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Research article

Isolation of endophytic bacteria from plant basil (*Ocimum sanctum* L.) as antibacterials against s*taphylococcus aureus*

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Abstract

Background: Endophytic bacteria are microorganisms that live inside plant tissues such as the basil. The endophytic bacteria could be used as a source of drug compounds by utilizing secondary metabolites from bacteria such as bacteriocin. The purpose of this study was to isolate and test the antibacterial activity of secondary metabolites of endophytic bacteria.
Materials and Methods: Methods used include the isolation of endophytic bacteria, identification of morphological (macroscopic and microscopic) and biochemistry, the determination of the growth curve, the extraction of secondary metabolites in the stationary phase, antibacterial activity test, test antibacterial properties, and screening of compounds active secondary metabolites of endophytic bacteria.
Results: Isolation of endophytic bacteria from leaves of basil, obtained two strain. Based on the results of morphological and biochemical identification, these isolates belong to the genus Enterobacter. The results of the screening of secondary metabolites of endophytic bacteria in the stationary phase the best results were obtained at the 26th hour with a diameter of 6.05 mm inhibition. Secondary metabolites found to have antibacterial activity against *Staphylococcus aureus* with MIC value of 256 ppm.
Conclusion: Endophytic bacteria isolated from the basil plant and have antibacterial activity against *Staphylococcus aureus.*

Introduction

Infectious diseases are one of the biggest health problems not only in Indonesia, but also in the whole world. In addition to the virus as the cause, the bacteria are also the main cause of infectious diseases [1]. Treatment for infectious disease problems is to use antibiotic drugs. But over time, are now commonly found bacteria that are resistant to antibiotics.

Resistance to antibiotics is due to many factors, especially by the use of antibiotics that are not appropriate. This resistance is a problem worldwide (pandemic) is no less important than the problem itself by bacterial infection. *Staphylococcus aureus* is one example of a bacterium that was resistant to penicillin, oxacillin and other beta-lactam antibiotics. In Asia, *Staphylococcus aureus* were found to be resistant to ciprofloxacin reached 37% [1]. In addition, *Staphylococcus aureus* is found in many cases of nosocomial infection.

Nosocomial infections are infections acquired from hospitals and attack sufferers who are in the process of nursing care [2]. Nosocomial infections are more common around the world, especially in poor countries and the developing countries. A study conducted by WHO in 2006 that about 8.7% of the 55 hospitals of 14 countries in Europe, Middle East, Southeast Asia and the Pacific found cases of nosocomial infections, particularly in Southeast Asia by 10% [3]. According to Nugrahaeni *et al.,* [3] states that the prevalence of nosocomial infections recorded is high at 6-16% with an average 9.8% in 2010. Such a huge impact caused by *Staphylococcus aureus* to health, it would require further treatment in controlling infections caused by bacteria. The development of drug compounds from nature is one step that can be taken to minimize morbidity and resistance problems.

The basil plant can be used as an alternative in the development of antimicrobial compounds. In addition, basil also has other activities besides antimicrobials such

as antiviral, antifungal, anti-inflammatory and anti-hypertension [4, 5]. In order to maintain the viability of a plant, the acquisition of bioactive compounds can be made efficient by the use of endophytic bacteria basil. The endophytic bacteria are microscopic living organisms that live inside plant tissues (xylem and phloem), leaves, roots, fruits, and stems included in the basil plant [6]. The endophytic bacteria have similar properties with its host, the possibility of secondary metabolites produced by endophytic bacteria of the basil plant also has antibacterial activity [7].

Utilization of endophytic bacteria is one of the techniques of biotechnology approaches in producing the active compound has several advantages, including faster to produce with a uniform quality, and can be produced on a large scale. Based on this background, has done research that aims to isolate and test the antibacterial activity of secondary metabolites of endophytic bacteria.

Experimental

Isolation and purification

Isolation of endophytic bacteria was conducted using a piece of plant and spread plate on NA medium, incubation for 2x24 hours at a temperature of 37 ^{0}C. Colonies that grow later subculture for purification [8,9].

