CHAPTER III

MATERIALS AND METHODS

1. Instruments, chemical agents, list names of tested strains, antibiotics culture media, reagents and buffers,

All materials name list were presented in APPENDIX A and B.

2. Sample collection, isolation, pH measurement and primary screening of actinomycetes

2.1 Sample collection

Soil samples from mangrove forests along Samut prakarn, Samut sakorn, Samut songkram, and Petchaburi provinces were collected and preserved at 4 °C.

2.2 Isolation and pH measurement of actinomycetes

Each of the soil samples was dried both at room temperature for 1 week and at 110°C for 1 h. The dried soil samples (0.5 g) were suspended in 4.5 ml of sterile distilled water. The room temperature dried soil suspensions were heated to 60-65 °C (15 min) to reduce non-thermotolerant microorganisms. Suspension of soil samples dried at both temperatures was serial diluted to 1:100 and 1:1000. The diluted soil samples (0.1 ml.) were spreaded on starch-casein nitrate agar (SCA) medium containing 15 μg/ml novobiocin and 25 μg/ml nistatin, and incubated at 30 °C for 14-21 days. Actinomycetes colonies were further purified by streak plate method on yeast extract-malt extract agar (YMA) medium, and incubated at 30 °C for 7-14 days. The purified cultures were maintained on YMA slant at 4°C for further studies.

The soil samples were dried at room temperature for 1 week. One gram of dried soil samples were suspended in 2.5 ml of sterile distilled water mix for 30 seconds, leave for 30 mins, gently mix 2-3 seconds measure pH and record data.

2.3 Primary screening of antimicrobial producing actinomycetes

All of the actinomycetes isolated were streaked separately on YMA medium, and incubated at 30°C for 10 days. Then six microorganisms tested; *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 16633, *Staphylococcus aureus* ATCC 6538P, *Micrococcus luteus* ATCC 9341, and *Candida albicans* ATCC 10231; were vertical streaked on each of the actinomycetes grown YMA plates. The plates were continuing incubated at 37°C for 24 hrs.

The antimicrobial producing actinomycetes exhibited inhibitory distance against microorganisms tested. The inhibitory distances were measured and recorded, and the actinomycetes were selected for further studies.

3. Identification and characterization of the isolated actinomycetes

3.1 Morphological and cultural characteristics

All actinomycetes isolated were cultivated on YMA plate by crosshatches streak technique (Shirling and Gottlieb, 1966) and incubated at 30°C for 14 days. Morphology of spore bearing hyphae, spore chain, and spore color were observed by simple inclined coverslip technique (William and Cross, 1971). Mature spore bearing hyphae of the selected isolates were also examined by scanning electron microscope (SEM) using method described by Deman, *et. al.* (1986). Cultural characteristics of all actinomycetes isolated grown on various agar media, yeast extract-malt extract agar (ISP medium no.2), oatmeal agar (ISP medium no.3), inorganic salts-starch agar (ISP medium no.4), glycerol asparagine agar (ISP medium no.5), tyrosine agar (ISP medium no.7) at 30°C for the 14 days were observed for colors of mature substrate mycelium, spore, and diffusible pigment using crosshatches streak technique (Shirling and

Gottlieb, 1966). The Jacal (Japan Color Reserch Institute) Color Card L2200 was used to specify their colony color.

3.2 Physiological and biochemical characteristics

3.2.1 Temperature and pH tolerance

All *Streptomyces* strains were tested for the effect of temperatures (10°C, 28°C, 45°C) and pHs (4.0, 5.0, 7.0, and 10.0) on their growth on YMA medium.

3.2.2 NaCl tolerance

All *Streptomyces* strains were tested by the use of YMA medium containing 2%, 4% or 6% NaCl.

3.2.3 Carbon sources utilization

Carbon sources utilization was determined by method described by Shirling and Gottlieb (1966). Basal agar medium, ISP-9 supplement with 0.3% Casamino acid was prepared and a carbon source was added to give concentration of approximately 1% (D-glucose (positive control), D-mannitol, L-rhamnose, Raffinose, L-arabinose, D-fructose, Glycerol, D-xylose, and sucrose) autoclave at 110°C for 10 min. Examination by comparing growth or a given carbon source with two controls, growth on basal medium alone, and growth on basal medium plus glucose was performed.

3.2.4 Starch hydrolysis

Antimicrobial producing strains were streaked on the surface of inorganic salts- starch agar plate (ISP-4) (Shirling and Gottlieb, 1996) and incubated at 28-30°C for 10 days. After incubation was complete, Gram's iodine solution was poured on the surface of the agar plate. If starch hydrolysis was present, a dark blue color did not appear.

