Chapter 3

Materials and Methods

Materials

1. Media (Appendix A)

1.1 Gas production test medium

1.2 de Man Rogosa Sharpe (MRS) medium (Merck®, Germany)

1.3 de Man Rogosa Sharpe (MRS) medium with bromocresol green

1.4 Luria Bertani (LB) medium

1.5 Luria Bertani (LB) medium with ampicillin (100 mg/ml)

1.6 Mueller-Hinton broth (MHB) medium (Merck®, Germany)

1.7 Modified MRS medium for fermentation of 22 carbohydrates and substrate utilization

1.8 Motility test medium

1.9 Tryptic Soy Broth (TSB) (Merck®, Germany)

2. Chemical reagents for DNA isolation

2.1 Alkaline SDS solution

2.2 Ethanol, absolute

2.3 Isopropanol

2.4 Lysis buffer pH 8.0

2.5 Lysozyme (70,000 units/mg)

2.6 Phenol:Chloroform:Isoamylalcohol = 50:50:1

2.7 Plasmid extraction kit (HiYield^{TM} Plasmid Mini Kit, RBC Bioscience, Taiwan).

2.8 Proteinase K (10 mg/ml)

2.9 Sodium acetate, 3M pH 4.8 and 5.2

2.10 TES buffer pH 8.0

3. Chemical reagents for agarose gel electrophoresis (Appendix B)

3.1 Agarose gel 0.8 and 1%

3.2 Ethidium bromide 1 mg/ml

3.3 Loading dye

3.4 Marker 100 bp, 1 kb, λDNA/*Eco*130I (*Sty*I) (Fermentas, USA)

3.5 TAE buffer 1X

4. Materials

4.1 Beaker 100, 250 and 500 ml

4.2 Centrifuge tube 15 and 50 ml

4.3 Cylinder 100, 500 and 1,000 ml

4.4 Duran bottle 250 and 500 ml

4.5 Durham tube

4.6 Glass bottle 15 and 30 ml

4.7 Glass slide

4.8 Microcentrifuge tube 1.5 ml

4.9 Micropipette 0.5-10.0, 10-100, 20-200, 50-200 and 200-1,000 µl

4.10 Paper disc diameter 6 mm (Macherey-Nagel, Germany)

4.11 Petri dish

4.12 Pipette 1, 5 and 10 ml

4.13 Pipette tip

4.14 Spatula

4.15 Test tube 12x100 and 16x150 mm

4.16 Wire loop

5. Laboratory Equipments

Table 3.1 Laboratory Equipments

Equipment	**Model**	**Manufacturer**	**Country**
Autoclave	SS-325	Tomy	Japan
Balance, 2-digit precision	ARC120	Ohaus	USA
Balance, 4-digit precision	AB 304-S	Mettler Toledo	Switzerland
Centrifuge	Harrier 18/80	Sanyo	UK
	Mickro 200R	Hettich	Germany
Compound Microscope	-	Olympus	USA
Electrophoresis gel system	EC320	Unitech	USA
Electrophoresis power supply	EC250-90	E-C Apparatus Corporation	USA
Freezer -20^{o}C	SF-C991 NG	Sanyo	Japan
Hot air oven	-	Heraeus	USA
Hot plate and magnetic stirrer	Cerastir	Clifton	UK
Incubator	-	Memmert	Germany
Lamina flow	TL2448	Holten LaminAir	Denmark

Table 3.1 Laboratory Equipment (continued)

Equipment	Model	Manufacturer	Country
pH meter	713 pH meter	Metrohm	Switzerland
Spectrophotometer	Genesys20	Thermo spectronic	USA
Thermal cycler	GeneAmp® PCR system 9700	Applied Biosystems	USA
UV transiluminator	Gene Flash	Syngene Bio Imaging	USA
Vortex mixer	VM-300	Gemmy Industrial Corporation	Taiwan
Water bath	TW12	Julabo	Germany

Methods

1. Isolation of *Lactobacillus* spp.

One gram of each 24 samples of fermented foods (Table 3.2) was inoculated into 5 ml of MRS broth (Merck®, Germany) containing bromocresol green as a pH indicator. After 24-48 hours of incubation at 37°C, only samples that change color of media from green to yellow were streaked on MRS agar plate containing bromocresol green and incubated at 37°C for 24-48 hours. Colonies of various sizes and shapes were randomly picked from agar plates for further Gram stain evaluation. All Gram-positive rods, catalase negative and non-motile bacteria were subcultured to obtain pure cultures. Each pure isolate was maintained at 4°C and -20°C for further studies.

