CHAPTER 4

Antifungal Activity of Eleven Endophytic Actinomycetes and the Selection of Effective Isolates

4.1 Introduction

Actinomycetes, belong to phylum of Actinobacteria, is group of filamentous gram positive bacteria. This phylum contains diverse groups of endophytic actinomycetes providing the verity of secondary metabolites. Some bioactive compounds have been reported to suppress the growth of pathogenic microorganisms including plant pathogens and to treat human diseases (Zin *et al*., 2007; Chaudhary *et al*., 2013). In addition, some of endophytic strains, group of *Streptomyces* in particularly, have ability to produce phytohormone, nitrogen fixation, antibiotic, siderophores, nutrient competition, and also induce of systemic disease resistance (Hallmann *et al*., 1997; Hasegawa *et al*., 2006; Rosenblueth and Martínez-Romero, 2006).

In this Chapter, the selected endophytic actinomycetes, eleven isolates, were investigated the production of metabolic secretes and antifungal metabolites, and the inhibitory activity of their antifungal metabolites on the pathogenic fungi *P. aphanidermatum*, *R. solani*, and *S. rolfsii* as the tested fungi.

The objectives of this chapter were as follows:

1. To investigate the production of hydrolytic enzymes of eleven selected isolates including GAR1, KAE1, HOU2, NEE1, COF1, COF4, COF6, ERY1, POL2, PRE5 and SOL1
2. To investigate the production of antifungal metabolites of eleven selected isolates on damping-off pathogenic fungi
3. To select the most effective endophytic actinomycetes on controlling damping-off pathogenic fungi

4.2 Materials and Methods

4.2.1 Antifungal metabolites

The antifungal activity of the selected endophytic actinomycetes was determined on constrain mycelial growth of tested fungi using double layer method, a modified method of Williams *et al*. (1989). Spore suspension, 10 μl (10^7 spores/ml) of each isolate, was inoculated using spot-inoculation method onto plates (9 cm) containing International Streptomyces Project-2 (ISP-2) agar and incubated at 30°C for 7 days. Control treatment was spotted by distilled water without the spore suspension. After incubation, growing colony and the surrounding agar (1.5 × 1.5 cm^2) was removed and refilled with 5 ml of semi-solid PDA overlaid onto the plate. A culture disc (5 × 5 mm) of *P. aphanidermatum*, *R. solani*, and *S. rolfsii*, was then separately cultured on the set agar, at the removal position, and incubated in the dark at room temperature 26 ± 4ºC (Figure 4.1). Fungal colony diameter was measured when the untreated control plate fully developed (Figure 4.2). Physical malformations of fungal mycelium were observed under light compound microscope. This experiment was conducted with four replications and repeated twice. The inhibition percentage was calculated by reference to the growth of the untreated control using the following formula:

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{Colony diameter in treatment}}{\text{Colony diameter in control}}\right) \times 100$$

The data were analyzed by SPSS version 16.0 using one-way ANOVA.

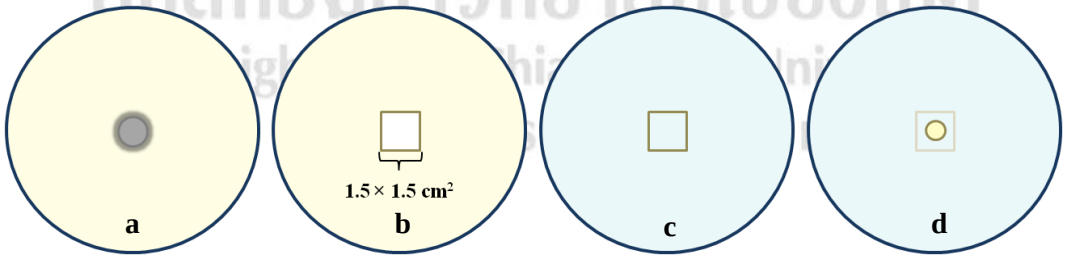

Figure 4.1 Double layer method was performed to determine the production of antifungal metabolites of the selected isolates.
(a) the spotted colony of selected isolate after incubation for 7 days
(b) the spotted colony is cut and removed
(c) refilled with semi-solid PDA
(d) a culture disc of the tested fungi is cultured on the set agar

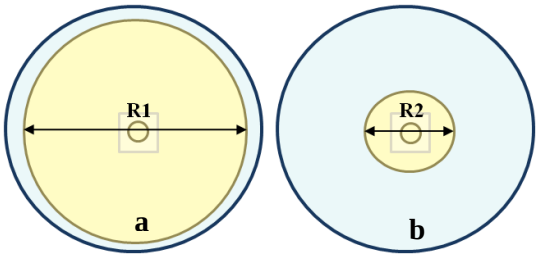

Figure 4.2 The inhibition percentage of the treated fungi is calculated comparing between
(a) the radial growth diameter of untreated control (R1) when grow fully Petri dish
(b) the radial growth diameter of treated fungi (R2)

4.2.2 Physiological studies

Eleven endophytic actinomycetes isolates were determined their physiological characteristics: the production of cellulase and phosphatase enzymes. Cellulase activity was investigated using carboxymethyl cellulose agar (CMC) medium and phosphatase activity was investigated using Czapek solution agar. Spore suspension, 10 µl (10^7 spores/ml) of each isolate, was sported on the center of the tested media in the Petri dishes and incubated in the dark at 30ºC. Cellulase activity of the isolates was determined after incubation for 7 days. The cultured medium was dyed with 0.1% Congo red for 5 minutes, washed 2 times with 1N NaCl, and measured diameter (cm) of the occurrence clear zone. Phosphatase activity was determined after incubation for 14 days by measuring diameter (cm) of the occurrence clear zone.

4.2.3 Morphological studies and identification of the selected endophytic actinomycetes

The selected isolates were preliminary characterized their growth behavior by culturing on International Streptomyces Project (ISP) according to Bergey's manual of systematic bacteriology (Williams *et al*., 1989). Single colony of the selected isolates was cultured on IMA-2, and a pure isolate was further cultured on ISP media incubated in the dark at 30ºC for 7-10 days. The color of aerial mycelium, substrate mycelium and diffusible pigment production of the isolates on each cultured medium were visually assessed by using Manual of Color Name (Kenkyojo, 1987).

