Composition and Characterization of Actinomycetes Isolated from Nipah Mangrove Sediment, Gastrointestinal and Fecal Pellets of Nipah Worm (*Namalycastis Rhodhocorde*)

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Abstract. Nipah worm (*Namalycastis rhodochorde*) belongs to the *Polychaeta* group that lived in the *Nypa fruticans* mangrove sediment in West Kalimantan. It has the potential to be cultivated because it has a high economic value in aquaculture. Feed aspect is an essential part of its cultivation, through improving the quality of feed by using probiotics isolated from its natural habitat, such as actinomycetes. These bacteria are known capable of producing secondary and primary metabolites. These metabolites are expected to increase immunity and biomass of worms. The purpose of this study was to isolate and determine the composition and characteristics of actinomycetes derived from mangrove sediment, gastrointestinal and faecal pellets of nipah worm that are potential as probiotic for feed formulation. Isolation and characterization of actinomycetes was conducted by pour plate method on SCA, ISP2, ISP3, ISP4, and ISP5 media; while characterization was by determining morphological characteristics of colonies, cell structure, and biochemical tests. Identification of the isolates referred to Bergey’s Manual of Determinative Bacteriology. The results showed that twelve isolates of actinomycetes had been found. Six isolates were from mangrove substrate, four isolates from fecal pellets and two isolates from the gastrointestinal tracts. All isolates were closely related to the genus *Streptomyces*.

1. Introduction
Nipah worm (*Namalycastis rhodochorde*) is a member species of Polychaeta occupied secondary mangrove waters. The worm lives in sediments around nipah trees (*Nypa fruticans*), and it is widespread in mangrove waters in West Kalimantan mangrove waters. Nipah worms have been used by the community as animal feed in aquaculture. This worm has potential to be developed because of its nutritional value, biomass, and high economic value. Nipah worm contains protein content >58% [1], which is beneficial for shrimp and fish farming. However, excessive exploitation of nipah worms and changes in the function of the Nipah mangrove ecosystem, causing a decline and the extinction of worm populations.

Cultivation is needed to fulfill the demand for nipah worms. Nutrition quality and disease prevention are the important keys to increase nipah worm production in aquaculture [2]. Our previous studies showed that the growth of nipah worm’s larvae was slow and easy to be attacked by parasitic diseases on a laboratory scale. The survival rates of nipah worm larvae were <20%. Forty segments of the worm's body were formed for around 3-4 months [3]. The previous study also showed that pathogenic bacteria were found in gastrointestinal nipah worms, which had hemolytic activity, and...
they inhibited the growth of worms [4]. Worm immunity can be increased by utilizing microflora capable of producing primary metabolites and antimicrobial compounds in the digestive tract. Actinomycetes are a group of microbes that produce both compounds. The products were known to have different abilities such as antimicrobials (antibiotic production), catalysts, anti-tumor, immunomodulators, and organic degradation ([5], [6], and [7]). More than half of the antibiotics are known, isolated from Actinomycetes [8], and most of them are from the genera Streptomyces and Micromonospora [9].

Nipah worms are a group of benthic organisms that eat organic material accumulated in sediments. Many soil-dwelling bacteria are known to produce secondary metabolites that are able to suppress other microorganisms that compete for the same resources [10]. It was known that actinomycetes are the most widely distributed microbes in the soil environment. For this reason, it was necessary to identify the types of microorganisms occupying the gastrointestinal tract, fecal pellets, and substrates such as actinomycetes. Furthermore, Actinomycetes can be utilized as fermenting agents of feed raw materials. The fermentation products were expected to prevent pathogenic bacterial infections and replace antibiotic use [4].

Information about the types of actinomycetes colonized the digestive tract, and faecal pellets of nipah worms (Namalycastis rhodochorde) and mangrove sediment and their ability to produce antimicrobial agents were beneficial. This information gave the opportunity to the development of nipah worm cultivation by using actinomycetes as a probiotic formulation of feed supplement products.

