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In Vitro Screening of Antagonistic Activity of Soil *Streptomyces* against Plant Pathogenic Fungi and Assessment of Its Characters

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Abstract Actinobacteria from the genus *Streptomyces* have been studied as potential biocontrol agents worldwide. In this study, a total of 35 isolates of Actinomycete like colonies were isolated from the rhizospheres of various plant species. These isolates were primary screened for antagonistic activity to inhibit mycelial growth of *Colletotrichum gloeosporioides*. The screen revealed that seven isolates of Actinomycete like colonies were good inhibitors of the fungal mycelial growth and were chosen for their antagonistic effects against *C. gloeosporioides* and *Biporalis maydis* by the dual culture method. The isolate that had the best inhibitory effect on both fungi was further evaluated for antagonistic activity against *C. capsici*, *Pyricularia* sp., *Fusarium* sp., *Curvularia* sp. and *Sclerotium roftsii*. The results showed that the selected isolate inhibited the mycelial growth of *C. capsici*, *Pyricularia* sp., *Fusarium* sp. and *Curvularia* sp. within the range of 65.5-91.6%, while it did not affect the mycelial growth of *S. roftsii*. Moreover, the culture filtrate of the selected isolate inhibited the mycelial growth of *C. gloeosporioides* and *B. maydis* under *in vitro* condition. Based on the 16S rDNA gene sequence, colony morphology, spore chain rearrangement and physiology, the best isolate belonged to the genus *Streptomyces* and assigned as *Streptomyces* sp. isolate SRF1. These findings suggest that the *Streptomyces* sp. isolate SRF1 is a potential candidate, with a broad antagonistic activity, for use as a biocontrol against plant pathogenic fungi.

Keywords: Antagonistic biocontrol agent, Plant pathogenic fungi, *Streptomyces*

Introduction

Plant pathogenic fungi are major causes of economically significant losses in many plants in agricultural production areas. These pathogens cause production yield losses of 25% in western countries and 50% in developing countries (Gohel *et al*., 2006). Therefore, chemical fungicides are used to control of these fungal pathogens under current agriculture practices (Prapagdee *et al*., 2008). However, there are concerns about

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chemical fungicides because they are not environmentally friendly and have harmful effects on humans and non-target organisms (Cook, 1993). Moreover, the extensive use of chemical fungicides can produce resistance in fungal strains (ŽivkoviĆ *et al*., 2010; Evangelista-Martínez, 2014). Therefore, the control of fungal plant diseases using non-chemical fungicides such as non-pathogenic microorganisms is very important and interesting because the non-pathogenic microorganisms are not as harmful to the environment as chemical fungicides (Moënne-Loccoz *et al*., 2001).

Antagonistic bacteria belonging to the genus *Streptomyces* are Gram positive soil bacteria that have been known for their potential to inhibit several plant pathogenic fungi responsible for major crop diseases (Joo, 2005; Lee *et al*., 2012; Evangelista-Martínez, 2014). Their antagonistic behavior to plant pathogenic fungi involved the production of bioactive compounds that are known as secondary metabolites (Trejo-Estrada et al, 1998; Ouhdouch *et al*., 2001). Recently, 60% of the bioactive compounds produced and developed for agricultural use have originated from *Streptomyces* species (Ilic *et al*., 2007). Some *Streptomyces* species have been reported as inhibiting the growth of plant pathogenic fungi (Taechowisan *et al*., 2005). *S. philanthi* RL-1-178 could inhibit *Sclerotium rolfsii* and *Ralstonia solanacearum*, the causal agents of *Sclerotium* root and stem rot and *Ralstonia* wilt of chili pepper, respectively (Boukaew *et al*., 2011). *Streptomyces* sp. CACIS-1.16CA has been reported to inhibit *Alternaria* sp., *Phytophtora capsici*, *Colletotrichum* sp. and *Rhizoctonia* sp. (Evangelista-Martínez, 2014). *Streptomyces* sp. A1022 has successfully been used as a biocontrol agent to protect pepper plants from anthracnose disease caused by *C. gloeosporioides* under greenhouse conditions (Lee *et al*., 2012). *Streptomyces* sp. CIMAP-A1 had potential antagonistic activity against some plant pathogenic fungi such as *Botrytis cinerea*, *Curvularia* sp. and *Sclerotinia sclerotiorum*. Moreover, the secondary metabolites produced from this strain have been used to inhibit spore germination and mycelial growth of the geminated spores of *A. alternate*, *C. acutatum*, *Curvularia andropogonis* and *Fusarium moniliforme* (Alam *et al*., 2012). The exponential culture filtrate of *S. hygroscopicus* SRA14 has been reported to inhibit the growth of *C. gloeosporioides* (Prapagdee *et al*., 2008).

