BIODIVERSITAS
Volume 23, Number 6, June 2022
Pages: 2957-2965

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230622

Antimicrobial activity of actinomycetes isolated from mangrove soil in Tuban, Indonesia

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Manuscript received: 30 November 2021. Revision accepted: 25 May 2022.

Abstract. *Fatimah, Suroiyah F, Solikha N, Rahayuningtyas ND, Surtiningsih T, Nurhariyati T, Ni'matuzahroh, Affandi M, Geraldi A, Thontowi A. 2022. Antimicrobial activity of actinomycetes isolated from mangrove soil in Tuban, Indonesia. Biodiversitas 23: 2957-2965.* This study aims to isolate, identify, and investigate the antimicrobial activity of actinomycetes isolated from mangrove soil of the Mangrove Center Tuban in Jenu Village, Tuban District, Indonesia. Soil sampling was carried out with a purposive sampling technique. The dry-heating and pour plate methods were used to isolate actinomycetes. Four isolates (LIB.2; LIA.4; LIA.9; and LIA.10) were selected and tested for their antimicrobial activities against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* ATCC 10231 using disc diffusion method. Colony morphology characterization and Gram staining were then conducted on the 4 isolates. The identification of LIB.2 was performed by biochemical assay and 16S rRNA gene sequence analysis. The results showed that crude antimicrobial extracts (CACs) of all isolates inhibited the growth of *E. coli* but not *S. aureus* and *C. albicans*. Isolate LIB.2 showed the highest inhibition zone with a diameter of 15,65 ± 2,33 mm, followed by LIA.9 (10,19 ± 2,78 mm), LIA.4 (9,80 ± 0,47), and LIA.10 (8,75 ± 0,42 mm). The most potential isolate was identified as a member of *Streptomyces* genera. Based on biochemical assay and 16S rRNA gene sequence analysis, LIB.2 was identified as *Streptomyces vellosus* strain LIB.2.

Keywords: Actinomycetes, antimicrobial compounds, human welfare, Mangrove Center Tuban, microbial diversity, *Streptomyces vellosus* strain LIB.2

INTRODUCTION

Mangroves, located in intertidal regions along tropical and subtropical coasts, are one of the world's most productive environments (Tan et al. 2018). According to Aida et al. (2016), mangrove ecosystems have high productivity in marine and coastal areas, one of which is supporting fishery resources. In addition, mangrove forests are places that can provide various ecosystem services for all organisms (Katili et al. 2017). The ecosystems are characterized by sandy or muddy soil with high salinity, relatively high tidal range, high average temperature, and strong wind (Ancheeva et al. 2019). Mangrove soils provide unique habitats for the growth of various microorganisms (Palla et al. 2018). Bacteria and fungi are the most dominant microorganism in mangroves which help maintain the productivity of the ecosystems by involving them in major nutrient cycles (Palit et al. 2022). Those microorganisms also have the potential to produce bioactive metabolites, which can be harnessed for industrial and medicinal purposes. Fungi isolated from mangrove soils were reported to produce industrial enzymes such as lipase, cellulase, protease, and pectinase and therapeutic compounds such as antimicrobials, antivirals, antioxidants, and anticancer (Gao et al. 2021; Sopalun et al. 2021). Meanwhile, bacterial strains isolated from mangrove soils produced antimicrobials, anti-inflammatory, and antioxidant compounds (Qureshi et al. 2020; Rajan et al. 2021).

One of the main bioactive compounds-producing bacterial groups in mangrove soils is actinomycetes. Actinomycetes are filamentous Gram-positive bacteria with a morphology resembling fungi with a large genome of more than 8 Mbp with high G + C content and biosynthetic gene clusters related to the biosynthesis of secondary metabolites (Law et al. 2019; Davies-Bolorunduro et al. 2021). Actinomycetes isolated from mangrove soils and sediments from various locations have the potential as antibiotics, anticancer, and antioxidant producers. A previous study by Indupalli et al. (2018) reported that *Saccharomonospora oceani* VJDS-3 isolated from mangrove ecosystems in Andhra Pradesh, India, produced antimicrobial methoxy ethyl cinnamate and antioxidant 4-methyl benzoic acid. *Streptomyces anandii* H41-59 isolated from mangroves in the coastal area of the South China Sea produced anticancer compounds, anandin A (Zhang et al. 2017).

