3. MATERIAL AND METHODS

A cross-sectional study was conducted from December 2006 to April 2007 with an overall aim to determine the microbiological quality of Dim sum (pork dumplings) sold in Chiang Mai, Thailand. The results of the microbiological analyses will be taken into consideration of the hygienic status of the production by comparison with the microbiological standard of Thailand.

3.1 Study design

3.1.1 Study area

The study area was located in Chiang Mai province (Northern Thailand). Samples have been randomly collected from the point of sale of pork dumplings according to the following criteria:

3.1.1.1 Superstores: packages of 3 well-known brands of frozen pork dumplings which manufactured by industrial enterprises were randomly collected from superstores.

1) Brand A
2) Brand B
3) Brand C

3.1.1.2 Small enterprises: cooked ready-to-eat pork dumplings which produced by local producers were randomly collected from 3 different sources of small enterprises (street vendors, markets and restaurants).

1) Street vendors
2) Markets
3) Restaurants

The microbiological analyses were carried out in the Central Laboratory of the Faculty of Veterinary Medicine, Chiang Mai University, Thailand.

3.1.2 Sampling strategy

3.1.2.1 Sample size determination

The sample size of 30 for each of the 6 groups of different sources was determined according to a statistical sampling scheme recommended for microbiological testing of foods published in the book Microorganism in Foods Volume 7(ICMSF, 2002). The design of the detailed sampling plan is given in figure 4.

3.1.2.2 Sample collection and sampling technique

180 samples have been randomly collected according to the following criteria:

1) Industrial enterprises

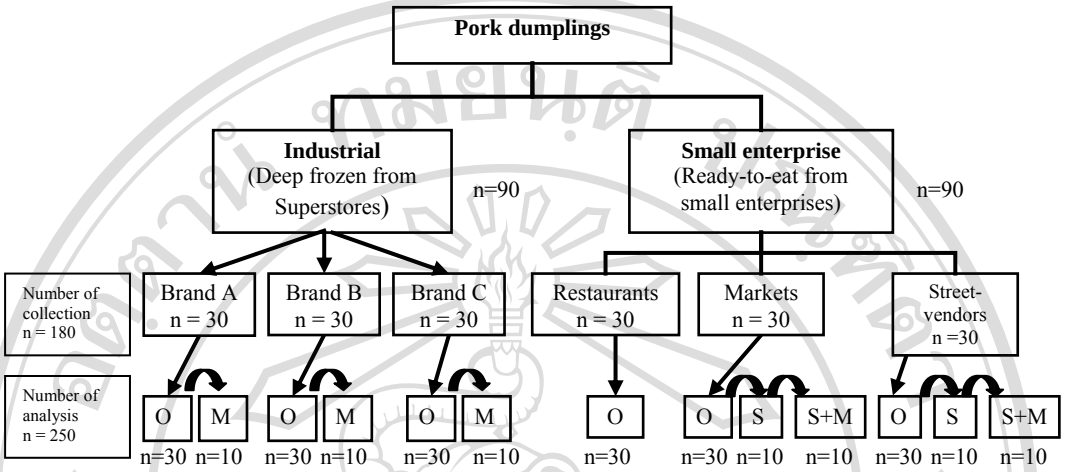
30 packs of frozen pork dumplings of brand A, brand B and brand C were randomly collected in different lots from 5 superstores. (1 pack of frozen pork dumplings equal to 1 sample). Additionally, 10 parallel samples were randomly selected from these 30 samples of each brand to study the effect of reheating.

