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Diversity of Actinomycetes at Several Forest Types in Wanagama I Yogyakarta and Their Potency as a Producer of Antifungal Compound

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Abstract

Actinomycetes are bacterial groups that produce many secondary metabolites, which different biological activities, such as antifungi, antibacteria, antivirus, antitumor, etc. Actinomycetes are widely distributed in soil and their diversity is influenced by type of forest. The aim of this study is to investigate diversity of actinomycetes in several forest types of Wanagama I forest in Yogyakarta and their potency as a producer of antifungal compound. Soil samples under the forest of *Tectona grandis*, *Swietenia macrophylla* King, *Bamboosa vulgaris*, *Melaleuca leucadendron*, and *Gliricidia maculata* were used as sources of soil bacteria. Bacteria and actinomycetes communities were analyzed through culture-independent approach by RISA and nested-PCR RISA using actinomycetes spesific primer (F243), respectively. Through culture-dependent approach, isolated actinomycetes diversity were analyzed by identification of morphology (colony and cell), genetic (BOX element by rep-PCR), and secondary metabolites (thin layer chromatography). In addition, isolates were assayed for their antifungal activity against *Saccharomyces cerevisae*, *Candida albicans*, *Fusarium oxysporum* and *Aspergillus flavus*. The presence of Polyketide Synthase-I (PKS-I) and NonRibosomal Peptide Synthetase (NRPS) genes were amplified by PCR to study their correlation with antifungal activity of the actinomycete isolates. The results showed that types of forest influence diversity of rhizobacteria especially actinomycetes. According to culture-independent approach, relatively, community of rhizobacteria from the highest were soil under the forest of *B. vulgaris*, *G. maculata*, *T. grandis*, *S. macrophylla* King, and *M. leucadendron*, respectively. Meanwhile, community of actinomycetes from the highest were soil under the forest of *G. maculata*, *B. vulgaris*, *M. leucadendron*, *S. macrophylla* King, and *T. grandis*, respectively. Fourty-three morphologically different isolates were found by using culture-dependent approach consisting of 17 isolates were found in soil under the forest of *M. leucadedron*, each of 9 isolates in *G. maculata* and *T. grandis*, 6 isolates in *S. macrophylla* King. and 2 isolates in *B. vulgaris*. More diversity of secondary metabolites were observed in soil actinomycetes under the forest of *M. leucadendron*. Of the 43 isolates, 100% were active against *S. cerevisae*, 37.20% against *C. albicans*, 95.30% against *F. oxysporum*, and 83.70% against *A. flavus*. Antifungal activity of actinomycete isolates did not always have correlation with the presence of PKS-I and NRPS.

Keyword : Wanagama I Forest, soil bacterial diversity, actinomycetes, ribosomal intergenic spacer analysis, antifungal activity

Introduction

The soil is a complex habitat for many microorganism, such as bacteria, fungi, protozoa, and algae. Bacteria are the most abundant micoorganisms in soil, attaining populations in excess of one hundred million (10^8) to one hundred billion (10^9) individuals per gram of soil. One of soil bacteria is actinomycetes, numbering 10^6 to 10^7 g^{-1} soil (Sylvia *et al*., 2005).

Actinomycetes are bacterial groups that produce many secondary metabolites, which have different biological activities, such as antibacteria, antifungi, antivirus,

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antiparasitic, antitumor, and immunosuppresive actions. Those bacteria also involve in transformation of organic compounds (Kononova, 1966; Kuster, 1967; Kusner, 1968; Huntzens, 1972). Decomposition of plant and animal residues by producing extracelluler enzymes, such as cellulose, khitinase, and peroxidase lignin (McCarthy, 1987; Crawford, 1988; Wong *et al*., 1991). In addition, actinomycetes involve in degradation of toxic compounds (McCarthy and Williams, 1992).

