Antifungal Activity against the Growth of Aflatoxin Producing Fungi from Soil Actinobacteria

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***Abstract*—Aflatoxins are one of the highly concerning fungal toxins, which can cause the serious problems to human and animal health such as hepatotoxicity, teratogenicity, and immunotoxicity. *Aspergillus flavus* is the potential aflatoxin producer and it has usually been isolated from aflatoxin-contaminated crops. The biological compounds with the outstanding antifungal activity for inhibiting the growth of aflatoxin producing fungi still require. This experiment was attempt to isolate antifungal producing actinomycete from soil. Thirty isolates of soil actinobacteria were tested for an ability to inhibit the growth of *A. flavus* IMI 242684 using dual culture test and agar well diffusion assay. Primary screening results from dual culture test showed that 6 isolates of actinobacteria, N39Wh, N44Cr, N38Gr, LP14Wh, N13Gy, and N42Wh exhibited antifungal activity against the growth of *A. flavus* and with percent inhibition in the range of 21.42 to 42.85% then, their activity were confirmed by agar well diffusion assay. The freeze-dried supernatant from isolate N39Wh at concentration of 250 mg/mL showed the strongest antifungal activity with inhibitory zone of 17.25 mm followed by isolate N44Cr, LP14Wh, N38Gr, N13Gy, and N42Wh which produced the inhibition zone at 14.31 mm, 13.71mm, 13.50mm, 11.53mm, and 10.15mm, respectively.**

***Index Terms*—aflatoxins, *Aspergillus flavus*, actinobacteria, antifungal**

I. Introduction

Mycotoxins are the secondary metabolites produced by fungi that are poisonous or toxic to mammals. The effects of mycotoxins on human or animal health vary widely depend upon the composition of organic compounds in their structures [1]. Various kinds of mycotoxins have been documented such as aflatoxins, citrinin, patulin, penicillic acid, tenuazonic acid, ochratoxin A, cytochalasins, deoxynivalenol, fumonisins, and zearalenone. Most of the mycotoxin in these groups are produced by the fungi in the genus of *Aspergillus*, *Penicillium*, and *Fusarium*. Among of these, aflatoxins are one of the highly toxigenicity mycotoxins produced by fungi in the genus of *Aspergillus*, mainly *Aspergillus flavus* and *Aspergillus parasiticus* [2], [3]. The contamination of aflatoxins in agricultural commodities provides the negative impact not only on health but also on economy as well [4]. The agricultural commodities that are usually encounter with aflatoxins contamination are cereals (maize, sorghum, pearl millet. rice, and wheat), spices (chilies, black pepper, coriander, turmeric, and ginger), oilseeds (groundnut, soybean, sunflower, and cottonseed), tree nuts (almond, pistachio, and walnut), milk (human and animal), and butter [3]. Among 18 different types of aflatoxins, aflatoxin B1 from *A. flavus* is the most potent toxicity to human and it has usually been isolated from feed and foodstuffs that are improperly storage, especially in tropical and subtropical climates. Aflatoxins are heat stable substances so that the thermal treatment processes such as boiling, baking, frying, and roasting cannot completely destroy the contaminated aflatoxins [5]. Consumption of aflatoxins contaminated food can lead to serious health problems to human and animals by causing hepatotoxicity, teratogenicity, immunotoxicity, and cancer. [3]. According to the guideline of Food and Agricultural Organization (FAO) and World Health Organization (WHO) about the allowable levels of total aflatoxins in food and feed substrate, the acceptable level of total aflatoxins in agricultural commodities is not higher than 20 ppb [6]. Actinomycetes are aerobic, spore forming Gram positive bacteria and they show some characteristics like fungi such as the formation of spore, substrate mycelium and aerial mycelium. These bacteria exist in various habitat especially in soil. More than 5,000 biological active compounds have isolated from actinobacteria and contributed to the development of 90% of commercial antibiotics [7]. Therefore, this study aim to determine the efficiency of soil actinobacteria for inhibiting the growth of *A. flavus* IMI 242684, by dual culture test and agar diffusion assay. Moreover, the result from this experiment will be used as the primary date for developing the microbial fungicide. Because, the best strategy to prevent the contamination of aflatoxins in agricultural commodities is inhibition the growth of

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aflatoxigenic fungi before the level of aflatoxins reached to the acceptable level.