2) Small enterprises

a) 30 samples of cooked ready-to-eat pork dumplings were randomly collected from 30 street vendors. (Normally, Vendors sell 10-20 pork dumplings in a plastic bag depending on a customer's order, therefore 1 plastic bag contained 10-20 pork dumplings equal to 1 sample). Additionally, 10 parallel samples were randomly selected and were stored at room temperature for 6-8 hours to simulate consumer's behavior and to study the effect of reheating.

b) 30 samples of cooked ready-to-eat pork dumplings were randomly collected from 30 vendors in 18 markets around Chiang Mai province. Additionally, 10 parallel samples were randomly selected and the protocol was repeated as mentioned in a)

c) 30 samples of cooked ready-to-eat pork dumplings were randomly collected from 30 restaurants.



O = Original samples

S = parallel samples/stored at room temperature (6-8 h)

M = parallel samples/microwave heated

Figure 4 Flow chart of sampling design

3.2 Sample preparation

3.2.1 Industrial enterprises

3.2.1.1 A pack of frozen pork dumplings consists of 10-12 pieces weighing 12-18 g /piece. Every pack of samples underwent certain procedures according to the following protocol: core temperature and pH were measured and then 5 pork dumplings were randomly selected and pooled together; after that 25 g of sample were taken for microbiological analysis.

3.2.1.2 A parallel sample was reheated by microwave according to the recommendation on the package. After that, a sensory test, a temperature measurement and a pH determination were performed. 5 reheated pork dumplings were pooled together and a 25 g sample was taken for microbiological analysis.

3.2.2 Small enterprises

3.2.2.1 Street vendor and market enterprises

1) Ready-to-eat pork dumplings vary in size depending on the vendors. A sensory test, a temperature measurement and a pH determination were performed. 5 pork dumplings were randomly selected and pooled together. A 25 g sample was taken for microbiological analysis.

2) Parallel samples were randomly selected and stored at room temperature for 6-8 hours. The temperature and pH measurements were performed after storage and then the samples were divided into 2 portions: one portion was immediately taken for the microbiological analysis, the other portion was reheated by microwave, and after that temperature and pH measurements were done followed by the microbiological analysis.

3.2.2.2 Restaurants

Ready-to-eat pork dumplings which are sold in restaurants consist of 4-6 pieces/serving and 18-20 g/piece. A sensory test, temperature and pH measurement was performed. All of the pork dumplings/serving were pooled together and a 25 g sample was taken for microbiological analysis.

3.3 Microbiological analyses

3.3.1 Preparation of the initial suspension according to ISO 6887-1

For the microbiological analysis the analytical unit (25g) was added to 225 ml of sterile 0.1% (w/v) peptone water (Merck®) and homogenized in a stomacher (IUL instrument) for 1 minute at medium speed and room temperature. Serial decimal 10-fold dilutions were done by transfer of 1 ml of initial suspension (10^{-1}) into a tube containing 9 ml of sterile 0.1% (w/v) peptone water (Merck®). Then the mixture was

homogenized to make 10^{-2} dilution. To prepare the further decimal dilutions, 1 ml of the 10^{-2} dilution was transferred into a tube containing 9 ml of sterile 0.1 % (w/v) peptone water (Merck®) to make 10^{-3} dilution. These operations were repeated by using a new sterile pipette to obtain 10^{-4} and 10^{-5} dilutions.

3.3.2 Enumeration of total aerobic mesophilic bacteria (ISO 4833, 2003)

The plating was done on Plate Count Agar (PCA, Merck®) as nutrient medium from the prepared dilutions (10^{-1} to 10^{-5}) by a duplicated pour plate method. The colonies that formed after incubation at 30 ºC for 48 hours under aerobic conditions were counted (ISO 4833, 2003).

