BIODIVERSITY OF ANTIBIOTIC-PRODUCING SOIL BACTERIA FROM YOGYAKARTA SPECIAL PROVINCE INDONESIA

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Original Article

BIODIVERSITY OF ANTIBIOTIC-PRODUCING SOIL BACTERIA FROM YOGYAKARTA SPECIAL PROVINCE INDONESIA

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ABSTRACT

Objective: The present study aims to evaluate the diversity of antibiotic-producing soil bacteria from Java, Indonesia.

Methods: An agar diffusion method to select the active isolates against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 was used. Furthermore, a combination approach consisting thin layer chromatography-bioautography (TLC-bioautography) analysis and restriction fragment length polymorphism (RFLP) analysis using HaeIII (BsuRI) restriction enzyme digestion were applied.

Results: Out of 19 isolates obtained, 12 isolates exhibited antibacterial activity and subjected further analysis. Based on the R_f value of active spots obtained by TLC-bioautography, the isolates were classified into three groups i.e. isolates with the R_f value of 0.2-0.3 such as KP13 on *E. coli*, isolates with the R_f value of 0.4-0.6 such as LP6, P301 and T25A on *S. aureus* as well as P301 on *E. coli* and isolates with the R_f value of 0.7-0.9 such as J3, J4, J5, J7, JS and TL and LP on *S. aureus* as well as J3, J4, J5, J7, JS, KP13 and TL on *E. coli*. Moreover, based on RFLP profile the isolates were assigned into three groups at the similarity level of 56 %. The isolate KP13 was assigned as Group A, whereas the isolates TL, LP6 and J4 were assigned as Group B and the isolates LP, P301, T25A, P302, JS, J7, J5 and J3 were assigned as Group C. Interestingly, the isolate KP13 was absolutely different from others pointed by zero of % similarity and might produce more than one antibacterial compounds.

Conclusion: The soil bacteria isolates are differentiated into three groups and the KP13 isolate shows zero % similarity to the other isolates.

Keywords: Soil bacteria, Diversity, NRPS, TLC-bioautography, Antibiotic.

INTRODUCTION

There is no doubt that antibiotics play an important role against infectious diseases since last 80 y [1]. The use of antibiotics in the management of the infectious diseases have saved millions of lives worldwide, however emerging and increasing resistance to antibiotics have limited their use. On the other hand, there is a lack of concerted efforts to unearth new antibiotics to take on the menace antimicrobial resistance [2–5]. Therefore, the need for new antibiotics are urgently needed, particularly those directed against multidrug-resistant bacteria [1, 2, 6].

It is well-known fact that until now soil bacteria have been the source for most of the antibiotics. The soil bacteria have made a phenomenal contribution to the antibiotic drug discovery and development over last seven decades [7]. Thousands of bioactive compounds originated from soil bacteria have been isolated. Moreover, hundreds of these bioactive compounds are now used in clinical fields as antibacterial, antifungal, antiparasitic and anticancer agents. Examples include chloramphenicol, erythromycin, neomycin, streptomycin, nystatin, tetracycline and amphotericin B [8, 9]. The soil bacteria are still being the chief natural antibiotic producers [1].

Traditionally, the screening of antibiotic-producing microorganism is mainly based on bioassays, which are limited by the screening models used and culture conditions resulting in lack of related bioactive compounds production [10]. Moreover, the bioassay approach are a complex, time-consuming and expensive endeavor. To date, the combined genomic approach with thin layer chromatography (TLC) fingerprint are widely used to identify microorganisms with the potential to synthesize efficiently bioactive compounds [10–14].

Non-Ribosomal Peptide Synthetase (NRPS) genes identification are widely applied to identify soil bacteria with potential bioactive compounds. These genes encode NRPS which involved in the synthesis of a large number of non-ribosomal peptides bioactive compounds produced by microorganisms [15,16]. Thin layer chromatography fingerprint is a simple tool to separate one compound to the others. This tool is also widely used to identify or distinguish bioactive compounds produced by microorganisms. In addition, it can be used to diverse microorganism based on their products [17–19]. In this study, identification of NRPS genes in combination with TLC fingerprint was used to identify soil bacteria isolates from Central Java, Indonesia.

