CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemical reagents

Chemical	Company
Agar	O.V. Chemical
Agarose	-
Ammonium citrate	Sigma
Bile salts (Ox gall)	HIMEDIA
Bromocresol purple	Fisher Scientific
Chloroform	LAB-SCAN
di-Potassium hydrogen phosphate	Scharlau
EDTA	Fisher Scientific
Ethanol	MERCK
Ethidium bromide	Plusone
Gelatin	M.C. Chiny center
Glacial acetic acid	LAB-SCAN
Glucose	Fluka
Lysozyme	Sigma
$MgCl_2$	Fermentas
$MnSO_4 \cdot 4H_2O$	Analar
$MgSO_4 \cdot 7H_2O$	Univar
NaCl	Univar
NaOH	Univar

Chemical	Company
Nucleotide(dNTP)	Fermemtas
Phenol	Fisher Scientific
Sodium acetate	Fisher Scientific
Tag polymerase	Fermentas
This-base	Fisher Scientific
This-HCL	Fisher Scientific
Tween-80	Labcham
Yeast extract	Scharlau

3.1.2 Equipments

Equipments	Company
Analytical balance (4 digit)	OERTING
Analytical balance (2 digit)	OERTING
Autoclave Model ACV-3167	IWAKI
Shaker Model ES-W	KUHNER
Hot air oven	MEMMERT
Incubator	LAB-THERM
Laminar Air Flow	AUGUSTA
Light microscope	OLYMPUS
Micro Pipette	Bio Pette and Gentry
pH Meter	CONSORT
UV-Vis spectophotometer	SHIMADZU
Vortex mixer	BOHEMIA
Water Bath	MEMMERT

3.1.3 Fecal sample

Fifty- five feces samples were collected from 2 to 4 months healthy infant.

3.2 Methods

3.2.1 Isolation and Screening of lactic acid bacteria.

The 10 grams of each fifty-five feces were collected from between 2 and 4 months of healthy infant. The sample was divided into two parts one for stored in 65% glycerol solution and kept at -80^{o}C (ratio of sample : glycerol solution = 1:1) and another was used for ten fold serial dilution. The 100 µl of appropriate dilutions were spread on MRS agar plus 0.04 g/l. bromocresol purple as indicator and incubated at 37^{o}C for 24 - 48 hours. After that, single colony from each isolate was tested on replica plate of MRS agar plus 10 g/l. of calcium carbonate, and incubated at 37^{o}C for 24 hours. Lactic Acid Bacteria showed clear zone on this media. The colonies were sub-cultured in MRS broth and re-streaked onto MRS agar to ensure purity. The purified LAB were maintained at -80°C in glycerol solution and kept on MRS agar slant at 4^{o}C for further studies.

3.2.2 Selection of acid tolerant lactic acid bacteria

The selected isolates from 3.2.1 were 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 count on MRS agar. The samples were incubated at 37^{o}C for 1 to 7 days. After incubated for about 7 days, turbidity were observed for various pH tolerant Lactic Acid Bacteria. The viable cell population was determined by dilution plate count on MRS agar after 24 - 48 hours of incubation (Erkkila and Petaja, 2000).

3.2.3 Selection of acid and bile tolerant lactic acid bacteria

An 0.25 ml of acid tolerant 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 2, 3 and 4 pH value : (adjusted pH value by using 1 N HCl). 0.15 and 0.30% of bile salts prepared from ox gall (HIMEDIA) was also added into the tubes of different pH values. 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 hours on MRS agar (pH 6.2) incubated for 48 hours at 37^{o}C (Erkkila and Petaja, 2000).

3.2.4 Identification of acid and bile lactic acid bacterial strains

The selected isolates were identified to the genus level following the criteria of Axelsson (1993) using the morphological, phenotypic and biochemical methods. Cultures were microscopically examined for Gram stain, cell size, cell arrangement and catalase production. Then tested for growth at different temperature, at different starting pH , at different NaCl concentrations and production of CO_2 from glucose. Finally, sequencing and comparison of 16S rRNA gene were the method for identify of LAB strains.

Morphological, phenotypic and biochemical methods (Axelsson, 1993).

Gram's stain

Selected isolates were sub-cultured in MRS agar (slant) and incubated at 37^{0}C for 24 hrs. One loop of selected isolates were placed on the slide and allowed to air dry. The area of inoculation was approximately 1 cm^{2}. Slides were heat fixed and crystal violet were added for 1 min. Then, rinsed and added iodine solution for 1 min. Decolorized by 95% alcohol and washed with distilled water. After that, added safranin O for 1 min and washed again with distilled water. Gram-stained slides were examined with a light microscope.

Catalase test

Selected isolates were sub-cultured in MRS agar (slant) and incubated at 37^0C for 24 hrs. One loop of selected isolates were placed on the slide and added 3% hydrogen peroxide. The air bubbles were observed. Appearance of air bubble was recorded a positive result.

CO_2 production

Selected isolates were sub-cultured in MRS broth with small tube and incubated at 37^0C for 24 hrs for 48-72 hrs. Heterofermentative strain would produced gas in small tube but Homofermentative bacteria not produce gas in small tube.

Growth at 10^0C and 45^0C

Selected isolates were sub-cultured in MRS broth and incubated at 10^0C and 45^0C for 48-72 hrs. Turbidity were observed.

Growth at 6.5% and 18% NaCl

Selected isolates were sub-cultured in MRS broth with 6.5% and 18% NaCl and incubated at 37^0C for 48-72 hrs. Turbidity were observed.

Growth at pH 4.4 and 9.6

Selected isolates were sub-cultured in MRS broth pH 4.4 and 9.6 that adjusted pH either by 1N HCl or 1N NaOH. Then, incubated at 37^0C for 48-72 hrs. Turbidity were observed.

3.2.4.2 16S rRNA gene analysis

DNA Extraction

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

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)

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.

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.

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).