The dishes containing 15 to 300 colonies were examined. The calculation of total aerobic mesophilic bacteria was done as follows;

1. General case

Calculation of APC was done according to the following formula:

$$N = \frac{\sum C}{[(n_1 \times 1) + (n_2 \times 0.1)] \times (d)}$$

Where

N = Number of colonies per gram of product

$\sum C$ = Sum of all colonies on all plates counted

n_1= Number of plates in first dilution counted

n_2 = Number of plates in second dilution counted

d = Dilution from which the first counts were obtained

2. Estimation of low numbers

2.1 If the two dishes contained less than 15 colonies, the formula was simplified and only the arithmetical mean was used for calculation.

$$N = y/d$$

Where

y = arithmetical mean of the colonies counted on two dishes

d = the dilution factor of the initial suspension

2.2 If the two dishes did not contain any colonies, the results are to be expressed as follows:

- Less than 1/d aerobic bacteria per gram

Where d is the dilution factor of the initial suspension

3.3.3 Enumeration of mesophilic lactic acid bacteria (ISO 15214, 1998)

The plating was performed into MRS medium (de Man, Rogosa and Sharpe, Merck®) from the prepared dilutions (10^{-1} to 10^{-3}) by a duplicated pour plate method. The colonies were counted after incubation at 30 ºC for 48 hours under anaerobic conditions by double-layer MRS medium (ISO 15214, 1998).

The calculation of mesophilic lactic acid bacteria followed the same rules as written in 3.3.2

3.3.4 Enumeration of *Enterobacteriaceae* (ISO 7402, 1993)

The number of *Enterobacteriaceae* was determined by the three tubes most probable number (MPN) method. E.E broth (Buffered Briliant Green Bile Glucose broth, Oxoid®) and VRBG (Violet Red Bile Glucose gar, Oxoid®) were used as the culture media. 5 of the typical pink to red colonies or colourless, mucoid colonies which have developed in the VRBG plates after incubation at 37 ºC for 24 hours were randomly selected for biochemical confirmation after subculturing in Nutrient Agar (NA, Merck®). The number of tubes which showed a negative reaction in the oxidase test and a positive fermentation test was registered as positive for *Enterobacteriaceae*. MPN of *Enterobacteriaceae*/g were calculated using the MPN tables (de Man, J.C. MPN tables. ISO 7402, 1993).

Oxidase reaction

Using the platinum/iridium loop, wire or glass rod, a portion of each well-isolated colony was taken and streaked on a filter paper moistened with the oxidase reagent (Fluka®, UK). The test was considered negative when the colour of the filter paper did not turn to dark within 10 seconds.

Fermentation test

The selected-colonies from nutrient agar were stabbed into tubes containing Glucose Agar (bromocresol purple as indicator) and then incubated at 37 ºC for 24 hours. If a yellow colour occurs throughout the contents of the tube, the reaction was regarded as positive. Most strains produce gas.

