N_2-FIXING BACTERIAL COMMUNITY IN LOW AND HIGH INPUT WHEAT (*Triticum aestivum* L.) AGROECOSYSTEMS

Thesis

SUBMITTED TO THE

G.B. Pant University of Agriculture & Technology
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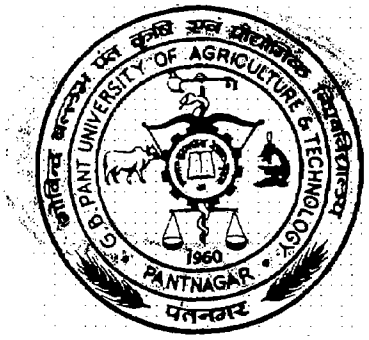

By:

Kawal Jeet

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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" To the sovereign God who rules all the things well"

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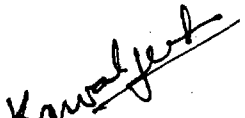

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Certificate

This is to certify that the thesis entitled "N_2-FIXING BACTERIAL COMMUNITY IN LOW AND HIGH INPUT WHEAT (*Triticum aestivum* L.) AGROECOSYSTEMS" submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** with major in **Microbiology** and minor in **Molecular Biology and Biotechnology** of the college of Post-Graduate Studies, G.B. Pant University of Agriculture and Technology, Pantnagar, is a record of bona fide research carried out by **Ms. Kawal Jeet, Id. No. 22924,** under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation and source of literature have been duly acknowledged.

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Certificate

We, the undersigned, members of the Advisory Committee of, **Ms. Kawal Jeet, Id. No. 22924,** a candidate for the degree of **Doctor of Philosophy** with major in **Microbiology** and minor in **Molecular Biology and Biotechnology,** agree that the thesis entitled "**N_2-FIXING BACTERIAL COMMUNITY IN LOW AND HIGH INPUT WHEAT (*Triticum aestivum* L.) AGROECOSYSTEMS**" may be submitted in partial fulfillment of the requirements for the degree.

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CONTENTS

INTRODUCTION

Chapter 1 *Introduction*

Wheat continues to be the most dynamic sector in the world grain production, especially in Asian subtropics in which India ranks second largest producer preceded only by China. Over the past decades crop production has kept pace with increased population but lately there has been either stagnation or decline in yield. On the other hand world population is growing at a rapid pace and is expected to reach 10 billion mark by 2050 with anticipated increase to occur largely in Africa, Latin America and Asia. Presently these regions have already serious problems with respect to food production access to food, water scarcity and nutritional disorders/ malnutrition **(Cakmak, 2002)**. More than 800 million people living in the developing countries are undernourished due to inadequate food availability. The problem is particularly widespread in Asia; about 70% of undernourished people globally live in Asia, predominantly in India and China.

The Green Revolution in agriculture is one of the most successful human achievement of this century in the country. This revolution resulted in global food security and played an important role in transforming the developing countries. To feed a world with huge increase in population and to sustain the well being of human, a large increase in food production must be achieved. The expected increase in world population will result in a serious pressure on the existing agricultural land via urbanization and intensification of crop production **(Alexandratos, 1995)**. The world today needs a second

'Green' or more appropriately, an 'Evergreen' Revolution in order to increase food production by 50% in the next 20 years to sustain the increase in population **(Leisinger, 1999; Vasil, 1998)**. Chemical fertilizers have played a significant role in the Green Revolution but their excessive use has led to reduction in soil fertility and the associated environmental degradation. **Ahmed (1995)** stated that the use of chemical fertilizers is reaching its theoretical maximum and beyond this there is likely to be no further increase in yields.

Nitrogen (N) is the most limiting nutrient for crop yields, and N fertilizers are an expensive input in agriculture costing more than US$45 billion per year globally **(Kush and Bennett, 1992)**. After N, Phosphorous (P) is the major plant growth limiting nutrient despite being abundant in soils in both inorganic and organic forms **(Gyaneshwar *et al.*, 2002)**. The excessive use of fertilizer raises problems due to adverse effects on the environment such as eutrophication of surface water, pollution of drinking water and gaseous emission causing global warming in high input areas in contrast to states of India like U.P., M.P., Bihar and Uttaranchal, where marginal farmers are not able to meet expensive fertilizers due to economic reasons; quite a large component of this community is also dependent on seasonal rains alone for cultivation of various crops. Efforts have been made to develop effective microbial strains using molecular genetic techniques and screening programmes based on large gene pool of individual isolates. A large percentage of total

aerobic heterotrophic microbial population in plant root zone including wheat is diazotrophic since free-living N_2-fixers utilize atmospheric nitrogen hence they affect the soil nutrient status which in turn influences the plant growth. Field inoculation with *Azospirillum* sp. has been widely evaluated worldwide and it has been concluded that these bacteria are capable of promoting yield of agricultural important crops in various soil types **(Okon and Labandera-Gonzalez, 1994)**. In wheat, a non-tropical cereal, it has been assayed widely for field inoculation, showing in many cases significant yield increases **(Boddey *et al.*, 1986; Millet *et al.*, 1985)**. However, response to inoculation has not always been successful, and the factors affecting the crop response are not well understood **(Okon and Labandera-Gonzalez, 1994)**. Several diazotrophic bacteria belonging to the genera *Acetobacter, Alcaligens, Azoarcus, Azospirillum, Enterobacter, Herbaspirillum, Pseudomonas,* and *Zoogloea* have been isolated from rice and other members of the family Gramineae **(Dobereiner, 1992; Reinhold-Hurek *et al.*, 1993; Baldani *et al.*, 1996; Hassan *et al.*, 1998)**.

Enhancement of crop yields of cereals by field inoculation with nitrogen-fixing bacteria has been reported extensively **(Boddey *et al.*, 1991; Urquiaga *et al.*, 1992; Dobereiner *et al.*, 1993)**. Yield increase is attributed to biological N_2-fixation and also to the production of plant growth substances, siderophore production, enzyme production like acid and alkaline phosphatase which

solubilize insoluble forms of phosphorous, and to extensive root colonization by such bacteria.

Although data is available which allow predictions to be made for improved selection of rhizosphere competent strains, study of rhizosphere ecology is fundamental to the introduction of the microbial inoculants for improved soil and plant health. The potential for developing a reliable strategy for selecting rhizosphere competent bacteria would improve by understanding the community diversity and population dynamics of dominant forms in the rhizosphere milieu.

Due to continued practice of rice-wheat rotation seriously impairment in soil health has been observed. Soil biotic factors may also be contributing towards decline in productivity in rice-wheat systems. Cropping systems where rotation is not followed by any leguminous crop often face serious soil health problems in the long run and this is especially strongly reflected in the nutrient status of nitrogen and phosphorus. Studies of the rhizospheric N_2-fixing microflora have until now suffered from the use of largely selective media for population profiles and subsequent isolation; however, it is now widely believed that only a small fraction of nitrogen fixing bacteria are actually culturable **(Ueda *et al.*, 1995)**. Different growth requirements resulting from varying pathological properties of such bacteria precludes their simultaneous cultivation through such methodologies. In addition, the likelihood that some of these microorganisms are non-

culturable makes a general cultural approach for evaluating N_2-fixing population nearly impractical **(Widmer *et al.*, 1999)**. Recent tools of molecular ecology have provided methods for analyzing environmental DNA extracts for specific gene pools including those of nitrogen-fixing forms.

Considering the above background of N_2-fixing bacteria in wheat rhizosphere, the following goals were specifically set for the present study.

OBJECTIVES

1. Analysis of *nif*H gene pool complexity in rhizosphere under, low input vs high input soil systems, at different stages of crop growth.
2. *In situ* evolution of effective isolate(s) on N_2-fixation ability and plant growth.
3. Detailed analysis of the dominant forms to arrive at phylogenetic relationships.

REVIEW OF LITERATURE

Chapter 2 Review of Literature

Wheat (*Triticum aestivum* L.) is an important cereal of India which is cultivated as a major crop in the Northern parts of the country. The rotation is followed with rice cultivation. About 24 million ha of the cultivated land in the Asian subtropics is occupied by rice-wheat rotation which has emerged as a major production system in the Indo-Gangetic plains of South-Asia; the system occupies about 13.5 million ha **(Ladha *et al.*, 2000)**. The continued intensive practice of this crop rotation system has seriously impaired crop yield **(Duxbury *et al.*, 2000; Yadav *et al.*, 2000)** and soil health besides the problems of alkanity/salinity, deterioration of soil texture, loss of soil nutrients that has lead to new pests, diseases and weed problems, and has consequently resulted in low production levels. The signs of deterioration in agricultural production systems are already evident in other productive systems on account of abiotic and biotic stresses.

Bhandari *et al.* (2002) suggested that long-term experiments (LTE) provide a system for examining changes in crop yield and soil nutrient levels. Several long-term rice-wheat experiments were conducted in South Asia but most studies were restricted to simple yield trend analysis without recourse to periodic sampling strategy **(Nambiar, 1994; Duxbury *et al.*, 2000; Yadav *et al.*, 2000;)**. A long-term experiment was laid out in 1983 at the experimental farm of Punjab Agricultural University (PAU), Ludhiana by

Bhandari *et al.* (2002) in which soil nutrient levels (C, N, P, K status, and P and K balances), grain yield trends and crop nutrient removal were determined in 14 years rice-wheat rotation. Recommended level of N, P, K were supplemented with N through farmyard manure (FYM), wheat chopped straw (WCS) of Sesbania (*Sesbania cannabina* Linn. and Merrill). Soil parameters were analyzed in soil samples collected periodically from 1988-1999. Rice yield declined from 0.07 to 0.13 Mg ha^{-1} yr^{-1} dependent on treatment whereas wheat yields declined by 0.04 Mg ha^{-1} yr^{-1} with applications of 75% and 100% NPK fertilizers but were maintained over 14-yr period in the other treatments **(Bhandari *et al.*, 2002)**.

The evident decline in yield on long-term experimental plots coupled to stagnating farmer yields, productivity limited growth rates and degrading soil and water resources have raised questions about the sustainability of rice-wheat rotation systems unless corrective measures are not immediately taken up. Experiments at Pantnagar, India have also shown decline in yields in intensive rice-wheat systems when input levels were kept constant.

It is hypothesized that the main cause of decline in crop yields has been a reduction in the natural ability of soil to provide nitrogen by interactions between organic matter and soil microbes. Soil biological factors also appear to contribute towards decline in productivity in rice-wheat system. Solarization trails in Nepal have shown that the incidence of root nematode in rice and root necrosis in

wheat were negatively correlated with yield, indicating that underground pathogens were atleast partly responsible for reduction in yields.

The microbial populations in soil are known to be complex **(Kuske *et al.*, 1997)** and contains $10^3 - 10^4$ different genomes g^{-1} soil dry weight **(Borneman *et al.*, 1996)**. Microorganisms are therefore an essential component in functioning and sustainability of all natural ecosystems. In fact they are essential even for man-made ecosystems but are frequently ignored due to their small size and methodological difficulties associated with their detection. The central role of microorganisms in ecosystem processes, including biogeochemical cycling of nutrients, biodegradation **(Prosser, 2002)** and plant growth, makes it both unwise and dangerous to ignore any aspect of their ecology.

A large percentage of the total aerobic heterotrophic microbial population in plant root zone including wheat is diazotrophic. Since free-living N_2-fixers utilize atmospheric nitrogen hence it affects the soil nutrient status which in turn influences the total microbial community structure and thus indirectly affects plant growth.

Biological Nitrogen Fixation (BNF)

Biological nitrogen-fixation is carried out by nitrogenase enzyme which plays a critical role in the global nitrogen cycle as it catalyzes the reduction of atmospheric dinitrogen to ammonia. This enzyme is present in a large number of organisms, ranging from symbiotic and free-living bacteria to cyanobacteria **(Bursey and Burgess, 1998)**.

An abbreviated list of nitrogen-fixing organisms is given in Table 2.1, presenting a variety of prokaryotes, both anaerobic and aerobic, which fix nitrogen.

Table 2.1: Some Nitrogen-fixing organisms

Free-living aerobes		
Chemoorganotrophs	**Phototrophs**	**Chemolithotrophs**
Bacteria:		
Azotobacter spp.	Cyanobacteria (various but not all)	*Alcaligens*
Azomonas		*Thiobacillus* spp.
Klebsiella		*Streptomyces thermoautotrophicus*
Beijerinckia		
Bacillus polymyxa		
Mycobacterium flavum		
Azospirillum lipoferum		
Citrobacter freundii		
Acetobacter diazotrophicus		
Methylomonas		
Methylococcus		
Free-living anaerobes		
Chemoorganotrophs	**Phototrophs**	**Chemolithotrophs**
Bacteria :	**Bacteria:**	**Archaea:**
Clostridium spp.	*Chromatium*	*Methanosarcina*
Desulfovibrio	*Chlorobium*	*Methanococcus*
Desulfotomaculum	*Thiocapsa*	
	Rhodospirillum	
	Rhodopseudomonas	
	Rhodomicrobium	
	Rhodopila	
	Rhodobacter	
	Heliobacterium	
	Heliobacillus	
	Heliophilum	
Symbiotic		
Leguminous plants	**Non-leguminous plants**	
Soybeans, peas, clover, locust, and so on, in association with a bacterium of the genus *Azorhizobium, Bradyrhizobium, Rhizobium,* or *Sinorhizobium*	*Alnus, Myrica, Cenothus, Comptonia, Casuarina;* in association with actinomycetes of the genus *Frankia*	

Nitrogenase has been extensively studied in the soil bacterium *Azotobacter vinelandii* **(Burgess and Lowe, 1996; Howard and Rees, 1996)**. This enzyme consists of two oxygenlabile metalloproteins of which one is the molybdenum iron (MoFe) protein also called molybdoferredoxin a Mr ≈240,000 $\alpha_2\beta_2$ heterodimer encoded by the *nif*D and *nif*K genes. This protein has been crystallographically characterized by itself **(Kim and Rees, 1992)** and in a complex with the second nitrogenase protein **(Schindelin *et al.*, 1997)**. The nitrogenase protein from the obligate anaerobe, *Clostridium pasteurianum* has also been characterized by X-ray crystallography **(Kim *et al.*, 1993)**. The resulting crystallographic structures have confirmed the existence of two distinct and interesting types of metal clusters. One of these is called the iron-molybdenum cofactor (FeMo cofactor) which is actually a (Mo-7Fe-9S-homocitrate) cluster **(Kim and Rees, 1992; Chan *et al.*, 1993)** and is thought to be the substrate reduction site **(Burgess and Lowe, 1996)**. The second is a (8Fe-7S) cluster which is also called P-cluster and appears to accept electrons as they are transferred to MoFe protein **(Howard *et al.*, 1996; Peters *et al.*, 1997)**.

The second protein in the nitrogenase enzyme is the iron (Fe) protein also called azoferredoxin of Mr ≈60,000, a α_2 homodimeric protein which is encoded by *nif* H gene. **Georgiadis *et al.* (1992)** have characterized this protein by crystallography; it forms complex with

the MoFe protein **(Schindelin *et al.*, 1997)**. The crystallographic structure of Fe protein shows that it contains a single (4Fe-4S) cluster bound by two cysteinyl sulfurs from each identical subunit. The Fe protein acts as a reductant that is able to transfer electrons to MoFe protein in such a manner that the MoFe protein is then able to use them for substrate reduction.

All N_2- fixing proteobacteria studied so far synthesize the conventional FeMo cofactor containing nitrogenase encoded by *nif* genes, but a few also contain alternative nitrogenase enzymes with metals other than Mo **(Rudnick *et al.*, 1997)**. One contains a FeV-cofactor and the other a Fe-cofactor. The subunits of these enzymes are encoded by genes *vnf* and *anf*, respectively. *Azotobacter vinelandii* harbors three genetically distinct nitrogenase systems that are differentially expressed depending on the availability of metals in the medium: a *nif*-encoded Mo-containing nitrogenase, a *vnf*-encoded V-containing nitrogenase, and an *anf*-encoded iron only nitrogenease **(Bishop and Premakumar, 1992)**. In addition to *nif*, *A. paspali* also contains both the alternative enzymes while *A. chroococcum* contains only *vnf* genes. On the other hand, *Rhodobacter capsulatus* and *Rhodospirillum rubrum* contain only *anf* genes, in addition to *nif* **(Mosepohl and Klipp, 1996)**. Recently, **Ruttimann-Johnson *et al.* (2003)** have identified a gene from *Azotobacter vinelandii* whose product exhibits primary sequence similarity to NifY, NafY, NifX, and VnfX family of proteins, which are required for effective V-dependent diazotrophic

growth. As this gene was located down-stream of *vnf* K in an arrangement similar to the relative organization of the *nif* K and *nif* Y genes, it was designated *vnf*Y. VnfY has a role in maturation of the V-dependant dinitrogenase, with a specific role in the formation of the V-containing cofactor and/or the insertion into apodinitrogenase.

Evolution

Regarding origin and evolution of the nitrogen-fixing system, two issues are highly relevant i.e., scattered distribution among the prokaryotes and considerable structural uniformity of nitrogenase gene and proteins. This has given rise to speculation that the emergence of diazotrophy may be a relatively recent evolutionary event implying horizontal genetic transfer of *nif* among pre-existing strains **(Postgate, 1974; Puhler *et al.*, 1979)**. In contrast, an alternative view point is that nitrogenase is an ancient enzyme **(Kleiner *et al.*, 1976)** like hydrogeanse or ferredoxins, the fixation of nitrogen may not necessarilly have been the original function of the nitrogenase, but it could have been a detoxifying enzyme reducing substrates such as cyanides and/or cyanogens that might have been toxic to primitive life **(Silver and Postgate, 1973)**. **Hennecke *et al* (1985)** suggested that *nif* genes could have evolved to a larger degree in a similar fashion as the bacteria which carry them. This interpretation goes speak against the idea of a recent lateral distribution of *nif* genes among microorganisms. According to this thought, the possibility of

horizontal *nif* gene transfer at very early stages of prokaryotic evolution can not be completely ruled out.

Mechanism of Nitrogenase

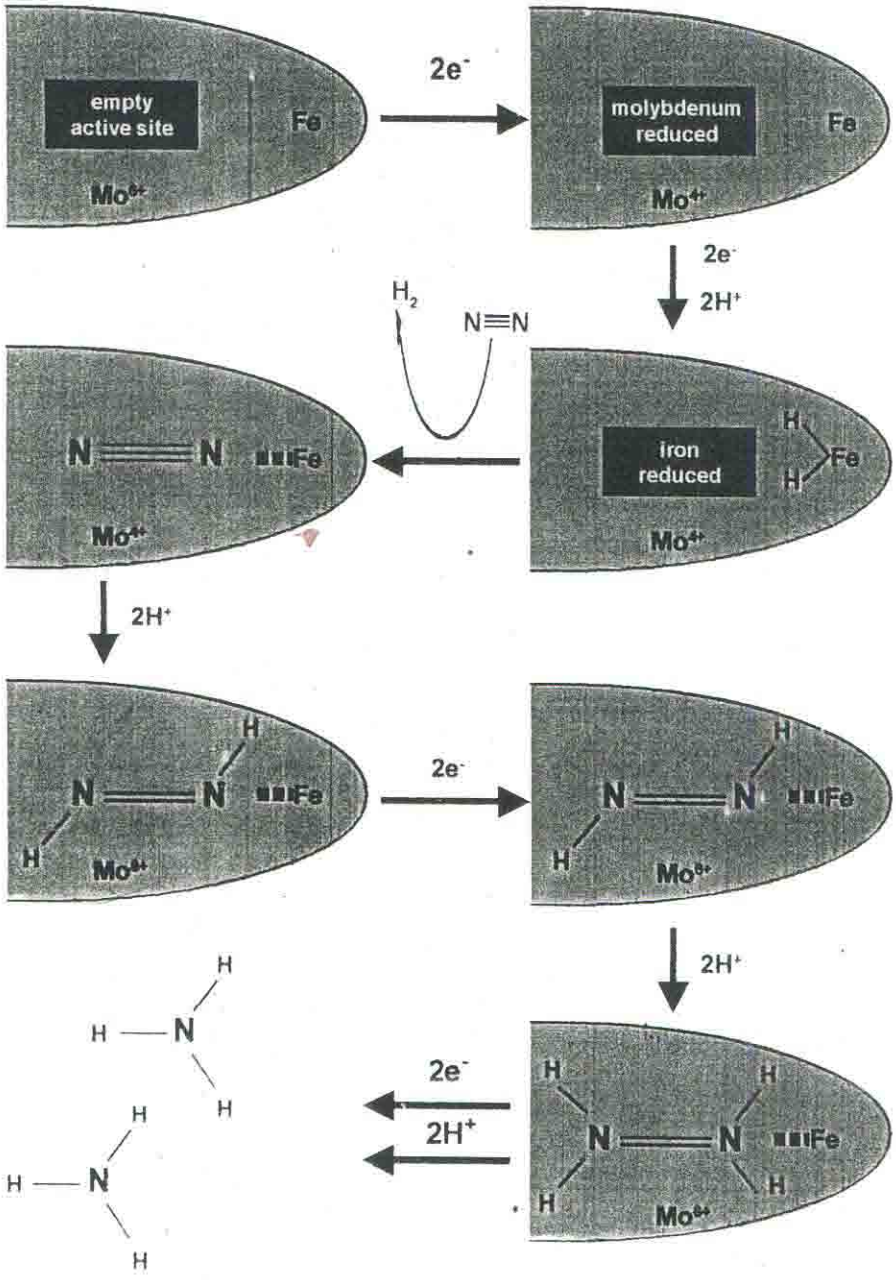
Nitrogenase can reduce many small molecules with triple bonds in addition to nitrogen. In the biological fixation process, N_2 is reduced to ammonium which is further converted to organic form. The reduction process is catalyzed by the enzyme complex nitrogenase, which consists of two separate proteins called dinitrogenase and dinitrogenase reductase. Nitrogenase enzyme is not very fast (the turn over number is around 50 moles/min per mol of Mo) and so about 2-5% of the total cell protein (http:www.science.siu.edu/ microbiology/ micr 425/ 425 Notes/12-NitrFix.html). The reaction $N_2 + 3H_2 \rightarrow 2NH_3$ actually releases energy. The overall ΔG for the reaction is about -8 Kcal/mole. However the first step, breaking up the triple bond is extremely unfavorable:

$N_2 + H_2 = N_2H_2$ $\Delta G^0 \approx +50$ Kcal/mole

$N_2 + 2\,e^- + 2H^+ = N_2H_2$ $E^0 = -1200$ mV

Thus nitrogenase has to carry out a single step which needs a reductant with a redox potential of -1200 mV. Local pH, alteration in solvation etc. can bring this down to about -1000 mV but even though this potential is much more negative than any other biological redox potential. The redox potential of dinitrogenase reductase in -290 mV. When ATP binds to this protein its redox potential is lowered to -400 mV. ATP binding alters the conformation of dinitrogenase reductase

NITROGENASE MECHANISM



and lowers its reduction potential, allowing it to interact with dinitrogenase. Thus ATP must be hydrolyzed for reduced dinitrogenase reductase to reduce dinitrogenase. Two ATPs are hydrolyzed per electron transferred or 4ATP/2e. 4ATP yields approximately -30 Kcal which is equivalent to 750 mV per pair of electrons, which along with E^0 of dinitrogenase reductase just over the 1000 mV negative-the correct value.

Electron flow in nitrogen fixation

In nitrogenase electron flows in following sequence:

Electron donor → dinitrogenase reductase → dinitrogenase → N_2. The electrons for nitrogen reduction are transferred to dinitrogenase reductase from ferredoxin or flavodoxin, low potential iron-sulfur proteins. In *Clostridium pasteurinum,* ferredoxin is the electron donor and is reduced by phosphoclastic splitting of pyruvate to acetyl-CoA + CO_2. In each cycle of electron transfer, dinitrogenase reductase is reduced by ferredoxin/flavodoxin and binds two molecules of ATP. As the electron transfer to dinitrogenase, the ATP is hydrolyzed and dinitrogenase reductase dissociates from dinitrogenase and begins another cycle of reduction and ATP binding. When appropriately reduced, dinitrogenase then reduces N_2 to NH_3 (Fig. 2.2). Although only six electrons are necessary to reduce N_2 to $2NH_3$, eight electrons are actually consumed in the process, two electrons being lost as hydrogen (H_2), for each mole of N_2 reduced.

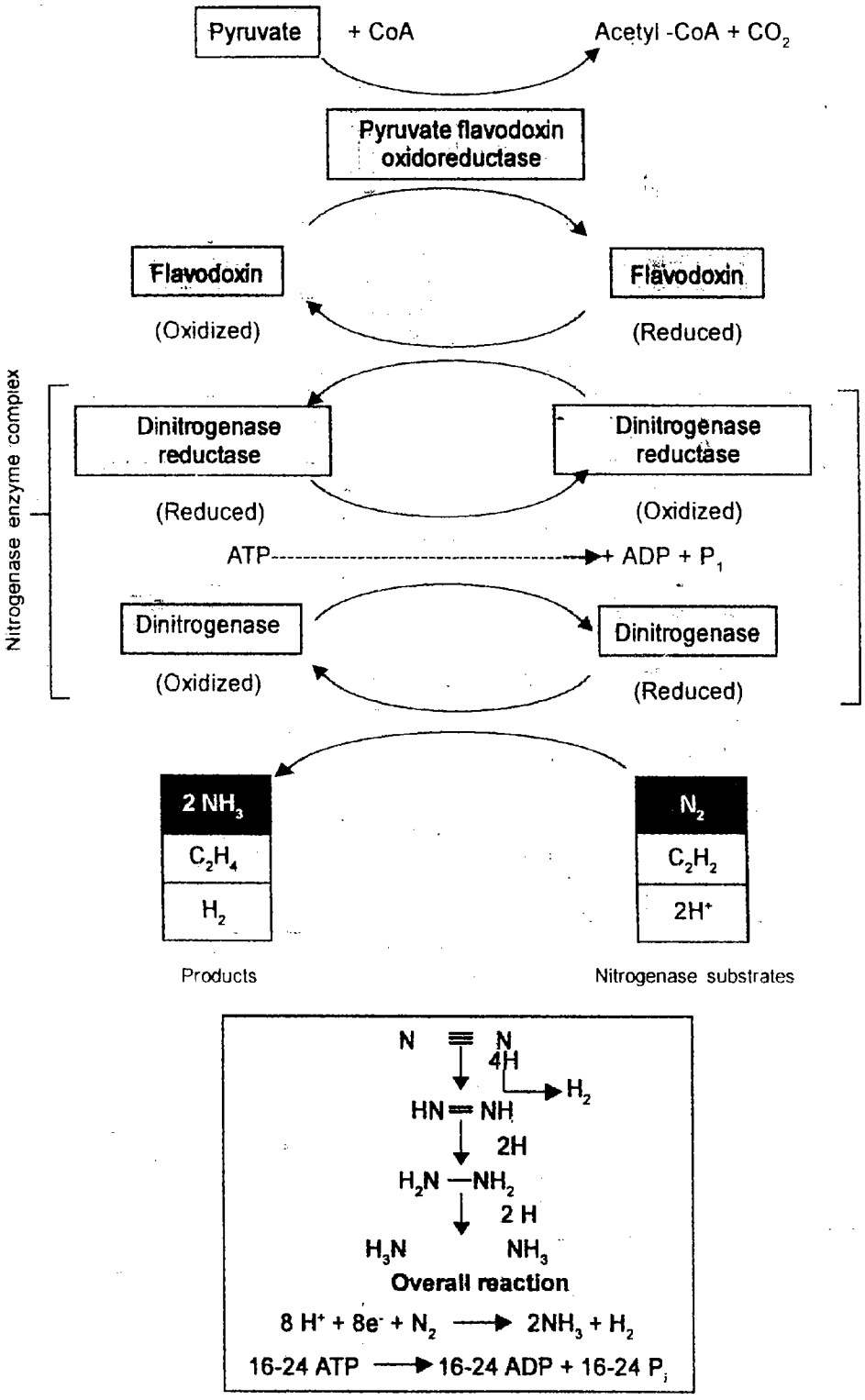


Fig. 2.2 : Nitrogenase system : Steps in reduction of N_2 to NH_3

Genetics and Regulation of Nitrogen Fixation

The genes for dinitrogenase and dinitrogenase reductase in *Klebsiella pneumoniae,* a well-studied N_2 fixer, are part of a complex regulation (a large network of operons) called the *nif* regulon (Fig.2.3); the *K. pneumoniae nif* regulon spans 24 kb of DNA and contains 20 genes arranged in several transcriptional units (Fig.2.3). In addition to nitrogenase structural genes, the genes for FeMo-co, genes controlling the electron transport proteins, and a number of regulatory genes are also present in the *nif* regulon. Dinitrogenase is a complex protein made up of two subunits, α (products of *nif*D gene) and β (product of the *nif*K gene), each of which is present in two copies. Dinitrogenase reductase is a protein dimer consisting of two identical subunits, the product of *nif*H. FeMo-co is synthesized through the participation of several genes, including *nif*N, V, Z, W, E, and B as well as Q, which encode a product involved in molybdenum processing. The *nif*A gene encodes a positive regulatory protein that serves to activate transcription of other *nif* genes.

Regulation of *nif*A Gene

The expression of *nif* genes depends on the habitat where bacterial community resides. Intercellular communication via diffusible chemical signals is very well described for bacteria besides its function to modulate a number of cellular processes. The perception and interpretation of these signals enables bacteria to sense their habitat, leading to coordinate expression of genes. The result of this

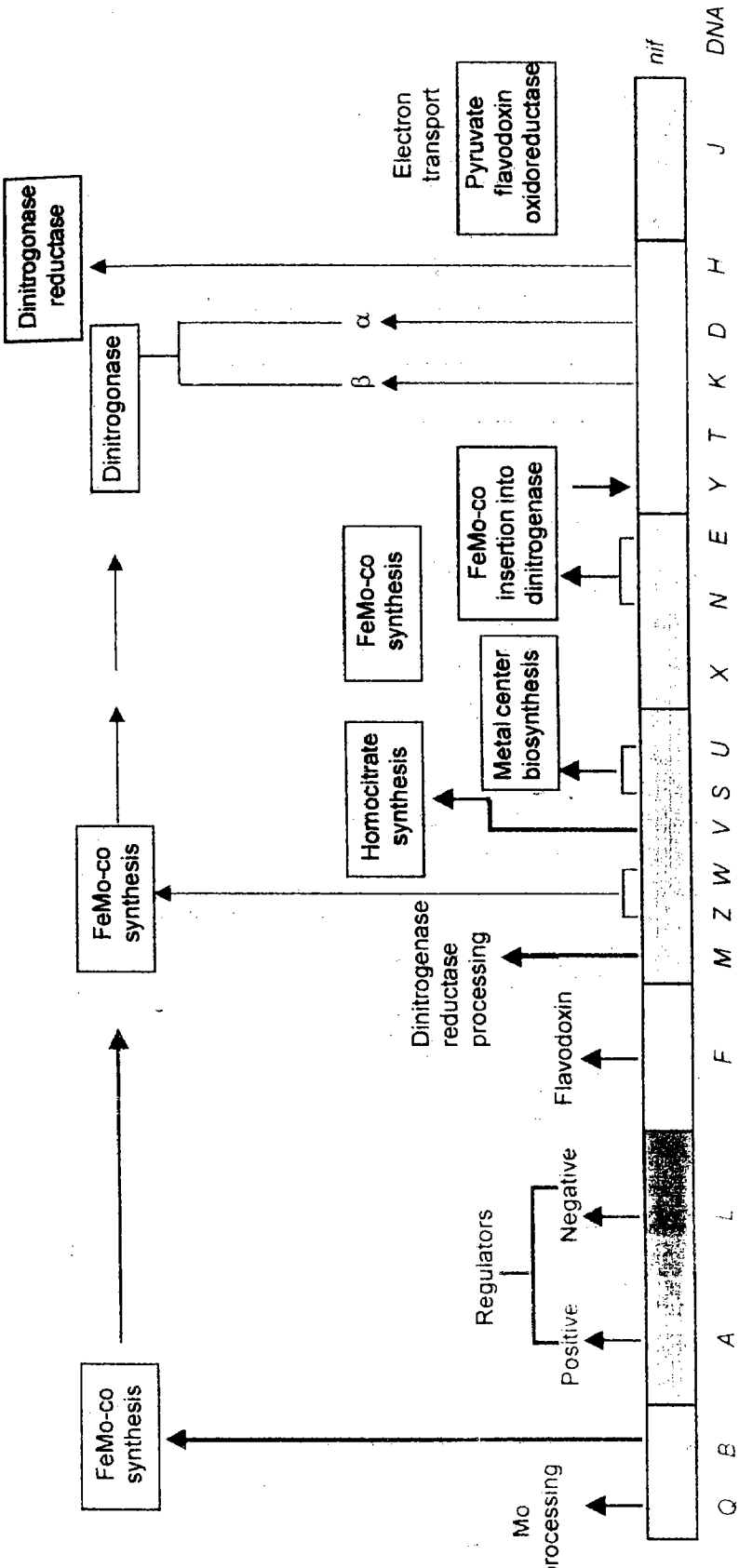


Fig. 2.3 : The Genetic structure of *nif* regulon in *Klebsiella pneumoniae*

communication is appropriate response of the bacterial community to its environment **(Winans and Bassler, 2002)**. Bacteria belonging to the genera *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium,* and *Sinorhizobium* and have the ability to infect the roots of leguminous plants, with the formation of nodules and thus establish a nitrogen-fixing symbiosis **(Loh and Stacey, 2003)**. On the other hand, *Herbaspirillum seropedicae,* an endophytic diazotrophic bacterium has been found in the tissues of members of Gramineae, such as wheat, rice, and maize **(Baldani *et al.*, 1986)**. Infection by *H. seropedicae* occurs by binding of bacteria to root surface and proliferation in the secondary roots at sites of root surface damage, this is followed by penetration and aggregation of the bacteria in intercellular spaces and vascular bundles, followed by colonization and establishment in the xylem vessels **(James *et al.*, 2002)**. Nitrogen fixation in this organism occurs under microaerophilic conditions and is regulated at both, the level of synthesis and the level of activity of NifA protein, in response to the levels of fixed nitrogen and oxygen **(Fischer, 1994)**. In members of the gamma subclass of the *Proteobacteria* (γ-proteobacteria), regulation of transcriptional activity of NifA protein by these two effectors involves NifL protein, which forms an inactive complex with NifA in presence of high levels of ammonium or oxygen. In *H. seropedicae* and *Azospirillum brasilense* (β- and α-proteobacteria, respectively), the Nif A protein is directly inactivated in response to increased levels of fixed nitrogen and oxygen **(Arsene *et al.*, 1996;**

Souza *et al.*, 1999). Although the mechanism of Nif A activity control in these two groups of bacteria differs, the signalling pathways leading to ammonium response, have similarities **(Monteiro *et al.*, 2003)**. In *Klebsiella pneumoniae,* another protein called GlnK protein is required to relieve the inhibitory effect of Nif L or Nif A under nitrogen-fixing conditions **(Arcondeguy *et al.*, 1999; He *et al.*, 1998; Jack *et al.*, 1999)**. In contrast, in *Azotobacter vinelandii,* the GlnK protein is required for ammonium dependent inhibition of NifA by NifL **(Little *et al.*, 2000; Little *et al.*, 2002; Rudnik *et al.*, 2002)**. In *A. brasilense* and *H. seropedicae,* which do not contain Nif L, the PII protein, the product of *gln* B, is necessary to relieve auto-inhibition and ammonium control of Nif A activity by its N-terminal domain **(Arsene *et al.*, 1996; Benelli *et al.*, 1997; Souza *et al.*, 1999)**. The signalling pathway for control of Nif A activity in response to oxygen in even less clear. In γ-proteobacteria, the Nif L protein is reversibly oxidized, and forms a transcriptionally inactive complex with Nif A **(Hill *et al.*, 1996)**. In some other studies it was found that sodium dithionite could reduce Nif L of *A. vinelandii in vivo,* resulting in a protein that was unable to complex with Nif A. Although *in vivo* Nif L-reducing and oxidizing species have not been defined yet **(Macheroux *et al.*, 1998; Klopprogge and Schmitz, 1999; Grabbe *et al.*, 2001)**, it has been suggested that a heme protein may be involved **(Hill *et al.*, 1996)**.The Nif A protein from rhizobia, *A. brasilense* and *H. seropedicae* are not active in presence of high oxygen concentrations

and require iron for *in vivo* activation of *nif* gene promotors, suggesting that Nif A proteins of this class can sense oxygen directly **(Fischer *et al.*, 1988; Arsene *et al.*, 1996; Monteiro *et al.*, 1999; Souza *et al.*, 1999)**. Alternatively, an iron-containing signal transducer is a Fnr protein. This transcriptional regulator is responsible for switching-off aerobic metabolisms to anaerobic metabolism, and response towards molecular oxygen **(Unden and Schirawski, 1997)**. However, an O_2-tolerant nitrogenase from *Streptomyces thermoautotrophicus* utilizes superoxide as an electron donor **(Ribbe *et al.*, 1997)** that has fundamentally changed the thinking about the interrelation between N_2-fixation and O_2. Oxygenic phototrophs such as cyanobacteria produce superoxide in great abundance and researchers have been obsessed with the idea that how do these organisms keep the superoxide away from the nitrogenase **(Gallon, 1992; Bergman *et al.*, 1997)**. It is therefore ironical that, in at least one diazotroph, superoxide production may actually benefit N_2-fixation **(Gallon, 2001)**. In theory, a nitrogenase in an organism such as *S. thermoautotrophicus* could easily couple itself directly with the photosynthesis **(Ribbe *et al.*, 1997)**.

