CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemical Reagents

Proteinase K (Amershem), Catalase (Sigma), β - glycerophosphate (Fluga), *Tag* polymerase (Fermentus), Lysozyme (Sigma), Nucleotide (dNTP) (Fermentus), $MgCl_2$, Ethidium bromide (Plusone), Agarose, Ethanol, Chloroform (LAB-SCAN), Glacial acetic acid (LAB-SCAN), This-HCL (Fisher), EDTA (Fisher), This-base (Fisher), Phenol (Fisher), NaOH (Univa)

3.1.2 Equipments

Autoclave Model ACV-3167 (Hiriyama), Centrifuge (Eppendrof), Micro Pipette (Bio Pette and Gentry), Hot Air Oven (Memmert), Laminar Air Flow (Augusta), Incubator (LAB-Therm), pH Meter (Consort), Shaker (Kuhner), Water Bath (Memmert), Vortex Mixer (Vortex-2-Genie), Spectrophotometer (Spectronic Instruments) and Concentrator (Eppendrof Concentrator 5301)

3.1.3 Fermented Vegetable and Fruit Products

Ten kind of a various fermented vegetable and fruit products were collected different markets in Northern of Thailand. (Data Shown in table 3.1)

Table 3.1 Ten kind of Northern of Thai fermented vegetable and fruit products.

Fermented vegetable and fruit products	No. of collected sample
Pickled of green cabbage	10
Pickled of bamboo shoots	7
Pickled of soybean	6
Pickled of wild spider flower	6
Pickled of chinese radish	6
Pickled of garlic	3
Pickled of camellia olefera	3
Pickled of spanish plum	3
Pickled of santol	3
Pickled of mango	3
Total	**50**

* source of markets were analysed shown in appendix B

3.1.4 Indicator microorganisms

The indicator microorganism, *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 microorganism culture

The indicator microorganisms were growed with nutrient agar (NA) 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.

3.2.2 Isolation of lactic acid bacteria

Ten grams of fermented vegetable and fruit products were suspended with 90 ml of 0.85% NaCl was homogeniously for 10 fold serial dilution. The 100 µl of each diluent was spreaded on MRS agar supplemented with 0.004% bromocresol purple and incubated at 37^0C for 24 hours. The yellow colonies were picked up and transferred to MRS broth and incubated at 37^0C for 48 hours. The 40 µl of culture were applied to paper disc and laid on sureface of MRS agar plus $CaCO_3$. After incubated 24 hours at 37^0C clear zone showed around the paper disc contained acid producing bacteria.

3.2.3 Isolation of antimicrobial substance producing lactic acid bacteria from fermented vegetable and fruit 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 37^0C for 48 hours, 1.5 ml of cells cultured were centrifuged at 4^0C and 13,000 rpm for 15 minutes. The supernatant was filtered with 0.22 µm nitrocellulose filler membrane.

The NA plus each indicator microorganisms, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella enteritidis* and *Escherichia coli* TISTR 73 was prepared by grown each indicator microorganism in nutrient broth at 37^0C for 24 hours then spreaded 100 µl each of indicator microorganism on nutrient agar plates prior to use.

The 40 µl filtrated was applied to 5 mm diameter paper disc which laid on the surface of NA plus indicator microorganism. The diameter of the inhibition zone was measured after incubation 37^0C for 24 hours. LAB which produce antimicrobial substance were show clear zone around the paper disc. 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.

2. Selection antimicrobial substance producing LAB

For the selection of antimicrobial substance by cultured the antimicrobial LAB substance producing for 48 hours in 5 ml MRS broth at 37^0C. The 1.5 ml of cultured was centrifugeg at 13,000 rpm for 15 minute and 1 ml of cell-free supernatant was concentrated by speed vacuum to volume 0.1 ml. The concentrated supernatant divide into two part one was adjusted to pH 7 with 3 N NaOH and another was non-adjusted pH value. Each solution were drop on disc which liad on NA containing with test indicator microorganisms and incubated at 37^0C for

24 hours. LAB witching 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

The selected antimicrobial substance producing LAB were grown in 5 ml of MRS broth at 37^0C for 48 hours, 1.5 ml of the cultured were centrifuged at 13,000 rpm for 15 minutes at 4^0C and 1 ml of cleared supernatant was concentrated to volume 0.1 ml by speed vacuum and used for antimicrobial activity test against four indicator microorganism testing.

