KONG CHIA HUOI B.Sc. (Hons) Biotechnology 2024

ISOLATION AND CHARACTERISATION OF FREE-LIVING NITROGEN FIXING BACTERIA FROM SOIL ENVIRONMENT

KONG CHIA HUOI

BACHELOR OF SCIENCE (HONS) BIOTECHNOLOGY

FACULTY OF SCIENCE
UNIVERSITI TUNKU ABDUL RAHMAN
APRIL 2024

ISOLATION AND CHARACTERISATION OF FREE-LIVING NITROGEN FIXING BACTERIA FROM SOIL ENVIRONMENT

By

KONG CHIA HUOI

A project report submitted to the Department of Biological Science

Faculty of Science

Universiti Tunku Abdul Rahman

In partial fulfilment of the requirements for the degree of

Bachelor of Science (HONS) Biotechnology

April 2024

ABSTRACT

ISOLATION AND CHARACTERISATION OF FREE-LIVING NITROGEN FIXING BACTERIA FROM SOIL ENVIRONMENT

Kong Chia Huoi

Biological nitrogen fixation is a vital process in the nitrogen cycle which is performed by diazotrophs. Diazotrophs use nitrogenase enzymes to catalyse the conversion of dinitrogen gas into ammonia, which can be absorbed by plants. This present study aimed to isolate and characterise the free-living nitrogen fixing bacteria from the soil samples collected in Kampar. The locations involved were Zone F and Zone P in the UTAR Kampar campus as well as Eastlake areas. The nitrogen fixing bacteria from the collected soil samples were isolated using Burk's medium. The bacterial isolates were characterised through Gram staining and the Gram-negative bacterial isolates were subjected to selected biochemical tests for the identification of *Enterobacteriaceae*. All the twenty bacterial isolates were screened for the *nif*H gene, but only three of them showed an approximate 360 bp amplicon after the PCR amplification using n*if*H primers. As such, the three bacterial isolates, N-6, E-I, and E-VI were selected for 16S rRNA sequencing, and the BLASTN results showed that they were identified as *Bacillus luciferensis*, *Dickeya zeae*, and *Kosakonia oryzendophytica*, respectively. However, the BLASTX results showed that the *nif*H gene was only present in E-I and E-VI.

ACKNOWLEDGEMENTS

Firstly, I would like to express my heartfelt gratitude to my supervisor, Asst. Prof. Dr. Choo Quok Cheong, for his guidance, patience, and valuable advice throughout my final year project. I am sincerely grateful to the lab officers from the Faculty of Science for their assistance and guidance whenever I am in need. Next, I would like to thank my friends, Wee Ching Hui, Ngoi Thong Yee, and Tan Yimin, for their helping hands and support during this period. Lastly, my deepest appreciation is to my beloved parents for their unwavering support, encouragement, and understanding. Without them, it would be tough for me to solve all the problems met and complete my final year project successfully.

DECLARATION

I hereby declare that this final year 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.

KONG CHIA HUOI

APPROVAL SHEET

This final year project report entitled "**ISOLATION AND CHARACTERISATION OF FREE-LIVING NITROGEN FIXING BACTERIA FROM SOIL ENVIRONMENT**" was prepared by KONG CHIA HUOI and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biotechnology at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Asst. Prof. Dr. Choo Quok Cheong) Date: 8 May 2024

Supervisor

Department of Biological Science

Faculty of Science

Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: 3 April 2024

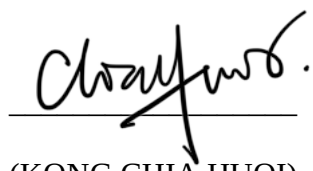
PERMISSION SHEET

It is hereby certified that **KONG CHIA HUOI** (ID No: **21ADB04964**) has completed this final year project report entitled "**ISOLATION AND CHARACTERISATION OF FREE-LIVING NITROGEN FIXING BACTERIA FROM SOIL ENVIRONMENT**" under the supervision of Asst. Prof. 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 report in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

I hereby give permission to my supervisor to write and prepare manuscript of these research findings for publishing in any form, if I did not prepare it within six (6) months time from this date provided that my name is included as one of the authors for this article. Arrangement of the name depends on my supervisor.

Yours truly,



(KONG CHIA HUOI)

TABLE OF CONTENTS

		Page
ABSTRACT		**ii**
ACKNOWLEDGEMENTS		**iii**
DECLARATION		**iv**
APPROVAL SHEET		**v**
PERMISSION SHEET		**vi**
TABLE OF CONTENTS		**vii**
LIST OF TABLES		**x**
LIST OF FIGURES		**xii**
LIST OF ABBREVIATIONS		**xiii**
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	
2.1	Soil Environment	4
2.1.1	Soil Microbiome	4
2.2	Diazotrophs	6
2.2.1	Free-Living Nitrogen Fixing Bacteria	7
2.2.2	Symbiotic Nitrogen Fixing Bacteria	8
2.3	The Family *Enterobacteriaceae*	9
2.4	Nitrogenase	9
2.4.1	Conventional Nitrogenases	9
2.4.2	Alternative Nitrogenases	11
2.5	Factors Affecting Nitrogenase Activity	12
2.6	Isolation of Nitrogen Fixing Bacteria Using Culture-Dependent Method	13
2.7	Detection and Screening Methods	14

2.7.1 Conventional Bacterial Detection Method 14
2.7.2 Molecular Detection Method 15
2.8 The 16S rRNA Gene, a Universal Marker of Prokaryotes 17
2.9 The *nif*H Gene, a Gene Marker of Diazotrophs 18

3 MATERIALS AND METHODS

3.1 Materials and Preparation 19
3.2 Soil Sampling Sites 21
3.2.1 Collection of Soil Samples 21
3.3 Cultivation and Isolation of Soil Bacteria 23
3.4 Preliminary Characterisation of Bacterial Isolates 24
3.4.1 Gram Staining 24
3.4.2 Catalase Test 25
3.4.3 Oxidase Test 25
3.4.4 Oxidation-Fermentation (OF) Test 25
3.5 Total Bacterial DNA Extraction 26
3.6 PCR Amplification of *nif*H Gene 27
3.7 PCR Amplification of 16S rRNA Gene 29
3.8 Purification of PCR Products 30
3.8.1 Purification of *nif*H and 16S rRNA Gene PCR Products 31
3.9 DNA Sequencing 32
3.9.1 Analysis of Nucleotide Sequences 32

4 RESULTS

4.1 Cultivation and Isolation of Soil Bacteria 33
4.2 Preliminary Characterisation of Bacterial Isolates 36
4.2.1 Gram Staining 36
4.2.2 Catalase Test 39
4.2.3 Oxidase Test 39
4.2.4 Oxidation-Fermentation (OF) Test 39
4.3 Total DNA Extraction 42

4.4 PCR Amplification and Purification 43
4.5 DNA Sequencing and BLAST Analyses 45

5 DISCUSSION

5.1 Cultivation and Isolation of Soil Bacteria 47
5.1.1 Burk's Medium 47
5.2 Preliminary Characterisation of Bacterial Isolates 48
5.2.1 Gram-Positive and Gram-Negative Bacteria Ratio in Soil 48
5.2.2 Biochemical Tests for Gram-Negative Bacteria 49
5.3 Total DNA Extraction 49
5.4 PCR Amplification of *nif*H Gene 50
5.5 Purification of PCR Products 50
5.6 DNA Sequencing and BLAST Analyses 51
5.6.1 Bacterial Isolate N-6 51
5.6.2 Bacterial Isolate E-I 52
5.6.3 Bacterial Isolate E-VI 54
5.7 False-Positive Results 55
5.7.1 Growth of Non-Diazotrophic Bacteria on Nitrogen-Free Medium 55
5.7.2 Gram Staining 56
5.8 Future Studies 57

6 CONCLUSION 58

REFERENCES 59

APPENDICES 74

LIST OF TABLES

	Table	Page
3.1	List of used materials and their respective manufacturers.	19
3.2	Culture medium preparation protocols.	20
3.3	Oligonucleotide sequences of *nifH1* and *nifH2*.	27
3.4	Each composition volume for *nif*H gene PCR amplification.	28
3.5	Optimised parameters for *nif*H gene PCR amplification.	28
3.6	Oligonucleotide sequences of 16S F and 16S R.	29
3.7	Each composition volume for 16S rRNA gene PCR amplification.	30
3.8	Optimised parameters for 16S rRNA gene PCR amplification.	30
4.1	Colony morphological observations of bacteria isolated from soil samples collected in the UTAR Kampar campus (Sample 1 and 2) and the Eastlake area (Sample 3 and 4) on Burk's agar.	34
4.2	Microscopic observations of bacteria isolated from soil samples collected in the UTAR Kampar campus (Sample 1 and 2) and the Eastlake area (Sample 3 and 4) after Gram staining.	37
4.3	Observations of catalase test of Gram-negative bacteria.	40
4.4	Observations of oxidase test of Gram-negative bacteria.	40

4.5	Observations of oxidation-fermentation (OF) test of Gram-negative bacteria.	41
4.6	Concentration and purity of the extracted bacterial total DNA.	42
4.7	Concentration and purity of purified PCR products (~360 bp).	43
4.8	Concentration and purity of purified PCR products (~1500 bp).	44
4.9	The BLASTX analysis for partial *nif*H gene sequences compared to the most identical known protein sequences in the GenBank database.	46
4.10	The BLASTN analysis for partial 16S rRNA gene sequences compared to the most identical nucleotide sequences of known bacteria in the GenBank database.	46

LIST OF FIGURES

	Figure	Page
2.1	The structure of the molybdenum nitrogenase, containing MoFe protein and Fe protein [adapted from Seefeldt, Hoffman and Dean (2009)].	10
3.1	Soil sampling sites viewed on Google Maps, a) Sample 1, b) Sample 2, c) Sample 3, and d) Sample 4.	22
4.1	Colony morphology of D-1 bacteria grown on a) Burk's agar and b) LB agar. D-1 bacterial colonies appeared white on Burk's agar while creamy on LB agar.	33
4.2	The 2% (w/v) agarose gel image of the purified PCR product amplified with *nif*H primers for N-6, E-I, and E-VI. Lane 1: 100bp DNA ladder, 0.2 μg (Vivantis); Lane 2: N-6; Lane 3: E-I; Lane 4: E-VI.	43
4.3	The 0.7% (w/v) agarose gel image of the purified PCR product amplified with 16S primers for N-6, E-I, and E-VI. Lane 1: 1 kb DNA ladder, 0.2 μg (GeneDireX); Lane 2: N-6; Lane 3: E-I; Lane 4: E-VI.	44

LIST OF ABBREVIATIONS

anf	Iron-only nitrogenase gene
ARA	Acetylene reduction assay
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic acid
Fe	Iron
FeFe	Iron-iron
GN	Gram-negative
GP	Gram-positive
LB	Luria broth
MoFe	Molybdenum-iron
NCBI	National Centre for Biotechnology Information
nif	Nitrogen fixation gene
OF	Oxidation-fermentation
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
T_m	Melting temperature
UTAR	Universiti Tunku Abdul Rahman
VFe	Vanadium-iron
vnf	Vanadium nitrogenase gene

CHAPTER 1

INTRODUCTION

Every living organism on Earth requires nitrogen to survive. Nitrogen is required for producing biomolecules such as nucleic acids and proteins. Plants acquire nitrogen from the soil, while animals and humans obtain nitrogen from the food they consume. Nitrogen element is abundantly found in gaseous form in the air, where nearly 80% of the atmosphere is occupied by dinitrogen gas. However, the majority of living organisms are unable to access and utilise gaseous nitrogen. In fact, dinitrogen gas needs to be converted into ammonia or nitrate in the soil for the primary producers to absorb and utilise (Morgan and Connolly, 2013). The conversion process is known as nitrogen fixation, where dinitrogen gas is transformed into biologically available nitrogen by specific prokaryotes (Bernhard, 2010). This process is very crucial in the nitrogen cycle to enhance the primary production in the ecosystems.

Since biologically available nitrogen is limited in the soil, farmers will apply nitrogenous fertilisers in their plantations to supply nitrogen resources to the crops. In this case, crop production can be enhanced to fulfil the food requirements of the fast-growing population. However, chemical fertilisers have polluted the environment in significant amounts instead of being fully utilised by the plants. Increased biologically available nitrogen will pollute the water

sources, affecting the natural global nitrogen cycle and causing climate change, which will eventually lead to the imbalance of the ecosystems (United Union Environment Programme (UNEP), 2024). Furthermore, large amounts of nitrogen accumulated in the soil environment will cause nutrient imbalance in plants, hence negatively impacting plant growth (Bernhard, 2010). Excessive nitrogenous fertilisers can also lead to soil acidification, polluting the soil and inhibiting plant growth (Zhou et al., 2021). To enhance sufficient reactive nitrogen in the soil, an ideal way is through biological nitrogen fixation. By applying nitrogen fixing microbes to the soil, the amount of fixed nitrogen can be maintained at a desired level but not to the extreme. Therefore, the application of effective nitrogen fixers is a potential replacement for chemical nitrogenous fertilisers.

Researchers commonly target the *nif*H gene, which encodes nitrogenase reductase, to investigate the potential of the bacterial isolates to fix nitrogen (Zehr and Turner, 2001). There are many universal and group-specific *nif*H primers designed to be used to amplify the *nif*H gene in most of the diazotrophs (Gaby and Buckley, 2012). To further identify the species of the nitrogen fixing bacteria, DNA sequencing of the 16S rRNA gene is an ideal determination method, because this gene is a universal marker for identifying bacterial species (Lane et al., 1985).

The objectives of this project include the following:

- To isolate nitrogen fixing bacteria from soil samples collected in Kampar using nitrogen-free media.
- To characterise the bacterial isolates through Gram staining and to classify the Gram-negative bacterial isolates as *Enterobacteriaceae* through selected biochemical tests.
- To amplify and detect the *nif*H homologous genes using PCR.
- To identify the bacterial species through the analysis of the 16S rRNA sequences using the BLASTN programme.

CHAPTER 2

LITERATURE REVIEW

2.1 Soil Environment

The soil environment consists of many components, including minerals, organic matter, a variety of organisms, liquids, and gases. Soil is a living resource that serves as a basis for life, therefore, it is crucial for both humans and the ecosystem. Generally, the characteristic of the soil determines their corresponding types and functions. In detail, physical characteristics include soil structure, water movement, temperature, and pH; the soil's biological environment is related to all the organisms living in the soil; whereas available nutrient is the main focus of chemical characteristics of the soil (Al-Kaisi et al., 2017). On the other hand, climate change and human activities could have a great impact on the soil environment, leading to various effects, such as reduced soil fertility, declined nutrient cycling, and decreased microbial diversity (Doula and Sarris, 2016).

2.1.1 Soil Microbiome

A wide variety of microorganisms live in the soil environment, including bacteria, fungi, archaea, and protozoa, which make up the soil microbiome. They are responsible for the degradation of organic matter and biogeochemical cycling, particularly the nitrogen cycle, to ensure the availability of soil mineral nutrients

to the plants (Basu et al., 2021). Thus, soil microbiome plays a vital role in enhancing soil fertility and stimulating plant growth. Additionally, some bacterial species can form associative interactions with the rhizosphere through plant-microbe interactions (Hayat et al., 2010). Plant-microbe interactions might be mutualistic, harmful, or neutral, depending on the microbes involved, but beneficially, these interactions could facilitate carbon and nitrogen sequestration (Velmourougane, Saxena and Prasanna, 2017). In addition, the soil microbiome can assist in the pathogen defense of plants by producing antibiotics and also enhance stress tolerance by secreting certain enzymes (Abdul Rahman, Abdul Hamid and Nadarajah, 2021).

Besides, the composition of microbial communities in the soil environment could be altered by various factors. In fact, microbial population in the soil depends on the soil depth, available nutrients, soil pH, oxygen concentration, soil texture, etc (Bhattarai, Bhattarai and Pandey, 2015). Moreover, studies also found that the growing plant species could have a significant influence on the soil microbiome (Tkacz et al., 2020; Hannula et al., 2021). For instance, bacterial communities of forest soils are found to be responsive to climate change and increased levels of carbon dioxide in the atmosphere (Lladó, López-Mondéjar and Baldrian, 2017). However, the response varies in different forest ecosystems. Hence, the composition of soil microbiome varies in different soil types and areas, where different plant species are growing.

