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Characteristics and antibacterial potency of actinomycetes isolated from nypa palm litter

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Abstract. *Kurniatuhadi R, Setyawati TR, Yanti AH, Fadhila A. 2024. Characteristics and antibacterial potency of actinomycetes isolated from nypa palm litter. Biodiversitas 25: 3449-3459.* Actinomycetes are ubiquitous bacteria which are known to produce secondary metabolites with bioprospecting potential as antibacterial agents. This research aimed to qualitatively determine the density and diversity of this group, as well as the antibacterial potential of actinomycetes from nypa palm (*Nypa fruticans* (Thunb.) Wurmb.) litter. Isolation was performed using the pretreatment pour plate method, which involved wet heat treatment using a selective starch casein agar (SCA) medium. Actinomycetes density was calculated using the standard plate count (SPC). The isolated microorganisms where characterized based on macroscopic, microscopic characters, and biochemical tests. Potential antibacterial activity against *Aeromonas* sp. NrBF9 was qualitatively assessed employing the cross-streak method. The results showed that actinomycetes accounted for 11% of the total bacterial density. Macroscopic screening of the isolates' colony characteristics revealed the successful retrieval and culture of eight isolates. All isolates exhibited similar characteristics to those of the genus *Pseudonocardia*. Six out of the eight isolates, coded as TCN1, TCN2, TCN4, TCN5, TCN6, and TCN7, could inhibit the activity of *Aeromonas* sp. NrBF9, resulting in the formation of a clear zone surrounding of colonies. The six isolates of *Pseudonocardia* with antibacterial activity have the potential to be developed as feed additives for nypa palm worm aquaculture.

Keywords: Actinobacteria, long nipah worm, pre-heat treatment isolation, macrohabitat of polychaete

INTRODUCTION

The nypa palm (*Nypa fruticans* (Thunb.) Wurmb.), also known as the nipah palm, is a dominant palm tree species found in the waters of the Kakap River estuary in the Kubu Raya District of West Kalimantan, Indonesia. Nypa palm trees in estuaries facilitate a transition between freshwater and seawater ecosystems, leading to the formation of sediment. The sediment surrounding nypa palm trees exhibits high productivity because of its high organic matter content (Kurniatuhadi et al. 2023). The estuary environment has fluctuating salinity conditions that only specific organisms, such as polychaete worms, can live in (Junardi 2021).

One of the polychaete worms found in the roots of nypa palm mangroves is the nypa palm worm (*Namalycastis rhodochorde* Glasby, Miura, Nishi, Junardi), known for immersing itself in mud (Junardi 2021). Members of the Polychaeta class are widely utilized as a supplementary aquaculture feed due to its potential to enhance shrimp gastrointestinal health (Setyawati et al. 2020). The nutritional value of polychaetes makes them a valuable feed source in aquaculture, leading to high market demand for these worms (Junardi 2021).

Efforts are being made to support the survival of the nypa palm worm include laboratory-scale cultivation to meet the demand for nypa palm worms. However, current efforts to cultivate palm worms are facing challenges due to high mortality in the early stages of development. The larval survival rate is less than 20%, and the juvenile growth rate is 20.77% (Setyawati et al. 2020; Junardi 2021). The duration required for the maturation to the adult stage (40 segments) in aquaculture spans six months, a significantly extended period in contrast to the growth timeline observed in its natural habitat (Setyawati et al. 2020). One solution for successfully cultivating palm worms involves adding probiotics to the feed to enhance the worms' digestibility. The search for probiotic sources has been the subject of research conducted by Setyawati et al. (2020), involving the screening and selection of bacteria isolated from the gastrointestinal tract. A bacterium within the *Aeromonas* sp. NrBF9 was suspected to have pathogenic activity. Symptoms observed following *in vivo* injection in a pathogenicity test included the appearance of wounds on the body surface, broken body parts, and paleness. Probiotic feed can be explored using palm worm substrates, such as actinomycetes. Actinomycetes are bacteria that produce natural products, including antibiotics, antifungals, antivirals, anticancer agents, enzymes, and other compounds (Katili and Retnowati 2017).

The composition and antibacterial potency of actinomycetes isolated from the nypa palm mangrove substrate are not well understood. Previous research by Yanti et al. (2020) focused on isolating actinomycetes from nypa palm mangrove sediments, gastrointestinal pellets, and the feces of nypa palm worms without heat pretreatment obtained 12 isolates belonging to the genus *Streptomyces*. Lee (2014) isolated actinomycetes from mangrove sediments

in the Peninsular Malaysia area, tested their antimicrobial properties, and demonstrated that actinomycetes could produce potent active metabolites. Actinomycetes isolated from the mangroves and sediments of the Cochin estuary in India revealed the presence of isolates with the code ER7. These actinomycetes exhibited antimicrobial activity against *Bacillus* sp. (Rosmine and Varghese 2016).

The comprehensive characterization of colonies at a macroscopic level, combined with the examination of the biochemistry and physiology of actinomycetes isolates obtained from mangrove environments, is imperative for the systematic classification and identification of genera and species. These attributes offer valuable insights into the extensive diversity found within the actinomycetales order, even when certain species are grouped within the same genus. Several research studies have highlighted the global importance of the actinomycetales order owing to its broad distribution and its capacity to produce a variety of diffusible pigments and antibiotic compound (Sengupta et al. 2015; Perez-Coral et al. 2022). Actinomycetes exhibit a wide range of morphological characteristics, including diverse colors and mycelial structures, and they can manifest aerial mycelium, substrate mycelium, and diffusible pigments, depending on the species (Perez-Coral et al. 2022). Therefore, investigations related to the characterization of native actinomycetes should involve thorough biological, biochemical, morphological, and molecular analyses due to the impact of environmental factors (Law et al. 2017).

