CHAPTER III

EXPERIMENT

1. Sample collection, isolation and primary screening of actinomycetes

1.1 Sample collection and isolation of strains

Ninety eight soil samples collected at 3-8 centimeter deep (Tortora *et al.*, 1995) of natural soils such as forest soil and mountain soil in Chiangrai, Nan, Phatthalung, Satun, Songkhla, Chaiyaphum, and Trat provinces, Thailand (Table 4.1) were preserved at 4 $^{\circ}$C until the process of isolation. A 1.0 g of each soil sample was suspended in sterile distilled water (4.5 ml), heated at 55 $^{\circ}$C for 6 minutes and was diluted to 1:1 and 1:10 dilutions. Each dilution (0.1 ml) was spreaded on surface of Modified Starch Casein Nitrate Agar plates and Potato Starch-Glycerol Agar plates (Appendix A) plus 10 μg/ml of tetracycline for the selection of *Streptomyces* species and plus 50 μg/ml of novobiocin for the selection of *Kitasatospora* species (Tajima *et al.*, 2001; Takahashi and Omura, 2003). Agar plates were incubated at 30°C for 7-14 days depended on the growth of each isolate. The different colonies were pick up and streaked for purification on Yeast Extract- Malt Extract Agar plate (YMA, Appendix A). The plates were incubated at 30°C for 7-14 days. Colonies were transferred to cultivate on YMA slants incubated at 30 $^{\circ}$C for14 days. Stock cultures on slant were kept in cold room at 4 $^{\circ}$C.

1.2 Primary screening of antimicrobial activity of the strains

All of the strains were streaked on the edge of YMA plates which were then incubated at 30°C for 14 days. Six test microorganisms, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, *Micrococcus luteus* ATCC 9341, *Escherichia coli* ATCC 25922 on Nutrient Agar (NA) (Appendix A) slants and *Candida albicans* ATCC 10231 on Sabourad Dextrose Agar slants (SDA) (Appendix A) were vertical streaked from the edges of each strain. The plates were incubated for 24 hr. at 37 $^{\circ}$C. The clear inhibition distances of each test microorganisms were measured.

1.3 Bioautographic method on TLC plate

Crude extract of strain S3-1 was use in bioautographic method for showing the R_f value that have active spot. The developed thin layer chromatography plate is placed on the agar plate that has been inoculated with the test organisms. The diffused substances from the chromatographic layer will be inhibited to the test microorganisms on agar plate. The clear inhibition distances of each test microorganisms were observed after incubation for 1-2 days.

2. Identification methods

Morphological, cultural, physiological, and biochemical properties of microorganisms were determined by the methods as described in the International *Streptomyces* Project (ISP) (Shirling and Gottieb, 1966; Arai, 1975).

2.1 Morphological and cultural characteristics

2.1.1 Morphological characteristics

All strains were grown on YMA plate (Appendix A) by crosshatches streak technique (Shirling and Gottlieb, 1966) and incubated at 30°C for 10-14 days. Spore bearing hyphae, spore chain, and spore morphology of them were studied by using simple inclined coverslip technique (William and Cross, 1971). The spores and mycelia were observed under scanning electron microscope (SEM) using the method of Deman and Gupta, 1986.

2.1.2 Cultural characteristics

Cultural characteristics were studied based on the colors of mature aerial mycelium, substrate mycelium, spore, and diffusible soluble pigment using crosshatch streak (Shirling and Gottlieb, 1966). The strains were cultured on five different agar media, YMA, Oatmeal, Tyrosine, Glycerol-Asparagine Agar and Inorganic Salt-Starch Agar (Appendix A). They were incubated at 30°C for 7-14 days. The color of the reverse (under) side of the mass growth of substrate mycelium on five media, spore color and cultural characteristics were observed. The Jacal Color Card L2200 (Japan Color Research Institute) was used in the determination of the color designations of colonies.

2.2. Physiological and biochemical characteristics

The 18 strains which showed antimicrobial activity against many test microorganisms or showed wide inhibition zone of primary screening at least 20.1 mm (+++) against one test microorganisms were selected for further study. The physiological and biochemical characteristics were studied for all of the selected strains.

2.2.1 Melanin production

The selected strains were cultivated on a Tyrosine Agar plate (Appendix A) and incubated at 30°C for 4 days which was then compared with uninoculated plate. The colony forming a brown or black diffusible pigment was recorded as positive (+). Absence of brown or black colors, or total absence of diffusible pigment, was recorded as negative (-) melanin formation.

