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Isolation of Actinomycetes from Sugarcane (*Saccharum Officinarum*) Rhizosphere and the Ability to Produce Antibiotic

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Abstract*—Actinomycetes are well-known microorganism as secondary metabolites producers which serve as antibiotics. In this study, sugar cane (*Saccharum officinarum*) rhizosphere soil samples were collected from Piyungan, Bantul, Yogyakarta. The aims of this study were to obtain Actinomycetes isolates from sugar cane (*Saccharum officinarum*) rhizosphere which potentially produce antibiotics and to analyse the Thin Layer Chromatography (TLC) profile of ethyl acetate extract of active Actinomycetes isolates. The method used in primary screening to evaluate Actinomycetes antibacterial activity from the sugar cane rhizosphere are agar block method and cup-plate method, and the extract activity assay used Kirby-Bauer method. Determination of antibiotic activity was observed by measuring the diameter of inhibition zone. Extract component was analyzed by Thin Layer Chromatography (TLC). The results of this research was found 39 isolates of Actinomycetes classified into 7 groups based on color grouping. The result of agar block test was revealed that among 39 isolates, four isolates (10.25%) were able to inhibit Staphylococcus aureus. They were Te.234, Te.235, Te.236, and Te.325 isolates. Inhibition zone of crude extracts from these isolates against Staphylococcus aureus were Te.234=7.67 mm, Te.235=3.75 mm, Te.236=12.42 mm, and Te.325=19.16 mm. However, none of these isolates were able to inhibit Escherichia coli. Results of TLC profile showed that the suspected compounds contained in ethyl acetate extract of culture broth are alkaloid, polypeptide, steroid, alcohol, phenol and ketone/aldehyde compounds. Based on the results, it can be concluded that Actinomycetes can be isolated from sugar cane rhizosphere which has potential as an antibiotic producer by narrow spectrum to inhibit Gram-positive bacteria such as Staphylococcus aureus.

Keywords—actinomycetes, isolation, rhizosphere, antibiotic, sugar cane, TLC profile

I. INTRODUCTION

Actinomycetes is a group of organisms widespread in nature, and play a significant role in the future of biotechnology, because of their importance as producers of vitamins, enzymes, antitumor agents, immune modifying agents and, mainly, antibiotic compounds [1]. Almost 80% of the world's antibiotics are produced by Actinomycetes, mostly from the genera Streptomyces and Micromonospora [2]. At least 5,000 documented bioactive compounds are known as being produced by Streptomycetes [3]. Most Streptomyces and Actinobacteria produce a diverse array of antibiotics, including aminoglycosides, macrolides, β-lactams, peptides, polyenes, polyethers, and tetracycline. These antibiotics have been applied in the medical areas, agriculture, and research.

Present time, high incidence of infectious disease in Indonesia, may cause increasing the use of antibiotics. Frequent use of antibiotic can trigger bacterial resistance. Because, any use of antibiotics can increase selective pressure of evolution process and thereby increasing the relative numbers of resistant bacteria and allowing for further growth [4]. This can cause infectious diseases to not be easily cured. Therefore, exploration of new antibiotics is an important thing to do to overcome this. The first step to finding a new antibiotic is to explore antibiotic-producing microbes. In this case, Actinomycetes has become the microbial of choice for isolation because it has become the largest source of antibiotics.

Actinomycetes are the most widely distributed groups of microorganisms in nature, especially in rhizosphere soils. Actinomycetes may constitute 40% of the total microbial bacterial population in grasses soils, i.e. from the family Poaceae. Besides rice, wheat, and corn, sugarcane is also a member of the family Poaceae [5]. To date, isolation of Actinomycetes from the rhizosphere of sugarcane has never been carried out. New isolation areas have the potential to produce new microbes and / or antibiotics. Hence, isolation of Actinomycetes from sugarcane rhizosphere is an important thing to do.