Actinomycetes are believed to be involved in the degradation of nypa palm fronds and are associated with the survival of palm worms in their natural habitat. Exploration and characterization are required to obtain a diverse range of actinomycetes from nypa palm mangrove litter in the Kakap River area, Kubu Raya District. The isolation of actinomycetes for the development of nypa palm worm culture can yield antibacterial agents against palm worm pathogens and a source of secondary metabolites for use as immunomodulators in nypa palm worm feed formula. Therefore, this research was aimed to explore the diversity of actinomycetes originating from nypa palm litter in the habitat of the nypa palm worm (*N. rhodochorde*) and its screening for potential antibacterial compounds. The wet-heat treatment isolation process was used to selectively isolate actinomycetes, which grow slowly and inhibit the growth of unwanted bacteria.

MATERIALS AND METHODS

Study area

Nypa palm litter sampling was conducted in Sungai Kakap Village, which is part of the Sungai Kakap Sub-district in the Kubu Raya District of West Kalimantan Province, Indonesia. Sungai Kakap Sub-district covers an area of approximately 13.27 square kilometers. The research site was about 1 km from an *Avicennia* mangrove. Litter samples were collected from a secondary mangrove area at the mouth of the Kakap River (Figure 1). The research location was dominated by nypa palm trees (*N. fruticans*), which provide the habitat for the nypa palm worm (*N. rhodochorde*). Sampling was performed in nine spots (Figure 1.A), namely location point 1 (00°03'04.4"S, 109°09'59.6"E), point 2 (00°03'05.2"S, 109°10'00.1"E), point 3 (00°03'06.0"S, 109°10'01.4"E), point 4 (00°03'05.8"S, 109°10'01.6"E), point 5 (00°03'05.5"S, 109°10'01.9"E), point 6 (00°03'04.4"S, 109°10'00.5"E), point 7 (00°03'03.6"S, 109°09'59.9"E), point 8 (00°03'04.1"S, 109°09'59.7"E), and point 9 (00°03'04.7"S, 109°10'00.3"E).

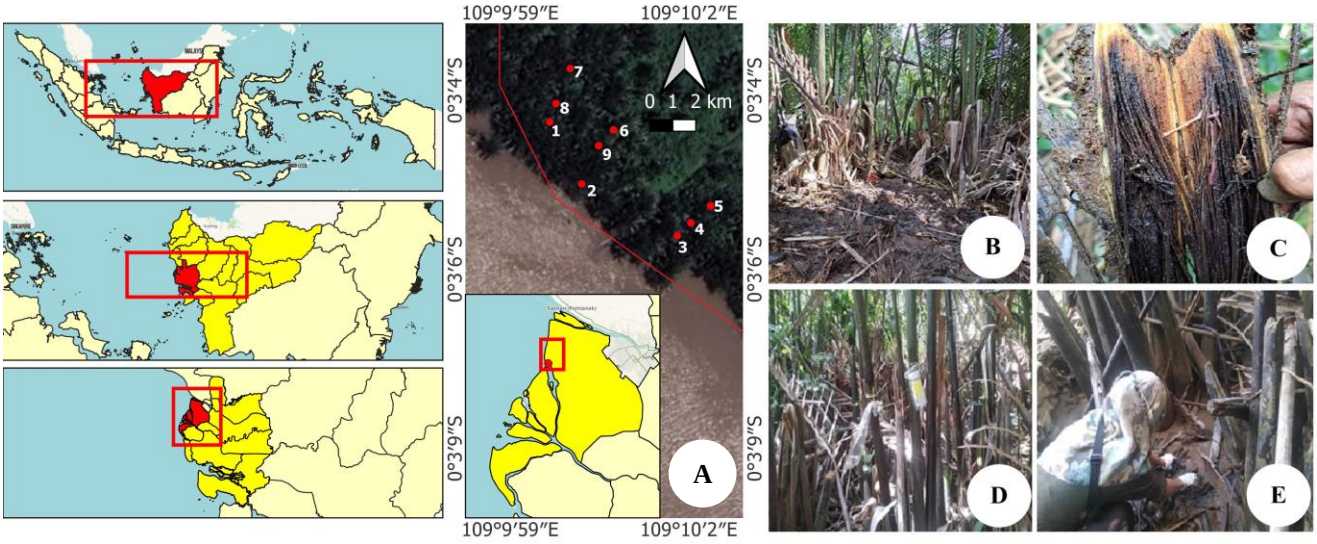


Figure 1. Location of the Sungai Kakap Estuary of Sungai Kakap Sub-district, Kubu Raya District, West Kalimantan Province, Indonesia. The sampling sites were dominated by *Nypa fruticans* (B, D, and E) and polychaetes, especially *Namalycastis rhodochorde*, on the substrate and litter (C)

Litter sample collection and pretreatment

Before sampling nypa palm litter, a survey was conducted to determine the sampling plot. Purposive sampling was performed in the mangrove area, which is dominated by nypa palm plants (*N. fruticans*). The collected litter comprised palm leaf midrib litter that had been decomposed on the surface of the mud substrate, characterized by soft and dark brown fronds. Five replicates were collected, and the samples were placed in sterilized bags. The collected samples were then chopped and placed in containers. Wet heat treatment was performed by weighing 10 g of the sample, suspending it in 90 mL of sterile saline, and then stirring it using a rotary shaker at 170 rpm for 30 min. Pretreatment heating was performed in an oven at 60°C for 20 min (Abidin et al. 2016; Meenakshi et al. 2024).

