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Screening Antimicrobial Activity of Actinomycetes Isolated from Raja Ampat, West Papua, Indonesia

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Abstract

In the framework of exploitation of antimicrobial activity of Actinomycetes in Papua, one hundred isolates of Actinomycetes isolated from soil and leaf litter samples from various ecosystems in Batanta and Salawati Island, Raja Ampat, West Papua were screened. We obtained 200 crude extracts from 100 isolates based on two extraction phases. Nonpolar metabolites were extracted by ethyl acetate : methanol (4:1) solvent while the polar metabolites were concentrated using a freeze-drying method. Based on the agar dilution method, a total of 43 from 200(21.5%) crude extracts have antimicrobial activity against bacteria and yeasts (*Escherichia coli* NBRC 14237, *Bacillus subtilis* NBRC 3134, *Staphylococcus aureus* NBRC 13276, *Micrococcus luteus* NBRC 1367, *Candida albicans* NBRC 1594 and *Saccharomyces cerevisiae* NBRC 10217). Some crude extracts showed anti-Gram negative (1.5%), anti-Gram positive (17%) and antifungal (17%) activities. Crude metabolites which were extracted using ethyl acetate : methanol were more effective on antimicrobial activity (35%) compared with water extraction (17%). Five most potential isolates (BL-13-5, BL-06-5, BL-14-2, BL-22-3, and Sl-36-1) were identified based on 16S rRNA gene sequence data. Sequence similarity search by BLAST program revealed that they show sequence similarities to *Streptomyces kanamyceticus* (92%), *Streptomyces verne* (92%), *Streptomyces narbonensis* (92%), *Streptomyces malachitofuscus* (98%), and *Streptomyces hygroscopicus* (96%), respectively.

Abstrak

Penapisan Aktivitas Antimikroba dari Actinomycetes yang Diisolasi dari Raja Ampat, Papua Barat, Indonesia. Dalam rangka pencarian aktivitas antimikroba dari aktinomycetes di Papua, sebanyak seratus isolat Actinomycetes yang berasal dari tanah dan serasah dari beberapa ekosistem di Pulau Batanta dan Salawati, Papua Barat telah diuji. Sebanyak 200 ekstrak dari 100 isolat Actinomycetes telah diperoleh melalui dua tahap ekstraksi. Metabolit non polar diekstraksi menggunakan pelarut etil asetat : metanol (4:1), sedangkan metabolit polar diperoleh dari pemekatan medium menggunakan metode kering beku. Berdasarkan hasil pengujian menggunakan metode difusi agar, sebanyak 43 dari 200 ekstrak (21,5%) memiliki aktivitas antimikroba terhadap bakteri dan khamir (*Escherichia coli* NBRC 14237, *Bacillus subtilis* NBRC 3134, *Staphylococcus aureus* NBRC 13276, *Micrococcus luteus* NBRC 1367, *Candida albicans* NBRC 1594, dan *Saccharomyces cerevisiae* NBRC 10217). Hasil penelitian menunjukkan beberapa ekstrak Actinomycetes memiliki aktivitas anti bakteri gram negatif (1,5%), anti bakteri gram positif (17%), dan anti fungi (17%). Metabolit yang diekstraksi dengan pelarut etil asetat : metanol lebih aktif (35%) dibandingkan dengan pelarut air (17%). Sebanyak lima isolat yang memiliki aktivitas antimikroba tertinggi (BL-13-5, BL-06-5, BL-14-2, BL-22-3, dan Sl-36-1) diidentifikasi berdasarkan data sekuen gen 16S rRNA. Berdasarkan hasil pencarian homologi dengan program BLAST, diperoleh homologi spesies berturut-turut adalah *Streptomyces kanamyceticus* (92%), *Streptomyces verne* (92%), *Streptomyces narbonensis* (92%), *Streptomyces malachitofuscus* (98%), dan *Streptomyces hygroscopicus* (96%).

Keywords: 16S rRNA gene, actinomycetes, antimicrobes, crude extracts, Raja Ampat

1. Introduction

Actinomycetes are a kind of bacterial group that is intensively studied because of its antimicrobial activity. Actinomycetes have the highest diversity in soil and perform a major function as a decomposer and a bioactive compound producer. Among microorganisms, Actinomycetes are one of the most attractive sources of

all types of bioactive metabolites that have important applications in human medicine [1-2]. Antibiotics are major secondary metabolites produced by Actinomycetes. Actinomycetes produce more than 75% of the 10,000 antibiotics which have been found [3-4]. On the other hand, infectious diseases and resistant strains are increasing progressively due to antibiotics treatments. Therefore, efforts to find new antibiotics are crucial.

