

Isolation, Purification, and Characterization of Antimicrobial Substances from Endophytic Actinomycetes

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Abstract

Antimicrobial active substances produced by endophytic actinomycetes were isolated and purified. Plant samples were obtained from four different medicinal plants namely *Curcuma domestica*, *Phaleria macrocarpa*, *Isotoma longiflora*, and *Symplocos cocchinensis*. Isolation of actinomycetes was conducted using HV agar with the addition of cycloheximide, nystatin, nalidixic acid, and rifamycin. A total of 21 actinomycete isolates were obtained and tested for antimicrobial activity against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC27853, and *Bacillus subtilis* ATCC 66923. Among the isolates, isolate KY01 was the most active to Gram-positive and Gram-negative bacteria. Morphological observation and identification using 16S rRNA gene sequence showed that the isolate KY01 was similar to *Streptomyces antibioticus*. An active compound from the isolate KY01 was produced using yeast peptone medium. The active compound was purified using silica-gel-column chromatography and preparative high performance liquid chromatography (HPLC). A single peak of the active compound was detected with HPLC and LCMS, which showed a retention time of 26.6 min and molecular weight (MW) 906.4474 g/mol, respectively.

Abstrak

Isolasi, Purifikasi, dan Karakterisasi Senyawa Antimikroba dari Aktinomisetes Endofit. Isolasi dan purifikasi senyawa aktif anti mikroba yang dihasilkan oleh aktinomisetes endofit telah dilakukan. Sampel tanaman diperoleh dari empat jenis tanaman obat yaitu *Curcuma domestica*, *Phaleria macrocarpa*, *Isotoma longiflora*, dan *Symplocos cocchinensis*. Isolasi aktinomistes endofit dilakukan dengan menggunakan medium HV agar dengan penambahan antibiotik sikloheksimida, nistatin, asam nalidik, dan rifamisin. Sebanyak 21 isolat aktinomisetes diperoleh dari sampel tanaman obat yang selanjutnya diuji aktivitas antimikroba terhadap *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC27853, dan *Bacillus subtilis* ATCC 66923. Hasil pengujian pada 21 isolat yang diperoleh, isolat KY01 menunjukkan aktivitas antimikroba paling kuat terhadap bakteri Gram-positif maupun bakteri Gram-negatif. Pengamatan secara morfologi dan identifikasi menggunakan *sequence gen 16 rRNA* menunjukkan bahwa isolat KY01 memiliki kemiripan dengan *Streptomyces antibioticus*. Produksi senyawa aktif isolat KY01 dengan kultur cair menggunakan medium yeast pepton. Purifikasi senyawa aktif dari ekstrak fermentasi dilakukan menggunakan kromatografi kolom silica gel dan HPLC preparatif. Hasil identifikasi menggunakan HPLC menunjukkan puncak dengan waktu retensi 26,6 menit dan hasil karakterisasi senyawa aktif menggunakan LCMS menunjukkan bobot molekul senyawa aktif sebesar (MW) 906,4474 g/mol.

Keywords: endophytic actinomycetes, isolation, screening, antimicrobial activity, purification

1. Introduction

Microbial endophytes are organisms that colonize living internal tissues of plants and live in a symbiotic or mutual relationship with the host. Endophytes are ubiquitous in all plant species on Earth and contribute to their host plants by producing abundant metabolites that are harnessed for plant defense and endurance. Studies

on the interaction and functions of endophytes inside their hosts have addressed the ecological relevance of endophytes. Since they occupy unique biological niches, endophytes are an exceptional source of novel bioactive products for exploitation in medicine, agriculture, and industry. To date, several antibacterial, antifungal, and antiviral compounds, antioxidants, and cytotoxic substances have been isolated from endophytes, and

screening newer endophytes for their functional roles is a promising method for overcoming the increasing threat of drug-resistant strains of human and plant pathogens [1]. It has been rationalized that plants that have an ethno-botanical history are likely candidates since the medical uses to which the plant may have been selected relates more to its population of endophytes than to the plant's biochemistry [2].

The most frequently isolated endophytes are fungi; however, Gram-positive and -negative bacteria can be found as endophytes [3]. Endophytic microorganisms are found in every plant on Earth. These organisms inhabit the internal part of the plants and have various relationships ranging from symbiotic to pathogenic. Endophytes may contribute to their host plant by producing a plethora of substances that provide protection and ultimately survival to the plant. In addition, some endophytic microorganisms can produce valuable pharmaceutical substances of biotechnological interest [4].

