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An Actinomycetes Producing Anticandida Isolated from Cajuput Rhizosphere: Partial Identification of Isolates and Amplification of *pks-I* genes

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Abstract

Actinomycetes have been the most prolific producer of various kinds of antifungal metabolites, and many of them are described as being produced by polyketide synthetases (*pks*). We present strain of Actinomycetes producing anticandida isolated from rhizosphere plant for amplification of Pks-I genes. The isolate was obtained from Wanagama I Forest UGM Yogyakarta. Gene of seven isolates, from total of 173 isolates, were amplified using degenerate primer to detect the presence of *pks* genes. One strain that is named *Streptomyces* sp. GMR-22 was partialy identified as anticandida producing actinomycete. The strain shown the strongest activity against *Candida albicans*. Based on bioautography assay, one spot active with R_f 0.57 was appeared as bright yellow by cerrium sulphate but it was and not visible on UV_{254} and $_{366}$ lights.

Key words : *pks* genes, anticandida, *Streptomyces* sp GMR-22, rep-PCR, cajuput rhizosphere

Introduction

The fungal infections is reported increasing by 400% over the past two decades, commonly caused by *Candida* (Nedal, 2007; Georgopapadakou and Walsh, 1994). The increasing of mycosis has important implications for health care costs in the community (Ghannoum and Rice, 1999). Broad spectrum antifungal agents are important in todays medical field due to a significant increasing in numbers of immunosuppressive patients. Therefore, it is very important to develop new antifungal agent with improved pharmacological properties, both natural bioactive product and synthesized ones. The history of new drugs discovery showed that novel antibiotic agents have found from actinomycetes (Badji *et al*., 2006).

Soil actinomycetes, especially belong to genus *Streptomyces* have been focused on intensive research for the past decades because the strain are prolific producer of active metabolite natural product (Jongrungruangchok *et al*., 2006). It has been estimated that approximately two-thirds of natural accurring antibiotics have been isolated from actinomycetes (Lemriss *et al*., 2003).

In recent years, the screening of new strain has been exponentially increased, but the number of novel compounds has decreased in the same proportion. This problem is due to old molecules that were being rediscovered by conventional procedures that are use

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today (Ketela *et al*., 2002). Detection of gene sequences involved in synthesis of secondary metabolites especially antifungal polyketide has already been described in different taxonomic groups of Actinomycetes (Sacido and Genilloud, 2004). This method may represent alternative approach to be focused on screening of a strain with the highest potency on producing polyketide substances.

A broad range of bioactive secondary metabolite polyketides and peptide substances which were applied in medical, pharmaceutical and agriculture especially antifungal are synthesized by type-I polyketide synthases (*pks*). Polyketides are natural product assembled or biosynthesized from condensation of repeating ketide or acetate units (Mayer *et al*., 2007; Moore and Hertweck, 2002). Genes encoding *pks* have been identified and characterized for a rapidly growing number of biologically active compounds. Type I of *pks* are multifunctional enzymes, whereas type II of *pks* are composed of single function enzymes that associated to form a complex. However, some polyketide products require the coordinated function from several type I enzyme in a consecutive building process (Walsh, 2004). The purpose of this research was to study about the amplification of *pks* gene from actinomycetes which producing anticandida substances.

Materials and Methods

Isolation of actinomycetes from soils

Actinomycetes was isolated from the medicinal plant rhizosphere soil that were collected by dilution agar plating method using Starch Nitrate Agar suplemented with cycloheximide (50 mg/l, dissolved in 70% EtOH) as described by Ahmed (2007). A plant species, cajuput *(Melaleuca leucadendron* L*)* was selected for the study. Sample were collected from Wanagama Forest in Daerah Istimewa Yogyakarta Province on 2007. Starch Nitrate Agar (SNA) medium was used for bacteria screening and isolation which consisted of starch soluble 20g, NaCl 0.5g, KNO_3 1 g, $K_2HPO_4.3H_2O$ 0.5g, $MgSO_4.7H_2O$ 0.5g, $FeSO_4.7H_2O$ 0.01 g, agar 15-20g, aquadest 1 l pH (7.2-7.4). Sample (about 10 g) of air-dried soil were mixed with sterile destilated water (100 ml). The soil suspension were heated in waterbath at 70°C for 1 h, then homogenized by shaking at 200 rpm for 30 min at 30°C. One mililiter of soil mixtures were transferred to 9 ml of sterile destilated water and subsequently to final dilution 10^{-6} . About 0.1 ml of final dilution was spreaded to the media surface by using drigalsky stick. Plates were incubated at 30°C up to 20 days. Actinomycetes colonies with different morphologies were selected and transferred to Starch Nitrate Agar slant for taxonomic studies and determination of antifungal activity.

