Chapter 3

Materials and Methods

I The isolation methods

A. Sampling of cave soil[79]

Thirty soil samples were collected from various caves in central region of Thailand. Fresh soil was taken from the soil surface and down to a depth of 6 cm. The samples were placed in the laboratory under favorable condition of temperature, at 4°C in refrigerator.

Table 1 The cave soil samples and their sources

Sample number	Cave	Province
1-5	Mungkorn-tong	Kanchanaburi
6-10	Kao-lam	Kanchanaburi
11-15	Sarika	Rajburi
16-20	Jom-pon	Rajburi
21-20	Kao-bin	Rajburi
26-30	Pothi-sat	Saraburi

B. Isolation and collection of cultures[80,81,82]

In order to isolate antibiotic-producing Actinomycetes, 10 gm of each soil sample was suspended in sterile distilled water

to make 1:10, 1:100, 1:1000 and 1:10000 dilutions. One ml portion of soil dilution was plated with potato dextrose agar (plus nystatin 50 µg/ml) to determine the total count.

0.1 ml of the soil suspension was applied to the other agar surface by pipette, then spread across the surface with sterile glass rod to isolate the selected colonies.

Two sets of each soil sample were studied. One was incubated at 28-30°C for 3-5 days under aerobic condition and the other was under anaerobic condition in gas pak jar.

The isolated colonies of actinomycetes were smooth or lichenoid, hard and densely textured, raised and adhering to the medium. They were usually covered completely or partially, in form of spots or concentric rings, by aerial mycelium, which might be variously pigmented. Such a colony was subcultured and purified by streaking, before making stock cultures on the potato-dextrose agar slants and kept in a cold room at 4°C.

II Screening of cultures for antibiotic production[83,84]

The pure cultures were streaked in a narrow band across the centers of the nutrient agar plates, then incubated at 28-30°C for five days or until growth and, possibly, sporulation had occurred. Six test organisms were then streaked from the edges of the plates up to but not touching the growth, they were as follows :-

Staphylococcus aureus	ATCC 6538-P
Bacillus subtilis	ATCC 6633

Streptococcus faecalis	Department of Microbiology, Faculty of Medicine, Chulalongkorn Hospital
Escherichia coli	ATCC 10534
Pseudomonas aeruginosa	NCTC 10612
Candida albicans	ATCC 10231

All test organisms were cultured overnight on nutrient agar slants at 37°C, except *C. albicans*, it was cultured on sabouraud dextrose agar slant. They were washed from the agar surface with normal saline solution to make the suspensions before use.

The assay plates were further incubated at 35°-37°C to allow growth of the test organisms. The clear inhibition distances over which the growth of each test organism has been inhibited by antibiotic in the vicinity of the actinomycetes was observed. Only those actinomycetes that have produced antibiotics with interesting microbial inhibition spectra were retained for further testing. A photograph was taken to provide a permanent record of the inhibitory spectrum of each strain.

III Taxonomic studies of selected strain

Morphological and physiological properties of a selected strain (strain ST-13-2)were determined by media and methods described by Shirling and Gottlieb[24] along with several supplementary tests.

A. Morphological characterization

a) Culture media for morphological studies

The "standard" culture media were medium 2 (yeast extract-malt extract agar),medium 3 (oatmeal agar), medium 4 (inorganic

salts-starch agar) and medium 5 (glycerol-asparagine agar). They were cooled to about 50°C and poured 7 plates of each medium using 25 ml of medium in each 9 cm diameter sterile petri dish. Poured media were held for a minimum of 24 hr at 25-28°C to promote moderate drying and to check sterility before inoculation.

b) Inoculation of plates for morphological studies

The suspension of spores or mycelium was prepared by adding 3-5 ml of sterilized distilled water to the stock slant and using a sterile wire loop to get the spores or mycelial fragments. Then 0.05 ml of it was inoculated onto the agar surface near one edge of the petri dish of each medium.

The flame-sterilized wire loop was used to make 5 equally spaced streaks across the plate, dipping the loop into the inoculum prior to each streak and cross streaks made without picking up additional inoculum.

The plates were incubated in the dark at 25°-28°C. For each culture observed two plates 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.

c) Determination of micromorphological characteristics

1. Direct light microscopic examination

Determine the characteristics of the culture surface on opened dishes of the crosshatched culture. Use a magnification, 100x-700x to establish the presence (or absence) of chains of

spores. Determine the number of spores at the end of mature hyphae, the form of the spore chain and spore-bearing hyphae, and other special morphological observations.

