CHAPTER III

MATERIALS AND METHODS

1. Isolation of *Streptomyces strains*

Twenty four soil samples were collected from various sources in Thailand (Krabi, Chaiyaphoom, Chanthaburi, Chon Buri, Lampang Kanchanaburi, Nong kai, Ratchaburi, Chachoengsao, Srakaew), as shown in Table 9.

Five hundred milligrams of each dried soil sample was mixed with 1.5 ml of steriled distilled water and was kept at 55 °C 6 minutes and diluted to 1:10, 1:100, 1:1,000, 1:10,000 dilutions. One ml of each soil suspension was spreaded on two kinds of isolation media agar plates, potato carrot agar (PCA) and sodium casienate agar (SCA) with sterile glass rod. Each soil sample was incubated at 30 °C for 7-14 days and *Streptomyces* colonies were picked up and streaked onto an agar plate of yeast extract malt extract medium for culture purification. Stock cultures were made on the YM slants, and kept in a cold room at 4 °C.

2. Screening of antibiotics

The cultures were streaked across the centers of the YM plates, then incubated at 30 °C for seven days or until growth. Four test organisms, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231 were streaked from the edges of the plates.

All test organisms were cultured 24 hours on agar slants at 37 °C, except *Candida albicans* ATCC 10231, which was cultured on sabouraud dextrose agar slant at 30 °C. They were washed from the agar surface with normal saline solution to make the suspensions before use.

The plates were incubated at 37 °C to allow growth of the test organisms. The clear inhibition distances of each test organism was observed.

3. Identification of strains

Morphological, cultural, physiological and biochemical characteristics of strains were determined by media and methods described by Shirling and Gottlieb (1966).

3.1 Morphological and cultural characteristics

3.1.1 Culture media for morphological studies

The "standard" culture media for morphological studies for all cultures were medium 2 (yeast extract-malt extract agar); medium 3 (oatmeal agar); medium 4 (inorganic salts-starch agar); and medium 5 (glycerol-asparagine agar).

All media were cooled to about 50 °C and dispensed aseptically into sterile petri dishes and 7 plates were prepared for each medium using 25 ml of medium in 9 cm diameter. Poured media were held for a minimum of 24 hours at 25-28 °C to promote moderate drying and to check sterility before inoculation.

3.1.2 Inoculation of plates for morphological studies

Spores or mycelia were transferred from a stock culture slant to one of test tube containing 3-5 ml of steriled distilled water and suspended by using a steriled wire loop to make a very turbid suspension 0.05 ml of the inoculum was placed onto the agar surface near one edge of the petri dish (1 drop from a 1 ml serological pipette).

A flame-sterilized wire loop was used to make 5 equally-spaced streaks across the plate. The loop was dipped into the pool of inoculum prior to each streak

and crosshatch streaks were made on the agar plates. The plates were incubated in the dark at 25-28 °C. For each culture was observed of each medium after 7 days, two at the end of 14 days, and two at the end of 21 days. One extra plate was inoculated in case of accident.

3.1.3 Determination of morphological characteristics

3.1.3.1 Direct light microscopic examination

The characteristics of the spore-bearing hyphae and spore chains were determined by direct microscopic examination of the culture surface on opened dishes of the crosshatched cultures. An adequate magnification was used to establish the presence (or absence) of chains of spores. The number of spores at the end of mature hyphae of 3-10 or more than 10, and the form of the spore chain and spore-bearing hyphae were determined.

3.1.3.2 Color determinations

Color determinations were made for the mass color of mature, sporulating aerial surface growth, for the color of the substrate mycelium (reverse side) and diffusible soluble pigments other than melanins.

3.1.3.3 Scanning electron microscopic examination of spore morphology and surface

The same crosshatch petri dish cultures prepared for observation under the light microscope were used. The culture was cut to some small cubes (3-5 mm^2) and then were primary fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 at room temperature for 1 hour in refrigerator for overnight. Thus they were washed with phosphate buffer 10-15 minutes, 3 times and were treated with secondary fixative, a 1 % solution of OsO_4 in phosphate buffer, pH 7.2 for 1-2 hours, and washed in the same process before drying.

The specimens were dehydrated through a graded ethanol series (30%, 50%, 70%, 90% and absolute ethanol, 10-15 minutes 2 times in each) and finally dried in the critical point dryer. The specimen was fixed to a stub and coated with a thin film of gold by Ion sputter.

The spore chains were observed and photographed in the scanning electron microscope at high magnification of 5000x-10,000x. Spore surface was characterized as smooth, spiny, hairy and warty.

