, represents positive electrostatic potential, such that it has an ability to receive electrons from electron rich species.

HOMO region has high energy electron, assisting in the internal excitation of water molecule. While LUMO region is ready to receive or donate electron, helping in further reaction. With these electron areas in water, plays a key role in its chemistry and behavior.

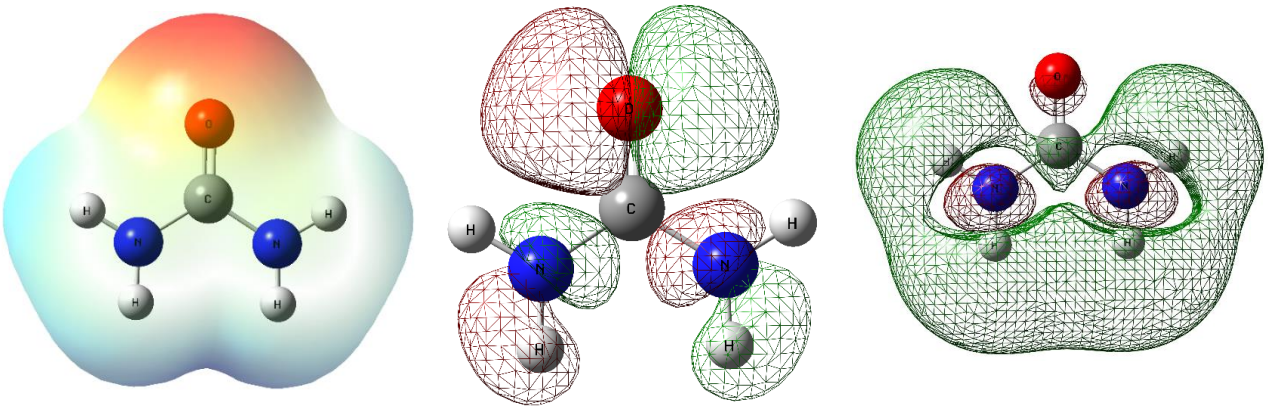

Fig 17: MEP, HOMO and LUMO graphical analysis of urea.

Similar is the case with urea, oxygen atom share an area with red color, indicating the atom with negative electrostatic potential. This oxygen atom being high in electron density will form chemical interaction with low electron carrying species or atoms. While, hydrogen atom bonded with the nitrogen atom show positive electrostatic potential, therefore having an ability to receive electron form the species with high electron density with in the molecule or between other chemical species.

Looking on the MEP of cobalt oxyhydroxide nanoparticle, the region around Co-O bond bears zero electrostatic potential as is represented by green in color. While oxygen atom bonded with Co via double bond shows prominent red color area. Therefore, indicating the region with high electron density. While, hydrogen bonded with other oxygen atom represent the area with positive electrostatic potential.

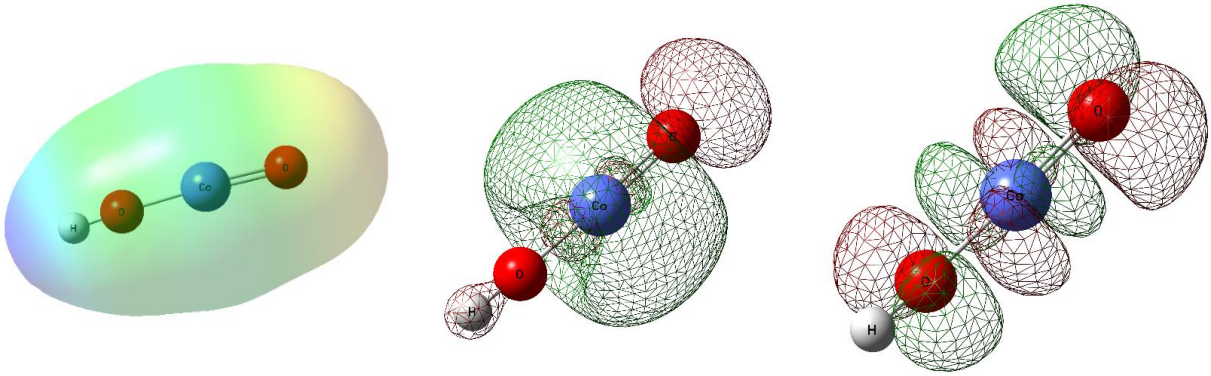

Fig 18: MEP, HOMO and LUMO graphical analysis of cobalt oxyhydroxide.

Based on this MEP analysis, HOMO and LUMO region of any molecules we can study about the reactive areas of any compounds based on the electron and proton interactions shows chemical and biological activity.

