CHAPTER 4

RESULTS

4.1 Metagenomics Sequencing and Comparisons Among Mangrove Soil 1, 2 and 3

This research was conducted to develop a biofertilizer containing the mixing of specific mangrove-associated bacteria that could increase the growth rate and protein contents of duckweed plants. At the start of this study, mWGS sequencing technology was used to gain insights into species biodiversity, functions, and pathways within three soil samples collected from the freshwater riverine area of Lukut mangrove. With the rapid advancements in sequencing and informatics technologies, metagenomic investigations using Next Generation Sequencing (NGS) have become increasingly renowned. NGS, as a fundamental strategy for probing community diversity and characteristics, is gaining prominence due to its capacity to generate vast amounts of data and abundant information. In addition, there are limited studies regarding the effect of beneficial microbes on the growth and performance of duckweed cultivated in an open-air system.

Hence, this chapter comprises of: 1) Result of soil samples from mangrove swamps in Sungai Lukut including amplicon analysis from mWGS, taxonomic diversity, structural comparison and functional analysis of bacterial communities, metabolism pathway and number of elements in mangrove soil (soil 1, 2 and 3); 2) Bacterial identification using 16s rRNA gene sequencing analysis including morphology, phylogenetic tree of isolated bacterial species after screened on three different agar (Jensen's, Aleksandrow and Pikovskaya's agar).; 3) Total Plate Count (TPC) of soil inoculated with biofertilizer; 4) Nutrient analysis of biofertilizer Set A, B, C and control.; 5) Result of the duckweed growth after grown on medium containing biofertilizer Set A, B, C and control; and 6) Protein amount extracted from duckweed plants after grown on medium containing biofertilizers Set A, B, C and control. This study comprises three major objectives which were to identify a wide range of microbial species and function of genes from three soil samples collected at Sungai Lukut, Negeri Sembilan, Malaysia using metagenomic mWGS, identify selected bacterial species which grown on selective media which are nitrogen-fixing bacteria, potassium solubilizing bacteria and phosphorus solubilizing bacteria by using Polymerase Chain Reaction (PCR) and sequencing on 16s rRNA gene and determine the selected bacterial species that can affect duckweed plant through growth rate and protein contents analysis.Metagenomics sequencing and comparisons among soil 1, 2 and 3

4.1.1 DNA Quality and Purity For mWGS

DNA purity and quality are crucial as it greatly impacts the success of the analysis. Three samples, soil 1, 2 and 3 were analysed for their purity and quality. The good quality DNA will have an A_{260}/A_{280} of 1.7 – 2.0. The results of the DNA purity and quality were displayed in Table 4.1.

Table 4.1: DNA Purity and Quality of Three Soil Sample (Soil 1, 2 And 3) Determined by Nanodrop.

Samples	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/µL)	Vol (µL)
Soil 1	1.932	2.050	125.65	30
Soil 2	1.940	1.441	197.80	30
Soil 3	1.904	0.788	76.05	30

Note: Purity of OD260/280 (NanoDrop™) = 1.8 – 2.0

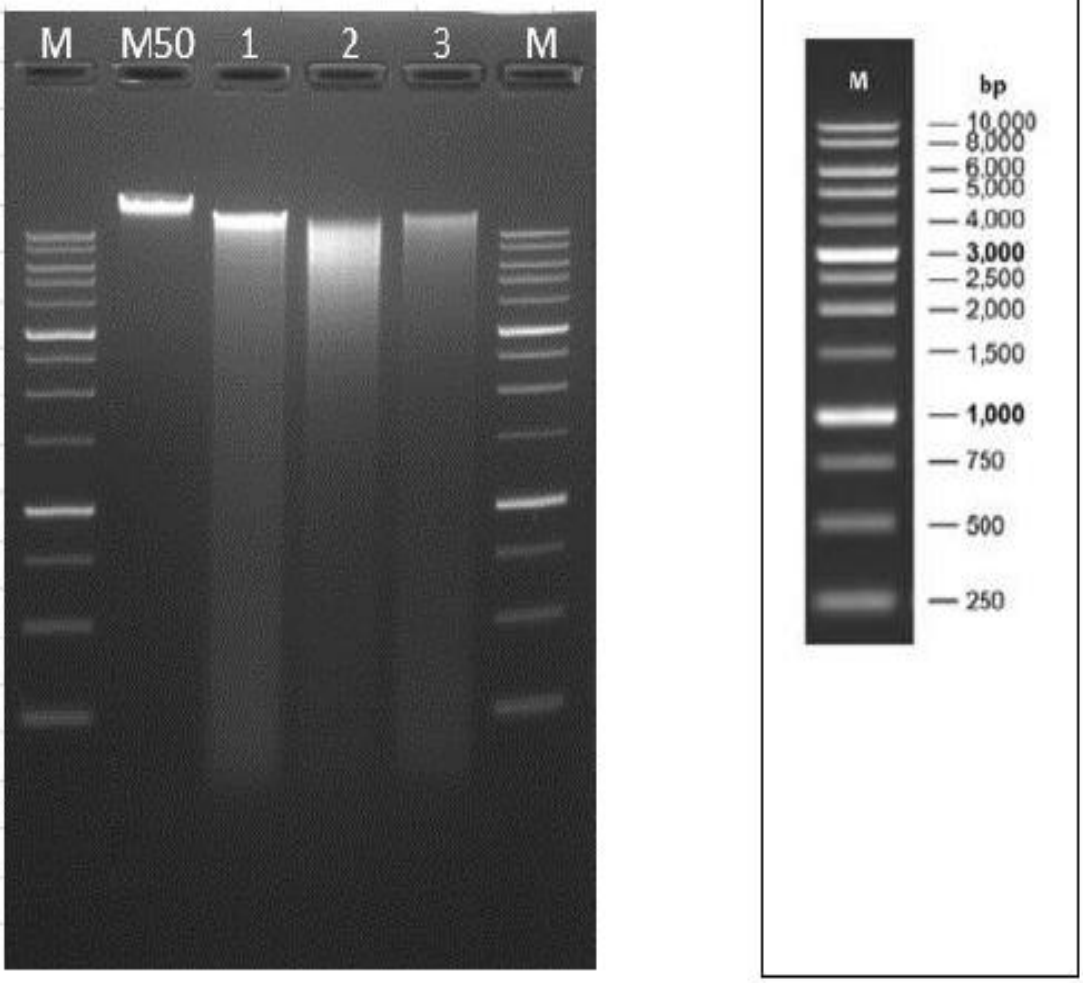


Figure 4.1 : Agarose Gel of Soil 1, 2 and 3 Using Electrophoresis

Note: M represents DNA ladder. Lanes M50 represents DNA marker. Lanes 1, 2 and 3 represents in soil 1, 2 and 3 respectively.

4.1.2 Amplicon Analysis by mWGS

A total of 3.4 Mbp of sequencing reads were obtained from the three samples of soil 1, soil 2 and soil 3, with an average length of 816.6 bases and the total length of is 2250 Mbp. The average lengths of soil 1, soil 2 and soil 3 are 777 bp, 747 bp and 926 bp respectively. A maximum number of scaftig is 14 907 bp for soil 1, 32 291 bp for soil 2 and 760 908 bp for soil 3. The sequencing data showed 64.61 of GC percent. The Venn diagram is shown in Figure 4.2, the number of core genome for the three soil samples are 53 908, of which the number exclusive of the core genome for soil 1 is 241 556 genes, soil 2 is 580 197 genes, and soil 3 is 1 266 352 genes.

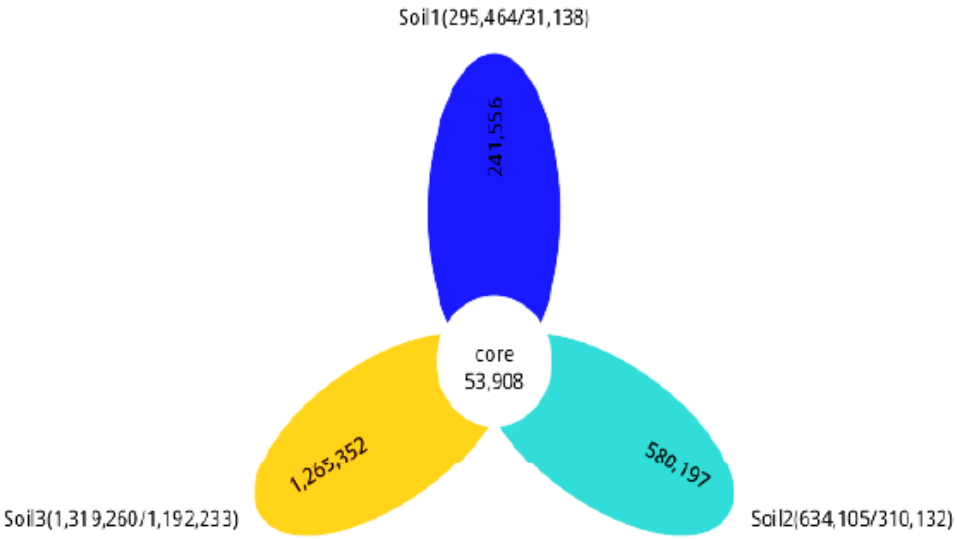


Figure 4.2 : Core-Pan Genome Figure of Soil 1, 2 and 3 from Sungai Lukut

Notes: The middle circle indicates the number of core genome. The two numbers in parentheses indicate the number contained in the soil and the peculiar gene number in soil respectively. The number in the sector indicates the gene number exclusive of core-genome in the soil.

