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ACID PRODUCTION AND PROBIOTIC PROPERTIES OF SELECTED LACTIC ACID BACTERIA

Miss Tanatip Thamacharoensuk

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Pharmaceutical Chemistry and
Natural Products

Department of Food and Pharmaceutical Chemistry
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ได้ทำการคัดกรองการผลิตกรดแลกติกของแบคทีเรียจำนวน 36 ไอโซเลทที่แยกจากเปลือกไม้และคัดกรองคุณสมบัติโปรไบติกของแบคทีเรียกรดแลกติกจำนวน 41 ไอโซเลทจากมูลสัตว์ อุจจาระมนุษย์สุขภาพดี อาหารหมัก ซีอิ๊วและหญ้าหมัก พบว่าไอโซเลทที่คัดเลือกทั้งหมด 52 ไอโซเลทอยู่ในสกุล *Lactobacillus* (12 สายพันธุ์) *Enterococcus* (14 สายพันธุ์) *Pediococcus* (7 สายพันธุ์) *Lactococcus* (1 สายพันธุ์) *Weissella* (7 สายพันธุ์) และ *Sporolactobacillus* (11 สายพันธุ์) และพิสูจน์เอกลักษณ์ได้เป็น *L. futasaii* (2 สายพันธุ์), *L. acidipiscis* (2 สายพันธุ์), แต่ละสายพันธุ์ของ *L. namurensis*, *L. farraginis*, *L. mucosae*, *L. paracasei* subsp. *tolerans*, และ *L. salivarius*, *L. pentosus* (3 สายพันธุ์), *E. hirae* (10 สายพันธุ์), *E. avium* (4 สายพันธุ์), *P. pentosaceus* (4 สายพันธุ์), *P. acidilactici* (3 สายพันธุ์), *Lc. garvieae* (1 สายพันธุ์), *W. paramesenteroides* (3 สายพันธุ์), *W. thailandensis* (2 สายพันธุ์), *W. cibaria* (1 สายพันธุ์), *W. confusa* (1 สายพันธุ์), *S. nakayamae* subsp. *nakayamae* (3 สายพันธุ์), *S. terrae* (2 สายพันธุ์), *S. kofuensis* (1 สายพันธุ์), *S. inulinus* (3 สายพันธุ์) และสองสายพันธุ์ BK92^T และ BK117-1^T ที่แยกได้จากเปลือกไม้ของต้นพะยอมและแคแสดถูกเสนอเป็นแบคทีเรียสปีชีส์ใหม่ชื่อว่า *Sporolactobacillus shoreae* sp.nov. และ *Sporolactobacillus spathodeae* sp.nov. ตามลำดับ โดยอาศัยลักษณะทางฟีโนไทป์อนุกรมวิธานทางเคมีและจีโนไทป์โดยการวิเคราะห์ลำดับเบสในช่วง 16S rRNA gene *S. inulinus* BK65-3 และสายพันธุ์ BK70-3 ผลิตกรดแลกติกความเข้มข้น 101.42 และ 117.85 กรัมต่อลิตรตามลำดับ มีค่าความบริสุทธิ์เชิงแสงของกรดดีแลกติกถึง 100 เปอร์เซนต์จากน้ำตาลกลูโคสที่ความเข้มข้น 120 กรัมต่อลิตร ภาวะที่เหมาะสมในการผลิตกรดของ *S. inulinus* BK65-3 คือกลูโคส 140 กรัมต่อลิตรที่ 48 ชั่วโมงไม่เขย่า จะให้ความเข้มข้นของกรดแลกติก 131.45 กรัมต่อลิตร ค่าปริมาณผลผลิต 93.89 เปอร์เซ็นต์ และอัตราการผลิต 2.74 กรัมต่อลิตรต่อชั่วโมง ในขณะที่ภาวะเดียวกัน *S. inulinus* BK70-3 ต้องการการเขย่าให้ความเข้มข้นของกรดแลกติก 133.79 กรัมต่อลิตร ค่าปริมาณผลผลิต 95.56 เปอร์เซ็นต์และอัตราการผลิต 2.79 กรัมต่อลิตรต่อชั่วโมง อัตราการผลิตของ *S. inulinus* BK70-3 จะเพิ่มขึ้นเป็น 2.93 กรัมต่อลิตรต่อชั่วโมงเมื่อเลี้ยงในถังหมักขนาด 5 ลิตร และพบว่า *S. inulinus* BK70-3 สามารถทนต่อความเข้มข้นของกลูโคสได้สูงถึง 200 กรัมต่อลิตร *S. inulinus* BK70-3 จึงเป็นสายพันธุ์ที่ดีในการผลิตกรดแลกติก *L. acidipiscis* SR7-1 และ *L. farraginis* SL4-1 แสดงฤทธิ์เป็นพิษต่อเซลล์มะเร็งลำไส้ใหญ่ (Caco-2 cells) โดยไม่มีฤทธิ์เป็นพิษต่อเซลล์ปกติ (Vero cells) สายพันธุ์ทั้งหมดไม่แสดงฤทธิ์เป็นพิษต่อเซลล์มะเร็งเม็ดเลือดขาว (U937 cells) แบคทีเรีย 15 สายพันธุ์ ได้แก่ *L. futasaii* PC72-4, *P. pentosaceus* PC73-3, *L. futasaii* KC74-1, *L. acidipiscis* PC75-2, *L. namurensis* KC78-5, *W. thailandensis* PC79-5, *W. thailandensis* KC81-2, *W. cibaria* PC86-2, *L. acidipiscis* SL4-1, *L. farraginis* SR7-1, *L. mucosae* SL7-2, *L. paracasei* subsp. *tolerans* MSMC39-5, *W. confusa* MSMC57-2, *W. paramesenteroides* MSMC 63-2 และ *L. salivarius* MSMC120-2 กระตุ้นการสร้าง IL-12 ในช่วง 1,585.23 ± 7.80 ถึง 430.65 ± 35.02 pg/ml ซึ่งมากกว่า *L. plantarum* NRIC 1067 โดยสายพันธุ์ทั้งหมดทนต่อน้ำดีที่ความเข้มข้น 1 เปอร์เซ็นต์ *P. pentosaceus* PC73-3, *L. namurensis* KC78-5, *L. farraginis* SL4-1 และ *L. mucosae* SL7-2 เป็นสายพันธุ์ที่ทนกรดที่พีเอช 3 และสามารถยึดเกาะต่อเซลล์ผนังลำไส้มากกว่า *L. rhamnosus* GG ซึ่งเป็นสายพันธ์ควบคุม ดังนั้น *P. pentosaceus* PC73-3, *L. namurensis* KC78-5, *L. farraginis* SL4-1 และ *L. mucosae* SL7-2 มีคุณสมบัติเป็นโพรไบโอติกที่มีศักยภาพในการป้องกันมะเร็งโดยกระตุ้นการสร้าง IL-12 โดยเฉพาะ *L. farraginis* SL4-1 อาจนำมาใช้ในมะเร็งลำไส้ใหญ่เนื่องจากฤทธิ์เป็นพิษต่อเซลล์มะเร็งลำไส้ใหญ่

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TANATIP THAMACHAROENSUK: ACID PRODUCTION AND PROBIOTIC PROPERTIES OF SELECTED LACTIC ACID BACTERIA. ADVISOR: PROF. SOMBOON TANASUPAWAT, Ph.D., CO-ADVISOR: ASST. PROF. NUTTHA THONGCHUL, Ph.D., KENTARO KODAMA, Ph.D., 186 pp.

Thirty-six isolates of lactic acid bacteria (LAB) from tree barks were screened for lactic acid production and forty-one isolates from animal feces, healthy human feces, fermented foods, soy sauce mash and silages were screened for the probiotic properties. The selected isolates (52 isolates) were belonged to the genera *Lactobacillus* (12 strains), *Enterococcus* (14 strains), *Pediococcus* (7 strains), *Lactococcus* (1 strain), *Weissella* (7 strains) and *Sporolactobacillus* (11 strains), and they were identified as *L. futasaii* (2 strains), *L. acidipiscis* (2 strains), each strain of *L. namurensis*, *L. farraginis*, *L. mucosae*, *L. paracasei* subsp. *tolerans* and *L. salivarius*, *L. pentosus* (3 strains), *E. hirae* (10 strains), *E. avium* (4 strains), *P. pentosaceus* (4 strains), *P. acidilactici* (3 strains), *Lc. garvieae* (1 strain), *W. paramesenteroides* (3 strains), *W. thailandensis* (2 strains), *W. cibaria* (1 strain), *W. confusa* (1 strain), *S. nakayamae* subsp. *nakayamae* (3 strains), *S. terrae* (2 strains), *S. kofuensis* (1 strain) *S. inulinus* (3 strains) and two strains $BK92^T$ and $BK117\text{-}1^T$ isolated from White-Meranti and African Tulip tree barks were proposed as novel species as *Sporolactobacillus shoreae* sp.nov. and *Sporolactobacillus spathodeae* sp.nov., respectively based on their phenotypic, chemotaxonomic and genotypic characteristics including 16S rRNA gene sequence analysis. *S. inulinus* BK65-3 and BK70-3 was found to produce 101.42 g/L and 117.85 g/L of lactic acid, respectively with high optical purity of D-lactic acid (100%ee) from 120 g/L of glucose concentrations. The optimum condition of lactic acid production of *S. inulinus* BK65-3 was 140 g/L of glucose at 48 h without agitation and provided lactic acid of 131.45 g/L, yield of 93.89%, and productivity of 2.74 g/L.h while at same condition *S. inulinus* BK70-3 was required agitation and provided lactic acid of 133.79g/L, yield of 95.56% and productivity of 2.79 g/L.h. The productivity of *S. inulinus* BK70-3 was increased to 2.93 when cultivated in 5-L fermentor. *S. inulinus* BK70-3 could tolerant at high glucose concentrations up to 200 g/L. The strain *S. inulinus* BK70-3 could be considered as the good strain for lactic acid production. *L. acidipiscis* SR7-1 and *L. farraginis* SL4-1 showed cytotoxic effects against colorectal cancer cell lines (Caco-2 cells) with non-toxicity to normal cell lines (Vero cells). All isolates showed no cytotoxic effects against leukemic U937 cells. Fifteen strains including *L. futasaii* PC72-4, *P. pentosaceus* PC73-3, *L. futasaii* KC74-1, *L. acidipiscis* PC75-2, *L. namurensis* KC78-5, *W. thailandensis* PC79-5, *W. thailandensis* KC81-2, *W. cibaria* PC86-2, *L. acidipiscis* SL4-1, *L. farraginis* SR7-1, *L. mucosae* SL7-2, *L. paracasei* subsp. *tolerans* MSMC39-5, *W. confusa* MSMC57-2, *W. paramesenteroides* MSMC63-2, and *L. salivarius* MSMC120-2 induced IL-12 production ranged from 1,585.23 ± 7.80 to 430.65 ± 35.02pg/ml that was higher than *L. plantarum* NRIC 1067. All strains were tolerated in 1% bile. *P. pentosaceus* PC73-3, *L. namurensis* KC78-5, *L. farraginis* SL4-1 and *L. mucosae* SL7-2 were acid tolerated to pH 3 and showed higher adhesion ability as compared to positive control *L. rhamnosus* GG. Thus, *P. pentosaceus* PC73-3, *L. namurensis* KC78-5, *L. farraginis* SL4-1 and *L. mucosae* SL7-2 were the potential probiotics for prevention of cancers by stimulation of IL-12 production, especially, *L. farraginis* SL4-1 might be useful in colorectal cancer due to their cytotoxic effects against colorectal cancer.

Department:	Food and Pharmaceutical Chemistry	Student's Signature ....
Field of Study:	Pharmaceutical Chemistry and Natural Products	Advisor's Signature ....
		Co-Advisor's Signature ....
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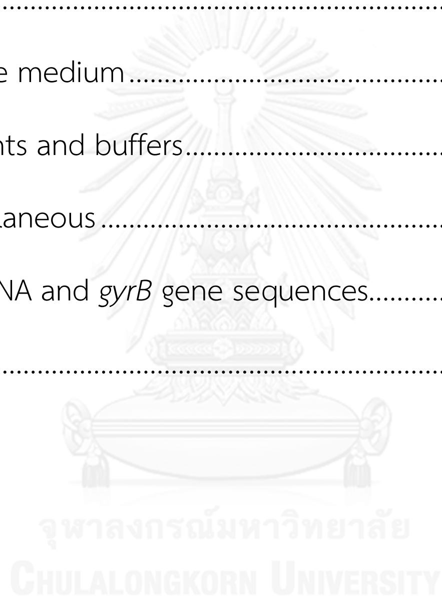
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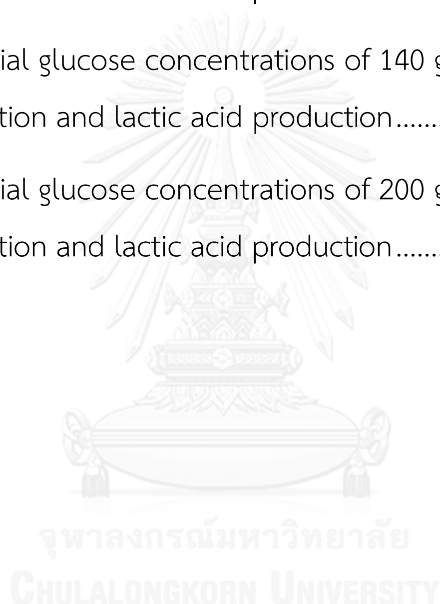
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LIST OF ABBREVIATIONS AND SYMBOLS

AAD	=	Antibiotic-associated diarrhea
AIDS	=	Acquired Immune Deficiency Syndrome
ASR	=	Annual statistics report
ATP	=	Adenosine tri phosphate
bp	=	Base pairs
°C	=	Degree Celsius
Caco-2 cells	=	Human colon adenocarcinoma cell lines ATCC HTB-37
$CaCO_3$	=	Calcium carbonate
CDAD	=	*Clostridium difficile*-associated disease
CFU	=	Colony forming unit
CO_2	=	Carbon dioxide
CTLs	=	Cytotoxic T lymphocytes cells
$CuSo_4$	=	Copper (II) sulfate
D(-)	=	Dextrorotation
DMEM	=	Dulbecco Modified Eagle medium
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
ELISA	=	Enzyme-linked immunosorbent assay
EMP pathway	=	Embden-Meyerhof-Parnas pathway
F	=	Forward
FAME	=	Fatty acid methyl ester
FBS	=	Fetal bovine serum

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g	=	Gram
μg	=	Microgram
G+C	=	Guanine-plus-cytosine
GRAS	=	Generally recognized as safe
GYP	=	Glucose yeast peptone medium
gyrB	=	DNA gyrase subunit B
h	=	Hour
HCl	=	Hydrochloric acid
H_2O	=	Water
H_2SO_4	=	Sulfuric acid
HPLC	=	High performance liquid chromatography
IBD	=	Inflammatory bowel disease
IBS	=	Irritable bowel syndrome
IFN-γ	=	Interferon-gamma
IgA	=	Immunoglobulin A
IL-12	=	Interleukin-12
KH_2PO_4	=	Potassium phosphate monobasic
K_2HPO_4	=	Potassium phosphate dibasic
L(+)	=	Levorotation
L	=	Liter
Log	=	logarithm
LA	=	Lactic acid
LAB	=	Lactic acid bacteria
LDH	=	Lactate dehydrogenase

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LGG	=	*Lactobacillus rhamnosus* GG
M	=	Molar
M199	=	Medium 199
MEGA	=	Molecular evolutionary genetics analysis
meso-DAP	=	*meso*-Diaminopimelic acid
mg	=	Milligram
MHC	=	Major histocompatibility complex
min	=	Minute
MK	=	Menaquinone
mL	=	Milliliter
mm	=	Millimeter
mM	=	Millimolar
µL	=	Microliter
µm	=	Micrometer
MRS	=	deMan-Rogosa-Sharpe
MTT	=	3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NSS	=	Normal saline solution
NK cells	=	Natural killer cells
nm	=	Nanometer
nov.	=	Novel
OD	=	Optical density
O.P.	=	Optical purity
%	=	Percentage

%ee	=	Percentage of enantiomer excess
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
pg	=	Picrogram
PLA	=	Polylactic acid
PLDA	=	Poly (D-lactide)
PLLA	=	Poly (L-lactide)
P	=	Productivities
R	=	Reverse
rpm	=	Round per minute
RPMI 1640	=	Roswell Park Memorial Institute medium number 1640
rRNA	=	Ribosomal ribonucleic acid
SCFA	=	Short chain fatty acids
SEM	=	Scanning electron microscope
sp.	=	Species
Th-1	=	T helper-1
TLC	=	Thin layer chromatography
TMB	=	3,3',5,5'-Tetramethylbenzidine
TNF-α	=	Tumor necrosis factor-alpha
U	=	Units
U937 cells	=	Human monocytic leukemic cell lines ATCC CRL-2367
Vero cells	=	African green monkey kidney fibroblasts; ATCC CCL-81
Y	=	Yields

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CHAPTER I
INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive, catalase-negative bacteria that consist of several genera, *Lactobacillus, Lactococcus (Lc.), Enterococcus, Pediococcus, Leuconostoc, Streptococcus, Oenococcus, Sporolactobacillus, Aerococcus, Carnobacterium, Tetragenococcus, Vagococcus,* and *Weissela* (Lahtinen *et al.*, 2011). They are recognized by their ability to produce lactic acid and are commonly distributed in nature such as fermented foods, soil, decomposing plants as well as found in human and animal digestive systems (Parente *et al.*, 2001; Heilig *et al.*, 2002; Callon *et al.*, 2004).

LAB can be divided into two groups based on their fermentative characteristics; homofermentative and heterofermentative LAB. Homofermentative LAB produced almost exclusively lactic acid from glucose while heterofermentative LAB produced lactic acid and other byproduct such as CO_2 etc. (Saeed A & Salam A, 2013). Lactic acid is generally used in many industries such as food, beverage, medical, pharmaceutical, leather and textile industries. Optical purity of lactic acid was used as a raw material for the production of polylactic acid in plastic industries. They provide several advantages properties such as biodegradability, thermoplasticity and high tensile strength. They are used in specialty medical or drug controlled release products (Rasal *et al.*, 2010). According to the environmental concerning, there has been an increasing interest in environmental-friendly biodegradable plastics such as polylactic acid (Reddy *et al.,* 2008). However, stereoisomer of lactic acid depends on the chemical or biological processes. Microorganisms have distinct enzymatic reactions that provide optical purity of D- or L- or racemic lactic acid (Wee *et al.*, 2006).

In addition, LAB are known as “probiotics” which have the potential health and nutritional benefits. They can provide relief for lactose intolerant and reduce travel and infective diarrhea. Evidence for other benefits such as remodeling of microbial communities, suppression of pathogens, stimulation the immune system by

up-regulation of anti-inflammatory factors and suppression of pro-inflammatory factors or suppression of cancer remain to be established (Sanders *et al.*, 2007; O'Flaherty & Klaenhammer, 2010). LAB such as *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Pediococcus* and *Enterococcus* are usually used as probiotics for human and animal. They might be prepared one or mix of LAB, particularly *Lactobacillus* that are used as the component in commercial dairy products (Kailasapathy, 2013). Therefore, isolation and identification of lactic acid bacteria to find the new species or specific strains showing high lactic acid production with high optical purity or showing the probiotic properties are objectives in this research.

The main objectives of this research are as follows:

1. To isolate and select lactic acid bacteria that specifically produce D-lactic acid
2. To isolate and screen lactic acid bacteria possessing probiotic properties
3. To identify the selected lactic acid bacteria using phenotypic, chemotaxonomic characteristics and 16s rRNA gene sequence analysis
4. To select the lactic acid bacteria strains showing high lactic acid production with high optical purity as well as to determine its optimization
5. To select the lactic acid bacteria strains showing probiotic properties in acid and bile tolerance as well as in inhibition of cancer cells proliferation

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CHAPTER II
LITERATURE REVIEW

2.1 Taxonomy and metabolism of lactic acid bacteria

Lactic acid bacteria (LAB) were first systematically classified by Orla-Jensen in 1919 into six genera based on morphology, sugar fermentation and some physiological characteristics. However, LAB have currently been expanded to the large groups as shown in Figure 2.1 based on molecular characteristics such as mol% G + C content, ribosomal RNA sequencing (rRNA) and DNA hybridization studies (Axelsson, 2004).

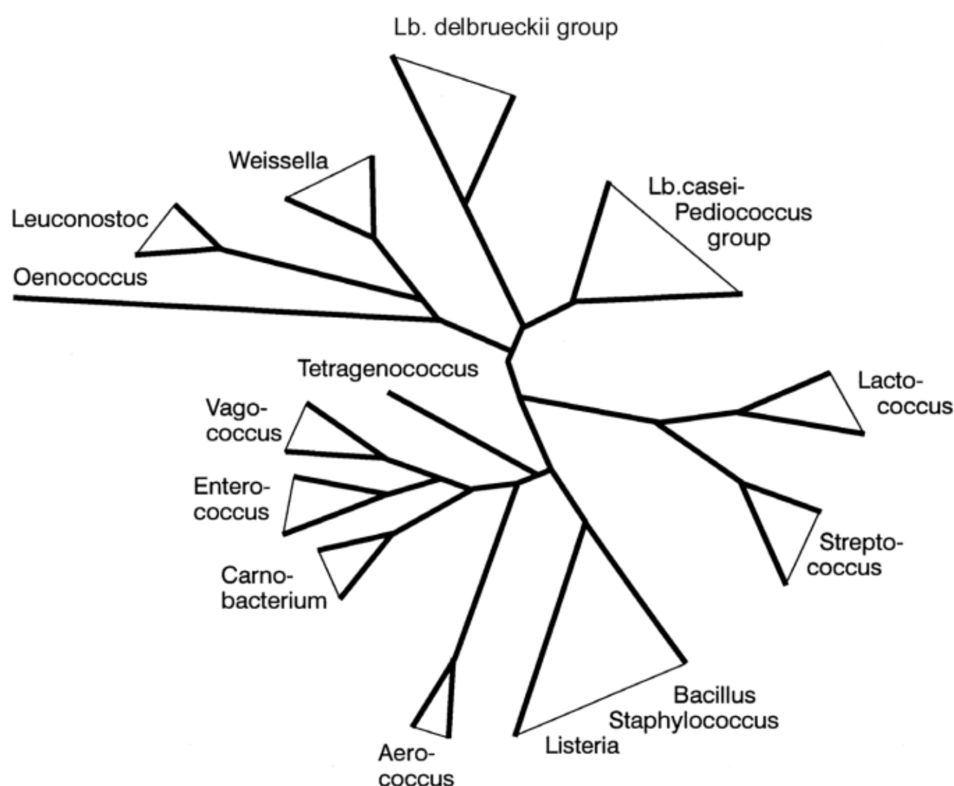

Figure 2.1 The diagram of unrooted phylogenetic tree of the lactic acid bacteria.
Note: Evolutionary distances are estimated (Axelsson, 2004).

LAB are defined as a group of microaerophilic, Gram-positive organisms which produce lactic acid as the major end product. They are differentiated from other organisms by their ability to ferment glucose to lactic acid, hence the name, lactic acid bacteria. Common genera of LAB and their differential characteristics are shown in Table 2.1. They were belonged to the phylum *Firmicutes* which mostly comprised of *Lactobacillus, Streptococcus, Enterococcus, Pediococcus, Lactococcus, Leuconostoc, Aerococcus, Oenococcus, Carnobacterium, Vagococcus, Tetragenococcus* and *Weisella* (Axelsson, 2004). The genus *Lactobacillus* is the largest of these genera, comprising 214 described species and 29 subspecies (http://www.bacterio.cict.fr/l/lactobacillus.html, 4/9/15). *Bifidobacterium* and *Sporolactobacillus* that are spore forming bacteria are also in the members of the LAB. The typical characteristics of LAB are Gram-positive, low-GC, usually non-motile, non-spore forming, mostly catalase-negative and varying in shape from long, slender rods to short coccobacilli, commonly found in chains and are able to produce primarily lactic acid. The G+C content in their DNA less than 50 mol%. They lack the ability to synthesize cytochromes and porphyrins and therefore cannot generate ATP by creation of a proton gradient. These organisms can only obtain ATP by fermentation, usually of sugars. They have smaller colonies of 2 – 3 mm and can grow at temperatures from 5 to 45 °C, most strains able to grow at pH 4.4 and tolerant to acidic conditions. The growth is optimum at pH 5.5 – 6.5. Moreover, they can grow under anaerobic conditions and also grow in aerobic conditions. They are heterotrophic and have limited biosynthetic ability, requiring a complex nutrition. A rich medium is usually employed when cultivation of lactic acid bacteria (Lahtinen *et al.*, 2011). These multiple requirements restrict to their habitats, however, they are widely distribute in environments where carbohydrates are available, such as food (dairy products, fermented meat, sourdoughs, vegetables, fruits, beverages), respiratory, GI and genital tracts of animals and humans, and in plant material as shown in Table 2.2 (Tohno *et al.,* 2012; Cizeikiene *et al.*, 2013; Rubio *et al.*, 2014). They are extensively used in the manufacture of food products and industrial fermentations and also closely associated with the health-promoting probiotic (Clarke *et al.*, 2012; Gerez *et al.*, 2013).

Table 2.1 Genera of LAB and their differential characteristics (Lahtinen *et al.*, 2011)

Family	Genera	Characteristics									
		Shape	CO_2 from glucose	Growth at 10 0C	Growth at 45 0C	Growth in 6.5% NaCl	Growth in 18% NaCl	Growth at pH 4.4	Growth at pH 9.6	Fermentation	Type of lactic acid
Aerococcaceae	*Aerococcus*	Cocci (tetrads)	-	+	-	+	-	-	+	Homo	L
Carnobacteriaceae	*Carnobacterium*	Rods	-	+	-	ND	-	ND	-	Homo	L
Enterococcaceae	*Enterococcus*	Cocci	-	+	+	+	-	+	+	Homo	L
	Vagococcus	Cocci	ND	+	-	-	-	ND	-	Homo	L
Lactobacillaceae	*Lactobacillus*	Rods	Variable	Variable	Variable	Variable	-	Variable	-	Homo, Hetero	D,L,DL
	Pediococcus	Cocci (tetrads)	-	Variable	Variable	Variable	-	+	-	Homo	L,DL
Leuconostocaceae	*Leuconostoc*	Cocci	+	+	-	Variable	-	Variable	-	Hetero	D
	Weisella	Cocci	+	+	-	Variable	-	Variable	-	Hetero	D,DL
Streptococcaceae	*Lactococcus*	Cocci	-	+	-	-	-	Variable	-	Homo	L
	Streptococcus	Cocci	-	-	Variable	-	-	-	-	Homo	L

ND, no data

Table 2.2 Habitats of species of LAB

Habitat	Source	Species	References
Gastrointestinal tract	Human	*L. ruminis, L. crispatus, L. gasseri, L. plantarum, L. acidophilus, L. delbrueckii* and *L. paracasei*	Vaughan *et al.,* 2005
	Poultry	*E. faecium*, *E. faecalis*, *S. gallolyticus*, *L. murinus*, *L. reuteri and L. ingluviei*	Nazef *et al.,* 2008
	Piglet	*L. sobrius*, *L. amylovorus*, *L. salivarius, L. johnsonii* and *L. gasseri*	Pieper *et al.,* 2008
	Cattle	*S. bovis*, *W. paramesenteroides, Lc. lactis* subsp *lactis, E. faecium*, *E. hirae*, *E. mundtii, L. mucosae*, *L. plantarum*, and *L. reuteri*	Espeche *et al.,* 2009
	Dog	*L. plantarum L. pentosus, L. murinus, L. reuteri, L. casei, L. fermentum, L. mucosae, L. rhamnosus, L. salivarius, P. acidilactici, W. cibaria* and *W. confusa*	Beasley *et al.,* 2006
Mucosal membranes:			
Oral cavity	Human	*L. gasseri*, *L. vaginalis*, *L. paracasei*, *S. intermedius, S. mitis, S. oralis, S. mutans*, *S. salivarius and S. gordonii*	Dal Bello & Hertel, 2006; Aas *et al.,* 2008
	Rat	*L. casei* and *S. mutans*	Michalek *et al.,* 1981
	Calf	*E. faecium*	Maldonado *et al.,* 2012
Vagina	Human	*L. crispatus, L. fermentum, L. gasseri, L. jensenii, L. reuteri, L. salivarius, L. vaginalis,* and *P. acidilactici*	Jin *et al.,* 2007
	Bovine	*L. fermentum* and *L. rhamnosus*	Otero *et al.,* 2006

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Table 2.2 Habitats of species of LAB (Continued)

Habitat	Source	Species	References
Fermented food:			
Fermented vegetable	Fermented olives	*Leuc. mesenteroides, L. plantarum*, *L. pentosus*, *L. paraplantarum* and *L. paracasei* subsp. *paracasei*	Argyri *et al.,* 2013
	Kimchi	*L. acidophilus*, *L. casei*, *L. plantarum*, *L. sakei* and *Leuc mesenteroides*	Chang *et al.,* 2010
	Fermented cassava	*L. plantarum, L. brevis, L. fermentum, L. pentosus* and *W. cibaria,*	Kostinek *et al.,* 2005; 2007
	Sauerkraut	*Leuc. mesenteroides, L. plantarum, P. pentosaceus*, *L. brevis, Leuc. citreum, Leuc. argentinum, L. paraplantarum, L. coryniformis*, and *Weissella* sp.	Plengvidhya *et al.,* 2007
Sourdough	Sourdough	*L. brevis*, *L. fermentum*, *L. paralimentarius*, *L. plantarum*, and *L. pontis L. frumenti*, *L. panis*, and *L. reuteri*.	De Vuyst & Vancanneyt, 2007
Fermented meat	Sausage	*L. plantarum*, *L. sakei*, *L. rhamnosus, L. curvatus, L. brevis* and *W. cibaria*	Drosinos *et al.,* 2007; Pringsulaka *et al.,* 2012
Fermented fish	Pla-som	*Lc. garvieae*, *S. bovis*, *W. cibaria*, *P. pentosaceus*, *L. plantarum*, and *L. fermentum*	Kopermsub & Yunchalard, 2010

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Table 2.2 Habitats of species of LAB (Continued)

Habitat	Source	Species	References
Dairy products	Cheese	*E. faecalis*, *Lc. lactis* subsp. *lactis*, *Leuc. mesenteroides, Leuc. pseudomesenteroides, L. paracasei and L. plantarum*	Gonzalez *et al.,* 2007; Nikolic *et al.,* 2008
	Fermented milk	*L. helveticus*, *L. kefiranofaciens, L. delbrueckii* subsp.*bulgaricus*, and *S. thermophiles*	Chammas *et al.,* 2006; Watanabe *et al.,* 2008; Thirabunyanon *et al.,* 2009
	Yoghurt	*L. acidophilus*, *L. delbrueckii* ssp. bulgaricus, and *S. thermophilus*	Gaus *et al.,* 2006
Silage	Corn stover	*L. plantarum*, *L. pentosus, L. brevis*, *Leuc. lactis*, *W. cibaria* and *E. mundtii*	Pang *et al.,* 2011
	Grass silage	*E. gallinarum*, *L. acidipiscis, L. curvatus,* Lc. *garvieae*, *L. coryniformis Lc. lactis* subsp. *cremoris*, *Leuc. pseudomesenteroides*, *P. acidilactici*, and *P. pentosaceus*,	Tohno *et al.,* 2012
Beverages	Wine	*L. buchneri, L. hilgardii, L. plantarum, L. paracasei, L. casei, L. collinoides, L. brevis, L. mali, P. parvulus, P. pentosaceus, Leuc. mesenteroides, O. oeni*	Landete *et al.,* 2005; Jin *et al.,* 2008
	Beer	*L. brevis*, *L. lindneri*, *P. damnosus*, *P. acidilactici, L. delbrueckii* ssp. *delbrueckii* and *L. delbrueckii* ssp. *bulgaricus*	Suzuki *et al.,* 2005; Sawadogo-Lingani *et al.,* 2007
Fruit	Fruit	*L. plantarum, L. agilis*, *L. bifermentans*, *L. hilgardii*, *L. fructosus*, *L. fermentum* and *Streptococcus* spp	Nyanga *et al.,* 2007

LAB can be divided into two groups, heterofermentative and homofermentative LAB based on metabolism (Figure 2.2). Homofermentative LAB are streptococci, enterococci, lactococci, pediococci homofermentative lactobacilli and *Sporolactobacillus*. They produce almost exclusively lactic acid more than 85% from glucose through Embden-Meyerhof-Parnas pathway (EMP pathway) by the splitting of fructose 1,6-bisphosphate with aldolase enzyme into two triose phosphate moieties which are further converted to lactate. This pathway generates 2 mol of lactic acid and 2 mol of ATP per one mole of glucose metabolized. This pathway produces more acid from a fixed amount of glucose than heterofermentative metabolism (Saeed A & Salam A, 2013). Heterofermentative LAB are *Weisella, Leuconostoc* and heterofermentative lactobacilli. They produce only 50% lactic acid and other byproduct such as CO_2, acetic acid and/or ethanol from glucose through phosphoketolase pathway which is initiated by the oxidation of glucose 6-phosphate to gluconate 6-phosphate followed by decarboxylation and splitting of the resulting pentose 5-phosphate into a C-2 and a C-3 moiety. One mole of glucose generates 1 mol of lactic acid, 1 mol of ethanol, 1 mol of CO_2 and 1 mol of ATP. The ratio of acetate and ethanol depend on the redox potential of the system (Saeed A & Salam A, 2013).

