CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

1. Fresh noni fruit (Meungmai Market, Chaing Mai)
2. Distilled water (Polestar, Thailand)
3. Cane sugar / sucrose (Mitrphol™, Thailand)
4. 5 l plastic bottles (Polyethylene theraphtharate, PET)
5. Commercial noni juices (Siam Noni™: Suprederm, Thailand)
6. Fermented noni juices (Lookyothamachart™, Thailand)

3.2 Microbiological examination equipments

1. Plastic petri dishes (Hycon, USA)
2. Inoculating loops
3. Inoculating needles
4. Microscopic slides
5. Aluminium foil
6. Test tubes 16x150 ml with plastic caps (Pyrex, USA)
7. Screw-capped tubes
8. Bunsen burners
9. Beakers (Pyrex, USA)
10. Measuring cylinders (Pyrex, USA)
11. Erlenmeyer flasks (Pyrex, USA)
12. Stirrer rods
13. Forceps
14. A burette (Pyrex, USA)
15. Sterile pipettes (1 and 10 ml) (KIMAX, USA)
16. Duran bottles (Schott Duran, Germany)

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17. An incubator at 30°C (Haereous, England)
18. An incubator at 37°C (Mammert, USA)
19. A colony counters (Chiltern, England)
20. An autoclave (Gallenkamp, England)
21. A hot air oven (Mammert: Model ULM-400, USA)
22. An analytical balance (Mettler-Toledo: Model AG204, USA)
23. A kitchen pot
24. A vortex mixer (Vortex genie 2: Model G 560E, USA)
25. Durham tubes
26. A blender (Hitashi: Model VA-MILL, Thailand)
27. A pH meter (Hanna Instrument, Italy)
28. Anaerobic jar (Merck, Germany)
29. A water bath (Memmert: Model L 4999, Germany)
30. A Laminar flow cabinet

3.3 Media and Reagents

1. Potato Dextrose Agar (PDA) (Merck, Germany)
2. Plate Count Agar (PCA) (Merck, Germany)
3. Tryptic Soy Agar (TSA) (Merck, Germany)
4. McConkey Agar (Merck, Germany)
5. de Man, Rogosa and Sharp (MRS) Agar (Merck, Germany)
6. Lauryl Sulfate Tryptose (LST) Broth (Merck, Germany)
7. Brilliant Green Lactose Bile Broth (BGLBB) (Merck, Germany)
8. Mannitol Egg Yolk Polymyxin (MYP) Agar (Merck, Germany)
9. Tryptose Sulfite Cycloserine (TSC) Agar (Merck, Germany)
10. Peptone (BD, France)
11. 70% alcohol (O.V. Chemical Ltd., Thailand)
12. 0.85% NaCl (Merck, Germany)
13. NaOH (Merck, Germany)
14. Phenolphthalein (Merck, Germany)
15. Non fat dry milk (Merck, Germany)

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16. Anaerocult A (Merck, Germany)
17. Gram's iodine solution (Merck, Germany)
18. Crystal violet solution (Merck, Germany)
19. Safranin solution (Merck, Germany)
20. 95% alcohol (Merck, Germany)
21. Tartaric acid (Fluka, Switzerland)

3.4 Research Design Scope and Method

3.4.1 Examination of noni fruit, commercial noni juice and commercial fermented noni juice

Fresh noni fruits were bought from Moungmai Market. These fruits were washed, air-dried and aseptically cut into small pieces on sterile aluminium foil that was carried out inside a laminar air flow cabinet. The small pieces of noni fruit were transferred into a blender and blended into a homogenous solution. The microbiological and chemical composition (details in section 3.5) of the fresh noni fruit together with a commercial brand of noni juice and a commercial fermented noni juice were then examined (Srimuang, 2004). To find the correct number of microorganisms in petri dishes, noni, noni juice and fermented noni juice samples were diluted using a decimal dilution series. The first 10^{-1} dilution was done by suspending or homogenizing 10 ml of noni juice with 90 ml of 0.1% peptone solution at pH 6.8-7.0 in a 100 ml sterile container. The amount of 1 ml of this mixture contained 0.1 ml of noni juice sample. Subsequent dilutions from this mixture were done using a similar procedure up to 10^{-6} dilution (AOAC, 2000). Noni fruit, commercial noni juice and commercial fermented noni juice samples were then examined for their microbiological qualities by pour plate. The pour plate technique was done in duplicate for each sample dilution and carried out for:

1. Total Plate Count (TPC) using Plate Count Agar (PCA) (AOAC, 2000).
2. Yeast and mould using Potato Dextrose Agar (PDA) (AOAC, 2000).
3. Gram Negative bacteria using McConkey Agar (AOAC, 2000).

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4. Lactic acid bacteria using de Man, Rogosa and Sharp (MRS) Agar (AOAC, 2000).