2.2 Diazotrophs

Dinitrogen gas occupies almost 80% of the atmosphere. In this diatomic gas, the triple covalent bond between the two nitrogen atoms is very stable and strong, making it nearly inert. Therefore, the breakdown of the dinitrogen molecule requires a large amount of energy (Ogunseitan, 2005). As a result, many organisms are not able to acquire nitrogen sources directly from dinitrogen gas as they cannot afford the excessive energy requirement. Exceptionally, a group of microorganisms designated as diazotrophs, is responsible for the biological nitrogen fixation on the Earth, providing biologically available nitrogen sources in the soil for plants. In brief, they possess the nitrogenase enzyme to convert atmospheric nitrogen into biologically accessible nitrogen, such as ammonia or nitrate (Bernhard, 2010). Additionally, these specific microbes not only enhance soil fertility but a small portion of them, called diazotrophic plankton, is found to have increased ocean fertility, through fixing nitrogen and dissolving organic nitrogen in the seawater (Bonnet et al., 2023).

In the soil environment, diazotrophs can be classified into two subgroups, one lives independently in the soil, while another can develop symbiotic relationships with plant roots. Other than fixing atmospheric nitrogen, diazotrophs can stimulate plant growth by secreting phytohormones, solubilising inorganic phosphate, and mineralising organic phosphate (Dobbelaere, Vanderleyden and Okon, 2003). The common species of phytohormone-secreting diazotrophs include *Azospirillum*, *Azotobacter*, *Klebsiella*, and *Rhizobium* (Imran et al., 2021). On top of that, these four bacterial species are

able to solubilise inorganic phosphate in the soil to assist the uptake of phosphate by the plants (Seshadri, 2000; Sridevi and Mallaiah, 2009; Jha, Saxena and Sharma, 2013; Nosrati et al., 2014). Moreover, nitrogen fixing microbes can produce antibiotics and promote systemic resistance of the plants to protect the plants from detrimental impacts caused by the pathogens (Dobbelaere, Vanderleyden and Okon, 2003). Owing to these beneficial abilities, nitrogen fixing bacteria are beneficial to be included in the production of biofertilizers to enhance plant growth in nutrient-deficient land, without causing environmental pollution issues (Ritika and Utpal, 2014).

2.2.1 Free-Living Nitrogen Fixing Bacteria

Free-living nitrogen fixing bacteria are found in the soil and rhizosphere, usually deep into the ground where the concentration of oxygen is relatively lower. This is due to the sensitivity of nitrogenase towards oxygen, where its enzymatic activity reaches optimal in low levels or the absence of oxygen (Gallon, 1981). To prevent the irreversible deleterious effects caused by the oxygen present in the soil environment, the free-living nitrogen fixing bacteria must possess strategies to avoid or minimize the contact of their nitrogenase with oxygen. For instance, the bacteria will increase their respiration rates and substrate utilisation to decrease oxygen concentration in the surroundings (Smercina et al., 2019). Furthermore, since they live independently in the soil, they have to acquire energy sources through the oxidation of organic molecules or decomposition, whereas some of them are chemolithotrophic, which utilise inorganic compounds. As a result, the carbon sources available in the soil can greatly affect

the nitrogen fixation process carried out by these free-living bacteria (Smercina et al., 2019). Besides, the common free-living nitrogen fixing bacterial species mostly belong to the genus *Azotobacter*, *Bacillus*, *Clostridium*, *Klebsiella*, *Pseudomonas*, and the phylum Cyanobacteria (Orr et al., 2011; Wagner, 2011). In fact, some specific nitrogen fixing cyanobacteria are also able to fix the carbon source from the environment through photosynthesis (Orr et al., 2011).

2.2.2 Symbiotic Nitrogen Fixing Bacteria

Symbiotic nitrogen fixing bacteria have an associative relationship with the plants. This type of nitrogen fixing bacteria forms nodules at the roots after infecting the host plant, serving as a platform for nitrogen fixation (Kawaka, 2022). In return for providing nitrogen sources, the plant supplies carbon sources acquired through photosynthesis to the symbiotic bacteria, as their energy source used to fix the atmospheric nitrogen. Additionally, symbiotic nitrogen fixing bacteria are confined in the nodules, where there is a diffusion barrier in the cortex to restrict the penetration of oxygen (Kawaka, 2022). Therefore, their nitrogenases are protected from oxygen. For instance, this symbiotic relationship is mostly found in leguminous plants where rhizobia are hosted (Mylona, Pawlowski and Bisseling, 1995). Moreover, the genus *Frankia* was found to be associated with actinorhizal plants (Rascio and La Rocca, 2013).

2.3 The Family *Enterobacteriaceae*

Bacteria from the family *Enterobacteriaceae* can be found in the environment, including soil and water, as well as the human gut microbiome. Under this family, several species from the common genera *Enterobacter* and *Klebsiella* are nitrogen fixers (Ladha, Barraquio and Watanabe, 1983; Lin et al., 2012; Ghorai and Ghosh, 2023). *Enterobacteriaceae* consists of Gram-negative rod-shaped bacteria that are facultatively anaerobic, which utilise D-glucose as their energy source in both aerobic and anaerobic conditions (Farmer, Farmer and Holmes, 2010). Additionally, they have the characteristics of catalase-positive and oxidase-negative, but with some exceptions (Octavia and Lan, 2014).

2.4 Nitrogenase

Nitrogenase is an enzyme complex that is found in diazotrophs. This enzyme reduces atmospheric nitrogen into ammonia, playing a crucial role in the process of biological nitrogen fixation. Basically, nitrogenase can be divided into two distinct groups, which are conventional nitrogenases and alternative nitrogenases.

2.4.1 Conventional Nitrogenases

The most studied conventional nitrogenase is the molybdenum nitrogenase. The molybdenum nitrogenase complex consists of two major protein components, a molybdenum-iron protein (MoFe protein) and an iron protein (Fe protein), as shown in Figure 2.1. MoFe protein is encoded by the *nif*DK genes, whereas Fe

protein is a reductase, encoded by the *nif*H gene. Two different subunit pairs ($\alpha_2\beta_2$), a Fe_4S_3 cluster, and a $MoFe_3S_3$ cluster form the heterotetrametric structure of the MoFe protein (Seefeldt, Hoffman and Dean, 2009). On the other hand, the Fe protein is a homodimer (γ_2) that contains a P cluster. There are two Fe proteins associated with a MoFe protein, each binds to one αβ-unit. The Fe protein is responsible for transferring electrons to the MoFe protein to reduce the nitrogen at the active sites (Burgess and Lowe, 1996). To be able to do so, the Fe protein has to biosynthesise the FeMo cofactor and then insert the preformed FeMo cofactor into the FeMo protein. In fact, these two processes do not require the transportation of electrons.

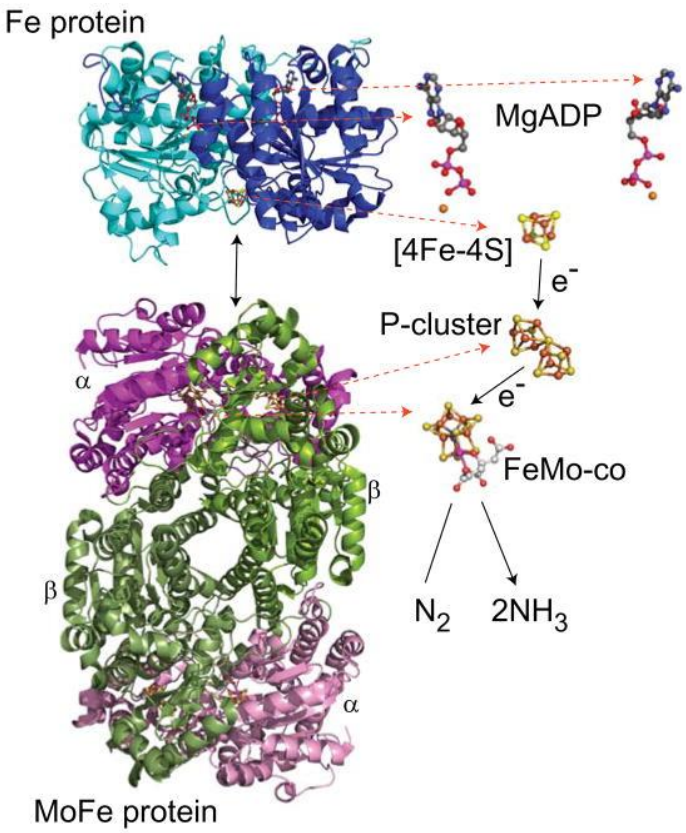


Figure 2.1: The structure of the molybdenum nitrogenase, containing MoFe protein and Fe protein [adapted from Seefeldt, Hoffman and Dean (2009)].

2.4.2 Alternative Nitrogenases

In the biological fixation process, it was assumed that the enzyme which catalyse the reduction of atmospheric nitrogen to ammonia is only limited to conventional nitrogenase. This assumption was no longer relevant since Bishop, Jarlenski and Hetherington (1980) discovered the ability of *nif*⁻ strain *Azotobacter vinelandii* to fix nitrogen without the catalysation by molybdenum nitrogenase. Since then, the hypothesis regarding the existence of alternative nitrogenases started to be proven by researchers among the members of the *Azotobacter* genus. For instance, *A. chroococcum* (Robson, 1979), *A. vinelandii* (Hales et al., 1986), *A. Beijerinckii,* and *A. nigricans* (Fallik, Chan and Robson, 1991). The detected alternative nitrogenase was named vanadium nitrogenase.

Soon after the discovery of the first alternative nitrogenase in *A. vinelandii,* Chisnell, Premakumar and Bishop (1988) successfully purified the second alternative nitrogenase from *nif*HDK deletion strain of the same bacterial species, in the absence of molybdenum and vanadium. A study by Müller et al. (1992) classified the nitrogenase that only contains iron but neither molybdenum nor vanadium, as iron-only nitrogenase. In brief, vanadium nitrogenase and iron-only nitrogenase are both alternative nitrogenase, encoded by the *vnf* (Robson et al., 1989) and *anf* (Mylona et al., 1996) genes, respectively.

The enzyme structure of both vanadium and iron-only nitrogenases is similar to the molybdenum nitrogenase, where the Fe protein is present in all forms, but the MoFe protein is replaced by VFe and FeFe protein, respectively (Hales et al., 1986; Eady, 1996). The mechanisms and phylogenies of the three forms of nitrogenase are related (Dos Santos et al., 2012). Therefore, due to the conservation of Fe protein among the three discovered nitrogenases, it is suggested that their enzymatic function is similar.

2.5 Factors Affecting Nitrogenase Activity

The composition of soil minerals is one of the factors that affect nitrogenase activity (Omar and Abd-Alla, 1992). In fact, essential elements such as molybdenum, iron, calcium, magnesium, and potassium must be present in the soil environment for optimal growth of the diazotrophs (Stella et al., 2010). Besides, neutral soil pH is ideal for diazotrophs to live and perform biological nitrogen fixation optimally (Schubert, Mengel and Schubert, 1990). Rich carbon sources in the soil can also enhance nitrogenase activity as the reduction of dinitrogen gas requires large energy consumption (Ogunseitan, 2005).

Besides, the presence of external nitrogen inhibits the nitrogen fixation process (Tan, Ui and Amir, 2015). An investigation using *Clostridium pasteurianum* reported the absence of nitrogenase activity in the bacterial cells that were cultured with excess ammonia (Daesch and Mortenson, 1972). Moreover, nitrate

was found to suppress nitrogen fixation in *Azotobacter* after a certain adaptation period (Wilson, Hull and Burris, 1943).

Since nitrogenase activity is sensitive to oxygen, diazotrophs only perform nitrogen fixation under low levels or absence of oxygen (Gallon, 1981). However, the biological nitrogen fixation process requires a large amount of ATP to be generated through respiration, where oxygen is responsible as an electron acceptor. In this case, diazotrophs have developed effective strategies to allow their access to oxygen, meanwhile, to prevent the contact of oxygen with their nitrogenase enzymes (Soto-Urzúa and Baca, 2001). For instance, diazotrophs can form a protective barrier surrounding their nitrogenases to prevent the penetration of oxygen or modify the conformation of their nitrogenases, making the enzymes resistant to the inactivation caused by oxygen. Furthermore, some diazotrophs are able to compensate for the inactivation of nitrogenase by synthesising new nitrogenases continually.

2.6 Isolation of Nitrogen Fixing Bacteria Using Culture-Dependent Method

Nitrogen fixing bacteria possess the ability to fix atmospheric nitrogen into ammonia and utilise it for their growth. Thus, the cultivation of nitrogen fixing bacteria does not require the supplementation of nitrogen in the culture media. Nitrogen-free media are specifically formulated to promote the selective growth of nitrogen fixing bacteria, whereas the non-diazotrophic bacteria are unable to survive as they are dependent on external nitrogen sources. The need for changes

in the formulations to optimise the isolation procedures of different diazotrophs contributed to the development of several types of nitrogen-free media, such as Burk's medium, Jenson's medium, and Ashby's sucrose media (Burk and Lineweaver, 1930; Jensen, 1942; Subba Rao, 1977). In fact, all nitrogen-free media are similar in composition.

Most importantly, carbon sources such as sucrose, glucose, or mannitol need to be supplied in the media to support the metabolic activities of the bacteria. Free-living nitrogen fixing bacteria usually utilize sucrose, D-mannose, and L-arabinose as their carbon source to perform metabolism (Stella et al., 2010). Elements essential for cell growth such as molybdenum, iron, magnesium, calcium, and potassium are also included in the composition of nitrogen-free media as inorganic salts. Molybdenum is supplied as sodium molybdate, and its presence is effective in enhancing the efficiency of the nitrogen fixers in fixing nitrogen (Ranganayaki and Mohan, 1981). In fact, molybdenum deficiencies can affect the activity of molybdenum nitrogenase, restricting the smoothness of the biological nitrogen fixation process (Hernandez, George and Rubio, 2009).

2.7 Detection and Screening Methods

2.7.1 Conventional Bacterial Detection Method

The conventional ways to detect bacteria include the cultivation of bacteria using enrichment media followed by bacterial isolation with selective media. Subsequently, Gram staining is performed on the bacterial colonies growing on

the selective agar plates, and the biochemical properties of the isolates are also studied (Gugliandolo et al., 2011). These traditional methods are economical and easy to conduct, but they are time-consuming due to the bacterial incubation time, and are labour-intensive, where a lot of procedures are included (Kim and Kim, 2021). Furthermore, unreliable results might be generated because some species of bacteria are non-cultivable in the laboratory, resulting in no formation of the respective colonies (Oliver, 2010). In this case, the identification of the variety of bacteria from a sample might not be precise.

2.7.2 Molecular Detection Method

To compensate for the limitations of the conventional methods, many effective rapid methods have been developed, such as nucleic acid-based, biosensor-based, and immunological-based methods (Law et al., 2015). Among them, polymerase chain reaction (PCR) is a widely used methodology for the amplification and detection of targeted DNA sequences (Barghouthi, 2011). PCR technique is very sensitive and able to generate accurate results in a short time in comparison with the cultured-based bacterial detection methods (Magistrado, 2001). Additionally, bacterial identification is not only limited to the genera as the species of the bacteria can be determined by sending the PCR amplicons for DNA sequencing. Moreover, molecular techniques have provided a platform for researchers in the discovery of new bacterial genera or species. However, PCR amplification of target DNA might be affected by cell lysate contained in the sample due to the DNA extraction procedures, as well as the degradation of the nucleic acids (Wilson, 1997). Hence, the purity of the template DNA is crucial for obtaining

desired amplicons. Besides, the melting temperature of the primer used is important, especially when the PCR reaction involves two primers, in order to optimise the annealing temperature which is suitable for the binding of both primers to the template DNA. In fact, the composition of the PCR mixture and the PCR parameters should be optimized to enhance the quality of the amplification results (Lorenz, 2012).