In general, actinomycetes are known to be the main producers of antibiotics. More than 80% of currently in-use antibiotics, such as tetracycline, macrolide,

chloramphenicol, nucleosides, and polyenes, are produced by actinomycetes (Ibnouf 2021; Raja and Prabakarana 2011; Shrestha et al. 2021). Members of genus streptomyces, in particular, were reported as the main antibiotic producers among them (Berdy et al. 2012). Antibiotics are molecular compounds used to treat and prevent diseases from bacterial infections (De Simeis et al. 2021). They are the cornerstone of modern medicine used in healthcare. However, the efficacy of antibiotics is uncertain due to the emerging cases of antibiotic resistance (Raval and Sahay 2021). Thus, exploring novel antibiotics-producing microorganisms, mainly actinomycetes, is critical to combat antibiotic resistance. Among the currently reported efforts is the isolation of echinomycin-producing *Streptomyces* sp. B475 from Maowei Sea Mangrove Reserve, China, and actinomycin X2 and actinomycin D-producing *Streptomyces smyrnaeus* UKAQ_23 from mangrove sediment in Jubail, Saudi Arabia (Lu et al. 2019; Qureshi et al. 2021).

The actinomycetes biodiversity in mangrove ecosystems, in particular in Indonesia, is still underexplored (Li et al. 2019; Hamed et al. 2021). It is unfortunate because, with a total area of 4.25 million ha, mangrove forests in Indonesia are the largest globally, representing 20% of the world's mangroves (Poedjirahajoe et al. 2019). One of the mangrove ecosystems in Indonesia is Mangrove Center Tuban, located in the coastal area of Jenu Village, Tuban, Indonesia. However, there is no report on antibiotics-producing actinomycetes isolated from the Mangrove Center. Therefore, we performed the first bioprospecting of antimicrobial compounds-producing actinomycetes from the Mangrove Center Tuban. In this study, we conducted soil sampling for actinomycetes isolation from the farthest area (the location I) and the nearest (Estuary area, location II) from the sea. The actinomycetes isolated and their antimicrobial activities were expected to vary due to the different physiochemical conditions of the sampling sites (Sengupta et al. 2015).

MATERIALS AND METHODS

Procedures

Collection of soil sample

Soil samples were taken from the mangrove soil in Mangrove Center Tuban in Jenu Village, Tuban District, East Java, Indonesia (Figure 1), using the purposive sampling method at 2 different locations, namely location I by taking 10 sample points and location II by taking 8 sample points. At each sampling site, 100 g of soil was taken. Samples were taken using a soil bore at 0-15 cm depth (Wahab et al. 2015) and then put in sterile plastic bags. The collected sample was analyzed for physicochemical parameters, including humidity (70-82%), temperature (27-28°C), salinity (0-15%), and soil pH (4-6.4) (Zhang et al. 2021).

Pre-treatment of soil samples

Soil samples were dried and heated to remove unwanted Gram-negative bacteria for 1 week. Samples that have been air-dried at room temperature, then put in an aluminum cup and then heated in an oven at a temperature of 65°C for 30 minutes (Sweetline et al. 2012; Daquioag et al. 2021).

Actinomycetes isolation

The soil samples were suspended in sterile distilled water (1:9 w/v) and inoculated into Starch Casein Agar (SCA) media supplemented with 0.1% chloramphenicol and 0.1% griseofulvin using the pour plate method (Krismawati et al. 2015). The samples were then incubated for 1-4 weeks at 27°C (Sweetline et al. 2012).

Antimicrobial activity assay

Four actinomycetes isolates were selected for antimicrobial activity assay. The isolates were grown in Nutrient Broth (NB media) for 1 week at 32ºC. Supernatants containing Crude antimicrobial compounds (CACs) were harvested by centrifugation at 6000 rpm for 10 minutes. An antimicrobial assay was conducted using the disc diffusion method against *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923.

