CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemical Reagents

Name of Chemical Reagents	Production Company
Agar	O.V. Chemical
Bromocresol purple	Fisher Scientific
Glucose	Fluka
$MnSO_4 \cdot 4H_2O$	Analar
$MgSO_4 \cdot 7H_2O$	Univar
di-Potassium hydrogen phosphate	Scharlau
Yeast extract	Scharlau
Sodium acetate	Fisher Scientific
Ammonium citrate	Sigma
Tween-80	Labcham
Gelatin	M.C. Chiny center
Beef extract	Himedia
NaCl	Univar
Proteinase K	Amershem
Catalase	Sigma
Ox gall	Himedia
β-glyerophosphate	Fluka
Tag DNA polymerase	Fermentus
Lysozyme	Sigma
Nucleotide(dNTP)	Fermentus

$MgCl_2$	Fermentus
Ethidium bromide	Plusone
Agarose	-
Ethanol	Merck
Chloroform	Lab-scan
Glalcial acetic acid	Lab-scan
This-HCL	Fisher
EDTA	Fisher
This-base	Fisher
Phenol	Fisher
NaOH	Univar

3.1.2 Equipments

Names of Equipments	**Production Company**
Autoclave Model ACV-3167	Hiriyama
Beaker (50, 100, 250, 500 and 1000 ml)	Pyrex
Centrifuge	Eppendorf
Cylinder (100, 500 and 1000 ml)	Pyrex
Flask (250 ml)	Pyrex
Micro Pipette (5 ml, 20 µl, 200 µl, 1000 µl)	Bio Pette and Gentry
Hot Air Oven	Memmert
Incubator	LAB-THERM
Laminar Air Flow	Augusta
Loop	-
Needle	-
Petri Dish	Pyrex
pH Meter	Consort
Shaker	Kuhner
Test tube	Pyrex
Volumetric Flask (100 ml)	Pyrex
Vortex Mixer	Vortex-2-Genie

Water Baht	Memmert
Test paper	Advance
Concentrator	Eppendorf Concentrator
Ultraviolet (UV) transilluminator	-
Gel box	-
PCR thermocycle	Biored

3.1.3 Fermented meat products

Fifty samples of a various fermented meat products were collected, Plaa-raa, sour fermented fish (Plaa-som), Plaa-jom Mum and Nham from different markets in Thailand were analysed. They included and other products.

3.1.4 Indicator microorganisms

The indicator microorganisms, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella enteritidis* and *Escherichia coli* TISTR 73 were obtained from Faculty of Science Chiang Mai University, Biotechnology Department Faculty of Agro-Industry Chiang Mai University and Microbiological Resources Centre (MIRCEN).

3.2 Methods

3.2.1 Indicator microorganisms culture

The indicator microorganisms were growed with nutrient agar (NA) (Atlas, 1946) at 37°C for 24 h. The cultured slants were stored at 4 °C and subcultured every two weeks. The indicator microorganisms which use for screening of antimicrobial substance producing lactic acid bacteria were prepared by transfer the microorganisms from 24 h culture slant to 50 ml of nutrient Broth (NB) and incubated in water bath shaker at 37°C for 24 h. The optical density of culture was observed at 600 nm. The high optical density culture was diluted to $OD_{0.5}$ with nutrient broth

which the viable cells of that density is equal to 1.0×10^5 cells/ml when calculate by the standard curve.

3.2.2 Isolation of Lactic acid bacteria

Ten grams of fermented meat products were suspended with 90 ml of 0.85% NaCl homogeniously for 10 fold serial dilutions. The 100 µl of each diluent was spreaded on MRS agar supplemented with 0.004% bromocresol purple and incubated at 37°C for 24 h. The yellow colonies were picked up and transferred to MRS broth and incubated at 37°C for 48 h. The 0.4 µl of culture were applied to paper disc and laid on surface of MRS agar plus $CaCO_3$. After incubated 24 h at 37°C the paperdisc which showed clear zone around their own disc contained acid producing bacteria.

3.2.3 Isolation of antimicrobial substance producing lactic acid bacteria from fermented meat products

3.2.3.1 Pretest by paper disc diffusion method

1. Screening of antimicrobial substance producing LAB

LAB were grown in 5 ml MRS broth at 37°C for 48 h, 1.5 ml of the cells cultured were centrifuged at 4°C and 13,000 rpm for 15 minutes. The supernatant was filtered with 0.22-µm nitrocellulose filter membrane. The 0.4 µl of filtrated was applied to 5 mm diameter paper disc which laid on surface of NA plus indicator microorganism. The NA plus each indicator microorganisms, *Salmonella enteritidis*, *Bacillus cereus*, *Staphylococcus aureus* and *Escherichia coli* TISTR 73 was prepared by grown each indicator microorganism in nutrient broth at 37°C for 24 h. Then spreaded 100 µl each of indicator microorganism prior to use. The paper disc diffusion method described by Koneman *et al*. (1983) was used with some modification and this method was used for screening of antimicrobial activity. The

diameter of the inhibition zone was measured after incubation at 37°C for 24 h. LAB which produce antimicrobial substance were showed clear zone around the paper disc.

