SELECTION OF LACTIC ACID BACTERIA FOR D-LACTIC ACID PRODUCTION FROM CASSAVA STARCH

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SELECTION OF LACTIC ACID BACTERIA FOR D-LACTIC ACID PRODUCTION FROM CASSAVA STARCH

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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สุดารัตน์ พราหมณ์แก้ว : การคัดเลือกแบคทีเรียกรดแล็กติกเพื่อการผลิตกรดดี-แล็กติกจากแป้งมันสำปะหลัง (SELECTION OF LACTIC ACID BACTERIA FOR D-LACTIC ACID PRODUCTION FROM CASSAVA STARCH)
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กรดดี-แล็กติกเป็นมอนอเมอร์ที่มีความสำคัญในอุตสาหกรรมพลาสติกชีวภาพ เนื่องจากใช้เพื่อผลิตพอลิดี-แล็กติกแอสิด เพื่อเป็นส่วนผสมในการปรับปรุงความเสถียรต่อความร้อนของพอลิแอล-แล็กติกแอสิด ซึ่งเป็นองค์ประกอบหลักของพลาสติกย่อยสลายได้ทางชีวภาพประเภทหนึ่ง การศึกษาครั้งนี้เพื่อคัดเลือกสายพันธุ์ของแบคทีเรียกรดแล็กติกที่มีศักยภาพในการผลิตกรดดี-แล็กติกจากแป้งมันสำปะหลังซึ่งเป็นวัตถุดิบราคาถูก จากการทดสอบความสามารถในการผลิตกรดดี-แล็กติกของแบคทีเรียกรดแล็กติกที่แยกจากแหล่งธรรมชาติจำนวน 306 ไอโซเลท พบว่ามีจำนวน 121 ไอโซเลท ที่สามารถผลิตกรดแล็กติกในอาหารเลี้ยงเชื้อที่มีน้ำตาลกลูโคสปริมาณ 20 กรัมต่อลิตร เป็นแหล่งคาร์บอนที่ให้ค่าสภาพกรดทั้งหมดของอาหารภายหลังการเลี้ยงเชื้อที่อุณหภูมิ 35 องศาเซลเซียส เป็นเวลา 48 ชั่วโมง ในช่วงร้อยละ 0.053 ถึง 1.77 แบคทีเรียที่พบว่าสามารถผลิตกรดดี-แล็กติกที่มีความบริสุทธิ์เชิงแสงของกรดมากกว่าร้อยละ 90 มีจำนวน 7 ไอโซเลท (รหัสไอโซเลท WR73 CWMC2-5 CWMC1-3 CWMR1-5 CWR2-16 LF1 และ PSMS1-5) ซึ่งผลิตกรดดี-แล็กติกได้ในปริมาณสูงเท่ากับ 2.00 17.94 15.88 15.84 15.16 10.42 และ 10.16 กรัมต่อลิตร ตามลำดับ และมีเพียงไอโซเลท WR73 ที่สามารถใช้แป้งมันสำปะหลังเป็นแหล่งคาร์บอนสำหรับผลิตกรดดี-แล็กติกที่มีความบริสุทธิ์เชิงแสงของกรดมากกว่าร้อยละ 99 ได้ ซึ่งเมื่อระบุชนิดของแบคทีเรียกรดแล็กติกไอโซเลท WR73 ด้วยลักษณะทางสัณฐานและสรีรวิทยาพบว่ามีความเหมือนกับ *Lactobacillus delbrueckii* subsp. *delbrueckii* ร้อยละ 97.1 และมีความเหมือนของลำดับนิวคลีโอไทด์ของ 16S rRNA gene (1450 คู่เบส) ร้อยละ 77 เท่ากันเมื่อเทียบกับ *Lactobacillus delbrueckii* DSM 20074^{T} *Lactobacillus coryniformis* DSM 20001^{T} *Carnobacterium* sp. MARL 15 และ *Carnobacterium pleistocenium* FTR 1 จากฐานข้อมูล GenBank สหรัฐอเมริกา จึงสามารถระบุได้เพียงว่าไอโซเลท WR73 เป็นแบคทีเรียในสกุล *Lactobacillus* และได้เลือกแบคทีเรียไอโซเลทนี้มาศึกษาสภาวะที่เหมาะสมต่อการเจริญและการผลิตกรดแล็กติก พบว่าอาหารเลี้ยงเชื้อที่เหมาะสมต่อการผลิตกรดประกอบด้วยส่วนผสมหลักคือ แป้งมันสำปะหลัง ทริปโตน และกากยีสต์ที่เหลือจากการหมักเบียร์ที่ความเข้มข้น 30.0 3.0 และ 3.0 กรัมต่อลิตร ตามลำดับ มีค่าความเป็นกรด-ด่างของอาหารเริ่มต้นเท่ากับ 7.0 และที่อุณหภูมิ 35 องศาเซลเซียส จากนั้นได้ทดลองผลิตกรดดี-แล็กติกในถังหมักที่มีอาหารเลี้ยงเชื้อตามส่วนประกอบที่เหมาะสมจากการศึกษา ปริมาตร 5.0 ลิตร ภายใต้

สภาวะการเลี้ยงเชื้อที่เหมาะสม พบผลผลิตกรดดี-แล็กติกปริมาณสูงสุด 19.75 กรัมต่อลิตร เมื่อเลี้ยงเชื้อเป็นเวลา 48 ชั่วโมง อัตราการเจริญจำเพาะสูงสุดของแบคทีเรียมีค่าเท่ากับ 0.93 ต่อชั่วโมง กรดดี-แล็กติกที่ผลิตได้นี้ สามารถสกัดแยกจากอาหารเลี้ยงเชื้อแป้งมันสำปะหลังราคาถูกนี้ได้ง่าย ด้วยกรรมวิธีการตกผลึกให้ได้เกลือของแมกนีเซียม ข้อมูลที่ได้จากการศึกษานี้เป็นแนวทางสำคัญในการผลิตกรดดี-แล็กติกที่มีความบริสุทธิ์เชิงแสงของกรดมากกว่าร้อยละ 99 ซึ่งเป็นที่ต้องการสำหรับการผลิตพอลิดี-แล็กติกแอสิด ด้วยกรรมวิธีการผลิตกรดที่มีต้นทุนต่ำ

สาขาวิชาจุลชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

SUDARAT PRAMKAEW : SELECTION OF LACTIC ACID BACTERIA FOR D-LACTIC ACID PRODUCTION FROM CASSAVA STARCH.
THESIS ADVISOR : ASST. PROF. SUREELAK RODTONG, Ph.D. 185 PP.

D-LACTIC ACID PRODUCTION/LACTIC ACID BACTERIA/CASSAVA STARCH

D-Lactic acid is one of desirable monomers to be used for the production of poly(D-lactic acid), PDLA, in bioplastics industry. PDLA is useful for improving thermostability of poly(L-lactic acid), PLLA, which is the main component of poly(lactic acid), PLA, biodegradable plastics. In this study, three hundred and six isolates of lactic acid bacteria were screened for their capability to produce D-lactic acid. These bacteria were isolated from their natural habitats. One hundred and twenty one from the total of 306 isolates could produce lactic acid from glucose at total acidity ranging from 0.053-1.77%. Only seven isolates, codes WR73, CWMC2-5, CWMC1-3, CWMR1-5, CWR2-16, LF1, and PSMS1-5, were able to produce D-lactic acid with optical purity >90% at high concentrations of 2.00, 17.94, 15.88, 15.84, 15.16, 10.42, and 10.16 g/l, respectively. And only one isolate, WR73, could utilize a cheap raw material, cassava starch, and produce optically pure D-lactic acid. The isolate was identified as belonging to the genus *Lactobacillus* according to its morphological and physiological characteristics which had 97.1% identity to *Lactobacillus delbrueckii* subsp. *delbrueckii*. Also its 16S rRNA gene sequence (1450 bp) had 77% homology compared to either *Lactobacillus delbrueckii* DSM 20074^{T}, *Lactobacillus coryniformis* DSM 20001^{T}, *Carnobacterium* sp. MARL 15 or

Carnobacterium pleistocenium FTR 1 from GenBank database, U.S.A. Isolate WR73 was then selected for optimization of its growth and lactic acid production conditions. The suitable medium for both growth and lactic acid production was found to composed of cassava starch, tryptone, and spent of brewery yeast sludge at concentrations of 30.0, 3.0, and 3.0 g/l, respectively, as main ingredients. Optimum pH and temperature were at 7.0 and 35°C. The isolate was tested for its D-lactic acid production from cassava starch using 5.0 l of the optimized medium in a controlled fermenter under optimum conditions. At 48 h of cultivation, the maximum D-lactic acid yield of 19.75 g/l ($Y_{LA/S}$, of 66.51%) with >99% optical purity were achieved. The strain had its specific growth rate (μ_{max}) of 0.93 h^{-1}. The acid product could be simply purified from the inexpensive optimized medium by crystallization as magnesium D-lactate. Data from this study are useful for optically pure D-lactic acid production from low-cost substrate, which is necessary for biopolymer production.

School of Microbiology Student's Signature_____

Academic Year 2010 Advisor's Signature_____

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LIST OF ABBREVIATIONS

ADI	=	Arginine deiminase
ANOVA	=	Analysis of varience
AOAC	=	Association of Official Analytical Chemists
ATP	=	Adenosine triphosphate
BLAST	=	Basic local alignment search tool
bp	=	Base pair
°C	=	Degree Celsius
$CaCO_3$	=	Calcium carbonate
CFU	=	Colony forming unit
CK	=	Carbamate kinase
CO_2	=	Carbon dioxide gas
Co., Ltd.	=	Limited company
C_p	=	Specific heat
CRD	=	Completely Randomized Design
$CuSO_4$	=	Copper(II) sulfate
dATP	=	Deoxyadenosine triphosphate
dCTP	=	Deoxycytidine triphosphate
dGTP	=	Deoxyguanosine triphosphate
dNTPs	=	Deoxynucleoside triphosphate
dTTP	=	Deoxythymidine triphosphate

LIST OF ABBREVIATIONS (Continued)

DMRT	=	Duncan's Multiple Range Test
DNA	=	Deoxyribonucleic acid
DSC	=	Differential scanning calorimetry
EDTA	=	Ethylenediaminetetraacetic acid
e.g.	=	For example
et al.	=	et alia (and others)
EU	=	European Union
$FeSO_4$	=	Ferrous sulfate
g	=	Gram
GPC	=	Gel permeation chromatography
h	=	Hour
H_2	=	Hydrogen gas
ΔH_c	=	Heat of combustion
HCl	=	Hydrochloric acid
H_2O	=	Water
HPLC	=	High performance liquid chromatography
lb	=	Pound
i.e.	=	That is
Inc.	=	Incorporation
Kb	=	Kilobase
kDa	=	Kilodaltons
K_2HPO_4	=	Dipotassium hydrogen phosphate

LIST OF ABBREVIATIONS (Continued)

(k) J	=	(Kilo) joule
KPL	=	Kirkegaard and Perry Laboratories
l	=	Liter
M	=	Molar
MC	=	MRS containing 0.5% $CaCO_3$
$MgSO_4$	=	Magnesium sulfate
min	=	Minute
ml	=	Milliliter
mM	=	Millimolar
mm	=	Millimeter
$MnSO_4$	=	Manganese sulfate
mol	=	Mole
MRS	=	Man Rogosa Sharpe
N	=	Normal
N_2	=	Nitrogen gas
NaCl	=	Sodium chloride
NAD^+	=	Nicotinamide adenine dinucleotide
NADH	=	Reduced NAD
nm	=	Nano metre
OCT	=	Ornithine carbamoyl-transferase
OD	=	Optical density
%	=	Percentage

LIST OF ABBREVIATIONS (Continued)

P	=	P-value
PCR	=	Polymerase chain reaction
PDLA	=	Poly(D-lactic acid)
pK_a	=	Acid dissociation constant
PLA	=	Poly(lactic acid)
PLLA	=	Poly(L-lactic acid)
PTS	=	Phosphotransferase system
RAM	=	Rogasa Agar Modified medium
rpm	=	Round per minute
rRNA	=	Ribosomal ribonucleic acid
SD	=	Standard deviation
SDS	=	Sodium dodecylsulphate
sec	=	Second
SEM	=	Scanning electron microscopy
SSC	=	Standard saline citrate
SSF	=	Simultaneous saccharification and fermentation
subsp.	=	Subspecies
T_m	=	Melting temperature
TSBS	=	Trypticase soy broth supplemented with 2% NaCl
U	=	Unit
U.K.	=	United Kingdom
µl	=	Microliter

LIST OF ABBREVIATIONS (Continued)

μm	=	Micrometer
U.S.A.	=	United States of America
UV	=	Ultraviolet
v/v	=	Volume by volume
w/v	=	Weight by volume

CHAPTER I

INTRODUCTION

1.1 Introduction

Lactic acid bacteria produce lactic acid as the major end product during carbohydrate fermentation, and can generate adenosine triphosphate (ATP) from the fermentation of sugar (Axelsson, 2004). They are found in nutrient-rich environments such as fermented food, milk, meat, and gastrointestinal tract of human and animals (Wood and Holzapfel, 1995). Two enantiomers of lactic acid, D-form and L-form, have been naturally found. D-Lactic acid is a mirror image of L-lactic acid, which can be soluble in water. It exhibits low volatility (Narayanan *et al*., 2004). D-Lactic acid can be produced by either chemical synthesis or microbial fermentation (John *et al*., 2009). The biological method has the advantage that an optically pure D-lactic acid can be obtained by choosing a specific strain of microorganism, whereas chemical synthesis always results in a racemic mixture of lactic acid, which is a major disadvantage for the application of pure lactic acid form (Ryu *et al*., 2003; John *et al*., 2009). D-Lactic acid is mainly used in the bioplastic industry (Benthin and Villadsen, 1995; Sawai *et al*., 2007). Poly(D-lactic acid) (PDLA) obtained from the polymerization of D-lactic acid, is an important polymer because it could improve the thermostability of poly(L-lactic acid) (PLLA) by the stereocomplex formation (Ikada *et al*., 1987; Tsuji and Fukui, 2003). Recently, it was reported that an equimolar blend PLLA and PDLA generated a racemic crystal called stereo-complex

poly(lactic acid), which was more heat-resistant than the PLLA homo-polymer due to the high melting temperature (Sawai *et al*., 2007). PLLA is biodegradable aliphatic polyester, which has been usually prepared by dehydration of L-lactic acid into relatively low molecular weight polyester, and is converted into L-lactide by depolymerization. Then, purified L-lactide monomer is converted into PLLA by catalytic ring-opening polymerization (Kharas *et al*., 1994). The aliphatic polyester has a crystallinity of around 37%, a glass transition temperature between 50-80°C and a melting temperature around 175°C (Kharas *et al*., 1994; Lunt, 1998). PLLA is sensitive to heat, especially at temperature higher than 190°C (Kharas *et al*., 1994). However, the melting temperature of PLLA can be increased to 230°C by physically blending the polymer with PDLA (Ikada *et al*., 1987). This finding has attracted interest in the production of D-lactic acid. D-Lactic acid has been reported to be produced by several species of lactic acid bacteria, particularly *Lactobacillus delbrueckii* (Manome *et al*., 1998; Tanaka *et al*., 2006; Calabia *et al*., 2007), *Lactobacillus coryniformis* subsp. *torquens* (Manome *et al*., 1998; Yàñez *et al*., 2003), *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc carnosum*, *Leuconostoc fallax* (Manome *et al*., 1998), *Lactobacillus bulgaricus* (Benthin and Villadsen, 1995), as well as genetically modified *Lactobacillus plantarum* (Okano *et al*., 2009; 2010). However, potential microorganisms for D-lactic acid production from cheap raw materials are still desirable for the application of the acid in biopolymer production. Based on inexpensive and abundant agricultural products in Thailand, cassava starch was reported to be a potential substrate for lactic acid production from bacterial fermentation process (Rodtong and Ishizaki, 2003). Cassava (*Manihot esculent*

Crantz) is a perennial plant widely grown in some tropical countries, including Thailand, as one of the most important commercial crops. There are one million hectares devoted to cassava planting in Thailand. Approximately 40 percent of the cassava produced in Thailand is processed into cassava starch (FAO, 2009). Cassava starch is, thus, become the interesting carbon source for D-lactic acid production. This research focused on screening and selection of lactic acid bacteria for D-lactic acid production from cassava starch.

1.2 Research objectives

The objectives of this research are as follows:

1. To screen and select the potential strain of lactic acid bacteria for D-lactic acid production from cassava starch.

2. To optimize some conditions for D-lactic acid production and produce D-lactic acid using the optimized conditions by the selected strain.

3. To identify the selected strain.

4. To preliminarily investigate the extraction and purification of D-lactic acid for further application.

1.3 Research hypothesis

D-Lactic acid could be potentially produced by specific strains of lactic acid bacteria using abundant agricultural products such as cassava starch as a raw material. The acid could be extracted and purified from the inexpensive optimized medium for application in biopolymer production.

1.4 Scope of the study

At least 300 lactic acid bacterial isolates obtained from stock cultures of the Microbial Culture Collection Laboratory at Suranaree University of Technology, were be screened and selected for D-lactic acid production. The selected isolate was identified by morphological, physiological and genetical characteristics. Some optimum production conditions (particularly nutrient sources using cassava starch as a sole carbon source, nitrogen source, pH, temperature, % inoculum size) for D-lactic acid production were determined. The acid product was preliminarily extracted and purified from the inexpensive optimized medium.

1.5 Expected results

The potential strain for D-lactic acid production from cassava starch is obtained. Optimum conditions for D-lactic acid production are achieved. The selected strain is identified. The purified acid is separated from the inexpensive optimized medium, and could be applied for biopolymer production.

CHAPTER II

LITERATURE REVIEW

2.1 Lactic acid bacteria

2.1.1 Taxonomy

Typical lactic acid bacteria are Gram-positive, non-spore forming, catalase-negative, devoid of cytochromes, anaerobic but aerotolerant cocci or rods that are acid-tolerant and produce lactic acid as the major end product during sugar fermentation (Axelsson, 2004). However, under certain conditions, some lactic acid bacteria do not display all these characteristics. They generally lack catalase and cytochromes when grown in laboratory growth media, which lack heme. Therefore, the lactic acid bacteria do not posses an electron transport chain and rely on fermentation to generate energy (Axelsson, 2004). Since they do not use oxygen in their energy production, lactic acid bacteria grow under anaerobic conditions, but they can also grow in the presence of oxygen. They are protected from oxygen by-products (e.g. H_2O_2) because they have peroxidases. These organisms are aerotolerant anaerobes. Because of the low energy yields, lactic acid bacteria often grow more slowly than microbes capable of respiration, and produce smaller colonies of 2-3 mm.

Lactic acid bacteria were classified in the phylum Firmicutes comprised of 21 genera which are *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosicoccus*, *Dolosigranulum*, *Enterococcus*, *Eremococcus*, *Facklamia*, *Globicatella*, *Helcococcus*, *Ignavigranum*,

Lactococcus, *Lactosphaera*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Axelsson, 2004). *Lactobacillus* is the largest genus, comprising around 80 recognized species. The classification of lactic acid bacteria into different genera is largely based on their cell morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentration, acid or alkaline tolerance, chemotaxonomic markers such as fatty acid composition, constituents of the cell wall, and phylogenetic relationships. A summary of the differentiation of lactic acid bacteria genera with classical phenotypic tests is shown in Table 2.1.

2.1.2 Morphology

2.1.2.1 Cell morphology

Cell morphology is important in the current descriptions of the lactic acid bacteria genera. The bacteria can be divided into 2 groups: rods (*Lactobacillus* and *Carnobacterium*) and cocci (*Leuconostoc*, *Streptococcus*, *Alloiococcus*, *Dolosicoccus*, *Dolosigranulum*, *Enterococcus*, *Eremococcus*, *Facklamia*, *Globicatella*, *Helcococcus*, *Ignavigranum*, *Lactococcus*, *Lactosphaera*, *Oenococcus*, *Vagococcus*, *Aerococcus*, *Pediococcus*, and *Tetragenococcus*) (Axelsson, 2004). Cell morphology of the rods is long, slender rods to coccobacilli, which cell size varies between 0.5-1.2×1.0-11.0 µm, and arrange in chains (Table 2.2). The Gram-positive coccus group has spherical cell ranging in diameter from 0.5-3.5 µm, which occur singly or in pairs, chains and tetrads (Table 2.2). Examples of cell morphology are revealed in Figures 2.1 and 2.2. One exception is relatively described genus *Weissella*, which can include both cocci and rods (Collins *et al*., 1993). Furthermore, cell division in two perpendicular directions in a single plane

leading to tetrad formation is used as a key characteristic in the differentiation of the cocci. The tetrad-forming genera are *Aerococcus*, *Pediococcus*, and *Tetragenococcus.*

2.1.2.2 Colony morphology

Because of the low energy yields, lactic acid bacteria often grow slowly, and produce small colonies about 2-3 mm diameter (Reddy *et al*., 2008). Lactic acid bacteria grow tremendously fast when supplied with an abundance of nutrients. Different genera of lactic acid bacteria produce different-looking colony, some colonies may be colored, some colonies are circular in shape, and others are irregular. The characteristics of a colony such as shape, size, pigmentation, elevation, and margin, are termed the colony morphology, which is useful for the identification of lactic acid bacteria. Colony morphology of some species of lactic acid bacteria are revealed in Table 2.3.

2.1.3 Metabolism of lactic acid bacteria

2.1.3.1 Carbohydrate metabolism

According to Somkuti (2000), lactic acid bacteria prefer an environment rich in simple carbohydrates. The bacteria are unable to syntersize ATP by respiration, but could obtain ATP from substrate level phosphorylation of carbohydrate fermentation (Axelsson, 2004). Hexoses are mainly degraded to lactate (homofermentatives) or to lactate and additional products such as acetate, ethanol, carbon dioxide (CO_2), formate or succinate (heterofermentatives) (Figure 2.3). Sugar transport across the cytoplasmic membrane is driven by an ATP-dependent permease system in several species of *Lactobacillus* and *Leuconostoc*, and *Streptococcus thermophilus*. In all mesophilic *Lactococcus* and *Lactobacillus casei*, transmembrane transport of sugars requires phosphorylation and the process is dependent on the

phosphoenol pyruvate phosphotransferase system. For lack of an adequate level of galactokinase activity, certain thermotolerant lactobacilli (*L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis*, *L. acidophilus*) and *Streptococcus thermophilus* metabolize only the glucose moiety of lactose, while in *Lactococcus*, the galactose moiety of lactose is metabolized by the tagatose-6-phosphate pathway (Figure 2.4). Some lactic acid bacteria could also transport galactose by a permease system and this hexose process through the Leloir pathway (Figure 2.5).

Lactic acid fermentation with emphasis to use starch or starchy substrates was also reported. In most cases, starch cannot be used by lactic acid bacteria directly, and the large starch macromolecules have to be split into glucose molecules by enzymatic hydrolysis. Bioconversion of polysaccharide carbohydrate materials to lactic acid can be made much more effective by coupling the enzymatic hydrolysis of substrates and microbial fermentation of the derived glucose, which has been successfully employed for lactic acid production from raw starch materials (Reddy *et al*., 2008). Amylolytic lactic acid bacteria can ferment different types of amylaceous raw material, such as corn (Nakamura, 1981), potato, cassava (Giraud *et al*., 1991) and different starchy substrates (Vishnu *et al*., 2002) due to the ability of their α-amylase to partially hydrolyze raw starch. In commercial scale, glucose addition is an expensive alternative. The use of a cheaper source of carbon, such as starch, the most abundantly available raw material on earth next to cellulose, in combination with amylolytic lactic acid bacteria may help to decrease the cost of the overall fermentation process. Amylolytic lactic acid bacteria can convert starch directly into lactic acid (Figure 2.6). Development of production strains which ferment starch to lactic acid in a single step is necessary to make the process economical.

Many reports emphasize on fungi producing enzymes to degrade raw starch like *Rhizopus oryzae* (Bergmann *et al*., 1988), but least work is done on isolation of amylolytic lactic acid bacterial strains (Figuerao *et al*., 1995; Morlon-Guyot *et al*., 1998). Some strains of *Lactobacillus* produce extracellular amylase and ferment starch directly to lactic acid. Very few bacteria have been reported so far for direct fermentation of starch to lactic acid, such as *L. manihotivorans* OND 32, *L. manihotivorans* LMG 18010^{T}, *L. fermentum ogi* E 1, *L. fermentum* MW 2, *L. fermentum* K 9, *L. amylovorus* ATCC33622^{T}, *L. amylovorus* B 4542, *L. amylovorus*, *L. amylophilus* JCIM 1125^{T}, *L. amylophilus* B 4437, *L. amylophilus* GV 6, *L. acidophilus*, *L. fermentum* L 9, *L. plantarum* A 6, *L. plantarum* LMG 18053^{T}, *L. plantarum* NCIM 2084^{T}, *S. bovis* 148, *Lactobacillus* sp. TH 165, *Leuconostoc* St3-28, *L. cellobiosus*, *S. macedonicus*, and *L. amylolyticus* (Reddy *et al*., 2008).

2.1.3.2 Nitrogen metabolism

The nutritionally fastidious satisfy their requirements for peptides and amino acids as nitrogen sources through the activities of protease and peptidase enzymes, which are accompanied by di- and tri-peptide and amino acid transport system (Axelsson, 2004). In the best-studied lactococcal model, peptides generated by the membrane-bound proteinase system, are translocated into the cell interior by peptide transport systems, where they are further hydrolyzed to amino acids by substrate-specific peptidases (Figure 2.7), which enter amino acid catabolism for ATP generation (Figure 2.8).

Table 2.1 Differential characteristics of lactic acid bacteria.

Character	Rods		Cocci							
	Carnob.[a]	*Lactob.*[a]	*Aeroc.*[a]	*Enteroc.*[a]	*Lactoc.*[a] *Vagoc.*	*Leucon.*[a] *Oenoc.*	*Pedioc.*[a]	*Streptoc.*[a]	*Tetragenoc.*[a]	*Weissella*[a,b]
Tetrad formation	-	-	+	-	-	-	+	-	+	-
CO_2 from glucose[c]	-[d]	±	-	-	-	+	-	-	-	+
Growth at 10°C	+	±	+	+	+	+	±	-	+	+
Growth at 45°C	-	±	-	+	-	-	±	±	-	-
Growth at 6.5% NaCl	ND[e]	±	+	+	-	±	±	-	+	±
Growth at 18% NaCl	-	-	-	-	-	-	-	-	+	-
Growth at pH 4.4	ND	±	-	+	±	±	+	-	-	±
Growth at pH 9.6	-	-	+	+	-	-	-	-	+	-
Lactic acid[f]	L	D, L, DL[g]	L	L	L	D	L, DL[g]	L	L	D, DL[g]

+, positive; -, negative; ±, response varies between species; ND, not determined.

[a] *Lactob.*, *Lactobacillus*; *Leucon.*, *Leuconostoc*; *Pedioc.*, *Pediococcus*; *Streptoc.*, *Streptococcus*; *Aeroc.*, *Aerococcus*; *Carnob.*, *Carnobacterium*; *Enteroc.*, *Enterococcus*; *Lactoc.*, *Lactococcus*; *Oenoc.*, *Oenococcus*; *Tetragenoc.*, *Tetragenococcus*; *Vagoc.*, *Vagococcus*.

[b] *Weissella* strains may also be rod-shaped.

[c] Test for homo- or heterofermentation of glucose; negative and positive denotes homofermentative and heterofermentative, respectively.

[d] Small amounts of CO_2 can be produced, depending on media.

[e] No growth in 8% NaCl has been reported.

[f] Configuration of lactic acid produced from glucose.

[g] Production of D-, L- or DL-lactic acid varies between species.

Source: Axelsson (2004).

Table 2.2 Cell morphology, cell sizes, and cell arrangements of some lactic acid bacteria.

Genus	Cell morphology	Cell size (µm)	Cell arrangement	References
Lactobacillus	Rods and coccobacilli	0.5-1.2×1.0-11	Single, pairs, and chains	Hammes and Vogel (1995)
Carnobacterium	Slender rods	0.5-0.7×1.1-3.0	Single and pairs	Schillinger and Holzapfel (1995)
Leuconostoc	Cocci	0.9-1.2	Single, pairs, and chains	Dellaglio *et al.* (1995)
Streptococcus	Cocci or ovoid	0.8-1.2	Single, pairs, and chains	Hardie and Whiley (1995)
Enterococcus	Cocci or ovoid	0.8-2.0×0.6-2.5	Single, pairs, and chains	Devriese and Pot (1995)
Lactococcus	Cocci or ovoid	0.5-1.0	Single, pairs, and chains	Teuber (1995)
Pediococcus	Cocci	0.36-1.43	Tetrads and pairs	Simpson and Taguchi (1995)
Aerococcus	Cocci	1.0-2.0	Tetrads and pairs	Simpson and Taguchi (1995)

Table 2.3 Colony morphology of some lactic acid bacteria on specific media and growth conditions.

Genus	Medium[a]	Colony morphology	Colony size (diameter)	Cultivation time (day)	Growth conditions	Reference
Lactobacillus	MRS agar	White, smooth, and convex	1.0-2.0 mm	2	Anaerobic, 35-37°C	Hammes and Vogel (1995)
	Glucose-blood- liver agar	Brown, rough surface, round form, umbonate, and erosed edge	0.7-2.5 mm	2	Anaerobic, 37°C	Hammes and Vogel (1995)
	Homohiochii agar	White smooth, round, convex, and slimy	2.0 mm	4	Anaerobic, 30°C	Hammes and Vogel (1995)
	KPL agar	White, smooth to rough, circular or irregular, and convex	0.5-3.0 µm	10	Anaerobic, 30°C	Hammes and Vogel (1995)
	Sanfrancisco agar	Rough, circular, plateaux with irregular border, smooth, convex centre, translucent, and grayish	1.0-2.0 mm	2-5	Anaerobic, 30°C	Hammes and Vogel (1995)
	Briggs agar	Circular to irregular, convex, opaque, yellowish white, rough, and undulate	0.3-1.5 mm	14	Anaerobic, 30°C	Hammes and Vogel (1995)

Table 2.3 (Continued) Colony morphology of some lactic acid bacteria on specific media and growth conditions.

Genus	Medium[a]	Colony morphology	Colony size (diameter)	Cultivation time (day)	Growth condition	References
Carnobacterium	TSBS agar	Circular raised and entire	1.0-2.0 mm	1	Anaerobic, 23°C	Schillinger and Holzapfel (1995)
Leuconostoc	MRS agar	Greyish-white smooth and round convex	1.0-1.5 mm	2	Anaerobic, 25°C	Dellaglio *et al.* (1995)
*Streptococcu*s	Blood agar	Circular entire, convex, opaque, shiny smooth, and non-pigment	2.0 mm	2	Anaerobic, 37°C	Hardie and Whiley (1995)
Enterococcus	Blood agar	Smooth circular, entire, and non-pigment	1.0-2.0 mm	1	Anaerobic, 35-37°C	Devriese and Pot (1995)
Pediococcus	MRS agar	Grayish- white, convex, circular, and entire	1.0 mm	1	Anaerobic, 37°C	Simpson and Taguchi (1995)
Aerococcus	Blood agar	Non-pigment and circular	< 1.0 mm	1	Anaerobic, 37°C	Simpson and Taguchi (1995)

[a] MRS agar**,** de Man, Rogosa and Sharp agar**;** TSBS agar**,** trypticase soy broth supplemented with 2% NaCl**;** KPL agar, Kirkegaard and Perry Laboratories agar.

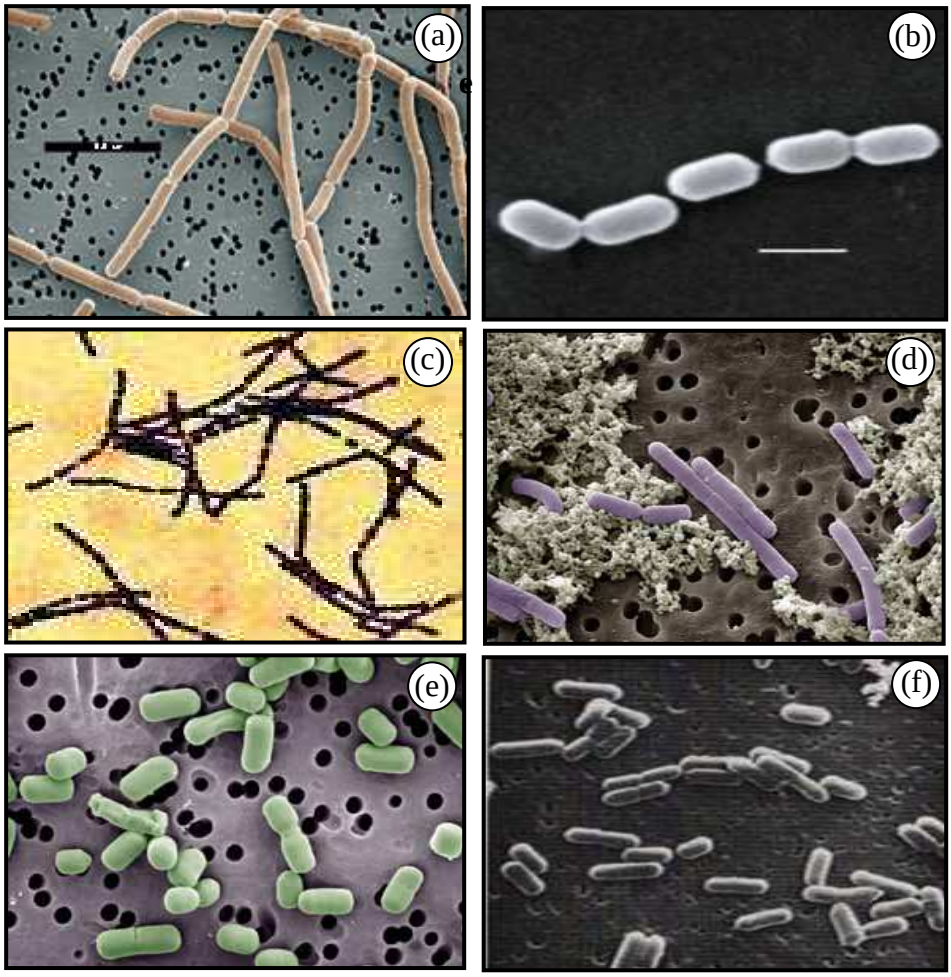


Figure 2.1 Cell morphology of Gram-positive rod lactic acid bacteria: a, *Lactobacillus delbrueckii*; b, *Lactobacillus gasseri*; c, *Lactobacillus bulgaricus*; d, *Lactobacillus casei*; e, *Lactobacillus. brevis*; f, *Carnobacterium piscicola.*

Source: Schillinger and Holzapeel (1995).

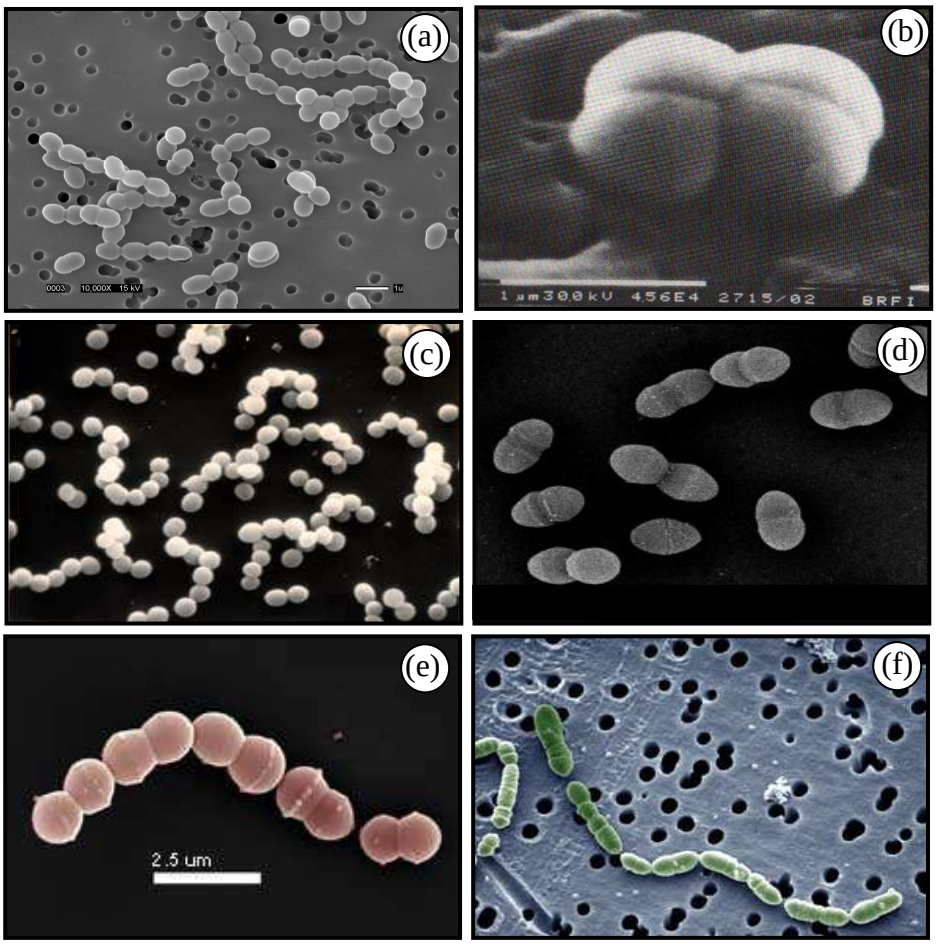


Figure 2.2 Cell morphology of Gram-positive coccus lactic acid bacteria: a, *Leuconostoc mesenteroides*; b, *Pediococcus pentosaceus*; c, *Lactococcus lactis* subsp. *cremoris*; d, *Enterococcus* spp.; e, *Streptococcus thermophilus*; f, *Oenococcus oeni.*

Source: Schillinger and Holzapeel (1995).

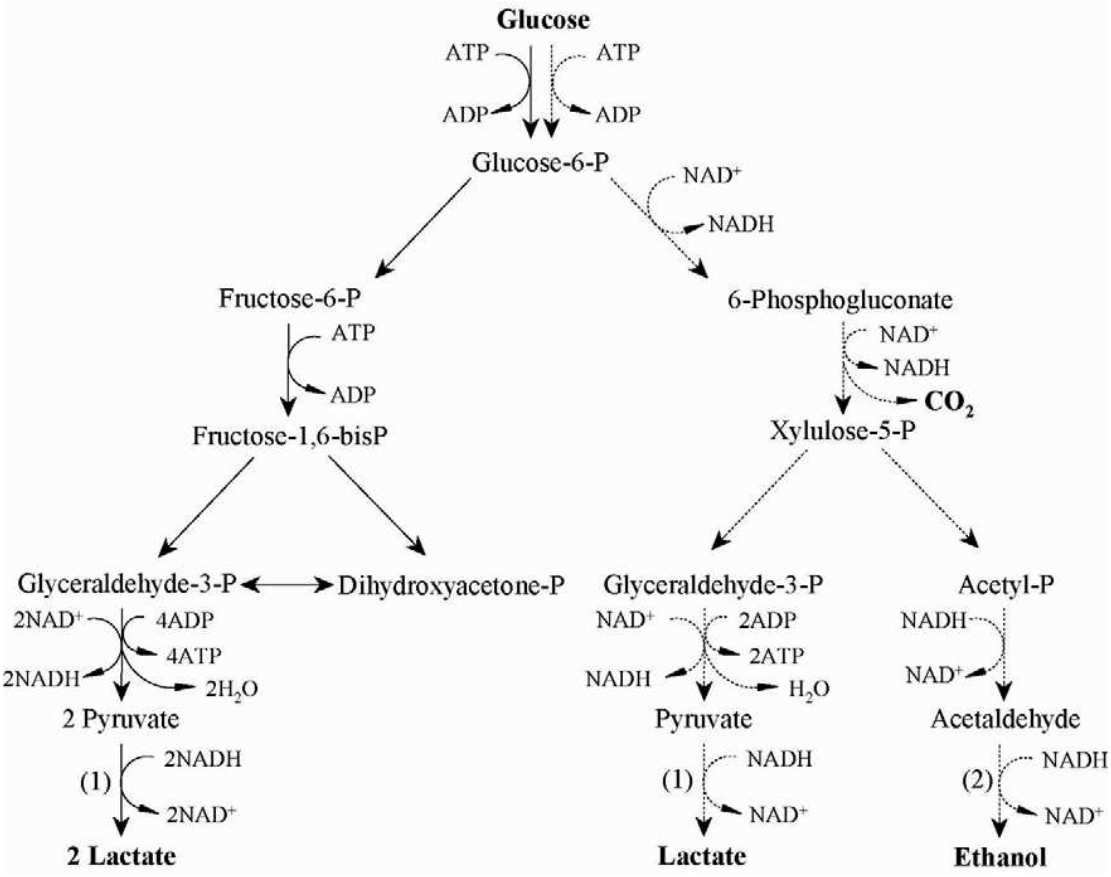


Figure 2.3 Metabolic pathways of homofermentative (solid line) and heterofermentative (dotted line) lactic acid bacteria: P, phosphate; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; NAD+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide (reduced form); (1), lactate dehydrogenase; (2), alcohol dehydrogenase.

Source: Pal *et al*. (2009).

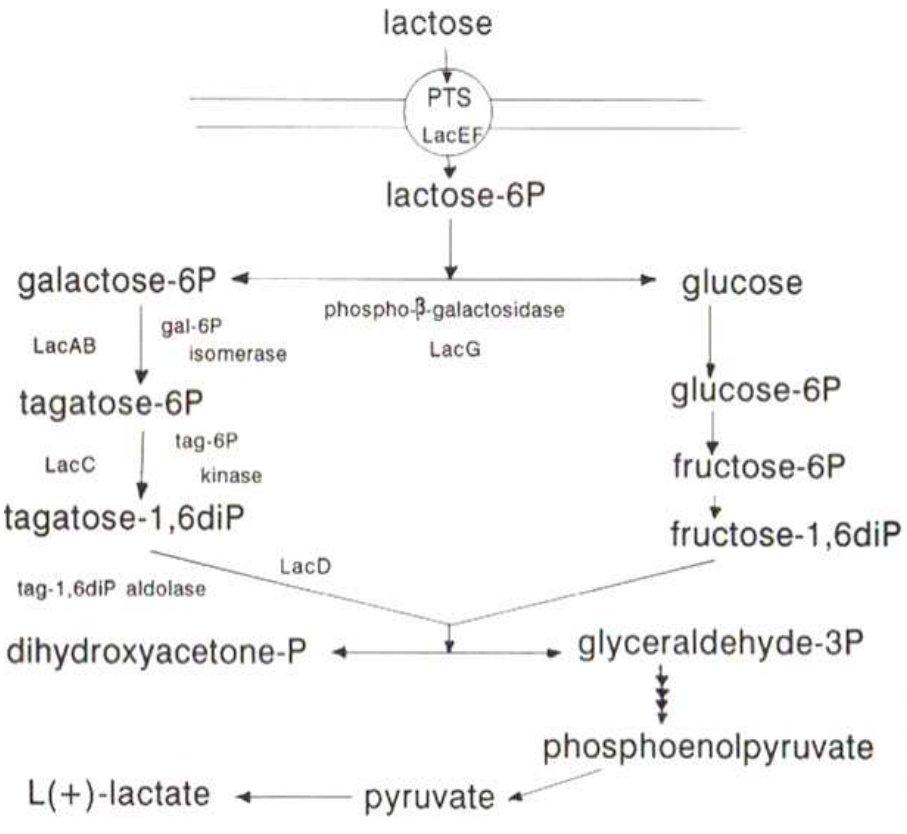


Figure 2.4 Pathway of lactose utilization by industrial *Lactococcus lactis*. This strain contains a lactose PTS, lactose enters the cytoplasm as lactose 6-phosphate. Then lactose 6-phosphate is cleaved by phospho-β-galactosidase to yield glucose and galactose-6-phosphate. Glucose is phosphorylated by glucokinase and metabolized through the glycolytic pathway, whereas galactose-6-phosphate is metabolized through the tagatose-6-phosphate pathway. The enzyme system of the lactose PTS and phosphor-β-galactosidase are generally inducible and repressed by glucose.

