CHAPTER 3 MATERIALS AND METHODOLOGY

3.1 Equipments and Instruments

1. Analytical balance, METTLER TOLEDO Model AG204, Switzerland
2. Laminar flow cabinet, DWYER Series 0325, USA
3. Hot air incubator, MEMMERT Model ULM500 Series TS8000, Germany
4. Refrigerator, Sanyo, Japan
5. Autoclave, BECTHAI and HIRAYAMA Model HA300D, Japan
6. Vortex mixer, Scientific industries Model genie 2, USA
7. Bench Top Centrifuge, HITACHI himac Model CT6D, Japan
8. Incubator Shaker, NEW BRUNWICK SCIENTIFIC, USA
9. 96-microwell plate, Costar, USA.
10. Auto pipette multichannel volume 120 microlitter, Blo, USA
11. Auto pipette volume 20 microlitter, Autopipette, USA
12. Auto pipette volume 200 microlitter, Autopipette, USA
13. Auto pipette volume 1000 microlitter, Autopipette, USA
14. Balance accuracy 0.0001 Grams, Metter Toledo Model AG204, Switzerland
15. Balance accuracy 0.01 Grams, Metter Toledo Model GG4002-S, Switzerland
16. Digital microscope, Dino-Lite Model AM413ZT, Taiwan

3.2 Media culture and Chemical Reagents

1. Tryptic Soy Agar (TSA), Difco, France
2. Tryptic Soy Broth (TSB), Difco, France
3. Lactose broth (LB), Hi-media, India
4. Buffered listeria enrichment broth (BLEB), Oxoid, England

5. Nutrient broth (NB), Scharlau Chemis, Spain
6. Yeast extract, Himedia Laboratories, India
7. Lab lemco powder, Oxoid, England
8. Peptone A, US Biologocal, USA
9. Proteose, US Biologocal, USA
10. D-mannitol, US Biological, USA
11. Tryptose, US Biologocal, USA
12. Lactose, ACROS organics, USA
13. Sodium Chloride (NaCl), UNIVAR, New Zealand
14. Nalidixic acid, ACROS organics, USA
15. Lithium chloride, US Biological, USA
16. Acriflavine, ACROS organics, USA
17. Polymycin B, US Biological, USA
18. Ethanol, CARLO ERBA Reagent, France

3.3 Bacterial Strains

1. *Listeria innocua* (from Department of Medical Science; DMST, Thailand)
2. *Escherichia coli* (from Department of Medical Science; DMST, Thailand)
3. *Staphylococcus aureus* (from Department of Medical Science; DMST, Thailand)

3.4 Sample Preparation

3.4.1 Strain Preparation

L. innocua

In this study used *L. innocua* instead of *L. monocytogenes, due to L. innocua* is non-pathogenic. And also it is physiologically close to *L. monocytogenes*, and both can

occur in the same food products (Cornu et al, 2002). Single colony of *L. innocua* was grown in TSB (100 mL) and incubated at 37 ^{o}C (200 rpm, 24 h)

E. coli

Single colony of *E. coli* was grown in TSB (100 mL) and incubated at 37 ^{o}C (200 rpm, 12 h)

S. aureus

Single colony of *S. aureus* was grown in TSB (100 mL) and incubated at 37 ^{o}C (200 rpm, 24 h) (Yamaki et al., 2011).

All strains were incubated to reach around 10^{9}-10^{10} CFU/mL. This initial cell was use in examination the interaction of conventional inhibitors on growth and selectivity of *Listeria* spp. For the investigation an efficient non selective enrichment protocol all strains were prepared serial dilution to desired dilution around 2-3 log CFU/mL and use this as the initial cell.

3.4.2 Media Preparation

3.4.2.1 Investigation an Efficient Non Selective Enrichment Protocol

The growth media, Nutrient Broth (NB), Lactose Broth (LB), Tryptic soy Broth (TSB), Buffered *Listeria* enrichment Broth base (BLEB), Fraser Broth base (FB) and Palcam Broth base (PB) that prepared according to manufacturer's formulas (table 3.2) were used to study the effect of non selective medium to the growth of bacteria. Two hundred microlitre of desired *Listeria* concentration was pipetted into 20 mL of non selective broth from each condition to Duran bottle. These samples were analyzed 11 times: 0, 2, 4, 6, 8, 10, 12, 14, 16, 20 and 24 h.