By using conventional, O_2-sensitive nitrogenase, preliminary progress has already been made towards successfully introducing the ability to fix N_2 into chloroplasts of the crop plants **(Merrick and Dixon, 1994; Dixon *et al.*, 1997)**. But there are definite problems to be solved in maintaining fully functional enzyme in such an oxygen-

rich environment. There is therefore great potential for exploiting the O_2-stable nitrogenase from *S. thermoautotrophicus* in such studies.

After the discovery of this novel nitrogenase, the possibility of existence of similar systems in other organisms including oxygenic phototrophs, has become higher. **Postgate (1987)** has referred as "ghosts", those potential nitrogen fixing organisms that have not been fully investigated and include eukaryotes as well as prokaryotes. These organisms were originally suspected to be diazotrophs because they could grow in the apparent absence of any nitrogen source other than atmospheric N_2, but they did not have any *nif* H genes and were therefore unable to reduce acetylene. In *S. thermoautotrophicus nif* genes were found to be absent; the nitrogenase is encoded by different genes and moreover, it is unable to catalyse acetylene reduction **(Ribbe *et al.*, 1997)**. Therefore it seems to be a good case for re-examining the diazotrophic status of the so-called 'ghosts' of yesteryears.

Community Dynamics

Microbial communities are considered to be volatile regarding species composition and activities. Both, biotic and abiotic interactions, such as competition and predation, play an important role in community dynamics **(Piceno and Lovell, 2000a)**. Competition between bacterial species for limiting resources has been extensively studied in the laboratory microcosms **(Kolb and Martin, 1988; Ka *et al.*, 1994; Blunenroth and Wagner-Dobler, 1998)**. These

interactions ultimately influence the microbial community composition and structure in natural ecosystems.

Plant rhizosphere/ endorhizosphere/ rhizoplane are ideal locations for studies of competitive interactions of bacteria on account of availability of resource rich environment. These locations are dynamic in which microbial communities have access to an elevated supply of carbon and energy-rich materials from plant roots, relative to neighbouring bulk soil and sediments **(Campbell and Greaves, 1990)**. It is anticipated that such communities supported by high level of carbon and energy resources, are capable of both quantitative and qualitative changes in composition over very short time frames. Rhizosphere environment is also relatively static physically, and can resist the potentially confounding effects of naturally occurring disturbances on microbial community compositions or activities **(Piceno and Lovell, 2000b)**. Rhizosphere microbial community composition and structure is influenced by both competition and predation **(Kolb and Martin, 1988; Zuberer, 1990)**. Experiments examining competitive interactions in natural systems are however rare, and a majority of information on microbial competition has therefore come from laboratory experiments **(Fredrickson and Stephanopoulos, 1981)**. While complexity of natural systems poses substantial obstacle to such studies, the true significance of competitive interactions in microbial community dynamics can only be addressed by *in situ* analysis. This is particularly true of bacterial

interactions because of their relatively rapid doubling time, large diversity **(Woese, 1994; Amann *et al.*, 1995; Fuhrman and Compbell, 1998)**, heterogenous microenvironments, and susceptibility to factors, such as predation and viral lysis, that are characteristic of natural systems harbouring bacterial populations.

A large component of the total aerobic heterotrophic bacterial population in root zone is diazotrophic **(Bally *et al.*, 1983; Watanabe and Barraquio, 1979; Oyaizu-Masuchi and Komagota, 1988)** which provide a major biological source of nitrogen in natural ecosystems. Diazotrophs can provide their own nitrogen which is often the growth limiting nutrient in soil, and thus may have a competitive advantage in such an environment given the fact that there is sufficient energy to support nitrogen fixation. The population of diazotrophs **(Hermann *et al.*, 1993)** and their activity **(Whiting *et al.*, 1986)** are tightly linked to primary production of plants to provide the required carbon and energy resources to permit fixation of atmospheric nitrogen; if this capacity become less demanding, their competitiveness can decrease **(Kolb and Martin, 1988)**. Most studies of associative nitrogen-fixation have focused on crops of agronomic interest such as rice and sugar cane **(Ueda *et al.*, 1995; Engelhard *et al.*, 2000; Steenhoudt and Vanderleyden, 2000)**, where considerable inputs of fertilizer are required for plant growth. Only few studies have aimed to understand fixation under nitrogen limiting natural ecosystems **(Piceno *et al.*, 1999; Bagwell and Lovell, 2000; Piceno and Lovell, 2000 a,b)**.

Molinia coerula, a perennial grass which occurs mainly in acidic peat bog and slightly basic littoral meadows represents an example of oligotrophic environments **(Leps, 1999)**. The grass is hemicryptophytic in nature and possesses root system which ensures its survival during the cold season. **Hamelin *et al.* (2002)** have hypothesized that biological dinitrogen fixation could provide a valuable source of nitrogen for microbial and plant nutrition, under such conditions.

It is estimated that only 0.1-1% of bacterial cells in soil are culturable in the currently used media **(Amann *et al.*, 1995)**. Different molecular methods are considered to give a more accurate image of the total bacterial diversity in natural systems. These approaches can be applied to functional genes also, such as nitrogen-fixing genes. The *nif* H gene has been widely used to detect the nitrogen fixing bacteria in the natural environments **(Zehr and McReynolds, 1989; Ueda *et al.*, 1995; Ohkuma *et al.*, 1999; Piceno *et al.*, 1999; Widmer *et al.*, 1999; Zani *et al.*, 2000; Poly *et al.*, 2001)**. Although nitrogen-fixing bacteria are very diverse, *nif*H genes have evolved on the pattern of 16S rRNA genes and thus can be used as a molecular evolutionary marker **(Young, 1992)**.

All *nif* H genes fall into one of the four clusters **(Chien and Zinder, 1994)**, cluster I includes standard molybdenum nitrogenase from cyanobacteria and proteobacteria (α, β and γ), as well as γ proteobacterial *vnf* H; cluster II includes methanogen nitrogenases

and bacterial *anf* H; cluster III includes nitrogenases from diverse anaerobic bacteria such as clostridia (low G+C,gram positive) and sulfate reducers (δ-proteobacteria), which is an example of the *Nif*H phylogeny deviating from the 16S rRNA phylogeny; cluster IV includes divergent nitrogenase from archaea.

The highly conserved nature of the *nif* H gene makes it an ideal molecular tool for determining the potential of biological nitrogen fixation in any natural environments **(Zehr and Capone, 1996)**. Since diazotrophy is distributed widely and inconsistently among prokaryotes, it is difficult to assess the distribution and diversity of diazotrophs based solely on 16S rRNA phylogenetic diversity studies. However, it is an interesting aspect of nitrogen-fixation that while it is so widely recognized, the distribution is randomly throughout the microbial taxa. However, as proposed by **Young (1992)** the distribution of N_2-fixation capability could simply have been overlooked because of preconceived notion that N_2-fixation is a special capability. Designing PCR primers that amplify the *nif* H gene and sequencing the amplified genes can identify the assemblage of microorganisms capable of nitrogen fixation in any environment. This approach has been applied to environment that are nitrogen limited or are known to support nitrogen-fixation, such as oligotrophic oceans **(Zehr *et al.*, 1998)**, marine microbial mats **(Zehr *et al.*, 1995; 1999)**, modern marine stromatolites **(Steppe *et al.*, 2001)**, rice roots **(Ueda *et al.*, 1995; Engelhard *et al.*, 2000)**, termite hind guts

(Ohkuma *et al.*, 1999) and tropical sea grass beds **(Bagwell *et al.*, 2002)**.

Zehr and McReynolds (1989) used degenerated oligonucleotides for amplification of *nif*H gene from the marine cyanobacterium *Trichodesmium thiebautii.* However, there has been doubt about the identity of the organism responsible for nitrogen fixation in *Trichodesmium* aggregates, since it has never been maintained in axenic cultures. By using degenerated primers, it is possible to amplify clone, and sequence a segment of *nif* H gene from a natural assemblage of *T. thiebautii.* The DNA and presumed amino acid sequence showed that the gene was most closely related to that of *Anabaena* spp., another cyanobacterium. The DNA sequence of *T. theibautii* was 66-79% similar to the corresponding nucleotide sequence of the *nif* H gene from an *Anabaena* sp. **(Mevarech *et al.*, 1980)**, *Rhizobium meliloti* **(Torok and Kondorosi, 1981)**, *Clostridium pasteurianum* **(Chen *et al.*, 1986)**, *Azotobacter vinelandii* **(Brigle *et al.*, 1985)**, *Klebsiella pneumoniae* **(Scott *et al.*, 1981; Sundaresan and Ausubel, 1981)** and *Rhodobacter capsulatus* **(Schumann *et al.*, 1986)** where as the deduced amino acid sequence was 76-84% similar.

Although the amino acid sequence of the *nif* H gene is highly conserved, there are many positions where amino acid residue variability can be found between the species. Overall 42 variable amino acid positions in 359 bp segment are present in which 8 are

unique to *C. pasteurianum* of the remaining 34 positions, there are 41 amino acids which occur in a specific position in only one species other than *T. thiebautii.* The amino acids in the variable region of *T.thiebautii* sequence match the unique amino acid residues most often used by *Anabaena* sp. No exact matches have been found with *K. pneumoniae* and *A. vinelandii* at unique amino acid residues. In another study **Kirshtein *et al.* (1991)** used degenerated oligonucleotide primers for amplification of highly conserved region of *nif* H gene from N_2-fixing bacterium closely associated with the marine filamentous cyanobacterium *Trichodesmium* sp., a heterotrophic isolate from the root/rhizome of the seagrass *Ruppia maritima,* the heterocystous fresh water cyanobacterium *Anabaena oscillarioides.* DNA was extracted directly from the rhizosphere of roots of sea grass (*Halodule wrightii*) followed by cloning and sequencing. The technique was shown to be useful for detection of particular N_2-fixing microorganisms; data obtained on DNA sequences of *nif* H gene has provided information about taxonomic groups of N_2-fixing microorganisms. **Ben-Porath and Zehr (1994)** amplified a 359-bp fragment of *nif*H to distinguish cyanobacterial, eubacterial and archaebacterial *nif*H genes, as well as to distinguish heterocystous from non heterocystous *nif*H genes, that a DNA sequence of 359-bp segment was sufficiently variable for differentiation. These workers suggested that amplified *nif*H fragments can be used as DNA probes to differentiate between species, although there was substantial cross-

reactivity between the *nif*H amplification products of some strains. While DNA sequences provide the most-definitive information about detection, characterization and taxonomy of diazotrophs, but ecological studies would be greatly facilitated if suitable probes could be developed to more rapidly characterize communities at least prior to cloning and sequencing of *nif*H amplification products.

Ohkuma *et al.* (1996) demonstrated that there are diverse N_2-fixation genes within the symbiotic microbial community in termite gut. Phylogentic analysis of the clonally isolated *nif*H genes demonstrate that some of this diversity may result from the presence of multiple copies of *nif*H genes within a single organism, since some N_2-fixing microorganisms have alternative nitrogenase genes and several copies of *nif*H. For instance, *Clostridium pasteurianum* has six copies of *nif*H gene including an alternative nitrogenase gene. However, most of the termite *nif*H sequences are more distantly related to each other than are multiple copies within the same organisms, like *Clostridium pasteurianum* and *Azotobacter vinelandii.* Therefore it can be concluded that the natural community have strikingly diverse *nif*H sequences representing diverse N_2-fixing organisms, including yet unidentified forms.

The existence of *nif*H sequences does not always mean that the N_2-fixing activity is being expressed by the respective organisms, since nitrogenase in regulated at the transcriptional and post transcriptional levels **(Dean and Jacobson, 1992)**. It must also be

stated that the distribution of *nif*H sequences as final clones may not reflect the real distribution of *nif*H genes in the original microbial community, since there are some differences in the efficiencies of DNA extraction, PCR amplification and cloning. Hybridization experiments using specific probe are profitable in ecological studies of the natural microbial community and sequences described in different studies can be useful in the design of specific probes. Analysis of *nif*H together with rRNA is fruitful in giving information concerning the nitrogen economy and ecology.

Several new researches are now employing reverse transcriptase PCR (RT-PCR) to characterize *nif*H community in the natural environments. This technique permits assay of cells that are actively expressing specific genes at the time of sampling **(Kowalchuk *et al.*, 1999; Noda *et al.*, 1999)**. A nested reverse transcriptase PCR (RT-PCR) method was used by **Zani *et al.*, 2000** to assess the expression of nitrogenase in mesooligotrophic Lake George. These workers found several diazotrophs in Lake George samples that included cyanobacteria, α-proteobacteria, and a novel diazotrophic proteobacterial clade that expressed *nif*H transcripts; all of the bacteria detected had type I nitrogenase, and exhibited no sequences in group II, III, or IV. The expression of *nif*H transcripts does not necessarily indicate that bacteria were actively fixing atm. N_2; it does however provide information about bacteria that could have been fixing nitrogen and suggests prevailing nitrogen-fixing conditions for

the existing phylotypes. Since the available microorganisms expressed nitrogenase in a typical phosphorus-limited environment, it suggested that they could have been limited by multiple nutrients or that were limited by different nutrients in the same environment.

Mehta *et al.* (2003) have provided genetic evidence of potential nitrogen fixers in a hydrothermal vent environments for the first time. In order to prove the role of biological nitrogen fixation in nitrogen cycling in the subseafloor associated with unsedimented hydrothermal vent fluid, these workers analyzed a total of 120 *nif*H sequences from four samples: a nitrogen-poor diffuse vent named Marker 33 on Axial volcano, was sampled twice over a period of 1 year as its temperature decreased; a nitrogen rich diffuse vent Puffer environment, and a deep sea water with no detectable hydrothermal plume signals. Subfloor *nif* H genes from nitrogen-poor diffuse vent and nitrogen-rich diffuse vent were related to anaerobic clostridia and sulfate reducers. Other *nif*H genes included proteobacteria and divergent Archaea and were unique to the vent samples. All of the *nif*H genes from the deep-sea water samples were most closely related to the thermophilic anaerobic archaeon, *Methanococcus thermolithotrophicus*. The only difference in the *nif*H populations between the high ammonia Puffer environment and the low-ammonia Marker 33 vent were the presence of the phylogenetic group which was comprised of nine sequences from Marker 33 and contained no sequences from the other environment.

This could be an example of nitrogen availability selecting for different nitrogen fixing microorganisms in nitrogen poor environment.

Fixation of atmospheric nitrogen by free living soil microorganisms is considered a minor source of bioavailable nitrogen as compared to systems such as the *Rhizobium*-legume and *Frankia*-alder symbiosis **(Peoples and Craswell, 1992; Kennedy and Islam, 2001)**. However, most soils have the capacity for asymbiotic N_2-fixation **(Roper *et al.*, 1994; Widmer *et al.*, 1999; Lovell *et al.*, 2000; Shaffer *et al.*, 2000; Poly *et al.*, 2001;)** and sustains potential diazotrophs belonging to different bacterial groups such as proteobacteria, the cyanobacteria and *Firmicutes* **(Widmer *et al.*, 1999; Poly *et al.*, 2001)**. Anthrapogenic activities have led to increased nitrogen fertilization and atmospheric deposition of bioavailable nitrogen (NH_4^+, NO_3^-), thus reducing the extent of N-limitation in many ecosystems. On the contrary, nitrogen fixation ability of free-living diazotrophs can be induced by increasing the C/N ratios or availability of suitable easily consumable 'C' sources **(Limmer and Darke, 1996; Keeling *et al.*, 1998)**, like decaying plant material or root exudates **(Peoples and Craswell, 1992)**. Organic amendments in agricultural systems may also induce nitrogen-limited conditions **(Ocio *et al.*, 1991; Roper and Ladha, 1995)**. A more detailed understanding of the dynamics of N_2-fixing populations, the activities of specific groups, and the conditions required for induction of N_2-fixing ability in soil can aid in devising and optimizing nitrogen

management strategies for sustainable low-input agriculture and forestry. The strategies based on deliberate release **(Becking, 1992; Kennedy and Islam, 2001)** or stimulation of the natural diazotroph populations **(Kanungo *et al.*, 1997; Keeling *et al.*, 1998)** have yielded variable results.

In order to study gene expression in soil, a robust protocol for extraction of total RNA is essential. However only a small number of studies have been reported for successful analysis of mRNA isolated from soil or sediments **(Fleming *et al.*, 1998; Mendum *et al.*, 1998; Miskin *et al.*, 1999; Hurt *et al.*, 2001; Barke *et al.*, 2002)**. There are no reports that explain any direct correlation between the expression of genes involved in diazotrophy and assay of nitrogenase activity in soil. Moreover, in order to reliably detect mRNA, high density inoculation or very active communities are required. In soil microbiological research, reliable extraction of mRNA from soil is still considered a challenge **(Greer *et al.*, 2001)**. **Burgmann *et al.* (2003)** described an effective total mRNA extraction protocol from *Azotobacter vinelandii,* an aerobic free-living soil diazotroph. In this study, *Azotobacter vinelandii* growing in sterile soil and liquid culture served as a model system for *nif* H expression, wherein sucrose served as the carbon source and indirectly provided nitrogen-limited conditions; amendments of NH_4NO_3 were used to suppress nitrogen fixation. This new approach revealed that *nif*H gene expression was positively correlated with nitrogen fixation activity in both, soil and liquid

culture, therefore it can be a powerful tool for studying specific regulation of gene expression directly in soil and other natural environmental conditions.

The contiguous environments which differ in some important physicochemical parameters support microbial communities differing in species composition **(Ferris *et al.*, 1996; Teske *et al.*, 1996)**. These communities are quite sensitive to disturbance **(Ferris *et al.*, 1997)**. **Currin and Paerl (1998)** stated that in some cases the species composition and/or species diversity changes with changing environmental conditions. The other workers **(Felske and Akkermans, 1998; Piceno *et al.*, 1999)** indicated that the 'rhizosphere effect' may not just support locally elevated microbial biomass and activity, but also promotes microbial community stability and persistence within microenvironments. Based on this hypothesis, **Piceno *et al.* (1999)** assessed diazotroph assemblage composition in rhizosphere sediments from the tall and short form *Spartina alterniflora* growth zones over an annual cycle. DNA extraction, nitrogenase assays and several chemical parameters were analyzed at different time periods. Nitrogenase activity differed between zones and within a zone over an annual period. Soluble sulphide concentrations were higher in the short form *S. alterniflora* zone on all sampling time except one and differed within both zones on different samplings. Denaturating gradient gel electrophoresis (DGGE) profiles of *nif*H amplicons showed minor differences among sampling dates and

between sample zones, but the overall banding pattern was remarkably consistent. In another study, nutrient addition effects on rhizosphere diazotroph assemblage composition in a salt marsh grass were assessed *in situ* **(Piceno and Lovell, 2000b)**. The plots of short form *S. alterniflora* were amended with nitrogen (16.3 g m^{-1}) or nitrogen (16.3 g m^{-1}) and phosphorus (18.0 g m^{-1}) for either 2 weeks or 8 weeks. DGGE profiles of *nif*H amplicons from the short-term experiments (2 and 8 weeks) were compared to profiles from control (unamended) and from long-term (>10 y) nutrient amended plots for diazotroph assemblage composition. The banding pattern of DGGE profiles were very similar throughout the short-term experiments and control while one band that was prominent in control plots was not found in long-term nutrient addition plots, suggested that diazotrophy may provide a competitive advantage for some species in this system. These results indicate substantial short-term stability of the diazotroph assemblage composition, but the potential for change in the face of long-term changes in nutrient availability. **Piceno and Lovell (2000a)** also studied plant resource allocation effects on rhizosphere diazotroph assemblage composition. They hypothesized that alteration in plant productivity changes the availability of carbon/energy resources to rhizosphere microbiota which inturn was expected to intensify competition for the remaining carbon supply and could cause the loss of poor competitors from the assemblage. They clipped the leaves or shaded plots of salt marsh *S. alterniflora,* to shift

plant carbon resource allocation from the rhizosphere to the above ground shoots. Pore water parameters (pH, salinity, H_2S, NH_4^+) and acetylene reduction rates did not differ significantly among treatment and DGGE profiles of *nif*H sequences were also very similar across the control and experimental treatments, indicating that no detectable diazotroph species were displaced from the assemblage. Therefore, it could be concluded that rhizosphere diazotrophs are able to compete successfully against nondiazotrophs, in spite of the high energy requirements of nitrogen fixation and the species composition of the diazotroph assemblage in the *S. alterniflora* rhizosphere was stable in the face of short-term but potentially high impact-variation in carbon resource availability.

In soil microbiology, it is not yet fully understood what drives diversity in soil and how, armed with such knowledge agricultural productivity and land management can be improved by manipulating microbial diversity **(O'Donnell *et al.*, 2001)**.

Table 2.2: Factors influencing microbial diversity in soils (Adapted from Kennedy and Gewin, 1997)

Environmental factors	Soil factors	Anthropogenic factors
Landscape	Cation exchange	Crop
Soil type	Capacity	pH
Parent material	Organic matter	Fertilizers
Rainfall		Rotations
Microbes		Pesticides
Vegetation		Tillage

The seasonal changes and management influence the size, dynamics and therefore the ability of the microbial biomass to supply nutrients **(Brookes *et al.*, 1985; O' Donnell *et al.*, 1994; Wu *et al.*, 1994; He *et al.*, 1996; Wu *et al.*, 2000)**. However, the knowledge is still rudimentary about the way by which such factors influence microbial community structure in soils and whether the changes that occur influence significantly on soil processes. According to **Stewart (1991)**, the effect of fertilizers on microbial communities has potentially important implications for sustainable agriculture. Fertilizers interact with microbial communities in soils in a number of ways either promoting growth directly providing nutrients or indirectly by stimulating plant growth and enhancing root C flow **(Buyanovsky and Wagner, 1987)**. Alternatively, soil acidification due to fertilizer inputs may limit microbial growth and activity in soil **(Shiel and Rimmer, 1988; Khonje *et al.*, 1989; Macrae *et al.*, 1999)**. A little is known about the effects of agrochemicals and pH on the complexity of microbial communities in soil trophic web **(O'Donnell *et al.*, 2001)**. **Kennedy and Gewin (1997)** identified a number of soil and climatic factors which influence microbial diversity in soils (Table 2.2). The pH and crop cover were described as important anthropogenic factors influencing microbial diversity in soil ecosystems. *In situ* manipulation and bioengineering of important soil process such as nutrient supply and retention can be possible if the impact of management on microbial diversity and how such drivers compete with environmental

factors such as climate is known. **O'Donnell *et al.* (2001)** investigated the impact of fertilizer management on the size, activity and structure of microbial communities in the long term hay meadow trials. The plots were receiving essentially the same fertilizer inputs since established in 1897. The obtained data by fumigation extraction procedures **(O'Donnell *et al.*, 1994; Wu *et al.*, 1994, 2000)** showed clearly that fertilizer inputs do impact on the size of the microbial biomass in different plots. The biomass C levels in these plots remained relatively stable inspite of the time of fertilizer amendment or the changing conditions through spring, summer, autumn and winter but the amount of microbial biomass was significantly higher in the manured plots than in either the control or in the plots treated with mineral fertilizers. The studies showed that fertilizer management does impact on the size of the microbial biomass in soils but provide little information on the impact of fertilizer inputs on the structure of microbial communities. **O'Donnell (2001)** showed that in spite of apparent spatial distribution of community DNA, there was no readily discernible differences in community structure between the rhizosphere soil and the non-rhizosphere soil when analyzed using 16S consensus or actinomycete primers followed by DGGE **(Macrae, 1998)**. The apparent similar DGGE profiles of diversity in soils can mask differences in the communities and therefore need to be interpreted cautiously. Similarities in DGGE community profiles **(Heuer *et al.*, 1997; Duineveld *et al.*, 1998; Felske and**

Akkermans, 1998) have been shown where one might reasonably have expected to see significant differences. The clone libraries derived from the rhizosphere soils are phylogenetically less diverse than those of the bulk soils, for *Brassica napus*, dominated by sequences of the *Bacillus megaterium* group **(Macrae, 1998). Marschner *et al.* (2002)** studied spatial and temporal dynamics of the microbial community structure in the rhizosphere of white lupin. DNA was extracted from the non-cluster roots, the young, mature and senescent cluster roots with adhering soils on days 21, 35 and 51 and DGGE of 16S rDNA, digitization of the band patterns and multivariate analysis was performed to assess the bacterial community structure. The results showed that the bacterial community structure changes with plant age in all root zones. The bacterial communities of the cluster roots were cluster age and plant age dependent and different from those of the non-cluster roots. Another experiment based on root exudates and DGGE showed that both bacterial and eukaryotic (18S rDNA) community structure changed with organic acid exudation. It was concluded that the bacterial community structure in the rhizosphere of white lupin is highly dynamic both spatially and temporarily.

Several studies have suggested that elevated atmospheric CO_2 concentration can also alter the composition of soil microbial communities due to changes in the amount and/or composition of plant material input into the soil **(O'Neill *et al.*, 1987; Rogers *et al.*, 1992)**. The population of *R. leguminosarum* bv. *trifolii* in the

rhizosphere of white clover plants increased two folds when subjected to elevated atmospheric CO_2 concentration whereas in the rhizosphere of ryegrass, no change was observed **(Schortemeyer *et al.*, 1996)**. Authors suggested that the increase in population of *R. leguminosarum* bv *trifolii* was due to plant derived signal molecules (root exudates) which were selectively enhanced under elevated CO_2. **Montealegre *et al.* (2000)** in another study showed a shift in the community composition of *R. leguminosarum* bv *trifolii* due to increased CO_2 concentration.

Montealegre *et al.* (2002) used phospholipids analysis (PLFA) to assess the microbial community composition in rhizosphere soil from white clover plants grown under ambient and elevated CO_2. Results indicated clear differences between microbial communities in rhizosphere and bulk soil in response to elevated CO_2 concentration. However, several studies showed no alteration in microbial structure due to increased CO_2 concentrations **(Zak *et al.*, 2000)**. Relationships between physicochemical properties of soil and microbiological properties could originate from specialization of microorganisms to different ecological niches and individual population of soil organisms may also have responded differently to soil environmental conditions. Dynamics of a soil microbial community under spring wheat was studied under a range of soil conditions such as no-till and chisel-till management by **Petersen *et al.* (2002)**. Results of PLFA, chemical analysis and microbiological

properties showed that adaptation to environmental stresses was partly responsible for the microbial dynamics, tillage practice had little effect on the relationships between soil conditions and microbiological properties. The analysis of microbial community attributes under a variety of realistic growth conditions provided evidence that microbial community was modified by environmental conditions during growing season.

The complexity of the *nif*H gene pool was examined with bulk DNA extracted from forest plant litter and soil by nested PCR followed by restriction fragment length polymorphisms (RFLPs) of *nif*H amplicons **(Widmer *et al.*, 1999)**. The resulted RFLPs of PCR products were reproducibly different than the RFLPs of PCR products from the underlying soil. The characteristic differences were found during the entire sampling period between May and September. 42 *nif*H clones were obtained from forest litter library which were grouped into different groups and among 64 *nif*H clones obtained from forest soil library 13 different patterns were found. Only two of the RFLP patterns were common in both litter and soil, indicating that there were major differences between nitrogen-fixing microbial community. Overall 20 distinctive *nif*H *Hae*III RFLPs were obtained, five of the *nif*H DNA patterns were dominant in forest litter having characteristic fragments of 237-303 bp length, whereas soil samples contained primarily seven other patterns 131-188 bp length.

Shaffer *et al.* (2000) in another study reported the same 237-303 bp fragments characteristic for forest litter which could generally not be detected in plant litter or soil samples collected from clearcut sites adjoining the forest sites. These fragments (237-303 bp) were consistently found in the litter at this DF forest site over 16 months and were also obtained from litter at 12 other DF forest or recent (<2 yrs) clearcut sites. Whereas, trace to none of these fragments were found in 6 clearcut (5-10 yrs) or different forest types. The authors suggested that logging practice in DF forests that creates a clearcut removes a unique gene pool of nitrogen-fixing microorganisms which could potentially contribute more to nitrogen-fixation in forest litter than litter from natural or invasive plants that grow in clearcut sites **(Shaffer *et al.*, 1998)**.

The structure and composition of the *nif*H gene pools of six different soils in relation to management, plant cover and physiochemical properties and five soil fractions extracted from soil were studied through RFLP of PCR amplified *nif*H amplicons **(Poly *et al.*, 2001)**. Principle component analysis revealed two soil groups: the first group included the two cultivated soils and the forest soil and second group, consisting of three soils under permanent pasture. The *nif*H gene pool of the second group exhibited a more distinctive composition that of gene pool of the first group of soils. The results revealed that the observed differences in *nif*H gene pool structure among various soils can not be explained by the measured

physicochemical characteristics, whereas most studies reported the influence of soil physics **(Riffkin *et al.*, 1999)** and chemical properties **(Giller *et al.*, 1998)** on diazotrophic activity. The discrepancy in the findings suggested that diazotrophic activity and diazotrophic community structure are not similarly affected by soil properties. **Poly *et al.* (2001)** suggested that long term cultivation with tillage, fertilizer amendment, pesticide treatments or specific plant cover would results in a structure of the *nif*H gene pool different from permanent pasture. The composition of the *nif*H gene pool varied both on a large scale (among soils) and on a microscale (among microenvironments isolated from one soil). Soil management was found to be the dominant parameter which influences the genetic structure in the unfractionated soils by controlling inorganic nitrogen content and its fluctuation.

Effect of Inoculated Bacteria

Existence of complex interactions is known between wheat genotype and different *Azospirillum* spp. and nitrogen-fixing bacteria that proliferate in the rhizosphere of plant, fixing nitrogen, and transferring it to plant. **Saubidet and Barneix (1998)** reported that in wheat, different *A. brasilense* strains colonize differently a single cultivar, and that the same strain colonize in different numbers when inoculated to different cultivars.

Field inoculation with *Azospirillum* sp. has been widely evaluated worldwide in different cultures, and it has been concluded

that these bacteria are capable of promoting yield of the agricultural important crops in different soils and climatic conditions **(Okon and Labandera-Gonzalez, 1994)**. In wheat significant yield increase has been reported widely for field inoculation **(Mertens and Hess, 1984; Millet *et al.*, 1985; Boddey *et al.*, 1986)** but response to inoculation has not always been successful, because the factors affecting the crop response are not well understood **(Okon and Labandera-Gonzalez, 1994)**.The establishment of *Azospirillum* in the roots is a critical step towards an effective plant growth promotion **(Okon and Kapulnik, 1986; Bashan and Holguin, 1997)**. **Bashan and Holguin (1997)** suggested that attachment of *A. brasilense* to wheat roots proceeds in two phases, an adsorption phase and anchoring phase, where the bacteria become irreversibly bound to the roots, involving surface polysaccharides and lectins. The variations in the field inoculation results suggested that an important environmental/ bacteria-plant association interaction must exist. Different environmental factors in root colonization of wheat rhizosphere by *A. brasilense* have been studied, such as pH **(New and Kennedy, 1989)**, nitrogen supply **(Vande Broek *et al.*, 1993)** and soil conditions **(Bashan, 1999)**. **Saubidet *et al.* (2002)** studied the colonization of wheat roots by efficient *A. brasilense* strain BNH-10, under different N supplies, in disinfected and non-disinfected soil in order to understand the effects of bacterial inoculation on plant growth, and its interaction with environment. The plant grown in the

disinfected soil showed higher biomass and 'N' content in tissue than those in the non-disinfected soil and at maturity the inoculated plants showed higher grain yield and protein concentration also as compared to uninoculated.