Determination of antimicrobial substance production was performed by the agar well diffusion method modifier from Tagg and McGiven (1971). MRS agar, 20 ml, was mixed with $1x10^5$ cells/ml of a 24 hours culture of the indicator microorganism 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 5µl at different β-glycerophosphate concentration 0.1, 0.2, 0.3, 0.4 and 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 37^0C for 24 hours and examined for formation of inhibition zones. LAB which produced antimicrobial substance show clear zone around the well was selected.

2. Determination of bacteriocin-producing LAB

The isolate of LAB were screened for bacteriocin producing against a rang 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 4^0C for 15 minutes, 1.5 ml of cells free-supernatant was

concentrated to volume 0.1 ml by speed vacuum. The 20 µl concentrated supernatance supplement with 5 µl of 0.5 g/ml (w/v) β-glycerophosphate (final conc) and 5 µl of Proteinase K 1mg/ml at 37^0C for 2 hours. After incubation, the enzymes were inactivated by heat at 100^0C for 20 min before added each well of NA or MRS agar plus indicator microorganisms. Plates were incubated at 37^0C for 24 hours. Bacteriocin containing supernatant was disappeared when compare with non-heat supernatant

3.2.4 Acid and Bile Tolerance Test

For screening the effect of acid tolerance, A 100 µl of bacterial suspension was inculated into 5 ml. sterile MRS broth tubes with pH values 2, 3 and 4 (adjusted using 1N HCL/NaOH). The control consisted of 100 µl of the bacterial suspension added to MRS broth at pH 6.2 (adjusted using 1N HCL/NaOH) and incubated for 24 h at 37^0C. After incubation viable organisms were counted on MRS agar after 24 h at 37^0C.

Tolerance to bile salts was determined at 37^0C by inoculation of fresh cultures in MRS broth containing 0.15%, 0.3% (Ox-bile, Oxoid) and the control not containing bile acids. Resistance was assessed in terms of viable colony counts, enumerated after incubation for 0, 0.5, 1, 2.5 and 4 h. (modified of Erkkila and Petaja., 2000 ; modified of Kim and Worobo, 2000)

3.2.5 Biochemical characters

3.2.5.1 Gram stain

Isolated LAB cultured in slant MRS agar and incubated at 37 ^{0}C for 24 hrs. Take 1 loop of LAB from slant MRS agar streak on slide, then drop crystal violet for 1 min. after that pour and drop iodine solution for 1 min. Pour and drop alcohol and distilled water, then drop saflanin O for 1 min. clean with distilled water. After that check morphology under light microscope. (Chalat, 1999)

3.2.5.2 Catalase test

Isolated LAB cultured in slant MRS agar and incubated at 37 ^{0}C for 24 hrs. Take 1 loop of LAB from slant MRS agar on slide, drop Hydrogen peroxide 3% , if see air bubble shown the result positive but non exchang shown the result negative. (Chalat, 1999)

3.2.5.3 Oxidase test

Isolated LAB cultured in MRS broth added small tube and incubated at 37 ^{0}C for 48-72 hrs. Heterofermentative bacteria were shown gas in small tube but Homofermentative bacteria disappear gas in small tube. (Chalat, 1999)

3.2.5.4 Growth at 10^{0}C and 45^{0}C

Isolated LAB cultured in MRS broth incubated at 10^{0}C and 45^{0}C for 48-72 hrs., then observe density. (Chalat, 1999)

3.2.5.5 Growth at NaCl 6.5% and 18%

Isolated LAB cultured in MRS broth added NaCl 6.5% and 18% incubated at 37^{0}C for 48-72 hrs., then observe density. (Chalat, 1999)

3.2.5.6 Growth at pH 4.4 and 9.6

Isolated LAB cultured in MRS broth were adiusted pH to 4.4 and 9.6 with 1N HCL and 1N NaOH, incubated at 37^{0}C for 48-72 hrs., then observe density. (Toit *et al*., 1998)

3.2.6 16S-rRNA gene analysis

3.2.6.1 DNA Extraction

Three isolates were grown overnight in the appropriate MRS broth at 37°C. The saturated culture was used for genomic DNA extracttion, as

described by Anderson and Mckay (1983). In Table 3.2 show details of genomic DNA extraction.

Table 3.2 DNA Extracetion 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)

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.

3.2.6.2 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.

3.2.6.3 Sequencing of 16s-rRNA gene and phylogenetic tree gene 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 software (Thompson *et al*, 1994).