Isolation of actinomycetes

The media used in this research included starch casein agar (SCA), gelatine nutritive agar, urea Christensen's agar, Simmons' citrate agar, triple sugar iron agar (TSIA), starch agar, and liquid sugar fermentation medium. SCA (18 g agar, 0.02 g $CaCO_3$, 0.3 g casein, 0.01 g $FeSO_4.7H_2O$, 2 g K_2HPO_4, 2 g KNO_3, 0.05 g $MgSO_4.7H_2O$, 2 g NaCl, and 10 g soluble starch in 1 L distilled water); urea Christensen's agar (1 g dextrose, 1 g peptone, 5 g NaCl, 2 g KH_2PO_4, 0.012 g phenol red, and 15 g agar-agar in 1 L distilled water); Simmons' citrate agar (2.25 g citrate agar powder in 100 mL distilled water); TSIA (6.5 g TSIA powder in 100 mL distilled water); starch agar (5 g peptone, 3 g beef extract, 2 g starch, and 30 g agar in 1 L distilled water) were prepared and heated to boiling point using a hot-stir plate. All media were then placed in Erlenmeyer flasks and sterilized using an autoclave at 121°C for 15 min. In Christensen Urease media, an exception arises where the urea substance is combined after sterilizing the agar and distilled water. The aseptic procedure involves mixing urea, distilled water, and sterile agar (Sivanandhini et al. 2015; Qi et al. 2019; Yanti et al. 2020).

Actinomycetes were isolated from nypa palm litter using the serial dilution and pour plate method (Yanti et al. 2020). Serial dilutions were carried out at a 1:10 ratio. Specifically, 1 mL of the pretreated nypa palm litter sample was added to 9 mL of sterile saline solution, resulting in a 10^{-1} dilution. Subsequently, 1 mL of the 10^{-1} dilution was mixed with 9 mL of distilled water to create a 10^{-2} dilution, and so on up to 10^{-4}. One milliliter of each dilution was inoculated and poured using the pour plate method into 20 ml of SCA, in triplicate. Homogenization was achieved by creating a figure-eight pattern. The plates were incubated at 30°C for 6 days.

Purification and characterization of actinomycetes

The expanding actinomycetes colonies were examined morphologically. Their morphology was distinct, and colonies were purified by selecting the actinomycetes colonies and inoculating them on SCA using the streak plate method, followed by an incubation period of 5 days at 30°C (Yanti et al. 2020). The morphological characteristics of actinomycetes, as described by "Bergey's Manual of Determinative Bacteriology" (1994), were assessed based on features such as shape, the color of mature spores, the structure of colony edges, the color of the bottom of the colony, the surface texture of the colony, conidia, and pigment formation. Different characteristics were encoded. Characterization was performed using cultures that were incubated for 3, 7, and 14 days (Katili and Retnowati 2017; Yanti et al. 2020).

Gram staining

Hyphal staining was performed using the Gram staining method, which involved inoculating actinomycetes isolates with a loop needle. Aerial hyphae were spreaded over a glass surface moistened with distilled water and then flattened and allowed to air-dry. The next step involved dripping crystal violet onto the sample and allowing it to sit for 1 min. Then, the sample was rinsed with distilled water, and iodine was dripped on and allowed to sit for 1 min. Next, the sample was rinsed with distilled water and then with 95% ethanol until the color faded. The next step involved dripping safranin onto the sample and allowing it to sit for 45 s. Then, the sample was rinsed with distilled water and left to air-dry. Finally, the sample was observed under a microscope at a 1000x magnification (Cappuccino and Sherman 2014).

Biochemical analysis of actinomycetes

Gelatin liquefaction test

The gelatin test was performed by preparing a gelatin medium, inoculating actinomycetes therein, and storing the sample at 26°C for 48 h. After incubation, the medium containing actinomycetes isolates were stored in a refrigerator at 4°C for ±30 min. Melted media indicated a positive reaction, whereas, media that remained solid indicated a negative reaction. Incubation was repeated for 5 days. The cooling process was performed for 30 min to observe liquefaction (Cappuccino and Sherman 2014).

Catalase test

Actinomycete isolates were collected using a loop and transferred to the surface of a glass slide. The isolate was then treated with one drop of 3% H_2O_2. The presence of air bubbles in approximately 5-10 s indicated a positive result, whereas, negative results were denoted by the absence of bubbles (Varghese and Joy 2014).

Urease test

The urease test was performed aseptically using Christensen's urea agar. Actinomycetes isolates were inoculated onto the slanted surface of the medium. Next, the cells were incubated at 30°C. Color changes were observed at 24-48 h. A positive urease test result was indicated by a change in the medium from yellow to pink (Svane et al. 2020; Yanti et al. 2020).

Citrate utilization test

Citrate testing was performed using slanted Simmons' citrate agar. Actinomycete isolates were inoculated in the medium with a loop, using the puncture and scratch method. The cells were incubated at 30°C for 24-48 h. Positive reaction results were indicated by the medium

turning blue, whereas, negative results were denoted by the medium remaining green (Yanti et al. 2020).

Test of carbohydrate metabolism and hydrogen sulfide (H_2S) production

Actinomycetes isolates were inoculated in a TSIA medium with a loop, using the puncture and streak method. The tubes were then incubated for 48 h at 30°C. A positive reaction indicating fermented glucose was marked by yellow on the butt (base) and red on the slant (slant). Reactions that resulted in yellow in both butt and slant indicated lactose and sucrose fermentation. Gas formation was indicated by cracks and bubbles and a dark bottom (Yanti et al. 2020).

Sugar fermentation test

The sugar fermentation test was performed on glucose, lactose, fructose, mannitol, and inulin media. Bacteria were inoculated into test tubes containing each sugar medium. The cells were then incubated for 24 h at 30°C. Positive results were indicated by a change in the color of the medium to yellow and CO_2 bubble production. Negative results were indicated by no change in the color of the medium (Yanti et al. 2020).

