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Polyphasic Identification of Bacterial Endophytes Isolated from *Rhizophoramucronata* in Combado, Maasin City

In partial fulfilment of the 4th Quarter requirements for S&T Research 1

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ABSTRACT

The Philippines is known for its astounding biodiversity. Numerous species of flora and fauna are known to inhabit the Philippine marine and terrestrial domains. True to its highly diverse nature, the country hosts more than 50 percent of the total mangrove species around the world. Mangrove extracts are known sources of antifungal, antiviral, anti-cancer and anti-diabetic compounds. Furthermore, they may pose a great potential in hosting a multitude of microorganisms with promising therapeutic capabilities. Prior studies have shown that the plethora of benefits mangrove possess can be attributed to the microorgranisms of its microbiome, or known as the endophytes. This study isolated bacterial endophytes from a mangrove tree, which was later on identified as Rhizophoramucronata, from a mangrove forest in Combado, Maasin City, Southern Leyte. A total of 15 colonies were primarily selected and isolated, which were narrowed down to 10 morphologically distinct isolates. These isolates were characterized through polyphasic identification which included the morphological characterization and molecular techniques. For the morphology, the isolates were characterized through colony morphology and microscopic observation of the Gram's stain. For the molecular techniques, the DNA of the isolates were isolated and the 16s rRNA gene was amplified. Each of the morphospecies was sent to Macrogen in South Korea for DNA sequencing. The sequences received were then analyzed and made into phylogenetic trees to show the relationship between these bacterial species and other species of bacteria. A total of eight bacterial endophytes were identified. Of the eight, three belonged to the genus Bacillus, three to the genus Staphylococcus, and two to the genus Lysinibacillus. Results show that the bacterial endophytes isolated from Rhizophoramucronata were Lysinibacillus fusiformis, Staphylococcus haemolyticus, Staphylococcus epidermidis, Bacillus aryabhattai, Bacillus licheniformis, Bacillus amyloliquefaciens and Lysinibacillusboronitolerans.

Keywords: *16s rRNA gene, bacterial endophyte, DNA barcoding, mangrove, molecular identification, phylogenetic tree, polyphasicidentification, Rhizophoramucronata*

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CHAPTER 1
THE PROBLEM AND ITS SETTING

Background of the Study

Endophytes are microorganisms which live within plant tissues without harming them. It is believed that each plant on earth is a host to different endophyte species. In fact, there is an estimated 1 million species of endophytes. Nevertheless, only some of them are identified, described, and characterized (Gayathri et al., 2010). In addition, endophytes are known to produce secondary metabolites which have practical applications most especially in drug discovery, biofuel, bioremediation, and in agriculture. In this study, bacterial endophytes will be collected from a mangrove tree in Combado, Maasin City through the help of Dr. Primavera's mangrove field guide. They will be isolated in pure culture and identified through gram staining, DNA extraction, and phylogenetic analysis. Mangrove trees were chosen as it is believed that plants from unique and biodiverse environmental settings can be a promising source of endophytes producing new bioactive metabolites. Mangroves are salt tolerant plants and are one of the world's most productive ecosystem considering its ecological significance. Furthermore, its extracts have also shown bioactivity against plant, animal, and human pathogens (Eldeen, 2014). The 16s rRNA gene of the bacterial isolates will be amplified and sent to Korea for sequencing. The sequences will then be matched with a database to identify them and to come up with their respective phylogenetic trees through the use of computer softwares such as BLAST and MEGA.

Statement of the Problem

Main Problem: What are the different species of bacterial endophytes that can be isolated from *Rhizophoramucronata* in Combado, Maasin City?

Sub-problems:

1. What are the morphological characteristics of each bacterial isolate in terms of:

1.1 Shape

1.2 Elevation

1.3 Margin

1.4 Texture

1.5 Optical Property

1.6 Pigmentation

1.7 Size

1.8 Appearance

1.9 Form

2. Which bacterial isolates are gram-positive and which are gram-negative?
3. Which bacterial species does each isolatemost likely belong according to the BLAST results?
4. Which bacterial species does each isolate most likely belong according to the maximum likelihood method?

Significance of the Study

The research gap this study longs to address is the largely unknown information we have about the bacterial endophytes that can be isolated from a single species in Combado, Maasin City and their bioprospecting potential. This will be one, if not the first, of the pioneering studies regarding the identification of bacterial endophytes in Combado, Maasin City mangroves. This will serve as a preliminary step for future studies that will test the biological activities of these bacterial endophytes such as their antimicrobial, anti-inflammatory, cytotoxicity, and antioxidant properties. Furthermore, the study will put into record the different species of bacteria found in the mangrove forest of Combado, Maasin City. Lastly, it will also satisfy the need to describe and make copies of the different bacteria before they are extinct.

Scope and Limitations

Furthermore, the samples to be collected will only be from a single mangrove tree in Combado, Maasin City to control the number of bacterial endophytes to be yielded from the experiment, which will make it easier to accommodate each isolate within a month.

Definition of Terms

bioprospecting- to search for substances that are produced by living organisms and may be of medicinal or commercial value

secondary metabolites- small organic molecules produced by an organism that are not essential for their growth, development, and reproduction

cytotoxicity- ability of a substance to have a toxic effect on cells

CHAPTER 2
REVIEW OF RELATED LITERATURE

The plant endosphere is a home of a diverse group of microorganisms. Microorganisms which are found within plant tissues are called endophytes. These microorganisms could range from archaea, fungi, protists, and bacteria among others (Kandel, 2017). However, bacterial endophytes are the subjects of interest in this study.

Bacterial endophytes, like most of other endophytes, do not cause harm to their plant hosts but instead establish a mutualistic relationship with them (Rosenblueth& Romero, 2006). Endophytic bacteria in a single plant host are not only limited to a single kind of bacteria but comprise several species, or even genera. Recognition of 'true' endophytic bacteria requires the isolation from surface-disinfected tissues and microscopic visualization of the bacteria inside the plant tissues. Moreover, most endophytes appear to originate in a plant's rhizosphere or phyllosphere (Ryan, 2007).

As stated earlier, endophytes establish a mutualistic relationship with their host. In fact, bacterial endophytes can promote plant growth and even serve as biocontrol agents. In addition, they also produce a plethora of secondary metabolites and natural products which could be used in agriculture, medicine, and even in industry. Furthermore, the removal of soil contaminants is also an exceptional ability of these endophytes which contribute to soil fertility. Furthermore, the use of bacterial endophytes for biotechnological applications such as phytoremediation and biofuel production is becoming an increasing field of interest (Ryan, 2007).

In line with this, a study was conducted by Jayvee Cruz (2015) and his team from the Philippine Rice Research Institute which isolated and screened bacterial endophytes from *Nypafruticans*, or commonly known as a nipa palm. The said plant was chosen since a sustainable high productivity is observed from this plant. This high productivity of the plant was attributed to its indefinite mechanisms for maintaining fertility. Endophytic bacteria were believed to be involved in this indefinite mechanism of the palm. Therefore, their study longed to isolate and identify endophytic bacteria from the said palm. After, they have also screened the endophytes for possible plant growth promoting traits. Specifically, the traits for the production of indole acetic acid (IAA), phosphate solubilization, nitrogen fixation, and starch hydrolysis were taken into consideration. As a result of their study, it was found out that all bacterial isolates produced IAA in culture. Ten of the isolates dissolved precipitated tricalcium phosphate. Five isolates were nitrogen-fixing while four

were able to hydrolyze starch. The result of their study proved the various applications of bacterial endophytes most especially in terms of promoting plant growth (Cruz, 2015).

A similar study was also conducted by Monica Rosenblueth and Esperanza Martinez-Romero (2006) from the Universidad Nacional Automa de Mexico in Cuernavaca, Mexico. They longed to study the interaction of bacterial endophytes and their hosts. As found by their study, some endophytes are seed-borne, but some have mechanisms to colonize. Nevertheless, it is worth noting that those endophytes which are unable to produce proteins necessary for colonization are impaired in the colonization process. In addition, they emphasized that plant genes expressed in the presence of endophytes could provide clues how the endophytes affect the plant. It was however found out that some human pathogens such as salmonella could also become endophytic. Moreover, they are not removed by disinfection procedures that eliminate superficially occurring bacteria (Rosenblueth et al, 2006). As a consequence, delivery of endophytes to the environment should be carefully evaluated to avoid introducing pathogens.

On the other hand, plants from unique and biodiverse environmental settings can be a promising source of endophytes producing new bioactive metabolites. With all this information on hand, mangroves were chosen in this study. This is because mangroves are salt tolerant plants and are one of the world's most productive ecosystem considering its ecological significance. Furthermore, its extracts have also shown bioactivity against plant, animal, and human pathogens (Eldeen, 2014).

One of the most important coastal ecosystems in the world in terms of primary production and coastal protection is the "rainforest by the sea" or the mangrove forest. Mangroves are facultative halophytes that have the ability to grow in either fresh or saltwater depending on which is available (Estomata&Abit, 2011). It grows along tidal mudflats and shallow coastal area extending along rivers and their tributaries.