The taxonomic positions of the selected isolates were identified using 16S rRNA gene sequencing. Single colony of the selected endophytic actinomycetes isolates were cultured on IMA-2 medium, incubated in the dark at 30ºC for 7 days and their spore mass were collected in centrifuge tube (1 ml). TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) 100-500 μL was added into the vial tube that contained selected spore mass. The tubes were centrifuge at 10000-12000 rpm for 10 minute and the supernatant was collected and used as DNA template. Fragment amplification of 16S rRNA gene of the selected isolate was carried out by using two primers: 11F, 350F, 785F, 1099F and 11F, 350F, 685F, 1099F, 1542R, at 30 PCR cycles. The genome sequencing was compared to the GenBank, EMBL, DDBJ, PDB database by program BLASTN 2.2.27+.

4.3 Results

4.3.1 The production of hydrolytic enzymes

Eleven selected endophytic actinomycetes isolates were evaluated their ability to produce cellulase and phosphatase enzymes. The activity of hydrolytic enzymes (cellulase/phosphatase) is shown in Table 4.1. The results showed that 3 isolates, COF6, ERY1 and POL2, produced cellulase enzyme (Figure 4.3); and 4 isolates, COF6, ERY1, NEE1 and POL2, produced phosphatase enzyme (Figure 4.4).

Table 4.1 Clear zone in diameter of cellulase and phosphatase enzymes produced by the isolates after cultured in carboxymethyl cellulose agar and Czapek's solution for 7 and 14 days, respectively

isolates	**Clear zone (cm)**	
	cellulase enzyme	**phosphatase enzyme**
COF6	3.20	1.67
ERY1	3.62	1.48
POL2	3.92	1.62
NEE1	0	1.12

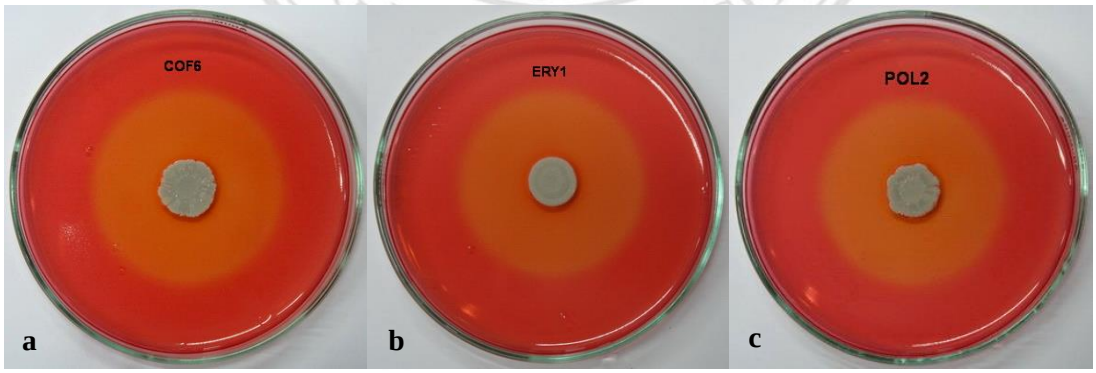

Figure 4.3 The production of enzymatic cellulose hydrolysis on carboxymethyl cellulose agar medium after incubation for 7 days of the isolates
(a) COF6 (b) ERY1 (c) POL2

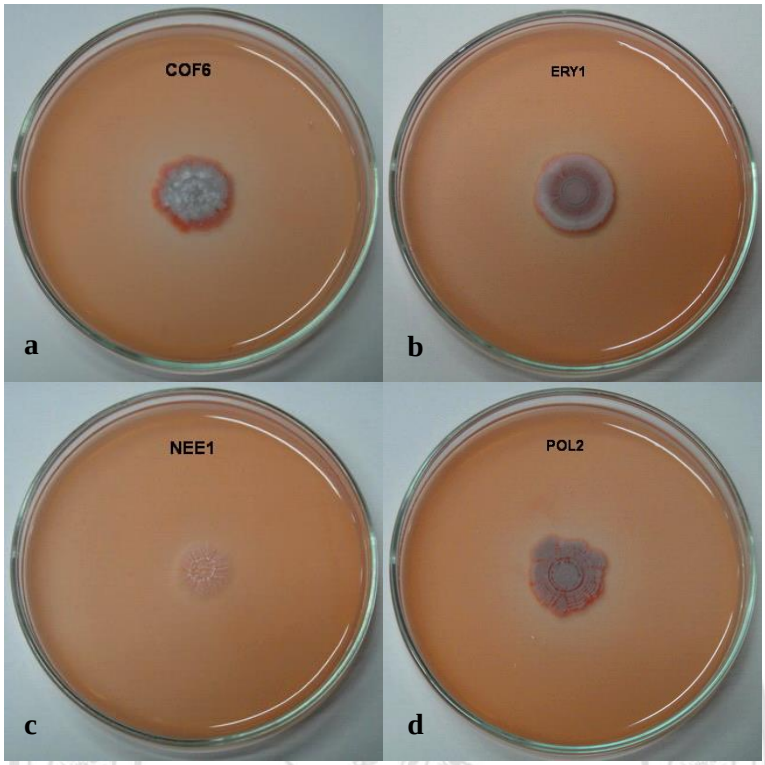

Figure 4.4 The production of enzymatic phosphate hydrolysis on Czapek solution agar after incubation for 14 days of the isolates
(a) COF6 (b) ERY1
(c) NEE1 (d) POL2

4.3.2 Antifungal activity

The inhibition percentage of antifungal metabolites produced by eleven selected endophytic actinomycetes on the growth of damping-off pathogenic fungi is shown in Table 4.2. The results revealed that each isolate exhibited different inhibitory activities on each test pathogenic fungi.