Given that potentially novel strains of *Streptomyces* have been isolated, it is important that the researchers should be reliably identified the species they are analyzing. In recent years, amplification and sequencing of the 16S rDNA region has successfully been used and is particularly effective for characterization and identification of the *Streptomyces* genus (Evangelista-Martínez, 2014; Kim *et al*., 2006). Kim *et al*. (2006) successfully used the 16S rDNA sequence to identify the soil Actinomycete strain VC-A46^{T} and assign as a novel *S. cheonanensis* sp. nov. Choi *et al*.

(2009) identified potential soil bacteria as *S. griseofuscus* CNU-A91231 based on the sequence of the 16S rDNA gene.

The present study aims to isolate antagonistic *Streptomyces* from soil and to investigate its potential to inhibit the growth of plant pathogenic fungi under *in vitro* conditions. The potential isolate was identified by its 16S rDNA gene sequence, while some biochemical and physiological properties were also investigated.

Materials and methods

Isolation of antagonistic Streptomyces

Soil samples were collected from the rhizospheres of banana plants, rice fields and soil used for the cultivation of mango and sentol plants. Each composite sample consisted of 5 individual samples taken from an area of 30 m^2. Approximately 50 g of each individual soil sample were taken from a depth of 10 cm below the top of the soil surface. The individual samples were mixed in double ziplock bags, and the composite soil samples were air dried at ambient temperature for 2-3 days. Ten grams of the dried soil was suspended in 100 mL of sterile distilled water and incubated at 40^oC for 30 min. The mixture was serially diluted up to 10^{-4}. One hundred milliliters of each dilution was pipetted and spread on the surface of arginine-glycerol mineral salt agar (AGMA) in duplicate. The plates were incubated at 37^oC for 14 days prior to selection of the Actinomycete like colonies (Holt et al., 2000). Selected colonies were subcultured onto half potato dextrose agar (HPDA) for the production of pure bacterial strains. The pure cultures were kept on HPDA slants at 4^oC and in 20% (v/v) glycerol at -80^oC (Kaewkla and Franco, 2013).

Isolation of plant pathogenic fungi

Plant pathogenic fungi (*Colletotrichum gloeosporioides*, *C. capsici*, *Biporalis maydis*, *Pyricularia* sp., *Fusarium* sp., *Curvularia* sp. and *Sclerotium roftsii*) were isolated from infected host plants that showed typical disease symptoms using the tissue transplanting technique. Infected tissues were cut at the infection site and then surface sterilized in 1% NaOCl solution for 2 min. The infected tissues were washed with sterile distilled water twice before being blotted dry on sterilize filter paper. Then, the infected tissues were placed onto water agar (WA) and incubated at 28^oC for 2-3 days. The emerging colonies were subcultured on PDA and incubated at 28^oC for 7 days. The fungi were identified based on conidia morphological characteristics and confirmed by carrying out pathogenicity tests in strict conformity with Koch's postulates.

Primary screening of antagonistic Streptomyces

All isolates of Actinomycete like colonies were initially screened for antagonistic activity against *C. gloeosporioides* using the dual culture method with 90 mm Petri dishes containing 20 ml of PDA medium. One loopful of each isolate of Actinomycete like colonies was streaked on half a PDA plate and incubated at 37^{o}C for 5 days before being placed with the fungal hypha tips (7mm-diameter) from the colony margins of *C. gloeosporioides* on the opposite side at a 30 mm distance. The plate was then incubated at 28^{o}C for 5 days. Each dual culture had three replications. The isolates that inhibited the mycelial growth of the plant pathogenic fungi were selected as candidate antagonistic biocontrol agents for further study.