4.1.3 Taxonomic Diversity of The Bacterial Communities in Mangrove Soil 1, 2 and 3

Based on sequence analysis, at the Genus level, OTUs could be classified into 10 genera in soil 1, soil 2 and soil 3. Of them, the phyla *Bradyrhizobium, Nocardioides, Methyloceanibacter, Pseudolabrys, Mycobacterium, Mycolicibacterium, Sphingomonas, Streptomyces, Solirubrobacter, Hyphomicrobium* and others were present in soil 1, soil 2 and soil 3. As shown in Figure 4.3, *Bradyrhizobium* was the major phyla in soil 3 which is 17.10% while the rest of two soil samples: soil 1 (3.18%) and soil 2 (3.33%) followed by soil 1: *Pseudolabrys* (2.75%), soil 2: *Mycobacterium* (2.11%), and soil 3: *Nocardioides* (3.78%) and soil 1: *Mycobacterium*, (2.01%), soil 2: *Hyphomicrobium* (0.56%), and *Mycolicibacterium*: soil 3 (1.86%). Lastly, the ten most dominant species were *Bradyrhizobium sp.* CCBAU 43298, *Desulfobacteraceae bacterium, Psseudolabrys taiwanensis, Methylocenibacter superfactus, Solirubacterales bacterium* 70-9, *Deltaproterobacterium* CSP 1-8, *bacterium, Myxococcales bacterium*, and *Mycolicibacterium moriokense.*

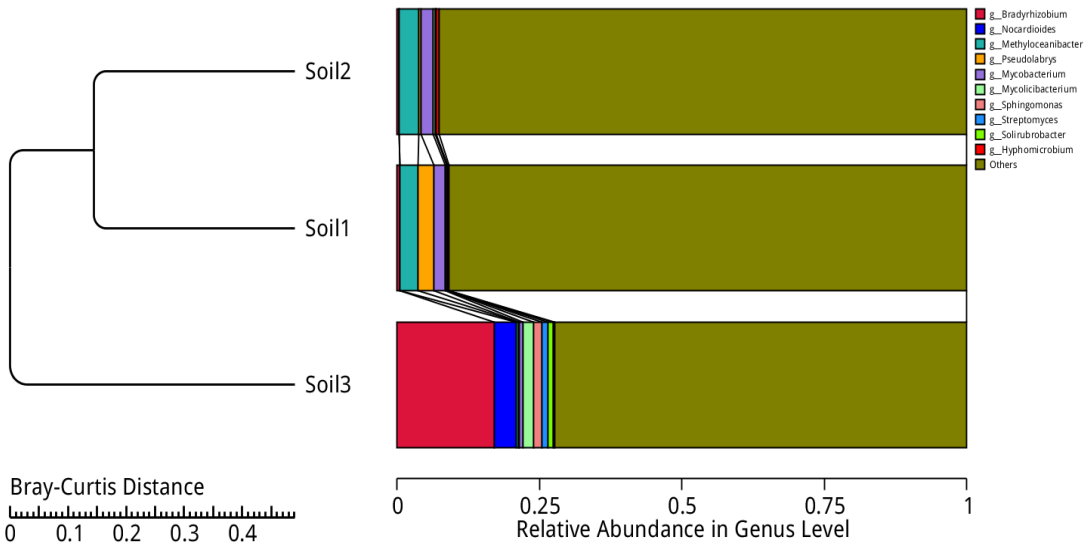


Figure 4.3 : Taxonomic Diversity of Microorganism in Collected Soil 1, 2 And 3. The Bray-Curtis distance shows relative abundance of genus level of three mangrove soil.

4.1.4 Structural Comparison of Bacterial Communities in Mangrove Soil 1, 2 and 3

The thirty-five most abundant genera were used to draw a heat map diagram (Figure 4.4). As shown in the heat map, six phyla were present at higher proportion of the heatmap: *Actinobacteria, Chloroflexi, Gemmatimonadetes, Nitrospirae, Proteobacteria* and *Thaumarchaeota*. *Psedolabrys* were comparatively higher than other genera in soil 1 while the least are *Tetasphaera* and *Phycicoccus*. Three known genera in phyla *Proteobacteria* (*Desulfofustis, Woeseia* and *Hypomicrobium*) were comparatively more abundant in soil 2. In addition, the major abundance of the 26 genera in soil 3 were: *(Actinobacteria: Tetrasphaera, Phycicoccus, Microbacterium, Microlunatus, Frankia, Mycolicibacterium, Solirubrobacter, Conexibacter, Nocardioides, Actinomadura, Marmoricola, Aeromicrobium, Agromyces, Pseudonocardia, Streptomyces* and *Micromonospora), (Chloroflexi: Kouleothrix); (Nitrospirae: Nitrospira,); (Proteobacteria: Afipia, Lysobacter, Mesorhizobium, Bradyrhizobium, Devosia, Steroidobacter and Spingomonas),* and *(Thaurmarchaeota:, Candidatus Nitrosocosmicus*)).

Heatmap of the thirty-five most abundant species in the three soil samples were also drawn (Figure 4.5). In soil 1, bacterial species are mostly from *Proteobacteria* which include *Pseudolabyrs taiwanensis, Pseudolabrys sp.* Root162, *Desulfobacterales bacterium* SG8_35_2, *Desulfobacterales bacterium* S5133MH16, and *Myxococcales bacterium* SG8_38. Moreover, two most abundant bacteria are found in soil 2 which are *Woeseia oceani* and *Proteobacteria bacterium.* Two phyla, *Actinobacteria* and *Proteobacteria* are most abundant in soil 3 which are *Solirubrobacterales bacterium 70-*

9, Mycolicibacterium rhodesiae, Mycolocibacterium moriokaense, Solirubrobacter sp. URHD0082, *Solirubrobacter soli, Sphingomonas sp.* URHD0007, *Sphingomonas sp.* URHD0057, *Frankia sp.* Iso899, *Bradyrhizobium sp.* CCBAU 43298, *Bradyrhizobium manausense,* and *Bradyrhizobium liaoningense*.

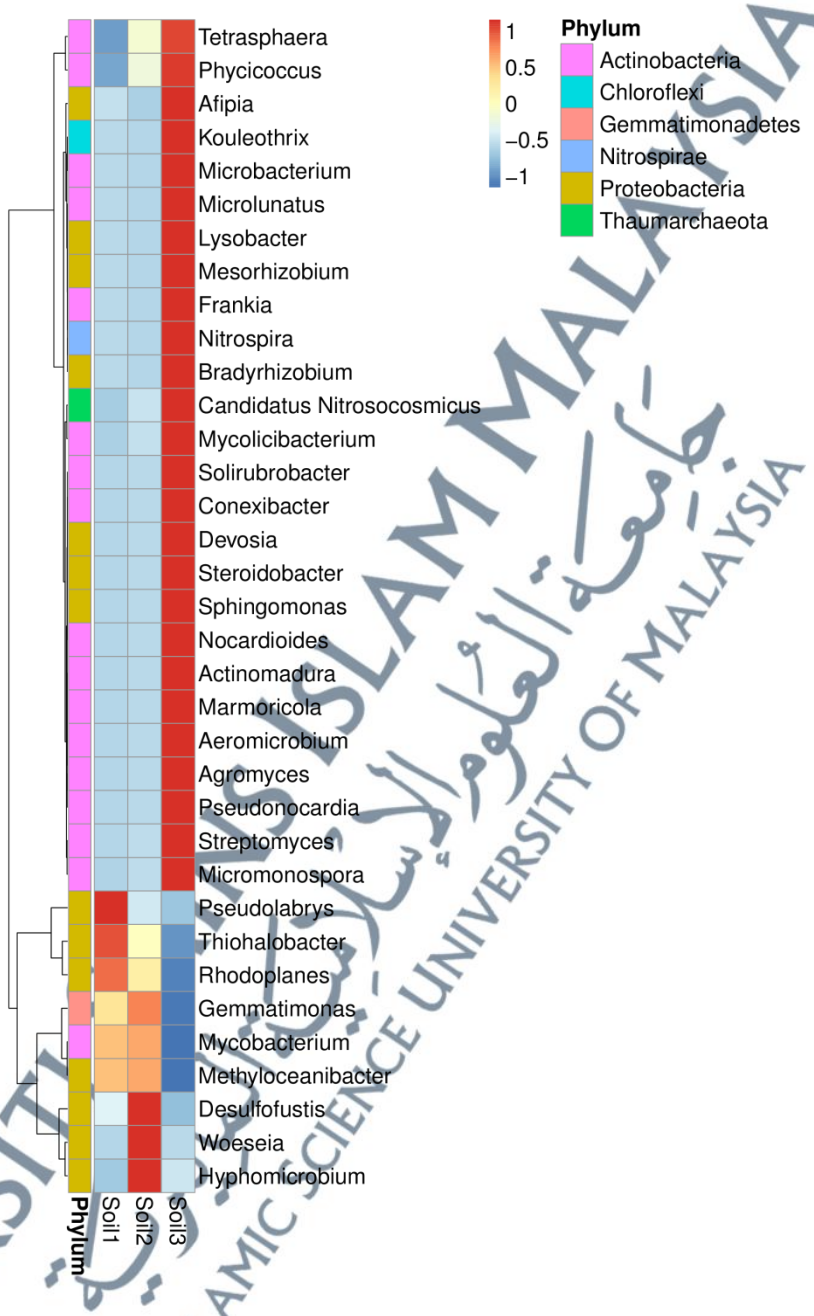


Figure 4.4 : Clustering Result Visualized in The Heat Map of Genus Level Obtained from The Analysis of mWGS of Soil 1, 2 and 3 from Sungai Lukut