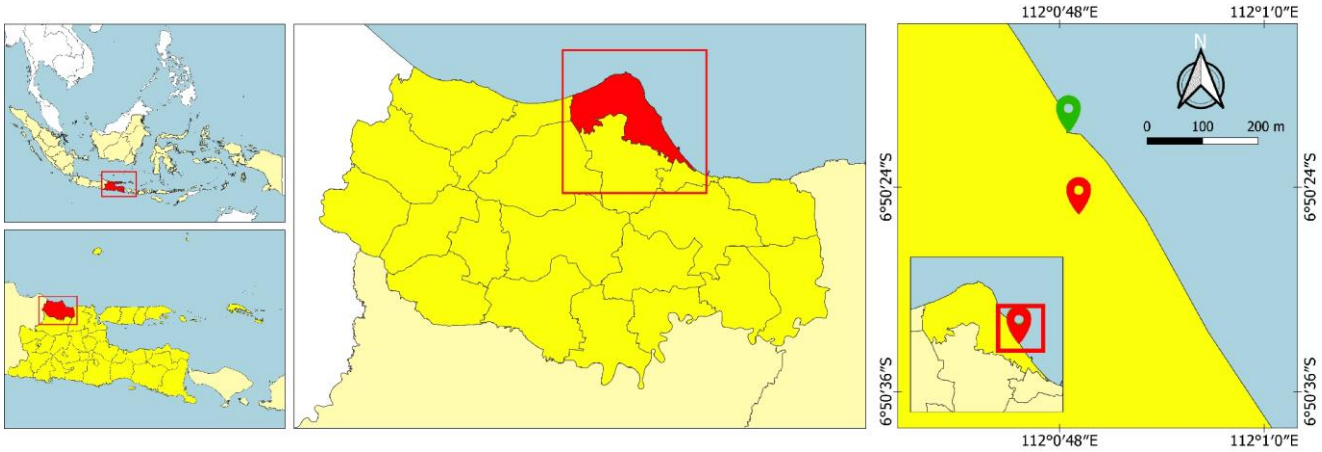

Figure 1. Map of sampling locations; the yellow pin indicates location I (LI), and the red pin indicates location II (LII)

In addition, the antifungal assay was performed against *Candida albicans* ATCC 10231 that grew in a Potato Dextrose Broth (PDB) media. One ml of each test microorganism (OD_{600nm} = 0.1) was mixed with 15 mL Mueller Hinton Agar (MHA) medium in a sterile petri dish. Twenty µl of the supernatant from each actinomycete isolate were loaded on the paper disk and put onto the solidified inoculated MHA. The Petri dishes were then incubated for 48 hours at 30-35°C. The inhibition zone diameter was measured using a caliper. This assay was carried out in triplicate (Wahab et al. 2015).

Characterization and identification

Colony characterization and Gram staining were conducted on the 4 selected actinomycetes isolates (Sweetline et al. 2012). In addition, biochemical identification using Microbact identification kit GNB 12A/B, 24E (Oxoid). Finally, molecular identification was conducted on the actinomycete with the highest antimicrobial activity.

For molecular identification, the genomic DNA of the selected isolate was extracted using Wizard Genomic DNA Purification Kit (Promega). First, amplification of the 16S rRNA gene was conducted by Polymerase Chain Reaction (PCR) using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') (Fatimah et al. 2016). PCR product was then visualized using UV Transilluminator. The PCR product was then sent to 1st Base (Malaysia) for purification and Sanger sequencing.

16S rRNA gene sequence analysis

The 16S rRNA gene sequences were trimmed to remove low-quality DNA sections (<15%), and gaps were removed using the Bioedit software ver 7.2.5. The sequence consensus obtained was then compared with the sequences from the GenBank database accessed through the National Center of Biotechnology Information (NCBI) BLAST software (Li et al. 2017). A phylogenetic tree was then constructed using the Neighbor-Joining method, with Bootstrap analysis 1,000 times in MEGA software ver. 11 (Tamura et al. 2021).

RESULTS AND DISCUSSION

Sampling sites

The Mangrove Center Tuban is an artificial conservative area. Vegetation is dominated by *Rhizophora* sp. Soil samples used in this study were collected from location I (105 m from the sea) and location II (40 m from the sea) (Figure 1) in Mangrove Center Tuban.

