NITROGEN FIXING BIOFERTILIZER FOR CARBON STORAGE

M.Sc. Thesis

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Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

A dissertation submitted as the partial fulfilment of the requirement for M.Sc. degree in Biotechnology

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RECOMMENDATION

This is to certify that **Ms. Anju Tamang** has successfully completed this thesis work entitled **"NITROGFEN FIXING BIOFERTILIZER FOR CARBON STORAGE"** under my supervision.

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

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CERTIFICATE OF EVALUTATION

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DECLARATION

I, hereby, declare that the thesis report entiled **"NITROGFEN FIXING BIOFERTILIZER FOR CARBON STORAGE"** submitted by me, Anju Tamang (T.U. Regd. No. : 5-1-8-5-2008), to Tribhuvan University, Kirtipur, Nepal with supervision of Dr. Pramod Aryal and Prof .Dr. Rajani Malla for partial fulfilment of requirement for the degree of M.Sc. in Biotechnology is a record of confide research work carried out by me. I further declare that the work reported in this thesis has not been submitted elsewhere, either in a part or in full, for the award of any other degree of diploma.

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Anju Tamang

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LIST OF ACRONYMS

NADPH	Nicotinamide Adenine Diphosphate(H)
Fd	Ferrodoxin
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
EFFs	Environmentally friendly fertilizers
BNF	Biological Nitrogen fixation
SNF	Symbiotic nitrogen fixation
PGPR	Plant growth promoting rhizobacterium
PHAs	Polyhydroxyalkanoates
PHB	Poly-beta hydroxybutyric acid
GR	Green Revolution
IAA	Indole-3-acetic acid
μM	Micro molar
UV	Ultraviolet
VBNC	Viable but not culturable
DNA	Deoxyribo Nucleic Acid
MoFe	Molybdenum-iron
GTP	Guanosine triphosphate
PTS^{Ntr}	Nitrogen-related phosphotransferase system
TCA	Tricarboxylic acid cycle
CCR	Carbon catabolite repression
SB	Sudan black
AFU	Arbitrary fluorescence units
MFI	Median fluorescence intensity
PCR	Polymerase chain reaction
16S rRNA	Svedberg's unit ribosomal Ribonucleic Acid
NJ	Neighbor joining

LB	Luria Bertani
DDW	Double Distilled Water
NCBI	National Center for Biotechnology Information
bp	Base Pairs
mL	Milliter
MRVP	Methyl Red Voges Proskauer
SIM	Sulfide Indole Motility
TSI	Triple Sugar Iron Agar
mM	Millimolar
EPS	Extracellular polysaccharide
T_m	Melting temperature
OTUs	Operational taxonomic units
VBNC	Viable but not culturable
IVET	Vivo expression technology
MLST	Multilocus sequence typing
MLSA	Multilocus sequence analysis
rpoB	β-subunit of RNA polymerase
gyrB	β-subunit of gyrase
EBP	Enhancer-binding protein
UAS	Upstream activator sequences
PTS^{Ntr}	Nitrogen-related phosphotransferase system
PHB^+	PHB producer
PHB^-	PHB non-producer

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ABSTRACT

Nitrogen is primarily required by plants for their growth and dinitrogen as it exists has very stable triple bond which requires relatively high amount of energy to cleave making it unusable by plants. Therefore, despite its availability in large excess; are still unavailable for the crops and has to undergo biological nitrogen fixation so as to be absorbed by them. To meet the nutritional demand of plants nitrogen (N) is employed in the form of fertilizers in agricultural field. Long-term use of synthetic chemical fertilizers has resulted in soil acidification, poor soil aggregate stability and low levels of micronutrients. Further, it aggravates the environmental pollution through denitrification (N_2 and N_2O) and volatilization (NH_3) potentially contributing in global warming, stratospheric ozone layer depletion and acid deposition. Hence, alternative for these are sought in the form of biofertilizer that minimizes the potential hazardous effects to environment. Further, steep increase in production and utilization of polymers has resulted in significant burden on solid waste management followed by rapid depletion of fossil fuels used in production of such polymers. Moreover, synthetic biodegradable plastics are actually not truly biodegradable as they are labelled imposing harsh impacts on ecological degradation. Therefore, green alternative for these are bioplastics and biocomposites which when discarded in soil and landfills are usually acted upon by microbes such as bacteria, fungi, algae generating CO_2, CH_4, cellular components and other products. PHB in particular is polymer of industrial importance and has found to contain properties like biodegradability, thermoplasticity along with other traits similar to petrochemical plastics of recent usage. The preliminary identification of isolates via biochemical tests were followed by molecular characterization that involved PCR amplification, gel electrophoresis and DNA sequence analysis. When subjected to PCR amplification, the universal primer used yielded an amplicon of the expected size ~1500 bp. The sample A17 showed 98% similarity with the gene cluster sequence of *Azotobacter* species available from GenBank database while P8 depicted 99% concurrence with *Pseudomonas* species. The sequence obtained by DNA sequencing was further characterized by multiple sequencing alignment. The clustering approach called neighbor joining (NJ) method was used for the reconstruction of phylogenetic trees that yielded unrooted tree. The isolates of *A. vinelandii* and *P. fluorescens* were presented as nitrogen fixing species spectrophotometrically and as PHB producer using Flow cytometry that utilizes FlowJo software to quantify the PHB. The amount of fixed nitrogen was found to be as high as 21.8395 gm/L for A17 while the value of 1.8199 gm/L was achieved for P8 for 144 hours of incubation. PHB was quantified under different condition and maximum production was recorded at nitrogen limiting condition. The bacterial PHB content can directly be correlated with median fluorescence intensity expressed in AFU, Arbitrary Fluorescence Units. For P8 maximum value of 688.7505 AFU has been achieved with median 57.4 where 99.9% of the population produced PHB. Similarly for A17 maximum value of 545.1923 AFU of PHB was obtained with the median value of 30.0 where 93.6% of the total population were actively producing PHB. The isolates which possess the capability to fix the nitrogen along with its ability to store the carbon source as PHB was obtained. The amount of fixed nitrogen and PHB produced by the isolates established them as potent candidate for both biofertlizer as well as biopolymer producer.

Keywords: Biofertilizer, biopolymer, nitrogen fixation, PHB, spectrophotometry, Flow cytometry

CHAPTER 1

INTRODUCTION

1.1 Background

Human civilization has always depend upon and existed on ground of agriculture. Further civilization has been considered as product of agricultural Neolithic revolution. This fact has elaborated by H.G. Wells with the averment “civilization was the agricultural surplus.”

Plants sustain its life through nourishment and enrichment provided in the form of various manure and fertilizers. Inorganic and organic fertilizers are the source to maintain the growth condition (Ginting *et al*., 2003). Various nutritional demands are met by continually applying manure which provides nitrogen (N), potassium (K), Phosphorous (P), calcium (Ca) and magnesium (Mg) content in soil (Watts *et al*., 2010). Among the various elements required for essential growth of plants nitrogen is one of the chief elements. Inorganic nitrogen compounds i.e. ammonium, nitrate and nitrite (NH_4^+, NO_3^-, NO_2^-) constitute only 5% of the total nitrogen present in soil but still they are the majority of the nitrogenous form that are absorbed by the plants (Brady *et al*., 2008).

1.2 Nitrogen as an essential elemental constituent

Nitrogen is the most abundant and common element present on earth and atmosphere constituting about 78% of the atmospheric gas in dinitrogen gaseous form (N_2). Dinitrogen as it exists has very stable triple bond which requires relatively high amount of energy to cleave making it unusable for living organisms including faunal species. Therefore despite its availability in large excess; are still unavailable for the crops and has to undergo biological nitrogen fixation so as to be absorbed by them with following reaction.

$$N_2 + 8H^+ + 8\,e^- \longrightarrow 2NH_3 + H_2 \qquad 147\ \mathrm{Kcalmol^{-1}}$$

The ammonia (NH_3) thus produced from this reaction gets dissolved into water at physiological pH to form ammonium ion which are readily absorbed by the plants. The reaction involved is

$$NH_3 + H_2O \longrightarrow NH_4^+ + OH^- \qquad pK_a = 9.25$$

An alternative way for nitrogen fixation is atmospherics nitrogen fixation that involves lightening event which provides sufficient energy to convert water vapor and oxygen into highly reactive hydroxyl free radicals, free hydrogen atoms and free oxygen atoms. Such free radicals then attack dinitrogen in the atmosphere to yield nitric acid (HNO_3). Nitric acid thus formed reaches the soil through by the process of rain ultimately dissociating into nitrate (NO_3^-) which can be utilized by plants for nitrogenous sources (Bloom, 2015).

The various processes involving reaction that interconverts the N forms within plants and microorganisms are the energy intensive for instance (Guerrero MG, 1981):

$NO_3^- + NAD(P)H + H^+ \longrightarrow NO_2^- + NAD(P)^+ + H2O$ 34 $Kcalmol^{-1}$

Where NAD(P)H indicates either NADH or NADPH.

$NO_{2-} + 6Fd_{red} + 8H^+ \longrightarrow NH_4^+ + 6Fd_{ox} + 2H_2O$ 103.5 $Kcalmol^{-1}$

Where Fd is ferrodoxin and the subscripts red and ox stand for reduced and oxidized respectively.

$NH_4^+ + glutamate + ATP \longrightarrow Glutamine + ADP + P_i$ 4.3 $Kcalmol^{-1}$

$Glutamine + 2\text{-}oxoglutarae + (NADH + H^+ \text{ or } Fd_{red}) \longrightarrow 2glutamine + (NAD^+ \text{ or } Fdox)$
13.4 $Kcalmol^{-1}$

When assimilated into glutamine and glutamate, nitrogen is incorporated into other amino acids via transamination and thus incorporated nitrogen in amino acids is finally incorporated into other organic nitrogen compounds such as purines and pyrimidines (Bloom, 2015).

1.3 Nitrogen sources

Nitrogen required by the plants as primary nutrient is present in various forms in soil which limits plant productivity and frequently represents a major constraint to food production worldwide (Jones *et al.*, 2013). Higher plants utilizes organic form of nitrogen (N), ammonium ion (NH_4^+) and nitrate (NO_3^-) as nitrogen source in accordance with their relative availability in soil (Britto DT, 2013). Microorganism present in soil however also uses these forms of nitrogen making it difficult for the plants to compete with them in utilization of organic N (Nosholm *et al.* 2009, Kuzyakov *et al.*, 2013). Plants are proved to be incompetent and competition becomes fierce between plants and microorganism for adsorption of soil NH_4^+ because ammonium ion adsorbs onto cation exchange complex and because microorganisms uses NH_4^+ as energy source via nitrification (microbial conversion of NH_4^+ to NO_3^-) in addition to its utilization as nitrogen source. Hence NO_3^- has found to be major N source for most of the higher plants (Marshner H, Marshner P, 2012).

NO_3^- is very important N source for the plants not only for those growing in location where higher soil NO_3^- concentration but also for those growing in deficient and low concentrated environment. Even N fixation has been shown to be ceased in those plants with potential symbiotic N fixing capabilities when NO_3^- is present in rhizosphere (Cabeza R *et al*., 2014). Forest soil has NH_4^+ as the major N source which displays high rates of gross nitrification indicating small but ecologically important NO_3^- pool (Stark *et al*., 1997).

However large disproportionate relation exists in terms of energy and NO_3^- dependence. Large amount of energy is required to assimilate NO_3^- into organic N compound. It is evident plant expends about 25% of their total energy in shoots (Bloom *et al*., 1989) and roots (Bloom *et al*., 1992) for NO_3^- assimilation both day (Cousins *et al*., 2004) and night (Rubio-Asensio *et al*.,2015) but organic N compounds constitute less than 2 % of plant dry mass. Hence so as to extenuate the expenditure of the energy; plants show unique mechanism that other organisms are incapable of performing called photorespiration (Bloom, 2014).

In addition to using NO_3^- as N source for higher plants organic N, NH_4^+ are also utilized by them. Most organisms exhibit this behavior however microorganisms prefer organic N forms first followed by higher energy inorganic N compound NH_4^+ over NO_3^-. Further adsorption and assimilation of NO_3^- over organic N, NH_4^+ is exhibited only in absence of organic N form by bacteria, cyanobacteria, fungi and phytoplankton (Bloom, 2015).

1.4 Synthetic Nitrogen Fertilizers

To meet the nitrogen demand of the plants supplemental nitrogen (N) is employed in the form of fertilizers in agricultural field by the help of which plant grows faster or N enters natural ecosystem in the form of atmospheric deposition (Asagi *et al*., 2009). Development of synthetic fertilizer has revolutionized the agriculture increasing the crop yield and harvest in great extent. Application of synthetic chemical fertilizer had gradually become the main measure to improve the soil fertility. Among growth limiting factors application of N in the form of chemical fertilizer has visible effects on yield increase during long term cultivation compared with no N treatment (Geng *et al*., 2015).

Among the various fertilizers urea is the cheapest and most commonly used chemical fertilizer. Urea is alkaline hydrolyzing fertilizer which subsequently increases the pH of the soil zone of its contact and intensifies the denitrification process to release of large volume of N_2O along with NH_3 and NO_x (Mulveny *et al*., 1997). Having the large surface area, the usage of urea has arisen many problems because only 30-45% of the applied prilled urea is used by the plant while rest of the chemicals are lost in air or water contaminating these systems and affecting the both floral and faunal lives (Dong *et al*., 2012). Other many types of losses are also influenced by the method of application of such fertilizer. While deep placement of urea minimizes many problems such as inefficiencies, run off, volatilization losses and emission of nitrous oxide however the process is found to be labor intensive (Rahman *et al*., 2016).

Chemical fertilizer has widely and extensively been used which creates immediate nitrogen availability facilitating improvement in low soil fertility. However with long-term use of such fertilizers has resulted in soil acidification, poor soil aggregate stability and low levels of micronutrients (Wang et al., 2017). Long term use of nitrogen fertilizer (NPK) alone or in combination with organic manure markedly decreases the soil bulk density and increases total soil porosity and for prolonged carbon sequestration, usage of organic fertilizers are highly recommendable (Chaudhary, 2017).

Increase in N application in the form of synthetic fertilizer obviously increase the yield but it in long term it aggravates the environmental pollution through denitrification (N_2 and N_2O) and volatilization (NH_3) (Watanabe *et al*., 2009, Zhao *et al*., 2009). Gaseous N_2O, NH_3 and NO_x have adverse effects on environment and potentially contributing in global warming, stratospheric ozone layer depletion and acid deposition (Khalil, 2010).

Despite the fact that farmers typically prefers mineral fertilizers to organic amendments, the importance of using alternative to these fertilizers to improve crop quality and restore soil fertility is recognized (Biau *et al*., 2012; Ghosh *et al*., 2012).

Because of the increasing concern for global warming and environmental issues an alternative to these chemical fertilizers has to be sought after. More efficient, ecofriendly and economic alternative should be encouraged and should be practiced and promoted to increase soil organic carbon content which not only improves physical and chemical properties of soil but also helps in carbon sequestration.

1.5 An alternative to synthetic nitrogen fertilizers

To address the problem caused by synthetic fertilizers such as water eutrophication and toxicity, groundwater pollution, air pollution, soil quality degradation and even the eco system change; an alternative to these are being searched since long and still going on (Tilman *et al*.,2002; Khan *et al*. 2008). To maintain the sustainability of modern agriculture a new approach is being extensively researched (Wen *et al*. 2017).

Various strategies have exercised to increase efficiency of fertilizers so as to minimize and eliminate their negative impacts on the environment such as improving fertilizer, application methods (e.g.: use of split or localize application), precision fertilization, fertigation-fertilization via irrigation systems and the use of environmentally friendly fertilizers (Shaviv, 1993; Timilsena *et al*., 2015).

Environmentally friendly fertilizers (EFFs) improve nutrient efficiency and minimize leaching and volatilization losses of fertilizers and also reduce environmental hazards. They reduce environmental pollution resulting from nutrient losses by retarding or even controlling the release of nutrients to soil and often referred as enhanced efficiency fertilizers (Timilsena *et al*., 2015).

The other positive aspects of choosing these ecofriendly fertilizers include: decrease in NO_x emission, Increase in organic matter in soil, improvement in water retention and water holding capacity and adjustment of soil pH (Chen, 2017).

Thus usage of biofertilizers as green manure or organic fertilizer is one of the best alternatives to synthetic fertilizers. Application of organic fertilizers in soil initiates the mineralization process thereby producing the inorganic nitrogen which is to be absorbed by the plants (Liu *et al*., 2014). However rate of mineralization process are governed by multiple factors viz. soil properties, microorganisms, temperature, agricultural management, water content along with type of organic fertilizers used (Griffin, 2008, Dessureault-Rompre *et al*., 2010, Fan *et al*., 2010, Lobell, D.B, 2007).

Many different species of microorganisms has been used previously which had great biological N fixation (BNF) ability as biofertilizers or green manure and were more cost efficient. Because of their sustainable supplementation and associated improvement of soil fertility they are the best substitute for synthetic fertilizer. Practice of using N fixing microorganisms as biofertilizers is encouraged over synthetic one for net economic benefit to minimize N loss and especially NH_3 volatilization (Yao, 2018).

Many studies has revealed several secondary metabolites produced by endophytic bacteria are involved in a broad spectrum of roles which ranges from having biotechnological potential to producing antibiotics, acting as biological control agents against plant pathogens along with promoting plant growth (Penca *et al*., 2018).

Moreover one of the crucial element sulphur (S) involved in symbiotic nitrogen fixation (SNF), such as protein and protein cofactors, their need can be fulfilled by thiols synthesized in nodules contributing to S-economy of the whole plant. Hence N fixing nodules with N fixing bacteria are also the source of reduced –S, triggered global programming of sulfate uptake, reduction and assimilation at both the transcriptional and metabolite levels (Kalloniati *et al*., 2015).

Thus these various nitrogen fixing, plant growth promoting bacteria are definitely the biotechnological tool of the maximum potential to promote agricultural development in short term. Beside their nitrogen fixing ability, their production of phytohormones also promotes plant growth. Further, the intensity of plant growth promotion, including transfer of fixed nitrogen from these associative bacteria to the plant depends on effective interaction of the plant genotype and bacterial species (Souza *et al*., 2013).

The utilization of plant growth promoting or/and N fixing bacteria as an alternative to synthetic bacteria to improve productivity of important cereals along with addressing concerning environmental issues is a reality. Many promising number of researches has enhanced our understanding on the bacterial physiology and the mechanisms of bacteria-plant interaction. Among these bacteria *Azotobacter vinelandii* and *Pseudomonas fluorescens* are promising.

Azotobacter vinelandii in particular has characteristic properties that can overcome the obligation of low oxygen concentration environment for the function of nitrogenase. As a free bacterium it possess very much evolved enzymes and transporters to minimize the loss of fixed nitrogen to the surrounding environment making it best candidate for effective N fixing bacterium and its use in biofertilizers scheme (Barney *et al*., 2015).

As a part of optimized cultivation; application of plant growth promoting rhizobacterium (PGPR), *Pseudomonas fluorescens has* been practiced to improve yield and quality of the fruit. These beneficial biotic stimuli are also able to induce plant secondary metabolism using chemical elicitors. These PGPR are found to trigger systemic changes during the plant growth which results in increased crop fitness either by stimulating plant growth or by preventing disease (Kumar, 2012). Reliable modifications of secondary metabolism by PGPR provides a way to add nutritional value to crop and contribute to nutritional security and are of commercial interest (Gracia-Seco *et al*. 2015).

These both bacteria *Azotobacter vinelandii* and *Pseudomonas fluorescens* are amenable to genetic studies and manipulation, and much has been known about their nitrogen metabolism and regulation of nitrogen fixation. Many researches and studies have allowed progress in understanding of molecular aspect of plant-bacterium interaction. The knowledge gathered and information available for these bacteria and their systems permits the genetic manipulation and further study to maximize the benefits of plant growth promoting bacteria.

1.6 Polymers and their alternative

Human life today without a polymer is unimaginable and both natural and synthetic polymers are directly involved in comfort and facilitation of human life and are responsible for life itself. From commodities used in daily basis to medication, nutrition, communication, transportation, irrigation, buildings, clothing and highways polymers are used almost everywhere and play essential and ubiquitous role (Namazi, 2017).

The polymer is simply a macromolecule with high molecular weight between 10000 - 1000000 g/mol that consist of several structural units usually bound together by covalent bond (Elias, 1997). Polymers are obtained through chemical reaction of monomers that has ability to react with another molecule to form the same type or another type in the suitable condition to form the polymer chain. The process occurring naturally has resulted in natural polymers (cellulose, starch, and natural rubber) while synthetic polymers are artificial (Namazi, 2017).

These synthetic plastic though improves quality of human life replacing commodity like glasses or paper and has comprehensive features such as strength, lightness and durability, the usage of it is problematic for its accumulation as waste is creating a global environmental issue and has resulted in a significant burden on solid waste management. Further, rapid depletion of fossil fuels has prompted in developing alternative approach that will address unceasing human demands for polymers which will be more ecofriendly (Możejko-Ciesielska and Kiewisz, 2016). Hence bioplastics and biocomposites production of biopolymers are the green alternative and potential substitute for conventional non-degradable plastics.

Bioplastics or biopolymers are simply materials that degrade into carbon dioxide, water and biomass by the action of living organisms or enzymes. These materials when discarded in soil and landfills are usually acted upon by microbes such as bacteria, fungi, algae generating CO_2, CH_4, cellular components and other products, as stipulated in "American Standard for Testing and Methods" (ASTM D-833) (Silva and Garcia-Cruz; 2014).

Among the various groups of biopolymers, polyhydroxyalkanoates (PHAs) which are the polyesters of hydroxyalkanoates (PHAs); are the most well-known and are synthesized by numerous bacteria as carbon and energy storage compounds. These are of particular interest because they possess thermoplastic characteristics and resemble synthetic polymers to a larger extent (Nehra *et al.*, 2015). Compared to “so-called” biodegradable plastics made synthetically plastics produced from PHAs have been reported to be truly biodegradable in both aerobic and anaerobic environments (Page, 1995). Belonging to the class of PHAs Poly-beta hydroxybutyric acid (PHB) is the most popular and the best characterized polymer synthesized by microbes under unfavorable conditions such as limitation of some essential nutrients or excess availability of carbon source and they serve as energy reserve materials in these microbes similar to fat in humans or starch in plants (Lee, 1995).

1.7 Poly-β-Hydroxybutyrate (PHB) and its production by bacteria

Poly- β -hydroxybutyrate (PHB) is polymer of industrial importance and is formed by the linear polymerization of 3-hydroxyacid monomer units. This polymer so far has found to contain properties like biodegradability, thermoplasticity along with other traits similar to petrochemical plastics of recent usage (Akarraonye *et al*., 2010). The properties of pure PHB proportionate to greater extent with commonly used bulk plastic for instance polypropylene. This natural biopolymer is unique in the sense that it incorporates three exception features as: (i) thermoplastic processability; (ii) 100% resistance to water and moisture; and (iii) 100% biodegradability (Díaz-Barrera *et al*., 2016). Therefore it allows its applications as potential green alternative that possess the qualities of common plastics and aids in new waste management strategies thereby addressing the global environmental concern.

The various taxonomically different strains of microbes have been found to synthesize PHB and microbes such as *Azotobacter, Bacillus, Pseudomonas, Rhizobium, Methylotroph* are able to generate PHB up to 30 – 80% of their dry cell weight (Lafferty *et al*., 1988). In large number of plants rhizospheric soil layer is colonized by numerous bacterial and many of them are able to accumulate polyhydroxybutyrate as energy and carbon sources, which make it a source to isolate PHB producers (Nehra *et al*., 2015).

PHB accumulation in microbes is a natural system of storing energy and carbon in response to the environmental conditions or physiological stress, usually when nutrient supplies like nitrogen or phosphate are imbalanced (Díaz-Barrera *et al*., 2016). They are biosynthesized under conditions of carbon excess and limiting concentration of essential growth nutrients while those collected biopolymers are depolymerized when the carbon source is exhausted, degradation products can be used as a source of carbon and energy materials (Możejko-Ciesielska and Kiewisz, 2016). For many of the PHB producers a condition of nitrogen limitation is key factor for PHB accumulation but for others such as *Azotobacte*r genus, oxygen limitation is a key factor in polymer production.

Azotobacter vinelandii synthesizes three molecules of important biotechnological and biochemical applications one of them being PHB while other two are the extracellular polysaccharide alginate, sidephores compounds which is responsible for the mucoid phenotype of the strain. (R. El-Shanshoury *et al*., 2013). Beside PHB it also produces polymers of industrial importance: the alkylresorcinols 5-n-heneicosylresorcinol, 5-n-tricosylresorcinol, and their galactoside derivatives and these compounds are related to the morphological differentiation through which it transforms into desiccation-resistant cysts (Segura, 2003).

1.8 Rationale of the study

A biofertilizer is better replacement of synthetic fertilizer to meet the supplemental nitrogen demand of plants. To address the exigent dispute of water eutrophication and toxicity, groundwater pollution, air pollution, soil quality degradation and even the eco system change; a pursuit of cost effective and ecofriendly biofertilizer is of immediate concern.

Further, compounds which are accumulated in bacteria that functions primarily as energy reserves has wider scope for its utility in polymer science. The compounds such as glycogen and polyhydroxybutyrate are accumulated particularly in the stationary phase when growth is limited by a deficiency of some factor other than the carbon and energy source. The majority of compounds accumulated as carbon reserves also serves as alternative reserves depending upon the bacterial species and upon the nature of the carbon and energy source in the environment (Wilkinson, 2017). Those most commonly implicated as specialized carbon and energy reserve, particularly, polyhydroxybutyrate (PHB) has spacious purview in terms of applications.

Polymer science that encompasses researches in multiple disciplines including chemistry, physics, and engineering is indeed most rapidly increasing and imperative field that is earnestly looking for the biodegradable polymers. The pervasive use of the polymers, without which life seems impossible has definitely prompted the revelation of sources through which these biodegradable polymers could be obtained. The usage in wide range, from routine commodities to medication, nutrition, communication, transportation, irrigation, buildings, clothing and highways, polymers are used almost everywhere and play essential and ubiquitous role (Namazi, 2017). Hence, an attempt was made to reveal the abilities of some strains to produce these reserves, PHB. The study solely aims to couple two of the most decisive areas: agriculture and polymer industries.

1.9 Objectives

1.9.1 General Objectives

To study the nitrogen fixing biofertilizers for its carbon storage capabilities.

1.9.2 Specific Objectives

1. To isolate the bacteria: *Azotobacter vinelandii* and *Pseudomonas fluorescens*; from soil sample.
2. To subject isolated bacteria to biochemical tests for preliminary identification.
3. To perform molecular characterization, PCR followed by gene sequencing for confirmation of isolated bacteria.
4. To detect the ability of isolated bacteria to fix the atmospheric nitrogen using Nessler’s method and to quantify the fixed form of nitrogen, ammonium, spectrophotometrically.
5. To inspect the PHB producing abilities of isolated bacteria using Sudan Black and to further quantify the PHB produced, via flow cytometry using fluorescent dye, Nile red.