For the establishment of an effective association, attachment of bacteria to roots is an important and necessary condition. In case of *Azospirillium* two attachment phases were described that differ it in firmness and in the metabolites mediating these processes **(Michiels *et al.*, 1991)**. The first phase is associated with the hydrophobic proteins at the bacterial surface which is rapid, weak and reversible **(Castellanos *et al.*, 1998)** and is mediated by polar flagellum **(Croes *et al.*, 1993)**. The second phase called anchoring is characterized by the formation of fibrils and is affected by polysaccharides **(Michiels *et al.*, 1990; 1991)**. Plant lectins are known to play an important role in attachment by fulfilling an acceptor role for bacterial-surface polysaccharides **(Del Gallo *et al.*, 1989; Konnova *et al.*, 1994; Karpati *et al.*, 1995)**. **Yegorenkova *et al.* (2001)** studied the dynamics of adsorption of the nitrogen-fixing soil bacteria *Azospirillum brasilense* 75 and 80 and *A. brasilense* Sp 245 to the roots of seedlings of common spring wheat in relation to inoculum size, period of incubation and with the roots and bacterial growth phase. With increasing size of inoculum and time of contact the number of root attached cells were also increased.

The change in pH of rhizosphere of several plant species have been demonstrated as result of imbalances in cation/ anion uptake **(Marschner *et al.*, 1986)**. The process is also affected by root exudates transport process and release of H^+ associated with root growth **(Werner, 1992)**. Proton extrusion from wheat roots is an important phenomenon of proton efflux from the root is directly related to important physiological processes, i.e. the mineral uptake **(Nye, 1981; Bashan and Levanony, 1989; Werner, 1992)** and plant cell enlargement **(O' Neil and Scott, 1983; Werner, 1992)**. Proton efflux has been correlated with root extension **(Pilet *et al.*, 1983; Werner, 1992)**, geotropism **(Mulky and Evans, 1981)**, iron **(Romheld and Marschner, 1981)** or Phosphorus **(Hedley *et al.*, 1983)** deficiency, nutrition by ammonium or nitrate **(Bashan and Levanony, 1989)**. Plant growth promoting bacteria also affect the proton efflux **(Bashan, 1990; Bashan *et al.*, 1992; Bertrand *et al.*, 2000)**. *A. brasilense* is also reported to cause enhancement of proton efflux in wheat **(Bashan *et al.*, 1989; Bashan, 1990)**, cowpea and soybean **(Bashan *et al.*, 1992)**. However, root growth and proton efflux is stimulated by azospirille **(Bashan and Holguin, 1997)**.

Amooaghaie *et al.* (2002) conducted an experiment to find out the correlation between these two-phenomenon and their relation with rate of colonization of root and found that compatible strains are necessary for increasing of proton efflux and root extension in wheat cultivars. Several diazotrophic bacteria belonging to the genera

Acetobacter, Alcaligenes, Azoarcus, Azospirillum, Enterobacter, Herbaspirillum, Pseudomonas, Zoogloea have been isolated from rice and other members of the family Gramineae **(You *et al.*, 1991; Dobereiner, 1992; Reinhold-Hurek *et al.*, 1993; Malik *et al.*, 1994; Baldani *et al.*, 1996; Hassan *et al.*, 1998)**. *Acetabacter, Azoarcus, and Herbaspirillum* have been considered as endophytes because of their occurrence in plant tissues **(James and Olivers, 1998).** Enhancement of crop yields of cereals by inoculation with nitrogen-fixing bacteria have been observed in many field **(Boddey *et al.*, 1991; Urquiaga *et al.*, 1992; Dobereiner *et al.*, 1993)**. Yield increase is attributed to biological N_2 fixation and also to the production of plant growth substances by colonizing bacteria.

MATERIALS AND METHODS

3.1 FIELD SITE

Field sites for present study were located in village Bhawanipur, Dist. Badaun (longitude 78^0 30'E, latitude 28^0 15'N, sea level 278.5 m, average rainfall 820 mm, average temp: lower 1-6^{0}C, maximum 42-48^{0}C, humidity 30-40%, average moisture on 15 bar 3.5%, average moisture on 1/3 bar 20.5%), Uttar Pradesh. The selection of fields was made on the basis of fertilizer input and grain yield. Three fields were selected: (i) Low input-low yield (SLL), (ii) Low input-high yield (SLH) and (iii) High input-high yield (SHH). SLL and SHH fields were practiced as raised bed/ plain bed to observe the impact of flooded conditions on the diazotrophic community. Over the past 20 years, rice-wheat rotation had been practiced in these fields, which are largely rainfed. The farmers are cultivating wheat variety UP-2338 for the last several years.

3.2 SAMPLING

Soil samples were collected at 0 d (pre-sowing) and after 140 d (post-harvesting) from the upper 0-15 cm profile for analysis of soil nutrient and indigenous bacterial population, samples were collected randomly from five different sites, from a 0.5×0.5 m^2 area.

For assessment of culturable bacterial and *in situ* community dynamics plants were uprooted at 25 d (crown root initiation), 45 d (tillering), 90 d (flowering) and 120 d (maturity) stages of wheat

growth; 0 d and 140 d samples were also collected for analysis. Ten plants along with roots and rhizospheric soil were uprooted from five different places in each field and samples from the same field were pooled together. Samples were transported to the laboratory on dry ice. For cultural analysis, samples were stored at 4°C; for *in situ* community analysis, at -80°C and for enzyme assays at -20°C.

For analysis of C, N, P and K, and micronutrients soil was air dried.

3.2.1 Soil Parameters

Soil pH, organic C **(Walkey and Black, 1934)**, total N and total P **(Jackson, 1973)**, available N **(Subhiah and Asija, 1956)**, available P **(Olsen *et al.*, 1954)**, and exchangeable K **(Schollenberger and Simon, 1945)** were analyzed. Micronutrient (Zn^{++}, Mn^{++}, Cu^{++}, Fe^{++}) analysis was carried out by Atomic Absorption Spectrophotometry **(Lindsay and Norvell, 1978)**.

3.2.2 Bacterial Population Dynamics and Recovery of Bacteria

For assessment of bacterial dynamics in bulk soil (BS), rhizosphere (RS) and rhizoplane/ endorhizosphere (RE) fractions, dilution plating was performed an Angle's medium, Jensen's agar, and yeast extract mannitol agar (YEMA).

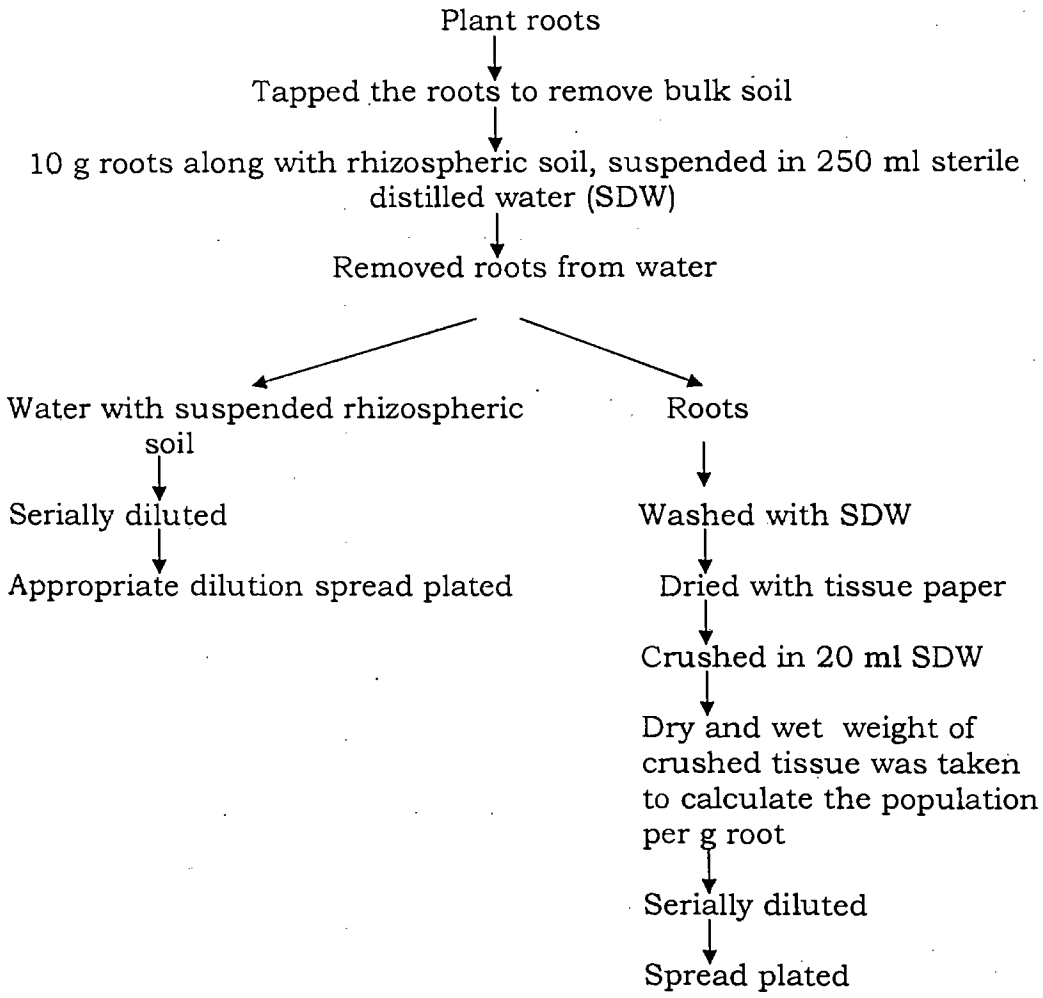
3.2.3 Plating of Bulk Soil

A sample of 10 g bulk soil as suspended in 100 ml of 0.85% saline and shaken vigorously at 150 rpm at 15°C for 1 h in order to detach bacteria from soil particles. After shaking, it was serially diluted and 100 µl of 10^5, 10^6 dilutions were spread plated on Angle's

medium for total bacterial counts; 10^2, 10^3 dilutions on Jensen's agar medium for all free-living nitrogen fixers, and 10^4, 10^5 dilutions on YEMA for rhizobial counts. Plating was done in triplicates. Plates were incubated at 28±1°C in a BOD incubator. Colonies on Angle's medium were counted after 48 and 72 h, on YEMA after 48 h, and on Jensen's after 4 d.

3.2.4 Rhizosphere (RS) and Rhizoplane/Endorhizosphere (RE)

For enumeration and isolation of bacteria from these fractions, following steps were employed:



3.2.5 Isolation, Purification and Preservation

Isolates from Jensen's medium were picked up from RS/RE fractions of 45 d sample and restreaked on the same medium for pure culture. Pure cultures were maintained on ½ strength Jensen's slants and preserved in 20% glycerol at -80°C.

3.3 SOIL ENZYME ASSAYS

Soil enzyme activity is a measure of microbial population structure and functions. Therefore, dehydrogenase, and acid and alkaline phosphomonoesterase (phosphatase) activities were measured for assessment of population dynamics.

3.3.1 Dehydrogenase Activity

Soil dehydrogenase activity was assayed to assess the biological activity of the sample. Dehydrogenase activity was measured according to modified method of **Thalman (1968)** in terms of trimethyl formazan (TPF) using triphenyltetrazolium chloride (TTC) as substrate.

Reagents

- Tris buffer (0.1 M; pH 7.4)
- Substrate solution (0.7%)
- Standard stock solution (10 mg TPF ml^{-1})
- Working standard solution (0.1 mg TPF ml^{-1})

Procedure

5 g moist soil in 4 tubes (30 ml capacity)

↓

Added 5 ml of substrate solution in 3 tubes; to 1 tube, 5 ml of Tris buffer (control), (0.1 M; pH 7.4)

↓

Mixed, closed the tubes with rubber stoppers and incubated for 16 h at 25°C

↓

25 ml acetone was added to both the samples and control in order to extract TPF

↓

Mixed thoroughly and kept on shaker for 2 h in dark

↓

Contents were filtered in a semi dark room

↓

O.D. of filtrate was taken at 546 nm within 1 h

Calibration Curve

Pipetted 0 (reagent blank), 1, 2, 5, 7 and 10 ml of working standard (0.1 mg TPF ml^{-1}) into 6 tubes, diluted to 30 ml with acetone. Calibration standard correspond to 0, 100, 200, 500, 700, and 1000 µg TPF. Regression values was calculated.

Calculation for Dehydrogenase

The level (µg) of TPF in the filtrate were determined from the calibration curve.

$$\text{Dehydrogenase activity} = \frac{(S-C)\times 100}{5\times \%\ dm} = \mu g\ TPF g^{-1}\ dm\ 16\ h^{-1}$$

S→mean value of sample (µg TPF)

C→mean value of control (µg TPF)

100% dm^{-1} factor for soil dry matter

5→initial soil weight

3.3.2 Acid/Alkaline Phosphomonoesterase Activity

Acid and alkaline phosphatase activity was measured in terms of release of p-nitrophenol (pNP) using p-nitrophenyl phosphate as substrate **(Tabatabai and Bremner, 1969).**

Reagents

- Modified universal buffer stock solution

12.1 g	Tris (hydroxymethyl) aminomethane
11.6 g	Maleic acid
14.0 g	Citric acid monohydrate
6.3 g	Boric acid in 1 M NaOH
1000 ml	Distilled water
Kept at 4^{0}C	

- Working buffer solution for acid phosphatase (pH 6.5)
- 200 ml modified universal buffer was diluted to 1000 ml and adjusted to pH 6.5
- Working buffer solution for alkaline phosphatase (pH 11.0)
- Same as for acid phosphatase, pH was adjusted to 11.0
- Substrate solution for acid phosphatase (115 mM) in working buffer solution (pH 6.5)
- Substrate solution for alkaline phosphatase (115 mM) in working buffers solution (pH 11.0)
- Calcium chloride (0.5 M)
- NaOH (0.5 M)
- Standard stock solution (1 mg pNP ml^{-1})
- Working standard solution (20 μg pNP ml^{-1})

Procedure

1 g moist soil in 4 flasks

↓

Added 1 ml desired substrate solution (either acid or alkaline) +4 ml corresponding working buffer;

4 ml working buffer in residual flask (control)

↓

Shaken briefly, closed and incubated for 1h at 37°C

↓

Added 1 ml $CaCl_2$ solution (0.5 M)

+

4 ml NaOH (0.5 M) to both sample and control

↓

Pippetted 1 ml of the used substrate solution to control

↓

Added 90 ml of distilled water to both samples and control

↓

Shaken briefly and filtered

↓

Extinction of yellow colour intensity was measured at 400 nm against blank

Calibration standards

Pipetted 0 (reagent blank) 1, 2, 3, 4 and 5 ml of working standard (20 µg pNP ml^{-1}) into six tubes, the volume was adjusted to 5 ml with distilled water; added 1ml of calcium chloride (0.5 M) and 4 ml NaOH (0.5 M), mixed thoroughly and filtered through two folded filters. Absorbance was taken at 400 nm on DU spectrophotometer, calibration standards corresponded to 0, 20, 40 60, 90 and 100 µg of p-nitrophenol.

Calculation for Phosphomonoesterase Activity

The activity is expressed as µg p-nitrophenol (pNP) per g dry matter and incubation time. The conc. of pNP in samples and control was calculated from the calibration curve.

$$\text{Phosphomonoesterase activity} = \frac{(S-C)\times 10\times 100}{\%\ \text{dm}} = \mu g\ pNP g^{-1}\ dm\ h^{-1}$$

S→mean value of sample (µg pNP)

C→mean value of control (µg pNP)

100% dm^{-1} factor for soil dry matter

3.4 SOIL DNA EXTRACTION WITH FAST PREP MACHINE

DNA was extracted from rhizospheric soil from all fields at different stages of wheat growth using Fast DNA SPIN KIT (for soil) following the manufacturer's (BIO 101, Inc.) protocol.

***In situ nif* H Community Dynamics**

A large number of microorganisms are diazotorphic and possess *nif*H gene. Although *nif*H is considered as a marker gene in diazotrophic population, but still is very diverse among them. Hence, degenerate primers were used to amplify *nif* H gene from soil nitrogen-fixing community.

*nif*H regions were amplified by using hemi-nested PCR according to modified protocol of **Widmer *et al.* (1999)**. Three primers (Microsynth, Balgach, Switzerland), *nif*H (for A) and *nif*H (rev) were used for first step PCR and *nif* H (for B) and *nif* H (rev) for second step heminested PCR. The reverse primer was modified by adding a 40 bp

GC-clamp generally used for denaturing gel electrophroesis, although the property of GC-clamp was not used. But the size of second PCR product was 40 bp longer than expected (according to the position of the primer on *Azotobacter vinelandii nifH* gene). The description of the primers is as follows:

*nif*H (for A)
5′- GCI WTI TAY GGN AAR GGN GG- 3′
(size, 20 mer; position, 19-38; degeneracy, 128 times)

*nif*H (rev)
5′- CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG
GCR TAI ABN GCC ATC ATY TC- 3′
(size, 20 mer with 40 bp GC-clamp; position, 463-482;
degeneracy, 48 times)

*nif*H (for B)
5′- GGI TGI GAY CCN AAV GCN GA- 3′
(size, 20 mer; position, 112-131; degeneracy, 96 times)

⇒ DNA sequence degeneracies are indicated by using the International Union of Pure and Applied Chemistry Conventions **(Liebecq, 1992)**.

R → A/G; Y → C/T; W → A/T; V → A/C/G;
B → C/G/T; N → A/C/G/T

Inosine (I) was used to reduce the degeneracy of the primer by replacing four fold-degenerate positions (N) in the 5′ portions (N) because it can pair with all the four nucleotides.

First Step-PCR

Reaction was set for 20 µl by mixing the reagents in the following concentrations :

Buffer	1X
$MgCl_2$	1.5 mM
dNTPs	0.2 mM
nif H (for A)	0.5 µM
nif H (rev)	0.5 µM
Taq polymerase	1 U (Genei, Bangalore, India).

Reaction conditions for first step PCR

Step 1	Initial denaturation	94°C	4:30 min
Step 2	Denaturation	94°C	0:45 min
Step 3	Annealing	50°C	0:30 min
Step 4	Extension	72°C	0:20 min
Step 5	29 cycles from step 2		
Step 6	Final extension	72°C	10:0 min

Second Step Hemi-nested PCR

Reaction was set for 50 µl volume by mixing the reagents in following concentration:

Buffer	1X
$MgCl_2$	1.5 mM
dNTPs	0.2 mM
nif H (for B)	0.5 µM
nif H (rev)	0.5 µM
Taq polymerase	1 U (Genei, Bangalore, India).

Reaction conditions for Hemi-nested PCR

Step 1	Initial denaturation	94°C	4:30 min
Step 2	Denaturation	94°C	0:45 min
Step 3	Annealing	50°C	0:30 min
Step 4	Extension	72°C	0:20 min
Step 5	34 cycles from step 2		
Step 6	Final extension	72°C	10:0 min

Amplified product was checked on 1% agarose gel (0.5 mg/ml EtBr) by electrophoresis.

Restriction Fragment Length Polymorphism (RFLP)

To analyze the time, input and practice dependent changes in the *nif*H community, *nif*H amplicons from all samples (BS, RS) were subjected to double digestion with two restriction endonucleases (tetra-cutturs) viz. *Alu* I and *Rsa* I for 8 h at 37°C. The reaction was set for 25 µl, as follows:

10 X buffer	2.5 µl
Alu I (10 U/µl)	0.2 µl
Rsa I (10 U/µl)	0.2 µl
TDW	7.1 µl
PCR product	17.0 µl

Polyacrylamide Gel Electrophoresis (PAGE)

Digested DNA samples were analyzed by electrophoresis in 8.5% polyacrylamide gel (30 :1). The electrophoresis conditions were 80 V

for 1 h followed by 60 V for 7 h in 1X Tris-borate-EDTA buffer. The gel was stained by ethidium bromide (0.5 μg/ml) for 20 min and visualized under UV.

For the verification of consistency of patterns, each sample was analyzed at least twice.

3.5 FUNCTIONAL CHARACTERIZATION OF ISOLATES

For functional characterization of bacterial isolates, growth under different conditions, siderophore production, phosphatase activity, and antagonisms against phytopathogens were evaluated.

Growth Characteristics

Isolates were checked for their ability to grow on different nitrogen free media and under different/ conditions viz., aerobic/microaerophillic.

3.5.1 Ability to Grow in N-free Media

Pure cultures were streaked on N- free **Dworkin and Foster (1958)** minimal DF and LGI medium along with control medium supplemented with $(NH_4)_2SO_4$ (0.2%). Plates were incubated at 28±1°C till growth appeared. In DF medium, agarose was used as the solidifying agent.

3.5.2 Ability to Grow under Aerobic/ Microaerophilic Conditions

Pure cultures were streaked on N-free ABpy medium along with control medium supplemented with NH_4NO_3 (1%) as nitrogen source. Plates were incubated in the normal O_2 atmosphere and in 2% O_2 at 28°C till the growth appeared.

3.5.3 Siderophore Production

Pure cultures were spot inoculated on chrom-azurol S (CAS) plates **(Schwyn and Neilands,1987)** and incubated at 28±1^{0}C in BOD for 3 d. Orange halo zone around culture indicated positive test for siderophore production.

3.5.4 Phosphatase Activity

Phosphatase activity was checked on tryptose phosphate agar (TPA) supplemented with 2 mg ml^{-1} of the substrate, tetra sodium salt of phenolphthalein diphosphate and 0.05 mg ml^{-1} methyl green as indicator **(Rodriguez *et al.*, 2000)**. Pure culture was spot inoculated on TPA and incubated at 28^{0}C for 3 d. Positive isolate turned dark green in colour.

3.5.5 Antagonistic Property

Antagonistic property of isolates was checked against three phytopathogens, *Helminthosporium sativum, Fusarium oxysporum* and *Pythium* sp. by dual culture bioassay. A 1 mm agar disc was removed from actively growing fungus on potato dextrose agar (PDA) and placed on PDA : NA (1:1 w/w) plates. Test bacterial isolates were spot inoculated towards periphery of the plate which were incubated at 28±1^{0}C for 4 d. Inhibition of fungus growth, if any, around the test bacterial culture was measured.

3.6 MOLECULAR CHARACTERIZATION OF BACTERIAL ISOLATES

Molecular characterization of the bacterial isolates was carried out by 16S rDNA amplification followed by amplified ribosomal DNA restriction analysis (ARDRA).

3.6.1 Genomic DNA Extraction

Genomic DNA from isolates was extracted by a modified method of **Bazzicalupo and Fani (1994).**

Reagents

1. Tris-Cl (0.1 M, pH 6.8)
2. Tris –EDTA (pH 8.0)
3. Sodium dodecyl sulphate (SDS) (10%)
4. NaCl (5 M)
5. Cetyl-trimethyl ammonium bromide (10%)
6. Proteinase K (20 mg/ml)
7. Phenol : chloroform (1:1 v/v)
8. Chloroform : Isoamyl alcohol (24 :1 v/v)
9. Rnase (10 mg ml^{-1})
10. Ethanol
11. Tris-acetate EDTA (TAE)(1X)
12. Loading dye (6 X)
13. Ethidium bromide (10 mg/ml)

Extraction protocol

1. A single bacterial colony was streaked on LB agar plate and incubated at 28±1^{0}C for 18 h.

2. The culture was scrapped using a loop and suspended in 1.5 ml of Tris-Cl (0.1 M, pH 6.8).
3. Suspension centrifuged at 6000 rpm for 5 min and resulting pellet was washed with Tris-Cl to remove any polysaccharides.
4. Pellet was resuspended in 567 µl TE (pH 8.0), added 30 µl SDS (10%) and 3 µl proteinase K (20 mg ml^{-1}).
5. Mixed the content by gentle inversion of tubes and incubated at 37°C for 1-2 h till solution become clear.
6. 100 µl of 5 M NaCl and 80 µl 10% CTAB were added and mixed contents by inversion; samples incubated at 65°C for 10 min.
7. Added 600 µl of phenol : chloroform (1 :1) and mixed gently by inversion, centrifuged at 11,000 rpm for 5 min.
8. Upper aqueous phase was collected to which were added 800 µl of chloroform : isoamyl alcohlol (24:1); contents mixed by inversion and finally centrifuged at 11,000 rpm for 10 min at 4°C.
9. Upper aqueous phase was collected in Eppendorf tubes; 2 µl RNase (10 mg ml^{-1}) was added and tubes incubated for 30 min at 37°C.
10. Double volume of chilled absolute ethanol was added and tubes kept overnight at -20°C.
11. Contents of tubes centrifuged at 11,000 rpm for 10 min at 4°C. Supernatant was discarded and pellet was washed with 70% chilled ethanol.

12. Finally, pellets were air dried and suspended in TE (pH 8.0).

3.6.2 Quantification of DNA

Extracted DNA was electrophoresed on 0.8% agarose gel at 80 V and visualized on UV transilluminator. The quantity and purity of DNA was checked by its absorbance at 260/280 on DU 640B Beckman spectrophotometer.

Amplification of 16S rDNA

Amplification of 16S rDNA was performed on the genomic DNA of the forty four bacterial isolates recovered from Jensen's medium along with 16 standard cultures, of which 11 were nitrogenase positive. Two universal eubacterial primers were used:

Forward

GM3f-5'AGAGTTTGATCMTGG- 3'

Reverse

GM4r-5'TACCTTGTTACGACTT- 3'

50 µl PCR reaction was set with the final concentration of reagents as follows:

Buffer	1X
$MgCl_2$	2.0 mM
dNTPs	0.25 mM
Primer	0.25 µM each
Taq polymerase	1 U (Genei, Bangalore, India)
Template	10-15 ng

PCR Reaction Conditions

Step 1	Initial denaturation	94°C	3 min
Step 2	Denaturation	94°C	1 min
Step 3	Annealing	64°C	1 min
Step 4	Extension	74°C	1 min
Step 5	3 cycles from step 2		
Step 6	Denaturation	94°C	1 min
Step 7	Annealing	53°C	1 min
Step 8	Extension	74°C	1 min
Step 9	2 cycles from step 2		
Step 10	Denaturation	94°C	1 min
Step 11	Annealing	52°C	1 min
Step 12	Extension	74°C	1 min
Step 13	20 cycles from step 10		

The PCR was performed on a PTC-200 thermal cycler (M.J. Research). The amplified product was visualized on 0.8% agarose under UV transilluminator.

3.6.3 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Two restriction endonucleases (both tetra-cutturs) viz., *Taq* I and *Hae* III (MBI, Fermentas) were used for analysis, as per the following conditions:

10 X buffer	2.0 µl
Enzyme (10 U/µl)	0.1 µl
TDW	5.9 µl
DNA	12.0 µl

The mixture was incubated for 4 h at 37°C for *Hae* III and 65°C for *Taq* I. Restriction products were analyzed on 2.5% agarose gel. Electrophoresis was carried out at 60 V for 4-5 h.

3.7 SOIL MICROCOSM EXPERIMENT FOR PLANT GROWTH PROMOTION

Plant growth promotion in soil microcosm was studied under non sterile soil conditions employing all forty four isolates and sixteen standard bacterial cultures. Twenty one isolates were recovered from SLL, ten from SLH and thirteen from SHH. Overall 60 treatments were used in microcosm experiment.

Table 3.1: Bacterial Isolates used for Treatments

	Isolate/ Catalogue No.
Standard culture	*Alcaligenes faecalis* Neu 1033, *Aquaspirillum dispar* Neu 1046, *Azospirillum brasilense* Sp.7, *Azotobacter beijerenckii, A. chroococcum, A. vinelandii, Citrobacter freundii* Neu 1020, *Enterobacter aerogens* Neu 1036, *Enterobacter cloacae* Neu 1027, *Escherichia coli* Neu 1006, *Klebsiella oxytoca* Neu 30, *Klebsiella terrigena* Neu 1103, *Paenibacillus polymyxa* Neu 25, *Pseudomonas fluorescens* Neu 1043, *Rhizobium leguminosarum* DSM 30132, *Xanthobacter autotrophicus* Neu 2137
SLL	SLL3, SLL4, SLL5, SLL22, SLL27, SLL29, SLL32, SLL34, SLL42, SLL45, SLL55, SLL68, SLL71, SLLRE23, SLLRE30, SLLRE39, SLLRE43, SLLRE45, SLLRE48, SLLRE58, SLLRE62
SLH	SLH2, SLH8, SLH10, SLH19, SLH25, SLH34, SLH39, SLH46, SLH51, SLH52
SHH	SHH2, SHH15, SHH16, SHH17, SHH29, SHH33, SHH36, SHH29, SHH59, SHH68, SHH72, SHH79, SHH80

The distribution was 13 from SLL(RS), 8 from SLL(RE), 10 from SLH(RS), 13 from SHH(RS). Sixteen standards were used, all were positive for nitrogenase except *Alcaligenes faecalis* Neu 1033, *Enterobacter aerogens* Neu 1036, *E. cloacae* Neu 1027, *Escherichia coli* Neu 1006 and *Pseudomonas fluorescens* Neu 1043.

3.7.1 Potting Mixture

The original field soil from Bhawanipur was used which was loamy sand (sand, 72%; silt, 14%; clay, 8-10%). SLL : SLH were in the ratio of 1:1 (w/w)

3.7.2 Experimental Design

The experiment was carried out under net house conditions in the Department. Five replicates for each treatment were placed in a randomized design. Total of 60 bacterial treatments were used in the study described in Table 3.1

Pot size	:	750 g soil volume per pot
Wheat variety	:	UP 2338
Plants per pot	:	4

3.7.3 Inoculum Preparation

Pure bacterial cultures were grown in ½ strength nutrient agar at 28±1°C for 24 h. Cultures were suspended in 0.85% saline to maintain an OD to 1.0 at 600 nm.

3.7.4 Seed Bacterization

Carboxymethyl cellulose (0.1% CMC) was mixed in culture broth to allow cells to adhere properly to seed surface. One ml culture was added to 1 g seed, mixed properly and air dried for 3 h before sowing.

3.7.5 Sowing and Watering

Five seeds were sown per pot. Thinning was done to four plants after germination. Watering of plants was done on alternate days to maintain moisture.

3.7.6 Observation

Plants were harvested at 30th day of sowing and observed for the following parameters.

Growth parameters

(i) Plant height
- Root and shoot length were measured

(ii) Plant weight
- Root and shoot fresh and dry weight were recorded.

3.8 SOIL ENZYME ACTIVITY

The effect of introduced bacteria on indigenous population was measured in terms of different soil enzyme activity. For enzyme analysis rhizospheric soil and loose adhared soil were taken. Dehydrogenase and acid and alkaline phosphomonoesterase activities were monitored as described in section 3.3.

3.9 TOTAL SHOOT N AND P

Total N and P in plant shoot tissue of selected treatments were analyzed. Total N was assayed using semi automatic nitrogen

analyzer, Gerhardt, Germany, according to **Jackson (1973)**. Total phosphorous was analyzed according to the method given by **Jackson (1973)**.

3.10 ACETYLENE REDUCTION ASSAY

The promising isolates were checked for nitrogenase activity **(Hardy *et al.*, 1968b)**.

Procedure

1. A loopful culture was streaked on semi-solid slant of Jensen's medium.
2. Cultures incubated at 28^{0}C for 5 d.
3. Cotton plugs were replaced with air tight serum stoppers.
4. 10% of air in the tubes was replaced with acetylene.
5. Tubes were incubated at 28±1^{0}C for 16-24 h till growth appeared.
6. 1 ml of gas sample was drawn from the tube and injected into gas chromatograph for ethylene estimation.
7. Values for 1 ml standard ethylene gas were recorded.
8. Total protein of in culture samples from the tube was estimated as per following steps:

⇒ The culture was collected in 2 ml of 2 N NaOH.

⇒ Cell suspension was kept in boiling water bath for 10 min.

⇒ Tubes were cooled, neutralized with 2 ml 2N HCl and protein content estimated by Bradford's method.

9. C_2H_4 produced was calculated using the following formulae.

n moles of C_2H_4 produced hr^{-1} mg^{-1} protein

$$= \frac{C \times Ps \times As \times V}{Pstd \times Astd \times T \times P}$$

Where,

C = Concentration of ethylene in standard in n moles

Ps = Peak height of sample

As = Attenuation used for sample

Pstd = Peak height of standard

T = Time of incubation in hrs.

P = Protein content of bacterial growth on slant in mg

V =volume of air space in the assay vial.

3.11 *nif* H AMPLIFICATION OF ISOLATES

*nif*H amplification of promising isolates was performed with degenerate primers as described in section 3.4.

3.12 IDENTIFICATION OF ISOLATES

Identification of promising isolates was performed by FAMES analysis.

3.13 SEQUENCING

*nif*H positive isolate was subjected to partial 16S rDNA sequencing (ABI 310 genetic analyzer, Applied Biosystem).

3.12 STATISTICAL ANALYSIS

Statistical analysis was performed for the data collected from microcosm experiment. Growth parameters and soil enzymes were analyzed by ANOVA. Correlation was determined for growth parameters, soil enzymes, total N and total P content of treated plants.

RESULTS

4.1 SOIL PARAMETERS

Soil parameters for all the fields with respect to pH, organic carbon, organic matter, total 'N', available 'N', total 'P', available 'P' and micronutrients were analyzed. The results are summarized in Table 4.1a and 4.1b.

There was a marginal difference in organic carbon content of SLL PF/RB (0.61/0.62) and SLH (0.64) but % organic 'C' was comparatively high in the SHH PF/RB (0.83/0.84) as compared to the former. Nearly similar trend was observed in case of total 'N' although available 'N' level was comparable among various fields. There was clearcut difference in total 'P' within the experimental fields (Table 4.1a). Total 'P' level observed was highest in SHH PF/RB (1672.67/1686.68 kg ha^{-1}) followed by SLH (1040.66 kg ha^{-1}). Available 'P' content of SHH PF/RB fields was almost 3 times as much as SLL PF/RB and twice as much as SLH. Exchangeable potassium level was highest in SLH (190.61 kg ha^{-1}) followed by SHH PF/RB (183.80 and 170.19 kg ha^{-1} respectively).

