POTENTIALITY OF LACTIC ACID BACTERIA FOR L-LACTIC ACID PRODUCTION FROM TAPIOCA STARCH

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ศักยภาพของแบคทีเรียกรดแล็กติกในการผลิตกรดแอล-แล็กติกจากแป้งมันสำปะหลัง

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POTENTIALITY OF LACTIC ACID BACTERIA FOR L-LACTIC ACID PRODUCTION FROM TAPIOCA 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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กรดแอล-แล็กติกเป็นสารเคมีที่ละลายน้ำได้ดีและใช้ประโยชน์ทั้งในอุตสาหกรรมอาหาร เครื่องดื่ม เครื่องสำอาง และยา รวมถึงการผลิตพลาสติกย่อยสลายได้ทางชีวภาพซึ่งต้องการกรดแอล-แล็กติกความบริสุทธิ์เชิงแสงสูงมากกว่าร้อยละ 99 จึงเป็นกรดชนิดที่มีความต้องการสูงในปัจจุบัน แต่การผลิตกรดแอล-แล็กติกที่คุ้มทุนยังคงอาศัยกระบวนการทางชีวภาพที่ใช้น้ำตาลกลูโคสเป็นวัตถุดิบหลัก การใช้แป้งซึ่งมีราคาถูกกว่าน้ำตาลเป็นวัตถุดิบสามารถช่วยลดต้นทุนการผลิตได้ แต่ต้องค้นหาจุลินทรีย์ที่มีศักยภาพในการใช้แป้งและผลิตกรดได้ด้วย การศึกษานี้จึงเน้นการคัดเลือกแบคทีเรียกรดแล็กติกที่สามารถผลิตกรดแอล-แล็กติกได้โดยตรงจากแป้งมันสำปะหลัง และศึกษาสภาวะที่เหมาะสมในการผลิตกรดแอล-แล็กติกที่มีความบริสุทธิ์เชิงแสงสูงจากอาหารแป้งมันสำปะหลัง จากการทดสอบความสามารถในการผลิตกรดแอล-แล็กติกของแบคทีเรียกรดแล็กติกที่แยกได้จากแหล่งที่พบเชื้อตามธรรมชาติจำนวน 280 ไอโซเลท พบว่ามีจำนวน 128 ไอโซเลท ที่สามารถผลิตกรดแล็กติกได้ในความเข้มข้นช่วง 0.91-8.60 กรัมต่อลิตร ที่มีความบริสุทธิ์เชิงแสงของกรดแอล-แล็กติกมากกว่าร้อยละ 95 เมื่อเลี้ยงเชื้อในอาหารเหลวที่มีน้ำตาลกลูโคสร้อยละ 2 แบคทีเรียเหล่านี้ไม่สร้างก๊าซคาร์บอนไดออกไซด์จากกระบวนการหมัก จึงได้เลือกแบคทีเรีย 2 ไอโซเลท (CAR134 และ SUT513) ที่ผลิตกรดแอล-แล็กติกได้สูงเท่ากับ 7.89 และ 8.60 กรัมต่อลิตร ตามลำดับ และสามารถย่อยแป้งมันสำปะหลังได้ เมื่อศึกษาเพื่อระบุชนิดของแบคทีเรียด้วยลักษณะทางสัณฐานวิทยา สรีรวิทยา และการหาลำดับนิวคลีโอไทด์ของ 16S rDNA พบว่าแบคทีเรียทั้ง 2 ไอโซเลท จัดอยู่ในสกุล *Streptococcus* ที่ต่างสายพันธุ์กัน ซึ่งเมื่อศึกษาสภาวะที่เหมาะสมต่อการผลิตกรดแอล-แล็กติกจากแป้งมันสำปะหลัง พบว่าส่วนประกอบหลักของอาหารเลี้ยงเชื้อที่เหมาะสมต่อการผลิตกรดของไอโซเลท CAR134 และ SUT513ประกอบด้วยแป้งมันสำปะหลัง 30 และ 30 กรัมต่อลิตร ยีสต์แห้งเหลือจากอุตสาหกรรมการผลิตเบียร์ 3.0 และ 5.0 กรัมต่อลิตร และทริปโตน 4.0 และ 2.5 กรัมต่อลิตร ตามลำดับ ที่ค่าความเป็นกรด-ด่างเริ่มต้นของอาหารเลี้ยงเชื้อเท่ากับ 7.0 เมื่อทดลองผลิตกรดแอล-แล็กติกในถังหมักที่บรรจุอาหารปริมาตร 5 ลิตร ควบคุมสภาวะให้เหมาะสมต่อการเจริญที่ 35 องศาเซลเซียส แบคทีเรียไอโซเลท CAR134 และ SUT513 สามารถผลิตกรดแอล-แล็กติกที่มีความบริสุทธิ์เชิงแสงมากกว่าร้อยละ 99 ได้ความเข้มข้นสูงสุดที่ 32.70 และ 38.90 กรัมต่อลิตร เมื่อเลี้ยงเชื้อเป็นเวลา 38 และ 28 ชั่วโมง ตามลำดับ คิดเป็น

ผลผลิตกรดแอล-แล็กติก ร้อยละ 92.15 และ 99.74 อัตราการผลิตกรดแล็กติกสูงสุดเท่ากับ 1.41 และ 1.61 กรัมต่อลิตรต่อชั่วโมง อัตราการเจริญจำเพาะสูงสุดเท่ากับ 0.27 และ 0.51 ต่อชั่วโมง ตามลำดับ กรดแอล-แล็กติกในน้ำหมัก (อาหารแป้งมันสำปะหลังที่ผ่านการเลี้ยงเชื้อ) สามารถทำบริสุทธิ์ได้ง่ายด้วยการตกผลึกด้วยการเติมเกลือแคลเซียมคลอไรด์ และได้ความเข้มข้นของกรดบริสุทธิ์ที่มีความบริสุทธิ์เชิงแสงร้อยละ 100 เท่ากับ 57.0 และ 64.2 กรัมต่อลิตร ตามลำดับ กรดแอล-แล็กติกความบริสุทธิ์เชิงแสงสูงที่ผลิตได้นี้เป็นประโยชน์อย่างยิ่งในการใช้เพื่อผลิตพลาสติกย่อยสลายได้ทางชีวภาพ

สาขาวิชาจุลชีววิทยา
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ลายมือชื่อนักศึกษา ชุบล ฟักุลเงิน
ลายมือชื่ออาจารย์ที่ปรึกษา

YUBON PIKUL-NGOEN : POTENTIALITY OF LACTIC ACID BACTERIA FOR L-LACTIC ACID PRODUCTION FROM TAPIOCA STARCH. THESIS ADVISOR : ASST. PROF. SUREELAK RODTONG, Ph.D. 190 PP.

LACTIC ACID BACTERIA/L-LACTIC ACID/TAPIOCA STARCH/BIOPOLYMER

L-Lactic acid is a water soluble chemical widely used in food, beverage, cosmetic, and pharmaceutical industries. The high optical purity (>99%) of the acid is also desired for biodegradable plastics production. Thus, the high demand of L-lactic acid is currently faced but the economical production of the acid is still relied on bioconversion of sugars, particularly glucose. Starchy materials, low-cost substrates compared to sugars, are an alternative raw material but the suitable microorganism capable of both utilizing starch and producing L-lactic acid has to be applied. This study emphasized on screening of potential lactic acid bacteria for producing L-lactic acid with high optical purity from tapioca starch and optimizing the acid production conditions. A total of 280 lactic acid strains isolated from their natural habitats were tested for L-lactic acid production using liquid medium containing 2% glucose. One hundred and twenty-eight were found to producing lactic acid at concentrations in the range of 0.91-8.60 g/l with >95% optical purity of L-lactic acid. These bacteria were homofermentative. Two starch-utilizing isolates (CAR134 and SUT513) producing L-lactic acid at concentrations of 7.89 and 8.60 g/l respectively, were then selected for the acid production from tapioca starch. The two strains were identified as belonging to different strains of the genus *Streptococcus* according to their morphological and

physiological characteristics, and 16S rRNA gene sequence. For optimization of its growth and lactic acid production conditions, the suitable media for both growth and L-lactic acid production of isolates CAR134 and SUT513 were found to composed of main ingredients as follows: 30 and 30 g/l (dry weight) of tapioca starch, 3.0 and 5.0 g/l of spent brewer's yeast, and 4.0 and 2.5 g/l of tryptone, respectively, at the initial pH of 7.0. When lactic acid fermentation was performed in a bioreactor containing 5 l of the optimized media under optimal temperature at 35°C, the strains CAR134 and SUT513 could produce the maximum L-lactic acid concentrations of 32.70 and 38.90 g/l with >99% optical purity after cultivation for 38 and 28 h, respectively. The two strains (CAR134 and SUT513) could produce L-lactic acid yield ($Y_{LA/S}$) of 92.15 and 99.64% with productivity of 1.41 and 1.61 g/l.h, and specific growth rates (μ_{max}) of 0.27 and 0.51 h^{-1}, respectively. The acid product could be simply purified from the inexpensive optimized tapioca starch media by crystallization using calcium chloride, which resulted in purified L-lactic acid (100% optical purity) of 57.0 and 64.2 g/l for isolates CAR134 and SUT513 respectively. L-Lactic acid with high optical purity is very useful for the production of biodegradable plastics.

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LIST OF ABBREVIATIONS

ATP	=	Adenosine triphosphate
bp	=	Base pair
BSA	=	Bovine serum albumin
CFU	=	Colony forming unit
CO_2	=	Carbon dioxide
°C	=	Degree Celsius
Da	=	Dalton
dATP	=	Deoxyadenosine triphosphate
dCTP	=	Deoxycytidine triphosphate
dGTP	=	Deoxyguanosine triphosphate
dNTPs	=	Deoxynucleoside triphosphate
dTTP	=	Deoxythymidine triphosphate
DNA	=	Deoxyribonucleic acid
EDTA	=	Ethylenediaminetetraacetic acid
e.g.	=	for example
et al.	=	et alia (and others)
g	=	Gram
FDA	=	Food and Drug Administration
GRAS	=	Generally recognized as safe
h	=	Hour

LIST OF ABBREVIATIONS (Continued)

HCl	=	Hydrochloric acid
HPLC	=	High pressure liquid chromatography
Kb	=	Kilobase
kDa	=	Kilodalton
l	=	Liter
LA	=	Lactic acid
D-LA	=	D-Lactic acid
L-LA	=	L-Lactic acid
LAB	=	Lactic acid bacteria
M	=	Molar
mM	=	Millimolar
mg	=	Milligram
min	=	Minute
ml	=	Milliliter
mm	=	Millimeter
MRS	=	De Man, Rogosa and Sharpe medium
ng	=	Nanogram
nm	=	Nanometer
OD	=	Optical density
%	=	Percentage
GPa	=	Giga Pascal
PCR	=	Polymerase chain reaction

LIST OF ABBREVIATIONS (Continued)

PDLA	=	Poly(D-lactic acid)
PLA	=	Poly(L-lactic acid)
PLLA	=	Poly(L-lactic acid)
RAM	=	Rogosa agar modified medium
RNA	=	Ribonucleic acid
rpm	=	Round per minute
rDNA	=	Ribosomal deoxyribonucleic acid
sec	=	Second
subsp.	=	Subspecies
µg	=	Microgram
µl	=	Microliter
U.S.A.	=	United States of America
UV	=	Ultraviolet
v/v	=	Volume by volume

Bacteria genera

A.	=	*Aerococcus*
E.	=	*Enterococcus*
L.	=	*Lactobacillus*
Lc.	=	*Lactococcus*
Leu.	=	*Leuconostoc*
P.	=	*Pediococcus*
S.	=	*Streptococcus*

CHAPTER I

INTRODUCTION

1.1 Introduction

Lactic acid bacteria (LAB) are characterized by a relatively simple sugar fermentation pathway that, by definition, results in the formation of lactic acid. LAB are naturally found in nutrient-rich environments such as plant, fermented food, milk, meat, and intestinal tracts of human and animals (Hofvendahl and Hägerdal, 2000). There are two optical isomers of lactic acid, L-lactic acid and D-lactic acid. L-Lactic acid can be manufactured by either chemical synthesis or microbial fermentation. Racemic DL-lactic acid is always produced by chemical synthesis from petrochemical resources. An optically pure L(+)- or D(-)-lactic acid can be obtained by microbial fermentation of renewable resources when the appropriate microorganism that can produce only one of the isomers is selected (Hofvendahl and Hägerdal, 2000). L-Lactic acid is considered to be one of the most useful chemicals used in food (as a preservative, acidulant, and flavouring agent), chemical, textile, and pharmaceutical industries (Åkerberg and Zacchi, 2000). It also functions as the main monomer for the production of biodegradable poly(L-lactic acid) (PLLA or PLA), which is well-known as a sustainable bioplastic material (Datta *et al*., 1995). The optical purity of lactic acid is very important for the biopolymer production. The worldwide demand for lactic acid is estimated roughly to be 130,000 to 150,000 tonnes per year (Wee *et al*., 2006). However, the global consumption of lactic acid is expected to increase rapidly

in the near future. NatureWorks LLC, a major PLLA manufacturer established in the United States of America (U.S.A.), expects that the global PLLA market may increase to 500,000 tonnes per year by 2010 (Wee *et al*., 2006). Most widely used substrates for the production of lactic acid by fermentation are refined sugars, particularly glucose, which are expensive. Several species of LAB in genera *Carnobacterium*, *Aerococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Pediococcus*, *Tetragenococcus*, and *Vagococcus* could potentially produce L-lactic acid from glucose (Axelsson, 2004). Lactic acid is also produced from abundant and renewable substances such as whey, molasses, beet and cane sugar, and starch (Vishnu *et al*., 2002). Tapioca starch, a cheap agricultural product in Thailand, has also been reported to be used for the production of lactic acid without pretreatment by enzymic saccharification to glucose (Rodtong and Ishizaki, 2003). This research focused on screening, selection, and comparison of L-lactic acid production from tapioca starch by LAB.

1.2 Research objectives

1) To screen, select, and compare lactic acid bacterial isolates for their L-lactic acid production capability from tapioca starch.

2) To identify the potential L-lactic acid-producing strains.

3) To optimize medium compositions for L-lactic acid production from a selected potential strain.

4) To preliminarily investigate methods for the extraction and purification of L-lactic acid from tapioca starch fermentation medium.

1.3 Research hypotheses

L-Lactic acid could be potentially produced by specific strains of lactic acid bacteria using tapioca starch, an abundant agricultural product in Thailand. The acid could be extracted and purified from the inexpensive optimized medium for industrial application.

1.4 Scope and limitations of the study

Lactic acid bacteria obtained from stock cultures of the Microbial Culture Collection and Applications Research Unit, Suranaree University of Technology, were screened and selected for L-lactic acid production from glucose. The selected strains were tested for their tapioca utilization and L-lactic acid production from the starch. The potential strains were identified. Medium compositions for L-lactic acid production from a selected strain were optimized. The suitable method for L-lactic acid extraction and purification from starch medium after fermentation was preliminary investigated.

1.5 Expected results

Potential strains for L-lactic acid production from tapioca starch be obtained. Data of the medium compositions and the information of extraction and purification method for L-lactic acid production from the optimized starch medium after lactic acid fermentation would be achieved.

CHAPTER II

LITERATURE REVIEW

2.1 Lactic acid bacteria

2.1.1 Taxonomy

Lactic acid bacteria (LAB) are Gram-positive; cocci or rods; anaerobic, microaerophilic, or aero-tolerant; and catalase negative. They produce lactic acid as the major end product during fermentation of carbohydrate. Generally, LAB are mesophilic microorganisms that grow in the temperature range of 10 to 45°C. However, some of the LAB reported as thermophilic that can grow at high temperature of 45°C. With respect to growth pH, some can grow as low as 3.2, some as high as 9.6, and most can grow in the pH range of 4.0-4.5 (Axelsson, 2004). Twenty one genera of LAB have been reported including *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosicoccus*, *Dolosigranulum*, *Enterococcus*, *Eremococcus*, *Facklamia*, *Globicatella*, *Helcococcus*, *Ignavigranum*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Axelsson, 2004). The classification of LAB 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 (Axelsson, 2004). A summary of the differentiation of LAB genera with classical physiological 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 LAB genera. The bacteria can be divided into 2 groups: rods (*Lactobacillus* and *Carnobacterium*) and cocci (*Aerococcus*, *Alloiococcus*, *Dolosicoccus*, *Dolosigranulum*, *Enterococcus*, *Eremococcus*, *Facklamia*, *Globicatella*, *Helcococcus*, *Ignavigranum*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*) (Axelsson, 2004). Cell morphology of the cocci have spherical cells ranging in diameter from 0.5-3.5 µm, which occur singly or in pairs, chain and tetrads. Some cocci that are sometimes oval or even short rods and occur as coccobacilli, are classified in the genus *Leuconostoc*. The Gram-positive rod group was long, slender rods to coccobacilli, which variable in size and range from 0.5-1.2×1.0-11.0 µm, and cells are arranged in chains. One exception is the relatively recently described the genus *Weissella*, which is the first genus in LAB group that, by definition, 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* (Axelsson, 2004).

Table 2.1 Characteristics for different lactic acid bacterial genera.

Character	Rods		Cocci							
	Carno bacterium	*Lacto bacillus*	*Aero coccus*	*Entero coccus*	*Lacto coccus, Vago coccus*	*Leuco nostoc, Oeno coccus*	*Pedio coccus*	*Strepto coccus*	*Tetrageno coccus*	*Weissella*[a]
Tetrad formation	-	-	+	-	-	-	+	-	+	-
CO_2 from glucose[b]	-[c]	±	-	-	-	+	-	-	-	+
Growth at 10°C	+	±	+	+	+	+	±	-	+	+
Growth at 45°C	-	±	-	+	-	-	±	±	-	-
Growth at 6.5% NaCl	ND[d]	±	+	+	-	±	±	-	+	±
Growth at 18% NaCl	-	-	-	-	-	-	-	-	+	-
Growth at pH 4.4	ND	±	-	+	±	±	+	-	-	±
Growth at pH 9.6	-	-	+	+	-	-	-	-	+	-
Lactic acid[e]	L	D, L, DL[f]	L	L	L	D	L, DL[f]	L	L	D, DL[f]

+, positive; -, negative; ±, response varies between species; ND, not determined.
[a]: *Weissella* strains may also be rod-shaped.
[b]: Test for homo- or heterofermentation of glucose; negative and positive denotes homofermentative and heterofermentative, respectively.
[c]: Small amounts of CO_2 can be produced, depending on media.
[d]: No growth in 8% NaCl has been reported.
[e]: Configuration of lactic acid produced from glucose.
[f]: Production of D-, L- or DL-lactic acid varies between species.
Source: Axelsson (2004).

2.1.2.2 Colony morphology

Because of the low energy yields, LAB often more grow slowly than microbes capable of respiration, and produce smaller colonies. Colony size of LAB varies during growth, depending on species and growth form (medium, cultivation time, and growth condition) (Holzapfel and Wood, 1995). LAB grow tremendously fast when supplied with an abundance of nutrients. Different genera of LAB will produce different colony appearance. Some colonies may be colored. Some colonies are circular in shape, and others are irregular.

2.1.3 Habitats

LAB have complex nutrient requirements, due to their limited ability to synthesize B-vitamins and amino acids (Axelsson, 2004). Therefore, they are naturally found in nutrient-rich environments such as plants, foods (dairy products, fermented meat, sourdough, fermented vegetables, silage, beverages), sewage, and also in the genital, intestinal and respiratory tracts of human and animals (Hofvendahl and Hägerdal, 2000) (Table 2.2).

2.1.4 Carbohydrate metabolism of lactic acid bacteria

LAB are chemotrophs that obtain their energy from chemical substances. LAB 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 degraded mainly to lactic acid (homolactic fermentation) or to lactic acid and additional products such as acetic acid, ethanol, carbon dioxide (CO_2) (heterolactic

Table 2.2 Habitats of some lactic acid bacterial species.

Habitat	Source	Species[a]	Reference
Human and animals:			
Intestinal tract	Human and animals	*L. acidophilus*	Hansen and Mocquot (1970)
		L. reuteri	O'Sullivan *et al*. (2009)
		L. gasseri	
		L. salivarius	
	Human and chicken	*L. salivarius*	Rogosa *et al*. (1953)
	Poultry, cattle, dogs and cat	*E. faecium*	Holzapfel and Wood (1995)
Intestine	Chicken	*L. gallinarum*	Fujisawa *et al*. (1992)
		L. acidophilus	Morishita *et al*. (1971)
	Mouse	*L. delbrueckii*	Tannock (1990)
	Rat, foal, piglet and dog pup	*E. hirae*	Holzapfel and Wood (1995)
	Pig	*L. brevis*	Tannock (1990)
		L. fermentum	
	Cow	*S. bovis*	Narita *et al*. (2004)
		L. sakei	O'Sullivan *et al*. (2009)
Vagina	Human	*L. acidophilus*	Hansen and Mocquot (1970)
		L. crispatus	Hammes and Vogel (1995)
Faeces	Human	*L. crispatus*	Hammes and Vogel (1995)
		L. reuteri	
		L. johnsonii	O'Sullivan *et al*. (2009)
		L. acidophilus	
	Chicken, mice and pig	*L. johnsonii*	Fujisawa *et al*. (1992)
	Cow	*L. casei*	Holzapfel and Wood (1995)
Saliva	Human	*L. plantarum*	O'Sullivan *et al*. (2009)

Table 2.2 (Continued) Habitats of some lactic acid bacterial species.

Habitat	Source	Species[a]	Reference
Fermented food and feed:			
Dairy products	Yoghurt	*L. delbrueckii* subsp. *bulgaricus*	Orla-Jensen (1919); Weiss and Schillinger (1984); O'Sullivan *et al*. (2009)
		S. thermophilus	O'Sullivan *et al*. (2009)
	Cheese	*L. delbrueckii* subsp. *bulgaricus*	Orla-Jensen (1919);
		L. delbrueckii subsp. *lactis*	Weiss and Schillinger (1984)
		L. helveticus	Bergey *et al*. (1925); O'Sullivan *et al*. (2009)
	Fermented milk	*L. delbrueckii* subsp. *lactis*	Orla-Jensen (1919); Weiss and Schillinger (1984)
Fermented vegetable	Swine waste-corn	*L. amylophilus*	Nakamura and Cromwell (1979)
	Cattle waste-corn	*L. amylovourus*	Nakamura (1981)
	Sorghum ogi	*L. brevis*	Adesokan *et al*. (2009)
		L. fermentum	
	Burukuku	*L. delbruiekii*	
		Leu. messenterroides	
	Retted cassava (fufu)	*L. plantarum*	
		L. casei	
	Maize ogi	*L. acidophillus*	
	Corn starch production waste	*L. amylophilus*	Altaf *et al*. (2005)
	Fermented sorghum product	*L. plantarum* subsp. *argentoratensis*	Correia *et al*. (2010)
		L. brevis	
		L. paracasei subsp. *paracasei*	

Table 2.2 (Continued) Habitats of some lactic acid bacterial species.

Habitat	Source	Species[a]	Reference
Fermented food and feed:			
Fermented vegetable	Fermented sorghum product	*L. fermentum*	Correia *et al*. (2010)
		P. pentosaceus	
		S. thermophilus	
Fermented meat	Dry sausage	*L. curvatus*	Hammes and Vogel (1995)
	Sour dough	*L. reuteri*	Hammes and Vogel (1995)
		Lc. lactis subsp. *lactis*	Petrov *et al*. (2008)
Silage	Corn silage	*L. casei*	Cai *et al*. (2007)
		P. acidilactici	Torriani *et al.* (1987)
	Grass silage	*L. plantarum*	Ruser (1989)
		L. casei	
		L. graminis	Holzapfel and Wood (1995)
Spoiled food:			
Beverages	Apple juice	*L. mali*	Carr and Davies (1970)
	Wine must	*L. mali*	Carr and Davies (1970)
	Beer	*P. dextrinicus*	Russel and Walker (1953)
		L. casei	
		L. brevis	
Raw milk	Cow	*Lc. lactis*	Schleifer *et al*. (1985)
		Lc. cremoris	
		L. casei	Orla-Jensn (1919)
		S. bovis	
		A. viridans	Devriese *et al.* (1999)

[a]: *A.*, *Aerococcus*; *E.*, *Enterococcus*; *L.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *Leu.*, *Leuconostoc*; *P.*, *Pediococcus*; *S.*, *Streptococcus*.

fermentation) (Figure 2.1). 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 *L. casei*, transmembrane transport of sugars requires phosphorylation and the process is dependent on the phosphoenol pyruvate phosphotransferase (PEP/PTS) system. The biochemical pathway of lactose which is the basis of the industrial application of lactococci has been elucidated, certain thermotolerant lactobacilli (*L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis*, *L. acidophilus*) and *S. thermophilus* metabolize only the glucose moiety of lactose, while in lactococci, the galactose moiety of lactose is metabolized by the tagatose-6-phosphate pathway (Figure 2.2). Some LAB could also transport galactose by a permease system and process this hexose through the Leloir pathway (Figure 2.3).

2.1.5 Starch-utilizing and lactic acid-producing bacteria

Starch is one of the most common storage compounds, and generally consists of 25% amylose and 75% amylopectin (Moat *et al*., 2002). Amylose is a long and unbranched chain of glucose in α-(1,4) linkage whereas amylopectin is a highly branched form of starch in which the backbone consists of glucose chains in α-(1,4) linkage with α-(1,6) linkages at the branch points. Amylose can be hydrolyzed by α-amylase, which cleaves the α-(1,4) linkages to yield a mixture of α-glucose and α-maltose. Amylose is also hydrolyzed by β-amylase producing β-maltose. These enzymes also hydrolyze amylopectin to yield glucose, maltose, and a branched core, but it is not completely degradation. The α-(1,6) linkage in branch is hydrolyzed by α-(1,6)-glucosidase.