MATERIALS AND METHODS

Soil sample preparation and bacterial isolation

The soil samples were collected from Seplawan cave and rhizosphere of ginger plant (*Zingiber officinale*), eucalyptus plant (*Melaleuca leucadendra*), galanga plant (*Alpinia galanga*), rice plant (*Oryza sativa*) and tin plant (*Ficus carica*) from Yogyakarta Special Province, Indonesia and its surroundings. Samples were taken at a depth of 10-15 cm into the clean sterile plastic bag. Each sample was then diluted in 0.9 % (w/v) NaCl with $10^{-1}$to 10^{-4} dilution series and spread on SNA (Starch Nitrate Agar) medium containing cycloheximide [20] and incubated at room temperature for 7 d. The colony which looked different was purified on SNA medium and incubated at room temperature until grew many of uniform bacterial colonies of each isolated bacteria.

Fermentation procedure

The fermentation was carried out using the method as described by Hamdin *et al.* [20] with modification. One loop of bacterial colonies was inoculated in 5 ml of Starch-Nitrate Broth medium and incubated on a rotary shaker at 200-250 rpm for 5 d at room temperature. The culture was then inoculated into 100 ml Liquid Starch-Nitrate in 500 ml Erlenmeyer and incubated at room temperature shaker for 14 d. Then the culture was transferred to a conical tube and centrifuged at 3000 rpm 15 min. The supernatant was taken as a source of secondary metabolites.

Extraction of secondary metabolites

The supernatant containing secondary metabolites were extracted with ethyl acetate (1: 1) (v/v) by shaking for 10 min. The top layer was taken and inserted in tubes. The extraction was carried out until the solvent got colourless. Ethyl acetate phase was collected and passed through a column that has been filled with Na_2SO_4. Afterward, the filtrate was evaporated to get dry extract and stored in the refrigerator for the next test.

Thin layer chromatography (TLC)

A total of 1.25 mg of ethyl acetate extracts was diluted in methanol then eluted in TLC system. The TLC was performed with the stationary phase of silica gel F254 (E. Merck) and a mobile phase of chloroform-methanol (7: 3) (E. Merck). The chromatographic spots were detected using ultraviolet ray at 254 and 366 nm.

Preparation of test bacteria

One loop of bacterial colonies of *Staphylococcus aureus* *Escherichia coli* were grown in 1 ml of BHI broth medium and incubated for 24 37 °C. Subsequently, it was taken 100 µl and put into 1 ml of BHI and then incubated for 4 h at 37 °C. After incubation, it was diluted with 0.9 % NaCl to turbidity equal to the Mc Farland standard (10^8 CFU/ml). The test bacterial suspension was spread on Mueller-Hinton medium [21].

Determination of antibacterial activity

Antibacterial activity was carried out using cup plate method. Each well on Mueller Hinton agar medium having been given the t bacteria was filled with 50 µl of culture broth. Afterwards, the each well was incubated at 37 °C for 18-24 h. The existence of sterile zone around the wells referred that the culture broth has antibacterial activity [22, 23]. In addition, this method was also applied to examine the antibacterial activity of ethyl acetate extract. The extract was previously suspended in 10 % DMSO to be a concentration of 20 % (w/v). The 50 µl of each suspension was put into the well.

The bioautography against *S. aureus* and *E. coli* of TLC plate containing chromatogram patches was carried out on Mueller Hinton agar medium. The examination was performed by touching the TLC plate for 30 min on Mueller Hinton agar me previously cultivated the tested bacteria. Subsequently, it was incubated at 37 °C for 18-24 h. The appearance of the sterile zone (no bacterial colonies) after incubation reveals that the chromatogram spots contain antibiotic compounds [24].

Genomic DNA extraction

Extraction of bacterial genomic DNA was done according to literature method [25–27] with modificatio isolated bacterium was grown for 4 d at 30 °C with shaking in 250 ml flask containing 70 ml of SNB medium. The pellet of 1 ml isolate culture was harveste centrifugation (5000 rpm 10 min) and washed twice using TE buffer [100 mM Tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 1.5 M NaCl]. Then, lysozyme (50 µl, 10 mg/ml) was mixed with the pellet and incubated at 37 °C for 1 h. The ble was further incubated at 65 °C with SDS [50 µl, 20 % (w/v)] for 2 h. The mixture was then centrifuged at 6000 rpm for 10 min. a ard; the supernatant was precipitated by NaCl (50 µl, 5M) and incubated at room temperature for 2 h. After centrifugation at 13 000 rpm for 10 min, the DNA was separated from the aqueous phase by adding cooled is panol (50 µl) and keep in the freezer (-20 °C) overnight. The mixture was centrifuged at 13 000 rpm for 5 min. The precipitate DNA was cleaned with 70 % ethanol (50 µl) and the supernatant was then removed. The purity of DNA solution was re-suspended with TE buffer (50 µl) and then checked under ultra voilet ray at 260 and 280 nm.

