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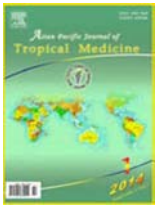

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Screening and study of antifungal activity of leaf litter actinomycetes isolated from Ternate Island, Indonesia

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ABSTRACT

Objective: To characterize abundance of leaf litter actinomycetes from Ternate Island and to assess the antifungal activity of actinomycetes isolates against *Candida albicans*, *Saccharomyces cerevisiae (S. cerevisiae)*, and *Aspergillus niger*.
Methods: Actinomycetes were isolated from leaf litter of *Durio* species, *Syzygium aromaticum*, *Piper betle*, *Myristica fragrans*, or *Pandanus* species and unknown plants. Actinomycetes isolates were cultured in a liquid medium. Bioactive compounds were extracted and tested against fungal using Beury-Kirby method with modification. Minimum inhibitor concentration and cell leakages were conducted. Actinomycetes that produced the highest antifungal activity were indentified using molecular sequence data in 16S rRNA gene.
Results: Out of 50 selected isolates, two isolates MG-500-1-4 and SR-2-2 has highest activity against *S. cerevisiae*. Concentration of material containing nucleic acids, proteins, Ca^{+} and K^{+} ions and morphological observations indicated that extracts of MG-500-1-4 and SR-2-2 caused cell leakage and invagination of *S. cerevisiae* cells. Based on 16S rRNA gene identification, MG-500-1-4 and SR-2-2 isolates are similar to *Streptomyces misakiensis* and *Streptomyces tricolor* respectively.
Conclusions: Ternate Island contains interesting biodiversity of actinomycetes that has potential use in agriculture, fisheries, and human health to reduce problem of fungal pathogen.

1. Introduction

Ternate Island (Moluccas) is one of eastern Indonesian islands located in Wallace biogeographical region. These regions are meeting-point of the biota of two major geographical regions: Australia and Oriental region. Ternate has a large number of species that are unique to the area and is recognized as a biodiversity hotspot[1]. The diversity of microbes of Ternate Island is expected to be more higher than that of plants. This is based on the prediction of ratio of unique species and vascular plant of 6:1[2]. Despite the richness of fauna and flora of Ternate Island, there is only a few information about microbial diversity. Microbial diversity of the island is, therefore, intriguing to study. Actinomycetes belong to a microbial group that is still interesting to explore until now due to their potency to produce active substances against drug resistance microbes[3]. In agriculture, actinomycetes are promising to be used for biocontrol against fungal pathogen of plant[4], and bacterial pathogen in aquaculture[5]. Fish pathogenic fungi is also a problem in aquaculture[6]. Fungal diseases candidiasis and aspergillosis are infections caused by fungi, leading to increasing morbidity and mortality[7]. Thus, actinomyctes having activity against harmful fungi are useful not only for agriculture and aquaculture but also for human health.

In an ecosystem, leaf litter is an important source of microbes including actinomycetes[8]. Moreover, it is also reported that actinomycetes could be antagonist to decomposer fungi including fast growing genera *Mucor*, *Penicillium*, *Trichoderma* and moderately slow-growing genera *Cladosporium* and *Mortierella*[9]. The aim of this research is to obtain information about actinomycetes abundance from leaf litters in Ternate

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Island and to reveal their potential activity against yeast *Candida albicans* (*C. albicans*), *Saccharomyces cerevisiae* (*S. cerevisiae*) and filamentous fungi *Aspergilus niger* (*A. niger*).

2. Material and methods

2.1. Sample site of actinomycetes isolation

Leaf litter samples were obtained from Ternate Island, mainly in Gamalama Mountain, Northern Moluccas, Indonesia. Sites for sampling had been taken according to the altitude and canopy of plantation (monoculture) or vegetation (heterogenic mixture) of 5 species: *Durio* species, *Syzygium aromaticum (S. aromaticum)*, *Piper betle (P. betle)*, *Myristica fragrans*, or *Pandanus* species and others (mixture of unknown plant species) respectively.

