3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents and Media

1. Sabouraud dextrose agar (SDA,BBL®)
2. Trypticase soy agar (TSA, Oxoid®)
3. Gram staining solutions (1- 4) :morphological identification of bacteria
   1) Crystal violet
   2) Gram's iodine
   3) Acetone-alcohol decolorizer
   4) Safranin, 1 g/l (0.1% w/v)
4. Lactophenol cotton blue: morphological identification of fungi
5. 3% Hydrogen peroxide
6. 1:4 diluted plasma
7. Glucose phosphate peptone water
8. Simmon's citrate medium
9. Lactose egg yolk milk agar
10. Motility indole urea (MIU) medium
11. Nitrate broth
12. Peptone water sugar
13. Starch peptone water

14. Sodium hydroxide (NaOH)

15. Hydrochloric acid (HCl)

3.1.2 Standard species of bacteria for the positive and negative control in biochemical tests

1. *Staphylococcus aureus*: positive catalase and coagulase control
2. Streptococcus species: negative catalase control
3. *Enterobacter aerogenes* : positive V-P control
4. *Klebsiella pneumoniae* : positive citrate control
5. *Escherichia coli (E. coli)*: negative V-P and citrate control, positive indole and nitrate control
6. *Pseudomonas. maltophila* : positive maltose fermentation control
7. *Pseudomons. aeruginosa* : negative nitrate and maltose fermentation control
8. *Aeromonas hydrophiea* : positive motility and gelatin hydrolysis control
9. *Shigella species* : negative motility control
10. *Clostridium perfringens* : positive lecithinase production control
11. *C. sporogens* : negative lecithinase production control
12. *Bacillus subtilis* : positive starch hydrolysis control

3.1.3 Instruments

1. Air Sampler , a modified model of Andersen Sampler made by Nikken Bio-Medical Laboratory, Japan (Fig. 1)
2. QUEBEC Colony Counter, made by American Optical Corporation
3. Compound Microscope

Fig. 1 Apparatus (Nikken Bio-Medical Laboratory, Japan)

4. Dry-wet thermometer

5. Petri dishes

6. Plastic bags

7. Masking tape

8. Autoclave

9. Hot air oven

10.Refrigerator

11.Balances

12.pH meter

13.Test-tube holders

14.Bacterial filter

15.Microscopic slides

16.Glasswares : Flasks, Test tubes

3.2 Methods

3.2.1 Air Sampling

1. Selection of study site

Chiang Mai city within the moat was selected as the study site because it is residential and business area with relatively high density of population. The people in this area have more opportunity to be exposed to airborne microorganisms.

2. Description of study site

The Chiang Mai city area within the moat is an area of about 4 km^2 from 2076000 to 2078000mN (meter North) and from 498000 to 500000mE (meter East) according to the Universal Transformation Mapping system (UTM). This area was divided into 12 grids varying in size from 0.135 to 0.27 km^2 (Fig.2).

3. Preparation of air sampling

The TSA and SDA were prepared and tested for sterility before air samplings were carried out. The commercial products of both media were available for air sampler. Sampling sites were surveyed before the sampling day with the help of local people. Air sampler was checked before each sampling trip.