The number of actinomycetes in soil is influenced by many environmental factors such as soil type, soil depth, vegetation type, pH, moisture content, and aeration (Lacey, 1973; Hunter-Cevera and Eveleigh, 1990; Labeda and Shearer, 1990). The quality and quantity of soil organic matter that is contributed by a plant, components of root exudates, and soil macrofauna community influence actinomycetes diversity. Rhizosphere and soil with relatively high levels of organic matter is suitable habitat for actinomycetes growth (Yurnaliza, 2001). Henis (1986) pointed out that the number of actinomycetes in soil is positively correlated with the level of organic matter. Crawford *et al*. (1993) demonstrated that number of actinomycetes in the rhizosphere can be found twice to thrice when compared with non-rhizosphere. It is caused by root of plant producing exudates as nutrition sources for pathogenic and non-pathogenic microorganisms (Lynch, 1983), but interaction of root plant and bacteria can be negatively by secretion components of root exudates inhibiting growth of some bacteria.

Diversity of soil actinomycetes has a positively impact for exploration of novel bioactive campounds which many biological activities. Bouizgarne *et al*. (2006) explained that in the Rhizosphere can be found actinomycetes species which potent as producer antifungal compounds. Isocianin is produced by Streptomyces from roots of *Argania spinosa* L. showing in vitro antifungal activity against *Fusarium oxysporum* f.sp. *albedinis*, *Verticillium dahliae*, *Candida albicans*, dan *Saccharomyces cerevisae* (Bouizgarne *et al*., 2006). On the other hand, antifungal compounds is also produced by rare actinomycetes, such as *Micromonospora*, *Dactylosporangium*, *Streptosporangium*, *Actinomadura*, and *Nocardioform* actinomycetes (Lee dan Hwang, 2002). *The Antibiotic Literature Database (ABL)* reported that 46.3% rare actinomycetes was found, 40.4% was unknown of actinomycetes genus (Lazzarini *et al*., 2000). It described that the number of actinomycetes which potent as producers of novel bioactive substances are very abundant in nature.

Actinomycetes diversity can be analyzed by using culture methods, but only a small part of microbial diversity can be detected. The cultivability values reported range from 0,001 – 15% depending on the environment (Amann *et al*., 1995). On the other hand, culture independent approach by amplification 16S rRNA genes from environmental samples has revealed that 7 – 64% of the amplified sequences originated from uncultured microorganisms (Kuske *et a*l., 1997; Zhou *et al*., 1997). For example, the forest soil, only 100 of bacteria species is detectable by culture dependent, but with culture independent approach about of 4.000 bacterial genomes can be isolated (Torsvik *et al*., 2002).

Using both culture-independent and culture-dependent approaches, the aims of the present study were to examine the effect of forest type in Wanagama I to diversity of actinomycetes and to find actinomycete isolates that have potency as a producer of antifungal compounds.

Materials and Methods

Soil Sampling

A total of 10 soil samples were collected from 5 different locations of various forest type in Wanagama I Yogyakarta by using transect method (*Bamboosa vulgaris*, *Gliricidia maculata*, *Tectona grandis*, *Melaluela leucadendron*, and *Swietenia macrophylla* King). The soil samples randomly selected 2 replicate at each of forest type (0-20 cm in depth). Furthermore, soils were air-dried at room temperature and stored at 20°C until used for furher experiments.

Diversity analysis by Culture-indepenent approach

Isolation of soil bacteria genome was analyzed by Gabor (Gabor *et al*., 2002) methods. 0.5 g (dry weight) of soil was resuspended in 750 ìl lysis buffer (100 mM Tris, 100 mM EDTA, 150 mM NaCl, 1% CTAB, pH 8) and 40 µl lysozyme (60 mg/ml) was added to a Eppendorf tube. The soil suspension was homogenized and incubated at 37°C for 60 min. Each sample was added 200 µl 20% SDS and incubated at 68°C for 120 min. The soil suspension was centrifuged for 10 min at 13.000 rpm. Carefully, supernatant was transfered to a new Eppendorf tube. The supernatant was added chloroform (1 : 1) and centrifuged at 13.000 rpm for 10 min. The aqueous (upper) phase was transferred to a new Eppendorf tube and added 0.6 volumes of isopropanol. Furthermore, it was centrifuged at 13.000 rpm for 5 min. The supernatant was discarded and rinsed with 1 ml 70% ethanol. The pellet was air-dried at room temperature and dissolved in 25 µl TE buffer (100 mM Tris and 1 mM EDTA, pH 8). The DNA was purified by Phenol : Chloroform : isoamyl alcohol (25 : 24 : 1) (Ausubel et al., 1995). Furthermore, it was purified by SpinPrep™ Gel DNA Kit.