Endophytic actinomycetes in particular are considered potential sources of bioactive compounds and various novel compounds [5]. Actinomycetes are the main source of antibiotics, and endophytic actinomycetes isolated from medicinal plants have considerable development potential. New actinomycetes have been found in the tissue of medicinal plants [6-9]. Moreover, most endophytic actinomycetes in medicinal plants can produce important compounds, and some have a new chemical structure [10-12]. Actinomycetes, especially the *Streptomyces* species, are valuable economic and biotechnological bacteria that provide more than two thirds of the antibiotics and bioactive compounds used today [13]. Endophytic actinomycetes associated with plants also play an important role in protecting their host from phytopathogenic invasion [14]. The present study was designed to screen endophytic filamentous actinomycetes from four medicinal plants in Padang, West Sumatra province, and assess their antibacterial activities for preliminary antibacterial activity.

2. Methods

Sample collections. Stems, roots, and leaves of *Curcuma domestica*, *Phaleria macrocarpa*, *Isotoma longiflora*, and *Symplocos cochinchinensis* were collected in Padang, West Sumatra province. Plant samples were kept in plastic bags and stored at 4 °C until isolation.

Isolation of endophytic actinomycetes. Plant materials were cut into small pieces (1 × 1 cm). The samples were washed by running tap water for 1-2 min to remove soil particles and then surface sterilized with 70% ethanol for 10 min and 1% sodium hypochlorite for 15 min. The plant materials were then rinsed with sterilized water three times before soaking in 10% (w/v) NaHCO₃

solution for 5 min. Then the final washed solution was spread in Humic Acid-Vitamin (HV) agar containing 100 µg/ml cycloheximide, 25 µg/ml nystatin, 100 µg/ml nalidixic acid, and 5 µg/ml rifamycin. Endophytic actinomycetes were observed after incubation at 28 °C for 1-4 weeks.

Actinomycetes were identified with gene 16S rRNA. DNA was isolated using the FastPrep kit (MP Biomedical) for DNA isolation. The pellet was lysed using a lysing matrix, combined with 1 ml, and homogenized using a FastPrep instrument for 40 sec at 4500 rpm. PCR was performed to amplify the DNA using forward primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3'), primers described by Turner *et al.* 1999 [15]. The PCR mixture containing 8F and 1492R primers was added to the DNA solution. PCR amplification was performed using GoTaq Green (Promega), 1 µl primer 9F and 1 µl primer 1492R, and 20 µl DNA template. The final volume was 50 µl. PCR amplification was performed using the Takara Thermal Cycler. The program was as follows: 30 cycles 96 °C for 2 min as the pre-denaturation step, 30 cycles of 96 °C for the 45 sec denaturation step, 30 cycles of 60 °C for the 20 sec annealing step, 30 cycles of 68 °C for the 1 min polymerization step, and 68 °C for the 2 min post-polymerization. The PCR products were visualized using gel electrophoresis on 1% agarose (Qiagen, Germany) and compared with 1 kb DNA ladder (Fermentas, Germany).

The PCR product was purified using a Gel/DNA extraction kit. The 16S rRNA gene obtained was submitted to the DNA sequencing facility, Genetic Laboratory, Biotech Centre. A Big Dye Terminator V 3.1 cycle sequencing kit was used to sequence the DNA. The DNA was then run in an automated DNA sequencer using capillary electrophoresis (ABI 300 genetic analyzer). The sequence was compared to the database available at NCBI using the BLAST search engine.

Determination of antimicrobial activities. Antimicrobial activity was monitored with the agar diffusion paper-disc (6 mm) method. Discs were dripped with a methanol solution of extract, dried, and then placed over the agar surface plates freshly inoculated with either *E. coli* ATCC 25922, *S. aureus* ATCC25923, *B. subtilis* ATCC 66923, or *P. aeruginosa* ATCC27853 as the organisms test. Suspensions of the organisms test were adjusted to 10⁶ cfu/ml. The most potent isolates were noted for each organism test, based on the mean diameter of the inhibition zones [16].