Mangroves provide tremendous values and benefits to mankind and other organisms. They are a source of valuable plant products that can be used as food, traditional herbal medicine, and other wood and forest products. The earliest uses of mangroves date back to 1230 where Rhizophora seedlings were used as food in times of famine, cure to sore mouth and in the production of fuel, tannin, dye and wine (Saranraj&Sujitha, 2015).

Mangroves are shown to be significant ecologically. They are important in maintaining and building the soil, as a reservoir in tertiary assimilation of waste, and in the global cycle of carbon dioxide, nitrogen, and sulphur. Furthermore, they play significant role in the stabilization of coastal, promoting land accretion,

fixation of mud banks and in the dissipation of winds, tidal and wave energy. Mangroves also create unique ecological environments that host rich assemblages of species. They serve as nesting grounds for hundreds of bird species and as home to a wide variety of reptile, amphibians, mammals, fish, crabs, shrimps, mollusks and other invertebrates (Garcia, Malabrigo&Gevaña, 2014).

Mangrove plants have exhibited uses in folklore medicines. Plant extracts were used for centuries as a method for treating several heath disorders. Pharmaceutical potential of mangrove plants including antibacterial and antifungal properties have been reported. Extracts from mangroves and mangrove-dependent species have proven activity against human, animal and plant pathogens. Preliminary studies demonstrated that the mangrove plant extracts have anti-bacterial against pathogenic bacterial strains, as well as, antibiotic resistant strains such as Staphylococcus sp. and Proteus sp. (Saranraj&Sujitha, 2015). Mangrove extracts can also be possible sources of mosquito larvicides, antifungal, antiviral, anti-cancer and anti-diabetic compounds.

Until now, more than 200 bioactive metabolites have been isolated from true mangroves of tropical and subtropical populations (Saranraj&Sujitha, 2015). According to their chemical structure, most of the isolated compounds belong to steroids, triterpenes, saponins, flavonoids, alkaloids, tannins, and phenolics which have a wide range of therapeutic possibilities. The plethora of benefits mangroves possess can be attributed to the microorganisms of its microbiome. Classifying the different microorganisms can be the gateway into finding out the wide array of its uses.

Classification of microorganisms on the basis of traditional microbiological methods (morphological, physiological and biochemical) creates a blurred image about their taxonomic status and thus needs further clarification (Prakash et al., 2007). Hence, the classification of microorganisms should be based on a more realistic and reliable approach. For this reason, the methods currently applied also include the complete 16S rRNA gene sequencing and its comparative analysis to phylogenetic trees, DNA-DNA hybridization studies with related organisms, analyses of molecular markers and signature pattern(s), biochemical assays, physiological and morphological tests (Prakash et al., 2007). The combination of these multiple approaches is known as the 'polyphasic approach'.

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of

time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Janda & Abbott, 2007).

The 16S rRNA can be extracted from multiple organisms and compared using softwares such as BLAST and MEGA. The data can be used to create phylogenetic trees and other visual models through softwares such as FigTree and MrBayes. Phylogenetic trees based on the 16S rRNA show the differences in base composition, tempo of evolution, unequal rates of evolution in different regions of the rRNA genes, and selection of sequence stretches analyzed (Prakash et al., 2007). The evolutionary relationships between the different organisms can be more easily understood through the creation of a phylogenetic tree.

Throughout the years, microorganisms have posed potentials in causing human diseases. Therefore, the screening of antibacterial activity and other pharmaceutical values of vast medicinal plants have been used as remedies for human diseases. Among these are the different parts of mangroves and mangrove associates are widely used in the world. The marine world offers an extremely rich resource for important compounds of structurally novel and biologically active metabolites.

The Philippines has a very rich biodiversity in terms of number and percentage. It ranks fifth globally in terms of the number of plant species and 5% of the world's flora (Garcia, 2013). In mangroves alone, the country holds at least 50% mangrove species of the world's approximately 65 species. Being archipelagic in nature, a large part of the population of the Philippines depend on mangroves for food, livelihood, and shelter derived from mangrove ecosystems. More than half of the country's 1500 towns and 42, 000 villages depend on these marine habitats for food and other goods and services (Garcia, Malabrigo&Gevaña, 2014). On the eastern coast of the Samar Island, the mangrove forest plays an important role in the protection of the coastline for coconut plantations. Camacho et al. (2011) also wrote that Banacon Island in the Province of Bohol had shown the carbon sink potential of mangroves in the Philippines.

Aside from aforementioned, the City of Maasin of Southern Leyte is reported for the biodiversity of mangroves within its vicinity. The city is characterized by relatively flat lands along coastal areas (Province of Southern Leyte, 2013). Mangroves, locally in the Philippines known as bakhaw or bakawan, have been recorded of rich biodiversity in Maasin which have a plethora of uses, values, and benefits.

CHAPTER 3
METHODOLOGY

As indicated in the title, this chapter encapsulates the research methodology of the study. Specifically, this includes the research design, the collection of the plant samples, the preparation, culturing, and isolation of endophytic bacterial strains, the morphological characterization of the bacterial isolates, as well as the molecular identification of the isolates. Furthermore, also included herein are the various statistical treatments which will be used for the collected data in order to answer the problems or questions posed.

RESEARCH DESIGN

Since there was no further examination or analysis of the data collected was done in this study, the researchers chose a descriptive research design. The main purpose of the study was to purely gather the data about bacterial endophytes found from the *Rhizophoramucronata* in Combado, Maasin City.

COLLECTION OF SAMPLES

Samples from the different parts of a single mangrove tree were collected from Cambado, Maasin City. Before performing the collection, of plant materials, a letter of request was sent to appropriate authorities to ask permission.

Seeking Permission from the appropriate authorities

A letter of request was written addressed to the Maasin City Local Government Unit and the Department of Environment and Natural Resources requesting for permission to collect samples. The procedures and requirements for the request were personally inquired in their respective offices.

Field Collection of the Plant Materials

Healthy and mature plant parts including twigs, leaves, and roots were randomly collected from a single mangrove plant in Brgy. Combado, Maasin City, Southern Leyte. Moreover, the host plant was chosen randomly and was then identified using Dr. Primavera's mangrove field guide and Yong's Comparative Guide to Asian Mangroves. It was ensured that the host plant had observable fruits and flowers to identify its species.

PREPARATION, CULTURING, AND ISOLATION OF ENDOPHYTIC BACTERIAL STRAINS

The plant samples were treated in 75% ethanol and then rinsed in water to remove debris on the surface. Moreover, the agar and broth media were also prepared and dispensed into the petri dishes and test tubes

respectively. Subsequently, the plant tissues were plated onto the dishes. The bacterial colonies were then isolated in pure culture.

Preparation of Plant Samples and Surface Sterilization

The plant samples were washed in running distilled water to remove debris on the surface. In accordance to the method previously described by I.M.S. Eldeen, the samples were treated with 75% ethanol for 1 minute and then immersed in sodium hypochlorite for 10 minutes. Subsequently, they were re-immersed in 75% ethanol for 30 seconds. Afterward, they were rinsed three times with sterile water, dried, and sliced into small parts around 3-5 mm each (Eldeen, 2014).

Preparation of Agar and Broth Media

13 g of nutrient broth powder and 15 g of agar agar powder was dissolved in 1 liter of distilled water and placed in a media bottle. The mixture was brought to a boil inside the autoclave to dissolve the powder completely. Approximately 20-25 mL of agar was then dispensed into each of the sterile petri dish.

25 g of Luria Bertani Broth powder was dissolved in 1 liter of distilled water and then placed in a media bottle. The mixture was also brought to a boil inside the autoclave to dissolve the powder completely. The broth was then dispensed into sterile test tubes each containing 4.5 mL (Preparation of Media and Cultures, 2019).

Growing the Bacterial Endophytes on a Mixed Culture

Aseptically, five fragments of each sample were plated on nutrient agar. For every plant part, five petri dishes were plated with five fragments. These petri dishes were incubated at 37°Cfor 24 hours (Eldeen, 2014).

Transferring the Bacterial Endophytes into Broth

Aseptically, the colonies exhibiting different morphological characteristics were selected and transferred to the broth. The test tubes were then incubated at 37°C for 24 hours.

Transferring the Bacterial Colonies into Pure Cultures

Using an inoculating loop, the bacteria were transferred from the broth into pure culture by re-streaking it on the petri dishes containing agar using the quadrant streak method. The plates were incubated at 37°Cfor 24 hours. When an individual colony was already be observed in each plate, the colonies were then transferred to a broth. After, 500 µL of the liquid culture was added to 500 µL of the 50% glycerol in a 2 mL screw top

tube and they were mixed gently. The 50% glycerol was made by diluting 100% glycerol in dH_2O. Subsequently, the plates were refrigerated at 4℃ while the glycerol stock tubes were frozen to -80℃ for storage (Nair, 2015).

MORPHOLOGICAL CHARACTERIZATION OF THE BACTERIAL ISOLATES

The different bacterial isolates were characterized morphologically, thus focusing on the form and structure. The bacterial isolates were Gram stained in order to differentiate the isolate into either a Gram positive or Gram negative bacteria. The isolates were also observed in their plates. Shape, margin, elevation, size, texture, appearance, pigmentation, and optical property of each bacterial colony will be recorded.

