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Isolation and Characterization of a Novel Endophytic *Streptomyces* SUK 06 with Antimicrobial Activity from Malaysian Plant

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Abstract: In search of new antimicrobial agents, samples of plant stem which were originally used as traditional medicine were collected from various parts of peninsular Malaysia. The isolate, namely SUK 06 from *Thottea grandiflora* (family-Aristolchieaceae) was cultured on International Streptomyces Project medium (ISP 2). Macroscopically, the bacteria surface appeared to be smooth with granule texture, irregular folding, dry and chalky and showed a substrate mycelium with grey in color. By scanning electron microscope, this isolate had a unique spiral filament structure. Molecular biological studies on the 16S rRNA revealed that it was distinct from all other genetic accessions of *Streptomycetes* in GenBank, but bear some genetic similarity to other *Streptomycetes*. The secondary metabolites extracted by ethyl acetate showed demonstrable killing activity against one or more pathogenic bacteria and inhibition zones (mm) were as followed: *Bacilus subtilis* (17 mm), *Pseudomonas aeruginosa* ATCC 27853 (20 mm), *Bacillus cereus* (22 mm), *Pleisiomonas shigelloides* (20 mm) and MRSA ATCC 700699 (37 mm). Nevertheless, there were some antifungal activity measured as a percentage of inhibition (%) against *Fusarium solani* (62%), *Aspergillus fumigatus* (44%), *Pythium ultimum* (20%), *Phytophthora erythroseptica* (23%) and *Geothrichum candidum* (23%).

Key words: Endophytes, *Streptomycetes*, 16S rRNA, MRSA, antimicrobial, *Fusarium solani*

INTRODUCTION

Streptomyces which is characterized as Gram positive, filamentous bacteria with high G+C contents was formerly found as saprophyte that dwells in the soil. It's an organism that produces a variety of metabolite particularly antimicrobial that was used either in medicinal therapies or as research tool. Extensive research have been carried out in the last decades to isolate *Streptomyces* from soil and while still yielding new drugs, the numbers of novel compound have been dwindling (Palaez, 2006). In order to avoid the redundancy of finding the same compound and to overcome the emerging of new drug resistance in many bacterial pathogens, new untapped source of bioactive products must be discovered. It is now obvious that these filamentous bacteria exist as endophytes within the living tissues of certain higher plants (Ezra *et al*., 2004; Castillo *et al*., 2006). The complex interaction has gained our interest because it can reveal new source of novel drugs which can be used in either agriculture or medicinal field. (Cao *et al*., 2004; Castillo *et al*., 2003, 2002; Strobel, 2002; Strobel *et al*., 2005).

A fast and accurate method has been widely used to identify newly isolated endophytes using 16S rDNA gene sequence analysis. This technique is important to identify organisms which are slow-growing, fastidious and where identification by conventional methods is time consuming along with subjective interpretation such as identifying *Streptomycetes* sp. Castillo *et al*. (2003) had succeeded in identifying *Streptomyces* sp. NRRL 30566, which produced novel antibiotic Kakadumycins using this method. When used with previously described phenotypic data, the addition of information from 16S rRNA can be applied to reestablish existing bacteria taxonomy (Kim and Goodfellow, 2002).

Previous report showed that plants representing families were present during the era of the ancient

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Gondwanaland, yielded numerous novel bioactive endophytic *Streptomycetes* (Castillo *et al*., 2007). Thus, it appears likely that other biologically active Streptomycete isolates exist in plants throughout the world. In this study, isolation of endophytic Streptomycetes have been carried out from the ethno botanical plants that hold feature to healing wounds, skin infection, or curing fever. This study illustrates the details on the isolation and bioactivities of this novel endophytic *Streptomyces* SUK 06 and showed that some of the bioactive products were extractable with organic solvents.

MATERIALS AND METHODS

Plants selection and location: *Thottea grandiflora* stem and some root samples were collected from the province of Bukit Bauk in Terengganu State situated at the east coast of peninsular Malaysia. This plant was traditionally used as postpartum tonic, cure fever and malarial infection (Mat-Salleh and Latiff, 2002) A few portions of the stems with length of approximately 10 cm was cut, tagged and stored in a clean plastic bag. A specimen voucher number also given and submitted to Universiti Kebangsaan Malaysia (UKM) botanical collection.