Characterization of endophytic bacterial isolates

Characterization of microscopic observation (simple and gram staining) and biochemical tests include starch hydrolysis, proteolysis, tests gelatin, carbohydrate fermentation, catalase, lactose fermentation, VP test, citric test, test methyl red.

Growth curve determination (Fermentation)

Bacterial isolates were obtained, entered into erlenmeyer already containing TSB (Trypticase Soy Broth) medium and sub-cultured to medium NB. Incubation at 25^{0}C for 24 hours. Measurements at 0 hours performed during the start inoculated into the culture medium NB. Data were taken every interval of 2 hours and absorbance was measured using a spectrophotometer at a wavelength of 600 nm [10,11].

Extraction of secondary metabolites

Fermentation result then centrifuged at a rotation speed of 10000 rpm for 15 minutes. Supernatant was extracted using a solvent ethyl acetate [12], whereas bacterial cells were extracted using a sonicator for 15 minutes at the speed of a wave of 20 kHz.

Antibacterial activity test method microdilution broth

The concentration of microbes used 0.5 McFarland (10^8 CFU/ml). Then inoculated into micro-dilution plates containing NB media and extraction. Plates were incubated at 37^{0}C for 24 hours then observed clear section. The smallest concentration where no microbial growth is expressed as the MIC. Then the results are clear inoculated into solid media and observed clear zone formed. The lowest concentration that kills microbes designated as MBC [13].

Determination of the bacteriostatic properties or bakteriosida

Prepare 10 reaction tubes, enter 1 mL of the extract of fermented into 9 tubes and 100 mL of the bacterial suspension. Then add 9 mL media NB (Nutrient Broth). Prepare positive control 1 tube filled with media NB (Nutrient Broth) with the addition of 100 mL of the bacterial suspension. Incubate at a temperature of 36.5 ± 1°C. Take snippets at time 0, 1, 2, 3, 4, 5, 6, 7, 8 hours. Then measure the wavelength of maximum absorbance at 600 nm [14].

Secondary metabolite screening compounds

Secondary Metabolites Compound screening is a test done to determine the chemical constituents contained in secondary metabolites (supernatant).

Results and Discussion

Isolation and purification

A total of 2 isolates of endophytic bacteria have been isolated from the leaves of the basil plant. The endophytic bacteria from growing basil leaves start to look after the sample was grown on NA media during 2x24 hours. Based on the isolation method used, the number of endophytic bacteria obtained using a spread plate method is higher than the methods of plant piece. The endophytic bacteria grow more limited methods of plant piece, because the plant is cut small samples and immediately placed on a surface. Isolates of media spread plate then have to do the purification step (Figure 1).

Figure 1. Bacterial Isolates Endophytic purification results (isolates-01 and isolates-02)

Screening of endophytic bacteria

Bacterial isolates were obtained is then carried out initial screening. Initial screening aims to get isolates endophytic bacteria have potential as an antibacterial. In this stage, the test bacteria used are *Staphylococcus aureus* using disc diffusion method. Based on observations from the two isolates in the can (01 isolates and isolates-02) only isolates-01 that showed activity against *Staphylococcus aureus*.

Characterization of endophytic bacterial isolates

Macroscopically, colonies of bacteria can endophytic in yellowish white, endophytic bacteria isolates have varying shapes and colors, but generally single colony purification results endophyte had a yellowish white color. Based on microscopic observation, simple and gram staining, obtained bacterial form of bacillus (rod) and belongs to the Gram-negative (Figure 2).

Figure 2. The results of Gram staining using differential dye

Biochemistry test

The results of biochemical testing on isolates of endophytic bacteria-01 (Table 1) can be identified to the genus Enterobacter (According to Bergey's Mannual of Determinative Bacteriology 1974).

