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Antibacterial Activity of Butanol Extract from Cell Free Fermentation Broth of *Streptomyces spp.*Isolated from Vegetable Plantation Soil.

Isnaeni[1]*, Mega FERDINA WARSITO[1], Noor ERMA NASUTION[1], Ni Made MERTHANIASIH[2], Achmad TOTO POERNOMO[1], Diana FAIRUZ[1], and Astrid HANIFAH[1].

[1]Department of Pharmaceutical Chemistry Faculty of Pharmacy, Airlangga University, Jl. Dharmawangsa Dalam Surabaya 60286, Indonesia
[2]Department of Microbiology Faculty of Medicine, Airlangga University, Jl. Prof. Mustopo, Surabaya 60286, Indonesia

ABSTRACT

Thirty five *Streptomyces spp.* have been isolated from vegetable plantation soil collected from Sidoarjo, East Java, Indonesia. The isolation and fermentation process to obtain antibacterial substances were performed in the International Streptomyces Projects (ISP)-4 media on rotary shaker at 150 rpm, 28^{o}C for seven days. In vitro antibacterial properties testing of one day to five days free cell fermentation broth (CFFB) of the *Streptomyces* isolates have been carried out by diffusion agar method using Gram positive and Gram negative bacteria as test microorganisms. The aims of this research are to extract the CFFB of the potential isolates by n-butanol and screen in vitro anti bacterial activities of the extract. It was found that the n-butanol extract of the CFFB of *Streptomyces* B2 and K2 strain showed their activities against *Methicillin-resistant Staphylococcus aureus* (MRSA) ATCC 12323701 with MIC of 0,1 and 0,5 ppm respectively. *Streptomyces* B10 and K6 strain showed their potential activities against *Bacilus subtilis* ATCC 6633 and *Escherichia coli* ATCC 25922 with MIC 0,273 and 2,93 respectively. Both isolates are also active against *Mycobacterium tuberculosis* H37Rv and patients isolate strain.

Keywords: Antibacterial activity, *Streptomyces spp.*, n-butanol extract, cell free of fermentation broth

Corresponding author

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INTRODUCTION

Belong to Actinomycetes, characterization of *Streptomyces* spp. are on transition between bacteria and fungus [1]. As a filamentous bacteria, *Streptomyces* spp. life in widely habitat like vegetable plantation, mangrove soil, and marine [2, 3, 4], most of them aerobically. A large number of *Streptomyces* spp. has been known as antibiotic production agents. Available drugs 70% have been isolated from actinomycetes; of which 60% are clinical used and applied for agriculture. *Streptomyces spp* produce about 75% of commercially and medically useful antibiotics that are natural origin such as erythromycin, Neomycin, tetracycline and Cefoxitin [5]. Chen et al [6] have discovered novel potent anti-tuberculosis from marine Streptomyces, they called actinomycin X2 and actinomycin D. In the previous study, isolation and identification of antimicrobial producing actinomycetes have been performed and potential isolate characterized as *Streptomyces macrospores* [5]. Several isolates showed growth inhibition activities against *Staphylococcus aureus* 96, *Staphylococcus aureus* 2940, *Escherichia coli* 739, *Candida albicans* 237, *Pseudomonas aeroginosa* 2453 and *Bacillus megaterium* 287. The hospital cultures used for the test were *Staphylococcus* spp, *Escherichia coli*, *Bacillus* spp, *Candida spp*, *Aspergillus spp*, *Salmonella spp*, *Shigella spp* and *Streptococcus spp*. [7]. This research gave great attention to discover active agent as anti pathogenic bacteria, especially *Mycobacterium tuberculosis* H37Rv and patients isolate strain from hospital. On the other hands, Multi Drug Resistant (MDR) like *Methicillin-resistant Staphylococcus aureus* (MRSA) ATCC 12323701 is the important target for novel antibiotic activity test. Isnaeni et al (2016) have reported that CFFB of *Streptomyces spp*. isolated from vegetable plantation soil, Sidoarjo, East Java, Indonesia showed potential activity against MTB and MRSA. In this research, butanol extract of the CFFB will be proved their antibacterial activities.

