https://doi.org/10.48048/tis.2022.375

Antifungal Activity of *Streptomyces spectabilis* SP-O2 against Aflatoxin Producing Mold, *Aspergillus flavus*

Rattikarn Uasoontornnop[1], Kerkchai Junniam[2], Phetcharat Maseechan[3], Nuchjarin Lanongkan[3], Kruawan Chotelersak[1], Kwannan Nantavisai[1], Piyatida Tangteerawatana[1], Maneekarn Namsa-Aid[2] and Srisombat Puttikamonkul[1,*]

[1]*Department of Microbiology, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110, Thailand*
[2]*Department of Chemistry, Faculty of Sciences, Srinakharinwirot University, Bangkok 10110, Thailand*
[3]*Department of Microbiology, Faculty of Sciences, Srinakharinwirot University, Bangkok 10110, Thailand*

([*]Corresponding author's e-mail: srisombat@g.swu.ac.th)

Received: 8 November 2021, Revised: 15 December 2021, Accepted: 23 December 2021, Published: 4 November 2022

Abstract

Among soil isolated species of actinomycetes bacteria that affected the growth of aflatoxin producing mold; *Aspergillus flavus* TISTR 3041, the isolate SP-O2 showed a promising inhibitory activity on the dual culture assay. This isolated bacterium was characterized on various International Streptomyces Project media. The macroscopic morphological analysis showed mycelium, sporogenesis, and orangish pink of colonial pigment. The scanning electron microscope (SEM) photograph showed that the sporogenesis has occurred by shrinking of both sides of aerial mycelium. After the sporogenesis has been completed, the fragmentation of altered mycelium started from the tips of aerial mycelium to produce 0.8-1 micrometer fragmented spores. The 16S rRNA sequences analysis revealed that isolate SP-O2 shared a 99.93 % (1 mismatch/1436 nt) similarity with *Streptomyces spectabilis* NBRC 13424 AB184393. Co-culture liquid assay was analyzed for the aflatoxin produced by *A. flavus* in the presence of *Streptomyces spectabilis* SP-O2. The result showed that UV-visualized band of aflatoxin B1 was not detected by co-cultivation of *Streptomyces spectabilis* SP-O2 with *A. flavus* by thin layer chromatography compared to the control. Indeed, fungal growth of *A. flavus* was destroyed by the antifungal substance produced by *Streptomyces spectabilis* SP-O2 in the liquid culture. Furthermore, biocontrol of contaminated *A. flavus* was conducted on peanut kernels and the results showed that *Streptomyces spectabilis* SP-O2 has antifungal activity against the presence of *A. flavus*. Together, these results suggested that further investigation on the development of biocontrol use of *Streptomyces spectabilis* SP-O2 to overcome the contamination of aflatoxin producing mold in the feedstock and agricultural-based industry is needed.

Keywords: *Streptomyces spectabilis*, *Aspergillus flavus*, Aflatoxin, Antifungal activity, Biocontrol

Introduction

Aflatoxins are mycotoxins contaminating a large fraction of the world's food, including maize, cereals, groundnuts and tree nuts. To protect the consumer from the harmful effects of mycotoxins, the regulations have been established in many countries [1]. Aflatoxins are the complex aromatic compound produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* contaminated a variety of staple foods, particularly maize and groundnuts, in low-income countries [2,3]. Aflatoxins are naturally occurring compounds known to be potent human carcinogens, including AFB1, AFB2, AFG1 and AFG2. The most abundant secondary metabolite of aflatoxin found is aflatoxin B1 (AFB1). AFB1 is metabolized by liver enzyme cytochrome P450 to an unstable molecule, AFB1-8,9-epoxide, which intercalates more readily into DNA, forming active carcinogen called AFB1-DNA adducts [3-5]. *A. flavus* and *A. niger* are soil-inhabiting, filamentous fungi that saprophytically utilizes a wide range of organic substrates. Moreover, the agricultural countries found those fungi accumulate at different stages including preharvest, harvest, processing and handling where tropical regions with high humidity and temperature promote fungal growth [6].

The current strategies employed to control AFB1 are various physical and chemical methods. However, these practical applications have limitation by causing nutritional loss, sensory quality

reduction and high cost of equipment. Moreover, the application of synthetic fungicides including triazoles and acylalanines are commonly used in agriculture for fungal disease control. However, those fungicides have involved in the development of resistant varieties and adverse effects on human health and beneficial microflora [7-9]. Therefore, an effective and safe method has increased the emphasis for biological control, which ultimately reduces the concentration of chemical pesticides. Antifungal compounds were isolated from bacteria i.e., *Streptomyces* sp., *Bacillus cereus* subsp. *thuringiensis* significantly higher activity than the standard fungicide bavistin against agriculturally important fungi [8,10].

Given that *Streptomyces* are among the most numerous and ubiquitous soil bacteria responsible for producing most natural antibiotics used in human and veterinary medicine, these Gram-positive bacteria exhibit complex multicellular development of filament and sporulation. The genome sequence of *S. coelicolor*, representative of the genus, has revealed much about the many adaptations of this model actinomycete to live in the highly competitive soil environment. Each actinomycete isolate has potential 10 - 20 secondary metabolites encoding genes [11]. *Streptomyces* genus consisting of characterized species have provided over two third of naturally occurring antibiotics discovered and continue to be major source of various bioactive compounds such as antibiotics, fungicides, enzymes, pigments, plant promoting, antioxidant and anticancer [8,10,12-18].

Even though antifungal compounds against *A. flavus* have been described in *Streptomyces yanglinensis* 3-10, *Streptomyces alboflavus* TD-1, *Streptomyces philanthi* RL-1-178 and *Streptomyces roseolus*, other species possess promising activity are still in attempt to develop for the potent biocontrol [13,17,19,20]. Therefore, screening, isolation, and characterization of promising isolates of actinomycetes producing potential antibiotics and other therapeutics have been an intensive focus on research [21]. Our aims were to discover microbial resources from soil environment and evaluate their values in biocontrol of aflatoxin producing mold in the agricultural produces and feedstock.