Source: Wood and Holzapfel (1995).

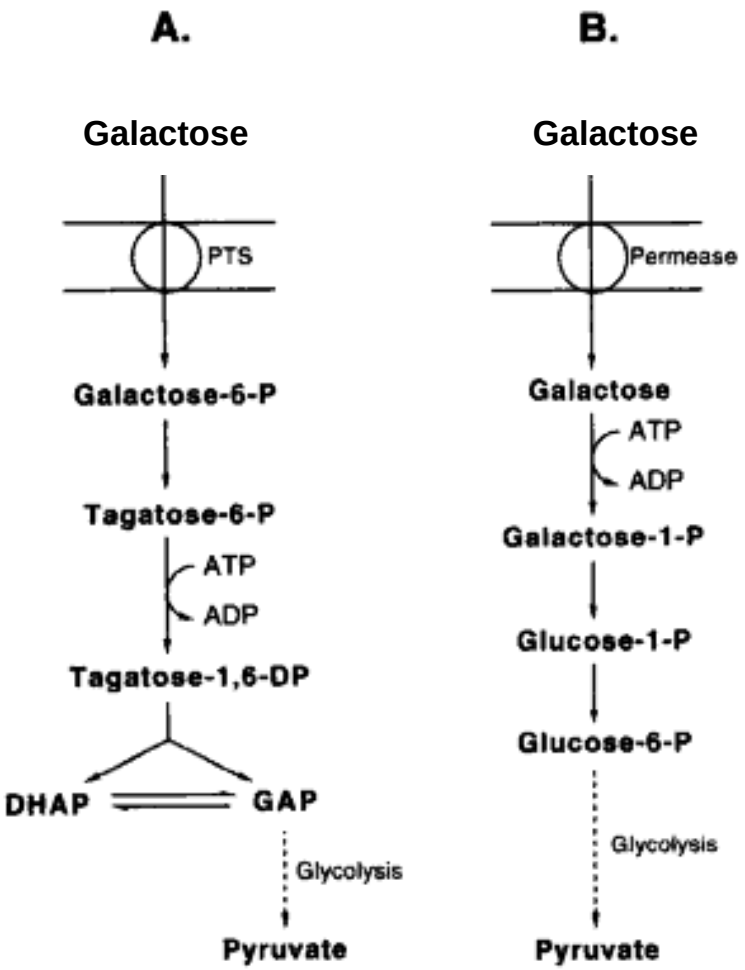


Figure 2.5 Galactose metabolism in lactic acid bacteria. A, tagatose-6-phosphate pathway; B, Leloir pathway.

Source: Axelsson (2004).

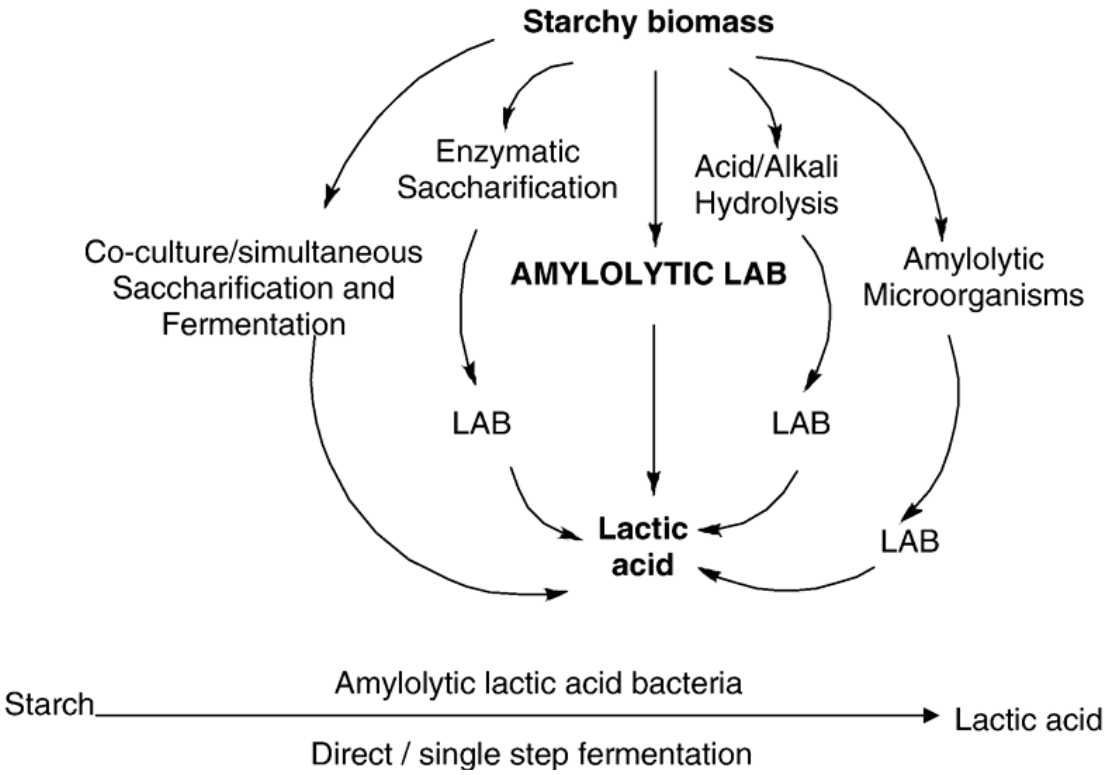


Figure 2.6 Schematic representation of lactic acid production from starch.

Source: Reddy *et al*. (2008).

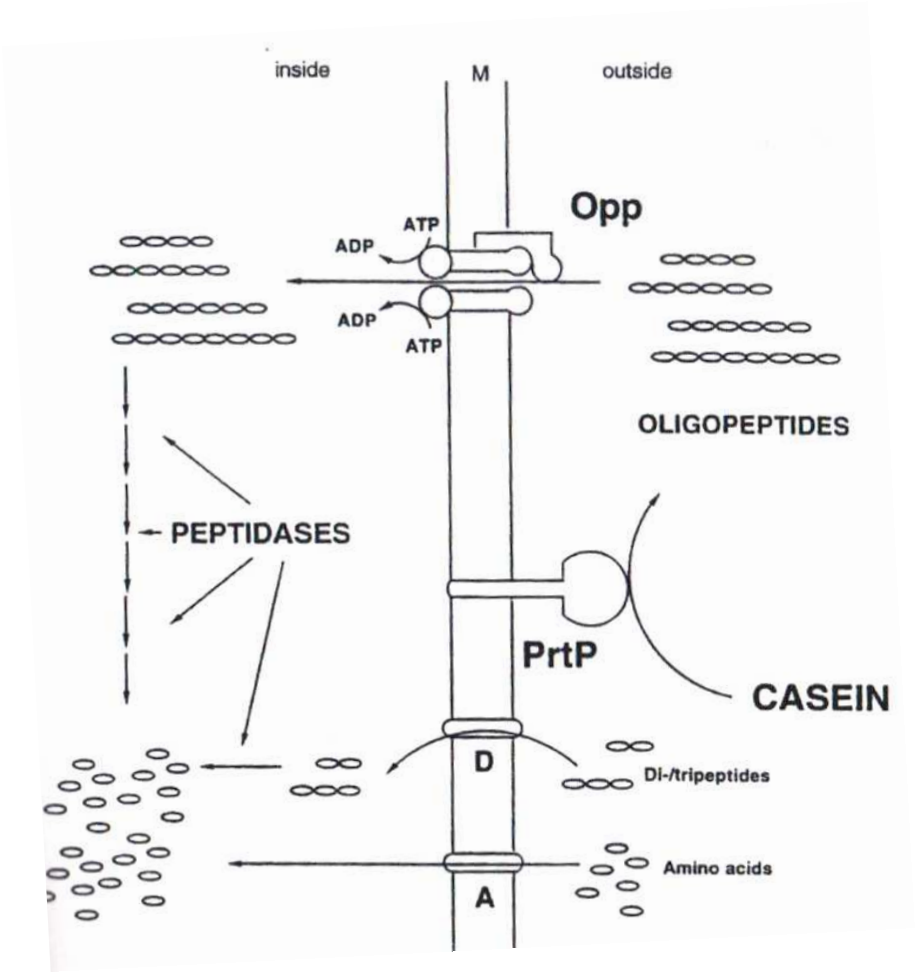


Figure 2.7 Model of the proteolytic pathway in *Lactococcus lactis.* Included transport of di- and tri-peptides and free amino acid, but note that these contribute very little to the total growth of lactococci in milk. PrtP, membrane-anchored proteinase; Opp, oligopeptide transport system; D, di-/tri-peptide transport system (s); A, amino acid transport system (s); M, cytoplasmic membrane.

Source: Axelsson (2004).

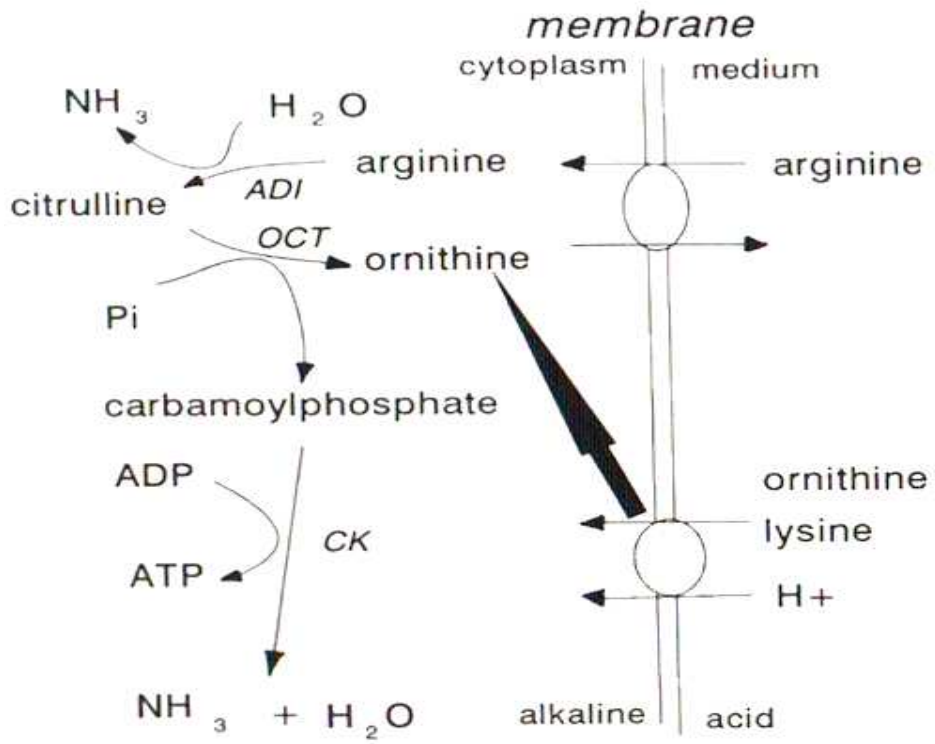


Figure 2.8 Arginine/ornithine antiport and the arginine deiminase pathway in *Lactococcus lactis* subsp. *lactis*. Accumulation of ornithine (lysine) via the Δp-driven lysine transport system is also shown. ADI, arginine deiminase; OCT, ornithine carbamoyl-transferase; CK, carbamate kinase.

Source: Teuber (1995).

2.1.4 Ecology of lactic acid bacteria

Lactic acid bacteria have complex nutrient requirements, due to their limited ability to synthesize B-vitamins and amino acids (Axelsson, 2004). Therefore, they are found in foods and silages such as dairy products, fermented meat, sour dough, fermented vegetables, corn silage, grass silage, beverages, and also found in the gastrointestinal tract, oral cavity, intestine, vagina, and faeces of humans and animals (Wood and Holzapfel,9 1995) (Table 2.4).

Table 2.4 Habitats of some lactic acid bacterial species.

Habitat	Source	Species[a]	Reference
Human and animals:			
Gastrointestinal tract	Human	*L. reuteri*	Shornikova *et al*. (1997); Devriese *et al.* (1991)
		E. faecium	Shornikova *et al*. (1997); Devriese *et al.* (1991)
		E. faecalis	Shornikova *et al*. (1997); Devriese *et al.* (1991)
	Poultry	*E. faecium*	Shornikova *et al*. (1997); Devriese *et al.* (1991)
	Cattle	*E. faecium*	Shornikova *et al*. (1997); Devriese *et al.* (1991)
	Dog	*E. faecium*	Devriese *et al.* (1992)
	Cat	*E. faecium*	Devriese *et al.* (1992)
Mucosal membranes:			
Oral cavity	Human	*S. constellatus*	Holdeman and Moore (1974)
		S. intermedius	Holdeman and Moore (1974)
		S. mitis	Holdeman and Moore (1974)
		S. oralis	Andrewes and Horder (1906)
		S. mutans	Andrewes and Horder (1906)
	Wild rat	*S. mutans*	Bridge and Sneath (1982)
	Hamster	*S. mutans*	Bratthall (1970)
Intestine	Human	*L. acidophilus*	Bratthall (1970)
		L. rhamnosus	Bratthall (1970)

Table 2.4 (Continued) Habitats of some lactic acid bacterial species.

Habitat	Source	Species[a]	Reference
Mucosal membranes:			
Intestine	Human	*L. plantarum*	Hansen and Mocquot (1970)
		L. brevis	Hansen and Mocquot (1970)
		L. salivarius	Collins *et al.* (1989)
	Dog	*L. acidophilus*	Bergey *et al.* (1923)
	Rat	*E. hirae*	Hammes and Vogel (1995)
	Foal	*E. hirae*	Rogosa *et al.* (1953)
	Piglet	*E. hirae*	Hammes and Vogel (1995)
	Dog pup	*E. hirae*	Devriese and Pot (1995)
	Pig	*E. hirae*	Devriese and Pot (1995)
	Rainbow	*S. thoraltensis*	Devriese and Pot (1995)
	Trout	*S. intestinalis*	Devriese and Pot (1995)
	Carp	*C. piscicola*	Devriese and Pot (1995)
	Salmon	*C. piscicola*	Hardie and Whiley (1995)
	Channel catfish	*C. piscicola*	Hardie and Whiley (1995)
Vagina	Human	*L. crispatus*	Hammes and Vogel (1995)
	Sow	*L. johnsonii*	Hammes and Vogel (1995)
		S. hyovaginalis	Hardie and Whiley (1995)
		S. thoraltensis	Hardie and Whiley (1995)
Faeces	Human	*L. crispatus*	Hammes and Vogel (1995)
		L. reuteri	Hammes and Vogel (1995)
		S. bovis	Hardie and Whiley (1995)
		S. intestinalis	Hardie and Whiley (1995)

Table 2.4 (Continued) Habitats of some lactic acid bacterial species.

Habitat	Source	Species[a]	Reference
Mucosal membranes:			
Faeces	Chicken	*L. johnsonii*	Hammes and Vogel (1995)
	Mice	*L. johnsonii*	Hammes and Vogel (1995)
	Pig	*L. johnsonii*	Hammes and Vogel (1995)
		S. bovis	Hardie and Whiley (1995)
		S. intestinalis	Hardie and Whiley (1995)
	Hamster	*L. hamsteri*	Hammes and Vogel (1995)
	Cow	*L. casei*	Hammes and Vogel (1995)
		L. coryniformis	Hammes and Vogel (1995)
		L. plantarum	Hammes and Vogel (1995)
	Cow	*L. brevis*	Hammes and Vogel (1995)
Fermented food and feed:			
Fermented vegetable	Fermented cucumber	*L. plantarum*	Daeschel *et al.* (1990)
	Fermented cassava	*L. plantarum*	Kostinek *et al.* (2005)
	Sauerkraut	*Leuc. mesenteroides*	Dellaglio *et al.* (1995)
		L. brevis	Dellaglio *et al.* (1995)
		L. plantarum	Dellaglio *et al.* (1995)
Sour dough	Sour dough	*L. curvatus*	Dellaglio *et al.* (1995)
		L. sakei	Dellaglio *et al.* (1995)
		L. reuteri	Hammes and Vogel (1995)
		L. brevis	Hammes and Vogel (1995)
		L. fermentum	Hammes and Vogel (1995)
		L. plantarum	Hammes and Vogel (1995)
Fermented meat	Dry sausage	*L. sakei*	Hammes and Vogel (1995)

Table 2.4 (Continued) Habitats of some lactic acid bacterial species.

Habitat	Source	Species[a]	Reference
Fermented meat	Dry sausage	*L. curvatus*	Hammes and Vogel (1995)
		L. plantarum	Hammes and Vogel (1995)
		L. farciminis	Hammes and Vogel (1995)
Fermented food and feed:			
Fermented meat	Fermented sausage	*P. pentosaceus*	Simpson and Taguchi (1995)
		L. alimentarius	Hammes and Vogel (1995)
	Semi-dry sausage	*P. pentosaceus*	Garvie (1986)
Dairy products	Cheese	*E. faecalis*	López-Díaz *et al.* (2000)
		E. faecium	López-Díaz *et al.* (2000)
		E. durans	López-Díaz *et al.* (2000)
		E. avium	López-Díaz *et al.* (2000)
		Lc. lactis	López-Díaz *et al.* (2000)
		L. plantarum	López-Díaz *et al.* (2000)
		L. casei	López-Díaz *et al.* (2000)
		L. rhamnosus	López-Díaz *et al.* (2000)
		Leuc. mesenteroides subsp. *dextranicum*	López-Díaz *et al.* (2000)
		Leuc. mesenteroides subsp. *mesenteroides*	López-Díaz *et al.* (2000)
		Leuc. paramesenteroides	López-Díaz *et al.* (2000)
	Fermented milk	*L. acidophilus*	Mercenier *et al.* (2003)
		L. rhamnosus	Mercenier *et al.* (2003)
		L. reuteri	Mercenier *et al.* (2003)
		L. casei	Mercenier *et al.* (2003)

Table 2.4 (Continued) Habitats of some lactic acid bacterial species.

Habitat	Source	Species[a]	Reference
Fermented food and feed:			
Dairy products	Fermented milk	*L. plantarum*	Mercenier *et al.* (2003)
		L. johnsonii	Mercenier *et al.* (2003)
		L. crispatus	Mercenier *et al.* (2003)
		L. paracasei	Mercenier *et al.* (2003)
		L. gasseri	Mercenier *et al.* (2003)
	Yoghurt	*L. bulgaricus*	Talon *et al.* (2002)
		S. thermophilus	Talon *et al.* (2002)
		L. acidophilus	Talon *et al.* (2002)
	Sour cream	*W. confusa*	Van Der Meulen *et al.* (2007)
		W. cibaria	Van Der Meulen *et al.* (2007)
Silage	Corn silage	*L. casei*	Cai *et al.* (2007)
		P. acidilactici	Torriani *et al.* (1987)
	Grass silage	*L. plantarum*	Hammes and Vogel (1995)
		E. faecium	Devriese and Pot (1995)
Spoiled food:			
Raw milk	Cow	*Lc. lactis*	Schleifer *et al.* (1985)
		Lc. cremoris	Schleifer *et al.* (1985)
		L. casei	Orla-Jensn (1919)
		S. bovis	Orla-Jensn (1919)
		S. uberis	Orla-Jensn (1919)
		A. viridans	Devriese *et al.* (1999)
		E. faecium	Sherman and Wing (1937)

Table 2.4 (Continued) Habitats of some lactic acid bacterial species.

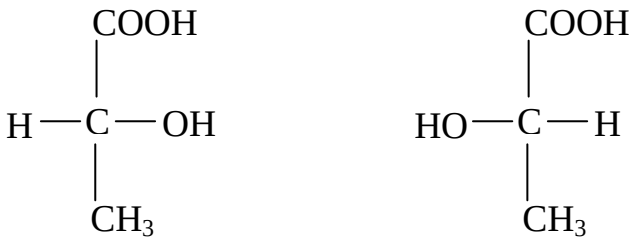
Habitat	Source	Species[a]	Reference
Spoiled food:			
Raw milk	Cow	*E. durans*	Sherman and Wing (1937)
Meat	Vacuum-packaged meat	*C. divergens*	Shaw and Harding (1984)
		C. piscicola	Morishita and Shiromizu (1986)
		L. sakei	Schillinger and Lucke (1986)
		L. curvatus	Schillinger and Lucke (1986)
		Leuc. gelidum	Collins *et al.* (1987)
		Leuc. carnosum	Collins *et al.* (1987)
	Vacuum-packaged beef	*Lc. raffinolactis*	Schillinger and Lucke (1986)
	Vacuum-packaged refrigerated beef	*L. algidus*	Kato *et al.* (2000)
	Modified atmosphere packaged poultry meat	*Lc. raffinolactis*	Barakat *et al.* (2000)
Beverages	Wine	*Leuc. oenos*	Dellaglio *et al.* (1995)
		Leuc. mesenteroides	Dellaglio *et al.* (1995)
		L. plantarum	Dellaglio *et al.* (1995)
		P. damnosus	Dellaglio *et al.* (1995)
	Beer	*P. dextrinicus*	Simpson and Taguchi (1995)
		L. casei	Hammes and Vogel (1995)
		L. brevis	Hammes and Vogel (1995)
		L. malefermentans	Hammes and Vogel (1995)
	Grape fruit juice	*L. plantarum*	Kelly *et al.* (1996)

[a] *L.*, *Lactobacillus*; *P.*, *Pediococcus*; *Leuc.*, *Leuconostoc*; *S.*, *Streptococcus*; *Lc.*, *Lactococcus*; *C.*, *Carnobacterium*; *A.*, *Aerococcus*; *E.*, *Enterococcus*; *W.*, *Weissella*.

2.2 D-Lactic acid

2.2.1 Chemical structure and property

D-Lactic acid, 2-hydroxypropionic acid, is a mirror image of L-lactic acid which could be soluble in water (Narayanan *et al*., 2004; John *et al*., 2007). It exhibits low volatility, and has chemical formula of $C_3H_6O_3$ (Figure 2.9). The L-form diverge the D- form in its effect on polarized light. For L-lactic acid, the plane is rotated in a clockwise (dextro) direction, whereas the D-form rotates the plane in a anticlockwise (laevo) direction. Since lactic acid has high reactivity due to containing both hydroxyl (-OH) and carboxyl (-COOH) groups. In solution, lactic acid can lose a proton from the acidic group, producing the lactate ion $CH_3CH(OH)COO^-$. The lactate ion could be precipitated with salts solution such as $MgCl_2$ (Benthin and Villadsen, 1995), $Ca(OH)_2$, and $CaCO_3$ (Narayanan *et al*., 2004). However, $MgCl_2$ is added to the supernatant to neutralize the acid produced and produce a magnesium salt of the acid which could improve purification method of lactic aid fermentation (Benthin and Villadsen, 1995).



D(-)-Lactic acid **L(+)-Lactic acid**

Figure 2.9 Enantiomers of lactic acid; D(-)-lactic acid and L(+)-lactic acid.

Source: Kharas *et al.* (1994).

Other properties of lactic acid and isomer of lactic acid are summarized in Table 2.5. Both enantiomers of lactic acid have the same physical properties.

Table 2.5 Physical properties of lactic acid.

Property	Value
Molecular weight	90.08 g/mol
Melting point	L: 53°C D: 53°C
Boiling point	122°C at 14 mm Hg 82°C at 0.5 mm Hg
Dissociation constant, K_a at 25°C	1.37×10^{4}
Acidity (pK_a)	3.85
Heat of combustion, ΔH_c	1361 KJ/mole
Specific heat, C_p at 20°C	190 J/mole/°C

Source: Narayanan *et al.* (2004).

2.2.2 Sources of D-lactic acid

Lactic acid is commercially produced either by chemical synthesis or by microbial fermentation. Approximately 90% of the total lactic acid produced worldwide is by bacterial fermentation and the rest is produced synthetically by the hydrolysis of lactonitrile (John *et al*., 2009). The chemical synthesis of lactic acid always results in racemic mixture of lactic acid, which is a major disadvantage. The biotechnological production of lactic acid offers several advantages compared to chemical synthesis like low cost of substrates, low production temperature, and low energy consumption. High product specificity is yet another advantage of lactic acid fermentation, as it produces a desired stereoisomer, optically pure L-(+)- or D-(−)-lactic acid (Hofvendahl and Hahn-Hägerdal, 2000; John *et al*., 2009).

2.2.2.1 Chemical synthesis

D-Lactic acid can be produced by either chemical synthesis or microbial fermentation. The chemical synthesis always results in a racemic mixture of lactic acid, and was represented by reactions described in Figure 2.10 (Narayanan *et al.*, 2004).

(a) Addition of hydrogen cyanide

$$\underset{\text{Acetaldehyde}}{CH_3CHO} + \underset{\text{Hydrogen cyanide}}{HCN} \xrightarrow{\text{catalyst}} \underset{\text{Lactonitrile}}{CH_3CHOHCN}$$

(b) Hydrolysis by H_2SO_4

$$\underset{\text{Lactonitrile}}{CH_3CHOHCN} + H_2O + \underset{\text{Sulphuric acid}}{1/2H_2SO_4} \longrightarrow \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Ammonium salt}}{1/2(NH_4)_2SO_4}$$

(c) Esterification

$$\underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Methanol}}{CH_3OH} \longrightarrow \underset{\text{Methyl lactate}}{CH_3CHOHCOOCH_3} + H_2O$$

(d) Hydrolysis by H_2O

$$\underset{\text{Methyl lactate}}{CH_3CHOHCOOCH_3} + H_2O \longrightarrow \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Methanol}}{CH_3OH}$$

Figure 2.10 Lactic acid production process via chemical synthesis.
Source: Narayanan *et al.* (2004).

The commercial process for lactic acid synthesis is based on lactonitrile. Hydrogen cyanide is added to acetaldehyde in the presence of a base to produce lactonitrile. This reaction occurs in liquid phase at high atmospheric pressures. The crude lactonitrile is recovered and purified by distillation. It is hydrolyzed to lactic acid, either by concentrated HCl or by H_2SO_4 to produce the

corresponding ammonium salt and lactic acid. Lactic acid is esterified with methanol to produce methyl lactate, which is removed and purified by distillation and hydrolyzed by water under acid catalyst to produce lactic acid and the methanol, which is recycled.

2.2.2.2 Microbial fermentation

Several organisms could produce lactic acid such as lactic acid bacteria and molds, particularly in the genus *Rhizopus*, *Mucor*, and *Monilia* (Narayanan *et al*., 2004). These fungal strains produce only L-lactic acid aerobically from glucose, sucrose or starch. Whereas, lactic acid bacteria could produce either D- or L-lactic acid or racemic mixture of lactic acid by fermentation depending on the species being used. About 90% of literatures reported on lactic acid production, were focused on bacterial fermentation (Zhao *et al*., 2010). Lactic acid can be produced from sugars or sugar containing hydrolyzates or the single-step conversion of starchy or cellulosic wastes by direct conversion by amylolytic lactic acid-producing microorganisms or by the simultaneous hydrolysis and fermentation by adding enzymes and inoculum together. The configuration of lactic acid produced by fermentation is dependent upon the stereospecificity of the lactate dehydrogenase possessed by the organism. D-Lactic acid-producing bacteria contain D-lactate dehydrogenase (D-LDH), which is a key enzyme converting pyruvate to D-lactic acid. Some lactic acid bacterial strains have been studied for their D-lactic acid production capabilities. Examples are *Lactobacillus delbrueckii*, *Lactobacillus coryniformis* subsp. *torquens*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc carnosum*, and *Leuconostoc fallax* (Manome *et al*., 1998). In addition, Yáñez *et al.* (2003) reported that *Lactobacillus*

coryniformis subsp. *torquens* could produce D-lactic acid from filter paper by simultaneous saccharification and fermentation (SSF) with adding enzymes for cellulose hydrolysis. *Lactobacills delbrueckii* JCM 1148^{T} was also reported to produce D-lactic acid from sugarcane molasses, sugarcane juice, and sugar beet juice (Calabia and Tokiwa, 2007). Recently, Okano *et al*. (2009a) reported that *Lactobacillus plantarum* NCIMB 8826^{T} *ldhL1*: *amyA*, a strain replacing the *ldhL1* gene with an *amyA*-secreting expression cassette from *Streptococcus bovis* 148, could directly produce D-lactic acid from raw corn starch. Moreover, Okano *et al*. (2009b) reported that optically pure D-lactic acid fermentation from arabinose was achieved by using the *Lactobacillus plantarum* NCIMB 8826^{T} Δ*ldhL1*. The mutant strain also had ability to direct fermentation of an optical pure D-lactic acid from cellulosic materials (Okano *et al*., 2010). While, Joshi *et al*. (2010) reported the production of D-lactic acid from sucrose, molasses and cellobiose using the mutant strain of *Lactobacillus lactis* NCIM 2368^{T}.

2.2.3 Application of D-lactic acid

PDLA is important to be applied to blend with PLLA in biopolymer production (Zhao *et al*., 2010). PLLA is a biodegradable polymer, and approved for use in food packaging in several countries, particularly United States of America (U.S.A.), European Union *(*EU*)* countries, and Japan (Narayanan *et al*., 2004). The polymer has increased in demand in recent years. Though L-lactic acid can be polymerized to give crystalline PLLA suited to commercial uses (Sodegard and Stolt, 2002), its application is limited by its low melting point. Thermal stability of PLA is not sufficiently high to some applications as an alternative of commercial polymers. Thermal processes such as melt molding and spinning causes thermal degradation of

PLLA (Tsuji and Fukui, 2003). However, PLLA could form stereocomplex crystallites with PDLA in solution or during crystallization from the melt, which could enhance the mechanical performance, thermal stability, and hydrolysis-resistance of PLLA-based materials. Therefore widen its applications as an alternative of commercial polymers and drug delivery systems (Wang and Mano, 2008). This finding made D-lactic acid more important.

PDLA which obtained by polymerization of D-lactic acid, was found to improve the thermostability of PLLA by the stereocomplex formation (Tsuji and Fukui, 2003). Ikada *et al.* (1987), and also reported that the 1:1 blend of PLLA and PDLA produced a stereocomplex with T_m around 230°C higher than melting temperature of PLLA (T_m around 175°C). Tsuji (2000, 2002) investigated the *in vitro* hydrolysis of well-stereo-complexed 1:1 blend and non-blended films from PLLA and PDLA, and found the reason for the difference in hydrolysis behaviors between the well-stereo-complexed 1:1 blend and non-blended films. For this investigation, the films were prepared from PLLA and PDLA both having a medium molecular weight $M_W = 1.5\times10^5$ by solvent evaporation method and their hydrolysis in phosphate-buffered solution (pH = 7.4) at 37°C. Hydrolysis of the 1:1 blend and non-blended films was performed up to 30 months, and the hydrolyzed films were studied using gel permeation chromatography (GPC), tensile tests, differential scanning calorimetry (DSC), scanning electron microscopy (SEM), optical polarizing microscopy, X-ray diffractometry, and gravimetry. It was found that the rate of reduction in molecular weight, tensile strength, Young's modulus, melting temperature, and mass remaining of the films in the course of hydrolysis was lower for the well-stereo-complexed 1:1 blend film than the non-blended films. The induction period until the start of decrease

in tensile strength, Young's modulus, and mass remaining were longer for the well-stereo-complexed 1:1 blend film than for the non-blended films. These findings strongly suggest that the well-stereo-complexed 1:1 blend film is more hydrolysis-resistant than the non-blended. This is probably due to the peculiar strong interaction between L- and D-lactyl unit sequences in the amorphous state, resulting in the future decreased interaction of PLLA or PDLA chains and water molecules. However, the peculiar strong interaction between PLLA and PDLA chains may have caused the retarded proteinase K-catalyzed hydrolysis of the PLLA/PDLA blend film compared with that expected from the hydrolysis rates of non-blended PLLA and PDLA films.

PDLA acted as a nucleating agent of PLLA, thereby increasing the crystallization rate (Yamane and Sasai, 2003). The role of stereocomplex as a nucleating agent and the crystallization of homo PLLA was initiated in instantaneous homogeneous nucleation (Figure 2.11). When the blends were cooled from 200°C to 120°C, the size of the spherulites decreased and the number of the spherulites increased significantly with PDLA content. It is clear that the blends with higher PDLA content (5% PDLA) have a higher number of nucleation sites. These nucleation sites are stereocomplex crystallites with 3/1 helix in conformation and surrounded by PLLA crystalline phase.

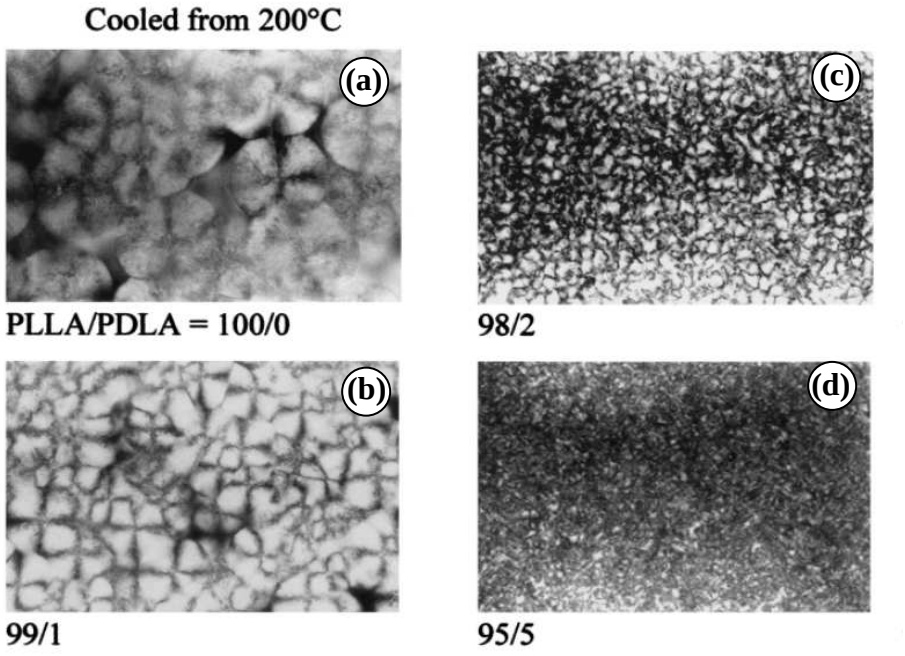


Figure 2.11 The polarized optical micrographs of the spherulites (A: pure PLLA, B to D: PLLA blend with 1%, 2%, and 5% of PDLA, respectively) grown at 120°C observed in the blends which contained PDLA with a molecular weight of 1.2×10^5 after cooling from 200°C.

Source: Yamane and Sasai (2003).

2.2.4 Production of D-lactic acid by bacterial fermentation

2.2.4.1 Substrates

The choice of substrates to be used for D-lactic acid production depends on the microorganisms studied because microorganisms have different sugar transport systems. Carbohydrates are the common substrates for microbial fermentation. It has been reported that generally lactic acid bacteria could ferment glucose via different pathways. However, glucose is economically unfavorable for D-lactic acid fermentation. A major concern in D-lactic acid fermentation was to

reduce the cost of raw materials which accounted for more than 34% of total production-cost (Akerberg and Zacchi, 2000). Cheap raw materials are necessary for the feasible economic production of lactic acid because polymer producers and other industrial users usually require large quantities of lactic acid at a relatively low cost. D-Lactic acid production could provide low production cost, when agricultural products or waste cellulosic substrates such as rice bran, rice starch, sugarcane molasses, sugarcane juice, sugar beet juice, and filter paper were used as substrates for D-lactic acid fermentation (Table 2.6).

2.2.4.2 Bacterial strains

Some species of lactic acid bacteria have been studied for their D-lactic acid production capabilities. Examples are *Lactobacillus delbrueckii*, *Lactobacillus coryniformis* subsp. *torquens*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc carnosum*, *Leuconostoc fallax* (Manome *et al*., 1998), and *Lactobacillus bulgaricus* (Benthin and Villadsen, 1995). These species have D-lactate dehydrogenase (D-LDH) which converts pyruvate to D-lactic acid during sugars fermentation.

Table 2.6 D-Lactic acid production by lactic acid bacteria using different substrates.

Substrates concentration	Microorganisms[a]	D-Lactic acid purity (%)[b]	Reference
Glucose (10 g/l)	*L. delbrueckii*	87.2	Manome *et al.* (1998)
	L. coryniformis subsp. *torquens*	92.2	
	Leuc. mesenteroides subsp. *mesenteroides*	95.7	
	Leuc. mesenteroides subsp. *dextranicum*	91.5	
	Leuc. carnosum	94.0	
	Leuc. fallax	86.1	
Glucose (100 g/l)	*L. plantarum* NCIMB 8826 Δ*ldhL1*/pCUSαA	99.7	Okano *et al.* (2009b)
Lactose (100 g/l)	*L. bulgaricus*	99.9	Benthin and Villadsen (1995)
Filter paper (33 g/l)	*L. coryniformis* subsp. *torquens*	100.0	Yáñez *et al.* (2003)
Rice starch (100 g/l, converted value as maltose)	*L. delbrueckii*	97.5	Fukushima *et al.* (2004)
Rice bran (100 g/l)	*L. delbrueckii*	95.0	Tanaka *et al.* (2006)
Sugarcane molasses (119 g/l)	*L. delbrueckii*	97.1	Calabia and Tokiwa (2007)
Sugarcane juice (133 g/l)	*L. delbrueckii*	98.3	
Sugar beet juice (105 g/l)	*L. delbrueckii*	97.5	
Rice powder (100 g/l, converted value as maltose)	*L. delbrueckii* LD 0028	97.5	Lee (2007)
Raw corn starch (100 g/l)	*L. plantarum* NCIMB 8826 Δ*ldhL1*/pCUSαA	99.6	Okano *et al.* (2009b)
Cellooligisaccharides (2 g/l)	*L. plantarum* NCIMB 8826 Δ*ldhL1*/pCUSαA	99.5	Okano *et al.* (2010)
Hydrolyzed cane sugar (150 g/l)	*L. lactis* NCIM2368	98.0	Joshi *et al.* (2010)

[a] *L.*, *Lactobacillus*; *Leuc.*, *Leuconostoc.*

[b] D-Lactic acid purity (%) = $\left(1-\frac{\text{L - lactic acid}}{\text{total lactic acid}}\right)\times 100$.

2.2.4.3 Fermentation process

A) Fermentation conditions

The amount of D-lactic acid, which are produced by lactic acid bacteria is strongly influenced by cultures and fermentation conditions. Some main factors affecting the D-lactic acid production as follow:

1A) Chemical factors

Lactic acid bacteria typically have complex nutritional requirements, due to their limited ability to synthesize their own growth factors such as vitamins B and amino acids. They require some elements for growth, such as carbon and nitrogen sources, in the form of carbohydrates, amino acids, vitamins, and minerals (Axelsson, 2004; Wee *et al*., 2006). They are several growth-stimulation factors that have a considerable effect on the production rate of lactic acid (Wee *et al*., 2006).

Compositions of the medium (carbon and nitrogen sources, minerals and growth factors) are known to have impact on D-lactic acid production.

The carbohydrates utilized for D-lactic acid manufacture are generally derived from by products of agricultural processes (e.g., rice bran) and sugars: glucose, sucrose (from cane or beet sugar) and lactose (from cheese whey or whey permeate). A number of literatures reported on the capability of several bacterial strains to produce D-lactic acid by using sugars, rice bran, and filter paper as a carbon source (Table 2.6).

The nutritionally fastidious bacteria satisfy their requirements for peptides and amino acids as a nitrogen source through the activities

of protease and peptidase enzymes. Yeast extract, peptone, tryptone, and meat extract are the most important medium components for lactic acid fermentation. These compounds are principal growth factors for lactic acid bacteria. Timbuntam *et al*. (2006) tried various nitrogen sources like silkworm larvae, yeast autolyzate, dry yeast, and shrimp waste as a replacement of yeast extract in cane juice medium. At the same concentration of nitrogen sources (1% w/v), addition of silkworm larvae, yeast autolyzate, and shrimp waste all led to increases in lactic acid production more than that addition of yeast extract.

2A) Physical factors

pH is an easily manipulated variable in the process and it has a very strong impact on the cell response and metabolism. From the production standpoint, pH control is absolutely required to achieve essential of lactic acid concentrations for an economical process. In general terms, lactic acid bacteria tolerate pH values between 3.4 and 8.0, but growth and production mostly occur between pH 5.4 and 6.4, with the optimum pH being strain-dependent (Kharas *et al*., 1994). A pH range of 6.0-6.5 has been reported optimal for lactic acid production using *Lactobacillus casei* strain (Krischke *et al*., 1991). However, pH 5.5 has been used for lactic acid production using *Lactobacillus helveticus* by Ghaly *et al.* (2004). The optimum pH for D-lactic acid production generally ranges between 5 and 7. Manome *et al*. (1998) showed that the optimal D-lactic acid production for *Lactobacillus delbrueckii* was at 37°C and pH 6.8. To avoid drop of pH during fermentation, salt solution is added to the medium for maintaining pH between 5.0 and 7.0.

The temperature is also one of the important factors, which influences the activity of metabolic/cell enzymes. Enzymes are most active at optimum temperature and enzymatic reaction proceeds at maximum rate. However, below and above optimal temperature, reaction rate is decreased, which causes the problems in cell metabolism (Panesar *et al*., 2010). Effects of temperature on D-lactic acid production are highly variable, and are depend on the strain being used and the experimental conditions. The optimal temperature for growth of lactic acid bacteria varies between genera from 20 to 45°C (Wood *et al*., 1995). *Lactobacillus helveticus* and *Lactobacillus acidophilus* could use in a temperature range of 37-45°C. Krischke *et al.* (1991) used 37°C temperature for lactic acid production using *Lactobacillus casei*. In addition, a temperature of 28°C has also been reported optimal for *Lactobacillus casei* (Nabi *et al*., 2004). There are a number of literatures reported on D-lactic acid production using a variety of temperatures, for example, 30°C for *Lactobacillus coryniformis* subsp. *torquens*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc carnosum*, and *Leuconostoc fallax* (Manome *et al*., 1998), 37°C for *Lactobacillus delbrueckii*, *Lactobacillus bulgaricus* (Benthin and Villadsen, 1995; Manome *et al*., 1998; Tanaka *et al*., 2006), 39°C for *Lactobacillus coryniformis* subsp. *torquens* (Yáñez *et al*., 2003), and 40°C for *Lactobacillus delbrueckii* (Calabia *et al*., 2007).