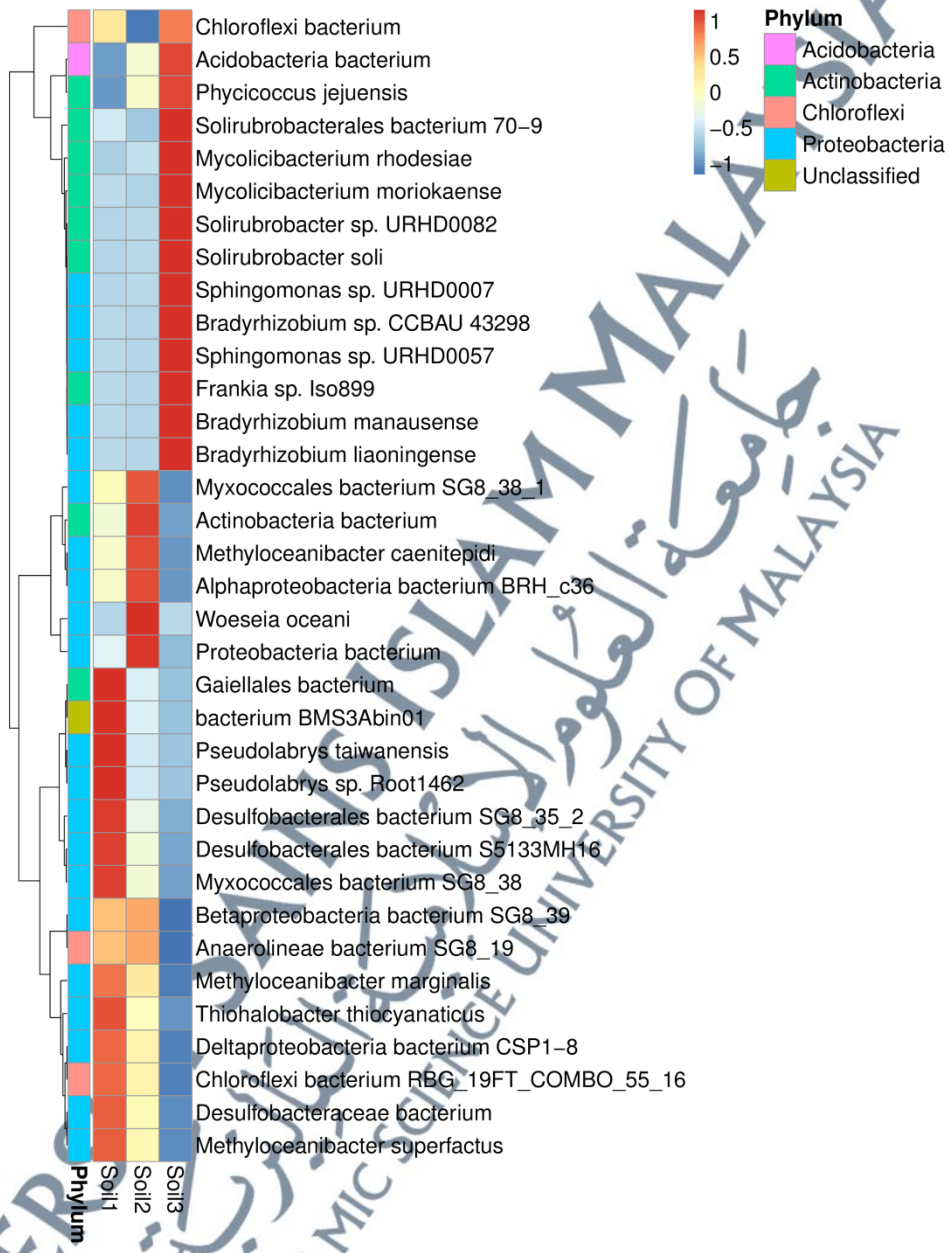


Figure 4.5 : Clustering Result Visualized in The Heat Map of Species Level Obtained from The Analysis of mWGS of Soil 1, 2 and 3 from Sungai Lukut

4.1.5 Functional Analysis Of Bacterial Communities In Mangrove Soil 1, 2 and 3

Functional analysis of bacterial communities showed the most abundant genes found in the soil of the rhizosphere of *R. mucronata, A. officinalis*, and *N. fruticans* based on the KEGG database (Figure 4.6). Three samples from the soil rhizosphere contain the genes that function in carbon, methane, nitrogen, and sulphur pathways, followed by atrazine and dioxin degradation.

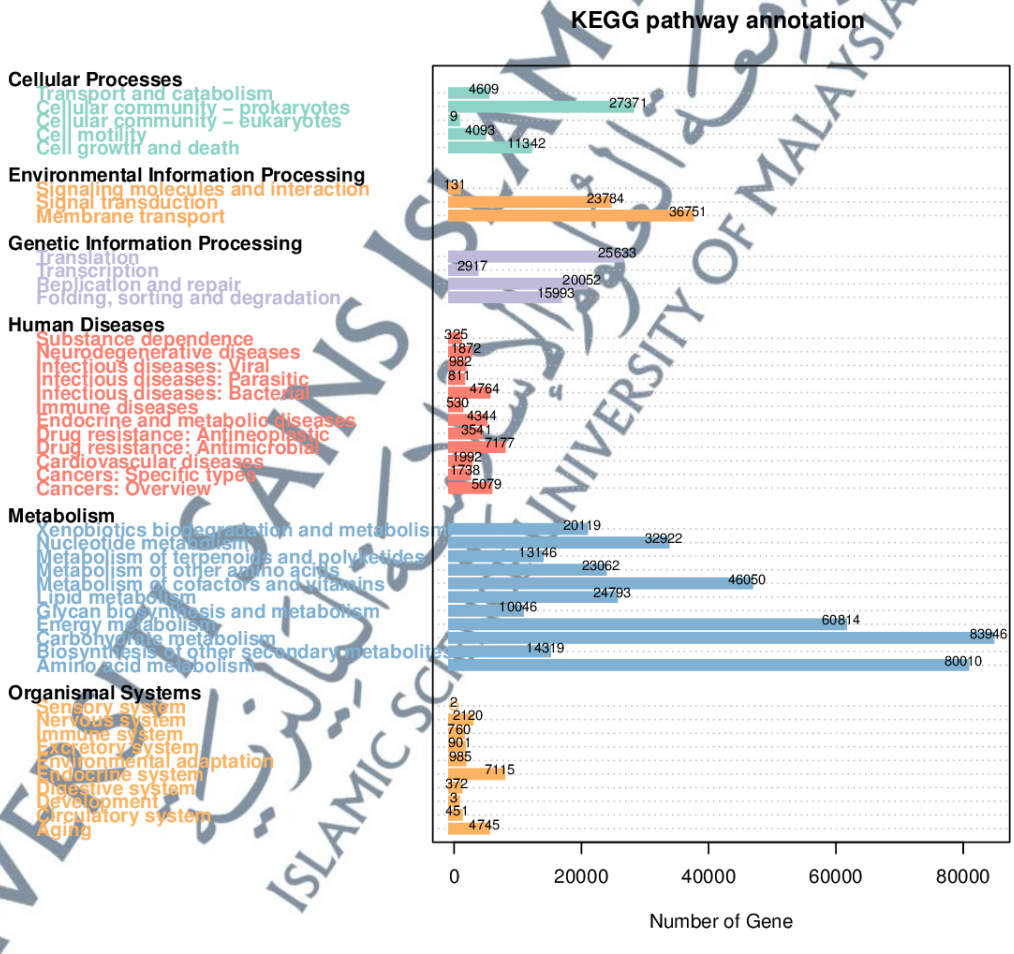


Figure 4.6 : Cartogram of Annotated Gene Number from The Unique Genes Annotation Result.
The Function Annotation Was Performed Based on the KEGG database.

4.1.5.1 Mechanism of Carbon Fixation Metabolism and The Genes and Bacteria Involved

In the reductive citric acid cycle, a crucial metabolic pathway for carbon fixation in prokaryotes, carbon dioxide is reduced and assimilated into organic compounds. In mangrove soil samples of 1, 2, and 3, several key genes participate in this process as shown in APPENDIX A. The cycle initiates with the enzyme pyruvate dehydrogenase (PDH) complex, including MDH. Malate dehydrogenase (MDH) is found in all three soils, and its presence suggests its involvement in the conversion of oxaloacetate to malate, a critical step in the cycle. The fumarase genes (*fumA*, *fumB*, *fumC*) present in soil 2 and 3 encode enzymes responsible for the interconversion of fumarate and malate, contributing to the cycle's efficient functioning. Similarly, in soil 2, the succinate dehydrogenase genes (*sdhA*, *sdhB*, *sdhC*, *sdhD*) and fumarate reductase genes (*frdA*, *frdB*, *frdC*, *frdD*) are found, indicating their participation in the conversion of succinate to fumarate and vice versa.

Moreover, soil 2 and 3 harbor tricarboxylic acid cycle genes such as trans-aconitate hydratase (*tfrA*, *tfrB*), succinyl-CoA synthetase (*sucD*, *sucC*), and isocitrate dehydrogenase (IDH1, IDH2), which collectively catalyze the formation of succinate, succinyl-CoA, and isocitrate, respectively. These genes are integral to generating the cycle's intermediate products. The presence of genes like *acnA* and *acnB* in all three soils highlights the participation of aconitase in the conversion of isocitrate to α-ketoglutarate. Similarly, ACL genes (*aclA*, *aclB*) in all three soils imply their role in the formation of acetyl-CoA, a precursor for the cycle. Additionally, the presence of *ccsA*, *ccsB*, and *ccl*

genes in all three soils suggests the involvement of citrate lyase in facilitating the cleavage of citrate into oxaloacetate and acetyl-CoA. Genes such as *porA*, *porD*, and *porC* found in all three soils encode enzymes that aid in the conversion of α-ketoglutarate to succinyl-CoA, an essential step in the reductive citric acid cycle. Lastly, genes like *ppdK*, *ppc*, and PC found across all three soils play pivotal roles in the overall operation of the cycle, contributing to the regeneration of oxaloacetate and the completion of the carbon fixation process.

Numerous carbon-fixing bacteria have been identified within the rhizosphere soil of Sungai Lukut, Negeri Sembilan. Although Proteobacteria are ubiquitous across all soil samples, their highest prevalence is observed in soil 2 and 3. Within the Proteobacteria group, *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* are present in all three soil types, while *Deltaproteobacteria* are least abundant in soil 3. Noteworthy genera include *Psedolabrys* (present in soil 1), *Methyloceanibacter* (present in soil 1 and 2), *Hypomicrobium* and *Nitrospinae* (present in soil 2), and *Nocardiodes*, *Streptomyces*, *Sphingomonas*, *Psedonocardia*, and *Bradyrhizobium* (present in soil 3). In this study, the KEGG database highlights the presence of bacteria involved in carbon fixation pathway were *Woeseia oceani*, *Methyloceanibacter superfactus, Thiohalobacter thiocyanaticus, Phycicoccus jejuensis, Solirubrobacter soli, Solirubrobacter sp*. URHD0082, and *Pseudolabrys taiwanensis.*

4.1.5.2 Mechanism of Methane Metabolism and The Genes and Bacteria Involved

The mechanism of the methane metabolism from KEGG database is shown in APPENDIX B. Methane metabolism in methanotrophic cells begins with a hydroxylation reaction from methane oxidation into methanol catalysed by methane monooxygenase encoded by the genes MMO (*mmoB, mmoC, mmoD, mmoX, mmoY,* and *mmoZ*). The enzyme coenzyme M methyltransferase catalyses the transfer of a methyl group from corrinoid protein, where it is bound to the cobalt cofactor, forming methyl-CoM, the substrate for genes *mcrA, mcrB, mcrC, mcrD,* and *mcrG*. Finally, the enzyme methanophenazine oxidoreductase catalyses the final step in methanogenesis by regenerating Coenzyme M and Coenzyme B.

