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Original Article

ANTIMICROBIAL ACTIVITY OF *Streptomyces* spp. ISOLATES FROM VEGETABLE PLANTATION SOIL

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ABSTRACT

Fifteen *Streptomyces* isolates were isolated from soil in some different location on vegetable plantation at agriculture standard condition. The isolates were assessed for their antibacterial activity against *Mycobacterium tuberculosis* (MTB) ATCC H37RV and mycobacterial which isolated from Dr. Soetomo Hospital patients in Surabaya. The International *Streptomyces* Project 4 (ISP4) and Middlebrook 7H9 (MB7H9) wwere used as growth or fermentation medium. The screening of inhibition activity was performed using turbidimetry and spot-test on agar medium. Results shown that 33.3% of the isolates (5 isolates) have anti-mycobacterial activities. The first line anti tuberculosis drug rifampicin, (RIF), ethambutol (EMB), isoniazid (INH), and pyrazinamide (PZA) were used as standards or positive controls with concentration 20 ppm. Optical density of crude fermentation broth concentrated from five isolates relatively lower than five anti-tuberculosis drug activity standard, although their activities against some microbial were similar to the standard at spot-test. The most efficient isolate shown anti-mycobacterial activity was *Streptomyces* B10 which identified as *Streptomyces violaceousniger*. In addition, fatty acid methyl ester (FAME) profile of gas chromatography-mass spectrometry chromatogram of each isolates were studied and compared to *Streptomyces* spp.

Keywords: *Anti-mycobacterial, Mycobacterium tuberculosis, Streptomyces spp.*

INTRODUCTION

Anti-infection drug development of disease caused by *Mycobacterium tuberculosis* (MTB) is on Indonesia priority list. Tuberculosis (TB) is a chronic infectious disease which becoming a global epidemic. Indonesia is a country with worlds' fifth biggest TB cases. Estimation of TB prevalence is 566.000 cases or it means 244 cases in 100.000 of population.

The availability of antibiotic raw materials is one of limitation in TB drug development, which more than 96% of it were imported (Zignol et al., 2006; Isnaeni et al., 2013). The dependence of antibiotic raw material in Indonesia should be stopped. Multi Drug Resistant of MTB strain caused the anti-tuberculosis drugs are not effective to combat MTB, there should be an alternative solution to solve this. Supply of natural, semi-synthetic, and fully synthetic antibiotic raw material isolates which already resisted to antibiotic were not equal to its demand. Science and technology development in anti-tuberculosis drug exploration is expected can solve the problem, but it not supported with the technology availability yet. On the other hand, Indonesia has abundant natural resources which contain antimicrobial active compound. These natural resources can be obtained from garden, farm field, community residence, volcanic area, water resources (river, lake, and sea), composted organic matter, also trash (Zignol, 2006).

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This study was designed to explore the *Streptomyces spp.* potential as antibiotic because of its richness of active compounds (Abouwarda and El-Wafa, 2011; Isnaeni et al, 2014). Streptomycin is an antibiotic which had been used as anti-tuberculosis drug and first isolated by Waksman (1943) from *Streptomyces griseus.* Streptomycin use is not in the first option to cure TB anymore because of its resistance and h igh toxicity (Isnaeni, 1998). Recent study of *Streptomyces spp.* antibiotic shown that there are anti-tuberculosis activity on actinomycin X2 and actinomycin D isolated from *Streptomyces* MS449; which live in sea (Chen, 2012). *Streptomyces* has wide range of habitat, but called as soil bacteria because of its geosmin smell which identic with soil also mostly found in soil.

Secretion of active metabolite compound variation is depending on S*treptomycin* habitat. This study screened the antimicrobial potential of *Streptomyces* spp. isolated from vegetable plantation soil in Krian, Sidoarjo. Previously, the similar research was performed from garden, volcanic soil (Semeru mountain), and soil from composted trash. Fatty acid methyl ester (FAME) profile of the isolates was also reported (Isnaeni et al., 2013). All isolates reported have exhibited antimicrobial activities and showed similar profile in term of some FAME components, like pentadecanoic acid metil ester, hexadecanoic acid metil ester, and cyclopropaneoctanoic acid metil ester.