Detection of the inhibitory activity of actinomycetes bacteria against Aeromonas *sp. NrBF9*

Aeromonas sp. NrBF9 was isolated from the gastrointestinal tract of the palm worm (*N. rhodochorde*). This bacterium is reported to have hemolytic activity and the highest DNAse index among all isolated bacteria, indicating its pathogenic nature (Setyawati et al. 2020). The growth of pathogenic bacteria can be inhibited by identifying agents that can impede their development. The initial screening was performed using the cross-streak method. A pure culture of *Aeromonas* sp. NrBF9 was cultivated on nutrient agar (NA) and incubated for 24 h. The actinomycetes isolate was inoculated by streaking it on one side of the Petri dish and covering one-third of the dish with SCA. The plate was incubated for 3 days at 26°C (Nofiani et al. 2022). After the 3-day incubation period, *Aeromonas* sp. NrBF9 was streaked along a 4.5-cm line on the side of the plate, perpendicular to the actinomycetes isolate. The plate was incubated at 30°C for 24 h. Actinomycetes isolates with the potential to act as antagonists were identified by the formation of an inhibition zone where *Aeromonas* sp. NrBF9 failed to grow. The actinomycetes isolates were observed for 7 days.

RESULTS AND DISCUSSION

Composition and characteristics of actinomycetes isolated from nypa palm litter

Actinomycetes and non-actinomycetes bacteria isolated from nypa palm mangrove litter grew on SCA medium by day 3rd after incubation and exhibited colony morphology distinct from non-actinomycetes bacteria. Actinomycetes isolates had a characteristic cottony surface with colony diameters ranging from 0.7 to 3 mm (Table 1; Figure 2), while non-actinomycetes bacteria had a smooth surface. The population density of actinomycetes bacteria was found to be lower than that of non-actinomycetes bacteria.

The density of actinomycetes, as determined through the replication of the isolation procedure, constituted 20% of the total density of bacterial colonies observed on the SCA medium. Following the initial isolation phase, 113 isolates exhibiting characteristics suggestive of actinomycetes colonies were identified. Subsequent purification procedures yielded only eight isolates suitable for further cultivation. The characteristics and potential of these pure isolates were analyzed in-depth. The eight isolates were designated TCN1, TCN2, TCN3, TCN4, TCN5, TCN6, TCN7, and TCN8.

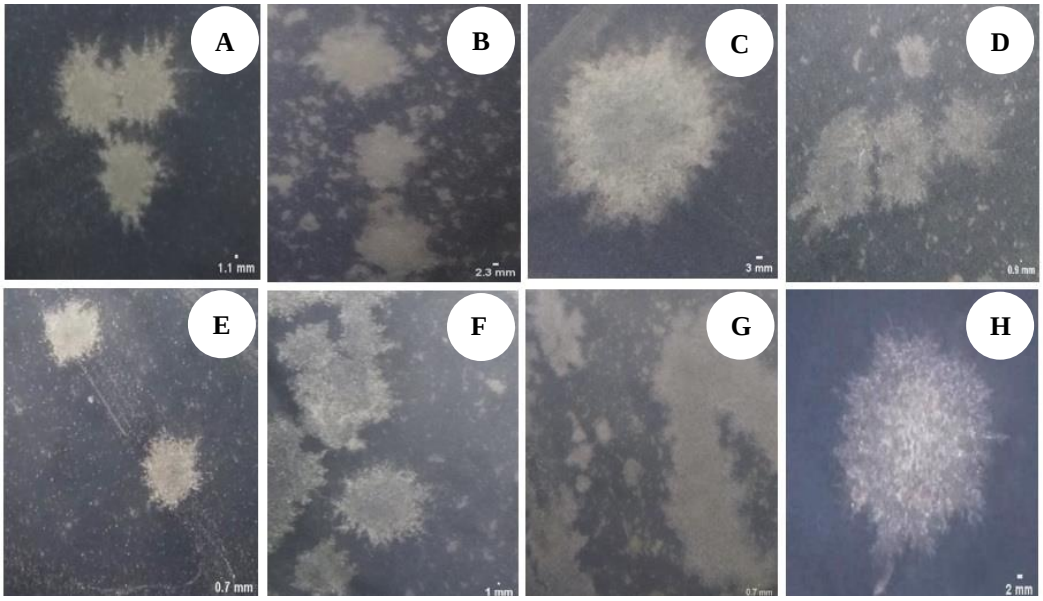


Figure 2. Macro-colony characteristics of A. TCN1; B. TCN2; C. TCN3; D. TCN4; E. TCN5; F. TCN6; G. TCN7; and H. TCN8 isolates

Table 1. Colony, cell, and biochemical characteristics of actinomycetes isolates from nypa palm mangrove litter

Characteristics	Isolates							
	TCN1	**TCN2**	**TCN3**	**TCN4**	**TCN5**	**TCN6**	**TCN7**	**TCN8**
Colony morphology								
Shape	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular
Margin	Filament	Filament	Filament	Filament	Filament	Filament	Filament	Filament
Elevation	Raised	Raised	Raised	Raised	Raised	Raised	Raised	Convex
Surface	Cottony	Cottony	Cottony	Cottony	Cottony	Cottony	Cottony	Cottony
Aerial hyphae color	White	White	White	White	White	White	White	White
Substrate color	White	White	White	White	White	White	White	White
Diffusible pigment	-	-	-	Bluish	Bluish	Bluish	-	Bluish
Diameter (mm)	1.1	2.3	3	0.9	0.7	1.0	0.7	2.0
Cell/microscopic features								
Gram staining	+	+	+	+	+	+	+	+
Branched hyphae	Branched	Branched	Branched	Branched	Branched	Branched	Branched	Branched
Conidial shape	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval
Conidial size (µm)	6.1	5.1	6.9	6	6.2	5.9	5.3	5.5
Motility	-	-	-	-	-	-	-	-
Biochemistry								
Glucose	-	-	-	-	+	-	-	+
Lactose	-	-	-	-	-	-	-	-
Sucrose	-	-	+	-	+	-	-	-
Mannitol	+	-	+	-	+	+	+	+
Inulin	-	-	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-
Catalase activity	+	+	+	+	+	+	+	+
Urease activity	+	+	+	+	+	+	+	+
Gelatin liquefaction	-	-	-	+	+	-	-	+
Genus	*Pseudonocardia*	*Pseudonocardia*	*Pseudonocardia*	*Pseudonocardia*	*Pseudonocardia*	*Pseudonocardia*	*Pseudonocardia*	*Pseudonocardia*