1.10 Research hypothesis

1.10.1 Null hypothesis

The isolated bacteria will not be able to exhibit nitrogen fixing ability and will not store the polyhydroxybutyrate (PHB) as carbon source.

1.10.2 Alternative hypothesis

The isolated bacteria will exhibit nitrogen fixing ability and store the polyhydroxybutyrate (PHB) as carbon source.

CHAPTER- 2

LITERATURE REVIEW

2.1 Background

Ever increasing global population with its rising food demand requires fulfillment of food security thus highlighting the significance and gravity of agriculture sector. Recent studies has shown that about a billion people are devoid of adequate amount of food and nutrition worldwide and the agricultural production has to be doubled so as to cater the needs of expected 9 billion people in coming 2050 AD. Currently, more than 870 million people are chronically starving imposing a challenge on agricultural production. Hence, to address the production shortfalls; our concern should be focused on devising a new tool, technology, or to adopt new strategies to increase production sustainably maintaining at the same time, environmental sustainability and economic opportunity.

The Green Revolution (GR) technology adoption increased wide varieties of crop yield per hector increasing 12-13% food supply between 1960 to 2000. Later, crop genetic improvement mainly focused on producing high-yielding varieties that resulted in early maturity. However, an alternative improved inputs like fertilizers, irrigation and pesticides were also critical part of GR intervention. The utilization of chemical fertilizers and pesticides to increase the production during the GR periods had adverse effect on environment later on by affecting soil fertility, water hardness, development of insect resistance, increasing toxic residue in food chain and animal feed etc. This environmental concern had led today’s agricultural trend towards organic approaches. Hence addressing this complex challenge of today’s world, it’s crucial to harness the best of scientific knowledge and technological breakthrough. Therefore biofertilizers and biopolymers are today’s green alternatives (Raja, 2013).

2.2 Biofertilizer and Biofertilizer strains

Biofertilizers are the preparations containing active or latent strains of soil microorganism, either bacteria alone or in combination with algae or fungi that increases the plant availability and uptake of mineral nutrients by their interactions in the rhizosphere when applied through seed or soil (Paul *et al.*, 2013). Generally they contain free-living microorganism associated with root surface but they can also be endophytes which are microbes with the ability to colonize the intracellular or even extracellular spaces of plant tissues without causing the damage to the host plant. Biofertilizers are considered one of the major components of integrated nutrient management for being cost effective and renewable source of plant nutrients to supplement and/or replace the chemical fertilizers so as to maintain sustainable agriculture. They are known stimulate certain microbial processes in the soil that reinforce the extent of availability of nutrients in a form easily assimilated by plants.

Biofertilizers are found to boost the nutrient uptake and quality attributes with the reduction in inorganic fertilizer dose and raising the economy associated with agriculture (Talwar and Singh, 2017).

Because of such beneficial effects a wide variety of plant growth promoting rhizobacteria (PGPR) has been identified and well characterized. Davidson reviewed molecular biology and plant genetics of plant growth promoting bacteria which benefited the plant directly or indirectly by antagonizing a phytopathogen or removing a growth inhibitor.

2.3 Rhizosphere and Rhizospheric Bacteria

The volume of soil directly influenced by the presence of roots of living plant or the compartment of soil influenced by the root is termed as rhizosphere (Kumar *et al*., 2012). Lorez Hiltner in 1904 first introduced the concept of rhizosphere where soil environment attached to the roots is considered as the range of microbial abundance and activity due to the presence of root exudates and rhizodeposits (Hiltner, 1904). In more simplified notion, rhizosphere is a thin layer of soil region in the vicinity of plant roots which is very significant and active as this region provides the area for intense microbial activities, root activities and metabolism (Shanmugam *et al*., 2015).

Rhizosphere is furnished with massive viable microbial population that can exert beneficial, neutral to detrimental effects on the host plants. Such root colonizing bacteria or rhizobacteria that exerts beneficial effects on the growth of the host plant via direct and indirect mechanisms are plant growth promoting rhizobacteria (Juanda, 2005). This plant microbe interaction in the rhizosphere is the key for enhancing the plant health and soil fertility (Khan, 2006).

PGPR strains use one or more direct or indirect mechanism for plant growth and these mechanisms can be active simultaneously or independently at different stages of such plant growth (Ahmad *et al*., 2008). The direct mode of enhancing the plant growth by PGPR includes: fixation of atmospheric nitrogen, solublization of minerals such as phosphorus, production of siderophores, and synthesis of plant growth hormones viz. indole-3-acetic acid (IAA), gibberellic acid, cytokinins, and ethylene (Nelson, 2004). On the other hand indirect mode of action comprises of biological control of plant pathogens and deleterious microbes through the production of antibiotics, lytic enzymes, hydrogen cyanide, catalase and siderophores or through competition for nutrients and space promoting plant health and growth indicated by increased seedling emergence, vigor and yield (Khan, 2006).

The microorganisms being commonly used as biofertilizers are nitrogen fixing soil bacteria (*Rhizobium, Azotobacter*), nitrogen-fixing cyanobacteria (*Anabaena*), and Phosphate solubilizing bacteria (*Pseudomonas putida*) and arbuscular mycorrhizal fungi (Brar *et al*., 2012).

The finest and familiar PGPR are biological nitrogen fixers (BNF) which directly obtains N from the atmosphere and convert them into organic forms; readily useable by plants. These include the members of the genus *Rhizobium, Azospirillum* and blue green algae. Rhizobia are symbiotically associated with legumes and nitrogen fixation occurs at the site where bacterium resides i.e. roots and nodules (Cruz, 1993). The genus *Azospirillum* is rhizobacteria associated with monocots and dicots such as grasses, wheat, maize and *Brassica chinesis* and has many N-fixing species (Bashan *et al*. 1990, Singh *et al*. 2011). *Azospirillum* strains have been isolated repeatedly from rice and has also been sequenced for the strain *Azospirillum* sp. B510 (Razie *et al*., 2008; Kaneko *et al*., 2010).

The appreciable N fixation has been reported in *Azotobacter* spp. and *Azospirillum* spp. in the rice crop rhizosphere (Sison, 1999). However, Okon and Labandera-Gonzales established the fact that main effect of *Azospirillum* spp. is the stimulation of the density and length of root hairs, the rate of appearance of later roots, and the root surface area triggered by phytohormones (Okon and Labendera-Gonzales, 1994).

After nitrogen fixation, phosphate solubilization is crucial for plant growth as large proportion of soluble inorganic phosphate added to the soil is fixed as insoluble forms soon after their application and becomes unavailable for the plant (Rodriquez and Fraga, 1999). Several soil bacteria strains specifically belonging to genera *Bacillus* and *Pseudomonas* hold competency in transforming the insoluble forms to soluble form by secreting organic acids such as formic acid, acidic propionic, lactic, glycolic, fumaric and succinic acids (Vazquez *et al*., 2000).

The other decisive genus PGPR is *Trichoderma,* a common fungus present in nearly all soils. They are known to enhance plant growth and crop yield by attacking plant pathogens, parasitizing other fungi and hastening the mineralization of organic matter thus releasing nutrients from soil organic materials (Harman, 2000; Yedidia *et al*., 2001, Cuevas VC, 1991).

Based on these literatures, it can be assumed that biofertilizers could offer an opportunity for increasing yields, productivity and resource use efficiency. So keeping all this in view, the present study focuses on specifically 2 of the important plant growth promoting strains from the rhizospheric soils viz. *Azotobacter vinelandii* and *Pseudomonas florescence* .

The basis of selection for these strains lays on the fact that former along with the nitrogen fixing properties, mineral solubilization and phytohormones production also shows reduction in heavy metal toxicity while later species removes deleterious microorganisms and chemicals from environment by antagonism of phytopathogens It has well known fact that Pseudomonas are capable of producing antibiotics along with production of fluorescent siderophores and hence their name, which contribute to the fact that they possess high affinity to Fe^{+++} that specifically enhance their acquisition of iron by binding to membrane bound siderophores receptors. This highly efficient metal chelation mechanism that scavenges iron creating an iron deficient environment deleterious to fungal growth is very helpful for plant health and growth (Davison, 1998).

Hence these studied genre, *Azotobacter* spp. and *Pseudomonas* spp. represent a group of bacteria that can exert positive effects on the stimulation and plant growth promotion in multiple crops and additionally possess the capacity of asymbiotic nitrogen fixation along with biological control of pathogens.

2.4 *Azotobacter* species

2.4.1 Description and significance

Azotobacter is a genus of free-living diazotrophic bacteria whose resting stage is cyst. The genus *Azotobacter* stands out from the other genera of the family (*Azomonas, Beijerinckia* and *Derixa*) by the ability of its members to from metabolically dormant cysts after exponential growth or upon induction of vegetative cells with specific reagents (Lin *et al*., 1968).

Principally found in neutral to alkaline soils in aquatic environment, can also be found on some plants. It has several metabolic potentials, including atmospheric nitrogen fixation by conversion to ammonia. Their unique system of three distinct nitrogenase enzymes makes these bacteria of particular interest for better understanding of nitrogen fixation and its role as biofertilizers. Azotobacter spp. has found to possess highest metabolic rate of any organisms.

Although *A. chroococcum* is considered the genus type species, the majority of the studies in this genus have been carried out on *A. vinelandii* besides *A. beijernckii* and *A. paspali* ,the other, species of the genus (Sadoff, 1975).

Along with nitrogen fixation *A. vinelandii* are found to accumulate polyhydroxybutyrate (PHB) in vegetative cells (Stevenson *et al*., 1966) depending on the carbon source and its concentration or other culture conditions (Senior *et al*., 1972; Stockdale *et al*., 1968), which is industrially important. It also has characteristics such as production of plant growth hormones and antibiotics (Gonzalez-Lopez *et al*., 1986) as well as additional industrially important substances like extracellular polysaccharide (EPS) alignate and siderophores compounds (Diaz-Barrera *et al*., 2010),

2.4.2 Cell structure and metabolism

Azotobacters cells are gram negative, predominantly bluntly large rod shaped to oval, at least 2 microns in diameter. They can live singly, in chains, or in clumps, and may or may not be mobile by flagella. Their resting stage is spent as a thick-walled cyst, which protects the organism from harsh climates (Rodelas *et al*.,1999) .

A. vinelandii cells are however prolate spheroids measuring approximately 2 × 5 μm and thus comprising of the volume at least 10 times larger compared to *Escherichia coli* cells. It is an obligate aerobe which grows with a generation time of 2.5 to 3 hours in Burk's nitrogen free buffer (Wilson *et al*., 1952). *A. vinelandii* cultures are found to grow on mannitol, rhamnose and other carbohydrates, as well as variety of other organic acids and certain alcohols (Lin *et al*., 1968) however when cells are grown on plates of Burk's N-free agar containing sublethal levels (0.2%) of n- butanol, growth is slow but complete encystment occurs in 120 hours (Tichan *et al*., 1962; Winogradsky, 1938; Wyss *et al*., 1961).

Azotobacter spps. are of great interest for their unique mode of metabolism by which they can fix nitrogen aerobically. The unique and prominent ability of high respiration rates by the cell allow the normally oxygen-sensitive nitrogenase to experience limited oxygen exposure. They are also capable of producing a protein that protects the nitrogenase for sudden oxygen-provoked stress. The other individualistic trait of Azotobacter is its ability to synthesize not just one, but three nitrogenase. Further, PHB is a polymeric form of β-hydroxybutyrate (BHB) that accumulates during vegetative growth while BHB is a normal metabolite of *A. vinelandii.* Glucose metabolism is thought to be regulated by PHB by reductions in the inhibitory levels of reduced pyridine nucleotide cofactors (senior *et al*., 1971).

2.4.3 Genome structure

Genetic analysis and manipulation is a powerful tool for better understanding of various cellular functions and metabolisms. *A. vinelandii* contains approximately 10 times as much DNA per cell as E. coli however because of its larger size; the DNA to protein ratio in both the organisms remains almost the same. Early exponential phase A. vinelandii cells contain four nucleoids as seen in nuclear staining (Pochon *et al*., 1948) but this number reduces to one at the time of last cell division during the early stages of encystment (Sadoff, 1971).

Guanine and cytosine (G+C) content of 65% is found in highly purified DNA of *A. vinelandii* and is homogeneous in CsCl density gradients prepared in an analytical centrifuge.However, material has a bimodal distribution of G+C content with one component (30 to 40% of the total) possessing a T_m (temperature at which 50% of a given class of DNA has melted) 5 C lower than the other as reveled by the analysis of a large number of DNA melting studies. The average G+C determined by this procedure is also 65% (Sadoff, 1975). The bouyant density and melting data suggest that the DNA is a heteropolymer of a single density class with a sufficiently large number of G+C regions to affect its melting characteristics. The existence of such regions has been suggested from the results of studies of partial hydrolysis of *A. vinelandii* DNA (Khan *et al*., 1972).

The DNA of *Azotobacter spp.* display many similarities, in terms of gene type and recognition factors, to the DNA of Escherichia coli. Genetic information can be transferred between Azotobacters or to other bacteria by way of conjugation or transformation. The renaturation of purified, sheared, and denaturated DNA extracted from a given prokaryote would be expected to occur with second order kinetics because the chromosome of each species is, essentially, a unique DNA sequence (Britten *et al*., 1966; Britten *et al*., 1968).

The principal difficulty in conducting genetic studies in *A. vinelandii* is the production and selection of mutants by those various techniques applicable to *E. coli, B. subtilis*, and a wide variety of other organisms. The difficulty in such studies is thought to be due to the organism's possessing an unusual DNA repair apparatus, a unique segregation system, an unusual nuclear complement, or more than one of these properties.

2.5 *Pseudomonas* species

2.5.1 Description and significance

Pseudomonas bacteria with tolerance of a variety of physical conditions are most commonly found in soil, marshes, coastal marine habitats and plant and animal tissues. *P. fluorescens* is commonly found soil bacteria and abundant operational taxonomic units (OTUs) in rhizosphere samples (Gottel *et al*., 2011; Brown *et al*., 2012). The genus *Pseudomonas* contains more than 140 species and most of them are found to be saprophytic. With different species of this bacteria behaving as opportunistic pathogens; endotoxin acts as major virulence factor in bacteremia and septic shock (Iglewski BH, 1996).

P. fluorescens having number of encoded antimicrobial mechanism plays a vital role in promoting plant health and has widely been studied as an environmental microbe (Silby *et al*., 2009; Feazel *et al*., 2009; Hass *et al*., 2005). It also possesses a number of functional traits that facilitates in production of bioactive secondary metabolites, siderophores, and a type III secretion system, the ability to form biofilms and the plasticity of some strains to adopt to growth at higher temperatures (Laursen *et al*., 2004, Weller *et al*., 2007; Mavrodi *et al*., 2006). Members of the *P. fluorescens* species are successful colonizers in a wide range of environments and habitats due to diverse functional abilities. Functional range and environmental niches of the the *Pseudomonas* genus include plant interactions through plant growth promotion and plant diseases resistance, colonization via production of biofilms, host defenses, antibiotic production and resistance, microbial competition through biocontrol, production of novel secondary metabolites, bioremediation and control of pollutants and environmental protection via UV, desiccation etc (Silby *et al*., 2011).

2.5.2 Cell structure and metabolism

Pseudomonas species are Gram-negative, aerobic bacilli with certain strains being capable of using nitrate instead of oxygen as final electron accepter during cellular respiration and are motile rods measuring 0.5 to 0.8, µm by 1.5 to 3.0 µm. It has multiple flagella however; single polar flagellum aids the motility for the bacterium. Species are distinguished by biochemical and DNA hybridization tests. Antisera to lipopolysaccharide and outer membrane proteins show cross-reactivity among serovars(Iglewski BH, 1996). *P. fluorescens* are unable to ferment glucose and are chemoorganotrophic and grow at a pH between 4 and 8. Isolates of *P. fluorescens* from non-mammalian samples allows the growth in the temperature range of 4-32^{0}C (Moore *et al*., 2006) while those isolated from mammals and humans has elevated growth range upto 37^{0}C (Donnarumma *et al*., 2010; Sperandio *et al*., 2012).

P. fluorescens can also be found in an antagonistic relationship with eukaryotic microbes that includes oomycetes and amoeba where antagonistic relationship with amoeba potentially reflects conserved mechanism that are also used with macrophages as has been hypothesized for other bacteria (Cosson *et al*., 2008). *P. fluorescens* grows in basic media that are rich in peptide containing medium with 0.1 to 1.0% (wt/vol) energy source for instance nutrient broth/agar and tryptic soy broth/agar where other members of *Pseudomonas* genus also shows optimum growth (Moore *et al*., 2006). Increased production of fluorescent siderophores can achieved in selective media also called pigment enhancing media such as King's A and B media , Pseudosel agar medium and Pseudomonas agar F medium with deficient iron content which allows for detection of natural fluorescence (King *et al*., 1954). These media are found to contain additional compounds such as potassium, magnesium, and/or cetrimide that further enable selective growth of *P. fluorescens* and cetrimide in particular helps to inhibit the growth of non-*Pseudomonas* microbial flora (Lowbury *et al*., 1955).

Despite the difficulty in isolation of particular species of the *Pseudomonas* genus because of their similar phenotypic traits and similar growth conditions, pigment production can be utilized to achieve the discrimination against isolates from different groups for this is specific trait and varies by species groups as for example: the blue-green pyocyanin , readily produced by *P. aeruginosa* strains is typically not produced by strains of the *P. fluorescens* species.The environmental isolates of *P. fluorescens* are readily cultivated in laboratory by use of standard culturing techniques at a lower temperature range 5^0C to 32^0C using a simple medium with a carbon source and aerobic incubation for 24 to 48 hours (Moore *et al*., 2006).

The ability of *P. fluorescens* to become viable but not culturable (VBNC) could explain the phenomenon in which *P. fluorescens* can be found more frequently in human lung metagenomic DNA and VBNC state is hypothesized to be a survival strategy that allows bacteria to prersist in harsh environment (Oliver, 2005).

2.5.3 Genome structure

Isolates classified as *P. fluorescens* has undergone extensive renaming and reorganization, consistent with the high degree of genomic diversity within the species in past few decades (Peix *et al*., 2009)). Exploitation of these bacteria requires an improved understanding of the determinants of ecological performance, specifically persistence and in vivo expression technology (IVET) promoter trapping strategies were devised and implemented to identify the plant and soil induced genes (Silby *et al*., 2009).

Multiple conserved genes or housekeeping genes measures the evolutionary distance between the species or strains and are necessary for the basic functions of the cell and are obligatory in all bacterial cells under comparisons (Byun *et al*., 1999; Savli *et al*., 2003; Dagerhamn *et al*., 2008). Multilocus sequence typing (MLST) and multilocus sequence analysis (MLSA) are the techniques based on the concepts of genetic evolution at multiple conserved genes “housekeeping genes” (Chan *et al*., 2001; Urwin *et al*., 2003; Sullivan *et al*., 2005). Housekeeping genes that are used in classifying Pseudomonas species include rpoD (α-subunit of RNA polymerase), rpoB (β-subunit of RNA polymerase), and gyrB (β-subunit of gyrase) responsible for negative super coiling of DNA during replication (Tayeb *et al*., 2008; Adekambi *et al*., 2009; Yamamoto *et al*., 2000). Members of the *Pseudomonas* genus were identified with the combination of these three housekeeping genes and the 16S rRNA that generated the phylogenetic tree (Anzai *et al*., 2000; Mulet *et al*., 2010). This analysis helped in stating the fact that *P. fluorescens* contained the widest range of genetic diversity (Mulet *et al*., 2010).

Total number of genes found across all strains is called pan-genome and study of this size of this pan-genome could be one of the approaches for studying the level of genetic diversity between *P. fluorescens* strains. Pan-genome of *P. fluorescens* bacteria contained 13,782 genes which is much larger compared to pan genome of *P. aeruginosa* which has 7,824 genes. While performing all-against-all comparison of 14 *Pseudomonas* genomes across multiple species using reciprocal FASTA approach a "core genome" of 2,789 genes was there with the *P. fluorescens* species however only 20 were unique to the species itself within the *Pseudomonas* genus and those encode protens involved in regulation, biofilm formation or unknown functions (Loper *et al*., 2012).

The level of genetic similarities between the strains is higher with in each clade of *P. fluorescens* species with 4,188, 3,729 and 3,893 shared conserved domains between the members of clades 1, 2 and 3 respectively (Loper *et al*., 2012). The clad designation also provides information about functional differences between clusters of *P. fluorescens* including the presence/absence and type of type III secretion (T3SS) which is a molecular "needle" complex utilized by bacteria to inject bacterial proteins into host cells (Salmond *et al*., 1993 and Loper et la, 2012). The genes for a T3SS are found only in clades 2 and 3, not in clade 1, while biosynthesis gene cluster to produce hydrogen cyanide, a volatile molecule used to kill off competing bacteria is found only in clades 1 and 2 (Ramette *et al*., 2003; Gross *et al*., 2009; Loper *et al*., 2012). Genes found in every clade, such as those for the siderophore pyoverdine; reflect functional categories that are generally preserved across the Pseudomonas genus as a whole (Loper *et al*., 2012).

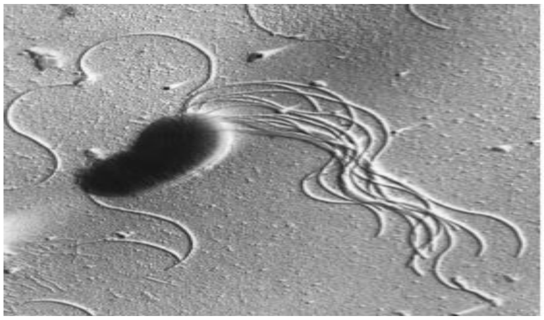


Fig 1: Scanning electron micrograph of *P. fluorescens* (Scales *et al*., 2014).

Plant growth promotion by *P. fluorescens* is exhibited via molecular mechanism that include export of organic acids and siderophores that facilitate dissolution of P in soils (Kurek *et al*., 2013, Giles *et al*., 2014, Pastor *et al*., 2014) and secretion of phosphatases and phytases that mineralize organic P in the plant rhizosphere (Giles *et al*., 2014, Pastor *et al*., 2014). *Pseudomonas* strains exhibit nutrient environment dependent plant growth promotion effects and this strain specific effect on plants can be attributed to the differences in

Pseudomonas transportomes (Silby *et al*. 2009, Larsen *et al*., 2015). Transportome is relative capacity to transport specific substances across the cell membrane as a function of the set of transmembrane transporters in the genome. These transportomic capacities of *Pseudomonas* strains and their correlation with measured phenotypic and biochemical parameters would highlight potential molecular mechanisms underlying the rhizosphere community's specific contributions to plant's acclimation to stress, C sequestration capacity and the maintenance of productivity under suboptimal conditions (Shinde *et al*., 2017).

2.6 Nitrogen fixation

The growth of all organisms including plants heavily depends on the availability of minerals and one of such chief nutrient is nitrogen which is required in large excess as an essential component of many cellular constituents such as proteins and nucleic acids and is required in plant development and limiting factor in plant growth (Santi *et al.*, 2013). There is an abundant supply of nitrogen in earth's atmosphere in the form of dinitrogen gas that makes up about 80% of atmosphere however plants are unable to directly access N_2 for it has triple bond between the two combining nitrogen atoms that requires the large amount of energy for breakdown of the bond making it inert.

Plants absorb the available nitrogen in the soil through their roots in the form of ammonium and nitrates. Nitrogen must be "fixed" or combined in the form of ammonium (NH_4) or nitrate (NO_3) ions before it could be used for growth of the plant. The weathering of rocks releases these ions so slowly that it has a negligible effect on the availability of fixed nitrogen. The limited bioavailability of nitrogen and the dependence of crop growth on this element have generated the demand for N-based fertilizers (Dobermann, 2007; Westhoff, 2009). Since large portion of the N-based fertilizers applied to soils remains unused by the plants because of the inefficient application, it has generated the nitrate contamination of soils and ground water which in turn has generated health hazards and compromising agricultural sustainability. Further, manufacturing of N fertilizers are more energy consuming compared to either P or K fertilizers requiring more than 6 times the energy than required by the either of later ones (Da Silva *et al*., 1978).

Hence microorganism have central role in almost all aspects of nitrogen availability and thus for life support on earth. A comparatively very small amount of ammonia is produced by lightning and industrially it is produced by the Haber-Bosch process, using iron-based catalyst at very high pressure followed by fairly high temperature but major conversion of N_2 into ammonia and hence into proteins is achieved by microbes in the process called nitrogen fixation.

A process of conversion of atmospheric N_2 to NH_3, a form that can be readily utilized by plants is called biological nitrogen fixation (BNF) which can be performed only by some prokaryotes (Lam *et al*. 1996; Franche *et al*., 2009). Those bacteria that have the ability to fix the atmospheric nitrogen are called diazotrophs and they encode nitrogenase enzyme, that catalyses the conversion of N_2 gas to ammonia. The nitrogenase complex is highly conserved in free-living and symbiotic diazotrophs (Santi *et al*., 2013).

2.7 Nitrogenase

Biological nitrogen fixation where conversion of N_2 to NH_3 occurs is an essential process in the global nitrogen cycle which is crucial in sustaining the life on earth (Smil, 2001). Nitrogen fixation has always been considered fundamentally important for the multiple reasons that include its significance in global nutrition along with relevance of nitrogenase as model system for examining processes such as multiple electron oxidation reduction reactions, complex biological metal assembly, and even nucleotide-dependent signal transduction (Peters and Szilagyi, 2006). By this process major portion of metabolically accessible nitrogen enters the biosphere, and this whole process is activated in presence of enzyme called “nitrogenases”. These metal based catalysts utilize high energy input in the form of ATP, but functions at room temperature and atmospheric pressure (Burgess *et al*., 1996). Nitrogenases are exclusively produced by microbes and three major families have been identified and four types of nitrogenases are known. These are mechanistically related; however their catalytic components are genetically distinct and are unique for their composition of active site metalloclusters (Eady, 1996; Ribbe *et al*., 1997).Amidst, Mo-dependent nitrogenase (FeMo cofactor) is most abundant and extensively studied (Burgess *et al*., 1996; Rees *et al*., 2000). The FeMo cofactor comprises of molybdenum (Mo) in addition to iron (Fe), sulfur (S), R-homocitrate, and a light atom of unknown identity called “X” (Einsle *et al*., 2002).

Molybdenum nitrogenase contains two components or in other words requires the participation of two separately purified protein partners which are known as Fe protein or nitrogen reductase and the MoFe protein or dinitrogenase, which under the ideal condition catalyzes the following reaction whose limiting stoichiometry can be represented by the chemical equation:

$$N_2 + 8e^- + 16MgATP + 8H^+ \longrightarrow 2NH_3 + H_2 + 16MgADP + 16Pi$$

The Fe protein or dinitrogen reductase or component II is 60kD homodimer that contains a single bridging $[4Fe\text{-}4S]^{2+/1+}$ cubane cluster and two MgATP binding sites and involved in MgATP hydrolysis and electron transfer to the substrate reduction component, 230 KD MoFe protein heterotetramer. This MoFe protein contains two types of metal clusters: The

P clustures and the FeMo-cofactors (FeMo-cos). FeMo cofactor bound in the MoFe protein having composition [7Fe-Mo9S-homocitrate-X] is the site of substrate binding and reduction in nitrogenase (Benton and Peters, 2004; Tezcan *et al*., 2005).