2.2.2 Carbon utilization

Basal agar medium (Appendix A) was prepared and a carbon source was added to give a concentration of approximately 1%. After autoclave at 110°C for 10 min, the mixture was agitated and 25 ml of this mixture was poured into 9 cm diameter petri dish. Carbon sources and controls required for the test are repeated below:

No carbon source (negative control), D-glucose (positive control), Glycerol, L-arabinose, Sucrose, D-xylose, D-manitol, D-fructose, Sucrose, Melibiose, Rhamnose, Raffinose.

The plates were observed at 10-14 days. Growth on given carbon source was always compared with the two controls; growth on basal medium alone and growth on basal medium plus glucose. Result were recorded as follows:

Strong positive utilization (++) when growth on tested carbon in basal medium was equal to or greater than growth on basal medium plus glucose. Positive utilization (+) when growth on tested carbon was significantly better than on basal medium without carbon, but somewhat less than on glucose. Utilization doubtful (+/-) when growth on tested carbon was only slightly better than on the basal medium without carbon and significantly less than on glucose. Utilization negative (-) when growth was similar to or less than growth on the basal medium without carbon.

2.2.3 Acid production from carbohydrates (Gordon *et al.*, 1974)

The selected strains were inoculated into Basal Inorganic Nitrogen medium (Appendix A). The pH value of this medium was adjusted to 7.0 before the addition of 0.04% solution of bromocresol purple. After this agar medium was sterilized by autoclaving, 0.5 ml of a 10%

solution of each carbohydrate was added aseptically to the tubes. Cultures on slants were observed after 7 and 28 days of incubation at 30°C. If acid productions were present, the slants would become yellow.

2.2.4 Hydrolysis of esculin (Gordon *et al.*, 1974)

The selected strains were inoculated into Esculin broth (Appendix A), incubated at 30°C, and observed for growth and blackening of the medium at 2 and 4 weeks. A tube of the same broth without esculin was also inoculated and used as a control.

2.2.5 Nitrate reduction

The selected strains were inoculated into Peptone KNO_3 (Appendix A) and incubated at 30°C for 4-6 days. On forth day, 1 ml of culture was transferred into a test tube and three drops of sulfanilic acid reagent and two drops of *N,N*-dimethyl-1-naphthylamine solution were sequentially added. If nitrate were present, the mixture would become pink to red.

2.2.6 Starch hydrolysis

The selected strains were streaked on the surface of Inorganic salts-Starch agar plate (Appendix A) and incubated at 30°C for 10 days. After incubation, Gram's iodine solution was poured on the surface of agar plate. If the starch hydrolysis was present, a dark blue color did not appear.

2.2.7 Gelatin liquefaction

The selected strains were inoculated into the test tube of Bouillon Gelatin broth (Appendix A) and incubated at 30°C for 21 days. The inoculated tube was compared with control when places both tubes at 20 °C for 30 min. If the gelatin was hydrolyzed, it was liquid not solid.

2.2.8 Milk coagulation and milk peptonization

The selected strains were inoculated in tube of sterile 10% Skim milk (Appendix A) in distilled water and incubated at 30°C for 7- 14 days. If milk was peptonized, milk would be converted to clear solution. If milk was coagulated, milk would precipitate.

2.2.9 NaCl tolerance

The selected strains were streaked on YMA plates (Appendix A) to which NaCl concentrations were added to give concentrations of 2, 4 and 6%. The plates were incubated at 30°C for 7-14 days. The result was recorded as positive if the culture grew.

2.2.10 Temperature tolerance

The selected strains were streaked on YMA plates (Appendix A) and were incubated at 10, 28 and 45 ^{o}C for 7-14 days. The result was recorded as positive if the culture grew.

2.2.11 pH tolerance

The selected strains were streaked on YMA plates (Appendix A) which separately adjusted pH to 4, 5, 7, 9, and 10. The plates were incubated at 30^{o}C for 7-14 days. The result was recorded as positive if the culture grew.

2.3 Chemotaxonomic characteristics

The selected strains were cultivated on Yeast Extract- Malt extract broth on rotary shaker 200 rpm at 30^{o}C for 4 days. Cells were collected by centrifugation and were dried by lyophilization.

