Nano-biochemical Approach for 'One Pot' Reduction of Atmospheric N_2 and CO_2 for Bio-fuel and Bio-fertilizer

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Glossary Acronyms

µ	Micro
ATP	Adenosine Tri Phosphate
BLAST	Basic Local Alignment Search Tool
Bp	Base Pairs
DNA	Deoxyribonucleic Acid
Dntp	Deoxyribonucleotidetriphosphate
DMSO	Dimethylsulfoxide
EDTA	Ethylene Diamine Tetra Acetic Acid
L	Litre
LB	Luria Bertani
M	Milli
M	Molar
Min	Minute
NADPH	Nicotinamide Adenosine Diphosphate(H)
NCBI	National Center For Biotechnology Information
NEB	New England's Biolabs
OD_{600}	Optical Density At 600nm
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
Rpm	Revolutions Per Minute
RT	Room Temperature
Tm	Melting Temperature
Ta	Annealing Temperature
UV	Ultraviolet
IPCC	The International Panel Of Climate Change
DDW	Double Distilled Water
16S rRNA	Svedberg's unit ribosomal Ribonucleic Acid
TCA	Tricarboxylic Acid
LB	Luria Bertani
ET	Electron transfer

Mn	Manganese
Mo	Molybdenum
Zn	Zinc
Gdh	Gluamate dehydrogenase
XRD	X-Ray Diffraction
FTIR	Faurior transform infra-red
SEM	Scanning Electron Microscopy
TEM	Transmission Electron microscopy
CNT	Carbon nanotube
DNS	Di-nitro Salicylic Acid
PEM	Proton Exchange Membrane
mA	Milli Ampere
Mg	Milligram

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ABSTRACT

Taming the global warming and air pollution is one of the major issues till date and alternative reduced nitrogen carbon sources for developing bio-fertilizer and bio-fuel, respectively, is highly sought. Carbon dioxide reduction without mimicking the plant photosynthesis, serving the reactions in dark would be of love as it can overcome the burden of exposing light sources in vessel. Bacteria were isolated from Nepalease prehistoric nature soils (rhizosphere of tree fern) that included extracellular electron transfer bacteria *Geobacter sps,* diazotrophic *Azotobacter sps, Pseudomonas sps* and diazotrophic autotroph *Azospirillium sps* on the basis of Carbon Catabolite Repression (CCR). Syntrophic growth of microbial consortium was developed for reducing nitrogen (Nessler's test for ammonium) and carbon (Molisch's test). The reduced carbon was quantified by DNS method. Ethanol was detected (Dichromate oxidation method) by fermenting with commercially available yeast. The residue after ethanol production can be used as Nitrogen fertilizer since these diazotrophs can fix atmospheric N_2 upon application in soil. In addition, the CO_2 reduction potential will increase soil carbon content supporting the growth of other microbes in amending soil. Biogenic silica nanoparticles also synthesized from rice husk char for augmenting reduction rate in syntrophic growth. Synthesized silica nanoparticles were characterized by XRD, FTIR and SEM. Since silica is the most abundant minerals, functionalization of Si with ammonium group as a nitrogenous fertilizer to substitute urea was done. The developed technology could be further developed to mitigate global warming through nitrogen fixation to substitute urea and non-photosynthetic CO_2 reduction for bio-fuel and others.

Keywords: Bacterial consortium, Syntrophic growth, CO_2 reduction, Nitrogen fixation, Electrotrophs and Electricity, Biogenic Silica nanoparticle, Bio-ethanol, Bio-fertilizer**.**

1. INTRODUCTION

1.1 Background

Greenhouse effect is a natural phenomenon which warms up the earth but when amount of greenhouse gases like Carbon dioxide, Nitrous oxide, Methane, water vapour et cetera are increased by anthropogenic activities the earth captures more heat because of radiating Sun's infrared rays that have longer wavelengths obtained after striking on earth are absorbed readily by the greenhouse gases and is able to form 'thermal blanket' to the Earth (IPCC, 1990). The global annual temperature has increased at an average rate of 0.07°C (0.13°F) per decade since 1880 and at an average rate of 0.17°C (0.31°F) per decade since 1970 (NOAA, 2018). Moreover, annual temperature increment up to 2015 was found to be 0.90°C but in 2016 it rose by 0.94°C. This shows the increment of global temperature is increasing very rapidly and is thought to cause detrimental condition to environment.

The increasing global population that is predicted to reach nine billion by 2050 from recent seven billion plus is staining resources for food, feed and fuel (Sasson, 2012). It has been recently stated that if the present global population is to enjoy European standard of living the earth could sustainably support only about 2 billion people (http://www.worldpopulationbalance.org). Thus, sustainable alternatives are to be found. Alternative to depleting fossil fuel (Hook *et al*., 2010) and also its effect in global warming (IEA, 2010) is a must and bio-fuel could be an alternative (Balat and Balat 2009). However, the present day cereal based biofuel (FAO 2008) cannot sustain the food and feed demand (http://www.fao.org/fileadmin/templates/wsfs/docs/Issues_papers/HLEF2050) and the works on next generation fuel using lignocellulosic biomass could be alternative to cereal based. However, the land requirement for the lignocellulosic biomass could be a limiting factor (Limayem and Ricke, 2012). The land used for cultivation would be required for food production (Godfray *et al*., 2010) but all the agricultural waste could not be good source for alternative fuel (Sarkar *et al*., 2012). The forest could not be reduced to cultivate energy crops such as switchgrass. Thus, alternative mechanism of producing reduced carbon sources could be better option so that the marginalized land and sky above them could be used.

The main purpose of the study is taming the global warming caused by anthropogenic emission of carbon dioxide and nitrous oxide in controlling the air pollution of environment. Continuous production of carbon dioxide and carbon monoxide through vehicles and chemical industries has increased the CO_2 emission. In 2007 it was 28.8 Gt that is predicted to grow to 40.3 Gt by 2030 and 50 Gt by 2050 (Hu *et. al.,* 2018). In addition, chemically synthesized nitrogenous fertilizers are the source of contamination

of ground water and soil (https://www.sciencedaily.com/releases/2013/10/131021211720.htm). Application of the nitrogenous fertilizer is one of the main sources of nitrous oxide release through N-cycle (Thomson *et al*., 2012). Nitrous Oxide is also one of the major causes of global warming because there are lots of nitrogenous fertilizers used in agriculture and the addition of nitrate and ammonium fertilizers enhances release of the Nitrous oxide in the atmosphere at a rate of 0.25% per year (IPCC, 1190).

Adverse effect like monsoon flood in South Asia, severe drought in Africa, wildfire across world, Artic sea ice cover is at its low level, ocean more warmer and acidic than recorded in history are assumed to be the effect of global warming (NOAA, 2016). Thus, it is high time to explore or take the right action towards remedy for the global warming and pollution via using natural recycled resources. These crucial environment issues are no more blame game so each has to do from their own side. As of today, solar energy remains the most abundant renewable energy resource available to us but the limiting factor is its storage and low intensity of light make us increasing dependency on fossil fuel and it might create energy crisis in coming future. It is highly desirable to use for solar energy is powering fuel generation by water splitting, where chemical fuels (e.g., H_2) can be produced and stored. Some successful examples have been reported in the literature using heterogeneous photo-catalysts for visible light-driven water splitting (Zou *et al.,* 2001; Maeda *et al.,* 2006).

In addition, there has been rapid progress in mimicking natural photosynthesis, and an exploding body of research in this area holds much promise for improving our understanding of the natural systems and reducing the costs of solar energy conversion. Knowledge gained from research in photosynthesis has potential to greatly facilitate the development of efficient devices leading to the production of affordable and energy-rich fuels from natural sunlight. However, grand challenges remain, including the discovery of inexpensive, robust, and efficient water-oxidation catalysts. In addition, limited success has been achieved in coupling single-photon charge separation with well-defined homogenous catalysts (McConnell *et al*., 2010).

Actually the robust solution often could be found from nature. Before the lives were possible in the earth, totally there has been adverse situation (http://www.bbc.com/earth/story/20150701-the-origin-of-the-air-we-breathe) but to cope with the situation CO_2 got reduced and evolved oxygenic environment to make conditions possible for aerobic living organisms. Looking into modes of CO_2 reduction the CO_2 reduction in Crenarchaeota is through reverse Kreb Cycle. The Kreb Cycle is major energy producers in most of the aerobic organisms (Buchanan and Arnon., 1990) and thus is clear indication of evolutionary perspective of CO_2 reduction. This could be further substantiated because Euryarcheota such as Autotrophic *Archaeoglobales*

(Vorholt *et al*., 1995*)* reduce CO_2 through reductive acetyl CO-A Pathway anaerobically, the critical compound produced in the pathway Acetyl-CoA is assimilated via pyruvate synthase for leading to reductive Kreb or rTCA cycle (Berg 2011). Both of these mechanisms are energized by inorganic energy sources like oxidation of ammonia, hydrogen sulphide or elemental sulphur and Oxygen or metal ion used as electron acceptor (Barton, Fardeau, and Fauque, 2014). On the other hand, archea use CO_2 in the atmosphere for the fixation of Carbon by involving highly modified metabolic pathway, 3-hydroxypropionate/4-hydroxybutyrate cycle (Berg *et al.,* 2007) or Calvin Cycle, the cycle is important in photosynthetic dark reduction of CO_2 (Diwan and Joyce 2005). These modes of CO_2 reduction clearly indicate metamorphosis of CO_2 reduction that might have given rise to Calvin cycle through RuBisCo enzyme (Cooper and Geoffrey 2000), the most abundant CO_2 reducing enzyme found in several phyla (Dhingra, Portis and Daniell 2004).

This could be supported because lithotrophs fix CO_2 through Calvin cycle (Kuenen, 2009). They are either bacteria or archea that consume rocks for the electron rich reduced inorganic compounds for their energy source and are also called chemolithotrophs. Chemolithotrops mostly includes sulphur oxidizers, nitrifying bacteria, iron oxidizers, hydrogen oxidizers and mostly found around hydrothermal vent and other location where inorganic substrates are abundantly found. Presence of Calvin cycle in two different phyla and energy source being different than the Sunlight indicates that there must have been evolutionary perspective of this. This can be presumed because, Archea have not been reported to perform photosynthesis (Bryant and Frigaard., 2006) though Phototrophic archea Halobacteria has been reported but they have no obligatory rule having ability to be photosynthetic, giving rise to photoheterotroph. Thus, there are of two types of phototrophs: photoheterotrophs depends on light energy for production of ATP but obtained carbon from environmentally available organic compounds and photoautotrophs those includes green plant and cyanobacteria (Campbell *et al*., 2008). This clearly indicate that the photoautotrophy might have evolved from the combination of Calvin cycle and photoheterotrophy of ATP generation since most of the ATP generation in aerobic organism is through proton motive force across the membrane and these proton being generated in Kreb cycle (Berg, Tymoczko and Stryer., 2002) giving link to Crenachaeota, Euryarcheota, Archea and Phototrophic archea Halobacteria.

This link can be corroborated because there are some hydrogen sulphide oxidizing Cyanobacteria (Cohen 1989) to obtain energy for CO_2 reduction akin to lithotrophs (Friedrich *et al*., 2001) but most of them are photosynthetic and use Calvin cycle. Cyanobacteria, bacteria that obtain energy through photosynthesis, created the conditions in the planets early atmosphere that directed the evolution of aerobic metabolism and eukaryotic photosynthesis. They have microcompartment called as

carboxysomes having RuBisCo enzyme as well as the enzyme carbonic anhydrase used for metabolic channelling to help the local CO_2 concentration to increase RuBisCo enzyme activity. Separated compartments in thylakoid phycobilisomes acting as light harvesting antennae absorb light having 450nm to 660nm wavelength. Almost highly energized electrons come from oxidation of water and CO_2 is reduced to form carbohydrates through Calvin Cycle. Anyway they are considered as first creator of oxygenic environment and also known to reduce nitrogen and Carbon dioxide under aerobic condition though have modification in system during the process (Hamilton *et al*., 2016). Moreover, cyanobacteria are also diazotrops that fix nitrogen as well as carbon fixation through Calvin Cycle (Stal and Lucas, 2015.)

The linkage for CO_2 reduction and oxygenic environment evolution could be somewhere linked between archea that reduce CO_2 by using inorganic substrates without Sunlight but that system could not have been sufficient thus cyanobacteria evolved with advanced mechanism of CO_2 reduction in oxygenic environment using Sunlight available. Moreover, cyanobacteria are diazotrophs that fix atmospheric nitrogen (Fay P, 1992). However, the nitrogenase enzyme is labile (Robson, 1979) for oxygen and cyanobacteria have two compartments to reduce CO_2 that evolves oxygen and N_2 that works in absence of oxygen (Kerfeld, Heinhorst and Cannon, 2010). This opens an intriguing fact that could there be any linkage between diazotrophy and autotrophy where non-compartmentalized reduction of both CO_2 and N_2 could be feasible in the evolution and could there be any linkage of these reductions without Sunlight to sustain any life forms and what could be the source of energy. Since gaseous N_2 in atmosphere is not easily utilized major forms of life (Haselkorn and Buikema, 1992) but nitrogen is critical element in all living organisms (https://www.sciencelearn.org.nz/resources/960-the-nitrogen-cycle) diazotrophy must have evolved to sustain life forms.

As diazotrophs are those microorganisms that have the ability to reduce molecular (N_2) gas into ammonium ion (Burris, 1966) with high rate of biological nitrogen fixation in terrestrial environment (Cleveland *et al*., 1999) that can be utilized by microorganisms and plants. Several types of diazotrophs are found from endophytic, symbiotic, free living rhizospheric (Naher *et al.,* 2013) and associative (Santoyo *et al*., 2016) indicating their critical role in plant growth in natural environment. .

Most of these diazotrophs rely on root exudates (Döbereiner *et.al*., 1993) or other forms of reduced organic carbon source (Kuyper, Marchant and Kartal, 2011) for their growth. Moreover, some of these have ability to mineralize different rocks (Uroz *et al*., 2009) or oxidize metals (Ullah *et al*., 2015) indicating their ability to mobilize electron as energy source. This leads to the probability of evolutionary linkage with electrotrophs. Some electrotrophs like *Geobacter sulfureducens* have the ability to fix atmospheric nitrogen. All these organisms are facultative anaerobic (Hallberg, González-Toril and Johnson,

2010) indicating they can survive in poor oxygen conditions, too. Moreover, some have acetate as preferred reduced carbon source (Widdel, (1987) compared to glucose in several microorganism (Görke and Stülke, 2008). This indicates that these organisms have CO_2 fixation ability to through reversible Entner-Doudoroff Pathway for making macromolecules like amino and nucleic acids. Thus, this opens an avenue where bacteria should also have evolved diazotrophic autotrophy in fixing both CO_2 and N_2 for their growth. Some diazotrophs that might have evolved chemoautolithotrophy to autotrophy through Calvin cycle and in *Rhodospirillium rubrum* RuBisCo enzyme is found is also diazotrophic (Tabita *et al*., 2007). However, its autotrophy or photosynthetic mechanism and diazotrophy is only observed during anaerobic growth.

The intriguing fact that the diazotrophs have special mechanisms to protect nitrogenase from oxygen (Berman-Frank, Lundgren and Falkowski, 2003) till today indicates that CO_2 reduction that evolved from chemolithotrophy, reverse Kreb cycle to present day RuBisCo system must have evolved from anoxygenic environment. The photosynthetic organisms use Sunlight and differ in use of light wave length that plant use 680nm and 700nm whereas photosynthetic bacteria have special pair 760 nm, 840 nm and 870 nm and 960nm (Berg, Tymoczko and Stryer, 2002) to generate proton and electron but CO_2 fixation occurs in dark reaction center with RuBisCo (Silverstein and Alvin, 2008) that is labile to oxygen (Carmo-Silva *et al*., 2010) there must have been some evolutionary lineage where CO_2 could have been reduced in dark without any forms of light. The striking fact that some *Azospirillum* sps. such as *Rhodospirillium centenum* and *Azospirillium lipoferum* have RuBisCo gene in their chromosome (Sant'Anna *et al*., 2009) and are diazotrophs (Vessey, 2003). Some of them, *R. centenum* and *A. lipoferum* are reported to show autotrophy (Orlova *et al*., 2016). In addition, *A. thiophilum* shows litotrophic autotrophy and has enzymes for Calvin cycle indicating presence of RuBisCo (Orlova *et al*., 2016).

This indicates that syntrophic growth environment between chemolithotrophs and diazotrophs could have supported CO_2 and N_2 reduction which eventually merged genetically to give rise to diazotrophic autotroph that gave evolutionary perspective for RuBisCo system in all phyla and since organic carbon sources could be provided with those organisms including plants the diazotrophs eventually lost autotrophic gene (highly energy demanding (Gong, Cai and Li, 2016)). But, what could be evolutionary perspective for photosynthetic mechanism? The presence of RuBisCo gene in some *Azospirillum* sps indicates whether this is vestige only or has ability to reduce CO_2 contrary to photosynthetic mechanism. The presence of electrotrophic diazotroph or fast respiring diazotroph(Camil and Diaz, 2016)that consume oxygen or photosynthetic anaerobic diazotrophic autotroph it was presumed that diazotrophic RuBisCo containing organism could potentially reduce CO_2 in dark and syntrophic growth system was developed.

In addition, the Earth's most abundant material silicon dioxide that is plant stimulant could enhance CO_2 reduction rate in the present system because different semiconductor like titanium dioxide (TiO_2) have been used for the oxidation of water into oxygen and hydrogen in artificial photosystem because it has large band gap (more than 3ev) suitable for the water splitting (Barber and Tran, 2013) it can be presumed that silicon would support in water splitting as it is also a good semiconductor which has also more than 3eV band gap and ionic form of silicates could also help the mobilization of extra cellular electron and proton produced by electrotroph, *Geobacter sulfureducens*. Thus, silica nanoparticles were prepared and investigated.

In the present study the developed syntrophic growth or mixotrophic syntrophic growth with Si nanoparticles could support the growth of four different diazotrophic bacteria without any nitrogen or carbon sources without any light forms including Sunlight. The reduced nitrogen and carbon sources were further used to make bio-ethanol without any supplement with glucose, cellulose or other forms of reduced carbon source. The technology has been invented, though still in primitive form, where nitrogen and carbon can be simultaneously reduced in "One Pot" system without any light forms. This can abate the problem of harvesting Sunlight for water splitting (Bolton, Strickler and Connolly., 1985) horizontal farming of algal mass (Xu *et al*., 2009) or land use for energy crop (Strapasson *et al*., 2017) or cadmium sulphide mediated microbial CO_2 reduction (Iravani 2014). The technology has to be further engineered to increase CO_2 reduction as has been performed for carboxysome (Yeates *et al*., 2008). Moreover, the present system can be used to supplement reduced nitrogen source in cellulose mediated bio-fuel industry as nitrogen content of lignocellulosic biomass is lost during the pretreatment in minimizing the cost and the bacterial consortium could be developed as bio-fertilizer to substitute chemical nitrogen fertilizer. This would help in taming global warming caused by burning of fossil fuel or use of methane for urea production and N_2O released during nitrogen cycle of applied fertilizer. "One Pot" reduction of carbon and nitrogen has been devised that could not only substitute chemical nitrogenous fertilizer but could also store carbon in soil after application because of its ability to reduce CO_2. The increased CO_2 reduction in soil could support growth of other microbiome and make soil healthier.

1.2 Current Studies

Carbon and nitrogen fixation with growth rate of cyanobacteria is affected by light exposure, with carbon fixation depicting positive while with nitrogen fixation showing negative relationship with increasing light doses. The shortest light period has given maximal N2 fixation while longest light period with photosynthetic active radiation (400-700 nm) has given maximum photosynthetic carbon fixation (Cai and Gao, 2015)

indicating that “One Pot” nitrogen and carbon reduction could be impaired. Thus, in the present study this was achieved without use of any light sources.

In addition, recently, Daniel Nocera (2018), Harvard University’s Patterson Rockwood Professor of Energy, has developed a cell composed of a silicon sandwich which has a cobalt catalyst on top and Nickel –Molybdenum-Zinc alloy on the bottom that was able to split water molecule into hydrogen and oxygen thus can mimic photosynthesis which is most efficient. Plant can convert about 1 per cent of sunlight into energy whereas manmade the technology could produce at least 10 times better results and also achieved a solar energy to hydrogen efficiency of more than 10 per cent and the schematic illustration is represented in Figure 1.1. (https://www.scientificamerican.com/custom-media/pictet/besting-nature/). However, the Sunlight could be stumbling block because the system would require horizontal platform to harvest the light. In our study, the syntrophic growth system with electrotrophic diazotroph was also included for efficient electron transfer and Si nanoparticle and mineral mixture containing Mn and Mo, ZN was used in growth medium for dark reduction of both nitrogen and carbon. The hydrogen molecules thus generated could be oxidized to proton and electron by hydrolase of diazotroph or oxidation by electrotroph and could be used by autotroph to reduce CO2.

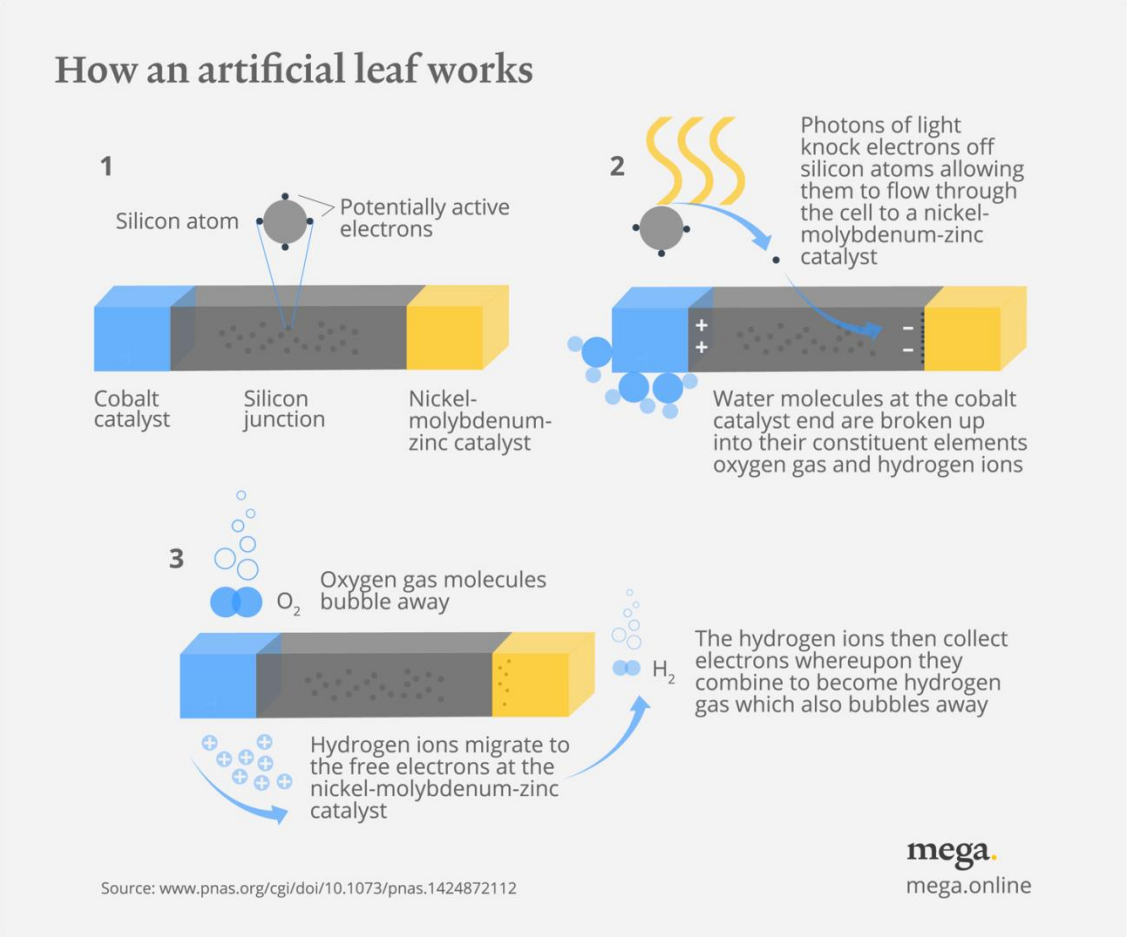


Figure 1.1: Working Mechanism of Artificial Leaf for Mimicking Photosynthesis

1.3 Hypothesis

The world is facing ever increasing challenges for climate change due to rapid population growth, industrialization and urbanization so that different unpredicted events like erratic rainfall, depletion of snow from mountain ranges, glaciers have been occurred, so taming the global warming as well as pollution, wish to exploring the natural phenomenon that exist in nature for the sequestration of carbon as well as Nitrogen via microorganism so that following hypothesis are predicted:

Null Hypothesis (H_0):

Before the diazotrophic photoautotrophy has been developed, there could not be microbial system to fix atmospheric CO_2 as well as N_2 without sunlight

Alternative Hypothesis (H_1):

Before the Diazotrophic photoautotrophy has been developed, there could be microbial system to fix atmospheric CO_2 as well as N_2 without sunlight

1.4 Objectives of the study

1.4.1 Broad objectives

- Mixotrophic growth system intricating nanoparticles and syntrophic consortium of diazotrophs for CO_2 and N_2 reduction to develop as bioethanol and biofertilizer.

1.4.2 Specific Objectives

- To develop a protocol to isolate the Electrotrophs, Diazotrophs and characterization of them
- To test the electricity generation from the isolated *Geobacter* sp.
- To test CO_2 and N_2 fixation at media by Putative *Azospirillium* and its effect on syntrophic growth
- To convert the captured carbohydrates into bioethanol and use of bacterial consortium as biofertilizer
- To extract silica nanoparticles and functionalization with nitrogen for making nitrogenous fertilizer

1.5 Rationale of study

The study give idea about how to adapt and mitigate the climate change and global warming by finding out the preliminary data of lowering carbon dioxide and capture with the help of microflora which has been isolated. If this one gives preliminary scientific data then one could establish industry and then can contribute not only the field of environment also in the field of world's economy by changing the system of dependency on chemical fertilizer as well as fuel industry. The study help to explore technology development and transfer in the climate change adaptation.

2. LITERATURE REVIEW

2.1 Carbon-dioxide and Nitrous oxide: cause of global warming

Global warming is thought to due to anthropogenic emission of greenhouse gases (GHG), mainly carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4) is greater than natural (Solar and Volcanic) is at an unprecedented level in past two millennia (Joos and Spahni, 2008). The atmospheric warming by emitted CO_2 accumulated is mostly due to readiative forcing is relatively irreversible even the emission is control as it takes hundreds of years to compensate due to slowing of heat in ocean (Solomon *et al*., 2009). Thus, the emission rate has to be decreased sooner so that whatever environmental losses we might have made could not be further detrimental to the Earth and human civilization. The largest CO_2 emitters in 2012 were reported to be China (about 29%), secondly United States shares 16%, followed by European Union with 11%, whereas India had 6%, with cumulative of Russian Federation to be at 5% and Japan shared 3.8%. Following graph has shown the status of CO_2 emission in the world (Olivier *et al.,* 2012).

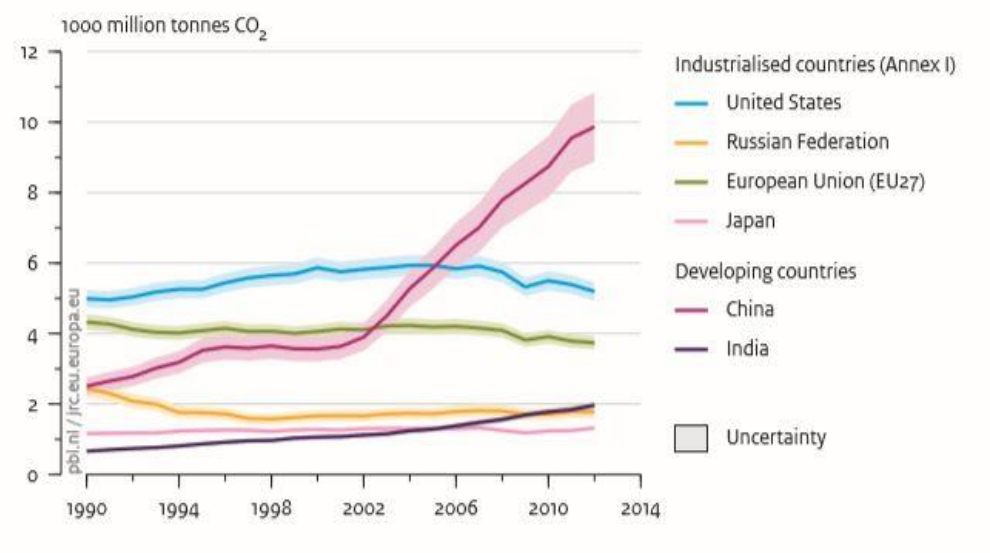


Figure 2.1: Global CO_2 emissions per region from fossil-fuel and cement production

About 90% of CO_2 emission is contributed from the fossil fuel combustion. As reviewed in (IPCC, 2014), in general, natural gas ~15 kg C/GJ) per unit of energy hold roughly half the amount of carbon (C) compared to coal (~26 kg C/GJ). Thus, the combustion of coal produces about 75% more CO_2 than that of natural gas. Coal combustion globally is responsible for 43% of CO_2 emissions from fossil-fuel combustion, with 28% emitted from coal-fired power plants. Industry, in particular iron and steel manufacturing, is the second largest source.

Nitrous oxides (N_2O) are also one of the causes of global warming. Per year nitrous oxide is increased at the rate of 0.25% (IPCC 1990). The Journal of Nature Geoscience mentioned that nitrous oxide is known as 'laughing gas' ranked in third major source behind carbon-dioxide and methane in contributing to global warming and effectiveness of nitrous oxide in trapping heat is 310 times faster than carbon dioxide where 60% of the nitrous oxide in atmosphere is produced itself in nature (https://www.triplepundit.com/2010/04/nitrous-oxides-global-warming-impact-no laughing-matter/).

Agricultural soils are the main source of nitrous oxide emission to the nature because all ammonium and nitrate fertilizer nitrogenous fertilizer are not used. Some are lost as nitrous oxide. The emission of nitrous oxide by the nitrification and denitrification depends on the content of N in the soil (Akiyama *et al*., 2000). Nitrification is dominant process and the gas diffusion is greater allow the emission of oxidized form of nitric oxide (NO) in dry and aerated soils but in moist soils gas diffusion and aeration is small and NO get chance to react with oxygen at atmosphere and is released as N_2O (Davidson *et al*., 2013). Urea is one of the most used synthetic nitrogenous fertilizers whose consumption has increased in these years very rapidly. Global chemical nitrogenous fertilizer production and consumption indicated that China will be the highest consumer nation as depicted in Figure 2.2 (Yahya *et al.,* 2018) where the production rise is consistent in recent years but it is projected to rise exponentially,

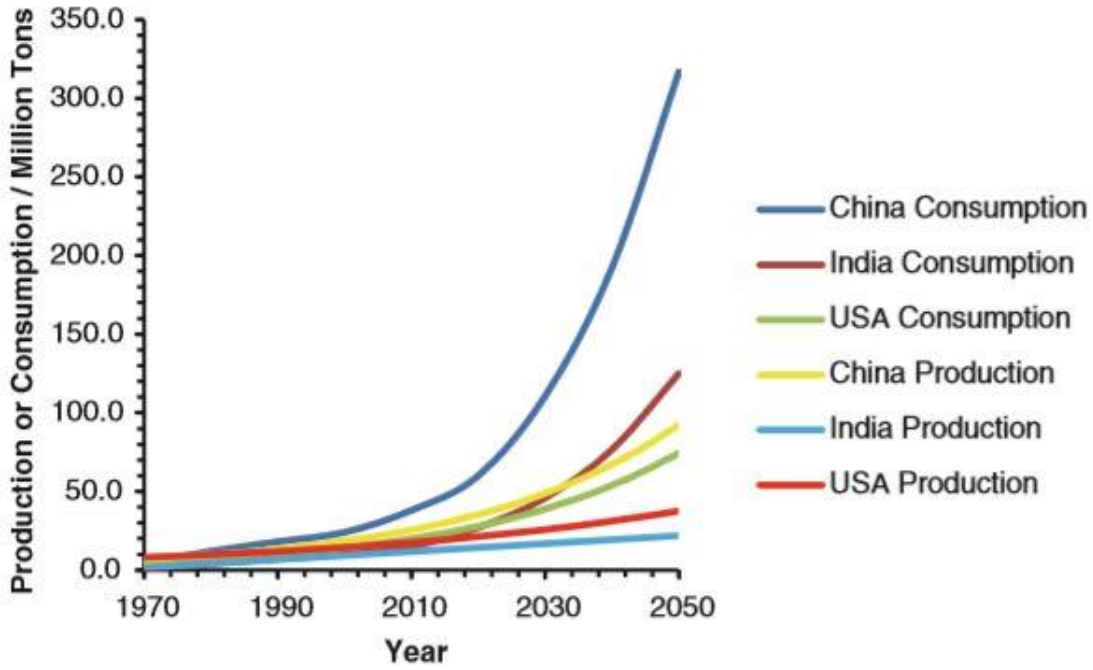


Figure 2.2: Production and consumption of nitrogenous fertilizer versus the year of China, India and the USA (Yahya *et. al.,* 2018).

2.2 RuBisCo

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) is the major enzyme assimilating CO_2 into the biosphere (andersson and Backlund, 2008). It is the most abundant enzyme found in most autotrophic organism, ranging from diverse prokaryotes, including photosynthetic and chemolithotrophic bacteria and archea to eukaryotic algae and higher plants, as it compromises about 50% of the soluble protein in the leaves or as specific bacteria (Ellis, 1979). There are almost four forms of RuBisCo that is Form I, II, III and IV or RuBisCo like protein whereas first three involved in the carboxylation and oxygenation of ribulose 1,5-bisphosphate while last one does not catalyse either of these reaction (Tabita *et al*., 2007). All RuBisCo enzymes are multimeric as shown in Figure 2.3.

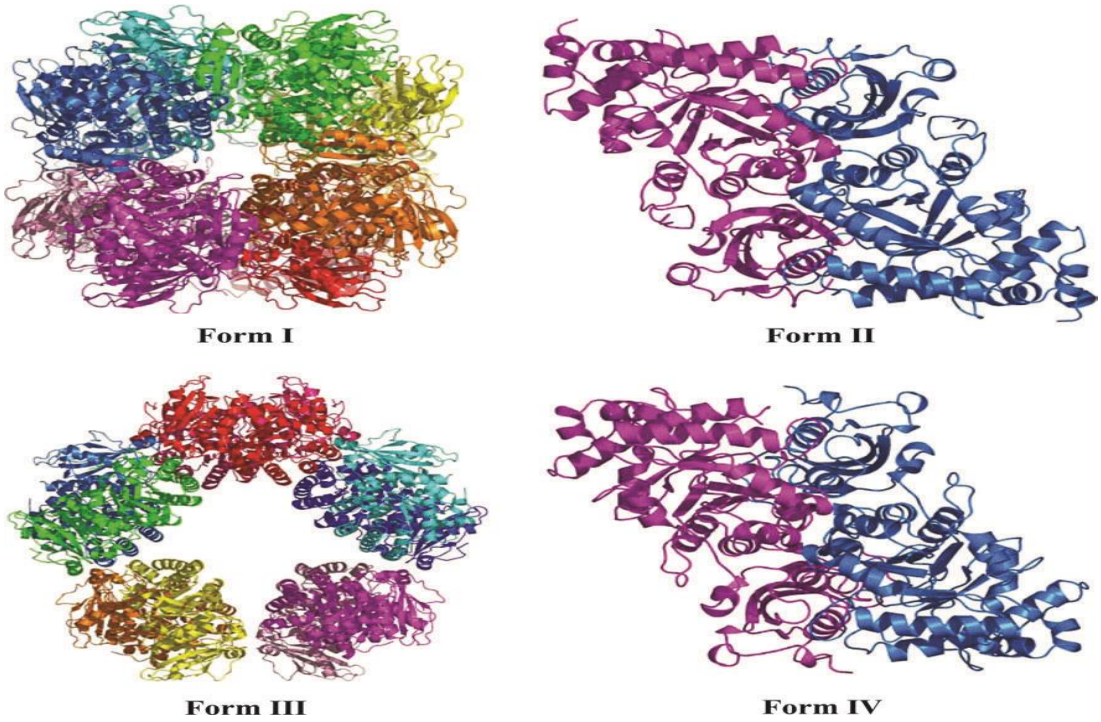


Figure 2.3: Representative structures of different forms of RuBisCo.

All forms are comprised of dimers of catalytic large subunits. Form I is comprised of four dimers with small subunits decorating the top and bottom of the L8 octomeric core. Only form I has small subunits. Form II is comprised of dimers of L, ranging from L2–8 depending on the source. Form III is found only in some archaea and is comprised of dimers of L in either an L2 or (L2)5 arrangements as above. Form IV (the RuBisCo -like Protein or RLP) appears thus far always to have an L2 structure (Li *et al*., 2005; F. R. Tabita *et al*., 2007).

Two different types of subunits occur: catalytic large (L, 50-55 kDa), and small (S, 12-18 kDa) subunits. The activity of enzyme depends on the temperature, substrate and

concentration. From the structural point of view an important distinction between different molecular forms of RuBisCo depends on the presence or absence of the small subunit.

Form I

The most common form (form I) of RuBisCo is composed of large and small subunits in a hexadecameric structure, L8S8 (Figure. 2.3). The molecule exhibits local 422 symmetries and consists of a core of four L2 dimers arranged around a four-fold axis, capped at each end by four small subunits. The small subunit is not essential for catalysis because *Synechococcus* have only large subunit octamer still possess carboxylase activity and undoubted specificity (Andrews, 1988).

Form I RuBisCo is present in most chemoautotrophic bacteria, cyanobacteria, red and brown algae, and in all higher plants. The incorporation of a small subunit in RuBisCo, giving rise to the form I enzymes, might have occurred at one specific stage in RuBisCo evolution. Based on amino acid sequences of the form I enzymes, a distinction have been made between green-type enzymes (forms I A and B from cyanobacteria, eukaryotic algae and higher plants) and red-type enzymes (forms I C and D from non-green algae and phototropic bacteria).

Form II

The form II enzyme is a dimer of large subunits (L2) and lacks small subunits (Fig. 2.3). The form II enzyme was initially discovered in purple non-sulphur bacteria, but has also been found in several chemoautotropic bacteria and in eukaryotic dinoflagellates. This has interesting implications for evolutionary relationships. Several non-sulphur phototropic bacteria such as *Rhodobacter sphaeroides*, *R. capsulatus*, several *Thiobacillus* sp., and *Hydrogenovibrio marinus* contain both form I and form II enzymes. The first crystal structure of RuBisCo was from the dimeric form II enzyme from *Rhodospirillum rubrum* (Tabita *et al*., 2007).

Form III

Archaea possessing a type III RuBisCo are all anaerobic and it is most likely that only the carboxylase activity of RuBisCo, and not the competitive oxygenase activity (by which RuBP reacts with O2 to prepare one molecule of 3-phosphoglycerate and one molecule of 2-phosphoglycolate), is biologically relevant in these strains (Sato *et al*., 2007)The protein is homodecamer, consisting of five dimer units which form a ring-like pentagonal structure. This arrangement is essential for its high thermostability. In contrast to form I RuBisCo, the form III RuBisCo is composed solely of large subunits (Ezaki *et al*., 1999).