3.2. Physiological and biochemical characteristics

3.2.1 Melanin production

3.2.1.1 Culture media and inoculation for melanin production

Production of melanoid pigments was determined on agar slants of medium 6, and medium 7 (Shinobu's modification of Masumoto's tyrosine agar). Cultures used as inoculum source should be less than 3 weeks old. A heavy inoculum of spores and aerial mycelium picked up on a standard wire loop was used. This inoculum was streaked on the surface of the agar slant. Each experimental culture was inoculated onto 2 slants of medium 7.

3.2.1.2 Observations and interpretations

Malanoid pigments on medium 6 and medium 7 were observed after 2 and 4 days. Inoculated tubes were compared with uninoculated controls. Cultures forming a greenish brown to brown to black diffusible by other color were recorded as positive (+). Absence of brown to black colors, or total absence of diffusible pigment, was recorded as negative (-) for melanoid pigment production.

3.2.2 Carbon Utilization

Basal medium and carbon sources were used. Detailed instructions for medium 9 (Pridham and Gottlieab carbon utilization medium) including carbon source sterilization are given on appendix.

After autoclaved the basal agar medium was cooled to 60 °C and sterile carbon source was added aseptically to give a concentration of approximately 1%. The mixture was agitated and 25 ml of medium per dish was poured into 9 cm petri dishes. Plates of each carbon compound was prepared in duplicate for each culture to be tested. The medium was stored in refrigerator.

Carbon sources and controls required for the test are repeated below:

- No carbon source (negative control)
- D-glucose (positive control)
- L-arabinose
- Sucrose
- D-xylose
- D-mannitol
- D-fructose
- Rhamnose
- Raffinose

Five ml of turbid suspension of spores or mycelium in sterile water was prepared. The suspension was centrifuged, and the supernatant broth was decanted. Sterile distilled water (or sterile 0.85 % NaCl) was added to restore the original volume in the centrifuge tube. The washed sediment was mixed and resuspended with a sterile rod or pipette 3 times. The amount of sediment from different cultures were compared. When amount of sediment is much less than normal amount, proportionately less water was used for the final resuspension). The resuspended inoculum was used at once or within 3 hours to inoculate carbon utilization tests. The

uninoculated plates were dried at room temperature for 4 hours after freshly poured or after removal from refrigerator storage. Approximately 0.05 ml of washed inoculum (1 drop from a sterile 1ml serological pipette or dropping pipette) was placed onto one edge of the agar surface. The drop was streaked straight across the dish. A second drop was repeated. Plates were inoculated in duplicate. Only one culture was used per plate to avoid false positive due to cross feeding. The plates were observed at 10-16 days. Growth on a given carbon source was always compared with the two controls; growth on basal medium alone and growth on basal medium plus glucose. Results 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 basal medium without carbon (utilization was always recorded as negative if growth was not better than no carbon control.)

3.2.3 Nitrate reduction

Cultures in peptone KNO_3 broth (appendix) were tested for the presence of nitrite after 5, 10 and 14 days of incubation at 28° C. One ml of each broth culture was withdrawn aseptically and mixed with 3 drops of each of the following two solutions; (1) sulphanilic acid (2) dimethel-D naphthylamine 2 drops. The appearance of red colour indicated the presence of nitrite. After 14 days of incubation, nitrate was demonstrated by a red colour after the addition of 4-5 mg of zinc dust to the previously tested for nitrite. When a positive test for nitrates was obtained, The tubes

were incubated further, and then the qualitative test for ammonia by Nessler's solution was performed (Gray and Parkinson, 1968). If ammonia was present, a yellow-brown color developed in the medium.

3.2.4 Starch hydrolysis

The culture was streaked in band across the centers of the inorganic salt starch agar plate and incubated at 30 °C for 7 days. After incubation was complete, the surface of the plate was flooded with dilute Gram's iodine solution. After 15 to 30 minutes, the unchanged starch became dark blue, while a clear zone underneath and around the growth indicated the hydrolysis of the starch (Gray and Parkinson, 1968).

3.2.5 Gelatin liquefaction

The culture was stabbed in the Bouillon gelatin tube and incubated at 30 °C for 7 days. After incubation was complete, the tube was kept in refrigerator at 4 °C for 30 minutes. The result was positive if the culture tube was hydrolyzed compared with the control tube.

3.2.6 Litmus milk reduction

Two tubes of litmus milk was inoculated with the culture and incubated at 28-30 °C, and daily observation was performed through a ten day period as following :- a) reduce of litmus, b) milk coagulation, c) milk peptonization, d) gas production and e) any changes in pH according to indicator.