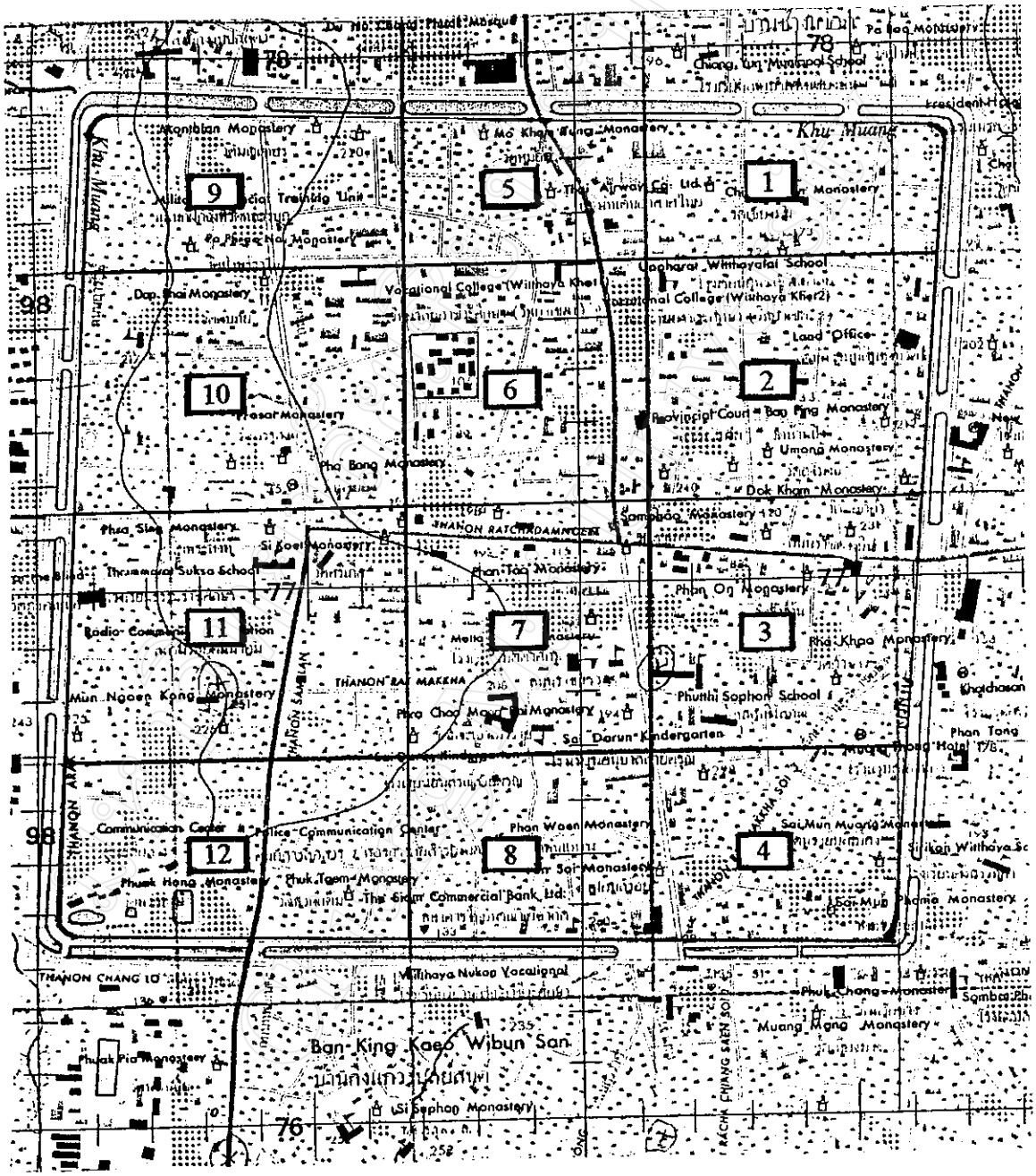

Fig.2 The map of study area

4. Sampling strategies and methods

Six air samples, three samples for airborne bacteria and three samples for airborne fungi, were collected from each grid in the rainy season (September and October 1996) as well as in the cool season (January 1997).

In the rainy season, the sedimentary (Open Plate) method was chosen to take air samples. Three points were selected in one grid and two petri dishes were used at one point i.e. one plate for bacteria and another plate for fungi. Therefore six petri dishes were used for one grid and 72 dishes for the whole study area. The sampling time was 10 minutes for all air samples.

In addition to this regular sampling, a repeated sampling by sedimentary method was carried out at grid 6 and 7. Twelve samples were taken in this repeated sampling. An air sampler was also used for air sampling at grid 3 and grid 4. This air sampler is a modified style of Andersen Sampler with one stage and 37 holes. The operation parameter was an air flow of 50 liters per minute(standard condition). The sampling time was 1 minute for bacteria and 30 seconds for fungi in the rainy season. Two sampling points were selected in each grid. Two plates were used to take air sample at one point i.e. one plate for bacteria and another plate for fungi, thus 8 samples were taken by the air sampler in the rainy season.

In the cool season, the same air sampler was used with the same operation condition except the sampling time was 30 seconds for bacteria. Six air samples were taken at one point in each gird i.e. three samples for bacteria and three samples for fungi.

The sampling height was one meter from the ground for all air samples. During air sampling, the weather parameters were recorded including dry and wet temperature. The sampling period was from 10 a.m. to 2 p.m. in every sampling day.

3.2.2 The Numeration and Identification of Bacteria and Fungi

1. Bacteria

The TSA plates were incubated at 37 °C for one day. The colonies in each plate were counted under colony counter. The macroscopic features were observed and recorded. Different colonies were selected for Gram staining and observed under microscope. Some biochemical tests were used for identification of the genera and species .

The procedure for identification of Gram positive rods is shown in Figure 3 (Cheesbrough, 1985).

