Gram Positive Antibacterial Activity of Ethyl Acetate Extract of *Penicillium* sp. LBKURCC34’s Growth Media Stimulated by *Staphylococcus aureus*

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Abstract. This study was aimed to evaluate the antimicrobial activity of local isolate of peat swamp soil strain, *Penicillium* sp. LBKURCC34 on Gram positive pathogen, *B. subtilis* ATCC11774 and *S. epidermidis* ATCC12228. The fresh strain of *Penicillium* sp. LBKURCC34 and *Staphylococcus aureus* was added into fermentation media and incubated for 14 days. The media extracted with ethyl acetate and evaporated, then the concentrate dissolved in methanol. Antimicrobe test was performed by the disc diffusion method and the resazurin-based turbidometric method toward Gram positive bacterial pathogens (*Bacillus subtilis* ATCC11774 and *Staphylococcus epidermidis* ATCC12228). The crude extract could inhibit the growth of these pathogen microorganism. The extract with biotic stimulation has higher activity than the extract without biotic stimulation. It could give 10% higher activity against *Bacillus subtilis* ATCC11774 compared with positive control, amoxicillin. This fungal strain is potential as antibiotics producer.

1. Introduction

Antibiotics have long been on the first row in fighting against microbial infection. Intensive research on antibiotics has been underway for many years ago. Recent studies have dealt with the synthesis of large numbers of antibiotics over the past three decades and the search for new antibiotics from natural products such secondary metabolites from microorganism and plant. The synthesis antibiotics caused complacency about the threat of antibiotics resistance [1]. Furthermore, the discovery of new antibiotics has lagged behind with the development of antibiotics-resistant infectious microorganism [2][3]. Therefore, the search for novel antibiotics from natural origins especially microorganism is an example of successful bioprespecting. Some of the most well known extrolites that are produced by microorganism are species in *Penicillium*

This strain is the most famous and economically important being penicillins produced by *Penicillium* sp. The discovery and development of penicillin from *Penicillium notatum* by Sir Alexander Fleming represent one of the most important developments in the annals of medical history. This discovery came by accident and serendipity when the petri dish of *Penicillium* gave clear zone onto *Staphylococci* growth area [4]. Since then penicillin being the greatest weapon yet developed to combat infectious diseases. Exploration *Penicillium* potency from various places especially from extreme and understudied ecosystem became one strategy to look for novel bioactive compound [3].
Indonesian tropical peat forest is one an example of a challenging habitat (acidic, waterlogged and nutrient poor environment) as well as has enormous microbial diversity. Our previous study has isolated several *Penicillium* strains from natural forest of biosphere reserve area of Giam Siak kecil – Bukit Batu Riau which are named by culture collection; LBKURCC 29, 30, 34, 38, and 39. The screening of antibiotic extrolites from these strains resulted in the most potential strain was *Penicillium* sp. LBKURCC34 [5]. Unfortunately, the extract from the fungus only gave 50-70% activity compared to positive control amoxicillin. To increase the activity, in this paper, modification of antibiotic production method was applied. The medium of local isolate, *Penicillium* sp. LBKURCC34, was added by *Staphylococcus aureus*, then the media was extracted by ethyl acetate after incubation 14 days. This study carried out to explore Gram positive antibacterial activity as preliminary investigation. While *Bacillus subtilis* was chosen due to this species capable to grow within diverse environments including the gastrointestinal tracts of animals, *Staphylococcus epidermidis* is an innocuous commensal microorganism on the human skin. These species are widely used for antimicrobial assessment.

2. Methodology

2.1. Preparation of Media and Fungal and Bacterial Inoculum

2.1.1. *Potato Dextrose Agar (PDA)*. PDA (Merck Cat. No.1.10130) liquid was obtained by dilution of 39 g of PDA powder into 1 L of aquadest. This media was autoclaved for 15 minutes at 121°C. After cooling to 50°C, 5 mL of 1% sterile citric acid was added to every 1 L PDA to inhibit the growth of bacteria. Media (15 ml) were dispensed into petri dishes, and allowed to solidify. This solid media was used for preparing fresh *Penicillium* sp. LBKURCC34.