Materials and methods

Media, microorganisms and cultivation conditions

Aflatoxin producing *A. flavus* TISTR 3041 was obtained from the Thailand Institute of Scientific and Technological Research (TISTR) Phathumthani, Thailand [22]. They were grown on Potato Dextrose Agar (PDA) (HIMEDIA, INDIA) at 28 °C for 7 days, while the isolated actinomycetes bacteria were cultivated on Yeast Malt Agar (YMA; ISP medium No.2) (HIMEDIA, INDIA) at 28 °C for 14 days.

Isolation of actinobacteria

Streptomyces spp. were isolated from various soil samples collected from dirty soil near a water source. We aimed to induce the expression of genes involved in the secondary metabolism of *Streptomyces* spp. and to inhibit other well-grown bacteria. Thus, a standard 10-fold dilution plate method was performed on PDA supplemented with Chloramphenicol; 50 µg/mL (below the MIC). To obtain the pure culture, the isolated actinobacteria were cultured on YMA supplemented with Chloramphenicol; 50 µg/mL at 28 °C and store as spore suspension in 20% (v/v) glycerol at –20 °C for subsequent investigation.

Morphology of *Streptomyces spectabilis* SP-O2

Cultural characteristics of *Streptomyces spectabilis* SP-O2 were examined on various International Streptomyces Project (ISP) media [23,24], including Yeast Malt Agar (ISP2 or YMA), oatmeal agar (ISP3), inorganic salts-starch agar (ISP4), glycerol-asparagine agar (ISP5), peptone yeast-iron agar (ISP6), and tyrosine agar (ISP7). Production of melanin was tested on the ISP6 and ISP7 media. The growth capability, pigment production and color of both aerial and substrate mycelia were recorded every day after incubation at 28 °C for 14 days. The macroscopic morphology of their colonies was visualized by stereomicroscope.

Screening for antifungal activity

Dual culture agar assay was used to screen the potential isolates having antifungal activity. Briefly, actinomycetes bacteria and *A. flavus* TISTR 3041 were harvested in 0.01 % Tween 80 by scraping with a sterile cotton swab and filtered through cotton wool. The fungal conidia were counted and diluted to 1×10^5 conidia/mL whereas the obtained spores of actinobacteria were difficult to count by hemacytometer and required plate count method. The 50 µL of 1×10^4 conidia was used for the dual culture agar assay by inoculating fungal spores into 0.6 mm-diameter (1×10^4 conidia) well at the center of PDA plate. The

isolated *Streptomyces* sp. was inoculated at equal amount into 4 wells around the well containing target fungus. Control well was added with 50 µL of liquid Potato Dextrose Broth at equally spaced with the periphery well of the Dual culture agar assay [15]. Three replicates were prepared for each isolated actinobacteria. The growth inhibition of *A. flavus* TISTR 3041 was measured after 14 days at 28 °C.

Identification of aflatoxin degradation

The inoculum of *Streptomyces spectabilis* SP-O2 was cultivated in 50 mL of Tryptone Yeast Extract Broth (ISP Medium No. 1 broth) (HIMEDIA, INDIA) at 200 rpm, 28 °C for 48 h. One mL of concentrated inoculum was added into 10 mL of ISP1 broth supplemented with 6 µg/mL aflatoxin B1(FERMENTEK) [25] compared to the culture control without aflatoxin B1 and the aflatoxin B1 control, and incubated at 200 rpm, 28 °C for 48 h in the shaker. Five microlitres of each filtrate mixture was spotted and dried onto silica gel 60 F254 TLC plates (Merck, Germany). Chloroform/acetone/acetic acid (40:10:1 by volume) was used as the developing solvent and aflatoxin fluorescence was detected by viewing under ultraviolet light (365 nm).

Replacement of pure aflatoxin B1 with aflatoxin producing *A. flavus* TISTR 3041, the 1×10^5 spores inoculum of fungus were grown in 50 mL of PDB at 200 rpm, 28 °C for 24 h. One mL of each concentrated inoculum (fungus, actinobacteria) was added into 10 mL of ISP1 broth and incubated at 200 rpm, 28 °C for 48 h in the shaker. Two separate control tubes including the *A. flavus* TISTR 3041 and *Streptomyces spectabilis* SP-O2 alone were compared on TLC plates. Three different experiments were conducted separately.

Co-culture liquid assay

To confirm the antifungal activity, the co-culture liquid assay was used. The *A. flavus* TISTR 3041 and *Streptomyces spectabilis* SP-O2 were co-cultivation in 10 mL of ISP1 broth as described above (Identification of Aflatoxin Degradation). The co-culture pellets were transferred onto ISP2 and PDA for verification of fungal viability. The experiment was repeated 3 times.

Antifungal activity on sterilized peanuts

Peanut kernels were sterilized by autoclave condition (121 °C, 15 min) instead of surface sterilization as modified from the inhibitory effect on *A. flavus* measured by Shakeel *et al*. [17]. Fifteen g of sterilized peanut kernels were mixed with 2 mL of spores suspension of the isolate SP-O2 (1×10^5 spores/mL) and incubated at 28 °C for 0-7 days prior the co-cultivation with 1×10^5 spores of *A. flavus*. For the control treatment, 2 mL of sterile 0.01% tween 80 was added to the sterilized peanuts. The treatment plates were gently mixed by hand for the complete liquid absorption of the kernels. After 5 days of incubation at 28 °C, the growth of *A. flavus* on kernels was evaluated.