The sampled soil characteristics are presented in Table 1. Locations I and II were divided into locations I-A and II-A with a pH range of 5-6 and locations I-B and II-B with a pH of 4-4,8, which is relatively acidic.

Isolation of actinomycetes from mangrove soil

Four isolates of actinomycetes were obtained from the sampling site of location I, and none of the isolates were obtained from location II (Table 2.). The LIB.2 isolate indicated a unique finding that actinomycetes could grow on acidic soil. The four isolates have various macroscopic and microscopic characteristics (Table 3). The colony morphology of the isolates is shown in Figure 2, and the results of Gram-staining are shown in Figure 3. Four actinomycetes isolates have different colony morphological characters (Table 3). Generally, macroscopic characteristics of actinomycetes colonies are small, dry, wrinkly, fibroma, or velvety on the surface. In this study, the LIA.4 isolate was dry, and the LIA.9 isolate had a fibrotic surface; the LIA.10 isolate was a small wrinkle, and the LIB.2 isolate was velvety. Moreover, the aerial mycelium of LIB.2 was grey, and LIA.4 was white, while LIA.9 was white with filaments, and LIA.10 was yellow with scraper edges.

Screening of antimicrobial activity

The actinomycetes isolated from the mangrove soil from Jenu, Tuban, can inhibit the growth of *E. coli* ATCC 25922 but could not inhibit the growth of *S. aureus* ATCC 25923 and *C. albicans* ATCC 10231 (Table 4). It might de due to the specific ability of the isolates to inhibit the growth of Gram-negative bacteria, namely *E. coli*. The LIB.2 isolate has the largest diameter of inhibition against *E. coli* ATCC 25922 (15.65 ± 2.33 mm), while LIA.10 isolate has the lowest inhibitory zone (8.75 ± 0.42 mm).

The initial screening test results showed that LIB. 2 has the highest inhibitory zone, as indicated by the size of the clear zone. The inhibitory zone of LIB. 2 was compared to the negative control (NB) (Figure 5) and positive control (chloramphenicol, tetracycline, and amoxicillin) (Figure 6).

Table 2. Actinomycetes isolated from mangrove soil in Jenu, Tuban, Indonesia

Location	Code of isolates	Amount
LI-A	LIA.4 LIA.9 LIA.10	3
LI-B	LIB.2	1
LII-A	-	0
LII-B	-	0
Total		4

Table 1. Characteristics of the soil sample

Location	Coordinates	Soil physicochemical parameters			
		pH	Temp. (°C)	Salinity (%)	Moisture (%)
LI-A	S 06°50.413'-S 06°50.438'; S 112°00.804'-E 112°00.828'	6.3 ± 0.1	27.6 ± 0.5	9.1 ± 5.1	7.4 ± 0.5
LI-B	S 06°50.427'; E 112°00.819'	4.0 ± 0.0	28.0 ± 0.0	15.0 ± 0.0	8.0 ± 0.0
LII-A	S 06°50.344'-S 06°50.352'; E 112°00.801'-E 112°00.820'	5.9 ± 0.5	28.7 ± 0.5	0.0 ± 0.0	8.0 ± 0.0
LII-B	S 06°50.347'; E 112°00.809'	4.8 ± 0.0	29.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0

Table 3. Characteristics of Actinomycetes isolates from mangrove soil in Jenu Tuban, Indonesia

Code	Macroscopic			Microscopic		
	Color of aerial mycelium	The color of substrate mycelium	Change the color of medium	Gram	Spore	The shape of the spore chain
LIB.2	Gray	Gray	No	+ (purple)	Yes	*Streptomyces*/Spiral
LIA.4	White	Pink	Pink	+ (purple)	Yes	*Streptomyces*/Straight
LIA.9	White	Blackish Green	No	+ (purple)	Yes	*Nocardia*
LIA.10	Yellow	Yellow	Yellow	+ (purple)	Yes	*Nocardia*

The inhibitory zone of LIB.2 was not as large as the inhibitory zone produced by the positive control (Table 5).