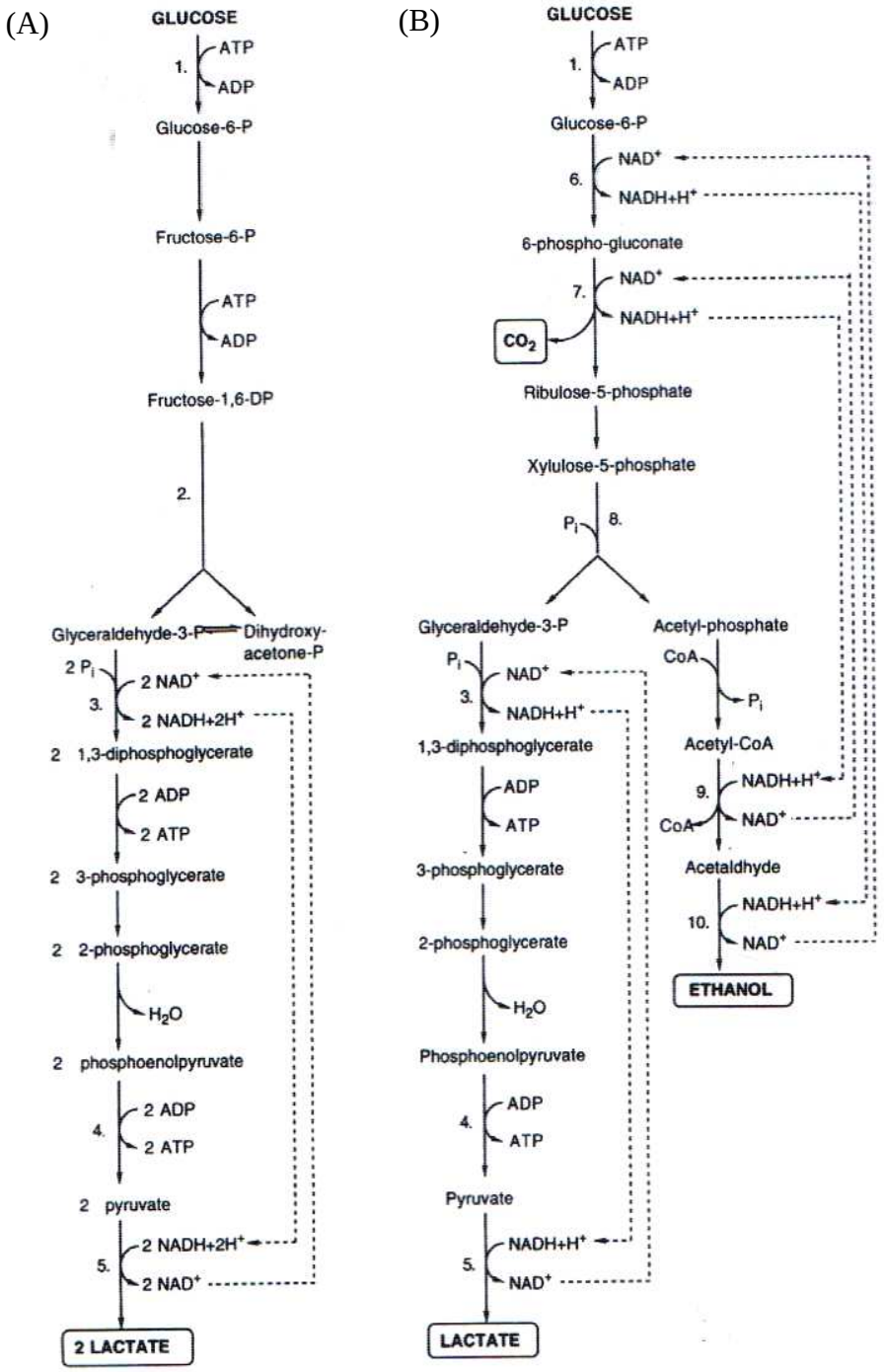


Figure 2.1 Major fermentation pathways of glucose: (A) homolactic fermentation (Glycolysis pathway); (B) heterolactic fermentation (Phosphoketolase pathway). 1, Glucokinase; 2, Fructose-1,6-diphosphate aldolase; 3, Glyceradehyde-3-phosphate dehydrogenase; 4, Pyruvate kinase; 5, Lactate dehydrogenase; 6, Glucose-6-phosphate dehydrogenase; 7, 6-Phosphogluconate dehydrogenase; 8, Phosphoketolase; 9, Acetaldehyde dehydrogenase; 10, Alcohol dehydrogenase.
Source: Axelsson (2004).

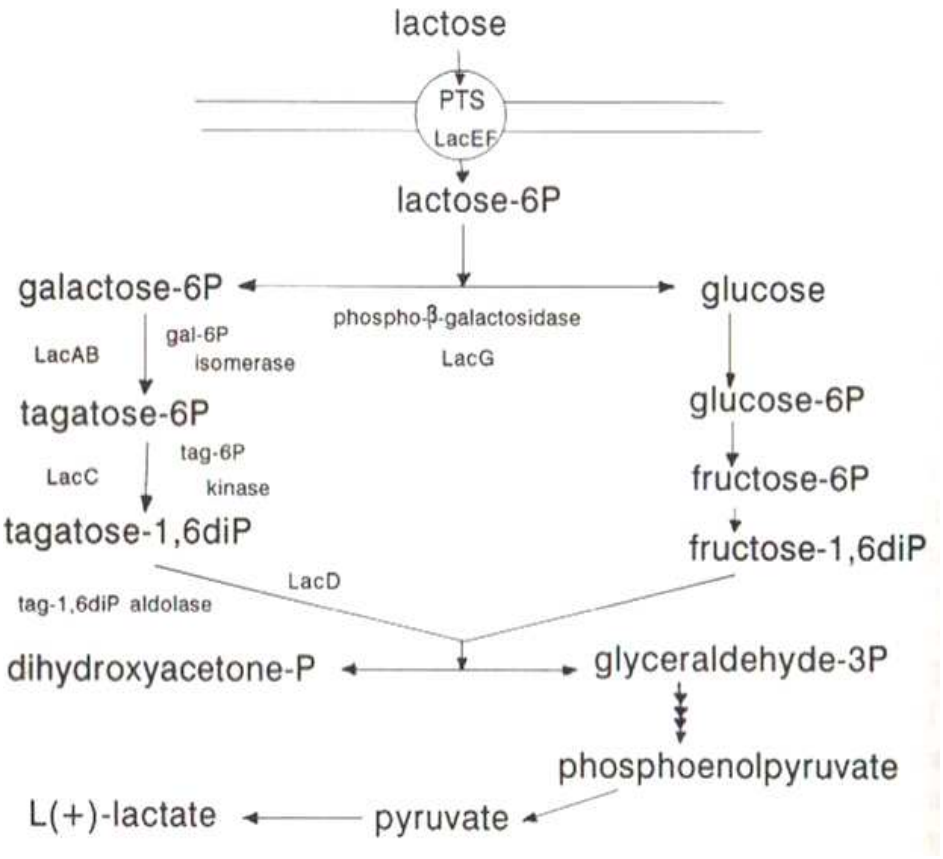


Figure 2.2 Pathway of L(+)-lactic acid production from lactose by an industrial *Lactococcus lactis* strain. This strain contains a lactose PTS, lactose enters the cytoplasm as lactose 6-phosphate. Then lactose 6-phosphate is cleaved by phospho-*β*-galactosidase to yield glucose and galactose-6-phosphate. 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 phospho-*β*-galactosidase are generally inducible and repressed by glucose.

Source: Holzapfel and Wood (1995).

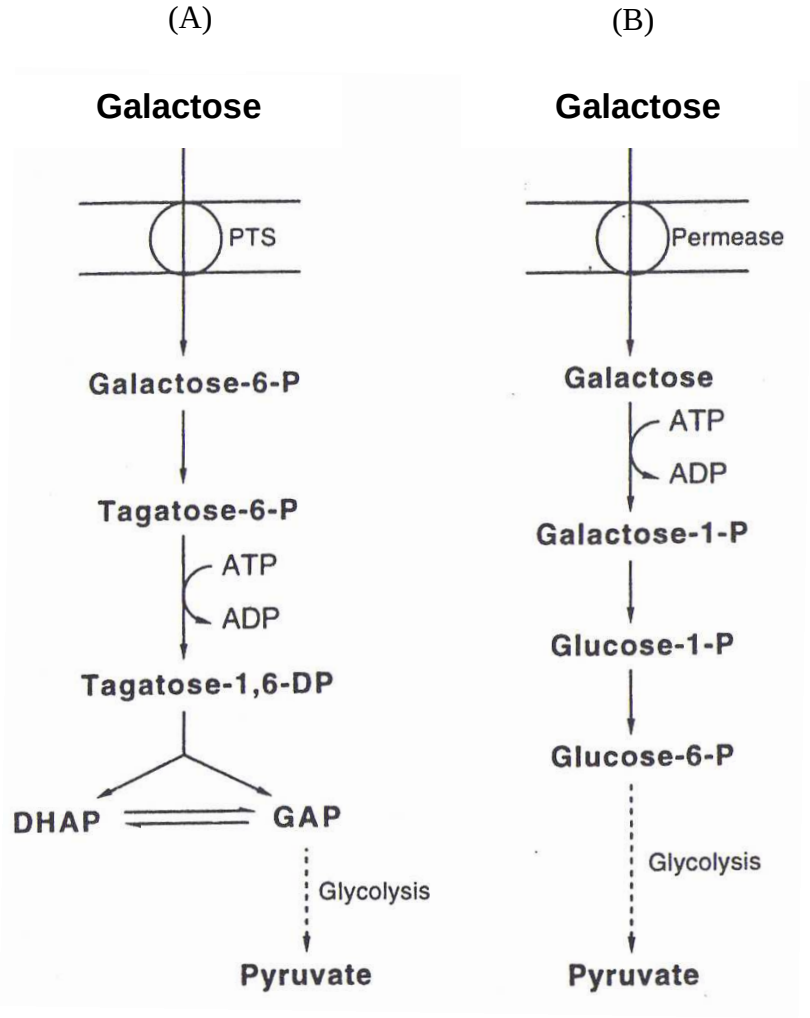


Figure 2.3 Galactose metabolism in lactic acid bacteria: tagatose-6-phosphate pathway (A); Leloir pathway (B).

Source: Axelsson (2004).

Thus, the combined action of *α*-(1,6)-glucosidase and *α*-amylase is required to completely degrade amylopectin to glucose and maltose (Sansit, 2004).

Lactic acid fermentation with emphasis on the use of starch or starchy substrates, was also reported. In most cases, starch cannot be used by LAB directly, and the large starch macromolecules are converted into glucose molecules by

treatment with acid or enzymes. 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). The hydrolysis occurs in two steps. First, the insoluble starch has to be liquefied at high temperatures (80-85°C) using an amylase, and, secondly, the liquefied starch has to be degraded down to glucose by a glucoamylase at medium temperatures (55°C) (Venus, 2006). Amylolytic LAB can ferment different types of amylaceous raw material, such as potato starch (Giraud *et al*., 1991; Petrov *et al*., 2008), corn starch (Nakamura, 1981; Zhang and Cheryan, 1991; Mercier *et al*., 1992; Vishnu *et al*., 2000; Narita *et al*. 2004; John *et al*., 2007), sago starch (Shibata *et al*., 2007), sorghum flour, wheat flour, cassava flour, rice flour and barley flour (Vishnu *et al*., 2002) due to the ability of their *α*-amylases to partially hydrolyze raw starch. Amylolytic LAB utilize starchy biomass and convert into lactic acid in a single step fermentation. Although amylolytic LAB are able to simultaneously hydrolyze and ferment starch to lactic acid, however, only a few amylolytic LAB have been reported on their lactic acid production ability, such as *Lactobacillus plantarum* (Giraud *et al*., 1991; 1994; Panda and Ray, 2008), *L. manihotivorans* (Guyot *et al*., 2000; Ohkouchi and Inoue, 2006), *L. amylophilus* (Yumoto and Ikeda, 1995; Vishnu *et al*., 2000; 2002; John *et al*., 2007; Yen and Kang, 2010), *L. amylovorus* (Zhang and Cheryan, 1991; 1994; Linko *et al*., 1996; Nagarjun *et al*., 2005), *L. fermentum* Ogi E1 (Santoyo *et al*., 2003), *Lactococcus lactis* subsp. *lactis* (Petrov *et al*., 2008), *Enterococcus faecium* (Shibata *et al*., 2007), and *Streptococcus bovis* (Narita *et al*., 2004).

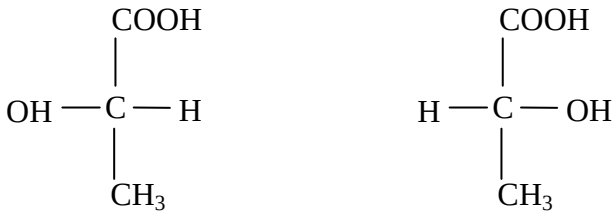
2.2 L-Lactic acid

L-Lactic acid (2-hydroxypropanoic acid), is the most widely occurring natural organic acid in many foods, both naturally or as a product of *in situ* microbial fermentation (as in sauerkraut, yogurt, buttermilk, sourdough breads, and many other fermented foods). Lactic acid is also a major metabolic intermediate in most living organisms (Datta *et al*., 1995).

2.2.1 Chemical structure and property of L-lactic acid

L-Lactic acid is a mirror image of D-lactic acid which could be soluble in water (Narayanan *et al*., 2004). It exhibits low volatility and has chemical formula of $C_3H_6O_3$ (Figure 2.4). L(+)-Lactic acid differs from D(-)-lactic acid 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 salt solution such as $Ca(OH)_2$ and $CaCO_3$ (Narayanan *et al*., 2004). Excess $CaCO_3$ is added to the supernatant to neutralize the acid produced and produce a calcium salt of the acid in the broth. The broth containing calcium lactate could improve purification method of lactic acid fermentation (Datta *et al*., 1995).

Other properties of lactic acid and isomer of lactic acid are summarized in Table 2.3. Both enantiomers of lactic acid have the same physical properties.



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

Figure 2.4 Enantiomers of lactic acid; L(+)-lactic acid and D(-)-lactic acid.

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

Table 2.3 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	$1.37x10^{-4}$
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).

The melting point of L(+)-lactic acid is reported at 52.8°C, and that of racemic lactic acid is 16.8 to 25.5°C. The pure isomers form colorless monoclinic crystals having a melting point of 54°C. A synthetic racemic lactic acid prepared by mixing equal quantities of the D(-)- and L(+)-isomers melts in the range of 28 to 33°C. Lactic acid is a weak acid that both isomers and the racemic mixture have the same dissociation constants and pK_a (Lockwood *et al*., 1965).

The presence of two functional groups, hydroxyl and carboxyl, permits a wide variety of chemical reactions for lactic acid. The primary classes of these reactions are oxidation, reduction, condensation, and substitutions.

2.2.2 Sources of L-lactic acid

L-Lactic acid can be manufactured by either chemical synthesis or microbial fermentation, both are used for commercial production. For example, in Japan, lactic acid is manufactured synthetically by means of the lactonitrile route by Musashino Chemical Co. Carbohydrate fermentation technology is used by Archer Daniels Midland Company (ADM) and NatureWorks LLC (Datta and Tsai, 1996).

2.2.2.1 Chemical synthesis

The chemical synthesis routes produce only the racemic lactic acid. The commercial process is based on lactonitrile, which used to be a byproduct from acrylonitrile synthesis (Figure 2.5). Lactonitrile produced by combining of hydrogen cyanide reaction and occurs at atmospheric pressures. The crude lactonitrile is then recovered and purified by distillation and is hydrolyzed to lactic acid by using either concentrated hydrochloric or sulfuric acid, producing the corresponding ammonium salt as a byproduct. This crude lactic acid is esterified with methanol, producing methyl lactate, which is recovered and purified by distillation, and hydrolyzed by water under acid catalysts to produce lactic acid, which is further concentrated, purified, and shipped under different product classifications. The raw materials and processing costs do not lend support to this chemical synthesis approach

for large-scale, low-cost manufacturing in the future (Datta and Tsai, 1996). This process is represented by the following reactions:

(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

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

(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 2.5 Lactic acid production process via chemical synthesis.

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

There are other possible routes for chemically synthesizing of lactic acid, for example: oxidation of propylene glycol; reaction of acetaldehyde, carbon monoxide, and water at elevated temperatures and pressures; hydrolysis of chloropropionic acid (prepared by chlorination of propionic acid), and nitric acid oxidation of propylene. However, none of these routes has led to technically and economically viable processes (Datta *et al*., 1995).

2.2.2.2 Microbial fermentation

Microorganisms that can produce lactic acid, can be divided into two groups: bacteria and fungi. Zhou *et al*. (1999) reported that *Rhizopus oryzae* ATCC 52311 utilize glucose aerobically to produce only L-lactic acid. However in industrial fermentations, the use of various species of *Lactobacillus* is preferred owing to higher rates of metabolism and increased yields. The microorganisms selected for investigations of the biotechnological production of L-lactic acid are listed in Table 2.4.

Table 2.4 Potential L-lactic acid producing-microorganisms.

Microorganism	**Substrate concentration (g/l)**	**L-Lactic acid concentration (g/l)**	**Reference**
Rhizopus oryzae ATCC 52311	Glucose (94)	83.0	Zhou *et al*. (1999)
Rhizopus oryzae	Glucose (120)	112.0	Efremenko *et al*. (2006)
Bacillus sp. 2-6	Glucose (121.3)	118.0	Qin *et al*. (2009)
Aspergillus niger SL-09 and *Lactobacillus* sp. G-02	Jerusalem artichoke flour (200)	120.5	Ge *et al*. (2009)
Entercoccus faecalis RKY1	Glucose (100)	97.0	Yun *et al*. (2003)
Lactobacillus delbrueckii subsp. *lactis* DSM20073	Glucose (72)	52.0	Michelson *et al*. (2006)
Lactobacillus casei	Glucose (140)	112.5	Ding and Tan (2006)
Lactobacillus delbrueckii NCIM 2365	Hydrolyzed cane sugar (150)	135.0	Kadam *et al*. (2006)
Lactobacillus amylophilus GV6	Cassava starch (60)	33.6	Vishnu *et al*. (2002)
	Corn starch (60)	49.0	Vishnu *et al*. (2000)
Lactobacillus plantarum MTCC1407	Sweet potato flour (55)	23.86	Panda and Ray (2008)
Lactococcus lactis subsp. *lactis* B84	Soluble potato starch (18)	5.5	Petrov *et al*. (2008)
Streptococcus bovis 148	Corn starch (20)	14.7	Narita *et al*. (2004)

LAB could produce either L- or D-lactic acid or racemic mixture of lactic acid by fermentation depending on species. Lactate dehydrogenase is a key enzyme in lactic acid fermentation by most LAB. Most bacterial species possess only one lactate dehydrogenase gene (Rodtong and Ishizaki, 2003). L-Lactic acid-producing bacteria contain L-lactate dehydrogenase (L-LDH) which is a key enzyme converting pyruvate to L-lactic acid.

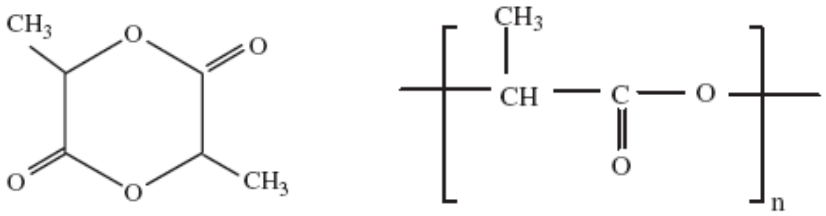
2.2.3 Industrial application of L-lactic acid

L-Lactic acid is a versatile chemical having a wide range of applications in food, pharmaceutical, leather, textile industries. It also functions as the monomer for the biodegradable plastics (John *et al*., 2007).

2.2.3.1 Biodegradable plastics

Biodegradable poly(L-lactic acid) (PLLA or PLA) is a biodegradable polymer approved for use in food packaging in several countries, particularly U.S.A., European Union countries, and Japan (Narayanan *et al*., 2004). There is an increased interest in degradable plastics because of environmental concerns over plastics disposal. Conventional plastic materials are not easily degraded in the environment because of their high molecular weight and hydrophobic character (Kharas *et al*., 1994). As a kind of biodegradable polymer, PLLA might become a potential environmentally friendly substitute of non-biodegradable plastics derived from petrochemicals (Lu *et al*., 2009). PLLA is aliphatic polyesters and belongs to the α-hydroxy group, which is the most readily biodegradable thermoplastics material (Kharas *et al*., 1994). L-Lactic acid contains both hydroxyl and carboxylic acid

functional groups. This substance can thus undergo self-esterification to form a cyclic dimer, L-lactide and a linear polymer, poly(L-lactide) (Figure 2.6) (Lockwood *et al*., 1965).



L-Lactide, (Cyclic dimer)

Poly(L-lactic acid) (PLLA), (Linear polymer)

Figure 2.6 Structure of L-lactide and poly(L-lactic acid).

Source: Nair and Laurencin (2007).

The conversion of L-lactic acid to high-molecular weight PLLA is achieved by ring-opening polymerization, polycondensation or other methods (chain extension, grafting). The ring-opening polymerization has been prepared by dehydration of L-lactic acid into relatively low molecular weight polyester which is converted into L-lactide by depolymerization. Then, purified L-lactide monomer is converted into poly(L-lactide) by catalytic ring-opening polymerization (Kharas *et al*., 1994). The polycondensation prepared by dehydration of L-lactic acid by condensation, followed by chain extention (Södergård and Stolt, 2002; Narayanan *et al*., 2004).

PLLA is a semi crystalline polymer (approximately 37% crystallinity) and the degree of crystallinity depends on the molecular weight and polymer processing parameters. It has a glass transition temperature of 60-65°C and

a melting temperature of approximately 175°C (Middleton and Tipton, 2000). It is sensitive to heat, especially at temperatures higher than 190°C (Kharas *et al*., 1994). PLLA has a good tensile strength, low extension and a high modulus (approximately 4.8 Giga Pascal, GPa) (Nair and Laurencin, 2007). However, the thermal stability of PLLA is not sufficiently high to some applications as an alternative of commercial polymers. Thermal processes such as melt molding and spinning cause of thermal degradation of PLLA (Tsuji and Fukui, 2003). The melting temperature of PLLA can be increased to 230°C by physically blending the polymer with poly(D-lactic acid) (PDLA) (Ikada *et al*., 1987). It also finds applications in the preparation of biodegradable polymers for medical uses such as surgical sutures, prostheses, and controlled drug delivery systems (Wee *et al*., 2006).

However, being more hydrophobic than polyglycolide, the degradation rate of PLLA is very low. It has been reported that high molecular weight PLLA can take between 2 and 5.6 years for total resorption *in vivo* (Bergsma *et al*., 1995; Middleton and Tipton, 2000). The rate of degradation, however, depends on the degree of polymer crystallinity as well as the porosity of the matrix.

2.2.3.2 Food industry

The major use of L-lactic acid is in food and food-related applications. Food and food-related applications account for approximately 85% of the demand for lactic acid whereas the non-food industrial applications account for only 15% of the demand. As a food acidulant, lactic acid has a mild acidic taste in contrast to other food acids. Lactic acid is non-volatile, odorless, and is classified as generally recognized as safe (GRAS) for use as a general purpose food additive by the

FDA in the U.S.A. and other regulatory agencies elsewhere. It is a very good preservative and pickling agent for sauerkraut, olives, and pickled vegetables. It is used as acidulant, flavoring, pH buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods, such as candy, bread and bakery products, soft drinks, soups, sherbets, dairy products, beer, jams and jellies, mayonnaise, and processed eggs, often in conjunction with other acidulants. An emerging new use for lactic acid or its salts is in the disinfection and packaging of carcasses, particularly those of poultry and fish, where the addition of aqueous solutions of lactic acid and its salts during the processing increased shelf life and reduced the growth of anaerobic spoilage organisms such as *Clostridium botulinum*. A large fraction (>50%) of the lactic acid for food-related uses goes to produce emulsifying agents used in foods, particularly for bakery goods (Datta *et al*., 1995).

2.2.3.3 Pharmaceutical and cosmetic applications

The water-retaining capacity of lactic acid makes it suitable for use as moisturizer in cosmetic formulations. The ability of lactic acid to suppress the formation of tyrosinase is responsible for its effects like skin lightening and rejuvenation. As humectants, the lactates are often superior to natural products and more effective then polyols (Datta *et al*., 1995). Ethyl lactate is the active ingredient in many anti-acne preparations. The natural occurrence of lactic acid in human body makes it very useful as an active ingredient in cosmetics (Wee *et al*., 2006).

L-Lactic acid is used in pharmaceutical industry as a very important ingredient. Pharmaceutical industry shows presence for L-lactic acid because the D-isomer is not metabolized by human body. Lactic acid and its salts have

been mentioned for various medical uses. They provide the energy and volume for blood besides regulation of pH. Lactic acid involves medical applications as an intermediate for pharmaceutical manufacture, for adjusting the pH of preparations and in tropical wart medications (Wee *et al*., 2006).

2.2.3.4 Other industries

Lactic acid is also used in some other industries. For example, technical-grade lactic acid is extensively used in leather tanning industries as an acidulant for deliming hides and in vegetable tanning. Lactic acid is used as descaling agent, solvent, cleaning agent, slow acid-releasing agent and humectants in a variety of technical processes (John *et al*., 2007).

L-Lactic acid could be potentially used for the manufacturing of large-volume oxygenated chemicals, such as propylene glycol, propylene oxide, acrylic acid, and acrylate esters, and other chemical intermediates such as lactate ester plasticizers. The advances made in hydrogenolysis technology can be further developed and integrated to make propylene glycol from lactic acid in the future (Datta and Henry, 2006).

2.2.4 Production of L-lactic acid by lactic acid bacteria

L-Lactic acid is the most important organic acid produced by LAB. Several species in genera *Carnobacterium*, *Aerococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Pediococcus*, *Tetragenococcus*, and *Vagococcus* could potentially produce L-lactic acid (Table 2.1) (Axelsson, 2004). Fermentative production of lactic acid from biomass has recently been reviewed besides other

reviews on microbiological production and biotechnological production of L-lactic acid (Datta *et al*., 1995; Wee *et al*., 2006; John *et al*., 2007). Development of production strains which ferment starch to lactic acid in a single step is necessary to make the process economical. Amylolytic LAB can convert the starch directly into lactic acid (Reddy *et al*., 2008). A few bacteria have been reported so far for direct fermentation of starch to lactic acid.

2.2.4.1 Substrates

A number of different substrates have been used for the fermentative production of lactic acid by LAB. Since LAB are unable to synthesize ATP by respiration, and that have lactic acid as the major end product from energy-conserving fermentation of sugars. The pure product is obtained when a pure sugar is fermented, resulting in the reduction of purification costs. However, this is economically unfavorable, because pure sugars are expensive, compared to lactic acid produced. Instead waste products from agriculture and forestry are utilized (Hofvendahl and Hahn-Hägerdal, 2000). In order to produce lactic acid to be feasible, cheap raw materials are necessary, because polymer producers and other industrial users usually require large quantities of lactic acid at a relatively low cost. Raw materials for lactic acid production should have the following characteristics: low cost, low levels of contaminants, rapid production rate, high yield, little or no byproduct formation, ability to be fermented with little or no pretreatment, and year-round availability. When refined materials are used for production, the costs for product purification should be significantly reduced. However, this is still economically unfavourable because the refined carbohydrates are so expensive that

they eventually result in higher production costs (Hofvendahl and Hahn-Hägerdal, 2000). Therefore, there have been many attempts to screen for cheap raw materials for the economical production of lactic acid (Wee *et al*., 2006). Reports in the literature of recent investigations are listed in Table 2.5.

