CHAPTER IV

RESULTS

1. Isolation of Endophytic Fungi

Fifty-eight isolates of endophytic fungi were obtained from six species of Thai medicinal plants, as shown in Table 3. Most of them were isolated from limb. Only leaf of *Canangium odoratum* Bail. was found to harbor the fungal endophyte. No fungal endophyte was found in samples collected from 4 species, i.e. *Abrus precatorius* Linn., *Cassia alata* Linn, *Datura metel* Linn., and *Puntica granatum* Linn.

Table 3 Number of endophytic fungi isolated from leaves and limbs of Thai medicinal plants.

Family	Scientific name	Thai name	Isolate code	Number of isolate		
				Leaf	Limb	Total
Annonaceae	*Canangium odoratum* Bail.	กระดังงาไทย	*Codo*	5	5	10
Euphorbiaceae	*Sauropus androgynus* Merr.	ผักหวานบ้าน	*Sand*	0	11	11
Lythraceae	*Lawsonia inermis* Linn.	เทียนกิ่ง	*Line*	0	12	12
Piperaceae	*Piper sarmentosum* Roxb.	ชะพลู	*Psar*	0	6	6
Rutaceae	*Hesperethusa crenulata* Roem.	พญายา	*Hcre*	0	11	11
	Murraya paniculata (L.) Jack	แก้ว	*Mpan*	0	8	8

2. Antimicrobial Activities of Isolated Endophytic Fungi

By dual-culture agar diffusion assay, 43 isolates (74.14%) of endophytic fungus isolates were found to show antimicrobial activities. The inhibition zone around the agar cube of endophytic fungus isolate was observed. The number of active endophytic fungus isolates exhibiting activities against *S. aureus, E. faecalis, B. subtilis, E. coli*, and *P. aeruginosa* were 18 (41.86%), 14 (32.56%), 20 (46.51%), 3 (6.98%), and 1 (2.33%) isolates, respectively, as shown in Figure 1. Anti-*C. albicans*, anti-*S. cerevisiae*, and anti-*T. mentagrophytes* activities were evident in 13 (30.23%), 5 (11.63%), and 32 (74.42%) isolates, respectively. Figure 2-5 demonstrated antimicrobial activities against *S. aureus*, *P. aeruginosa*, *C. albicans* and *T. mentagrophytes* of endophytic fungus isolates from dual-culture agar diffusion assay.

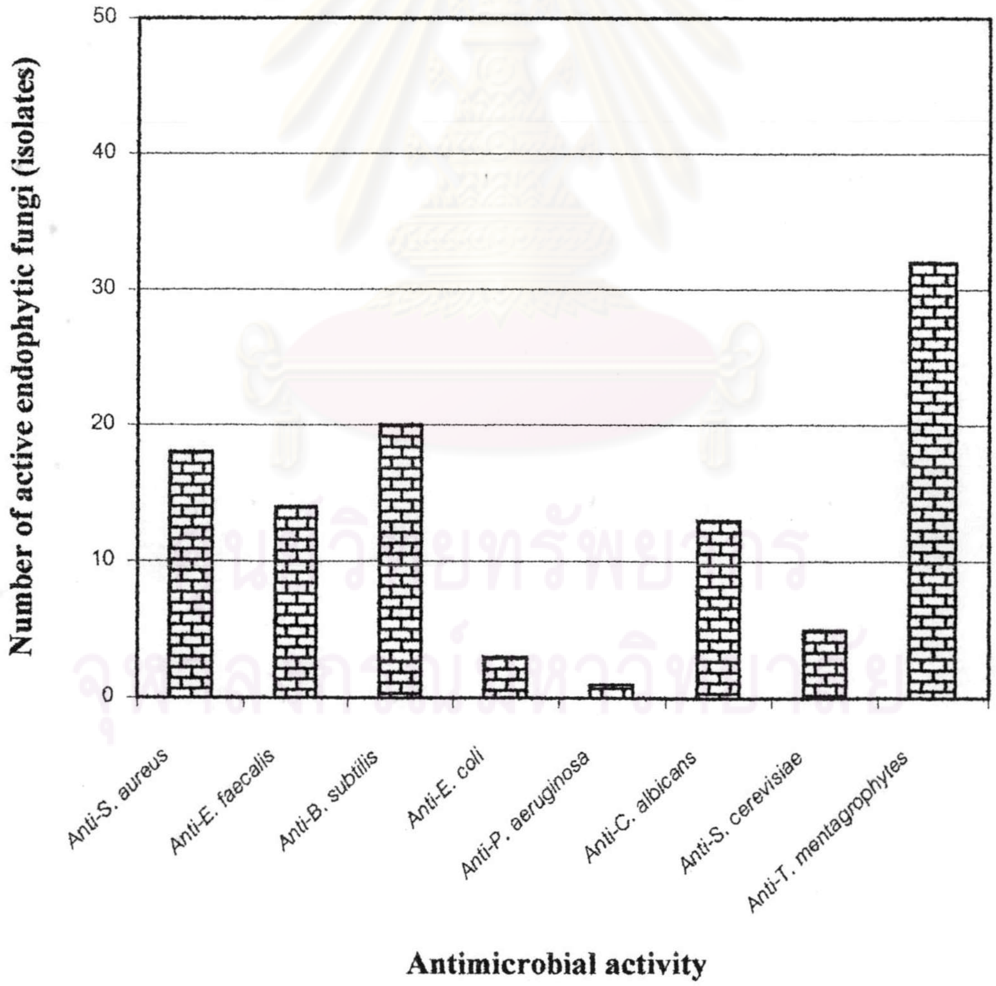


Figure 1 Numbers of endophytic fungus isolates exhibiting antimicrobial activities

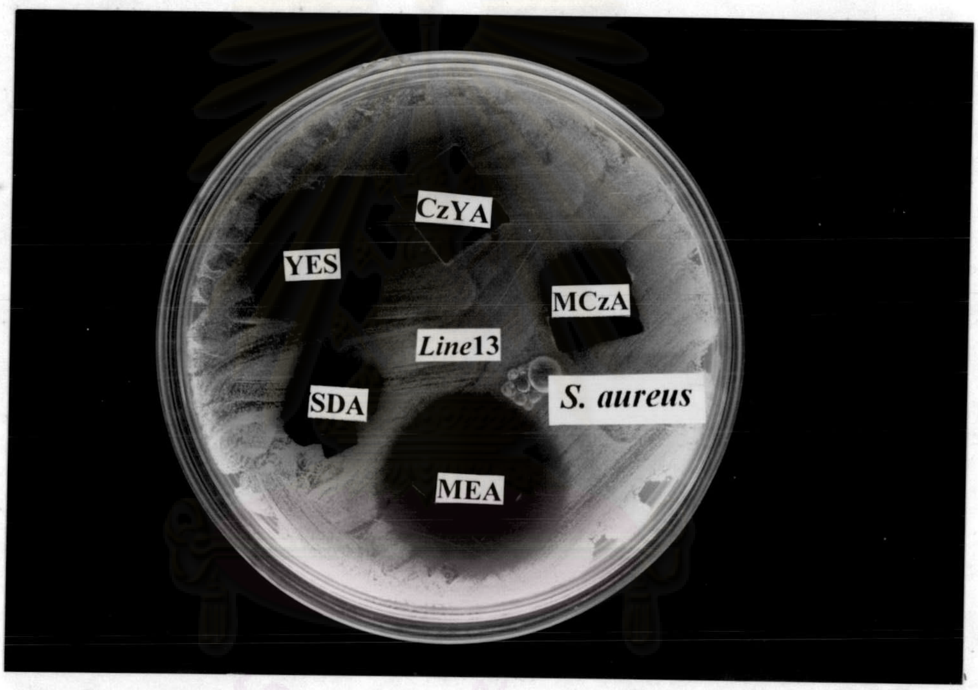


Figure 2 Dual-culture agar diffusion assay exhibiting anti-*S. aureus* activity of endophytic fungus isolate *Line* 13 grown on malt extract agar.

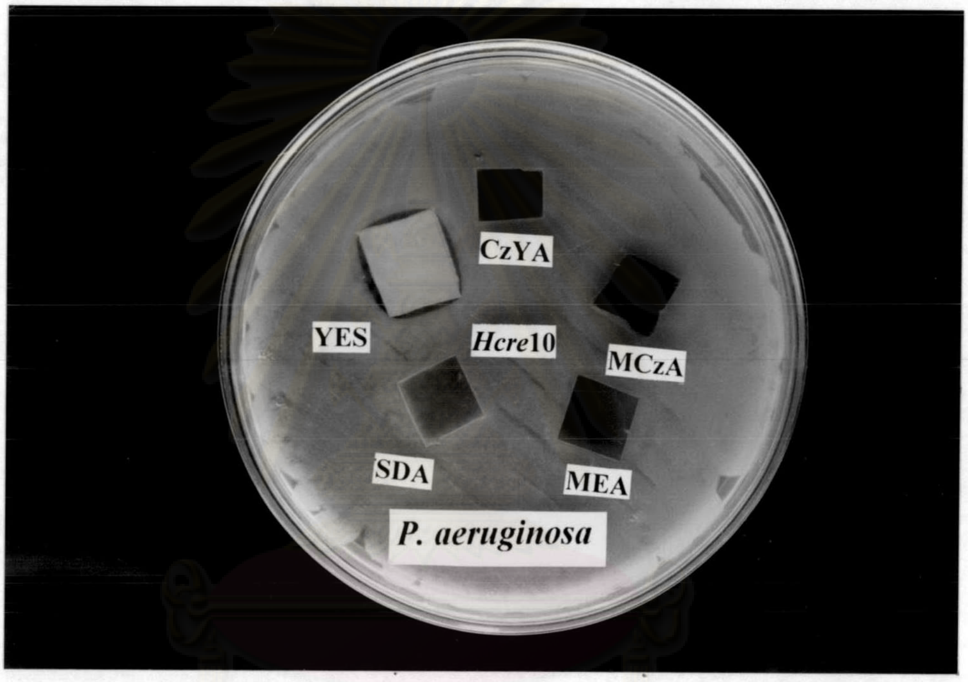


Figure 3 Dual-culture agar diffusion assay exhibiting anti-*P. aeruginosa* activity of endophytic fungus isolate *Hcre* 10 grown on yeast extract sucrose agar.

