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Fermentation and Antimicrobial Activity assay from *Aspergillus fumigatus* Isolated from Corncob

[1,2]Friardi Ismed, [1]Annisa Nofriani, [1]Rezi Sri Haryenti, [1]Anita Elsya and[1,2*]Akmal Djamaan

[1]Faculty of Pharmacy, University of Andalas, Padang, Indonesia
[2]Laboratory of Biota Sumatra, University of Andalas, Padang, Indonesia

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ABSTRACT

Fermentation and antimicrobial activity assay from Aspergillus fumigatus isolated from corncob has been carried out. The purpose of this study is to isolate A. fumigatus from corncoband then fermentation to produce secondary metabolites. Fermentation process was conducted using corncob as liquid medium at the temperature of 30^{0}C, agitation 150 rpm, and pH of medium 6. Antimicrobial activity assay and characterization of secondary metabolites were done by agar-diffusion methods, UV-Visible, and infrared spectroscopy method. Extraction of secondary metabolites produced using liquid-liquid extraction method. The purification of the antibacterial compound was used by preparative thin layer chromatography method. Results showed that the optimum condition of fermentation A. fumigates was of 8 days. Antimicrobial activity assay against Staphylococcus aureus, Escherichia coli and Candida albicans indicated that the antibacterial activity of isolated compounds and no antifungal activity observed. It is also found that from UV-Vis spectrophotometer result of fourth isolated compounds that had antibacterial activity showed the maximum wavelength were of 201.80nm, 202.60nm, 203.20nm and 202.00nm respectively. Infrared spectroscopy result the compound AB1and AB2 consist of OH groups, stretch C=C, C – H and C = O, while in the AB3 and AB4compounds there is a stretch of C = O.

Keywords: *Aspergillus fumigatus*, corncob, fermentation, antimicrobial.

INTRODUCTION

There are potential areas for development of corn that widely available in Indonesia, especially on dry land outside Java, such as Sumatra, Borneo, New Guinea and Sulawesi. There are about 6.96 million hectares of land located in14 provinces has the potential for development of corn[1]. This corn can be made become many variety of foods, but leaves waste such as corncobs. Corncobis a major part of the corn waste. Of the weight of corn, 40-50% of them are estimated as corncob [2]. It was stacked in large volumes, it will cause environmental pollution. Pollution among these corn cobs is the growth of fungus.

Natural products from microorganisms and plants play an important role in drug discovery, where sales of these products more than 30% in the worldwide [3]. Microbial secondary metabolites has been regarded as one of the best resources for the discovery of new drugs [4]. Until early 2013, more than 42,000 natural compounds have been known to isolated from microorganisms and high level fungus [5]. From twenty types of the most widely prescribed drugs, six of its produced from fungi [6].

A. fumigatus is a fungus that grows on corn cobs. Research into the potential of secondary metabolites of *A. fumigatus* have been done by Furtado *et al*., (2005) [6] which examined the potential of *A. fumigatus* produces secondary metabolites as antimicrobial, and Coleman *et al*., (2011) [7], suggests that antimicrobial compounds from *A. fumigatus* is gliotoxin. Research on secondary metabolites and its potential as an antimicrobial of *A. fumigates* grow rapidly until now. In 1943, Menzel *et al*. [8] have done with the isolation of antibacterial compounds from *A. fumigatus*, which gliotoxin and fumigacin. Shaaban *et al*., (2013) [9] has done with structure elucidation of secondary metabolites produced by *A. fumigatus* and test the bioactivity against bacteria, fungi and microalgae. From these studies it appears that the result of extract test showed high antimicrobial activity against *Bacillus subtilis*, *Streptomyces virido chromogenes*, microalgae *Chlorella vulgaris, C. sorokiniana* and *Scenedesmussubspicatus*. While 2 single compound showed activity against *B.subtilis, Staphylococcus aureus, Candida albicans* and *Mucormiehi.*

In this paper, we reported the isolation of *A.fumigatus* from corn cob waste, doing fermentation with corn cob cooking water enriched with minerals. Aim of this fermentation is producing secondary metabolites. Secondary metabolites expected from this research are secondary metabolites that active as antimicrobial. This study was conducted to obtain isolates antimicrobial secondary metabolites produced by *A. fumigatus*. Further examination is characterized by thin layer chromatography, ultraviolet-visible spectrophotometer and infrared spectroscopy.