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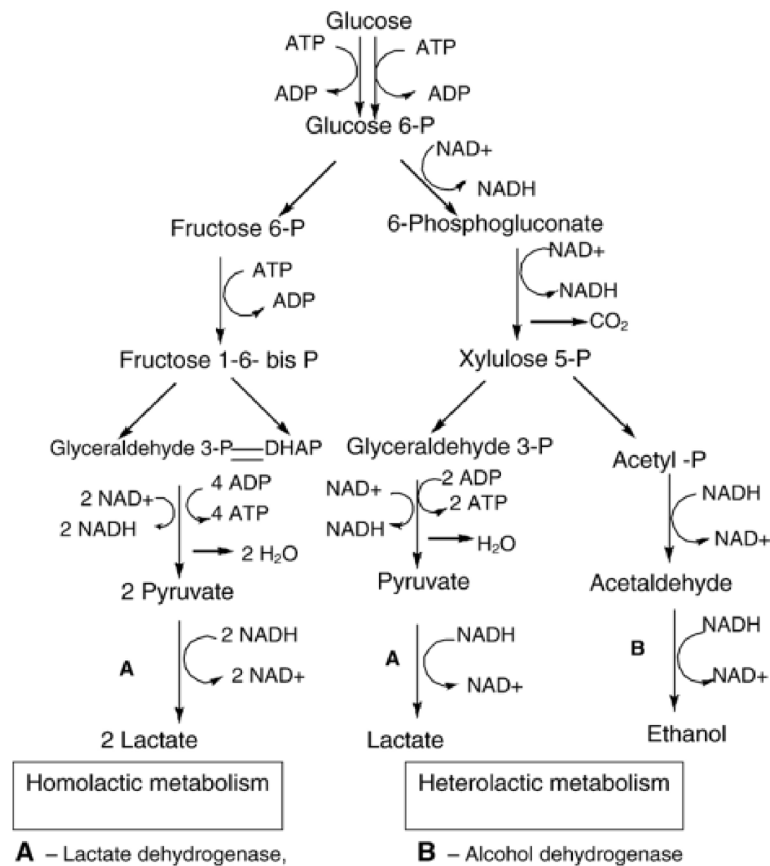

Figure 2.2 Metabolism of lactic acid bacteria (Reddy *et al.,* 2008)

Lactic acid (2-hydroxypropanoic acid) is one of the smallest organic active molecules. It is classified as Generally Recognized As Safe (GRAS) for use as a general purpose food additive by U.S. FDA. The chemical formula is $C_3H_6O_3$. There are two type of lactic acid stereoisomer, L(+) and D(-) form as shown in Figure 2.3 (Vaidya *et al.*, 2005).

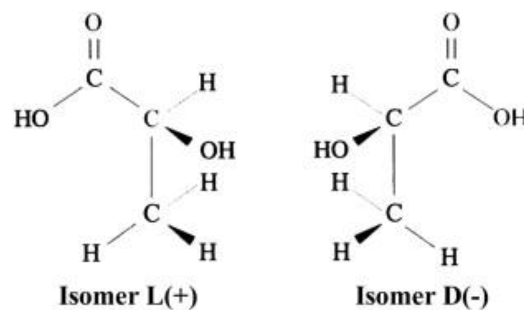

Figure 2.3 Lactic acid stereoisomers (Martinez *et al.,* 2013)

2.2 Lactic acid production

Lactic acid is manufactured either by chemical synthesis or microbial fermentations. It is the important organic acid produced by lactic acid bacteria (LAB) fermentation. Lactic acid fermentation by LAB can be resulting in optically stereoisomer L-, D- and DL-racemic mixture of lactic acid. While, chemical synthesis can be resulting in particularly racemic DL-lactic acid as shown in Figure 2.4 (Wee *et al.*, 2006). Raw materials such as whey, molasses, starch waste, beet, cane sugar and other carbohydrate rich materials are used as renewable resources for lactic acid production whereas chemical synthesis derived from petrochemicals that are expensive cost. It’s not surprising that production of lactic acid by microbial fermentation have been advantage over chemical synthesis. (Reddy *et al.*, 2008).

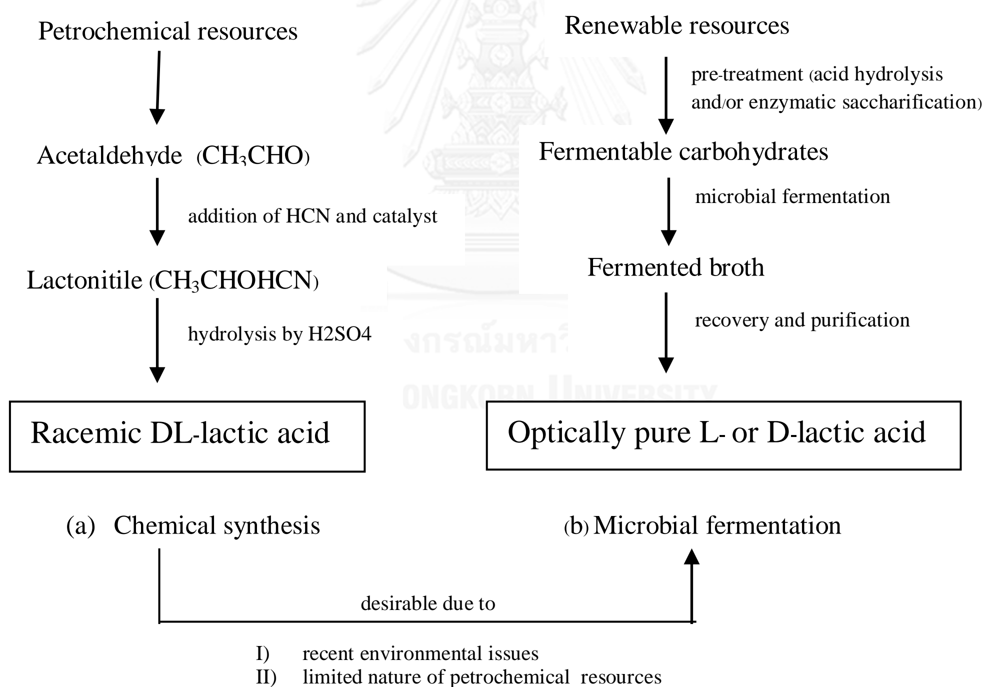

Figure 2.4 Overview of the two manufacturing methods of lactic acid; (a) chemical synthesis and (b) microbial fermentation (Wee *et al.,* 2006)

Currently, lactic acid is applied in a wide variety of industrial applications such as food, beverage, medical, pharmaceutical, leather and textile industries as well as chemical feed stock etc. The isomer L-lactic acid is the preferred useful in food and pharmaceutical industries because humans have particularly L-lactate dehydrogenase that can metabolizes only L-lactic acid (Reddy *et al.,* 2008).

Lactic acid has long been used in food and beverage industries. Most lactic acid is used as emulsifying agent in baking foods, especially esters of lactic acid such as stearoyl-2-lactylate, glyceryl lactostearate, glyceryl lactopalmitate. It is also used as flavoring agent or inhibitor of bacterial spoilage in a many process of foods such as candy, breads, soft drinks, soups, sherbets, dairy products, beer, jams and mayonnaise (John *et al.*, 2007).

It is also an advantage in medical and pharmaceutical applications such as topical ointments, lotions, humectants. Calcium lactate can be used as anti-caries agent and for calcium deficiency therapy. For medical applications, lactic acid based polymers (PLA) can be used as sutures, orthopedic implants and drug delivery systems. These polymers are biodegradable thermoplastics, transparent and their degradation can be controlled by adjusting the isomeric composition, and molecular weight. Poly L-lactic acid can help in controlled release drug and also is approved for restore facial lipoatrophy in HIV patient and non-HIV to correction of nasolabial folds and other facial wrinkles (Södergård & Stolt, 2002; Datta & Henry, 2006; Palm *et al.*, 2010). For pharmaceutical formulations, the water retaining capacity of lactate is used as moisturizer in cosmetic formulations. Moreover, lactic acid is used as skin-lightening associated with the suppression of enzyme tyrosinase. Ethyl lactate is the active ingredient in many anti-acne preparations (Wee *et al.*, 2006).

Lactic acid is used as an acidulant for deliming hides and in vegetable tanning in leather tanning industries. It is also used as solvent, descaling agent, cleaning agent and humectants in a variety of processes. Moreover, it is the most important feedstock monomer for chemical conversions to potentially useful chemicals such as propionic acid, acetic acid, acrylic acid (Datta & Henry, 2006).

In Bioplastic industries, lactic acid is used as a raw material for synthesis of biodegradable plastic, polylactic acid (PLA) sometimes called polylactide. PLA is an interesting polymer because it can be derived from renewable resources and provides a biodegradable polymers alternative to synthetic ones derived from petrochemical sources. The replacement of existing synthetic polymers by biodegradable polymers is also significantly decrease waste disposal problems. The lactic acid polymers have many advantages properties such as biodegradability, thermo plasticity, high tensile strength (Rasal *et al.*, 2010). The physical properties of PLA depend on the composition isomers, especially optically pure lactic acid (Lim *et al.*, 2008). Commercial PLA are usually synthesized from lactide monomers, the cyclic dimer of lactic acid. Polymerization of L-lactide results in poly (L-lactide) (PLLA) and polymerization of D-lactide results in poly (D-lactide) (PDLA). PLLA has a crystallinity of around 37%, a glass transition temperature ~ 67 °C, a melting temperature between 173-178 °C and sensitive to heat, especially at temperature higher than 190°C. However, the melting temperature of PLLA can be increased 40-50 °C and its heat resistant temperature can be increased up to 60°C by physically blending the polymer with PDLA. The mixture of PDLA and PLLA has a highly regular stereocomplex with increased crystallinity. Maximum stability temperature is occurred when blending 1:1 ratio of PLLA and PDLA. They exhibit highly thermostability, slow degradation, highly strength with high modulus that suitable for synthesis polymer carried out at not less than 200 °C. They are used in many applications such as medical products in orthopedic fixation (pins, rods, ligaments etc.), cardiovascular applications (stents, grafts etc.), dental applications, intestinal applications, sutures and matrices for drug delivery (Södergård & Stolt, 2002; H. Li & Huneault, 2007; Nampoothiri *et al.*, 2010).

The majority of current commercial PLA is poly (meso-lactide), which is a mix of L-lactide (> 95%) and D-lactide (<5%). Poly (meso-lactide) can be used in a wide range of applications, such as film- and tray- packaging, bottle packaging. This type of PLA is no stereochemical structure. It is highly amorphous, does not rotate polarized light (L. Shen *et al.*, 2010). Properties of PLA depend on the component isomers,

processing temperature, annealing time and molecular weight. Polylactide is a clear, colorless thermoplastic, many respects is similar to polystyrene. PLA can be processed like most thermoplastics into fiber and film. In general, PLA are soluble in dioxane, acetonitrile, chloroform, methylene chloride, 1,1,2-trichloroethane and dichloroacetic acid. Ethyl benzene, toluene, acetone and tetrahydrofuran only partly dissolve polylactides when cold, though they are readily soluble in these solvents when heated to boiling temperatures. All PLA are insoluble in water, some alcohols and alkanes (Nampoothiri *et al.,* 2010).

Furthermore, lactate is the end-products of bacterial fermentative reactions in the colon of human. They were produced as minor but significant substance. They have been effect on many different physiological processes including regulation of denovo lipogenesis and cholesterol biogenesis in the liver, acting as an energy source for human cells in the intestine and in other organs including muscle, brain, heart and liver, regulating production of gut hormones involved in satiety, impacting on fat storage and adipocyte leptin and adipokine production, and possibly impacting on thermogenesis in muscle (Scheppach, 1994; Q. Shen *et al.*, 2012). Other significant end products of carbohydrate fermentation in colon are acetate, propionate, butyrate, ethanol, succinate, formate, valerate and caproate. (Salminen *et al.*, 1998).

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2.3 Parameters of lactic acid fermentation

The effectiveness of a fermentation process of lactic acid production depends upon many parameters such as microorganism, nutrient sources, fermentation process, temperature, and pH etc. and can be measured as the concentrations of lactic acid produced, lactic acid yield and productivity. Adjusting several parameters could improve lactic acid concentrations, yields, productivity and optical purity of lactic acid, or provide fast fermentation rate, low cost, reduction of substrates nutrition and avoid byproduct formation (Hofvendahl & Hahn–Hägerdal, 2000; Abdel-Rahman *et al.*, 2013).

- Microorganisms

Lactic acid can be produced by several microorganisms such as bacteria, fungi, yeast, cyanobacteria and algae. Lactic acid bacteria (LAB) are well known as efficient lactic acid producers, especially in the genus *Lactobacillus* and *Streptococcus* which were interesting more than other microorganisms. They metabolized glucose via EMP pathway or phosphoketolase pathway to produce lactic acid as mentioned earlier and so called homofermentative and heterofermentative LAB, respectively. In industries, lactic acid production is favorable used homofermentative LAB because they produce lactic acid as the major end product. The preferred homofermentative LAB are *Lactobacillus delbkueckii* subsp. *lactis*, *L. delbrueckii* spp. *bulgaricus*, *L. helveticus*, *L. acidophilus, L. amylophilus, L. amylovorus, Streptococcus thermophilus, Lactococcus lactis*, *L. casei*, *L. paracasei, L. pentosus, L. plantarum and L. rhamnosus* have also been reported (Nolasco-Hipolito *et al.*, 2002; Tango & Ghaly, 2002; G. Bustos *et al.*, 2004; Rojan *et al.*, 2005; John *et al.*, 2006). Recently, *Sporolactobacillus* is increasing interest in lactic acid production. They are homofermentative LAB that have the ability to produce an optical purity D-lactic acid. For some applications, such as PLA synthesis, a racemic mixture of composition or an optically pure product is desirable. *Sporolactobacillus inulinus* sp. strain CASD produces high D-lactic acid concentrations with high productivity and optical purity (Wang *et al.,* 2011). This strain also produces D-lactic acid increasingly when using two-reactor repeated batch fermentation compared with one-reactor system (Zhao *et al.*, 2010). Combination of strains in fermentation may enhance lactic acid production because of several metabolic pathways for the utilization of substances (Cui *et al.*, 2011). Sometimes genetic engineering can be used to alter the properties of microorganism resulted in better lactic acid production (Okano *et al.*, 2009).

- Nutrient sources

LAB require a complex nutrition such as carbohydrates, sugar, peptides, amino acids, vitamins and minerals. Carbon and nitrogen sources are major factors which impact economic production of lactic acid. Refined sugars have been a traditional substrate for lactic acid production. They provided pure lactic acid product that is advantageous in lower purification costs as well as pretreatment cost of substrate. However, extensive studies in nowadays are trendy to use renewable resources such as byproducts from agriculture, food industries, and natural unutilized biomasses. This reason is because of refined sugars are expensive resulting in higher scale-up cost (Abdel-Rahman, *et al.,* 2013). Refined sugars usually used such as glucose, sucrose, lactose and maltose. Comparison of various carbon have been exhibited that glucose provided higher lactic acid concentrations and yields compared with other sugars. Xylose, galactose, arabinose, lactose, and fructose were less effective (Hofvendahl & Hahn–Hägerdal, 2000; Vaidya *et al.*, 2005). Nitrogen is also an essential source for microbial growth and lactic acid production. Peptone and yeast extract are significant nitrogen sources for lactic acid production. Alternative nitrogen supplement might increase incubation times or provided low lactic acid product. Yeast extract alone at high concentrations gave higher lactic acid production than low amounts of yeast extract and peptone (Altaf *et al.*, 2007).

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- pH

The fermentation processes are associated with several factors. pH is one of the major factors in the production of lactic acid. Although LAB is known to tolerate lower pH, the optimal pH for lactic acid production with high productivity is narrow range, preferably between pH 5.5 to 6.5. In fermentation process, pH is set at the beginning and then left to decrease due to acid production. The pH value is needed to maintain at a constant during fermentation. Neutralizing agents such as $CaCO_3$, NaOH, $Ca(OH)_2$, $Mg(OH)_2$ or NH_4OH were used to control the pH. Maintain pH at

constant gave higher lactic acid concentrations, yield and productivity compared with uncontrolled pH (Martinez *et al.*, 2013).

- Temperature

The temperature had effect on the bacterial growth and lactic acid production. The optimal temperature growth for mesophilic and thermophilic LAB had difference. Selected undesirable temperature may affect the lactic acid production and yield. For example, *L. delbrueckii* is a mesophilic bacteria, which have optimum temperature growth between 20 and 40 °C and thus the most efficient lactic acid production at 37°C and highest lactic acid concentrations was obtained at same temperature. Lactic acid production will be decreased when temperature below 30°C or over 40°C (Idris & Suzana, 2006). Also, *Lc. lactis* and *L. rhamnosus* showed the highest production at 37 °C. Some strains such as *L. amylophilus*, which is known to grow at 15°C but not at 45°C, gave the highest production and yield at 35°C. In some cases, same temperature gave the high lactic acid concentrations, yield and productivity while some cases did not. However, the preferred LAB was used in several industrial process should be able to produce high lactic acid at higher temperatures in order to reduced contamination risk during fermentation (Hofvendahl & Hahn-Hägerdal, 2000).

- Fermentation process

Selection of fermentation process depends on the respect to the type of substrate, microbial growth, and viscosity of fermentation broth. The process of fermentations include batch, fed-batch, repeated or semicontinuous and continuous fermentation. Each operation has different advantages and disadvantages. Batch fermentation is advantages in simple operation, high product concentrations, reduced risk of contamination but low productivity and might be substrate and/or end product inhibition. Fed-batch fermentation has advantages in solving substrate inhibition problem, providing high product concentrations but still occur end product

inhibition and quite difficult to conduct optimal design. Repeated batch fermentation was advantages in saving labor and time processes, achieving high growth rates but requirement of special devices. Continuous fermentation has advantages in high productivity and can control growth rates but utilization of the carbon source is incomplete (Abdel-Rahman, *et al.,* 2013). If the substrate is expensive, batch or repeated batch fermentation is appropriate because they provided the maximum yield. If investment costs are high, continuous operation is considered because of the maximum productivity. High productivity is obtained by recycling the cells that provide a high cell density without reducing the yield. Whereas, cell immobilization provides high cell density as well as improvement of both productivity and yield (John *et al.,* 2007).

- Optical purity

Lactic acid can be produced as in form of L(+) and D(-) or DL-racemic mixture of lactic acid. The types of stereoisomer depend on process either chemical synthesis or microbial fermentation as mentioned in earlier. In LAB fermentation, lactate dehydrogenase (LDH) is an important enzyme which determines the stereoisomer of the lactic acid (Zheng *et al*., 2012). L- lactate dehydrogenase L(+)-LDH and D- lactate dehydrogenase D(-)-LDH, which specific to produce L (+) form or D (-) form lactic acid or its DL-racemic mixture. Few species provide DL-lactate by using lactate racemase enzyme. *L. curvatus, L. paracasei, L. plantarum* WCFS1 *and L. sakei* 23k have been reported about lactate racemase. *L. casei, L. amylophilus, L. rhamnosus* and *L. salivarius* have especially L- lactate dehydrogenase produced L (+) lactic acid. *L. delbrueckii* subsp. *Bulgaricus, L. lactis, L. coryniformis, Leuconostoc* and *Sporolactobacillus* have also especially D- lactate dehydrogenase produced D (-) lactic acid. *L. helveticus* and *L. plantarum* have two enzymes produce a racemic mixture (Garvie, 1980; Zheng *et al.*, 2012). Strains, substrate, pH, temperature and aeration had influence on the isomer purity of lactic acid in the microbial fermentation. Zhang *et al.* (2008) reported that the variation of the isomer were found when pH, temperature and fermentation time were changed.

The effect of fermentation parameters on the lactic acid production are shown in Table 2.3

Table 2.3 Effect of fermentation parameters on the lactic acid production

Microorganisms	Type	Substrate	Fermentation mode	LA produced (g/L)	Isomer	Optical purity (%ee)	Yield	Productivity (g/L/h)	References
L. manihotivorans LMG 18010	Wild type	Starch	Batch	12.6	L	98.0	0.67	0.50	Guyot *et al.*, 2000
Enterococcus faecalis RKY1	Wild type	Glucose	Batch	143.8	L	99.0	0.96	5.14	Wee *et al.*, 2003
L. plantarum NCIMB 8826	Wild type	Glucose	Batch	86.0	D/L	12.6	0.89	4.51	Okano *et al.*, 2009
L. plantarum ΔldhL1	Mutant	Glucose	Batch	86.6	D	99.7	0.89	4.54	
L. paracasei subsp. *paracasei* CHB2121	Wild type	Glucose	Batch	94.8	L	96.6	0.93	4.46	Moon *et al.*, 2012
L. coryniformis sub. torquens ATCC 25600	Wild type	Microalgae	SSF + Batch	36.6	D	95.8-99.6	0.46	1.02	Nguyen *et al.*, 2012
L. delbrueckii subsp. *lactic* QU41	Wild type	Glucose	Batch	87.4	D	99.9	1.01	0.52	Tashiro *et al.*, 2011
S. inulinus ATCC15538	Wild type	Glucose	Flask	38.9	D	-	0.64	0.57	Zheng *et al.*, 2010
S. inulinus F3-4	Mutant (Genome shuffling)	Glucose	Flask	93.4	D	-	0.95	1.37	
Sporolactobacillus sp. DX12	Wild type	Glucose	Flask	40.7	D	96	-	-	Xu *et al.*, 2010
Sporolactobacillus sp. Y2-8	Mutant (Nitrogen ion beam implantation)	Glucose	Flask	120-143.6	D	99.08	-	-	

SSF = Simultaneous saccharification and fermentation; LA = Lactic acid concentration

Table 2.3 Effect of fermentation parameters on the lactic acid production (Continued)

Microorganisms	Type	Substrate	Fermentation mode	LA produced (g/L)	Isomer	Optical purity (%ee)	Yield	Productivity (g/L/h)	References
S. inulinus Y2-8	Mutant	Glucose	Batch	120	D	99.1	ND	1.0	Zheng *et al.*, 2012
S.laevolacticus JCM2513	Wild type	Sugarcane	Cont	67	D	99.8	0.96	12.2	
S. inulinus JCM6014	Wild type	Sugarcane	Cont	64	D	98.8	0.96	8.9	Sawai *et al.*, 2011
S. terrae ST316	Wild type	Sugarcane	Cont	62	D	85	-	5.9	
S. inulinus CASD	Wild type	Peanut + Glucose	Fed batch	207	D	99.3	0.93	3.8	Wang *et al.*, 2011; Yu *et al.*, 2011
S. inulinus CASD	Wild type	Glucose	Rep batch	79.2-87.3	D	-	0.94-0.99	-	Zhao *et al.*, 2010; Yu *et al.*, 2011
S. leavolacticus DSM442	Wild type	Glucose	Fed batch	144.4	D	99.3	0.96	4.13	Li *et al.*, 2013
S. inulinus Y2-8	Mutant	Glucose	Flask	104.11	D	-	0.77	0.62	Zheng *et al.*, 2014
		Glucose +$MnSO_4$	Flask	115.07	D	-	0.81	0.68	
		Glucose + KH_2PO_4	Flask	68.91	D	-	0.62	0.41	
Sporolactobacillus sp. YBS1-5	Mutant (ARTP breeding method)	Glucose	Flask	125	D	99	-	1.39	Sun *et al.*, 2014
			Fed batch	127	D	-	-	1.72	
S.laevolacticus SK5-2	Wild type	Glucose	Flask	112.45	D	98.79	0.94	1.56	Prasirtsak, 2011

ARTP = Atmospheric and room temperature plasma; Rep batch = Repeated batch; Cont = Continuous fermentation; LA = Lactic acid concentration

2.4 Probiotics

- Definition of probiotics

The root of the word probiotic is derived from the Greek language. The word pro is meaning "for" and biotic, meaning "life”, probiotic, meaning “for life”. In 1965, Lilly and Stillwell were the first to propose the term probiotic to describe “substances secreted by one microorganism that stimulates the growth of another microorganism” and that was contrasted with the word antibiotic. In 1974, Parker improved the meaning of the probiotic by adding the word “organisms” to the definition, explained probiotics as “Organisms and substances which have a beneficial effect on the host by contributing to its intestinal microbial balance”. The definition was greatly improved by Fuller in 1989, described probiotic as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". This definition was very close to that it is used today. They emphasized the viability of microorganisms and have a beneficial effect on host. There were other definitions of probiotic in later, mostly broadened the definition of probiotics. However, some aspects of the proposed definition are not justified. The term probiotics which generally accepted was described by FAO/WHO whose defined probiotics as “live microorganisms, which, when consumed in adequate amounts, confer a health benefit on the host” (Schrezenmeir & de Vrese, 2001). In fact, early the term probiotic was established, probiotics have long been used in fermented foods. Metchnikoff in 1907 was the first to suggest that ingested bacteria, in the form of yogurt and other fermented foods, could have a benefit effect on the gastrointestinal health. He believed that lactobacilli involved human health and longevity however his theories of healthfulness and longevity through consumption of lactobacilli were disputed because of unexpected consumer and insufficient scientific based evidence (Guarner *et al.*, 2008).

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- Criteria for probiotic bacteria selection

Many criteria for selection of probiotics bacteria that have been shown in Table 2.4. However, the important criteria generally based on some specific activity in nutritional or health benefit, viability and safety used. According to viability, desirable characteristics include the ability to survive transit through the stomach and small intestine, and also can colonize in the human gastrointestinal tract. Other characteristics might be survival during manufacturing. The assessment of viable probiotic *in vitro* tests was evaluated by acid and bile tolerance and intestinal epithelial adhesion. According to safety, acceptable criteria include non-pathogenic, non-toxic, generally recognized as safe (GRAS) microorganism and should be normal inhabitant for the target purposes such as human origin for human probiotics. Other parameters were assessed within under laboratory that effecting of beneficial health such as production of antimicrobial substances, inhibition of enteric pathogens, antibiotic resistance, stimulation of immune responses or ability to influence metabolic activities e.g. cholesterol assimilation, lactase activity, and vitamin production (Dunne *et al.*, 2001; Tuomola *et al.*, 2001; Harish & Varghese, 2006; Gaggia *et al.*, 2010).

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Table 2.4 Expected characteristics and safety criteria of probiotics

Appropriateness	
i.	Accurate taxonomic identification
ii.	Normal inhabitant of the species targeted
iii.	Nontoxic, nonpathogenic
Technological suitability	
iv.	Amenable to mass production and storage: adequate growth, recovery, concentrations, freezing, dehydration, storage, and distribution
v.	Viability at high populations (preferred at 10^{6} –10^{8})
vi.	Stability of desired characteristics during culture preparation, storage, and delivery
vii.	Provides desirable organoleptic qualities (or no undesirable qualities)
viii.	Genetically stable
ix.	Genetically amenable
Competitiveness	
x.	Survival, colonization and being metabolically active in the targeted site
xi.	Resistant to bile
xii.	Resistant to acid
xiii.	Competition with the resident microbiota
xiv.	Adhesion to epithelium or mucus
Performance and functionality	
xv	Ability to exert at least one scientifically-supported health-promoting properties
xvi.	Antagonistic toward pathogenic bacteria
xvii.	Production of antimicrobial substances
xviii.	Modulation of immune responses
xix.	Antimutagenic
xx.	Anticarcinogenic
xxi.	Production of bioactive compounds

(Modified from Klaenhammer & Kullen, 1999; Gaggia *et al.,* 2010)

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- Microorganisms considered as probiotics

The most interested microorganisms used as probiotics are lactic acid bacteria (LAB), especially in genus *Lactobacillus* and *Bifidobacterium*. Other genera of LAB used as probiotics including genus *Streptococcus*, *Pediococcus*, *Enterococcus*. Moreover, some fungi and yeast such as *Saccharomyces* sp., *Aspergillus* sp. and *Torulopsis* sp. have also been used as exhibit in Table 2.5 (Holzapfel *et al.*, 2001; Kailasapathy, 2013). Probiotic preparations might contain one species or combination of various microorganisms, particularly *Lactobacillus* that are component in fermented dairy products. *Lactobacillus* and *Bifidobacterium* have been greatly interested because of their possession of intestinal normal flora and perception of GRAS status. The beneficial effects of *Lactobacillus* and *Bifidobacterium* have been several investigated and achieved well-documentation with positive effect on human health (Liévin-Le Moal & Servin, 2014; Tojo *et al.*, 2014; Patten & Laws, 2015; Toiviainen *et al.*, 2015). Also, Bacterial probiotics usually used for animal include strains of the *Lactobacillus, Bacillus, Pediococcus, Enterococcus* and yeasts. They are used to improve animal health, promote growth rates and increased production of milk and eggs (Bernardeau & Vernoux, 2013).

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Table 2.5 Microorganisms considered as probiotics

	Species
Lactobacillus	*L. acidophilus**, *L. plantarum**, *L. casei**, *L. paracasei, L. reuteri**, *L. amylovorus**, *L. gallinarum**, *L. gasseri, L. crispatus**, *L. rhamnosus**, *L. brevis**, *L. delbreuckii* subspecies *bulgaricus, L. fermentum**, *L. helveticus* and *L. johnsonii*
Bifidobacterium	*B. lacti***s, B. bifidum, B. longum**, *B. infantis, B. breve*, *B. animalis**, *B. thermophilum**, *B. essensis, B. laterosporus* and *B. adolescentis*
Other lactic acid bacteria	*Enterococcus faecalis**, *E. faecium**, *Lactococcus lactis**, *Leuconstoc mesenteroides**, *Pediococcus acidilactici**, *P. pentosaceus**, *Streptococcus thermophilus Streptococcus cremoris, S. diacetylactis, S. intermedius,* and *S. salivarius*
Non-lactic acid bacteria	*Bacillus cereus* var. *toyoi**, *Escherichia coli* strain *nissle, Saccharomyces cerevisiae**, *Saccharomyces boulardii* and *Propionibacterium freudeneichii**

*Main application for animals

(Modified from Holzapfel *et al.,* 2001; Ouwehand *et al.,* 2002; Gaggìa *et al.,* 2010; Kailasapathy, K., 2013)

- Health benefits of probiotics

Due to the incidence of illnesses that may be caused by deficiency of intestinal normal flora and the antibiotic resistance in microorganisms has been become increasingly, alternative strategies need to be developed for management. One of these is using the potential probiotic therapy to improve the numbers and activities of normal flora promoting good health properties (Rolfe, 2000). Probiotics have been numerous investigated for their benefit in prophylaxis and therapeutic several diseases (Table 2.6). The effectiveness of probiotics is based on the knowledge that normal flora can protect humans against infection and disturbance of this flora can increase susceptibility to diseases. Role of probiotics include improvement of intestinal health, enhancement of the immune response, and cancer prevention. They prevent gastrointestinal tract infections caused by rotavirus,

enterotoxigenic *E. coli*, *Shigella*, *Salmonella*, *Vibrio cholera*, antibiotic- associated diarrhea including *Clostridium difficile*–associated disease, *Helicobacter pylori* gastroenteritis, irritable bowel syndrome, inflammatory bowel disease including Crohn's disease and ulcerative colitis (Rupa & Mine, 2012). Probiotics are recommended for clinical prevention and therapeutic uses based on the level of efficacy outcome as shown in Table 2.7.