Morfological characteristic

Specimen for microscopic observation were prepared by cover slip culture methods as follows : colony from a stock culture of isolates was spreaded on SNA medium then followed by buried cover glass slip in media, incubated at 30°C for 1 week. The mycelium structure, color and arrangement of spora was observed by microscope (400x magnification). The cultural characteristic of strain were examined on various ISP media after incubated for 2 weeks at 30°C. Determination of culture were performed using the methods described by Shirling and Gottlieb (1966).

Detection of in vitro anticandida activity

Anticandida activity of these isolates was carried out by dual assay method in Malt Extract Agar (MEA) (Aghighi *et al*., 2005). Isolates were grown on SNA plates for 7 days at 37°C, then a cylinder borer (8 mm in diameter) was cut out of the actinomycetes colony and placed on the test media (MEA) which had inoculated with tested fungal *Candida albicans*. The plate was incubated for 3 days at 35°C and the degree of inhibition to

test fungal growth were measured. A plate with no actinomycetes inoculant was used as a control. The diameter of inhibition zone was determined by lack of fungal growth that was surrounded cylinder borer contained actinomycetes inoculant. Inhibition zone were then differed as strong inhibition (>40 mm), moderate inhibition (<40-30 mm) and weak inhibition (<20mm).

Extraction of the anticandida

Anticandida production was observed by 10 days of incubation and the broth of fermentation media was centrifuged at 3500 rpm for 15 min to separate the biomasss. Different solvents were used for extraction of the antifungal compounds, such as n-hexane, benzene, chloroform, and ethylacetate. Supernatant of the culture was added to solvent with 1:1 v/v proportion, then shaked for 25 min. The solvent was separated using funnel and evaporated to obtain the maserate. The maserate was concentrated by rotary evaporator and dried with anhydrous Na_2SO_4 (Augustine *et al*., 2005)

TLC-Bioautography of antifungal

The active fraction of the isolate was determined by using Thin Layer Chromatography-Bioautography as described by Frandberg *et al.*(2000). The TLC was performed using 0.2 mm-thick silica gel plates (Silica gel 60, F_{254}, Merck). Ten microliters of fraction were applied to the plates, then developed with $CHCl_3$:EtOAc:MeOH, (5:4:1) as solvent system. After the solvent system was evaporated from the TLC plate, the plate was then placed on media agar culture which had inoculated with test fungi. The plates were kept in a refrigerator for 2 h and then incubated at 35°C for 2 days.

Genomic DNA extraction

Extraction of genomic DNA was done with method as described by Song *et al.* (2004) and Badji *et al.*(2006). Isolates of actinomycetes were grown for 4 days at 30°C with shaking in 250 ml flask containing 70 ml of ISP2 medium. Pellet of isolates was harvested by centrifugation (3.000 rpm 15 min) and washed twice with aquabidest. About 300 mg of mycelia was used for DNA extraction as follows : the sample was dispersed in 400 µl TE and resuspended with 400 µl SET buffer, 50 µl lysozyme (10 mg/ml), 20 µl proteinase K (20 mg/ml) was added and incubated at 37°C for 1 h. RNAase A solution was added, and the suspension incubated at 65°C for 10 min. The lysate was extracted with an equal volume of phenol and centrifuged (13.000 rpm 10 min). The aqueous layer was re-extracted with phenol (1:1v/v), and then by chloroform (1:1v/v). DNA was recovered from the aqueous phase by adding cooled ethanol prior to centrifugation. The precipitated DNA was cleaned with 100 µl ethanol 70% and then supernatan was removed. The purity of DNA solution checked spectrophotometrically at 260 and 280 nm.