2.1.2. *Potato Dextrose Broth (PDB)*. PDB (Merck Cat No.1.08339, 24 g) powder was diluted into 1 L of aquadest. Each 50 ml PDB media in 250 ml erlenmayer flask was autoclaved for 15 minutes at 121°C. This media was used for initial inoculum of *Penicillium* sp. LBKURCC34 preparation.

2.1.3. *Initial inoculum of Penicillium sp. LBKURCC34 preparation*. PDB media (50 ml in 250 ml erlenmayer flask) was added spores of *Penicillium* sp. LBKURCC34 inoculum at a initial spore concentration of 7 x 10^12 spores/ml (OD 0.34). This initial inoculum was incubated at 30°C for 7 days on a rotary shaker (125 rpm).

2.1.4. *Media for Antibiotic Production*. a different media with the following composition (pH 5.6-5.8): glucose 2%, yeast extract 0.5%, peptone 0.5%, MgSO_4 0.05%, KH_2PO_4 0.1% was autoclaved for 15 minutes at 121°C, and continued by adding initial inoculum of *Penicillium* sp. LBKURCC34.

2.1.5. *Nutrient Agar (NA)*. NA (Merck Cat. No.1.05450) was prepared by dilution of 28 g of NA powder into 1 L of Aquadet. This media was autoclaved for 15 minutes at 121°C. After cooling to 50°C, dispensed into tube and allowed to solidify. This solid media was used for preparing fresh bacterial pathogen *Staphylococcus aureus* as elicitor on antibiotic production and antibacterial activity test.

2.1.6. *Nutrient Broth (NB)*. NB (Merck Cat. No.1.06649) liquid was obtained by dilution of 13 g of NB powder in to 1 L of aquadest, then autoclaved for 15 minutes at 121°C. This media was used for preparation of *Staphylococcus aureus* as elicitor on antibiotic production.

2.2. *Antibiotic production by Penicillium sp. LBKURCC34 with and without stimulated of elicitor Staphylococcus aureus*

Antibiotics production was performed based on Lee et al., method [6] with some modification. There were two of 1 L flask that contained production media. Firstly, fresh inoculum of *Penicillium* sp. 
LBKURCC34 were inoculated into both media for antibiotic production. Incubation was continued with shaking for 5 minutes everyday at 30°C for 14 days (without pathogen addition – called Inoculum A). On Day 3, pathogen bacterial, fresh *S. aureus* in nutrient broth media 5 ml (OD 0.1) was added to another production flask (with pathogen addition – called Inoculum B). After 14 days fermentation, the mycelium was separated from the medium (Inoculum A and Inoculum B) by filtration using a Whatman® GF/C filter (CAT No. 1822-055). The filtrates were extracted twice with ethyl acetate (media filtrates – ethyl acetate 2: 1). The ethyl acetate extracts were then combined, dried with anhydrous Na$_2$SO$_4$, and the ethyl acetate evaporated at room temperature in a rotary vacuum evaporator. Residue obtained from every 1 l fermentation media was dissolved in 1 ml methanol (MeOH). It resulted in Extract A and Extract B.

### 2.3. Antibiotic activity screening

To determine the antibiotic activity of the extracts against Gram positive pathogens (*B. subtilis* ATCC11774 and *S. epidermidis* ATCC12228), the extracts were tested using the disc diffusion and the resazurin-based turbidimetric methods.

- The disc diffusion: Ten μl of redissolved ethyl acetate extracts of *Penicillium sp.* LBKURCC34 fermentation medium (Extract A and Extract B) in MeOH (Merck 1.06009.1000, pa.) were applied to sterile 6-mm antibiotic blank disc and left to dry at room temperature (30°C), before transferring to a agar plates that has been spreaded with *B. subtilis* ATCC11774 and *S. epidermidis* ATCC12228. The cultures were incubated at 37°C for one day, and the inhibition zones were measured (mm, started the edge of disc). The assays were performed in triplicates. As positive controls, ten μl of a 3 μg/μl Amoxicillin (Sigma A8523-5G) in MeOH were used. As negative controls 10 μl of the solvent MeOH were used.