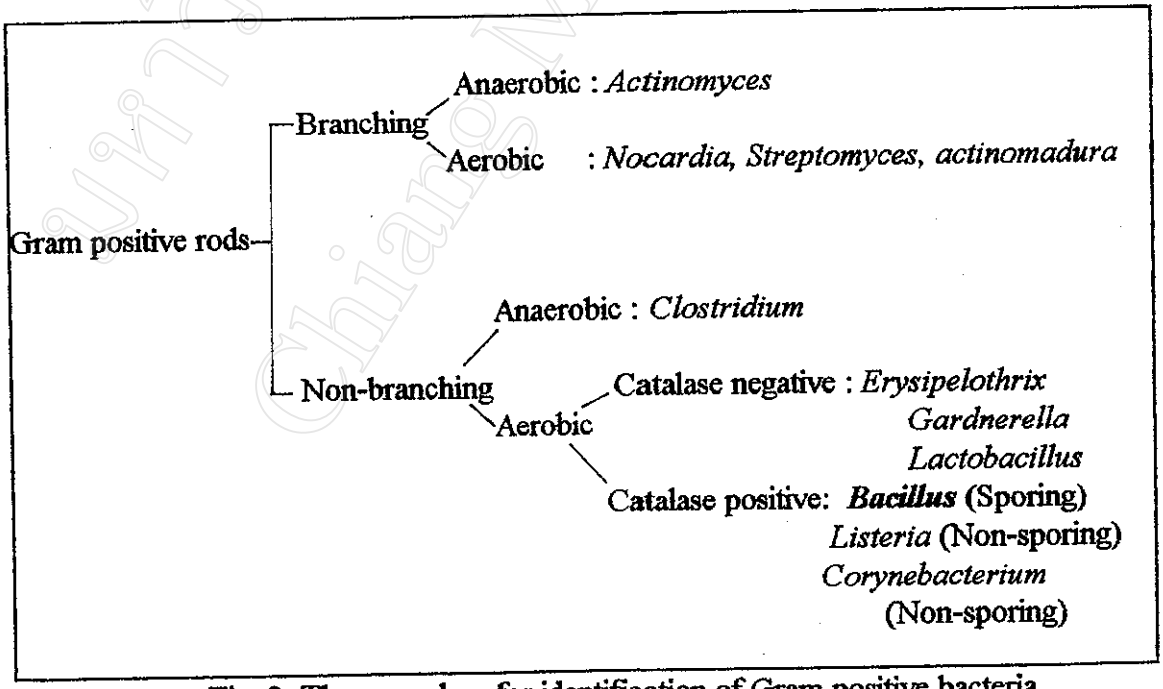

Fig. 3 The procedure for identification of Gram positive bacteria

(Cheesbrough, 1985).

The chart of biochemical tests for identification of *Bacillus* spp. is shown in Figure 4 (Koneman *et al.*, 1994) .

The procedure for identification of Gram positive cocci is shown as follow (Cheesbrough, 1985):

Gram positive cocci
- Catalase positive ---> *Staphylococcus*
- Catalase negative ---> *Streptococcus*

Anaerobic cocci

The biochemical tests for identification of *Staphylococcus* spp. are shown as follows (Koneman et al,1994) :

Staphylococcus
- Coagulase
  - \+ S.*aureus*
  - \- Novobiocin resistance
    - \+ S.*saprophylious*
    - \- CNS (coagulase negative staphylococci)

2. Fungi

The SDA plates were incubated at room temperature for three days. The colonies in each plate were also counted. Each different colony was described and recorded. The fungi were identified by subculturing on SDA using slide culture technique. The slides were incubated for 2-7 days and stained with cotton blue. The microscopic features of fungal spores and mycelium were observed under microscope.