Scanning electron microscopic analysis

Surface topography of *Streptomyces spectabilis* SP-O2 was performed on gold coated samples that had been previously dehydrated using an analytical Scanning Electron Microscope (SEM) (JEOL JSM-6390LV) at the faculty of dentistry, Srinakharinwirot University. Briefly, the slide culture of actinobacteria was prepared by inclined coverslip ($22\times22mm^2$) in 10 days grown on YMA at 28 °C and further culture for another 18 days. The cultured coverslip was fixed with 2.5 % glutaraldehyde (Sigma-Aldrich) in 1× phosphate buffer solution (PBS, pH 7.4) at 4 °C for 24 h, then washed with PBS and water, 15 min each. The samples were dehydrated with alcohol series (30, 50, 70, 80, 90, 100, 100, 100 % ethanol), 20 min each. Last step of sample drying was done in hexamethyldisilazane (HMDS; Sigma-Aldrich).

Molecular identification and phylogenetic analysis

The DNA of the isolate SP-O2 was extracted and 16S rDNA gene was amplified by PCR. The 2 primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') or 800R (5'-TAC CAG GGT ATC TAA TCC-3') and 518F (5'-CCA GCA GCC GCG GTA ATA CG-3') or 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') for single strand 16S rDNA sequencing, and 4 primers of 27F, 518F, 800R and 1492R for double strands 16S rDNA sequencing were used. The nucleotide sequences obtained from all primers were assembled using Cap contig assembly program, an accessory application in BioEdit (Biological sequence alignment editor) Program (http://www.mbio.ncsu.edu/ BioEdit/BioEdit.html). The identification of phylogenetic neighbors was initially carried out by the BLASTN [26] program against 16S rDNA sequence database of validly published prokaryote. The sequences with the highest scores were calculated pairwise sequence similarity using global alignment algorithm [27]. For phylogenetic

tree, the obtained sequences were analyzed by multiple alignments using CLUSTAL X version 2.0.11. The alignment was modified by eliminate gaps and ambiguous nucleotides prior to the construct a phylogenetic tree. The phylogenetic tree was constructed with neighbor-joining in the MEGA X software [28]. The confidence values of individual branches in the phylogenetic tree were determined with 1,000 replications.

Results and discussion

Actinobacteria isolation and antifungal activity

Aflatoxin is a major problem in the food processing industry which can be prevented and reduced by controlling the contamination of the causative fungus, *A. flavus* [29]. There are several techniques to prevent mold and extend the shelf life of food before consumption. All of which reduce the nutritional value and fungicides is prohibited in food due to an adverse effect to consumers health including its involvement in the resistance strains of fungi in the environment [9]. Therefore, the need to develop the biological agents created by microorganisms is an alternative approach that ensures food safety. Fourteen promising actinobacteria with different morphological colonies were isolated and continued antifungal activity analysis. Among them, only the isolate SP-O2 showed the promising growth inhibition against *A. flavus* TISTR 3041. The dual culture agar assay revealed that *A. flavus* TISTR 3041 was unable to grow toward the isolate SP-O2 inoculated wells in comparison to PDB control well (**Figure 1**). Our main hypothesis was to isolate actinomycetes with antifungal activity against aflatoxin producing *A. flavus* TISTR 3041. The isolate SP-O2 was a potential isolate obtained from the dirty soil sample near water source.

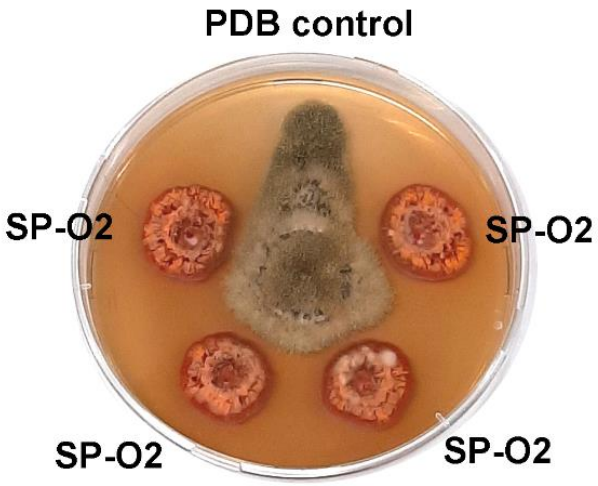
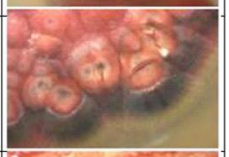
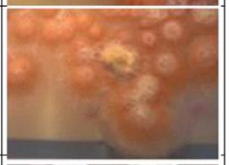


Figure 1 Dual culture agar assay showing antifungal activity of the isolate SP-O2. Plate inoculated with *A. flavus* TISTR 3041 at the center and surrounded with this potential isolate. PDB was a control in same plate.

Morphological characteristics of SP-O2

For the biocontrol development, it is important to characterize the morphology of *Streptomyces* sp. isolate SP-O2 on available ISP media and sporogenesis through scanning electron microscope. Growth ability and characteristics including vegetative, aerial mycelia and soluble pigments of *Streptomyces* sp. isolate SP-O2were evaluated on different ISP media for 10 days at 28 °C. **Table 1** showed that this isolate SP-O2 can grow well on all tested ISP media. However, the color of vegetative and aerial mycelia was different, and it secreted black soluble only on the ISP6 media.

Table 1 Culture characteristics of *Streptomyces* sp. SP-O2 (10 days).

Medium	Aerial mycelium	Vegetative mycelium	Soluble pigment	Growth	Colony
YMA	light orange	orangish pink	none	+++	
ISP 3	orangish red	reddish pink	none	+++	
ISP 4	light red	dark orange	none	+++	
ISP 5	light orange	orange	none	+++	
ISP 6	light gray	grayish black	black	++	
ISP 7	light orange	orange	none	+++	

The macroscopic morphological analysis of the isolate SP-O2 under stereomicroscope showed that its colony resembled an orangish pink flower (**Figure 2**(**A**)). The SEM photograph of *Streptomyces* sp. isolate SP-O2 grown on YMA at 28 °C for 18 days showed the sporogenesis that had occurred by shrinking of both sides of Rectiflexibiles aerial mycelium. After the sporogenesis had been completed, the fragmentation of altered mycelium started from the tips of aerial mycelium to produce 0.8-1 micrometer fragmented smooth and cylindrical spores (**Figure 2**(**B**)). The isolate SP-O2 grew well on the ISP media and produced flower-like colonies without soluble pigment unless it was cultivated on ISP6 media, the black pigment of melanin was produced which similar to the previous characterized *Streptomyces spectabilis* SNF4435 [30]. The vegetative and aerial mycelium were orangish pink color among all ISP media. At least 2 weeks of cultivation, SEM photograph suggested that spores were generated through the fragmentation of altered mycelium started from the tips of aerial mycelium to produce 0.8-1 micrometer fragmented smooth and cylindrical spores. Moreover, we noticed that the sporogenesis had occurred by shrinking of both sides of aerial mycelium and subsequently fragmenting was occurred.