Repetitive extragenic palindromic-PCR (rep-PCR)

Repetitive DNA fingerprinting was performed on all isolates following the method of 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 as follow: 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. Data from electrophoresis of PAGE results was subjected to Unweight Pair Group with Mathematical Average (UPGMA) analysis.

Amplification of pks gene

The *pks* genes were amplified from purified DNA of the strain as decribed previously (Sacido and Genilloud, 2004; Petrosyan *et al*, 2003) using a commersial kit (Mega Mix Blue) and primers K1F (5'TSAAGTCSAACATCGGBCA3') and M6R (5'CGCAGGTTSCSGTACCAGTA3'). The condition of amplified fragment was as follow : pre-denaturation of the target DNA at 98°C for 3 min followed by 30 cycles at 94°C for 1 min, primer annealing at 57°C for 1 min, and primer extension at 72°C for 5 min, the reaction mixture was held at 72°C for 5 min and then cooled at to 4°C. Detection of amplification gene products were analyzed by electrophoresis in 1.5% (w/v) agarose gels stained with ethidium bromide.

Results and Discussion

Isolation of actinomycetes from soils

In this study, actinomycetes isolate was isolated from rhizosphere plant of cajuput, because previous investigations support that the extent of Actinomycetes are higher in roots area of medicinal plant. Taechowisan *et al.* (2005) isolated antifungal producing *Streptomyces* from the root of Zingiberaceae. Bouizgarne *et al.* (2006) found a novel of isochainin from a strain *Streptomyces* sp. isolated from rhizosphere soil of the indigenous Moroccan plant *Argania spinosa* L. Cao *et al.* (2004) reported that endophytic actinomycetes from roots and leaves of banana (*Musa acuminata*) plants showed their activities against *Fusarium oxysporum f.sp. cubence*. Merckx *et al.* (1987) indicated that the rhizosphere represents a unique biological niche that supported abundance and diversity of saprophytic microorganisms because high input of organic materials derived from the plant roots and root exudates.

One hundred and seventy three strains were isolated and resulted differences on colony morphology. Among 27 strains that produced anticandida substances, only 7 strains were selected and used for amplification of *pks-I* genes. One strain (*Streptomyces* GMR-22) showed a strong producing anticandida and used as a primary candidate for study. In the Table 1, anticandida activity of 7 isolates on Malt Extract agar medium are summarized.

Table 1. Anticandida activity of selected actinomycetes against *Candida albicans*

Name of Isolates	Diameter of inhibition zone (mm)
GMR-9	34.6
GMR-17	35.3
GMR-18	40.8
GMR-19	30.5
GMR-22	**45.2**
GMR-27	41.3
GMR-36	40.6

strong inhibition (>40 mm), <40-30 mm (moderate inhibition) and <20mm (weak inhibition).

Strain GMR-22 showed strongest activity against *C.albicans* both unicelluler and multicelluler fungi (Figure 1). Based on these tests, GMR-22 was selected as the final candidate for screening of bioactive subtances (anticandida) because it fulfilled all of necessary criteria, such as : fast growth on many media, high inhibition activity and broad antifungal spectrum (data not shown).

Culture characteristic of GMR-22 are revealed abundant to moderate on several media except Nutrient Agar medium (Table 2). Powdery aerial mycelia formed and turned to white grayish when mature on all media. Soluble pigment was not produced on any media tested; melanin was not produced.

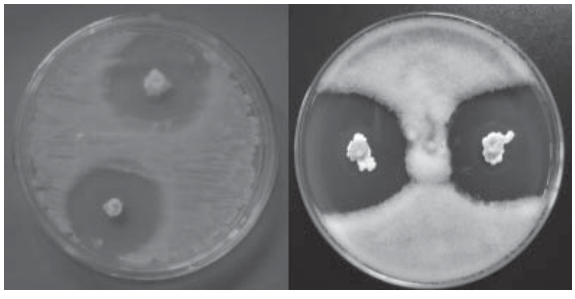

Figure 1. Zones of growth inhibition caused by metabolites of *Streptomyces sp.GMR-22* grown on medium agar for 3 and 7 days. A. *Candida albicans* B. *Tricoderma resei*