High resolution structures of the Fe proteins, the MoFe protein and the Fe potein-MoFe protein complex has resolved many issues and allowed examination of fundamental aspects of nitrogenase mechanism that includes way of MgATP binding and established the fact that hydrolysis is responsible for intercomponent electron transfer. However, determination of location and process of substrate binding to the FeMo cofactor during nitrogenase catalysis is still ambiguous (Seefeldt *et al*., 2004). Several complicating features of nitrogenase are hindering the approach of understanding interaction of N_2 with nitrogenase active site that requires N_2 or some partially reduced form of N_2 trapped at the active site of FeMo cofactor at a sufficient concentration and in a state that is responsive to structural or biophysical examination. Consequently, all methods of characterization of N_2 or intermediate-bound states have utilized freeze or chemical quenching of nitrogenase during turnover. Major drawback of this approach is relatively low concentration of trapped states because of the many possible states that are in equilibrium. Therefore substrate binding mechanism is combination of different indirect approaches that includes:

1. Synthesis and reactivity of FeMo cofactor model compounds.

2. Development of theoretical models based on the structure of the FeMo cofactor

3. Examination of the effect on catalytic activity elicited by amino acid substitution or by modification of the composition of the FeMo cofactor

4. Examination of interaction of alternative substrates and inhibitors with the FeMo cofactor using kinetic and spectroscopic methods

These approaches have ultimately given rise to development of two different views of substrate binding and the catalytic mechanism of the nitrogenase. In first view, mechanism involves direct participation of Mo and (R) - homocitrate while in other binding occurs at a [4fe-4s] face of the FeMo cofactor (Seefeldt *et al*., 2004).

In order to bind N_2 by MoFe protein; a condition called “turnover” is essential in presence of electron donor Fe protein and MgATP for it cannot bind N_2 at resting state and initiates only after three or more electrons have accumulated within the MoFe protein (Thorneley *et al*., 1985). During turnover, nucleotide bound Fe protein which is reduced form, docks with the MoFe protein. This phenomenon induces MgATP hydrolysis thereby transferring one electron and eventually two components of protein dissociate (Howard and Rees, 1994; Seefeldt *et al*., 1997). This cycle needs to repeat itself for multiple times with consecutive Fe protein-MoFe protein association and dissociation during catalysis as multiple electrons are required for substrate reduction and MoFe protein must have ability to sustain number of levels of reduction.

The MoFe protein is heterotetramer ($\alpha_2\beta_2$), where each αβ unit contains two novel metalloclusters involved in electron transfer and substrate binding and acts as catalytic unit (Kim *et al*., 1992). Among two one is termed as P cluster which is [8Fe-7S] cluster located near an αβ unit pseudosymmetric interface that provides the Fe protein docking surface (schindelin *et al*. 1997; Chiu *et al*., 2001) and other is called FeMo cofactor [7Fe-9S-Mo-X-homocitrate], where X is proposed to be an N atom (Dance, 2003; Lee *et al*., 2003; Lovell *et al*., 2003; Hinnemann *et al*. 2003). The P cluster initially receives the electron delivered from the Fe protein which is then transferred to the FeMo cofactor which provides the substrate binding or reduction site as shown by complementary genetics (McLean *et al*., 1981) and biochemical studies (Shah and Brill, 1977).

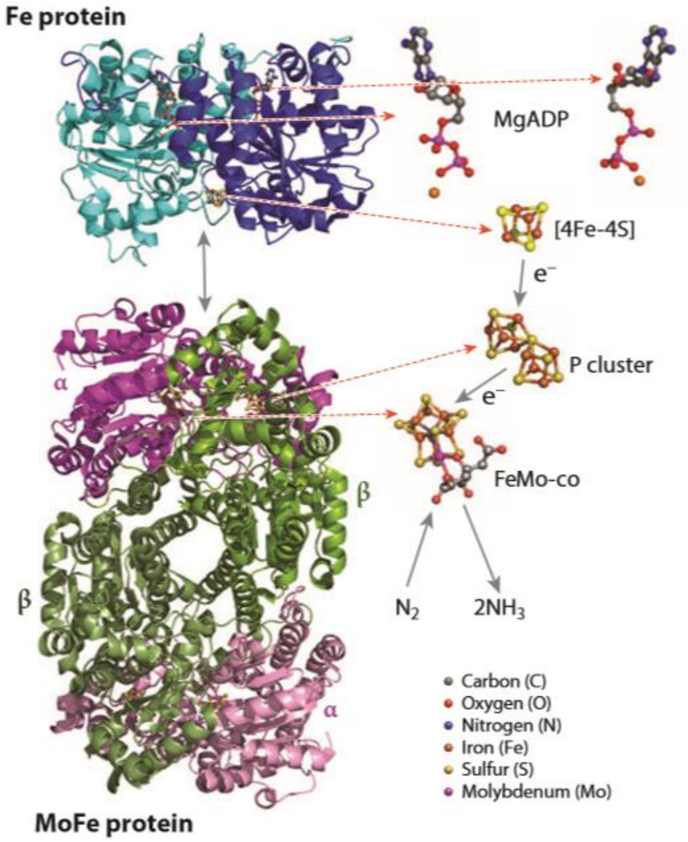


Fig 2: Structures of the nitrogenase molybdenum-iron (MoFe) and Fe proteins. (Seefeldt *et al*., 2009)

Above pictures shows the structure of nitrogenase where MoFe protein is depicted as $\alpha_2\beta_2$ tetramer with the- and β- subunits and Fe protein is shown as γ_2 dimer with subunits of different color. A MoFe protein binds two Fe proteins, with each αβ- unit acting as catalytic unit and one Fe protein is associated with one αβ-unit of the MoFe protein. The relative positions and stuctures of two bound magnesium ADP (MgADP) molecules, the Fe protein [4Fe-4S] cluster, the MoFe protein P cluster (8Fe-7S), and the FeMo cofactor (FeMo-co) (7Fe-Mo-9S-homocitrate-X) are also shown in figure where electrons flow from the [4Fe-4S] cluster to the P cluster to the FeMo-co (Seefeldt *et al*., 2009).

2.8 The site of N_2 binding and reduction

FeMo protein is site for substrate binding that contains two novel metalloclusters called P cluster ([8Fe-7S]) and the FeMo cofactor ([7Fe-Mo-9S-homocitrate-X]) and each one of them are contained separately within αβ- unit thus allowing FeMo protein to have two catalytic units (Kim and Rees, 1992).

2.8.1 FeMo Cofactor

It is the site of substrate binding and reduction and consists of a transition metal –S framework and one molecule of R-homocitrate (Hoover *et al*., 1987). Each inorganic sulphides bridge at 3 sides while two subcultures [4Fe-3S] and [3Fe-Mo-3S] are connected by atom X at the corner (Einsle *et al*., 2002). 2-hydroxy and 2- carboxy groups of R-homocitrate coordinates it with Mo atom. The presence of light atom X though confirmed at the center of the FeMo cofactor its identity is still unknown however electron density of it, is consistent with atoms like N, C or O (Christiansen *et al*., 1998; Lee *et al*., 2003; Yang *et al*., 2005; Lukoyanov *et al*., 2007). FeMo cofactor is fastened to the MoFe protein at both ends through $\alpha\text{-}275^{Cys}$ to a Fe atom and with $\alpha\text{-}442^{His}$ to the Mo atom. To establish the FeMo cofactor as substrate binding site various arguments have been established based on following evidences:

1. Electron-nuclear double resonance (ENDOR) spectroscopic reveals that in CO-inhibited MoFe protein CO binds to the FeMo cofactor but not to P cluster (Pollock *et al*., 1995; Christie *et al*., 1996; Lee *et al*., 1997; Lee and Hales*et al*., 1997; Lee *et al*., 1999).

2. When MoFe cofactor is altered structurally by substituting the homocitrate with citrate, MoFe protein exhibits altered catalytic properties (Imperial *et al*., 1989; Madden *et al*., 1990; Madden *et al*., 1991).

3. Mutant strains devoid of FeMo cofactor and are incapable of reducing substrate can be made active with the addition of isolated FeMo cofactor (Christiansen *et al*., 1998; Ribbe *et al*., 2002).

4. Substitutions of amino acids in the FeMo cofactor –binding environment alters both catalytic and spectroscopic properties of the altered MoFe protein (Dos Santos *et al*., 2005).

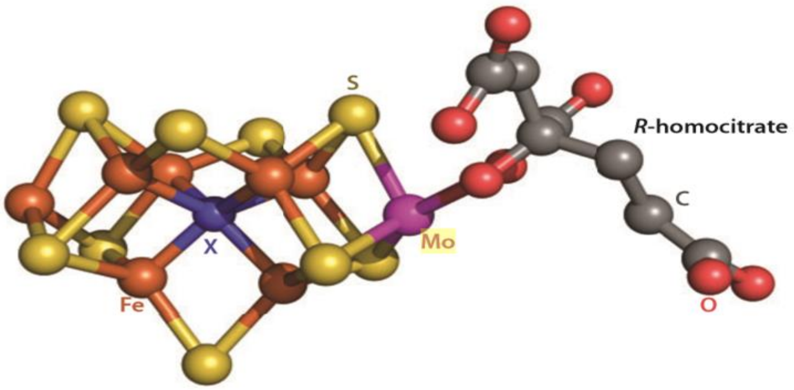


Fig 3: Structure of the iron-molybdenum (FeMo) cofactor of nitrogenase (Seefeldt *et al*., 2009).

2.8.2 P cluster and its role in catalysis

The P clusters which are [8Fe-7S] clusters contained within αβ-unit are thought to mediate electron transfer between the Fe protein and the substrate reduction site of the FeMo cofactor (Seefeldt *et al*., 2009). It is only known naturally occurring [Fe-S] cluster that contains serinate-O (β-188^{Ser}) and amide-N (α-88^{Cys}) ligands along with typical cysteinate-S ligands (Kim *et al*., 1992; Peters *et al*., 1997). Two subclusters [4Fe-4S] are linked at one corner by μ_6-sulpfide that gives it unique overall structure and can undergo structural rearrangement upon oxidation (P^{OX}) from resting state which is dithionite reduced state (P^N). This rearrangement allows displacement of both the serinate-O and amide-N ligands (Peters *et al*., 1997). Different oxidation states of P cluster can be obtained by treating MoFe protein with electron transfer agents and in resting state (P^N), all of the Fe atoms are in the ferrous oxidation state (Lindahl *et al*., 1988; yoo *et al*., 2000). Since reduction of the P^N state to a hypothetical P^R state has never been observed only P^N more oxidized states are utilized during catalysis. P^N state can be oxidized sequentially to P^{1+}, P^{2+} and P^{3+} oxidation states (P^{OX}) with involvement of 3 electrons. Since $P^{3+/2+}$ redox couple is not reversible in vitro only $P^{1+/N}$ and $P^{2+/1+}$ couples are involved in P cluster electron transfer with possibility of transfer of one or two electrons from P cluster to the FeMo cofactor during catalysis. Based on these information working model suggested involves transfer of an electron to the FeMo cofactor upon association of the Fe protein with the MoFe protein from P cluster in its all ferrous resting (P^N) state and this phenomenon is quickly followed by re –reduction of the oxidized P cluster to the P^N state by electron transfer from the Fe protein (Seefeldt *et al*., 2009).

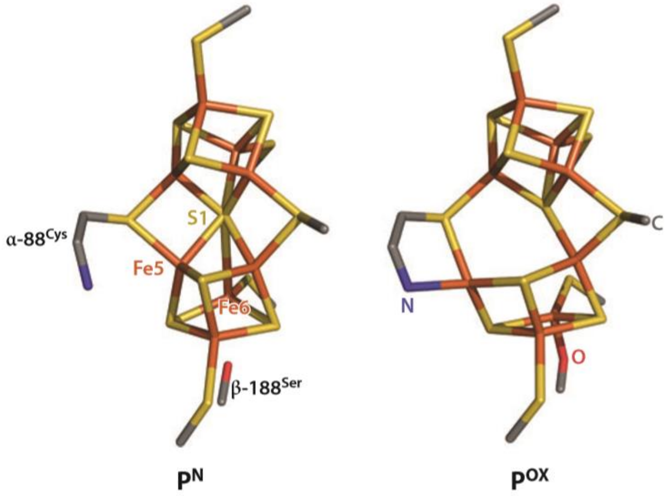


Fig 4: Structures of the P cluster [8Fe-7S] in the reduced (P^N) and oxidized (P^{OX}) states (Seefeldt *et al*., 2009).

2.9 Reduction of N_2 by nitrogenase

The reduction of N_2 by enzyme nitrogenase involves somewhat complicated interactions and exchange of electrons between the two components of the enzyme and is further obscured by the involvements of agents like MgATP and protons (Howard *et al*., 1994; Howard *et al*., 1996; Seefeldt *et al*., 2004; Rees *et al*., 2005; Dos Santos *et al*., 2005). Although molecular details of this multistep and multicomponent system are not fully understood it can be simplified into following subheadings.

2.9.1 Fe protein cycle

The homodimeric Fe protein accommodates one nucleotide (MgATP/MgADP) binding site buried within each subunit and a single [4Fe-4S] cluster that bridges the two subunits (Georgiadis *et al*., 1992). The Fe protein cycle operates in three- state cycle that shows participation of the Fe protein in the nitrogenase mechanism. In this cycle, reduced Fe protein transiently associates with the MoFe protein (Hageman *et al*., 1978). The reduced Fe protein has two bound MgATP molecules and its [4Fe-4S] cluster is in the 1^+ oxidation state. All along this process of association, the two MgATP molecules get hydrolyzed and are converted to two MgADP molecules and single electron is transferred from the Fe protein [4Fe-4S] cluster into the MoFe protein. The oxidized Fe protein ([4Fe-4S] $^{2+}$) thus produced with two bound MgADP molecules then dissociates from the MoFe protein which is rate limiting step of the catalysis (Thorneley *et al*., 1985).The released Fe protein is then regenerated with two steps: by replacement of MgADP with MgATP and the reduction of ([4Fe-4S] $^{2+}$) cluster to 1^+ oxidation state (Lowe *et al*., 1995). The physiological reluctant for the Fe protein however depends on the organism with reduced ferrodoxin or flavodoxin being the most common (Mortenson *et al*., 1964, Martin *et al*., 1989).

2.9.2 MoFe protein cycle

For the catalysis, the FeMo cofactor of MoFe protein must accept eight electrons while single electron is delivered in each Fe protein binding event thus interlocking the Fe protein cycle (1- electron cycle) and MoFe cycle (8- electron cycle) where former drives the MoFe protein to successively reduced state (Seefeldt *et al*., 2009). Thorneley and Lowe has developed a nomenclature for intermediate catalytic states of MoFe protein that successively accumulates electron with corresponding reduction of N_2 where number of electrons (and protons) added to the MoFe protein (E) is denoted with “n” and MoFe protein thus proceeds through states from E_0 to E_8 during N_2 fixation before returning to the resting (E_0) state (Thorneley *et al*., 1985). This model of the nitrogenase mechanism gives rise to several important conclusions such as

1. Before binding of nitrogen there must be accumulation of 3 or 4 electrons with the MoFe protein indicating E_3 or E_4 states.
2. Binding of nitrogen to the MoFe protein releases H_2.
3. In the absence of nitrogen, the MoFe protein will reduce protons and would only access low En states with production of H_2.

Along with nitrogen and protons, nitrogenase can also reduce number of small compounds containing unsaturated bond system with reduction of acetylene (C_2H_2) to ethylene (C_2H_4) being the most common (Dilworth *et al*., 1966; Burgess *et al*., 1996). One of the noticeable facts is that acetylene binds to a less reduced E state (E_2) while N_2 which binds to higher reduced states (E_3, E_4) which makes N_2 competitive inhibitor of acetylene reduction. By the similar reason when both N_2 and acetylene are present, the later appears to be noncompetitive inhibitor for the reduction of the former though both compounds are likely to bind to the same site on the FeMo cofactor (Dos Santos *et al*., 2005).

2.10 Mechanism of biological nitrogen fixation

There are two bases for the discussion of nitrogenase mechanism: 1) Kinetic model that describes the multistep reduction of N_2 to NH_3 and its implications for the stoichiometry of this reaction, 2) strategies and procedure that enables the trapping of catalytic intermediates and their characterization by advanced paramagnetic resonance techniques (Hoffman *et al.*, 2014).

Kinetics and stoichiometry of nitrogenase mechanism:

Lowe and Thorneley and their coworkers have the major contribution in "kinetic" foundation of nitrogenase mechanism. The mechanistic approach involved the kinetic study through steady-state, stopped-flow and freeze-quench kinetics measurement and this model describes the kinetics of transformations among catalytic intermediates (denoted E_n) where n is the number of steps of electrons/protons delivery to MoFe protein of nitrogenase enzyme(Thorneley *et al.*, 1985, Burgess *et al.*, 1996; Roth *et al.*, 2010).

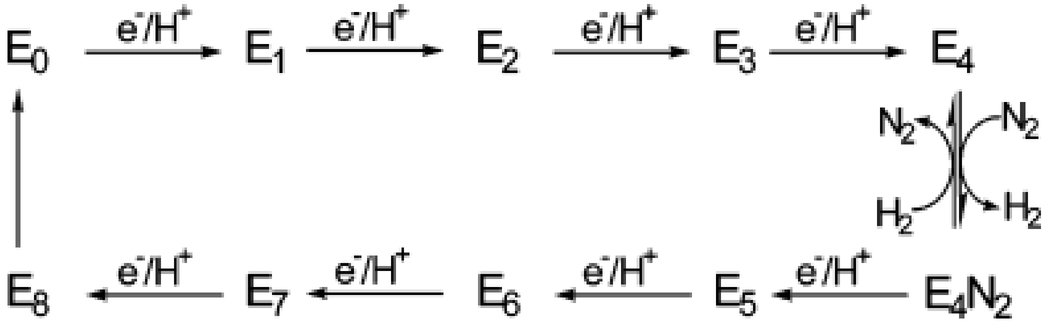


Fig 5: Simplified LT kinetic scheme highlighting correlated electron/proton delivery (Hoffmann *et al.*, 2014)

Biological nitrogen fixation is represented by the following equation, where one mole of nitrogen with 16 moles of ATP and supply of electrons and protons (hydrogen ions) produces two moles of ammonia.

$N_2 + 8H^+ + 8e^- + 16\ ATP = 2NH_3 + H_2 + 16ADP + 16\ Pi$

The reaction is catalyzed by the enzyme nitrogenase consisting of two proteins: an iron protein (Fe protein) and molybdenum-iron protein (MoFe protein) and occur while N_2 is bound to the nitrogenase enzyme complex. The Fe proteins are reduced by electrons donated by ferrodoxin and reduced Fe protein binds ATP. Binding and hydrolysis of two MgATP species within the Fe protein drives the electron to get transferred from Fe protein to MoFe protein (Duval *et al*., 2013) and after the delivery of its electron the Fe protein gets released which is the rate determining step of the whole catalysis process (Thorneley *et al*., 1985). The reduced molybdenum-iron protein thus formed donates electrons to N_2 producing HN=HN. In two further cycles of this process that requires electrons donated by ferrodoxin HN=HN is reduced to H_2N-H_2N and finally reduced to $2NH_3$. Depending on the type of microbes, the reduced ferrodoxin which is supplier of electrons for the process is generated by photosynthesis respiration or fermentation.

The reaction shows the involvement of ATP and obligatory formation of 1 mol of H_2 per mole of N_2 reduced which appears as "waste" of 2 reducing equivalents and four ATP per N_2 reduced (Thorneley *et al*., 1985, Burgess *et al*., 1996). Recent studies have succeeded in characterizing E_n intermediate states beyond the resting state E_0 (Seefeldt *et al*., 2009; Hoffman *et al*., 2014) which enabled integration of a reaction pathway and kinetic scheme which is central to development of mechanism based on the properties of catalytic intermediates (Hoffmann *et al*., 2014).

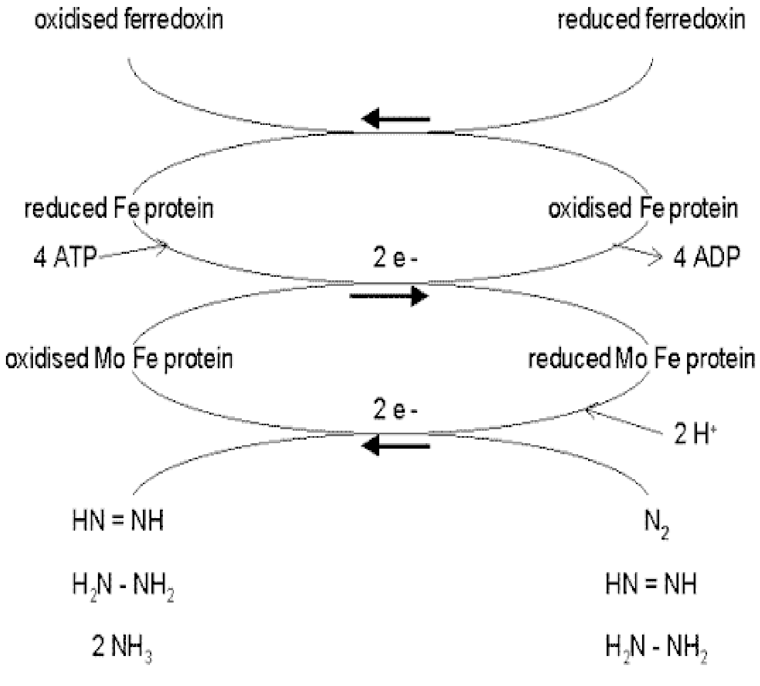


Fig 6: Atmospheric nitrogen fixation by diazotrophs

2.10.1 Regulation of nitrogen fixation

Despite the fact that there exist little apparent variation in the sequences and structures of nitrogenases, there appear to be as many nitrogenase-regulation schemes as there are nitrogen fixing species, however all characterized diazotrophs regulate nitrogenase at the transcriptional level at the same time smaller group also retain a fast acting post translational regulation system. Regulation is described in molecular basis with the consideration of functional and structural characteristics of the proteins responsible for control of nitrogen fixation (Halbleib and Ludden, 2000).

The protein with the site of substrate reduction is nitrogenase-iron (MoFe) protein, also called dinitrogenase or component I while the obligate electron donor to MoFe protein is nitrogenase iron protein (Fe Protein) also called dinitrogenase reductase or component II. The genes encoding MoFe protein and Fe protein along with accessory genes for electron transfer proteins, metal cluster synthesis and regulation consists of nif regulation (Dean and Jacobsen, 1992). MoFe protein is a 230-KDa $\alpha_2\beta_2$ tetramer of the nifD and nifK gene products (Howard and Rees, 1996) while Fe protein is a 64-kDa α_2 dimer of the nifH gene product (Angove *et al*., 1997).

Azotobacter vinelandii allows easiness for the study of complexities of nitrogen fixation and its regulation performed by free living diazotrophs for it is well equipped with structural and mechanistic information for nitrogenase system and is tractable to genetic manipulation (Rubio and Ludden, 2008; Schwarz *et al*., 2009; Seefeldt *et al*., 2009). Genetic complexities of *A. vinelandii* shows 82 genes whose products are known or predicted and are organized into at least 16 different transcriptional units. This organism is able to produce three different types of nitrogenases that are distinguished by their metal content of the active sites, are designated as 'Nif' , 'Vnf', and 'Anf'. In addition to the iron and sulphur required for the assembly of the metal-sulphur cofactors associated with all three systems, the Nif system typically requires molybdenum; Vnf system requires vanadium while Anf system does not require either molybdenum or vanadium for its activity (Joerger and Bhishop, 1988; Joerger *et al*., 1989).

Regulation of the various nitrogenase systems in response to a demand for fixed nitrogen and transition metal availability occurs at the transcriptional level with the respective participation of the nitrogen-fixation-specific regulatory proteins NifA/NifL, VnfA and AnfA (Walmsley *et al*., 1994; Martinez-Argudo *et al*., 2005). Espression of certain nif-specific genes (nifU, nifS, nifV,nifB) is required to sustain the activity of all three system which is an interesting aspect of transcription regulation of nitrogen fixation in A. vinelandii (Joerger *et al*., 1989; Kennedy and Dean 1992; Drummond *et al*., 1996). Cellular nitrogen metabolism that involves the Ntr-specific and associated regulatory proteins has also been controlled using the expression from the nitrogenase system (Walmsley *et al*., 1994).

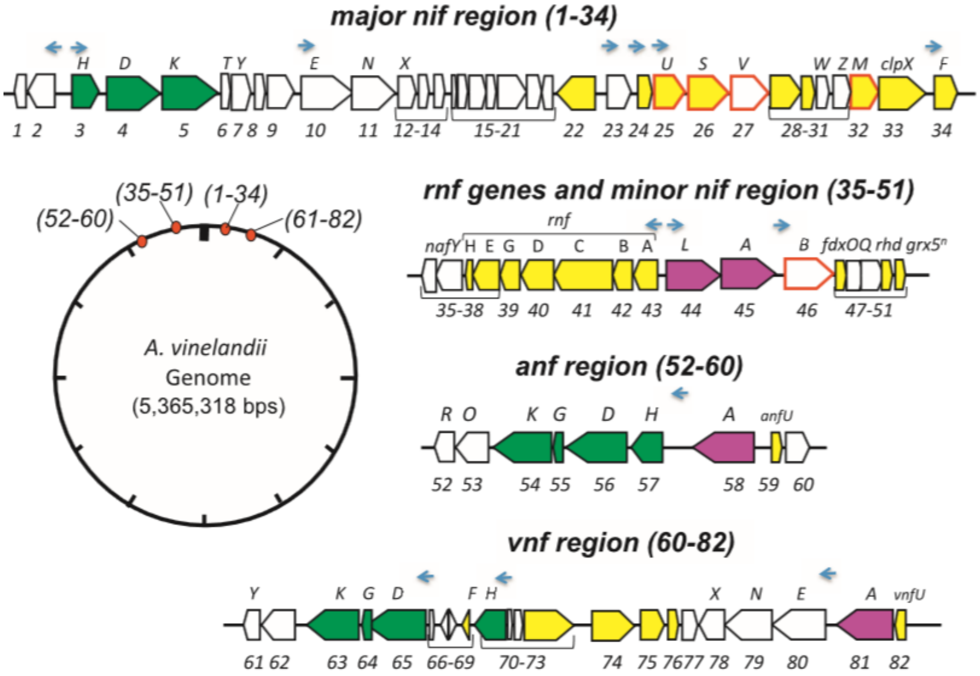


Fig 7: Genes encoding products known or predicted to be involved in nitrogen fixation in *A. vinelandii* (Santos and Dean, 2011).