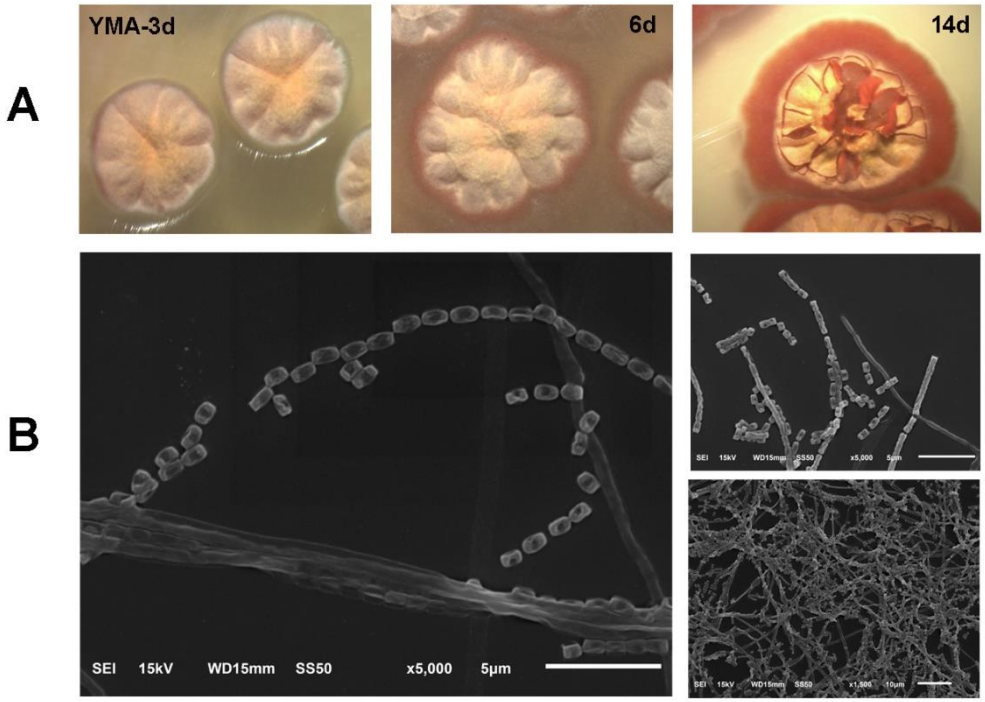


Figure 2 Macroscopic morphology of the *Streptomyces* sp. isolate SP-O2 on YMA plate at 3, 6and 14 days (A) and SEM microscopic morphology of mycelium and approximately 0.8-1 micrometre smooth and cylindrical spores (B). The fragmentation of mycelium and massive interweaving mycelium were also observed.

Inhibition of aflatoxin production and fungal growth

The co-culture liquid assay indicated that *Streptomyces* sp. isolate SP-O2was unable to degrade the pure aflatoxin B1. Nevertheless, the result of co-cultivation of *Streptomyces* sp. isolate SP-O2 and *A. flavus* TISTR 3041 suggested that aflatoxin producing fungus was killed and unable to produce aflatoxin when visualizing under ultraviolet light on TLC plate (**Figure 3**(**B**)). The antifungal activity results conducted by dual culture agar assay and co-culture liquid assay consistently suggesting the fungicide property of secreted metabolites produced by *Streptomyces* sp. isolate SP-O2. In contrast to viability of the isolate SP-O2, post cultivation of the tissue pellets of *A. flavus* TISTR 3041 and *Streptomyces* sp. isolate SP-O2 on the YMA and PDA media were unable to recover the fungus. The 2 different appearances of co-cultivate were spotted on each side of each medium. However, there was no mold growing on any spotted area (**Figure 3**(**C**)). Although, we anticipated that the isolate SP-O2 may possess aflatoxin degradation, the TLC plate of pure aflatoxin B1 indicated that *Streptomyces* sp. isolate SP-O2 was not directly involved in the degradation of aflatoxin as found in AFB1 degrading bacterial strain such as *Rhodococcus erythropolis*, and *Stenotrophomonas maltophilia* [31,32]. The absence of aflatoxin in the mixture obtained from co-culture of SP-O2 and *A. flavus* on TLC plate was due to the defective fungus in the co-cultivation sample (**Figure 3**(**C**)). *A. flavus* showed few bands of derivative aflatoxin including AFB1, AFB2, AFG1 and AFG2 different from the AFB1 control lane [33]. Consequently, this data suggested that *Streptomyces* sp. isolate SP-O2 was a promising isolate for the potent biocontrol development in suppressing postharvest contaminated by *A. flavus*.

