Antifungal Activity from Co-Culture of a Local Fungus of Tropical Peat Swamp Soil, *Penicillium* sp. LBKURCC34 with Gram-Negative and Gram-Positive Bacteria

Yuana Nurulita*, Yuharmen, Andy Dahliati, Yum Eryanti, Supridianto, Khairulinas, Yuli Haryani, & Titania Tjandrawati Nugroho

Chemistry Department, Faculty of Mathematics and Natural Sciences, University of Riau, Pekanbaru 28293

*Corresponding author: ynurulita@lecturer.unri.ac.id

Abstract. Microorganism is important producer of novel bioactive natural products, particularly in the field of drug discovery. Co-culture methods is one of powerful emerging tools for enhancing the chemical diversity of microorganisms. This research used the local culture collection of *Penicillium* sp. LBKURCC34, the fungus isolated from peat soil of primary forest at Giam Siak Kecil Bukit Batu (GSKBB) - Biosphere Reserve in Riau Province, to produce secondary metabolites secreted to the growth media that was cultivated by two different Gram of bacterial pathogen, *Escherichia coli* and *Staphylococcus aureus*. The 14 days fermentation was carried out and the media was extracted with ethyl acetate. The ethyl acetate crude extract was evaporated, then the concentrate was dissolved in methanol. Antifungal, *Candida albicans* test was performed by the disc diffusion and the resazurin-based turbidimetric method. This study found that the crude extract of the co-culture with *S. aureus* could inhibit *C. albicans* growth, while that extract of the co-culture with *E.coli* could not do. The value of minimum inhibitory concentration (MIC) of the potential extract was less than the positive control, Ketoconazole. It only has potency as bacteriostatic extract.

1. Introduction

Recently, the antifungal resistance is an worldwide problem due to the increase in microbial antibiotic resistance in clinical settings and the limited findings of new antibiotic compounds. Resistance to antifungals is a major health problem with an antifungal resistant rate of dispersion not supported by the discovery of new antifungal compounds [1]. Pathogenic fungi are stronger and existing antifungal compounds no longer have any effect. the resazurin-based turbidimetric method.

Natural products are the main source for developing of drugs and other chemical agents. One of the resourceful method to find out new native compounds especially from microorganisms, is by mixing several microorganisms in one culture production media (called co-culture method). This method can increase the productivity of known compounds [2] as well as discover new compounds. Furthermore, on the side of biotechnology, co-culture fermentation could increase the yield as well as improve the control of product qualities and the prospect of applying cheaper substrates [3]. Several compounds have been known as an active compound that produced by co-culture fermentation such as emericellamides A and B [4], mycotoxin [5], and 2,5-diketopiperazines [6].

In this study, co-culture of potential local isolate of peat swamp soil, *Penicillium* sp. LBKURCC34 was reported with modification of fermentation media using co-culture with two different Gram of
bacterial pathogen, *Staphylococcus aureus* (represent as Gram-positive bacterium) and *Escherichia coli* (represent as Gram-negative bacterium). The activity of antifungal, *Candida albicans* was assessed using diffusion method (disc) and the potential extract was measured to find out its minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC).

2. Materials and methods

2.1. Antibiotics production of fungal strain stimulated by pathogen bacteria

Antibiotics production was performed using co-culture of fresh isolates of *Penicillium sp.* LBKURCC34 and bacterial pathogen of *S. aureus* and *E. coli* as elicitors. Generally, the method was based on Lee et al., [7] with some modification. Firstly, fresh spores of *Penicillium sp.* LBKURCC34 were inoculated into flasks with an initial spore concentration of 7 x 10^12 spores/ml (OD 0.34). The flasks contained Potato Dextrose (PD) medium (50 ml). This initial inoculum was incubated at 30°C for 7 days on a rotary shaker (125-150 rpm). At day 7, these two flasks of 50 ml PD initial inoculum and biomass that had grown were transferred to new flasks containing 1 liter of production media. The composition of 1 liter of production media (pH 5.6-5.8) included glucose 20 g, yeast extract 5 g, peptone 5 g, MgSO_4_0.5 g, and KH_2PO_4_1 g. Incubation was continued with shaking for 5 minutes everyday at 30°C for 14 days incubation. Pathogen bacterial elicitors were added on Day 3 which was prepared by 1 loop of pathogen colonies of *S. aureus* and *E. coli* had been inoculated into tubes with 5 ml of nutrient broth (NB), then incubated for 18-24 hours until they reached OD 1.0.