Ribosomal Intergenic Spacer Analysis (RISA) and nested-PCR

The intergenic spacer region of bacteria DNA was amplified in 10 µl PCR mixtures diversity of 2 µl sterile distilled water, 1 µl each of 1406F and 23sR primer (25 pmol/ µl), 1 µl DNA (25 ng/µl), and 5 µl Mega Mix Royal. All reagents were combined and heated at 95°C for 5 min. Thirty cycles of PCR were performed with an Thermocycler (BioRad) at 92 °C for 1 min, 50°C for 1,5 min, and 68°C for 8 min, followed by elongation at 68°C for 8 min. Community of actinomycetes was analyzed by nested-PCR. First, the 16S rRNA gene is amplified with actinomycetes specific primer (F243) and 23sR. Second, the product of first amplification were used as template for a second PCR with primer pair 1406F-23sR. The composition and condition PCR was performed same with RISA of bacteria community. The product of RISA and nested-PCR were analyzed in 8% Polyacrilamide Gel Electrophoresis (PAGE).

Diveristy Actinomycete by Culture dependent Approach

Isolation and Characterization of actinomycetes in soils

The medium used for isolating and characterizing actinomycetes was starch nitrate agar (20.0 g starch nitrate, 1.0 g KNO_3, 0.5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g NaCl, 0.01 g $FeSO_4.7H_2O$, 20.0 g bacteriological agar, and 1 L H2O) (Waksman, 1961). Actinomycetes from soil samples had been isolated by pour plating technique on starch nitrate agar after serial dilution with 0,85% NaCl. Dry colonies of actinomycetes were selected and isolated. The isolates were characterized by morphological methods. The microscopic characterization was done by hanging drop. The mycelium structure was observed through the oil immersion (40X).

Genomic DNA extraction

Mycelia (5 ml) grown in a starch nitrate were centrifuged, rinsed with TE and resuspended in 0.4 ml SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5). Lysozyme was added about of 50 µl (10 mg/ml) and incubated at 37°C for 1 h. Then 50 µl 10% SDS was added and incubated at 65°C with occasional inversion for 1 h. About of 50 µl 5 M NaCl was added and incubated at 65°C for 1 h. Chloroform 400 ìl was added and incubated at room temperature for 30 min with frequent inversion. The mixture was centrifuged at 13.000 rpm for 10 min and the aquoeus phase was trasnferred to a new tube. Chromosomal DNA was precipitated by the addition of 1 vol 2-propanol with gentle inversion. The DNA was transferred to a new tube, rinsed with 70% ethanol, dried and dissolved in a suitable volume of TE buffer. Samples were extracted in the same volume of phenol/chloroform/isoamyl alcohol (25:24:1) and precipited with 0.1 vol 3M sodium acetate. The pellets were washed with 70% ethanol, dried and dissolved in TE buffer.

Repetitive extragenic palindromic-PCR (rep-PCR)

Repetitive DNA fingerprinting was performed on all the isolates following the method of Sadowsky (Sadowsky *et al.*, 1996). The PCR primer BOXA1R derived from the repetitive sequences (5'-CTACGGCAAGGCGACGCTGACG -3') was used to amplify the DNA samples. The PCR mixture contained 3 µl H_2O, 1 µl DNA genome of actinomycetes as template (50 ng/µl), 1 µl BOX A1R primer (15 pmol), and 5 µl Mega Mix Royal (MMR). The following PCR condition was performed: Predenaturing (95°C, 5 min), 30 cycles of 94°C for 1 min, 53°C for 1.5 min, 68°C for 1 min, and a final extension step at 68°C for 10 min. The PCR product was visualized by 8% PAGE.

Profile of secondary metabolites of actinomycete isolates

Loopful of selected actinomycetes on slant agar were inoculated into 5 ml soluble Starch nitrate broth. Then it was incubated in a rotary shaker at 150 rpm for 7 days at room temperature. The culture was inoculated into 50 ml starch nitrate broth in 500 ml Erlen meyer flask. The flask was incubated on shaker at room temperature for 7 days. The culture was transferred into 50 ml sterile conical tubes and centrifuged at 3000 rpm for 15 min. Futhermore, the supernatant was used as a source of antifungal metabolites.