The first PCR amplification of the *nif*H gene from environmental samples with a pair of degenerate primers was done by (Kirshtein, Paerl and Zehr, 1991). Since then, numerous primer pairs have been designed to optimise the detection of *nif*H genes from different environmental samples, such as soils, roots, and estuarine samples (Angel et al., 2018). For instance, the detection of the *nif*H gene in rhizospheres was done to investigate the relationship between the diversity of *nif*H genes and the amount of nitrogenous fertilizer applied (Coelho et al., 2009). In addition, a group of researchers used the real-time qPCR technique to detect the *nif*H gene of *Methanobrevibacter smithii* in water samples containing sewage input, to study the potential of this methanogenic archaea as an indicator of sewage pollution (Johnston et al., 2010). A review from Gaby and Buckley (2012) stated that there is a total of 15 universal *nif*H primers that can successfully amplify the *nif*H gene in more than 90% of nitrogen fixers, whereas there are also several *nif*H primer pairs that are specific to certain groups of prokaryotes. Their analysis was done based on an aligned database of 23,847 *nif*H sequences.

2.8 The 16S rRNA Gene, a Universal Marker of Prokaryotes

The 16S rRNA gene is widely used to identify bacterial species as it is found in all prokaryotes (Woese, 1987). The 16S rRNA gene has an approximate size of 1500 bp, encoding the 16S ribosomal RNA which forms the 30S subunit of prokaryotic ribosomes. The sequences of the 16S rRNA gene are highly conserved, where the variations within the region can determine the bacterial genera or species. Bacterial identification is done by comparing the sequence of the PCR-amplified 16S rRNA gene with the sequences in the database (Clarridge, 2004). Besides, the 16S rRNA gene is very stable and its size is sufficient for bioinformatic purposes, contributing to the universal use of the gene to identify bacterial species (Janda and Abbott, 2007). In addition, the evolution of bacteria can be examined by analysing the minor random changes in the sequence of the 16S rRNA gene.

16S rRNA sequencing can be performed to investigate the diversity of the bacterial communities present in the environment, which includes the soil. For instance, researchers performed 16S rRNA sequencing to study the bacterial diversity between humus soil and mineral soil of evergreen broad-leaved forests in China (Chan et al., 2006). Besides, the identification of bacteria living in the rhizosphere can also be done through 16S rRNA sequencing, to examine the bacterial communities in the natural and post-mining soil (Azaroual et al., 2022). On the other hand, the 16S rRNA gene can be utilised to determine the species of bacteria that are present in the water bodies (Nakatsu, Byappanahalli and

Nevers, 2019) as well as the human microbiome, such as oral, lung, and gut microbiomes (Ames et al., 2017).

2.9 The *nif*H Gene, a Gene Marker of Diazotrophs

16S rRNA sequencing only provides general information regarding the bacterial species, but it cannot relate to the potential ability of the bacteria to fix nitrogen. Thus, a specific gene marker must be detected to assist with the identification of nitrogen fixing bacteria, which is the *nif*H gene. The *nif*H gene is directly linked to biological nitrogen fixation as it encodes the dinitrogenase reductase subunit of a nitrogenase enzyme, while another subunit is encoded by the *nif*D and *nif*K genes. Among these genes, the *nif*H gene is mostly targeted to determine the potential nitrogen fixing ability of unknown prokaryotes, as it is highly conserved among diazotrophs (Raymond et al., 2004). Moreover, the evolutionary history of a diazotroph can be studied based on the phylogeny generated through the analysis of its *nif*H gene, as it agrees with the respective 16S rRNA phylogeny (Zehr et al., 2003).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and Preparation

Table 3.1 lists the materials used in this project, while Table 3.2 shows the protocols for the preparation of culture media.

Table 3.1: List of used materials and their respective manufacturers.

Material	**Manufacturer**
Burk's medium powder	HiMedia Laboratories
Agar powder, glucose	Chem Soln
Luria agar powder	Condalab
OF basal medium (Hugh & Leifson)	Liofilchem
2 × *Taq* master mix	Vazyme
100 bp DNA ladder	Vivantis
1 kb DNA ladder	GeneDireX
Degenerate primers (nifH1, nifH2, 16S F and 16S R)	Integrated DNA Technologies
Agarose powder	First Base Laboratories

Table 3.2: Culture medium preparation protocols.

Culture Medium	Preparation Protocols
Burk's agar medium	A weight of 21.3 g Burk's medium powder and 18 g agar powder were dissolved with 1 L of distilled water in a screw cap bottle. After autoclaving, the medium was poured into petri dishes and allowed to solidify at room temperature. The agar plates were dried overnight at room temperature before use.
Luria agar (Lennox)	A weight of 35 g Luria agar powder was dissolved with 1 L of distilled water in a screw cap bottle. After autoclaving, the medium was poured into petri dishes and allowed to solidify at room temperature. The agar plates were dried overnight at room temperature before use.
Oxidation and fermentation medium	A weight of 9.8 g OF basal medium powder and a volume of 100 mL of 10% D-glucose solution was dissolved with 900 mL of distilled water. The medium was poured into 15 mL centrifuge tubes until 3/5 of the tube. The tubes were autoclaved before use.

3.2 Soil Sampling Sites

A total of four soil samples were collected from four locations. Sample 1 was collected from an undisturbed area opposite Zone F in the UTAR Kampar campus (4°20'13.1"N 101°08'38.9"E). Sample 2 was obtained from an undisturbed area opposite Zone P in the campus (4°20'20.2"N 101°08'08.4"E). Sample 3 was collected from undisturbed land near the East Lake (4°20'10.4"N 101°08'53.6"E), whereas another undisturbed land in the East Lake area (4°20'06.0"N 101°09'00.3"E) designated as Sample 4 was collected. The maps of the soil sampling sites are shown in Figure 3.1.

3.2.1 Collection of Soil Samples

A spade which was cleaned with 70% (v/v) ethanol was used to collect the soil samples. The soil was collected at a depth of approximately 10 cm and was then transferred into a sterile 50 mL centrifuge tube. The tube was kept in an ice box until further processing.

Figure 3.1: Soil sampling sites viewed on Google Maps, a) Sample 1, b) Sample 2, c) Sample 3, and d) Sample 4.

3.3 Cultivation and Isolation of Soil Bacteria

Two grams of each soil sample was suspended with 20 mL of sterile distilled water in a sterile 50 mL centrifuge tube. Ten-fold serial dilution was performed on the soil mixture using sterile distilled water until 10^{-3}-fold dilution was obtained. Next, 100 μL of each serially diluted soil mixture was spread onto Burk's agar plates. The plates were then incubated at 30 °C overnight.

The bacterial growth was observed after overnight incubation. The colonies with different morphology were differentiated and streaked onto new Burk's agar plates. After incubating at 30 °C overnight, the isolated bacterial colonies were then subcultured onto LB agar plates for the maintenance of bacterial culture. Similarly, the LB agar plates were incubated at 30 °C overnight and were then kept at 4 °C.

The bacteria isolated from Sample 1 were named D1, D2, D3, and D4, whereas the bacteria isolated from Sample 2 were N1, N2, N3, N4, N5, and N6. Sample 3 gave four bacterial isolates which were designated as E1, E2, E3, and E4, while the bacterial isolates obtained from Sample 4 were named EI, EII, EIII, EIV, EV, and EVI.

3.4 Preliminary Characterisation of Bacterial Isolates

The morphological characteristics of the bacterial colonies on Burk's agar were observed and recorded. The characteristics involved were colour, shape, margin, and elevation. Subsequently, Gram staining was performed on all the bacterial isolates. Then, the Gram-negative bacteria were selected and proceeded with additional biochemical tests for the classification of *Enterobacteriaceae*. The biochemical tests included catalase, oxidase, and oxidation-fermentation tests.

3.4.1 Gram Staining

A single bacterial colony was mixed and spread with a drop of distilled water on the microscopic slide to form a thin film. The film was air-dried and heat-fixed over the bunsen burner. Next, the smear was stained with crystal violet for 30 seconds and rinsed with distilled water to remove the excessive stain. Subsequently, the smear was covered with iodine and allowed to sit for 30 seconds before rinsing with distilled water. The smear was then decolourised by dropping the decolourising reagent on the tilted slide for 10 seconds. The slide was rinsed with distilled water again. After that, the smear was counterstained by flooding the slide with safranin for another 30 seconds. After rinsing the slide with distilled water, the slide was blotted dry with tissue paper. Lastly, the slide was examined under an oil-immersion objective using a light microscope. The colour and shape of the bacterial cells were recorded.

3.4.2 Catalase Test

A few drops of 3% hydrogen peroxide were placed on a microscopic slide. Then, a loopful of a single bacterial colony was transferred to the reagent. The formation of bubbles was recorded. A positive result (catalase production) refers to immediate bubble formation, whereas no bubbling is considered a negative result.

3.4.3 Oxidase Test

A loopful of a single bacterial colony was transferred onto a piece of filter paper with an inoculating loop. A drop of oxidase test reagent was dropped onto the colony. The colour change to dark blue on the filter paper indicates a positive result (oxidase production), whereas no colour change shows a negative result.

3.4.4 Oxidation-Fermentation (OF) Test

A loopful of a single bacterial colony was stabbed into two tubes of OF media supplemented with 1% glucose solution. One of the tubes was added with a few drops of mineral oil to prevent the penetration of oxygen. All the tubes were capped and then incubated at 30°C. The colour change was observed and recorded after 2 days of incubation.

3.5 Total Bacterial DNA Extraction

The fast-boiling method was used to extract the total bacterial DNA of all the bacterial isolates (Dashti et al., 2009). First of all, two loops of the bacterial colony were mixed with 200 μL of sterile deionised water in a 1.5 mL microcentrifuge tube. The mixture was then centrifuged at 13,000 × g for 2 minutes. Next, the supernatant was carefully discarded and another 200 μL of sterile deionised water was added to the pellet. The pellet was resuspended and the mixture was centrifuged at 13,000 × g for 2 minutes. Subsequently, the supernatant was discarded, and the remaining pellet was resuspended with 100 μL of sterile deionised water. After that, the tube was placed in the heat block at a temperature of 95 °C for 15 minutes. Then, the tube was immediately transferred into ice and allowed to stay for 5 minutes. After cooling, the mixture was centrifuged again at 13,000 × g for 6 minutes. In the next step, the supernatant was transferred into another clean 1.5 mL microcentrifuge tube. Eventually, the concentration of the extracted total bacterial DNA was measured with the NanoDrop spectrophotometer. The DNA was then stored in the -20 °C freezer until further use.

3.6 PCR Amplification of *nif*H Gene

The PCR amplification was performed using *nifH1* and *nifH2* which are both degenerate primers that specifically target the conserved region of the *nif*H gene within the bacterial DNA. The region that will be targeted has a size of approximately 360 bp. The oligonucleotide sequences of both degenerate primers used in this PCR amplification are shown in Table 3.3. The PCR reagent mixture consists of 2 × *Taq* master mix (Vazyme), 1 μM forward primer (*nifH1*), 1 μM reverse primer (*nifH2*), and DNA templates. In addition, a negative control was prepared by replacing the DNA templates with sterile deionised water. Table 3.4 shows the volume of each composition contained in the PCR reagent mixture. Besides, the optimised parameters for PCR amplification of the *nif*H gene are shown in Table 3.5. After PCR amplification, the PCR product was assessed on 2% (w/v) agarose gel.

Table 3.3: Oligonucleotide sequences of *nifH1* and *nifH2*.

Degenerate primers	Sequences	Expected size
nifH1 (forward primer)	5'- CTG YGA YCC NAA RGC NGA -3'	~ 360 bp
nifH2 (reverse primer)	5'- GDN GCC ATC ATY TCN CC -3'	
Degeneracy code	R=A or G; Y=T or C; D=A, T or G; N=A, T, C or G	

Table 3.4: Each composition volume for *nif*H gene PCR amplification.

Composition	Single Tube Volume (μL)
2 × *Taq* master mix	5
nifH1 (10 μM)	1
nifH2 (10 μM)	1
DNA template	1
Sterile deionised water	2

Table 3.5: Optimised parameters for *nif*H gene PCR amplification.

Stage	Temperature (°C)	Duration (min)	Cycle
Initial denaturation	94	5	1
Denaturation	94	1	30
Annealing	58	1	
Extension	72	1	
Final extension	72	10	1

3.7 PCR Amplification of 16S rRNA Gene

The bacterial isolates that showed a band of approximately 360 bp after PCR amplification of the *nif*H gene would be used for subsequent PCR amplification for their corresponding 16S rRNA gene. The 16S F and 16S R are the degenerate primers used in this PCR amplification, targeting a conserved region of 16S rRNA gene. Table 3.6 shows the oligonucleotide sequences of both primers. Similarly, the PCR reagent mixture consists of 2 × *Taq* master mix (Vazyme), 0.4 μM forward primer (16S F), 0.4 μM reverse primer (16S R), DNA templates, and sterile deionised water. Table 3.7 lists the volume of each composition in the reagent mixture, and the PCR parameters are shown in Table 3.8. After amplification, the PCR product was assessed on 0.7% (w/v) agarose gel.

Table 3.6: Oligonucleotide sequences of 16S F and 16S R.

Degenerate primers	Sequences	Expected size
16S F (forward primer)	5'- AGA GTT TGA TYM TGG CTC AG -3'	~ 1,500 bp
16S R (reverse primer)	5'- TRA CGG SCR GTG TGT A -3'	
Degeneracy code	R=A or G; Y=T or C; D=A, T or G; N=A, T, C or G	

Table 3.7: Each composition volume for 16S rRNA gene PCR amplification.

Composition	Single Tube Volume (μL)
2 × *Taq* master mix	5
16S F (10 μM)	0.4
16S R (10 μM)	0.4
DNA template	0.5
Sterile deionised water	3.7

Table 3.8: Optimised parameters for 16S rRNA gene PCR amplification.

Stage	Temperature (°C)	Duration (min)	Cycle
Initial denaturation	94	5	1
Denaturation	94	1	30
Annealing	50	1	
Extension	72	1	
Final extension	72	10	1

3.8 Purification of PCR Products

The PCR products of both *nif*H and 16S rRNA genes were purified using the QIAquick gel extraction kit (Qiagen). After the purification process, the concentration of the purified DNA was measured using the NanoDrop spectrophotometer.

3.8.1 Purification of *nif*H and 16S rRNA Gene PCR Products

Firstly, the PCR product was loaded in a 2% (w/v) agarose gel (Qiagen). The DNA fragment with an approximate size of 360 bp was excised from the agarose gel with a clean scalpel. The excised gel slice was cut into smaller pieces and weighed in a clean 1.5 mL microcentrifuge tube. After that, 3 volumes of Buffer QG to 1 volume of gel were added into the tube. The tube was then placed in a heat block at 50 °C for 10 minutes. During the incubation, the tube was inverted several times to help dissolve the gel completely. Subsequently, 1 gel volume of isopropanol was added and mixed with the dissolved gel mixture. Next, a QIAquick spin column was placed in a provided 2 mL collection tube. The mixture was applied to the column and centrifuged at 10,000 × *g* for 2 minutes. The following step was to remove the flow-through, and then an additional 0.5 mL of Buffer QG was added to the column. The collection tube was centrifuged again for 2 minutes at 10,000 × *g*. Again, the flow-through was discarded and 0.75 mL of Buffer PE was added to the column and left to stand for 4 minutes before centrifuging at 10,000 × *g* for 2 minutes. An additional 2-minute centrifugation was performed after the flow-through was discarded to completely remove the residual ethanol. Subsequently, the QIAquick column was transferred into a clean 1.5 mL microcentrifuge tube. A volume of 30 μL of sterile deionised water was added to the centre of the membrane to elute the purified DNA. The column was stood for 5 minutes and centrifuged at 10,000 × *g* for 2 minutes.

3.9 DNA Sequencing

The purified PCR products of both targeted genes were outsourced to Apical Scientific for direct sequencing.