After that, other steps in methane metabolism involve the acetyl-CoA pathway. Methanol is then oxidised into formaldehyde by methanol dehydrogenase (MDH), which can be further oxidised or assimilated as a substrate for cell carbon fixation pathways. Oxidation of methanol is catalysed by methanol dehydrogenase (*mdh1, mdh2, mxaA, mxaC, mxaD, mxaF, mxaJ, mxaK,* and *mxaL*). The conversion of formaldehyde to formate is by *fdhA*. After that, formate dehydrogenase (FDH) produced carbon dioxide (CO_2) from formate. Then, ferredoxin is reduced to form acetyl-CoA encoded by the gene *cdhA*. The genes of *porA, porB, porD, porC,* and *porG* are involved in the production of pyruvate by ferredoxin 2-oxidoreductase. Operating the cycles creates pyruvate and then acetyl-CoA, key intermediates for further cell metabolism.

The genes of MMO are abundant in soil 3. Following that, soil coenzyme M methyltransferase (*mtaA*) is less abundant in soil 3. Then, soil 1, soil 2, and soil 3 have the most abundant genes of heterodisulfide reductase (*hdrA, hdrB, hdrC, hdrD,* and *hdrE*), with these genes forming coenzyme-B. The tetrahydromethanopterin S-methyltransferase (*mtrA, mtrB, mtrC, mtrD, mtrE, mtrF, mtrG,* and *mtrH*) is involved in the formation of coenzyme-M, and these genes are present in soil 2 and soil 3. The gene *fdhA*, which produces formate, is also present in soil 2 and soil 3. The gene FDH, which is present to produce carbon dioxide, is abundant in all soils. Following that, the gene *cdhA* is the least abundant in soil 1 and soil 2. Lastly, CoA-acetylation is encoded by the genes *porA, porB, porD,* and *porC* and is found to be the most abundant in soils 1, soil 2, and soil 3.

The representatives of methanotroph can only be found in one phylum which is Proteobacteria. Methane oxidizing bacteria (MOB) that belongs to *Proteobacteria* are found in three groups: *Gammaproteobacteria, Betaproteobacteria,* and *Alphaproteobacteria*. *Gammaproteobacteria* include a wide range of MOB, such as *Methylosarcina, Methylocaldum, and Methylobacter* from soil 3 in comparison to *Betaproteobacteria* that includes *Methylibium, Methylonera,* and *Methyloversatilis* from soil 3. *Alphaproteobacteria* are only includes *Methylocystis* which are present in soil 2 and soil 3 and *Methyloceanibacter* which present in soil 1, 2 and 3. Result from this study showed that all of the MMO were most closely related to those Methanotrophs of (*Methylocystis sp., Meythlopila sp., Methylosarcina lacus, and Methyloceanibacter methanicus)* and those of Methylotroph (*Methylobacterium nodulans, Methylobacterium*

sp. 17SD2-17, *Methylibium sp.*, *Methylotenera sp.* 24-45-7, *Methylocaldum sp.*, *Methyloversatilis universalis*, *Methyloceanibacter caenitepidi*, *Methyloceanibacter marginalis*, *Methyloceanibacter sp.* wino2, *Methyloceanibacter superfactus* and *Hyphomicrobium sp.*).

4.1.5.3 Mechanism of Nitrogen Metabolism and The Genes and Bacteria Involved

According to the KEGG metabolism pathway showed in APPENNDIX C, the N-fixing bacteria generally begin by converting the nitrogen to ammonia through ATP hydrolysis by dinitrogen oxidoreductase, encoded by the nitrogenase molybdenum-iron protein (*nifDKH*) and nitrogenase delta subunit (*anfG*). Then, bacteria generally convert the ammonia to nitrite by ferricytochrome-c oxidoreductase encoded by the genes hydroxylamine dehydrogenase (*hao*) and nitrite reductase (*nrfA*), followed by nitrification reactions by ferredoxin-nitrate reductase (*narB*), which convert nitrite sequentially to nitrate.

The nitrogen metabolism pathway is encoded by the genes *nifDKH* and *anfG*, and it is abundant in soil 1, 2, and 3. Both genes are in charge of ATP hydrolysis formation. Only soil 2 and soil 3 contain ferredoxin-nitrite reductase (*nirA*), which produces ammonia. Both *nirB* and *nirD* genes are also in charge of producing ammonia, which is abundant in soils 1, 2, and 3. The gene *nrfA*, which is present in soil 2 and 3, is required for the conversion of nitrite to ammonia and conversely. The genes of *narB* are present in

soil 3, as it can produce nitrite or nitrate. In the nitrification reaction, *hao* is the most abundant in soil 2 and soil 3.

In this study, nitrogen-fixing bacteria involved in nitrogen metabolism pathway are belonged to *Alphaproteobacteria (*which abundant in soil 2 and 3*)* and *Gammaproteobacteria (*which abundant in soil 1 and 2*)*, indicating that the nitrogen-fixing group in this phlyum is predominant in the Sungai Lukut mangrove ecosystem. A number of nitrogen-fixing bacteria, identified as members of the genera *Eubyza, Nitriliruptor, Nitrospina, Nitrospira, Nitrobacters, Nitratireductors, Nitrosomonas, Nitrococcus, Nisea, Thioalbus, Thioalkalivibrio, Thriothrix,* and others have been found in the rhizosphere of mangrove. N-fixing bacteria are found to be the least abundant in soil 1 which are *Nitrococcus* and *Thioalbus* followed by the rhizosphere bacteria in soil 2 which are *Eubyza, Nitrospina, Nitrosomonas* and *Thiotrix.* The genera that are detected the most in soil 3 are *Eubyza, Nitriliruptor, Nitrospira, Nitrobacters, Nitrareductors, Nitrosomonas, Nisea, and Thioalbus.* Lastly, *Thioalkalivibrio* has been discovered in all soil samples (soil 1, 2 and 3). Nitrogen-fixing bacteria that are found to be abundant in mangrove soil in Sungai Lukut include *Euzebya rosea, Euzebya tangerina, Nitriliruptor alkaliphilus, Nitrospina gracilis, Nitrospira defluvii, Nitrospira japonica, Nitrospira lenta, Nitrospira moscoviensis, Nitrospira sp.* CG24D, *Nitrobacter sp.* Nb-311A, *Hyphomicrobium denitrificans, Hyphomicrobium nitrativorans, Nitratireductor basaltis, Nitratireductor indicus, Nisaea denitrificans, Sulfuricella sp.* T08, *Nitrosomonas mobilis, Nitrosomonas sp.* Nm143, *Thiobacillus sp.* 65-29, *Nitrosococcus halophilus,* and *Nitrosococcus watsonii.*

4.1.5.4 Mechanism of Sulfur Metabolism and The Genes and Bacteria Involved

Some inorganic forms of reduced sulfur, mainly sulphide (H_2S/HS) and elemental sulphur (S), can be oxidised by sulphur oxidising bacteria. According to the sulphur pathway from KEGG showed in APPENDIX D, the dissimilarity sulphate oxidation reaction begins by converting sulphide to sulfite in the presence of sulfite reductase (*cysI, cysJ, sir*) and dissimilatory sulfite reductase (*dsrA, dsrB*) genes, followed by the formation of adenosine-5'-phosphosulfate (APS) catalyse. Then, sulphate adenylyltransferase catalyses APS encoded by sulphate adenylyltransferase (SAT and *met3*) genes to produce sulfate. The genes of *cysJ, cysI, sir, dsrA,* and *dsrB* are abundantly formed during dissimilatory sulphate reduction and oxidation in soil 1, 2, and 3. Finally, SUOX are less abundant in soil than sulphite and sulphate 3. This gene is also involved in sulfite oxidase deficiency (H01237). Besides, all soil contains *soeA*, *soeB*, and *soeC*, and the genes are present in soil 1, soil 2, and soil 3.

Proteobacteria are present in all soils, but they are found the most in soil 2 and 3. Among Proteobacteria, both *Betaproteobacteria* and *Gammaproteobacteria* are discovered in soil 1, 2 and 3, while *Deltaproteobacteria* is only detected in soil 2 and 3. *Desulfatibacillum, Desulfobulbus, Desulfocapsa, Desulfofustis, Desulfuromonas, Desulfomonile*, and *Desulfacinum* are the genus found in this study. All of the genus is only present in soil 2. The rhizosphere bacteria that are involved in sulphur mechanism in KEGG pathway are *Desulfatibacillum alkenivorans, Desulfocapsa sulfexigens, Desulfofustis sp., Dulfuromonas sp., Desulfobulbus sp., Desulfopila aestuarii,*

Sulfurifustis variabilis, Sulfuricaulis limicola, Desulfofustis glycolicus, Desulfospira joergensenii, and *Desulfomonile tiedjei.*

4.1.5.5 Mechanism of Atrazine Degradation Metabolism and The Genes and Bacteria Involved

As shown in atrazine degradation pathway in KEGG database (APPENDIX E), atrazine is first catalysed by atrazine chlorohydrolase (*atzA*) to produce hydroxyatrazine (HA), which is then catalysed by hydroxydechloroatrazine ethylaminohydrolase (*atzB*) to produce N-isopropyl amide, which is then hydrolyzed by N-Isopropylammelide isopropylaminohydrolase (*atzC*). Four genes are involved in the atrazine degradation pathway, which are cyanuric acid amidohydrolase (*atzD),* biuret amidohydrolase (*atzE),* allophanate hydrolase (*atzF),* and URE. The genes of *atzD* are rich only in soil type 3, as it produces 1-carboxybiuret from cyanuric acid. Besides, the *atzE* gene is present in soil 1, soil 2, and soil 3 as it hydrolyzes urea-1-carboxylate by 1-carboxybiuret hydrolase. Lastly, the genes of *atzF* and *URE* are also abundant in soil 1, soil 2, and soil 3 as they form biuret and urea, respectively.