Photograph typical morphology of the sporulating hyphae.

2. Color determinations

Color determinations were recorded only for mature cultures. Describe the aerial mass color, the color of substrate mycelium (reverse color), and the soluble colors other than melanoid pigmentation.

3. Scanning electron microscopic examination[85,86]

Use the same crosshatch petri dish culture prepared for observation under the light microscopic. The culture was cut to some small cubes (3-5 mm^3) and then were primary fixed in a 4% solution of paraformaldehyde in 0.1 M phosphate buffer pH 7.2 at room temperature for 2 hr. After that they were washed 3 times with buffer and were treated with secondary fixative, a 1% solution of osmium tetroxide in buffer, and washed in the same process before drying.

The specimens were dehydrated through a graded ethanol series (35%, 70%, 95% and absolute ethanol, 20 min in each) and finally in the critical point dryer. Fix the specimen to a stub and coat with a thin film of gold by Sputter coater.

The spore chains were observed and photographed in the scanning electon microscope at high magnification of 5000x-10,000x.

B. Physiological characteristics

a) Melanin production

1. Culture media for melanin production

Determine production of melanoid pigments on agar slants of medium 1 (tryptone-yeast extract agar), medium 6 (peptone iron agar, Difco, supplemented with 0.1% yeast extract), and medium 7 (Shinobu's modification of Masumoto's tyrosine agar).

2. Inoculation of slants for melanin production

The culture used as inoculum source was less than 3 weeks old. Streak a heavy inoculum of spores and aerial mycelium on the surface of 2 slants of each medium.

The agar slants were incubated at 25 -28^{o}C. Observed melanoid pigments after 48 hr and 96 hr.

3. Interpretation for melanin productions

Compare inoculated tubes with uninoculated controls. Culture forming a greenish brown to black diffusible pigment or a distinct brown pigment modified by other color was recorded as positive (+). Absence of brown to black colors, or total absence of diffusible pigment, was recorded as negative (-) for melanoid pigment production.

b) Carbon utilization

1. Basal media and carbon sources

Detailed instructions for medium 8 (Pridham and Gottlieb carbon utilization medium) including carbon source sterilization were given in Appendix II.

After autoclaving the basal agar medium, cool it to 60°C and add sterile carbon source aseptically to give a concentration of approximately 1%. Agitate the mixture and pour 25 ml of medium per dish into 9 cm Petri dishes. Prepare duplicate plates of each carbon compound for each culture to be tested.

Carbon sources and controls required for the test were repeated below :-

No carbon source (negative control)

D-glucose (positive control)

L-arabinose	D-mannose
D-xylose	Sucrose
D-fructose	Raffinose
Inositol	D-mannitol
L-rhamnose	Salicin
D-galactose	Cellulose

2. Procedures for carbon utilization tests

2.1 Preparation of washed inoculum

Prepare 5 ml of suspension of spores or mycelium in sterile water and transfer to 50 ml of medium 1 (tryptone-yeast extract broth) in a 250 ml Erlenmeyer flask. The flask was incubated for 48 hr at 25-28°C on a mechanical shaker. Use vigorous agitation with sterile glass beads to break up the 48-hour growth.

Centrifuge the suspension at 3000 rpm for 30 min. Decant the supernatant broth and resuspend the sediment with sterile distilled water. Repeat the centrifugation. Add sterile distilled water to the washed sediment to restore the original volume

and use the inoculum for carbon utilization tests within 3 hr.

2.2 Inoculation for carbon utilization tests

The uninoculated plates were dried by leaving them at room temperature for 4 hr after they are freshly poured.

Place approximately 0.05 ml of washed inoculum onto an edge of the agar surface. Streak the drop straight across the dish and repeat with a second drop. Inoculate duplicate plates. (Use only one culture per plate to avoid false positive due to cross feeding).

The plates were incubated at 25-28^{o}C and observed at 10-16 days.

2.3 Interpretation for carbon utilization tests

Compare growth on a given carbon souce with the two controls; growth on basal medium alone, and growth on basal medium plus glucose.

Record results as follows:

2.3.1 Strongly positive utilization (++), when growth on tested carbon in basal medium is equal to or greater than growth on basal medium plus glucose.

2.3.2 Positive utilization (+), when growth on tested carbon is significantly better than on the basal medium without carbon, but somewhat less than on the basal medium plus glucose.