3.2.5 Gelatin liquefaction

Antimicrobial producing strains were inoculated into test tube of Bouillon gelatin broth (Arai, 1975) and incubated at 28-30°C for 21 days. The inoculated tube was compared with non-inoculate control when placed both tubes at 20°C for 30 min. If the gelatin was hydrolyzed, it became liquid, not solidify.

3.2.6 Nitrate reduction

Antimicrobial producing strains were inoculated into Peptone KNO_3 broth (Arai, 1975) and incubated at 28-30°C for 4-6 days. On the forth day, 1 mL of the culture was transferred into a test tube and two drops of the sulfanilic acid reagent, and then by three drops of N,N-dimethy1-1-naphthylamine solution were added. If nitrites were present, the mixture would become pink to red.

3.2.7 Milk coagulation and milk peptonization

Antimicrobial producing strains were inoculated in tube of sterile 10 % skim milk in distilled water and incubated at 28-30°C for 7-14 days. If milk was peptonized, milk would be converted to clear solution. If milk was coagulated, milk would precipitate.

The melanin formation and hydrogen sulfide production were examined in tyrosine agar and peptone iron agar supplemented with 0.1% (w/v) yeast extract and incubated for 21 days, respectively.

3.3 Chemotaxonomic characteristics (Komagata and Suzuki, 1987)

Actinomycetes isolates were inoculated into 500 ml Erlenmeyer flask containing 100 ml of YMB medium and incubated on a rotary shaker (150-200 rpm) at 30°C for 4 days. Cells were collected by centrifugation at 4°C, 12,000 rpm for 5 min.

3.3.1 Diaminopimelic acid analysis

Chemical analysis of cell wall was carried out by the method described by Komagata and Suzuki (1987). Dried cells (10mg) of the selected isolates were hydrolyzed with 6N HCL at 100°C for 3 h. The hydrolyzed solution was filtered and evaporated. The 400 μL of distilled water was added into dried sample. Diaminopimelic acid (DAP) isomers in the whole cell hydrolysates were separated by thin layer chromatography using cellulose plate (Merck No.5577). DL-DAP standard (0.01 M) and whole cell hydrolysates of two known strains that contained *meso*- and L-DAP were used as authentic DAP isomers. TLC was developed by methanol:water:6N hydrochloric acid:pyridine solvent system (80:17.5:1.5:10 w/v). The spots were visualized by spraying with 0.5% ninhydrin in water-saturated n-butanol followed by heating at 100°C for 2-3 minutes. The spots gradually disappeared in a few minutes.

3.3.2 Menaquinone analysis

Dried cells (100-500 mg) were extracted with chloroform:MeOH (2:1) for 3 hr. 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 then developed by 100% benzene and the band of menaquinone band was detected by using a UV lamp (254 nm). The menaquinone was filtered and dried it up with N_2 gas. The menaquinone sample was analyzed by HPLC.

3.3.3 DNA isolation and purification

DNA of the selected isolates grown in YMB containing 0.2% glycine were extracted and purified by method described by Tamaoka (1994). Cells were harvested and suspended in 10 mL of saline-EDTA buffer pH8.0. The cell suspension was mixed with 20 mg of lysozyme and incubated at 37°C for 30 min followed by the incubation period of 10 min at 50°C with 10% SDS. The phenol

extraction was then carried out by adding an equal volume of phenol: chloroform (1:1) to the sample for removal of protein and other debris. The upper layer of the mixture was collected after centrifugation at 10000 rpm for 10 min. Chromosomal DNA was precipitated with two volumes of ice cold absolute ethanol. DNA was dissolved with 0.1xSSC and treated with RNase and proteinase K solution at 37°C for 1 h for removal of RNA and protein, respectively. Chromosomal DNA was stored in 0.1xSSC at 4°C

3.3.4 DNA base composition analysis

The 10 μL of heated DNA (1mg/mL) was hydrolyzed with 10 μL nuclease P_1 at 50°C for 1 h and followed by the incubation period of 1 h at 37°C with 10μL of alkaline phosphatase. The hydrolyzed DNA was determined using the HPLC method of Tamaoka & Komagata (1984). An equimolar mixture of nucleotides was used as the quantitative standard for analysis of DNA base composition.