In above figure different colors are used to specify the genes that encode a product involved in nitrogen fixation in *A vinelandii*. Genes encoding products involved in the catalytic reduction of N2 (green), genes encoding regulatory proteins (purple), genes encoding products required for all three nitrogenase systems (red border), and genes encoding products having known or predicted functions that are similar to housekeeping functions provided by the products of genes located elsewhere on the genome (yellow). The origin of replication is indicated as a dark bar in the circular genome (Dos Santos and Dean, 2011).

Most present day biological nitrogen fixation is catalyzed by molybdenum nitrogenase (Mo) encoded by nifHDK which is an oxygen sensitive metalloenzyme complex made up of Fe protein which in turn is product of nifH and the MoFe protein that is product of nifDK (Burgess *et al*., 1996).

2.10.2 NifA

The positively acting regulatory protein, NifA is required to activate transcription of the other nif genes necessary for nitrogenase structure and activity. Repression of nitrogenase synthesis is achieved by preventing either the activity or the synthesis of NifA by two separate mechanisms (Bali *et al*., 1992). NifA has a domain architecture which is similar to other members of the enhancer-binding protein (EBP) family.

In EBP family central conserved AAA$^+$ ATPase domain is flanked by an amino-terminal regulatory domain and a carboxy-terminal DNA-binding domain, which contains a helix–turn–helix motif that is required for recognition of the upstream activator sequences (UAS) enhancer-like elements. A ubiquitous signaling module which is found in all kingdoms of life is GAF domain. In some cases it has been found to bind small molecules including cyclic nucleotides and formate. This GAF domain is found in N –terminal regions of NifA proteins (Ho *et al*., 2000). This domain has been shown to have a role in regulating NifA activity however in many cases the precise mechanism of regulation is ambiguous and seemingly has diverse role in various diazotrophs (Dixon and Kahn, 2004). The GAF domain of A. vinelandii NifA binds 2-oxoglutarate to modulate the activity of the AAA$^+$ domain in response to a second regulatory protein, NifL however binding of 2-oxoglutarate to the GAF domain *of Klebsiella 32neumonia* NifA has not been observed (Little and Dixon; 2003). In opposition, regulation of NifA activity in response to concentration of fixed nitrogen and possible interaction with signal transduction protein GlnB is exhibited by GAF domains of NifA proteins from diazotrophic porteobacteria *Azospirillum brasilense* (Arsene *et al*., 1996; 1999).

The nifA gene is adjacent to and downstream of nifL, and two of these genes are coexpressed (Dixon, 1988). The function of NifL protein is to inactivate NifA in presence of ammonium even in trace levels and transcription of nifLA requires the phosphorylated NtrC protein, which is dephosphorylated and remains inactive in cells grown with ammonium. Subunits of conventional molybdenum nitrogenase are encoded by the nifHDK genes and are located in a large cluster of nif genes which includes in order nifIDKTYENXUSVWZMF (Jacobson *et al*., 1989).

In several diazotrophs, the expression of nifHDK requires the NifA protein as an activator (Bennett *et al*., 1988; Santero *et al*., 1988). The nifA gene of *A. vinelandii* is not linked to the major nif genes and located upstream of the nifQB genes in a cluster (Bennett *et al*., 1988; Joerger and Bishop 1988). Although ntrC is present in *A. vinelandii* as part of a glnA-ntrBC gene cluster however note necessary for the expression of nitrogen fixation genes (Toukdarian *et al*., 1986, 1990). The DNA sequence of 300 bp upstream of nifA contained a partial open reading frame encoding a protein with the C-terminal regions (Bali *et al*., 1992).

The product of the rpoN (or ntrA or glnF) is Sigma factor 54 which is associated with RNA polymerase which recognizes the NifA activated nif promoters. The sigma recognition sequence includes the conserved bases GC at -12. -13 and GG at -24, -25 base pairs upstream from the transcription start site. Transcription and DNA strand separation (closed to open –complex formation) is preceded by NifA interaction with bound RNA polymerase and concomitant ATP hydrolysis. The binding of NifA to the upstream activator site (UAS) approximately 80-150 base paris further upstream intensifies its local concentration. The UAS contains the conserved sequence TGT-N_{10}-ACA (Balnco *et al*., 1993).

Although product of NifA is required only for the synthesis of the nitrogenase -1 but not for the nitrogenase-2 and nitrogenase-3 in *A vinelandii*; growth data indicates that nifA is required for a factor (or factors) necessary for maximal diazotrophic growth under Mo and V-deficient conditions. *A vinelandii* has two nifA-like genes, designated anfA and vnfA and the product of anfA appears to be required for expression of nitrogenase-3 and the product of vnfA appears to be required for the synthesis of nitrogenase-2 (Joerger *et al*., 1989).

2.10.3 Transcriptional regulation of nitrogenase

At transcriptional level nitrogen fixation is regulated in response to environmental oxygen and ammonium levels. Nitrogenase components being oxygen labile happens to be beneficial for bacteria to repress transcription at higher oxygen levels. Another beneficial aspect of it lies in the fact that it represses metabolically expensive nitrogenase system when the cellular level of fixed nitrogen is sufficiently high. The extent to which transcription is affected by each stimulus is characteristic of the specific diazotrophs. Nitrogenase expression in symbiotic diazotrophs is found to be prominently insensitive to ammonium because of suppression of ammonium level by export of ammonium to their symbiont and hence expression of nif genes in free living diazotrophs is more sensitive to cellular ammonium levels (Merrick, 1992).

The ideal model for nitrogen assimilation and the nitrogenase mechanism is derived from *Klebsiella pneumoniae* and in this model considers NifA (the nifA gene product), a σ^{54} (γpoN gene product) dependent transcriptional activator responsible for control of all major nif gene cluster transcription. The gene products of ntrBC gene contain global two component transcriptional activator system responsible for cellular nitrogen regulation and control the transcription of nifA (Merrick, 1992). In the paradigm system of *K pneumoniae*, the nifA gene is cotranscribed with nifL, which encodes a redox and nitrogen responsive regulatory flavoprotein (NifL). NifL effectively adds another level of regulation in response to oxygen and fixed nitrogen and acts as a negative regulator of NifA. Additional sensitivity of oxidized NifL to the presence of nucleotides in vitro increases inhibition especially in response to ADP (Hill *et al*., 1996).

Deviations from the K. pneumonia paradigm is well exhibited in others diazotrophs and in *A. vinelandii* expression of nifA is not under the control of the ntrBC gene products and uncertainity lies if nifA expression is under nitrogen control. Redox dependent control of nifA expression in *Rhizobioum meliloti* occurs in response to fixL and fixJ, which encode a two component regulatory system responsive to oxygen (Merrick, 1992). This system seemingly replaced the ntrBC control and R. meliloti also lacks NifL but NifA still appears to be inhibited by oxygen stimulus (Krey *et al*., 1992). Likewise, *Rhodobacter capsulatus* lacks NifL and contains nif-related genes analogous to ntrBC but expression of a γpoN-like gene is found to be sensitive to oxygen and fixed nitrogen status (Merrick, 1992). In addition *Rhodobacter capsulatus* contains two copies of nifA that respond distinctly to ammonium (Klipp and Paschen, 1998). Hence nitrogenase transcriptional control mechanism is specific for particular diazotrophs.

2.10.4 Post-translational regulation of nitrogenase

Nitrogen fixation being metabolically demanding process there exist additional layer of nitrogenase regulation in a few free living diazotrophs, in order to prevent unproductive nitrogen fixation during energy limiting or nitrogen sufficient conditions. In this process of post translational regulation nitrogenase complex is rapidly, reversibly inactivated by ADP –ribosylation of Fe protein. The system of ADP –ribosylation has been identified in *Rhodospirillum rubrum a*nd *Rhodobacter capsulatus, Azospirillum brasilense* and *Azospirillum lipoferum* and *Chromatium vinosum* (Ludden and Roberts, 1989).

The post translational nitrogenase regulation was first identified in R rubrum and hence remains the model organism for the paradigm system and basic model for ADP –ribosylation in *R. rubrum* shows the roles of the NAD^+ dependent enzyme, dinitrogenase reductase ADP-ribosyltransferase (DRAT) and its partner, dinitrogenase reductase activating glycohydrolase (DRAG). In *R. rubrum* ADP ribosylation of Fe protein occurs by the formation of an α-N-glycosidic bond between the guanidino nitrogen atom of arginine and the terminal ribose of ADP –ribose at Arg101. This ADP –ribose is identical structurally to the modifying groups of attached by bacterial ADP –ribosylating toxins (Halbleib and Ludden, 2000).

The transfer of electron is not prevented between complexed Fe protein and MoFe protein rather association between them is prevented in presence of ADP –ribose group. ADP ribosylated Fe protein differs from the unmodified Fe protein only in sense that two subunits of inactive Fe protein dimer are not equivalent because ADP –ribosylation occurs on only one subunit and this modified Fe proteins retain the native [Fe_4S_4] cluster which can be chemically oxidized and reduced along with oxygen labiality of the active Fe protein. Modified Fe protein still retains the ability to bind MgATP and to undergo the conformational change that gives access of the [Fe_4S_4] cluster to chelators despite its inability to hydrolyze MgATP (Ludden and Roberts, 1989). It still plays the role in synthesis and insertion of FeMo-co into MoFe protein (Shah *et al*., 1988).

The genes encoding DRAT (drat) and DRAG (drag) are cotranscribed from a non–nif operon that includes third gene (drag) of unknown function. The configuration of the draTGB operon is conserved in *A. brasilense* and *M. lipoferum* (Inoue *et al*., 1996) however R. capsulate lacks draB (Masepohl *et al*., 1993). DRAT is 30-kDa monomer with no measurable activity with other arginine residues or water as the ADP –ribose acceptor and has high specificity toward oxidized, MgADP –bound Fe protein (Ludden and Roberts, 1989; Halblieb *et al*., 2000). The structural domains of DRAG is expected to be similar and some key residues are conserved though amino acid sequence is not highly similar to bacterial toxins and surprisingly, the Fe proteins from K. pneumonia and A vinelandii that lack the dra operon are better substrate for *R. rubrum* than the *R. rubrum* protein itself. DRT doesn't catalyze the reverse or gylcohydrolytic reactions instead dinitrogenase reductase-activating glycohydrolase (DRAG) catalyses removal of the ADP –ribose group restoring fully active Fe protein with an intact Arg101 side chain (Ludden and Roberts, 1989).

DRAG is a 32-kDa monomeric binuclear manganese enzyme capable of cleaving the α-N-gycosidic bond of a number of analogs of ADP –ribosylarginine however only the MgATP bound form of ADP –ribosylated Fe protein which is reduced form but not the MgADP bound or nucleotide –free form , is the substrate for DRAG (Ludden and Roberts, 1989; Shah *et al*., 1988). The both DRAT and DRAG is thought to bind with the same surface of Fe protein and MoFe protein that is well depicted by the inhibition of cellular nitrogenase activity by over expressed DRAT (Grunwald *et al*., 1995). Both of these enzymes are regulated in vivo because it is believed that the regulatory signals involve either negative effectors or known assay components (Grunwald *et al*., 1995; Kanemoto *et al*., 1984; Liang *et al*., 1991).

Cellular fluctuations in ATP and ADP levels during the inactivation/activation cycles are insufficient to result the dramatic nitrogenase activity regulation despite the fact DRAT and DRAG have opposite specificities for MgADP- and MgATP –bound Fe protein(Ludden and Roberts, 1989). Both of these enzyme DRAT and DRAG is believed to be regulated by sensing the cellular energy and redox status directly from the state of Fe protein based on the sensitivity of both of these enzyme toward the redox state of Fe protein (Halbleib *et al*., 2000) and cellular NAD^+ concentration has also been proposed for positive effector of DRAT (Nore´n *et al*., 1997). Regulation by weak and reversible binding of Fe^{2+} or Mn^{2+} ion in DRAG is expected on the basis of alteration of divalent cation affinities by unregulated variant of DRAG.

The nitrogenase-inactivating conditions of nitrogen sufficiency (NH_4^+) and energy limitation (darkness) give rise to convergent signal transduction pathways (Kanemoto *et al*., 1984) but merging of pathways remains unclear. Intermediary role of glutamine is suggested by the perturbation of both responses when inhibition of glutamine synthetase takes place keeping the cellular concentration of glutamine relatively unaffected by the modification and demodification of Fe protein (Kanemoto and Ludden, 1987). The effect on ammonia response appears to be independent of the darkness response. This observation was made by genetically perturbing the nitrogen control genes (glnB,ntrBC) and results obtained doesn't support the model of closely related signal transduction pathways (Zhang *et al*., 1992).

The response of DRAT and DRAG activities to exogenous inactivation effectors is not species specific and they appear to sense a global regulatory signal, present even in nitrogen fixing bacteria that lack the dra operon (Zhang *et al*., 1992).

Apart from the paradigm systems regulatory mechanisms of specific organism may confirm the different layers of nitrogenase regulation mechanism.

2.11 Polyhydorxybutyrate (PHB) in reference to *Azotobacter vinelandii*

A. vinelandii and *Pseudomonas* has the potential to produce relatively large amounts of the polymer of high molecular weight on cheap substrates combined with easy extraction process (Galindo *et al*., 2007). This is an unique attribute which is demonstrated under unbalanced growth conditions, which are also exhibited by wide range of bacterial and archaeal species to form carbon and energy reserve materials. A polymer of the polyhydroxyalkanoates (PHAs) family of polyesters accumulated in the form of intracellular granules to store carbon and reducing equivalents are poly(β-hydroxybutyrate) (PHB) with over 140 possible constituent monomers that have been traditionally classified as short chain length (C4 and C5) and medium chain length (≥C6) hydroxyalkanoates (Aldor and Keasling. 2003).

PHB are prime candidates for commodity and specialty commercial plastic production and are also model compounds for metabolic engineering. Besides role as polymer which is biodegradable and biocompatible thermoplastic, PHB is involved in supporting nitrogen fixation (Segura and Espin, 1998).

Although some or all monomeric constituents of numerous bio based polyesters like polylactic acid and 3GT (polymer of 1,3 propanediol and terephthalic acid) are produced by microbial fermentation they are synthesized chemically. However, PHAs and their unnatural polythoester analogs are assembled and accumulated in vivo and can be manipulated by metabolic engineering. The metabolic pathways operating in the cell and external carbon source control the host's PHA synthase (polymerase) and hydroxyacyl-CoA thioester precursors supplied to the enzyme and these in turn control the monomeric composition of the biopolymer. The biosynthetic routes to PHA monomers compete with and/or rely on the various important pathways that include tricarboxylic acid (TCA) cycle, fatty acid degradation (b-oxidation) and fatty acid biosynthesis for precursors, and involve central metabolites such as acetyl-CoA and cofactors such as NADPH. The monomeric composition of the PHA provides insight into the metabolism that was involved in polyester biosynthesis (Aldor and Keasling. 2003).

PHB is accumulated as a result of depletion of nitrogen, phosphorous or oxygen to form carbon and energy reserve material. Various nitrogen-rich media containing casein hydrolysate, yeast extract, tryptone, casamino acids, corn steep liquor and collagen hydrolysate have been used for PHB production (Lee and Chang, 1995; Bormann *et al*., 1998; Khanna and Srivastava, 2005; Chaijamrus and Udpuay, 2008) and it has been found carbon-substrate rich media such as molasses, whey, hemicelluloses, palm oil, starch, glucose, fructose, sucrose, maltose, gluconate or glycerol accumulates PHB in bacterial cells and can be used as substrates for PHB production (Page, 1989; Page and Knosp, 1989; Quagliano *et al*., 1994; Alias and Tan, 2005; Chaijamrus and Udpuay, 2008; Kishk, 2009).

Surprisingly unrefined carbon sources such as corn syrup, cane molasses, beet molasses, or malt extract not only supports PHB formation but comparable or even better yield can be obtained from them compared to refined sugars. The development of a low cost process to produce biopolymer with properties similar or superior to petrochemical plastics is crucial for the large scale production of microbial PHB (El-Shanshoury *et al*., 2013).

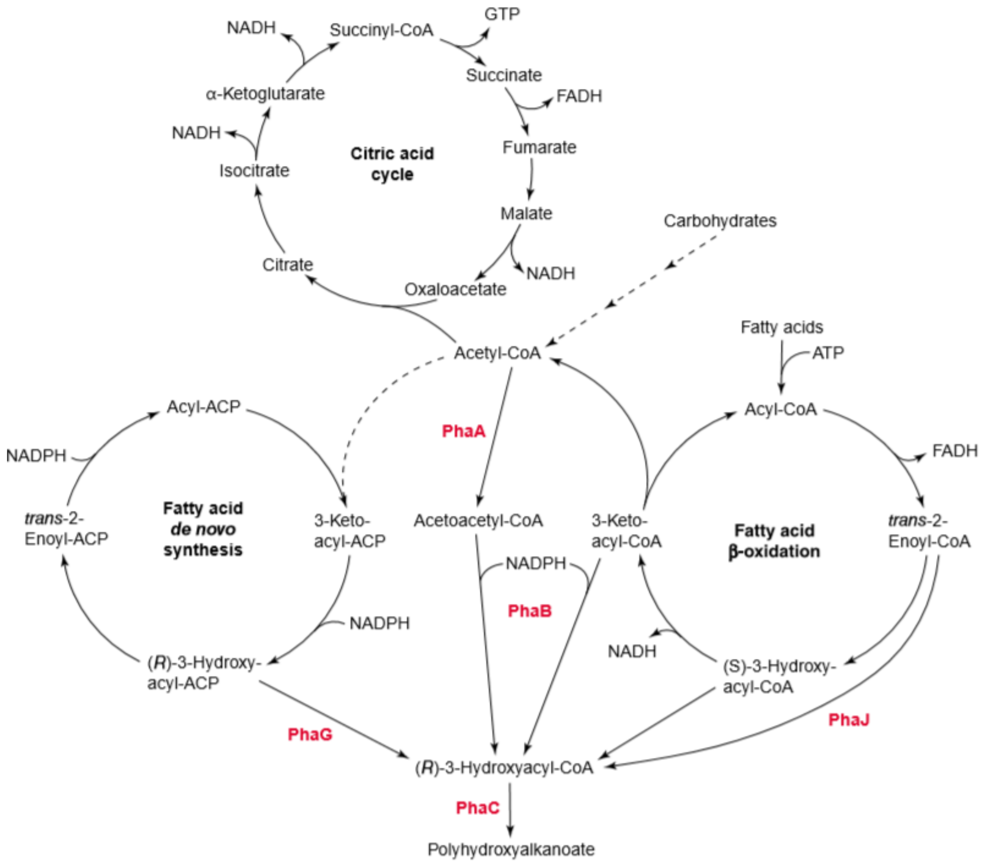


Fig 8: PHA biosynthesis in the context of microbial metabolism

2.11.1 PHB biosynthesis

The PHB is in general accumulated by bacterium in response to interference in its growth, mainly due to lack nutrients such as nitrogen, phosphorus, magnesium and oxygen, and the presence of an excess carbon source (Burns *et al*., 2007). In *A. vinelandii* PHB production involves three enzymes viz. β-ketothiolase, acetoacetyl-CoA reductase and PHB synthase. Initially the enzyme β-ketothiolase promotes condensation of two acetyl-CoA molecules to generate acetoacetyl-CoA. After that, acetoacetyl-CoA gets reduced by acetoacetyl-CoA reductase using NADPH, producing D(-)-β-hydroxybutyryl-CoA and finally this gets polymerized by PHB synthase producing PHB (Segura *et al*., 2003). The control point in the process is the major activity of enzyme β-ketothiolase and this is activated when the acetyl-CoA concentration is high, a situation that results from the accumulation of NADH or NADPH in response to low oxygen concentration in the medium. Enzymes of Krebs cycle citrate synthase and isocitrate dehydrogenase are inhibited by these metabolites since they reduce the carbon flow into the cycle and leads to an upsurge of acetyl-CoA concentration, a fact that stimulates the β-ketothiolase activity. The enzyme activity of acetoacetyl-CoA reductase which catalyzes the second step of the process is also supported by these conditions (Page *et al*., 1992). The structural genes responsible for PHB synthesis in *A.*

vinelandii are phbA gene encoding the β-ketothiolase and phbC and phbB genes codifying the second and third enzyme, respectively (Segura *et al*., 2003).

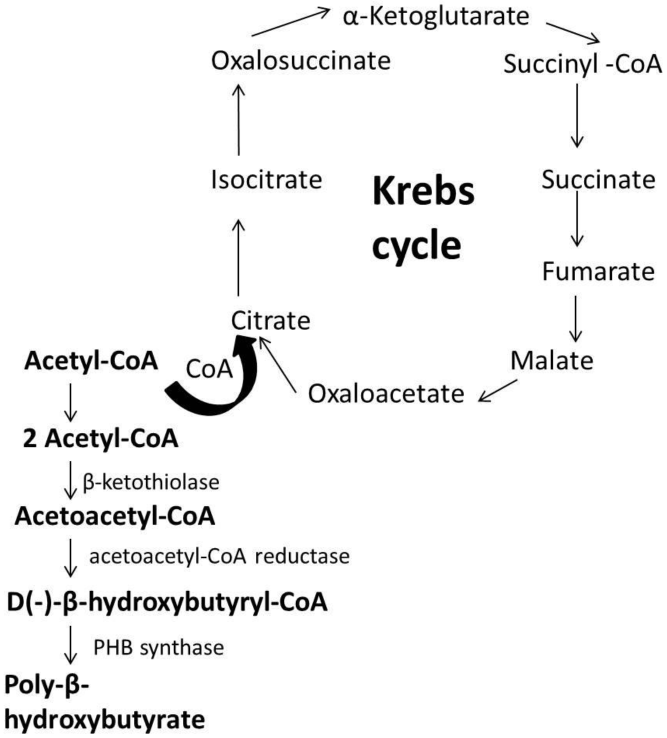


Fig 9: Polyhydroxybutyrate biosynthesis and Krebs cycle by *Azotobacter vinelandii* adapted from Segura *et al*., (2003).

2.11.2 PHB biosynthesis regulation

PHB synthesis in *Azotobacter* species is controlled post transcriptionally at the level of β-ketothiloase activity that catalyses the first step of PHB synthesis (Senior and Dawes, 1971). Additionally, PHB synthesis in *A. vinelandii* may also be controlled at the transcriptional level on the basis of presence of phbR. The phbR gene is upstream of and in the opposite direction of the phbBAC biosynthetic gene cluster that encodes PhbR, a transcriptional activator which is a member of the AraC family of activators and transcription of the phbBAC biosynthetic operon is initiated from two overlapping promoters, p_B1 and p_B2 (Peralta-Gil *et al*., 2002).

An enzymatic activities involved in PHB synthesis are coded by the PHB biosynthetic gene cluster phbBAC that regulates β- ketothiolase (PhbA), acetoacetyl-CoA reductase (PhbB), and PHB synthase (PhbC), in *A. vinelandii* (Pettinari *et al*., 2001). In the same DNA region where phbBAC genes are found, other genes related to PHB synthesis were also found:

phbR, which codes for a member of the AraC family of transcriptional activators; phbP, coding for a putative granule-associated protein, and phbF, a putative regulator of the phbP (Galindo *et al*, 2007).

A gene phbR coding for a protein exhibiting identity to transcriptional activators of the XylS/AraC family was identified in *A. vinelandii* and is linked to the phbBAC. It is also present in *Pseudomonas* sp.strain61-3 and is located 310 nucleotides upstream and in the direction opposite that of the phb biosynthetic cluster (Peralta-Gil *et al*, 2002). This is indeed a positive regulatory factor for the PHB biosynthetic genes (Matsusaki *et al*., 1998). More than 100 proteins from different bacteria are included in AraC family and most members are transcription activator that binds to specific 18-to 20-bp sequences at target promoters. The AcaC C-terminal domain of these amino acids constitutes the DNA binding domain that contains two helix-turn-helix DNA binding regions (Gallegos *et al*., 1997). The conservation of this DNA binding domain allows the other family members to bind to their targets in similar manner. PhbR and RpoS act as activators of phbB and phbR. PhbR binds specifically to the direct repeated sequence TGTCACCAA-N_4-CACTA present in the phbB promoter region (Hernandez-Eligio *et al*., 2012).

In *Azotobacter vinelandii* regulation of PHB synthesis is made more complex with the involvement of different groups of genes in addition to phbR and rpoS such as non-phosphorylated form of the IIA^{Ntr} protein of the nitrogen-related photransferase system encoded by ptsN and inactivation of the two-component system gacS and gacA genes. The former is found to be involved in negative regulation of phbR and phbBAC expression (Noguez *et al*., 2008) while later is found to impair alginate and PHB production (Castaneda *et al*., 2001).

The other regulatory systems are also found to be involved along with the allosteric control of the first biosynthetic enzyme, β-ketothiolase, by the CoA/acetyl-CoA ratio in *A. vinelandii*. Two overlapping promoters pB1 and pB2 allows the location to initiate the transcription of phbBAC biosynthetic operon and transcription of the PHB biosynthetic operon from the pB1 promoter is activated by PhbR, encoded by phbR whereas transcription from pB2 is dependent on the sigma factor RpoS and increases during the stationary phase of growth. Transcription of phbR itself also starts from two promoters, pR1 and pR2. In addition, there exist an indirect way where transcription from pR2 is induced during the stationary phase and is dependent on RpoS (Peralta-Gil *et al*., 2002).

Therefore regulation model is proposed as: While in balanced growth conditions where cells grows exponentially inhibition of the activity of β-ketothiolase present in cells occurs with low transcription of phbBAC caused by both the lack of RpoS, which affects transcription from one of the promoters of the PHB biosynthetic operon, and by the low concentraction of PhbR, whose transcription is also partially dependent on this sigma factor (Peralta-Gil *et al*., 2002). Further; when entered into stationary phase stimulation of phbBAC operon occurs as result of increase in transcription of phbR preceded by the increase in transcription of rpoS. Moreover, during stationary phase activity of TCA cycle slows down allowing an increase in the acetyl-CoA/CoA ratio, which relieves the inhibitory effect on β-ketothiolase (Galindo *et al*, 2007).

In addidtion; two component global regulatory GacS/GacA system formed by sensor kinase GacS and its corresponding response regulator GacA is also involved in the control of PHB production in *A. vinelandii* (Castaneda *et al*., 2000; 2001) and PHB production is diminished by either gacS or gacA mutations. The model suggests GacA in its phosphorylated form to be a positive regulator for PHB synthesis and is required for transcription of rpoS (Castaneda *et al*., 2001).

Likewise, the nitrogen-related phosphotransferase system (PTS^{Ntr}) is also involved in the control of PHB synthesis. This system is homologous to the phosphoenol pyruvate-sugar phosphotransferase system (PTS) found in a number of bacterial genera that mediates the glucose and other carbohydrates uptake and concomitant phosphorylation. In *A. vinelandii* PHB production is found to lower when mutation occurs on encoding enzyme I^{Ntr} (Segura and Espin, 1998). This regulation occurs via phosphate relay where negative regulator of PHB synthesis is enzyme I^{Ntr} autophosphorylates and is apparent teriminal phosphoryl acceptor that uses phosphoenolpyruvate, and IIA^{Ntr} protein (Galindo *et al*., 2007).