II. Materials and Methods

A. Isolation of Actinobacteria

Soil samples were collected from Nan and Lampang provinces, Thailand. The 50 g of blended soil were pretreated with heating at 100 ℃ for 1 hr. Then, 1g of the sample was transferred into 9mL of basic lauryl sulfate solution and ten-fold serial dilution was performed. The diluted solutions were plated on soil extract agar which were composed of 0.05% w/v $CaSO_4.2H_2O$, 0.025% w/v $Ca(NO_3)_2.4H_2O$, 0.005% w/v $MgSO_4.7H_2O$, 0.03% w/v K_2SO_4, 0.002% w/v KH_2PO_4, 0.01% w/v $NaHCO_3$, 0.002% w/v $CaCl_2.2H_2O$, 0.01% w/v yeast extract, 0.01% w/v casamino acids, 0.02% w/v glucose, 10% v/v soil extract (1kg of humic soil in 1L of distillation water), 1.8% w/v microbiological agar, 0.1% v/v vitamin mixture (0.05% w/v p-aminobenzoic acid, 0.05% w/v calcium pantothenate, 0.05% w/v inositol, 0.05% w/v niacin, 0.05% w/v pyridoxine HCl, 0.05% w/v riboflavin, 0.05% w/v thiamine HCl, 0.025% w/v biotin), 0.0025 % w/v nalixidic acid, 0.005% w/v cycloheximide, 0.0001% w/v terninafin, and 0.03% v/v trace element mixture (0.4% w/v $CaCl_2.H_2O$, 0.2% w/v $ZnSO_4.7H_2O$, 0.01% w/v $Na_2B_4O_7.10H_2O$, 0.5% w/v $Fe.SO_4.7H_2O$, 0.005% w/v Kl, 0.05% $CoCl_2.6H_2O$, 0.02% w/v $CuSO_4.5H_2O$, 0.2% w/v $MnCl_2.4H_2O$, 0.005% w/v $Na_2MoO_4.2H_2O$, and 0.1% v/v H_2SO_4). The plates were incubated at room temperature for 14 days and the colonies of actinobacteria were selected and transferred to yeast extract-malt extract agar (0.4% w/v glucose, 0.4% w/v yeast extract, 1% w/v malt extract, 1.8% w/v microbiological agar) for further experiments.

B. Dual Culture Test

For dual culture test, an agar disc at 6mm in diameter of 4 days old culture of A. flavus IMI 242684 was transferred onto potato dextrose agar (PDA) and was placed 3cm away from the periphery of petri dish. An agar disc at 6mm in diameter of 7 days old of actinobacteria was placed 3cm apart from A. flavus IMI 242684 on the opposite. The plate was incubated at room temperature for 7 days. The antifungal activity of actinobacteria was evaluated by percent of inhibition, which was calculated based on the following formula,

$$\% \text{ of inhibition} = (R1-R2)/R1 \times 100$$

R1 = Radial growth of A. flavus without antagonistic interaction (control)

R2 = Radial growth of A. flavus with antagonistic interaction (treatment)

C. Preparation of Fungal Spore Suspension

The spore suspension of A. flavus IMI 242684 was prepared by growing of the fungi on PDA at room temperature for 14 days. The 10 mL of sterile tween 80 at concentration of 0.05% v/v was added into the plate in order to detach the spores, then the suspension was filtrated through sterile cotton wool. The numbers of spores were determined by haemacytometer.

D. Preparation of Freeze-Dried Supernatant

The antifungal producing strain of actinobacteria was activated on yeast-malt extract agar for 14 days after that the single colony was transferred into yeast-malt extract broth (pH = 7.4) at 25 ℃ for 14 days with shaking. The cell free supernatant was collected by centrifugation at 8,000 rpm for 10 min at 4 ℃ and sterilized by filtration using 0.22 μm syringe filter. Then, the sterilized supernatant was concentrated by freeze dying. The frozen sample was freeze-dried for 18hr and preserved at -20 ℃ for further experiment.

E. Agar Well Diffusion Assay

The antifungal activity of freeze-dried supernatant were determined by agar well diffusion assay. The freeze-dried sample was dissolved in sterile distilled water in order to make the various concentrations (100, 200, and 250mg/mL). Twenty milliliters of molten PDA were poured into $_{90}$ mm sterile petri dish and left to set (agar base) then, the 10mL of molten PDA inoculated with spore of *A. flavus* IMI 242684 at concentration of 10^8 spores/mL was overlaid over the surface of agar base. After the medium was solidified, a well of 7.5mm in diameter was made by sterilized cork-borer and the 100 μL of tested solution at each concentration was loaded into well. Antifungal activity was observed after incubated at room temperature for 5 days. The zone of inhibition were measured using vernier caliper. All experiments were performed in triplicate.