Isolating endophytic *Streptomycete* sp.: Each plant stem samples were subjected to surface sterilization treatment to eliminate the contaminating microflora. This procedure includes sequential immersion of the stems in 70% alcohol for 5 min, 0.9% NaOCl for 10 min and 95% alcohol for 30 sec (Zin *et al*., 2007b). Using aseptic technique the bark of the stems was removed and the inner stem tissues were cut into small pieces sized 0.5×0.5 cm. The tissue was then placed onto Starch Casein Agar (SCA) surfaces which have been supplemented with 50 μg mL^{-1} Cycloheximide, 50 μg mL^{-1} Nystatin and 20 μg mL^{-1} Nalidixic acid. The petri dish was sealed and incubated at 28°C. Observation was done for a few weeks and bacterial growth on the agar or from stem tissue was re-cultured on International Streptomyces Project medium (ISP 2) agar for morphology and pigment observation. The organism appearing as actinomycetes, yielding an earthy odor in culture, seemed as likely candidates as potential streptomycetes. It was cultured on nutrient agar and stored in sterile 15% glycerol water at -70°C. It was kept in the living microbe collection at UKM and labeled as SUK 06.

Microscopy observation: Microscopy observation was done using light and Scanning Electron Microscopy (SEM). Light microscopy observation was carried out to observe the morphology of SUK 06 using Gram staining and SEM for observation of spore morphology on agar. The colony of SUK 06 was fixed in 10% (v/v) Formalin Phosphate Buffered and after washing with PBS, it was then dehydration in serial ascending of alcohol concentration from 50% to absolute alcohol (15 min for each concentration except for absolute alcohol in which the dehydration was done twice). The samples were then processed by procedures described by Zin *et al*. (2007a). It was then critical-point dried, gold coated and images were recorded using Philips XL 30 SEM.

DNA extraction of *Streptomyces* SUK 06: For DNA extraction *Streptomyces* SUK 06 was cultured in 10 mL nutrient broth for 4 to 7 day so that satisfactory amount of bacteria cells was obtained. The whole culture was centrifuged at 10,000 g for 20 min and the supernatant was completely discarded. The DNA extraction protocol was based on Conn and Franco (2004), The DNA collected was dissolved in 50 μL sterile water and stored at -20°C until further use.

Amplification and sequencing 16S rRNA of *Streptomyces* SUK 06: The extracted DNA was quantified using spectrophotometer at A_{260}. PCR amplification was based on Coombs and Franco (2003) with modification which using primers 27f (5-AGAGTTTGATCMTGGCTCAG-3) and 1492r (5-TACGGYTACCTTGTTACGA3) (First Base Lab, Selangor, Malaysia). It was carried out in 50 μL mixture comprising 5 μL 27 f (10 pmol), 5 μL 1492r (10 pmol), 5 μL 10X PCR buffer, 1 μL DNTP 10 mM, 3 μL $MgCl_2$ 25 mM, 1 μL 5U μL Taq polymerase, 5 μL template DNA and 25 μL sterile H_2O. The temperature profile for PCR were: 94°C for 8 min; followed by 30 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 2 min and then final elongation at 72°C for 10 min. The PCR products were purified by using a Wizard PCR Prep DNA purification kit (Promega). After purification, cloning of amplified 16S rDNA was carried out using Invitrogen pCR®8/GW/TOPO®TA Cloning® kit. The procedure employed was based on manufacturer instruction. Plasmid extraction from transformant was done using miniprep plasmid extraction method from Sambrook *et al*. (1989). The plasmid then sequenced bidirectional 27f, 704f (5-GTAGCGGTGAAATGCGTAGA-3), 765r (5-CTGTTTGCTCCCCACGCTTTC-3) and 1492r primers (First Base Lab, Selangor, Malaysia). The sequence obtained were analyzed using CLC Free Workbench 4.0 software and compared to the other streptomycete gene sequences in GenBank by using the BLAST software (blastn) at the National Center of Biotechnolgy Information (NCBI) web site (http://www.ncbi.nih.gov/).

Bioassay of *Streptomyces* SUK6: *Streptomyces* SUK 06 was grown onto nutrient agar for 14 days. Then aseptically, a plug with diameter of 1 cm of the grown *Streptomyces* SUK 06 was transferred onto another nutrient agar which has been previously plated with test bacteria. The test bacteria used were *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis*, *Enterococus fecalis*, *Bacillus cereus*, *Salmonella aberdeen* NCTC74, *Escherichia coli* NCTC 11601, *Pleisiomonas shigelloides* and MRSA ATCC 700699. The test fungi were as followed: *Fusarium solani*, *Aspergillus fumigatus*, *Pythium ultimum*, *Thrichoderma*, *Phytophthora erythroseptica* and *Geothrichum candidum*. The inhibition zones were observed for 24 and 48 h in which Oxacillin was used as a positive control.

