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Screening of Actinomycetes Producing an ATPase Inhibitor of *Japanese Encephalitis Virus* RNA Helicase from Soil and Leaf Litter Samples

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Actinomycetes are commercially important microorganisms for the production of antibiotics, enzymes, inhibitors of enzymes, and other bioactive secondary metabolites. Some 853 isolates of actinomycetes were isolated from soil and leaf litter samples in Kupang NTT and Enrekang, South Sulawesi. Those isolates were then tested for inhibition of ATPase activity of RNA helicase from *Japanese encephalitis virus* (JEV), in order to identify a drug candidate for the treatment of JEV infection. Results revealed that 14 isolates have relatively high inhibition-activity on JEV ATPase activity of the JEV-RNA-helicase, which range from approximately 40.0-50.0% inhibition. The highest inhibition-activity was identified in *Actinoplanes philippinensis* 5-849 with 49. 9% of inhibition-activity and *Streptomyces chartreusis* 5-095 with 49.2% of inhibition-activity.

Key words: actinomycetes, *Japanese encephalitis virus,* JEV, RNA helicase, inhibitor

Aktinomiset merupakan mikroorganisme yang penting secara komersial untuk produksi antibiotik, enzim, enzim inhibitor, dan metabolit sekunder bioaktif. Sejumlah 853 isolat aktinomiset telah diisolasi dari sampel tanah dan serasah di Kupang (Nusa Tenggara Timur) dan Enrekang (Sulawesi Selatan). Isolat diuji kemampuannya untuk menghambat aktivitas ATP-ase dari RNA helikase *Japanese ensefalitis virus* (JEV), dalam rangka mencari kandidat obat bagi pengobatan infeksi JEV. Hasil penelitian menunjukkan ada 14 isolat yang memiliki aktivitas inhibisi 40-50% terhadap aktivitas ATPase dari RNA helikase-JEV, di antaranya ialah *Actinoplanes philippinensis* 5-849 dan *Streptomyces chartreusis* 5-095.

Kata kunci: aktinomiset, virus, *Japanese encephalitis virus*, JEV, RNA helikase, inhibitor

Japanese encephalitis virus (JEV) is one of the most prevalent causative agents of viral encephalitis with high morbidity and mortality. Approximately 50 000 human cases occur annually in Asia. In nature, JEV is transmitted between vertebrates by the mosquito *Culex tritaeniorynchus*, and causes infect approximately 10% of the susceptible population in Southeast Asian countries each year. In Indonesia, particularly, JEV is endemic in Kalimantan, Bali, Nusa Tenggara, Sulawesi, Maluku, Papua, and Lombok (Spicer 1997). This virus is recognized by the World Health Organization as a major threat to public health because of the high incidence of clinical infections, and approximately thirty percent of which are fatal and half result in neuropsychiatric sequelae. JEV belongs to genus *flavivirus* in the family *Flaviviridae*, whose members include several pathogens of humans and animals. Although vaccines have been developed since 1960, unfortunately no effective drug is clinically available so far. Several efforts have been performed to find a drug as a candidate for treatment of JEV infection, including finding inhibitors of enzymes which are essential for JEV replication such as protease, RNA helicase and RNA polymerase (Borowski *et al*. 2002).

Some studies recently discovered drug candidates for *flavivirus*, particularly *Hepatitis C virus* (HCV) and JEV, through RNA helicase inhibitor screening. Hatsu *et al*. (2002) noted that secondary metabolites from broth cultures of *Streptomyces* sp. could be act as inhibitor to RNA helicase of JEV. In general, the drug candidates were chemical substances such as ribavirin-5-triphosphate (Borowski *et al*. 2001) and SCH16, a derivatives of N-methylisatin-β-thiosemicarbazone (MIBT) (Sebastian *et al*. 2008) which were able to completely inhibit *in vitro* JEV and *West Nile virus* (WNV) replication, however the studies on the inhibition by natural products is still limited.

Actinomycetes are commonly found in natural substrates such as soils and litters, and play a significant role in the degradation of naturally organic substances (Williams *et al*. 1984). Therefore, actinomycetes have the ability to produce large amounts of secondary metabolites such as antibiotics, enzymes, enzyme inhibitors, and other bioactive compounds. About 70% of presently commercial antibiotics were found in secondary metabolites produced from actinomycetes (Miyadoh 1993).