2.2. Extraction

After 14 days of fermentation, the mycelium was separated from the medium (Inoculum A for *S. aureus* addition and Inoculum B for *E. coli* addition) by filtration using a Whatman® GF/C filter (CAT No. 1822-055). The filtrates were extracted with ethyl acetate (media filtrates – ethyl acetate 2:1). The ethyl acetate extracts were dried with evaporation at room temperature in a rotary vacuum evaporator. The residue obtained from every 1 l fermentation media was dissolved in 1 ml methanol (MeOH). It resulted in Extract A (co-culture with *S. aureus*) and Extract B (co-culture with *E. coli*).

2.3. Antifungal activity screening against *Candida albicans*, using the disc diffusion and the resazurin-based turbidometric methods.

2.3.1. The disc diffusion. Ethyl acetate extracts of co-culture: Extract A and Extract B were dissolved in MeOH (Merck 1.06009.1000, pa.) based on the concentration calculation. That extracts were applied to a sterile 6-mm antibiotic blank disc and left to dry at room temperature (30°C), before transferring to agar plate that have been spread with *Candida albicans*. The cultures were incubated at 37°C for 1-3 days, 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.

2.3.2. The resazurin-based turbidometric method: This method was based on Teh et al. [8]. Resazurin solution was prepared by dissolving 0.0675 g in 10 ml sterile distilled water, then mixed by vortex mixer to ensure homogeneity. The solution was sterilised using a syringe filter 0.2 µm and kept in a brown bottle. In a 96-well flat-bottom microtitrator plate, for pathogen fungal culture, the assay composed triplicate of Extract A and B, control positive amoxicillin, control positive tetracycline, and control negative dimethyl sulfoxide (DMSO). In this project, 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 140 µl muller hinton broth (MHB), sample 20 µl, pathogen bacteria 20 µl (in MHB after 1 day incubation with OD 0.1). The solution was homogenized. The next day, resazurin solution 20 µl was added, then the plate was incubated for 48-36 hours. The lowest concentration prior to colour change was considered as the minimum inhibitory concentration (MIC). The solution 100 µl on MIC well was spread on the
nutrient agar plate, then incubated for 2-5 days. Minimum fungicidal concentration (MFC) was considered to the concentration that there was no fungal growth on the plate.

3. Result and discussion

The development of the fermentation method employed more than one microbial strain to constitute synthetic co-culture in order to improve bioproduction has been applied in many biotechnology approaches and widely studied. It has been reported in several projects that the method has been successful to enhance metabolite productivity. Nonaka et al [2] combined *Penicillium pinophilum* FKI-5653 with *Trichoderma harzianum* FKI-5655. While Onaka et al., [9] mixed *Streptomyces* species with mycolic-acid containing bacteria, Schroechk et al. [10] revealed that biosynthesis of archetypal polyketides in *Aspergillus nidulans* could be stimulated by 58 soil-dwelling actinomycetes. Furthermore the capacity of *Penicillium* sp. LBKURCC34 (local isolate from peat swamp soil of Riau), to produce active compounds among other isolates has also been proven [11]. To increase the productivity and the potency to find new compounds from this isolate, this study has tried to modify the fermentation method using elicitor biotic, bacterium of Gram-negative and Gram-positive. After 14 days of incubation in production media, bacteria biotic elicitor changed the appearance of fermentation media. Media *Penicillium* sp. LBKURCC34 in single cultivation showed clear yellowish media (Picture is not showed). Compared with that media, mixed of bacteria and *Penicillium* sp. LBKURCC34, resulted in cloudy yellowish media (Figure 1). Precipitation of bacteria could be found at the bottom of the flask. After 14 days of incubation, the media was extracted by ethyl acetate after filtration from mycelium.