MATERIALS AND METHODS

Procurement *A. fumigatus*
The corn cobs cut into small pieces with knife. Then, left edits in the open area for a week until found growth of fungus.

Isolationand Purification of *A. fumigatus*
Green fungus that grows on corn cob then isolated into PDA medium in a Petri dish. Fungi were incubated in an incubator at25-30^{0}Cfor 5-7 days. If found growth of different colonies macroscopically, it must be separated into another Petri dish to obtain a single fungal isolates.

Identification
Identification carried out macroscopically and microscopically. Macroscopic observation includes direct visual observation of the fungal colonies, while the microscopic observation by using microscope to look at the form of spores of the fungus with the help of reagent lactophenol methylene blue.

The manufacturing medium of water boiled of corn cobs
200g of corn cobs cut into pieces and put into1000ml boiling water for 15 minutes. Then, sterilization the medium boiling water of its and 3% glucose, 0.5% $CaCO_3$, 0.1% $FeSO_4$, 0.2% $MgSO_4$, $ZnSO_4$0.01%, 0.2% $NaNO_3$ with autoclave at a temperature of 121ºC and 15lbs of pressure or about 15 minutes, then mixing aseptic condition. pH fermentation media was adjusted at 6.

Fermentation Process
Two step cultures were perform to fermentation process. First, *A. fumigates* which had been grown on PDA medium then transferred into100 mL of water boiled corn cob media that have been made (liquid media). Incubated at temperature of30^{0}C, agitation was 150 rpm for 72 hours.

Then, 5 mL inoculums pour aseptically into100mL of the fermentation medium into 6 Erlenmeyer flask 250-ml. Incubation at 30^{0}C of temperatures, agitation was 150 rpm for 48, 96, 144, 192, 240 and 288 hours. After the fermentation process is complete, separate the biomass and the measure pH of media.

Fractionation
Filtrate media remaining as results of separation with biomass was then fractionated with ethyl acetate to obtain crude fraction of ethyl acetate.

Separation and Purification of Compounds
The components contained in ethyl acetate fraction was monitored by thin layer chromatography. Results of the appearance of stains under 254nm UV lamp, with mobile phase DCM: methanol (9:1). Further separation using

preparative TLC with silica G60F_{254}as stationary phase and DCM: methanol(9:1) as mobile phase. Scraped each ribbon stains that looked and then reconstituted with ethyl acetate then precipitated the silica. Take the ethyl acetate portion and dried to obtain isolates compounds.

Antimicrobial Activity Test with Bioautography Method

The antimicrobial activity of the crude fraction was evaluated by using bioautography assay against bacteria *Staphylococcus aureus, Escherichia coli* and the yeast *Candida albicans.*

The crude fraction were dissolved (30 mg/mL) in ethyl acetate, from which 4µLwere applied on silica gel G60 F254 thin layer chromatography plate (Merck® Silica Gel60F_{254}). Eluted using a mobile phase dichloromethane: methanol (9:1).After elution, the plates were carefully dried for complete removal of the solvents.

TLC plates which have been developed are placed on potato dextrose agar medium (15ml) for detection of antifungal activity and on nutrient agar medium (15ml) for the detection of antibacterial activity. For the detection of antifungal activity, 5 mL PDA suspended with 1mL spore was poured on a TLC plate. For the detection of antibacterial activity, 5 mL NA media with 1mL of bacterial suspension was poured on a TLC plate.

Then Petri dishes were stored at 4^0C for diffusion for 3hours. Then the plates were incubated at room temperature or 24 hours for bacteria and 4 days for fungi. After incubation, the inhibition zone is checked using a1 mg/5mL iodotetra zolium chloride reagen

Identification and Characterization of Separation Compound

Identification and characterization of isolated compounds includes examining thin layer chromatography used was dichloromethane:methanol(9:1),UV-Vis spectrophotometer at wavelengths between 200-800nm and infrared spectroscopy in the wave number 600-4000cm^{-1}

RESULTS AND DISCUSSION

A. fumigates isolated from corncob. The macroscopic observation it showed colony of green with texture glanular smooth as velvet and this grow thcan be seen after approximately seven days. The microscopic observation using reagents lachtophenol methylene blue, showed that fungi has hypha septate, stalk-the stalk conidiofor that long with a large head(conidia). There is a single spore and chain. So that the identification results conclude that this fungusis *A. fumigatus.*