2.3.3 Utilization doubtful (±), when growth on tested carbon is only slightly better than on the basal medium without carbon and significantly less than on the basal medium plus glucose.

2.3.4 Utilization negative (-), when growth is similar to or less than growth on basal medium without carbon. (Always record utilization as negative if growth is not better than no-carbon control).

C) Biochemical reactions[87]

The culture used as inoculum was less than 3 weeks old.

1. Nitrate reduction

Inoculate two nitrate broth tube with the culture and incubate at 28-30°C for 4 to 6 days. On the forth day, tranfer 1 ml of the medium into a test tube and add three drops of the sulfanilic acid reagent, followed by two drops of dimethyl-α-napthylamine solution. If nitrites were present, the mixture would become pink. When a positive test for nitrates has been obtained, incubated the tubes further, and then perform the qualitative test for ammonia by Nessler's solution. If ammonia was present, a yellow-brown color developed in the medium.

2. Starch hydrolysis

Streak the culture in a band across the centers of the starch agar plate and incubate at 28-30°C for 5 days. After incubation was complete, flood the surface of the plate with dilute Gram's iodine solution. If starch hydrolysis was present, a dark-blue color was not appeared.

3. Gelatin liquefaction

Streak the culture in a band across the centers of the 0.4% nutrient gelatin agar and incubate at 28-30°C for 5 days. After incubation was complete, flood the surface of the plate

with saturated ammonium sulfate solution. Whenever, the gelatin has been hydrolyzed, turbidity did not appear.

4. Litmus milk reduction

Inoculate two tubes of litmus milk with the culture and incubate at 28-30°C, and observe daily through a ten day peroid as following :- a) reduction of litmus, b) milk coagulation, c) milk peptonization, d) gas production, and e) any changes in pH according to the indicator.

5. Casein decomposition

Melt a nutrient agar tall and allow it to cool to about 45°C. Aseptically pipette 1 ml of sterile fresh skim milk into the tall, throughly mixed, and poured into a sterile Petri dish. After the agar has hardened, make a single streak across the center of the plate with the culture and incubate at 28-30°C for 5 days. Observed the clear zone if there was casein decomposition.

6. Tyrosine and Xanthine decomposition

Streak the culture in a band across the centers of the tyrosine agar plate and xanthine agar plate. The plates were incubated at 28-30°C for 5 days. If the decomposition was present, the clear zone was resulted.

IV Development of methods for submerged-culture antibiotics production[82,83]

A. Development of media[10]

1. Liquid media

The selected antibiotic producing actinomycetes strain ST-13-2 which showed broad spectrum and strong inhibition when tested

on solid media was tested for ability to produce antibiotic in 3 kinds of liquid media : glucose peptone medium, glucose soybean medium, and maltose soybean medium.

Thirty-five milliliters of each liquid medium in 250 ml Erlenmeyer flask with 20 glass beads was used in this test.

2. Inoculation for antibiotic production

The inoculum was prepared in the same manner as describe in the title number III. A.b (page 32).

Each liquid medium was inoculated with 0.5 ml of the inoculum and was incubated on a rotary shaker (180 rpm, 28°C) for 3,5 and 7 days. By these time, 5 ml of each culture fluid was taken aseptically to assay for antibiotic activity against 6 test organisms by agar diffusion method.

3. Antibiotic assay(88)

3.1 Preparation of inoculum

The fresh cultures of each test organism on antibiotic medium no. 1 slants that had been incubated at 37°C for 16-18 hr were used. They were washed out with sterile NSS and standardized by determining the dilution that would permit 25% light transmission at wave length 525 nm.

3.2 Preparation of assay plate

The flasks with 100 ml medium no.1 were melted then cooled to 48°C, and inoculated with 0.5 ml of each inoculum suspension. After through mixing avoiding air bubbles, the 20 ml of agar

was poured into 9 cm sterile petri dishes. A sterile cork borer with 6 mm diameter was used to press upon the hardened agar to make 5 sharp circles to give holes after removing the agar within the circles.

3.3 Assay procedures

The inoculated agar plate were filled each hole with 50 µl of each fermented liquid medium from IV. A.2

The plates were left 1 hr at room temperature for diffusion into the agar medium. Then, they were incubated at 37°C for 16-18 hr. Compare the diameters of the inhibition-zone by measuring with a sliding caliper.