Extraction of antifungal metabolites was performed by using ethyl acetate. The supernatant was transferred to a separating flask. Ethyl acetate was added with a ratio of 1:1(v/v) and shaked vigorously for 10 min. The top layer is transferred by Pasteur pipette to a clean glass tube. Ethyl acetate extraction was done twice. The supernatant was collected and passed throughout a column containing sodium sulfate and the filtrate was evaporated until the volume becomes 1ml and kept in the refrigerator until further experiments.

Secondary metabolites analysis by Thin Layer Chromatography (TLC)

Fifteen microliter of ethyl acetate extract were spotted on the Silica gel 60F254 (Merck) with solvent system chloroform: ethyl acetate : acetic acid (7:2:0.1%) (v/v). The separated spots were visulized by densitometry to determine the presence the different substances.

Determination of antifungal activity

Fourty-three isolates of actinomycetes were screened for their antifungal activity against *Saccharomyces cerevisae*, *Candida albicans*, *Fusarium oxysporum*, and *Aspergillus flavus* by using swab method. Malt extract agar (20.0 g malt extract, 20.0 g glu-

cose, 1.0 g peptone, and 20.0 g bacteriological agar, and 1 L H_2O) was used as medium of *S. cerevisae* and *C. albicans*. Potato Dextrose Agar (200 g potato, 20.0 g glucose, 20.0 g bacteriological agar, and 1 L H_2O) was used as medium of *F. oxysporum* and *A. flavus*, respectively. Five microliter of ethyl acetate extract was diluted in 10 ìl ethyl acetate and then loaded into paper disk. The paper disk was placed into petridish which has been swabbed with each of the fungi. The plates were incubated at room temperature for 24 h. The diameter of inhibition (clear) zone was measured to determine the extent of antifungal activity.

Analysis of soil bacteria diversity

Data from electrophoresis of PAGE results was subjected to Unweight Pair Group with Mathematical Average (UPGMA) analysis.

Results and Discussion

Diversity Analysis by Culture-Independent Approach

The result showed that types of forest determined diversity of rhizobacteria and especially actinomycetes. According to culture-independent approaches, community of rhizobacteria from the highest was detected from soil under the forest of *B. vulgaris*, *G. maculata*, *T. grandis*, *S. macrophylla* King, and *M. leucadendron* respectively in Figure 1.

Figure 1 suggests that the forest of *B. vulgaris* might be a particularly good source of diverse rhizobacteria.

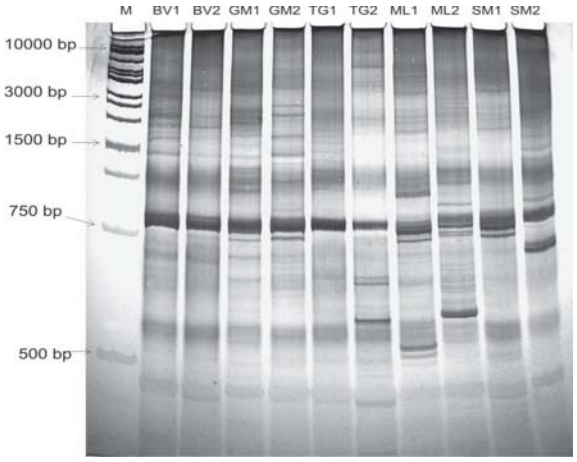

Figure 1. The Profil of rhizobacteria diversity in soil under the forest of *B. vulgaris* (BV), *G. maculata* (GM), *T. grandis* (TG), *M. leucadendron* (ML), and S. *macrophylla* King (SM) analyzed by RISA. M = 1 kb DNA ladder marker; 1-2: repetitions.

Dendrogram of RISA the five of forest types (BV, GM, TG, ML, and SM) is represented in Figure 2. Similarity coefficient of dendrogram described the distances of similarity. Figure 2 shows that community of rhizobacteria was varied in soils under the different forest types, except community of rhizobacteria under the forest of *B. vulgaris*, suggesting that members of this community was grouped in one cluster.

