



Research Article

Antifungal Potency of Secondary Metabolites Produced by Endophytic Bacteria against Pathogenic Fungi *Pyricularia oryzae* Cav.

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ABSTRACT

Blast disease caused by *Pyricularia oryzae* Cav. is one of the most important diseases on rice. One of the alternative controlling methods in *P. oryzae* is biological control through the utilization of secondary metabolites produced by endophytic bacteria. The study aimed to determine the antifungal potency of secondary metabolites produced by rice endophytic bacteria against *P. oryzae*. The experiment was conducted using 9 endophytic bacteria isolated from rice (Os1, Os2, Os3, Os4, Os5, Os6, Os7, Os8, and Os10). Each isolates were grown in ISP2 liquid media and the secondary metabolites compounds were extracted using two different solvents; methanol and ethyl acetate : methanol (4:1) (v/v). The effect of secondary metabolites was tested using agar well diffusion method. The results demonstrated that the secondary metabolites extracted by both solvents have antifungal effect on the growth of *P. oryzae*. The highest growth inhibition was shown by secondary metabolites extracted by ethyl acetate : methanol (4:1) from Os1 (42%) and Os3 (39%). Antifungal activity of the secondary metabolites was indicated by the formation of clear zone. HPLC (High Performance Liquid Chromatography) analysis showed the differences of peaks and retention time between secondary metabolites produced by Os1 and Os3 which has antifungal activity, whereas the secondary metabolites produced by Os10 that did not show the antifungal activity.

Keywords: antifungal, endophytic bacteria, HPLC, *P. oryzae*, secondary metabolites

INTRODUCTION

Blast disease caused by *Pyricularia oryzae* Cav. is one of the most important plant diseases on rice. In Indonesia, *P. oryzae* attacks reached 19,629 ha of 12,883,578 ha of rice yields area in 2009 (Prayudi, 2009). Control strategies for blast must be carried out in order to maintain the quality and quantity of rice yields. The common effort to control blast is the use of fungicides (Srivastava *et al.*, 2017). However, it is commonly known that the extensive use of fungicides has led to negative impacts, including the emergence of pathogens resistant population, accumulation of toxic residues, and negative effects on human health and environment (Sehajpal *et al.*, 2009).

One of the eco-friendly control techniques is the use of biopesticides. Biopesticide is defined as a formulation contains certain bioactive compounds that are potent antibiotics (Schumann & D'Arcy,

2012). Endophytic bacteria are known as producer of biologically active compounds. Secondary metabolites produced by endophytic bacteria are reported able to enhance the plant growth and to inhibit the plant diseases development (Ortiz-Castro *et al.*, 2009). In addition, secondary metabolites from endophytic bacteria also have been reported to have the ability in producing growth regulators, providing nitrogen and phosphate to plants which contribute to stimulate and to enhance plant resistance (Ikeda *et al.*, 2010).

Widiyanti *et al.* (2017) isolated several endophytic bacteria from healthy rice plant isolates which were able to inhibit the growth of several pathogenic fungi of rice including *P. oryzae*. Furthermore, Ainun (2018) reported the inhibitory mechanism shown by those isolates was related to its ability to produce chitinase and HCN. However, it is not known yet whether endophytic bacterial isolates produce secondary metabolites which can inhibit the

growth of *P. oryzae*. This study reported the ability of endophytic bacteria in producing antifungal secondary metabolites against *P. oryzae*.

MATERIALS AND METHODS

Maintenance of Endophytic Bacteria

Endophytic bacteria isolates were isolated from healthy rice plants variety IR64. The endophytic bacteria isolates were successfully isolated and morphologically characterized in various growing media. The bacteria demonstrated a strong inhibition activity against *P. oryzae* (Widiantini *et al.*, 2017). The isolates of endophytic bacteria Os1, Os2, Os3, Os4, Os5, Os6, Os7, Os8, and Os10 refreshed on solid ISP2 (International Streptomyces Project 2) media (4 g yeast extract, 10 g malt extract, 4 g D-glucose, 2% agar, 1 l RO water, pH was adjusted to $7,2 \pm 0,2$) from 30% glycerol stock.