Antibiotics which are secondary metabolites are mostly semipolar to nonpolar compounds. This compound can be extracted from culture broth using ethyl acetate solvent [6]. Based on biosynthesis pathway, antibiotics from Actinomycetes can be classified into 2 large groups, i.e. polyketides derivate and polypeptides derivate. Therefore, the objective of the present study was to isolate Actinomycetes from the sugar cane (*Saccharum officinarum*) rhizosphere in Piyungan, Bantul, Yogyakarta, and to determine antibacterial activity of the ethyl acetate extracts of culture broth and determine their compounds.

II. MATERIALS AND METHOD

A. Materials

Sugar cane (*Saccharum officinarum*) rhizosphere, Starch Nitrat Agar (SNA) medium (soluble starch 20 g; sodium chloride 0.5 g; KNO3 1 g; K2HPO4.3H2O 0.5 g; MgSO4.7H2O 0.5 g; FeSO4.7H2O 0.01 g; agar 20 g; Aquades ad 1000 ml (pH 7.2-7.4)), Starch Nitrat Broth (SNB) medium, Mueller Hinton Agar (MHA) medium, Brain Heart Infusion (BHI) medium, NaCl 0.9%, aquadest, chloramphenicol, ethyl acetate, chloroform, methanol, Silica gel GF 254 and also Staphylococcus aureus ATCC 25923 as well as Escherichia coli ATCC 25922.

B. Methods

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1) Sample Collection and Processing

The rhizosphere soils (3 samples) were collected from sugar cane plantation in Piyungan, Bantul, Yogyakarta from a depth of 5 cm during the dry season in 2015. The soils were collected in sterile plastic bags which were serially named as Te.1, Te.2, Te.3 and then brought to the laboratory and dried overnight at 28°C to eliminate the bacterial growth and to reduce water contents. From each dried soil sample, 1 g was taken and mixed separately with 9 ml of sterile water, vortex in 5 minutes. The suspension was heated at 50°C in 10 minutes, then vortex in 5 minutes. The supernatant was serially diluted with sterile water into 10-2 – 10-4.

2) Selection and Isolation of Actinomycetes

A dilution series of 10-2–10-4 of rhizosphere sample are plated onto SNA supplemented with cycloheximide 75µg / mL. Plates were incubated at 28 ° C for up to 14 days [7]. The colonies showed typical morphology of Actinomycetes such as dry powder and fungus-like those selected and sub cultured on noncycloheximide SNA medium. After that, the plates were incubated at 28 ° C for up to 14 days.

3) Classification and Identification of Actinomycetes

Classification of Actinomycetes was conducted based on the differences of the colony characters, e.g. size, shape (both in elevation and margin), color or pigmentation, texture and consistency. Then, it was carried out isolates grouping based on their aerial mycelium (AM), vegetative mycelium (VM) colors and pigments diffused into the media [8].

4) Screening for Antibiotic Potential with Agar Blocks Method

Cylindrical pieces diameter 6 mm were cut out from purified isolates of Actinomycetes. The blocks were placed on Mueller Hinton media inoculated with the test-microorganisms Staphylococcus aureus and Escherichia coli (108 cells/ml). The plates were placed in refrigeration for 14-18 hours so that the antibacterial substance can diffuse to the medium. Then, they were incubated for 24 hours at 37°C. The antibacterial activity was measured in mm of the sterile zone [9].

*5) F*ermentation

A loop of isolates having antibacterial activity were cultured in 50ml SNB (Starch Nitrate Broth) and incubated at 25-30 °C for 5-10 days with stirring. After 5 days, the culture was scaled up in SNB 500ml and incubated at 25-30 °C for 5-14 days. After 14 days, Actinomycetes cultures were filtered using Whatman filter paper for further assay [10].