Identification of the isolate with the highest antibacterial activity

LIB.2 isolate was the isolate with the highest inhibitory zone against *E. coli*; therefore, it was further investigated to determine its genera. The results of the biochemical assay of the LIB.2 isolate are presented in Table 6. The LIB.2 isolate is a Gram-positive rod bacteria with a gray aerial mycelium and spiral-shaped spore chain and produced catalase. Based on physiological characteristics, the LIB.2 isolate had a similarity of 92.8% with the genus *Streptomyces* (Bergey and Holt 2000).

DNA Sequencing

The 16S rDNA amplification of the LIB.2 showed that the sequence length was around 1500 bp (Figure 5). The BioEdit program showed a sequence of around 1401 bp. Based on BLAST analysis, LIB.2 showed the highest homology with *Streptomyces vellosus* strain HR 29 (KT438921.1) with a percentage identity value of 100%. Therefore, the LIB.2 strain was classified into the Genera of *Streptomyces* and 100% similar to the *S. vellosus* strain HR 29 (KT438921.1) (Figure 6).

Table 4. The inhibitory zone of Actinomycetes against *Escherichia coli, Staphylococcus aureus,* and *Candida albicans*

Code of isolate	Diameter of the inhibitory zone (mm)		
	E. coli ATCC 25922	*S. aureus* ATCC 25923	*C. albicans* ATCC 10231
LIB.2	15.65 ± 2.33	0	0
LIA.4	9.80 ± 0.46	0	0
LIA.9	10.19 ± 2,78	0	0
LIA.10	8.75 ± 0.42	0	0

Table 5. The inhibitory zone of LIB.2 and positive control against *Escherichia coli* ATCC 25922

Sample	The average diameter of the inhibitory zone (mm)
LIB.2	11.60 ± 1.98
Chloramphenicol 0,01%	17.44 ± 0.05
Tetracycline 0,01%	22.73 ± 2.30
Amoxicillin 0,01%	29.81 ± 0.69

Table 6. Physiological characteristics of LIB.2 isolate

Tests	Results
Gram	+
Color of aerial mycelium	Gray
The shape of the spore chain	Spiral
Catalase	+
Motility	-
Glucose	-
Xylose	-
Arabinose	-
Rhamnose	-
Raffinose	-
Mannitol	-
Inositol	-
Salicin	-
Sucrose	-

Note: +: present; -: absent

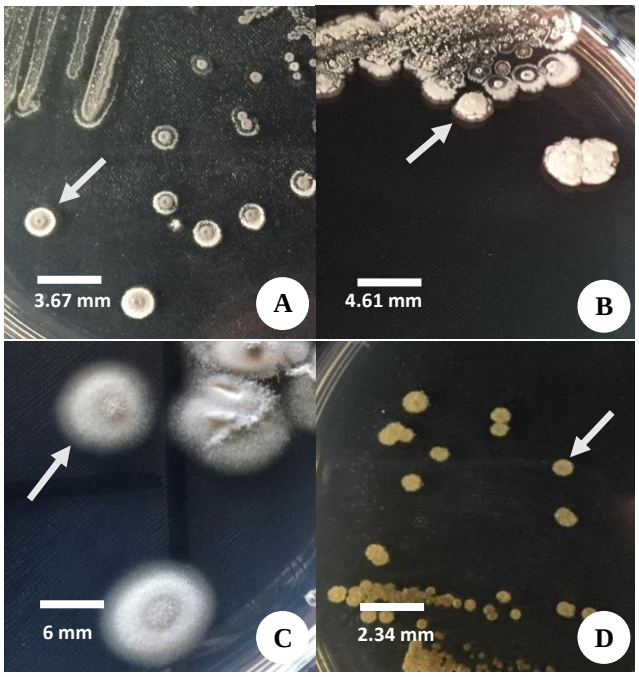

Figure 2. Macroscopic characteristics of actinomycetes isolated from location I. The white arrows indicate the single colonies of (A) LIB.2, (B) LIA.4, (C) LIA.9, and (D) LIA.10. The diameter of each isolate is shown in the bottom right corner of each unit (cm)