Raja Ampat, West Papua, Indonesia, is known as one of the world's biodiversity hotspots. It has a unique biogeographical history and is located between the Asia and Australia plates, making it an interesting place to explore biodiversity. Raja Ampat is known as a region with a high diversity of flora and fauna [5-6]. However, there is only limited information about its microbial diversity and their antimicrobial activity. Up to now, the information on microbial diversity in this region has only been reported by Nurkanto in 2009 [7]. Comprehensive research on Raja Ampat's microbial diversity has never been conducted.

In this paper, we describe the screening of Actinomycetes isolates from soil and leaf litters in Raja Ampat, West Papua and identify the potential isolates based on 16S rDNA sequence data.

2. Methods

Actinomycetes fermentation and metabolites extraction. Isolates were cultured in 100 ml of Actino Medium No. 1 (Daigo, Japan) containing 5 g/l poly peptone, 3 g/l yeast extract, at pH 7.2. Incubation was conducted for 7 days at 28 ^{o}C with a 100 rpm rotary shaker. Crude antimicrobial compound was recovered from the culture's filtrate by using a solvent extraction with ethyl acetate : methanol (4:1). Ethyl acetate : methanol was added to the filtrate in the ratio 1:1 (v/v) and shaken vigorously for 20 min. The organic layers were collected and the organic solvent was dehydrated in a vacuum evaporator at 40 ^{o}C to obtain a gummy crude extract.

Medium residues were extracted based on Rispail *et al.* [8] method with some modifications. Medium residue after non-polar extraction was centrifuged at 7000 rpm, and the supernatants were dried with a freeze dryer for 48 h**.** Metabolites obtained were analyzed using thin layer chromatography (TLC) (gel silica $GF_{254,}$ Merck) using the solvent system dichloromethane : methanol (15:1). Then, TLC chromatogram was analysed with UV 254 nm and sprayed with 1% $CeSO_4$ in 10% H_2SO_4.

Antimicrobial assay. Antimicrobial activity of crude extract produced by Actinomycetes were determined by the disk paper diffusion method [9-10]. Inhibition zones were expressed as diameter (mm) and measured after incubation at 37 ^{o}C for 24 h, which was repeated twice [11]. The target organisms used for antimicrobial activities were *Escherichia coli* NBRC 14237*, Bacillus subtilis* NBRC 3134*, Staphylococcus aureus* NBRC 13276*, Micrococcus luteus* NBRC 1367, *Candida albicans* NBRC 1594 and *Saccharomyces cerevisiae* NBRC 10217. We used Mueler Hinton agar (Difco) as a medium for antibacterial and Sabouraud agar (Difco) as a medium for antifungal activity. Organic extracts were diluted with 1% DMSO and filtered with 0.22 µm (Milipore). Water extracts were diluted in sterile aquadest and filtered. For a negative control antimicrobial test, we used sterile DMSO 1% for organic extracts, and sterile distillated water for water extracts. Chloramphenicol and erythromycin were used for anti-bacterial positive control, nystatin and kabicidin for anti-fungal positive control in 1000 µg/ml concentration.

Genomic DNA extraction and amplification. Potential isolates which have antimicrobial activity were identified using 16S rRNA gene sequence Genomic DNA extraction was conducted by Intragene matrix kit (Biorad) in accordance with the manufacturer's instructions.

The 16S rRNA gene of Actinomycetes were amplified using primers 9F (5`GAGTTTGATCCTGGCTCG) and 1510R (5`GGCTACCTTGTTACGACTT). Volume total of each PCR reaction was 25 µl, containing 2.5 µl DNA template, 1.25 µl of 20 pmol of each primer, 12.5 µL GoTaqgreen master mix (Promega) and 7.5 µl deionized water. PCR was performed in Thermalcycler (Takara Shuzo Co., Ltd., Shiga, Japan) under the following conditions: 3 min at 94 ºC, followed by 35 cycles of 1 min at 94 ºC, 1 min at 56 ºC, 2 min at 72 ºC, and then final extension at 72 ºC for 7 min. The PCR reaction mix was analyzed by agarose gel electrophoresis using Mupid Mini Cell (exu) in 1% agarose, tris-EDTA buffer, 100 V for 20 min. Standard 100 bp DNA ladder (Promega) was used as DNA marker. Then the gel was soaked at 1 µl/100 ml ethydium bromide for 15 min. Visualization of PCR products was conducted using UV transilluminator (Bioinstrument, ATTO).