All the soils were on the alkaline side with a pH high of 7.4 in SLH. Cu^{++} concentration in SLH (3.02 ppm) was highest and about 1.5 times greater than SLLPF/RB and 2 times that of SHHPF/RB. In SHH field, a clearcut impact of practice was observed in Cu^{++} conc. i.e. SHH PF (1.36 ppm) and SHH RB (1.65 ppm). Zn^{++} concentration was highest

in SLH (1.62 ppm) followed by SHH RB (1.15 ppm); this was twice as high as that in SHH PF (0.59 ppm).

Highest concentration of Mn^{++} was recorded in SLH (24.11 ppm) as compared to SLLPF/RB (17.13 ppm and 18.54 ppm) and SHH PF/RB (19.25 ppm and 20.00 ppm). The available Fe^{++} concentration was 42.66 ppm in SHH RB which was about twice the levels observed in SLL and SLH field soils (Table 4.1b).

4.2 DYNAMICS OF CULTURABLE BACTERIAL POPULATIONS

For the assessment of input/ practice dependent and wheat growth stage-dependent changes in bacterial populations, dilution plating was performed for bulk soil (BS), rhizosphere (RS) and rhizoplane/ endorhizosphere (RE) fractions.

Total bacterial population for all the five fields (SLL PF; SLL RB; SLH; SHH PF; SHH RB) was observed on Angle's Medium (Fig.4.1a) whereas free living N_2-fixers (Fig. 4.1b) and *Rhizobium* on Jensen's agar and YEMA (Fig. 4.1c), respectively.

4.2.1 Bacterial Population in Bulk Soil

4.2.1.1 Total

At 0 d, total bacterial population was of the order, 10^6 cfu g^{-1} soil in all five fields; maximum population (2.15×10^6 cfu/2.35×10^6 cfu) was observed in SHH PF/RB. After harvest of crop at 140d, the total bacterial population remained almost at the same initial level (Fig. 4.2).

Table 4.1a: Macronutrient status of experimental fields

Fields	Organic (%)	Organic matter (%)	N (Kg ha^{-1})		P (Kg ha^{-1})		Exchangeable potassium (Kg ha^{-1})
			Total N	Available N	Total P	Available P	
SLLPF	0.61±0.01	1.06±0.02	1198.22±31.92	95.75±1.91	918.99±79.87	11.44±0.26	136.15±11.76
SLLRB	0.62±0.01	1.08±0.02	1205.19±43.51	96.24±1.91	923.82±69.11	11.98±0.71	149.76±11.79
SLH	0.64±0.01	1.11±0.03	1219.12±60.33	99.41±3.80	1040.66±59.47	15.39±0.18	190.61±11.79
SHHPF	0.83±0.01	1.42±0.03	1581.37±43.51	100.77±0.48	1672.67±143.15	28.49±1.07	170.19±11.79
SHHRB	0.84±0.04	1.45±0.07	1602.27±31.92	100.87±0.91	1686.68±57.38	30.65±1.82	183.80±20.42

Table 4.1b: Chemical and Micronutrient status of experimental fields

Fields	pH	Micronutrient Conc. (ppm)			
		Cu	Mn	Fe	Zn
SLLPF	7.2	1.65±0.03	18.54±0.33	22.14±0.58	0.62±0.03
SLLRB	7.2	1.65±0.06	17.13±0.37	22.56±0.58	0.58±0.01
SLH	7.4	3.02±0.20	24.11±01.66	20.366±1.76	1.62±0.08
SHHPF	7.3	1.36±0.01	19.25±0.27	38.31±0.23	0.59±0.02
SHHRB	7.3	1.65±0.04	20.00±0.40	42.66±0.61	1.15±0.04

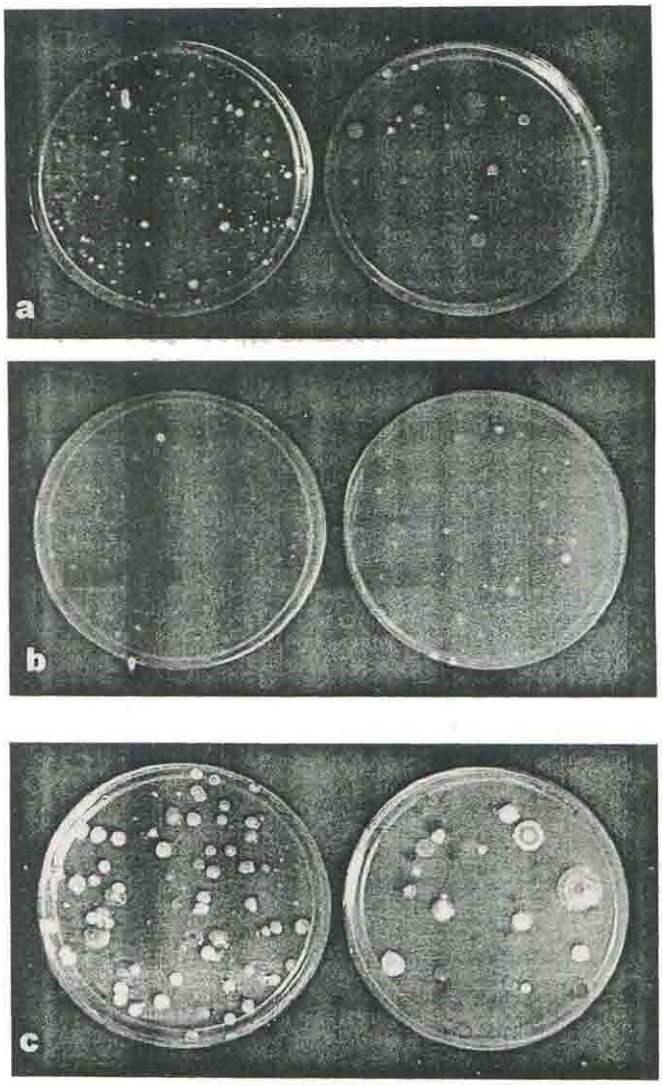


Fig. 4.1 : Rhizospheric culturable diversity
(A) Total bacterial population on Angle's medium
(B) Free living N_2 fixing bacterial population on Jensen's medium
(C) *Rhizobium* population on YEMA

4.2.1.2 Free living N_2-fixers

Free- living N_2-fixers on Jensen's medium were greater in SLH (1.05×10^4 cfu g^{-1} soil) compared to SLL and SHH PF/RB (10^3 cfu). At 140 d the population was of the order, 10^4 cfu g^{-1} in various soil systems (Fig. 4.3).

4.2.1.3 *Rhizobium*

Rhizobium population was assessed on YEMA and was of the order, 10^5 cfu in various soil systems at 0 d; this declined to ~10^4 cfu g^{-1} of soil after 140 d. Maximum population was observed in SHH RB at 0 as well as at 140d (Fig. 4.4).

4.2.2 Plant Growth Associated Bacterial Population Dynamics

Over the period of crop growth, rhizosphere (RS) and rhizoplane/ endorhizosphere (RE) fractions were analyzed for total bacterial, free- living N_2-fixers and *Rhizobium* counts.

4.2.2.1 Total bacterial population structure

In all soil systems, a similar trend was observed for total bacterial population. The population increased from 25 d upto 90 d. Maximum population was established in SHH RB rhizospheric samples (6.94×10^7 cfu) which declined later by approximate one log unit (Fig. 4.5a).

Closely similar RS population dynamics were followed in RE fractions. A gradual increase was observed from 25 d upto 90 d in RE samples. Maximum level was 6.25×10^7 cfu g^{-1} root tissue in SHH RB RE fraction (Fig. 4.5b).

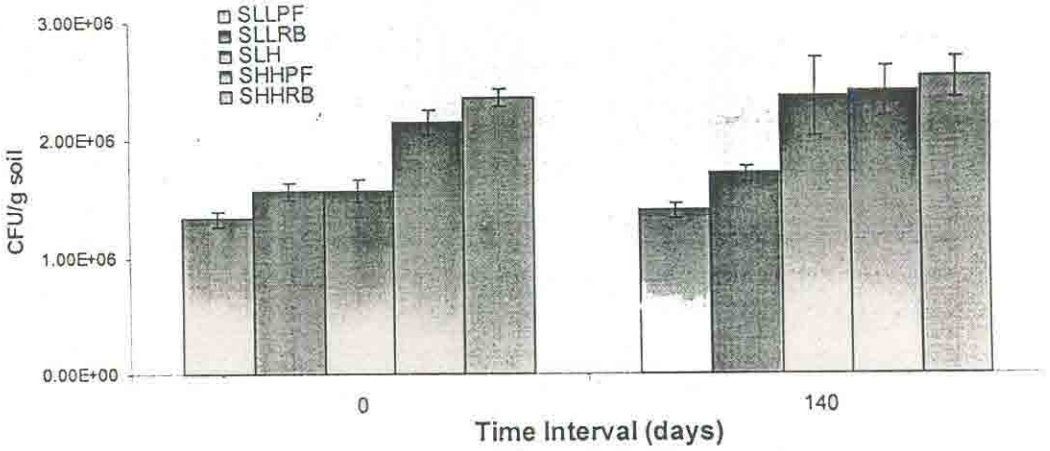


Fig 4.2 : Total bacterial population in bulk soil

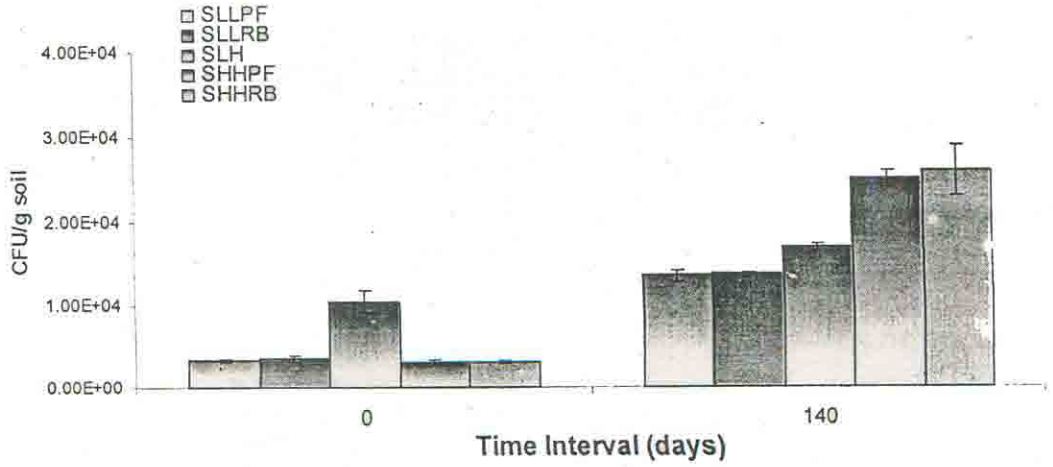


Fig 4.3 : Free living nitrogen fixing population in bulk soil

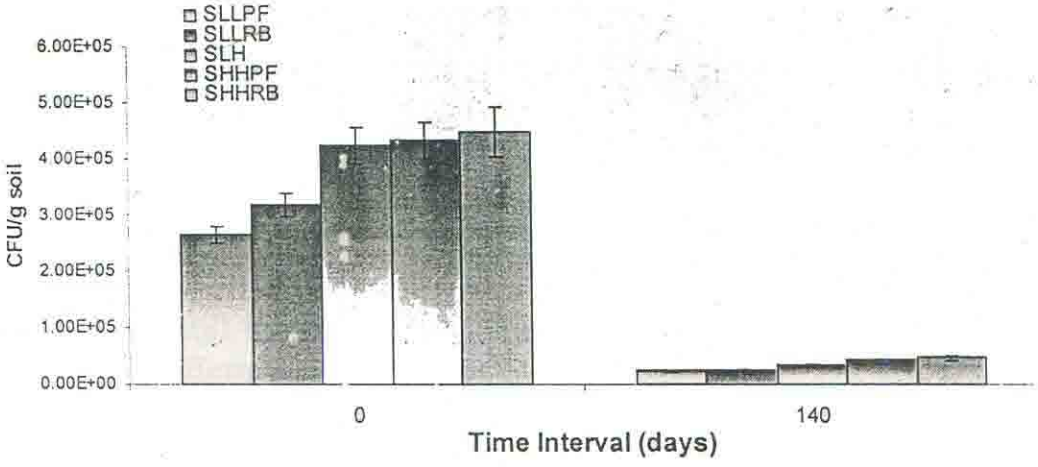


Fig 4.4 : *Rhizobium* population in bulk soil

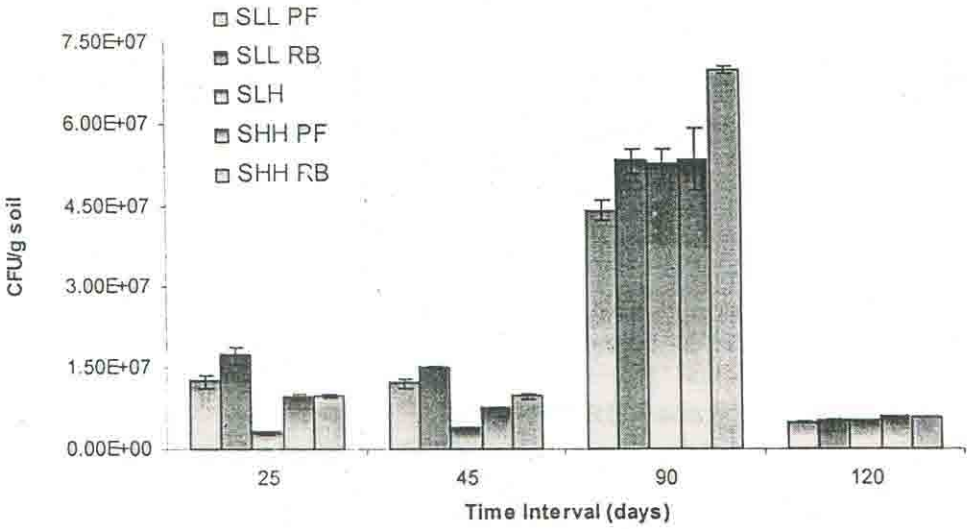


Fig. 4.5a: Total bacterial population dynamics in wheat RS at different growth stages

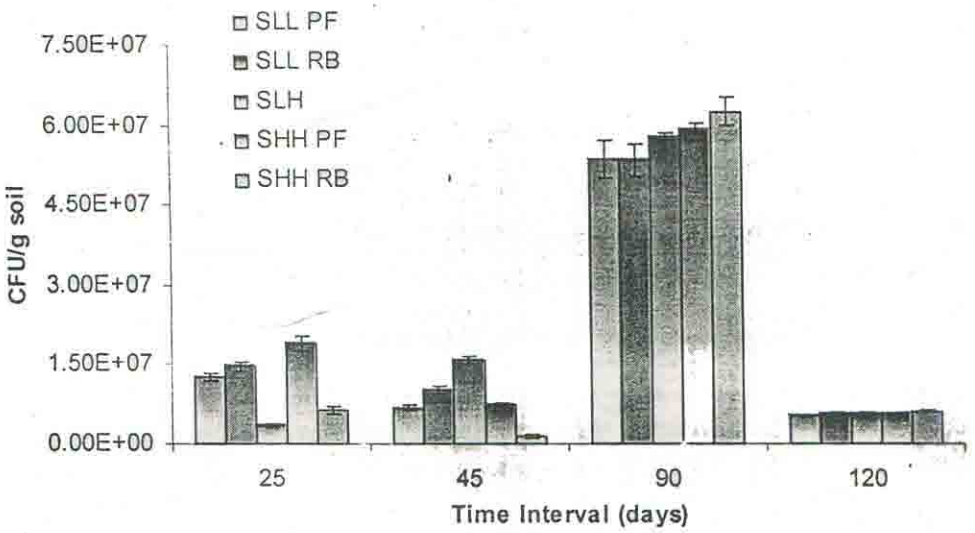


Fig. 4.5b: Total bacterial population dynamics in wheat RE at different growth stages

4.2.2.2 Free- living N_2-fixers

Population of free- living N_2-fixers gradually increased upto 90 d in all rhizosphere (RS) field samples (Fig.4.6a). Maximum population was established in SHH RB system (5.6×10^5 cfu g^{-1} soil).

In SLL PF/RB RE field samples, maximum population at 45 d was of the order, 10^5 cfu g^{-1} root tissue which decreased at later growth stage in SLH and SHH PF/RB; maximum population was established at 90 d (Fig. 4.6b).

4.2.2.3 *Rhizobium*

In all fields maximum *Rhizobium* population ~10^6 cfu g^{-1} soil was established within 45 d and remained almost constant upto 90d (Fig. 4.7a), followed by a decrease.

In SLH RE fraction, maximum population was established at 45 d (4.77×10^6 cfu g^{-1} root weight). Closely similar population dynamics was followed by RE fractions as RS (Fig. 4.7b).

4.3 SOIL ENZYME ACTIVITY

Biologically active nature of soil and influence of change in cropping practice was also assessed by measuring dehydrogenase and acid and alkaline phosphomonoesterase activities. Changes in enzyme activity were measured in bulk soil i.e. 0d/140d and rhizospheric/zone between rhizosphere and bulk soil at different growth stages of wheat growth.

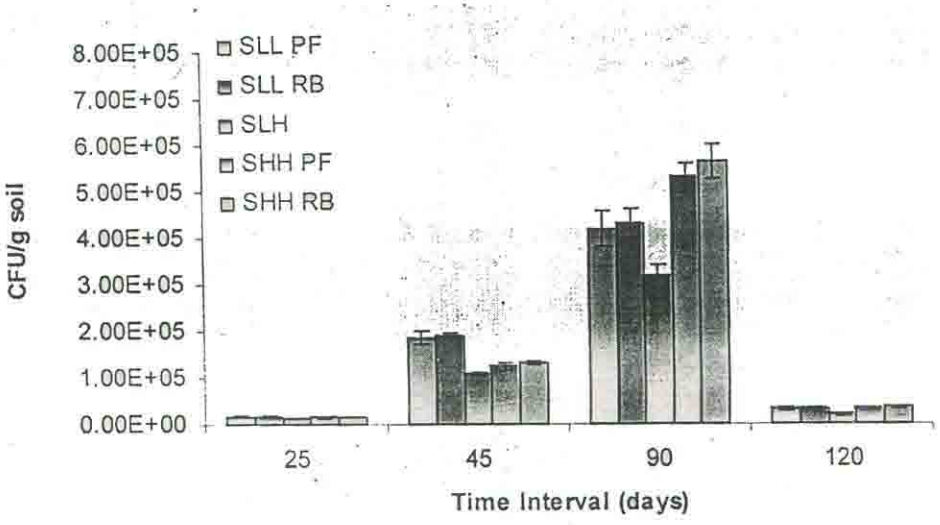


Fig. 4.6a: Dynamics of free living N2-fixers in wheat RS at different growth stages

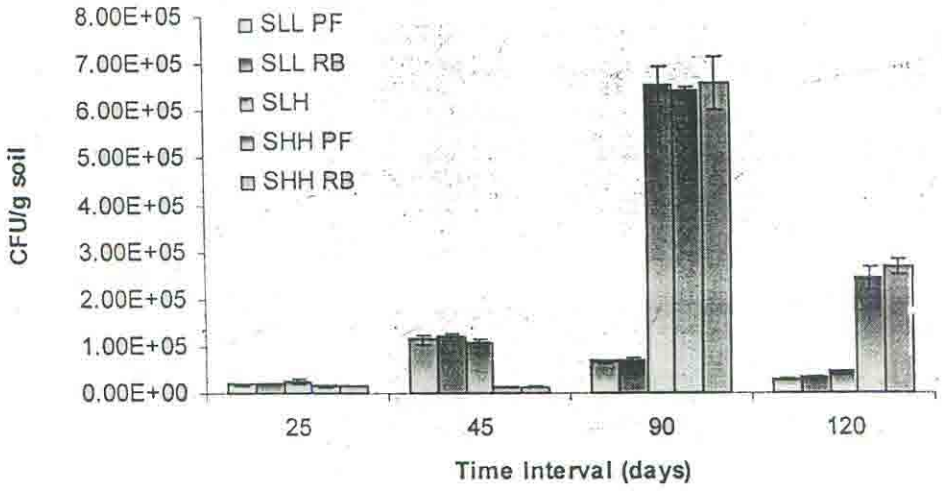


Fig. 4.6b: Dynamics of free living N2-fixers in wheat RE at different growth stages

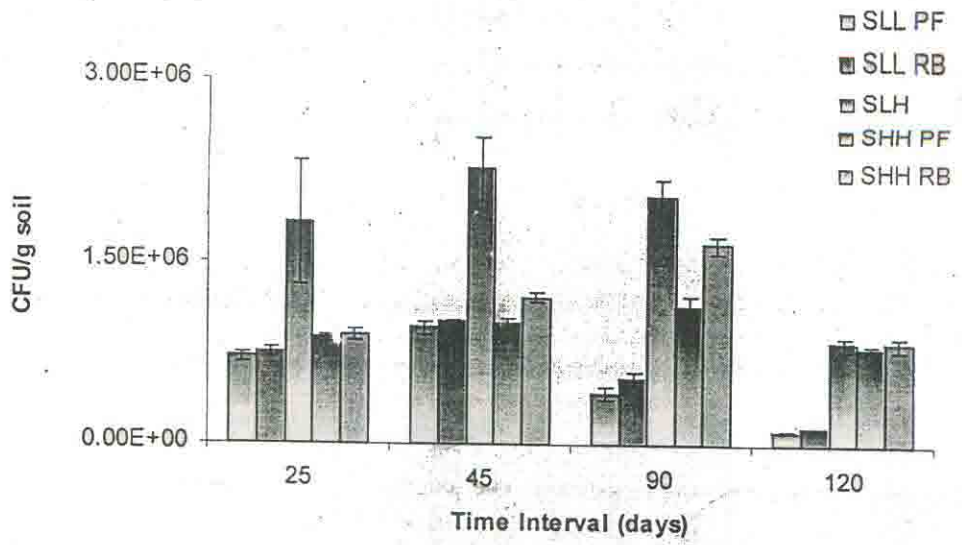


Fig. 4.7a: Dynamics of *Rhizobium* in wheat RS at different growth stages

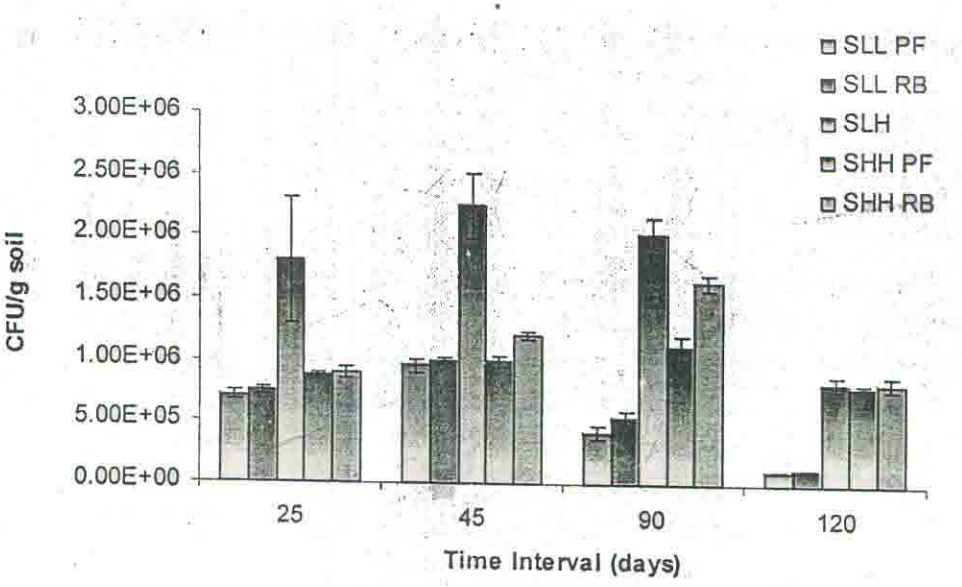


Fig. 4.7b: Dynamics of *Rhizobium* in wheat RE at different growth stages

4.3.1 Dehydrogenase Activity

Dehydrogenase activity increased with age of the plant and reached maximum at 90 d in all field samples followed by a decline. Maximum activity (171.29 µg TPF g^{-1}. dm. $16h^{-1}$) was found in 90 d samples of SHH RB (Fig. 4.8).

4.3.2 Acid Phosphomonoesterase Activity

Acid phosphomonoesterase activity was maximum in SHH PF/RB fields (463/473 µg pNP g^{-1}.dm.h^{-1}) at 45 d which were significantly higher than other fields (Fig.4.9). The activity increased with plant age and attained maximum level at 45d in SLH and SHH PF/RB; it attained a maxima in SLL PF/RB after 90d.

4.3.3 Alkaline Phosphomonoesterase Activity

A similar trend like dehydrogenase was observed in alkaline phosphomonoesterase activity (Fig. 4.10). The activity was significantly higher in SHH PF/RB field samples (498.09/505.15 pNP g^{-1} dm.h^{-1}). In all field samples, highest activity was observed at 90 d (Fig.4.9).

4.4 *In situ nif* H Community Dynamics

Extracted soil DNA was amplified (411bp) with degenerate primers so that most of the *nif*H population could be recovered. Amplified product was double digested with two restriction enzymes: *Alu* I and *Rsa* I. The resultant *nif*H profiles of 0, 25, 45, 120 and 140 d were analyzed.

Time/ Input dependent change

Unrestricted 411 bp *nif*H amplicon appeared at 25 d in SLL PF/RB and was observed upto 90 d; in SLH field it was present at 45d

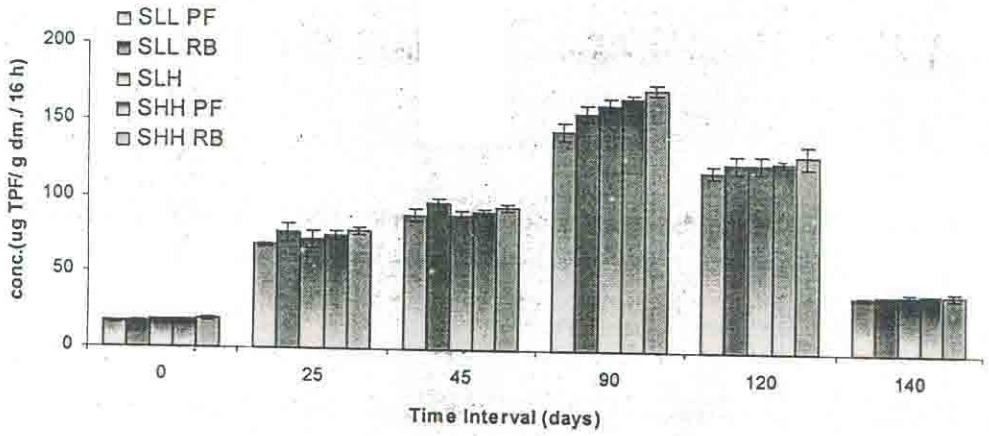


Fig. 4.8: Dynamics of dehydrogenase activity during different growth stages of wheat

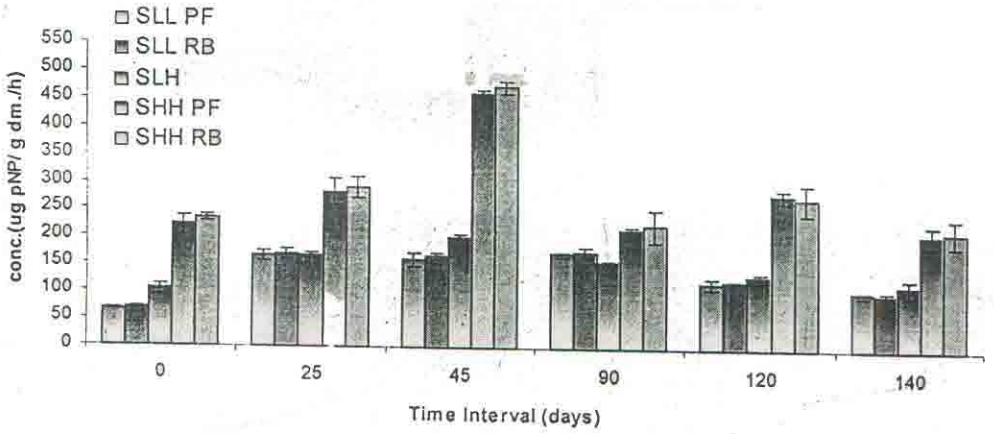


Fig. 4.9: Dynamics of acid phosphatase activity during different growth stages of wheat

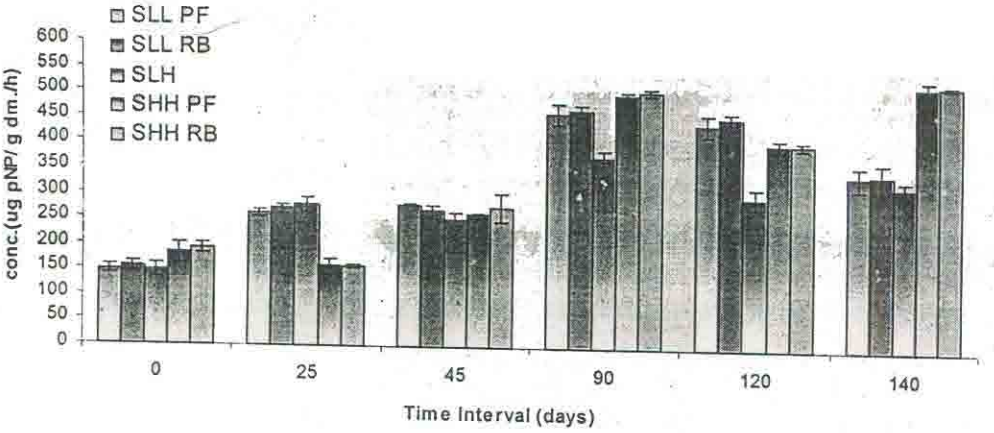


Fig. 4.10: Dynamics of alkaline phosphatase activity during different growth stages of wheat

only. At 0 day, a 280 bp fragment was present in SLH and SHHPF/RB but at 25 d this band was not observed SHHPF/RB fields (Fig. 4.11, 4.12 & 4.13).

This band persisted in SLH samples upto 140d; in addition, two new fragments of 151 and 130 bp were also detected at 45 d which could be seen upto 90 d. furthermore, a 235 bp fragment was also detected in SLH upto 25 d. At 120 day a band of 225 bp was observed in SLH, SHHPF/RB which was present until 140 d; a new fragment of 59 bp was observed only at 120d.

Plain field Vs raised bed system

A band of 189 bp was observed in plain beds of SLL and SHH field at 25 d which continued 140 d. Whereas as a 161 bp fragment was specific to raised fields of SLL and SHH. It appeared at 25 d and persisted upto 140d.

Bands of fragment size 253 bp, 120 bp and 49 bp were present from 0 to 140 d irrespective of the input system practice (Table 4.2). These band can be used as a marker in the study of *nif*H gene pool.

4.5 FUNCTIONAL CHARACTERISTICS OF ISOLATES

Growth characteristics

Bacterial isolates recovered from various fields on Jensen's agar were picked up on the basis of distinguishable or morphotypes and analyzed for various characteristics including growth in N-free media. All isolates were able to grow in different nitrogen-free media supplied with various carbon sources as also in media amended with salts of ammonium, under normal O_2 and low O_2 atm.