Gram Staining

A slide of cell samples were made, one for each bacterial isolate, while applying the aseptic techniques. From the broth, it was done using an inoculating loop while from the plate, it was done using an inoculating needle. However, for bacteria from the glycerol stock tube, it was done by scraping some of the frozen bacteria off of the top using a sterile loop. The sampleswere heat fixed by carefully passing the slide over a flame. The primary stain (Crystal Violet) was then added to each of the sample and the samples wereallowed to set for 1 minute. Afterward, the slides were rinsed. The mordant (Gram's Iodine) was then added and was allowed to set for 2 minutes. Subsequently, the sampleswere rinsed and were rinsed with alcohol for 30 seconds. The glass slides were then rinsed with water. The secondary stain (safranin) was added and allowed to set for 1 minute. Lastly, the sampleswere rinsed gently with water (Bruckner, 2016).

Describing Bacterial Cells and Colonies

The slides were viewed under the microscope to determine whether the bacteria were Gram negative or Gram positive. Gram positive bacteria look purple/violet under the microscope while Gram negative bacteria appear pink.

Furthermore, the bacterial colonies were characterized based on their form (circular, irregular, filamentous, or rhizoid), elevation (raised, convex, flat, umbonate, crateriform) and margin (entire, undulate, filiform, curled, or lobate). The color, size, shape, appearance, texture and optical property of the bacterial colonies were all observed and recorded.

MOLECULAR IDENTIFICATION OF THE BACTERIAL ISOLATES

The 16s ribosomal RNA gene of all bacterial isolates were analyzed in order to identify the different isolates. The DNA of each isolate was extracted, and the 16s rRNAwas amplified through Polymerase Chain

Reaction (PCR). Agarose Gel Electrophoresis was then performed in order to check the quality and quantity of the amplified genes. All sequences were sent to Korea for DNA sequencing.

Extraction of the 16s rRNA Bacterial Gene

1.5 mL of the bacterial isolate was placed in a 2 mL microcentrifuge tube. The tubes were then centrifuged at a maximum speed of 1 minute. The supernatant was removed and another 1.5 mL of the bacterial isolate was further added into the tube. The tubes were centrifuged again at the same setting and the supernatant was removed again. 200 µL of water was added to the microcentrifuge tube and mixed well. The mixture was then transferred to a PCR tube and was boiled for 15 minutes. Afterward, the PCR tubes were centrifuged at maximum speed for 2 minutes. Lastly, 50 µL of the supernatant was transferred to another microcentrifuge tube (Eldeen, 2014).

Amplification of the 16s rRNA through Polymerase Chain Reaction

2 µL of the DNA was placed to the prepared master mixes. Subsequently, the PCR was ran on a thermocycler with the following settings: 44℃ for 5 minutes, 30 cycles of 94℃ for 1 minute, 55℃ for 1 minute, 72℃ for 1 minute, and 72℃ for 10 minutes (Eldeen, 2014).

Evaluation of the PCR Products through Agarose Gel Electrophoresis

1.5 g of agarose was weighed and mixed with 100 mL of buffer (TBE) in a microwavable flask. The mixture was microwaved for 1-3 minutes or until the agarose was completely dissolved. The agarose solution will be cooled down to about 50℃. After, the agarose gel was poured into a gel tray with the well comb in place and was left to solidify. Once solidified, the agarose gel was placed into the electrophoresis unit. The electrophoresis unit was then filled with buffer until the gel is completely covered.

Subsequently, 2 µL of loading dye was mixed with 5 µL of each DNA samples using a micropipette on the surface of a parafilm. The mixtures was then loaded onto the wells but the first well was left empty. This is because the first well was loaded with a molecular weight ladder. The gel was ran at 80-150 V until the dye line was approximately 75-80% of the way down the gel. It took around 1-1.5 hours. Lastly, the gel was removed from the electrophoresis chamber and was viewed under UV light, usually using a UV transilluminator, to visualize the bands or the DNA fragments (Agarose Gel Electrophoresis, 2018).

Sequencing of the PCR Products

Upon successful gene amplification, the PCR products was sent to the Macrogen Laboratory in the Republic of South of Korea for DNA sequencing.

STATISTICAL TREATMENT

In order to analyze the sequences for maximum likelihood, different softwares namely MEGA and BLAST will be used. The sequences will be matched with a database to identify them and to come up with their respective phylogenetic trees. The results of the phylogenetic trees in correlation with the morphological characteristics will serve as the basis of the identity of the isolates. The softwares performs different statistical computations for maximum likelihood, as follows:

One of the softwares used for the analysis of maximum likelihood is BLAST. According to the NCBI (2019), the Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. The RNA sequences gathered from the endophytes are placed on the software to be analyzed. BLAST works by detecting local alignments between sequences that work the best. The BLAST computers start with a small set of three letters, which they call the “query word". The level of similarity can be based on multiple scoring systems (Portfield, 2014). The usual scoring system of identity score will be used in this study.

The “identity or identity score” determines the extent to which two sequences have the same residues at the same positions in an alignment, often expressed as percentage (Fassler& Cooper, 2011)

The other software which will be used is MEGA. The computer program package, Molecular Evolutionary Genetics Analysis (MEGA) is a desktop application designed for comparative analysis of homologous gene sequences either from multigene families or from different specie with emphasis on inferring evolutionary relationships and patterns of DNA and protein evolution (Kumar et al., 2008). Through the software's analysis, evolutionary distances are measured, and the phylogenies are constructed. MEGA software aims to serve the purposes of extracting valuable information from nucleotide or protein sequence for statistical assessment and biological data mining (Khan, 2017). It allows users to infer evolutionary relationships of homologous sequences, explore basic statistical properties of genes and estimate neutral and selective evolutionary divergence among sequences.

The software offers many methods of estimating the evolutionary from nucleotide and amino acid sequence data. In this software, the methods for estimating evolutionary distances from nucleotide and amino acid sequence data would include the three different methods of phylogenetic inference (UPGMA, neighborjoining and maximum parsimony) and two statistical tests of topological differences (Kumar et al., 2008).

MEGA contains visual modules for browsing, editing and computing basic statistical quantities for the input data. The software can allow the user to calculate base frequencies and relative synonymous codon usage for all positions across all selected sequences or for only positions they highlight. These basic statistical quantities are necessary to assess the DNA and protein sequence variability, location of positions that harbor evolutionary change and inequality of the usage of 4 nucleotides, 20 amino acid residues and 64 codons (Kumar et al., 2008).

Computing evolutionary relationship by means of graphical representation of phylogenies using branching tree like diagram is one of the core applications of MEGA. In order to create phylogenetic trees, the sequences of each endophyte isolated must be retrieved. These sequences will be aligned. A data subset containing nearly any combination of sequences including groups, domains, and genes is constructed. A process called complete deletion or pair wise deletion will follow, and data sub setting and transformation in MEGA is accomplished automatically by codon extraction from the selected data subsets and its translation, if needed. The trees created are then evaluated and represented. It is important to conduct statistical test for evaluating phylogenetic tree. MEGA runs a statistical re-sampling process called bootstrapping (Khan, 2017). It is a process done to check the reliability of the trees created through measuring the probability of branch recovery if the taxa were to be sampled again. Its values are typically from 1000 repeated calculations and values >70% is acceptable.

ISSN No:-2456-2165

CHAPTER 4
RESULTS AND DISCUSSION

This chapter presents the data collected and the results of the statistical analysis. The chapter, to be more specific, will include the table for morphological characteristics, the table and figures of the Gram's stain, the table of identity scores, and the figures of the phylogenetic trees.

RESULTS

A single tree of *Rhizophoramucronata* served as the host of the bacterial endophytes. The bacterial endophytes isolated were taken from the tree's leaves, twigs and roots. A total of 15 colonies were primarily selected and isolated from 15 culture media. Among them, 10 morphologically distinct isolates were selected for detail study towards identification based on colony morphology and microscopic observation. (Table 1)

Table 1. Part of *Rhizophoramucronata* where bacterial endophytes are taken

Bacterial Isolate No.	Source of Isolate
1	Leaves
2	Leaves
3	Leaves
4	Leaves
5	Leaves
6	Leaves
7	Twigs
8	Twigs
9	Leaves
10	Leaves

As the first step in identifying the species of each bacterial endophyte isolated, the morphological characteristics of each were observed and recorded. Colonies of the selected isolates were characterized based on shape, margin, elevation, size, texture, appearance, pigmentation, and optical property as reflected in Table 2.

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Table 2. Morphological characteristics of the species of bacterial endophytes isolated as observed on a compound microscope

Bacterial Isolate No.	**Shape**	**Margin**	**Elevation**	**Size**	**Texture**	**Appearance**	**Pigmentation**	**Optical Property**
1	Circular	Undulate	Raised	Small	Rough	Glistening	Non-pigmented	Transparent
2	Irregular	Undulate	Flat	Moderate	Smooth	Dull	Non-pigmented	Opaque
3	Circular	Entire	Raised	Small	Smooth	Glistening	Non-pigmented	Transparent
4	Circular	Entire	Raised	Moderate	Smooth	Glistening	Non-pigmented	Transparent
5	Circular	Entire	Raised	Small	Smooth	Glistening	Non-pigmented	Transparent
6	Irregular	Undulate	Raised	Small	Rough	Glistening	Non-pigmented	Transparent
7	Irregular	Curled	Flat	Small	Rough	Dull	Non-pigmented	Transparent
8	Circular	Entire	Raised	Moderate	Smooth	Glistening	Non-pigmented	Transparent
9	Circular	Entire	Raised	Moderate	Smooth	Glistening	Non-pigmented	Transparent
10	Circular	Entire	Flat	Punctiform	Rough	Dull	Non-pigmented	Transparent

Subsequently, the 10 isolates were stained using the standard procedures of Gram's staining. The slides were then viewed through a microscope as shown in Figures 1-10.