3.2.7 NaCl tolerance test

The culture was streaked in a band across the centers of the ISP 2 medium which was added sodium chloride 4%, 7%, 10% and 13% respectively. The plates were incubated at 30 °C for 7-14 days. The result was recorded as positive if the culture grew.

3.2.8 Optimum temperature for growth

The culture was streaked in a band across the centers of the ISP 2 medium. The plates were incubated at 30 °C, 37 °C, 42 °C and 55 °C respectively for 7-14 days. The colony on agar surface was present if the culture grew.

4. Antibiotics production

4.1 Organism

Streptomyces sp. CB 5-3 was cultivated on YM media (see appendix) at 28 °C for 7 days to 1 month and kept in refrigerator at 4 °C.

4.2 Method of cultivation

Streptomyces sp. CB 5-3 colony was grown on YM slant for 7-14 days at 30 °C then the mycelia were inoculated into 500 ml Erlenmeyer flask containing 250 ml of GBP medium. This seed culture was incubated at 28 °C for 7 days on rotary shaker (200 rpm). Ten ml of the above seeds were inoculated into 250 ml of strile PY media (autoclaved at 121 °C for 20 minutes) in 500 ml Erlenmeyer flask to yield 2 % of inoculum. The culture medium was incubated at 28 °C on rotary shaker (200 rpm) for 14 days, and then the culture broth was collected by centrifugation.

4.3 Antibiotic assay

Active fractions were detected by agar disc diffusion method according to Mannual of Chemical Microbiology (Murray, 1995).

4.4 Effect of pHs on antibiotic production

The appropiate medium (120 ml) was fermented as described in 4.2 and the pHs was adjusted to 6.0, 6.25, 6.5, 6.75 and 7.0. After 14 days the fermentation broth was detected for antibiotic production by agar disc diffusion method using *C. albicans* ATCC 10231 as the test organism, and the optimum pH was selected for fermentation.

5. Separation of compounds

General separation techniques

5.1 Chromatography

5.1.1 Analytical thin layer chromatography

Adsorbent	:	Silica gel 60 F-254 precoated plate (E. Merck No.1.0715)
Layer thickness	:	200 μm for precoated TLC plate
Technique	:	one way, ascending
Distance	:	5 cm
Temperature	:	room temperature 25-30 °C
Detection	:	1) visual detection under daylight. 2) visual detection under ultraviolet light at the wavelengths of 254 and 365 nm.

5.1.2 Preparative Thin-Layer Chromatography(PLC)

Adsorbent : Silica gel 60 F-254 precoated plate (E. Merck No.1.0715)

Layer thickness : 200 μm for precoated TLC plate

Technique : one way, ascending

Distance : 15 cm

Temperature : room temperature 25-30 °C

Detection : visual detection under ultraviolet light at the wavelengths of 254 and 365 nm.

Plate size : 10 x 20 cm for preparative TLC plates

5.1.3 Column chromatography

5.1.3.1 Silica gel chromatography

Adsorbent : Silica gel 60 (number 9385) particle size 0.040-0.063 mm (230-400 mesh ASTM) (E. Merck)

Packing : Dry packing

Application of the sample : The sample was triturated with silica gel, dried and loaded on the top of the column.

Examination of eluates : Fractions were examined in the same manner as thin layer chromatography.

Solvent : ethyl acetate, methanol and water (6:4:1)

5.1.3.2 Gel filtration chromatography

Adsorbent : Sephadex LH-20

Packing : The adsorbent was suspended in the solvent and left for swelling for approximate 24 hours, and then poured into the column and allowed to settle properly.

Application of the sample extract : The sample was dissolved in a small volume of solvent and carefully loaded, on top of the column.

Examination of eluates : Fraction were examined by TLC using visual detection under ultraviolet light at wavelengths of 254 and 365 nm. Similar fractions were combined and evaporated to dryness under reduced pressure.

Solvent : methanol

5.1.3.3 Flash column chromatography

Adsorbent : Polystyrene (MCI Gel)
High porous Polymer CHP 20 P (75 x 150) Lot No. 4F511 Mitsubishi Chemical Corporation

Packing : The adsorbent was wet-packed after being suspended in methanol. The slurry of adsorbent was poured into the column, then allowed to settle overnight.

Sample loading : The sample was dissolved in a small volume of the solvent, triturated with adsorbent, air dried and loaded onto the top of the column.

Solvent

All of organic solvents used in this work were commercial grade and were redistilled prior to use.

6. Isolation of compounds

Twenty liters of PY fermentation broth of *Streptomyces* sp. CB 5-3 was centrifuged at 5,000 rpm for 20 minutes and then partitioned with 250 ml of isobutanol 60 times. The isobutanol layer was evaporated under reduced pressure to yield 16.55 g of isobutanol extract. A small portion of this fraction was kept as reference.