METHODS

Streptomyces spp. Isolation

Soil samples were taken from agribusiness vegetable farm with kale (K), spinach (B), and corn (J) plantations. Soil were taken randomly using aseptic method in 10-20 cm soil depth from surface with assumption that farm management is correlate to agribusiness management standard in term of soil nutrient (Isnaeni et al, 2014). Soil sample was taken 10 gram and diluted in 90 ml of saline solution (NaCl 0.9%) then mixed by using vortex for 15 minutes. Then 1 ml of suspension was put into a sterile petri dish, added with CISP-4 medium (Difco), and homogenized. Samples were incubated on 28±2°C temperature for 4 days. Suspected *Streptomyces* sp. colony was isolated based on morphology and geosmin smell using Ose needle which touched into the colony then streak it to ISP-4 agar surface. Colony then incubated in the same temperature (Zignol et al., 2006).

Antimicrobial Potential Screening

Agar diffusion method of antimicrobial screening was modified by using agar medium as reservoir (Isnaeni, 1998). *Streptomyces* spp. culture on the agar medium was taken out by using stainless pipe (0.8 cm in diameter) on 4 days after incubation, then placed the colonies containing agar in agar medium surface inoculated with *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* (25% of transmittance in 580-600 nm) respectively.

Active *Streptomyces* spp. Fermentation

Isolates which shown activity were inoculated to 50 ml of ISP-4 liquid medium, then incubated in rotary shaker 150rpm with 28±2°C temperature for 4 days. Fermentation broth was centrifuged, supernatant was separated and dried using freeze dryer machine until obtained powder (Zignol et al., 2006).

Mycobacterium tuberculosis Preparation

Mycobacterium tuberculosis strain ATCC H37RV was obtained from Medical Microbiology Department Airlangga University and RS Dr. Soetomo TB patients. Culture was inoculated to liquid Middle Broke medium and incubated until reach the optical density (OD) in accordance with Mc Farland's nephelometer standard No. 1 (Abouwarda and El-Wafa, 2011)

Inhibition Zone of Streptomyces spp. Fermentation supernatant

Powder which obtained from *Streptomyces spp.* fermentation supernatant were diluted in 5 ml of sterile water then filtered using filter membrane to obtain solution as testing material. Each of solution was pippeted 1 ml, put in 4 ml of Middle Broke liquid medium which inoculated with *Mycobacterium sp.*, then incubated in 28±2°C temperature for 28 days. Negative control was non-inoculated Middle Broke medium, positive control was Middle Broke medium which inoculated with with *Eschericia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans.* Standard solutions were use as comparison EMB, PZA, INH, and RIF with 20 ppm concentration (Zignol et al., 2006).

Streptomyces B10 Identification

Identification of *Streptomyces* B10 was referred to Shirling and Gottlieb method (1966), Mythili (2011) and using determination key of Bergey's Manual (1986).

RESULTS

The ability of 35 *Streptomyces* sp. isolates in inhibition of Gram positive and negative bacteria also fungi growth is shown in Table 1. Streptomycin 20 ppm solution was used as positive control (K+). The data was obtained from average of 3 times of observation. Screening of supernatant concentrate activity results was shown in Table 2.

Table 1. Inhibition potential of *Streptomyces spp.* isolates from Indonesia soil on *Eschericia coli*(E), *Staphylococcus aureus (*S), *Pseudomonas aeruginosa*(P.), *and Candida albicans*(C).

Isolate Code	Diameter of inhibition zone (mm)				Isolate Code	Diameter of inhibition zone (mm)		
	S	*E*	*P*	*C*		*S*	*E*	*P*
B1	11.45	10.85	10.49	0.00	K9	0.00	0.00	0.00
B2	25.40	25.50	26.25	10.91	K10	22.84	22.28	22.49
B3	12.88	14.48	14.30	0.00	K11	11.90	10.97	0.00
B4	9.99	0.00	9.89	13.66	J1	0.00	0.00	0.00
B5	0.00	0.00	0.00	0.00	J2	0.00	0.00	0.00
B6	14.44	0.00	9.08	11.75	J3	23.12	22.44	23.01
B7	17.01	16.33	16.91	12.63	J4	15.05	0.00	0.00
B8	0.00	0.00	0.00	0.00	J5	0.00	0.00	0.00
B9	12.05	22.38	10.15	0.00	J6	0.00	0.00	0.00
B10	**16.73**	**15.12**	**16.37**	**13.58**	**J7**	**21.01**	**20.18**	**20.08**
K1	17.17	15.71	14.78	11.80	J8	11.93	11.71	12.77
K2	16.36	14.87	15.38	13.24	J9	0.00	0.00	0.00
K3	17.32	14.46	16.46	13.38	**J10**	**19.06**	**18.65**	**19.66**
K4	0.00	0.00	0.00	0.00	J11	12.88	12.54	13.13
K5	18.2	17.36	18.84	0.00	**J12**	**12.83**	**13.05**	**12.91**
K6	**15.76**	**13.63**	**15.34**	11.86	J13	21.73	19.88	20.96
K7	13.88	14.01	13.88	9.55	J14	0.00	0.00	0.00
K8	11.08	9.95	11.51	0.00	K(+)	18.19	17.08	18.78