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Furthermore, Indonesian actinomycetes have not been explored yet for screening of useful bioactive compounds such as inhibitors of JEV replication. Therefore, a study to find a novel strain with a novel secondary metabolite useful for pharmaceutical and industrial applications is important. In this study, we isolated actinomycetes from soil and leaf litter samples and screened the actinomycetes for inhibitor of ATPase activity of JEV RNA helicase.

MATERIALS AND METHODS

Microorganisms. The 853 isolated actinomycetes used for testing for inhibition-of ATPase activity of JEV RNA helicase were obtained from soil and leaf litter samples in Kupang, Nusa Tenggara Timur, and Enrekang South Sulawesi, Indonesia under collaborative research between Indonesian Institute of Sciences (LIPI), Indonesia and National Institute of Technology & Evaluation (NITE), Japan in 2005. The project have been done from 2004-2009. (Widyastuti and Ando 2009). In the project in 2005**,** SDS-yeast extract (SDS-YE) method (Hayakawa & Nonomura 1987), rehydration-centrifugation (RC) method (Hayakawa *et al.* 2000), and oil separation system (OSS) method were used in this study as referenced in Widyastuti and Ando (2009). All the isolates have been identified at the genus level by sequencing of their 16S rDNA at the project in 2005 as stated in Widyastuti and Ando (2009). In this study, the compiling 16S rDNA data of the isolates based on their isolation sources and isolation methods were done.

Growth Medium and Production Medium. Yeast extract-starch agar (YS agar) (Nonomura and Ohara 1969) was used as growth medium and contained 2 g yeast extract, 10 g soluble starch, 15 g agar added to 1 L distilled water and adjust pH to 7.3.

International *Streptomyces* project 2 (ISP 2) medium was used as production medium for screening the JEV ATPase inhibitor from actinomycetes, and contained 4 g yeast extract, 10 g malt extract, 4 g dextrose, 20 g agar added to 1 L distilled water and adjust pH to 7.3.

Expression and Purification of JEV NS3 Proteins. The expression plasmids containing JEV NS3 genes were independently transformed into *E. coli* BL21 (DE3) pLysS cells (Stratagene, La Jolla, CA, USA). After the IPTG induction at 37 °C for 3 h, the cells were harvested by centrifugation at 8000 rpm. The harvested cells were resuspended in buffer B (10 mM Tris HCl buffer (pH 8.5), 100 mM NaCl, 0.25% v/v Tween 20), and disrupted by sonication for 5 min on ice. The soluble fraction of the cell lysate was mixed with TALON metal affinity resin (Clontech, Palo Alto, CA, USA). After gentle mixing for 1 h at 4 °C, the resin was collected by a brief centrifugation and washed with buffer B. Resin-bound protein was eluted with 2 volumes of buffer B containing 400 mM imidazole. Eluted protein fractions were dialyzed against dialysis buffer (10 mM Tris HCl (pH 8.5), 100 mM NaCl, 10% v/v glycerol) at 4 °C. Purified JEV NS3 proteins from the transformant *E. coli* were then used as substrate helicase enzyme for ATPase assay (Utama *et al*. 2000a).

Colorimetric ATPase Assay. The amount of phosphate moiety released from ATP was measured. A 50 µL per well of reaction mixture containing 10 mM MOPS buffer (pH 6.5), 2 mM ATP, 1 mM $MgCl_2$, purified NS3 protein (0.8 pmol) and 5 µL per well of culture supernatant as inhibitor substances was incubated in a 96−well microtiter plate at room temperature for 30 min. The reaction was stopped by adding 100 µL per well of dye solution (water:0.081% malachite green:5.7% ammonium molybdate in 6 N HCl:2.3% polyvinyl alcohol = 2:2:1:1, v/v). After the addition of 25 µL per well of 30% sodium citrate, the absorbance at 620 nm with a reference wavelength at 492 nm was measured.

Percentage of inhibition (%) was measured using the equation (A-I) /A x 100, which A was absorbance without added inhibitor substances, and "I" was absorbance with added inhibitor substances (Hatsu *et al*. 2002). In this assay the amount of free phosphate moiety released from ATP was measured via absorbance of RNA helicase enzyme. We have measured the change of absorbance with and without added inhibitor substances. If there was no inhibitory effect from the supernatant of actinomycetes, the JEV RNA helicase enzyme would continue to hydrolyze ATP to ADP and inorganic phosphate released from this reaction will be bounded with malachite green and ammonium molibdate made a coloured complex compound of phosphomolybdate-malachite green. The absorbance of this coloured complex compound were detected using spectrophotometer.