MATERIALS AND METHODS

Tested samples

Several butanol extracts derived from Cell Free Fermentation Broth (CFFB) and streptomycin (Meiji) standard solution were tested. The CFFB were obtained by fermentation processes of *Streptomyces sp*. B10, K6, B2, K2, K10, J3, J7, and J13 isolated from vegetable plantation soils, Sidoarjo, Indonesia that previously reported by Isnaeni et al [8]. The isolates were selected by previous antimicrobial activity screening using modified diffusion method against at least one of the two bacterial strains used during the testing (Fig. 1). The organic solvents used (butanol, methanol, P.A. – Merck) to extract, dissolve, and dilute the active compounds. For the diffusion method, the solvent used was methanol or water.

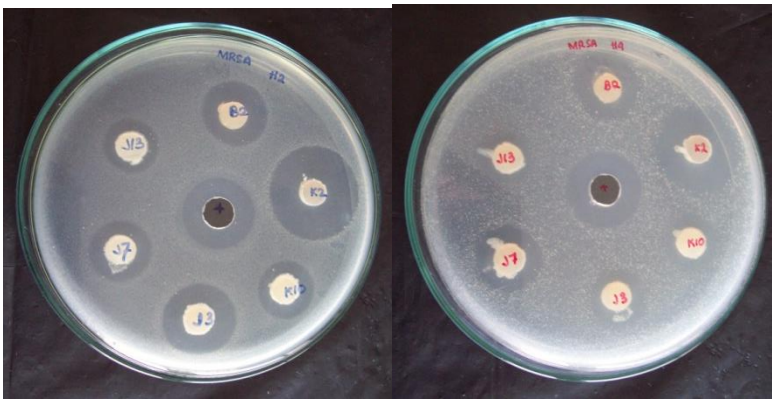

Figure 1: Growth inhibition activities of B2, K2, K10, J3, J7, and J13 strain grown on ISP-4 medium for 4 days against MRSA on media Nutrient agar after incubation 24 hours.

Test-bacteria

The antibacterial activity of the butanol extracts were assessed against following bacteria species: *Staphylococcus aureus* ATCC 25923 (American Type Culture Collection, Rockville, MD) and *Escherichia coli* ATCC 25922, Bacillus subitil ATCC 6633, *Methicillin-resistant Staphylococcus aureus* (MRSA) 12323701 (was obtained from Dr Soetomo hospital) prepared in Nutrient Broth (NB), taken from stock culture in Nutrient Agar

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(NA) Media (Oxoid). Overnight cultures were kept for 24 h at 35ºC ± 1ºC and the purity of cultures was checked after 8 h of incubation. After 24h of incubation, bacterial suspension (inoculum) was diluted with sterile physiological solution to obtain OD258 nm values of 25% with culture media [9]. *Mycobacterium tuberculosis* (MTB) H37Rv (ATCC 27294) and isolated strains from DR Soetomo hospital (Px) were used here for bioactivity screening and assays. The MTB H37Rv were grown at 37 °C to mid-log phase in Middlebrook 7H9 (Oxoid) broth supplemented with 10 % OADC enrichment (Becton Dickinson), 0.05 % Tween-80, and 0.2 % glycerol. The cultures were then diluted to a bacterial suspension with OD600 values of 0.025 with culture media (6).

Agar diffusion well

The agar diffusion well was performed for antibacterial activity testing against all bacterial tests, except MTB. The bacterial inoculum (15 µL) was added to 15 mL NB media, mixed vigorously by vortex at 45ºC, uniformly spreading in using pour plate method on a sterile Petri dish (20 cm in diameter) NA agar. Several serial dilutions yielded concentrations of 20-100 ppm for activity screening of the extracts and nine serial dilutions yielded concentrations of 5, 4, 3, 2, 1, 0.5, 0.1, 0.05, 0.01 ppm for MIC determination. Concentration of the samples varied depending on the *Streptomyces* isolates tested. 50 µL of each sample solution were added to each of the wells [10] mm diameter holes cut in the agar gel, 20 mm apart from one another). The plates were incubated for 24 h at 36ºC ± 1ºC, under aerobic conditions. After incubation, diameter of inhibition zone of the bacterial growth around the reservoir was measured in mm. Tests were performed in duplicate [10].