Liquid culture for production of secondary metabolite. An established slant of isolate was inoculated in a 250 ml flask containing 50 ml of vegetative medium (YEME medium) consisting of bacto peptone 5 g/l, yeast extract 3 g/l, malt extract 3

g/l, glucose 3 g/l, and demineral water 50 ml. The pH value of the medium was adjusted to 7.6 before sterilization. The flask was incubated at 30 °C for 2 days in an incubator-shaker. Fifty milliliters of the culture was transferred to 1,000 ml of the fermentation medium. The fermentation medium consisted of glucose 4 g/l, bacto peptone 15 g/l, yeast extract 3 g/l, Fe (III) citrate hydrate 0.3 g/l, and dematerialized water 1000 ml [17]. The pH value of the medium was adjusted to 7.6 before sterilization. The fermentation was carried out at 30 °C for 5 days in an incubator-shaker.

Purification of active compound. The culture broth was centrifuged at 14000 ×g for 15 min. The broth supernatants were divided and extracted using ethyl acetate as the solvent. The supernatant and the organic solvent were mixed thoroughly by shaking them in a 1 l capacity separating funnel and then allowed to stand for 30 min. Two layers were separated, the aqueous layer and the organic layer, which contained the solvent and the antimicrobial agent. The organic layer was concentrated by evaporation under vacuum to the least possible volume, after dehydration with anhydrous Na₂SO₄. The aqueous layer was re-extracted, and the organic layer added to the above organic layer. The organic layer was concentrated by repeated cycles of evaporation under vacuum. The dry extract of the supernatant was purified using silica gel column chromatography. Dry extract was injected onto the column and then eluted stepwise with the chloroform-methanol solvent system as follows: First, the column was eluted with 100% chloroform (Fraction 1). Then the chloroform was reduced by 10% in each fraction while the methanol was increased by 10% in each fraction, until the methanol percentage was 100%. Thirty fractions were collected (each 20 ml) and then concentrated and dried to test the antimicrobial activity. The active fractions obtained from the chromatography column were further purified with preparative high performance liquid chromatography (HPLC) and identified using LCMS.

3. Results and Discussion

Isolation of endophytic actinomycetes. Several colonies of endophytic actinomycetes were morphologically observed on HV agar medium spread after 1-4 weeks of incubation. Hyphal growth of endophytic actinomycetes was also detected on the surface of the stem, root, and leaf materials. Only one endophytic were obtained from the leaf *I. longiflora* sample. After the endophytes were purified, 21 isolates of endophytic actinomycetes were obtained: five isolates derived from *C. domestica*, six isolates derived from *P. macrocarpa*, six isolates derived from *I. longiflora*, and four isolates derived from *S. cocchinensis* (Table 1). The results indicated various types of endophytic actinomycetes were obtained from four different plant samples.

Table 1. Endophytic Actinomycetes Isolated from Four Medicinal Plants

Name of plant	Number of endophytic actinomycetes		
	Stem	root	leaf
<i>Curcuma domestica</i>	3	2	0
<i>Phaleria macrocarpa</i>	4	2	0
<i>Isotoma longiflora</i>	4	1	1
<i>Symplocos cocchinensis</i>	2	2	0

Most of the isolates were obtained from stem and root materials; one isolate was obtained from leaf material. No growing colony from the leaf sample was caused by penetration of the ethanol and sodium hypochlorite into the thin leaf tissue in the surface sterilization process. Priya 2012 [18] found most isolates were obtained from stems and roots, and obtained no endophytic actinomycetes from leaf samples.

Due to host-endophyte coevolution, some plants that produce bioactive natural products have associated endophytes that produce the same natural products [19]. Since the microbial sources of bioactive compounds are easier and more economic for large-scale production than plant sources, the discovery that rare, valuable plant products might also be produced by their endophytic microorganisms is of special pharmacological interest [20]. A well-known example is the anticancer agent taxol found in yew tree species (*Taxus* sp.). Stierle and Strobel isolated and characterized a novel taxol-producing fungus *Taxomyces andreanae* from the yew *Taxus brevifolia* [21]. Thus, when searching for novel, endophyte-based drugs, surveying traditional medicinal plants for bioactive metabolites that may be produced by their associated endophytes is a particularly fruitful approach [22-24]. *C. domestica*, *P. macrocarpa*, *I. longiflora*, and *S. cocchinensis*, the samples in this study, are important components of traditional Indonesian medicinal practices and have recently been used as commercial herbal medicines.