Table 2.6 Clinical effects of some probiotic bacteria

Species	Clinical effects	References
P. acidilactici	- Reduced incidence of diarrhea - Inhibited the growth of tumor-like germ cells - Modulate intestinal bacterial communities - Stimulate some aspects of the nonspecific immune response - Suppress autoimmune encephalomyelitis by inducing IL-10-producing regulatory T cells	Casey *et al.,* 2007 Ferguson *et al.,* 2010 Takata *et al.,* 2011
L. acidophilus	- Immune enhancing - Balancing of intestinal microflora - Reduce risk of Antibiotic-Associated Diarrhea and *Clostridium difficile*-	Gao *et al.,* 2009 Leyer *et al.,* 2009

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Species	Clinical effects	References
	Associated Diarrhea in Adult Patients - Reduce fever, rhinorrhea, and cough incidence and duration	
L. delbrueckii	- Stimulation of immune system - Resistance to *Listeria monocytogenes* infection	Dos Santos *et al.,* 2011
L. johnsonii	- Reduced *E. coli* O78K80 and *C. perfringens* colonization - Modulation of intestinal microflora - Immune enhancement - Adjuvant in *Helicobacter pylori* treatment	La Ragione *et al.,* 2004
S. thermophilus	- Modulate the immune system via induced anti-inflammatory IL-10 production - Protective effect against oxidative stress	Latvala *et al.,* 2011 Wang *et al.,* 2015
L. plantarum	- Modulation of immunity - Reducing the level of LDL-cholesterol - Antimicrobial activity - Anti-oxidative stress - Induction of natural killer activity against cancer cells	Horinaka *et al.,* 2011 Mikelsaar *et al.,* 2014
L. rhamnosus GG	- Reduction of antibiotic-	X.-Q. Li *et al.,* 2012

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Species	Clinical effects	References
	associated diarrhea - Treatment and prevention of rotavirus and acute diarrhea - Immune response modulation - Preventation of colorectal carcinogenesis	Escamilla *et al.,* 2012
L. reuteri	- Reduced rotavirus diarrhea in children - Treatment of acute diarrhea in children - Prevention of Hypercholesterolemia	Francavilla *et al.,* 2012 Azevedo *et al.,* 2012 Jones *et al.,* 2012
L. casei Shirota	- Reduced respiratory symptoms - Increases natural killer cell activity in smokers	Reale *et al.,* 2012 Van Puyenbroeck *et al.,* 2012

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Table 2.7 Recommendations for Probiotic Use

Clinical Condition	Effectiveness	Strain	Dose
Diarrhea			
Infectious childhood—treatment	A	*S. boulardii*	10^{9} ufc × 3/d
		LGG	10^{10} –10^{11} ufc
		L. reuteri SD2112	10^{10} –10^{11} ufc × 2/d
Prevention of infection	B	*S. boulardii, LGG*	
Prevention of AAD	A	*S. boulardii*	10^{9} ufc × 2/d or 3 × 10^{10} ufc × 1/d
		LGG	10^{10} –10^{11} ufc × 1-2/d
		Combination of *L. casei* DN114 G01+ *L. bulgaricus* + *S. thermophilus*	10^{10} ufc × 2/d
Prevention of recurrent CDAD	B/C	*S. boulardii, LGG*	
Prevention of CDAD	B/C	*LGG, S. boulardii*	
Inflammatory bowel disease (IBD)			
Pouchitis			
Preventing and maintaining remission	A	VSL#3	4.5 × 10^{11} ufc × 2/d
Induce remission	C	VSL#3	
Ulcerative colitis			
Inducing remission	B	*E. coli* Nissle, VSL#3	
Maintenance	A	*E. coli* Nissle	5 × 10^{10} × 2/d
		VSL#3	

Clinical Condition	Effectiveness	Strain	Dose
Crohn's	C	*E. coli* Nissle, *S. boulardii*, *LGG*	
Irritable bowel syndrome (IBS)	B	*B. infantis* B5624	4.5×10^{11} ufc $\times$ 2/d
	C	VSL#3 *B. animalis*, *L. plantarum* 299V	
Necrotizing Enterocolitis	B	*L. acidophilus* NCDO1748	10^{9} ufc $\times$ 2/d
		B. bifidium NCDO1453	3.5×10^{8} ufc x 1/d
Immune Response	A	*LGG*, *L. acidophilus* LAFT1, *L. plantarum*, *B. lactis*, *L. johnsonii*	
Allergy			
Atopic eczema associated with cow's milk allergy Treatment	A	*LGG*, *B. lactis*	
Prevention	A	*LGG*, *B. lactis*	
Radiation Enteritis			
	C	VSL#3, *L. acidophilus*	
Vaginosis and Vaginitis			
	C	*L. acidophilus*, *L. rhamnosus* GR-1, *L. reuteri* RC14	

AAD indicates antibiotic-associated diarrhea; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; CDAD, *Clostridium difficile*-associated diarrhea; LGG, *Lactobacillus rhamnosus* GG; VSL#3 including *L. casei, L. plantarum, L. acidophilus, L. delbrueckii* subsp. *bulgaricus, B. longum, B. breve, B. infantis* and *S. salivarius* subsp. *thermophilus*. "A" recommendation is based on strong, positive, well-conducted, controlled studies in the primary literature, not abstract form. "B" recommendation is based on positive, controlled studies but the presence of some negative studies. "C" recommendation is based on some positive studies but clearly an inadequate amount of work to establish the certainty of "A" or "B". (Modified from Aureli *et al.,* 2011; Floch *et al.,* 2011)

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Immune response

Roles of probiotics on the immune response are supported by several reviewed reports (McCracken *et al.*, 1999; Macfarlane & Cummings, 2002; McNaught *et al.*, 2005; Madsen, 2006). They suggested that probiotics are useful in enhancing the immune response *in vitro*, animal and human studies. The effect of probiotics on immune responses can be influenced by strains-specific probiotic mediated through several aspects of specific and nonspecific immune responses, involving both B (humoral immunity) and T lymphocytes (cell-mediated immunity) (Parvez *et al.*, 2006). The intrinsic properties of probiotics to modulate the immune system such as activating macrophages, increasing natural killer cell activity, increasing cytokines excretion, and increasing immunoglobulins-secreting cells (Perdigón *et al.*, 1991; Ouwehand *et al.*, 2002). These abilities to stimulate immune responses are useful in very specific diseases, for example, immunodeficiency diseases (e.g. cancer, AIDS and leukemia), autoimmune diseases (e.g. allergies and rheumatoid arthritis) and upper respiratory tract infections. LAB showed positive results especially in prevention and reduction of the severity of respiratory infections, atopic eczema and dermatitis. The activity of prevention of respiratory infections is due to increasing IgA-secreting cells in the bronchial mucosa. In regular smokers, positive effects were also found by reduced natural killer cells (NK cells) activity (Aureli *et al.*, 2011; Quinto *et al.*, 2014). In addition, probiotics might improve the immunogenicity by adjuvant with orally administered vaccines such as rotavirus, polio, cholera and influenza (Quinto *et al.,* 2014). Probiotics that have been reported as having positive effect on immune response are *L. casei* Shirota, *L. rhamnosus* GG, *L. acidophilus*, *L. plantarum*, *L. johnsonii* and *Bifidobacterium lactis*. However, the immune response might enhance when administered of two or more probiotics such as administered with combination of *Lactobacillus* and *Bifidobacteria* (Cunningham-Rundles *et al.*, 2000). *L. casei* Shirota and *L. rhamnosus* GG have been well-documented effective to enhance the host defense by induce Interleukin-12 (IL-12) cytokine (Heufler *et al.*, 1996; Christensen *et al.*, 2002; Shida *et al.*, 2011). It is a potential Th-1-associated cytokine, produced primarily by antigen-presenting cells such as macrophage and dendritic

cells. They enhance cell-mediated immunity through modulation of cytotoxic T lymphocytes cells (CTLs) and NK cells (Christensen *et al.*, 2002). A key role of IL-12 is the induction of IFN-γ which displays an important function against viral infection (Romani *et al.*, 1997). Recent studies showed another functions of IL-12 with involving in antitumor activity (Colombo & Trinchieri, 2002; Trinchieri, 2003; Kortylewski *et al.*, 2009). The ability to suppress tumor growth may associate with induction of Major histocompatibility complex (MHC) expression on tumor cells, suppression of tumor angiogenesis and stimulation of cytotoxic activity (Trinchieri, 2003). The anti-tumor activity of IL-12 has been demonstrated in several murine tumor models (Brunda *et al.*, 1993; Schultz *et al.*, 2000; Elzaouk *et al.*, 2006). They showed the inhibition of local tumor growth as well as prevention of metastasis formation (Elzaouk *et al.*, 2006). Probiotics *L. casei* Shirota and *L. plantarum* exhibited the properties of antitumor by impaired IL-12 response (Murosaki *et al.*, 2000). Wang *et al.,* (2014) reported that *L. plantarum* 70810 possessed strong antitumor activity in colon cancer cell.

Cancer

Cancer is one of the major leading causes of morbidity and mortality in worldwide. They are characterized by an abnormal growth of cells with rapidly divide, invade and/or spread throughout the body and thus healthy organ damage. There are many types of cancer upon to a part of organs including cervical, colorectal, breast, liver, lung, prostate cancer and also blood cancer that are mostly affect white blood cells abnormally called leukemia. Leukemia is the fifth leading cause of cancer mortality in male and the sixth of that in female in US. Colorectal cancer is the third prevalence, mortality and incident of cancer subordinated of prostate, lung cancer in male and breast, lung cancer in women in US (Siegel *et al.*, 2014). In Thailand, leukemia is the sixth leading cause of cancer in male and few incidents in female (Figure 2.5). The statistics of colorectal cancer is quite no different among Thailand and US, the third and the fifth incidence of cancer in male and female, respectively (Figure 2.6) (Khuhaprema, 2012). Diet consumption may lead a

contribution of colorectal cancer risk. Evidence also supports that the colonic microflora are involved in the etiology of colorectal cancer (Fotiadis *et al.*, 2008). Lactic acid bacteria and their substances have many beneficial effects in the cancer. Studies on the effect of LAB consumption indicated that LAB might reduce the risk, incidence and number of colorectal cancer (Rafter, 2004). These effects may be involved the inhibition of mutagenic activity by decrease levels of several enzymes such as β-glucuronidase, nitroreductase, and azoreductase that can convert pro-carcinogens to carcinogens (Uccello *et al.*, 2012). Studies of LAB have been shown that *L. acidophilus* and *L. rhamnosus* GG decreased colonic enzymes, like β-glucuronidase and nitro-reductase (De Moreno de LeBlanc & Perdigón, 2005; Verma & Shukla, 2013). Most animal and human studies indicate that LAB decreases fecal enzyme levels that may be involved in formation of carcinogens (Marteau *et al.*, 1990; Commane *et al.*, 2005). Moreover, the immune system play an important role for regulation of cancer in body involved with tumor development and suppression. LAB induce strong adjuvant effects and enhance the immune system of the host including modulation of cell-mediated immune responses, modulation of cytokine pathways, and regulation of interleukins and tumor necrosis factors against tumor (Matsuguchi *et al.*, 2003; Uccello *et al.*, 2012). Apoptosis may be the one of an action mode of probiotics in cancer (J. Y. Kim *et al.*, 2002; Iyer *et al.*, 2008). They are responsible for deletion of cells in normal tissues. LAB and their soluble compound may interact directly with tumor cells and inhibited their growth or provided cell death (Commane *et al.*, 2005). Escamilla *et al.* (2012) reported that the significant effect of LAB supernatant to reduce the growth and viability of the human colon cancer cell line in culture. Puertollano *et al.* (2013) showed the cytotoxic properties of *L. plantarum* supernatants on leukemia tumor cell line.

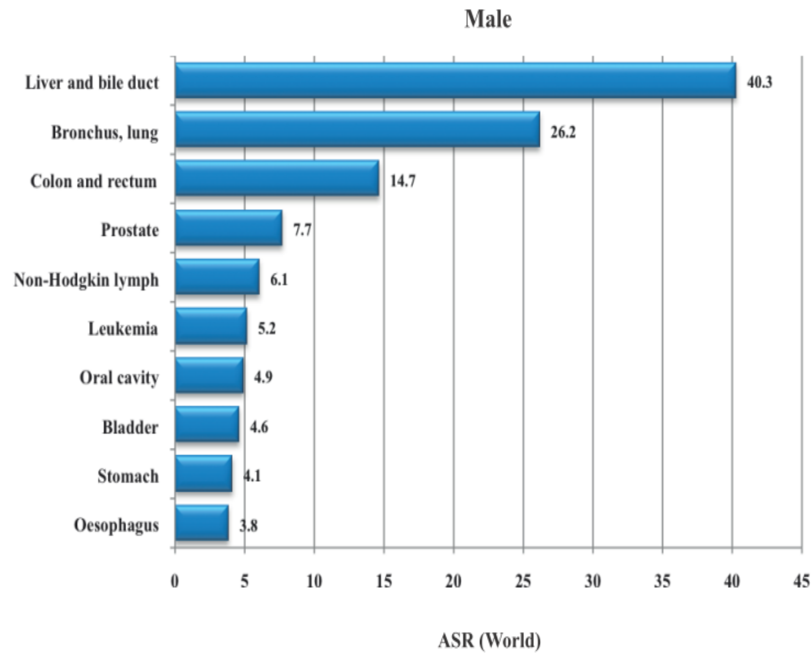

Figure 2.5 Ten leading cancer types in male, Thailand, 2008

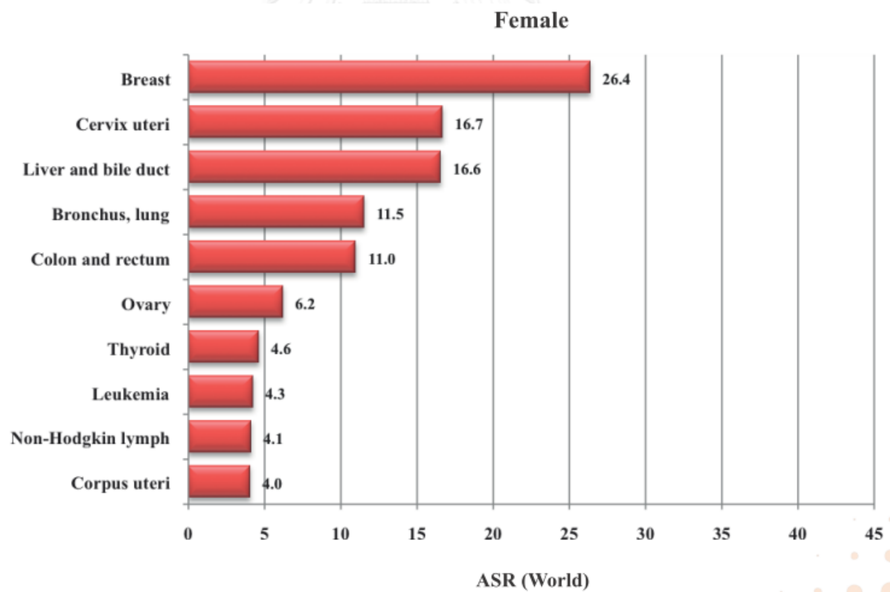

Figure 2.6 Ten leading cancer types in female, Thailand, 2008

Abbreviations: ASR; Annual statistics report expressed as percentage per 100,000 populations.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

Lactic acid production

1. Glucose ($C_6H_{12}O_6$) (Merck, Germany)
2. Yeast extract (Difco, USA)
3. Peptone (Difco, USA)
4. Calcium carbonate ($CaCO_3$) (Sigma, Germany)
5. Potassium phosphate monobasic (KH_2PO_4) (Riedel-de Haen, Germany)
6. Potassium phosphate dibasic (K_2HPO_4) (Merck, Germany)
7. Electronic balance ML3002E/01 (Metter Toledo AG, Switzerland)
8. Spectrophotometers UV mini-1240 (Shimadzu, Japan)
9. pH meter AB15 (Fisher Scientific, Singapore)
10. Rotary incubator shaker G25 (New Brunswick Sciencetific, USA)
11. Autoclave KT-40L (ALP, Japan)
12. High Performance Liquid Chromatography Shimadzu LC-6A (Shimadzu, Japan)
13. Centrifuge KR-20000T (Kubota, Japan)
14. D- and L-lactate dehydrogenases (Boehringer, Germany)

Cell culture

1. Cell lines: Human colon adenocarcinoma cell lines (Caco-2 cells) ATCC HTB-37
2. Cell lines: Human monocytic leukemic cell lines (U937 cells) ATCC CRL-2367

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3. Cell lines: African green monkey kidney epithelial cell lines (Vero cells)
4. Fetal bovine serum (Gibco-Invitrogen, USA)
5. Penicillin-Streptomycin (Gibco-Invitrogen, USA)
6. Dulbecco Modified Eagle medium (DMEM) (Gibco-Invitrogen, USA)
7. Roswell Park Memorial Institute medium number 1640 (RPMI 1640) (Gibco-Invitrogen, USA)
8. Medium 199 (M199) (Gibco-Invitrogen, USA)
9. Oxgall or bovine bile (Sigma, USA)
10. 3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, Germany)
11. Dimethyl sulfoxide (DMSO) (Fisher Scientific, India)
12. 25% Trysin (Gibco-Invitrogen, USA)
13. IL-12-specific ELISA kit (OptEIA; BD Pharmingen, USA)
14. Hemocytometer (Hausser Scientific, USA)
15. Counter (Fisher Scientific, USA)
16. 96-well tissue culture plates (Corning, USA)
17. 24-well tissue culture plates (Corning, USA)
18. 0.22 μm pore size filter Millipore (MA, USA)
19. Multi-Detection Microplate Reader (BioTek® Synergy HT, USA)
20. Speed vacuum (Rotational Vacuum Concentrator RVC 2-18, Germany)

Bacterial culture

1. deMan-Rogosa-Sharpe (MRS) broth (Difco, USA)
2. Agar (Difco, USA)

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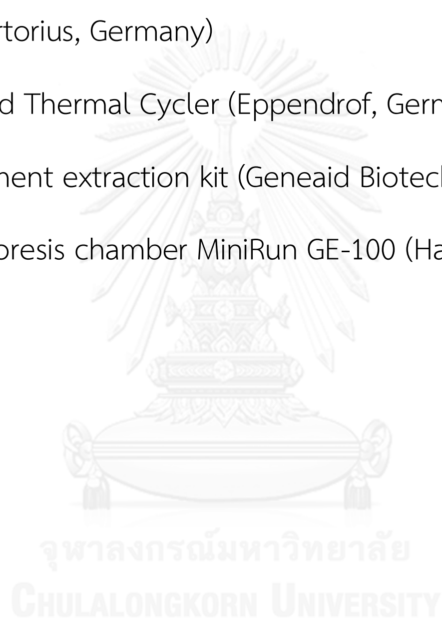
3. Anaerobic GasPak (Mitsubishi, Japan)
4. Microscope CHS model (Olympus, Japan)
5. Electronic balance (Sartorius, Germany)
6. pH meter (Mettler toledo, Italy)
7. Incubator (Memmert, Germany)
8. Autoclave HA-3D model (Hirayama, Japan)
9. Larminar flow hood BV-126 (ISSCO, Thailand)
10. Centrifuge (Sartorius, Germany)
11. PCR Authorized Thermal Cycler (Eppendrof, Germany)
12. PCR DNA fragment extraction kit (Geneaid Biotech, Taiwan)
13. Gel electrophoresis chamber MiniRun GE-100 (Handzhou Bioer technology, China)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3.2 Methods

3.2.1 Sample collection and isolation method

Forty-five samples were collected from various sources in different districts of Thailand including animal feces (12 samples), fermented foods (8 samples) and tree bark (25 samples). Four isolates of healthy human feces which were received ethical approval (SWUEC 37/2551) by a research ethics committee were kindly provided from Dr. Malai Taweechotipatr (Srinakharinwirot University, Bangkok) whereas two isolates of silages and 1 isolate of soy sauce mash were kindly provided from Dr. Somboon Tanasupawat (Chulalongkorn University, Bangkok). 0.25 g of fresh sample from tree bark were taken directly for cultivation in 5 mL GYP broth (Appendix A) and were incubated at 30 °C for 72 h under anaerobic condition by using an anaerobic GasPak (Mitsubishi, Japan). One drop (50 μL) of broth was transferred and streaked on GYP agar plate containing calcium carbonate (Appendix A) and was incubated at the same condition. One gram of the feces samples and fermented foods were primarily diluted in 0.85% normal saline solution (NSS). Serial dilutions of the suspension were prepared by the 10-fold dilution method for decreasing the number of bacterial cells. 200 μL of each serial dilution were spread onto MRS agar containing 0.5% $CaCO_3$ and incubated at 37 °C for 48-72 h under anaerobic condition. LAB isolates were selected by the presence of a clear zone around the colonies and were picked up for purification based on difference colonies morphology. Pure cultures were selected and maintained in MRS broth, in freezer and were lyophilized for further studies.

3.2.2 Screening of lactic acid production

The isomer of lactic acid was preliminarily screened by enzymatically following the method of Tanasupawat *et al.* (2007) which using D- and L-lactate dehydrogenases. D-lactic acid producing bacteria were selected for fermentation.

- Fermentation of lactic acid bacteria

Lactic acid production was determined as described by Prasirtsak (2011). Briefly, LAB were inoculated onto GYP slant and incubated at 37°C for 2 days under anaerobic condition. The bacterial cells were adjusted the optical density approximately 0.3-0.4 at OD 600 nm. 1% v/v of bacterial cells were added to 50 mL of preculture medium (Appendix A) and incubated at 37°C under anaerobic condition. After 26 h incubation, 2% v/v of preculture medium were transferred to 50 mL of fermentation medium (Appendix A) and incubated at 37°C for 72 h under anaerobic condition.

- Analysis of total lactic acid and optical purity of lactic acid

After fermentation, 1 mL of culture broths were collected and centrifuged at 10,000 rpm for 10 min. The supernatants were diluted in sterile Milli-Q water and filtrated with cellulose acetate filter for total and optical purity of lactic acid measurement. Lactic acid, residual glucose concentrations and optical purity of lactic acid were analyzed using High Performance Liquid Chromatography (HPLC) as conditions described below.

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- Conditions for total lactic acid and residual glucose concentration analysis

Column	Biorad Aminex HPX-87H ion exclusion organic acid column (300 mm × 7.8 mm)
Mobile phase	0.005 M H_2SO_4
Temperature	45 °C
Flow rate	0.6 ml/min
Detector	Refractive index detector RID-6A (Shimadzu, Japan)
Time	25 min
Volume	15 μL
Standard concentrations	0.25, 0.50, 1.00, 1.50 and 2.00 g/L

- Conditions for optical purity of lactic acid analysis

Column	Sumi Chiral OA-5000 (150 mm x 4.6 mm)
Mobile phase	1 mM $CuSO_4$
Temperature	40 ^{o}C
Flow rate	1.0 ml/min
Detector	UV detector 254 nm
Time	30 min
Volume	5 μL
Standard concentrations	0.25, 0.50, 1.00, 1.50 and 2.00 g/L

Lactic acid, acetic acid, glucose and ethanol were used as the standard for total lactic acid analysis. L-Lactic acid and D-lactic acid were used as the standard for optical purity of lactic acid analysis. The retention times of L-lactic acid and D-lactic acid were 18.9 and 24.1, respectively. LAB which produced high lactic acid and optical purity were selected for further experiments. The percentage of optical purity of lactic acid, percentage of yield and productivity were calculated as seen in Appendix C.

- Optimization of lactic acid production in flask scale

To obtain the efficient lactic acid production and optical purity of lactic acid, the effect of initial glucose concentrations, fermentation time and shaking on lactic acid production were investigated.

- Effect of initial glucose concentrations on lactic acid production

The optimum concentrations of glucose were investigated by varying the glucose concentrations at 100, 120 and 140 g/L. Total lactic acid and optical purity of lactic acid were analyzed as described above.

- Effect of fermentation time on lactic acid production

The optimum time of fermentation were investigated by varying time at 0, 24, 30, 48 and 72 h. Total lactic acid and optical purity of lactic acid were analyzed as described above.

- Effect of shaking on lactic acid production

The culture fermentation with or without shaking were investigated to determine the optimum condition of fermentation. Shaking was conducted at 100 rpm. Total lactic acid and optical purity of lactic acid were analyzed as described above.

- Optimization of lactic acid production in bacth fermentor

The batch fermentation of selected LAB was performed at 3-L working volume in a 5-L fermentor. Briefly, LAB were inoculated onto GYP slant and incubated at 37°C for 2 days under anaerobic condition. The bacterial cells were adjusted the optical density approximately 0.3-0.4 at OD 600 nm. 1% v/v of bacterial cells were added to 50 mL of GYP preculture medium in flask and incubated at 37°C under anaerobic condition for 26 h. 2% v/v of preculture medium were transferred to 5-L fermentor and carried out at 37°C under anaerobic condition. The optimum concentrations of glucose were investigated in fermentor by varying the glucose concentrations at 100, 140 and 200 g/L. Samples were collected every 3 h for 48 h to analyze total lactic acid, optical purity of lactic acid and cell biomass.

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3.2.3 Screening of probiotic properties

- Preparation of LAB supernatant

The supernatants were prepared as follows: The culture was cultivated into MRS broth and incubated at 37 ^{o}C overnight under anaerobic condition. The culture was adjusted the bacterial concentrations to 10^8 CFU/ml and added into MRS broth, incubated at 37 ^{o}C for 48 h under anaerobic condition. The supernatants were collected by centrifugation at 4,000 rpm for 10 min and filtrated with 0.22 μm membrane filter. The supernatant was evaporated to dryness with speed vacuum. The pellets were re-suspended with an equal volume of serum-free culture media and kept at -20 ^{o}C for further experiments.

- Cytotoxicity assays

The cytotoxicity of selected lactic acid bacteria against tumor cell line were determined as described by Chimchang (2012). MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed to evaluate the cytotoxic effect of supernatant of selected LAB on cancer cell lines including Caco-2 colon carcinoma cells (ATCC HTB-37) and U937 human monocytic leukemic cells (ATCCCRL-2367™) as compare to the Vero normal cells from African monkey kidney cells. Briefly, Caco-2 and Vero cells were cultured in Dulbecco modified Eagle's minimal essential medium (DMEM) and Medium 199 (M199), respectively. U937 cells were cultured in Roswell Park Memorial Institute Medium (RPMI 1640). All culture media were supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (0.1 mg/ml), and incubated at 37 °C with humidified atmosphere containing 5% CO_2. Caco-2, U937 and Vero cells were seeded in 96-well plates at a final density of 5.9 x 10^3, 5 x 10^4 and 1.2 x 10^4 cells per well, respectively. Caco-2 and Vero cells were incubated at 37°C overnight before treatment with samples. 10% v/v of LAB supernatants were treated into cell line cultures and incubated at 37°C for 24 h in 5% CO_2 incubator. After incubation, MTT solutions were added to each well and incubated at 37°C for 3 h in 5% CO_2 incubator. The culture media of Caco-2 and

Vero cells were discarded and dimethyl sulfoxide (DMSO) was added and mixed gently, whereas U937 cells added DMSO without supernatant discarded. The optical density was measured at 595 nm using a microplate reader. Cells and MRS broth with cells were used as control. This experiment was conducted in triplicate of three independent assays. The percentage of cell viability was calculated as follows:

$$\text{The percentage of cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

- Preparation of viable LAB

The viable of bacterial strains were prepared as follows: bacterial cells were cultivated into MRS broth at 37 ^{o}C for 24 h under anaerobic condition and adjusted to 10^{8} CFU/ml. Bacterial cells were harvested by centrifugation at 10,000 rpm for 10 min, washed three times in phosphate saline buffer (1XPBS), re-suspended in sterilized deionized water, and then lyophilized. Before using, bacterial lyophilization was re-suspended with PBS and adjusted to 10^{8} CFU/ml.

- Induction of Interleukin (IL)-12 cytokine production

Interleukin (IL)-12 productions were determined according to the method of Kimoto-Nira *et al.* (2012). Briefly, murine macrophage cell lines J774A.1 were routinely grown in DMEM supplemented with 10% FBS and antibiotics. The J774A.1 cells were seeded in 24-well plates at a concentrations of 2.5×10^{5} cells per well. Cells were incubated with or without the viable LAB (20 % v/v) at 37 ^{o}C in a 5% CO_2 air atmosphere for 24 h. The supernatants were collected by centrifugation at 2,000 rpm for 3 min and used for IL-12 measurements. This experiment was conducted in triplicate.

- Interleukin (IL)-12 measurements

The concentrations of IL-12 from J774A.1 cells supernatants were determined by IL-12-specific ELISA kit according to the manufacturer's protocol. Briefly, anti-mouse IL-12 was coated overnight in 96-well plates and then fixed with 10% FBS in PBS for 1 h. The sample supernatants were added and incubated for 2 h. The plate was washed with PBS. Biotinylated anti-mouse IL-12 and Streptavidin-horseradish peroxidase were used for detection of IL-12. The color change of 3,3',5,5'-Tetramethylbenzidine (TMB) was measured at 450 nm using a Microplate Reader. The recombinant mouse IL-12 (p40) was used as a standard in this assay. The concentrations of IL-12 was interpolated from a standard curve and expressed as pg/ml of culture medium.

- Acid tolerance

Screening for acid tolerance of the LAB were determined according to the method of Hyronimus *et al.* (2000). Bacterial cells were cultivated into MRS broth at 37 ^{o}C overnight under anaerobic condition. Each strain of 10^{8} CFU/ml concentrations was inoculated into MRS broth at various pH values (pH 2.0 and 3.0) adjusted with hydrochloric acid. The cultures were incubated at 37°C for 3 h under anaerobic condition and conducted in duplicates. After incubation, the cultures were performed 10-fold serial dilution in phosphate buffer (0.1 M, pH 7.2). 0.1 ml of each serial dilution was transferred onto MRS agar by spreading plate method and incubated at 37 °C for 24-48 h under anaerobic condition. Total viable counts were determined before incubation and after 3 h incubation and expressed as the $\log_{10}$ of colonies grown on MRS agar. Unadjusted pH MRS broth (pH 6.5 ± 0.2) was used as a control.

- Bile tolerance

Bile tolerance of selected LAB were determined according to the method of Lee *et al.* (2011). Bacterial cells were cultivated into MRS broth at 37 °C overnight under anaerobic condition. Bacterial cells at 10^8 CFU/ml concentrations were inoculated into MRS broth with various concentrations of bile salt (0.3%, 0.5% and 1.0%) and without bile salt (control culture) and were incubated at 37°C for 3 h under anaerobic condition. After that, 10-fold serial dilution in phosphate buffer (0.1 M, pH 7.2) was performed. 0.1 ml of each serial dilution was transferred onto MRS agar by spreading plate method and incubated at 37 °C for 24-48 h under anaerobic condition. Total viable counts were determined before incubation and after 3 h incubation and expressed as the $\log_{10}$ of colonies grown on MRS agar. The experiment was performed in duplicates

- Adhesion assays

The adhesion of LAB were examined using Caco-2 intestinal cells by modification of the methods of Fernandez *et al.* (2003) and Bustos *et al.* (2012). Briefly, Caco-2 cells were routinely grown in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. Monolayers of Caco-2 cells were seeded at a concentrations of 5×10^5 cells per well in 24-well plate and incubated at 37 °C in a 5% CO_2 air atmosphere. Cells were used at late post-confluence culture after 15 days, with a change of medium every two days. Just before use about 2 h, the monolayer was washed twice with PBS and DMEM without FBS and antibiotics were added to each well. Bacterial cells were prepared as follows: LAB were grown overnight in MRS broth, washed twice with PBS, resuspended in DMEM without FBS and antibiotics to give the bacterial concentrations of 10^8 CFU/ml. The suspensions were added to well plate containing Caco-2 cells and then incubated at 37 °C in a 5% CO_2 air atmosphere for 1 h. The cell cultures were washed twice with PBS and then Caco-2 cells were lysed by addition of 0.05% Triton-X100 solution for 10 min. The number of viable LAB adhesion was determined by plating serial dilutions on

MRS agar plates. *L. rhamnosus* GG was used as a positive control and adhesion assays were conducted in duplicate.

- Statistical analysis

Statistical analysis was conducted using Student's unpaired t-test and one way ANOVA of Tukey method (SPSS 22.0). A probability of $P < 0.05$ was considered to be significant.

3.2.4 Identification methods

- Phenotypic characteristics

Phenotypic characteristics including morphological such as shape, spore-forming, cell arrangement, Gram reaction and cultural characteristics such as color, size and shape were determined as described by Tanasupawat *et al.* (1992) . Physiological and biochemical characteristics such as sugar utilization, nitrate reduction, arginine hydrolysis, temperature for growth, pH for growth, and NaCl tolerance were also determined by the method of Tanasupawat *et al.* (1998) .

- Chemotaxonomic characteristics

Chemotaxonomic characteristics including cell wall peptidoglycan, isoprenoid quinone and cellular fatty acids were analyzed. The cell wall peptidoglycan was determined using TLC method as described by Staneck and Roberts (1974). Isoprenoid quinone was extracted from the freeze-dried cells as described by Collins *et al.* (1977) and was analyzed using high-performance liquid chromatography (HPLC). The cellular fatty acids were determined by preparing the fatty acid methyl esters (Sakamoto et al., 2002) and were analyzed using gas chromatography according to the instructions of the Microbial Indentification System (MIDI).

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- Genotypic characteristics

Genomic DNA was extracted as described by Saito and Miura (1963). The 16S rRNA gene sequences were amplified by polymerase chain reaction (PCR) with the following primers (Universal primers): 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 518F (5'-CCAGCAGCCGCGGTAATACG-3'), 800R (5'-TACCAGGGTATCTAATCC-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') and were sequenced by Macrogen®, Korea using universal primers as described above. The similarity of 16S rRNA gene sequences were determined using BLAST software compared to EzTaxon-e database (Kim *et al.,* 2012). Multiple alignments of sequences were performed by CLUSTAL X version 1.81 in BioEdit software. The alignment was modified by eliminate gaps and ambiguous nucleotides prior to the construct a phylogenetic tree. The phylogenetic tree was constructed with neighbor-joining (Saitou & Nei, 1987) in the MEGA 5 software (Tamura et al. 2011). The confidence values of individual branches in the phylogenetic tree were determined with 1000 replications (Felsenstein, 1985). The *gyrB* gene sequences were also performed using primers; UP1Gi (5'-GAAGTCATCACCGTTCTGCAYGSIGGIGGIAARTTYGG-3') and GBlgc-R (5'-GTGAGTAACAATGTACGDATRTGIGCICCRTCIAC-3'). The primers UP1s (5'-GAAGTCATCACCGTTCTGCA-3') and GBlgc-Rs (5'-GTGAGTAACAATGTACG-3') were used as primers for sequencing of *gyrB* gene (Matsuo *et al.*, 2006; Fujita *et al.*, 2010). The phylogenetic tree based on the *gyrB* gene sequences was constructed as similar to 16S rRNA gene sequences. The DNA G + C content was determined using HPLC as described by Kitahara *et al.* (2010). DNA–DNA hybridization was performed using photobiotin-labelled DNA probes in microplates and analyzed by fluorometric method as described by Ezaki *et al.* (1989). The experiment was conducted with eight replications.

CHAPTER IV
RESULTS AND DISCUSSIONS

4.1 Isolation of lactic acid bacteria

Seventy-seven lactic acid bacteria were obtained from various sources in Thailand (Table 4.1). Thirty-six isolates were isolated from tree barks and were screened for the lactic acid production while forty-one isolates were screened for the probiotic properties. Twenty-six isolates were isolated from wild elephant feces (22 isolates) and buffalo feces (4 isolates). Eight isolates were isolated from Thai fermented foods including fermented fish "Pla-chom" (3 isolates) and "Pla-ra" (2 isolates), fermented shrimp "Kung-chom" (3 isolates). Two isolates of silages and one isolate of soy sauce mash were obtained from Dr. Somboon Tanasupawat (Chulalongkorn University, Bangkok). Four isolates of healthy human feces were obtained from Dr. Malai Taweechotipatr (Srinakharinwirot University, Bangkok).