Cheap raw materials, such as starchy and cellulosic materials, whey, and molasses, have been used for lactic acid production (Hofvendahl and Hahn-Hägerdal, 2000). Among these, starchy and cellulosic materials are currently receiving a great deal of attention, because they are cheap, abundant, and renewable (Richter and Berthold, 1998). The starchy materials used for lactic acid production include sorghum flour (Richter and Träger, 1994; Vishnu *et al*., 2002), wheat flour (Hofvendahl and Hahn-Hägerdal, 1997; Oh *et al*., 2005), corn starch (Vishnu *et al*., 2000; Narita *et al*. 2004; Oh *et al*., 2005; John *et al*., 2007), cassava starch (Xiaodong *et al*., 1997; Vishnu *et al*., 2002), sago starch (Shibata *et al*., 2007), potato starch (Giraud *et al*., 1991; Yun *et al*., 2004; Petrov *et al*., 2008), rice flour (Vishnu *et al*., 2002; Yun *et al*., 2004), and barley (Linko and Javanainen, 1996; Vishnu *et al*., 2002). These materials have to be hydrolyzed into fermentable sugars before fermentation, because they consist mainly of α(1,4)- and α(1,6)-linked glucose (Richter and Träger, 1994; Hofvendahl and Hahn-Hägerdal, 1997; Oh *et al*., 2005). This hydrolysis can be carried out simultaneously with fermentation.

Some industrial waste products, such as whey and molasses, are of interest for common substrates for lactic acid production. Whey is a major byproduct of the dairy industry, and it contains lactose, protein, fat, and mineral salts. For complete utilization of whey lactose, it is necessary to supplement whey with an additional nitrogen source (Hofvendahl and Hahn-Hägerdal, 2000). Schepers *et al*.

Table 2.5 Substrates for L-lactic acid production.

Substrate concentration (g/l)	**Microorganism[a]**	**Fermentation mode**	**Fermentation time**	**L-Lactic acid concentration (g/l)**	**Reference**
Glucose (72)	*L. delbrueckii* subsp. *lactis* DSM20073	Batch	24 h	52.0	Michelson *et al.* (2006)
Sorghum flour (60)	*L. amylophilus* GV6	Batch	4 days	29.4	Vishnu *et al.* (2002)
Sugar molasses (130)	*E. faecalis* RKY1	Batch	12 h	65.1	Wee *et al.* (2004)
Hydrolyzed wheat flour (200)	*E. faecalis* RKY1	Batch	24 h	102.0	Oh *et al.* (2005)
Hydrolyzed corn starch (200)		Batch	24 h	63.5	
Cassava flour (60)	*L. amylovorus* GV6	Batch	4 days	33.6	Vishnu *et al.* (2002)
Rice bran (300)	*Lactobacillus* sp. RKY2	Batch	12 h	129.0	Yun *et al.* (2004)
(60)	*L. amylophilus* GV6	Batch	4 days	30.9	Vishnu *et al.* (2002)
Barley starch (170)	*L. casei* NRRL B-441	Batch	47 h	162.0	Linko and Javanainen (1996)
(60)	*L. amylophilus* GV6	Batch	4 days	27.3	Vishnu *et al.* (2002)
Sago starch (20)	*E. faecium* No.78	Batch	24 h	16.6	Shibata *et al.* (2007)
	L. amylovorus JCM 1126	Batch	24 h	14.3	
	L. manhotivorans JCM 12514	Batch	24 h	11.0	
Sweet potato starch (20)	*L. amylophilus* BCRC 14055	Batch	120 h	21.62	Yen and King (2010)
Soluble starch (15.2)	*L. amylophilus* GV6	Batch	48 h	13.5	Altaf *et al.* (2007b)
Soluble potato starch (18)	*Lc. lactis* subsp. *lactis* B84	Batch	6 days	5.5	Petrov *et al.* (2008)

[a]: *E.*, *Enterococcus*; *L.*, *Lactobacillus*; *Lc.*, *Lactococcus*.

(2002) supplemented whey with yeast extract for rapid production of lactic acid with *Lactobacillus helveticus*. Also, there have been several attempts to produce lactic acid from whey by batch culture of *L. casei* (Büyükkilci and Harsa, 2004). Molasses is a waste product from the sugar manufacturing process, and it usually contains a large amount of sucrose (Hofvendahl and Hahn-Hägerdal, 2000). *Lactobacillus delbrueckii* and *Enterococcus faecalis* have been used for lactic acid production from molasses (Kotzanmanidis *et al*., 2002).

2.2.4.2 Production process

A) Factors affecting L-lactic acid fermentation

Parameters including microorganism, carbon and nitrogen sources, fermentation mode, pH, and temperature affect the fermentative L-lactic acid production. The potential production of the acid is compared in terms of L-lactic acid concentration, yield, and productivity. Also byproduct formation and isomer of L-lactic acid have been reported (Holvendahl and Hahn-Hägerdal, 2000).

The amount of L-lactic acid produced by LAB are strongly influenced by cultures and fermentation conditions. Some main factors affecting the L-lactic acid production are as follows:

a) Chemical factors

LAB typically have complex nutritional requirements, due to their limited ability to synthesize their own growth factors such as B vitamins 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). There 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 L-lactic acid production. Carbon sources are mainly carbohydrates utilized for L-lactic acid manufacture which are generally derived from sucrose (from syrups, juices and molasses), lactose (from whey), maltose (produced by specific enzymatic starch conversion processes), glucose (from starch conversion processes). A number of literatures reported on the capability of several bacterial strains to produce L-lactic acid by using molasses, whey, starchy, and cellulosic materials. The starchy materials used for lactic acid production include wheat, corn, sago, potato, rye, sweet sorghum, wheat, tapioca, potato, rice, and barley as a carbon source.

A number of nitrogenous materials like whey permeate, yeast extract, malt sprouts, malt combing nuts, grass extract, peptones, beef extract, casein hydrolysate, corn steep liquor, soybean hydrolysate with supplementation of vitamins to supplement carbohydrate sources to give fast and heavy growth have been studied. However, yeast extract seems to be the most effective supplement **(**Narayanan *et al*., 2004). The compound is a principal growth factor for LAB. Some low-cost nutrients, such as soy protein hydrolyzate (Hsieh *et al*., 1999), byproducts from malting industry (Hujanen and Linko, 1996; Pauli and Fitzpatrick, 2002; Fitzpatrick *et al*., 2003), bacterial extract (Rivas *et al*., 2004; Gao *et al*., 2006), ram horn protein hydrolyzate (Kurbanoglu and Kurbanoglu, 2003), fish wastes (Martone *et al*., 2005; Gao *et al*., 2007), corn steep liquor (Rivas *et al*., 2004; Lee, 2005; Oh *et al*., 2005), whey protein hydrolyzate (Fitzpatrick and O'Keeffe, 2001), red lentil flour

and bakers yeast (Altaf *et al*., 2005, 2006, 2007a, and 2007b), and rice bran (Yun *et al*., 2004; Gao *et al*., 2008), have been put under screening for lactic acid production.

b) Physical factors

The main physical factors reported to influence lactic acid fermentation are pH and temperature. 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 the lactic acid concentrations essential for an economical process. In general, LAB can 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). The optimum pH for cell growth and lactic acid production of *Lactobacillus plantarum* was shown to be between 5.0 and 6.8 (Yumoto and Ikeda, 1995; Fu and Mathews, 1999; Ray *et al*., 2009). For *L. amylophilus* (Vishnu *et al*., 2000) and *L. lactis* (Bai *et al*., 2004) could produce lactic acid at pH 6.5. For *L. manihotivorans* had optimum pH at 5.0-6.0 (Guyot *et al*., 2000; Ohkouchi and Inoue, 2006). *Lactococcus lactis* subsp. *lactis* could produce lactic acid at pH 6.0 (Petrov *et al*., 2008). The strain *Enterococcus faecium* was produced lactic acid of pH 6.5 (Shibata *et al*., 2007). For *Streptococcus bovis* grew at pH between 5.8-9.6 with the optimum pH at 5.5-6.0 (Narita *et al*., 2004; Yuwono and Kokugan, 2008).

Most LAB grow best at temperatures between 37 and 42°C (Kharas *et al*., 1994). Effects of temperature on L-lactic acid production are highly variable, and are depend on the strain being used and the experimental conditions.

The effect of temperature on the production of L-lactic acid has only been studied in a few reports. The temperature giving the highest productivity was in some cases lower than the temperature resulting in high L-lactic acid concentration and yield (Hofvendahl and Hahn-Hägerdal, 2000), whereas, in others, the same temperature gave the best results in all categories (Hujanen and Linko, 1996). For *L. amylophilus*, which is known to grow at 15°C but not at 45°C (Hammes and Vogel, 1995), the optimal temperatures were 25 and 35°C for the maximum productivity and yield, respectively. Strain *L. amylophilus* showed its optimum temperature at 30°C (Yen and King, 2010). For *L. casei* and *L. paracasei*, their optimal temperatures were reported to be between 37 and 44°C (Richter and Träger, 1994), which is contradictory to the information that the strains grow at 15°C but not at 45°C (Hammes and Vogel, 1995). For strain *L. casei* LA-04-1, its optimal temperature was reported at 42°C (Ding and Tan, 2006). In agreement with previous observations, *Lactococcus lactis* and *L. rhamnosus* exhibited the highest yields and productivities at 33 to 35°C and 41 to 45°C, respectively (Hujanen and Linko, 1996). For strain *Lactococcus lactis* subsp. lactis, the optimal temperature was reported at 33°C (Petrov *et al*., 2008). For the optimal temperature was reported to be 37°C of *L. casei* (Linko and Javanainen, 1996), *L. amylophilus* (Vishnu *et al*., 2000), *L. helventicus* (Aeschlimann and Von Stockar, 1990), *L. amylovorus* (Shibata *et al*., 2007) and *L. lactis* (Bai *et al*., 2004). For strain *Enterococcus faecium* was produced lactic acid at 30°C (Shibata *et al*., 2007). For *Streptococcus*, its generally grows within the range 20-42°C (Hardie and Whiley, 1995). A strain of *Streptococcus bovis*, the optimal temperature was reported to be 37-39°C (Narita *et al*., 2004; Yuwono and Kokugan, 2008).

B) Process configuration

Batch, fed-batch, repeated batch, and continuous fermentations are the most frequently used process for lactic acid production. 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 (Hujanen and Linko, 1996; Holvendahl and Hahn-Hägerdal, 2000). Reports in the literature of recent studies on the biotechnological production of lactic acid by different fermentation approaches are listed in Table 2.6.

For example, *Enterococcus faecium* No.78 was selected for L-lactic acid production using sago starch in batch and continuous fermentations. During fermentation of the batch process, the bacterium fermented sugar to create pyruvate then pyruvate was converted to L-lactic acid via L-lactate dehydrogenase under anaerobic condition. L-Lactic acid could also be produced from starchy substrates by the simultaneous saccharification and fermentation (SSF). Such a combined process of converting enzymically liquefied starch to glucose with α-amylase and glucoamylase, and glucose to lactic acid by LAB, would be expected to decrease the total process time, and the capital and operating costs (Linko and Javanainen, 1996).

The process of lactic acid production from starch was achieved by the use of amylolytic LAB, possessing extracellular amylase activity. Very few reports are available on isolation of amylolytic LAB for single step fermentation of inexpensive complex carbohydrates (starch) to lactic acid. Use of efficient amylolytic lactic acid-producing bacteria were eliminated saccharification costs of substrate thereby reducing the production cost (Reddy *et al*., 2008).

Table 2.6 L-Lactic acid production by lactic acid bacteria using different fermentation modes.

Microorganism[a]	Fermentation mode	Fermentation time	Substrate concentration (g/l)	L-Lactic acid concentration (g/l)	Reference
L. casei subsp. *rhamnosus*	Batch	50 h	Glucose (50)	57.0	Olmos-Dichara *et al.* (1997)
L. rhamnosus LA-04-1	Batch	25 h	Glucose (130)	90.0	Li *et al.* (2010)
	Fed-batch	63 h	Glucose (770)	170.0	
L. casei LA-04-1	Batch	84 h	Glucose (140)	112.5	Ding and Tan (2006)
	Fed-batch	84 h	Glucose (850)	180.0	
Lc. lactis subsp. *lactis* ATCC 19435	Batch	48 h	Glucose (85)	65.0	Åkerberg *et al.* (1998)
L. lactis BME5-18M	Batch	112 h	Glucose (150)	150.2	Bai *et al.* (2004)
	Fed-batch	80 h	Glucose (600)	161.2	
L. amylovorus GV6	Batch	96 h	Cassava flour (60)	33.6	Vishnu *et al.* (2002)
			Sorghum flour (60)	29.4	
			Wheat flour (60)	29.9	
			Rice flour (60)	30.9	
			Barley starch (60)	27.3	
L. amylophilus BCRC 14055	Bacth	120 h	Sweet potato starch (40)	29.1	Yen and King (2010)
	Fed-bacth	84 h	Sweet potato starch (70)	43.7	
E. faecium No.78	Batch	24 h	Sago starch (20)	16.6	Shibasa *et al.* (2007)
	Continuous	84 h		17.9	
E. faecalis RKY1	Batch	24 h	Hydrolyzed wheat flour (200) + corn steep liquor (15)	102.7	Oh *et al.* (2005)

[a]: *E.*, *Enterococcus*; *L.*, *Lactobacillus*; *Lc.*, *Lactococcus*.

2.2.5 Extraction and purification of L-lactic acid from fermentation medium

The extraction and purification of L-lactic acid from fermentation broth are important for obtaining L-lactic acid. Several methods are available for the purification of lactic acid from fermentation media. Lactic acid is removed from its fermentation broth by a series of separation steps, such as precipitation, filtration, acidification, purification using activated carbon, evaporation and crystallization (Yi *et al*., 2008). The classical methods are based on precipitation, extraction or distillation (Lazarova and Peeva, 1994; Vaccari *et al*., 1993). The extraction and purification steps consisted of a series of successive precipitation with $CaCO_3$, butanol esterification, purification by carbon columns, ion exchange, and evaporated (Datta *et al*., 1995). Recently, various attempts have been carried out to remove the lactic acid simultaneously as it is formed. Extraction method is some of the viable alternative for separation of lactic acid from the fermentation broths. The lactic acid separation by extraction was studied with trioctyl amine in methyl isobutyl ketone (Choudhury and Swaminathan, 1998), tripropylamine dissolved in isoamyl alcohol (Uslu *et al*., 2009), 1-decylaldehyde in tri-n-decylamine (Gao *et al*., 2009). Hano *et al*. (1993) studied the reactive extraction of lactic acid from the fermented broth. They indicated that *in situ* extraction was possible with the use of di-noctyl-amine and with adjustment of the fermentation broth to a pH 5.0 by ammonia.

Iyer and Lee (1999) attempted to extract lactic acid simultaneously with the use of a two-zone fermentor-extractor system. The system was operated under a fed-batch mode with *in situ* removal of lactic acid by solvent extraction. Kim *et al*. (2000) proposed the recovery process of lactic acid using two distillation columns.

They used two Oldershaw columns and reboilers for fractionation of methanol and reactions. Sun *et al*. (2006) involved extraction and purification of lactic acid from fermentation broth by esterification and hydrolysis method. Eggeman and Verser (2005) studied the extraction by acidification, esterification and hydrogenolysis.

Nanofiltration membranes and ion exchange resins were occasionally coupled with the bioreactor for *in situ* removal of lactic acid (Wee *et al*., 2006). Electrodialysis fermentation with ion exchange membranes was often used for *in situ* removal of lactic acid (Nomura *et al*., 1998). Min-Tian *et al*. (2005) had previously developed a continuous electrodialysis fermentation system for the production of lactic acid. In their study, the system of electrodialysis fermentation with a level meter was the most efficient system, and a higher yield could be obtained if the glucose concentration in the broth could be controlled to remain at a lower level. Reactive extraction can selectively remove lactic acid from the fermentation broth, and may combine with a modified two-phase electro-electrodialysis (Yi *et al*., 2008).

Ion exchange used in bioseparation for lactic acid recovery based on ion exchange have been reported. Evangelista and Nikolov (1996) recovered lactic acid from fermentation broth using weak base anion exchangers (MWA-1, IRA-35, VI-15). The pH was maintained below lactic acid pK_a for its adsorption, with the fermentation broth being acidified using a cation exchange resin. Ye *et al*. (1996) also proposed a process for lactic acid production combining a membrane bioreactor and membrane filtration. Lactate was recovered from clarified fermentation broth using an anion exchanger (Amberlite IRA-400). The operating procedure was similar to that described by Srivastava *et al*. (1992) as they used the same ion exchanger. Vaccari *et al*. (1993) recovered lactic acid from clarified fermentation broth using an anionic

resin (Amberlite IRA-420). The eluate from the anionic resin contained ammonium lactate, which was treated with a strong cation-exchange resin (Amberlite IR-120), yielding a lactic acid solution that was subsequently concentrated. Cao *et al*. (2002) studied lactic acid recovery using a strongly basic ion exchanger (Amberlite IRA-400). They worked with lactic acid solutions and fermentation broths at different pH values (2 and 5). Ataei and Vasheghani-Farahai (2008) recovered lactic acid from fermentation broth using an anionic resin (Amberlite IRA-400) and cationic resin (Amberlite IRA-120) at pH 6.1. González *et al*. (2006) reported the purification of lactic acid from fermentation broth using weak anion exchanger (Lewatit S3428) and treating with a strong cation resin (Lewatit S2568H) at pH below the pK_a of lactic acid (3.86).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals, reagents, and media

3.1.1 Screening and selection of lactic acid bacteria for L-lactic acid production

The medium used for cultivation, screening and selection were De Man, Rogosa and Sharpe medium (MRS), MRS medium containing 0.5% calcium carbonate, Rogosa agar with modification medium (RAM) (Rodtong and Ishizaki, 2003), M17 medium (Atlas, 2004), and modified M17 medium.

Chemicals used for media preparation were D-glucose, proteose peptone, beef extract, yeast extract, tryptone, soy peptone, calcium carbonate, and skim milk (Himedia, Hi-Media Laboratories Pvt Ltd, India); Tween 80 (ACRŌS organics, Acros Organics, USA); tri-ammonium citrate and disodium β-glycerophophate (Fluka, Sigma-Aldrich Chemical Company, U.S.A.); sodium acetate, magnesium sulfate, manganese sulfate, dextrose, ascobic acid and di-potassium hydrogen phosphate (Carlo Erba Reagenti, Montedison group, Italy); iron(II) sulphate (BDH, BDH Labolatory supplies, England); and lactose (Merck, Merck Chemicals, Germany). Tapioca starch was purchased from Sanguan Wongse Industries Co., Ltd, Thailand, and spent brewer's yeast was obtained from Boonrawd brewery Co., Ltd., Thailand.

Chemicals and reagents used for L-lactic acid determination were sodium hydroxide, hydrochloric acid, copper(II) sulphate, ethanol alcohol and sulfuric

acid (Carlo Erba); phenol (BDH); analytical grade glucose (Merck); analytical grade L-lactic acid and analytical grade D-lactic acid (Sigma, Sigma-Aldrich Chemical Company, U.S.A.); and 4-phenylphenol (ACRŌS organics).

3.1.2 Extraction and purification of L-lactic acid

Chemicals used for L-lactic acid extraction were calcium chloride (Fluka); sulfuric acid (Carlo Erba); phenol (BDH); and activated carbon (Merck). Quick Start™ Bradford Protein Assay was product of Bio-Rad (Hercules, CA, U.S.A.).

3.1.3 Identification of lactic acid bacteria

3.1.3.1 Morphological and physiological characterization

Chemicals used for morphological and physiological characterization were crystal violet (POCH, POCH SA, Poland); iodine, potassium iodide, ethanol, safranin O, paraffin oil, hydrogen peroxide and sodium chloride (Carlo Erba); malachite green (Riedel-deHaën, Honeywell Riedel-deHaën, Germany); gelatin (Labchem, Ajax Finechem, New Zealand); skim milk (Himedia); and tetramethyl-p-phenylenediamine dihydrochloride (Fluka). For biochemical characterization, API 50 CH/CHL strips (bioMérieux, bioMérieux Industry, France) were also used.

3.1.3.2 Molecular characterization of 16S ribosomal RNA gene

Chemicals and reagents used for genomic DNA extraction were lysozyme, absolute ethanol and sodium chloride (Merck); tris-base and tris-HCl (Promega, Promega Corporation, U.S.A.); phenol (BDH), sodium citrate, isopropyl

alcohol and chloroform (Carlo Erba); sodium dodecylsulphate (SDS) (Fluka); and ethylenediaminetetraacetic acid (EDTA) (Sigma).

Chemicals and reagents used for genomic DNA detection were tris-(hydroxymethyl)-aminomethane (USB^{TM}, Amersham International, England); boric acid (Carlo Erba); bromophenol blue (USB); sucrose (Merck); ethidium bromide (Promega); ethylene-diaminetetraacetic acid (Sigma); and LE Agarose (Seakem, Cambrex Bio Science Rockland, Inc., USA).

Reagents used for polymerase chain reaction (PCR) amplification were PCR buffer, $MgCl_2$ solution and *Taq* DNA polymerase (Invitrogen, Invitrogen life technologies, U.S.A.); and dNTPs (dATP, dCTP, dGTP, and dTTP) (Promega). The oligonucleotide primers were ordered from the Ward Medic Ltd., Partnership, Thailand. The molecular weight markers were purchased from Invitrogen.

3.2 Instrumentation

All instruments required for the selection of starch-utilizing and lactic acid-producing bacteria, optimization of L-lactic acid production conditions, production of L-lactic acid using the optimum conditions, preliminary extraction and purification of L-lactic acid and identification of selected isolates of L-lactic acid-producing bacteria were located in the Instrument Buildings of the Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima Province, Thailand.

3.3 Screening of lactic acid bacteria for L-lactic acid production from glucose

To obtain the L-lactic acid-producing bacterial strains, lactic acid bacteria obtained, were tested for their potential production of L-form acid using glucose the main substrate (Rodtong, 2001).

3.3.1 Microorganisms

At least 150 isolates of lactic acid bacteria (LAB) were obtained from stock cultures of the Microbial Culture Collection and Applications Research Unit, Suranaree University of Technology. The bacteria were cultured using their isolation media according to data provided from the Research Unit, and kept at -80°C with the addition of skim milk to 5% (v/v) final concentration until use. The media MRS broth (Appendix A1.1), RAM broth (Appendix A1.9), MRS broth with the addition of calcium carbonate (Appendix A1.4) and M17 broth (Appendix A1.7) were used as culture main medium. For cell propagation procedure, the stock cultures were taken from -80°C freezer, thawed at room temperature. The isolates were inoculated in the same isolation medium containing 2% glucose. One hundred μl of each culture were used to inoculate 3 ml of cultured medium broth. After incubation at 35°C for 24 h in anaerobic chamber with a gas mixture of CO_2:H_2:N_2 (5:5:90%) (Shel LAB, Sheldon Manufacturing, Inc, U.S.A.). The culture was streaked onto same cultured medium agar (MRS agar, Appendix A1.2; RAM agar, Appendix A1.10; MC agar, Appendix A1.5; M17 agar, Appendix A1.8), and incubated under the same conditions for 48 h. Then, a single colony was sub cultured for further study.

3.3.2 L-Lactic acid production from glucose

L-Lactic acid-producing strains were screened for their lactic acid production. One loopful of each selected isolate grown for 48 h at 35°C on the suitable agar medium was inoculated into 15 ml of MRS broth containing 2% glucose in 20 ml test tube and incubated at 35°C for 24 h under anaerobic condition without shaking or agitating. After incubation, the bacterial growth was spectrometer measured at 600 nm (A_{600}) using SmartSpec™ 3000 spectrophotometer (BioRad). Fermentation broth was centrifuged at 4,500 rpm for 20 min (Labofuge 400R, Heraeus Instruments, Heraeus Instruments GmbH, Germany) at 4°C to separate bacterial cells. The pH of cultured medium was measured using pH meter (CCMP 510 pH Conductivity Meter, WPA, Biochom Ltd, England). Titratable acidity (% lactic acid) of the medium was also determined by titration method (AOAC International, 2000) with 0.1 N NaOH using phenolphthalein as pH indicator. Concentration and optical purity of lactic acid produced, was detected using high performance liquid chromatograph (HPLC) (HP 1200, Agilent Technology Inc., U.S.A.), 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., U.S.A.) was eluted with 0.005 M $CuSO_4$ as a mobile phase at a flow rate of 0.7 ml/min, and the column temperature was maintained at 35°C. Each selected strain was also tested for gas production capability using 5 ml of MRS broth containing 2% glucose with Durham tube, and incubated at 35°C for 24 h under anaerobic condition for distinguish between homofermentatives and heterofermentatives (Gül *et al.*, 2005). The homolactic bacterial isolates capable of L-lactic acid production in high concentration with optical purity >95% were selected.

3.4 Selection of lactic acid bacterial isolates and comparison for their L-lactic acid production from tapioca starch

To obtain potential bacterial isolates that could directly produce L-lactic acid from tapioca starch, both starch hydrolysis and lactic acid production capabilities were tested and evaluated.

3.4.1 Investigation of tapioca starch hydrolysis

Lactic acid bacterial isolates were tested for their starch utilization capabilities on modified MRS, MRS with addition of calcium carbonate, or M17 agar containing 1% tapioca starch (Appendix A1.2, A1.5, and A1.8) or RAM containing 1% tapioca starch (Appendix A1.10) depending on their isolation and cultivation media. The bacteria grown on cultured agar for 48 h were spotted in duplicate onto the surface of each test medium. Isolates that produced wide clear zone on the agar plate after incubating at 35°C for 48 h, and reacting with iodine solution (Iverson and Millis, 1974) were selected for testing lactic acid production from tapioca starch using liquid media.

3.4.2 L-Lactic acid production from tapioca starch

Starch-utilizing bacteria were tested for their L-lactic acid production capabilities using RAM broth containing 1% tapioca starch, and compared to MRS broth containing 2% glucose. One loopful of the selected isolates grown on RAM agar for 48 h, was inoculated into 15 ml of RAM broth. The inoculated RAM broth was incubated at 35°C for 18 h. The bacterial growth was monitored spectrophotometically at 600 nm (A_{600}). Then 2% (v/v) of the cultures (10^6 CFU/ml)

were inoculated into 15 ml of RAM medium in 20 ml test tube (duplicate set of 10 tubes). The incubation was performed at 35°C for 72 h under anaerobic condition without shaking or agitating. Samples (two tubes) were taken at each time interval at 0, 6, 12, 18, 24, 30, 36, 48, 60 and 72 h. The bacterial growth was monitored spectrophotometically at 600 nm (A_{600}) and viable cell counts (CFU/ml) were determined by plating serial dilutions of bacterial suspension on RAM agar. Plates were incubated anaerobically at 35°C for 48 h.

For the detection of L-lactic acid, fermentation broth was centrifuged at 8,000 rpm for 10 min at 4°C to separate bacterial cells. The acid production was estimated from pH reduction and titratable acidity (%lactic acid), then total lactic acid was determined by colorimetric assay (Kimberley and Taylor, 1996). Concentrations and optical purity of L-lactic acid were analyzed using HPLC. Total sugars was also determined by the phenol-sulfuric acid method (Dubois *et al*., 1956), using glucose as a standard.