2. Selection of antimicrobial substance producing LAB

For the selection of antimicrobial substance by cultured the antimicrobial substance producing LAB in 5 ml MRS broth at 37°C for 48 h. The 1.5 ml of cultured was centrifuged at 13,000 rpm for 15 minutes and 1 ml of cell- free supernatant was concentrated by speed vacuum to volume 100 µl. The concentrated supernatant divide into two parts one was adjusted to pH 7 with 3 N NaOH and another was non-adjusted pH value. Each solution were drop on disc which lied on NA containing with test indicator microorganisms and incubated at 37°C for 24 h. LAB which produce extracellular antimicrobial substance were show clear zone around the paper disc.

3.2.3.2 Confirm test by agar well diffusion method

1. Determination of antimicrobial substance producing LAB

The selected antimicrobial substance producing LAB were grown in 5 ml of MRS broth at 37°C for 48 h, 1.5 ml of the cultured were centrifuged at 13,000 rpm for 15 minutes at 4°C and 1 ml of cleared supernatant was concentrated to volume 100 µl by speed vacuum and used for antimicrobial activity against four indicator microorganisms testing.

Determination of antimicrobial substance production was performed by the agar well diffusion method modified from Tagg and McGiven (1971). MRS agar, 20 ml, was mixed with 1×10^5 cell/ml of a 24 h culture of the indicator microoganism and poured into sterile petri dish. After solidification, wells were perforated with a steriled 5 mm cork borer.

The concentrated supernatant, 25 µl, supplement with 5 µl at β-glycerophosphate concentrations 0.5 g/ml (w/v) for elimination of any inhibition due to pH reduction cause by organic acid production (Bromberg *et al*, 2004), was

placed into each well. All plates were then incubated at 37°C for 24 h and examined for formation of inhibition zones. LAB which produced antimicrobial substance show clear zone around the well was selected.

2. Screening of bacteriocin-producing LAB

The isolate of LAB were screened for bacteriocin production against a range of indicator microorganisms using the agar well diffusion method as described above. After cells were removed from the growth medium by centrifurge at 13,000 rpm and 4°C for 15 minutes, 1 ml of cell free-supernatant was concentrated to 100 µl by speed vacuum. Twenty microliter of concentrated supernatant supplemented with 5 µl of 0.5 g/ml (w/v) β-glycerophosphate and 5 µl of 1 mg/ml proteinase K were incubated at 37°C for 2 h. After incubation, the enzymes were inactivated by heat at 100 °C for 20 min before added to each well of MRS agar plus indicator microorganism. Plates were incubated at 37°C for 24 h. Bacteriocin containing supernatant was disappeared when compared with non-heat supernatant.

3.2.4 Selection of acid tolerant lactic acid bacteria

The selected isolate of LAB were produced bacteriocin and subcultured in MRS broth. Then, the cultures were inoculated in 5 ml of MRS broth pH 2, 3 and 4 that adjusted pH value by 1N HCl. The initial bacterial concentration was 8.9-9.1 log CFU/ml and was checked by viable cell population determination on MRS agar. The samples were incubated at 37^{o}C for 24 h. After incubated for about 24 h, turbidity were observed for various pH tolerance LAB. The viable cell population was determined by dilution and plates counting on MRS agar after 24 - 48 h of incubation (Erkkila and Petaja, 2000).

3.2.5 Selection of bile tolerant lactic acid bacteria

An 100 µl of LAB suspension (8.9-9.1 log CFU/ml) was inoculated into 5 ml of sterile MRS broth. The experimental series contained MRS broth tubes

with following 0.15 and 0.30% of bile salts prepared from ox gall (HIMEDIA) was also added into the tubes. Then, the tubes were incubated at 37^{o}C and the viable bacteria were counted after exposure for 0, 0.5, 1.0, 2.5 and 4.0 h on MRS agar (pH 6.2) incubated for 48 h at 37^{o}C (modified from Erkkila and Petaja, 2000).

3.2.6 Characterization of bacteriocin-producing LAB

Bacteriocin producing strains were Gram-stained and catalase activity was tested by spotting colonies with 3% hydrogen peroxide.