By the same, Fnr-like regulatory protein called CydR has also been argued to regulate PHB synthesis in *A. vinelandii*. The transcription of gene cydAB which codes for cytochrome bd terminal oxidase required for aerotolerant nitrogen fixation is repressed by protein CydR however in presence of oxygen its DNA binding capacity is diminished thereby derepressing the transcription of cydAB (Wu *et al*., 2000). During the exponential growth cydR mutant overexpresses β-ketothiolase and acetoacetyl-CoA reductase, and accumulates PHB and role of of CydR in the control of PHB synthesis is assumed to be related to the redox state of the cell though mechanism and its relationship with other regulatory systems is still unclear (Galindo *et al*., 2007).

Beyond genetic regulation oxygen limitation positively controls PHB synthesis at metabolic level (Senior *et al*. 1972). During oxygen limitation and carbon excess, NADPH increases and inhibits the activities of the tricarboxylic acid (TCA) cycle enzymes, citrate synthase and isocitrate dehydrogenase. The inhibition of these (TCA) cycle enzymes causes an increase in acetyl-CoA to feed the PHB synthesis pathway (Senior and Dawes 1971). Further, this phenomenon of inhibition reduces the quantity of CoA, which is an inhibitor of the β-ketothiolase that catalyzes the first step of the PHB biosynthetic pathway. Additionally, the inhibition of citrate synthase and isocitrate dehydrogenase causes an increase in acetyl-CoA to feed the PHB synthesis pathway (Senior and Dawes1973). This relationship between TCA cycle and PHB synthesis in *A. vinelandii* is supported by mutation in pycA (encoding pyruvate carboxylase subunit A) that causes expected slowdown in the TCA cycle activity due to low oxaloacetate concentration, resulting in a higher availability of acetyl-CoA for the synthesis of PHB(Segura and Espin, 2004).

In case of high oxygen concentration, acetyl-CoA enters the TCA cycle, and β-ketothiolase is accordingly inhibited by CoA, thereby preventing PHB synthesis (Castillo *et al*., 2013).

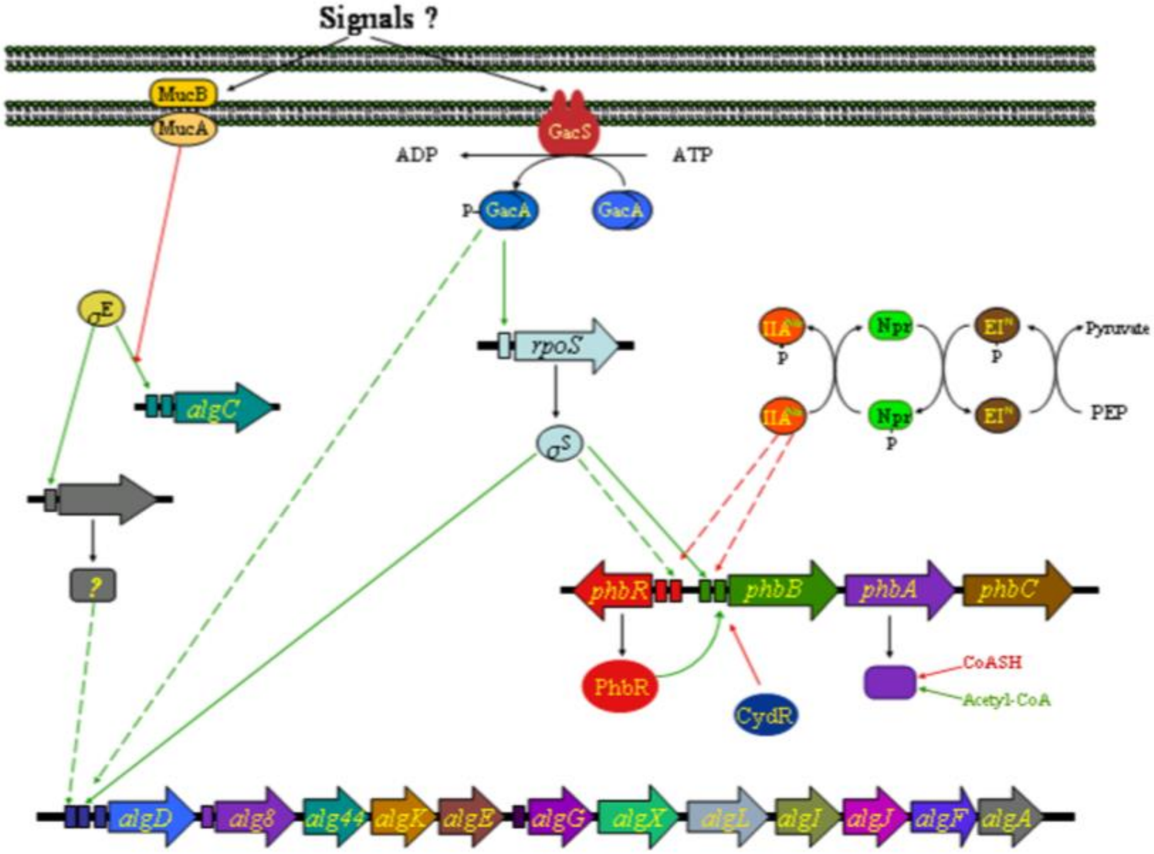


Fig 10: Organization of the A. vinelandii alginate and PHB biosynthetic genes (Galindo *et al*., 2007).

CHAPTER-3

MATERIALS AND METHODS

3.1 Materials Required

A bacteriological media, reagents, chemicals, equipment along with materials used during research are enlisted in Appendix I.

3.2 Study site

The isolates used in the study were collected mainly from Panchase and Vadaure of Kaski district of western Nepal. The geographical location of the site has furnished the place with abundance and diversity in floral and faunal entities. The site with lush green forest receives a significant downpour throughout the year which enables vegetation to proliferate from subtropical to temperate forest.

The habitat of the studied isolates (*Azotobacter vinelandii and Pseudomonas fluorescens*) being soil and their pervasive nature allow them to be found in ground which are neutral to weakly basic. Several members of these isolates are found to be associated with plants hence rhizospheric soil from the roots of tree fern were gathered to isolate the organisms.

3.3 Sample collection

The total of 5 different samples were collected for the isolation process. The soil samples were collected few inches deeper from the surface, mechanically using spades and spatula and then retained in zip lock plastic bag. The samples were stored in refrigerator at 4^0C until use. The samples were then transferred into Jensen media until growth were visible and then finally availed for isolation.

(A) (B)

Fig 11: (A) site of sample collection, (B) Collection of sample

3.4 Isolation and Screening

3.4.1 Isolation of *Azotobacter vinelandii* and *Pseudomonas fluorescens*

The soil samples previously collected were incubated in Jensen media (appendix-I) containing sodium acetate as C-source so as to achieve the desired growth. After an adequate growth were observed, 100 µL of the broth containing bacterial growth were transferred into Jensen media with top agar. After a thorough mixing this top agar was then poured into bottom agar. Finally, the inoculated petri dishes were incubated at 28^0C until oval, dew drop colonies were observed.

3.4.2 Screening of *Azotobacter vinelandii*

The colonies so obtained in isolated process were picked and streaked on Jensen media incorporating ethylene glycol as sole C-source. Both *Azotobacte*r and *Pseudomonas* are known to utilize the ethylene glycol as C- source for the survival. Finally screening of former is achieved through transfer of culture, sustaining the life in ethylene glycol, to Jensen media containing rhamnose where only *Azotobacter* can survive with utilization of this form of carbohydrate as the energy source.

3.4.3 Screening of *Pseudomonas fluorescens*

In order to differentiate the *Pseudomonas* species from *Azotobacter*, the culture obtained in Jensen media containing ethylene glycol as C- source in preceding experiment was transferred to Jensen media furnished with toluene as sole C-source. Since, only *Pseudomonas* species could sustain their life in media containing toluene, segregation is achieved leading to screening of the desired bacteria.

After the isolation and screening of bacteria of interest, they were further subjected to various morphological as well as biochemical tests.

3.5 Morphological characterization of isolates

The colonies procured after the isolation for both the samples were analyzed visually and for each of the separate samples obtained. Those colonies were selected for further inoculation which exhibited independent and distinct growth. From these screened media, multiple colonies were selected and streaked on separate plates which are then used for morphological as well as biochemical analysis of both the isolates. The new samples thus obtained are subjected to basic observations like: shape, size, color and the visual properties. The gram staining was also performed to apprehend the cell composition of the isolates.

3.5.1 Gram staining

The process involved aseptic transfer of small amount of colony to a loop full of water placed on slide followed by air drying and preparation of quality smear(too heavy or too light cell concentration was avoided) which was then heat fixed. After that smear was flooded with crystal violet for 1 minute. The slide was then washed in gentle and indirect stream of distilled water using wash bottle. Following slide was then flooded with the mordant: Gram's iodine and allowed to set for minute and again washed with water. Thenceforth, slide was flooded with decolorizing agent alcohol till it ran clear from the slide. Finally, slide was flooded with counterstain safranin and was allowed to retain for a minute. Washing the slide again with water till no color appeared on effluent followed by drying, the results were observed under oil immersion (100x) using a Bright field microscope.

Ultimately different observations were reported from gram smear that included morphology of bacteria present, their numbers and the colors of the bacteria. The gram reaction of bacteria indicated their cell wall composition. Gram positive cell wall contain thick layer of peptidoglycan with numerous teichoic acid cross linking which resists the decolorization and stains purple while gram-negative cells have thin layers of peptidoglycan, one to three layers deep with a slightly different structure than the peptidoglycan of gram-positive cells and stains pink.

3.6 Biochemical characterization of isolates

The biochemical tests are decisive methods availed amidst many and are the fastest developing trend in bacterial identification. These tests not only shortens the time required to identify the microbes but also reduces costs, ensure and enhances the accuracy of identification of an unknown samples.

The Bergey's Manual of Determinative Bacteriology was followed in order to classify the isolates of *Azotobacter* and *Pseudomonas* using various biochemical tests. The routine biochemical tests used in the study are mentioned below.

3.6.1 Catalase test

The catalase test is used to determine whether or not, the bacterial isolates are capable of producing catalase enzyme that neutralize toxic forms of oxygen metabolites; hydrogen peroxide. The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme while microorganisms that live in oxygenated environments possess them.

In order to carry out the catalase test, a drop of 3% H_2O_2 was placed on opposite ends of clean and grease-free glass slides. Using a sterile wooden stick (toothpick), a colony of the bacterial culture was transferred to the drop of 3% H_2O_2. Effervescence marked by bubbles was examined. The lack of catalase is evident by a lack of or weak bubble production and was considered as negative result while rapid evolution of oxygen was considered as catalase positive result.

3.6.2 Oxidase test

The oxidase test separates the bacteria that produces Cytochrome C Oxidase enzyme with non-producers. This enzyme is a part of bacterial electron transport chain and bacteria with this enzyme are capable of utilizing oxygen as terminal electron acceptor in respiration. The oxidase negative result indicates the absence of cytochrome c oxidase that oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product, therefore in absence of such enzyme the reagent remains reduced and is colorless.

For the catalase test, a small portion of the bacterial culture was taken using a sterile wooden stick and rubbed over the oxidase disc (pre-soaked with 1% tetra methyl-p-phenylenediaminedihydrochloride). Development of purple or blue coloration was examined.

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

Methyl Red (MR) test differentiates the isolates on the basis their ability to perform mixed acid fermentation when glucose is supplied. Variety and amount of fermentation yield produced in anaerobic glucose fermentation by bacteria are one of the imperative taxonomic characteristic that provides the basis for differentiation among various genera of enteric bacteria.

The pH range at which methyl red act, is considerably lower than other indicators used in bacteriologic culture media because of which isolates are required to produce large quantities of acid from carbohydrate substrate being used. Using this indicator, medium produces color yellow above pH 5.1 and red at pH 4.4. Hence, when culture medium develops red color, indicates MR positive result while culture medium retaining the yellow color marks the negative outcome because of less amount of acid being produced from the fermentation of glucose.

Voges-Proskauer (MR-VP) test indicates the presence of acetoin in bacterial culture medium. Depending on the enzyme system possessed by various bacteria, pyruvic acid-crucial compound obtained in fermentative degradation of glucose-is further metabolized through metablic pathways resulting in production of acetoin (acetyl methyl carbinol), a neutral-reacting end product. In the presence of atmospheric oxygen and 40% potassium hydroxide, acetoin is converted to diacetyl, and alpha-naphthol serves as a catalyst to bring out a red complex indicating the positive result within 15 minutes or more. However, the test shouldn't be read after an hour for Voges-Proskauer cultures may produce a copper like color, potentially resulting in a false positive interpretation.

So as to perform MR-VP test, MR-VP medium were first labeled with the name of the microorganism, then the microorganisms were aseptically inoculated in each tube and incubated at 28°C. After 48 hr, 5-6 drops of MR reagent was added to the incubated tubes labellsed MR and color change was observed.

For VP, Barritt's reagent A and B in the ratio of 3:1 were added in the incubated tubes labelled VP and shaken to provide oxygen. The color change was noticed after 20-30 min of incubation.

3.6.4 SIM (Sulphur Indole Motility) Test

SIM test utilizes SIM medium that enables determination of three characteristics by which bacteria can be classified. H_2S production is indicated by peptonized iron and sodium thiosulphate as an indicator where H_2S reacts with peptonized iron to form black precipitate of ferrous sulphide (Sulkin *et al*, 1940; Sosa, 1943). The organism that are motile further intensifies the H_2S reaction.

Motility detection becomes feasible because of semisolid nature of medium. The motility is confirmed when growth radiates out from the central stab line. Those with motility grow away from line of inoculation showing diffused growth while non –motile grow along the stab line.

The splitting of amino acid tryptophan to indole is detected by indole test that involves the addition of chemical reagents following the incubation period. Tryptophan from peptone degenerates into indole only in specific bacteria and presence of indole is detected by Kovac's or Ehrlich's reagent which contains 4 (p)-dimethylamino benzaldehyde, this reacts with indole to produce a red coloured compound.

To carry out the SIM test, isolated colonies from an 18-24 hour culture on agar media was inoculated in SIM media by stabbing the center of the medium to a depth of 1/2 inch. The medium was incubated aerobically at 28^0C for 18-24 hours. After incubation, the tubes were observed for H_2S production and motility. Once H_2S and motility reaction were recorded, three drops of Kovacs Reagent was applied to the surface of the medium. Development of a pink to red color was observed for positive indole result while no change in color would indicate negative result.

3.6.5 Citrate utilization

Citrate utilization test inquires the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic ammonium dihydrogen phosphate ($NH_4H_2PO_4$) is the sole fixed nitrogen source. This test distinguishes the members of bacteria based on their metabolic byproducts. When citrate, an organic acid, is used as a carbon source, alkaline carbonates and bicarbonates are produced. Additionally, ammonium hydroxide is produced when the ammonium salts in the medium are used as the sole nitrogen source leading to alkalinization of media which is detected by bromothymol blue indicator.The bromothymol blue pH indicator is a deep forest green at neutral pH while at alkaline pH to above 7.6, bromothymol blue changes to blue indicating citrate positive result. Those bacteria which do not utilize citrate as the sole carbon and energy source will not be able to grow on the Simmons citrate medium, thus a citrate-negative test culture will be virtually indistinguishable from an uninoculated slant and will appear green.

To peform citrate test, Simmon's citrate agar slants were prepared and labeled with the name of the bacteria. Then the bacteria samples were aseptically inoculated on the surface of the medium in a zig-zag pattern. The tubes were incubated at 28°C for 24-48 hr and the color change in the medium was observed.

3.6.6 Sugar fermentation test

The sugar fermentation test confirms the competence of bacteria to ferment a specific carbohydrate. It also inspects presence of acid and/or gas produced from carbohydrate fermentation. This Carbohydrate fermentation patterns differentiates the bacterial groups and species. Different single carbohydrate source such as Glucose, Lactose, Sucrose or any other carbohydrate are used in basal medium along with pH indicator such as phenol red to detect the lowering of the pH of the medium due to acid production. Small inverted tubes called Durham tube is also immersed in the medium to test for the production of the gas (hydrogen or carbondioxide).

In order to carry out sugar fermentation test, peptone water containing different carbohydrate source along with indicator were inoculated with the organisms and incubated at 28^0C for 24 hours. Inverted Durham tubes were immersed in media to collect any gas that might be given off as an end product of fermentation. The tubes were observed for change in color and compared with control media.

3.6.7 Urease test

Urease test investigates the ability of bacteria to synthesize urease enzyme that splits the urea in presence of water to release ammonia and carbon dioxide. Urease if present hydrolyses the urea, and ammonia and carbon dioxide thus produced combines themselves along with water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink. Hence in urease positive test, the color of the slant changes from light orange to magenta. However if organism do not produce urease the medium retains original color.

In order to perform urease test, the urea medium was inoculated with a loopful of culture of the test organism. The cap of the tube was left loosely and incubated at 28^{0}C for 18-25 hours. Finally change in color of the medium was observed and recorded.

3.6.8 TSIA (Triple Sugar Iron Agar) test

This test differentiates the bacteria on the basis of their ability to ferment different sugars viz. lactose, glucose, sucrose and their ability to produce gas.

To carry out TSIA test, the top of a well-isolated colony was touched with a sterilized straight inoculation needle. The colony was inoculated in the TSI Agar by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant. The cap of the tube was left on loosely and incubated at 28^{0}C in ambient air for 18 to 24 hour. Inferences are drawn based on the following facts

- 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 peoptone is a major constituent of TSI Agar).
- If H_2S is produced, the black color of ferrous sulfide is seen.

Following observations were made and results were assigned accordingly:

1. Alkaline slant/no change in butt (K/NC); Red/Red = glucose, lactose and sucrose non-fermenter
2. Alkaline slant/Alkaline butt (K/K); Red/Red = glucose, lactose and sucrose non-fermenter

3. Alkaline slant/acidic butt (K/A); Red/Yellow = glucose fermentation only, gas (+ or -), H_2S (+ or -)
4. Acidic slant/acidic butt (A/A); Yellow/Yellow = glucose, lactose and/or sucrose fermenter gas (+ or -), H_2S (+ or -).

3.7 Molecular Characterization of the isolates

3.7.1 Preparation of cell

The isolated bacterial colony was inoculated in 2 ml of LB medium and incubated overnight at 28^0C. 1.5 ml of so cultured bacterial sample was transferred to sterilized Eppendorf tube and centrifuged at 5000 rpm for 5 minutes at 4^0C. Instantly, supernatant was discarded leaving only cell pellet by aspiration and to this remaining previously grown bacterial culture was added. This culture was again centrifuged at 5000 rpm for 5 minutes. Again the supernatant was discarded without disturbing the cell pellet.

3.7.2 Cell lysis

Then cell pellet obtained in previous process was re-suspended in 450 µL of TE1 buffer by gentle pipetting. The solution was then subdivided into two half consisting of 255 µL each and then transferred into fresh sterilized Eppendorf tubes. To both of the tubes 180 µL of lysozyme (1mg/ml) was added and incubated at 37^0C for 30 minutes. These tubes were repeatedly inverted every 5 minutes for proper cell lysis during the incubation period. After that 45 µL STEP solution was added to each of the tubes and subjected to ice incubation for 45 minutes till the solution became clear due to cell lysis incorporating the gentle inversion during the incubation period.

3.7.3 Separation of genomic DNA

To the solution obtained from cell lysis, equal volume of chilled phenol (450 µL) was added and mixed by vortexing. The mixture was then centrifuged at 13000 rpm for 10 minutes that separates the mixture into two layers. Among them upper aqueous layer containing DNA was transferred into fresh sterilized Eppendorf tube without disturbing the lower organic phase. To the aqueous solution with DNA, equal volume of mixture solution with ratio chilled phenol: chloroform: isoamyl alcohol (25: 24: 1) was added and mixed by vortexing followed by centrifugation at 13000 rpm for 10 minutes. Again, aqueous layer was collected in fresh Eppendorf tube and to it added equal volume of chloroform followed by vortexing. After mixing, the solution is subjected to centrifugation at 13000 rpm for 2 minutes. The aqueous obtained in this case was again collected in fresh Eppendrof tube for further process.

3.7.4 Genomic DNA recovery

To the aqueous solution (450 µL) containing the genomic DNA, 100 µL of 3M chilled sodium acetate with pH 5.2 was added and double volume of 95% ethanol (i.e. 1100 µL) was added and incubated at -20°C for 30 minutes. The mixture was then centrifuged at 13000 rpm for 20 minutes at 4°C. The supernatant was poured off and the pellet was washed with 250 µL of 70% ethanol without disturbing the pellet. The solution was then centrifuged at 13000 rpm for 10 min at 4°C. After draining the supernatant, the remaining ethanol was removed by keeping the tube open at room temperature for 5-10 minutes. To make sure DNA pellet did not over dry it was not allowed to expose in air for longer duration to evaporate alcohol. The genomic DNA was then re-suspended in 100 µL of autoclaved milliQ water or TE buffer of pH 8.0 and stored at -20°C until use.

3.8 Genomic DNA sequencing

The extracted DNA samples were subjected PCR amplification using 16s rRNA universal primer and gel run was done on the PCR product so obtained. The bands present on gel were visualized for their sharpness by placing them on Gel Doc. The PCR product that possessed the sharpest band were selected for DNA sequencing.

3.8.1 Sequence Editing Alignment

Base call for the chromatograms obtained for each region was achieved via PHRED quality score (Ewing & Green, 1998b). The original forward and reverse raw sequences were assembled and edited in Sequencer v. 4.1.4 (GeneCodes Corporation, USA) to estimate the quality of generated sequence traces. Minimum match percentage 70 and minimum overlap 20 was maintained to assemble the sequences. Removal of gaps and ambiguous nucleotides was done manually and Contig were constantly observed during the process. Further editing of aligned sequences were achieved via comparison with reference sequence (www.ncbi.nlm.nih.gov/blast) where peaks of the chromatograms of both forward and reverse sequences were closely scrutinized. The text format of assembled consensus contig was then imported in Bioedit v.7. All candidate 16s rRNA sequences were aligned by ClusterW, (multiple sequence alignment tools) in Bioedit using default parameters. The both primer end was delineated from the alignment matrix. Primer excluded 16s rRNA sequences were exported for further analysis.

3.8.2 Phylogeny Inference

Neighbor-joining (NJ) method was used for the construction of phylogeny tree in MEGA v.7.0.14. Uniform distribution rate was applied while constructing such evolutionary tree and P-distance was used as genetic measure keeping negative branch length to zero. Reliability of tree was enhanced using typically 1000 replicates of bootstrap.

The scale used for estimation of node support was as follows:

BS 50–74% (weak bootstrap support) and 75-100% for strong support (reference needed search specifically in case of bacterial)

The bootstrap value of ≥50% for clades was considered to indicate the efficiency of tree resolution and identification was regarded successful when monophyletic clades with bootstrap value >50% was achieved in phylogenetic tree constructed.

3.9 Determination of nitrogen fixation using Nessler's reagent

Presence of ammonia and ammonium salts are detected by Nessler's reagent, an alkaline solution of potassium tetraiodomercurate with potassium hydroxide, which turns the media yellow.

To determine the nitrogen fixation, bacterial samples were cultured in Jensen media and incubated for 48 hours. Then after, 100 µL of sample was taken and diluted with 850 µL of distilled water. The solution was then treated with 50 µL of Nessler's reagent and appearance of yellow color was observed (Cappuccino and Sherman, 1992).

The samples that showed yellow coloration indicating presence of ammonia and ammonium salts were used for quantification of those nitrogenous compound using spectrophotometer. The absorbance were recorded for each different samples at a wavelength of 435 nm.

A standard curve was obtained using known concentration of ammonium hydroxide (NH_4OH) and taking the corresponding absorbance at wavelength of 435 nm. A series of different diluted ammonium hydroxide solutions of known concentration (0.11mM, 0.15mM, 0.25mM, 0.35mM, 0.45mm, 0.5mM and 1.0mM) were used to obtain their corresponding absorbance and a standard curve was drawn.

Finally, the concentration of ammonia and their salts produced by different samples were calculated using the standard curve.

3.10 Determination and quantification of PHB (polyhydroxybutyrate)

3.10.1 Detection of PHB producing strain

The single colony of isolates grown in Jensen media was taken and smear was prepared on the glass slide. It was then heat fixed and stained with 0.3% (w/v in 70% ethanol) solution of

Sudan Black B and allowed to stay for 10 minutes. The smear was then decolorized by immersing the slide in xylene. Then after it was counterstained with safranin and was allowed dry for 10 seconds. The slide was then observed under oil immersion (100x) using a Bright field microscope. The bacterial cells that appeared bluish black were PHB positive and were identified as PHB producing strains while others were marked as non PHB producers.

3.10.2 Quantification of PHB

The PHB thus produced by bacterial strains were then quantified. For this, bacteria were grown under different condition such as: N limiting condition and N sufficient condition where in both cases glucose, present as carbon source, was representing C excess condition. Similarly, bacteria was also grown under normal condition without changing the media composition where carbon source was sodium acetate. The OD was constantly checked during the incubation period and sample was taken for quantification after each of the sample maintained the OD of 0.5.

1 ml of each sample obtained from above procedure was taken and centrifuged at 10000 rpm for 5 minutes at room temperature. The pellet so obtained was then washed with 500 µL of 1X PBS (Phosphate buffer saline) and again centrifuged at 10000 rpm for 2 minutes. 500 µL of permeabilisation solution was then added to the pellet obtained followed by centrifugation at 10000 rpm for 5 minutes. The pellet obtained in this case was treated with 1 ml of 1X PBS. The dilution of 1:100 was achieved using 1X PBS and 1 ml of this diluted bacterial suspension was mixed thoroughly through vortexing for few seconds. This diluted bacterial suspension was then mixed with 20 µL of nile red solution of strength 1mM and mixed well through vortexing for few seconds and incubated at room temperature for 30 minutes protecting it from light radiation. Finally the prepared samples were analyzed using flow cytometry (Lagares *et al*., 2017).

3.10.2.1 Set up of flow cytometer for measurement

The flow cytometer was cleaned up using 1X PBS and in order to clearly identify bacterial population in FSC× SSC dot plots, FSC and SSC channel voltages were set to E02 and 300 respectively. The samples were then acquired in flow cytometer and sample flow rate was set at the low flow position. Then FL2 channel voltage was adjusted to maximize the difference of median values corresponding to PHB^+ and PHB^- bacterial population. By observing the dot plots, samples were acquired till utmost 50,000 events per sample were recorded. Finally, the data obtained were analyzed using a software FlowJo. The desired population of bacteria loaded with PHB in each sample was gated and its corresponding histogram was obtained. Histogram of FSC vs FL2-H delivers median fluorescence intensity (MFI) expressed in AFU (Arbitrary fluorescence units) which linearly correlates with the bacterial PHB content. The population of bacteria loaded with PHB in each sample was identified by comparing the corresponding MFI with that of the bacteria that were analyzed as a negative control. All these observations were performed in triplicates for the accuracy and reliability of measurement.