Many bacteria that are responsible in atrazine degradation are discovered in mangrove soil from Sungai Lukut, Negeri Sembilan include *Actinobacteria, Chloroflexi* and *Proteobacteria*. Some of the genus that belong to *Actinobacteria* are *Mycobacterium, Gordonia, Rhodococcus, Tetrasphaera, Clavibacter, Friedmaniella, Nocardiodes, Pseudonocardiodes, Streptomyces*, and *Solirubrobacter,* while the genus *Caldilinea* and

Kouleothrix are belong to *Chloroflexi.* Among bacteria, *Proteobacteria* are present the most which include *Alphaproteobacteria,* and *Betaproteobacteria.* The genus of *Alphaproteobacteria* that are present in soil 1 and 3 are *Rhodoplanes* and *Pseudolabrys.* Besides, only *Methyloceanibacter* are present in both soil 2 and 3. Moreover, soil 3 are abundant of genus *Bradyrhizobium, Rhizobium, Devosia, Hyphomicrobium,* and *Pseudorhodoplanes.* The genus of *Betaproteobacteria* are only present in soil 3 which are *Caenimonas, Variovorax, Rhizobacter*, *Bovinethylophilus,* and *Nitrosomonas.* The KEGG database indicated that atrazine degrading bacteria present is *Mycobacterium sp.* ACS1612, *Friedmanilla flava, Pseudonocardia thermophila, Solirubrobacter soli, Kouleothrix aurantiaca, Methyloceanibacter marginalis, Pseudorhodoplanes sinuspersici,* and *Pseudolabrys taiwanensis.*

4.1.5.6 Mechanism of Dioxin Degradation Metabolism and The Genes and Bacteria Involved

According to the KEGG pathway showed in APPENDIX F, in aerobic biodegradation, polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are usually initiated by an angular dioxygenase system that attacks a ring adjacent to the ether oxygen bridging the two rings, yielding 2,2′,3-trihydroxydiphenyl ether or 2,2′,3-trihydroxybiphenylIn the second step, 2,2′,3-trihydroxydiphenylether was converted into 2-hydroxy-6-oxo-6-(2-hydroxyphenoxy)-hexa-2,4-dienoate (HOHPDA) by 2,2′,3-trihydroxybiphenyl dioxygenases. In the final

step, 2-Hydroxy-6-oxo-6-(2-hydroxyphenoxy)-hexa-2,4-dienoate hydrolysis will yield catechol or salicylate as the final substrate for benzoate degradation.

The 2,3-hydroxilating of the biphenyl results in the formation of 2,3-Dihydro-2,3-dihydroxybiphenyls during PCB degradation. The enzyme 2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase catalyzes the second step in the biphenyl degradation pathway. Following that, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPD) hydrolase hydrolyzed HOHPDA to produce 2-hydroxy-2,4-pentadienoate, which is a substrate for benzoate degradation and a lower pathway to pyruvate metabolism and the TCA cycle.

Different gene products interact with each other to exert biological functions. There are several genes involved in the atrazine degradation pathway, including *dbfA1, dbfB, praC, xylH, dmpH, xylI, nahK, mhpD, mhpE, mhpF, bphAa, bphA1, bphA,* and *bphC*. The gene *dbfA1* is involved in the first step of PCB degradation and it is present in soil 3. Furthermore, the hydrolysis of HOHPDA is encoded by *dbfB* genes, which are found in soil 3. Genes of *praC* and *xylH* are found in soil 1, 2, and 3 and are involved in the production of catechol. The genes *dmpH, xylI,* and *nahK* are involved in 2-oxopent-4-enoate-forming and are found to be most abundant in soil 2 and soil 3. Besides, the formation of (2Z)-2-hydroxypenta-2,4-dienoate and 4-hydroxy-2-oxopentanoate is encoded by the genes *mhpD* and *mhpE*, respectively, and is present in soil 2 and soil 3. CoA-acetylation is solubilized by acetaldehyde dehydrogenase encoded by the genes of *mhpF*, which are abundant in soil 1, 2, and 3. Finally, only soil 3 is rich in *bphAa, bphA1, bphA,* and *bphC* genes because it is involved in the formation of (1S, 2R).-3-

phenylcyclohexa-3,5-diene-1,2-diol and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate which are essential for pyruvate metabolism and the TCA cycle.

Most of the bacteria that are involved in dioxin degradation pathway from KEGG database are from *Actinobacteria* and *Proteobacteria* (particularly *Alphaprotetobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*). *Actinobacteria* are found to be more abundant in soil 3 but less abundant in soil 1 and 2. The bacteria that present in soil 2 and 3 are from the genus *Mycobacterium, Mycolicibacterium, Nocardia, Eubyza*, *Rhodococcus, Phycicoccus, Blastococcus, Geodermatophilus, Instraporangium, Tetrasphaera, Agromyces, Athrobacter, Nocardioides, Pseudunocardia, Streptomyces,* and *Conexibacte*r. While *Actinoplanes* and *Eubyza* are only present in soil 1. Besides, the genus of *Afipia, Bradyrhizobium, Devosia, Rhodoplanes, Methyloceanibacter Pseudorhodoplanes, Nicella, Novosphigobium* and *Spingomonas* from *Alphaproteobacteria* are present in soil 2 and 3. Meanwhile, the only genus presents in soil 1, 2 and 3 are *Pseudolabys.* Moreover, *Comamonas* from *Betaproteobacteria*, and *Woeseia* and *Lysobacter* from *Gammaproteobacteria* are only present in soil 3. The bacteria that are present the most in dioxin degradation pathway are *Mycolicibacterium morikaense, Eubyza tangerina*. *Pseudolabrys taiwanensis.*

4.1.6 Comparative Analysis of EggNOG AND CaZY of Bacterial Diversity

Metagenomes provide insight into the physiology of a community by clarifying the collective functions that are encoded in the genomes of organisms that make up the community. Function annotation based on the KEGG, as shown in Figure 4.7 (a)(b), reveals that most of the core genes found in mangrove soil are involved in metabolic pathways. The highest genes are involved in carbohydrate metabolism and followed by amino acid metabolism. Both soil 1 and soil 2 demonstrated the highest levels of energy production and conversion, comprising 5.38% and 5.19% respectively. Conversely, soil 3 displayed the highest proportion in amino acid transport at 5.36%. In addition, the functional annotation based on eggNOG shows the genome isolated from the soil of mangrove trees' rhizosphere mostly contains genes that are associated with energy production and conversion, amino acid transport, metabolism, replication, recombination and repair, as well as carbohydrate transport and metabolism pathways (Figure 4.7 (a)). In this study, the functional annotation based on the CAZy reveals six CAZy main functions as follows: Glycoside Hydrolase (GH), Glycosyl Transferase (GT), Polysaccharide Lyase (PL), Carbohydrate Esterases (CE), Auxiliary Activities (AA) and Carbohydrate-Binding Modules (CBM) were found in collected mangrove soil (Figure 4.7 (b)). Glycoside hydrolases exhibited the highest representation, with percentages of 0.57% in soil 1, 0.50% in soil 2, and 0.66% in soil 3.

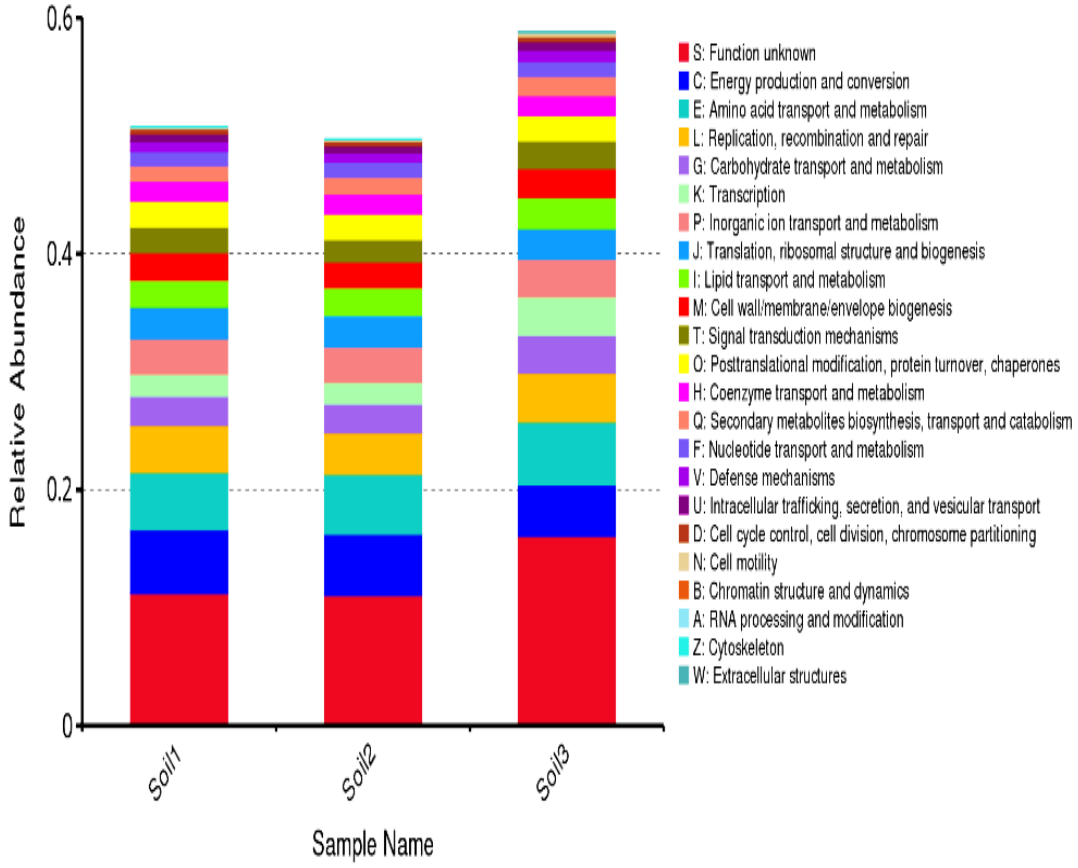


Figure 4.7 : Cartogram of The Relative Abundance Genes Found in Genome DNA Isolated from Soil 1, 2 And 3.
The functional annotation was performed based on the eggNOG database.