6) Screening for Antibiotic Potential with Well Diffusion Method

Wells of 6 mm diameter were punched on the plate of Mueller Hinton medium inoculated with the test-microorganisms Staphylococcus aureus and Escherichia coli (108 cells/ml) using a sterile cork borer. Fifty microliters of filtrates were transferred into respectively labelled wells. The plates were then left for 2 hours at 2-8°C so that the antibacterial substance can diffuse well. Thereafter they were incubated for 24 hours at 37°C. The antibacterial activity was measured in mm sterile zone [5].

7) Extraction of Antibiotic Compounds

At the end of the fermentation period (14 days), the culture broth (1 L) was concentrated at 50°C until the volume merely ±100ml. Equals volume of culture filtrate and ethyl acetate (1:1) were taken in a sterile separatory funnel and agitated well. The ethyl acetate phase that contains an antibiotic agent was separated from the aqueous phase. It was evaporated using rotatory evaporation, and transferred into evaporating dishes to dried in water bath at <60°C. The dried precipitate was stored into Eppendorf tube for further test [11].

8) Screening for Antibiotic Potential with Kirby Bauer Method

In vitro antibacterial activity of ethyl acetate extracts of culture broth was performed by the disc diffusion technique (Kirby Bauer). The discs were prepared by dissolving 20µl extract on the sterilized paper discs (6 mm in diameter). Chloramphenicol (30µg/disc) was used as positive control. As a negative control, a blank disc impregnated with ethyl acetate. The test disc, and both controls were placed on Mueller Hinton medium inoculated with the test-microorganisms Staphylococcus aureus and Escherichia coli (108 cells/ml) and then left in a refrigerator at 2-8°C for 2 hours in order to diffuse antibacterial substance. Thereafter they were incubated for 24 hours at 37°C. The antibacterial activity was determined by measuring the zone of inhibition in mm.

9) Thin Layer Chromatography (TLC) Profile

Ten milligram crude ethyl acetate extract was dissolved with 200 µl methanol and loaded about 20µl on the silica gel F254 plates with the help of capillary tube. Chloroform: methanol (70:30) was used as the solvent system. Chloroform: methanol (70:30) was used as the solvent system. Spots were obtained after the solvent get eluted over 8 cm. After drying, the silica plates were visualized under UV254, UV366 and were sprayed with Ninhydrin, Dragendorff, 2,4-dinitrophenylhydrazin (2,4-DNPH) and vanillin-sulfuric acid reagents. Then the stained plates were heated to about 105 °C for 15 minutes till maximal coloration. The Rf value of the compound was also determined after running thin layer chromatography.

III. Result and Discussion

A total of 39 isolates of Actinomycetes were isolated as pure culture from 3 sugar cane soil samples in Piyungan, Bantul, Yogyakarta. Inoculation was carried out in SNA medium, this medium seems to be specific for Actinomycetes, and it contains soluble starch as a source of carbon (C), mineral salts such KNO3; K2HPO4.3H2O; MgSO4.7H2O and FeSO4.7H2O as a source of minerals for the enzyme function, and NaCl to raise the osmotic pressure and maintain the chemical balance for support bacterial growth [7].

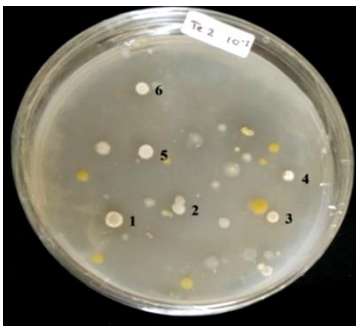

Fig. 1. Inoculation results of 10-3 serial dilution of soil sample code Te.2 in SNA medium.

The morphological macroscopic appearance of the colonies was powdery, dry, chalk-like, hard and difficult to scrap textures which are the characteristics of Actinomycetes and the shape was circle with convex elevation (Fig 1). After 14 days of incubation in purification step, the colonies were observed with white, brown, and orange mycelium colors. Some of them produced brownish and grayish pigmentations (Fig 2). Among 39 isolates, 7 groups were classified based on the color grouping method (Table 1).