RESULTS

Actinomycetes Isolates. Of the 853 isolates of actinomycetes used in this study, 529 isolates were defined as a group of *Streptomyces* (Family *Streptomycetaceae*), and 324 isolates as a group of non-*Streptomyces* or so called rare actinomycetes (other family in the order *Actinomycetales*). Further, from the group of non-*Streptomyces*, 381 have zoospore (motile actinomycetes) and 153 do not have zoospore (non-motile actinomycetes). Based on results in origin, 716 were isolated from soil and 137 were isolated from leaf litter samples. Some 488 isolates from the soil sources

were identified as belonging to the family *Streptomycetaceae* and 228 isolates were identified as group of non-*Streptomyces*. A total of 137 isolates obtained from leaf litter samples were divided into 41 isolates of the family *Streptomycetaceae* and 96 isolates of the group of non-*Streptomyces* group (Table 1). This result revealed that the genus *Streptomyces* was the dominant actinomycetes in the soils used. Based on results of the isolation methods, 468 were isolated by SDS-YE method resulted in *Streptomyces* as the major genus, 286 were isolated by RC method resulted *Actinoplanes* as the major genus, and 99 were isolated by oil separation method which resulted *Streptomyces* as the major genus.

Twenty eight genera were found in Kupang, Nusa Tenggara Timur and 40 genera were found in Enrekang. Furthermore, 33 genera were found in soils samples and 12 genera were found in litter samples. The genera of *Dietzia, Kocuria, Planomonospora, and Sphaerosporangium* were only found in *Kupang, and the genera of Actinokineospora, Cellulomonas, Dermatophilus, Geodermatophilus, Agromyces, Virgosporangium,* and *Planotetraspora were only found in Enrekang* (Table 2). The genus *Streptomyces*, *Actinoplanes*, and *Nonomuraea* were abundant in soil samples and the genus *Actinoplanes*, *Streptomyces*, and *Kinesoporia* were abundant in litter samples.

Screening of Actinomycetes which Have Inhibition-Activity on ATPase of JEV RNA Helicase. All actinomycetes isolates obtained from soil and leaf litter samples in Kupang and Enrekang were then screened for their ability to inhibit ATPase activity of JEV RNA helicase. Of the 853 actinomycete isolates, 14 isolates produced metabolites having inhibitory activity as high as 40-50%. Thirty-five isolates demonstrated inhibition to 30-40% and 37 isolates showed 20-30% inhibitory activity (Fig 1).

Of the 14 isolates actinomycetes which were found to have the highest potency as antiviral compound were presented in Table 3, the bioactive compounds produced from secondary metabolites were released in broth cultures. The highest inhibition-activity was identified in *Actinoplanes philippinensis* 5-849 with 49. 9% of inhibition-activity and *Streptomyces chartreusis*. 5-095 with 49.2% of inhibition-activity.

Table 1 Actinomycetes isolated from leaf litter and soil samples

Sampling source	Sampling site	Σ isolate	*Streptomyces*	Non-*Streptomyces*	
				Motile	Non-motile
Litter	Kupang	59	31	23	5
	Enrekang	78	10	54	14
Soil	Kupang	318	210	52	56
	Enrekang	398	278	42	78

Compiling data from Final Report of Collaborative Research Project in 2005 between LIPI-NITE 1 (Widyastuti and Ando 2009).

Table 2 Genera of actinomycetes found in soil and leaf litter samples

Soil	*Actinokineospora*	-	2	2
	Actinoplanes	47	25	72
	Catenuloplanes	3	7	10
	Couchioplanes	1	2	3
	Dactylosporangium	1	2	3
	Planotetraspora	-	4	4
	Actinomadura	1	4	5
	Agromyces	-	1	1
	Cellulomonas	-	1	1
	Cryptosporangium	7	11	18
	Dermatophilus	-	1	1
	Dietzia	1	-	1
	Geodermatophilus	-	1	1
	Isoptericola	1	2	3
	Kocuria	1	-	1
	Kribbella	3	13	16
	Microbispora	-	1	1
	Micromonospora	7	5	12
	Mycobacterium	7	6	13
	Nocardia	2	10	12
	Nocardioides	1	4	5
	Nonomuraea	16	11	27
	Planomonospora	-	4	4
	Planobispora	1	-	1
	Promicromonospora	2	2	4
	Pseudonocardia	3	1	4
	Saccharothrix	1	1	2
	Sphaerosporangium	1	-	1
	Streptacidiphilus	-	2	2
	Streptomyces	210	272	482
	Streptosporangium	1	1	2
	Virgosporangium	-	1	1
	Verrucosispora	-	1	1
Leaf litter	*Actinoplanes*	10	45	55
	Catenuloplanes	-	1	1
	Couchioplanes	-	2	2
	Dactylosporangium	1	-	1
	Kineosporia	12	6	18
	Amycolatopsis	-	1	1
	Cryptosporangium	-	11	11
	Krasilnikovia	1	1	2
	Micromonospora	2	1	3
	Nocardia	-	1	1
	Promicromonospora	-	1	1
	Streptomyces	31	10	41
Total				853