Form IV

Although strongly related to RuBisCo family, it lacks the conserved Lys active site in position 327, which is replaced by an Arg residue, suggesting that it may catalyze enolization but not carboxylation. It may involve in sulfur metabolism and oxidative stress response (Hanson and Tabita, 2001).

It is suggested that microbes synthesize RuBisCo molecules that differ from the typical plant paradigm clearly, three separate bona fide forms of RuBisCo (forms I, II, and III) have now been described, each of which catalyzes the carboxylation or oxygenation of RuBP, albeit for potentially different physiological purpose (F Robert Tabita, 1999).

In addition to RuBisCo, there are some proteins they are termed RuBisCo like protein (RLPs). Both of these do possess functional similarities in that both proteins catalyze reactions using analogous substrates in both cases via an initial enolization-type reaction.

The RuBisCo homolog from *Chlorobium tepidum* was termed the RuBisCo -like protein (RLP) and categorized as form IV RuBisCo (Hanson and Tabita, 2001). Different studies confirmed the role of *C. tepidum* RLP in sulfur metabolism (thiosulfate-oxidation), and its disruption is reported to create a general stress response (Thomas, Hanson and Tabita, 2003). Furthermore, in *Bacillus subtilis*, based on phylogenetic analyses of currently available RLP sequences, genetic studies and biochemical analyses suggested that its RLP (or YkrW/MtnW) participates in a methionine salvage pathway and catalyzes the enolization of the RuBP analog 2,3-diketo5-methylthiopentyl-1-P (Tabita *et al.*, 2007).

Table 2.1 RuBisCo and RuBisCo like protein lineages and phylogenetic distribution

Lineage	Subunit composition	Rubisco activity	Rubisco active site	Phylogenetic distribution
I-A	L_8S_8	+	+	α, β, γ-Proteobacteria, Cyanobacteria, Prochlorales, Sargasso Sea metagenome, GOS metagenome
I-B	L_8S_8	+	+	Cyanobacteria, Prochlorales, Eukaryotes-Viridiplantae (Streptophyta, Chlorophyta), Euglenozoa, Sargasso Sea metagenome
I-C	L_8S_8	+	+	α, β -Proteobacteria, Chloroflexi
I-D	L_8S_8	+	+	α, β, γ-Proteobacteria, Eukaryotes-stramenopiles, Rhodophyta, Haptophyceae
II	L_2 and L_n	+	+	α, β, γ-Proteobacteria, Eukaryotes-Alveolata (Dinophyceae)
III	L_2 and $(L_2)_5$ (L_n?)	+	+	Methanogenic and thermophilic crenarchaeota, thermophilic and halophilic euryarchaeota
IV-NonPhoto	?	?	-	α, β, γ-Proteobacteria, Chloroflexi
IV-DeepYkr	?	-	-	α-Proteobacteria, Clostridia, Non-methanogenic euryarchaeota, Eukaryotes-*Ostreococcus tauri*
IV-AMC	?	?	-	Acid mine drainage microbial consortium
IV-GOS	?	?	-	GOS sequence collection
IV-Photo	L_2	-	-	α, β -Proteobacteria, Chlorobia
IV-YkrW	L_2	-	-	Firmicutes, acid mine drainage microbial consortium

Though there are many natural ways of CO_2 fixing pathway but anthropogenic emission of carbon dioxide should be decrease and create a carbon sequestration technique because it is urgent need of the world due to global warming. The biological CO_2 fixing gets much attention due to bio-refinery approach from which biofuels and value added chemical can be obtained (Cuellar-Bermudez *et al.,* 2015).

Hu *et al*.,(2018) have done engineered into autotrophic *Synechococcus elongatus* and heterotrophic CO_2 fixing *E. coli* phosphoenolpyruvate carboxykinase (PCK) pathway for malate production with ATP generation to provide a novel method for replenishing ATP to enhance CO_2 fixation and engineering CO_2 –fixing pathway to improve the production of value added chemicals. However, microbial production of value added chemicals from CO_2 faces some challenges, mainly economic, because of efficient ATP supply for CO_2 fixing pathway and installing of new pathway with biosynthetic pathways.

2.3 CO_2 Fixation

It is the process of converting inorganic carbon to organic carbon via living organisms. There are altogether six natural different pathways that are known to date for the carbon fixation. They are Calvin-Benson-Bassham cycle, the 3-hydroxypropionate cycle, the Wood-Ljungdahl pathway, the reductive tri-carboxylic acid (TCA) cycle, the dicarboxylate/4-hydroxybutyrate cycle, and the 3-hydroxypropionate-4-hydroxybutyrate cycle (Ducat and Silver, 2012). The Calvin cycle, the 3-hydroxypropionate cycle, and 3-hydroxypropionate-4-hydroxybutyrate cycle are aerobic, while the others pathways are anaerobic pathways because of the presence of certain oxygen-sensitive enzymes. The schematic diagram of six natural pathway of carbon fixation is illustrated in Figure 2.3.

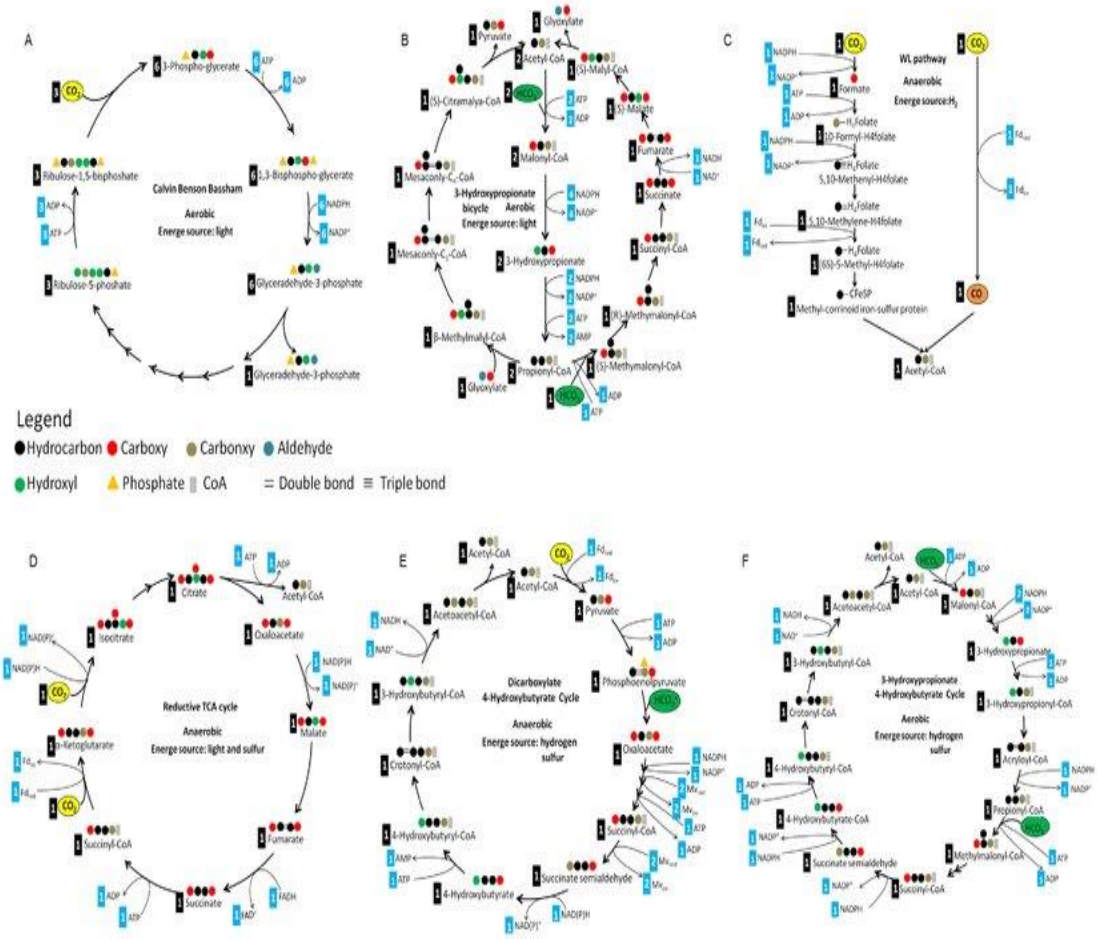


Fig. 2.4: Natural CO_2-fixation pathways - A: Calvin cycle; B: hydroxypropionate cycle; C: Wood Ljungdahl pathway; D: reductive TCA cycle; E: dicarboxylate/4-hydroxybutyrate cycle; F: 3-hydroxypropionate/4-hydroxybutyrate cycle

2.3.1 Aerobic CO_2 Fixation Pathway

A. Calvin-Benson-Bassham cycle

This is the most important CO_2 fixation pathway in nature from which all crop biomasses obtain their reduced carbon. It exists widely in plants, algae, cyanobacteria, and other organisms and most of these are associated with the photon driven water hydrolysis (Krapp, Quick, and Stitt, 1991). Calvin cycle converts three molecules of CO_2 to one molecule of glyceraldehyde 3-phosphate, with the consumption of nine ATP molecules and six nicotinamide adenine dinucleotide phosphate (NAD(P)H) molecules (Gong *et al*., 2016). It is the highest energy-consuming pathway among all six natural CO_2-fixation pathways. RuBisCo is the rate-limiting enzyme in this cycle, with an average activity of 3.5 µmol min–1 mg–1 (Bar-Even *et al*., 2010).

B. Hydroxypropionate cycle

The 3-hydroxypropionate cycle (Figure 2.3 B) exists in photosynthetic green non-sulfur bacteria and is driven by light, most complex, containing 16 enzymatic reaction steps that are catalyzed by 13 enzymes. In contrast to the Calvin cycle, which converts CO_2 to glyceraldehyde 3-phosphate, this cycle converts three molecules of HCO_3– into one molecule of pyruvate, with the addition of five ATP and NAD(P)H molecules. There are two CO_2-fixing enzymes in this cycle: acetyl-CoA carboxylase and propionyl-CoA carboxylase, catalyze CO_2 fixation (Ishii *et al*., 2004).

C. 3-hydroxypropionate/4-hydroxybutyrate cycle

Archaeal aerobic CO_2-fixation pathway discovered in 2007 is the 3-hydroxypropionate-4-hydroxybutyrate cycle, which is driven by sulfur and hydrogen. This cycle synthesizes one molecule of acetyl coenzyme A from two molecules of HCO3–, four molecules of ATP, and four equal molecules of NAD(P)H. The two CO2 fixing enzymes used are the same as those of the 3-hydroxypropionate cycle (Berg *et al*., 2010).

2.3.2. Anaerobic CO_2 Fixation Pathway

D. WoodLjungdahl pathway

The Wood-Ljungdahl pathway (Figure 2.4 C), which exists mainly in acetate-producing anaerobes, was identified in the 1970s by Harland G. Wood and Lars G. Ljungdahl (Ragsdale, 2008) and uses hydrogen as its energy source. It is the only non-cycle CO_2-fixation pathway, contains the fewest reaction steps, and consumes the least amount of energy. This pathway converts two molecules of CO_2 (or one molecule of CO_2 and one molecule of carbon monoxide) into one molecule of acetyl coenzyme A, using one ATP and four NAD(P)H molecules. It is therefore called the anaerobic acetyl coenzyme A pathway (Drake, 1994).

E. Reductive TCA cycle

The reductive TCA cycle (Figure 2.4 D) exists in photosynthetic green sulfur bacteria and anaerobic bacteria. This cycle generates one molecule of acetyl coenzyme A via two molecules of CO_2, with the consumption of two ATP and four NAD(P)H molecules (Evans *et al*., 1966; Kim *et al* , 1992)The two CO_2-fixing enzymes in this cycle are α-keto glutarate synthase and isocitrate dehydrogenase. The enzyme α-ketoglutarate synthase is strictly anaerobic, with unknown activity. Isocitrate dehydrogenase has the highest activity amongst all CO_2-fixing enzyme (Berg *et al*., 2010).

F. Dicarboxylate/4-hydroxybutyratecycle

The archaeal anaerobic CO_2-fixation pathway—the dicarboxylate/4-hydroxybutyrate cycle (Figure 2.4E)—was discovered in 2008. This cycle uses sulfur and hydrogen as energy sources (Huber *et al*., 2008). One molecule each of CO_2 and HCO_3– are used to synthesize one molecule of acetyl coenzymeA consuming three ATP and four NAD(P)H molecules. The CO_2-fixing enzymes in this cycle are pyruvate synthase and phosphoenol pyruvate carboxylase. Pyruvate synthase is strictly anaerobic enzyme which coverts acetyl Co-A to pyruvate. It is reported that the Km of phosphoenol pyruvate carboxylase to HCO_3–is the smallest amongst all carboxylases (Berg, Kockelkorn, Buckel, and Fuchs, 2007; Huber *et al*., 2008).

Table2.2: Summary of Six Natural CO_2 Fixation Pathway

	Organisms	Energy	Species	Reaction numbers	Total reaction equations	ATP/CO_2 (mol/mol)	NAD(P)Ha /CO_2 (mol/mol)	CO_2-fixing enzymes	Specific activity μmol min^{-1} mg^{-1} (CO_2/HCO_3^-)	Reference
A	Plant Algae Cyanobac-teria	Light	Maize *Scenedesmus* sp. *Synechocystis* sp.	13	$3CO_2$+9ATP+ 6NAD(P)H→ GA-3P+ 9ADP+6NAD(P)$^+$+8Pi	3	2	RuBisCO (EC: 2.1.1.127)	3.5	(Bar-Even et al., 2010; Calvin, 1949; Calvin and Massini, 1952)
B	Green nonsulfur bacteria	Light	*Chloroflexus aurantiacus*	16	$3HCO_3^-$+5ATP+ 5NAD(P)H→Pyruvate+ 3ADP+2AMP+3Pi+ 2PPi+5NAD(P)$^+$	1.67	1.67	Acetyl-CoA carboxylase (EC: 6.4.1.2) Propionyl-CoA carboxylase (EC: 6.4.1.3)	18 30	(Bar-Even et al., 2010; Herter et al., 2001; STRAUSS and FUCHS, 1993)
C	Anaerobic bacteria	Hydro-gen	*Clostridium ljungdahlii*	8	$2CO_2$+ATP+2NAD(P)H+ $2Fd_{red}$+CoASH→AcCoA+ ADP+Pi+2NADP$^+$+$2Fd_{ox}$	0.5	2	Formate dehydrogenase (EC: 1.2.1.2) CO dehydro-genate/Acetyl-CoA synthase (EC: 2.3.1.169)	2.34 0.46	(Drake, 1994; Ragsdale, 1997)
D	Green sulfur bacteria	Light Sulfur	*Chlorobiumthio-sulfatophilum*	9	$2CO_2$+2ATP+2NAD(P)H+ FADH+Fd_{red}+CoASH→ AcCoA+2ADP+2Pi+ 2NAD(P)$^+$+FAD$^+$+Fd_{ox}	1	2	2-Oxoglutarate synthase (EC: 1.2.7.3) Isocitrate dehydrogenase (EC: 1.1.1.87)	– 53	(Bar-Even et al., 2010; Evans et al., 1966; Kim et al., 1992)
E	Archaea	Hydro-gen Sulfur	*Ignicoccus hospitalis*	14	CO_2+HCO_3^-+3ATP+ NAD(P)H+Fd_{red}+4MV_{red}+ CoASH→AcCoA+2ADP+ AMP+2Pi+2PPi+NAD(P)$^+$ +Fd_{ox}+ 4MV_{ox}	1.5	2	Pyruvate synthase (EC: 1.2.7.1) Phospho-enolpyruvate car-boxylase (EC: 4.1.1.31)	– 35	(Bar-Even et al., 2010; Huber et al., 2008)
F	Archaea	Hydro-gen Sulfur	*Metallosphaera sedula*	16	2HCO_3^-+4ATP+ 4NAD(P)H+CoASH→ AcCoA+3ADP+3Pi+ AMP+PPi+4NADP$^+$	2	2	Acetyl-CoA carboxylase (EC: 6.4.1.2) Propionyl-CoA carboxylase (EC: 6.4.1.3)	18 30	(Bar-Even et al., 2010; Berg et al., 2007)

2.4 Nitrogen Fixation

The chemical processes by which atmospheric nitrogen is converted into ammonia or nitrogenous compound by means of microorganisms as a part of nitrogen cycle is commonly said nitrogen (N_2) fixation. N_2 fixation occurs in three different ways: (i) through geochemical processes such as lightning, (ii) biologically through the action of the enzyme, nitrogenase, found only in a select group of microorganisms, and (iii) industrially through the Haber–Bosch process. About 60 % of synthetic nitrogen fertilizers are presently used for cereals, with irrigated rice production accounting for approximately 10 % of the use. Since >20 % (Da Silva *et al* , 1978) of the fertilizer applied is actually used by plants, the inefficient use of nitrogen contributes to nitrate contamination of soils and ground water, leading to health hazards and compromising agricultural sustainability. Moreover, manufacturing N fertilizer requires six times more energy than that needed to produce either P or K fertilizers (Da Silva *et al* , 1978).

Burgess and Lowe in 1996 stated that diazotrophs encode nitrogenase, the enzyme complex that catalyzes the conversion of N_2 gas to ammonia. The nitrogenase complex is highly conserved in free-living and symbiotic diazotrophs and structure and function of nitrogenase enzyme complexes involved in nitrogen fixation have several salient features. Foremost, nitrogenase is a two-component system composed of the MoFe protein (also called dinitrogenase or component I) and the electron-transfer Fe protein (also called dinitrogenase reductase or component II) (Bulen and LeComte, 1966). For this, to complete the reduction it requires a reducing source and MgATP are required for catalysis (Mortenson, 1964). The reduction happens through single electron transfer and MgATP hydrolysis where Fe protein and MoFe protein associate and dissociate in a catalytic cycle and the ultimate N_2 reducing MoFe protein contains two metal clusters: the iron–molybdenum cofactor (FeMo-co), which provides the active site for substrate binding and reduction, and P-cluster, involved in electron transfer from the Fe protein to FeMo-co (Hoffman *et al*., 2014). Crystallographic structures for both Fe51 and MoFe32 proteins have been resolved, the alternative V- and Fe-type nitrogenases, in which the Mo of FeMo-co is replaced by V or Fe have been also discovered (Andersson *et al*., 2006).

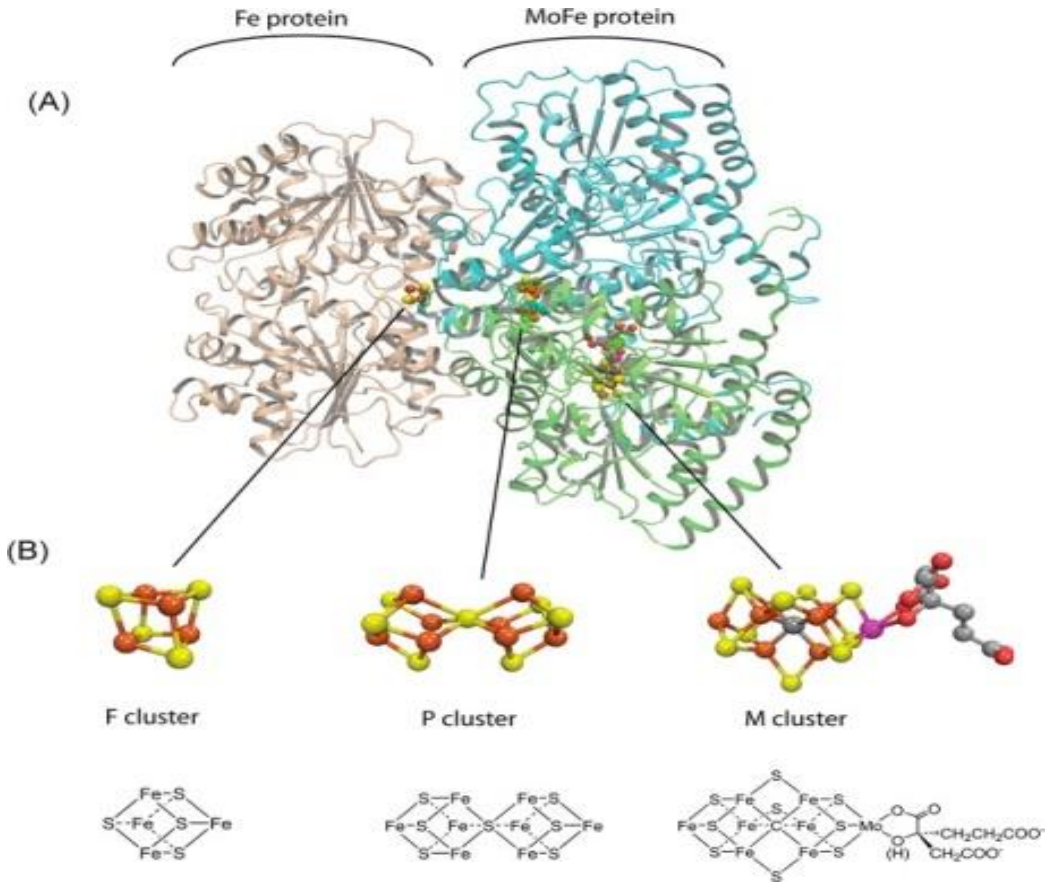


Figure 2.5: Molybdenum Nitrogenase. (A) One catalytic half of the Fe protein: MoFe protein complex with the Fe protein homodimer shown in tan, the MoFe protein α subunit in green, and the β subunit in cyan. (B) Space filling and stick models for the 4Fe–4S cluster (F), P-cluster (P), and FeMo-co (M) (Hoffman *et al*., 2014)

Schematically the mechanism is explained in Figure 2.5. Mainly during nitrogen deprivation conditions the diazotrophs sense depletion of glutamine inside the cells expresses *nif* operon that is repressed by *nif*R upon derepression by *nif*A. Upon expression of *nif* operon the proton and electron is provided by NADPH which upon oxidation reduces ferrodoxin that then subsequently transfer the electron to Fe-S cluster. This reduced Fe-S cluster then transfer the electron to P-cluster and subsequently Fe-Mo-co finally reduces one molecule of nitrogen at the expense of eight electron and proton giving two molecules of ammonia and one molecule of hydrogen. Since ammonia is toxic to the cell, this is either converted to ammonium or nitrate ion (Turk-Kubo *et al*., 2012) and these ions are used by the cells for their nitrogen demand. In addition, the energy consuming this process cannot compensate for the hydrogen molecule to be released as gas so the diazotrophs have (NiFe) hydogenases, the enzymes that catalyze the oxidation of hydrogen into protons and electron, so the proton can be used as energy source. The *hup*F and *Hup*K genes are found only in hydrogenase clusters in the presence of oxygen from the bacteria like *Rhizobium leguminosarum*, *Azotbacter*

vinelandii, Rhodsssopseudomonas palustris, Rhodobacter sphaeroides ATCC17029 etcetera (Albareda *et al*., 2012).Though nitrogen reduction is highly energy requiring step but still the diazotrophs have managed to recycle the hydrogen molecule. Some interests are there whether nitrogenase system could be exploited for hydrogen generation as fuel sources (Hallenbeck, 2012).

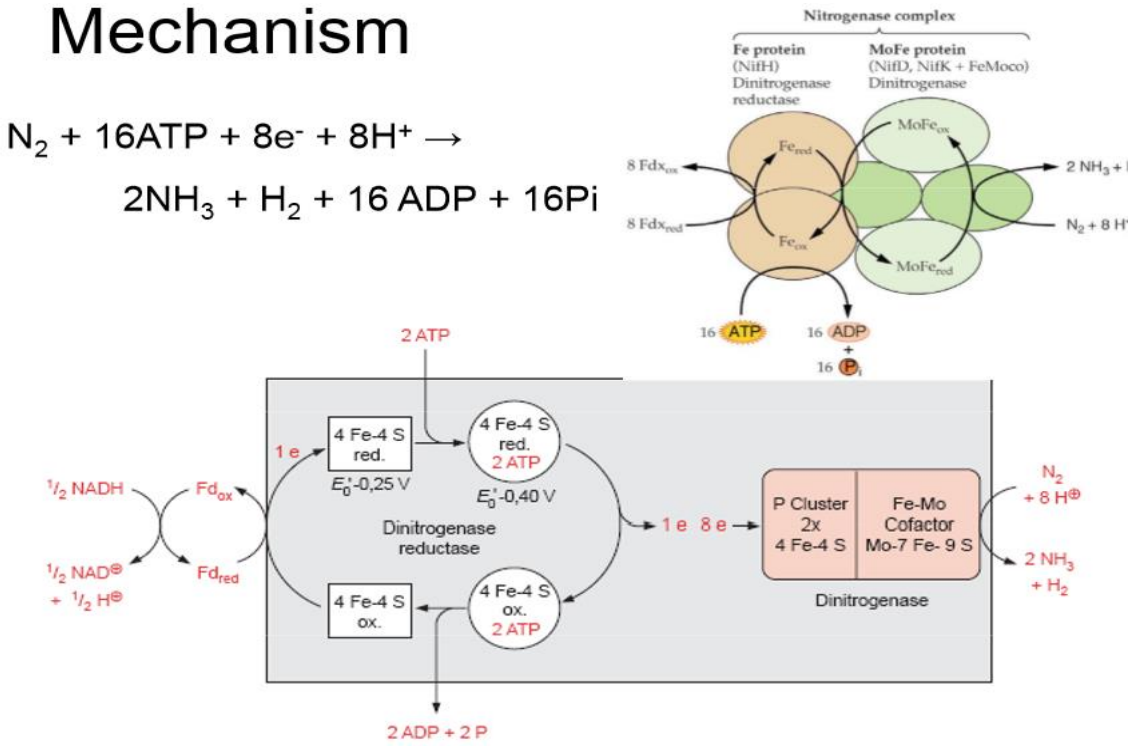


Figure 2.6 Mechanism of Nitrogen Fixation

2.5 Nitrogen Fixing Diazotrophs

Free-living nitrogen fixing bacteria can convert atmospheric nitrogen into ammonia from which they make amino acids and proteins. There are two types of nitrogen fixing bacteria: free living bacteria which fixes 30% of N_2 and symbiotic bacteria which fixes 70% of N_2 (Bezdicek and DF, 1998). *Azotobacter* species are free living aerobic bacteria, *Azospirillium* is free associative bacteria whereas purple nonsulfur bacteria, Green sulphur bacteria are free living anaerobic bacteria (Tejera *et al*., 2005). During the fixation of Nitrogen at the soil, there is no problem that *Azospirillium* can enter roots of plant and fix nitrogen and can find its way into the host plant and suggested that fixed nitrogen passed to the host immediately after fixation or only after death and breakdown of the bacterial cells (Oken *et. al.,* 1983)

2.6 *Azospirillium* and RuBisCo gene

Proteobacteria are known to adapt in environments with medium to high CO_2 making them an interesting candidate to explore CO_2 reduction. The presence of O_2 (in general, RuBisCo also have affinity to O_2 and high levels of this molecule can inhibit CO_2 fixation) may limit the ability. So far, there have been no reports showing that *A. amazonense* has autotrophic behaviour but possess the main genes cluster of Calvin-Benson-Basham cycle: genes *cbbL* and *cbbS*, and they encode the large and small subunits of RuBisCo (Sant'Anna *et al*., 2009). However, from the *Azospirillum* group, at least *Rhodospirillium centenum* and *Azospirillium lipoferum* are known to be capable of growing autotrophically by means of RuBisCO (Hartmann and Baldani, 2006).

Unlike *Azospirillum* sps B510 and *A. brasilense* Sp245, which do not contain Form I or II of RuBisCos ("true" RuBisCo s) genetic coded in their genomes. The RuBisCo phylogenetic reconstruction also indicated the close relationship of the *A. amazonense* enzyme with those from members of the family *Bradyrhizobiaceae* (order *Rhizobiales*) (figure 2.8), namely *Rhodopseudomonas palustris* and *Bradyrhizobium* spp. In fact, the genetic organization of the carbon-fixation makes these organisms to be part of the evolutionary process. The diversity between the *Azospirillium* species and RuBisCo gene in them also shows significance of this species in evolution.

This family might be mostly aerobic or microaerobic and phylogenetic tree clearly shows a split between these orders. The outermost clade containing all the *Azospirillum* species divides it in two main subclades: one containing *A. amazonense , A. irakense, Rhodocista pekingensis* and *R. centenum* while another containing the other *Azospirillum* species (Fani *et al*., 1995). Among the genus, *A. amazonense* having genes encoding those enzymes for glycolysis likely able to consume carbohydrates via glycolysis but no activity of 6-phosphofructokinase and fructose bisphosphate so most of the gram negative aerobic bacteria glycolysis are inoperative, which follow the Entner-Doudoroff pathway (ED pathway) (Martinez-Drets *et al*., 1985) and this catabolic feature should be experimentally verified when pursuing the search for similar organisms. But this characteristics gives additional evolutionary prospects that most of the enzymes involved in ED pathway are reversible and if CO_2 is reduced by RuBisCo that would ultimately give glyceraldehyde-3-phosphate (GAP) then this ED pathway can support in taking the sugar molecules to both pathways of TCA and pentose phosphate resulting in precursors of amino acids, nucleic acids, cofactors and lipids for biosynthesis of cellular building blocks as observed cyanobacters, autotrophic bacteria, algae, phytoplanktons and plants.

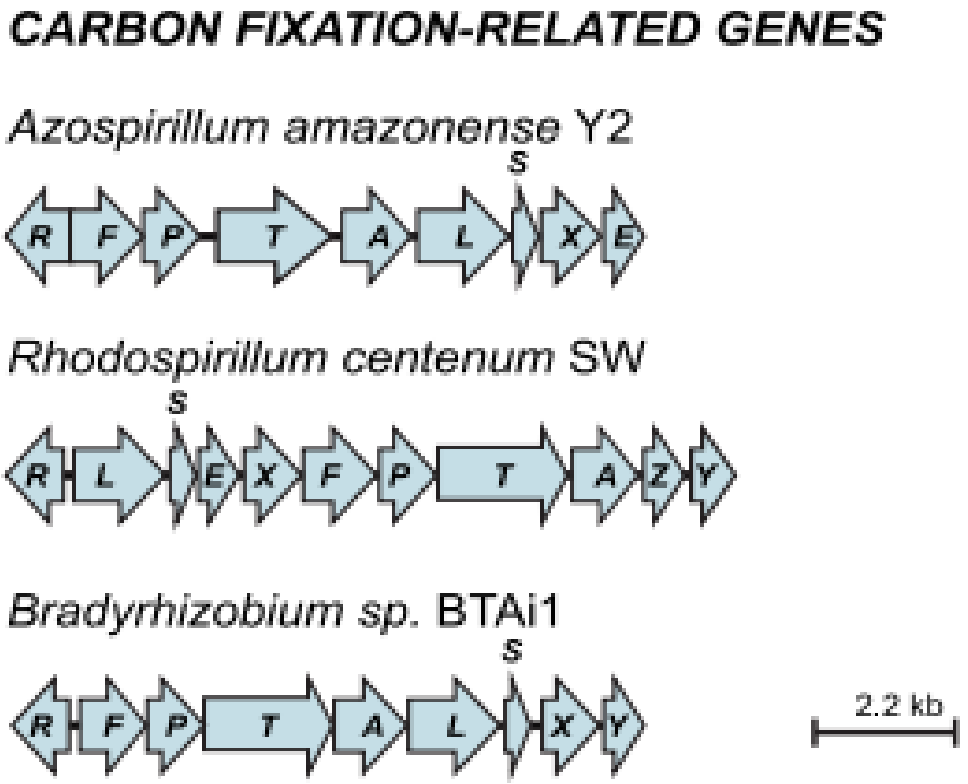


Figure 2.7 Organization of the carbon fixation gene cluster across different species. Arrows represent genes and their respective direction of transcription

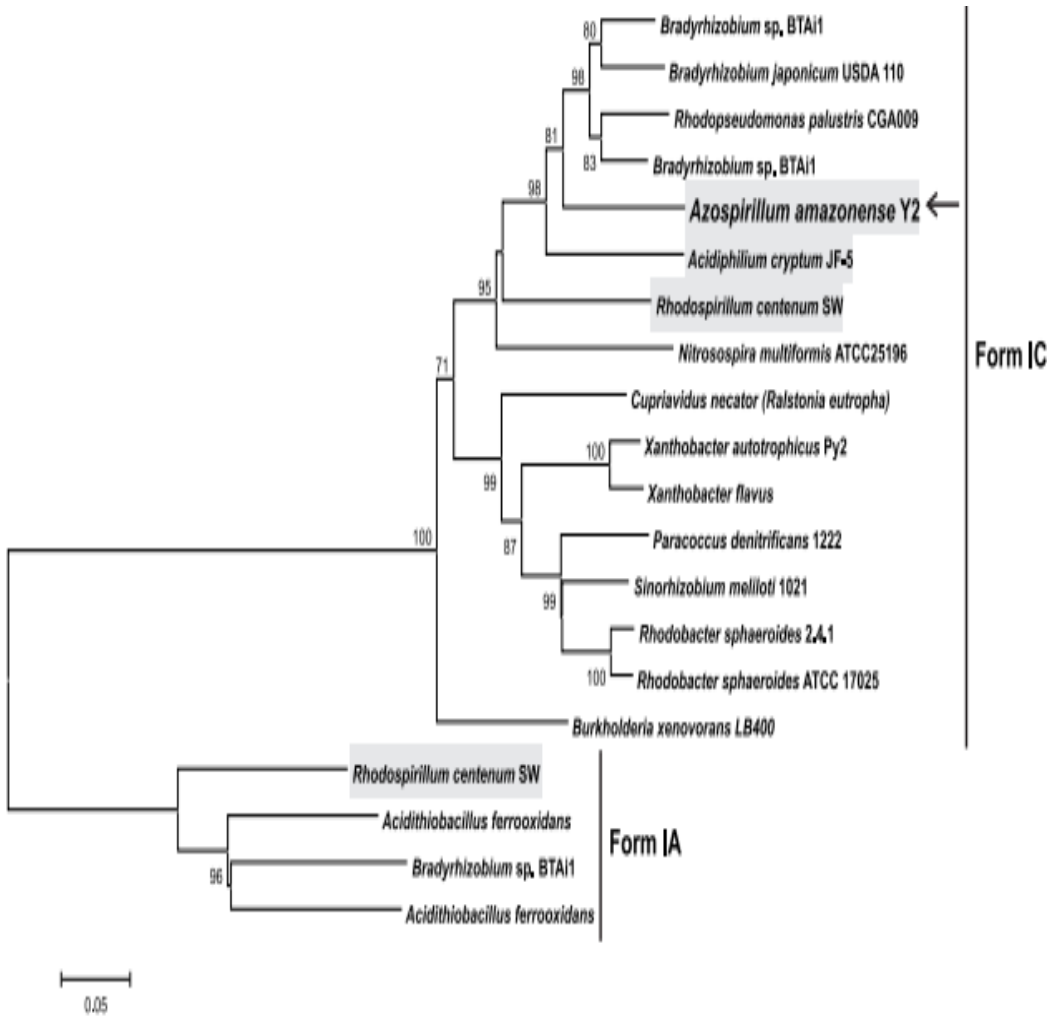


Figure 2.8 Phylogenetic Tree Based on the Concatenated Cbbl-Cbbs Rubisco Amino Acid Sequences

This genus of *Azospirillium* from alpha-proteobacteria is free living, nitrogen fixing bacteria mostly found in rhizosphere of plants like rice, maize, living in soils of tropical, subtropical and temperate regions of all over the world, contributed to the growth of rice plants by means of biological nitrogen fixation, showing its potential for use as an agricultural inoculant (Bashan and de-Bashan, 2010). They are able to utilize distinct nitrogen sources, including ammonia, nitrate, nitrite, dinitrogen and amino acids (Steenhoudt and Vanderleyden, 2000) because the conversion of nitrogen compounds to ammonia expends more energy therefore the metabolic pathways implicated in this process are strictly regulated to minimize energy waste (Hartmann and Zimmer, 1994).

In all diazotrophic species of the Proteobacteria examined so far, the transcriptional activator *Nif*A and the sigma N alternative RNA polymerase-associated factor are the master regulators of nitrogen fixation genes (Dixon and Kahn, 2004). Sequence motifs similar to the consensus region of sigma N and *Nif*A binding sites are present upstream of

the *nif*H gene, the homolog of "orf2"(indicated by the number 7 in Figure 2.9) of the orf2nifUSVorf4 cluster from *A. brasilense* and the *nif*B gene (Frazzon and Schrank, 1998).

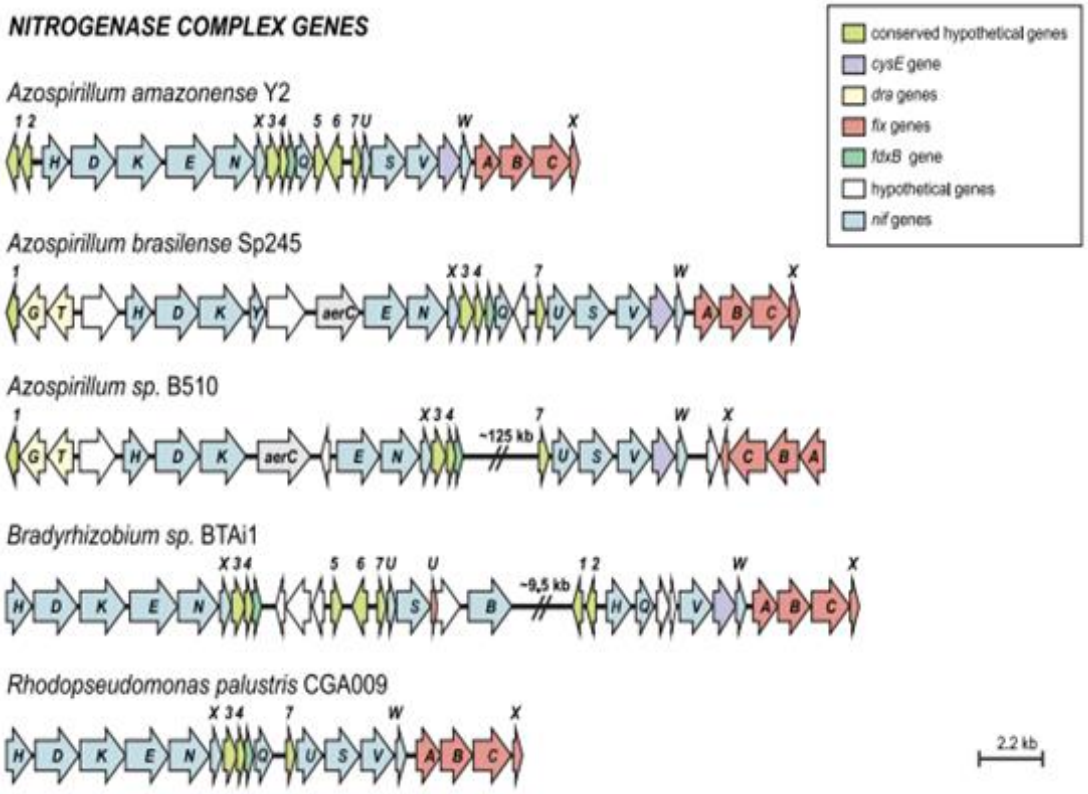


Figure. 2.9: Organization of nitrogen fixation gene cluster across different species

The *fix* genes are essential for nitrogen fixation process, namely *fix*ABCX, *fix*LJfixK and *fix*NOQP-fixGHIS. The *fix*ABCX genes from *A. amazonense*, responsible for electron transfer to nitrogenase, are located downstream of the *nif*W gene (figure 2.9). The operon *fix*ABCX is regulated by the NifA protein in *A. brasilense* (Sperotto *et al*., 2004)and *Rhizobium* sps (David *et al*., 1988) because it is tightly associated with the *nif* cluster.