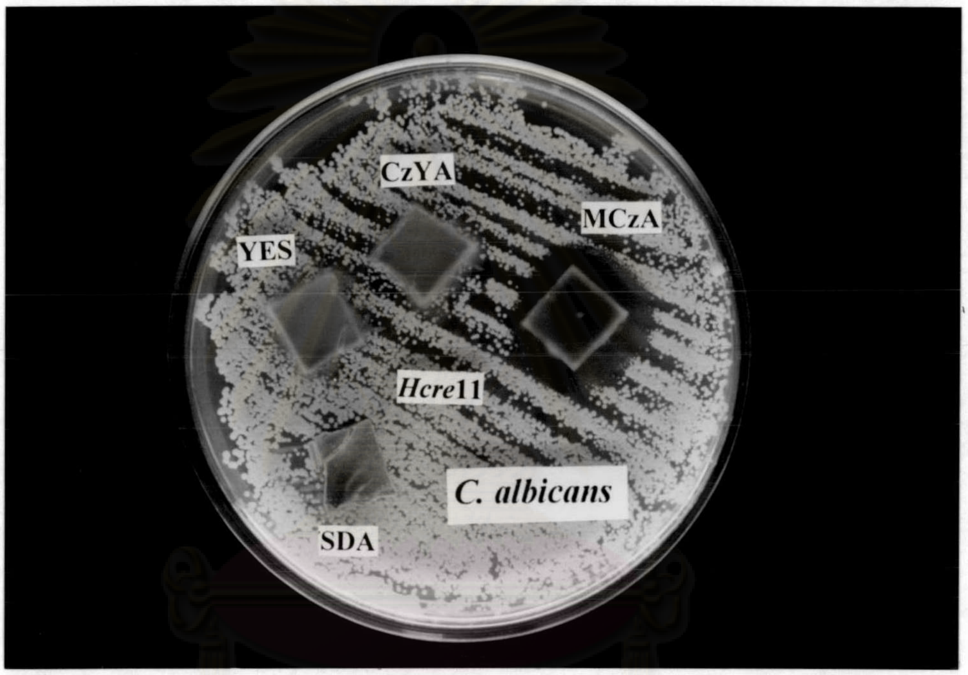


Figure 4 Dual-culture agar diffusion assay exhibiting anti-*C. albicans* activity of endophytic fungus isolate *Hcre* 11 grown on malt Czapek agar.

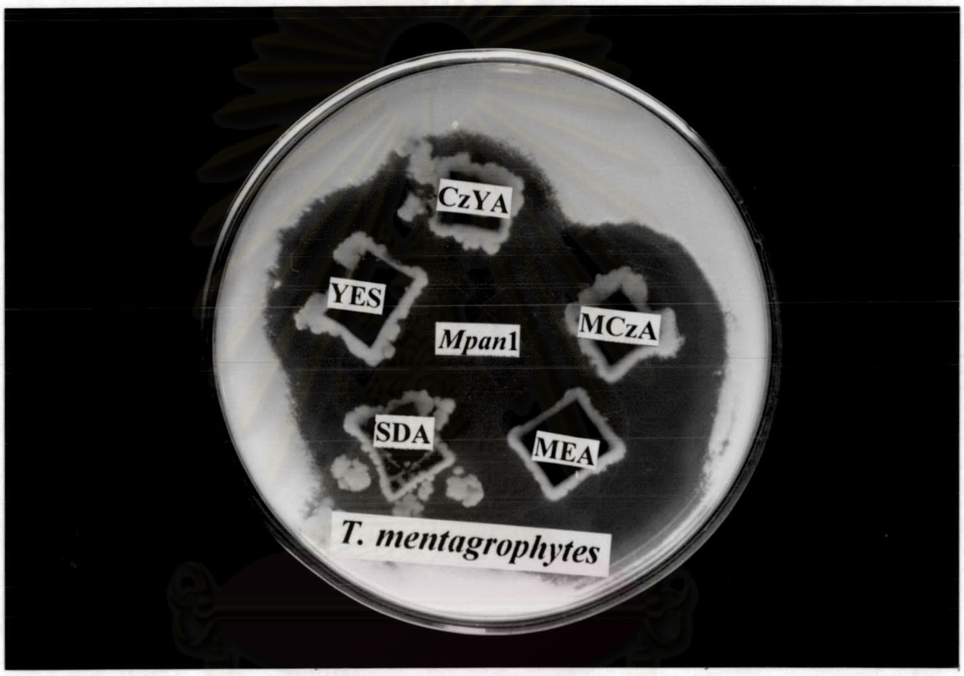


Figure 5 Dual-culture agar diffusion assay exhibiting anti-*T. mentagrophytes* activity of endophytic fungus isolate *Mpan* 01 grown on Czapek yeast autolysate agar, malt Czapek agar, malt extract agar, Sabouraud's dextrose agar and yeast extract sucrose agar.

3. Effect of Culture Media on Antimicrobial Activities of Endophytic Fungus Isolates

It was found that culture media (CzYA, MCzA, MEA, SDA and YES) affected both type and intensity of antimicrobial activities of endophytic fungus isolates, as shown in Table 9 in Appendix. Figure 6 summarized the variation in number of fungal endophyte isolates that exhibited antimicrobial activities when they were cultivated on different media. Anti-*E. coli* and anti-*P. aeruginosa* activities were culture medium specific. They were observed only in active isolates grown on MEA and YES, respectively. The highest numbers of active isolates against *S. aureus*, *B. subtilis*, *C. albicans* and *S. cerevisiae* were found when they were grown on MEA. The same highest numbers of active endophytic fungus isolates with anti-*E. faecalis* activity were demonstrated when they were grown on MCzA and MEA.

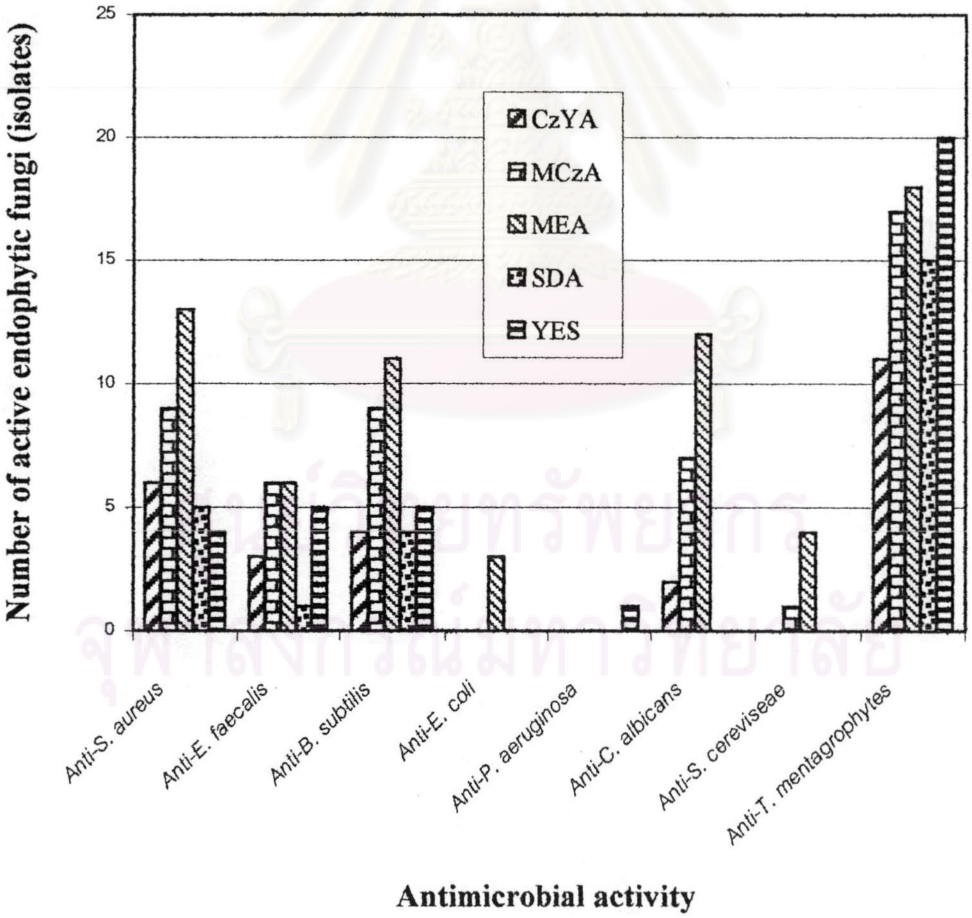


Figure 6 Numbers of active endophytic fungus isolates demonstrating activities against test microorganisms when cultured on different media

Anti-*C. albicans* and anti-*S. cerevisiae* activities could not be observed in culture grown on SDA and YES. It was found that 2 active isolates showed anti-*C. albicans* activitiy when they were grown on CzYA. For anti-*T. mentagrophytes* activity, the numbers of active isolates were the highest and the lowest when they were cultivated on YES and CzYA, respectively.

4. Stability of Antimicrobial Phenotype of Active Endophytic Fungus Isolates

A total of 100, 17, 56 and 27 active endophytic fungus isolates, that were kept in stock culture, were re-examined for their antimicrobial activities against *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *C. albicans* ATCC 10231 and *S. cerevisiae* ATCC 9763, respectively, as shown in Table 10-17 in Appendix. It was found that 29 (29.00%), 4 (23.53.%), 4 (7.14%), and 1 (3.70%) isolates of endophytic fungus isolates were still active against *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *C. albicans* ATCC 10231, and *S. cerevisiae* ATCC 9763, respectively, as shown in Figure 7.