3.5 Optimization of some conditions for L-lactic acid production

To determine the optimal conditions for L-lactic acid production by the selected bacterial strains, the suitable composition of RAM medium was investigated. The cultivation conditions for L-lactic acid detection obtained from section 3.4 was used.

3.5.1 Concentrations of tapioca starch

To obtain the suitable concentration of tapioca starch, a cheap and abundant carbon source, various concentrations of the starchy raw material (10, 15, 20, 25, 30, 35, and 40 g/l) were applied to RAM medium. Inoculum for lactic acid

production was prepared by inoculating one loopful of the selected isolate grown on RAM agar at 35°C for 48 h into 25 ml of RAM broth. The inoculated broth was incubated at 35°C for 18 h under anaerobic condition without shaking or agitating. The bacterial growth was monitored spectrophotometically at 600 nm (A_{600}). Then 2% (v/v) of the starter culture (approximately 10^6 CFU/ml) were inoculated into 100 ml of the RAM medium in 125 ml Erlenmeyer flask, then incubated at 35°C under anaerobic condition for 48 h. The viable cell counts (CFU/ml) were determined by plating serial dilutions of bacterial suspension on RAM agar, and incubated anaerobically at 35°C for 48 h. L-Lactic acid accumulated in RAM medium containing different concentrations of tapioca starch was evaluated. It was found that RAM medium containing 30 g/l of tapioca starch could provide the high L-lactic acid concentration of 6.86 g/l. The medium was selected for further investigation.

3.5.2 Types and concentrations of nitrogen sources

Types and concentrations of nitrogen source of fermentation medium were investigated. Two types of nitrogen source, tryptone and yeast extract, were optimized. The medium containing 30 g/l of tapioca starch was used to test using 50 ml fermentation medium in 125 ml Erlenmeyer flask.

3.5.2.1 Tryptone

Tryptone was efficiently used as a nitrogen source for lactic acid production by LAB. Various concentrations of tryptone (2.0, 2.5, 3.0, 4.0, and 5.0 g/l) were investigated to obtain the optimal concentration.

3.5.2.2 Spent brewer's yeast

Yeast extract was applied as the growth factor. Also various concentrations of yeast extract (1, 2, and 3 g/l) were added into fermentation medium, then it was replaced by spent brewer's yeast at concentrations of 3, 5 and 10 g/l. Components of media containing yeast extract and spent brewer's yeast were described in Table 3.1. The optimum concentrations of yeast extract and spent brewer's yeast in tapioca starch medium were determined.

Table 3.1 Components of optimized RAM media.

Component	Component concentration (g/l) of medium no.					
	1*	2	3	4	5	6
Tapioca starch	Optimal concentration from section 3.5.1					
Tryptone	Optimal concentration from section 3.5.2.1					
Yeast extract	3.00	2.00	1.00	-	-	-
Spent brewer's yeast	-	3.00	3.00	3.00	5.00	10.00
Dipotassium phosphate	6.00	6.00	6.00	6.00	6.00	6.00
$MgSO_4.7H_2O$	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

* Initial medium composition.

3.5.3 Initial pH of fermentation medium

The initial pH of the optimized fermentation medium containing suitable concentration of carbon and nitrogen source for L-lactic acid production was studied. The medium was adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0 and 8.0 using 1 N HCl and 1 N NaOH before sterilization and inoculation of selected bacteria. Inoculum was

prepared as described in section 3.5.1, and inoculated into 50 ml of the medium in 125 ml Erlenmeyer flask.

3.5.4 Cultivation temperature

The suitable temperature for L-lactic acid production was investigated from various incubation temperatures (25, 30, 35, 37, 40, and 45°C) using the suitable medium resulted from sections 3.5.1-3.5.3. The technique for inoculum preparation as described in section 3.5.1, and were inoculated into 50 ml of the medium in 125 ml Erlenmeyer flask.

3.5.5 Inoculum size

The inoculum size for the L-lactic acid production was studied. Various inoculum sizes (1, 2, 3, 4, and 5%) were applied in the suitable medium resulted from sections 3.5.1-3.5.3, and cultivated at optimum temperature from section 3.5.4. The technique for inoculum preparation as described in section 3.5.1, and were inoculated into 50 ml of the medium in 125 ml Erlenmeyer flask.

3.6 Production of L-lactic acid from tapioca starch

Lactic acid production by selected isolates were carried out in 6.6 l controlled fermentor (Biostat B Quatro, Braun Sartorius, Melsungen, Germany) containing 5.0 l of the optimized fermentation medium. The medium was sterilized in an autoclave at 121°C for 30 min. The suitable inoculum size (v/v) containing approximately 10^6 CFU/ml was inoculated into the optimized medium, and cultivated at the optimum temperature and pH controlled by automatic addition of 5 N NaOH. Temperature, pH,

and agitation were computer-controlled and monitored on line using MFCS SCADA Software. Changes in L-lactic acid concentration, starch concentration, and bacterial growth were measured at each time interval during 48 h cultivation as described in section 3.4.

3.7 Preliminary investigation for L-lactic acid extraction and purification from the optimized medium

L-Lactic acid was purified from fermentation broth by the method as described by Benthin and Villadsen (1995). Cells and residual starch were removed from fermentation medium by centrifugation at 10,000×g for 15 min at 4°C (BECKMAN COULTER, Beckman Coulter Inc, U.S.A.). The medium was heated for protein precipitation and separated the protein using filter paper (Whatman no. 4). The medium was heated for protein precipitation and separated the protein using filter paper (Whatman no. 4). A solution of $CaCl_2$ was added to the medium (0.5 mol Ca/mol lactic acid). The solution was concentrated to one-fourth by evaporation at 80-90°C. Then, L-lactate was crystallized from the solution at 4°C for 24 h. By centrifugation, 4,000×g for 10 min, a supernatant and two precipitate fractions were obtained. The lower precipitate was more light-coloured and was the lactate salt. The top precipitate contained very little L-lactate and was presumably inorganic salts (e.g. magnesium/calcium phosphate). The top precipitate was removed with a spoon (a transparent centrifugation tube was necessary). The lower precipitate was washed with 1 v/v distilled water at 0°C and dissolved in distilled water at 90°C (the same volume as for crystallization). pH was then adjusted to 2.5 with concentrated sulfuric acid and mixing for 5 min. The L-lactate solution was treated with activated carbon.

3.8 Identification of the selected lactic acid bacterial isolates

The selected lactic acid bacterial isolates potentially produced L-lactic acid were identified by their morphological and physiological characteristics (Holt *et al*., 1994) and 16S rRNA gene sequence (Weisburg *et al*., 1991).

3.8.1 Morphological characterization

Colony morphology of selected isolates was observed on RAM agar at 35°C for 48 h under anaerobic condition. Cell morphology was detected by Gram staining of 18 h cultures grown on RAM agar. Endospore was examined as described by Cappuccino and Sherman (1999).

3.8.2 Physiological characterization

3.8.2.1 pH, temperature, and salt tolerance tests

Bacterial isolates were tested for growth at different pH (4.0, 4.4, 4.8, 5.0, 6.0, 7.0, 8.0, and 9.6), temperatures (5, 10, 15, 20, 30, 35, 37, 40, 42, 45, 50, and 55°C), and salt concentrations (0, 3.0, 4.0, 6.5, 8.0, 10.0, and 18.0% NaCl), into MRS (Appendix A1.1) and RAM (Appendix A1.9) broth. For pH tolerance test, only the pH of the broth was varied while the temperature was fixed at 35°C. For growth temperature test, only cultivation temperature was varied while pH of the medium was fixed at 7.0. For growth salt at different concentration, only the NaCl concentration of the broth was varied while pH of medium and cultivation temperature were fixed at 7.0 and 35°C respectively. The inoculum of 0.1 ml (approximate 10^6 CFU/ml) was aseptically transferred to 5 ml of MRS or RAM broth.

The optical density at 600 nm wavelength was used for evaluating bacterial growth. Each treatment was tested in duplicate.

3.8.2.2 Carbohydrate fermentation

Carbohydrate fermentation was detected using API 50 CH/CHL strip (bioMérieux, bioMérieux Industry, France) which contained tests for carbohydrate assimilation and fermentation of 49 different compounds (and a control). After inoculation with bacterial cultures, the strips were incubated at 37°C for 48 h. Then the APILAB Plus software version 5.0 from bioMérieux and Analytab Products' computer database were used for comparison of carbohydrate assimilation and/or fermentation patterns.

3.8.2.3 Starch hydrolysis

RAM agar (Appendix A1.10) and MRS agar (Appendix A1.2) containing 1% tapioca starch was used for starch hydrolysis testes at 35°C for 48 h under anaerobic condition. The agar plates were then flooded with iodine solution (Appendix A2.1). Blue color of the agar around colony of the test organism indicated negative result (no starch hydrolysis), while a clear zone around colony of the test organism indicated positive result (positive starch hydrolysis).

3.8.2.4 Arginine hydrolysis

Bacterial isolates were tested for arginine hydrolysis in MRS and RAM broth without beef extract but containing 0.05% glucose and 0.3% arginine, and 0.2% sodium citrate replacing ammonium citrate. One loopful of bacterial cells

was aseptically transferred to 5 ml of the medium and was incubated at 35°C for 48 h under anaerobic condition. After incubation, the culture was examined for the presence of ammonia in the medium. Ammonia was detected by addition of Nessler's reagent to the culture. Ammonia in the medium was react with these reagents to produce a yellow color.

3.8.2.5 Casein hydrolysis

Modified MRS and RAM agar containing 1% skim milk was used for casein hydrolysis test, pure cultures of bacteria were point-inoculated onto these agar plates and incubated at 35°C for 48 h under anaerobic condition. Clear zones of proteolysis around colonies were the positive reaction for the proteolytic test while a negative reaction were had not clear zone around the colony (negative hydrolysis).

3.8.2.6 Gelatin hydrolysis

Each bacterium to be tested was stabbed into deep gelatin medium (Appendix A1.3). The inoculated gelatin medium was incubated at 35°C for 48 h under anaerobic condition. Gelatin hydrolysis was indicated by liquification of the medium after the tube was kept at 4°C for 20-30 minutes.

3.8.2.7 Motility

Each bacterium to be tested was stabbed into deep tube of motility medium (Appendix A1.6). The inoculated motility medium was incubated at 35°C for 48 h under anaerobic condition. During growth, motile bacteria could

migrate from the line of inoculation to form a dense turbidity in the surrounding medium. Non-motile bacteria were grown only along the line of the inoculation.

3.8.2.8 Catalase test

Bacterial cells (late log phase cultures) were transferred to the surface of a glass slide. 1 or 2 drops of 3% hydrogen peroxide were added over cells. Rapid appearance of gas bubbles indicated the positive result of catalase test.

3.8.2.9 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 A2.3). Late log phase culture of bacteria 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.

3.8.2.10 Gas production

Bacterial isolates were tested for gas production in MRS (Appendix A1.1) broth without tri-ammonium citrate. One loopful of bacterial cells was aseptically transferred to 5 ml of MRS broth with durham tube and incubated at 35°C for 48 h under anaerobic condition. Heterofermentatives produced gas but homofermentatives were failure to produce gas.

3.8.3 Molecular characterization of 16S ribosomal RNA gene

3.8.3.1 Extraction of genomic DNA

Genomic DNA extraction from lactic acid bacterium isolates was performed as described by Tamaoka (1994). Wet cells of bacteria were harvested for cultured broth and resuspended in lysis buffer containing 0.75 mg/ml of lysozyme, and kept 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 A2.4) solution and then a 1 ml portion of 10 ml of 10% SDS were 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) was 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 pellet was washed with 70% ethanol and washed with 99% ethanol. The DNA was air died then dissolved in 200 µl of 0.1×SSC (pH 7.0) (Appendix A2.2). Genomic DNA was detected in 0.8% agarose gel electrophoresis (BioRad, BioRad Laboratories, Italy), stained with ethidium bromide, and examined under UV transilluminator (BioRad). The concentration of DNA was measured by SmartSpec™ 3000 spectrophotometer at 260 nm (BioRad) 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.

3.8.3.2 Amplification of 16S ribosomal RNA gene

Amplification of 16S ribosomal RNA gene was performed by polymerase chain reaction (PCR) using fD1 and rP2 primers (Weisburg *et al*., 1991) as forward and reverse to obtain approximately 1,500 bp of the gene. The PCR amplification reaction was performed in 50 µl mixture containing 200 ng of bacterial DNA, 5 µl of 10× reaction buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 1.5 µl of 25 mM $MgCl_2$, 1.0 µl of dNTPs mixture (dATP, dCTP, dGTP, dTTP at 2 mM of each concentration) 1.0 µl of each primer (fD1 and rP2) (20 mM of each concentration), and 0.3 µl of *Taq* DNA polymerase (5 units/µl), and adjusted volume to 50 µl with deionized water. The program of amplification consisted of 1 cycle of 95°C for 2 min; 35 cycles of 95°C for 45 sec, 55°C for 45 sec, 72°C for 2 min; and the final cycle of 72°C for 7 min. The PCR reactions were carried out in the automated thermal cycle (Thermo electron corporation P×2 Thermal Cycler, Bioscience Technologies Division, Waltham, MA, U.S.A.). The PCR amplified products were examined by electrophoresis using 1% agarose and stained with ethidium bromide. The size of PCR products was compared with 1 Kb plus DNA ladder. The separated PCR products were observed under short wavelength UV light.

3.8.3.3 Sequencing of ribosomal DNA

Nucleotide sequence of the gene obtained from ABI 3730xl DNA analyzer (Model 373, U.S.A.) was analyzed and converted to single letter code in text file format by the Chromas 1.56 program (Technelysium Pty. Ltd). The sequence was also corrected by manual inspection of the chromatogram. All alignments were examined and manually optimized with the BioEdit program (North

Carolina State University, U.S.A.). The sequence was compared to local alignment search from GenBank database using the BLAST (Basic Local Alignment Search Tool) program of the National Center for Biotechnological Information (NCBI). (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The sequence information was then imported into the CLUSTAL X software program (Hitachi Software Engineering Co.) for assembly and alignment. Phylogenetic tree was inferred using the Maximum Pasimony method with software MEGA version 4 (Kumar *et al*., 2004). The stability relationships were evaluated by a boot strap analysis of 1000 replications.

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	Weisburg *et al*. (1991)
Sequencing forward primer	TAACTACGTGCCAGCAGCC	515-533	Udomsil (2008)
rP2	ACGGCTACCTTGTTACGACTT	1491-1511	Weisburg *et al*. (1991)

[a]: *Escherichia coli* numbering

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Screening of lactic acid bacteria for L-lactic acid production from glucose

A total of 280 isolates of LAB obtained from stock cultures of the Microbial Culture Collection and Applications Research Unit, Suranaree University of Technology, and isolated from their natural habitats, were then tested for their L-lactic acid production using MRS broth containing 2% glucose. One loopful of each bacterial isolate was incubated into 15 ml of the medium, then incubated under anaerobic condition at 35°C for 24 h. The bacterial growth was determined spectrophotometically at 600 nm (A_{600}). L-Lactic acid was harvested in supernatant by centrifugation for cell separation. The supernatant having pH range between 3.5-5.6, was determined for titratable acidity (% lactic acid). The titratable acidity in the range of 0.476-1.887% was found. The concentration and optical purity of L-lactic acid isomer were detected using HPLC. It was found that 160 isolates could produce L-lactic acid in the range of 0.91-19.12 g/l with optical purity >95%, and 120 isolates were produced DL-lactic acid in the range of 0.24-18.25 g/l (optical purity lower than 95%). Homolactic acid-producing bacterial isolates were detected using MRS broth with durham tube. One hundred and ninety-six isolates were found to produce acid without gas (Appendices B1-B4).

From a total of 280 isolates studied, 192 isolates were Gram-positive rods having cell sizes between 0.31-0.41×0.60-0.65 μm and 0.03-0.53×0.76-2.0 μm, 88 isolates were Gram-positive cocci having cell sizes of 0.38-0.51 μm, which occurred singly, in pairs, or in short chains. Their colony morphology was observed after incubating on MRS agar medium after 48 h incubation under anaerobic condition. Circular and irregular colonies with entire and undulate margins; and flat, low convex, convex and umbonate were found. These colonies included punctiform, small, moderate and large colonies with 0.1-4.0 mm in diameters.

Lactic acid bacteria generate ATP from the fermentation of sugar resulting in the production of lactic acid as the major end product during carbohydrate fermentation due to the bacteria unable to synthesize ATP by respiration (Axelsson, 2004). Refined sugars such as glucose and sucrose are the most commonly used substrates for commercial production of lactic acid by fermentation processes (Vishnu *et al*., 2002). Yun *et al*. (2003) studied types of carbohydrates for *Enterococcus faecalis* RKY1 cultivating at 38°C. The medium containing 30 g/l glucose was found to be the best medium for lactic acid production when compared to the medium containing the same concentration of either fructose, maltose, galactose, lactose, glycerol, xylose, whey, or starch. Galactose was metabolized into formic and acetic acids as major end products, whereas xylose, glycerol, whey, and starch were poorly utilized. When the bacterium was grown on fermentation medium containing either glucose, fructose, or maltose. It produced lactic acid with the high yield of 16.80-18.18 g/l comparable to the yield of lactic acid to total organic acids of 0.86-0.91 g/g, through homofermentative pathway. However, organic acids other than lactic acid were rarely produced. In this study, our results also showed that MRS

medium containing glucose provided the clear detection of lactic acid-producing strains. Isolates capable of producing high concentration of L-lactic acid were then selected for the further investigation.

4.2 Selection of lactic acid bacterial isolates and comparison for their L-lactic acid production from tapioca starch

Starch-utilizing and lactic acid-producing bacterial isolates obtained from stock cultures, were tested for their capability to utilize tapioca starch and produce lactic acid according to previous reports (Rodtong, 2001; Rodtong and Ishizaki, 2003).

4.2.1 Investigation of tapioca starch hydrolysis

All lactic acid-producing bacterial isolates tested were able to utilize tapioca starch after point inoculating onto either modified MRS or RAM agar medium and incubating at 35°C for 48 h under anaerobic conditions. One hundred and twenty-eight out of 280 isolates could utilize tapioca starch, which were observed from clear zoned (0.1-1.7 cm in diameters) surrounding bacterial colonies resulted from iodine reaction.

These 128 isolates could utilize tapioca starch and produce L-lactic acid at concentrations of 0.91-8.60 g/l with optical purity >95%, and they were also homofermentatives. Two isolates, codes SUT513 and CAR134, produced the highest lactic acid concentration of 8.60 and 7.89 g/l respectively, from tapioca starch 10 g/l (Appendices B1-B4 and Figure 4.1). In this study, most of lactic acid-producing bacteria were selected from isolates growing well in RAM medium, which implied that these bacteria had their ability to break down starch during lactic acid

fermentation process. The two isolates exhibiting the highest L-lactic acid concentration were then selected for further identification and lactic acid production.

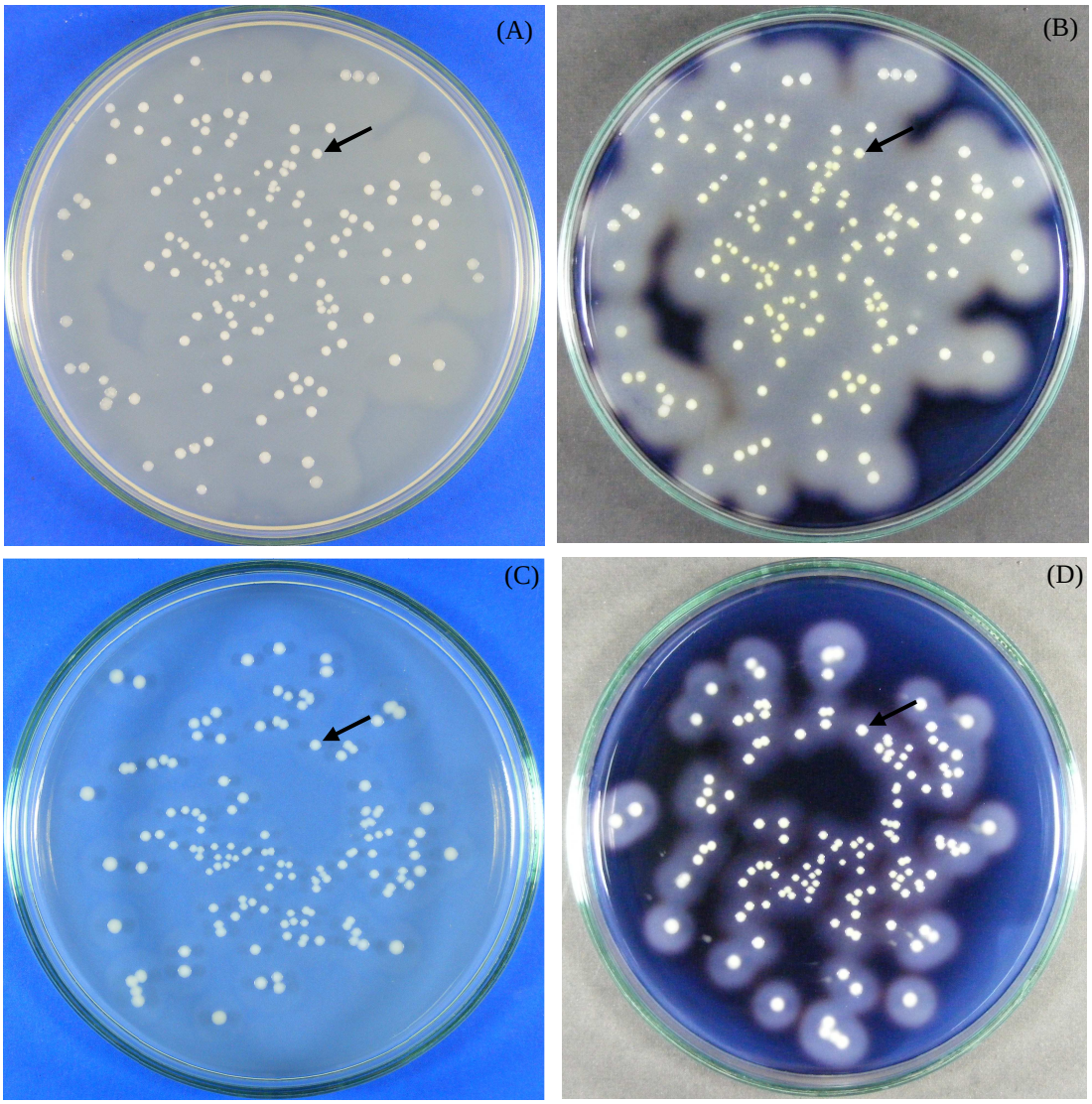


Figure 4.1 Colonies of the selected bacteria obtained from fermentation medium at 48 h cultivation and spreaded on RAM agar: isolates SUT513 (A and B); CAR134 (C and D); these colonies produced enzyme to hydrolyze tapioca starch as shown by the reaction of iodine (B and D).

4.2.2 L-Lactic acid production from tapioca starch

Starch-utilizing isolates SUT513 and CAR134 were selected for evaluating their potential production of lactic acid from tapioca starch compared to glucose using RAM medium containing 10 g/l of tapioca starch and MRS medium containing 20 g/l of glucose respectively. Inoculums of the two bacterial isolates were prepared, and inoculated into the media, then incubated under anaerobic condition at 35°C for 72 h. Isolate SUT513 grew well in both glucose and tapioca starch media. Its cell concentration was about 10^9 CFU/ml after cultivating 12 h, and became to stationary phase until 72 h. The isolate SUT513 could produce acid resulting in decreasing pH of the media from 6.25 to 4.09 for MRS broth containing glucose as a carbon source, and 7.10 to 4.05 for RAM broth containing tapioca starch as a carbon source, at 12 h of fermentation. Titratable acidity ranging from 0.366-0.653% and 0.416-0.559%, and the highest total lactic acid of 6.53 and 5.10 g/l with the maximum concentrations of L-lactic acid of 6.86 and 5.24 g/l with optical purity >99% for glucose and tapioca starch media, respectively, were detected (Figure 4.2A).

For isolate CAR134, it also grew well in both glucose and tapioca starch media. Its cell concentration was about 10^9 CFU/ml after cultivating 6 h, but droped to 10^5 and 10^4 CFU/ml after 18 h respectively. pH of the media decreased from 6.25 to 4.04 for MRS broth containing glucose and 7.10 to 4.21 for RAM broth containing tapioca starch, respectively, at 12 h fermentation time. Because, the pH during growth was not controlled and the bacterial metabolism was affected from acid accumulation. Lactic acid inhibit bacterial growth because, as the external pH declines, the acid is protonized as soon as it is exported out of the bacteria. Unchanged, it diffuses back into the cell and dissociates due to the higher intracellular pH. The cell then has to use

ATP to pump out protons, and energy eventually is depleted causing growth stop and the bacteria die (Mussatto *et al*., 2008). The total acids were range 0.347-0.698% and 0.386-0.500%, the highest concentrations of total lactic acid were 6.70 and 4.55 g/l, and the maximum concentrations of L-lactic acid were 6.82 and 4.55 g/l with optical purity >99%, respectively (Figure 4.2B).

L-Lactic acid produced by isolates SUT513 and CAR134 was at concentrations of 6.86 and 6.70 g/l with lactic acid yield of 0.90 and 0.52 g/g, and productivity of 0.12 and 0.14 g/l.h, when 2% glucose was used as major carbon source (Appendices B5-B8 and Figure 4.2). The lactic acid productivities obtained from 2% glucose were about 0.01 and 0.06% higher than that of 1% tapioca starch respectively. The amount of glucose used in the fermentation medium was 50% higher than tapioca starch but L-lactic acid yields and productivities were not much different after 72 h cultivation. Theses results reveal that isolates SUT513 and CAR134 could efficiently hydrolyze tapioca starch to glucose, then glucose was immediately converted to L-lactic acid. The two isolates, SUT513 and CAR134, are very efficient for the direct production of L-lactic acid with high optical purity from tapioca starch. The results also suggest that tapioca starch composing of starch (85.53%), moisture content (11.95%), fiber content (0.18%), total nitrogen content (0.35%), fat content (1.64%) and ash content (0.35%), can be a potential substrate for L-lactic acid production.

