CHAPTER 3
MATERIALS AND METHODS

3.1 Materials, Chemical Reagents and Equipments

3.1.1 Materials

1. Soybean Chiangmai type 60
2. Tomato fruits
3. Reconstituted Skimmed Milk (Mission, Swiss)
4. Sugar (Mitpol, Thailand)
5. *Lactobaccilus acidophilus* (Christian Hansen, Denmark)
6. *Bifidobacterium bifidum* (Christian Hansen, Denmark)
7. *Streptococcus thermophilus* (894 ATCC 19258) (TISTR) (Thailand Institute of Scientific and Technological Reseearch, Thailand)
8. *Lactobaccilus bulgaricus* (892 ATCC 11842) (TISTR) (Thailand Institute of Scientific and Technological Reseearch, Thailand)
9. Kappa-carragenan (Nakarai Chemicals Ltd., Japan)
10. Sodium-carboxymethylcellulose (CMC) (Nakarai Chemicals Ltd., Japan)
11. Guar gum (Wendt-Chemie, Germany)
12. Fibersol-2 (The East Asiatic Public Company Ltd. (EAC), Thailand)
13. Fructo-oligosaccharide (Greenlife, USA)

3.1.2 Chemical Reagents

1. Sodium bicarbonate 0.5 M pH 9.0 ($NaHCO_3$) (O.V. Chemical, Chiang Mai, Thailand)
2. Sulphuric acid (H_2S0_4) (Merck, Germany)

3. Sodium sulphate ($NaSO_4$) (Merck, Germany)
4. Copper sulphate (Cu_2SO_4) (Merck, Germany)
5. Bromogresol green (Fluka, Switzerland)
6. Ethyl alcohol (Merck, Germany)
7. Sodium hydroxide (NaOH) (Merck, Germany)
8. Hydrochoric acid (HCL) (Merck, Germany)
9. Phenolpthalein indicator (Merck, Germany)
10. Maximum Recovery Diluent (MRD) (Oxoid, England)
11. M-17 agar (Oxoid, England)
12. de Man Rogosa Sharp (MRS) agar (Oxoid, England)
13. Plate Count Agar (Merck, Germany)
14. Potato Dextrose Agar (Merck, Germany)
15. Lauryl Broth (Himedia, India)
16. Lactose (Difco, USA)
17. Yeast extract (Merck, Germany)
18. Casamino acids (Difco, USA)
19. Fructose (Fluka, Switzerland)
20. Potassium dihydrogen phosphate (USA)
21. Tween 80 (Fluka, Switzerland)
22. Soytone (Difco, USA)
23. Peptone from casein (Merck, Germany)
24. Agar (Himedia, India)
25. Tryptone (Difco, USA)

3.1.3 Equipments

1. An electric juice blender (Central, Thailand)
2. A blender (National, Thailand)
3. A refrigerator (Whirpool, Thailand)

4. A hot air oven model ULM 500 (Memmert, Germany)
5. An incubator model BE 400 (Memmert, Germany)
6. A waterbath (Memmert model WB 10 L1, Germany)
7. A pH-meter model C830 (Consort, Belgium)
8. Autopipettes (Pipetman, Germany)
9. A refractometer (Atago, Japan)
10. A vortex model Ginie2 (Scientific, USA)
11. An autoclave (Gallenkamp, England)
12. A muffle furnance (Gallenkamp, England)
13. A Brookfield Viscometer (Brookfied Enginerring Labs, Germany)
14. A Bostwick consistometer (USA)

3.2 Methods

3.2.1 Starter culture propagation

Starter culture propagation was applied from a method of Sankhavadhana (2001).

3.2.1.1 Stock culture preparation preparing litmus milk that contains 16% skimmed milk powder, 2% litmus solution, 0.3% yeast extract and 0.2 g calcium carbonate. After that, transferring 10 ml of the solution into a test tube with a size of 15 × 160 mm and then sterilize the solution at 121^{0}C for 15 min. Followed by cooling to 37^{0}C in cool water at room temperatures and inoculating the sterilized solution with a starter culture. Incubating the inoculated solution at 37^{0}C for 24 h. The stock cultures were kept at 5^{0}C after the incubation. The cultures had a shelf-life for 2 weeks, therefore the starter cultures were refreshed regularly.

