DIVERSITY OF NITROGENASE (*nif*H) GENE POOL IN YAM PLANTATION FIELD OF C4 AGRICULTURAL LAND, UTAR PERAK CAMPUS

By

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ABSTRACT

DIVERSITY OF NITROGENASE (*nif*H) GENE POOL

IN YAM PLANTATION FIELD OF C4 AGRICULTURAL LAND,

UTAR PERAK CAMPUS

Biological nitrogen fixation is a major route for the conversion of atmospheric nitrogen to ammonia, a process mediated exclusively by prokaryotic microorganisms known as diazotrophs. In this study, free-living, nitrogen-fixing microbial population in agricultural soil of yam plantation field in C4 agricultural land in Universiti Tunku Abdul Rahman was analyzed using culture-independent methodology. The complexity of *nif*H gene pool was assessed by performing nested PCR on the extracted total microbial DNA. Amplified PCR products were cloned into pGEM-T Easy Vector and transformed into competent *Escherichia coli* JM109 bacterial cells. Recombinant clones were assessed using colony PCR and alkaline lysis. A total of twenty-three recombinant plasmids were purified and their nucleotide sequences determined. Majority of the homologous *nif*H sequences were shown to have high similarity to partial *nif*H gene from unculturable microorganisms. Many of the *nif*H sequences also demonstrated high percentage of identity with α- and δ-Proteobacteria, mostly represented by diazotrophs with close resemblance with *Bradyrhizobium japonicum* and

Anaeromyxobacter sp. Fw109-5, respectively. The phylogenetic analysis based on the translated *nif*H sequences demonstrated that twenty recombinant clones obtained in this study were categorized into δ-Proteobacteria (Cluster I). The other NifH sequences were clustered within Cyanobacteira and Gram-positive bacteria with both high and low GC content. This study demonstrated the predominance of δ-Proteobacteria in the studied soil sampling site from a broad range of free-living diazotrophs.

ACKNOWLEDGEMENT

Apart from the efforts of myself, the success of this project depends largely on the encouragement and guidelines of many others. I take this opportunity to express my gratitude to the people who have been instrumental in the successful completion of this project. I would like to express my sincere gratitude to my supervisor, Assistant Professor Dr Choo Quok Cheong for the continuous support of my research project with his patience, motivation, enthusiasm, and immense knowledge. Besides my supervisor, I would like to thank the rest of my fellow labmates: Chen Zi Xin, Kan Yin Ying, Joanne Chan and Ooi Shao Yin in promoting a stimulating and welcoming academic and social environment. Last but not the least, I would like to thank my family: my parents Liew Chee Khuan and Chang Lee Kiang for giving birth to me at the first place and supporting me spiritually throughout my life.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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LIEW BOON YEE

APPROVAL SHEET

This project report entitled "DIVERSITY OF NITROGENASE (*nif*H) GENE POOL IN YAM PLANTATION FIELD OF C4 AGRICULTURAL LAND, UTAR PERAK CAMPUS" was prepared by LIEW BOON YEE and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biotechnology at Universiti Tunku Abdul Rahman.

Approved by:

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PERMISSION SHEET

It is hereby certified that LIEW BOON YEE (ID No: 11ADB01675) has completed this final year project entitled "DIVERSITY OF NITROGENASE (*nif*H) GENE POOL IN YAM PLANTATION FIELD OF C4 AGRICULTURAL LAND, UTAR PERAK CAMPUS" under the supervision of Dr Choo Quok Cheong (Supervisor) from the Department of Biological Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(LIEW BOON YEE)

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LIST OF ABBREVIATIONS

A	Absorbance
ADP	Adenosine diphosphate
anf	Alternative nitrogen fixation gene
ARISA	Automated ribosomal intergenic spacer analysis
ATP	Adenosine triphosphate
Blast	Basic Local Alignment Search Tool
bp	Base pair
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
FAOSTAT	Food and Agriculture Organization of the United Nations
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
ExPaSy	Expert Protein Analysis System
FLT	fluorescently labeled terminal
IITA	International Institute of Tropical Agriculture
IPTG	Isopropyl-β-D-thiogalactopyranoside
MCS	Multiple cloning site
MEGA	Molecular Evolutionary Genetics Analysis
MoFe	Molybdenum iron

N_2	Nitrogen
NH_3	Ammonia
NCBI	National Center for Biotechnology Information
nif	Nitrogen fixation gene
NifH	Translated *nifH* sequence
OD	Optical density
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
SOM	Soil organic matter
SON	Soil organic nitrogen
TGGE	Temperature gradient gel electrophoresis
UTAR	University Tunku Abdul Rahman
vnf	Vanadium nitrogenase gene
WMO	World Meteorological Organization
X-Gal	5-bromo-4-chloro-3-indolyl- β-D-thiogalactopyranoside

CHAPTER 1

INTRODUCTION

Yam is the third most important agricultural crop in tropical regions of West Africa, Central America, the Caribbean, Pacific Islands and Southeast Asia (Srivastava, et al., 2009). Agricultural soils are heterogeneous environments that affect soil microbial growth, causing evolution of diverse soil bacterial species. The structure and diversity of soil microbial communities play a critical role in the function and long-term sustainability of soils (Kennedy, 1999). However, forest conversion to cropping land typically resulted in declining soil organic matter which is related to decrease in microbial biomass and activity (Islam and Weil, 2000).

In agricultural field, plants require relatively high levels of nitrogen for the production of biomass or yield. Although an estimated 3.9×10^5 metric tons of nitrogen is available from the atmosphere, nitrogen is the chemical element in limited supply relative to the needs of organisms. Molecular nitrogen is inert and useless for most living organisms and their involvement in atmospheric chemical reaction is limited (Ogunseitan, 2005). Four microbial processes dominate the biogeochemical cycling of nitrogen, which are nitrogen fixation, nitrification, denitrification and nitrogen mineralization.

Nitrogen fixation is a biological process where atmospheric nitrogen is converted to ammonia in the biosphere. This renders their essential role in maintaining soil fertility and agricultural production. The process of biological nitrogen fixation is carried out by prokaryotes, providing Earth's ecosystems with about 200 million tons of nitrogen per year (Rascio and Rocca, 2008). Besides, this process offers advantages over the use of expensive ammonium-based fertilizer nitrogen. They include higher efficiency in the utilization of nitrogen by the plant, the minimization of nitrogen leaching, and the reduction of soil and water contamination (Peoples, Herridge and Ladha, 1995).

Biological nitrogen fixation is performed by phylogenetically diverse groups of prokaryotic microorganisms belonging to the domains of Bacteria and Archea (Eady, 1991). This process is carried out by symbiotic bacteria belonging to few phylogenetic groups. Non-symbiotic, free-living nitrogen-fixing microbes that cover a wider range of phylogenetic groups of microorganisms also take part in nitrogen fixation process independent of other microorganisms. These free-living diazotrophs exert a significant impact on agricultural sector by fixing nitrogen elements, yet quantification of their potential has not been confirmed.

