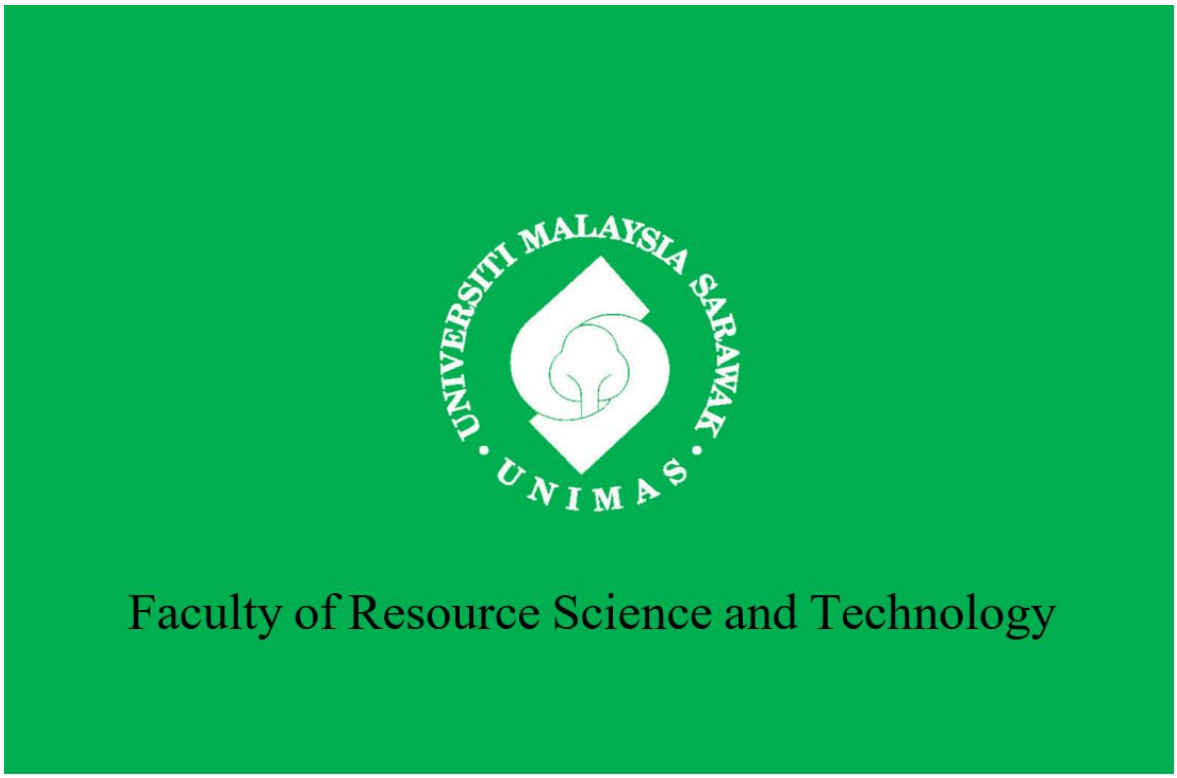

Identification and Characterization of Bacteria Communities in Peat Soil of Paddy Farms in Lundu, Sarawak

Josephine Elvina Binti Suching (72287)

Bachelor of Science with Honours
(Resource Biotechonology)
2022

Identification and Characterization of Bacteria Communities in Peat Soil of Paddy Farms in Lundu, Sarawak

Josephine Elvina Binti Suching (72287)

A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours
(Resource Biotechnology)

SUPERVISOR: DR. SAMUEL LIHAN

Programme of Resource Biotechnology
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UNIVERSITI MALAYSIA SARAWAK
2022

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Identification and Characterization of Bacteria Communities in Peat Soil of Paddy Farms in Lundu, Sarawak