Data source: Compiling data from Final Report of Collaborative Research Project in 2005 between LIPI-NITE 1 (Widyastuti and Ando 2009).

DISCUSSION

Actinomycetes are prokaryotic microorganisms belonging to a group of Gram positive bacteria that have high G+C contents, are saprophytic, produce large amount of sporangia and stable mycelium. Actinomycetes are widely distributed in nature and in man-made environments, and play an important role in the degradation of organic matter. They are also well known as a rich source of antibiotics and bioactive

Table 3 Actinomycetes have highest inhibitory activity to ATPase activity of JEV RNA helicase enzyme isolated from soils and leaf litter samples in Kupang and Enrekang

Isolate	Inhibition (%)	Name of isolate
5-087	45.1	*Streptomyces floridae*
5-095	49.2	*S. chartreusis*
5-096	43.5	*S. marokkonensis*
5-119	47.0	*Streptomyces Smarlab 330204*
5-124	46.0	*S. violens*
5-217	40.9	*S. albus*
5-218	44.3	*S. ginsengisoli*
5-224	41.0	*S. albus*
5-264	43.6	*S. cyanoalbus*
5-304	44.2	*S. durhamensis*
5-320	41.4	*S. pulveraceus*
5-800	42.3	*S. badius*
5-849	49.9	*Actinoplanes philippinensis*
5-1036	40.4	*Kribbella flavida*

Table 4 Actinomycetes having inhibitory activity to ATPase activity of JEV RNA helicase enzyme isolated from soils and leaf litter samples at Kupang and Enrekang

Inhibition (%)	Number of Isolates
0	594
0.01-10	96
10.01-20	77
20.01-30	37
30.01-40	35
40.01-50	14

molecules, and are of considerable importance in industry (Seong *et al*. 2001). Many are well known for their economic importance as producers of biologically active substances, such as antibiotics, vitamins, and enzymes (Basilio *et al*. 2003).

This report revealed as stated also in Widyastuti and Ando (2009), that species diversity in the soil samples (33 genera) are higher when compared with the litter samples (12 genera), this is in line with the review of Hayakawa (2008) that states soil is the natural habitat of actinomycetes. The genus *Streptomyces* was widely distributed in Kupang, Nusa Tenggara Timur, and Enrekang both in sampling sources. As noted by Hayakawa *et al.* (2000), rare actinomycetes were divided in two groups according to the type of zoospora produced, non-motile actinomycetes and motile actinomycetes. Non-motile actinomycetes formed non-flagellated zoospores, such as *Nocardia*, *Micromonospora,* etc. and motile actinomycetes formed flagellated zoospores. Examples of motile zoospora are *Actinokineospora, Actinoplanes, Catenuloplanes, Couchioplanes, Dactilosporangium,* and *Planotetraspora.* Actinomycetes isolated from Enrekang soils and leaf litter samples were abundant with rare actinomycetes from the genus *Actinoplanes*.

Based on the isolation method as stated in Widyastuti and Ando (2009), SDS YE methods were useful for isolation of the genus *Streptomyces*. On the other hand, RC methods gave an abundance of the genus *Actinoplanes*. Further, in this research it can be seen that the actinomycetes originating from litter sample was dominated by motile actinomycetes (the genus *Actinoplanes*), while from the soil was dominated by non-motile actinomycetes (the genus *Streptomyces*) (Table 1). The RC method is a simple enrichment method incorporating differential centrifugation for the isolation of motile actinomycetes (Hayakawa *et al*. 2000). The phosphate buffer-soil extract solution liberates motile zoospores and centrifugation eliminates *Streptomyces* and other non-motile actinomycetes from the supernatant, thereby facilitating selective growth of motile actinomycetes on the isolation plates subsequent to inoculation (Hayakawa 2008). The OSS method is a selective isolation methods expected to favour actinomycetes from the genus *Rhodococcus*, but unfortunately in this study this genus is not obtained. Using OSS methods, the genus *Mycobacterium was found*. This may be because the *Mycobacterium* isolates were putatively closely related with the genus *Rhodococcus* on the basis of their mycolic acid profiles.