Nitrogen-fixing diazotrophs possess the multi-enzyme complex nitrogenase, which is composed of two metalloproteins: the nitrogenase iron (Fe) protein or nitrogenase reductase and the nitrogenase molybdenum-iron (Mo-Fe) protein or

dinitrogenase. As a functional gene marker, *nif*H gene encodes for Fe protein has the advantage for providing evidence for potential nitrogen fixation in soil (Young, 1992). The *nif*H gene had largely been studied by culture-independent approaches which provided a more complete picture of the diazotrophic community than culture-based approaches (Su, et al., 2006). This is due to the fact that majority of bacteria are not readily cultured using standard laboratory media, and thus culture-independent techniques are required to access the genetic diversity of most environmental samples (Giovannoni, et al., 1990).

In this study, the phylogenetic diversity of *nif*H gene pool revealing diazotrophic community of agricultural soil was accessed using culture-independent methodology. To gain insight to the diversity of *nif*H genes in agricultural soil, *nif*H genes within the soil of yam plantation area was investigated. The objectives of this study include:

- To assess the genetic diversity of culture-independent diazotrophs in agricultural soil sample using molecular approach
- To identify novel *nif*H homologous sequences from non-culturable diazotrophs
- To examine distribution of soil nitrogen-fixing diazotrophs using molecular phylogenetic analysis

Chapter 2

LITERATURE REVIEW

2.1 Properties of Agricultural Soil

Soil is a complex, loose, terrestrial surface material containing three components. They include weathered fragments of parent rock in various stages of breakdown, water, and minerals and organic compounds resulting from the decay of dead plants and animals (Stiling, 2012). Most of soil-forming processes such as litter fall tend to act from the top down. Soil develops a vertical structure known as soil profile composing five horizons (O, A, E, B and C). Soil horizons are set apart from other soil layers by differences in physical and chemical composition or organic structure (Strahler and Strahler, 1992).

Agricultural soils are heterogeneous environments that affect soil microbial growth, rendering soil bacteria diversity. An ideal agricultural soils are balanced in contributions from mineral components (sand: 0.05–2 mm, silt: 0.002–0.05 mm, clay: <0.002 mm), soil organic matter (SOM), air, and water. The balanced contributions of these components allowed water retention and drainage, oxygen in the root zone, nutrients to facilitate crop growth and provide physical support for plants (Parikh and James, 2012). However, the distribution of these soil

components in a particular soil is influenced by the five factors for soil formation: parent material, time, climate, organisms, and topography (Jenny, 1941).

Soil pH is often a major factor regulating organic matter turnover and inorganic nitrogen production in agricultural soils (Kemmitta, et al., 2006). It regulates soil nutrient bioavailability, vegetation community structure, plant primary productivity and a range of soil processes including soil microbial community structure and activity (Robson, 1989). However, soil pH needs to be maintained within a range of 6.5 to 7.0 for optimization of plants nutrient uptake (Howell, 1997).

Nitrogen mineralized from soil organic nitrogen (SON) makes important contribution to nitrogen required by agricultural crops. This may account for more than 50% of total crop nitrogen uptake (Stevens, Hoeft and Mulvaney, 2005; Nyiraneza, et al., 2010). The changes of SOM and SON are dependent on farming practices such as fertilization, composting and incorporation of crop residues, crop rotations and soil utilization, with these factors usually interacting with one another over a long period time (Huang, et al., 2007).

2.2 Global Yam Plantation

Yam (*Dioscorea* spp.) is a monocotyledon tropical plant which produces underground or aerial tubers. They are mainly cultivated on a large scale in West Africa, the Caribbean and Southeast Asia as the major source of calories for the peoples of the tropics (Ikediobi and Igboanusi, 1983). The most economically important yam species are *Dioscorea rotundata* (white yam), *D. cayenensis* (yellow yam), *D. alata* (water yam), *D. opposite* (Chinese yam), *D. bulbifera* (air potato), *D. esculenta* (lesser yam), *D. dumetorum* (bitter yam) and *D. trifida* (cush-cush yam).

Yams are farmed on about 5 million hectares in about 47 countries in tropical and subtropical regions of the world (IITA, 2009). The world average annual yield of yams was 12.86 tonnes per hectare, according to data published by Food and Agriculture Organization of the United Nations (FAOSTAT) in 2012. Based on FAOSTAT figures in 2012, there was 38 million metric tons of yam production in Nigeria, which served as the largest yam producer so far.

Crop management plays an important role in procuring high yields in tropical tuber crops (Lebot, 2009). Yam cultivation is generally limited by high costs of planting material and labour, decreasing soil fertility, inadequate yield potential of varieties, and increasing levels of field, storage pests and diseases associated with intensification of cultivation. As yams are high-nutrient-demanding species, the

major constraint for enhancing yam productivity is low soil fertility, both in terms of macronutrient and micronutrient deficiency (O'Sullivan and Ernest, 2007). Based on the study of Diby, et al. (2011), nutrient use efficiencies and agronomic efficiency indicated the impact of nitrogen source in promoting yields of yams especially under non-fertilized conditions.

2.3 Nitrogen: An Essential Element of Earth System

Nitrogen commonly exists in the form of nitrogen (N_2) as a colourless, odourless and inert gas that makes up 78% of the atmosphere (Kubiszewski, 2010). Other reservoirs of nitrogen compounds are within soils and the sediments of lakes, rivers, and oceans, surface water, groundwater and biomass of living organisms (Reece, et al., 2011).

All forms of life require nitrogen for the synthesis of proteins and other important biochemicals. Thus, nitrogen is often the limiting nutrient for plant and microbial growth in soils, particularly in agricultural production. In natural systems, nitrogen source for plant growth comes from the soil, rainfall or other atmospheric deposition, or through biological nitrogen fixation (Sylvia, et al., 2005).

Nitrogen cycle consists of a series of oxidation-reduction reaction of nitrogen-containing compound. Nitrogen gas in the atmosphere is converted into ammonia through the biological nitrogen fixation process. Plants take up the nitrates and ammonia by assimilation and conversion into organic nitrogen. When living organisms excrete waste, die and decompose, organic nitrogen is broken down and converted into ammonia by saprobiotic bacteria through ammonification process. Some ammonia is consumed by plants and the remaining in the soil is converted into nitrates by nitrifying bacteria. These nitrates were then stored in humus or leached into groundwater or converted into nitrogen gas and returned to the atmosphere through denitrification process (Gordon, 2005).

Global nitrogen cycle has been significantly altered over the past century due to consequence of anthropogenic inputs such as such as agricultural or industrial processes. Excess nitrogen in the environment is associated with many large-scale environmental concerns, including eutrophication of surface waters, toxic algae blooms, hypoxia, acid rain, nitrogen saturation in forests, and global warming (Meunier, 2013). Global atmospheric nitrous oxide mole fractions have increased from pre-industrial value of 270nmolmol^{-1} to 325nmolmol^{-1} in 2012 (WMO, 2013). Based on Environmental Protection Agency (EPA) data, agricultural soil contributed 8% higher gas emission in year 2011 than 1990 in United States.

2.4 Biological Nitrogen Fixation

Biological transformation of nitrogen compound in biosphere is highly dependent on the activities of diverse assemblages of microorganisms, such as bacteria, archaea, and fungi (Bernhard, 2012). Nitrogen gas is transformed to its biologically available form via nitrogen fixation by bacteria residing in soils and aquatic environments, or living symbiotically with plants. The global quantity of nitrogen fixed annually by natural ecosystems is estimated to be approximately 100 teragram (Tg) per year for terrestrial ecosystems (Chapin, Matson and Vitousek, 2011).