The central regulators of nitrogen metabolism are the PII proteins. Three PII homolog genes (*glnB*, *glnK* and *glnK*2) have been found in the *A. amazonense* genome (Leigh and Dodsworth, 2007). The *glnK* gene and *glnB* gene have ortholog counterparts in *Azospirillum* spp. B510, *A. brasilense* Sp245 and *R. centenum*. The *glnK* gene is upstream of the *aat* gene (aminotransferase) and the *glnB* is upstream of the *glnA* gene (glutamine synthetase). The third gene, *gln*K2, which is located downstream of the *amt*B gene, is absent in *A. brasilense* Sp245, *Azospirillum* spp. B510 and *R. centenum* although this genetic association is frequently found in diverse prokaryotes (Sant'Anna *et al*., 2009).

In symbiotic diazotrophs, the transcription of *fix* genes involves the oxygen-responsive FixLJ where the *FixL* protein, in the absence of oxygen, autophosphorylates and transfers

phosphate group to *FixJ* and phosphorylated *FixJ* activates the expression of *FixK*, which then activates the transcription of genes required for microaerobic growth (Dixon and Kahn, 2004).

Dinitrogen reduction requires high levels of energy under microaerobic conditions so that the *fixNOQP* and *fixGHIS* genes encode membrane-bound cytochromeC oxidase and the redox process-coupled cation pump, respectively, which are definitely involved in respiration under microaerobic conditions, supplying energy for nitrogen fixation (Preisig *et al*., 1996).

Moreover, bacteria of the genus *Azospirillum* also produce high levels of poly-b-hydroxybutyrate (PHB) utilized the energy and carbon storage source under nutritional stress conditions (Kadouri *et al*., 2003). The essential genes for PHB biosynthesis are present in the *A. amazonense* genome: phbA (bketothiolase), phbB (aceto acetyl coenzyme A reductase) and phbC (PHB synthase). Also, the phaZ gene that encodes a PHB depolymerase , the first enzyme of the PHB degradation pathway, is also found in its genome (Sant'Anna *et al*., 2011) This further signifies its evolutionary importance to deposit reduced carbon in polymeric form when CO_2 reduction rate could be higher than the assimilation in biomass or to preserve for future use.

2.7 Genus Azotobacter

Bacteria of genus *Azotobacter* belongs in to gamma-subclass of the proteobacteria, gram negative, free living diazotroph, various shapes from rods to spheres nature. *Azotobacter* respires aerobically, receiving energy from redox reactions, using organic compounds as electron donors. *Azotobacter* can use a variety of carbohydrates, alcohols and salts of organic acids as sources of carbon (Gomare *et al*., 2013). *Azotobacter* can fix at least 10 µg of nitrogen per gram of glucose consumed *(*Gomare *et al*., 2013). *Azotobacter* plays an important role in the nitrogen cycle in nature as it possesses a variety of metabolic functions (Sahoo *et al*., 2013). They have mentioned that besides playing role in nitrogen fixation, *Azotobacter* has the capacity to produce vitamins such as thiamine and riboflavin and plant hormones *viz.,* indole acetic acid (IAA), gibberellins (GA) and cytokinins (CK) (Revallis *et al.,* 2000).

Azotobacter which donot need any host plant can be used as biofertilizer and can supplied 15-20 kg/ha N per year and also can produce antifungal compounds and increase germination vigour in young plant (Chen, 2006). Foliar application of biofertilizers especially Azotobacter could safely be used with half the normal dose of chemical nitrogen fertilizer to improve mulberry leaf production. The combination of Azotobacter with the Beijerinckia has further potential for the improvement in mulberry leaf production (Sudhakar *et al*., 2000). Azotobacteria genus synthesizes auxins, cytokinins, and GA–like substances, and these growth materials are the primary

substances controlling the enhanced growth. These hormonal substances, which originate from the rhizosphere or root surface, affect the growth of the closely associated higher plants (Wani *et al*., 2013).

Azotobacter vinelandii is a well-known of free living nitrogen fixers as well as siderophore. Siderophores means Fe-chelating molecules that are secreted by bacteria which serve to transport iron across cell membrane (Neilands, 1995). It is found in the iron rich soil and produces three iron –binding compounds under Fe-limited growth conditions. They are white fluorescent, blue-white fluorescent and yellow green fluorescent which chelates for Fe^{2+}, Fe^{3+}, MoO_4^{2-}, $V0_3^{2-}$, and $W0_4^{2-}$ (Knosp *et al*., 1984)

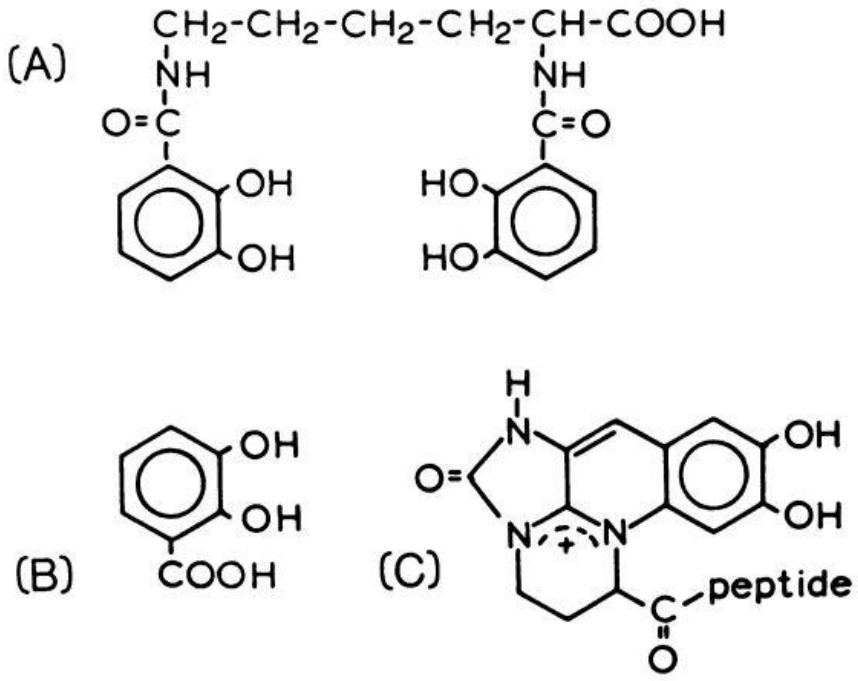


Figure 2.10 Iron-binding compounds produced by *A. vinelandii*. (A) The white-green fluorescent (N,N'-bis-(2,3-dihyroxybenzoyl)-L-lysine) (B) The blue-white fluorescent (2,3-dihydroxybenzoic acid) (C) The yellow-green fluorescent (o-dihydroxy quinoline-peptide) (Knosp *et al*., 1984)

Azotobacter as siderophores have a potential to mineralize different rocks that means ferric iron at rock and grain surfaces in deserts is dissolved by siderophores that are produced by indigenous microorganisms Azotobacter, fungi and algae (Knosp *et al*., 1984). Siderophores are high affinity biochemical chelating agents that microorganisms secrete to obtain iron from their environment for metabolic purposes (Neilands, 1995). There are two principal kinds of siderophores: hydroxamates, which are produced by many of the fungi, and catechols, which are produced by all classes of bacteria, except perhaps the lactobacilli (Adams *et al*., 1992; Neilands, 1995).

2.8 Genus Pseudomonas

Bacteria of genus *Pseudomonas* belongs to gamma proteobacteria family Pseudomonadaceae containing 191 validly described species among which *P. aruginosa* is opportunistic human pathogen, *P. syringae* is plant pathogen and *P.fluorescens* is the plant growth promoting rhizobials (PGPR). As bio-fertlizer the interest in *P. fluorescens* as an ecofriendly N-fertilization has been pursued (Hol *et al*., 2013). *P. fluorescens* species complex are Gram-negative, motile rods that are primarily aerobic, unable to ferment glucose, and chemoorganotrophic and grow at a pH between 4 and 8 (Scales *et al*., 2014). They are obligate aerobes but capable of using nitrate instead of oxygen as a final electron acceptor during cellular respiration (Moore *et al*., 2006).

P. fluorescens produces secondary metabolites that allow it to successfully competing microorganisms. Examples include phenazine, hydrogen cyanide (HCN) 2,4-diacetylphloroglucinol (DAPG), rhizoxin, and pyoluteorin (Keel *et al*., 1996; Ramette *et al*., 2003; Mavrodi *et al*., 2008;). Phenazines are pigmented compounds that have antitumor, antimalarial, antiparasitic, and antimicrobial activities. Molecular mechanisms of plant growth promoting plant defence by *P. fluorescens* have been elucidated. *P. fluorescens* is most known as a root colonizer. They have mutualistic interactions, which provide benefits such as increasing nutrients, producing hormones, increasing tolerance to abiotic stresses (water, temperature, heavy metals) or biotic stresses (pests and pathogens) (Jäderlund *et al*., 2008; Saravanakumar *et al*., 2009; Senthilraja *et al*, 2010). The effect of 2,4-diacetylphloroglucinol (2,4-DAPG), a secondary metabolite from *P. fluorescens*, on *Azospirillum* gene expression have been found to up-regulate genes involved in several traits related to root colonization and growth promotion. Co-inoculation of *P. fluorescens* and *Azospirillum* stimulated root growth in spring wheat (Combes-Meynet *et al*., 2011; Garbeva *et al*., 2011). They also found that changes in gene expression in *P. fluorescens* when exposed to three other rhizobacteria: *Bacillus* sp., *Brevundimonas* sp. or *Pedobacter* sp. Interestingly, *P. fluorescens* had specific responses to the different competitors; two species increased antimicrobial metabolite production by *P. fluorescens* but Bacillus did not (Garbeva *et al*., 2011). Thus, designing syntrophic growth system and formulating bio-fertilizer consisting of different bacteria could be an alternative to chemical fertilizers.

2.9 Genus *Geobacter*

Bacteria of genus *Geobacter* belongs to delta Proteobacteria of family Geobacteraceae. They are mostly facultative aerobe, non- motile, non-spore forming, Gram negative, rod shaped bacteria which have ability to oxidize organic compounds, metals including iron, radioactive metals and petroleum compound too (Childers *et al*., 2002). It uses acetate as electron donor (Caccavo *et al*., 1994). Under anaerobic environment, it generates

extracellular electrons which have potential to reduce various compounds and metals. Multihaem c-type cytochromes(c-Cyts) are the major electron carrier proteins which are located in bacterial cell envelope. It has two major electron transporting c-Cyts, which are OmcE and OmcS. They transfer electrons to the metals in external environment (Lloyd, 2003) through these c-Cyts that facilitate the electron transfer (ET) from the Quinone pool inside the membrane to outer membrane. It has potential in bioremediation, biofertilization along with power generation in Microbial fuel cells (MFC) (Bond and Lovley, 2003).

The ability of *Geobacter* species to fix atmospheric nitrogen is an important metabolic feature for these applications. Unlike the regulatory mechanisms known in other nitrogen-fixing microorganisms, nitrogen-fixation gene regulation in *Geobacter sulfurreducens* is controlled by two two-component His–Asp phosphorelay systems (Ueki and Lovley., 2010).

One of these systems appears to be the master regulatory system that activates transcription of the majority of nitrogen-fixation genes and represses a gene encoding glutamate dehydrogenase during nitrogen fixation. The other system whose expression is directly activated by the master regulatory system appears to control by anti-termination of the expression of a subset of the nitrogen-fixation genes whose transcription is activated by the master regulatory system and whose promoter contains transcription termination signals.

Among the genes up-regulated during nitrogen fixation, gnfK (*Geobacter* nitrogen fixation histidine kinase) and gnfR (*Geobacter* nitrogen fixation response regulator) encoding a histidine kinase and a response regulator in the two-component system have been studied for their putative role in nitrogen fixation gene regulation (Ueki and Lovley, 2010). In addition, nifH, which encodes a nitrogenase iron protein, and *glnB*, which encodes a nitrogen regulatory protein PII, have been also studied as they are well known to be involved in nitrogen fixation in other bacteria. GnfM (*Geobacter* nitrogen fixation master regulator) has been found to have DNA-binding activity at the promoter region of gnfK, nifH, and *glnB* as well as to the promoter region of gdhA that appears to be repressed during nitrogen fixation (Methé *et al*., 2005). The GnfM-binding sites are located upstream of the RpoN-dependent promoter in gnfK, suggesting that GnfM functions as a transcriptional activator. In gdhA, the GnfM-binding sites are located upstream of the RpoN-dependent promoter as well as at the region overlapping the RpoN-dependent promoter, suggesting that GnfM functions as a transcriptional repressor for gdhA. A sequence analysis of upstream regions of the nitrogen-fixation genes with an RpoN-dependent promoter by the authors revealed similar sequences

with those in gnfK and gdhA, which may function as a binding site of GnfM.

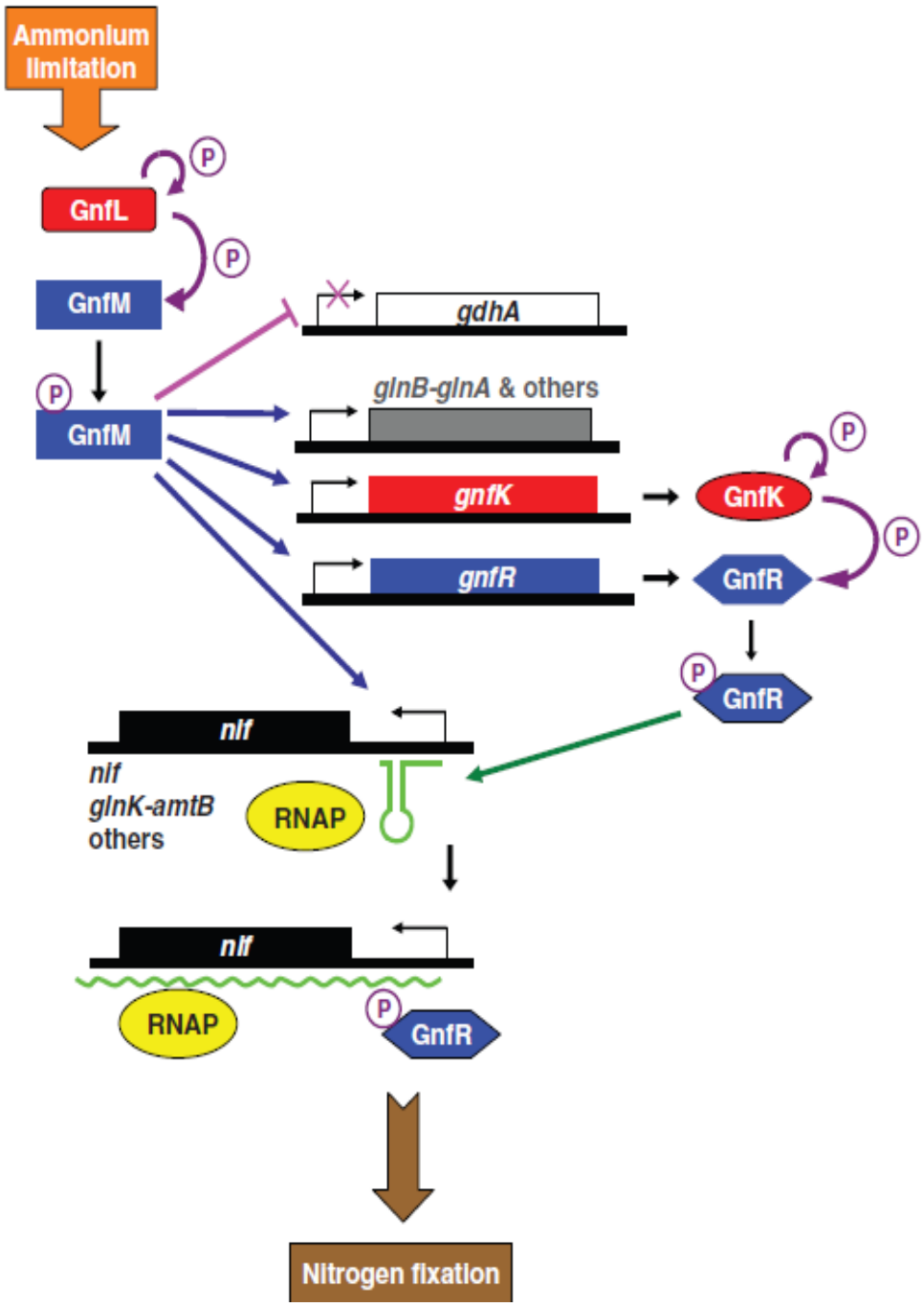


Figure 2.11 Model for the regulation of gene expression during nitrogen fixation in G. *sulfurreducens* (Ueki and Lovley, 2010)

Thus, GnfM functions as a transcription activator for gnfK and a repressor for gdhA and these activities of GnfM are modulated by phosphorylation via GnfL. The majority of nitrogen-fixation genes appear to be controlled by the sigma factor RpoN and the two-component system consisting of the histidine kinase GnfL and the response regulator GnfM, which also belongs to the enhancer-binding protein (EBP) family (Ueki and Lovley,

2010). In addition to nitrogen metabolism, RpoN controls genes involved in a wide range of cellular processes such as fumarate respiration, Fe (III) reduction, and pili and flagella biosynthesis in G. *sulfurreducens*.

2.10 Syntrophic Consortia

Microbes are ubiquitous in nature; discharge a variety of metabolites and assist the growth of other microbes in a community and syntrophy is a mutualism that governs the metabolism and growth of diverse microbes in natural and engineered ecosystem. Particularly, Syntrophy is interspecies interaction that is based on providing trophic benefits for both partners (McInerney *et al*., 2009).

A representative example of syntrophy is found in methanogenic communities, where reducing equivalents, e.g., hydrogen and formate, transfer between syntrophic partners.

A well-characterized syntrophic interaction occurs between fermentative bacteria (syntrophs) and methanogenic archaea (methanogens), which cooperatively transform organic compounds, such as volatile fatty acids (VFA, including butyrate, propionate, and acetate) into methane. This syntrophic interaction is based on the transfer of reducing equivalents, such as hydrogen and formate, between these microbes and is also termed "interspecies electron transfer (IET)" (Schink, 1997)

A recent study by Rotaru *et al*. (2014) reported that *Methanosarcina barkeri* is also able to accept electrons from *G. metallireducens* through the formation of cell aggregates. The study also revealed that when *M. barkeri* was grown in co-cultures with hydrogen-producing *Pelobacter carbinolicus*, *M. barkeri* utilized hydrogen as an electron donor for carbon dioxide reduction, but did not aggregate with *Pelobactor carbinolicus*. These observations demonstrate that close physical contact is needed for direct IET via electric current but not for interspecies hydrogen transfer.

Fluorescence *in situ* hybridization has revealed that the two *Geobacter* species are closely associated with each other within the aggregates. Notably, cell growth and ethanol consumption proceeded even when co-cultures were inoculated with a *G. sulfurreducens* strain that was unable to utilize hydrogen and formate due to deletion of the genes encoding formate dehydrogenase (*fdnG*) and uptake hydrogenase (*hybL*), demonstrating that syntrophic ethanol metabolism by *G. metallireducens* and *G. sulfurreducens* can occur without interspecies transfer of hydrogen and/or formate. In addition, it is found that a mutation that enhances the production of OmcS, a pili-associated c-type cytochrome that promotes electron transfer to insoluble Fe(III) oxides (Mehta *et al*.,2005) and electrodes, was selectively introduced into the genome of *G. sulfurreducens* under co-culture conditions, resulting in the acceleration of aggregate formation. In contrast, deletion of the gene encoding OmcS or the structural pilin protein

PilA impaired cell growth in the co-cultures. These findings suggest that IET between these *Geobacter* species in cell aggregates occurs via electrical networks comprised of conductive pili and extracellular cytochromes (Kouzuma *et al*., 2015).

2.11 Mixotrophic growth

Mixotrophic is a nutritional strategy between autotrophic and heterotrophic feeding autotrophy can manufactured organic materials from inorganic sources and heterotrophs rely on outsources of organic food materials. Autotrophs might be chemotrophs and phototrophs: chemotrophs use energy from specific inorganic molecules and phototrophy use energy from sunlight. Though combination of phototrophy and phagotrophy are possible, mixotrophic is reserved for the combination of phototrophy and phagotrophy. A mixotrophic organism assimilates dissolved carbon and nitrogen from the environment and turns it into a biomass and energy via the autotrophic pathway. When the organism dies, its biomass becomes available in the form of dead biomass through their heterotrophic pathway. It is often found planktonic protists with freshwater and sea habitats (Troost *et al*., 2005).

2.12 Silica nanoparticles

Silicon dioxide nanoparticles, also known as silica nanoparticles or nanosilica, have more stability; low toxicity and ability to be functionalized with a range of molecules and silica are found as quartz.

Nano-silica particles are divided into P-type and S-type according to their structure. The P-type particles are characterized by numerous nanopores having a pore rate of 0.61 ml/g. The S-type particles have a comparatively smaller surface area. The P-type nano-silica particles exhibit a higher ultraviolet reflectivity when compared to the S-type. Silicon belongs to Block P, Period 3 while oxygen belongs to Block P, Period 2 of the periodic table, they are also use as additive for rubber and plastics having melting point 1600 degree centigrade and boiling point 2230 degree centigrade (AZoM, 2001).

Yearly, 600 million tons of rice paddy is produced, 20% of rice paddy is husk, is an agricultural waste which may cause air pollution as well as soil pollution. Sodium silicate imply as a silicon source in industrial production of silica. Though, sodium silicate produced by smelting quartz sand and sodium carbonate at 1300˚C not only requires a large quantity of energy, but also further purification (Affandi *et al*., 2009). In spite, low temperature extraction of amorphous silica from plant biomass yields high quality, environmental friendly and cost effective product as opposed to the high energy processing of the inorganics. It is well known that certain plants, including Equisetaceae, Graminae, Cyperaceae and Poaceae, contain high levels of biogenic silica in the form of hydrated silica ($SiO_2. nH_2O$) deposited in the tissue.

The chemical composition of Rice Husk (RH) is similar to that of many common organic fibers, containing cellulose, lignin, hemicelluloses, and silica, which is the primary component of ash. After burning, the organic composition is decomposed and rice husk ash (RHA) is obtained. RHA is one of the most silica-rich raw materials containing about 90% to 98% silica (Yunusa *et al*., 2016) and some amount of metallic impurities (after complete combustion) among the family of other agro-wastes. It is important that the silica in RHA exists in the amorphous state and has high surface area. Because of these features, silica has many applications, such as sources for synthetic adsorption material, carriers, medical additives, fillers in composite materials, etc., and demonstrates advantages when achieved at nanometer size.

Silica is a polymer of silicic acid consisting of inter-linked SiO_4 units in a tetrahedral fashion with the general formula SiO_2. In nature, it exists as sand, glass, quartz, etc. Naturally occurring silica is crystalline, whereas synthetically obtained silica is amorphous in nature. Silica used in chemical applications is synthesized from either silicate solution or silane reagents.

There are different methods to prepare silica nanoparticles. Adam *et al*., (2011) synthesized spherical nanosilica from agricultural biomass as RH via the sol–gel method. The resulting silica particles have shown agglomerates with an average dimension of 15 to 91 nm. (Jal *et al*., 2004) synthesized nanosilica through the precipitation method, and the resulting nanosilica is found to have a particle size of 50 nm in dimension. However, the sol–gel technique is the most common method for silica synthesis. It involves simultaneous hydrolysis and condensation reaction. In this process, a sol of sodium silicate or silicon alkoxide or halide gets converted into a polymeric network of gel. During silica synthesis by sol–gel process under certain conditions like restriction of gel growth, silica gets precipitated. In such preparation, the steps involved are coagulation and precipitation from silica solution. Anyways, sol-gel techniques are low cost production of silica nanoparticles from where ultrafine powders on a nanoscale and with homogenous particle size distribution could be found (Thuc, and Thuc 2013). And it also provides a way of solving the problem of agrowaste and air pollution due to Rice husk ash/Char.

2.13 Biofuel and Biofertilizer

Biofuels are those fuels obtained directly from plants or indirectly from agricultural, commercial or industrial wastes. Bioethanol is one of the biofuel made by fermentation mostly from carbohydrates and lingo cellulosic biomass. In 2010 biofuel production reached 105 billons litres and it provided 2.7% of the world's fuels for road transport (Worldwatch, 2011). Currently, there is a lot of emphasis on using crop residues, e.g. stover, straws, hulls, stems, and stalks (Lau and Dale, 2009) as well as common weeds present in the southern part of the United States as energy crops. For example the state

of Oklahoma, USA launched a major initiative towards using switch grass, a common weed within the state, for ethanol production. Other US states (e.g. Minnesota) are looking into collectively using multiple plants within their tall grass prairie ecosystems as the feedstock for energy production (an approach that has been called low-impact high diversity (Tilman *et al*., 2006).

Biofertilizer are the substance which contain living microorganisms which colonizes the rhizosphere or interior of the plant and promotes the growth by increasing the avaiolability of primary nutrients to host plant. Actually Plant Growth Promoting Rhizobacteria (PGPR) as biofertilizer reinforce the nutrient status of host plant into five ways: 1) biological N_2 fixation 2) increasing the availability of nutrients in the rhizosphere 3) intensify the root surface area 4) heighten up other beneficial symbioses of the host and 5) combine the mode of action (Vessey, 2003).

The most studied and longest exploited PGPR are the rhizobia for their ability to fix N_2 in their legume hosts and commercially rhizobia inoculants for use on legume crops first introduced in the 1890s (Fred *et al*.,1933) but despite that associative N_2 fixation of non-legume crop plants were exploit early 1970s and 1980s via biological nitrogen fixation (BNF) led to agronomically significant levels in most crops. For instance, *Azospirillium brasilense* believed once act as PGPR via BNF but growth promoting effects originate predominately from other mechanisms too (Vessey, 2003). Effect of PGPR as biofertilizer is mentioned on the given table with references.

Table 2.3: Plant Growth Promoting Rhizobacteria (PGPR) showing their stimulation of plant growth is related to their ability to fix N_2.

PGPR	Relationship to host	Host crops	Sample References
Azospirillum Sp.	Rhizospheric	Maize Rice Wheat	de Salamone *et al*., 1996 Malik *et al*., 1997 Boddey *et al*., 1986
Azoarcus sp.	Endophytic	Kallar grass Sorghum Rice	Hurek *et al*., 2002 Stein *et al*., 1997 Egener *et al*., 1999
Azotobacter sp.	Rhizospheric	Maize Wheat	Pandey *et al*., 1998 Mrkovacki and Milic, 2001
Bacillus polymyxa	Rhizospheric	Wheat	Omar *et al*., 1996
Cyanobacteria	# Rhizospheric	Rice Wheat	Hashem, 2001 Obreht *et al*., 1993
Herbaspirillum sp.	Endophytic	Rice Sorghum Sugarcane	James *et al*., 2002 James *et al*., 1997 Pimentel *et al*., 1991

#Numerous species: predominantly of the genera of Anaebena and Nostoc (Vessey, 2003)

3. MATERIALS and METHODOLOGY

3.1 Materials

The sources of chemicals, material employed in this study are from following sources: Mastermix and DNA ladder 100bp are provided from Chigen, China.

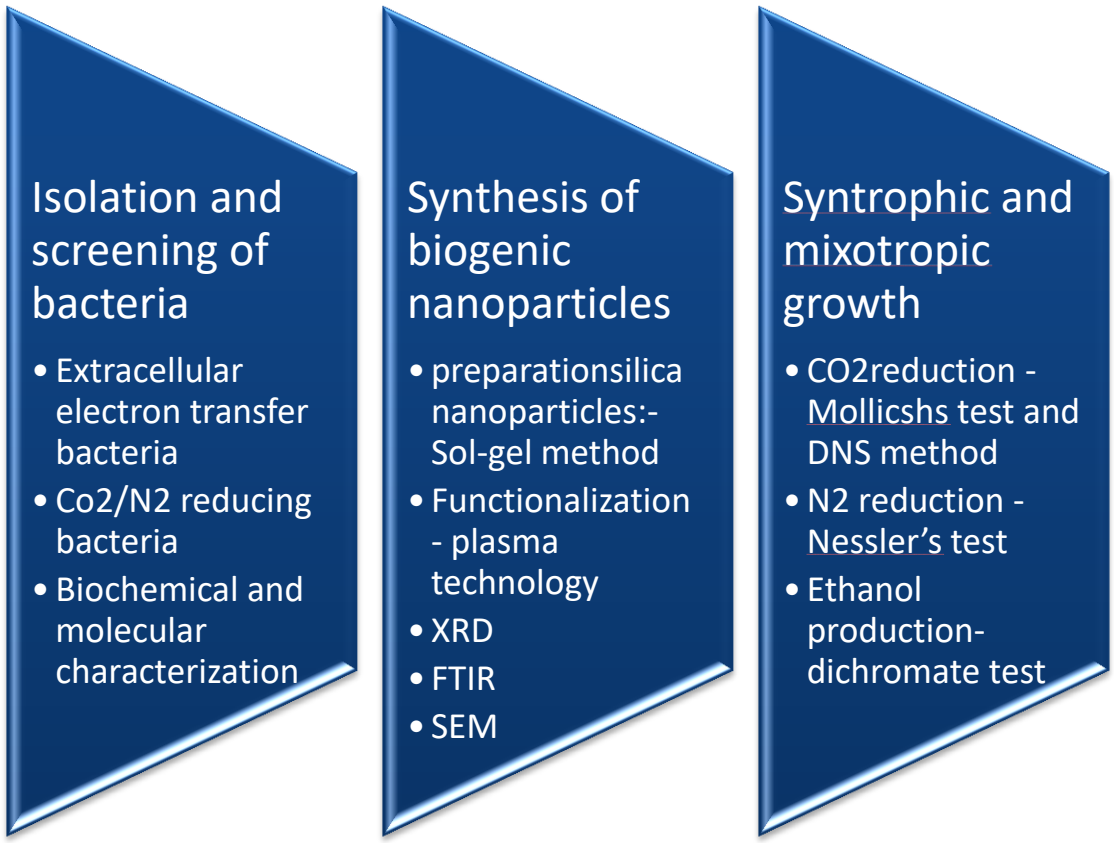
Plasma technology and materials for functionalization are provided by Kathmandu University.

Muffle furnace, heating mantle and related material are provided by RECAST, Kirtipur.

The results of FTIR are performed from Central Department of Chemistry, Tribhuwan University, XRD from IIT-Guwahati, Chemical Engineering Department India and SEM from Martin Luther University, Halle, Germany.

Remaining chemicals like sodium meta-silicate, media which are needed for bacterial growth, sulphuric acid, hydrochloric acid, acetic acid, absolute ethanol including other machine are provided by laboratory of department of biotechnology (especially all reagents were purchased from Himedia, India Pvt.Ltd)

3.2 Methodology



3.2.1 Consortium of bacterial with silica

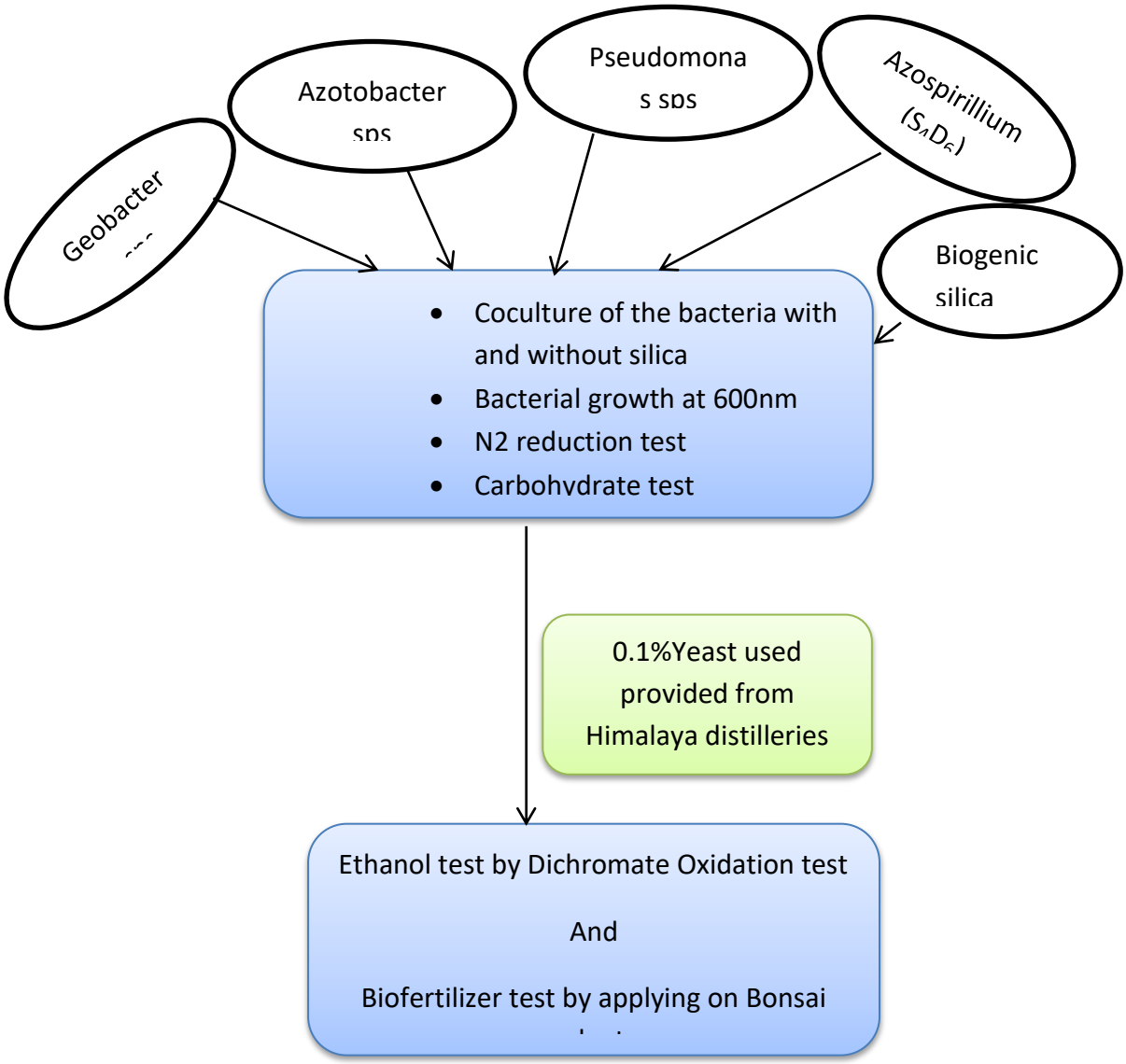


Figure 3.1: Diagrammatic sketch of methods and methodology

3.2.2 Sample Collection

Soil samples were collected from Panchase, Kaski from around 1500 to 2700m altitude above sea level. The reason for selection of this region was presumed to be potent for the presence of prehistoric, environmental friendly bacteria which was favourable for herbivorous vegetation and was the place where presence of tree-fern give the possibility of finding marine or inorganic substrates bacteria using as an energy sources similarly, some samples were collected from stagnant water from Panchase lake, kaski for searching of variety of water living bacteria which could use dissolving Hydrogen as energy source for their metabolism.

Figure 3.2: Collecting soil samples under tree fern at Bhadaure, Kaski

Soil samples and their GPS location are mentioned in the table 3.1.

Table 3.1: Soil Sample Collection from Kaski, Nepal

SN	Sample name	GPS(Latitude\longitude)	Altitude(m)	Soil temperature(C)
1	Bhadaure 1	La:28.26754 Lo:83.82673	1522	17
2	Bhadaure 2	La:28.267338 Lo:83.826682	1524	13
3	Sample 3	La:28.259037 Lo:83.810941419	1419	9
4	Sample 4	La:28.258817 Lo:83.811249	1401	10
5	River sample 5	La:28.261742 Lo:83.78355	1352	
6	Swapyland 6	La:28.248928 Lo:83.78355	1963	
7	Panchase lake7	La:28.234082 Lo:83.787713	2287	
8	Way to panchase	La:28.23283 Lo:83.790479	2347	20
9	Sidhane kaski		1600	23

3.2.3 Isolation and screening of bacteria

3.2.3.1 Isolation of *Geobacter, Azobacter* and *Pseudomonas*

Soil samples were brought from tree-fern rhizospheric region below 10 cm of Panchase Kaski about 1500m to 2600m and swampy land near to pond. Each soil samples were enriched at GS-15 modified media (Appendix 4) without nitrogen and adding 0.05mM sodium acetate as carbon source and creating anaerobic condition and incubated at 28°C for 2-3 days. Then 100 µl inoculum from each sample was mixed with 10ml top agar (0.8%) of GS-15 media and poured on the bottom agar (1.5%) GS-15 media and again incubated at 28°C for 2 days. Single colony was picked at another GS-15 media plate and incubated for 2 days at same condition. Then isolated colony was streaked on 0.05mM Aniline GS-15 media and incubated again for two days then isolated colony streaked on both 0.05Mm sodium acetate GS-15 media and 0.05mM ethylene Glycol GS-15 then incubated at 28°C for 3 days. Those lived at 0.05mM sodium acetate GS-15 media and died at 0.05mM Ethylene glycol Gs-15 were putative *Geobacter* sps. Those who survived at Ethylene glycol might be *Azobacter* or Pseudomonas which further were separated on the basis of carbon catabolite repression method. Those lived at Ethylene glycol again streaked on 0.05mM toluene and incubated for 3 days at 28 °C and observed the lived and died bacteria, those lived bacteria would be *Pseudomonas*. Same experiment done second time but at that time, there was no creation of anaerobic condition still growth was seen accordingly.