2.3.1 Cell wall analysis

The chemical analysis of cell wall diaminopimelic acid (DAP) isomers were carried out by method of Kutzner, 1981. Dried whole-cell (10 mg) of selected strains were hydrolyzed with 6N HCl at 100 ^{o}C for 18 hours. DAP isomers were separated by Thin Layer Chromatography on cellulose plate (Merck No. 1.05577). The standard of *meso*-DAP (0.01 M) was applied for reference purposes. The solvent system to developed TLC is methanol-water-10 N HCl-pyridine (80:17.5:2.5:10, v/v). The spots were visualized by spraying with 0.2% ninhydrin solution in water-saturated n-butanol followed by heating at 100^{o}C for 5 minutes. DAP isomers appear as dark-green spots with R_f 0.29 (L-isomer) and 0.24 (*meso* and DL-isomer). Spots will gradually disappear in a few minutes.

2.3.2 Menaquinone analysis (Komagata and Suzuki, 1987)

Dried cells (100-500 mg) were extracted with chloroform : methanol (2:1) for 2-3 h. The suspension was then filtered and dried under rotary evaporator. The dried sample was dissolved with a small amount of acetone and applied onto a silica gel TLC (Merck no.1.05744). The applied TLC was developed by petroleum-diethyl ether (85:15, v/v) and the band of menaquinone was detected under a UV lamp 254 nm. The menaquinone band was scraped and dissolved with methanol (HPLC grade). The suspension was filtered and dried it up with N_2 gas. The menaquinone sample was analyzed by HPLC.

2.3.3 DNA base composition analysis (Yamada and Komagata, 1970; Tamaoka, 1994)

DNA was isolated from cells grown in Yeast Extract- Malt Extract broth with 0.2 % glycine for 3-4 days. Cells were harvested and suspended in 10 ml of saline-EDTA buffer pH 8.0 (Appendix B). The cell suspension was inoculated with 12 mg of lysozyme at 37°C for 2 hours followed by incubation with Tris-SDS and heat at 55°C for 10 min. The phenol extraction was then carried out by adding an equal volume of phenol:chloroform (1:1) (Appendix B) to the sample for removal of protein and other debris. The upper layer of mixture was collected after centrifugation at 12,000 rpm for 10 min. DNA was precipitated with ice cold 95 % ethanol. DNA was dissolved with 0.1 x SSC (Appendix B) and treated with RNase A and RNase T_1 solution (Appendix B) at 37 °C for 1 hr for the removal of RNA. DNA was stored in 0.1 x SSC at 4 °C. The 10 µl of DNA (1 mg/ml) was hydrolyzed with 10 µl nuclease P_1 at 50 °C for 1 h and followed by reacting with 10 µl of alkalines phosphatase incubated at 37 °C for 1 h. The hydrolyzed DNA was applied to HPLC. An equimolar mixture of nucleotides was used as the quantitative standard for analysis of DNA base composition.

3. 16S rDNA sequence analysis and phylogenetic tree construction

3.1 16S rDNA amplication by PCR

The isolated DNA was used for 16S rDNA amplication by PCR.

The PCR was performed in a total volume of 50 µl containing

- 1 µl of DNA sample
- 0.5 µl of Taq DNA polymerase
- 5 µl of 10 x polymerase buffer
- 5 µl of dNTP mixture
- 0.5 µl of forward primer
- 0.5 µl of reverse primer
- 37.5 µl of Millique water

A DNA Thermal Cycle (Gene Amp PCR System 2400: Perkin Elmer) was used with a temperature profile of 3 min at 95 °C followed by 30 cycles of 30 sec at 95 °C (denaturing of DNA), 15 sec at 55°C (primer annealing), and 1 min at 72 °C (polymerization). At the end of the cycle, the reaction mixture was kept at 72 °C for 5 min and then cooled to 4°C. The 1.5 kb amplified 16S rDNA fragment was separated by agarose gel electrophoresis. The PCR amplified products were analyzed by running 5 µl of reaction mixture on a 1% agarose gel in Tris-acetate EDTA buffer (Appendix B). Agarose gel was stained in an ethidium bromide solution (0.5 mg/ml) and examined under UV- transilluminator to visualize the amplified 16S rDNA band.