B. Determination of optimum pH and temperature

1. Preparation of liquid media

The selected liquid medium from development of media from IV. A was dispensed into 3 sets of the 500 ml Erlenmeyer flasks, each set had 6 flasks containing 130 ml of the medium. The pH of the medium in each set was adjusted to pH 4,5,6,7,8, and 9 with either 1N NaOH or 1N H_2SO_4 before sterilization.

2. Preparation of seed medium

Prepare 3.0 ml of turbid suspension of mycelial growth from strain ST-13-2 in sterile water and inoculate into a 500 ml Erlenmeyer flask containing 130 ml of the selected liquid media (pH 7.0). The flask was incubated on a rotary shaker (300 rpm, 28°C) for 3 days.

3. Fermentation

A 5.0 ml aliquot of the culture from the seed medium was inoculated into each the 500 ml Erlenmeyer flask describe in B.1.

Fermentation was carried out for each set at 23°, 30° and 33°C with agitation (300 rpm).

The pH of the culture fluid was measured and the antibiotic production during fermentation was monitored by agar diffusion method using *S. aureus* ATCC 6539-P as the test organism. An example of a typical time course of the fermentation in each 500 ml Erlenmeyer flask was determined every day until the antibiotic activity was decreased.

V Determination of antibiotic substances by thin-layer chromatography (TLC)[89,90]

A. Preparation of antibiotics

1. Standard antibiotics[91]

The known antibiotics used as reference standard antibiotics were penicillin G,kanamycin,cefotaxime, cloxacillin, tobramycin, bacitracin, doxycycline,framycetin, erythromycin.

Concentration of each standard solution to be applied to chromatoplate was calculated from minimal inhibitory concentration (MIC) and the volume applied on to each spot was about 0.02 ml.

2. Unknown antibiotics[92]

The streptomyces strain ST-13-2 was fermented in suitable condition (from IV) to provide high yield of antibiotics in broth culture and then prepared as follow :-

a) The whole fermentation broth (6 ml) were extracted with 3 ml of butanol, alone or in the presence of either 0.6 ml of 6N HCl or 0.5 ml of 2N NH_4OH. A 0.02 ml sample of each butanolic extract was

applied to the chromatoplate by means of a micropipette.

b) 10 ml of isopropanol was shaken with 6 ml of whole broth for 15 min, then centrifuged. A 0.04 ml sample of the supernatant was applied to the chromatoplate by means of a micropipette.

c) A 10 ml sample of the supernatant from the isopropanolic solution was dried *in vacuo*. The residue was triturated twice with 1 ml of ethanol. To the material insoluble in ethanol were added 2 ml of water and 2 ml of acetone. After centrifugation of the resulting suspension, a 0.02 ml sample of the supernatant and a 0.02 ml sample of the earlier ethanolic solution were applied to chromatoplates.

B. Thin-layer chromatography[89]

1. Preparation of TLC plate

Technique	:	One way, ascending
Absorbent	:	Silica gel 60 G (7731) 30 gm/60 ml of distilled water
Plate size	:	20 cm x 20 cm and 10 cm x 20 cm
Layer thickness	:	250 μ
Activation	:	Air dried for 15 min and then heating in a hot air oven at 105°C for 1 hr
Distance for development	:	15 cm

2. Solvent systems for TLC[69]

The chromatoplates were developed in solvent systems as follows :-

Table 2 Solvent systems for TLC

System	Component	Ratio
1	Butanol : acetic acid : water	3:1:1
2	Chloroform : methanol : 17% ammonia	2:1:1
3	Propanol : pyridine : acetic acid : water	15:10:3:10
4	Ethanol : conc. ammonia: water	8:1:1
5	Ethanol : water	4:1
6	Ethylacetate : methanol	100:15

3. Detection of compound on TLC plate[90]

3.1 Microbiological detection (bioautograph)

A filter paper (Whatman No 1), 20 cm x 20 cm, was moistened with solvent and pressed firmly on to the chromatoplate by means of another glass plate. After 10-15 min, the paper was removed, laid immidiately on to the inoculated *S.aureus* agar plate (after drying if it had been moistened with an organic solvent) and left for 10-15 min. It was then removed and the agar plate allowed to incubate for 18 hr. The inhibition zones are located and theirs positions marked in the corresponding places on the chromatoplate.

3.2 Chemical detection through specific colour reactions

The chromogenic reagents employed was a 10% permanganate solution, followed in 10 min by the application of a 0.2% bromphenol blue solution.