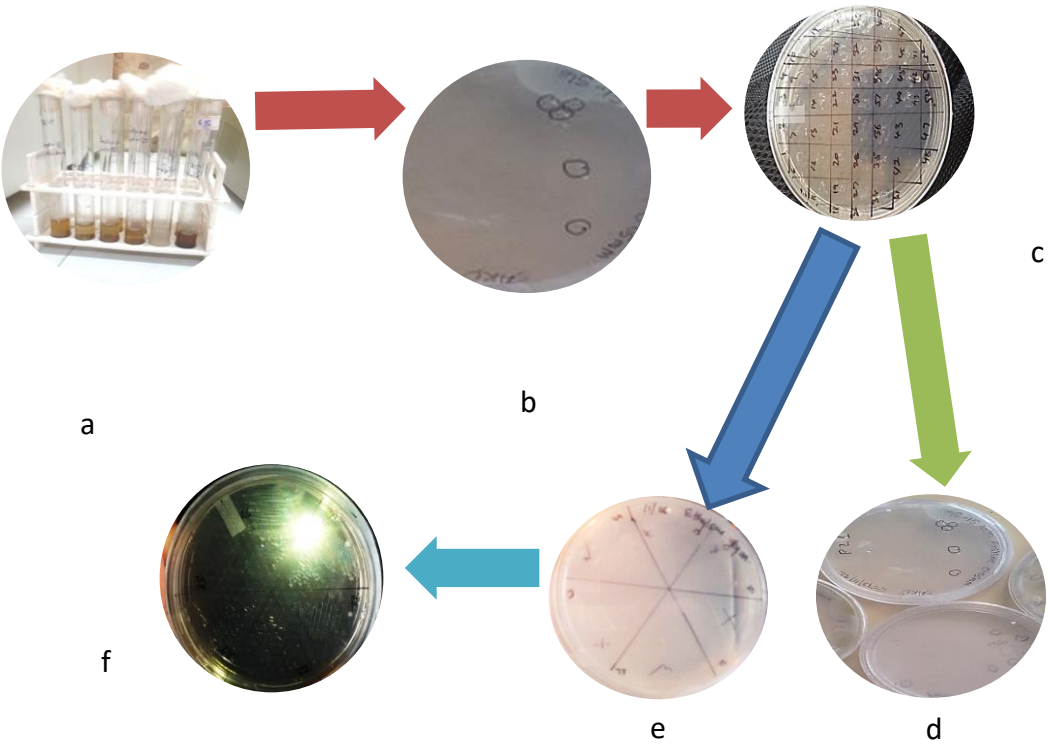


Figure 3.3: Flow Diagram of Isolation and Screening of Bacteria

(a) Enrichment for G*eobacter,* GS-15 media without nitrogen and adding 0.05mM sodium acetate was prepared and inoculate 0.5gm soil and incubate at 28C for two days.
(b) 100 µl inoculum from each sample was mixed with 10ml top agar (0.8%) of GS-15media and pour plated on the bottom agar (1.5%) GS-15 media and again incubated at 28°C for 2 days.
(c) Single colony was picked at another GS-15 media plate and incubated for 2 days at same condition.
(d) Isolated colony was streaked on 0.05mM Aniline Gs-15 media and incubated again for two days then isolated colony streaked on both 0.05Mm sodium acetate GS-15 media and 0.05mM ethylene Glycol GS-15 then incubated at 28°C for 3 days.
(e) Lived bacteria on ethylene glycol were either *Azotobact*er or *Pseudomonas*
(f) Again lived bacteria on ethylene glycol were inoculated 0.05 mM toluene and incubated for 3 days that grew on toluene were putative *Pseudomonas sps* and died ones were *Azotobacter sps.*

3.2.3.2 Isolation and screening of nitrogen and carbon-dioxide reducing bacteria

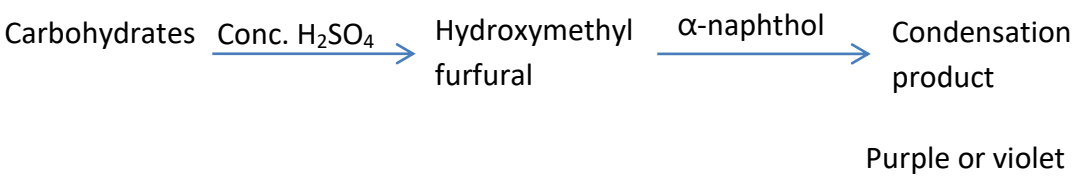
Isolation and screening of N_2 and CO_2 reducing bacteria from soil sample was done by my colleague (Anju Tamang) and one of the students (Susma Khadka) of my supervisor from another institute. The sample (S_4D_6) as Putative *Azospirillium* sps was provided to me. Later on I had reproduced them on nitrogen and carbon free media termed as NCF media. NCF media composition was given at appendix 2.

3.2.3.3 Qualitative test for presence of carbohydrate on NCF media

NCF media was prepared with pH 6.8 and pH 4.5 and autoclaved at 121°C with 15lbs for 15 min. Then 1 loopful glycerol stock of given sample was inoculated to the cooled media at sterile laminar hood and incubated for 3 days at 28°C. Then after 3 days, growth rate observed at spectrophotometer at 600nm. After observing OD 0.08 to 0.4 angstrom, the bacterial culture on different pH were centrifuged at 5000 rpm for 5min for pelleting the bacteria and supernatant was taken for following qualitative test:

3.2.3.3.1 Molisch's test

This is a general test for all carbohydrates. Conc. H_2SO_4 hydrolyses glycosidic bonds to yield monosacchaides which in the presence of an acid get dehydrated to form furfural and its derivatives. These products react with sulphonated α-naphthol to give a purple complex. Polysaccharides and glycoprotein also give a positive reaction.

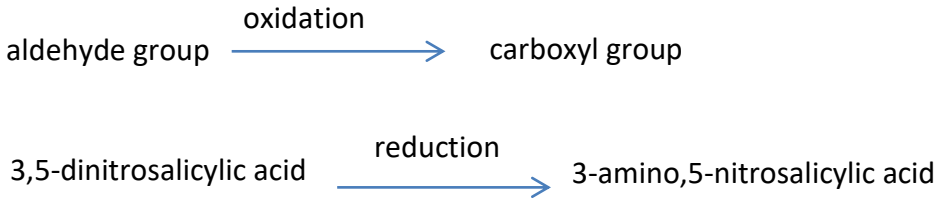


1ml supernatant of the sample was taken in test tube and 1 drop of 5% α-naphthol was added then, about 1ml conc. H_2SO_4 was added from the wall of test tube, purple colour was obtained whether there was presence of bacteria.

3.2.3.4 Quantitative test for carbohydrates present on NCF media

3.2.3.4.1 DNS test (3,5-dinitrosalicylic acid)

The presence of free carbonyl group is determined by this method which is also called reducing sugar. This involves the oxidation of the CHO functional group present for example glucose, Fructose. Simultaneously 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino-5-nitrosalicylic acid under alkaline condition.



Because dissolved oxygen can interfere with glucose oxidation, sulphite which itself is not necessary for the color reaction, is added in the reagent to absorb the dissolved oxygen.

Although this is a convenient and relatively inexpensive method, due to the relatively low specificity, one must run blanks diligently if the colorimetric results are to be interpreted correctly and accurately. One can determine the background absorption on the original cellulose substrate solution by adding cellulase, immediately stopping the reaction, and measuring the absorbance, i.e. following exactly the same procedures for the actual samples. When the effects of extraneous compounds are not known, one can effectively include a so-called internal standard by first fully developing the color for the unknown sample; then, a known amount of sugar is added to this sample. The increase in the absorbance upon the second color development is equivalent to the incremental amount of sugar added (Miller, 1959).

3.2.3.4.2 Calibration curve preparation for reducing carbohydrates

10mg/ml of glucose stock solution was prepared. Then 2ml of glucose with different concentration starting from 10µg/ml-100µg/ml were prepared with blank (H_2O). They were treated with 2ml DNS (3,5 dinitrosalicylic acid) and left for bathing for 5min at boiling temperature of water and then cooled them. After cooling, 2ml of 40% Rochelle's salt (sodium potassium tartarate) was added then absorbance at 540nm was observed and calibration curve for carbohydrates was obtained.

3.2.3.4.3. Carbohydrate test for the samples

2ml of Supernatant of NFC media only was taken for blank and 2ml of supernatant from each of the bacterial samples were taken separately in different vials and same process was repeated as for calibration curve for carbohydrate preparation and absorbance was taken at 540nm.

3.2.3.4.5 Nitrogen reduction test by Nessler's test

3.2.3.4.5.1Calibration curve for biological nitrogen reduction

Standard curve was plotted by taking the UV absorbance at 435nm of different concentration (0.1µg/ml- 1µg/ml) 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.

The 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 colour indicated positive test for nitrogen source or biological nitrogen fixation.

3.2.3.4.5.2 Quantification of Nitrogen in the Samples

Biological nitrogen fixation test was carried out by culturing the organism in NCF media. A loop full of bacteria was inoculated in 10ml NCF media and incubated at 28°C for three days. After three days, production of ammonia by the bacteria was tested by the reaction of incubated bacterial sample with Nessler's reagent.

Quantification was done by centrifuging 1ml NCF media with incubated bacteria at 5000 rpm for 5 minutes and the supernatant was collected for spectrophotometric analysis. For this, 2ml solution containing 1500µl of distilled water, 400 µl cultured supernatant and 100µl Nessler's reagent and absorbance was observed at 435nm. The amount ammonia production was calculated using standard curve.

3.2.3.4.6 Biochemical test for the putative *Geobacter*

3.2.3.4.6.1 Gram Staining

The isolated single colony was used to streak fresh plates and the colonies in these fresh plates were selected using a sterile inoculating loop and transferred to a clean slide with a drop of sterile water on it. The samples were air-dried onto the slides and heat-fixed by passing through a Bunsen burner flame several times. Dyes were obtained from BD-

BBL. Each smear was covered first with crystal violet dye for one minute and washed with water. Next, the smear was overlayed with stabilized gram iodine for thirty seconds and again rinsed with water. The smear was next washed with gram decolourizer for ten seconds until all violet dye was removed. Finally, gram saffranin was placed over the smear for one minute and rinsed with water. Slides were examined under 100X oil immersion microscopy (Winn and Koneman, 2006). At this point, the morphology (rods or cocci) and grouping (clusters, chains, pairs, tetrads, etc.) of the cells were recorded as well as the Gram stain results. Smears appearing to be predominantly blue or black were labelled as "Gram-positive," and indicated the retention of crystal violet dye in a thick peptidoglycan layer of the cell wall. Predominantly pink or red smears were labelled "Gram-negative" because crystal violet was not retained due to a thin layer of peptidoglycan, contained within the outer membrane of the cell wall of gram negative bacteria (Nester, 2007). Based on the gram stain and cell morphology results, diagnostic tests were selected on a sample-by sample basis.

3.2.3.4.6.2 Catalase Test

Catalase, an enzyme which breaks down hydrogen peroxide, is frequently found in aerobic and facultative anaerobic bacteria because hydrogen peroxide is a potentially toxic by-product of aerobic respiration. To test for the presence of catalase, isolated colonies were selected and transferred via a sterile glass rod or tooth pick to a clean slide. Hydrogen peroxide (3%, Scholar Chemistry, Lot AD-5209) was applied drop wise to the bacterial colony on the slide. If the bacteria contained catalase, hydrogen peroxide was converted to water and oxygen gas, causing bubbles to appear on the slide. Catalase negative bacteria produced no reaction (Alexander and Strete, 2001).

3.2.3.4.6.3 Methyl Red-Voges- Proskauer (MR-VP) test

The methyl red test is used to determine organisms that ferment glucose to a stable acid end product in a great degree, lowering the pH of the system despite the presence of buffer. Media contained peptone, glucose, and a phosphate buffer. Broth was inoculated and incubated at 30^{o}C for 2 days to allow stable acids to be produced. At the end of the fifth day, methyl red indicator was added. Methyl red indicator is red at pH less than 4.4 and yellow at pH above 6.0, so a red color means result was positive, and a yellow result means negative (Leboffe and Pierce, 2012).

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3.2.3.4.6.4 Motility, Indole production and Hydrogen sulfide production

Indole is one of the degradation products from the metabolism of the amino acid tryptophan. Bacteria which possess the enzyme tryptophanase are capable of cleaving tryptophan to produce indole pyruvic acid and ammonia. Indole production is indicated

by the formation of a pink to red colour after the addition of three to four drops of Kovacs' reagent to the surface of the medium and gentle shaking (Cappuccino and Sherman, 2002).

Sulfur-Indole Motility (SIM) agar media was melted, dispensed in tubes and then autoclaved. On setting, each of the tubes were inoculated by stabbing with the all isolates respectively in duplicate for replication then incubated at 72°C and 55°C for 48 h. Two uninoculated tubes were used as controls. Presence of indole was detected by addition of Kovac's reagent to 48 h cultures of the isolates according to the protocol of (Benson, 2002). A negative reaction was indicated by the formation of a yellow colour. The ability of isolates to produce hydrogensulfide from substrates was indicated by a black colour while the absence of black colour in the media indicated negative results. Lack of motility was detected by the confinement.

3.2.3.4.6.5 Citrate utilization

Some micro-organisms utilize citrate as a sole source of energy in the absence of fermentable glucose. Citrate permease in bacteria facilitates the transportation of citrate into the cell where it is acted on by citrate enzyme to produce oxalo-acetic acid and acetate, which are converted to pyruvic acid and carbon dioxide making the medium alkaline due to carbon dioxide combining with sodium and water to form sodium carbonate that changes bromothymol blue from green to blue (Cappuccino and Sherman, 2002).

The ability of the isolates to use citrate as carbon source for their energy was investigated by Simmons citrate agar slants as previously described (Benson, 2002; Williams *et al*., 1989). Simmons citrate agar was boiled to melt. The medium was then dispensed in tubes and autoclaved. On cooling, the slants were inoculated by streaking with each of the isolates in duplicate for replication and incubated at 72°C for 48 h.

Uninoculated tube with the same medium served as control. Utilization of citrate was indicated by colour change from green to deep blue, while for the negative isolates the colour remained green (Cappuccino and Sherman, 2002).

3.2.3.4.6.6 Oxidative/Fermentative Test (O/F)

The oxidative-fermentative test determines if certain gram-negative rods metabolize glucose by fermentation or aerobic respiration (oxidatively). During the anaerobic process of fermentation, pyruvate is converted to a variety of mixed acids depending on the type of fermentation. The high concentration of acid produced during fermentation will turn the bromothymol blue indicator in OF media from green to yellow in the presence or absence of Oxygen.

Certain non-fermenting gram-negative bacteria metabolize glucose using aerobic respiration and therefore only produce a small amount of weak acids during glycolysis and Krebs cycle. The decrease amount of peptone and increase amount of glucose facilitates the detection of weak acids thus produced. Dipotassium phosphate buffer is added to further promote acid detection.

O/F Test is used to determine if gram-negative bacteria metabolize carbohydrates oxidatively, by fermentation, or are nonsacchrolytic (have no ability to use the carbohydrate in the media) (https://microbeonline.com/oxidative-fermentative-test-principle-procedure-results).

3.2.3.4.6.7 Urease test

Many organisms especially those that infect the urinary tract, have an urease enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink.

3.2.3.4.6.8 Triple Sugar Iron Agar Test (TSI)

If lactose (or sucrose) is fermented, a large amount of acid is produced, which turns the phenol red indicator yellow both in butt and in the slant. Some organisms generate gases, which produces bubbles/cracks on the medium.

If lactose is not fermented but the small amount of glucose is, the oxygen deficient butt will be yellow (remember that butt comparatively have more glucose compared to slant i.e. more media more glucose), but on the slant the acid (less acid as media in slant is very less) will be oxidized to carbon-dioxide and water by the organism and the slant will be red (alkaline or neutral pH).

If neither lactose/sucrose nor glucose is fermented, both the butt and the slant will be red. The slant can become a deeper red-purple (more alkaline) as a result of production of ammonia from the oxidative deamination of amino acids (remember peptone is a major constituents of TSI Agar).

If H2S is produced, the black color of ferrous sulfide is seen.

3.2.3.4.6.9 SIM Test

SIM tests the ability of an organism to do several things: reduce sulfur, produce indole and swim through the agar (be motile). SIM is commonly used to differentiate members of *Enterobacteriaceae*.

SIM medium contains nutrients, iron, and sodium thiosulfate. One of the nutrients is peptone, which contains amino acids, including tryptophan.

If an organism can reduce sulfur to hydrogen sulfide, the hydrogen sulfide will combine with the iron to form ferric sulfide, which is a black precipitate. If there is any blackening of the medium, it indicates the reduction of sulphur and is a positive result.

The sulfur and motility test results should be determined before you perform the indole test.

Some bacteria possess the ability to produce the enzyme tryptophanase, which hydrolyzes tryptophan. The end products of this hydrolyzation are indole, pyruvic acid, and ammonia, by way of deamination. The Kovac's reagent that you add to the SIM medium to test for indole contains hydrochloric acid, p-dimethylaminobenzaldehyde (DMABA), and n-amyl alcohol. DMABA reacts with indole to produce a red quinoidal compound. If the reagent turns red, the indole test is positive.

3.2.4 Molecular Characterization of Putative *Geobacter* Species

3.2.4.1 Genomic DNA isolation

3.2.4.1.1 Preparation of cells

Putative *Geobacter sulfurreducens* were revived respectively and streaked on LB agar plate and incubated overnight at 37°C. Then, a single isolated colony was inoculated in 2 ml of LB medium and incubated at 37°C for 12 hours. From this overnight culture 1.5 ml was transferred to sterilized eppendorf tube and centrifuged at 5,000 rpm for 5 minutes at 4°C. Immediately, supernatant was discarded by aspiration and remaining overnight culture (0.5 ml) was also added to same tube containing the cell pellet and centrifuged again at 5,000 rpm for 5 minutes at 4°C. Then, supernatant was removed as much as possible without disturbing the cell pellet.

3.2.4.1.2 Cell Lysis

The cell pellet was re-suspended in 450 µl of TE1 buffer by gentle pipetting. The solution was split into two fresh sterilized eppendorf tubes by transferring 225 µl of above suspension to each tube. To each tube 180 µl of lysozyme (1 mg/ml) was added. Both tubes were incubated at 37°C for 30 minutes gently mixing the solution by inverting the tube every 5 minutes for proper cell lysis. Then 45 µl of STEP solution was added in both tubes and incubated at 37°C for 45 minutes or until the solution became clear due to cell lysis, with gentle inversion in between the ice incubation period. Equal volume of chilled phenol (450 µl) was added and mixed well. The mixture was centrifuged at 13,000 rpm for 10 minutes. The upper aqueous layer containing DNA was transferred to fresh sterilized eppendorf tube without carryover of lower organic phase. Again, equal volume

of chilled phenol: chloroform: isoamyl alcohol (25:24:1) was added to above aqueous solution, mixed well and centrifuged at 13,000 rpm for 10 minutes at 4°C. After collection of aqueous layer in a fresh tube, equal volume of chloroform was added and mixed. The mixture was then centrifuged at 13,000 rpm for 2 minutes and the aqueous phase was collected in a fresh eppendrof tube.

3.2.4.1.3 Genomic DNA recovery

To the aqueous solution (450 µl) containing genomic DNA, 100 µl of 3M chilled sodium acetate (pH 5.2) and double volume of 95% ethanol (1,100 µl) was added and incubated at -20°C for 30 minutes. Then the mixture was centrifuged at 13,000 rpm for 20 minutes at 4°C. The supernatant was poured off and pellet was washed with 250 µl of 70% ethanol without disturbing the pellet. Then the solution was centrifuged at 13,000 rpm for 10 minutes at 4°C. After draining the supernatant, remaining ethanol was removed by keeping the tube open in room temperature for 5- 10 minutes. Care was taken not to over dry the DNA pellet. The genomic DNA was re-suspended in 100 µl TE buffer (pH 8.0) and stored at -20°C until use.

3.2.4.2 DNA quantification

Isolated DNA from each sample was quantified from nanodrop at biotechnology laboratory. It was give directly DNA purity = A260 / A280 and concentration of genomic DNA.

3.2.4.3 Amplification of gene present at *Geobacter* species.

3.2.4.3.1 Primer designing

Primer were designed manually using different web based tools discussed below for meeting the parameters according to the general guidelines mentioned in appendix using the nucleotide sequence deposited in the Gene Bank for *Geobacter sulfurreducens* 16s rRNA, omcJ (2685380).To meet the general criteria of primer designing regarding length, melting temperature I, GC content, secondary structure, self and hetero dimer etc. BLAST- search (non-redundant database in NCBI) was performed for each primer to ensure that the designed primers would not prime to non-specific regions. *In-silico* PCR amplification was performed to examine the performance of the designed primer. The basic information of designed primers is presented in Appendix

3.2.4.3.2 PCR amplification

The chromosomal DNA sample isolated from *Geobacter sulfurreducens,* used as the template DNA for PCR amplification using specifically designed primer sand chromosomal DNA of these organisms were used for amplification of 16srRNA and specific gene. The DNA sample was quantified spectrophotometrically and diluted in 1X

TAE to prepare template DNA with concentration of 50ng/µl. PCR amplification was performed with TIANGEN 2X Tag PCR Master Mix DNA polymerase. The general protocol was followed to prepare the reaction mixtures and to determine PCR conditions are given as:

Table 3.2: Polymerase Chain Reaction Condition

S.N	STEP	TEMPERATURE	TIME	CYCLE
1.	Denaturing temperature	95°	5’	1
1.	Denaturing	95°	1’	5
2.	Annealing	52°(16srna)	1’	
3.	Extension	72°	0.5’	
1	Denaturing	95°	1’	25
2	Annealing	54°	1’	
3	Extension	72°	0.5’	
1	Final extension	72°	5’	1
2	Hold	4°	A	

Table 3.3: Polymerase chain reaction mixture

S.N	Components	Volume (total volume 10 µl)
1	DNA template(50ng/µl)	1 µl
2	Master mix (2X)	5 µl
3	Forward primer (10pmole)	1 µl
4	Reverse primer (10pmole)	1 µl
5	Nuclease free water	2 µl

The two step reaction conditions were used for PCR amplification. For the first 5 PCR cycles, the annealing temperature targeted to only priming region (3’-end nucleotide complimentary to template DNA) was used whereas for rest 25 PCR cycles, annealing temperature (Tm– 5°C) was determined regarding the whole primer sequence. This strategy increases overall PCR efficiency and yield of PCR product. PCR was performed using Thermal cycler: My Gene Series Peltier Thermal Cycler Model. Then 5 µl of PCR product was subjected to 1% agarose gel electrophoresis along with 100bp TIANGEN DNA ladder in a separate well and visualization was done in Gel Documentation System.

3.2.5 Electricity production and N_2 reduction test for putative *Geobacter* species

Extracellular electron transfer from bacteria was tested by the microbial fuel cell (MFC) as an electron donor for anode electrode. Material and pretreatment of membrane procedure mentioned at appendix 9. Here only operational method was explained.

3.2.5.1 Construction of MFC

The MFC set was set up by joining the two plastic bottles each having capacity of 1000ml via a glass tube with help of a rubber gasket sterilized by 70% ethanol. Nafion117 was used as a permeable membrane. The graphite felt was used as anode at anodic chamber where 5% culture of one of the putative *Geobacter* was kept. At the cathodic chamber, 700ml of 0.1M pH 7.6 phosphate buffer was kept where platinum wire was inserted as an electron acceptor, the wires arising from the anode and cathode compartment were connected to the multimeter and open circuit voltage was observed. Voltage production was observed for 7 days at the interval of half hour.

3.2.5.2 Nitrogen reduction test for putative *Geobacter*

Quantification was done by centrifuging 1ml NCF media with incubated bacteria at 5000rpm for 5 minutes and the supernatant was gained for spectrophotometric analysis. For this, 2ml solution containing 1500µl of distilled water, 400 µl cultured supernatant and 100µl Nessler's reagent and absorbance was observed at 435nm. The amount ammonia production was calculated using standard curve.

3.2.6 Nanoparticle synthesis

3.2.6.1 Synthesis of Biogenic silica nanoparticles from rice husk char

Sol- gel method

Silica nanoparticles were synthesized from agricultural waste material i.e. rice husk char which contain cellulose, lignin, hemicellulose and silica. After burning rice husk char it is called Rice Husk Ash (RHA) almost containing about 90 % to 98% silica and some metal impurities. RHA exists in amorphous stage and possess high surface area which is better for using as sources of synthetic adsorption, carriers, medical additives, fillers in composite materials, drug delivers, etc (Le *et al*., 2013).

Common name of silica are silicic acid, quartz, glass, sand, anhydrous form of silicic acid, silicon dioxide etc. Actually silica is a polymer of silicic acid consisting interlinked of $(SiO4\}^{4-}$ units which is tetrahedral structure. Naturally occurred silica are crystalline whereas synthetically obtained silica are being amorphous mostly used in making silane and silicate solution (Bergna and Roberts, 2006).

Among the various method, Sol-gel is easy and fast technique to synthesize nanoparticle which involve simultaneous hydrolysis and condensation reaction where metal impurities are removed by treating acid and a sol of sodium silicate prepared by using alkaline solution and gets converted into a polymeric network of gel, and by using coagulation and precipitation technique the gel gets precipitated. From this method reliable nano-sized about average dimension 15 to 91 nm can be obtained (Le *et al*., 2013).

Biogenic silica nanoparticle synthesis was run thoroughly from rice husk char. Rice husk was washed with distilled water for three times and was dried on hot air Oven at 120^{o}C. Dried rice husk was burned at 600^{o}C in Muffle furnace for 6-8 hrs. Complete burn of rice husk resulted in white ash indicating the complete burning of carbon. 10g rice husk ash (RHA) was kept in Round bottom Flask with 30%w/v H_2SO_4 and 10% v/v HCl, then it was boiled at 80^{O}C on heating mantle fitted with reflux condenser for 4 hours. Acid treated solution was then filtered with Whattman filter paper No 1. Washing was done with DDW till the filtrate reached pH 7.0. Then acid washed RHA in the filter paper was scooped out with plastic spoon.

The scooped RHA transferred in to borosil round bottom flask and treated with 1M NaOH for 6 hours at 80^{o}C on heating mantle for solubilization in the form of sodium silicate. Sodium silicate solution was then filtered through Whatman no.1 filterpaper. After collecting Sodium Silicate solution, warm DDW was passed through remaining residues for complete extraction of sodium silicate solution.

Then sodium silicate solution was titrated with 1N HCl until pH 7.0 when silica gel started to form and was aged for 18 hrs. After aging, gel was washed by agitating it in 100 ml of DDW using magnetic stirrer for 10 minutes. Then solution was centrifuged at 3,000 rpm for 15 minutes and supernatant was discarded. Gel was collected with help of glass rod. These washing steps were repeated for three times. Gel was dried at 110^{o}C for overnight. The dried material was presumed to be silica nanoparticles and was collected in polypropylene Falcon tubes that was then stored in desiccator to prevent absorption of any moisture. This is the standard protocol (Thuc and Thuc, 2013)

Figure 3.4: Silica Nanoparticles Preparation

Again protocol has been changed for the synthesis of silica nanoparticles from rice husk. Instead of HCl, phosphoric acid (H_3PO_4) was chosen and gelling was done by glacial acetic acid in spite of HCl.

3.2.6.2 Characterization of silica nanoparticles

3.2.6.2.1 X-Ray Diffraction

X-ray helps to recognize the synthesized particle whether amorphous or crystalline. It was done in the lab of India at the range of diffraction angle (2θ) with the range 5 to 80. It is an analytical tool for separating of matter in nature by using Bragg's equation $n\lambda=2d \sin\theta$ where n is an integer, λ is characteristics of wavelength of X-rays ,d is inter-planar distance between rows of atoms and θ is the angle of X-ray beam with respect of these planes. Diffraction pattern can be thought as a chemical fingerprint, and chemical identification (Toraya, 2016).

3.2.6.2.2 Scanning Electron Microscopy (SEM)

SEM is the technique that produces images of sample by scanning the surface of sample by using of focused beam of electrons. The main features of SEM are to use of high energy electrons to minimize the effects of surface contamination of sample, oblique

scanning, direct viewing at low magnification and useful in the determination of size distribution (surface area) of the specimens (McMullan 1953).

3.2.6.2.3 Fourier Transform Infrared Spectroscopy

FTIR stands for Fourier transform infrared, infrared radiation is passed through a sample such emissive or absorptive spectrum of solid or liquid or gas resulting signal at detector gives off a molecular fingerprint of the sample helps to identify or quantify the unknown sample.

FTIR spectrometer has several advantages than conventional dispersive IR spectrometer having improved signal-to-noise ratio of spectrum, high accuracy wavenumber, and short scan time of all frequencies, high resolution (0.1 to 0.005 cm^{-1}) and error in a range of ± 0.01cm^{-1}. So Fourier transform converts output of detector into an interpretable spectrum and produce pattern that provides the structural insights.

FTIR specially use in chemical application like identification of functional group of organic compounds and inorganic ions.

3.2.6.3 Quantification of biogenic silica nanoparticles from rice husk char

3.2.6.3.1 Calibration Curve for Molybdate Reactive Silica

The standard curve to quantify the isolated silica nanoparticles was prepared following the Indian standard.

First 0.473 g of sodium metasilicate nanohydrate (Na_2SiO_3. $9H_2O$) was dissolved in 100 ml of DDW. Then 10 ml of this stock solution was diluted to 500ml in DDW to give the solution concentration 20µg of SiO_2/ml.

Then different volumes of this solution were taken consecutively (5ml, 10ml and so on) to prepare 50 ml of silica solution constituting SiO_2 from 100µg to 1,000µg. To each 50ml solution, 1ml of 1:1 of hydrochloric acid and Water and 2 ml ammonium molybdate reagent was added in quick succession. The solutions were mixed by inverting at least six times and let to stand for 5-10 minutes. After that 2ml of oxalic acid solution was added and mixed well. The yellow color appeared as the reaction of silica to molybdic acid. The intensity of color was detected at 410 nm after 2min but before 15 minutes after addition of oxalic acid. The blank for this was made using 50 ml DDW in place of silica solution following similar protocols of addition of other reagents.

3.2.6.3.1.2 Quantification of molebdate reactive silica

Quantification of concentration of molybdate reactive silica in biogenic nanosilica produced was performed following the protocol described:

The synthesized silica nanoparticles were again solubilized in 100 ml of 1N NaOH to give 1g/l sodium silicate solution. Then solutions were diluted with DDW and reagents to give calculated total silica (but not reactive silica) concentration of 200 µg and 500 µg per 50 ml solution. The absorbance of these unknown solutions were determined spectrophotometrically to calculate total amount of reactive silica by using formula derived from calibration curve with constants obtained from standard sodium silicate solution.

3.10.3.3 Bacterial growth at residues came from acid treatment during synthesis of biogenic silica

Different concentration 0.1mM, 0.2mM, 0.3mM, 0.4mM, 0.5mM were used instead of mineral mix at preparation of GS-15 media and a loop full of fresh culture of putative *Geobacter* samples were cultured and blank and positive culture also prepared and incubated at 37°C for three days and bacterial growth was checked observing optical density at 600nm.

3.2.6.4 Functionalization of biogenic silica

Biogenic silica both obtaining from standard protocol and modified protocol were treated with plasma technology where they were run with high voltage 6000V. Silica treated with different condition like as: on vacuum, distilled water and atmospheric nitrogen where all molecules become at gaseous state and bonding each other and give a new product of interest. Here, our intension was functionalization of the nitrogen group into the silica (SiO_2) which could be used as nitrogenous fertilizer. The confirmation of functionalization was checked by FTIR.

3.2.7 Consortium of bacterial culture

3.2.7.1 Preparation of bacterial culture

Fresh cultures of putative *Geobacter, Azotobacter, Pseudomonas* and *Azospirilium* were prepared from glycerol stock to LB media and incubated at 28°C for overnight. 100ml NCF media was prepared with pH 6.8 and 4.5 at different conical flask then distribute 10 ml NCF media on each culture tube (preparing two set of culture media with pH 6.8 and 4.5) and subjected for autoclaved. Then autoclaved media were ready for inoculation of different consortium of the bacteria and biogenic silica was also added to some vial of culture to see the effect of its. A loop full bacteria was inoculated both media having pH 6.8 and 4.5 and incubated at 28°C for 3 days.

3.2.7.2 Biological nitrogen reduction and carbohydrate reduction test

At 3rd day, nitrogen test by Nessler's reagent and carbohydrate test by DNS and Rochelle's salt

The procedures were performed same as mention above topic number 3.2.3.4.2.

3.2.7.3 Ethanol test

Acidified potassium dichromate ($K_2Cr_2O_7$) solution is also used to distinguish between primary, secondary and tertiary alcohols.

So this method can be used determination of ethanol based on oxidation of ethanol by reacting with excess of acidic potassium dichromate solution.

$$3C_2H_5OH + 8H_2SO_4 + 2K_2CR_2O_7 \longrightarrow 3C_2H_5COOH + 2CR_2(SO_4) + 11H_2O + 2K_2SO_4$$

When ethanol is present in an aqueous solution, chromium ions oxidize ethanol, and these ions are reduced from the +6 oxidation state to +3, changing the color from orange to green (Seo *et al*., 2009; Miah *et al*., 2017)

This study aimed to use a convenient technique for measurement of alcohol from co-culture of the bacteria and comparison of between them that which combination are given better result in the production of ethanol.

To the 3rd day samples of different consortium of bacteria with pH 4.5, 0.1% yeast was added and again incubated at 28°C for three days.

At 7th day, the consortium of bacteria with yeast was centrifuged at 5000 rpm for 5 min and supernatants were taken as samples for ethanol test.

3.2.7.4 Calibration Curve for Absolute Ethanol and Quantification of Ethanol from Samples

Dichromate oxidation method

Potassium dichromate was prepared by dissolving 10 gm potassium dichromate in 100ml of 5M H_2SO_4. 10 Different concentration of standard ethanol were prepared at different test tube and each sample was taken separately (about 1 ml). 1 ml Tri-n-butyl phosphate (TBP) was added and then vortexed for 10 min and allowed to stand for separate layer or were centrifuged at 12000 rpm for 5 min. About 750µl of upper TBP layers were taken in new microtubes then equivalent amount of potassium dichromate solution use and then vortexed for 10 min and then centrifuged for 5min which allow to separation of layers. Lower aqueous layer was separated and absorbance at 595nm was taken. A standard graph of ethanol between concentration and absorbance was plotted and concentration of ethanol of unknown sample was determined accordingly.

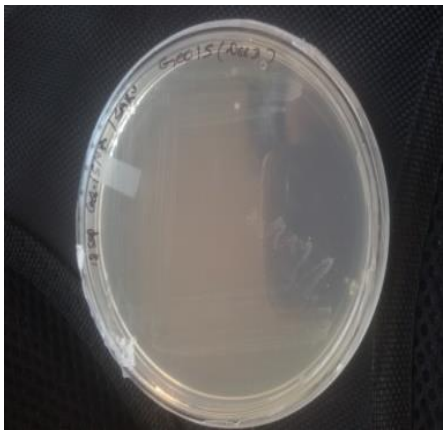
4. RESULTS and DISCUSSION

4.1 Isolation and Screening of Isolated Bacteria

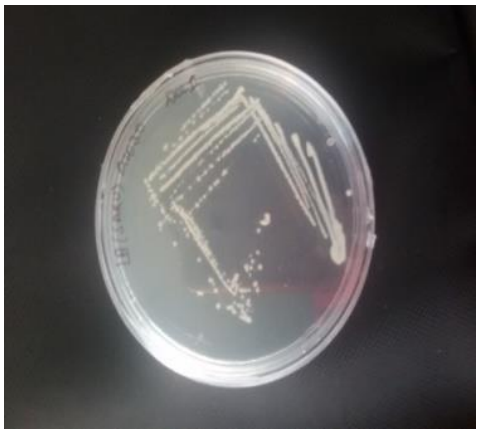
Total 43 samples were isolated and screened 18 samples as putative *Geobacter sps*, 4 putative *Azotobacter sps and 18 putative Pseudomonas sps* based on Carbon catabolite repression method. The putative *Geobacter sps* were preliminary confirmed by biochemical test.

Table 4.1 List of Isolated Bacteria from Soil Samples

SN	Sample name	Number of isolated colony	Putative Geobacter	Putative Azotobacter	Putative Pseudomonas
1	Bhaduare1	1-3	1,2		
2	Bhadaure2	4-12	4,5,8,9,12	11 ,30	7
3	S3	13-19	13,14,16,17,18		15,19
4	S4	20-24	22,	23,24	20,21
5	River sample S5R	25-30	25		26,27,28,29,10
6	Way to panchase SW6	31-35	-		31,32,33,34,35
7	Panchase lake PL1	36-40	-		36,37,38,39
8	Way to panchase lake WPL	41-42	40,41,42		
9	Sidhane kaski	43	43		



Putative *Geobacter* sps



Putative *Azotobacter* aps

Putative *Pseudomonas* sps

Putative Azospirillum sps (Taken from colleague, Anju Tamang)

Figure 4.1 Different isolated bacteria from different soil samples

Bacteria are isolated on the basis of carbon catabolite repression which is also mentioned in the paper; Görke and Stülke, 2008. In our study, For *Geobacter sulfurreducens*, collected soil sample was enriched with GS-15 media and incubated at 28°C for 3 days to create anaerobic conditions as this bacterium is known to survive in anaerobic condition (Thomas, 2006; Rollefson *et al*., 2009). Then 100 µl sample was mixed in top agar containing GS-15 medium and was poured in petriplate (bottom agar) consisting of GS-15 media as described for *Geobacter* (Lovley *et al*., 1993). Further screening process was done with the isolated colonies. Three different bacteria were screened according to our purpose. As *Geobacter sulfurreducens* is nitrogen fixing (Ueki and Lovley, 2010) so it was grown on nitrogen free acetate medium to rule out from other non-nitrogen fixing acetate utilizing bacteria.

Further selection was done by culturing *Geobacter sulfurreducens* on aniline as sole carbon source since it has been described that this bacterium can breakdown aniline and use it as carbon source (Kazumi *et. al*., 1995). Moreover, *Geobacter sulfurreducens* can utilize aniline in anaerobic condition, anaerobic growth on aniline (0.05mM) N_2 free medium to rule out other aniline utilizing non nitrogen fixing bacteria such as *Frateuria* (Murakami *et. al.*, 2003) and *Rhodococcus* (Zhuang *et al*., 2007). Furthermore, it was validated by growing in 0.05mM ethylene glycol nitrogen free medium (GS-15) to rule out from ethylene glycol utilizing organism like *Azotobacter* (Townsend *et. al.*, 2005) and *Pseudomonas* (Mückschel *et al*., 2012). Blast results of ethylene glycol utilizing gene, tartronate semi-aldehyde synthase (Gcl), revealed that there is no such homologue in *Geobacter* indicating absence of this gene to utilize ethylene glycol. Hence those isolates that could not survive in this media confer the isolated organism could be *Geobacter* sps. And those survive on ethylene glycol streaked on toluene media where only

Pseudomonas survive (Zylstra and Gibson, 1989) because of presence of genes that are able to degrade the toluene from the media (de Bont, 1998). Among *Pseudomonas* sps, *P. putida, P. aruginosa* and *P. testoterovani, P. acidovorani* showed the efflux system of toluene and that of *P. putida* is schematically presented (Figure 4.2).

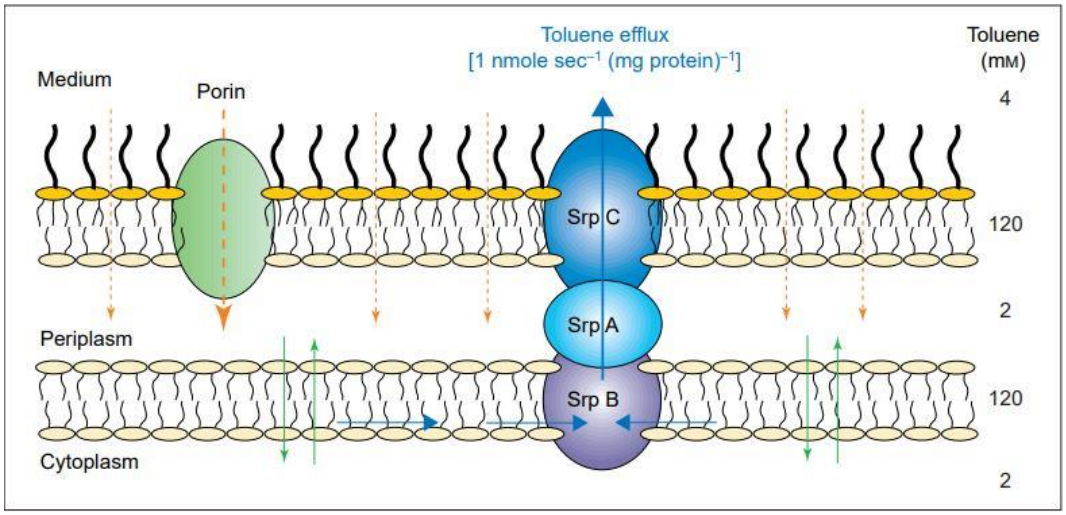


Figure 4.2 : Schematic Diagram Of Toulene Import And Efflux In *Pseudomonas putida*.