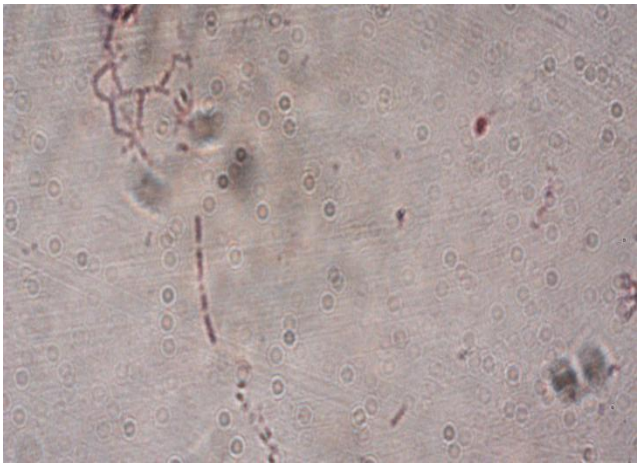

Figure 1. Photomicrograph showing endophytic bacterial cells of isolate MBE03 under a light microscope using oil immersion (100X magnification).

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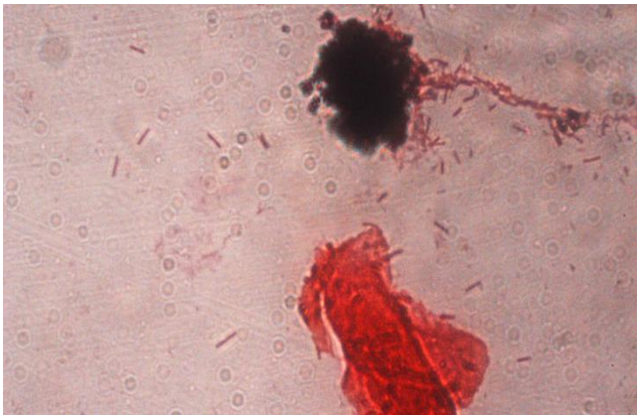

Figure 2. Photomicrograph showing endophytic bacterial cells of isolate MBE04 under a light microscope using oil immersion (1000X magnification).

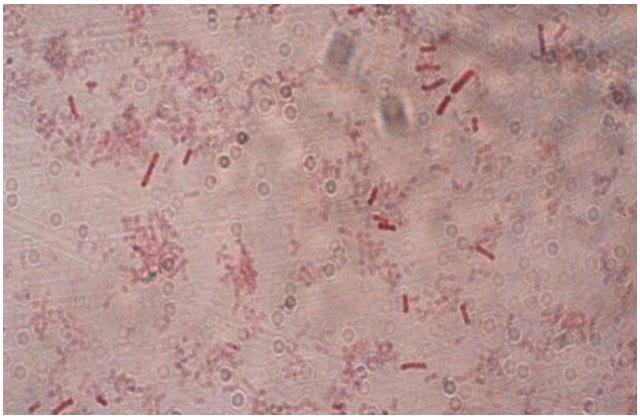

Figure 3. Photomicrograph showing endophytic bacterial cells of isolate MBE06 under a light microscope using oil immersion (1000X magnification).

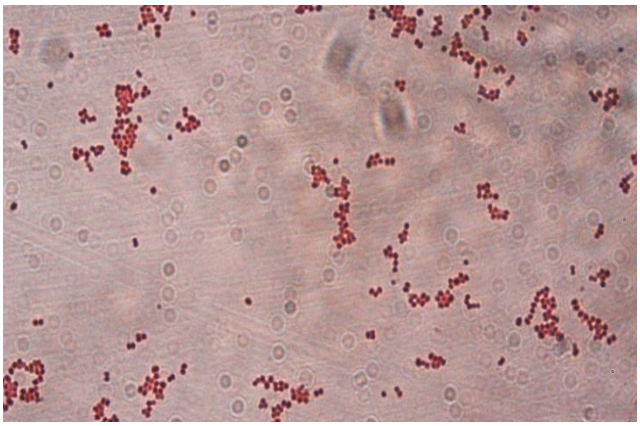

Figure 4. Photomicrograph showing endophytic bacterial cells of isolate MBE05 under a light microscope using oil immersion (1000X magnification).

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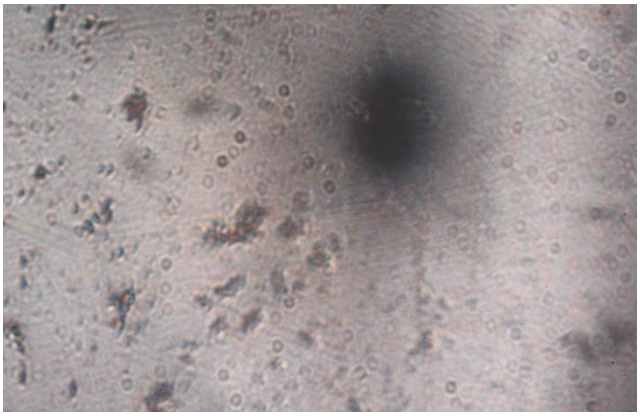

Figure 5. Photomicrograph showing endophytic bacterial cells of isolate MBE07 under a light microscope using oil immersion (1000X magnification).

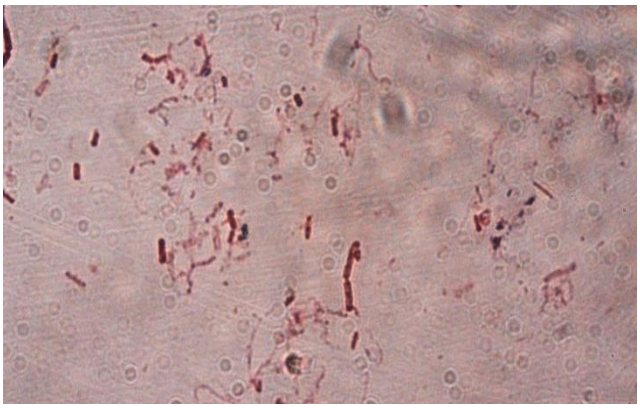

Figure 6. Photomicrograph showing endophytic bacterial cells of isolate MBE08 under a light microscope using oil immersion (1000X magnification).

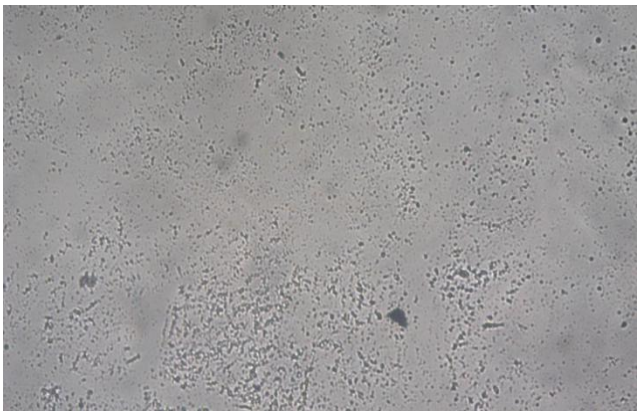

Figure 7. Photomicrograph showing endophytic bacterial cells of isolate MBE11 under a light microscope using high power objective (400X magnification).

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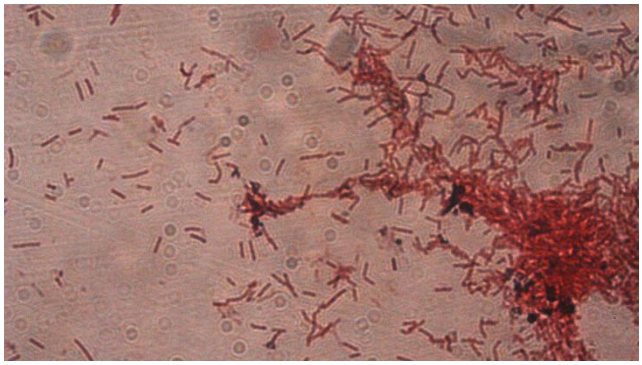

Figure 8. Photomicrograph showing endophytic bacterial cells of isolate MBE13 under a light microscope using oil immersion (1000X magnification).

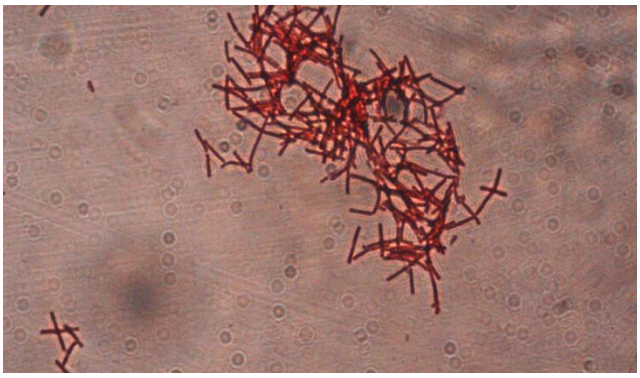

Figure 9. Photomicrograph showing endophytic bacterial cells of isolate MBE14 under a light microscope using oil immersion (1000X magnification).