2. Methods

2.1 Study Area

Nipah mangrove mud, fecal pellets, and nipah worms were sampled from secondary mangrove areas located in the estuary of the Kakap River, Sungai Kakap Village, Kubu Raya Regency, West Kalimantan Province. The distance of the sampling location to the Avicennia mangrove area was about 1 km. Mangrove vegetation was dominated by nipah trees (Nypa fruticans) as the main habitat of nipah worms (Namalycastis rhodochorde). Sediment samples were collected from eight different stations of the area with an area of sampling area of 500 m².

![Figure 1](image_url). Location of sampling sites, Sungai Kakap Village, Kubu Raya Regency, West Kalimantan.
2.2 Sampling nipah worm, fecal pellets, and nipah mangrove sediment
Sampling of Nipah worm, its fecal pellets, and mangrove sediment was carried out at eight stations in Sungai Kakap mangrove area (Fig. 1), and it used a purposive random sampling method. The mangrove sediments around the nipah wormhole were taken using a 1 inch diameter drilled-pipe of paralon. The drilled-pipe was inserted at several points of sediment with a depth of about 75 cm (based on the nipah worm), the sediment in the drilled-pipe was then transferred into a sterile container for isolating actinomycetes in the laboratory. Nipah worm fecal pellets found on the surface of the sediment were taken using a sterilized spoon.

2.3 Worm surgery and intestinal obstruction
The nipah worms before being dissected were rinsed first using distilled water, then anesthetized with 5% alcohol. The weak nipah worms were then placed in the surgical cavity and performed surgically to be taken in the contents of the digestive tract.

2.4 Isolation of Actinomycetes from nipah mangrove sediment, gastrointestinal and faecal pellets of nipah worm
Isolation of Actinomycetes bacteria from the digestive tract of the nipah worm was carried out by making serial dilution and pour plate method [11]. The gastrovascular cavity of the nipah worm was dissected by a sterile dissecting set. Ten g of each of fecal samples, nipah worm intestine, and mangrove sediment was suspended in 90 ml of sterile saline solution and then agitated at a speed of 120 rpm for 30 minutes in a rotary shaker. Serial dilutions were made by taking 1 ml of each suspension and then mixing it with 9 ml of sterile saline buffer solution (10⁻¹). Serial dilution was carried out until a 10⁻⁵ dilution rate. As much as 1 ml of the last three dilutions were inoculated onto Starch Casein Agar (fulfill with composition), then incubated for 48 hours at 37° C. Bacterial colonies were re-cultured to obtain a pure culture.

2.5 Determining the density of actinomycetes
Actinomycetes density of each sample was determined based on the plate count standard method on starch casein agar [12].

2.6 Characterization of Actinomycetes isolates on Differential Agar Medium
All bacterial isolates were cultured on Starch Casein Agar (ISP1), Inorganic Salt Starch Agar (ISP4), Oatmeal agar (ISP3), and Glycerol Asparagine Agar (ISP5). The growth of bacterial colonies was observed for morphological characters, including shape, the color of mature spores, the color of lower colonies, the surface texture of colonies, presence or absence of aerial mycelium and substrate mycelium, and pigment formation. The characterization was carried out on the cultures incubated for the seventh and fourteenth days.

2.7 Characterization cell morphology and biochemical properties of Actinomycetes
Characterization of cell morphology included Gram stain and conidia shape, and biochemical character tests include catalase activity test, H₂S formation test, citrate utilization, gelatin liquefaction, and utilization of carbohydrates.

2.8 Data analysis
Characterization of the bacterial isolates was based on the morphology of colony, cell and mycelium, and characteristics of biochemistry and nutrition with referred to Bergey's Manual of Determinative Bacteriology.
3. Results
Either actinomycetes or non-actinomycetes bacteria from samples of mangrove sediment, fecal pellets, and intestinal worms were able to grow on SCA medium after three-days of incubation. Isolates of Actinomycetes bacteria can be distinguished from other groups of bacteria by observing colony characters that have a texture like cotton or powdery. The population density of actinomycetes bacteria was lower than non-actinomycetes bacteria, with a proportion of 14% (Fig. 2).