Nitrogen gas is reduced to ammonia (NH_3) with biological nitrogen fixation process under aerobic conditions, where nitrogen-fixing species oxidize sugar to acquire energy. Despite of enormous amount of nitrogen gas in the atmosphere, oxidation of nitrogen gas to nitrate is non-favorable in nature. This is because the triple bond between nitrogen atoms is very stable, requiring tremendous energy to break it apart, rendering nitrogen fixation an “energy expensive” process (Coyne, 1999). Biological nitrogen fixation is considered to increase soil acidity indirectly, after fixed nitrogen is transformed by ammonification and nitrification (Paul and Clark, 1996).

Nitrogen-fixing systems possess four fundamental requirements, which include enzyme nitrogenase, strong reductant such as reduced ferredoxin, ATP and oxygen-free conditions (Garret and Grisham, 2010). Three characteristics composing bacterial nitrogen fixation are large population of microorganisms consistent with observed nitrogen fixation rates, a rapid cell formation indicating nitrogen fixation is linked to growth and lastly, nitrogen must be atmospheric (Havelka, Boyle and Hardy, 1982).

2.5 Nitrogenase: A Key Enzyme Involved in Biological Nitrogen Fixation

Biological reduction of nitrogen to ammonia is catalyzed by highly conserved nitrogenase complex which is present in the cytoplasm of the bacteroids. Four classes of nitrogenase have been identified, and they include Mo-nitrogenase, V-nitrogenase, Fe-only nitrogenase, and nitrogenase from *Streptomyces thermoautotrophicus* (Hu and Ribbe, 2011). Out of these nitrogenases, Mo-nitrogenase is the best studied class of nitrogenase.

Mo-nitrogenase enzyme consists of two proteins: MoFe protein serves as dinitrogenase, a $\alpha_2\beta_2$ tetramer of 230 kDa, and Fe protein served as dinitrogenase reductase, a 63 kDa γ_2 homodimer (Duyvis, Wassink and Haaker, 1996). MoFe prtein contains P-cluster (Fe_8S_7) that is located at the α/β-subunit interface and a FeMo cofactor (FeMoco) that is buried within the α-subunit (Lee, Hu and Ribbe, 2009). However, Fe protein contains a single Fe_4S_4 cluster that creates

α_2-homodimer bridge between two identical subunits (Buchanan, Gruissem and Jones, 2009).

Nitrogenase catalysis involves repeated association and dissociation between Fe and MoFe proteins. MoFe protein provides a catalytic center for nitrogen reduction, while Fe protein functions as an ATP-dependent reductase for MoFe protein by providing high-energy electrons (Nomata, et al., 2006). Electrons are finally transferred to nitrogen at the catalytic site of MoFe protein, resulting in the formation of ammonia molecules.

The overall stochiometry of biological nitrogen fixation using nitrogenase can be represented by the following equation.

$$N_2 + 8H + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16\ P_i$$

According to the above chemical equation, two ATP are required for each electron transferred from dinitrogenase reductase to dinitrogenase. Thus, a total of 16 molecules of ATP are needed. Burris and Roberts (1993) postulated that probably 20-30 ATP are required under natural conditions as the process is less significant than observed under optimum laboratory conditions.

2.6 Free-living Diazotrophs in Soil Environment

Natural ecosystems rely on the biological conversion of atmospheric nitrogen for plant and microbial growth in the absence of modern fertilizers or animal wastes. Microbe uses atmospheric nitrogen as their source of nitrogen for growth is called diazotroph (Sylvia, et al., 2003). Distribution of diazotrophs in soil may be influenced by soil texture, nitrogen levels in the soil and the vegetation of the environment as well as a host of other physical, chemical and biological factors (Bardgett, et al., 1999).

Nitrogen-fixing diazotrophs are categorized as obligate anaerobes (e.g. *Clostridium pasteurianum*), facultative anaerobes (e.g. *Klebsiella pneumonia*), photosynthetic bacteria (e.g. *Rhodobacter capsulatus*), cyanobacteria (e.g. A*nabena*), obligate aerobes (e.g. *Azotobacter vinelandii*) and methanogens (e.g. *Methanosarcina barkeri*). Diazotrophs are widely distributed among the Prokarya and Archea with more than 100 species reported (Rosch, Mergel and Bothe, 2002). They include members of α-, β-, γ-, and δ-Proteobacteria, Firmicutes, Cyanobacteria (blue-green algae), and Archaea with most of them are uncultivated (Dixon and Kahn, 2004). However, it has been shown that heterotrophic diazotrophs are the most common nitrogen-fixing microbes (Zehr, et al., 2003).

Although the majority of nitrogen fixation occurs through symbiotic association of bacteria with the roots of leguminous plants, inputs from free-living diazotrophs are an important contributor to fertility in many environments (Ropper, Marschke and Smith, 1989). However, Devevre and Horwath (2000) suggested that the larger and sustained microbial biomass generally found under anaerobic compared to aerobic conditions may immobilize more nitrogen. The ecological impact of free-living diazotroph activity is disputable with estimates of activity varying widely from 0 to 60 kg N ha^{-1} yr^{-1} in cropland and natural ecosystems (Day, et al., 1975; Cleveland, et al., 1999, Gupta, Roper and Roget, 2006).

2.7 Methods to Assess Soil Microbial Diversity

2.7.1 Culture-Dependent Method

Culture-based techniques are useful in understanding the physiological potential of isolated microorganisms as they are easy, fast and inexpensive and provide information on the viable, heterotrophic component of the population (Kirk, et al., 2004). Traditional analysis of soil microbial communities relied on culturing techniques using a variety of culture media in order to maximize the recovery of different microbial species (Hill, et al., 2000). Although there are modifications of culture media to maximize the recovery of diverse microbial groups from soils, it was estimated that less than 0.1% of the microorganisms found in typical agriculture soils are culturable (Atlas and Bartha, 1998).

Developing culture-dependent protocols that identify unique bacterial operational taxonomic units (OTUs) is an important research topic in soil bacterial ecology. Ellis, et al. (2003) reported that culturable bacteria are important to soil ecosystem functions because of their higher total biomass and metabolic activity. Therefore, culturable bacteria may provide an ecologically relevant complement to culture-independent community characterizations and serve as responsive indicators of physical, chemical and biological changes in the soil environment (Edenborn and Sexstone, 2007).

Culture-dependent methods demonstrated some disadvantages where microorganisms can only be cultivated when their metabolic and physiological requirements can be reproduced *in vitro*. Problems with using culturing for community analysis arise from the fact that an artificial homogenous medium typically allows growth of only a small fraction of the organisms (Carraro, et al., 2011). Furthermore, enumerating bacteria from traditional microbial culturing techniques may produce inaccurate results under investigation of complex microbial communities (Besnard, Federighi and Cappelier, 2000).

2.7.2 Culture-Independent Method

The unidentified populations of non-culturable bacteria are often neglected in routine culture-dependent analyses (Janssen, et al., 2002). Nucleic acid-based molecular methods, especially in polymerase chain reaction (PCR) have revolutionized the study of soil microbial ecology which is previously constrained by its inability to culture the majority of cells detected using direct microscopic observation (Hirsch, Mauchline and Clarka, 2010).