Tabel 2. Cultural characteristics of strain *Streptomyces sp*.GMR-22

Medium	Colony Color	Growth	Aerial Mycelium	Substrate Mycelium
ISP2	Brown	Abundant/Good	White- Grayish	Black
ISP3	Gray	Moderate	Gray	White
ISP4	Gray	Moderate	Black	Creamy
ISP5	White	Moderate	White-Grayish	Grayish
SNA	Brown	Abundant	White	Light Yellow
Bennett	Brown	Abundant	White	White
Nutrient Agar	White	Poorly	Yellowish	Gray
Gzapek's Agar	White	Moderate	White	White
TSA	White	Moderate	White	White

Detection of in vitro anticandida activity

Initial studies in shake flask determined a medium that was promising for producing anticandida. Starch Nitrate broth and Bennett medium were using for fermentation medium, with addition of peptone and KNO_3 (data not shown). The anticandida substance of GMR-22 was extracted from supernatant using different solvent, all solvent fractions showed inhibition activity except ethylacetate fraction (Table 3), although most of antifungal antibiotics were extracted using ethyl acetate (Franco and Countinho, 1991). The anticandida properties of GMR-22 strain have not been determined as active component until purification is conducted.

The bioautography assay showed the best separation was reached by using ethylacetate and $CHCl_3$ as mobile phase. Bioautography on TLC revealed one clearing zones from the chloroform semi-purified extract (after column chromatography vacum) designated as R_f 0.57 were shown to be active spot against *C.albicans*. This indication showed that the strain contained bioactive components that inhibition effect for *C.albicans* growth. The analyzis of active spot on chromatograms by cerrium sulfate appear as bright yellow spot but not visible on UV_{254} and $_{366}$ light.

Table 3. Different solvent was used for extraction of anticandida from *Streptomyces sp* GMR-22

Solvents	Zone of Inhibition
n-Hexane	++
Benzene	+
Chloroform	+++
Ethylacetate	-

(+++) = >20 mm (++) = < 20 mm (+) = <10 mm
(-) = no inhibition

Repetitive Extragenic Palyndromic PCR (rep-PCR)

Genetic diversity of actinomycetes isolates from soil sample represented on BOX element variation were shown in Figure 2. Seventeen of a total 173 isolates produced antifungal substances based on antagonistic activity toward several tested fungi. Similarity coefisient dendrogram of isolates represented that 13 isolates were genetically diversed when analyzed using rep-PCR (Figure 3). From 17 isolates that were analyzed, 15 different similarity pattern were differentiated. Isolate of GMR-5/6 or GMR-22/27 were yielding similarity pattern on BOX element variation. The similarity in DNA fingerprints indicates that BOX repetitive

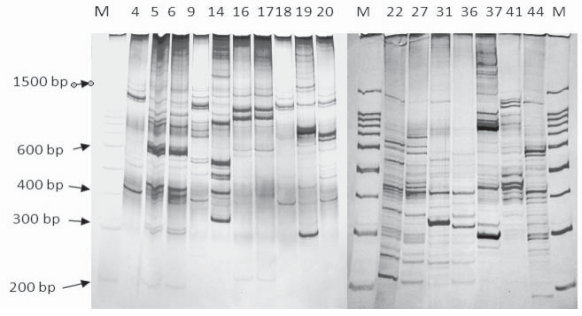

Figure 2. Genetic diversity of actinomycetes isolated from soil sample of *M. leucadendron* were analyzed by rep-PCR with BOXA1R primer. M : 200 bp DNA ladder, 4-44: name of isolates