Starch-degrading amylolytic LAB have been reported. And the capacity of the bacteria to convert starch into lactic acid is known, but it is not a common characteristic (Giraud *et al*., 1991). Narita *et al*. (2004) compared lactic acid fermentation from glucose to raw corn starch by *Streptococcus bovis* 148 using batch

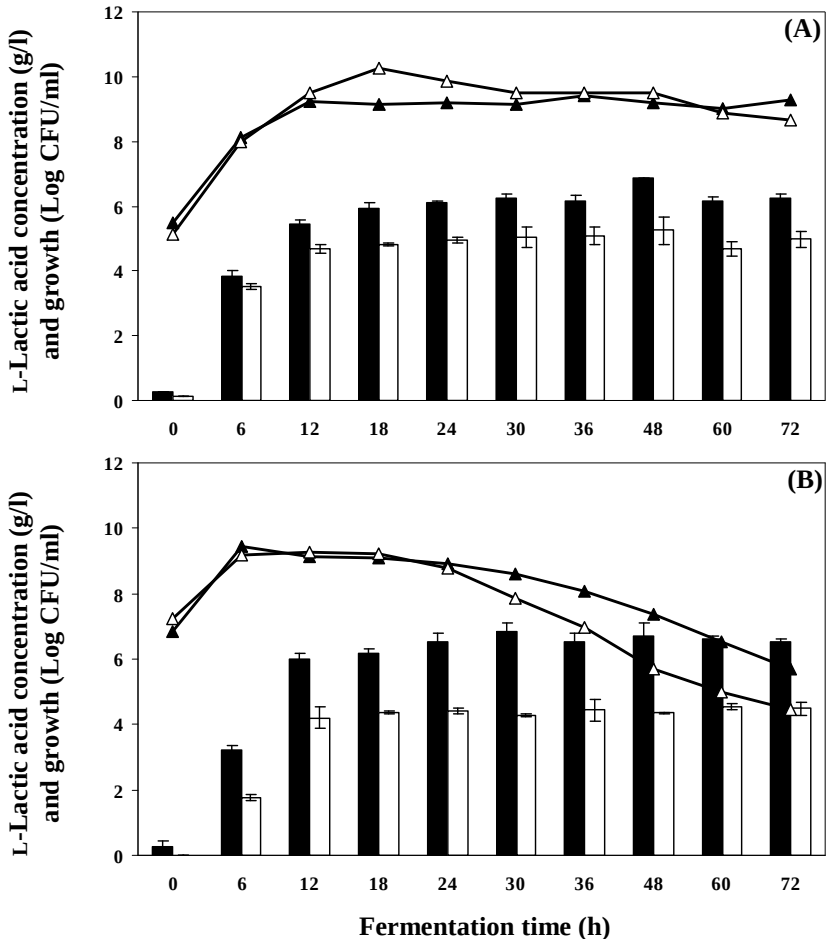


Figure 4.2 Comparison of growth (Log CFU/ml) in MRS medium containing 2% glucose (▲) and RAM medium containing 1% tapioca starch (△), and L-lactic acid (g/l) produced in MRS containing 2% glucose (■) and RAM containing 1% tapioca starch (□) by selected strains SUT513 (A) and CAR134 (B) after incubation at 35°C for 72 h.

fermentation at 37°C. Raw corn starch (20 g/l) provided similar lactic acid yield and production rate to glucose. The *S. bovis* strain efficiently hydrolyzed raw corn starch to glucose, which was immediately converted to L-lactic acid. *Streptococcus bovis* 148 could produce 14.73 g/l L-lactic acid with 95.6% optical purity. *Enterococcus*

faecalis RKY1 was reported to produce lactic acid of 18.18 g/l from 20 g/l glucose. The starch was poorly fermented into lactic acid by *E. faecalis* RKY1, although a few cell growths were observed at 38°C (Yun *et al*., 2003).

Enterococcus faecium No. 78 could produce L-lactic acid from various substrates including starch at 30°C. The strain could produce L-lactic acid 18.6, 15.4, 16.6, 13.2, 12.4 and 14.3 g/l from 20 g/l of glucose, soluble starch, sago starch, corn starch, wheat starch and potato starch, respectively. Yield of lactic acid with optical purity of 98.6% from sago starch was higher than glucose and other varities of starch (Shibata *et al*., 2007). In this study, glucose could be replaced with tapioca starch for the main carbon source for L-lactic acid production by strains SUT513 and CAR134. These isolates were then selected for the acid production from tapioca starch, a low cost substrate compared to sugar, the common raw material.

4.3 Optimization of some conditions for L-lactic acid production

To obtain the maximum production of L-lactic acid and low production cost, some components of RAM medium and production conditions including carbon source, nitrogen source, initial pH of medium, incubation temperature, and initial inoculum size for culturing the selected L-lactic acid-producing isolates were investigated. RAM medium composed of 1% tapioca starch, 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±0.2 prior to sterilization by autoclaving.

4.3.1 Concentrations of tapioca starch

The selected lactic acid-producing isolates were able to utilize tapioca starch when culturing in RAM medium. Glucose was replaced with tapioca starch at various concentrations (10, 15, 20, 25, 30, 35, and 40 g/l), to obtain the optimal concentration. Initial cell counts of isolates SUT513 and CAR134 were approximately 10^6 CFU/ml. After cultivating for 48 h, the bacterium growth and lactic acid produced were detected (Figures 4.3A and 4.3B). The relationship between the concentration of substrate used and the concentration of L-lactic acid produced was found. Substrate concentration from 10 g/l to 30 g/l (dry weight) provided the increase in L-lactic acid yield. Both isolates showed the highest L-lactic acid concentration at 30 g/l (dry weight) of tapioca starch applied to RAM broth. When added tapioca starch at concentrations of 35 and 40 g/l to fermentation medium, amounts of L-lactic acid produced, did not change. However, lactic acid productivity decreased when adding tapioca starch more than 30 g/l (Figure 4.3). This could be probably due to substrate inhibition in batch fermentations. The highest L-lactic acid concentrations of 5.55 g/l and 5.22 g/l (Figures 4.3A and 4.3B) were observed from strains SUT513 and CAR134 respectively (Appendices C1-C2). L-Lactic acid produced had an optical purity of >99%. Therefore, the optimum concentration of tapioca starch was considered to be 30 g/l for lactic acid production from isolates SUT513 and CAR134.

For an economic process, amylolytic microorganisms with high starch conversion to L-lactic acid could be needed. The direct lactic acid production by *Lactobacillus manihotivorans* LMG18011^T from soluble starch and food wastes was reported (Ohkouchi and Inoue, 2006). L-Lactic acid (19.5 g) could be produced from 200 g food wastes. Lactic acid production by *L. plantarum* NCIM 2084 could produce

72.9 g/l lactic acid from 100 g/l of liquefied starch (Krishnan *et al*., 1998). *Lactobacillus amylophilus* NRRL B4437 produced 29 g/l of lactic acid from 45 g/l of corn starch and *L. amylovorous* converted 120 g/l liquefied starch to 92.5 g/l lactic acid (Zhang and Cheryan, 1991; Mercier *et al*., 1992). *Lactobacillus amylophilus* JCM 1125 also produced lactic acid of 53.4 g/l from 100 g/l liquefied starch (Yumoto and Ikeda, 1995) and *L. amylophilus* GV6 produced 49.0 and 76.2 g/l L-lactic acid from 60 g/l corn starch and 90 g/l soluble starch, respectively (Vishnu *et al*., 2000). The amylolytic strain could produce lactic acid from 60 g/l of all fours: 38.1 g/l from cassava flour, 35.9 g/l from rice flour, 33.2 g/l from wheat flour, 33.0 g/l from sorghum flour, and 31.8 g/l from barley flour (Vishnu *et al*., 2002). Yen and King (2010) reported results of batch operation with 20, 40, and 60 g/l sweet potato starch resulting in 21.62, 29.09, and 37.16 g/l of lactic acid by *L. amylophilus* BCRC 14055 at 30°C. *Lactococcus lactis* subsp. *lactis* B84, was capable of utilizing soluble potato starch as a sole carbon source and produced L-lactic acid. The acid concentration of 5.5 g/l could be produced from 18 g/l starch in batch fermentation at 33°C, agitation 200 rpm and pH 6.0 for 6 days. Starch was completely hydrolyzed after 6 days of fermentation (Petrov *et al*., 2008). The *Streptococcus bovis* 148 was also found to directly produce lactic acid from 20 g/l of corn starch yielding the maximum lactic acid concentration of 14.73 g/l (Narita *et al*., 2004). Shibata *et al*. (2007) reported that *Enterococcus faecium* No.78 could produce 16.6 g/l lactic acid from 20 g/l sago starch. The strain could produce lactic acid higher than *L. amylovorus* JCM 1126 and *L. manihotivorans* JCM 12514, which produced lactic acid at concentrations of 14.3 and 11.0 g/l respectively.

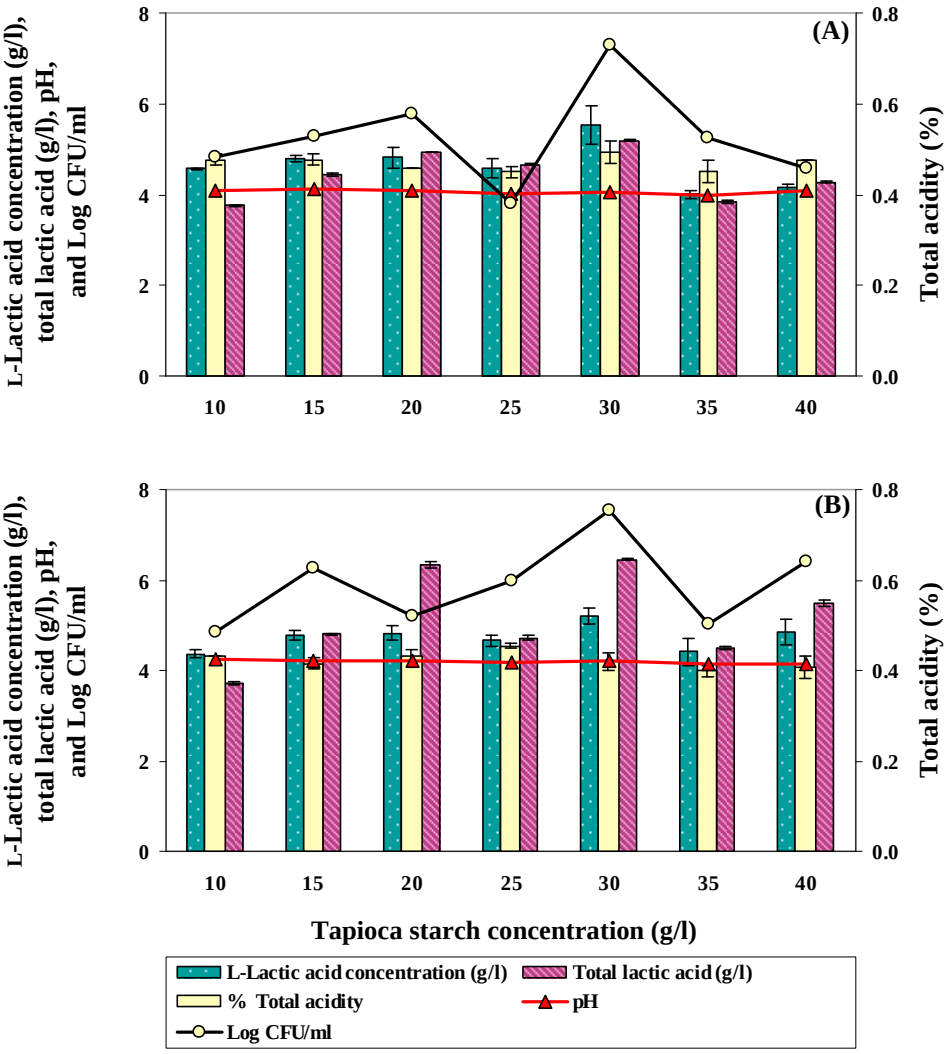


Figure 4.3 Effect of tapioca starch concentrations (10, 15, 20, 25, 30, 35, and 40 g/l) on bacterial growth and L-lactic acid production by SUT513 (A) and CAR134 (B) in modified RAM medium at an initial pH of 7.0 and incubated at 35°C for 48 h.

4.3.2 Types and concentrations of nitrogen sources

Nitrogen source is another crucial substrate for lactic acid production. Since two components, tryptone and yeast extract, of RAM medium could be served as nitrogen source by bacterial isolates SUT513 and CAR134. The price of the two compounds was still high when compared to some other types of nitrogen sources such as spent brewer's yeast. The medium used for investigating nitrogen sources composed of 3% tapioca starch, 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±0.2.

4.3.2.1 Tryptone

Tryptone (5 g/l) is added as a nitrogen source in RAM medium. Various concentrations of the compound (2.0, 2.5, 3.0, 4.0, and 5.0 g/l) were applied in RAM medium containing 30.0 g/l (dry weight) of tapioca starch for evaluating L-lactic acid production yield. After cultivating the isolates SUT513 and CAR134 for 48 h, the bacteria grew well in the media containing 2.0-5.0 g/l of tryptone. Their cell counts were from approximately 10^6 CFU/ml to 10^5 and 10^6 CFU/ml respectively. The maximum L-lactic acid concentration was also obtained from RAM media containing 2.5 and 4.0 g/l of tryptone for isolates SUT513 and CAR134. For isolate CAR134, L-lactic acid was increased with the increase in tryptone concentrations from 2.0 to 4.0 g/l. The selected bacterial strains SUT513 and CAR134 could produce the maximum concentration of L-lactic acid at 5.16 and 5.12 g/l (Figures 4.4A and 4.4B) in media containing tapioca starch at concentration of 30.0 g/l (dry weight) (Appendices C3-C4). L-Lactic acid produced had an optical purity of >99%. Thus,

RAM broths containing 2.5 and 4.0 g/l of tryptone were for isolates SUT513 and CAR134 respectively.

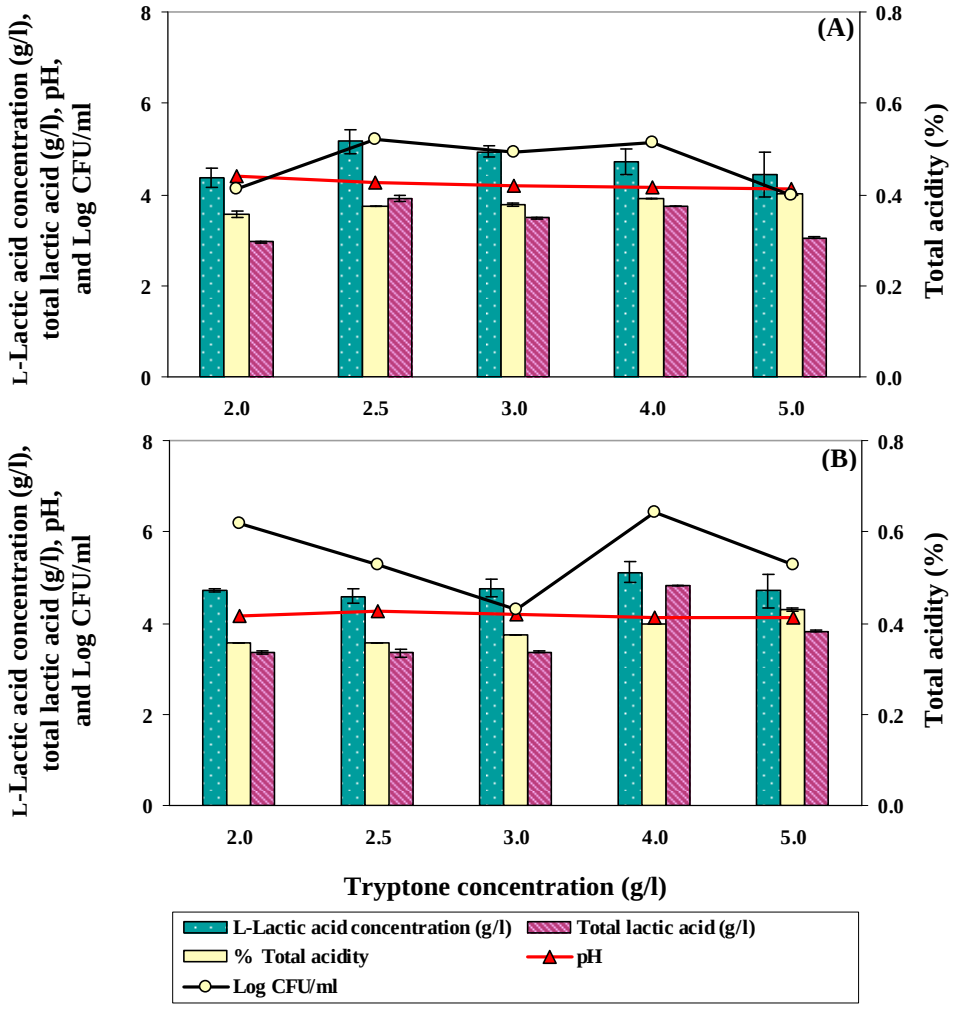


Figure 4.4 Effect of tryptone concentrations (2.0, 2.5, 3.0, 4.0, and 5.0 g/l) on growth and L-lactic acid production by bacterial isolates SUT513 (A) and CAR134 (B) in modified RAM medium at an initial pH of 7.0 and incubated at 35°C for 48 h.

As effects of various nitrogen sources on cell growth and lactic acid production were investigated, the more expensive nitrogen source leads to higher lactic acid yields and the increase in acid production cost. The suitable nitrogen source for industrial application should be available with low cost. A few reports tried to investigate yeast extract and tryptone in MRS medium for lactic acid production, *Lactobacillus casei* YIT 9018, for example (Oh *et al*., 1995). The response surface methodology (RSM) was used for investigating tryptone for growth and lactic acid production by *L. casei* YIT 9018. The optimum conditions were found to be as follows: 3.04% tryptone, 0.892% yeast extract, 1.58% glucose, 0% Tween 80, and incubation temperature at 35°C.

4.3.2.2 Spent brewer's yeast

Most LAB require a wide range of growth factors including amino acids, specific minerals, vitamins, fatty acids, purines and pyrinmidines for their growth and biological activity (Li *et al*., 2006). Yeast extract has been reported to be the most important medium component for lactic acid fermentation. It is a costly source of nitrogen and growth factors. In this study, the original RAM medium contained 3.0 g/l of yeast extract. Various concentrations of the component (1.0, 2.0, and 3.0 g/l) were applied in RAM medium containing 30 g/l (dry weight) of tapioca starch and 2.5 g/l (for isolate SUT513) and 4.0 g/l (for isolate CAR134) of tryptone respectively. To obtain the low cost medium, yeast extract was tested to be reported by spent brewer's yeast obtained from Boonrawd brewery Co., Ltd., Thailand. Three concentrations (3.0, 5.0, and 10.0 g/l) of spent brewer's yeast (Table 4.1) were used to prepare fermentation medium. After cultivating the isolates SUT513 and CAR134 in

media containing spent brewer's yeast for 48 h, very good growth and production of L-lactic acid were found. Yeast extract could be completely replaced by 3.0 g/l of spent brewer's yeast in the medium for isolate CAR134. The maximum concentration of L-lactic acid was 5.34 g/l (Figure 4.5B) in medium containing 30.0 g/l (dry weight) tapioca starch with 4.0 g/l of tryptone. When 3.0 g/l spent brewer's yeast was used as a nitrogen source, fermentation showed a little higher yield than that with 3.0 g/l yeast extract (Appendix C6).

Table 4.1 Medium constituents for the investigation of effects of yeast extract and spent brewer's yeast on lactic acid production by strains SUT513 and CAR134.

Component	Component concentration (g/l) of medium no.					
	1*	2	3	4	5	6
Tryptone						
SUT513	2.50	2.50	2.50	2.50	2.50	2.50
CAR134	4.00	4.00	4.00	4.00	4.00	4.00
Yeast extract	3.00	2.00	1.00	-	-	-
Spent brewer's yeast	-	3.00	3.00	3.00	5.00	10.00
Dipotassium phosphate	6.00	6.00	6.00	6.00	6.00	6.00
$MgSO_4.7H_2O$	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
Tapioca starch	30.00	30.00	30.00	30.00	30.00	30.00

* Initial medium composition.

For isolate SUT513, L-lactic acid concentration of 5.16 g/l was obtained in the fermentation medium containing 30 g/l tapioca starch, 3.0 g/l yeast extract, and 2.5 g/l tryptone. The yield and productivity of L-lactic acid were 1.05 g/g and 0.11 g/l.h respectively, from the media containing 30.0 g/l (dry weight) tapioca starch supplemented 5.0 g/l of spent brewer's yeast and 2.5 g/l of tryptone. L-Lactic acid concentration was 4.82 g/l in 48 h (Appendix C5). Yield and productivity of L-lactic acid were 0.76 g/g and 0.10 g/l.h respectively (Figure 4.5A). Spent brewer's yeast, byproduct of yeast fermentation process, could support similar lactic acid productivity to yeast extract. Medium no. 5 (Table 4.1) was then chosen for further optimization. Spent brewer's yeast used contained starch (75.53%), moisture content (10.62%), fiber content (5.21%), total nitrogen content (6.21%), fat content (2.37%) and ash content (5.18%). Usually, minerals and vitamins are found in spent brewer's yeast (Duarte *et al*., 2008). The minerals include aluminum, barium, calcium, chromium, cobalt, copper, iron, magnesium, manganese, phosphorus, potassium, selenium, silicon, sodium, strontium, sulfur, zinc, and silicon. The vitamins include biotin, choline, folic acid, niacin, pantothenic acid, riboflavin, thiamine, and pyridoxine. Amino acid compositions (of total protein) include alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine (Duarte *et al*., 2008). These components of spent brewer's yeast could support growth and lactic acid production of LAB. The addition 10.0 g/l of spent brewer's yeast to optimal medium could not increase lactic acid yield of both isolates SUT513 and CAR134. The acid produced was only L-lactic acid isomer having >99% optical purity.

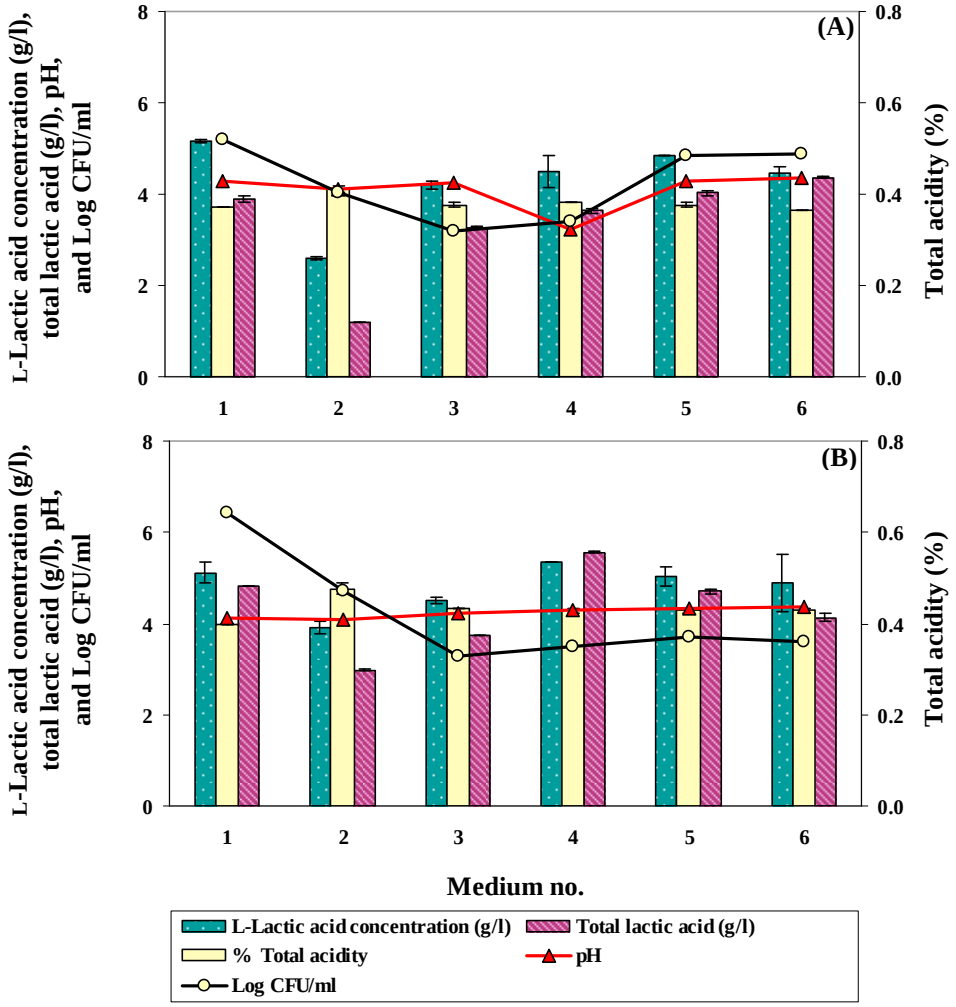


Figure 4.5 Effects of yeast extract and spent brewer's yeast (Table 4.1) on growth and L-lactic acid production by bacterial isolates SUT513 (A) and CAR134 (B) in modified RAM medium at the initial pH of 7.0 and incubated at 35°C for 48 h.

In the economic analysis of lactic acid fermentation, the largest contributor was found to be yeast extract accounting for about 38% of medium cost (Teleyadi and Cheryan, 1995; Altaf *et al*., 2007b). The addition of nutrients with higher concentrations generally had a positive effect on the lactic acid production.

Generally, MRS medium, which contains yeast extract, peptone and meat extract, was superior to yeast extract, which in turn was better than malt extract. This reflects the complex nutrient demands of LAB, being fastidious because of limited biosynthesis capacity (van Niel and Hahn-Hägerdal, 1999). For example, *Lactobacillus rhamnosus* NBRC 3863 could produce lactic acid at 42°C and pH 6.0 in batch fermentation providing with 100 g/l glucose supplemented with 1.7% fish wastes and 0.6% spent cells. The fermentation efficiency was similar to that using 1.5% yeast extract (Gao *et al*., 2007). However, when 0.3% yeast extract was combined with 30 g/l rice bran hydrolysate, lactic acid productivity became 1.6 times higher than that of the control fermentation using 1.5% yeast extract (Gao *et al*., 2008). Kwon *et al*. (2000) reported that 15 g/l yeast extract could be successfully replaced with 19.3 g/l soytone supplemented with vitamins, resulting in a production of 125 g/l lactic acid from 150 g/l glucose in batch fermentation at 42°C and pH 6.0 by *L. rhamnosus* ATCC 10863. The volumetric productivity and lactate yield were 2.27 g/l.h and 92% respectively, which were higher than those of 15 g/l yeast extract. Altaf *et al*. (2007a) reported the production of L-lactic acid by *L. amylophilus* GV6 at 37°C using 0.8% red lentil flour supplemented with 1% baker's yeast. And the possibility to replace more expensive commercial nitrogen sources, peptone and yeast extract in MRS medium containing 1% of soluble starch with red lentil flour and baker's yeast. The maximum lactic acid production of 13.5 g from 15.2 g soluble starch was obtained for 48 h, with 92% lactic acid yield efficiency.

