CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Culture media

(1) De Man, Rogasa and Sharpe agar (MRS broth and agar)

(2) Glucose Yeast Peptone (GYP)

Note: the composition and preparation of this media were shown in Appendix A

3.1.2 Chemical reagents

Name of chemical reagents	Company
Agar	O.V.
Ammonium citrate	SIGMA
Beef extract	HIMEDIA
Bromocresol purple	FLUKA
$CaCl_2$	UNIVAR
Ethanol	MERCK
$FeSO_4$. $7H_2O$	UNIVAR
Gelatin	M.C. CHINY CENTER
Glucose	FLUKA
HCl	J.T. BAKER
KCI	MERCK
K_2HPO_4	SCHARLAU

$MgSO_4 \cdot 7H_2O$	UNIVAR
$MnSO_4 \cdot 4H_2O$	ANALAR
NaCl	UNIVAR
$NaH_2PO_4 . H_2O$	MERCK
$Na_2HPO_4 . 12H_2O$	MERCK
Ox Bile, Bacteriological	HIMEDIA.
Peptone	BIOMARK™
Sodium acetate	FISHER SCIENTIFIC
Sodium alginate	FLUKA
Tween-80	LABCHAM
Yeast extract	SCHARLAU
κ-carrageenan	FLUKA

3.1.3 Equipments

Name of Equipments	**Company**
Analytical balance (2 digits)	OERTING
Autoclave	IWAJKI Model ACV-3167
Autopipette (P1000, P200)	GITSON
Beaker	PYREX
Computer	HP
Cover glass	-
Cylinder	PYREX
Duran	DURAN
Hood	TOPLAB
Laminar Air Flow	AUGUSTA
Light microscope	ZEISS
Loop	-
Magnetic bar	-
Magnetic stirrer	LIO LAB LIMITED
Microcentrifuge	HETTICH

Petri dish	PYREX
pH Meter	CONSORT
Refrigerated centrifuge	SORVALL (Model Super T21)
Slide	-
Spreader	-
Test tube	PYREX
Vortex FS-16	BIOSAN
Vortex mixer	VORTEX-2 GENIE
Water bath	MEMMENT

3.1.4 Microorganism

Lactobacillus fermentum 2311 M in this study was obtained from Srikanjana Klayraung (Department of Biology, Faculty of Science, Maejo University). This strain was collected from fermented tea leaves. The culture was cultured by MRS medium. The cells were harvested by centrifugation at 6,000 rpm at 4 ^{o}C for 15 min and washed with sterile 0.85% NaCl solution.

3.2 Methods

3.2.1 Optimization of alginate beads size

The optimization of microbead size was studied by vary the agitation time from 20 to 60 minutes by emulsion technique in 3.2.2 (without bacteria cells) (Krasaekoopt, 2003).

3.2.2 Microencapsulation of *Lactobacillus fermentum* 2311M with alginate

Two percent of sodium alginate solution (w/v) was prepared. Five milliliters of alginate solution mixed with 1 ml of cells suspended solution (2 x10^{10} cells/ml) before added to 50 ml of vegetable oil in a beaker and stirred for 20-50 min. Then, 100 ml of 0.1 M calcium chloride solution was added to hardening

of microcapsules and breaking the emulsion. The hardened microcapsules were harvested by centrifugation at 1,600 rpm, at 4ºC for 15 min and washed with distilled water. The microcapsules were collected and stored at 4ºC (Mandal *et al*., 2006) and observed under the simple light microscope (Ouyang *et al*., 2004). Then, checked cells leak in $CaCl_2$ solution and container for encapsulated cells. Finally, viable cells were estimated by plate counts on GYP agar.

3.2.3 Survival of alginate beads and free cells at low pH conditions

Encapsulated cells and free cells were incubated in 0.08 M HCl solution containing 0.2% NaCl at pH 1.5 and 6.5, respectively. The samples were incubated for 3 h and taken at 0, 1 and 3 h. Then, the sample were harvested by centrifugation at 12,000 rpm at 4 ^{o}C for 1 min. Next, the capsules and free cells were washed with $CaCl_2$ solution for alginate beads and 0.85% NaCl solution for free cells. Finally, added phosphate buffer solution (0.1M, pH 7.1± 0.2) to alginate beads for depolymerization of the capsules at room temperature for 30 min (Mandal *et al*., 2006) and 0.85% NaCl solution to free cells for suspension. Finally, viable cells were estimated by plate counts on GYP agar.

3.2.4 Survival of alginate beads and free cells at bile salt solution conditions

Encapsulated cells and free cells were incubated in bile salt solution at 0%, 0.15% and 0.3% concentration, respectively. Next, the samples were incubated for 3 h and taken at 0, 0.5, 1, 1.5, 2, 2.5, 3 h. Then, the capsules and free cells were harvested by centrifugation at 12,000 rpm at 4 ^{o}C for 1 min. After that, the capsules and free cells were washed with $CaCl_2$ solution for alginate beads and 0.85% NaCl solution for free cells. Finally, added phosphate buffer solution (0.1M, pH 7.1± 0.2) to alginate beads for depolymerization of the capsules at room temperature for 30 min (Modified by Clark and Martin, 1994) and 0.85% NaCl solution to free cells for suspension. Finally, viable cells were estimated by plate counts on GYP agar.