Table 2: Energy of highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) with Gap energy in kcal/mol.

Molecules	**HOMO (kcal/mol)**	**LUMO (kcal/mol)**	**Energy Gap (kcal/mol)**
Carbon-dioxide	-234.01	-2.20	231.81
Water	-181.73306	33.13	214.86
Urea	-154.86	33.89	188.75
Cobalt oxyhydroxide	-163.20	-84.51	78.69

4.6 Cobalt oxyhydroxide Nanoparticle Synthesis

Nanoparticle was synthesized using the first protocol with slight modifications (Patil *et. al*., 2016).

We devised another set of protocol in which we used Sodium percarbonate as oxidizing agent instead of 30% Sodium hydroxide. The whole protocol was same in other regards. The yield of nanoparticle was noted and X-ray diffraction and FT-IR results of synthesized nanoparticle were analyzed. The nanoparticle synthesized was thus weighed and stored for further use.

4.6.1 Characterization of synthesized nanoparticle

Fourier transfer infrared characterization of cobalt nanoparticle

FT-IR is generally done to find out the correct empirical formula for any compound. The characteristics peaks found corresponds to different types of interaction between atoms present in the compound. For our sample peaks at different location were seen. The presence of O-H bond is seen by the presence of peak at wavenumber 3408. The presence of Co-O vibrations is indicated by the peak at wavenumber 586 and 667. Similarly, presence of water molecule or hydroxide in cobalt colloids is observed at around wavenumber of 1649. The presence of these peaks when compared with the standards give us evidence to support the fact the FTIR results for synthesized nanoparticle are comparable (Jagadale et. al., 2012).

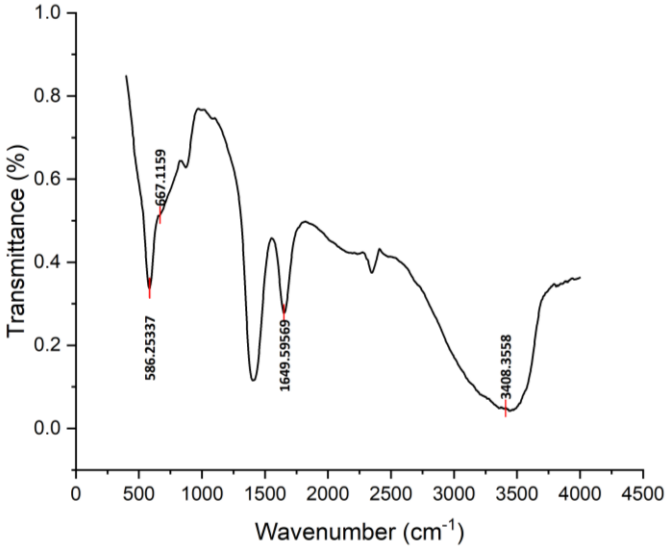

Fig 19: FTIR of cobalt sample with reference paper with transmittance (%T) via wavenumber (per cm).

4.7 Syntrophic culture in NCF media with varied concentration of cobalt nanoparticle

NCF media with Syntrophic culture with different concentration of cobalt nanoparticles were incubated and the bacterial growth pattern was observed for 7 days. The bacterial growth having 1.5mg/100ml cobalt nanoparticle and the bacterial culture without nanoparticles showed the significant growth on the 4th day of incubation.

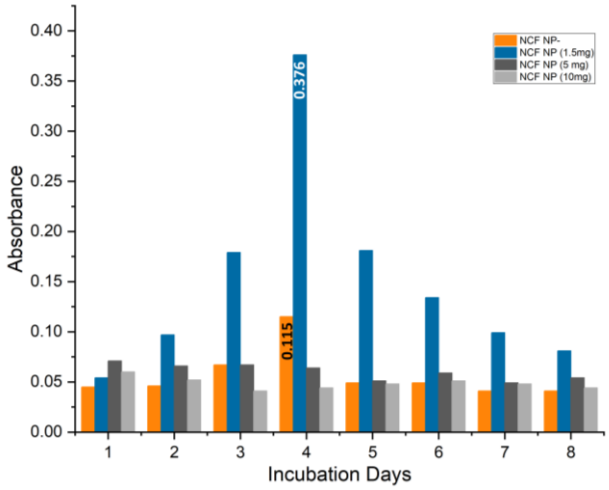

Fig 20: Syntrophic culture in NCF media with varied concentration of cobalt nanoparticle

However, the bacterial culture having 5mg/100ml and 10mg/100ml nanoparticles showed declining growth patter thus, suggesting the concentration might have played toxic effect to growing consortium.