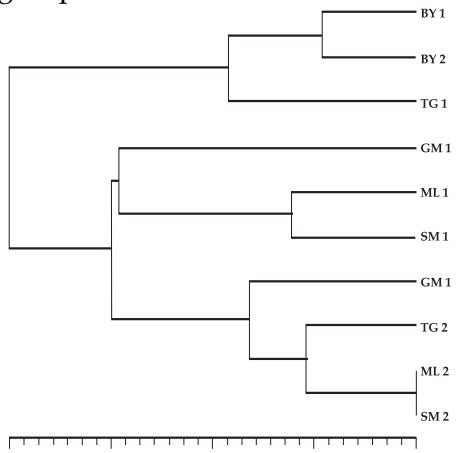

Figure. 2. Dendrogram of rhizobakteri community based on result of RISA in soil under the forest of *B. vulgaris* (BV), *G. maculata* (GM), *T. grandis* (TG), *M. leucadendron* (ML), and *S. macrophylla* King. (SM). Clusters were determined using UPGMA. 1-2 : repetitions

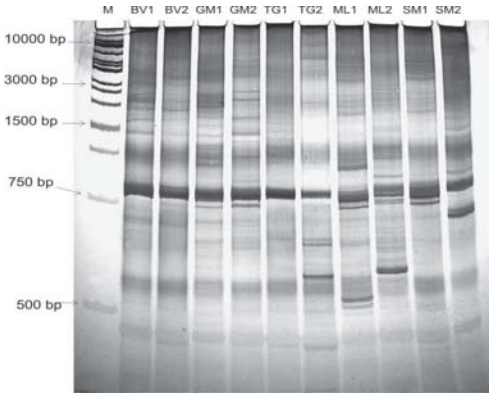

Figure 3. The profil of actinomycetes diversity in soil under the forest of *B. vulgaris* (BV), *G. maculata* (GM), *T. grandis* (TG), *M. leucadendron* (ML), and S. *macrophylla* King (SM) analyzed by nested-PCR RISA with actinomycetes specific primer F243. M = 1 kb DNA ladder marker; 1-2: repetitions.

Meanwhile, the community of actinomycetes from the highest were soils under the forest of *G. maculata*, *B. vulgaris*, *M. leucadendron*, *S. macrophylla* King, and *T. grandis*, respectively (Fig. 3). Figure 3 suggests that the highest actinomycetes community in soil under the forest of G. maculata may be supported by high content of organic matter and exudates secreted by the roots. Actinomycetes generally increased in number with an increase in the organic matter content of the soil (Henis, 1986). Hayakawa *et al*. (1988) also demonstrated that actinomycetes were abundant in soils with a higher organic matter content.

Dendrogram of nested-PCR RISA results for the five of forest types (BV, GM, TG, ML, and SM) is represented in Fig. 4. Figure 4 shows that community of actinomycetes was different in soils under the different forest types, except community of rhizobacteria under the forest of *B. vulgaris* and *G. maculata*. These results indicated that actinomycetes inhabiting under the two forest was dominated by certain actinomycetes grouped in one cluster.

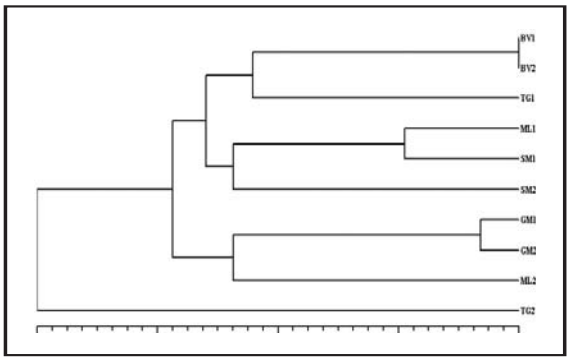

Figure. 4. Dendrogram of actinomycetes community based on nested-PCR RISA in soil under the forest of

Diversity analysis by culture-dependent approach

Culturable actinomycetes in various forest types of Wanagama I Yogyakarta, were isolated from soils under the forest of B. vulgaris, G. maculata, T. grandis, M. leucadendron, and S. macrophylla King. Analysis of isolated actinomycetes diversity was investigated by the identification of morphology (colony and cell), genetic (BOX element by rep-PCR), and secondary metabolites (thin layer chromatography). In addition, actinomycete isolates were assayed for their antifungal activity against S. cerevisae, C. albicans, F. oxysporum and A. flavus. The presence of Polyketide Synthase-I (PKS-I) and Non-Ribosomal Peptide Synthetase (NRPS) genes were amplified by degenerated primers to understand their correlation with antifungal activity of actinomycetes isolates.