PCR products purification. PCR products were purified by the PEG precipitation using Hiraishi *et al.* [12] method and some modifications. Briefly, a 25 µl aliquot of the PCR product solution was mixed with 15 µl PEG solution (40% PEG 6000 and 10 mM $MgCl_2$) and 6 µl 3 M sodium acetate, shaken gently for 10 min at room temperature and centrifuged at 10.000 xg for 15 min. The supernatant was removed carefully by pipetting and the precipitated DNA was then washed twice with 85% ethanol and redissolved in 10 µl sterile distilled water. Purified 16S rDNAs were stored at −20 °C until analysis.

Cycle sequencing. Cycle sequencing was conducted using a single primer, 9F. Each reaction of PCR cycle sequencing consisted of 1 µl primer 9F (5 pmol), 1 µl of purified PCR product, 0.2 µl of Big Dye Terminator sequence premix kit (Applied Biosystems Inc., Warrington, UK), 5 times sequence buffer 2 µl and deionized water up to 10 µl. Amplification was performed for 30 cycles: 20 sec 96 ºC, 10 sec 96 ºC, 5 sec 50 ºC, and 4 min 60 ºC for final extension.

The first step for sequence preparation was purification of PCR cycle sequencing products. As a total of 10 µl cycle sequence product was mixed with 1 µl 3M Na-acetate and 25 µl absolute ethanol. Then, the solution was centrifuged at 16.000 xg for 20 min. The supernatant was removed, and then the pellet was washed using 70% ethanol and re-centrifuged for 5 min. The supernatant was removed and 5 µl of HiDi-Formamide (Applied Biosystems Inc., Warrington, UK) was added to the pellet and mixed. Before loading to the sequencer sample tray, all samples were boiled at 95 ºC for 2 min and cooled on ice immediately.

Sequencing and data analysis. Sequence determination was performed using an automated DNA sequencer (3130 genetic analyzer ABI PRISM, Applied Biosystem, Hitachi, USA). The 16S rDNA of five isolates were sequenced partially (500 bp) using primers 520F dan 920R. Nucleotide sequences of the 16S rRNA gene of Actinomycetes isolates were determined and the similarity of each sequence was compared to the reference species of bacteria available in genomic database banks (Genbank), using the NCBI BLAST data library available at the website: *http://www.ncbi.nlm.nih.gov* [13].

Phylogenetic analyses of Actinomycetes isolates and other 16S rDNAs of the highest similar sequences obtained from Genbank were carried out. The phylogenetic tree was constructed by the Neighbour-Joining method [14] using Clustal X ver. 1.83 software [15-16].

3. Results and Discussion

We used 100 selected isolates for antimicrobial assay based on two extraction methods. The extracts were divided into organic and water extracts. We obtained 200 crude extracts from those 100 selected isolates. Organic extracts were obtained from ethyl acetate : methanol extraction, and water extracts were obtained from freeze-dried process on the remaining medium. Our result showed that 43 extracts have antimicrobial activities (Table 1). We used positive and negative control (Table 2).

The high antibiotic activities were observed in seven isolates: BL-13-5, BL-06-5, BL-14-2, BL-22-3, BL-22-2, BL-22-5, and Sl-36-1.Three isolates e.g., BL-22-2, BL-22-3, and BL-22-5 were collected from the same host sample, *Piper betle* leaf litter. It is known that leaves of *Piper betle* produce metabolites which have antibacterial activity [17-19].

Based on two extraction phases, we obtained two kind of metabolite characters (polar and non polar). Our results showed that there was a difference in the pattern of antimicrobial activities among 100 isolates. The crude metabolites extracted using ethyl acetate : methanol showed to be more effective with antimicrobial activities than those extracted using water solvent (Fig. 1).

Actinomycetes's metabolites contained in paper disc diffused on agar in the petri dish. The antimicrobial activity was observed as clear zone surrounding colonies (Fig. 2). Simultaneously, some sensitive microbes could not grow well or died because of certain concentrations of Actinomycetes metabolites.