Table 3.2 Fermented foods used for bacterial isolation

No.	Sample	Collecting place
1	Pla-Ra	Kad-Tonpayom, Chiang Mai
2	Pickling fish	Kad-Tonpayom, Chiang Mai
3	Pak-kad-dong (sour)	Kad-Tonpayom, Chiang Mai
4	Pak-kad-dong (sweet)	Kad-Tonpayom, Chiang Mai
5	Nor-mai-dong	Kad-Tonpayom, Chiang Mai
6	Pickling crab	Kad-Tonpayom, Chiang Mai
7	Nham	Kad-Tonpayom, Chiang Mai
8	Kimchi	Course 215481 Industrial Microbiology
9	Nham	Kad-Lungmor, Chiang Mai
10	Sausages (sour)	Kad-Lungmor, Chiang Mai
11	EM	Terex brand
12	Pak-kad-dong (sour)	Railway station market, Phitsanuloke
13	Nor-mai-dong	Railway station market, Phitsanuloke
14	Tao-jeaw	Railway station market, Phitsanuloke
15	Pla-som	Railway station market, Phitsanuloke
16	Nham	Railway station market, Phitsanuloke
17	Pla-Ra	Railway station market, Phitsanuloke
18	Miang	Kad-Luang, Chiang Mai
19	Pak-kad-dong (sour)	Kad-Luang, Chiang Mai
20	Nor-mai-dong	Kad-Luang, Chiang Mai
21	Pla-Som	Kad-Kongkong, Chiang Mai
22	Nham	Kad-Kongkong, Chiang Mai
23	Yogurt	Dutchmill brand
24	Drinking yogurt	Betagen brand

2. Isolation of plasmids

For plasmid isolation, the alkaline extraction procedure was modified from a technique described by O'Sullivan and Klaenhammer (1993). Briefly, 10 ml

overnight culture was harvested and pellets were resuspened in 200 µl of 25% sucrose containing 30 mg/ml lysozyme (70,000 units/mg). After incubation for 1 hour at 37°C, cells were lysed with 400 µl of alkaline SDS solution (3% SDS and 0.2 M NaOH) and incubated at room temperature for 7 minutes then 300 µl of ice-cold 3M sodium acetate (pH 4.8) were added. After centrifugation at 13,000 rpm for 15 minutes, 650 µl of isopropanol were added in the supernatant followed by centrifugation at 13,000 rpm for 15 minutes. The supernatant was extracted with 350 µl of phenol:chloroform:isoamylalcohol (50:50:1) and precipitated by 1 ml of ice-cold absolute ethanol prior to dissolve plasmid DNA in 50 µl distilled water. Only plasmid containing isolates were repeatly extracted by a plasmid extraction kit (HiYield™ Plasmid Mini Kit, RBC Bioscience, Taiwan) according to the manufacturer's instructions. For DNA detection, 1% agarose gel electrophoresis was performed as described by Sambrook *et al*. (1989). All plasmid DNA were maintained at -20°C for further studies.

3. Bacterial identification

3.1 Conventional method

All bacterial isolated were evaluated their macroscopic and microscopic morphologies. Biochemical characterizations were tested including catalase test, motility test, growth at 15 and 45°C, gas production and fermentation of 22 carbohydrates according to Bergey's manual of systematic bacteriology (Kandler and Weiss, 1986) as follows: amygdalin, arabinose, cellobiose, esculin, fructose, galactose, glucose, gluconate, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose and xylose.

3.2 Molecular identification by 16S rRNA gene determination

Only plasmid containing isolates were confirmed using 16S rRNA gene determination. Genomic DNA of each bacterial isolate containing plasmid was extracted by the method of Martin-Platero *et al.* (2007) as the following: 1 ml of overnight culture were harvested, resuspened in 100 µl of TES buffer (10% sucrose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 10 mg/ml freshly made lysozyme and 40 µg/ml RNase) and incubated for 30 minutes at 37°C in water bath. The protoplast cells were immediately lysed by adding 600 µl of lysis buffer (100 mM Tris-HCl pH 8.0, 100 mM EDTA, 10mM NaCl and 1% SDS) for 15 minutes at room temperature. The lysate were treated with 10 µl of proteinase K (10 mg/ml) for 15 minutes at 37°C. After incubation at 80°C for 5 minutes before cooling down to room temperature for 5-10 minutes, 200 µl of sodium acetate (3 M, pH 5.2) were added, chilled on ice for 15 minutes and centrifuged. The supernatant was taken to a new tube and 600 µl of isopropanol were added to precipitate the DNA. Finally, genomic DNAs were dissolved in distilled water. All genomic DNAs were maintained at -20°C for further studies.