There are mainly two types of molecular analysis for microbial communities study using DNA extracted from soil. The partial community DNA analysis determines only parts of the information by focusing on genome sequences targeted and amplified by PCR, whereas whole community DNA analysis focuses on all genetic information contained in extracted soil microbial DNA (Ranjard, Poly and Nazaret, 2000).

Recently, novel genes encoding important catabolic enzymes were isolated from non-culturable microorganisms using metagenomic approaches (Lee, et al., 2004). Culture-independent methods involve different molecular techniques and they include temperature gradient gel electrophoresis (TGGE), restriction fragment length polymorphism (RFLP) and clone library analysis.

An indirect approach for assessing potential biological nitrogen fixation function is the characterization of the diazotroph populations by molecular methods due to present of non-culturable diazotrophs (Roszak and Colwell, 1987). Culture-independent strategies have predominately focused on using the *nif*H gene to study diazotroph diversity. The amplification of *nif*H gene from DNA samples with a variety of primers targeting different *nif*H gene populations were investigated (Rapley, 2006).

2.8 Molecular Approaches in *nif*H gene study

2.8.1 Nested PCR Amplification of *nif*H gene

The *nif*H gene is one of the oldest existing functional genes in the history of gene evolution. The phylogeny among bacteria based on this gene is reportedly to be in agreement with that inferred 16S rRNA gene, demonstrating that *nif*H could be considered a good marker for diazotrophic community structure (Borneman, et al., 1996; Hennecke, et al., 1985; Ueda, et al., 1995; Zehr, et al., 1995). Nowadays, *nif*H gene has been used for the determination of the diversity and characterization of nitrogen fixation genes in natural soil microbial communities (Ben-Porath and Zehr, 1994).

Being the most conserved gene in the *nif* operon, the degeneracy of the genetic code has resulted in significant variability *nif*H sequences at the nucleotide level (Zehr and McReynolds, 1989). For effective 'universal' amplification, the primers must be highly degenerate in order to successfully capture the full diversity of the diazotrophic community (Widmer, et al., 1999). The use of degenerate primers reduces the number of mismatches between template and primer sequences and in effect reduces bias. PCR bias using 16S as a model gene have shown differences in GC content to cause bias but no similar bias has been detected when using the *nif*H primers *nif*Hl and *nif*H2 (PoIz and Cavanaugh, 1998; Tan, Reinhold-Hurek and Hurek, 2003; Diallo, Reinhold-Hurek and Hurek, 2008).

Nested PCR can enhance the sensitivity and specificity of PCR amplification by allowing first round of amplification with less stringent external primers, followed by a second round with internal primers designed to recognize specific regions within the initial amplicon (Haqqi, et al., 1988). In such way, non-specific annealing of primer is minimized. This is particularly useful when organisms or genes are detected in environmental samples with exact specificity or uniqueness of the primers is unknown (Steffan and Atlas, 1988). Application of *nif*H-specific nested PCR to soil microbial DNA also effectively diluted putative PCR inhibitors to acceptable levels and allowed successful amplification (Rosado, et al., 1998).

2.8.2 Molecular Phylogenetic Analysis of *nif*H Gene

Molecular phylogenetics applies a combination of molecular and statistical techniques to infer evolutionary relationships among organisms or genes (Dowell, 2008). Gene duplication and recruitment have probably occurred several times during the evolution of nitrogenase, the current distribution of which has been influenced by gene loss and horizontal gene transfer (Raymond, et al., 2004). Due to the enormous phylogenetic differences among nitrogen fixers, *nif*H gene sequences have diverged considerably and DNA sequences encoding conserved protein regions may differ due to codon redundancy for most amino acids (Zehr and McReynolds, 1989).

Different phylogenetic clusters of *nif*H gene were shown and they include Cluster I, II, III and IV. Cluster I includes standard molybdenum nitrogenases from cyanobacteria, α-, β- and γ-proteobacteria and γ-proteobacterial *vnf*H. Cluster II involves methanogen nitrogenases and bacterial *anf*H. Cluster III includes nitrogenases from diverse anaerobic bacteria such as clostridia (low GC, gram-positive bacteria) and sulfate reducers (δ-proteobacteria), which is an example of *nif*H phylogeny deviating from the 16S rRNA phylogeny. Cluster IV however, includes divergent nitrogenases from archea (Mehta, Butterfield and Baross, 2003). Figure 2.1 illustrates a phylogeny of *nif*H gene based on sequence analysis of clone.