F. Molecular Identification Based on 16s rRNA Sequence

An isolate that exhibited the best antifungal activity was subjected to 16S rRNA sequence analysis for identification of its genus and species. The genomic DNA was extracted using HipureTM Bacterial Genomic DNA Purification Kit from HiMedia Laboratory Pvt. Ltd. The 16s rRNA gene was amplified by the universal primers, 27F and 1492R. The amplification was accomplished in 50 μL reaction mixtures: 20mM Tris-HCl, 10mM $(NH_4)_2SO_4$, 10 mM KCl, 2mM Mg_2SO_4, 0.1% TritonX-100, 20 pmol of each primer, 0.25mM each of four dNTP, 0.5 U of *Taq* DNA polymerase (NEB, USA) and 10ng of DNA template. The thermocycling program was started with initial denaturation at 94 ℃ for 3 min and followed by 35 thermal cycles of 94 ℃ for 30s, 55 ℃ for 60s, and 72 ℃ for 90s. Finally, the final extension was done at 72 ℃ for 3 min. The PCR products were separated by 1.0% agarose gel electrophoresis and the amplicon (~1.5 kb) was purified. The nucleotide sequences were analyzed and compared with the 16S rRNA sequences of bacterial type strains in EzbioCloud database (www.ezbiocloud.net/eztzxon). Moreover, the evolutionary pattern of actinobacteria was calculated according to the distance matrix methods. The statistical algorithm that was selected to construct the phylogenetic tree was Neighbor-joining assay. The evolutionary analysis was performed in MEGA7 program.

III. Results and Discussion

Totally 30 isolates of soil actinobacteria were obtained from soil sample of Nan province for 14 isolates (N3Gy, N4Cr, N5Br, N7Wh, N13Ye, N17Gy, N21Gr, N30Wg, N33Wg, N37Wh, N38Gr, N39Wh, N42Wh, and N44Cr), and Lampang province for 16 isolates (LP4Gy, LP5Gy, LP7Gr, LP11Ye, LP12Cr, LP13Cr, LP14Wh, LP18Wh, LP19Wh, LP21Wh, LP24Gr, LP25Gr, LP53Wg, LP55Cr, LP57Cr, and LP60Wg).

Figure 1. A dual culture test between N39Wh *A. flavus* IMI24684

The results from dual culture test revealed that five isolates from soil sample of Nan province (N13Gy, N38Gr, N39Wh, N42Wh, and N44Cr) and one isolate from soil sample of Lampang province (LP14Wh) could inhibit the mycelial radial growth of *A. flavus* IMI 24684. An isolate N39Wh exhibited the strongest antifungal activity with percent inhibition at 42.85 (Fig. 1) followed by isolate N44Cr, N38Gr, LP14Wh, N13Gy, and N42Wh with percent inhibition at 41.42, 40.00, 35.71, 34.28, and 21.42, respectively as presented in Table I. In order to confirm their antifungal activity, the concentrated cell free supernatant from 6 isolates at concentration of 100mg/mL, 200mg/mL, and 250mg/mL were subjected to agar well diffusion assay and the results found that the antifungal activity could be detected only at the concentration of 250mg/mL for all samples. The concentrated cell free supernatant from isolate N39Wh exhibited the strongest antifungal activity with the inhibition zone of 17.5mm in diameter, followed by isolate N44Cr, LP14Wh, N38Gr, N13Gy, and N42Wh with inhibition zone at 14.31mm, 13.71mm, 13.50mm, 11.53, and 10.15mm, respectively (Table II).

TABLE I. The Percent Inhibition of Mycelial Growth

Isolate	Radial growth (mm)	% of inhibition
LP14Wh	27	35.71
N13Gy	27.6	34.28
N38Gr	25.2	40.00
N39Wh	24.0	42.85
N42Wh	33.0	21.42
N44Cr	24.6	41.42

TABLE II. Antifungal Activity from Agar Diffusion Assay

Isolate	Concentration of freeze-dried sample (mg/mL)	Zone of inhibition (mm)
LP14Wh	100	0
	200	0
	250	13.71
N13Gy	100	0
	200	0
	250	11.53
N38Gr	100	0
	200	0
	250	13.50
N39Wh	100	0
	200	0
	250	17.50
N42Wh	100	0
	200	0
	250	10.15
N44Cr	100	0
	200	0
	250	14.31

From the results of dual culture test and agar well diffusion assay, the isolate N39Wh was the outstanding strain for inhibiting the growth of *A. flavus* IMI 242684 and it was selected to identify the genus and species by 16s rRNA gene sequencing. The result found that nucleotide sequences of 16s rRNA gene from isolate N39Wh was similar to the nucleotide sequences of 16S rRNA gene of *Streptomyces rochei* NBRLB-2410, *Streptomyces plicatus* NBRC-13071, and *Streptomyces enissocaesilis* NRRLB-13071 with 99.61% identity as the results presented in Table III.