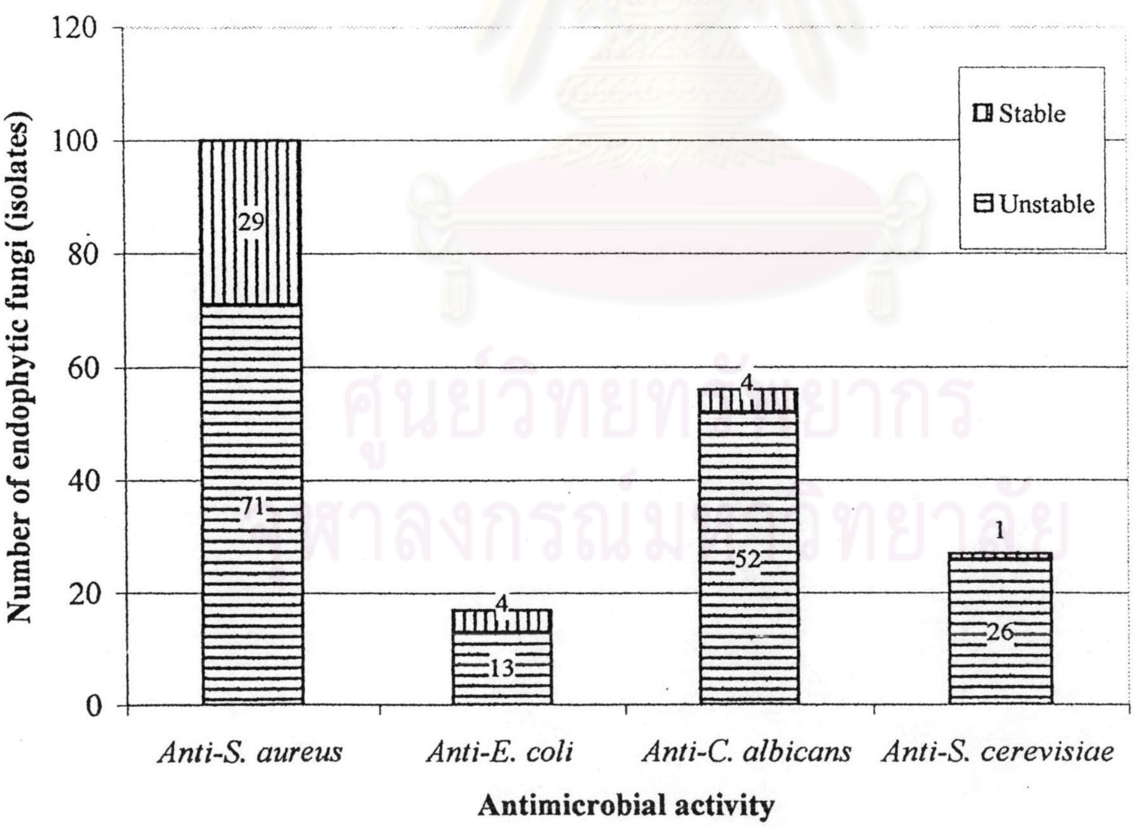


Figure 7 Numbers of endophytic fungus isolates, previously observed as active, exhibiting stable and unstable antimicrobial activities.

5. Susceptibility of Selected Mutants

Based on MICs demonstrated in Table 4, the low susceptible mutants of *S. aureus* and *E. coli* could be selected by the gradient plate technique. The variation in susceptibility of selected mutant to various antibiotics was observed. The MICs of the used antibiotics for selected *S. aureus* mutants were in the range of 8-128 times the MICs for wildtype strain, except erythromycin (1,024 times) and rifampin (2.56×10^5 times). For selected *E. coli* mutants, the MICs of the used antibiotics were in the range of 8-64 times the MICs for wildtype strain. Low susceptible *C. albicans* and *S. cerevisiae* mutants to nystatin were also selected. The selected mutants responded to nystatin with MICs of 8 μg/ml (8×MIC for wildtype strain) and 4 μg/ml (4×MIC for wildtype strain) for *C. albicans* and *S. cerevisiae*, respectively.

As shown in Table 5, most of the selected low susceptible *S. aureus*, except strain with low susceptibility to tetracycline (Te^L), resisted to the respective antibiotic that was used in selection. It was found that each mutant showed cross-resistance to other antibiotic. Kanamycin resistant (K^R) *S. aureus* was cross-resistance to the highest number of antibiotics. For *E. coli*, each selected low susceptible strain was found to be resistant to the respective antibiotic, as shown in Table 6. Cross-resistance was also observed in some selected *E. coli* mutants. Some representative agar-diffusion assay plates, exhibiting antibiotic resistance of the selected mutants, were shown in Figure 8-17.

Table 4 Minimum inhibitory concentrations (MICs) (μg/ml) of antibiotic for wildtype strains and the selected mutants.

Antibiotics	MICs (μg/ml)			
	S. aureus		*E. coli*	
	ATCC 29213	Mutant	ATCC 25922	Mutant
Bacitrain	128	16,384	ND	ND
Cephalosporin C	128	2,048	32	1,024
Chloramphenicol	8	128	8	512
D-cycloserine	64	512	64	512
Erythromycin	1	1,024	ND	ND
Kanamycin	4	64	4	128
Lincomycin	4	128	ND	ND
Penicillin G	1	16	ND	ND
Polymyxin B	ND	ND	8	128
Rifampicin	0.008	2,048	16	256
Tetracycline	0.25	4	2	32

ND = not determined

Table 5 The inhibition zone diameters (mm) of *S. aureus* ATCC 29213 and mutant strains around the antibiotic disks

Antibiotic disk	Inhibition zone diameters (mm) of *S. aureus*[a]										
	ATCC 29213	B^R	CEC^R	C^R	DCY^R	E^R	K^R	Li^R	P^R	RA^R	Te^L
Bacitracin (10 μg/disk)	19.9	0	0	10.3	10.4	17.3	8.3[b]	7.4[b]	11.0	16.1	0
Cephalosporin C (100 μg/disk)	19.9	15.5	0	28.9	0	17.0	24.7	24.8	17.2	0	13.9
Chloramphenicol (30 μg/disk)	23.0	14.9	19.3	10.4[b]	21.6	20.3	21.9	22.2	21.4	22.8	21.9
D-cycloserine (100 μg/disk)	24.7	21.8	22.1	0	0	23.4	0	0	0	23.8	23.1
Erythromycin (15 μg/disk)	28.9	0	23.7	22.8	25.8	0	27.7	24.5	27.2	24.5	27.6
Kanamycin (30 μg/disk)	25.8	11.1[b]	27.3	27.7	22.1	23.5	11.3[b]	29.5	26.9	24.9	24.5
Lincomycin (10 μg/disk)	23.5	22.3	0	0	16.1	0	0	0	0	21.8	23.1
Penicillin G (10 μg/disk)	36.6	0	29.8	30.3	27.7[b]	33.2	26.9[b]	26.0[b]	0	31.1	30.2
Rifampin (0.75 μg/disk)	23.9	24.9	17.3	18.9	23.9	26.9	9.7[b]	18.0	8.6[b]	0	24.8
Tetracycline (30 μg/disk)	27.6	9.7[b]	26.5	23.9	25.7	28.2	25.7	28.3	29.1	25.9	23.4

[a] ATCC 29213 = *Staphylococcus aureus* ATCC 29213

B^R = Bacitracin resistant *S. aureus*

CEC^R = Cephalosporin C resistant *S. aureus*

C^R = Chloramphenicol resistant *S. aureus*

DCY^R = D-cycloserine resistant *S. aureus*

E^R = Erythromycin resistant *S. aureus*

K^R = Kanamycin resistant *S. aureus*

Li^R = Lincomycin resistant *S. aureus*

P^R = Penicillin G resistant *S. aureus*

RA^R = Rifampin resistant *S. aureus*

Te^L = Tetracycline low susceptible *S. aureus*

[b] Inhibition zone diameter was in the range of resistant strain as recommended in the interpretive standards (33)

Table 6 The inhibition zone diameters (mm) of *E. coli* ATCC 25922 and mutant strains around the antibiotic disks

Antibiotic disk	Inhibition zone diameters (mm) of *E. coli*[a]							
	ATCC 25922	CEC^R	C^R	DCY^R	K^R	PB^R	RA^R	Te^R
Cephalosporin C (100 μg/disk)	25.4	0	20.2	0	23.4	22.0	0	0
Chloramphenicol (30 μg/disk)	26.2	20.4	0	21.5	27.0	21.9	24.9	26.6
D-cycloserine (100 μg/disk)	20.3	21.7	16.8	0	23.0	21.5	19.8	20.4
Kanamycin (30μg/disk)	24.6	18.3	0	24.2	0	26.6	18.9	24.9
Polymyxin B (300 μg/ml)	24.6	13.1	15.4	12.2	13.8	0	10.9	15.3
Rifampin (75 μg/disk)	19.9	26.4	27.8	14.6	30.7	27.2	0	18.9
Tetracycline (30 μg/disk)	15.5	21.7	11.1[b]	20.0	36.3	28.5	23.0	0

[a] ATCC 25922 = *Escherichia coli* ATCC 25922

CEC^R = Cephalosporin C resistant *E. coli*

C^R = Chloramphenicol resistant *E. coli*

DCY^R = D-cycloserine resistant *E. coli*

K^R = Kanamycin resistant *E. coli*

PB^R = Polymyxin B resistant *E. coli*

RA^R = Rifampin resistant *E. coli*

Te^R = Tetracycline resistant *E. coli*

[b] Inhibition zone diameter was in the range of resistant strain as recommended in the interpretive standards (33)

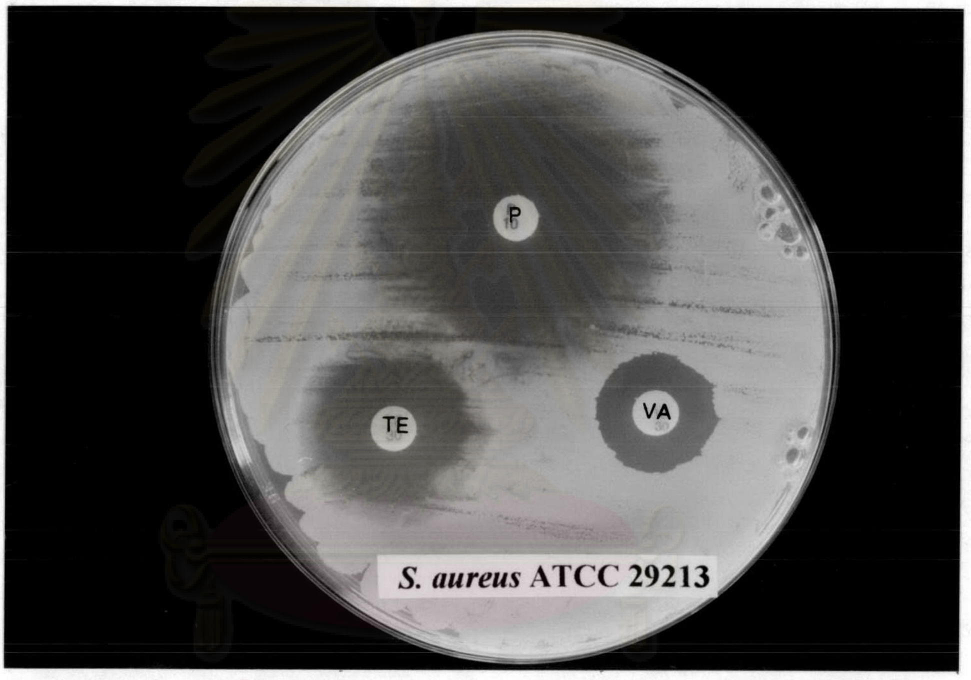


Figure 8 Antibiotic disk-agar diffusion assay demonstrating inhibition zone of *Staphylococcus aureus* ATCC 29213 around penicillin G, tetracycline and vancomycin.