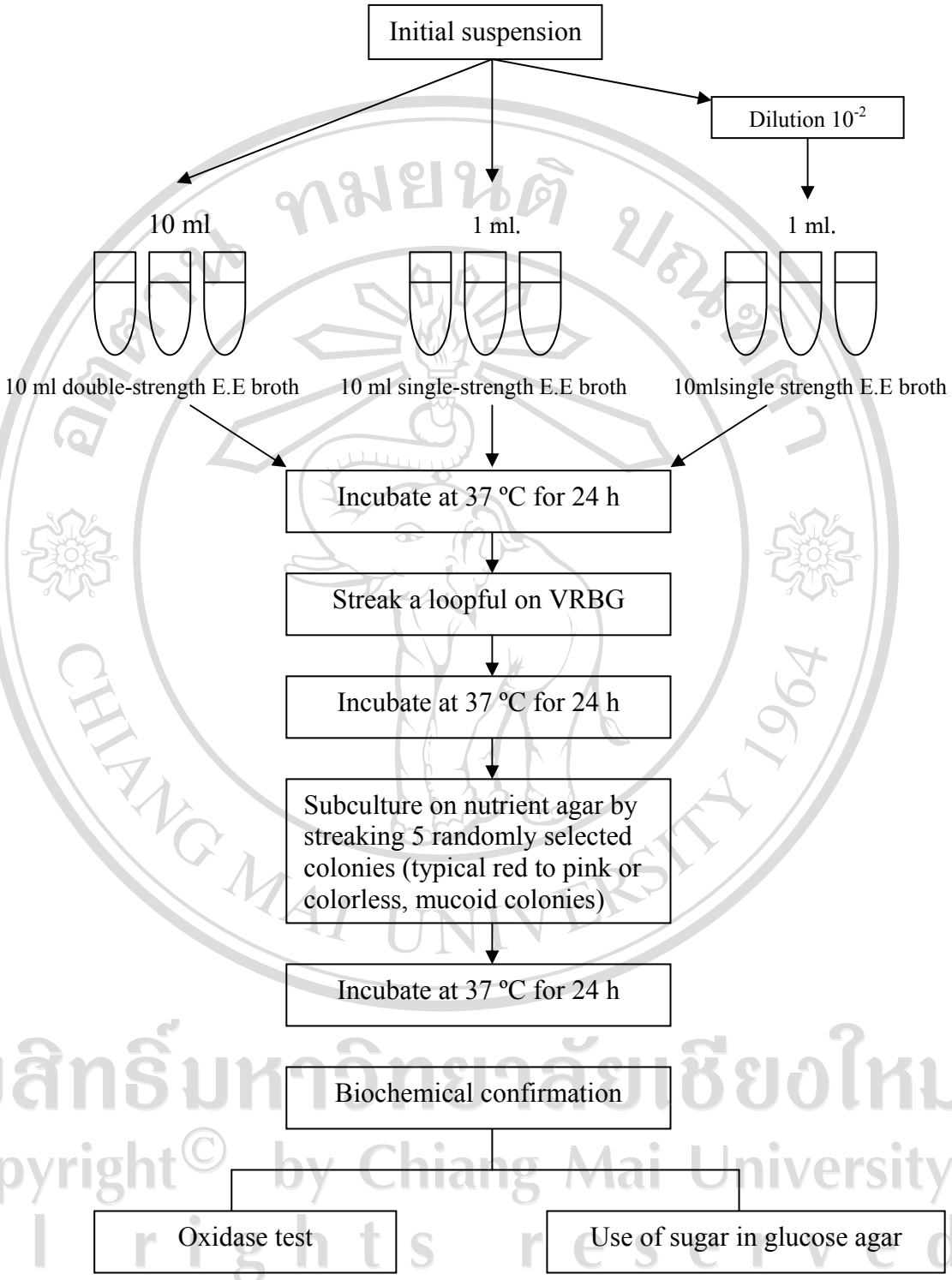


Figure 5 Flow chart of enumeration of *Enterobacteriaceae*

3.3.5 Enumeration of coliforms (ISO 4831, 1991)

The total numbers of coliforms were determined by the three tubes most probable number (MPN) method. Lauryl Sulphate Tryptose broth (Merck®) was used as selective enrichment medium and Brilliant Green Lactose Bile Broth (Merck®) was used as confirmation medium. The number of tubes that showed gas formation in the BGLB-confirmation-broth was counted. The probable numbers of coliforms were calculated according to the MPN tables (de Man, J.C. MPN tables. ISO 4831, 1991)