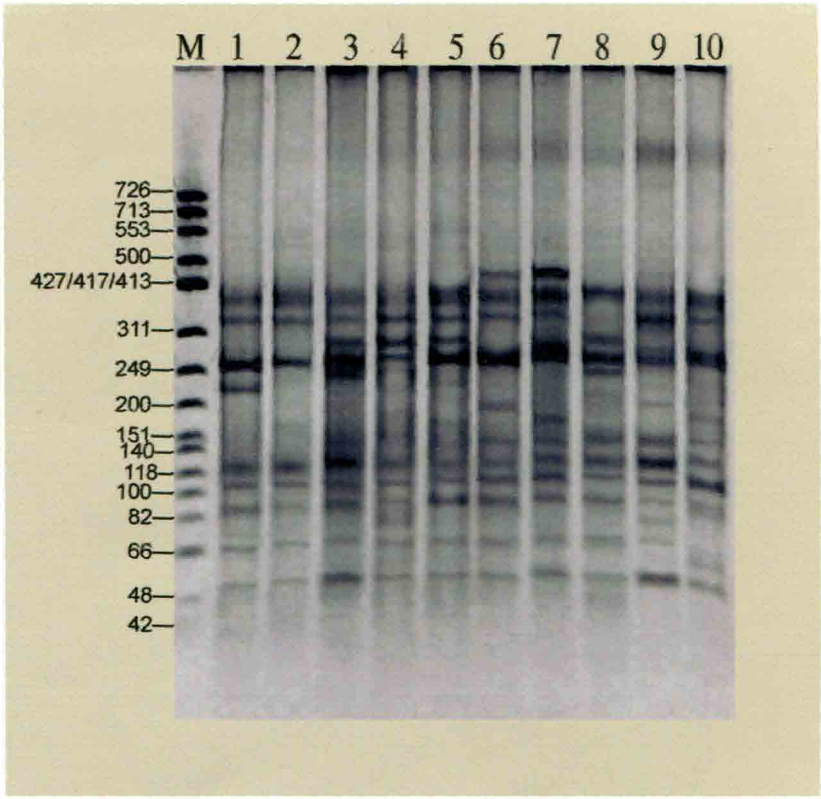


Fig.:4.11 : *nif*H profile of wheat rhizosperic soil at different stages of growth L : Lane 1-5, 0 d samples, Lane 1: SLLPF; Lane 2 : SLLRB; Lane 3 : SLH; Lane 4 : SHHPF; Lane 5 : SHHRB; Lanes 6 -10, 25 d sample, Lane 6 : SLLPF; Lane 7 : SLLRB; Lane 8 : SLH; Lane 9 : SHHPF; Lane 10 : SHHRB

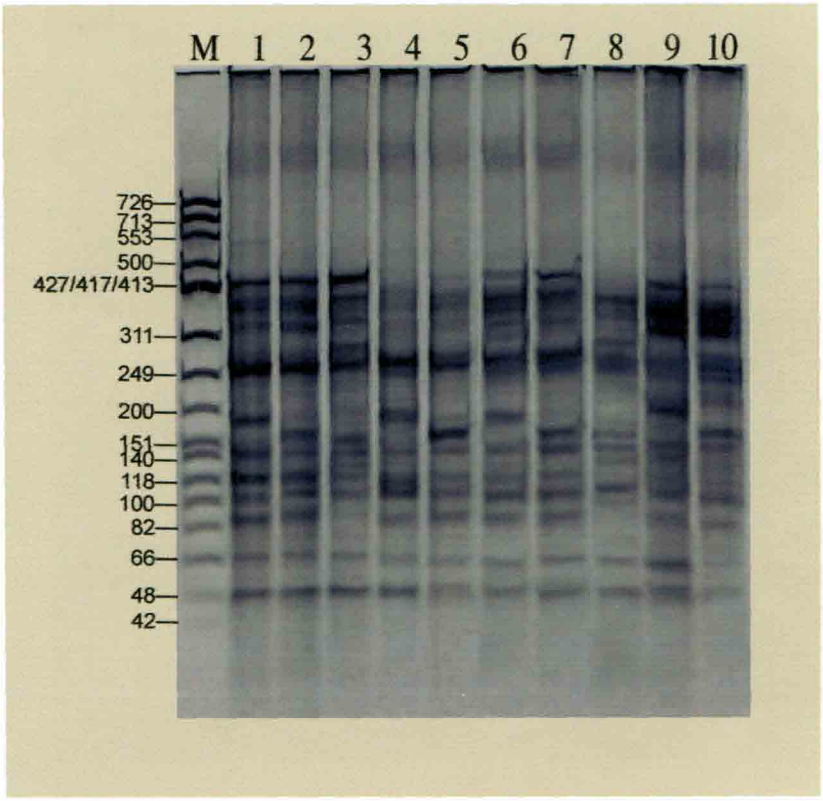


Fig.:4.12 : *nif*H profile of wheat rhizosperic soil at different stages of growth L : Lane 1-5, 45 d samples, Lane 1: SLLPF; Lane 2 : SLLRB; Lane 3 : SLH; Lane 4 : SHHPF; Lane 5 : SHHRB; Lanes 6 -10, 90 d sample, Lane 6 : SLLPF; Lane 7 : SLLRB; Lane 8 : SLH; Lane 9 : SHHPF; Lane 10 : SHHRB

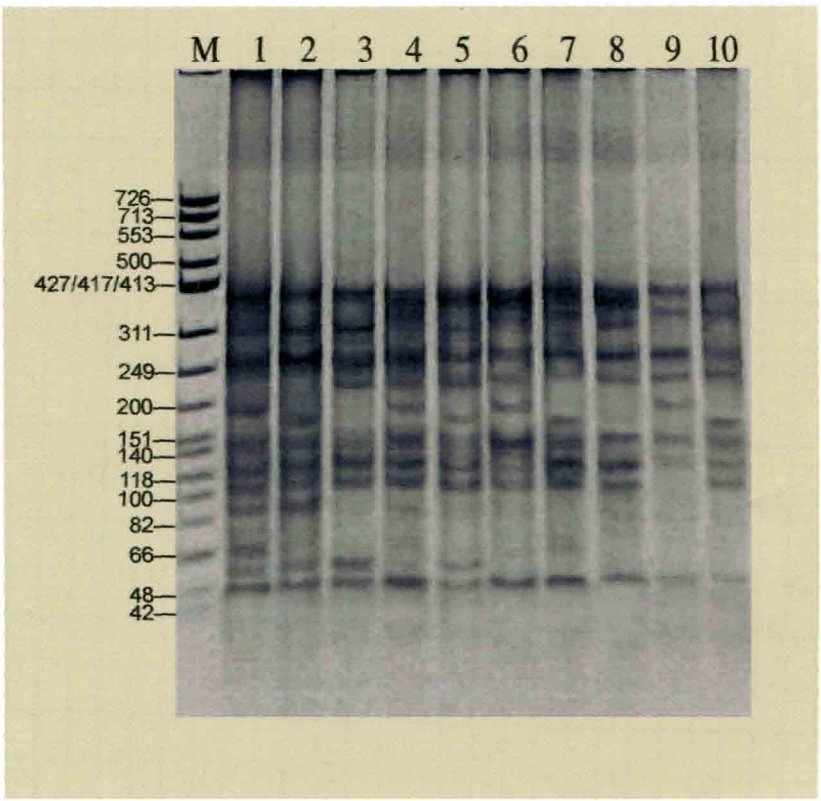


Fig.:4.13 : *nif*H profile of wheat rhizosperic soil at different stages of growth L : Lane 1-5, 120 d samples, Lane 1: SLLPF; Lane 2 : SLLRB; Lane 3 : SLH; Lane 4 : SHHPF; Lane 5 : SHHRB; Lanes 6 -10, 140 d sample, Lane 6 : SLLPF; Lane 7 : SLLRB; Lane 8 : SLH; Lane 9 : SHHPF; Lane 10 : SHHRB

Table 4.2: Restriction profiles of *nif* H community in wheat rhizospheric soil at different growth stages

Days	Fields	Restriction profiles (band size in bp) *Alu* I/ *Rsa*I															
		4[illegible]	280	381	314	253	189	161	151	144	130	120	104	82	67	59	49
0	SLLPF	-	-	+	+	+	-	-	-	-	-	+/-	+/-	+	+	-	+
	SLLRB	-	-	+	+	+	-	-	-	-	-	+/-	+/-	+	+	-	+
	SLH	-	+	+	+	+	-	-	-	+	-	+	+/-	+	+	-	+
	SHHPF	-	+	+	+	+	-	-	-	-	-	+/-	+/-	+	+	-	+
	SHHRB	-	++	-	+	-	-	-	-	-	-	+/-	+/-	+	+	-	+
25	SLLPF	+/-	-	+	+	+	+	-	-	+	-	+	+	+	+	-	+
	SLLRB	+	-	+	+	+	-	+	-	+	-	+	+	+	+	-	+
	SLH	-	+	+	-	+	-	-	-	+	-	+	+	+	+	-	+
	SHHPF	-	-	+	+	+	+	-	-	+	-	+	+	+/-	+	-	+
	SHHRB	-	-	+	+	+	-	+	-	+	-	+	+	+	+	-	+
45	SLLPF	+	-	+	+	+	+	-	-	+	-	+	+	+	+/-	-	+
	SLLRB	+	-	+	+	+	-	+	-	+	-	+	+	+	+/-	-	+
	SLH	+	+	+	+/-	+	-	-	+	+	+	+/-	+/-	+/-	+/-	-	+
	SHHPF	-	-	+/-	-	+	+	-	+	+	+	+/-	+/-	+/-	+/-	-	+
	SHHRB	-	-	+/-	-	+	-	+	-	+	-	+/-	+/-	+/-	+	-	+
90	SLLPF	+	-	+	+	+	+	-	-	+	-	+/-	+	+	+	-	+
	SLLRB	+	-	+	+	+	-	+	-	+	-	+/-	+	+	+	-	+
	SLH	-	+	+	+/-	+	-	-	+	+	+	+	+	-	+	-	+
	SHHPF	+	-	+	+	+	+	-	-	+	-	+	+	+	+	-	+
	SHHRB	+	-	+	+	+	-	+	-	+	-	+/-	+	+/-	+	*	+
120	SLLPF	-	-	+	+	+	+	-	-+	-	+	+	+	+	+	+	+
	SLLRB	-	-	+	+	+	-	+	-	+	-	+	+	+	-	+	+
	SLH	-	-	+	+	+	-	-	-	+	-	+	+	-	-	+	+
	SHHPF	-	-	+	-	+	+	-	-	+	-	+	+	+/-	+	+	+
	SHHRB	-	-	+	+/-	+	-	+	-	+	-	+	+	-	-	+	+
140	SLLPF	-	-	+	-	+	+	-	-	+	-	+	+	+	-	-	+
	SLLRB	-	-	+	-	+	-	+	-	+	-	+	+	+	-	-	+
	SLH	-	-	+	+	+	-	-	-	+	-	+	+	-	-	-	+
	SHHPF	-	-	+	-	+	+	-	-	+	-	+	+	-	-	-	+
	SHHRB	-	-	+	-	+	-	+	-	+	-	+	+	-	-	-	+

235 bp was present in SLH only in 0 d and 25 d; 225 bp was appeared at 120 d in SLH, SHH PF and SHHRB and 140 d it was present it all fields

- : absent +: present +/-: weak

4.5.1 Growth in N-free Media

All isolates were grown in DF minimal medium supplemented with/without $(NH_4)_2SO_4$; colony growth was variable for the isolates. In ammonium amended medium, the colonies appeared in 3 d whereas in without ammonium, they required in 5 d for appearance.

In LGI medium all isolates were able to grow in 2d.

4.5.2 Growth under Aerobic/Microaerophilic Conditions

ABpy medium in which pyruvate served as carbon source, was used to perform this experiment. In ABpy medium supplemented with/without NH_4NO_3 and normal O_2 atm, the growth rates were different for bacteria. In ammonium supplemented medium growth appeared after 3 d whereas in ammonium-free medium it appeared after 6 d of incubation. Growth in low O_2 atm (2%) in ammonium supplemented ABpy medium was similar to that in normal O_2 atm. However, in NH_4NO_3-free medium, bacteria were able to grow in 4 d, reflecting that nitrogenase enzyme was much more active in low O_2, supporting growth in absence of NH_4NO_3.

4.5.3 Siderophore Production

A slight orange halozone appeared around spotted cultures of SLL45, SLL22 and SHH79. Rest of the isolates were negative for siderophore test.

4.5.4 Phosphatase Activity

All 44 isolates except SLL3, SLL45, SLLRE23 and SLLRE62 were positive for phosphatase activity.

4.5.5 Antagonistic Property

No isolate showed any antagonism against *Pythium sp.* Isolates SLL22, SLL71 SHH36 and SLL68 and standard *Azotobacter beijerenckii* showed antagonisms towards *Fusarium oxysporum* and *Helminthosporium sativum* whereas *Paenibacillus polymyxa* Neu 25 was found to be antagonistic against *Helminthosporium sativum* (Table 4.3; Fig. 4.14).

Table 4.3: Antagonsitic potential of bacterial isolates recovered from wheat fields

Sl. No.	Origin/ Name of bacteria	Zone of inhibition (mm)	
		Helminthosporium sativum	***Fusarium oxysporum***
1.	SLL71	30	15
2.	SLL 22	24	24
3.	SHH 36	28	20
4.	SLL 68	24	18
5.	*P. polymyxa* Neu25	22	-
6.	*A. beijerenckii*	30	20

4.6 MOLECULAR CHARACTERIZATION OF ISOLATES

Genomic DNA of all the 44 isolates and 16 standards was extracted and amplified for 16S rDNA followed by ARDRA. The 16S rDNA product was 1.43 kb.

ARDRA

Amplified 16S rDNA of all the 44 isolates restricted with 2-tetracutters i.e. *Taq*I and *Hae*III resulted in 2-4 bands of 200-900 bp (Fig. 4.15a & b). Restrictions profiles were analyzed by UPGMA cluster analysis using NTSYSpc 2.02i software (Fig.4.16). All the bacterial

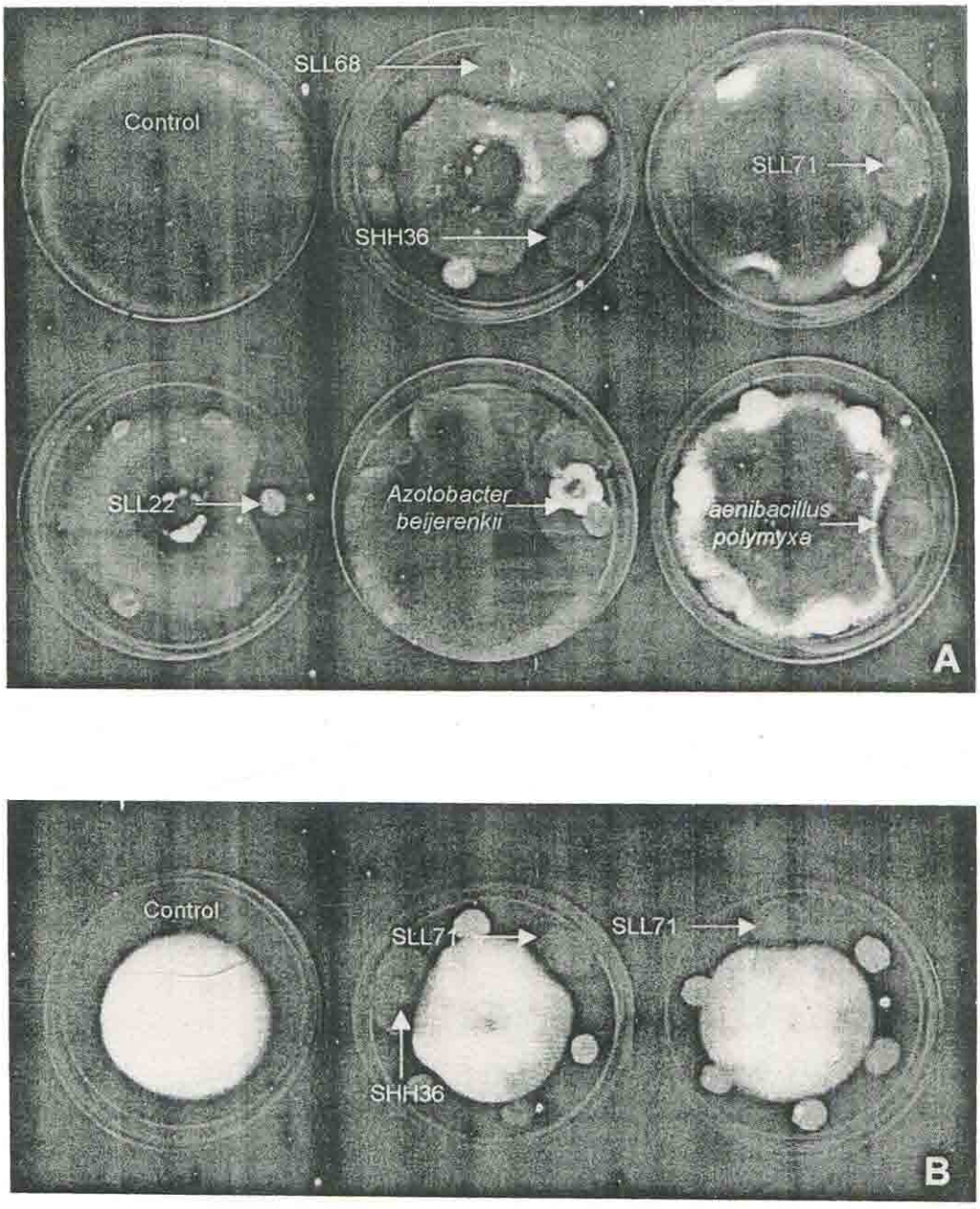


Fig. 4.14 : Screening of bacterial isolates for antifungal activity against
(A) *Helminthosporium sativum*
(B) *Fusarium oxysporum*

Fig. 4.15 a&b: Restriction profiles of bacterial isolates

From Lane 1 – 44

Lane 1	SLL32
Lane 2	SHH33
Lane 3	SLL3
Lane 4	SLL5
Lane 5	SLL34
Lane 6	SLL42
Lane 7	SLLRE23
Lane 8	SLLRE39
Lane 9	SLLRE43
Lane 10	SLLRE48
Lane 11	SLLRE58
Lane 12	SLH2
Lane 13	SLH8
Lane 14	SLH10
Lane 15	SLH39
Lane 16	SLH46
Lane 17	SLH51
Lane 18	SLH59
Lane 19	SLL27
Lane 20	SLL71
Lane 21	SLL45
Lane 22	SLL55
Lane 23	SLL4
Lane 24	SLL22
Lane 25	SLL68
Lane 26	SHH36
Lane 27	SLH19
Lane 29	SLH34
Lane 30	SLH25
Lane 31	SLLRE30
Lane 32	SHH29
Lane 33	SLRE62
Lane 34	SLL29
Lane 35	SHH15
Lane 36	SHH16
Lane 37	SHH17
Lane 38	SHH53
Lane 39	SHH68
Lane 40	SHH72
Lane 41	SHH79
Lane 42	SHH80
Lane 43	SHH2
Lane 44	SLH52

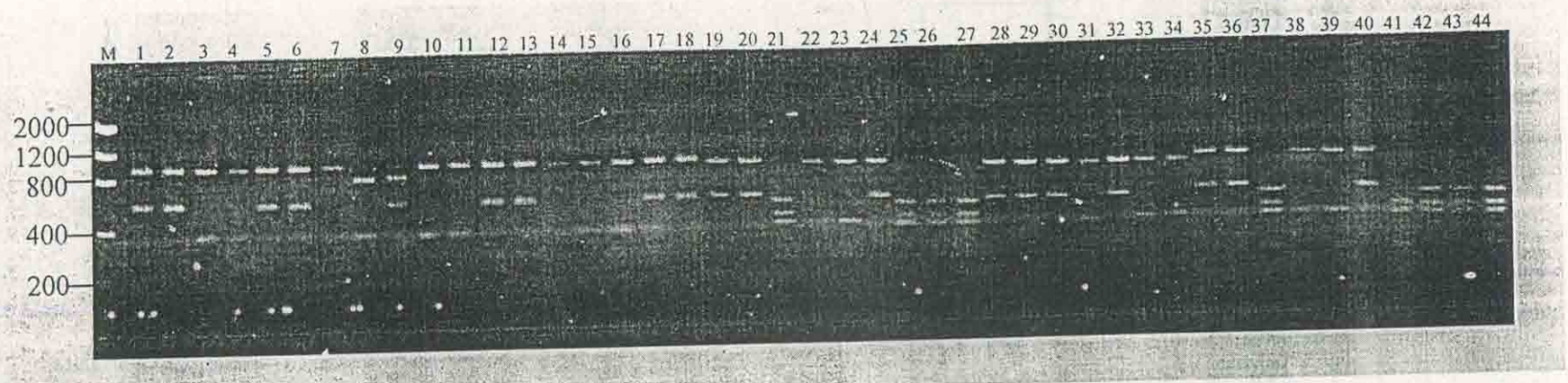


Fig 4.15a : ARDRA of bacterial isolates with restriction endonuclease *Taq*I

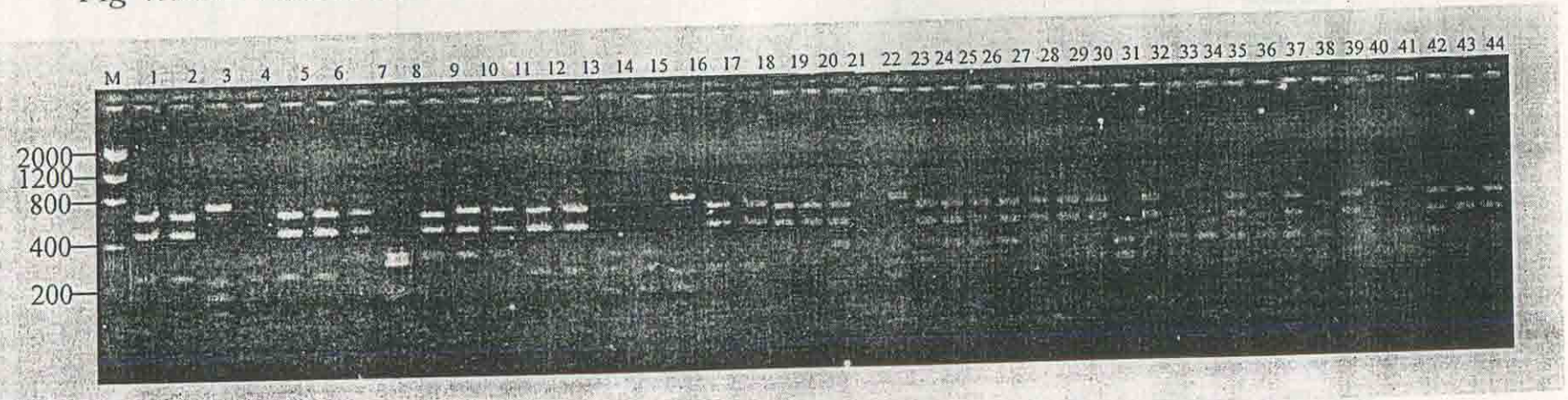


Fig 4.15b : ARDRA of bacterial isolates with restriction endonuclease *Hae*III

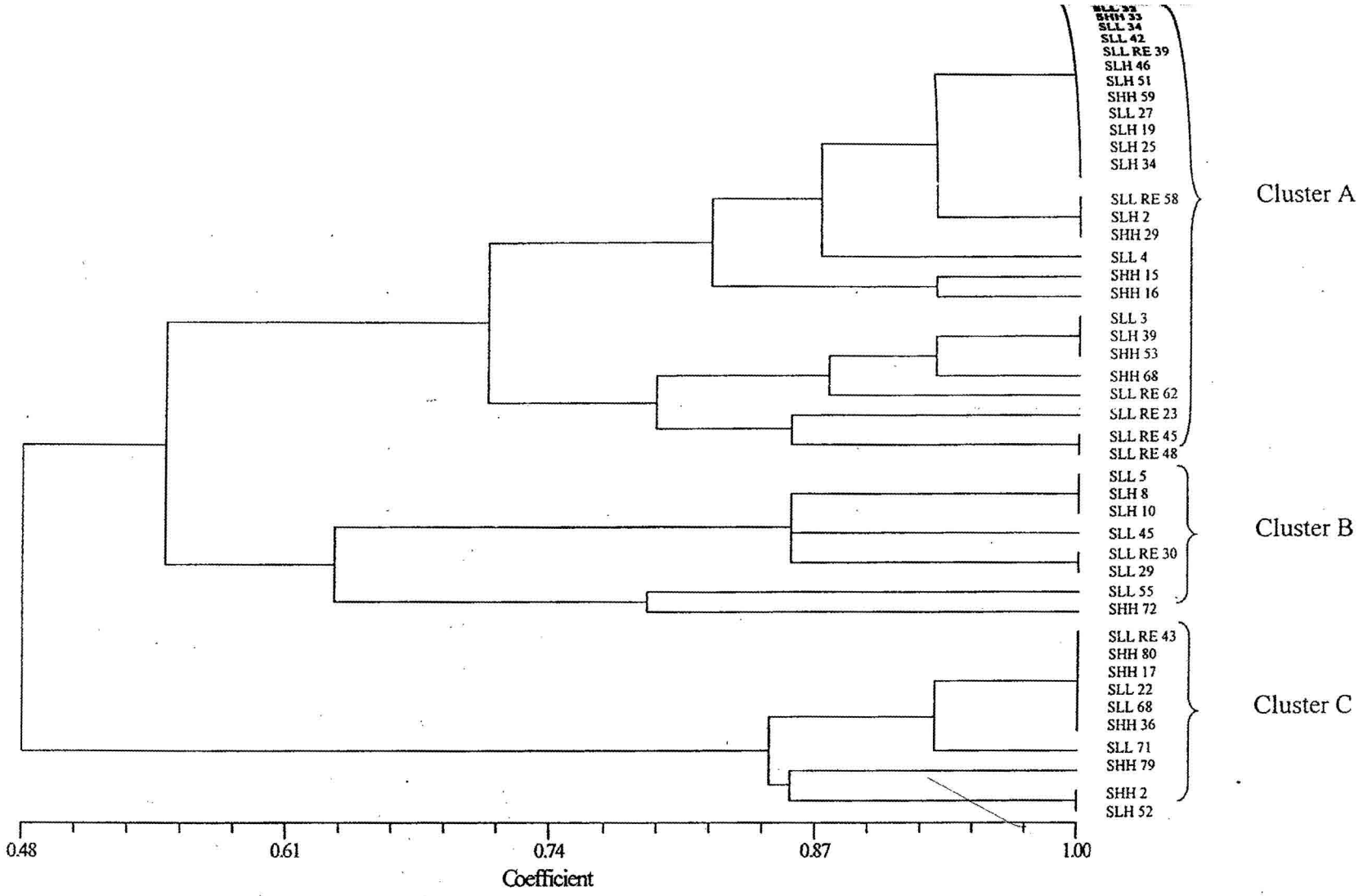


Fig 4.16 : UPGMA Cluster analysis of ARDRA profile of bacterial isolates using Jaccard's similarity coefficient

grouped into three major clusters A, B and C. Cluster A and B exhibited 55% similarity level whereas cluster C showed lowest similarity (48%) with both of these clusters. Fourteen promising isolates were distributed in different clusters. SHH59, SLL27, SLH19, SLH25, SLH34, SHH29 and SLL4 were placed in cluster A. Cluster B included SLL45, SLLRE30 and SLL55. SLL22, SLL68, SHH36 and SLL71 were grouped in cluster C.

4.7 SOIL MICROCOSM EXPERIMENT

Soil microcosm experiment was performed to assess the growth promotory potential of isolates.

Plant Growth

Plant growth was recorded after 30 d of seed bacterization in terms of shoot/ root length and shoot/ root fresh and dry weight.

4.7.1 Shoot Length

Shoot length was significantly increased by all bacterial treatments. Maximum increase was observed in *Xanthobacter autotrophicus* Neu 2137 treatment (31.83 cm) which was statistically comparable to *Rhizobium leguminosarum* DSM 30132, SLL45, SLL71, SLH51 and SHH79 (Table 4.4).

4.7.2 Shoot Fresh Weight

Maximum shoot fresh weight (1.58 g) was recorded in SLH 19 treated plants as compared to uninoculated control (0.78 g) (Table 4.4) shoot weight was positively influenced by all bacterial treatments except SLL3, SLLRE45, SLH2 and SHH72; SHH33 and SHH80 treated plants were comparable to non-bacterized control (Table 4.4).

4.7.3 Shoot Dry Weight

Compared to control, shoot dry weight was increased in all treatments except SLL3, SLLRE45, SLH2 and SHH72 (Table 4.4). Maximum shoot dry weight (0.215 g) was observed in SLH19.

Root Growth

At 30 d, the influence of different bacterial isolates was also observed for root length, and fresh and dry weight.

4.7.4 Root Length

Maximum root length (5.24 cm) was observed in SLH39 which was statistically equal to *K. terrigena* Neu 1103, SLL27, SLL45, SLH25, SLH34, SLH52, SHH29, SHH36, SHH68 (Table 4.4). In treatments SLL3, SLL34, SLLRE23, SLLRE43, SLLRE45, SLLRE58, SLLRE62, SLH2 a negative influence on root length was observed compared to control. Minimum root length (3.57 cm) was recorded in SLLRE45.

4.7.5 Root Fresh Weight

At 30 d maximum root fresh weight (0.168 g) was attained in SLLRE48 and SHH59 that was significant and at par with SLL45, SHH15, SHH33 and SHH68. Treatment SLLRE45 was found to be marginally inhibitory (0.10g) compared to control (0.106g) (Table 4.4).

4.7.6 Root Dry Weight

Maximum dry weight (0.046 g) was observed in SLH19 and SHH59 treatments (Table 4.4). Dry root weight of plants treated with *Pseudomonas fluorescens* Neu 1043, SLL3, SLL4, SLL5, SLL22,

Table 4.4: Plant growth parameters after 30 d of inoculation

Isolate/Catalogue No.	Fresh shoot weight (g)	Dry shoot weight (g)	Shoot length (cm)	Fresh root weight (g)	Dry root weight (g)	Root length (cm)
Control	0.78	0.104	21.79	0.106	0.037	4.23
Alcaligenes faecalis Neu 1033	1.18	0.163	28.19	0.112	0.040	4.29
Aquaspirillum dispar Neu 1046	1.12	0.157	29.40	0.113	0.042	4.22
Azospirillum brasilense Sp7	1.30	0.156	31.63	0.127	0.042	5.23
Azotobacter beijerenckii	1.18	0.161	25.50	0.126	0.042	4.37
Azotobacter chrococcum	1.31	0.180	29.40	0.144	0.042	4.30
Azotobacter vinelandii	1.31	0.176	27.50	0.146	0.044	4.35
Citrobacter freundii Neu 1020	1.17	0.160	27.00	0.121	0.041	1.10
Enterobacter aerogens Neu 1036	0.77	0.105	27.75	0.126	0.043	4.28
Enterobacter cloacae Neu 1027	1.08	0.150	27.75	0.110	0.041	3.90
Escherichia coli Neu 1006	0.97	0.128	28.31	0.116	0.040	3.73
Klebsiella oxytoca Neu 30	1.18	0.161	27.06	0.135	0.043	4.72
Klebsiella terrigena Neu 1103	1.03	0.140	29.03	0.144	0.040	4.93
Paenibacillus polymyxa Neu 25	1.03	0.138	29.60	0.101	0.041	4.33
Pseudomonas fluorescens Neu 1043	1.05	0.148	28.25	0.134	0.043	4.74
Rhizobium leguminosarum DSM 30132	1.22	0.166	31.00	0.110	0.039	4.79
Xanthobacter autotrophicus Neu 2137	1.29	0.178	31.81	0.128	0.042	4.78
SLL3	0.55	0.074	23.13	0.148	0.043	3.98
SLL4	1.31	0.178	28.38	0.159	0.045	4.59
SLL5	1.16	0.159	29.21	0.116	0.043	4.20
SLL22	1.27	0.163	28.69	0.134	0.044	4.32
SLL27	1.31	0.178	24.75	0.141	0.039	5.20
SLL29	1.32	0.181	22.76	0.131	0.041	4.73
SLL32	1.31	0.181	27.25	0.160	0.044	4.29
SLL34	1.07	0.143	27.50	0.148	0.038	3.93
SLL42	1.31	0.176	28.88	0.154	0.043	4.42
SLL45	1.43	0.187	30.81	0.165	0.044	5.08
SLL55	1.40	0.185	30.13	0.141	0.042	4.68
SLL68	1.38	0.185	28.25	0.158	0.043	4.89
SLL71	1.38	0.183	31.31	0.148	0.042	4.67
SLLRE23	1.14	0.157	26.38	0.121	0.041	3.88
SLLRE30	1.35	0.181	27.06	0.125	0.041	4.30
SLLRE39	0.99	0.131	25.88	0.125	0.040	4.70
SLLRE43	0.88	0.116	28.25	0.109	0.034	3.63
SLLRE45	0.18	0.024	23.44	0.100	0.034	3.57
SLLRE48	1.30	0.176	26.90	0.168	0.043	4.78
SLLRE58	1.30	0.177	24.88	0.149	0.041	4.03
SLLRE62	0.94	0.128	24.75	0.106	0.036	3.83
SLLH2	0.65	0.085	27.13	0.117	0.035	4.00
SLH8	1.16	0.154	28.36	0.131	0.042	4.35
SLH10	1.31	0.179	26.75	0.134	0.042	4.79
SLH19	1.58	0.215	28.50	0.153	0.046	4.80
SLH25	1.39	0.191	27.86	0.149	0.042	5.22
SLH34	1.42	0.187	28.53	0.132	0.042	5.14
SLH39	1.36	0.181	27.56	0.122	0.043	5.24
SLH46	0.96	0.131	27.63	0.125	0.043	4.90
SLH51	1.30	0.173	30.50	0.116	0.041	4.67
SLH52	1.44	0.192	26.99	0.149	0.046	5.00
SHH2	1.19	0.163	27.31	0.119	0.043	4.59
SHH15	1.18	0.158	28.88	0.164	0.043	4.45
SHH16	1.06	0.140	28.56	0.125	0.044	4.74
SHH17	0.97	0.139	25.96	0.118	0.042	4.45
SHH29	1.45	0.187	28.38	0.127	0.038	5.10
SHH33	0.79	0.104	26.00	0.162	0.045	4.43
SHH36	1.35	0.178	24.88	0.156	0.046	5.00
SHH53	1.03	0.134	28.50	0.117	0.039	4.42
SHH59	1.44	0.189	27.50	0.168	0.046	4.19
SHH68	1.13	0.149	29.44	0.167	0.043	5.02
SHH72	0.65	0.090	24.11	0.136	0.042	4.23
SHH79	1.09	0.140	30.76	0.134	0.037	4.62
SHH80	0.75	0.102	24.69	0.155	0.041	4.42
Cd at 5%	0.070	0.70	1.49	0.005	0.003	0.21

SLL32, SLL42, SLL68, SLLRE48, SLH39, SLH46, SHH2, SHH15, SHH16, SHH33 and SHH68 was statistically equal to treatments having maximum dry root weight, the value ranged from 0.043 to 0.045 g except SLLRE 43 and SLLRE45 which were significantly lower than control (0.037 g).

Conclusion

In soil microcosm experiment, all 59 treatments were better than control except SLL RE45 in which an inhibitory effect on plant growth was observed. On the basis of plant growth parameters and soil enzymes analysis, 14 isolates were found to be positive. Plants from these treatments and along with three standards, *Azotobacter chroococcum*, *Citrobacter freundii* Neu 1020 and *Xanthobacter autotrophicus* Neu 2137 were analyzed for total N and P and respective bacteria were tested for nitrogenase activity. The selected isolates were SLL4, SLL22, SLL27, SLL45, SLL55, SLL68, SLL71, SLLRE30, SLH19, SLH25, SLH34, SHH29 and SHH59.

4.8 SOIL ENZYME ACTIVITY

To assess the effect of introduced bacterial population on indigenous populations, soil enzyme activity was measured in terms of dehydrogenase, acid/ alkaline phosphomonoesterase.

4.8.1 Dehydrogenase Activity

Maximum dehydrogenase activity was observed in SHH29 treatment (233.42 μg TPF g^{-1}.dm.$16h^{-1}$). Dehydrogenase activity was significantly higher in all bacterial treatments as compared to uninoculated control (Table 4.5).

4.8.2 Acid Phosphomonoesterase Activity

Maximum acid phosphomonoesterase activity was found in SLH 19 treated soil (265.26 µg pNP $g^{-1}.dm.h^{-1}$). This was comparable to the activity observed in microcosms developed with standards, *Pseudomonas fluorescens* Neu 1043 and *Azospirillum brasilense* Sp7. (Table 4.5).

4.8.3 Alkaline Phosphomonoesterase Activity

Maximum alkaline phosphomonoesterase activity was observed in *Pseudomonas fluorescens* Neu 1043 treated plants (416.52 µg pNP $g^{-1}.dm.h^{-1}$). This was statistically comparable to that observed with SLL27, SHH59, SLL4 *Klebsiella oxytoca* Neu 30, SLL55 and SHH29 (Table 4.5) and was significantly higher than control (296.79 µg pNP $g^{-1}.dm.h^{-1}$).