The bacterial consortium without nanoparticle in the media shows small peak as compared to the bacterial culture containing 1.5mg/100ml of nanoparticle. We can presume that *Geobacter* spp , extract electrons from metals present in the media and generate electrons which enhances the mechanism of water splitting resulting in producing proton and electrons. These electron reduces carbon through Calvin cycle by the carbon reducing bacteria (Champine *et. al*., 2000).

Whereas the bacterial culture containing 1.5mg/100ml nanoparticle shows the significant growth on the 4th day. It can be assumed that the addition of cobalt nanoparticles enhances the water slitting more effectively which acts as catalyst.

Thus to enhance the carbon reduction or fixation we have to optimized the media with cobalt nanoparticles for enhancing the water splitting as cobalt possesses distinctive attributes, including exceptional electrical conductivity, thermal stability, unique electronic properties, chemical stability, and remarkable catalytic performances. These characteristics render cobalt-based catalysts highly promising materials for applications in carbon dioxide reduction reactions (CO_2RR). As an abundant transition metal found in the Earth's crust, cobalt presents itself as a splendid alternative to noble metals like platinum (Pt), iridium (Ir), ruthenium (Ru), and others (Usman *et. al*., 2021).

4.8 Media optimization using different bacterial consortium

NCF with different number of syntrophic culture and its growth pattern was observed to optimize the media. The syntrophic bacterial cultural with four organism has the significant growth pattern as compared to the bacterial consortium having six organism on the 4th day of incubation but when we observed the bacterial consortium containing six different organism showed the small peak as compared to the another consortium. From this we can assume that the *Bacillus* Spp. present in the consortium which are fast-growing, Gram-positive, aerobic bacterium, might have consumed the reduced carbon fixed by bacteria present in the consortium for producing various compounds like cysteine, SAM, vitamins, inositol, acetoin, hyaluronan and other chemical (Su *et. al*., 2020). Whereas, the other bacteria present in the consortium could not get enough reduce carbon to perform metabolic function thus resulting slow growth.

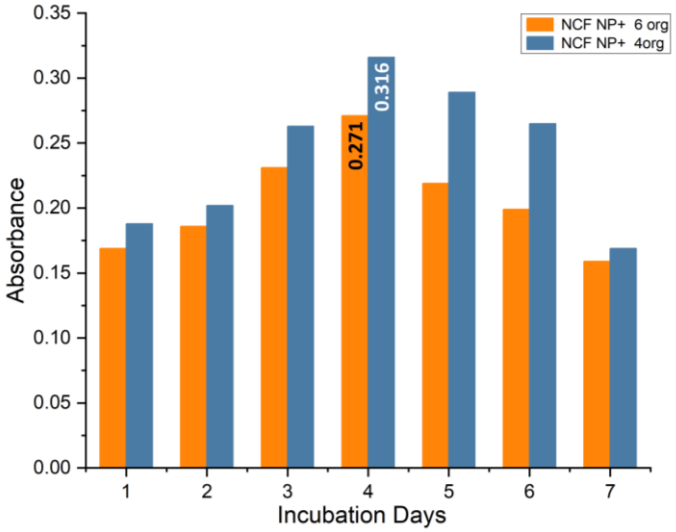

Fig 21: Growth comparison between the consortium of 4 and 6 different bacteria against the incubation days.

However, the bacterial consortium containing four bacteria reduces higher carbon on the 4th days of incubation thus resulting highest peak. These syntrophic vessel contain the Azospirillum spp which have the carbon dioxide reducing enzyme RuBisCo. This enzyme helps in reducing the carbon dioxide that were dissolved in the media from the air while preparing the media. Cobalt nanoparticle present in the media helps in splitting the water that releases electron and proton which are used by Azospirillum for reduction carbon dioxide. *Azotobacter* is fast respiring bacteria which help in increasing the carbon dioxide reduction as the oxygen is consumed by the *Azotobacter* to protect the nitrogenase enzyme.

Likewise *Pseudomonas* release hydrogen per nitrogen fixed. Hydrogenase helps in breakdown of released hydrogen into proton and electron which are consumed by the bacteria present in the media for increasing carbon dioxide reducing.

4.9 Detection of reduced carbon in media

Various qualitative tests are employed for the analysis of carbohydrates, including the Molisch test, Anthrone test, Benedict's test, Silver mirror test (Tollen's reagent), Iodine test, Barfoed's test, and Resorcinol (Seliwanoff's test). These tests rely on the formation of specific complexes during reactions, which are detected by observing changes in the color of the sample solution (Pooja *et. al*., 2022). Among them only Molisch's test and Anthrone's test was performed for the qualitative detection of reduced carbon in the media.