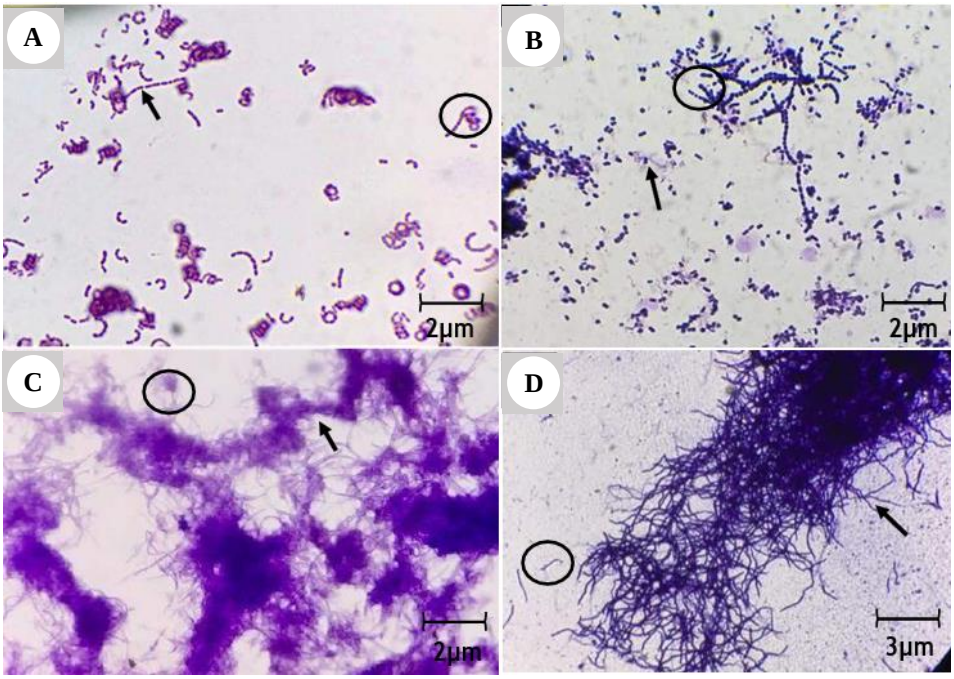

Figure 3. Microscopic characteristics of actinomycetes isolated from mangrove soil obtained in Jenu, Tuban by Gram staining. The arrow shows the spore chain, and the circle indicates the aerial hyphae. (A) LIB.2, (B) LIA.4, (C) LIA.9, and (D) LIA.10

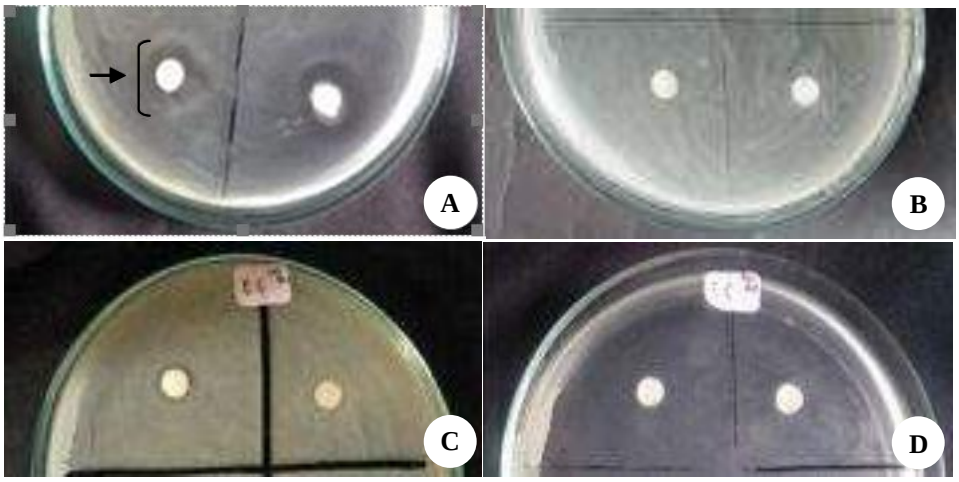

Figure 4. The inhibitory zone (➶) of actinomycetes against *E. coli* ATCC 25922 *by* disk diffusion method. The code of (A) LIB.2, (B) LIA.4, (C) LIA.9, and (D) LIA.10

