

Actinomycetes of *Orthosipon stamineus* rhizosphere as producer of antibacterial compound against multidrug resistant bacteria

H Rante, R Yulianty, Usmar, N Djide, Subehan, R Burhamzah, M B Prasad

Laboratory of Microbiology Pharmacy, Faculty of Pharmacy, Universitas Hasanuddin, Makassar, Indonesia

E-mail: h_rante@yahoo.co.id

Abstract. The increasing case of antibiotic resistance has become an important problem to be faced in treating the infection diseases. The diversities of microbia, especially actinomycetes bacteria which originated from rizosphere soil of medicinal plant, has opened a chance for discovering the metabolites which can be used in solving the antibiotic resistant pathogenic bacteria problems. The aim of this research was to isolate the actinobacteria originated from medicinal plant rizosphere of *Orthosipon stamineus* as the producer of anti-multidrug resistances bacteria compounds. Three isolates of actinomycetes has been isolated from *Orthosipon stamineus* rhizosphere named KC3-1, KC3-2 and KC3-3. One isolate (KC3-3) showed big activity in inhibiting the test microbes by antagonistic test of actinomycetes isolates against *Staphylococcus aureus* and *Eschericia coli* antibiotic resistant bacteria. Furthermore, the KC3-3 isolate was fermented in Starch Nitrate Broth (SNB) medium for 14 days. The supernatant and the biomass of the fermentation yield were separated. The supernatant were extracted using ethyl acetate as the solvent and the biomass were extracted using methanol. The antibacterial activity test of ethyl acetate and methanol extract revealed that the extracts can inhibit the bacteria test up to 5% concentration. The ethyl acetate and methanol extracts can inhibit the bacteria test up to 5% concentration.

Keywords: Actinomycetes, *Orthosipon stamineus* rizosphere, antibacterial

1. Introduction

Study of discovering a new antimicrobial compound which is safe and potential is one of a challenge in pharmaceutical industry nowadays, especially in related to the increasing of oportunistic infection to the host [1]. The presence of antibiotic and other chemotherapeutic compounds becoming a relief thingeither nowadays or in the future, however it's still not enough yet. The main problem is there is a must for the presence of new medical agents which have a better potency in comparison to what have existed today [2]. One of effort which can be done is doing a screening of bioactive compound towards any organisms or doing a modification towards the compounds which have existed today.

Actinomycetes were a group of bacteria which is the most antibiotic-producing (70%) compared to fungi (20%) and bacteria (10%) [3], and have an important role in environment because it can doing biotransformatinal and metabolism processes in a vast range. Beside, this bacteria is also the main microbia in micro-soil ecosystem [4] and it is the source of bioactive compound which is widely



distributed in marine and terrestrial habitat [5]. Actinomycetes is abundant surrounds the high level plant rooting [6] and in rhizosphere soil approaching 40% in the total of soil microflora. Proteobacteria and actinomycetes were dominant population derived from any special of plant rhizosphere [7]. Microorganism abundant in rhizosphere because plant roots excrete exudate which contain organic compound such as amino acid, saccharine, and organic acid which were nutrients for the microorganism. Composition and the pattern of root exudate, plant species and soil type influencing the activity and the population of rhizosphere microbes.

2. Materials and Methods

2.1. Sampling

Rhizosphere soil samples were taken from medicinal plants in Makassar City of South Sulawesi Province. Samples were taken around root in 5-25 cm depth from ground level using sterile stainless still tools and then stored in tightly sealed containers which have been preserved with 70% alcohol, then taken to the laboratory for use in the study.

2.2. *Actinomycetes isolation*

The isolation of actinomycetes from soil samples was done by the method of spread and pour plate to obtain a single colony. Each dilution result was taken 1 mL and then spread into a Petri dish then SNA medium were poured then incubated at 30°C for 14 days. The colonies that grow and show actinomycetes are re-isolated to obtain a single colony. The pure colony was then inoculated into SNA media for use in subsequent tests

2.3. *Testing of antibacterial activity with antagonistic test*

Determination of antimicrobial activity was done by all isolates of actinomycetes grown into SNA media, then 7 days incubation actinomycetes on SNA media were placed on the surface of NA (Nutrient Agar) media which had been inoculated by test bacteria on separate plate for each test bacteria, then Incubated for 24 hours at 37°C. Each isolate was observed for its ability to inhibit the test bacteria characterized by the formation of clear zones around the isolates. A stable isolate forming a clear zone at 2 times test was chosen as the isolate for further testing

2.4. *Fermentation of antibacterial active isolate*

The pre-culture of the active isolates were made in a 500 mL erlenmeyer flask containing 100 mL of SNA liquid medium and incubated at 28°C for 3 days. Pre-culture (starter) is transferred into a 500 mL Erlenmeyer containing 100 mL of the same medium. Fermentation was carried out at a temperature of 28°C for 14 days under the conditions of the agitated at a rate of 150 rpm.