![Figure 2](image)

**Figure 2.** Colonies of Actinomycetes and non-actinomycetes bacteria on SCA medium. White arrow was colony of Actinomycetes isolate and brown arrow was non-actinomycetes bacteria (left) comparison of the percentage of Actinomycetes from the total number of microbes growing on agar (right)

The Actinomycetes bacterial density isolated from intestinal worms was higher compared to mangrove sediment and worm feces samples, which was $9.5 \times 10^3$ CFU/g (Table 1). The results found twelve isolates of Actinomycetes based on different colony morphological characters (Fig. 3).

| No. | Sample                        | Density (cfu/g) |
|-----|-------------------------------|----------------|
| 1   | Nipah mangrove sediment       | $3.1 \times 10^3$ |
| 2   | Nipah worm fecal pellets      | $3.6 \times 10^1$ |
| 3   | Nipah worm gastrointestinal   | $9.5 \times 10^2$ |

Twelve pure culture isolates were coded based on the origin of the sample. Six isolates of mangrove sludge samples were coded as NrASA (NrASA1 - NrASA6), four isolates of fecal samples were coded as NrAFA1 - NrAFA4, and two isolates of intestinal samples of nipah worms were as NrAGA1 and NrAGA2. All of the isolates grew very well on the four different media, namely YMEA (ISP2), OA (ISP3), ISSA (ISP4), and GAA (ISP5) with incubation temperature at 30° and 35° C. The twelve isolates showed different characteristics especially the shape and surface of the bacterial colonies (Fig. 3). Their differences were also exhibited when growing on the four different media. The differences of all isolates were also found in mature spore color, colony shape and colony surface texture, presence or absence of substrate and aerial mycelium, and the presence or absence of diffusible pigments and exudate (Table 2).
Table 2. Cultural characteristics of Actinomycetes isolated from mangrove sediment, fecal pellets, and gastrointestinal of nipah worm (*Namalycastis rhodochorde*) on different media.

| Codes       | Character of Colonies |
|-------------|-----------------------|
|             | Spores color | substrate color | shape | surface texture | Aer/sub mycelia | pigment | D. colony (mm) | secrete/ecsudate |
| NrAFA1      | SCA          | brown           | gray   | round           | powdery        | d/d      | nd             | 6               |
|             | ISSA         | brown           | brown  | round           | powdery        | d/d      | nd             | 6               |
|             | GAA          | brown           | brown  | round           | powdery        | d/d      | nd             | 10              |
|             | OA           | brown           | brown  | round           | powdery        | d/d      | nd             | 9               |
| NrAFA2      | SCA          | brown           | gray   | round           | powdery        | d/d      | nd             | 6               |
|             | ISSA         | brown           | gray   | round           | powdery        | d/d      | d (low)        | 6               |
|             | GAA          | brown           | yellowish-white | round | powdery        | d/d      | nd             | 15              |
|             | OA           | brown           | brown  | round           | powdery        | d/d      | d (low)        | 6               |
| NrAFA3      | SCA          | brown           | yellowish-white | round | powdery        | d/d      | nd             | 5               |
|             | ISSA         | brown           | dark   | yellowish white | round         | powdery  | d/d             | 6               |
|             | GAA          | brown           | brownish | round       | powdery        | d/d      | nd             | 6               |
|             | OA           | brown           | brownish | round       | powdery        | d/d      | nd             | 9               |
| NrAFA4      | SCA          | gray            | white  | round           | cottony        | d/d      | nd             | 5.5             |
|             | ISSA         | brownie         | yellowish-white | round | powdery        | d/d      | nd             | 5               |
|             | GAA          | brownish         | yellowish-white | round | powdery        | d/d      | nd             | 6               |
|             | OA           | brown           | brown  | round           | powdery        | d/d      | nd             | 9               |
| NrASA1      | SCA          | white, gray     | dark yellow | irregular | cottony        | d/d      | d (high)       | 4               |
|             | ISSA         | brown           | yellowish-white | irregular | powdery        | d/d      | nd             | 4               |
|             | GAA          | gray            | dark yellow | irregular | powdery        | d/d      | d (high)       | 4               |
|             | OA           | brown           | dark yellow | irregular | powdery        | d/d      | d (high)       | 8               |
| NrASA2      | SCA          | brown           | white  | round           | powdery        | d/d      | nd             | 5               |
|             | ISSA         | brown           | gray   | round           | powdery        | d/d      | nd             | 5               |
|             | GAA          | brown           | brownish | round       | powdery        | d/d      | nd             | 5               |
|             | OA           | brown           | brownish | round       | powdery        | d/d      | nd             | 7               |
| NrASA3      | SCA          | brown           | yellow | round           | powdery        | d/d      | d (low)        | 6               |
|             | ISSA         | brown           | white  | round           | powdery        | d/d      | nd             | 6               |
|             | GAA          | brown           | yellow | round           | powdery        | d/d      | d (mod)        | 5               |
|             | OA           | brown           | brown  | round           | powdery        | d/d      | d (low)        | 4               |
| NrASA4      | SCA          | brown           | white  | round           | powdery        | d/d      | nd             | 7               |
|             | ISSA         | brown           | yellowish-white | round       | powdery        | d/d      | nd             | 7               |
|             | GAA          | brown           | yellow | round           | powdery        | d/d      | d (mod)        | 9               |
|             | OA           | brown           | yellow | round           | powdery        | d/d      | d (high)       | 6               |
The color of mature spores or aerial mycelium and substrate mycelia were very varied. Almost all bacterial isolates had gray to brown aerial mycelia, but NrASA3 isolates that growing on ISP4 medium had greenish mycelium. Seven isolates produced pigments that were spread in agar media (Tab. 2). The color of the pigment detected on the agar surface was yellow, with color variations from pale yellow to dark yellow. Dissolved pigments were produced by all culture isolates when growing in GAA and OA media. NrASA1 and NrASA4 isolates were bacterial isolates producing high-intensity diffusible pigments. Seven pure actinomycetes isolates were able to produce brown-colored exudates on the surface of the colony. The exudates appeared on the aerial mycelium had a consistency like sticky liquid and water droplets in the middle of the colony. However, pigments or exudates mostly only appeared in colonies that grew on ISSA or OA media.