Fermentation, extraction and assay procedures: A few blocks of ISP 2 containing *Streptomyces* SUK 06 were inoculated into 30 mL of ISP 4 broth and incubated at room temperature 28°C for 7 days and eventually transferred onto 300 mL nutrient broth and incubated in rotary shaker for 21 days at room temperature (Zin *et al*., 2007b). Culture filtrates were extracted with three half-volume of ethyl acetate. The organic phase was pooled followed by evaporation using rotavapor (model Eyela Rotary Vacuum Evaporator N-N series) at 40°C. The crude extract was dissolved in methanol and was then used for bioactivity against test bacteria and fungi. Known amounts of the dried extract taken up in methanol were then placed directly onto a spot on a PDA plate, dried in the hood and then the test fungi was placed 1.0 cm away from the test spot and eventually evaluated for growth by measuring hyphal development in contrast to the control plate. The data was expressed as percentage of linear growth as compared to the control and the test was performed in triplicate. The method is repeated for bacteria assay but the plate was carefully plated with test bacteria after adding the extract. It was incubated at 37°C for 24 h and zone of inhibition zone was measured.

RESULTS AND DISCUSSION

Endophytic *Streptomyces* SUK 06 from *Thottea grandiflora*: *Streptomyces* sp. designated as SUK 06 was obtained from the stem of *Thottea grandiflora* after 2 weeks incubation on SCA. The macroscopic characterization was as followed: substrate mycelium emerged after four days incubation and formation of mycelium on the 6th day. The aerial formation was white in color then gradually changed into grey. The elevated colony appeared to be fine edge, chalky, dry, irregular folding and produced some brown pigmentation (Fig. 1). The observation under light microscope showed branching filament and Gram positive bacteria (Fig. 2). SEM observation revealed that the spore ornamentation was spiral (*Spira*) with the diameter sized ranged 0.5 um to 0.7 μm (Fig. 3) and this is not a common occurrence among endophytic Streptomycetes from previously reported by Castillo *et al*. (2005). Molecular analysis of 16S rRNA showed 98% similarity with *Streptomyces misawanensis*, *Streptomyces fulvoviolaceus* and *Streptomyces ginsengisoli* (Table 1).

Fig. 1: Colony of *Streptomyces* SUK 06 on ISP2 agar

Fig. 2: *Streptomyces* SUK 06 under light microscope (1000X)

Table 1: Identification of *Streptomyces* SUK 06 based on partial sequencing of 1455 bp region of 16S rRNA gene sequence and other related *Streptomyces* in Gene Bank

Species	Score (bits)	Similarity to 16S rRNA (%)
Streptomyces misawanensis AB184533.1	2503	98
Streptomyces fulvoviolaceus AB184573.1	2487	98
Streptomyces ginsengisoli AB245393.1	2478	98
Streptomyces diastatochromogenes subsp. *Luteus* AB184503.1	2425	97
Streptomyces myxogenes AB184484.1	2425	97
Streptomyces niveoruber strain NRRL B-2724 DQ445796.1	2420	97
Streptomyces fimbriatus AB184444.1	2352	96
Streptomyces lavendulae EF371426.1	2344	96
Streptomyces eurythermus D63870.1	2197	95
Streptomyces glomeratus AJ781754.1	2191	95