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ABSTRACT

Bacteria play a vital role in controlling biogeochemical cycles in the peat soil ecosystem. Most of the studies on soil bacteria focus mainly on the paddy farms in Indochina Peninsula including Laos, Vietnam, and Thailand while bacteria diversity is still poorly known in Malaysia, especially in Borneo Sarawak. The risk associated with the microbes in paddy farm is not well documented. This study was conducted to isolate and characterize the bacteria species from paddy fields located in various places in Lundu, Sarawak including Kampung Simboh Farm 1, Kampung Simboh Farm 2, Kampung Sukam, and Kampung Pueh. Four soil samples were collected randomly from each site. One gram of the soil sample was diluted in 9 mL of 0.80% (w/v) normal saline solution. The diluted sample was plated on Nutrient Agar plates and incubated at 28 °C for 24 h. A total of eleven bacteria isolates were then identified using 16S rRNA analysis. The physiological and biochemical of bacteria species were characterized by using Gram Staining and tested against eight antibiotics. The result showed that the most dominant bacterial taxa found with different abundances in all study sites were Firmicutes and followed by *γ-proteobacteria*. Gram-positive bacteria species were more prevalent in the peat soil ecosystem compared to Gram-negative bacteria. The antibiotic susceptibility testing showed that 100% of bacterial isolates are susceptible to Norfloxacin (NOR,10 µg). This study suggests that bacterial abundances and interaction between bacterial species promote and maintain a functional agroecosystem which improves crop productivity as well as plant nutrition and health. On another hand, the bacteria found in the farm may pose a health risk to those who are in direct contact with the soil in the farms, especially the farmers.

Keywords: Peat soil, paddy farms, bacterial community,16S rRNA analysis, characterization

ABSTRAK

Bakteria memainkan peranan penting dalam mengawal biogeokimia dalam ekosistem tanah gambut.Kebanyakan kajian tertumpu pada sawah padi di Peninsula Indochina termasuklah Laos,Vietnam, dan Thailand manakala kepelbagaian bakteria di Malaysia terutamanya Sarawak Borneo masih kurang dikaji. Mikrob yang berisiko di sawah padi tidak didokumentasikan dengan baik. Kajian ini dilakukan bertujuan untuk mengasingkan dan mencirikan spesies bakteria dari sawah padi di pelbagai kawasan di Lundu, Sarawak seperti Kampung Simboh Sawah 1, Kampung Simboh Sawah 2, Kampung Sukam, dan Kampung Pueh. Empat sampel tanah gambut telah dikumpul secara rawak dari tempat kajian. Satu gram sampel tanah telah dicair secara bersiri dalam 9 mL larutan garam biasa. Sampel yang dicairkan disapu rata atas agar-agar nutrien dan diinkubasi pada suhu 28 °C selama 24 jam. Sebelas asingan spesies bakteria telah dikenal pasti menggunakan analisis 16S rRNA. Fisiologikal dan biokimia species bakteria telah dicirikan menggunakan Pewarnaan Gram dan diuji terhadap lapan antibiotik. Keputusan menunjukkan Firmicutes merupakan taksa yang paling dominan dijumpai dalam perbezaan kelimpahan dari semua tempat kajian dan diikuti oleh taksa γ-proteobakteria. Species gram-positif bakteria berleluasa dalam ekosistem tanah gambut berbanding kepada spesies gram-negatif bakteria. Ujian Suseptibiliti Antibiotik telah menunjukkan 100% asingan bakteria ialah lemah terhadap Norfloxacin (NOR, 10 µg) antibiotik. Kajian ini menunjukkan bahawa kelimpahan bakteria dan interaksi antara spesies bakteria menggalakkan dan mengekalkan fungsi agroekosistem yang membantu meningkatkan daya pengeluaran tanaman bersamaan nutrisi dan kesihatan tumbuhan. Selain itu, bakteria yang dijumpai di kebun boleh menimbulkan risiko kesihatan terhadap sesiapa yang berkontak rapat dengan tanah di kebun, terutamanya para petani.

Kata kunci: Tanah gambut, sawah padi, komuniti bakteria, analisis 16S rRNA, pencirian

