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ANTIBACTERIAL CAPACITY OF *Streptomyces* ISOLATE FROM A MANGROVE PLANT RHIZOSPHERE *Avicennia marina*

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ABSTRACT

This research was conducted to obtain *Streptomyces* isolates from *Avicennia marina* rhizosphere capable of inhibiting *E. coli* and *S. aureus* growth, to investigate the capability and the characteristics of its antibacterial compound. This study completed the isolation by applying pour plate method on SCN agar medium. Antagonistic screening and selection processes were carried out by diffusion and dilution methods. Observation on the characteristic of the antibacterial compound applied was TLC method and MIC assay. This research confirmed the antibacterial compound capability by applying bioautography assay. Parameters measured consisted of inhibition zone diameter, Rf value on a bioautography plate, and the lowest concentration capable of inhibiting bacterial growth. Out of 16 isolates of *Streptomyces* obtained, *Streptomyces* 404 showed higher antagonistic activity than others. Inhibition zone diameter reached 20–25 mm in *E. coli* and *S. aureus* growth, respectively. TLC assay showed three spots in which two of them confirmed antibacterial activity in the bioautography assay that yielded Rf values of 0.47 for *E. coli* and 0.72 for *S. aureus*, while MIC assay showed that the lowest extract concentration inhibited bacterial growth was 20%.

KEY WORDS: bioautography assay, *Streptomyces*, antibacterial activity, Rf value, MIC

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INTRODUCTION

Actinomycetes are soil-borne microbes capable of producing antibacterial compounds. They are Gram-positive, filamentous, and saprophytic bacteria (Oskay *et al.*, 2004). An example of these microbes is *Streptomyces* (Thakur, 2011). Antibacterial compounds produced by *Streptomyces* are streptomycin, aureomycin, chloromycetin, tramycin, erythromycin, and magnamycin. These compounds have varied potentials and specification (Holt *et al.*, 2000).

Explorations of *Streptomyces* have been widely done, due to the high demand for antibacterial compounds, and triggered by the emergence of resistance problems of pathogenic microbes toward antibiotics. The exploration has been done in various environments, even in a unique habitat.

Mangrove forest is a possible environment for the source of *Streptomyces*. It has rich organic substances that very likely to be the source of antibacterial producing actinomycetes (Alanis, 2005). Mangrove forest of Segara Anakan, Cilacap, is an intertidal zone influenced by the sea tidal waves. According to Suryono (2006), various mangrove species grew according to zonation, the distance from where they grew to the shore.

Avicennia marina was a species that grow in the closest distance to sea waters with higher environment salinity compared to other mangrove vegetation (Sukmarani *et al.*, 2008). The explorations of *Streptomyces* in mangrove area is crucial because this bacteria has a superior ability to survive the intertidal zone, thus considered to have some specific potential related to primary and secondary metabolite productions (Sathya and Ushadevi, 2009).

The assay of antibacterial activity could be done with diffusion and dilution method towards Gram-negative bacteria, *Escherichia coli*, and Gram-positive bacteria, *Staphylococcus aureus*. The species have different cell wall structure located on the peptidoglycan layer. Thus, different responses toward antibacterial compounds were expected. The mechanisms of microorganism growth inhibition included cell wall synthesis inhibition, a distraction of cell membrane permeability, and blockage of replication, transcription, and translation of bacterial genetic materials (Oskay, 2004). The advance assay of antibacterial compounds is bioautographic test towards chromatography results, to test the antibacterial ability of antibacterial compounds (Oedjijono *et al.*, 1993; Patil *et al.*,2013).

Based on this review, the objectives of this research were to obtain antibacterial producing *Streptomyces* isolate from the *Avicennia marina* rhizosphere, and to examine the antibacterial ability and antibacterial compounds characters produced.

METHOD

The samples were *A. marina* rhizosphere soils of site E46 and E40 Segara Anakan, Cilacap. *Streptomyces* was isolated with pour plate method on solid medium, Starch Casein Agar (SCN), and incubated at room temperature for 5–7 days. The isolates were then characterized morphologically and biochemically according to Holt *et al.* (2000).

Screening and selection of antibacterial producing isolates were done by diffusion method in Nutrient Agar (NA) medium. The isolates were incubated in liquid SCN medium. After eight days, the cultures were filtered through Whatman paper. The filtrate of 15 µl volume was dropped on a paper disc 6 mm, and placed on spread cultures of *E. coli* and *S. aureus*. The cultures were incubated for 24–48 hours at a temperature of 37°C. Parameters that evaluated were Φ of inhibition zone. *Streptomyces* producing the widest inhibition was selected for the next assay.

