Chapter 3

Materials and Methods

3.1 Raw material

3.1.1 White rice, KhumKar brand from Tesco Lotus, Chiang Mai

3.1.2 Brown rice, KhumKar brand from Tesco Lotus, Chiang Mai

3.1.3 Black glutinous rice, Nan province from Tesco Lotus, Chiang Mai

3.1.4 Brown sugar, Mitr Phol Gold, Mitr Phol, Thailand

3.1.5 Honey from longan flower, Doi Khum brand, Thailand

3.2 Probiotic microorganism

Lactobacillus acidophilus TISTR 450 from Thailand Institute of Scientific and Technological Research that was donated by a Japanese researcher.

3.3 Equipment

3.3.1 An autoclave (Tomy SS-325, USA)

3.3.2 Auto pipettes (Gilson, France)

3.3.3 1,000 ml clear bottles

3.3.4 A 25 ml titration burette (HBG, Germany)

3.3.5 Filtered cloths

3.3.6 1,000 ml measuring cylinders

3.3.7 100 ml measuring cylinders

3.3.8 125 ml erlenmeyer flasks

3.3.9 250 ml erlenmeyer flasks

3.3.10 A gas cooker (National, Thailand)

3.3.11 A big spoon (Seagull, Thailand)

3.3.12 Kitchen pots (Croccodile, Thailand)

3.3.13 0-100^{O}C mercury thermometer (Fisher, UK)

3.3.14 A microplate reader (Dynex Technology, USA)

3.3.15 An oven (Gallenkamp, England)

3.3.16 A pH meter (Metrohm, Switzerland)

3.3.17 Measurement pipettes (10 ml) (HBG, Germany)

3.3.18 A hand refractometer (ATAGO N-1E, Japan) measurement range 0-32^{O}Brix

3.3.19 200 ml and 500 ml volumetric flasks

3.3.20 A Brookfield viscometer (Brookfield, England)

3.3.21 A colorimeter (Minolta, Japan)

3.3.22 A light microscope (Olympus, Japan)

3.3.23 A vernier caliper (Vis, Poland)

3.3.24 A centrifuge (Sanyo HARRIE 18180, Japan)

3.3.25 Ocular and stage micrometer (Olympus, Japan)

3.3.26 A colony counter (American optical company, USA)

3.3.27 Dilution bottles

3.3.28 A hot air oven (Binder, UK)

3.3.29 An incubator room at 37^{O}C

3.3.30 A laminar flow cabinet (Astec Microflow model ABS 1200, UK)

3.3.31 Glass petri dishes

3.3.32 A stomacher (Seward, England).

3.3.33 A vortex mixer (Gemmy Industrial, Taiwan).

3.3.35 An analytical balance (Mettler, Switzerland).

3.3.36 An analytical balance (Ohaus, USA).

3.3.37 A blender (Moulinex, France)

3.3.38 A cold room storage at 6^{o}C

3.3.39 Needle 0.40x12 mm 27G (Nipro, Japan).

3.3.40 10 ml syringe (Nipro, Japan).

3.3.41 120 ml white bottles

3.3.42 Test tubes (5 and 10 ml) (Pyrex, USA).

3.3.43 Filter paper no. 40 (Whatman, Egland).

3.4 Chemical reagent

3.4.1 Calcium chloride (Merck, Germany)

3.4.2 Distilled water

3.4.3 Hydrochloric acid (HCl) (Merck, Germany)

3.4.4 Lactic acid (Fluka, UK)

3.4.5 Phenolphthalein (BHD, England)

3.4.6 Potassium hydroxide (KOH) (Merck, Germany)

3.4.7 Sodium alginate (Fluka, UK)

3.4.8 Sodium chloride (NaCl) (J.T. Baker, Mexico)

3.4.9 Sodium citrate (Fisher Scientific, UK)

3.4.10 Sodium hydroxide (NaOH) (Merck, Germany)

3.5 Microbiological media

3.5.1 de Man, Rogosa and Sharpe broth (Diffco, USA)