TABLE OF CONTENT

DECLARATION i

ACKNOWLEDGMENT iii

ABSTRACT iv

ABSTRAK iv

TABLE OF CONTENT v

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF ABBREVIATIONS ix

CHAPTER 1: INTRODUCTION 1

1.1 Study of Background 1

1.2 Objectives 3

CHAPTER 2: LITERATURE REVIEW 4

2.1 Significant of Paddy 4

2.1 Bacterial Composition in Peat Soil 5

2.2 The Role of Bacterial Community in Peat Soil 5

2.2.1 Nitrogen-fixing Bacteria 5

2.2.2 Decomposition of Rice Straw 7

2.3 Soilborne Pathogens in Paddy Farm 7

CHAPTER 3: MATERIALS AND METHODS 10

3.1 Sample Collection 10

3.2 Isolation of Pure Culturable Bacteria from Peat Soil 11

3.3 DNA Extraction from Pure Bacteria 12

3.4 Identification of Bacterial Species in Soil Samples 13

3.4.1 16S rRNA PCR Sequencing 13

3.4.2 DNA Sequencing and Blasting 14

3.5 Characterization of Soil Bacteria 15

3.5.1 Physiological Characteristics of Bacterial Strains 15

3.5.2 Antibiotic Susceptibility Testing 15

CHAPTER 4: RESULTS 17

4.1 Isolation of Pure Bacteria 17

4.1.1 Bacteria Culture on Nutrient Agar 17

4.2 Identification of Bacterial Species in Soil Sample 18

4.2.1 16S rRNA Sequencing 18

4.2.2 Dominant Phyla 19

4.3 Characterization of Soil Bacteria Species 21
4.3.1 Physiological Characteristics of Bacterial Species 21
4.3.2 Antibiotic Susceptibility Testing 25
CHAPTER FIVE: DISCUSSION 27
5.1 The Diversity of Microbes 27
5.2 The Soil Pathogen and Antibiotic Resistance 30
CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS 32
CHAPTER SEVEN: REFERENCES 34
CHAPTER EIGHT: APPENDICES 39
Appendix A: Figures of Bacterial Growth on Nutrient Agar 39
Appendix B: Pure Bacteria Isolate on Nutrient Agar 41
Appendix B: Figures of Antibiotic Susceptibility Test of Bacteria Isolates 43

LIST OF TABLES

TABLE		PAGE
Table 3.1	Soil samples of paddy farms from Lundu, Sarawak	10
Table 3.2	Study locations of paddy farms in Lundu, Sarawak	11
Table 3.3	Soil samples of bacteria labelling	12
Table 3.4	PCR reaction mixture	13
Table 3.5	Universal primers for amplification of 16S rRNA gene fragments	14
Table 3.6	Antibiotic selection by using Kirby-Bauer disc diffusion	16
Table 4.1	Abundances of bacteria based on the number of isolates identified using the 16S rRNA gene	19
Table 4.2	Gram staining of soil bacterial samples	22
Table 4.3	Number of isolates susceptible and resistant to the selected antimicrobial agents	26

LIST OF FIGURES

FIGURE		PAGE
Figure 4.1	Serial dilution up to 10^{-3} and 10^{-4} dilution factor of peat soil samples from KSK	17
Figure 4.2	Sub-cultured bacterial sample in nutrient agar after incubation of 16-24 hours	18
Figure 4.3	The result of 16s rRNA PCR sequencing	18
Figure 4.4	Percentage of bacterial phyla identified based on 16S rRNA amplification in (a) KSF1, (b) KSF2 &KSK, and (c) PH	20
Figure 4.5	Eight antibiotics testing against 11 isolates of soil bacteria	25

LIST OF ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
δ	Omega
°C	Degree celsius
%	Percentage
µg	Micrograms
µm	Micrometres
µL	Microlitres
16S	16 Subunits
27f	27 forward
517r	517 reverse
BLAST	Basic Local Alignment Search Tool
CFU	Colony-forming unit
Cm	Centimetres
C	Current
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphate synthesis
g	Gram
kb	Kilobase
L	Litre
ml	Millilitres
mM	Millimetres

MgCl2	Magnesium chloride
mcr	Mobilized Colistin Resistance
MHA	Muller-Hinton agar
MHB	Mullet-Hinton broth
MPN/g	Most probable number per gram
NCBI	National Centre for Biotechnology Information
*nif*H	Nitrogen Fixing gene
NA	Nutrient agar
N	Nitrogen
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
rpm	Rotation per minute
Tm	Melting temperature
V	Voltage
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 Study Background

Rice is the primary staple food and crop in Southeast Asia including Malaysia with the production volume of rice in 2020 at about 1.51 million metric tons (Hirschman, 2022). Firdaus *et al.* (2020) stated that paddy is also the primary source of income and livelihood for the paddy farming community, particularly for small-scale farmers and landless agricultural workers. About 40% of farmers' income is derived solely from paddy production. Due to an acute food shortage, Sarawak imported 145,468 metric tonnes of rice in 2021. As a result, rice production decreased from 142,000 metric tonnes in 2012 to 89,400 metric tonnes by 2021. In Sarawak, about 38% of rice production meets the state's demand (Borneo Post Online, 2022).