The antibacterial substance was produced on liquid SCN mediums with 7, 14, 21, and 28 days incubation at room temperature. The evaluated parameters were inhibition zone diameter of rough extract, mycelium biomass (X), filtrates pH, Rf and MIC values. Filtrates were extracted with ethyl acetate and methanol solution (4:1). The extracts were diluted in various concentrations (1%, 2.5%, 5%, 10%, 20%, 40%, 80%, 100%), and then the antibacterial ability was tested with diffusion method. After 24 hours incubation at 37°C, inhibition zone diameters were measured. MIC assay was done by mixing 0.8 mL NB medium, 0.1 mL *S. aureus* and *E. coli* cultures, and 0.1 mL solution of antibacterial compound extracts in various concentrations. These cultures were incubated for 24–48 hours at 37°C. The observation was focused on the turbidity of the cultures. MIC value was the lowest concentration to inhibit bacterial growth which was indicated by the clear culture broth.

The characters of antibacterial compounds were observed with TLC using eluant acetic acid, butanol, and distilled water (3:1:1). Antibacterial compounds were placed (15 µL), sprayed with ninhydrin 1% diluted in NaOH 1% solvent, to obtain the compound spots. TLC plate containing spots was used for the bioautography test. It was placed in a sterile petri dish, added with agar medium, and 100 µL bacterial liquid cultures with spread method, then incubated for 24 hours at 37°C. The spots containing antibacterial compounds did not show growth of the tested bacteria, measured by the Rf value as a character of antibacterial compounds.

RESULTS AND DISCUSSION

There was 16 *Streptomyces* isolated *A. marina* rhizosphere, 12 from site E40 and four from site E46. They have diverse morphological and biochemical characters. The differences between isolates were the presence of aerial mycelium, substrate mycelium (color), and melanoid pigment. According to Holt *et al.* (2000), the isolates produced a hard colony, powdery surface, wrinkle and lichenoid mycelium, with 2–3 mm of colony diameter. Aerial mycelium had straight and bent or spiral shape to support spores arrangement. Its colors were red, purple, brown, white, yellow and gray. The colony secreted black exudates and fused pigment with black color. Thakur *et al.* (2011) reported that almost all *Streptomyces* isolates found in mangrove rhizosphere had substrate mycelium and aerial mycelium. Substrate mycelium served to strengthen bacteria to survive and absorb nutrition, and aerial mycelium functioned for sporangium reproduction. Amal *et al.* (2011) stated that there was the various color of melanoid pigment and that mycelium produced by *Streptomyces* colony were blue, purple, red, yellow, green, brown, and black. The pigment which was undiffused in the medium appeared in its substrate and aerial mycelium.

The results of the biochemical test showed that almost all isolates were able to use sugar, as tested in the sole carbon source. IMViC test responded positively to all isolates, except for VP test. Holt *et al.*, (2000) described 150 isolates of *Streptomyces*; and showed several key characters as references for genus and species determination. The characters included mycelium shape, positive response to casein (as N source) test, carbon source tests (monosaccharides, disaccharides, and polysaccharides), reduce nitrates test, positive catalase test, and gelatin suspension survival.

Culture filtrate of *Streptomyces* isolates E404 had the greatest inhibitory activity towards *E. coli* and *S. aureus* with an inhibition zone of 12.5–13.0 mm in diameter. This isolate also had the highest growth rate (X = 119.2 mg) (Table 1). The biomass production corresponded to enzyme and secondary metabolite productions in which isolate with the greatest inhibitory value produced the most biomass (Waites *et al.*,2001).

Table 1. The average diameter of inhibition zone (IZ) of bacteria by culture filtrates and *Streptomyces* isolates biomass (X)

No	Isolate Code	ΦIZ (mm)		X (mg)
		S.a	*E. c*	
1	E461	10.0	12.0	101.5
2	E401	9.0	7.0	30.9
3	E402	7.0	8.0	91.9
4	E403	9.0	8.0	83.3
5	E462	8.0	9.0	63.7
6	E404	13.0	12.5	119.2
7	E405	8.0	7.0	63.6
8	E463	9.0	8.0	106.3
9	E406	12.5	7.0	102.3
10	E407	10.5	10.0	84.4
11	E408	9.0	8.0	102.1
12	E409	11.5	7.0	86.8
13	E4010	10.0	7.0	106.3
14	E4011	11.0	8.0	75.6
15	E4012	8.0	8.0	63.7
16	E464	1.0	8.0	103.5

Culture filtrates inhibited the pathogenic bacteria growth (Table 2). The longer the incubation period, the wider the diameter of the inhibition zone because of a larger amount of antibacterial compounds produced by isolate E404.

Table 2. The average diameter of inhibition zone of isolate E404 culture filtrate towards *E. coli* and *S. aureus* by different incubation periods.

No	Incubation Period (Day)	ZH (mm)		pH	X (mg)
		Sa	*Ec*		
1	0	0	0.0	7.19	4.5
2	7	9	8.5	6.80	40.4
3	14	17	20.0	6.73	25.5
4	21	20	18.0	6.90	13.6
5	28	25	23.0	8.23	12.0

The cultures were in the exponential phase during seven days of incubation, then idiophase, the phase of secondary metabolites production. Thus, during the incubation period, high X value but little ZH was obtained (40.4 mg). Secondary metabolites antibacterial compounds accumulated during 28 days incubation period. During this period, there was no biomass increase, but inhibition zone became wider than previously.

