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Isolation of *Actinomycetes* from mangrove sediments at Ujung Pangkah, Gresik, Indonesia

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ABSTRACT

This research was conducted to get actinomycetes isolate from mangrove sediments at Ujung Pangkah in Gresik-Indonesia. Sediment samples were taken from mangrove rhizosphere in Ujung Pangkah on 3 different location sampling areas. Ten grams of soil sample was accurately weighed and transferred to add 100 mL of sterile NaCl 0.9% mixed well for 10 minutes. The resultant solution was serially diluted up to 10^{-12} (10^{-3}, 10^{-4} until 10^{-12}) with NaCl 0.9%. One millilitre of each intermediate dilution (10^{-3}, 10^{-4} until 10^{-12}) was added to 10 mL of sterile molten Starch Casein Agar medium which has been supplemented using both chloramphenicol and griseofulvin each one 0.05 ppm individually in separate flasks. The plates were incubated for the growth of actinomycetes colonies at 28°C and observed intermittently during incubation. After 7 days of incubation, the colonies showing the characteristics of actinomycetes (rough, chalky, powdery appearance radiating growth and leathery texture) were observed. Identification of actinomycetes isolates was conducted by observing macroscopic characteristic of the colony, microscopic of conidial, bacterial cell and the ability of bacteria to resist from acid alcohol. Identification was done based on Bergey's manual of determinative if bacteriology. The result of this research obtained 9 different isolates actinomycetes. The 9 isolate was identified as 3 genera from actinomycetes. The genera of isolate obtained are *Micromonospora*, *Nocardia* and *Streptomyces*.

Key words: *Actinomycetes isolate, Incubation, Bacteriology*

Introduction

Mangroves are saltwater forest ecosystems which is found in tropical and sub-tropical regions throughout the world (Huang *et al*., 2008). Mangrove ecosystems are characterized by their surface soil being flooded by water where salinity and surface fluctuations are affected by tides. Mangrove soils have a unique character for the growth of various kinds of microorganisms that play an important role in degrading the soil. Several factors that affect mangrove ecosystems cause microorganisms to adapt by producing unique metabolites, namely primary and secondary metabolites (Thatoi *et al.*, 2013)

Some groups of microorganisms that can be found in mangrove ecosystems include fungi, bacteria, algae and protozoa. Microbial groups in tropical mangrove forests consist of 91 % bacteria and fungi, 7 % algae and 2 % protozoa (Palla *et al.*, 2018). According to Naikpatil and Rathod (Naikpatil and Rathod, 2011), actinomycetes make up 10 – 15 % of microbial communities in the soil. In the mangrove ecosystem to date only 5 % of microbes have been chemically isolated and examined and belong to groups of bacteria, fungi and actinomycetes (Xu, 2015).

The order actinomycetes or actinomycetales are members of the Actinobacteria groups. Actinomycetes are a group of Gram-positive bacteria and have high G and C content (^ 55 %) in their DNA

(Naikpatil and Rathod, 2011), This organism has a stucture that resembles a fungus. Only after DNA testing, this group is classified as bacteria.

This bacterium has many important roles in various industries because of its ability to produce a number of diverse metabolite compounds. These metabolite compounds have benefits such as, antibiotics, antifungal, antiviral, anticancer, enzymes, immunosuppressants and other compounds that are beneficial in industry (Xu *et al.*, 2014). On this basis actinomycetes are widely used as medicinal ingredients in tackling diseases. In addition, actinomycetes also have an important role in soil mineralization, nutrient immobilization, antibiosis and plant growth prometer production (Sonia *et al.*, 2011) which later can also be applied to agriculture. Aquatic actinomysetes have been shown to have an important role in the discovery of several new bioactive compounds such as research conducted by Huang *et al.* (Huang *et al.*, 2008) rifamycin from Micromonospora, Fehling *et al.* (Feling *et al.*, 2003) found salinosporamide-A as an anticancer metabolite of the Salinispora strain, Marinomisin from *Marinophilus sp.* and much more. According to Anzai *et al.* (2008) out of 22,500 biologically active compounds, 45 % are derived from actinomycetes.

Most actinomycetes are organisms that live freely and are widely distributed in nature both in terrestial and aquatic ecosystems. Among various ecosystems, very little research has been carried out to obtain actinomycetes isolates from mangrove ecosystems, one of which is the Ujung Pangkah Mangrove ecosystem in Gresik. Ujung Pangkah Mangrove Ecosystem is one of the river mouths of Bengawan Solo. Based on fields observations conducted by Rudianto (Rudianto, 2014) in this ecosystem there are many industrial waste contaminants that are channeled into watersheds that produce chemical wastes such a hydrocarbons and heavy metals.

