CHAPTER 3

MATERIAL AND METHODS

3.1 Samples Collection

Soil samples were collected at the depth of 5 cm at the freshwater riverine mangrove at Sungai Lukut, Negeri Sembilan, Malaysia (coordinate 2° 35' 25.2342" N, 101° 48' 9.831" E) on 20th February 2020, during the low tide at three sampling points. Soil 1 and soil 2 were collected in the dimension 5 m x 5 m where *Rhizophora mucronata* and *Avicennia officinalis* trees are populated at that area. Meanwhile, soil 3 was collected at riverbank which is near *Nypa fruticans* tree and 50 m distance from soil 1 and soil 2. The samples were placed in 50 mL falcon tube, transported in an ice box, then stored at -20°C for a month before being transferred to -80°C until ready for analysis.

3.2 Isolation of DNA from Soil Microbiome for Metagenomics Analysis

DNA was extracted from 1 g of soil samples by using the QIAGEN Power Soil Pro Kit. The method of DNA extraction was followed as in manufacturer's guidelines. Subsequently, the extracted DNA was checked for their purity and quality using

NanoDrop (BioDrop) by loading the sample volume of 0.5µL into the microvolume sample port. Then, the results of the DNA purity and quality were recorded. Next, the extracted DNA was separated on 1.0% (w/v) agarose gel for visualisation as performed using the Biorad Universal Hood II Gel Doc System located at Institute of Fatwa and Halal (IFFAH), USIM. The high quality of DNA was sent to Centre for Chemical Biology (CCB) Universiti Sains Malaysia (USM) for metagenomics analysis.

3.2.1 Genome Sequencing and Assembly

The tight and highly reproducible DNA fragment distribution was provided by Covaris Sonicator using the mechanical shearing technique. Metagenome was assembled based on clean data from Illumina sequencing data. The library was constructed and quantified by using adapter addition and Agilent 2100/qPCR.

In bioinformatics analysis, samples passing quality control were assembled initially using an optimized MEGAHIT protocol. The Scaffolds were cut off at "N" to get fragments without "N", called Scaftigs (Mende et al., 2012; Nielsen et al., 2014), which is a continuous sequence within scaffolds. Clean data of all samples were mapped to assemble Scaftigs using Soap 2.21, and unutilized paired-end (PE) reads were collected with mapping parameters are –u, -2, -m 200. Next, mixed assembly was conducted on the utilized reads with the same assemble parameter. The scaftigs of each sample and mixed assembled, which were less than 500 bp, were trimmed (Mende et al., 2012; Nielsen et al.,

2014; Qin et al., 2010). Here, effective scaftigs were used for gene prediction and abundance analysis.

3.2.2 Gene Prediction and Abundance Analysis

In gene prediction and abundance analysis, scaftigs (>=500bp) were used for Open Reading Frame (ORF) prediction by MetaGeneMark (Mende et al., 2012) using single and mixed samples. The ORFs less than 100 nt (Mende et al. 2012; Qin et al. 2010) were trimmed. The ORFs were dereplicated by Cluster Database at High Identity (CD-HIT) (Li and Godzik, 2006; Fu et al., 2012) with parameters –c 0.95, -G 0, -aS 09, -g 1 and –d 0 to generate gene catalogues. The gene catalogues contain the non-redundant continuous gene encoding the nucleic acid sequence called genes. Dereplicating by default was used as an identity with 95%, while the coverage was 90%. Here, the longest gene was chosen as the representative gene (unigene). Clean data were mapped to the gene catalogue using SoapAligner to calculate the quality of the data with parameters –m 200, -x 400, identity ≥ 95%. Lastly, the gene abundance was calculated on the total number of mapped reads and gene length. The computation formula was as shown in Equation 1 (Karlsson et al., 2012; Cotillard et al., 2013; Le Chatelier et al., 2013; Oh et al., 2014; Zeller et al., 2014).

$$G_k - \frac{R_k}{L_k} \cdot \frac{1}{\sum_{i-1}^{n} \frac{R_i}{L_i}} \quad \text{........ (Equation 1)}$$

Where: G = Gene abundance

R = Number of mapping reads

L = The length of gene

3.2.3 Taxonomy and Function Annotation

Metagenomic reads were compared with the database of taxonomically informative gene families to annotate each metagenomic homolog. The function of the coding sequence was inferred based on its similarity to sequences in the databases from the Kyoto Encyclopedia of Genes and Genomes (KEGG), Non-supervised Orthologous Groups (eggNOG) and Carbohydrate-Active enzymes Database (CAZy). The clustering analysis shown in the heatmap figure was carried out based on the taxonomic abundance table and function abundance table. Multivariate statistical analysis and comparative analysis of metabolic pathways were performed to explore species composition and functional composition differences between soil 1, soil 2 and soil 3.