Table 4.2 Antifungal activity of the eleven endophytic actinomycete isolates on the growth of damping-off pathogenic fungi after incubation for 3 days

Isolates	[1]Inhibition percentage		
	Pythium aphanidermatum	***Rhizoctonia solani***	***Sclerotium rolfsii***
GAR1	75.84 BC[2]	54.59 A	100 A
KAE1	79.03 B	56.80 A	100 A
HOU2	0 E	0 C	6.33 D
NEE1	77.50 B	55.83 A	100 A
COF1	25.42 D	15.14 B	100 A
COF4	0 E	20.00 B	11.16 C
COF6	0 E	0 C	0 E
ERY1	84.31 A	0 C	62.34 B
POL2	72.92 C	58.05 A	100 A
PRE5	78.47 B	60.14 A	100 A
SOL1	77.92 B	57.22 A	100 A
$LSD_{0.01}$	3.9587	8.1678	1.8862

[1] Inhibition percentage was calculated by referent to the growth of control treatment.
[2] The same letter in the same column are not significantly different at $LSD_{0.01}$.

1) *Pythium aphanidermatum*

P. aphanidermatum was strongly inhibited by isolate ERY1 at 84.31% followed by KAE1 and PRE5 at 79.03% and 78.47%, respectively (Table 4.2). The fungus showed abnormal growth: mycelial tip compassed and colony growth as flat surface on medium compared with untreated control (Figure 4.5 and Figure 4.6).

In the case of isolate ERY1, the antifungal metabolites produced by the isolate had the highest inhibitory activity on the growth of *P. aphanidermatum*. The fungus showed a very small growth and no fluffy mycelia when compared with control treatment. The fungus did not produce sporangium or any other reproduction and also showed the aggregation of mycelial protoplasm under light compound microscope (Figure 4.6). In addition to isolate ERY1, the production of sporangia of *P. aphanidermatum* was observed when treated with isolate PRE5, but the sporangium showed the lysis of sporangial membrane, the swollen mycelia and the aggregated protoplasm (Figure 4.6). In addition, all treatments were further incubated for 15 days and we found that the antifungal metabolites from the effective isolates still showed strong inhibitory activities.

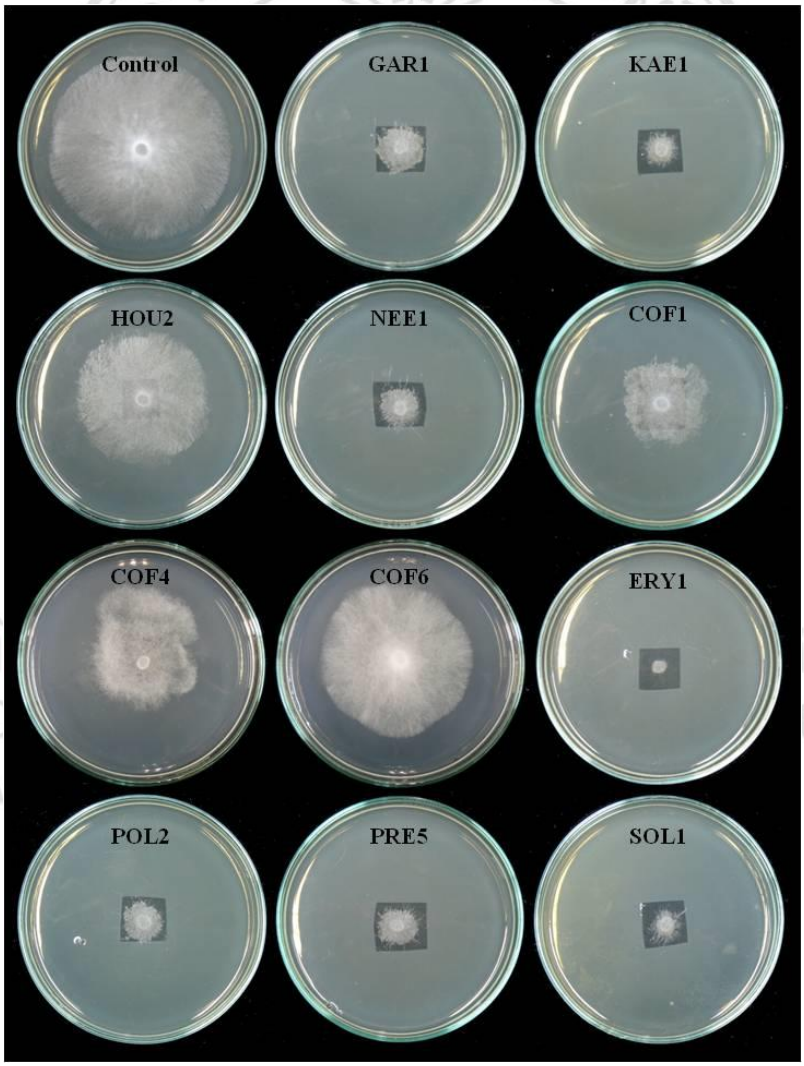

Figure 4.5 Inhibitory activity of antifungal metabolites produced by eleven isolates of endophytic actinomycetes on the growth of *Pythium aphanidermatum* compared with control treatment after incubation for 1 day

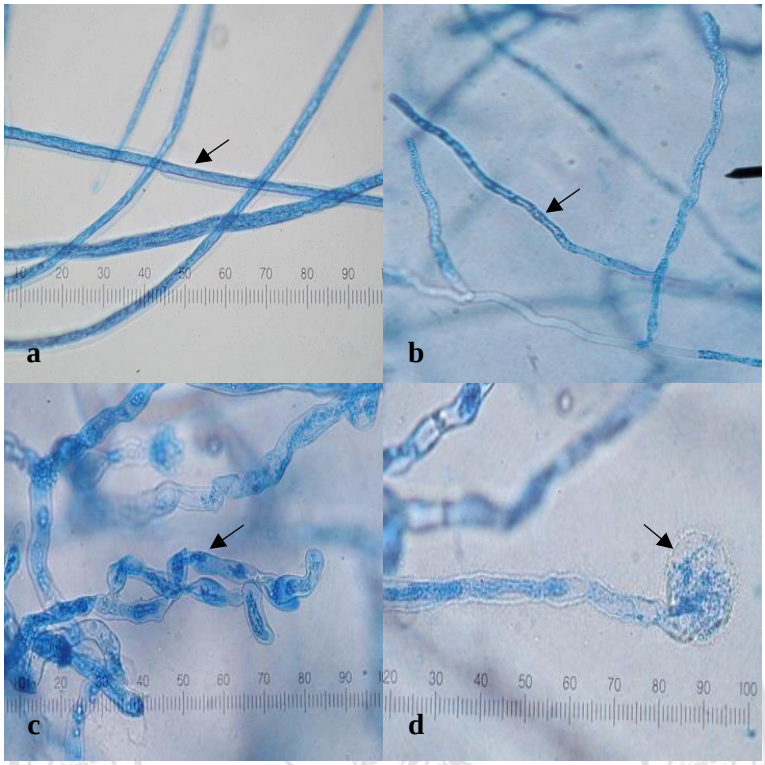

Figure 4.6 Mycelial growth of *Pythium aphanidermatum*
(a) control treatment
(b) mycelial protoplasm aggregation after treatment with antifungal metabolites produced by isolates ERY1
(c) swollen mycelia and aggregated protoplasm after treatment with antifungal metabolites produced by isolates PRE5
(d) cell lysis of sporangial membrane