Isolation of actinomycetes

Actinomycetes grew well on the starch nitrate agar medium. Fourty-three morphologically different isolates were obtained from various types of forest by using a culture-dependent approach (Figure 5). The higher of number of morphologically different isolates were isolated from soil under the forest of *M. leucadendron* and lowest under the forest of *B. vulgaris*. Figure

demonstrated that soils under the forest of Wanagama I Yogyakarta was dominated by unculturable actinomycetes. This results was agree with Amman *et al*. (1995), who explained that actinomycetes diversity can be analyzed by culture methods, but only a small part of microbial diversity is detected. The cultivability values range from 0,001 – 15%, depending on the environment factors.

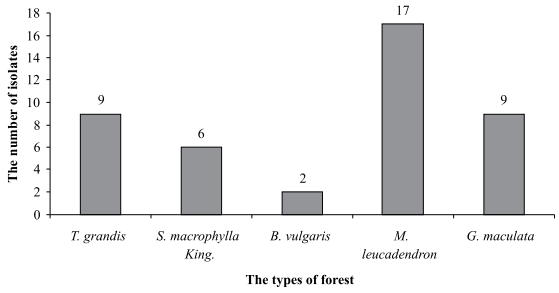

Figure 5. The number of different morfologically actinomycetes isolates from soils under the forets types of Wanagama I Yogyakarta

Genetic diversity of actinomycete isolates

Investigation results of genetic diversity of actinomycete isolates are presented in Figure 6.

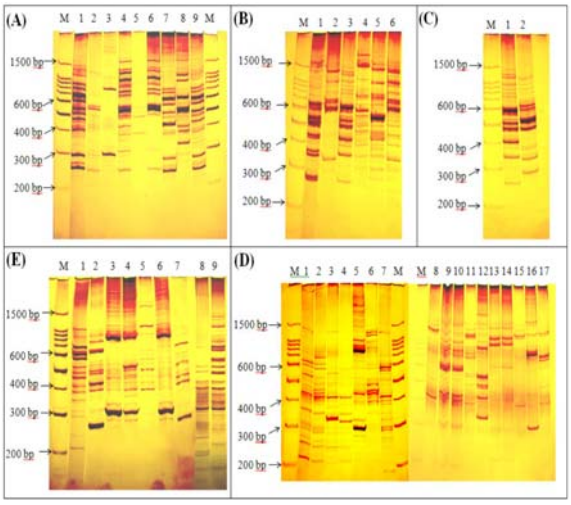

Figure 6. Genetic diversity of actinomycetes isolated from soil under the forest of *T. grandis* (**A**), *S. macrophylla* King. (**B**), *B. vulgaris* (**C**), *M. leucadendron* (**D**), and *G. maculata* (**E**) were analyzed by rep-PCR with BOXA1R primer. M : 100 bp DNA ladder, 1-17 : name of isolates

The similarity in DNA fingerprints indicates that BOX repetitive sequences of the bacteria were located at the same position in chromosome. It, therefore, could be predicted to have a close relationships. Nevertheless, each soil under the forest types had morphologically different isolates. These indicated that BOXA1R primer might not be suitable to amplify BOX sequences of every actinomycetes species.

Nuzliani *et al*. (2006) had analyzed genetic diversity of six bacteria isolates using rep-PCR and they found three DNA fingerprints. Genersch and Otten (2003) explained that the DNA fingerprint by rep-PCR were useful for classification of bacteria subtype or strain. Carson *et al*. (2003) had proved that DNA fingerprint by rep-PCR was accurate, reproducible and efficient method as compared to ribotyping technique.