Note: (-) indicates a negative or undetectable result; (+) indicates a positive or detectable result; (nd) means not documented in the source

The observations of colony characteristics revealed similarities in their macroscopic features. The eight isolates with the codes TCN1, TCN2, TCN3, TCN4, TCN5, TCN6, TCN7, and TCN8 shared many similarities. They exhibited an irregular shape with thread-like edges, a cotton-like surface, and white front and back colors (Figure 2). Morphological differences were evident in elevation, pigment diffusion, and diameter of colony. Isolates TCN1 to TCN7 exhibited a raised surface, while TCN8 was convex. Diffusible pigment was only produced by isolates TCN4, TCN5, TCN6, and TCN8 (Table 1; Figure 3). The colonies shared irregular shapes, edges resembling threads (filaments), and surfaces resembling cotton (cottony). Both the upper and lower sides of the colonies exhibited a white coloration; however, a color alteration was observed in the upper part or aerial hyphae of seven isolates (excluding the actinomycetes isolate with the code TCN8), transitioning from white to grayish after the seventh day of incubation (Figure 2).

Actinomycetes isolates observed under 1000× magnification exhibited similarities in Gram staining, classifying them as Gram-positive bacteria. They displayed a branched hyphal structure and conidial shape characterized as oval. The conidia size ranged between 5.3 and 6.9, with no observed motility. Among the isolates, only TCN1 showed septa, while TCN2, TCN3, TCN4, TCN5, TCN6, TCN7, and TCN8 displayed segmented or fragmented hyphae, as illustrated in Figure 4.

Biochemical tests revealed several similarities, including negative results in the lactose, inulin, citrate utilization, and H_2S production tests and positive results in the catalase and urease activity tests. The biochemical tests indicating differences in fermentation included the glucose, sucrose, mannitol, and gelatin liquefaction tests (Table 1). Three isolates, namely TCN4, TCN5, and TCN8, have been identified as capable of gelatin liquefaction. Among these, only TCN5 and TCN8 exhibited positive results in glucose fermentation, while TCN3 and TCN5 showed positive results for sucrose fermentation. Most isolates demonstrated the ability to ferment mannitol, except for TCN2 and TCN4. Notably, TCN5 is the only isolate that tested positive for the fermentation of three types of carbohydrates and gelatin liquefaction.

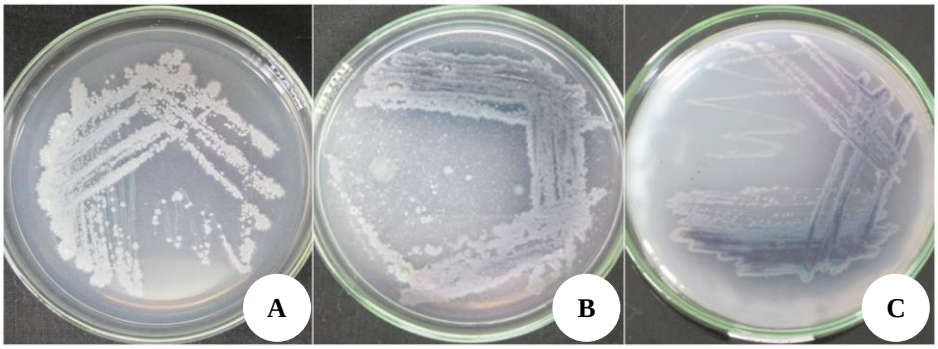


Figure 3. Visualization of TCN-coded actinomycetes colonies on the third day of A. Incubation; B. Seventh day; C. Fourteenth day. On the seventh day, the purple pigment appeared, becoming increasingly bluish by the fourteenth day

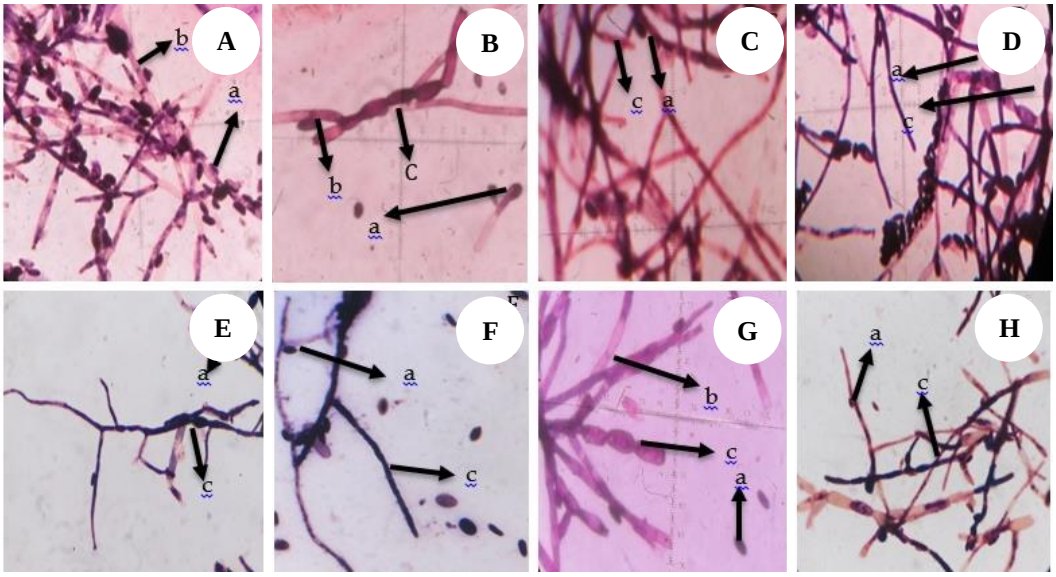


Figure 4. Microscopic characteristics of A. TCN1; B. TCN2; C. TCN3; D. TCN4; E. TCN5; F. TCN6; G. TCN7; H. TCN8. a. Conidia; b. Septa of hyphae; c. Fragmentation of hyphae

The characterization of the eight isolates identified by the codes TCN1, TCN2, TCN3, TCN4, TCN5, TCN6, TCN7, and TCN8 revealed several similarities in macroscopic colonies, hyphae, and biochemical tests. Therefore the characteristics of the actinomycetes isolated from nypa palm litter closely resemble those of the genus *Pseudonocardia*.