Optical density changing pattern was shown on positive control (KP) which consisted only by MB medium and MTB (10^7 cfu/mL) from week 0 and has been increased on week 1. Cells were reach stationary phase on week 2 until week 4 (Figure 2). Inhibition activity was shown on 5 species from 15 cultured species. They are K6, B10, J7, J10, and J12 which have lower OD than KP (Table 2). Colony shapes and color of *Streptomyces spp.* isolates were shown on Figure 1. The colonies have variation on color, they are white, gray, and pink which specific to each colony. All of the colonies gave geosmin smell, and leathery or powdery appearance. Microscopically, they produce branched filaments that may be long or short, depending on the species.

The effect of testing solution and first line anti-tuberculosis drug additions were shown on Figure 2. It was found that there is decreasing of OD in observation weeks. Inhibition potency of B10 was not significantly different with INH, PZA, and EMB, but it was significantly different with RIF.

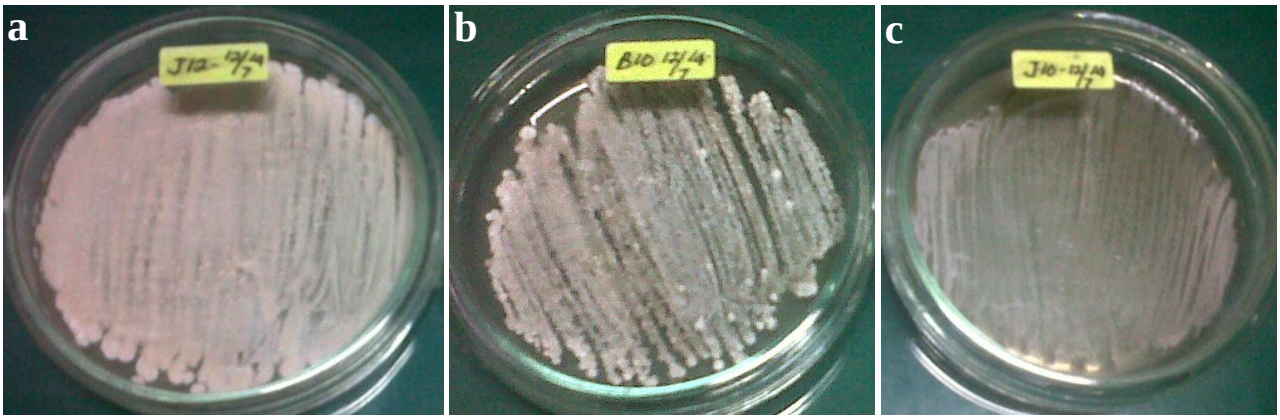

Figure 1. *Streptomyces* spp. culture on ISP-4 agar medium in day 4 (Isolate a= J12; b = B10; c = J10)

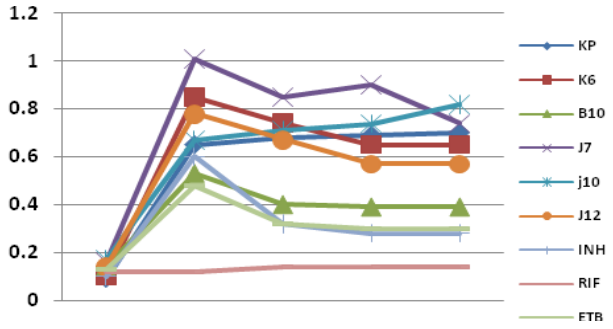

Figure 2. Optical density of MTB ATCC H37Rv culture on MB medium after week 4 with testing solution and antituberculosis drug . KP = MB medium + MTB; K6 = MB medium + MTB + 1 ml of K6 supernatant concentrate; B10 = MB medium + MTB + 1 ml of B10 supernatant concentrate; J7 = MB medium + MTB + 1 ml of J7 supernatant concentrate; J10 = MB medium + MTB + 1 ml of J10 supernatant concentrate; J12 = MB medium + MTB + 1 ml of J12 supernatant concentrate; INH = MB medium + MTB + 1 ml of 20 ppm INH; RIF = MB medium + MTB + 1 ml of RIF 20 ppm; EMB = MB medium + MTB + 1 ml of EMB 20 ppm.