Secondary metabolites secondary of actinomycete isolates

Diversity of secondary metabolites actinomycete isolates is illustrated in Figure 7. Figure 7 shows densitogram of secondary metabolites of selected isolates at UV 254 nm. The results showed that secondary metabolites were produced by actinomycetes from soil under the forest of *M. leucadendron* were more diverse than other forest types. This is represented by many peaks describing many secondary metabolites. Therefore, these results suggested that the determination of the diversity of bioactive compounds may be more clearly observed using scanning densitometry at UV 366 nm.

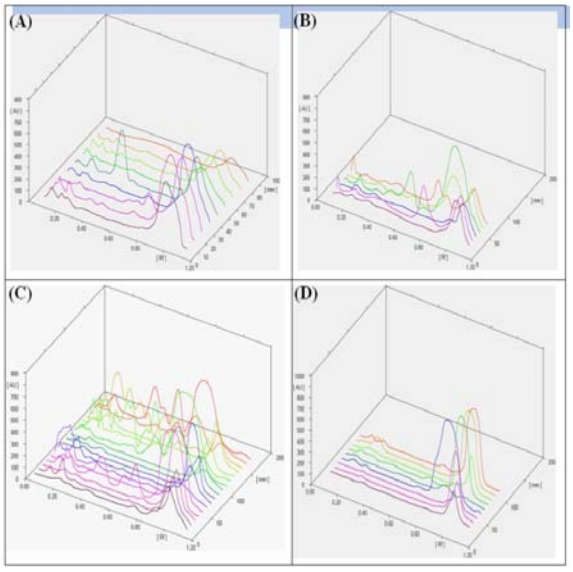

Figure 7. Diversity of secondary metabolites of actinomycete isolates analyzed by TLC. Secondary metabolites were extracted with ethyl acetate and visualized by UV 254 nm. **(A)** : profil of secondary metabolites diversity of isolates were isolated in soil under the forest of *T. grandis*, **(B)** : the forest of *S. macrophylla* King, **(C)** : the forest of *M. leucadendron*, and **(D)**: the forest of *G. maculata*.

Antifungal activity of actinomycete isolates

Actinomycetes have been evaluated as a source of biocontrol agents or antibiotic compounds based on their distribution in various habitats (Lee and Hwang, 2002). In this study, actinomycetes were isolated from soils under the forest of Wanagama I Yogyakarta had potency as a producer of antifungal compounds (Table 1). Among the 43 actinomycete isolates, 43 isolates (100%) had antagonistic activity against *Saccharomyces cerevisae*, 16 isolates (37.20%) against *Candida albicans*, 41 isolates (95.30%) against *Fusarium oxysporum*, and 36 isolates (83.70%) against *Aspergillus flavus*. In addition, secondary metabolites produced by all actinomycete isolates had antifungal activity at least against two fungies, moreover secondary metabolites were produced by several isolates had antifungal activity to all of fungi.

Table 1. Potency of actinomycetyes as antifungal producer from various types of forest

No	The types of forest	The number of actinomycete isolates which have antifungal activity (%)			
		S. cerevisae	*C. albicans*	*F. oxysporum*	*A. flavus*
1	*T. grandis*	100	22.20	100	88.90
2	*S. macrophylla*	100	16.70	83.30	66.70
3	*T. grandis*	100	100	100	100
4	*M. leucadendron*	100	41.20	94.10	82.35
5	G. *maculata*	100	44.40	100	88.90
	Total of isolates	100	37.20	95.30	83.70

Secondary metabolites were extracted with ethyl acetate. Solvents : ethyl acetate : chloroform : acetic acid (7: 2: 0.1%).

Detection of PKS-I and NRPS genes

The presence of Polyketide Synthase-I (PKS-I) and NonRibosomal Peptide Synthetase (NRPS) genes were detected by degenerated primers to understand their correlation with antifungal activity of actinomycetes isolates. Among the 43 isolates, NRPS gene were found in 25 isolates and both genes were only found in 1 isolate (data not shown), whereas the antifungal activity of each isolates was not significantly different. The data indicated that there was no always correlation between antifungal activity and the presence of PKS-I and NRPS genes. The absence of amplicons indicated that some of the isolates lack PKS-I and NRPS genes, the specific degenerate primer pairs might not be suitable to amplify these genes. Furthermore, not only PKS-I or NRPS gene is involved in the biosynthesis of bioactive secondary metabolites (Fingking and Marahiel, 2004).

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