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ISOLATION AND ENDOPHYTES ANTIMICROBIAL ACTIVITIES FROM SHIITAKE MUSHROOM (*LENTINULA EDODES*) IN BACTERIA AND FUNGI

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ABSTRACT

Endophytes produce secondary metabolite compounds has great potential for the development of new drugs. Endophytes could be produced from various plants one of which is shiitake mushrooms (*Lentinulaedodes*). The aims of this study to isolate and assay endophytic activity of shiitake mushrooms against bacteria and fungi. The method were used in this experimental includes paper disc method and microdilution broth. The result of endophytic bacteria isolation was obtained by three isolate bacteria suspected of isolate S1 (*Bacillus cereus*), isolate S2 (*Staphylococcus sp*), isolate S3 (*Bacillus macerans*) and isolate of endophytic fungi obtained 1 isolate suspected isolate F1 (Penicillium). The results of antibacterial activity assay showed that the largest inhibitory zone diameter against *Escherichia coli* was found in isolate S1 (30000 ppm) with diameter of 10,14 ± 0,63 mm and to *Staphylococcus aureus* in isolate S3 (11000 ppm) with diameter 9,19 ± 0, 68 mm. The results of antifungal activity assay showed that the largest inhibitory zone diameter of *Candida albicans* was found in concentration of 34000 ppm with diameter 6.45 ± 0.35 mm. MIC and MBC values of antibacterial assay was found in isolate S1 concentrations more than 32000 ppm, isolate S2 more than 4000 ppm and isolate S3 more than 14000 ppm. MIC and MFC values of antifungal assay was found in concentration of 2125 ppm. The conclusion result of endophytic assay as an antimicrobial activity expressed quite well.

INTRODUCTION

Infectious diseases caused by bacteria and fungi in developing countries likeIndonesia are still the highest contributor to morbidity and mortality. It is still a big problem and a serious challenge for Indonesia to find alternative antibiotic treatment in order to reduce the high incidence of the disease (Utami, 2005). Infectious diseases are caused by several microorganisms such as bacteria, viruses, parasites, and fungi that enter and multiply in the body (Jawetz et al., 2005). There are several types of pathogenicbacterial and fungal that can infect humans including*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*and *Candida albicans* (Leboffe, 2011).There were approximately 9.2 million people affected by infectious diseases and around 1.5 million people died (WHO, 2013). In Indonesia, there was an increase in the prevalence of infectious diseases in 2013 including pneumonia around 2.7%, pulmonary tuberculosis0.4%, and hepatitis

1.2%. Upper Respiratory Tract Infection(ARI) of 1.9% and diarrheais still the main cause of death in infants (Riskesdas, 2013). As for fungal infections, based on "*The Fungal Research Trust. How common are fungal diseases? Fungal Research Trust 20th Anniversary meeting. London june 18th 2011*"in 2011 there were 140,000,000 cases per year of fungal infections caused by species *Micosporum*, 1.500.000.000 fungal infection cases per year are caused by *Candida* species and 1,000,000 yeast infection cases per year caused by *Aspergillus*species (Vandeputte et al., 2012).Indonesia has many kinds of plants that are nutritious for health, one of them is shiitake mushroom (*Lentinulaedodes*). Shiitake mushroom (*Lentinulaedodes*) is a fungus that is very popular in the world, especially Japan. Shiitake mushrooms are also commonly referred to Chinese Black Mushroom.Besides known as foodstuffs, thisfungus also has efficacy as a medicine such as antifungal, antioxidants, antimicrobial, and also antilipidemic(Zembron, 2013). The antibacterial activity conducted against *Propionibacterium acnes*, *Staphylococcus epidermidis*and *Staphylococcus aureus* at concentrations of 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 ppm. The best MIC value obtained in fraction of ethyl acetate and n-hexane against the test bacterium at a concentration of 256 ppm. KBM value of the n-hexane fraction against *S. aureus* at a concentration of 512 ppm and ethyl acetate fraction against *S. aureus* and *S. epidermidis* at a concentration of 512 ppm. SEM test results showed the presence of antibacterial activity which is indicated by a change of cell morphology, their lumps and their cell wall frown on *P. acnes* were exposed to ethyl acetate fraction (Sukmawati et al, 2019).Chemical studies of natural materialson bioactive compounds (secondary metabolites) derived from plants, especially medicinal plants, have been widely reported in Indonesia. However, the chemical research of natural substances on secondary metabolites of microorganisms such as bacteria or fungi found in medicinal plants is still not much or even no research conducted and reported in Indonesia. One of secondary metabolites, which has great potential for development of new drugs is endophytic fungi. Endophytic fungi can produces various functional compounds such as anti-cancer compounds, antiviral, antibacterial, antifungal, plant growth hormones, insecticides and others,both with the host or not but have biological activities similar to bioactive compounds produced by the host (Fisher and Petrini, 1987; Strobel and Daesy, 2003).