To amplify the 16S rRNA gene by PCR, LacbF/LacbR primers were used following the method of Corsetti *et al*. (2004). The primer LacbF was 5′-TGCCTAATACATGCAAGT-3′ and LacbR was 5′-CTTGTTACGACTTCACCC-3′. The 50 µl PCR mixtures contained 2 µl of DNA template (50 ng of DNA), 1 µl of dNTP (2.5 mM each), 5 µl of $MgCl_2$ (25 mM) (Fermentas, USA), 0.5 µl of each primer (100 mM) (Pacific Science, Thailand), 0.5 µl of *Taq* DNA polymerase (Fermentas, USA), 5 µl of 10X *Taq* DNA polymerase buffer (Fermentas, USA) and 35.5 µl of sterile distilled water. PCR amplifications of the 16S rRNA were

performed using the GeneAmp® PCR System 9700 (Applied Biosystems, USA). The PCR program comprised an initial template denaturation step for 2 minutes at 94°C followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing for 1 minute at 49°C and extension for 1 minute at 72°C. The final extension step was 7 minutes at 72°C.

PCR products were separated by 1% agarose gel electrophoresis and were purified by the NucleoSpin® Extract II (Macherey-Nagel, Germany). DNA sequencing reactions were performed by Macrogen (Korea). The 1.5 kb nucleotide sequences of the 16S rRNA gene obtained were subjected to BLAST analysis (Altschul *et al*., 1990) with the NCBI database (http://www.ncbi.nlm.nih.gov). Sequences were aligned with representative *Lactobacillus* spp. 16S rRNA gene sequences and phylogenetic tree was constructed using the Molecular Evolution Genetics Analysis (MEGA) software version 4.0 (Tamura *et al*., 2007).

The nucleotide sequences of 16S rRNA gene of 12 plasmid containing *Lactobacillus* spp. have been deposited in the GenBank database under the Accession No. GQ900597, GQ900598, GQ900599, GQ900600, GQ900601, GQ900602, GQ900603, GQ900604, GQ900605, GQ900606, GQ900607 and GQ900608 (Appendix C).

4. Characterization of plasmids containing *Lactobacillus* spp.

4.1 Antibiotic susceptibility test

Lactobacillus isolates that contained plasmid DNA were studied for their antibiotic susceptibility by a paper disc diffusion method. Tests were performed with 21 antibiotics (Oxoid®, UK) (Table 3.3). Agar plates were inoculated and incubated

according to NCCLS guidelines (National Committee for Clinical Laboratory Standards, 1984). These guidelines were modified for lactobacilli, where Mueller-Hinton agar was replaced by MRS agar. Inhibition zones were measured using a ruler accurate to a millimeter. The results were expressed as susceptible (S), moderately susceptible (MS) and resistant (R), and interpreted as described by Charteris *et al*. (1998).

Table 3.3 Antibiotics used in this study

No.	Antibiotic	Concentration per disc	
1	Ampicillin	10	µg
2	Bacitracin	10	units
3	Cefotaxime	30	µg
4	Cefoxitin	30	µg
5	Cephalothin	30	µg
6	Chloramphenicol	30	µg
7	Ciprofloxacin	5	µg
8	Clindamycin	2	µg
9	Erythromycin	15	µg
10	Fusidic acid	10	µg
11	Gentamycin	10	µg
12	Kanamycin	10	µg
13	Nalidixic acid	30	µg
14	Nitrofurantoin	300	µg
15	Norfloxacin	10	µg
16	Penicillin G	10	units
17	Polymyxin B	300	units
18	Rifampicin	5	µg
19	Streptomycin	10	µg
20	Tetracycline	30	µg
21	Vancomycin	30	µg

4.2 Bacteriocin production test

Plasmid containing *Lactobacillus* spp. were determined for their bacteriocin production by a well-diffusion technique (Dave and Shah, 1997). Briefly, Mueller-Hinton Agar (MHA) with 0.9% agar held at 45°C was inoculated with 1% of active culture of the indicator strains; *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus* and *Staphylococcus epidermidis*. About 25 ml of melted agar were poured into a sterile Petri dish and wells were punctured in the solidified agar using a sterile bottom of pasteur pipette having 6.0 mm diameter. Cell-free extract of 48 hour grown culture of plasmid containing *Lactobacillus* spp. were collected by centrifugation at 6,000 rpm for 10 minutes. The supernatant of each isolates was divided in to two portions: (A) untreated, (B) neutralized to pH 6.0 with 1M NaOH following by ultrafiltration to concentrate the bacteriocins using Amicon® Ultra-4 centrifugal filter devices (MWCO=10,000 Da) (Millipore, USA). Wells were filled with 50 µl of the above treated and untreated supernatants. The agar plates were left for 2 hours at room temperature for diffusion of the test material into the agar. The plates were incubated at 37°C for 24-48 hours and zones of inhibition were measured.