Anti MTB testing

The in vitro activity of compounds against MTB H37Rv was determined qualitatively in a Middlebrook 7H9 agar plate contained one mL water solution of dried butanol extract. The butanol extract was derived from 250 mg dried CFFB of B10 or K6 isolate extracted by 5 mL of n-BuOH, evaporated and dissolved in water to gain 2 mg/mL. For primary screening, aliquots (80 µl) of the bacterial suspension were spotted on the surface of each testing media. Rifampycin standard solution (20 ppm) served as the positive control, while sterile water served as the blank control. The plates were then incubated at 37ºC for 28 days, and the MTB growths were observed as previously carried out by Isnaeni et al. [6].

RESULTS AND DISCUSSIONS

Streptomycetes have high potential to produce secondary metabolite such as antibiotics [11], anthelminthic enzymes, herbicides [12], anti-cancer drugs [13], growth factors like vitamin B12 [14] and immune modulators [15]. In this research, great attention was given for antibacterial activities. In this research great attention was paid for antibacterial activity testing.

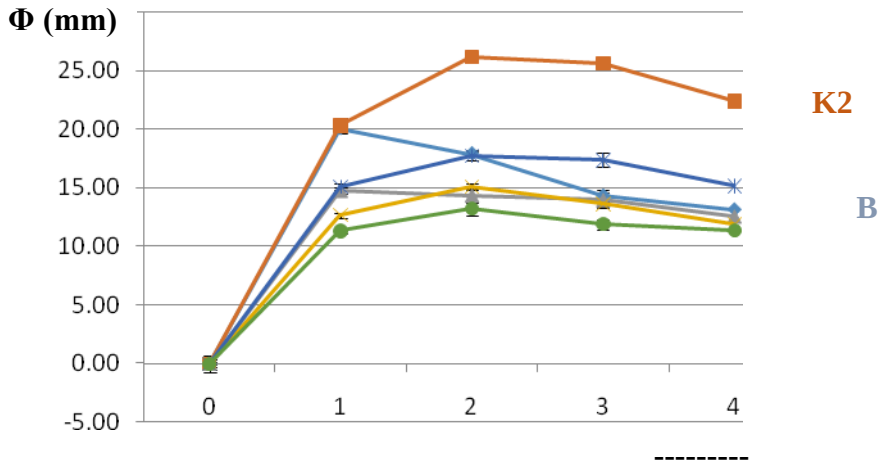

Figure 2: Curve of growth inhibition activities of Cell Free Fermentation Broth of B2 and K2 strain on ISP-4 medium for 4 days against MRSA (compared to other strain).

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Table 1: Growth inhibition activities of CFFB extract of *Streptomyces* spp. against MRSA 12323701

Sample	Conc. (ppm)	Replication					Mean	SD	RSD
		I	II	III	IV	V			
Streptomisin (K+)	100	23.90	23.65	22.90	22.85	23.50	23.36	0.47	1.99%
Hexane (K-)		0.00	0.00	0.00	0.00	0.00	0.00	0.00	-
Ethyl acetate (K-)		0.00	0.00	0.00	0.00	0.00	0.00	0.00	-
Ethanol (K-)		0.00	0.00	0.00	0.00	0.00	0.00	0.00	-
Hexane									
B2	**1000**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**-**
K2	1000	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-
Ethyl acetate									
B2	1000	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-
K2	1000	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-
n-butanol									
B2	20	19.95	19.65	19.25	20.35	20.25	19.89	0.45	2.27%
K2	100	16.05	15.80	15.95	16.25	15.50	15.91	0.28	1.77%
CFFB									
B2	1000	16.95	16.95	16.85	17.10	16.60	16.89	0.19	1.10%
K2	10	13.45	14.00	13.80	14.20	13.60	13.81	0.30	2.18%

