ISOLATION AND CLASSIFICATION OF ANTIMICROBIAL PRODUCING MICROORGANISMS FROM MANGROVES IN SABAH

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ABSTRACT

Mangroves ecosystem is one of the extreme environments targeted in research to discover novel actinomycetes and their novel compounds. The extreme conditions such as the salinity and tidal gradient in the mangroves may trigger metabolic adaptations of the microorganisms especially actinomycetes that could result in valuable metabolites productions. Thus, soil samples are collected from the mangroves area in Sabah to isolate and classify the microorganisms. The soil samples collected are cultured onto 4 different types of agar media (STMS, BD, GYM Streptomyces and TSA), incubated at 28 ℃ and observed for 30 days. The bacteria with distinct morphology are isolated and maintained on GYM Streptomyces agar. Then, the DNA of isolated bacteria are extracted using phenol-chloroform, PCR amplified, sequenced and aligned to construct the phylogenetic trees. The diversity of the microorganisms in mangrove ecosystem are determined through the DNA identification.

Key words: Mangroves ecosystem, microorganisms, diversity

INTRODUCTION

The prevention or treatment of diseases constantly need the discovery of novel and useful bioactive compounds, which can be isolated from natural sources such as plants and microorganisms. These bioactive compounds can either show antibiotics, anti-inflammatory or antioxidant activities. In other words, the discovery of these compounds can help people to live longer and reduce the mortality rate due to infectious or chronic diseases. Recently, researchers has been exploring the natural sources to discover novel drugs. Microorganisms have been focussed because many studies found that microorganisms from different ecosystems have shown some potentials for human use as many interesting compounds have been derived from them (Law *et al.*; Ser *et al.*).

Actinobacteria are a heterogeneous group of Gram-positive bacteria with high guanine (G) and cytosine (C) ratio in their DNA, which is more than 55 mol %. They have characteristics similar to bacteria and fungi, but they also possess enough distinctive features to delimit them into a different category. Besides, they are found everywhere in aquatic and terrestrial ecosystem. Majority of them are saprophytic and soil-dwelling organisms, but some of them can survive in fresh and salt water, and the air. They are abundant in soil with densities of 10^6 to 10^9 cells per gram of soil. In addition, streptomycetes cover over 95 % of all the actinomycetes strains isolated from the soil. Most of the species do not cause harm to animals and plants, while some are important pathogens (Takahashi and Nakashima, 2018).

The probability of discovering novel biologically active molecules from various known soil bacteria have reduced due to the occurrence of saturation effect. Moreover, the known Actinobacteria isolated from various environments are found to produce common compounds. Besides, new natural products and chemical compounds are critically demanded in pharmacology due to the appearance of multidrug resistant pathogenic bacteria such as MRSA and fungus. Consequently, poorly exploited areas such as the mangrove environments are explored to discover novel Actinobacteria and novel metabolites (Lee *et al.*, 2014).

The mangrove ecosystem yields commercial forest products, supports coastal fisheries and protects coastlines as it is one of the world's dynamic environments. Recently, the mangrove microorganisms' resources have been targeted in research by considering that the salinity and tidal gradient in the mangrove can trigger metabolic adaptations that could result in valuable metabolites productions (Lee *et al.*, 2014). Several studies have discovered novel Actinobacteria from the mangrove environments, demonstrated by the isolation of *Streptomyces pluripotens* (Lee *et al.*, 2014), *Streptomyces antioxidants* (Ser *et al.*, 2016), *Streptomyces colonosanans* (Law *et al.*, 2017), *Streptomyces gilvigriseus* (Ser *et al.*, 2015) and *Streptomyces malaysiense* (Ser *et al.*, 2016). Many actinomycetes isolates from marine environments contain polyketide synthetase (PKS) and nonribosomal polyketide synthetase (NRPS) pathways, which are the characteristics of secondary metabolites pathway (Salomon *et al.*, 2004).