3.2.5 Storage of alginate beads at temperature 4 °C, 8 °C and 20 °C as compared to free cells for 3 months

The alginate beads were kept in 0.1 M $CaCl_2$ solution and free cells kept in 0.85% NaCl solution, respectively. Encapsulated cells and free cells were taken every week for 3 month by centrifugation at 12,000 rpm at 4 °C for 1 min. Then, phosphate buffer solution (0.1M, pH 7.1±0.2) was added to encapsulated cells for depolymerization of the capsule at room temperature for 30 min and 0.85% NaCl solution to free cells for suspension. Finally, viable cells were estimated by plate counts on GYP agar (Modified by Krasaekoopt *et al*., 2006).

3.2.6 Optimization of κ-carrageenan beads size

The optimization of microbead size was studied by vary the agitation time from 20 to 60 minutes by emulsion technique in 3.2.8 (without bacteria cells) (Krasaekoopt, 2003).

3.2.7 Microencapsulation of *Lactobacillus fermentum* 2311M with κ-carrageenan

κ-carrageenan solution containing 0.9% NaCl (w/v) and 0.5% of κ-carrageenan (w/v) was prepared. The κ-carrageenan solution was boiled and cooled down to 40-45 ºC. The κ-carrageenan-cell mixture prepared from 3 ml of κ-carrageenan solution and 1 ml of cell suspension (2×10^{10} cell/ml) before added to 50 ml of vegetable oil (40 ºC) and stirred for 20-50 min. Then, 150 ml of 0.3 M potassium chloride solution was added for microcapsules hardening. The hardened microcapsules were harvested by centrifugation at 1,600 rpm, at 4 ºC for 15 min and washed with potassium chloride (Adhikari, 2000). The microcapsules were observed under the simple light microscope (Ouyang *et al*., 2004) and stored at 4 ºC for further studies. After, checked cells leak in KCl solution and container for encapsulated cells. Finally, viable cells were estimated by plate counts on GYP agar.

3.2.8 Survival of κ-carrageenan beads and free cells at low pH conditions

Encapsulated cells and free cells were incubated in 0.08 M HCl solution containing 0.2% NaCl at pH 1.5 and 6.5, respectively. The samples were incubated for 3 h and taken at 0, 1 and 3 h. Then, the sample were harvested by centrifugation at 12,000 rpm at 4 ^{o}C for 1 min. Next, the capsules and free cells were washed with KCl solution for κ-carrageenan beads and 0.85% NaCl solution for free cells. Finally, added 0.9% NaCl to κ-carrageenan beads for depolymerization of the capsules at room temperature for 20 min and 0.85% NaCl solution to free cells for suspension. Finally, viable cells were estimated by plate counts on GYP agar (Modified by Tsen *et al*., 2004; Mandal *et al*., 2006).

3.2.9 Survival of κ-carrageenan beads and free cells at bile salt solution conditions

Encapsulated cells and free cells were incubated in bile salt solution at 0%, 0.15% and 0.3% concentration, respectively. Next, the samples were incubated for 3 h and taken at 0, 0.5, 1, 1.5, 2, 2.5, 3 h. Then, the capsules and free cells were harvested by centrifugation at 12,000 rpm at 4 ^{o}C for 1 min. After that, the capsules and free cells were washed with KCl solution for κ-carrageenan beads and 0.85% NaCl solution for free cells. Finally, added 0.9% NaCl to κ-carrageenan beads for depolymerization of the capsules at room temperature for 20 min and 0.85% NaCl solution to free cells for suspension. Finally, viable cells were estimated by plate counts on GYP agar (Modified by Tsen *et al*., 2004; Clark and Martin, 1994).

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3.2.10 Storage of κ-carrageenan beads at temperature 4 ^{o}C, 8 ^{o}C and 20 ^{o}C as compared to free cells for 3 months

The κ-carrageenan beads were kept in 0.05M KCl solution and free cells kept in 0.85% NaCl solution, respectively. Encapsulated cells and free cells were taken every week for 3 month by centrifugation at 12,000 rpm at 4 ^{o}C for 1 min. Then, 0.9% NaCl solution was added to encapsulated cells for depolymerization of the capsule at room temperature for 20 min and 0.85% NaCl solution to free cells for suspension. Finally, viable cells were estimated by plate counts on GYP agar (Modified by Audet *et al*., 1988).

3.2.11 Analysis of Data

The data were analyzed by Statistical Analysis System (Ver.7.0). Analysis of variance was carried out using 'General Linear Models' of SYSTAT version 7.0 and treatment means were compared by Fisher's Least Significant Difference at 5% level.