In the figure, an equilibrium situation with an external concentration of toluene is set up with 4mM and Cytoplasmic toluene concentration with 2mM. Toluene enters via outer membrane. Though it is not clear from where toluene passes via whether porin or diffuses, efflux of toluene is suggested from inner leaflet of cytoplasmic membrane srpABC group. The srp ABC genes are for the regulation of solvent resistance pump of toulene in *P. putida.* The homologous genes were also reported *P. aeruginosa* (Srikumar, Li and Poole, 1997). So, such type of homologs have to be present in our isolates of putative *Pseudomonas* species. This was the first time report where such reduced carbon source variation has been carried to isolate any bacteria that has been developed on the works of Machchhendra Thapa thesis works. Thus, new insights and protocol has been optimised and devised in the laboratory which could provide better method for the isolation and screening of Geobacter, Azotobacter and Pseudomonas from the massive soil sample.

4.2 Biochemical test of Isolates:

4.2.1 Gram staining of putative *Geobacter*

Microscopic study of Putative *Geobacter* has shown pinkish color that means the isolated one are gram negative. They are rod shape and non-motile. From this we can conclude they have very thin peptidoglycan which would be dissolves when alcohol is added in the protocol of Gram staining. Some of strain also obtained purple color that might be due to contamination of other microorganisms otherwise obtaining almost are

gram negative bacteria. This is in concordance to the reported characteristics of *Geobacter* sps (lovely *et al*., 1993).

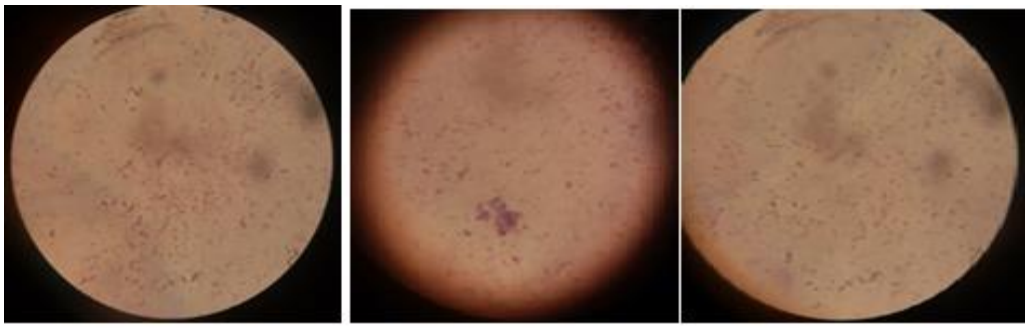


Figure 4.3: Gram staining of isolated Putative Geobacter: Gram negative due to pinkish color

4.2.2 Catalase test

All the isolates were seen catalase positive indicating that they had catalase enzyme. Catalase mediates the breakdown of hydrogen peroxide (H_2O_2) into oxygen and water. Almost all aerobics and facultative anaerobes show positive towards the catalase test. From the experiment it can be thought that the isolated one were either facultative anaerobic or aerobic bacteria.

Figure 4.4 Catalase test of putative *Geobacter* sps

4.2.3 Citrate test

Citrate ($C_6H_4O_7^{4-}$) is used as a carbon and energy source and alkaline carbonates and bicarbonates are produced. Growth of bacteria turned bromothymol blue indicator to green blue because with an increase of medium pH above 7.6 the color changes into blue. All the putative *Geobacter* (19 samples) showed positive towards citrate test meaning that they could use citrate as carbon and energy source with breakdown into oxaloacetate and acetate.

Figure 4.5 Citrate test of isolated Geobacter sps; positive test towards citrate test

Uninoculated culture tube with the same medium served as control.

4.2.4 Methyl Red-Voges Proskauer test (MR-VP Test)

Methyl red test

Methyl Red (MR) test is a biochemical test performed on bacterial species to detect the ability of an organism to produce stable acids end products (Mixed-acid fermentation) from supplied glucose. All the isolates of putative *Geobacter* sps were negative towards MR test because there was no color change from yellow to red.

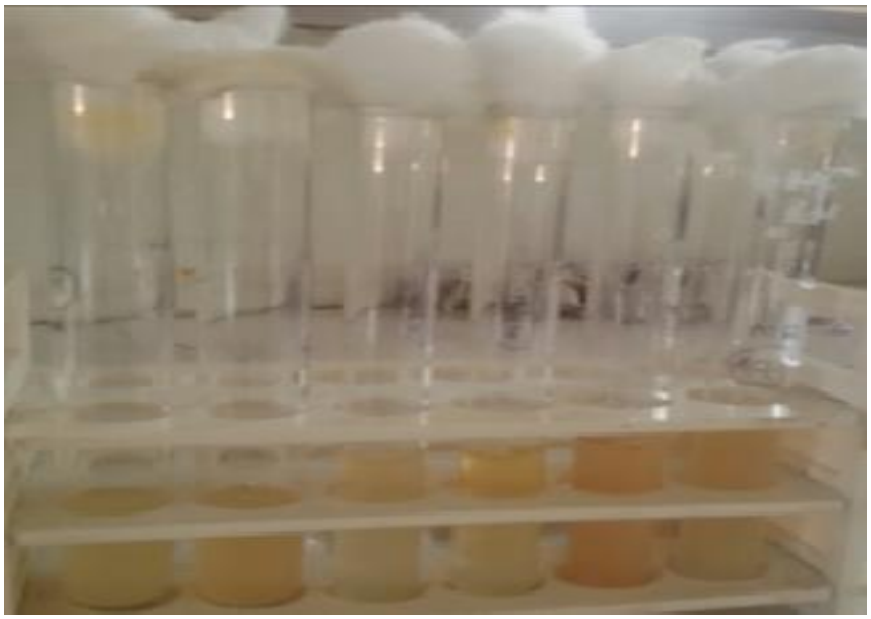


Figure 4.6 Methyl Red test of isolated *Geobacter* sps; no color changed of media

Voges- Proskaur test

Voges–Proskauer (VP) test is used to detect acetyl-methyl-carbinol (acetoin) in a bacterial broth culture. The test was performed by adding alpha-naphthol and potassium hydroxide to the Voges-Proskauer broth that inoculated with bacteria. A cherry red color indicates a positive result, while a yellow-brown color indicates a negative result. All the putative *Geobacter* sps had shown positive test.

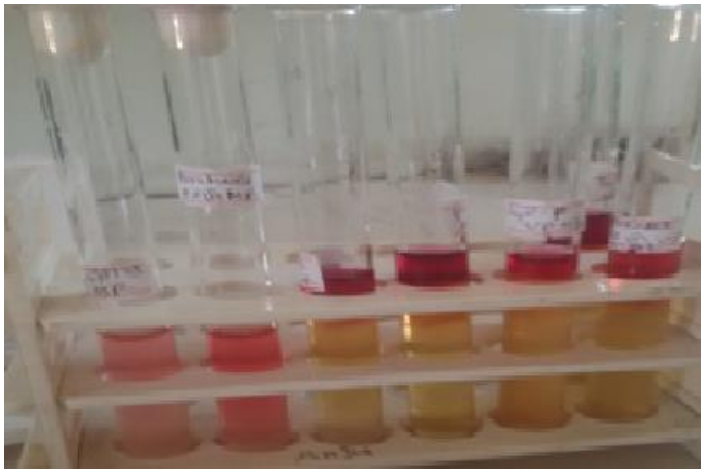


Figure 4.7 Voges- Proskaur tests of isolated *Geobacter* sps

4.2.5 Oxidative/Fermentative Test (O/F)

The oxidative-fermentative test determines if certain gram-negative rods metabolize glucose by fermentation or aerobic respiration (oxidatively). During the anaerobic process of fermentation, pyruvate is converted to a variety of mixed acids depending on the type of fermentation.

Figure 4.8: Oxidative-Fermentative test of isolated putative *Geobacter* sps.

4.2.6 Urease test

All the putative *Geobacter* had shown negative result which mean there was absent of urease enzyme with them. If they have urease enzyme, they converts the urea to ammonia and color change the media from yellow to pink. There was no any kind of urease like enzyme was reported from *Geobacter sps.*

Figure 4.9: Urease test; no color changed

4.2.7 SIM test

In the experiment, SIM media contained ferrous ammonium sulphate and sodium thiosulfate which both serve as indicator for the production of hydrogensulfide (H_2S) and when a black precipitate is detected in the media then it is presumed to be positive. Isolates no. 12, 16, 17 and 43 were found to be hydrogen sulphide producer while remaining were gave negative results. For indole test, sample no, 1, 2. 4, 8, 22 had shown indole positive because they had red band at the top of medium when Kovac's reagent was added on them and remaining did not turn into red so thus were indole negative but their motility were not tested further but if the putative ones were *Geobacter* they should show motility.

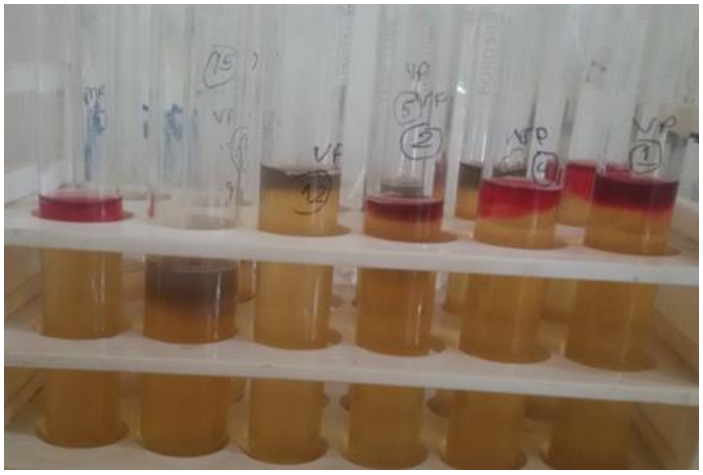


Figure 4.10: SIM test; black precipitate due to H_2S and red band due to indole Positive of media after inoculation of putative *Geobacter*

4.2.8 Triple sugar iron (TSI) test

TSI is a semi solid media having slant and butt. But of most of the media were red that means no sucrose was fermented but slant was turned into yellow that means they produce acid at top as it was slight red meaning the acid was oxidized to CO_2 and water by organism and become red (alkaline or neutral). Isolates no. 2, 4, 5,8,9 14, 16, 17, 22 and 25 had produced gases and seen cracks but Media was not turned into black so could not be sure whether that produce gas was hydrogen sulphide (H_2S) or other gases. It might be carbon-dioxide and water.

Figure 4.11: Triple iron sugar (TSI) test of putative *Geobacter* sps

According to this experiment, the isolated putative *Geobacter* are gram negative, facultative anaerobes, hydrogen sulphide reducer, citrate utilizer as well as extracellular electron donor which coincide with literature where their characteristics are mentioned as: they are gram negative, can grow with Oxygen (Lin, Coppi and Lovley, 2004), citrate and acetate oxidiser (Caccavo *et al*., 1994), are keep in δ-proteobacteria (Lin, Coppi and Lovley, 2004). Following table 4.2 shows the status of isolated putative *Geobacter* sps.

Test Sample	Gram - Staining	Catalase Test	O/F Test	Indole	H_2S	Gas	Motility	Citrate	Remark
1	+	+	F	+	_	_	-	+	Rejected
2	_	+	F	+	_	+	-	+	
4	_	+	F	+	+	+	-	+	putative
5	_	+	F	_	_	+	-	+	
8	_	+	F	+	_	+	-	+	
9	_		F			+	-		
12	_	+	F	_	+	-	-	+	Putative
13	_						-		Putative
14	_		F	_	_	+	-	+	
16	_	+	F	_	+	+	-	+	Putative
17	_	+	F	_	+	+	-	+	Putative
18	_						-		Putative
22	_	+	F	+	_	+	-	+	Putative
25	+	+	F	_	_	+	-	+	Rejected
40	_						-		Putative
41	_						-		
42	+	_	F	_	_	_	-	+	Rejected
43	_	+	F	_	+	_	-	+	Putative

Table 4.2 Summary Biochemical Tests of Putative *Geobacter sps.*

4.3 Molecular characterization of Putative Geobacter

DNA extraction of six isolates was done at 0.8% agarose gel electrophoresis (Figure 4.12) and DNA quantification was also performed (Table 4.3).

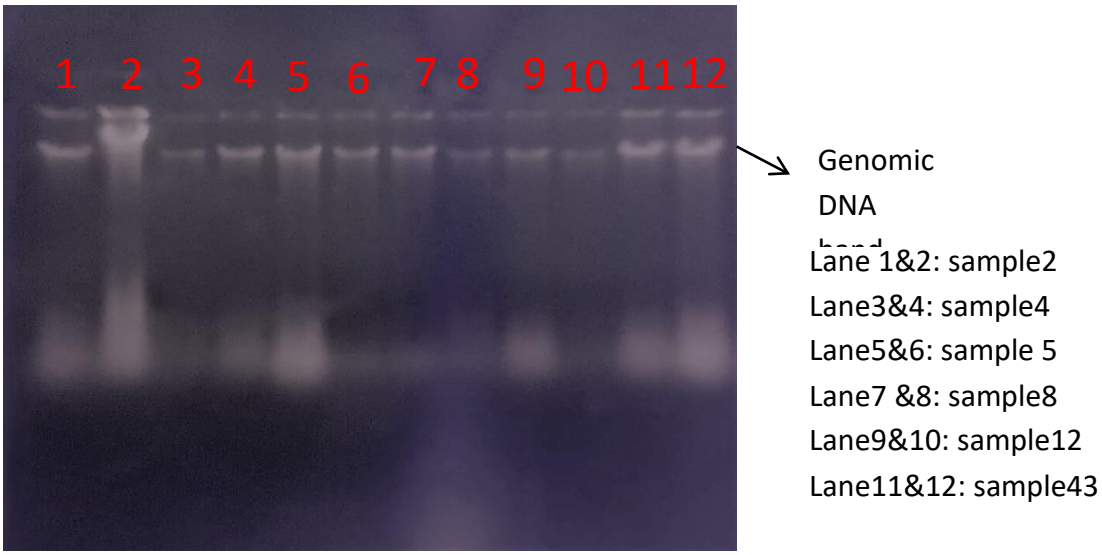


Figure 4.12: 0.8% Agarose gel electrophoresis of DNA samples

Table 4.3: concentration of DNA of the isolated Putative *Geobacter* sps

samples	Nucleic acid concentration(ng/µl)	$OD_{260/280}$
1	86.03	1.78
2	221.52	2.03
4	53.02	2.16
5	296.86	2.08
8	204.99	2.10
9	40.52	2.77
12	77.24	2.00
13	220.00	_
14	105.00	
16	65.01	_
17	78.19	1.41
18	100.00	_
22	75.90	_
25	202.02	_
40	105.00	1.87
41	46.20	_
42	99.02	_
43	170.51	2.03

The concentration of DNA of the samples were determined by Nanodrop, highest DNA concentration were found of the samples number of 2, 5, 8 and 25 with respective concentration 221.52, 296.00, 204.99 and 202.02. Most samples like 2, 4, 5, 12, 43 having the ratio of Optical density 260/280 are more than 1.8 which denotes the protein contamination that might be due to lack of proper working of proteinase K enzyme. Some of the samples like 1, 17, 40 have A260/280 less than 1.8 which means there is contamination of RNA and this could be possible due to no use of Rnase during the extraction. The extracted DNA samples were diluted and used for Polymerase Chain Reaction (PCR).

Universal primers 16S rRNA was used for the molecular characterization, Forward →5'CTA GTT GGT AGG GTA ATG GC 3' and Reverse → 5' GCA CCC TCC GTA TTA CCG 3' sequence were chosen from NCBI gene bank and had been using web tools Oligocalc and Oligoanalizer, their self–polymerisation, stability, mfold, GC content etc. were checked and *in silico*-PCR had done for their theoretical validity and experiment was also done and being amplified the bacterial gene with 16S rRNA means the these gene are proven that they are bacterial gene. Similarly, specific gene found in *Geobacter sulfurreducens*, OmcJ, was amplified choosing forward and reverse primers as sequence Forward 5' CTA GTT GGT AGG GTA ATG GC 3' and Reverse 5' GCA CCC TCC GTA TTA CCG 3' and have got significant results.

4.3.1 Primer design and performed polymerase chain reaction

Sample 17 and 12 have to be *Geobacter sulfurreducens* and we could say these isolated bacteria were non pathogenic and environment friendly. In the similar way remaining isolates could be confirmed. Confirmation of putative *Azospirillum* (N_2 and CO_2) isolates was through biochemical test and molecular characterization was performed by my collegue (Anju Tamang).

The product size of universal primer was found to be 300 bps which is in accordace with In-silico PCR analysis . Similarly, the product size of specific gene omcJ was obtained around 280 bps which is also in accordance with In-Silico PCR data analysis. This gives us confirmatory that our isolated *Geobacter* species contained that extracellular elcetron transfer gene and come close to those isolated ones are *Geobacter sulfurreducens*.

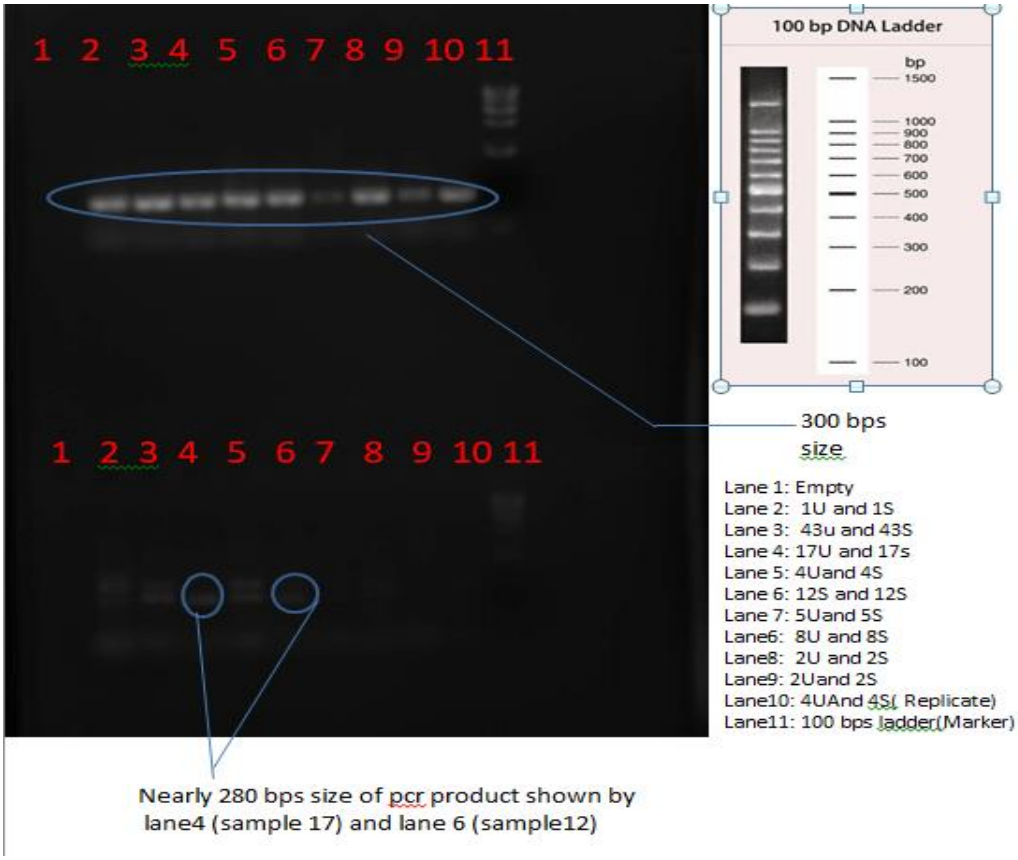


Figure 4.13: PCR product size of G. sulfurreducens at 1% agarose gel documentation

4.4 Electricity Production with One of *Geobacter* Isolates

Geobacter sps sample no 12 was used for electricity production and after two hour set up of culture at microbial fuel cell had given high rate production of electron that was 318 mV which would be transfer into graphite felt and complete the open circuit. It also denotes the bacterium was extracellular electron transfer bacteria.

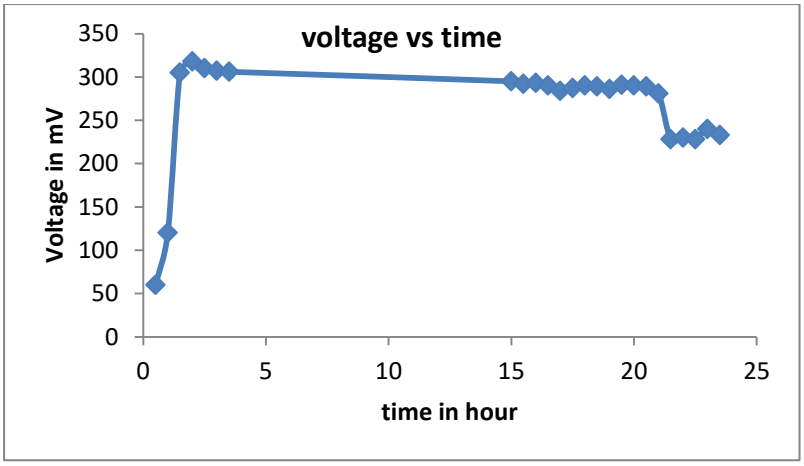


Figure 4.14: Electricity production by one of the isolated *Geobacter* sps

In the MFC, anode and cathode were fixed between two chambers, cathodic chamber contain cathode as platinum wire; electrode for the transfer of electron getting from anodic chamber.

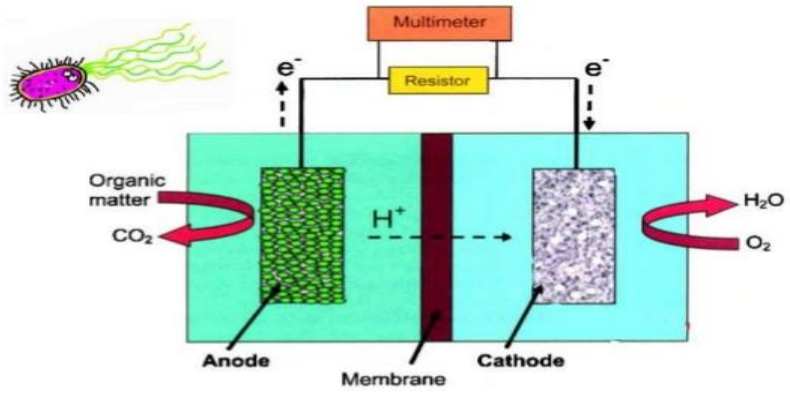


Figure 4.15: Schematic Diagram of Microbial Fuel Cell.

At cathodic chamber, phosphate buffer with pH 8.5 was put and then mouth of each chamber was closed with sterilized cotton so that there was no any contact with atmosphere and then voltage was measured with help of voltmeter without fitting resistance (R). At anodic chamber fresh culture of putative *Geobacter sulfurreducence* (5%) were left to grow and release of electrons for completing the electric circuits. Sample no. 12 was able to produce electricity. Research (Nevin *et al.*, 2009) has been reported that Geobacter sulfurreducens can produce 8.01 to14.82 mA current. So that it could be one of the alternatives of renewable source of electricity. For more yield, we could do chromosomal engineering with the gene that are responsible for extracellular electron transfer at *Geobacter sulfurreducens.*

There has been reported that Geobacter species have has pili which can locate Fe (III) oxides and establish contact with Fe (III) oxides so that generated electron from NADH

in inner membrane and quinone pool should be transfer into inner periplasmic cytochrome-c (Vandieken *et al.,* 2012) then into outer periplasmic cytochromes-c where OmcE, OmcS and omcJ genes transfer the electron to the pili which reduces the metal oxide with help of aromatic amino acid (Lloyd, J. 2003).

4.5 Calibration curve for ammonium quantification by Nessler's reagent

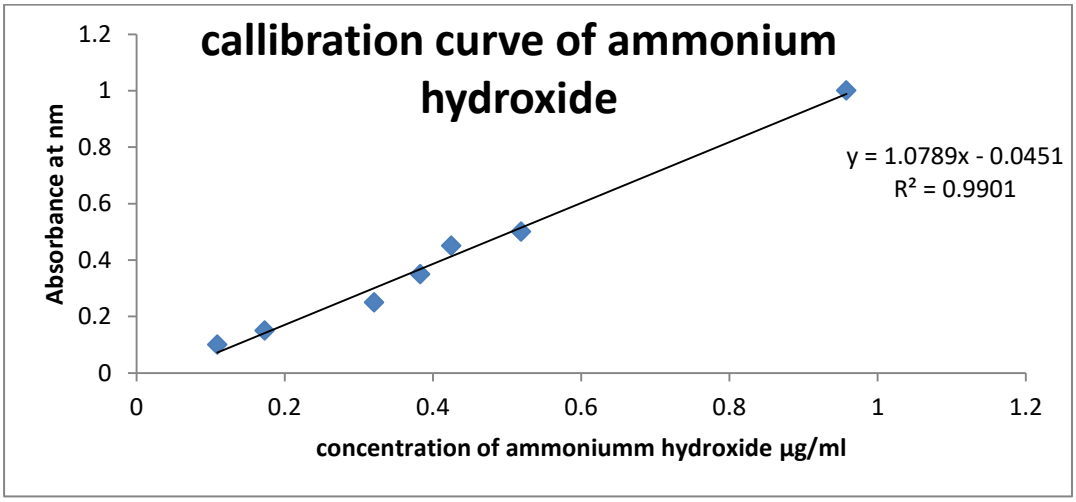


Figure 4.16: Standard Calibration Curve for Quantification of Ammonium Ion during Nitrogen Fixation

Calibration linearity curve was observed using different levels of calibration standards in the range 0.1 µg/ml to 0.6µg/ml including blank ammonium hydroxide (NH_4OH). It shows the linearity with correlation coefficient of 0.9901. Use of Nessler's reagent in the standards has formulated brown color to yellow in different intensities which was measured by UV-visible spectrophotometer and it was found that ammonium hydroxide gave a maximum lemda λ_{max} at 435 nm.

4.6 Electron Transfer Bacteria (Putative *Geobacter* sps) For Biological Nitrogen Fixation

Among the 18 samples of putative *Geobacter*, sample no. 12 has depicted highest concentration of production of ammonia. It was found to be 0.3 mg/ml. As we have known that *Geobacter sulfurreducens* are electron transfer bacteria as well as nitrogen fixer. As mentioned above it has two component systems, His-Asp phosphorelay system, activates the transcription of majority of nitrogen fixation and represses a gene encoding glutamate dehydrogenase during nitrogen fixation.

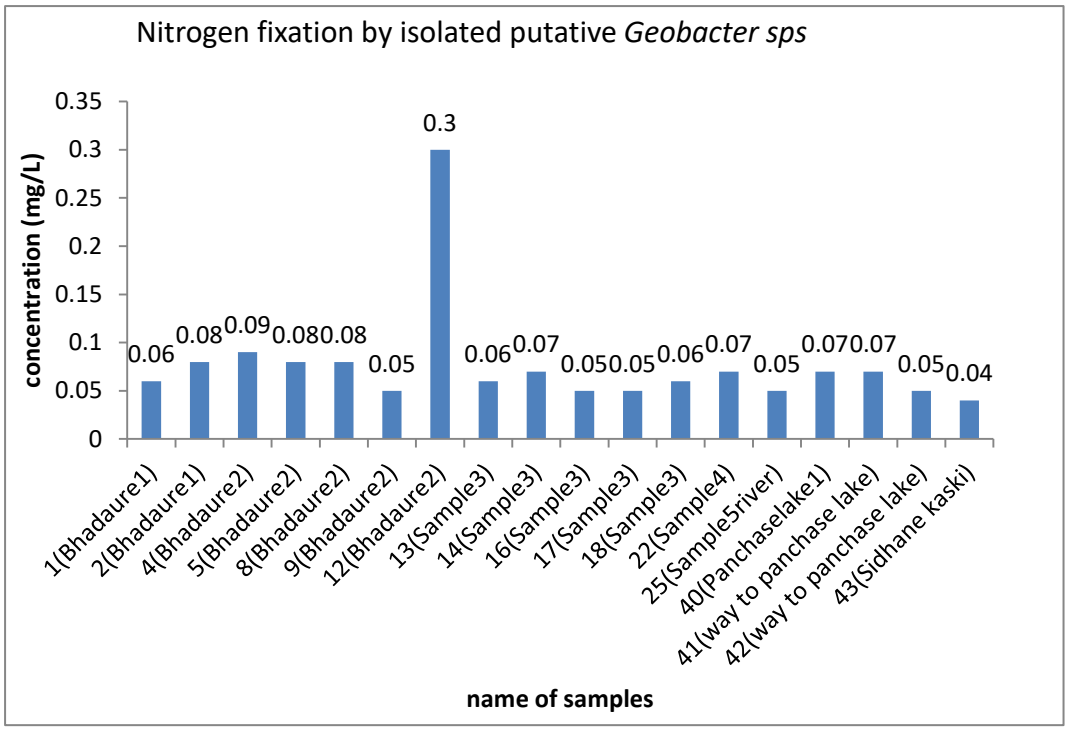


Figure 4.17: Nitrogen fixation by isolated putative *Geobacter* sps

In the media of Gs-15, the media composition has been modified as from previously described protocol and both are mentioned in Appendix 3 and Appendix 4.

Nitrogen source has been omitted at GS-15 modified media and provided atmospheric nitrogen source from atmosphere and the bacterial cultures of different *Geobacter sps* has been survived and given significance growth rate. It means cultured ones are able to fix nitrogen on the media but not in significant amount. Further for more yield, clonning of gdh gene and nif genes could be done.

4.7 Synthesis of Biogenic Silica

Biogenic silica was extracted sidewise by a widely used method 'sol-gel method'. Fine, porous, adsorptive silica particles was obtained after it was over-dried at 110°centigrade overnight. It was reconfirmed by SEM, FTIR and XRD from which its average size, functional group present in it and its nature would be determined respectively.

4.7.1 Characterization of biogenic silica particles

4.7.1.1 X-Ray Diffraction

As X-ray diffraction is a tool for determining the atomic and molecular structure of a crystal which follow the Bragg's law (Hummel, 2011).
$2d\sin\theta=n\lambda$, where n is a positive integers, λ is the wavelength of incident wave, θ is scattering angle, d is path difference between two waves.
A crystal is a composition of periodically arranged in a 3D space while amorphous do not show periodicity and atoms are randomly distributed in 3D space so scattering of X-rays by atoms is the point to be considered. When there is periodic arrangement of atoms the X-rays will be scattered only in certain directions when they hit the formal lattice planes, which will cause high intensity peaks. Moreover, in amorphous phase, X-rays are scattered at many directions leading to a large bump distributed in a wide range that is 2θ instead of high intensity narrower peaks (Ladd, Palmer and Palmer 1985).

A broad peak centered about at the diffraction angle (2θ) 22.475°, without any sharp peak (Figure 4.17) and no any regular repetition of such peaks clearly indicated that the isolated silica nanoparticles were amorphous in nature. There is no any sharp line obtained which is in agreement with the characteristics of amorphous silica. The diffraction angle (2θ) detected by XRD is nearly same as Kalapathy, 2000, where silica nanoparticles form RHA having a broad X-ray diffraction pattern with amorphous solid nature broad peak centered at 22.159° at this angle.

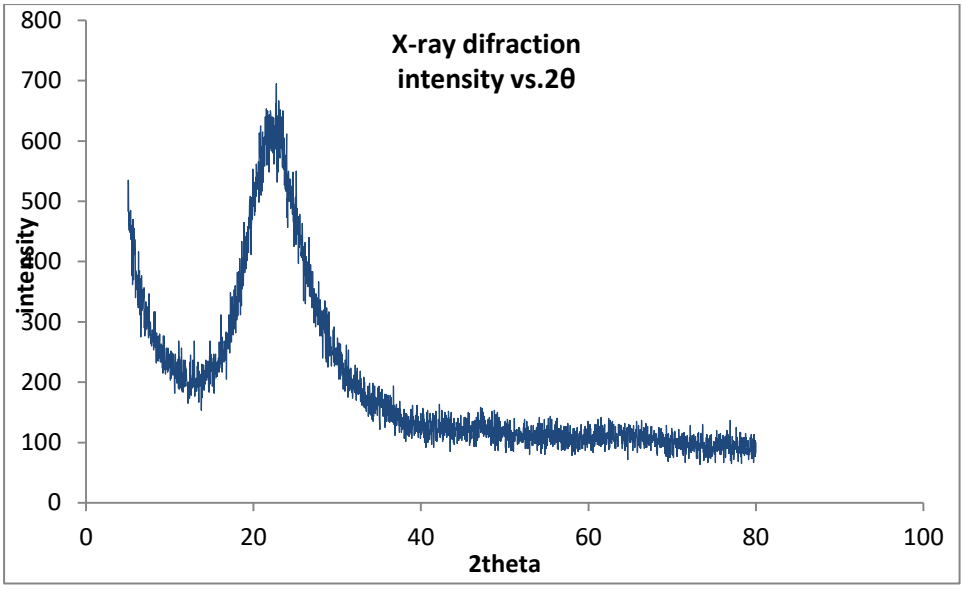


Figure 4.18: XRD spectra of biogenic silica treating with HCl and H_2S0_4 gelling by HCl (sample1)

The structural analysis and particle size of presumed silica nanoparticles were performed on the basis of Bragg's phenomenon and Debye Scherrer's equation

$$\tau = k\lambda/ß\cos\theta$$

where τ is the mean size of ordered crystalline domains, K is constant that adjust a dimensionless shape factor and its value denotes as 0.9 that is put in close proximity 1, λ is another constant denoted to adjust extra the X-ray wavelength where its value is 0.1541 for the present device, ß is the Full Width at Half the Maximum intensity (FWHM) or peak width and θ is the Bragg's angle here θ= 2θ/2 where 2θ is the value of highest intensity peak. The average size of sensitized silica sample S1 was to be determined as 1.074 nm. This result comes up Hassan *et al*., 2014 near to the value of agreements where its value has given 20nm to 25nm, with applying the same procedure.
Hence, it can be generalized that extracted silica sample (S1) by sol-gel method is clearly nano-sized and amorphous in nature.

4.7.1.2 X-ray diffraction of silica sample treating HCl/H_2SO_4 gelling by acetic acid

The X-ray diffraction pattern of Sample 2 (S2) does not have any remarkable peak due to some noises. However, if we consider the average mean curve and its pattern shows peaks near diffraction angle of 21.679°. The average sizes of particles were calculated taking the denoted peak and it was found to be 5.505nm.

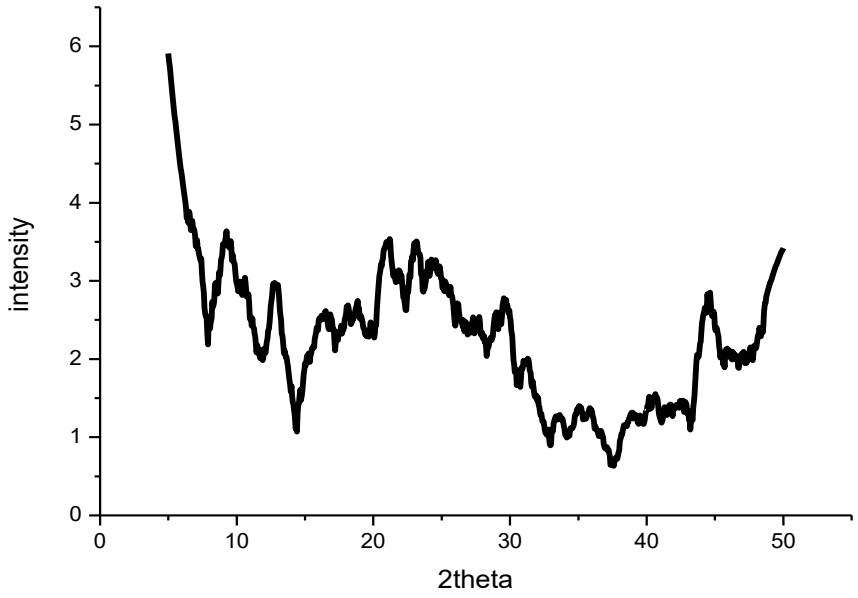


Figure 4.19 XRD spectra of biogenic silica treating with HCl and H_2S0_4 gelling by acetic acid (sample2)

Apparently, this curve has various noises. So, synthesized particle could not be obtained as in amorphous nature completely. It was seen that the sample was mixture of crystalline and amorphous in nature too. This could be because of gelling by acetic acid the nature of silica change its formed or there should be contamination of other impurities during the process of XRD so the XRD could not be consistent.

4.7.1.3 X-Ray Diffraction of Silica Sample Treating H_2SO_4/H_3PO_4 Gelling By Acetic Acid

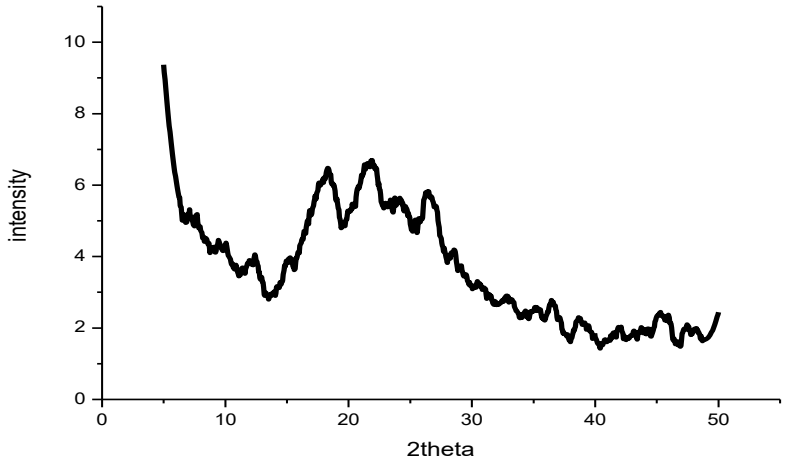


Figure 4.20: XRD spectra of biogenic silica treating with H_3PO_4 and H_2SO_4 gelling by acetic acid (sample3)

The above XRD result shows that Sample (S3) is also clearly following the regular pattern as the sample S1. It also shows the broad peaks near refraction angle 21.0361°. Then,

taking the overall nature of XRD data, the particle size was calculated and it was found to be 10.686 nm. This result has very good similarity as the research done by (Thuc and Thuc 2013) and also it show the amorphous in nature. It is because, instead of some contamination and noise, its overall pattern is identical to the XRD of commercial silica.