This research was conducted to isolate and identify actinomycetes from Ujung Pangkah Mangrove ecosystem, Gresik Regency, Indonesia. Actinomycetes in this ecosystem are thought to have the potential to produce primary and secondary metabolites. Actinomycetes will produce primary and seconday metabolites in extreme conditions such as the Ujung Pangkah mangrove ecosytem, which is polluted by various types of waste. The results of isolation from this study can be used for further research such as screening for primary and secondary metabolite that produce actinomycetes.

Materials and Methods

Research Samples

The materials used in this study were mangrove sediments, Starch-Casein Agar, NaCl 0.9%, violet crystals, safranin, acetone alcohol, lugol, aquades, 70 % alcohol, and emersion oil.

Sterilization of Tools and Materials

Sterilization of tools made of glass and materials using an autoclave with a 121^{O} C temperature and 2 atm of pressure for 15 minutes. Oze sterilized using bunsen combustion fire, while the equipment that cannot stand the heat is sterilized using 70 % alcohol (Cappuccino and Sherman, 2013)

Sampling

Soil samples taken at several points refer to research conducted by Fatiqin (Fatiqin, 2011) which divides into three sampling areas which are far from the sea, approaching the sea, and dealing with the sea. Sampling was carried out at three locations (red dots) in the Ujung Pangkah mangrove ecosystem in accordance with the three point image. Each location was taken two samples from adjacent points each of 5 g. Samples are taken using pipes with a 5 – 20 cm depth from the surface where microbial activity can generally be found (Priyadarshini *et al.*, 2016). Samples were taken around the mangrove's roots because the microbes concentrationaround the roots

Fig. 1. Viewing of sampling locations in the Ujung Pangkah mangrove ecosystem. Note: 1. Location facing the sea, 2. Location close to the sea, 3. Location far from the sea.

is generally greater than in far areas from the roots (Arifiyanto, 2018). The samples obtained are stored in a sample glass and covered with aluminum foil and than put in a ice box to be brought to the laboratory.

Characterization of Soil Samples

Soil sample identification aims to get the soil samples characteristics. This identification includes pH of the soil, soil color, and soil temperature. The identification is carried out at the sampling location.

Total of Plate Numbers Calculation

Samples were taken as much as ten g and suspended using physiological NaCI into the Erlenmeyer flask until reaching 100 mL volume and shaken for 10 minutes. Samples are diluted in stages starting from 10^{-3} to 10^{-12} dilution. Samples that have been carried out dilution taken as much as 1 mL and dropped on a Petri dish then SCA media that has been added griseofulvin and chloramphenicol 0.05 ppm respectively poured on a cup. Then, incubate the patri dish at 28 °C for 7 days (Palla *et al.*, 2018) Collonies are calculated to determine ALT with formula:

Number of bacteria = Number of colonies x 1 / diluent factor

The media provisions that are used as the ALT basic calculation are petri containing 30-300 CFU / ml colonies.

Actinomycetes Isolation

The isolation of actinomycetes begins by taking the colony from culture on the calculation of the total plate number by taking it using sterile ose and applying it to the surface of the media using the quadrant swipe method. The taken colony is a seperate colony and does not overlap with other colonies and choose different colors and shapes. Than the petri dish are incubated at 28 °C for 7 days. This treatment can be repeated to get a similar colony (Palla *et al.*, 2018).

Purification and Storage of *Actinomycetes*

Colonies that have been purely identified macroscopically are suspected actinomycetes bacteria despited into oblique SCA media. The media is was incubated at 28 °C for 7-14 days and growth was observed (Palla *et al.*, 2018). Media that have been incubated and there is growth of actinomycetes bacteria are stored in refrigerator.

Identification

The actinomycetes colony was observed morphologically and microscopically by reffering to Bergey's manual of determinative bacteriology.

Macroscopic colony

Characterization of mummies culture that had been incubated for 7-14 days at 28 °C was observed morphology of actinomycetes colony based on shape, color, texture, growth time and mycelium colony as well as the ability of actinomycetes to give a characteristic soil odor. Colonie that have the same color and shape are grouped.

Microscopic colony

Observations that based on using light microscopy by Shirling and Gottlieb (1966) method, isolates were grouped based on the form of air mycelium which can be observed by Gram staining. In Gram staining can be observed types of Grams, the shape of bacteria and the presence or absence of spores. Acid-resistant staining is also done to find out some groups of actinomycetes that are resistant to acid resistant staining.