3.9.1 Analysis of Nucleotide Sequences

The electropherograms of partial *nif*H and 16S rRNA gene sequences were analysed using Chromas software. DNA sequences were generated by overlapping the sequences from the forward and reverse sequences. The reverse sequence was converted to its corresponding reverse complement form before overlapping with the forward sequence. Lastly, the edited *nif*H gene sequences were analysed using the BLASTX programme, while the analysis of the 16S rRNA gene sequences was done using the BLASTN programme. Both programmes were developed by the National Centre for Biotechnology Information (NCBI).

CHAPTER 4

RESULTS

4.1 Cultivation and Isolation of Soil Bacteria

A total of twenty bacterial isolates were isolated from four different locations in Kampar, Perak. In the UTAR Kampar campus, four bacterial isolates were obtained from an undisturbed area opposite Zone F (Sample 1), and another six isolates were from an area opposite Zone P (Sample 2). For the soil samples collected from the Eastlake area, Sample 3 contributed to four bacterial isolates while Sample 4 gave six isolates. The bacterial colonies that grew on Burk's agar medium showed slightly different morphology compared to the colonies grown on LB agar. The distinct morphological characteristics of bacterial colonies were observed on Burk's agar plates and listed in Table 4.1. Figure 4.1 shows the different morphology of D-1 bacteria grown on Burk's agar and LB agar.

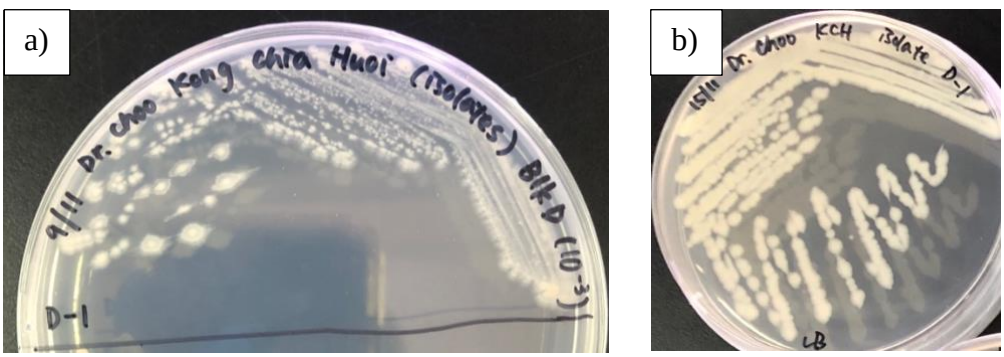


Figure 4.1: Colony morphology of D-1 bacteria grown on a) Burk's agar and b) LB agar. D-1 bacterial colonies appeared white on Burk's agar while creamy on LB agar.

Table 4.1: Colony morphological observations of bacteria isolated from soil samples collected in the UTAR Kampar campus (Sample 1 and 2) and the Eastlake area (Sample 3 and 4) on Burk's agar.

Location	Sample	Bacterial isolate	Colour	Shape	Margin	Elevation
Opposite Zone F (in campus)	1	D-1	White	Irregular	Undulate	Flat
		D-2	Yellow	Round	Entire	Raised
		D-3	White	Irregular	Undulate	Flat
		D-4	Cream	Filamentous	Filamentous	Convex
Opposite Zone P (in campus)	2	N-1	White	Irregular	Undulate	Flat
		N-2	Yellow	Curled	Entire	Raised
		N-3	Cream	Filamentous	Entire	Convex
		N-4	White	Irregular	Undulate	Flat
		N-5	Yellow	Round	Entire	Raised
		N-6	White	Irregular	Undulate	Flat

Table 4.1 Continued.

Location	Sample	Bacterial isolate	Colour	Shape	Margin	Elevation
Eastlake area	3	E-1	Yellow	Round	Entire	Convex
		E-2	White	Round	Entire	Raised
		E-3	Cream	Irregular	Undulate	Flat
		E-4	Translucent	Irregular	Entire	Raised
	4	E-I	Cream	Irregular	Undulate	Flat
		E-II	Cream	Irregular	Undulate	Flat
		E-III	Cream	Filamentous	Entire	Raised
		E-IV	Yellow	Round	Entire	Raised
		E-V	Translucent	Irregular	Undulate	Flat
		E-VI	White	Round	Entire	Convex

4.2 Preliminary Characterisation of Bacterial Isolates

4.2.1 Gram Staining

The colour and shape of the isolated bacteria were observed under an oil-immersion objective using a light microscope after Gram staining procedures. Gram-positive bacteria appeared purple in colour whereas Gram-negative bacteria appeared pink. Table 4.2 lists the microscopic observations of the bacteria isolated from collected soil samples (Sample 1 - 4) after Gram staining. The composition of Gram-positive and Gram-negative bacteria in Sample 1 was 50% each. Sample 2 consisted of 83% Gram-positive bacteria and 17% Gram-negative bacteria. Bacteria isolated from Samples 3 and 4 were all Gram-positive.

Table 4.2: Microscopic observations of bacteria isolated from soil samples collected in the UTAR Kampar campus (Sample 1 and 2) and the Eastlake area (Sample 3 and 4) after Gram staining.

Location	Sample	Bacterial isolate	Gram staining	Morphology
Opposite Zone F (in campus)	1	D-1	Gram-positive	Coccobacillus
		D-2	Gram-negative	Coccobacillus
		D-3	Gram-positive	Streptobacillus
		D-4	Gram-negative	Coccobacillus
Opposite Zone P (in campus)	2	N-1	Gram-positive	Bacillus
		N-2	Gram-positive	Coccobacillus
		N-3	Gram-positive	Coccobacillus
		N-4	Gram-positive	Streptobacillus
		N-5	Gram-positive	Bacillus
		N-6	Gram-negative	Streptobacillus

Table 4.2 Continued.

Location	Sample	Bacterial isolate	Gram staining	Morphology
Eastlake area	3	E-1	Gram-positive	Coccobacillus
		E-2	Gram-positive	Coccus
		E-3	Gram-positive	Streptobacillus
		E-4	Gram-positive	Coccus
	4	E-I	Gram-positive	Streptobacillus
		E-II	Gram-positive	Bacillus
		E-III	Gram-positive	Streptobacillus
		E-IV	Gram-positive	Bacillus
		E-V	Gram-positive	Streptobacillus
		E-VI	Gram-positive	Coccus

4.2.2 Catalase Test

Two out of the three Gram-negative bacteria could form bubbles when 3% hydrogen peroxide was applied to the bacterial cells, indicating a positive result. The two bacterial isolates were designated as D-4 and N-6, and isolated from Sample 1 and 2 respectively. D-2 bacteria from Sample 1 could not produce catalase, thus showing a negative result where no bubbles were formed. The observations of the catalase test are listed in Table 4.3.

4.2.3 Oxidase Test

Only N-6 bacteria isolated from Sample 2 turned dark blue when the oxidase reagent was added, whereas the other two Gram-negative bacteria from Sample 1 showed negative result with no colour change observed. The observations of the oxidase test are tabulated in Table 4.4.

4.2.4 Oxidation-Fermentation (OF) Test

The two Gram-negative bacteria isolated from Sample 1 are classified as glucose fermenters. They were able to catalyse glucose under aerobic and anaerobic conditions, where both tubes of OF media turned yellow after incubation. N-6 bacteria from Sample 2 is non-saccharolytic because it could not utilise glucose as a substrate, and thus the OF media tubes remained green. The observations of the OF test are shown in Table 4.5.

Table 4.3: Observations of catalase test of Gram-negative bacteria.

Location	Sample	Bacterial isolate	Bubble formation	Result
Opposite Zone F (in campus)	1	D-2	No bubble formed	Negative
		D-4	Bubbles formed	Positive
Opposite Zone P (in campus)	2	N-6	Bubbles formed	Positive

Table 4.4: Observations of oxidase test of Gram-negative bacteria.

Location	Sample	Bacterial isolate	Colour	Result
Opposite Zone F (in campus)	1	D-2	No colour change	Negative
		D-4	No colour change	Negative
Opposite Zone P (in campus)	2	N-6	Dark blue formed	Positive

Table 4.5: Observations of oxidation-fermentation (OF) test of Gram-negative bacteria.

Location	Sample	Bacterial isolate	Colour		Result
			Aerobic condition	Anaerobic condition	
Opposite Zone F (in campus)	1	D-2	Yellow	Yellow	Fermentative
		D-4	Yellow	Yellow	Fermentative
Opposite Zone P (in campus)	2	N-6	Green	Green	Non-saccharolytic

4.3 Total DNA Extraction

The total DNA of the bacterial isolates was extracted through the fast-boiling method. The respective concentrations and purity of the extracted DNA are shown in Table 4.6.

Table 4.6: Concentration and purity of the extracted bacterial total DNA.

Bacterial isolate	Concentration (ng/μL)	Purity (A_{260}/A_{280})
D-1	223.3	2.05
D-2	159.0	1.96
D-3	314.7	2.14
D-4	211.3	2.13
N-1	222.5	2.09
N-2	167.2	1.87
N-3	130.4	1.86
N-4	520.3	2.18
N-5	108.6	2.06
N-6	183.1	1.86
E-1	80.4	2.09
E-2	255.7	2.12
E-3	403.4	2.13
E-4	327.1	2.02
E-I	721.8	2.16
E-II	570.1	2.19
E-III	142.9	2.15
E-IV	125.1	1.97
E-V	757.1	2.23
E-VI	943.5	2.05

4.4 PCR Amplification and Purification

After PCR amplification using a pair of *nif*H primers, only three bacterial isolates showed an approximate 360 bp DNA band on 2% (w/v) agarose gel. Therefore, these three bacteria were selected for further analysis. The bacteria include N-6 from Sample 2, as well as E-I and E-VI from Sample 4. The gel image of the purified PCR products is shown in Figure 4.2. The respective concentration and purity are listed in Table 4.7.

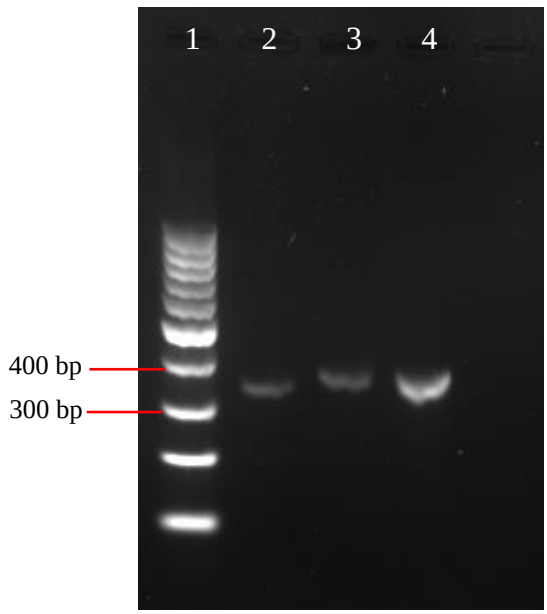


Figure 4.2: The 2% (w/v) agarose gel image of the purified PCR product amplified with *nif*H primers for N-6, E-I, and E-VI. Lane 1: 100 bp DNA ladder, 0.2 µg (Vivantis); Lane 2: N-6; Lane 3: E-I; Lane 4: E-VI.

Table 4.7: Concentration and purity of purified PCR products (~360 bp).

Bacterial isolate	Concentration (ng/µL)	Purity (A_{260}/A_{280})
N-6	10.7	1.58
E-I	9.2	1.87
E-VI	16.4	1.88

Bacterial isolates N-6, E-I, and E-VI were selected for subsequent PCR amplification of their corresponding 16S rRNA gene. All three isolates obtained a single band with an approximate size of 1500 bp on 0.7% (w/v) agarose gel. Figure 4.3 is the gel image of the purified PCR product. The respective concentration and purity are recorded in Table 4.8.

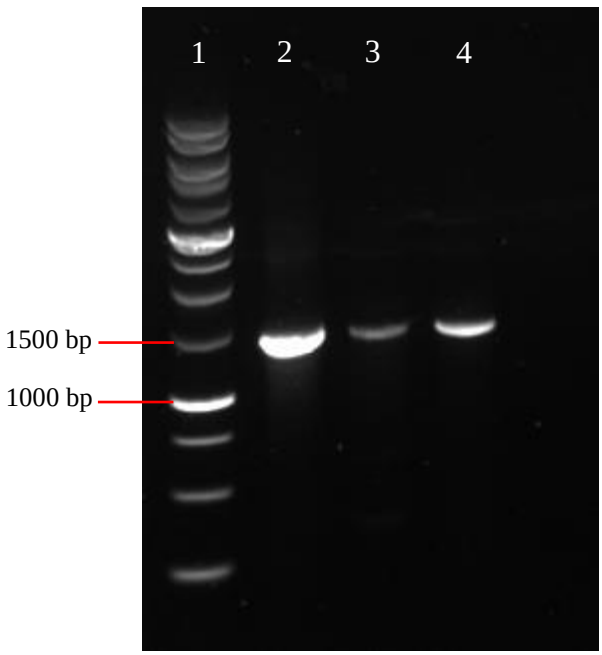


Figure 4.3: The 0.7% (w/v) agarose gel image of the purified PCR product amplified with 16S primers for N-6, E-I, and E-VI. Lane 1: 1 kb DNA ladder, 0.2 µg (GeneDireX); Lane 2: N-6; Lane 3: E-I; Lane 4: E-VI.

Table 4.8: Concentration and purity of purified PCR products (~1500 bp).

Bacterial isolate	Concentration (ng/µL)	Purity (A_{260}/A_{280})
N-6	107.7	1.89
E-I	27.1	1.59
E-VI	45.0	1.95

4.5 DNA Sequencing and BLAST Analyses

For the *nif*H gene, the electropherogram of N-6 displayed a poor sequencing result, however, the electropherograms of both E-I and E-VI showed a good result, as shown in Appendix A. The reverse-complement sequences of N-6 could not overlap with its forward sequences. Thus, a BLASTX search was performed on both forward and reverse-complement *nif*H sequences of N-6, to compare with the sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov). However, the forward sequences failed to be analysed. Furthermore, E-I and E-VI were only sent to sequence their forward sequences, hence, their forward sequences were directly subjected to BLASTX search after trimming. The highest-matched sequence identities from the sequence database were summarised in Table 4.9.

On the other hand, the electropherograms of the 16S rRNA gene for three bacterial isolates revealed good sequencing results, as shown in Appendix B. In addition, a longer sequence could be formed when the reverse-complement sequences overlapped with the forward sequences after trimming. The edited sequences were subjected to a BLASTN search to find the most similar nucleotide sequences in the GenBank database. The sequence identities with the highest percentage from the database are listed in Table 4.10.

Table 4.9: The BLASTX analysis for partial *nif*H gene sequences compared to the most identical known protein sequences in the GenBank database.

Sample	Identities	Score (bits)	E-value	Highest BLASTX identity	Accession
N-6	38/58 (66%)	78.2	$2e^{-13}$	glycoside hydrolase family 13 protein [*Bacillaceae*]	WP_069032965.1
E-I	95/97 (98%)	188	3e-59	dinitrogen reductase, partial	AFD32292.1
E-VI	94/97 (97%)	191	6e-60	nitrogenase reductase protein, partial [*Enterobacter* sp.]	QCF45230.1

Table 4.10: The BLASTN analysis for partial 16S rRNA gene sequences compared to the most identical nucleotide sequences of known bacteria in the GenBank database.