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Table 4.1 Isolation sources and isolate number

Isolation source	Province	Number of samples	Isolate no.	Number of isolates	Purposes
Tree bark	Chulalongkorn university, Bangkok	17	BK58-2, BK58-3, BK59-2, BK62-2, BK65-1, BK65-3, BK66-2, BK69, BK70-1, BK70-2, BK70-3, BK108, BK109-1, BK110-2, BK113, BK115-2, BK116-1, BK116-2, BK116-3, BK117-1, BK117-2, BK118, BK119 and BK120-4	24	Lactic acid production
Tree bark	Rayong	8	BK90-1, BK90-2, BK91-2, BK92, BK95-2, BK95-3, BK96-2, BK98-2, BK103-1, BK103-2, BK103-3 and BK105-2	12	
Elephant feces	Patchaburi	10	EL1-1, EL1-2, EL1-3, EL2-1, EL2-2, EL2-3, EL3-1, EL4-1, EL4-2, EL4-3, EL5-1, EL5-2, EL5-3, EL6-1, EL6-2, EL7-1, EL7-2, EL8-1, EL8-2, EL9-1, EL10-1 and EL10-2	22	Probiotic properties
Buffalo feces	Mahasarakham	2	BF13-1, BF13-2, BF13-3 and BF14-1	4	
Fermented fish "Pla-chom"	Surin	3	PC72-4, PC73-3, and PC75-2	3	
Fermented fish "Pla-ra"	Buriram	2	PC79-5, and PC86-2	2	
Fermented shrimp "Kung-chom"	Buriram	3	KC74-1, KC78-5 and KC81-2	3	
Soy sauce mash	Bangkok	-	SR7-1	1	
Silage	Bangkok	-	SL4-1 and SL7-2	2	
Healthy human feces	Bangkok	-	MSMC39-5, MSMC57-2, MSMC63-2 and MSMC120-2	4	

4.2 Screening of lactic acid production

Thirty-six isolates from tree barks were cultivated into GYP medium and were evaluated the lactic acid production (Appendix C, Table 1). This experiment was conducted using 120 g/L of initial glucose concentrations at 37°C under anaerobic condition for 72 h. The isolates which showed high lactic acid concentrations and low residual glucose were selected to determine the optical purity of lactic acid. The results were found that nine isolates including BK58-2, BK58-3, BK59-2, BK65-1, BK65-3, BK66-2, BK70-1, BK70-2 and BK70-3 produced high lactic acid concentrations and all glucose were consumed or almost terminated. Nine isolates showed the optical purity of lactic acid both D-lactic acid and DL-lactic acid (Table 4.2). The lactic acid concentrations, residual glucose concentrations, %yield, productivity and optical purity of isolates were shown in Figure 4.1-4.3. The isolate BK70-2 showed the highest lactic acid production with the lactic acid concentrations, % yield and productivity as 118.51 g/L, 98.76% and 1.65 g/L.h, respectively. The isolate BK70-3 showed high lactic acid production similar to BK70-2 with the lactic acid concentrations, % yield and productivity as 117.85 g/L, 98.21% and 1.64 g/L.h, respectively. However, the stereoisomer of lactic acid of BK70-2 and BK70-3 was different. The isolate BK70-2 produced DL-lactic acid while BK70-3 produced D-lactic acid. Only five isolates produced pure optical purity of D-lactic acid with vary degree as follows: BK59-2 (100 %ee), BK65-1 (91.64 %ee), BK65-3 (100 %ee), BK66-2 (89.24 %ee) and BK70-3 (100 %ee). The isolates BK65-3 and BK70-3 which produced very high lactic acid concentrations and also showed high optical purity of D-lactic acid were selected to find the optimum condition for lactic acid production.

Table 4.2 Lactic acid production of isolates

Isolate no.	Lactic acid production			Residual glucose (g/L)	Isomer (% Optical purity)
	Final lactic acid (g/L)	% Yield	Productivity (g/L.h)		
BK58-2	89.60	89.92	1.24	20.36	DL
BK58-3	114.61	95.51	1.59	0	DL
BK59-2	88.01	93.08	1.22	25.45	D(100)
BK65-1	113.78	94.82	1.58	0	D(91.64)
BK65-3	101.42	84.52	1.41	0	D(100)
BK66-2	105.18	87.65	1.46	0	D(89.24)
BK70-1	108.14	90.12	1.50	0	DL
BK70-2	118.51	98.76	1.65	0	DL
BK70-3	117.85	98.21	1.64	0	D(100)

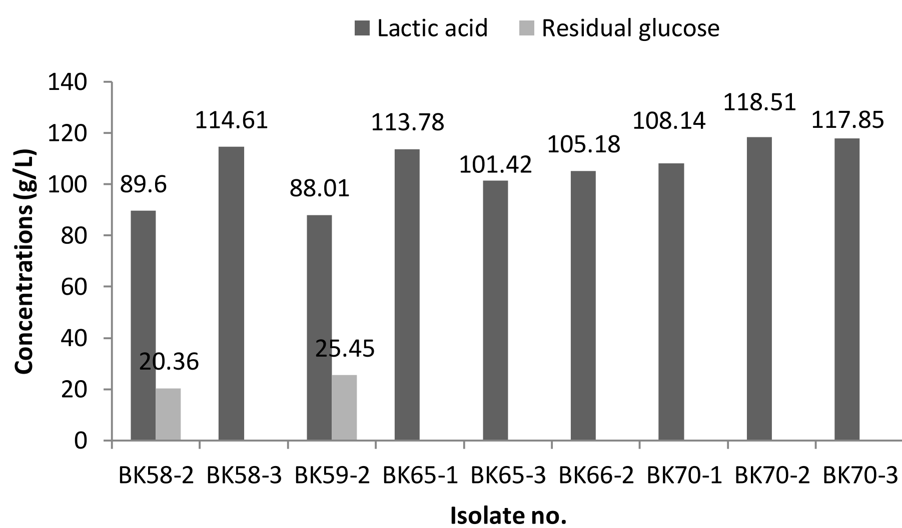

Figure 4.1 Lactic acid and residual glucose concentrations of isolates

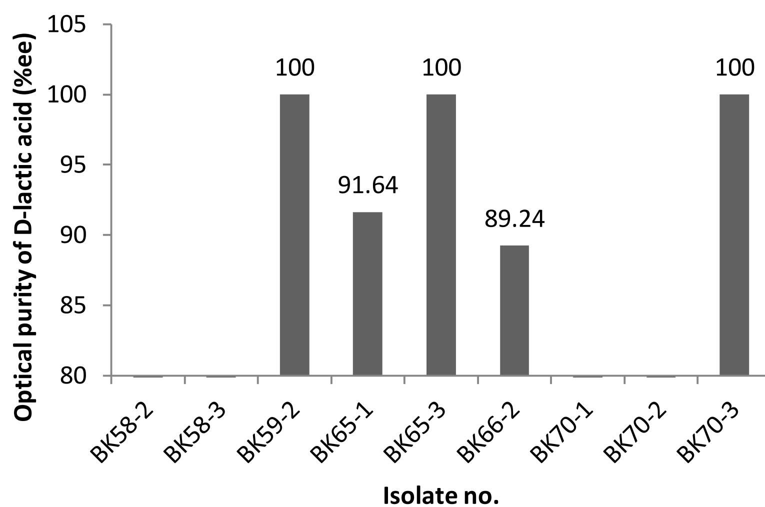

Figure 4.2 Optical purity of D-lactic acid of isolates

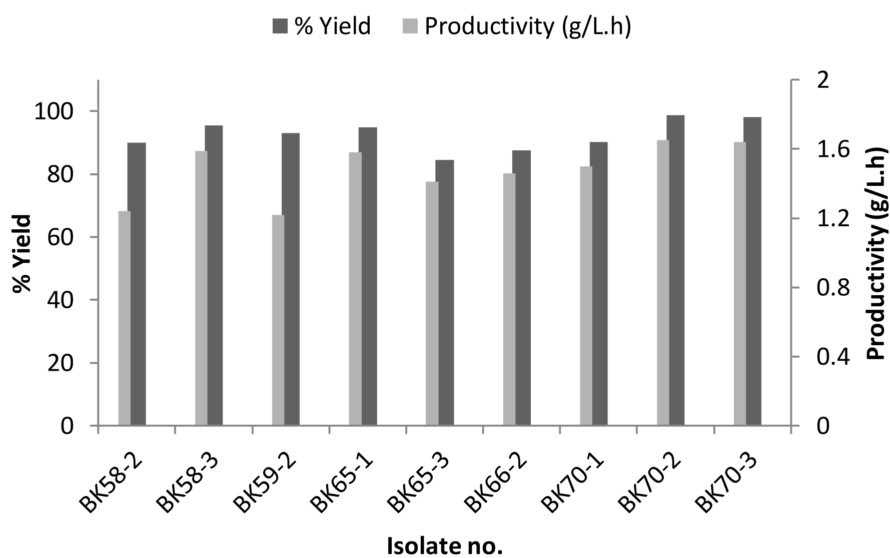

Figure 4.3 Percentage of yield and productivity of lactic acid of isolates

- Optimization of lactic acid production of selected isolates in flask scale

The isolates BK65-3 and BK70-3 were selected to find the optimum condition for lactic acid production. Lactic acid production depends on several factors such as substrate, time and shaking. The effect of initial glucose concentrations were evaluated for provide the highest lactic acid concentrations with no effect on bacterial cells. The fermentation time was evaluated in order to find the optimum time which provided highest productivity and also the effect of shaking on lactic acid production was evaluated.

The effect of initial glucose concentrations of isolates BK65-3 and BK70-3 at 100, 120 and 140 g/L on lactic acid production was exhibited in Figures 4.4 and 4.5, respectively. The experiment was conducted for 72 h. The results of both isolates showed that the concentrations of lactic acid increased in the proportion with glucose concentrations. Residual glucose concentrations were not found in all concentrations. The optical purity of D-lactic acid was varied with different glucose concentrations. In contrast with the productivity, it was increased from 1.33 g/L.h to 1.87 g/L.h in BK65-3 and was increased from 1.37 g/L.h to 1.88 g/L.h when initial glucose concentrations were raised (Figure 4.6-4.7). The initial glucose concentrations at 140 g/L were selected for further experiment.

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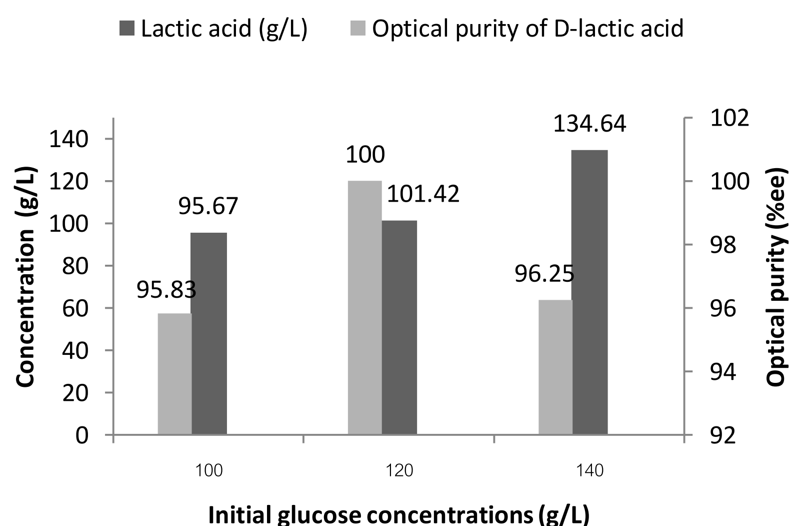

Figure 4.4 Effect of initial glucose concentrations of the isolate BK65-3

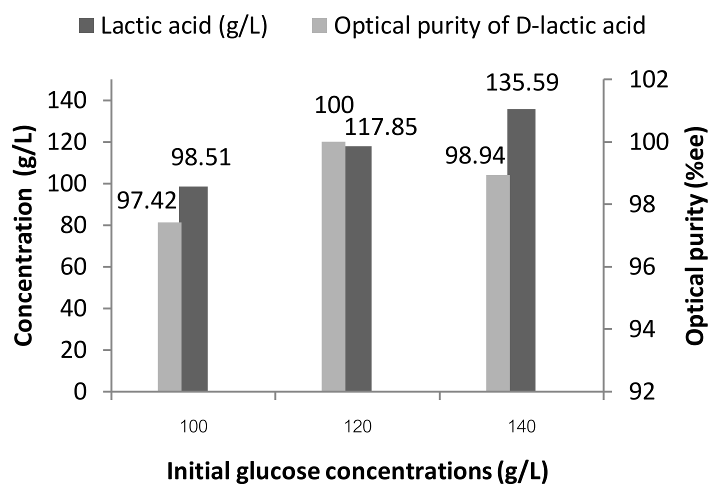

Figure 4.5 Effect of initial glucose concentrations of the isolate BK70-3

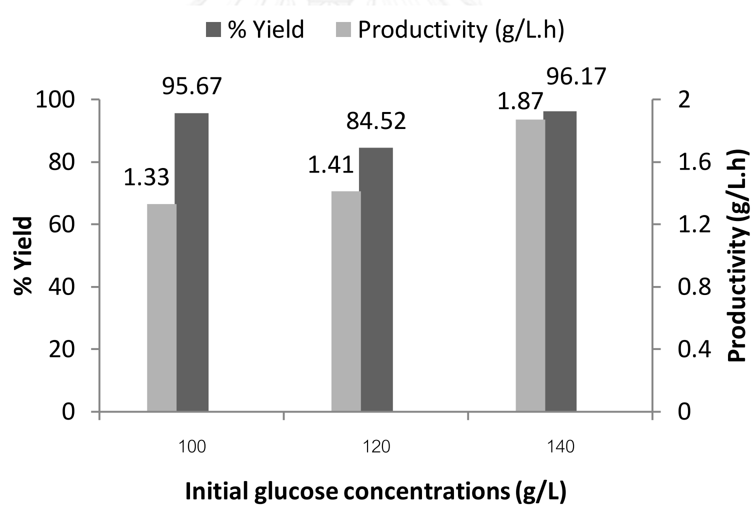

Figure 4.6 Percentage of yield and productivity of lactic acid of the isolate BK65-3 at various initial glucose concentrations

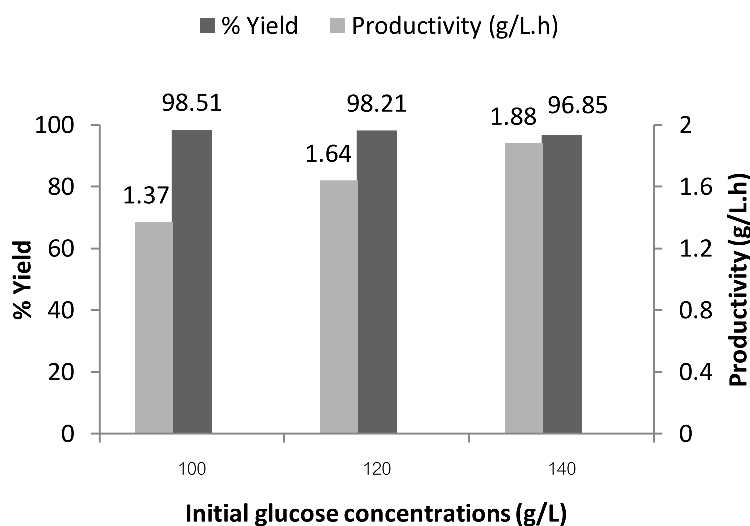

Figure 4.7 Percentage of yield and productivity of lactic acid of the isolate BK70-3 at various initial glucose concentrations

The optimum of fermentation time of isolates BK65-3 and BK70-3 was determined at 0, 24, 48 and 72 h. The results were found that both isolates can utilize all 140 g/L of glucose concentrations within 48 h (Figure 4.8). The lactic acid concentrations at that time of the isolates BK65-3 and BK70-3 were 131.45 and 122.09 g/L, respectively, while the lactic acid concentrations at 72 h of BK65-3 and BK70-3 were 134.64 and 135.59 g/L, respectively (Figure 4.9). The optical purity of D-lactic acid was varied when fermentation time was changed. In BK65-3, the optical purity of D-lactic acid at 24, 48 and 72 h was 100, 97.26 and 96.25%ee, respectively. In BK70-3, the optical purity of D-lactic acid at 24, 48 and 72 h was 100, 99.17 and 98.94%ee, respectively. The highest productivity of both isolates was found at 48 h fermentation with 2.74 g/L.h of productivity in BK65-3 and 2.54 g/L.h of productivity in BK70-3 (Figure 4.10). Fermentation at 48 h was selected as the optimum of fermentation time for further experiment.

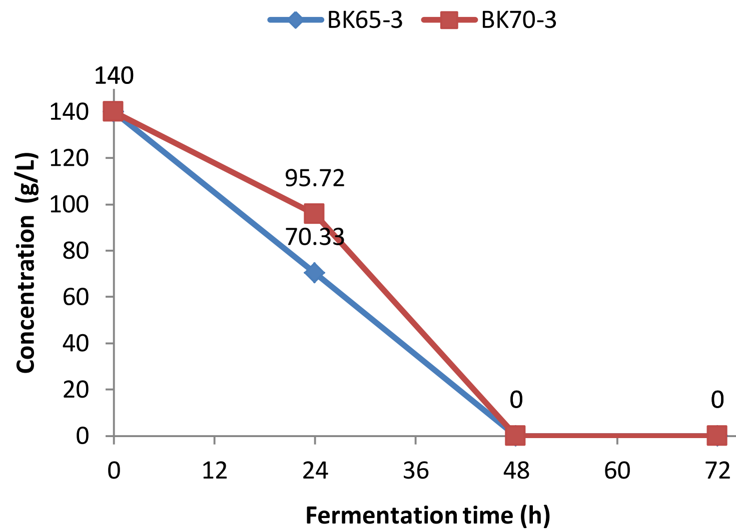

Figure 4.8 Glucose consumption of isolates BK65-3 and BK70-3 after 72 h fermentation

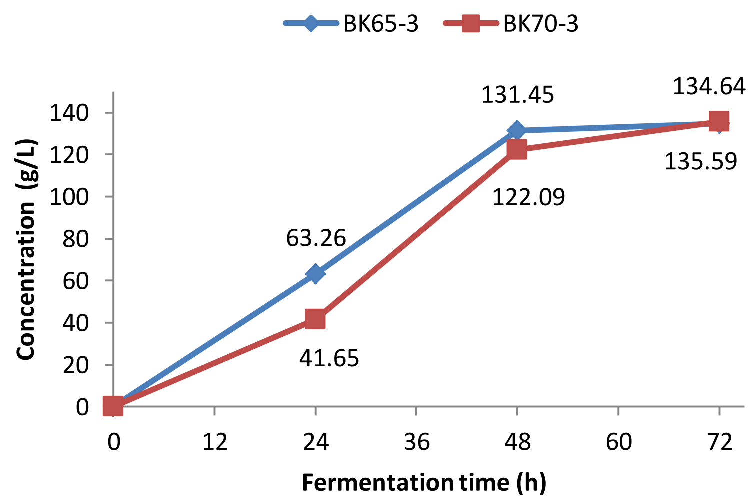

Figure 4.9 Lactic acid production of isolates BK65-3 and BK70-3 after 72 h fermentation

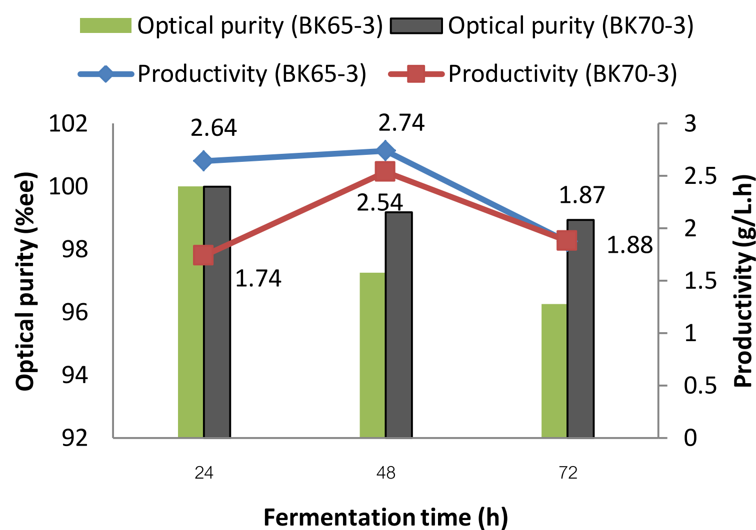

Figure 4.10 Optical purity of D-lactic acid and productivity of isolates BK65-3 and BK70-3 at 24, 48 and 72 h

According to previously described in this experiment, we carried out the fermentation without shaking. However, the lactic acid production might be affected by shaking. The effect of shaking on lactic acid production was evaluated in this study. The results showed that all glucose concentrations were utilized after 48 h in BK65-3 and BK70-3 either shaking or without shaking (Figure 4.11). At 48 h fermentation, the lactic acid concentrations of BK65-3 without shaking (131.45 g/L) were found higher than that of BK70-3, 122.09 g/L. Conversely, the lactic acid concentrations of BK70-3 with shaking (133.79 g/L) were found higher than that of BK65-3, 121.46 g/L, respectively (Figure 4.12). The isolate BK70-3 was selected to scale up in 5-L fermentor since they provided high lactic acid concentrations under shaking and suitable for agitation in fermentor. Yield, productivity and optical purity of D-lactic acid of BK70-3 with shaking at 48 h were 95.56%, 2.79 g/L.h and 98.53 %ee, respectively (data not shown).

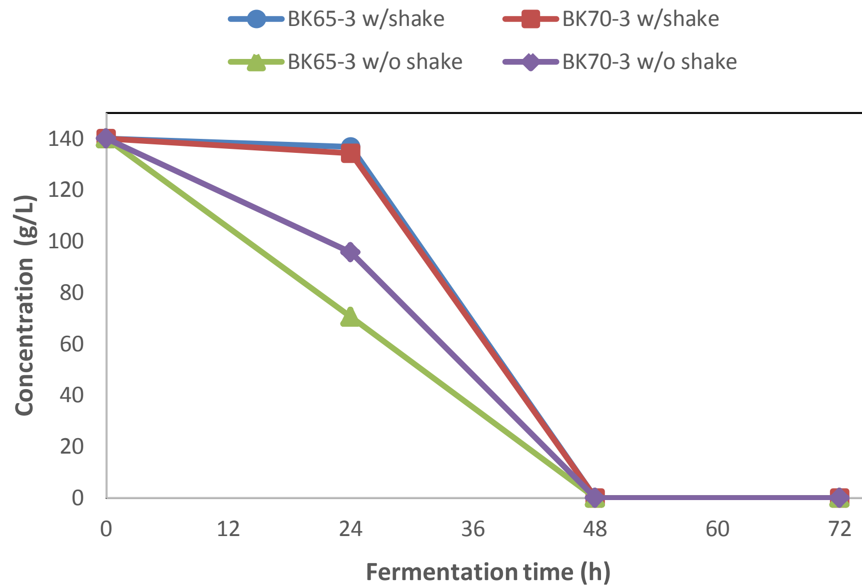

Figure 4.11 Effect of shaking on the glucose consumption of isolates BK65-3 and BK70-3 after 72 h fermentation (w/shake, with shake; w/o, without shake)

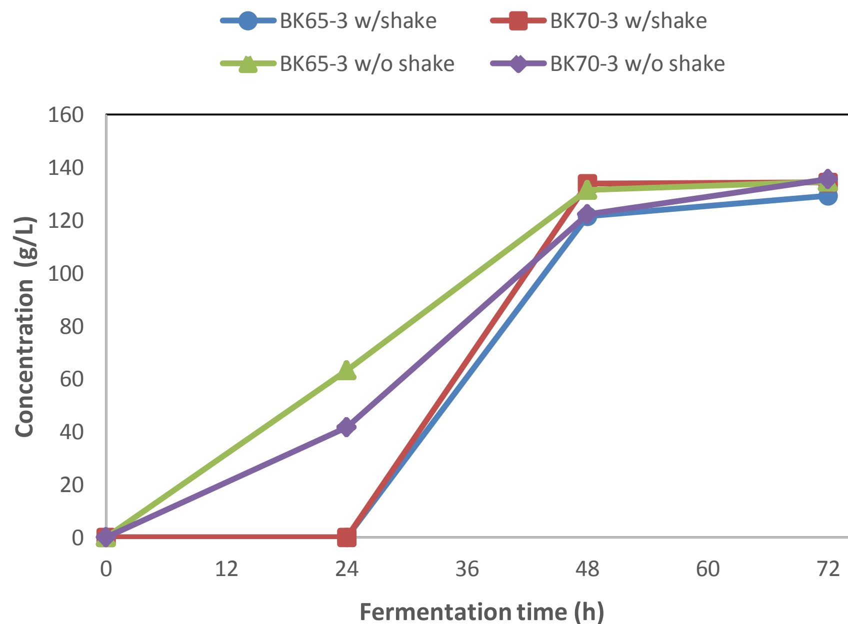

Figure 4.12 Effect of shaking on the lactic acid concentrations of isolates BK65-3 and BK70-3 after 72 h fermentation (w/shake, with shake; w/o, without shake)

- Optimization of lactic acid production of BK70-3 in batch fermentor

The isolate BK70-3 was selected to evaluate the optimum condition of lactic acid production in batch 5-L fermentor with the presence of increasing concentrations of glucose. The initial glucose concentrations up to 200 g/L were used to investigate glucose tolerance of isolate BK70-3. The results showed that the lactic acid produced increased with the increase of initial glucose concentrations. At glucose concentrations 100 g/L, all glucose was utilized within 36 h and also cell growth reached to maximum at 36 h while the maximum lactic acid was achieved at 45 h (Figure 4.13) with 93.55 g/L of lactic acid concentrations, 91.38% of yield, 2.08 g/L.h of productivity and 100%ee of optical purity of D-lactic acid (Appendix C, Table 2). At glucose concentrations 140 g/L, all glucose was utilized within 42 h and also cell growth reached to maximum at 42 h while the maximum lactic acid was achieved at 45 h (Figure 4.14) with 131.63 g/L of lactic acid concentrations, 94.02% of yield, 2.93 g/L.h of productivity and 98.89%ee of optical purity of D-lactic acid (Appendix C, Table 3). The trend of glucose consumption at 100 and 140 g/L, and lactic acid production in fermentor were found similar to that of in flask scale exception with the productivity. The productivity of BK70-3 was improved in fermentor from 2.79 to 2.93 g/L.h when using 140 g/L of glucose concentrations. At glucose concentrations 200 g/L, the isolate BK70-3 could not utilize all glucose within 48 h (Figure 4.15 and Appendix C, Table 4). However, glucose consumption and lactic acid production might be increased by increasing of fermentation time (Figure 4.16-4.17). The results revealed that the growth rates of BK70-3 at 100 and 140 g/L of glucose concentrations were similar while increasing of glucose concentration up to 200 g/L resulting in a decrease in growth rate (Figure 4.18-4.19). All glucose concentrations showed lag phase about 18 h might be due to small inoculum size. Therefore, the optimum glucose concentrations for lactic acid production of BK70-3 in batch fermentor were 140 g/L at 45 h.

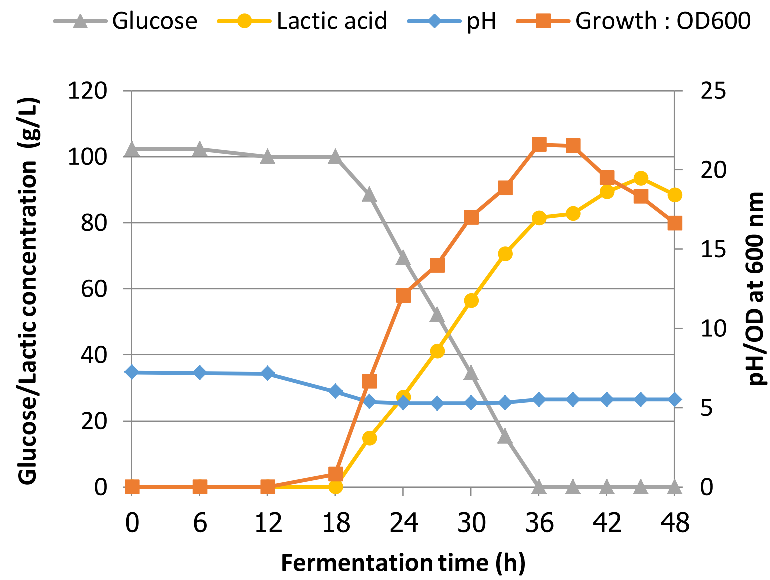

Figure 4.13 Effects of initial glucose concentrations of 100 g/L on the cell growth, pH, glucose consumption and lactic acid production

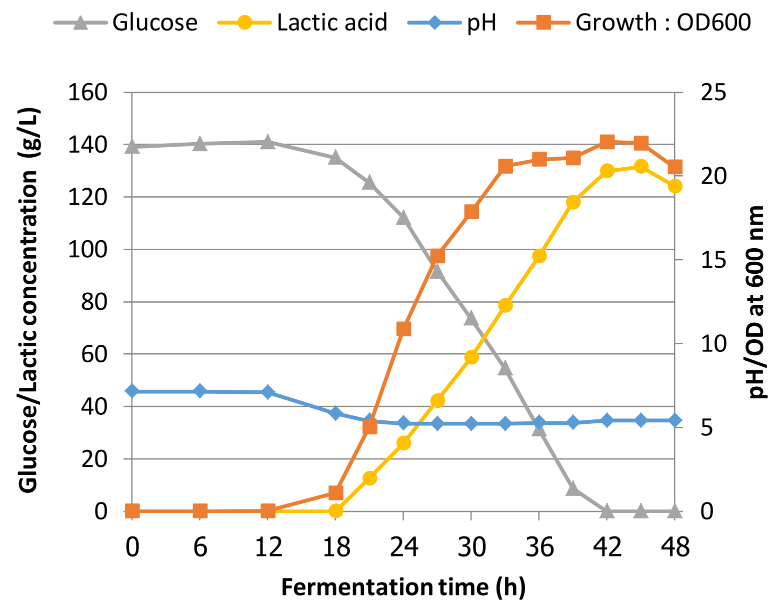

Figure 4.14 Effects of initial glucose concentrations of 140 g/L on the cell growth, pH, glucose consumption and lactic acid production

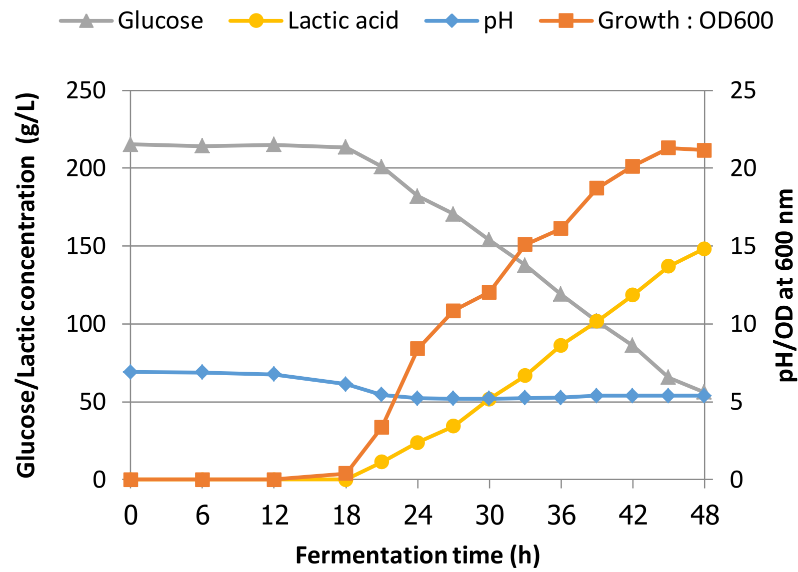

Figure 4.15 Effects of initial glucose concentrations of 200 g/L on the cell growth, pH, glucose consumption and lactic acid production

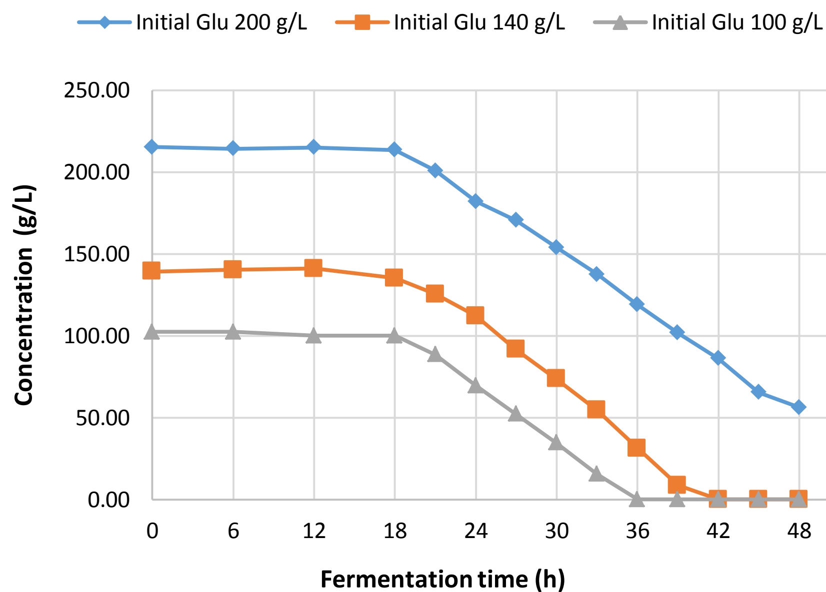

Figure 4.16 Effect of various initial glucose concentrations on the glucose consumption

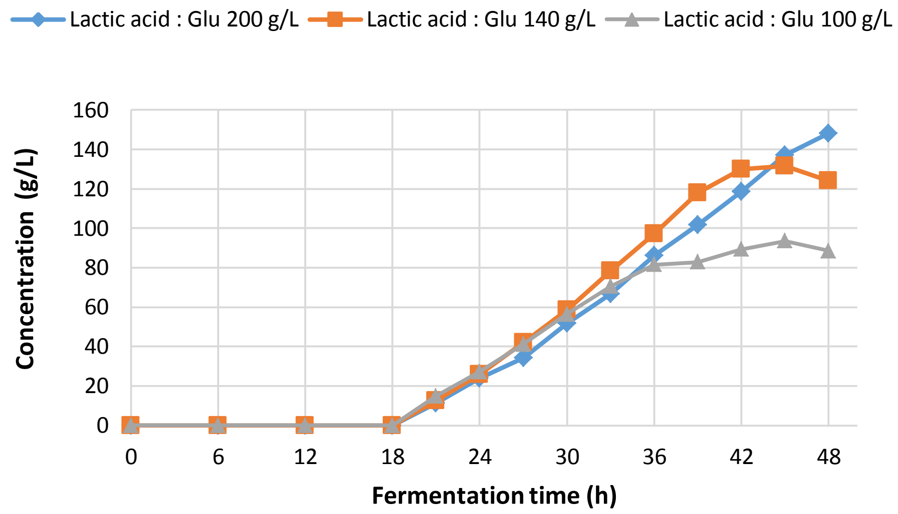

Figure 4.17 Effect of various initial glucose concentrations on the lactic acid concentration

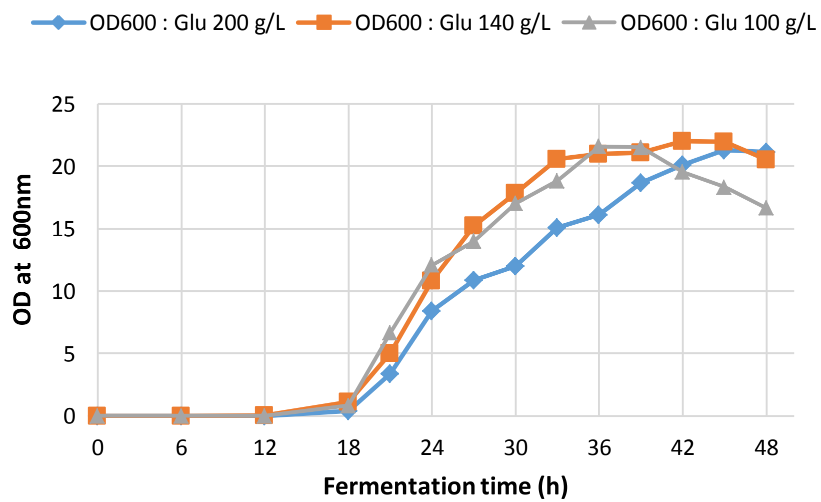

Figure 4.18 Effect of various initial glucose concentrations on the cell growth

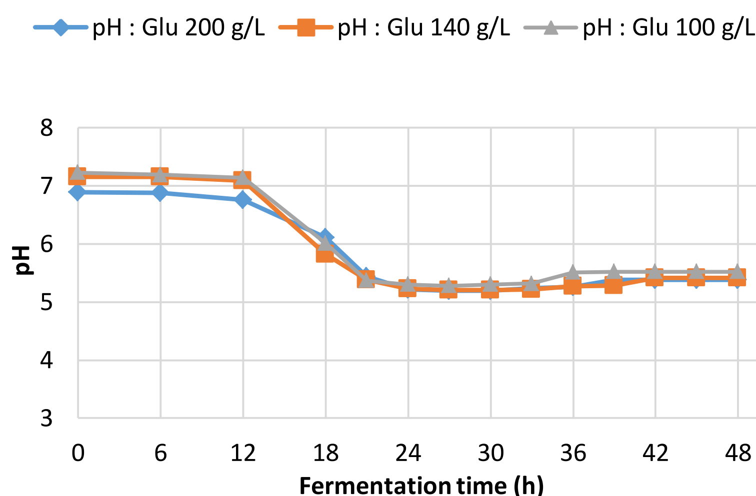

Figure 4.19 Effect of various initial glucose concentrations on the pH

4.3 Identification of selected lactic acid bacteria from tree barks

Nine isolates, BK58-2, BK58-3, BK59-2, BK65-1, BK65-3, BK66-2, BK70-1, BK70-2 and BK70-3 which showed high lactic acid production were identified using the phenotypic characteristics, chemotypic characteristics and 16S rRNA gene sequencing. They were Gram-positive rods, catalase-negative, anaerobically or microaerophilic, sporulation, no gas formation, nitrate reduction and arginine hydrolysis are negative. Colonies were typical circular, smooth and white in color. The cell wall peptidoglycan were *meso*-diaminopimelic acid. Lactic acid production was found to be only D- or DL- lactic acid. Based on the phenotypic, chemotaxonomic and genotypic characteristics, all isolates were belong to the genus *Sporolactobacillus* and were separated into four groups.