Amplificat and RFLP of NRPS gene

The NRPS genes were amplified from purified DNA of the strain as cribed previously [15] using a commercial kit (Fermentas®) and primers A3F (5'-GCSTACSYSATSTACACSTCSGG-3'), A7R (5'-SASGTCVCCSGTSCGGTAS-3'). The condition of ampl d gene fragment included pre-denaturation of the target DNA at 95 °C for 5 min followed by 35 cycles at 95 °C for 0.5 min, primer annealing at 58 °C for 2 min and primer extension at 72 ° r 4 min. The final reaction mixture was held at 72 °C for 10 min. The PCR product was exposed by electrophoresis u 1 % (w/v) agarose gels stained with ethidium brom Each PCR product was digested with HaeIII (BsuRI). The digests were incubated at 37 °C for 3 h. After digestion, the RFLP patterns were sc ned using 2 % agarose gel electrophoresis. Subsequently, the degree of similarity between strains was calculated by percent similarity, and clustering was performed statistically by the un weighted pair group method using average linkages (UPGMA) using MVSP 3.2.

RESULTS

Antibacterial activity

Nineteen soil bacteria have been isolated, and the antibacterial vity of the supernatant and ethyl acetate extract of culture broth of the isolates against *S. aureus* and *E. coli* has been ev ed (table 1). Among 19 the supernatants, six supernatants were active against *S. aureus* with a diameter of inhibition zone varied from 7.2 to 11.5 mm and 11 supernatants were active against *E. coli* (6.5-11.5 mm). However, the antibacterial activity of the supernatants were not similar to thyl acetate extracts. Out of 19 the ethyl acetate extra 7 extracts were active against *S. aureus* (8.8-12.8 mm) and only 10 extracts were active against *E. coli* (11.8 mm). The supernatant of J4 isolate had the highest activity against both *S. aureus* and *E. coli* (11.5 mm). For the ethyl acetate extract, the highest activity against *S. aureus* was shown by the GST isolate (12.8 mm), whereas the highest activity against *E. coli* was shown by J4 isolate (21.3 mm).

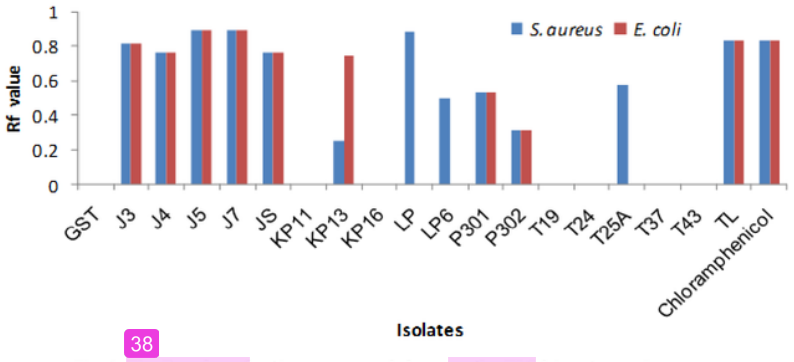

Fig. 1: The R_f value of active compounds base on the TLC-bioautography assay

Table 1: Antibacterial activity of supernatants and ethyl acetate extracts of the culture broth against *S. aureus* and *E. coli*

S. No.	Isolates	*Diameter of inhibition (mm)			
		Supernatants		Ethyl acetate extracts	
		S. aureus	*E. coli*	*S. aureus*	*E. coli*
1	GST	9.25±0.29	-	12.75±0.87	-
2	J3	-	-	11.75±0.50	12.75±0.29
3	J4	-	-	11.75±0.87	21.25±0.96
4	J5	-	7.00±0.00	11.50±0.00	11.75±0.87
5	J7	8.38±0.48	8.38±1.03	11.63±0.63	11.75±0.65
6	JS	9.00±0	7.00±0.00	11.50±1.68	12.25±0.50
7	KP11	-	-	13.00±0.41	-
8	KP13	-	-	13.00±0.82	11.25±0.29
9	KP16	-	7.50±0.58	11.00±0.58	11.25±1.04
10	LP	-	-	10.75±0.5	-
11	LP6	-	8.00±1.22	8.75±0.29	-
12	P301	-	11.50±1.29	-	-
13	P302	-	6.50±0.00	-	-
14	T19	-	-	8.00±0.41	-
15	T24	11.5±0.91	11.50±0.71	11.50±0.58	14.50±0.91
16	T25A	-	-	12.25±0.50	-
17	T37	-	6.50±0.00	9.25±0.65	14.75±0.50
18	T43	7.13±0.63	8.75±1.19	8.75±0.65	15.25±0.29
19	TL	10.00±0.82	9.38±1.32	12.00±0.71	-
20	Chloramphenicol			33.50±1.91	35.00±1.15