Sample	Identities	Score (bits)	E-value	Highest BLASTN identity	Accession
N-6	1299/1319 (98%)	2326	0.0	*Bacillus luciferensis*	DQ870692.1
E-I	1291/1304 (99%)	2337	0.0	*Dickeya zeae*	CP006929.1
E-VI	1301/1303 (99%)	2394	0.0	*Kosakonia oryzendophytica*	MN428218.1

CHAPTER 5

DISCUSSION

5.1 Cultivation and Isolation of Soil Bacteria

5.1.1 Burk's Medium

Burk's medium contains sucrose as the carbon source and other important elements such as magnesium, potassium, calcium, molybdenum, etc in the form of mineral salts (HiMedia Laboratories, 2020). It was originally formulated to isolate and cultivate *Azotobacter* species (Burk and Lineweaver, 1930). However, other bacterial species have also been successfully isolated using Burk's medium as well. For instance, Burk's agar medium added with yeast extract could be used to isolate *Clostridium butyricum* from small mammal droppings, with incubation under anaerobic conditions at 30 °C (Li and Maser, 1986). *Bacillus subtilis* was also successfully isolated from paddy soil using Burk's medium with incubation at 27 °C for 7 days (Hadija et al., 2021). Furthermore, *Pseudomonas* sp. present in agricultural soil samples was isolated on Burk's agar medium after two-day incubation at 30 °C (Ha et al., 2018). In 2019, a group of researchers successfully isolated *Paraburkholderia tropica*, *Paenibacillus cineris*, *Bacillus aryabhttai*, *Bacillus megaterium*, and *Klebsiella pneumonia* from different cropping systems using Burk's medium (Xa and Nghia, 2019). In brief, Burk's medium is capable of isolating various species of nitrogen fixing bacteria from environmental samples under different incubation conditions.

5.2 Preliminary Characterisation of Bacterial Isolates

5.2.1 Gram-Positive and Gram-Negative Bacteria Ratio in Soil

Based on the results of Gram staining, the ratio of Gram-positive (GP) bacteria is much higher than Gram-negative (GN) bacteria among the bacteria isolated from different soil samples. In a total of twenty bacterial isolates, 85% are GP bacteria whereas GN bacteria only account for 15%, referring to Table 4.2. In fact, the GP:GN ratio is dependent on the form of carbon sources available in the soil (Fanin et al., 2019). GP bacteria are usually associated with complex carbon compounds (carbonyls) that are obtained from soil organic matter, whereas GN bacteria prefer simple carbon compounds acquired from plants, such as alkyl. Other factors, such as soil properties (Xue et al., 2018), application of fertilisers, and species of plants (Silva and Nahas, 2002) can also alter the ratio of GP and GN bacteria in the soil environment. Therefore, the GP:GN ratio varies among the soil types from different locations.

Furthermore, the cell wall of both GP and GN bacteria contains a peptidoglycan layer, but an outer membrane is present above the peptidoglycan layer of GN bacteria. This outer membrane serves as a protective barrier for the bacterial cell (Silhavy, Kahne and Walker, 2010). The lipopolysaccharides (LPS) present in the outer membrane make the cell surface hydrophilic, restricting the penetration of deleterious hydrophobic substances. Furthermore, in response to environmental stress, GN bacteria can regulate their gene expression for adaptation (Dorman, 2009). Generally, GN bacteria are more tolerant to external stress and harsh conditions, hence preferably found in such environments.

5.2.2 Biochemical Tests for Gram-Negative Bacteria

In this project, three biochemical tests (catalase, oxidase, and oxidation-fermentation tests) were performed for the presumptive categorisation of Gram-negative bacteria belonging to the *Enterobacteriaceae* family. Based on the results of selected biochemical tests tabulated in Tables 4.3, 4.4, and 4.5, only isolate D-4 showed the general characteristics of *Enterobacteriaceae*, which is catalase-positive, oxidase-negative and fermentative.

5.3 Total DNA Extraction

The fast-boiling method was performed to extract total bacterial DNA. During the process, heating and cooling steps resulted in damage to the bacterial cell walls and cell membranes, releasing the DNA in the aqueous environment. It is a simple, economical, and rapid method to extract DNA for molecular techniques, with reasonably good quality DNA (Dashti et al., 2009). The extracted DNA might contain other cytoplasmic substances, but it will not cause any significant interferences in this study. According to Table 4.6, the concentration of the extracted DNA ranged between 80.4 ng/μL to 943.5 ng/μL, whereas the DNA purity ranged from 1.86 to 2.23. The purity of DNA is referred to as the ratio of absorbance at 260 nm and 280 nm. A pure DNA has a ratio of ~1.8. A lower ratio indicates protein contamination whereas a higher ratio indicates RNA contamination (Lucena-Aguilar et al., 2016).

5.4 PCR Amplification of *nif*H Gene

In this project, only three bacterial isolates (N-6, E-I, and E-VI) showed a band of approximately 360 bp after PCR amplification of the *nif*H gene, as shown in Figure 4.2. Thus, only three bacterial isolates out of the twenty can be considered potential nitrogen fixers. In fact, 85% of the bacterial isolates were able to grow on a nitrogen-free medium although no amplification of the expected size 360 bp was observed.

5.5 Purification of PCR Products

In this study, the purification of PCR products was done using the Qiagen QIAquick commercial gel extraction kit. This process is crucial to be performed before the PCR products are sent for direct sequencing. In order to generate good sequencing results, a purified PCR product is required where the interfering compounds such as the primers, enzymes, and nucleotides are removed. After the purification process, agarose gel electrophoresis was performed on the purified PCR products. A single band of approximately 360 bp was observed for the purified PCR products amplified using *nif*H primers (Figure 4.2), whereas a distinct band of about 1500 bp was observed for the purified PCR products amplified using 16S primers (Figure 4.3). According to Tables 4.7 and 4.8, the concentration of the PCR products ranged from 9.2 ng/μL to 107.7 ng/μL, while the purity ranged between 1.58 and 1.95. The concentration and purity of purified PCR products are crucial for reliable results in DNA sequencing. PCR products with higher purity would yield sequencing results of good quality, as there would not be background noises contributed by the impurities.

5.6 DNA Sequencing and BLAST Analyses

5.6.1 Bacterial Isolate N-6

Bacterial isolate N-6 was isolated from Sample 2 which was collected at an undisturbed area opposite Zone P in the UTAR Kampar campus. Based on the BLASTN result, the 16S rRNA gene sequences of N-6 showed the closest relationship to *Bacillus luciferensis* with 98% identities. On the other hand, the resultant PCR fragment using *nif*H primers yielded a band of approximately 360 bp, which the respective BLASTX analysis showed 66% identities to glycoside hydrolase. In fact, the BLASTX analysis is non-satisfactory due to the presence of background noises, as indicated in Appendix A (a). On top of that, the PCR amplicons were not the targeted *nif*H gene. The PCR amplification of the non-targeted DNA fragment might be due to the lower annealing temperature used in the PCR reaction. Non-specific binding between primers and the template DNA tends to occur when the annealing temperature is too low. For N-6, the optimised annealing temperature was determined to be 48 °C, where a clear distinct band of approximately 360 bp was observed. The gene coding for glycoside hydrolase may have some sequence similarity with *nif*H degenerate primers, resulting in the amplification of the targeted 360 bp DNA fragment. The non-targeted gene sequences of glycoside hydrolase may not have aligned perfectly with the *nif*H primer sequences, binding could still occur even if there are some unpaired bases within the sequences, particularly at lower annealing temperatures. As a result, the non-targeted 360 bp DNA fragment was PCR-amplified.

B. luciferensis was first discovered in volcanic soil by Logan et al. (2002). It is a Gram-positive rod-shaped bacterium, but it will become Gram-negative during cultivation at 30 °C within 24 hours. *B. luciferensis* is oxidase-positive, catalase-positive, and facultatively anaerobic (Reimer et al., 2022). However, it cannot utilise glucose as a substrate. From these descriptions, the results of Gram staining and all three biochemical tests for N-6 in this present study are consistent.

B. luciferensis has not been reported to possess nitrogen fixing ability. Although this bacterium may not be a potential nitrogen fixer, other species of *Bacillus* such as *B. megaterium*, *B. flexus*, *B. circulans*, *B. mycoides*, *B. marisflavi* and *B. cereus* are found to be able to fix nitrogen (Ding et al., 2005; Yousuf et al., 2017; Singh et al., 2020b).

5.6.2 Bacterial Isolate E-I

Bacterial isolate E-I was obtained from Sample 4 which was collected from an undisturbed land in the Eastlake area. BLASTN analysis showed the 16S rRNA gene sequences of E-I with the highest similarity to *Dickeya zeae* with 99% identities. On top of that, the BLASTX result of the *nif*H translated sequence corresponded to dinitrogen reductase with 98% identities. The sequencing results are reliable as evenly-spaced peaks were observed from the respective electropherogram, as shown in Appendix A (b). Additionally, the annealing temperature of the *nif*H gene PCR amplification for E-I was optimised at 58 °C,

comparatively higher than the temperature optimised for N-6. In this case, the *nif*H primers should be highly complementary to the target gene as they could still bind to the target sequences at such a high annealing temperature and specifically amplify the target DNA fragment. Furthermore, the melting temperature (T_m) of the forward primer (*nifH1*) at 58.3 °C is relatively close to the optimised annealing temperature.

Dickeya sp. was first described as a genus of Gram-negative rod-shaped bacteria that can utilise glucose in both aerobic and anaerobic conditions (Samson et al., 2005). By referring to Table 4.2, the Gram staining result of isolate E-I did not agree with the 16S rRNA gene sequencing result, as it showed Gram-negative. Due to the false Gram staining result, biochemical tests were not carried out on E-I. The result of biochemical tests should indicate that E-I belongs to the family *Enterobacteriaceae*.

Additionally, the *nif*H gene was successfully detected in *D. zeae* (Boluk et al., 2021) as well as other members such as *D. dadantii* (Itoh et al., 2019) and *D. solani* (Malgorzata, 2015). Although some *Dickeya* species are known to fix nitrogen, all members from the genus *Dickeya* are identified as a plant pathogen that infects various plants to cause vascular wilts or soft rots (Samson et al., 2005).

5.6.3 Bacterial Isolate E-VI

Similar to bacterial isolate E-I, E-VI was isolated from Sample 4. BLASTN analysis of the 16S rRNA gene sequences of E-VI demonstrated significant alignment with the sequences of *Kosakonia oryzendophytica* with 99% identities. Using BLASTX analysis, the translated 360 bp gene fragment corresponded to the nitrogenase reductase, with an identity of 97%. Similar to E-I, the sequencing result is reliable since the spaces between the peaks were evenly distributed, as observed from the electropherogram shown in Appendix A (c). The optimised annealing temperature to PCR-amplify the *nif*H gene from E-VI was the same as E-I, which was 58 °C. Due to the complementary sequences between the n*if*H primers and the targeted *nif*H sequence, specific binding could occur. Therefore, the targeted *nif*H gene is amplified at a relatively high annealing temperature of 58 °C.

Kosakonia oryzendophytica was previously known as *Enterobacter oryzendophyticus* (Hardoim et al., 2013) and was reclassified by Li et al. (2016) based on distinctions in some partial gene sequences. *K. oryzendophytica* belongs to the *Enterobacteriaceae* family and is a Gram-negative rod-shaped bacterium that is facultatively anaerobic, catalase-positive, and oxidase-negative. Due to the false result of Gram staining (Table 4.2), the biochemical tests were not performed on E-VI.

The presence of *nif*H genes in *K. oryzendophytica* (Hardoim et al., 2013), *K. oryzae* (Peng et al., 2009), *K. radicincitans* (Singh et al., 2020a), and *K. arachidis* (Singh et al., 2021) has been reported. In addition, *K. oryzae* was found to promote the growth of rice plants, whereas *K. radicincitans* and *K. arachidis* can enhance the growth of sugarcane.

5.7 False-Positive Results

5.7.1 Growth of Non-Diazotrophic Bacteria on Nitrogen-Free Medium

Theoretically, non-diazotrophs should not grow on nitrogen-free media due to the absence of available nitrogen sources. However, it is very difficult to eliminate traces of nitrogen in nitrogen-free media, as the glassware or distilled water might contain minimal nitrogen sources. The impurities might also be present in commercial agar powder, which are challenging to completely remove during the purification of agar (Scholten and Pierik, 1998). Hence, trace nitrogen present in the Burk's agar might have supported the survival of 85% of the bacterial isolates that are not diazotrophs in this project. Similar to a study by Chowdhury et al. (2007), most bacteria isolated from the nitrogen-free media were later identified as non-diazotrophs, as no amplicon of approximately 360 bp was observed after PCR amplification using *nif*H primers. These bacteria are classified as nitrogen-scavenging bacteria that can take advantage of the nitrogen traces or even the fixed nitrogen from diazotrophs on the same culture plate (Hurek et al., 1989). Furthermore, they can easily acquire atmospheric ammonia when growing on an agar surface as their nitrogen source (Hill and

Postgate, 1969). Therefore, molecular detection methods must be performed to avoid false-positive results.

5.7.2 Gram Staining

The false-positive Gram staining results are considered an issue in this project. Table 4.2 shows that bacterial isolates E-I and E-VI were identified as Gram-positive bacteria after proper Gram staining procedures. However, the 16S rRNA gene sequences of E-I and E-VI demonstrated as *Dickeya zeae* and *Kosakonia oryzendophytica*, respectively. In fact, both species are Gram-negative bacteria that belong to the family *Enterobacteriaceae*. In theory, the decolourising agent is applied to the smear to dissolve the outer membrane of Gram-negative bacteria. In this case, the crystal violet dye can be released, and the cells can be then counterstained with Safranin to appear pink. However, insufficient exposure time of the bacterial smear with the decolourising agent might lead to the retaining of crystal violet in the cells. Additionally, a thick smear preparation might be another contributing factor. The crystal violet might be difficult to release from the Gram-negative bacterial cells due to the overlapping of cells (Oethinger et al., 2011). Therefore, a false-positive result is observed.

5.8 Future Studies

To facilitate the isolation of diazotrophs instead of the nitrogen-scavengers, the nitrogen-free media could be added with a bromothymol blue indicator. Colour change from green to blue could be observed around the colonies of nitrogen-fixers, due to the pH change caused by the accumulated ammonium ions in the culture medium (Hingole and Pathak, 2016). In this case, the nitrogen fixers could be directly selected from the culture plates to avoid the waste of time and materials for the following screening procedures. Additionally, the acetylene reduction assay (ARA) could be performed to determine the presence of nitrogenase among the potential bacterial isolates. Nitrogenase enzymes not only reduce dinitrogen gas into ammonia but also catalyse the reduction of acetylene into ethylene. Through ARA, the formation of ethylene is detected using the gas chromatography technique (Montes-Luz et al., 2023). On the other hand, the detection of the *nif*D or *nif*K gene concurrently with the *nif*H gene could be considered. The *nif*DK genes encode another essential component of the nitrogenase. Therefore, the presence of two respective bands of *nif* genes could provide a more reliable determination of potential diazotrophs.

CHAPTER 6

CONCLUSION

Among twenty bacteria isolated from four sampling sites in this project, only three of them demonstrated the presence of the approximate 360 bp band after PCR amplification using *nif*H primers. The respective 16S rRNA genes of the three bacteria, designated as N-6, E-I, and E-VI, were PCR-amplified and sequenced. The BLASTN results of N-6 showed the highest similarity to the 16S rRNA gene sequence of *Bacillus luciferensis*, but the detection of the *nif*H gene was not successful in N-6. In fact, this species has not been reported to possess nitrogen fixing ability. On the other hand, the 16S rRNA gene sequence of E-I highly matches the sequence of *Dickeya zeae*, whereas the result for E-VI corresponded to *Kosakonia oryzendophytica*. Certain strains of these two bacteria species are nitrogen fixing bacteria. In fact, the BLASTX results of the amplified *nif*H gene for both bacteria corresponded to nitrogenase reductase. In this study, obtaining false-positive results has been a concern in the bacterial isolation and Gram staining procedures. For the isolation of potential diazotrophs, a nitrogen-free media supplemented with the bromothymol blue indicator could effectively prevent false-positive results, whereas the false-positive Gram staining results could be avoided by preparing a thin smear and wisely increasing the duration of the decolourisation step. In future studies, the acetylene reduction assay and the detection of the *nif*DK genes along with the *nif*H gene could be performed, to efficiently determine the potential diazotrophs.

REFERENCES

Abdul Rahman, N.S.N., Abdul Hamid, N.W. and Nadarajah, K., 2021. Effects of abiotic stress on soil microbiome. *International Journal of Molecular Sciences*, [e-journal] 22(16). https://doi.org/10.3390/ijms22169036.

Al-Kaisi, M.M., Lal, R., Olson, K.R. and Lowery, B., 2017. Fundamentals and functions of soil environment. *Soil Health and Intensification of Agroecosytems*, [e-journal]. pp.1–23. https://doi.org/10.1016/B978-0-12-805317-1.00001-4.