Isolation of chemical constituents from Streptomyces sp. CB 5-3

From the bioactivity screening test, the isobutanol extract exhibited antifungal (*Candida albicans*) activity.

6.1 Isolation of fraction D4

The isobutanol extract (16.55 g) was fractionated by Silica gel flash column chromatography (1.5 × 90 cm) eluted with ethyl acetate: methanol: water (6:4:1). Each 25 ml eluted was collected to give 91 eluates, A1/1-41 and A2/1-50.

The eluates were checked by TLC using the same solvent system as developing solvent and eluates which gave similar chromatographic pattern were combined as shown in Table 6.

Table 6 Combined fractions from the isobutanol extract

Fraction	Weight (mg)	Fraction	Weight (mg)
A1/6-8	647.8	A2/6-11	896.4
A1/9-23	1670.9	A2/12-40	1073.2

Fraction A1/6, A1/9-23, A2/6-11 and A2/12-40 was fractionated by gel filtration chromatography (2.5 × 80 cm) using Sephadex LH-20 as the adsorbent. Each 50 ml fraction was obtained by using methanol as eluent. The fractions were combined according to their TLC patterns to give these fraction as shown in Table 7.

Table 7 Combined fractions from fraction A1/6-8, A1/9-23, A2/6-11 and A2/12-40

Fraction	Weight (mg)	Fraction	Weight (mg)	Fraction	Weight (mg)	Fraction	Weight (mg)
A2/6-11		A1/6-8		A2/12-40		A1/9-23	
B1/6	34.7	B2/6	105.4	B3/1	23.1	B4/1	151.1
		B2/7	73.3	B3/4	13.1	B4/5	41.0

Fraction B1/6, B2/6, B2/7, B3/1, B3/4, B4/1 and B4/5 was fractionated by flash column chromatography (1.5 × 3 cm) using Polystyrene (MCI gel) as the adsorbent. Each 2 ml fraction was obtained by using methanol as eluent. The fractions were combined according to their TLC patterns to give these fraction as shown in Table 8.

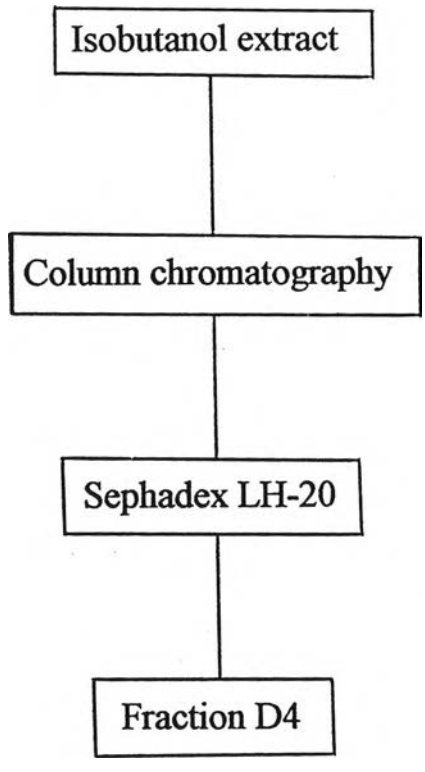
Table 8 Combined fraction from fraction B1/6, B2/6, B2/7, B3/1, B3/4, B4/1 and B4/5

Fraction	weight (mg)	Fraction	Weight (mg)	Fraction	Weight (mg)
B2/7+B4/5		B1/6+B2/6+B3/4		B3/1+B4/1	
C2/7	24.2	C3/7-8	25.1	C4/7-8	1320.0
Fraction	Weight (mg)	Fraction	Weight (mg)	Fraction	Weight (mg)
C2/7+C3/7-8		C4/7-8 (I)		C4/7-8 (II)	
D1/6-9	21.3	D2/5-6	135.1	D3/6-7	303.3

Fraction C2/7, C3/7-8 and C4/7-8 was fractionated by flash column chromatography (1.5 × 3 cm) using Polystyrene (MCI gel) as the adsorbent. Each 2 ml fraction was obtained by using ethyl acetate : methanol (1:1) as eluent.

Fraction D1/6-9, D2/5-6 and D3/6-7 were combined to give fraction D4 (459.7 mg). It showed activity against *C. albicans* by using agar disc diffusion method (R_f 0.7 (ethyl acetate : methanol : water = 6 : 4 : 1)).

Isolation of fraction D4



Scheme 1 Isolation of isobutanol extract from *Streptomyces* sp. CB 5-3