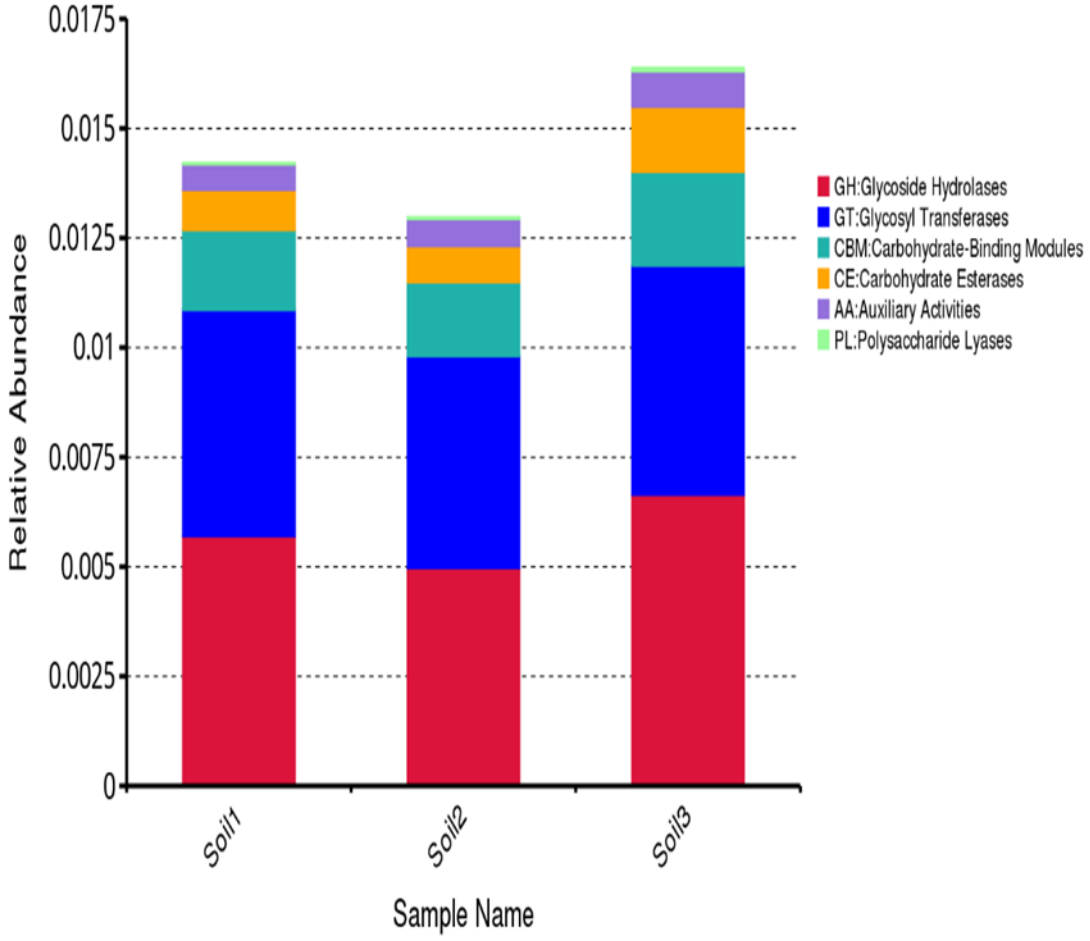
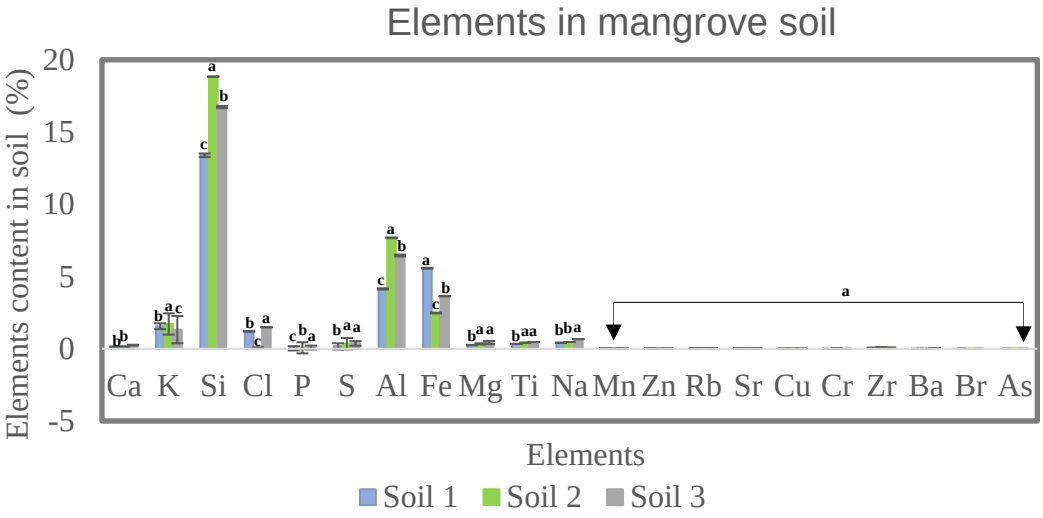


Figure 4.8 : Cartogram of The Relative Abundance Genes Found in Genome DNA Isolated Soil 1, 2 And 3.
The functional annotation was performed based on the CAZy database.

4.2 **Chemicals Elements Present in Mangrove Soil**

The mangrove soil was analysed to determine the element using X-ray fluorescence (XRF). Figure 4.8 (a) shows soil elements in collected mangrove soils. Mangrove soil from soil 1, 2, and 3 contain the highest amounts of silicone (P = 0.000), which are 13.39%, 18.83%, and 16.73% respectively. The element aluminium showed the second highest amount (P = 0.000) in soil 1, 2, and 3 with 4.14%, 7.68%, and 6.46% respectively. Iron was the third most abundant element (P = 0.000) in mangrove soils 1, 2, and 3, accounting for 5.57%, 2.48%, and 3.64%, respectively. Mangrove soil of 1, 2 and 3 has no significant difference in potassium and phosphorus as both of the element are low which were potassium (1.56% in soil 1, 1.72% in soil 2, and 1.33% in soil 3) and phosphorus (0.03% in soil 1, 0.07% in soil 2, and 0.09% in soil 3). Furthermore, sulphate levels (P = 0.000) in mangrove soils 1, 2, and 3 were 0.17%, 0.35%, and 0.37%, respectively. After that, the amounts of manganese, zinc, rubidium, copper, cesium, chromium, zirconium, bromine, and arsenic are not significantly different in the range of 0.00% to 0.05%. Figure 4.8 (b) shows the N, P and K elements in collected mangrove soils. Analysis of chemical elements content in mangrove soils reveals that smallest tracing element was P and followed by N. K element was the highest element presence in mangrove soil and slightly increase (P=0.003) in soil 2 compared to the soil 1 and soil 3.

(a)



(b)

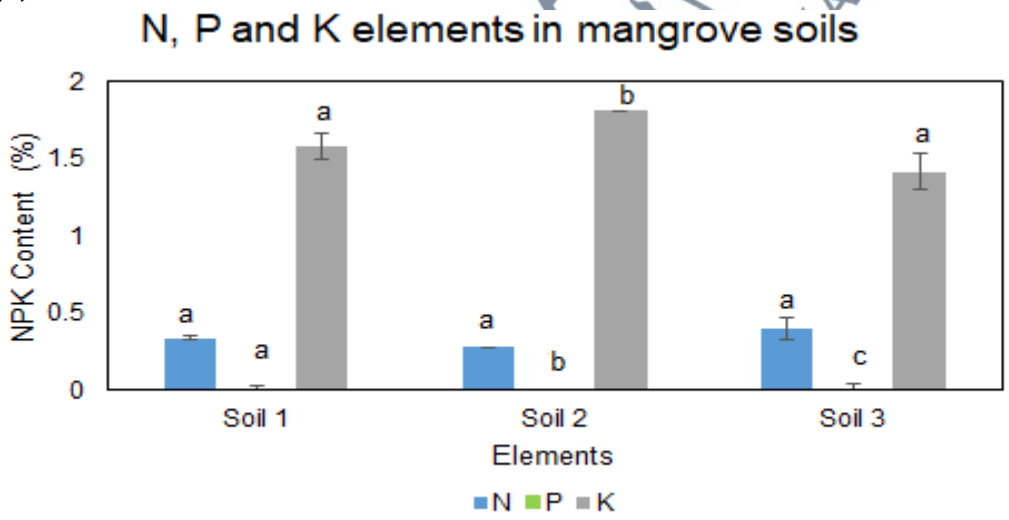


Figure 4.9 : The Presence of (a) Ca = calcium, K = potassium, Si = silicone, Cl = chlorine, P = phosphorus, Al = aluminium, Fe = Iron, Mg = magnesium, Ti = titanium, Na = sodium, Mn = manganese, Zn = zinc, Rb = rubidium, Sr = strontium, Cu = copper, Ce = cesium, Cr = chromium, Zr = zirconium, Br = bromine, As = arsenic) elements in mangrove soil 1, 2 and 3 of Sungai Lukut.
(b) Nitrogen (N), Phosphate (P) and Potassium (K) elements in mangrove soil. All data are mean± standard deviations (n=3). Means that do that share a letter between samples are significantly different ($P < 0.05$) based on Tukey 95% simultaneous confidence intervals.

4.3 Bacterial Identification in Mangrove Soil Using 16s rRNA Gene Sequencing Analysis

4.3.1 Morphology of The Bacteria Isolated from The Mangrove Soil

As the first step in identifying the species of each bacterial isolate, the morphological characteristics of each were observed and recorded. Colonies of the selected isolates were characterised based on shape, texture, and appearance. The ability of isolated strains to fix nitrogen was determined on Jensen's agar. Visible colony growth on the agar indicated positive nitrogen fixation, whereas bacteria that could not fix nitrogen were unable to grow on this medium. J1, J2, and J3 indicate nitrogen-fixing bacteria. Three of the nitrogen-fixing bacteria appeared as round, light yellow, yellow, and milky white colonies on Jensen's agar. The abilities of isolated strains to solubilize inorganic phosphorus and potassium were screened on Pikovskaya's agar and Aleksandrow agar, as the key indicators. Potassium-solubilizing bacteria are labelled as A1, A2, and A3, and phosphate-solubilizing bacteria go by the names P1, P2, and P3. The potassium- and phosphate-solubilizing activity of the isolates was qualitatively evaluated by the formation of halos (clear zones) around the colonies growing on Pikovskaya's medium. The isolated bacteria corresponding to Jensen's agar, Aleksandrow agar and Pikovskaya's agar were shown in Figure 4.9.