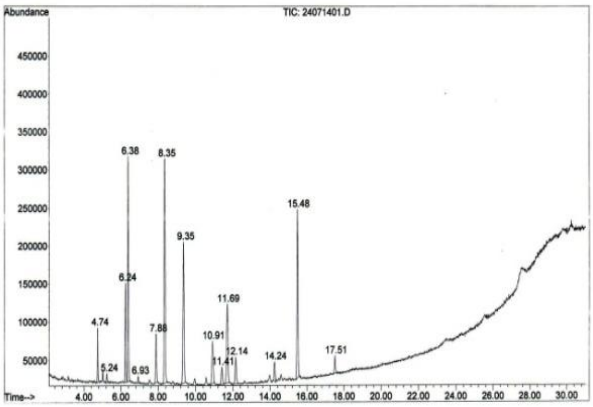

Figure 3. Fatty acid methyl ester chromatogram of *Streptomyces* B10

Fermentation results of *Streptomyces* sp. isolates on ISP-4 medium shown that there are 5 isolates which prospective to be used as anti-tuberculosis drug. They are B10, J7, J10, J12, dan K6. Isolate with J10 code was shown increasing activity and was confirmed using spotting test on MB agar with positive result that it can inhibit the MTB H37Rv (kode Px1) growth. J10 has been shown the same pattern as RIF which inhibit *Mycobacterium* Px-1 and Px-2. Rif and J10 isolates was not inhibit Px-3 and Px-4 growth (Figure 4).

All of the first line drugs with 20 ppm concentration (above of Minimum Inhibition Concentration) where shown brief result on week 2. The highest potential has been shown by rifamycin. Biochemical test results shown; that B10 has high activity; which confirmed that B10 is similar to *Streptomyces violaceousniger*character. The PCR analysis of 5 isolates shown the same thick band; which will be used as base of 16S r-RNA analysis. It will give the information of their correlation in phylogenetic tree.

Fatty acid methyl ester was identified to classify each species based on their cell fatty acid. *Streptomyces* activity can be analyzed by Gas Chromatophic Mass spectrophotometric method. Based on their fatty acid profile and their ability to inhibit *M. tuberculosis* H37Rv growth, those isolates has been shown the similarity to

Streptomyces spp. (Figure 3). It is necessary to do further study to make sure the correlation between anti-tuberculosis activity and their fatty acid profile.

Table 2. Optical Density of *Streptomyces spp*. fermentation for 4 weeks in MB medium with first line antituberculosis drugs