3.2 16S rDNA sequence

The amplified 16S rDNA was used as templates for sequencing with big dye terminator sequencing Kit (Perkin Elmer) and analyzed by AB1377 automated DNA sequencer (Perkin Elmer). The sequencing reaction for each sample was performed in DNA Thermal Cycler (Gene Amp PCR System 2400; Perkin Elmer) with a temperature profile of 30 sec at 96 ^{o}C followed by 25 cycles of 10 sec at 96 ^{o}C (denaturing of DNA), 5 sec at 50 ^{o}C (primer annealing), and 4 min at 60 ^{o}C (polymerization). Sequencing for each sample is carried out in both forward and reverse directions (Appendix C).

3.3 16S rDNA sequence analysis and phylogenetic tree construction

Homology search was performed using the standard BLAST sequence similarity searching program version 2.2.1 from the web server http://www.ncbi.nlm.nih.gov/BLAST/ against previously reported sequence at the GenBank/EMBL/DDBJ database. The sequence was multiply aligned with selected sequences obtained from the three main databases by using the CLUSTAL W version 1.81. The alignment was manually verified and adjusted prior to the construction of phylogenetic tree. The phylogenetic tree was constructed by using neighbor-joining (Saitou and Nei, 1987) in the MEGA program version 2.1 (Kumar *et al.*, 2001). The confidence values of branches of the phylogenetic tree were determined using the bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The values for sequences similarity among the closest strains were calculated manually after pairwise alignments obtained using the CLUSTAL X program (Thompson *et al.*, 1997). Gaps and ambiguous nucleotides were eliminated from calculations.

3.4 DNA-DNA hybridization

DNA for DNA-DNA hybridization was purified as mentioned above. The spectrophotometric method for DNA quantitation was used to determine both concentration and relative purity of DNA in a solution. Two aborption spectra (A_{260} and A_{280}) was used. The DNA is suitable for DNA-DNA hybridization if the ratio of A_{260}/A_{280} is between 0.56-0.59.

3.4.1 DNA labeling probe with photobiotin

A 10 µl of DNA solution (1 mg/ml) and 15 µl of photobiotin solution (1 mg/ml) were mixed in an eppendorf tube and the irradiated with sunlamp for 30 min on ice. After irradiation, free photobiotin was removed by adding 100 µl of 0.1 M Tris-HCl buffer pH 9.0, and 100 µl of n-

butanol into biotinylated DNA solution. The solution was then mixed and centrifuged at 12,000 rpm for 20 seconds. The upper layer was removed. Again 100 µl of n-butanol was added, and the solution mixed, centrifuged and the upper layer was removed. After centrifugation at 12,000 rpm for 20 seconds, the upper layer was removed. The biotinylated DNA solution was boiled for 15 min and then immediately cooled in ice. The solution was sonicated for 3 min and dissolved in 10 ml of hybridization solution (Appendix B).

3.4.2 Photobiotin labeling DNA-DNA hybridization

The photobiotin labeling DNA-DNA hybridization was performed by the method described by Ezaki *et al.*, (1989). DNA (10 µg) of selected strains, type strain and reference DNA (Calf thymus) were boiled for 10 min and immediately cooled in ice. Then 500 µl of 2 x PBS (Appendix B), 100 µl of 0.1 $MgCl_2$ and sterile distilled water were added to make a total volume of 1 ml and mixed well. 100 µl of a heat denatured DNA solution was added to microdilution wells and fixed by incubation at 37 ^{o}C for 2 hours. After incubation, the DNA solution was removed, and 200 µl of prehybridization solution (Appendix B) was added to microdilution wells. Microdilution plate was incubated at 54^{o}C (hybridization temperature) for 1 hour. The prehybridization solution was removed from the wells and replaced with 100 µl of a hybridization solution mixture containing biotinylated DNA. Microdilution plate was incubated at 54^{o}C (hybridization temperature) for 15 hours.

3.4.3 Detection of biotin-containing hybrids

After hybridization, the microdilution wells were washed three times with 200 µl of 0.2x SSC buffer. Then 100 µl of Streptavidin-Peroxidase solution (solution I) (Appendix B) was added and incubated at 30 ^{o}C for 10 min. Solution I (Appendix B) was removed from the wells and replaced with 100 µl of solution II (Appendix B). Microdilution plate was incubated at 37 ^{o}C for 30 min. After incubation, microdilution plate was washed three times with 200 µl of PBS. 100 µl of solution III (Appendix B) was added, and the plate was incubated at 37 ^{o}C for 10 min. The enzyme reaction was stopped with 100 µl of 2M H_2SO_4 (Appendix B). The absorbance was measured at 450 nm with Microplate Reader (Microplate Manager 4.0 Bio-Rad Laboratories, Inc.) and calculated for the value of percentage DNA homology.