4.3.2 Bacterial Identification Using 16s rRNA Gene Sequencing Analysis

Sequences of the 16S rRNA gene are a powerful and most commonly used tool in bacterial identification at both the genus and species levels and in inferring phylogenetic relationships. The phylogenetic tree was constructed with Mega X software using maximum likelihood with a 1000 bootstrap model using Tamura-Nei model. BLAST analysis revealed that all the sequences were bacteria in nature. The taxonomic identification through the analysis of the bacterial 16S rRNA gene amplified from nine isolates revealed nine unique bacterial phylotypes (sequencing similarity, 98–100%) (Table 4.2) and Figure 4.10 represent phylogenetic tree of bacterial species isolated from Jensen's agar, Alesandrow agar and Pikovskaya's agar. These strains belong to 6 different genera including *Bacillus, Acinetobacter, Brachybacterium, Enterobacter, Klebsiella* and *Paenibacillus*. Among the isolates, three bacterial strains from Jensen's agar were detected, namely; *Acinetobacter radioresistens* (J1), *Brachybacterium paraconglomeratum* (J2), *Enterobacter cloacae* (J3). *Acinetobacter radioresistens* strain OsEp Plm 15B15 (MT367790.1) showed 99.25% identity. Besides that, *Brachybacterium paraconglomeratum* strain *AS53* (MT214268.1) showed 99.65% identity and similarity values of 99.62% with *Brachybacterium sp.* (OK326000.1). Likewise, *Enterobacter cloacae* strain SUK83 (KY908479.1) from the same agar had 99.63% identity (Figure 4.10).

Phylogenetic analysis in Figure 4.10 showed 3 different strains from Aleksandrow agar which determines *Klebsiella quasipneumoniae* (A1), *Bacillus tropicus* (A2) and *Paenibacillus pasadenensis* (A3) *with* 98 - 99% identity. First bacteria from Aleksandrow

agar, *Klebsiella quasipneumoniae* strain cjy02 (MN177200.1) had 99.70% identity. Besides that, *Bacillus cereus* (LC530606.1) which had 98.67% identity. *Paenibacillus pasadenensis* strain zp09 (KM100367.1) which had 99.33% identity. Analysis of 16S rRNA gene sequence identified three strain from Pikovskaya's agar with two of them had a same species which are *Bacillus cereus* (P1 and P2) and *Bacillus thuringiensis* (P3). One of the novel strains from Pikovskaya's agar was *Bacillus cereus* strain R1 (MN213372.1) showed sequence similarity values of 99.34%. Second, the bacteria had the closest relatives with *Bacillus cereus* strain E1 (OP597695.1). Besides that, *Bacillus thuringiensis* strain PDKV Bt I-3 (OP209990.1) showed sequence similarity values of 99.74% (Figure 4.10).

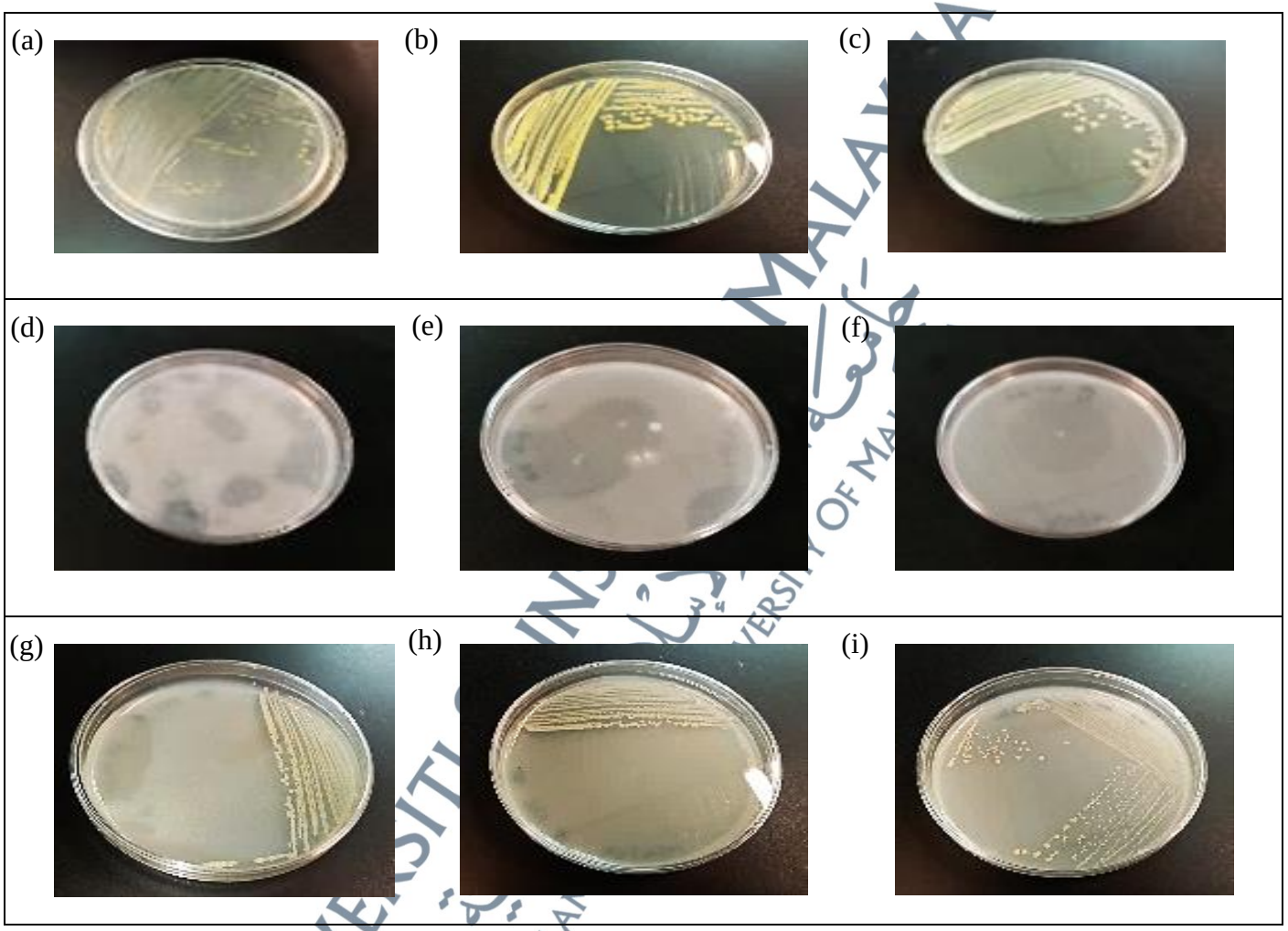


Figure 4.10 : (a), (b) and (c) Represents Bacteria Colonies on Jensen's Agar. (d), (e), and (f) Represents Bacteria Colonies on Aleksandrow agar. Lastly, (g), (h) and (i) Represents Bacteria Colonies on Pikovskaya's Agar.

Table 4.2 : List of The Bacterial Species Obtained from Soil 1, 2 and 3 by Using 16s rRNA Sequencing.
The morphology along with query cover, percent identity and accession number are included in the table.

Samples	Identity	Query cover	Percent identity	Accession number
J1	*Acinetobacter radioresistens* strain OsEp Plm 15B15	100 %	99.25 %	MT_367790.1
J2	*Brachybacterium paraconglomeratum strain AS53*	99 %	99.65 %	MT_214268.1
J3	*Enterobacter cloacae* strain SUK83	99 %	99.63 %	KY_908479.1
A1	*Klebsiella quasipneumoniae* strain cjy02	100 %	99.70 %	MN_177200.1
A2	*Bacillus tropicus* strain WSB89	98 %	98.67 %	OP_630954.1
A3	*Paenibacillus pasadenensis* strain zp09	100 %	99.33 %	KM_100367.1
P1	*Bacillus cereus* strain R1	99 %	99.34 %	MN_213372.1
P2	*Bacillus cereus* strain E1	100 %	100 %	OP_597695.1
P3	*Bacillus thuringiensis* strain PDKV Bt I-3	100 %	99.74 %	OP_209990.1

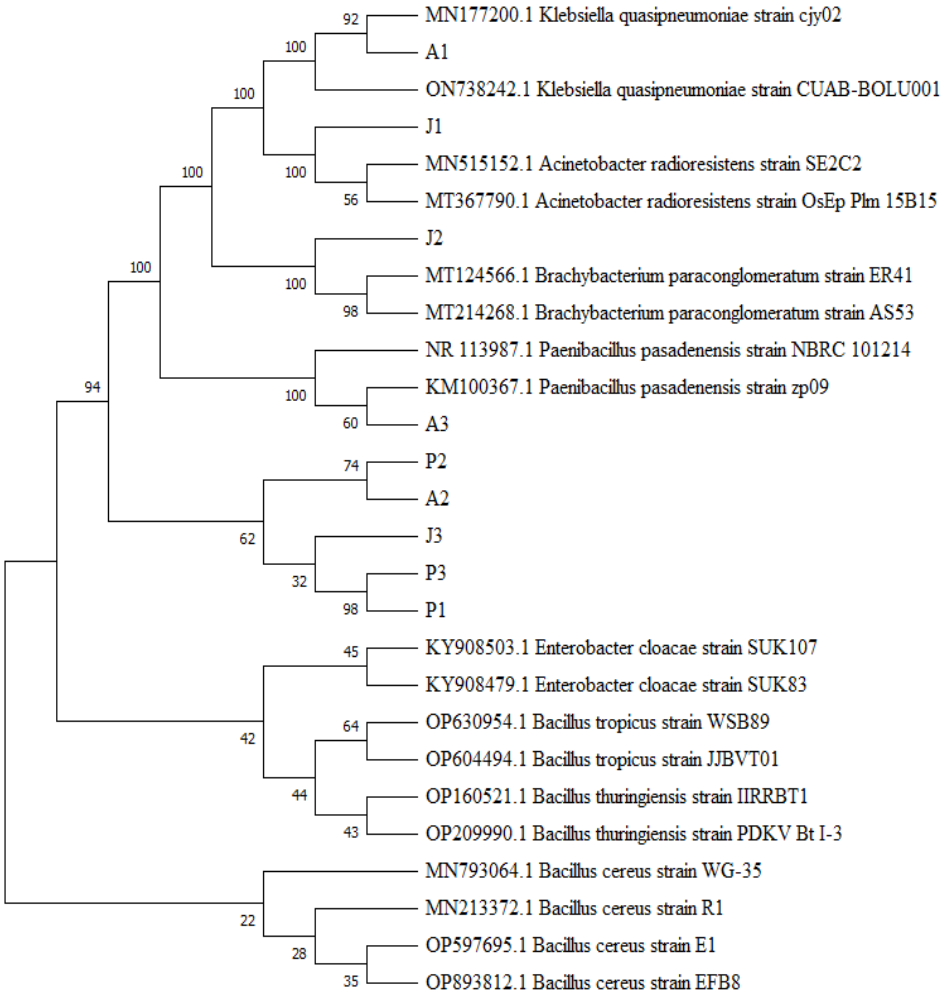


Figure 4.11 : Phylogenetic Tree of Bacterial Species Isolated from Jensen's Agar, Aleksandrow Agar, and Pikovskaya's Agar.
The DNA of the bacteria were extracted, which then being sequenced with 16s rRNA gene sequencing and BLAST sequence analysis with NCBI.