B) Process configuration

Batch, fed-batch, and continuous fermentations are the most frequently used methods for lactic acid production.

1B) Batch fermentation

Batch fermentation is one of the methods used in industrial production of lactic acid, where the sterile growth medium is inoculated with the microorganism, and no additional growth medium is added. D-Lactic acid is most commonly produced in the batch fermentation under the best fermentation conditions. For batch fermentation of lactic acid production by lactic acid bacteria, the best result obtained in the published patent with glucose as the substrate is 115 g/l for final D-lactic acid concentration (Voleskow *et al*., 1984). Sugarcane juice (as the substrate) 118 g/l of D-lactic acid with productivity 1.66 g/l.h (Calabia and Tokiwa, 2007). Recently, the highest D-lactic acid concentration (120 g/l) with productivity of 1.0 g/l.h in batch fermentation was observed with 200 g cane sugar/l (Joshi *et al*., 2010). While the batch fermentation of *Corynebacterium glutamicum* Δ*ldhA*/pCRB204, the D-lactic acid concentration also reached 120 g/l (Okino *et al*., 2008). Batch fermentation has been the process used industrially for L- and D-lactic acid production. However, lactic acid concentration and productivity could also be decreased due to the inhibition by high substrate concentration and low pH of the medium. Several reports proposed fed-batch and continuous culture techniques to eliminate this restriction by providing an essentially invariant microbial environment.

2B) Continuous fermentation

In continuous cultures, cells can be maintained at a constant physiological state and growth rate. Membrane cell-recycle systems coupled with the repeated-batch and continuous cultures have proven to be efficient in lactic acid production. Some researches developed a bioreactor combining conventional electrodialysis and bipolar membrane electrodialysis for product removal and pH

control in lactic acid fermentation. Wee and Ryu (2009) conducted continuous D-lactic acid fermentations using lignocellulosic hydrolyzates and corn steep liquor as inexpensive raw materials in a 2.5 l bioreactor system by *Lactobacillus* sp. With lignocellulosic hydrolyzates, the lactic acid productivity was maximum (6.7 g/l.h) at a dilution rate of 0.16 h^{-1} under the cell-recycle continuous fermentation conditions, which D-lactic acid concentration was 42.0 g/l. The cell-recycle continuous fermentation of lignocellulosic hydrolyzates yielded a lactic acid productivity of 6.7 g/l.h for a dilution rate of 0.16 h^{-1} using 30 g/l of corn steep liquor and 1.5 g/l of yeast extract as nutrients. The productivity (6.7 g/l.h) acquired by the cell-recycle continuous fermentation of lignocellulosic hydrolyzates was 1.6 times higher than the lactic acid productivity in the continuous fermentation without cell-recycle system.

3B) Fed-batch fermentation

Fed-batch culture is a batch culture fed continuously or sequentially with substrate without the removal of fermentation medium, which is beneficial when changing nutrient concentrations affect the productivity and biomass of the desired product (Lee *et al*., 1999; Roukas and Kotzekidou, 1998). Higher lactic acid concentrations may be obtained in batch and fed-batch cultures than in continuous cultures, whereas higher productivity may be achieved by the use of continuous cultures. For example, Ding and Tan (2006) developed a fed-batch fermentation process with different feeding strategies: pulse fed-batch, constant feed rate fed-batch, constant residual glucose concentration fed-batch, and exponential fed-batch, for enhancing the production of L-lactic acid from glucose by *Lactobacillus casei* LA-04-1. All experiments were carried out in a 5 l fermenter with an initial broth volume of 2.2 l at 42°C, and agitation speed at 150 rpm. Exponential fed-batch

culture exhibited the effective process for L-lactic acid production. After cultivating the bacterial strain for 84 h, the maximum L-lactic acid concentration, obtained in the exponential feeding glucose solution with 850 g/l and 1% of yeast extract, was 180 g/l which was 90.3% for L-lactic acid yield and 2.14 g/l.h for productivity. The exponential fed-batch culture showed 56.5% improvement in L-lactic acid production, compared to the traditional batch culture in which 112.5 g/l for final L-lactic acid were achieved from glucose concentration of 140 g/l and using 25% NH_4OH for controlling pH at 6.25.

2.2.5 Extraction and purification

The extraction and purification of D-lactic acid from fermentation broth are important for obtaining D-lactic acid. Several methods are available for the purification of lactic acid from fermentation media. The classical methods are based on precipitation, extraction or distillation (Vaccari *et al*., 1993; Lazarova and Peeva, 1994). Methods based on ion-exchange or electrodialysis have also been investigated (Vaccari *et al*., 1993). Solvent extraction and purification by crystallization of D-lactic acid from lactose fermentation using *Lactobacillus bulgaricus* were described by Benthin and Villadsen (1995). The extraction and purification steps consisted of successive precipitation with Mg salt, butanol extraction and purification by crystallization. Each step of the purifications is documented by the yield of D-lactic acid and purity of the D-lactic acid by measurement of the elements N and P. Nitrogen-containing compounds in the fermentation medium are mainly amino acids and peptides. These compounds are highly soluble and are, therefore, effectively removed in the crystallization step. The extraction step is very effective in removing phosphorus because phosphorus is negatively charged. After completion of the

purification, the overall yield of D-lactic acid was 72% and the purity was more than 99%. Contaminations in the final D-lactic acid with nitrogen, phosphorus and L-lactic acid were only 0.032% (w/w), 0.018% (w/w) and 0.04% (w/w), respectively.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals, reagents, and media

Reagents and media used in each steps were as follows:

3.1.1 Screening, selection, and production of D-lactic acid

The microbiological medium used for culturing lactic acid bacteria were De Man, Rogosa Sharpe (MRS) medium (Appendix A3.1) purchased from Himedia (Himedia laboratories, India) and modified Rogasa agar medium (Appendix A3.3; Rodtong and Ishizaki, 2003). Tryptone and yeast extract were purchased from Himedia (Hi-Media Laboratories Pvt Ltd, Mumbi, India). Di-potassium hydrogen phosphate anhydrous, manganese sulphate monohydrate, Iron (II) sulphate, D-glucose anhydrous, and magnesium sulfate monohydrate were obtained from Carlo erba (Carlo Erba Reagenti, Milan, Italy). Cassava starch was purchased from Sanguan Wongse Industries Co., Ltd., (Nakhon Ratchasima, Thailand). Proteose peptone, yeast extract, and beef extract were purchased from Himedia (Himedia laboratories). tri-Ammonium citrate, di-potassium hydrogen orthophosphate, and sodium acetate were obtained from Carlo erba (Carlo Erba Reagenti). Spent of brewery yeast sludge used for optimization and production of D-lactic acid was purchased from Boonrawd brewery Co., Ltd., (Khon Kaen, Thailand). Standards of D-lactic acid and L-lactic acid for analysis of lactic acid configurations are products of Fluka and Supelco (Sigma-Aldrich Chemical Company, Missouri, U.S.A.). Copper (II) sulphate which used for

mobile phase for the detection of lactic acid purchased from Carlo erba (Carlo Erba Reagenti).

3.1.2 Lactic acid bacterial identification

3.1.2.1 Biochemical characterization

Media and reagents for testing carbohydrate fermentation of lactic acid bacteria were API 50 CHL medium and API 50 CH strips (Bio-Mérieux, bioMérieux, Inc., Marcy-l'Étoile, France).

3.1.2.2 Genetic characterization

Isopropyl alcohol (Merck KGaA, Darmstadt, Germany) was used to precipitate genomic DNA; 70% ethanol (Merck) to wash genomic DNA pellet. Agarose (Promega, U.S.A.) was used to prepare gel for electrophoresis. Reagents used for polymerase chain reaction (PCR) amplification: PCR buffer, $MgCl_2$ solution, dNTPs (dATP, dCTP, dGTP, and dTTP) and *Taq* DNA polymerase, were purchased from Invitrogen (Invitrogen, Invitrogen life technologies, Carlsbad, U.S.A.). Oligonucleotide primers were ordered from the Science Pacific Company, Ltd. (Thailand).

3.1.3 Extraction and purification of D-lactic acid

Analytical grade of magnesium chloride for D-lactic acid crystallization was purchased from Asia Pacific Chemicals Limited, (Ajax, Taren Point, Australia). Sulphuric acid (96%) for acidification was purchased from Carlo erba (Carlo Erba Reagenti). Activated carbon for decolourization was bought from Merck (Merck KGaA).

3.2 Instrumentations

All instruments required for screening and selection of D-lactic acid-producing lactic acid bacteria, optimization of some D-lactic acid production conditions, production of D-lactic acid using optimum conditions, D-lactic acid purification, and identification of the selected isolate(s) of D-lactic acid-producing bacteria, are located at the Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

3.3 The approximate analysis of cassava starch and spent of brewery yeast sludge

The approximate analysis of cassava starch and spent of brewery yeast sludge including moisture content, fiber, total nitrogen, fat, ash, and available carbohydrate contents were conducted according to AOAC International (2000).

3.4 Screening and selection of lactic acid bacteria for D-lactic acid production

3.4.1 Screening of lactic acid bacteria

3.4.1.1 Bacteria

A least 300 isolates of lactic acid bacteria were obtained from stock cultures of the Microbial Culture Collection Laboratory, Institute of Science, Suranaree University of Technology. These isolates were kept in freezer at -80°C.

3.4.1.2 Cultivation of bacteria for screening of lactic acid production capability

The stock cultures were taken from -80°C, thawed at room temperature. Two hundred microliters of each culture were inoculated into 2 ml of either MRS broth (De Man, 1960), MC broth (MRS agar containing 0.5% $CaCO_3$), or RAM broth (Rogasa agar modified medium) (Appendix A3.1-3.3). After incubation at 35°C for 24 h under anaerobic conditions in anaerobic chamber (Shel LAB, Shelden Manufacturing Inc, U.S.A.) supplied with a gas mixture of CO_2:H_2:N_2 (5:5:90%), the culture was streaked onto the same media as broth and incubated at 35°C for 48h under anaerobic conditions. Single colonies were purified by successive streaking on these media. Then, a single colony of pure culture was collected for further study.

3.4.1.3 Test for acid and gas production using liquid medium containing glucose

A least 300 isolates of lactic acid bacteria were tested for lactic acid and gas production using MRS and RAM broth containing 2% (w/v) of glucose (Appendix A3.1 and A3.3). For production of lactic acid, one loopful of the selected isolate grew at 35°C for 48 h on MRS agar and RAM agar, was inoculated into 15 ml test tube containing 10 ml of MRS broth and RAM broth, then incubated at 35°C for 48 h under anaerobic conditions. The bacterial growth was monitored spectrophotometically at 600 nm (A_{600}). Then, Acid produced from glucose was examined by pH values measurement and total acidity expressed as percent lactic acid according to AOAC International (2000). Bacterial cells were removed from cultured broth by centrifuging at 10,000×g for 10 min at 4°C. The supernatant was used to determine total acidity by titration with 0.1 N NaOH to pH of 8.2. The isolate(s) capable of producing high total acidity were selected. The production of gas from glucose was examined in 5 ml of MRS broth or RAM broth in a test tube containing a

Durham tube, and incubated at 35°C for 24 h under anaerobic conditions. The production of gas from glucose was used for distinguishing between homofermentatives and heterofermentatives.

3.4.2 Selection of D-lactic acid production isolates, and test for cassava starch utilization using agar medium

3.4.2.1 Selection of D-lactic acid production isolates

After cultivating bacterial isolate(s) in 15 ml test tube containing 10 ml of MRS broth or RAM broth containing 2% glucose, at 35°C for 24 h under anaerobic conditions. Cells were removed from the broth by centrifuging at 10,000×g for 10 min at 4°C. The supernatant was filtered through a 0.45 μm membrane filter, and diluted 500 times with deionized water to concentration of 0.01 to 1.0 g/l to detect for D-lactic acid using high performance liquid chromatography (HPLC) (HP 1200, Agilent Technology Inc., U.S.A.) equipped with a Chiral Astec CLC-L column (5 μm, 4.6 mm×15 cm, Sigma Chemical Co., U.S.A.) (Tanaka *et al*., 2006). Mobile phase (0.005 M $CuSO_4$) was run isocratically at a flow rate of 0.7 ml/min. Injection volume was 10 μl. Lactic acid was detected by UV detector at 254 nm (Manome *et al*., 1998). The isolate(s) capable of producing D-lactic acid in high concentration were selected.

3.4.2.2 Investigation of cassava starch utilization using agar medium

The selected of D-lactic acid bacterial isolate(s) was tasted for its cassava starch utilization capability by point inoculating on RAM or MRS agar containing 1% of cassava starch as a carbon source, and incubated at 35°C for 48 h

under anaerobic conditions. Starch-hydrolyzing isolate(s) produced clear zone surrounding bacterial colonies on the agar plate after the addition of 1% iodine solution. Then, the isolate was selected for optimization of D-lactic acid production conditions.

3.5 Optimization of some conditions for D-lactic acid production

To obtain the efficient D-lactic acid production, some optimal conditions for cultivation the selected isolate(s) were investigated. Varies concentrations of carbon source and nitrogen source of RAM medium (section 3.4.1) was tested. Suitable initial pH of the culture medium, cultivation temperatures, and inoculum sizes were also investigated.

3.5.1 Concentrations of cassava starch

Carbon source is another crucial chemical factor affecting D-lactic acid production. To lactic acid production cost, cassava starch, a cheap and abundant raw material, was tested as the main carbon source in RAM medium (Appendix A3.5) at various concentrations (10, 15, 20, 25, 30, 35, and 40 g/l) to achieve the optimal concentration. D-Lactic acid-producing isolate at 2% (v/v) inoculum size (approximately 10^6 CFU/ml) was inoculated into 100 ml of the medium in 125 ml flask, and then incubated at 35°C for 48 h under anaerobic conditions. Bacterial growth was measured by plating serial dilutions of bacterial suspension on RAM agar. Plates were incubated anaerobically at 35°C for 48 h in anaerobic chamber (Shel LAB). The pH was measured using pH meter. Total acidity of the culture broth was determined according to AOAC International (2000). D-Lactic acid was determined by high performance liquid chromatography (HPLC). Total sugars were

determined by the phenol-sulphuric acid method (Dubois *et al*., 1956). The concentration of cassava starch that provided the highest concentration of D-lactic acid was chosen for further investigation.

3.5.2 Concentrations of tryptone

The optimum concentration of nitrogen source contained in the lactic acid fermentation medium was investigated. Tryptone in RAM medium composition was served as the main nitrogen source. Various concentrations of tryptone (2.0, 2.5, 3.0, 4.0, and 5.0 g/l) were added to the RAM medium (section 3.5.1) to obtain the optimal concentration. D-Lactic acid-producing isolate at 2% (v/v) inoculum size (approximately 10^6 CFU/ml) was inoculated into 50 ml of the medium in 125 ml flask, and then incubated at 35°C for 48 h under anaerobic conditions. Bacterial growth and acid production were monitored as described in section 3.5.1.

3.5.3 Sources and concentrations of growth factors

Lactic acid bacteria are generally fastidious organisms, which require complex nutrients such as amino acids and vitamins for cell growth (Oh *et al*., 2003). Yeast extract, the most commonly used nitrogen source and growth factors, provides complex nutrients for lactic acid bacteria (Vickroy, 1985). The optimum concentration of yeast extract was investigated using the medium containing the optimum concentrations of cassava starch and tryptone (sections 3.5.1-3.5.2). Spent of brewery yeast sludge, the cheap nitrogen and growth factor sources, was also used to replace yeast extract. Yeast extract and spent of brewery yeast sludge at concentrations of 1-10 g/l (Table 3.1) were applied for D-lactic acid production by the selected isolate. Bacterial growth and acid production were monitored as described in section 3.5.1.

Table 3.1 Components of media for investigation of the suitable source and concentrations of growth factors for D-lactic acid production.

Component	Component concentrations (g/l) of medium number					
	1	**2**	**3**	**4**	**5**	**6**
Cassava starch (dry weight)	30.00	30.00	30.00	30.00	30.00	30.00
Tryptone	3.00	3.00	3.00	3.00	3.00	3.00
K_2HPO_4	6.00	6.00	6.00	6.00	6.00	6.00
Yeast extract	3.00	2.00	1.00	0.00	0.00	0.00
Spent of brewery yeast sludge	0.00	3.00	3.00	3.00	5.00	10.00
$MgSO_4.7H_2O$	0.57	0.57	0.57	0.57	0.57	0.57
$MnSO_4.4H_2O$	0.12	0.12	0.12	0.12	0.12	0.12
$FeSO_4.7H_2O$	0.03	0.03	0.03	0.03	0.03	0.03
pH 7.0±0.2 at 25°C						

3.5.4 Initial pH of D-lactic acid production medium

The initial pH of the optimized medium for the D-lactic acid production by the selected isolate were studied using the medium containing optimum concentrations of carbon, nitrogen, and growth factor sources (sections 3.5.1-3.5.3). The initial pH of the medium was adjusted to 5.0, 5.5, 6.0, 6.5, 7.0, and 8.0 using 1 N HCl and 1 N NaOH, and used for culturing the selected D-lactic acid-producing isolate(s). Bacterial growth and acid production were monitored as described in section 3.5.1.

3.5.5 Cultivation temperatures

The suitable temperature for D-lactic acid production was investigated. Various cultivation temperatures; 25, 30, 35, 37, 40, and 45°C, were performed for the cultivation of the selected D-lactic acid-producing isolate(s) in the suitable medium

resulted from sections 3.5.1-3.5.4. Bacterial growth and acid production were monitored as described in section 3.5.1.

3.5.6 Inoculum sizes

The amount of inoculum size could affect growth and D-lactic acid production. Various inoulum sizes was studied for the production of D-lactic acid by the selected D-lactic acid-producing isolate(s) in the suitable medium, which resulted from section 3.5.1-3.5.5 at various % (v/v) inoculum sizes; 1, 2, 3, 4, and 5%. Bacterial growth and acid production were monitored as described in section 3.5.1.

3.6 Production of D-lactic acid using the optimized production conditions

D-Lactic acid was produced using the optimized production conditions in a 6.6-l jar fermenter (Biostat® *B* plus, Germany) containing 5.0 l of fermentation medium. The optimized medium was sterilized at 121°C for 35 min, then inoculated with the suitable inoculum size (approximately 10^6 CFU/ml) of the selected isolate, and cultivated at the optimum temperature. The agitation speed was maintained at 200 rpm to insure completely mixing the fermentation broth. The culture pH was kept constant at the optimum pH during fermentation through automatic addition of 5 N NaOH. The temperature, pH, and agitation were computer-controlled and monitored on line using MFCS SCADA Software (Sartorius, Germany). During fermentation experiments, bacterial growth, pH, total acidity, total sugars, and D-lactic acid concentration were measured at various time intervals.

3.7 Preliminary investigation of D-lactic acid extraction and purification from the optimized medium

After completion of the fermentation under optimum conditions using cassava starch as a raw material in section 3.6, D-lactic acid was extracted and purified according to the method as described by Benthin and Villadsen (1995).

3.8 Identification of the selected lactic acid bacterial isolate(s)

The D-lactic acid-producing bacterial isolate(s) that produce high amount of D-lactic acid were identified using morphological and physiological characteristics, and 16S ribosomal RNA (rRNA) gene sequence.

3.8.1 Morphological and physiological characterization

3.8.1.1 Morphological characteristics

The D-Lactic acid-producing bacterial isolate(s) were grown on MRS and RAM agar at 35°C under anaerobic conditions for 48 h. Cell morphology and cell arrangement were observed by Gram staining (Cappuccino and Sherman, 1999).

3.8.1.2 Physiological characteristics

A Biochemical characteristics

Biochemical reactions were conducted following the standard determinative bacteriology procedure (Shaw and Harding, 1984; Holt *et al*., 1994; Cappuccino and Sherman, 1999).

1) Catalase test

Catalase activity was determined by transferring 24-48 h culture from MRS or RAM agar to a slide glass. Rapid gas formation after dropping 3% hydrogen peroxide (H_2O_2) (Appendix A2.7) indicated a positive result.

2) Oxidase test

The filter paper (Whatman no. 4) was placed into a petridish and wet with 0.5 ml of 1% tetramethyl-p-phenylenediamine dihydrochloride (Appendix A2.6). The bacterial cells grew on MRS or RAM agar for 24-48 h, were streaked onto the reagent zone of the filter paper. The development of a deep blue color at the inoculation site within 5-10 seconds indicated a positive result of oxidase test.

3) Gas production from glucose

The production of gas from glucose was assayed by growing the bacteria in MRS or RAM broth containing Durham tube for 24-48 h at 35°C under anaerobic conditions. Gas entrapped in Durham tube, represented the positive gas production.

4) Casein hydrolysis

Hydrolysis of casein was tested by cultivating the point inoculation colony on MRS agar or RAM agar added 1% skim milk at the final concentration (Appendix A4.3, Appendix A4.4) and incubating at 35°C for 2 days under anaerobic conditions. A positive reaction of the proteolytic test was indicated by clear zone around colony.

5) Starch hydrolysis

Hydrolysis of starch was tested by cultivating the point inoculation colony on MRS agar or RAM agar added 1% soluble starch (Appendix A4.1, Appendix A4.2) and incubating at 35°C for 2 days under anaerobic conditions. Starch hydrolysis was visualized by adding iodine solution to the plate. A clear zone around colony indicated the positive result.

6) Arginine hydrolysis

Hydrolysis of arginine was tested using MRS or RAM broth without beef extract. The medium was added 0.05% glucose, 0.3% arginine, 0.2% sodium citrate, and ammonium citrate. One loopful of the 24-48 h culture was inoculated into the medium, and incubated at 35°C for 1 to 3 days under anaerobic conditions. Ammonia was detected using Nessler's reagent (Shaw and Harding, 1984).

7) Gelatin hydrolysis

The 24-48 h culture was stabbed into the gelatin medium, and incubated at 35°C for 1 day (Appendix A4.7, Appendix A4.8). Gelatin hydrolysis was indicated by liquefying of the medium after the tube was kept at 4°C for 15-30 minutes.

8) Sugar fermentation patterns

Sugar fermentation patterns were determined by the API 50 CH/CHL (Biomérieux, RCS Lyon, France) according to the manufacturer instructions. Then the API LAB Plus software version 5.0 (bioMérieux and Analytab Products' computer database) were used for comparing carbohydrate assimilation and/ or fermentation patterns.

B Effects of salt concentration, pH, and temperature on growth

For the investigation of effects of salt (sodium chloride) concentration, pH of medium, and temperature, on growth of the selected isolate, MRS or RAM broth was used as the basic medium.

The inoculum size of 2% (approximate 10^6 CFU/ml) of an overnight culture was inoculated into 5 ml of MRS or RAM broth containing different concentrations (0, 3.0, 4.0, 6.5, 8.0, 10.0, and 18.0%) of NaCl, and incubated at 35°C for 1 to 3 days under anaerobic conditions.

For the effect of pH, MRS or RAM broth was prepared and adjusted to pH of 4.0, 4.4, 4.8, 5.0, 6.0, 6.5, 7.0, 8.0, and 9.6, then inoculated with an overnight culture of the selected isolates. The culture was then incubated at 35°C for 1 to 3 days under anaerobic conditions. Growth of the bacteria was spectrophotometric measurement at A_{600}.

For optimum temperature, the inoculum size of 2% was added into 5 ml of MRS broth, pH 6.5 and incubated at various temperatures 5, 10, 15, 20, 25, 30, 35, 37, 40, 42, 45, 50, and 55°C for 1 to 3 days under anaerobic conditions.

C Lactic acid configuration

The selected isolate(s) was grown in 5 ml MRS or RAM broth containing 2% glucose, and incubated at 35°C for 24 h under anaerobic conditions. The cells were removed by centrifuging at 10,000×g for 10 min at 4°C. Lactic acid configuration in the supernatant was determined using HPLC as described in section 3.4.2.

3.8.2 Nucleotide sequence of 16S ribosomal RNA gene

Nucleotide sequence of 16S rRNA gene was used for genetic characterization involving three major steps; genomic DNA extraction, amplification of 16S ribosomal RNA gene, and sequencing of the 16S rRNA gene.

3.8.2.1 Extraction of genomic DNA

Genomic DNA of the selected D-lactic acid-producing isolate was extracted and purified using the method of Tamaoka (1994). One loopful of the selected isolate was inoculated into 120 ml of RAM broth supplement with 0.8-1.5% glycine (Yamada and Komagata, 1970), and incubated at 35°C for 12-18 h under anaerobic conditions. Bacterial cells at the late exponential phase were harvested by centrifugation at 12,000 rpm at 4°C for 2 min. The supernatant was discarded, and the cell pellet was washed twice with saline-EDTA (pH 8.0) (Appendix A1.4). Wet cells were suspended in 0.75 mg/ml of lysozyme in a centrifuge tube, and incubated the suspension at 37°C for 30 min. The cells were suspended in a 1 ml portion of 4 ml of Tris-NaCl (pH 9.0) (Appendix A1.2) solution and then a 1 ml portion of 10 ml of 10% SDS was added, mixed well, and incubated the suspension to 60°C for 5 min. Then, a 1 ml portion of 3 ml of phenol: chloroform (1:1) were added, gently mixed for 1 min, and centrifuged at 10,000×g at 4°C for 10 min. The top supernatant was transferred to a fresh centrifuge tube. While stirring the solution with a glass rod, the twice volumes of cold ethanol were added and spooled the DNA with a glass rod. The DNA was air died and then dissolved in 200 µl of 0.1×SSC (pH 7.0) (Appendix A1.7). Genomic DNA was detected in 0.8% agarose gel electrophoresis (Bio-Rad, Milan, Italy), stained with ethidium bromide (1 mg/ml) (Appendix A1.10), and examined under UV transilluminator (Bio-Rad). The concentration of DNA was measured by SmartSpec™

3000 spectrophotometer at 260 nm (Bio-Rad) and DNA purity with respect to contaminants, such as protein, was calculated from the ratio of optical density at A_{260}/A_{280}. Pure DNA has an A_{260}/A_{280} ratio of 1.8-2.0. The conversion factor for determination of DNA concentration is 1.0 OD_{260} = 50 µg/ml of double stranded DNA. Then, DNA solution was maintained at -20°C until use.

3.8.2.2 Amplification of the 16S ribosomal RNA gene

Polymerase Chain Reaction (PCR) was performed using Thermoelectron corporation P×2 Thermal Cycler (Bioscience Technologies Division, U.S.A.) with a primary heating step for 2 min at 95°C, followed by 35 cycles of denaturation for 45 sec at 95°C, annealing for 45 sec at 55°C, and extension for 2 min at 72°C which was followed by a final extension at 72°C for 7 min. Amplification reaction was prepared in the total volume of 25 µl of reaction mixture contained 2 µl of genomic DNA, 13.5 µl of MilliQ water, 2.5 µl of 10X PCR buffer (Invitrogen), 2.5 µl of 25 mM $MgCl_2$, 2.5 µl of 2 mM dNTPs mixture (Invitrogen), 1.0 µl of each primer (fD1 and rP2) (10.0 pmoles/ µl), and 0.5 µl of *Taq* DNA polymerase (5U/µl) (Invitrogen). The primer sequences were shown in Table 3.2. The PCR amplified products were examined by electrophoresis in 1.0% agarose and stained with bromide (Sigma) (1 mg/ml). The size of PCR product was compared with 1 Kb DNA ladder (Invitrogen). The expected size of amplified DNA fragments was approximately 1500 bp.

3.8.2.3 Analysis of 16S rRNA gene sequence

Sequencing of 16S rRNA gene was performed using primers fD1, rP2, and a forward primer (Table 3.2). Nucleotide sequence data obtained from DNA sequencing software ABI377 using Automated DNA Sequencer were converted

to single letter code in text file format by the Chromas 1.56 program. The sequence was also corrected by manual inspection of the chromatogram, then compared to closely relatives available in GenBank databases [http://www.ncbi.nlm.nih.gov/] using standard nucleotide-nucleotide BLAST pragram [blastn] to ascertain their closest relatives.

Table 3.2 Oligonucleotide primers used for PCR amplification and sequencing of 16S rRNA gene.

Primer	**Primer sequence (5' to 3')**	**Target region[a]**	**Reference**
fD1	AGAGTTTGATCCTGGCTCAG	8-27	Wilson *et al.* (1991)
rP2	ACGGCTACCTTGTTACGACTT	1490-1511	Wilson *et al.* (1991)
Forward primer	TAACTACGTGCCAGCAGCC	515-533	Udomsil (2008)

[a] *Escherichia coli* numbering.

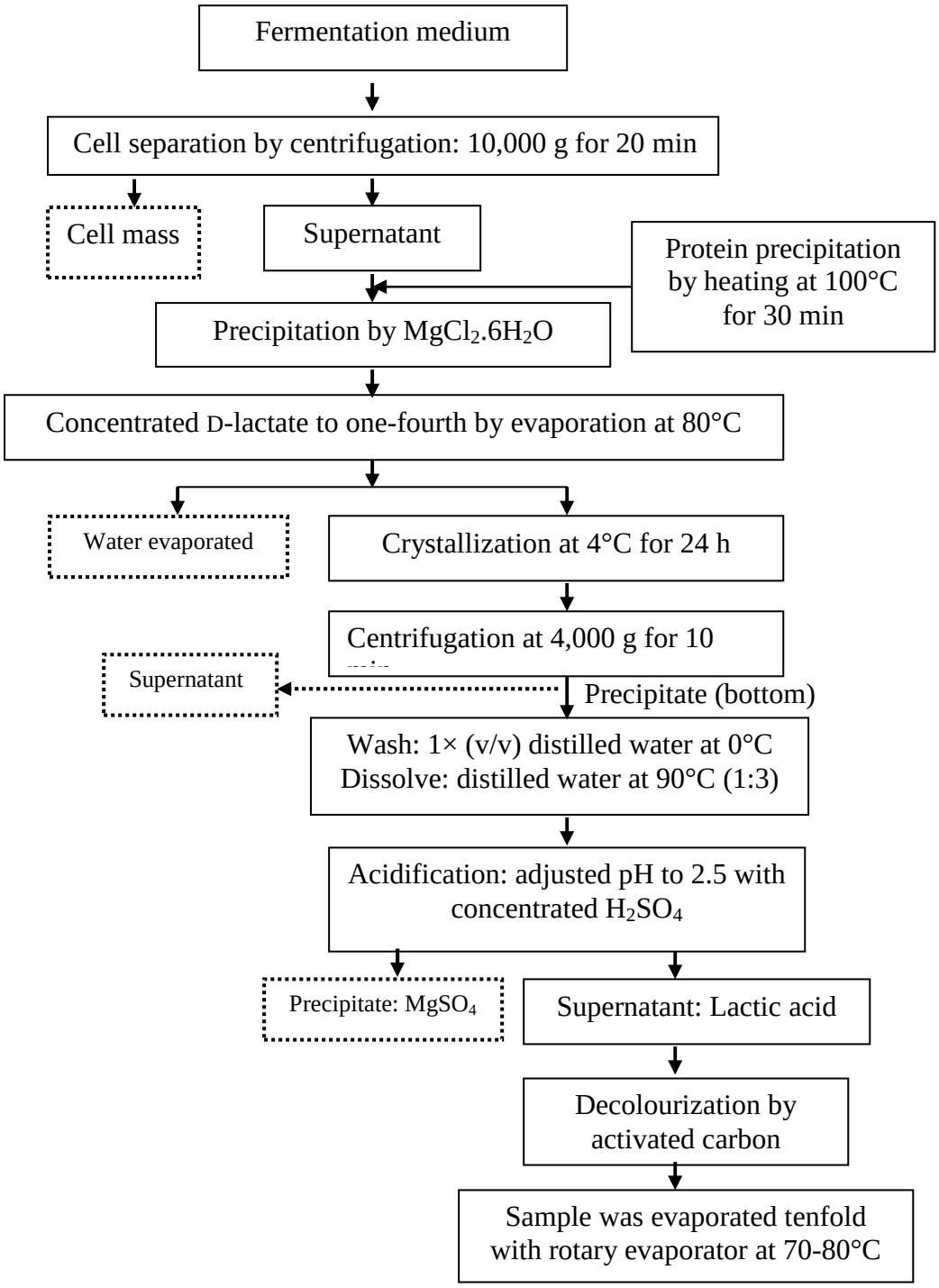


Figure 3.1 Flow chart of the D-lactic acid extraction and purification from the fermentation medium.

Modified from: Benthin and Villadsen (2000).

3.9 Statistical analysis

The optimization of some D-lactic acid production conditions was analyzed in duplicates. Statistical analysis was evaluated in Completely Randomized Design (CRD). Analysis of Variance (ANOVA) and means comparison by Duncan's Multiple Range Test (DMRT) were used to determine differences between mean at $P<0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 The approximate analysis of cassava starch and spent of brewery yeast sludge

Physical and chemical composition of cassava starch and spent of brewery yeast including moisture, fiber, total nitrogen, fat, ash, and available carbohydrate contents were determined (Table 4.1). Spent of brewery yeast sludge obtained from Boonrawd brewery Co., Ltd., was used as nitrogen source for D-lactic acid production. Spent of brewery yeast sludge had high total nitrogen content (6.21±0.054%) (Table 4.1). Cassava starch obtained from Sanguan Wongse Industries Co., Ltd., contained of 85.53% available carbohydrate and it also included total nitrogen content (0.35±0.023%). Southgate (1991) reported that cassava starch consisted of 17-23% amylose and 77-83% amylopectin. Amylose is a long and unbranched chain of glucose, whereas amylopectin is a highly branched form of starch in which the backbone consists of glucose. Cassava starch was used as carbon source for acid production.

Table 4.1 Compositions analysis results of cassava starch and spent of brewery yeast sludge.

Component	Cassava starch	Spent of brewery yeast sludge
Available carbohydrate (%)	85.53	75.53
Moisture content (%)	11.95±0.057	10.62±0.185
Total nitrogen content (%)	0.35±0.023	6.21±0.054
Ash content(%)	0.35±0.014	5.18±0.056
Fiber content (%)	0.18±0.014	5.21±0.403
Fat content (%)	1.64±0.063	2.37±0.169

Data are mean ± standard deviation (SD) for duplicate data.

4.2 Screening and selection of lactic acid bacteria for D-lactic acid production

4.2.1 Screening of lactic acid bacteria for D-lactic acid production

Three hundred and six isolates of lactic acid bacteria obtained from stock cultures of the Microbial Culture Collection Laboratory, Suranaree University of Technology, were screened for lactic acid production in MRS and RAM medium using glucose as the main carbon source (Rodtong and Ishizaki, 2003; Gül *et al*., 2005). One hundred and twenty-one of lactic acid bacterial isolates (Appendix B1, Appendix B2) were selected for further investigation based on their D-lactic acid production ability. These isolates provided the concentration of total acidity expressed as lactic acid ranging from 0.053-1.77% corresponding to the decrease in pH of 6.5-7.0 to pH of 4.21-4.89. Organic acid such as lactic acid from microbial metabolic activities causes the decrease in pH value. Gül *et al.* (2005) indicated that a carbon source, such as glucose, which typically required for lactic acid production of lactic

acid bacteria, and they also produced gas from glucose for distinguish between homofermentative and heterofermentative.

4.2.2 Selection of D-lactic acid-producing isolates and test for cassava starch utilization using agar medium

4.2.2.1 Selection of D-lactic acid-producing isolates

From the screening step, one hundred and twenty-one out of 306 isolates (Appendix B1, Appendix B2) were selected for D-lactic acid detection using HPLC via a comparison of retention times of the standard D- and L-lactic acid. Only seven isolates, WR73, CWMC2-5, CWMC1-3, CWMR1-5, CWR2-16, LF1, and PSMS1-5, were able to produce D-lactic acid with optical purity > 90% at high concentrations of 2.00, 17.94, 15.88, 15.84, 15.16, 10.42, and 10.16 g/l, respectively (Table 4.2) when cultivated in MRS medium containing 2% of glucose at 35°C for 24 h under anaerobic conditions. These isolates were selected for further investigation.

4.2.2.2 Investigation on cassava starch utilization using agar medium

The selected bacterial isolates produced D-lactic acid (optical purity > 90%) from glucose, were tasted for their cassava starch utilization capabilities on RAM agar containing 1% cassava starch as a carbon source. It found that only one isolate, WR73, could utilize cassava starch. It provided clear zone around colonies when 1% iodine solution was added onto the RAM agar (Figure 4.1). Whereas six isolates: CWMC2-5, CWMC1-3, CWMR1-5, CWR2-16, LF1, and PSMS1-5, did not express their starch utilization activities. Several D-lactic acid-producing strains have been reported to be non-starch-utilizing strains. These strains include *Lactobacillus*

delbrueckii subsp. *delbrueckii*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus jensenii*, *Lactobacillus coryniformis* subsp. *torquens*, *Lactobacillus viridescens* (Kandler and Weiss, 1986) *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc oenos*, *Leuconostoc pseudomesenteroides*, *Leuconostoc mesenteraides* subsp. *cremoris*, *Leuconostoc gelidum*, *Leuconostoc citreum* (Dellaglio *et al.*, 1995), *Weissella thailandensis* (Tanasupawat *et al*., 1998), *Weissella hellenica* (Collins *et al*., 1993), *Weissella koreensis* (Lee *et al*., 2002), and *Weissella soli* (Magnusson *et al*., 2002). Therefore, only isolate WR73 capable of produce D-lactic acid (with optical purity > 99.9%) and could utilize cassava starch was selected for D-lactic acid production.

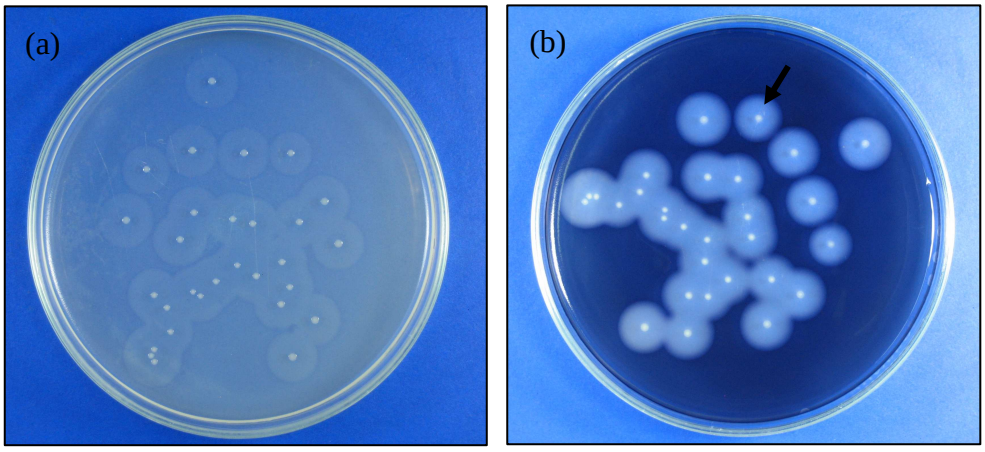


Figure 4.1 Growth and cassava starch hydrolysis of the selected bacterial isolate WR73: (a) Growth on RAM medium containing 1% cassava starch and (b) Positive cassava starch hydrolysis colony (arrow) after adding iodine solution onto the RAM agar surface of (a).

Table 4.2 Lactic acid production by 7 selected isolates using fermentation medium containing 2% of glucose and incubation at 35°C for 24 h under anaerobic conditions.

Bacterial isolate code	Culture medium	Gas from glucose	pH of cultured broth	Growth (A_{600})	Total acidity (%)[a]	Lactic acid concentration (g/l)[b]		Optical purity (%)[c]	
						D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid
WR73	RAM	Negative	4.63	0.112	0.36	2.00	0.00	100.00	0.00
CWR2-16	MRS	Negative	3.58	0.473	1.17	15.16	1.50	91.00	9.00
CWMC1-3	MRS	Negative	3.50	0.445	1.29	15.88	1.50	91.37	8.63
CWMC2-5	MRS	Negative	3.39	0.511	1.36	17.94	2.00	90.00	10.00
CWMR1-5	MRS	Negative	3.36	0.511	1.27	15.84	1.50	91.35	8.65
LF1	MRS	Positive	3.86	0.245	0.82	10.42	0.00	100.00	0.00
PSMS1-5	MRS	Positive	3.83	0.247	0.86	10.16	0.00	100.00	0.00

[a] Titration method (AOAC International, 2000).
[b] HPLC analysis (Tanaka *et al*., 2007).
[c] Optical purity of D-lactic acid = (1-(L-lactic acid / total lactic acid)) × 100.
[d] D or L, the isomer recorded makes up 90% or more of total lactic acid; DL, 25-75% of total lactic acid are of the L- configuration.

4.3 Optimization of some conditions for D-lactic acid production

To obtain the maximum production of D-lactic acid from inexpensive medium, some components (cassava starch and nitrogen source concentrations) of D-lactic acid screening medium and the acid production conditions (initial pH of culture medium, incubation temperatures, and suitable inoculum sizes) were investigated to obtain the optimum concentrations.

4.3.1 Concentrations of cassava starch

The suitable concentration of cassava starch for D-lactic acid production by the selected isolate(s) was determined using RAM medium containing 10, 15, 20, 25, 30, 35, and 40 g/l of cassava starch. Results showed that the D-lactic acid concentration increased with the increase in cassava starch concentration up to 30 g/l (Figure 4.2 and Table 4.3). The maximum D-lactic acid (3.45±0.15 g/l) was obtained at 48 h fermentation with an initial cassava starch concentration of 30 g/l (p<0.05). D-Lactic acid concentration decreased when cassava starch was higher than 30 g/l. This may due to inhibition by high substrate concentration (Figure 4.2). Substrate inhibition in lactic acid fermentation was also reported for *Lactococcus lactis* subsp. *lactis* on glucose (Åkerberg *et al*., 1998), *Lactobacillus bulgaricus* on lactose (Mehaia and Cheryan, 1987), and *Lactobacillus delbrueckii* on sucrose (Monteagudo *et al*., 1994). Some of the productions of D-lactic acid have been reported. *Bacillus* (*Lactobacillus*) *laevolacticus* produced D-lactic acid at a yield of 97% from 50 g/l of glucose in a chemostat with controlling pH at 6.0 (De Boer *et al.*, 1990). *Lactobacillus bulgaricus* Lb-12 produced 40 g/l of D-lactic acid from 100 g/l of lactose monohydrate at pH 6.0. The strain consumed only the glucose moiety of lactose (Benthin and Villadsen, 1995).

Table 4.3 Growth and D-lactic acid production of bacterial isolate WR73 after cultivation in 100 ml of RAM broth containing various concentrations of cassava starch at 35°C for 48 h.