Molisch test

The Molisch test serves as a preliminary method to discern between carbohydrates and non-carbohydrates in a given sample. It operates on the principle of dehydration reactions to identify carbohydrate content. During the test, concentrated sulfuric acid is added to the sample, causing carbohydrates to undergo dehydration and transform into aldehydes. These aldehydes, such as furfural or hydroxymethylfurfural, are generated from the dehydration of pentoses or hexoses. The Molisch reagent, containing α-naphthol, then reacts with these aldehydes, resulting in the formation of a purple-colored condensation product. This distinct color change indicates the presence of carbohydrates in the sample, making the Molisch test a useful tool in carbohydrate analysis (Pooja *et. al*., 2022).

When the reduced carbon present in the NCF media was reacted with α-Naphthol which upon reacting with H_2SO_4, finally gives furfural which gives purple or violet colour ring structure as colour thus indicating there is the presence of reduced carbon or free CHO as a carbon source in the media.

As the media is free of nitrogen and carbon source, thus we can assume that the organism present in the media reduces or fix the carbon dioxide from air in the media for assimilating cellular processes.

Anthrone test

The Anthrone test provides a rapid and convenient means for quantifying carbohydrates, regardless of whether they are free or bound to lipids or proteins. This method is widely applicable across various types of carbohydrates, making it a versatile tool in carbohydrate analysis. By utilizing Anthrone reagent, which reacts specifically with carbohydrates, the test facilitates quick and efficient detection and quantification.

On reaction with conc. H_2SO_4 and the reduced carbon present in the media give the furfural which upon reacting with anthrone reagent gives blue green colour thus indicating that the organism present in the media reduces the carbon for their growth and to carry out the cellular processes.

As the media is free of nitrogen and carbon source, thus we can assume that the organism present in the media reduces or fix the carbon dioxide from air in the media for assimilating cellular processes. The Anthrone test is a method to ascertain the glucose content within a solution. This test involves the reaction of sugar with concentrated sulfuric acid (H_2SO_4),

resulting in the formation of furfural. Subsequently, furfural reacts with anthrone, yielding a distinctive blue-green coloration. During experimentation, the culture supernatant of the media exhibited a green coloration. This observation corroborated the results obtained from the Molisch's test, thereby suggesting that the isolate in question has the capability to utilize both gases for its growth. Consequently, it implies that upon further development as a bio-fertilizer, this isolate would not only mitigate these gases for its support but also foster the growth of other microorganisms within its ecological niche. This, in turn, would contribute to maintaining a high biomass in the soil by offering both reduced nitrogen and carbon sources.

Hence, the prospect of formulating a biofertilizer consortium through a syntrophic growth system, as proposed by Stams & Plugge, 2009, emerges as a viable avenue. Given the qualitative nature of the Anthrone test, the presence of reduced carbon sources in the media necessitated further analysis, which was accomplished through the Anthrone's test for quantification.

As the carbon dioxide reduction is high energy demanding process bacteria therefore uses energy from the hydrogen that are released during nitrogen fixation catalyzed by nitrogenase. Nitrogenase is an enzyme complex which is made up of two metal components, dinitrogenase MoFe (molybdenum-iron protein) serving as the catalytic component and dinitrogenase reductase (Fe protein). Nitrogenase is inactivated in aerobic environment because it is extremely O_2 sensitive.

Certain nitrogen-fixing microorganisms have developed strategies to mitigate the inhibitory or toxic effects of oxygen. For instance, some diazotrophic bacteria exclusively fix nitrogen under anaerobic or microaerophilic conditions to ensure low oxygen levels within the cell. Others, like *Azotobacter*, exhibit heightened respiratory rates as a mechanism to counteract the presence of oxygen. These adaptations enable nitrogen-fixing bacteria to thrive in environments with varying oxygen concentrations, facilitating efficient nitrogen fixation processes (Soumare *et. al.*, 2020).

Biological nitrogen fixation demands significant energy, with 16 ATP molecules used to break down one N_2 molecule, plus an additional 12 ATP molecules for NH^{4+} assimilation and transport, totaling 28 ATP molecules. For every gram of nitrogen gained, nodulating plants must supply their bacterial partners with 12 grams of glucose. However, despite this energy cost, biological nitrogen fixation remains more efficient than the Haber-Bosch process. Unlike the energy-intensive Haber-Bosch method, which requires temperatures of 400-500 °C and

high pressure, biological nitrogen fixation occurs under ambient conditions, making it a more sustainable and environmentally friendly option for nitrogen production (Soumare *et. al*., 2020).