Positive significant correlation was observed between plant growth parameters and soil enzymes except one i.e. between shoot length and alkaline phosphomonoesterase enzyme activity (Table 4.6).

Table 4.6: The correlation coefficients (r) between soil enzymes and plant growth parameters

Plant growth parameters	Correlation dehydrogenase	Acid phosphomonoesterase	Alkaline phosphomonoesterase
Fresh Shoot wt.	0.515**	0.629**	0.553*
Dry shoot wt.	0.482**	0.599**	0.526**
Shoot length	0 414**	0.345*	Ns
Fresh root wt.	0.397**	0.369**	0.456**
Dry root wt.	0.472**	0.495*	0.374**
Root length	0545*	0.608*	0.517**

**Significant at 1%; *Significant at 5%; ns: non significant

Table 4.5: Soil enzyme activity in wheat rhizospheric soil after 30 d of inoculation

Isolate/Catalogue No.	Dehydrogenase activity (µg TPF g^{-1}. dm. $16h^{-1}$)	Acid phosphomono-esterase activity (µg pNP g^{-1}. dm. h^{-1})	Alkaline phosphomono-esterase activity (µg pNP g^{-1}. dm. h^{-1})
Control	140.80	229.20	296.79
Alcaligenes faecalis Neu 1033	191.56	241.44	310.22
Aquaspirillum dispar Neu 1046	190.76	238.27	300.71
Azospirillum brasilense Sp7	221.32	248.63	382.50
Azotobacter beijerenckii	196.18	245.29	299.95
Azotobacter chrococcum	207.10	237.09	303.33
Azotobacter vinelandii	200.34	239.51	301.95
Citrobacter freundii Neu 1020	211.34	241.66	325.32
Enterobacter aerogens Neu 1036	196.56	232.94	291.75
Enterobacter cloacae Neu 1027	207.50	236.25	320.61
Escherichia coli Neu 1006	204.39	231.14	330.36
Klebsiella oxytoca Neu 30	220.11	242.85	398.05
Klebsiella terrigena Neu 1103	213.22	235.80	375.52
Paenibacillus polymyxa Neu 25	207.20	237.11	304.66
Pseudomonas fluorescens Neu 1043	220.84	249.02	416.52
Rhizobium leguminosarum DSM 30132	209.38	239.71	306.95
Xanthobacter autotrophicus Neu 2137	202.61	234.09	304.26
SLL3	205.87	234.34	304.88
SLL4	221.37	246.47	400.77
SLL5	206.05	236.83	304.98
SLL22	225.00	253.90	387.39
SLL27	211.10	248.97	409.75
SLL29	213.73	239.11	376.23
SLL32	214.40	236.79	381.37
SLL34	209.95	224.10	312.30
SLL42	210.85	241.09	374.30
SLL45	224.70	249.80	371.54
SLL55	223.10	257.72	395.54
SLL68	223.53	258.86	385.71
SLL71	227.82	250.85	366.04
SLLRE23	191.92	230.00	296.18
SLLRE30	222.78	240.80	329.53
SLLRE39	193.91	226.51	286.81
SLLRE43	189.90	233.47	305.72
SLLRE45	193.99	230.64	286.48
SLLRE48	190.61	228.56	298.11
SLLRE58	195.28	231.28	314.73
SLLRE62	195.32	221.28	306.32
SLLH2	203.76	219.52	304.55
SLH8	219.65	241.14	311.91
SLH10	225.97	241.66	342.68
SLH19	232.77	265.26	370.46
SLH25	232.28	247.55	373.14
SLH34	229.78	257.32	371.59
SLH39	232.13	247.44	314.59
SLH46	224.40	230.78	324.82
SLH51	230.54	239.36	304.81
SLH52	219.39	241.41	351.68
SHH2	223.39	232.39	355.55
SHH15	214.91	242.36	330.43
SHH16	210.00	241.09	334.95
SHH17	197.47	236.94	298.57
SHH29	233.42	252.77	394.74
SHH33	216.14	236.09	314.69
SHH36	229.03	255.25	370.69
SHH53	227.42	241.33	315.63
SHH59	232.49	244.25	406.02
SHH68	232.55	251.27	327.93
SHH72	215.43	235.30	305.66
SHH79	205.12	237.75	323.33
SHH80	198.59	233.73	313.90
Cd at 5%	7.74	8.89	10.75

4.9 TOTAL N, P AND NITROGENASE

Total N, P in plant shoots of selected treatments were analyzed and nitrogenase activity of respective isolates was determined.

4.9.1 Total Nitrogen

Total N in shoot tissue of selected treatments was determined. Maximum N (3.11%) was assimilated in SLH19 treatments compared to untreated plants (2.49%). Nitrogen content was higher in all treated plants except in SLLRE30 (2.51%) that was comparable to uninoculated control (Table 4.7). Total N was positively correlated with shoot biomass and soil enzymes (Table 4.8).

4.9.2 Total Phosphorus

Total P increased in all treatments as compared to uninoculated plants (Table 4.7). Maximum phosphorus (0.51%) was accumulated in SLH25 as compared to uninoculated (0.37%). Positive significant correlation was observed between shoot biomass and soil enzyme (Table 4.8).

Table 4.8: Correlation coefficients between shoot biomass, total N, P, and soil enzymes

	Fresh shoot wt.	Dry shoot wt.	Dehydrogenase activity	Acid phosphomono-esterase	Alkaline Phosphomono -esterase
Total N	0.45	0.45	0.59	0.54	0.35
Total P	0.41	0.43	0.46	0.38	0.44

4.9.3 Acetylene Reduction Assay

Acetylene reduction assay for selected isolates and three standards was performed. Only one isolate showed significant nitrogenase activity (Table 4.7). Three standards i.e. *Azotobacter*

Table 4.7: Total N and P in wheat shoot tissue and nitrogenase activity of respective isolates

Isolate No.	Total N (%)	Total P (%)	Nitrogenase activity (n mole C_2H_4 mg^{-1} protein. 12 h^{-1})
Control	2.49±0.06	0.37±0.02	-
Azotobacter chroococcum	2.80±0.04	0.44±0.04	+/-
Citrobacter freundii Neu 1020	2.58±0.05	0.37±0.03	+/-
Xanthobacter autotropicus Neu 2137	2.62±0.04	0.40±0.02	+/-
SLL 4	2.86±0.06	0.48±0.02	+/-
SLL 22	2.94±0.012	0.47±0.03	+/-
SLL 27	2.70±0.07	0.45±0.03	+/-
SLL 45	2.62±0.14	0.38±0.04	+/-
SLL 55	2.75±0.17	0.43±0.04	4.43 ± 0.52
SLL 68	2.70±0.07	0.40±0.03	+/-
SLL 71	2.87±0.07	0.42±0.04	+/-
SLLRE 30	2.51±0.05	0.40±0.03	+/-
SLH 19	3.11±0.09	0.50±0.03	+/-
SLH 25	3.07±0.09	0.51±0.03	+/-
SLH 34	2.95±0.10	0.48±0.02	+/-
SHH 29	2.77±0.09	0.42±0.02	+/-
SHH 36	3.01±0.10	0.45±0.02	+/-
SHH 59	2.85±0.10	0.47±0.02	+/-

chroococcum, Citrobacter freundii Neu 1020 and *Xanthobacter autotrophicus* Neu 2137 taken as positive control did not show any significant activity under the experimental conditions employed (Table 4.7).

4.10 *nif* H AMPLIFICATION OF ISOLATES

Only SLL55 isolate could be amplified with *nif*H degenerate primers. An amplicon of 411 bp was obtained.

4.11 IDENTIFICATION OF PROMISING ISOLATES

All 14 promising isolates were identified through fatty acid methyl esters analysis on a MIS system (Appendix I). Their entry names with similarity index (SI) are shown in Table 4.9. The FAMES profile of isolate showed low SI of 0.094 with *Micrococcus-lylae* GC subgroup B, no match was found with other libraries. Isolates SLL22 showed a SI of 0.522 with *Bacillus-amyloliquefaciens* and 0.405 with *Bacillus subtilis.* A SI of 0.146 was found for isolate SLL27 with *Brevibacillus laterosporus*: a SI value of 0.162 and match with *Bacillus megaterium* was shown. The FAMES profile of SLL45 showed a low SI value of 0.012 only with one organism i.e. *Kocuria varians.* The FAMES profile of nitrogenase positive isolates SLL55 showed SI of 0.273 with *Pseudomonas chlororaphis* and 0.245 with *Pseudomonas putida-* biotype B. Isolate SLL68 was weakly similar (SI 0.49) with *Micrococcus lyae* GC subgroup B. Isolate SLL71 showed a SI of 0.347 with *Bacillus subtilis.* Isolate SLLRE30 recovered from wheat rhizoplane/ endorhizosphere showed low similarity (SI 0.078) with

Table 4.9: Identified entry names of bacteria with Sim index

Isolate name	Origin	Matches		
		Library	Sim index	Entry Name
SLL4	Wheat Rh	TSBA40 4.10	0.094	*Micrococcus lylae*-GC subgroup B
			0.092	*Brevibacillus laterosporous* (*Bacillus*)
			0.084	*Bacilus megaterium* GC subgroup A
		ACTIN1 3.80		No match
		CLIN40 4.10		No match
		YST28 3.80		No match
SLL22	Wheat Rh	TSBA40 4.10	0.522	*Bacillus amyloliquefaciens* (*Bacillus subtilis* group)
			0.419	*Bacillus subtilis*
			0.383	*Bacillus marinus*
			0.284	*Paenibacillus macerans*- GC subgroup A (*Bacillus*)
			0.263	*Paenibacillus gordonae* (*Bacillus gordonae*)
		ACTIN1 3.80		No match
		CLIN40 4.10		*Bacillus subtilis*
		YST28 3.80		No match
SLL27	Wheat Rh	TSBA40 4.10	0.146	*Brevibacillus laterosporus* (*Bacillus*)
			0.144	*Bacillus –megaterium* GC subgroup A
			0.098	*Micrococcus-lylae*-GC subgroup B
		ACTIN1 3.80		No match
		CLIN40 4.10	0.162	*Bacillus megaterium*
		YST28 3.80		No match
SLL45	Wheat Rh	TSBA40 4.10	0.012	*Kocuria-varians* (*Micrococcus*)
		ACTIN1 3.80		No match
		CLIN40 4.10		No match
		YST28 3.80		No match
SLL55	Wheat Rh	TSBA40 4.10	0.273	*Pseudomonas chlororaphis* (*Pseudomonas aureofaciens*)
			0.222	*Pseudomonas syringae-syringae*
			0.183	*Pseudomonas putida* biotype A
		ACTIN1 3.80		No match
		CLIN40 4.10	0.245	*Pseudomonas putida* biotype B
			0.136	*Pseudomonas putida* biotype A
		YST28 3.80		No match
SLL68	Wheat Rh	TSBA40 4.10	0.049	*Micrococcus lylae* GC subgroup B
		ACTIN1 3.80		No match
		CLIN40 4.10		No match
		YST28 3.80		No match
SLL71	Wheat Rh	TSBA40 4.10	0.347	*Bacillus subtilis*
			0.261	*Paenibacillus macerans* GC subgroup A (*Bacillus*)
			0.257	*Bacillus licheniformis* (*Bacillus subtilis* subgroup)
			0.197	*Bacillus amyloliquefaciens* (*Bacillus subtilis* group
			0.187	*Bacillus lentimorbus*
		ACTIN1 3.80		No match
		CLIN40 4.10	0.319	*Bacillus subtilis*
		YST28 3.80		No match

SLLRE 30	Wheat RE	TSBA40 4.10	0.078	*Bacillus coagulans*
			0.069	*Microbacterium-lique faciens (Aureobacterium liquefaciens)*
			0.042	*Brevibacterium casei*
		ACTIN1 3.80		No match
		CLIN40 4.10		No match
		YST28 3.80		No match
SLH19	Wheat Rh	TSBA40 4.10	0.162	*Bacillus megaterium* GC subgroup A
			0.154	*Brevibacillus laterosporus (Bacillus)*
			0.082	*Micrococcus lylae* GC subgroup B
		ACTIN1 3.80		No match
		CLIN40 4.10	0.156	*Bacillus megaterium*
		YST28 3.80		No match
SLH25	Wheat Rh	TSBA40 4.10	0.207	*Brevibacillus laterosporus (Bacillus)*
			0.187	*Bacillus megaterium* GC subgroup A
			0.142	*Micrococcus lylae* –GC subgroup B
		ACTIN1 3.80		No match
		CLIN40 4.00	0.176	*Bacillus megaterium*
		YST28 3.80		No match
SLH34	Wheat Rh	TSBA40 4.10	0.239	*Bacillus megaterium* GC subgroup A
			0.208	*Brevibacillus laterosporus (Bacillus)*
		ACTIN1 3.80		No match
		CLIN40 4.00	0.235	*Bacillus megaterium*
		YST28 3.80		No match
SHH29	Wheat Rh	TSBA40 4.10	0.077	*Bacillus lentimorbus*
				Cellulomonas turbata (Oerskovia turbata)
		ACTIN1 3.80		No match
		CLIN40 4.00		No match
		YST28 3.80		No match
SHH36	Wheat Rh	TSBA40 4.10	0.536	*Bacillus amyloliquefaciens (Bacillus subtilis group)*
		ACTIN1 3.80		No match
		CLIN40 4.00	0.656	*Bacillus subtilis*
		YST28 3.80		No match
SHH59	Wheat Rh	TSBA40 4.10	0.145	*Bacillus megaterium* GC subgroup A
			0.139	*Brevibacillus laterosporus (Bacillus)*
			0.079	*Bacillus lentimorbus*
			0.078	*Bacillus flexus*
			0.075	*Micrococcus lylae* –GC subgroup B
		ACTIN1 3.80		No match
		CLIN40 4.00	0.125	*Bacillus megaterium*
		YST28 3.80		No match

SLL: Low input low yield field
SLH: Low input high yield field
SHH: High input high yield field
Rh: Rhizosphere
RE: Rhiozoplane/ endorhizosphere

Bacillus coagulans. FAMES profile of SLH19 showed SI of 0.162 with *Bacillus megaterium*. GC subgroup A and 0.156 with *Bacillus megaterium*. Isolate SLH25 had a similarity value of 0.207 with *Brevibacillus laterosporus* and 0.176 with *Bacillus megaterium*. FAMES profile of SLH34 had a SI of 0.239 with *Bacillus megaterium* GC subgroup A. A low SI of 0.077 with *Bacillus lentimorbus* was found for SHH29. The FAMES profile of SHH36 showed high similarity of 0.593 with *Bacillus subtilis* and isolate SHH 59 showed low similarity index (0.145) with *Bacillus megaterium* GC-subgroup A.

4.11 16S rDNA SEQUENCING

Partial 16S rDNA (515 bases) sequence of *nif* H positive isolate SLL55 was sequenced and searched in Ribosomal Database Project (RDP) and BLAST search. Partial 16S rDNA sequencing showed maximum similarity (89.01%) with UNCLASSIFIED/UNALIGNED PSU85869 0.890135 *Pseudomonas* sp. IC038 16S ribosomal RNA gene (partial sequence) & U85869 0.8901357 *Pseudomonas* IC038str.IC038 of PS.AZOTOFORMANS_SUBGROUP through RDP search and 86.8% with many *Pseudomonas* sp. through BLAST search (Appendix II). The partial 16S rDNA sequence of isolate is given in Fig.4.17

5'-AGGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATGACTAGGAATCTGCCTGGTAGTGGGG
GATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATC
AGATGAGCCTAGGTCGGATTACGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGACAGG
ATGATCAGTCACACTGGTAACTGTAGACACGGTCCAGACTCCTACGGGGAGGCAGCAGTGGGGAATATTGGACAATGG
GCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGG
TTGTAGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATA
CAGAGGGTGCCAAGCCGTTAATCGAATTACTGGGCCGTAAAACCCC-3

Fig. 4.17 : Partial 16S rDNA sequence of SLL55

DISCUSSION

An increase in the number and activity of microbes in the vicinity of plant roots, a phenomenon known as the rhizosphere effect, was first described by **Hiltner (1904)**. The rhizosphere is commonly perceived as a site with high levels of microbial activity and large numbers of bacteria. It is defined as the part of soil under the direct influence of plant roots and it is generally assumed that microbes in this region use soluble compounds released by plant roots as their major nutrient sources; this ability is the nutritional basis of rhizosphere colonization **(Lugtenberg *et al.*, 1999)**. In the present study, microbial counts in all fields increased with plant age. After 90 d the population declined in all field rhizosphere/ rhizoplane-endorhizosphere irrespective of the input system/ practice. **Piceno *et al.* (1999)** suggested that the "rhizosphere effect" may not just support locally elevated microbial biomass and activity, but could also promote microbial community stability and persistence of community within this important microenvironment.

A significant part of the organic material photosynthesized in the aerial organs of the plant is released by the living roots into the soil, a process known as rhizodeposition. The matter so deposited comprises, among others the freed cap cells, polysaccharides, soluble secretions and lysates **(Whipps, 1990)**. These materials serve as not only substrates to nearby microorganisms, but they also induce changes in the physiochemical characteristics of the surrounding soil,

such as acidity, moisture, electrical conductivity, redox potential **(Lynch, 1990)** and oxygen availability **(Hojberg and Sorensen, 1993)**. Several studies have indicated that the structural and functional diversity of rhizosphere populations is affected by the plant species due to differences in root exudation and rhizodeposition in different root zones **(Sorensen, 1997; Jaeger *et al.*, 1999)**. Furthermore, the soil type, growth stages, cropping practices and other environmental factors **(De Leif *et al.*, 1994; Westover *et al.*, 1997; Lupwayi *et al.*, 1998)** seem to influence the composition of the microbial community in the rhizosphere. Rhizosphere microorganisms exert strong effects on plant growth and health by nutrient solubilization, N_2-fixation, or the production of plant hormones **(Hoflich *et al.*, 1994; Patten and Glick, 1996)**.

The initial total counts in bulk soil of all fields were of ~106 cfu which increased 1.5 times at 90 d. The free-living nitrogen fixers population was ~103 cfu which increased gradually and a level of 105 cfu in various fields. *Rhizobium* population also increased by one order. This data is supported by the findings of **Campbell and Greaves (1990)** that in general, the microbial densities in the rhizosphere, which are commonly measured by determining the number of cfu or total bacteria per gram of rhizosphere soil, are 2 or 3 orders of magnitude greater than the microbial densities in bulk soil. However, high densities do not necessarily indicate that the level of activity of all of the microorganisms associated with the rhizosphere throughout the

entire root system are high. In fact, the rhizosphere has been recognized as an oligotrophic environment which contains minute cells whose growth is limited by the lack of substrates **(Foster, 1988)**. In present study also the microbial population declined after 90 d because of low level of root exudation.

The bacterial count data was supported by the soil enzyme activity. Dehydrogenase activity was gradually increased from 0d upto 90 d which declined afterwards. The same pattern was followed by alkaline phosphomonoesterase where the activity increased upto 90d followed by declined in all except SHHPF/ SHHRB. Acid phosphomonesterase activity was also increased with plant age.

A comparison of growth behaviour of isolates under normal and low O_2 conditions in ammonium supplemented ABpy medium indicated that under ammonium sufficient conditions, bacteria did not fix N. However, under low O_2 atm in limited supply of ammonium there was fixation of atmospheric N. **Limmer and Darke (1998)** suggested that N_2-fixation has an extremely high energy of activation and was therefore expensive relative to the energy demands of the cell. A plentiful supply of ammonium prevents nitrogen fixation in diazotrophs as nitrogenase enzyme is not synthesized if there is sufficient or excess supply of fixed nitrogen in the environment **(Rudnick *et al.*, 1997)**. The mechanisms by which cells respond to ammonium have been studied in several members of the proteobacteria, including species from three of four subgroups of this

major taxon. In all of them, NifA is a transcriptional activator of expression of all other *nif* genes, the product of which is required for synthesis of active nitrogenase. NifA is either inactivated or not synthesized in ammonium grown cultures of proteobacteial diaztorophs **(Rudnick *et al.*, 1997). Fishcer (1994)** reported that nitrogen fixation in *Herbaspirillum seropedicae,* an endophytic diazotrophic bacterium of members of the Gramineae, occurred under microaerobic conditions and was regulated at the level of synthesis and the activity of the NifA protein in response to fixed nitrogen and oxygen concentrations. In members of the gamma subclass of proteobacteria, regulation of the transcription at activity of the NifA protein by these two effectors involves NifL protein, which forms an inactive complex with NifA in presence of high levels of ammonium or oxygen **(Monteiro *et al.*, 2003). Souza *et al.* (1999)** reported that in *Herbaspirillum seropedicae* and *Azospirillum brasilense* (beta and alpha-proteobacteria, respectively) the NifA protein was directly inactivated in response to increased levels of fixed nitrogen and oxygen. The signaling pathway for control of NifA activity in response to oxygen is however less clear.

Rhizospheric bacteria can enhance plant growth by a number of different mechanisms **(Glick, 1995; Glick *et al.*, 1999)**. The positive correlation coefficients between plant biomass and dehydrogenase and acid and alkaline phosphomonesterases suggest that plant growth is increased by enhanced microbial activities in the vicinity of

rhizosphere. Acid and alkaline phosphomonoesterase are known to solubilize organic and inorganic phosphate. **Rodriguez and Fraga (1999)** reported that one of the plant growth promoting mechanisms involves solubilization of inorganic and organic phosphates from soil making phosphorus available for plant assimilation. A substantial fraction of the phosphorus in the soil is in the form of poorly soluble phosphate to be assimilable by the plant **(Rodriguez *et al.*, 2000)**. The secretion of organic acids by at least some rhizobacteria, causes localized lowering of the soil pH and a concomitant enhancement of phosphate diffusion **(Drew, 1990)**. Inorganic phosphates in soil may become more available for uptake by the roots of plants. Organic phosphates are solubilized by bacteria with the help of phosphatase enzyme, especially acid phosphatase, which play a major role in organic phosphate solubilization in soil **(Goldstein, 1994; Rodriguez and Fraga, 1999)**. Positive correlation between shoot biomass, dehydrogenase, acid/alkaline phosphomonoesterase, total P and total N content suggested that increased biomass was due to increased activity of soil enzymes. **Kucey *et al.* (1989)** reported that microorganisms involved in P solubilization are better scavengers of soluble P and can enhance plant growth by increasing the efficiency of biological nitrogen fixation, with associated increase in availability of other trace elements such as Fe, Zn etc. substances. **Saubidet *et al.* (2002)** reported that wheat plants inoculated with *Azospirillum brasilense* showed higher biomass, grain yield and N content and

higher grain protein than uninoculated control and suggested that *A. brasilense* increased plant growth by stimulating nitrogen uptake by roots.

*nif*H profile of wheat rhizosphere of all fields at different time periods showed variation in profiles with respect to time/ input system/ practice (Table 4.2). SLH field profile was much more different from other fields since it exhibited some different fragments of *nif*H amplicons, and their appearance was time dependent. The fragments appeared at a particular growth stage of wheat. The pH, exchangeable K and Zn, Cu of the SLH field was significantly higher than other fields. **Kennedy and Gewin (1997)** identified a number of soil and climatic factors which influenced microbial diversity in soil. Soil pH, cation exchange capacity, vegetation etc. influence the microbial diversity. In SLH field, some wild leguminous crops were seen; it could have influenced the microbial diversity. There was a clear cut difference in the *nif*H profile of plain field and raised bed system. A band of 189 bp was observed in raised bed of SLL and SHH fields at 25 d and remained upto 140 d whereas a 161 bp fragment was specifically present in SLL and SHH raised bed at 25 d and persisted upto 140 d. **Kennedy and Gewin (1997)** reported some anthropogenic factors like crop, fertilizers, rotations, pesticides and tillage which affected, the diversity of microbes. Bands 253, 120, 49 bp in *nif*H profile of all fields were common and were present at all the growth stages of wheat. These can be used as a marker for *in situ nif*H study

of these fields. These results suggest that inspite of having different *nif*H profiles, the rhizosphere of a particular crop also promotes microbial community stability and persistence of community within this important microenvironment.

Mahaffee and Kloepper (1997) reported that microbial community structure changes across a gradient from the plant root to surrounding bulk soil in terms of relative abundance, even though membership (i.e. species composition) was shared across this gradient. On the other hand a recent investigation has shown compositional stability of the diazotrophs assemblage over a 1-year period in *Spartina* rhizosphere **(Piceno *et al.*, 1999)**. This study provided indirect evidence of stability of diazotrophs assemblage during altered carbon availability, since plant productivity varied seasonally.

RFLP analysis of *nif*H PCR products from environmental samples is a powerful tool for assessing the presence and diversity of nitrogen-fixing microorganisms in natural ecosystem. Although this approach does not directly allow evaluation of functional aspects of the nitrogen-fixing populations in a sample, structural information on the gene pool and the potential for nitrogen fixation can be assessed. The number and positions of the RFLP fragments reflects the diversity and heterogeneity of the nitrogen-fixing populations in a sample **(Widmer *et al.*, 1999)**. The RFLP analysis performed in this study yielded highly reproducible patterns over time and revealed clear differences between the *nif*H gene pool present in the litter and soil

from the Dougles fir forest site. **Shaffer *et al.* (2000)** studied the temporal and spatial distribution of the *nif*H gene of N_2-fixing bacteria in forests and clear cuts in Western Oregon and found that five of the *nif*H DNA pattern of RFLP were dominant type in DF litter with characteristic fragments of 237-303 bp length; samples from soil contained primarily seven other pattern, 131-188 bp length and suggested that 237-303 bp fragments characteristic of fir forest litter could generally not be collected in clearcuts that adjoin the forest site.

All 14 promising isolates identified by FAMES analysis in this study exhibited low similarity index (SI) with the organisms available in FAMES library; isolates SLL22 and SHH36 were an exception with SI above 0.50. Isolate SLL22 has been identified as *Bacillus amyloliquefaciens* (*Bacillus subtilis* group) and SHH36 as, *Bacillus subtilis*. The other organisms that were nitrogenase positive could not be identified through MIS analysis (Table 4.16).

Only one organism SLL55 out of 14 isolates showed significant nitrogenase activity (ARA) in given *in vitro* conditions, even standard cultures were failed to show any significant nitrogenase activity. The result suggested that given conditions for ARA were not suitable for weak strains of nitrogen fixers. Isolate SLL55 could also be amplified with degenerate *nif*H primers. A nested reverse transcriptase PCR (RT-PCR) method was used by **Zani *et al.*, 2000** to assess the expression of nitrogenase in mesooligotrophic Lake George. These workers found several diazotrophs in Lake George samples that

included cyanobacteria, α-proteobacteria, and a novel diazotrophic proteobacterial clade that expressed *nif*H transcripts; all of the bacteria detected had type I nitrogenase, and exhibited no sequences in group II, III, or IV. The expression of *nif*H transcripts does not necessarily indicate that bacteria were actively fixing atm. N_2; it does however provide information about bacteria that could have been fixing nitrogen and suggests prevailing nitrogen-fixing conditions for the existing phylotypes. Since the available microorganisms expressed nitrogenase in a typical phosphorus-limited environment, it suggested that they could have been limited by multiple nutrients or that were limited by different nutrients in the same environment.

Partial 16 S rDNA sequencing of SLL55 showed maximum similarity (89.01%) with UNCLASSIFIED/UNALIGNED PSU85869 0.890135 *Pseudomonas* sp. IC038 16S ribosomal RNA gene (partial sequence) & U85869 0.8901357 *Pseudomonas* IC038str.IC038 of PS.AZOTOFORMANS_SUBGROUP through Ribosomal Database Project search and 86.8% with many *Pseudomonas* sp. through BLAST Search. Isolate SLL55 is found to be new for nitrogen fixation. **Berge *et al.* (1991)** sequenced the *rrs* gene of the nitrogen-fixing strains which were isolated from the rhizosphere of wheat (TOD45) and maize (RSA19) and were identified as *Bacillus circulans* (group 2) by phenotypic characterization (API 50 CH). Since nitrogen fixation among aerobic endosphore forming bacilli is a physiological character of major importance both for taxonomic and phylogenetic purposes and for ecological assessment, the type strains of some representative

species of the genera *Bacillus* and *Paenibacillus* were analyzed for nitrogenase activity and presence of *nif*H gene.

Measurement of nitrogen gains through micro-Kjeldahl yielded positive results in *P. polymyxa* **(Rhodes-Robert, 1981)**. These workers concluded that *P. polymyxa* type strain was nitrogen fixing, and that suitable conditions for demonstrating acetylene reduction remained to be elucidated.

Although a *nif*H gene was detected in *P. polymyxa* type strain ATCC842^T (as well as LMG13294^T), and in strain TOD45, only weak nitrogenase activity was measured **(Achouak *et al.*, 1999)**. Weak acetylene-reduction activity for the type strains of *Paenibacillus larvae, p. amylolyticus, P. lautus, P. macquariensis* and *P. peoriae* was found but no detectable *nif*H amplicons were observed. A similar case was reported for *Streptomyces thermoautotrophicus* **(Gadkari *et al.*, 1997)**. However, O_2-tolrant nitogenase from *S. thermoautotrophicus* utilized superoxide as an electron donor **(Ribbe *et al.*, 1997)** that has fundamentally changed the thinking about the interrelation between N_2-fixation and O_2. After the discovery of this novel nitrogenase, the possibility of the existence of similar system in other organisms has become higher. **Postgate (1987)** had referred as "ghosts", those potential nitrogen fixing organisms that remain to be fully investigated. These organisms were originally suspected to be diazotrophs because they could grow in the apparent absence of any nitrogen source other than atm. N_2, but do not have any *nif* H genes and are therefore

unable to reduce acelylene. In *S. thermoautotrophicus nif* genes were found to be absent; the nitrogenase is encoded by different genes and moreover, it is unable to catalyse acetylene reduction **(Ribbe *et al.*, 1997)**. Therefore it seems to be a good case for rexamining the diazotophic status of the so-called "ghosts". It appears that this study has provided some isolates that belong to the so-called category of "ghosts" and require further investigations to arrive at their true capacity to fix nitrogen.

SUMMARY

In situ nif H profiles of wheat rhizosphere of selected fields were analyzed at different growth stages to assess whether input / practice/growth stages of plant had any impact of diversity structure and function. Soil DNA was extracted and amplified with *nif*H degenerate primers and resultant product was double digested with *Alu*I and *Rsa*I. *nif* H profile of various fields showed that,

1. There was time/ input dependent change in the *nif*H community of various fields i.e. SLL PF/RB, SLH and SHH PF/RB.
2. Comparative profiles showed that rhizosphere of SLH field had much more different N_2 - fixing community as compared to other fields; bands of fragment size 235, 225 & 130 bp appeared at different time periods.
3. Clearcut impact of plain vs raised bed practice on *nif*H community was observed in various fields. The band pattern suggested that irrespective of input system some nitrogen-fixing populations were specific to plain field (band of 189 bp) and some to raised bed (band of 161 bp).
4. Presence of few bands i.e. 253, 120 & 49 bp throughout the wheat growth irrespective of practice/time/input system suggested that these bands can be used as marker for *nif* H community.

The selected experimental fields were low in available N & P. To find promising bioinoculants as an alternative to expensive chemicals, 44 isolates were recovered from Jensen's medium at 45 d and screened for their plant growth promotory potential. All isolates used

in the present study were able to grow in N-free media which had different 'C' sources and in microaerophilic conditions.

PCR amplification for 16S rDNA was performed for all the isolates and restricted with two restriction enzymes i.e. *Taq*I and *Hae* III for clustering. Six major clusters were formed in *Taq*I restriction profile and eight major clusters in *Hae*III. Microcosm experiment results were as follows:

1. Dehydrogenase activity, acid and alkaline phosphomonoesterase activity were increased as compared to control.
2. Plants inoculated with these bacterial isolates had higher biomass
3. A total of 14 treatments were selected on the basis of plant growth parameters and enzyme activity for shoot N & P and their respective isolates for nitrogenase activity only one isolates showed significant nitrogenase activity (ARA) and could be amplified with degenerate *nif* H primers.
4. A positive correlation was observed between plant growth parameters, soil enzymes, shoot N & P.
5. Correlation data suggests that bacterial isolates improved plant growth through plant growth promontory properties.

FAMES analysis was performed for identification of selected 14 isolates. Only two isolates SLL22 and SHH36 showed significant similarity index of 0.522 and 0.656 with *Bacillus amyloliquefaciens* and *Bacillus subtilis* respectively. The FAMES profile of *nif*H positive

isolates SLL55 showed SI of 0.273 with *Pseudomonas chlororaphis* and 0.245 with *Pseudomonas putida*- biotype B.

Partial 16S rDNA sequencing of SLL55 showed maximum similarity (89.01%) with UNCLASSIFIED/UNALIGNED PSU85869 0.890135 *Pseudomonas* sp. IC038 16S ribosomal RNA gene (partial sequence) and U85869 0.8901357 *Pseudomonas* IC038str.IC038 of PS.AZOTOFORMANS_SUBGROUP through Ribosomal Database Project search and 86.8% with many *Pseudomonas* sp. through BLAST search.

Isolate SLL55 is found to be new for nitrogen fixation.

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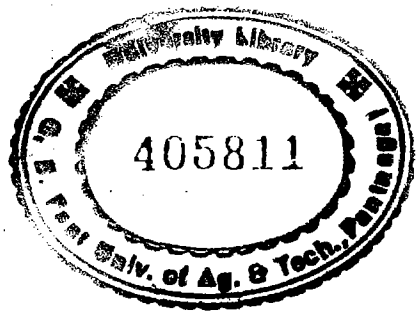
Zuberer, D.A. 1990. Soil and rhizosphere aspects of N_2-fixing plant-microbe associations. In: Lynch, J.M. (ed). The rhizosphere. John Wiley & Sons, Ltd, New York, pp. 317-353.