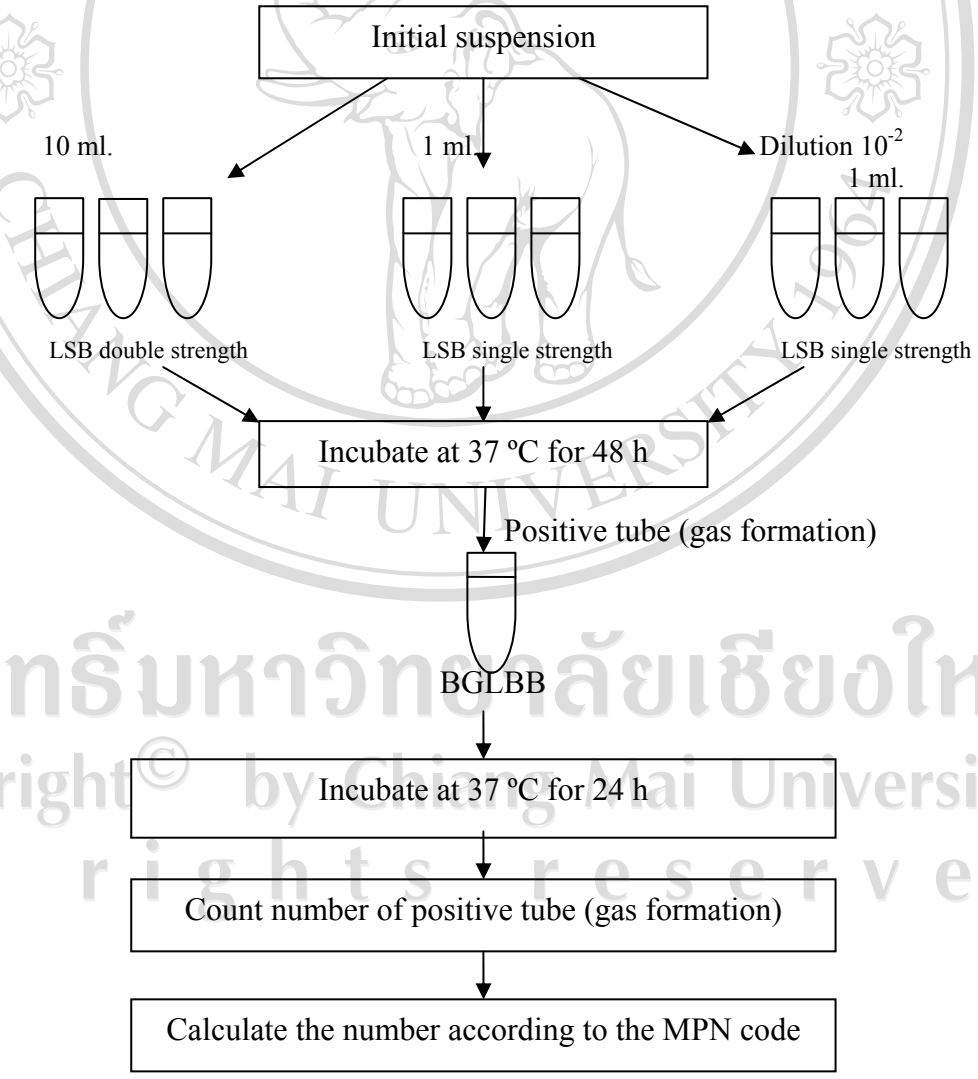


Figure 6 Flow chart of enumeration of coliforms

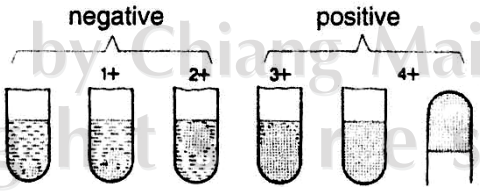
3.3.6 Enumeration of coagulase-positive staphylococci
(ISO 6888-1, 1997)

A duplicated spread plate method was performed using Baird-Parker Agar (BPA,Merck®) prepared by adding sterile egg yolk to a tellurite emulsion (Bacto™ , Difco®). After incubation at 37 ºC for 48 hours, the coagulase test was applied to typical black-grey, bright, smooth colonies with clear zones. The colony count was determined according to ISO 6888-1(1997).

Coagulase test (Confirmation test)

Typical colonies inoculated were transferred to a tube of Brain-Heart Infusion broth (Merck®) with a sterile wire and then incubated at 37 °C for 24 hours. After that, 0.1 ml of each culture Brain-Heart Infusion broth was added to 0.3 ml of rabbit plasma (Bactident@Coagulase, Merck®) in sterile tubes and incubated in a water bath at 37 °C. The tube contents were checked every hour for coagulation by gently tipping to the side.