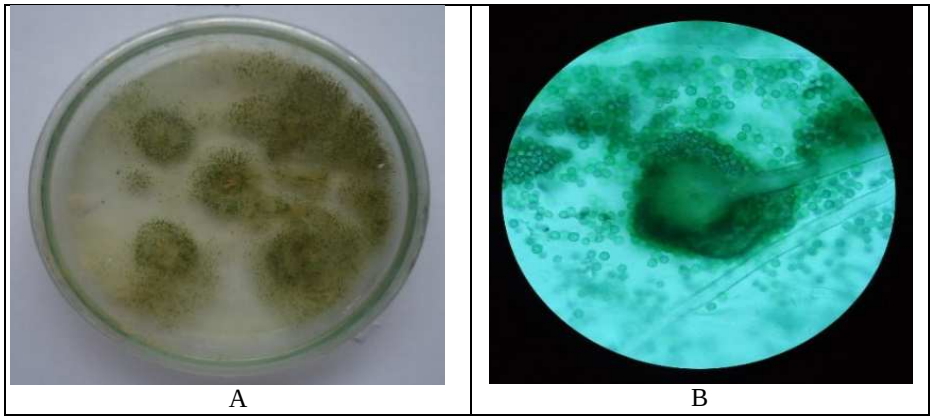

Figure 1. Observation of macroscopic (A) and microscopic (B) of *A. fumigates* isolated from corn cobs

A. fumigates then fermented to produce secondary metabolites. The initial inoculum which carried out the first growth of the fungus in fermentation media was then incubated at 30^0C, agitation was of 150rpm for 72 hours. The 30^0C temperature chosen considering be the optimum temperature for the production of metabolites [6].

After 144 hours of fermentation, changing of fermentation media which originally white becomes a little dull yellow. The changing of the color is getting stronger in the next fermentation time up to 288 hours fermentation media has become brown color. This indicated that the secondary metabolites compound in the medium have been produced [11].

After 48,96, 144, 192, 240 and 288 hours fermentation, the biomass was separated from the growth media. The biomass obtained was 436,7 mg; 708,8 mg; 720,8mg; 1634,1mg; 837,2 mg and 945.2 mg, respectively. A decrease in pH of fermentation medium was also measured and the results was pH 5.6 at 48 hours of fermentation; 5.3 at 96 hours; 5.2 to 144 hours; 4.5 to 192 hours; 4.3 to 240 hours and a pH of 4.2 at 288 hours of fermentation.

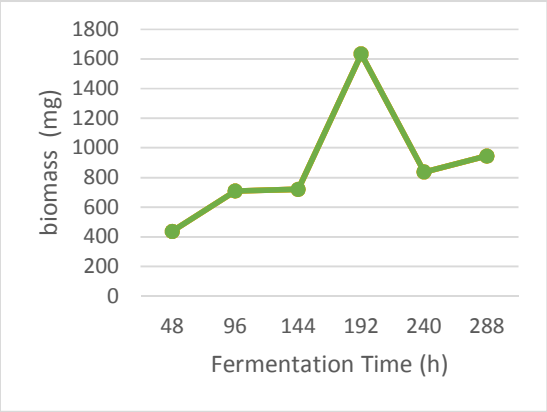

Figure 2. The relationship between fermentation time and biomass produced

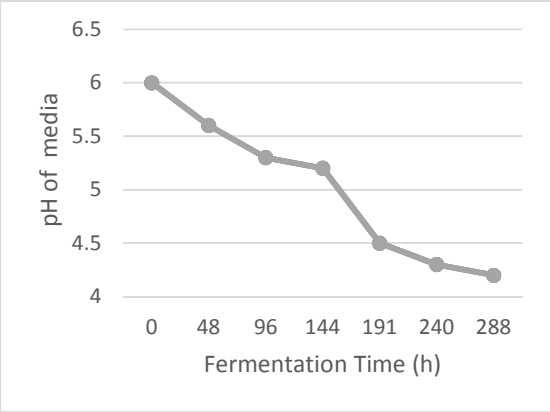

Figure 3. The relationship between fermentation time and decrease of pH of media

A decrease in the pH of the fermentation medium due to the production of organic acids, among which fumaric acid and lactic acid by this fungus[12]. A decrease in the pH of the media and the increase in biomass indicate the role of both on the production of metabolites. These results indicate that the metabolites synthesized in its infancy. The more cells that grow, the metabolite produced is increasing along with the metabolites are excreted out of the cell [13].