Detection of inhibitory activity against *Aeromonas* sp. NrBF9

The evaluation of actinomycetes isolates' activity against the pathogenic bacteria *Aeromonas* sp. NrBF9 revealed that six actinomycetes isolates demonstrated the ability to inhibit the growth of *Aeromonas* sp. NrBF9 bacteria within a 24-hour period. The TCN5 isolate exhibited inhibitory effects on *Aeromonas* sp. NrBF9 through the antibiosis mechanism consistently observed (Table 2). The inhibitory activity was assessed by observing areas where *Aeromonas* sp. NrBF9 bacteria failed to grow in the presence of actinomycetes isolates and the test bacteria (Figure 5.B), as well as by evaluating the competitive interactions between the bacteria (Figure 5.B.c).

Pseudonocardia sp. TCN5 isolate demonstrated a sustained presence of a clear zone for up to 168 hours post cross-streak treatment, exhibiting consistent inhibitory activity against *Aeromonas* sp. NrBF9 within a good activity category. In contrast, the other four isolates showed a reduction in the clear zone size by 48 hours. Moreover, *Pseudonocardia* sp. TCN4 began to exhibit a decrease in antibacterial efficacy at 72 hours, evident from the merging of the streak area between *Pseudonocardia* and *Aeromonas*. Notably, *Pseudonocardia* sp. TCN8 was the sole isolate that lacked antibiotic activity, without a clear zone appearing 24 hours after testing.

The width of the clear zone surrounding the streak between *Pseudonocardia* sp. TCN5 and *Aeromonas* sp. NrBF9 corresponds to the diffusion width of the purple diffusible pigment after 24 hours of incubation. The diffusible pigment observed on the agar surface exhibits a purple color (Figure 5.A). Subsequent to 168 hours of incubation, a noticeable color transition from the diffusible pigment to dark blue occurs. Additionally, the invasion of the *Pseudonocardia* sp. TCN5 colony is evident as it covers the streak of the *Aeromonas* sp. NrBF9 colony (Figure 5.B).

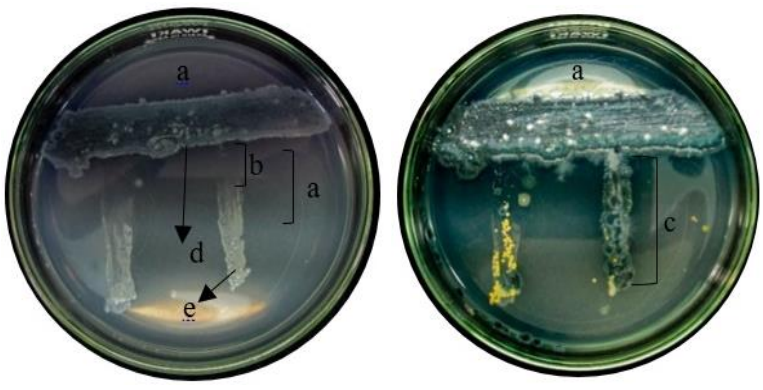
Discussion

Cultivating actinomycetes from extreme environments, particularly saline habitats, poses significant challenges due to this group's slow growth rate. This complexity was evident during the isolation and purification of actinomycetes from palm leaf midrib litter in the present work. Among 113 isolates suspected to be actinomycetes, only eight successfully grew and were purified. Traditional isolation methods require careful consideration of factors such as selecting appropriate sources for filtering, utilizing selective media, optimizing culture conditions, and identifying potential colonies during primary isolation. The selection of suitable media and growth conditions is crucial; as existing media are typically designed for specific microbial genera or species. As stated in other studies on microbial discovery, when working with environmental samples containing diverse microbial populations, the choice of media and growth conditions influences the enrichment of certain populations over others (El Karkouri et al. 2019).

Table 2. Inhibitory activity of actinomycetes bacterial isolates against *Aeromonas* sp. NrBF9

Isolate code	Antibacterial activity (in hours)						
	24	48	72	96	120	144	168
TCN1	+	K+	K+	K+	K+	K+	K+
TCN2	+	K+	K+	K+	K+	K+	K+
TCN3	-	K+	K+	K+	K+	K+	K+
TCN4	++	+	K+	K+	K+	K+	K+
TCN5	++	+++	+++	+++	+++	+++	+++
TCN6	+	K+	K+	K+	K+	K+	K+
TCN7	+	K+	K+	K+	K+	K+	K+
TCN8	-	-	-	K+	K+	K+	K+

Notes: (-): indicates no inhibitory activity; (+): indicates weak activity (<25% inhibition); (++): indicates moderate activity (25-50% inhibition); (+++): indicates good activity (>50% inhibition); (K+): indicates competition



A **B**

Figure 5. The inhibitory activity of *Pseudonocardia* spp. against *Aeromonas* sp. NrBF9. A. 24-hour isolate; B. 168-hour isolate. a. Diffusible pigment; b. Inhibitory activity of actinomycetes isolates against *Aeromonas* sp. NrBF9; c. Competition; d. Actinomycetes (*Pseudonocardia*) colony; e. *Aeromonas* sp. NrBF9 colony

Previous research (Yanti et al. 2020) has effectively identified actinomycetes belonging to the genus *Streptomyces* in the mud substrate of the nypa palm worm habitat without subjecting the samples to heating pretreatment. The specimens analyzed in this investigation were obtained from the decomposed nypa palm frond litter within the mud substrate of the nyppa palm worm habitat. The primary objective of the comparative analysis was to ascertain potential variations in the composition of actinomycetes when isolated from decomposed nypa palm frond litter.