Mangroves area in Sabah covers for 59 % of the country's total and 7.6 % of the global total. Some forest formations in Sabah was extended from coastal mangroves at sea level up to inland sub-alpine vegetation on Mount Kinabalu. Mangroves are one of the significant resources to the state and are legally protected under the Sabah Forest Enactment via the gazettement of forest reserves. Approximately 95.7 % or 338,049.12 ha of 341,000 ha of Sabah mangroves are gazetted as Mangrove Forest Reserves (Meng, 2016).

The demand of new antibiotics increases drastically due to the emergence of multidrug-resistant pathogens. The natural products produced by Actinobacteria have specific structures which undergo antimicrobial reaction toward the pathogens. However, a lot of secondary metabolites found exhibited toxic nature which limited their consumptions and so more novel secondary metabolites are ought to be extracted from novel Actinobacteria which were searched from less exploited extreme environment especially mangrove ecosystem in Asia. Hence, the aim of this study is to isolate and classify antimicrobial producing microorganisms especially Actinobacteria from mangroves in Sabah. The diversity of the microorganisms in the mangrove ecosytem is determined through the DNA identification. Besides, novel bacteria are ought to be discovered and their antimicrobial activities will be tested. Bacteria with positive antimicrobial activities will contain novel compounds which can be used to discover new drug.

LITERATURE REVIEW

Classification of Actinobacteria

The phylum Actinobacteria are characterised into 6 classes including Actinobacteria, Acidimicrobiia, Coriobacteriia, Nitriliruptoria, Rubrobacteria and Thermoleophilia. Subsequently, the class Actinobacteria are further characterised into 16 orders called Actinomycetales, Actinopolysporales, Bifidobacteriales, Catenulisporales, Corynebacteriales, Frankiales, Glycomycetales, Jiangellales, Kineosporiales, Micrococcales, Micromonosporales, Propionibacteriales, Pseudonocardiales, Streptomycetales, Stretosporangiales and Incertae sedis (Whitman *et al.*, 2012).

Adaptation of Actinobacteria in Mangrove Ecosystems

Mangrove ecosystem provides environment with extreme condition such as low pH and high salinity, which are not favourable for most of the microorganisms. However, extremophiles bacteria such as Actinobacteria are able to survive by adapting cellular and metabolically. Acidophilic Actinobacteria encounter higher pH gradient compared to neurophiles which leads to difficulty in maintaining circumneutral intracellular pH. Thus, they adapt to maintain the intracellular pH by few possible mechanisms. For instance, they can inhibit the influx of proton into the cytoplasm by reducing the permeability of their cell membrane and pore size in membrane channels, or by generating a positive transmembrane electric potential inside the cell which is created by a Donnan potential. Furthermore, they can remove the protons by pumping out the excess protons from the cytoplasm using transporters, or by cytoplasmic buffering which is helped by buffer molecules that have basic amino acids such as arginine, histidine and lysine. Moreover, they can use acetic or lactic acid to uncouple proton at low pH, and use chaperons to protect the DNA and proteins as the chaperones involve in protein refolding (Stan-Lotter and Fendrihan, 2017).

Halophilic Actinobacteria in mangrove ecosystem need to maintain the osmotic balance with the environment through specialized cellular and enzymatic adaptations to avoid from dehydrating, contracting and destructing in both structure and function. Hence, they have adapted to enhance the osmotic regulation between the cytoplasm and the external environment by using two different strategies which are high-salt-in strategy and low-salt-in, organic-solute-in strategy. For the high-salt-in strategy, the cells accumulate inorganic salts inside the cytoplasm by passively transport the Cl- ions from the environment into the cytoplasm with the help of Cl^- associated channel protein. For the low-salt-in, organic-solute-in strategy, the cells accumulate compatible organic solute inside the cytoplasm instead of the inorganic salts. The moderate halophiles which grow in environment with fluctuating salinity need this strategy as the compatible organic solutes (osmolytes) can prevent the cell proteins from denaturation and improve the tolerance of the cells towards the fluctuations in the saline environment (Edbeib *et al.*, 2016). Many organic compounds including amino acids, betaines, ectoines, N-acelated diamino acids, N-derivatized carboxamides of glutamines, polyols and sugar can work as compatible solutes in the halophiles (Cimerman *et al.*, 2018).