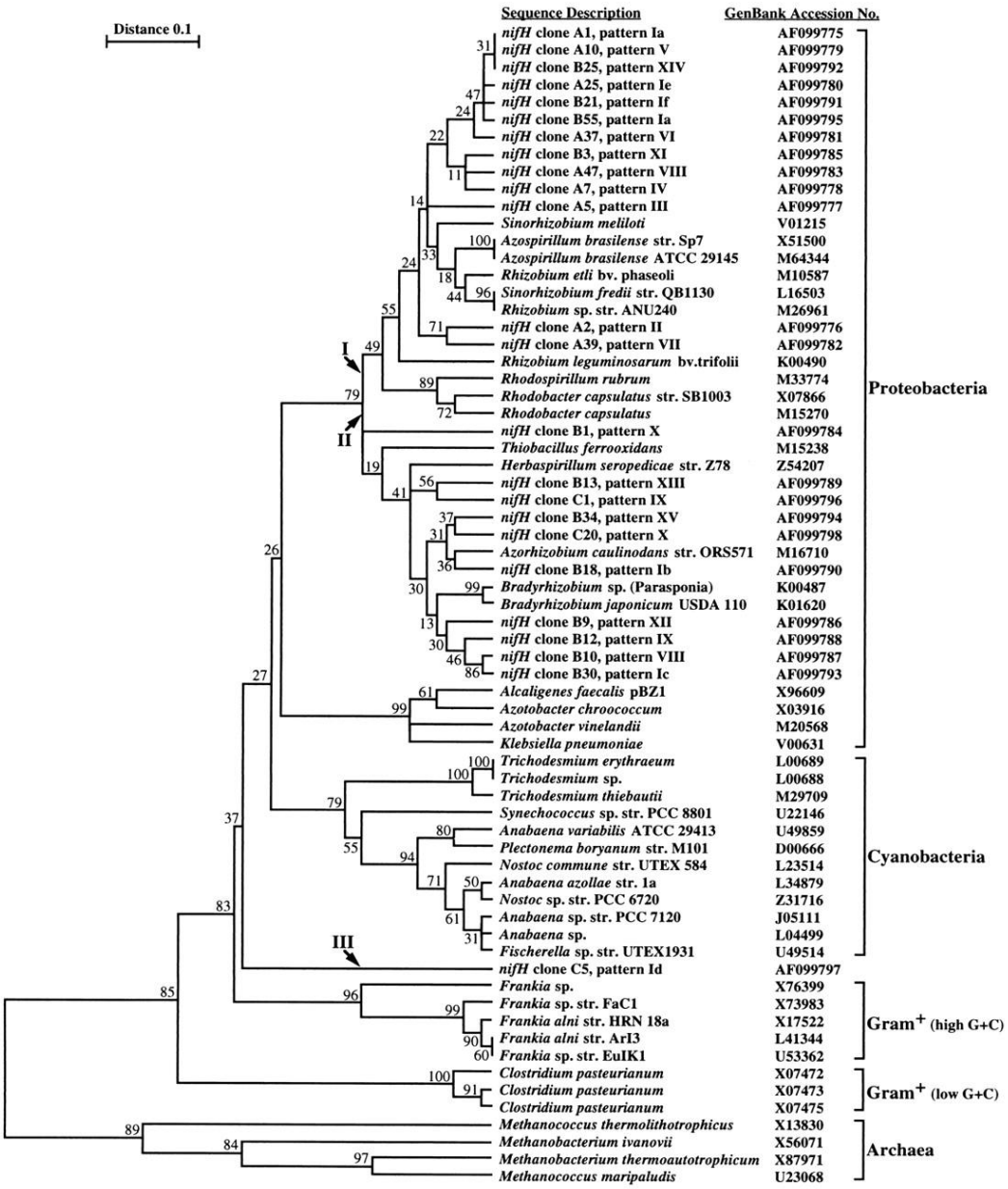

Figure 2.1: Representative of NifH phylogenetic tree. This contructed *nif*H phylogeny is adapted from the study of Widmer et al. (1999) regarding the analysis of *nif*H gene pool complexity in soil and litter in a Douglas fir forest. An alignment of a 110-amino-acid portion of all 67 sequences was used for phylogenetic analysis with TREECON. Pairwise protein sequence distances and unweighted pair group with mathematical average cluster analysis of 100 bootstrap samplings were used to determine the phylogenetic relationships of the 24 new *nif*H sequences and 43 known sequences retrieved from GenBank. Each of the *nif*H sequences determined in Widmer's study is identified by the clone designation (e.g., clone A1) and the corresponding HaeIII RFLP pattern (e.g., pattern Ia). Arrows I, II, and III indicate the three branches where the *nif*H clones clustered.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Agricultural Soil Sample

An agricultural soil sample was chosen in this study. The soil sample obtained from a yam plantation field in C4 agricultural land, University Tunku Abdul Rahman (UTAR) Kampar, Perak.

3.1.2 Materials Used in the Study

All materials used in this study were provided by Department of Biological Science in UTAR (Perak campus). Chemical media or reagents were available from final year project lab, microbiology lab and molecular biology lab. List of essential materials and commercial kits were shown in Table 3.1 and bacterial strains and plasmids used were tabulated in Table 3.2.

Table 3.1: List of materials used and their particular sources.

Materials	Source/ Reference
6x DNA Loading Dye, Gene Ruler 100bp DNA ladder, Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Fermentas
5x Green GoTaq Flexi Buffer, GoTaq Flexi DNA Polymerase, T4 DNA Ligase 10x Buffer, T4 DNA Ligase, pGEM-T Easy Vector, Buffer H, *Eco*RI	Promega
25mM Magnesium chloride ($MgCl_2$), 10x *Taq* buffer, DNA polymerase (recombinant), T7 and SP6 promoter primer, *nif*H degenerate primer (*nif*H1, *nif*H2, *nif*H3 & *nif*H4), Agarose powder	1st Base
VC 1kb DNA Ladder	Vivantis
5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal)	EMD Chemicals Inc.
10mM dNTP-mix	Nano Helix
LB broth, LB agar, Sodium Hydroxide, Potassium Acetate, Glacial Acetate Acid	Merck KGaA
Sodium dodecyl sulfate (SDS)	Fisher Scientific
D-glucose, Tris-HCl	Amresco
Ampicillin powder	Bio Basic Inc.
Ethylenediaminetetraacetic acid (EDTA)	QREC (Asia) Sdn. Bhd.
Commercial kit used:	
PowerSoil® DNA Isolation Kit	MO BIO Laboratories Inc.
QIAquick™ Gel Extraction Kit	QIAGEN
Wizard® Plus SV Minipreps DNA Purification System	Promega

Table 3.2: Bacteria strains and plasmids used in this study.

Name	Description	Source/ Reference
Bacteria strains:		
E. coli JM109	*E. coli* JM109 is a cloning strain that is used for the generation of plasmid DNA and blue/white screening. Genotype: *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (rk–, mk+), *rel*A1, *sup*E44, Δ(*lac*-proAB), [F', *tra*D36, *pro*AB, *laqIqZ*ΔM15]	Promega
Plasmids:		
pGEM®-T Easy Vectors	Plasmid size: 3015bp High-copy-number vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region (MCS) within *lac*Z gene. The *lacZ*α encodes for α-peptide of enzyme β-galactosidase which allows blue/white selection of recombinants by insertional inactivation. Restriction site within the multiple cloning region allows release of insert by single-enzyme digestion.	Promega
pUC19	Plasmid size: 2686bp Commonly used plasmid cloning vector in E.coli with pMB1 ori, ampicillin selection marker and multiple cloning site within *lacZ*α gene. The pUC19 plasmid used as positive control in transformation process.	

3.1.3 Preparation of media and reagents

Preparation methods of media and reagents were shown in Table 3.3.

Table 3.3: Preparation methods of media and reagents.

Media/ Reagent	Preparation
LB broth	An amount of 5g of medium powder was added into Schott bottle and topped up with 200mL ddH_2O. Solution was sent for autoclaving and stored at room temperature.
LB agar	An amount of 8g of medium powder was added into Schott bottle and topped up with 400mL ddH_2O. Solution was sent for autoclaving and stored in 70 ℃ oven before pour plate process.
Ampicillin stock solution (50mg/mL)	An amount of 0.05g of ampicillin powder was dissolved in 1mL ddH_2O and filter-sterilized using 0.22 µm membrane filter. 1.5mL microcentrifuge tube contained sterile ampicillin solution was wrapped with aluminium foil and stored at -20 ℃.
IPTG solution (100mM)	An amount of 23.8mg of IPTG powder was mixed with 1mL ddH_2O and filter-sterilized into 1.5mL microcentrifuge tube wrapped with aluminium foil. Solution was stored at -20 ℃.
X-Gal solution (50mg/mL)	An amount of 50mg X-Gal powder was dissolved in 1mL DMSO solution and directly added into 1.5mL microcentrifuge tube wrapped with aluminium foil. Solution was stored at -20 ℃.
Lysozyme solution (10mg/mL)	An amount of 10mg lysozyme powder was dissolved in 1mL 10mM Tris-Cl. Solution was stored at -20 ℃.
Alkaline lysis solution I	2.5mL of 1M Tris-Cl, 2.5mL of 0.5M EDTA and 10mL of 0.5M D-glucose solution was added into Schott bottle and topped up to 100mL with ddH_2O. Solution was stored at room temperature.
Alkaline lysis solution II	1mL of 10% SDS and 0.2mL of 10M NaOH solution was added into 15mL tube and topped up to 10mL with ddH_2O. Solution was freshly prepared.
Alkaline lysis solution III	60mL of 5M potassium acetate and 11.5mL glacial acetic acid was added into Schott bottle and 100mL with ddH_2O. Solution was stored at 4 ℃.