APPENDICES

File C:\SHERLOCK\RAW\E03C01.530\A0151019.D
Sherlock Id. 10

29

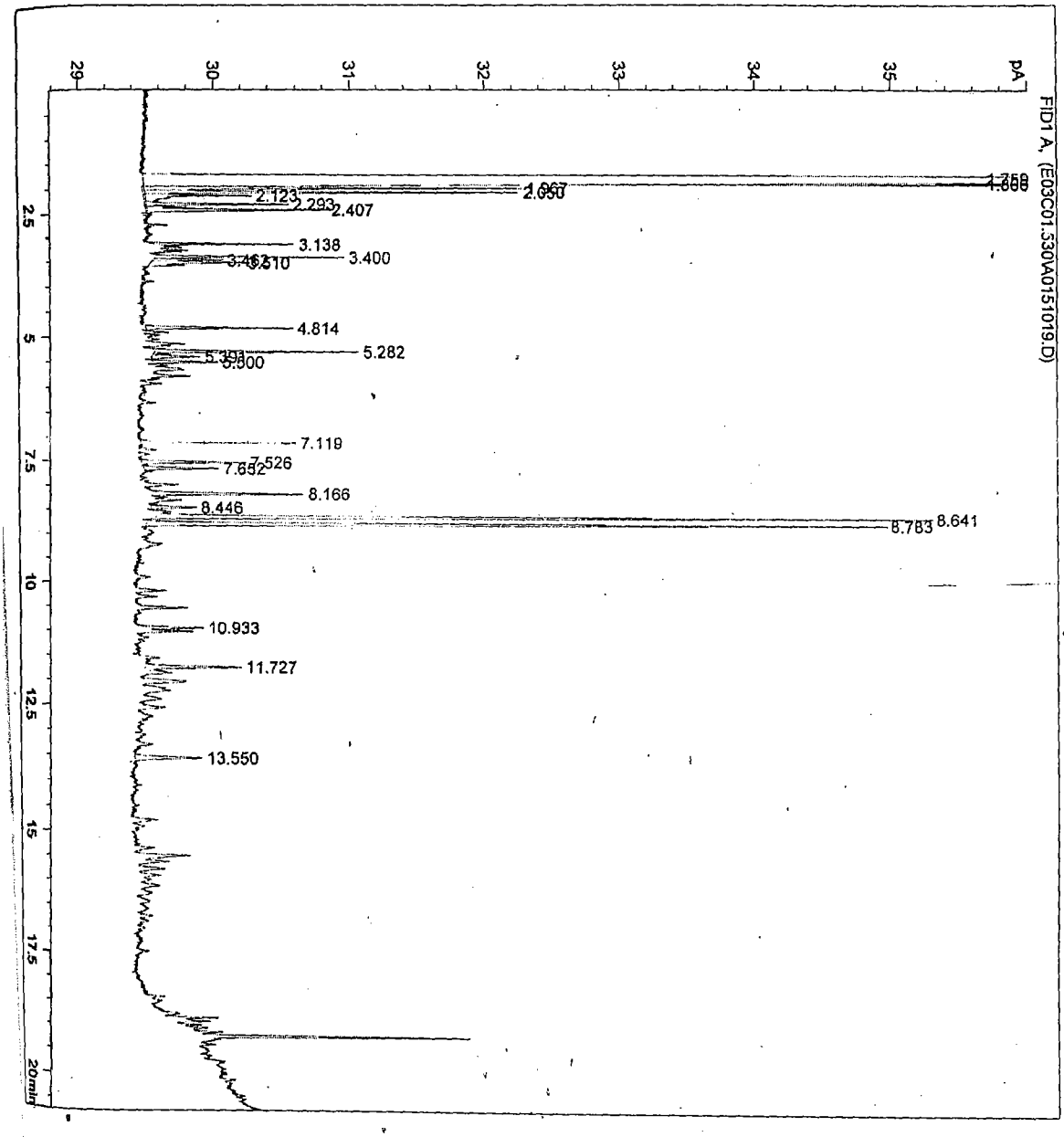
Sample Name: 1019

APPENDIX I

Injection Date : 12/1/03 6:31:45 PM
Sample Name : 1019
Acq. Operator : S.MayilRaj
Location : Vial 13
Inj : 1
Inj Volume : 2 µl

Method : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed : 12/1/03 6:28:32 PM by S.MayilRaj
MIDI Aerobe method saved on ChemStation Version 4.02
Switched to new integration algorithm 11-Nov-98

Isolate SLL4



*** End of Report ***

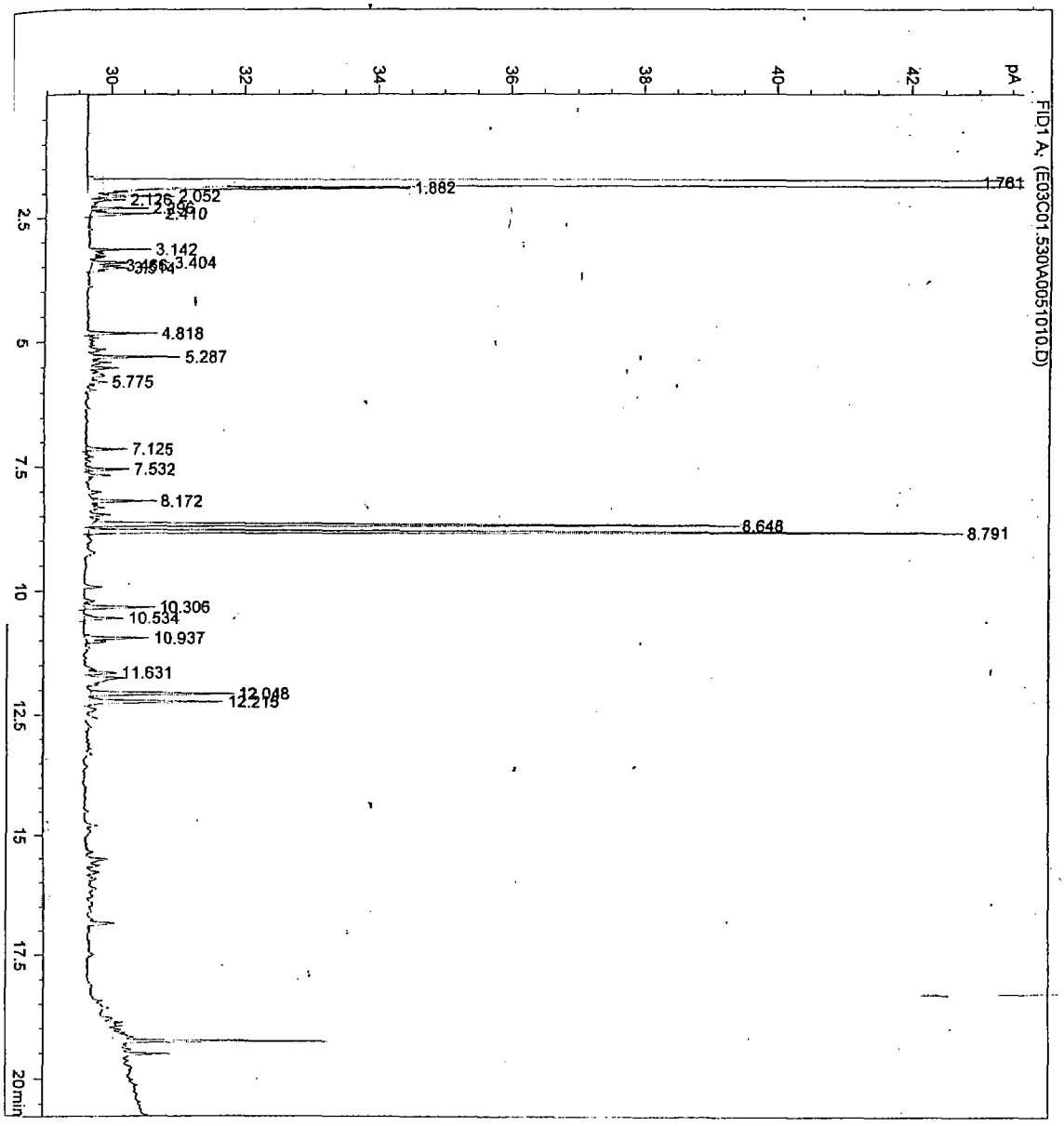
File C:\SHERLOCK\RAW\E03C01.530\A0051010.D 20 Sample Name: 1010
Sherlock Id: 1

Kawal
21.1.04

Injection Date : 12/1/03 2:24:53 PM
Sample Name : 1010 Location : Vial 4
Acq. Operator : S.MayilRaj Inj : 1
Inj Volume : 2 µl

Method : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed : 12/1/03 2:21:38 PM by S.MayilRaj
MIDI Aerobe method saved on ChemStation Version 4.02
Switched to new integration algorithm 11-Nov-98

Isolate SLL22



*** End of Report ***

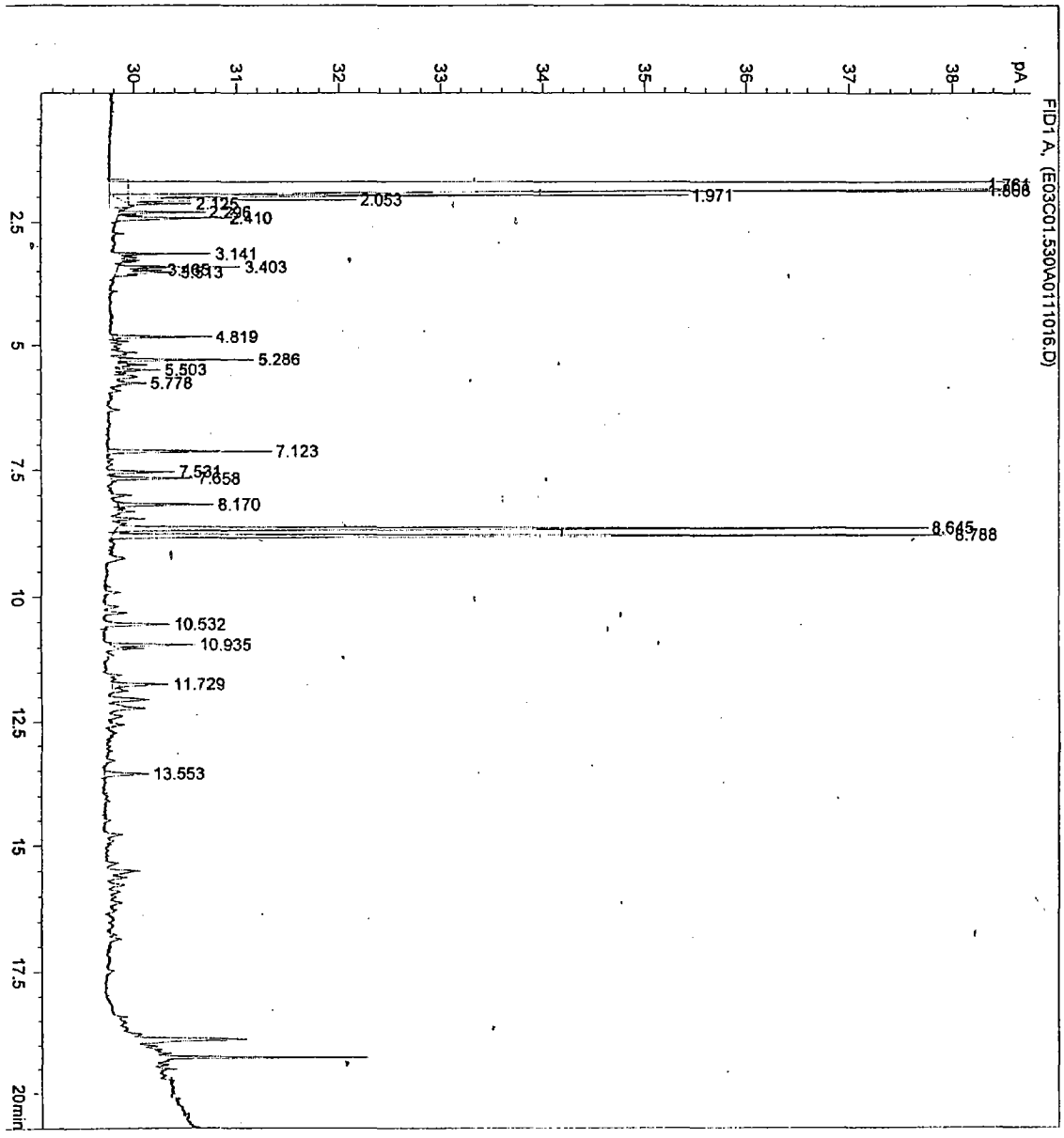
strument 1 12/1/03 2:45:38 PM S.MayilRaj

File C:\SHERLOCK\RAW\E03C01.530\A0111016.D Sample Name: 1(
Sherlock Id: 7

26

Injection Date : 12/1/03 4:53:05 PM
Sample Name : 1016 Location : Vial 10
Acq. Operator : S.MayilRaj Inj : 1
Inj Volume : 2 µl
Method : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed : 12/1/03 4:49:45 PM by S.MayilRaj
MIDI Aerobe method saved on ChemStation Version 4.02
Switched to new integration algorithm 11-Nov-98

Isolate SLL27



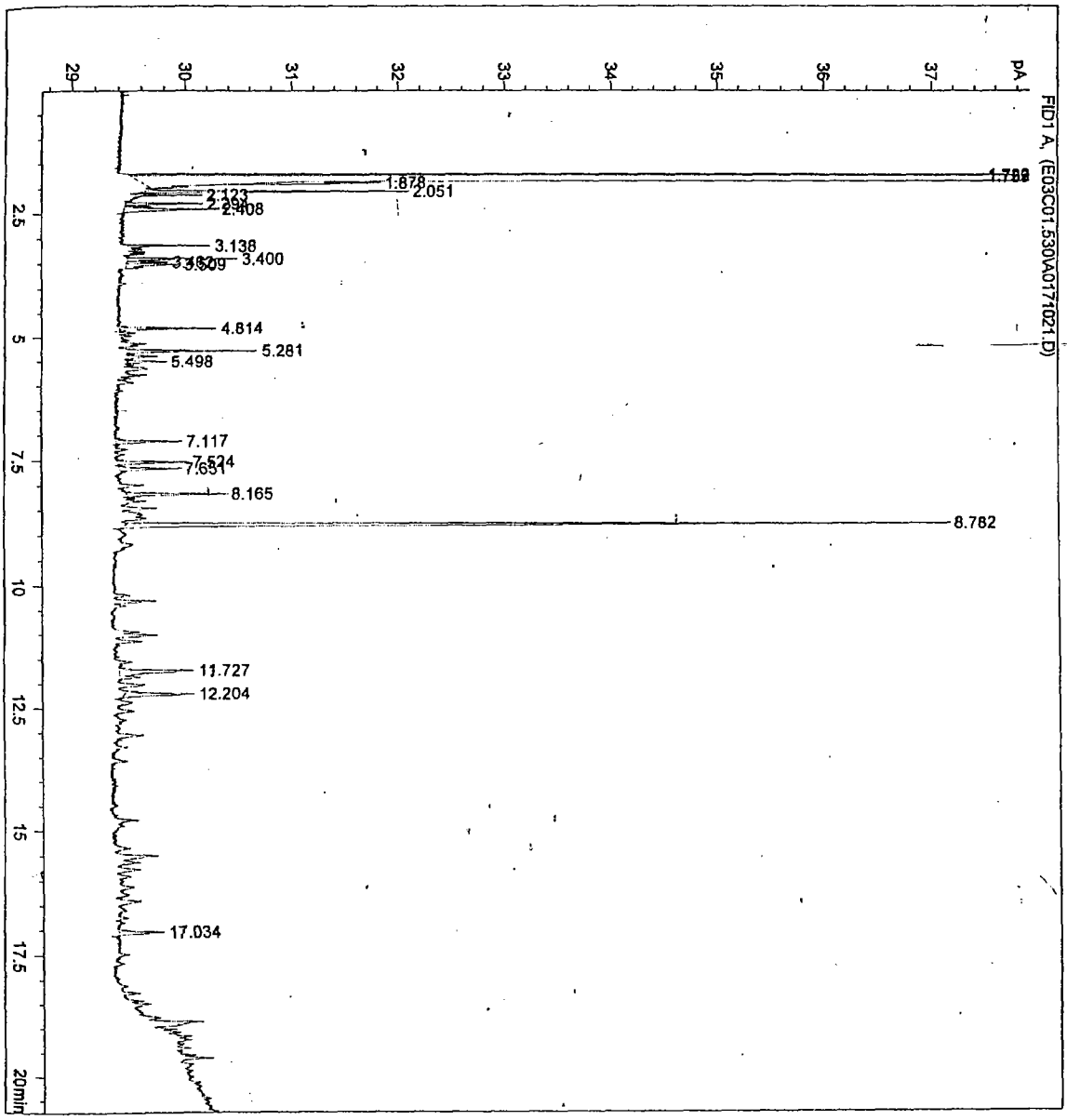
*** End of Report ***

File C:\SHERLOCK\RAW\E03C01.530\A0171021.D 31 Sample Name: 1021
Sherlock Id: 12

Injection Date : 12/1/03 7:21:09 PM
Sample Name : 1021 Location : Vial 15
Acq. Operator : S.MayilRaj Inj : 1
Inj Volume : 2 µl

Method : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed : 12/1/03 7:17:53 PM by S.MayilRaj
MIDI Aerobe method saved on ChemStation Version 4.02
Switched to new integration algorithm 11-Nov-98

Isolate SLL45



*** End of Report ***

ata File C:\SHERLOCK\RAW\E03C01.530\A0061011.D 21 Sample Name: 1011
Sherlock Id: 2

==========

Injection Date : 12/1/03 2:49:41 PM
Sample Name : 1011 Location : Vial 5
Acq. Operator : S.MayilRaj Inj : 1
Inj Volume : 2 µl

Method : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed : 12/1/03 2:46:10 PM by S.MayilRaj
MIDI Aerobe method saved on ChemStation Version 4.02 **Isolate SLL55**
Switched to new integration algorithm 11-Nov-98

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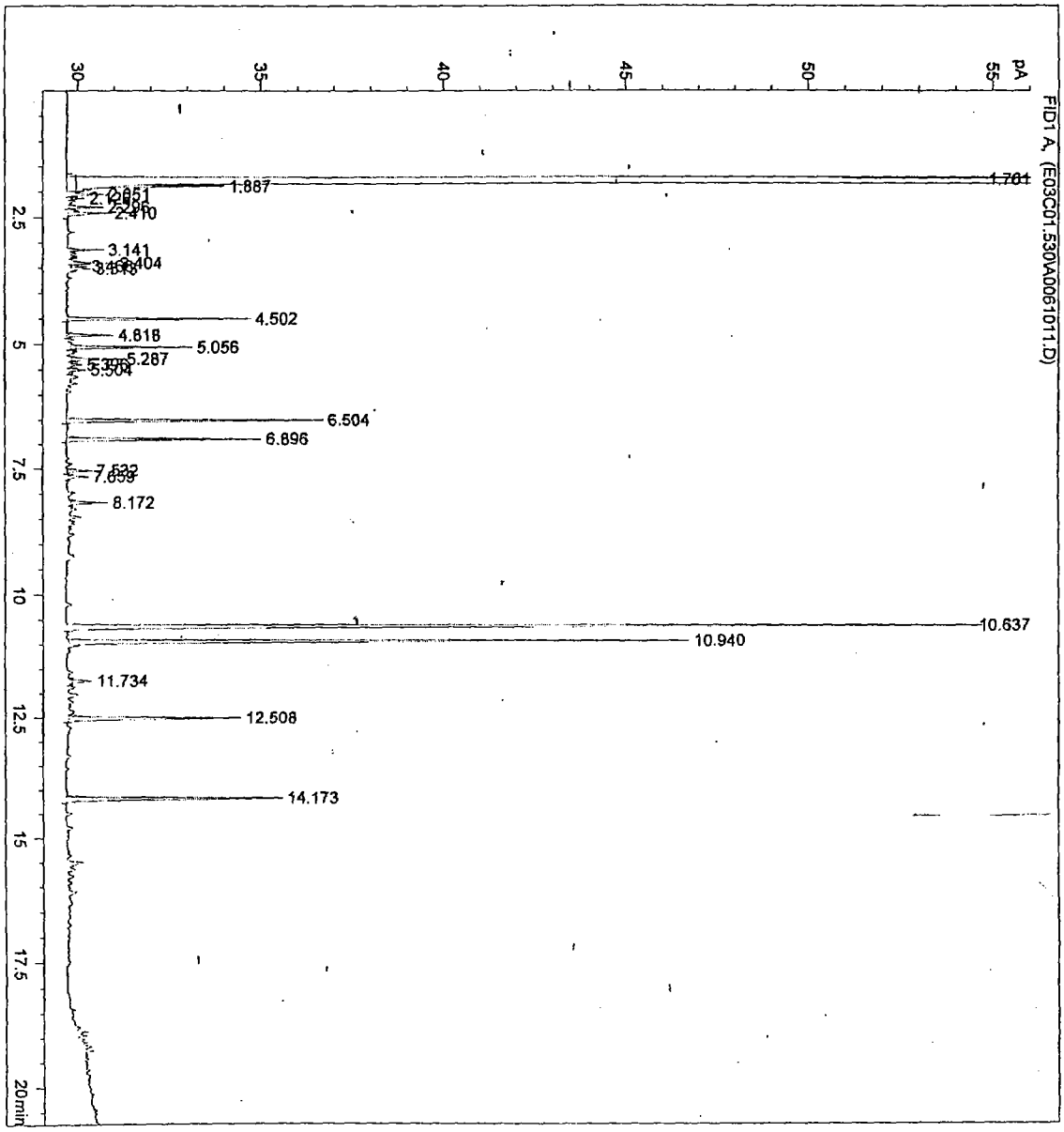
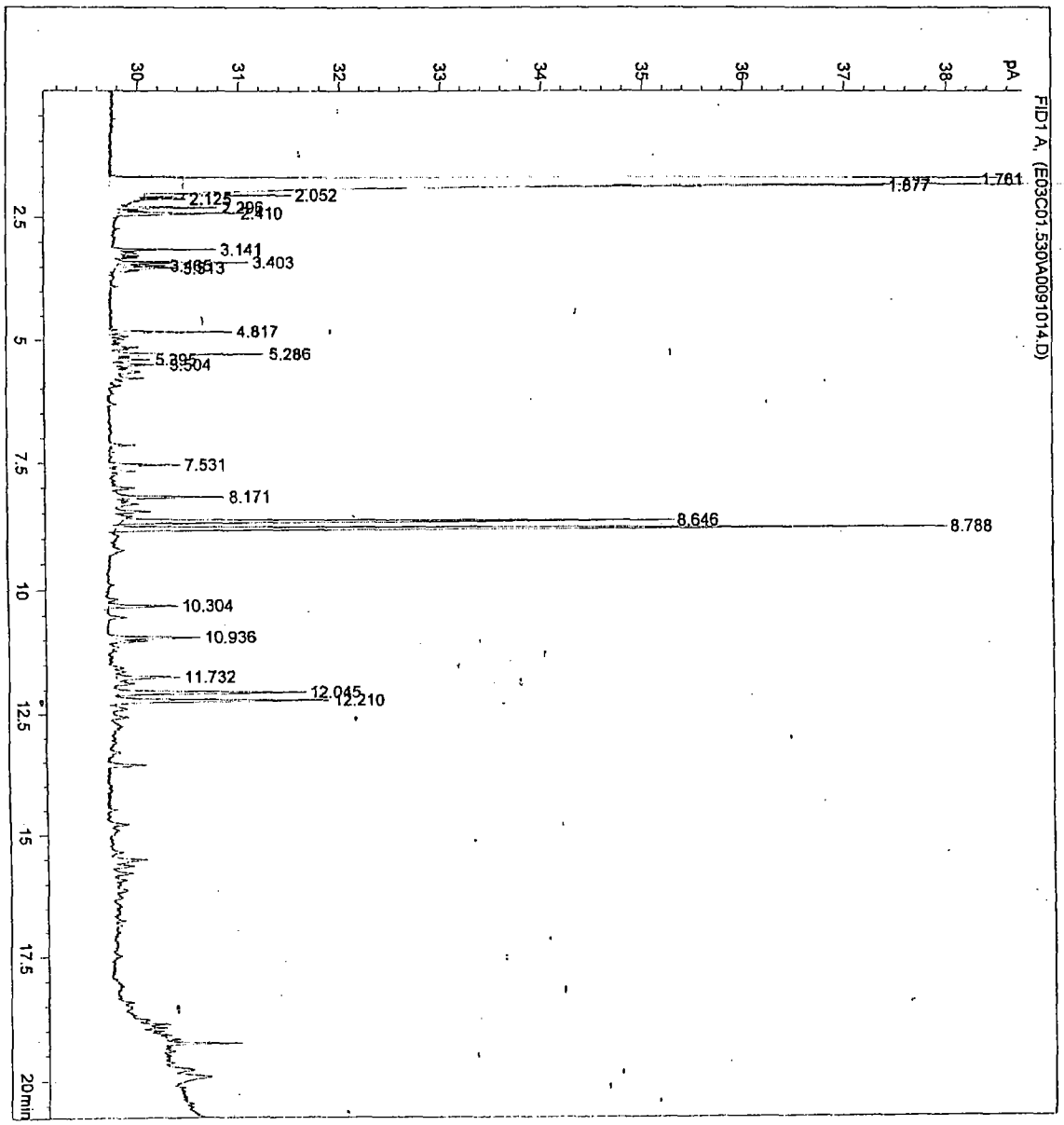


*** End of Report ***

ıta File C:\SHERLOCK\RAW\E03C01.530\A0091014.D 24 Sample Name: 1014
Sherlock Id: 5

Injection Date : 12/1/03 4:03:39 PM
Sample Name : 1014 Location : Vial 8
Acq. Operator : S.MayilRaj Inj : 1
Inj Volume : 2 µl
Method : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed : 12/1/03 4:00:25 PM by S.MayilRaj
MIDI Aerobe method saved on ChemStation Version 4.02
Switched to new integration algorithm 11-Nov-98

Isolate SLL71



*** End of Report ***

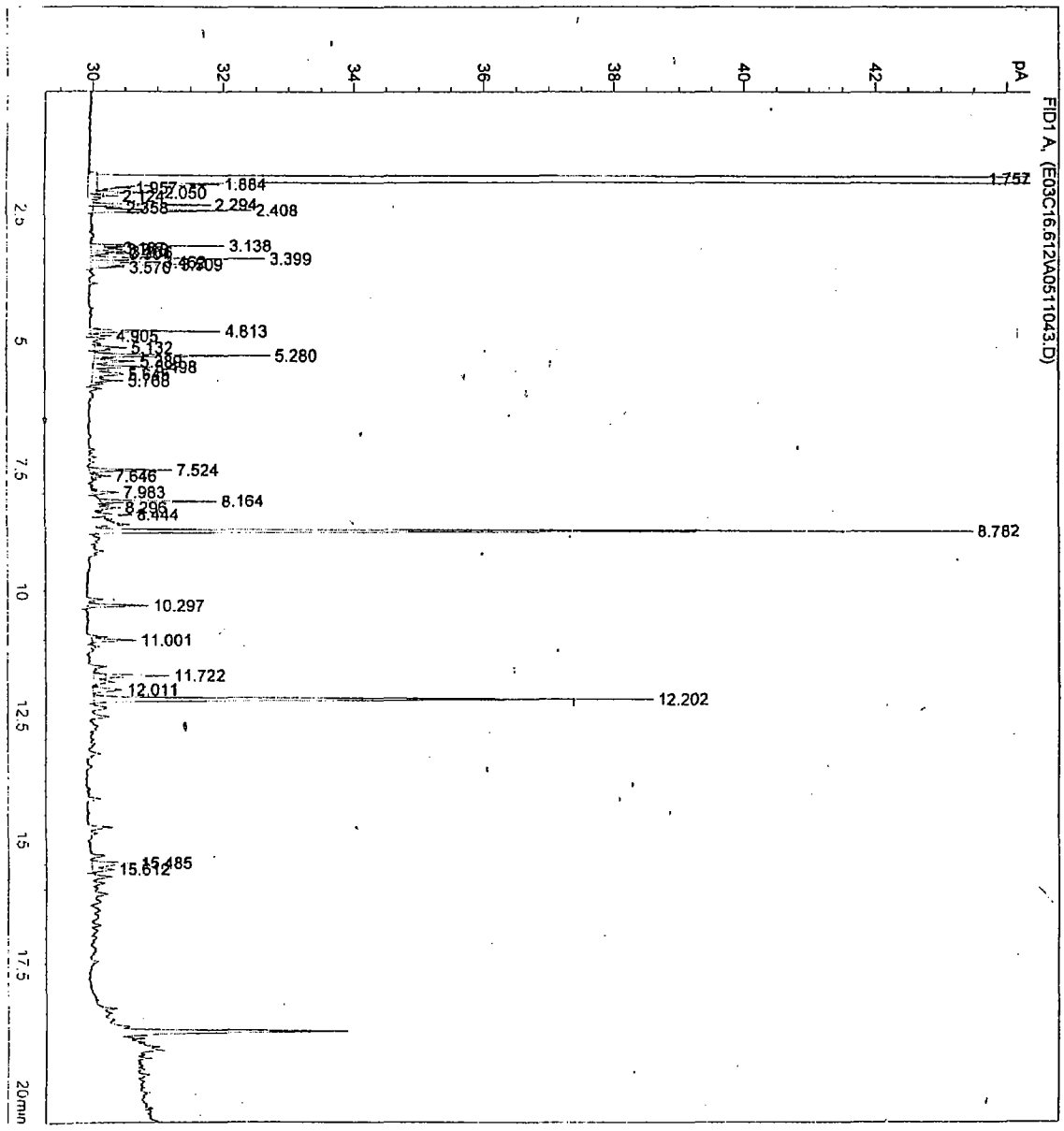
Data File C:\SHERLOCK\RAW\E03C16.612\A0511043.D
Sherlock Id: 14

Sample Name: 1043

Injection Date : 12/17/03 5:35:39 PM
Sample Name : 1043
Acq. Operator : S.MayilRaj
Location : Vial 45
Inj : 1
Inj Volume : 2 µl

Method : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed : 12/17/03 5:32:16 PM by S.MayilRaj
MIDI Aerobe method saved on ChemStation Version 4.02
Switched to new integration algorithm 11-Nov-98

Isolate SLLRE30



*** End of Report ***

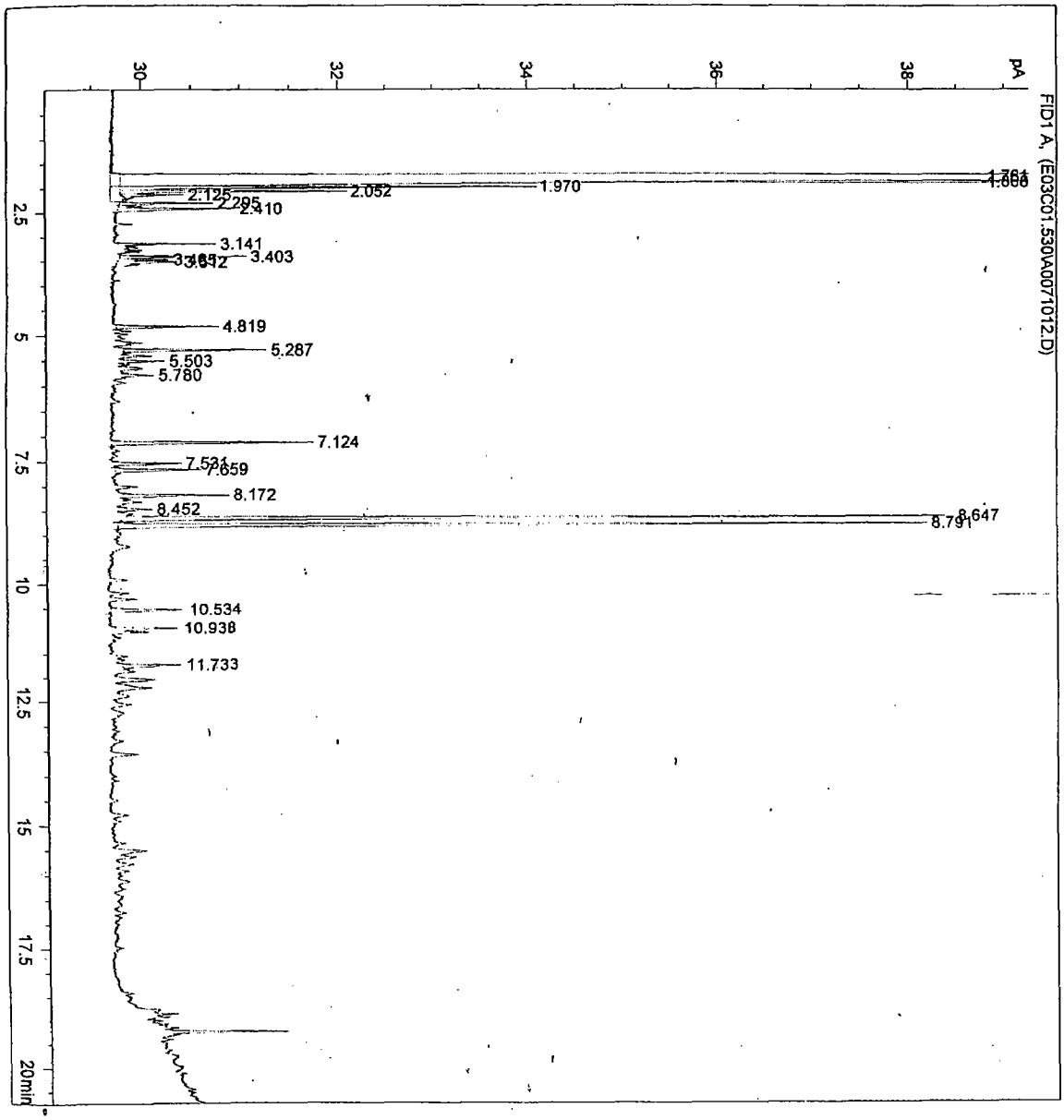
Data File C:\SHERLOCK\RAW\E03C01.530\A0071012.D Sample Name: 1012
Sherlock Id: 3

22

Injection Date : 12/1/03 3:14:23 PM
Sample Name : 1012 Location : Vial 6
Acq. Operator : S.MayilRaj Inj : 1
Inj Volume : 2 µl

Method : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed : 12/1/03 3:10:59 PM by S.MayilRaj
MIDI Aerobe method saved on ChemStation Version 4.02
Switched to new integration algorithm 11-Nov-98