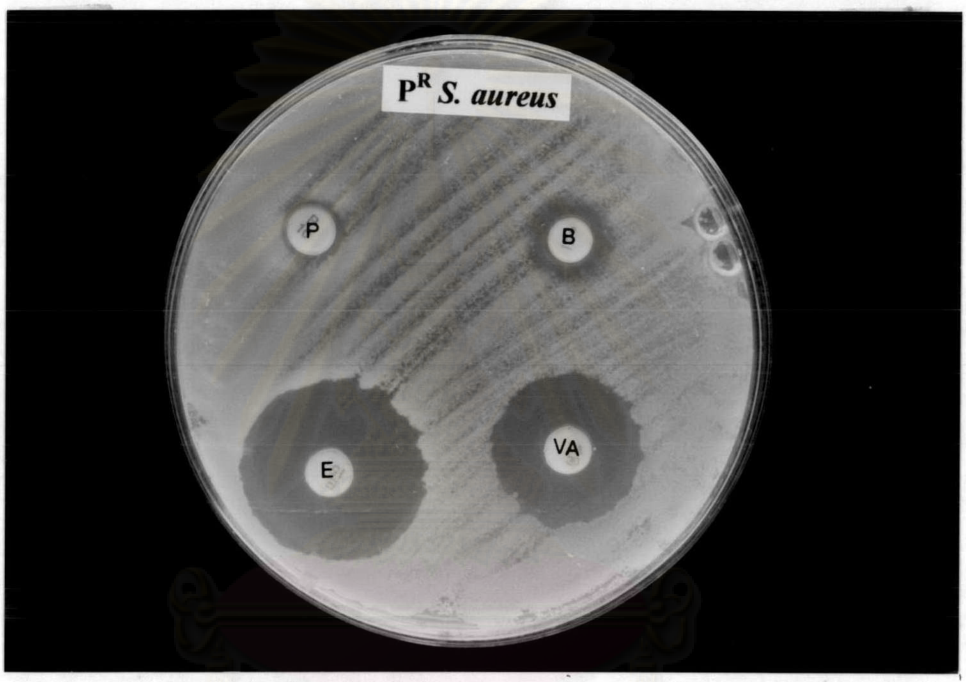


Figure 9 Antibiotic disk-agar diffusion assay demonstrating resistance of *S. aureus* mutant to penicillin G.

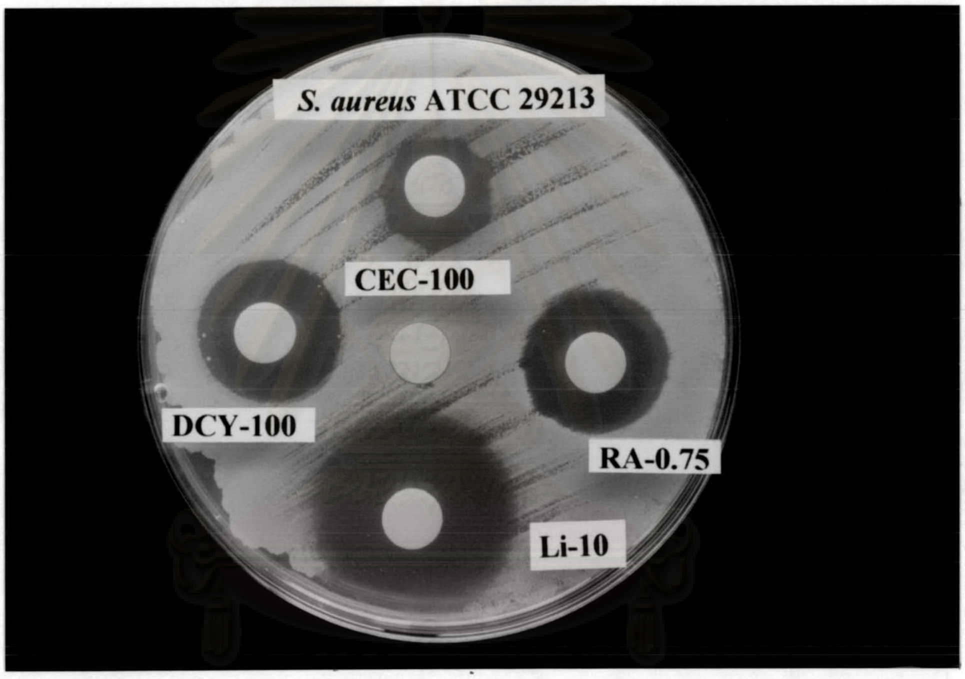


Figure 10 Antibiotic disk-agar diffusion assay demonstrating inhibition zone of *Staphylococcus aureus* ATCC 29213 around cephalosporin C, D-cycloserine, lincomycin and rifampin antibiotic disks.

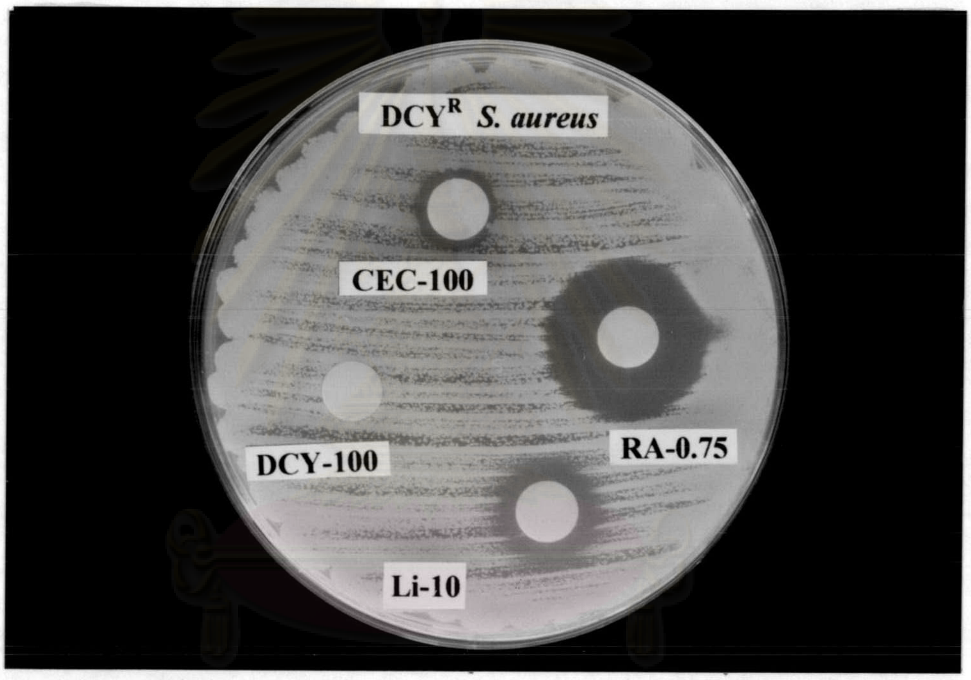


Figure 11 Antibiotic disk-agar diffusion assay demonstrating resistance of *S. aureus* mutant to D-cycloserine.

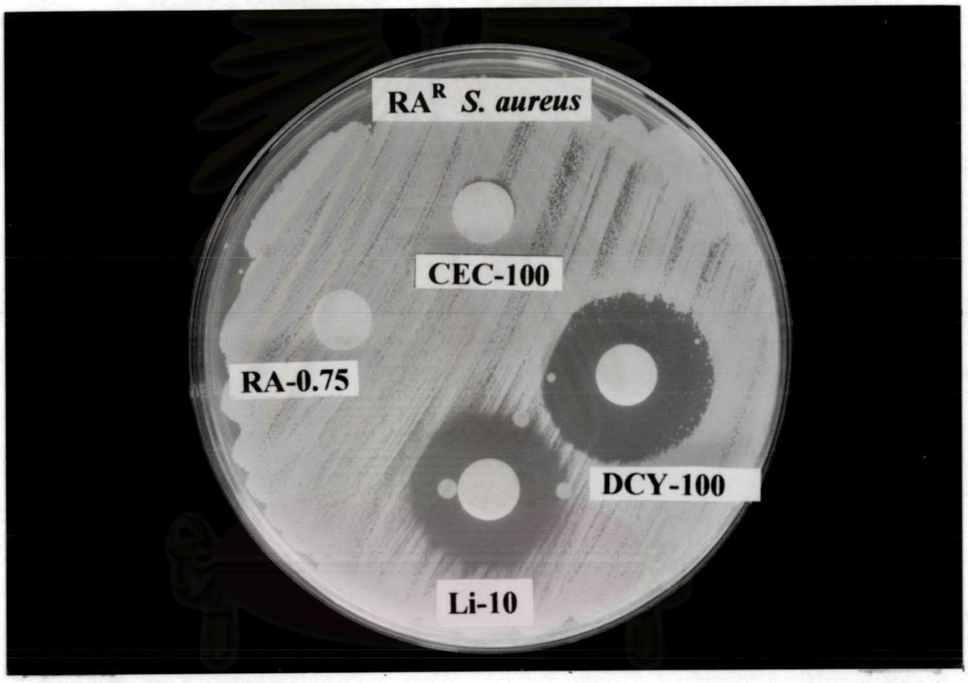


Figure 12 Antibiotic disk-agar diffusion assay demonstrating resistance of *S. aureus* mutant to rifampin.