3.2.1.2 Mother cultures preparation preparing milk for the fermentation of mother cultures using 16% skimmed milk and 0.1% yeast extract. Mixing the ingredients

with distilled water and transferring a solution volume of 100 ml into a 250 ml bottle and sterilize at 121^{0}C for 5 min. After that cooling to 37^{0}C in cool water, inoculating the sterile medium with 2% (v/v) of the stock culture and incubating at 37^{0}C for 36 h. Finally, keeping the culture at 5^{0}C. The culture had a shelf-life for 7 days. The number of *S. thermophilus* and *L. bulgaricus* in mother culthers was 10.14 ± 0.13 and 10.05 ± 0.01 log CFU/ml.

3.2.2 Soymilk preparation

Washing soybean Chiangmai type 60 seed with distilled water and soaking the beans in 0.5 M sodium bicarbonate at pH 9.0 with a ratio of $NaHCO_3$: soybean of 4:1 at room temperature for 6-8 h. Blending the soybean seeds with boiled distilled water for 1-3 min. Cooling the soybean seeds at room temperature and blending them in a waring blender using hot distilled water (at 80^{0}C) with a ratio of 9:1 (w/w) for water and soybean, respectively, for 3 min. Filtering the blended solution through a double-layered cheese cloth. A solution that passed the filtered cloth was recognized as soymilk (Yotsombut, 1984 and Kamaly 1997).

3.2.3 Soy milk yogurt preparation

Mixing a basic formula of soymilk ingredients, containing reconstituted skimmed milk, stabilizer and sugar. Adding the mixture ingredients into soymilk (from the section 3.2.2) and pasteurizing the mix solution at 85°C for 30 min. Directly after the pasteurization, cooling the soymilk solution to 43°C and adding with 2% (w/v) yogurt starter cultures consisted of *S. thermophilus* and *L. bulgaricus* at ratio of 1:1. Incubating the soymilk solution with the yogurt cultures at 43°C for 6 h to produce soymilk yogurt (Lourens - Hattingh and Viljoen, 2001).

3.2.4 The production of soymilk yogurt with an addition of probiotic bacterium

Preparing soymilk yogurt according to the procedure in the section 3.2.3. Aseptically adding 2% (w/v) probiotic bacterium (either *B. bifidum* or *L. acidophilus*) into the soymilk yogurt. Cooling and keeping the soymilk yogurt added with a probiotic bacterium at 4^0C.

3.2.5 Study growth curves of each probiotic bacteria

3.2.5.1 Samples preparation

Mixing dried ingredients of a basic formula for soymilk yogurt containing 6% (w/v) reconstituted skimmed milk and 5% (w/v) sugar. Adding the ingredient mixture into 15 ml (w/v) soymilk that was prepared according to the procedure in the section 3.2.2. Heating the soymilk solution at a pasteurization condition of 85°C for 30 min. Directly after the pasteurization process, cooling the soymilk solution to 43°C and adding 2% (w/v) commercial yogurt starter cultures (*S. thermophilus* and *L. bulgaricus* at a ratio of 1:1) together with 2% (w/v) a probiotic culture (either *B. bifidum or L. acidophilus*) to produce soymilk yogurts. Incubating the soymilk solution with yogurt and probiotic bacteria at 43°C for 42 h. During the incubation period, soymilk or soymilk yogurt samples were taken at 0, 2, 4, 6, 8, 10, 12, 15, 18, 24, 36 and 42 h to be analyzed. The experiment was done for 3 replication.

3.2.5.2 Analysis for the study of the growth curves of probiotic bacteria

Chemical qualities

1. pH measurement using a pH–meter.

2. Total titratable acidity according to a method of AOAC (AOAC, 1998).

Microbiological qualities

Total viable counts of added cultures in soymilk or soymilk yogurt samples were enumerated using a spread plate method. For the yogurt bacteria, de Man Rogosa Sharp (MRS) agar and M-17 agar were employed to enumerate *L. bulgaricus* and *S. thermophilus*, respectively, and incubated anaerobically and aerobically, respectively, at 37°C for 48 h (IDF standard, 1997). The probiotic bacteria (both *B. bifidum* and *L. acididophilus*) were monitored using a modified Homofermentative Heterofermentative Differential (HHD) agar and anaerobic incubated at 37°C for 72 h (Lourens-Hattingh and Viljoen, 2001). The results of microbial enumeration were reported as colony forming units/ml (CFU/ml).

3.2.6 A basic formula soymilk yogurt

3.2.6.1 A basic formula soymilk yogurt preparation

A production of a soymilk yogurt basic formula following the section 3.2.3 was prepared after producing soymilk using a method that was adapted from Yotsombut (1984) and Kamaly (1997) (section 3.2.2). For the soymilk yogurt production processes, the processing steps were followed the method of Lourens-Hattingh and Viljoen (2001). After producing the soymilk yogurt, the yogurt was stored at 4°C and representative

samples were taken on 0, 7, 14 and 21 days of storage to be analyzed for different parameters. The experiment was done for 3 replication.