Table 1. Test Results of Biochemistry

Isolat Code	Test	Result
Isolat – 01	Hydrolysis of starch	+
	Catalase	+
	Hydrolysis Gelatin	+
	Fermentation of Carbohydrates	+
	Fermentation of Lactose	+
	Voges Preskuer	-
	Motility	+
	H_2S	-
	Indol	-
	Simmons Sitrat	-
	Metil Red	-

Determination of optimum fermentation time

The timing of fermentation aim to get the harvest optimum in obtaining secondary metabolites produced by endophytic bacteria. Making the growth curve in the present study using the method of turbidimetry by measuring the Optical Density (OD) every 2 hours with a wavelength of 600 nm. Secondary metabolites of endophytic bacteria will be issued by the time it reached the stationary phase.

Based on observations in Figure 3, the phase lag starting from hour to hour-0 up to 4th by the increase in the number of bacterial cells was slow. It can be influenced by several factors, namely the species of bacteria, and the initial conditions of the bacterial cell before entering the new settings [15].

Exponential phase starting at the 4th and ended at the 16th hour. In this phase, bacterial cells begin to reproduce themselves by splitting themselves asexually [16]. Stationary phase starting at the 18th till the 26th, characterized by the addition of bacteria, the number of cells that grow in proportion to the number of cells that die. This happens, because the source of nutrition in the medium begins to decline so that endophytic bacteria would produce secondary metabolites as a defense against extreme environmental conditions [17].

The ability of secondary metabolites that act as an antibacterial can be identified by screening the stationary phase. The selected stationary time eg at the 18th, 22nd, and 26th hours. The stationary phase screening process to test the antibacterial of supernatant and bacterial cells to test bacteria *Staphylococcus aureus* (Tables 2 and 3).

Table 2. Screening Stationary Phase (supernatant)

Inhibition Zone (cm)	Time (hour)
0.525	18
0.565	22
0.605	26

Table 3. Screening Stationary Phase Cells Bacteria

Inhibition Zone (cm)	Time (hour)
0.510	18
-	22
0.610	26

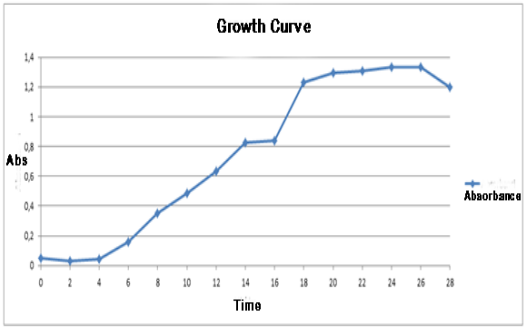

Figure 3. Growth curve of Isolat-01

Based on the results pengamtan that potential as an antibacterial metabolite is at the end of the stationary phase (26th Hour). The results are consistent with the

statement of Pelczar *et al.,* [18] which explains that the antimicrobial secondary metabolites produced by microorganism at the end of the stationary phase. This is because the secondary metabolites disintetis at the end of the life cycle of cell growth that is on the stationary phase in which the cell population at that time numbered anyway. The number of cells are living and the dead is directly proportional to each other and also synthetic secondary metabolites that occur as a result of the start decreasing nutrients contained in a growth medium such microorganisms. The limitations that cause secondary metabolites released by microorganisms in an effort to maintain its life cycle.

The next phase is the phase of death. Death phase occurs when there are no more nutrients available in the medium, resulting in a decrease in the number of bacterial populations.

Extraction of secondary metabolites

The weight of the extract (supernatant) gained as much as 0.2 grams and the weight of the extract (bacterial cells) gained as much as 0.001 grams. Weight of secondary metabolites extract obtained is then used as an antibacterial activity test.

Antibacterial activity testing

In testing the antibacterial activity by using microdilution method, the result that the extract metabolites have a fairly good activity against test bacteria *Staphylococcus aureus* with the acquisition of MIC value of 256 ppm (Table 4 and Figure 4). Antibacterial activity of the extract metabolites into the category of moderate antibacterial activity. As for the antibiotic tetracycline has a MIC with a concentration of 2 ppm. This was stated by Holetz [19], which states that the strength of antibacterial activity with microdilution method has four categories. For MIC less than 100 ppm expressed these extracts have antibacterial activity either; 100-500 ppm expressed these extracts have antibacterial activity being; 500-1000 ppm whereas otherwise weak antibacterial activity.