Ames, N.J., Ranucci, A., Moriyama, B. and Wallen, G.R., 2017. The human microbiome and understanding the 16S rRNA gene in translational nursing science. *Nursing Research*, [e-journal] 66(2), pp.184–197. https://doi.org/10.1097/NNR.0000000000000212.

Angel, R., Nepel, M., Panhölzl, C., Schmidt, H., Herbold, C.W., Eichorst, S.A. and Woebken, D., 2018. Evaluation of primers targeting the diazotroph functional gene and development of NifMAP – a bioinformatics pipeline for analyzing *nif*H amplicon data. *Frontiers in Microbiology*, [e-journal] 9. https://doi.org/10.3389/fmicb.2018.00703.

Azaroual, S.E., Kasmi, Y., Aasfar, A., El Arroussi, H., Zeroual, Y., El Kadiri, Y., Zrhidri, A., Elfahime, E., Sefiani, A. and Meftah Kadmiri, I., 2022. Investigation of bacterial diversity using 16S rRNA sequencing and prediction of its functionalities in Moroccan phosphate mine ecosystem. *Scientific Reports*, [e-journal] 12(1). https://doi.org/10.1038/s41598-022-07765-5.

Barghouthi, S.A., 2011. A universal method for the identification of bacteria based on general PCR primers. *Indian Journal of Microbiology*, [e-journal] 51, pp.430–444. https://doi.org/10.1007/s12088-011-0122-5.

Basu, S., Kumar, G., Chhabra, S. and Prasad, R., 2021. Role of soil microbes in biogeochemical cycle for enhancing soil fertility. *New and Future Developments in Microbial Biotechnology and Bioengineering*, [e-journal] pp.149–157. https://doi.org/10.1016/B978-0-444-64325-4.00013-4.

Bernhard, A., 2010. The nitrogen cycle: Processes, players, and human impact. *Nature Education*, [online] Available at: <https://www.nature.com/scitable/knowledge/library/the-nitrogen-cycle-processes-players-and-human-15644632/> [Accessed 1 February 2024].

Bhattarai, A., Bhattarai, B. and Pandey, S., 2015. Variation of soil microbial population in different soil horizons. *Journal of Microbiology & Experimentation*, [e-journal] 2(2), pp.75–78. https://doi.org/10.15406/jmen.2015.02.00044.

Bishop, P.E., Jarlenski, D.M. and Hetherington, D.R., 1980. Evidence for an alternative nitrogen fixation system in *Azotobacter vinelandii*. *Proceedings of the National Academy of Sciences*, [e-journal] 77(12), pp.7342–7346. https://doi.org/10.1073/pnas.77.12.7342.

Boluk, G., Arizala, D., Dobhal, S., Zhang, J., Hu, J., Alvarez, A.M. and Arif, M., 2021. Genomic and phenotypic biology of novel strains of *Dickeya zeae* isolated from pineapple and taro in Hawaii: Insights into genome plasticity, pathogenicity, and virulence determinants. *Frontiers in Plant Science*, [e-journal] 12. https://doi.org/10.3389/fpls.2021.663851.

Bonnet, S., Benavides, M., Le Moigne, F.A.C., Camps, M., Torremocha, A., Grosso, O., Dimier, C., Spungin, D., Berman-Frank, I., Garczarek, L. and Cornejo-Castillo, F.M., 2023. Diazotrophs are overlooked contributors to carbon and nitrogen export to the deep ocean. *The International Society for Microbial Ecology (ISME) Journal*, [e-journal] 17(1), pp.47–58. https://doi.org/10.1038/s41396-022-01319-3.

Burgess, B.K. and Lowe, D.J., 1996. Mechanism of molybdenum nitrogenase. *Chemical Reviews*, [e-journal] 96(7), pp.2983–3012. https://doi.org/10.1021/cr950055x.

Burk, D. and Lineweaver, H., 1930. The influence of fixed nitrogen on *Azotobacter*. *Journal of Bacteriology*, [e-journal] 19(6), pp.389–414. https://doi.org/10.1128/jb.19.6.389-414.1930.

Chan, O.C., Yang, X., Fu, Y., Feng, Z., Sha, L., Casper, P. and Zou, X., 2006. 16S rRNA gene analyses of bacterial community structures in the soils of evergreen broad-leaved forests in south-west China. *Federation of European Microbiological Societies (FEMS) Microbiology Ecology*, [e-journal] 58(2), pp.247–259. https://doi.org/10.1111/j.1574-6941.2006.00156.x.

Chisnell, J.R., Premakumar, R. and Bishop, P.E., 1988. Purification of a second alternative nitrogenase from a *nif*HDK deletion strain of *Azotobacter vinelandii*. *Journal of Bacteriology*, [e-journal] 170(1), pp.27–33. https://doi.org/10.1128/jb.170.1.27-33.1988.

Chowdhury, S.P., Schmid, M., Hartmann, A. and Tripathi, A.K., 2007. Identification of diazotrophs in the culturable bacterial community associated with roots of *Lasiurus sindicus*, a perennial grass of Thar Desert, India. *Microbial Ecology*, [e-journal] 54, pp.82–90. https://doi.org/10.1007/s00248-006-9174-1.

Clarridge, J.E., 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*, [e-journal] 17(4), pp.840–862. https://doi.org/10.1128/CMR.17.4.840-862.2004.

Coelho, M.R.R., Marriel, I.E., Jenkins, S.N., Lanyon, C. V., Seldin, L. and O'Donnell, A.G., 2009. Molecular detection and quantification of *nif*H gene sequences in the rhizosphere of sorghum (*Sorghum bicolor*) sown with two levels of nitrogen fertilizer. *Applied Soil Ecology*, [e-journal] 42(1), pp.48–53. https://doi.org/10.1016/j.apsoil.2009.01.010.

Daesch, G. and Mortenson, L.E., 1972. Effect of ammonia on the synthesis and function of the N_2-fixing enzyme system in *Clostridium pasteurianum*. *Journal of Bacteriology*, [e-journal] 110(1), pp.103–109. https://doi.org/10.1128/jb.110.1.103-109.1972.

Dashti, A.A., Jadaon, M.M., Abdulsamad, A.M. and Dashti, H.M., 2009. Heat treatment of bacteria: A simple method of DNA extraction for molecular techniques. *The Journal of the Kuwait Medical Association*, [online] Available at: <https://www.researchgate.net/publication/266888615_Heat_Treatment_of_Bacteria_A_Simple_Method_of_DNA_Extraction_for_Molecular_Techniques> [Accessed 14 January 2024].

Ding, Y., Wang, J., Liu, Y. and Chen, S., 2005. Isolation and identification of nitrogen-fixing bacilli from plant rhizospheres in Beijing region. *Journal of Applied Microbiology*, [e-journal] 99(5), pp.1271–1281. https://doi.org/10.1111/j.1365-2672.2005.02738.x.

Dobbelaere, S., Vanderleyden, J. and Okon, Y., 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. *Critical Reviews in Plant Sciences*, [e-journal] 22(2), pp.107–149. https://doi.org/10.1080/713610853.

Dorman, C.J., 2009. Global regulators and environmental adaptation in Gram-negative pathogens. *Clinical Microbiology and Infection*, [e-journal] 15(1), pp.47–50. https://doi.org/10.1111/j.1469-0691.2008.02684.x.

Dos Santos, P.C., Fang, Z., Mason, S.W., Setubal, J.C. and Dixon, R., 2012. Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. *BioMed Central (BMC) Genomics*, [online] Available at: <http://www.biomedcentral.com/1471-2164/13/162> [Accessed 5 February 2024].

Doula, M.K. and Sarris, A., 2016. Soil environment. *Environment and Development*, [e-journal] pp.213–286. https://doi.org/10.1016/B978-0-444-62733-9.00004-6.

Eady, R.R., 1996. Structure−function relationships of alternative nitrogenases. *Chemical Reviews*, [e-journal] 96(7), pp.3013–3030. https://doi.org/10.1021/cr950057h.

Fallik, E., Chan, Y.K. and Robson, R.L., 1991. Detection of alternative nitrogenases in aerobic Gram-negative nitrogen-fixing bacteria. *Journal of Bacteriology*, [e-journal] 173(1), pp.365–371. https://doi.org/10.1128/jb.173.1.365-371.1991.

Fanin, N., Kardol, P., Farrell, M., Nilsson, M.-C., Gundale, M.J. and Wardle, D.A., 2019. The ratio of Gram-positive to Gram-negative bacterial PLFA markers as an indicator of carbon availability in organic soils. *Soil Biology and Biochemistry*, [e-journal] 128, pp.111–114. https://doi.org/10.1016/j.soilbio.2018.10.010.

Farmer, J.J., Farmer, M.K. and Holmes, B., 2010. The *Enterobacteriaceae*: General characteristics. *Topley & Wilson's Microbiology and Microbial Infections*, [e-journal] pp.2–43. https://doi.org/10.1002/9780470688618.taw0051.

Gaby, J.C. and Buckley, D.H., 2012. A comprehensive evaluation of PCR primers to amplify the *nif*H gene of nitrogenase. *Public Library of Science (PLOS) One*, [e-journal] 7(7). https://doi.org/10.1371/journal.pone.0042149.

Gallon, J.R., 1981. The oxygen sensitivity of nitrogenase: A problem for biochemists and micro-organisms. *Trends in Biochemical Sciences*, [e-journal] 6, pp.19–23. https://doi.org/10.1016/0968-0004(81)90008-6.

Ghorai, P. and Ghosh, D., 2023. Screening and isolation of potential nitrogen-fixing *Enterobacter* sp. GG1 from mangrove soil with its accelerated impact on green chili plant (*Capsicum frutescens* L.) growth amelioration. *Journal of Applied Biology & Biotechnology*, [e-journal] 11(6). https://doi.org/10.7324/JABB.2023.139674.

Gugliandolo, C., Lentini, V., Spanò, A. and Maugeri, T.L., 2011. Conventional and molecular methods to detect bacterial pathogens in mussels. *Letters in Applied Microbiology*, [e-journal] 52(1), pp.15–21. https://doi.org/10.1111/j.1472-765X.2010.02959.x.

Ha, T.T.T., Lam, T.T., Huyen, N.T. and Canh, N.X., 2018. Characterization and identification of nitrogen-fixing bacteria isolated from agricultural soil. *Vietnam Journal of Science, Technology and Engineering*, [e-journal] 60(3). https://doi.org/10.31276/VJSTE.60(3).48.

Hadija, Kuswinanti, T., Jayadi, M. and Larekeng, S.H., 2021. Isolation, characterization and identification of nitrogen fixing bacteria with organic fertilizer applications in paddy soil. *IOP Conference Series: Earth and Environmental Science*, [e-journal] 807(2). https://doi.org/10.1088/1755-1315/807/2/022024.

Hales, B.J., Case, E.E., Morningstar, J.E., Dzeda, M.F. and Mauterer, L.A., 1986. Isolation of a new vanadium-containing nitrogenase from *Azotobacter vinelandii*. *Biochemistry*, [e-journal] 25(23), pp.7251–7255. https://doi.org/10.1021/bi00371a001.

Hannula, S.E., Heinen, R., Huberty, M., Steinauer, K., De Long, J.R., Jongen, R. and Bezemer, T.M., 2021. Persistence of plant-mediated microbial soil legacy effects in soil and inside roots. *Nature Communications*, [e-journal] 12. https://doi.org/10.1038/s41467-021-25971-z.

Hardoim, P.R., Nazir, R., Sessitsch, A., Elhottová, D., Korenblum, E., Van Overbeek, L.S. and Van Elsas, J.D., 2013. The new species *Enterobacter oryziphilus* sp. nov. and *Enterobacter oryzendophyticus* sp. nov. are key inhabitants of the endosphere of rice. *BioMed Central (BMC) Microbiology*, [e-journal] 13. https://doi.org/10.1186/1471-2180-13-164.

Hayat, R., Ali, S., Amara, U., Khalid, R. and Ahmed, I., 2010. Soil beneficial bacteria and their role in plant growth promotion: A review. *Annals of Microbiology*, [e-journal] 60, pp.579–598. https://doi.org/10.1007/s13213-010-0117-1.

Hernandez, J.A., George, S.J. and Rubio, L.M., 2009. Molybdenum trafficking for nitrogen fixation. *Biochemistry*, [e-journal] 48(41), pp.9711–9721. https://doi.org/10.1021/bi901217p.

Hill, S. and Postgate, J.R., 1969. Failure of putative nitrogen-fixing bacteria to fix nitrogen. *The Journal of General Microbiology*, [e-journal] 58(2). https://doi.org/10.1099/00221287-58-2-277.

HiMedia Laboratories, 2020. *Burk's medium*. [online] Available at: <www.himedialabs.com> [Accessed 14 January 2024].

Hingole, S.S. and Pathak, A.P., 2016. Isolation of halotolerant plant growth promoting *Klebsiella pneumoniae* from Tuppa, Nanded, Maharashtra. *International Journal of Innovative Biological Research*, [e-journal] 5(1), pp.5–9. https://doi.org/10.1111/ijibr.v5i1.104.

Hurek, T., Reinhold, B., Grimm, B., Fendrik, I. and Niemann, E.-G., 1989. Occurrence of effective nitrogen-scavenging bacteria in the rhizosphere of kallar grass. In: Skinner, F.A., Boddey, R.M., Fendrik, I. (eds). *Nitrogen Fixation with Non-Legumes*. Dordrecht: Springer Netherlands, pp.321–330. https://doi.org/10.1007/978-94-009-0889-5_36.

Imran, A., Hakim, S., Tariq, M., Nawaz, M.S., Laraib, I., Gulzar, U., Hanif, M.K., Siddique, M.J., Hayat, M., Fraz, A. and Ahmad, M., 2021. Diazotrophs for lowering nitrogen pollution crises: Looking deep into the roots. *Frontiers in Microbiology*, [e-journal] 12. https://doi.org/10.3389/fmicb.2021.637815.

Itoh, K., Ohashi, K., Yakai, N., Adachi, F. and Hayashi, S., 2019. Changes in acetylene reduction activities and *nif*H genes associated with field-grown sweet potatoes with different nursery farmers and cultivars. *Horticulturae*, [e-journal] 5(3). https://doi.org/10.3390/horticulturae5030053.

Janda, J.M. and Abbott, S.L., 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *Journal of Clinical Microbiology*, [e-journal] 45(9), pp.2761–2764. https://doi.org/10.1128/JCM.01228-07.

Jensen, H.L., 1942. Nitrogen fixation in leguminous plants. II. Is symbiotic nitrogen fixation influenced by *Azotobacter*? *Proceedings of The Linnean Society of New South Wales*, pp.205–212.

Jha, A., Saxena, J. and Sharma, V., 2013. Investigation on phosphate solubilization potential of agricultural soil bacteria as affected by different phosphorus sources, temperature, salt, and pH. *Communications in Soil Science and Plant Analysis*, [e-journal] 44(16), pp.2443–2458. https://doi.org/10.1080/00103624.2013.803557.

Johnston, C., Ufnar, J.A., Griffith, J.F., Gooch, J.A. and Stewart, J.R., 2010. A real-time qPCR assay for the detection of the *nif*H gene of *Methanobrevibacter smithii*, a potential indicator of sewage pollution. *Journal of Applied Microbiology*, [e-journal] 109(6), pp.1946–1956. https://doi.org/10.1111/j.1365-2672.2010.04824.x.

Kawaka, F., 2022. Characterization of symbiotic and nitrogen fixing bacteria. *Applied Microbiology and Biotechnology (AMB) Express*, [e-journal] 12. https://doi.org/10.1186/s13568-022-01441-7.

Kim, S. and Kim, S., 2021. Bacterial pathogen detection by conventional culture-based and recent alternative (polymerase chain reaction, isothermal amplification, enzyme linked immunosorbent assay, bacteriophage amplification, and gold nanoparticle aggregation) methods in food samples: A review. *Journal of Food Safety*, [e-journal] 41(1). https://doi.org/10.1111/jfs.12870.