Antimicrobial activity assay. Within 21 endophytic actinomycetes, three isolates were active against the test bacteria. In the biological screening, antimicrobial activity against *E. coli* ATCC 25922, *S. aureus* ATCC25923, *B. subtilis* ATCC 66923, and *P. aeruginosa* ATCC27853 was exhibited by isolate KY01; the zones of inhibition were 13, 15, 16, and 13 mm, respectively (Table 2). Isolate MD02 inhibited *S. aureus* ATCC25923, *B. subtilis* ATCC 66923; the zones of inhibition were 9 and 10 mm, respectively. Isolate KD04 inhibited *S. aureus* ATCC25923, *B. subtilis* ATCC 66923; the zones of inhibition were 9 and 11 mm, respectively.

The strong inhibitory activity of these strains against various pathogens suggested that these endophytic actinobacteria may be potential candidates for produc

Table 2. Antibacterial Activity of Endophytic Actinomycetes

Source of plant	Code of isolate	Zone of inhibition (mm)			
		<i>E. coli</i> ATCC 25922	<i>S. aureus</i> ATCC25923	<i>B. subtilis</i> ATCC 66923	<i>P. aeruginosa</i> ATCC27853
<i>Curcuma domestica</i>	KY01	13	15	16	13
	KY02	-	-	-	-
	KY03	-	-	-	-
	KY04	-	-	-	-
	KY05	-	-	-	-
<i>Phaleria macrocarpa</i>	MD01	-	-	-	-
	MD02	-	9	10	-
	MD03	-	-	-	-
	MD04	-	-	-	-
	MD05	-	-	-	-
	MD06	-	-	-	-
<i>Isotoma longiflora</i>	SK01	-	-	-	-
	SK02	-	-	-	-
	SK03	-	-	-	-
	SK04	-	-	-	-
	SK05	-	-	-	-
	SK06	-	-	-	-
<i>Symplocos cochinchinensis</i>	KD01	-	-	-	-
	KD02	-	-	-	-
	KD03	-	-	-	-
	KD04	-	-	-	-

bioactive compounds. One endophytic actinomycetes isolated from *C. domestica*, KY01 was selected to produce antibacterial compounds because it showed higher activity on the antibacterial test.

Identification of selected isolate using 16S rRNA (partial sequence of isolate KY01). Identification using 16S rRNA revealed that isolate KY01 was 100% homologous to *Streptomyces antibioticus*, class *Actinobacteria*, order *Actinomycetales*, family *Streptomycetaceae*, and genus *Streptomyces*. Morphology observation showed a grey, glossy surface that was circular with folding hyphae with some antenna (aerial hyphae) arising vertically. In the beginning of growth, a single globular colony was formed, and then the hyphae expanded further.

Streptomyces is a genus of actinomycetes that morphologically resemble fungi and physiologically resemble bacteria. Subsequent growth of *Streptomyces* colonies as they spread over the agar surface is thought to follow similar kinetics to filamentous fungi [25]. The colony growth of the *Streptomyces* is initiated when a spore germinates, giving rise to one or more long multinucleoid filaments. These filaments elongate and branch repeatedly, originating a vegetative mycelium (substrate mycelium) that develops over and into the

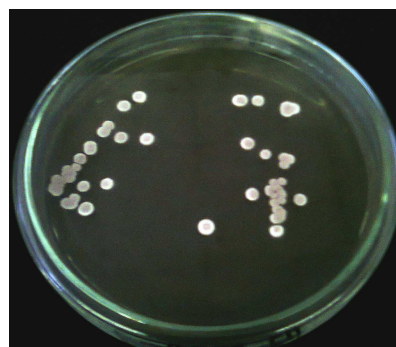


Figure 1. Morphology of Isolate KY01 Identified as *S. antibioticus*

culture medium [26]. Previous research showed that *S. antibioticus* produced actinomycin [27]. Atta 2010 [28] found the active compound with molecular formula $C_{20}H_{24}O_4$ produced by *S. antibioticus* AZ-Z710, but the molecular structure of the active compound has not been found.

Four furanones (butenolides) were isolated from the fermentation broth of *S. antibioticus* TU 99 and in preliminary tests have been shown to be biologically active [29].