5. Proteolytic bacteria using Tryptic Soy Agar (TSA) with 10% (w/v) non fat dry milk (Busta *et al*, 1984).

6. Spore-forming bacteria [*Bacillus* spp. using Mannitol Egg Yolk Polymyxin (MYP) Agar and *Clostridium* spp. using Tryptose Sulfite Cycloserine (TSC) agar)] (AOAC, 2000).

Coliform bacteria using MPN method (AOAC, 2000).

Chemical analysis: total titratable acidity by a titration method (AOAC, 2000) and pH value using a pH meter. The analysis was done in triplicate for each sample.

3.4.2 Production of fermented noni juices

Fresh noni fruits were bought from Moungmai Market, washed and cut into small pieces (noni fruit was cut diagonally with a thickness approximately 5 mm). After that, the noni fruits were filled into 5 l plastic bottles and added with cane sugar and distilled water in a ratio of 3:1:10 for noni fruits, sugar and distilled water, respectively (Anonymous, 2003). All the ingredients were mixed properly and incubated at room temperature for 9 months to ferment the noni fruit using a natural fermentation. During this incubation period, samples of noni juice were separated on 0, 2, 4, 6, 8, 10, 12, 24, and 36 weeks to be analyzed for their microbiological and chemical changes. The method of microbiological examination and chemical analysis were similar to the section 3.4.1. A flowchart for the production of fermented noni juices could be seen in Figure 3.1.

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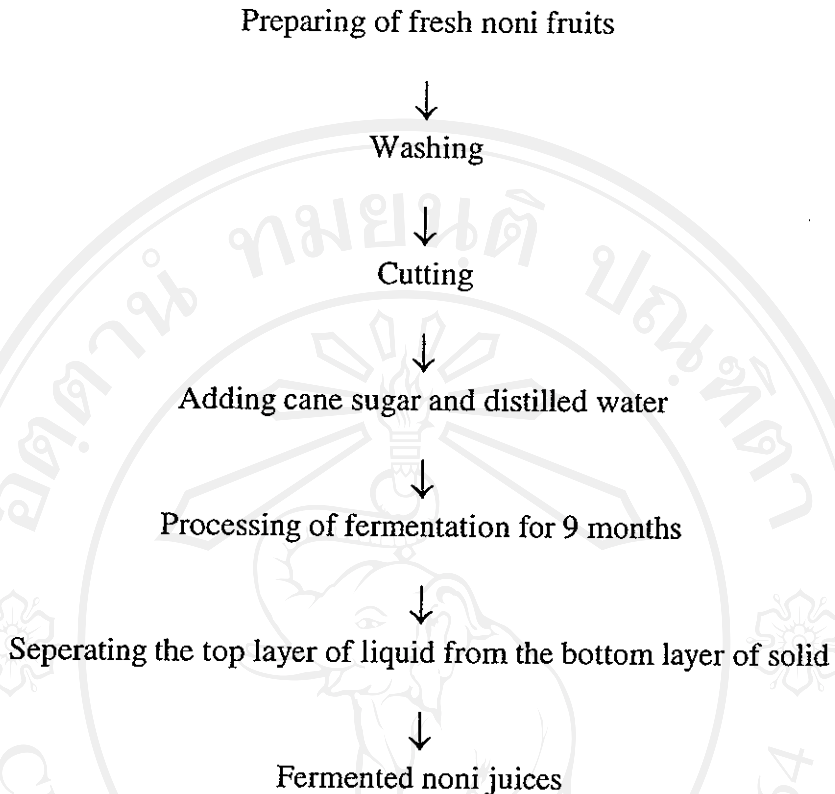

Figure 3.1 A production flowchart of fermented noni juices in a laboratory scale (Anonymous, 2003)

3.4.3 Effect of heat treatment on the microbiological quality of noni juices

Fresh noni fruits were bought from Meungmai Market, washed and cut into small pieces (noni fruit was cut diagonally with a thickness approximately 1 cm). The fruits were then added with distilled water at a ratio of 1:1 (w/v) for noni fruit and distilled water, respectively. The fruit and distilled water were blended together into a homogenous solution using a blender and filtered by sterile double layer cloths to produce noni juices. To give flavor to the juice, salt at a concentration of 1% (w/v) and 5% (w/v) sugar were also added. After mixed properly, the juices were pasteurized using 3 different heat treatments, which were at 64°C for 5 min, at 72°C for 1 min and at 80°C for 15 s (Srimuang, 2004). For the pasteurization treatments, they were conducted using test tubes in a controlled temperature waterbath. After the pasteurization, noni juices were cooled down in cool water. A batch of noni juice

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was also boiled at 100°C for 10 min as a reference for the traditional method commonly applied for noni juices. The boiling was done using a kitchen pot. Following the heat treatment, the noni juices were cooled down using cool water. Heat treated noni juices were then examined for their microbiological qualities. The method of microbiological examination was similar to the section 3.4.1. A flowchart for the production of heat-treated noni juices was displayed in Figure 3.2.