MATERIALS AND METHODS

Tools and Materials: The tools used in this study were petri dish, erlenmeyer, sterile scissors, incubators, ose needle, Whatmanno. 42 paper disc, 42 Whatmanno. 42 filter paper, ruler, sterile knife, centrifugator, shaker, test tube, tube rack, spiritus burners, analytical balance, pipette, spatula, glass beaker, measuring cup, stirring bar, bath, autoclave, fatty cotton, yarn mattress, micropipette, LAF, tip, heat-resistant plastic, sterile tissue. The materials used in this study were 70% alcohol, comparative antimicrobials (Tetracycline and Ketoconazole), distilled water sterile, shiitake mushrooms (*Lentinulaedodes*), medianutrientagar, potatos dextrose agar, media nutrient broth, potatos dextrose broth, mediahydrolysis agar starches, mediaagar lipids, media agar peptone, media gelatin, media litmus milk, media sim agar,simmon's citrate agar media, MRVP media, broth urease test media, carbohydrate media (lactose, dextrose, sucrose), 1% sodium hypochlorite.Bacteria samples used in this study were *Escherichia coli* and *Staphylococcus aureus*, and fungi samples used in this study is *Candida albicans*.

Method:

Isolation: Cutshiitake mushrooms that have been washed with running water and then soaked in a solution of 70% alcohol for ± 3 minutes, cleaned with a solution of Sodium Hypochlorite 1% for ± 5 minutes. Then dried with sterile wipes and washed again with 70% alcohol for ± 0.5 min, followed by sterile distilled water 3 times. Shiitake mushroomsare cut in 1cm × 2cm using a sterile knife. The shiitake mushroom pieces were inoculated onto petri dishes containing

PDA and incubated at room temperature for 1-2 weeks and to a petri dish containing NA and incubated at 37 ° C for 1-3 days.

Antimicrobial activity test: Testing the antimicrobial endophytic activityof shiitake mushrooms was carried out against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* using paper disc and microdilutionmethods.

Test Activity of Endophytic Secondary Metabolites against Microbes Test by Paper DiscMethod:

Test activity test of endophytic secondary metabolites against *C. albicans, S. aureu*s and *E. coli*was done by placing a paper disc Whatman no. 42 with diameter of 6 mm that had been soaked in the culture endophyticfungal and bacteria supernatant for 30 minutes on a PDA medium containing isolates of *C. albicans*and on NA medium containing isolates of *S. aureus* and *E. coli*. Each culture contains microbes as much as 10^6cfu/mL. The cultures were incubated at room temperature for endophytic fungi and at 37°C for endophytic bacteria for 24 hours. After an incubation period was completed, the observation of inhibition zone is formed and measured in diameter.

Test Activity of Endophytic Secondary Metabolites Against Microbial Test with*Microdilution* Method:

A total of 100 µL of PotatosDextroseBroth /Nutrient Broth (for fungal test / bacteriatest) is included in the micro plate in the first column (as a negative control). 5 µL fungal or bacterial suspensions added to 10 mL PotatosDextroseBroth / Nutrient Broth then stirred with a vortex. A total of 100 µL of the mixture was put in a micro plate in the second column until the twelfth. In the twelfth column, 100 µL of a antibiotic solution/ secondary metabolites of endophyticis added with a particular concentration then homogenized. From the twelfth column, 100 µL is taken and then transferred to the eleventh column. The dilution is continue until the third column will have the smallest concentration. Plates were incubated at 25 ° C for 3 x 24 hours for mold, 37 ° C for 24 hours for bacteria and then observed for clear parts (no microbial growth).The smallest concentration where microbial growth is not seen is defined as the MIC. A total of 5 µL aliquots from each clear portion weretransferredtoPotatosDextroseAgar/Nutrient Agar and incubated at 25 ° C for 3 x 24 hours for mold, 37 ° C for 24 hours for the bacteria and then observed. The lowest concentration where there is no microbial growth set as a MinimumFungicideConcentration (MFC) and Minimum Bactericidal Concentration (MBC).Plants that are used is shiitake mushroom with the Latin name *Lentinulaedodes* (Berk) Pegler of family Marasmiaceae with No. Determination B-3595/IPH.3./KS/XII/2017 whichisolated using the spread plant method. The advantage of this method is the result of growth obtained can be better. 3 (three) endophytic bacteria isolates and 1 (one) endophytic fungi isolate were obtained from the isolation process of shiitake mushrooms.(CLSI,2012)