In vitro antagonistic activity assay

The selected antagonistic biocontrol agents were tested for their antagonistic activity against *C. gloeosporioides*, *C. capsici*, *B. maydis*, *Pyricularia* sp., *Fusarium* sp., *Curvularia* sp. and *S. roftsii* using the dual culture method as described in the initially screening test with the only alteration being that the incubation period of the dual culture plate was changed to seven days at 28^{o}C. The control plate consisted of individual cultures of the pathogen. After seven days, the dual culture plates were evaluated for antagonistic activity that reduced the pathogen colony expansion. The percentage of mycelial growth reduction (PGI) was calculated using the formula:

$$PGI\ (\%) = KR\text{-}R1/KR \times 100,$$

where KR represents the fungal growth radius (mm) of the control culture and R1 represents the fungal growth radius distance (mm) in the direction of the *Streptomyces* growth (ŽivkoviĆ *et al*., 2010). The PGI was categorized from 0 to 4, where 0 = no growth inhibition; 1 = 1-25% growth inhibition; 2 =26-50% growth inhibition; 3 = 51-75% growth inhibition; and 4 = 76-100% growth inhibition. The data from each experiment were analyzed with an analysis of variance, and means were compared by Duncan's Multiple Range Test (DMRT) (at $P = 0.05$).

Effect of culture filtrate on mycelial growth of plant pathogenic fungi

A disk of the mycelium of the selected antagonistic biocontrol agent was inoculated in a 250 mL Erlenmeyer flask containing 100 mL potato dextrose broth (PDB). The flask was incubated for 7 days at 37ºC on a rotary shaker at 150 rpm. The culture filtrate was collected and the cells and spores were removed by centrifugation (Tomy MX-301, Japan) at 9,100 g

for 15 min. The culture filtrate was filtered through a 0.2 µm filter before being diluted with twofold dilutions in PDB medium for a 5 mL total volume. The diluted culture filtrates were incubated with a mycelial disk of the plant pathogenic fungi *C. gloeosporioides* (isolates Cg1 and Cg2) and *B. maydis* (isolates Bm1 and Bm2) for 7 days at 28ºC. The control tube contained 5 mL PDB that was incubated with the individual plant pathogenic fungi. The mycelial growth was categorized on a scale of 0 to 2, where 0 = no mycelial growth, 1 = growth limited around mycelial disk and 2 = mycelia overgrows into liquid medium (Alvindia and Natsuaki, 2008). The experiment was done with three replications.

Morphological characterizations of selected antagonistic biocontrol agent isolate SRF1

The selected antagonistic biocontrol agent isolate SRF1 was culture on various culture media consists of AGMA, Czapek dox agar (CDA) and yeast extract malt extract agar (YEME) at 37ºC and the colony features of the isolate were examined daily for 14 days. The spore chain arrangement of the isolate was prepared using the slide culture method (Cross, 1989). After sporulation, the spore chains were measured under a light microscope.

Salt tolerance and streptomycin resistance tests of selected antagonistic biocontrol agent isolate SRF1

The selected antagonistic biocontrol agent isolate SRF1 was cultured on YEME medium containing 0 to 10% (w/v) NaCl for salt tolerance testing, and cultured on HPDA containing 50 and 100 ppm of streptomycin for streptomycin resistance testing. The inoculated medium was incubated at 37ºC and the emerging colony was examined daily for 14 days.

Molecular identification of the best isolate by 16S rDNA sequence analysis

The selected antagonistic biocontrol agent isolate SRF1 was cultured in PDB medium for 7 days at 37ºC on a rotary shaker at 150 rpm. The cells harvested from the PDB were homogenized with liquid nitrogen in a mortar and pestle before being transferred to a microcentrifuge tube, and then the genomic DNA was extracted using a DNA extraction kit (Vivantis, Malaysia). DNA samples were checked on 1% agarose gel electrophoresis and stored at -20 ^{o}C.