3.2 Soil Sampling

3.2.1 Sampling Site

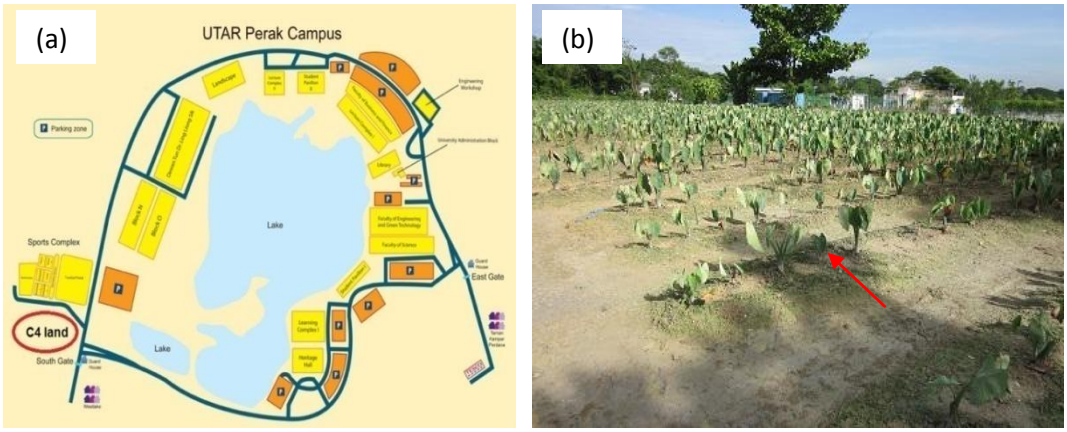

Figure 3.1: Site of soil sampling in this study. (a) Map of UTAR Perak campus, with C4 land circled in red. (b) Red arrow showed the region of soil sampling, as nitrogen fixing bacteria was mostly found in rhizosphere area.

The figure above showed the site of soil sampling. The selected sampling site was located at C4 land area of UTAR Perak Campus in Kampar with latitude of 4.336395 and longitude of 101.14203. Agriculture soil from yam plantation field was obtained in month of October 2013. As a tropical country, Malaysia has constantly high temperatures, high humidity, relatively light winds and abundant rainfall throughout the year. The average temperature of October in Kampar is about 27 ℃ with high precipitation of 341 mm.

3.2.2 Soil Sample Collection

During soil sampling, approximately 10g of agriculture soil was collected 10cm from the soil surface and transferred into 50mL collection tube using a sterile spatula. Topsoil (or Horizon A) is the upper, outermost layer of soil within 5-20cm from the top. It has the highest concentration of organic matter and microorganisms where most of the Earth's biological soil activity occurs (Thompson and Goyne, 2012). The collection tube with soil sample was then placed into ice box.

3.3 Soil Microbial DNA Isolation

Soil microbial DNA was isolated from 1.0g of agriculture soil sample using a commercially available PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc) according to manufacturer's instructions. In summary, soil sample was put into Powersoil Bead Tube which contains buffer to disperse soil particles, protect DNA and dissolve humic acid. Several solutions were added to cause cell lysis and precipitate non-DNA material. After centrifugation process, supernatant was transferred to spin filter. Through centrifugation process, soil microbial DNA was bound to silica membrane and was eluted by 100 µL ddH_2O. Isolated DNA was stored in -20 ℃ freezer. The presence of isolated soil microbial DNA was detected by gel electrophoresis using 1.0% of agarose gel. Besides, nano spectrophotometer was used to determine DNA concentration and purity using 260/280 and 260/230 ratio.

3.4 Nested PCR Amplification

3.4.1 Amplification of *nif*H gene with Degenerated Primers

Amplification of *nif*H gene from a large complex mixture of soil microbial DNA was carried out with nested PCR, involving two sets of degenerate primer: outer primer (*nif*H3 and *nif*H4) and inner primer (*nif*H1 and *nif*H2). In primary PCR, *nif*H4 (forward) and *nif*H3 (reverse) primers originated from *Azotobacter vinelandii* annealed to outer region of the target gene, generating about 470bp of DNA fragment (Zani, et al., 2000). In secondary PCR, *nif*H1 (forward) and *nif*H2 (reverse) primers which nucleotide sequences were proposed by Zehr and McReynolds (1989) annealed within the target DNA region and produced about 360bp of DNA fragment. The *nif*H primer sequences were tabulated in Table 3.3.

Table 3.4: Primer sequence of *nif*H gene amplification.

Amplification	Primer Name	Primer Sequence
Primary PCR	*nif*H4 (forward)	5' – TTYTAYGGNAARGGNGG – 3'
	*nif*H3 (reverse)	5' – ATRTTRTTNGCNGCRTA – 3'
Secondary PCR	*nif*H1 (forward)	5' – CTGYGAYCCNAARGCNGA – 3'
	*nif*H2 (reverse)	5' – GDNGCCATCATYTCNCC – 3'

DNA sequence degeneracy: Y = T/C; R = A/G; D = A/G/T; N = A/C/G/T

3.4.2 Parameter of Nested PCR Amplification

The PCR amplification utilized the primary and secondary PCR approach. In primary PCR, total soil microbial DNA was used as DNA template. As for the secondary PCR, primary PCR product was used as DNA template. PCR mixture with a total volume of 25μL was prepared. They consisted of the following: 1x PCR buffer, 1.5mM $MgCl_2$, 0.2mM dNTP, 400nM of each forward and reverse primer, 0.81ng/μL of DNA template, 0.04U/μL *Taq* DNA polymerase and ddH_2O. Similar concentration was used for secondary PCR as well. A negative control replacing DNA template with ddH_2O was prepared to ensure the absence of contamination. The PCR parameter for primary and secondary PCR was indicated in Table 3.5. PCR mixture was hold in thermocycler under 4℃ before taken out.

A volume of 5μL of each PCR products were electrophoresed in 1.5% agarose gel to verify the presence of DNA fragment with size of 360bp. PCR products corresponding to DNA with band size of 360bp was stored in freezer in -20℃ until used.

Table 3.5: Primary and secondary (nested) PCR cycling condition.

Primary PCR			
Steps	**Temperature (℃)**	**Time (minute)**	**Cycle number**
Initial denaturation	94	5	1
Denaturation	94	1	2 – 30
Annealing	45	1	
Extension	72	1	
Final extension	72	10	31

Secondary PCR			
Steps	**Temperature (℃)**	**Time (minute)**	**Cycle number**
Initial denaturation	94	5	1
Denaturation	94	0.5	2 – 30
Annealing	55	0.5	
Extension	72	0.5	
Final extension	72	10	31

3.4.3 Purification of PCR product

The secondary PCR product was electrophoresed in 1.5% agarose gel. Through UV transilluminator, target DNA was excised from agarose gel with a sterile scalpel. Target DNA was gel-purified via QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's protocol. Purified target DNA was eletrophoresed in 1.5% agarose gel to confirm the presence of 360bp target DNA.

3.5 Molecular Cloning of 360bp Gene Fragment

3.5.1 Ligation of Purified DNA into Cloning Vector

The purified DNA was ligated into pGEM-T Easy Vector (Promega) according to manufacturer's instructions. Ligation mixture was prepared as shown in Table 3.6. The mixture was then incubated at 4℃ overnight to get maximum number of transformants.

Table 3.6: Material and volume used in ligation mixture.