Peat soils are formed by 30% of organic matter accumulation and contained an essential reservoir of terrestrial natural carbon and nitrogen. The organic matter breakdown is facilitated by the different species of bacteria found in peat soil. About 63% of the peatland in Sarawak makes it the largest peatland in Malaysia (Khing, 2016). Peatland is critical to the country's socioeconomic well-being Sarawak, by utilization of agricultural lands for paddy plantation. Paddy farms in Lundu, Sarawak are surrounded by peat soil which is composed unique ecosystem with an abundance of bacterial communities which play a pivotal role in soil ecosystem processes and functions. The most common activities carried out by microorganisms in paddy fields such as methanogenesis and methane oxidation benefit the atmospheric and soil

ecosystem. In addition, the bacterial community serves a crucial role in global carbon sequestration, nutrient retention, and nitrogen fixation in the peat soil (Islam & Wright, 2004).

The microbial community contributes to soil processes such as decomposition, mutualization, and pathogens. Naturally occurring bacteria in soil promote the growth of the agricultural crop by having different types of functional groups across the soil ecosystem such as diazotrophs, rhizotrophs, decomposers, mutualists, and lithotrophs. The peat soil of paddy farm has high contained organic materials composed of a diverse group of bacteria that are determined by the activity of soil bacteria like decomposition of rice straw and other organic matters. Paddy farm undergoes a harvesting season annually, the decomposition of rice straw which brings beneficial effects to the soil ecology and maintains the bacterial functions for the next season of paddy plantation.

However, there is a potential disease associated with soil bacteria in paddy farms than affects human health. Bacterial species such as *Bacillus cereus* are found naturally in decomposed organic matter. The pathogenic properties of this bacterium cause gastroenteritis. It has spores for germination in the host and contaminates animals and plants which then the plant will be consumed by humans. Rice is the primary food which consume daily in Southeast Asia; however, ingestion of contaminated rice can cause food poisoning in humans caused by *B. cereus* from the soil. The utilization of agrochemicals to mitigate the plant diseases contribute to the distribution of antibiotic-resistance bacteria in the soil ecosystem, arise global health concerns (Udikovic-Kolic *et al.*, 2014). Antibiotic-resistant bacterial strains can be transmitted from contaminated harvested products to humans including farmers and

their families, indirectly via skin inoculation and ingestion of contaminated food. World Health Organization [WHO] (2021) reported *Klebsiella pneumoniae* is one of the most threatening antibiotic-resistance bacteria present in the soil and showed high rates of resistance to ciprofloxacin varying from 4.1% to 79.4%. *K. pneumoniae* causes urinary tract disease in humans when come into contact with the infected plant.

Other than recent studies that have been published related to bacterial diversity in the paddy farm is still limited in Borneo Sarawak as these studies were mainly focused on the paddy field in Indochina Peninsula. Hence, in the present study, the isolation, and characterization of the bacteria community from peat soil of paddy farms were carried out by four samples from different locations in Lundu, Sarawak. In this research, molecular technology like 16S rDNA-PCR was used capable to identify and characterize a broad spectrum of bacteria species within the most dominant phylum found in peat soil of paddy farms.