4.10 Quantification of Reduced carbon source (RCS) in the media

DNS test was carried out to detect the presence of reduced sugar in the media containing two different bacterial consortium. Reduced carbon have the free aldehyde and ketone functional groups. From the figure, we can observed that the reduced carbon released by bacterial consortium containing four and six organism was found to reduce 45.75 µg/ml of carbon into the media.

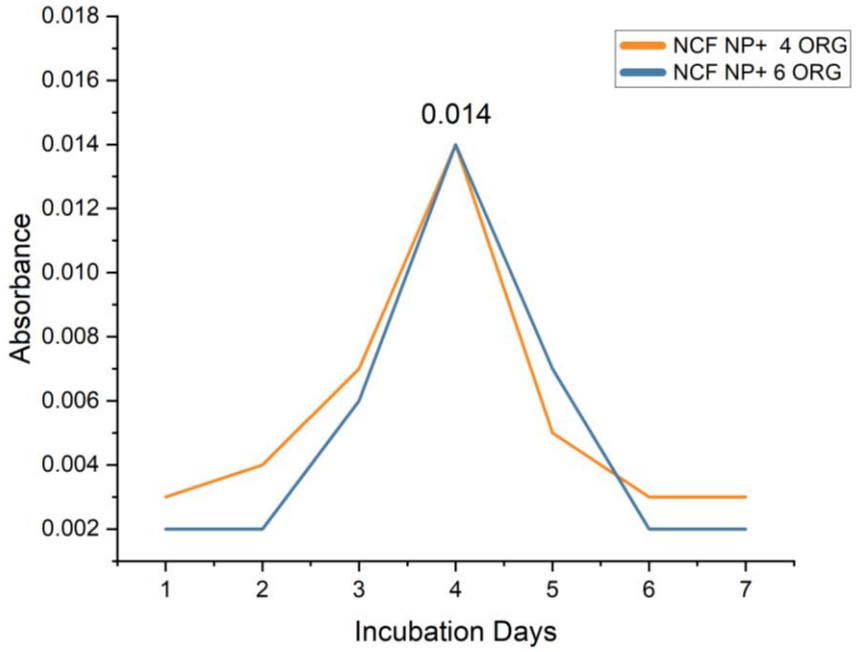

Fig 22: Reduced carbon estimation between the consortium, expressed as absorbance

With the increasing microbial growth, the ability to reduce carbon into the media also increases. Therefore, at the 4th day of incubation with the optimum growth the concentration of the carbon reduced in the media was also found optimum.

4.11 Quantification of Reduced nitrogen in the media

Nessler's test was carried out to detect the presence of ammonia or ammonium compound in the media. The amount of ammonium ion in the media was done by comparing the amount of ammonium ion in the media with the calibration curve drawn using ammonium hydroxide with Nessler's reagent.

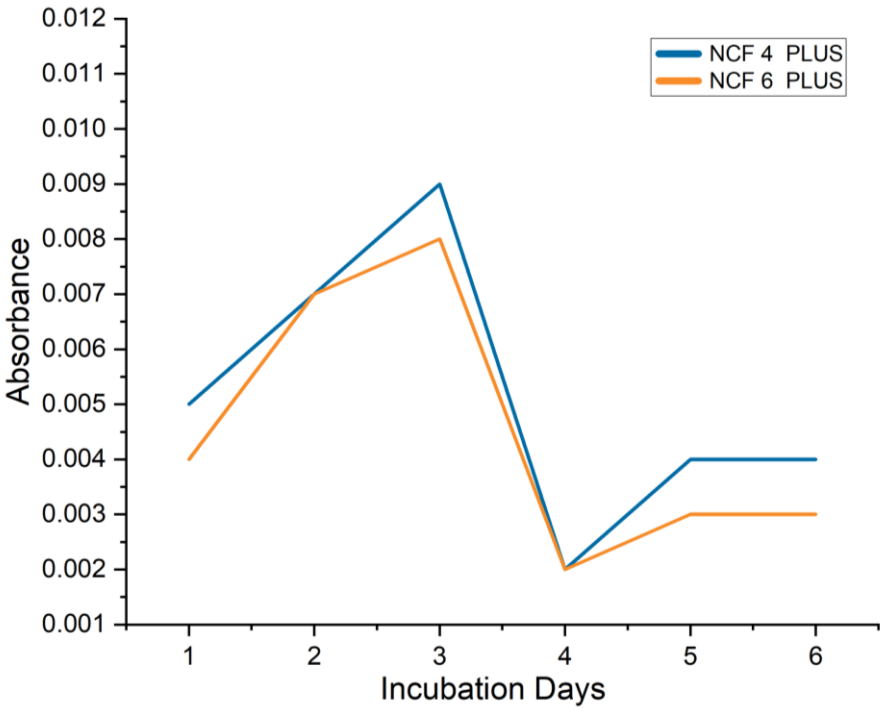

Fig 23: Reduced nitrogen estimation between the consortium expressed as absorbance.