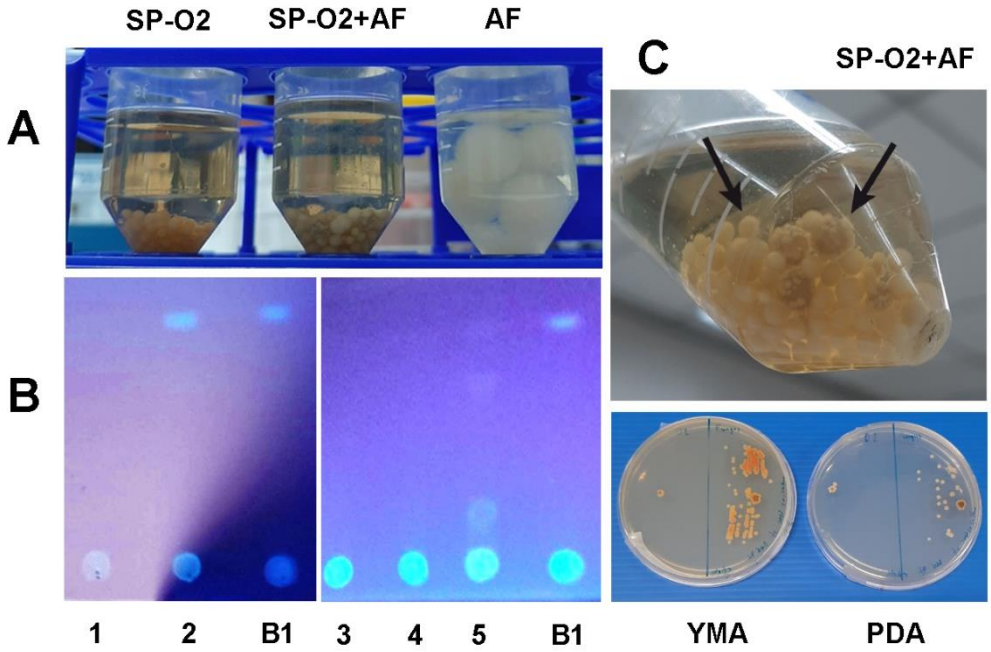


Figure 3 Co-culture assay was performed for evaluation of aflatoxin and fungal growth inhibition ability of *Streptomyces* sp. isolate SP-O2, AF; *A. flavus* TISTR 3041 (A). The filtrate mixture of each reaction was spotted on TLC plate and monitor the visualized band of aflatoxin compared to aflatoxin B1 control (B1), 1,3; SP-O2 control, 2; a culture of SP-O2 with B1, 4; a co-culture of SP-O2 and AF, 5; AF control (B). Two different appearances of co-culture pellets (arrows) were cultivated on YMA and PDA plates for evaluation of *A. flavus* cell death; the small white pellet resembling SP-O2 control was spotted on the left side and the rough pellet with the darkened color of co-culture was streaked on the right side of both media (C).

Inhibition of fungal growth on peanut kernels assay

Given that antifungal compound produced by *Streptomyces* sp. isolate SP-O2 killed *A. flavus* TISTR 3041 in the co-culture liquid assay, its ability was also demonstrated by the peanut kernels assay. The inhibitory effect of *Streptomyces* sp. isolate SP-O2 against the fungal growth on the peanuts was at least 6 days of pre-incubation compared to the control (**Figure 4**). The results showed that the grayish color was observed on the kernels of such treatment instead of greenish spores formation as presented on the control plate without the *Streptomyces* sp. isolate SP-O2. The longer cultivation of the *Streptomyces* sp. isolate SP-O2 increased the inhibitory effect of fungal growth. In addition, the peanut kernels assay showed that *Streptomyces* sp. isolate SP-O2 had ability to kill the fungus contaminated on the agricultural produces and feedstock. A comparison of our results with previous studies on *A. flavus* inhibition revealed that our isolate has similar potential of fungicide property to *S. yanglinensis* 3-10 isolated from a healthy rice leaf grown in the field near Wuhan, China [17]. Interestingly, spores of *S. yanglinensis* 3-10 can suppress the aflatoxin production at 46.41 % inhibition whereas the higher efficiency of inhibition was observed by crude extract. It emphasizes that further investigation of bioactive metabolite of *Streptomyces* sp. isolate SP-O2 must be elucidated for better comparison. Besides, *Streptomyces roseolus* had been studied for potential use as a biocontrol agent against aflatoxin B1 contamination. The investigator had shown that the reduced concentrations of aflatoxin B1 and kojic acid in the co-cultures with *A. flavus* were respectively correlated with the downregulation of the aflatoxin B1 gene cluster and *koj*R gene expression. However, the treated fungus demonstrated hyper sporulation phenotype correlated with the overexpression of genes involved in fungal development [13]. These emphasize that aflatoxin degrading bacteria may not be the ultimate choice if the fungus growth is highly activated and plays a key role on crop disease, shorten shelf life, and spoilage. Nevertheless, the fungicidal activity observed in *Streptomyces* sp. isolate SP-O2 causing the destruction of aflatoxin producing mold; *A. flavus* TISTR 3041 which resulted in the absence of aflatoxin metabolite.

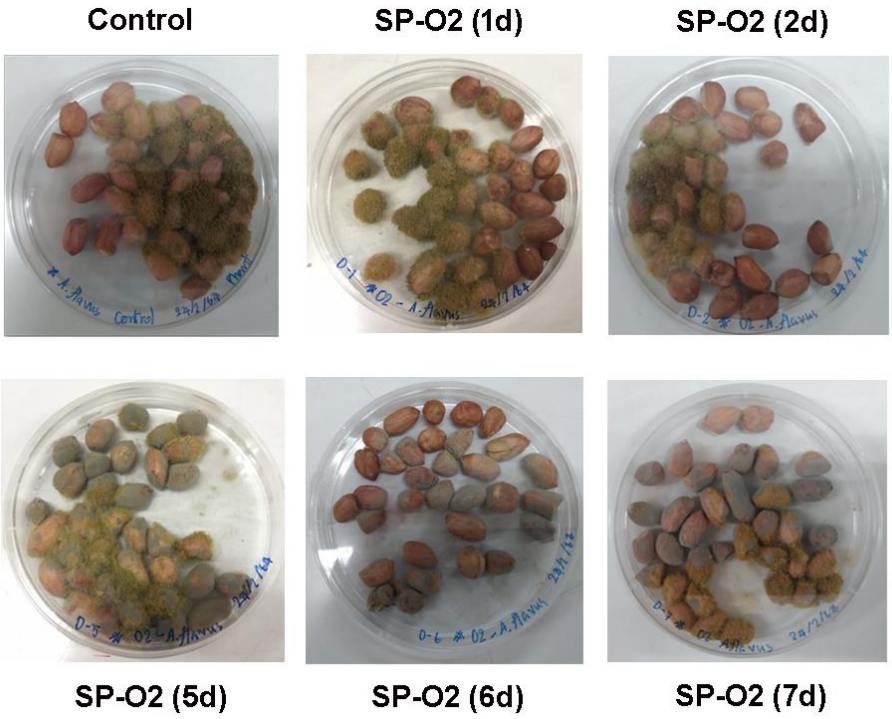


Figure 4 Inhibition of fungal growth on peanut kernels incubated with *Streptomyces* sp. isolate SP-O2. The *A. flavus* TISTR 3041 control, and co-cultivation of fungus with pre-cultured SP-O2 for 1, 2, 5, 6 and 7 days, respectively.