Inhibitory activity of the antibacterial rough extract compounds of isolate 404 towards *E. coli* and *S. aureus* was unstable (known as bacteriostatic). The high concentration of the extract showed low inhibitory effect. Cultures produced inhibition zone (clear zone) of > 16–20 mm were characterized as the moderate class (Nedialkova & Naidenova 2004). Polarity and saturation of antibiotic after dilution caused the diffusion inhibition in the medium (Fauziah, 2010). Preparations in obtaining rough extracts of antibiotic affected the ability of antibiotic compounds. Evaporation and heating steps might decrease the antibacterial compound ability which might not be the case in the screening step (Andrews, 2000).

Table 3. The results of antibacterial rough extract tests for *Streptomyces* 404 with dilution method

Extract Concentration	Inhibition			
	S. aureus		*E. coli*	
	24 hours	48 hours	24 hours	48 hours
1 %	-	-	-	-
2.5 %	-	-	-	-
5 %	-	-	-	-
10 %	+	-	-	-
20 %	+	+	+	-
40 %	+	+	+	+
80 %	+	+	+	+
100 %	+	+	+	+

The assay of antibacterial activity with dilution method showed that the highest concentration of 20% towards both *E. coli* and *S. aureus*. (Table 3). Some factors, such as antibiotic group, work mechanism, and pathogenic bacterial resistance influenced inhibitory activity.

The thin layer chromatography test separated compounds of the extract (Figure 1). There were three spots, indicating fractions with different distances to origin spot. In TLC, the solvent used, types of movement and static phase determined the test results (Lade *et al.*, 2014). Maatoui *et al.*, (2014) reported that *Streptomyces* could produce more than one active compounds that corresponded to the fractions formed in chromatography.

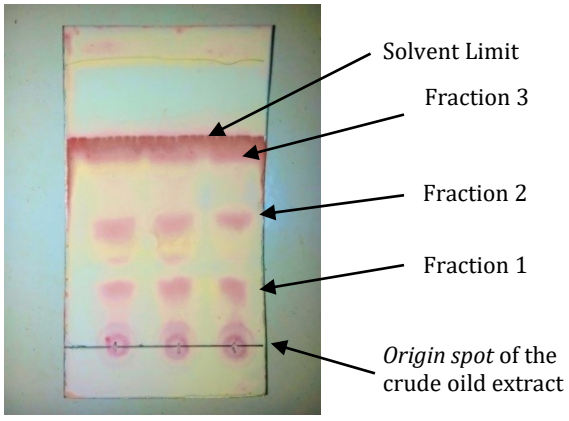

Figure 1. Separation of dissolved compounds with thin layer chromatography (TLC) method

Bioautography showed the compounds that inhibited *E. coli* and *S. aureus* the growth had different Rf value. This difference explained that *Streptomyces* isolate 404 produced two types of antibacterial compounds (Figure 2). Bioautography is a test for activity of antimicrobial compounds in situ along with TLC test. Compounds on the TLC plate diffused with agar containing microbes. Thus the inhibition effect was seen on the microbial growth (Oedjijono *et al.*, 1993; Patin *et al.*, 2013). Active compound with Rf value of 0.47 inhibited *E. coli*, while that with Rf value 0.72 inhibit *S. aureus*. *Streptomyces* could produce more than one active compound in every life phase (Kishore, 2011).

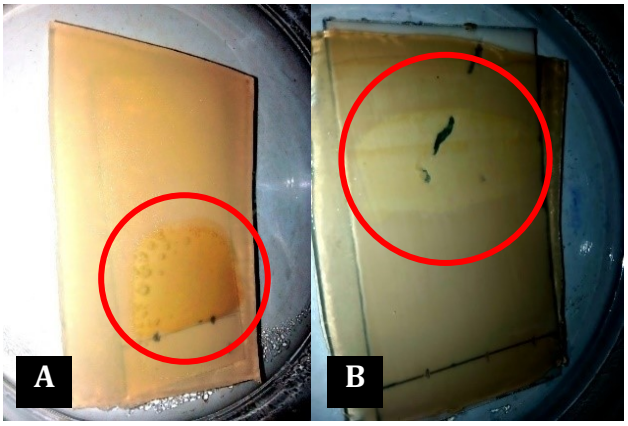

Figure 2. Bioautography results on (a) *E. coli,* (b) *S. aureus*

CONCLUSION AND SUGGESTION

There were 16 isolates of *Streptomyces* obtained from *A. marina* rhizosphere. The *Streptomyces* isolate E404 showed antibacterial activity towards *E. coli* and *S. aureus* indicated by the bioautography test. The Rf characterized *Streptomyces* secondary metabolites at the values of 0.47 and 0.72.

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