2.5. *Extraction of metabolite compound*

After fermentation for 14 days, microbial growth medium is filtered to separate biomass and fermentation liquids. The fermentation fluid is extracted 2 times with ethyl acetate (1: 1 v/v) solvent in a separating funnel for 20 min while the biomass is extracted with methanol. The extract obtained was evaporated and then stored in the desiccator for use in the next test.

2.6. *Antibacterial activity test*

One culture of rejuvenated bacteria on NA (Nutrient Agar) media was suspended into a tube containing 5 mL of NB (Nutrient Broth) medium and incubated for 24 hours at 37 C. The suspension of the bacteria was diluted using 0.9% sterile NaCl to its turbidity equivalent to the standard 0.5 McFarland solution (bacterial population 1×10^7 CFU/mL - 1×10^8 CFU/mL). The composition of McFarland's standard solution is BaCl₂ 0.048 M 0.5 mL and H₂SO₄ 0.18 M 99.5 mL.

Antibacterial activity was determined by bioassay method based on [1] and [8] against test bacteria. The extract obtained was tested for the test bacteria in the following way: extract of ethyl acetate with certain concentration dissolved in organic solvent. A total of 20 µL maserate which has been known for its concentrate is inserted into the disc paper (6 mm in diameter). After all solvents have

evaporated further disc paper is placed on the surface of the media that has been inoculated with test bacteria with streak method. All plates were incubated for 24 hours at 37°C, observed the presence of antibacterial activity characterized by a zone of growth inhibition of bacteria around the disc paper.

3. Results and Discussion

3.1. Isolation and selection of *Orthosipon stamineus* rhizosphere actinomycetes

Isolation of actinomycetes from rhizosphere *Orthosipon stamineus* was performed using the spread plate method. Using general media for actinomycetes isolation SNA (Strach Nitrate Agar). Starch Nitrate, starch casein, and Glycerolglycine are the most suitable medium for isolating actinomycetes bacteria, but Starch Nitrate is the most commonly used medium because it can isolate different types of actinomycetes based on different pigmentation and morphological characters so that the Starch medium Nitrate is the most suitable culture medium [9].

In order to distinguish between actinomycetes isolates and bacterial and fungal contaminants, we observed the morphological characteristics of actinomycetes, actinomycetes colonies showed a consistency of powders and adherence strongly to the medium and had slow growth, in contrast to other bacterial colonies that had slimy surfaces and growth fast. Actinomycetes isolates grown on SNA media are characterized by colonies that resemble fungal colonies but are more compact.

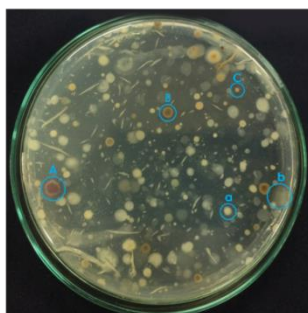


Figure 1. Microbia colony (A, B, C: Actinomycetes colony; a: Bacteria colony; b: Fungi colony)

The three isolates of actinomycetes obtained were separated and purified by streak on SNA medium then named KC3-1, KC3-2 and KC3-3 (Figure 2).

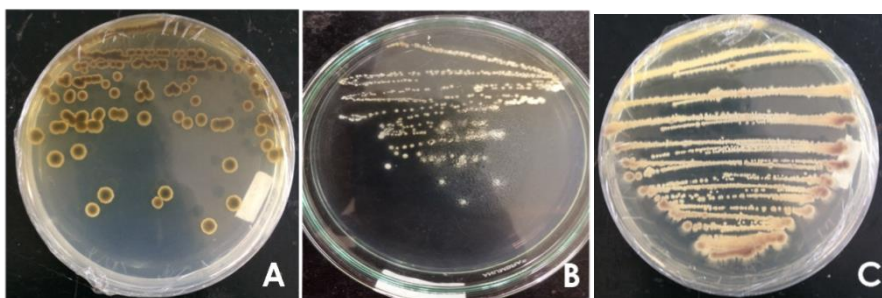


Figure 2. Actinomycetes isolate A : Actinomycetes isolate KC3-1; B. Actinomycetes isolate KC3-2; C. Actinomycetes isolate KC3-3

3.2. Antagonistic test of actinomycetes isolate

Antagonistic test is a qualitative test that is aimed to know the antibacterial activity of the pure actinomycetes isolates. The method used in antagonistic test was *agar block* method. Of the three isolates tested, only two isolates showed an inhibitor of *S. aureus* and *E. coli* bacteria, namely KC3-1 and KC3-3 isolates characterized by clear zones on agar that already contained test microbes. And isolate KC3-3 indicating the largest antibacterial activity so that this isolate is continued into the fermentation stage.