Batch fermentation containing glucose supplemented with corn steep liquor and acid-hydrolysate of soybean meal as an alternative to yeast extract was performed by *L. casei* LA-04-1 at 42°C for L-lactic acid production. Most of the

initially existing 140 g/l of glucose was utilized and lactic acid concentration of 112.5 g/l was obtained (Li *et al*., 2006). *Enterococcus faecalis* RKY1 was reported to produce lactic acid with 102 g/l.h from medium contained 200 g/l of whole wheat flour hydrolyzate, 15 g/l of corn steep liquor, and 1.5 g/l of yeast extract at 38°C and pH 7.0 (Oh *et al*., 2005). Thus, in this study, the medium no. 5 in Table 4.1 (for SUT513) and medium no. 4 in Table 4.1 (for CAR134) were used to determine the suitable initial pH as well as the optimal temperature to obtain the maximum L-lactic acid production. Spent brewer's yeast could successfully replace yeast extract to reduce the cost of fermentation medium.

4.3.3 Initial pH of the culture medium

To obtain the maximum L-lactic acid production by cultivating the isolates SUT513 and CAR134, the initial pH of the suitable medium from section 4.3.2 was varied at pH 5.0, 5.5, 6.0, 6.5, 7.0 and 8.0. Isolates SUT513 and CAR134 could produce L-lactic acid in the medium adjusting initial pH at pH 5.0-8.0 (Figure 4.6). To produce lactic acid economically by direct bioconversion from starchy substrates, lactic acid productivity in relation to initial pH, which could influence the saccharification of starch, was investigated. The optimum initial pH was found to occur between 7.0 and 8.0. Below pH 7.0, strain SUT513 could not completely convert tapioca starch to lactic acid. This observation supports the conclusion that hydrolysis of starch did not occur at pH 5.0-6.5. With the initial pH of medium at 8.0, the production of lactic acid was slightly lower than at initial pH 7.0. In *Lactococcus lactis* subsp. *lactis* C2, it has been suggested that the utilization of starch was not affected at pH 4.5, but that uptake of carbohydrate could be inhibited by increased

proton levels (Yokota *et al*., 1995). However, the rapid drop in culture pH <4.0 during logarithmic growth phase should be prevented, since it is suggested to cause inhibition of lactic acid production. The highest L-lactic acid concentration of 4.82 g/l (Figure 4.6A) and 5.34 g/l (Figure 4.6B) produced by strains SUT513 and CAR134 respectively, was observed (Appendices C7-C8). L-Lactic acid obtained had an optical purity of >99%. Therefore, the initial pH of medium at 7.0 was chosen for further optimization.

The effect of pH on lactic acid production has been studied by cultivating the isolates *Lactobacillus plantarum*, *L. amylophilus* GV6 and *L. lactis* BME5-18M at pH between 5.0 and 7.0. The optimum pH for cell growth and lactic acid production of *L plantarum* was showed to be between 5.0 and 6.8 (Yumoto and Ikeda, 1995; Fu and Mathews, 1999; Ray *et al*., 2009). *Lactobacillus amylophilus* GV6 (Vishnu *et al*., 2000) and *L. lactis* BME5-18M (Bai *et al*., 2004) could produce lactic acid at pH 6.5. *Lactobacillus manihotivorans* LGM18010^T had optimum pH at 6.0 (Guyot *et al*., 2000) whereas *L. manihotivorans* LGM18011^T exhibited the maximum activity for growth at pH 5.0 (Ohkouchi and Inoue, 2006). *Lactococcus lactis* subsp. *lactis* B84 could produce lactic acid at pH 6.0 (Petrov *et al*., 2008). *Enterococcus faecium* No.78 was produced lactic acid at pH 6.5 (Shibata *et al*., 2007). Narita *et al*. (2004) reported that *Streptococcus bovis* 148 grew at pH between 5.8 and 9.6 with its optimum pH at 6.0, whereas *S. bovis* JMC 5802 showed its optimum pH for lactic acid production at 5.5 (Yuwono and Kokugan, 2008). Two selected strains grew and showed high L-lactic acid concentration in the medium adjusting pH at 7.0. The two selected strains could be used as starter cultures for lactic acid production.

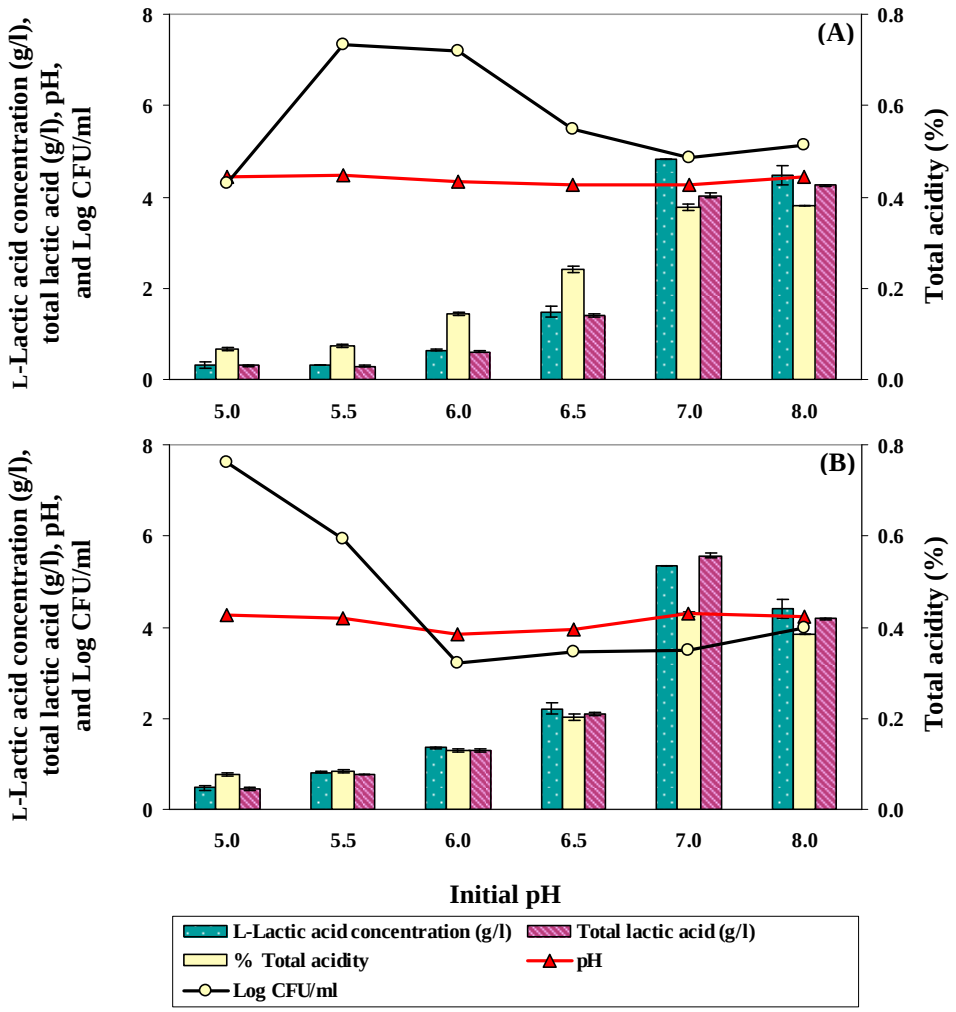


Figure 4.6 Effect of initial pH of fermentation medium (5.0, 5.5, 6.0, 6.5, 7.0, and 8.0) on bacterial growth and L-lactic acid production by SUT513 (A) and CAR134 (B) in modified RAM medium at an initial pH of 7.0 and incubated at 35°C for 48 h.

4.3.4 Cultivation temperature

The optimal temperature for L-lactic acid production was determined by cultivating the isolates SUT513 and CAR134 in the optimized medium with the pH adjusting to 7.0 for 48 h. The incubation temperatures were varied at 25, 30, 35, 37, 40 and 45°C based on the range of their growth temperatures. The production of L-lactic acid from starch was achieved at various temperatures (Figure 4.7). Results showed that the isolate SUT513 had ability to produce L-lactic acid when it was cultivated in optimal medium at 25-37°C. But the L-lactic acid concentration decreased when the cultivation temperature of medium was increased to 40 and 45°C. For the isolate CAR134, it could produce lactic acid when incubated at both 35 and 37°C. But the L-lactic acid concentration decreased when cultivated at temperatures of 25, 30, 40 and 45°C. Both strains could produce the maximum L-lactic acid concentration when cultivated at 35°C. The highest L-lactic acid production of 4.82 g/l (Figure 4.7A) and 5.34 g/l (Figure 4.7B) was observed by strains SUT513 and CAR134 respectively (Appendices C9-C10). L-Lactic acid produced had its optical purity of >99%. Thus, the optimal temperature for L-lactic acid production of the selected isolates was 35°C.

The temperature stimulating the highest productivity was in some cases lower than the temperature resulting in the highest lactic acid concentration and yield. For *L. amylophilus*, the optimal temperatures were 25 and 35°C for the maximum productivity and yield respectively (Hammes and Vogel, 1995). *Lactobacillus amylophilus* BCRC 14055 showed the optimum temperature for lactic acid production at 30°C (Yen and King, 2010). For *L. casei* and *L. paracasei* the optimal temperature for lactic acid production was reported to be between 37 and 44°C respectively

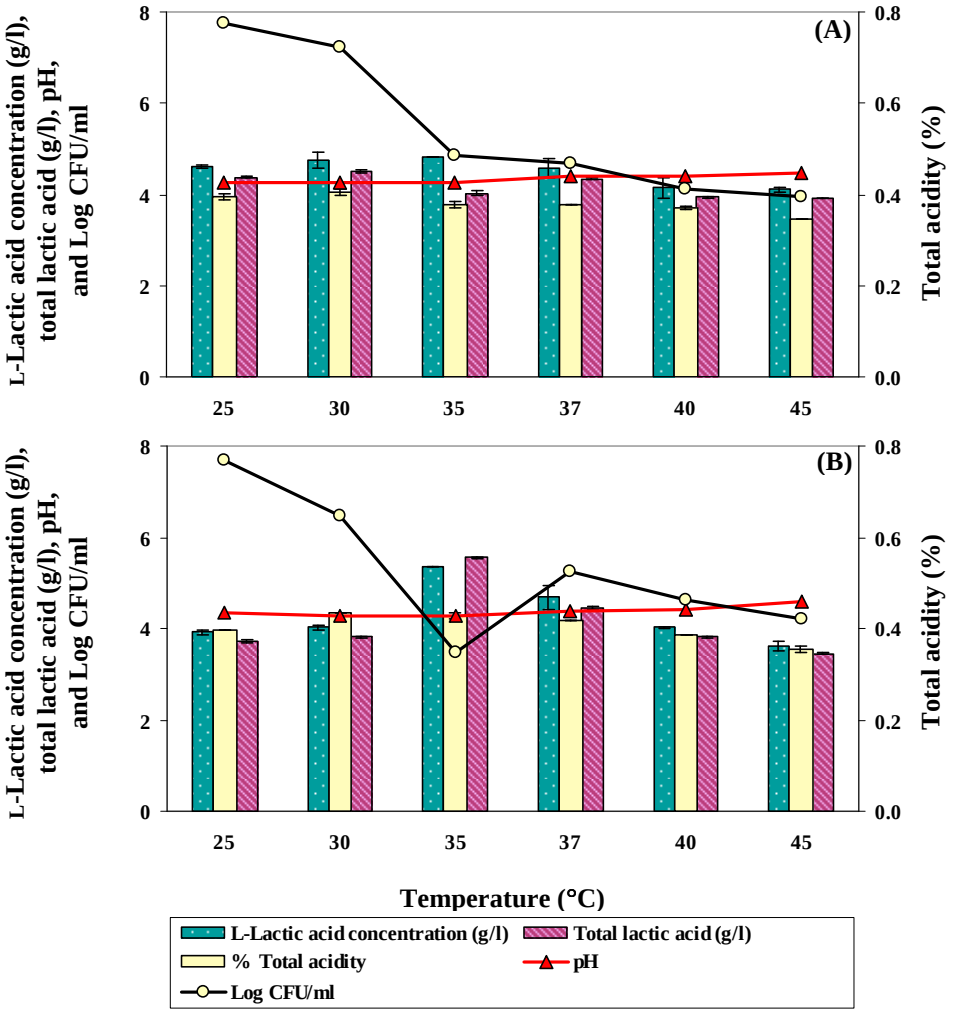


Figure 4.7 Effect of cultivation temperatures (25, 30, 35, 37, 40, and 45°C) on growth and L-lactic acid production of bacterial isolates SUT513 (A) and CAR134 (B) in modified RAM medium at an initial pH of 7.0 and incubated at 35°C for 48 h.

(Richter and Träger, 1994). In agreement with previous observations, *Lactococcus lactis* and *L. rhamnosus* exhibited the highest yields and productivities at 33 to 35°C and 41 to 45°C respectively (Hujanen and Linko, 1996). For *L. plantarum* MTCC 1407, it was produced lactic acid at 35°C (Ray *et al*., 2009). The optimal temperature

was reported to be 37°C for *L. casei* NRRL B441 (Linko and Javanainen, 1996), *L. amylophilus* GV6 (Vishnu *et al*., 2000), *L. helventicus* (Aeschlimann and Stockar, 1999), *L. lactis* BME5-18M (Bai *et al*., 2004), and *Streptococcus bovis* 148 (Narita *et al*., 2004). Whereas the optimal temperature for lactic acid production by *S. bovis* JCM 5802 was at 39°C (Yuwono and Kokugan, 2008). For *Lactococcus lactis* subsp. *lactis* B84 the optimal temperature for lactic acid production was reported to be 33°C (Petrov *et al*., 2008). The strain *Enterococcus faecium* No.78 was produced lactic acid at 30°C (Shibata *et al*., 2007). Therefore, temperature was a critical parameter that should be controlled in order to obtain the maximum lactic acid production.

4.3.5 Inoculum size

The amount of inoculum for L-lactic acid production was investigated by cultivating the isolates SUT513 and CAR134 in their optimized media with adjusting the initial pH at pH 7.0 and incubating at 35°C for 48 h. Inoculum sizes of 1, 2, 3, 4 and 5% of the starter culture containing approximately 10^6 CFU/ml were tested. Results showed that the high L-lactic acid yield was obtained when 1, 2, 3 and 5% of inoculum size were applied. But the acid concentration was a little bit increased when the 4% inoculum size was added into the fermentation medium. Thus, the 1% inoculum size was chosen for the production of lactic acid. The highest L-lactic acid production of 3.90 g/l (Figure 4.8A) and 4.69 g/l (Figure 4.8B) was observed by the strains SUT513 and CAR134, respectively. L-Lactic acid produced had its optical purity of >99% (Appendices C11-C12).

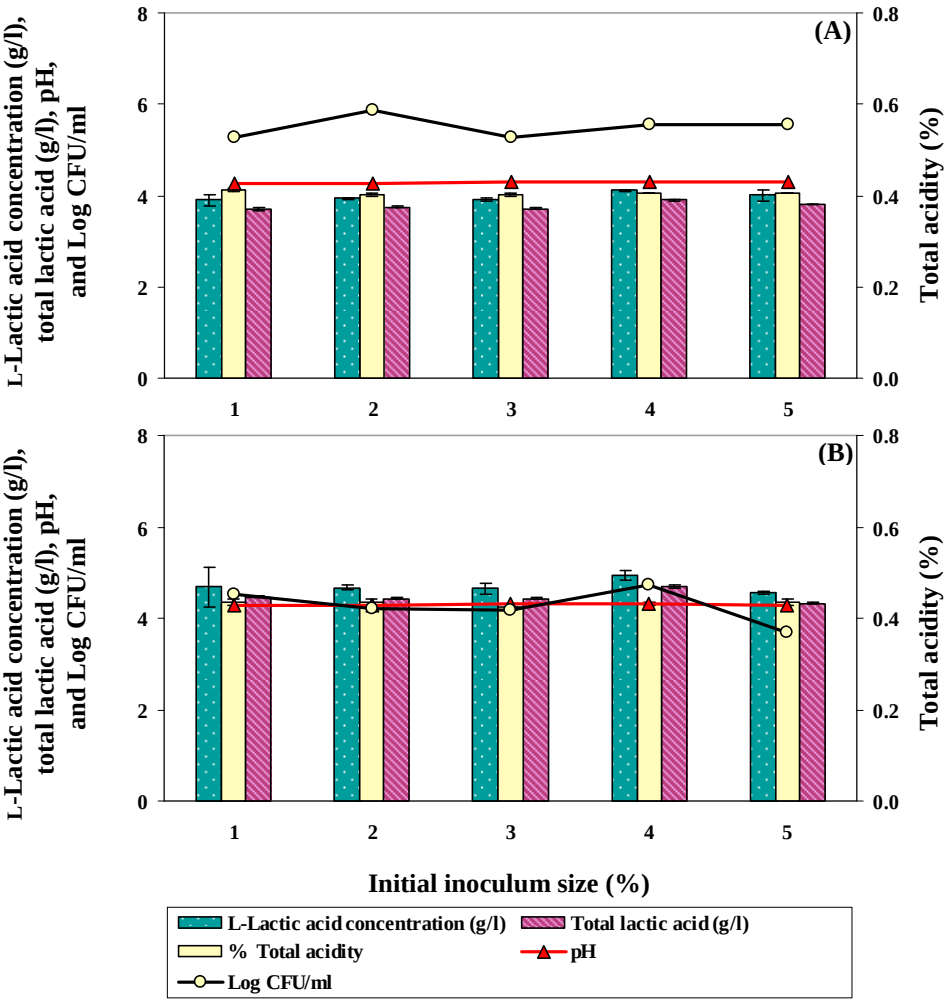


Figure 4.8 Effect of initial inoculum sizes (1, 2, 3, 4, and 5%) on L-lactic acid production by bacterial isolates SUT513 (A) and CAR134 (B) in modified RAM medium at an initial pH of 7.0 and incubated at 35°C for 48 h.

The effect of initial inoculum size on the production of lactic acid has only been studied in a few reports. Ray *et al*. (2009) found that the addition of *L. plantarum* MTCC 1407 at five levels (1, 2, 3, 4, and 5%) of inoculum volumes, 2% level was found to be the best for lactic acid production and inoculum levels higher

than 2% had obtained adverse effect. In case of semi-solid fermentation, the inoculum level varies according to the initial sugar or starch content used in the fermentation (John *et al*., 2007). Because growth of the microorganisms in simultaneous saccharification and fermentation (SSF) depends on the substrate: moisture ratio in correlation with environmental factors like pH and temperature. John *et al*. (2006) reported a similar inoculum volume level in lactic acid production from agro-wastes using *L. delbrueckii* as an inoculant. However, Linko and Javanainen (1996) reported that *L. casei* produced lactic acid when 10% inoculum was added. The highest lactic acid yield (98%) was achieved in 47 h from barley starch (130 g/l) simultaneously liquefied, saccharified, and fermented. Lactic acid production from barley starch with the 20% inoculum was also much slower than with a well-balanced simultaneous liquefaction, saccharification, and fermentation using the 10% inoculum.

4.4 Production of L-lactic acid from tapioca starch

L-Lactic acid production was performed in a 6.6 l controlled fermentor containing 5.0 l optimal medium containing 30 g/l (dry weight) of tapioca starch (Table 4.2). The cultivation temperature and pH were automatically controlled at 35°C and pH 7.0 and the agitation speed was maintained at 200 rpm for 48 h. An inoculum containing approximately 10^6 CFU/ml was inoculated into the suitable medium at 1% (v/v) size. Fermentation medium was taken at each time interval for measurement of bacterial growth, pH of the medium, remained substrate, and lactic acid concentration. The strain SUT513 was started L-lactic acid production at 4 h after inoculation and continuously increased until 28 h, which reached the maximum L-lactic acid yield of 38.9 g/l. The profile of cell growth and pH change during the

lactic acid fermentation was recorded (Figure 4.9A). Bacterial growth was related to the increase in lactic acid concentration. The maximum cell growth was obtained as 1.58×10^{13} CFU/ml for 12 h. Tapioca starch was completely consumed within 48 h. For substrate consumption, the complete consumption of tapioca starch was found within 20 h of fermentation. The result showed that available substrate limited lactic acid production. The strain could produce L-lactic acid yield ($Y_{LA/S}$) of 0.9964 g/g (99.64%), production rate (P_{LA}) of 1.61 g/l.h and specific growth rate (μ_{max}) of 0.51 h^{-1} (Table 4.3). L-Lactic acid yield of 99.64% based on tapioca starch was produced by isolate SUT513. Spent brewer's yeast could affect production because some amino acid could be used for fermentation process. In addition, some amino acid can converse to pyruvate and increase lactic acid yield (Campbell *et al*., 2006).

For the strain CAR134, L-lactic acid was started to produce at 6 h after inoculation and continuously increased until 38 h, which reached the maximum L-lactic acid yield of 32.70 g/l (Figure 4.9B). Bacterial growth was related to the increase in lactic acid. The maximum growth was obtained as 5.25×10^{11} CFU/ml for 14 h. Tapioca starch (30 g/l) was completely consumed within 22 h of fermentation. The result showed that the available substrate limited lactic acid production. CAR134 had its specific growth rate (μ_{max}) of 0.27 h^{-1}, lactic acid productivity (P_{LA}) of 1.41 g/l.h, and lactic acid yield ($Y_{LA/S}$) of 0.9215 g/g or 92.15% (Table 4.3). The acid produced was only L-lactic acid isomer (optical purity of >99%) (Appendices E3 and C13-C14).

Table 4.2 Components of RAM and optimized media for lactic acid production by bacterial strains SUT513 and CAR134.

Component	RAM medium (g/l)	Optimized medium (g/l) for isolate	
		SUT513	CAR134
Tapioca starch	10.00	30.00	30.00
Tryptone (Pancreatic digest of casein)	5.00	2.50	4.00
Yeast extract	3.00	-	-
Spent brewer's yeast	-	5.00	3.00
Dipotassium phosphate	6.00	6.00	6.00
$MgSO_4.7H_2O$	0.57	0.57	0.57
$MnSO_4.4H_2O$	0.12	0.12	0.12
$FeSO_4.7H_2O$	0.03	0.03	0.03

Table 4.3 Comparison of direct L-lactic acid fermentation from tapioca starch by isolated SUT513 and CAR134.

Parameter	Isolate code	
	SUT513	CAR134
Maximum bacterial growth (CFU/ml)	1.58×10^{13} (12 h)	5.25×10^{11} (14 h)
Maximum L-lactic acid concentration (g/l)	38.90 (28 h)	32.70 (38 h)
Specific growth rate (μ_{max}) (h^{-1})	0.51	0.27
Productivity (P_{LA}) (g/l.h)	1.61	1.41
Lactic acid yield ($Y_{LA/S}$) (%)	99.64	92.15
Optical purity of L-lactic acid (%)	>99	>99

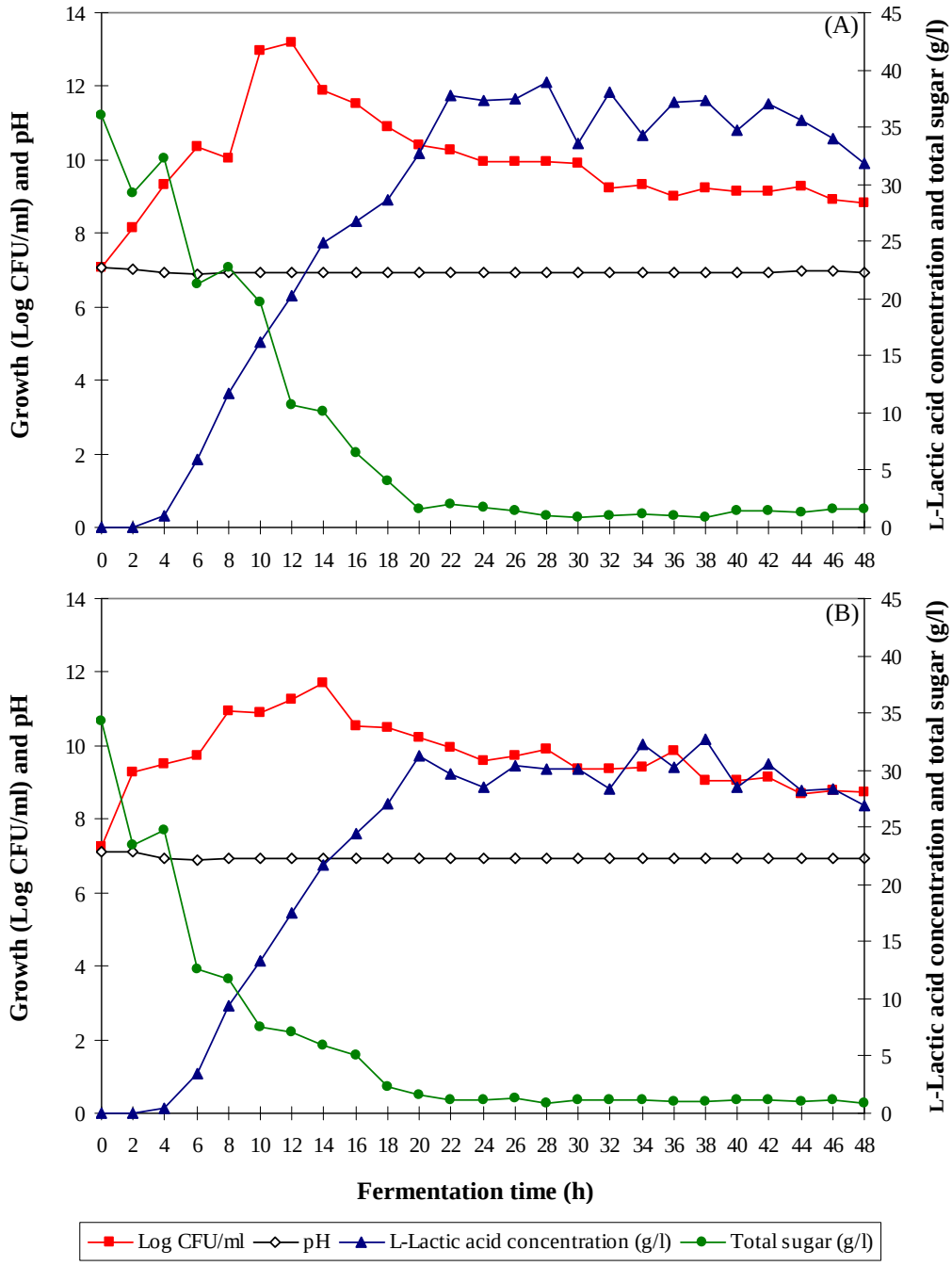


Figure 4.9 L-Lactic acid production by isolates SUT513 (A) and CAR134 (B) in 5 l optimized medium containing 30 g/l (dry weight) of tapioca starch in a controlled bioreactor at 35°C and pH 7.0.