16S rDNA gene sequence comparisons and phylogenetic analysis

A BLAST search of the GenBank database using 1436 bp 16S rDNA gene sequence of the *Streptomyces* sp. isolate SP-O2 showed its similarity to that of many members of the genus *Streptomyces*. A phylogenetic tree based on 16S rDNA gene sequences of members of the genus *Streptomyces* was constructed according to the bootstrap test of neighbor-joining algorithm method. Phylogenetic analysis indicated that the *Streptomyces* sp. isolate SP-O2 consistently falls into a clade together with *Streptomyces spectabilis* NBRC 13424 AB184393 (GenBank; accession No. AB184393, 99.93% similarity) (**Figure 5**). *Streptomyces spectabilis* can produce many types of antibiotics with high antibacterial activity, including spectinomycin, streptovaricin, and some other metabolites [34,35], and has a high application value in the pharmaceutical industry. However, there are no reports on its activity against soil-borne plant diseases or used in agriculture [36]. Therefore, screening, isolation, and characterization of promising isolates of *Streptomyces* producing potential antifungal activity and other therapeutics have been an intensive focus on our research. In addition, the taxonomy of *Streptomyces* sp. isolate SP-O2 resembled the published literature of type strain *S. spectabilis* NRRL 2494 and *S. spectabilis* SNF4435 [30,37]. However, the limitation of SEM morphology of *S. spectabilis* SNF4435 was not clearly seen the fragmentation of Rectiflexibiles mycelium into spores as we observed in our isolate. Surprisingly, 16S rDNA analysis demonstrated 99.93 % similarity to *S. spectabilis* NBRC 13424 AB184393 as same as published report in Chen *etal.*[36] but *Streptomyces* sp. CB-75 exhibited much different to our isolate including the characteristics on ISP media and SEM photography of aerial mycelium and spores morphology. Moreover, this previous published of *Streptomyces* sp. CB-75 showed broad-spectrum antifungal activity against 11 plant pathogenic fungi not including *A. flavus*.

The 16S rDNA sequencing is a powerful method of identification that offers a useful alternative due to the given ribosomal small subunit existing universally among bacteria and includes species-specific variability regions. Additionally, a previous study by Kataoka *et al.*[38] demonstrated that partial nucleotide sequences (120 bp) of the 16S rRNA gene containing a variable α region was able to differentiate 8 major clusters of category I in Bergey's Manual of Systematic Bacteriology. The phylogenetic relationships of the type strains in the *S. fulvissimus* major cluster including *S. fulvissimus*,

S. aureoverticillatus, *S. spectabilis*, and *S. longispororuber* were distinctly separated. According to our phylogenetic tree of the 16S rDNA complete sequences (1436 bp), the data suggests that our newly isolated *Streptomyces* sp. SP-O2 belongs to *Streptomyces spectabilis* SP-O2.