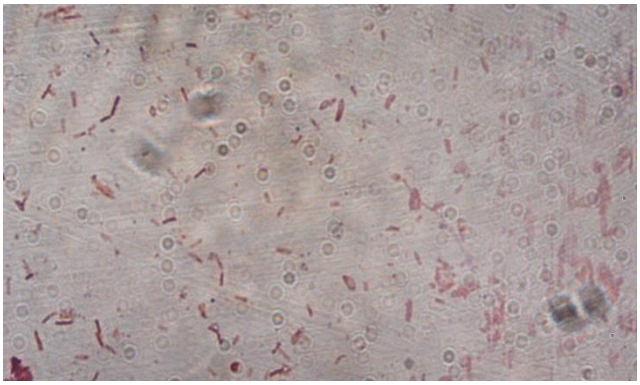

Figure 10. Photomicrograph showing endophytic bacterial cells of isolate MBE15 under a light microscope using high power objective (400X magnification).

Based on the result of the phylogenetic analysis, existing literature suggested that seven of the isolates that were successfully identified were Gram positive while only one Gram negative as summarized in Table 3.

Table 3. Classification of the bacterial isolates into Gram positive (+) or Gram negative (-).

Isolate	**Classification**
MBE03 (*Lysinibacillus fusiformis*)	-
MBE05 (*Staphylococcus haemolyticus*)	+
MBE07 (*Staphylococcus epidermidis*)	+
MBE08 (*Bacillus aryabhattai*)	+
MBE11 (*Bacillus licheniformis*)	+
MBE13 (*Bacillus amyloliquefaciens*)	+
MBE14 (*Lysinibacillusboronitolerans*)	+
MBE15 (*Staphylococcus epidermidis*)	+

Each of the morphospecies was sent to Macrogen in South Korea for DNA sequencing. The sequences received were then analyzed and made into phylogenetic trees to show the relationship between these bacterial species and other species of bacteria.

By polyphasic identification, the bacterial endophytes were classified to the species level. There are a total of eight identified bacterial endophytes. Of the eight, three belonged to the genus *Bacillus,* three to the genus *Staphylococcus,* and two to the genus *Lysinibacillus*. Each figure from 11 to 18 shows the phylogenetic tree of one bacterial endophyte.

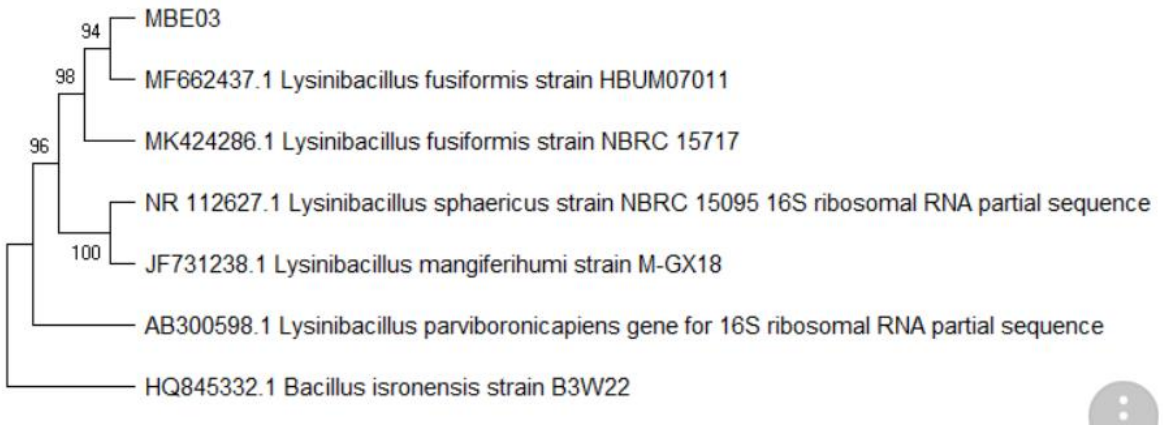

Figure 11. Phylogenetic tree for MBE 03 identified as*Lysinibacillus fusiformis*. Node values represent the maximum likelihood bootstrap values.

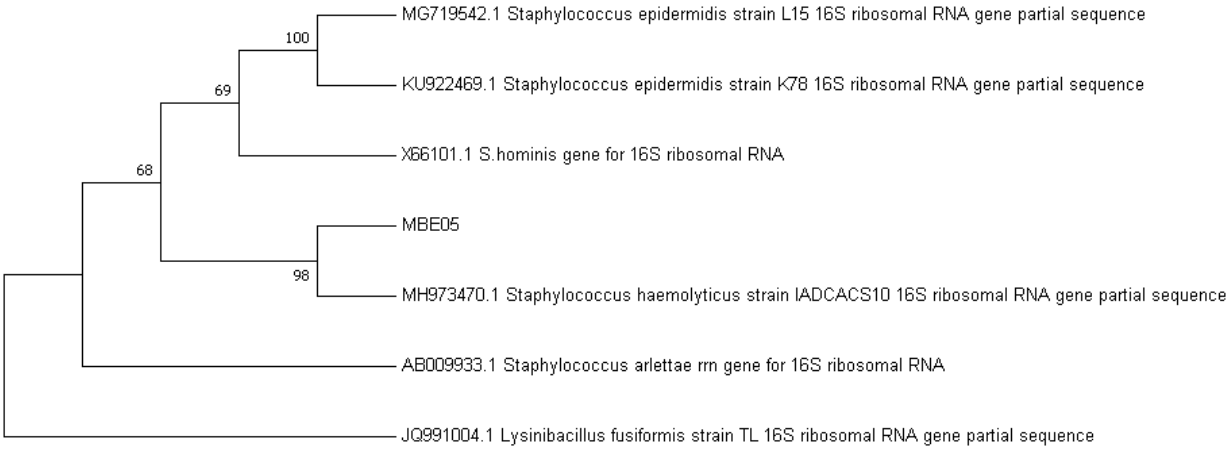

Figure 12. Phylogenetic tree for MBE 05 identified as *Staphylococcus haemolyticus*. Node values represent the maximum likelihood bootstrap values.

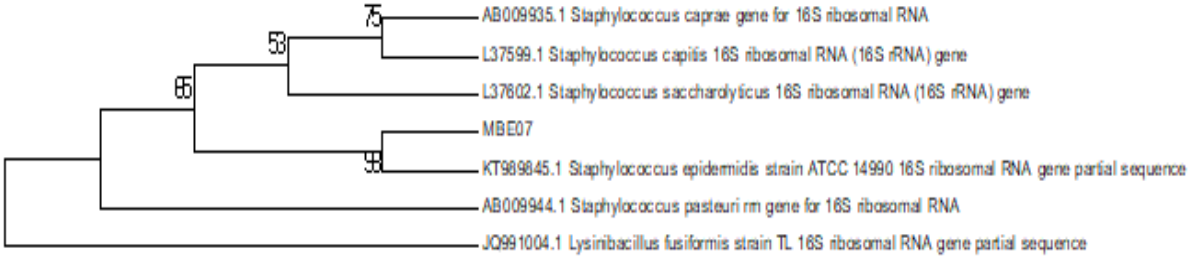

Figure 13. Phylogenetic tree for MBE 07 identified as *Staphylococcus epidermidis*. Node values represent the maximum likelihood bootstrap values.

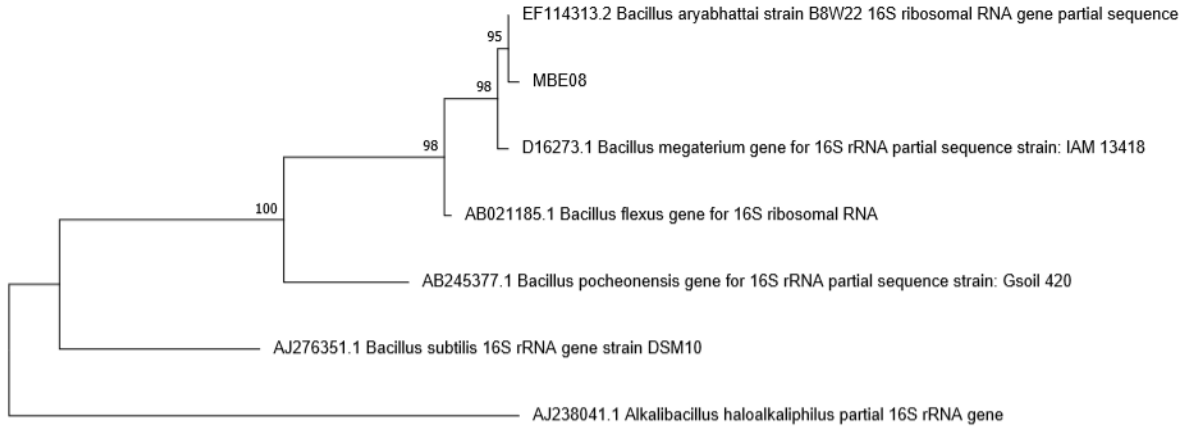

Figure 14. Phylogenetic tree for MBE 08 identified *Bacillus aryabhattai*. Node values represent the maximum likelihood bootstrap values.