Five of the most potential isolates (BL-13-5, BL-06-5, BL-14-2, BL-22-3, and Sl-36-1) which showed the highest antimicrobes were identified. Based on partial sequence (500 nt) of 16S rDNA using primers 520F and 920R, homology search by BLAST program showed that they show similarities to *Streptomyces kanamyceticus* (92%), *Streptomyces verne* (92%), *Streptomyces narbonensis* (92%), *Streptomyces*

Table 1. Antimicrobial Activities from 100 Actinomycetes Isolates

Microorganism	Number of Antimicrobial Activities (n,%) n=100
E. coli	3 (3)
M. luteus	31 (31)
B. subtilis	19 (19)
S. aureus	19 (19)
S. cerevisiae	34 (34)
C. albicans	4 (4)

Table 2. Negative and Positive Control for Antimicrobial Test

Micro organism	Negative Control		Positive Control			
	DM	DW	CL	ER	NY	KB
E. coli	-	-	+	+	-	-
M. luteus	-	-	+	+	-	-
B. subtilis	-	-	+	+	-	-
S. aureus	-	-	+	+	-	-
S. cerevisiae	-	-	-	-	+	+
C. albicans	-	-	-	-	+	+

DM = 1% DMSO; DW = Destilated water; CL = chloramphenicol; ER = Erythomycin; NY = Nystatin; KB = Kabicidin

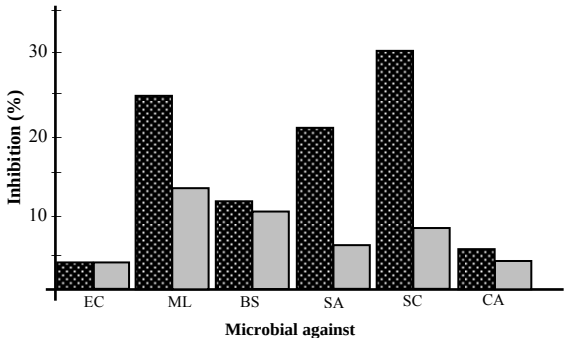

Figure 1. Antimicrobials Activity based on Two Extraction Methods (EC= *E. coli*; ML= *M. luteus*; BS= *B. subtilis*; SA= *S. aureus*; SC= *S. cerevisiae*; CA= *C. albicans*); Organic Solvent (▩), Water Solvent (□)

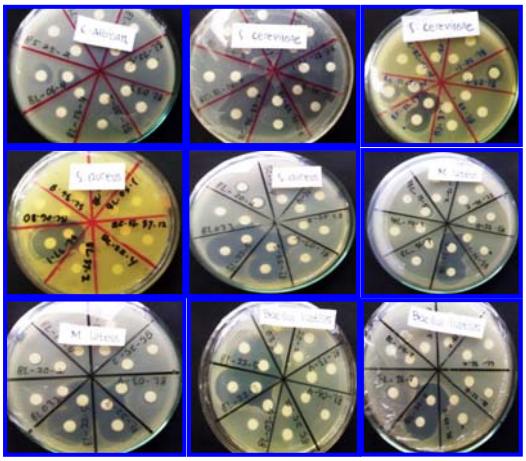

Figure 2. Clear Zone from Antimicrobial Activity Produced by Actinomycetes

malachitofuscus (98%), and *Streptomyces hygroscopicus* (96%), respectively. According to the species guideline for Actinomycetes based on 16S rDNA sequence data, homology less than 98% indicated new taxa [20-21]. Our results indicated that isolates BL-13-5, BL-06-5, BL-14-2, and Sl-36-1 have homology less than 98% compared with their closest species in NCBI gene bank. However, the sequences were based on a partial sequence of 16S rRNA gene. We need to determine the full sequence of 16S rRNA gene, chemotaxonomy, physiological tests and so on to confirm their identity.

Phylogenetic trees of selected isolates is shown in Fig 3. According to the result of phylogenetic analysis, five Actinomycetes isolates belong to the genus *Streptomyces*. They were separated into different clusters. Among the five isolates, the isolate BL-22-3 was closely related with *S. malachitofuscus.* The position of isolates BL-06-5 and BL-14-2 were clustered together and separated with other species. The two isolate BL-13-5 was separated from their closely related species *S. kanamyceticus* and *S. fradiae*. Isolate SL-36-1 also was separated with its closest species.

Based on the low homology value between five isolates to their closely related species and phylogenetic analyses, we assumed that isolates BL-13-5, BL-06-5, BL-14-2, and SL-36-1 belong to new taxa although we need further study to elucidate their identities.

Metabolite produced by *Streptomyces* BL-13-5 had the ability against bacteria *M. luteus* and yeast *S. cereviseae*. As shown in Fig 3, the closest species to isolate BL-13-5 is *S. kanamyceticus*. *S. kanamyceticus* produced kanamycin antibiotic [22] against some bacterial pathogen by interacting with the 30S subunit of prokaryotic ribosomes and indirectly inhibits translocation during protein synthesis [23-24].