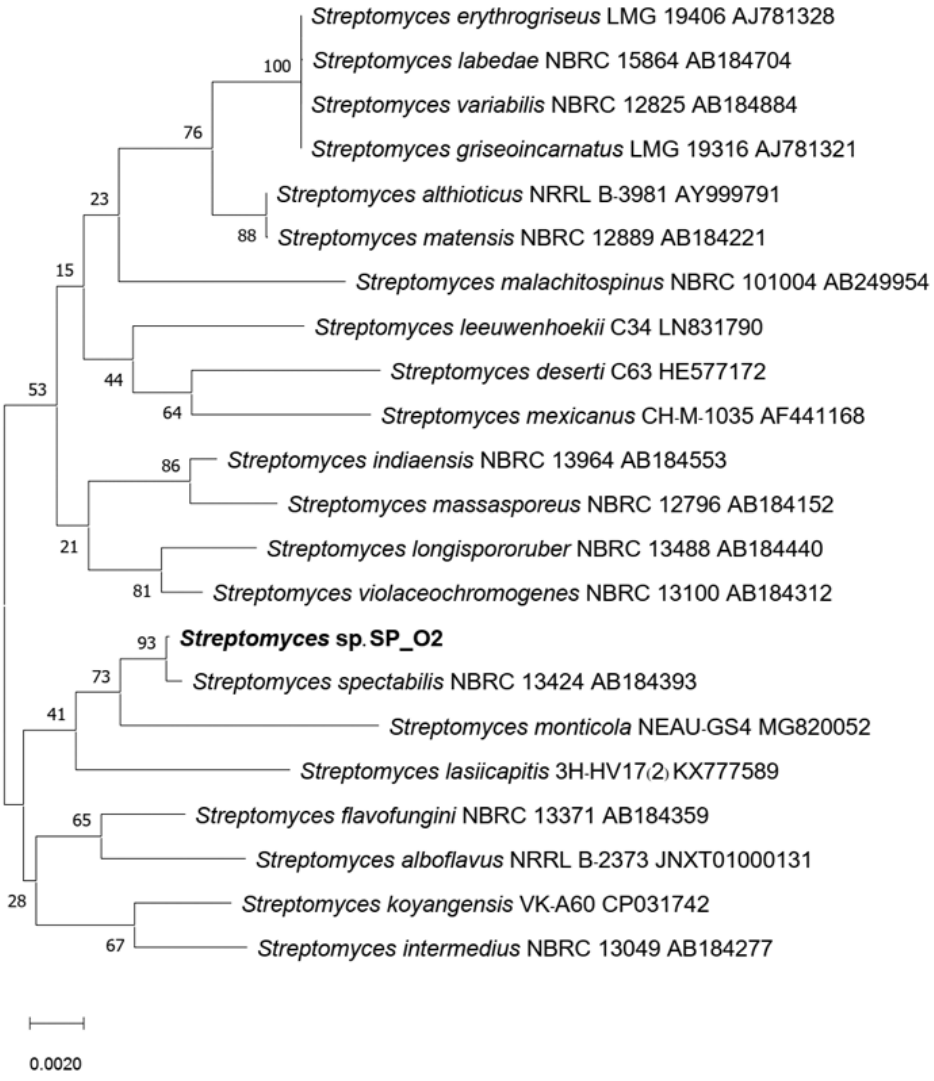


Figure 5 Neighbour-joining phylogenetic tree of the 16S rDNA sequences showing the relationships between *Streptomyces* sp. isolate SP-O2 and related species of the genus *Streptomyces*. Bar, 0.002 substitutions per nucleotide position.

Conclusions

The isolated actinobacteria in this study was identified as *Streptomyces spectabilis* SP-O2 based on its morphological characteristics, physiological properties and molecular identification. It exhibited the promising fungicidal activity against *A. flavus* in the dual culture agar assay, co-culture liquid assay and peanut kernels assay with consistency results. Strikingly, the secreted metabolites producing by *Streptomyces spectabilis* SP-O2 play a crucial role on the fungal killing instead of aflatoxin degradation ability. Therefore, this promising isolate must be further investigated on the killing mechanism and biocontrol development of using in controlling of aflatoxin producing mold in the agricultural produces and feedstock.

Acknowledgements

This study was supported by a research grant from HRH Princess Mahachakri Sirindhorn Medical Center, Faculty of Medicine, Srinakharinwirot University (Contract No. 428/2562). We thank the Department of Microbiology, Faculty of Medicine, Srinakharinwirot University for providing the instruments and facilities. Moreover, we also thank the Faculty of Dentistry, Srinakharinwirot University for SEM service.

References

[1] HPV Egmond, RC Schothorst and MA Jonker. Regulations relating to mycotoxins in food: Perspectives in a global and European context. *Anal. Bioanal. Chem*. 2007; **389**, 147-57.

[2] GA Payne and MP Brown.Genetics and physiology of aflatoxin biosynthesis. *Annu. Rev. Phytopathol*. 1998; **36**, 329-62.

[3] CP Wild and YY Gong. Mycotoxins and human disease: A largely ignored global health issue. *Carcinogenesis* 2010; **31**, 71-82.

[4] M Carvajal-Moreno. Metabolic changes of aflatoxin B1 to become an active carcinogen and the control of this toxin. *Immunome Res.* 2015; **11**, 104.

[5] IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. *IARC Monogr.Eval.Carcinog.Risks Hum.* 2002; **82**, 1-556.

[6] G Perrone, A Susca, G Cozzi, K Ehrlich, J Varga, JC Frisvad, M Meijer, P Noonim, W Mahakarnchanakul and RA Samson. Biodiversity of *Aspergillus* species in some important agricultural products. *Stud. Mycol*. 2007; **59**, 53-66.

[7] EA Emmert and J Handelsman. Biocontrol of plant disease: A (gram-) positive perspective. *FEMS Microbiol. Lett*. 1999; **171**, 1-9.

[8] T Kaur, R Rani and RK Manhas.Biocontrol and plant growth promoting potential of phylogenetically new *Streptomyces* sp. MR14 of rhizospheric origin. *AMB Express*2019; **9**, 125.

[9] PE Verweij, E Snelders, GHJ Kema, E Mellado and WJG Melchers. Azole resistance in *Aspergillus fumigatus*: A side-effect of environmental fungicide use? *Lancet Infect. Dis*. 2009; **9**, 789-95.

[10] SN Kumar, B Nambisan and C Mohandas. Purification and identification of two antifungal cyclic dipeptides from *Bacillus cereus* subsp. *thuringiensis* associated with a rhabditid entomopathogenic nematode especially against *Fusarium oxysporum*. *J. Enzyme Inhib. Med. Chem*. 2014; **29**, 190-7.

[11] SD Bentley, KF Chater, AM Cerdeño-Tárraga, GL Challis, NR Thomson, KD James, DE Harris, MA Quail, H Kieser, D Harper, A Bateman, S Brown, G Chandra, CW Chen, M Collins, A Cronin, A Fraser, A Goble, J Hidalgo, T Hornsby, … and DA Hopwood. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* 2002; **417**, 141-7.

[12] J Abraham and R Chauhan. Profiling of red pigment produced by *Streptomyces* sp. JAR6 and its bioactivity. *3 Biotech*2018; **8**, 22.

[13] I Caceres, SP Snini, O Puel and F Mathieu. *Streptomyces roseolus*, a promising biocontrol agent against *Aspergillus flavus*, the main aflatoxin B_1 producer. *Toxins*2018; **10**, 442.

[14] HM Kemung, LTH Tan, TM Khan, KG Chan, P Pusparajah, BH Gohand LH Lee. *Streptomyces* as a prominent resource of future anti-MRSA drugs. *Front. Microbiol*. 2018; **9**, 2221.

[15] D Qi, L Zou, D Zhou, Y Chen, Z Gao, R Feng, M Zhang, K Li, J Xieand W Wang. Taxonomy and broad-spectrum antifungal activity of *Streptomyces* sp. SCA3-4 isolated from rhizosphere soil of *Opuntia stricta*. *Front. Microbiol*. 2019; **10**, 1390.

[16] JN Selvakumar, SD Chandrasekaran and M Vaithilingam. Bio prospecting of marine-derived *Streptomycesspectabilis* VITJS10 and exploring its cytotoxicity against human liver cancer cell lines. *Pharmacogn. Mag*. 2015; **11**, S469-S473.