4. Fermentation of the selected strains for antimicrobial productions

A loopful of selected strains was inoculated into 100 ml of seed medium consisting of 0.4% glucose, 0.4% yeast extract, 1% malt extract, pH 7.3 in 250 ml Erlenmeyer flask. The flask was incubated on a rotary shaker 200 rpm at 28°C for 4 days after that 5 ml of the seed medium was transferred into 500 ml Erlenmeyer flasks containing 250 ml of the production medium consisting of 0.4% glucose, 0.4% yeast extract, 1% malt extract, 0.1% $CaCO_3$, pH 7.3. The flasks were incubated on a rotary shaker 200 rpm at 28°C for 7 days (Shirato and Motoyama, 1966).

5. Chromatographic techniques

5.1 Analytical thin-layer chromatography

Technique : One dimension ascending

Sample loading : The sample was dissolved in a small volume of the eluant and spot on TLC

Adsorbent : Silica gel GF_{254} coated on an aluminium sheet (E.Merck)

Layer thickness : 250 μm

Distance : 5 cm

Temperature : laboratory temperature (25-30°C)

Detection : 1. Visual detection under daylight

2. Visual detection under ultraviolet light at wavelengths of 254 and 365 nm.

5.2 Column chromatography

5.2.1 Quick column chromatography

Adsorbent : Silica gel (No. 7734), partical size 0.040-0.063 μm (E. Merck)

Packing method : The adsorbent was slurried in the eluant and poured into a column and allowed to settle overnight.

Sample loading : The sample was triturated with siliga gel, dried and loaded on top of the column

Detection : Fractions were examined by TLC technique in the same manner as described in section 5.1

5.2.2 Flash column chromatography

Adsorbent : Silica gel (No. 7734), partical size 0.040-0.063 μm (E. Merck)

Packing method : The adsorbent was slurried in the eluant and poured into a column and allowed to settle overnight.

Sample loading : The sample was dissolved in a small volume of the eluant and loaded on top of the column

Detection : Fractions were examined by TLC technique in the same manner as described in section 5.1

5.3 Solvents

Throughout this work, all commercial grade organic solvents were redistilled prior to use.

6. Extraction and fractionation of the extract of *S. spectabilis* S3-1

6.1 Extraction

The YM fermentation broth culture (20L) of *S. spectabilis* S3-1 was filtered through a glass funnel packed with Whatman No. 1. The filtrate was partitioned with ethyl acetate 3 times. The ethyl acetate layer was collected and concentrated under reduced pressure at 45 ^{o}C to yield 1.43 gram of the ethyl acetate extract (dark brown oily liquid) as shown in Scheme 3.1.

6.2 Fractionation

The dark brown oily liquid showed the yellow spot on TLC (Si Gel, 15% MeOH in CH_2Cl_2, R_f 0.8) as the major product. The main yellow spot was isolated by chromatographic techniques to obtain the pure compounds.

The crude extract of S3-1 was purified by quick column chromatography using silica gel as the adsorbent (column 4 x 4 cm.). The gradient of EtOAc : hexane was used as a mobile phase and methanol was finally used to elute the extract. Twenty five milliliters of fractions were collected. Combination of the fractions with similar chromatographic patterns (TLC Si Gel, 30%hexane in EtOAc) gave 8 fractions (S001, S002, S003, S004, S005, S006, S007, S008) as shown in Table 3.1 and Figure 3.1.