3.2.6.2 Analysis for a basic formula of soymilk yogurt

Physical qualities

1. Viscosity using a Brookfield viscometer.
2. Syneresis according to an adapted method of Wu *et al.* (2001).
3. Consistency using a Bostwick consistomer.

Chemical qualities

1. Total solid content according to a method of AOAC (AOAC, 2002).
2. pH measurement using a pH–meter.
3. Total titratable acidity according to a method of AOAC (AOAC, 1998).
4. TSS (Total Soluble Solid) using a hand refractometer.
5. Moisture content according to a method of AOAC (AOAC, 2000).

Microbiological qualities

Total viable counts of yogurt bacteria were monitored using a spread plate method. *S. thermophilus* and *L. bulgaricus* were grown on M-17 and MRS agar, respectively, and incubated anaerobically and aerobically, respectively, at 37°C for 48 h (IDF standard, 1997). Microbiological results were reported as colony forming units/ml (CFU/ml).

Statistical analysis

Collected data was analyzed statistically using an Analysis of Variance by applying Completely Randomized Design (CRD). If the F value was significantly different, the treatment means were further analyzed using a Duncan's multiple-range test (DMRT) (Pongsirikool, 2002). The statistical analysis was conducted using a SPSS program version 10.

3.2.7 Study important factor of soymilk yogurt ingredients that affected the viability of probiotic bacteria in soymilk yogurt during storage at 4^0C for 21 days

3.2.7.1 Screening the main factor of soymilk yogurt ingredients that affected the viability of probiotic bacteria in soymilk yogurt during storage at 4^0C for 21 days using a Plackett and Burman design

The Plackett and Burman design was an experimental design that was used to screen the main factors of soymilk yogurt ingredients that affected the viability of probiotic bacteria in soymilk yogurt during low storage temperature. The design was applied for soymilk yogurt that was added either with *L. acidophilus* or *B. bifidum*. The experimental plan for the Plackett and Burman design was displayed in Table 3.1. For the low and high levels of each soymilk yogurt ingredients/factors, they could be seen in Table 3.2 (Pongsirikool, 2002).

Table 3.1 A plot of Plackett and Burman design (N=12) to screen the main factors of soymilk yogurt ingredients that affected the viability of probiotic in soymilk yogurt

Experimental unit (N)	A	B	C	D	E	F	G	H	I	J	K
1	+	+	-	+	+	+	-	-	-	+	-
2	+	-	+	+	+	-	-	-	+	-	+
3	-	+	+	+	-	-	-	+	-	+	+
4	+	+	+	-	-	-	+	-	+	+	-
5	+	+	-	-	-	+	-	+	+	-	+
6	+	-	-	-	+	-	+	+	-	+	+
7	-	-	-	+	-	+	+	-	+	+	+
8	-	-	+	-	+	+	-	+	+	+	-
9	-	+	-	+	+	-	+	+	+	-	-
10	+	-	+	+	-	+	+	+	-	-	-
11	-	+	+	-	+	+	+	-	-	-	+
12	-	-	-	-	-	-	-	-	-	-	-

Note: A - G were soymilk yogurt ingredients/factors that were studied in this design

H - K were dummy variable

(-) for low level and (+) for high level

The soymilk yogurt ingredients/factors that were studied including

A	for	soymilk (%) (w/v)
B	for	sugar (%) (w/v)
C	for	Reconstituted Skimmed Milk (RSM) (%) (w/v)
D	for	kappa- carrageenan (%) (w/v)
E	for	sodium-carboxymethylcellulose (CMC) (%) (w/v)

F for guar gum (%) (w/v)

G for pH level (pH level of soymilk yogurt before an addition of probiotic bacteria)

Table 3.2 The low and high levels of the soymilk yogurt ingredients/factors on the Plackett and Burman design

Factor	Factor level (%)	
	Low level (-)	High level (+)
A	15	60
B	5	15
C	6	12
D	0	0.3
E	0	0.3
F	0	0.3
G	4.3	4.9

Note: Each of an experimental unit (N) to make soymilk yogurt was based on 1 lt.