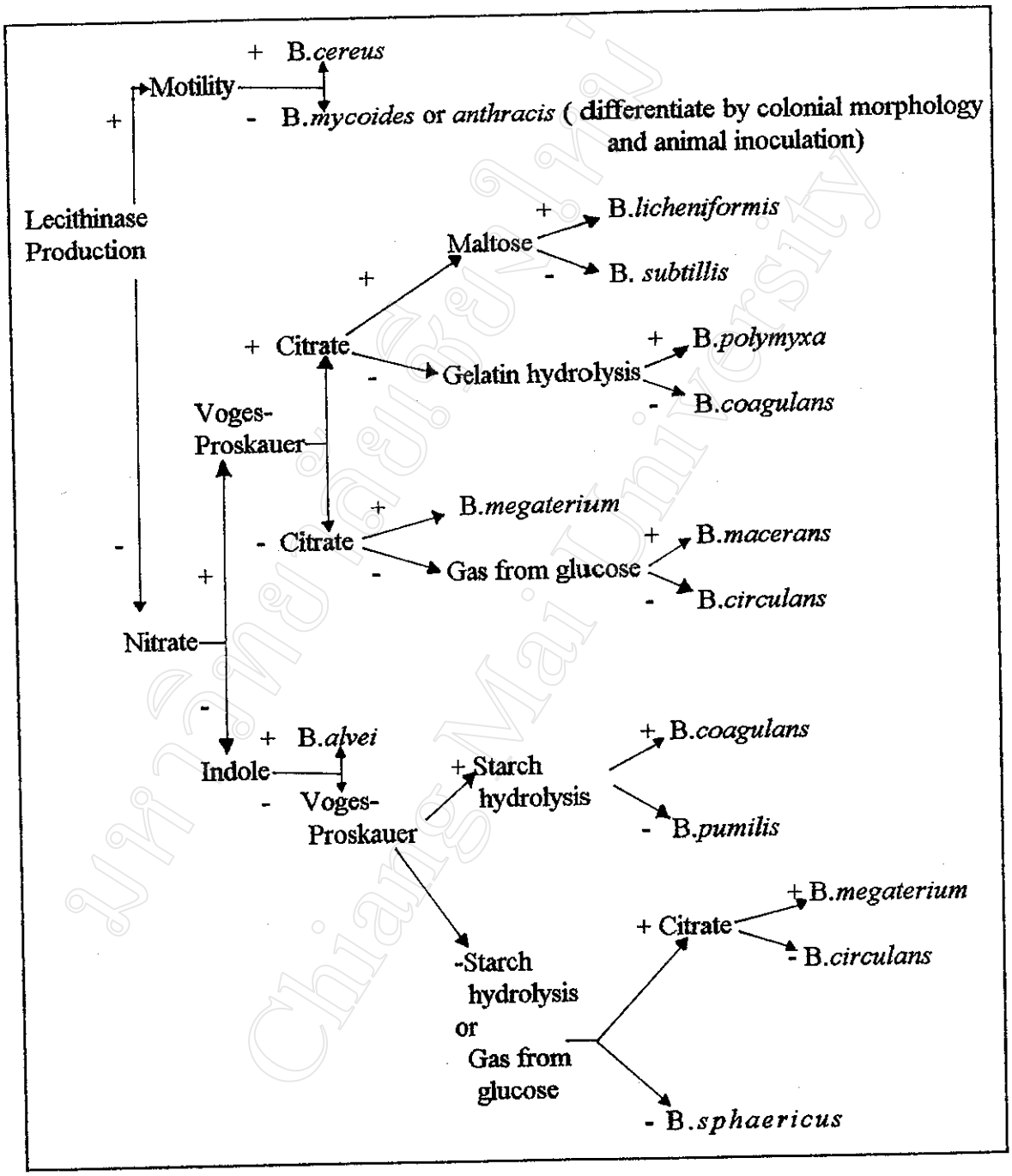

Fig 4. The chart of biochemical tests for identification of *Bacillus* spp.

(Koneman *et al.*, 1994)

3.2.3 Data Processing and Statistical Analysis

1. Data processing

The main work in data processing was to transform the data of colony count to the density or concentration of bacteria and fungi.

The formula for converting the colony count to density of bacteria and fungi in the rainy season (sedimentary method) was shown as :

Density (CFUs/dish/min.) = (Colony counts/dish) / 10 (1)

(Sampling time is 10 minutes for every air sample)

The formula for converting the the colony count to concentration of bacteria and fungi in the rainy and cool seasons (inertial method) was shown as :

Concentration (CFUs/m^3air) = (Colony counts/dish) X 20 (2)

(Sampling time is 1 mimute)

Concentration(CFUs/m^3 air)=(Colony counts/dish) X 40 (3)

(Sampling time is 30 seconds)

The air flow for sampling of bacteria and fungi was 50 liters/min.($1m^3$ = 1000 liters).

2. Statistical Analysis

The statistical analysis was carried out by using computer software i.e. SPSS (Statistical Package for Social Sciences) for MS WINDOWS Release 6.0. The descriptive statistics, analysis of variance (ANOVA) and t-test were used.

3.2.4 Risk Assessment

The risk assessment of airborne bacteria and fungi was done by the following steps in this study i.e. Step (1): classification of data; Step (2): scoring of data; Step (3):

risk assessment. Four parameters (the density of bacteria and fungi, the concentration of bacteria and fungi) were considered in this risk assessment. There were two homogeneous subsets in each parameter. In each parameter, there were three parts i.e. low end, overlay part and high end. The data were classified into three levels i.e. low, medium, and high density (or concentration) according to their position in homogeneous subsets of density (or concentration) of bacteria and fungi. The data belonging to the low end of subset 1 were grouped into low class while the data belonging to the high end of subset 2 were grouped into high class. The data belonging to overlay part of subset 1 and 2 were grouped into medium class. The rule for scoring of data was that the low class was 1 and the medium class was 2 as well as the high class was 3. The higher the score, the higher the risk. The risk level in each grid depended on the scores at the grid. When four parameters were considered in risk assessment, the average score for a grid was 8 meaning the medium risk. If the score of grid was less than 8, the risk of airborne bacteria and fungi at this grid was low level. If the score of grid was higher than 8, the risk of airborne bacteria and fungi at this grid was high level.