The pretreatment heating of nypa palm litter samples involved dry heating in an oven to inhibit the growth of other microbes and observe the outcomes of actinomycetes isolation. The results of the isolation after heat-pretreatment of nypa palm litter indicated that bacterial colonies with characteristics similar to actinomycetes colonies continued to thrive on agar medium. The physical treatment of samples for the isolation process using the dry heat method can be utilized to isolate various genera of actinomycetes. Actinomycetes spores demonstrate higher resistance to desiccation compared to the majority of bacteria. These actinomycetes spores can germinate after heat treatment, unlike other bacterial vegetative cells. Heat drying soil samples at a temperature of 120°C for 1 hour, as well as the simple act of air-drying soil, sediment, lichens, and samples, can effectively eliminate most undesirable bacteria. Additionally, it supports the growth of *Streptomyces* and other rare genera such as *Spirilliplanes*, *Actinomydura*, *Spirilliplanes*, and *Pseudonocardia* exclusively on the growth medium (Kumar and Jadeja 2016; Nofiani et al. 2022; Meenakshi et al. 2024).

Actinomycetes isolated from nypa palm litter tended to exhibit similar traits to those of the genus *Pseudonocardia* (Table 1). While the characteristics of the genus *Pseudonocardia* are not thoroughly described in books or scientific articles, several studies and literature reviews describe *Pseudonocardia* as catalase-positive, Gram-positive, non-motile, and producing diffusible pigment (Nikou et al. 2017; Riahi et al. 2021). *Pseudonocardia* is reported to originate from several types of substrates, including mangrove sediments, especially primary mangroves and marine sediments (Liu et al. 2015; Mangamuri et al. 2015; Zhang et al. 2017; Nofiani et al. 2022). Research conducted by Sharapova (2023) also identified *Pseudonocardia* in plant litter collected in Russia. *Pseudonocardia*, according to Kim and Goodfellow (2015), comprises aerobic actinomycetes with a yellowish to brownish substrate and white or yellowish-white aerial mycelia. The color of the substrate and aerial parts can vary, influenced by the media used.

The microscopic characteristics of *Pseudonocardia* include Gram positivity and branched hyphae. In this work, the conidia were oval and the hyphae were branched, with septa present in isolates TCN1, TCN2, and TCN7. In contrast, the hyphae were fragmented in isolates TCN3, TCN4, TCN5, TCN6, and TCN8. The traits of the Actinomycete isolated from nypa palm litter were similar to those of *Pseudonocardia*. Nofiani described the hyphae of the genus *Pseudonocardia* as segmented (Nofiani et al. 2022). Fragmentation of the mycelium may or may not be visible. Kim and Goodfellow (2015) reported that hyphae on the substrate branch and fragment into rod shapes. Aerial hyphae, when fragmented into oval or square chains, are equipped with two or more spores in chain form. The spores have a smooth surface and are produced by substrate mycelia or aerial mycelia originating from acropetal buds or through basipetal septation. Research conducted by Nofiani et al. (2022) showed that *Pseudonocardia* in aerial hyphae can branch, contain septa, and produce spores with an ovoid shape at the tip of the hyphae. *Pseudonocardia* can be found in plant residues and soil (Gao et al. 2018; Wang et al. 2023). *Pseudonocardia* was successfully isolated from plant litter collected from Ba Be Park, Vietnam, by Sakiyama et al. (2010).

The TCN4, TCN5, TCN6, and TCN7 isolates in this work could produce pigments that diffused into the culture medium. The pigment color in the four isolates tended to be blue or bluish (Table 1; Figure 3). According to Abidin et al. (2018) and Salim et al. (2017), actinomycetes are known for their ability to produce diffusible pigments in a variety of colors, including purple, yellow, blue, green, orange, and red. The color of the diffusible pigment produced by *Pseudonocardia* TCN4, TCN5, TCN6, and TCN8 differed from that of the *Pseudonocardia* isolated by Nofiani et al. (2022) from primary mangrove substrates, which exhibited a predominantly yellow color on Yeast Malt Agar (YMA). This difference may be due to the bacterial species and the use of media with different compositions (YMA and SCA). Pigment formation does not play a role in the growth of organisms but rather contributes to increased survival and competitiveness (Barka et al. 2016). The presence of soluble pigments is a crucial characteristic for identifying and distinguishing new species of actinomycetes (Li et al. 2016). Bacteria belonging to the *Pseudonocardia* genus exhibit key biochemical characteristics, including a positive response to the catalase test (Kim and Goodfellow 2015). Our results showed that all eight isolates were catalase-positive, as indicated by the formation of bubbles (Table 1). Microorganisms capable of using the catalase enzyme can decompose H_2O_2 into water and oxygen components. According to Cappuccino and Sherman (2014), the primary function of the catalase enzyme produced by bacteria is to facilitate the aerobic respiration process. The results of this study are similar to the research findings of Jafari et al. 2014 who showed that the catalase test yielded positive results for *Pseudonocardia* sp. JB05.

The urease test also showed positive results for all eight isolates (Table 1), as indicated by a color change in the medium from yellow to pink. The urease enzyme functions to break down nitrogen and carbon bonds, with the final product being the base ammonia (Cappuccino and Sherman 2014). The objective of breaking down urea compounds is to maintain stable nitrogen and carbon levels, supporting cellular activity and enabling bacteria to form various compounds for defense mechanisms (Svane et al. 2020). Positive urease test results were found for the species *Pseudonocardia abyssalis* and *Pseudonocardia petroleophila* (Parra et al. 2021).