The coagulase test was regarded as positive, if the volume of the clot occupied more than three-quarters of the original volume of the liquid. If the test was negative after 4-6 hours, the incubation of the tube was continued and a final assessment was made after 24 hours. As a control, 0.1 ml of sterile brain-heart infusion broth was added to the recommended quantity of rabbit plasma and incubated without inoculation.



Negative	no coagulation
1+ positive	a few small separate clots
2+ positive	a few small joined clots
3+ positive	large extensively coagulated clots
4+ positive	complete coagulation, contents do not shift when tube is inverted

Figure 7 Grades of coagulase reaction

A calculation of the number N of identified coagulase-positive staphylococci present in the test portion was done following the same rules as written in 3.3.2.

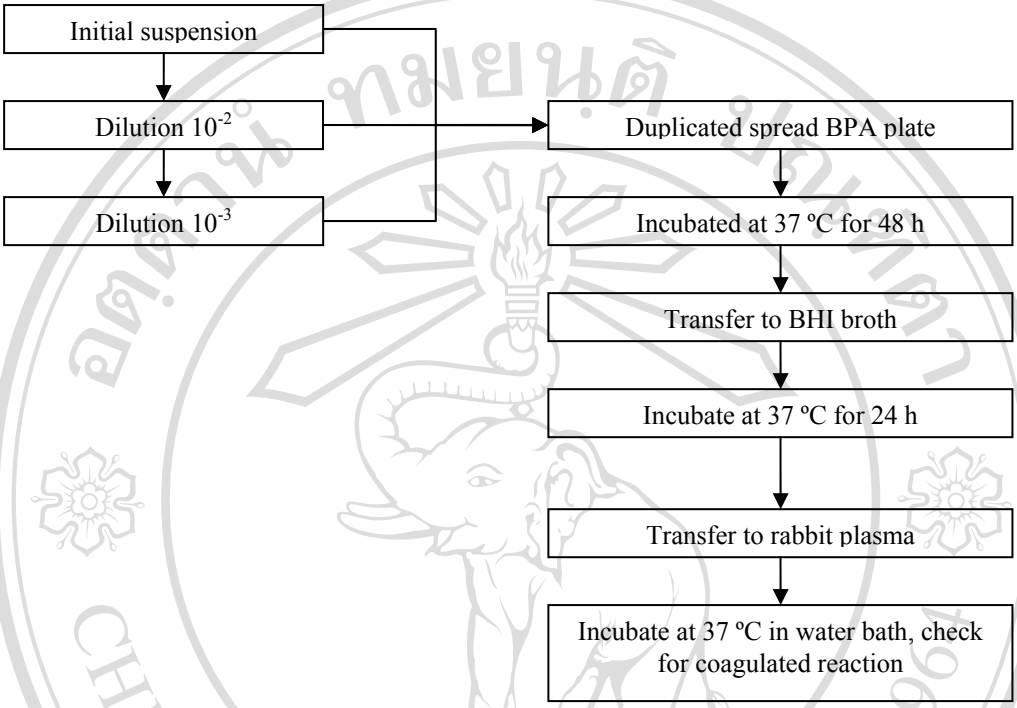


Figure 8 Flow chart of enumeration of coagulase-positive staphylococci

3.3.7 Enumeration of Pseudomonad (ISO 13720, 1995)

The duplicated spread plate method was performed using *Pseudomonas* CFC Selective Agar (Merck®) which contains cetrimide, fucidin and cephalotin. The inoculated medium was incubated at 25 ºC for 48 hours. All grown colonies are suspected *Pseudomonas spp*. and counted as such. For confirmation 5 randomly selected colonies were subcultured on Nutrient agar (Merck®). When they developed a positive oxidase reaction and showed growth only on the aerobic surface of Kligler's double sugar iron Agar, the result was registered as positive for *Pseudomonas spp*..