VI Laboratory evaluation of antibiotic producing to isolated pathogenic organisms from patients[93;94]

Four groups of organisms used were *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* that were derived from specimens of the patients in Siriraj Hospital and Bangkok Christian Hospital.

A. Discs and preparation of antibiotic from strain ST-13-2

1. Discs

The disc agar diffusion method was used to determine antimicrobial susceptibility test.

The standard antibiotic discs tested were follows :-

Amikacin	30 µg	(BioLab)	Batch.853
Cefotaxime	30 µg	(Difco)	Lot. 732393
Ceftriazone	30 µg	(BBL)	Lot. 308565
Cloxacillin	5 µg	(BRL)	Batch.16638
Erythromycin	15 µg	(Difco)	Lot. 724690
Gentamicin	10 µg	(BBL)	Lot. 501550
Moxalactam	30 µg	(Lilly)	Lot. 8 DG 87 A
Netilmicin	30 µg	(BBL)	Lot. 308502
Penicillin	10 Units	(BioLab)	Batch 8462
Tobramycin	10 µg	(Lilly)	Lot. 7 RN 37 A

2. Preparation of antibiotics from strain ST-13-2

The liquid media with high yield of antibiotics from strain ST-13-2 was divided and treated as follows :-

Part I The stock broth

Part II Dilute the stock broth to one fold (v/v) with sterile distilled water

Part III Lyophilize to dry powder and dissolved with sterile distilled water to make two and four fold(v/v) concentration of the stock broth

B. Preparation of culture medium and plates

Melt previously prepared and steriled Muller-Hinton agar medium pH 7.2-7.4, cool to 45°-50°C, and on a level surface, pour into 9 cm diameter petri dishes to a depth of 4 mm (about 20 ml of medium was required). The plates were dried for 1 hr at 37°C.

C. Preparation of inoculum

Thirty isolates of each groups of the organisms were separately inoculated into 5 ml of Soybean-casein digest broth and incubated for 6-8 hr at 37°C. Then standardize the culture to match a 0.5 turbidity standard of Mac Farland.

D. Inoculation of plates

Spread 0.1 ml of each standardized inoculum over the sterile surface of the agar medium in several directions. Allow the inoculated plates to dry at room temperature for about 5 min.

E. Assay procedure

Apply the susceptibility discs manually using aseptic precautions. Gently press each disc down to the surface of the inoculated

agar with sterile forceps and space the discs evenly so that they are 10-15 mm away from the edge of the plate and sufficienty separated from one another to avoid overlapping zones of inhibition. The standard antibiotic discs tested for each organisms were follows :-

Table 3 The antibiotic discs used for each test organisms.

Test organism	Antibiotic disc
S. aureus	Erythromycin Penicillin Cloxacillin Cefotaxime
E. coli	Gentamicin Tobramycin Ceftriazone Cefotaxime
Ps. aeruginosa	Netilmicin Amikacin Moxalactam Cefotaxime
K. pneumoniae	Gentamicin Tobramycin Ceftriazone Cefotaxime

Four stainless cylinder cups (0.6 cm x 1 cm) for the concentration of the antibiotics from strain ST-13-2 were also placed on each disc agar plate. The cylinder cups were filled with the solution by means of micropipette.

The plates were left at room temperature for 30 min, then incubated at 37°C for 16-18 hr.

F. Interpretation of the test results

The diameter of each inhibition zone was measured with a sliding calipers to the nearest mm, reading to the point of complete inhibition as judged by the unaided eye from the underside of the plate. The zone diameter interpretative standards of NCCLS[92] (the National Committee for Clinical Laboratory Standards) was used as shown in the table 4.

Table 4 Zone diameter interpretive standards

Antimicrobial agents	Disc Content	Zone Diameter of Inhibition (mm)		
		Resistant	Intermediate	Susceptible
Amikacin	30 µg	≤ 14	15-16	≥ 17
Cefotaxime	30 µg	≤ 14	15-22	≥ 23
Ceftriazone(95)	30 µg	≤ 12	13-15	≥ 16
Cloxacillin	5 µg	≤ 10	11-12	≥ 13
Erythromycin	15 µg	≤ 13	13-17	≥ 18
Gentamicin	10 µg	≤ 12	13-14	≥ 15
Moxalactam	30 µg	≤ 14	15-22	≥ 23
Netilmicin	30 µg	≤ 12	13-14	≥ 15
Penicillin G when testing Staphylococci	10units	≤ 28	-	≥ 29
Tobramycin	10 µg	≤ 12	13-14	≥ 15