The effect of pH control on lactic acid productivity has been reported. Without pH control, only 16.8 g lactic acid was produced from 50 g soluble starch by *Lactobacillus manihotivorans* LMG18011^T, and culture pH dropped to 3.5. With pH control at 5.0, the production of lactic acid increased to 40.7 g at 25°C (Ohkouchi and Inoue, 2006). The yield of lactic acid from starch by *L. manihotivorans* LMG18011^T with pH control at 5.0 was 2.5 times higher than without pH control. The culture pH was controlled at pH 5.0, the highest production and yield ($Y_{LA/S}$) with 98.5% optical purity were achieved. These results were distinctly different from those obtained with *L. manihotivorans* LMG18010^T (Guyot *et al*., 2000).

For *L. amylophilus* BCRC 14055, results from batch operation at different starch concentrations (20, 40, and 60 g/l) at 30°C showed that the batch with 20 g/l of initial starch provided the maximum productivity and the maximum yield of 0.31 g/l.h and 98% respectively (Yen and Kang, 2010). Batch operations with 20 and 60 g/l starch indicated the high starch concentrations leading to a slightly lower productivity, but largely decrease the yield. A slightly lower productivity in the batch operation with 60 g/l of starch could be the consequence of high lactic acid inhibition. These results were obviously different from those obtained from *L. amylophilus* (Yumoto and Ikeda, 1995), which was found that the lactic acid concentration could be as high as 37.16 g/l from the batch operating with 60 g/l starch, which might be high enough to reduce productivity. To increase productivity and lactic acid concentration, a starch-controlled fed-batch operation with 20 g/l of initial starch was performed and controlled pH at 5.3. The maximum productivity of 0.75 g/l.h and the yield of 69% were obtained from the fed-batch operation with starch controlled at 8±1 g/l.

However, *Enterococcus faecium* No. 78 was also tested for its lactic acid production from sago starch at 30°C and pH 6.5. The strain could produce L-lactic acid of 16.6 g/l from 20 g/l of starch (Shibata *et al*., 2007). It was superior to the other amylolytic LAB on the direct lactic acid fermentation with starches and produced lactic acid of high optical purity (98.6%). In the direct lactic acid fermentation from starch, continuous culture has hardly been reported. Continuous culture system with high cell density of *E. faecium* showed higher lactic acid productivity (3.04 g/l.h) than those of batch culture (1.10 g/l.h) and conventional continuous culture (1.56 g/l.h). Results from this study reveal that the isolates SUT513 and CAR134 have their potential for optically pure L-lactic acid production from tapioca starch. Amylolytic activity may involve with at least two enzymes. During fermentation the main activity is found with the cells. The existence of one extracellular and one cell-bound α-amylase has also been reported for some starch-hydrolyzing streptococci (Lindgren and Refai, 1984).

4.5 Preliminary investigation for L-lactic acid extraction and purification method from fermentation medium

L-Lactic acid produced by selected strains in the controlled fermentor from section 4.4 was extracted and purified. Tapioca starch fermentation medium, 600 ml with 38.90 g/l (for isolate SUT513) and 32.70 g/l (for isolate CAR134) L-lactic acid, after bacterial cell separation was heated to approximately 80-90°C to coagulate proteins which removed by filtration. L-Lactic acid was then precipitated from the cultured medium using $CaCl_2$ (0.5 mol of Ca/mole of lactic acid), the formed calcium lactate then concentrated under vacuum to 200 ml fermentation medium. After

crystallization, the main component contained in the fermentation mash was the salt of lactic acid (Ca lactate). The precipitated calcium lactate was separated from dissolved impurities by centrifugation. There were some loss of lactic acid in this step. Only lactic acid at concentrations of 18.89 and 15.89 g/l for isolates SUT513 and CAR134 respectively, were obtained. However, some impurities were still remained in the cake. The lactic acid could then be extracted from organic phase by backextraction with water. There was a loss of calcium lactate (14.80-20.45 g/l) along with the washes. After separating all insoluble components, the mash was acidified with 12 M sulfuric acid (37% in concentration) to liberate the lactic acid from its salt. Color of the acid solution was removed by treating with activated charcoal, resulting in a clear or lighter color compared to fermentation medium (Figure 4.10). The optical purity of L-lactic acid did not change during these purification steps. Purified L-lactic acid at concentrations of 64.2 and 57.0 g/l in 10 ml final volume with >99% optical purity for isolates SUT513 and CAR134 respectively, were achieved.

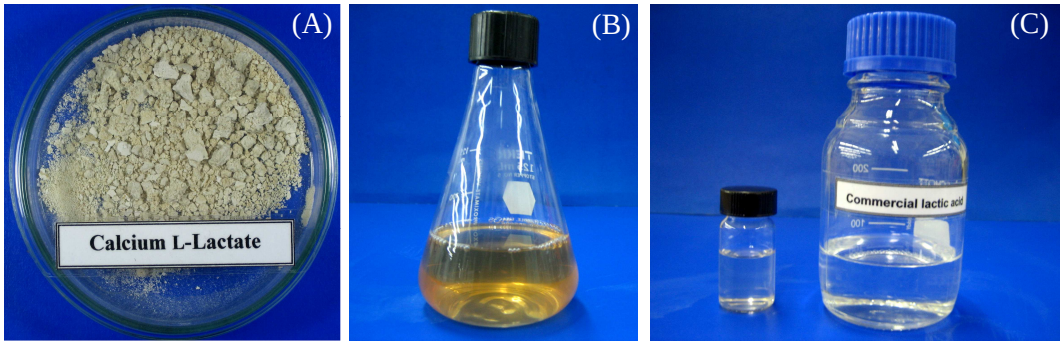


Figure 4.10 L-Lactic acid purification from tapioca starch fermentation broth: calcium L-lactate (A), purified L-lactic acid before decolorization (B), and purified L-lactic acid after decolorization compared to commercial lactic acid (C).

In fermentation broth, some organic acids (citric acid, lactic acid, formic acid, acetic acid, butyric acid, and propionic acid) that could be produced, were determined using a HPLC (Appendix E1). The total sugar content was also determined by the phenol-sulfuric acid method. After purification, about 99% of total sugars were removed.

Batch fermentation is traditionally performed with calcium hydroxide, but the regeneration of lactic acid results in the production of large amounts of solid calcium sulfate (Hofvendahl and Hahn-Hägerdal, 2000). Better alternatives are ammonia or calcium carbonate, leading to production of the fertilizer ammonium sulfate (Datta *et al*., 1995) or gaseous carbon dioxide, respectively. Continuous removal of the acid with extraction or electrodialysis results in even higher lactic acid concentrations and yields. The extracting material must be bio-compatible so as not to harm the organism, and one way of achieving is the aqueous two-phase systems (Yi *et al*., 2008), which provide good separation of lactic acid and cells when combined with a tertiary amine. González *et al*. (2006) studied the purification of lactic acid from fermentation broth using a weak anion exchanger (Lewatit S3428) and treated with a strong cation resin (Lewatit S2568H) at pH below the pK_a of lactic acid (3.86). The final purity of lactic acid was higher than 99%. Electrodialysis can be used in a pH controller producing the lactate anion (Min-Tian *et al*., 2005). Microbial cells are removed by filtration not to foul the membranes. The price of the membranes is presently a considerable drawback. Both aqueous two-phase systems and electrodialysis yield lactic acid, instead of lactate, which potentially could decrease the purification costs. For this study, a simple method was described for purification of optically pure L-lactic acid from cultured tapioca starch fermentation broth.

4.6 Identification of the selected lactic acid bacterial isolates

Many organisms can be identified on the basis of morphological characteristics. However, this approach is not reliable for all groups of organisms, including bacteria which possess limited morphological differentiation (Entis *et al*., 2001). Conventional methods for bacterial identification rely on results of biochemical tests and assimilation assays (Reva *et al*., 2001). Such physiological tests have been performed using traditional microbiological methods or commercially available kits such as API system. The API has been tested extensively with reported accuracy ranging from 90.2-93.0% (Entis *et al*., 2001). Therefore, the molecular technique especially, 16S rDNA sequence is needed to assist. The combination of morphological and physiological characterization, was also important, and should be used in combination with the molecular technique for accuracy identification.

4.6.1 Morphological and physiological characterization

Morphological and physiological characteristics of 4 selected bacterial strains exhibited the highest L-lactic acid concentration were studied. These isolates were Gram-positive, non-spore forming and non-motile. Three out of 4 isolates were ovoid cells, and only 1 isolate was cocci. All strains were homofermentative. Gas is not produced from D-glucose. The API-system was used for the characterization.

The selected strains CAR128, CAR134 and CAR135 were Gram-positive rods with the approximate cell sizes of 0.454-0.749×0.328-0.454 μm after cultivation on RAM agar containing 1% tapioca starch at 35°C for 18-24 h (Figure 4.11). They were non-motile and endospores were not observed after cultivation on RAM agar for 7 days. Colonies of CAR128, CAR134 and CAR135 were circular, low

convex with entire margin, white to cream color with 2.0-3.0 mm diameter after cultivation on RAM containing 1% tapioca starch at 35°C for 48 h (Figure 4.12). They were facultative anaerobic bacteria, grew in the presence of 0-4.0% NaCl. Strain CAR128 grew at 20-42°C and pH 6.0-8.0, while CAR134 and CAR135 grew at 20-45°C and pH 4.8-8.0. All selected strains showed catalase and oxidase negative. They were able to hydrolyze gelatin and skim milk. Isolates CAR128, CAR134 and CAR135 were also able to hydrolyze starch with wide clear zone (reacting with iodine) of 0.30, 0.70 and 0.75 cm diameter, respectively.

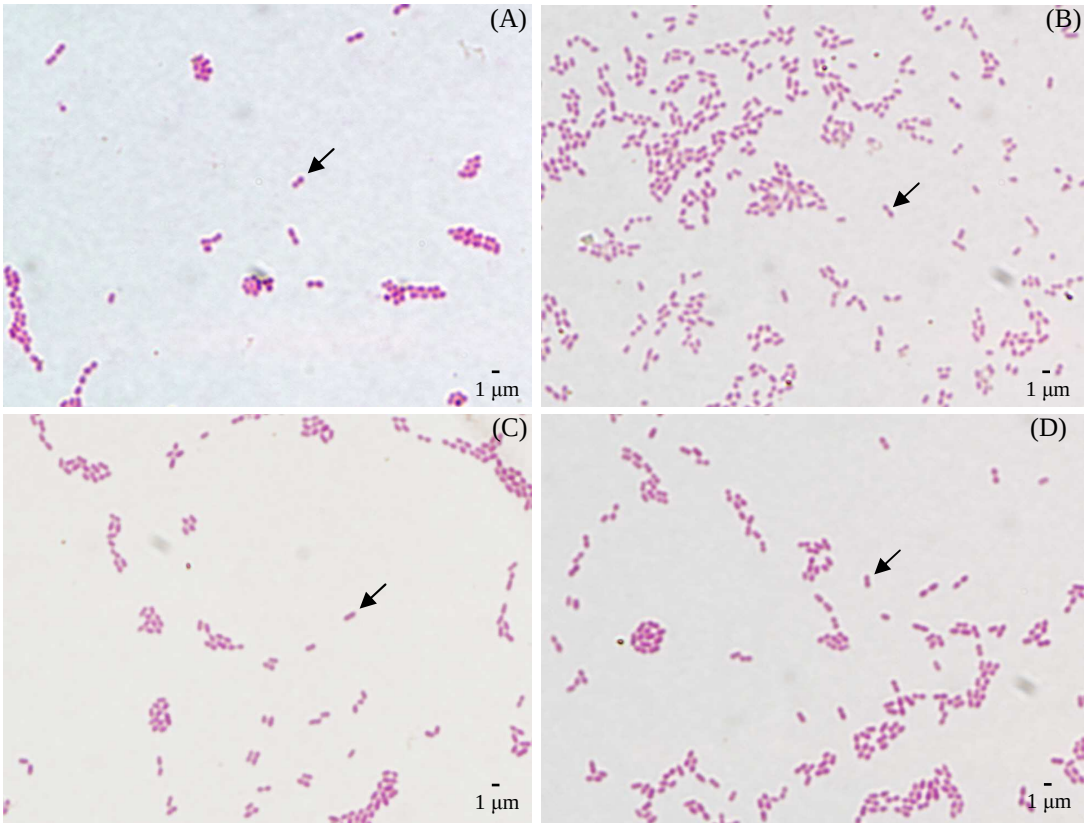


Figure 4.11 Gram stain of isolates SUT513 (A), CAR128 (B), CAR134 (C), and CAR135 (D), bright field microscopy (1,000×). Arrows indicate bacterial cell.

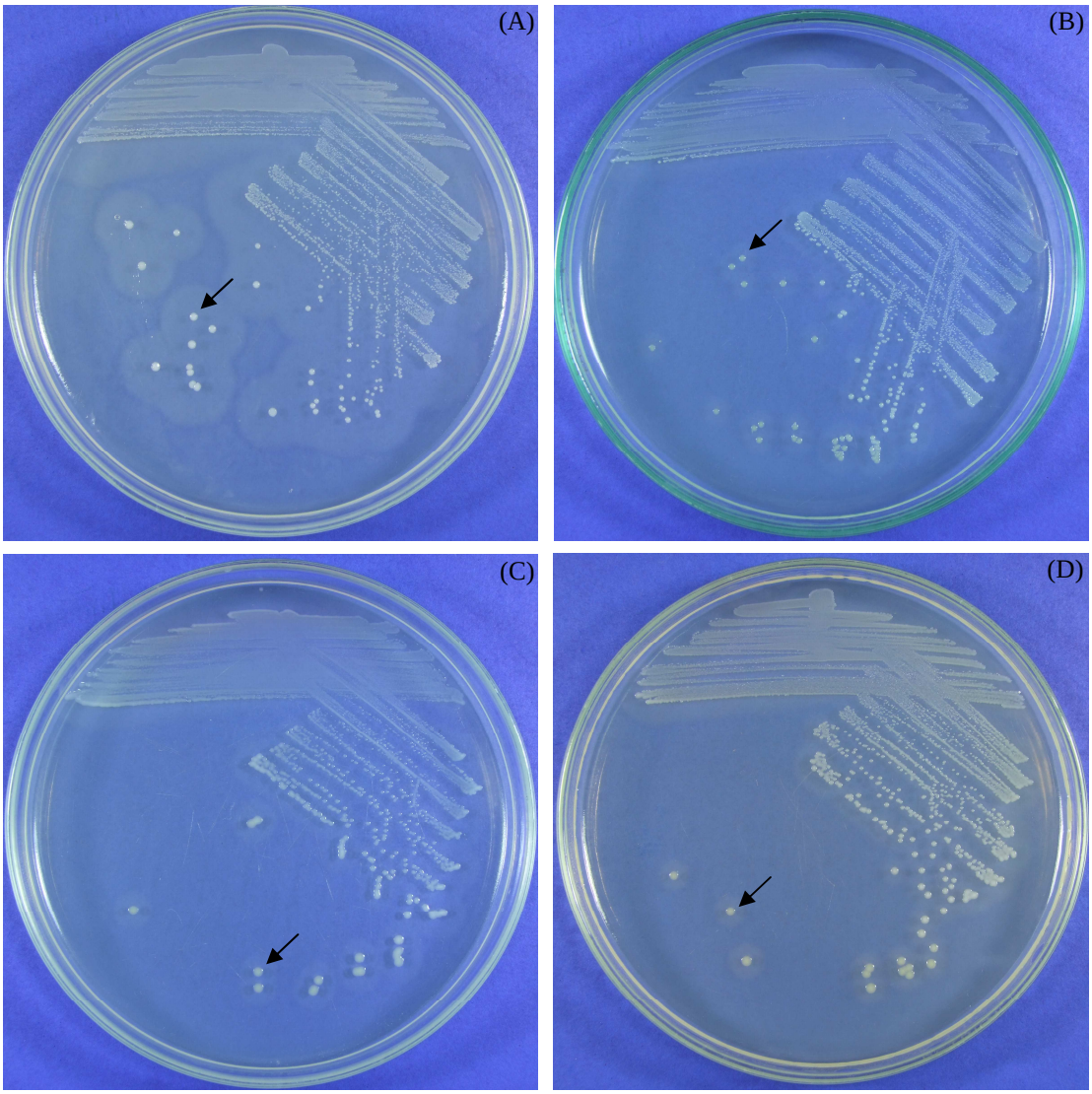


Figure 4.12 Colony morphology of isolates SUT513 (A); CAR128 (B); CAR134 (C); and CAR135 (D), on RAM agar containing 1% tapioca starch at 35°C for 48 h. Arrows indicate bacterial colonies.

Isolate SUT513 was Gram-positive coccus, non-spore forming and non-motile. The diameter of cell ranged from 0.38 to 0.51 μm after cultivation for 18-24 h at 35°C on RAM agar containing 1% tapioca starch. Colonies on RAM agar

containing 1% tapioca starch were circular, low convex with entire margin, and white color with 1.0-2.0 mm diameter after cultivation at 35°C for 48 h (Figure 4.12). The isolate SUT513 was catalase and oxidase negative, and grew at 0-3.0% NaCl, 20-45°C, and wide pH range of 5.0-8.0. They were facultative anaerobic bacteria. The isolate SUT513 was able to hydrolyze skim milk, but not hydrolyze gelatin. And it was able to hydrolyze starch with wide clear zone of 1.5 cm diameter (Figure 4.1).

All strains were identified based on carbohydrate assimilation and/or fermentation using API 50 CH/CHL system (bioMérieux). The results were compared in terms of the similarity percentages of carbohydrate assimilation and/or fermentation patterns with reference strains of the APILAB Plus software (version 5.0). Profiles of the selected isolates in API 50 CH gallery revealed that all strains could ferment D-glucose, D-galactose, D-fructose, D-mannose, D-maltose, D-lactose, D-saccharose (Sucrose), D-rafinose, *N*-acetylglucosamine, esculin, salicin, amidon (Starch) and hydrolyze glycogen. The isolates did not ferment glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-*β*D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-*α*D-mannopyranoside, methyl-*α*D-glucopyranoside, inulin, D-melezitose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, 2-ketogluconate, and 5-ketogluconate. Amygdalin, arbutin, D-cellobiose, D-melibiose, D-trehalose, and gentiobiose were fermented by strains CAR128, CAR134 and CAR135 but not by the strain SUT513. Strains CAR128 and CAR135 could weakly ferment Methyl-*α*D-glucopyranoside. They formed L-lactate from D-glucose but no gas was released, and they did not ferment neither gluconate nor pentose. They were considered as obligate homofermentatives. Morphological

and physiological characteristics of the selected strains are concluded in Table 4.4. These characteristics still had limitation for species identification. Isolates CAR128, CAR134 and CAR135 were biochemically identified as *Lactobacillus crispatus* with 99.9% similarity. Isolate SUT513 showed similar biochemical characteristics to *Lactobacillus acidophilus* 2 at 99.6% but they cell morphology was different. Isolates CAR128, CAR134, CAR135 and SUT513 were close to *Lactobacillus crispatus* and *Lactobacillus acidophilus* 2, but showed different phenotypic characteristics. Kandler and Weiss (1986) found that *Lactobacillus crispatus* and *Lactobacillus acidophilus* produced DL-lactic acid from D-glucose. However, our results indicated that CAR128, CAR134, CAR135 and SUT513 produced L-lactic acid from D-glucose, which was different from *Lactobacillus crispatus* and *Lactobacillus acidophilus*. Thus, morphological and physiological characteristics were not sufficient for identification of these isolates.

Table 4.4 General characteristics of 4 isolates of selected L-lactic acid-producing bacteria.

Characteristics	Bacterial strain			
	SUT513	CAR128	CAR134	CAR135
Cell shape	Cocci	Ovoid	Ovoid	Ovoid
Cell arrangement	Single, pairs, chains	Single, pairs, chains	Single, pairs, chains	Single, pairs, chains
Cell size (μm)	0.38-0.51	(0.30-0.43) × (0.44-0.75)	(0.32-0.45) × (0.45-0.74)	(0.31-0.45) × (0.48-0.76)
Gram	+	+	+	+
Spore forming	-	-	-	-
Aerobic growth	+	+	+	+
Anaerobic growth	+	+	+	+
Catalase test	-	-	-	-

Table 4.4 (Continued) General characteristics of 4 isolates of selected lactic acid-producing bacteria.

Characteristics	Bacterial strain			
	SUT513	CAR128	CAR134	CAR135
Oxidase test	-	-	-	-
Motility	-	-	-	-
Gas from D-glucose	-	-	-	-
Growth at 0% NaCl	+	+	+	+
3.0%	+	+	+	+
4.0%	-	+	+	+
6.5%	-	-	-	-
8.0%	-	-	-	-
10.0%	-	-	-	-
18.0%	-	-	-	-
Range (% NaCl)	0-3.0	0-4.0	0-4.0	0-4.0
Growth at 5°C	-	-	-	-
10°C	-	-	-	-
15°C	-	-	-	-
20°C	+	+	+	+
30°C	+	+	+	+
35°C	+	+	+	+
37°C	+	+	+	+
40°C	+	+	+	+
42°C	+	+	+	+
45°C	+	-	+	+
50°C	-	-	-	-
55°C	-	-	-	-
Range (°C)	20-45	20-42	20-45	20-45

Table 4.4 (Continued) General characteristics of 4 isolates of selected lactic acid-producing bacteria.

Characteristics	Bacterial strain			
	SUT513	CAR128	CAR134	CAR135
Growth at pH 4.0	-	-	-	-
4.4	-	-	-	-
4.8	-	-	+	+
5.0	+	-	+	+
6.0	+	+	+	+
6.5	+	+	+	+
7.0	+	+	+	+
8.0	+	+	+	+
9.6	-	-	-	-
Range pH	5.0-8.0	6.0-8.0	4.8-8.0	4.8-8.0
Hydolysis of:				
Starch	+	+	+	+
Skim milk	+	+	+	+
Gelatin	-	+	+	+
Arginine	-	-	-	-
Lactic acid configuration	L	L	L	L

4.6.2 Molecular characterization of 16S ribosomal RNA gene

From morphological and physiological characteristic results of selected bacterial strains, they could not be identified. Therefore, the 16S rRNA sequence analysis was performed to assist this identification. Genomic DNA of isolates SUT513 and CAR134 was extracted and purified (Figure 4.13), and used for 16S DNA amplification by PCR using fD1/rP2 primers.

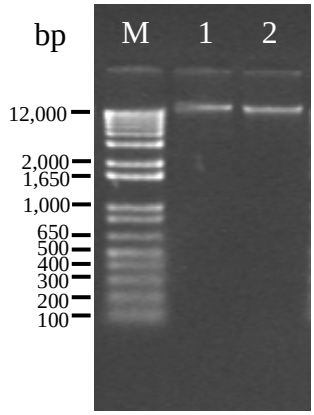


Figure 4.13 Agarose gel electrophoresis of genomic DNA extracted from two selected isolates of starch-utilizing and L-lactic acid-producing bacteria. Lanes: M, 1 Kb plus DNA ladder (Invitrogen) as a molecular weight marker; 1, bacteria isolates SUT513; and 2, CAR134.

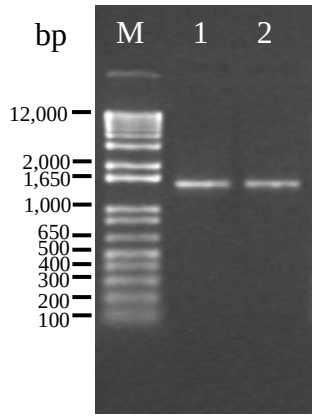


Figure 4.14 Agarose gel electrophoresis of PCR fragment obtained from the amplification of genomic DNA of selected isolates using primer fD1 and rP2. Lanes: M, 1 Kb plus DNA ladder (Invitrogen) as a molecular weight marker; 1, bacteria isolates SUT513; and 2, CAR134.

The length of amplified fragments of the two isolates were similar in size being approximately 1,500 bp (Figure 4.14). After sequencing of the DNA fragments, nucleotide sequences of the isolates SUT513 and CAR134 had 91-99% homology compared to *Streptococcus* species (Tables 4.5 and 4.6).

After sequencing, the nucleotide sequences of the PCR product (1,500 bp) were analyzed, and compared to local alignment that have been reported using BLAST version 2.2.9 program from GenBank database of the National Center for Biotechnological Information (NCBI). The 16S rDNA sequence, corresponding to positions 8-1420, alignment is given in Appendix G. Isolate SUT513 showed the highest similarity with CAR134 at relation value of 99% (Figure 4.15). The phylogenetic trees of isolates SUT513 and CAR134 were constructed based on 16S rRNA gene sequences using the neighbour-joining method and the maximum parsimony method by MEGA version 4 (Kumar *et al*., 2004) (Figure 4.16), which demonstrated that the two strains formed a tight clade closely related to *Streptococcus infantarius*, *S. lutetiensis*, *S. bovis* and *S. equinus* species from GenBank database, with similarity values of 99%. The stability relationships were evaluated by a boot strap analysis of 1,000 replications.

Table 4.5 Similarity of 16S rRNA gene sequences of SUT513 and CAR134 compared with *Streptococcus* species from NCBI nucleotide sequence database.

Bacterial isolate code	Length of sequence (nt)	Nucleotide sequence comparison, identification result and details				
		Closest relative*	Length of sequence (bp)	Sequence homology (%)	GenBank accession number	Isolation source/ remark of closest relative
SUT513	1466	*Streptococcus luteiensis* subsp. *infantarius* CIP 106107^T	1470	99	DQ232530	Human blood
		Streptococcus luteiensis subsp. *infantarius* CIP 106106^T	1470	99	DQ232529	Infant feces
		Streptococcus luteiensis CIP 106849^T	1470	99	DQ232532	Human
		Streptococcus bovis ATCC 27960^T	1500	98	AB002481	Swine
		Streptococcus bovis NCFB 2476^T	1539	98	AF396922	Ruminants
		Streptococcus bovis NCTC 11436^T	1517	98	AJ305257	Human blood
		Streptococcus equinus CIP 82.5^T	1469	98	DQ232522	Horse feces
		Streptococcus equinus NCDO 1037^T	1463	98	AF429765	Horse feces
		Streptococcus luteciae NEM 782^T	1461	98	AJ297215	Human isolate

Table 4.5 (Continued) similarity of 16S rRNA gene sequences of SUT513 and CAR134 compared with *Streptococcus* species from NCBI nucleotide sequence database.

Bacterial isolate code	Length of sequence (nt)	Nucleotide sequence comparison, identification result and details				
		Closest relative*	Length of sequence (bp)	Sequence homology (%)	GenBank accession number	Isolation source/ remark of closest relative
CAR134	1457	*Streptococcus luteiensis* subsp. *infantarius* CIP 106107^T	1470	99	DQ232530	Human blood
		Streptococcus luteiensis subsp. *infantarius* CIP 106106^T	1470	99	DQ232529	Infant feces
		Streptococcus luteiensis CIP 106849^T	1470	99	DQ232532	Human
		Streptococcus bovis ATCC 27960^T	1500	99	AB002481	Swine
		Streptococcus bovis NCFB 2476^T	1539	99	AF396922	Ruminants
		Streptococcus bovis NCTC 11436^T	1517	99	AJ305257	Human blood
		Streptococcus equinus CIP 82.5^T	1469	99	DQ232522	Equine feces
		Streptococcus equinus NCDO 1037^T	1463	99	AF429765	Horse feces
		Streptococcus luteciae NEM 782^T	1461	99	AJ297215	Human isolate

* ATCC, American Type Culture Collection; CIP, Collection de I'Institut Pasteur; NCDO, National Collection of Dairy Organism; NCFB, National Collection of Food Bacteria; NCTC, National Collection of Type Culture; NEM, Necker-Enfants Malades.