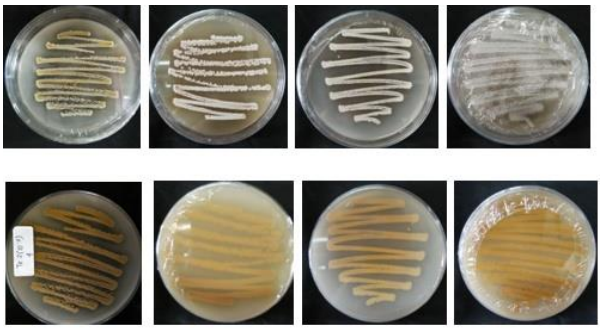

Fig. 2. Aerial mycelial view (upper picture) and reverse-side mycelial view (lower picture) of Te.234 (1), Te.235 (2), Te.236 (3), Te.325 (4) grown in SNA media after 14 days.

TABLE I. Color Grouping

No	AM	VM	P	∑	isolates
1	White	White	-	4	Te.241*, Te.121, Te.242, Te.322
2	White	Brown	-	8	Te.223, Te.232, Te.226, Te.227, Te.229, Te.233*, Te.327, Te.321
3	White	Brown	Greyish	7	Te.2210, Te.2211, Te.2212, Te.324, Te.123, Te.323, Te.231*
4	White	Brown	Brownish	8	Te.125, Te.221, Te.222, Te.224, Te.2213, Te.236, Te.325, Te.235*
5	Brown	Brown	-	7	Te.228, Te.234*, Te.243, Te.244, Te.122, Te.126, Te.124
6	Brown	Brown	Greyish	4	Te.225, Te.331, Te.332, Te.333*
7	Orange	Orange	Greyish	1	Te.326*

[a.] AM = Aerial mycelium, VM = Vegetative mycelium, P =Diffusible pigment, ∑ = total of isolates.

After isolation and purification were completed, the pure isolates then tested their antibacterial activity using agar-blocks method. As the results of primary screening, four (10.25%) Actinomycetes isolates were shown antibacterial activity against one test bacteria, S.aureus i.e. Te.234, Te.235, Te.236, and Te.325. However, none of entire isolates were able to inhibit Escherichia coli (Fig 3). Listari [12] has reported antibacterial testing of Actinomycetes isolates by agar block method could be more effective rather than well diffusion method. Nedialkova and Naidenova [9] also reported that 30% of the Actinomycetes strains had lost their inhibition potential perhaps due to the inconvenient liquid growth medium. Such results had been reported from other scientists too, which had found the activity reducing in comparison with that showed by the agar blocks method [13].

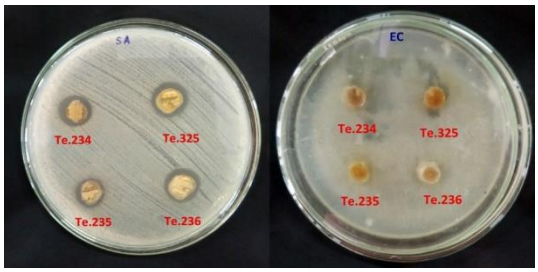

Fig. 3. Antibacterial activity of Te.234, Te.235, Te.236, and Te.325 by agar blocks method against Staphylococcus aureus (A) and Escherichia coli (B).

These active isolates then was sub cultured on 50 ml SNB (Starch Nitrate Broth) medium and get incubated for 5 days. During 5 days, Actinomycetes will pass through the lag and log phase. When bacteria get into lag phase, they will adapt themselves to growth conditions and not yet able to divide. While bacteria get into log phase, they will rapidly growing and dividing, their metabolic activity increase and the culture reach the maximum growth rate. Thus, it was expecting after 5 days of incubation, cultures might contain the most optimal number of Actinomycetes cells. After 5 days, culture was continued with a ratio 1:10 in SNB 500ml and 1000ml, then incubated at 25-30 °C for 14 days. Sulistyani and Akbar [7] reported Actinomycetes generally entered stationary phase in 14 days of incubation, which the microbes extracellularly released secondary metabolites as pigments and antibiotics. These metabolites could be diffused into the culture medium. Thus, the aim of this grading culture is to increase the number Actinomycetes cells in the culture medium, by increasing the number of cells, it is expected that secondary metabolites such as antibiotics produced more intense.