Material	Volume
10x Ligation buffer	1 µL
pGEM-T Easy Vector (50ng/µL)	1 µL
T4 DNA Ligase (3u/µL)	1 µL
Purified PCR product	7 µL

3.5.2 Preparation of *Escherichia coli* Competent Cells

E. coli JM109 strain was streaked on LB agar plate and incubated overnight at 37℃. A single colony was inoculated into 5mL LB broth and incubated overnight at 37℃ with 200rpm agitation. A volume of 500 µL of overnight inoculum was transferred into 20mL fresh LB medium. The inoculum was further agitated at 200rpm at 37℃. Absorbance was monitored 1.5 hours after and when OD_{600} reached 0.5-0.6A, the culture was centrifuged for 15min in 8000rpm. Supernatant was removed and the cell pellet was resuspended with 2mL 0.1M (cold) $CaCl_2$. The competent *E. coli* cells were subsequently incubated on ice water for 2 hours.

3.5.3 Transformation

Tubes containing ligation mixture, negative and positive controls were prepared for heat-shock transformation process. For ligation, 200µL of competent *E. coli* cells were transferred into pre-chilled 1.5mL microcentrifuge tube, followed by addition of 3µL ligation mixture. The tube was then mixed and incubated on ice for 1 hour. For positive control, 1µL of pUC19 was added into 100µL competent cells, whereas only 100µL of competent cells was used in negative control tube. After an hour of incubation, the three tubes were placed into water bath with temperature of 42℃ for 90 seconds. Then, all tubes were immediately transferred onto ice and incubated for additional 5 minutes. A volume of 800µL of LB broth was added into each tube and the mixtures were incubated at 37℃ at 200rpm for 45 minutes. The tubes were centrifuged at 6000rpm for 15 minutes, followed by removal of 800µL of supernatant from each tube. Cell pellet was resuspended with remaining LB broth and spread on LB agar plate supplemented with 50µg/mL of ampicillin, 20µL of 50mg/mL of X-Gal and 20µL of 100mM IPTG. The agar plates were incubated overnight at 37℃.

Colonies on ligation plate were patched on LB agar plate supplemented with similar concentrations of ampicillin, X-Gal and IPTG and incubate overnight at 37℃.

3.6 Screening of Recombinant Clones

3.6.1 Colony PCR

The presence of recombinant plasmids was detected using colony PCR. Each white colony was inoculated into PCR mixture using sterile toothpick. PCR mixture contained 1x PCR buffer, 1.5mM $MgCl_2$, 0.2mM dNTP, 400nM of each T7 and SP6 primer, 0.04U/µL *Taq* polymerase and topped to 25µL with ddH_2O. The colony PCR parameter was shown in Table 3.7. The T7 and SP6 primers annealed to T7 and SP6 RNA polymerase promoter sequence which flanked the multiple cloning region in pGEM-T Easy Vector. The colony PCR products were electrophoresed in 1.5% agarose gel to detect the presence of correct DNA insert.

Table 3.7 Colony PCR cycling condition.

Steps	Temperature (℃)	Time (minute)	Cycle number
Initial denaturation	96	3	1
Denaturation	96	0.5	2 - 30
Annealing	50	0.25	
Extension	60	0.25	
Final extension	60	5	31

3.6.2 Alkaline Lysis and Restriction Digestion

The following method is based on the protocol proposed by Birnboim and Doly in 1979. A single white colony was inoculated into 6mL LB broth supplemented with 50 µg/mL of ampicillin. The inoculum was incubated overnight at 37 ℃ with 200rpm agitation. *E. coli* cells were harvested by centrifugation at full speed for 15 minutes in a table-top centrifuge. Supernatant was discarded and the pellet was resuspended with 200 µL of Solution I. After transferring the mixture into sterile microcentrifuge tube, 10 µL lysozyme was added and gently mixed. Solution II (200 µL) was added and inverted gently to mix. The mixture was incubated on ice for 5 minutes until lysate became clear and viscous. After 300 µL of Solution III (cold) was added and gently mixed, the mixture was incubated for 5 minutes on ice. This was followed by centrifugation for 15 minutes at full speed in a table-top centrifuge. Supernatant was transferred into new sterile microcentrifuge tube and 700 µL of 95% ethanol was added, mix by invertion and incubated at room temperature for 15 minutes. The mixture was centrifuged for 5 minutes at full speed. After ethanol was discarded, pellet was washed with 70% ethanol and centrifuged for 5 minutes. Ethanol was aspirated and the pellet was air-dried. The pellet was resuspended in 50 µL ddH_2O and stored in -20 ℃. The presence of extracted plasmid DNA was detected using 1.0% agarose gel.

Extracted plasmid DNA from alkaline lysis process was digested with restriction enzyme *Eco*R1 (Promega) to verify the presence of target DNA in the recombinant plasmids. The restriction digestion reaction was set up as stated in Table 3.8 and incubated overnight at 37° C water bath.

Table 3.8: Component and volume used in restriction digestion mixture.

Component	Volume
10x Promega Buffer H	1 µL
Promega EcoR1 (12u/µl)	1 µL
Extracted plasmid	2 µL
ddH_2O	6 µL

3.7 Extraction of Recombinant Plasmid

Recombinant white colonies were used for plasmid extraction using Wizard Plus SV Minipreps DNA Purification System (Promega) based on manufacturer's protocol. Recombinant colonies were inoculated into 5mL LB broth containing 50 µg/mL of ampicillin and incubated overnight at 37 ℃ with 200rpm agitation. The next day, they were harvested by centrifugation at 10,000x*g* for 5 minutes. The extracted plasmids were eluted in 30 µL of ddH_2O and stored in -20 ℃. The extracted recombinant plasmid was detected using 1.0% agarose gel. Purity and concentration of DNA was determined by nanospectrophotometer.

3.8 DNA Sequencing and Phylogenetic Analysis

3.8.1 DNA Sequencing

Purified recombinant plasmids were outsourced to First Base Laboratories Sdn Bhd for one-pass DNA sequencing using T7 universal primer.

3.8.2 Analysis of Nucleotide Sequences

The *nif*H clone sequences were examined and edited manually using Sequence Scanner v1.0 (Applied Biosystem). The resulting nucleotide sequences were aligned with BlastX programme hosted on the National Center for Biotechnology Information (NCBI) website. BlastX is used to compare the newly determined DNA sequences against existing sequences in the NCBI non-redundant protein database. The software translates the nucleotide sequences and aligned the translated queries, based on local alignment algorithm, with known NifH polypeptide sequences. Hits with the highest score and lowest E-value were selected. The amino acid identities of bacterial strains with the nearest NifH polypeptide were identified.

3.8.3 Multiple Sequence Alignment

All nucleotide sequences that were verified to be *nif*H homologous gene were translated into their respective polypeptide sequences using ExPASy Proteomics tools. Compilation of the translated NifH polypeptide sequences were used to perform the multiple sequence alignment using ClustalX 2.1 (Larkin, et al., 2007).

3.8.4 Construction of Phylogenetic Tree

The multiple sequence alignment of the NifH polypeptide sequences were used to construct the phylogenetic tree, utilizing MEGA 6.0 software using neighbour-joining methodology (Tamura, et al., 2011). A total of 55 known NifH polypeptide sequences were retrieved from the GeneBank database hosted on NCBI (Table 3.9). The phylogenetic tree was analysed with 100 bootstrap values.