Furthermore, the fractionation then separated from biomass of this fungus. Fractionation was performed using ethyl acetate. Ethyl acetate is separated from the fermentation medium by using separating funnel. After fractionation was completed, the solvent was evaporated with rotary evaporator to obtain thick fraction.

Fractions were then analyzed using thin layer chromatography with mobile phase of dichloromethane:methanol (9:1). TLC results extract at 48, 96, 144, 192, 240 and 288 show the same amount of staining, the 6 stains. This indicates that the amount of compounds produced by all the extracts are the same. Extract with the highest biomass, which hextract at192 hours (H8) was selected for further analysis. Testing the antimicrobial activities carried out by using methods bioautography. Bioautography antimicrobial activity test by allowing evaluation of activity per compound[14]. This technique allows the localization of the antimicrobial activity directly in the chromatographic plate where organisms are applied [15].

Results of antimicrobial activity with bioautography method showed that the bacterium *S. aureus* had clear zone with Rf 0.41(stains 2); 0.46(stains 3); 0.62(stains 4) and 0.85(stains 6). While the *E.coli* bacteria showed clear zone with Rf0.41(stains 2) and 0.85(stains 6) and not seen any clear zone testing against *C. albicans*, it showed there is no presence of substances that are antifungal.

The results of bioautography activity could be seen the compound antimicrobial is compounds with stains 2,3,4 and 6 in plate chromatography with Rf 0.41; 0.46; 062 and 0.85 respectively. The fourth of these compounds then purified using preparative thin layer method. Crude fraction purification is done by using preparative TLC with silica $60F_{254}$ as stationary phase and dichloromethane: methanol (9:1) as mobile phase.

Fourth antibacterial compounds were then visible spectrum with ultraviolet-visible spectrophotometry and infrared spectroscopy. By using ultraviolet-visible spectrophotometry, it can be seen that the compound AB1with Rf 0.41 has a maximum wavelength of 201.8 nm, the compound AB2 with Rf0 .47 has maximum wavelength of 202.6 nm, AB3 compound with Rf 0.62 has the maximum wavelength of 203.2 nm, AB4 compound with Rf 0.85 has maximum wavelength 202.0 nm. This data serves only as a rough guide to the identification of functional groups in a molecule, because the wavelength of maximum is also influenced by the solvent and chemical molecular structure that contains the chromophore [16].

Results of infrared spectroscopy data obtained wave numbers which indicate the functional groups of compound. Infra red spectra of compound AB1 has OH in with wavenumber 3232.83 cm^{-1}, the aromatic ring wave number1557.44 cm^{-1}, CH bond at wave numbers 1381.07 cm^{-1} and1354.45 cm^{-1}, C-O at wave numbers 1107.32 cm^{-1} and the cyclohexane group at wave number 1015.81 cm^{-1}. At AB2 compounds has OH in with wave number 3232.83 cm^{-1}, the aromatic ring at wave number 1557.44 and1539.71cm^{-1}, CH bond at wave number 1415.93cm^{-1}, CO at wave number 1053.92 cm^{-1} and cyclohexane at 1023.05 cm^{-1} wave number. AB3 has CO at wavenumber 1059.12cm^{-1} [17].

CONCLUSION

The best cultivation conditions using corn cob as liquid media was of 192 hours or 8days which gained biomass was 1634.1 mg and extract was 228.5 mg. The result showed that the bioatography antimicrobial method against *S. aureus* showed that there are four active antibacterial compounds. Antimicrobial activity against *E. coli* showed that the are two compounds that has activity as antibacterial agent. No result showed that has antifungal compounds against *C.albicans.*

The results showed that characterization of UV-Vis spectrophotometer there were four antibacterial compounds with wavelength were 201.80 nm, 202.60 nm, 203.20 nm and 202.00 nm. Characterization for infrared spectroscopy there were compoundAB1andAB2 with functional groups were OH groups, strain C=C, CH and CO, while compound AB3 the functional group was stretch and AB4 was CO.

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