1.2 Objectives

The specific objectives of this study are:

1. To isolate a pure culture of bacteria in peat soil of paddy farms in Lundu, Sarawak
2. To identify the bacteria species by using 16S rRNA-PCR analysis
3. To characterize the physiological and biochemical of the bacteria community in the samples

CHAPTER 2

LITERATURE REVIEW

2.1 Significant of Paddy

Rice production in Malaysia was reported at 2,343.7 thousand tonnes in 2020. This records a decrease from the previous year which is 2,352.8 thousand tonnes (Department of Statistics Malaysia, 2021). However, a total of Malaysia's paddy cultivation area is 0.70 million hectares, where the lowest among Southeast Asia countries where Indonesia, Vietnam, and Thailand, the largest rice producers in the Southeast Asia, with total paddy cultivation area of 11.50, 7.54, and 10.83 million hectares, respectively (United States Department of Agriculture [USDA], 2020). The yield of paddy rice in Malaysia increased by 2.73 percent from 35,009 hg per hectare in 2019 to 35,968 hg per hectare in 2020. Since the decrease of 5.73 percent in 2017, paddy rice yield has decreased by 4.08 percent in 2020. Paddy is also the primary source of income and livelihood for paddy farmers, especially minority of farmers and landless agricultural employee. Paddy production accounts for around 40% of farmers' income. The majority of Malaysia's rice is imported from Thailand, Vietnam, Pakistan, and India (The Malaysian Reserve, 2022). In 2022, Vietnam was reported to export approximately 35,000 tonnes of rice to Malaysia (The Star, 2022).

Sarawak has known with the traditional rice cultivation which has commercial value. Sarawak rice production meet 38 % of state's demand. From approximately 130,000 hectares in 2012 to approximately 83,000 hectares in 2021, Sarawak's overall paddy farming area has declined drastically. The low rice self-sufficiency level (SSL) causes Sarawak to import 145,468 metric tonnes of rice in 2021. As a result, rice production decreased from 142,000 metric tonnes in 2012 to 89,400 metric tonnes by 2021 (Borneo Post Online, 2022). According to the

Department of Agriculture Sarawak (2012), about 70% of rice consumption were mostly from imported rice.

2.1 Bacterial Composition in Peat Soil

In rice fields in Southeast Asia, a diverse microbial flora of soil bacteria was discovered, as evidenced by several studies. In most cases, *Proteobacteria*, *Acidobacteria*, and *Chloroflexi* will constitute most of the bacterial populations found in rice fields (Ahn *et al.*, 2016). Members of the *Proteobacteria* division were the most numerous of these organisms, accounting for 37.8% of the total. This included α -*Proteobacteria* (13.5%), γ-*Proteobacteria* (12.2%), δ -*Proteobacteria* (6.8%), and β -*Proteobacteria* (5.4%). This was followed by the *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, and *Planctomycetes* division with 16.2%, 12.2%, 10.8%, 10.8%, 8.1%, respectively. There were fewer examples of the phyla *Firmicutes*, *Gemmatimonadetes*, and *Verrucomicrobia*. In a paddy field in Ningxia, the research uncovered a wide variety of soil bacteria from all over the world (Zhang *et al.*, 2008). According to the most current research conducted by Wang *et al.* (2021), the paddy field is home to *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Bacteroidota*, *Desulfobacteriota*, *Nitrospirota*, and *Myxococcota*. These phyla were shown to be the most prevalent in all the samples.

2.2 The Role of Bacterial Community in Peat Soil

2.2.1 Nitrogen-fixing Bacteria

The peat soil ecosystem serves crucial roles in carbon cycling and nitrogen fixation with the richness of carbon and nitrogen that is very beneficial to the balance of soil nutrient

composition for the growth of plants. Liu *et al*. (2018) demonstrated that carbon and nitrogen are essential for microbial growth, especially for diazotrophic bacteria. Nitrification is a crucial biogeochemical N-cycling and biological wastewater treatment process (Yi *et al*., 2019)`. There is a diverse microbial community in the peat soil ecosystem such as phylum *Firmicutes* that act as nitrogen fixers. *Bacillus cereus* is a member of the *Firmicutes* group which showed the most dominant species that is involved in nitrogen fixation (Azeem *et al*., 2021). An investigation by Azeem *et al.* (2020) indicated that there are several nitrogen-fixing bacteria communities associated with the legume crop. For instance, *Proteobacteria* contains member of *Burkholderiales*, *Xanthomonadales*, and the *Cyanobacteria* phylum. They also reported that there is a high similarity of *Proteobacteria, Actinobacteria*, and *Cyanobacterial* phyla that contain a broad spectrum of nitrogen-fixing bacteria members involving the improvement of nitrogen fixation in the soil ecosystem.