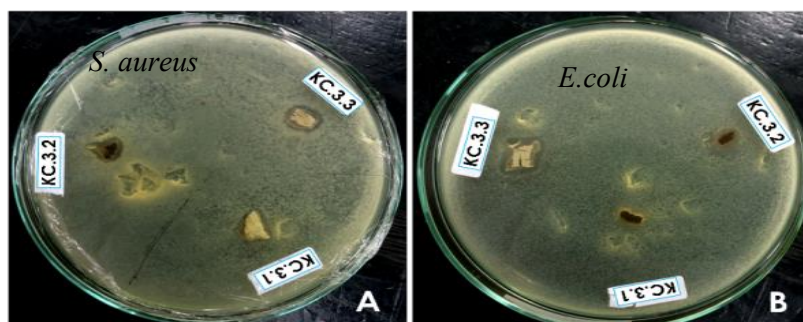


Figure 3. Antagonist test of isolate actinomycetes with zones of inhibition

3.3. Fermentation and antibacterial activity test

Fermentation was done to obtain the secondary metabolite from actinomycetes isolate. Fermentation was conducted on pure isolate which was 7 days old. Fermentation on KC3-3 isolate was conducted using SNB (*Starch Nitrate Broth*) medium and then incubated for 14 days. According to [10], On the 14th days of incubation, generally actinomycetes has entered the stationary phase. This is the phase of the bacteria do not show significant growth or the microorganism growth stop and the balance is present between the splitting cells and the dying cells. This is related to the nutrition which is reduced so there was a competition between microbes so that in this phase the microbes were producing antibacterial compound (secondary metabolite) to prevent the competition of nutrition and space. The fermentation yield (supernatant) were extracted using ethyl acetate twice (1:1 v/v), and biomass extracted using methanol. The extract obtained were tested for its antibacterial activity.

Antibacterial activity test result revealed that the supernatant and the biomass of fermentation extract of KC3-3 actinomycetes can inhibit the growth of test bacteria. The production of secondary metabolite of KC3-3 actinomycetes isolate was produce extracellularly to the fermentation media or supernatant, although in intracellular of the biomass was also present the secondary metabolite. This is based on antibacterial activity which was bigger than its supernatant liquid which has been extracted by organic solvent of ethyl acetate compared to the metabolite that is produced in biomass of cell (Figure 4). In the process of extracting fermentation or supernatant liquids is used ethyl acetate solvent, in addition to the greatest antibacterial activity in ethyl acetate extract also based on the research of [11] among the four types of solvents used hexane, chloroform, ethers and ethyl acetate, the most promising ethyl acetate extract as an antimicrobial with a large range of bacteria and fungi. [12] showed that metabolite extraction of actinomycetes using ethyl acetate was based on solubility and maximized antimicrobial activity. Ethyl acetate extract and methanol extract from fermentation of isolate actinomycetes KC3-3 were able to inhibit *S. aureus* and *E. coli* bacteria up to 5% (1 mg / paper disk). These results indicate that actinomycetes isolate of KC3-3 isolated from rhizosphere *Orthosipon stamineus* can be a potential antibiotic compounds producer.