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3.3 Screening of Nitrogen-Fixing Bactera, Potassium Solubilizing Bacteria and Phosphate Solubilizing Bacteria

1 g of soil samples were individually suspended in 10 mL of 0.85% of NaCl for inoculation into Jensen's agar (HiMedia Laboratories), Pikovskaya`s agar (HiMedia Laboratories) and Aleksandrow agar (HiMedia Laboratories). Jensen's agar, Pikovskaya`s agar and Aleksandrow agar were used to screen and culture nitrogen-fixing bacteria, phosphate solubilizing bacteria and potassium solubilizing bacteria respectively. The Jensen's medium was aerobically incubated at 27 ºC for 48 hours and at 35 ºC for 7 days for both Aleksandrow agar and Pikovskaya's agar after spreading the soil suspension (Bhavi et al., 2020; Bhullar et al., 2022; Chhetri et al., 2022).

3.4 Identification of Culture Bacteria DNA Extraction Using 16s Rrna Gene Sequence Analysis

Bacterial culture was prepared by proliferating the single colony from Jensen's agar, Pikovskaya`s agar and Aleksandrov agar in a nutrient broth under 28 ºC with 200 rpm shaking for 48 hours to obtain the optical density at 600 nm (Bae et al., 2013). DNA from the bacterial culture were extracted with PROMEGA Wizard® Genomic DNA Purification Kit. The DNA purity, integrity and quantity were determined using Nanodrop (Biodrop).

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After that, the samples were amplified by Polymerase Chain Rearction (PCR) (BIORAD T100^{TM} Thermal Cycler) using primer set of universal primer; 27F 5'-AGA GTT TGA TCC TGG CTC AG-3', 1492R GGT TAC CTT GTT ACG ACT T-3') (Zhou et al., 2009). The components for PCR reaction were 12.5µL of Go Taq® Green Master Mix 2X, 0.5µL of forward primer, 0.5µL of reverse primer, 5µL of DNA template and 6.5µL of nuclease-free water. Amplification was carried out on a Mastercycler nexus PCR cycler, programme to perform an initial denaturation at 94 ^{o}C for 5 min; 30 cycles of denaturation for 1 min at 94 ^{o}C, annealing for 1 min at 60 ^{o}C, and extension for 1 min at 72 ^{o}C; and the final extension at 72 ^{o}C for 10 min, followed by cooling to 4 ^{o}C until the sample is recovered (Fatima et al., 2011). Amplicons are then visualised with a UV transilluminator after resolving 5 µl of the products in the 1% agarose gel and staining with GelRed. Lastly, purified PCR products were subjected for DNA sequencing at Next Gene Scientific Sdn Bhd. Raw sequences were analysed based on NCBI sequence database (MT367790, MN515152, MT214268, MT124566, KY908503, KY908479, MN177200, ON738242, OP630954, OP604494, NR113987, KM100367, MN793064, MN213372, OP597695, OP893812). Then, MEGA X software was used to perform the sequence using CLUSTAL W alignment. The Maximum Likelihood technique based on the Tamura-Nei model was applied to infer the evolutionary history (Tamura et al., 2011).

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3.5 Development of Biofertilizer by Using Bio-Inoculate and Multipurpose Compose

Multipurpose compost was used as a carrier material for the development of biofertilizer. Multipurpose compost was packed in autoclave polythene covers and sealed using an electric sealer (3 g in 5 mL of water). The package was sterilized at 121°C for 20 minutes.

Nine bacterial isolates were cultured in nutrient broth and incubated at 28°C with 200 rpm shaking for 48 hours to obtain the optical density (OD) at 600 nm equals to 0.3 (Dicko et al., 2018). Bacterial cultures were then centrifuged at 11057 ×g at 4 ^{o}C for 15 minutes and resuspended in sterilized nutrient broth. The process was repeated twice. After that, the pellet was separated from the supernatant and the pellet was freeze dried for 2 days using a freeze dryer (LGJ-18 vacuum freeze dryer). Then, pre-sterilized multipurpose compost were inoculated with bacteria with a ratio of bacteria pellet to pre-sterilized commercial compost of 1: 50 as recommended by Stella et al. (2019).

The mixture was manually shaken by hands until the microbial inoculum was uniformly spread in multipurpose compost. Microbial inoculum and pre-sterilized multipurpose compost were packed in the polythene bag and was immediately sealed. The package containing pre-sterilized commercial compost without bacterial inoculation was used as a control. The packages were placed at 30°C for 7 days. After 7th day interval, biofertilizer pellets were tested for microbial survivability by determining viable cell counts and physicochemical analysis (Pathirana and Yapa, 2020).