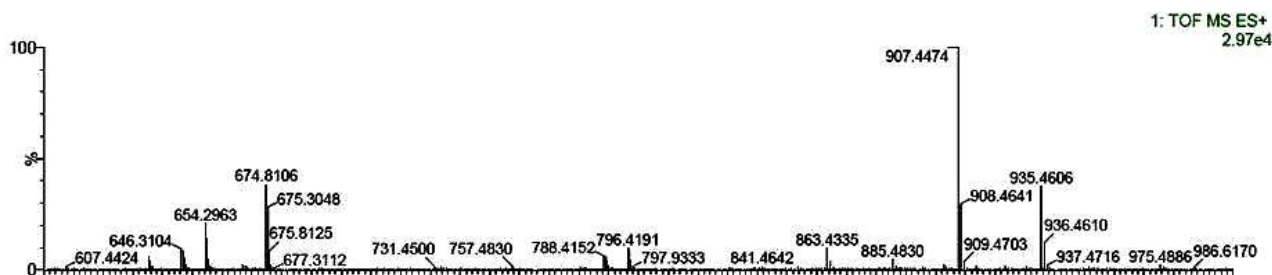


Figure 2. Spectrum of ESI-MS of Active Compound

Liquid culture and purification of active substance.

Liquid culture of isolate KY01 was carried out for 5 days by using a yeast-peptone medium. On the last day of culture, the medium color was darker and more viscous than on the first day. Many white granules were observed in the bottom of the flask. From a 5 l volume of culture, we obtained 4.32 g of dry biomass and after methanol extraction 2.45 g of extract. However, 0.31 g of ethyl acetate extract was obtained from the supernatant. Antibacterial activity assay of the broth extract against *E. coli* ATCC 25922, *S. aureus* ATCC25923, *B. subtilis* ATCC 66923, and *P. aeruginosa* ATCC27853 showed that the supernatant extract was active, but no activity (no clear zone formed) with the biomass extract was observed. This indicates that the isolate KY01 produced antimicrobial compounds through extracellular secretion. The supernatant extract was further purified using silica gel column chromatography and preparative HPLC.

The antibacterial activity test applied to all HPLC fractions showed that the peak around retention 26.5 min contained the active fraction. The fraction was collected and used for HPLC analysis. The chromatogram of the active fraction showed that the active fraction had a retention time of 26.5 min at gradient elution methanol-water 0-100% using analytical HPLC. The polarity difference of each compound and the interaction of each compound with the stationary phase become the principle of separation and purification. Reverse-phase chromatography for HPLC analysis uses a hydrophobic stationary phase and a polar (aqueous) mobile phase. As a result, hydrophobic molecules in the polar mobile phase tend to adsorb to the hydrophobic stationary phase, and hydrophilic molecules (high polarity molecule) in the mobile phase pass through the column and are eluted first. The peak around 26.5 min showed a semi-polar molecule.

The molecular weight of the active compound was determined with electrospray ionization mass spectrometry (ESI-MS). The ESI-MS spectra showed that this active compound has molecular weight $(MW+H)^+$ of 907.4474 g/mol or (MW) 906.4474 g/mol. The ESI-MS spectrum of the active compound is shown

in Figure 2. The chromatographic separation and mass spectrophotometer detection (LCMS) provided a large number of fragmentation pattern. Analysis can use ESI positive and negative charges. The negative ESI mode is characterized by the formation of the $[M-H]^-$ ion, and the positive ESI mode is characterized by the formation of the $[M+H]^+$ ion. This experiment used positive ESI. Figure 2 represents the typical fragmentation pattern of the bioactive compounds detected from the isolate KY01. HPLC coupled with LC-MS is one of the most powerful tools for detecting bioactive principles from natural products [30].

4. Conclusions

A total of 21 endophytic actinomycetes were obtained from four medicinal plants. One isolate (KY01) inhibited *E. coli* ATCC 25922, *S. aureus* ATCC25923, *B. subtilis* ATCC 66923 and *P. aeruginosa* ATCC27853, and two isolates (MD02 and KD04) inhibited *S. aureus* ATCC25923 and *B. subtilis* ATCC 66923. Selected isolate KY01 was identified as *S. antibioticus*, which produced an antibacterial compound with molecular weight (MW) 906.4474 g mol⁻¹. Active fraction HPLC chromatogram of the isolate KY01 extract showed a retention time of 26.5 min at gradient elution methanol-water 0-100%.

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