Isolate SLH19

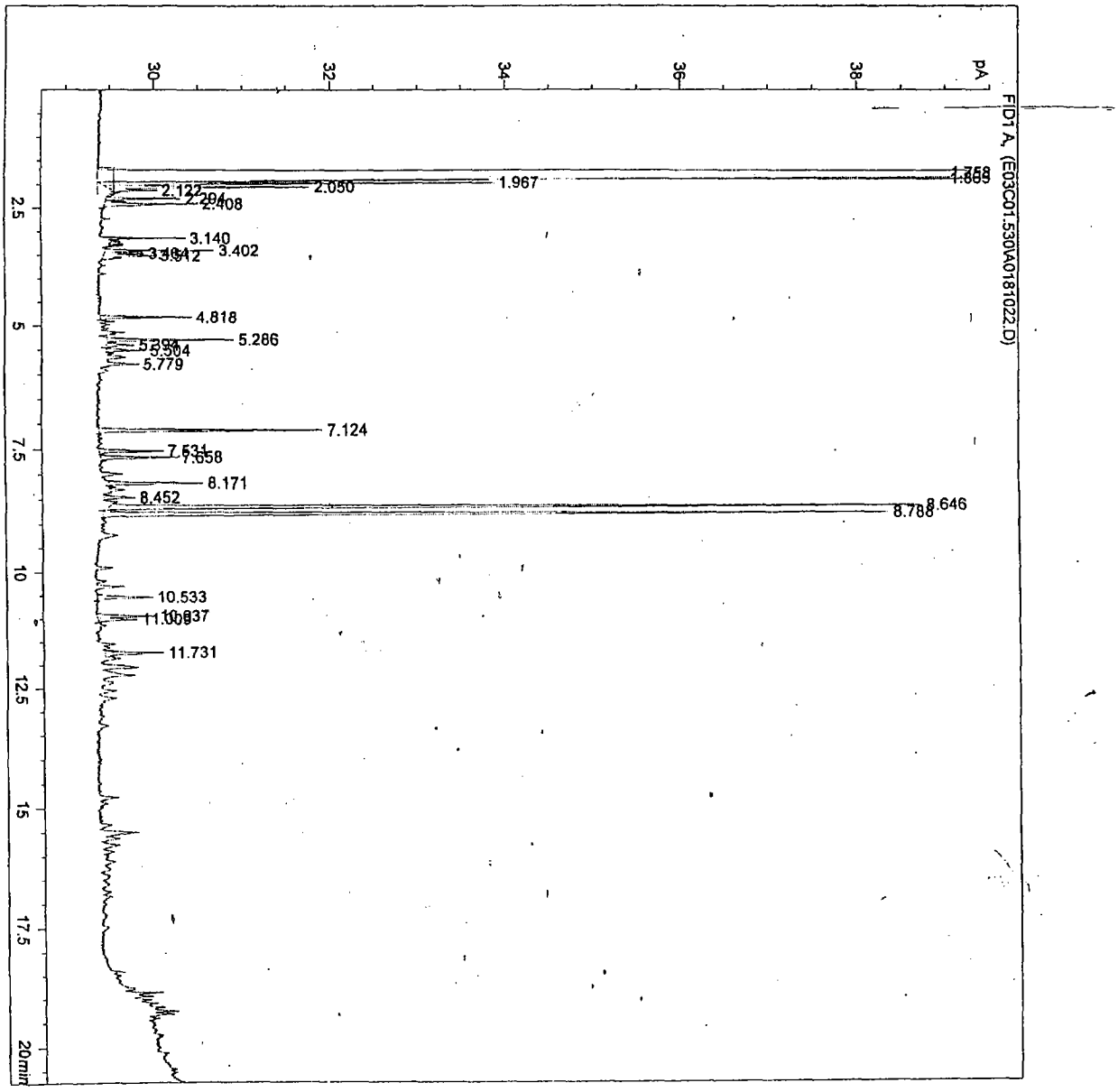


*** End of Report ***

ıta File C:\SHERLOCK\RAW\E03C01.530\A0181022.D 32 Sample Name: 1022
Sherlock Id: 13

Injection Date : 12/1/03 7:50:52 PM
Sample Name : 1022 Location : Vial 16
Acq. Operator : S.MayilRaj Inj : 1
Inj Volume : 2 µl
Method : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed : 12/1/03 7:50:01 PM by S.MayilRaj
MIDI Aerobe method saved on ChemStation Version 4.02
Switched to new integration algorithm 11-Nov-98

Isolate SLH34



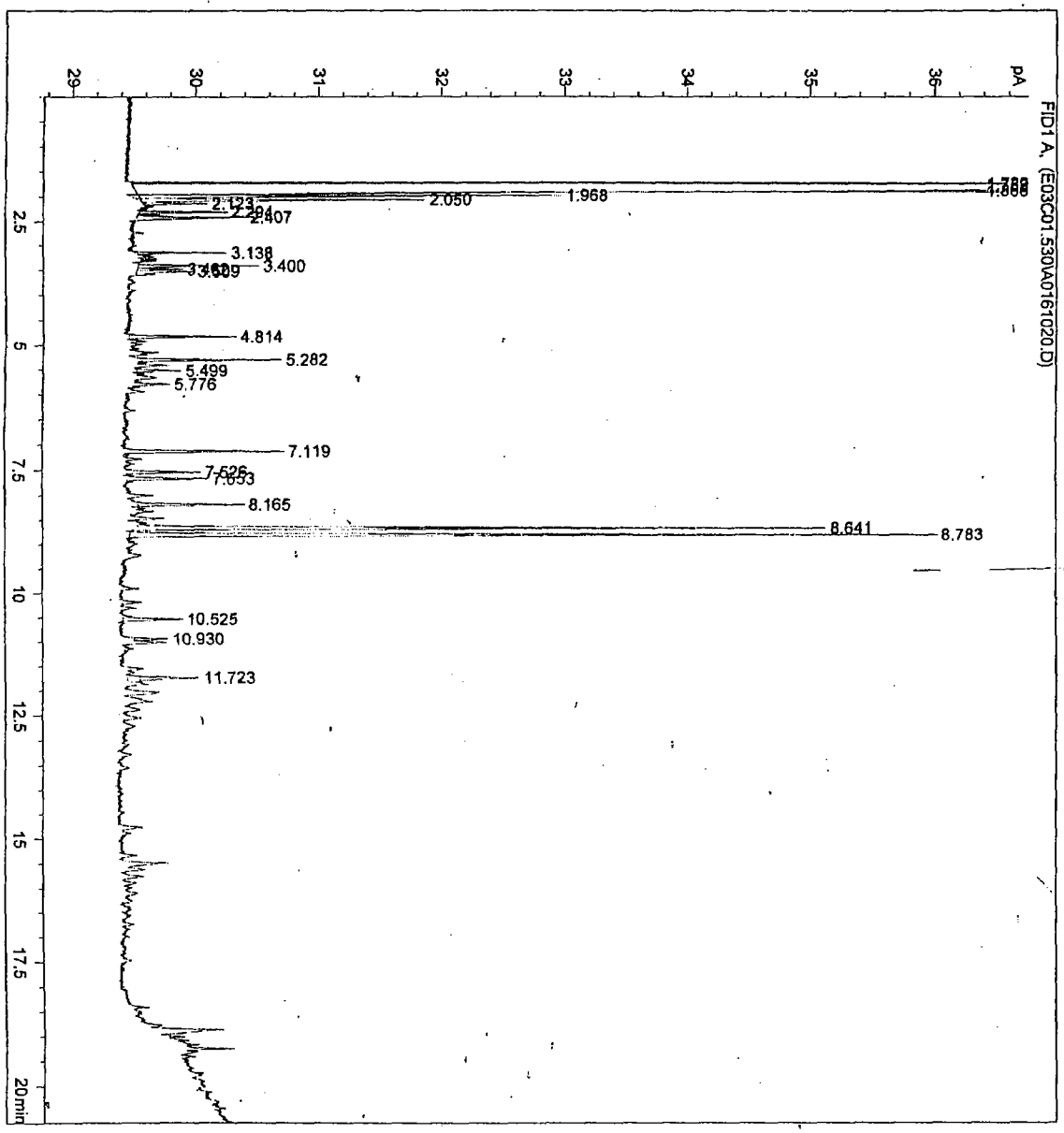
*** End of Report ***

ıstrument 1 12/1/03 8:11:37 PM S.MayilRaj

Data File C:\SHERLOCK\RAW\E03C01.530\A0161020.D 3D Sample Name: 1020
Sherlock Id: 11

Injection Date : 12/1/03 6:56:31 PM
Sample Name : 1020 Location : Vial 14
Acq. Operator : S.MayilRaj Inj : 1
Inj Volume : 2 µl
Method : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed : 12/1/03 6:53:04 PM by S.MayilRaj
MIDI Aerobe method saved on ChemStation Version 4.02
Switched to new integration algorithm 11-Nov-98

Isolate SLH25



*** End of Report ***

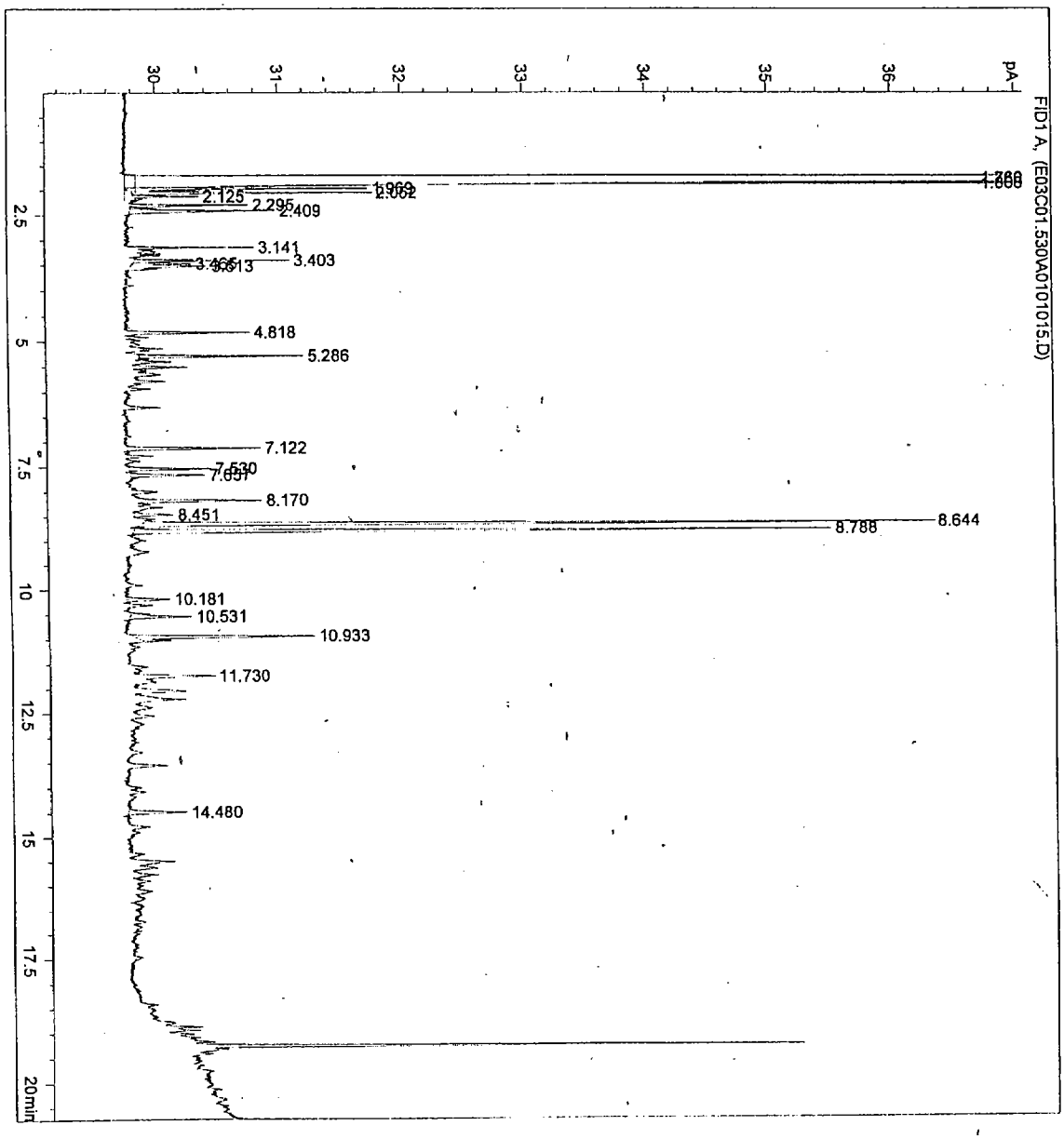
ata File C:\SHERLOCK\RAW\E03C01.530\A0101015.D
Sherlock Id: 6

25

Sample Name: 1015

Injection Date : 12/1/03 4:28:25 PM
Sample Name : 1015
Location : Vial 9
Acq. Operator : S.MayilRaj
Inj : 1
Inj Volume : 2 µl
Method : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed : 12/1/03 4:25:04 PM by S.MayilRaj
MIDI Aerobe method saved on ChemStation Version 4.02
Switched to new integration algorithm 11-Nov-98

Isolate SHH29



*** End of Report ***

Data File C:\SHERLOCK\RAW\E03C16.612\A0401033.D Sample Name: 1033

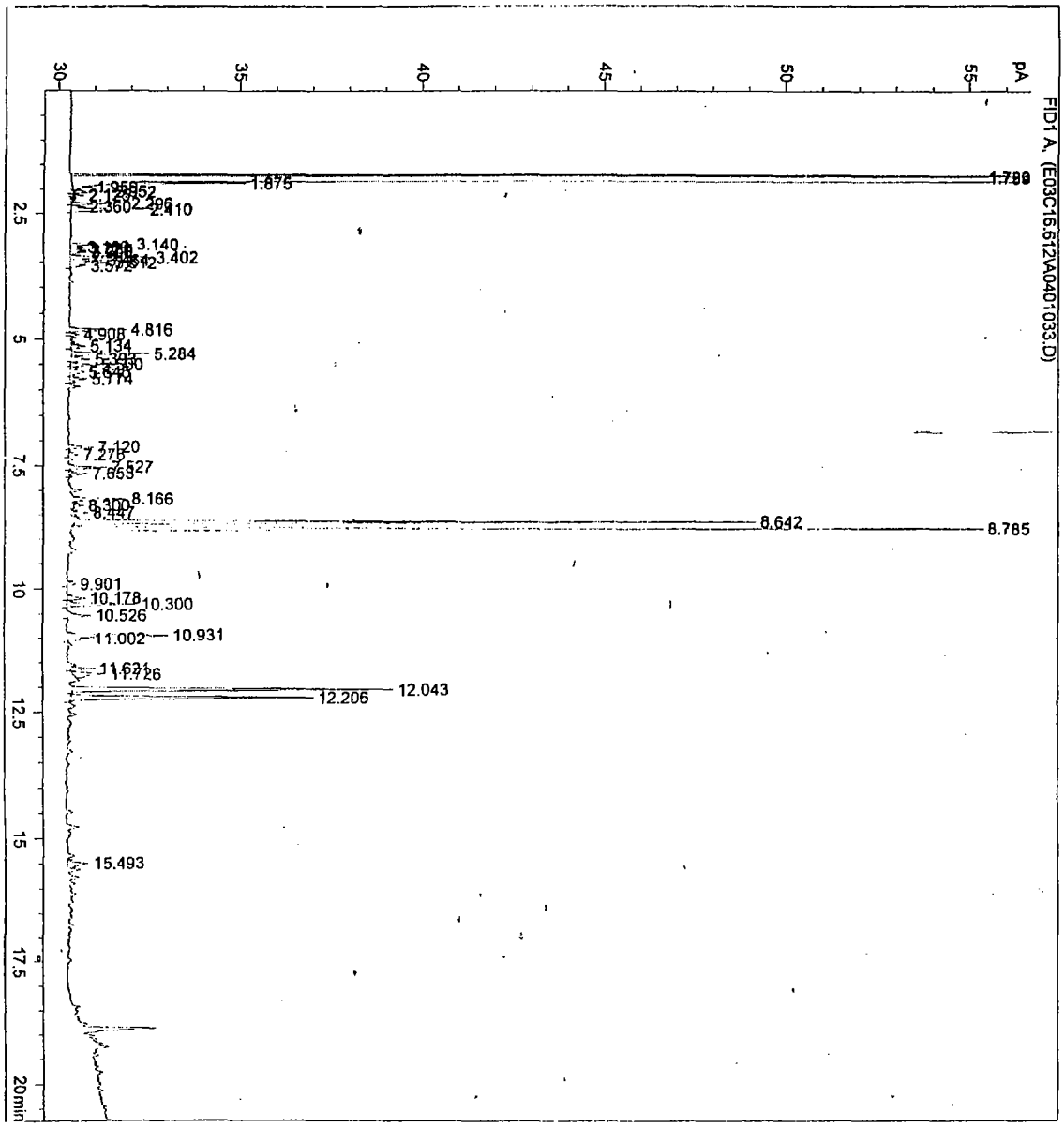
Sherlock Id: 4'

83

Injection Date : 12/17/03 1:04:03 PM
Sample Name : 1033 Location : Vial 35
Acq. Operator : S.MayilRaj Inj : 1
Inj Volume : 2 µl

Method : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed : 12/17/03 1:00:47 PM by S.MayilRaj
MIDI Aerobe method saved on ChemStation Version 4.02
Switched to new integration algorithm 11-Nov-98

Isolate SHH36



*** End of Report ***

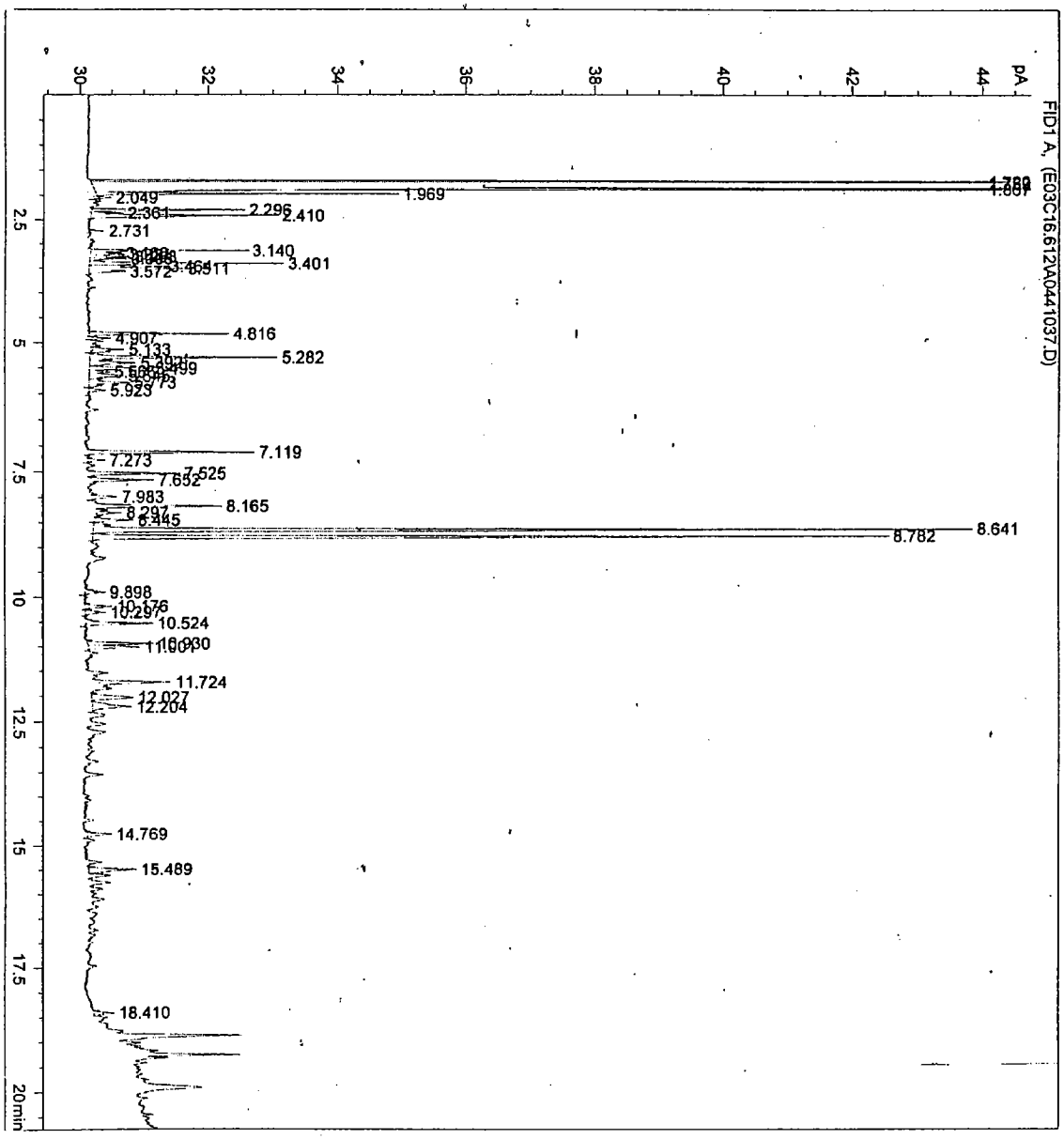
Data File C:\SHERLOCK\RAW\E03C16.612\A0441037.D Sample Name: 1037

Sherlock Id: 8

27

Injection Date : 12/17/03 2:42:45 PM
Sample Name : 1037 Location : Vial 39
Acq. Operator : S.MayilRaj Inj : 1
Inj Volume : 2 µl
Method : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed : 12/17/03 2:39:31 PM by S.MayilRaj
MIDI Aerobe method saved on ChemStation Version 4.02
Switched to new integration algorithm 11-Nov-98

Isolate SHH59



*** End of Report ***

APPENDIX II

RIBOSOMAL DATABASE PROJECT

Short-ID :raw_11006
Full name :raw_11006 [Unknown form], 514 bases, B0E7B9AB checksum.
Sequence :502 unique oligos.
RDP data :Small Subunit rRNAs with Prokaryotic tree
Comments:A minimum of 100 unique oligos required
:A total of 166 sequences were excluded
:34362 sequences were included in the search
:The screening was based on 7-base oligomers
Date :Sat Apr 24 07:58:02 2004
CPU time :0.63 seconds

BACTERIA

PROTEOBACTERIA

GAMMA_SUBDIVISION

PSEUDOMONAS_AND_RELATIVES

PSEUDOMONAS_SUBGROUP

PS.TOLAASII_SUBGROUP

AF064461 0.8781422Pseudomonas cedrella str. CFML 96-198
AB016428 0.8781370Pseudomonas putida ATCC 17472
AB013843 0.8451406str. 10-12.H
AJ002801 0.8451400str. 150

PS.AZOTOFORMANS_SUBGROUP

Ps.spS3_6 0.869 289Pseudomonas sp. str. S3.6
Ps.mucidol 0.8511440Pseudomonas mucidolens IAM 12406 (T)
AF074384 0.8881421Pseudomonas gessardii CIP 105469
Ps.azotofo 0.8531429Pseudomonas azotoformans IAM 1603 (T)
U85868 0.8801360ACAM 213
U85869 0.8901357Pseudomonas IC038 str. IC038
AF170731 0.8531379Pseudomonas QSSC1-9 str. QSSC1-9
Ps.synxant 0.8471428Pseudomonas synxantha IAM 12356
AB004760 0.8881347unnamed organism
AF057645 0.8881422Pseudomonas libaniensis CIP 105460

UNCLASSIFIED / UNALIGNED

PSU85869 0.8901357Pseudomonas sp. IC038 16S ribosomal RNA gene, partial sequence.
PSU85868 0.8801360Pseudomonas sp. ACAM213 16S ribosomal RNA gene, partial sequence.
AB022820 0.857 574str. POCPN-72.
AB022298 0.857 574str. POCPN-71.
AF175584 0.853 455clone WJGRT-6.
PSPAJ28010 0.8451400Pseudomonas sp. 16S rRNA gene, isolate 150.

BLAST SEARCH

gi|32527610|gb|AY321588.1|	Pseudomonas fluorescens 16S ribo...	868
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gi|32351737|gb|AY308044.1|	Pseudomonas sp. AZ22R11 16S ribo...	868
0.0		
gi|21898830|gb|AY121983.1|	Pseudomonas sp. RA12 16S ribosom...	868
0.0		
gi|15789150|gb|AY037718.1|	Uncultured earthworm cast bacter...	868
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gi|15789136|gb|AY037704.1|	Uncultured earthworm cast bacter...	868
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gi|15789132|gb|AY037700.1|	Uncultured earthworm cast bacter...	868
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gi|15789130|gb|AY037698.1|	Uncultured earthworm cast bacter...	868
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gi|15789121|gb|AY037689.1|	Uncultured earthworm cast bacter...	868
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gi|15789108|gb|AY037676.1|	Uncultured earthworm cast bacter...	868
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gi|15212589|gb|AY038710.1|	Uncultured proteobacterium clone...	868
0.0		
gi|15212588|gb|AY038715.1|	Uncultured proteobacterium clone...	868
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gi|14289550|gb|AF376232.1|AF376232	Uncultured bacterium ckn...	868
0.0		
gi|45477492|gb|AY510014.1|	Pseudomonas sp. LCY16 16S riboso...	868
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gi|45477491|gb|AY510013.1|	Pseudomonas sp. LCY11 16S riboso...	868
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gi|7025466|gb|AF228367.1|AF228367	Pseudomonas fluorescens b...	868
0.0		
gi|3309635|gb|AF074384.1|AF074384	Pseudomonas gessardii 16S...	868
0.0		
gi|3047379|gb|AF057645.1|AF057645	Pseudomonas libaniensis 1...	868
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gi|27753488|emb|AJ536421.1|PSP536421	Pseudomonas sp. 7:3 pa...	868
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gi|1850401|gb|U85869.1|PSU85869	Pseudomonas sp. IC038 16S r...	868
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gi|40737922|gb|AY439233.1|	Pseudomonas sp. GIC22 16S riboso...	868
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gi|37784581|gb|AY209180.1|	Pseudomonas sp. ED105-1 16S ribo...	868
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gi|2209052|dbj|AB004760.1|	Unidentified bacteria gene for 1...	868
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gi|22217941|emb|AJ244725.1|PCF244725	Pseudomonas cf. synxan...	868
0.0		
gi|32351740|gb|AY308052.1|	Pseudomonas sp. AZ22R3 16S ribos...	862
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gi|15789128|gb|AY037696.1|	Uncultured earthworm cast bacter...	862
0.0		
gi|28269052|gb|AF468404.1|	Arctic sea ice bacterium ARK9971...	860
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gi|37359288|gb|AY151820.1|	Pseudomonas sp. PM2 16S ribosoma...	860
0.0		

gi|30409077|gb|AY277894.1| Pseudomonas reactans 16S ribosom... 860
0.0
gi|22476474|gb|AY135932.1| Uncultured bacterium clone SG2-1... 860
0.0
gi|21726938|emb|AJ492830.1|PFU492830 Pseudomonas fulgida pa... 860
0.0
gi|15183115|gb|AY039441.1| Earthworm cast bacterium C27M1 1... 860
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gi|15789176|gb|AY037744.1| Uncultured earthworm cast bacter... 860
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gi|7920706|gb|AF255337.1| Pseudomonas reactans 16S ribosoma... 860
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gi|18150370|gb|AF388208.1|AF388208 Pseudomonas sp. NZ064 16... 860
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gi|17220747|gb|AY014829.1| Pseudomonas sp. NZ124 16S riboso... 860
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gi|17220745|gb|AY014827.1| Pseudomonas sp. NZ113 16S riboso... 860
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0.0
gi|17220741|gb|AY014823.1| Pseudomonas sp. NZ106 16S riboso... 860
0.0
gi|17220733|gb|AY014815.1| Pseudomonas sp. NZ065 16S riboso... 860
0.0
gi|27530751|dbj|AB074632.1| Uncultured gamma proteobacteriu... 860
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gi|3142690|gb|AF064461.1|AF064461 Pseudomonas cedrella 16S ... 860
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gi|44935448|gb|AY546087.1| Pseudomonas sp. DG01 16S ribosom... 860
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gi|13357195|gb|AF326356.1|AF326356 Pseudomonas sp. G2 16S r... 860
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gi|1850400|gb|U85868.1|PSU85868 Pseudomonas sp. ACAM213 16S... 860
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gi|10567509|gb|AF094738.1|AF094738 Pseudomonas putida strai... 860
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gi|10567500|gb|AF094729.1|AF094729 Pseudomonas fluorescens ... 860
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gi|9623379|gb|AF267911.1|AF267911 Pseudomonas synxantha 16S... 860
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gi|40019085|gb|AY486386.1| Pseudomonas synxantha strain AU2... 860
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gi|4220625|dbj|AB022820.1| Obligately oligotrophic bacteria... 860
0.0

gi|4200022|dbj|AB022298.1| Obligately oligotrophic bacteria... 860
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gi|3347670|dbj|AB016428.1| Pseudomonas putida gene for 16S ... 860
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gi|19568801|gb|AF479375.1| Glacial ice bacterium M3C4.1K-B3... 858
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gi|15789193|gb|AY037761.1| Uncultured earthworm cast bacter... 858
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gi|12056353|emb|AJ296562.1|UBA296562 Uncultured bacterium G... 858
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gi|28269039|gb|AF468391.1| Arctic sea ice bacterium ARK1006... 854
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gi|32351739|gb|AY308051.1| Pseudomonas sp. AZ22R2 16S ribos... 854
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gi|15789178|gb|AY037746.1| Uncultured earthworm cast bacter... 854
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gi|13940352|emb|AJ291844.1|PSP291844 Pseudomonas sp. IrT-R9... 854
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gi|33391994|gb|AY345369.1| Bacterium L3_Loihi 16S ribosomal... 852
0.0
gi|15789182|gb|AY037750.1| Uncultured earthworm cast bacter... 852
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gi|17864939|gb|AF448263.1|AF448263 Uncultured bacterium clo... 852
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gi|8980512|emb|AJ295374.1|UBA295374 Rape rhizosphere bacter... 852
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gi|15183076|gb|AY039425.1| Soil bacterium NS9 16S ribosomal... 850
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gi|40737953|gb|AY439264.1| Pseudomonas sp. GICR15be 16S rib... 850
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gi|13235572|emb|AJ301568.1|UGA301568 Uncultured gamma prote... 848
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gi|18092524|gb|AF451270.1| Pseudomonas sp. E102 16S ribosom... 844
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gi|13940351|emb|AJ291841.1|PSP291841 Pseudomonas sp. IrT-R5... 844
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gi|14701887|gb|AF389432.1|AF389432 Uncultured bacterium clo... 844
0.0
gi|4433345|dbj|D84025.1|PSEIAM24 Pseudomonas synxantha 16S ... 844
0.0
gi|20152172|gb|AY092072.1| Pseudomonas fluorescens strain 3... 842
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gi|15212590|gb|AY038717.1| Uncultured proteobacterium clone... 841
0.0
gi|13940350|emb|AJ291840.1|PSP291840 Pseudomonas sp. IrT-R5... 841
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0.0
gi|30527214|gb|AY275485.1| Pseudomonas fulgida isolate MSB2... 839
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gi|15637508|gb|AF408901.1|AF408901 Pseudomonas sp. NZCH11 1... 839
0.0

gi|15637506|gb|AF408899.1|AF408899 Pseudomonas sp. NZHA6 16... 839
0.0
gi|15637505|gb|AF408898.1|AF408898 Pseudomonas sp. NZHA8 16... 839
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gi|15637504|gb|AF408897.1|AF408897 Pseudomonas sp. NZHA3 16... 839
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gi|15637496|gb|AF408889.1|AF408889 Pseudomonas sp. NZHA1 16... 839
0.0
gi|45477490|gb|AY510012.1| Pseudomonas sp. LCY16 16S riboso... 839
0.0
gi|17864951|gb|AF448275.1|AF448275 Uncultured bacterium clo... 837
0.0
gi|5733742|gb|AF170731.1|AF170731 Pseudomonas sp. QSSC1-9 1... 837
0.0
gi|4433332|dbj|D84009.1|PSEIAM08 Pseudomonas azotoformans 1... 837
0.0
gi|14701905|gb|AF389450.1|AF389450 Uncultured bacterium clo... 833
0.0
gi|4433337|dbj|D84017.1|PSEIAM16 Pseudomonas mucidolens 16S... 833
0.0
gi|33111948|emb|AJ576245.1|PPU576245 Pseudomonas putida par... 831
0.0
gi|33391987|gb|AY345362.1| Bacterium Ph16 16S ribosomal RNA... 829
0.0
gi|19309781|emb|AJ314687.1|UBA314687 Uncultured bacterium p... 829
0.0
gi|15183110|gb|AY039437.1| Soil bacterium S94D1 16S ribosom... 825
0.0
gi|32351743|gb|AY308055.1| Pseudomonas reactans 16S ribosom... 821
0.0
gi|20336447|gb|AY093461.1| Uncultured bacterium clone MB-A2... 821
0.0
gi|26225096|gb|AY167969.1| Swine manure bacterium 37-8 16S ... 821
0.0

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ABSTRACT

In present study, the selected low and high input wheat experimental fields which were low in available N and P were studied. Diazotrophs are known to improve yield of wheat plants through various mechanisms. *In situ*, *nif*H profiles of wheat rhizosphere of selected fields showed time/input dependent changes in *nif*H community. Comparative profiles showed that SLH rhizosphere had much more different *nif*H community and plain vs raised bed practice had some specific N_2- fixing community irrespective of input systems. To improve the plant growth, 44 isolates were recovered from wheat rhizosphere on Jensen's medium and screened for their plant growth potential. A total of 14 isolates were selected on the basis of microcosm experiment. Plants from selected bacterial treatments were analyzed for shoot N & P isolates for nitrogenase activity. Only one isolates SLL55 was nitrogenase positive structurally (*nif* H amplification) and functionally (ARA). Correlation data between growth parameters, total N, P & soil enzymes suggests that bacterial isolates improved plant growth through plant growth promotory properties. The selected isolates were identified with FAMES. Only 2 isolates SLL 22 and SHH 36 were found to have significant similarity index of 0.522 & 0.656 with *Bacillus amyloliquefaciens* and *Bacillus*

subtilis. Partial 16S rDNA sequencing of SLL55 showed maximum similarity (89.01%) with UNCLASSIFIED/UNALIGNED PSU85869 0.890135 *Pseudomonas* sp. IC038 16S ribosomal RNA gene (partial sequence) & U85869 0.8901357 *Pseudomonas* IC038str.IC038 of PS.AZOTOFORMANS_SUBGROUP through Ribsomal Database Project search and 86.8% with many *Pseudomonas* sp. through BLAST Search.

Isolate SLL55 is found to be new for nitrogen fixation.

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