Further test results MIC, is the determination of value by growing part MBC clear over the medium NA (Nutrient Agar) with dispersive technique. The results obtained, extract the metabolites do not have the ability to kill of the test bacteria. It is characterized aliquots were grown on agar NA (Nutrient Agar) is overgrown by bacteria test.

Table 4. Antibacterial Activity Test microdilution method

Bacteria	Tetrasiklin (ppm)		extract metabolites (ppm)	
	MIC	MBC	MIC	MBC
Staphylococcus aureus	2 ppm	2 ppm	256	-

* MIC = Minimum Inhibitory Concentration; MBC = Minimum Bactericidal Concentration

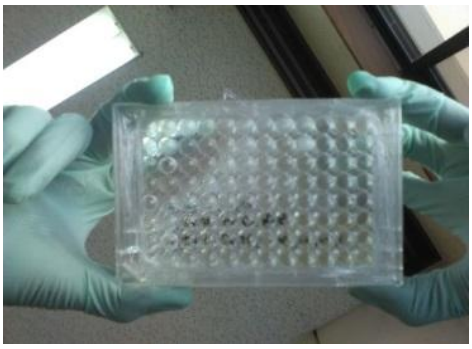

Figure 4. Antibacterial activity test Microdilusi method

Determination of antibacterial properties

Determination of antibiotic properties aims to determine the nature of the activity of secondary metabolites obtained. Measurements dilakukkan for 8 hours with the use of a therapeutic dose calculation to the antibiotic that is three times the drink in a day. In this test the extract of secondary metabolites used comparison drug tetracycline. Tetracycline has an activity to inhibit bacterial protein synthesis / bacteriostatic.

In testing the samples (extract of secondary metabolites) absorbance continued to increase as indicated by increasing absorbance value in getting. The increase occurred from hour to hour-0 until the 6th. Absorbance is beginning to look stable from hour to hour-7 to all 8. Results of the test sample are proportional to the comparator drug tetracycline. Absorbance is starting to look constantly at the clock 7th to 8th hour. From the test results it can be concluded that the extract of secondary metabolites thought to have properties as bacteriostatic against *Staphylococcus aureus* (Figure 5).

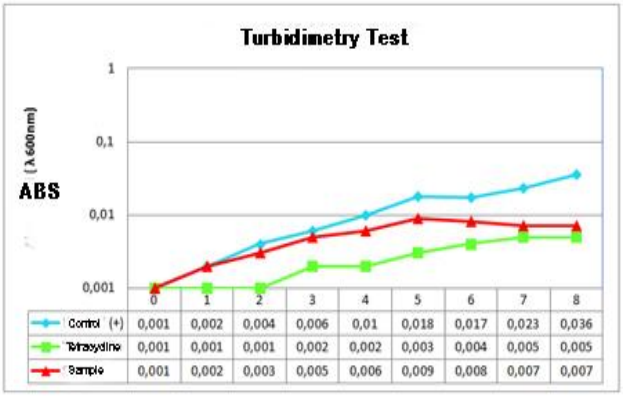

Figure 5. Graph determination antibacterial properties

Phytochemical screening

Results of phytochemical screening of secondary metabolites extract menjukan that secondary metabolites were found belonging to the class of alkaloids (Table 5). The results are marked with a color change to red brick after addition of reagents Dragendorff. One of the chemical constituents present in the basil plant is an alkaloid compound. The compounds are thought to have antimicrobial activity.

Table 5. Results of Secondary Metabolite Screening Phyto-chemicals

Testing group	Result
Alkaloids	+
Phenol	-
Steroid/ Terpenoids	-
Saponin	-

Conclusion

Endophytic bacteria isolates obtained from the basil plant as much as 2 isolates that allegedly belong to the genus Enterobacter. Secondary metabolites of endophytic bacteria from the basil plant has potential as an antibacterial with MIC value of 256 ppm and have antibacterial properties as bacteriostatic against *Staphylococcus aureus*.

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