Table 3.1 Non-selective formulas

Function	NB		LB		TSB		BLEB		FB		PB	
	Component	Conc. (g/L)	Component	Conc (g/L)	Component	Conc. (g/L)	Component	Conc. (g/L)	Component	Conc. (g/L)	Component	Conc. (g/L)
C source			Lactose	5	Dextrose	2.5					D-Mannitol	5
N source	Beef extract	3	Beef extract	3	Pancreatic digest of casein	17	Yeast extract	6	'Lab-Lemco' powder	5	Peptone	23
	Peptone	5	Peptone	5	Papaic digest of soybean meal	3			Proteose peptone	5	Yeast extract	1
									Tryptone	5		
									Yeast extract	5		
Buffer					Dipotassium phosphate	2.5	Potassium di-hydrogen orthophosphate	1.35	Di-sodium hydrogen phosphate	12		
							Disodium hydrogen orthophosphate	9.6	Potassium dihydrogen phosphate	1.35		
Salt					Sodium chloride	5			Sodium chloride	20		
Neutralizer											Polysorbate 80	2
											Soya lecithin	5
							TSB	30				

3.4.2.2 Examination of the Interaction of Conventional Inhibitors on Growth and Selectivity of *Listeria* spp.

After the effect of non selective medium on growth of bacteria was studied, the broth base was selected from previous experiment and added the conventional selective agent by varying type and concentration if inhibitor. One hundred twenty microlitre of desired *L. innocua, E. coli* and *S. aureus* concentration was transferred into 1.2 mL of different treatment medium from each condition to 96 deep well plate (Figure 3.1) then they were mixed by auto pipette. These samples were analyzed 10 times: 0, 2, 4, 6, 8, 10, 12, 16, 20 and 24 h.

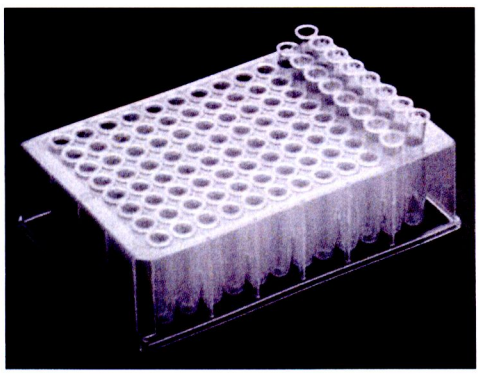

Figure 3.1 96 deep well plate

3.4.2.3 Optimization the Combination of the Effective Inhibitors

From previous experiment three select an efficient inhibitors and concentration to design experiment by using Minitab soft ware to design and analyze the response surface methodology. Box-behnken design was used to optimize the combination of inhibitors. Three selected selective agents including acriflavine, polymyxin B and lithium chloride were selected to determine the optimum combination. Treatments were prepared according the formulas from the experimental design showed in Table 3.1. Then the inhibitory effects were explored by using the number of initial cell count

minus the number of final cell count and transforming it into the percent inhibitory effect (Equation 3.1).

$$\left(\frac{Initial\ cell\ count - Final\ cell\ count}{Initial\ cell\ count}\right) \times 100 \qquad \text{(Equation 3.1)}$$

Table 3.2 Design of experiment by using Minitab

Order	Acriflavin (mg/L)	Polymyxin B (mg/L)	Lithium chloride (g/L)
1	7.5	17.5	30
2	7.5	17.5	30
3	5	25	30
4	5	17.5	20
5	5	17.5	40
6	10	25	30
7	7.5	10	20
8	10	10	30
9	7.5	25	20
10	7.5	10	40
11	5	10	30
12	7.5	25	40
13	7.5	17.5	30
14	10	17.5	40
15	10	17.5	20

3.5 Cell Enumeration

Initial cell number of each lot of recovered inoculums had been determined. Enumeration method was followed Standard Plate Count (SPC), spread plate method.

Diluted inoculums at dilutions 10^2 and 10^3 were re-shaked and transferred 5 µl aliquots onto surface of separated; duplicate Tryptic Soy Agar (TSA) on 96-microwell plate. Then the samples were incubated at 37°C for 24 h.

3.6 Growth Characteristics Determination Using Sigmoidal Function

Calculation procedures are as follow. Optical density data saved as text file were opened by using Microsoft excel 2007 program. Data were collected in one work sheet and sorted by cultivation condition (e.g., media types). Cultivation time data and optical density growth data of *Listeria* were transferred to SigmaPlot 10.0 program to estimate µmax. Growth curves were created and curve fitting were applied. Maximum specific growth rate was fixed into original built-in Sigmoid 4 Parameter (Equation 3.2).

$$y = \frac{a}{1+e^{\frac{-(x-x_0)}{b}}} \qquad \text{(Equation 3.2)}$$

y_0 = the amount of initial inoculation of *Listeria*

x_0 = the first derivative maximum of the function

a = maximal value of *Listeria* growth

b = slope of curve

μ_{max} = maximum specific growth rate, 1/b (h^{-1})

Curve Fit command would mathematically solve using real observed data, such that calculated 1/b (μ_{max}) might represent actual value reflecting experimental value.