Mechanisms of Producing Secondary Metabolites

The Polyketides (PKs) are synthesized by multifunctional enzymes named Polyketide synthases (PKSs) which are organized structurally into modules. A PKS module consists of three essential domains which are acyltransferase (AT), acyl carrier protein (ACP) and ß-ketoacyl synthase (KS), and four accessory domains which are ß-ketoacyl reductase (KR), dehydratase (DH), enoylreductase (ER) and methyltransferase (MT). The PKS modules carry out many enzymatic reactions to produce polyketides. AT catalyzes the attachment of the substrate (e.g., acetyl or malonyl) to the ACP, and KS catalyzes the condensation of substrates attached in ACP. Subsequently, KR reduces the keto ester, DH dehydrates the compound, and ER reduces the carbon-carbon double bond in the molecule (Risdian *et al.*, 2019).

The non-ribosomal peptides (NRPs) are synthesized by a group of multifunctional enzymes called non-ribosomal peptide synthases (NRPSs) which use proteins as templates to incorporate amino acids. The NRPSs are organized modularly and each module functions independently to complete a cycle of peptide elongation. Each module contains three domains which are adenylation (A) domain, peptidyl carrier protein (PCP) or thiolaton (T) domain, and condensation (C) domain. The biosynthesis of NRPs is carried out by few steps. Firstly, the A-domain recognizes and activates the amino acid substrate through adenylation using Mg-ATP, and produces an aminoacyl adenylated intermediate. Secondly, the PCP-domain binds the aminoacyl adenylated intermediate covalently to its 4'-phosphopantetheine (PP) cofactor to form a thioester called aminoacyl-S-PCP. Thirdly, the C-domain cayalyzes formation of the amide bonds between the carboxyl groups of the two cognate aminoacyl-S-PCPs to form a dipeptide. The growing peptide intermediate remain covalently bound to the PCPs during the entire elongation process. After the condensation step, thioesterase (TE) helps to release the linear intermediate peptide by hydrolysis or internal cyclization in bacteria (Du and Lou, 2010; Martínez-Núñez and López, 2016).

Application of Mangrove Actinobacteria

The secondary metabolites produced by Actinobacteria have specific structures which carry out antimicrobial reaction toward the pathogens. The Actinobacteria isolated from mangrove ecosystem which produce useful secondary metabolites in recent years are listed in Table 1.

Table 1: Mangrove Actinobacteria with their antimicrobial secondary metabolites

Actinobacteria	Source	Compound	Application	References
Streptomyces sp. GQ478246 and *Streptomyces sp.* HQ340165	Mangrove soil (Tamilnadu, India)	Alkaloids and Quinine	Antitumor, Apoptosis, Antibacterial	Ravikumar *et al.*, 2012
Streptomyces sp. RL23 and RL66	Mangrove soil (Japan)	JBIR-94	Antioxidative	Kawahara *et al.*, 2012
Streptomyces albogriseolus HA10002	Mangrove sediment (Hainan, China)	Fungichromin B	Antibacterial, Antifungal	Zeng *et al.*, 2013
Streptomyces sp. MA-12	Semi-mangrove plant *Myoporum bontiodes* (Guangdong Province, China)	Di-*O*-prenylated flavone	Antifungal	Ding *et al.*, 2013
Jishengella endophytica 161111	Mangrove plant *Acanthus illicifolius* (Hainan, China)	Pyrazine Derivatives	Anti-H1N1 virus	Wang *et al.*, 2014
Streptomyces sp. 219807	Mangrove soil (Sanya, Southeast China)	Halichoblelide D	Cytotoxic	Han *et al.*, 2016