3.4 16S rDNA sequence analysis and phylogenetic tree construction

16S rDNA of the selected isolates were amplified by Thermal cycle sequencing method (GeneAmp PCR System 2400; Applied Systems) according to the following program: 94°C (5 min) followed by 35 cycles of denaturation at 94°C (1 min), primer annealing at 50°C (1 min), and primer extension at 72°C (2 min). At the end of the cycles, the reaction mixture was kept at 72°C for 5 min and then cooled to 4°C. Forward and reverse primers were 5'.....3', and 3'......5', respectively. The 1500 kbp amplified 16S rDNA fragment was separated by agarose gel electrophoresis and purified by a Prep-A-Gene DNA purification kit (Bio-Rad Laboratories, Hercules, Calif). The 16S rDNA fragment was sequenced by an ABI PRISM BigDye Terminator Cycle sequencing Ready Reaction kit (Applied Biosystem) according to the manufacturer protocol, using the following primers, 5'.....3' and 3'......5'. The thermal cycling condition was as followed 96°C (30 s), followed by 30 cycles of denaturation at 96°C (10 s), primer annealing at 50°C (10 s), and primer extension at 60°C (3 min). The sequences were analyzed by an ABI PRISM 377 Genetic Analyser (Applied Biosystems)

at DNA Technology Laboratory (BIOTEC), Kasetsart University, Kamphaengsaen, and Nakornpathom.

The sequence homology search was analyzed by standard BLAST sequence similarity searching program version 2.2.1 available from http://www.ncbi.nim.gov/BLAST/:. The sequence was multiply aligned with selected sequence obtained from GenBank/EMBL/DDBJ database using CLUSTAL W program version 1.81 available from http://workbench.sdsc.ed/:. and the alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. Phylogenetic tree was constructed by neighbour-joining method (Saito and Nei, 1987) using MEGA program version 2.1 (Kumar, *et. al.* 2001). The confidence value of branch of the phylogenetic tree was determined by bootstrap analysis (Felsenstein, 1985) based on 1000 samplings. The value of sequence similarity among closest strains was calculated manually after pairwise alignment using the CLUSTAL W program version 1.81. Gaps and ambiguous nucleotides were eliminated from a calculation (Fitch, 1967).

4. Antimicrobial activity test (agar disc diffusion method)

Single colony of the 2 new actinomycetes species and 6 actinomycetes species showed interesting antimicrobial activity were inoculated into 500 ml Erlenmeyer flask containing 100 ml of YMB, and incubated at 30°C on a rotary shaker (150-200 rpm) for 4 days. Then 2 ml of each culture was transferred into a 500 ml Erlenmeyer flask containing 200 ml of production medium (YMB containing calcium carbonate), and incubated at the same above conditions for 10 days. The culture broths were filtered through Whatman filter paper and extracted with ethyl acetate. The ethyl acetate extracts were dried by rotary evaporator, dissolved in methanol then dried by vacuum dried. The antimicrobial activity of the extract were dissolved in methanol obtained were determined by an agar disc diffusion method (Lorian, 1980). Evaluation of the antimicrobial activity was performed against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 16633, *Staphylococcus aureus* ATCC 6538P, *Micrococcus luteus* ATCC 9341 and *Candida albicans* ATCC 10231. All tested bacteria were cultivated on Mueller-Hinton agar (MHA) slant whereas

the tested yeast strain was cultivated on Sabouraud dextrose agar (SDA) slant and incubated at 37°C for 24 hours. The cultures were washed from the agar slant by sterile normal saline solution. The cell suspensions were adjusted by McFarland NO 0.5, provided approximately $1x10^8$ CFU (colony forming unit/ml). Tested bacteria, and yeast were separately spreaded on Petri dish containing 20 ml of MHA, and SDA, respectively. A loopful of each tested microorganisms prepared was swabbed on the surface of MHA and SDA plates. Agar disc diffusion assay was performed by applying the methanol dissolved extract (1 mg) on a sterile paper disc (0.6 cm in diameter). The applied paper discs were completely air-dried at room temperature in a sterile Petri-dish. Then each dried discs was placed on the surface of MHA and SDA plates already swabbed by the tested microorganisms, and incubated at 37°C, 24 hours for bacteria and at 30°C, 48 hours for yeast. The diameters of inhibition zones were subsequently measured.