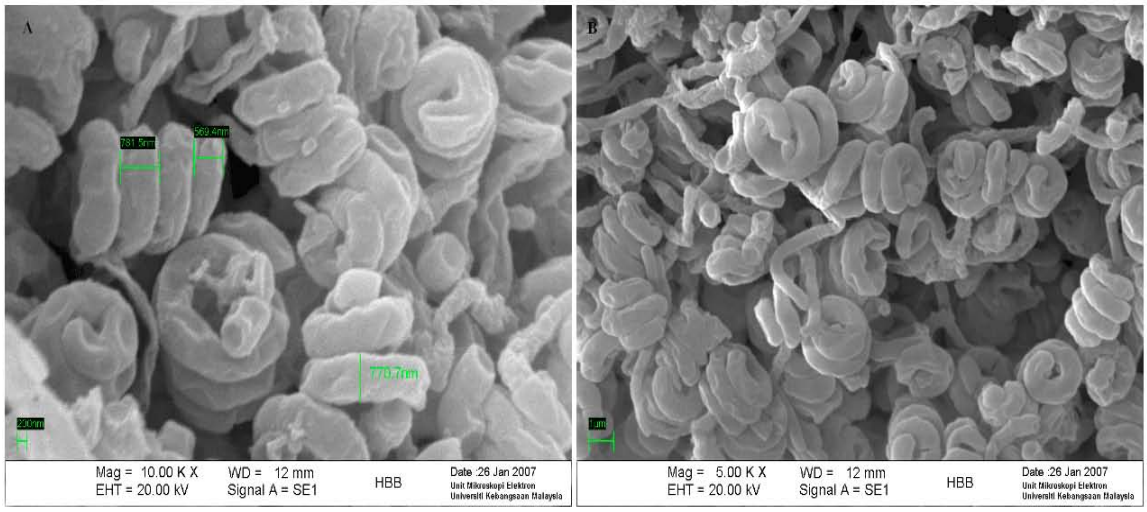

Fig. 3: Scanning electron microscopy of *Streptomyces* SUK 06, spores (A) and hyphae (B)

Table 2: Inhibitory activity of the ethyl acetate extracts of *Streptomyces* SUK 06 broth culture against various human-pathogenic bacterium

	Diameter of inhibition zone (mm)	
Test bacteria	Oxacillin	Crude extract SUK 06
Bacillus subtilis	17	17
Bacillus cereus	14	22
Pseudomonas aeruginosa ATCC 27853	0	20
MRSA ATCC 700699	43	37
Pleisiomonas shigelloides	33	20

Table 3: Inhibitory activity of the ethyl acetate extract of *Streptomyces* SUK 06 broth culture against various plant-pathogenic fungi

Test fungus	Inhibition (%)
Phytophthora erythroseptica	23
Trichoderma	43
Geothrichum candidum	23
Pythium ultimum	20
Fusarium solani	62
Aspergillus fumigatus	44

Bioassay of *Streptomyces* SUK 06: Biological activity of several endophytic *Streptomycetes* against the fungal test organisms has been reported but none was lethal to either Gram-positive or Gram negative bacteria (Zin *et al*., 2007b). In this study, *Streptomyces* SUK 06 was active against Gram positive bacterium rather than Gram negative bacterium. The strongest activity was towards MRSA ATCC 700699 and to the rest of the tested organisms, can be graded as susceptible to intermediate susceptibility (Table 2). Using Oxacillin as a positive control, the antibacterial test showed that the crude extract has at least equivalent activity to that of Oxacillin. In comparison of the effects towards *Bacillus subtilis*, the same inhibition effect which was intermediately susceptible was observed between Oxacillin and crude extract, whereas for *Bacillus cereus*, the Oxacillin produced smaller inhibition zone. The crude extract however, gave a larger inhibition zone that can be classified as susceptible. Interestingly, test against *Pseudomonas aeruginosa*, using crude extract did produce inhibition zone as large as 20 mm despite the fact that oxacillin did not produce any inhibition. When tested against *Pleisiomonas shigelloides* and MRSA, the crude extract produced a smaller inhibition zone compared to oxacillin but it can still be concluded as susceptible. Results against fungi showed that the strongest activity was towards *Fusarium solani* (Table 3). It is well known that *Fusarium* sp. is an agent that infects plants and causing root rot disease. For example *Fusarium solani* causes brown root rot on peanuts plants (Rojo *et al*., 2007) and *Fusarium oxysporum* causes fusarium wilt on banana (Cao *et al*., 2004) and these infections may cause low yield of crops. A lot of research has been done to find a good antagonistic agent for *Fusarium* sp. using various species of bacteria (Lim *et al*., 1991; Li *et al*., 2005; Pal *et al*., 2001). In this study, the crude extract of SUK6 was found to be capable of inhibiting *Fusarium solani* and this could be used as an indicator for its uses or further development of *Streptomyces* SUK6 as a biocontrol for infections due to *Fusarium* sp. This is of importance as Malaysia is an agroeconomic country seeking for environmental-friendly approach to control pathogen infection on economically valued crops such as banana. As for now, preliminary test revealed the potential of the extracts to inhibit several pathogenic bacteria and a few plant pathogenic fungi. Relationship between endophyte SUK 06 and the host *Thottea grandiflora* cannot be established at a current phase. In the meantime several reports on other endophyte using other plants has covered that the endophyte contributes to host survival against environmental hardiness, promotes production of growth

hormone and also increase resistance again other pathogenic microbes (Selosse *et al*., 2004; Strobel, 2003; Tokala *et al*., 2002; Coombs and Franco, 2003; Cao *et al*., 2003). On the other hand, this study will consequently serve as prelude for more comprehensive study on the properties of the bioactive product yielded by *Streptomyces* SUK 06. Further investigation on the biological and chemical properties to ascertain if it may have the potential to be used in biological control or as new drugs for medicinal usage is underway.

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