Group I consisted of three isolates, BK58-2, BK58-3 and BK66-2. Cells were rod-shaped and spore forming. The isolate grew at pH 4.5-8.5 and in 3% NaCl. No growth was found at pH 4 and in 6% NaCl. Acid is produced from D-fructose, maltose, D-mannitol, D-mannose, raffinose (mostly), sorbitol, sorbose (mostly), sucrose and trehalose but did not produce acid from amygdalin, L-arabinose,

cellobiose, D-galactose, gluconate (mostly), lactose, melibiose, methyl-α-D-glucoside, rhamnose, ribose, salicin and D-xylose. Variable acid production was found in gluconate, raffinose and sorbose in some isolates (Table 4.3). The 16S rRNA gene sequence of BK58-2, BK58-3 and BK66-2 showed 99.93% (1,404 bps), 99.86% (1,444 bps) and 99.93% (1,431 bps) similarity, respectively, to *Sporolactobacillus nakayamae* subsp. *nakayamae* JCM 3514^T (Figure 4.20). Therefore, they were identified as *S. nakayamae* subsp. *nakayamae* (Yanagida *et al.,* 1997).

Group II consisted of two isolates, BK70-1 and BK70-2. Cells were rod-shaped and spore forming. The isolate grew at pH 4.0-8.5 and in 3% and 6% NaCl. Acid is produced from cellobiose D-mannitol, methyl-α-D-glucoside, sorbose and sucrose but did not produce acid from amygdalin, L-arabinose, D-fructose, D-galactose, gluconate, lactose, maltose, D-mannose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, trehalose and D-xylose (Table 4.3). The 16S rRNA gene sequence of BK70-1 and BK70-2 showed 100% (1,436 bps) and 100% (1,430 bps) similarity to *Sporolactobacillus terrae* JCM 3516^T (Figure 4.20). Therefore, they were identified as *S. terrae* (Yanagida *et al.,* 1997).

Group III consisted of one isolates, BK59-2. Cells were rod-shaped and spore forming. The isolate grew at pH 4.0-8.5 and in 3% NaCl. Slightly growth was found in 6% NaCl. Acid is produced from D-fructose, D-mannose, sucrose, trehalose and D-xylose but did not produce acid from amygdalin, L-arabinose, cellobiose, D-galactose, gluconate, lactose, maltose, D-mannitol, melibiose, methyl-α-D-glucoside, raffinose, rhamnose, ribose, salicin, sorbitol and sorbose (Table 4.3). The 16S rRNA gene sequence of BK59-2 was 100% similarity of 1,404 bps to *Sporolactobacillus kofuensis* JCM 3419^T (Figure 4.20). Therefore, they were identified as *S. kofuensis* (Yanagida *et al.,* 1997).

Group IV consisted of three isolates, BK65-1, BK65-3 and BK70-3. Cells were rod-shaped and spore forming. The isolates grew at pH 4.5-8.5 and in 3% NaCl. No growth was found at pH 4 in 6% NaCl. Acid is produced from D-fructose, gluconate, D-mannose, raffinose, sucrose and trehalose but did not produce acid from amygdalin, L-arabinose, cellobiose, D-galactose, lactose, D-mannitol, melibiose,

rhamnose, ribose, salicin, sorbitol, sorbose and D-xylose. Variable acid production was found in maltose and methyl-α-D-glucoside (Table 4.3). The 16S rRNA gene sequence of BK65-1, BK65-3 and BK70-3 were 99.93% (1,484 bps), 99.86% (1,419 bps) and 99.86% (1,468 bps) similarity, respectively, to *Sporolactobacillus inulinus* NBRC 13595^{T} (Figure 4.20). Therefore, they were identified as *S. inulinus* (Kitahara & Suzuki, 1963).

Table 4.3 Characteristics of selected isolates from tree barks

Characteristics		Group I (3)	Group II (2)	Group III (1)	Group IV (3)
Cell form		Rods, endospore forming			
Colony		Circular, white			
Gram strain		+	+	+	+
Catalase production		-	-	-	-
Nitrate reduction		-	-	-	-
Arginine hydrolysis		-	-	-	-
Growth at 50 °C		-	-	-	-
Growth at	pH 4.0	-	+	+	-
	pH 4.5	+	+	+	+
	pH 8.0	+	+	+	+
	pH 8.5	+	+	+	+
Growth in	3% NaCl	+	+	+	+
	6% NaCl	-	+	+w	-
Acid produced from	Amygdalin	-	-	-	-
	L-Arabinose	-	-	-	-
	Cellobiose	-	+	-	-
	D-Fructose	+	-	+	+
	D-Galactose	-	-	-	-
	Gluconate	-(+1)	-	-	+
	Lactose	-	-	-	-
	Maltose	+	-	-	+(-1)
	D-Mannitol	+	+	-	-
	D-Mannose	+	-	+	+
	Melibiose	-	-	-	-
	Methyl-α-D-Glucoside	-	+	-	+ (-1)
	Raffinose	+(-1)	-	-	+
	Rhamnose	-	-	-	-
	Ribose	-	-	-	-
	Salicin	-	-	-	-
	Sorbitol	+	-	-	-
	Sorbose	+(-1)	+	-	-
	Sucrose	+	+	+	+
	Trehalose	+	-	+	+
	D-Xylose	-	-	+	-
Meso-Diaminopimelic acid		+	+	+	+

+ : positive reaction, +w : weakly positive reaction, - : negative reaction. Numbers in parentheses are the number of isolates showing the reaction.

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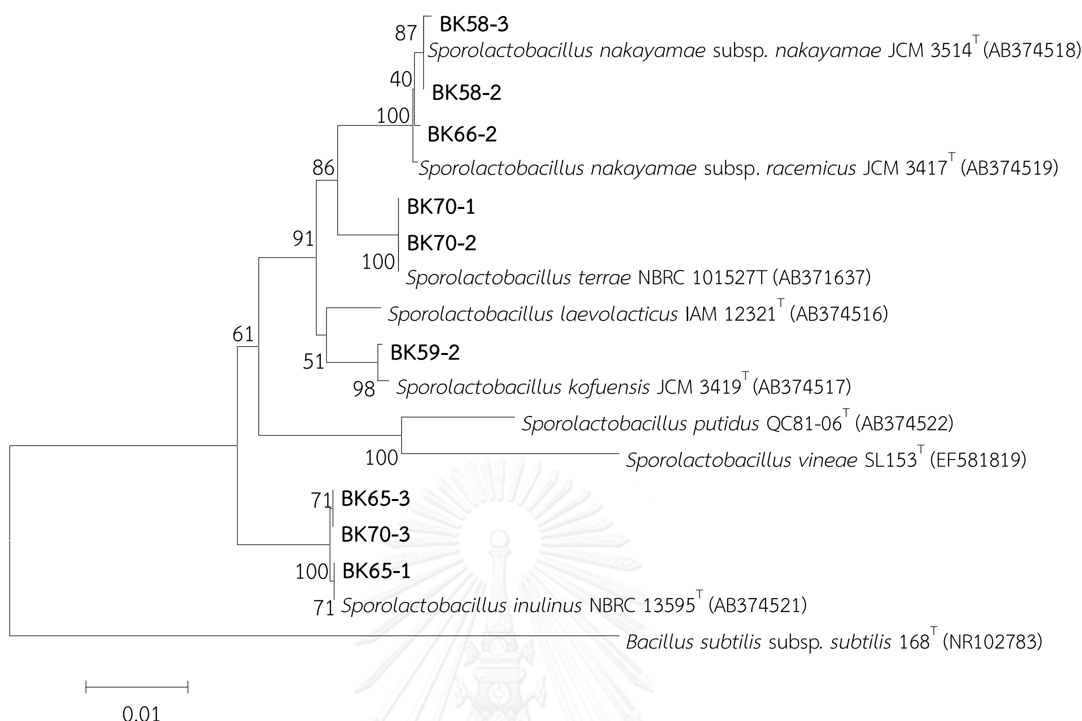

Figure 4.20 Phylogenetic relationships based on 16S rRNA gene sequences using the neighbour-joining method showing the position of selected isolates from tree bark. Bar, 0.01 substitutions per nucleotide position.

In this study, nine strains isolated from tree barks identified as *Sporolactobacillus,* produced D and DL-lactic acid homofermentatively as reported by Lantinen *et al.* (2011). The isolates were consistent with the characteristics of the genus *Sporolactobacillus* (Kitahara & Suzuki, 1963; Yanagida *et al.,* 1997; Hatayama *et al.,* 2006; Chang *et al.,* 2008; Fujita *et al.,* 2010). Two strains consisted of *Sporolactobacillus inulinus* BK65-3 and *S. inulinus* BK70-3 produced high lactic acid concentrations with high optical purity of D-lactic acid (100%) which exceeds the requirement of lactate polymerization process. Poly(D-lactic acid) have been increasing interested in recent years. Blending of poly (D-lactic acid) and poly(L-lactic acid) can improve the polymer properties such as melting points, provide highly thermostability, slow degradation, highly strength with high modulus (Tsuji, 2005). However, there are few studies on the microbial production of D-lactic acid as

compared to L-lactic acid. LAB strains, *L. coryniformis*, *L. delbrueckii*, engineered*Lplantarum* and *Leuconostoc mesenteroides* B512 have been reported in D-lactic production (Butos *et al.,* 2004; Okano *et al.,* 2009; Coelho *et al.,* 2011; Nakano *et al.,* 2012) and in *Sporolactobacillus* strains were still few reports. Fukushima *et al.* (2004) reported that *S. inulinus* was the potential D-lactic acid producer with highly optical purity. Recent studies of *S. inulinus* on the D-lactic production were investigated by Zheng *et al.* (2012; 2014) and Bai *et al.* (2015). In this study, *S. inulinus* BK65-3 produced high lactic acid (101.42 g/L), %yield (84.52%), productivity (1.41 g/L.h) and optical purity of D-lactic acid (100%ee) while *S. inulinus* BK70-3 produced high lactic acid (117.85 g/L), %yield (98.21%), productivity (1.64 g/L.h) and optical purity of D-lactic acid (100%ee) when using 120 g/L of initial glucose concentrations. *S. inulinus* BK70-3 showed high lactic acid production in comparison with previous reports which showed the lactic acid production of *S. laevolacticus* SK5-2 with lactic acid (112.45 g/L), %yield (93.71%), productivity (1.56 g/L.h) and optical purity of D-lactic acid (98.79%ee) at the same condition (Prasirtsak, 2011). The highest lactic acid concentrations of *S. inulinus* BK65-3 and strain BK70-3 were obtained when using 140 g/L of glucose concentrations and provided 134.64 g/L and 135.59 g/L of lactic acid, respectively. The optimum condition of D-lactic acid production of *S. inulinus* BK65-3 was 140 g/L of glucose concentrations at 48 h without shaking and provided lactic acid concentrations (131.45 g/L), %yield (93.89%), productivity (2.74 g/L.h) and optical purity of D-lactic acid (97.26%ee) while optimum condition of D-lactic acid production of *S. inulinus* BK70-3 was found at same condition as strain BK65-3 but without shaking and provided lactic acid concentrations (133.79 g/L), %yield (95.56%) productivity (2.79 g/L.h) and optical purity of D-lactic acid (98.53%ee). These results of *S. inulinus* BK65-3 and strain BK70-3 in flask scale were found greater than previous studies which showed in Table 4.4. The optical purity of D-lactic acid of *S. inulinus* BK65-3 and strain BK70-3 was decreased from 100%ee to 97.26%ee and 98.53%ee, respectively when fermentation parameters such as glucose concentrations, fermentation time and shaking were changed. Sawai *et al.* (2011) reported that optical purity of D-lactic acid in *S. inulinus* was increased by increasing of fermentation time because the isomerization reaction

for L-lactic acid to D-lactic was increased. Although the effect of other factor on the optical purity of D-lactic acid is not elucidated, changing of optical purity may be involved either the D-lactate dehydrogenase gene or enzyme which was responsible for the specific D-lactic acid production or involved in isomerization reaction (Wang *et al.,* 2014). In 5-L fermentor, *S. inulinus* BK70-3 was selected to optimize the lactic acid production because they provided high lactic acid concentration and suitable for agitation in fermentor due to their tolerance when shaking. The optimum conditions of lactic acid production of *S. inulinus* BK70-3 was 140 g/L of glucose concentrations at 45 h and provided lactic acid concentrations (131.63 g/L), %yield (94.02%), productivity (2.93 g/L.h) and optical purity of D-lactic acid (98.89%ee). The production of lactic acid in fermentor was found similar in flask scale exception with the productivity which was improved (Table 4.4). The fermentation time was quite long due to long lag phase fermentation (about 18 h), however, fermentation time could be improved with the increasing of initial inoculum concentrations (Panesar *et al.,* 2010). Furthermore, *S. inulinus* BK70-3 could tolerant at high glucose concentrations up to 200 g/L in batch fermentor which was interesting finding because cell growth and lactic acid production were usually absolute inhibited at high glucose concentrations as reports in the study of Li *et al.* (2013). However, the effect of high glucose concentrations on the lactic acid production of *S. inulinus* BK70-3 were observed. The results showed that the cell growth, glucose consumption and lactic production rate were decreased in 200 g/L of glucose concentrations due to inhibition by high substrate concentration, a conventional property of batch fermentation. Therefore, the glucose concentrations of 140 g/L were suitable for lactic acid production of *S. inulinus* BK70-3 in batch fermentor. The strain *S. inulinus* BK70-3 could be considered as the efficient strain for lactic acid production.

Table 4.4 Comparison of lactic acid production by *Sporolactobacillus* sp. between previous studies and this study

Microorganisms	Substrate	Fermentation mode	LA produced (g/L)	Isomer	Optical purity (%ee)	Yield	Productivity (g/L/h)	References
S. inulinus Y2-8	Glucose	Batch	120	D	99.1	-	1.0	Zheng *et al.*, 2012
S.laevolacticus JCM2513	Sugarcane	Cont	67	D	99.8	0.96	12.2	
S. inulinus JCM6014	Sugarcane	Cont	64	D	98.8	0.96	8.9	Sawai *et al.*, 2011
S. terrae ST316	Sugarcane	Cont	62	D	85	-	5.9	
S. inulinus CASD	Peanut + Glucose	Fed batch	207	D	99.3	0.93	3.8	Wang *et al.*, 2011
S. inulinus CASD	Glucose	Rep batch	79.2-87.3	D	-	0.94-0.99	-	Zhao *et al.*, 2010
S. leavolacticus DSM442	Glucose	Fed batch	144.4	D	99.3	0.96	4.13	Li *et al.*, 2013
S. inulinus Y2-8	Glucose	Flask	104.11	D	-	0.77	0.62	Zheng *et al.*, 2014
Sporolactobacillus sp. YBS1-5	Glucose	Flask	125	D	99	-	1.39	Sun *et al.*, 2014
		Fed batch	127	D	-	-	1.72	
S.laevolacticus SK5-2	Glucose	Flask	112.45	D	98.79	0.94	1.56	Prasirtsak, 2011
*S. Inulinus*BK65-3	Glucose	Flask	131.45	D	97.26	0.94	2.74	
*S. Inulinus*BK70-3	Glucose	Flask	133.79	D	98.53	0.96	2.79	In this study
		Bacth	131.63	D	98.89	0.94	2.93	

Rep batch = Repeated batch; Cont = Continuous fermentation; LA = Lactic acid concentration

Furthermore, two Gram-stain positive, endospore-forming lactic acid bacteria, designated BK92^T and BK117-1^T, which were isolated from tree barks were proposed as novel species of the genus *Sporolactobacillus*. Strain BK92^T was isolated from White-Meranti (*Shorea talura* Roxb.) tree bark in Rayong province, Thailand. Strain BK117-1^T was isolated from African Tulip (*Spathodea campanulata* P.Beauv.) tree bark in Bangkok, Thailand. They were Gram-stain positive and facultatively anaerobic bacteria. Colonies were circular, smooth and white. Cells were motile and endospore-forming rod-shaped (Figure 4.21). They produced D-lactic acid. They grew at 20-40 °C, pH 4.5-8.0 and 1-2% (w/v) NaCl but did not grow at 42, 45, 50 °C, pH 3.0, 4.0 and 3, 5, 7 and 9% (w/v) NaCl. The optimum growth occurred at 30 °C and pH 6.0-7.0. They were negative for catalase, oxidase and nitrate reduction. The API 50 CH and API ZYM results of strains BK92^T and BK117-1^T were shown in Table 4.5. Differential characteristics of strain BK92^T and BK117-1^T were found in growth at 20 °C, in 2 % NaCl, some carbohydrate utilization and enzyme activity including D-cellobiose, esculin, sucrose, D-tagatose and cysteine arylamidase.

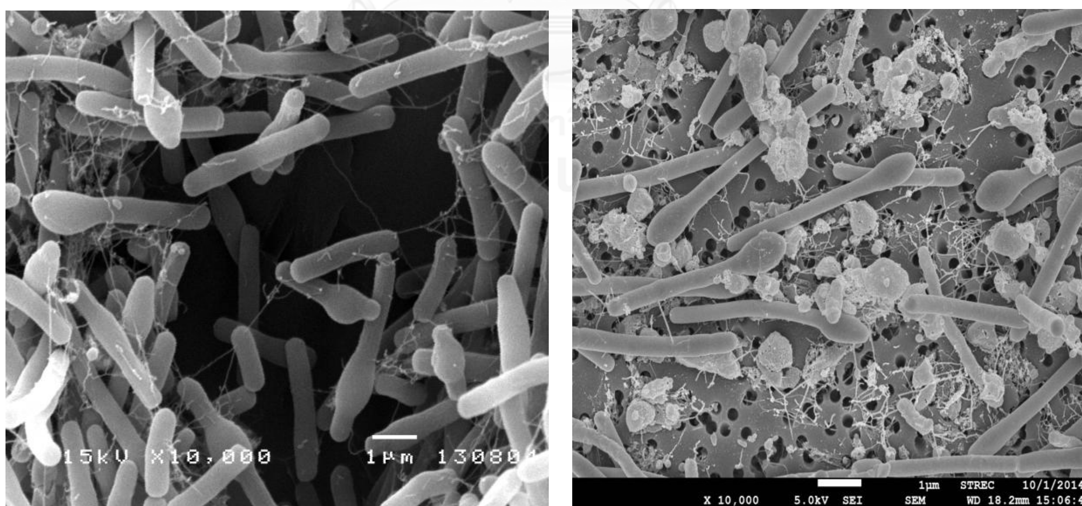

Figure 4.21 Scanning electron micrographs of strains BK92^T (a) and BK117-1^T (b) grown on modified GYP agar for 7 days

Both of them contained *meso*-diaminopimelic acid in cell wall type peptidoglycan. The results were consistent with the characteristics of the genus *Sporolactobacillus* (De Vos, P. *et al.,* 2009). The menaquinone (MK-7) was found in strains BK92^T and BK117-1^T. The major fatty acids of strain BK92^T were anteiso-C17:0 (61.5%) and anteiso-C15:0 (11.6%) while strain BK117-1^T were anteiso-C15:0 (33.8%) and anteiso-C17:0 (30.4%) (Table 4.6). The results were in agreement to the cellular fatty acid of the genus *Sporolactobacillus* which were comprised anteiso-C15:0 and anteiso-C17:0 predominantly. The G + C contents of DNA strains BK92^T and BK117-1^T were 46.6 mol% and 47.4 mol%, respectively, that were in the range (43 to 50 mol%) of the genus *Sporolactobacillus* (De Vos, P. *et al.,* 2009).

Table 4.5 Differential characteristics of strain BK92^T, BK117-1^T and *Sporolactobacillus* species

Strains: 1, BK92^T; 2, BK117-1^T; 3, *S. putidus* JCM 15325^T; 4, *S. vineae* JCM 14637^T; 5, *S. inulinus* JCM 6014^T; 6, *S. terrae* JCM 3516^T; 7, *S. nakayamae* subsp. *nakayamae* JCM 3514^T; 8, *S. kofuensis* JCM 3419^T; 9, *S. nakayamae* subsp. *racemicus* JCM 3417^T; 10, *S. laevolacticus* JCM 2513^T. All phenotypic data were determined in this study. +, positive; w, weak positive; -, negative reaction.

Characteristics	1	2	3	4	5	6	7	8	9	10
Growth temperature range (° C)	25-40	20-40	30-45	25-42	20-45	20-45	20-40	20-42	20-42	20-40
Optimum growth temperature (° C)	30	30	37	37	30	30	30	30	30	30
pH range for growth	4.5-8.0	4.5-8.0	5.0-8.0	4.5-9.0	4.5-9.0	4.5-9.0	4.5-9.0	4.5-9.0	6.0-9.0	4.5-9.0
Optimum pH for growth	6.0	6.0-7.0	5.0	5.0	6.0-7.0	6.0-7.0	5.0-6.0	6.0-7.0	8.0-9.0	6.0-7.0
Growth in 2 % NaCl	w	-	-	+	+	+	+	+	+	w
3 % NaCl	-	-	-	+	+	+	+	+	+	-
Acid production from:										
Amidon	-	-	-	-	-	-	-	-	-	w
Amygdalin	-	-	-	-	-	-	-	-	+	w
D-Cellobiose	+	-	-	-	-	-	-	-	+	-
Esculin	-	+	-	-	-	-	-	-	+	-
D-Galactose	+	+	+	-	-	-	-	-	+	w
Inulin	-	-	-	-	w	-	-	-	-	w
D-Lactose	-	-	-	-	-	-	-	-	-	w
D-Maltose	-	-	w	+	-	-	+	-	+	+
D-Mannitol	-	-	+	w	+	+	+	+	+	+
D-Melezitose	-	-	-	-	-	-	-	-	-	+
D-Melibiose	-	-	-	-	-	-	-	-	+	+
Methyl-α D-Glucopyranoside	-	-	-	+	+	-	-	-	-	w
D-Raffinose	-	-	-	-	w	-	w	-	+	+
D-Saccharose (sucrose)	+	-	+	+	+	+	+	+	+	+
Salicin	-	-	-	-	-	-	-	-	w	-

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Table 4.5 Differential characteristics of strain BK92^T, BK117-1^T and *Sporolactobacillus* species (Continued)

Strains: 1, BK92^T; 2, BK117-1^T; 3, *S. putidus* JCM 15325^T; 4, *S. vineae* JCM 14637^T; 5, *S. inulinus* JCM 6014^T; 6, *S. terrae* JCM 3516^T; 7, *S. nakayamae* subsp. *nakayamae* JCM 3514^T; 8, *S. kofuensis* JCM 3419^T; 9, *S. nakayamae* subsp. *racemicus* JCM 3417^T; 10, *S. laevolacticus* JCM 2513^T. All phenotypic data were determined in this study. +, positive; w, weak positive; -, negative reaction.

Characteristics	1	2	3	4	5	6	7	8	9	10
Acid production from:										
D-Sorbitol	-	-	-	w	+	-	+	-	-	+
L-Sorbose	-	-	-	+	-	+	+	-	+	+
D-Tagatose	+	-	+	-	+	+	+	+	+	+
D-Trehalose	-	-	+	w	+	+	+	+	+	+
D-Turanose	-	-	w	w	w	-	w	-	-	-
Enzyme activity:										
Acid phosphatase	w	w	+	w	w	w	-	-	w	-
Cysteine arylamidase	w	-	-	-	+	-	-	-	w	-
Esterase (C-4)	-	-	-	-	-	-	-	-	w	-
α-Galactosidase	-	-	-	-	-	-	-	-	+	-
β-Galactosidase	-	-	-	-	-	-	-	-	+	w
α-Glucosidase	-	-	+	w	-	-	-	-	w	-
Leucine arylamidase	+	+	w	+	+	+	+	-	+	+
Naphthol-AS-BI-phosphohydrolase	-	-	-	-	+	-	+	-	w	-
Valine arylamidase	+	+	-	+	+	+	+	-	+	-

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Table 4.6 Cellular fatty acid composition of strains BK92[T], BK117-1[T] and *Sporolactobacillus* species

Strains: 1, BK92[T]; 2, BK117-1[T]; 3, *S. putidus* JCM 15325[T]; 4, *S. vineae* JCM 14637[T]; 5, *S. inulinus* JCM 6014[T]; 6, *S. terrae* JCM 3516[T]; 7, *S. nakayamae* subsp. *nakayamae* JCM 3514[T]; 8, *S. kofuensis* JCM 3419[T]; 9, *S. nakayamae* subsp. *racemicus* JCM 3417[T]; 10, *S. laevolacticus* JCM 2513[T]. All data are shown in a percentage of the total fatty acids. -, not detected.

Fatty acid	1	2	3	4	5	6	7	8	9	10
$C_{14:0}$	-	-	-	0.3	-	0.4	-	-	-	0.4
$C_{16:0}$	5.8	6.7	4.4	5.7	6.8	7.0	6.1	5.5	4.4	5.2
$C_{18:0}$	-	-	-	-	-	0.9	-	-	-	-
iso-$C_{14:0}$	-	0.9	-	-	-	0.6	-	-	1.1	0.7
iso-$C_{15:0}$	4.5	9.7	4.2	15.2	7.5	10.7	5.1	10.3	10.7	11.2
iso-$C_{16:0}$	5.0	3.6	5.1	4.9	2.7	5.7	4.0	6.2	5.4	7.2
iso-$C_{17:0}$	3.4	4.3	5.1	7.6	2.4	7.2	2.4	3.9	4.6	4.6
anteiso-$C_{15:0}$	11.6	33.8	12.7	11.0	31.3	20.4	30.9	27.0	36.4	30.2
anteiso-$C_{17:0}$	61.5	30.4	65.8	50.7	44.7	33.6	38.7	44.5	33.6	36.1
$C_{18:1}$ ω9c	8.2	9.6	2.8	4.2	4.6	12.3	11.6	2.8	3.8	4.4
Summed feature 10	-	1.0	-	0.4	-	1.3	1.3	-	-	-

Based on the EzTaxon-e blast analysis, strains BK92[T] and BK117-1[T] were the members of the genus *Sporolactobacillus*. They exhibited 97.1% 16S rRNA gene sequence similarity between BK92[T] and BK117-1[T]. Strain BK92[T] (1,372 bps) showed 97.7 % 16S rRNA gene sequence similarity with *S. putidus* QC81-06[T], 96.9 % with *S. terrae* JCM 3516[T], 96.6% with *S. vineae* SL153[T], 96.4% with *S. kofuensis* JCM 3419[T] and *S. laevolacticus* IAM 12321[T], 96.1% with *S. nakayamae* subsp. *nakayamae* JCM 3514[T], 96.0% with *S. nakayamae* subsp. *racemicus* JCM 3417[T] and 95.9 % with *S. inulinus* NBRC 13595[T]. Strain BK117-1[T] (1,351 bps) showed 97.1 % 16S rRNA gene sequence similarity with *S. putidus* QC81-06[T] and *S. terrae* JCM 3516[T], 96.9 % with *S. kofuensis* JCM 3419[T], 96.8% with *S. nakayamae* subsp. *nakayamae* JCM 3514[T], 96.7% with *S. nakayamae* subsp. *racemicus* JCM 3417[T] and *S. laevolacticus* IAM 12321[T], 96.3 % with *S. inulinus* NBRC 13595[T] and 95.0% with *S. vineae* SL153[T]. The

neighbour-joining phylogenetic tree based on 16S rRNA gene sequence (Figure 4.22) indicated that strains BK92^{T} and BK117-1^{T} formed a distinct branch within the clade of the genus *Sporolactobacillus*. They showed the closest relatives with the *S. putidus* QC81-06^{T} and *S. vineae* SL153^{T}. According to *gyrB* gene sequence analysis, strains BK92^{T} (1,096 bps) and BK117-1^{T} (1,039 bps) exhibited 74.0 % identical. Strain BK92^{T} showed 80.9 % *gyrB* gene sequence similarity with *S. vineae* SL153^{T}, 78.0% *S. putidus* QC81-06^{T}, 75.6% with *S. kofuensis* JCM 3419^{T}, 75.5% with *S. laevolacticus* IAM 12321^{T}, 75.4% with *S. nakayamae* subsp. *racemicus* JCM 3417^{T}, 74.9% with *S. nakayamae* subsp. *nakayamae* JCM 3514^{T}, 74.7% *S. terrae* JCM 3516^{T} and 73.5% with *S. inulinus* NBRC 13595^{T}. Strain BK117-1^{T} showed 77.0 % *gyrB* gene sequence similarity with *S. kofuensis* JCM 3419^{T}, 76.3% with *S. inulinus* NBRC 13595^{T}, 75.0% with *S. vineae* SL153^{T}, 75.7% with *S. laevolacticus* IAM 12321^{T} and *S. terrae* JCM 3516^{T}, 75.5% with *S. putidus* QC81-06^{T}, 75.3% with *S. nakayamae* subsp. *racemicus* JCM 3417^{T}, and 75.0% with *S. nakayamae* subsp. *nakayamae* JCM 3514^{T}. The *gyrB* gene phylogenetic tree using neighbor-joining was showed in Figure 4.23. They exhibited similar topologies with the 16S rRNA gene phylogenetic tree. Strains BK92^{T} and BK117-1^{T} showed separately phylogenetic position with related species.