  The resazurin-based turbidometric method [7]: Resazurin solution was prepared by dissolving 0.0675 g in 10 ml sterile distilled water, then a vortex mixer was used to mix the solution to ensure homogeneity. The solution was sterilised using syringe filter 0.2 μm and kept in a brown bottle. In a 96-well flat bottom microtiter plate, for each pathogen bacterial culture, the assay composed duplicate of Extract A and B, control positive amoxicillin, control positif tetracycline, and control negative dimethyl sulfoxide (DMSO). Each extract was used 12 concentration of 4000; 2000; 1000; 500; 250; 125; 62.5; 31.25; 15.62; 7.81; 3.91; and 1.95 μg/ml. Each well contained 20 μl of pathogen bacteria 20 μl (in MHB after 1 day incubation with OD 0.1). Solution was homogenized and incubated for 1 day. Next day, resazurin solution 20 μl was added, then the plate was incubated for 24 hours. The lowest concentration prior to colour change was considered as the minimum inhibitory concentration (MIC). The solution 100 μl on MIC well was spreaded on nutrient agar plate, then incubated for 1 day. Minimum bactericidal concentration (MBC) was considered to the concentration that there was no bacterial growth on plate.

### 3. Results and Discussion

During this study, biotic stimulation (*Staphylococcus aureus*) was chosen as the elicitor to stress the fungi which boosted up its antibacterial exhibition in comparison to the normal growing strain. The fermentation of the *Penicillium* sp. LBKURCC34 in antibiotic production media was added by pathogen bacteria, *Staphylococcus aureus* at the mid-exponential growth phase, Day 3 [8]. There were different colour of the media with and without pathogen bacteria, *S. aureus*. While the media without pathogen addition gave clear dark yellowish solution, the media with pathogen addition gave cloudy yellowish solution (pictures not shown). Pathogen bacteria influence the appearance of the fermentation process.

The ethyl acetate extracts of antibiotic production media (Extract A and B) were assessed their potency as antibacteria against Gram positive bacteria *B. subtilis* ATCC11774 and *S. epidermidis* ATCC12228 using disc diffusion method. This well-known procedure that is used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing offers many advantages [9].
There are simple methods with low cost as well as could test enormous numbers of microorganism antimicrobial agents and easily interpret the results. This assay revealed that the extract B gave better activity than the extract A against these two pathogens (28 – 43% higher) (Table 1). The extract B also had compounds that could inhibit the pathogens better than positive control, amoxicillin against \textit{S. epidermidis} ATCC12228 (higher 10% in the same concentration, 38 µg/disc, (Table 2)). It seemed that \textit{Staphylococcus aureus} forced \textit{Penicillium} sp. LBKURCC34 to secrete more active antibacterial compounds. Need further investigation to compare the composition of these extracts.

**Table 1.** The antibiotic activity of ethyl acetate extract of \textit{Penicillium} sp. LBKURCC34 fermentation medium against Gram positive bacteria using disc diffusion method.

| No | Sample  | Clear Zone (mm) | \textit{B. subtilis} ATCC11774 | \textit{S. epidermidis} ATCC12228 |
|----|---------|-----------------|---------------------------------|-----------------------------------|
| 1  | Extract A | 19 µg/disc | 6.00 ± 0.00                     | 6.00 ± 0.00                       |
|    |         | 38 µg/disc | 6.53 ± 0.31                     | 7.03 ± 0.15                       |
|    |         | 57 µg/disc | 6.83 ± 0.15                     | 7.90 ± 0.10                       |
| 2  | Extract B | 19 µg/disc | 8.40 ± 0.20                     | 8.87 ± 0.11                       |
|    |         | 38 µg/disc | 11.63 ± 0.15                    | 10.53 ± 0.21                      |
|    |         | 57 µg/disc | 12.10 ± 1.05                    | 10.77 ± 0.90                      |
| 3  | Amoxicillin | 38 µg/disc | 10.50 ± 1.37                    | 16.50 ± 3.19                      |

The extract A was fermentation without \textit{S. aureus} addition. The extract B was fermentation with \textit{S. aureus} addition.