Antibacterial Activity Assay

The test results using the paper disk method show that endophytic bacteria can inhibit the growth of pathogenic bacteria with different abilities. Bacterial isolates S1 has a concentration that produced the greatest inhibitory diameter of the bacteria test which is 30,000 ppm with a inhibitory diameter of 10.14 ± 0.63 to bacteria *E. coli* and 20,000 ppm with a inhibitory diameter of 9.03 ± 1.34 against *S. aureus*, S2 isolates had concentration that produced the largest inhibitory diameter of the bacteria test at 500 ppm with a diameter of 8.70 ± 0.97 to bacteria *E. coli* and 3000 ppm with a inhibitory diameter of 7.95 ± 1.25 against *S. aureus* and S3 isolates had a concentration that produced the largest inhibitory diameter of the bacteria test at 5,000 ppm with a diameter of 8.99 ± 1.15 to bacteria *E. coli* and 11,000 ppm with a inhibitory diameter of 9.19 ± 0.68 against *S. aureus*. According to antibiotic sensitivity standard (Sharma et al., 2009), although there was a inhibitory zone formed, *Escherichia coli* and *Staphylococcus aureus* showed low

sensitivity to the secondary metabolites of bacterial isolates S1, S2 and S3.The test results using microdilution method can be seen from the negative controls (first column) whichonly contains media, showingthe form of a solution does not change color, which remains as before, means there is no microbial growth. The procedures performed during the test can be said aseptic and capable toprovide the correct test results. The test resultsof S1, S2 and S3 showthat the solution in column 12 to column 3 becomes cloudy, which means that concentration of these isolates have not been able to inhibit the growth of pathogenic bacteria, it is estimated that the minimum inhibitory concentration (MIC) for bacterial isolates S1 was concentrated more than 32000 ppm.

Table 1: Isolation of Entophytic Bacteria and Fungi

Isolate	Shape	Colour	Elevation	Side	Information
S1	Wrinkles	White	Convex	Wavy	Shiny
S2	Wrinkles	White	Convex	Wave	Shiny
S3	Circle	White	Convex	Slick	Shiny

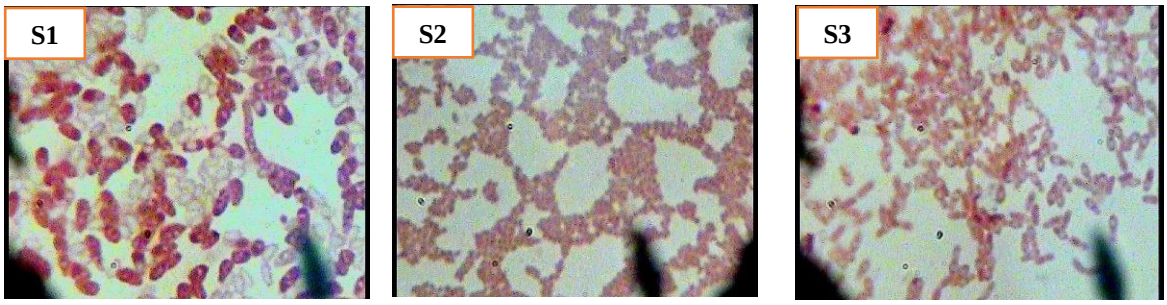

Figure 1. The results of endophytic bacteria gram stain (S1) Gram positive bacilli bacterial cells, (S2) Gram positive cocci bacterial cells, (S3) Gram negative bacilli bacterial cells.