Chapter-4

RESULTS AND DISCUSSION

4.1 Screening of bacterial isolates

The foundation of isolation and screening of bacterial isolates was based on the paradigm of the cellular regulation process, the carbon catabolite repression (CCR) in which bacteria preferentially utilizes one of the carbon sources, most frequently glucose, when facilitated with multiple choices of carbon sources (Deutscher J., 2008). So as to achieve the selective isolation of *Azotobacter* and *Pseudomonas*, the soil sample was incubated at 28^{O}C for 48 hours after enriching with Jensen medium containing sodium acetate. As mentioned in literature, when grown in medium containing both acetate and glucose these bacteria shows diauxic growth and preferentially uses acetate as primary carbon source (Quiroz-Rocha *et al*, 2017). 100 µl sample from so prepared culture beforehand was mixed in top agar followed by pouring in petridish containing Jensen media with ethylene glycol. Since, *Pseudomonas* and *Azotoabacter* are provided with ability to utilize ethylene glycol as a sole source of carbon and energy under oxic as well as anoxic conditions serving as platform from which establish themselves a whole-cell biocatalyst for the production of glyoxylic acid from ethylene glycol (Mückschel *et al*, 2012). Uniformly, 40 different isolates were taken from this plate and streaked in toluene containing plates in pursuance of obtaining *Pseudomonas* only leaving behind the *Azotobacter* that wouldn't survive such environment. *Pseudomonas* by far has been established as exceptionally solvent tolerant through various mechanism and thrives in the presence of organic solvents such as toluene which facilitates it with ability to produce industrially relevant chemicals as well (Volkers *et al*, 2015). Hereby, the colonies that grew in this medium with toluene were marked as *Pseudomonas* and those succumbed were separated as *Azotobacter*. Futher, those *Azotobacter* were separately grown on Jensen media containing Rhamnose as sole carbon source. The putative *Azotobacter* were grown in such media with Rhamnonse considering the insights drawn from literature that affirms presence of an alternative pathway of L-rhamnose metabolism, which is different from the known bacterial pathway in this particular bacterium (Watanabe *et al*., 2008).

Fig 12: Bacteria grown in Jensen media with sodium acetate

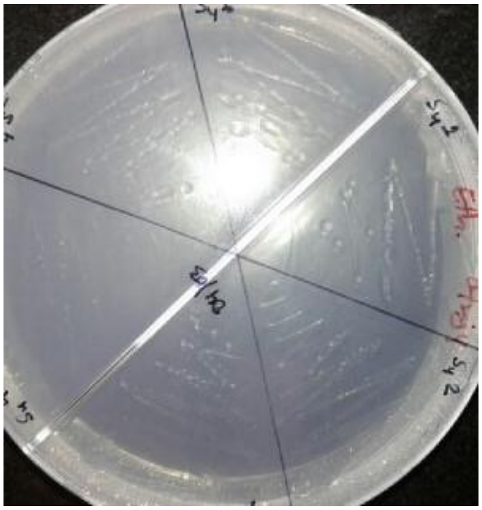


Fig 13: Bacteria grown in Jensen media with ethylene glycol

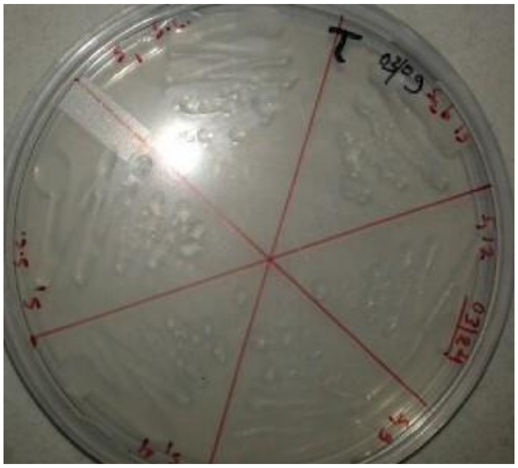


Fig 14: Bacteria grown in Jensen media with toluene

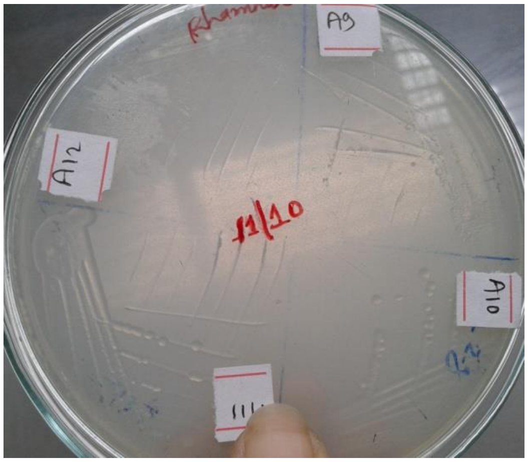


Fig 15: Bacteria grown in Jensen media with Rhamnose

4.2 Morphological Screening

Cell morphology is diverse in large extent and are not just trivial secondary characteristic but are of greater value (Young, 2006). Bacteria with abundance of cellular forms are capable of changing shape during their life cycle with functional significance. Shape varies at the levels of cell geometry, size and surface appendages both placement and number and it relates directly to survival in diverse environment (Yang *et al*, 2016). Bacterial morphology studies are of greater relevance as the shape affects critical biological functions that includes nutrient acquisition, motility, dispersion, stress resistance and interactions with other organisms (Teeseling *et al*., 2017).

Morphological screening of bacterial colonies as well as bacterium itself provides an opportunity to probe the preliminary identification of the putative bacteria. In addition to other biological functions bacterial morphology promotes adherence to biotic and abiotic surfaces, survival under low-nutrient or stressful conditions, evasion of mammalian complement deposition and efficient dispersal through mucous barriers and tissues (Yang *et al*, 2016).

Further, bacteria are subjected to general cataloging through visualization method that divides the bacteria into two very general classes; Gram positive and Gram negative based on their cell wall composition. Gram negative organism have thin peptidoglycan layer(1-2 layers) and have additional lipopolysaccharide layer while those Gram positive possess thick and multilayered (40 layers) peptidoglycan enabling them to retain its purple color in contrast to pink or red color of Gram negative bacteria due to counterstain (Nagata *et al*, 2010). This relatively simple and inexpensive technique allows screening of isolates of interests with information of cell wall composition, cell membrane permeability and also aids in drawing the inferences about the physiological characteristics that putative organism of interest may possess (Bartholomew *et al*, 1952). Out of 36 initial isolates 21 were screened as putative *Azotobacter* while 15 isolates were deemed as putative *Pseudomonas*. Among 21 putative *Azotobacte*r 4 were identified as Gram negative cocci while 17 were recognized as Gram negative rods. Equivalently, in dispersion through 15 putative *Pseudomonas*, 5 of the isolates were found to be Gram positive cocci while remaining 10 isolates were identified as Gram negative that comprises of both rods and cocci in which former shape dominates in larger extent

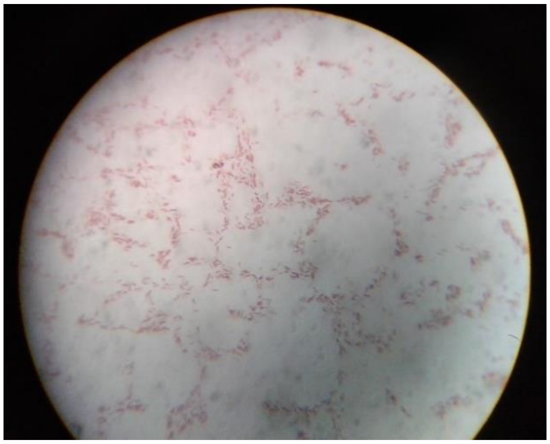


Fig 16: Gram staining of putative *Azotobacter spp*

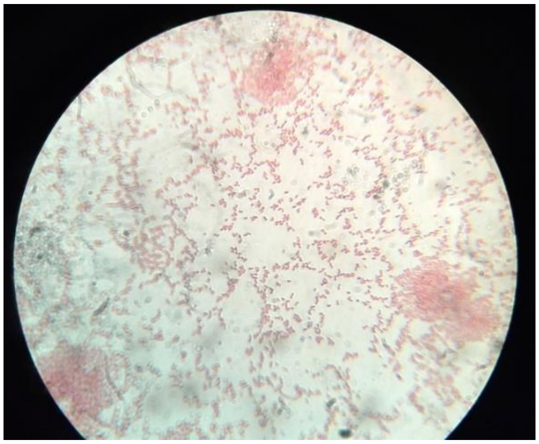


Fig 17: Gram staining of putative *Pseudomonas spp*

Table 1: Morphological characteristics of different isolates from Panchase, Sidhane and Bhadaure

SN	Sample	Cell Shape	Gram Reaction
1	A1	Cocci	Positive
2	A2	Rod	Negative
3	A3	Rod	Negative
4	A4	Rod	Negative
5	A5	Cocci	Negative
6	A8	Rod	Negative
7	A10	Rod	Negative
8	A11	Rod	Negative
9	A12	Rod	Negative
10	A13	Rod	Negative
11	A15	Rod	Negative
12	A16	Rod	Negative
13	A17	Rod	Negative
14	A18	Rod	Negative
15	A19	Rod	Negative
16	A20	Rod	Negative
17	A21	Rod	Negative
18	A22	Rod	Negative
19	A23	Cocci	Positive
20	A24	Cocci	Positive
21	A25	Cocci	Positive
22	P1	Rod	Negative
23	P2	Cocci	Positive
24	P3	Rod	Negative
25	P4	Cocci	Positive
26	P5	Rod	Negative
27	P6	Rod	Negative
28	P7	Cocci	Negative
29	P8	Rod	Negative
30	P9	Rod	Negative
31	P10	Cocci	Positive
32	P11	Rod	Negative
33	P12	Rod	Negative
34	P13	Rod	Negative
35	P14	Cocci	Positive
36	P15	Cocci	Positive

4.3 Biochemical Characterization

Single colony of isolates which exhibited Gram negative results in previous morphological Screening were further subjected to different biochemical tests to reveal further biological characteristics of isolates of interest. Based on previous characterization, 17 isolates of putative *Azotobacter* and 10 isolates of putative *Pseudomonas* were analyzed using different biochemical tests. These tests not only reduced the time required to identify the microbes but also minimized the costs, ensured and enhanced the accuracy of identification of an bacterial samples.

The Bergey's Manual of Determinative Bacteriology was followed in order to classify the isolates of *Azotobacter* and *Pseudomonas* using various biochemical tests. The routine biochemical tests were used in no particular order and the results were tabulated as follows:

Table 2: Biochemical characterization of different isolates

Test/ Sample	**Motility**	**Oxidase**	**O/F Test**	**Catalase**	**Urease**
A2	+	-	F	+	-
A3	+	-	F	+	-
A4	-	+	F	-	-
A5	+	-	F	+	-
A8	+	-	F	+	-
A10	-	+	F	+	-
A11	+	-	F	+	-
A12	+	+	F	+	-
A13	-	+	F	+	+
A15	+	-	F	+	+
A16	-	-	F	+	-
A17	+	+	F	+	-
A18	-	+	F	+	+
A19	+	+	O	+	+
A20	+	-	F	-	-
A21	-	+	O	+	-
A22	+	-	F	+	-
P1	-	+	O	+	-
P3	-	-	O	+	+
P5	-	+	O	+	-

P6	+	-	F	+	-
P7	-	-	F	-	-
P8	+	+	O	+	-
P9	+	+	O	-	-
P11	-	-	O	+	-
P12	+	+	F	-	-
P13	+	-	O	+	-

(+) = positive, (-) = negative, O =Oxidative F=Fermentative, R = red, Y = yellow, K = alkali, A = acid, K/A = only glucose fermenter, A/A = glucose, lactose/sucrose fermenter

Table 3: Additional biochemical characterization of different isolates

Sample	**IMViC**				**TSIA**				
	Indole	MR	VP	Citrate	Butt	Slant	Gas Production	H_2S Production	Result
A2	+	-	-	-	R	Y	+	-	K/A
A3	+	+	-	+	R	Y	-	-	K/A
A4	-	+	-	+	Y	Y	+	+	A/A
A5	+	-	-	+	R	Y	-	-	K/A
A8	+	-	-	+	R	Y	-	+	K/A
A10	-	+	-	+	Y	Y	+	-	A/A
A11	-	-	+	-	Y	Y	+	+	A/A
A12	+	-	-	+	R	Y	-	-	K/A
A13	+	-	-	-	Y	Y	+	+	A/A
A15	-	-	+	+	Y	Y	+	+	A/A
A16	+	+	-	+	R	Y	-	-	K/A
A17	+	-	-	+	Y	Y	-	-	A/A
A18	-	-	-	+	Y	Y	-	-	A/A
A19	-	-	+	-	R	Y	+	+	K/A
A20	+	-	-	+	Y	Y	-	-	A/A
A21	-	-	-	+	Y	Y	-	-	A/A
A22	+	+	-	+	R	Y	-	-	K/A
P1	-	+	+	+	R	Y	-	+	K/A
P3	-	-	-	+	R	Y	-	-	K/A
P5	-	+	+	+	R	Y	-	-	K/A

P6	+	-	-	+	Y	Y	+	-	A/A
P7	-			-	R	Y	-	+	K/A
P8	-	-	-	+	R	Y	+	-	K/A
P9	-	-	-	-	R	Y	-	-	K/A
P11	+	+	-	+	Y	Y	+	+	A/A
P12	-	+	+	-	Y	Y	+	+	A/A
P13	+	-	-	+	R	Y	-	-	K/A

(+) = positive, (-) = negative, O =Oxidative F=Fermentative, R = red, Y = yellow, K = alkali, A = acid, K/A = only glucose fermenter, A/A = glucose, lactose/sucrose fermenter

4.4 Molecular Characterization

The characterization and identification of bacteria was done using two of the molecular methods namely: Polymerase chain reaction (PCR) amplification and DNA sequence analysis.

4.4.1 PCR amplification

The four samples that produced the best results in previous assessments, were further scrutinized by PCR amplification for their actuality as bacterial cells. The universal primer used yielded an amplicon of the expected size ~1500 bp as shown in the figure below:

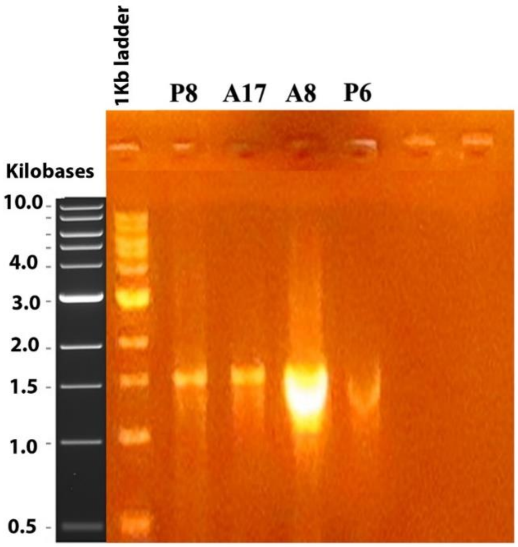


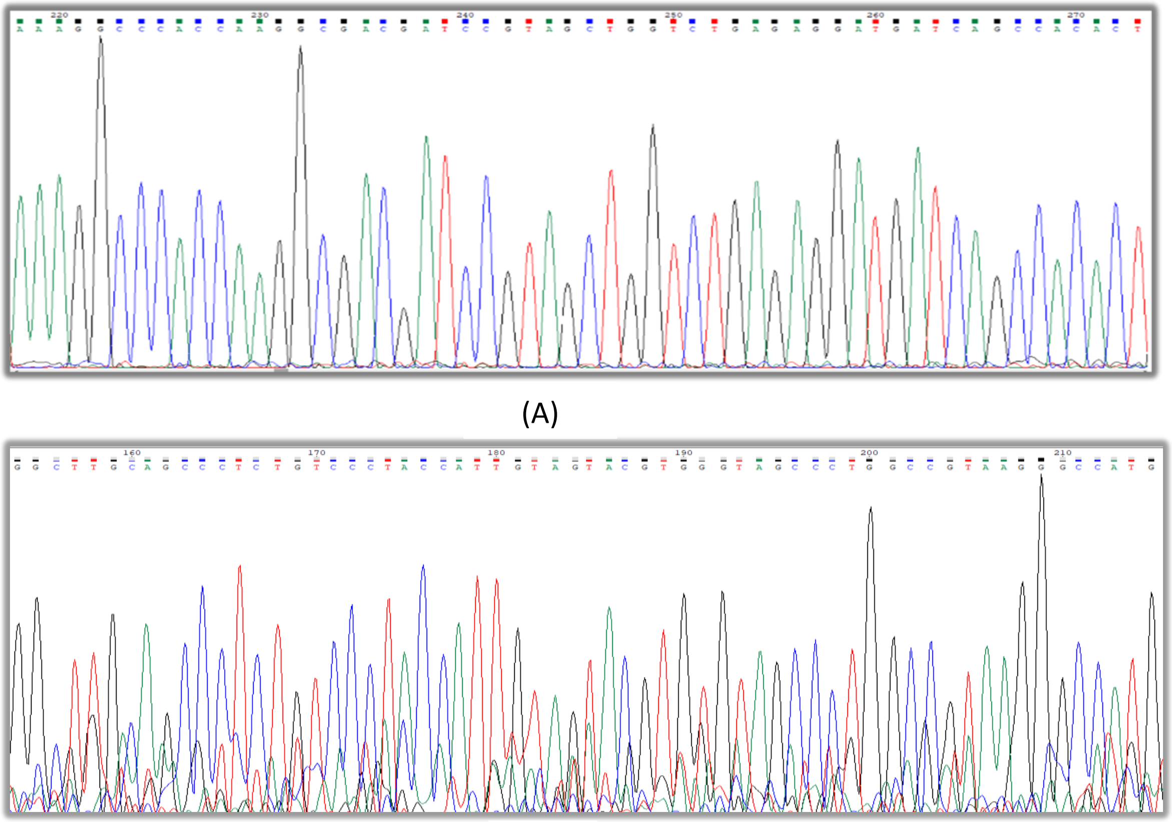
Fig 18: Gel electrophoresis performed on PCR product of 16s rRNA gene of putative *Azobacter* (A17 and A8) and *Pseudomonas* (P8 and P6) using universal primer

The gel electrophoresis picture verified all the samples to be of bacteria and two samples; A17 and P8 that showed the prominent DNA band were further analyzed by DNA sequencing method.

4.4.2 DNA sequence analysis

DNA sequence data answers many types of questions since DNA sequences differ considerably between species and between individuals within a species. DNA sequences are extensively used for identification and was used for the same purpose here as well.

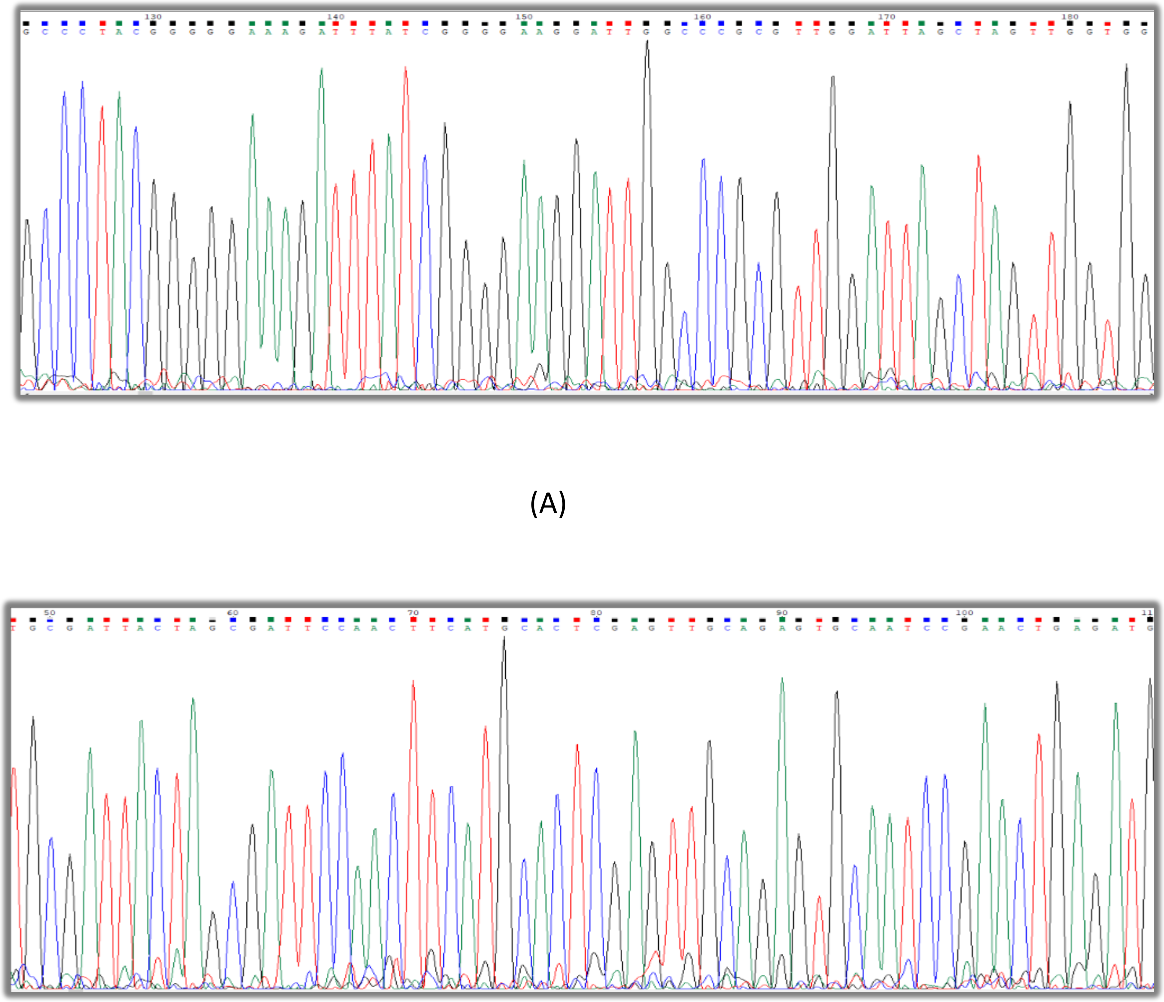
The PCR product of the isolates were further characterized by DNA sequence analysis to confirm the bacterial species. The sequence read obtained showed maximum resemblance with the presumed respective bacteria. The sample A17 showed 98% similarity with the gene cluster sequence of *Azotobacter* species available from GenBank database while P8 depicted 99% concurrence with *Pseudomonas* species. The blast hits retained significant e-value of 0.0. The chromatogram obtained from sequencing are shown in figure below:



(A)

(B)

Fig 19: Chromatogram of sample P8 (A) for forward primer sequence (B) for reverse primer sequence



(A)

(B)

Fig 20: Chromatogram of sample A17 (A) for forward primer sequence (B) for reverse primer sequence

The both chromatograms; forward as well as reverse for each of the samples P8 and A17 shows scanty baseline noise and therefore real peaks are still easy to call. However, noise in case of reverse primer sequence of sample P8 might have resulted because sample itself may have been dim. All in all, chromatograms obtained are devoid of polymorphism and confirms no disputes with the samples. Hence these results were further characterized by multiple sequence alignment.

4.4.3 Multiple sequence alignment

The sequence obtained by DNA sequencing was further characterized by multiple sequencing alignment. The highly similar sequence obtained in BLAST search indicate a significant alignment of the putative DNA sequence to 16s rRNA genes of *Pseudomonas spp* for P8 and *Azotobacter spp* for A17 samples. The clustering approach called neighbor joining (NJ) method was used for the reconstruction of phylogenetic trees that yielded unrooted tree with branched lengths as follows:

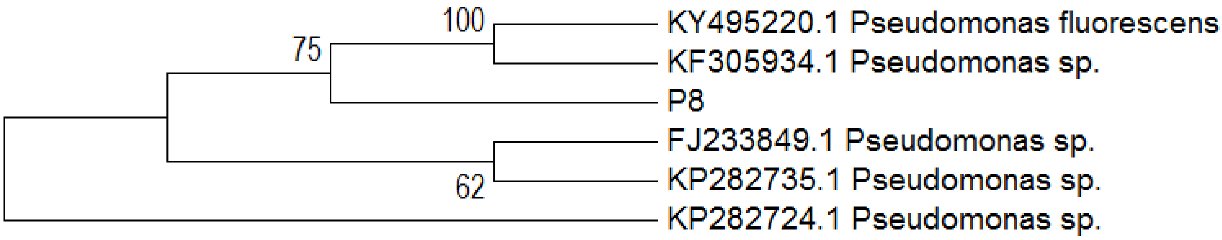


Fig 21: 16s rRNA tree generated with the neighbor joining method with 1000 bootstrap resamplings for P8.

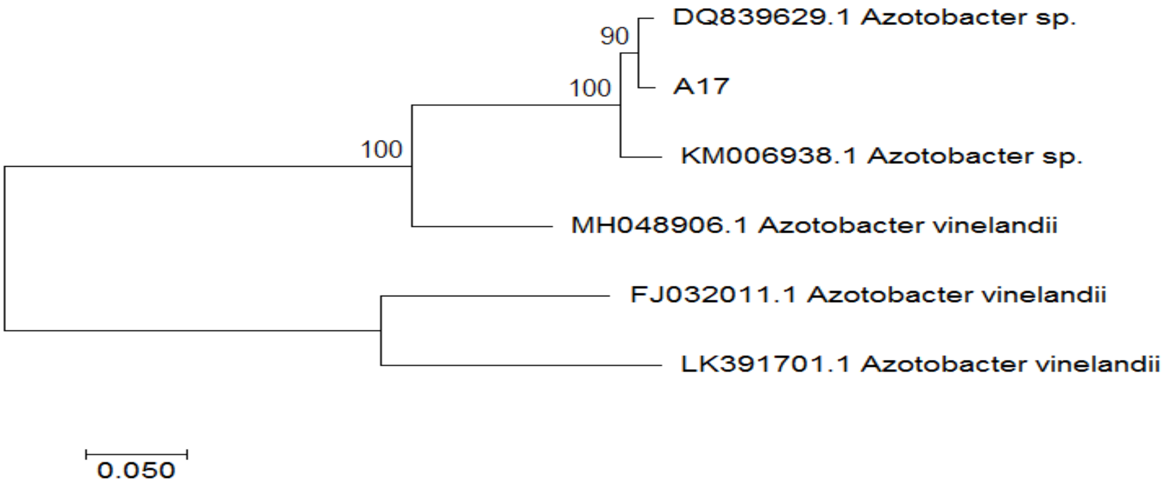


Fig 22: 16s rRNA tree generated with the neighbor joining method with 1000 bootstrap resampling for A17.

The trees above shows the evolutionary relationship of isolate P8 with the *Pseudomonas* genus while relationship of isolate A17 with the *Azotobacter* genus based on their 16s rRNA sequences. In above figures branch with 100% support indicates certainty that is the species with in it were always found together as a cluster. Hence other sequences belong to that cluster.