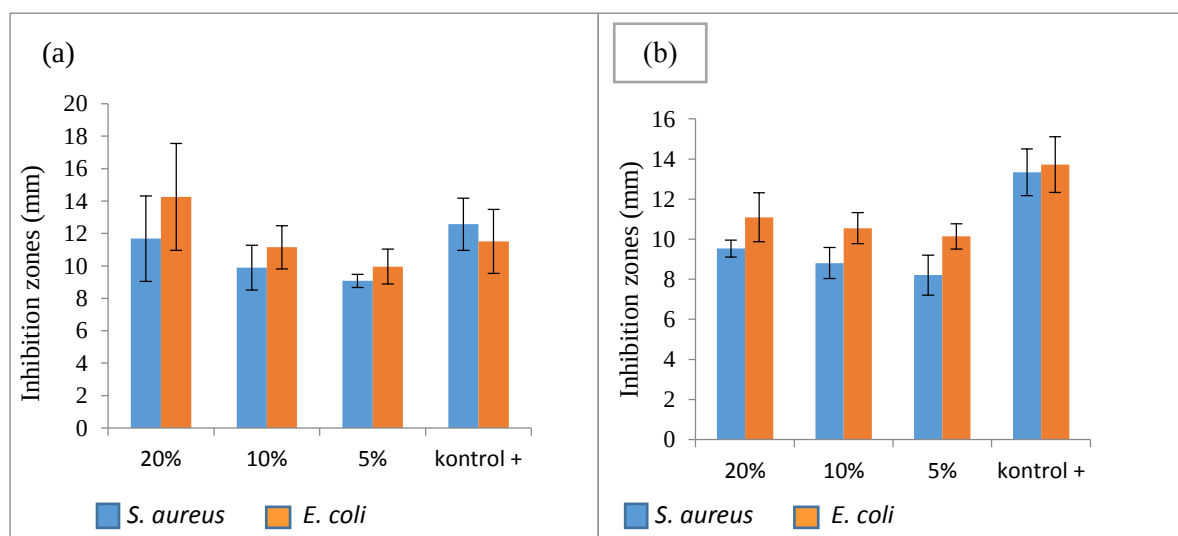


Figure 4. Result of antibacterial test of isolate actinomycetes KC3-3, (a) Ethyl acetate extract from supernatant, (b) Methanol extract from biomass.

4. Conclusions

Three bacteria isolates which isolated from *Orthosipon stamineus* rhizosphere were in group of actinomycetes named KC3-1, KC3-2 dan KC3-3. Actinomycetes of KC3-3 isolate was able to produce secondary metabolite which was active against *S. aureus* and *E. coli* up to 5 % concentration which were equivalent to 1 mg/paperdisk.

Acknowledgement

The authors thank to Ministry of Research, Technology and Higher Education of The Republic of Indonesia which has funding this project in PUPT 2017 scheme.

References

- [1] Badji B, Zitouni A, Mathieu F, Lebrihi A and Sabaou N 2006 Antimicrobial compounds produced by *Actinomadura* sp AC104 isolated from an algerian sahara *Soil Can. J. Microbiol.* **55**(4) 373-382.
- [2] Allen N E and Nicas T I 2003 Mechanism of action of oritavancin and related glycopeptides antibiotics *FEMS Microbiology Review* **26** 511-532.
- [3] Atlas R 1997 *Principle of Microbiology* (USA: Wmc. Brown Publisher).
- [4] Thangapandian V, Ponmurugan P and Ponmuruga N K 2007 Actinomycetes diversity in the rhizosphere soils of different medicinal plants in Kolly Hills-Tamil Nadu, India, for secondary metabolite production *Asian J. Plant. Sci.* **6** 66-70.
- [5] Pathom-Aree W, Stach J E M, Ward A C, Horikoshi K, Bull A T and Goodfellow M 2006 Diversity of actinomycetes isolated from challenger deep sediment (10,898 m) from the Mariana Trench *Extremophiles* 2006a **10** 181-9.
- [6] Oskay M, Tamer A U and Azeri C 2004 Antibacterial activity of some actinomycetes isolated from farming soils of Turkey *Afric. J. of Biotech.* **3**(9) 441-446.

- [7] Singh L S, Buruah I and Bora T C 2006 Actinomycetes of Loktat habitat: Isolation & screening for antimicrobial activity *Biotechnology* **5**(2) 217-221.
- [8] Pandey B, Ghimire P and Agrawal V P 2004 Studies on the antibacterial activity of actinomycetes isolated from the Khumbu Region of Mt. Everest. International Conference on the Great Himalaya-yas: Climate, Health, Ecology, Management and Conservation, Kathmandu. Kathmandu University and the Aquatic Ecosystem Health and Management Society, Canada. January 12-15, 2004.
- [9] Ghanem N B, Sabry S A, El-Sherif Z M, Abu El-Ela G A 2000 Isolation and enumeration of marine actinomycetes from seawater and sediments in Alexandria *J. Gen. Appl. Microbiol.* **46** 105–111.
- [10] Mulyadi and Nanik S 2013 Aktivitas cairan kultur 12 Isolat actinomycetes terhadap bakteri resisten *KESMAS* **7**(2) 55-112.
- [11] Arasu M V, Duraip&iyan V, Agastian P, Ignacimuthu S 2009 In vitro antimicrobial activity of *Streptomyces* spp. Eri-3 isolated from Western Ghats Rock Soil (India) *J. de Mycologie Medicale* **19** 22-28.
- [12] Alim Al-Bari M A, Sayeed M A, Rahman M S, Mossadik M A 2006 Characterization and antimicrobial activities of a phthalic acid derivative produced by *Streptomyces bangladeshiensis*: A novel species collected in Bangladesh *Research J. Med. Medical. Sci.* **1**(2) 77-81.