Results and Discussion

Characterization of Soil Samples Results

Mangrove soil samples were taken at three different sampling locations, which are far from the sea, approaching the sea, and dealing directly with the sea. The sample is taken at 10.00 AM until 12.00 PM. Each sample location has a pH level that not much different, which is obtained ± 6.8 average. The average temperature obtained is ± 28.5 °C. Soil samples obtained are quite clay at locations far from the sea. Samples at this location are dominated by mud. Contrast with the two samples taken from locations close to the sea and those dealing directly with the sea which has a slightly sandy consistency.

Soil samples taken from three points of the sampling location are mixed so, the average soil pH is ± 6.9. Generally actinomycetes are intolerant of acids and the amount decreases at pH 5.0. In theory, actinomycetes are suitable to grow in the pH range of 6.5–8.0 (Subba Rao, 1994). Based on Kumar *et al.* (2014) in the mangrove ecosystem, the number of actinomycetes colonies counted the most in samples with an alkaline pH (± 7.5).

The temperature of the three sampling locations

obtained a range between 27 °C-29 °C. The optimal temperature for actinomycetes growth is between 25°C-30°C (Subba Rao., 1994). However, Barka et al. (Barka *et al.*, 2016) has slightly different results. The optimal temperature range is a temperature between 25 ° C-37 ° C. Temperature is known to have an influence on metabolism and bacterial growth. When the temperature increases, the rate of chemical reactions in the bacteria also increases. At a certain temperature point, the growth rate does not increase when the temperature rises. This is caused by protein denaturation when peptide bonds start to break from the tertiary and quaternary structures of proteins (Wheelis, 2011). Ujung Pangkah mangrove ecosystem has an optimal temperature range for actinomycetes growth, so that actinomycetes can grow well in this ecosystem.

Calculation of Total Plate Numbers

Soil samples obtained from three locations were mixed and the calculation of the total plate count on the Starch-Casein Agar medium. Bacterial colonies were counted using a colony counter. This calculation is carried out in the Bacteriology laboratory of the Pharmaceutical Research Services in Microbiology Labaratory, Faculty of Veterinary Medicine Universitas Airlangga. The results of the calculation of bacterial colonies obtained data as follows :

10^{-3}= ~;10^{-4} = ~; 10^{-5} = 520; 10^{-6}= 118;10^{-7} = 22; 10^{-8}= 8;10^{-9}= 7;10^{-10}= 1; 10^{-11} = 3; 10^{-12}4~ = 5.

The results can be calculated the number of bacteria per milliliter in the sample as follows :

Number of bacteria = number of colonies x 1 / diluent factor

$= 118 \times 1/10^{-6}$

$= 118000000$

$= 1{,}2 \times 10^{8}$

In this soil sample, the number of bacteria per milliliter of sample was 120,000,000 bacteria isolated using SCA media. This study found 13 isolates suspected of actinomycetes from 43 isolates and identified only 9 different isolates. Data obtained from the calculation of total plate numbers could not shown a picture of the actual number of actinomycetes because they are still mixed with other bacteria. According to Palla *et al.* (2018) actinomycetes that live in mangrove ecosystems are indeed not as many as in terrestrial areas. The abundance of actinomycetes is influenced by the moisture level of the soil sample. Soils filled with water are not suitable for growth of actinomycetes, whereas soils in arid and semi-arid regions can maintain large populations due to spore resistance to drought (Subba Rao, 1994).

Isolation and Identification of *Actinomycetes* Bacteria

Actinomycetes bacteria were isolated on SCA media. The isolated bacteria were taken from the calculation of the total plate count. Intake of the colony is carried out in separate colonies. Bacterial isolation is repeated until a pure colony is obtained. The isolation was obtained as many as 43 isolates and 13 isolates were thought to be an actinomycetes group and consisted of 9 different isolates. Following are the macroscopic observations of colonies which are thought to be a group of actinomycetes bacteria :

Microscopic identification is also established by observing cell shapes and Gram types. Gram staining can also be used to determine differences in the structure of actinomycetes and fungi. Actinomycetes are Gram-positive which have branched hyphae that often develop into mycelium and have a rod shape (Chakraborty, 2015). The results of these observations indicate that 13 isolates that were thought to belong to the actinomycetes group were Gram-positive and rod-shaped. Pratiwi (Chakraborty, 2015) explains, the structure of bacterial cells, the cell wall of Gram-positive bacteria contains a thick layer of peptidoglycan so that it can

Table 1. The macroscopic observations of the colonies suspected actinomycetes group