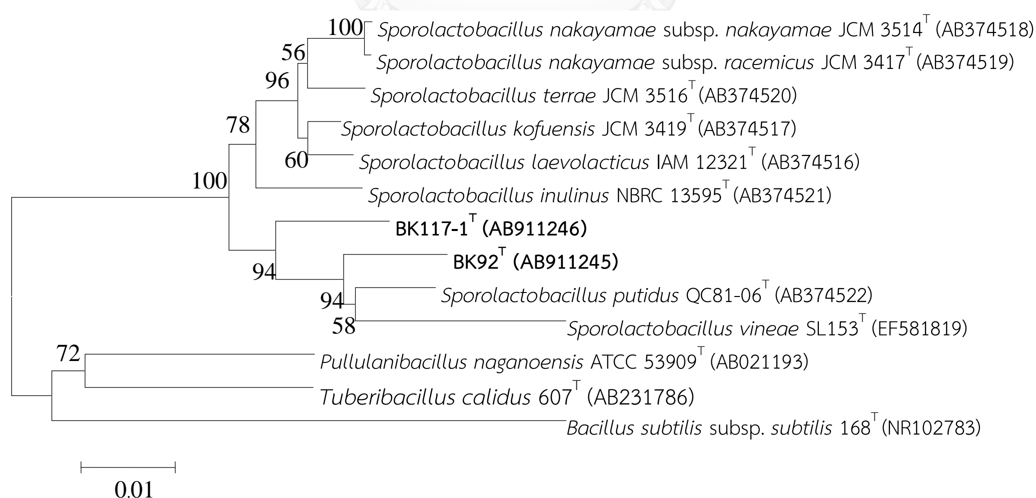

Figure 4.22 Phylogenetic relationships based on neighbour-joining analysis of 16S rRNA gene sequences of strains BK92^{T}, BK117-1^{T} and related *Sporolactobacillus* species. Closely related genera are used as the outgroup. Bar 0.01 substitutions per nucleotide position. Bootstrap values are expressed as percentages of 1000 replications

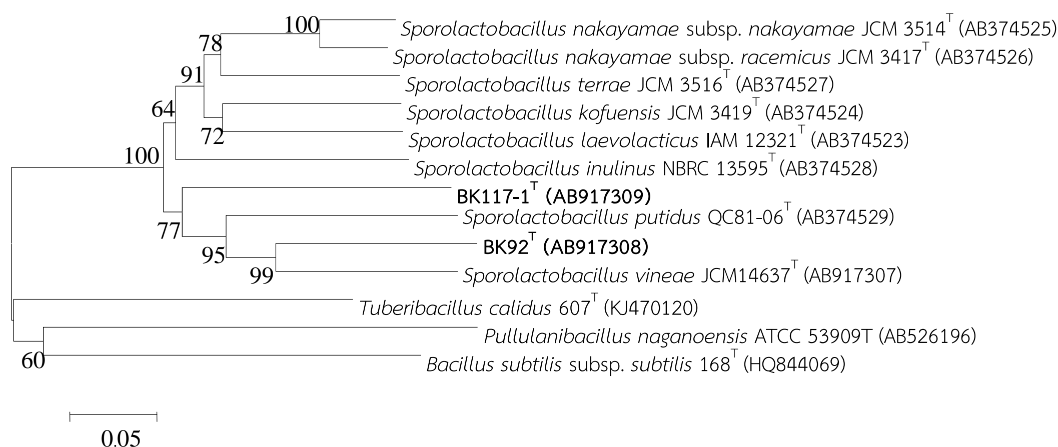

Figure 4.23 Phylogenetic relationships based on neighbour-joining analysis of *gyrB* gene sequences of strains BK92^{T}, BK117-1^{T} and related *Sporolactobacillus* species. Closely related genera are used as the outgroup. Bar 0.05 substitutions per nucleotide position.

The strain BK92^{T} showed 8.0 and 8.0% DNA-DNA relatedness to *S. putidus* QC81-06^{T} and to *S. vineae* SL153^{T}, while strain BK117-1^{T} exhibited 5.0 and 8.0% DNA-DNA relatedness to *S. putidus* QC81-06T and to *S. vineae* SL153^{T}. Strains *S. putidus* QC81-06^{T} and *S. vineae* SL153^{T} exhibited reciprocally low DNA-DNA relatedness (7-11%) to strains BK92^{T} and BK117-1^{T}. The DNA-DNA relatedness of strains BK92^{T} and BK117-1^{T} with their closest phylogenetic neighbours was well below the 70% cut-off point recommended for the assignment of the strains to the same genomic species (Wayne *et al.,* 1987). Comparative DNA-DNA relatedness (7-21%) between BK92^{T} and BK117-1^{T} revealed that they were also distinct species.

On the basis of phenotypic, genetic characteristics and phylogenetic analysis, including DNA-DNA relatedness, they provided clear evidence that strains BK92^{T} and BK117-1^{T} represent novel species of the genus *Sporolactobacillus*, for which the names *Sporolactobacillus shoreae* sp.nov. and *Sporolactobacillus spathodeae* sp. nov., respectively, are proposed.

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Description of *Sporolactobacillus shoreae* sp.nov.

Sporolactobacillus shoreae (sho' re.ae. N.L. n. Shorea scientific name of White-Meranti, the source of isolation of the type strain).

Cells are Gram-stain positive, facultative anaerobic, motile, endospore-forming straight rods with rounded end (0.4-0.6 x 1.5-6.6 µm). Colonies are circular, smooth, white and approximately 1.0-2.0 mm in diameter after 72 h. The endospores are oval and terminal or subterminal with swollen sporangia. Produces D-lactic acid from glucose. Catalase, oxidase and nitrate reduction are negative. Grows facultative anaerobically on MRS agar or GYP agar plates and grows well on GYP agar at 30 °C. Grows at 25-40 °C (optimally at 30 °C), at pH 4.5-7.5 (optimally at pH 6.0) and in 1% (w/v) NaCl. Grows slightly in 2% (w/v) NaCl but not at 42, 45, 50 °C, pH 8.0, 9.0 and 3, 5, 7, 9% (w/v) NaCl. Acid is produced from D-cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, N-acetyl glucosamine (weakly), sucrose and D-tagatose but not from D-adonitol, amidon, D- arabinose, L-arabinose, D-arabitol, L-arabitol, arbutin, amygdalin, dulcitol, erythritol, esculin, D- fucose, L-fucose, gentiobiose, glycerol, glycogen, inositol, inulin, D-lactose, D-lyxose, D-maltose, D-mannitol, D-melezitose, D-melibiose, methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside, methyl-β-D-xylopyranoside, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate, D-raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, L-sorbose, D-trehalose, D-turanose, xylitol, D- xylose and L-xylose. In the API ZYM system, positive reactions for acid phosphatase (weakly), cysteine arylamidase (weakly), leucine arylamidase and valine arylamidase but negative for alkaline phosphatase, chymotrypsin, C-4 esterase, C-8 esterase, C-14 lipase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucosaminidase, α-glucosidase, β-glucosidase, β-glucuronidase, α-mannosidase, Naphthol-AS-BI-phosphohydrolase, N-acetyl-β-glucosaminidase, phosphoamidase and trypsin. The predominant fatty acids are anteiso-C17:0 and anteiso-C15:0. Peptidoglycan contains *meso*-diaminopimelic acid. Menaquinone with seven units (MK-7) is detected. The DNA G + C content of the type strain is 46.56 mol%. The type strain is BK92^T (=JCM 19541^T =LMG 28365^T =PCU 336^T =TISTR 2234^T).

Description of *Sporolactobacillus spathodeae* sp.nov.

Sporolactobacillus spathodeae (spatho' de.ae. N.L. n. Spathodea scientific name of African Tulip, the source of isolation of the type strain).

Cells are Gram-stain positive, facultative anaerobic, motile, endospore-forming straight rods with rounded end (0.3-0.4 x 1.5-6.1 µm). Colonies are circular, smooth, white and approximately 2.0 mm. in diameter after 72 h. Grows facultative anaerobically on MRS agar or GYP agar plates but grows well on GYP agar at 30 ^{o}C. The endospores are oval and terminal with swollen sporangia. Produces D-lactic acid from glucose. Catalase, oxidase and nitrate reduction are negative. Growth at 20-40 °C (optimally at 30 °C), pH 4.5-8.0 (optimally at pH 6.0-7.0) and 1% (w/v) NaCl but not at 42, 45, 50 °C, pH 3.0, 4.0, 9.0 and 2, 3, 5, 7% and 9% (w/v) NaCl. Hydrolysis of esculin. Acid is produced from D-galactose, D-glucose, D-fructose, D-mannose and N-acetyl glucosamine (weakly) but not from D-adonitol, amidon, D- and L-arabinose, D-arabitol, L-arabitol, arbutin, amygdalin, D-cellobiose, dulcitol, erythritol, D-fucose, L-fucose, gentiobiose, glycerol, glycogen, inositol, inulin, D-lactose, D-lyxose, D-maltose, D-mannitol, D-melezitose, D-melibiose, methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside, methyl-ß-D-xylopyranoside, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate, D-raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, L-sorbose, sucrose, D-tagatose, D-trehalose, D-turanose, xylitol, D-xylose, L-xylose. In the API ZYM system, positive for acid phosphatase (weakly), leucine arylamidase and valine arylamidase but negative for alkaline phosphatase, chymotrypsin, cysteine arylamidase, C-4 esterase, C-8 esterase, C-14 lipase, α-fucosidase α- and β-galactosidase, β-glucosaminidase, α- glucosidase, β-glucosidase, β-glucuronidase, α-mannosidase, Naphthol-AS-BI-phosphohydrolase, N-acetyl-β-glucosaminidase, phosphoamidase and trypsin. The predominant fatty acids are anteiso-C15:0 and anteiso-C17:0. Peptidoglycan contains *meso*-diaminopimelic acid. Menaquinone with seven units (MK-7) is detected. The DNA G + C content of the type strain is 47.41 mol%. The type strain is BK117-1^{T} (= JCM 19542^{T}=LMG 28366^{T} =PCU 337^{T} =TISTR 2235^{T}).

In lactic acid production, two novel species, *S. shoreae* BK92[T] and *S. spathodeae* BK117-1[T], showed low lactic acid concentrations and could not utilized glucose concentrations at 120 g/L (Appendix C, Table 1). Therefore, they were not appropiated for the lactic acid production. Interestingly, many *Sporolactobacillus* was isolated from tree barks in Thailand in this study. As previous reports, *Sporolactobacillus* were isolated from chicken feed (Kitahara & Suzuki, 1963), soil and some traditional fermentation beverages and juices (Yanagida *et al.,* 1997; Chang *et al.,* 2008; Fujita *et al.,* 2010). Also, few studies have been reported *Sporolactobacillus* from tree barks. There has been only one report and does not emphasize in *Sporolactobacillus* (Prasirtsak *et al.,* 2013). Therefore, tree barks could be considered as a source of *Sporolactobacillus* isolates.

4.4 Screening of probiotic properties

Forty-one isolates from animal feces, human feces, Thai fermented foods, soy sauce mash and silages were cultivated into MRS medium and evaluated the probiotic properties including the cytotoxicity against cancer cell lines, stimulation of Interleukin (IL)-12 production, the ability of acid and bile tolerance, and adhesion ability of selected lactic acid bacteria.

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- The effect of LAB on the cytotoxicity against cancer cell lines

Forty-one supernatants of isolates including EL1-1, EL1-2, EL1-3, EL2-1, EL2-2, EL2-3, EL3-1, EL4-1, EL4-2, EL4-3, EL5-1, EL5-2, EL5-3, EL6-1, EL6-2, EL7-1, EL7-2, EL8-1, EL8-2, EL9-1, EL10-1, EL10-2, BF13-1, BF13-2, BF13-3, BF14-1, PC72-4, PC73-3, KC74-1, PC75-2, KC78-5, PC79-5, KC81-2, PC86-2, SR7-1, SL4-1, SL7-2, MSMC39-5, MSMC57-2, MSMC63-2 and MSMC120-2 were screened for the cytotoxicity against cancer cell lines. Two type of cancer cell lines; human monocyte leukemic cell lines (U937 cells) and human colorectal adenocarcinoma cell lines (Caco-2 cells) were used to determine the cytotoxic effect by using MTT assay. The effect of LAB supernatants on the cytotoxicity against U937 cells and Caco-2 cells were exhibited

in Table 4.7. The value below at 80% of cell viability was considered as cytotoxic effect. The results showed that no LAB isolate had the cytotoxic effect against U937 cells. The percentage of U937 cells viability of LAB isolates were found in between 80 ± 8.67 to 106.36 ± 4.93 and showed no significant differences as compared with MRS control (Figure 4.24). In contrast with Caco-2 cells, 2 of 41 isolates showed the cell viability less than 80% and had the cytotoxic effect against Caco-2 cells. SR7-1 and SL4-1 showed cytotoxic effect against Caco-2 cells with the percentage of cell viability of 73.88 ± 2.40 and 77.16 ± 0.88, respectively which were significant differences as compared with MRS control (Figure 4.25).

Table 4.7 Effects of LAB supernatants on the cytotoxicity against U937 cells and Caco-2 cells

Isolate no.	Cytotoxic effect		Cell viability (%)	
	U937 cells	Caco-2 cells	U937 cells	Caco-2 cells
MRS broth	-	-	99.71 ± 2.02	91.61 ± 3.33
EL1-1	-	-	93.91 ± 5.98	84.43 ± 2.43
EL1-2	-	-	94.90 ± 3.63	91.97 ± 4.05
EL1-3	-	-	101.06 ± 6.26	98.16 ± 3.31
EL2-1	-	-	93.38 ± 4.57	94.86 ± 2.27
EL2-2	-	-	81.36 ± 9.53	92.70 ± 3.50
EL2-3	-	-	91.92 ± 5.84	90.08 ± 1.85
EL3-1	-	-	80 ± 8.67	89.86 ± 1.93
EL4-1	-	-	80.17 ± 3.59	92.80 ± 4.92
EL4-2	-	-	81.56 ± 4.77	89.79 ± 1.64
EL4-3	-	-	81.66 ± 1.59	88.48 ± 3.01

-, negative effect.

The data were expressed as mean ± SEM of three independent experiments

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Table 4.7 Effects of LAB supernatants on the cytotoxicity against U937 cells and Caco-2 cells (continued)

Isolate no.	Cytotoxic effect		Cell viability (%)	
	U937 cells	Caco-2 cells	U937 cells	Caco-2 cells
EL5-1	-	-	94.21 ± 3.35	92.36 ± 2.66
EL5-2	-	-	82.42 ± 7.43	88.00 ± 8.66
EL5-3	-	-	97.88 ± 2.76	83.80 ± 2.51
EL6-1	-	-	80.03 ± 0.92	90.18 ± 4.48
EL6-2	-	-	87.29 ± 2.53	96.17 ± 3.66
EL7-1	-	-	95.00 ± 4.05	89.62 ± 1.72
EL7-2	-	-	96.36 ± 2.80	95.95 ± 1.65
EL8-1	-	-	80.40 ± 2.65	91.61 ± 3.45
EL8-2	-	-	80.89 ± 3.11	84.43 ± 5.98
EL9-1	-	-	80.70 ± 5.05	91.97 ± 2.58
EL10-1	-	-	87.42 ± 9.26	98.16 ± 3.12
EL10-2	-	-	80.66 ± 2.03	94.86 ± 3.61
BF13-1	-	-	101.22 ± 1.90	92.70 ± 3.88
BF13-2	-	-	86.323 ± 5.59	90.08 ± 3.93
BF13-3	-	-	92.68 ± 3.97	89.86 ± 2.46
BF14-1	-	-	99.74 ± 2.89	92.80 ± 1.90

-, negative effect

The data were expressed as mean ± SEM of three independent experiments

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Table 4.7 Effects of LAB supernatants on the cytotoxicity against U937 cells and Caco-2 cells (continued)

Isolate no.	Cytotoxic effect		Cell viability (%)	
	U937 cells	Caco-2 cells	U937 cells	Caco-2 cells
PC72-4	-	-	98.65 ± 6.06	107.26 ± 13.33
PC73-3	-	-	93.64 ± 7.29	100.11 ± 11.99
KC74-1	-	-	105.02 ± 10.25	110.43 ± 4.35
PC75-2	-	-	103.52 ± 2.92	91.38 ± 4.16
KC78-5	-	-	106.21 ± 4.34	91.38 ± 4.81
PC79-5	-	-	100.30 ± 8.21	96.03 ± 7.33
KC81-2	-	-	102.17 ± 4.78	80.27 ± 2.69
PC86-2	-	-	96.03 ± 1.70	97.51 ± 8.16
SR7-1	-	+	98.95 ± 9.14	73.88 ± 2.40
SL4-1	-	+	97.83 ± 1.32	77.16 ± 0.88
SL7-2	-	-	92.67 ± 4.29	82.09 ± 3.02
MSMC39-5	-	-	105.24 ± 4.93	108.28 ± 5.04
MSMC57-2	-	-	106.36 ± 4.93	94.78 ± 7.35
MSMC63-2	-	-	97.98 ± 2.36	84.13 ± 6.79
MSMC120-2	-	-	105.91 ± 5.18	112.13 ± 6.51

+, positive effect; -, negative effect

The data were expressed as mean ± SEM of three independent experiments

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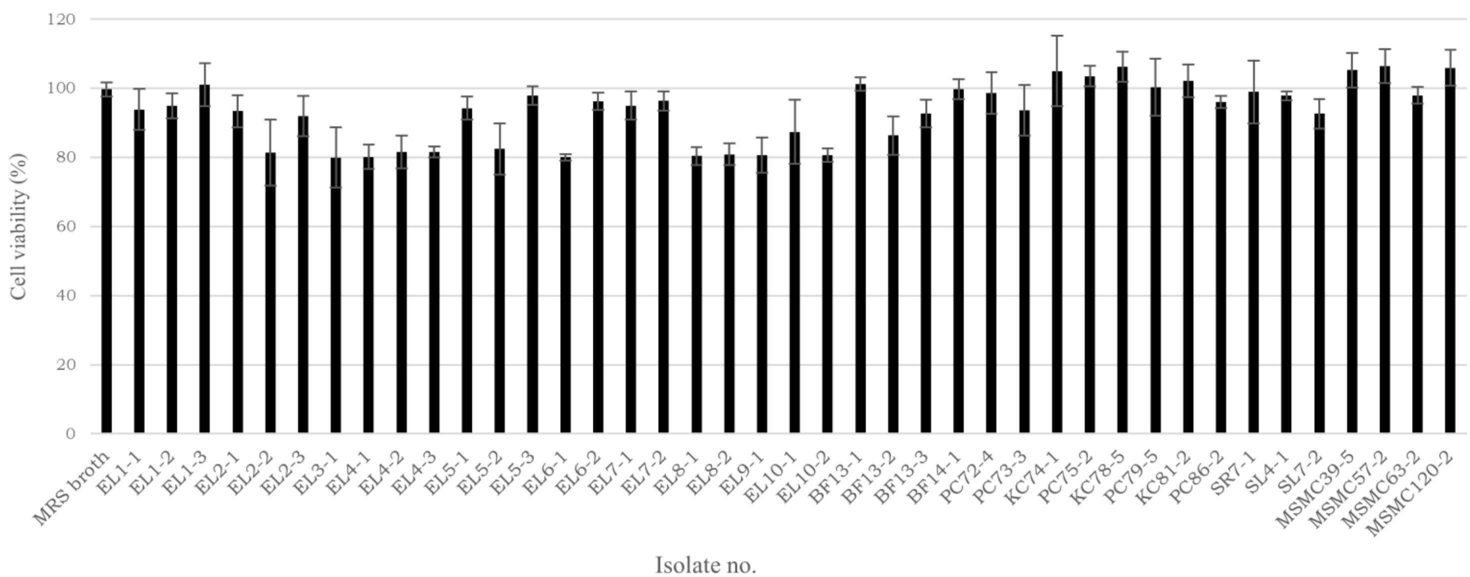

Figure 4.24 Effect of LAB supernatants on the cell viability of U937 cells

Bar indicated standard error of mean (SEM)

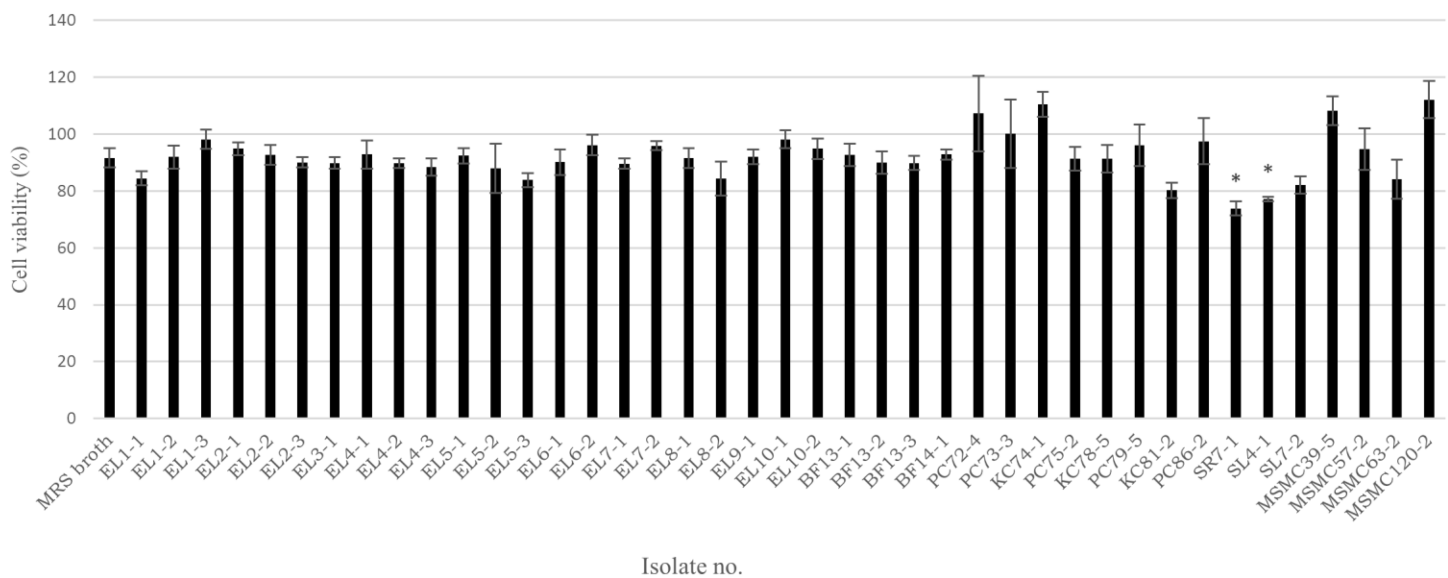

Figure 4.25 Effect of LAB supernatants on the cell viability of Caco-2 cells.

Bar indicated standard error of mean (SEM). Asterisks denote significant differences as compared with MRS control *(p<0.01)

The isolates SR7-1 and SL4-1 which showed the cytotoxic effect against Caco-2 cells were selected to test with Vero normal cells. The effect of SR7-1 and SL4-1 on the cytotoxicity and cell viability against Vero cells were shown in Table 4.8. They showed no cytotoxic effect against Vero cells with the percentage of cell viability of 94.24 ± 3.17 and 90.55 ± 2.12, respectively and no significant differences as compared with MRS control (Figure 4.26).

Table 4.8 Effect of LAB supernatants on the cytotoxicity against Vero cells.

Isolate no.	Cytotoxic effect	Cell viability (%)
MRS broth	-	89.93 ± 6.46
SR7-1	-	94.24 ± 3.17
SL4-1	-	90.55 ± 2.12

-, negative effect

The data were expressed as mean ± SEM of three independent experiments

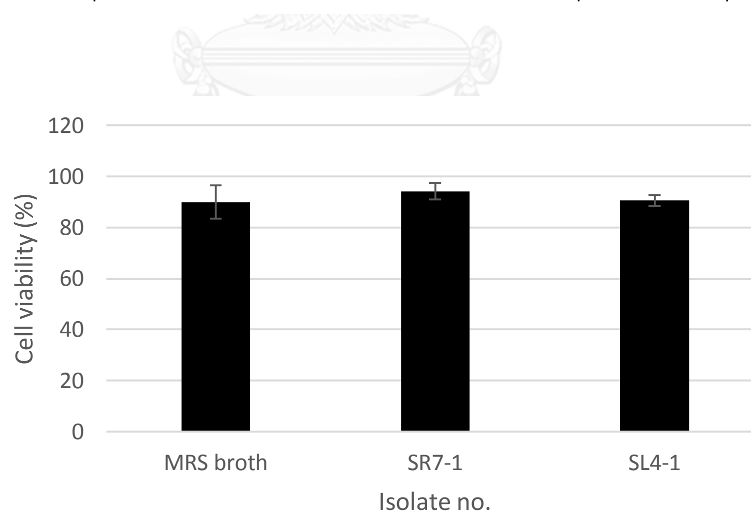

Figure 4.26 Effect of LAB supernatants on the cell viability of Vero cells

Bar indicated standard error of mean (SEM) of three independent experiments

- The effect of selected LAB on the induction of Interleukin (IL)-12

Fifteen isolates of LAB isolated from Thai fermented foods, soy sauce mash, silage and healthy human feces were selected to evaluate the induction of Interleukin (IL)-12 production. The concentrations of IL-12 was determined by using cytokine-specific sandwich ELISA and interpolated from a standard curve as shown in Appendix C, Figure 1. The concentrations of IL-12 were expressed as pg/ml of culture medium. The results revealed that all isolates had the ability to induce IL-12 production with significantly different from control (-28.20 ± 1.69 pg/ml), and were likewise compared to positive control *L. plantarum* NRIC 1067 (170.28 ± 31.84 pg/ml). They produced IL-12 cytokine with distinct degrees as shown in Table 4.9 and Figure 4.27. The isolate PC86-2 showed the greatest ability to induce IL-12 production (1585.23 ± 7.80 pg/ml) while isolate MSMC39-5 showed the lowest ability to induce IL-12 production (430.65 ± 35.02 pg/ml). The ability to induce IL-12 production of isolates was followed by PC86-2, KC81-2, MSMC63-2, PC79-5, MSMC57-2, SR7-1, KC74-1, PC73-3, MSMC120-2, SL7-2, PC75-2, PC72-4, KC78-5, SL4-1 and MSMC39-5 with IL-12 concentrations of 1585.23 ± 7.80, 1454.64 ± 27.89, 1396.15 ± 13.87, 1311.25 ± 33.31, 1164.29 ± 25.73, 1098.18 ± 55.60, 1084.41 ± 26.90, 1062.28 ± 12.61, 795.04 ± 15.34, 780.30 ± 58.23, 727.15 ± 12.25, 658.05 ± 10.93, 654.24 ± 18.54, 440.61 ± 14.21 and 430.65 ± 35.02 pg/ml, respectively.

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Table 4.9 Induction of Interleukin (IL)-12 production of the isolates

Isolate no.	IL-12 concentrations (pg/ml)
Control	-28.20 ± 1.69
PC72-4	658.05 ± 10.93
PC73-3	1062.28 ± 12.61
KC74-1	1084.41 ± 26.90
PC75-2	727.15 ± 12.25
KC78-5	654.24 ± 18.54
PC79-5	1311.25 ± 33.31
KC81-2	1454.64 ± 27.89
PC86-2	1585.23 ± 7.80
SR7-1	1098.18 ± 55.60
SL4-1	440.61 ± 14.21
SL7-2	780.30 ± 58.23
MSMC39-5	430.65 ± 35.02
MSMC57-2	1164.29 ± 25.73
MSMC63-2	1396.15 ± 13.87
MSMC120-2	795.04 ± 15.34
L. plantarum NRIC 1067	170.28 ± 31.84

Control, phosphate buffer saline (1XPBS); positive control, *L. plantarum* NRIC 1067. The data were expressed as mean ± SEM of three independent experiments.

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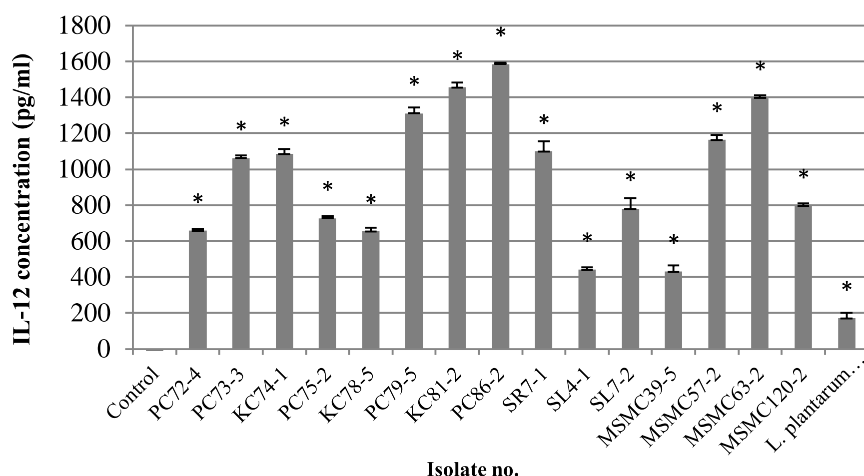

Figure 4.27 Effect of selected isolates on IL-12 production. Bar indicated standard error of mean (SEM). Asterisks denote significant differences as compared with control and positive control *L. plantarum* NRIC 1067 *($p<0.01$)

- Acid tolerance

Fifteen isolates which showed the ability to induce IL-12 production, and showed cytotoxicity against Caco-2 cells with no cytotoxic effect against normal Vero cell lines were selected for assessment of their ability to tolerate at low pH (2.0 and 3.0). The effect of acidic environment on the survival of selected isolates was exhibited in Table 4.10 and Figure 4.28. The results showed that none of all isolates were able to survive at pH 2. Five isolates including PC73-3, KC78-5, SL7-2, MSMC39-5 and MSMC120-2 were able to survive at pH 3 having decrease 0.09, 1.02, 0.17, 1.05 and 0.41 log CFU/ml, respectively as compared to MRS control. The isolate SL4-1 showed strong tolerance at pH 3 having an increase of 0.3 log CFU/ml as compared to MRS control.

Table 4.10 Survival of selected isolates after incubation for 3 h at pH 2 and pH 3

Isolate no.	Number of bacteria (CFU/ml)			Number of bacteria (log CFU/ml)		
	MRS	pH2	pH3	MRS	pH2	pH3
PC72-4	1.00E+06	0.00E+00	0.00E+00	6.00	0.00	0.00
PC73-3	1.06E+07	0.00E+00	8.60E+06	7.03	0.00	6.93
KC74-1	3.00E+06	0.00E+00	0.00E+00	6.48	0.00	0.00
PC75-2	2.83E+07	0.00E+00	0.00E+00	7.45	0.00	0.00
KC78-5	6.55E+06	0.00E+00	6.20E+05	6.82	0.00	5.79
PC79-5	3.26E+07	0.00E+00	0.00E+00	7.51	0.00	0.00
KC81-2	3.95E+07	0.00E+00	0.00E+00	7.60	0.00	0.00
PC86-2	3.80E+07	0.00E+00	0.00E+00	7.58	0.00	0.00
SR7-1	7.73E+07	0.00E+00	0.00E+00	7.89	0.00	0.00
SL4-1	4.50E+06	0.00E+00	9.05E+06	6.65	0.00	6.96
SL7-2	1.50E+06	0.00E+00	1.02E+06	6.18	0.00	6.01
MSMC39-5	2.60E+07	0.00E+00	2.30E+06	7.41	0.00	6.36
MSMC57-2	2.87E+07	0.00E+00	0.00E+00	7.46	0.00	0.00
MSMC63-2	1.75E+07	0.00E+00	0.00E+00	7.24	0.00	0.00
MSMC120-2	1.88E+07	0.00E+00	7.25E+06	7.27	0.00	6.86

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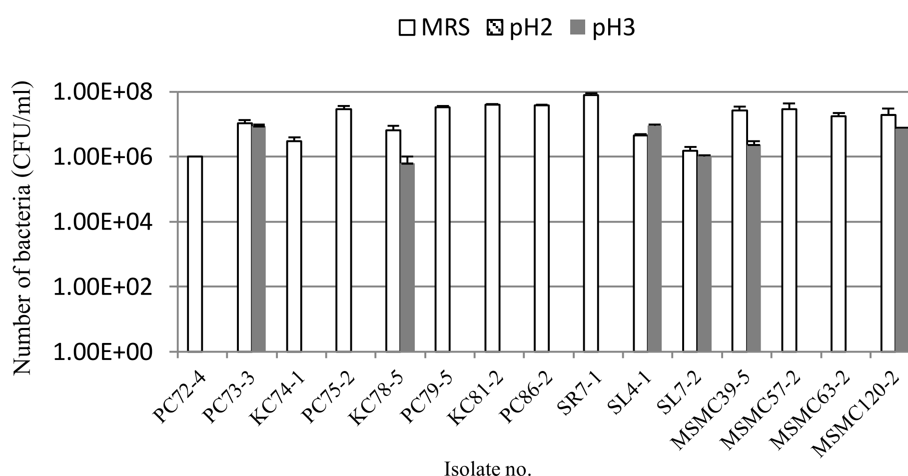

Figure 4.28 Effect of acidic environment on the survival of selected isolates

The data were analyzed after 3 h of incubation in duplicate. Bar indicated standard error of mean (SEM).