Extraction of Secondary Metabolites on ISP2 Liquid Media

Endophytic bacteria isolates were cultured on ISP2 liquid media. Two loops full of the endophytic bacteria isolates were transferred into Erlenmeyer flask containing 100 ml sterile liquid ISP2 media. Endophytic bacteria were incubated for 10 days at shaking incubator at 120 rpm in room temperature. Then, the optical density values were determined. The endophytic bacteria suspension was centrifuged for 30 minutes at 6000 rpm to separate the bacterial cells from the supernatant. The supernatant was extracted using two types of solvents, 100% methanol and mixture of ethyl acetate and methanol (4:1) (v/v). The supernatant was then evaporated using a rotary evaporator at 45°C and pressure of 218 mbar until the solvent completely evaporated. The filtrate was re-suspended in 1 ml of sterile RO water and filtered through a 0.2 µm pore size of membrane filter.

Screening for Antifungal Activity of Secondary Metabolites against P. oryzae

Antifungal activity of secondary metabolites against *P. oryzae* was tested using agar well diffusion method. Approximately 20 ml PDA media was poured into 9 cm diameter petri dishes. After the media set, a 6 mm diameter well was made using cork borer. As much as 30 µl of the filtrate was pipetted into the well. A 6 mm in diameter of plug of *P. oryzae* was placed 3 cm away from the well. Plates were incubated at room temperature.

The antifungal activity was observed by measuring the radial growth of *P. oryzae* colony. The effect of the filtrate against the mycelia of *P. oryzae* was also observed under the microscope. The percentage of inhibition in radial growth (PIRG) was calculated using the following formula:

$$\text{PIRG} = \frac{R1-R2}{R1} \times 100\%$$

Description:

R1 = radial colony of *P. oryzae* in the control treatment

R2 = radial colony of *P. oryzae* in the treatment plate

Thin Layer Chromatography (TLC) Analysis

The filtrates which prepared earlier were further analyzed by the TLC method. Each filtrate was blotted on Silica gel 60 F264 plate size 10 cm×5 cm (Whatman). Dichloromethane and methanol (9: 1) (v/v) was used as solvent (Dewi *et al.*, 2017). TLC plates were removed from the chamber immediately before the solvent reached 1 cm from the top of the plates. TLC plates were sprayed with butanol, acetic acid, and water with (4: 1: 5) (v/v/v). Then the plate was visualized under the UV light. The appeared stains were marked using a pencil in which the R_f (Retention factor) value were calculated.

Analysis of High Performance Liquid Chromatography (HPLC)

The HPLC analysis was started with preparation of Os extract samples. The samples analyzed by HPLC were only 3 secondary metabolite extracts of Os1, Os3, and Os10. The selection of these samples was based on the antifungal activity of the extract against pathogenic fungi *P. oryzae*. Following the antifungal activity test, the metabolite extract which has high antifungal activity was the extract Os3 isolate, the moderate antifungal activity was the extract Os1 isolate, and no antifungal activity was the extract of Os10 isolate.

Chromatographic separation required 10 µl of each sample solution. The sample was injected into a 10 µl size vial. HPLC separation was done using C-18 column and the pump system used was a gradient system of milli Q water and methanol.

RESULTS AND DISCUSSION

The antifungal activity of secondary metabolites produced by endophytic bacteria extracted with two different solvents was tested against *P. oryzae* and

showed different results. Secondary metabolites extracted with ethyl acetate and methanol (4:1) showed a higher percentage of inhibition compared to the percent inhibition of secondary metabolites extracted with methanol only. The highest percentage inhibition on secondary metabolites from ethyl acetate extraction mixed with methanol reached 42%, while the highest inhibition percentage in secondary metabolites from methanol extraction only reached 38% (Table 1).

When the filtrate was able to inhibit the growth of *P. oryzae*, smaller than control radial colony of *P. oryzae* was formed, leaving gap which called as inhibition zone. The effectiveness of the secondary metabolites in inhibiting the growth of pathogen detected by the size of the inhibition zone. The greater inhibition zone was formed, the more potent of the compound in inhibiting the growth of pathogens (Barry *et al.*, 1979). Not all of the endophytic bacteria isolates were able to produce antifungal compound against *P. oryzae*. Five isolates (Os1, Os3, Os4, Os5 and Os6) demonstrated antifungal activity when extracted using both methanol and methanol-ethyl acetate solvent.