The partial small 16S rDNA gene was amplified using the primers fD1 (5'-AGAGTTTGATCCTGGCTC-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg *et al*., 1991). The temperature profiles for the PCR cycles were as follows: denaturation at

94°C for 5 min, 57°C for 2 min and 72°C for 2 min, which was followed by 29 cycles at 94°C for 2 min, 50°C for 30 sec and 72°C for 2 min, and a final extension reaction at 72°C for 10 min. The amplification was carried out in a Thermal cycler (Applied Biosystems, USA) in 50 µl of polymerase chain reaction (PCR) mixture containing 100 ng of total DNA, 0.1 mM of each dNTP, 2.5 unit of *Tag* DNA polymerase (Invitrogen, USA) in 5 µl of 10x *Tag* buffer, 2 mM $MgCl_2$, 0.2 mM of each primer and the final volume was adjusted with sterile deionized water. The PCR products obtained were purified with a Gel/PCR DNA Fragments extraction kit (Geneaid, USA)**.** Sequencing was performed by Macrogen Advancing through Genomics (Macrogen Inc., Korea). Sequence data of the partial 16S rDNA gene was compared with sequences in the National Center for Biotechnology Information data bank. The novel sequence (accession number KM068791) and 16S rDNA gene sequences of the reference strains obtained from GenBank were aligned using ClustalW (www.genome.jp/tools/clustalw/). Phylogenetic analyses were performed using Mega 4 (Tamura et al., 2007) and a Neighbor Joining tree (NJ tree) was constructed (bootstrap replicates = 1000; seed = 64,238) using the Kimura 2 parameter method for pairwise deletion at uniform rates. *Bacillus subtilis* was used as an outgroup.

Results and Discussion

Isolation and selection of antagonistic Streptomyces

The actinobacteria have been used as a natural source of new antibiotics. Many researchers have focused on the isolation of new species from unexplored ecological niches because they may produce new bioactive compounds (Evangelista-Martínez, 2014). In recent years, soil has been used as the isolation source of new Actinomyces, as in Kim *et al*. (2006) that isolated a novel *S. cheonanensis* sp. nov. with antifungal activity from a soil sample collected from Cheonan, Korea. In this study, a total of 35 isolates of Actinomycete like colonies were isolated from four soil samples collected from the northeast of Thailand. The aerial mycelium observed was in the color range of white, grey, brown and black. All the isolates were evaluated for their *in vitro* antagonistic activity against the plant pathogenic fungi *C. gloeosporioides*. Seven isolates of the Actinomycete like colonies consisting of four isolates from soil planted with mango (SM1, SM5, SM6 and SM8) and three isolates from soil planted with bananas (SB8), soil planted with sentol (SS1) and a rice field (SRF1) showed inhibition zones of at least 15 mm and were selected for further studies.

In vitro antagonistic activity assay

Seven selected Actinomycete like colonies were assayed against the plant pathogenic fungi *C. gloeosporioides* isolates Cg1 and Cg2 and *B. maydis* isolates Bm1 and Bm2. The results showed that all the selected isolates inhibited the growth of the tested fungi in the range 70.4-100.0%. The selected Actinomycete like colonies isolate SRF1 showed the highest percentage of mycelial growth reduction in the tested fungi, which was significantly greater than with the other isolates (Table 1). The inhibition zone produced by the isolate SRF1 varied from 28-30 mm (Fig. 1). Therefore, the isolate SRF1 was chosen for further evaluation of the antagonistic activity against *C. capsici*, *Pyricularia* sp., *Fusarium* sp., *Curvularia* sp. and *S. roftsii*. The results showed that isolate SRF1 inhibited the mycelial growth of *C. capsici*, *Pyricularia* sp., *Fusarium* sp. and *Curvularia* sp. in the range of 65.5-91.6%, while there was no effect on the mycelial growth of *S. roftsii* (Table 2). These findings correlate with those of Alam *et al*. (2012) who found that *Streptomyces* sp. CIMAP-A1 showed the greatest antagonistic activity against a wide spectrum of plant pathogenic fungi such as *Stemphylium* sp., *B. cinerea*, *Colletotrichum* spp. and *Curvularia* spp., while weak antagonistic activity was observed against *R. solani*. Evangelista-Martínez (2014) found that the percentage of fungal growth inhibition of *Alternaria* sp., *Fusarium* sp., *P. capsici* and *R. solani* by *Streptomyces* sp., CACIS-1.16A ranged from 47.6% to 61.5%. Therefore, the antifungal activity of *Streptomyces* may be dependent on the strain of antagonistic *Streptomyces* and plant pathogenic fungi that are tested.