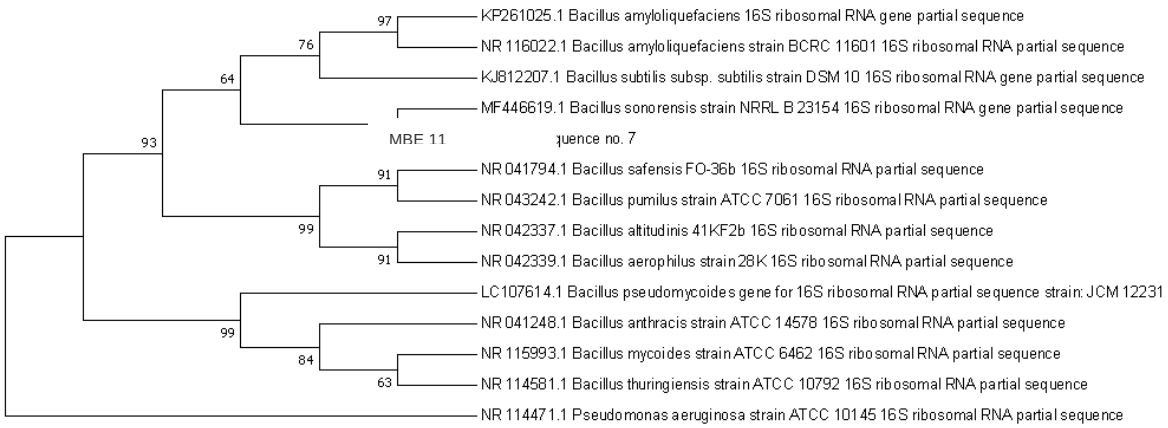

Figure 15. Phylogenetic tree for MBE 11 identified as *Bacillus sonorensis*. Node values represent the maximum likelihood bootstrap values.

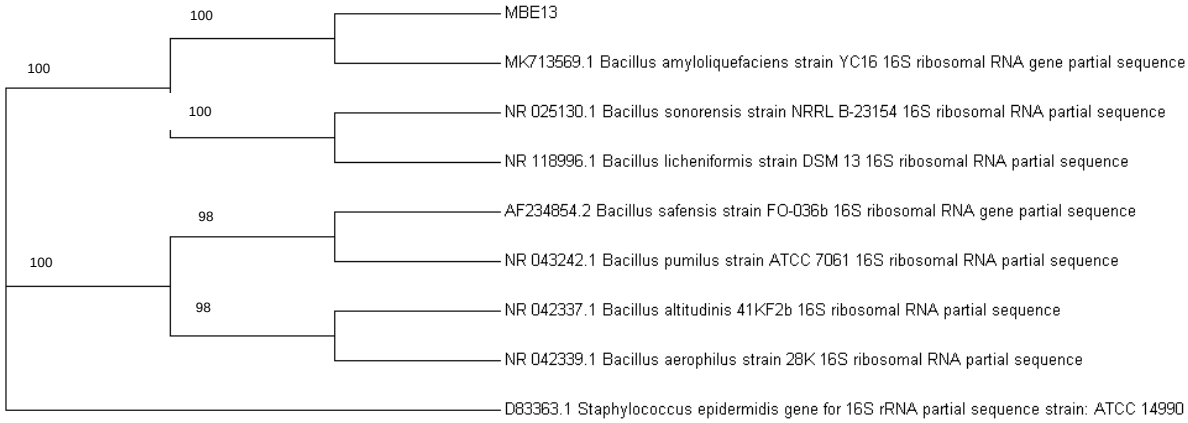

Figure 16. Phylogenetic tree for MBE 13 identified as *Bacillus amyloliquefaciens*. Node values represent the maximum likelihood bootstrap values.

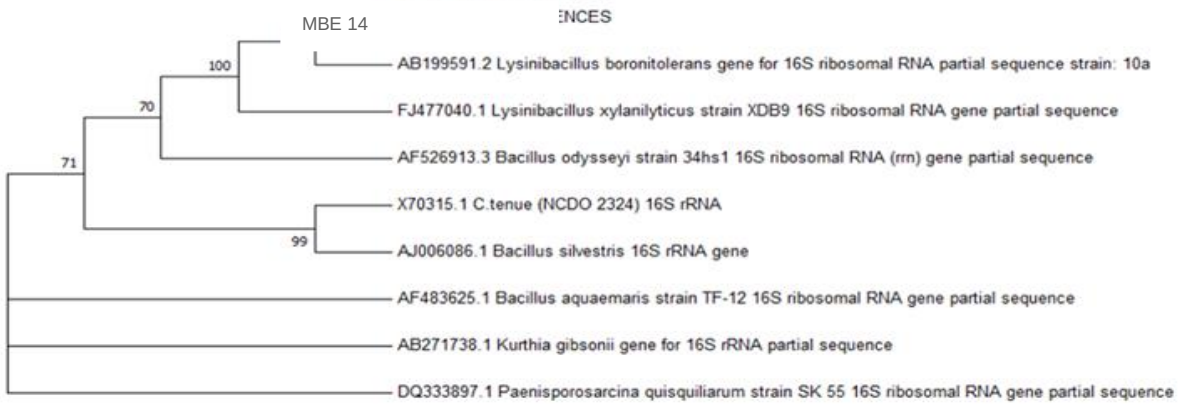

Figure 17. Phylogenetic tree for MBE 14 identified as*Lysinibacillusboronitolerans*. Node values represent the maximum likelihood bootstrap values.

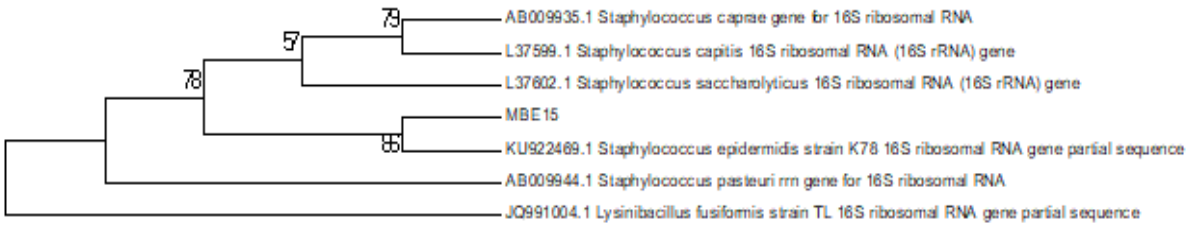

Figure 18. Phylogenetic tree for MBE 15 identified as *Staphylococcus epidermis*. Node values represent the maximum likelihood bootstrap values.

DISCUSSION

Ten morphologically distinct bacterial endophytes were isolated from the single mangrove host. Table 1 showed that out of ten isolates, eight were isolated from the leaves of the host. The remaining two isolates were from the twigs of the host.

As observed in table 2, the shapes of the bacterial endophytes isolated were circular, irregular and filamentous. Circular was the most observed shape, as the colonies of bacterial isolates 1, 3, 4, 8, 9 and 10 were circular. The colonies of bacterial isolates 2, 6 and 7 were observed to be irregular. On the other hand, among all the bacterial isolates, bacterial isolate 4 had a different shape. Bacterial isolate 4 was the only isolate that is filamentous in shape.

Similar to its shape, the margin of bacterial isolate 4 was filamentous. On the other hand, entire was the margin observed for most of the bacterial isolate circular in shape. Bacterial isolate 1, although circular in shape, was observed to have an undulate margin. Bacterial isolates 2 and 6 also had undulate margins. Bacterial isolate 7 had a curled margin, the only bacterial isolate with curled margin among all other.

Apart from the shape and margin, the elevation of the colonies of each bacterial isolate was also observed. Based on the table, flat and raised were the two values of elevation recorded, and the two values of elevation was equal in terms of the number of isolates that possess the value. Five bacterial isolates were raised, while the other five bacterial isolates were flat, in elevation. Bacterial isolates 1, 3, 5, 6 and 9 were observed to have a raised elevation. On the other hand, bacterial isolates, 2, 4, 7, 8 and 10 were flat in elevation.

The sizes of the colonies of the bacterial isolates were characterized as shown in the table. The sizes of the colonies range from punctiform to moderate. Bacterial isolate 10 was the only bacterial isolate punctiform in size. Bacterial isolates 1, 3, 5, 6 and 7 were small in size. Moderately sized bacterial isolates, on the other hand, were bacterial isolates 2, 4, 8 and 9. The texture of the bacterial isolates were also observed. As

observed in the table, the texture was either rough or smooth. Similar to elevation, the two values, rough and smooth, have equal number of bacterial isolates having the characteristic. The bacterial isolates that are rough in size include bacterial isolates 1, 4, 6, 7 and 10. The rest of the bacterial isolates were smooth.

Similar to the texture and elevation, there were also two values for the appearance. The appearance of the bacterial isolates was either glistening or dull. Six bacterial isolates, namely bacterial isolates 1, 3, 5, 6, 8 and 9, appeared glistening. The other four bacterial isolates appeared dull. In addition to the morphological characteristics mentioned, the pigmentation of each bacterial isolate was observed. All the bacterial isolates are non-pigmented, which means the bacterial isolates possessed no color.