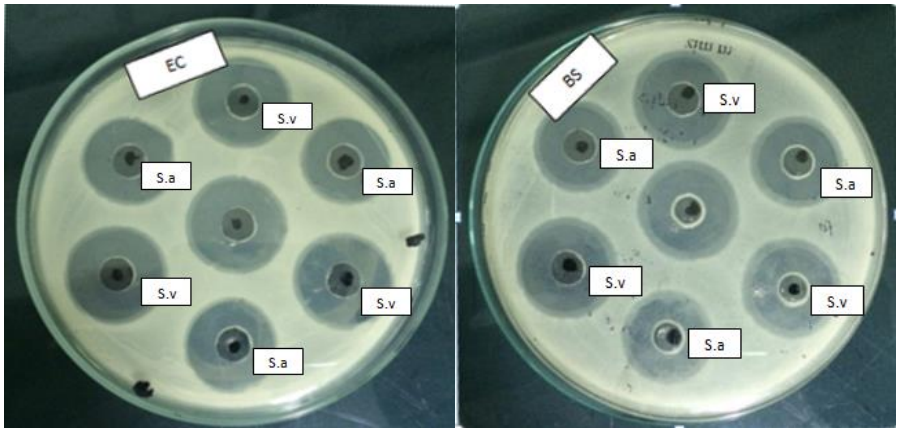

Figure 3: Growth inhibition activities of *n*-butanol extract of CFFB of *Streptomyces sp B10 strain* (Sv) and K6 strain (S.a) against *Eschericia coli* ATCC-25922 and *Bacillus subtilis* ATCC-6633

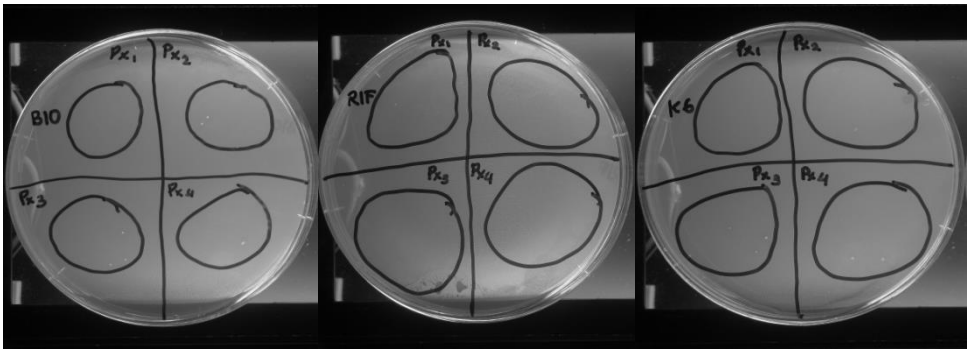

Figure 4: Growth inhibition activities of n-butanol extract of CFFB of Streptomyces sp B10 (A), Rifampicin 20 ppm (B), and K6 (C) againt *Mycobacterium tuberculosis* H37Rv (PX1) and patient isolates (PX2, PX3 and PX4).

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Table 2: Time course data of fermentation of active substances production from *Streptomyces* sp. B10 and K6 strain against *Bacillus subtilis* ATCC 6633

	Days	Diameter of inhibition zone (mm)			
		Replic. I	Replic. II	Replic. III	Mean±SD
K6	1	3,10	3,15	3,00	3,08±0,08
	2	3,45	3,20	3,20	3,28±0,14
	3	3,85	3,90	3,70	3,82±0,10
	4	4,15	4,15	4,10	4,13±0,03
	5	6,30	6,25	6,40	6,32±0,08
	6	4,60	4,40	4,45	4,48±0,10
	7	4,05	4,05	4,10	4,07±0,03
B10	1	5,05	5,00	5,00	5,02±0,03
	2	5,30	5,45	5,35	5,37±0,08
	3	8,45	8,30	8,40	8,38±0,08
	4	4,90	4,85	5,00	4,92±0,08
	5	3,70	3,60	3,65	3,65±0,05
	6	2,60	2,55	2,60	2,58±0,03
	7	2,05	2,15	2,25	2,15±0,10

