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Separation and Identification of Antibacterial Compounds in The Butanol-Methanol Extract of Streptomyces Sp. B10 Metabolite with TLC-Densitometry

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Abstract: ***Streptomyces sp. B10 is the isolate of soil samples in the agri-business area of Krian, Sidoarjo, which has great potential as antibacterial. Butanol extract of fermented broth of Streptomyces sp. B10 indicates an antibacterial activity. In the previous study, metabolite separation of the butanol-methanol extract of Streptomyces sp. B10 metabolite by Thin Layer Chromatography (TLC) method has not been satisfied yet.This study aims to establish an appropriate mobile phase to properly separate the compounds in the butanol-methanol extract of Streptomyces sp. B10 metabolite in the elution process using High-Performance Thin Layer Chromatography (HPTLC) or Reversed-phase Thin Layer Chromatography (TLC). Furthermore, this study also aims to discover the number of compounds in the butanol-methanol extract of Streptomyces sp. B10 metabolite which can be separated by HPTLC or reversed-phase TLC. Lastly, it is to determine the relative concentration of each compound in the butanol-methanol extract of Streptomyces sp. B10 metabolite is based on the comparison of measurement results area with a densitometer.The type of research to be conducted was experimental research. This study separated the compounds in butanol-methanol extract of Streptomyces sp. B10 metabolites. It would be optimized with TLC using RP-TLC, HPTLC, and the eluent optimization to get better results and determine the compound proportion contained in the extract with densitometry.High-Performance Thin Layer Chromatography of 0.1 M KOH impregnation with a mobile phase of methanol: water (6:4 v/v) could be used for the separation of the butanol-methanol extract of Streptomyces sp. B10 metabolite.***
It is necessary to research with other methods or instruments to determine the structural composition of compounds in the extracts and determine the types of antimicrobial compounds produced by Streptomyces sp. metabolite.

Keywords: densitometry separation, Streptomyces sp. B10, thin layer chromatography

1. INTRODUCTION

Streptomyces is a genus of Gram-positive bacteria in the form of filaments, such as fungi, which can live in a variety of environmental conditions. *Streptomyces* have a hyphae layer which can distinguish between the chain and spore phases of these bacteria (1,2). The most interesting aspect about *Streptomyces* is its ability to produce bioactive secondary metabolites,

which serve as antifungal, antiviral, antitumor, antihypertensive, as well as antibiotics and immunosuppressants. The secondary metabolites can be useful ingredients for medicines or agriculture (3).

Butanol is the best solvent for extracting antibacterial active compounds from *Streptomyces sp.* metabolite. Butanol extract of *Streptomyces sp.* B10 fermentation has an antibacterial power against the growth of *Escherichia coli* and *Bacillus subtilis* (4). Thin Layer Chromatography (TLC) is the simplest chromatographic method, which only requires the eluents or solvent developers and coated plates to produce the separations both quantitatively and semi-quantitatively (5,6). The principle of compound separation of thin layer chromatography is carried out by adsorption and partition. Adsorption is the process of analyte absorption in the surface area of the stationary phase, while the partition is the analyte ability in the solution to be distributed into the solvent or mobile phase used. The solvents used have the nature of not interfering or few interfering (7).

Impregnation can also be applied to obtain a better separation (8). Impregnation is a technique performed on the TLC stationary phase to achieve a better chromatography result. This includes changing the mode or selectivity of the chromatographic system, increasing the chromatography performance, and improving detection capabilities (9).

This research will optimize the process of separating compounds in the butanol-methanol extract of *Streptomyces sp.* B10 metabolites using HPTLC or the reversed-phase TLC to separate it better. This research is also expected to determine the relative concentrations of compounds contained in the butanol-methanol extract of *Streptomyces sp.* B10 metabolite is based on an area comparison using a densitometer.

This study aims to establish an appropriate mobile phase to properly separate the compounds in the butanol-methanol extract of *Streptomyces sp.* B10 metabolites in the elution process using HPTLC or the reversed-phase TLC. This research also aims to discover the number of compounds in the butanol-methanol extract of *Streptomyces sp.* B10 metabolite which can be separated by HPTLC or reversed-phase TLC. Another aim is to determine the relative concentration of each compound in the butanol-methanol extract of *Streptomyces sp.* B10 metabolite is based on the comparison of measurement results area with a densitometer.

This research is expected to provide information about the appropriate mobile phase and can be used for the compound separation in the butanol-methanol extract of *Streptomyces sp.* B10 metabolite, and as supporting research to obtain active isolates to antibiotics produced by *Streptomyces sp.* B10. B10.