The last morphological characteristic shown in the table is the optical property. The values for the optical property were opaque transparent. Bacterial isolates 2 and 4 have opaque value for optical property. The rest of the bacterial isolates were transparent.

Based on the results, different bacterial endophytes were isolated. This proves plants are homes to diverse group of microorganism such the endophytes (Kandelet.al, 2017).
Among the eight successfully identified isolates, seven were Gram positive while only one was Gram negative. This means that seven species had a thick layer of peptidoglycan which had the capability of retaining a crystal violet stain in a standard Gram staining procedure. On the other hand, only one species had a thin peptidoglycan layer which would retain the safranin stain (Curr, 2005). Moreover, Gram positive bacteria were known to appear purple under the microscope while Gram negative bacteria were known to appear pink (Bruckner, 2016). These results conform to a similar study conducted by Eldeen and his team from the Institute of Marine Biotechnology in Malaysia as 16 of the bacterial endophytes they have isolated from a mangrove tree were Gram positive whereas only 5 were Gram negative (Eldeen et al., 2014).

Of the eight isolated bacterial endophytes three of which belong to the genus Bacillus. The genus *Bacillus* encompasses a great diversity of strains (Gordon, 1973). The dominance of *Bacillus* in the Mangrove plant indicates a role in plant defense against pathogens (Bibi et al., 2017). One such bacteria found was *Bacillus aryabhattai*, it has been established to survive in harsh conditions for a significant number of time (Park et al., 2017). One of the other bacterial endophyte belonging to this genus is *Bacillus amyloliquefacien*. Samples collected from the mangrove forests of Andaman & Nicobar islands yielded a mosquitocidal bacterium found to be *Bacillus amyloliquefaciens*. Lastly there was *Bacillus sonorensis*; a strand of this species was found from two mangrove plants *Cyperusconglomeratus* and *Halopeplisperfoliata* (Bibi et al., 2017).

The other three isolated bacterial endophytes all belong to the genus *Staphylococcus*. Two of the bacterial endophytes are found to be the same species, *Staphylococcus epidermis*. Studies conducted by Berg and his team, Vendan and his team, and Kai and his team stated that *S. epidermidis* had functions in plant protection, plant growth and development abilities. Apart from this, presence of *S. epidermidis* as a major member of microbiome of bryophytes attest plants as natural and primitive habitats of this bacterium (Opelt, Berg, & Berg, 2007). The other species of *Staphylococcus* is *Staphylococcus haemolyticus. Staphylococcus haemolyticus* is one of the most frequent aetiological factors of staphylococcal infections (Czekaj, Ciszewski, &Szewczyk, 2015)

The last two of the remaining species of bacterial endophytes both belong to the genus *Lysinibacillus*. Those remaining endophytes to be more specific are *Lysinibacllus fusiformis* and *Lysinibacillusboronitolerans*. A species of *Lysinibacillus fusiformis* was found in theSundarban mangroves of West Bengal, India (Tabao&Moasalud, 2010). Both of these species were also found in the digestive tracks of, *Sphaeromaterebrans*, a mangrove-boring isopod (Carren, James, Leila, & Jared, 2013).

CHAPTER 5
SUMMARY OF FINDINGS, CONCLUSION, AND RECOMMENDATION

This chapter presents the findings, conclusion, and recommendations of the study. To be more specific, it will include the significance of the study, the answers the objectives of the study, summary of data gathered, a conclusion, and recommendations.

SUMMARY OF FINDINGS

There have been studies regarding both endophytes and mangroves but so far no information whatsoever exists about the bacterial endophytes found in *Rhizophoramucronata,* a species of mangrove tree, located in the mangrove forest of Combado, Maasin City. Furthermore, there are only a number of studies conducted that reaches to the species level of identification of bacterial endophytes isolated from the Philippines. The main objective of this study was to identify the different species of bacterial endophytes that can be isolated from a mangrove in Combado, Maasin City. While, the secondary objectives were to find out the morphological characteristics, whether it was gram-positive or gram-negative, and the accession numbers and identity scores of isolated endophytes. During this study, surface sterilization, agar and broth preparation, sample isolation, morphological characterization, gram staining, 16s rRNA extraction, amplification, and evaluation, and DNA sequencing were conducted in order to gain the data needed for the polyphasic identification of the bacterial endophytes.

Eight of the ten bacterial endophytes isolated from the randomly selected *R. mucronata*were identified to be *Lysinibacillusfusimoris, Staphylococcus haemolyticus, Staphylococcus epidermis, Bacillus aryabhattai, Bacillus sonorensis, Bacillus amyloliquefaciens,* and *Lysinibacillusboronitolerans.*

The eight bacterial endophytes' accession numbers and identification scores were also identified. *Lysinibacillusfusimoris*had an accession number of MF662437.1 and an identity score of 100 percent , *Staphylococcus haemolyticus* had an accession number of MH973470.1 and an identity score of 99.93 percent , *Staphylococcus epidermis* had an accession number of KT989845.1 and an identity score of 99.83 percent, another *Staphylococcus epidermis* had anaccession number of KT989845.1 and an identity score of 99.83 percent, *Bacillus aryabhattai*had an accession number of EF114313.2 and an identity score of 99.79 percent, *Bacillus sonorensis*had an accession number of MF446619.1 and an identity score of 99.93 percent, *Bacillus amyloliquefaciens*had an accession number of MK713569.1 and an identity score of 100 percent, and *Lysinibacillusboronitolerans*had an accession number of AB199591.2 and an identity score of 99.79 percent.

Most of the observed colony morphology also correlated with some studies.These include *Staphylococcus epidermidis* who was previously described as Gram positive and non-pigmented. In addition, *Lysinibacillusboronitolerans* was also reported to be circular and entire. *Lysinibacillus fusiformis*on the other hand, was previously described as non-pigmented and rough. Lastly, Bacillus licheniformis was also previously described as non-pigmented, irregular, and rough.

All important data this study gathered include the species of the isolated bacterial endophytes and their morphological characteristics, classification of cell wall constituent (gram-positive or gram-negative), accession number, and identity score.

CONCLUSION

Based on the results of the study and the conditions when the study was conducted the researchers arrived at the following conclusions: there were eight distinct species of bacterial endophytes that were isolated from one mangrove tree host identified as Rhizophoramucronata. Six of these eight species were isolated from the leaves while the other two were isolated from the twigs. As shown in the phylogenetic analysis using the Molecular Evolutionary Genetic Analysis (MEGA), two were from the genus Lysinibacillus, three were from the genus Bacillus, andthreewere from the genus Staphylococcus. Specifically, the eight were identified as Lysinibacillus fusiformis, Staphylococcus haemolyticus, Bacillus aryabhattai, Bacillus licheniformis, Bacillus amyloliquefaciens, Lysinibacillusboronitolerans, and two were Staphylococcus epidermidis. These isolates had best matches in BLAST Analysis to bacterial strains with accession numbers of MF662437.1, MH973470.1, KT989845.1, EF114313.2, MF446619.1, MK713569.1, AB199591.2, and KU922469.1 respectively. Moreover, seven of these endophytic strains were found to be Gram positive while only one, Lysinibacillus fusiformis, was found to be Gram negative.

RECOMMENDATIONS

The study was conducted in order to identify the bacterial endophytes present in the mangrove trees of Combado, Maasin City. The plant parts, which the bacterial endophytes were isolated from, came from a single mangrove plant randomly chosen among the mangrove trees in the mangrove forest. As part of the recommendations, the number of mangrove trees used as host could be increased. Increasing the number of mangrove trees can possibly contribute to an increase in the number of bacterial endophytes isolated.

For other researchers conducting studies similar to this study, researchers shall ensure that the host plants are healthy and mature. Researchers shall also ensure that the host plants have visible fruits and flowers to aid in the identification of the host plants. Dr. Primavera’s mangrove field guide and other similar resources can be

used to identify the species of mangrove tree used host. Moreover, other researchers shall follow the recommended procedures. Bacterial endophytes shall be isolated within 24 hours. Aseptic techniques shall be observed at all times.

In Gram-staining, future researchers shall take note that Gram-stains should be observed under the microscope immediately after. Results can be affected by the quality of the Gram-stains. When slides are not newly Gram-stained, it would be hard to determine whether the isolate is Gram-positive or Gram-negative. Furthermore, a good backbone tree shall be used in generating phylogenetic trees. A good backbone tree will help the researchers locate the best match of the organism, and thus, identify the bacterial isolates until genus and species level.

Due to the limited time of experimentation, the study was not able to continue with the testing of the biological activities of the identified bacterial endophytes. However, the results of the study can serve as preliminary step for future studies that will test the biological activities. The researchers recommend that future researchers seek to test the biological activities of the identified bacterial endophytes. Tests for biological activities such antimicrobial, antioxidant, anti-inflammatory, and cytotoxicity can be conducted. Furthermore, future researchers could also study the interaction of the bacterial endophytes and their host. In this way, future researchers can provide clues on how these bacterial endophytes affect their host plants.Mangrove trees are one of the most important coastal ecosystems in the world, and its extracts have also shown bioactivity against plant, animal and human pathogens (Eldeen, 2014). By conducting such studies, information regarding mangrove plants will be added and introduced.