Cassava starch (%)	Growth		Lactic acid			Total sugars (g/l)[d]
	CFU/ml	Log CFU/ ml	Total acidity (%)[a]	D-Lactic acid (g/l)[b]	Yield ($Y_{p/s}$)[c] (%)	
1.0	3.4×10^6	6.53[e]	0.29±0.02[ab]	3.15±0.0[b]	78.30±0.33[a]	4.96±0.84[e]
1.5	4.3×10^6	6.63[f]	0.29±0.00[ab]	3.24±0.03[b]	54.85±0.72 [b]	8.66±3.28[de]
2.0	9.2×10^6	6.96[g]	0.29±0.01[ab]	3.20±0.07[b]	59.68±1.40 [b]	13.33±0.15[cd]
2.5	7.7×10^5	5.89[d]	0.29±0.00[ab]	3.16±0.05[b]	34.51±0.49 [c]	14.69±0.82[c]
3.0	4.7×10^5	5.67[a]	0.30±0.01[a]	3.45±0.15[a]	35.10±1.55 [c]	18.47±1.43[bc]
3.5	5.4×10^5	5.73[b]	0.27±0.01[b]	3.14±0.01[b]	29.89±0.10 [c]	22.42±4.39[ab]
4.0	5.7×10^5	5.76[c]	0.27±0.01[b]	3.25±0.05[b]	21.13±0.34 [c]	26.04±0.19[a]

Data are mean± standard deviation (SD) for duplicate data, letters indicate significant difference within the same column ($P<0.05$).
[a] Titration method (AOAC International, 2000).
[b] HPLC analysis (Tanaka *et al*., 2007).
[c] D-Lactic acid yield (%) = [D-Lactic acid produced/total sugar consumed] ×100.
[d] Colorimeteric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

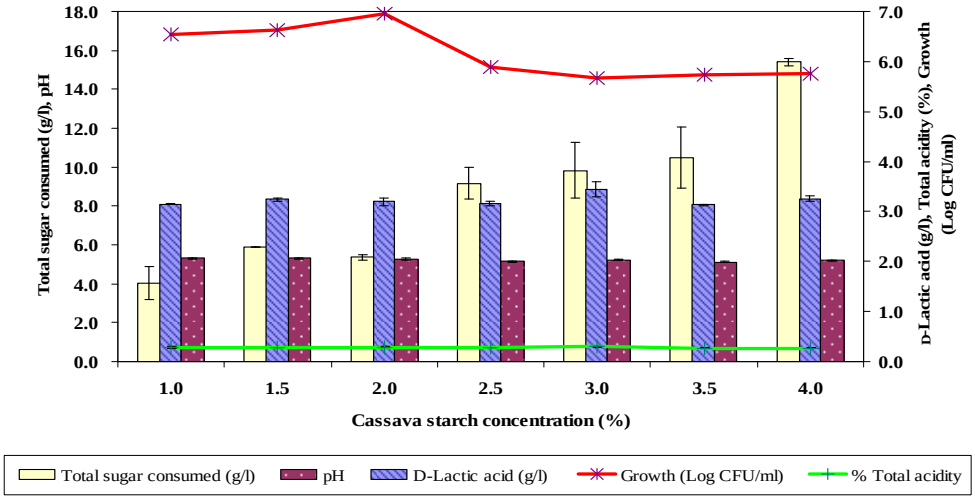


Figure 4.2 Growth and D-lactic acid production by bacterial isolate WR73 after cultivation in 100 ml of RAM broth containing various cassava starch concentrations at 35°C for 48 h.

Lactobacillus coryniformis subsp. *torquens* produced 39 g/l of D-lactic acid from 40 g/l of glucose at pH 6.4 (González-Varay *et al*., 1996). Metabolically engineered *Escherichia coli*, which is deficient in the phosphotransacetylase and phosphoenolpyruvate carboxylase, produced D-lactic acid homofermentatively at pH 7.0 (Chang *et al*., 1999). D-Lactic acid production by *Lactobacillus delbrueckii* from rice saccharificate, which is prepared from white rice by enzymatic hydrolysis, has been developed (Fukushima *et al*., 2004). In this process, two strains of *Lactobacillus delbrueckii* (LD 0025 and LD 0028) could ferment the rice saccharificate and produce D-lactic acid in high conversion. Particularly, the strain LD 0028 produced high yield of D-lactic acid (70%), and presented highly optical purity (>97.5) in the large scale fermentation. Cassava starch which is inexpensive and abundantly available raw material when compared to sucrose, glucose, and lactose. The cost of analytical grade sugar such as glucose was about 100 times higher than that of cassava starch. Thus, 30 g/l (dry weight) of cassava starch concentration was chosen to be used as the inexpensive carbon source in RAM medium for D-lactic acid production by the isolate WR73.

4.3.2 Concentrations of tryptone

Lactic acid bacteria are generally fastidious organisms, which require complex nutrients such as amino acids and vitamins for cell growth (Axelsson, 2004). Tryptone in RAM medium could be served as a nitrogen source, varied concentrations of tryptone (g/l): 0, 5, 10, 15, and 20, were added to the RAM medium containing 30 g/l of cassava starch (equivalent to 31.08 g/l of total sugar). After cultivating isolate WR73 for 48 h at 35°C under anaerobic conditions, the maximum yield of 3.19±0.01 g/l of D-lactic acid was produced with the bacterial growth of

7.8×10^6 CFU/ml when 3.0 g/l of tryptone concentration was added in the fermentation medium (Table 4.4 and Figure 4.3). The results suggested that tryptone at the concentration of 3.0 g/l was suitable for D-lactic acid production by the isolate WR73. This concentration was then selected for the future experiments.

4.3.3 Sources and concentrations of growth factors

Yeast extract is one of the most commonly used as growth factors which provided complex nutrients particularly nitrogen source for lactic acid bacteria (Nancib *et al*., 2005). However, yeast extract is an expensive material to be used in the industrial process. The spent of brewery yeast sludge could be used to replace yeast extract, thus the spent of brewery yeast sludge at initial concentrations of 0, 3, 5, and 10 g/l, and yeast extract at initial concentrations of 0, 1, 2, and 3 g/l were added to RAM fermentation medium. Results showed that D-lactic acid concentration increased when the spent of brewery yeast sludge concentration up to 5.0 g/l was supplemented (Table 4.5 and Figure 4.4). The highest D-lactic acid concentration (3.35±0.00 g/l) was obtained in only medium with 5.0 g/l spent of brewery yeast sludge (Table 4.5). RAM medium containing 3.0 g/l spent of brewery yeast sludge gave similar D-lactic acid concentration (3.31±0.03 g/l) to the medium containing the mixture of 1.0 g/l of yeast extract and 3.0 g/l of spent of brewery yeast sludge (Table 4.5). These media also provided higher bacterial counts ($1.0\text{-}4.1\times10^6$ CFU/ml) than the medium containing 5.0 g/l of spent of brewery yeast sludge (9.3×10^5 CFU/ml). In addition, the media containing yeast extract gave better growth of isolate WR73 than the media without yeast extract. The results suggested that yeast extract was excellent nutrient for the bacterial growth.

Table 4.4 Growth and D-lactic acid production of bacterial isolate WR73 when cultivated in 50 ml of RAM broth containing various tryptone concentrations at 35°C for 48 h.

Tryptone (g/l)	Growth		Lactic acid			Total sugars (g/l)[d]
	CFU/ml	Log CFU/ml	Total acidity (%)[a]	D-Lactic acid (g/l)[b]	Yield ($Y_{p/s}$)[c] (%)	
2.00	5.1×10^6	6.71[b]	0.27±0.01[c]	3.09±0.05[a]	40.91±0.71[a]	20.75±0.11[a]
2.50	7.6×10^6	6.88[a]	0.28±0.01[bc]	3.15±0.08[a]	50.23±1.06[a]	20.50±4.81[a]
3.00	7.8×10^6	6.89[a]	0.29±0.01[b]	3.19±0.01[a]	47.08±0.08[a]	20.83±3.21[a]
4.00	3.0×10^6	6.48[c]	0.34±0.00[a]	3.10±0.03[a]	51.48±0.42[a]	21.83±2.40[a]
5.00	3.3×10^5	5.51[d]	0.33±0.01[a]	3.12±0.18[a]	55.92±1.11[a]	22.34±2.21[a]

Data are mean± standard deviation (SD) for duplicate data, letters indicate significant difference within the same column ($P<0.05$).
[a] Titration method (AOAC International, 2000).
[b] HPLC analysis (Tanaka *et al*., 2007).
[c] D-Lactic acid yield (%) = [D-Lactic acid produced/total sugar consumed] ×100.
[d] Colorimeteric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

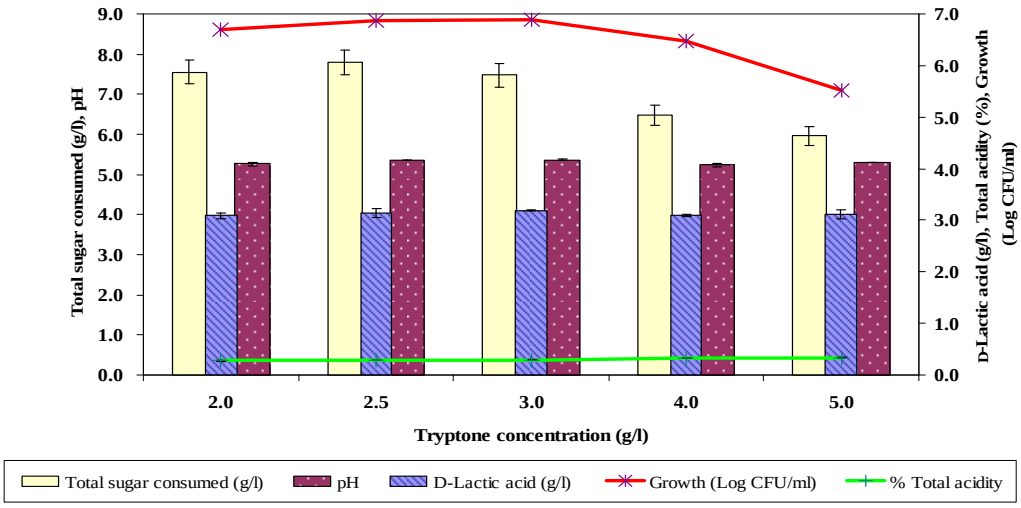


Figure 4.3 Growth and D-lactic acid production by bacterial isolate WR73 after cultivation in 50 ml of RAM broth containing various tryptone concentration at 35°C for 48 h.

Table 4.5 Growth and D-lactic acid production of bacterial isolate WR73 when cultivated in 50 ml of RAM broth containing various concentrations of yeast extract and spent of brewery yeast sludge at 35°C for 48 h.

Yeast extract (g/l)	Spent of brewery yeast sludge (g/l)	Growth		Lactic acid			Total sugars (g/l)[d]
		CFU/ ml	Log CFU/ ml	Total acidity (%)[a]	D-Lactic acid (g/l)[b]	Yield ($Y_{p/s}$)[c] (%)	
3.00	0.00	7.8×10^6	6.89[a]	0.29± 0.01[d]	3.19± 0.01[b]	40.91± 1.33[a]	20.83± 3.21[a]
2.00	3.00	3.9×10^6	6.59[b]	0.37± 0.01[a]	3.03± 0.01[a]	29.48± 7.45[ab]	20.48± 2.71[a]
1.00	3.00	4.1×10^6	6.61[c]	0.34± 0.01[bc]	3.31± 0.00[c]	23.09± 2.35[b]	16.67± 1.45[a]
0.00	3.00	1.0×10^6	6.01[d]	0.34± 0.01[bc]	3.31± 0.03[c]	25.15± 6.45[b]	17.48± 3.36[a]
0.00	5.00	9.3×10^5	5.97[e]	0.35± 0.01[b]	3.35± 0.00[d]	23.11± 0.86[b]	16.56± 0.53[a]
0.00	10.00	7.9×10^5	5.90[f]	0.33± 0.00[c]	3.33± 0.00[cd]	27.72± 7.24[b]	18.64± 3.24[a]

Data are mean± standard deviation (SD) for duplicate data.
[a] Titration method (AOAC International, 2000).
[b] HPLC analysis (Tanaka *et al*., 2007).
[c] D-Lactic acid yield (%) = [D-Lactic acid produced/total sugar consumed] ×100.
[d] Colorimeteric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

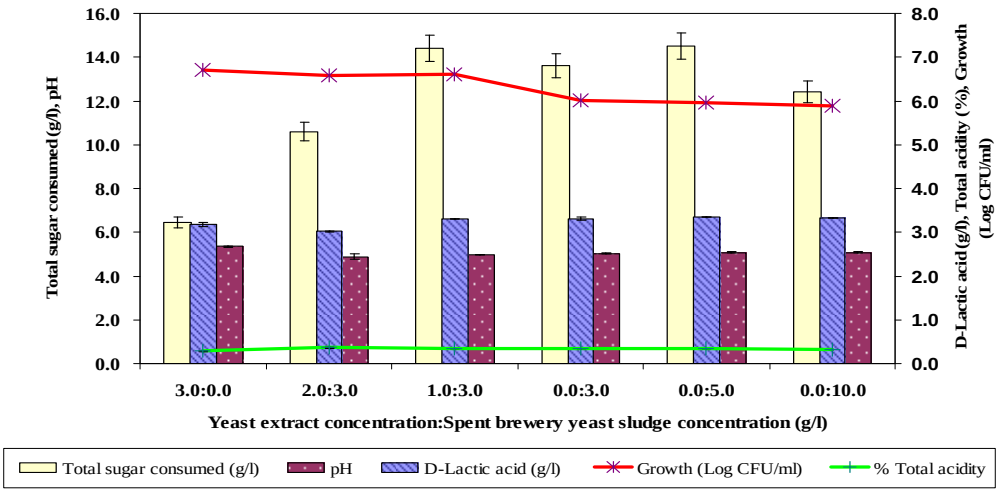


Figure 4.4 Growth and D-lactic acid production by bacterial isolate WR73 after cultivation in 50 ml of RAM broth containing various concentrations of yeast extract and spent of brewery yeast sludge at 35°C for 48 h.

The importance of yeast extract for lactic acid bacteria was reported. The main contribution of yeast extract in the medium for lactic acid bacterial growth could be the compositions of purine, pyrimidine, and B-vitamins for lactic acid bacteria (Nancib *et al*., 2005). Some other sources for the D-lactic acid production have been reported. Nancib *et al.* (2005) studied the effect of different nitrogen sources on growth of *Lactobacillus casei* subsp. *rhamnosus*. Ammonium sulfate in combination with the required vitamins could be a good alternative nutrient source to costly yeast extract. Timbantam *et al.* (2006) found that the addition of low cost of nitrogen source such as 1% (w/v) of silk worm larvae, yeast autolysate, and shrimp waste provided higher lactic acid concentration (11.7-16.6 g/l of lactic acid) than medium supplemented with 0.5-1.0% yeast extract (10.0 g/l lactic acid). On the other hand, yeast extract was rather expensive (accounted for about 38% of total medium cost) to hinder its use in large quantities in lactic acid production (Altaf *et al*., 2007). In conclusion, spent of brewery yeast sludge could be an alternative to costly yeast extract, and spent of brewery yeast sludge at the concentration of 3.0 g/l was selected for replacing yeast extract for D-lactic acid production by the isolate WR73.

4.3.4 Initial pH of D-lactic acid production medium

The effect of pH of fermentation medium on D-lactic acid production was evaluated by using the optimized medium at initial pH in the range of 5.0-8.0. Results showed that pH 7.0 was found to provide better condition than other pHs, and the 58.88±5.90% (P<0.05) yield ($Y_{LA/S}$) of D-lactic acid were achieved (Table 4.6 and Figure 4.5). While the initial pH of 5.0 gave the lower concentration of D-lactic acid than at the initial pH of 5.5-8.0 (P<0.05) (Table 4.6). The uptake of carbohydrate could be inhibited by increasing proton levels (Yokota *et al*., 1995).

Table 4.6 Effect of initial pH of the optimized RAM broth on growth and D-lactic acid production of bacterial isolate WR73 when cultivated in 50 ml of the medium at 35°C for 48 h.

pH	Growth		Lactic acid			Total sugars (g/l)[d]
	CFU/ml	Log CFU/ml	Total acidity (%)[a]	D-Lactic acid (g/l)[b]	Yield ($Y_{p/s}$)[c] (%)	
5.0	1.0×10^2	2.00[f]	0.08±0.00[e]	2.58±0.00[a]	44.86±2.08[b]	25.33±0.27[a]
5.5	2.4×10^3	3.38[e]	0.13±0.01[d]	2.81±0.07[b]	39.04±8.61[bc]	23.88±1.72[a]
6.0	1.0×10^5	5.01[a]	0.20±0.03[c]	2.91±0.05[b]	32.66±4.77[c]	22.18±1.14[a]
6.5	4.0×10^4	4.60[b]	0.23±0.01[b]	3.07±0.01[c]	43.62±3.48[bc]	24.04±0.57[a]
7.0	4.7×10^4	4.54[c]	0.33±0.01[a]	3.65±0.11[e]	58.88±5.9[a]	24.87±4.66[a]
8.0	3.5×10^4	3.67[d]	0.33±0.00[a]	3.50±0.04[d]	60.94±8.4[a]	25.33±5.00[a]

Data are mean± standard deviation (SD) for duplicate data, letters indicate significant difference within the same column ($P<0.05$).
[a] Titration method (AOAC International, 2000).
[b] HPLC analysis (Tanaka *et al*., 2007).
[c] D-Lactic acid yield (%) = [D-Lactic acid produced/total sugar consumed] ×100.
[d] Colorimeteric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

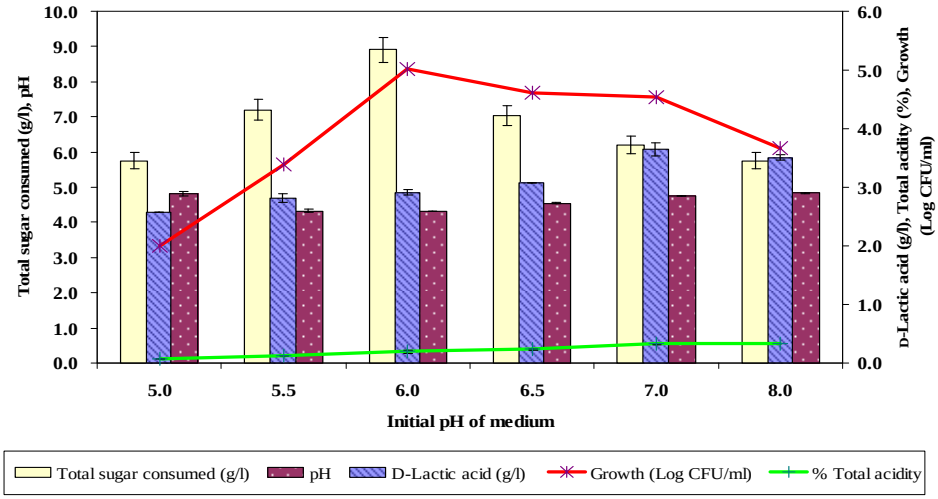


Figure 4.5 Effect of initial pH of the modified RAM broth on growth and D-lactic acid production by bacterial isolate WR73 after cultivation in 50 ml of the medium at 35°C for 48 h.

Panesar *et al.* (2010) reported that the hydrogen ion concentration of medium has the maximum influence on microbial growth. The pH affects at least two aspects of microbial cells, i.e. functioning of its enzymes and the transport of nutrients into the cell. It limits the synthesis of metabolic enzymes responsible for the synthesis of new protoplasm. pH values also affect RNA and protein synthesis. When microorganisms were grown on either side of their optimum pH range, their lag phases might be increased. Manome *et al*. (1998) showed that the optimal conditions for D-lactic acid production by *Lactobacillus delbrueckii* was at 37°C and pH 6.8. In general, lactic acid bacteria would tolerate pH values between 3.4 and 8.0, but growth and lactic acid production mostly occur between pH 5.4 and 6.4, with the optimum pH being strain-dependent (Kharas *et al*., 1994). From these observations, the initial pH of fermentation medium at 7.0 was considered to be the optimum pH for the maximum D-lactic acid production of isolate WR73. For further investigation, the initial pH of the fermentation medium was adjusted to 7.0.

4.3.5 Cultivation temperatures

The optimal temperature for D-lactic acid production was determined by cultivating the isolate WR73 in the optimized medium at optimum pH for 48 h. The incubation temperatures were varied at 25.0, 30.0, 35.0, 37.0, 40.0, and 45.0°C based on the range of its growth temperatures. Results showed that the highest bacterial growth (3.1×10^5 CFU/ml) and D-lactic acid yield (53.34±3.06%) were obtained when the cultivating medium at 35°C ($P<0.05$) (Table 4.7 and Figure 4.6). In addition, bacterial growth and D-lactic acid production declined when the temperature increased from 35°C to 45°C. The temperature is also one of the important factors, which influences the activity of metabolic/cell enzymes. Enzymes are mostly active at

the optimum growth temperature and enzymatic reaction proceeds at the maximum rate. However, below and above optimal temperature, reaction rate is decreased, which causes the problems in cell metabolism. The optimal temperature for growth of lactic acid bacteria varies between genera from 20 to 45°C (Wood and Holzapte, 1995). Buchta (1983) reported that temperature of 45°C was suitable for lactic acid fermentation by *Lactobacillus delbrueckii* and *Lactobacillus bulgaricus*. *Lactobacillus helveticus* and *Lactobacillus acidophilus* could grow at temperatures ranging from 37-45°C (Wood and Holzapte, 1995). Krischke *et al.* (1991) reported a temperature at 37°C was used for lactic acid production using *Lactobacillus casei*. However, a temperature at 28°C has also been reported to be optimal for *Lactobacillus casei* in a separate study (Nabi *et al*., 2004). A variety of temperatures were reported to be suitable for on D-lactic acid production, for example, 30°C for *Lactobacillus coryniformis* subsp. *torquens*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc carnosum*, and *Leuconostoc fallax* (Manome *et al*., 1998), 37°C for *Lactobacillus delbrueckii* and *Lactobacillus bulgaricus* (Benthin and Villadsen, 1995; Manome *et al*., 1998; Tanaka *et al*., 2006), 39°C for *Lactobacillus coryniformis* subsp. *torquens* (Yáñez *et al*., 2003) and 40°C for *Lactobacillus delbrueckii* (Calabia *et al*., 2007). From these results, the temperature at 35°C was suitable for D-lactic acid production by the isolate WR73.

Table 4.7 Effect of cultivation temperatures on growth and D-lactic acid production of bacterial isolate WR73 when cultivated in 50 ml RAM broth containing 30 g/l of cassava starch with the optimum initial pH for 48 h.

Temperature (°C)	Growth		Lactic acid			Total sugars (g/l)[d]
	CFU/ml	Log CFU/ml	Total acidity (%)[a]	D-Lactic acid (g/l)[b]	Yield ($Y_{p/s}$)[c] (%)	
25.0	3.0×10^5	5.48[a]	0.27±0.02[ab]	3.60±0.02[b]	26.11±0.11[e]	17.29±0.04[d]
30.0	2.1×10^5	5.33[b]	0.31±0.00[a]	3.72±0.07[a]	40.46±3.91[d]	21.88±0.73[c]
35.0	3.1×10^5	5.49[a]	0.30±0.01[a]	3.74±0.01[a]	53.34±3.06[a]	24.07±0.38[a]
37.0	2.0×10^4	4.31[c]	0.28±0.01[a]	3.61±0.03[b]	44.49±3.90[c]	22.96±0.65[b]
40.0	5.0×10^3	3.70[d]	0.27±0.02[ab]	3.54±0.01[b]	53.18±4.7[a]	24.42±0.57[a]
45.0	3.0×10^3	3.48[e]	0.22±0.05[b]	3.52±0.06[b]	50.64±1.2[b]	24.12±1.83[a]

Data are mean± standard deviation (SD) for duplicate data, letters indicate significant difference within the same column ($P<0.05$).
[a] Titration method (AOAC International, 2000).
[b] HPLC analysis (Tanaka *et al*., 2007).
[c] D-Lactic acid yield (%) = [D-Lactic acid produced/total sugar consumed] ×100.
[d] Colorimeteric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

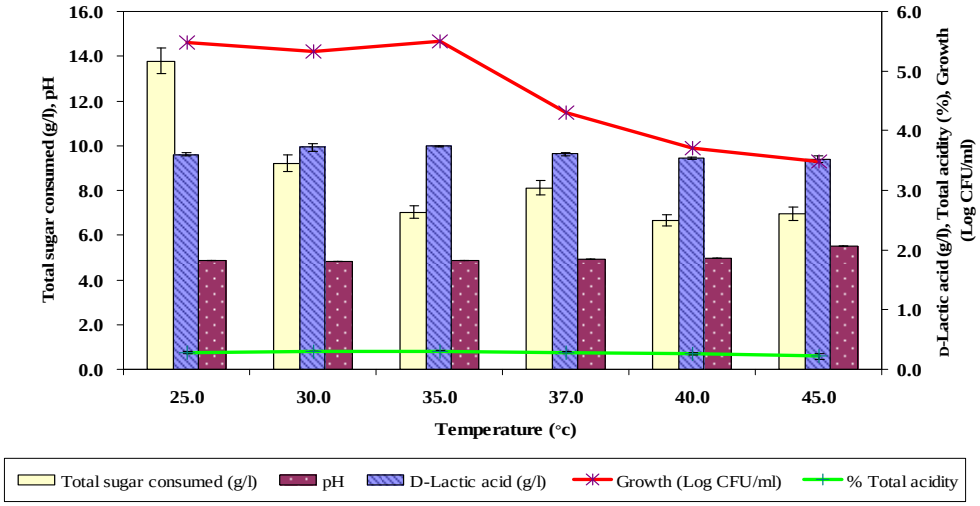


Figure 4.6 Effect of cultivation temperatures on growth and D-lactic acid production by bacterial isolate WR73 when cultivation in 50 ml of RAM broth containing 30 g/l of cassava starch with optimum initial pH at various temperatures for 48 h.

4.3.6 Inoculum sizes

The influence of inoculum size on D-lactic acid production was studied, different inoculum sizes (1-5%, v/v) were separately added to the optimized medium. Bacterial growth and D-lactic acid production increased when inoculum size was increased up to 2% (v/v) (Table 4.8 and Figure 4.7), and inoculum sizes at 1-5% was insignificant difference on the lactic acid yields (D-lactic acid yield, ($Y_{LA/S}$), of 54.84±3.16% and P>0.05). From these results, 1% (v/v) of inoculum size could be considered to be optimal for achieving the maximum D-lactic acid production (3.67±0.08 g/l). However, the utilization of 2% (v/v) of inoculum size for the lactic acid production has been reported (Roy *et al*., 1986; Gandhi *et al*., 2000). The higher inoculum size (3%, v/v) has also been used for lactic acid production (Chiarini *et al*., 1992). Thus, 1% (v/v) of inoculum size was selected for D-lactic acid production by the isolate WR73.

Table 4.8 Effect of inoculum sizes on growth and D-lactic acid production of bacterial isolate WR73 when cultivated in 50 ml RAM broth containing 30 g/l of cassava starch at the optimum temperature for 48 h.

Inoculum (%)	Growth		Lactic acid			Total sugars (g/l)[d]
	CFU/ ml	Log CFU/ml	Total acidity (%)[a]	D-Lactic acid (g/l)[b]	Yield ($Y_{p/s}$)[c] (%)	
1	2.0×10^4	4.30[d]	0.28± 0.01[a]	3.67± 0.08[a]	54.84± 3.16[b]	25.95±3.13[a]
2	3.4×10^4	4.53[a]	0.29± 0.01[a]	3.79± 0.29[a]	60.85± 4.46[a]	26.41±3.47 [a]
3	3.1×10^4	4.49[b]	0.29± 0.02[a]	3.65± 0.15[a]	42.86± 1.22[c]	24.12±1.83[a]
4	2.2×10^4	4.34[c]	0.30± 0.01[a]	3.57± 0.05[a]	43.34± 2.42[c]	24.42±0.57[a]
5	2.3×10^4	4.35[c]	0.30± 0.01[a]	3.71± 0.05[a]	43.15± 3.44[c]	24.04±0.57[a]

Data are mean± standard deviation (SD) for duplicate data, letters indicate significant difference within the same column (P<0.05).

[a] Titration method (AOAC International, 2000).
[b] HPLC analysis (Tanaka *et al*., 2007).
[c] D-Lactic acid yield (%) = [D-Lactic acid produced/total sugar consumed] ×100.
[d] Colorimeteric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

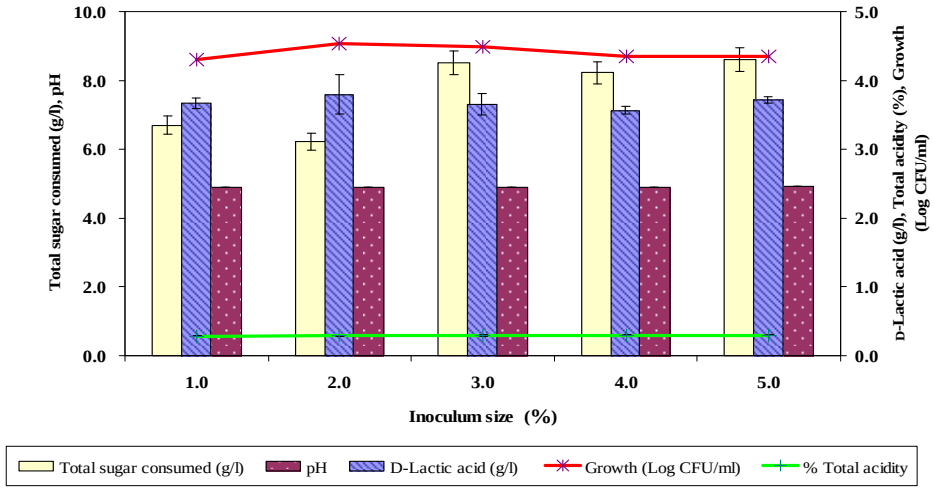


Figure 4.7 Effect of inoculum sizes on growth and D-lactic acid production by bacterial isolate WR73 when cultivation in 50 ml of RAM broth containing various inoculum sizes at 35°C for 48 h.

Table 4.9 Composition of the optimized medium for D-lactic acid production by bacterial isolate WR73.

Component	Component concentrations (g/l)
Cassava starch (dry weight)	30.00
Tryptone (pancreatic digest of casein)	3.00
Yeast extract	0.00
Spent of brewery yeast sludge	3.00
K_2HPO_4	6.00
$MgSO_4.7H_2O$	0.57
$MnSO_4.4H_2O$	0.12
$FeSO_4.7H_2O$	0.03
pH 7.0±0.2 at 25°C	

4.4 Production of D-lactic acid using the optimized production conditions

The production of D-lactic acid by isolate WR73 was investigated using a 6.6 l controlled fermenter containing 5.0 l of the optimized medium (Table 4.9), and 5 N NaOH as the neutralizing agent. To keep the medium in the fermenter homogeneous and protect oxygen dissolved into the medium, agitation was performed at 200 rpm. The fermentation medium was inoculated with 1.0% (v/v) of a late log phase culture (approximately 10^6 CFU/ml). Temperature and pH of the cultivation medium were kept constant at 35°C and 7.0 respectively for 48 h. The fermentation profiles, in terms of pH, D-lactic acid production, total sugars, and cell growth were detected at time intervals. It was found that D-lactic acid production started at 3 h after inoculation and continuously increased until 48 h (Figure 4.9), while the turbidity of medium was increased due to bacterial growth during fermentation (Figure 4.8). The bacterium grew rapidly during 6 h cultivation. The maximum bacterial count (5.3×10^{10} CFU/ml) was also obtained at 6 h of fermentation (Table 4.11 and Figure 4.9), and the bacterium had specific growth rate of 0.93 h^{-1}. The maximum D-lactic acid yield of 19.75 g/l (D-lactic acid yield, ($Y_{LA/S}$), of 66.51%) was achieved after 48 h (Table 4.10). The optical purity of D-lactic acid was shown to be >99.9% by HPLC analysis. L-Lactic acid was not found in fermentation medium till 48 h of cultivation. Results showed that isolate WR73 could use cassava starch without supplementation of any enzyme (α-amylase, β-amylase, and pullulanase) for D-lactic acid production. D-Lactic acid was reported to be produced by several strains of lactic acid bacteria. *Lactobacillus bulgaricus* Lb-12 produced 40 g/l of D-lactic acid from 100 g/l of lactose monohydrate at pH 6.0, and the optical purity was more than 99%. The strain

consumed only the glucose moiety of lactose (Benthin and Villadsen, 1995). Yáñez *et al.* (2003) reported that *Lactobacillus coryniformis* subsp. *torquens* ATCC 25600 could produce D-lactic acid (0.89 g of D-lactic acid/g cellulose) from 33.0 g/l of filter paper by simultaneous saccharification and fermentation (SSF) with the addition of cellulolytic enzymes for cellulose hydrolysis. The optical purity of D-lactic acid was 95.0%. *Lactobacillus delbrueckii* JCM 1148 was also reported for the production of D-lactic acid at yield concentrations of 107 g/l, 120 g/l, and 84 g/l from sugarcane molasses (119 g/l of total sugar), sugarcane juice (133 g/l of total sugar), and sugar beet juice (105 g/l of total sugar), respectively (Calabia and Tokiwa, 2007). *Lactobacillus delbrueckii* JCM 1148 also provided D-lactic acid with optical purity in the range of 97.2-98.3%. *Lactobacillus delbrueckii* could produce D-lactic acid from rice saccharificate prepared from white rice by enzymatic hydrolysis (α-amylase, β-amylase, and pullulanase (Fukushima *et al*., 2004). In this process, two strains of *Lactobacillus delbrueckii* (LD 0025 and LD 0028) could ferment the rice saccharificate to produce D-lactic acid in high conversion. Particularly, the strain LD 0028 D-lactic acid with high optical purity (>97.5%) was obtained in high yield (70%) in the large scale fermentation. Therefore, the production of highly optical purity of D-lactic acid by isolate WR73 from cassava starch without enzymatic hydrolysis should have benefit for the manufacture poly(D-lactic acid) PDLA.

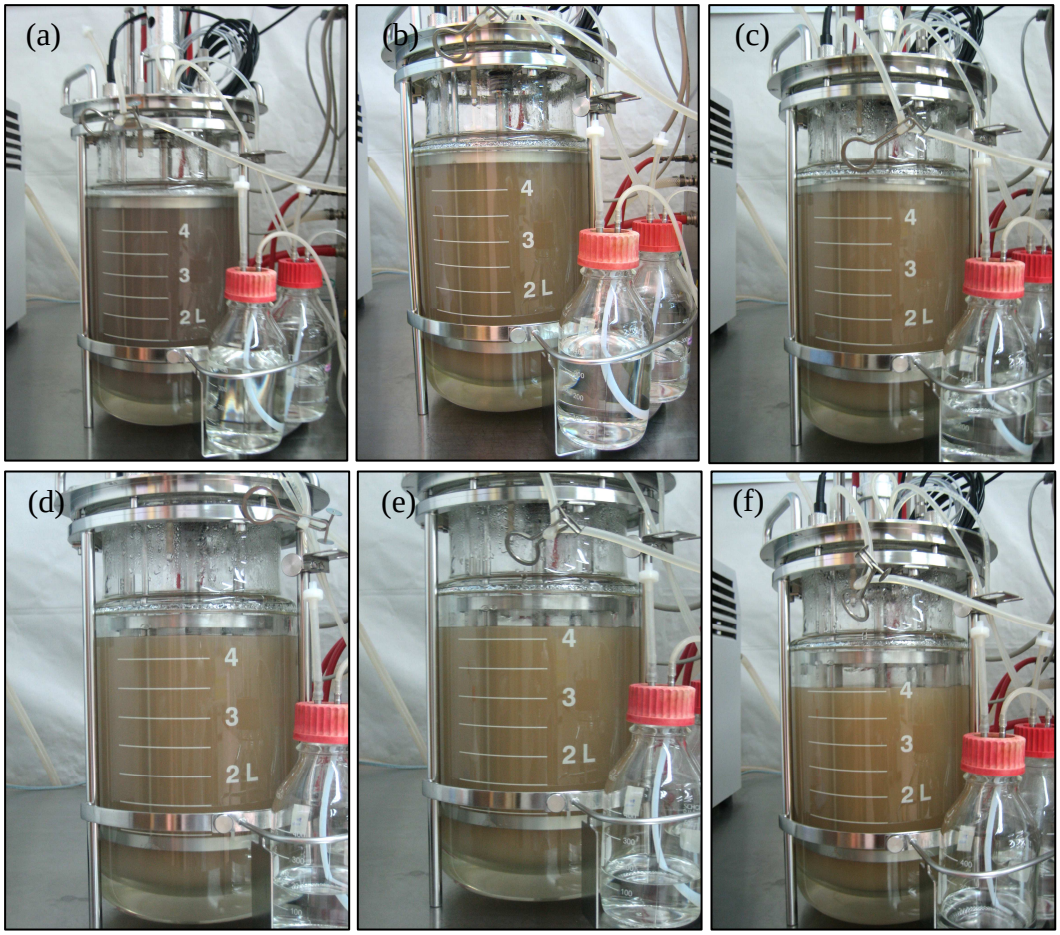


Figure 4.8 Fermentation medium containing 30 g/l of cassava starch for lactic acid production in bioreactor (5.0 l in capacity, Biostat® Bplus): (a) Initial medium at 0 h, (b) medium after inoculating starter culture for 6 h, (c) medium after inoculating starter culture for 12 h, (d) medium after inoculating starter culture for 24 h, (e) medium after inoculating starter culture for 36 h and, and (f) medium after inoculating starter culture for 48 h.

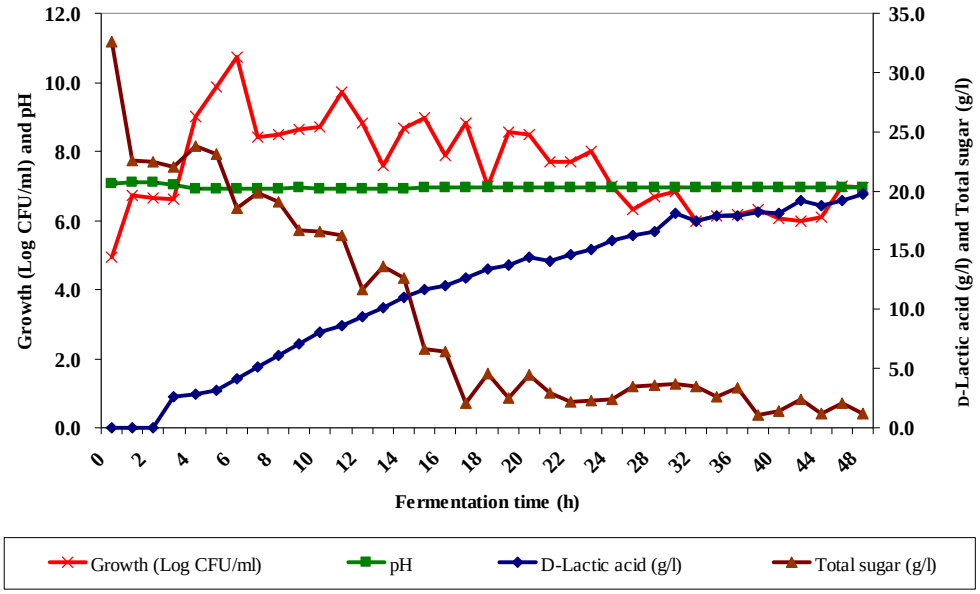


Figure 4.9 Growth and D-lactic acid production by isolate WR73 at 35°C for 48 h in 5.0 l of the optimized medium containing 30 g/l of cassava starch at the initial pH of 7.0.

Table 4.10 Specific growth rate and D-lactic acid production by isolate WR73 in 5.0 l of optimized medium containing 30 g/l of cassava starch at the initial pH of 7.0 in 6.6 l fermenter at 35°C for 48 h.

Specific growth rate (μ_{max}) (h^{-1})	D-Lactic acid concentration (LA_{max}) (g/l)	Lactic acid yield ($Y_{LA/S}$)	
		(g/g)	(%)
0.93	19.75	0.67	66.51

Table 4.11 D-Lactic acid production by bacterial isolate WR73 after cultivation in 5.0 l of optimum RAM broth containing 30 g/l of cassava starch at initial pH of 7.0 in 6.6 l fermenter at 35°C for 48 h.