**Table 2. Continuation**

|          | Spores color | Substrate color    | Shape | Surface Texture | Aer/Sub Mycelia | Pigment | D. Colony (mm) | Secreted Exudate |
|----------|--------------|--------------------|-------|----------------|----------------|---------|----------------|------------------|
| NrASA5   |              |                    |       |                |                |         |                |                  |
| SCA      | brown        | yellowish-white    | round | powdery        | d/d            | nd      | 5              | nd               |
| ISSA     | brown        | yellowish-white    | round | powdery        | d/d            | nd      | 5              | nd               |
| GAA      | brown        | yellowish-white    | round | powdery        | d/d            | nd      | 4              | nd               |
| OA       | brown        | gray               | round | powdery        | d/d            | nd      | 8              | nd               |
| NrASA6   |              |                    |       |                |                |         |                |                  |
| SCA      | brownish     | yellow             | round | powdery        | d/d            | d (low) | 7              | nd               |
| ISSA     | brown        | white              | round | powdery        | d/d            | nd      | 7              | d (center)       |
| GAA      | brown        | yellowish-white    | round | powdery        | d/d            | nd      | 3              | nd               |
| OA       | brown        | brown              | round | powdery        | d/d            | nd      | 5              | nd               |
| NrAGA1   |              |                    |       |                |                |         |                |                  |
| SCA      | dark gray    | yellowish-white    | round | powdery        | d/d            | nd      | 4              | nd               |
| ISSA     | grayness     | yellowish-white    | round | powdery        | d/d            | nd      | 4              | nd               |
| GAA      | grayness     | yellowish white    | round | powdery        | d/d            | d (low) | 3              | nd               |
| OA       | grayness     | yellow             | round | powdery        | d/d            | d (low) | 5              | nd               |
| NrAGA2   |              |                    |       |                |                |         |                |                  |
| SCA      | dark gray    | yellowish-white    | round | powdery        | d/d            | nd      | 3              | nd               |
| ISSA     | grayness     | yellowish-white    | round | powdery        | d/d            | nd      | 4              | nd               |
| GAA      | grayness     | yellowish white    | round | powdery        | d/d            | d (low) | 3              | nd               |
| OA       | grayness     | yellow             | round | powdery        | d/d            | d (low) | 5              | nd               |

Information: d = detected; nd = not detected; mod = moderate
Figure 3. Diversity of structural characteristics of Actinomycetes colonies isolated from mangrove sediment, fecal pellets, and gastrointestinal of nipah worm growing on different media, including different characters of aerial mycelia surface, margin and elevation, the color of mature spore, presence of exudates and surface pigments, and the texture of colonies.