Oxidase test

A portion of each well-isolated colony was streaked onto a filter paper moistened with the oxidase reagent. The test was considered as positive when the colour of the filter paper has turned to dark in 10 seconds.

Use of sugar in Kligler's agar

A wire was used to streak the slant surface of the agar and then the wire was stabbed into the butt to the bottom of the agar. The temperature and time of incubation were 25 ºC for 24 hours. The test was considered as positive when growth occurred only on the aerobic surface of Kligler's double sugar iron Agar

The number, N, of identified microorganisms present in the test sample was calculated according to the formula written in 3.3.2.

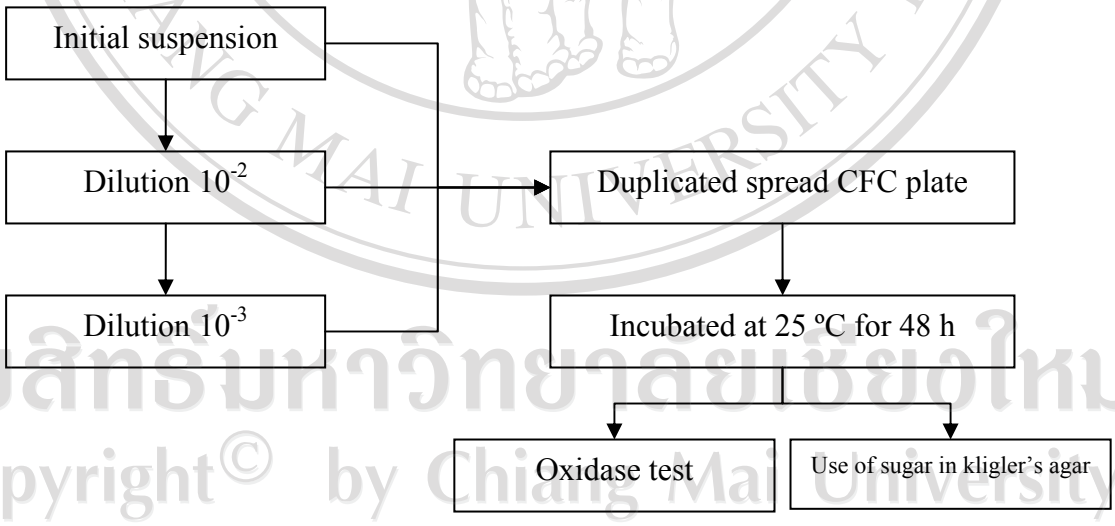


Figure 9 Flow chart of enumeration of *Pseudomonas spp.*

3.4 Additional analyses

3.4.1 Temperature measurement

A food grade thermometer (DeltaTRAK®) was used to determine the core temperature of the samples at the moment of sampling, after storage and after reheating.

3.4.2 pH measurement

pH value was determined by a food grade pH meter (CyberScan, EUTECH) immediately after sampling, after storage and after reheating.

3.4.3 Sensory test

The sensory test was performed by the investigator to detect deviation from the normal sensory profile.

3.5 Statistical analysis

The statistical software SPSS 14.0 for Windows was used for statistical analysis. The median of microbial counts were determined. The log-transformed microbiological results were used for descriptive statistics of aerobic bacteria, lactic acid bacteria, coagulase-positive staphylococci and *Pseudomonas spp.* For the *Enterobacteriaceae* and coliforms, the MPN/g results were calculated. A Mann-Whitney U test was applied for comparison between industrial samples and small enterprise samples. A Kruskal –Wallis test was applied for comparison among 3 brands of industrial and among 3 different sources (street vendor, market and restaurant) of small enterprises to find out whether significant differences exist. A Wilcoxon Signed Ranks test and a Friedman test were used for the comparison between the sample in the original state and after reheating of industrial samples and small enterprise samples respectively to detect significant differences.