Fermentation time (h)	Growth		pH	Total acidity (%)[a]	D-Lactic acid (g/l)[b]	Sugar concentration (g/l)	
	CFU/ml	Log CFU/ml				Total sugars[c]	Total sugar consumed
0	8.3×10^{4}	4.92	7.08	0.00	0.00	32.65	0.00
1	5.2×10^{6}	6.72	7.11	0.00	0.00	22.55	10.10
2	4.6×10^{6}	6.66	7.11	0.00	0.00	22.47	10.18
3	4.1×10^{6}	6.61	7.03	0.00	2.65	22.07	10.58
4	1.0×10^{9}	9.00	6.93	0.06	2.80	23.77	8.88
5	7.3×10^{9}	9.86	6.93	0.21	3.13	23.11	9.54
6	5.3×10^{10}	10.72	6.93	0.40	4.10	18.58	14.07
7	2.6×10^{8}	8.41	6.91	0.57	5.09	19.79	12.86
8	3.0×10^{8}	8.48	6.92	0.72	6.12	19.08	13.57
9	4.5×10^{8}	8.65	6.95	0.86	7.12	16.66	15.99
10	5.0×10^{8}	8.70	6.93	0.97	8.06	16.53	16.12
11	5.3×10^{9}	9.72	6.92	1.08	8.64	16.27	16.38
12	6.8×10^{8}	8.83	6.93	1.17	9.40	11.65	21.00
13	4.0×10^{7}	7.60	6.93	1.26	10.19	13.61	19.04
14	4.6×10^{8}	8.66	6.93	1.35	10.96	12.65	20.00
15	9.4×10^{8}	8.97	6.94	1.41	11.68	6.66	25.99
16	8.0×10^{7}	7.90	6.94	1.49	11.94	6.45	26.20
17	6.5×10^{8}	8.81	6.94	1.54	12.64	2.08	30.57
18	1.0×10^{7}	7.00	6.94	1.59	13.41	4.63	28.02
19	3.5×10^{8}	8.54	6.94	1.65	13.69	2.49	30.16
20	3.0×10^{8}	8.48	6.94	1.68	14.40	4.46	28.19
21	5.0×10^{7}	7.70	6.94	1.73	14.07	2.95	29.70
22	5.0×10^{7}	7.70	6.94	1.76	14.65	2.16	30.49
23	1.0×10^{8}	8.00	6.94	1.80	15.06	2.27	30.38
24	1.0×10^{7}	7.00	6.94	1.83	15.76	2.39	30.26
26	2.0×10^{6}	6.30	6.94	1.88	16.29	3.45	29.20
28	5.0×10^{6}	6.70	6.94	1.94	16.55	3.65	29.00
30	7.0×10^{6}	6.85	6.94	1.98	18.14	3.69	28.96
32	1.0×10^{6}	6.00	6.94	2.03	17.48	3.53	29.12
34	1.3×10^{6}	6.11	6.94	2.06	17.85	2.64	30.01
36	1.5×10^{6}	6.18	6.95	2.11	17.88	3.41	29.24
38	2.0×10^{6}	6.30	6.95	2.14	18.18	1.06	31.59
40	1.1×10^{6}	6.04	6.94	2.18	18.07	1.41	31.24
42	1.0×10^{6}	6.00	6.94	2.21	19.15	2.43	30.22
44	1.2×10^{6}	6.08	6.94	2.25	18.72	1.21	31.44
46	9.7×10^{6}	6.99	6.94	2.28	19.24	2.09	30.56
48	9.0×10^{6}	6.95	6.95	2.30	19.75	1.16	31.49

[a] Titration method (AOAC International, 2000).
[b] HPLC analysis (Tanaka *et al*., 2007).
[c] Colorimeteric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

4.5 Preliminary investigation of D-lactic acid extraction and purification from the optimized medium

A 600 ml of fermentation broth was used for the purification of D-lactic acid by D-lactate crystallization. Magnesium ion was chosen for the crystallization process (Figure 4.10). Magnesium salt has a larger difference between solubilities at 0°C and 100°C (Benthin and Villadsen, 1995). The purity of D-lactic acid is obtained from the complex fermentation medium by various steps. Cells were removed from the fermentation medium by centrifugation. Nitrogen-containing compounds in the fermentation medium are mainly amino acids and peptides. These compounds are highly soluble and are therefore effectively removed in the crystallization step (Benthin and Villadsen, 1995). This process, protein was precipitated and filtrated by heating at 100°C for 30 min. Magnesium D-lactate which obtained by crystallization was acidified with sulfuric acid for lactic acid liberation from its salt. Finally, coloring components was removed by activated charcoal. The D-lactic acid concentration of each of separation (Figure 4.11) was determined. The final concentration of purified D-lactic acid at 52.1 g/l with 99.9% optical purity of D-lactic acid was achieved from 600 ml of fermentation broth (initial D-lactic acid concentration at 19.75 g/l), and provided 2.29% of purified D-lactic acid yield with 5 ml of final purified D-lactic acid. The loss primarily occurred at crystallization step and appeared % loss of D-lactic acid (97.71%). After decolourization by activated charcoal, the color of purified D-lactic acid was clear which was similar to commercial lactic acid (Figure 4.10). Total sugars were not found after purification. However, yield of D-lactic acid decreased with the increase of purification steps. The results indicated that, the yield of D-lactic acid had loss at all purification steps. The concentration of

the D-lactic acid obtained in the present procedure was lower than the commercially available D-lactic acid. Several developed methods have been introduced for lactic acid purification such as esterification (Sun *et al*., 2006), ion-exchange based method (Cao *et al*., 2002; Vaccari *et al*., 1993), membrane based method (Choi *et al*., 2002), and electrodialysis (Habova *et al*., 2004; Li *et al*., 2004; Min-Tian and Koide, 2004). The choice of the separation process should be based on the efficient and economically usage of these extractants (Kharas *et al*., 1994). Thus, purification of D-lactic acid has to be further developed in order to increase the concentration of D-lactic acid in order to meet the requirement of biopolymer production.

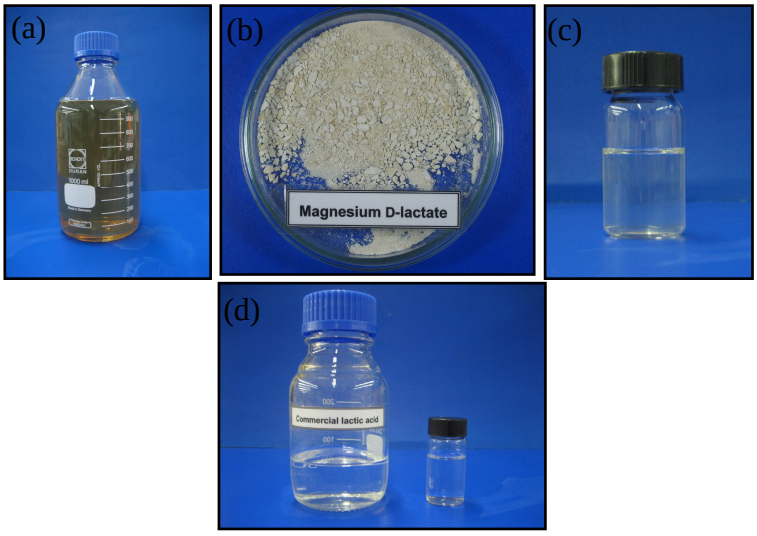


Figure 4.10 D-Lactic acid purified from cassava starch fermentation medium: (a) fermentation medium after bacterial cell separation, (b) magnesium D-lactate obtained from precipitation by $MgCl_2.6H_2O$, (c) the purified D-lactic acid before decolourization, and (d) the purified D-lactic acid after decolourization.

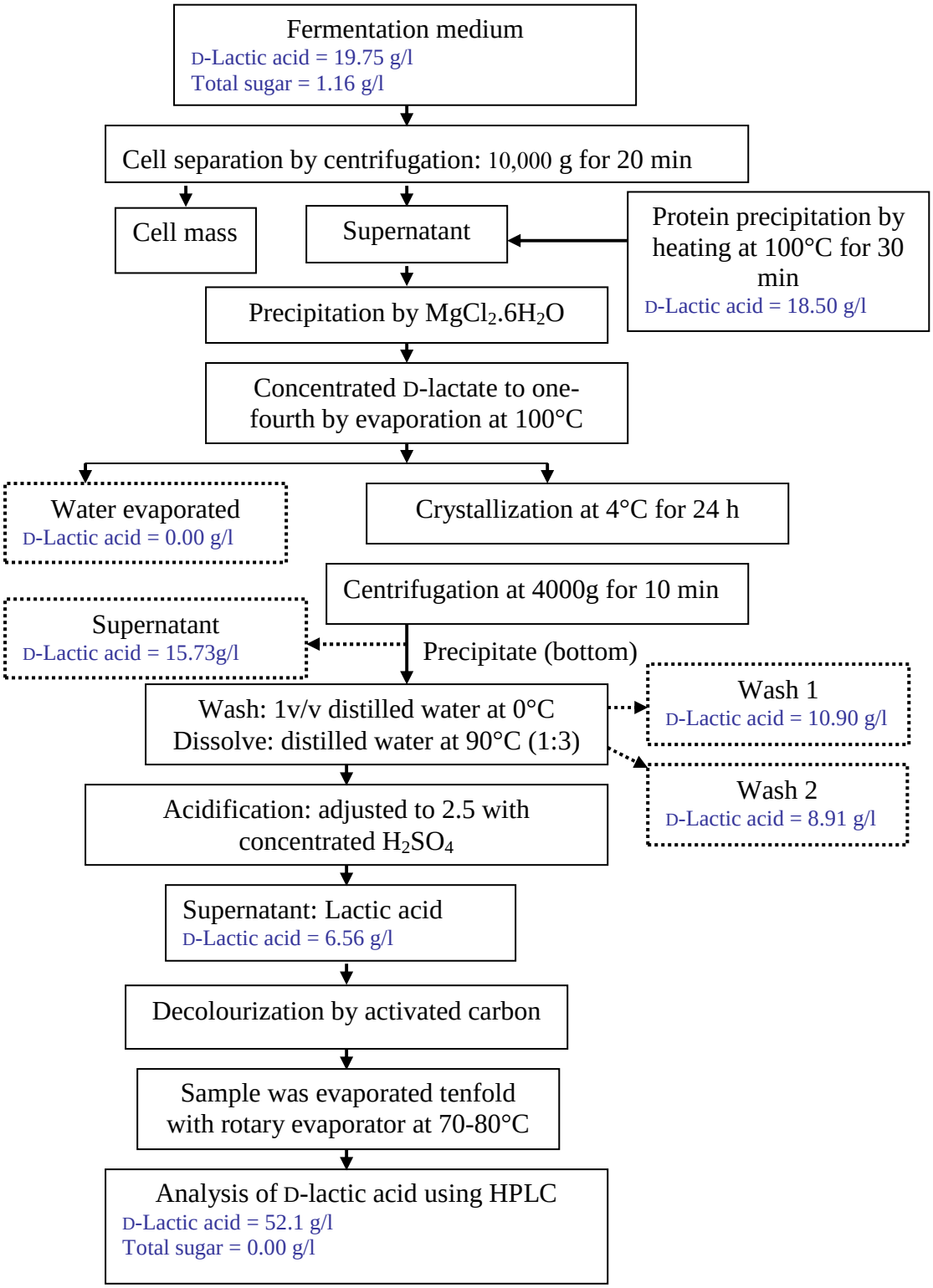


Figure 4.11 D-Lactic acid recovery at each procedure after completion of the fermentation.

4.6 Identification of the selected lactic acid bacterial isolate(s)

4.6.1 Morphological and physiological characterization

Five lactic acid bacterial isolates (WR73, CWR2-16, CWMC1-3, CWMC2-5, and CWMR1-5) were selected for identification according to their D-lactic acid production abilities (section 4.2). These selected isolates had colonies grown on RAM or MRS agar, and were Gram-positive, rods, non-spore forming, oxidase, and catalase negative (Table 4.12 and Figure 4.12). Cells of isolate WR73 had size of 0.53-0.66×0.77-1.06 µm after cultivation on RAM agar containing 1% cassava starch at 35°C for 18 h under anaerobic conditions (Figure 4.13). Isolates CWR2-16, CWMC1-3, CWMC2-5, and CWMR1-5 had their cell sizes of 0.39-0.69×2.36-5.93, 0.20-0.62×1.73-6.01, 0.39-0.57×1.58-7.27, and 0.39-0.69×1.84-5.50 µm, respectively, when cultivated on MRS agar at 35°C for 18 h under anaerobic conditions (Figure 4.13). All of the selected isolates did not digest casein and gelatin, but could produced D-lactic acid from glucose. Isolate WR73 was motile and could utilize starch, whereas isolates CWR2-16, CWMC1-3, CWMC2-5, and CWMR1-5 were non-motile and could not hydrolyze starch. Isolate WR73 had its optimum growth temperature in the range of 30-40°C, optimal pH at 5.0-8.0, and not grew at 3.0-18.0% NaCl, while isolates CWR2-16, CWMC1-3, CWMC2-5, and CWMR1-5 had their optimum growth temperatures at 20-50°C and optimal pH 4.8-8.0, and could grow at 0.0-4.0% NaCl. Morphological and biochemical characteristics of the five lactic acid bacterial isolates WR73, CWR2-16, CWMR1-5, CWMC2-5, and CWMC1-3 were similar to genus *Lactobacillus* (Table 4.12). The selected isolates WR73, CWR2-16, and CWMR1-5 were identified as *Lactobacillus delbrueckii* subsp.

delbrueckii with preciseness at 97.1, 94.8, and 94.8% homology, respectively, according to their biochemical characteristics (API 50CH/CHL, Biomérieux) (Table 4.14), but showed different morphological and physiological characteristics from *Lactobacillus delbrueckii* (Table 4.13). According to Kandler and Weiss (1986), *Lactobacillus delbrueckii* was non-motile and could not hydrolyze starch and gelatin. Thus, results of morphological and physiological characteristics were not sufficient for identifying these isolates. Isolate WR73 was chosen for further characterization using 16S ribosomal RNA sequencing technique because the strain could produce high optical purity of D-lactic acid from starch.

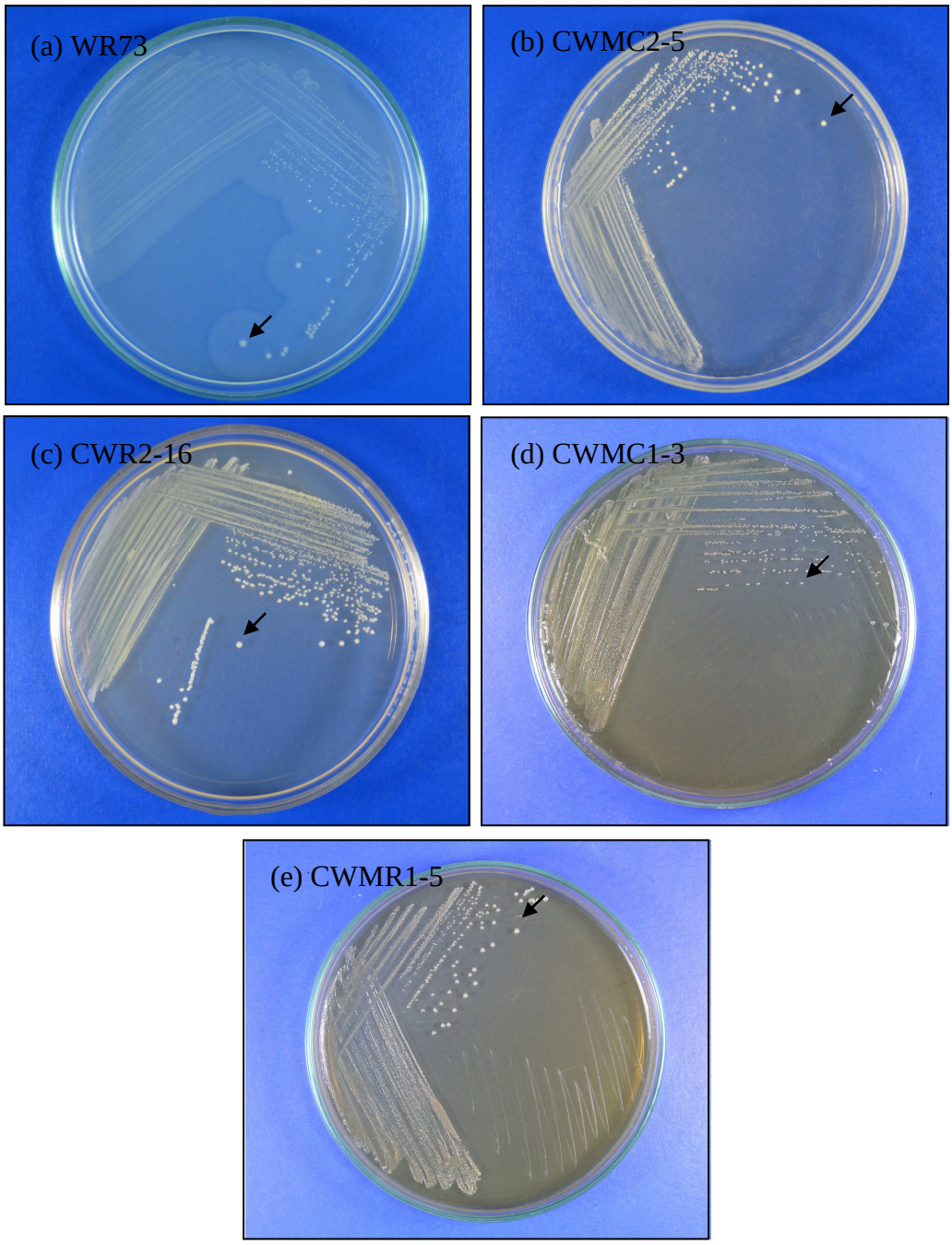


Figure 4.12 Colony morphology of selected lactic acid bacterial isolates grew on RAM (a) and MRS (b, c, d, e) agar under anaerobic conditions at 35°C for 48 h: (a) WR73, (b) CWMC2-5, (c) CWR2-16, (d) CWMC1-3, and (e) CWMR1-5.

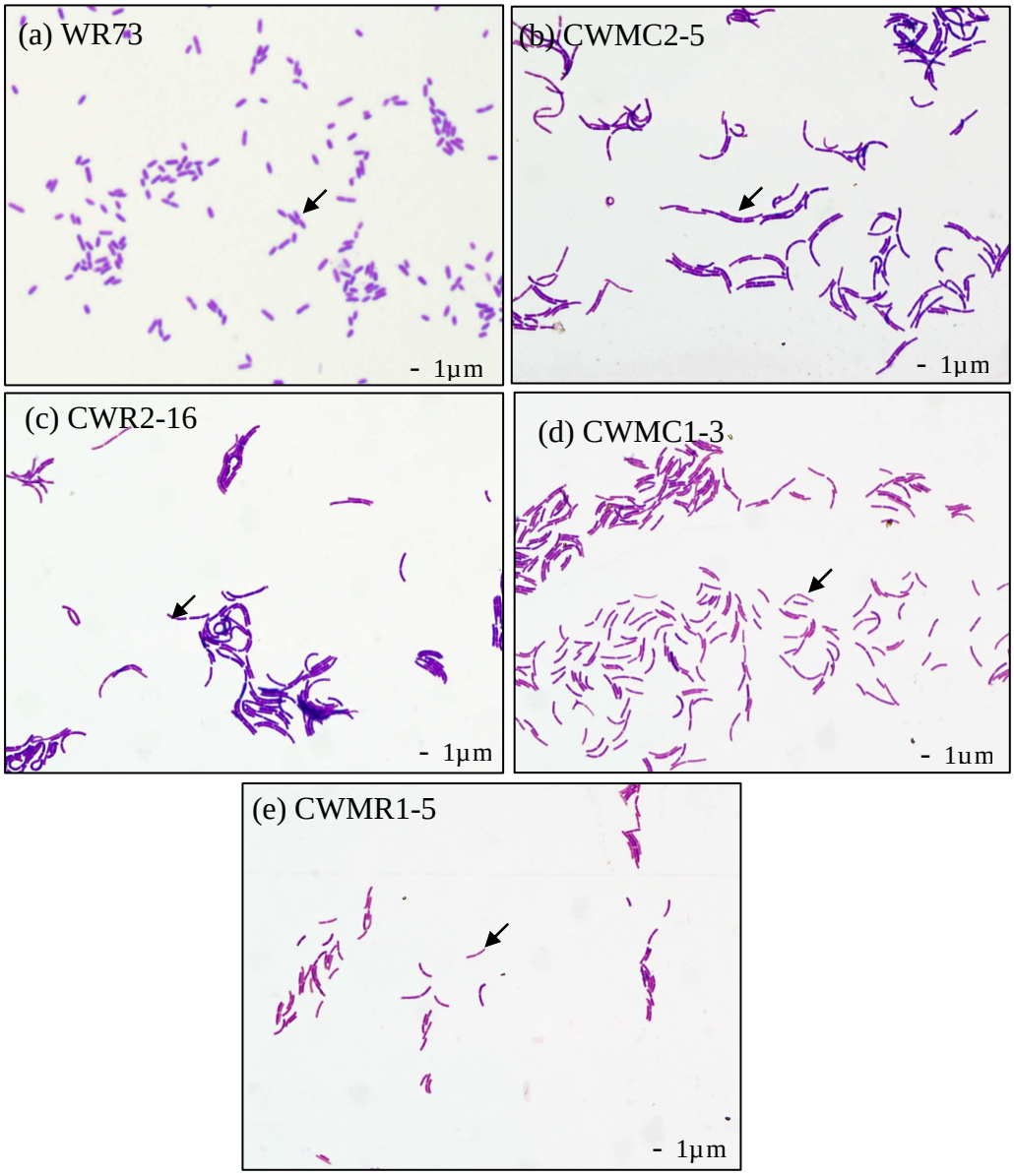


Figure 4.13 Cell morphology from gram staining of selected lactic acid bacterial isolate grew on RAM and MRS agar under anaerobic conditions at 35°C for 18 h: (a) WR73, (b) CWMC2-5, (c) CWR2-16, (d) CWMC1-3, and (e) CWMR1-5 (Bar = 1 µm, 100×magnification.

Table 4.12 Comparison of morphological and physiological characteristics of the selected lactic acid bacteria and some closely related genera.

Characteristics	Isolate number					Lactic acid bacterial genus			
	WR73	CWR2-16	CWMC1-3	CWMC2-5	CWMR1-5	*Lactob.*[a]	*Carnob.*[a]	*Weissella*	*Leucon.*[a]
Colony shape	White, smooth, circular, flat, entire edge	White, smooth, circular, flat, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, flat, entire edge	Convex, without pigment, entire edge, opaque	White, convex, shiny, circular	Smooth, round, grayish convex, entire edge	Smooth, round convex, grayish white
Colony diameter (mm)	1.1	1.0	1.0	1.0	1.2	2.0-5.0	1.0-2.0	ND	1.0
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods, usually straight, sometime cocco-bacilli	Slender straight rods	Rods, cocci	Cocci
Cell arrangement	Single, pair	Single, pair, chains	Single, pair, chains	Single, pair, chains	Single, pair, chains	Single, pair, chains	Single, pair, chains	Single, pair, chains	Pair, chains
Cell size (μm)	(0.53-0.66) ×(0.77-1.06)	(0.39-0.69) ×(2.36-5.93)	(0.20-0.62) ×(1.73-6.01)	(0.39-0.57) ×(1.58-7.27)	(0.39-0.69) ×(1.84-5.50)	(0.5-1.2) ×(1.0-10.0)	(0.5-0.7) ×(1.0-2.0)	0.5-0.7	(0.5-0.7) ×(0.7-1.2)
Gram stain	+	+	+	+	+	+	+	+	+
Spore formation	-	-	-	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	-	-	-
Motility	+	-	-	-	-	-[c]	D	-	-

Table 4.12 (Continued) Comparison of morphological and physiological characteristics of the selected lactic acid bacteria and some closely related genera.

Characteristics	Isolate number					Lactic acid bacterial genus			
	WR73	CWR2-16	CWMC1-3	CWMC2-5	CWMR1-5	*Lactob.*[a]	*Carnob.*[a]	*Weissella*	*Leucon.*[a]
Major fermentation products from utilizable carbohydrates anaerobically	Lactic acid	Lactic acid	Lactic acid	Lactic acid	Lactic acid	Mainly lactic acid; may give some acetic acid, ethanol, CO_2	Lactic acid; may give some acetic acid, formic, ethanol, CO_2	Mainly lactic acid	Lactic acid, ethanol
Lactic acid[f]	D	D	D	D	D	D, DL, L[g]	L	D, DL[g]	D
CO_2 production from glucose[h]	-	-	-	-	-	±	-[d]	+	+
Hydrolysis of:									
Starch	+	-	-	-	-	±	±	-	±[b]
Gelatin	-	-	-	-	-	-	NA	NA	NA
Arginine	-	+	+	-	-	±	+	±	-
Casein	-	-	-	-	-	±	NA	NA	-
Optimum growth temperature (°C)	30-40	20-50	20-50	20-50	20-50	30-40	30	30	20-30
Growth at 10°C	-	-	-	-	-	±	+	+	+
Growth at 45°C	+	+	+	+	+	±	-	-	-
Growth at 6.5% NaCl	-	-	-	-	-	±	NA[e]	±	±
Growth at 18% NaCl	-	-	-	-	-	-	-	-	-

Table 4.12 (Continued) Comparison of morphological and physiological characteristics of the selected lactic acid bacteria and some closely related genera.

Characteristics	Isolate number					Lactic acid bacterial genus			
	WR73	CWR2-16	CWMC1-3	CWMC2-5	CWMR1-5	*Lactob.*[a]	*Carnob.*[a]	*Weissella*	*Leucon.*[a]
Growth at pH 4.4	-	+	+	+	+	±	NA	±	±
Growth at pH 9.6	-	-	-	-	-	-	-	-	-
Habitat	Cassava starch production waste					Widespread in fermentable materials, rarely pathogenic	Food products; one species is a pathogen of fish	Fermented food, soil	Widely distributed on plants and in dairy and other food products

+, positive; -, negative; ±, response varies between species; ND, not determined.
[a] *Lactob*., *Lactobacillus*; *Carnob*., *Carnobacterium*; *Leucon*., *Leuconostoc*.
[c] Rarely motile.
[h] Test for homo- or heterofermentation of glucose; negative and positive denotes homofermentative and heterofermentative, respectively.
[d] Small amounts of CO_2 can be produced, depending on media.
[e] No growth in 8% NaCl has been reported.
[f] Configuration of lactic acid produced from glucose.
[g] Production of D-, L- or DL-lactic acid varies between species.
[b] 11-89% of strains are positive.
D = Substantial proportion of species differ.
Source: Axelsson (2004): Holt *et al.* (1994); Collins *et al*. (1993).

Table 4.13 Comparison of morphological and physiological characteristics of the selected lactic acid bacteria and some closely related strains.

Characteristics	Isolate number					Type strain		
	WR73	CWR2-16	CWMC1-3	CWMC2-5	CWMR1-5	*L. delbrueckii* [a]	*W. hellenica*[b]	*Leuc. fallax*[c]
Colony shape	White, smooth circular, flat, entire edge	White, smooth, circular, flat, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, convex, entire edge	White, smooth, circular, flat, entire edge	NA	NA	Smooth, round, grayish
Colony diameter (mm)	1.1	1.0	1.0	1.0	1.2	NA	NA	1.0
Cell shape	Ovoid	Rods	Rods	Rods	Rods	Rods	Cocci	Cocci
Cell arrangement	Single, pair	Single, pair, chains	Single, pair, chains	Single, pair, chains	Single, pair, chains	Single, pair, chains	NA	Pair, chains
Cell size (μm)	(0.53-0.66) ×(0.77-1.06)	(0.39-0.69) ×(2.36-5.93)	(0.20-0.62) ×(1.73-6.01)	(0.39-0.57) ×(1.58-7.27)	(0.39-0.69) ×(1.84-5.50)	(0.5-0.8) × (2.0-9.0)	NA	NA
Gram stain	+	+	+	+	+	+	+	+
Spore formation	-	-	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	-	-
Motility	+	-	-	-	-	-	-	-
CO_2 production	-	-	-	-	-	-	+	+
Hydrolysis of:								
Starch	+	-	-	-	-	-	NA	NA
Gelatin	-	-	-	-	-	-	NA	NA
Arginine	-	+	+	-	-	NA	-	-
Casein	-	-	-	-	-	-	NA	NA

Table 4.13 (Continued) Comparison of morphological and physiological characteristics of the selected lactic acid bacteria and some closely related strains.

Characteristics	Isolate number					Type strain		
	WR73	CWR2-16	CWMC1-3	CWMC2-5	CWMR1-5	*L. delbrueckii* [a]	*W. hellenica*[b]	*Leuc. fallax*[c]
Growth at:								
5°C	-	-	-	-	-	-	-	-
10°C	-	-	-	-	-	-	+	+
15°C	-	-	-	-	-	-	NA	+
20°C	++	++++	++++	++++	++++	+	NA	+
30°C	+++	++++	++++	++++	++++	+	NA	+
35°C	+++	++++	++++	++++	++++	+	NA	+
37°C	+++	++++	++++	++++	++++	+	NA	+
40°C	+++	++++	++++	++++	++++	+	NA	NA
42°C	+	++++	++++	++++	++++	+	NA	NA
45°C	+	++++	++++	++++	++++	+	-	-
50°C	-	++++	++++	++++	++++	NA	-	-
55°C	-	-	-	-	+	NA	-	-
Growth at:								
pH 4.0	-	++	+	+	+	NA	NA	NA
pH 4.4	-	+++	+	+	+	NA	NA	NA
pH 4.8	-	++++	++++	++++	++++	NA	NA	NA
pH 5.0	+++	++++	++++	++++	++++	+	NA	NA
pH 6.0	++++	++++	++++	++++	++++	+	NA	NA
pH 6.5	++++	++++	++++	++++	++++	+	NA	NA
pH 7.0	++++	++++	++++	++++	++++	+	NA	NA
pH 8.0	++++	++++	++++	++++	++++	NA	NA	NA

Table 4.13 (Continued) Comparison of morphological and physiological characteristics of the selected lactic acid bacteria and some closely related strains.

Characteristics	Isolate number					Type strain		
	WR73	CWR2-16	CWMC1-3	CWMC2-5	CWMR1-5	*L. delbrueckii* [a]	*W. hellenica*[b]	*Leuc. fallax*[c]
Growth at:								
pH 9.6	-	-	-	-	-	-	-	-
Growth at:								
0.0% NaCl	++++	++++	++++	++++	++++	+	NA	NA
3.0% NaCl	-	++++	++++	++++	++++	NA	NA	NA
4.0% NaCl	-	++++	++++	++++	++++	NA	NA	NA
6.5% NaCl	-	-	-	-	-	NA	NA	NA
8.0% NaCl	-	-	-	-	-	NA	NA	NA
10.0% NaCl	-	-	-	-	-	NA	-	
18.0% NaCl	-	-	-	-	-	-	-	-
Acid from:								
Glycerol	-	-	NT	NT	-	NA	NA	NA
Erythritol	-	-	NT	NT	-	NA	NA	NA
D-Arabinose	-	-	NT	NT	-	-	+	-
L-Arabinose	-	-	NT	NT	-	-	+	-
D-Ribose	-	+	NT	NT	+	-	+	+
D-Xylose	-	-	NT	NT	-	-	+	-
L- Xylose	-	-	NT	NT	-	-	+	-
D-Adonitol	-	-	NT	NT	-	NA	NA	NA
Methyl-βD-xylopyranoside	-	-	NT	NT	-	NA	NA	NA
D-Galactose	-	-	NT	NT	-	NA	+	-
D-Glucose	+	+	NT	NT	+	+	+	+
D-Fructose	+	+	NT	NT	+	+	+	+

Table 4.13 (Continued) Comparison of morphological and physiological characteristics of the selected lactic acid bacteria and some closely related strains.

Characteristics	Isolate number					Type strain		
	WR73	CWR2-16	CWMC1-3	CWMC2-5	CWMR1-5	*L. delbrueckii* [a]	*W. hellenica*[b]	*Leuc. fallax*[c]
Acid from:								
D-Mannose	+	+	NT	NT	+	+	+	+
L-Sorbose	-	-	NT	NT	-	NA	NA	NA
L-Rhamnose	-	-	NT	NT	-	-	NA	NA
Dulcitol	-	-	NT	NT	-	NA	NA	NA
Inositol	-	-	NT	NT	-	NA	NA	NA
D-Mannitol	-	-	NT	NT	-	-	-	d
D-Sorbitol	-	-	NT	NT	-	+	NA	NA
Methyl-αD-Mannopyranoside	-	-	NT	NT	-	NA	NA	NA
Methyl-αD-Glucopyranoside	-	-	NT	NT	-	NA	NA	NA
N-Acetylglucosamine	-	+	NT	NT	+	NA	NA	NA
Amygdaline	-	-	NT	NT	-	NA	NA	NA
Arbutin	-	-	NT	NT	-	NA	NA	-
Esculin	+	+	NT	NT	+	NA	NA	NA
Salicin	-	+	NT	NT	+	NA	+	+
D-Cellobiose	-	-	NT	NT	-	NA	+	-
D-Maltose	-	+	NT	NT	+	NA	+	+
D-Lactose	-	-	NT	NT	-	NA	-	-
D-Melibiose	-	-	NT	NT	-	-	-	-
D-Saccharose (Sucrose)	+	+	NT	NT	+	NA	-	+

Table 4.13 (Continued) Comparison of morphological and physiological characteristics of the selected lactic acid bacteria and some closely related strains.

Characteristics	Isolate number					Type strain		
	WR73	CWR2-16	CWMC1-3	CWMC2-5	CWMR1-5	*L. delbrueckii* [a]	*W. hellenica*[b]	*Leuc. fallax*[c]
Acid from:								
D-Trehalose	-	+	NT	NT	+	NA	-	d
Inulin	-	-	NT	NT	-	NA	NA	NA
D-Melezitose	-	-	NT	NT	-	-	NA	NA
D-Raffinose	-	-	NT	NT	-	-	-	-
Amidon (Starch)	+	-	NT	NT	-	NA	NA	NA
Glycogen	-	-	NT	NT	-	NA	NA	NA
Xylitol	-	-	NT	NT	-	NA	NA	NA
Gentiobiose	-	-	NT	NT	-	NA	NA	NA
D-Turanose	-	-	NT	NT	-	NA	NA	NA
D-Lyxose	-	-	NT	NT	-	NA	NA	NA
D-Tagatose	-	-	NT	NT	-	NA	NA	NA
D-Fucose	-	-	NT	NT	-	NA	NA	NA
L-Fucose	-	-	NT	NT	-	NA	NA	NA
D-Arabitol	-	-	NT	NT	-	NA	NA	NA
L-Arabitol	-	-	NT	NT	-	NA	NA	NA
Potassiumgluconate	-	-	NT	NT	-	NA	NA	NA
Potassium 2-ketogluconate	-	-	NT	NT	-	NA	NA	NA
Potassium 5-ketogluconate	-	-	NT	NT	-	NA	NA	NA
Configuration of lactic acid	D	D	D	D	D	D	D	D

Note: +1 to +4, A_{600} in the range of 0.1-0.2, 0.2-0.4, 0.5-0.9, and ≥ 1.0, respectively.

d = 11-89% strains positive; +, positive; -, negative.
NA = Not available.
NT = Not tested.
[a] Holt *et al.* (1994).
[b] Tanasupawat *et al.* (2000).
[c] Wood and Holzaptel (1995).

Table 4.14 Identification of selected lactic acid bacterial isolates according to their biochemical characteristics (API 50CH/CHL, Biomérieux).

Isolate code	Identification (% identity) According to API 50CH/CHL database
WR73	*Lactobacillus delbrueckii* subsp. *delbrueckii* (97.1%)
CWR2-16	*Lactobacillus delbrueckii* subsp. *delbrueckii* (94.8%)
CWMR1-5	*Lactobacillus delbrueckii* subsp. *delbrueckii* (94.8%)

4.6.2 Sequencing of 16S ribosomal RNA gene

The bacterial isolate WR73 was further characterized for 16S ribosomal RNA sequence. The gene was amplified from genomic DNA using fD1/rP2 primer (Weisburg *et al*., 1991). The expected size of amplified DNA fragment was approximate 1500 bp (Figure 4.14).

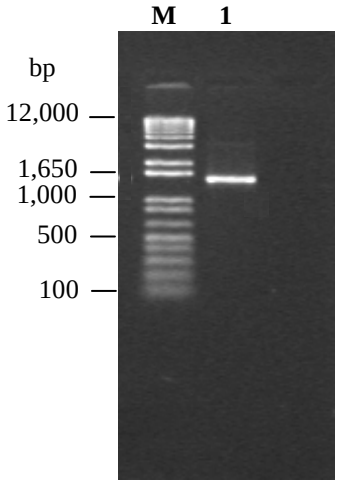


Figure 4.14 Gel electrophoresis of PCR product obtained from the amplification of 16S rRNA gene from strain WR73 using primers fD1 and rP2: Lanes: M, Molecular weight marker (1 kb plus DNA ladder, Invitrogen) and 1, bacterial isolate WR73.

After sequencing, the amplified 16S rRNA gene fragment of the bacterial isolate WR73 was aligned (Figure 4.15). The length of the 16S rDNA sequences from WR73 was 1450 bp. The 16S rDNA sequence was compared with GenBank database using standard nucleotide-nucleotide BLAST program to ascertain their closest relatives. The percentages of 16S rDNA sequence similarity of WR73 compared to *Lactobacillus delbrueckii* DSM 20074^{T}, *Lactobacillus coryniformis* DSM 20001^{T}, *Carnobacterium* sp. MARL 15^{T}, *Carnobacterium pleistocenium* FTR 1^{T}, *Weissella hellenica* NRIC 0203^{T}, *Leuconostoc fallax* DSM 20189^{T} were 77% (Tables 4.15 and 4.16). The isolate WR73 showed low level of DNA similarity (75-76%) compared to other isolates. The results suggested that the isolate WR73 closely related to species belonging to genera *Lactobacillus*, *Carnobacterium*, *Weissella*, and *Leuconostoc*. When phylogenetic tree based on the 16S rDNA sequences was constructed using the neighbour-joining method with software MAGA version 4.0 (Tamura *et al*., 2007), the isolate WR73 was clearly separated from *Lactobacillus delbrueckii* and other species in genera *Carnobacterium*, *Leuconostoc*, and *Weissella*. However, based on morphological and physiological characteristics of the isolate WR73, (Table 4.12), which were rod shape cells, catalase negative, homo D-lactic acid production from glucose, optimum growth temperature at 30-40°C (no growth at 10°C), and amylase-positive, it was identified as belonging to the genus *Lactobacillus*.

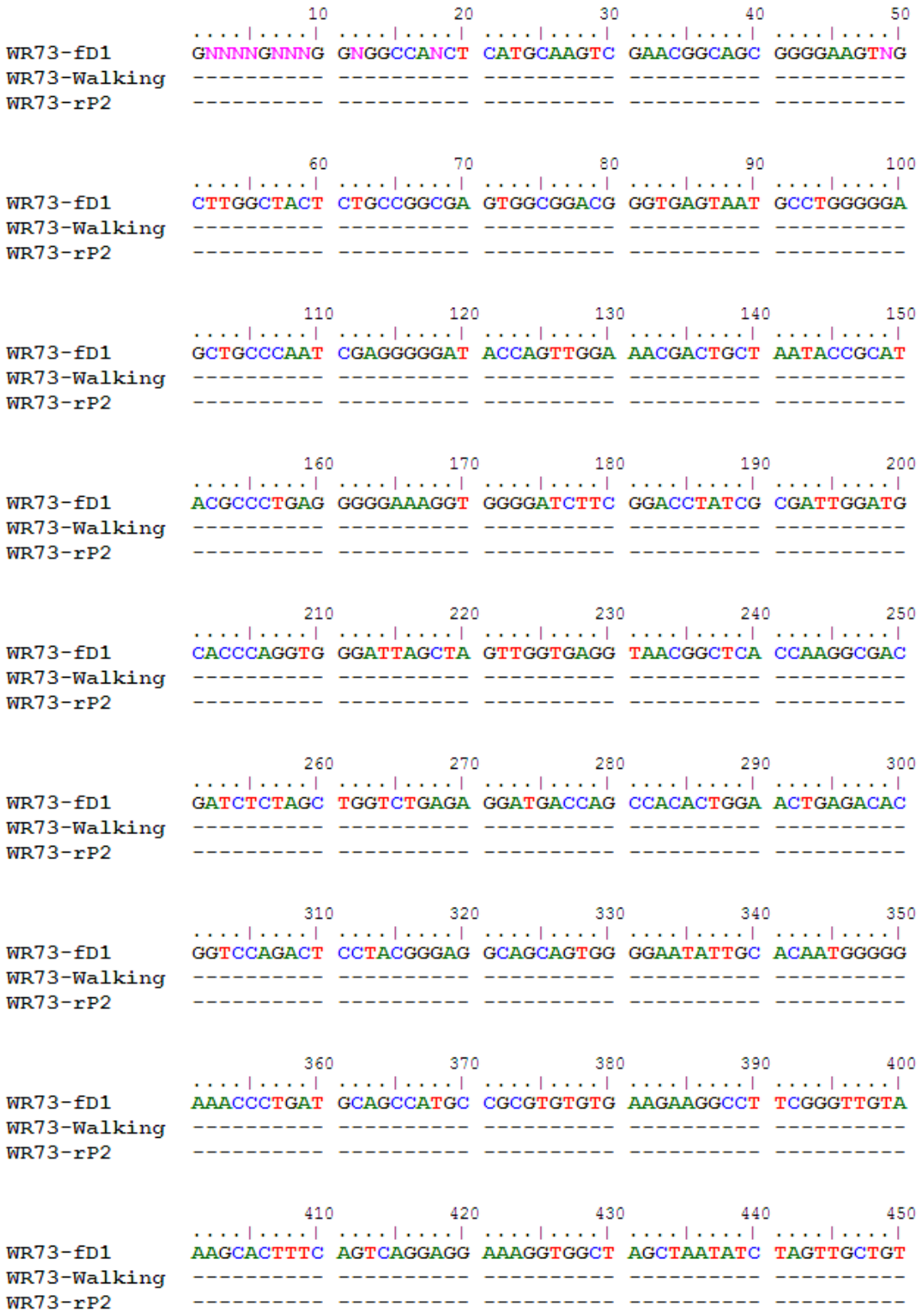


Figure 4.15 Sequence alignment of parts of 16S rRNA gene of bacterial isolate WR73 by using ClustalW and BioEdit programs.

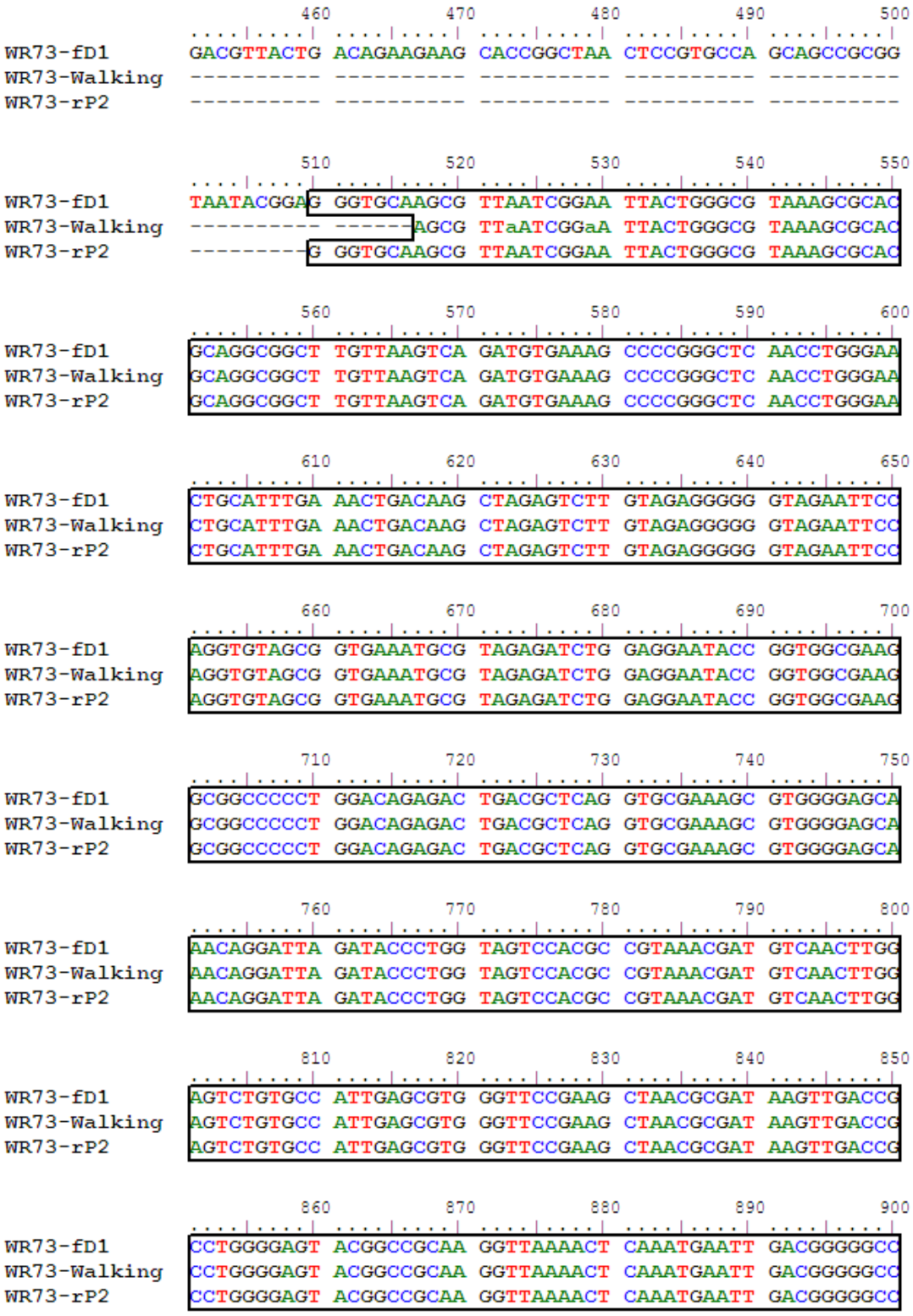


Figure 4.15 (Continued) Sequence alignment of parts of 16S rRNA gene of bacterial isolate WR73 by using ClustalW and BioEdit programs.