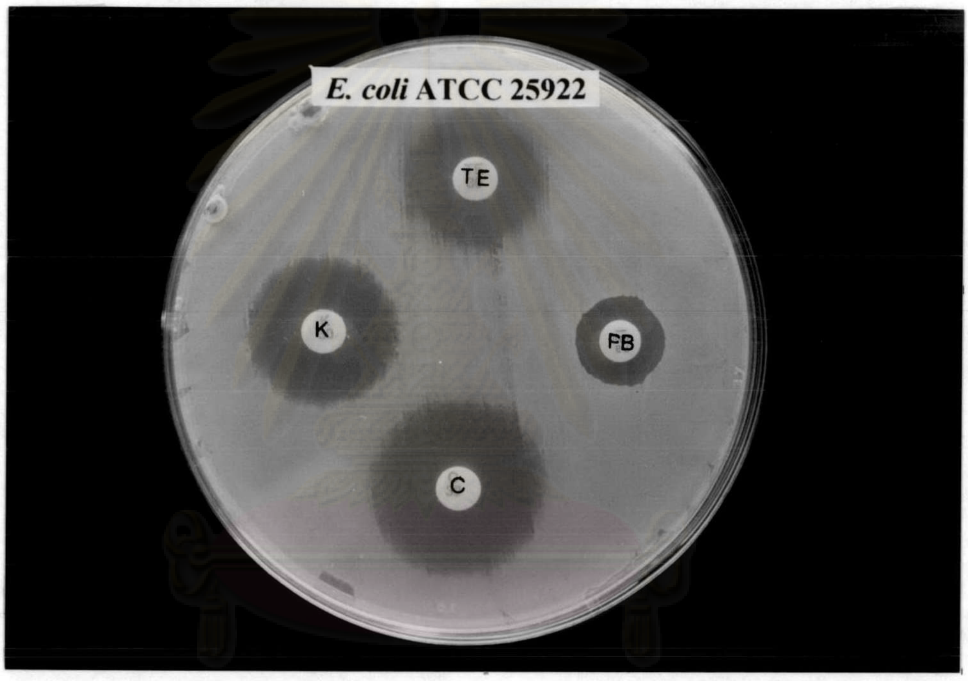


Figure 13 Antibiotic disk-agar diffusion assay demonstrating inhibition zone of *Escherichia coli* ATCC 25922 around chloramphenicol, kanamycin, polymyxin B and tetracycline antibiotic disks.

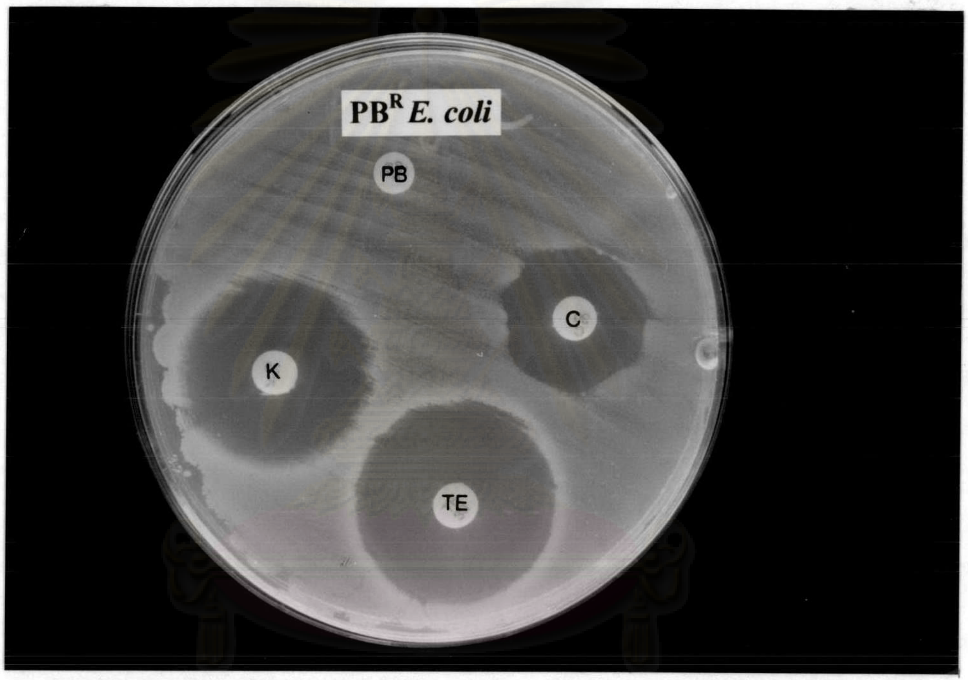


Figure 14 Antibiotic disk-agar diffusion assay demonstrating resistance of *E. coli* mutant to polymyxin B.

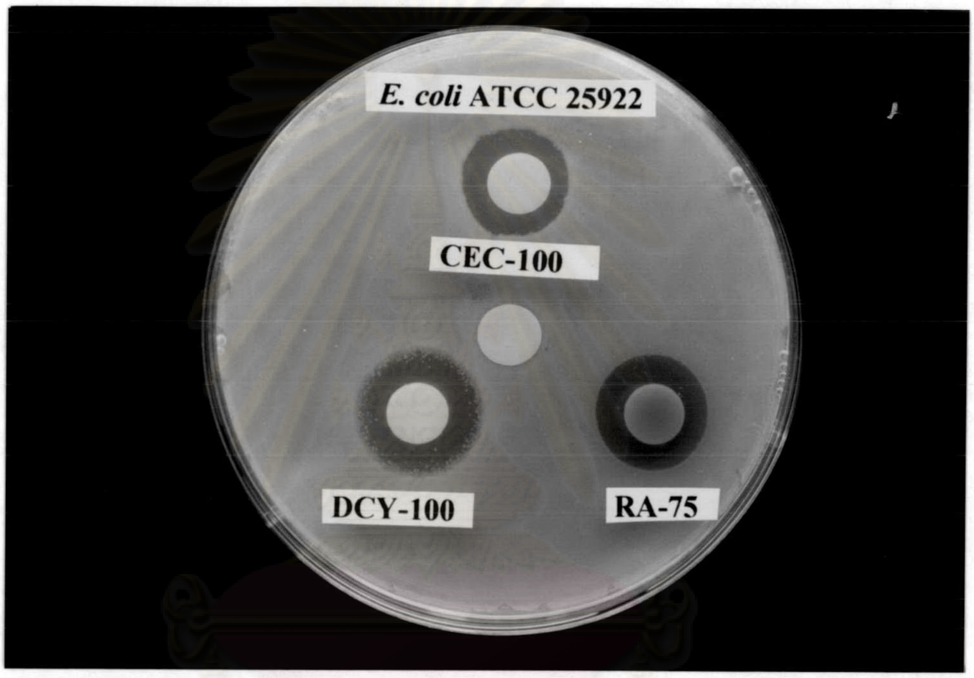


Figure 15 Antibiotic disk-agar diffusion assay demonstrating inhibition zone of *Escherichia coli* ATCC 25922 around cephalosporin C, D-cycloserine and rifampin antibiotic disks.

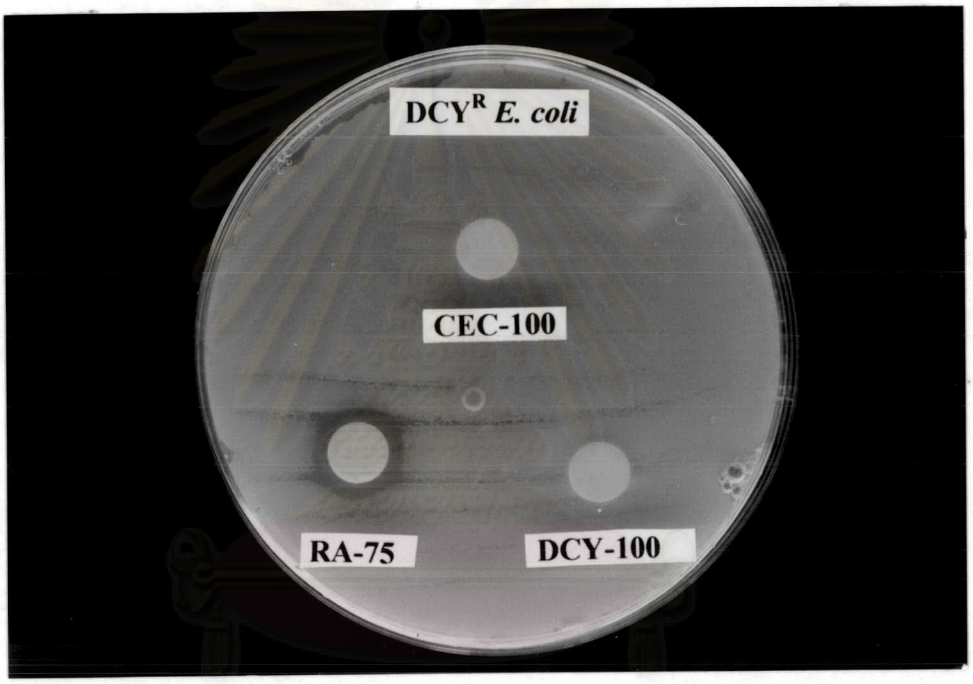


Figure 16 Antibiotic disk-agar diffusion assay demonstrating resistance of *E. coli* mutant to D-cycloserine.

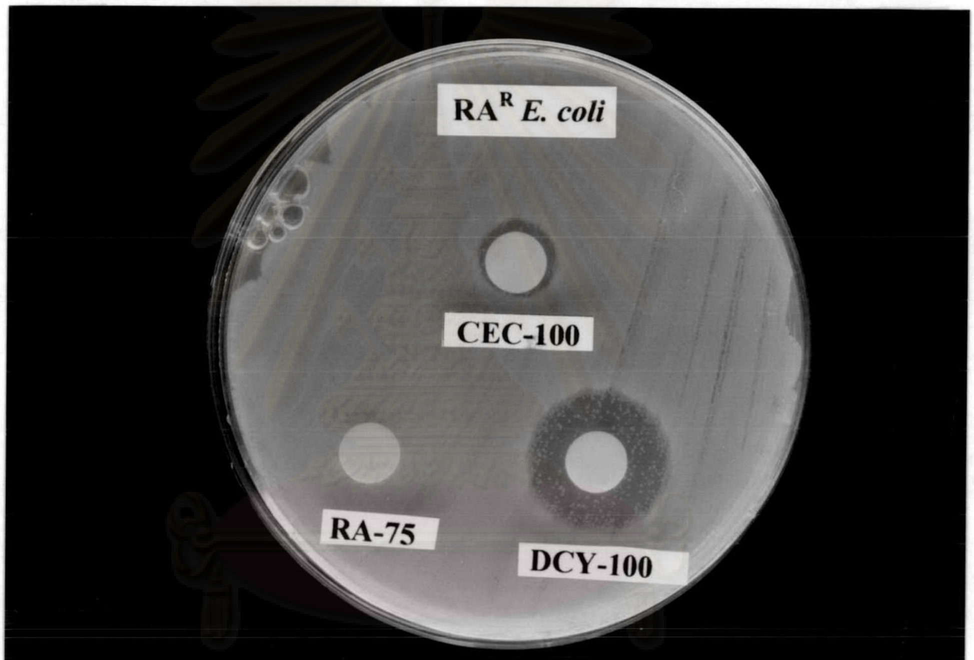


Figure 17 Antibiotic disk-agar diffusion assay demonstrating resistance of *E. coli* mutant to rifampin.