Table 3.9: Representative of known diazotrophs and their corresponding accession numbers of the *nif*H protein sequences.

Source Description	Accession No.	Group
Azospirillum brasilense	CAA35868	Alpha-proteobacteria
Acidithiobacillus ferrooxidans	AAA27374	Gamma-proteobacteria
Alcaligenes faecalis	CAA65427	Beta-proteobacteria
Anaeromyxobacter sp. Fw109-5	ABS27227	Delta-proteobacteria
Azotobacter chroococcum	AAA22140	Gamma-proteobacteria
Azotobacter vinelandii	AAA64709	Gamma-proteobacteria
Bradyrhizobium elkanii	ABG74604	Alpha-proteobacteria
Bradyrhizobium japonicum	AAG60754	Alpha-proteobacteria
Bradyrhizobium sp. RSA3	ACO58678	Alpha-proteobacteria
Burkholderia mimosarum	AAT06092	Beta-proteobacteria
Chroococcidiopsis thermalis PCC 7203	AAQ99146	Cyanobacteria
Clostridium cellulovorans 743B	ADL52532	Low G+C firmicute
Clostridium pasteurianum	AAT37644	Low G+C firmicute
Clostridium pasteurianum 2	P54800	Low G+C firmicute
Clostridium pasteurianum 3	P09553	Low G+C firmicute
Clostridium pasteurianum 4	P22548	Low G+C firmicute
Clostridium pasteurianum 5	P09554	Low G+C firmicute
Clostridium pasteurianum 6	P09555	Low G+C firmicute
Cyanothece sp. ATCC 51142	AAB61408	Cyanobacteria
Cyanobacterium sp. NBRC 102756	AFP43337	Cyanobacteria
Cyanobacterium PCC 7702	WP_017320752	Cyanobacteria

Table 3.9: (Continued)

Source Description	Accession No.	Group
Desulfatibacillum alkenivorans	WP_012610655	Delta-proteobacteria
Desulfovibrio africanus str. Walvis Bay	YP_005050775	Delta-proteobacteria
Desulfovibrio aespoeensis	WP_013513968	Delta-proteobacteria
Desulfovibrio vulgaris	WP_015946240	Delta-proteobacteria
Fischerella sp. JSC-11	EHC09729	Cyanobacteria
Frankia sp. EuIK1	AAC18640	High G+C firmicute
Frankia sp. CcI3	YP_483563	High G+C firmicute
Geobacter bemidjiensis Bem	ACH39087	Delta-proteobacteria
Geobacter sp. M21	ACT18197	Delta-proteobacteria
Geobacter uraniireducens Rf4	ABQ25379	Delta-proteobacteria
Geobacter sulfurreducens KN400	ADI85572	Delta-proteobacteria
Geobacter metallireducens GS-15	ABB30904	Delta-proteobacteria
Gluconacetobacter diazotrophicus PAl 5	AAD05046	Alpha-proteobacteria
Herbaspirillum seropedicae SmR1	CAA90932	Beta-proteobacteria
Klebsiella pneumonia	AFV52053	Gamma-proteobacteria
Leptolyngbya boryana	WP_017289029	Cyanobacteria
Methanococcus maripaludis	AAC45512	Archaea
Methanosarcina barkeri	CAA39552	Archaea

Table 3.9: (Continued)

Source Description	Accession No.	Group
Methanothermobacter thermautotrophicus	CAA61216	Archaea
Methanothermobacter thermautotrophicus 2	P08625	Archaea
Methanothermobacter marburgensis	WP_013294983	Archaea
Methanothermobacter thermautotrophicus strain Delta H	O26739	Archaea
Methylobacter luteus	CAD91849	Gamma-proteobacteria
Methylocystis sp. SC2	YP_006592767	Alpha-proteobacteria
Nostoc sp. PCC7120	CAA24729	Cyanobacteria
Paenibacillus durus 3	CAC27795	Low G+C firmicute
Pelobacter carbinolicus DSM 2380	ABA89338	Delta-proteobacteria
Pelobacter propionicus DSM 2379	ABL01060	Delta-proteobacteria
Rhizobium leguminosarum	ACO90393	Alpha-proteobacteria
Rhizobium tropici	ABD73338	Alpha-proteobacteria
Rhizobium sp. NCHA22	ABB88850	Alpha-proteobacteria
Rhodobacter sphaeroides	AAB86864	Alpha-proteobacteria
Sinorhizobium fredii	ABG74606	Alpha-proteobacteria
Trichodesmium erythraeum IMS101	AAD03796	Cyanobacteria

CHAPTER 4

RESULTS

4.1 Total Soil Microbial DNA Extraction

According to the gel image of Figure 4.1, the DNA fragment of the extracted total soil microbial DNA was larger than 10kb. Using nanospectrophotometer, results of DNA concentration and purity were tabulated in Table 4.1. The extracted DNA showed a value of 2.0 using 260/280 ratio, indicating the presence of RNA. A value of 1.05 in 260/230 ratio demonstrated the presence of residual humic acid in purified DNA.

Table 4.1: Concentration and purity of total microbial DNA isolated from soil sample using nanospectrophotometric measurement.

Parameter	Extracted DNA Data
DNA concentration	20.2 ng/μL
260/280 ratio	2.00
260/230 ratio	1.05

CHAPTER 5

DISCUSSION

5.1 Soil Sampling

5.1.1 Selection of Sampling Site

In this study, soil sample was collected from yam plantation within the agricultural land during the month of October 2013 which was during rainy season. Seasonal and temporal shifts in rainfall can have a large impact on the diversity, abundance, and responsiveness of soil microbial communities (Hullar, Kaplan and Stahl, 2006). Based on the study of Cregger, et al. (2012) on the effect of precipitation towards soil microbial community, it was observed that the community structure and abundance were more sensitive toward fluctuations in seasonal rainfall than constant precipitation treatments. Soil microbial community can quickly respond to changes in soil water potential after the rewetting of a dry soil to trigger soil respiration and microbial activity (Chou, et al., 2008). As a result, a higher possibility to get a complete picture of nifH gene pool in the sampling site was observed.

Soil microbial community in agricultural ecosystem is always exposed to long-term and frequent soil disturbance, resulting in lower diversity, different composition of soil microbial assemblages, and the resistance of community

CHAPTER 6

CONCLUSION

A total of 23 recombinant plasmids were obtained from the yam plantation within the agricultural soil sample of University Tunku Abdul Rahman (UTAR), Kampar, Perak. Based on NifH phylogenetic tree, majority of recombinant clones are classified as δ-proteobacteria, which is a subdivision of proteobacteria in Cluster I. A small number of recombinant clones are classified into cyanobacteria, low G+C gram-positive bacteria and high G+C gram-positive bacteria. Twenty recombinant clones revealed a high degree of identity with δ-proteobacteria, which are free-living diazotrophs. This indicated the relatively low diversity with predominance of δ-proteobacteria. However, a complete view of *nif*H gene pool in agricultural land remained unclear due to limitation number of *nif*H homologous sequences studied, thus further studies and characterization are required.

Even though a limited number of partial *nif*H sequences were obtained in this project, these sequences represent the ever expanding *nif*H sequence database. Thus, these *nif*H homologous sequences will be beneficial in providing references for future similar studies. This will further lead to a better understanding of diazotroph community in agricultural land.

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