Discussion

The physical and chemical components of soil greatly affect the diversity of actinomycetes (Raval and Nirmal 2021). Actinomycetes have been empirically proven to be a source of bioactive substances, i.e., antibacterial, antifungal, antiviral, and antiparasitic activity (Raval and Nirmal 2021). Several actinomycetes that produce antimicrobial compounds are aerobic chemoorganotrophic, have oxidative metabolites, and use various carbon sources as energy for their growth (Bertrand et al. 2015). The optimum temperature for the growth of actinomycetes ranges from 28-32°C. Tiwari et al. (2021) reported that the optimal mesophilic growth of actinomycetes was in the range of 28-32ºC. Actinomycetes can grow in the pH range of 4-5 and grow optimally in 6.5-8.0 (Prasad et al. 2015). The actinomycetes in this study were isolated from acidic soil with a pH of 4. Four actinomycetes were successfully isolated from location I, which had a pH of 4-6 with a salinity of 2%-15%. However, there were no actinomycetes at location II, which had a salinity of 0% with the same pH. It indicated that pH is not the only factor affecting actinomycetes' growth. The density of actinomycetes was affected by environmental factors, including temperature, pH, salinity, dissolved oxygen, dissolved nitrate, dissolved nitrite, dissolved phosphate, and dissolved ammonia. Environmental conditions can also limit actinomycetes diversity in each location (Prasad et al. 2015).

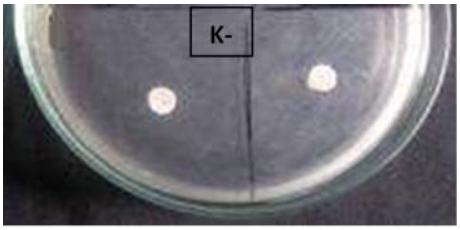

Figure 5. Negative control (NB)

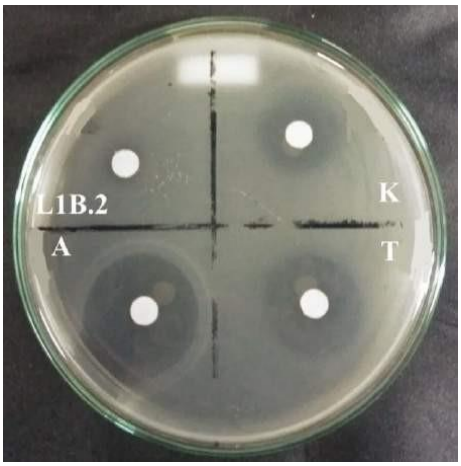

Figure 6. Inhibitory zone LIB.2 and positive control, A: Amoxicillin, K: Chloramphenicol, T: Tetracycline against *E. coli* ATCC 25922

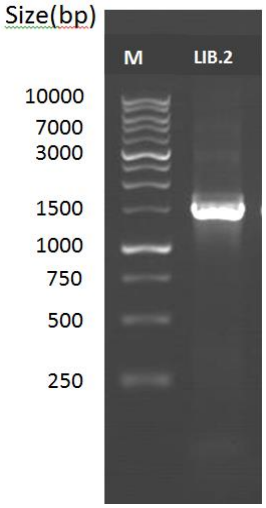

Figure 7. Electrophoregram of 16S rRNA gene in 0.8 % agarose gel. M = 1kb DNA Ladder (bp); LIB.2 = 16S rRNA gene of the LIB.2 isolate (1500 bp)

The physical and chemical properties of the soil affect the presence and distribution of the habitat of actinomycetes (Selim et al. 2021). Actinomycetes are mostly found in coastal soil and wet areas. It is due to the specific environmental conditions that support the growth and reproduction of actinomycetes. Marine actinomyces have an important role in biological applications such as antimicrobial, anticancer, antifungal, and enzyme producers (Selim et al. 2021). However, several environmental conditions, such as high salinity, acidic conditions, and very high temperature, are not suitable for the growth of actinomycetes. Nevertheless, some actinomycetes are found in marine soils, which may have the right conditions and nutrients for their growth. Actinomycetes produce optimal antibiotics under suitable environmental (Shrestha et al. 2021; Raval and Nirmal 2021).