Group	Mycelium Aerial Color	Mycelium Substrate Color	Number of Isolate	Isolate Name
1	Bluish white	Yellowish white	2	C21B, C34A
2	Pale white	Pale white	2	C25A, C25B
3	Bright white	Yellowish white	2	C31C, C101B
4	Pale white	Brown	2	C36BB, C48AC
5	Grey	Grey	1	C42A
6	Pale white	White	2	C44A, C102C
7	White	Red	1	C101C
8	Reddish white	White	1	C51B

form a rigid structure, and there is theatric acid which contains alcohol and phosphate. So when there is a purple-iodine crystal complex that enters a Gram-positive bacterial cell it cannot be washed away by alcohol because of the presence of a strong peptidoglycan layer on the cell wall. In Gram staining, spores can also be observed. Gram staining actually only colors vegetative cells from bacteria. The presence of these spores can be marked by the presence of parts that are not stained with Gram staining. However, Gram staining cannot be used to detect free sepora as in dead bacteria. As the result that bacteria has died, there are no vegetative cells that can be colored by Gram staining. Acid-resistant staining in this study was used to determine several genera from the actinomycetes group such as nocardia and mycobacterium. In acid-resistant staining could be known types of bacteria that have thick wax walls which resistant to discoloration using acid alcohol. Bacteria that are resistant to acid alcohol discoloration will be painted red, while those that are not acid resistant are painted blue.

Grouping of *Actinomycetes* Isolates

The grouping of actinomycetes was done by observing color and shape of the colony. This method was carried out to facilitate identification by classifying isolates based on the color of aerial mycelium and mycelium substrate. The morphology of actinomycetes growing on the media can be used to identify the characteristics of actinomycetes, but this information cannot be placed up to the specific genus (Basavaraj, 2010). The results of this grouping obtained 9 different isolates. Microscopic observations were made to observe the similarity of the cell structure of each group. This microscopic observation can also be used to determine the genus of bacteria. Group A was thought to be a group of actinomycetaceae family. In this family is characterized by the presence of bacterial cells that experience fragmentation into small rod shapes. After acid resistant staining, it is suspected that group one is a group of the nocardia genus. The nocardia genus belongs to the actinomycetaceae group which is resistant to acid-resistant staining as described in Bergey's Manual of Determinative Bacteriology. Morphological formation of group one also has similarities with the genus nocardia. Microscopic observations show the formation of spores in group one, so that it can be used to differentiate with the genus mycobacterium which are both acid resistant but do not produce spores and have slightly different structures.

Group B has members of groups 2, 3, 5, 6 and 8. This group is thought to be a group of the streptomycetaceae family and belongs to the genus Streptomyces. Streptomyces has a characteristic that is the location of the conidia within the mycelium forming a chain that distinguishes it from other genera in a family. Mycelium streptomyces has a characteristic that is not fragmented so that long spore chains are formed. These streptomyces species can be identified based on the color of the colony in certain media according to Bergey's Manual of Determinative Bacteriology. The colony's color identification must also be supported by observing conidial shapes.

Group C consists of two groups, 4 and 7. This

Table 2. Microscopic description analysis.

Number	Isolate	Gram	Spora	Acid resistant	Form of mycelium
1	C21B	+	+	+	Partially segmented and partially separated
2	C25A	+	+	-	Segmented
3	C25B	+	+	-	Segmented
4	C31C	+	+	-	Segmented
5	C34A	+	+	+	Partially segmented and partially separated
6	C36BB	+	+	-	Not segmented
7	C42A	+	+	-	Segmented
8	C44A	+	+	-	Segmented
9	C48AC	+	+	-	Not segmented
10	C51B	+	+	-	Segmented
11	C101B	+	+	-	Segmented
12	C101C	+	+	-	Not segmented
13	C102C	+	+	-	Segmented

group is thought to be a genus of micromonospores. The micromonospora genus has the characteristic that there is no septa in the mycelium. Spores of the genus micromonospora are located in conidia which attach to simple conidiophores on the surface of the mycelium. This genus has similar structure with genus thermoactinomyces in one family. The difference between the two genera is that the geno thermoactinomyces can only grow at temperatures of 50 °C-65 °C. In this study the media was incubated at 28 °C, so it can be ascertained that the growing colonies are a group of the genus micromonospora.

Conclusion

Based on the isolation of actinomycetes from mangrove sediments at the Ujung Pangkah Gresik-Indonesia, 13 isolates were obtained consisting of three genera. The three isolates consisted of genera Streptomyces, nocardia and micromonospora. The genus Streptomyces and micromonospora belong to the same family, streptomycetaceae, while the genus nocardia belongs to the family actinomycetaceae. Most of the isolates observed were identified as genus Streptomycetes with 7 isolates. The genus nocardia consists of 2 isolates and 4 micromonospores.

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