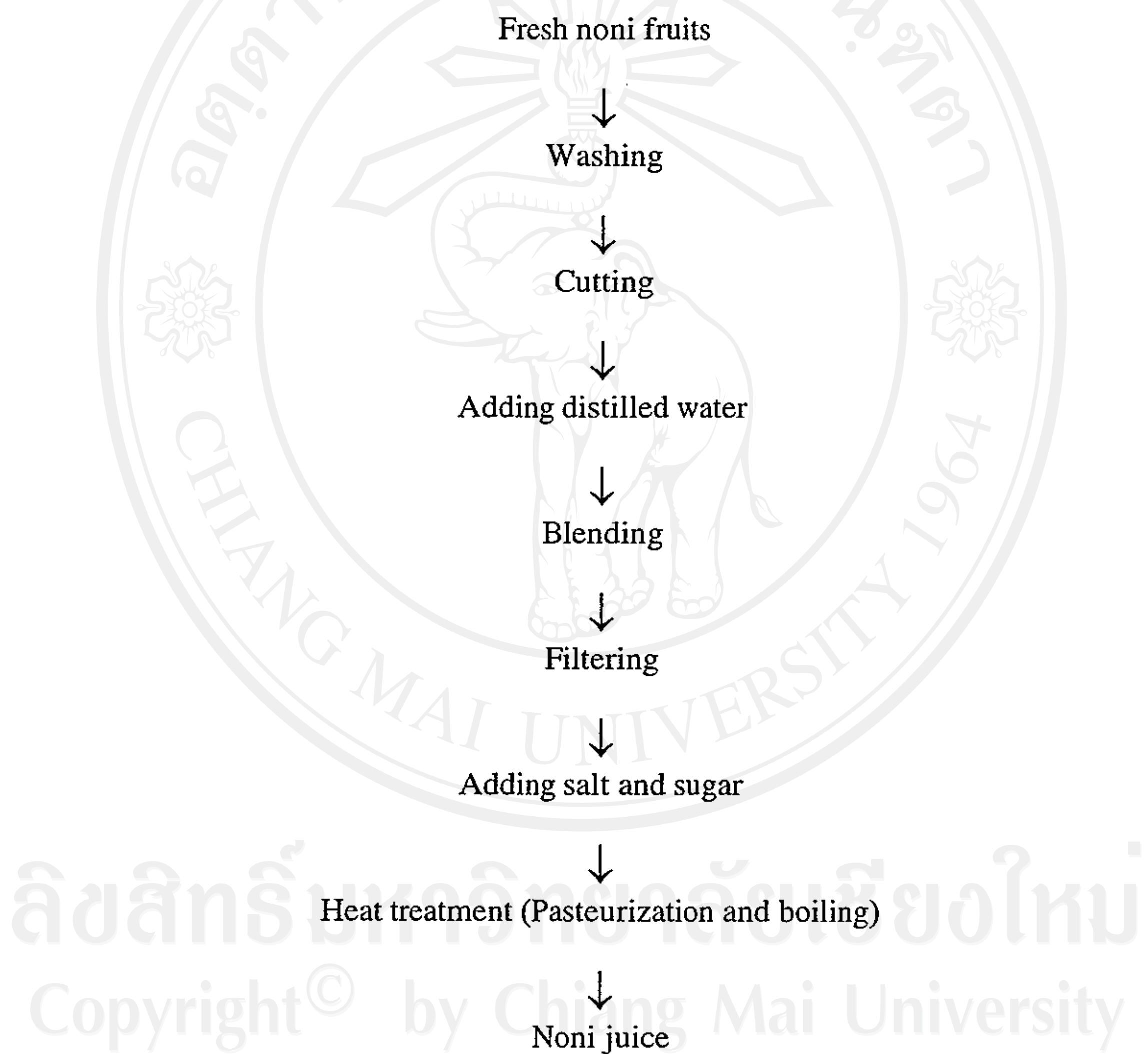

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Figure 3.2 A Diagram of noni juices production in the laboratory scale (Anonymous, 2003)

3.4.4 The effect of storage conditions on the microbiological quality of noni juices

To examine the quality of noni juices during storage periods, different heat treated noni juices were produced using heat treatment conditions of 64°C for 15 min, 72°C for 1 s, 80°C for 15 s and boiling (at 100°C) for 10 min. The method to produce these noni juices was similar to the section 3.4.3. After the heat treatment, the noni juices were immediately put in 200 ml sterile flasks and packed aseptically. Each of heat-treated noni juices were then stored at 4°C and room temperature for 21 days. During the storage period, samples of noni juices were examined for their microbiological properties on 0, 7, 14 and 21 days of storage. The method of microbiological examination was similar to the section 3.4.1.

3.4.5 Statistical analysis

Each experiment was independently done for 2 replication. All microbiological enumerations were done in duplicate. Collected data was analyzed using Analysis of variance (ANOVA) by applying a Completely Randomized Design (CRD). Treatment means were compared using Duncan New Multiple Range Test (DMRT) (Pongsririkul, 2001) A significance of difference was defined at the 95% level of confidence ($p \leq 0.05$). All the statistical analysis was conducted using a SPSS solfware version10 (SPSS Inc, USA).

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