4.7.2 Fourier Transfer Infra-Red (FTIR)

FTIR of Silica sample 1

Sample 1 was the silica sample which was treated with HCL and H_2SO_4 for removing halides and was gelling by HCl exactly following standard protocol. Above FTIR figure confirmed that presence of Si-O-Si (silica) the sample. In this figure, 470 cm^{-1} and 850 cm^{-1} Si-O-Si and Si-O-Si (siloxane) respectively. At the wave number 930 cm^{-1} is Si-OH (silanol), and near 1100cm^{-1} indicates sharp peak of Si-O-Si group. At 1600 cm^{-1} and 3500cm^{-1} H-O-H and OH had seen respectively. From this analysis we might confirmed that presence of silica in our isolated compounds.

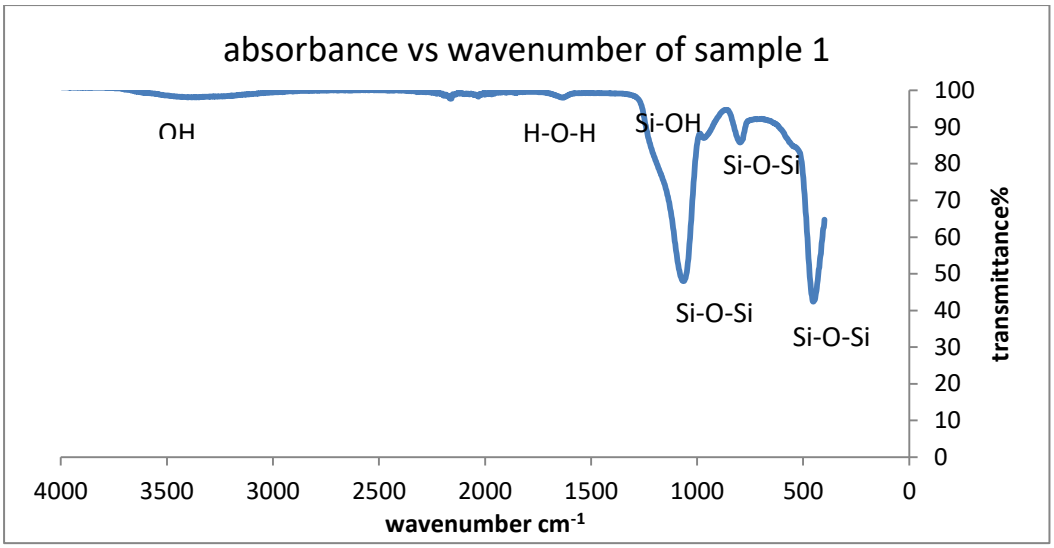


Figure 4.21 FTIR of Silica sample 1

FTIR of sample 2

This was the figure of FTIR of sample 2, silica was prepared by treating HCL and H_2SO_4 and gelling by acetic acid. Siloxane (Si-O-Si) group were found at 470cm^{-1}, 810cm^{-1}, 1100 cm^{-1} and Silanol (SiOH) group was found at 950cm^{-1}. Actually there was no difference was found in the FTIR though changed in protocol. Here Acetic acid (CH_3COOH) was used in the gelling of sodium silicate instead of HCl. It could be give a sense that there was no any effect when changing the gelling medium. The reason that we used mild acetic acid apart from strong acid HCl was that if we could that remaining filtrate could use in the bacterial growth as supplement in the medium.

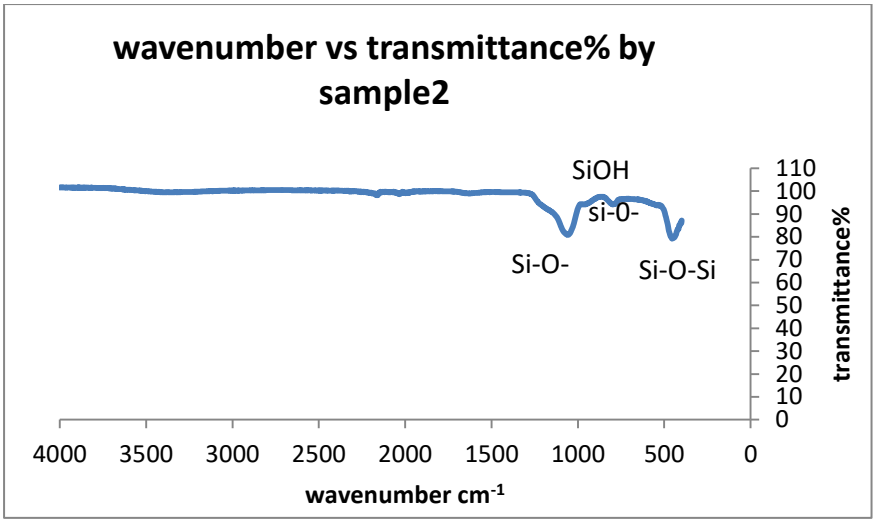


Figure 4.22: FTIR of Silica sample 2

FTIR of Silica sample 3

This was the FTIR figure of sample 3. Sample 3 was the silica which was prepared by treating Sulphuric acid (H_2SO_4) and phosphoric acid (H_3PO_4) and gelling by Acetic acid (CH_3COOH). Here also the results of FTIR also got same that is siloxane (Si-O-Si) group were found at 470cm^{-1}, 810cm^{-1} and 1100cm^{-1} and silanol (Si-OH) was found at 950cm^{-1}. Phosphoric acid was used instead of Hydrochloric acid (HCl). Phosphates and sulfates group could be nutrient source for the bacterial growth and an experiment was done by using remaining filtrate solution in the bacterial medium and incubated at the different *Geobacter* sp at different concentration. Unfortunately, we were unable to get the expected growth of bacteria from the modified filtrate solution.

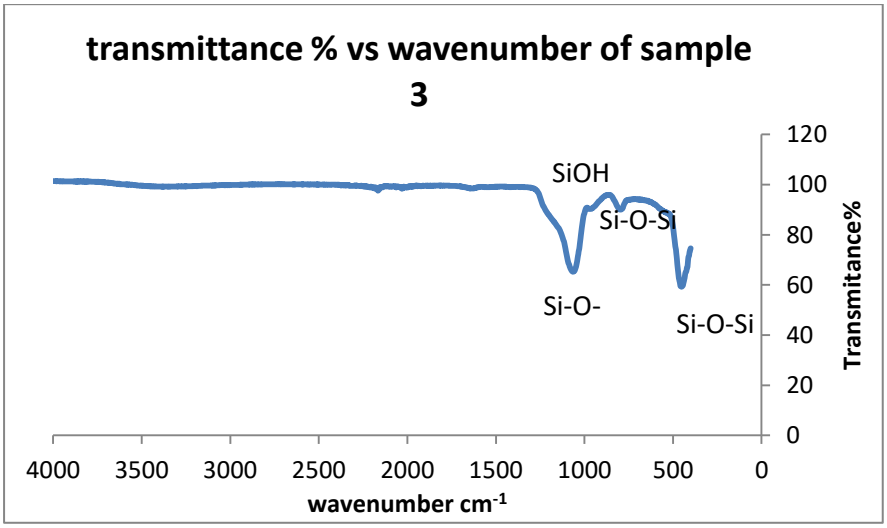


Figure 4.23: FTIR of silica sample 3

4.7.3 Scanning Electron Microscope (SEM)

The synthesized samples were also characterized by scanning electron microscopy at Martin Luther, University of Halle, Germany in order to study structural morphology. The detail discussion on each samples have been mentioned below.

Silica sample1 (S1)

After treating hydrochloric acid and sulphuric acid, sodium hydroxide and gelling by hydrochloric acid, characterization was done by SEM

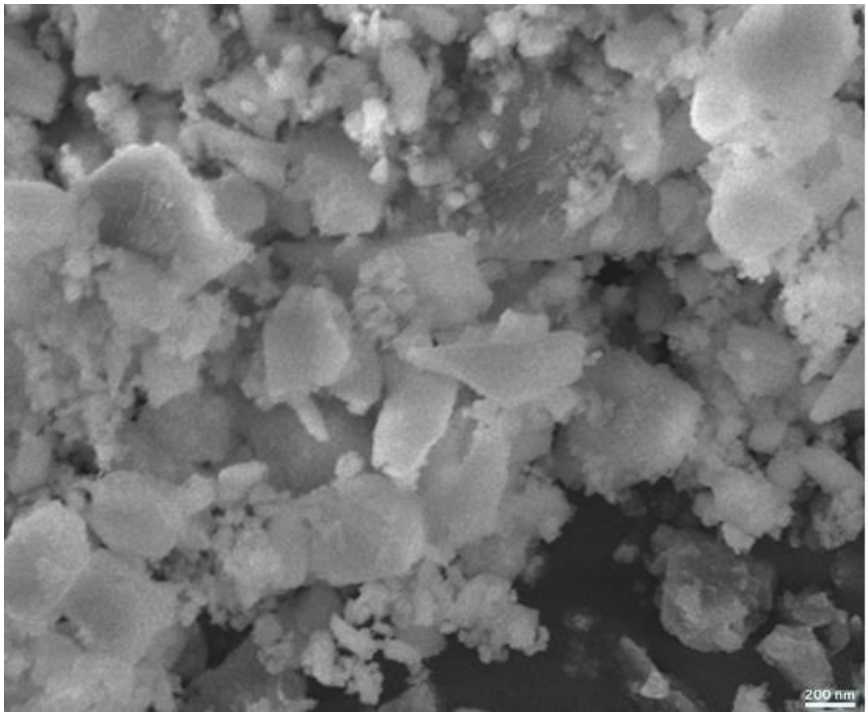


Figure 4.24: SEM of silica nanoparticles sample 1

This picture is the SEM of silica sample (S1) having 200nm micrometer standard bar. This pictures shows that synthesized particles are distinctly in amorphous in nature and are in the range of Nano size. Although, particles were clumping in size, they show the clear collection of individual nanoparticles. The main reasons behind these clumping might be due to low temperature drying, 110° centigrade.

Silica sample 2

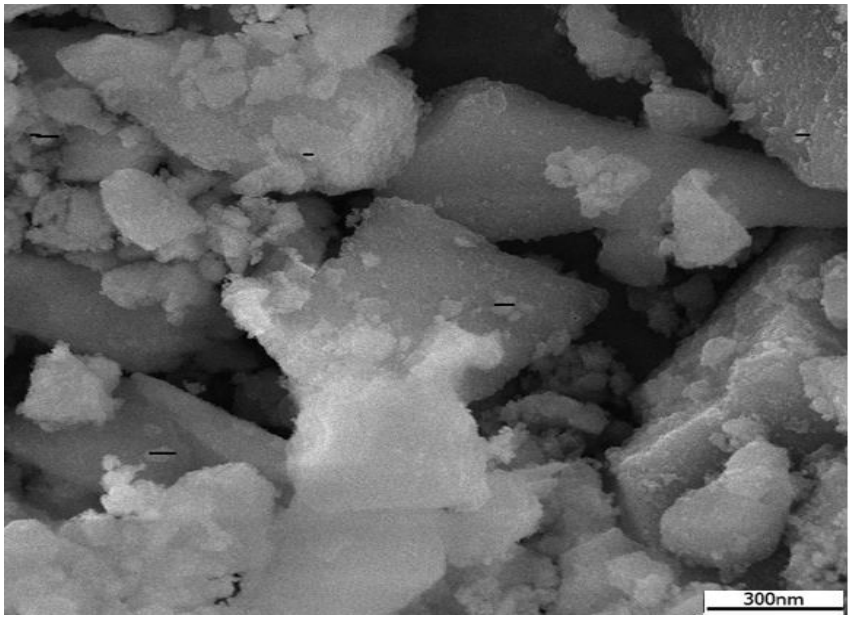


Figure 4.25: SEM of silica sample (S2)

As preceding this figure, these were amorphous solid nature and the size of silica sample (S2) is also in nanometre range. This sample was quite similar if protocol had not been changed but here a few modifications had been done that was in the use of reagent i.e. Acetic acid was used in gelling of silica from sodium silicates instead of sulphuric acid. This was done because of use of remain waste solution could be used as mineral solution for the microbial growth.

Silica sample 3 (S3)

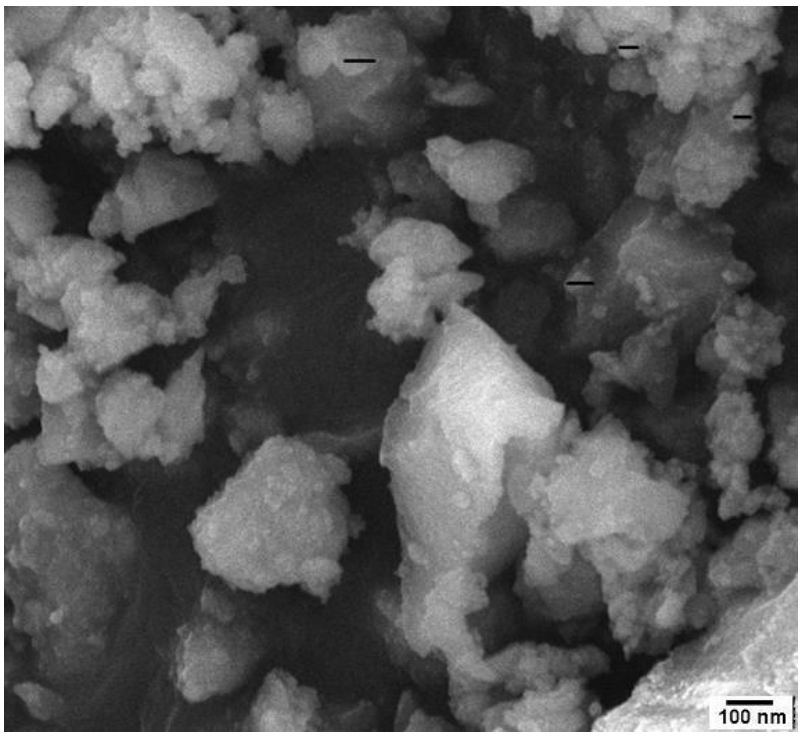


Figure 4.26: SEM of silica sample 3

This was the SEM picture of silica (S3) having standard bar 100nm. To analyse the picture, the average size of the completely modified silica nanoparticles were found to be in nano meter range which are slightly bigger than the particles size of sample 1 (S1).The morphological change in size of these three samples; S1, S2 and S3 as given by X-ray diffraction and Scanning Electron Microscopy can be correlated as in the table given below

Sample	Grain Size(nm) by XRD	Grain Size (nm) by SEM
S1	1.074	2.90
S2	5.505	6.19
S3	10.686	6.21

The samples; S1, S2 and S3 had been synthesised by using three various parameter. Hence, surface structural and particle size have been varied. The sizes of nano-sized particles are being increased while dealing with HCl/H_2SO_4 and gelling with acetic acid and H_3PO_4/H_2SO_4 and gelling with acetic acid respectively. More instantly, SEM images clearly show that grain size of S1 is smallest and it is increasing on S2 and S3. This nano size structures are similar to the result performed by X- ray diffraction analysis.

Furthermore, the structural analysis on SEM images say that synthesized particles are clearly amorphous in nature, it is because the clumps are made up by the sharing of many particles which have not been arranged in regular geometrical shapes.

Here phosphoric acid was used apart from hydrochloric acid and the acetic acid for gelling the silica so that waste from the isolation could be used as nutrient source as minerals source. As we know, Rice husk ash contained SiO_2=80-90% Alumina=1- 2.5% Ferric oxide=0.5% and remaining are trace elements (Ghosh and Bhattacherjee 2013).

Comparing three different silica sample S1, S2 And S3 we have got different size in nanoparticles and go for reason that we could get nanoparticles from sol gel method easily even we could do modification. In S1, nanoparticles size got nearly in range of suitable nano-range that is 1 to 100) nm. In S2 we got the little bigger nano-size this is due to adsorbent of acetic acid in pore of silica surface. Similarly, the size of S3 sample found as largest among S1 and S2.

Scanning electron microscope of silica sample give a generalized view of size of particles further more confirmation transmission electron microscopy could be done because the

spot size and the interaction volume are both large compared to the distances between atoms, so the resolution of the SEM is not high enough to image individual atoms, as is possible with transmission electron microscope (TEM).

4.8 Quantification of molybdate reactive biogenic silica

4.8.1 Calibration Curve

Calibration curve was prepared with concentration ranging from 2µg/ml to 20 µg/ml include the blank and absorbance was measured and finally got standard curve for quantification of biogenic silica. This result shows the linearity with correlation coefficient 0.9651.

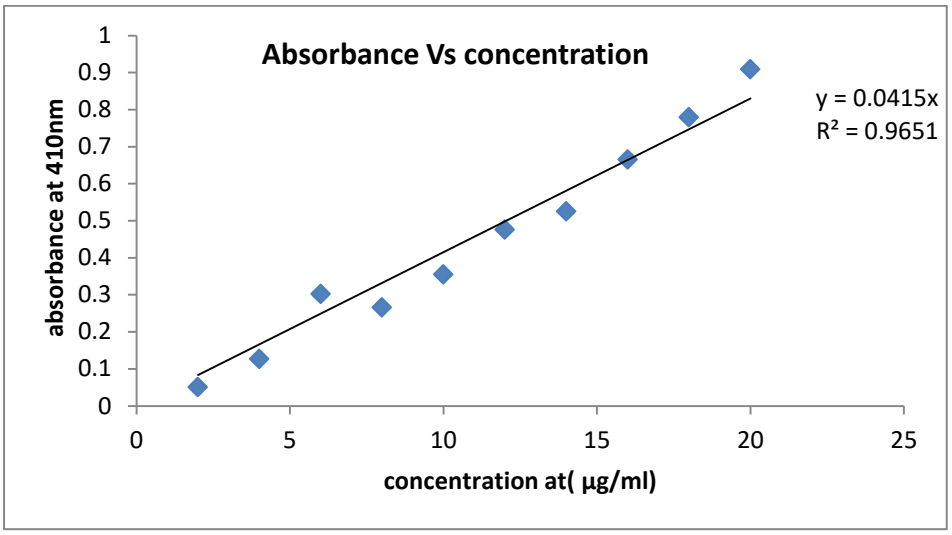


Figure 4.27: standard calibration curve for quantification of silica present in unknown samples

Then after isolated silica obtaining from standard protocol and modified protocol, their initial weight and final weight are compared. The samples are compared each other as well as previous sample which was done by the similar work from the one of the student of my supervisor. The silica percentage from standard protocol which was done in my lab has better result than previous one. Sample 1 has high percentage of silica it is increased by 20%. This could be possible due to higher accuracy in practice or good burning of rice husk so that other impurities like carbon, good elimination of minerals by Hydrochloric acid and sulphuric acid. The following table might be given in cystal clear view about the obtained silica.

Table 4.4 Silica Quantification from Synthesized Biogenic Silica from RHA

SN	Sample	Initial weight	final weight	Absorbance	Percentage of silica
1	Roji sample(std)(previous)			0.729	73%

2	$HCl+H_2SO_4$:HCl(sample 1)	5gm	2.95	0.934	93%
3	$HCl+H_2SO_4$:Acetic acid(sample 2)	5gm	2.90	0.611	61%
4	$H_3P0_4+H_2SO_4$:HCl	5gm	3.03	0.548	55%
5.	$H_3P0_4+H_2SO_4$:Acetic acid (sample 3)	5gm	3.45	0.536	54%

Final weight of silica obtained from different protocol are analysed as: From the literature rice husk contain about 20 to 30 % rice husk ash and this ash gave about 90 to 98% silica.

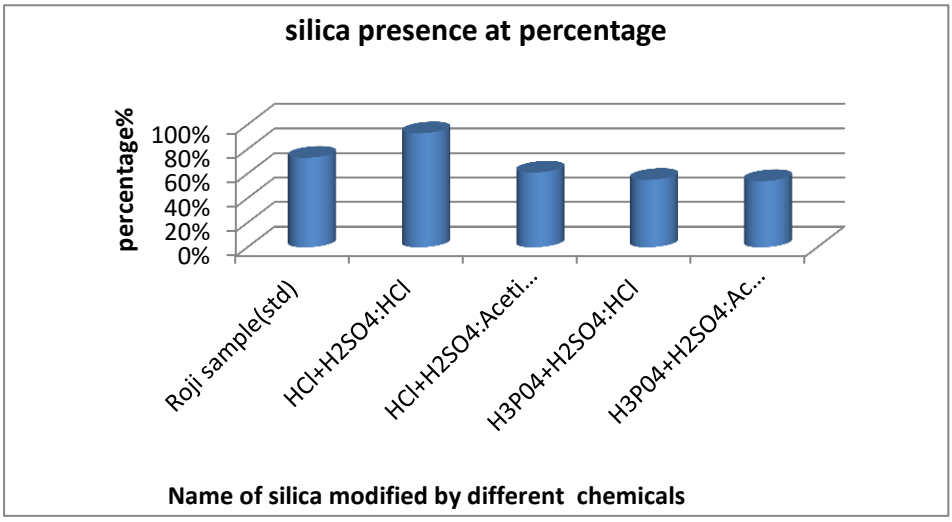


Figure 4.28: Silica Present at Synthesized Biogenic Silica

The final weight of 5gm ash treated with acid for demineralization and made soluble by alkaline and gelling with different kinds of acid so their final weight also became vary in their weight. Actually, the weight of ash treated with phosphoric acid and sulphuric acid and gelling by acetic acid gives more higher yield but presence of silica was low it was found to be only 54% so there might be addition of phosphates group and thus increase the final weight. To overview the data, standard one i.e. RHA treated with HCl and H_2SO_4 and gelling by HCl gave a high yield of silica than others and it was found to be 93% which has shown if higher yield needed then standard protocol was better. The figure no. 4.27 also made a crystal clear view about the percentage yield of silica from the isolated ones.

4.8.2 Plasma treated silica samples at 6000 V

Table 4.5 Different condition implied during functionalization of silica nanoparticles

SN	Sample name with features		Condition	Silica quantification (410)	Nesslers test (435)
a.	$HCl+H_2SO_4$:HCl	A1	Vaccum	0.435	0.166
b.	$H_3P0_4+H_2SO_4$:HCl	A3	Vaccum	0.031	0.029
c	$H_3P0_4+H_2SO_4$:Acetic acid	A4	Vaccum	0.338	0.002
d	$HCl+H_2SO_4$:HCl	C1	Vaccum+water	0.439	0.040
e.	$HCl+H_2SO_4$:acetic acid	C2	Vaccum+water	0.051	0.023
f.	$H_3P0_4+H_2SO_4$:Acetic acid	C4	Vaccum+water	0.252	0.054
g.	$HCl+H_2SO_4$:HCl	D1	Atmospheric nitrogen+water	0.053	0.033
h.	$HCl+H_2SO_4$:acetic acid	D2	Atmospheric nitrogen+water	0.048	0.044
i	$H_3P0_4+H_2SO_4$:Acetic acid	D4	Atmospheric nitrogen+water	0.073	0.022

Plasma technology is a technique at which high voltage energy is continuously given to substance so that they turn into gaseous states and created charged particles used to bond materials together and to change surface properties according our needs.in the low pressure gas is excited by supplied energy in a vacuum results zestful ions and electrons so that surface can be change efficaciously.

Chemical reaction of the ionized gas with surface of matter can occurred, there are two types of plasma system that are low pressure and atmospheric, here atmospheric plasma system have been discussed where gas is excited by high voltage under atmospheric pressure so that plasma is sparked. It is used especially pre-treatment of different surfaces of metal, polymer, ceramic and glass as well as silica. Many surface properties can be obtained, environmentally friendly process easily applicable independent of geometry, small part, powders, tubes etc.

As atmospheric plasma system have been used for our functionalization of atmospheric nitrogen into silica nanoparticles, silica is a porous nature though it has high surface area, nitrogen group can be adsorbed or chemically can be bonded for this we had tried Fourier transfer infrared spectroscopy each plasma treated sample we have found noise of at the graph of nitrogen group at the range of wavenumber 2100 cm^{-1}.

Characterization of plasma treated samples through FTIR

Sample A1, C1 and D1

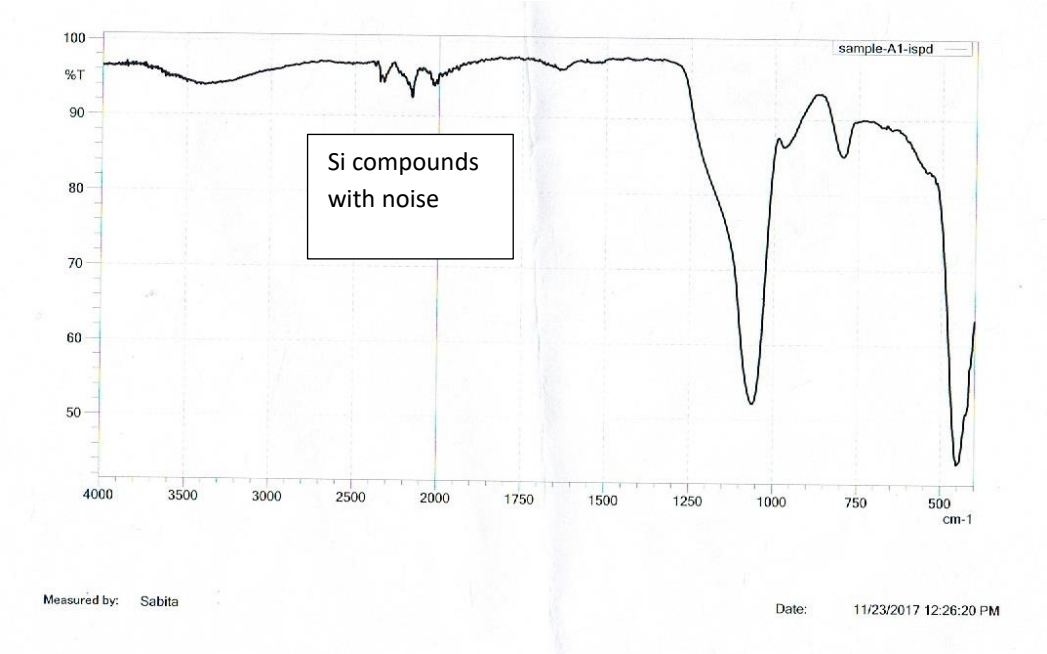


Figure 4.29: FTIR of Functionalized Silica Sample A1

A1 Sample was a sample of isolated silica nanoparticles which prepared as described Stober method. It was treated with HCl and H_2SO_4 for demineralization and soluble alkaline sodium silicate gelling by HCl. This sample was taken as control which was used for functionalization in the plasma without any air (vacuum). As usual, bending Si-O-Si, symmetric Si-O, stretching Si-O-H,and asymmetric stretching Si-O-Si found at 478cm^{-1}, 798cm^{-1}, 960cm^{-1}, 1100cm^{-1}, respectively (Sumathi and Thenmozhi, 2016). Except that there was found some noise too like nitrile groups or carbon-carbon bond at the range of 2000cm^{-1} to 2300cm^{-1}. These could be due presence of carbon-dioxide during the scanning of Fourier transformation or might be due to incomplete burn of rice husk ash so that carbon might have seen present.

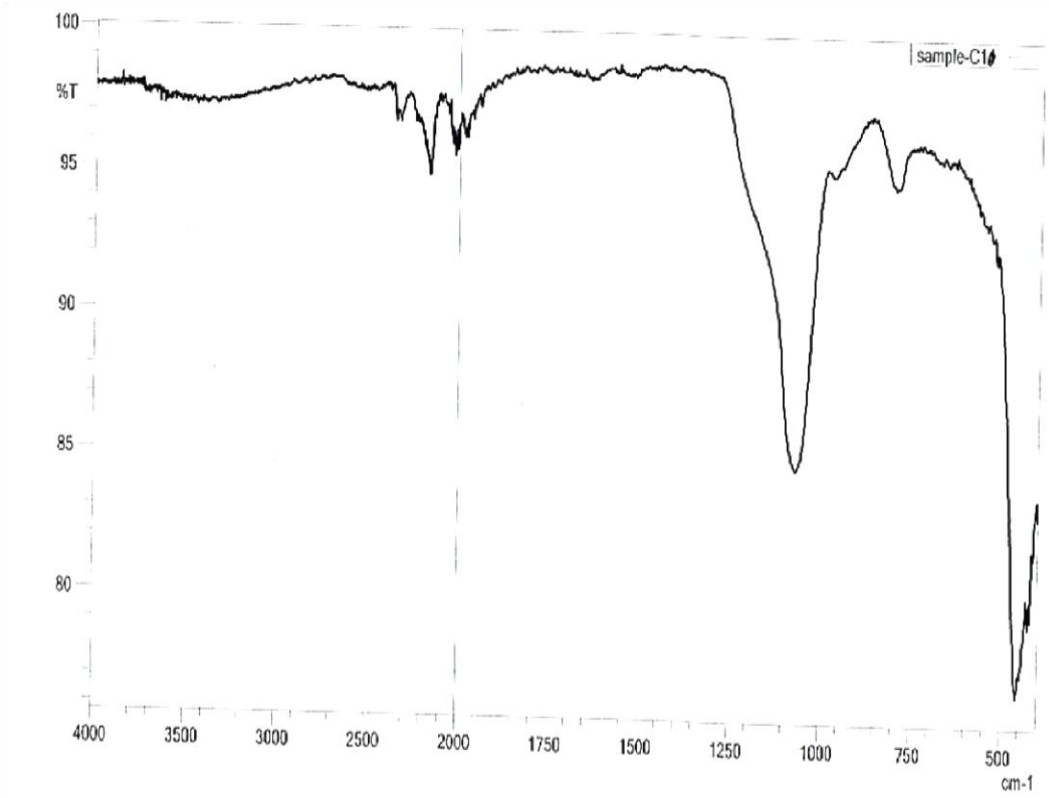


Figure 4.30: FTIR of Functionalized Silica Sample C1

In this figure, here was also same silicon functional group were seen at as mentioned above figure 4.20. Here Sample C1 describes as same as Sample A1 but water molecule was included while had done of plasma at 6000V and after FTIR results some more noise are found along with Hydrogen molecule near 1600cm^{-1}. That could suggest that water molecule could be added at the surface area of silica particles so that it could be used as adsorbent .There might be chemisorption should be happened.

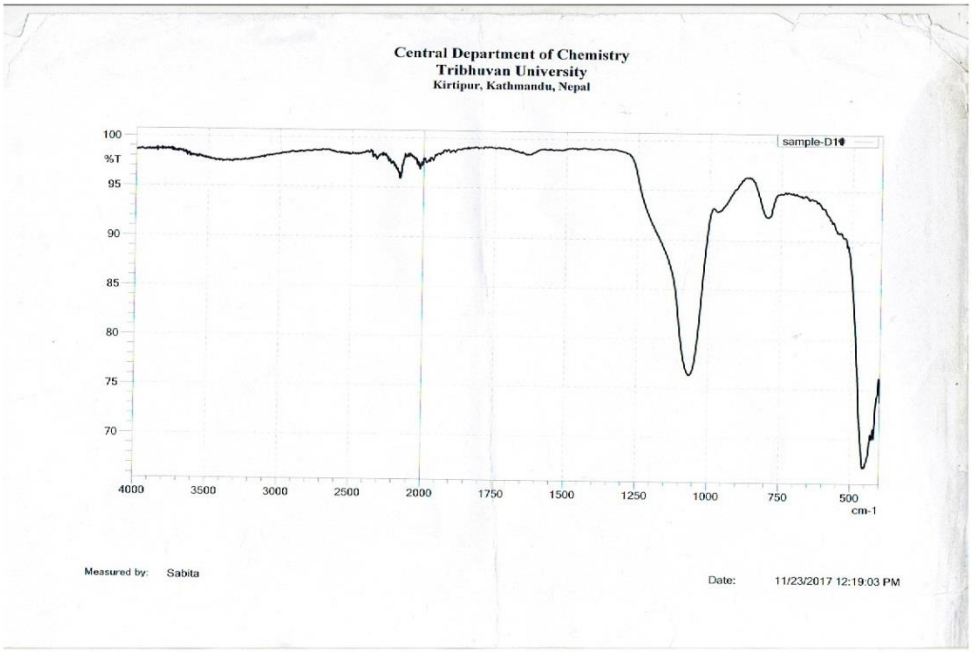


Figure 4.31: FTIR of Functionalized Silica Sample D1.

In this figure, sample D1 describes as same as sample A1 but the condition applied here was little bit differed that was atmospheric nitrogen was provided along moisture through air in plasma technology. Though there was no peculiar differences was obtained than in sample A1 and Sample C1, some more intensifies weak bands were seen in the form of noise like nitrile and N-H group at the range of 2000cm^{-1} to 2300cm^{-1}. Actually the possible reason that more intensifies clear band were not seen or noise with nitrogen group found in the possible range might because nitrogen source already present in our water source through dissolved air.

Comparison between samples A4, C4 and D4

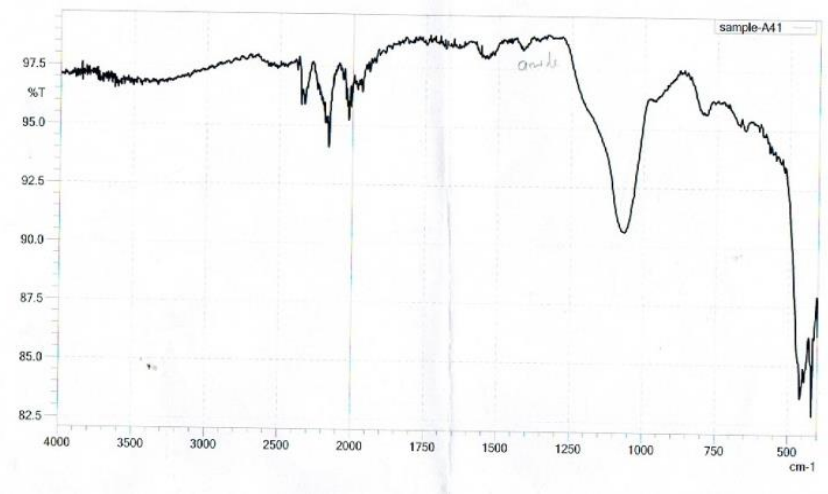


Figure 4.32: FTIR of functionalized silica sample A4

In this figure, sample A4 was the sample of isolated silica by obtaining the modification of standard protocol. Phosphoric acid (H_3PO_4) and Sulphuric acid (H_2SO_4) were used for demineralization and Acetic acid used for the gelling of sodium silicate and treated it with plasma at 6000V and it was taken as control for further experiment.

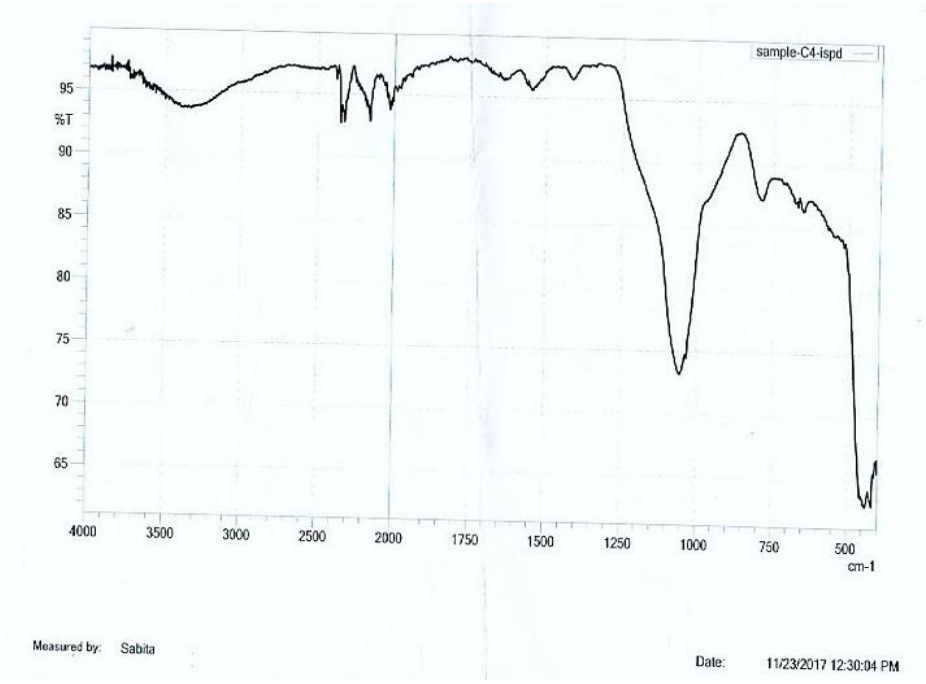


Figure 4.33: FTIR of Functionalized Silica Sample C4.

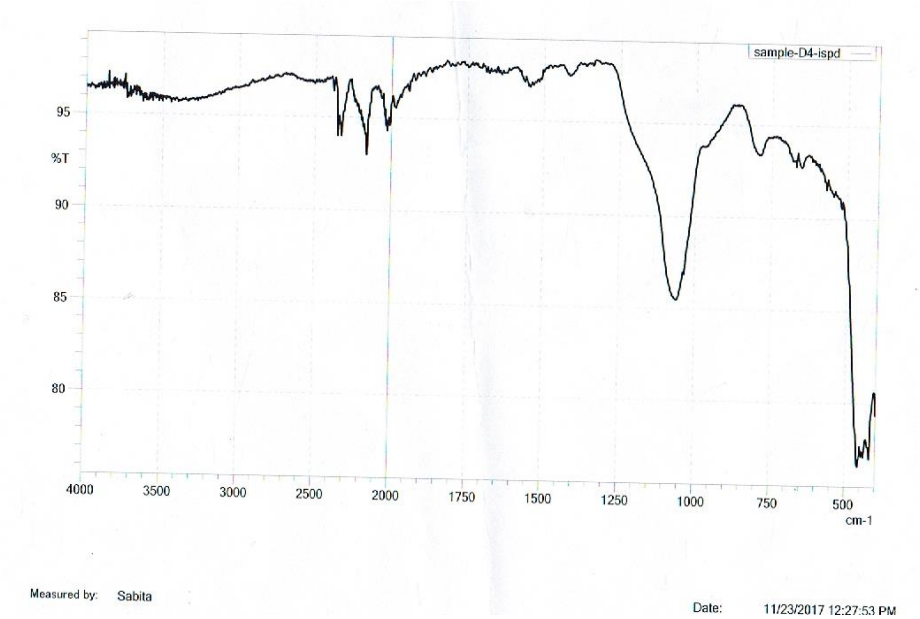


Figure 4.34: FTIR of functionalized silica sample D4

The sample C4 and D4 were as same as D1 in preparation phase but use of only water molecule and water molecule with atmospheric nitrogen in the respective samples. OH

group were present in the sample treated with water. There was no significance difference found between them. This could be due to either already presence of moisture in the sample.

In sample D4, the samples were treated with atmospheric nitrogen with moisture; as they contained silanol (Si-OH), silane (Si-O-Si) and hydroxyl (OH) group, gave indication of presence of inorganic nitrogen group near finger printing region about (500-1500) cm^{-1}.

As like sodium metasilicate, it was presumed that preparation of ammonium metaslicate by treating with plasma would be possible but depend upon external factors like different gases CO_2, O_2 etc. In addition, functionalization might be disturbed by poor adsorption. More noises were seen might be due to incomplete formation of ions of input compound in the plasma tank or 30 second of exposure of high voltage current not sufficient for the formation of bond, besides these, surface of silica could not able to provide sufficient surface area.