2. METHODS

The research was conducted using an experimental study (10,11). The substances used in this study included frozen supernatant fermented by *Streptomyces sp.* B10, which was the research result of Mantiningrum (12), the collection of the Faculty of Pharmacy, Airlangga University, distilled water, butanol p.a. (Merck), methanol p.a. (Merck), isopropanol p.a. (Merck), KH2PO4 (Merck), potassium hydroxide (Merck), formic acid (Merck). Fermented supernatant *Streptomyces sp.* B10 frozen in the freezer was used as an extract powder using a freeze dryer for 24 hours. Dry powder was then weighed 500 mg and extracted with 10 ml of butanol-methanol (1:1 v/v) solvent. Then, it was extracted by a vortex and centrifuged at 3,000 rpm for 10 minutes. The supernatant obtained was inserted into a vial that previously had been weighed, then evaporated to dryness (12). The tools used in this study included glassware, analytical balance (Sartorius), micropipette (Gilson MK 25019), autoclave (HL340 series vertical type stream sterilizer), vortex (Thermo Scientific), centrifuge (Hermle Z36 HK), freeze dryer (Christ Beta 1-8 K), oven (Sonita), densitometer (CAMAG/SHIMADZU), chromatographic vessels, silica gel TLC 60 F_{254} (Merck), Reversed-Phase TLC of silica gel 60

F_{254} (Merck) Merck), silica gel HPTLC 60 F_{254}(Merck). The study was conducted in the Microbiology Practicum Room of Faculty of Pharmacy, Universitas Airlangga from January 2018 to July 2018. In the thin-layer chromatography test of the butanol-methanol extract of fermented filtrate from *Streptomyces sp.* B10, the elution process, was carried out with a mobile phase of methanol-water with various comparisons (12). If it still was not found a proper separation, it was optimized with other mobile phases that had the appropriate polarity.

3. RESULTS

The repetitions or replications under separation conditions were carried out using the HPTLC plate of Silica gel F254 of KOH 0.1 M Impregnation mobile phase methanol: water (6:4 v/v). The replication results indicated that the second and third repetitions produced the same Rf of 0.79 and 0.69, respectively, with the same resolution, equal to 1.11. From those three data, Rf obtained an average of 0.80 and 0.70 and an average resolution of 1.33 (as shown in Table 1.).

Table 1**.** The separation result of secondary metabolites of *Streptomyces sp.* B10 in butanol-methanol extract using silica gel HPTLC F254 of 0.1 M KOH Impregnation with a mobile phase of methanol: water (6:4 v/v)

Replication No.	**Number of stains**	**Rf**	**Resolution**
I	2	0.825 0.725	1.78
II	2	0.79 0.69	1.11
III	2	0.79 0.69	1.11
Average		Rf 1 = 0.80 Rf 2 = 0.70	1.33

After the preparation of the percentage of the area, obtained from the densitogram of *Streptomyces sp.* B10 secondary metabolite in the butanol-methanol extract, as seen in Table 2, the results indicated that the average area reached 75.8% for the first stain and 24.1% for the second stain. Therefore, the ratio of relative concentrations between more polar compounds and less polar compounds indicated 3:1.

Table 2. The average and percentage ratio of densitogram area of the secondary metabolite of Streptomyces sp. B10 in the butanol-methanol extract

Replication No.	Stain Rf of 0.80 (%)	Stain Rf of 0.70 (%)
I	74.8	25.1
II	78.1	21.8
III	74.5	25.4
Average	75.8	24.1
Ratio	3	1

4. DISCUSSION

Streptomyces sp. B10 was the isolation result from agribusiness land in the Krian area, Sidoarjo. In the previous study, the separation of compounds in the butanol-methanol metabolite of *Streptomyces sp.* B10 utilized the Thin Layer Chromatography (TLC) method with methanol mobile phase:water (6:4 v/v), and two stains were obtained. However, in that study, a perfect separation had not yet been acquired (12). Accordingly, the study was conducted to identify the appropriate conditions for separating active compounds in butanol-methanol extract fermented by *Streptomyces sp.* B10 by utilizing the TLC method so that a better separation was acquired.

The first step in this research was carried out by making the butanol-methanol extract of *Streptomyces sp.* B10 metabolite. The powder resulted was yellowish-brown and had a distinctive odor, but the powder could change color to brown in the storage process. The color changes that occur might result from manganese (Mn), contained in the media, and used during the fermentation process of *Streptomyces sp.* B10 Manganese could bind oxygen from the air so that the powder got wet and changed color (13). Thus, the container used in storing the powder should be dry and tightly closed so that the air could not enter. Afterwards, the dry powder was extracted with butanol-methanol (1:1 v/v) and evaporated to obtain a clear yellow concentrated extract with a consistency like oil. The use of extraction solvents was based upon previous research, which suggested that butanol was the best solvent in extracting active antibacterial compounds from *Streptomyces sp.* B10 (4).