2) *Rhizoctonia solani*

R. solani was strongly inhibited by isolate PRE5 at 60.14% followed by POL2 and SOL1 at 58.05% and 57.22%, respectively (Table 4.2). *R. solani* was insensitive to the antifungal metabolites when compared to *P. aphanidermatum* and *S. rolfsii*. The fungus showed abnormal growth behavior, such as colony cluster, hyphal tip aggregated and penetrated into the medium (Figure 4.7 and Figure 4.8).

In the case of isolate PRE5, the antifungal metabolites produced by the isolate caused a thick of mycelial membrane and the frequency branching of mycelia (Figure 4.8). Besides, the abnormal growth of mycelia treated with the antifungal metabolites produced by isolate POL2 and SOL1 showed like a tie of mycelia (Figure 4.8). In addition, only little increase of the fungal growth was observed after incubation for 15 days, in contrast to control treatment.

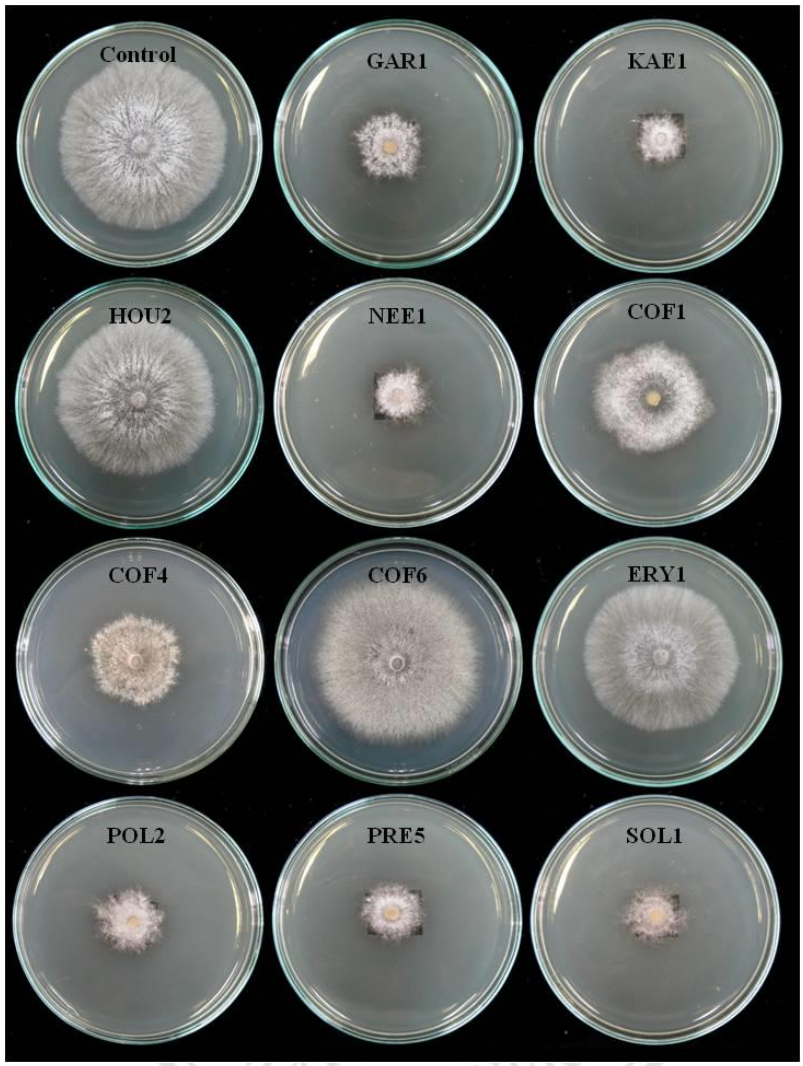

Figure 4.7 Inhibitory activity of antifungal metabolites produced by eleven isolates of endophytic actinomycetes on the growth of *Rhizoctonia solani* compared with control treatment after incubation for 2 days

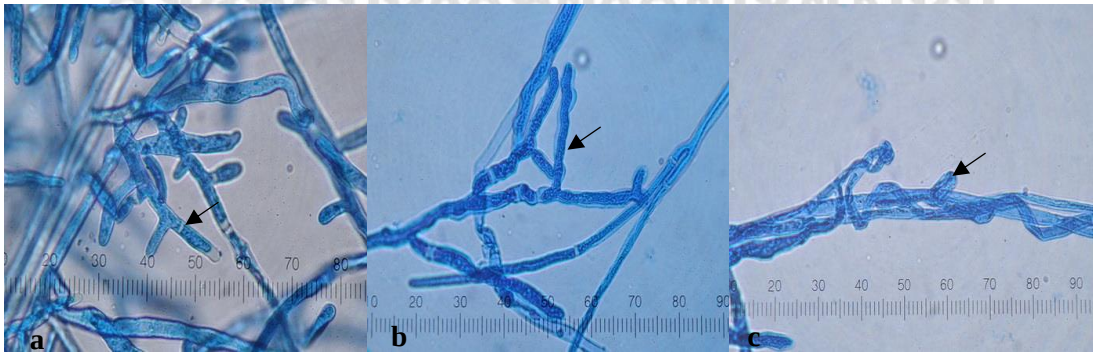

Figure 4.8 Mycelial growth of *Rhizoctonia solani*
(a) control treatment
(b) a thick of mycelial membrane after treatment with antifungal metabolites produced by isolates PRE5
(c) mycelial tie after treatment with antifungal metabolites produced by isolates POL2 and SOL1

3) *Sclerotium rolfsii*

S. rolfsii was completely inhibited by isolates GAR1, KAR1, NEE1, COF1, POL2, PRE5 and SOL1 at 100%, followed by isolate ERY1 at 62.34% (Table 4.2 and Figure 4.9). These results indicated that *S. rolfsii* was the most sensitive to the antifungal metabolites compared to *P. aphanidermatum* and *R. solfsii*. In the case of completely inhibition, we found that the mycelia of *S. rolfsii* could not touch the medium that contained antifungal metabolites, the mycelial growth was found only on its culture disc (Figure 4.10).