6. Antimicrobial Activities of Active Endophytic Fungus Isolates Against Low Susceptible Test Strains

The active endophytic fungi were tested against the low susceptible strains by dual-culture agar diffusion assay. Activities against the resistant *E. coli* mutants, the low susceptible *C. albicans* and *S. cerevisiae* mutants could not be found in any endophytic fungus isolates. Only five isolates, i.e. isolate *Bore* 04 (isolated from *Bixa orellana* Linn.), *Ccoc* 08 (isolated from *Carissa cochinchinensis* Pierre), *Cfis* 01A (isolated from *Cassia fistula* Linn.), *Line* 13 (isolated from *Lawsonia inermis* Linn.) and *Oind* 05A (isolated from *Oroxylum indicum* Vent.), were found to be active against the selected *S. aureus* mutant, as shown in Table 7. Isolate *Line* 13 showed the highest activity. It could inhibit bacitracin^{R}, chloramphenicol^{R}, erythromycin^{R}, lincomycin^{R}, rifampin^{R} and tetracycline^{L} strains. Isolate *Oind* 05A also inhibited these strains except bacitracin^{R} strain.

MICs of crude extracts obtained from culture broths of these active isolates were shown in Table 8. Isolate *Bore* 04 was found to be active equally against *S. aureus* ATCC 29213 and the resistant strains with MIC of 1.024 mg/ml. Comparing to activity against the reference strain, the equal activities against bacitracin^{R} and lincomycin^{R} were observed in isolate *Cfis* 01A and isolate *Line* 13, respectively. Lower activities against other test mutants than against the reference strain were demonstrated in isolates *Ccoc* 08, *Cfis* 01A, *Line* 13 and *Oind* 05A. MICs of the crude extracts for the test mutants were higher than those for the reference strain in the range of 2-4 times.

Table 7 Inhibition zone (mm) measured from the agar block of endophytic fungus culture exhibiting antimicrobial activities against selected *S. aureus* mutants

S. aureus	Inhibition zone (mm)				
	Bore 04	*Ccoc* 08	*Cfis* 01A	*Line* 13	*Oind* 05A
ATCC 29213	1	1	1	2	3.1
Bacitracin^R	1	2	2.25	3.5	0
Chloramphenicol^R	0	1	0	3.2	1.8
Erythromycin^R	1.7	0	1	2.4	1.8
Lincomycin^R	1	0	0	3.0	2.2
Rifampicin^R	0	0	0	3.0	2
Tetracycline^L	2	0	0	3.4	2.6

Table 8 The MICs (µg/ml) of crude extracts that were obtained from active endophytic fungus isolates exhibiting activities against *S.aureus* mutants

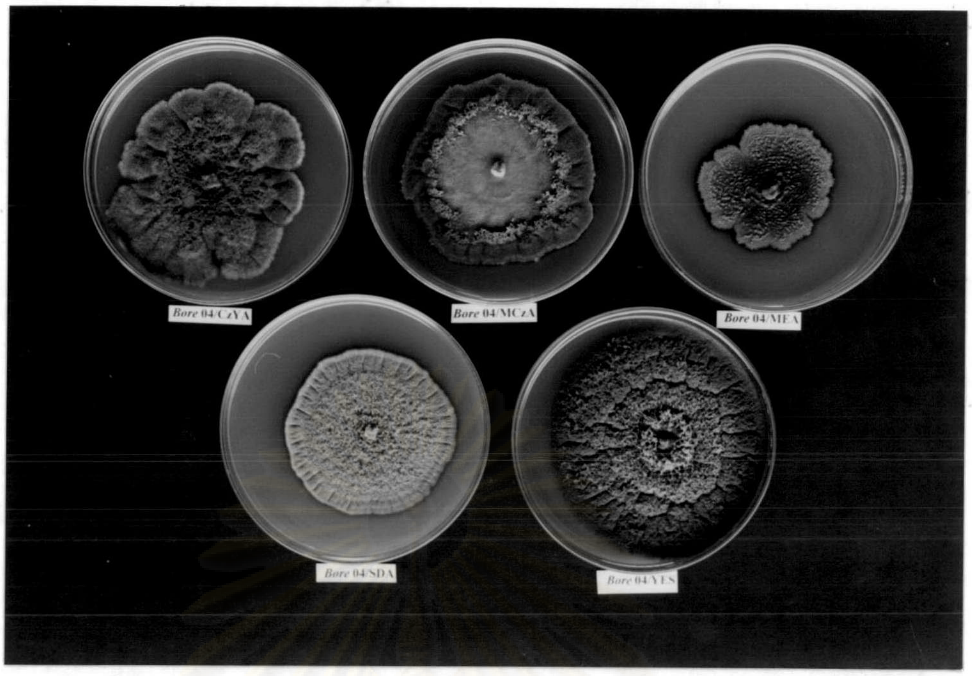
S. aureus	MICs (µg/ml)				
	Bore 04	*Ccoc* 08	*Cfis* 01A	*Line* 13	*Oind* 05A
ATCC 29213	1,024	128	256	512	256
Bacitracin^R	1,024	256	256	1,024	ND
Chloramphenicol^R	ND	256	ND	1,024	512
Erythromycin^R	1,024	ND	1,024	1,024	1,024
Lincomycin^R	1,024	ND	ND	512	512
Rifampicin^R	1,024	ND	ND	1,024	1,024
Tetracycline^L	ND	ND	ND	1,024	1,024

ND = Not determined

7. Identification of Selected Endophytic Fungus Isolates

Colony morphology of endophytic fungus isolates *Bore* 04, *Ccoc* 08, *Cfis* 01A, *Line* 13, and *Oind* 05A grew on 5 different media at 30 °C for 14 days as shown in Figure 18-22. All isolates, except *Line* 13, did not produce conidia or spore even grown on banana leaf agar and banana leaf agar with 1% vitamin B complex for 2 months. Production of conidia was observed in isolate *Line* 13 grown on PDA. Based on its microscopic morphology, as shown in Figure 23-25, isolate *Line* 13 was identified as *Alternaria* sp. Sexual stage development of isolate *Line* 13 could not be achieved when grown on banana leaf agar and banana leaf agar with 1% vitamin B complex.

A



B

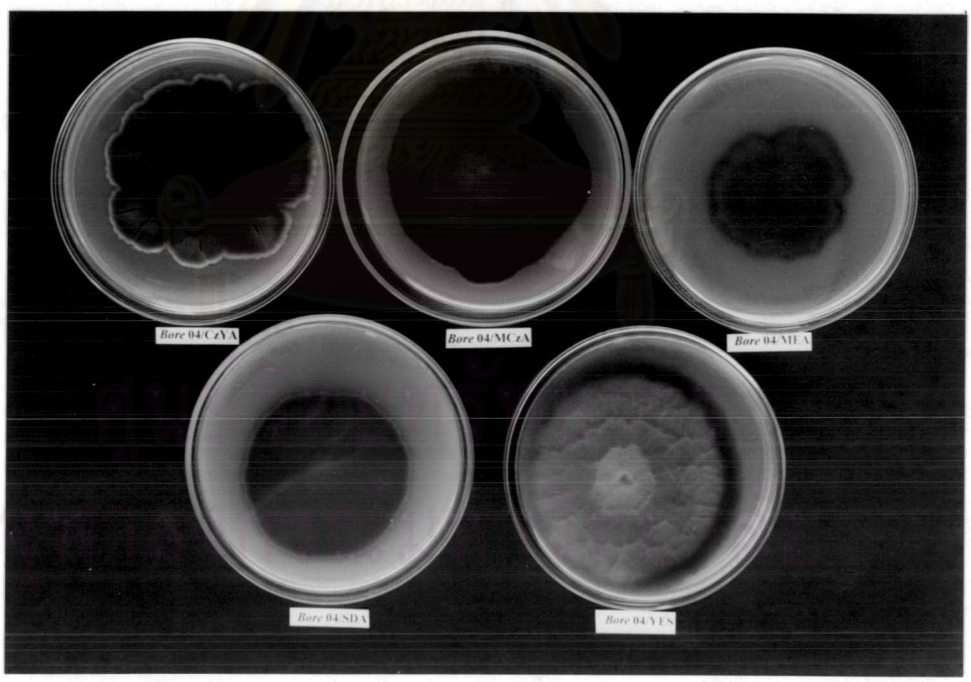
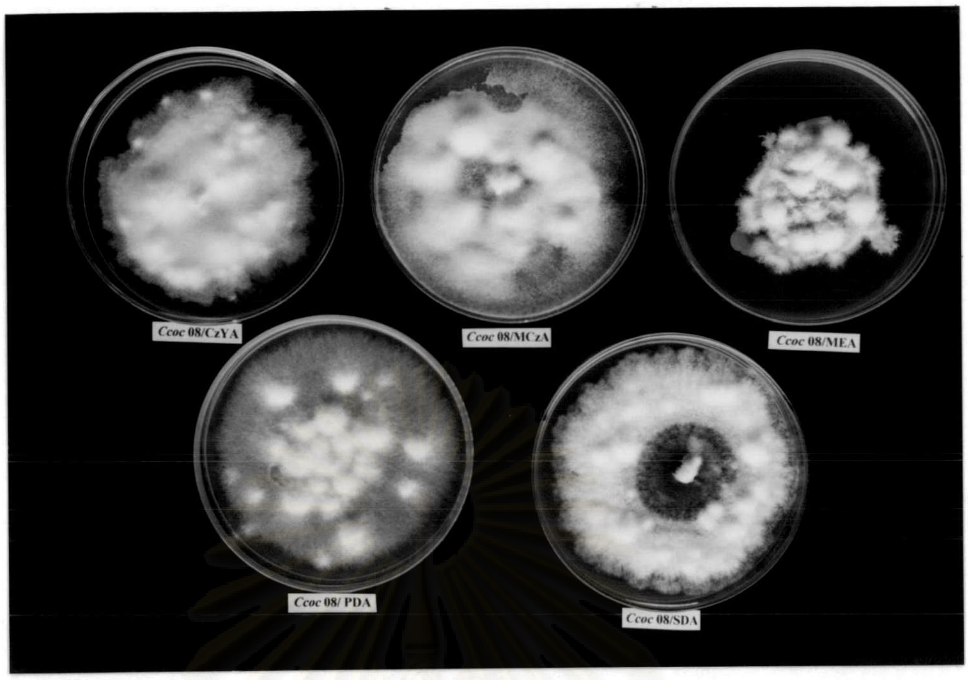