3.5.2 MRS-sorbitol medium (Dave and Shah, 1996 and Atlas, 1993) composed with peptone (Diffco, USA), agar (Helicopter, Thailand), beef extract (Merck, Germany), sodium acetate.$3H_2O$ (Merck, Germany), yeast extract (Diffco, USA), K_2HPO_4 (Fisher Scientific, UK), triammonium citrate (Fluka chemika, Switzerland), $MgSO_4.7H_2O$ (Fluka chemika, Switzerland), $MnSO_4.4H_2O$ (Univar, New Zealand) and sorbitol (Aldrich chemical, U.S.A)

3.5.3 Potato dextrose agar (PDA) media (Atlas, 1993)

3.5.4 Plate count agar (Diffco, USA)

3.6 Methods

3.6.1 Preparation of *Lactobacillus acidophilus* TISTR 450

L. acidophilus TISTR 450 was grown and maintained in MRS agar and refreshed in 10 ml MRS broth at 37°C for 24 hours. Viable cells were prepared by transferring 1 ml of *L. acidophilus* stock culture into 99 ml of MRS broth and incubated at 37°C for 16-18 hours. The cultures were then centrifuged at 4000 g for 10 minutes at 25°C and washed twice with sterile 0.85% NaCl solution. The harvested cells had a concentration about 10^8-10^9 CFU/ml (Murga *et al.*, 1998).

3.6.2 Preparation of rice milks

Rice milks were prepared by adding rice with a specific amount of distilled water. The mixtures were boiled for 30 minutes, followed by a filtration through a

double layer filtered cloth in order to remove solid residues in the solution. Amounts of 1,000 ml of the filtrates recognized as rice milks were then filled in glass bottles to be pasteurized at 80±3°C for 20 minutes (the heating time was around 30 minutes for 1 l rice milk sample) (วรชัย, 2546).

3.6.3 Rice and rice milk composition analysis

The chemical compositions of rice and rice milks were analysed for

3.6.3.1 Ash (AOAC, 2000)

3.6.3.2 Carbohydrate (AOAC, 2000)

3.6.3.3 Fiber (AOAC, 2000)

3.6.3.4 Lipid (AOAC, 2000)

3.6.3.5 Moisture content (AOAC, 2000)

3.6.3.6 Protein (AOAC, 2000)

3.6.3.7 Reducing sugar (Miller, 1959)

3.6.3.8 Total sugar (James, 1995)

3.6.4 The effect of types and concentrations of rice milks on the survival of *L. acidophilus* during storage at chilled temperature

Different types of rice including white, brown and black glutinous rice were investigated. Each rice type was added with distilled water to obtain concentrations of 5, 7 and 10% (w/v). Each rice milk was then pasteurized at 80±3°C for 20 minutes (วรชัย, 2546). After cooling down the heat milk to 40-45°C in cool water, viable cell of *L. acidophilus* at concentrations of 10^8 – 10^9 CFU/ml were added into each rice milk, mixed thoroughly and stored at 6°C for 15 days.

Analysis of rice milk sample during storage included:

3.6.4.1 Physical analysis

(1) Viscosity by a Brookfield viscometer (Brookfield, U.S.A)

(2) Color analysis by a colorimeter (Minolta, Japan)

3.6.4.2 Microbiological analysis

(1) Total plate count for all viable microorganisms in rice milks using PCA medium (AOAC, 1998)

Total bacteria were counted from duplicate samples of each rice milk production batch at 3 days interval during 15 days storage at $6^{O}C$. Each rice milk sample was mixed thoroughly and a representative sample of 1 ml from each milk sample was 10 fold serially diluted (10^4 to 10^6) in 0.85% sodium chloride. Enumeration was carried out using a pour plate technique on PCA medium. Plates were incubated at $37^{O}C$ for 72 hours. Plates containing 25 – 250 colonies were enumerated, recorded as colony forming units (CFU) per ml and calculated to log CFU/ml.