As from the graph we can observed that on the 3th day of incubation absorbance of the ammonium ions on the media was maximum.

The amount of ammonium ions released by bacteria consortium containing four organism in media was found to be 2.466667 mM. Whereas, the ammonium ions released by the bacterial consortium containing six organism in per liter of media was found to be 2.466667 mM. The media is free of nitrogen but, while preparing the media the atmospheric nitrogen dissolved in the media from the air. the bacterial nitrogen fixing gene gets activated, thus concentration of ammonium ions in the media increases on the 3^{rd} days of incubation.

As the media have high concentration of fix nitrogen in the media the nitrogen fixation is repressed in the bacteria because nitrogen fixation required high energy therefore after the 3th day of incubation the ammonium ion concentration in the media is slowly decreasing.

If we observed the graph after 4th day of incubation the ammonium ion concentration of the media is slowly increasing. this might occur as a result of consuming the available ammonium ion by bacterial consortium thus bacteria sense the need of fixed nitrogen in the media therefore the bacterial activates the nitrogen fixing gene.

G. sulfurreducens employs distinctive control mechanisms in regulating nitrogen-fixing genes expression. The GnfL/GnfM system, a master regulator, triggers the activation of most nitrogen-fixation genes while simultaneously suppressing gdhA, responsible for encoding

glutamate dehydrogenase, when nitrogen-fixing conditions prevail. Moreover, it is likely to stimulate gdhA transcription under conditions where ample ammonium is present.

Another key system, GnfK/GnfR, which becomes active during nitrogen fixation under the influence of GnfL/GnfM, governs a specific set of nitrogen-fixation genes through transcription antitermination. These genes possess transcription termination signals in their promoters and are under the regulatory control of the GnfL/GnfM system. These regulatory pathways intricately manage the expression of nitrogen-fixation genes in *G. sulfurreducens* based on nitrogen availability, ensuring an appropriate cellular response given the resource-intensive nature of nitrogen fixation (Ueki & Lovley, 2010b).

4.12 Bacterial Consortium in MFC

A microbial fuel cell (MFC) harnesses the power of microorganisms such as algae and bacteria to break down pollutants via anaerobic oxidation, with the generation of electrons in the process. Thus, generated electrons travel through an external circuit to produce electricity, driven by the anaerobic respiration and metabolism of electrochemically active bacteria. Oxygen commonly acts as the electron acceptor at the cathode. Consequently, MFCs transform the chemical energy of organic matter into electrical energy, utilizing microorganisms as biocatalysts (Moinee and Sanzida, 2021). Compared to traditional low-temperature fuel cells, MFCs have a distinct advantage in utilizing complex organic contaminants as fuel in the anode chamber. They function at room temperature and a neutral pH, with specific bacteria starting the oxidation of contaminants at relatively low voltages (Logan *et. al*., 2008).

MFCs provide a more energy-efficient solution compared to conventional wastewater treatment plants by generating electricity from organic matter present in wastewater, thus offsetting the energy requirements of such treatment facilities.

The anodic chamber contained phosphate buffer and the cathodic chamber contain the syntrophic culture of four different bacterial species. Likewise, in another MFC, the anodic chamber contained the phosphate buffer but the cathodic chamber contained the six different bacterial consortium and MFC were incubated at the room temperature with external 3 volt. After incubation, the growth rate was observed everyday by reading in spectrophotometer at 600nm for optical density of bacteria. The result obtained showed that the bacterial consortium had the optimum growth on the 4th day of incubation. But when we compare the peak between

two bacterial consortium, then the bacterial consortium containing six organism showed the highest peak.