Based on characteristics of cell morphology, biochemistry, and nutrition showed that properties of the twelve bacteria were Gram-positive, mycelia have branched air mycelium with spiral-shaped ends and long chain-shaped spores (Figure 4a & 4c), produce catalase enzyme, used glucose as a carbon source, and able to decompose gelatine. However, a few isolates were able to produce H$_2$S (4 isolates), used citrate (2 isolates) and lactose (NrAGA1) as carbon sources (Table 3).

Table 3. Morphological, biochemical and nutritional characteristics of Actinomycetes isolated from mangrove sediment, fecal pellets, and gastrointestinal of nipah worm (Namalycastis rhodohoride).

| Isolate Codes | Gram stain | Spore chain | Mycelia | Catalase | H/$S$ prod. | Citrate utz | Gelatine liq. | Glucose utz | Lactose utz | Sucrose utz | Starch lys |
|---------------|------------|-------------|---------|----------|-------------|-------------|---------------|-------------|-------------|-------------|------------|
| NrAFA1        | pos        | lch         | branch  | pos      | pos         | neg         | neg            | pos         | neg         | pos         | pos        |
| NrAFA2        | pos        | lch         | branch  | pos      | neg         | neg         | neg            | pos         | neg         | neg         | pos        |
| NrAFA3        | pos        | lch         | branch  | pos      | neg         | neg         | pos            | pos         | neg         | neg         | pos        |
| NrAFA4        | pos        | lch         | branch  | pos      | neg         | neg         | neg            | pos         | neg         | neg         | pos        |
| NrASA1        | pos        | lch         | branch  | pos      | neg         | neg         | neg            | pos         | neg         | neg         | pos        |
| NrASA2        | pos        | lch         | branch  | pos      | neg         | neg         | neg            | pos         | neg         | neg         | pos        |
| NrASA3        | pos        | lch         | branch  | pos      | neg         | neg         | pos            | pos         | neg         | neg         | pos        |
| NrASA4        | pos        | lch         | branch  | pos      | neg         | neg         | neg            | pos         | neg         | neg         | pos        |
| NrASA5        | pos        | lch         | branch  | pos      | neg         | neg         | neg            | pos         | neg         | neg         | pos        |
| NrASA6        | pos        | lch         | branch  | pos      | neg         | neg         | neg            | pos         | neg         | neg         | pos        |
| NrAGA1        | pos        | lch         | branch  | pos      | neg         | pos         | neg            | pos         | pos         | pos         | pos        |
| NrAGA2        | pos        | lch         | branch  | pos      | neg         | pos         | neg            | pos         | neg         | neg         | pos        |

(Note: lch= long chain; pos= positive result; neg= negative result)
Based on characterizations (Table 2, Table 3, Fig. 4f, Fig. 4g) and referred to Bergey’s Manual of Determinative Bacteriology showed that the twelve actinomycetes bacterial isolates had very close similarities with the actinomycetes group of the genus *Streptomyces*.

**Figure 4.** Spore chains and cell morphological characteristics of Actinomycetes isolated from mangrove sediment, fecal pellets and gastrointestinal of nipah worm: (a) substrate mycelia; (b) aerial mycelia; (c) spore-chain production in long-chain and fragmenting branched of aerial mycelia; (d) all spira and retina culi aperti type of spore chains; (e) diffusible pigment on NrASA1 and NrASA4 (left side).

The key character of all isolates which represented as a member of the genus *Streptomyces* could be seen from the form of lichenoid colonies. Most of all colonies of the 12 bacterial cultures showed lichenoid colonies on four different agar media (Fig. 3) and produced yellow diffusible pigments (Fig. 4e). In addition, aerial mycelia of the actinomycetes colony produced various pigments (Fig. 4h). Morphological spore characteristics of all isolates showed long spore chains and spiral and retinacullia formation. Those characteristics were closely related to the genus *Streptomyces* (Fig. 4b & 4d). However, further research is needed to determine the type and strain of the twelve isolates based on the 16S rRNA sequence character.