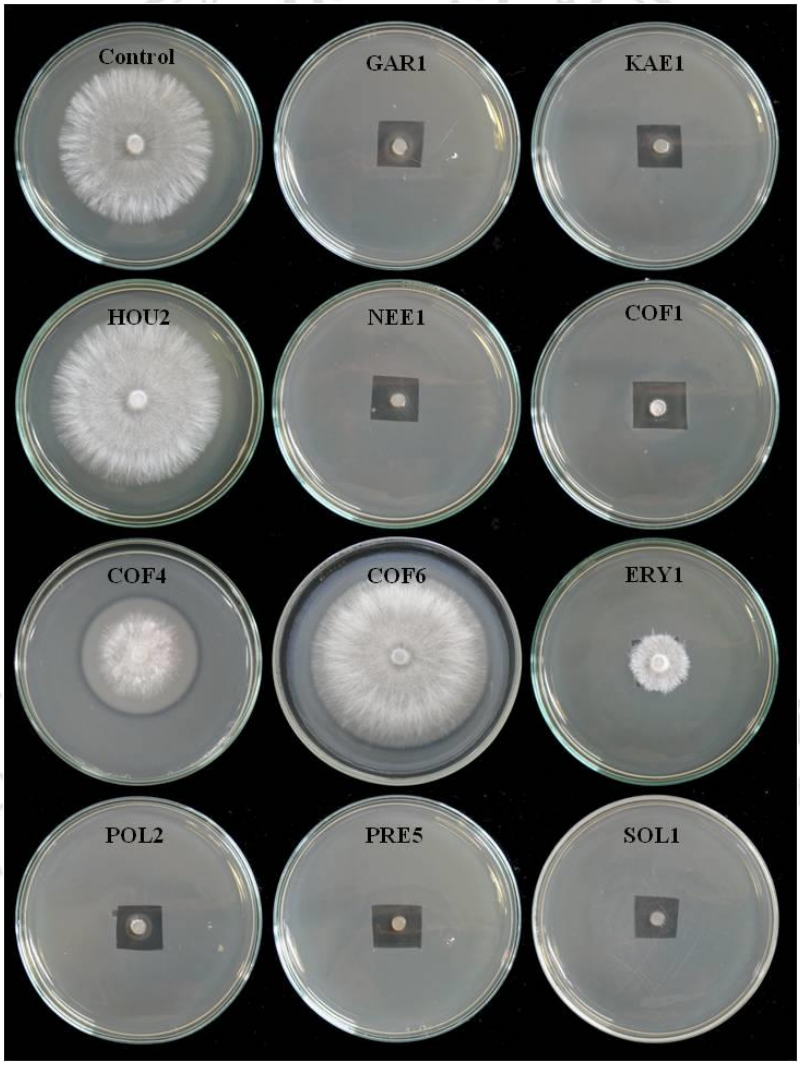

Figure 4.9 Inhibitory activity of antifungal metabolites produced by eleven isolates of endophytic actinomycetes on the growth of *Sclerotium rolfsii* compared with control treatment after incubation for 3 days

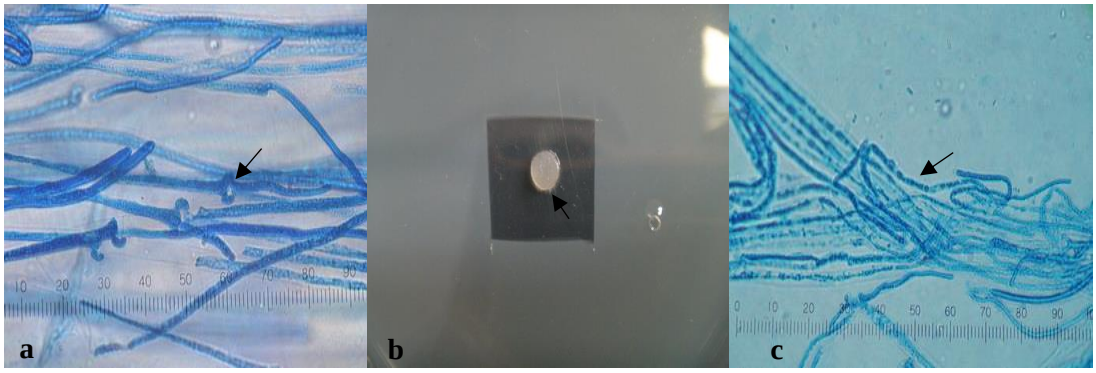

Figure 4.10 Mycelial growth of *Sclerotium rolfsii*
(a) control treatment
(b) a slight mycelial growth on its culture disc after treatment with antifungal metabolites produced by isolates PRE5
(c) mycelial protoplasm aggregation after treatment with antifungal metabolites produced by isolates ERY1

The results of antifungal activity showed that all selected isolates could produce antifungal metabolites and released into the medium in the Petri dishes. The isolates had different inhibitory activities on growth of the tested fungi, and the same isolate showed different inhibitory activities on the growth of *P. aphanidermatum*, *R. solani* and *S. rolfsii*. These may be involved in the capability to produce various types of antifungal metabolites. Data in this experiment led us to select the most effective isolates that were isolates ERY1 and PRE5 for the next studies. Thus, the isolates were studied their morphological characterization and identification as described below.