From this we can assumed this might have occurred because of external voltage supply which generates the proton and electron due to electrolysis which are then passed through electrode and proton exchange membrane, respectively. The *Geobacter* bacteria capture electrons from

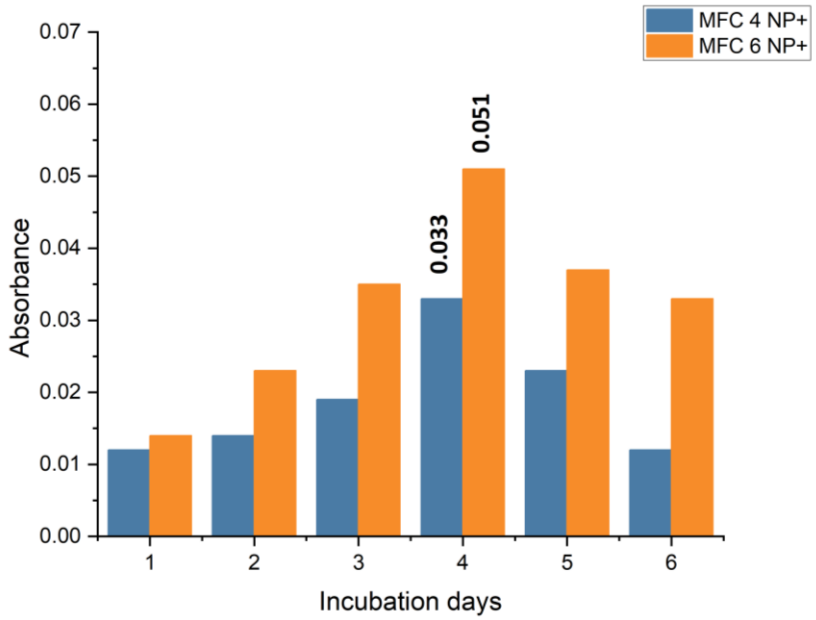

Fig 24: Growth absorbance of consortium in NCF media with different number of bacteria maintained in MFC

the electrode via nano-bio wires. These electrons would then be utilized by the bacteria for various metabolic processes, including the reduction of nitrogen (N_2) and carbon dioxide (CO_2). The bacterial consortium with six organism showed more growth in comparison with four organism. In this MFC setup continuous supply of electron remains available because of which continuous reduction of available CO_2 might have took place and *Bacillus* showing different growth pattern than available four organism, up took reduced carbohydrate. This might be the reason greater absorbance recorded in this 4th day of incubation in consortium with six group of organism.

Chapter 5

Summary

With the global population expected to approach 10 billion in the next 50 years, ensuring food security is a pressing challenge. Traditional agricultural practices involving chemical fertilizers and pesticides raise concerns regarding sustainability and safety. Thus, there's a rising interest in biofertilizers, offering sustainable and environmentally friendly alternatives for a resilient agricultural future.

Fossil fuels, dominant in global energy supply, emit GHGs like CO_2, contributing to climate change. Efforts to mitigate CO_2 levels involve global strategies, including CCS technology and biological carbon sequestration, converting CO_2 into biomass for environmental sustainability. Plant photosynthesis plays a crucial role in sequestering atmospheric CO_2, highlighting its significance in combating climate change.

Nitrogen, essential for plant growth, primarily exists as inaccessible dinitrogen in the atmosphere. Diazotrophs convert dinitrogen into soluble ammonia via nitrogen fixation, crucial for plant uptake. Molecular hydrogen serves as a specific inhibitor, while water and fixed nitrogen are key limiting factors for plant growth.

The diverse range of diazotrophs, from electrotrophic to autotrophic, suggests potential for a symbiotic growth system involving these organisms. Combining isolated strains like *Azospirillum*, *Azotobacter*, *Pseudomonas*, and *Geobacter* results in optimal growth, recommending *Geobacter* inclusion in nitrogen-fixing bio-fertilizer consortia. This synergistic approach not only substitutes nitrogenous fertilizers but also aids in CO_2 capture, promising sustainable agriculture and environmental benefits.

Certain *Azospirillum* strains possess RuBisCo enzyme and exhibit an autotrophic mechanism, capable of reducing CO2 even without a reduced carbon source, notably in dark conditions. Evidence of CO2 reduction was confirmed by the release of reduced carbon detected through Molisch's and Anthrone tests, underscoring *Azospirillum*'s potential for environmentally sustainable CO_2 reduction practices.

Azotobacter, a diazotroph, produces plant hormones such as indole acetic acids, gibberellic acids, naphthalene acetic acid, and vitamin B complex. These hormones play key roles in inhibiting root pathogens, promoting root growth, enhancing mineral uptake, and improving soil fertility.

MFC chamber with syntrophic culture, cobalt nanoparticle was used as a catalyst to enhance the water splitting thus releasing proton and electron which could been used to generate ATP and subsequent reduction of atmospheric N_2 and CO_2 dissolved in the media for enhancing microbial growth.