- Bile tolerance

Similar to acid tolerance test, 15 of LAB isolates which showed the ability to induce IL-12 production, and showed cytotoxicity against Caco-2 cells with no cytotoxic effect against normal Vero cell lines were selected for assessment of their ability to tolerate in various bile salt concentrations. The effect of bile environment on the survival of selected isolates was exhibited in Table 4.11 and Figure 4.29. The results showed that all isolates were able to survive in the presence of different percentages of bile salts with varying degree of bacterial viability. The viability of isolates were changed by slightly decreased in range of 0.09 to 0.75 log CFU/ml, while some isolates increased cell viability in range of 0.07 to 0.55 log CFU/ml as compared to MRS control. Four isolates including MSMC57-2, PC73-3, PC72-4 and PC79-5 showed strong tolerance in high bile salt by able to survive and increase of 0.54, 0.45, 0.29 and 0.21 log CFU/ml, respectively as compared to MRS control.

Table 4.11 Survival of selected isolates after incubation in MRS broth for 3 h at various bile salt concentrations

Isolate no.	Number of bacteria (CFU/ml)				Number of bacteria (log CFU/ml)			
	MRS	0.3% Bile	0.5% Bile	1.0% Bile	MRS	0.3% Bile	0.5% Bile	1.0% Bile
PC72-4	1.00E+06	3.45E+06	2.90E+06	1.95E+06	6.00	6.54	6.46	6.29
PC73-3	1.06E+07	3.32E+07	3.18E+07	3.01E+07	7.03	7.52	7.50	7.48
KC74-1	3.00E+06	1.50E+06	1.45E+06	9.00E+05	6.48	6.18	6.16	5.95
PC75-2	2.83E+07	1.59E+07	1.21E+07	1.04E+07	7.45	7.20	7.08	7.02
KC78-5	6.55E+06	4.30E+06	3.60E+06	3.25E+06	6.82	6.63	6.56	6.51
PC79-5	3.26E+07	9.80E+07	8.40E+07	5.25E+07	7.51	7.99	7.92	7.72
KC81-2	3.95E+07	2.18E+07	1.94E+07	1.52E+07	7.60	7.34	7.29	7.18
PC86-2	3.80E+07	9.80E+07	5.20E+07	1.95E+07	7.58	7.99	7.72	7.29
SR7-1	7.73E+07	6.27E+07	4.48E+07	3.73E+07	7.89	7.80	7.65	7.57
SL4-1	4.50E+06	2.55E+06	1.90E+06	1.55E+06	6.65	6.41	6.28	6.19
SL7-2	1.50E+06	1.78E+06	6.85E+05	3.00E+05	6.18	6.25	5.84	5.48
MSMC 39-5	2.60E+07	1.90E+07	1.75E+07	1.50E+07	7.41	7.28	7.24	7.18
MSMC 57-2	2.87E+07	3.22E+08	2.10E+08	1.01E+08	7.46	8.51	8.32	8.00
MSMC 63-2	1.75E+07	1.58E+07	5.80E+06	5.10E+06	7.24	7.20	6.76	6.71
MSMC 120-2	1.88E+07	8.10E+06	7.00E+06	3.35E+06	7.27	6.91	6.85	6.53

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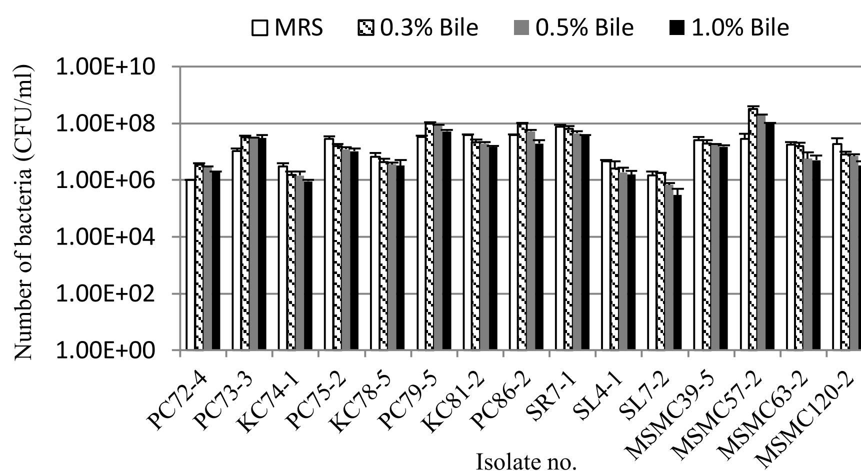

Figure 4.29 Effect of bile environment on the survival of selected isolates
The data were analyzed after 3 h of incubation in duplicate. Bar indicated standard error of mean (SEM)

- Adhesion assays

Six isolates which induced IL-12 production, and showed the cytotoxicity against Caco-2 cell lines and also showed the ability of acid tolerance at low pH and can survive in bile environment were selected to evaluate *in vitro* adhesion property. The adhesion ability of all isolates was shown in Table 4.12. Four of 6 isolates showed higher adhesion ability as compared to positive control *L. rhamnosus* GG (0.71 ± 0.18%) with the percentage of adhesion between 1.5 ± 0.65 to 10.22 ± 2.56. The isolate SL4-1 showed very high percentage of adhesion ability (10.22 ± 2.56%), followed by SL7-2 (2.55 ± 1.44%), PC73-3 (1.91 ± 1.2%) and KC78-5 (1.5 ± 0.65%). The remaining isolates, MSMC39-5 and MSMC120-2, showed low adhesion ability as compared to positive control *L. rhamnosus* GG with the percentage of adhesion ability of 0.03 ± 0.01 and 0.4 ± 0.13, respectively (Figure 4.30).

Table 4.12 Percentage of adhesion of isolates to Caco-2 cells. *L. rhamnosus* GG (LGG) was used as a positive control. Assay was conducted in duplicate of two independent experiments and expressed as mean ± SEM

Isolate no.	% Adhesion
SL4-1	10.22 ± 2.56
SL7-2	2.55 ± 1.44
MSMC39-5	0.03 ± 0.01
PC73-3	1.91 ± 1.2
KC78-5	1.5 ± 0.65
MSMC120-2	0.4 ± 0.13
L. rhamnosus GG	0.71 ± 0.18

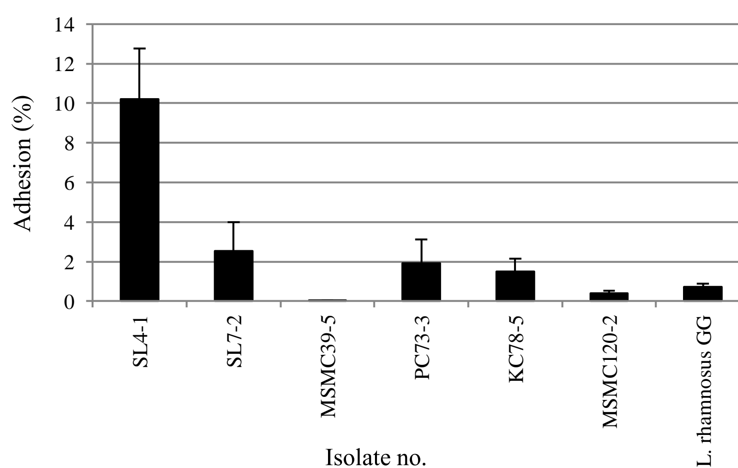

Figure 4.30 Adhesion ability to Caco-2 cell lines of selected isolates Bar indicated standard error of mean

4.5 Identification of selected lactic acid bacteria in probiotic properties

Fifteen isolates which showed probiotic properties were identified using the phenotypic characteristics, chemotypic characteristics and 16S rRNA gene sequencing. They were Gram-positive and catalase-negative. Five isolates were cocci in pairs or in chains that produced gas form glucose and one isolate was cocci with tetrad forming. The remaining isolates (9 isolates) were rods that did not produce gas. The phenotypic and chemotaxonomic characteristics including acid production from carbohydrates, nitrate reduction, arginine hydrolysis, and also growth at high temperatures, at different pH and NaCl concentrations of all isolates were shown in Table 4.13. Based on the genetic characteristics using 16S rRNA gene sequencing and phylogenetic analysis, nine isolates including PC72-4, KC74-1, PC75-2, SR7-1, KC78-5, SL4-1, SL7-2, MSMC39-5, and MSMC120-2 were belong to the genera *Lactobacillus.* The isolates PC72-4 and KC74-1 were identified as *L. futasaii* with 100% similarity of the 16S rRNA gene sequence (1,303 and 1,405 bps). The isolates SR7-1 and PC75-2 were identified as *L. acidipiscis* with 99.78% (1,372 bps) and 99.93% (1,400 bps) similarity of the 16S rRNA gene sequence, respectively. The isolates KC78-5, SL4-1, SL7-2, MSMC39-5, and MSMC120-2 were identified as *L. namurensis*, *L. farraginis*, *L. mucosae*, *L. paracasei* subsp. *tolerans* and *L. salivarius*, respectively with 99.64–100% similarity of the 16S rRNA gene sequence (1,398-1,420 bps). Five isolates including PC79-5, KC81-2, PC86-2, MSMC57-2 and MSMC63-2 were belonged to the genera *Weissella*. The isolates KC81-2 and PC79-5 were identified as *W. thailandensis* with 99.77% (1,371 bps) and 99.78% (1,394 bps) similarity of the 16S rRNA gene sequence, respectively. The isolates PC86-2, MSMC57-2 and MSMC63-2 were identified as *W. cibaria*, *W. confusa* and *W. Paramesenteroides,* respectively with 99.79-100% similarity of the 16S rRNA gene sequence (1,399-1,420 bps). One isolate PC73-3 was belonged to the genera *Pediococcus* and was identified as *P. pentosaceus* with 99.79% (1,436 bps) similarity of the 16S rRNA gene sequence (Figure 4.31).

Table 4.13 Characteristics of isolates from fermented foods, silage and human feces

Characteristics		Isolate no.							
		PC72-4	PC73-3	PC75-2	PC79-5	PC86-2	KC74-1	KC78-5	KC81-2
Cell form		Rods	Cocci	Rods	Cocci	Cocci	Rods	Rods	Cocci
Gas from Glucose		-	-	-	+	+	-	-	+
Nitrate reduction		-	-	-	-	-	-	-	-
Arginine hydrolysis		-	-	-	-	-	-	-	-
Growth at 50 °C		-	+	-	-	-	-	-	-
Growth at	pH 3.5	-	-	-	-	-	+	-	-
	pH 9.0	-	-	-	+	-	+	-	+
Growth in	3% NaCl	+	+	+	+	+	+	+	+
	6% NaCl	+	+	+	+	+	+	+	+
Acid production from:									
D-Amygdalin		-	+	+	-	+	+	-	-
L-Arabinose		-	+	+	+	+	-	-	+
Cellobiose		-	+	+	-	+	+	-	-
D-Fructose		+	+	+	+	+	+	+	+
D-Galactose		+	+	+	+	+	+	+	+
Gluconate		-	-	-	+	+	-	+	+
Lactose		-	-	+	-	-	-	-	-
Maltose		-	-	-	+	+	-	+	+
D-Mannitol		-	-	-	-	-	-	+	-
D-Mannose		+	+	+	+	+	+	-	+
Melibiose		-	-	-	+	-	-	+	+
Methyl-α-D-Glucoside		-	-	-	+	-	-	-	+
Raffinose		-	-	-	+	-	-	-	+
Rhamnose		-	+	-	-	-	-	-	-
Ribose		-	+	+	-	-	-	+	-
Salicin		-	+	+	-	+	+	-	-
Sorbitol		-	-	-	-	-	-	-	-
Sucrose		-	-	-	+	+	+	-	+
Trehalose		-	-	+	+	-	-	-	+
D-Xylose		-	-	-	-	+	-	-	-

+, positive; -, negative reaction.

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Table 4.13 Characteristics of isolates from fermented foods, silage and human feces (Continued)

Characteristics		Isolate no.						
		SR7-1	SL4-1	SL7-2	MSMC 39-5	MSMC 57-2	MSMC 63-2	MSMC 120-2
Cell form		Rods	Rods	Rods	Rods	Cocci	Cocci	Rods
Gas from Glucose		-	-	-	-	+	+	-
Nitrate reduction		-	-	-	-	-	-	-
Arginine hydrolysis		-	-	-	-	-	-	-
Growth at 50 °C		-	-	-	-	-	-	-
Growth at	pH 3.5	-	+	+	+	-	-	+
	pH 9.0	-	-	-	-	+	+	-
Growth in	3% NaCl	+	+	+	+	+	+	+
	6% NaCl	+	+	-	+	+	+	-
Acid production from:								
D-Amygdalin		-	-	-	+	+	-	-
L-Arabinose		+	+	+	-	-	+	-
Cellobiose		+	-	-	+	+	-	-
D-Fructose		+	+	+	+	+	+	+
D-Galactose		+	-	+	+	+	+	+
Gluconate		-	+	+	+	+	+	-
Lactose		-	-	-	+	+	+	+
Maltose		+	-	+	-	+	+	+
D-Mannitol		-	-	-	-	-	-	+
D-Mannose		+	-	-	+	+	+	+
Melibiose		-	-	+	-	+	+	+
Methyl-α-D-Glucoside		+	-	-	-	-	+	-
Raffinose		+	+	+	-	+	+	+
Rhamnose		-	-	-	-	-	-	+
Ribose		+	+	+	+	-	+	+
Salicin		-	-	-	+	+	-	+
Sorbitol		-	-	-	-	-	-	+
Sucrose		-	-	+	+	+	+	+
Trehalose		+	-	-	+	+	+	+
D-Xylose		-	+	+	-	+	+	-

+, positive; -, negative reaction.

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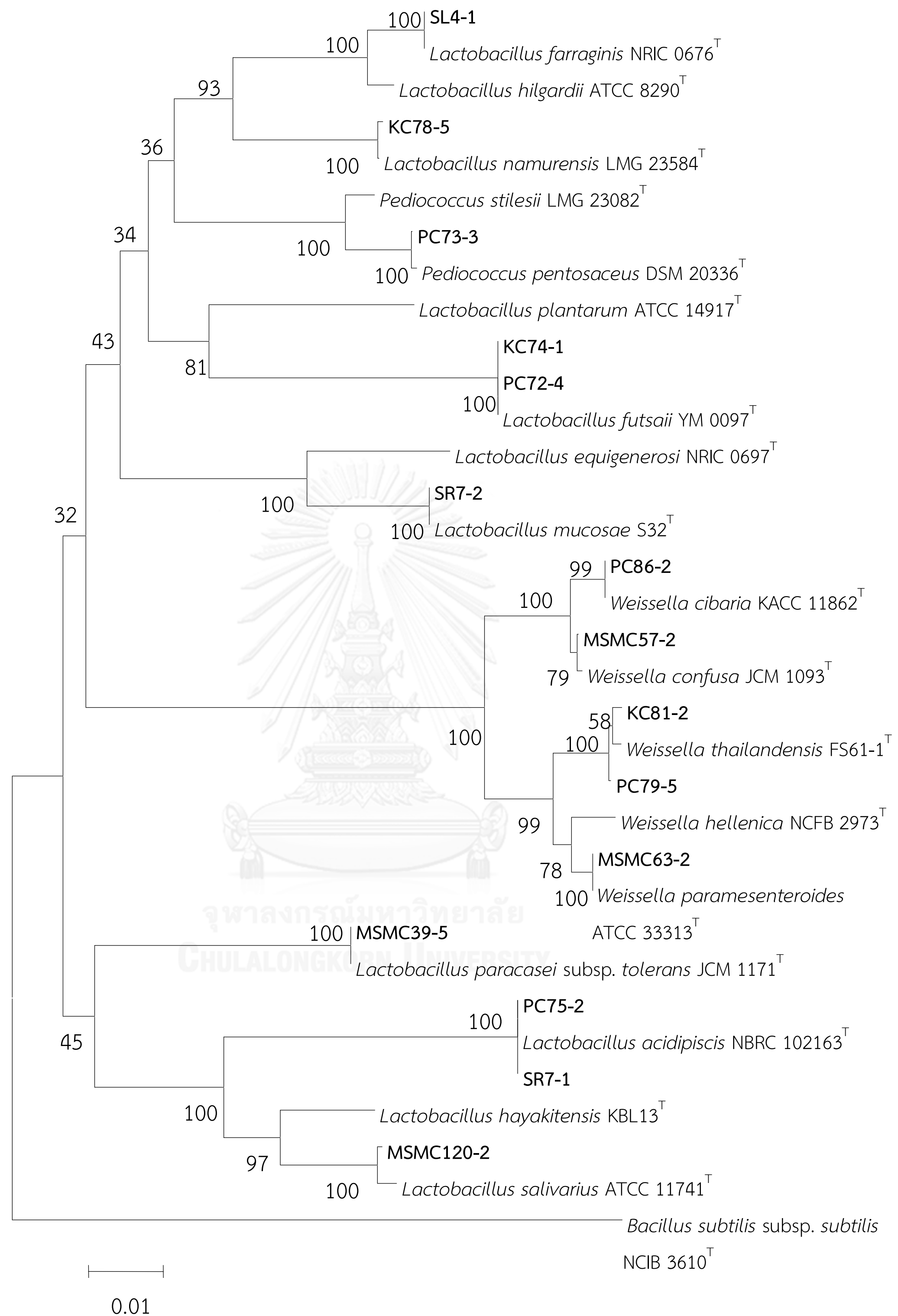

Figure 4.31 Phylogenetic relationships of isolates from fermented foods, silage and human feces based on 16s rRNA gene sequences. The phylogenetic was constructed by the neighbor-joining method. Bar, 0.01 substitutions per nucleotide position.

LAB are widely known as probiotic microorganisms with several beneficially health on the host, particularly bacterial strains in the genus of *Lactobacillus* which have been several investigated and achieved well-documentation (Floch *et al.,* 2011; Patten & Laws, 2015). The role of *Lactobacillus* on the prevention of cancer appears to be an increased interest. Zhang *et al.,* 2013 reported that *Lactobacillus salivarius* REN showed the anti-cancer activity in colorectal cancer in rat via several mechanisms such as inhibited 4-nitroquioline 1-oxide (4NQO) and dimethylhydrazine (DMH)-induced colorectal carcinogenesis, and also induced apoptosis. The supernatants of *L. acidophilus* and *L. casei* showed anti-cancer activity by decreasing of cell proliferation and increasing of cell apoptosis on Caco-2 cells (Soltan *et al.*, 2015). Additional research showed that the secretion of *L. acidophilus* 36YL exhibited the cytotoxic effect against several cancer types including human stomach carcinoma cell, human cervical carcinoma cell, human breast carcinoma cell and human colorectal carcinoma cell (Nami *et al.*, 2014). The soluble factor secretions of *L. reuteri* and *L rhamnosus* GG showed the anti-cancer activity against leukemia-derived cells by the induction of cell apoptosis (Iyer *et al.*, 2008; Chiu *et al.*, 2010). *L. acidipiscis* was first isolated from Thai fermented fish (Tanasupawat *et al.,* 2000) while *L. farraginis* was first isolated from a compost of distilled shochu residue in Japan (Endo and Okada, 2007). In this study, *L. acidipiscis* SR7-1 and *L. farraginis* SL4-1 were isolated from soy sauce mash and silage, respectively. We found that the supernatant of these two strains showed the anti-proliferation on Caco-2 cells with non-toxicity to normal cell lines (Vero cells) and previously studies have not yet been reported about anti-cancer properties of these two strains. The strains *L. acidipiscis* SR7-1 and *L. farraginis* SL4-1 could be used as a probiotic to prevent colorectal cancer development. Unfortunately, none of LAB in this study had the cytotoxic effect against leukemic U937 cells. Furthermore, the induction of Interleukin (IL)-12 productions was evaluated in this study. IL-12 is responsible for anti-cancer activity (Colombo & Trinchieri, 2002; Trinchieri, 2003; Kortylewski *et al.*, 2009). The results exhibited that all LAB isolated from Thai fermented foods, silage and healthy human feces including *L. futasaii* PC72-4, *P. pentosaceus* PC73-3, *L. futasaii* KC74-1, *L. acidipiscis* PC75-2, *L. namurensis* KC78-5,

W. thailandensis PC79-5, *W. thailandensis* KC81-2, *W. cibaria* PC86-2, *L. acidipiscis* SL4-1, *L. farraginis* SR7-1, *L. mucosae* SL7-2, *L. paracasei* subsp. *tolerans* MSMC39-5, *W. confusa* MSMC57-2, *W. paramesenteroides* MSMC63-2, and *L. salivarius* MSMC 120-2 showed the ability to induce IL-12 production with significantly superior effect as compared to *L. plantarum* NRIC 1067. *L. plantarum* have been reported as a potent inducer of IL-12 *in vitro* and *in vivo* studies (Murosaki *et al.,* 2000; Wang *et al.,* 2014). *W. cibaria* PC86-2 showed the greatest ability to induce IL-12 production. Our finding was consistent with the studies of Ahn *et al.* (2013) and Kwak *et al.* (2014) that showed the anticancer activity and immune modulating effect of *W. cibaria* which was isolated from kimchi, Korean traditional food. Additional the beneficial effect, probiotics should be high tolerance to acid and bile, able to adhere and colonize in the gastrointestinal tract (Dunne *et al.,* 2001). *P. pentosaceus* PC73-3, *L. namurensis* KC78-5*, L. farraginis* SL4-1, *L. mucosae* SL7-2, *L. paracasei* subsp. *tolerans* MSMC39-5, and *L. salivarius* MSMC120-2 showed the ability to tolerant at low pH. Interestingly, *L. farraginis* SL4-1 which had the cytotoxic effect against Caco-2 cells and also absence effect with normal cells as well as exhibited the ability to induce IL-12 production showed strongest tolerance in acidic environment and also able to tolerant in high bile salts. In bile tolerance test, all strains were tolerant in 0.3, 0.5 and 1% bile salts. Caco-2 cells express the morphological and physiological characteristics of human enterocytes and are widely used to study adhesion (Sambuy *et al.*, 2005). Six strains, *P. pentosaceus* PC73-3, *L. namurensis* KC78-5*, L. farraginis* SL4-1, *L. mucosae* SL7-2, *L. paracasei* subsp*. tolerans* MSMC39-5, and *L. salivarius* MSMC120-2, which showed positive effect on IL-12 stimulation as well as showed the ability of acid and bile tolerance were selected to evaluate *in vitro* adhesion. Four strains including *P. pentosaceus* PC73-3, *L. namurensis* KC78-5, *L. farraginis* SL4-1 and *L. mucosae* SL7-2 showed higher adhesion ability as compared to positive control *L. rhamnosus* GG. The probiotic *L. rhamnosus* GG has been well established to adhere on the intestinal epithelial cells (Lebeer *et al.*, 2012). The strain *L. farraginis* SL4-1 showed the highest adhesion capacity on Caco-2 cells. *P. pentosaceus* PC73-3, *L. namurensis* KC78-5, *L. farraginis* SL4-1 and *L. mucosae* SL7-2 which meet the criteria of probiotic properties such as acid and bile tolerance as well as adhesion ability

could be used as the potential probiotics for prevention of cancer by stimulation of IL-12 production. In this study, the strain *L. farraginis* SL4-1 was found as the interesting probiotic due to its several positive effects on the host health including anti-cancer activity of colorectal cancer and immunomodulation of IL-12.

Although the effects of twenty-six LAB from animal feces on probiotic properties were not found in this study, the diversity of LAB in elephant and buffalo feces was investigated. Previous studies showed the probiotic *L. fermentum, L. salivarius, L. plantarum,* and *L. reuteri* were isolated from pig feces (Yun *et al.,* 2009) and LAB were found from the foregut of the feral camel (Ghali *et al.,* 2011). However, there is no report of LAB from elephant and buffalo feces.

Twenty-six of LAB isolated from wild elephant and buffalo feces were divided into seven groups based on their phenotypic characteristic clustering and 16S rRNA gene sequence analysis (Figure 4.32). They were Gram-positive, catalase-negative, non-spore forming, non-motility, facultative aerobic and did not produce gas from glucose except the isolates in group VII. They did not reduce nitrate and did not grow at 50°C except the isolates in group IV (Table 4.14). They were belonged to the genera *Enterococcus, Pediococcus, Lactobacillus, Lactococcus* and *Weissella* based on 16S rRNA gene sequence and phylogenetic analysis (Figure 4.33).

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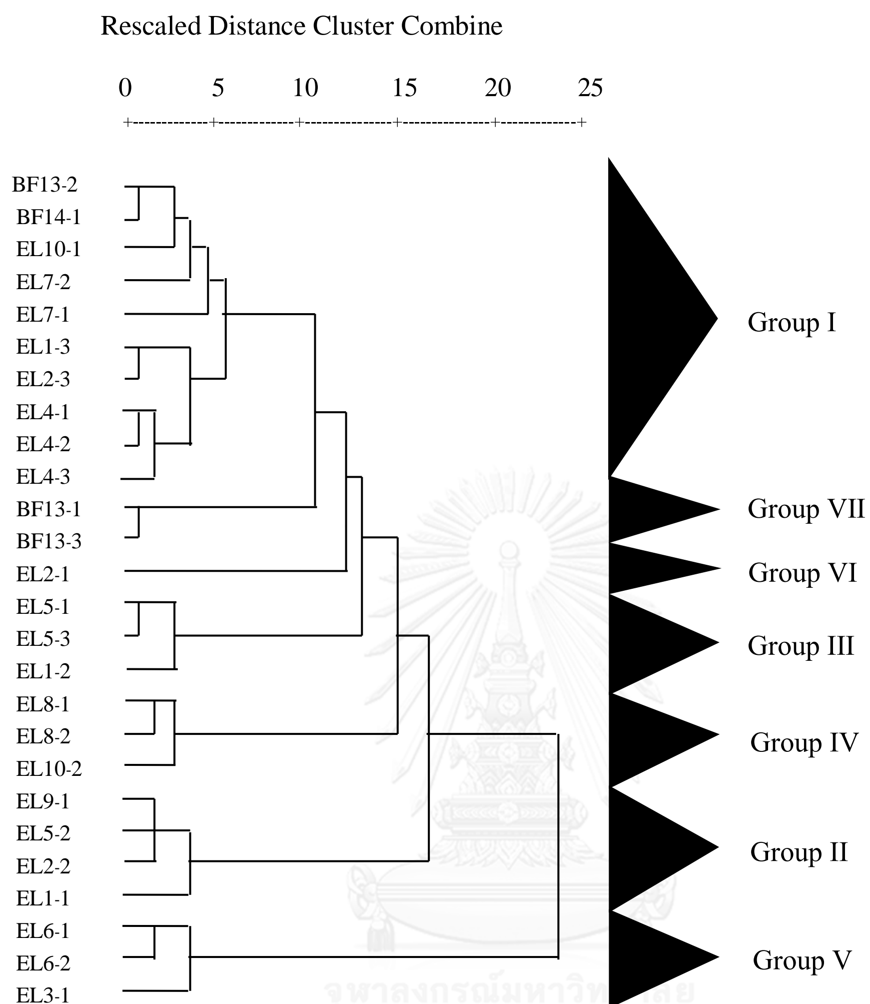

Figure 4.32 Dendogram using average linkage (between groups) showing the hierarchical cluster of isolates from animal feces based on their phenotypic characteristics.

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Group I consisted of ten isolates, which were EL1-3, EL2-3, EL4-1, EL4-2, EL4-3, EL7-1, EL7-2, EL10-1, BF13-2 and BF14-1. They were cocci in chains. They could hydrolyse arginine and grew at pH 8.5, 9.0 and in 3% NaCl but did not grow at pH 4.0. They did not contain *meso*-diaminopimelic acid in cell wall peptidoglycan. Variable growth was found in 6% NaCl and at pH 3.5. They fermented cellobiose, D-fructose, D-galactose, D-mannose, ribose and trehalose but did not ferment L-arabinose, gluconate, D-mannitol, methyl-α-D-glucoside and sorbitol. Variable fermentation was found in raffinose, lactose, maltose, melibiose, rhamnose, salicin, sucrose, and D-xylose (Table 4.15). The 16S rRNA gene sequence of representative strain in this group (EL4-1, EL4-3 and BF14-1) showed 100%, 100% and 99.93% similarity (1,404, 1,380 and 1,408 bps), respectively, to *E. hirae* ATCC 9790^T as shown in Figure 4.33.

Group II consisted of four isolates, which were EL1-1, EL2-2, EL5-2, EL9-1. They were cocci in chains. They grew at pH 8.5, 9.0 and in 3%, 6% NaCl. They did not contain *meso*-diaminopimelic acid in cell wall peptidoglycan. Variable growth was found at pH 3.5 and 4.0. They fermented raffinose, L-arabinose (weakly), cellobiose, D-fructose, D-galactose, gluconate (weakly), maltose, D-mannitol, D-mannose, melibiose, methyl-α-D-glucoside, rhamnose, ribose and sorbitol but did not ferment lactose, trehalose and D-xylose. Variable fermentation was found in salicin and sucrose (Table 4.15). The 16S rRNA gene sequence of representative strain in this group (EL1-1 and EL2-2) showed 99.86% and 99.78% similarity (1,393 and 1,384 bps), respectively, to *E. avium* NCFB 2369^T as shown in Figure 4.33.

Group III consisted of three isolates, which were EL1-2, EL5-1, EL5-3. They were tetrad forming cocci and did not contained *meso*-diaminopimelic acid in cell wall peptidoglycan. Hydrolysis of arginine was negative. They grew at pH 3.5 to pH 9.0 and in 3%, 6% NaCl but did not grow at 50 $^{\circ}$C. They fermented L-arabinose, cellobiose, D-fructose, D-galactose, maltose, D-mannose, melibiose, sucrose, salicin, trehalose and D-xylose but did not ferment raffinose, gluconate, methyl-α-D-glucoside, rhamnose or sorbitol. Variable fermentation was found in lactose, D-mannitol and ribose (Table 4.15). The 16S rRNA gene sequence of representative

strain in this group (EL1-2) showed 99.51% (1,434 bps) similarity to *P. pentosaceus* DSM 20336^T as shown in Figure 4.33.

Group IV consisted of three isolates, which were EL8-1, EL8-2, EL10-2. They were tetrad forming cocci and did not contain *meso*-diaminopimelic acid in cell wall peptidoglycan. They hydrolysed arginine and grew at 50 °C, at pH 4.0, 8.5 and in 3%, 6% NaCl but did not grow at pH 3.5 and 9.0. They fermented L-arabinose, cellobiose, D-fructose, D-galactose, D-mannose, ribose, salicin, trehalose and D-xylose and but did not ferment raffinose, gluconate, lactose, melibiose, methyl-α-D-glucoside, sorbitol and sucrose. Variable fermentation was found in maltose, D-mannitol and rhamnose (Table 4.15). The 16S rRNA gene sequence of representative strain in this group (EL8-2) showed 99.5% (1,411 bps) similarity to *P. acidilactici* DSM 20284^T as shown in Figure 4.33.

Group V consisted of three isolates, which were EL3-1, EL6-1, EL6-2. They were rod-shaped and contained *meso*-diaminopimelic acid in cell wall peptidoglycan. Hydrolysis of arginine was negative. They grew at pH 3.5 to pH 8.5, in 3% and 6% NaCl. Variable growth was found at pH 9. They almost fermented all carbohydrates including raffinose, L-arabinose, cellobiose, D-fructose, D-galactose, lactose, maltose, D-mannitol, D-mannose, melibiose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose and D-xylose. Variable fermentation was found in gluconate and methyl-α-D-glucoside (Table 4.15). The 16S rRNA gene sequence of representative strain in this group (EL3-1 and EL6-2) showed 99.93% and 100% similarity (1,434 and 1,399 bps), respectively, to *L. pentosus* JCM 1558^T as shown in Figure 4.33.

Group VI consisted of one isolate, EL2-1, was cocci in chains. This isoalte could hydrolyse arginine. It did not contain *meso*-diaminopimelic acid in cell wall peptidoglycan and did not grow at pH 3.5 but grew at pH 4.0 to pH 9.0, in 3% and 6% NaCl. The isolate fermented cellobiose, D-fructose, D-galactose, D-mannose, ribose, salicin and trehalose but did not ferment raffinose, L-arabinose, gluconate, lactose, maltose, D-mannitol, melibiose, methyl-α-D-glucoside, rhamnose, sorbitol,

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sucrose, or D-xylose (Table 4.15). The 16S rRNA gene sequence of strain EL2-1 in this isolate showed 100% (1,385 bps) similarity to *Lc. garvieae* ATCC 49156^{T} as in Figure 4.33.

Group VII consisted of two isolates, which were BF13-1, BF13-3. They were cocci in chains. The isolates produced gas from glucose. Hydrolysis of arginine was negative. They did not grow at pH 3.5, 4.0 and 9.0 but grew at pH 8.5, in 3% and 6% NaCl. They did not contain *meso*-Diaminopimelic acid. They fermented raffinose, L-arabinose, cellobiose, D-fructose, D-galactose, gluconate, lactose, maltose, D-mannose, melibiose, methyl-α-D-glucoside, sucrose and trehalose but did not ferment D-mannitol, rhamnose, ribose, salicin, sorbitol or D-xylose (Table 4.15). The 16S rRNA gene sequence of representative strain in this group (BF13-1) showed 99.93% (1,384 bps) similarity to *W. paramesenteroides* ATCC 33313^{T} as in Figure 4.33.