![Figure 1. Production media of co-culture Penicillium sp. LBKURCC34 with pathogen bacteria. (a) is co-culture with Staphylococcus aureus and (b) is co-culture with Escherichia coli.](image)

Dilution and diffusion methods are easy. They provide a high level of accuracy and reproducible result for antimicrobial tests [12]. Dilution method, fungal cells irreversibly reduced the blue dye resazurin to a pink resofurin by oxidoreductase [13]. In the investigation of the activity against *Candida albicans*, Extract A that is formed from co-culture of *Penicillium* sp. LBKURCC34 with *S. aureus* inhibited stronger than Extract B (co-culture of *Penicillium* sp. LBKURCC34 with *E. coli*) (Table 1). At the same concentration (38 µg/disc), this value was slightly lower than the inhibition value of positive control, amoxicillin, because amoxicillin actually has a better activity as antibacterial, not antifungal. The obtained results of the antifungal activity using diffusion and dilution method (MIC value, Table 2) confirmed that there was an activity on Extract A. Table 2 demonstrates the MIC of the extracts and the positive controls against *C. albicans*. It was observed higher MIC values of Extract A compared with Extract B and the positive control, amoxicillin and ketoconazole. Although the mechanism is not clear yet, it seems that Extract A perhaps could damage the cell walls of fungi [14].
Table 1. Antifungal activity of the extracts toward Candida albicans.

| Name of extract                     | Inhibition (mm) |
|-------------------------------------|-----------------|
|                                     | Extract A       | Extract A                      |
|                                     | (Co-culture A)  | (Co-culture S. aureus)         |
| Extract concentration 1 (57 µg/disc)| 8.50 ± 0.43     | 1.13 ± 0.02                    |
| Extract concentration 2 (38 µg/disc)| 7.80 ± 0.53     | 1.01 ± 0.02                    |
| Extract concentration 3 (19 µg/disc)| 0.20 ± 0.10     | 0.00 ± 0.00                    |
| Positive control, amoxicillin       | 8.10 ± 0.00     |                               |
| (38 µg/disc)                        |                 |                               |
| Positive control, ketoconazole      | 25.90 ± 0.36    |                               |
| (38 µg/disc)                        |                 |                               |
| Negative control (methanol)         | 0.00 ± 0.00     |                               |

Table 2. The values of MIC and MFC of the extract A towards Candida albicans.

| Name of extract                     | MIC (µg/ml) | MFC (µg/ml) |
|-------------------------------------|-------------|-------------|
| Extract A                           | 2000        | >4000       |
| Positive control, amoxicillin       | >4000       | >4000       |
| Positive control, ketoconazole      | 4000        | >4000       |

However, further investigation is needed to explore the potency of this extract as well as the method to confirm the promising of microbial isolate to contribute on finding new lead compounds as a new antifungal. A deeper study regarding this part is needed to investigate such as isolation and characterization of the active compounds and regulation of fungal – bacterial secondary metabolism in terms of the biosynthetic gene cluster (BCG) using genome mining method. Further work will be continuing to assess the potency of local fungus to produce candidate antifungal compounds.

4. Conclusion
The present investigation has shown that the co-culture fermentation of the fungi Penicillium sp. LBKURCC34 and S. aureus has the potency to produce bioactive as antibiotics, while the same method using E. coli as an elicitor biotic did not showed better potency. The research needs further investigation to identify the structure of active compounds using isolation and characterization of the active compounds.

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