METHODOLOGY

Sample collection, processing and isolation

Soil samples are collected from mangroves forest located in Sabah, Malaysia. Soil samples are collected at a depth of 20 cm from the top layer of the soil surface (after removing the top 2 to 3 cm). The samples are then placed into sterile Eppendorf tube using an aseptic metal trowel, kept in the dark for transport to the laboratory, and stored at -20 ℃ (Law *et al.*, 2017; Lee *et al.*, 2014). Each soil sample (5 g) is air-dried about 7 days and mixed with 50 mL sterile distilled water. Then the supernatant above the soil sample is serially diluted using sterile distilled water with 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions. The diluted samples with 10^{-2}, 10^{-3} and 10^{-4} dilutions are spread onto 4 different types of agar media (STMS, TSA, BD and GYM Streptomyces) supplemented with nalidixid acid (25 mg/mL), cycloheximide (40 mg/mL) and potassium dichromate (50 mg/mL). The plates are incubated at 28 ℃ and observed for 1 month. Single colonies of bacteria with distinct morphology are isolated and maintained on ISP 2 agar media at 28 ℃, and then stored in glycerol (80 %, v/v) at –20 ℃ for long term preservation (Tan *et al.*, 2015).

Genomic and Phylogenetic Analyses

The genomic DNA of bacteria is extracted using the Chelex-100 method (Zhou *et al.*, 2010). The single colonies of bacteria from the ISP2 agar media are picked up and put into a 1.5 mL Eppendorf tube. Then, sterile Chelex-100 (0.5 mL; 10 %, w/v) is added into the tube and it is boiled for 15 minutes. After cooling down, it is centrifuged at 12000 rpm for 5 minutes and kept at -20 ℃. The DNA extracted in the supernatant is used as template for PCR amplification of 16s rRNA. The reagents of reaction mixture (50 µL) for PCR include DNA template (2 µL), 2× EasyTaq PCR SuperMix solvent (25 µL), 10 µM primer 27F (1.5 µL), 10 µL primer 1492R (1.5 µL), and ddH_2O (20 µL). The PCR amplification is carried out using PCR System. The PCR process starts with initial denaturation at 95 ℃ for 5 minutes. Then, it is followed by 35 cycles of denaturation at 94 ℃ for 1 minute, binding of primer at 55 ℃ for 1 minute, and polymerization at 72 ℃ for 2 minutes. The process ends with extended polymerization at 72 ℃ for 10 minutes (Farris and Olson, 2007). The PCR product is sent to gene sequencing company for DNA sequencing. The DNA sequence is aligned using BioEdit with the corresponding sequences of the most closely related type strains of the same genus obtained from the EZBioCloud server with website address of https://www.ezbiocloud.net (Hall, 1999). Subsequently, the alignment is verified manually and adjusted prior to the reconstruction of phylogenetic trees. Phylogenetic trees are constructed with the neighbour-joining algorithms using MEGA 5.0 software (Saitou and Nei, 1987; Tamura *et al.*, 2011). Evolutionary distances for the neighbour-joining algorithms are computed using Kimura's two-parameter model (Kimura, 1983). Tree topologies are evaluated by bootstrap analysis based on 1000 replications (Felsenstein, 1985).

RESULTS AND DISCUSSION

There are 46 bacteria isolated from the mangrove soil. Based on pie chart in Figure 1, Actinobacteria covered 56 % from the isolated microorganisms, followed by Fermicutes (33 %) and Proteobacteria (11 %). This proves that Actinobacteria is the most abundant bacteria in the mangrove environment due to their ability to secrete secondary metabolites through polyketide synthase (PKS) and non-ribosomal peptide synthase mechanisms (Risdian *et al.*, 2019; Du and Lou, 2010; Martínez-Núñez and López, 2016). This is also related to pie chart in Figure 2 which represents the percentage of bacteria grown on different types of media. Gym streptomyces agar consists the most number of bacteria (39 %), followed by BD agar (28 %), STMS agar (22 %) and TSA agar (11%). This distribution is expected as GYM streptomyces agar and STMS agar are selective agar for streptomyces, which is most abundant Actinobacteria in soil, whereas BD agar is selective agar for Actinobacteria. Gym streptomyces agar has more number of bacteria compared to STMS agar as the carbon source from GYM streptomyces medium, which is glucose, is simpler compound compared to that from STMS medium, which is starch.