Table 1. Antifungal activity of seven isolates of selected Actinomycete like colonies against the plant pathogenic fungi *C. gloeosporioides* and *B. maydis*

Isolate	Radial growth inhibition (%)*			
	C. gloeosporioides		*B. maydis*	
	Isolate cg1	Isolate Cg2	Isolate Bm1	Isolate Bm2
SM1	88.15 b	88.89 b	95.55 a	98.42 a
SM5	76.30 cd	77.78 d	77.78 d	80.70 d
SM6	77.78 c	74.07 e	91.11 b	77.19 e
SM8	100.00 a	100.00 a	98.22 a	94.74 b
SS1	77.04 cd	84.44 c	82.22 c	89.29 c
SB8	87.41 b	70.37 f	82.22 c	80.70 d
SRF1	100.00 a	100.00 a	99.11 a	99.29 a

*Values labeled with the same letter in a column are not significantly different according to Duncan's multiple range test at P=0.05.

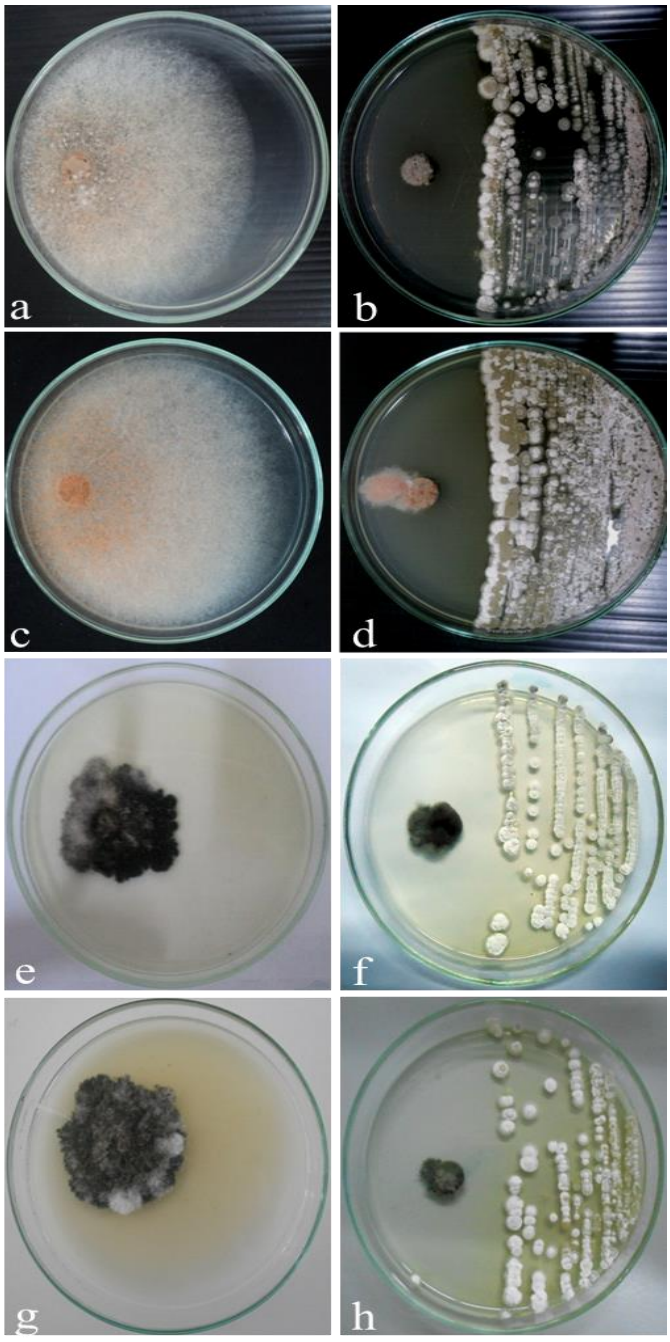

Figure 1. Antifungal activities of the Actinomycete like colonies isolate SRF1 against *C. gloeosporioides* isolate Cg1 (b) and Cg2 (d) and *B. maydis* isolate Bm1 (f) and Bm2 (h) by the dual culture method as well as a comparison with the individually cultured pathogens (a, c, e and g).