Kirshtein, J.D., Paerl, H.W. and Zehr, J., 1991. Amplification, cloning, and sequencing of a *nif*H segment from aquatic microorganisms and natural communities. *Applied and Environmental Microbiology*, [e-journal] 57(9), pp.2645–2650. https://doi.org/10.1128/aem.57.9.2645-2650.1991.

Ladha, J.K., Barraquio, W.L. and Watanabe, I., 1983. Isolation and identification of nitrogen-fixing *Enterobacter cloacae* and *Klebsiella planticola* associated with rice plants. *Canadian Journal of Microbiology*, [e-journal] 29(10), pp.1301–1308. https://doi.org/10.1139/m83-203.

Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L. and Pace, N.R., 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences*, [e-journal] 82(20), pp.6955–6959. https://doi.org/10.1073/pnas.82.20.6955.

Law, J.W.-F., Ab Mutalib, N.-S., Chan, K.-G. and Lee, L.-H., 2015. Rapid methods for the detection of foodborne bacterial pathogens: Principles, applications, advantages and limitations. *Frontiers in Microbiology*, [e-journal] 5. https://doi.org/10.3389/fmicb.2014.00770.

Li, C.Y. and Maser, C., 1986. *New and modified techniques for studying nitrogen-fixing bacteria in small mammal droppings*. [online] Available at: <https://web.archive.org/web/20180720084935id_/https://www.fs.fed.us/pnw/pubs/pnw_rn441.pdf> [Accessed 20 February 2024].

Li, C.Y., Zhou, Y.L., Ji, J. and Gu, C.T., 2016. Reclassification of *Enterobacter oryziphilus* and *Enterobacter oryzendophyticus* as *Kosakonia oryziphila* comb. nov. and *Kosakonia oryzendophytica* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, [e-journal] 66(8), pp.2780–2783. https://doi.org/10.1099/ijsem.0.001054.

Lin, L., Li, Z., Hu, C., Zhang, X., Chang, S., Yang, L., Li, Y. and An, Q., 2012. Plant growth-promoting nitrogen-fixing *Enterobacteria* are in association with sugarcane plants growing in Guangxi, China. *Microbes and Environments*, [e-journal] 27(4), pp.391–398. https://doi.org/10.1264/jsme2.ME11275.

Lladó, S., López-Mondéjar, R. and Baldrian, P., 2017. Forest soil bacteria: Diversity, involvement in ecosystem processes, and response to global change. *Microbiology and Molecular Biology Reviews*, [e-journal] 81(2). https://doi.org/10.1128/MMBR.00063-16.

Logan, N.A., Lebbe, L., Verhelst, A., Goris, J., Forsyth, G., Rodriguez-Diaz, M., Heyndrickx, M. and De Vos, P., 2002. *Bacillus luciferensis* sp. nov., from volcanic soil on Candlemas Island, South Sandwich archipelago. *International Journal of Systematic and Evolutionary Microbiology*, [e-journal] 52(6), pp.1985–1989. https://doi.org/10.1099/00207713-52-6-1985.

Lorenz, T.C., 2012. Polymerase chain reaction: Basic protocol plus troubleshooting and optimization strategies. *Journal of Visualized Experiments*, [e-journal] 63. https://doi.org/10.3791%2F3998.

Lucena-Aguilar, G., Sánchez-López, A.M., Barberán-Aceituno, C., Carrillo-Ávila, J.A., López-Guerrero, J.A. and Aguilar-Quesada, R., 2016. DNA source selection for downstream applications based on DNA quality indicators analysis. *Biopreservation and Biobanking*, [e-journal] 14(4), pp.264–270. https://doi.org/10.1089/bio.2015.0064.

Magistrado, P., 2001. Isolation and polymerase chain reaction-based detection of *Campylobacter jejuni* and *Campylobacter coli* from poultry in the Philippines. *International Journal of Food Microbiology*, [e-journal] 70(1–2), pp.197–206. https://doi.org/10.1016/S0168-1605(01)00537-2.

Malgorzata, G., 2015. *Characterization of Dickeya solani strains and identification of bacterial and plant signals involved in the induction of virulence*. PhD. University of Gdansk and Medical University of Gdansk.

Montes-Luz, B., Conrado, A.C., Ellingsen, J.K., Monteiro, R.A., de Souza, E.M. and Stacey, G., 2023. Acetylene reduction assay: A measure of nitrogenase activity in plants and bacteria. *Current Protocols*, [e-journal] 3(5). https://doi.org/10.1002/cpz1.766.

Morgan, J.B. and Connolly, E.L., 2013. Plant-soil interactions: Nutrient uptake. *Nature Education*, [online] Available at: <https://www.nature.com/scitable/knowledge/library/plant-soil-interactions-nutrient-uptake-105289112/> [Accessed 4 February 2024].

Müller, A., Schneider, K., Knüttel, K. and Hagen, W.R., 1992. EPR spectroscopic characterization of an ‘iron only] nitrogenase *S* = 3/2 spectrum of component 1 isolated from *Rhodobacter capsulatus*. *Federation of European Biochemical Societies (FEBS) Letters*, [e-journal] 303(1), pp.36–40. https://doi.org/10.1016/0014-5793(92)80472-S.

Mylona, P., Pawlowski, K. and Bisseling, T., 1995. Symbiotic nitrogen fixation. *The Plant Cell*, [e-journal] 7(7), p.869–885. https://doi.org/10.2307/3870043.

Mylona, P.V., Premakumar, R., Pau, R.N. and Bishop, P.E., 1996. Characteristics of *orf*1 and *orf*2 in the *anf*HDGK genomic region encoding nitrogenase 3 of *Azotobacter vinelandii*. *Journal of Bacteriology*, [e-journal] 178(1), pp.204–208. https://doi.org/10.1128/jb.178.1.204-208.1996.

Nakatsu, C.H., Byappanahalli, M.N. and Nevers, M.B., 2019. Bacterial community 16S rRNA gene sequencing characterizes riverine microbial impact on Lake Michigan. *Frontiers in Microbiology*, [e-journal] 10. https://doi.org/10.3389/fmicb.2019.00996.

Nosrati, R., Owlia, P., Saderi, H., Rasooli, I. and Malboobi, M.A., 2014. Phosphate solubilization characteristics of efficient nitrogen fixing soil *Azotobacter* strains. *Iranian Journal of Microbiology*, [online] Available at: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4367947/> [Accessed 4 February 2024].

Octavia, S. and Lan, R., 2014. The Family *Enterobacteriaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompsom, F. (eds). *The Prokaryotes*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp.225–286. https://doi.org/10.1007/978-3-642-38922-1_167.

Oethinger, M., Warner, D.K., Schindler, S.A., Kobayashi, H. and Bauer, T.W., 2011. Diagnosing periprosthetic infection: False-positive intraoperative Gram stains. *Clinical Orthopaedics & Related Research*, [e-journal] 469(4), pp.954–960. https://doi.org/10.1007/s11999-010-1589-9.

Ogunseitan, O., 2005. *Microbial diversity*. United Kingdom: Blackwell Publishing.

Oliver, J.D., 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. *Federation of European Microbiological Societies (FEMS) Microbiology Reviews*, [e-journal] 34(4), pp.415–425. https://doi.org/10.1111/j.1574-6976.2009.00200.x.

Omar, S.A. and Abd-Alla, M.H., 1992. Effect of pesticides on growth, respiration and nitrogenase activity of *Azotobacter* and *Azospirillum*. *World Journal of Microbiology & Biotechnology*, [e-journal] 8, pp.326–328. https://doi.org/10.1007/BF01201891.

Orr, C.H., James, A., Leifert, C., Cooper, J.M. and Cummings, S.P., 2011. Diversity and activity of free-living nitrogen-fixing bacteria and total bacteria in organic and conventionally managed soils. *Applied and Environmental Microbiology*, [e-journal] 77(3), pp.911–919. https://doi.org/10.1128/AEM.01250-10.

Peng, G., Zhang, W., Luo, H., Xie, H., Lai, W. and Tan, Z., 2009. *Enterobacter oryzae* sp. nov., a nitrogen-fixing bacterium isolated from the wild rice species *Oryza latifolia*. *International Journal of Systematic and Evolutionary Microbiology*, [e-journal] 59(7), pp.1650–1655. https://doi.org/10.1099/ijs.0.65484-0.

Qiagen, 2020. Protocol: QIAquick gel extraction using a microcentrifuge. In: *QIAquick Spin Handbook 01/2020*, pp.30–32.

Ranganayaki, S. and Mohan, C., 1981. Effect of sodium molybdate on microbial fixation of nitrogen. *Zeitschrift für allgemeine Mikrobiologie*, [e-journal] 21(8), pp.607–610. https://doi.org/10.1002/jobm.19810210807.

Rascio, N. and La Rocca, N., 2013. Biological nitrogen fixation. *Reference Module in Earth Systems and Environmental Sciences*, [e-journal] https://doi.org/10.1016/B978-0-12-409548-9.00685-0.

Raymond, J., Siefert, J.L., Staples, C.R. and Blankenship, R.E., 2004. The natural history of nitrogen fixation. *Molecular Biology and Evolution*, [e-journal] 21(3), pp.541–554. https://doi.org/10.1093/molbev/msh047.

Reimer, L.C., Sardà Carbasse, J., Koblitz, J., Ebeling, C., Podstawka, A. and Overmann, J., 2022. Bac*Dive* in 2022: The knowledge base for standardized bacterial and archaeal data. *Nucleic Acids Research*, [e-journal] 50(1), pp.741–746. https://doi.org/10.1093/nar/gkab961.

Ritika, B. and Utpal, D., 2014. Biofertilizer, a way towards organic agriculture: A review. *African Journal of Microbiology Research*, [e-journal] 8(24), pp.2332–2343. https://doi.org/10.5897/AJMR2013.6374.

Robson, R.L., 1979. Characterization of an oxygen-stable nitrogenase complex isolated from *Azotobacter chroococcum*. *Biochemical Journal*, [e-journal] 181(3), pp.569–575. https://doi.org/10.1042/bj1810569.

Robson, R.L., Woodley, P.R., Pau, R.N. and Eady, R.R., 1989. Structural genes for the vanadium nitrogenase from *Azotobacter chroococcum*. *The European Molecular Biology Organization (EMBO) Journal*, [e-journal] 8(4), pp.1217–1224. https://doi.org/10.1002/j.1460-2075.1989.tb03495.x.

Samson, R., Legendre, J.B., Christen, R., Saux, M.F.-L., Achouak, W. and Gardan, L., 2005. Transfer of *Pectobacterium chrysanthemi* (Burkholder et al. 1953) Brenner et al. 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen. nov. as *Dickeya chrysanthemi* comb. nov. and *Dickeya paradisiaca* comb. nov. and delineation of four novel species, *Dickeya dadantii* sp. nov., *Dickeya dianthicola* sp. nov., *Dickeya dieffenbachiae* sp. nov. and *Dickeya zeae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, [e-journal] 55(4), pp.1415–1427. https://doi.org/10.1099/ijs.0.02791-0.

Scholten, H.J. and Pierik, R.L.M., 1998. Agar as a gelling agent: Chemical and physical analysis. *Plant Cell Reports*, [e-journal] 17, pp.230–235. https://doi.org/10.1007/s002990050384.

Schubert, E., Mengel, K. and Schubert, S., 1990. Soil pH and calcium effect on nitrogen fixation and growth of board bean. *Agronomy Journal*, [e-journal] 82(5), pp.969–972. https://doi.org/10.2134/agronj1990.00021962008200050026x.

Seefeldt, L.C., Hoffman, B.M. and Dean, D.R., 2009. Mechanism of Mo-dependent nitrogenase. *Annual Review of Biochemistry*, [e-journal] 78, pp.701–722. https://doi.org/10.1146/annurev.biochem.78.070907.103812.

Seshadri, S., 2000. Solubilization of inorganic phosphates by *Azospirillum halopraeferans*. *Current Science*, [online] Available at: <https://www.researchgate.net/publication/285013288> [Accessed 4 February 2024].

Silhavy, T.J., Kahne, D. and Walker, S., 2010. The bacterial cell envelope. *Cold Spring Harbor Perspectives in Biology*, [e-journal] 2(5). https://doi.org/10.1101/cshperspect.a000414.

Silva, P. da and Nahas, E., 2002. Bacterial diversity in soil in response to different plans, phosphate fertilizers and liming. *Brazilian Journal of Microbiology*, [e-journal] 33(4). https://doi.org/10.1590/S1517-83822002000400005.

Singh, R.K., Singh, P., Guo, D.-J., Sharma, A., Li, D.-P., Li, X., Verma, K.K., Malviya, M.K., Song, X.-P., Lakshmanan, P., Yang, L.-T. and Li, Y.-R., 2021. Root-derived endophytic diazotrophic bacteria *Pantoea cypripedii* AF1 and *Kosakonia arachidis* EF1 promote nitrogen assimilation and growth in sugarcane. *Frontiers in Microbiology*, [e-journal] 12. https://doi.org/10.3389/fmicb.2021.774707.

Singh, R.K., Singh, P., Li, H.-B., Guo, D.-J., Song, Q.-Q., Yang, L.-T., Malviya, M.K., Song, X.-P. and Li, Y.-R., 2020a. Plant-PGPR interaction study of plant growth-promoting diazotrophs *Kosakonia radicincitans* BA1 and *Stenotrophomonas maltophilia* COA2 to enhance growth and stress-related gene expression in *Saccharum* spp. *Journal of Plant Interactions*, [e-journal] 15(1), pp.427–445. https://doi.org/10.1080/17429145.2020.1857857.

Singh, R.K., Singh, P., Li, H.B., Song, Q.Q., Guo, D.J., Solanki, M.K., Verma, K.K., Malviya, M.K., Song, X.P., Lakshmanan, P., Yang, L.T. and Li, Y.R., 2020b. Diversity of nitrogen-fixing rhizobacteria associated with sugarcane: A comprehensive study of plant-microbe interactions for growth enhancement in *Saccharum* spp. *BioMed Central (BMC) Plant Biology*, [e-journal] 20. https://doi.org/10.1186/s12870-020-02400-9.

Smercina, D.N., Evans, S.E., Friesen, M.L. and Tiemann, L.K., 2019. To fix or not to fix: Controls on free-living nitrogen fixation in the rhizosphere. *Applied and Environmental Microbiology*, [e-journal] 85(6). https://doi.org/10.1128/AEM.02546-18.

Soto-Urzúa, L. and Baca, B.E., 2001. Mechanisms for protecting nitrogenase from inactivation by oxygen. *Revista Latinoamericana de Microbiología*, [online] Available at: <https://pubmed.ncbi.nlm.nih.gov/17061570/> [Accessed 5 February 2024].

Sridevi, M. and Mallaiah, K. V., 2009. Phosphate solubilization by *Rhizobium* strains. *Indian Journal of Microbiology*, [e-journal] 49, pp.98–102. https://doi.org/10.1007/s12088-009-0005-1.

Stella, M., Suhaimi, M., Matthews, S. and Masduki, S., 2010. Selection of suitable growth medium for free-living diazotrophs isolated from compost. *Journal of Tropical Agriculture and Food Science*, [online] Available at: <http://jtafs.mardi.gov.my/jtafs/38-2/Free-living%20Diazotrophs.pdf> [Accessed 5 February 2024].

Subba Rao, N.S., 1977. *Soil microorganisms and plant growth*. New Delhi: Oxford & IBH Publishing Co.

Tan, T.Y., Ui, L.P. and Amir, H.A.G., 2015. Influence of external nitrogen on nitrogenase enzyme activity and auxin production in *Herbaspirillum seropedicae* (Z78). *Tropical Life Sciences Research*, [online] Available at: <https://pubmed.ncbi.nlm.nih.gov/26868594/> [Accessed 5 February 2024].

Tkacz, A., Bestion, E., Bo, Z., Hortala, M. and Poole, P.S., 2020. Influence of plant fraction, soil, and plant species on microbiota: A multikingdom comparison. *American Society for Microbiology*, [e-journal] 11(1). https://doi.org/10.1128/mBio.02785-19.