3.6 Microbial Survivability Test

One gram of each sample was mixed with 9 mL of sterile distilled water in ratio 1:9 and was allowed to mix thoroughly in a shaker for 1-2 hours. The suspension was serially diluted before dispensed into the agar plate and was incubated at 35°C for 24 hours. The number of bacterial growth on the plate was calculated using Equation 2.

$$Population\ density\ \left(\frac{CFU}{mL}\right) = \frac{Number\ of\ colonies \times Dilution\ factor}{Volume\ of\ culture\ plate} \quad \text{..... Equation 2}$$

3.7 N, P and K Determination in Mangrove Soil and Biofertilizer

Mangrove soil and biofertilizers were analysed for nitrogen, phosphorus, potassium after 7 days. Three samples of mangrove soil (soil 1, 2 and 3) with triplicate and three samples of biofertilizers and one control (without biofertilizer) with triplicate were used for this analysis. Nitrogen was measured using Kjedahl method. 1 g of soil samples were mixed with 10 mL of sulphuric acid (H_2SO_4). Then, one Kjedahl tablet was added to the sample solution. Next, the sample solution was digested them using Gerhardt Kjedahltem for 2 hours and 30 minutes and the samples were distilled and titrated using Gerhardt VAPODEST 500 around 3 minutes per sample.

Meanwhile, potassium and phosphorus were analyzed using X-ray Fluorescence (XRF) (Nazmin et al., 2019). In XRF determination (Bruker AXS, S8 Tiger, Karlsruhe, Germany), three soil samples and three set of formulated bio-fertilizers with control were

dried, homogenized and sieved at smaller particles sizes. Plastic cups that lined with 3.6 m thick Mylar polymer was used to hold samples and placed inside the XRF analyser. 7 g of soil samples were put into the plastic cups and covered with Mylar thin film. In operation conditions, the X-ray tube had a power of 15 W with a 50 kV generator. The spot size on the sample was typically 10×14 mm. The detector has a high resolution of 135 eV.

3.8 Duckweed (*Lemna Minor*) Growth Experiment

Duckweed *L. minor* growth experiment was conducted to evaluate the efficiency of formulated biofertilizers. Ten fronds of *L. minor* plants were sterilized using 70% ethanol, bleach and sterilized distilled water before transferred to container with width: 122mm x depth: 173mm x Height: 62mm that contains 200 mL of water and 25 g of control medium and three set of formulated biofertilizers (Set A, Set B and Set C). In this experiment, duckweed plants were grown at a greenhouse with temperature range between 26°C to 30°C. The numbers of duckweed fronds were recorded every 2 days in 15 days.

3.9 Quantification of Protein Content in Duckweed Fronds

Fresh *L. minor* was dried in the oven dryer (65°C for 24 hours) and ground into fine powder. Dried duckweed was soaked in sterilized distilled water in the ratio of 1: 10 (1 g

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of dried duckweed in 10 mL of distilled water) overnight to expand the cells before physical enforcement is applied to break the cells. Then, the soaking material and water was microwaved at 100 W for 15 minutes by using home-based microwave. The microwaved duckweed was filtered to separate the solids and green juice. The protein in green juice was quantified by using Bradford reagent and measured by using UV spectrophotometer (Varian Cary 50 Conc. UV-VIS Spectrophotometer). The protein quantification was followed the standard protocol of Quick Start™ Bradford Protein Assay. The standard protocol was performed using 1 mL cuvette assay formats. The samples were first diluted in test tubes which followed these concentrations (0, 0.125, 0.250, 0.500, 0.750, 1.000, 1.500 and 2.000) mg/mL. Each standard and diluted sample was pipetted into separate disposable cuvettes as followed in Table 3.1. The 1x dye reagent was added to each cuvette. The volume of standard, sample and 1x dye reagent for 1mL assay were as followed in Table 3.1. The cuvette with the mix of sample and reagent along with the mix of standard and 1x dye reagent were incubated for 5 minutes. Lastly, the UV spectrophotometer was set to 595 nm. The absorbance of the standard and samples were measured.

Table 3.1 : Volume of Standard, Sample and 1x Dye Reagent Using 1 mL Assay

Assay	Volume of standard and sample	Volume of 1x dye reagent
1 mL	20 µL	1 mL

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The protein of the duckweed was calculated using Equation 3.

$$y = mx + c \text{ .... (Equation 3)}$$

Where: y = absorbance at 595 nm.
x = protein concentration.

3.10 Statistical Analysis

All collected data of the element analysis, duckweed growth and amount of duckweed protein All collected data of the elements analysis, duckweed growth and amount of duckweed protein were analysed using MINITAB16 software (Minitab Ltd, Coventry, UK) and analysis of variance (ANOVA) available in the software. Mean difference analysis was conducted using the Tukey's method, with significant differences were defined between the sample means ($P < 0.05$).

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