The next step formed the process of separating the active compound in the butanol-methanol extract from the fermentation of *Streptomyces sp*. B10 that employs the PLC method. This method was applied as it was the simplest and most widely used chromatography method in the process of separating compounds (5). The results obtained for this separation condition were two stains with Rf 0.825 and 0.74, while the resulting resolution was still not adequately good at 1.08. This condition was likely attributed to the polarity of the mobile phase used, which was still not significantly suitable for the separation of compounds in the extract.

The next separation process used the Reversed-Phase Thin Layer Chromatography or RPTLC. The RPTLC is considered suitable for separating polar compounds and making solvent system optimization easy (14). Regarding the separation process under those conditions, only one stain was still visible from the tailings. The compounds could cause tailings that occurred in the extract, which were still firmly bound to the stationary phase, while the mobile phase continued to rise to produce a tail-stained. Therefore, the separation conditions using the RPTLC plate with a mobile phase of methanol: water (6:4 v/v) were not

considered suitable for the compound separation in the extract.

Subsequent separations use High-Performance Thin Layer Chromatography (HPTLC) plates. HPTLC is composed of adsorbents with smaller and uniform sized particles so that it is expected to produce a faster and better separation process (5). One stain was obtained in which the compounds in the extract could not be separated, and tailing was spotted. The second mobile phase used is methanol:water:formic acid (6:3.5:0.5 v/v). The addition of formic acid with higher polarity was expected to separate compounds in the extract better. However, the results obtained from this separation condition only revealed one large stain and tailing. The third mobile phase used was KH_2PO_4 by 7.5%. The results obtained by using the mobile phase only indicated one stain and tailing. The plate condition could cause this condition, or the polarity of the mobile phase used was still not suitable for the compound separation in the extract.

Due to the separation conditions using the HPTLC plate with the mobile phase, which was not suitable for the compound separation in the extract, impregnation of the HPTLC plate with 0.1 M KOH in methanol was carried out. The impregnation carried out in the stationary phase of TLC could change the model or selectivity of the chromatographic system, improve the performance of chromatography, and increase detection capabilities. The impregnation technique applied in this study was the pre-development technique. The pre-development technique is a simple technique in the preparation of TLC plate impregnation with the desired reagents (9). Based on the optimization results, the separation using HPTLC impregnated with 0.1 M KOH in the mobile phase of methanol: water (6:4 v/v) provided best results compared to other separation conditions because the resulting resolution was proportionate, above 1 (5) .

The ideal separation conditions were then repeated or replicated three times to identify the consistency of the resulting stains in order to obtain three data (Table 1). The second and third replications had a similar result, i.e., two stains with Rf 0.79 and 0.69 and the resolution of 1.11. From the three data collected, the Rf obtained was not much different, and the resulting resolution still fulfilled the requirements, i.e., more than 1 (5). The average Rf obtained reached 0.80 and 0.70, while the average resolution amounted to 1.33. Stains with larger Rf indicated that compounds were more polar than stains with smaller Rf.

The next stage was the observation using a densitometer with three times repetitions to determine the dominant compound contained in the butanol-methanol extract of *Streptomyces sp.* B10 metabolite. The HPTLC plates impregnated with 0.1 M KOH that had been eluted with a mobile phase of methanol:water (6:4 v/v) was scanned using a densitometer at the maximum wavelength of 254 nm. This step was also carried out to ensure the existence of two separate stains. The scanning process generated two peaks, high peak and lower peak. It indicated that the proportion of compounds with an average Rf of 0.80 was higher than those with an average Rf of 0.70. However, based on its separation, the two compounds had not been entirely separated because the second peak had appeared before the first peak reached the bottom.

After averaging the area percentage (Table 2), it resulted in 75.8% for stains with an average Rf of 0.80 and 24.1% for those with an average Rf of 0.70. From these averages, the ratio of compounds with Rf 0.80 and those with Rf 0.70 reached 3:1, and it could be concluded that compounds with Rf 0.80 had greater proportion in the butanol-methanol extract of *Streptomyces sp.* B10 metabolite.

5. CONCLUSION

Compounds in *Streptomyces sp.* B10 active metabolite in the butanol-methanol extract can be appropriately separated using HPTLC impregnated with 0.1 M KOH in a mobile phase of methanol:water (6:4 v/v). From the separation process, two active compounds are separated, with a resolution of 1.33 from the butanol-methanol extract of *Streptomyces sp.* B10

metabolite. It is separated using the HPTLC method impregnated with 0.1 M KOH in a mobile phase of methanol:water (6:4 v/v) with an average Rf of 0.80 and 0.70 respectively. Compounds with Rf 0.80 in the butanol-methanol extract of *Streptomyces sp.* B10 metabolite has a greater relative concentration than those with Rf 0.70 in a ratio of 3:1.

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