Table 2. Antifungal activity of the selected Actinomycete like colonies isolate SRF1 against plant pathogenic fungi

Plant pathogenic fungi	Host plant	Radial growth inhibition (%)	PGI category*
C. capsici	Chili	82.20	4
Pyricularia sp.	Rice	66.66	3
Fusarium sp.	Rice	91.66	4
Curvularia sp.	Rice	65.00	3
S. roftsii	Tomato	0.00	0

*The PGI was categorized from 0 to 4, where 0 = no growth inhibition; 1 = 1-25% growth inhibition; 2 =26-50% growth inhibition; 3 = 51-75% growth inhibition; and 4 = 76-100% growth inhibition

The important antifungal substances from *Streptomyces* spp. are the extracellular hydrolytic enzymes and antifungal compounds, such as antibiotics (Trejo-Estrada *et al*., 1998; Ouhdouch *et al*., 2001; Alam *et al*., 2012). These compounds are ingredients in cell-free culture filtrate that have been reported to inhibit the fungal mycelial growth and spore germination (Prapagdee *et al*., 2008; Alam *et al*., 2012). In this study, the cell-free culture filtrate of the isolate SRF1 inhibited the fungal mycelial growth of the plant pathogenic fungi *C. gloeosporioides* isolates Cg1 and Cg2 and *B. maydis* isolates Bm1 and Bm2 with varying sensitivities. The culture filtrate completely controlled the growth of the *C. gloeosporioides* isolates Cg1 and Cg2 at the dilution of 1:1, and completely controlled the growth of the *B. maydis* isolate Bm2 at the dilutions 1:1 and 1:2, whereas the culture filtrate showed partial effects on the growth of *B. maydis* isolate Bm1 at the dilutions 1:1 to 1:4 (Table 3). These findings correlate with those of Alam *et al*. (2012) who found that the inhibitory effect of the same concentration of culture filtrate of *Streptomyces* sp. CIMAP-A1 was invariable higher on *C. acutatum* (100%) and *C. andropogonis* (80-82%), whereas lower on *F. moniliforme* (30-32%). Soares *et al*. (2006) found that a high concentration of culture filtrate from *Streptomyces* sp. isolate AC26 gave a greater inhibition of the mycelial growth of *C. eragrostides* than a low concentration. Therefore, the evidence from the dual culture method and cell-free culture filtrate assay strongly suggest that the isolate SRF1 could be considered as a potential biocontrol agent to prevent or reduce plant diseases caused by plant pathogenic fungi.

Table 3. Antifungal activity of dilutions of the culture filtrate from the selected Actinomycete like colonies isolate SRF1 against the plant pathogenic fungi *C. gloeosporioides* and *B. maydis.*

Plant pathogenic fungi	Mycelium inhibition category*				
	1:1	1:2	1:4	1:6	Control
C. gloeosporioides Cg1	0	1	2	2	2
C. gloeosporioides Cg2	0	1	2	2	2
B. maydis Bm1	1	1	1	2	2
B. maydis Bm2	0	0	2	2	2

*0 = no mycelial growth, 1 = growth limited around mycelial disk and 2 = mycelia overgrows into liquid medium

Morphological and physiological properties as well as 16S rDNA sequence analysis of selected antagonistic biocontrol agent isolate SRF1

The growth of the *Streptomyces* sp. isolate SRF1 on three media was investigated. The results showed that the growth was good on the tested media, except for the CDA medium. The color of the aerial mycelium and substrate mycelium of the isolate was brown on the AGMA medium and white on the other tested media (Fig. 2). The spiral spore chain arrangement of the isolate was observed under a light microscope (Fig. 2). The isolate did not produce soluble pigments on the tested medium. The isolate could grow on YEME medium containing NaCl ranging from 1 to 7% at 37ºC. The isolate was sensitive to the antibiotic streptomycin.