Antimicrobes activity from *S. verne*, which is phylogenetically closely related to isolate BL-06-5, has never been reported. Our result showed that isolate BL-06-5 produced metabolite against fungal *S. cerevisiae* and *C. albicans.*

Streptomyces BL-14-2 is closely related to *S. narbonensis* based on 16S rRNA gene partial sequence.

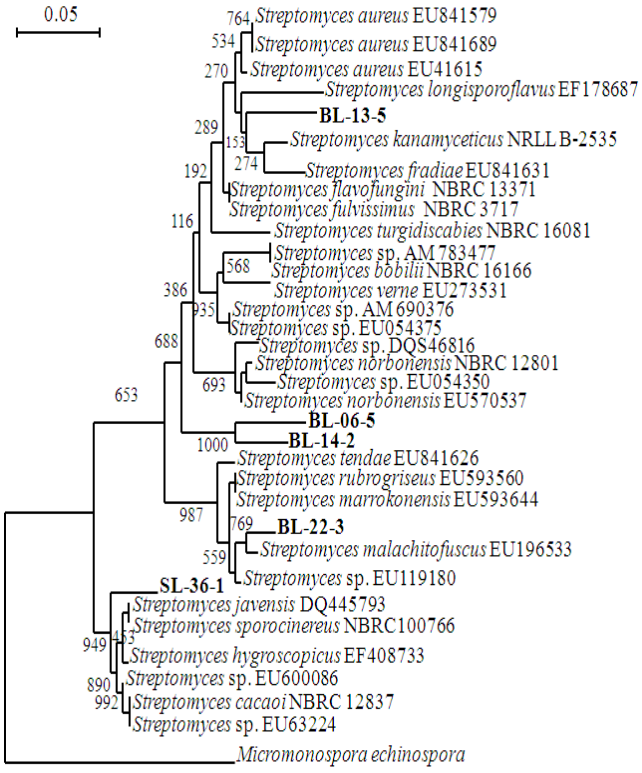

Figure 3. Phylogenetic Positions of Selected *Actinomycetes* Isolates based on Partial Sequences of 16S rDNA. As 500 bp were Compared. The Tree was Constructed by Neighbour-Joining Method [14]

A macrolide antibiotic from *S. narbonensis* is josamycin [25]. The drug has antimicrobial activity against a wide spectrum of bacterial pathogens. The mechanism of action of josamycin is via inhibition of bacterial protein biosynthesis by binding reversibly to the subunit 50S of the bacterial ribosome, thereby inhibiting translocation of peptidyl *t*RNA. This action is mainly bacteriostatic, but can also be bactericidal in high concentrations [25-26]. However, metabolites produced by isolate BL-14-2 are only active to gram positive bacteria (*B. subtilis* and *M. luteus*).

Isolate BL-22-3 showed the high homology with its closest related species, *S. malachitofuscus* (98%). Our result showed that isolate BL-22-3 produced metabolite which has wide activity against bacteria and fungi. The closest species, *S. malachitofuscus* was reported to have the ability as a wide spectrum antibiotic and antifungal [27-28].

Isolate Sl-36-1 has antifungal and anti-gram positive bacterial based on our investigation. Its closest relation to *S. hygroscopicus* (96%). This species is one of the famous Actinomycetes because of its valuable metabolites production. *S. hygroscopicus* produce immunosuppressant agents (sirolimus and ascomycin). The antibiotics geldanamycin [29], hygromycin B [30], nigericin [31] and validamycin [32] have been found in *S. hygroscopicus*. Indolocarbazoles, as an anticancer antibody can be found in *S. hygroscopicus* cultures [33]. Based on our screening, isolate SL-36-1 has a broad spectrum of antimicrobes.

4. Conclusions

We found that 43 from 200 crude extracts of 100 Actinomycetes isolates from Raja Ampat, West Papua have antimicrobial activities. Five of the most potential isolates (BL-13-5, BL-06-5, BL-14-2, BL-22-3, and Sl-36-1) have sequence similarities of 92% to *S. kanamyceticus*, 92% to *S. verne*, 92% to *S. narbonensis*, 98% to *S. malachitofuscus*, and 96% to *S. hygroscopicus*, respectively. This study provides comprehensive information on microbial prospects from Raja Ampat, West Papua, Indonesia and challenges the development of drug discovery.

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