Our analysis of screening for inhibitory activities to JEV RNA helicase enzymes showed a high proportion of inhibitory activity was produced by *Streptomyces* species. Seeking an inhibitor to JEV RNA helicase was performed through RNA-stimulated-ATPase-activity (ATP hydrolysis). Inhibition of ATP hydrolysis, was derived from an indirect inhibition of RNA helicase-mediated hydrolytic processing so that the energy performed from hydrolysis ATP was not available to unwind dsRNA JEV.

Colorimetric ATPase assay was chosen for detection of hydrolyzed ATP, because that was the easy and safe methods, without using radioactive compound which relatively unstable and caused radiation contamination (Utama *et al*. 2000b; Boguszewska-Chachulska *et al*. 2004). Robarts *et al*. 2004 and Welbourn *et al*. 2005 used colorimetric ATPase assay to detect ATP hydrolysis from RNA helicase JEV.

Screening inhibitors of JEV RNA helicase from actinomycetes showed a varied percentage of inhibition among the isolates, between 0% and 49.9% (Fig 1). The highest percentages of inhibition were 49.9% performed by isolate 5-849 identified as *Actinoplanes philippinensis* and 49.2% inhibition-activity obtained

from isolate 5-095 identified as *Streptomyces chartreusis* (Table 3). *Actinoplanes philippinensis* isolated from leaf litter samples at Enrekang was a rare actinomycetes. The role of rare actinomycetes as bioactive molecule sources became apparent as these organisms provided about 25% of the antibiotics of actinomycete origin reported during 1975 to 1980. Rare actinomycetes have usually been regarded as strains of actinomycetes whose isolation frequency by conventional methods is much lower than that of *Streptomycetes* strains.

Hatsu *et al*. (2002) noted that secondary metabolites from broth cultures of *Streptomyces* could act as inhibitor to RNA helicase enzyme of JEV. Helicase enzyme was a great potential target for the discovery new antiviral compounds because this enzyme is essential enzyme for replication of the virus. Helicase enzyme has a function as not only for helicase activity, but also RNA binding activity and RNA-stimulated-ATPase activity, both of these activities influence helicase activity. This function is interrupted, or at least depressed, by the existence of an ATPase inhibitor. Therefore this make the enzyme an attractive target for development new antiviral drugs, because an inhibitor of helicase enzyme could be due to the discovery of an inhibitor of RNA binding activity or an ATPase inhibitor.

In general, the active isolates showed an inhibition of ATPase activity of JEV RNA helicase enzymes were mainly produced from genus *Streptomyces*, although some of the non−*Streptomycetes* isolates also had inhibitory activity. Whether the activities being detected in these cases were due to single inhibitor acting on multiple microbial species, or were mixtures of compounds with different specificities, is unclear without chemical fractionation of the active extracts.

A diverse percentage of inhibition existed among the isolates, suggesting a varied ability of microorganisms to produce inhibitor compounds. *Streptomyces* are fast growing actinomycetes, will reach their stationary phase earlier at which stage the secondary metabolites are produced. Differences in incubation time to reach the stationary phase between *Streptomyces* and rare actinomycetes could act as a factor causing diverse inhibitory activity.

Of some of the antiviral candidates discovered, the drug candidates were proven *in vitro* and *in vivo*, such as BAY 57-1293 a compound which suppresses *Herpes simplex virus* (HSV) infection in the monkey (Betz *et al*. 2000). Some studies recently noted the discovery of *flavivirus* drug candidates through helicase-inhibitor-screening, particularly for HCV and JEV. In general, the drug candidates were chemically substances such as ribavirin -5"-triphosphate (Borowski *et al.* 2001), while the studies on naturally occuring inhibitors are rare or yet to be done. In the present study, we report on biologically active compound from actinomycetes which potentially have antiviral activity making them promising new drug candidates.

The existence of candidate strains with high inhibition of ATPase activity of JEV RNA helicase enzymes, suggests that Indonesian actinomycetes have not been thoroughly investigated and therefore have potential as a source of novel bioactive compounds. These results not only suggested the usefulness of Indonesian actinomycetes as screening sources of natural product based drug discovery programs, but also may be useful as basic data such as distribution studies.

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