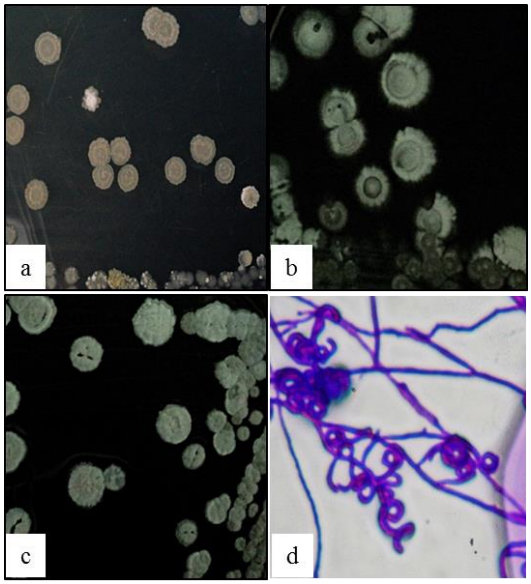

Figure 2. Colonies of the selected Actinomycete like colonies isolate SRF1 on AGMA (a), CDA (b) and YEME (c) and spiral spore chain arrangement observed at the magnification of 1000× using light microscope.

Identification of the bacterial isolates to the species level should be done so the potential strain can be identified. PCR techniques based on a particular genome such as the 16S rDNA gene are a reliable tool for the molecular taxonomy and identification of many bacteria (Janda and Abbott, 2007; Woo *et al*., 2008). In this study, the 16S rDNA gene analysis of the selected antagonistic isolate SRF1 was amplified using the universal primers fD1 and rP2. The PCR product of the isolate was about 1.5 kb which was shown to be part of the 16S rDNA gene once it was purified and sequenced. The sequences obtained were analyzed and compared with other species/strains of *Streptomyces* found in databases when performing a BLAST analysis. A fragment of the 16S rDNA gene consisting of 1,058 nucleotides was submitted to GenBank. A BLAST search in NCBI (www.ncbi.nih.gov/blast) showed that this sequence was very similar to many species of *Streptomyces* with 99% homology to *S. hygroscopicus* subsp. *hygroscopicus* (HQ244447), *S. endus* (AB564290), *S. sporocinereus* (FJ406115) and *S. demainii* (NR043723) as well as having 98% homology with *S. yogyakartensis* (NR117958) and *S. violaceusniger* (HQ244446). A phylogenetic tree was generated from 16 aligned sequences with similar characters that indicated the antagonistic isolate SRF1 was located in the same clade as the *Streptomyces* species (Fig. 3). Based on the molecular data from the 16S rDNA gene sequence, the bacterial morphology and physiology, we suggest that the isolate SRF1 belonged to the genus *Streptomyces*, which should be referred to as *Streptomyces* sp. isolate SRF1. However, we were unable to identify the isolate to the species level without further investigations.

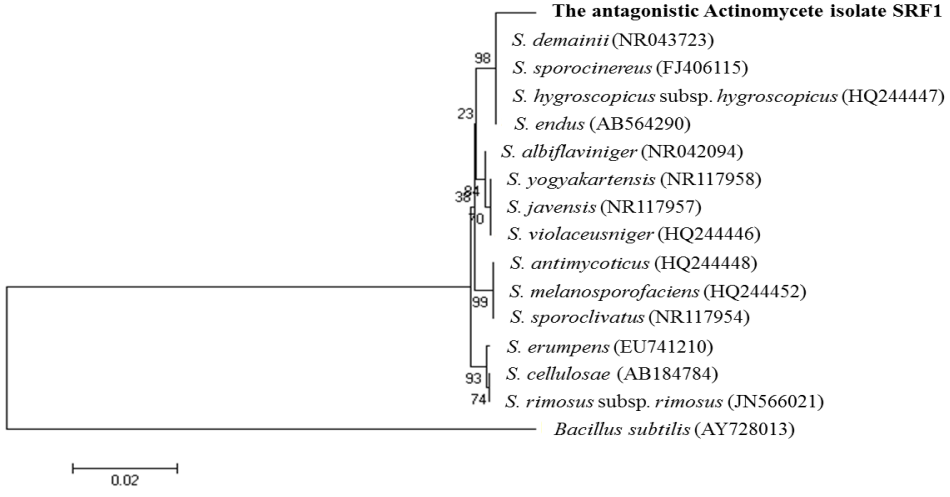

Figure 3. Phylogenetic relationship of the antagonistic Actinomycete isolate SRF1 and the 14 related *Streptomyces* species based on partial 16S rDNA gene sequences. The Neighbor Joining (NJ) tree was constructed using Mega 4. The percentages expressed above the branches are frequencies with which a given branch appeared in 1000 bootstrap replications when using the NJ method.

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