4. Discussion

Actinomycetes are a group of bacteria that play an important role in the decomposition of organic material in soil substrates and sediments. This group of bacteria is also known to have a wide habitat distribution, including aqueous sediments, dry soils, sediments with fluctuating salinity values, dried soils, air, and compost [[13], [14]]. Actinomycetes is known to have the ability to produce organic compounds or metabolites inhibiting the growth of pathogenic microbes [[15], [5]].

Actinomycetes are known as potential microbes because they can produce several enzymes, such as proteases, amylases, and cellulases, and the ability to produce antimicrobial compounds. This potential can be utilized in the cultivation nipah. This is based on the substrate and feed source of nipah worms, namely mangrove sediments and nipah plant litter, which are also a habitat for Actinomycetes. This relationship can be used as a parameter for the use of feed additives so that it can be the basis for determining formulations to assist the process of feed conversion or fermentation [[16], [17]]. [18] successfully isolated 42 Actinomycetes strains in the mangrove area of Kerala, India, while [19] also succeeded in isolating 25 actinomycetes isolates in the mangrove area of Torosiaje, Gorontalo Province. Based on the results of isolation, as many as 12 strains of Actinomycetes isolates were
successfully purified from mangrove nipah sediments in Kakap Village, Kubu Raya Regency, West Kalimantan Province. Morphological characterization of colonies, cells, biochemical and nutritional properties (Table 2 & Table 3) showed that the twelve isolates had characters that were very closely belong to the genus *Streptomyces*. 

The change of spore color from gray to dark brown is the characteristic of the genus *Streptomyces*. The form of spores in spira and retinaculliaperti formation has been confirmed as the property of the genus *Streptomyces* [20]. Isolates of NrASA 1 and NrASA4 produced diffusible dark yellow to brown pigments (Table 2 and Figure 4e) which was similar to the species *S. purpureus* and *S. griseoviridis*. The other isolates might have similarities to *S. antibioticus*, because they did not produce diffusible pigments, but produced H₂S, green and grayish mycelia aerials. However, molecular identification is needed to determine the exact species of the twelve bacterial isolates. 

*Streptomyces* spp. are often found in wet sediments such as mangrove sediments. [21] isolated Actinomycetes in the Kuantan mangrove area, Malaysia, which were dominated by the genus *Streptomyces*. [18] reported that 71% of the Actinomycetes found in Kerala, India, were *Streptomyces*. [22] stated that the marine biosphere and estuary are an Actinomycetes habitat. This correlates with organic matter derived from mangrove vegetation litter cover. However, [23] showed that the density of Actinomycetes originating from mangrove and estuary areas was lower than dried soil or terrestrial soils.

The density of Actinomycetes of mangrove nipah sediments was 3.1 × 10^3 CFU/g (Table 1). This number approaches the Actinomycetes density value obtained by [24] on Andaman Island, which was 3.29 × 10^3 CFU/g. The number of Actinomycetes density from fecal pellets and gastrointestinal nipah worms was lower than that of nipah mangrove sediments. Actinomycetes isolated from feces and digestive tract were the result of the entry of spores into the digestive tract through the process of eating. [25] stated that bacterial interactions that occurred in the digestive tract of worm came from the bacteria that are ingested from the soil and transit the gastrointestinal.

The presence of Actinomycetes is also thought to have a relationship between gastrointestinal characteristics and the availability of food types in the environment. Actinomycetes isolated from faecal pellets and gastrointestinal nipah worms reflected the relationship between eating behavior and the type of substrate in habitat. [26] found Actinomycetes from the digestive tract of termites that were known to have cellulolytic activity and consuming cellulose fiber. The relationship of feed behavior, type of substrate, microbe such as actinomycetes can be further developed through studies to support the cultivation of nipah worms.

### 5. Conclusion

Twelve Actinomycetes identified as a member of the genus *Streptomyces* spp. were successfully isolated from nipah mangrove sediments, faecal pellets, and gastrointestinal tract of nipah worm (*Namalycastis rhodochorde*) with their densities ranging from 3.6 × 10^1 to 3.1 × 10^3 CFU/g. The population of the bacterial isolates was affected by the behavior of nipah worm feed and the type of substrates.

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