Growth was assayed in MRS broth at 10 and 45°C as well as pH 4.4 and 9.6 incubated at 37°C. Salt tolerance was tested with 6.5 and 18% (w/v) NaCl in MRS broth. Production of CO_2 from glucose was tested in MRS broth containing Durhams tube (Axelsson, 1998).

3.2.7 16S rRNA gene analysis

3.2.7.1 DNA Extraction

Three isolates were grown overnight in the appropriate MRS broth at 37°C. The cultured cells were harvested for genomic DNA extraction, as described by Anderson and Mckay (1983). In Table 3.1 show the details of genomic DNA extraction.

Column 1 details the steps involved in a method, and columns 2 and 3 define the volumes of reagents used and other details for either screening or preparative plasmid purification protocols. The screening protocol was designed to be performed in a 1.5-ml Eppendorf centrifuge tube. All reagents were mixed immediately after addition by vortexing at low speed for 1 s, with the exception of the 3.0 N NaOH and the 2.0 M Tris-hydrochloride, pH 7.0. These reagents were mixed by inversion. Centrifugations were performed at room temperature in an Eppendorf centrifuge. The preparative protocol was usually performed in a 250-ml centrifuge bottle. All reagents were mixed by swirling. Shearing the lysate was performed by dispensing 13- to 15-ml portions of the lysate into screwcap test tubes (25 by 150

mm). Each tube was vortexed at full speed for 30 s, and the sheared lysates were pooled before denaturation. Isopropanol precipitates could be stored at 0 or -20°C overnight.

Table 3.1 DNA Extraction for lactic acid bacteria

Step	Details of following protocol:	
	Screening)[a] (1.5-10 ml)	Preparative (600 ml)[a]
Resuspend pelleted cells in buffer I..... .....379µl.		30 ml
Warm to 37°C, 5 min		
Add lysozyme.....96.5 µl		7.5 ml
(10 mg/ml in 25 mM Tris, pH 8.0)		
Incubate for 5 min at 37°C		
Add 0.25 M EDTA-50 mM Tris, pH 8.0.....48.2 ul		3.75 ml
Add sodium dodecyl sulfate (20%o [wt/vol]		
in 50 mM Tris-20 mM EDTA,..... 27.6 µ1		2.25 ml
Mix immediately Incubate for 5 to 10 min at 37°C to complete lysis		
Vortex..... 1.5-ml Eppendorf		15 ml per tube (25 by 150 mm)
at highest setting for 30 s in an appropriate tube		
Add fresh 3.0 N NaOH..... 27.6 µl		2.40 ml
Mix gently by intermittent inversion		
or swirling for 10 min..... Inversion		Swirl in 250-ml. centrifuge bottle
Add 2.0 M Tris-hydrochloride, pH 7.0 .....49.6 µl		3.90 ml
Continue gentle mixing for 3 min		
Add 5.0 MNaCl..... 71.7 µl		5.7 ml
Add phenol saturated with 3% NaCI;.....700 µl.		55.8 ml
mix thoroughly		
Centrifuge ..... 5 min		12,000 rpm
Remove upper phase and extract with chloroform-isoamyl alcohol		
(24:1) .....700 µl.		55.8 ml
Remove upper phase, precipitate with 1 vol of isopropanol		
Centrifuge ..... 5 min		12,000 rpm
Remove excess isopropanol and resuspend in 10 mM Tris-1 mM		
EDTA, pH 7.5 ..... 20 µl		1,200 µl

[a] The culture volume used in each protocol is indicated in parentheses.
Source : Anderson and Mckay (1983)

3.2.7.2 Amplification of 16S rRNA gene

Almost full-length 16S-rRNA genes were amplified by PCR using a pair of primer targeting for conserved regions of eubacteria, corresponding to position 8-27 (27f) and 1,490-1,511 (1492r) of the nucleotide sequences of the 16S rRNA gene in *Escherichia coli* (Brosius *et al*, 1978 and Weisburge *et al*, 1991). A typical reaction used the following program involving an initial denaturation of 5 min at 96°C, 35 cycle of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The final cycle was 72°C for 3 min and sample cooled down to 4°C. The PCR products were analyzed by electrophoresis on 1% (w/v) agarose gels in 1X TAE at 100V for 30 min. The gels were stained in ethidium bromide and observed on a UV transilluminator.

3.2.7.3 Sequencing of 16s-rRNA gene and phylogenetic tree analysis

The PCR products were sequence on both strand using primers 27f and 1492r aboved by Biogenomed CO., Ltd. The sequences were aligned with data in GeneBank using the Blast program. Phylogenetic analysis was performed with CLUSTAL W software (Thompson *et al*, 1994).