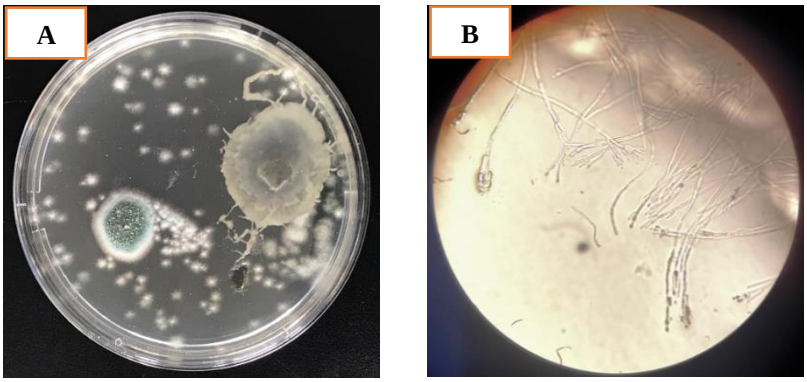

Figure 2. The result of macroscopic F1 isolates (A) is bluish green and isolates results F1 (B)microscopically show a conidial arrangement such as a broom

Table2. Biochemical Assay Results of Endophytic Bacterial Isolates

No	Biochemical Test	Result of Each Endophytic Bacteria Isolate		
		S1	S2	S3
1	Lipid Hydrolysis	-	+	+
2	Casein Hydrolysis	+	-	+
3	Starch Hydrolysis	+	-	-
4	Citrate	-	-	-
5	Lactose	-	-	-

6	Dextrose	+	-	-
7	Sucrose	+	+	-
8	Gelatin	-	-	-
9	Motility	+	-	+
10	H2S	-	-	-
11	Indol	-	-	-
12	MR	-	-	-
13	VP	-	-	+
14	Catalase	+	-	-
15	KOH String Test	Gram Positive	Gram Positive	Gram Negative

Table 3.Results Inhibitory Zone Diameter Endophytic Bacterial Isolates

Sample	**Concentrate (ppm)**	**Inhibitory Zone Diameter(mm) ± SD**	
		E. coli	S. aureus
Isolate S1	1000	7,83 ± 1,63	6,86 ± 1,06
	5000	7,25 ± 0,76	7,49 ± 0,65
	10000	6,31 ± 1,66	8,20 ± 1,04
	15000	7,18 ± 2,26	8,73 ± 0,85
	20000	8,25 ± 1,90	9,03 ± 1,34
	25000	9,70 ± 0,70	9,01 ± 0,64
	30000	10,14 ± 0,63	7,65 ± 0,68
	32000	8,88 ± 1,77	7,60 ± 0,39
Isolate S2	500	8,70 ± 0,97	5,94 ± 1,18
	1000	5,64 ± 1,11	6,33 ± 0,82
	1500	6,06 ± 0,38	7,84 ± 0,55
	2000	5,86 ± 0,46	7,61 ± 0,71
	2500	6,40 ± 0,62	7,33 ± 1,19
	3000	6,21 ± 0,71	7,95 ± 1,25
	3500	7,20 ± 1,00	7,55 ± 0,91
	4000	7,55 ± 0,59	6,83 ± 1,35
Isolate S3	1000	8,08 ± 1,03	7,81 ± 0,47
	2000	8,36 ± 0,88	8,68 ± 1,03
	3000	7,86 ± 0,98	6,61 ± 0,75
	4000	7,53 ± 1,05	7,00 ± 0,71
	5000	8,99 ± 1,15	7,98 ± 0,75
	8000	8,74 ± 1,18	8,95 ± 0,67
	11000	8,26 ± 0,68	9,19 ± 0,68
	14000	8,79 ± 1,54	9,19 ± 1,97
Control (+)	1000	21,55 ± 0	22,55 ± 0
Control (-)	-	4,00 ± 0	4,00 ± 0

Table 4.Test Results of Endophytic Bacterial Isolate by Microdilution Test

Bacteria	**Tetrasiklin (ppm)**		**Secondary Metabolites of Endophytic Bacteria**					
			S1		S2		S3	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Eschericia coli	2	2	> 32000	-	>4000	-	>14000	-
Staphylococcus aureus	2	2	> 32000	-	> 4000	-	> 14000	-