According to the 16s rRNA phylogenetic tree in Fig. 1, P8 has a close relationship with *Pseudomonas* species. The tree shows that the 16s rRNA sequence of this organism is very similar to *P. fluorescens* 16s rRNA sequences. Similarly, as reported in fig. 2, the isolate A17 has a very close relationship with *Azotobacter* species. It can be infered from the tree that the 16s rRNA sequence of A17 is very similar to *A. vinelandii* 16s rRNA sequences.

The bootstrap results in common interprets the probability that phylogenetic estimation represents the true phylogeny. Explicitly, under various conditions such as equal rates of change, symmetric phylogenies, and internodal change of <20% of the characters, bootstrap proportions of >70% usually correspond to a probability of >95% that the corresponding clade is real [Hillis *et al*, 1993]. Hence, from the above trees, the higher bootstrap value obtained for both of the samples confirms the respective samples to be the one that has been presumed. Therefore, P8 showed the identical similarities with *Pseudomonas fluorescens* while A17 confirmed such relationship with *Azotobacter vinelandii*.

4.5 Nitrogen fixation and ammonium quantification

Diazotrophs *Azotobacter* and Pseudomonas are known to fix the atmospheric nitrogen without symbiotic relations with plants, although some *Azotobacter* species are associated with plants (Kass *et al*., 1971). Concern with the quantitative analysis for the intermediates in biological N_2 fixation is centered on ammonia because it is the only demonstrated intermediate in the process (Burris, 1972). Ammonia is determined colorimetrically with the Nessler's reagent. So as to study the nitrogen fixation, 17 isolates of putative *Azotobacter* and 10 isolates of putative *Pseudomonas* cultured in nitrogen free medium were tested for presence of ammonium ions using Nessler's reagent and further quantified spectrophotometrically. The change in color of liquid culture to yellow indicated the presence of ammonium ion which then further quantified by taking absorbance at wavelength 435 nm peak since reaction mixture showed maxima at this wave length. The amount was quantified from the standard curve of NH_4OH taken at 430 nm. Among all the samples tested, one from each genus that showed the maximum ammonium production were studied for dependence of incubation time with ammonia production as well.

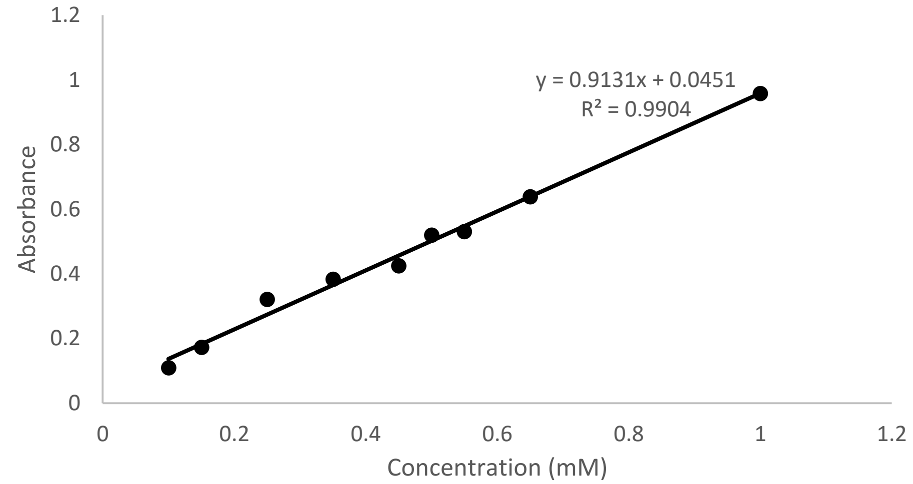


Fig 23: Standard curve of NH_4OH at 435 nm used for ammonium ion quantification of unknown samples

Table 4: Ammonium quantification of different putative *Azotobacter vinelandii and Pseudomonas fluorescens*

Sample	**Average Absorbance**	**Average Concentration (mg/L)**
A2	0.204	6.2095
A3	0.198	5.9685
A4	0.128	3.3043
A5	0.222	6.8819
A8	0.228	7.1103
A10	0.306	10.0789
A11	0.173	5.0170
A12	0.2	6.0446
A13	0.179	5.2580
A15	0.221	6.8692
A16	0.154	4.3065
A17	0.463	16.0543
A18	0.091	1.9087
A19	0.291	9.5080
A20	0.148	4.0655
A21	0.107	2.5177
A22	0.079	1.4393
P1	0.071	1.1475
P3	0.044	0.1326
P5	0.044	0.1199
P6	0.054	0.4878

P7	0.054	0.4878
P8	0.077	1.3759
P9	0.054	0.5005
P11	0.043	0.0945
P12	0.064	0.8684
P13	0.069	1.0587

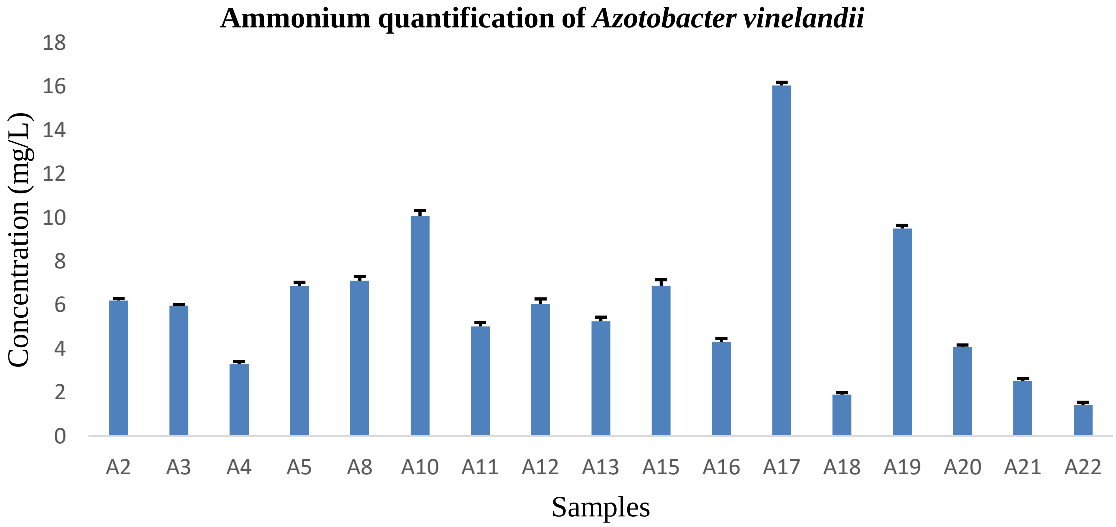


Fig 24: Ammonium quantification of different putative *Azotobacter* isolates

A17 showed the maximum value of 16.0543 gm/L of quantified ammonium while other isolates of putative Azotobacter remained quite insignificant compared to it. A10 and A19 also exhibits promising value of 10.0789 gm/L and 9.5080 gm/L of quantified ammonium ion. All these results were recorded for the incubation time of 96 hours.

Similar experiments were done on putative *Pseudomonas* species and following results of quantified ammonium were obtained which was well depicted in histogram below. In case of putative *Pseudomonas* maximum value of 1.3759 gm/L was achieved for 96 hour of incubation for sample P8. Comparable results were obtained for sample P1, P12 and P13 with value of 1.1475, 0.8684, and 1.0587 mg/L respectively while rest remain insignificant.

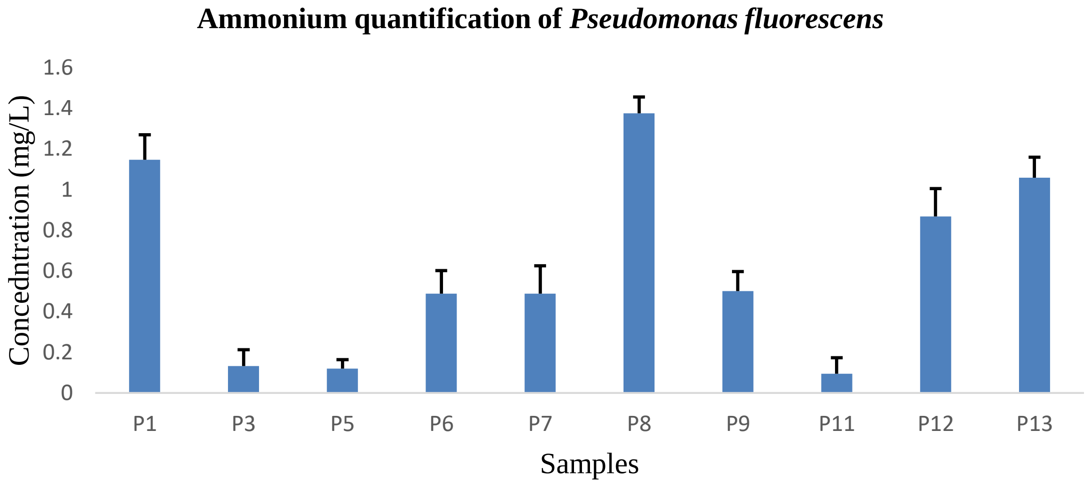
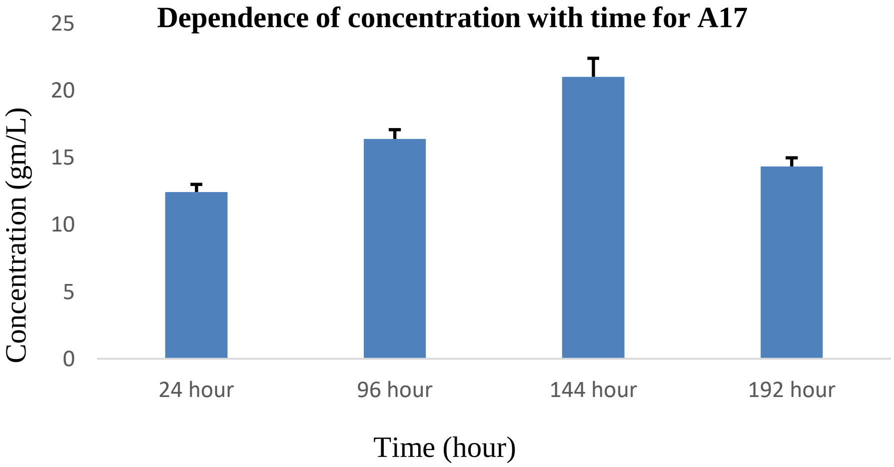
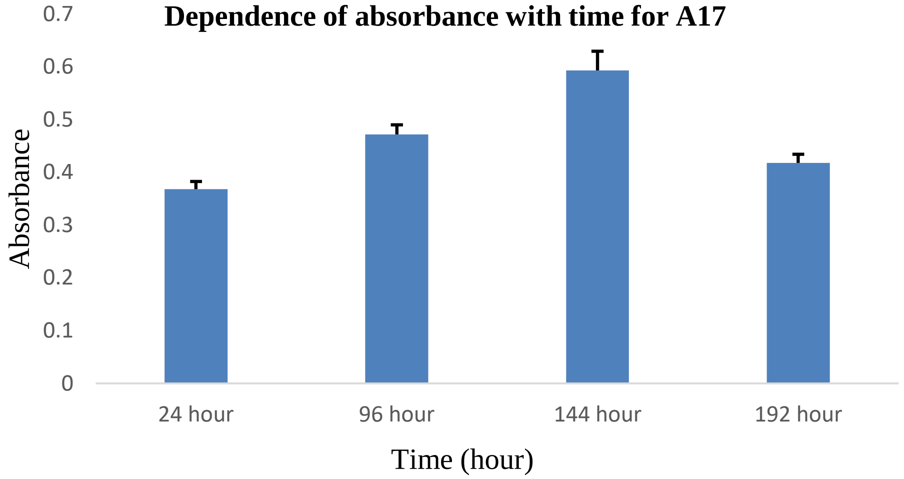


Fig 25: Ammonium quantification of different putative *Pseudomonas* isolates

Samples with highest ammonium production were studied for their dependence of the time of incubation with concentration of ammonium ion and hence eventually on the absorbance. Two isolates namely A17 of putative *Azotobacter* and P8 of putative *Pseudomonas* were selected based on their capacity of fixing nitrogen and converting them into ammonium ion.

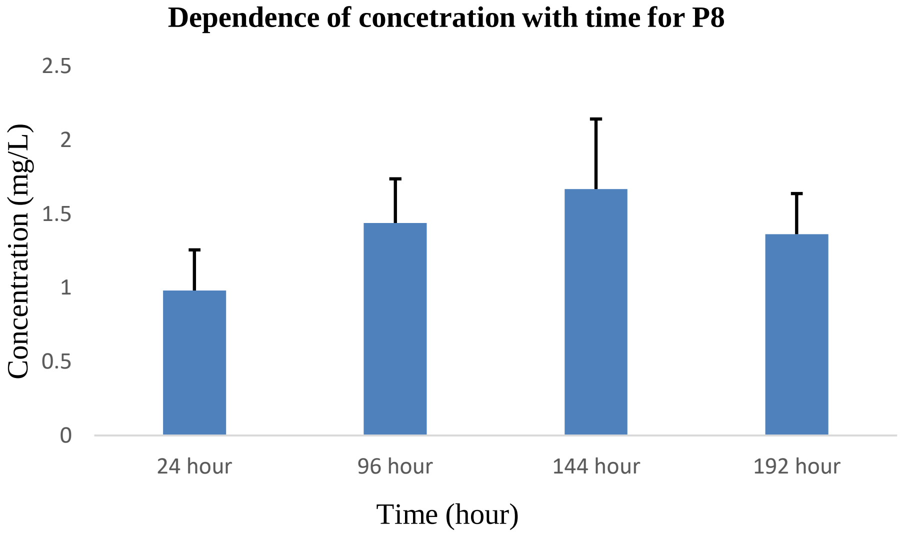


(A)

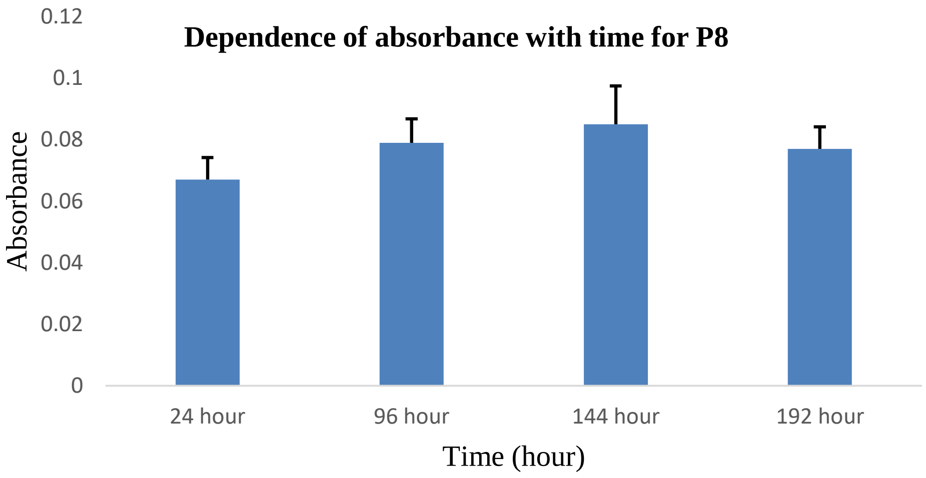


(B)

Fig 26: (A) Dependence of ammonium concentration and (B) absorbance with time of incubation for putative *Azotobacter*



(A)



(B)

Fig 27: (A) Dependence of ammonium concentration and (B) absorbance with time of incubation for putative *Pseudomonas*

While studying the dependence of concentration of quantified ammonium with time it was clearly observed that with prolonged incubation, the amount of quantified ammonium ion increased significantly reaching the maximum value of 21.8395024 gm/L in case of A17 and 1.8199667 gm/L in case of P8 for incubation period of 144 hours. Later on value of concentration tend to decrease for both of the bacteria following the typical bacterial growth curve.

4.6 PHB production

Azotobacter vinelandii has so far been found to assimilate different carbon sources as the sole carbon source for cell growth and some of them are in addition used for biopolymer production (Yoneyama *et al*., 2015). Then as well, synthesis of mcl-PHA has also been observed in fluorescent and non-fluorescent *Pseudomonas* species, whereas PHB production is not a common characteristic of *Pseudomonas spp*. Still PHB production in large extent has been reported in various works by many workers in past (Cantone *et al*., 2014).

4.6.1 Detection of PHB Production ability

The sample A17 and P8 were evaluated for the ability to produce PHB. The PHB production was verified by Sudan Black staining under light microscope. Bacterial cells appearing blue or black under the microscope were considered PHB producing (Wei *et al*, 2011). Both the samples showed the visible positive results regarding the process and were confirmed to be PHB producers.

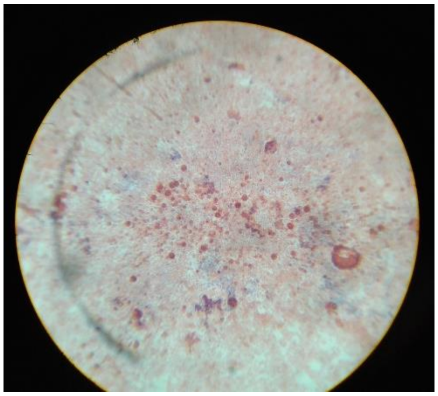


Fig 28: SB staining of *Azotobacter*

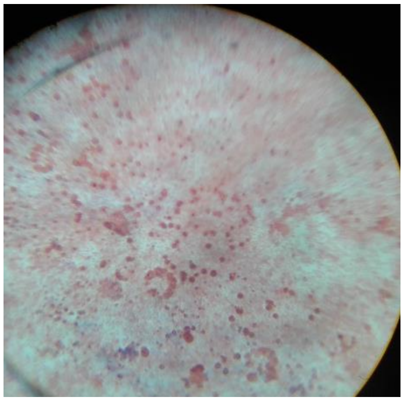


Fig 29: SB staining of *Pseudomonas*

4.6.2 PHB quantification using flow cytometry using Nile Red fluorescence

For determination of PHB an optimized method with every precaution possible to ensure high accuracy in flow cytometry was adapted (Lagares *et al*., 2017). Both samples (A17 and P8) with different conditions (varying media composition of N and C source) along with control (without Nile red staining) were analyzed by flow cytometer to evaluate maximum production of PHB. Two plots were obtained in the form of dot plots and histogram; of which former depicts the PHB stained population while later illustrates total event count against fluorescence intensity expressed in arbitrary fluorescence units (AFU) that was registered by the channel FL2. The median fluorescence intensity (MFI) values were for different bacterial population were also indicted in the corresponding histogram insets.

4.6.2.1 Analysis of sample P8 (*Pseudomonas fluorescens*)

A sample of *Pseudomonas fluorescens* was analyzed under different condition so as to attain the maximum production of PHB and to determine the limiting condition that would eventually check the rate of PHB production. To determine the nature of plot of non-staining condition that yield nonspecific fluorescence was also performed as follows:

PHB quantification under control condition (Nile red free)

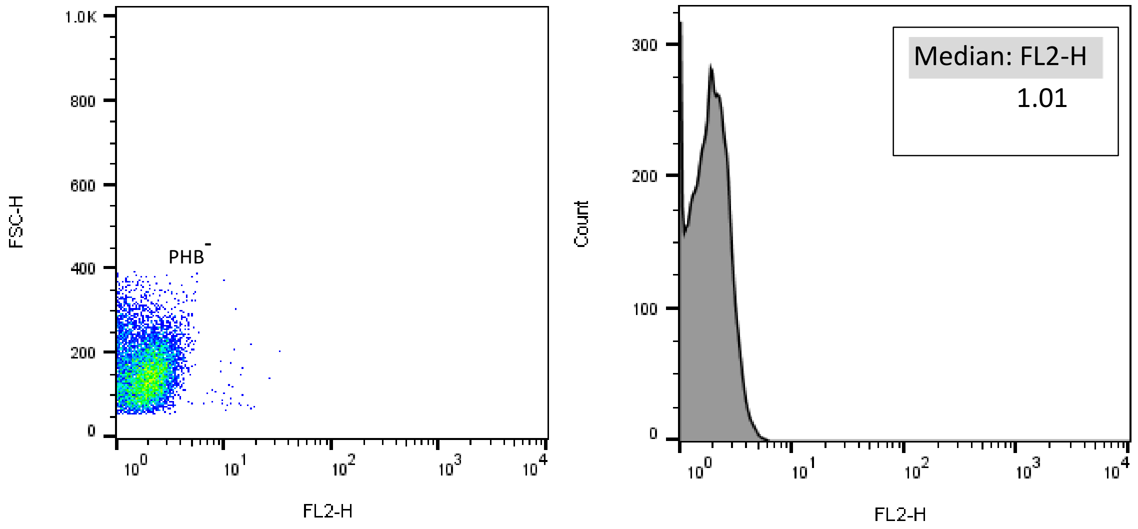


Fig 30: Dot plots (left panels) and histograms (right panels) from a sample of *Pseudomonas fluorescens* cells grown under controlled condition where PHB remains unstained in absence of Nile Red.

PHB quantification under normal condition (as in media composition)

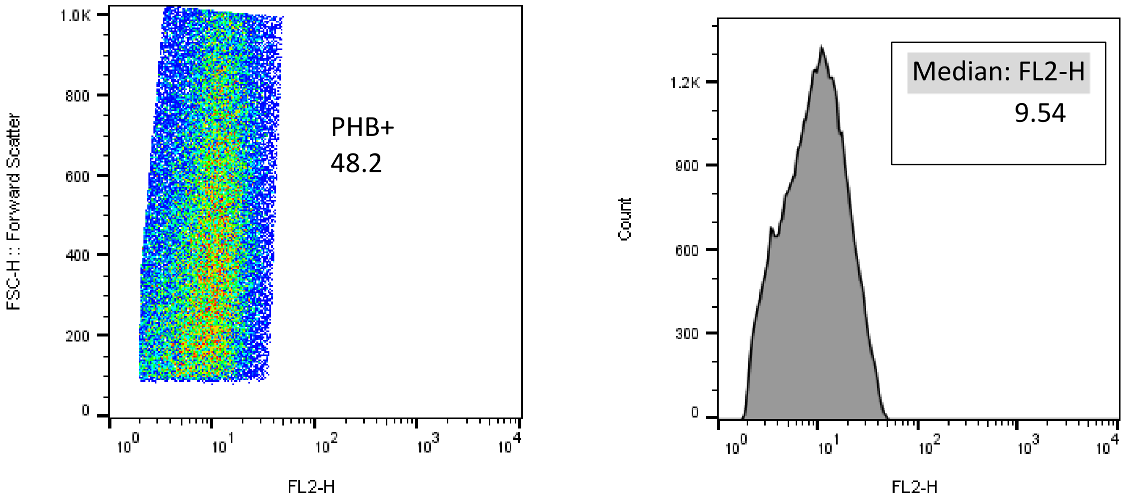


Fig 31: Dot plots (left panels) and histograms (right panels) from a sample of *Pseudomonas fluorescens* cells grown under normal condition without altering the media composition where PHB, if present, gets stained with Nile Red.

PHB quantification under C excess and N-limiting condition

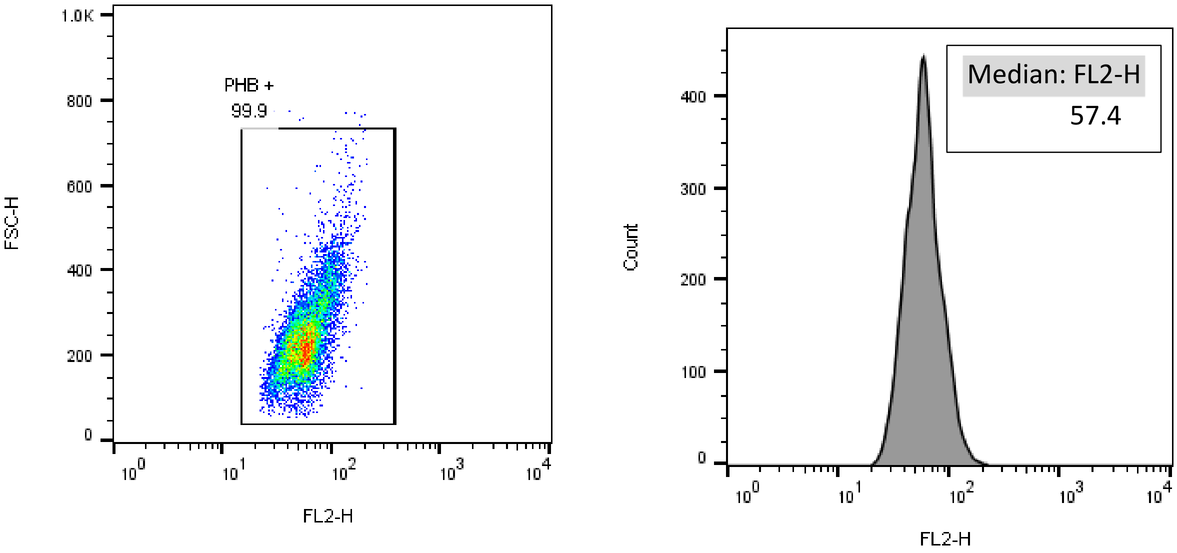


Fig 32: Dot plots (left panels) and histograms (right panels) from a sample of *Pseudomonas fluorescens* cells grown under nitrogen-limiting and carbon excess condition that enables PHB accumulation.

PHB quantification under C-excess and N-sufficient condition

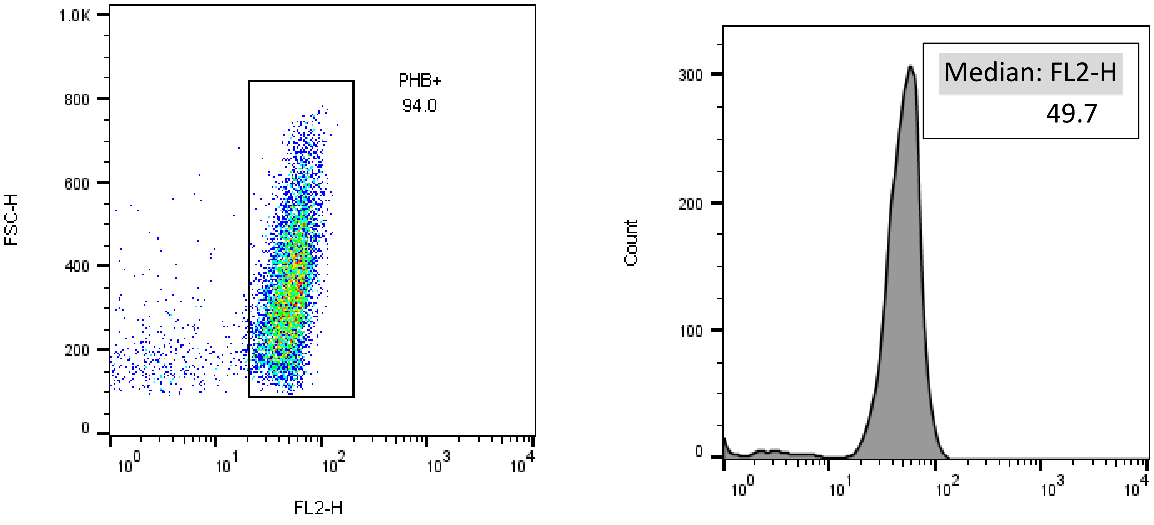


Fig 33: Dot plots (left panels) and histograms (right panels) from a sample of *Pseudomonas fluorescens* cells grown under nitrogen sufficient conditions as a negative control for PHB accumulation.