Figure 17 Obverse (A) and reverse (B) of colony morphology of endophytic fungus isolate *Bore* 04 grown on 5 different media at 30 °C for 14 days

A



B

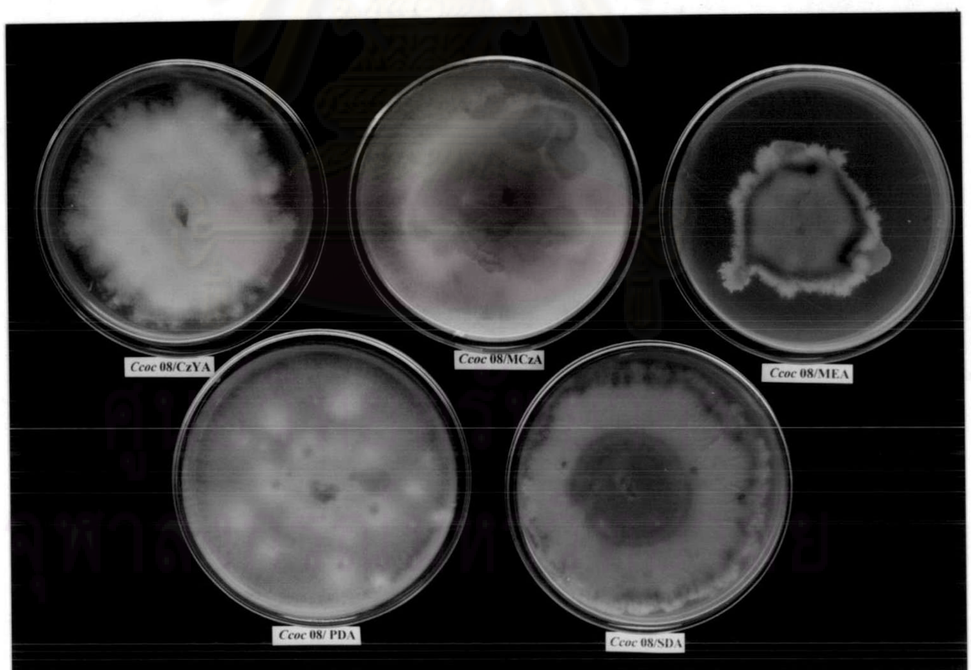
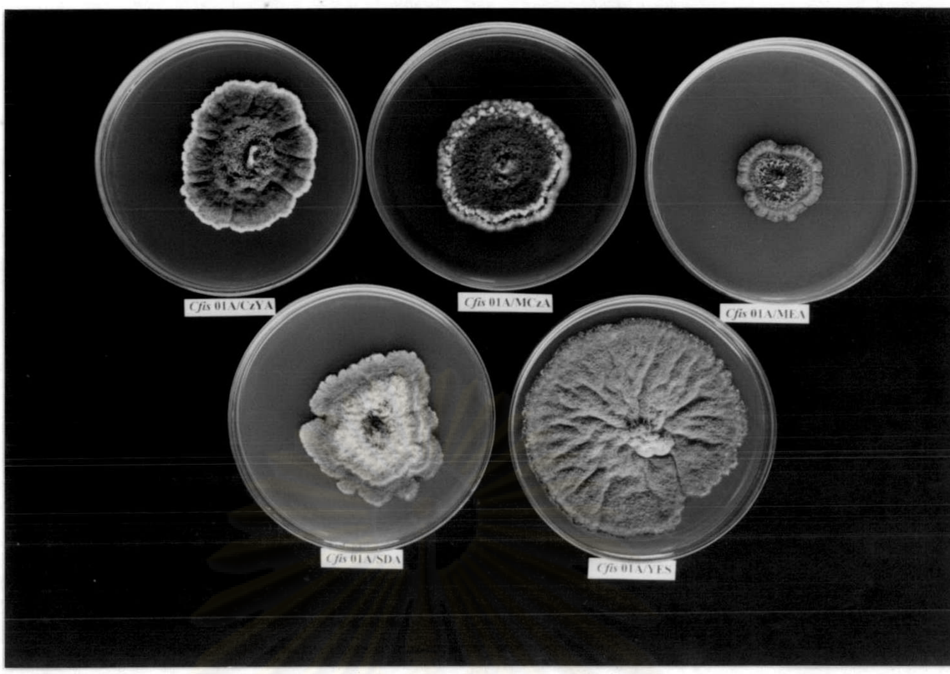


Figure 18 Obverse (A) and reverse (B) of colony morphology of endophytic fungus isolate *Ccoc* 08 grown on 5 different media at 30 °C for 14 days

A



B

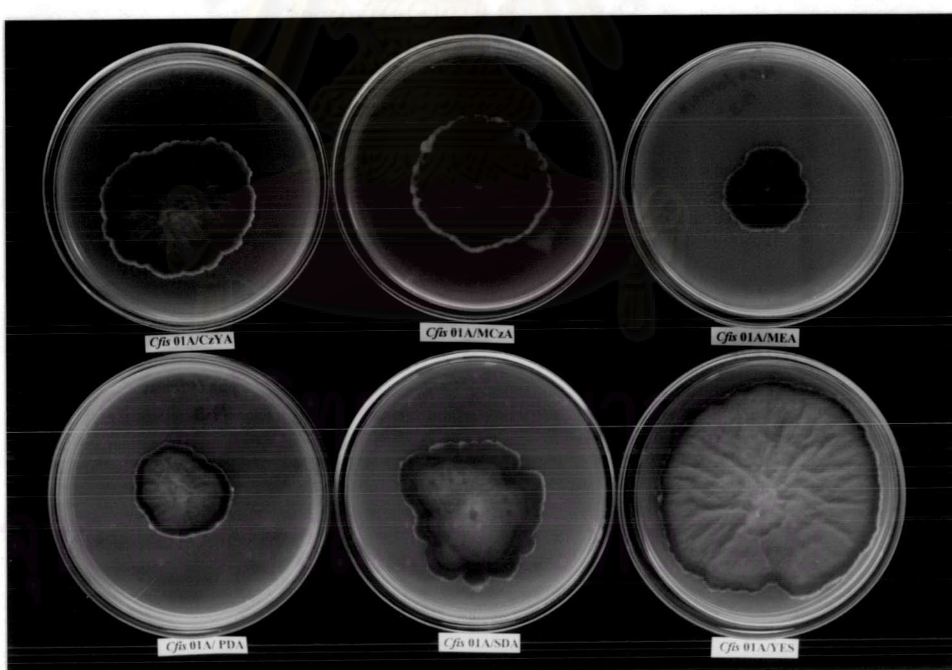
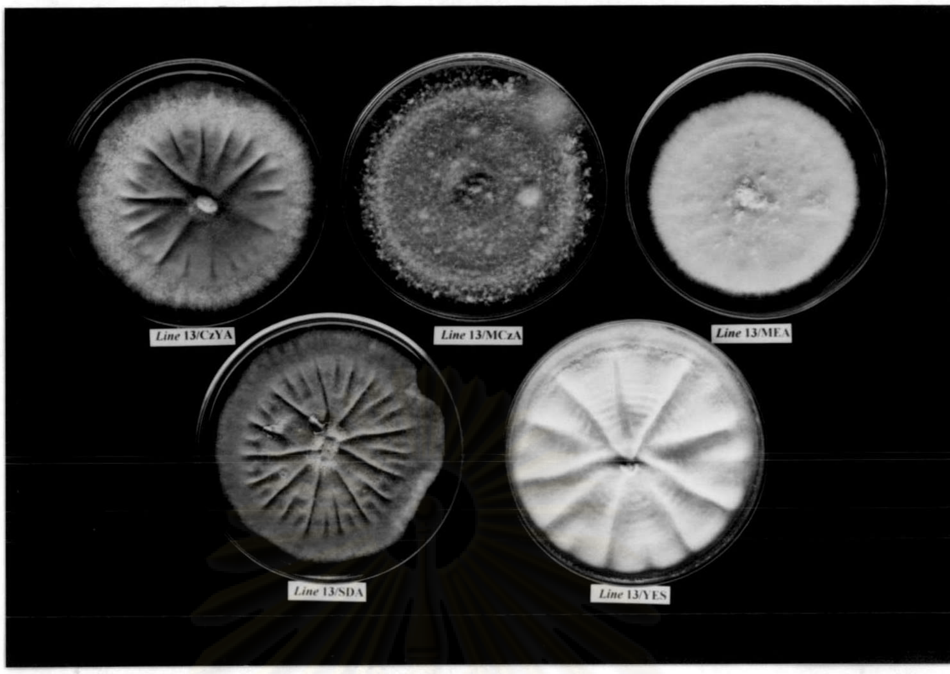


Figure 19 Obverse (A) and reverse (B) of colony morphology of endophytic fungus isolate *Cfis* 01A grown on 5 different media at 30 °C for 14 days.