4.3 Substrate utilization

Plasmid containing *Lactobacillus* spp. were grown in MRS broth at 37°C for 18-24 hours (OD_{660} = 1.0). One percent (w/v) of each substrate including soluble starch, CMC, colloidal chitin, inulin, gelatin, citrate, phenylalanine and tryptophan was added into the modified MRS broth to replace the conventional carbon source. Later, 2% of seed culture of each *Lactobacillus* isolate was inoculated and incubated at 37°C. Growth of the bacteria was determined by measuring culture pH and

turbidity of the culture broth at 660 nm every 24 hours for 3 days. Each plasmid containing *Lactobacillus* spp. was streaked on modified MRS agar with 1% of each substrates mentioned above. Occurence of clear zone was observed in each agar plate.

5. Characterization of plasmid

5.1 Digestion of plasmids by restriction enzymes

Extracted plasmids from 12 plasmid containing *Lactobacillus* spp. were digested with restriction enzymes; *Bam*HI, *Eco*RI and *Hin*dIII (Fermentas, USA) according to manufacturer's instructions; prior to be separated by electrophoresis on agarose gels. To determine plasmid profiles and dendrogram construction, all product digested were analyzed by using SPSS program (Version 16.0, SPSS Inc.).

5.2 Plasmid DNA sequencing

According to the conserved *rep* genes of plasmid in *Lactobacillus* spp., many cloning vector showed that use of *rep* gene from one *Lactobacillus* can be replicate in another lactobacilli when used as a cloning host. *Lactobacillus brevis* D11 was shown to harbour more than one plasmid and because of the appropriate size and high concentration of DNA, the smallest one named pSD11 which having a size of ca. 3.2 kb was selected to sequence. The plasmid pSD11 was extracted from agarose gel by the NucleoSpin® Extract II (Macherey-Nagel, Germany) followed by digestion with *Eco*RI (Fermentas, USA). The 1.7 kb fragment digested from *Eco*RI was cloned into the vector pUC19 (Fermentas, USA) and was transformed into a cloning host *E. coli* DH5α (Sambrook *et al.*, 1989). LB agar containing 100 mg/ml ampicillin, 100 mM IPTG and 50 mg/ml X-gal was used for selected the white colony. The recombinant

plasmid was sequenced with M13F(-20) 5′-GTAAAACGACGGCCAGT-3′ and M13R-pUC(-26) 5′-CAGGAAACAGCTATGAC-3′ universal primers (1st Base, Malaysia) and used as the starting point for sequence determination. The remaining part of the pSD11 was sequenced by primer walking. The primers designed for primer walking were listed in Table 3.4.

Table 3.4 Primers used for DNA sequencing

Number	Primer pairs	Sequence (5′⟶3′)
1	1.6F	GCTTGACGCTTGCTAACTCAAGC
	1.6R	CGCAGAGCCCAAACTTTACAAGG
2	1.6F_1	ACTTTGCCCGACTGCGATCG
	1.6R_1	GGTTCAACTTGGTAGAAAAGC
3	3.6F	ATATCACTGACAGCTGCTCG
	3.6R	CGTGATCAGCAGGAAATTGA

5.3 Nucleotide sequence analysis

Sequence annotation and database searches for similar sequences were performed using the BLAST at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) (Altschul *et al*., 1990). Analysis of the complete nucleotide sequence for open reading frame (ORF) was performed using the web-based NCBI ORF Finder program. Comparisons of DNA sequences or DNA-derived protein sequences were carried out using BLASTN, BLASTP and BLASTX programs (Altschul *et al*., 1990). Multiple sequence alignments were performed using the ClustalW at the European Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw) (Thompson *et al*., 1994).

5.4 Nucleotide sequence accession number

The complete nucleotide sequence of pSD11 has been deposited in the GenBank database under the Accession No. HQ622718 (Appendix D).