TABLE III. The Percent Similarity of Nucleotide Sequence

Bacteria	Strain number	Percent similarity
Streptomyces rochei	NRRL B-2410	99.61
Streptomyces plicatus	NBRC-13071	99.61
Streptomyces enissocaesilis	NRRL B-16365	99.61
Streptomyces geysiriensis	NBRC-15413	99.53
Streptomyces luteus	TRM-45540	99.53
Streptomyces mutabilis	NBRC-12800	99.53
Streptomyces vinaceusdrappus	NRRL-2363	99.45
Streptomyces djakartensis	NBRC-15409	99.30
Streptomyces tuirus	NBRC-15617	99.15
Streptomyces anandii	NRRL B-3590	98.91

Streptomyces aurantiogriseus	NBRC-12842	98.91
Streptomyces aeterosporus	NRRL B-24328	98.91
Streptomyces calvus	ISP-5010	98.91
Streptomyces levis	NBRC 15423	98.84
Streptomyces naganishii	NBRC-12892	98.84
Streptomyces brasiliensis	NBRC-101283	98.84
Streptomyces spiralis	NBRC-14215	98.84
Streptomyces zinciresistens	K42	98.84
Streptomyces virens	NBRC-15901	98.84
Streptomyces pilosus	NBRC-12807	98.76

The 16s rRNA sequence from actinobacteria isolate N39Wh was compared with the 16s rRNA sequences of 20 species in the genus of *Streptomyces*. The phylogenetic tree relationship was demonstrated in Fig. 2. According to the evolutionary pattern, the isolate N39Wh represented the closest evolutionary distance to *Streptomyces enissocaesillis* strain NRRL B-2410 and *Streptomyces rochei* strain NRRL B-2410. However, only the results of genotypic analysis based on the sequence of 16s rRNA were not sufficient to identify the specific species of isolate N39Wh, therefore genotypic characterization along with phenotypic and chemotypic characterizations were required for further experiment in order to distinguish isolate N39Wh from the closest species in the genus of *Streptomyces*.

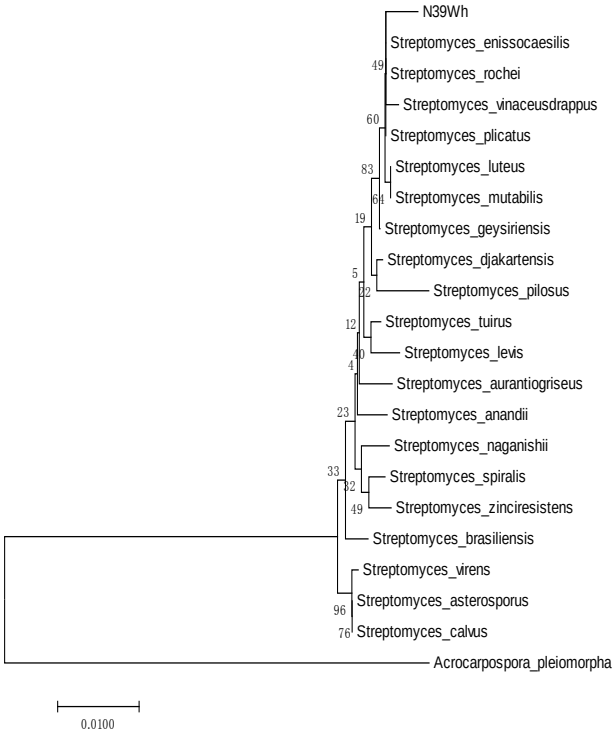

Figure 2. A phylogenetic tree demonstrates the evolutionary pattern of actinobacteria isolate N39Wh.

The antifungal activity from actinobacteria, especially in the genus of *Streptomyces* have been reported in many researches, the antifungal activity gained from the wide range of secondary metabolites and hydrolytic enzymes. Hydrolytic enzymes such as chitinase, β-1,3-glucanase, chitosanase, and protease play an important role in antifungal activity by breaking down complex of chitin and glucan in fungal cell wall [8], [9]. As stated in the previous report, the ethyl acetate extracts from culture broth of *S. rochei* could inhibit the growth of *Aspergillus fumigatus* and it was identified as butyrolactols-A [9]. Although, the antifungal activity against the growth of *A. flavus* has been detected from *S. rochei* but there were no document about the active compound and mechanisms for inhibiting the growth of *A. flavus*.

IV. Conclusoin and Future Tasks

According to the results of this experiment, the soil actinobacteria isolate N39Wh can produce the extra cellular substance that show the promising antifungal activity against the growth of *A. flavus* IMI 242684. However, it use high concentration for inhibiting the growth of tested fungi. Therefore, the chemical investigation methods are required to extract and identify the bioactive components of extracellular substance that are excreted from actinobacteria.

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