A



B

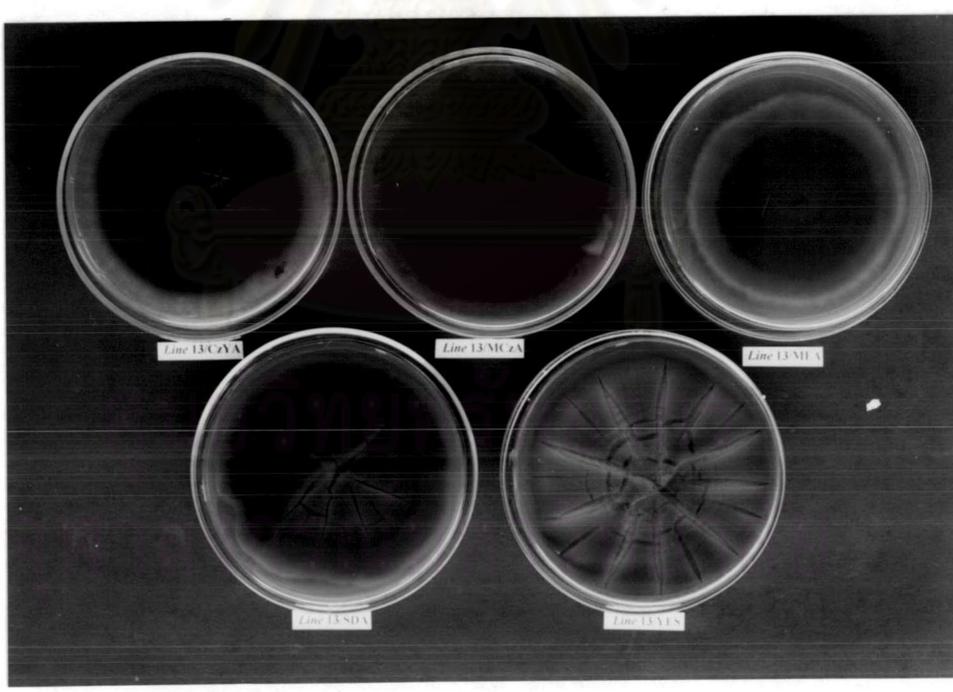


Figure 20 Obverse (A) and reverse (B) of colony morphology of endophytic fungus isolate *Line* 13 grown on 5 different media at 30 °C for 14 days.

A

B

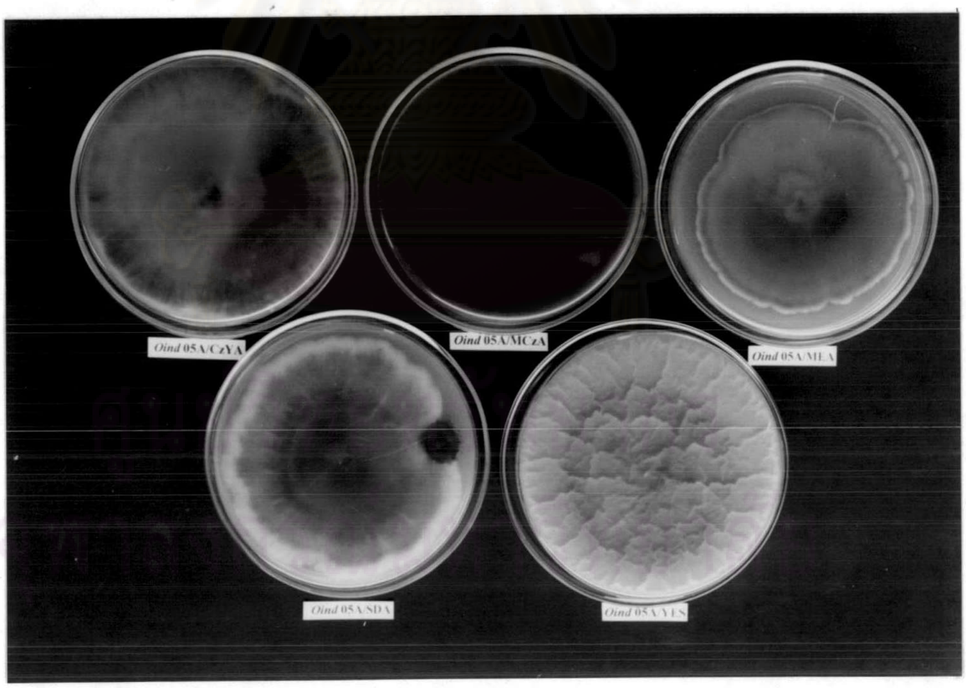


Figure 21 Obverse (A) and reverse (B) of colony morphology of endophytic fungus isolate *Oind* 05A grown on 5 different media at 30 °C for 14 days.

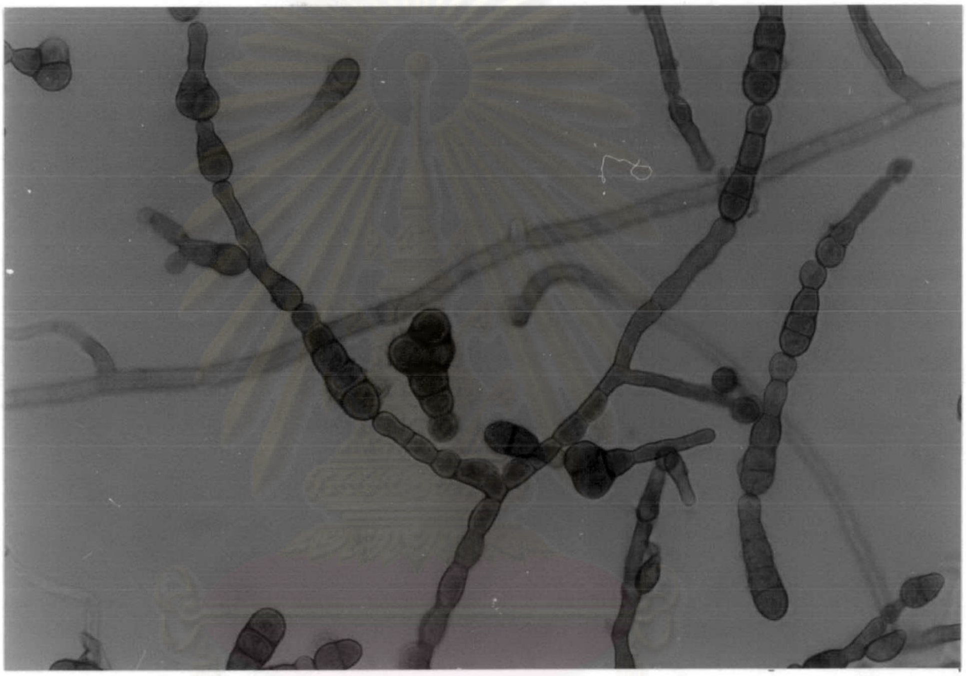


Figure 22 Microscopic morphology of endophytic fungus isolate *Line13* showing dematiaceous fungus with chain of conidia and sympodial conidiophore (x400)

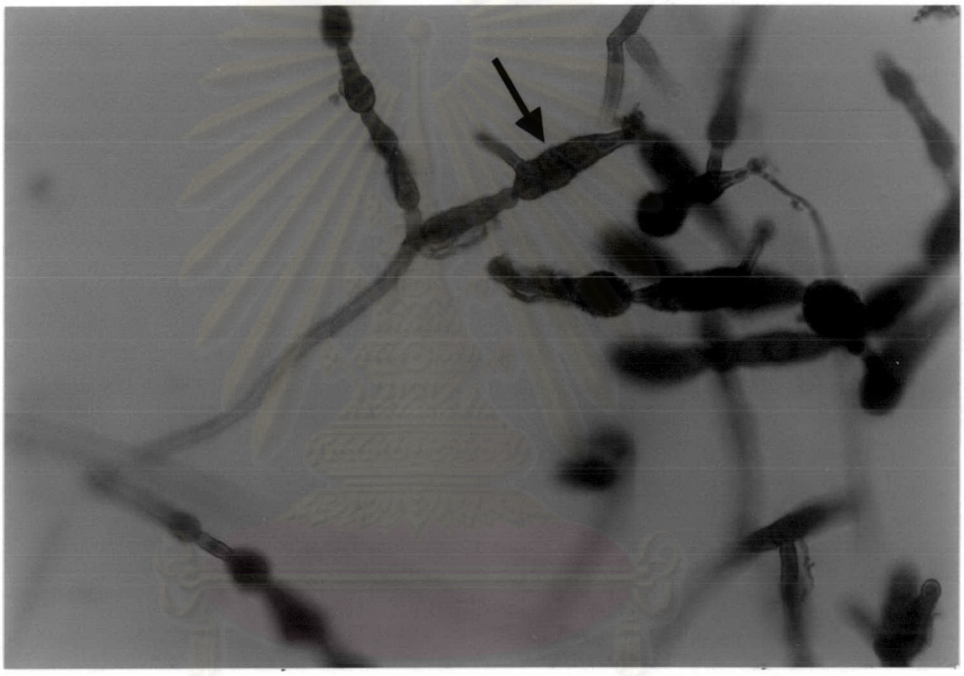


Figure 23 Microscopic morphology of endophytic fungus isolate *Line13* showing conidia in the form of dictyospore (arrow) and sympodial conidiophore (x400).

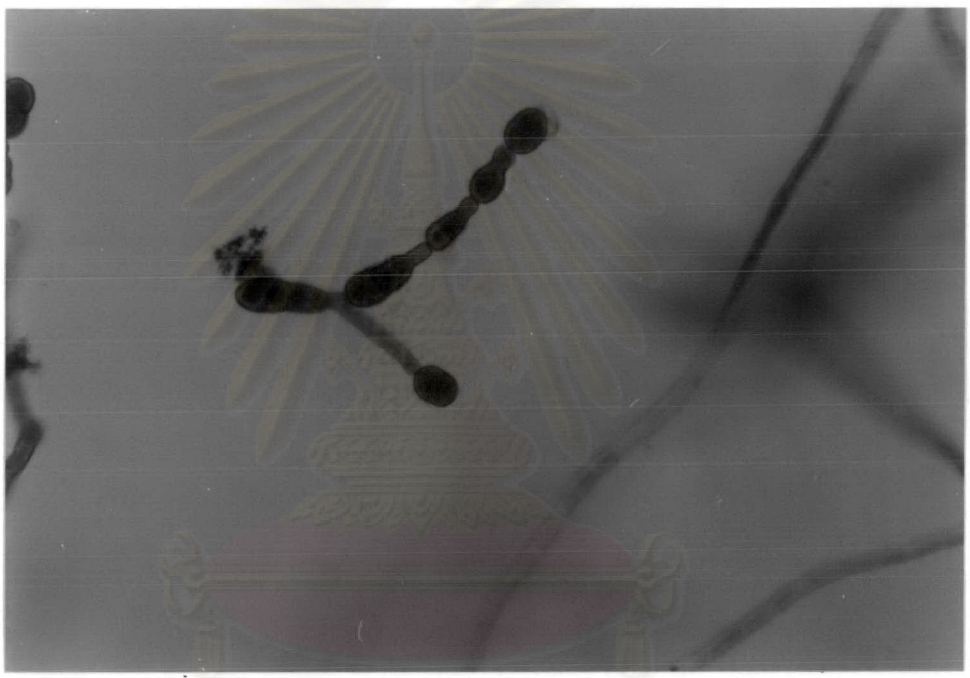


Figure 24 Microscopic morphology of endophytic fungus isolate *Line13* showing acropetal conidial development (×400).