Table 3.1 Fractions obtained from the crude extract of S3-1

Fraction code	Fraction	Mobile phase	Volume of mobile phase (ml)	Total weight (mg)
S001	1-5	Hexane	125	89.0
S002	6-10	5% EtOAc in hexane	125	86.0
S003	11	10% EtOAc in hexane	25	96.4
S004	12-19	10% EtOAc in hexane	200	217.0
	20-27	20% EtOAc in hexane	200	
	28-32	30% EtOAc in hexane	125	
S005	33-39	50% EtOAc in hexane	175	158.2
S006	40-45	50% EtOAc in hexane	150	92.8
S007	46-55	50% EtOAc in hexane	250	183.2
	56-58	70% EtOAc in hexane	75	
S008	59-63	90% EtOAc in hexane	125	504.0
	64-72	100% EtOAc	225	
	73-76	MeOH	100	

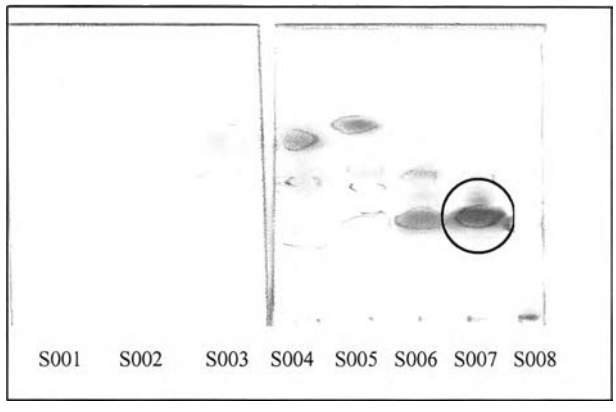


Figure 3.1 Chromatographic patterns of fractions obtained from the crude extract of S3-1 (TLC Si Gel, 30%hexane in EtOAc)

Fraction code S007 was selected to purify by flash column chromatography using silica gel as adsorbent (column 2 x 16 cm.). The 2% MeOH in CH_2Cl_2 was used as a mobile phase and methanol was finally used to elute the extract. Fifteen milliliters of fractions were collected. Combination of the fractions with similar chromatographic patterns (TLC Si Gel, 5%MeOH in CH_2Cl_2) gave 5 fractions (S009, S010, S011, S012, S013) as shown in Table 3.2 and Figure 3.2.

Table 3.2 Fractions obtained from S007

Fraction code	Fraction	Mobile phase	Volume of mobile phase (ml)	Total weight (mg)
S009	1-4	2 % MeOH in CH_2Cl_2	60	9.0
S010	5-6	2 % MeOH in CH_2Cl_2	30	51.3
S011	7-15	2 % MeOH in CH_2Cl_2	135	71.1
S012	16-27	2 % MeOH in CH_2Cl_2	180	24.8
S013	28-49	2 % MeOH in CH_2Cl_2	330	25.0
	50-55	MeOH	90	