In an earlier study, thirty five *Streptomyces spp*. isolated from vegetable plantation soil collected from Sidoarjo, East Java, Indonesia [6]. Antibacterial screening of twenty two of the isolates by modified diffusion agar method showed their inhibition activity against Gram positive and Gram negative bacteria [9]. The potential six strains of the isolates, namely B2, K2, K10, J3, J7, dan J13 produced more than 20 mm of zone inhibition diameter. Furthermore, identification of four days isolates against MRSA ATCC 12323701 indicated that the isolate B2 and K2 showed higher potency compared to the other isolates (Fig.1). The CFFB of the B2 and K2 was identified their activities against MRSA at one to four days fermentation. It was found that the peak activities obtained at four days fermentation (Table 1). The next step was extraction of the CFFB harvested at 24 hour and 72 hours of fermentation using n-hexane, ethyl acetate, and n-butanol, followed by evaporation of each extract and tested their activity against MRSA. Active compound was found in n-butanol extract exhibited by B2 and K2 isolates. The n-butanol of CFFB of *Streptomyces* sp. K2 and B2 were showed the higher activity compared to the other isolates (Fig. 2), although the activity of K2 isolates was higher than B2, with Minimum Inhibiton Concentration (MIC) at 0,1 ppm and 0,5 ppm respectively. On the other hand, the activity of the CFFB of *Streptomyces* sp. K2 in water was higher than B2 isolate.

The CFFB of B10 and K6 strain against Gram positive and Gram negative bacteria showed the maximum activities at three and five days fermentation respectively (Fig.3). The n-butanol extract of B10 strain inhibited the *Bacillus subtilis* ATCC 6633 and *Escherichia coli* ATCC 25922 growth, with MIC lower (0,73 ppm) than the K6 strain (2,93 ppm) (Fig. 4). Furthermore. In the previous research has been reported that n-butanol extract of the CFFB from B2 and K2 strains showed potential activities against MRSA 12323701 (Table 1). Growth inhibition testing against *Mycobacterium tuberculosis* H37Rv and patients isolates indicated that the B10 and K6 strains have showed significant activities (Fig.3). The growth inhibition activity of the CFFB of B10 strain was performed by turbidimetry method using nefelometer [6] and showed relatively higher than other six strains included K6 strain and its potency almost similar to ethambutol solution at 20 ppm, but still lower than rifampicin, pirazinamide and INH. There is great potential to develop B2, B10, K2, and K6 strains as sources of anti pathogenic bacteria event MDR bacteria. Additional research is required to optimize the antibacterial agent's production, especially for discovering anti infectious raw materials to overcome multi drug resistant and tuberculosis diseases. In the previous researches were reported the utilization of various media for producing both biomass and active metabolites from *Streptomyces* spp. [16, 17, 18]. Jennifer et al.[5] have been produced isolated antibacterial producing *Streptomyces* spp. using starch casein nitrate agar

medium. Identification of the potential *Streptomyces spp* as antimicrobial sources is the important thing to ensure a novel strains and antibiotics discovering. Taxonomically characterization on the basis of morphological and phenotypic characteristics was performed by standard method (5) indicated that B10 and K6 strain was closely associated with the type strain of *Streptomyces violaceousniger* and *Streptomyces antibioticus*. The strains have not been previously reported their anti mycobacterium activities. The *Streptomyces sp.* K2 was similar to K6 idetified as *Streptomyces antibioticus.* On the other hand, B2 were closely associated with the type strain of *Streptomyces purpureus*. Another method is required for rapid identification and differentiation of *Streptomyces* spp. by inoculating on selective media. One of the most promising methods recommended is biological molecular and the cellular fatty acid composition. Furthermore, isolation and purification of the active substances are very important step to gain novel antibiotic. Thin layer chromatography-bioautography method is appropriate method for both determination and bioassay purposes [19]. The active substances might be has another activities more than antibiotic as mentioned above.

CONCLUSION

In the course of our screening program for anti-bacterial activity of n-butanol extract of free cell fermentation broth (FCFB) of *Streptomyces* spp isolated from vegetable plantation soil grown in Sidoarjo, East Java we have found that seven isolates showed potentially opportunities to develop as antibacterial activity against Gram positive and Gram negative. Two of the *Streptomyces* spp isolates (K2 and B2 strain) were active against MRSA with MIC 0,5 ppm and 0,1 ppm respectively. On the other hand, another two isolates (B10 and K6 strain) were showed the same activities of growth inhibition against *Bacillus subtilis* ATCC 6633 and *Escherichia coli* ATCC 25922 with MIC 0,73 ppm and 2,93 ppm respectively. The activities screening of n-butanol extract from the CFFB of B10 and K6 strain indicated that both isolates showed growth inhibition activities against *Mycobacterium tuberculosis* H37Rv and patient isolates strain.

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