4.3.3 The morphology characterization and identification of the selected isolates

1) Isolate ERY1

Isolate ERY1 showed the aerial mycelia in gray, substrate mycelia in yellowish brown and no pigment was observed (Table 4.3 and Figure 4.11). Spore chain morphology of isolate ERY1 was rectiflexibiles type (Figure 4.12). Gram-staining of isolate ERY1 was gram-positive.

Table 4.3 The growth and cultural characteristic of *Streptomyces rochei* ERY1 on the various media

Media	***Growth**	**Color of:**		**Diffusible pigment production**
		Aerial mycelium	**Substrate mycelium**	
Yeast extract-malt extract agar (ISP-2)	++	Purplish white	Yellowish brown	None
Oatmeal agar (ISP-3)	+++	Brownish gray	Olive gray	None
Inorganic salts-starch agar (ISP-4)	++++	Grayish white	Yellowish white	None
Glycerol-asparagine agar (ISP-5)	+++	Light gray	Yellowish gray	None
Peptone-yeast extract-iron agar (ISP-6)	++	Yellowish white	Light reddish yellow	None
Tyrosine agar (ISP-7)	+++	Light gray	Yellowish brown	None
Potato Dextrose Agar (PDA)	++	Grayish white	Yellowish brown	None
Inhibitory Mold Agar-2 (IMA-2)	++++	Light gray	Soft greenish yellow	None

* +; sparse growth, ++; poor growth, +++; moderate growth, ++++; abundant

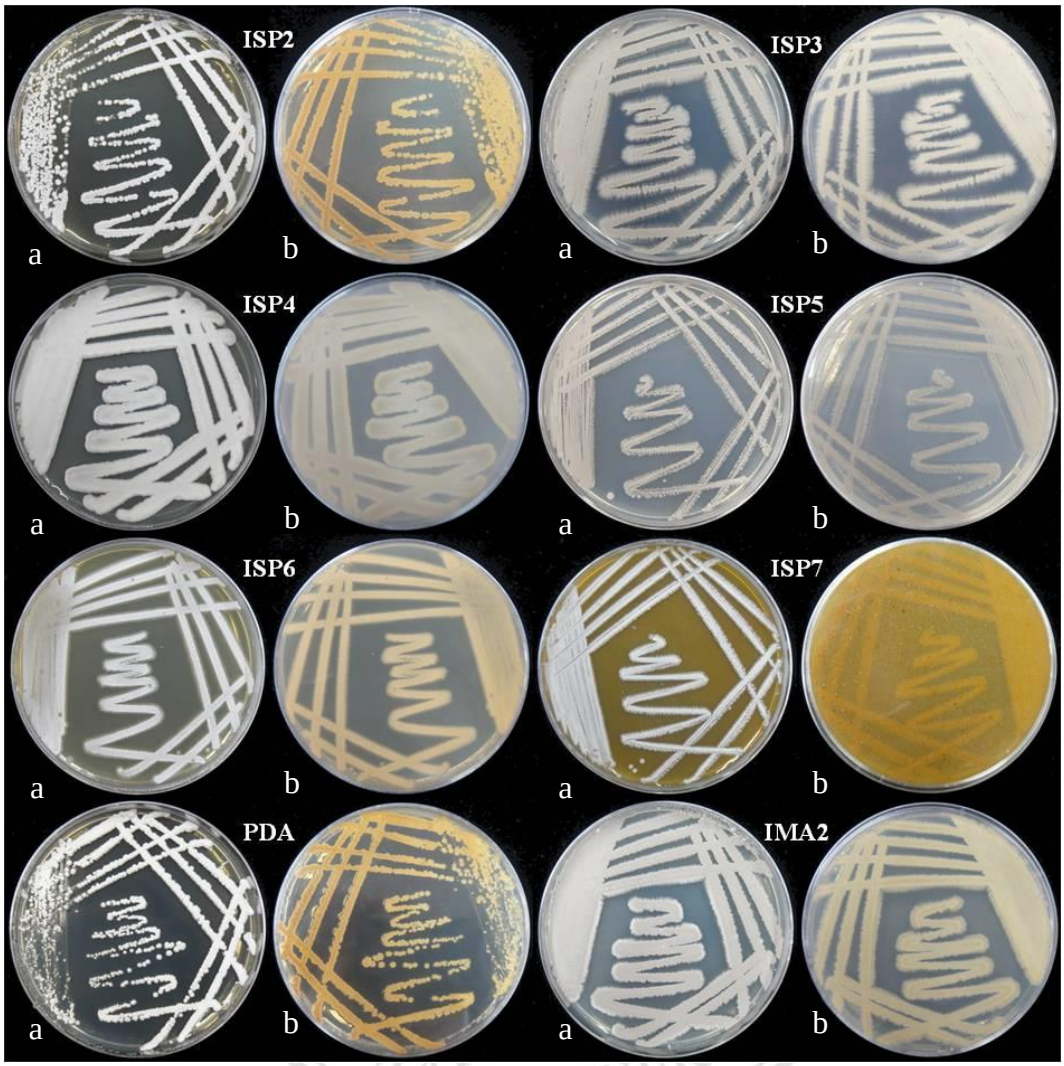

Figure 4.11 Cultural characteristics of *Streptomyces rochei* ERY1 on the various media
(a) on the surface of IMA2 medium in Petri dish
(b) under Petri dish after incubation in the dark at 30ºC for 7 days

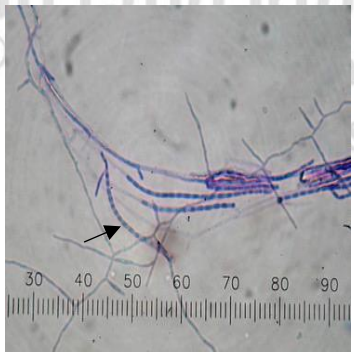

Figure 4.12 Rectiflexibiles type of spore chain of *Streptomyces rochei* ERY under light compound microscope at 1,000 magnification

The taxonomic position of the isolates was analyzed by 16S rRNA gene sequence analysis. Comparison of the sequencing with the database (GenBank, EMBL, DDBJ, PDB sequences) indicated that the isolate ERY1 was *Streptomyces rochei* isolate A-1 with 99% similarity (Table 4.4).