Broth cultures which contained secondary metabolites then tested their antibacterial activity using well diffusion. Based on the results, broth filtrate did not show any zone of inhibition in both test. bacteria. A negative result was expected because there is no substance having antibacterial activity. Another possibility is the incubation period of fermentation broth has not been optimized to produce secondary metabolites.

In this study, the ethyl acetate extract from SNB medium were subjected to antibacterial activity test (Fig 4) and chromatographic analysis (Fig 5-6). One liter fermentation broth isolates code Te.234, Te.235, Te.236 and Te.325 gave yields value of crude ethyl acetate extract, i.e. 0.402g/L, 0.353g/L, 0.282g/L and 0.751g/L respectively. With a same extraction method and used solvent, Jalaludeen et al [14] and Suthindhiran & Kannabiran's studies [15] showed that in a one liter fermentation broth, approximately obtain 0.1-0.7 g crude extract.

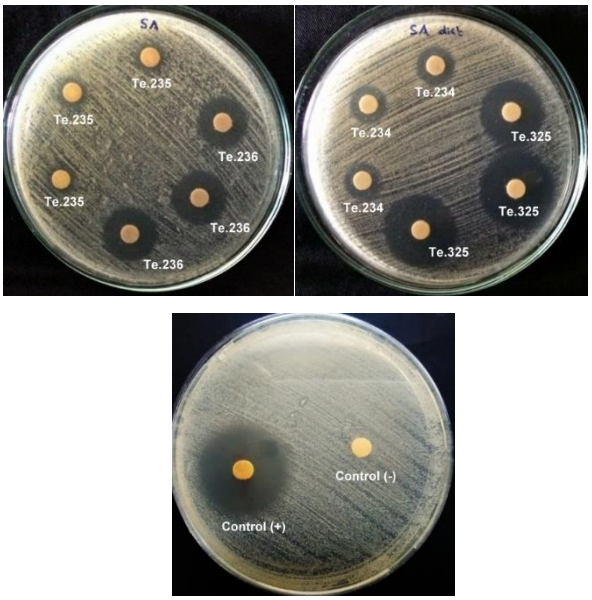

Fig. 4. Antibacterial activity of ethyl acetate extract of active culture broth against Staphylococcus aureus. Chloramphenicol (30µg/disc) as control (+) and ethyl acetate as control (-).

The results of ethyl acetate extract antibiotic activity gave a negative result against Escherichia coli. However, Stapthylococcus aureus showed a low to moderate sensitivity towards Te.234, Te.235 and Te.236. Te.325 was highly active against S.aureus giving a 19.16 mm zone of inhibition (ZOI) compared to the 24.50 mm ZOI of standard chloramphenicol (30µg/disc).

TLC analysis was done and sprayed with different reagents in order to determine the antibiotic compounds. TLC plates gave various spots under UV245 UV366 visualization, and it gave a positive result with Dragendorff, Ninhydrin, vanillin-sulfuric acid, and 2,4-dinitrophenylhydrazine (2,4-DNPH) spraying reagents. The Rf value was also determined in order to estimate the metabolites compounds in each spot. From four chromatogram plates, two of them are presented in Table IV-V i.e. Te.234 and Te.236 as well as Fig 5 (Te.234) and Fig 6 (Te.236).