(2) Viable cell count of *L. acidophilus* using MRS-sorbitol medium (Dave and Shah, 1996)

L. acidophilus was counted from duplicate samples of each rice milk production batch every 3 days interval during 15 days storage at $6^{O}C$. Each rice milk sample was mixed thoroughly and a sample amount of 1 ml of each milk sample was 10 fold serially diluted (10^4 to 10^7) in 0.85% sodium chloride. Enumeration was carried out using a drop plate technique on MRS-sorbitol medium. Plates were incubated anaerobically at $37^{O}C$ for 48 hours. Sample drops containing 5 to 25 colonies were enumerated, calculated as colony forming units (CFU) per ml and

adjusted to log CFU/ml. The highest survival of *L. acidophilus* in any experimental treatment within a specific experimental section was considered to be a suitable treatment that could maintain the viability of the probiotic bacterium in rice milks during storage at 6^{O}C and this experimental treatment would be further studied in the next experiment section.

(3) Viable cell counts of yeasts and moulds using PDA medium (AOAC, 1998)

Yeasts and moulds were counted from duplicate samples of each rice milk production batch every 7 days during 15 days storage. Each rice milk sample was mixed thoroughly and a sample amount of 1 ml of each milk sample was 10 fold serially diluted (10^3 to 10^5) in 0.85% sodium chloride. Enumeration was carried out using a spread plate technique on PDA medium and incubated at 37^{O}C for 5 days. Plates containing 10-150 colonies were enumerated, recorded as colony forming units (CFU) per ml and calculated to log CFU/ml.

3.6.4.3 Chemical analysis

(1) Total acidities (AOAC, 2000)

Ten milliliter of each sample was taken into a 125 ml Erlenmeyer flask. After adding 3 drops of phenolphthalein as an indicator, the sample was titrated with 0.01M NaOH until the solution color appeared pink. Results were expressed as percentage of lactic acid (lactic acid /100 ml sample).

(2) pH values by a pH meter (Metrohm, Switzerland)

A volume of 10 ml rice milk samples was transferred into 25 ml beaker. Into this sample, a pH meter probe was immersed for 5-8 minutes until the pH meter showed a constant pH value. The pH value shown by the pH meter was

record. Prior to the measurement of rice milk samples, the pH meter was calibrated using 2 standard buffer solutions, which were pH 4 and pH 7. The measurement of rice milk pH values was conducted every 3 days during 15 days storage at $6^{O}C$ and determined at room temperature.

(3) Total soluble solid by a refractometer (ATAGO N-1E, Japan)

The total soluble solid of rice milk samples were measured with a refractometer, and reported as $^{O}Brix$. The measurement of the total soluble solid was conducted every 3 days during 15 days at $6^{O}C$.

3.6.5 The effect of initial pH values and initial populations of *L. acidophilus* on the survival of the probiotic bacterium in rice milks during refrigerated storage

The most suitable rice type and concentration that supported the survival of *L. acidophilus* during refrigerated storage from the section 3.6.4 was used in this experiment. The pH of selected rice milk was adjusted to 4.5, 5.5 or 6.5 with 85% of lactic acid or 1 M sodium hydroxide prior to be pasteurized and inoculated with various amounts of *L. acidophilus*, 10^6 and 10^8 CFU/ml. The 6 different treatments of the rice milks were then stored at $6^{o}C$ for 15 days to monitor the survival of *L. acidophilus* in the rice milks. Analysis of the rice milks during storage followed the analysis procedures in the section 3.6.4.

3.6.6 The effect of different types and level of carbohydrate addition on the survival of *L. acidophilus* in rice milks during refrigerated storage

In this section, results from the section 3.6.5 for one initial pH value and one initial population of *L. acidophilus* were applied. After adjusted the pH of rice milks, the milks were added either with brown sugar or honey at concentrations of 0, 4 or 7% (w/v). The rice milks were then mixed thoroughly, pasteurized, cooled down, inoculated with *L. acidophilus* (from the section 3.6.5) and stored at 6^{O}C for 15 days.