Testing Material	Optical Density								
	0 days	**7 days**	**Δ (7-0)**	**14 days**	**Δ (14-0)**	**21 days**	**Δ (21-0)**	**28 days**	**Δ (28-0)**
Medium only	0.08	0.08	0.00	0.08	0.00	0.08	0.00	0.08	0.00
Medium + *M.tb*	0.09	0.65	0.56	0.68	0,59	0.69	0,60	0.70	0.61
K6	**0.10**	**0.85**	**0.75**	**0.74**	**0.64**	**0.65**	**0.55**	**0.65**	**0.55**
K2	0.11	1.64	1.53	1.70	1.59	1.60	1.49	1.49	1.38
K7	0.14	1.62	1.48	1.54	1.4	1.47	1.33	1.13	0.99
K10	0.31	2.15	1.84	2.30	1.99	2.29	1.98	2.01	1.70
K1	0.33	0.99	0,66	1.33	1.00	1.33	1.00	1.18	0.85
K3	0.13	1.01	0.88	1.19	1.06	1.18	1,05	1.77	1.64
B10	**0.14**	**0.53**	**0.39**	**0.40**	**0.26**	**0.39**	**0.25**	**0.39**	**0.25**
B7	0.21	1.74	1.53	1.58	1.37	2.12	1.91	3.11	2.90
B2	0.29	1.64	1.35	1.60	1.31	1.48	1.19	1.46	1.17
J10	**0.17**	**0.67**	**0.50**	**0.71**	**0.54**	**0.74**	**0.54**	**0.82**	**0.65**
J11	0.17	1.71	1.54	1.38	1.21	1.37	1.20	1.01	0.84
J13	0.11	1.30	1.19	1.04	0.93	0.92	0.81	0.84	0.73
J3	0.13	1.24	1.11	1.06	0.93	1.02	0.89	0.97	0.84
J12	**0.14**	**0.78**	**0.64**	**0.67**	**0.53**	**0.57**	**0.43**	**0.57**	**0.29**
J7	**0.16**	**1.01**	**0.85**	**0.90**	**0.74**	**0.74**	**0.58**	**0.73**	**0.57**
INH	0.10	0.60	0.50	0.32	0.22	0.28	0.18	0.28	0.18
RIF	0.12	0.12	0.00	0.14	0.02	0.14	0.02	0.14	0.02
ETB	0.13	0.48	0.35	0.32	0.19	0.30	0.17	0.30	0.17
PZA	0.11	0.47	0.36	0.32	0.21	0.29	0.18	0.23	0.12

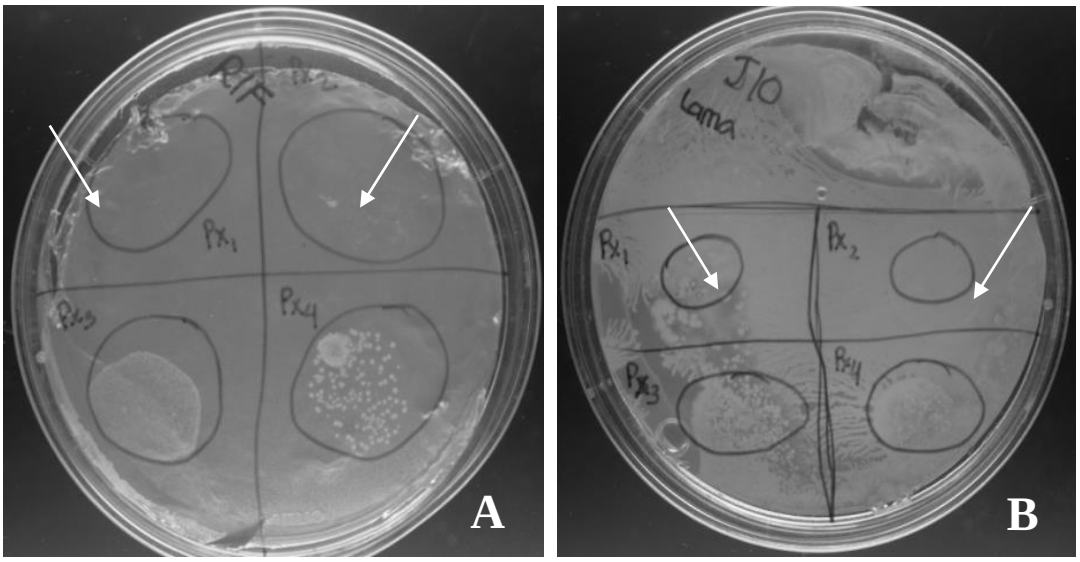

Figure 4. Inhibition of RIF solution 20ppm (A) and fermentation broth supernatant of *Streptomyces sp.* J10 (B) in MTB-H37Rv (PX-1), PX-2, PX-3, and PX-4 on MB medium after 21 days of incubation. There is no microbes growth on pointed zone.