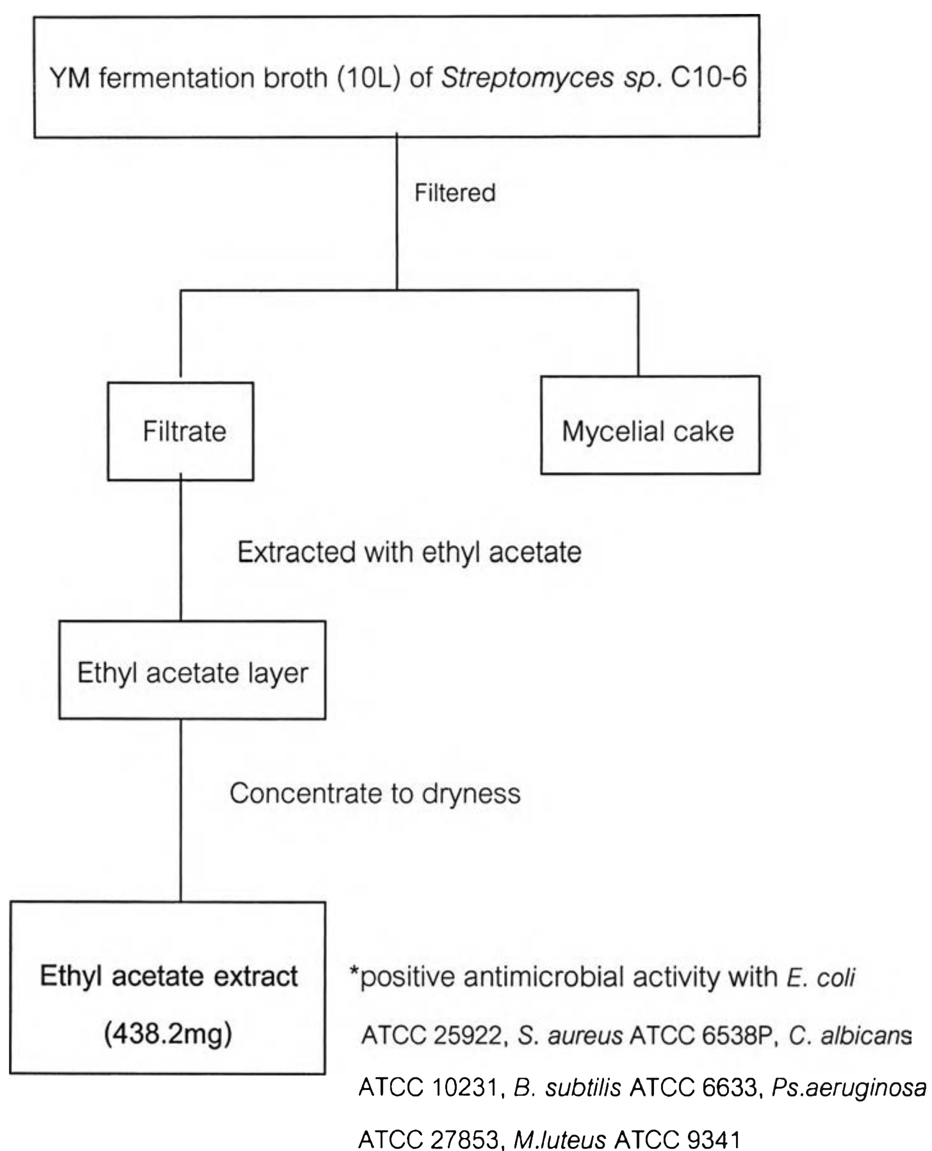
5. Extraction and isolation of antimicrobial products from *Streptomyces sp.* C10-6

The culture filtrates of new 2 actinomycetes species and 5 actinomycetes species possessing interesting antimicrobial activity were extracted with ethylacetate, rotary evaporation dried, dissolved in methanol, and vacuum dried as described above. The vacuum dried samples were re-dissolved in chloroform-*d* and subjected to nuclear magnetic resonance (NMR) analysis. Culture filtrate of strain C10-6 revealed interesting signals on the NMR spectrum, and was selected for chemical study.