[17] Q Shakeel, A Lyu, J Zhang, M Wu, G Li, T Hsiang and L Yang. Biocontrol of *Aspergillus flavus* on peanut kernels using *Streptomyces yanglinensis* 3-10. *Front. Microbiol*. 2018; **9**, 1049.

[18] B Zhang, G Cai, H Wang, D Li, X Yang, X An, X Zheng, Y Tian, W Zheng and T Zheng. *Streptomyces alboflavus* RPS and its novel and high algicidal activity against harmful algal bloom species *Phaeocystisglobosa*. *PLoS One* 2014; **9**, e92907.

[19] S Boukaew and P Prasertsan. Efficacy of volatile compounds from *Streptomyces philanthi* RL-1-178 as a biofumigant for controlling growth and aflatoxin production of the two aflatoxin-producing fungi on stored soybean seeds. *J. Appl. Microbiol*. 2020; **129**, 652-64.

[20] M Yang, L Lu, J Pang, Y Hu, Q Guo, Z Li, S Wu, H Liu and C Wang. Biocontrol activity of volatile organic compounds from *Streptomyces alboflavus* TD-1 against *Aspergillus flavus* growth and aflatoxin production. *J. Microbiol.* 2019; **57**, 396-404.

[21] A Janardhan, AP Kumar, B Viswanath, DVR Saigopal and G Narasimha. Production of bioactive compounds by actinomycetes and their antioxidant properties. *Biotechnol. Res. Int.* 2014; **2014**, 217030.

[22] P Sangmanee and T Hongpattarakere. Inhibitory of multiple antifungal components produced by *Lactobacillus plantarum* K35 on growth, aflatoxin production and ultrastructure alterations of *Aspergillus flavus* and *Aspergillus parasiticus*. *Food Control* 2014; **40**, 224-33.

[23] SK Augustine, SP Bhavsar, M Baserisalehi and BP Kapadnis. Isolation, characterization and optimization of antifungal activity of an actinomycete of soil origin. *Indian J. Exp. Biol.* 2004; **42**, 928-32.

[24] EB Shirling and D Gottlieb. Methods for characterization of *Streptomyces* species. *Int. J. Syst.Evol. Microbiol*. 1966; **16**, 313-40.

[25] MC Taylor, CJ Jackson, DB Tattersall, N French, TS Peat, J Newman, LJ Briggs, GV Lapalikar, PM Campbell, C Scott, RJ Russell and JG Oakeshott. Identification and characterization of two families of F420 H2-dependent reductases from Mycobacteria that catalyse aflatoxin degradation. *Mol. Microbiol*. 2010; **78**, 561-75.

[26] SF Altschul, TL Madden, AA Schäffer, J Zhang, Z Zhang, W Miller and DJ Lipman. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res*. 1997; **25**, 3389-402.

[27] EW Myers and W Miller. Optimal alignments in linear space. *Comput. Appl. Biosci*. 1988; **4**, 11-7.

[28] S Kumar, G Stecher, M Li, C Knyaz and K Tamura. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 2018; **35**, 1547-9.

[29] P Kumar, DK Mahato, M Kamle, TK Mohanta and SG Kang. Aflatoxins: A global concern for food safety, human health and their management. *Front. Microbiol*. 2016; **7**, 2170.

[30] K Kurosawa, K Takahashi and E Tsuda. SNF4435C and D, novel immunosuppressants produced by a strain of *Streptomyces spectabilis*. I. Taxonomy, fermentation, isolation and biological activities. *J. Antibiot*. 2001; **54**, 541-7.

[31] S Guan, C Ji, T Zhou, J Li, Q Ma and T Niu. Aflatoxin B1 degradation by *Stenotrophomonas maltophilia* and other microbes selected using coumarin medium. *Int. J. Mol. Sci.* 2008; **9**, 1489-503.

[32] OD Teniola, PA Addo, IM Brost, P Färber, KD Jany, JF Alberts, WHV Zyl, PS Steyn and WH Holzapfel. Degradation of aflatoxin B(1) by cell-free extracts of *Rhodococcus erythropolis* and *Mycobacterium fluoranthenivorans* sp. nov. DSM44556(T). *Int. J. Food Microbiol*. 2005; **105**, 111-7.

[33] S Alinezhad, M Tolouee, A Kamalzadeh, AA Motalebi, M Nazeri, M Yasemi, M Shams-Ghahfarokhi, R Tolouei and M Razzaghi-Abyaneh. Mycobiota and aflatoxin B_1 contamination of rainbow trout (*Oncorhinchus mykiss*) feed with emphasis to *Aspergillus* section *Flavi*. *Iranian J. Fish.* 2011; **10**, 363-74.

[34] KR Kim, TJ Kim and JW Suh. The gene cluster for spectinomycin biosynthesis and the aminoglycoside-resistance function of *spc*M in *Streptomyces spectabilis*. *Curr. Microbiol*. 2008; **57**, 371-4.

[35] D Spasova, N Vesselinova and R Gesheva. Comparative investigation of a streptovaricin-producing strain of *Streptomyces spectabilis* and its selectant. *Folia Microbiol.* 1997; **42**, 35-8.

[36] Y Chen, D Zhou, D Qi, Z Gao, J Xie and Y Luo. Growth promotion and disease suppression ability of a *Streptomyces* sp. CB-75 from banana rhizosphere soil. *Front. Microbiol*. 2017; **8**, 2704.

[37] EB Shirling and D Gottlieb. Cooperative description of type strains of *Streptomyces*. V. additional descriptions. *Int. J. Syst. Bacteriol.* 1972; **22**, 265-394.

[38] M Kataoka, K Ueda, T Kudo, T Seki and T Yoshida. Application of the variable region in 16S rDNA to create an index for rapid species identification in the genus *Streptomyces*. *FEMS Microbiol. Lett*. 1997; **151**, 249-55.