Major reason to perform these experiments were to look for the options of making nitrogenous silicate fertilizer to substitute urea that is nowadays vigorously used in the crop field which produces about 45% carbon-dioxide but excessive use of it could create problem to environment, increase the global temperature and water pollution also. In addition, silica are plant growth stimulant and also provide strength and immunity so it has been proved that silica is also need for the healthy growth of vegetables as well as crops. Effect of silica nanoparticles in the growth of plant had already been observed and it was seen effective to their growth (Ghalan and Raut 2016, Thesis work).

If ammonium metasilicate can be prepared then burden to depend on urea would be lesser and agro waste materials like rice husk ash could be re used and desertification by the ash could be reduced. Apart from this silica nanoparticle can be used in fillers, adsorptive materials, clay, quartz glass, chip of computer, carrier of drug delivery systems.

4.9 Carbon dioxide reduction

4.9.1 Growth of bacteria at Nitrogen and Carbon Free (NCF) media at 4.5 and 6.8 pH

Two conditions were provided for bacterial growth at pH 4.5 and pH 6.8. Better bacterial growth was obtained at pH 4.5 than that of pH6.8. The optimization of bacterial growth was done in different days. Absorbance was taken on 3^{rd}, 6^{th} and 9^{th} day which was detailed in Appendix 11. The better optical density at 600 was observed at 3^{rd} day for almost bacterial culture of different consortia. Among the pH 4.5, combination of following bacteria: *Azospirillium+ Geobacter (Azos+Geo)*, *Azospirillium+ Geobacter+*

Azotobacter+ Pseudomonas (Azos+Geo+Azo+Pse) and *Azospirillium+ Azotobacter+ Pseudomonas (Azos+Azo+Pse)* gave 0.334, 0.31, and 0.305 OD_{600} respectively which shows higher growth than others. This might be due to establishment of better coordination between them. At pH 6.8, combination of Azos+Geo+Azo gave highest growth that is OD_{600} 0.263. The increase in the absorbance (more turbidity) implies increase of bacterial growth. In the media there was no any carbon sources were added on it. From this, we can presume that atmospheric carbon dioxide has been reduced to carbon sources that could be used for energy generation and building block in making like RNA/DNA, phospholipids and amino acids. The bacterial consortium which have *Geobacter* have higher growth rate. As we have known from paper that it is electron donar as well as electron acceptor it could be enhancer to provide electron in the reducing equivalents and helps in the carbon fixation through calvin cycle in the carbon reducing bacteria. From this we can conclude that the combination of different bacteria have better growth rate than the single one and also they are able to grow at acidic condition and of course it is more preferential for our further experiment in production of biofuel that is bioethanol from the fixation of carbon at the media via microorganism which are isolated in the laboratory.

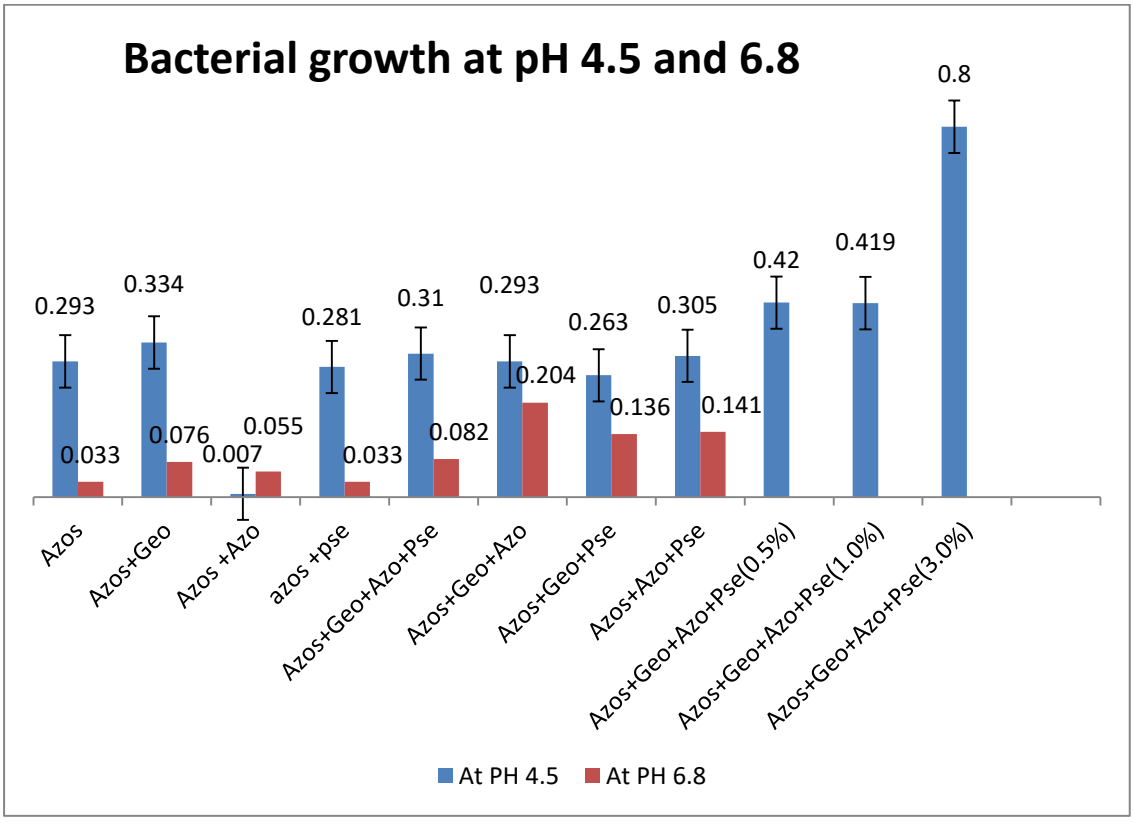


Figure 4.35: Growth of Different Consortia of Bacteria at pH4.5 and pH6.8

In addition, effect of silica nanoparticles on bacterial growth without any reduced carbon sources was also investigated. Different percentages like 0.5, 1, 3 % silica were

added on the consortia of *Azos+ Geo+ Azo+ pse.* This combination was selected because each have its important role in the build of biofertilizer for plant growth. At 3% silica with *Azos+ Geo+ Azo+ pse* gave OD_{600} 0.8A which is higher in rate we have done triplicate too of this but similar result is seen but the consortia with lactobacillus gave low growth rate. From this we more or less near to point that silica also have role in the bacterial culture of this experiment.

4.9.2 Qualitative test for carbohydrates of each of the samples

4.9.2.1 Molisch's test

A drop of 5% α Naphthol poured on about 3 ml broth sample and Conc.H_2SO_4 was added from wall of test tube and purple color was noted down.

Reaction

Carbohydrates $\xrightarrow{\text{Conc. } H_2SO_4}$ Hydroxymethyl furfural $\xrightarrow{\alpha\text{-naphthol}}$ condensation product (Purple or violet)

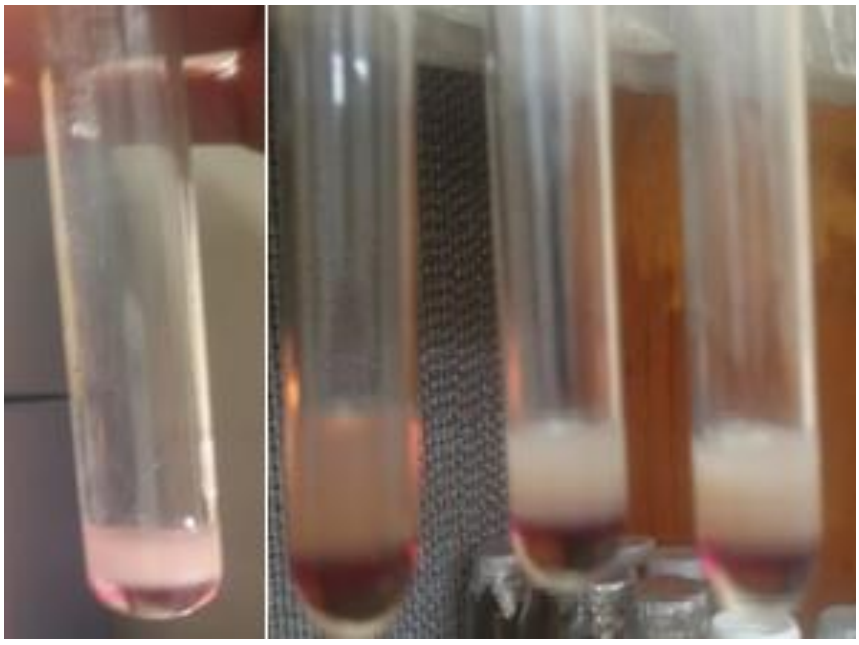


Figure 4.36: Nitrogen and carbon free media used (as protocol) chelating agent EDTA (Purple ring seen on the media which means the presence of carbohydrate or CHO group)

But EDTA is an ethylene diamine tetra acetic acid where carbon is present so modified protocol is used where EDTA is eliminated and pH is maintained by using dipotassium

hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) and ferrous sulfate ($FeSO_4$) are used.

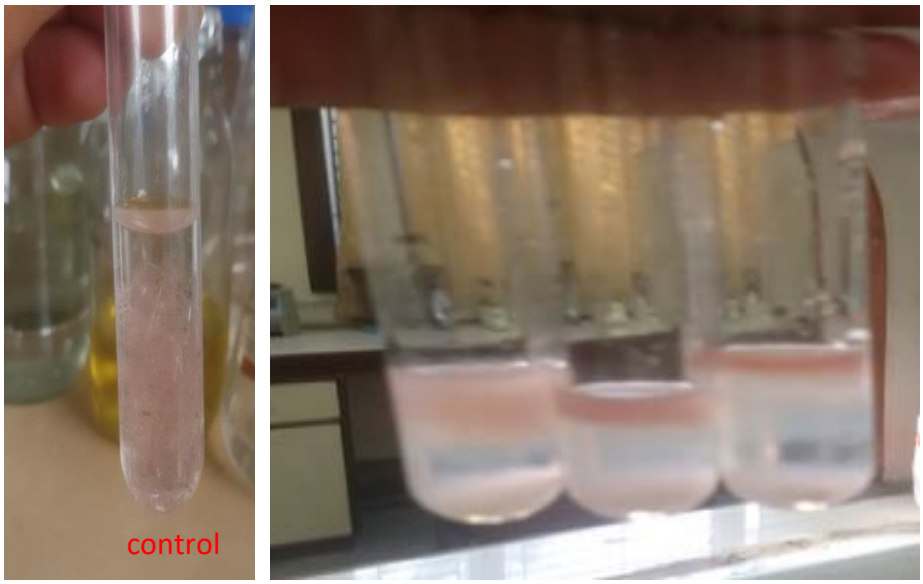


Figure 4.37: purplish or pinkish color seen on the NCF media without using EDTA

Faint purplish or violet color ring structure has been seen here and as control pure water was taken and same protocol was employed. Brownish crystals appeared only. In the experiment, the supernatant of the microbial growth culture was used after spinning at 10,000 rpm for 5 minutes to precipitate bacterial cells as pellet.

If there is free CHO (aldehyde group) in the media as a carbon source then only Molisch's test would give positive results otherwise no such ring like violet color would be obtained. This test is only preliminary test for reducing sugar that has free aldehyde and ketonic group (Devor 1950). At the terminal end of carbohydrates for further reaction. So in this media there must be fixed carbon from air via microorganism because there was no any alternative reduced carbon source was added in the media. Such experiments were done for three times and got the same results. Although a positive result from Molisch's test is only preliminary test so further DNS method has been used to quantify the reducing sugar present in the samples from yellow color formation and quantified by UV/visible double beam spectroscopy.

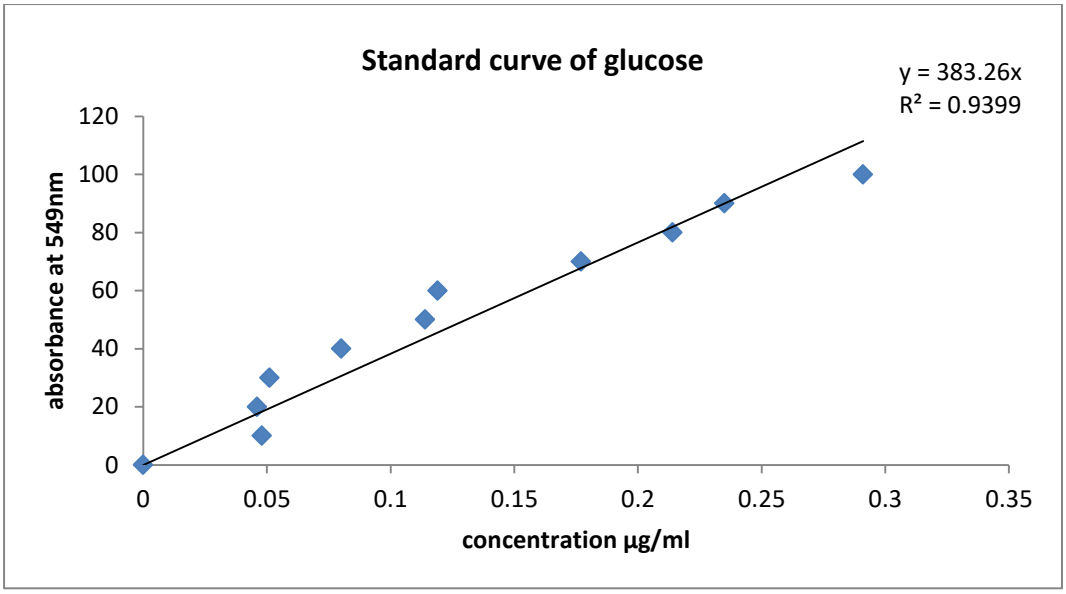


Figure 4.38: calibration curve for quantification of carbohydrates

This curve is the standard curve of glucose which gives test for the quantification of reducing sugar present in the unknown sample, by comparing the absorbance of unknown sample with the standard curve easily quantified the presence of carbon source and the straight line has value 0.9399 which denotes the reliability of the straight line.

4.9.2.2 Carbohydrate test from DNS reagent

Dinitrosalicyclic acid method was used for the determination of reducing sugar in the media and consortium of *Azos+Azo+Pse* has highest carbon-dioxide fixation rate that is 193.16 mg/L which is followed by second combination of *Azos+Geo+Azo* with 190.86mg/L. Consortium of all four bacteria, *Azos+Geo+Azo+Pse,* have lower effective to fix CO2 have 55.19mg/L than others and *Azospirillium (Azos)* only gave 19.92 mg/L which is relatively less than the combination one. As we know that Azospirillium have rubisco enzyme and can be an autotrophs and as well as Geobacter is extracellular electron donor and help to maintain pH of solution because it also can produce or hydronium ion. Again Azotobacter is siderophore chich can chelate un necessary iron and metal likewise psedudomonas is also strong colonizer and help to colonization of bacteria and might be enhances in the establishment of syntrophic growth so that it would be a reason that reducing carbon was found better in the consortia of *Azospirillium, Azotobacter, Geobacter* and *Pseudomonas*. In contrast we found that *Azos+Geo* or *Azos +Azo* was found to be 3% only here there mignt be electorn and hydrogen produce by geobacter could be used in the pH maintenance of the media for bacterial growth. The graph is plotted with error bar.

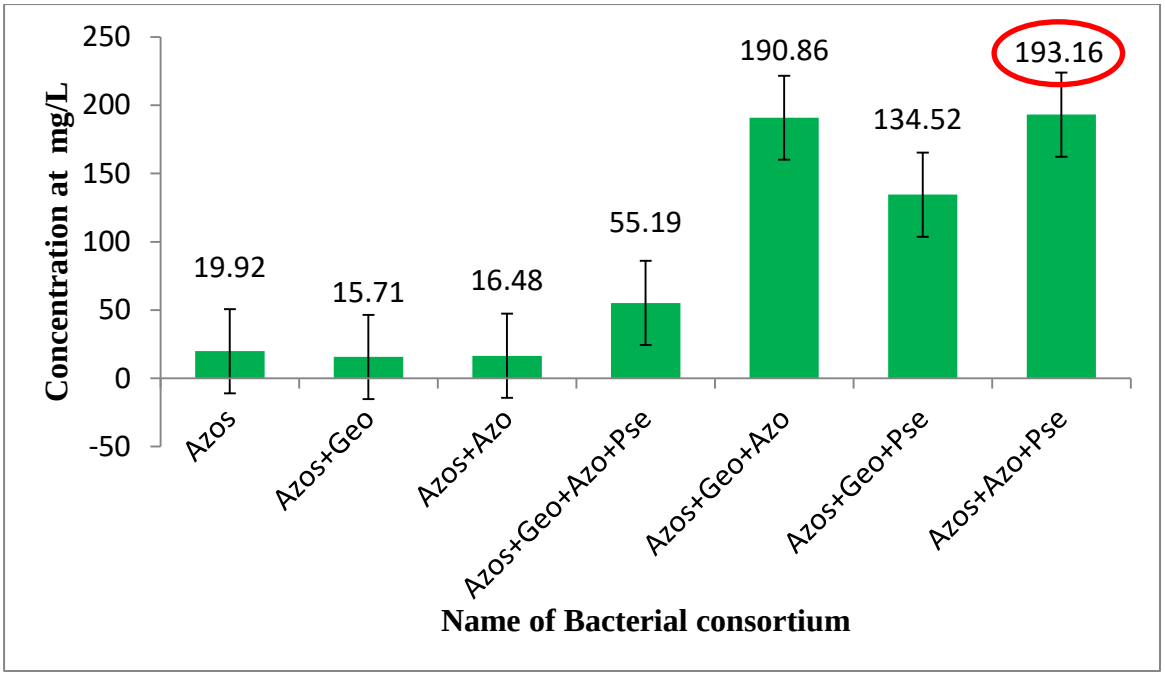


Figure 4.39: Concentration of Carbohydrates Fixed By Consortium of Bacteria

Putative *Azospirillium* (S_4D_6), for the first time has been reported for carbon fixation. However, the isolate has not been completely identified as *Azosprillium* sps. As we know *Azospirillium* is a microaerophillic, nitrogen fixing bacteria from the Rhodospirillaceae. *Rhodospirillium* is also from Rhodospirillaceae, an organism having RuBisCo enzyme and the free product of seduheptulose which can turn into Glucose- and fructose-6-phosphate in the presence of pentose phosphate isomerase have been reported (Westby, Cutshall and Vigil., 1983) (Sant'Anna 2011). Mainly *Azospirillium* depend on the glucose metabolism. Glycolysis occur in both aerobically connect TCA cycles and anaerobically connect fermentation pathways from which utilization of glucose to generate ATP, NADH and pyruvate etc (Peretó, 2011). Azospirillum can utilize different kinds of sugars, alcohol, organic acid as carbon sources and all the enzyme activities of the catabolic Embden-Meyerhof-Parnas pathway, the Entner-Doudoroff pathway (Westby, Cutshall and Vigil., 1983).

Though they use carbohydrates in their favorable condition, they can also grow chemolithoautotrophically. Azospirillium enables to grow chemoautotrophically even under nitrogen fixing condition using Hydrogen (H_2), generated by nitrogenase, as a sole electron donor, oxygen as an electron acceptor. Due to presence of Ribulose-1,5-bisphosphate carboxylase they can fix carbon-dioxide without light but expression of the uptake hydrogenase is enhanced by electron donor limitation and sensitive to oxygen , however, a higher concentration of oxygen was needed to support maximal activity of hydrogenase in comparison to nitrogenase (Dixon, 1972). The observation showed that decreased hydrogenase activity in the cultures of genus *Azospirillum brasilense* and

Azospirillum lipoferum grown in the presence of chelating agents such as EDTA could be increased only by the addition of Ni containing compounds.

The main quest is how *Azospirillium* is able to fix carbon dioxide as well as nitrogen without energy sources when in natural photosynthesis through Calvin Banson Bassam cycle energy for splitting water molecule is after capturing photon from appropriate wavelength of Sunlight. Putative *Azospirillium* isolate that has fixed carbondioxide because it grew in without giving any carbon sources in the NCF media. Although it could not be substantiated from the present works but it can be speculated that this putative isolate should use the electron and proton from water because the water molecule in the media could be in the ionic form because of auto-ionization of water (Geissler *et al*., 2001). And bacteria could have mechanism to take the proton and electron while during adapt to the environment. This can be presumed because the photon absorption by photosystem II is in a femtosecond (Klosek 1991) (Geissler *et al*., 2001) and in autoionization of water ($H_2O + H_2O \rightleftharpoons H_3O^+ + OH^-$) ionic components exist for a femtosecond and this could have been utilized. And there is also possibility of releasing of electron during the oxidation of metals combined with oxides or sulfates for example ferrous sulfate to ferric sulfate and hydrogen sulfate to elemental sulfur by the contact of atmospheric air (Reedy and Machin., 1923) or somehow enzyme present in bacteria. And in need bacteria could fix nitrogen from air with this reaction $N_2 + 8H+ + 8e^- + 16$ ATP = $2NH_3 + H_2 + 16ADP + 16$ Pi, (Henderson, Leigh and Pickett 1983) for this they might use ATP by using the carbohydrates which they formed during carbon fixation as like heterotrophs diazotrophs and proton utilization either from the water present in the media or hydrogenases present in the bacteria which break down the hydrogen molecule into proton and electron (Shafaat *et al*., 2013). [NiFe] hydrogenases are reported from diazotrophs like *Rhizobium leguminosarum*, *Rhodospirillum*, *Azotobacter vinelandii* etc. Azospirillum is also included into same family of R. rubrum that is rhodospirillaceae. There are eighteen gene involved (*hupSLCDEFGHIJKhypABFCDEX*) to synthesize an active hydrogenase. The hupF and hupK genes are found only in hydrogenase clusters from bacteria expressing hydrogenase in presence of oxygen. The hydrogenase large subunit contains the active center of the enzyme, a heterobimetallic [NiFe] cofactor unique in nature, in which the Fe atom is coordinated with two cyano and one carbonyl ligands; the hydrogenase small subunit contains three Fe-S clusters through which electrons are conducted either from H_2 to their primary acceptor (H_2 uptake), or to protons from their primary donor (H_2 evolution) (Okon and Labandera-Gonzalez, 1994). If there is limiting of ammonium production the regulatory gene regulate at the transcriptional level in a nifAL-type regulation at the production of it. And if the ammonia was supposed to toxic to bacteria during the nitrogen fixation, liquid ammonia undergoes autoionization similar to that of water $NH_3 + NH_3 = NH_4^+(l) + NH_2^-(l)$ (https://www.britannica.com/science/autoprotolysis) and in presence of water

ammonia undergoes as; $NH_3^+ + H_2O = NH_4^+ + OH$. So there should not be existed problem of autotoxicity of ammonia for them at the cultured media. All these are figure out schediametrically as:

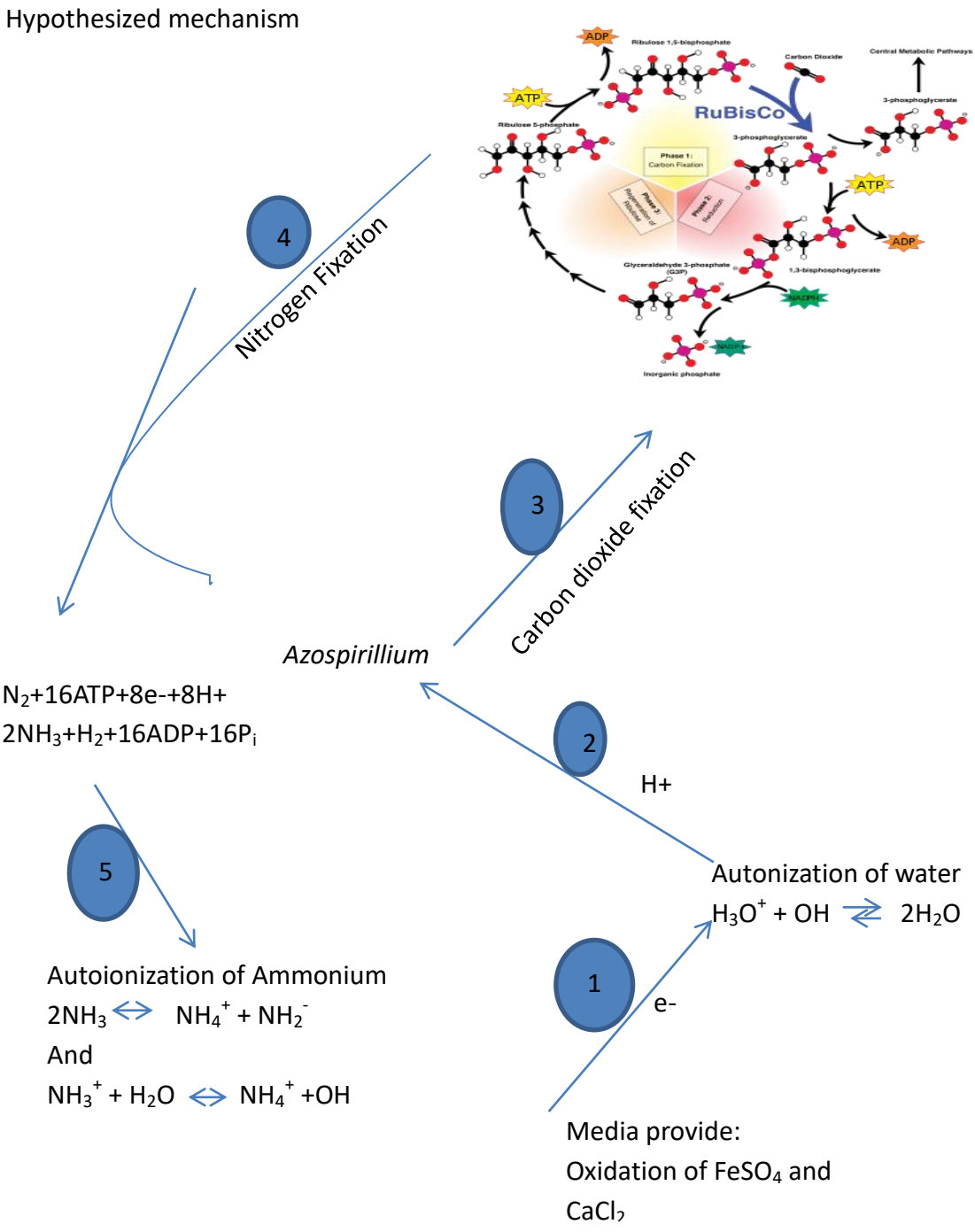


Figure 4.40: Proposing possible hypothesized mechanism

As well as *Azotobacter vinelandii* is the one of the fastest growing bacteria which could also fix the nitrogen from atmosphere. Again hydrogen molecule come from nitrogen fixation utilized by *Geobacter* sulfurreducens and utilize the carbon source fixed by the

Azospirillum species and also balances the proton (H+). They also produce extracellular electrons which also could be used as the energy source for the carbon fixation. Azospirillum could be produced reduced 3-carbon compounds via reductive tricarboxylic acid cycle and such produced carbon compound could be a carbohydrates source for all other bacteria. *Pseudomonas* uses as plant growth bacteria .After inoculation of tomato with endophytic *P. fluorescens* WCS417r, a thickening of the outer tangential and outermost part of the radial side of the first layer of cortical cell walls occurred when epidermal or hypodermal cells were colonized (Duijff *et al*., 1997). *Azospirillum* has also another benefit that it has been reported that A. brasilense released auxins, cytokinin and gibberellin. Increased number of root hairs and of lateral roots observed after inoculation with the bacteria and release of cytokinin, IAA (auxin) and gibberellin like substance by radioimmunoassay (Hartmann and Zimmer, 1994).

4.9.3 Nitrogen fixation by syntrophic growth tested by Nessler s reagent

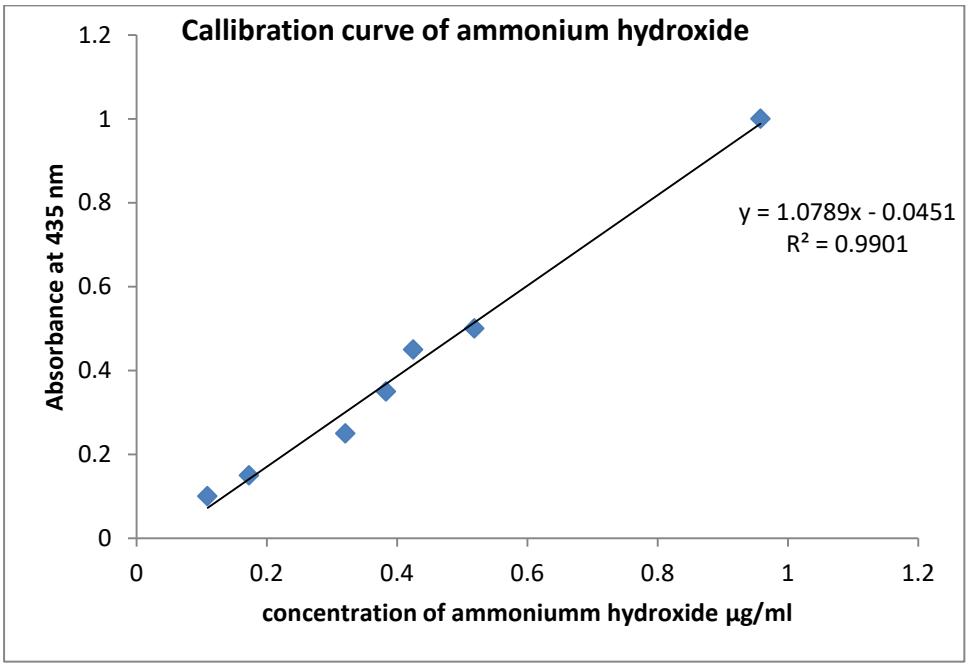


Figure 4.41: standard calibration curve of ammonium hydroxide

This is the standard curve of ammonium hydroxide raised from preparing of different concentration ranges from 0.1ug/ml to 1ug/ml. the equation of straight line was 1.0789x-o.0451 and its square mean root value was 0.99901 which implied to reliable. The blank was taken as water and proceed with same protocol.

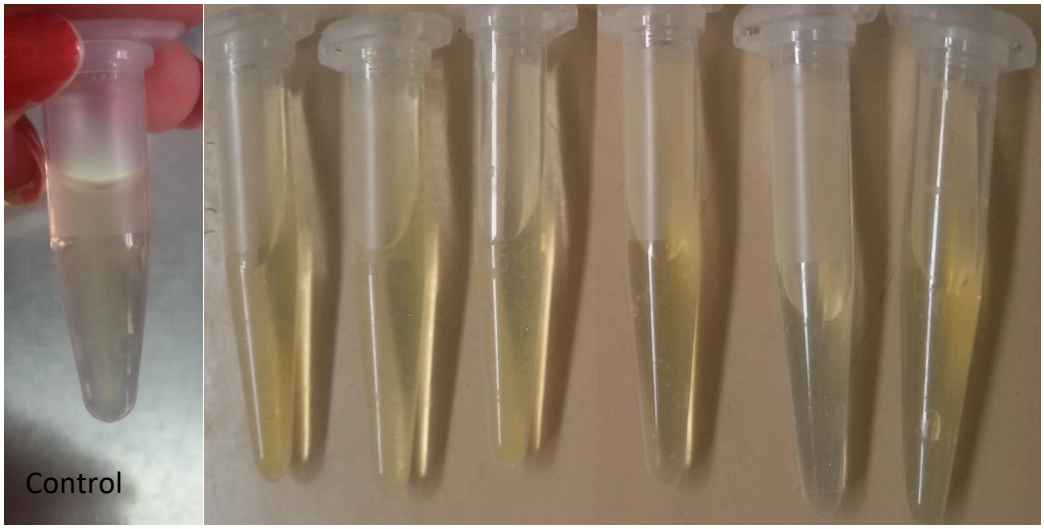


Figure 4.42: blank and supernatant of syntrophic bacterial grown cultures treated with Nessler's reagent.

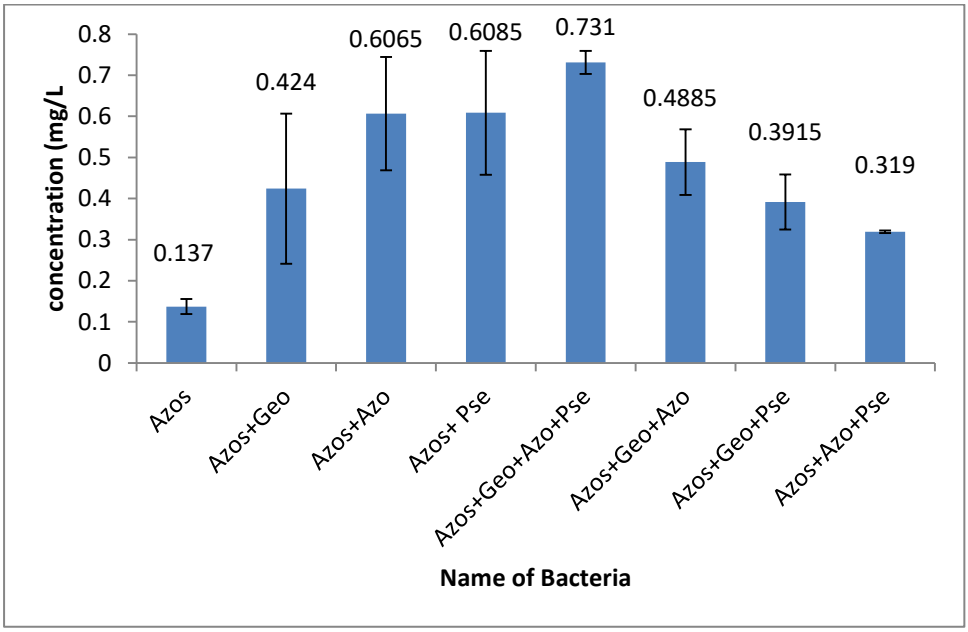


Figure 4.43: quantification of ammonium ion present at different syntrophic growth of bacteria

Among the isolates, *Geobacter*, *Azosprillium* and *Azotobacter* can fix nitrogen. In the experiment, combination of Azos+ Geo+ Azo+ pse gave the about 0.731mg/L ammonium ion on the nitrogen which was the highest yield of nitrogen in nitrogen and carbon free (NCF) media, *Azospirillium* sps gave only 0.137mg/L. The combination of Azos + Pse and Azos +Azo gave the same amount of ammonium ion that is 0.60 mg/L but the combination of Azos+ Azo+Pse produce only 0.319mg/L that means there was no

any effect of synergy. Combination of Azos +Geo gave 0.424mg/L whereas combination of Azos+Geo+Pse give lesser that is 0.391mg/L than that former one. Anyway combination of four isolated one is better to use for fixation of nitrogen.

All three Azospirillium, Geobacter and Azotobacter were diazotrops they have nif gene which can switch on the regulatory gene when they need nitrogen source from the atmosphere

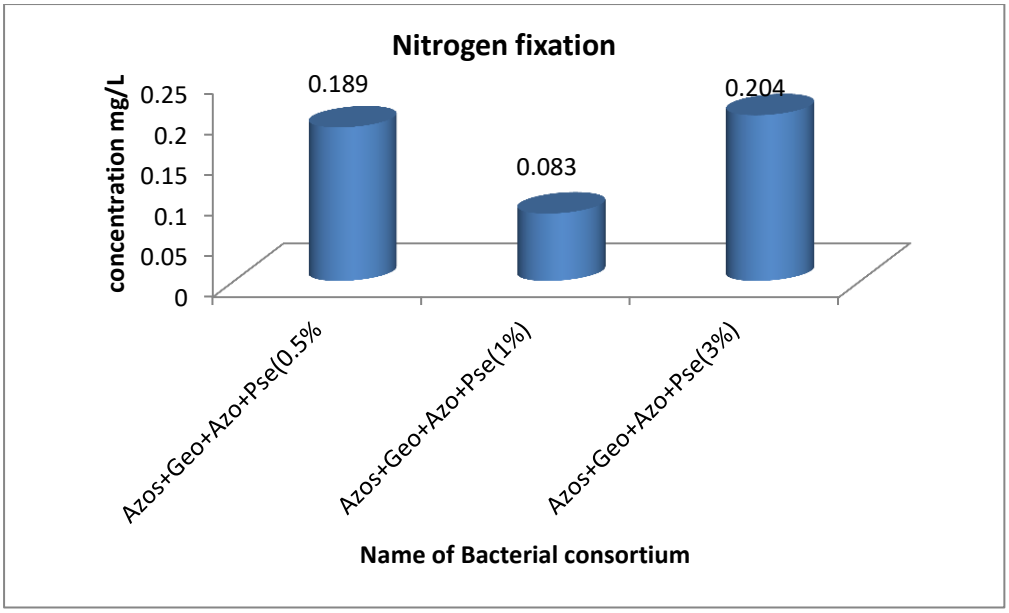


Figure 4.44: quantification of ammonium ion when silica nanoparticles added at the consortia

When biogenic silica added to different consortium of bacteria it worked to increase the rate of nitrogen fixation. The different percentage of silica that 0.5, 1, 3 were used in the combination of Azo+ Geo+ Azo+ Pse and they gave rise to 0.189mg/L, 0.083mg/L, 0.204mg/L respectively. At 0.5% and 3% rate of nitrogen fixation has seen high yield that means silica can help to increase the nitrogen fixation rate.

Silica is an amorphous in nature where it contains silicates unit SiO_4^- and other group like Si-OH, Si-O-Si might it help by providing high surface area and adsorption for nitrogen molecule could help the easy access of nitrogen for the microbial consortia and of course silica surface are porous in nature could help in electron transaction and proton (H^+) maintenance produced by *Geobacter* that's why this could be the reason to increase the rate of nitrogen fixation.

Actually it has been used different field, especially in agriculture its use are reported by researcher that improvement of seed germination *Lycopersicum esculentum* with the help of SiO_2.