Lastly, the identified bacterial endophytes can also be examined and tested for their probable uses in agriculture, medicine and industry. Moreover, the bacterial endophytes found in this study can be screened for the presence of secondary metabolites. Bacterial endophytes produce a plethora of secondary metabolites and natural products which could be used in agriculture, medicine and the industry (Ryan, et.al, 2008).

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REFERENCES

[1]. Agarose Gel Electrophoresis (2018). Retrieved from www.addgene.org/protocols/gel-electrophoresis/

[2]. Berg, G., Krechel, A., Ditz, M., Sikora, R. A., Ulrich, A., & Hallman, J. (2005). Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. FEMS Microbiology Ecology, 51(2), 215–229.

[3]. Bibi, F., Ullah, I., Akhtar, S., Yasir, M., Kensarah, E. A., Al-Ghamdi, A. A. K., &Azhar, E. I. (2017). Diversity and bioprospecting potential of rhizo and endophytic bacteria from two mangrove plants in Saudi Arabia. African Journal of Microbiology Research, 11(19), 729-739.

[4]. Bibi, F., Ullah, I., Alvi, S. A., Bakhsh, S. A., Yasir, M., Al-Ghamdi, A. A. K., &Azhar, E. I. (2017). Isolation, diversity, and biotechnological potential of rhizo-and endophytic bacteria associated with mangrove plants from Saudi Arabia. Genetics and Molecular Research, 16(2).

[5]. Bruneck, M. (2016, November 3). Gram Staining. Retrieved from serc.carleton.edu/microbelife/research_methods/microscopy/gramstain.html

[6]. Camacho, L., et al. (2011). Tree biomass and carbon stock of a community-managed mangrove forest in Bohol, Philippines. *Forest Science Techonology*, 7(4), pp. 161-167.

[7]. Carren, M. B., James, O., Leila, A., & Jared, O. B. (2013). Culturable gut microbiota of marine wood boring invertebrates Dicyathifermanni (wright, 1866), Sphaeromaterebrans (Bate, 1866) and Cirolana sp.

[8]. Cruz, J., Cadiente, M.K., Hoai Xuan, T., &Rasco Jr, E. (2015). Endophytic Bacteria Isolated from Nipa Palm (*Nypafruticans*) in Bulacan, Philippines. *Iamure International Journal of Ecology*, doi: 14.10.7718/ijec.v14i1.893

[9]. Curr, C. (2005). Gram staining.Current Protocols in Microbiology, 1(1). Retrieved from https://currentprotocols.onlinelibrary.wiley.com/doi/abs/10.1002/978047

[10]. 1729259.mca03cs00. doi: 10.1002/9780471729259.

[11]. Czekaj, T., Ciszewski, M., &Szewczyk, E. M. (2015). Staphylococcus haemolyticus- an emerging threat in the twilight of the antibiotics age. Microbiology, 161(11), 2061-2068.

[12]. Eldeen, E. (2014). Isolation of 12 Bacterial endophytes from some mangrove plants and determination of, antimicrobial properties of the isolates and the plant extracts. *International Journal of Phytomedicine*, 6, pp. 425-432. Retrieved from http://www.arjournals.org/index.php/ijpm/index

[13]. Estomata, N. &Abit, P. (2011). Growth and Survival of Mangrove Seedlings under Different Levels of Salinity and Drought Stress. *Annals of Tropical Research*, 339(2), pp. 107-129.

[14]. Fassler, J., & Cooper, P. (2011). BLAST glossary. BLAST Help.

[15]. Garcia, K.B., Malabrigo, P.L. &Gevaña, D. (2013). Philippines' Mangrove Ecosystem: Status Threats, and Conservation. *Mangrove Ecosystems of Asia*. doi: 10.1007/987-1-4614-8582-7

[16]. Gayathari, S., et al. (2010). Bioprospecting potential of fast growing endophytic bacteria from leaves of mangrove and salt-marsh plant species. *Indian Journal of Biotechnology*, 9(1), pp. 397-402. Retrieved from http://www.researchgate.net/publication/286203681_Bioprospecting_potential_of_fast_growing_endophytic_bacteria_from_leaves_of_mangrove_and_salt-marsh_plant_specis

[17]. Geetha, I., Manonmani, A. M., &Prabakaran, G. (2011). Bacillus amyloliquefaciens: a mosquitocidal bacterium from mangrove forests of Andaman & Nicobar islands, India. Actatropica, 120(3), 155-159.

[18]. Gordon, R. E., Haynes, W. C., Pang, C. H. N., & Smith, N. R. (1973). The genus bacillus. US Department of Agriculture handbook, (427), 109-26.

[19]. Janda, J. M., & Abbott, S. L. (2007). MINIREVIEW 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory . *Pluses , Perils , and Pitfalls*, 45(9), pp. 2761–2764. doi: https://doi.org/10.1128/JCM.01228-07

[20]. Kai, M., Effmert, U., Berg, G. &Piechulla, B. (2007). Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen Rhizoctoniasolani. Archives of microbiology, 187(5), 351–360.

[21]. Kandel, S. (2017). Bacterial Endophytes Colonization and Distribution within Plants. *Microorganisms*, 5(4), pp. 77-78. doi: doi.org/10.3390/microorganisms50040077

[22]. Khan, N. T. (2017). MEGA - Core of Phylogenetic Analysis in Molecular Evolutionary Genetics. *Phylogenetics Evolution Biology*, 5(2): 183. doi: 10.4172/2329-9002.1000183

[23]. Kumar, S., Nei, M., Dudley, J., & Tamura, K. (2008). MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics*, 9(4), pp. 299–306. doi: https://doi.org/10.1093/bib/bbn017

[24]. Laith, A.A. ,Najiah, M., Zain, S.M., Effendy, S.H.M. & Yang, M. (2008). *Natural Product Report*, 25, pp. 955-981.

[25]. Nair, V.V., et. al. (2015). Antibacterial and antioxidant potential of endophytic isolated from mangroves. *Der Pharmacia Lettre*, 7(12), pp. 53-57. Retrieved from http://scholarsresearchlibrary.com/archive.html

[26]. National Center for Biotechnology Information. Basic local alignment search tool. (2019). Retrieved from https://blast.ncbi.nlm.nih.gov/Blast.cgi

[27]. Opelt, K., Berg, C. & Berg, G. (2007).The bryophyte genus Sphagnum is a reservoir for powerful and extraordinary antagonists and potentially facultative human pathogens. FEMS microbiology ecology, 61(1), 38–53.

[28]. Park, Y.G., Mun, B.G., Kang, S.M., Hussain, A., Shahzad R., & et al. (2017) Bacillus aryabhattai SRB02 tolerates oxidative and nitrosative stress and promotes the growth of soybean by modulating the production of phytohormones. PLOS ONE 12(3): e0173203. https://doi.org/10.1371/journal.pone.0173203Portfield, A. (2013, July 23). How Does BLAST Work?. Retrieved from https://bitesizebio.com/21223/how-does-blast-work/

[29]. Prakash, O., et al. (2007). Polyphasic approach of bacterial classification fi – An overview of recent advances. *Indian Journal in Microbiology*, 47, pp. 98–108.

[30]. Preparation of Media and Cultures (2019). Retrieved from microbiologyonline.org/teachers/preparation-of-media-and-cultures

[31]. Province of Southern Leyte (2013). Province of Southern Leyte. Retrieved from www.southernleyte.gov.ph

[32]. Rambaut, A. (2007). FigTree, a graphical viewer of phylogenetic trees. Retrieved from http://tree. bio. ed. ac. uk/software/figtree

[33]. Ronquist, F. &Huelsenbeck, J. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* ,19 (12), pp. 1572-157.

[34]. Rosenblueth, M. & Romero, E. (2006). Bacterial Endophytes and Their Endophytes. *The American Phytopathological Society*, 19(8), pp. 827-837. doi: 10.1094/MPMI-19-0827

[35]. Ryan, R. (2007). Bacterial Endophytes: Recent Developments and Applications. *FEMS Microbiology Letters*, 281(1), pp. 127-137. doi: doi.org/10.1111/j.157 6968.2007.00918

[36]. Saranraj, P. &Sujitha, D. (2015). Mangrove Medicinal Plants: A Review. *American-Eurasian Journal of Toxicological Sciences*, 7(3), pp. 146-156.

[37]. Tabao, N. S. C. &Moasalud, R. G. (2010). Characterization and identification of high cellulase-producing bacterial strains from Philippine mangroves. Philippine .Journal of Systemic Biology, 4,13-20.

[38]. Vendan, R. T., Yu, Y. J., Lee, S. H. & Rhee, Y. H. (2010).Diversity of endophytic bacteria in ginseng and their potential for plant growth promotion. Journal of Microbiology, 48, 559–565.

[39]. Vespermann, A., Kai, M., &Piechulla, B. (2007).Rhizobacterial volatiles affect the growth of fungi and Arabidopsis thaliana. Appl. Enciron. Microbiol., 73(17), 5639-5641.

[40]. Yong, J. W. (2013). Comparative Guide to Asian Mangroves. *Swedish University of Agricultural Sciences.*