2.2. Isolation of actinomycetes

2.2.1. Fermentation and extracts preparation

Actinomycetes isolates were cultured in a liquid medium as follows. A loopful of mature slant culture was inoculated into a 50 mL culture tube containing 10 mL Actino medium No. 1 (Daigo, Japan) of the seed medium consisting of 5 g polypeptone, 3 g yeast extract and 1 L water, pH 7.2. The inoculated tubes were shaken on a rotary shaker (130 r/min) at 28 °C for 7 d. About 3 mL of the seed culture were transferred into a 500 mL flask containing 250 mL of the production medium consisting of glucose with same composition. The inoculated flasks were shaken on a rotary shaker (130 r/min) at 28 °C for 7 d. Then, 250 mL of ethyl acetate: methanol (4:1) was added to the flasks and they were shaken for 1 h. Mycelium was removed by filtration and centrifugation. The resulting supernatant without water was evaporated until dry powder. The powder extracts were resolved using acetone and used for bioassay.

2.2.2. Antifungal assay

Three strains of fungi/yeast tested in this screening were *C. albicans* NBRC 1594, *S. cerevisiae* NBRC 10217 and *A. niger*. Fungi and yeasts were cultured on fresh yeast malt agar. The inhibitory effect of the extract obtained from leaf litter actinomycetes was tested by a modified Beury-Kirby method with paper discs (5 mm diameter). Sterile paper discs were placed on the surface of assay plates, and 20 μL of extract were pipetted onto each disc and incubated at 35 °C. The inhibition zones around each disc were measured after 24 h.

2.2.3. Identification and phylogenetic analysis

Identification and phylogenetic analysis were conducted just for active actinomycetes isolates. Isolated strains were cultivated in 5 mL of Actino medium No. 1 broth (ISP2). Pellets were collected during early log phase growth. DNA extracted was conducted with GES method. About 0.5 μL DNA from extraction process was used as a temple for polymerase chain reaction (PCR) amplification of an approximately 1500 base segment of the 16S rDNA gene. The PCR primers were 20F (5′-GATTTTGATCCTGGCTCAG-3′) and 1500 R (5′-GTTACCTTGTTACGACTT-3′). The resulting PCR products were purified with polyethylene glycol precipitation[10]. The purified PCR products were sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems Inc., Foster, California) with the BigDye Terminator version 3.1 sequencing method. The sequencing primer used was primer 20F (5′-GATTTTGATCCTGGCTCAG-3′). The sequences were then assembled with BioEdit.

The 16S rDNA sequences inferred from the 16S rDNA sequences determinated as described above were aligment with 16S rDNA sequences from selected species from several related genera. Sequences of the reference strains were obtained from NCBI gene bank and were chosen based on a hight similarity rank with the strain from the actinobacteria along with a sequence to serve as an outgroup. Approximately 800 bases were included in the phylogenetic analysis, which was performed with the Clustal X 8.13 version and NJ plot win 95 computer program.

2.2.4. Minimum inhibitor concentration (MIC)

MIC value was determined based on broth micro dilution method[11,12]. Fungi were cultivated in Sabouraud broth at 35 °C, respectively until microbial concentration reached approximately 1×10^5 CFU/mL to 5×10^5 CFU/mL. We conducted 3 times repetition. Agar diffusion method for confirm MIC value was done. We used commercial antibiotic, such as antibacterial and antifungal, to compared the results.

2.2.5. Fungal cell leakages: protein, nucleic acid and ion

Cell leakage analysis was carried out based on measurement of content of material absorbing at 260 nm or 280 nm according to Lunde and Kubo[13], and ion K^+ and Ca^+ according to Prashar method[14], with some modifications. Yeast grew up in Saboroad medium for 24 h. About 10 mL suspension was added with Tween-80 and centrifuged at 3 500 r/min for 20 min. Supernatant