The nitrifying and denitrifying bacteria, including *Anaerolineaceae*, *Pseudarthrobacter*, *Bacillus*, and *Nitrospira*, were significantly enhanced in the paddy soil (Wang *et al*., 2021). It was discovered that *Bacillus* was a type of denitrifying bacteria of rice rhizosphere that could convert atmospheric nitrogen molecules into rice through adsorption (Yang *et al*., 2011). Several studies suggested that the most abundant diazotrophic bacteria were isolated from wetland rice farming. According to Fujii *et al.* (1987); Tou and Zhou (1989); Baldani and Dobereiner (1980), they discovered the diazotrophic bacterial species in the wetland rice, which is the most predominant including *Klebsiella oxytoca*, *Enterobacter cloacae*, and *Azospirillum*. Naher *et al.* (2009) isolated the most dominant diazotrophic bacteria including *Rhizobium, Burkholderia*, and *Corynebacterium* from the different soils of Tanjong Karang rice farming.

2.2.2 Decomposition of Rice Straw

Methanogenic bacteria communities are the main driver for the decomposition of the rice straw, which can ferment the carbohydrates into hydrogen, carbon dioxide, various fatty acids, and alcohols (Ji *et al*., 2018). Rice cultivation resulted in an increase in the total number of *Chloroflexi* species present. Most of the microorganisms that were found in *Chloroflexi* were strictly anaerobic bacteria. These bacteria were able to digest sugars and polysaccharides into organic acids and hydrogen which sped up the process of organic fields breaking down in paddy soil (Podosokorskaya *et al*., 2013). Based on Ji *et al*. (2018) performed the identification of the abundances of bacteria involved in the decomposition of the straw by using ILLUMINA sequencing of the bacterial 16S rRNA genes, showed *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Chlorobi*, *Actinobacteria*, and *Acidobacteria* are the most dominant phyla. It was discovered that *Proteobacteria* can take part in the biological cycle of vital mineral nutrients in the soil; a high concentration of Proteobacteria in rhizosphere soil is advantageous to the preservation of fertility and the growth of plants (Chauhdry *et al*., 2012).

2.3 Soilborne Pathogens in Paddy Farm

Several studies discovered that contaminated soil from paddy farm is a potential transmission route of *Burkholderia pseudomallei* known as the causative agent of melioidosis (Manivanh *et al*., 2017; Ong *et al*., 2017; Hsueh *et al*., 2018). Melioidosis is an endemic case reported with 165,000 human melioidosis cases per year, from which ~89,000 people die especially infected rice farmers occurred in Southeast Asia and northern Australia (Cheng & Currie., 2005; Inglis *et al*., 2001; Limmathurotsakul *et al*., 2016). In Malaysia, Abu Hassan *et al*. (2019) suggested that *B. pseudomallei* has highly associated with farming in Alor Setar, Kedah known as the

most extensive agriculture rice farming. Manivanh *et al.* (2017) reported the case of melioidosis caused by *B. pseudomallei* in a lowland paddy field in Laos which highly depend on a high soil water content and low total nitrogen, carbon, and organic matter content. Ong *et al.* (2017) also detected the presence of *B. pseudomallei* in a paddy farm in Ubon Ratchathani, northeast Thailand during heavy rain by isolating the bacteria from contaminated soil. The inhalation, ingestion, and skin inoculation of contaminated soil and water cause melioidosis in humans (Hsueh *et al*., 2018).

The peat soil is densely populated with bacterial, archaeal, and fungal organisms that are engaged in the breakdown of organic materials. Bacterial species such as *B. cereus* are found naturally in decomposed organic matter. The pathogenic properties of this bacterium cause gastroenteritis. It has spores for germination in the host and contaminates the rice, which when consumed by humans, thus causes food poisoning. There are several cases reported related to the contamination of *B. cereus* in rice that occurred in Indonesia, Pakistan, India, Malaysia, Belgium, America, Australia, Korea, Iran, China, and Nigeria (Lutpiatina,2020). Bilung *et al.* (2018) reported there is the presence of *B. cereus* in local unhusked (rough) rice samples from Sarawak, Malaysia. They found the number of *B. cereus* presence in all the collected rice samples approximately 1100 MPN/g. However, *B. cereus* developed resistance to multi drugs due to exposure of long-term application of synthetic chemicals such as chemicals fertilizers and pesticide thus create metal residues in soils. *B. megaterium* strain, *B. cereus* strain and *C. pseudoviolaceum* strains were isolated from contaminated soil samples of Cachar district of Assam, India, exert moderate resistance to Azithromycin, Ceftriaxone, Cefdinir, Rifampicin, Polymyxin and Co-trimoxazole. *Bacillus sp.* showed high tolerance to lead copper in contaminated soil (Nath *et al*., 2019).