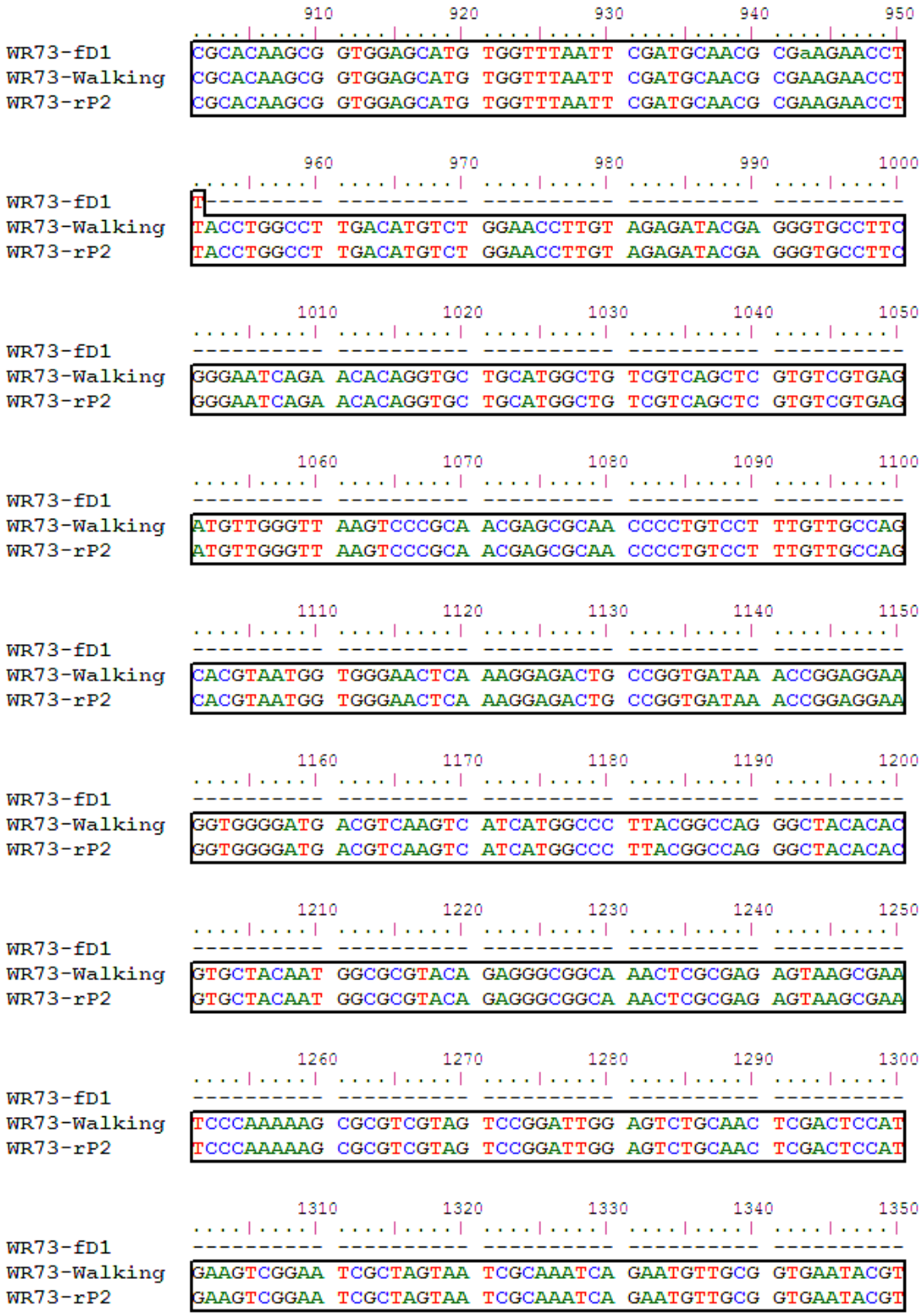


Figure 4.15 (Continued) Sequence alignment of parts of 16S rRNA gene of bacterial isolate WR73 by using ClustalW and BioEdit programs.

Figure 4.15 (Continued) Sequence alignment of parts of 16S rRNA gene of bacterial isolate WR73 by using ClustalW and BioEdit programs.

Table 4.15 16S rRNA gene sequence similarity and relation of the isolate WR73 and other Gram-positive rods from GenBank database.

Bacterial isolates	WR73	1	2	3	4	5	6
WR73	100						
1	76	100					
2	76	92	100				
3	77	93	99	100			
4	77	91	88	88	100		
5	77	89	87	87	92	100	
6	77	89	87	87	92	97	100

WR73: *Lactobacillus* sp. WR73, 1: *Lactobacillus jensenii* ATCC 25258^{T} (AF243176), 2: *Lactobacillus delbrueckii* NCDO 213^{T} (X52654), 3: *Lactobacillus delbrueckii* DSM 20074^{T} (M58814), 4: *Lactobacillus coryniformis* DSM 20001^{T} (M58813), 5: *Carnobacterium* sp. MARL 15^{T} (DQ343756), 6: *Carnobacterium pleistocenium* FTR 1^{T} (NR_025211).

Table 4.16 16S rRNA gene sequence similarity and relation of the isolate WR73 and other Gram-positive cocci from GenBank database.

Bacterial isolates	WR73	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
WR73	100																				
1	76	100																			
2	76	92	100																		
3	75	93	92	100																	
4	76	95	93	95	100																
5	76	90	87	90	90	100															
6	76	89	87	89	89	94	100														
7	75	92	89	91	91	92	92	100													
8	76	92	88	91	92	92	92	98	100												
9	75	93	89	92	92	93	92	96	98	100											
10	76	87	84	85	87	85	85	85	86	86	100										
11	76	85	84	83	84	83	84	84	84	84	92	100									
12	77	87	84	86	87	85	85	86	86	87	95	95	100								
13	77	86	83	85	86	83	83	84	84	84	90	88	91	100							
14	76	84	82	84	85	83	83	84	84	84	90	87	90	94	100						
15	75	82	83	81	82	81	81	81	81	81	87	87	87	91	95	100					
16	75	82	83	81	82	81	81	81	81	81	87	87	87	91	95	99	100				
17	76	86	83	85	87	85	84	85	85	85	88	84	87	86	86	82	82	100			
18	76	86	84	85	87	85	86	86	86	85	88	86	89	87	86	83	83	93	100		
19	76	86	84	86	86	86	86	86	85	86	90	87	90	86	86	83	83	88	88	100	
20	76	86	84	86	86	86	86	86	85	86	89	87	90	87	86	83	83	88	88	98	100

WR73: *Lactobacillus* sp. WR73, 1: *Streptococcus iniae* ATCC 29178^{T} (DQ303187), 2: *Streptococcus acidominimus* NCDO 2025^{T} (X58301), 3: *Streptococcus thermophilus* DSM 20617^{T} (X68418), 4: *Streptococcus suis* ATCC 43765^{T} (AB002525), 5: *Lactococcus lactis* subsp. *lactis* ATCC 13675^{T} (AB100790), 6: *Lactococcus garvieas* ATCC 49156^{T} (AF061005), 7: *Lactococcus raffinolactis* DSM 20443^{T} (EF694030), 8: *Lactococcus* sp. MARL 49^{T} (AY762111), 9: *Lactococcus plantarum* DSM 20686^{T} (EF694029), 10: *Weissella koreensis* JCM 11263^{T} (AY035891), 11: *Weissella thailandensis* NRIC 0296^{T} (AB023839), 12: *Weissella hellenica* NRIC 0203^{T} (AB023240), 13: *Leuconostoc fallax* DSM 20189^{T} (AF360738), 14: *Leuconostoc mesenteroides* NRIC 1541^{T} (AB023243), 15:

Leuconostoc carnosum NRIC 1722^{T} (AB022925), 16: *Leuconostoc carnosum* NCFB 2776^{T} (X95977), 17: *Aerococcus* sp. CCUG 28826^{T} (Y17318), 18: *Aerococcus viridans* ATCC 700406^{T} (AY707779), 19: *Pediococcus* sp. FUA 3226^{T} (GQ222394), 20: *Pediococcus stilesii* LMG 23082^{T} (AJ973157).

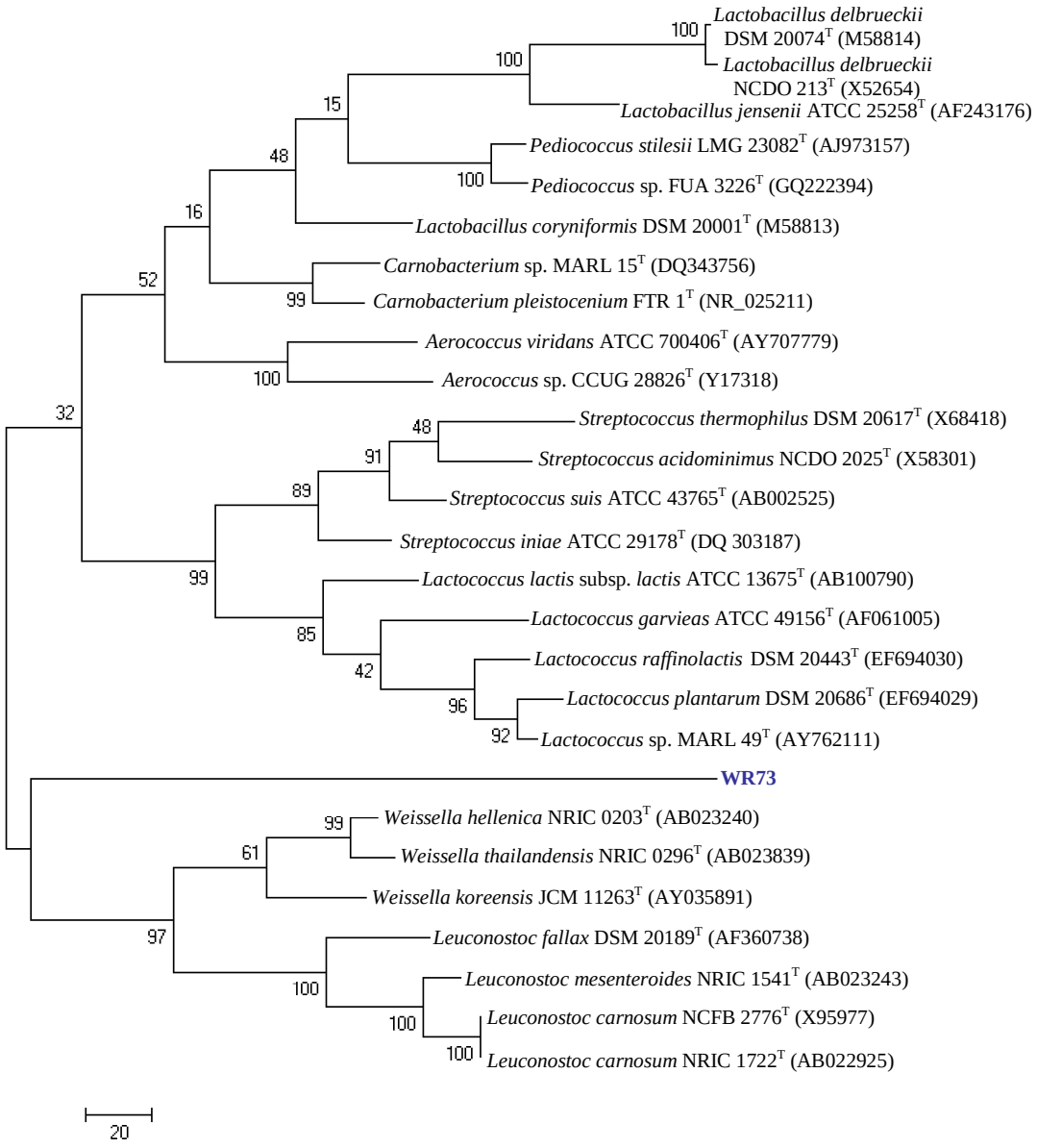


Figure 4.16 Phylogenetic tree showing strain WR73 and other related strains based on 16S rDNA sequences. Scale bar represents 20 substitutions per nucleotide position.

CHAPTER V

CONCLUSION

Three hundred and six isolates of lactic acid bacteria obtained from stock cultures of the Microbial Culture Collection Laboratory, Suranaree University of Technology, which were isolated from their natural habitats were screened for their D-lactic acid production using glucose as a carbon source. One hundred and twenty-one of lactic acid bacterial isolates from different habitats was selected based on their D-lactic acid production abilities, and these isolates provided the total acidity ranging from 0.053 to 1.77%. Seven isolates from a total of 306 isolates including of WR73, CWMC2-5, CWMC1-3, CWMR1-5, CWR2-16, LF1, and PSMS1-5 were able to produce D-lactic acid with optical purity >90% at high concentrations of 2.00, 17.94, 15.88, 15.84, 15.16, 10.42, and 10.16 g/l, respectively. Only one isolate, WR73, could utilize a cheap raw material, cassava starch, and produce optically pure D-lactic acid. (>99.9%). Isolate WR73 was selected for optimization of their D-lactic acid production conditions. Concentrations of carbon source, tryptone, yeast extract, spent of brewery yeast sludge, initial pH of the culture medium, cultivation temperature, and inoculum size were investigated to achieve the maximum D-lactic acid production. The suitable medium was found to compose of main ingredients as follows: 30.0 g/l of cassava starch, 3.0 g/l of tryptone, and 3.0 g/l of spent of brewery yeast sludge. The optimum inoculum size, pH, and temperature were 1%, 7.0, and 35°C, respectively. When D-lactic acid was produced lactic acid bacterial

isolate WR73 in a controlled fermenter containing 5.0 l of the optimized medium under optimum conditions. Isolate WR73 could produce optically pure D-lactic acid from cassava starch with the maximum D-lactic acid yield of 19.76 g/l (D-lactic acid yield, ($Y_{LA/S}$), of 66.51%) at 48 h of cultivation in a controlled fermenter containing 5.0 l of the suitable medium containing 30.0 g/l (dry weight) of cassava starch, 3.0 g/l of tryptone, 3.0 g/l of spent of brewery yeast sludge, 6.0 g/l of K_2HPO_4, 0.57 g/l of $MgSO_4.7H_2O$, 0.12 g/l $MnSO_4.4H_2O$, and 0.03 g/l of $FeSO_4.7H_2O$. This strain had its specific growth rate (μ_{max}) of 0.93 h^{-1}. Cassava starch could serve as a low-cost substrate for the production of high quality D-lactic acid for manufacturing of poly(D-lactic acid), PDLA, essential for successful application of the high-melting stereocomplex-type poly(lactic acid). D-Lactic acid was extracted and purified by magnesium D-lactate crystallization. The purified D-lactic acid concentration was 52.1 g/l, and the loss primary occurred at crystallization step. Moreover, the color of purified D-lactic acid was clear which was similar to commercial lactic acid after color removal by treatment with activated charcoal. The lactic acid bacterial isolate WR73 was identified using morphological, physiological, and 16S rRNA gene sequence. The strain WR73 was Gram-positive rod, catalase negative. It could produce homo D-lactic acid from glucose, and had its optimum growth temperature in the range of 30-40°C, optimal pH at 5.0-8.0, and not grew at 3.0-18.0% NaCl. It was motile and could utilize starch. The strain WR73 was identified as *Lactobacillus delbrueckii* subsp. *delbrueckii* with preciseness at 97.1% homology according to their biochemical characteristics (API 50CH/CHL, Biomérieux). From sequencing of 16S rDNA gene (1450 bp) amplified using fD1/rP2 primer, the strain WR73 was clearly separated from *Lactobacillus delbrueckii*, and other species in

genera *Carnobacterium*, *Leuconostoc*, and *Weissella*. However, the strain WR73 was still identified as belonging to the genus *Lactobacillus* by morphological and physiological characteristics, and 16S rRNA gene sequence.

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APPENDICES

APPENDIX A

CULTURE MEDIA AND REAGENTS

A.1 Chemicals, reagents and culture media used for 16S ribosomal RNA gene sequencing

1.1 Tris-EDTA (TE) Buffer

Tris-base	1.21	g
EDTA ($C_{10}H_{14}N_2O_8Na_2.2H_2O$)	0.37	g

The ingredients were dissolved and the final volume was adjusted to 1,000 ml with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches, after preparation.

1.2 Tris-NaCl

Tris-base	121.14	g
NaCl	5.84	g

The ingredients were dissolved and the final volume was adjusted to 1,000 ml with deionized water. Then, the pH was adjusted to 9.0 with 1 N HCl and 1N NaCl. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches, after preparation.

1.3 RNAase (10 mg/ml)

RNAase	10.00	mg

The RNAase was dissolved in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl and stored at -20°C.

1.4 Saline-EDTA (0.15 M NaCl+o.1 M EDTA)

NaCl	8.76	g
EDTA ($C_{10}H_{14}N_2O_8Na_2.2H_2O$)	37.22	g

The ingredients were dissolved and the final volume was adjusted to 1000 ml with deionized water. Then, the pH was adjusted to 8.0 with 1 N HCl and 1N NaCl. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches, after preparation.

1.5 SDS (10% w/v)

Sodium dodecylsulphate	10.00	g

The ingredients were dissolved and adjusted the volume to 100 ml with deionized water.

A.1.6 Phenol:Chloroform (1:1, v/v)

Crystalline phenol was liquidified in water bath at 65°C and mixed with chloroform in the ratio of 1:1 (v/v). The solution was stored in a light tight bottle.

1.7 20×SSC (20× standard saline citrate)

NaCl	17.50	g
Sodium citrate	8.80	g

The ingredients were dissolved and the final volume was adjusted to 1000 ml with deionized water. Then, the pH was adjusted to 7.0 with 1 N HCl and 1 N NaCl. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches after preparation.

1.8 Tris-borate (TBE) buffer (5X)

Tris-base	54.00	g
Boric acid	27.50	g
EDTA ($C_{10}H_{14}N_2O_8Na_2.2H_2O$)	0.37	g

The ingredients were dissolved and adjusted the volume to 1,000 ml with deionized water.

1.9 Gel loading buffer (6X)

Bromophenol blue	25.00	g

The dye was dissolved and adjusted the volume to 10 ml with 40% sucrose in water.

1.10 Ethidium bromide (10 mg/ml)

Ethidium bromide	1.00	g

The chemical was dissolved and adjusted the volume to 10 ml with sterilized deionized water.

A.2 Reagents used for identification of lactic acid bacteria

2.1 Acetone alcohol

Alcohol (95%)	700.00	ml
Acetone	300.00	ml

2.2 Crystal violet (Gram stain)

Crystal violet	2.00	g
Ethanol	20.00	g
Mixed thoroughly		
Ammonium oxalate (1% Aqueous solution)	80.00	ml

2.3 Hydrogen peroxide (3% solution)

Hydrogen peroxide (30%)	10.00	ml
Distilled water	100.00	ml

2.4 Iodine solution (Gram's iodine)

Iodine	1.00	g
Potassium iodide	2.00	g
Added distilled water and brought volume up to	300.00	ml

2.5 Safranin (Gram stain)

Safranin O (2.5% solution in 95% Ethanol)	10.00	ml
Distilled water	90.00	ml

2.6 Tetramethyl-p-phenylenediamine dihydrochloride (1%)

Tetramethyl-p-phenylenediamine dihydrochloride	1.00	g
Distilled water	100.00	ml

2.7 Malachite green

Malachite green	5.00	g
Distilled water	100.00	ml

A.3 Culture media for lactic acid bacterium culturing and D-lactic acid production

3.1 MRS medium (De Man, Rogosa and Sharpe Medium)

Proteose peptone	10.00	g
Beef extract	8.00	g
Yeast extract	4.00	g
Polysorbate 80 ($(NH_4)_3C_6H_5O_7$)	1.00	g
tri-Ammonium citrate	2.00	g
Sodium acetate trihydrate ($CH_3COONa.3H_2O$)	5.00	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.H_2O$	0.05	g
K_2HPO_4	2.00	g
Dextose	20.00	g

Final pH 6.2 ± 0.2 at 25°C

The compositions were suspended in 1,000 ml of distilled water. Bring to

the boil until dissolved completely. For solid medium was obtained by adding 15 g/l agar. Sterilization was done by autoclaving for 15 min at 121°C.

3.2 MC agar

The components were similar with MRS agar containing 0.5% $CaCO_3$. The medium was autoclaved at 121°C for 15 min.

3.3 Rogosa agar medium with modification (RAM) (Rodtong and Ishizaki, 2003)

Pancreatic digest of casein or Tryptone	5.00	g
Potassium hydrogen phosphate (K_2HPO_4)	6.00	g
Yeast extract	3.00	g
tri-Ammonium citrate	1.00	g
$MgSO_4.7H_2O$	0.57	g
$MnSO_4.4H_2O$	0.12	g
$FeSO_4.7H_2O$	0.03	g
Cassava starch	10.00	g

Final pH 7.0 ± 0.2 at 25°C

The compositions were suspended in 1,000 ml of distilled water. Bring to the boil until dissolved completely. For solid medium was obtained by adding 15 g/l agar. Sterilization was done by autoclaving for 15 min at 121°C.

A.4 Culture media for identification of lactic acid bacteria

4.1 MRS agar added 1% soluble starch (modified from MRS medium; Atlas and Parks, 1997)

Proteose peptone	10.00	g
Beef extract	8.00	g
Yeast extract	4.00	g
Polysorbate 80 ($(NH_4)_3C_6H_5O_7$)	1.00	g
tri-Ammonium citrate ($C_6H_{17}N_3O_7$)	2.00	g
Sodium acetate trihydrate ($CH_3COONa.3H_2O$)	5.00	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.H_2O$	0.05	g
K_2HPO_4	2.00	g
Soluble starch	10.00	g
Agar	15.00	g

Final pH 6.2 ± 0.2 at 25°C

The compositions were suspended in 1,000 ml of distilled water. Bring to the boil until dissolved completely. Sterilization was done by autoclaving for 15 min at 121°C.

4.2 RAM agar added 1% soluble starch (modified from RAM medium; Rodtong and Ishizaki, 2003)

Pancreatic digest of casein or tryptone	5.00	g
Potassium hydrogen phosphate (K_2HPO_4)	6.00	g

Yeast extract	3.00	g
tri-Ammonium citrate ($C_6H_{17}N_3O_7$)	1.00	g
$MgSO_4.7H_2O$	0.57	g
$MnSO_4.4H_2O$	0.12	g
$FeSO_4.7H_2O$	0.03	g
Soluble starch	10.00	g
Agar	15.00	g

Final pH 7.0 ± 0.2 at 25°C

The compositions were suspended in 1,000 ml of distilled water. Bring to the boil until dissolved completely. Sterilization was done by autoclaving for 15 min at 121°C.

4.3 MRS agar added 1% skim milk (modified from MRS medium; Atlas and Parks, 1997)

Proteose peptone	10.00	g
Beef extract	8.00	g
Yeast extract	4.00	g
Polysorbate 80 ($(NH_4)_3C_6H_5O_7$)	1.00	g
tri-Ammonium citrate ($C_6H_{17}N_3O_7$)	2.00	g
Sodium acetate trihydrate ($CH_3COONa.3H_2O$)	5.00	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.H_2O$	0.05	g
K_2HPO_4	2.00	g
Skim milk	10.00	g

Agar	15.00	g

Final pH 6.2 ± 0.2 at 25°C

The compositions were suspended in 1,000 ml of distilled water. Bring to the boil until dissolved completely. Sterilization was done by autoclaving for 15 min at 121°C.

4.4 RAM agar added 1% skim milk (modified from MRS medium; Atlas and Parks, 1997)

Pancreatic digest of casein or tryptone	5.00	g
Potassium hydrogen phosphate (K_2HPO_4)	6.00	g
Yeast extract	3.00	g
tri-Ammonium citrate ($C_6H_{17}N_3O_7$)	1.00	g
$MgSO_4.7H_2O$	0.57	g
$MnSO_4.4H_2O$	0.12	g
$FeSO_4.7H_2O$	0.03	g
Skim milk	10.00	g
Agar	15.00	g

Final pH 7.0 ± 0.2 at 25°C

The compositions were suspended in 1,000 ml of distilled water. Bring to the boil until dissolved completely. Sterilization was done by autoclaving for 15 min at 121°C.

4.5 Motility test medium (modified from MRS medium; Atlas and Parks, 1997)

Proteose peptone	10.00	g
Beef extract	8.00	g
Yeast extract	4.00	g
Polysorbate 80 ($(NH_4)_3C_6H_5O_7$)	1.00	g
tri-Ammonium citrate ($C_6H_{17}N_3O_7$)	2.00	g
Sodium acetate trihydrate ($CH_3COONa.3H_2O$)	5.00	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.H_2O$	0.05	g
K_2HPO_4	2.00	g
Dextose	20.00	g
Agar	3.00	g

Final pH 6.2 ± 0.2 at 25°C

The compositions were suspended in 1,000 ml of distilled water. Bring to the boil until dissolved completely. Sterilization was done by autoclaving for 15 min at 121°C.

4.6 Motility test medium (modified from RAM medium; Rodtong and Ishizaki, 2003)

Pancreatic digest of casein or tryptone	5.00	g
Potassium hydrogen phosphate (K_2HPO_4)	6.00	g
Yeast extract	3.00	g
tri-Ammonium citrate ($C_6H_{17}N_3O_7$)	1.00	g

$MgSO_4.7H_2O$	0.57	g
$MnSO_4.4H_2O$	0.12	g
$FeSO_4.7H_2O$	0.03	g
Dextose	20.00	g
Agar	3.00	g

Final pH 7.0 ± 0.2 at 25°C

The compositions were suspended in 1,000 ml of distilled water. Bring to the boil until dissolved completely. Sterilization was done by autoclaving for 15 min at 121°C.

4.7 Gelatin test medium (modified from MRS medium; Atlas and Parks, 1997)

Proteose peptone	10.00	g
Beef extract	8.00	g
Yeast extract	4.00	g
Polysorbate 80 ($(NH_4)_3C_6H_5O_7$)	1.00	g
tri-Ammonium citrate ($C_6H_{17}N_3O_7$)	2.00	g
Sodium acetate trihydrate ($CH_3COONa.3H_2O$)	5.00	g
$MgSO_4.7H_2O$	0.20	g
$MnSO_4.H_2O$	0.05	g
K_2HPO_4	2.00	g
Gelatin	96.00	g

Final pH 6.2 ± 0.2 at 25°C

The compositions were suspended in 1,000 ml of distilled water. Bring to the boil until dissolved completely. Sterilization was done by autoclaving for 15 min at 121°C.

4.8 Gelatin test medium (modified from RAM medium; Rodtong and Ishizaki, 2003)

Pancreatic digest of casein or tryptone	5.00	g
Potassium hydrogen phosphate (K_2HPO_4)	6.00	g
Yeast extract	3.00	g
tri-Ammonium citrate ($C_6H_{17}N_3O_7$)	1.00	g
$MgSO_4.7H_2O$	0.57	g
$MnSO_4.4H_2O$	0.12	g
$FeSO_4.7H_2O$	0.03	g
Gelatin	96.00	g

Final pH 7.0 ± 0.2 at 25°C

The compositions were suspended in 1,000 ml of distilled water. Bring to the boil until dissolved completely. Sterilization was done by autoclaving for 15 min at 121°C.

APPENDIX B

SCREENING OF ACID-PRODUCING ISOLATES

Table 1B Lactic acid production by lactic acid bacteria using MRS broth containing 2% of glucose when cultivated at 35°C for 24 h.

Bacterial isolate code	Gas from glucose	Growth (A_{600})	pH of cultured broth	Total acidity (%)[a]	Total lactic acid (g/l)	Lactic acid concentration (g/l)[b]		Optical purity (%)[c]		Isomer of lactic acid[d]
						D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	
LR4	Negative	0.754	3.24	1.12	12.82	5.85	6.96	45.68	54.32	DL
CWR2-3	Negative	0.412	3.84	0.50	7.08	2.88	4.21	40.61	59.39	DL
MYGYG MC9	Negative	0.811	4.21	1.77	24.25	15.36	8.89	63.36	36.64	DL
FLB 4	Negative	0.745	4.27	1.17	16.88	3.86	13.02	22.87	77.13	DL
M1-3-1	Negative	0.912	4.14	1.28	18.21	11.84	6.37	65.04	34.96	DL
M2D3-2	Negative	0.875	4.20	1.19	17.22	4.47	12.75	25.97	74.03	DL
FJPb MC 20	Negative	0.655	4.79	0.97	12.28	5.35	6.92	43.60	56.40	DL
R CZG6	Negative	0.211	5.64	0.32	11.34	5.03	6.31	44.37	55.62	DL
CWMR1-2	Positive	0.548	3.70	0.94	14.54	6.86	7.68	47.18	52.82	DL
CWMR2-1	Positive	0.621	3.78	0.90	13.84	3.45	10.39	24.93	75.07	DL

Table 1B (Continued) Lactic acid production by lactic acid bacteria using MRS broth containing 2% of glucose when cultivated at 35°C for 24 h.

Bacterial isolate code	Gas from glucose	Growth (A_{600})	pH of cultured broth	Total acidity (%)[a]	Total lactic acid (g/l)	Lactic acid concentration (g/l)[b]		Optical purity (%)[c]		Isomer of lactic acid[d]
						D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	
R CZG2	Negative	0.323	4.97	0.75	16.15	4.48	11.67	27.73	72.26	DL
R CZG5	Negative	0.465	4.78	0.68	11.07	3.62	7.46	32.65	67.35	DL
I4	Negative	0.652	4.64	0.87	19.32	5.05	14.27	26.13	73.87	DL
I9	Negative	0.605	4.73	0.89	8.51	4.23	4.28	49.71	50.29	DL
I1	Negative	0.625	4.68	0.93	16.06	4.56	11.49	28.43	71.57	DL
I13	Negative	0.753	4.68	0.94	18.96	7.65	11.30	40.36	59.64	DL
L10	Negative	0.701	4.38	0.82	15.13	4.28	10.86	28.25	71.75	DL
L12	Negative	0.797	4.36	0.85	16.69	4.48	12.22	26.81	73.19	DL
L5	Negative	0.731	4.30	0.93	16.21	4.13	12.07	25.51	74.49	DL
P9	Negative	0.722	4.28	0.92	11.07	3.07	8.00	27.70	72.30	DL
P10	Negative	0.620	4.34	0.96	16.64	4.54	12.10	27.29	72.71	DL
A1-1	Negative	0.675	4.59	0.87	14.78	4.37	10.42	29.53	70.47	DL
LC3	Positive	0.811	3.62	0.97	9.72	3.87	5.85	39.81	60.19	DL
CSR1-7	Positive	0.871	3.57	0.87	8.70	3.35	5.35	38.51	61.49	DL
CAMC4	Positive	0.561	3.73	0.90	14.45	7.84	6.60	54.26	45.74	DL
CAMC3	Positive	0.418	3.74	1.00	14.23	5.85	8.39	41.11	58.89	DL
CAMC5	Positive	0.634	3.72	0.92	13.24	8.27	4.97	62.46	37.54	DL

Table 1B (Continued) Lactic acid production by lactic acid bacteria using MRS broth containing 2% of glucose when cultivated at 35°C for 24 h.

Bacterial isolate code	Gas from glucose	Growth (A_{600})	pH of cultured broth	Total acidity (%)[a]	Total lactic acid (g/l)	Lactic acid concentration (g/l)[b]		Optical purity (%)[c]		Isomer of lactic acid[d]
						D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	
A1-2	Negative	0.612	4.57	0.91	14.23	3.91	10.33	27.45	72.55	DL
A2	Negative	0.542	4.62	0.85	17.32	4.55	12.77	26.25	73.75	DL
G4-1	Negative	0.903	4.59	1.06	16.37	4.23	12.14	25.86	74.14	DL
G3	Negative	0.912	4.54	1.00	17.93	4.91	13.02	27.39	72.62	DL
P12	Negative	0.766	4.70	0.87	16.02	4.33	11.69	27.01	72.99	DL
G4-2	Negative	0.642	4.65	0.92	15.48	4.06	11.42	26.20	73.80	DL
LP8	Negative	0.874	4.65	0.93	16.63	4.67	11.96	28.09	71.91	DL
CWR1-6	Negative	0.512	4.33	0.73	7.33	2.05	5.28	28.00	72.00	DL
LO10	Negative	0.749	4.73	0.86	15.57	4.39	11.17	28.21	71.78	DL
LP10	Negative	0.910	3.21	1.12	13.01	5.70	7.30	43.84	56.16	DL
CAR2	Negative	0.880	3.57	0.81	13.95	6.35	7.60	45.50	54.50	DL
CSR1-4	Negative	0.453	4.59	0.57	5.72	1.76	3.95	30.88	69.12	DL
CSR1-6	Negative	0.208	4.22	0.47	5.58	2.60	2.99	46.51	53.49	DL
CWMR2-2	Positive	0.517	3.67	0.95	14.58	6.86	7.71	47.05	52.95	DL
CWMC2-2	Positive	0.509	3.72	0.90	13.94	6.34	7.60	45.49	54.52	DL
CWMC2-1	Positive	0.452	3.71	0.92	14.69	6.65	8.04	45.27	54.73	DL
CWM2-1	Positive	0.611	3.69	1.00	14.75	7.08	7.68	48.00	52.00	DL

Table 1B (Continued) Lactic acid production by lactic acid bacteria using MRS broth containing 2% of glucose when cultivated at 35°C for 24 h.

Bacterial isolate code	Gas from glucose	Growth (A_{600})	pH of cultured broth	Total acidity (%)[a]	Total lactic acid (g/l)	Lactic acid concentration (g/l)[b]		Optical purity (%)[c]		Isomer of lactic acid[d]
						D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	
CAR5	Negative	0.294	4.26	0.40	5.75	2.43	3.33	42.20	57.80	DL
CWR1-2	Negative	0.239	4.68	0.30	4.37	2.03	2.34	46.42	53.58	DL
CWR1-1	Negative	0.277	4.24	0.50	7.52	2.64	4.88	35.13	64.87	DL
FLB36	Negative	0.912	4.23	1.20	17.55	4.38	13.17	24.96	75.04	DL
FJPb1	Negative	0.768	4.53	0.85	12.22	0.00	12.22	0.00	100.00	L
M2D3-2	Negative	0.779	4.20	1.19	17.22	4.47	12.75	25.97	74.03	DL
I2	Negative	0.749	4.75	0.84	17.43	4.21	13.22	24.15	75.85	DL
L11	Negative	0.532	4.36	0.85	14.87	3.06	11.81	20.59	79.50	DL
A1-3	Negative	0.502	4.56	0.85	15.01	3.74	11.27	24.93	75.07	DL
G5	Negative	0.413	4.73	8.01	12.19	2.98	9.22	24.40	75.59	DL
J4	Negative	0.547	4.50	0.85	14.06	3.17	10.90	22.53	77.47	DL
CWR1-4	Negative	0.281	3.84	0.52	4.43	0.00	4.43	0.00	100.00	L
CAR8	Negative	0.587	3.43	1.15	15.80	0.00	15.80	0.00	100.00	L
CSR2-3	Negative	0.258	5.15	0.20	6.16	0.00	0.20	0.00	100.00	L
CWM1-2	Positive	0.566	3.72	0.90	14.25	7.15	7.10	50.18	49.82	DL
CAMR8	Positive	0.741	3.64	1.00	13.51	6.40	7.11	47.38	52.62	DL
CWMR1-1	Positive	0.459	4.34	0.49	7.13	4.00	3.13	56.10	43.90	DL

Table 1B (Continued) Lactic acid production by lactic acid bacteria using MRS broth containing 2% of glucose when cultivated at 35°C for 24 h.

Bacterial isolate code	Gas from glucose	Growth (A_{600})	pH of cultured broth	Total acidity (%)[a]	Total lactic acid (g/l)	Lactic acid concentration (g/l)[b]		Optical purity (%)[c]		Isomer of lactic acid[d]
						D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	
CSR2-12	Negative	0.238	4.95	0.53	7.27	0.00	0.53	0.00	100.00	L
CSR2-14	Negative	0.246	5.21	0.21	6.04	0.00	0.21	0.00	100.00	L
CSR2-13	Negative	0.241	5.08	0.23	6.00	0.00	0.23	0.00	100.00	L
CSR2-16	Negative	0.246	5.11	0.20	6.21	0.00	0.20	0.00	100.00	L
CAR9	Negative	0.544	3.77	1.15	15.46	0.00	1.15	0.00	100.00	L
CAR13	Negative	0.948	3.54	1.19	19.91	0.00	1.19	0.00	100.00	L
CWR2-15	Negative	0.249	5.26	0.23	3.68	0.00	0.23	0.00	100.00	L
CAR10	Negative	0.827	3.83	0.85	11.96	0.04	11.91	0.33	99.67	L
CAMR1	Negative	0.815	3.53	1.12	15.69	0.06	15.53	0.38	99.62	L
CAMR5	Negative	0.868	3.59	1.06	15.47	0.29	15.18	1.87	98.13	L
CAMC2	Negative	0.806	3.65	0.99	14.80	0.00	14.80	0.00	100.00	L
CWMC1-2	Negative	0.805	3.62	1.03	16.28	0.78	15.49	4.79	95.21	L
CAM4	Negative	0.811	3.54	1.17	16.40	0.09	16.30	0.55	99.45	L
CAMR4	Positive	0.483	3.68	0.94	14.19	6.96	7.23	49.05	50.95	DL
CAMC1	Negative	0.913	3.30	1.58	11.47	7.69	3.78	67.04	32.96	DL
CAMR2-1	Negative	0.931	3.32	1.66	11.39	7.55	3.84	66.29	33.71	DL
R CZT1	Negative	0.213	5.09	0.21	3.99	2.08	1.92	52.02	47.98	DL

Table 1B (Continued) Lactic acid production by lactic acid bacteria using MRS broth containing 2% of glucose when cultivated at 35°C for 24 h.

Bacterial isolate code	Gas from glucose	Growth (A_{600})	pH of cultured broth	Total acidity (%)[a]	Total lactic acid (g/l)	Lactic acid concentration (g/l)[b]		Optical purity (%)[c]		Isomer of lactic acid[d]
						D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	
CAM3	Negative	0.745	3.44	1.37	18.99	0.15	18.84	0.79	99.21	L
CWMC1-1	Negative	0.264	4.37	0.59	4.14	0.00	4.14	0.00	100.00	L
CAR11	Negative	0.441	3.77	0.85	12.88	0.40	12.48	3.14	96.86	L
CAR12	Negative	0.485	3.62	1.03	16.48	0.75	15.72	4.55	95.45	L
CAMR6	Negative	0.426	3.90	0.81	11.08	0.00	11.08	0.00	100.00	L
CAMC10	Negative	0.621	3.46	1.28	16.99	0.33	16.66	1.94	98.06	L
CAMC9	Negative	0.557	3.51	1.29	13.50	0.00	13.50	0.00	100.00	L
CAM7	Negative	0.625	3.41	1.49	19.26	0.00	19.26	0.00	100.00	L
CAM8	Negative	0.521	3.54	1.16	15.89	0.16	15.73	1.01	98.99	L
CAM9	Negative	0.522	3.54	1.17	15.58	0.05	15.53	0.32	99.68	L
CWM1-5	Negative	0.122	5.57	0.20	4.30	0.12	4.19	2.79	97.21	L
RO NZT5	Negative	0.367	3.74	0.71	13.24	2.00	11.24	15.09	84.91	L
MRS NZF 10-2-1	Negative	0.504	3.33	0.72	12.69	1.45	11.23	11.45	88.55	L
CAMR3	Positive	0.633	3.73	0.92	13.76	6.31	7.46	45.86	54.14	DL
CAR14	Negative	0.935	3.67	0.91	16.13	6.60	9.53	40.92	59.08	DL
CAMR2-2	Negative	0.833	3.32	1.57	12.22	7.90	4.32	64.65	35.35	DL

Table 1B (Continued) Lactic acid production by lactic acid bacteria using MRS broth containing 2% of glucose when cultivated at 35°C for 24 h.

Bacterial isolate code	Gas from glucose	Growth (A_{600})	pH of cultured broth	Total acidity (%)[a]	Total lactic acid (g/l)	Lactic acid concentration (g/l)[b]		Optical purity (%)[c]		Isomer of lactic acid[d]
						D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	
R CZC1	Negative	0.202	4.00	0.33	6.70	1.25	5.45	18.69	81.31	L
R CZM1	Negative	0.231	3.78	0.47	7.27	1.45	5.83	19.90	80.10	L
LB2	Negative	0.505	4.38	0.65	6.78	0.00	6.78	0.00	100.00	L
MRS NZF 10	Negative	0.412	3.32	0.72	11.16	0.00	11.16	0.00	100.00	L
MRS NZT 20	Negative	0.441	3.52	0.74	14.06	2.12	11.95	15.06	84.94	L
CSR1-3	Negative	0.485	4.06	0.48	8.03	0.00	8.03	0.00	100.00	L
CAR3	Negative	0.501	3.75	0.58	9.86	0.00	9.86	0.00	100.00	L
CAR4	Negative	0.751	3.45	0.83	13.80	0.00	13.80	0.00	100.00	L
CAR6	Negative	0.497	3.87	0.59	10.33	0.00	10.33	0.00	100.00	L
CAR7	Negative	0.926	3.51	1.12	11.61	1.67	9.94	14.38	85.62	DL
LL4	Negative	0.564	4.42	0.76	7.62	1.46	6.16	19.18	80.82	DL
CSR2-1	Negative	0.522	4.69	0.77	7.70	1.44	6.26	18.70	81.30	DL
MRS NZF 2	Positive	0.245	4.16	0.42	8.50	2.38	6.12	28.00	72.00	DL
CWMR1-3	Negative	0.811	3.38	1.51	10.67	5.28	5.39	49.48	50.52	DL
CWR2-1	Positive	0.524	3.13	0.84	13.62	5.76	7.86	42.32	57.68	DL
R CZMU3	Positive	0.286	3.93	0.42	6.38	1.42	4.96	22.30	77.73	DL
LB5	Positive	0.223	3.64	0.76	7.68	2.60	5.08	33.86	66.14	DL

Table 1B (Continued) Lactic acid production by lactic acid bacteria using MRS broth containing 2% of glucose when cultivated at 35°C for 24 h.