The phylogenetic tree in Figure 3 shows the diversity and genetic relationship between the Actinobacteria. The four main order clusters (Classification) are Streptomycetales, Micromonosporales, Mycobacteriales and Microbacteriales. Streptomyces accounts 76.9 % (20 out of 26) among the Actinobacteria isolated. The statement made by Takahashi and Nakashima (2018), which is about streptomycetes cover the most of all the Actinobacteria strains isolated from the soil, is proven by the result. The diversity of the microorganisms is shown, as the isolated microorganisms form mangrove soil are not only restricted to Actinobacteria, but also consist of Fermicutes and Proteobacteria. The diversity of Actinobacteria is also shown as they consist of Streptomycetales, Micromonosporales, Mycobacteriales and Microbacteriales.

Figure 1: Types of bacteria isolated

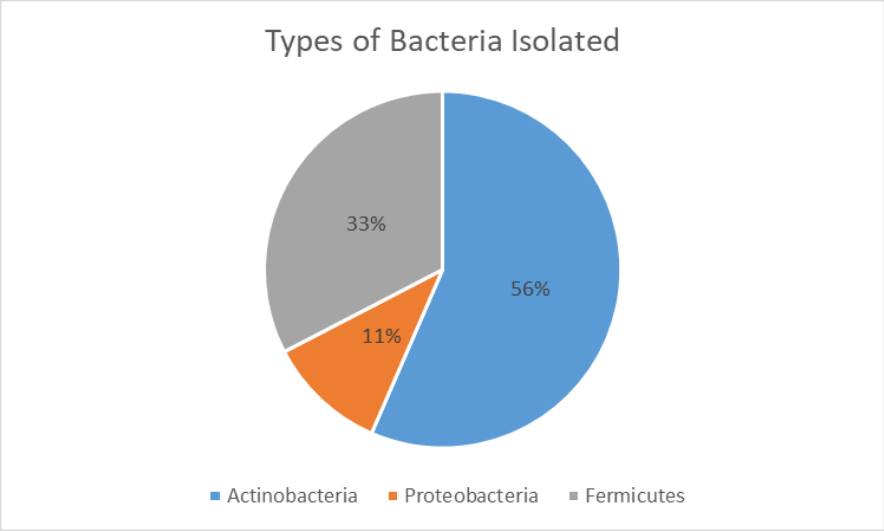


Figure 2: Bacteria Isolated from different media

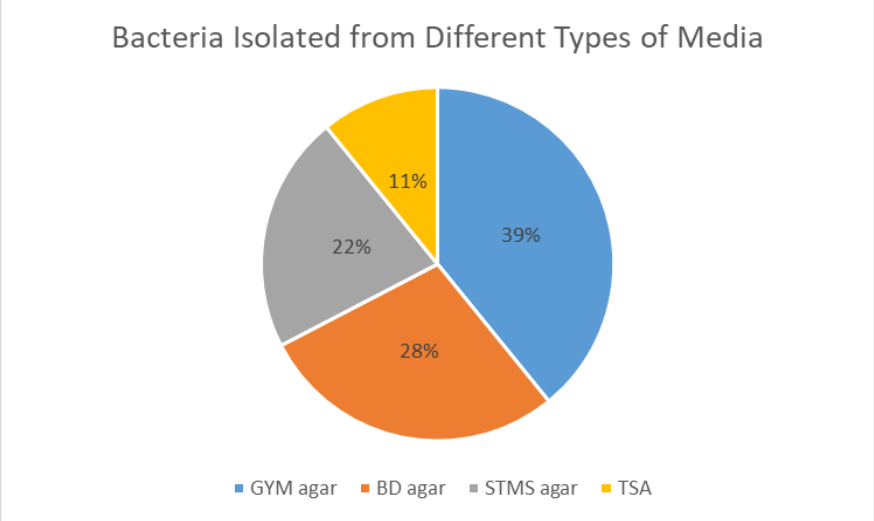