Table 4.14 Phenotypic characteristics of isolates from animal feces

Characteristics		Group I (10)	Group II (4)	Group III (3)	Group IV (3)	Group V (3)	Group VI (1)	Group VII (2)
Cell form		Cocci in chains		Tetrads		Rods	Cocci in chains	
Gas from Glucose		-	-	-	-	-	-	+
Nitrate reduction		-	-	-	-	-	-	-
Arginine hydrolysis		+	-	-	+	-	+	-
Growth at 50 °C		-	-	-	+	-	-	-
Growth at	pH 3.5	- (+3)	+(-2)	+	-	+	-	-
	pH 4.0	-	+(-2)	+	+	+	+	-
	pH 8.5	+	+	+	+	+	+	+
	pH 9.0	+	+	+	-	+(-1)	+	-
Growth in	6% NaCl	+ (-1)	+	+	+	+	+	+
Meso-Diaminopimelic acid		-	-	-	-	+	-	-
Lactic acid isomer		L	L	DL	DL	DL	L	D

+, positive; -, negative reaction; Numbers in parentheses indicate the number of isolates showing the reaction

Table 4.15 Acid production from carbohydrates of isolates from animal feces

Carbohydrate	Isolate in Group						
	I	II	III	IV	V	VI	VII
L-Arabinose	-	+w	+	+	+	-	+
Cellobiose	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+
Gluconate	-	+w	-	-	-(w1)	-	+
Lactose	+(-2)	-(+2)	-(+1)	-	+	-	+
Maltose	+(-3)	+	+	-(+1)	+	-	+
D-Mannitol	-	+	-(+1)	-(+1)	+	-	-
D-Mannose	+	+	+	+	+	+	+
Melibiose	+(-2)	+	+	-	+	-	+
Methyl-α-D-Glucoside	-	+	-	-	+(-1)	-	+
Raffinose	-(+1)	+	-	-	+	-	+
Rhamnose	-(+2)	+	-	+(-1)	+	-	-
Ribose	+	+	+(-1)	+	+	+	-
Salicin	+(-1)	-(+1)	+	+	+	+	-
Sorbitol	-	+	-	-	+	-	-
Sucrose	+(-4)	-(+1)	+	-	+	-	+
Trehalose	+	-	+	+	+	+	+
D-Xylose	-(+2)	-	+	+	+	-	-

+, positive; w, weak positive; -, negative reaction. Numbers in parentheses indicate the number of isolates showing the reaction

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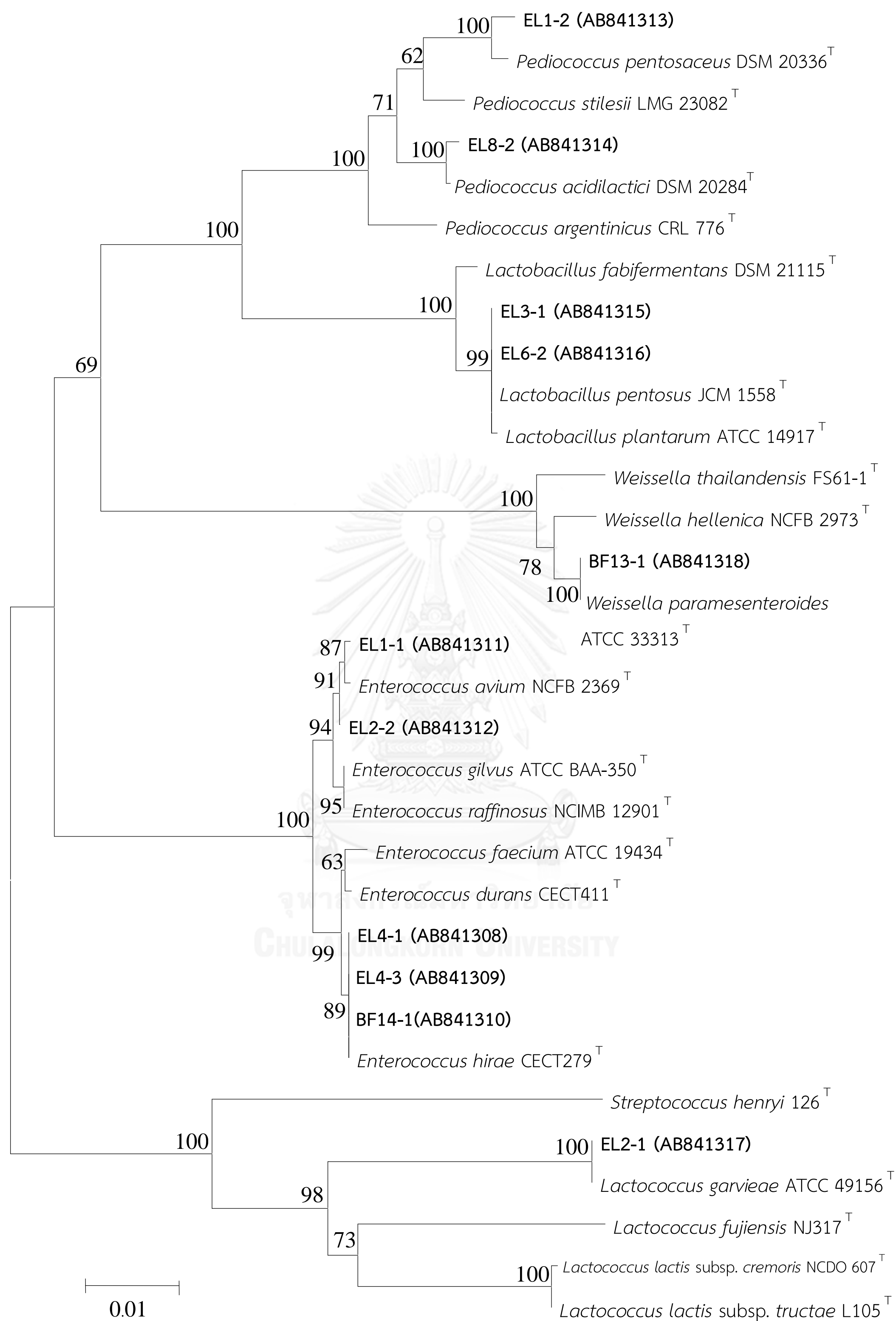

Figure 4.33 Phylogenetic relationships of isolates from animal feces based on 16s rRNA gene sequences. The phylogenetic was constructed by the neighbor-joining method. Bar, 0.01 substitutions per nucleotide position. Bootstrap expressed as percentages of 1000 replications

In this study, we found *E. hirae, E. avium, Lc. garvieae P. pentosaceus, P. acidilactici* and *L. pentosus* strains distributed in elephant feces and only *E. hirae* and *W. paramesenteroides* strains were in buffalo feces. Some strains of *E. hirae, E. avium* and *Lc. garvieae* were the opportunistic and nosocomial pathogens that infected in human (Watanabe *et al.,* 2011). The majority of LAB belonged to the genera *Enterococcus*, 38.46% were *E. hirae*. They were an unusual pathogen in humans causing urinary tract infection and acute pancreatitis (Bourafa *et al.,* 2015; Dicpinigaitis *et al.,* 2015).

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CHAPTER V
CONCLUSIONS

In this study, seventy-seven lactic acid bacteria were isolated and obtained from various sources in Thailand including animal feces, healthy human feces, fermented foods, soy sauce mash, silages and tree barks. Thirty-six isolates were evaluated for D-lactic acid production. While, forty-one isolates were evaluated for the probiotic properties such as the cytotoxic effect against cancer cell lines, stimulation of Interleukin (IL)-12 production, the ability of acid and bile tolerance, and adhesion ability. Fifty-two strains were selected and identified based on the phenotypic, chemotaxonomic and genotypic characteristics. They were identified as *Lactobacillus futasaii* (2 strains), *L. acidipiscis* (2 strains), *L. namurensis* (1 strain), *L. farraginis* (1 strain), *L. mucosae* (1 strain), *L. paracasei* subsp. *tolerans* (1 strain), *L. salivarius* (1 strain), *L. pentosus* (3 strains), *Enterococcus hirae* (10 strains), *E. avium* (4 strains), *Pediococcus pentosaceus* (4 strains), *P. acidilactici* (3 strains), *Lactococcus garvieae* (1 strain), *Weissella paramesenteroides* (3 strains), *W. thailandensis* (2 strains), *W. cibaria* (1 strain), *W. confusa* (1 strain), *Sporolactobacillus nakayamae* subsp. *nakayamae* (3 strains), *S. terrae* (2 strains), *S. kofuensis* (1 strain), *S. inulinus* (3 strains) and two strains isolated from tree barks, designated BK92^T and BK117-1^T, were proposed as *S. shoreae* sp. nov. and *S. spathodeae* sp. nov., respectively. *Sporolactobacillus* were homofermentative LAB which commonly produce exclusively D-lactic acid with high lactic acid concentrations. In lactic acid production, Nine strains from tree barks including BK58-2, BK58-3, BK59-2, BK65-1, BK65-3, BK66-2, BK70-1, BK70-2 and BK70-3 which were belonged to the genus *Sporolactobacillus* and were identified as *S. nakayamae* subsp. *nakayamae* (3 strains), *S. terrae* (2 strains), *S. kofuensis* (1 strain) and *S. inulinus* (3 strains) showed high lactic acid production while two novel species, *S. shoreae* BK92^T and *S. spathodeae* BK117-1^T showed low lactic acid production. Strains, *S. kofuensis* BK59-2, *S. inulinus* BK65-3 and strain BK70-3 produced high optical purity of D-lactic acid with 100 %ee and produced final lactic acid concentrations of 88.01 g/L, 101.42 g/L and 117.85 g/L, respectively when using 120 g/L of initial glucose concentrations for 72 h. The lactic

acid concentrations of *S. inulinus* BK65-3 and strain BK70-3 were increased when using 140 g/L of initial glucose concentrations to 134.64 g/L and 135.59 g/L, respectively. The highest productivity of *S. inulinus* BK65-3 and strain BK70-3 were obtained at 48 h. Thus, the optimum conditions of lactic acid production of *S. inulinus* BK65-3 was 140 g/L of glucose concentrations at 48 h without agitation and provided lactic acid concentrations (131.45 g/L), %yield (93.89%), productivity (2.74 g/L.h) and optical purity of D-lactic acid (97.26%ee) while at same condition *S. inulinus* BK70-3 was required agitation and provided lactic acid concentrations (133.79 g/L), %yield (95.56%) productivity (2.79 g/L.h) and optical purity of D-lactic acid (98.53%ee). The variation of optical purity of D-lactic acid of *S. inulinus* BK65-3 and strain BK70-3 were found when fermentation parameters such as glucose concentrations, fermentation time and shaking were changed. In batch fermentor, the optimum conditions of lactic acid production of *S. inulinus* BK70-3 was 140 g/L of glucose concentrations at 45 h and provided lactic acid concentrations (131.63 g/L), %yield (94.02%) and optical purity of D-lactic acid (98.89%ee). The productivity of *S. inulinus* BK70-3 was improved to 2.93 g/L.h. Furthermore, *S. inulinus* BK70-3 could tolerant at high glucose concentrations up to 200 g/L in fermentor. Therefore, *S. inulinus* BK70-3 was the potential strain for D-lactic acid production. In this study, we found that *Sporolactobacillus* species were distributed in several tree barks of Thailand and thus tree barks could be considered as a source of *Sporolactobacillus* isolates.

Two strains including *L. acidipiscis* SR7-1 and *L. farraginis* SL4-1 from soy sauce mash and silages, respectively showed cytotoxic effect against colorectal cancer cell lines (Caco-2 cells) with non-toxicity to normal cell lines (Vero cells). None of all isolates showed cytotoxic effect against leukemic U937 cells. Fifteen strains from healthy human feces, fermented foods, soy sauce mash and silages including *L. futasaii* PC72-4, *P. pentosaceus* PC73-3, *L. futasaii* KC74-1, *L. acidipiscis* PC75-2, *L. namurensis* KC78-5, *W. thailandensis* PC79-5, *W. thailandensis* KC81-2, *W. cibaria* PC86-2, *L. acidipiscis* SL4-1, *L. farraginis* SR7-1, *L. mucosae* SL7-2, *L. paracasei* subsp. *tolerans* MSMC39-5, *W. confusa* MSMC57-2,

W. paramesenteroides MSMC63-2, and *L. salivarius* MSMC120-2 showed the ability to induce IL-12 production with different degrees but significantly effect as compared to positive control *L. plantarum* NRIC 1067. None of 15 strains could tolerant at pH 2, but some strains including *P. pentosaceus* PC73-3, *L. namurensis* KC78-5, *L. farraginis* SL4-1, *L. mucosae* SL7-2, *L. paracasei* subsp. *tolerans* MSMC39-5, and *L. salivarius* MSMC120-2 could tolerant at pH 3. The tolerance of high bile was found in all strains. Four strains including *P. pentosaceus* PC73-3, *L. namurensis* KC78-5, *L. farraginis* SL4-1 and *L. mucosae* SL7-2 showed higher adhesion ability as compared to positive control *L. rhamnosus* GG. Therefore, *P. pentosaceus* PC73-3, *L. namurensis* KC78-5, *L. farraginis* SL4-1 and *L. mucosae* SL7-2 which meet the criteria of probiotic properties such as acid and bile tolerance as well as adhesion ability could be used as the potential probiotics for prevention of cancer by stimulation of IL-12 production. Especially, *L. farraginis* SL4-1 could be used as the potential probiotics for prevention of specific site such as colorectal cancer due to its anti-proliferation effect. Moreover, this is the first report on the identification of LAB diversity in elephant and buffalo feces as well as investigation of probiotic properties although the negative results were found in cytotoxic effect against cancer cell lines. Further works will be conducted on the useful of these isolates as probiotic strains in other application.

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APPENDIX

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APPENDIX A
Culture medium

GYP broth

Component	Amount	Unit
Glucose	10	g
Yeast extract	5	g
Peptone	5	g
KH_2PO_4	0.25	g
K_2HPO_4	0.25	g
Salt solution	10	g
Distilled water	1	L
pH 4.4		

GYP agar

Component	Amount	Unit
Glucose	10	g
Yeast extract	5	g
Peptone	5	g
KH_2PO_4	0.25	g
K_2HPO_4	0.25	g
Salt solution	10	g
$CaCO_3$	5	g
Distilled water	1	L
pH 6.8		
Agar	20	g

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Salt solution

$MgSO_4.7H_2O$	400	mg
$MnSO_4.5H_2O$	20	mg
$FeSO_4.7H_2O$	20	mg
NaCl	20	mg
Distilled water	10	mL

Preculture medium

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Glucose	10	g
Yeast extract	5	g
Peptone	5	g
KH_2PO_4	0.25	g
K_2HPO_4	0.25	g
Salt solution	10	g
$CaCO_3$	5	g
Distilled water	1	L
pH 6.8		

Fermentation medium

Glucose	120	g
Yeast extract	20	g
Peptone	10	g
KH_2PO_4	0.5	g
K_2HPO_4	0.5	g
Salt solution	20	g

$CaCO_3$	80	g
Distilled water	1	L
pH 6.8		

Glucose and $CaCO_3$ were added after sterilization.

Nitrate reduction medium

Yeast extract	5	g
Peptone	10	g
NaCl	10	g
KNO_3	1	g
Distilled water	1	L
pH 6.8		

Arginine medium

Peptone	1	g
NaCl	5	g
K_2HPO_4	0.3	g
L(+) arginine HCl	10	g
Phenol red	0.01	g
Distilled water	1	L
pH 6.8		
Agar	15	g

Spore formation medium A for strain BK92^T

Glucose	1	g
Yeast extract	10	g

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Peptone	10	g
10% KCl	10	mL
1.2% $MgSO_4.7H_2O$	10	mL
$CaCO_3$	5	g
Distilled water	1	L
pH 6.8		
Agar	15	g

Spore formation medium B for strain BK117-1^T

Glucose	1	g
Yeast extract	0.1	g
Peptone	0.1	g
KH_2PO_4	2.5	g
K_2HPO_4	2.5	g
$(NH_4)_2HPO_4$	1	g
$MgSO_4.7H_2O$	0.2	g
$FeSO_4.7H_2O$	0.1	g
$MnSO_4.7H_2O$	0.1	g
$CaCO_3$	5	g
Distilled water	1	L
pH 6.8		
Agar	15	g

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APPENDIX B
Reagents and buffers

0.85% NaCl

NaCl	0.85	g
Distilled water	100	mL

1X Phosphate buffer saline (PBS buffer)

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.24	g
Distilled water	1	L
pH 7.4		

0.005 mM H_2SO_4

Conc. H_2SO_4	556	µL
Distilled water	2	L

Filtration with 0.45 µm membrane filter and sonication for 30 min.

1 mM $CuSO_4$

CuSO4	0.5	g
Distilled water	2	L

Filtration with 0.45 µm membrane filter and sonication for 30 min.

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Standard solution for total lactic acid

Glucose	0.1	g
Lactate.$5H_2O$	0.1712	g
Ethanol	105.3	µL
Acetic acid	95	µL
Milli-Q water	50	mL

Nitrate reduction test reagents

Sulphanilic acid solution

Sulphanilic acid	0.8	g
5N Acetic acid	100	mL

***N*,*N*-dimethyl-1-naphthylamine solution**

N,*N*-dimethyl-1-naphthylamine	0.5	g
5N Acetic acid	100	mL

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Mixed indicator

Bromthymol blue	0.2	g
Neutral red	0.1	g
Ethanol	300	mL

PCR reaction mixture

	Stock	1 Volume (100 µL)
Forward Primer : 20F	10 pmol/µL	4
Reward Primer : 1530R	10 pmol/µL	4
10 x *Taq* buffer (NH_4SO_4 –$MgCl_2$)	10 x	10
dNTP	2.0 mM	2

$MgCl_2$	25 mM	8
Taq DNA polymerase	5 Unit/μL	0.5
Milli-Q water	-	66.5
Template	Undilute	5

1X Tris-acetate (TAE) buffer

50X Tris-acetate (TAE) buffer	20	mL
Distilled water	980	mL

Ethidium bromide solution (10 mg/mL)

Ethidium bromide	1	g
Distilled water	100	mL

0.8% Agarose gel

Agarose	0.8	g
Distilled water	100	mL

melt the mixture with the microwave.

Reagents and buffers for DNA-DNA hybridization

Pre-hybridization solution (10 mL)

20X SSC	1	mL
50X Denhardt's solution	1	mL
Formamide	5	mL
Sonicated salmon sperm DNA (10 mg/mL)	0.1	mL
Distilled water	2.9	mL

Hybridization solution (10 mL)

20X SSC	1	mL
50X Denhardt's solution	1	mL
Formamide	5	mL
Sonicated salmon sperm DNA (10 mg/mL)	0.1	mL
50% Dextran sulphate solution	0.5	mL
Distilled water	2.4	mL

PBS-BSA-Triton solution (10 mL)

BSA (Bovine serum albumin)	0.05	g
Triton X	10	µL
20X PBS	0.5	mL
Distilled water	9.5	mL

SABG (Streptoavidin-β-galactosidase) solution (10 mL)

PBS-BSA-Triton solution	10	mL
SABG	10	µL

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4-MUF (4 methyllumbelliferyl-*β*-D-galactoside) solution

4-MUF (10 mg/mL)	100	µL
1X PBS	10	mL

Freshly prepare

4-MUF (10 mg/mL)

4-MUF	1	mg
N-*N*-dimethylformamide	100	mL

20X Phosphate buffered saline (PBS)

Na_2HPO_4	28.8	g
NaCl	160	g
KH_2PO_4	4	g
KCl	4	g
Distilled water	1	L
pH 7.2-7.4		

1M Magnesium chloride ($MgCl_2$)

$MgCl_2$	92.5	g
Distilled water	1	L

Phosphate buffered saline-magnesium chloride (PBSMG) solution (10 mL)

20X PBS	0.5	mL
1M $MgCl_2$	1	mL
Distilled water	8.5	mL

Salmon sperm DNA (10 mg/mL)

Salmon sperm DNA	10	mg
TE buffer	1	mL

Dissolve salmon sperm DNA in TE buffer, boil the solution for 10 min, immediately cool in ice and sonicate for 3 min.

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20X Saline sodium citrate (SSC)

NaCl	175.3	g
Sodium citrate	88.2	g
Distilled water	1	L
pH 7.0		

1X Saline sodium citrate (SSC)

20X SSC	50	mL
Sterile distilled water	950	mL

APPENDIX C

Miscellaneous

Formulation of lactic acid production

1. Yield (%)

$$\% \text{ Yield} = \frac{\text{Total lactic acid concentrations (g/L)}}{\text{Initial glucose} - \text{Residual glucose (g/L)}} \times 100$$

2. Productivity (g/L.h)

$$\text{Productivity} = \frac{\text{Total lactic acid concentrations}}{\text{Fermentation time}}$$

3. Optical purity of lactic acid (%ee)

$$\% \text{ Optical purity} = \frac{\text{Area of D-lactic acid} - \text{Area of L-lactic acid}}{\text{Area of D-lactic acid} + \text{Area of L-lactic acid}} \times 100$$

Table 1 Screening of lactic acid production and optical purity of lactic acid

Isolate no.	Aminex HPX-87H – HPLC Concentrations (g/L)		Sumi chiral OA5000 - HPLC Concentrations (g/L)		Area		Optical purity of lactic acid (%ee)	Isomer type
	Glucose	Lactic acid	L-lactic acid	D-lactic acid	L-lactic acid	D-lactic acid		
BK58-2	20.36	89.60	47.01	43.24	704131	660798	ND	DL
BK58-3	0	114.61	62.83	57.19	999244	951876	ND	DL
BK59-2	25.45	88.01	0	86.156	0	1411846	100	D
BK62-2	87.99	12.81	ND	ND	ND	ND	ND	ND
BK65-1	0	113.78	5.56	111.42	83612	1916429	91.64	D
BK65-3	0	101.42	0	99.22	0	1769854	100	D
BK66-2	0	105.18	5.66	99.50	107605	1892457	89.24	D
BK69	74.96	51.63	ND	ND	ND	ND	ND	ND
BK70-1	0	108.14	58.59	53.94	912207	877108	ND	DL
BK70-2	0	118.51	64.87	61.72	102015	996650	ND	DL
BK70-3	0	117.85	0	114.84	0	2003623	100	D
BK90-1	70.98	51.89	ND	ND	ND	ND	ND	ND
BK90-2	118.26	4.31	ND	ND	ND	ND	ND	ND
BK91-2	119.66	0	ND	ND	ND	ND	ND	ND
BK92	111.45	0	ND	ND	ND	ND	ND	ND
BK95-2	110.87	14.29	ND	ND	ND	ND	ND	ND
BK95-3	84.50	45.25	ND	ND	ND	ND	ND	ND
BK96-2	91.51	14.75	ND	ND	ND	ND	ND	ND
BK98-2	84.79	47.52	ND	ND	ND	ND	ND	ND
BK103-1	12.72	90.55	ND	ND	ND	ND	ND	ND

Table 1 Screening of lactic acid production and optical purity of lactic acid (Continued)

Isolate no.	Aminex HPX-87H – HPLC		Sumi chiral OA5000 - HPLC				Optical purity of lactic acid (%ee)	Isomer type
	Concentrations (g/L)		Concentrations (g/L)		Area			
	Glucose	Lactic acid	L-lactic acid	D-lactic acid	L-lactic acid	D-lactic acid		
BK103-2	14.58	94.72	ND	ND	ND	ND	ND	ND
BK103-3	36.17	70.43	ND	ND	ND	ND	ND	ND
BK105-2	93.43	22.95	ND	ND	ND	ND	ND	ND
BK108	87.17	34.54	ND	ND	ND	ND	ND	ND
BK109-1	100.36	22.31	ND	ND	ND	ND	ND	ND
BK110-2	75.37	44.17	ND	ND	ND	ND	ND	ND
BK113	94.92	42.24	ND	ND	ND	ND	ND	ND
BK115-2	74.91	47.00	ND	ND	ND	ND	ND	ND
BK116-1	57.30	61.94	ND	ND	ND	ND	ND	ND
BK116-2	80.95	42.04	ND	ND	ND	ND	ND	ND
BK116-3	99.75	30.44	ND	ND	ND	ND	ND	ND
BK117-1	115.15	0	ND	ND	ND	ND	ND	ND
BK117-2	87.22	36.06	ND	ND	ND	ND	ND	ND
BK118	91.92	33.65	ND	ND	ND	ND	ND	ND
BK119	92.43	29.89	ND	ND	ND	ND	ND	ND
BK120-4	98.68	39.57	ND	ND	ND	ND	ND	ND

ND; Not detected

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Table 2 Effects of initial glucose concentrations of 100 g/L on the cell growth, pH, glucose consumption and lactic acid production

Time	pH	Growth at OD 600 nm	Glucose concentration (g/L)	Lactic acid concentration (g/L)	Productivity (g/L.h)	Yield (%)	D-LA (Area)	L-LA (Area)	Optical purity of D-lactic acid (%ee)
0	7.23	0	102.37	0	0	0	ND	ND	ND
6	7.19	0	102.38	0	0	0	ND	ND	ND
12	7.14	0	100.02	0	0	0	ND	ND	ND
18	6.01	0.82	100.01	0	0	0	ND	ND	ND
21	5.37	6.68	88.53	14.83	0.71	14.56	ND	ND	ND
24	5.30	12.10	69.48	27.25	1.14	26.80	ND	ND	ND
27	5.28	14.00	52.22	41.23	1.53	40.60	ND	ND	ND
30	5.30	17.02	34.54	56.40	1.88	55.54	ND	ND	ND
33	5.32	18.85	15.44	70.55	2.14	69.52	ND	ND	ND
36	5.51	21.60	0	81.55	2.27	80.40	1369854	1060	99.85
39	5.52	21.52	0	82.77	2.12	81.56	1412636	1661	99.77
42	5.52	19.53	0	89.36	2.13	87.29	1317764	0	100
45	5.52	18.33	0	93.55	2.08	91.38	1372144	0	100
48	5.52	16.67	0	88.48	1.84	86.43	1340166	11406	98.31

ND; Not detected

Table 3 Effects of initial glucose concentrations of 140 g/L on the cell growth, pH, glucose consumption and lactic acid production

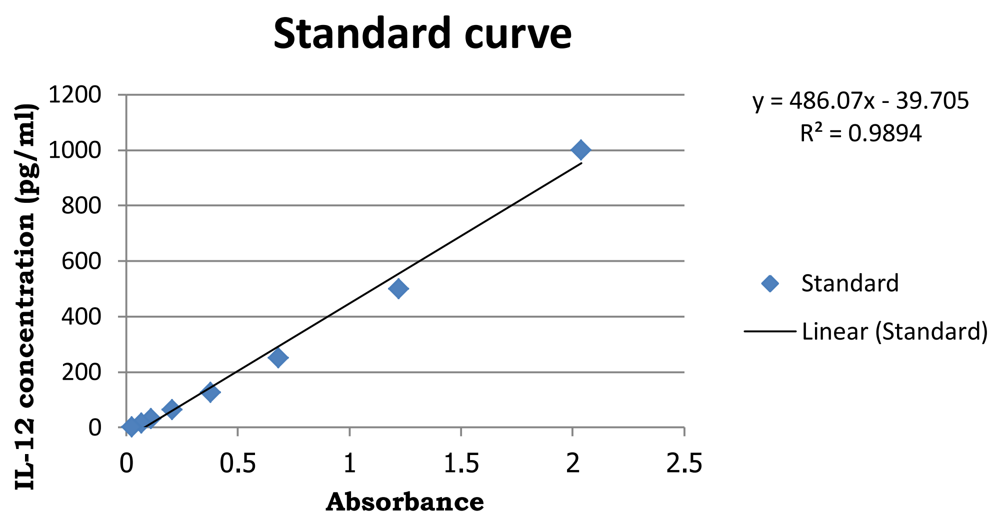
Time	pH	Growth at OD 600 nm	Glucose concentration (g/L)	Lactic acid concentration (g/L)	Productivity (g/L.h)	Yield (%)	D-LA (Area)	L-LA (Area)	Optical purity of D-lactic acid (%ee)
0	7.15	0	139.14	0	0	0	ND	ND	ND
6	7.15	0	140.28	0	0	0	ND	ND	ND
12	7.09	0	140.99	0	0	0	ND	ND	ND
18	5.82	1.09	135.04	0	0	0	ND	ND	ND
21	5.38	5.03	125.42	12.58	0.60	91.71	ND	ND	ND
24	5.23	10.85	112.10	25.92	1.08	95.85	ND	ND	ND
27	5.21	15.23	91.63	42.15	1.56	88.71	ND	ND	ND
30	5.21	17.85	73.65	58.63	1.95	89.53	ND	ND	ND
33	5.22	20.58	54.57	78.49	2.38	92.80	ND	ND	ND
36	5.27	20.98	31.25	97.31	2.70	90.19	1431464	0	100
39	5.28	21.08	8.61	118.03	3.03	90.42	1739789	0	100
42	5.41	22.03	0	129.93	3.09	93.38	1888632	0	100
45	5.41	21.95	0	131.63	2.93	94.02	2077486	14725	98.59
48	5.41	20.53	0	124.08	2.59	89.18	2005173	14931	98.52

ND; Not detected

Table 4 Effects of initial glucose concentrations of 200 g/L on the cell growth, pH, glucose consumption and lactic acid production

Time	pH	Growth at OD 600 nm	Glucose concentration (g/L)	Lactic acid concentration (g/L)	Productivity (g/L.h)	Yield (%)	D-LA (Area)	L-LA (Area)	Optical purity of D-lactic acid (%ee)
0	6.89	0	215.16	0	0	0	ND	ND	ND
6	6.88	0	214.28	0	0	0	ND	ND	ND
12	6.76	0	215.08	0	0	0	ND	ND	ND
18	6.11	0.39	213.37	0	0	0	ND	ND	ND
21	5.44	3.38	200.83	11.35	0.54	79.14	ND	ND	ND
24	5.22	8.40	181.99	23.74	0.99	71.55	ND	ND	ND
27	5.20	10.85	170.42	34.29	1.27	76.64	ND	ND	ND
30	5.19	12.03	153.83	51.83	1.73	84.50	ND	ND	ND
33	5.24	15.10	137.54	66.78	2.02	86.02	ND	ND	ND
36	5.26	16.13	118.90	86.31	2.40	89.66	1275631	0	100
39	5.38	18.70	101.65	101.65	2.61	89.55	1515304	0	100
42	5.38	20.13	86.04	118.54	2.82	91.80	1682646	0	100
45	5.38	21.30	65.44	137.06	3.05	91.54	1951976	0	100
48	5.38	21.15	56.15	148.22	3.09	93.21	2260232	5121	99.55

ND; Not detected



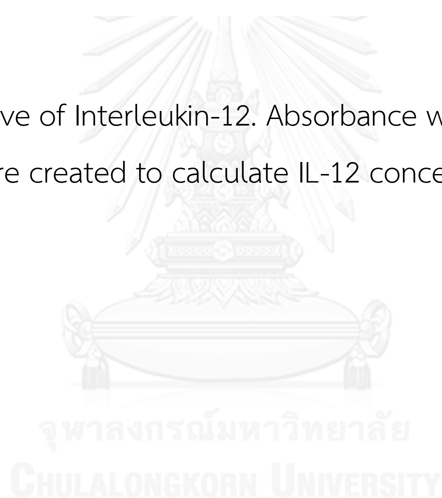


Figure 1 Standard curve of Interleukin-12. Absorbance was measured at 450 nm. The line and equation were created to calculate IL-12 concentrations of unknown samples.

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APPENDIX D

16S rRNA and *gyrB* gene sequences

16S rRNA gene sequences

>EL1-1 (AB841311)

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>EL1-2 (AB841313)

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>EL8-2 (AB841314)

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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

>BF13-1 (AB841318)

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>BF14-1 (AB841310)

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>PC72-4

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>PC73-3

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>KC74-1

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>PC75-2

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>KC78-5

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>PC79-5

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>KC81-2

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>PC86-2

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>SR7-1

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16S rRNA gene sequences of novel species

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GyrB gene sequences of novel species

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ATCACAGCCATGG

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PUBLICATIONS:

1. Thamacharoensuk, T., Thongchul, N., Taweechotipatr, M., Tolieng, V., Kodama, K., and Tanasupawat, S. (2013). Screening and characterization of lactic acid bacteria from animal faeces for probiotic properties. The Thai Journal of Veterinary Medicine, 43(4), 541.

2. Thamacharoensuk, T., Kitahara, M., Ohkuma, M., Thongchul, N., and Tanasupawat, S. (2015). Sporolactobacillus shoreae sp. nov. and Sporolactobacillus spathodeae sp. nov., two spore-forming lactic acid bacteria isolated from tree barks in Thailand. International Journal of Systematic and Evolutionary Microbiology, 65(4), 1220-1226.

POSTER PRESENTATIONS:

1. Thamacharoensuk, T., Thongchul, N., Taweechotipatr, M., Tolieng, V., Kodama, K. and Tanasupawat, S. 2011. Screening of lactic acid bacteria from animal faeces for production of optically pure L(+)- or D(-)-lactic acid and probiotic properties. The 23rd Annual Meeting of the Thai Society of Biotechnology, February 1-2, The Imperial Queen's Park Hotel, Bangkok, Thailand.

2. Thamacharoensuk, T., Thongchul, N., Tolieng, V., Kodama, K. and Tanasupawat, S. 2013. Characterization of optical purity D-lactic acid producing Sporolactobacillus strains isolated from tree bark. RGJ-Ph.D. Congress XIV, April 5-7, Jomtien Palm Beach Resort, Pattaya, Chonburi, Thailand.

3. Thamacharoensuk, T., Kitahara, M., Ohkuma, M., Thongchul, N., and Tanasupawat, S. 2014. Sporolactobacillus shoreae sp. nov. and Sporolactobacillus spathodeae sp. nov., two spore-forming lactic acid bacteria isolated from tree barks in Thailand. TSB International Forum 2014 “Green Bioprocess Engineering”, September 16-19, BITEC Bang Na Hall Conference, Bangkok, Thailand.

Chulalongkorn University