4.10 Ethanol test

4.10.1 Calibration curve for ethanol test

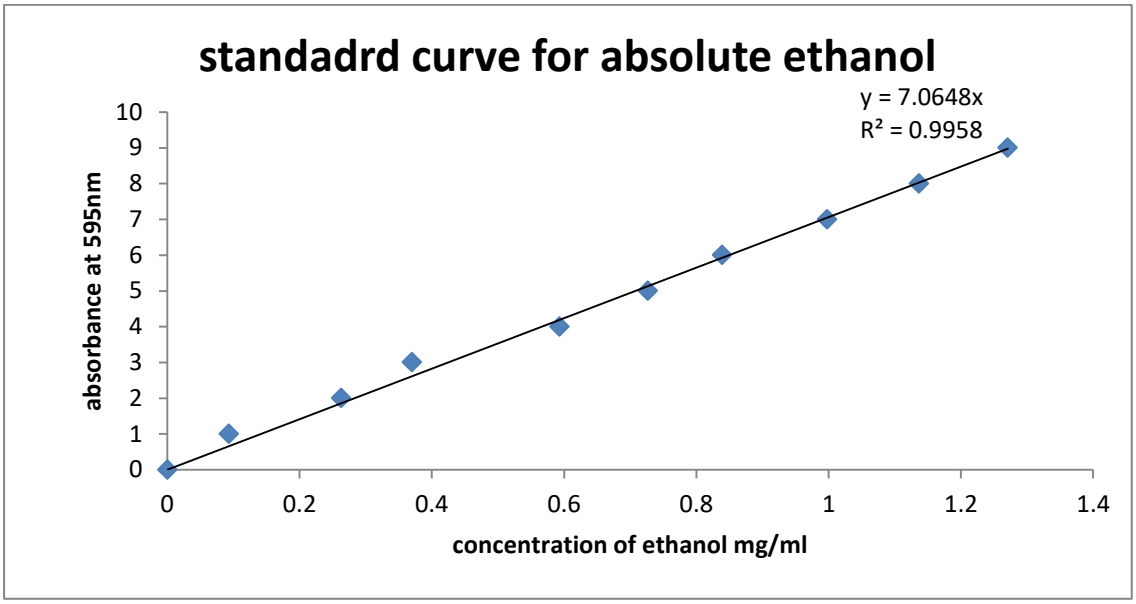
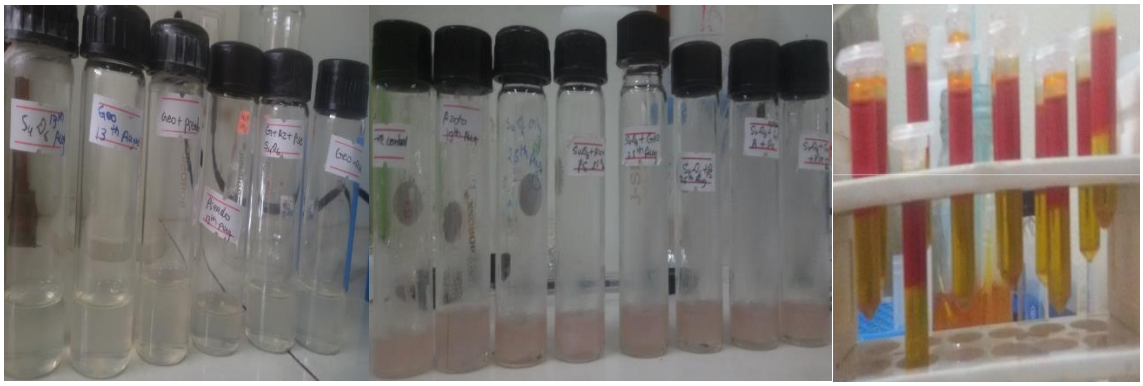


Figure 4.45: standard curve of ethanol for the quantification of ethanol from unknown samples

This is a standard curve of absolute alcohol having molecular formula C_2H_5OH straight line having y=7.0648x and square root value is 0.9958 which denotes the reliability of the standard curve. The blank was set with water (H_2O) and different types of concentration of ethanol alcohol ranges from 1mg/ml to 10mg/ml were picked up for the absorbance at 595nm.

4.10.2 Quantification of ethanol

In the next step, each consortium of bacterial samples was centrifuged at 5000 rpm for 5 minutes and supernatant were taken for estimation of alcohol content in it. For the positive sample, Potato Dextrose Broth (PDB) and Glucose was taken and then proceed through all over the process like fermentation with yeast and dichromate oxidation etc. Commercial yeast found in Nepalese market are used and potassium dichromate is preferred because dichromate is easily access in high purity and indefinitely stable in the air, when dichromate Cr (IV) turn into chromium product Cr(III) color changes from orange to greenish so spectra of color are analyzed through spectrophotometer easily.



A B C

Figure 4.46: A; Bacterial growth, B; Molisch's test and finally C; Ethanol test of consortium of bacteria through Dichromate oxidation

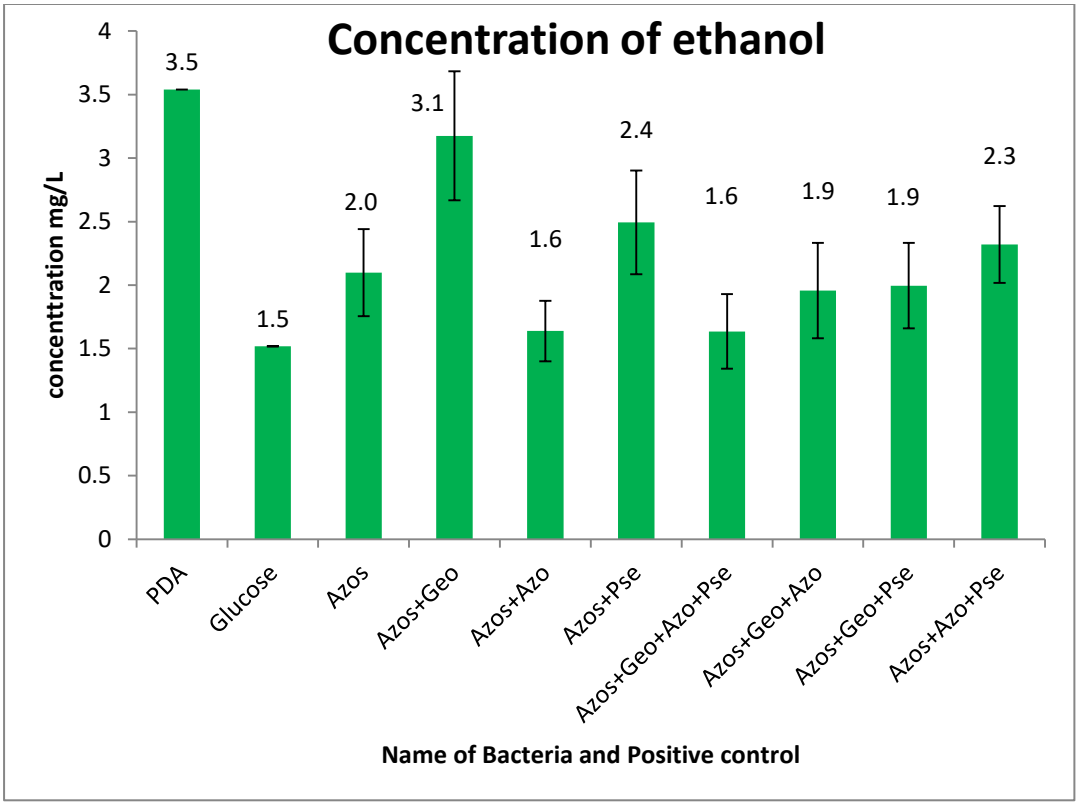


Figure 4.47 quantification of ethanol from the different consortia of bacteria

Positive control was taken potato dextrose broth (PDB) has highest rate of ethanol production that is 3.5mg/ml and glucose has 1.5 mg/ml. From the experiment, consortia of isolated one bacteria also can give the near of the range of ethanol production of PDB.

Different bacterial consortia of the isolates incubated with 0.1% commercial yeast with different percentage of silica nanoparticles for seven days at 28°C and bioethanol

production was estimated through dichromate oxidation method, oxidation state changes from +6 to +3, and color changes and absorption spectra for ethanol is maxima at 595nm and it is found to be highest for consortium *Azos+ G*eo with 3.1mg/ml and *Azos+ Geo+ Azo+ Pse* with 2.3mg/ml and others have also roles in production of ethanol. Effect of silica on the bacterial consortium also checked:

4.10.3 Effect of silica nanoparticles on concentration of ethanol production

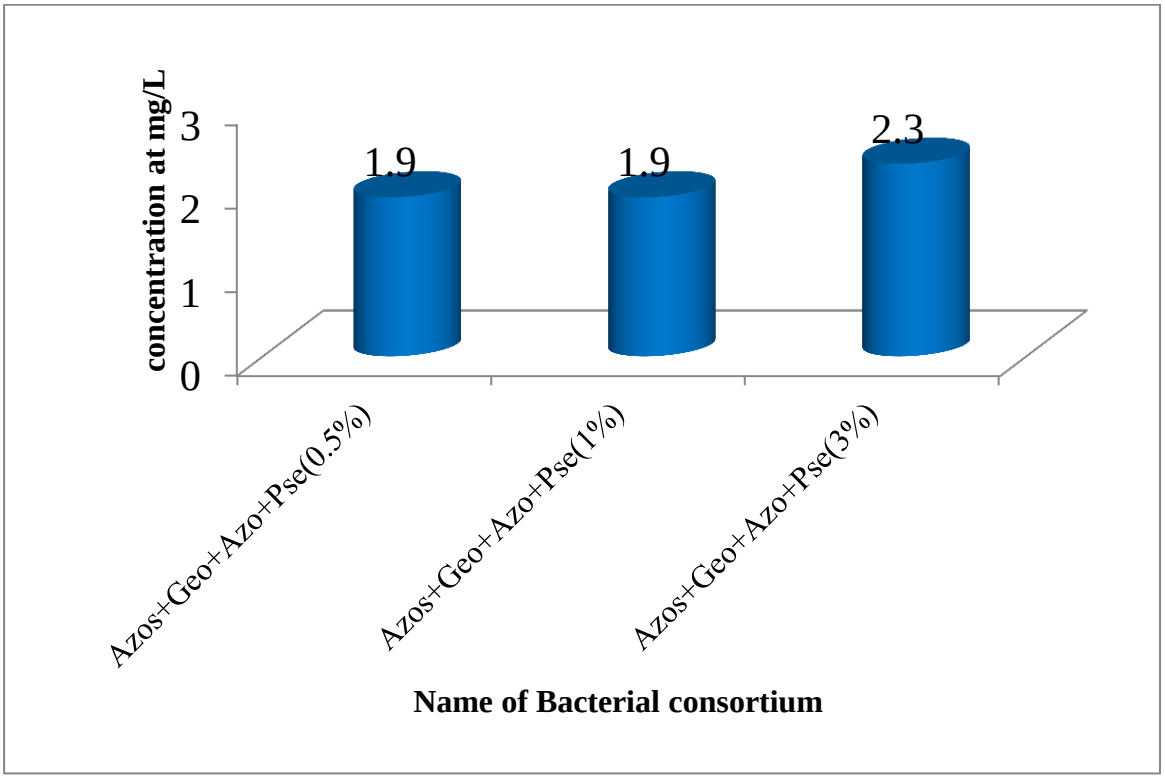


Figure 4.48: quantification of ethanol after adding silica.

The rate of ethanol production is increased by 1.25 times by using silica nanoparticles than in NCF media this might be due to electron may get space on porous nature of silica or chemical adsorption could be possible.

After the estimation of alcohol quantity we have done the distillation for the collection of alcohol from the carbon and nitrogen fixed media though we could not collect the ethanol, where carbon has been fermented into alcohol but the nitrogen content and the biomass of bacterial culture remaining in the solution of media and then we have applied such material or we could say nitrogenous biological fertilizer to the bonsai plant at pot system for one year. At the pot system there is only artificial soil which contain biochar, rock dust and the consortia of electrophs, diazotrophs carbon fixer: the isolated ones. Remaining solution (biofertilizer) has been tested after harvesting the ethanol portion.

4.10.4 Effect of Biofertilizer on Bonsai plant

Remaining solution (microbial consortia with artificial soil) named as Bio-fertilizer worked very well which were implied to the indoor bonsai plants. They have become healthy and greenery and well adapt to the provided situation. This is possible only when there is the sufficient nitrogen source occurred through nitrogen fixation and other necessary minerals. And there should be happening of carbon sequestration by autotrophs or use of dead biomass of organism for carbon source otherwise they could not show their healthy growth for upto 6-7 month continuously. After seven month they were become chlorosis (turn leaves into yellowing). This may be due to deficient of other nutrient like magnesium, zinc and most important minerals. Nitrogen and Carbon source from one pot could be provided from this technology for a long time if other parameters keep constant. The difference between upto 7 month and after 7 month at the bonsai plant could be observed in the figure 4.49. From this we could be come on a point that remaining solution can be an alternative of chemical fertilizer especially nitrogenous fertilizer.

Figure 4.49: Bonsai plant upto 7 month after applying remaining solution of different bacterial consortia at CDBT

Figure 4.50 Bonsai plant becoming wildering after applying remaining solution of different Bacterial consortia after 7 months

5. SUMMARY

The alternatives of fossil fuel and chemical fertilizer is highly sought to tame the global warming and air pollution. Reduction of carbon dioxide and nitrous oxide from the atmosphere is mainly performed by microorganism like cyanobacteria, nitrogen fixing bacteria. but not sufficient yet because of massive utilization of natural resources, Moreover, carbon dioxide reduction alone can be done by several phyla including plants. However, all the steps of carbon dioxide reduction require light sources for water splitting. Thus, carbon dioxide reduction without mimicking the plant photosynthesis that serves the reaction in dark would be of love as it can overcome the burden of exposing light sources in a reaction vessel full of bacteria in vertical harvesting or has to be done in horizontal platform that would require more land that could be scarce in future in response to population growth. Based on evolutionary perspective it was hypothesized that from chemolithotrophic autotrophy to photosynthetic autotrophy there must have been non-photosynthetic, albeit less efficient, that could reduce carbon dioxide.

Hence, different bacteria were isolated from Nepalese prehistoric nature soil, the rhizosphere of tree fern, screened for diazotrophic electrophic extracellular electron transferring *Geobacter sps,* diazotrophic *Azotobacter sps.* and *Pseudomonas sps.* along with diazotrophic autotroph *Azospirillium sps* on the basis of Carbon Catabolite Repression (CCR), initially performed by Machchhendra Thapa (B. Sc. Thesis, Purbanchal University, 2016) Anushruti Sangami (B. Sc. Thesis, Purbanchal University, 2016) and Sushma Khadka (B. Sc. Thesis, Purbanchal University, 2016), respectively.

Thus, this work focused on screening of diazotrophic electrotrophic bacteria. From nine different rhizospheric places around Panchase area having different altitudes, initially 43 isolates of different bacteria were isolated in nitrogen free and sodium acetate as reduced carbon source. When the isolated bacteria were recognized on the basis of CCR where their use of their preferred carbon sources like sodium acetate, aniline, ethylene glycol and toluene in nitrogen free media. Among them 18 were putative *Geobacter sps.* based on the fact that they grew in aniline containing media but not in ethylene glycol, 4 were putative *Azotobacter sps,* based on the facts that they grew in ethylene glycol containing but did not survive in toluene containing media and 18 were putative *Pseudomonas sps.* based on the fact that they grew in both ethylene glycol or toluene containing media and rest three had no growth further in other reduced carbon sources except sodium acetate. Thus, the putative *Geobacter* sps. were further pursued for characterization.

The individual isolates of *Geobacter* sps. were crossed checked biochemically followed by molecular characterization. Genomic DNA of each isolates of putative *Geobacter* sps.

were extracted and PCR amplification of 16s rRNA genomic DNA was done to confirm whether it is bacteria using universal primers. Once the isolates were reconfirmed to be bacteria they were further checked whether they are *Geobacter* sps. and PCR was performed of 16sRNA genomic DNA where the primer was designed in such a way that it only amplifies the sequence relevant to *Geobacter* sps. only. The specific gene, omcJ have done by designing the primer using different web based tool. All the samples were amplified by designed primers for 16s rRNA genomic DNA of *Geobacter* sps indicating that the isolates screened through designed protocol based on reduced carbon source utilization potential could be a new tool to screen *Geobacter* sps. from mixed samples like soil, water, sludge. These isolates were further subjected to identification of *Geobacter sulfureducence* by specific primer designed to amplify its specific gene omcJ (2685380) that is responsible for extra cellular electron transfer and 2 samples amplified confirming that the isolated putative *Geobacter* sps. were infact of Genus *Geobacter* and two were *Geobacter sulfureducence*. Moreover, the isolates representing *Geobacter Sulfureducens* physiologically confirmed for their ability to generate electricity in microbial fuel cell. Upon incubation of the isolate with sodium acetate as sole carbon source it generated electricity that was found to be 318 mV in voltmeter indicating that the isolates is in fact *Geobacter Sulfureducens*. Since all of the experiments were done in nitrogen free media, thus those organisms which can fix atmospheric nitrogen can survive and among the *Geobacter* sps only *Geobacter Sulfureducens* has the ability to fix nitrogen as it has nitrogenase system under two component system. Measuring nitrogen fixation potential in the nitrogen free GS-15 media it was found to be that highest amount of ammonium ion found in the culture media was 0.3 mg/ml 72 hr. This validates that the protocol designed to screen *Geobacter sulfureducence* from soil sample using different reduced carbon sources can putatively isolate the strain but further molecular validation is required and the primers designed could be used in future confirmatory experiments for screening of this strain. In addition, it can be speculated that screening of other diazotrophs could be done in similar manner and putative *Azotobacter* sps and *Pseudomonas* sps could be isolated.

Since the work was to develop a technology where both atmospheric nitrogen and carbon dioxide could be reduced simultaneously in “One Pot” system attempt was made. Based on earlier works of Sushma Khadga (B. Sc. Thesis, Purbanchal University, 2016) bacteria was isolated from the same soil sample exactly based on carbon repression method putative Azospirillium sps. isolated by the team member was tested for its diazotrophic autotrophy characteristics in dark it was found serendipitously that the isolate exhibited both features, opening an avenue for carbon dioxide reduction and storage.

At dark condition putative *Azospirillium* sps exhibited carbon and nitrogen fixation from the atmosphere on the carbon and nitrogen free media at pH 4.5 and 6.8 as indicated by

preliminary Molischs test and confirmatory by DNS test suggested that about 3% of reduced carbon was found in the medium. The photosynthetic organism the ultimate carbon dioxide reducing reaction occurs in dark and the photon from the Sunlight is used for water splitting to generate electron and proton that is required for carbon dioxide reduction and chemolithotrophs acquire electron from other sources the required proton and electron could have come either from water molecule itself or oxido-reduction of metals used in the media which could have helped in water splitting and generation of proton and electron required for reduction of both nitrogen and carbon dioxide. Since both the reactions are highly energy demanding the leaching of one molecule of hydrogen during nitrogen fixation cannot be overlooked and the diazotrophs has ability to oxidize hydrogen molecule to proton and electron it was presumed that syntrophic growth of different diazotrophs could enhance this reaction. Accordingly syntrophic growth among the four different diazotrophs mentioned above were coincubated or in combinations of them and it was found that the cells grew at both pH 4.5 and 6.8 in nitrogen and carbon free media (NCF) as observed from optical density at 600 nm (OD_{600}). The best combination for carbond dioxide reduction was growth among three diazotrophs, *Azospirillum* sps*, Azotobacter* sps and *Pseudomonas* sps that gave around 31% reduced carbon in the medium as calculated from DNS method. The best combination for nitrogen fixation was although found to be in the syntrophic growth containing all four including *Geobacter* sps and was found to contain 21% ammonium ion as calculated from Nessler's reaction.

The syntrophic growth system developed for the first time could be used as consortia of bacteria as bio-fertilizer that not only substitutes chemical nitrogenous fertilizer but also can store carbon dioxide in soil where the increased amount of reduced carbon sources could augment growth of soil microbiome. Moreover, carbon dioxide reduction potential could be further explored for bio-fuel and bio-plastic.

As silica, nickel, cobalt, molybdite have been reported to enhance water splitting upon exposure to photons, it was hypothesized that the electrophilic diazotroph *Geobacter* could transfer electron and increase water splitting rate in the presence of silica since other metals are present in the medium. Silica nanoparticles were extracted from rice husk char, major problem in managing in industries using thermal energy as rice husk is cheap source to burn. The protocol was sol gel method with slight modifications made. This was done because silica nanoparticles are known plant growth stimulant as well as having adsorptive role played in plant growth. They were characterized by XRD, FTIR and SEM technologies and found to be amorphous nature, Silanol, silane as functional groups, almost nanoparticles size in the range of (200-500) nm.

Modification was made to standard sol gel method by changing the nature of acids used in different steps so that the waste could be used as culture medium for the bacteria

mentioned above. Use of sulphuric acid would give sulphate, phosphoric acid would give phosphate and neutralizing with acetic acid would give sodium acetate as preferred sole reduced carbon source required for growth of bacteria. This would have minimized the cost of management of halogenic acids and sodium chloride generated during silica nanoparticle synthesis. Three types of silca were extracted by modifying the protocol. The concentration of pure silica in the isolated silica samples of were measured and among the three types of extraction protocols silica obtained by the standard sol gel method was found to be with higher yield (93%) than that of modified ones.

In addition, due to adsorptive nature and could remain tetrahedral structure of silicates (SiO_4^{4-}), functionalization of nitrogen group into the silicates was attempted to substitute urea from agriculture since both carbon and silicon are at similar position in the Periodic table and sodium metasilicates is available for research works. Functionalization was tried by low pressure plasma technology at Kathmandu University. Though no signification result was seen, some nitrogenous group were seen with hydrogen and carbon as N-H and C≡N around the 2300cm-^{1}. The presence of carbon in the sample indicated that complete combustion of the char did not take place and presence of this impurity might have had effect in its size. The nitrogen content present at silica samples was also determined by FTIR and spectrophotometrically. Though FTIR did not give sufficient proof of functionalization of nitrogen group during proceeding plasma technology, nitrogen content was shown by spectrophotometer at 435nm. This is the attempt for executing silica as a nitrogenous fertilizer after functionalization. Though expected results could not obtained, many literature have been reported of functionalization is possible. Hence, optimization of the protocol could be attempted.

As hypothesized above that the presence of silica could support increased water splitting potential, mixotrophic syntrophic growth of the bacteria with varied concentration of silica nanoparticle was performed. The effect of silica particles on the microbial consortia of the isolated bacteria was not lethal them and amazingly there was rather than any toxicity to the bacterial consortium growth there was higher yield of reduced carbon and nitrogen amount seen on the media. Combination of all four organisms containing consortial syntrophic growth had 38% ammonium ion in the medium.

Bioethanol production from this dark reduced carbonated and nitrated media was done using commercial yeast. As estimated of ethanol production by Dichromate method, the highest yield of ethanol production was observed on the NCF media after adding silica than without it. Consortia of *Azosspirillum* sps and Geobacter sps. had exhibited higher yield of about 3.1mg/ml of bio-ethanol. Among different consortia of bacteria growing in NCF media, consortium of all four had only 1.6mg/ml of bioethanol produced, as can be assumed the because the free reduced carbon in the medium was lower compared to

others, but when 3% silica was added in this consortium 2.3mg/ml of bioethanol was produced. At 3% silica the amount of ethanol production had been increased by 1.25 times than in lower and higher percentage of silica added.

As the innovation and prototype technology has been invented, it is suggested that the bacterial pellets along with yeast could be used as solid bio-fertilizer and the solution left after ethanol separation can be used as liquid fertilizer or aquaculture medium since presence of free ammonium and soluble reduced carbon would not give pyrolysis product during ethanol evaporation unlike present medium used for yeast growth and the foul odour coming from spent wash of ethanol industry. A small pot trial with bonsai plants as project has been performed using these and some other bacterial combination in artificial soil designed by our group which lacked any soil or any prior exposure to vegetation. All the plants tested survived for more than six months without any supplementation of chemical fertilizer or any other organic fertililzer but only irrigating with the water.

Though primitive, but this technology could be developed to robust technology and could be commercialized for the alternatives of fossil fuel and chemical fertilizer. The research would change the era of fossil fuel and chemical fertilizer from now onward if it would be commercialized. This technology could be a solution of global warming through nitrogen fixation to substitute urea and non-photosynthetic CO_2 reduction for bio-fuel and others.

6. CONCLUSION

A prototype technology has been developed that could potentially substitute chemical nitrogenous fertilizer and reduce carbon dioxide to make next generation bio-fuel. To our best knowledge, *Geobacter* sps has not been used as biofertilizer but some indication could be found in few literatures. Recent scenario demands that there is urgent need of remedy for the environmental pollution and to reduce the global warming. The global population that is expected to increase by approximately 3 billion by 2050 to above 9 billion would require higher energy and fossil fuel scarcity could propel social unrest or even war. Moreover, the environment pollution, rising of global temperature is of serious concern and should be minimized. Various methods such as synthetic biology and engineering the metabolic pathway of plant to yield more photosynthetic product have been explored for long time that could be useful for food production, however, for energy alternatives it would require horizontal space and land could be limiting. Alternatively, works on artificial leaf has been conducted without much success and fanfare to celebrate. Microbials have been explored but most of them, if not all, would require Sunlight and again horizontal growth would limit the use of technology. Nonetheless, here natural method has been discovered for the reduction of carbondioxide as well as nitrogen fixation in "One Pot" system by microorganism in dark and were isolated from the country thus not requiring any materials transfer from other countries. All these Electrotrophs, Diazotrops, Autotrophs work in tandem and enhance each other's works and resulting of carbon and nitrogen fixation. Here bacterial ethanol and biological nitrogen content as ammonium ion were estimated which elucidate the possibilities of establishment of industry of nitrogenous fertilizer along with lignocellulosic biomass based biofuel till carbon reduction is increased for business viability as fertilizer can generate capital and the liquid solution that is carbonated and nitrated can be used as nitrogen source since cellulose don't have any nitrogen and has to be supplemented from outside. For the idea to be commercially viable a small scale pilot project could be established and the university should have incubation center and funding.

RECOMMENDATION

- Isolated bacteria should be sent for sequencing for further 100% confirmation
- The yield of carbon fixation could be increased by bubbling of more carbondioxide during the growth of consortia of bacteria
- Chromosomal engineering could be done at the RuBisCo present in the putative *Azospirillium sps* samples
- Higher yield of Electricity production from *Geobacter sulfureducence,* further engineering study should be needed.

- The technology has to be promoted to flourishes as huge technological breakthrough to reduce carbon dioxide as well as nitrogen fixation at dark condition.
- For industrialization, pilot research should be conducted in sufficient area.

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APPENDICES

Appendix 1

Composition and preparation of different Microbiological culture Media

Nfb Medium	
DL Malic acid	5g
K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.2g
Nacl	0.1g
$CaCl_2.H_2O$	0.02g
Micronutrient solution	2ml
Bromothymol blue (0.5% in 0.2N KOH)	2ml
Fe(III)EDTA(1.64%)	4ml
Vitamin Solution	1ml
pH	6.8
For Semi Solid agar	0.5g
Solid agar	15g
Micronutrient Solution	**GMS/L**
$CuSO_4.5H_2O$	0.4
$ZnSO_4.7H_2O$	0.12
H_3PO_3	1.4
$Na_2MoO_4.2H_2O$	1
$MnSo_4.H_2O$	1.5
Vitamin solution	
Vitamin mix	mg/L
Biotin	2mg
Folic acid	2mg
Pyridoxine	10mg
Riboflavin	5mg
Thiamine	5mg
Nicotinic acid	5mg

Appendix 2

Modified Nfb media (termed as NCF media)

K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.2g
Nacl	0.1g
$CaCl_2.H_2O$	0.02g
Micronutrient solution	2ml
$FeSO_4$	0.05
pH	6.8
maintain by KH_2PO_4	

Micronutrient Solution	**GMS/L**
$CuSO_4.5H_2O$	0.4
$ZnSO_4.7H_2O$	0.12
H_3PO_3	1.4
$Na_2MoO_4.2H_2O$	1
$MnSo_4.H_2O$	1.5

Appendix 3

GS -15 MEDIA

Ingredients	Amount
Ammonium chloride	1.5gm
Sodium bicarbonate	2.5gm
Sodium dihydrogen phosphate	0.6gm
Potassium chloride	0.1gm
Sodium acetate	1.646gm
Mineral mix	10ml
Vitamin mix	10ml
Distilled water	1000ml

Mineral mix(1000ml)		Vitamin mix(1000ml)	
Ingredients	Amount	Ingredients	Amount
Nitrilotriacetic acid	1.5gm	Biotin	2mg
Magnesium sulphate	3.0gm	Folic acid	2mg
Manganese sulphate	0.5gm	Pyridoxine	10mg
Sodium chloride	1.0gm	Riboflavin	5mg
Ferrous sulphate	0.1gm	Thiamine	5mg
Calcium chloride	0.1gm	Nicotinic acid	5mg
Cobalt chloride	0.1gm	Pantothenic acid	5mg
Zinc chloride	0.13gm	Vitamin B12	0.1mg
Aluminum potassium sulphate	0.01gm	P-Aminobenzoic acid	5mg

Boric acid	0.01gm	Thioctic acid	5mg
Sodium molybdate	0.025gm	Sodium tungstate	0.025gm

Appendix 4

Composition of GS-15 modified media

Ingredients	Amount
Sodium bicarbonate	2.5gm
Sodium dihydrogen phosphate	0.6gm
Potassium chloride	0.1gm
Sodium acetate	1.646gm
Mineral mix	10ml
Vitamin mix	10ml
Distilled water	1000ml

Appendix 5

Preparation of Stock solutions, Buffers and Reagents

Tris-Cl (1 M, pH 7.5 and 8.0)

Tris-Cl buffer was prepared by adding 12.11 g of Tris base in 80 ml of MilliQ water and pH was adjusted to 7.5 or 8.0 by adding concentrated HCl. Then final volume was maintained to 100 ml. The solution was sterilized by autoclaving and stored at 4^0C.

EDTA (0.5 M, pH 8.0)

18.61 g of disodium EDTA.$2H_20$ was added to 80 ml of MilliQ water, stirred vigorously on magnetic stirrer and pH was adjusted to 8.0 with NaOH. The final volume of the solution was made upto 100 ml and sterilized by autoclaving and stored at 4^0C.

Tris EDTA (TE) buffers

pH 7.5
10 mM Tris-Cl (pH 7.5)
1 mM EDTA (pH 8.0)

pH 8.0
10 mM Tris-Cl (pH 8.0)
1 mM EDTA (pH 8.0)

Potassium Phosphate buffer (0.1 M, pH 7.0)

1 M K_2HPO_4	61.5 ml
1 M KH_2PO_4	38.5 ml
MilliQ water	upto 1000 ml

Nesslers reagent

Mercuric chloride	10.0 gm
Potassium iodide	7.0 gm
Sodium hydroxide	16.0 gm
Water (ammonia free)	100.0 ml
Final pH (at 25°C)	13.2±0.05

<u>Preparation of solutions for chromosomal DNA isolation</u>

TE1 solution

50 mM Tris-Cl (pH 7.5)
50 mM EDTA (pH 8.0)

STEP solution

50 mM Tris-Cl (pH 7.5)
0.2 mM EDTA (pH 8.0)
0.5% SDS
1 mg/ml Proteinase K

Lysozyme (1 mg/ml)

1 mg of lysozyme was dissolved in 10mM Tris-Cl (pH 8.0) with final volume of 1 ml. The solution was prepared immediately before use.

Proteinase K (20 mg/ml)

The lyophilized proteinase powder was dissolved at a concentration of 20 mg/ml in sterilized 50 mM Tris (pH 8.0), 1.5 mM calcium acetate. The solution was stored at -20^{0}C.

Sodium Acetate (3 M, pH 5.2)

40.83 g of sodium acetate.$3H_20$ was dissolved in 80 ml of MilliQ water and pH was adjusted to 5.2 with glacial acetic acid and final volume was made upto 100 ml. The solution was sterilized by autoclaving and stored at 4^0C.

Preparation of solutions for Agarose gel electrophoresis

10 X TAE Buffer

Tris base	4.84 g
Glacial acetic acid	1.142 ml
0.5 M EDTA (pH 8.0)	2 ml
MilliQ water	upto 100 ml

The working solution (0.5 X) of TAE buffer was prepared by diluting 17.5 ml of 10 X TAE stock solutions with MilliQ water to make the final volume upto 350 ml.

Ethidium Bromide (10 mg/ml)

100 mg of ethidium bromide was weighed and dissolved in 10 ml of MilliQ water. The solution was protected from sun light by wrapping with aluminum foil and stored at room temperature.

Primers

16 S rRNA *Geobacter*

Forward →5'CTA GTT GGT AGG GTA ATG GC 3'

Reverse → 5' GCA CCC TCC GTA TTA CCG 3'

Appendix 6

SN.	**Specification**		**Forward Primer**	**Reverse Primer**
			Oligo Calculator Results	
1	Annealing Sequence	Tm	52°C	52°C
		G + C	50%	61%
		Length	20	18
2	Tm	Basic	51.8°C	52.6°C
		Salt Adjusted	58.4°C	58.4°C
		Nearest Neighbor	49.23°C	50.88°C
3	G + C Content		50%	61%
4	Number of base pairs		20	18
5	Secondary Structure			
	Tm		16.2°C	16.8°C
	Dg		0.62 kcal/mol	0.5 kcal/mol

	3’ end	Free	Free
		DNA FOLD Results	
6	Secondary Structure		
	Tm	16.2°C	16.8°C
	Dg	0.60 kcal/mol	0.5 kcal/mol
	3, end	Free	Free
		Oligo Analyzer Results	
7	Self dimer: dG	-4.16 kcal/mol	-3.61 kcal/mol
8	Hetero dimer: dG	-9.08 kcal/mol	
9	Approximate Amplicon Size	298 bp	

Appendix 7

Geobacter sulfurreducens omc J gene

Forward 5’ CTA GTT GGT AGG GTA ATG GC 3’

Reverse 5’ GCA CCC TCC GTA TTA CCG 3’

SN.	**Specification**		**Forward Primer**	**Reverse Primer**
			Oligo Calculator Results	
1	Annealing Sequence	Tm	54°C	54°C
		G + C	58%	61%
		Length	19	18
2	Tm	Basic	53.2°C	52.6°C
		Salt Adjusted	59.5°C	58.2
		Nearest Neighbor	52.56°C	54.33
3	G + C Content		58%	61%
4	Number of base pairs		19	18
5	Secondary Structure			
	Tm		26.8°C	26.8°C
	dG		-0.12 kcal/mol	0.12 kcal/mol
	3’ end		Free	Free
			DNA FOLD Results	
6	Secondary Structure			
	Tm		26.8°C	26.8°C
	dG		-0.12 kcal/mol	0.12 kcal/mol
	3, end		Free	Free
			Oligo Analyzer Results	
7	Self dimer: dG		-9.82 kal/mol	
8	Hetero dimer: dG		-6.92 kcal/mol	

9	Approximate Amplicon Size	381

Appendix 8

1. <u>Isolation of Silica Nanoparticles(Sol-gel Method)</u>

Equipments

Muffle furnance("INDOSATI" Scientific Lab equipment)
Hot Air Oven
Magnetic Stirrer
Heating mental
Reflux condenser (BOROSIL)
Round Bottom flask (BOROSIL4400)
Centrifuge (Hugen)
Vortex Shaker

Chemicals

HCl
H_2SO_4
NaOH
Double Distilled Water(DDW)
Apparatus
Plastic spoon
Whatman filter paper no 1
Falcon Tube
Glass Rod
Silica porcelain
Heat resistant gloves

<u>2. Silica quantification</u>

Materials

UV spectrophotometry
Quartz cuvette
Tissue paper

Reagents

Sodium bicarbonate
Sulphuric acid 1N
Hydrochloric acid : water (1:1)
Ammonium molybdate reagent:
Dissolve 10 g ammonium molybdate [$(NH_4)_6Mo_7O_{24}.4H_2O$] in distilled water with stirring and gentle warming, and dilute to 100 ml. Filter, if necessary. Adjust to pH 7 to 8 with silica free ammonia or sodium hydroxide and store in polyethylene bottle to stabilize.

Oxalic acid solution: Dissolve 7. 5 g of oxalic acid in distilled water and dilute to 100 ml.

Stock silica solution: Dissolve 4.73 g of sodium metasilicate nonahydrate ($Na_2SiO_3.9H_2O$) in distilled water and dilute to 1000 ml

Standard silica solution: Dilute 10ml of stock solution to 1000ml with distilled water, 1.00ml = 10.0µg of SiO_2

Potassium chromate solution: Dissolve 630 mg of potassium chromate in distilled water and diltue to 1 litre.

Borax solution: Dissolve 10 g of sodium borate decahydrate in distilled water and dilute to 1 litre.

Appendix 9

Matetials and pretreatment of MFC

Platinum wire
MFC device
Voltmeter
Resistance
Electric wire
Carbon nanotube coated (CNT) anode

CNT electrode graphite: Treat graphite with absolute ethanol and sonicated for 15 min, follow by distilled water again sonicate for 15 min. Finally wash with 70% Acetone and ultrasonicated for 15 min at 25 degree centigrade Dry with vaccum for 12 hr then in oven for 12 hr at 60 degree centigrade

Treatment of plasma membrane: Remove impurities by boiling for 1hr in 3% H_2O_2, wash with deionized water and 0.5 M H_2SO_4, again with deionized water

Appendix 10

2. <u>Culture Media.</u>

2.1. Potato Dextrose Agar (PDA) (pH 4.5)

Potato(peeled)	200g
Dextrose	20g
Agar	15g
Distilled Water	1000ml

2.2. Tryptone broth

Tryptone	10g
NaCl	5g
$CaCl_2$	1ml

Distilled Water	1000ml

2.3. SIM Agar(pH 7.3)

Peptone	30g
Beef Extract	3g
Agar	3g
Ferrous ammonium sulphate	0.2g
Sodium thiosulphate	0.025g
Distilled Water	1000ml

2.4. Simmons citrate agar(pH 6.9)

Ammonium dihydrogen phosphate	1.0g
Dipotassium hydrogen phosphate	1.0g
Sodium chloride	5.0g
Sodium citrate	2.0g
Magnesium sulphate	0.2g
Bromo thymol blue	0.08g
Agar	15g
Distilled Water	1000ml

2.5. MR-VP broth(pH 6.9)

Peptone	7.0g
Potassium phosphate	5.0g
Dextrose	5.0g
Distilled Water	1000ml

3.6 Luria Broth

Casein enzymic hydrolysate	10.000
Yeast extract	5.000
Sodium chloride	5.000
Final pH (at 25°C)	7.0±0.2

Appendix 11

Test of Optical density at different day of different Bacterial consortia

S.N	S4D6	Geobacter	Azotobacter	Pseudomonas	OD at 600nm	2nd Set(3rd day)	6th day	9th day
1	✓				0.033	0.027	-0.007	-0.023
2	✓	✓			0.076	0.032	0.165	-0.019
3	✓		✓		0.055	0.340	0.191	-0.015
4	✓			✓	0.033	0.189	0.183	0.021
5	✓	✓	✓	✓	0.082	0.057	0.260	0.031
6	✓	✓	✓		0.204	0.341	0.114	-0.023
7	✓	✓		✓	0.136	0.061	0.225	0.049
8	✓		✓	✓	0.141	0.055	0.249	0.001

Appendix 12

Quantification of Ammonium ions fixed by Putative *Geobacter sulfurreducens*

S.N	Sample name Putative Geobacter isolates	Nessler's test absorbance at 435nm	mg/L ammonia production
1	1	0.020	0.06
2	2	0.039	0.08
3	4	0.046	0.09
4	5	0.038	0.08
5	8	0.040	0.08
6	9	0.012	0.05
7	12	0.229	0.30
8	13	0.017	0.06
9	14	0.035	0.07
10	16	0.004	0.05
11	17	0.007	0.05
12	18	0.019	0.06
13	22	0.032	0.07
14	25	0.004	0.05

15	40	0.030	0.07
16	41	0.034	0.07
17	42	0.008	0.05
18	43	0.001	0.04

Appendix 13

Nitrogen test from Nessler's reagent of different Bacterial consortia

S.N	S4D6	*Geobacter*	*Azotobacter*	*Pseudomonas*	OD at 435nm (1^{st})	OD at 435nm (2^{nd})
1	✓				0.124	0.150
3	✓	✓			0.553	0.295
4	✓		✓		0.704	0.509
5	✓			✓	0.715	0.502
6	✓	✓	✓	✓	0.751	0.711
7	✓	✓	✓		0.545	0.432
8	✓	✓		✓	0.344	0.439
9	✓		✓	✓	0.317	0.321