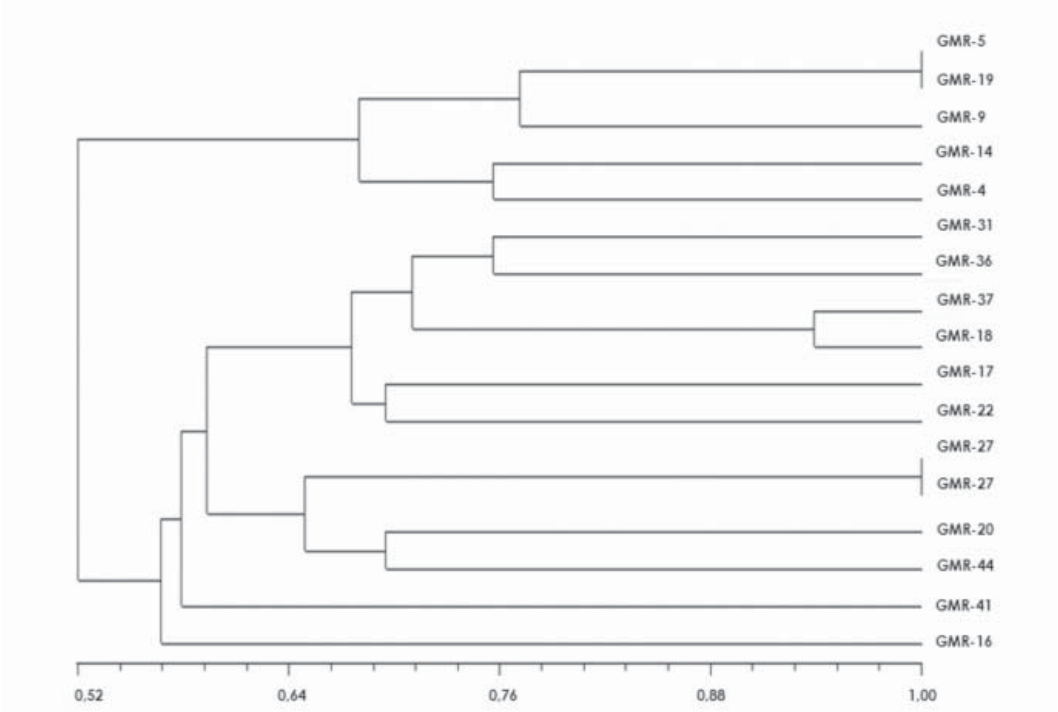

Figure 3. Dendrogram of Actinomycetes based on nested-PCR RISA in soil sample of *M. leucadendron*. Clusters were determined using UPGMA. 1-2 : repetitions.

sequences of the bacteria were located at the same position in chromosome. Therefore, it could be predicted that isolate GMR 5/6or GMR 22/27 had a close relationships. It was indicated that all isolates were different on molecular character except 4 isolates.

Amplification of pks genes

Seven isolates were selected from a total of 173 isolates for amplification of *pks* gene, which expected yield was 1400 bp (Figure 4). Preliminary morphologies studied indicated that isolates were belong to *Streptomyces* spp. The richest group of actinomycetes is represented by *Streptomyces* which have been extensively isolated by exploring antifungal and other biologically active substances. Therefore, all isolates were amplified on *pks* genes and all of those revealed that strains had genes expression for biosynthetic of poliketide substances.

The design of degenerated PCR primers specific for *pks-I* had been developed based on the alignment of known DNA sequences

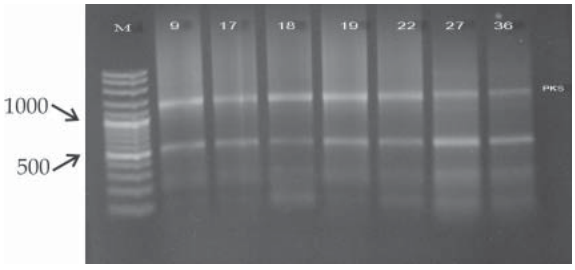

Figure 4. Agarose gel electrophoresis of Pks genes products from DNA isolated from Actinomycetes Lane 1: Ladder, Lane 2-8: Pks gene of isolates (GMR-9; GMR17; GMR18; GMR-19; GMR-22; GMR-27 and GMR-36)

of eleven modular *pks-I* biosynthetic clusters in actinomycetes (Sacido and Genilloud, 2004; Ayuoso *et al*., 2005). The PCR primer used to detect the presence of *pks* genes in the strain. However, the anticandida chemical structure could not be determined until the gene were sequenced.

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