United Union Environment Programme (UNEP), 2024. *What is nitrogen pollution?* [online] Available at: <https://www.unep.org/interactives/beat-nitrogen-pollution/#:~:text=and%20energy%20sectors.-,Nitrogen%20pollution%20is%20also%20the%20byproduct%20of%20burning%20fossil%20fuels,to%20deplete%20the%20ozone%20layer.> [Accessed 3 February 2024].

Velmourougane, K., Saxena, G. and Prasanna, R., 2017. Plant-microbe interactions in the rhizosphere: Mechanisms and their ecological benefits. In: Singh, D., Singh, H., Prabha, R. (eds). *Plant-Microbe Interactions in Agro-Ecological Perspectives*. Singapore: Springer Singapore. pp.193–219. https://doi.org/10.1007/978-981-10-6593-4_7.

Wagner, S.C., 2011. Biological nitrogen fixation. *Nature Education*, [online] Available at: <https://www.nature.com/scitable/knowledge/library/biological-nitrogen-fixation-23570419/> [Accessed 1 February 2024].

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology*, [e-journal] 63(10), pp.3741–3751. https://doi.org/10.1128%2Faem.63.10.3741-3751.1997.

Wilson, P.W., Hull, J.F. and Burris, R.H., 1943. Competition between free and combined nitrogen in nutrition of *Azotobacter*. *Proceedings of the National Academy of Sciences*, [e-journal] 29(9), pp.289–294. https://doi.org/10.1073/pnas.29.9.289.

Woese, C.R., 1987. Bacterial evolution. *Microbiology and Molecular Biology Reviews*, [e-journal] 51(2), pp.221–271. https://doi.org/10.1128/mr.51.2.221-271.1987.

Xa, L.T. and Nghia, N.K., 2019. Isolation and selection of biological nitrogen fixing and indole-3-acetic acid synthesizing bacteria from different cropping systems in Soc Trang province, Vietnam. *International Journal of Innovative Studies in Sciences and Engineering Technology*, [online] Available at: <https://www.researchgate.net/publication/343106699_Isolation_and_Selection_of_Biological_Nitrogen_Fixing_and_indole-3-_Acetic_Acid_Synthesizing_Bacteria_from_Different_Cropping_Systems_in_Soc_Trang_province_Vietnam> [Accessed 28 February 2024].

Xue, P.-P., Carrillo, Y., Pino, V., Minasny, B. and McBratney, Alex.B., 2018. Soil properties drive microbial community structure in a large scale transect in South Eastern Australia. *Scientific Reports*, [e-journal] 8. https://doi.org/10.1038/s41598-018-30005-8.

Yousuf, J., Thajudeen, J., Rahiman, M., Krishnankutty, S., P. Alikunj, A. and A. Abdulla, M.H., 2017. Nitrogen fixing potential of various heterotrophic *Bacillus* strains from a tropical estuary and adjacent coastal regions. *Journal of Basic Microbiology*, [e-journal] 57(11), pp.922–932. https://doi.org/10.1002/jobm.201700072.

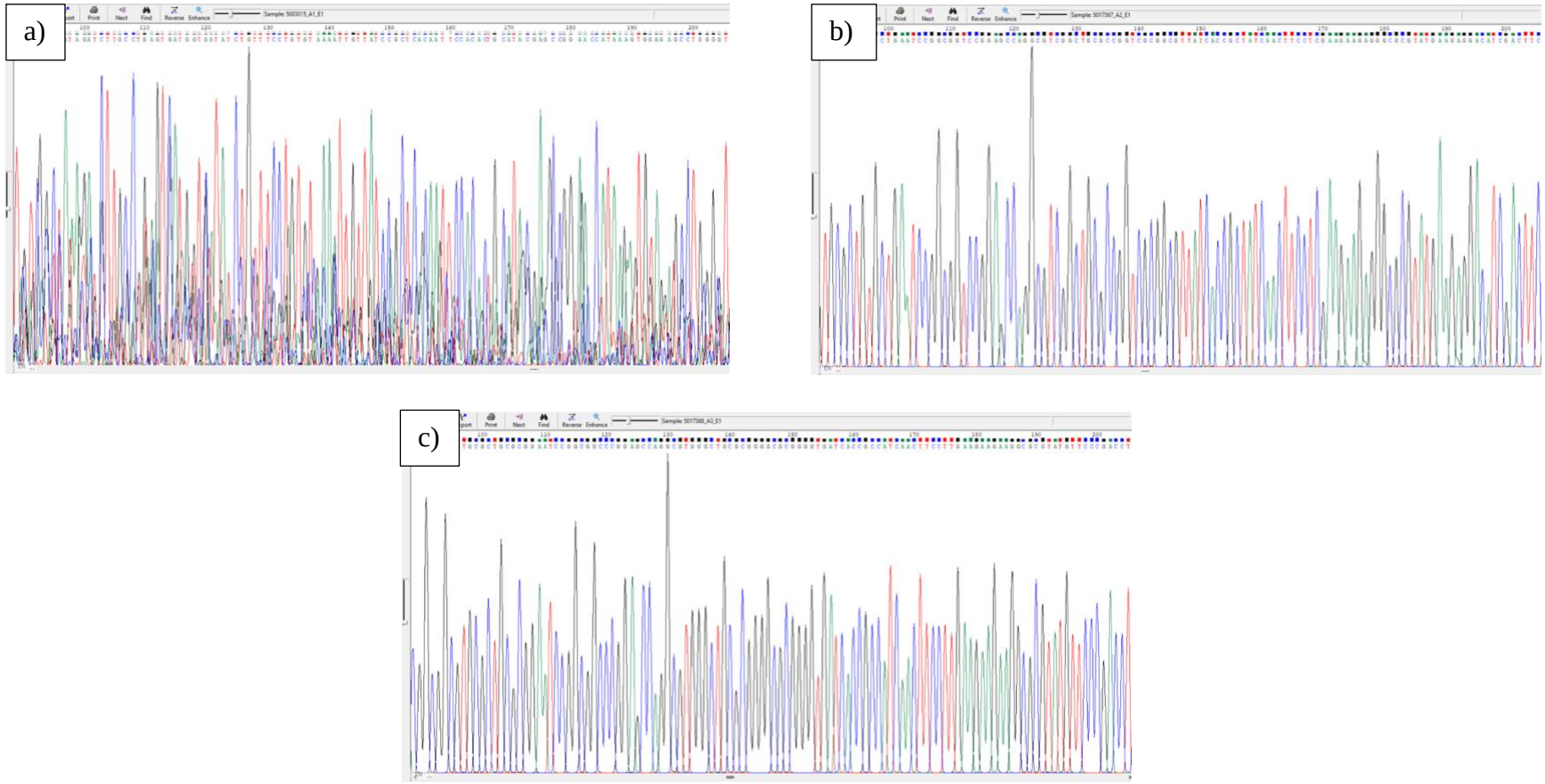
Zehr, J.P., Jenkins, B.D., Short, S.M. and Steward, G.F., 2003. Nitrogenase gene diversity and microbial community structure: A cross-system comparison. *Environmental Microbiology*, [e-journal] 5(7), pp.539–554. https://doi.org/10.1046/j.1462-2920.2003.00451.x.

Zehr, J.P. and Turner, P.J., 2001. Nitrogen fixation: Nitrogenase genes and gene expression. *Methods in Microbiology*, [e-journal] 30, pp.271–286. https://doi.org/10.1016/S0580-9517(01)30049-1.

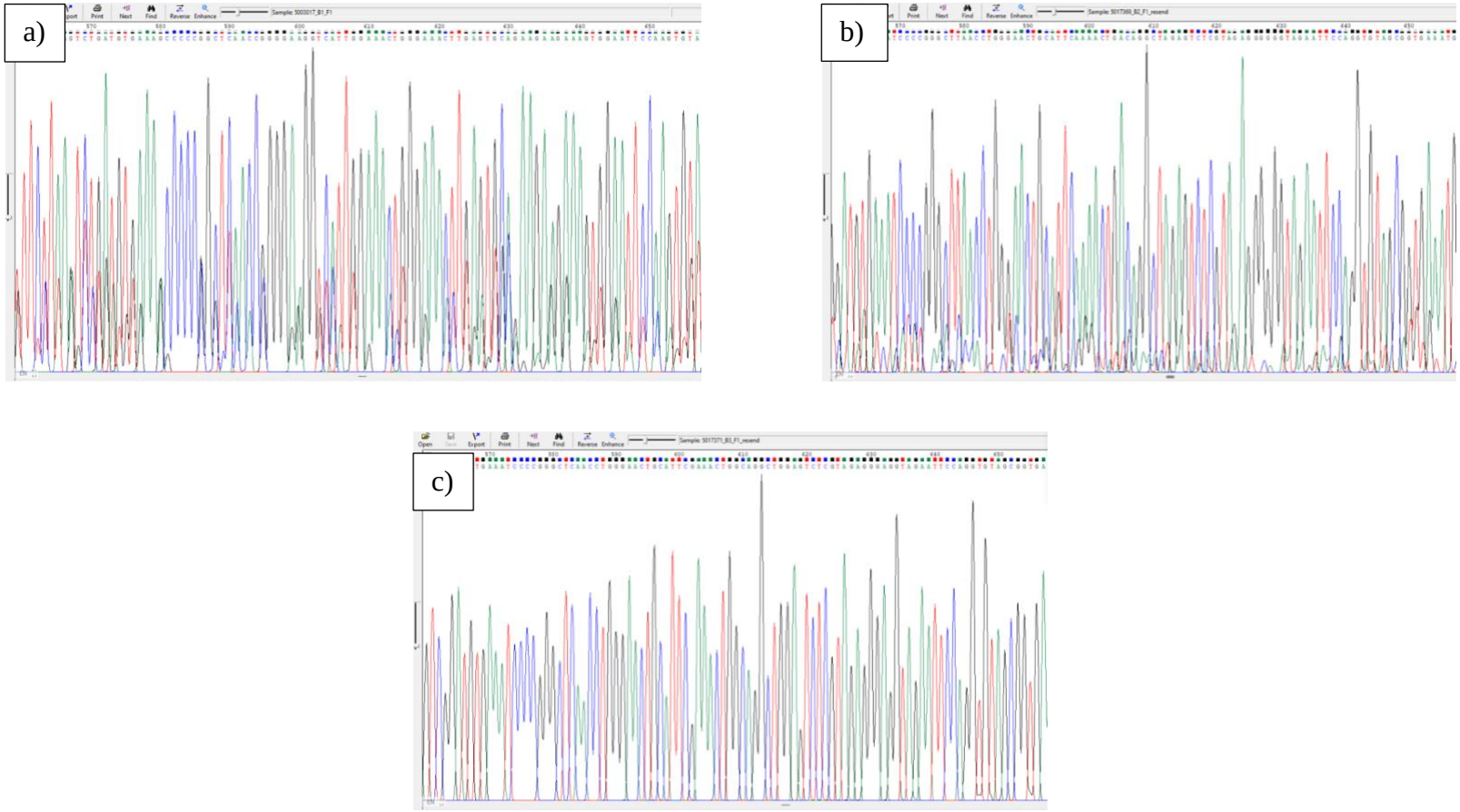
Zhou, J., Ma, M., Guan, D., Jiang, X., Zhang, N., Shu, F., Kong, Y. and Li, J., 2021. Nitrogen has a greater influence than phosphorus on the diazotrophic community in two successive crop seasons in Northeast China. *Scientific Reports*, [e-journal] 11(1). https://doi.org/10.1038/s41598-021-85829-8.

APPENDICES

Appendix A: Electropherograms of the *nif*H gene sequences of a) N-6, b) E-I, and c) E-VI, from 90 bp to 205 bp



Appendix B: Electropherograms of the 16S rRNA gene sequences of a) N-6, b) E-I, and c) E-VI, from 560 bp to 657 bp



Appendix C: The *nif*H gene sequences of bacterial isolates E-I and E-VI

>E-I_nifH1

TGGCGGCGGAAGTCGGCTCGGTGGAGTACCTGGAACTGGAGGACGTGCTGCAAATCGGCTACGGCGGCGTGCGCTGTGCTGAAT
CCGGCGGTCCGGAGCCAGGCGTCGGCTGCGCCGGTCGCGGCGTTATCACCGCTATCAACTTCCTCGAAGAAGAGGGCGCGTATG
AAGAGGACATCGACTTCGTGTTCTACGACGTGCTGGGCGACGTGGTGTGCGGCGGCTTCGCCATGCCTATCCGTGAAAACAAGG
CGCAGGAAATCTACATCGTTTGTTCCGGCGAGATGATGGCCCCA

>E-VI_nifH1

TTATGGAATGGCGGCAGAGTCGGTTCCGTAGAAGACCTGGAACTGGAAGACGTGCTGCAAATCGGTTACGGCGGCGTGCGCTGC
GCGGAATCCGGCGGCCCGGAGCCAGGCGTGGGCTGCGCGGGGCGCGGGGTGATCACCGCCATCAACTTCCTTGAAGAAGAAGG
CGCGTATGTTCCCGACCTCGATTTCGTGTTTTACGACGTGCTCGGCGACGTGGTGTGCGGCGGTTTCGCCATGCCGATCCGTGAA
AACAAGGCGCAGGAGATCTATATCGTCTGTTCCGGCGAAATGATGGCACCA

Appendix D: The 16S rRNA gene sequences of bacterial isolates N-6, E-I and E-VI

>N-6

CAGTCGAGCGGACTAATGGGAGCTTGCTCCCGTTAGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTACCTGTAAGACT
GGGATAACTTCGGGAAACCGGAGCTAATACCGGATGACATAAAGGAACTCCTGTTCCTTTATTGAAAGATGGCTTCGGCTATCAC
TTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGGAACGGCTCACCAAGGGGACCATGCGTAACCGACCTGAAAGGGTG
ATCGGCCACACTGGGACTGAAACACGGCCCAAACTCCTACGGGAGGCAGCAGTAAGGAATCTTCCGCAATGGACCAAAGTCTGA
CCGAACAACCCCCCGTGAGCCATGAAGGCCTTCCGGTCGTAAAGCTCTGTTGTTAAGGAAGAATAAGTGCTAATTGAATAAGCT
GGCACCTTGACCGTGACCTAACCAAAAAGCCACCGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTC
CGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGG
AAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAG
TGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCTGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAG
TACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGTGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGT
TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGC
ACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACA
CGTGCTACAATGGATAGTACAAAGGGTTGCAAGACCGCGAGGTGGAGCTAATCCCATAAAACTATTCTCAGTTCGGATTGTAGG
CTGCAACTCGCCTACATGAAGCCGGAATCACTAGTAATCGCGGATCAGCATGCCNCGNANGTNNNNNN

>E-I

TCGAGCGGCAGCGGGGGGAAGCTTGCTTCCCCGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAG
GGGGATAACTACTGGAAACGGTAGCTAATACCGCAAAATGTCGCAAGACCAAGGAGGGGGACCTTCGGGCCTCTTGCCATCGGA
TGTGCCCAGAGGGGATTAGCTAGTAAGTGGGGTAAAGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGACAGGATGACCAGCC
ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCACCC
ATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGGAGCAAGCTTAATACGTCTGTTCATT
GACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATGAC
TGGGCGTAAAGCGCACGCAGGCGGTCTGTTAAGTTGGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTCAAAACTGACA
GGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGG
CGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAA
ACGATGTCGATTTGGAGGTTGTGGTCTTGAACCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCA
AGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTAC
CTACTCTTGACATCCACAGGATCCGGCAGAGATGCTGGAGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTAGTCAG
CTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCACTTTGGGTGGGAACTCAAGG
GAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACTAGGGCTACACACGTGCTAC
AATGGCGTATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACT
CGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGNTNNCNGNNT

>E-VI

AGTCGGACGGTAGCACAGAGGAGCTTGCTCCTCCTGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATG
GAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATC
AGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGC
AGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCACAGC
AATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAA
TTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACT
GGCAGGCTGGAGTCTCGTAGAGGGAGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
AAGGCGGCCTCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT
GTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGG
CCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAAC
CTTACCTGGTCTTGACATCCACAGAAGTTTCCAGAGATGGGAATGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTC
AAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGT
GCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTG
CAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCANNTGCCNCNNGNNNC