CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Microbial cultures

Freeze-dried probiotics cultures of *L. acidophilus* (LA-5) and yoghurt starter cultures (a mixture of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*) were obtained from CHR-Hansen A/S (USA). These cultures were stored at -20^{o}C before used in any experiments.

3.1.2 Material for yoghurt manufacturing

- Reconstituted milk powder (Misssion P-PLUS Brand Thailand)
- Skimmed milk powder (Mission Brand Thailand)
- Carrageenan (Lab scan Co., Ltd, Thailand)

3.1.3 Maltodextrin

Maltodextrin with a DE value of 10 was supplied by Northern Chemical Co., Ltd, Thailand

3.1.4 Packaging material

- Polyethylene tetraphthalate/Polypropylene/Aluminum (PET/PP/Al) (Siam Pack Co., Ltd, Thailand)
- Nylon/Polyethylene (nylon/PE) (Siam Pack Co., Ltd, Thailand)

3.2 Chemicals and media

3.2.1 Chemical for immobilized process

- Sodium alginate solution (Sigma, Australia)
- Hi-maize starch (Sigma, Australia)
- $CaCl_2$ (Merck, Germany)

- Peptone solution (Lab scan Co., Ltd, Thailand)
- Phosphate buffer (Merck, Germany)

3.2.2 Chemical for analysis

- NaOH (Merck, Germany)
- Phenolphthalein (Lab scan Co., Ltd, Thailand)

3.2.3 Media for microorganism

- Maximum Recovery Diluent (MRD, Oxoid England)
- M-17 agar (Merck, Germany)
- deMan Rogosa Sharpe (MRS) agar (Merck, Germany)
- 100% glacial acetic acid (Lab scan Co., Ltd, Thailand)
- Maltose (Lab Chem Co., Ltd, Australia)
- Homofermentative and Heterofermentative Differential (HHD) agar that compose of 2.5% (g/l) of fructose (Fluka, Switzerland), 2.5% (g/l) of KH_2PO_4 (Merck, Germany), 10.0% (g/l) of soytone (Bacto ^{TM}, France), 1.5% (g/l) of peptone from casein (Merck, Germany), 3% (g/l) of casamino acid (Bacto ^{TM}, France), 1% (g/l) of Tween 80 (Chemical of highest quality, Mark, Japan), 1% (g/l) of yeast extract (Merck, Germany) and 15% (g/l) of agar (U&V Holding Thailand)

3.3 Equipment

- Centrifugation (Hettich, Germany)
- Syringe needle (24G (0.55x25 mm), Nipro Corporation, Japan)
- Refrigerator (Samsung, Thailand)
- Vacuum oven (Binder VD23, Germany)
- Spray drier model SDE 50 (J.C. Machinery and civil work Co., Ltd, Germany)
- Vernier caliper
- Balance (OHAUS Co., Ltd, USA)
- Bostwick consistometer
- Colorimeter (CR-300, Minota Co., Ltd, Japan)
- Magnetic stirrer (VELP Scientifica, Europe)
- Hot air oven (Termaks, Japan)

- Water bath (Memmert, Germany)
- pH-meter (CONSORT C830, CE, Belgium)
- a_w-meter (Series 3, AquaLab, USA)
- Hand refractometer (ATAGO, Japan)
- Incubator (Memmert, Germany)
- Autoclave (Gallenkarp, England)

3.4 Preparation for microorganism and encapsulation process

- M-MRS

Preparing 20% (w/v) of maltose solution was done by putting 20 g of maltose into a 100 ml warm distilled water. The warm water enhanced the solubility of the maltose. After maltose was dissolved completely in water, the solution was filtered using a 0.45 μm sterile filter and transferred into a sterile bottle to be kept at 4^{o}C before adding in MRS medium to replace to glucose. The MRS medium was composed of 10.0% (g/l) of peptone from casein (Merck, Germany), 5.0% (g/l) meat extract (Merck, Germany), 5.0% (g/l) of yeast extract (Merck, Germany), 1.0% (g/l) of Tween 80 (Chemical of highest quality, Mark, Japan), 2.0% (g/l) of KH_2PO_4 (Merck, Germany), 5.0% (g/l) sodium acetate (Lab scan Co., Ltd, Thailand), 2.0% (g/l) of KH_2PO_4 (Merck, Germany), 2.0% (g/l) triammonium citrate (Lab scan Co., Ltd, Thailand), 0.5% (g/l) of $MgSO_4$ ($7H_2O$)(Lab scan Co., Ltd, Thailand), 0.2% (g/l) of $MgSO_4$ ($4H_2O$) (Lab scan Co., Ltd, Thailand) and 15% (g/l) of agar (U&V Holding Thailand).The MRS medium was sterilized by an autoclave at 121^{o}C for 15 min. The cool medium was then added with 20% (w/v) filtered maltose, for example, the cool medium of 90 ml was added with 10 ml of maltose solution.

- 0.1 M $CaCl_2$

$$\text{The molarity of } CaCl_2 = \left(\frac{\text{Molecular wieght of } CaCl_2}{1{,}000 \text{ ml of distilled water}} \right)$$

The molecular weight of $CaCl_2$ is 111 g, so, 0.1M of $CaCl_2$ was done by mixing 111 g $CaCl_2$ into 1,000 ml of distilled water or added 11.1g $CaCl_2$ into 100 ml of distilled water. The $CaCl_2$ solution was sterilized by an autoclave at 121^{o}C for 15 min.

● 0.1% peptone solution

Add 0.1 g of peptone into 100 ml of distilled water, sterilize the solution by an autoclave at 121°C for 15 min and keep at 4°C.

● 10% hi-maize starch

Put 5 g of hi-maize starch into 50 ml of distilled water to get a concentration 10% (w/v). Sterilize this solution by an autoclave at 121°C for 15 min. When put a sterile hi-maize starch solution into 50 ml of 1.8% (w/v) sodium alginate below as
2.5 ml of 10% (w/v) hi-maize starch + 50 ml of alginate solution equally as 0.5%.
5.0 ml of 10% (w/v) hi-maize starch + 50 ml of alginate solution equally as 1.0%.
7.5 ml of 10% (w/v) hi-maize starch + 50 ml of alginate solution equally as 1.5%.
10 ml of 10% (w/v) hi-maize starch + 50 ml of alginate solution equally as 2.0%.

● 0.1 M, pH 7.0 phosphate buffer

Put 15.601 g of NaH_2PO_4 into 1,000 ml of distilled water to get 0.1 M, adjust pH by 1.0 M NaOH until the pH of solution is 7.0 and then sterilize by an autoclave at 121°C for 15 min with a subsequent storage at 4°C.

3.5 Research design and methods

3.5.1 Micro-encapsulation and cell release of bacteria

L. acidophilus cultures were immobilized using a calcium-alginate containing hi-maize starch (Sigma, Australia) as a filler material that followed a procedure of Chandramouli *et al*. (2004). Young culture of *L. acidophilus* was prepared by growing 0.1 g of the freeze dried culture in MRS broth (Merck, Germany) at 37°C for 24 h (Dave and Shah, 1996 and Lan-Szu and Weimer, 1998). The probiotic cells were harvested by centrifugation at 3000 x g for 15 min at 4°C (Hettich, Germany), washed twice with 0.1% (w/v) of sterile peptone solution (Chandramouli *et al*., 2004) and mixed with 1.8% (w/v) sodium alginate solution (Sigma, Australia) that sterilized at 121°C for 15 min. After the *L. acidophilus* inoculation, different levels of sterilized hi-maize starch at 0.5, 1.0, 1.5 and 2.0% (w/v) were added into the alginate solution and mixed homogenously. The cell suspension was then injected through a syringe needle (24G (0.55x25 mm), Nipro Corporation, Japan) into sterilized 0.1 M $CaCl_2$ (Krasaekoopt *et al*., 2003). The beads were left hardening for 30 min at room

temperature, rinsed with 0.1% of sterile peptone solution and kept at 4°C for 12 h before used in any experiment.

3.5.2 Yoghurt production

Basic yoghurt was prepared by mixing 15.5% (w/v) milk powder (Misssion P-PLUS Brand, Thailand), 10.0% (w/v) skimmed milk powder (Mission Brand, Thailand), 0.075% (w/v) carrageenan and 74.425% (w/v) distilled water following the procedure of Sankhavadhana (2001). After thoroughly mixed, the solution was heated to 82 ± 3°C for 5 min, cooled immediately to 45°C and aseptically added with 0.02% (w/v) freeze dried starter culture that was composed of *S. thermophilus* and *L. bulgaricus* at a ratio of 1:1. The yoghurt fermentation was carried out at 43°C for up to 6 h in which the yoghurt reached a pH value of 4.6. The fermentation process was arrested by immediately cooling the yoghurt to 4°C. For the *L. acidophilus* containing yoghurt, the probiotic cells were added as free cells or immobilized cells that were inoculated either before or after a fermentation process. The free cells of *L. acidophilus* were prepared by growing 0.1 g of the freeze dried culture in MRS broth at 37°C for 24 h (Dave and Shah, 1996 and Lan-Szu and Weimer, 1998). The probiotic cells were harvested by centrifugation at 3000 x g for 15 min at 4°C and washed twice with 0.1% of sterile peptone solution (Sheu and Marshall, 1993). The inoculum levels of *L. acidophilus* studied in this experiment were 0, 2, 4 and 8% (w/v). When the *L. acidophilus* added before a fermentation process, the bacterium was added together with the yoghurt starter culture, whereas the addition of *L. acidophilus* after a fermentation process was carried out before cooling the yoghurt to 4°C by mixing the bacterium into the yoghurt thoroughly with a gentle stirrer.

3.5.3 Vacuum drying

An amount of 70 ml of yoghurt was filled and spread aseptically into a 33.5 x 22.0 cm^2 tray before dried under a different condition in a vacuum oven. The drying condition of vacuum oven was an interaction between the drying temperatures (40 and 45°C) and the drying time (16, 20 and 24 h).

3.5.4 Production of yoghurt powder

Drying the yoghurt was conducted by a spray drier model SDE 50 manufactured by J.C. Machinery and civil work Co., Ltd. The type of atomizer used was a nozzle atomizer with a length of 44 cm together with an atomizer pressure at 15 psi, a co-current air flow and an air inlet temperature at 180^{o}C. Since the outlet temperature had a significant effect on the survival rate of microorganisms, the outlet temperature should be between 60 to 90^{o}C. Using an outlet air temperature lower than 60^{o}C would not produce dried powder and if the temperature was higher than 90^{o}C, it would produce powder with lower physical quality due to browning reaction (Kim and Bhowmik, 1990, Makarukpinyo, 1995 and To and Etzel, 1997). Therefore, different outlet temperatures of 75 ± 2, 80 ± 2, 85 ± 2 and 90 ± 2^{o}C were studied in this research to understand the survival rate of yoghurt and probiotic bacteria during a spray drying process. In addition, the total soluble solid of yoghurt that was initially 16 ± 2^{o}Brix (23.99% (w/v) total solid) was adjusted to 25% (w/v) total solid using 25% (w/v) maltodextrin solution (Desobry *et al*., 1997 and Cai and Corke, 2000) to produce a good drying rate during the spray drying process (Master, 1991).

3.5.5 Reconstitution of yoghurt powder

L. acidophilus containing yoghurt powder was mixed with distilled water at room temperature (30 ± 2^{o}C), warm water (50 ± 2^{o}C and 70 ± 2^{o}C) and hot water (90 ± 2^{o}C) at a ratio of 1:10 for the powder and the water, respectively, according to a procedure of Rardniyom, (2002). Different water temperatures were used to assess the survival of *L. acidophilus* and yoghurt starter bacteria in the reconstituted yoghurt solution.

3.5.6 Storage of yoghurt powder

L. acidophilus containing yoghurt powder was packed in 2 different materials of laminated pouches, which were Nylon/Polyethylene (nylon/PE) and Polyethylene tetraphthalate/Polypropylene/Aluminum (PET/PP/Al) and sealed as a vacuum packaging. The packed yoghurt powder was stored for 14 weeks either at room temperature, which was around 30 ± 5^{o}C or in a refrigerator at 4 ± 2^{o}C. During the storage period, samples of yoghurt powder were collected every 2 weeks for microbiological analysis and every 4 weeks for physical and chemical analyses.

3.5.7 Physical Analysis

3.5.7.1 Bead diameter, volume and density

To determine the diameter of calcium alginate-hi-maize beads, 25 randomly selected beads were measured by a vernier caliper. The bead diameter was then calculated to the bead volume using a formula of $4/3\pi\ r^3$. For the bead density, the volume of 50 randomly selected beads was measured by displacement of water in a 10 ml graduated cylinder. The beads were also weighted using a laboratory balance (OHAUS Co., Ltd, USA) to determine the bead density using a formula of Sun and Griffiths (2000);

$$\text{Bead density (g/ml)} = \left(\frac{\text{Total weight of 50 beads}}{\text{Total volume of 50 beads}} \right) \times 100$$

3.5.7.2 Consistency of yoghurt

The yoghurt consistency was measured by a Bostwick consistometer. The screener inside the consistometer was held and yoghurt sample was filled into the empty space behind the screener. The measurement was started when the screener was released and the yoghurt was run along the consistometer for 30 s (Gonzalez-Martinez *et al*., 2002). Consistency was determined by measuring the distance that was expressed in cm unit, over which the samples flowed in the Bostwick consistometer at 4^{o}C.

3.5.7.3 Whey drainage

Whey drainage was measured from the yoghurt sample using a syringe at the beginning and at the end of yoghurt storage period. The relative amount of whey drained off (in ml per 100 ml of initial sample) was calculated as the whey drainage (a modified method from Fiszman *et al*., 1999).

3.5.7.4 Color of yoghurt powder

An amount of 3 g yoghurt powder was transferred into a small plastic cup, rapped it and measured with a colorimeter (CR-300, Minota Co., Ltd, Japan).

3.5.7.5 Bulk density of yoghurt powder

An amount of 5 ± 0.1 g yoghurt powder was filled into a dried 10 ml cylinder, rapped gently the cylinder for 3 times and recorded the volume of the powder (Chitomarat, 2002).

$$\text{Bulk density (g/ml)} = \left(\frac{\text{Weight of yoghurt powder}}{\text{Volume of yohgurt powder}} \right)$$

3.5.7.6 Rehydration property of yoghurt powder

Yoghurt powder of 0.5 g (W_1) was transferred into a 100 ml beaker glass and added with 5 ml distilled water at 25°C. The solution was mixed by a magnetic stirrer (VELP Scienifica, Europe) for 1 min using a speed level of 7-8. The milk solution was then filtered by a Whatman filter paper no. 4 that was known for its weight (W_2), The filter paper was dried at 100°C for 4 h in a hot air oven (Termaks, Japan), cooled in a desiccator and weighed (W_3). The rehydration characteristic was determined by the following equation (Rardniyom, 2002);

$$\text{Rehydration (\%)} = \left(\frac{W_3 - W_2}{W_1} \right) \times 100$$

3.5.7.7 Solubility of dried yoghurt

The solubility property was figured out by transferring 1.0 g of yoghurt powder (W_1) into a 100 ml dried beaker glass and adding with 10 ml distilled water at room temperature. The powder was left undisturbed for 3 h at room temperature followed by keeping the beaker glass in a water bath (Memmert, Germany) previously set at 50°C for 30 min. The yoghurt solution was then centrifuged at 4,500 x g for 4 min (Hettich, Germany). The supernatant collected after the centrifuge was discarded. The beaker glass was washed with 10 ml distilled water at 50°C to collect the powder residues and added into the centrifuge tube that contained the powder residues from the first centrifuge process. The tube was centrifuged for the second time using a same condition and the supernatant was discarded. Into the tube, 10 ml of distilled water was added, mixed thoroughly and filtered the solution with a Whatman filter paper no. 4 that was previously weighed (W_2). Following the filtration, the filter paper was

dried at 100°C for 4 h, cooled in a desiccator and weighed again (W_3). The solubility of the yoghurt powder calculated using the following formula (Pearson, 1976);

$$\text{Solubility (\%)} = 100 - \left\{ \left(\frac{W_3 - W_2}{W_1} \right) \times 100 \right\}$$

3.5.7.8 Dispersibility of yoghurt powder

An amount of 0.5 g of yoghurt powder (W_1) was placed in a 100 ml beaker glass and added with 5 ml distilled water at 25°C. The powder was mixed gentle by a spatula for 1 min until no more fine particles was seen. The yoghurt solution was then filtered by a Whatman filter paper no. 4 that was known for its weight (W_2). The filter paper was dried at 100°C for 4 h by a hot air oven, cooled in a desiccator and weighed again (W_3). The yoghurt powder dispersibility was calculated by the following equation (Onwulata, 2005);

$$\text{Dispersibility (\%)} = \left\{ \left(\frac{W_3 - W_2}{W_1} \right) \times 100 \right\}$$

3.5.8 Chemical Analysis

3.5.8.1 pH values and total titratable activity

Measurement of pH values for yoghurt and yoghurt powder was conducted using a pH-meter (CONSORT C830, CE, Belgium). For the pH of the yoghurt powder, 1.0 g of the powder was mixed thoroughly with 10 ml distilled water before being measured for its pH value. For the total titratable acidity, 1 ml of yoghurt sample or 1 g of yoghurt powder diluted in 10 ml distilled water was titrated against with 0.1 M NaOH using a phenolphthalein indicator. The titration was ended with the development of pink color in the solution that persisted for more than 15 s (an AOAC method no. 947.05 (AOAC, 2000)). The total titratable acidity values were based on the calculation that 1 ml of 0.1 M NaOH was equaled with 0.009 g lactic acid.

3.5.8.2 Water activity, moisture content and total soluble solid

The water activity of yoghurt powder was determined by an a_w-meter (Series 3, AquaLab, USA). For the total soluble solid, one drop of yoghurt sample or 1 g of

yoghurt powder in 10 ml distilled water at room temperature was measured by a hand refractometer (ATAGO, Japan) The moisture content of yoghurt powder was carried out by drying 1 g of yoghurt powder in a hot air oven at 100°C for 4 h. The moisture content was then calculated using a following equation (an AOAC method no. 927.05 (AOAC, 2000)).

$$\text{Moisture content}(\%) = \left\{ \frac{(\text{Weight of filter paper and sample after heating}) - (\text{Weight of filter paper})}{\text{Weight of initial sample}} \right\} \times 100$$

3.5.9 Microbiological Analysis

3.5.9.1 Enumeration of *S. thermophilus*, *L. bugaricus* and free cells of *L. acidophilus*

Yoghurt or yoghurt powder samples were diluted using 10-fold dilution in Maximum Recovery Diluent (MRD, Oxoid, England) before being pour-plated using M-17 agar (Merck, Germany) and deMan Rogosa Sharpe (MRS) agar (Merck, Germany) adjusted to pH 5.2 using 100% glacial acetic acid (Lab scan Co., Ltd, Thailand) (Dave and Shah, 1996) for enumeration of *S. thermophilus* and *L. bulgaricus*, respectively (Tharmaraj and Shah, 2003). After the agar solidified, plates of *S. thermophilus* were incubated aerobically at 37°C for 48 h (Dave and Shah, 1996 and Tharmaraj and Shah, 2003), whereas plates for *L. bugaricus* enumeration were overlayed with MRS agar and incubated at 37°C for 72 h before being enumerated. For the *L. acidophilus* enumeration, diluted samples were spread-plated on MRS-maltose agar that used maltose (Lab Chem Co., Ltd, Australia) to replace glucose following the method of Kandler and Weiss (1984) and Rybka and Kailasapathy (1996) to selectively detect *L. acidophilus* in the presence of *L. bulgaricus*. Plates for *L. acidophilus* enumeration were incubated anaerobically at 37°C for 72 h (Rybka and Kailasapathy, 1996).

3.5.9.2 Enumeration of immobilized cells of *L. acidophilus* in calcium-alginate hi-maize starch beads.

An amount of 0.1 g beads was mixed with 10 ml phosphate buffer (0.1 M at pH 7.0) (Sultana *et al*., 2000 and Chandramouli *et al*., 2004) in a stomacher

(Sultana *et al*., 2000 and Kailasapathy, 2005) for 20 min at room temperature (Sheu and Marshall, 1993 and Chandramouli *et al*., 2004). After releasing the bacterium cells from the beads, the phosphate solution was 10-fold diluted in MRD. The *L. acidophilus* numbers were then enumerated using Homofermentative and Heterofermentative Differential (HHD) agar that composed of 2.5% (g/l) of fructose (Fluka, Switzerland), 2.5% (g/l) of KH_2PO_4 (Merck, Germany), 10.0% (g/l) of soytone (Bacto ^{TM}, France), 1.5% (g/l) of peptone from casein (Merck, Germany), 3% (g/l) of casamino acid (Bacto ^{TM}, France), 1% (g/l) of Tween 80 (Chemical of highest quality, Mark, Japan), 1% (g/l) of yeast extract (Merck, Germany) and 15% (g/l) of agar (U&V Holding Thailand) and incubated anaerobically at 37^{o}C for 72 h (McDonald *et al*., 1987). The cell recovery of *L. acidophilus* was estimated by applying the following formula.

$$\text{Cell recovery}(\%) = \left(\frac{\text{The number of } L.\ acidophilus \text{ before immobilized (log cfu/ml)}}{\text{The number of } L.\ acidophilus \text{ after immobilized (log cfu/ml)}} \right) \times 100$$

3.5.10 Statistical analysis

All results when possible were statistically analyzed by analysis of variance (SPSS ver 10.1, SPSS Inc., Chicago, USA). If the main effect was significantly different, the means were separated by the Duncan's multiple range test. The predetermined acceptable level of probability was 5% ($p<0.05$) for all the comparison (Montgomery, 2001).