Dragendorff is a color reagent to detect alkaloids and other nitrogen-containing compounds [16] by forming an orange-red colored following reaction with tertiary amines and quaternary ammonium compounds [17,18]. However, Habib [19] has reported false-positive alkaloid test using Dragendorff's reagent was found to result from reactions with nitrogenous (e.g. Peptides) or all non-nitrogenous compounds that were found to give a strong positive reaction with the reagent were compounds containing oxygen. Thus, the orange spots on the TLC plate, especially on Rf 0.93 in Te.234 and Rf 0.37; 0.42; 0.47; 0.56; 0.76; 0.86; 0.93 in Te.236 indicated that the extract was contained alkaloids or nitrogenous compounds such as peptides.

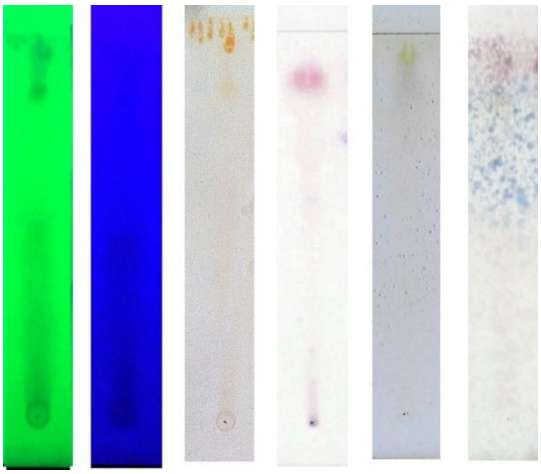

Fig. 5. Chromatogram of the ethyl acetate extract code Te.234 under UV254 (A), UV366 (B) visualization, and Dragendorff (C), Ninhydrin (D), 2,4-DNPH (E), vanilin/sulfuric acid (F) staining.

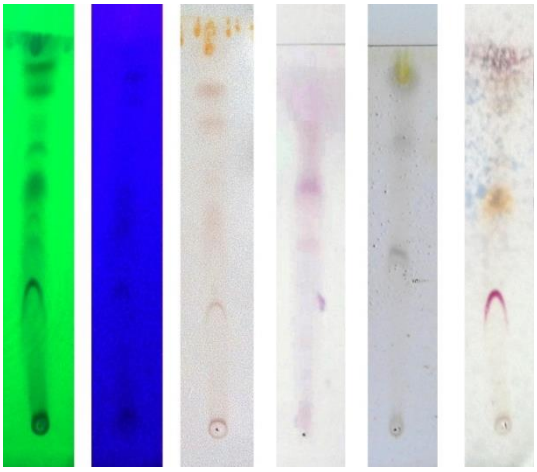

Fig. 6. Chromatogram of the ethyl acetate extract code Te.236 under UV254 (A), UV366 (B) visualization, and Dragendorff (C), Ninhydrin (D), 2,4-DNPH (E), vanilin/sulfuric acid (F) staining.

TABLE II. ANTIBACTERIAL ACTIVITY OF ETHYL ACETATE EXTRACTS OF ISOLATES TE.234, TE.235, TE.236, TE.325

Isolate Code	Inhibition zones (mm)	
	E. coli	*S. aureus*
Te.234	-	7.67±0.07
Te.235	-	3.75±0.05
Te.236	-	12.43±0.03
Te.325	-	19.16±0.45
Chloramphenicol (30 µg)	25.00	24.50
Ethyl Acetate	-	-

Ninhydrin is a chemical reagent used to detect ammonia or primary and secondary amines such amino acid or peptides. When reacting with these free amines, a red to purple color is produced (Gandjar & Rohman, 2007).