**Table 2.** Percentage of inhibition of ethyl acetate extract of \textit{Penicillium} sp. LBKURCC34 fermentation medium compared with positive control, amoxicillin (38 µg/disc)

| Pathogen       | Concentration of sample | Percentage of Inhibition compare to control (%) | Extract A | Extract B |
|----------------|-------------------------|-----------------------------------------------|-----------|-----------|
| \textit{B. subtilis} ATCC11774 | 19 µg/disc | 57.14                                         | 80.00     |           |
|                | 38 µg/disc | 62.19                                         | 110.76    |           |
|                | 57 µg/disc | 65.05                                         | 115.24    |           |
| Amoxicillin (38 µg/disc) |            | 100.00                                        | 100.00    |           |
| \textit{S. epidermidis} ATCC12228 | 1.9 µg/disc | 36.36                                         | 53.76     |           |
|                | 3.8 µg/disc | 42.60                                         | 63.82     |           |
|                | 5.7 µg/disc | 47.89                                         | 65.27     |           |
| Amoxicillin (3.8 µg/disc) |            | 100.00                                        | 100.00    |           |

The extract A was fermentation without \textit{S. aureus} addition. The extract B was fermentation with \textit{S. aureus} addition.

Due to the agar disc-diffusion method is not appropriate to determine the minimum inhibitory concentration (MIC) as well as could not distinguish bactericidal and bacteriostatics effects, further investigation was conducted using dilution method using resazurin as a dye. Resazurin (7-hydroxy-3H-phenoxazin -3-one 10-oxide) is a blue dye which can be irreversible reduced to a pink and highly red fluorescent substances, resofurin by oxidoreductase within viable cell [7]. This assay has been employed in many studies. MICs and minimum bactericidal concentrations (MBCs) on the extract A and B confirmed disc-diffusion result, the extract B gave higher activity than the extract A. While MIC value of the extract A seemed more than the concentration that had been used (>4000 µg/ml), MIC value of the extract B identified in 2000 µg/ml against \textit{B. subtilis} ATCC11774 and 1000 µg/ml against \textit{S. epidermidis} ATCC12228 (Table 3). However, the extract B could not kill the pathogen.
Tetracycline demonstrated that the most powerful component which is MIC value for these pathogens on 31.12 µg/ml and MBC value for *S. epidermidis* ATCC12228 on 500 µg/ml. The present study revealed probable development and use of novel and effective natural antibiotic extract from local isolate of Indonesian peat swamp forest soil. Further work of isolation and characterization of the bioactive compounds is greatly warranted to produce a detailed chemical profile from the extracts.

**Table 3.** MICs and MBCs of ethyl acetate extracts of *Penicillium* sp. LBKURCC34 fermentation medium and standard antibiotics amoxicillin and tetracycline against *Bacillus subtilis* ATCC11774 and *Staphylococcus epidermidis* ATCC12228

| Sample          | *B. subtilis* ATCC11774 | *S. epidermidis* ATCC12228 |
|-----------------|-------------------------|---------------------------|
|                 | MIC         | MBC        | MIC         | MBC        |
| Extract A       | >4000       | >4000      | >4000       | >4000      |
| Extract B       | 2000        | >4000      | 1000        | 2000       |
| Amoxicillin     | >4000       | >4000      | >4000       | >4000      |
| Tetracycline    | 31.12       | >4000      | 31.12       | 500        |

The extract A is fermentation without elicitor *S. aureus* addition. The extract B is fermentation with *S. aureus* addition. The concentration of MIC and MBC were in µg/ml.

4. Conclusion

The development of natural antibiotics would contribute to tackle antibiotic resistance bacteria problem. Exploration microorganism potency to produce active components could lead the investigation of lead compounds as new candidate antibiotic. This study demonstrated that *Staphylococcus aureus* stimulated the local isolate fungi, *Penicillium* sp. LBKURCC34 to produce and secrete more active extract to inhibit Gram positive bacteria; *B. subtilis* ATCC11774 and *S. epidermidis* ATCC12228. Further investigations such as isolation of the active antibacterial components from this production method are required to provide new effective natural antibiotics compounds and a detailed chemical profile from the extracts.

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