Bacterial isolate code	Gas from glucose	Growth (A_{600})	pH of cultured broth	Total acidity (%)[a]	Total lactic acid (g/l)	Lactic acid concentration (g/l)[b]		Optical purity (%)[c]		Isomer of lactic acid[d]
						D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	
LF5	Positive	0.744	3.47	0.74	11.30	3.95	7.36	34.91	65.09	DL
MRS NZF 10-2-2	Positive	0.625	3.49	0.66	11.09	1.71	9.37	15.47	84.53	DL
CWR2-10	Positive	0.871	3.84	0.87	14.57	6.44	8.13	44.2	55.80	DL

[a] Titration method (AOAC International, 2000).
[b] HPLC analysis (Tanaka *et al*., 2007).
[c] Optical purity of D-lactic acid = (1-(L-lactic acid / total lactic acid)) × 100.
[d] D or L, the isomer recorded makes up 90% or more of total lactic acid; DL, 25-75% of total lactic acid are of the L-configuration.

Table 2B Lactic acid production by lactic acid bacteria using RAM broth containing 2% of glucose when cultivated at 35°C for 24 h.

Bacterial isolate code	Gas from glucose	Growth (A_{600})	pH of cultured broth	Total acidity (%)[a]	Total lactic acid (g/l)	Lactic acid concentration (g/l)[b]		Optical purity (%)[c]		Isomer of lactic acid[d]
						D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	
CWR1-5	Negative	0.188	3.64	0.188	0.675	3.50	3.00	53.85	46.15	DL
CWR1-10	Negative	0.191	3.64	0.191	0.693	3.50	3.50	50.00	50.00	DL
CWR1-11	Negative	0.109	3.88	0.109	0.612	0.00	4.50	0.00	100.00	L
CWR1-13	Negative	0.110	3.56	0.110	0.621	2.50	3.00	45.45	54.55	DL
CWR1-17	Negative	0.149	3.45	0.149	0.666	4.00	3.00	57.14	42.86	DL
CWR1-20	Negative	0.107	3.79	0.107	0.504	0.00	4.00	0.00	100.00	L
CWR2-1	Negative	0.066	5.07	0.066	0.342	0.00	1.00	0.00	100.00	L
CWR2-3	Negative	0.069	4.71	0.069	0.342	0.00	1.50	0.00	100.00	L
CWR2-4	Negative	0.087	5.04	0.087	0.306	0.00	0.50	0.00	100.00	L
CWR2-8	Negative	0.053	4.89	0.053	0.306	0.00	1.00	0.00	100.00	L
CWR2-11	Negative	0.064	5.02	0.064	0.243	0.00	1.00	0.00	100.00	L
CWR2-13	Negative	0.055	5.04	0.055	0.297	0.00	0.50	0.00	100.00	L
CWR2-13	Negative	0.055	5.04	0.055	0.297	0.00	0.50	0.00	100.00	L
CCR2	Negative	0.019	5.01	0.019	0.306	0.00	1.00	0.00	100.00	L
CCR10	Negative	0.053	4.79	0.252	1.50	1.00	0.50	66.67	33.33	DL
RFR25	Negative	0.104	4.42	0.360	2.50	1.50	1.00	60.00	40.00	DL
RFR27	Negative	0.094	4.45	0.306	3.50	0.00	3.50	0.00	100.00	L
CAR5-2	Negative	0.177	3.61	0.576	6.00	0.00	6.00	0.00	100.00	L
CWR8-11	Negative	0.125	4.71	0.369	3.00	0.00	3.00	0.00	100.00	L

Table 2B (Continued) Lactic acid production by lactic acid bacteria using RAM broth containing 2% of glucose when cultivated at 35°C for 24 h.

Bacterial isolate code	Gas from glucose	Growth (A_{600})	pH of cultured broth	Growth (A600)	Total acidity (%)[a]	Total lactic acid (g/l)[b]	Lactic acid concentration (g/l)[b]		Optical purity (%)[c]		Isomer of lactic acid[d]
							D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	
CWR1-5	Negative	0.188	3.64	0.188	0.675	6.50	3.50	3.00	53.85	46.15	DL
CWR1-10	Negative	0.191	3.64	0.191	0.693	7.00	3.50	3.50	50.00	50.00	DL
CWR1-11	Negative	0.109	3.88	0.109	0.612	4.50	0.00	4.50	0.00	100.00	L
CWR1-13	Negative	0.110	3.56	0.110	0.621	5.50	2.50	3.00	45.45	54.55	DL
CWR1-17	Negative	0.149	3.45	0.149	0.666	7.00	4.00	3.00	57.14	42.86	DL
CWR1-20	Negative	0.107	3.79	0.107	0.504	4.00	0.00	4.00	0.00	100.00	L
CWR2-1	Negative	0.066	5.07	0.066	0.342	1.00	0.00	1.00	0.00	100.00	L
CWR2-3	Negative	0.069	4.71	0.069	0.342	1.50	0.00	1.50	0.00	100.00	L
CWR2-4	Negative	0.087	5.04	0.087	0.306	0.50	0.00	0.50	0.00	100.00	L
CWR2-8	Negative	0.053	4.89	0.053	0.306	1.00	0.00	1.00	0.00	100.00	L
CWR2-11	Negative	0.064	5.02	0.064	0.243	1.00	0.00	1.00	0.00	100.00	L
CWR9-1	Negative	0.539	2.92	0.539	1.071	15.00	9.00	6.00	60.00	40.00	DL
CWR9-2	Negative	0.496	2.92	0.496	1.188	16.00	9.50	6.50	59.38	40.63	DL
CWR9-4	Negative	0.542	3.01	0.542	1.098	14.50	9.00	5.50	62.07	37.93	DL
CWR9-7	Negative	0.058	3.01	0.058	0.954	13.50	8.00	5.50	59.26	40.74	DL
CAR10-2	Negative	0.365	2.96	0.365	1.134	16.50	9.50	7.00	57.58	42.42	DL
CAR10-3	Negative	0.680	2.88	0.680	1.152	16.00	9.50	6.50	59.38	40.63	DL

Table 2B (Continued Lactic acid production by lactic acid bacteria using RAM broth containing 2% of glucose when cultivated at 35°C for 24 h.

Bacterial isolate code	Gas from glucose	Growth (A_{600})	pH of cultured broth	Growth (A600)	Total acidity (%)[a]	Total lactic acid (g/l)[b]	Lactic acid concentration (g/l)[b]		Optical purity (%)[c]		Isomer of lactic acid[d]
							D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	
CWR2-18	Negative	0.048	5.10	0.048	0.306	1.00	0.00	1.00	0.00	100.00	L
CWR2-20	Negative	0.074	4.97	0.074	0.333	1.00	0.00	1.00	0.00	100.00	L
CWR2-25	Negative	0.085	4.88	0.085	0.333	1.50	0.00	1.50	0.00	100.00	L
CWR2-28	Negative	0.184	3.46	0.184	0.729	6.50	4.50	2.00	69.23	30.77	DL
CWR3-10	Negative	0.199	3.59	0.199	0.540	5.00	0.00	5.00	0.00	100.00	L
CWR3-11	Negative	0.153	3.69	0.153	0.594	5.50	0.00	5.50	0.00	100.00	L
CWR4-5	Negative	0.083	4.76	0.083	0.306	3.00	0.00	3.00	0.00	100.00	L
CWR4-14	Negative	0.161	3.78	0.161	0.522	5.00	0.00	5.00	0.00	100.00	L
CWR4-18	Negative	0.206	4.02	0.206	0.522	4.00	0.00	4.00	0.00	100.00	L
CWR4-21	Negative	0.141	3.95	0.141	0.504	4.50	0.00	4.50	0.00	100.00	L
CWR4-22	Negative	0.048	3.88	0.048	0.522	0.50	0.00	0.50	0.00	100.00	L
CAR10-4	Negative	0.579	2.89	0.579	1.188	15.00	9.00	6.00	60.00	40.00	DL
CAR26	Negative	0.268	3.50	0.268	0.936	15.50	1.50	14.00	9.68	90.32	L
CAR28	Negative	0.186	3.66	0.186	0.684	9.00	1.00	8.00	11.11	88.89	L
CAR29	Negative	0.269	3.20	0.269	0.792	13.50	1.00	12.50	7.41	92.59	L
CAR34	Negative	0.152	3.17	0.152	0.774	11.00	1.00	10.00	9.09	90.91	L

Table 2B (Continued) Lactic acid production by lactic acid bacteria using RAM broth containing 2% of glucose when cultivated at 35°C for 24 h.

Bacterial isolate code	Gas from glucose	Growth (A_{600})	pH of cultured broth	Growth (A600)	Total acidity (%)[a]	Total lactic acid (g/l)[b]	Lactic acid concentration (g/l)[b]		Optical purity (%)[c]		Isomer of lactic acid[d]
							D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	
CAR5-9	Negative	0.095	3.69	0.095	0.531	6.00	0.00	6.00	0.00	100.00	L
CAR5-11	Negative	0.122	3.63	0.122	0.693	6.50	3.50	3.00	53.85	46.15	DL
CAR5-20	Negative	0.017	3.57	0.017	0.684	8.50	4.50	4.00	52.94	47.06	DL
CAR5-22	Negative	0.087	3.75	0.087	0.666	7.50	4.00	3.50	53.33	46.67	DL
CAR5-29	Negative	0.107	3.18	0.107	1.044	14.50	9.00	5.50	62.07	37.93	DL
CAR5-32	Negative	0.112	3.98	0.112	0.711	8.00	4.00	4.00	50.00	50.00	DL
CAR5-35	Negative	0.168	3.55	0.168	0.729	6.00	3.00	3.00	50.00	50.00	DL
CAR8-1	Negative	0.199	3.97	0.199	0.441	4.50	2.50	2.00	55.56	44.44	DL
CAR8-5	Negative	0.395	3.18	0.395	0.810	10.00	5.50	4.50	55.00	45.00	DL
CAR8-7	Negative	0.310	3.20	0.310	0.738	10.50	6.00	4.50	57.14	42.86	DL
CWR8-10	Negative	0.266	3.94	0.266	0.522	4.00	2.50	1.50	62.50	37.50	DL

[a] Titration method (AOAC International, 2000).
[b] HPLC analysis (Tanaka *et al*., 2007).
[c] % Optical purity of D-lactic acid = (1-(L-lactic acid / total lactic acid)) × 100.
[d] D or L, the isomer recorded makes up 90% or more of total lactic acid; DL, 25-75% of total lactic acid are of the L-configuration.

APPENDIX C

STANDARD CURVES

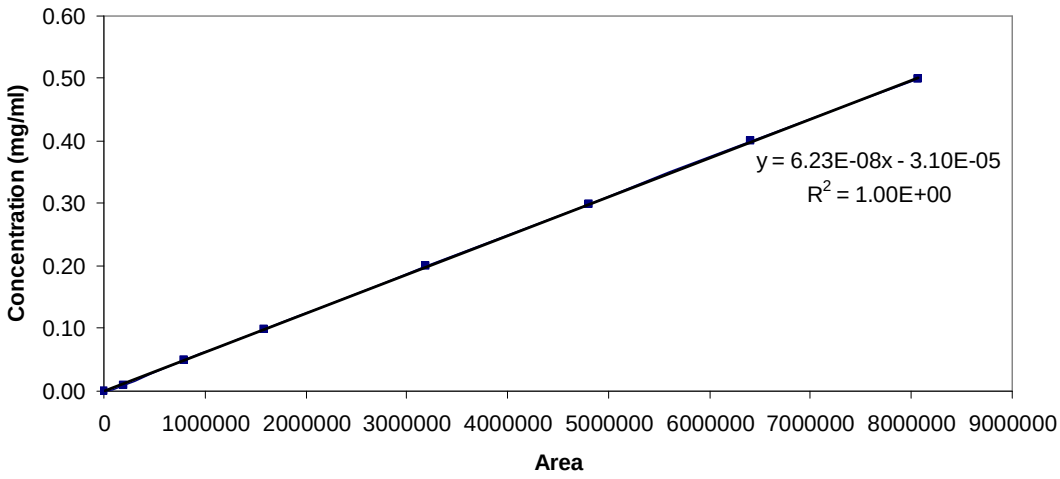

Figure 1C Standard curve of L-lactic acid using Chiral Astec CLC-L column with 0.005 M $CuSO_4$ mobile phase and UV detector at 254 nm.

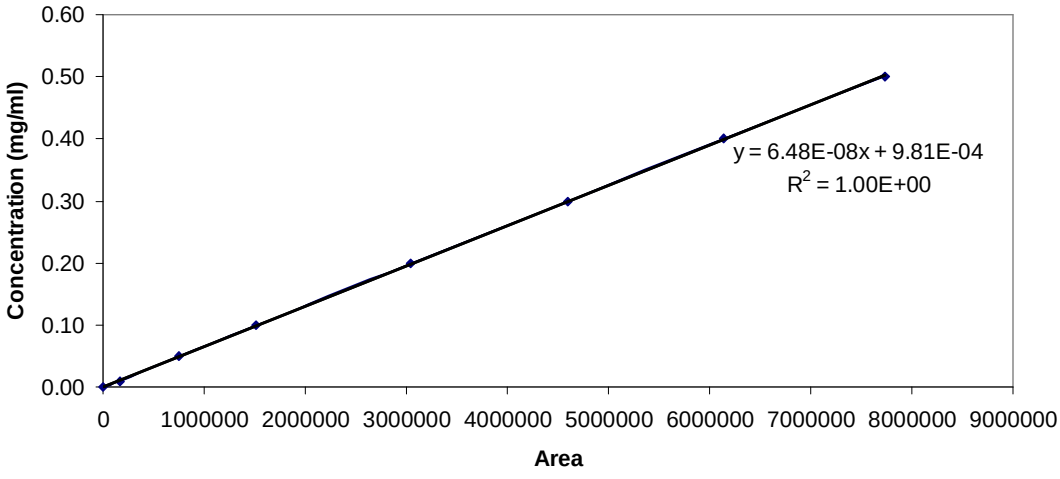

Figure 2C Standard curve of D-lactic acid using Chiral Astec CLC-L column with 0.005 M $CuSO_4$ mobile phase and UV detector at 254 nm.

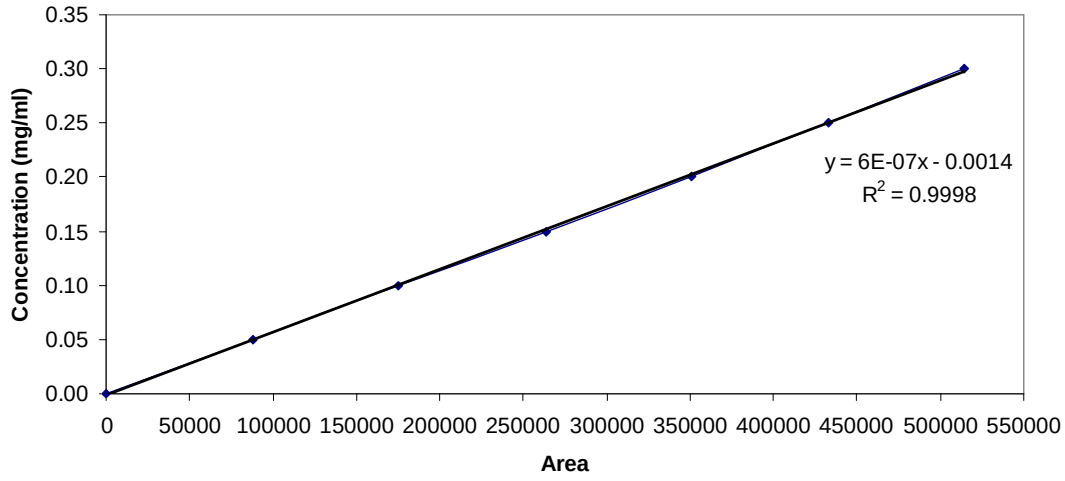

Figure 3C Standard curve of formic acid using Chiral Astec CLC-L column with 0.005 M $CuSO_4$ mobile phase and UV detector at 254 nm.

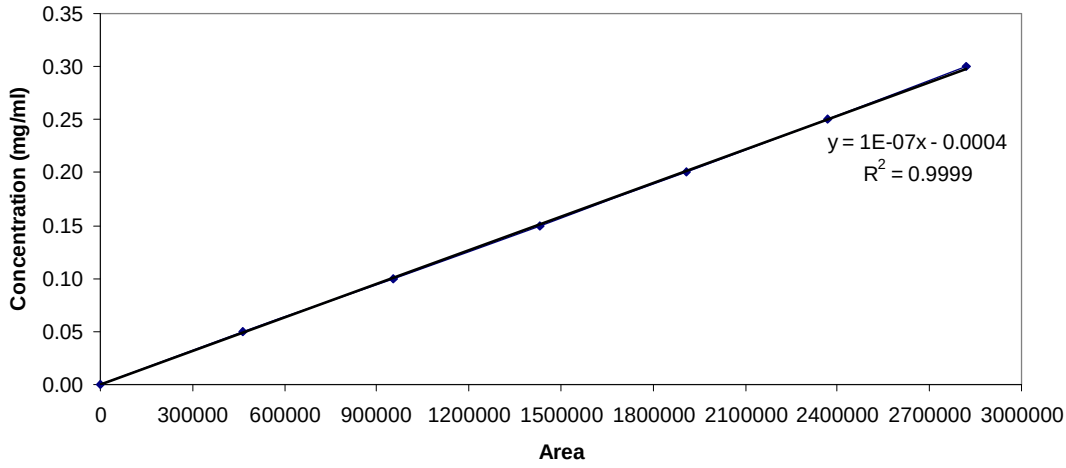

Figure 4C Standard curve of acetic acid using Chiral Astec CLC-L column with 0.005 M $CuSO_4$ mobile phase and UV detector at 254 nm.

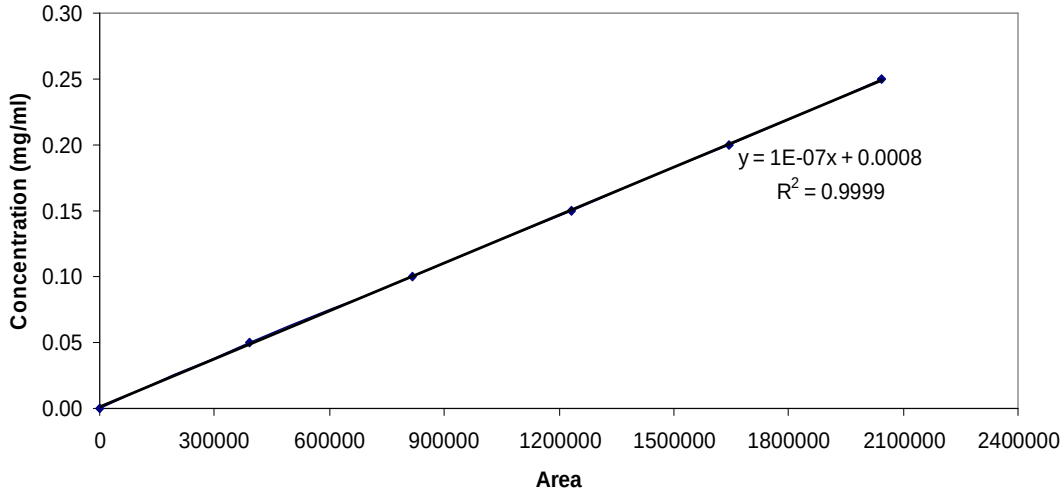

Figure 5C Standard curve of propionic acid using Chiral Astec CLC-L column with 0.005 M $CuSO_4$ mobile phase and UV detector at 254 nm.

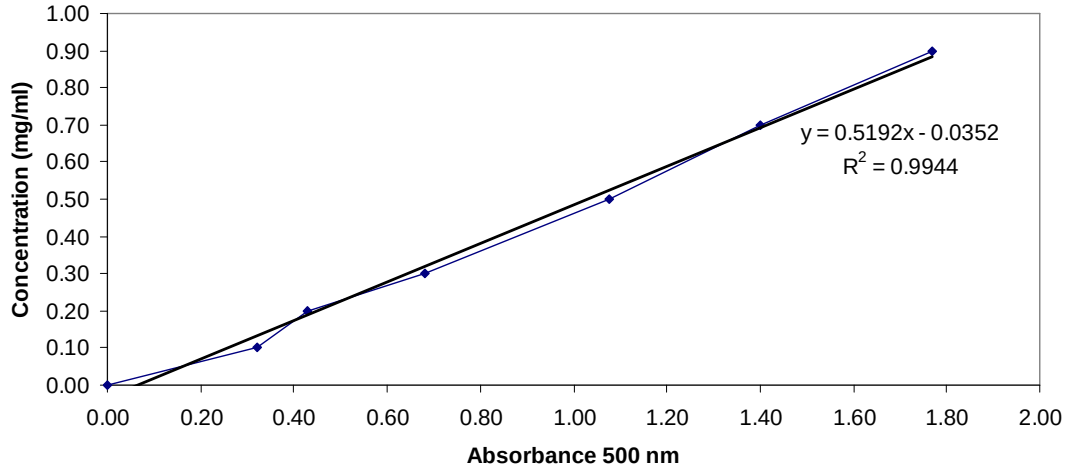

Figure 6C Standard curve of glucose according to phenol-sulfuric acid method.

APPENDIX D

HPLC CHROMATOGRAMS

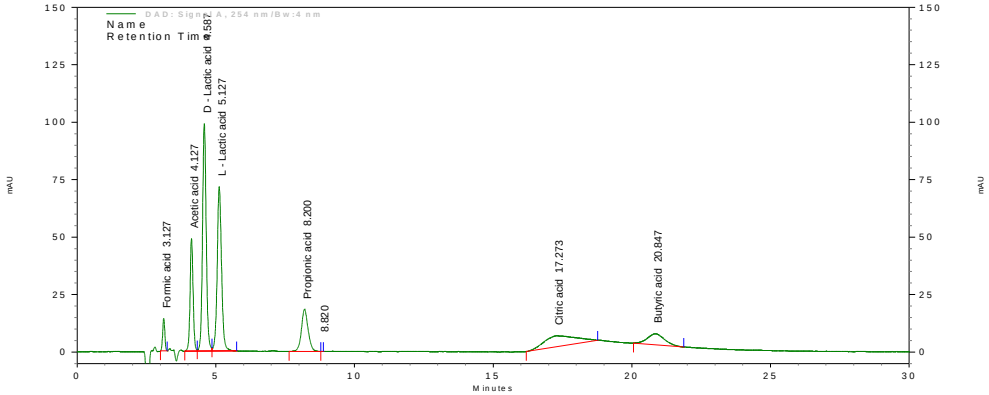

Figure 1D HPLC chromatogram of standards formic acid, acetic acid, D-lactic acid, L-lactic acid, propionic acid, citric acid, and butyric acid (0.1 mg/ml of each acids) using Chiral Astec CLC-L column with a 0.005 M $CuSo_4$ mobile phase and UV detector at 254 nm.

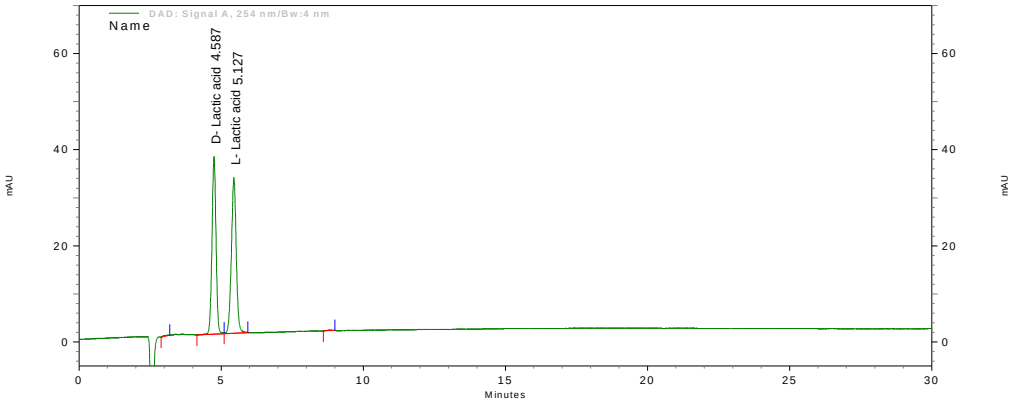

Figure 2D HPLC chromatogram of standards D-lactic acid and L-lactic acid (0.05 mg/ml) using Chiral Astec CLC-L column with a 0.005 M $CuSo_4$ mobile phase and UV detector at 254 nm.

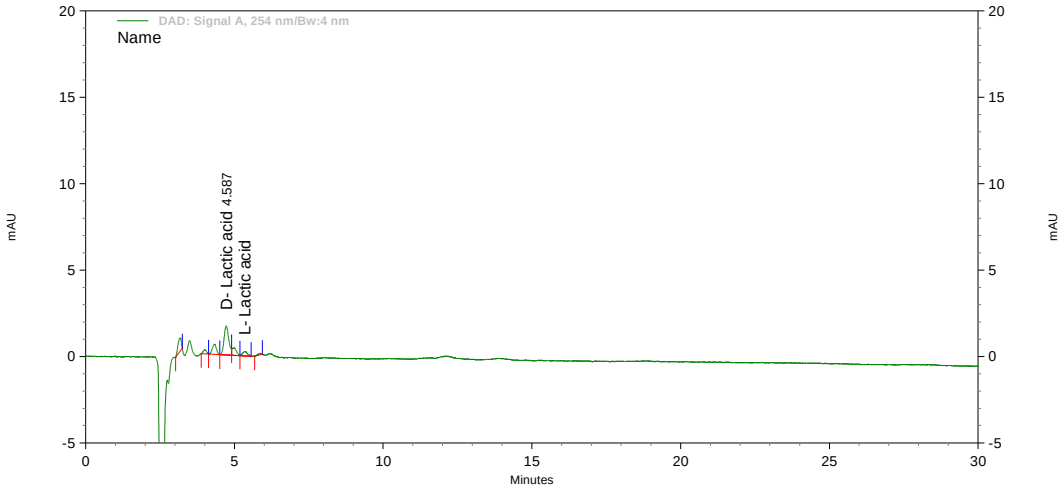

Figure 3D HPLC Chromatogram of D-lactic acid in RAM broth containing 2% glucose after cultivation of isolate WR73 under anaerobic conditions at 35°C for 24 h.

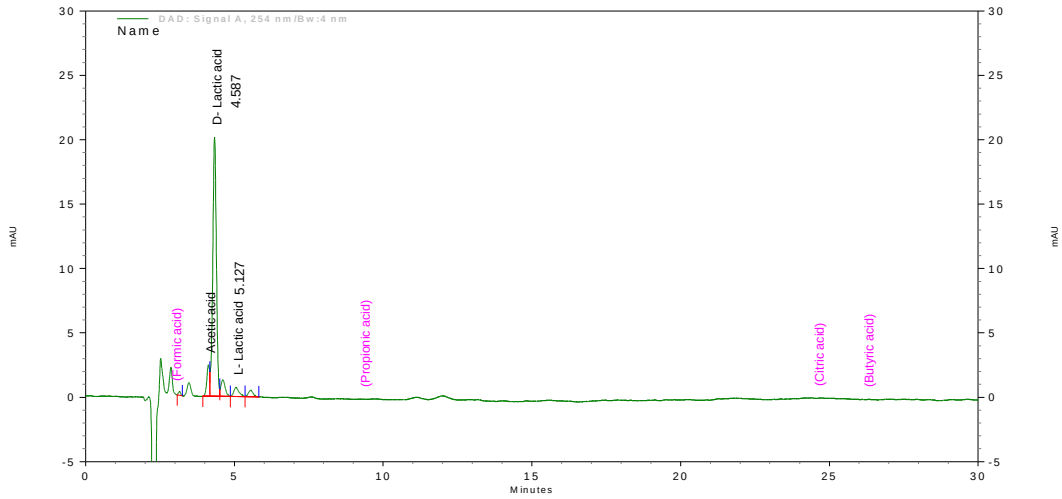

Figure 4D HPLC Chromatogram of D-lactic acid in MRS broth containing 2% glucose after cultivation of isolate CWR2-16 under anaerobic conditions at 35°C for 24 h.

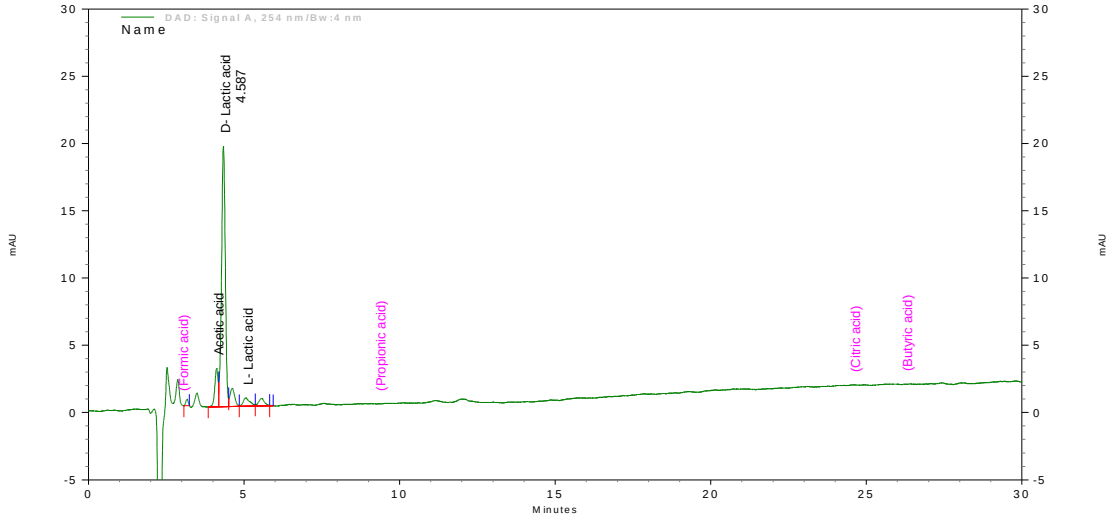

Figure 5D HPLC Chromatogram of D-lactic acid in MRS broth containing 2% glucose after cultivation of isolate CWMC2-5 under anaerobic conditions at 35°C for 24 h.

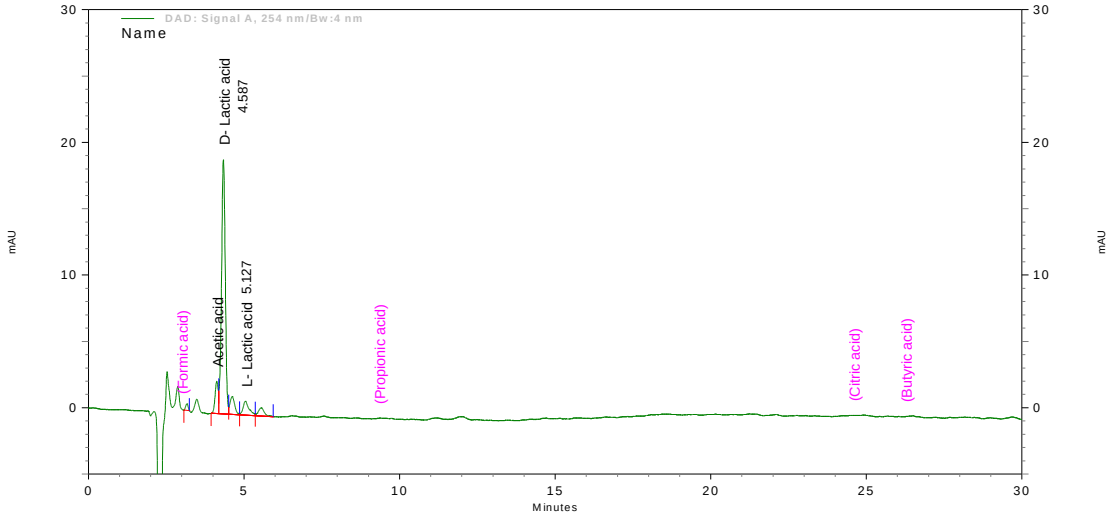

Figure 6D HPLC Chromatogram of D-lactic acid in MRS broth containing 2% glucose after cultivation of isolate CWMC1-3 under anaerobic conditions at 35°C for 24 h.

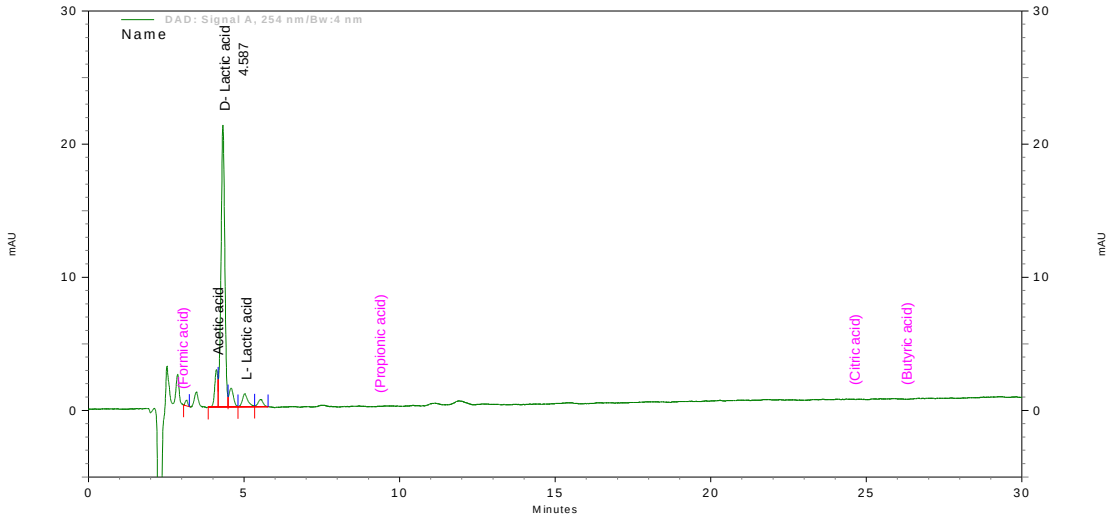

Figure 7D HPLC Chromatogram of D-lactic acid in MRS broth containing 2% glucose after cultivation of isolate CWMR1-5 under anaerobic conditions at 35°C for 24 h.

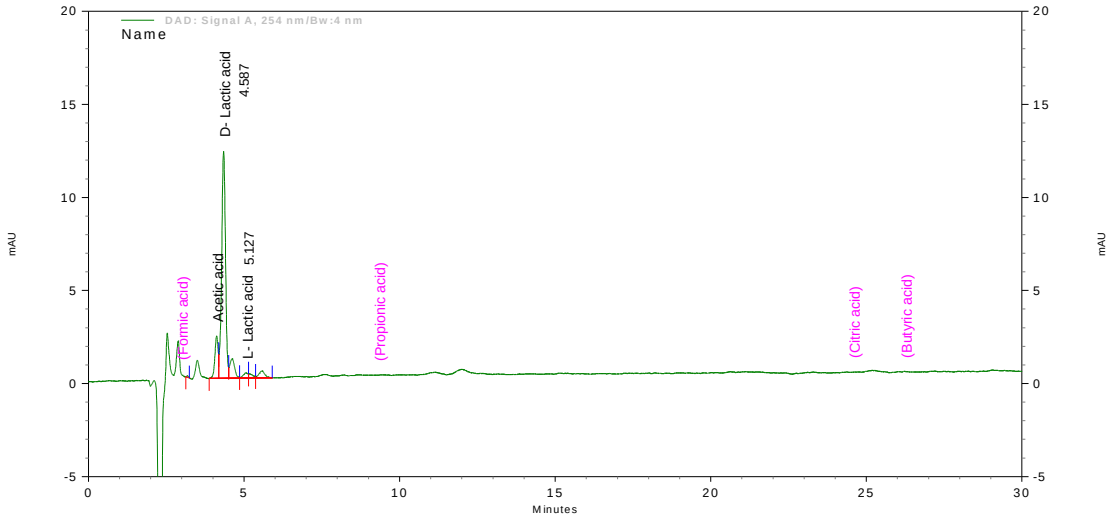

Figure 8D HPLC Chromatogram of D-lactic acid in MRS broth containing 2% glucose after cultivation of isolate LF1 under anaerobic conditions at 35°C for 24 h.

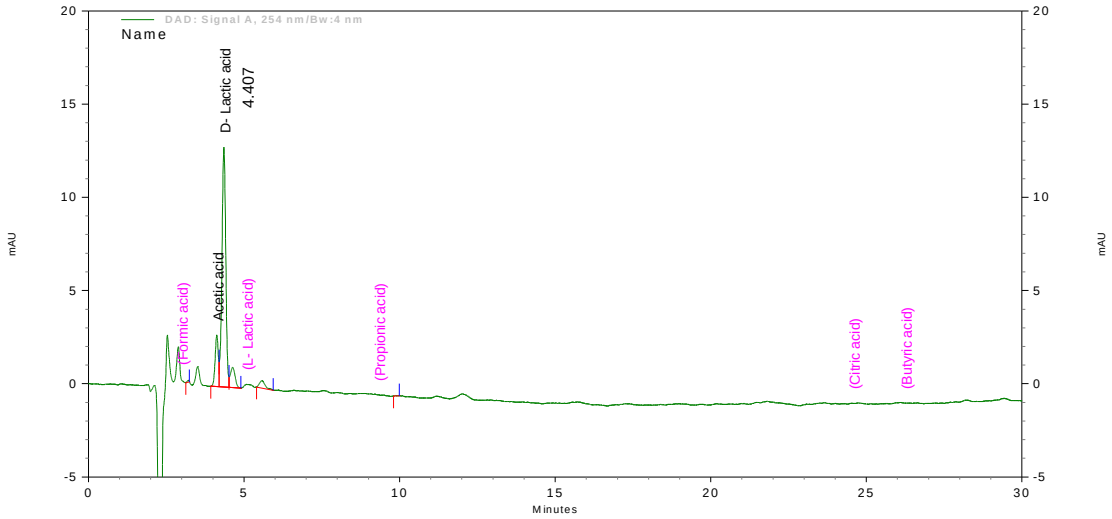

Figure 9D HPLC Chromatogram of D-lactic acid in MRS broth containing 2% glucose after cultivation of isolate PSMS1-5 under anaerobic conditions at 35°C for 24 h.

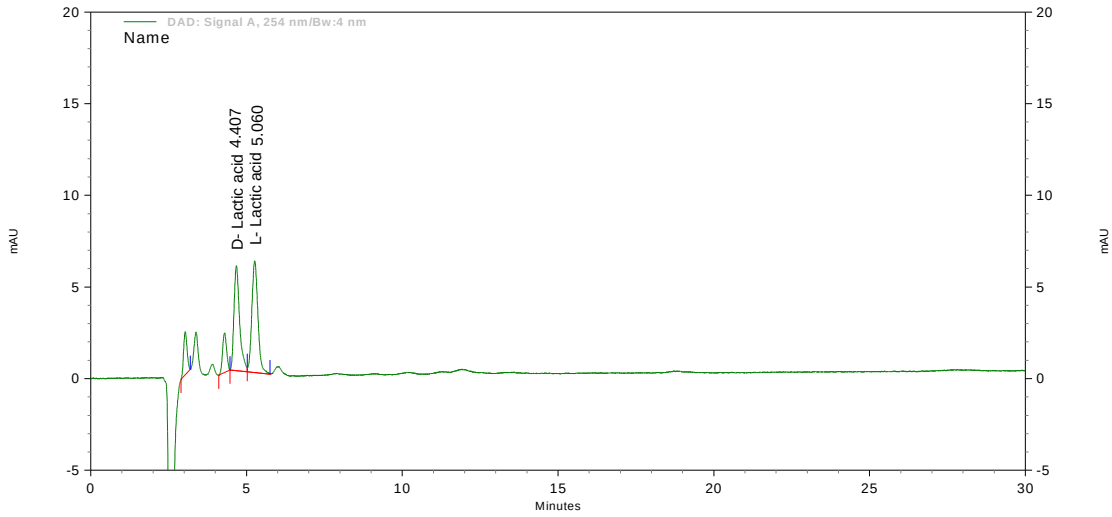

Figure 10D HPLC Chromatogram of DL-lactic acid in MRS broth containing 2% glucose after cultivation of isolate CWMR2-2 under anaerobic conditions at 35°C for 24 h.

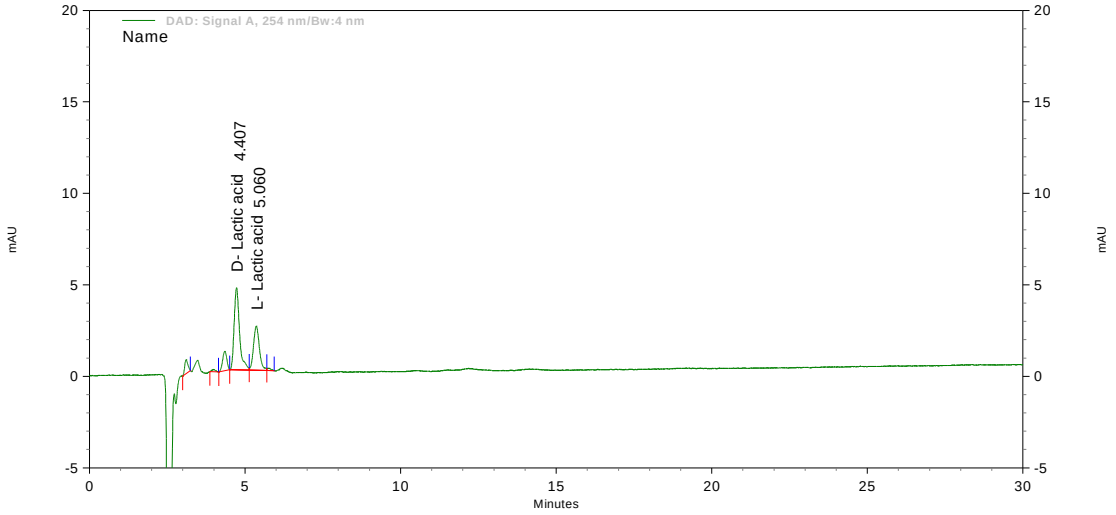

Figure 11D HPLC Chromatogram of DL-lactic acid in RAM broth containing 2% glucose after cultivation of isolate CWR2-28 under anaerobic conditions at 35°C for 24 h.