Analysis of the rice milks during storage, including total acidities, pH values, total soluble solid, total plate count and viable count of *L. acidophilus*, was carried out every 3 days. Whereas viscosity, color value and viable cell counts of yeasts and moulds were conducted weekly.

In addition, the amounts of reducing sugar (Miller, 1959) and invert sugar (Anonymous, 2005e and Miller, 1959) were analyzed every 3 days to monitor changes in the sugar contents of rice milks during low temperature storage.

3.6.7 The effect of an immobilization technique on the survival of *L. acidophilus* in rice milks during refrigerated storage

In this section, an immobilization technique by an extrusion method was carried out for *L. acidophilus* to access the possibility of the technique in improving the viability of the probiotic bacterium in rice milks during low storage temperature. Cells of *L. acidophilus* were grown in MRS broth at 37^{O}C for 16-18 hours to produce young cultures. These young cultures were then harvested by centrifuge at 4000 g for 10 min at 25°C, washed twice with sterile 0.85% NaCl solution and resuspended in 5 ml of 0.85% NaCl solution. Using the last solution, young cultures of *L. acidophilus*

were mixed with 20 ml of 2% (w/v) sodium alginate solution. The cell suspension was injected through a 0.40 x 12 mm needle into a 100 ml beaker containing 60 ml 0.05 M calcium chloride. The beads were allowed to stand for 30 minutes for gelification, rinsed with 0.85% NaCl and subsequently kept in sterile 0.85% NaCl solution. The entire process was carried out aseptically in a laminar flow chamber (Krasaekoopt *et al.*, 2004)

Rice milks were prepared according to the results in the sections 3.6.4 – 3.6.6. After pasteurization, the rice milks were inoculated with either free or encapsulated cells of *L. acidophilus* at an initial probiotic population according to the results in the section 3.6.5. The probiotic–added rice milks were then stored at 6°C for 15 days. During the storage period, different properties of rice milks were analysed regularly following the analysis procedure in the section 3.6.6. For microbiological analysis in rice milks with encapsulated *L. acidophilus* cells, 1 ml of rice milk sample with *L. acidophilus* beads was suspended in 9 ml of 1% (w/v) sterile sodium citrate solution at pH 6, followed by homogenizing in a stomacher for 5 minutes (Krasaekoopt *et al.*, 2004). The cultures were then diluted and plated at appropriate media to enumerate the number of *L. acidophilus*, total bacteria and yeast and mold as in the section 3.6.6.

3.6.7.1 Examination of alginate bead dimention

The dimensions of 152 randomly selected beads were determined using a light microscope and an ocular and stage micrometer (Sultana *et al.*, 2000). If the beads dimensions were more than 2 mm, the measurement by a vernier caliper was used instead.

3.6.7.2 Examination of bead density

Bead density was calculated by the following formula

Density of bead = mass/volume

Volume of sphere = (4/3) πr^3

3.6.7.3 Efficiency of cell release from the beads

For quantitative measurements of *L. acidophilus* viable cells by a drop plate method, it was necessary to solubilize the polymer beads to release the entrapped cells. To do this, one gram alginate beads was suspended in 9 ml of 1% (w/v) sterile sodium citrate solution at pH 6, followed by homogenizing in a stomacher for 5 min (Krasaekoopt *et al.*, 2004). The colony forming units (CFU/ml) were determined by the drop plate method on MRS-sorbitol agar plates and incubated anaerobically at 37^{O}C for 48 hours.

3.7 Experimental designs and statistical analysis

Collected data from the experimental sections 3.6.4, 3.6.5 and 3.6.6 was statistically analyzed by Analysis of Variance using a completely randomized factorial design with three replications. For the experimental section 3.6.7, a completely randomized design with three replications was applied. To determine differences between treatment means, a Least Significant Difference (LSD) test was employed (พิศมัย, 2547). All of the statistical analysis was conducted by using the SPSS statistical software version 12 (SPSS Inc., U.S.A).