TABLE III. TLC PROFILE OF ETHYL ACETATE EXTRACT CODE TE.234

Rf	UV-254	UV_{366}	Dragendorff	Ninhydrin	2,4-DNPH	Vanillin/sulfuric acid	Interpretation
0.28	Quench	Fluorescence	-	-	-	-	
0.93	Quench	-	Orange	Purple	-	-	Alkaloid, Peptide
0.97	Quench	-	-	-	Yellow	Violet	Ketones, Terpenoid/ Steroid

TABLE IV. TLC PROFILE OF ETHYL ACETATE EXTRACT CODE TE.236

Rf	UV-254	UV_{366}	Dragendorff	Ninhydrin	2,4-DNPH	Vanilin/ sulfuric acid	Interpretation
0.37	Quench	Fluorescence	Orange	Purple	-	Violet	Alkaloid, Peptide, Terpenoid /Steroid
0.42	Quench	-	Orange	-	-	-	Alkaloid
0.47	Quench	Fluorescence	Orange	Purple	Yellow	-	Alkaloid, Peptide, Ketones
0.56	Quench	Fluorescence	Orange	Purple	-	Brown	Alkaloid, Peptide, Phenol
0.76	Quench	-	Orange	-	-	-	Alkaloid
0.81	Quench	-	-	Purple	Yellow	-	Peptide, Ketones
0.86	Quench	Fluorescence	Orange	Purple	-	-	Alkaloid, Peptide
0.93	Quench	-	Orange	Purple	-	-	Alkaloid, Peptide
0.97	Quench	Fluorescence	-	-	Yellow	Violet	Ketones, Terpenoid /Steroid

Tertiary amines can not be detected on plates with Ninhidrin reagent, but it is detectable with Dragendorff's reagent [21]. So, these reagents could be synergistically to detect amines. Therefore, it can be concluded that the purple spots on the TLC plate, especially on Rf 0.93 in Te.234 and Rf 0.37; 0.47; 0.56; 0.81; 0.86; 0.93 in Te.236 is containing peptides compound. Ivanova et al [22] also used ninhydrin and Dragendorff's reagent to detect peptide in Streptomyces avidinii extract and reported that there was norophtalmic acid (y-glutamyl-alanyl-glycine), a tripeptide, in the extract.

The 2,4- Dinitrophenyl hydrazine can be used to specifically detect the carbonyl functionality of a ketone or aldehyde functional group and a positive test is signaled by a yellow, orange or red colors [23]. Thus, the yellow spots on the TLC plate, especially on Rf 0.97 in Te.234 and Rf 0.47; 0.81; 0.97 in Te.236 indicated that the extracts have a ketone or aldehyde-containing compounds. This reagent could be useful in order to supporting estimation of secondary

metabolites polyketides derivate, due to polyketides had a lot of ketone group.

Vanillin/sulfuric acid is a non-specific reagent to detect terpenoid or steroid compounds by giving a violet color [23]. Moreover, vanillin/sulfuric acid may also react with alcohols, phenols steroids, then produce red-brown, gray, seldom yellow color on the TLC plate [24]. Thus, the violet spots, especially on Rf 0.97 in Te.234 and Rf 0.37; 0.97 on Te.236 indicated that the extracts contain terpenoid/steroid compounds. Whereas, the brown spots on Rf 0.56 in Te.236 showed that isolates also contain alcohols and phenols. Rana and Salam [25] reported positive results was obtained by using vanillin/sulfuric acid on their metabolites extract, then it was interpreted the compound may be alcohol, phenol or steroid in nature.

In this study revealed the suspected compounds contained in ethyl acetate extracts are alkaloid, polypeptides, steroid, alcohol, phenol and ketone/aldehyde-containing compounds such as polyketides or sugar. Each compound has possibility as antibacterial. Hence, for proper identification of bioactive compound in extract it is necessary to perform bioautography analysis in the next study.

IV. CONCLUSION

A total of 39 Actinomycetes isolates can be isolated from sugar cane rhizosphere (*Saccharum officinarum*) in Piyungan, Bantul, Yogyakarta. The ethyl acetate extract of culture broth of four Actinomycetes isolates had antibacterial activity against S. aureus i.e. Te.234, Te.235, Te.236, and Te.325 isolates. They showed a narrow spectrum of antibacterial activity as it only inhibited gram positive bacteria. The compounds detected in the extract were alkaloid, polypeptide, steroid, alcohol, phenol and ketone/aldehyde.

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