Table 4.4 The similarity percentage of 16S rRNA gene sequence of endophytic actinomycete isolate ERY1 compared with the database

Accession	**Description**	**Total score**	**Query coverage**	**E value**	**Max ident**
GQ392058.1	*Streptomyces rochei* strain A-1 16S ribosomal RNA gene, partial sequence	2658	99%	0.0	99%
NR_041091.1	*Streptomyces rochei* strain NBRC 12908 16S ribosomal RNA, partial sequence >dbj|AB184237.1| *Streptomyces rochei* gene for 16S rRNA, partial sequence, strain: NBRC 12908	2612	97%	0.0	99%
AF503493.1	*Streptomyces tuirus* 16S ribosomal RNA, partial sequence	2608	99%	0.0	99%
AB184156.1	*Streptomyces mutabilis* gene for 16S rRNA, partial sequence, strain: NBRC 12800	2606	98%	0.0	99%
JN128892.1	*Streptomyces rochei* strain SM3 16S ribosomal RNA gene, partial sequence	2604	97%	0.0	99%
AF233338.1	*Streptomyces maritimus* 16S ribosomal RNA gene, partial sequence	2595	97%	0.0	99%
EU570372.1	*Streptomyces rochei* strain 173315 16S ribosomal RNA gene, partial sequence	2591	97%	0.0	99%

2) Isolate PRE5

Isolate PRE5 showed aerial mycelia in white, substrate mycelia in light yellow and no pigmentation was observed (Table 4.5 and Figure 4.13). Spore chain morphology of isolate PRE5 was spiral type (Figure 4.14). Gram-staining of isolate PRE5 was gram-positive.

Table 4.5 The growth and cultural characteristic of *Streptomyces albus* subsp. *albus* PRE5 on the various media

Media	***Growth**	**Color of:**		**Diffusible pigment production**
		Aerial mycelium	**Substrate mycelium**	
Yeast extract-malt extract agar (ISP-2)	++++	White	Dull yellow	None
Oatmeal agar (ISP-3)	+++	White	Yellowish white	None
Inorganic salts-starch agar (ISP-4)	+	White	White	None
Glycerol-asparagine agar (ISP-5)	+	White	White	None
Peptone-yeast extract-iron agar (ISP-6)	++++	White	Soft reddish yellow	None
Tyrosine agar (ISP-7)	+	White	White	None
Potato Dextrose Agar (PDA)	++++	White	Brownish gold	None
Inhibitory Mold Agar-2 (IMA-2)	++++	White	Light yellow	None

* +; sparse growth, ++; poor growth, +++; moderate growth, ++++; abundant

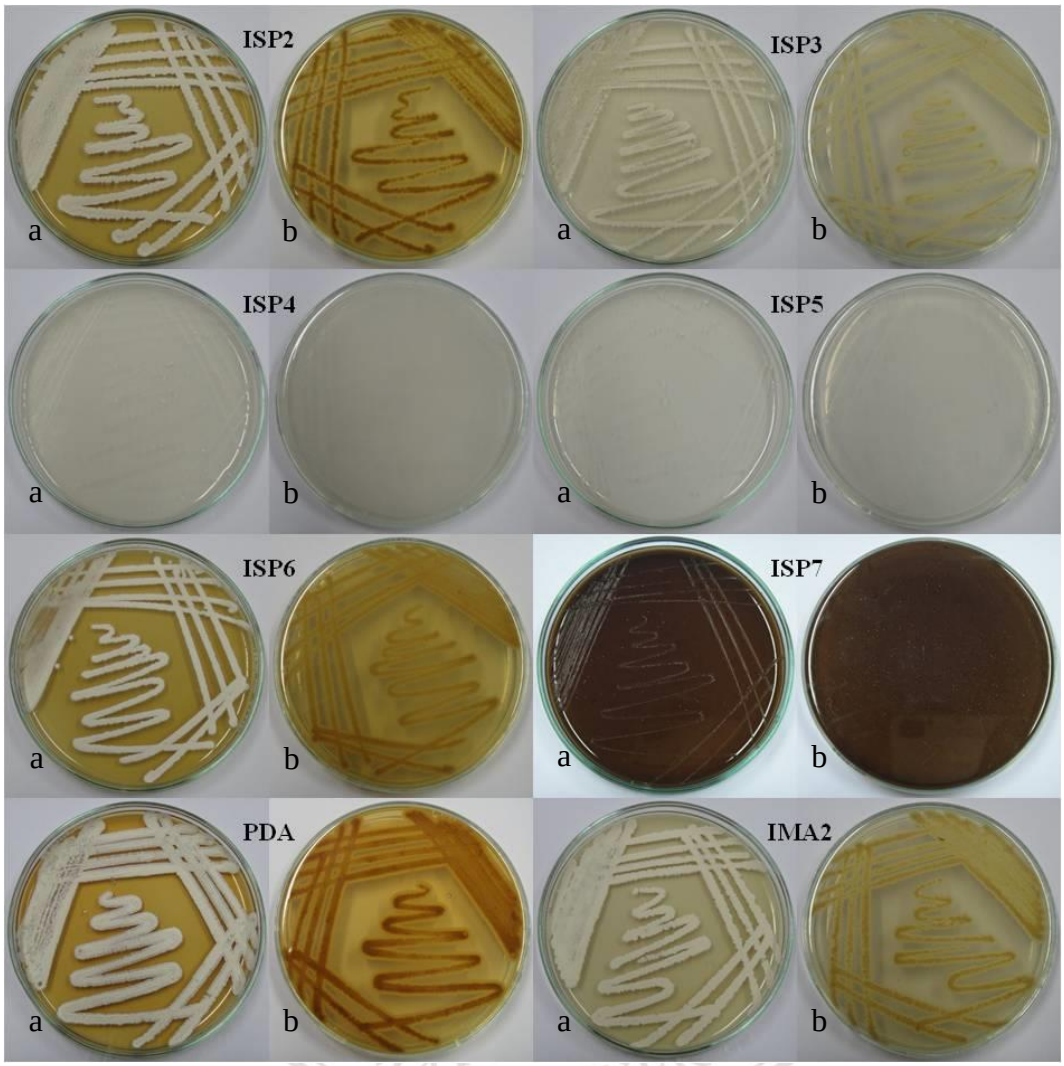

Figure 4.13 Cultural characteristics of *Streptomyces albus* subsp. *albus* PRE5 on the various media
(a) on the surface of IMA2 medium in Petri dish
(b) under Petri dish after incubation in the dark at 30ºC for 7 days