To determine which compound responsible for the antifungal activity against *P. oryzae*, TLC analysis was performed. The result demonstrated that the TLC system used in this study was unable to separate the compounds (Figure 1). The TLC analysis for all of the filtrates showed only one spot with the same Rf value although the antifungal activity of each

secondary metabolites were different. There were no stain completely separated on the TLC plates.

Analysis of secondary metabolite compounds was further carried out using HPLC. Three samples were run for the HPLC analysis (Figure 2). Those were Os1 and Os3 showed high antifungal activity whereas Os10 has no antifungal activity. Chromatogram patterns produced by Os1 and Os3 filtrates demonstrated some similar peaks. At the secondary metabolites extracted by ethyl acetate : methanol, similar peaks at the same retention time of 3.5 minutes, 5.5 minutes, and 7.0 minutes were shown at Os1 and Os3 sample. Similar peaks were also detected from Os1 and Os3 samples extracted with methanol. Those peaks were come out at 1.1 min, 1.4 min, 1.8 min, and 3.5 min. Those similar peaks that found both in Os1 and Os3 samples were not found at the Os10 filtrate.

The antifungal testing through agar well diffusion found the potential of the secondary metabolites produced by the endophytic bacterial isolates to inhibit and affect the growth of *P. oryzae*. Mycelia of *P. oryzae* were unable to grow normally when treated with filtrates produced by Os1, Os3, Os4, Os5 and Os6 compared to untreated. Although the inhibition rate was not high (<50% inhibition), this could be optimized through modification of nutritional and cultural conditions. Varieties of carbon source and nitrogen as well as elevated temperature and pH for culture condition has been reported to be able to raise the amount of antifungal compound produces by *Streptomyces* CTF9 (Sajid *et al.*, 2011).

Table 1. Radial growth of *Pyricularia oryzae* colonies and the percentage of growth inhibition due to the influence of secondary metabolite extract of rice plant endophytic bacteria extracted with two different solvents

Treatment	Methanol		Ethyl Acetate and Methanol	
	Radial growth of <i>P. oryzae</i> (cm)	Percent Inhibition (%)	Radial growth of <i>P. oryzae</i> (cm)	Percent Inhibition (%)
Control	3.00 a	-	3.30 cd	-
Os1	2.35 bcd	22	1.92 a	42
Os2	3.00 a	-	3.05 cd	8
Os3	1.85 d	38	2.00 a	39
Os4	2.10 cd	30	2.32 ab	30
Os5	2.83 ab	6	2.97 bc	10
Os6	2.30 bcd	23	2.90 abc	12
Os7	3.28 a	-	3.67 d	-
Os8	2.70 abc	10	3.72 d	-
Os10	3.20 a	-	3.67 d	-

Remarks: Means followed by the same letter in one column were not significantly different based on the Tukey's test at the level of 5%; (-) means no inhibition observed.

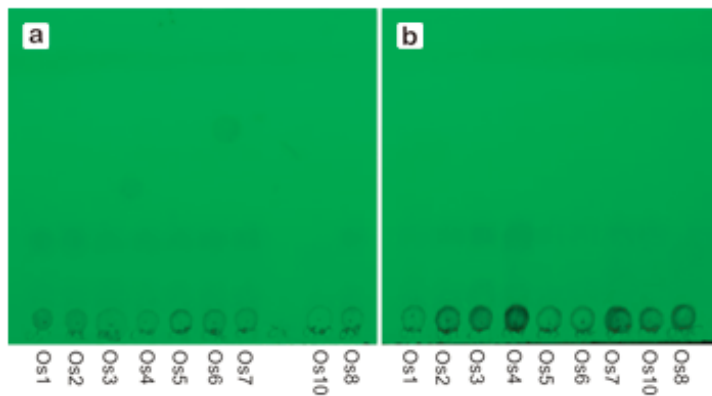


Figure 1. TLC analysis of secondary metabolites extract showed similar Rf value for all samples as it visualized under UV 354 nm, a) filtrates extracted using methanol and b) filtrates extracted using ethyl acetate:methanol (4:1)