3.2.7.1.1 Soymilk yogurt samples preparation in the Plackett and Burman design

Soymilk was prepared according to an adapted method of Yotsombut (1984) and Kamaly (1997) (section 3.2.2). The soymilk was then mixed properly with other soymilk yogurt ingredients following the experimental plan in the Tables 3.1 and 3.2 and produced to be soymilk yogurt based on a method of Lourens-Hattingh and Viljoen (2001) (section 3.2.3). The final soymilk yogurt was aseptically added with a probiotic bacterium, either *B. bifidum* or *L. acidophilus*, and stored at 4^0C. During the storage,

yogurt samples were taken on 0, 7, 14 and 21 days of storage to be analyzed for different parameters. The experiment was done for 3 replication.

3.2.7.1.2 Analysis of soymilk yogurt samples for the Plackett and Burman design

Physical qualities

1. Viscosity using a Brookfield viscometer.
2. Syneresis according to an adapted method of Wu *et al.* (2001).
3. Consistency using a Bostwick consistomer.

Chemical qualities

1. Total solid content according to a method of AOAC (AOAC, 2002).
2. pH measurement using a pH–meter.
3. Total titratable acidity according to a method of AOAC (AOAC, 1998).
4. TSS (Total Soluble Solid) using a hand refractometer.
5. Moisture content according to a method of AOAC (AOAC, 2000).

Microbiological qualities

Total viable counts of yogurt and probiotic bacteria were enumerated using a spread plate method. The yogurt bacteria of *L. bulgaricus* and *S. thermophilus* were grown on MRS and M-17 agar, respectively, and incubated anaerobically and aerobically, respectively, at 37°C for 48 h (IDF standard, 1997). The probiotic bacteria, which were *B. bifidum* and *L. acidophilus*, were monitored using a modified HHD agar and incubated

anaerobically at 37°C for 72 h (Lourens-Hattingh and Viljoen, 2001). The microbial results were reported as colony forming units/ml (CFU/ml).

Statistical analysis

Physical, chemical and microbiological data was analyzed statistically using a Plackett and Burman program in a statistical software of the SPSS program version 10. Differences between the treatment means were determined by applying a T-test method (Pongsirikool, 2002).

3.2.7.2 Study the optimal levels of the soymilk yogurt main factors to find the optimal formula for soymilk yogurt that could support the viability of probiotic bacteria in soymilk yogurt during low temperature storage for 21 days

After screening the main factors of soymilk yogurt ingredients that affected the viability of probiotic bacteria in soymilk yogurt, these main factors were further determined for their optimum levels. Results in the section 4.3.1.1 showed that there were 3 main factors that affected the viability of *B. bifidum* in soymilk yogurt. The optimum levels of these 3 factors were studied by applying a 2^3 factorial experiment in central composite design that could be seen in Table 3.3 and 3.4. For the viability of *L. acidophilus* in soymilk yogurt, there were 2 factors as were shown in the section 4.3.2.2. These 2 main factors were further investigated for their optimum levels using a 2^2 factorial experiment in central composite design, which was displayed in Tables 3.5 and 3.6.

Table 3.3 The levels for the main factors of soymilk yogurt added with *B. bifidum* in the 2^3 factorial experiment in central composite design

Main factors	Low level (-)	Center point (0)	High level (+)
RSM	3	6	9
Sugar	12	15	18
pH level	4.6	4.9	5.2

Table 3.4 The experimental plan for finding the suitable levels of the main factors of soymilk yogurt added with *B. bifidum* in the 2^3 factorial experiment in central composite design

Experimental units	Code	Main factor		
		RSM	Sugar	pH level
1	1	-1	-1	-1
2	a	+1	-1	-1
3	b	-1	+1	-1
4	c	-1	-1	+1
5	ab	+1	+1	-1
6	ac	+1	-1	+1
7	bc	-1	+1	+1
8	abc	+1	+1	+1
9	cp_1	0	0	0
10	cp_2	0	0	0
11	cp_3	0	0	0

3.2.7.2.1 Samples preparation

Soymilk yogurt samples were prepared according to the sections 3.2.2, 3.2.3 and 3.2.4. The ingredient composition of these yogurt samples followed the experiment results in the section 3.2.7.1 and Tables 3.3-3.6. After adding with probiotic bacteria, the yogurt samples were kept at 4^0C for 21 days. During the storage time, sampling was conducted on 0, 7, 14 and 21 days to be analyzed.