4.3.3 Microbial Content and N, P, K Analysis in Developed Biofertilizers

Next, the isolated bacteria were then grouped into three sets of bio-inoculants, with each exhibiting N-fixation, P and K solubilisation activities in three sets of formulated biofertilizers. The three sets of formulated biofertilizers were labeled as Set A, B, C and control. Set A consists of bio-inoculants *A. radioresistens, K. quasipneumonia* and *B. cereus*, Set B consists of bio-inoculants *B. paraconglomeratum, B. cereus* and *B. tropicus* and Set C consists of bio-inoculants *E. cloacae*, *P. pasadenensis* and *B. thuringiensis*. The three sets of bio-inoculants were then mixed with commercial compost. The three formulated biofertilizers were tested for microbial content and N, P and K analysis.

4.3.3.1 Microbial Survivability in Formulated Biofertilizers

Beneficial microbes are an active integrant of biofertilizers. Microbes in biofertilizers decrease themselves through their death, so an important condition is to include and keep a sufficient number of microbes. The bio-inoculate in biofertilizers can be tested for its survivability by total plate counting. The microbial survivability results indicate that the biofertilizer Sets A, B, and C had higher counts of viable microbes compared to the control group (Table 4.3). In comparison to the control group, the biofertilizer Sets A, B, and C demonstrated a significant difference (P=0.002) in microbial survivability. Furthermore, the outcomes highlighted that biofertilizer Set A outperforms Sets B and C in terms of enhancing microbial survivability. Based on one-way ANOVA table shown in APPENDIX G, a p-value suggests that there are significant

differences in microbial survivability among the control and biofertilizer sets (Set A, B and C).

Table 4.3 : Total Plate Count (TPC) (cfu/g) in Control and Biofertilizer Set A, B and C After 7 Days.

Note: The data shows the mean of colonies was normalized by $\log_{10}$ transformation. Means that do not share a letter between samples are significantly different ($P < 0.05$) based on Tukey 95% simultaneous confidence intervals.

Samples	Mean Log_{10} (cfu/g)
Control	$6.20 \times 10^{6}{}_{a}$
Set A	$8.80 \times 10^{6}{}_{b}$
Set B	$8.00 \times 10^{6}{}_{b}$
Set C	$8.30 \times 10^{6}{}_{b}$

4.3.3.2 N, P and K elements in formulated biofertilizer

Next, N, P and K content in the three sets of biofertilizers were analysed and compared with control (Figure 4.11). Analysis of chemical element contents in biofertilizers is also presented, in which K elements was the highest elements in three sets of biofertilizer and showed significantly increase (P=0.000) compared to control which is similar to N and P elements. However, P element was slightly increased in biofertilizer Set C compared to Set A and B, and the percentage of N element was similar in Set A and

Set C. The presence of mangrove-associated bacteria in biofertilizer is known to reflect the amount of N, P and K content.

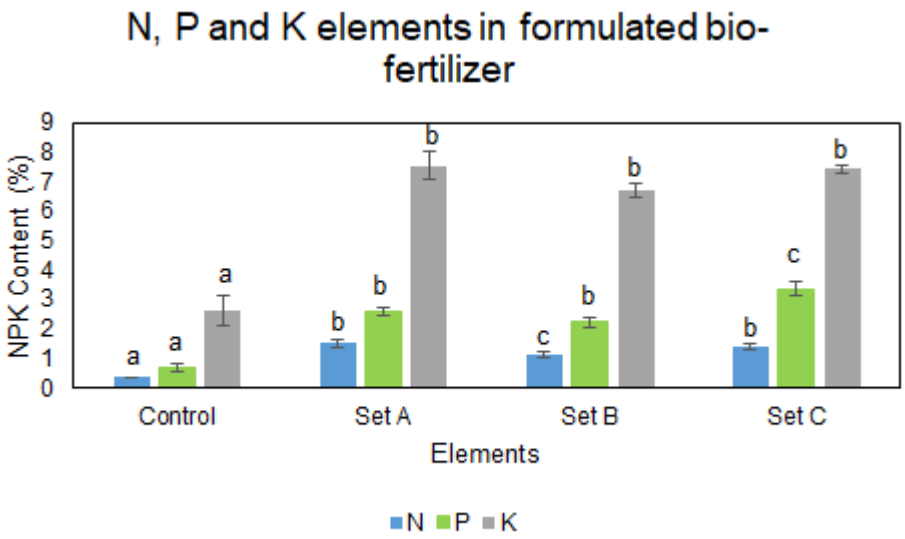


Figure 4.12 : Nitrogen (N), Phosphate (P) and Potassium (K) Elements in Control and Biofertilizer Set A, B and C.

Note: All data are mean ± standard deviations (n=3). Means that do not share a letter between samples are significantly different ($P < 0.05$) based on Tukey 95% simultaneous confidence intervals.

4.4 The Effect of Formulated Biofertilizers on The Growth of Duckweed and Its Protein Content

4.4.1 The Growth of Duckweed After Applying Biofertilizer Sets

The effectiveness of biofertilizer Set A, B and C compared to control on the growth of the duckweed plant is presented in Figure 4.12. The duckweed growth was evaluated in terms of the number of duckweed fronds. Relative to control, biofertilizer Set A, B and C exhibited a significant increase (P=0.000) of duckweed growth started on Day 3 until Day 15. The result from this analysis also showed that biofertilizer Set C is the most effective medium to boost duckweed growth compared to Set A and B. The result obtained from this experiment indicates that duckweed growth is correlated with the chemical element content in biofertilizer Set C (*E. cloacae*, *P. pasadenensis* and *B. thuringiensis*), which showed an increase of P compared to Set A and B.

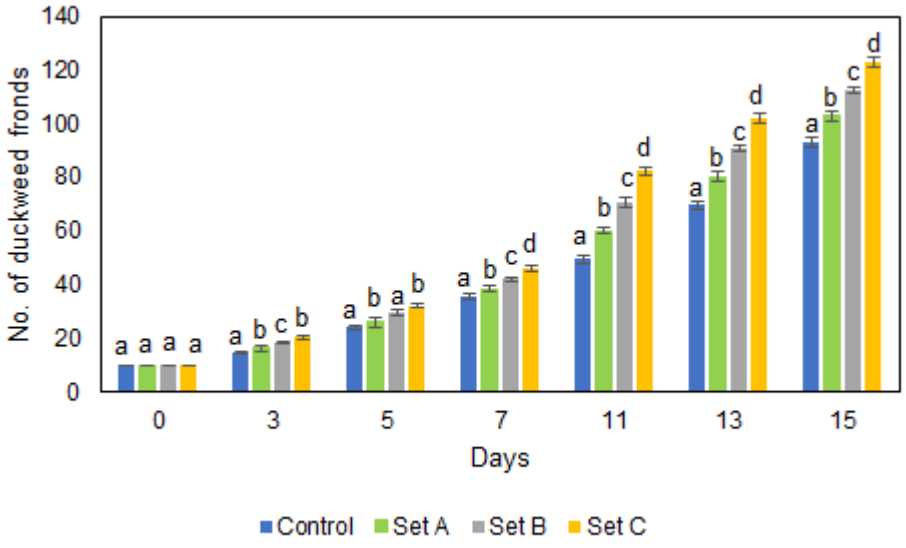


Figure 4.13 : The Growth of Duckweed in Control and Biofertilizer Set A, B and C After 15 Days.

Note: All data are mean ± standard deviations (n=3). Means that do not share a letter between samples are significantly different (P <0.05) based on Tukey 95% simultaneous confidence intervals.

4.4.2 Effect of Biofertilizer on The Duckweed Protein Content

The effectiveness of biofertilizer set A, B and C compared to control on the duckweed protein amount is presented in Figure 4.13. Overall, biofertilizer Set A, B and C displayed significant increase (P=0.000) in the amount of protein in duckweed plants compared to control. It indicates that the presence of mangrove-associated microorganisms in biofertilizer as duckweed growth medium is known to reflect the amount of protein in this plant.

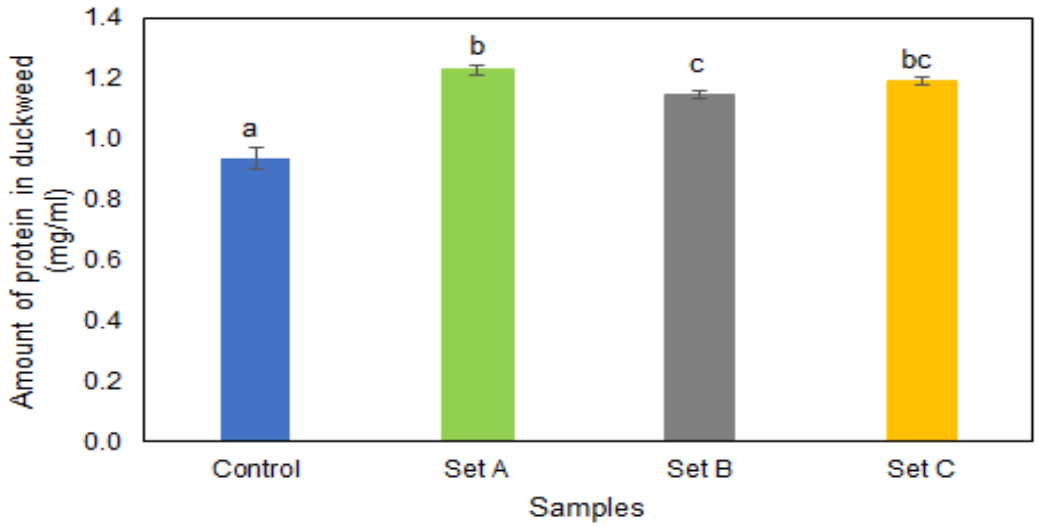


Figure 4.14 : Amount of Protein Harvested from Duckweed Grown in Control and Biofertilizer Set A, B and C in 15 Days.

Note: All data are mean ± standard deviations (n=3). Means that do not share a letter between samples are significantly different (P <0.05) based on Tukey 95% simultaneous confidence intervals.