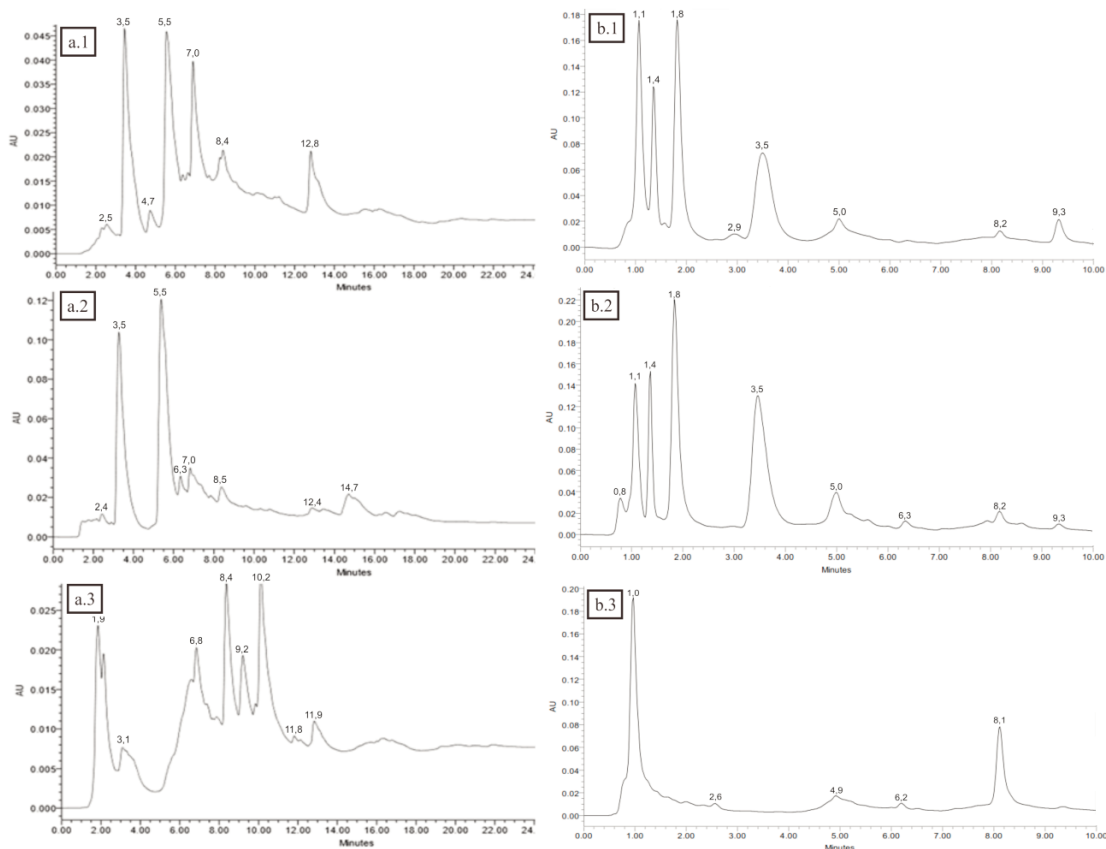


Figure 2. Chromatogram from HPLC analysis of samples Os 1 (1), Os3 (2) and Os10 (3) extracted by ethyl acetate and methanol (a) and methanol (b)

TLC is one of cheap and fast method for identifying the antifungal compound. The TLC bioautography following the compounds separation can distinguish which compound is responsible for antifungal activity (Suleimana *et al.*, 2009). However, in this study, attempt to separate the bioactive compounds using TLC was not successful. The solvent system used

was not able to separate the compounds. Nevertheless, following the HPLC analysis, we found that several similar peaks were detected from filtrates produced by both Os1 and Os3 but not in Os10. It can be assumed that the compound represented by those peaks were the compounds that responsible for antifungal activity. The HPLC analysis is one of

commonly used analytical tools for detecting various compounds in a crude mixture (Cieśła & Moaddel, 2016). However, it uses to identify the compound is limited to the availability of spectra in the database. Only compound within its spectra that available in the database that will be able to identified (Akpotu *et al.*, 2017). In this study, the detected active compounds were unable to be identified as not listed yet in the spectra database. Therefore, we are unable to identify the type of compounds produced by the endophytic bacterial isolates. Further study to identify the compound needs to be done by employing relatively novel and hyphenated techniques such as LC-NMR or HPLC-NMR.

CONCLUSION

Several bacterial endophyte isolates isolated from healthy rice plant demonstrated potential ability to produce antifungal compounds which were able to inhibit the growth of *P. oryzae*. Compound responsible for the antifungal activity was detected by using HPLC analysis. However, its identification of the type of antifungal compounds has not been elucidated. Further study needs to be conducted to identify the respective compounds

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