Table 3. Optical density of *Streptomyces spp*. fermentation broth on week 4 which has inhibition activity of *M. tuberculosis* growth

Code	Day 7	Day 14	Day 21	Day 28
KP	0.56	0.59	0.60	0.61
K6	0.75	0.64	0.55	0.55
B10	0.39	0.26	0.25	0.25
J7	0.85	0.74	0.58	0.57
J10	0.50	0.54	0.57	0.65*
J12	0.64	0.53	0.43	0.43
INH	0.50	0.21	0.18	0.18
RIF	0.00	0.02	0.02	0.02
ETB	0.35	0.19	0.17	0.17
PZA	0.36	0.21	0.18	0.12

It was found that 33,3% of the isolates (5 isolates) have anti-mycobacterial activities. Optical density of crude fermentation broth concentrated from the five isolates have exhibited inhibition potencies relatively lower than the first line anti tuberculosis drug rifampicin, (RIF), ethambutol (EMB), isoniazid (INH), and pyrazinamide (PZA) at 20 ppm concentration. Their activities against some microbial tests were similar. Spot-test activity of the five isolates against some MTB showed the similar result. The most efficient isolate shown anti-mycobacterial activity were *Streptomyces* B10 and *Streptomyces* K6; which identified as *Streptomyces violaceousniger* and *Streptomyces antibioticus* based on their morphologies and biochemical properties.

Table 4. Fatty acid fraction as a marker of *Streptomyces* antimicrobial activity

No	Retention Time (Rt, menit)	Fatty Acid	Position
1	8,65	Pentadecanoic acid 14-metil-metil ester	Appear as dominant peak
2	6,65	Tetradecanoic acid 12-metil-metil ester	
3	6,50	Pentadecanoic acid metil ester	
4	9,69	Hexadecanoic acid metil ester	
5	12,50	Cyclopropaneoctanoic Acid,2-Hexyl- Methyl Ester	
6	11,38	Cyclopropaneoctanoic acid metil ester	Appear as peak with antituberculosis activity
7	11.88	Heptadecanoic acid Methyl Ester	
8	12,05	Hexadecanoic Acid 14-Methyl-Methyl Ester	
9	4,92	Tetradecanoic acid methyl ester	
10	8,17	9-Hexadecenoic acid Methyl Ester	

DISCUSSION

Streptomycetes is one of the famous genus, because of their ability to produce more than half of the 10000 documented bioactive compounds, have offered over 50 years of interest to industry and academic (Annaliesa and Elizabeth, 2001). Some previous study has been shown that many active compounds were isolated from *Streptomyces* spp (Tanaka, and Omura, 1993; Kimand Hwang, 1998). Isnaeni (1998) has isolated a novel antibiotic of streptomycin derivate. This study is expected that the result can be used as anti-tuberculosis drug; which more potential than the first line anti-tuberculosis drug or can combined each other with more effective and low toxicity. The benefits of those active compounds have been proved by researchers (Abouwarda and El-Wafa, 2011; Chen, 2012). It will allow another researcher to explore drug's raw materials, such as from semi synthetic or fully synthetic process (Lefevre, 2004).

The next research will be focus on the extraction of the free cell supernatant from fermentation broth, isolation and purification of the active metabolite by using several organic solvent. A novel and potential antibiotic as anti TB are expected to be achievement. Isnaeni et al. (2014) have obtained buthanol extract from the free cell supernatant of the fermentation broth of two *Streptomyces* spp., which exhibited antibacterial activity against Multi Drug Resistance bacteria (Extended strain Beta Lactamase and Methicillin Resistance *Staphilococcus aureus*) .

Furthermore, due to the variety of morphological, cultural physiological, and biochemical characteristic, there are too difficult to define their taxonomy and species. In this study, the Streptomyces isolates were identified base on the morphological appearances, color and geosmin smell of the colonies, biochemical test and PCR. Naturally, Actinomycetes produce slender, branched filaments that develop into mycelia. They have aerial mycelia distinguishable from fungi and many species produce asexual spores called conidia (Mythili, 2011). Many approaches have been developed, such as 16SrRNA and cellular fatty acid composition (Ndowora et al., 1996, Gordana, 2000). Hopefully, a newly *Streptomyces* species will be developed and discovered from the five active isolates by studying the metabolite activity correlation with composition of the cellular fatty acid.

Species or metabolite discovery with anti-tuberculosis drug potential should be developed. Tripathi et al. (2004) has reported antibacterial and antifungal activity of *Streptomyces violaceusniger*, but anti-tuberculosis activity has not been founded. Kim et al (2005) have explored novel antimicrobial substance without explained its anti TB activity. Fermentation using the active *Streptomyces* spp isolates should be done for further study. The extraction method to obtain pure isolates can use polar, semi polar, and non-polar eluent. Bioautograpic thin layer chromatographic may also be developed as a simple and effective method to observed number and potency of the *Sterptomyces* spp. active compounds (Isnaeni, 2005).

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