3. Test Results Antifungal

Table 5. Results of Inhibitory Zone Diameter Endophytic Fungi Isolates

Samples	Concentration	Inhibition Zone Diameter (mm ± SD)
		Candida albicans
Isolates F1	4000	0
	8000	0
	12000	0
	16000	0
	20000	0
	24000	5.20 ± 0.14
	28000	5.65 ± 0.07
	34000	6.45 ± 0.35
Control (+)	1000	15.90 ± 1.41
Control (-)	-	4.00 ± 0

Table 6.The test results microdilution metabolites of endophytic fungi isolates

Fungi	Ketoconazole(Ppm)		Endophytic Fungi Secondary Metabolites (ppm)	
			isolates F1	
	MIC	MFC	MIC	MFC
Candida albicans	2	2	2125	2125

4. Phytochemicals screening results

Table 7.Phytochemicals Screening Results for Endophytic Bacterial Isolates

Phytochemical Screening Test	Result		
	S1	S2	S3
Alkaloids	-	-	-
Flavonoids	-	-	-
Quinone	-	-	-
Tanin	-	-	-
Saponin	-	-	-
Steroids / Triterpenoid	+	+	+

Information: (+) detected the existence of content(-) No contentwas detected

Table 8. Results of Endophytic Fungi Phytochemical Screening

Phytochemical Screening Test	Result
Alkaloids	-
Flavonoids	-
Quinone	-
Tanin	-
Saponin	-
Steroids / Triterpenoid	+

Information:(+) Detected the existence of content (-) No contentwas detected

Bacterial isolates S2 wasconcentrated more than 4000 ppm and bacterial isolates S3 was concentrated more than 14000 ppm.

Antifungal Activity Assay: Antifungal testing using paper disc method shows that endophytic fungi isolates can inhibit the growth of *Candida albicans* with different abilities. F1 fungi isolates which have the largest inhibitory diameters to *C. albicans*were foundat concentration of 34,000 ppm with inhibitory diameter of6.45 ± 0.35 able to inhibit the growth of *Candida albicans*. Endophytic fungi have the potential to produces metabolites are well marked by the formation of clear zone (inhibitory zone) around the discpaper.

Inhibitory zone is formed due to the endophytic fungi have the ability to produces extracellular compounds that are antifungal. The test results showed that antifungal activity of endophytic fungi shiitake mushrooms could be expected to produce secondary metabolites with the ptential as antifungal.Microdilution testing results can be seen from the negative controls (first column) that only contains media, showing the results in the form of a solution does not change color, which remains as before, means there is no microbial growth. The procedures performed during the test can be said aseptic and capable toprovide the correct test results. The test results of F1 isolates show that the solution in column 12 to column 8 become clear, which means at this concentrations F1 isolates can inhibit the growth of*Candida albicans*, while the column 7 to column 3 solution becomes cloudy, which means atthis concentrationF1 isolates have not been able to inhibit the growth of *Candida albicans*. It is estimated that the minimum inhibitory concentration for F1 isolates is at 2125 ppm. Thus, the antibacterial activity of secondary metabolites of F1 isolates has a weak antibacterial activity. Whereas Ketoconazole has a MIC value of 2 ppm. Furthermore, from the results of MIC test, MFC was determined by growing the clear part over the PDA medium (potatosDextrosa Agar) with a streak technique. The result, a secondary metabolite extract has the ability to kill seen from the fungitest. It is characterized by aliquots grown on PDA medium (PotatosDextrosaAgar) are not covered byfungi. The results for MFC was in 2125 ppm concentration. As for KFM ketoconazole is in a concentration of 2 ppm.

CONCLUSION

1. Thereis3 isolates of endophytic bacteria were obtained which is S1 isolates (*Bacillus cereus*), S2 isolates (*Staphylococcus sp*) and S3 isolates (*Bacillus maserans*) and 1 endophytic fungi isolate, F1 isolates (*Penicillium*).
2. Isolates of endophytic bacteria have antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* and isolates of endophytic fungi have antifungal activity against *Candida albicans*.
3. Secondary metabolites of endophytic bacteria isolates had MIC and MBC were varied as follows: S1 isolates is more than 32000 ppm, S2isolate is more than 4000 ppm and S3 isolates is more than 14000 ppm. MIC and MFC for secondary metabolites of endophytic fungal isolates F1 = 2125 ppm.

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