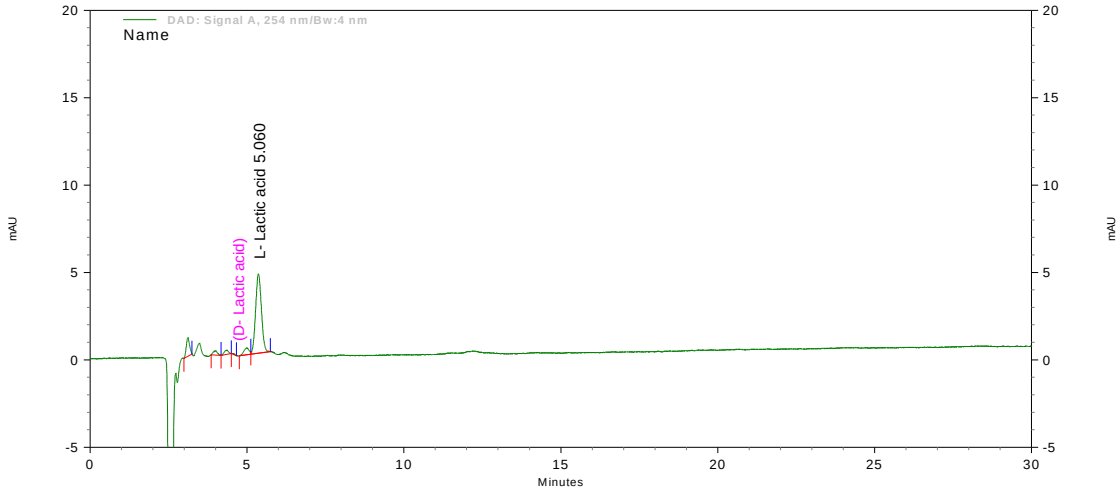

Figure 12D HPLC Chromatogram of L-lactic acid in RAM broth containing 2% glucose after cultivation of isolate CWR1-20 under anaerobic conditions at 35°C for 24 h.

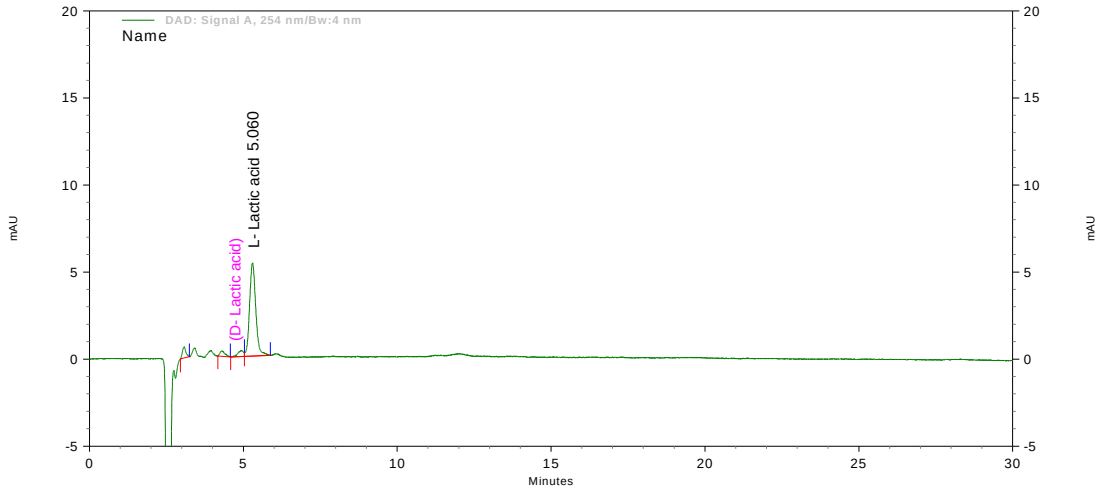

Figure 13D HPLC Chromatogram of L-lactic acid in RAM broth containing 2% glucose after cultivation of isolate CWR1-7 under anaerobic conditions at 35°C for 24 h.

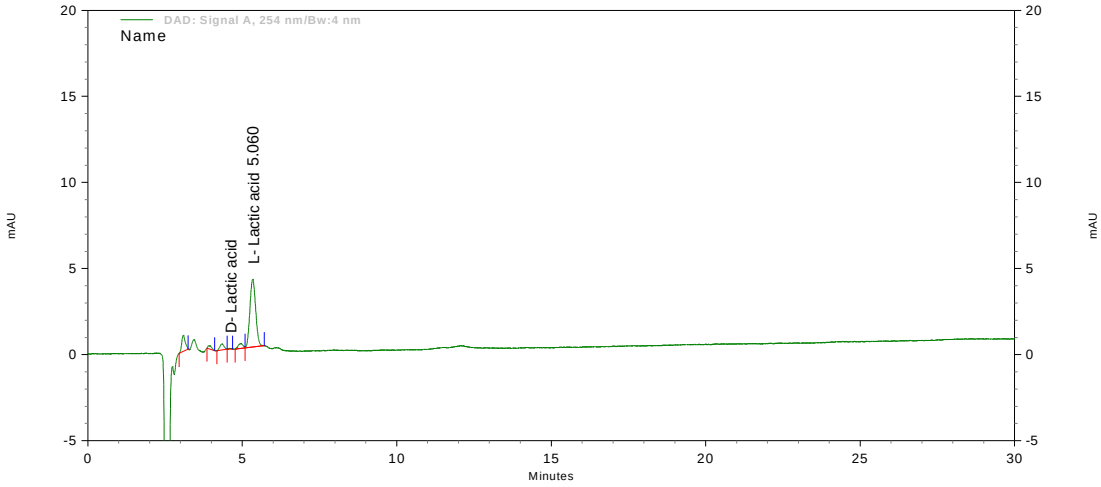

Figure 14D HPLC Chromatogram of L-lactic acid in RAM broth containing 2% glucose after cultivation of isolate CWR1-2 under anaerobic conditions at 35°C for 24 h.

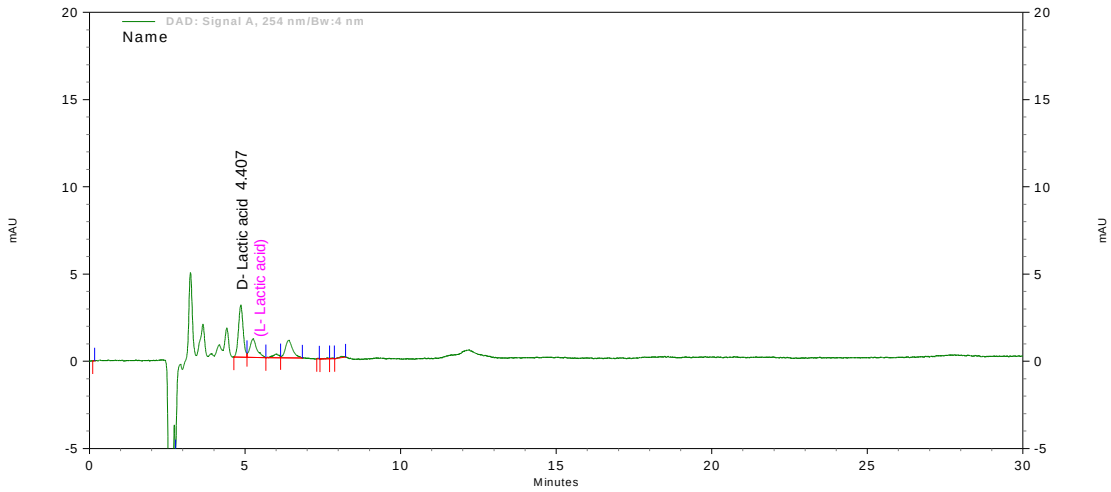

Figure 15D HPLC Chromatogram of D-lactic acid in RAM broth containing 3% cassava starch after cultivation of isolate WR73 under anaerobic conditions at 35°C for 48 h.

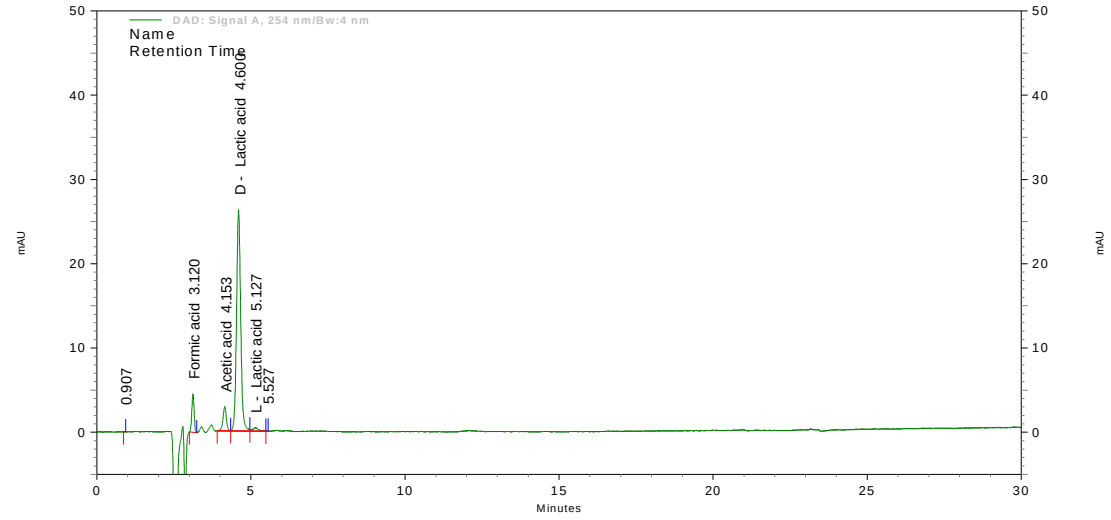

Figure 16D HPLC Chromatogram of D-lactic acid in 5.0 l of modified RAM medium containing 3% cassava starch with initial pH of 7.0 after cultivation of isolate WR73 at 35°C for 48 h.

APPENDIX E

NUCLEOTIDE SEQUENCE DATA

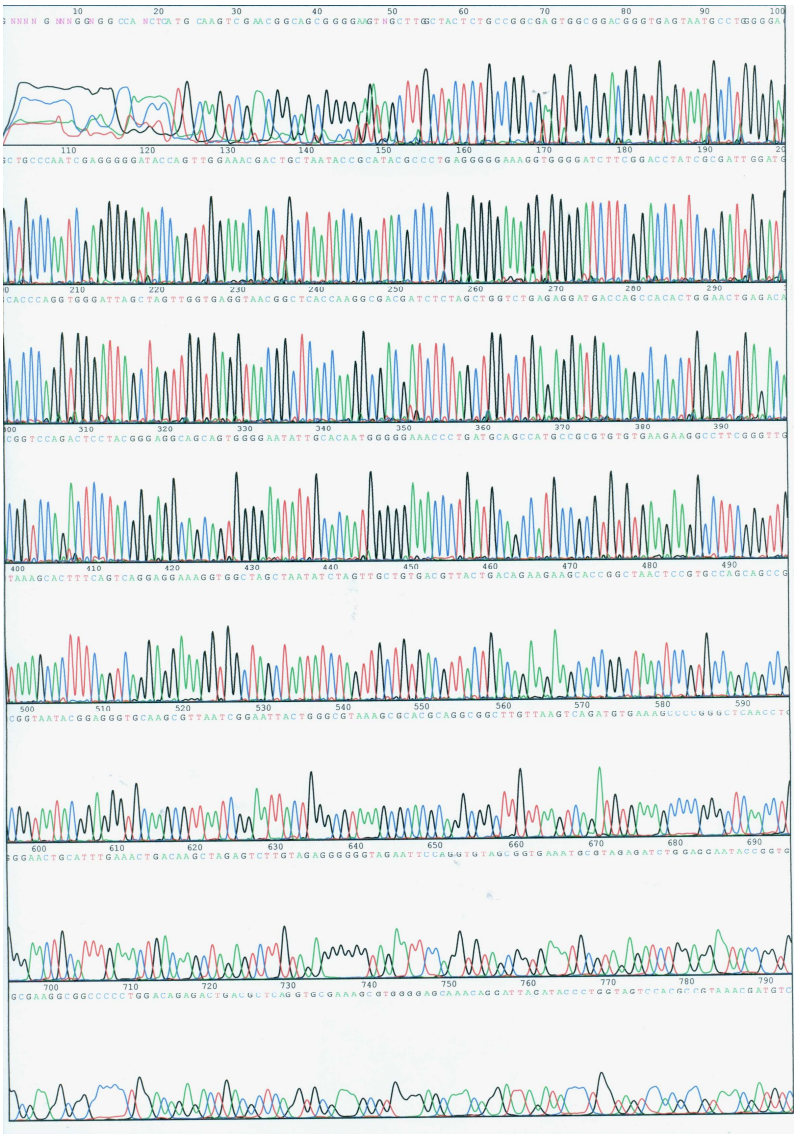

Figure 1E Sequence electrophenogram of bacterial isolate WR73 16S rDNA using fD1 primer.

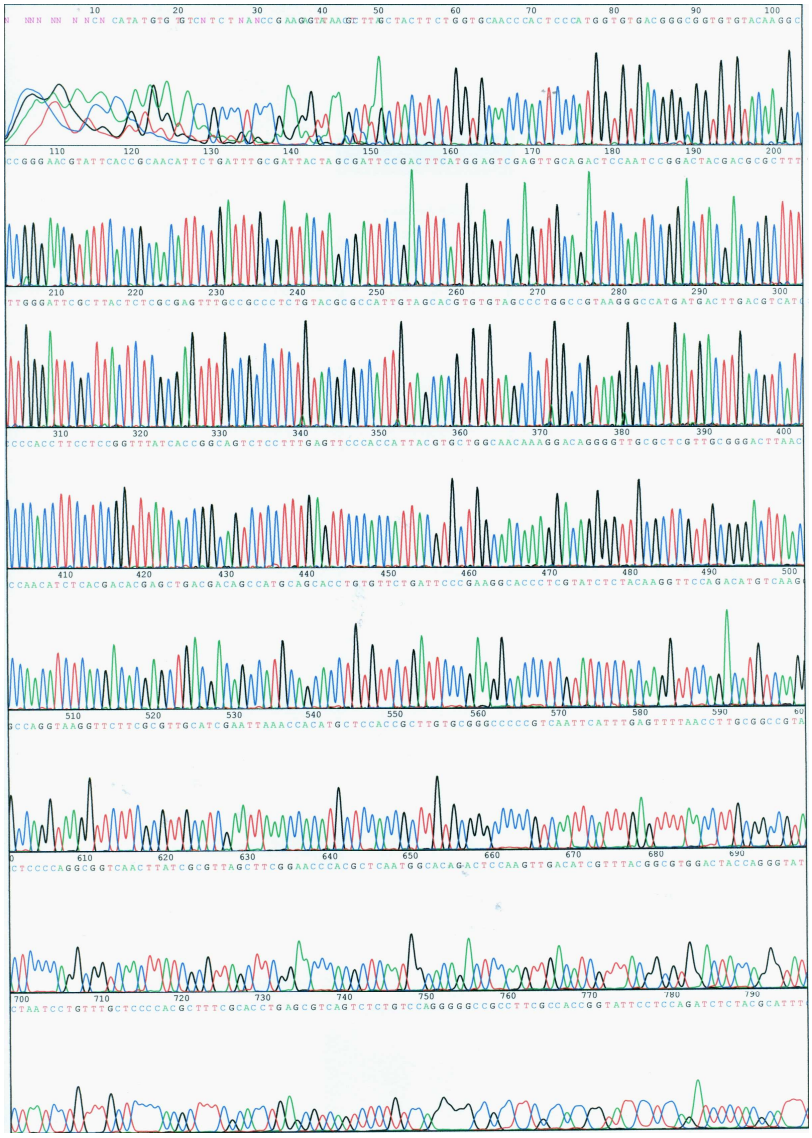

Figure 2E Sequence electrophenogram of bacterial isolate WR73 16S rDNA using rP2 primer.

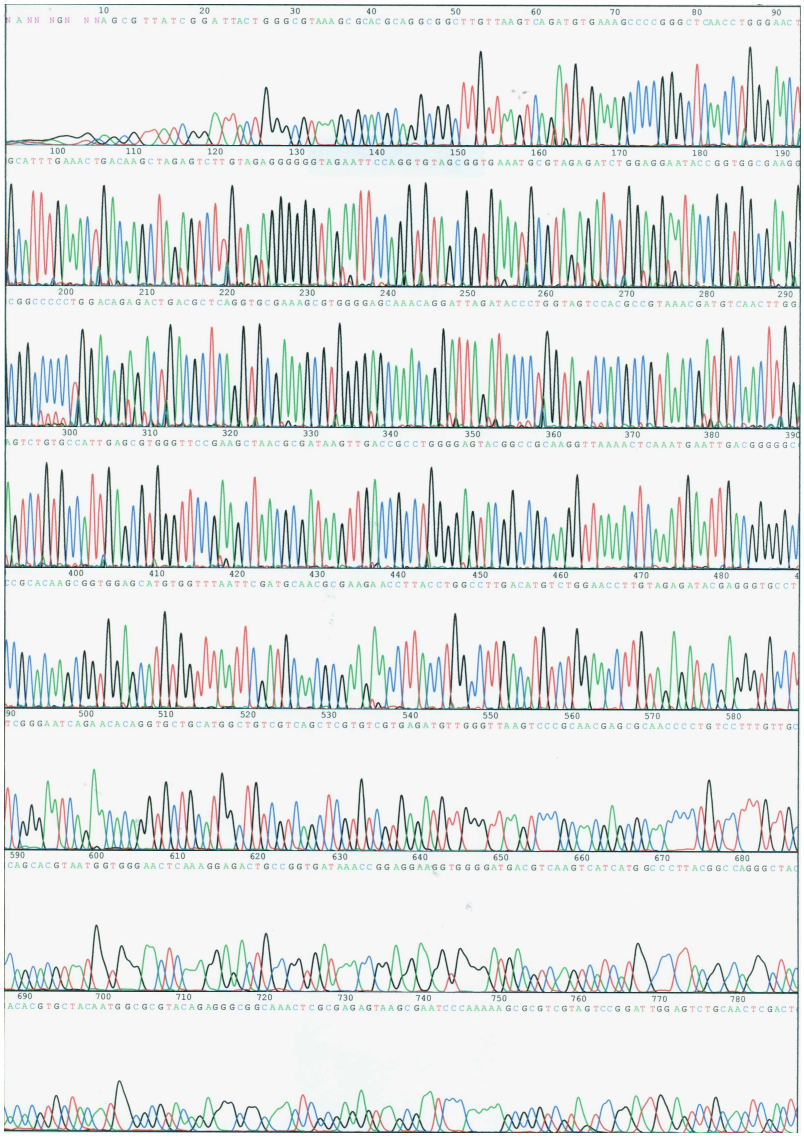

Figure 3E Sequence electrophenogram of bacterial isolate WR73 16S rDNA using walking forward primer.

APPENDIX F

LIST OF PRESENTATIONS

Poster Presentation

Pramkaew, S. and Rodtong, S. (2009). **Comparison of D-lactic acid production by Gram-positive rods and coccus of lactic acid bacteria**. The 21st Annual Meeting and International Conference of Thai Society for Biotechnology (TSB 2009), 24-25 September 2009, Queen Sirikit National Convention Center, Bangkok, Thailand.

Pramkaew, S. and Rodtong, S. (2009). **Investigation of D-and DL-lactic acid production by lactic acid bacteria isolated from cassava starch production waste**. The 2nd SUT Graduate Conference, 21-22 January 2009, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

Rodtong, S., Pramkaew, S., and Pananu, A. (2010). **Potential bacterial strains for D-lactic acid production from tapioca starch**. The 2nd Thai-Japan Bioplastics and Biobased Materials Symposium (AIST-NIA Joint Symposium), 9-11 September 2010, Impact Exhibition & Convention Center, Bangkok, Thailand.

Proceeding

Pramkaew, S. and Rodtong, S. (2010). **D-Lactic acid production from cassava starch by the starch-utilizing *Lactobacillus* sp. WR73**. The 22nd International Conference on Biotechnology for Healthy Living (TSB 2010), 20-22 October 2010, The Prince of Songkla University, Trang Campus, Thailand.

The 21st Annual Meeting and International Conference of Thai Society for Biotechnology
TSB 2009: "Biotechnology: A Solution to the Global Economic Crisis?"
24-25 September 2009: Queen Sirikit National Convention Center, Bangkok, Thailand

P-MF09

COMPARISON OF D-LACTIC ACID PRODUCTION BY GRAM-POSITIVE RODS AND COCCUS OF LACTIC ACID BACTERIA

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Lactic acid bacteria produce lactic acid as the major end product during carbohydrate fermentation. Two enantiomers of lactic acid, D-form and L-form, have been naturally found. D-Lactic acid has been of interest to be used in bioplastics industry. The acid is used to synthesize poly (D-lactic acid) that can improve the thermostability of poly(L-lactic acid), the main component of biodegradable plastics, by stereocomplex formation. Potential microorganisms for D-lactic acid production are still desirable for the production of the acid for biopolymer production. This research focused on the comparison of D-lactic acid production by Gram-positive rods and coccus of lactic acid bacteria by using glucose as substrate. One hundred and twenty five isolates of a total of 206 lactic acid bacterial isolates, and isolated from their natural habitats (cassava starch wastes, cassava pulp, intestinal tracts and faeces of animals) were detected for D-lactic acid production using high performance liquid chromatography. Six lactic acid bacterial isolates CWMC2-5, CWMC1-3, CWMR1-5, CWR2-16, LF1, and PSMS1-5, were able to produce D-lactic acids when cultivated in MRS broth containing 2% of glucose at 30°C for 24 h under anaerobic condition. Time course of D-lactic acid production of Gram-positive rods and coccus of selected isolates were investigated to obtain the suitable fermentation time for the maximum D-lactic acid production. It was found that high yield of D-lactic acid based on glucose consumption and production rate were obtained at 36 h incubation period. Gram-positive rods of isolates CWR2-16, CWMR1-5, CWMC2-5, CWMC1-3, and PSMS1-5 could produce high D-lactic acid yield of 99.01, 94.79, 93.60, 85.89, and 54.38 % respectively after incubation in MRS containing 2% glucose at 30°C for 36 h under anaerobic condition. Gram-positive coccus of isolate LF1 could produce high D-lactic acid yield of 53.07% after incubation in MRS at same conditions. In conclusion, Gram-positive rods have more potential to produce D-lactic acid than Gram-positive coccus. For the preliminary identification of D-lactic acid-producing bacteria, four D-Lactic acid bacterial isolates CWMC2-5, CWMC1-3, CWMR1-5, and CWR2-16 were similar to *Lactobacillus delbrueckii,* whereas LF1 and PSMS1-5 were similar to *Weissella thailandensis* and *Leuconostoc mesenteroides*, respectively based on some morphological and physiological characteristics.

Keywords: Comparison, Lactic acid bacteria, D-Lactic acid, Biopolymer

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Investigation of D-and DL-lactic acid production by lactic acid bacteria isolated from cassava starch production waste

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Lactic acid bacteria produce lactic acid as the major end product during carbohydrate fermentation. Two enantiomers of lactic acid, D-form and L-form, have been naturally found. D-Lactic acid has been of interest to be used in bioplastics industry. The acid is used to synthesize poly (D-lactic acid) that can improve the thermostability of poly (L-lactic acid), the main component of biodegradable plastics, by stereocomplex formation. Potential microorganisms for D-lactic acid production are still desirable for the production of the acid for biopolymer production. This research focused on the investigation of D-and DL-lactic acid production by lactic acid bacteria isolated from cassava starch production waste samples. A total of 96 lactic acid bacterium isolates was tested for their production of lactic acid in MRS medium supplemented with 2% of glucose at 30∘C for 24 h. Low pH values and high titratable acidity contents were used to screen potential lactic acid-producing strains. Then D- and DL-lactic acids were detected by high performance liquid chromatography. One and 33 isolates were able to produce D-and DL-lactic acids, respectively. D-Lactic acid-producing strain gave the maximum D-lactic acid yield of 9.92 g/l. For DL-lactic acid-producing strains, the potential strain produced D- and L-lactic acid yields of 4.28 and 4.17 g/l, respectively. Isolates producing high yields of D-lactic acid were selected for further investigation to obtain a potential strain for D-lactic acid production from cassava starch.

Keywords:

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Potential Bacterial Strains for D-Lactic Acid Production from Tapioca Starch

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Abstract

D-Lactic acid has been of interest to be used in bioplastics industry since it can be used to synthesize poly(D-lactic acid) that can improve the thermostability of poly(L-lactic acid), the main component of biodegradable plastics. Potential microorganisms are still desirable for the production of optically pure D-lactic acid. Two starch-utilizing lactic acid bacterial strains isolated from their habitats in Thailand, could produce D-lactic acid with >99.9% optical purity from tapioca starch, a cheap raw material. Conditions for growth and lactic acid production of the two strains were initially investigated. The suitable medium was found to compose of tapioca starch, tryptone, and spent brewer's yeast as main ingredients. The two bacterial strains had their specific growth rates (μ_{max}) of 0.89 and 0.93 h^{-1}, when cultivated in 5 L fermentation medium containing 20 and 30 g/L tapioca starch, respectively, in a 6.6 L bioreactor at 35°C. And D-lactic acid yields of 14.56 and 19.76 g/L ($Y_{LA/S}$, of 73.0 and 66.51%), respectively, with >99.9% optical purity were achieved at 48 h of cultivation. Results from this study reveal that these bacterial strains have their potential to directly produce high optical purity of D-lactic acid from tapioca starch.

Keywords: Lactic acid bacteria, D-Lactic acid, Tapioca starch

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D-Lactic acid production from cassava starch by the starch-utilizing *Lactobacillus* sp. WR73

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Abstract

D-Lactic acid was successfully produced for a cheap raw material, cassava starch, using the lactic acid bacterial strain WR73. The strain was identified as belonging to the genus *Lactobacillus* by physiological characteristics and 16S rDNA nucleotide sequence. Conditions for growth and lactic acid production of *Lactobacillus* sp. WR73 were optimized. The suitable medium was found to compose of main ingredients: cassava starch, tryptone, and spent of brewery yeast sludge at concentrations of 30.0, 3.0, and 3.0 g/l, respectively. Optimum pH and temperature for both growth and optically pure D-lactic acid production were at 7.0 and 35°C. The maximum D-lactic acid yield of 19.76 g/l ($Y_{LA/S}$, of 66.51%) with >99.9% optical purity were achieved at 48 h of cultivation in 5.0 l of the optimized medium in a controlled fermenter under optimum conditions with 1% inoculum size (approximately 10^6 cells/ml). This strain had its specific growth rate (μ_{max}) of 0.93 h^{-1}. Results from this study showed that cassava starch could serve as a low cost substrate for optically pure D-lactic acid production by *Lactobacillus* sp. WR73.

Keywords: *Lactobacillus* sp., Lactic acid bacteria, D-Lactic acid production, Cassava starch

Introduction

D-Lactic acid is one of the desirable monomers to be used for poly(D-lactic acid), PDLA, production in bioplastics industry. PDLA is useful for improvement the thermostability of poly(L-lactic acid), PLLA or PLA, which is the main component of biodegradable plastics (Zhao *et al*., 2010; Tsuji and Fukui, 2003). Presently, of the 80,000 ton of lactic acid produced annually worldwide about 90% is produced by lactic acid fermentation of sugars (John *et al*., 2009). The cost of raw material is one of the major factors in economics of production media for lactic acid fermentation. D-Lactic acid has been reported to be produced by several species of lactic acid bacteria, particularly *Lactobacillus delbrueckii* (Calabia *et al*., 2007; Tanaka *et al*., 2006; Manome *et al*., 1998), *Lactobacillus coryniformis* subsp. *torquens* (Yàñez *et al*., 2003; Manome *et al*., 1998), *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum, Leuconostoc carnosum, Leuconostoc fallax* (Manome *et al*., 1998), and *Lactobacillus bulgaricus* (Benthin and Villadsen, 1995). Potential microorganisms for the D-lactic acid production from cheap raw materials are still needed. In this study, D-lactic acid was successfully produced for a

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cheap raw material, cassava starch, using the lactic acid bacterial strain WR73, which was identified as belonging to the genus *Lactobacillus* by physiological characteristics and 16S rDNA nucleotide sequence.

Materials and Methods

Bacterial strain and media

D-Lactic acid production studies were performed using the selected starch-utilizing bacterial strain, *Lactobacillus* sp. WR73. Its stock culture was maintained at -80°C with the addition of 10% (v/v) skim milk. The bacterium was cultivated in modified Rogasa agar medium (RAM; Rodtong and Ishizaki, 2003) composing of 2% glucose, 0.2% tryptone, 0.6% K_2HPO_4, 0.3% yeast extract, 0.057% $MgSO_4.7H_2O$, 0.012% $MnSO_4.4H_2O$, and 0.003% $FeSO_4.7H_2O$ with the initial pH of 7.0. For solid medium, 1.5% (w/v) agar were added to the medium described above.

Raw materials

Cassava starch was purchased from Sanguanwongse industries Co., Ltd. (Nakhon Ratchasima, Thailand). The cassava starch contained carbohydrate (85.53%), moisture content (11.95%), fiber content (0.18%), total nitrogen content (0.35%), fat content (1.64%), and ash content (0.35%). Spent brewery yeast sludge was purchased from Boonrawd brewery Co., Ltd., (Khon Kaen, Thailand). The yeast sludge contained carbohydrate (75.53%), moisture content (10.62%), fiber content (5.21 %), total nitrogen content (6.21%), fat content (2.37%), and ash content (5.18%).

Analytical methods

Bacterial growth was determined as (CFU/ml) using RAM agar plates. The fermented broth was used for the determination of lactic acid and total sugar. % Total acidity was determined according to AOAC International (2000). The optical purity of lactic acid was analyzed by high performance liquid chromatography (HPLC) (HP 1200, Agilent Technology Inc., USA), equipped with a tunable UV detector set at 254 nm. A chiral Astec CLC-L column (5 μm, 4.6 mm×15 cm, Sigma Chemical Co., USA) was eluted with 0.005 M $CuSO_4$ as a mobile phase at a flow rate of 0.7 ml/min. The total sugar was determined by the phenol-sulphuric acid method (Dubois *et al*., 1956).

Characterization of the selected starch-utilizing bacterial strain

Morphological and physiological characterization of the selected starch-utilizing bacterial strain was conducted according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994), Collins *et al*. (1993), and Cappuccino and Sherman (1999). 16S Ribosomal RNA gene was amplified (Weisburg *et al*., 1991), and nucleotide sequence of the gene was analyzed using ABI 3730xl DNA analyzer (Model 373, USA).

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Production of D-lactic acid by the selected starch-utilizing bacterial strain

For the efficient production of D-lactic acid, cultivation conditions involving with medium composition, pH of the medium, growth and lactic acid production temperature, for *Lactobacillus* sp. WR73 were investigated. RAM medium was used for this study. The suitable inoculum size was also determined. Then, D-Lactic acid was produced using the optimized production conditions. A 6.6-l jar fermenter (Biostat® *B* plus, Germany) containing 5.0 l of fermentation medium was used. The optimized medium was sterilizer at 121°C for 35 min. Then suitable inoculum size of *Lactobacillus* sp. WR73 (approximately 10^6 CFU/ml) was added to the fermentation medium. The culture temperature was controlled at optimum temperature, and the agitation speed was maintained at 200 rpm to insure completely mixing the fermentation broth. The culture pH was kept constant at optimum pH during fermentation through automatic addition of 5 N NaOH. During fermentation experiments, bacterial growth, pH, total acidity, total sugars, and D-lactic acid concentration were measured at various time intervals.

Results and Discussion

Characterization of the selected starch-utilizing bacterial strain

The strain WR73 was selected from a total of 306 lactic acid bacterial strains isolated from their natural habitats. The strain WR73 can hydrolyze cassava starch on RAM agar (Figure 1), and produce D-lactic acid with >99.9% optical purity.

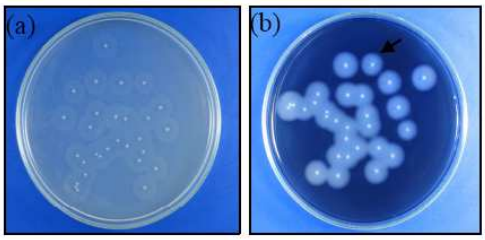

Figure 1. Growth and amylase expression of the selected bacterial isolate WR 73. (a) Growth on the agar medium containing 1% cassava starch, and (b) the addition of iodine solution onto the agar surface of (a). Arrows indicated positive colony of amylase expression

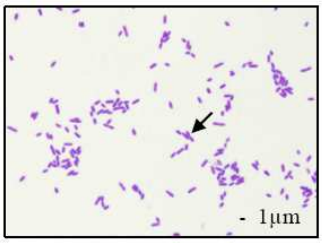

Figure 2. Cell morphology from Gram staining of *Lactobacillus* sp. WR73 cultivated on RAM agar at 35°C for 18 h under anaerobic conditions (Bar=1 µm)

Based on morphological and physiological characteristics of the strain WR73, (Figure 2 and Table 1), which were rod shape cells, catalase negative, homo D-lactic acid production from glucose, optimum growth temperature at 30-40°C (no growth at

10°C), amylase-positive, it was identified as belonging to the genus *Lactobacillus*. This strain had 97% similarity compared to *Lactobacillus delbrueckii* subsp. *delbrueckii* by biochemical characteristics. But its 16S rDNA sequence (1450 bp) amplified using fD1/rP2 primer, demonstrated that the strain WR73 was clearly separated from *Lactobacillus delbrueckii*, and other species in genera *Carnobacterium, Leuconostoc*, and *Weissella* (Figure 4).

Table 1. Characteristics of *Lactobacillus* sp. WR73 compared to related genera according to Holt *et al.* (1994) and Collins *et al.* (1993).

Characteristics	***Lactobacillus* sp. WR73**	***Lactobacillus***	***Carnobacterium***	***Weissella***	***Leuconostoc***
Cell shape	Rods	Rods, sometime cocco-bacilli	Rods	Rods, cocci	Cocci
Cell arrangement	Single, pairs	Single, pairs, and in chains	Single, pairs, and in chains	Single, pairs, and in chains	Pairs, in chains
Cell size (μm)	(0.53-0.66) ×(0.77-1.06)	(0.5-1.2) ×(1.0-10.0)	(0.5-0.7) ×(1.0-2.0)	0.5-0.7	(0.5-0.7) × (0.7-1.2)
Gram stain	+	+	+	+	+
Spore formation	-	-	-	-	-
Catalase test	-	-	-	-	-
Motility	+	-[c]	D	-	-
Lactic acid	D	D, DL, L[g]	L	D, DL[g]	D
CO_2 production	-	±	-[d]	+	+
Hydrolysis of:					
Starch	+	±	±	-	±[i]
Gelatin	-	-	ND	ND	ND
Arginine	-	±	+	±	-
Casein	-	±	ND	ND	-
Optimum growth temperature (°C)	30-40	30-40	30	30	20-30
Growth at 10°C	-	±	+	+	+
Growth at 45°C	+	±	-	-	-
Growth at 6.5% NaCl	-	±	ND	±	±
Growth at 18% NaCl	-	-	-	-	-
Growth at pH 4.4	-	±	ND	±	±
Growth at pH 9.6	-	-	-	-	-

+, positive; -, negative; ±, response varies between species; ND, not determined; D = Substantial proportion of species differ; [c] Rarely motile; [d] Small amounts of CO_2 can be produced, depending on media; [g] Production of D-, L- or DL-lactic acid varies between species; [i] 11-89% of strains are positive.

Production of D-lactic acid by the selected starch-utilizing bacterial strain

From optimization of some conditions for D-lactic acid production, different concentration of cassava starch; 10, 15, 20, 25, 30, 35, and 40 g/l was studied. After cultivating *Lactobacillus* sp.WR73 for 48 h at 35°C under anaerobic conditions, D-lactic acid concentration increased with the increase of initial cassava starch concentration up to 30 g/l. The maximum D-lactic acid (3.45±0.15 g/l) was obtained at 48 h fermentation with an initial cassava starch concentration of 30 g/l (p<0.05)

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(D-lactic acid yield, ($Y_{LA/S}$), of 35.10±1.55%) (Table 2). For optimum nitrogen concentrations in the optimized RAM medium, tryptone could be served as nitrogen source, varied concentrations of tryptone (g/l): 0, 5, 10, 15, and 20, were added to the medium containing 30 g/l of cassava starch. It was found that the strain WR73 could produce the maximum D-lactic acid of 3.19±0.01 g/l ($Y_{LA/S}$, 47.08±0.08 %) when using tryptone at concentration of 3.0 g/l. Moreover, the bacterial growth gave the maximum count (7.8×10^6 CFU/ml) at tryptone concentration of 3.0 g/l. In RAM medium supplement with 3.0 g/l spent brewery yeast sludge gave similar D-lactic acid concentration (3.31±0.03 g/l) compared with the medium supplied with 1.0 g/l yeast extract and 3.0 g/l spent brewery yeast sludge. Thus, spent brewery yeast sludge could be an alternative to costly yeast extract, and 3.0 g/l spent brewery yeast sludge was selected for D-lactic acid production by *Lactobacillus* sp. WR73.

The initial pH range of 5.0-8.0 of the fermentation medium was tested for D-lactic acid production. pH 7.0 was found to provide better condition than the other pHs, and the 58.88±5.90% ($P<0.05$) yield ($Y_{LA/S}$) of D-lactic acid were achieved. Various cultivation temperatures; 25, 30, 35, 37, 40, and 45°C, were provided for D-lactic acid production by *Lactobacillus* sp. WR73. Results showed that the highest bacterial growth (3.1×10^5 CFU/ml) and D-lactic acid yield (53.34±3.06%) were obtained when the cultivating medium at 35°C ($P<0.05$). The influence of different inoculum sizes (1-5%, v/v) on D-lactic acid production was studied. It was found that 1-5% inoculum sizes did not give the significant difference of lactic acid yields (D-lactic acid yield, ($Y_{LA/S}$), of 54.84±3.16% and $P>0.05$). Thus, 1 % (v/v) inoculum was considered for D-lactic acid production by the lactobacillus strain.

For the production of D-lactic acid by *Lactobacillus* sp. WR73 using the optimized production conditions, five liters of the medium containing 30.0 g/l (dry weight) of cassava starch, 3.0 g/l of tryptone, 3.0 g/l of spent brewery yeast sludge, 6.0 g/l of K_2HPO_4, 0.57 g/l of $MgSO_4.7H_2O$, 0.12 g/l $MnSO_4.4H_2O$, and 0.03 g/l of $FeSO_4.7H_2O$, were prepared in a 6.6 l fermenter. After inoculating 1.0% (v/v) of a late log phase of *Lactobacillus* sp. WR73 culture. Temperature and pH of the cultivation medium were controlled constantly at 35°C and 7.0 respectively. The bacterium grew rapidly during 6 h cultivation. The maximum bacterial count (5.3×10^{10} CFU/ml) was also obtained at 6 h of fermentation, and the bacterium had specific growth rate of 0.93 h^{-1}. The maximum D-lactic acid yield of 19.76 g/l (D-lactic acid yield, ($Y_{LA/S}$), of 66.51%) was achieved after 48 h. The optical purity of D-lactic acid was shown to be >99.9% by HPLC analysis. L-Lactic acid was not found in fermentation medium till 48 h of cultivation.

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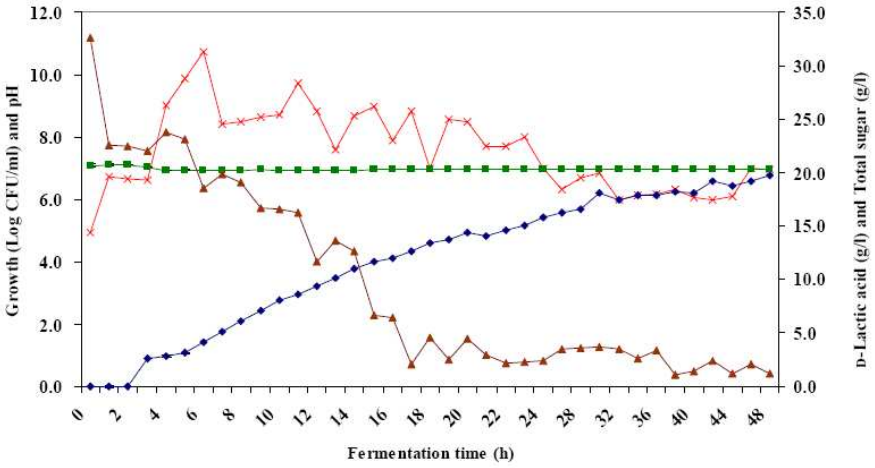

Figure 3. Growth and D-lactic acid production by *Lactobacillus* sp. WR73 in 5.0 l of optimized medium containing 30 g/l cassava starch at the initial pH of 7.0 and 35°C for 48 h. (x) growth; (■) pH; (◆) D-lactic acid; (▲) total sugar

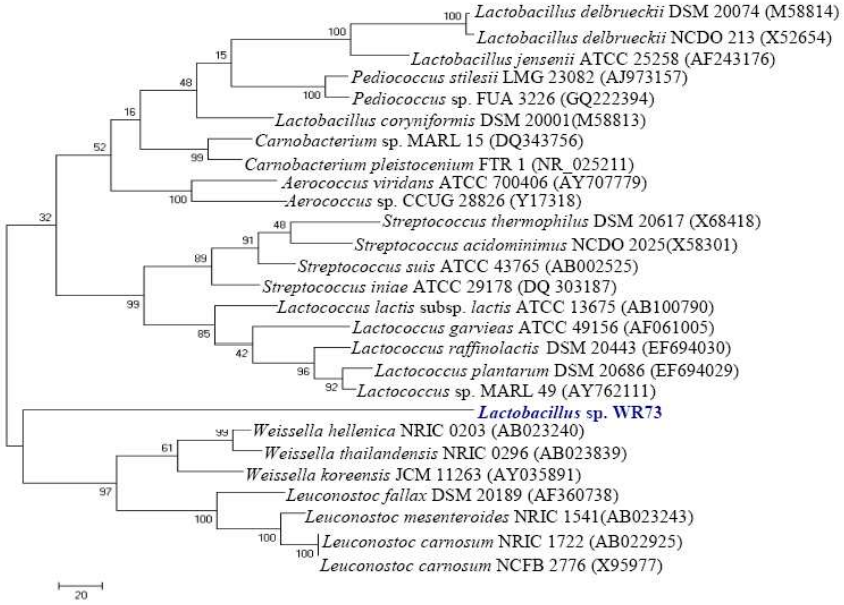

Figure 4. Phylogenetic tree showing positions of strain WR73, other *Lactobacillus* strains, and representative strains of other related genera based on 16S rDNA sequences. Scale bar represents 20 substitutions per nucleotide position

Conclusion

Lactobacillus sp.WR73 could produce optically pure D-lactic acid from cassava starch with the maximum D-lactic acid yield of 19.76 g/l (D-lactic acid yield, ($Y_{LA/S}$), of 66.51%) at 48 h of cultivation in a controlled fermenter containing 5.0 l of the suitable medium containing 30.0 g/l (dry weight) of cassava starch, 3.0 g/l of tryptone, 3.0 g/l of spent brewery yeast sludge, 6.0 g/l of K_2HPO_4, 0.57 g/l of $MgSO_4.7H_2O$, 0.12 g/l $MnSO_4.4H_2O$, and 0.03 g/l of $FeSO_4.7H_2O$. This strain had its specific growth rate (μ_{max}) of 0.93 h^{-1}. Cassava starch could serve as a low-cost substrate for the production of high quality D-lactic acid for manufacturing of poly(D-lactic acid), PDLA, essential for successful application of the high-melting of stereocomplex-type poly(lactic acid).

Acknowledgments

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