The results of the gelatin liquefaction test indicated that three isolates, TCN4, TCN5, and TCN8, could hydrolyze gelatin (Table 1). The isolates TCN1, TCN2, TCN3, TCN6, and TCN7 were found to be unable to melt gelatin. Gelatinase activity is indicated by liquefaction in bacterial cultures. Gelatin liquefaction is utilized to identify microorganisms capable of producing proteolytic enzymes, which are involved in breaking down proteins into amino acids (Cappuccino and Sherman 2014). Gelatin liquefaction was also observed in *Pseudonocardia abyssalis* (Parra et al. 2021).

The citrate test on the eight isolates showed negative results, with the medium remaining green throughout (Table 1). The citrate test is utilized to assess bacteria's capacity to use it as a carbon source of energy. Positive results are indicated by a color change in the medium from green to blue (Cappuccino and Sherman 2014). Research conducted by Nofiani et al. (2022) showed that isolates of *Pseudonocardia* sp. SMA1A also yielded a negative citrate test. All eight isolates also tested negative in the H_2S test (Table 1). H_2S production is indicated by the bacteria-inoculated medium turning black. The negative results indicated that the isolates were incapable of producing H_2S and iron sulfate in the final product of sulfate-reducing metabolism. H_2S is used to detect gas production by organisms and is commonly employed to identify the Enterobacteriaceae family (Cappuccino and Sherman 2014). This statement is in line with the research conducted by Jafari et al. (2014), in which the isolate *Pseudonocardia* sp. JB05 could not produce H_2S.

The results of the sugar fermentation tests varied, with no CO_2 bubbles forming in the Durham tube (Table 1). A positive reaction to glucose fermentation was observed in the TCN5 and TCN8 isolates, while sucrose fermentation was detected in isolates TCN3 and TCN5. Mannitol fermentation was present in isolates TCN1, TCN3, TCN5, TCN6, TCN7, and TCN8, as indicated by a color change from red to yellow. All eight isolates showed negative results in both the lactose and inulin fermentation tests. The sugar test is used to determine the ability of bacteria to utilize carbohydrates. Some microrganisms are capable of both anaerobic and aerobic fermentation, while others cannot properly oxidize glucose (Cappuccino and Sherman 2014). Testing the ability of *Pseudonocardia* isolates to utilize sugars, specifically glucose, yielded positive results. For instance, *Pseudonocardia alni* DSM 44104T and *Pseudonocardia serianimatus* showed positive results with sucrose, while *Pseudonocardia serianimatus*, *Pseudonocardia antitumoralis*, and *Pseudonocardia alni* DSM 44104T exhibited positive results with mannitol. Negative results were obtained in the lactose test for *Pseudonocardia autotrophica* DSM 535T, while negative results for inulin were observed for *Pseudonocardia alni* DSM 44104T.

Detection of the inhibitory activity of actinomycetes belonging to *Pseudonocardia* spp. against *Aeromonas* sp. NrBF9 showed that one isolate labeled TCN5 was capable of inhibition (Table 2). The samples were observed for 168 h to assess the isolates' capability to form a clear zone. The TCN5 also producing diffusible pigments (Table 1). Other studies have shown that various genera of actinomycetes, such as *Streptomyces*, *Nocardia*, and *Pseudonocardia*, can inhibit the growth of several bacteria, including *Bacillus*, *Aeromonas*, *Escherichia coli*, *Klebsiella*, and *Vibrio* (Rosmine and Varghese 2016; Nofiani et al. 2022; Putra et al. 2024). One isolates formed a clear zone (TCN5), which is thought to be the result of an antibiotic mechanism. Diffusible pigment production is a typical characteristic of actinomycetes that is related to the formation of antibiotics (Abidin et al. 2018). Similar research results were also reported by Putra et al. (2024) for *Streptomyces* sp. NrASA6, which can produce a diffusible pigment and inhibit *Aeromonas* sp. NrBF9, *E. coli*, and *Staphylococcus aureus* through an antibiotic mechanism.

The TCN1, TCN2, TCN3, TCN4, TCN6, TCN7, and TCN8 isolates did not form clear zones (Table 2). Their activity involved competition for space with *Aeromonas* sp. NrBF9 from 48 to 168 h. The TCN4, TCN6, and TCN8 formed diffusible pigments, but they were not antibiotics. These diffusible pigments are believed to have competitive properties. According to Card et al. (2016), competition refers to the ability of microorganisms to acquire resources and defend themselves against antagonistic microorganisms to control space and nutrients for self-preservation.

The bacterial isolates' behavior in antibacterial tests is influenced by the type of bacteria used. *Aeromonas* sp. NrBF9 is a Gram-negative bacterium. Gram-negative bacteria are known to have a more complex structure than Gram-positive bacteria, with the main difference being in the outer membrane, which is situated above the peptidoglycan. One mechanism of antibacterial activity against Gram-negative or Gram-positive bacteria is the destruction of the bacterial cell wall. Antibacterial compounds that penetrate through cross-linking mimic the peptide chain bound to the penicillin-binding protein. This process disrupts peptidoglycan synthesis and causes bacterial lysis (Kapoor 2017).

Based on the conducted research, the eight obtained isolates were suspected to belong to the genus *Pseudonocardia*. Assessing the potential of the isolates as antibacterials revealed that isolate TCN5, could inhibit *Aeromonas* sp. NrBF9 and exhibited antibiotic properties, while the isolates TCN1, TCN2, TCN3, TCN4, TCN6, TCN7, and TCN8 exhibited competitive properties. Research on further identification of these actinomycetes should continue, focusing on determining the optimal growth conditions for isolates, using molecular methods for species-level identification, and assessing the antibacterial potential of isolates exhibiting antibiosis. Many *Pseudonocardia* synthesize natural, bioactive compounds (Miyamoto et al. 2014; Won et al. 2017; Geddis et al. 2018; Subramani and Sipkema 2019), mediate the biodegradation of large amounts of industrially discharged environmental pollutants (Inoue et al. 2016; Yamamoto et al. 2018), and demonstrate great potential for application in human medicine, animal health, and crop protection (Riahi et al. 2021).

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