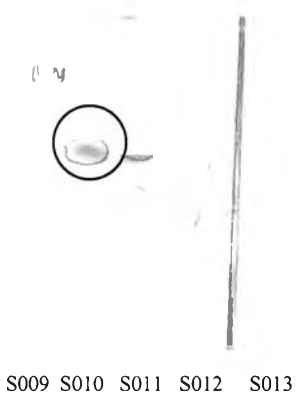
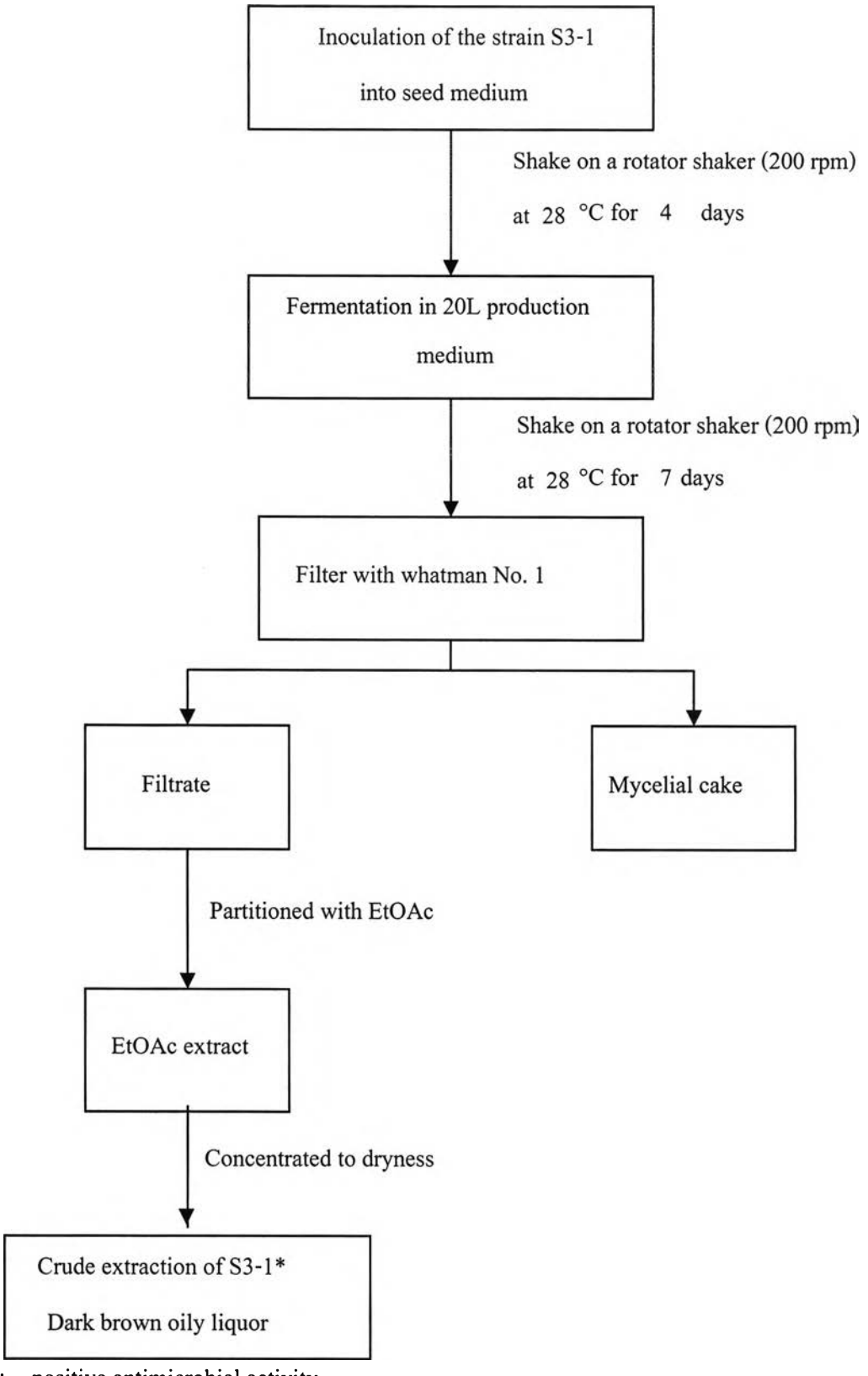


Figure 3.2 Chromatographic patterns of fractions obtained from S007 (TLC Si Gel, 5%MeOH in CH_2Cl_2)

All fractions code S001, S002, S003, S004, S005, S006, S007, S008, S009, S010, S011, S012, and S013 were monitored for antimicrobial activity by agar disc diffusion method.

7. Antimicrobial activity

The fermentation broth was filtered through Whatman filter paper No.1. The filtrate was partitioned with ethyl acetate 3 times. The ethyl acetate layer was collected and concentrated under reduced pressure at 45 ^{o}C. The antimicrobial activity of the isolate extracts was examined by an agar disc diffusion method (Lorian, 1991). Evaluation of the antimicrobial activity was performed against nine test microorganisms, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, Methicillin resistant *Staphylococcus aureus* 266, 269, 643, *Pseudomonas aeruginosa* ATCC 27853, *Micrococcus luteus* ATCC 9341, *Escherichia coli* ATCC 25922. The bacteria were cultivated on Mueller-Hinton Agar (MHA) slant at 37 ^{o}C for 24 hours while *Candida albicans* ATCC 10231 was cultivated on Sabourad Dextrose Agar (SDA) slant at 30^{o}C for 24 hours. The cell cultures were washed from an agar surface and suspended with normal saline solution and standardized to match a turbidity of McFarland standard No.0.5, providing approximately 1x10^{8} CFU (colony forming unit/ ml). A swab of each tested microorganism was spread on the surface of each plate. Test samples were dissolved in methanol solvent and then were applied on sterile paper disc (diameter 6 mm) 1 mg/disc and using methanol 20 µl for the disc control. These paper discs were left in a sterile Petri dish until the solvent was completely dried. The dried paper discs were placed on the surface of the inoculated agar plate and were incubated at 37^{o}C, 24 hours for bacteria and 48 hours for yeast. The diameter of inhibition zones were measured.



* positive antimicrobial activity

Scheme 3.1 Fermentation and extraction of YM fermentation broth of *S. spectabilis* S3-1