However, in this study, the salinities from both locations were not too high, but not many isolates could be obtained. The location I obtained four isolates (LIB.2, LIA.4, LIA.9, and LIA.10), while none of the isolates of actinomycetes were obtained in location II. The soil at location II had an unpleasant smell and a light brown color that may be unsuitable for actinomycetes.

A previous study by Hamed et al. (2021) reported that 10 isolates of actinomycetes strains were found in mangrove sediments in Egypt's red sea. Thirty actinomycetes have also been isolated from Antarctica (Silva et al. 2020). In comparison, Yanti et al. (2019) have isolated actinomycetes from the pelleted feces and digestive tract of Nipah worms. It proves that actinomycetes can be isolated from various environments as long as the environment meets the nutritional needs required by actinomycetes (Yanti et al. 2019).

The actinomycetes isolated from the mangrove soil of Jenu, Tuban belonging to *Streptomyces* and *Nocardia* genera (Table 3) were only able to inhibit the growth of *E. coli*, but not *S. aureus* and *C. albicans.* However, previous studies reported that actinomycetes could inhibit the growth of *Bacillus*, *Staphylococcus, E. coli, Klebsiella*, and *Pseudomonas* (Ozer et al. 2010; Malathi et al. 2021). Streptomyces is one of the genera of Actinomycetes that produces the most antibiotics. Antibiotics produced by *Streptomyces* are generally streptomycin produced by *S. griceus*, which can inhibit most Gram-negative bacteria. The genus Streptomyces spp. produces spectinomycin that inhibits the growth of *Mycobacterium tuberculosis*. Neomycin produced by S. *fradiae* produces broad-spectrum antibiotics. Tetracycline produced by *S. aurefaciens* inhibits Gram-positive and Gram-negative bacteria with a broad spectrum, such as Rickettsias. Erythromycin produced by *S. erythreus* inhibits Gram-negative bacteria. Chloramphenicol produced by *S. venezuelae* can have a narrow spectrum of antibiotics, and Streptomyces produces many more antibiotics. More than 60 types of antibiotics are produced from the genus Streptomyces (Nurkanto et al. 2008).

Figure 8. The phylogenetic tree of the LIB.2 isolate. Created using the MUSCLE alignment and Neighbor-Joining Method (Saitou and Nei 1987; Nei and Kumar 2000). *Halopolyspora alba* AFM 10251 (KF022042.1) was used as an outgroup strain. The phylogenetic tree was constructed using MEGA 11 (Tamura et al. 2021) and a scale of 0.01

The isolate which produced crude antimicrobial compounds (CACs) with the highest inhibition against *E. coli* was LIB.2. Molecular identification using the 16S rRNA gene revealed that strain LIB.2 showed the highest homology with *Streptomyces vellosus* strain HR 29 (KT438921.1) with a similarity value of 100%. *Streptomyces vellosus* strain HR 29 (KT438921.1) was reported to produce antibiotic lincomycin and antitumor and antioxidant compounds (Kim et al. 2008; Lee et al. 2015; Yang et al. 2021). However, lincomycin is known to be only active against Gram-positive bacteria. In contrast, CACs of *S. vellosus* LIB.2 in this study did not show any inhibition towards *S. aureus*. Therefore, it is suspected that *S. vellosus* strain LIB.2 produces antimicrobial compounds such as EA-371d and EA-371a produced by *S. vellosus* and is active against Gram-negative bacteria (Dey et al. 2022). Overall, this study found that the actinomycetes isolated from the mangrove soil in Jenu, Tuban, have antibacterial activity against *E. coli*. Therefore, the *Streptomyces vellosus* strain LIB.2 might be developed as an antibacterial agent. However, further research is needed to develop an optimization strategy to enhance the production of antibacterial substances from this strain.

ACKNOWLEDGEMENTS

The authors would like to thank Universitas Airlangga, Surabaya, Indonesia for funding the research through the *Penelitian Unggulan Fakultas* (PUF) 2021 scheme with contract number 2727/UN3.1.8 /PT/2021.

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