Each value represents mean±SD of four independent replicates

TLC-bioautography analysis

Furthermore, the ethyl acetate extract of the culture broths were subjected for TLC-bioautography analysis against *S. aureus* and *E. coli* (fig. 1). Among the 19 ethyl acetate extracts tested, 12 ethyl extracts active against *S. aureus* with the R_f value of active spots varied from 0.25 to 0.89 and only nine extracts active against *E. coli* with those R_f value varied from 0.30 to 0.89. Based on the R_f value, the isolates can be classified into three groups i.e. isolates with the R_f value of 0.2-0.3 such as KP13 on *E. coli*, isolates with the R_f value of 0.4-0.6 such as LP6, P301 and T25A on *S. aureus* as well as P301 on *E. coli* and isolates with the R_f value of 0.7-0.9 such as J3, J4, J5, J7, JS, TL and LP on *S. aureus* as well as J3, J4, J5, J7, JS, KP13 and TL on *E. coli*.

Among the 19 ethyl acetate extracts tested, the ethyl acetate extract of KP13 isolate exhibited different characteristic compared to the other extracts (fig. 1). The R_f value of KP13 on *S. aureus* (0.25) was different from that on *E. coli* (0.75) as shown in fig. 2. It was indicated that more than one antibacterial compounds might be produced by the KP13 isolate.

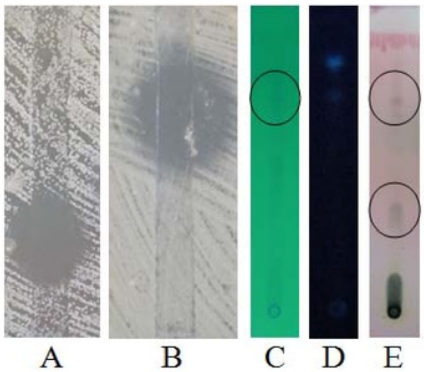

Fig. 2: Bioautographyanalysis of ethyl acetate extract of isolate KP13 against *S. aureus* (A) and *E. coli* (B), as well as the TLC result detected under UV 254 nm (C), UV 366 nm (D) and vanillin-sulfuric acid sprayed reagent (E)

Amplification and RFLP of NRPS gene

Twelve isolates showing antibacterial activity on bioautography analysis were then amplified on NRPS genes which expected yield was 700 bp. The result showed that all isolates had NRPS gene, except the isolated KP13. Furthermore, based on the RFLP profile of the NRPS genes (fig. 3) and the dendrogram profile (fig. 4), the isolates were assigned into three groups at the similarity level of 56 %. The isolate KP13 was assigned as Group A, whereas the isolates TL, LP6 and J4 were assigned as Group B and the isolates LP, P301, T25A, P302, JS, J7, J5 and J3 were assigned as Group C.