Chapter 6

Conclusion

Different bacterial were isolates and grown on the specific media. These isolates were able to fix atmospheric nitrogen and carbon-dioxide dissolved in the media. These isolates were biochemical characterized. Syntrophic culture with different bacterial consortium was performed. Cobalt nanoparticle was synthesized and characterized by XRD and FTIR. Additionally syntrophic culture with different concentration of cobalt nanoparticles was performed. MFC with syntrophic culture and cobalt nanoparticle was done by supplying 3 volt externally to observe the growth pattern. DFT analysis of carbon-dioxide, urea, cobalt oxyhydroxide and water was observed for understanding the electron properties of the molecules such that it will make easier for inferring the chemical and biological role with in this setup and study.

Chapter 7

Recommendations

- Experiment with different coatings or surface modifications for nanoparticles to prevent sedimentation and improve dispersion.
- Conduct field trials to assess the practical application of the technology in diverse agricultural settings.
- Develop integration strategies for seamless adoption of this technology into existing agricultural practices and infrastructure.
- Explore genetic engineering techniques to optimize the bacterial consortium's efficiency in nitrogen and carbon fixation and its interaction with nanoparticles.

Chapter 8

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Chapter 9

Appendix

9.1 Media Composition

NCF media composition

Ingredients	Composition
K_2HPO_4	0.5 g/l
$MgSO_4$	0.2 g/l
NaCl	0.1 g/l
$CaCl_2$	0.02 g/l
$FeSO_4$	0.05 g/l
Micronutrient solution	2 ml/l

Modified NCF media composition

Ingredients	Composition
K_2HPO_4	0.5 g/l
$MgSO_4$	0.2 g/l
NaCl	0.1 g/l
$CaCl_2$	0.02 g/l
$FeSO_4$	0.05 g/l
Micronutrient solution	2 ml/l
Cobalt Nanoparticle	15 mg/l

Micronutrient Solution (for 1000ml)

Ingredients	Composition
Nitrilotriacetic acid	1.5gm
Magnesium sulphate	3.0gm
Manganese sulphate	0.5gm
Sodium chloride	1.0gm
Ferrous sulphate	0.1gm
Calcium chloride	0.1gm
Cobalt chloride	0.1gm
Zinc chloride	0.13gm
Aluminum potassium sulphate	0.01gm
Boric acid	0.01gm
Sodium molybdate	0.025gm

9.2 Growth absorbance of consortium in NCF media with different concentration of nanoparticle

Incubation Days	NCF NP-	NCF NP (1.5mg)	NCF NP (5 mg)	NCF NP (10mg)
1	0.045	0.054	0.071	0.06
2	0.046	0.097	0.066	0.052
3	0.067	0.179	0.067	0.041
4	**0.115**	**0.376**	0.064	0.044
5	0.049	0.181	0.051	0.048
6	0.049	0.134	0.059	0.051
7	0.041	0.099	0.049	0.048
8	0.041	0.081	0.054	0.044

9.3 Growth absorbance of consortium in NCF media with different number of bacteria

Incubation Days	NCF NP+ 6 org	NCF NP+ 4org
1	0.156	0.169
2	0.175	0.186
3	0.179	0.231
4	**0.188**	**0.271**
5	0.19	0.219
6	0.191	0.199
7	0.145	0.159

9.4 Growth absorbance of consortium in NCF media with different number of bacteria maintained in MFC

Incubation Days	NCF NP+ 6 org	NCF NP+ 4org
1	0.013	0.013
2	0.016	0.034
3	0.021	0.038
4	**0.029**	**0.003**
5	0.015	0.005
6	0.016	0.009

9.5 Reduced nitrogen estimation between the consortium, expressed as absorbance and respective concentration

Incubation Days	Consortium of 4 organisms		Consortium of 6 organisms	
	Absorbance	**Concentration (mM)**	**Absorbance**	**Concentration (mM)**
1	0.005	2.59	0.004	2.55
2	0.007	2.675	0.007	2.6
3	0.009	2.75	0.008	2.7
4	**0.002**	**2.46**	**0.002**	**2.46**
5	0.004	2.55	0.003	2.5
6	0.004	2.55	0.003	2.5

9.6 Reduced carbon estimation between the consortium, expressed as absorbance and respective concentration

Incubation Days	Consortium of 4 organisms		Consortium of 6 organisms	
	Absorbance	**Concentration (µg/ml)**	**Absorbance**	**Concentration (µg/ml)**
1	0.003	18.25	0.002	15.75
2	0.004	20.75	0.002	15.75
3	0.007	28.25	0.006	25.75
4	**0.014**	**45.75**	**0.014**	**45.75**
5	0.005	23.25	0.007	28.25
6	0.003	18.25	0.002	15.75
7	0.003	18.25	0.002	15.75

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