Table 3.5 The levels for the main factors of soymilk yogurt added with *L. acidophilus* in the 2^2 factorial experiments in central composite design

Main factor	Low level (-)	Center point (0)	High level (+)
RSM	9	12	15
Sugar	12	15	18

Table 3.6 The experimental plan for finding the suitable levels of the main factors of soymilk yogurt added with *L. acidophilus* in the 2^2 factorial experiment in central composite design

Experiment al unit	Code	Main factor	
		RSM	Sugar
1	1	-1	-1
2	a	+1	-1
3	b	-1	+1
4	ab	+1	+1
5	cp_1	0	0
6	cp_2	0	0

3.2.7.2.2 Analysis of soymilk yogurt samples for the factorial experiment in central composite design

Physical qualities

1. Viscosity using a Brookfield viscometer.
2. Syneresis according to an adapted method of Wu *et al.* (2001).
3. Consistency using a Bostwick consistomer.

Chemical qualities

1. Total solid content according to a method of AOAC (AOAC, 2002).
2. pH measurement using a pH–meter.
3. Total titratable acidity according to a method of AOAC (AOAC, 1998).
4. TSS (Total Soluble Solid) using a hand refractometer.
5. Moisture content according to a method of AOAC (AOAC, 2000).

Microbiological qualities

Total viable counts of yogurt and probiotic bacteria were enumerated using a spread plate method. The yogurt bacteria of *L. bulgaricus* and *S. thermophilus* were grown on MRS and M-17 agar, respectively, and incubated anaerobically and aerobically, respectively, at 37°C for 48 h (IDF standard, 1997). The probiotic bacteria, which were *B. bifidum* and *L. acidophilus*, were monitored using a modified HHD agar and incubated anaerobically at 37°C for 72 h (Lourens-Hattingh and Viljoen, 2001). The microbial results were reported as colony forming units/ml (CFU/ml).

Statistical analysis

Data from different analyses was analyzed statistically by Analysis of Variance using a factorial experiment in central composite design. Differences between treatment means were determined by applying DMRT (Pongsirikool, 2002). All the statistical analysis was conducted using a SPSS program version 10.

3.2.8 Study the addition of nutrient compounds on the viablility of probiotic bacteria in soymilk yogurt during storage at 4°C for 21 days

3.2.8.1 Preparation of tomato extract (Harrigan, 1998)

Fresh tomato was extracted for its juice, following by a filtration through a cheese cloth. Into a 200 ml tomato juice that passed the filtration cloth, 10 g of tryptone and 10 g of yeast extract were added and mixed properly. The solution was than added with distilled water to 1000 ml and recognized as a tomato extract. After adjusting the tomato extract to pH 3.2 using 1N NaoH, the extract was sterilized at 121^0C for 15 min.

3.2.8.2 Samples preparation

Preparation of soymilk yogurt samples was conducted according to the sections 3.2.2, 3.2.3 and 3.2.4. Composition of soymilk yogurt ingredients followed the experiment result in the sections 3.2.7.1 and 3.2.7.2 together with an addition of 5% (w/v) nutrient compounds, which was fructo-oligosaccharides (FOS), fibersol-2 or tomato extract. The final soymilk yogurt products were kept at chilled temperature for 21 days. During the storage time, samples were taken on 0, 7, 14 and 21 days to be analyzed.

3.2.8.3 Analysis of soymilk yogurt samples with an addition of nutrient compounds

Physical qualities

1. Viscosity using a Brookfield viscometer.
2. Syneresis according to an adapted method of Wu *et al.* (2001).
3. Consistency using a Bostwick consistomer.

Chemical qualities

1. Total solid content according to a method of AOAC (AOAC, 2002).
2. pH measurement using a pH–meter.
3. Total titratable acidity according to a method of AOAC (AOAC, 1998).
4. TSS (Total Soluble Solid) using a hand refractometer.
5. Moisture content according to a method of AOAC (AOAC, 2000).

Microbiological qualities

Total viable counts of yogurt and probiotic bacteria were enumerated using a spread plate method. The yogurt bacteria of *L. bulgaricus* and *S. thermophilus* were grown on MRS and M-17 agar, respectively, and incubated anaerobically and aerobically, respectively, at 37°C for 48 h (IDF standard, 1997). The probiotic bacteria, which were *B. bifidum* and *L. acidophilus*, were monitored using a modified HHD agar and incubated anaerobically at 37°C for 72 h (Lourens-Hattingh and Viljoen, 2001). The microbial results were reported as colony forming units/ml (CFU/ml).

Statistical analysis

Data from different analyses was analyzed statistically by Analysis of Variance using a factorial experiment in central composite design. Differences between treatment means were determined by applying DMRT (Pongsirikool, 2002). All the statistical analysis was conducted using a SPSS program version 10.