4.6.2.2 PHB quantification of *Pseudomonas fluorescens* under different conditions

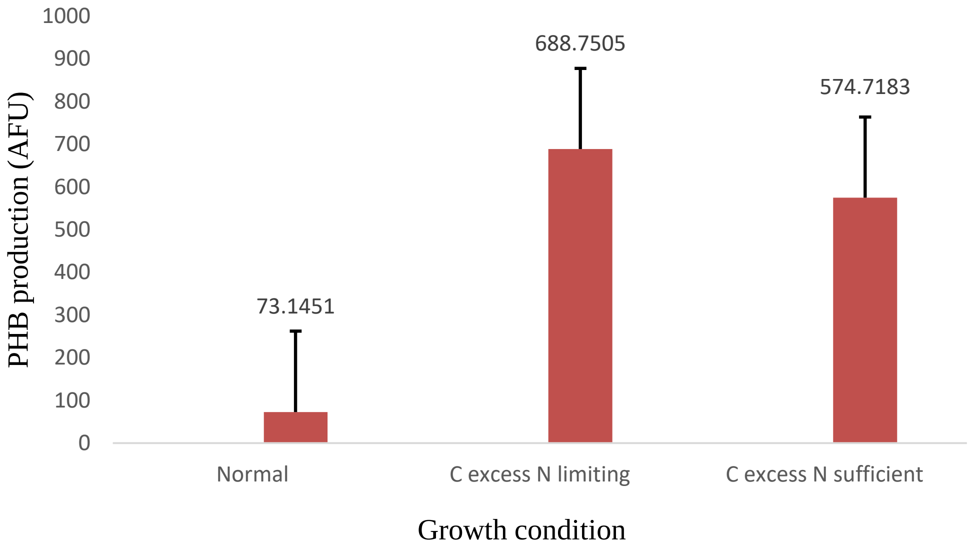


Fig 34: PHB production by *Pseudomonas fluorescens* under different conditions

A method based on flow cytometry of Nile red stained bacterial cells was established to quantify poly-3-hydroxybutyrate (PHB) production. Many works in the past have successfully testified a linear correlation between the amount of PHB and the fluorescence emission intensity of NR stained cells (Alves *et al*, 2017). The bacterial PHB content can directly be correlated with median fluorescence intensity (MFI; expressed in AFU, Arbitrary Fluorescence Units) from histograms of FL2-H. By comparing corresponding MFIs of bacteria that were analyzed as a negative control, population of bacteria loaded with PHB can easily be identified (Lagares *et al*., 2017).

In Fig 30, histogram depicts the nature of plot when bacteria with unstained PHB are subjected to quantification that showed the minimum median value of 1.01. In contrast to this, rest of the growth condition which were investigated for their maximum production of PHB has displayed larger median value proving Nile Red to be successful in staining PHB which then got quantified.

Fig 31 displays that under normal condition of growth where C source was sodium acetate; PHB production has found to be the minimum with 48.2% of the population producing such biopolymer. The median value of 9.54 which is much larger than median value of unstained sample suggests there is production and successful staining of PHB. Additionally, by comparing the histograms in Fig 32 and 33, it can be inferred that C excess and N limiting favors the maximum production of PHB where maximum value of 688.7505 AFU has been achieved with median 57.4 where 99.9% of the population produced PHB while in N sufficient condition with carbon excess only 94% of the PHB producers were identified with median value of 49.7 and PHB content of 574.7183 AFU. This validates the remark that PHB is accumulated under conditions of unbalanced growth as stated in previous works (Jendrossek *et al*, 2014). Hence, nutrient exhaustion and availability of carbon source has prompt the organism to switch on the accumulation of neutral polymer, the PHB, which enables it to store carbon and minimizes power usage (Lagares *et al*, 2017).

4.6.2.3 Analysis of sample A17 (*Azotobacter vinelandii*)

As in the case of *Pseudomonas* similar set of experiments were performed in the case of *A. vinelandii* that yielded following results which were then analyzed for determination of optimized condition of maximum PHB production.

PHB quantification under control condition (Nile red free)

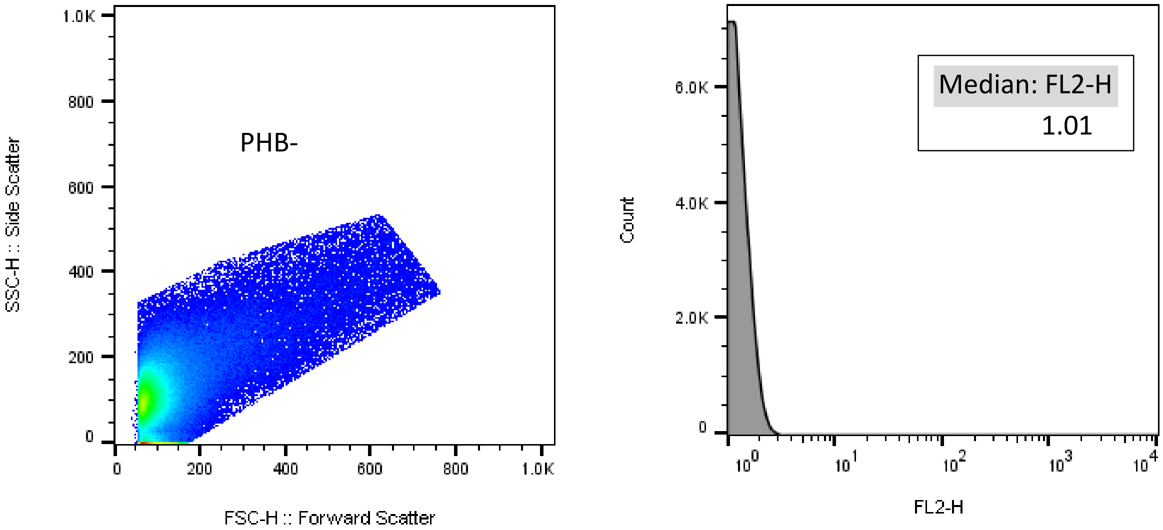


Fig 35: Dot plots (left panels) and histograms (right panels) from a sample of *Azotobacter vinelandii* grown under controlled condition where PHB remains unstained in absence of Nile Red.

PHB quantification under normal condition (as in media composition)

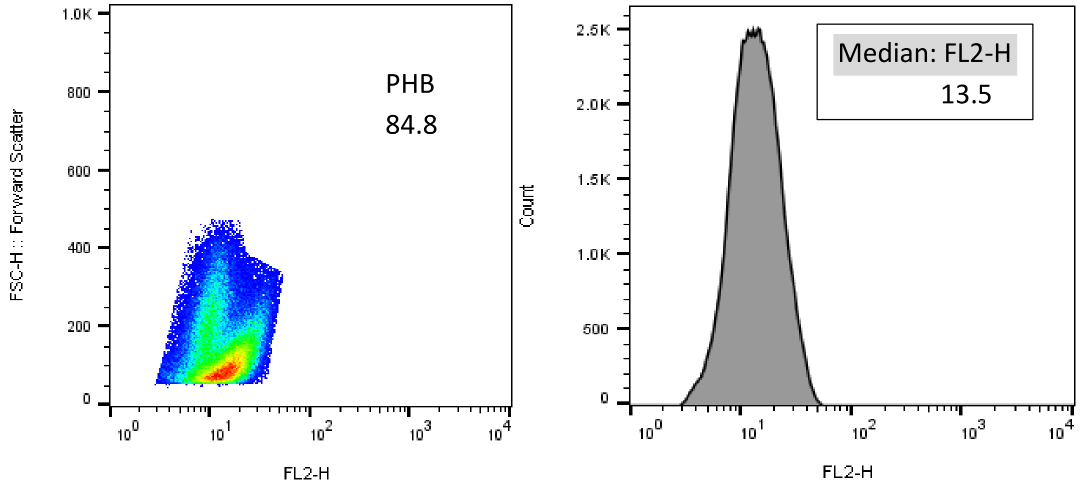


Fig 36: Dot plots (left panels) and histograms (right panels) from a sample of *Azotobacter vinelandii* grown under normal condition without altering the media composition where PHB, if present, gets stained with Nile Red.

PHB quantification under excess C and N-limiting condition

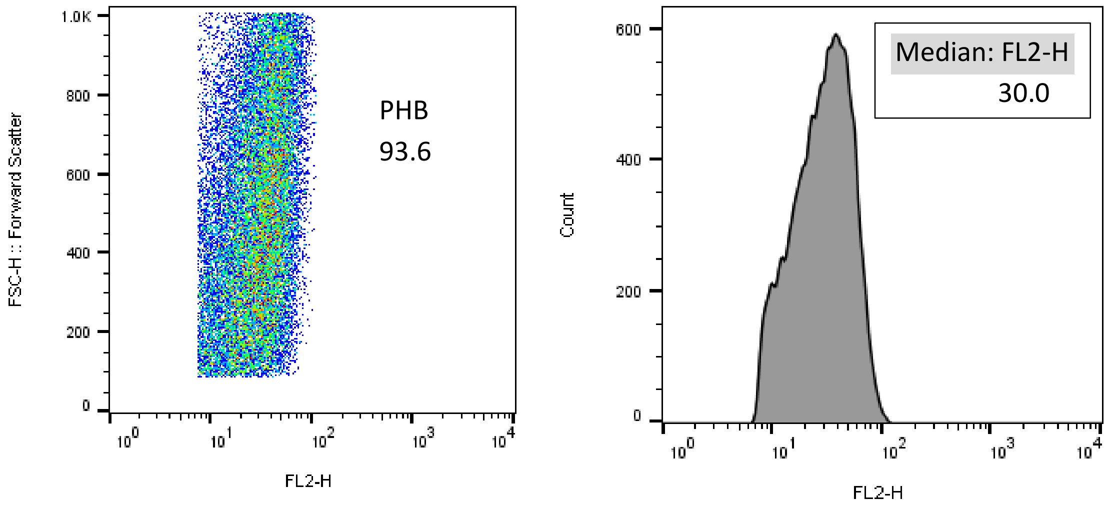


Fig 37: Dot plots (left panels) and histograms (right panels) from a sample of *Azotobacter vinelandii* grown under nitrogen-limiting and carbon excess condition that enables PHB accumulation.

PHB quantification under C-excess and N-sufficient condition

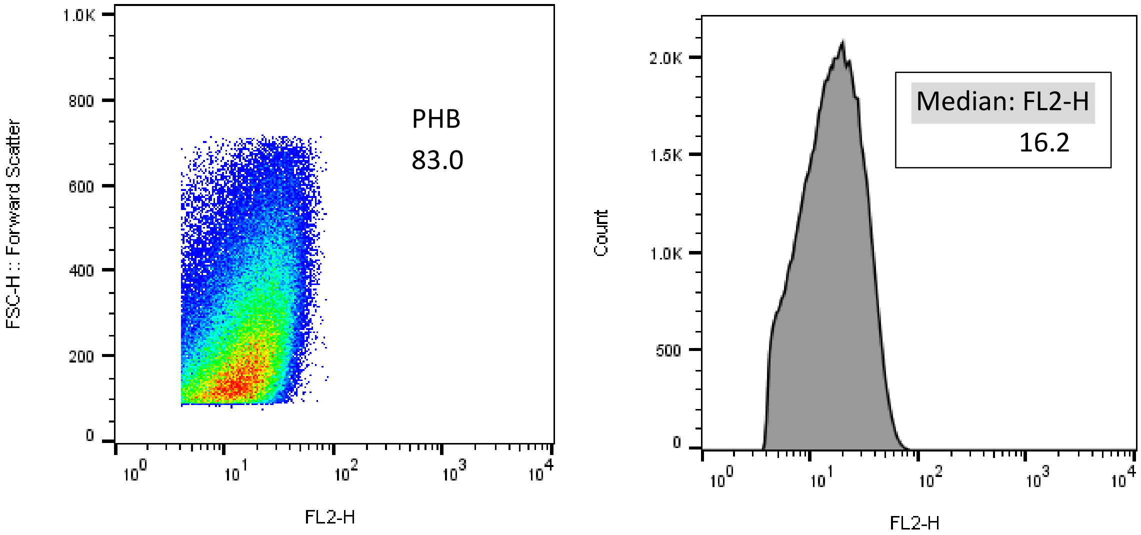


Fig 38: Dot plots (left panels) and histograms (right panels) from a sample of *Azotobacter vinelandii* grown under nitrogen sufficient conditions as a negative control for PHB accumulation.

4.6.2.4 PHB quantification of *Azotobacter vinelandii* under different conditions

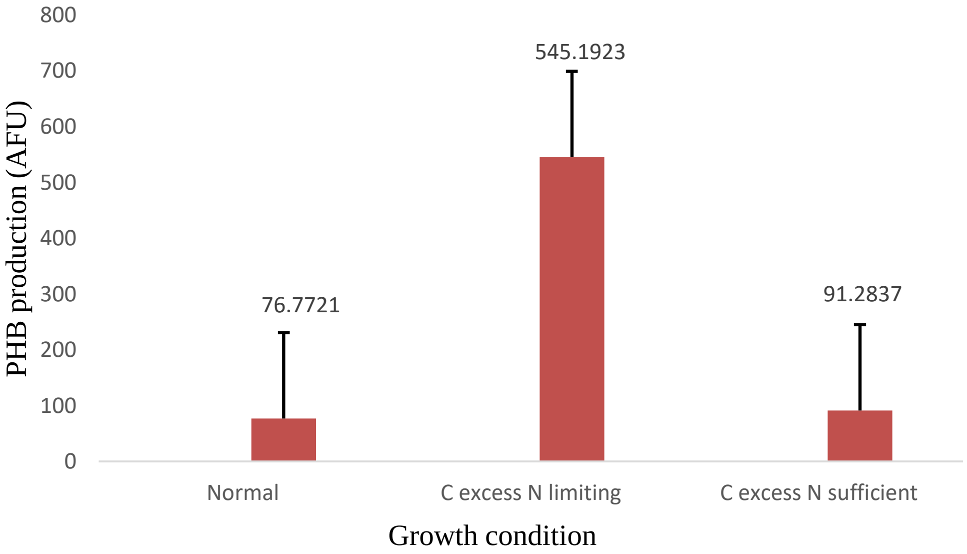


Fig 39: PHB production by *Azotobacter vinelandii* under different conditions.

The trend of PHB production remained indifferent in the case of *Azotobacter vinelandii*, however difference in amount of production is noteworthy

Fig 35 portrays a comparable result obtained for *Pseudomonas* when unstained PHB were quantified. Similar value of median 1.01 was observed that stood for statistical minima while for other growth conditions the values shifted to positive scale direction indicating successful staining of PHBs in the bacterial cells.

The normal condition of growth with sodium acetate as C source, PHB production was found to be the lowest however it is slightly more as compared to *Pseudomona*s under similar condition. The median value of 13.5 with 84.8% of the population producing PHBs contributed to larger yield as depicted by Fig. 36.

The analysis of Fig. 36, 37 and 38 confirms that optimum condition for the maximum production of PHB in the case of *Azotobacter* happens to be C excess and N limiting as obtained in the previous case. The maximum value of 545.1923 AFU of PHB was obtained with the median value of 30.0 where 93.6% of the total population were actively producing PHB. Nevertheless, this value remains modest relative to the value obtained for *Pseudomonas*. Further, in the case of N sufficient condition the value becomes appreciably trivial in the case of *Azotobacter* with production value of only 91.2837 AFU. But at the same time these results still maintains the claim of previous works that states unbalanced condition favors PHB production (Jendrossek *et al*, 2014).

Under the similar optimized condition for production of PHB, *Pseudomonas* was found to be more cost efficient compared to *Azotobacter*. However, later one still maintains the trend of production with satisfactory production under optimum condition.

CHAPTER 5

SUMMARY

An extensive search for replacement of synthetic fertilizer and polymer which is efficient and green has proven to be an elusive quest. Several researches has been done in past and some are still on the verge of finding out the methods for eliminating existing problems caused by synthetic fertilizers and polymers. Fertilizers and polymers are the two group of compounds whose use is unavoidable hence finding their alternative is the only choice. This work is entirely focused on filling up the gap or the inadequacy of such researches. Exploration of bacteria that can not only fix the atmospheric nitrogen acting as biofertilizer but can also store carbon source which can be utilized as biopolymer is the thematic idea of the whole research. To come up with such bacteria will serve as potential candidate prone to industrial application validating the purpose of the study.

In order to achieve the goal of finding out bacteria with nitrogen fixing ability along with its biopolymer producing capability, various subsequent experimentation and protocol were designed and followed. The initial collection of samples from soil and their preliminary identification via biochemical tests were done. The putative bacterial samples were then subjected to molecular identification. The PCR amplification followed by gel electrophoresis verified our samples to be of bacterial strain where universal primer yielded the amplicon of the expected size ~1500 bp. The DNA sequence analysis further verified and established the putative bacterial sample to be of the desired ones. Hence, these bacteria (*Azotobacter vinelandii* and *Pseudomonas fluorescens*) needed further examination for their ability to produce PHB and to fix atmospheric nitrogen.

Nitrogen is an essential nutrient for plants and all are not equipped with mechanism to fix direct atmospheric nitrogen into form which can be assimilated hence requires supplemental supply of them. The isolated bacteria thus was hypothesized to possess the mechanism which will benefit the plants that lack such nitrogen fixing ability. The isolated bacterial cells initially scrutinized using Nessler reagent which established them as nitrogen fixer. So further analysis involved, quantification of such fixed nitrogen spectrophotometrically. The quantified ammonium by both of the isolates displayed their credibility as nitrogen fixing biofertilizer.

Further aim of the study, to investigate the isolates for PHB production, was to address the existing global concern of managing solid waste because of synthetic polymer and to look for the alternative approach that doesn’t rely on fossil fuel to meet the ever increasing demand of polymers. Bioplastics and biopolymers, a better substitute for conventional non degradable plastics, were sought here in the study where PHB is already established biopolymer candidate produced by bacteria. To check such ability to produce PHB, Sudan Black was utilized to stain the cells which were then observed under oil immersion bright field microscope. The cells when appeared bluish black proved their competence as PHB producer.

Flow cytometry further studied the PHB producers and analyzed the data, using FlowJo software. The quantified amount of PHB produced by isolated samples established them as potent biopolymer producers that are suitable for industrial production.

Thus, the study confirms the isolated bacteria with strength to produce not only biopolymer but also fix the atmospheric nitrogen acting as biofertilizer. Hence, this study provides an alternate solution that meets global demand and at the same time settles global concern. When scaled up in industrial level, the findings of the study will act as milestone in an attempt to resolve the complications faced by agricultural sector and void suffered by polymer industries due to lack of biodegradable alternatives.

CHAPTER 6

CONCLUSION

Maintenance of sustainability of modern agriculture involves search for alternative approaches. Ever increasing food demands with limited land area necessitates the utilization of fertilizers which come with stumbling blocks. Sustainable supplementation and associated improvement of soil fertility with non-hazardous effects to environment, are the traits looked upon in fertilizer. To increase efficiency and at the same time minimize the negative impacts, is of prime intention while developing fertilizers. The external chemical supplant for nitrogen needs an alternative for same reason. Hence, the biofertilizers are long been sought for. Similarly, the demands of polymers depending upon its usage in wider range, is outrageous, hence their supplies. Such practice has led the problem in solid waste management and also to enhanced dependence on fossil fuels for their production, eventually leading to energy crisis. Hence, biodegradable plastics synthesized by bacteria will be of great significance. These would not only meet upsurge of the demands but will ensure the shift in energy source from fossil fuels, thereby providing aid in environmental conservation.

A study presumed the ability of concerned isolated bacteria to produce biopolymer, PHB and its potentiality to act as biofertilizer, fixing the atmospheric nitrogen. The research outcomes successfully established the isolated bacteria to possess the speculated abilities. The nitrogen fixing ability and the amount of fixed nitrogen by both of the bacteria approved their competence as biofertilizer. The results obtained from flow cytometry exhibited the prospect of the isolated bacteria as biopolymer producer.

Therefore, the study triumphantly presented the alternative solution for contemporary dispute. The dissertation provides a solid outlook and fills up the loophole present in studies of PHB producers and nitrogen fixers. It not only isolates and explores the abilities of potent bacteria but also advocates for its use in industrial scale.

Recommendations

- To maximize the nitrogen fixation, optimization of different growth conditions could be studied.
- The “nif” genes involved in nitrogen fixation could be studied for their engineering that could lead to better nitrogen fixing abilities.
- Different growth conditions could be studied and optimized for the maximum production of PHB
- Along with flow cytometry, GC (Gas chromatography) method is advisable for comparative study that will facilitate reliable quantification of PHB produced.

Flow cytometry further studied the PHB producers and analyzed the data, using FlowJo software. The quantified amount of PHB produced by isolated samples established them as potent biopolymer producers that are suitable for industrial production.

Thus, the study confirms the isolated bacteria with strength to produce not only biopolymer but also fix the atmospheric nitrogen acting as biofertilizer. Hence, this study provides an alternate solution that meets global demand and at the same time settles global concern. When scaled up in industrial level, the findings of the study will act as milestone in an attempt to resolve the complications faced by agricultural sector and void suffered by polymer industries due to lack of biodegradable alternatives.

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APPENDICES

APPENDIX –I

Jensen Media	**gm/L**
Sucrose	20
Dipotassium phosphate	1
Magnesium sulphate	0.5
Sodium chloride	0.5
Ferrous sulphate	0.1
Sodium molybdate	0.005
Calcium carbonate	2
Agar	15

Nesslers reagent	**gm/L**
Mercuric chloride	10
Potassium iodide	7
Sodium hydroxide	16
Water (ammonia free)	100mL
Final pH (at 25^0c)	13.2±0.05

Luria broth	**gm/L**
Casein enzymic hydrolysate	10
Yeast extract	5
Sodium chloride	5
Final pH (at 25^0c)	7±0.2

Preparation of solutions for chromosomal DNA extraction

TE1 solution

50 mM Tris-Cl (pH7.5)

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) to a 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. Then solution was stored at -20^{0}C.

Sodium Acetate (3 M, pH 5.2)

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

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 details and PCR condition for 16s rRNA gene amplification

Primer name	Tm	Sequence	Amplicon length(bp)
27F	56.4	AGAGTTTGATCMTGGCTCAG	1500
1492R	56.4	CGGTTACCTTGTTACGACTT	

PCR programme

Enzyme Deactivation-	95˚c	2min
Denaturation-	95˚c	30sec
Annealing	52˚c	30sec
Extension	72˚c	2.5min
Final extension	72˚c	5min

Final hold 4˚c

Total cycle: 35 cycle

APPENDIX II

Fig A: citrate utilization test

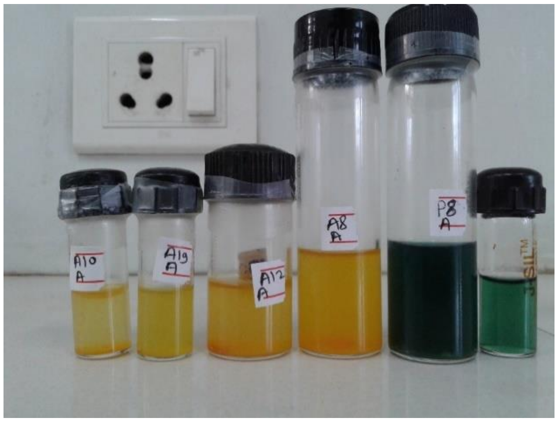

Fig B: Oxidative Fermentative test of isolates

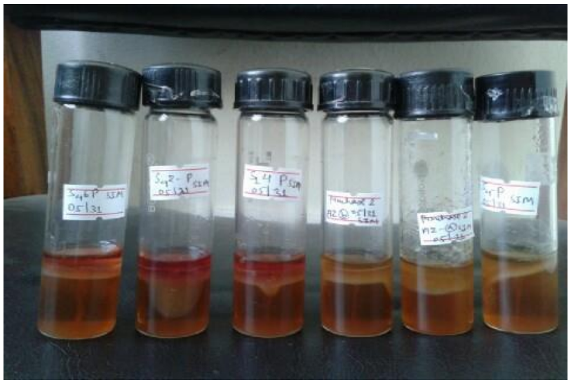

Fig C: SIM test of isolates

Fig D: TSIA test of isolates

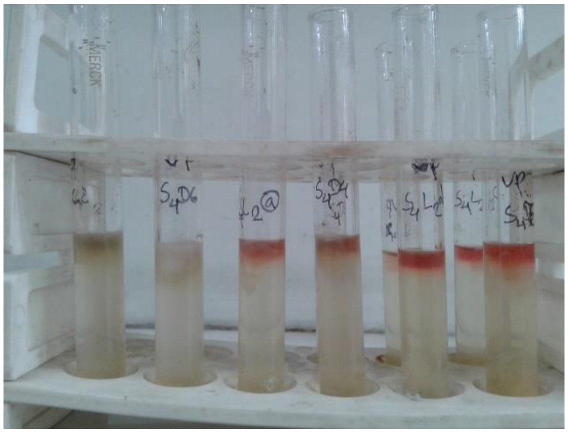

Fig E: Methyl red test of isolates

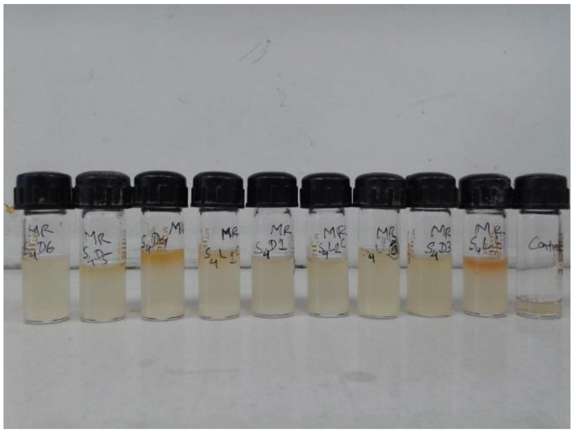

Fig F: Voges-Proskaur test of isolates

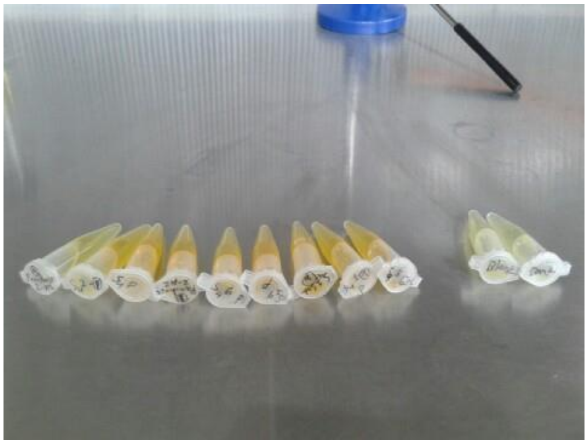

Fig G: Detection of fixed nitrogen assimilated by isolates