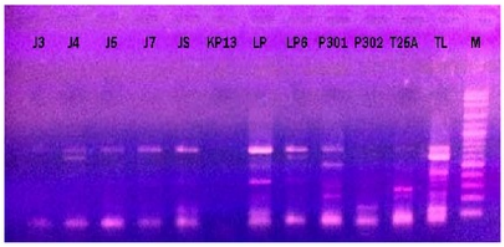

Fig. 3: Dendrogram of RFLP of NRPS gene sequence

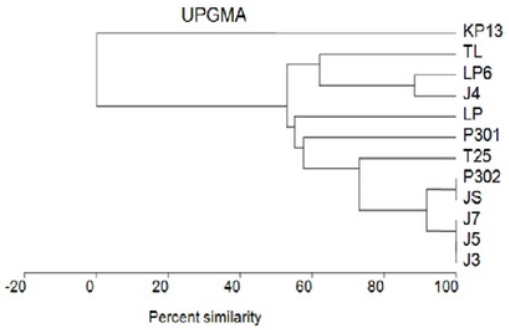

Fig. 4: Dendrogram of RFLP of NRPS gene sequence

DISCUSSION

This study was conducted to evaluate the diversity of antibiotic-producing soil bacteria from Yogyakarta Special Province, Indonesia based on the R_f values of active compounds in combination with the RFLP profile of NRPS gene sequence. The R_f values were determined using TLC-bioautography method. This method is sensitive for detection of active compounds even in the small amount [28]. Moreover, this method is also a simple tool to select and early dereplicate active compounds for further study [29, 30]. Therefore, TLC-bioautography method is often used for preliminary to detect active antimicrobial compounds from producing microorganisms' organic extracts, especially determination of the position the hot spot on TLC [19, 29, 31].

According to the R_f value, the isolates could be classified into three groups i.e. isolates that produced active compounds with the R_f value of 0.2-0.3, 0.4-0.6 and 0.7-0.9, respectively. The active compounds with the R_f value of 0.7-0.9 were closely related to the R_f value of chloramphenicol as the control, whereas the active compounds with the R_f value of 0.2-0.3 and 0.4-0.6 might be totally different with the chloramphenicol. However, the TLC-bioautography assay to determine the R_f value was performed using the single solvent system in this study. This system can not distinguish the active compounds having similar solubility. Further, TLC-bioautography assay will be performed using various solvent systems in order to distinguish the isolates that closely identical with potential antimicrobial activity.

The NRPS genes are involved in the synthesis of large number of non-ribosomal peptides of microorganisms [15, 16]. The RFLP profile of this gene sequence is frequently used to evaluate diversity and to distinguish among large soil bacteria populations [10–12, 14, 15, 26, 32–37]. This method is effective at dereplicating or identifying unique isolates. Therefore, combining the data derived from the TLC-bioautography method and RFLP profile would enable screening efforts to focus on the most potential isolates and increasing the chance of finding secondary metabolites with interesting antimicrobial activities.

According to the RFLP profile, the isolates could be classified into three groups at the similarity level of 56 % i.e. isolate KP13 was assigned as Group A, whereas the isolates TL, LP6 and J4 were assigned as Group B and the isolates LP, P301, T25A, P302, JS, J7, J5 and J3 were assigned as Group C. The isolate of Group A or the KP13 isolate was absolutely different from others as demonstrated by zero of % similarity. This isolate did not expressed the NRPS genes. However this isolate produced active compounds against both *S. aureus* and *E. coli*. Interestingly, the R_f value of the active compounds against *S. aureus* (0.25) was not similar from that on *E. coli* (0.75) indicating that this isolate produced two different active compounds. Furthermore, none of the NRPS genes in the KP13 isolate may be due to the fact that NRPS genes of this isolate were silent or these gene clusters of this isolate were incomplete.

The isolates of Group B or TL, LP6 and J4 isolates showed 56 % similarity to the Group C or LP, P301, T25A, P302, JS, J7, J5 and J3 isolates. Furthermore, the LP isolate showed 58 % similarity of RLFP to the other isolates of the Group C, whereas the J3, J5 and J7 isolates showed 100 % similarity and similar R_f values indicating that they were same isolates. The isolates P301, T25A, P302 and JS exhibited in the different group each other (fig. 4). As the previous study reported by Zhao *et al.* [32] and Li [10], no correlation between NRPS genes expression and antimicrobial activity of the isolates were observed in this study. There are isolates possessing the NRPS genes showing no antimicrobial activity and vice versa.

CONCLUSION

In conclusion, TLC-bioautography screening of active extracts produced by soil bacteria from Yogyakarta Special Province results in 12 ethyl acetate extracts active against *S. aureus* and *E. coli* with R_f values that can be classified into three groups namely isolates with the R_f value of 0.2-0.3, 0.4-0.6 and 0.7-0.9. Furthermore, screening based on the RFLP profile of the NRPS genes, the soil bacteria isolates are differentiated into three groups namely the group of KP13 isolate that shows zero % similarity to the other isolates and the group of TL, LP6 and J4 isolates that show 56 % similarity to the other group of LP, P301, T25A, P302, JS, J7, J5 and J3 isolates.

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CONFLICT OF INTERESTS

Declare none

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