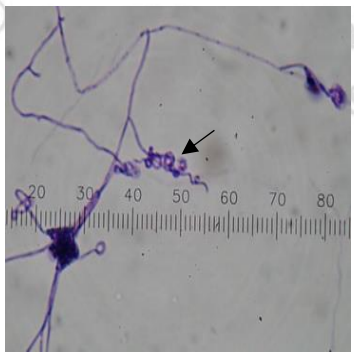

Figure 4.14 Spiral type of spore chain of *Streptomyces albus* subsp. *albus* PRE5 under light compound microscope at 1,000 magnification

The taxonomic position of the isolates was analyzed by 16S rRNA gene sequence analysis. Comparison of the sequencing with the database (GenBank, EMBL, DDBJ, PDB sequences) indicated that the isolate PRE5 was *Streptomyces albus* subsp. *albus* isolate DSM 40313^{T} with 99% similarity (Table 4.6).

Table 4.6 The similarity percentage of 16S rRNA gene sequence of endophytic actinomycete isolate PRE5 compared with the database

Accession	**Description**	**Total score**	**Query coverage**	**E value**	**Max ident**
NR_025615.1	Streptomyces albus subsp. albus strain DSM 40313 16S ribosomal RNA, partial sequence >emb|AJ621602.2| Streptomyces albus subsp. albus 16S rRNA gene, type strain DSM 40313T	2625	99%	0.0	99%
DQ026669.1	Streptomyces albus subsp. albus strain NRRL B-2365 16S ribosomal RNA gene, partial sequence	2606	98%	0.0	99%
AJ781753.1	Streptomyces gibsonii 16S rRNA gene, strain LMG 19912T	2591	98%	0.0	99%
AJ781366.1	Streptomyces rangoonensis 16S rRNA gene, type strain LMG 20295	2591	98%	0.0	99%
AB184781.1	Streptomyces albus subsp. albus gene for 16S rRNA, partial sequence, strain: NBRC 3710	2590	98%	0.0	99%
NR_041208.1	Streptomyces albus subsp. albus strain NBRC 3418 16S ribosomal RNA, partial sequence >dbj|AB184771.1| Streptomyces albus subsp. albus gene for 16S rRNA, partial sequence, strain: NBRC 3418	2590	98%	0.0	99%
AB184257.1	Streptomyces albus subsp. albus gene for 16S rRNA, partial sequence, strain: NBRC 13014	2584	98%	0.0	99%

4.4 Discussion

In this Chapter, antifungal metabolites production of the selected endophytic actinomycetes, 11 isolates from Chapter 3, was confirmed by removing the mass of the incubated isolates before testing antifungal activity on 3 pathogenic fungi: *P. aphanidermatum*, *R. solani*, and *S. rolfsii*. The results indicated that the selected isolates produced antifungal metabolites to inhibit the growth of the tested fungi. Eight isolates produced strong antifungal metabolites against *P. aphanidermatum*, especially isolate ERY1. Six isolates produced strong antifungal metabolites against *R. solani*, especially isolate PRE5. Eight isolates produced strong antifungal metabolites against *S. rolfsii*, especially POL2, PRE5 and SOL1. These may be due to the different production of antifungal metabolites and also the sensitivity of the tested fungi. The antifungal metabolites were not only inhibited the growth of tested fungi, but also caused the growth malformation of the fungi, such as lysis of hyphal, swelling, distortion and excessive branching of hyphae. A similar result has also been reported by Getha and Vikineswary, (2002). In addition to antifungal metabolites, the inhibition may also cause by the production of hydrolytic enzymes. The production of β-1,3-, β-1,4-, and β-1,6-glucanases could hydrolyze glucans and cause lysis of *Phytophthora* cell wall (Valois *et al*., 1996). In this study, cellulose hydrolytic enzymes were produced by isolates COF6, ERY1 and POL2, these may be contributed to cell lysis of *P. aphanidermatum*.

In this study, the isolates ERY1 and PRE5 were, however, selected because these two isolates showed the strong antifungal metabolites against the tested fungi, *P. aphanidermatum, R. solani*, and *S. rolfsii*, for at least 15 days, comparing with other isolates. The isolates ERY1 and PRE5 were identified by 16S rRNA to be *Streptomyces rochei*, and *Streptomyces albus* subsp. *albus*, respectively. *S. rochei* has been reported to inhibit the growth of *Phytophthora capsici* (Ezziyyani *et al*., 2004), *F. oxysporum* (Kanini *et al*., 2013), human pathogenic microorganisms, such as *Candida albicans*, *Staphylococcus aureus* and *Escherichia coli* (Reddy *et al.*, 2011), pathogens *Vibrio anguillarum, V. vulnificus, V. alginolitycus, Lactococcus garvieae, Streptococcus iniae, Carnobacterium piscícola,* and *Yersinia ruckeri* (León *et al.*, 2016). In postharvest, *S. rochei* could also reduce the resistance of ring rot disease caused by *Botryosphaeria dothidea* in apple fruits.

S. albus subsp. *albus* was reported to inhibit rice blast pathogens (Liao *et al*., 2015), and also use in an industrially as useful isolate and as a host for expression of secondary metabolite gene clusters (Olano *et al*., 2014). *S. albus* subsp. *albus* was also possess an antioxidant activity with pharmaceutically importance (Bhosale *et al*., 2015).

4.5 Conclusion

Eleven selected endophytic actinomycete isolates inhibited the growth of three pathogenic fungi: *P. aphanidermatum*, *R. solani*, and *S. rolfsii*. The results suggest that the antifungal metabolites produced by the isolates contributed to the fungal growth inhibition. The capacity to produce cellulose hydrolytic enzyme was found from the isolates COF6, ERY1 and POL2, and phosphate hydrolytic enzyme was found from the isolates COF6, ERY1, NEE1 and POL2. In this Chapter, however, only two potent isolates ERY1 and PRE5 were selected to determine their morphological characterization and identified as *Streptomyces rochei* and *Streptomyces albus* subsp. *albus*, respectively.