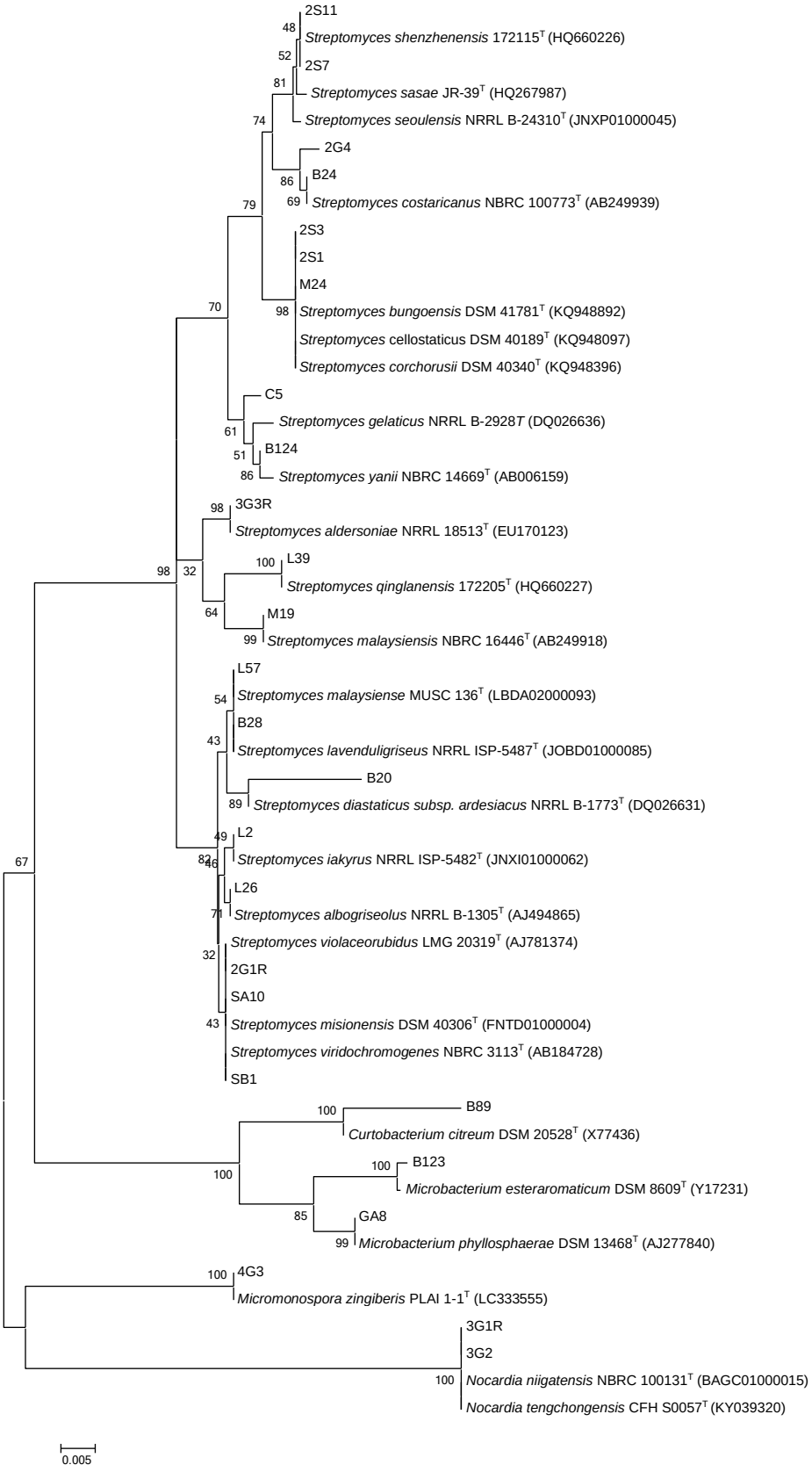


Figure 3: Phylogenetic tree of Actinobacteria from the mangrove soil



CONCLUSION

The microorganisms isolated from the mangrove soil showed diversity as they consist of Actinobacteria, Fermicutes and Proteobacteria. The diversity of Actinobacteria is also shown as they consist of Streptomycetales, Micromonosporales, Mycobacteriales and Microbacteriales. GYM streptomyces agar media has the most bacteria grown as it has simple organic compounds (Glucose, yeast extract and malt extract) to be utilized by Actinobacteria especially Streptomyces. There are more microorganisms will be isolated from the mangrove soil samples collected and the diversity will be widen. Novel Actinobacteria are not yet to be found from the samples but the search will be continued. Beisdes, the microorganisms identified will be testing for antimicrobial test to determine their potential to produce antibiotics.

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