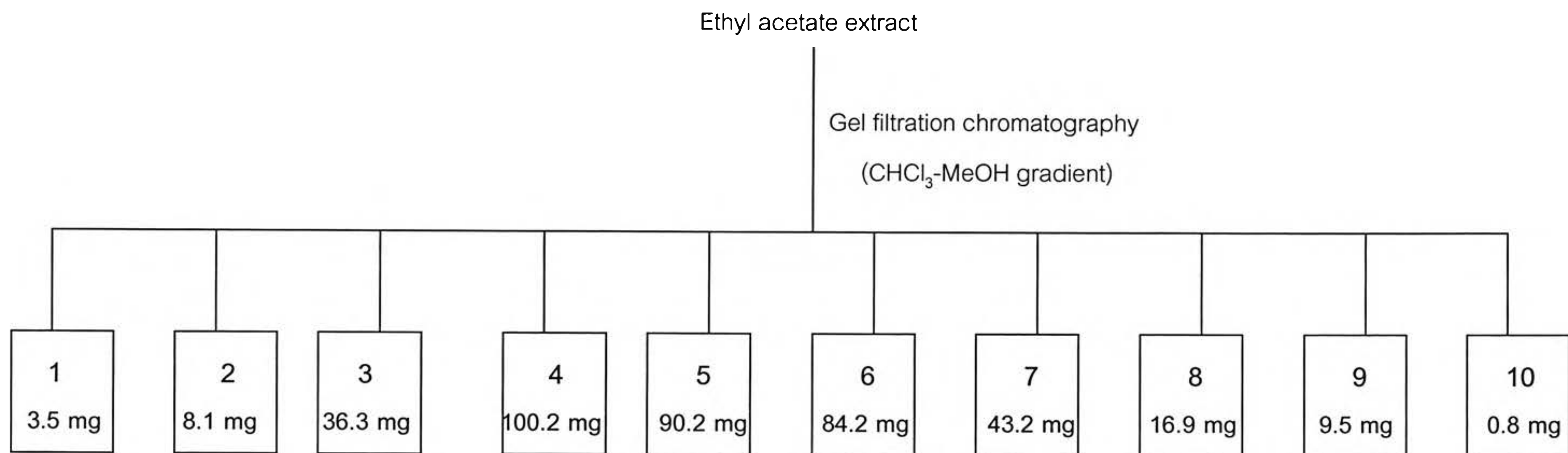
A loopful of *Streptomyces* sp. C10-6 was inoculated into 100 mL of YMB in a 500 mL Erlenmeyer flask, and incubated on a rotary shaker (200 rpm) at room temperature for 4 days. The vegetative seed obtained (2mL) was transferred into a 500 mL of Erlenmeyer flask containing 200 mL of YMB + 0.1%$CaCO_3$, and incubated at the same above conditions for 10 days. The YM fermentation broth (10L) of *Streptomyces* sp. C10-6 was filtered through a Buchner funnel. The filtrate was partitioned with ethyl acetate (10L*3). The ethyl acetate layer was collected and concentrated under

reduced pressure at 33°C to yield 438.2 mg of the ethyl acetate extract (dark brown oily liquid) as shown in Scheme 3.1.

The crude ethyl acetate extract (438.2mg) of *Streptomyces* sp. C10-6 was fractioned by gel filtration chromatography using siliga gel as an adsorbent as shown in Scheme 3.2. The gradient of MeOH in $CHCl_3$ was used as a mobile phase to give 10 fractions (1-10) (30 mL/ a fraction). Each fraction was further characterized by NMR and disc diffusion analysis against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 16633, *Staphylococcus aureus* ATCC 6538P, *Micrococcus luteus* ATCC 9341, and *Candida albicans* ATCC 10231.



Scheme 3.1 Extraction of the YM fermentation broth of *Streptomyces* sp. C10-6.



Scheme 3.2 Isolation of the ethyl acetate extract of *Streptomyces* sp. C10-6

6. Chemical study of antimicrobial agent

6.1 Column chromatography

6.1.1 Gel filtration chromatography

Adsorbent	: Sephadex LH-20 (Amersham Biosciences)
Packing method	: Sephadex LH-20 gel was suspened in an eluant and left standing overnight to swell prior to use, then poured into the column and allowed to settle.
Sample loading	: The sample was dissolved in a small volumn of an eluant (MeOH) and loaded on the top of column.
Detection	: Fractions were characterized by NMR and disc diffusion analysis.

6.2 Proton nuclear magnetic resonance spectroscopy (^{1}H NMR).

^{1}H NMR spectra of extracts and pure compounds as a purity check were recorded on a Bruker Gemini 2000 spectrometer operating at 200 MHz spectrometer (with $CDCl_3$, actone-d_6 and/or DMSO-d_6 as solvent). ^{1}H NMR spectra of pure compounds and all other NMR measurements were performed on Bruker AM-400 (400 MHz) NMR spectrometer operating at 400 MHz (^{1}H).

Spectra of pure compounds were processed using Bruker 1D WIN-NMR or 2D WIN-NMR software. They were calibrated using solvent signals (^{13}C: $CDCl_3$ 77.00 ppm, actone-d_6 30.50, 206.0 ppm and DMSO-d_6 39.5 ppm) or a signal of the portion of the partly or not deuterated solvent (^{1}H: $CHCl_3$ in $CDCl_3$ δ 7.26 ppm, acetone in acetone-d_6 δ d 2.05 ppm, water (H_2O) in acetone-d_6 $\delta \approx$ 2.8 ppm and DMSO-d_6 δ 2.50 ppm, and water (H_2O) in DMSO-d_6 δ 3.31 ppm).

6.3 Solvents

All solvents used in this research such as methanol, dichloromethane, ethyl acetate, and hexane were commercial grade and purified prior to use by distillation.

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