The prevalence of *Klebsiella pneumoniae* in the agriculture soils where the vast reservoir of antibiotic-resistance bacteria was reported about 82% were detected as multi-drug resistant. *K. pneumoniae* harbour β-lactam encoding genes which mean it is resistant to the different group of antibiotics including β-lactam antibiotics, aminoglycosides, ciprofloxacin, cotrimoxazole, carbapenem, piperacillin, and tazobactam (Aminul *et al.*, 2021). *K. pneumoniae* may remain in harvested crops and enter human food through uncooked food or handling the harvested crop causes urinary tract infections and pneumonia.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample Collection

This study was conducted on peat soil from a paddy farm located in Lundu, Kuching. Table 3.2 indicated that four samples of peat were taken from Farm 1 of Kampung Simboh, Farm 2 of Kampung Simboh, Kampung Sukam, and Kampung Pueh. A total of four peat soil samples were collected and stored in a plastic bag at 4°C inside a refrigerator prior to laboratory analysis. The soil samples were labelled as shown in Table 3.1. Sampling was taken place during the dry season.

Table 3.1: Soil samples of paddy farms from Lundu, Sarawak

Sample Code	Source
KSF1	Soil
KSF2	Soil
KSK	Soil
KPH	Soil

Table 3.2: Study locations of the paddy farms from Lundu, Sarawak

Sample Code	Location
KSF1	Kampung Simboh Farm 1
KSF2	Kampung Simboh Farm 2
KSK	Kampung Sukam
KPH	Kampung Pueh

3.2 Isolation of Pure Culturable Bacteria from Peat Soil

About 28 g of Nutrient agar (NA) was weighed and prepared in 1 L of the flask. The nutrient agar was poured into Petri dishes and let solidified then directly stored inside the fridge for isolation. The isolation of pure culture bacteria was carried out by dilution method and cultured on non-selective media. The non-selective media was a Nutrient agar (NA) (HiMedia Laboratories, Mumbai, India) used to culture the broad range of pure bacteria groups from the peat soil. According to the serial dilution method, about 0.80% (w/v) of saline solution was prepared and added about 9 mL into four tubes. In this study, approximately 1 g of soil containing culturable bacteria was soaked in 0.80% (w/v) of saline solution and serially diluted from $10^{-3}$to 10^{-4}, then inoculated on nutrient agar (HiMedia Laboratories, Mumbai, India), and incubated at 28 °C aerobically for 24 hours. The growing colonies were purified by using the streak method on nutrient agar (HiMedia Laboratories, Mumbai, India) and then incubated aerobically at 28 °C. The agar plates were labelled according to the sample code. The pure cultures were stored in the slanted nutrient agar (HiMedia Laboratories, Mumbai, India).

Table 3.3: Soil samples of bacteria labelling

No of Isolate	Bacterial Code
1	KSF1-1
2	KSF1-2
3	KSF2-1
4	KSF2-2
5	KSF2-3
6	KSK-1
7	KSK-2
8	KSK-3
9	KPH-1
10	KPH -2
11	KPH-3

KSF1: Kampung Simboh Farm 1, KSF2: Kampung Simboh Farm 2, KSK: Kampung Sukam, KPH: Kampung Pueh, 1: Colony 1, 2: Colony 2, 3: Colony 3

3.3 DNA Extraction from Pure Bacteria

Bacterial culture DNA was extracted from bacterial isolates by heating the bacterial suspension at 95 °C for ten minutes. Then the DNA samples were centrifuged at the maximum speed, 1350 rpm for 1 minute. About 2 μL of DNA supernatant of each sample was added into 38 μL of PCR master mix.