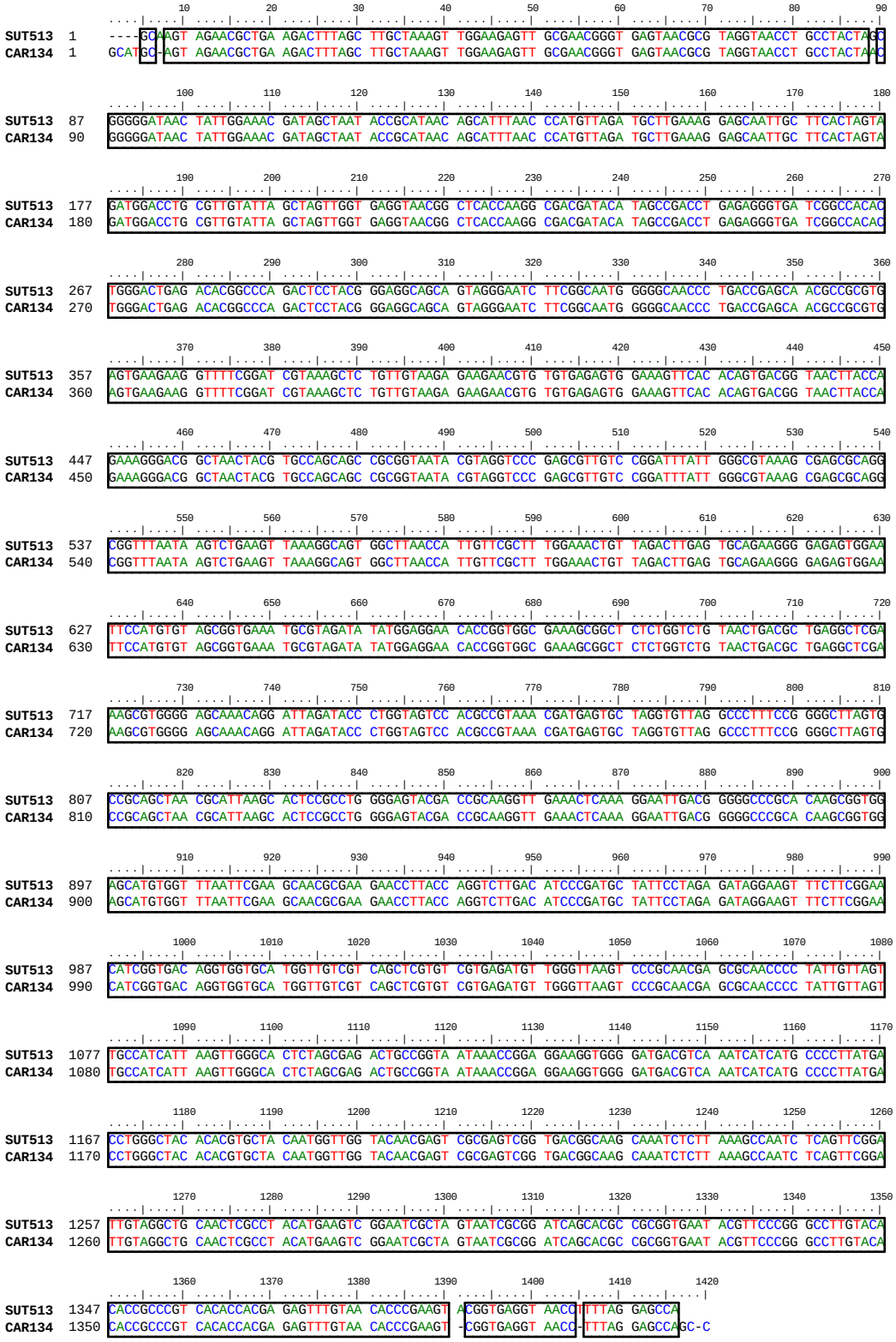


Figure 4.15 Sequence alignment of partial 16S rDNA amplified by fD1 and rP2 primers, of SUT513 and CAR134 using BioEdit program.

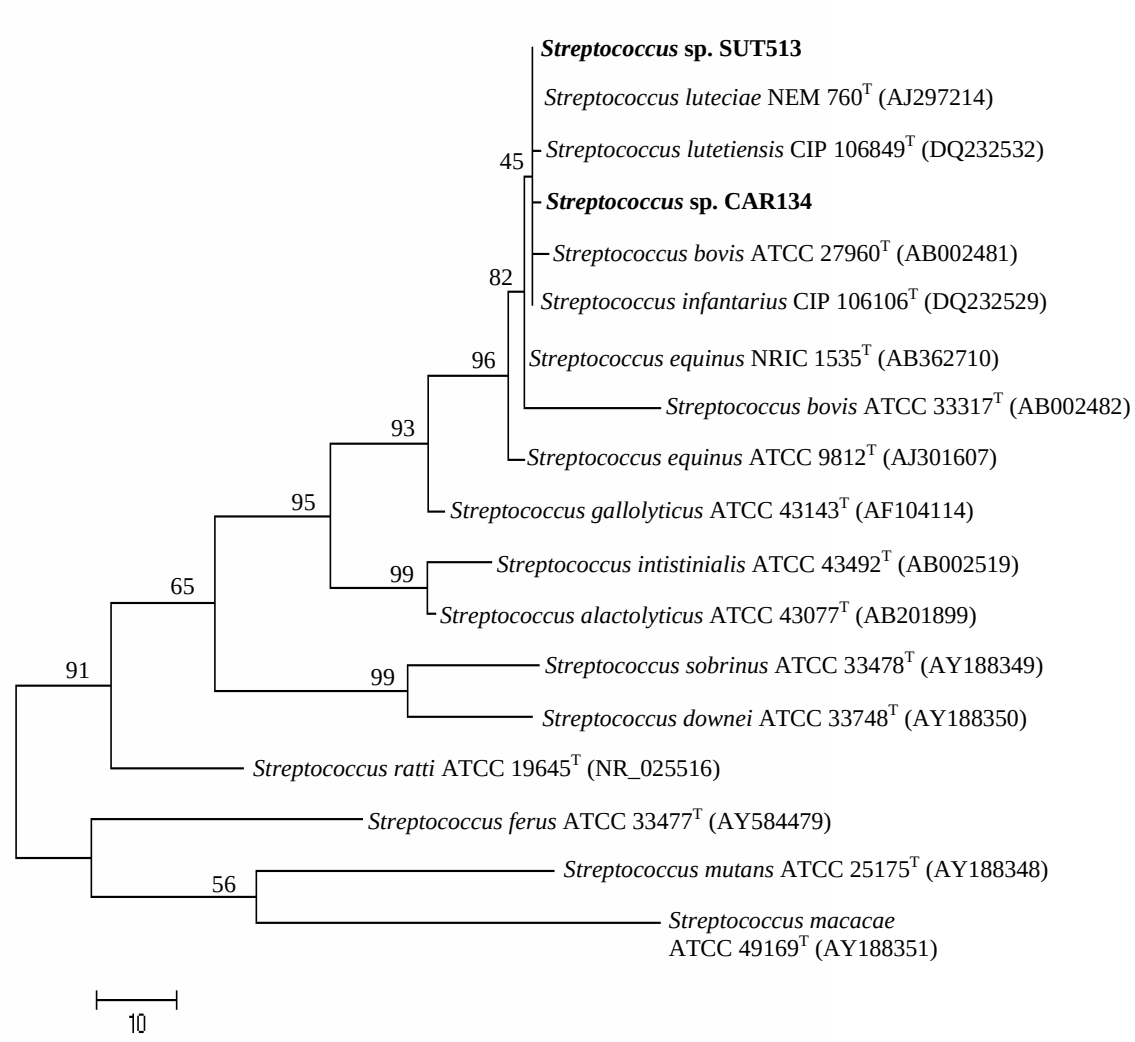


Figure 4.16 Phylogenetic tree of isolates SUT513 and CAR134, based on 16S rRNA gene sequences constructed by using the neighbour-joining method. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replication.

Table 4.6 16S rRNA gene sequence similarity of isolates SUT513 and CAR134 and related species.

Bacterial isolates	SUT513	CAR134	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SUT513	100																	
CAR134	99	100																
1	99	99	100															
2	99	99	99	100														
3	99	99	99	100	100													
4	98	98	98	98	98	100												
5	99	99	99	99	99	98	100											
6	96	96	96	96	96	95	96	100										
7	98	98	98	98	98	97	98	98	100									
8	98	98	99	98	98	97	99	95	97	100								
9	95	95	96	96	96	95	96	93	95	96	100							
10	96	96	96	96	96	95	96	93	94	96	97	100						
11	93	93	94	94	94	93	94	91	92	94	93	93	100					
12	93	93	93	93	93	92	93	91	92	94	93	93	97	100				
13	93	93	94	94	93	93	94	91	92	94	93	95	93	94	100			
14	92	92	92	92	92	91	92	90	91	92	92	92	93	93	93	100		
15	93	93	94	94	94	92	94	91	92	93	91	91	93	93	94	93	100	
16	91	91	91	91	91	90	91	89	90	91	90	90	90	91	91	92	91	100

SUT513: *Streptococcus* sp. SUT513, CAR134: *Streptococcus* sp. CAR134, 1: *Streptococcus infantarius* subsp. *Infantarius* CIP 106106 (DQ232529), 2: *Streptococcus lutetiensis* CIP 106849 (DQ232532), 3: *Streptococcus bovis* ATCC 27960 (AB002481), 4: *Streptococcus bovis* ATCC 33317 (AB002482), 5: *Streptococcus equinus* NRIC 1535 (AB362710), 6: *Streptococcus equinus* ATCC 9812 (AJ301607), 7: *Streptococcus luteciae* NEM 760 (AJ297214), 8: *Streptococcus gallolyticus* subsp. *gallolyticus* ATCC 43143 (AF104114), 9: *Streptococcus intestinalis* ACTT 43492 (AB002519), 10: *Streptococcus alactolyticus* ATCC 43077 (AF201899), 11: *Streptococcus downei* ATCC 33748 (AY188350), 12: *Streptococcus sobrinus* ATCC 33478 (AY188349), 13: *Streptococcus ratti* ATCC 19645 (NR_025516), 14: *Streptococcus ferus* ATCC 33477 (AY584479), 15: *Streptococcus mutans* ATCC 25175 (AY188348), 16: *Streptococcus macacae* ATCC 35911 (AY188351).

Table 4.7 Morphological and physiological characteristics of 4 selected isolates and their closer phylogenetic neighbours *Streptococcus bovis*, *S. equinus*, *S. infantarius* and *S. lutetiensis*.

Characteristics	Selected LAB isolate				Type strain			
	SUT513	CAR128	CAR134	CAR135	*S. bovis*[a]	*S. equinus*[a]	*S. infantarius*[b]	*S. lutetiensis*[c]
Cell shape	Cocci	Ovoid	Ovoid	Ovoid	Cocci	Cocci	Cocci	Cocci
Cell arrangement	Single, pairs, short chains	Single, pairs, short chains	Single, pairs, short chains	Single, pairs, short chains	Pairs, short chains	Pairs, short chains	Pairs, short chains	Pairs, short chains
Cell size (μm)	0.38-0.51	(0.30-0.43) × (0.44-0.75)	(0.32-0.45) × (0.45-0.74)	(0.31-0.45) × (0.48-0.76)	0.8-1.0	0.8-1.2	0.8-1.2	0.8-1.2
Gram	+	+	+	+	+	+	+	+
Spore forming	-	-	-	-	-	-	-	-
Aerobic growth	+	+	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-
Growth at 10°C	-	-	-	-	-	-	-	-
Growth at 45°C	+	-	+	+	-	+	-	-

Table 4.7 (Continued) Morphological and physiological characteristics of 4 selected isolates and their closer phylogenetic neighbours *Streptococcus bovis*, *S. equinus*, *S. infantarius* and *S. lutetiensis*.

Characteristics	Selected LAB isolate				Type strain			
	SUT513	CAR128	CAR134	CAR135	*S. bovis*[a]	*S. equinus*[a]	*S. infantarius*[b]	*S. lutetiensis*[c]
Growth at 6.5% NaCl	-	-	-	-	+	-	-	-
Growth at 18% NaCl	-	-	-	-	-	-	-	-
Growth at pH 4.4	-	-	-	-	-	-	-	-
Growth at pH 9.6	-	-	-	-	-	-	-	-
Gas from D-glucose	-	-	-	-	+	-	-	-
Hydolysis of:								
Starch	+	+	+	+	+	+	+	+
Skim milk	+	+	+	+	-	-	NA	NA
Gelatin	-	+	+	+	NA	NA	NA	NA
Arginine	-	-	-	-	-	-	-	-
Acid from:								
Glycerol	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	NA	NA	NA	NA
D-Arabinose	-	-	-	-	d	-	NA	NA
L-Arabinose	-	-	-	-	d	-	-	-

Table 4.7 (Continued) Morphological and physiological characteristics of 4 selected isolates and their closer phylogenetic neighbours *Streptococcus bovis*, *S. equinus*, *S. infantarius* and *S. lutetiensis*.

Characteristics	Selected LAB isolate				Type strain			
	SUT513	CAR128	CAR134	CAR135	*S. bovis*[a]	*S. equinus*[a]	*S. infantarius*[b]	*S. lutetiensis*[c]
D-Ribose	-	-	-	-	-	-	-	-
D-Xylose	-	-	-	-	d	-	NA	NA
L- Xylose	-	-	-	-	d	-	NA	NA
D-Adonitol	-	-	-	-	NA	NA	NA	NA
Methyl-*β*D-xylopyranoside	-	-	-	-	NA	NA	NA	NA
D-Galactose	+	+	+	+	+	+	NA	NA
D-Glucose	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	NA	NA
D-Mannose	+	+	+	+	+	NA	NA	NA
L-Sorbose	-	-	-	-	NA	NA	NA	NA
L-Rhamnose	-	-	-	-	(-)	-	-	NA
Dulcitol	-	-	-	-	NA	NA	NA	NA
Inositol	-	-	-	-	NA	NA	NA	NA
D-Mannitol	-	-	-	-	d	-	-	-

Table 4.7 (Continued) Morphological and physiological characteristics of 4 selected isolates and their closer phylogenetic neighbours *Streptococcus bovis*, *S. equinus*, *S. infantarius* and *S. lutetiensis*.

Carbohydrate	Selected LAB isolate				Type strain			
	SUT513	CAR128	CAR134	CAR135	*S. bovis*[a]	*S. equinus*[ab]	*S. infantarius*[b]	*S. lutetiensis*[c]
D-Sorbitol	-	-	-	-	d	-	-	-
Methyl-αD-mannopyranoside	-	-	-	-	(-)	-	NA	NA
Methyl-αD-glucopyranoside	-	w	-	w	+	+	+	+
N-Acetylglucosamine	+	+	+	+	(+)	-	-	-
Amygdalin	-	+	+	+	(+)	+	NA	NA
Arbutin	-	+	+	+	(+)	+	NA	NA
Esculin	+	+	+	+	+	NA	NA	NA
Salicin	+	+	+	+	(+)	(+)	+	NA
D-Cellobiose	-	+	+	+	NA	NA	NA	NA
D-Maltose	+	+	+	+	+	+	+	+
D-Lactose	+	+	+	+	(+)	(+)	+	+
D-Melibiose	-	+	+	+	(+)	(+)	+	-
D-Saccharose (Sucrose)	+	+	+	+	+	+	+	+
D-Trehalose	-	+	+	+	-	+	-	-

Table 4.7 (Continued) Morphological and physiological characteristics of 4 selected isolates and their closer phylogenetic neighbours *Streptococcus bovis*, *S. equinus*, *S. infantarius* and *S. lutetiensis*.

Characteristics	Selected LAB isolate				Type strain			
	SUT513	CAR128	CAR134	CAR135	*S. bovis*[a]	*S. equinus*[a]	*S. infantarius*[b]	*S. lutetiensis*[c]
Inulin	-	-	-	-	d	(+)	NA	NA
D-Melezitose	-	-	-	-	(-)	-	-	-
D-Rafinose	+	+	+	+	(+)	(+)	-	+
Amidon (Starch)	+	+	+	+	+	(+)	+	+
Glycogen	+	+	+	+	(+)	(+)	-	-
Xylitol	-	-	-	-	NA	NA	NA	NA
Gentiobiose	-	+	+	+	NA	NA	NA	NA
D-Turanose	-	-	-	-	NA	NA	NA	NA
D-Lyxose	-	-	-	-	NA	NA	NA	NA
D-Tagatose	-	-	-	-	(+)	-	-	-
D-Fucose	-	-	-	-	NA	NA	NA	NA
L-Fucose	-	-	-	-	NA	NA	NA	NA
D-Arabitol	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	NA	NA	NA	NA
Potassium gluconate	-	-	-	-	NA	NA	NA	NA

Table 4.7 (Continued) Morphological and physiological characteristics of 4 selected isolates and their closer phylogenetic neighbours *Streptococcus bovis*, *S. equinus*, *S. infantarius* and *S. lutetiensis*.

Characteristics	Selected LAB isolate				Type culture strain			
	SUT513	CAR128	CAR134	CAR135	*S. bovis*[a]	*S. equinus*[a]	*S. infantarius*[b]	*S. lutetiensis*[c]
Potassium 2-Ketogluconate	-	-	-	-	NA	NA	NA	NA
Potassium 5-Ketogluconate	-	-	-	-	NA	NA	NA	NA
Lactic acid configuration	L	L	L	L	L	L	L	L

+: Positive; -: negative; (+): 75-89% are positive; (-): 75-89% are negative; d: 11-89% strains positive; delayed reaction; w: weakly.
[a] : Hardie and Whiley (1995); [b]: Schlegel *et al*. (2000); [c]: Poyart *et al*. (2002).
NA = Not available.

CHAPTER V

CONCLUSIONS

Two hundred and eighty isolates of lactic acid bacteria were obtained from stock cultures of the Microbial Culture Collection and Applications Research Unit, Suranaree University of Technology. One hundred and ninety two isolates were Gram-positive rods, and 88 isolates were Gram-positive cocci occurring singly, in pairs or in chains. Colonies of the isolates on medium agar were punctiform, small, moderate and large with 0.1-4.0 mm in diameters with circular and irregular forms, and entire and undulate margins. These colony elevation of these colonies was flat, low convex, convex and umbonate. One hundred and twenty-eigth out of 280 isolates could utilize tapioca starch performing wide clear zones of 0.1-1.7 cm in diameters on RAM agar after reacting with iodine. These isolates were selected for testing L-lactic acid production using MRS broth containing 2% glucose. After incubating for 24 h, pH of the cultured broth was found to be between 3.5-5.6 corresponding to total acidity of 0.476-1.887%. One hundred and ninety-six out of 280 isolates were homofermentatives, and 160 out of 196 isolates were produced with L-lactic acid with optical purity >95% in the range of 0.91-19.12 g/l. One hundred and twenty-eight out of 160 isolates could utilize tapioca starch and produce L-lactic acid at concentrations ranging between 0.91 and 8.60 g/l. Two starch-utilizing isolates (CAR134 and SUT513) producing L-lactic acid at concentrations of 7.89 and 8.60 g/l respectively, were then selected for the acid production from tapioca starch. The two strains were

identified as belonging to different strains of the genus *Streptococcus* according to their morphological and physiological characteristics, and 16S rRNA gene sequence, corresponding to positions 8-1420, which demonstrated that the two strains formed a tight clade closely related to *Streptococcus infantarius*, *S. lutetiensis*, *S. bovis* and *S. equinus* species from GenBank database, with similarity values of 99%. For evaluation of L-lactic acid production from tapioca starch compared to glucose, isolates CAR134 and SUT513 produced lactic acid of 6.70 and 6.86 g/l in MRS broth containing 2% glucose, and 4.55 and 5.24 g/l in modified RAM broth containing 1% tapioca starch, respectively, after 48 h incubation. For optimization of bacterial growth and lactic acid production conditions, the suitable media for both growth and L-lactic acid production of isolates CAR134 and SUT513 were found to compose of main ingredients as follows: 30 and 30 g/l (dry weight) of tapioca starch, 3.0 and 5.0 g/l of spent brewer's yeast, and 4.0 and 2.5 g/l of tryptone, respectively, at the initial pH of 7.0. The optimum cultivation conditions were under anaerobic condition at 35°C with the inoculum size (10^6 CFU/ml) of 1% (v/v). The maximum concentrations of L-lactic acid of 5.34 and 5.16 g/l with optical purity >99% were obtained for isolates CAR134 and SUT513 respectively. When lactic acid fermentation was performed in a 6.6 l bioreactor containing 5 l of the optimized media under optimal cultivation conditions, strains CAR134 and SUT513 could produce the maximum L-lactic acid concentrations of 32.70 and 38.90 g/l with >99% optical purity after cultivation for 38 and 28 h, respectively. The two strains could produce L-lactic acid yield ($Y_{LA/S}$) of 92.15 and 99.64 % with productivity of 1.41 and 1.61 g/l.h, and specific growth rates (μ_{max}) of 0.27 and 0.51 h^{-1}, respectively. The acid product could be simply purified from the inexpensive optimized tapioca starch media by

crystallization using calcium chloride. The purification process resulted in purified L-lactic acid (100% optical purity) of 57.0 and 64.2 g/l in 10 ml total volume from 600 ml of fermentation broth, for isolates CAR134 and SUT513 respectively. From this study, the starch-utilizing and lactic acid-producing bacterium isolates CAR134 and SUT513 could directly produce L-lactic acid with 100% optical purity from tapioca starch, a cheap and abundant raw material in Thailand. L-Lactic acid with high optical purity is very useful for the production of biodegradable plastics.

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APPENDICES

APPENDIX A

CULTURE MEDIA AND REAGENTS

1. Culture media

All culture media were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches.

1.1 De Man, Rogosa and Sharpe broth (MRS broth)

Proteose peptone	10.00	g
Beef extract	8.00	g
Yeast extract	4.00	g
Tween 80 ($(NH_4)_3C_6H_5O_7$)	1.00	g
tri-Ammonium citrate ($CH_3COONa.3H_2O$)	2.00	g
Sodium acetate trihydrate	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
Distilled water added and brought up total volume to	1,000.00	ml
Final pH 6.2 ± 0.2 at 25°C		

1.2 De Man, Rogosa and Sharpe agar (MRS agar)

MRS medium was purchased from Himedia (Hi-Media Laboratories Pvt Ltd, India) and added with agar (15 g/l).

1.3 Gelatin test medium

The components were similar to MRS broth, and added with gelatin (96 g/l).

1.4 MRS broth containing 0.5% calcium carbonate

Proteose peptone	10.00	g
Beef extract	8.00	g
Yeast extract	4.00	g
Tween 80 ($(NH_4)_3C_6H_5O_7$)	1.00	g
tri-Ammonium citrate ($CH_3COONa.3H_2O$)	2.00	g
Sodium acetate trihydrate	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
$CaCO_3$	5.00	g
Distilled water added and brought up total volume to	1,000.00	ml
Final pH 6.2 ± 0.2 at 25°C		

1.5 MRS agar with addition of 0.5% calcium carbonate

The components were similar to MRS agar, and added with 0.5% $CaCO_3$.

1.6 Motility test medium (modified from MRS medium; Atlas, 2004)

The components were similar to MRS broth, and added with agar (3 g/l).

1.7 M17 broth (modified from M17 medium; Atlas, 2004)

Disodium β-glycerophosphate	19.00	g
Beef extract	5.00	g
Lactose	5.00	g
Glucose	5.00	g
Papaic digest of soybean meal or soy peptone	5.00	g
Yeast extract	2.50	g
Ascorbic acid	0.50	g
$MgSO_4.7H_2O$	0.25	g
Distilled water added and brought up total volume to	1,000.00	ml

Final pH 6.9±0.2 at 25°C

1.8 M17 medium (modified from M17 medium; Atlas, 2004)

The components were similar to M17 broth, and added with agar (11 g/l).

1.9 Rogosa broth with modification medium (RAM) (Rodtong and Ishizaki, 2003)

Pancreatic digest of casein or tryptone	5.00	g
Di-potassium hydrogen phosphate (K_2HPO_4)	6.00	g
Yeast extract	3.00	g
$MgSO_4.7H_2O$	0.57	g
$MnSO_4.4H_2O$	0.12	g
$FeSO_4.7H_2O$	0.03	g
Tapioca starch	10.00	g
Agar	15.00	g
Distilled water added and brought up total volume to	1,000.00	ml
Final pH 7.0 ± 0.2 at 25°C		

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

The components were similar to RAM broth and added with agar (15 g/l).

2. Reagents

2.1 **Iodine solution** (Gram's iodine)

Iodine	1.00	g
Potassium iodide	2.00	g
Distilled water added and brought up total volume to	300.00	ml

2.2 **20×SSC (20× standard saline citrate)**

NaCl	17.50	g
Sodium citrate	8.80	g
Distilled water added and brought up total volume to	1,000.00	ml

Final pH 7.0 ± 0.2 at 25°C

The solution was sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches. To prepare 0.1×SSC and 0.2×SSC, the 20×SSC was diluted to the desirable concentration.

2.3 **Tetramethyl-p-phenylenediamine dihydrochloride** (1%)

Tetramethyl-p-phenylenediamine dihydrochloride	1.00	g
Distilled water added and brought up total volume to	100.00	ml

2.4 Tris-NaCl (pH 9.0)

Tris-base	121.14	g
NaCl	5.84	g
Distilled water added and brought up total volume to	1,000.00	ml

Final pH 9.0 ± 0.2 at 25°C with 1 N NaOH

The solution was sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches.

APPENDIX B

SCREENING AND SELECTION OF L-LACTIC ACID-PRODUCING BACTERIA

Table B1 Screening of L-lactic acid-producing bacteria from isolates growing well in RAM medium, using MRS medium containing 2% glucose.

Isolate no.	Gas production	Growth (A600) (1:5, dilution)	pH	Total acidity (%)[a]	Starch hydrolysis	Lactic acid (LA) concentration (g/l)[b]		Optical purity of lactic acid (%)[c]	
						L-LA	D-LA	L-LA	D-LA
CAR18	Negative	0.118	4.82	0.87	Positive	3.00	0.00	100.0	0.0
CAR19	Negative	0.749	3.62	1.70	Negative	16.54	0.86	95.0	5.0
CAR20	Negative	0.384	4.48	1.29	Positive	4.66	0.00	100.0	0.0
CAR23	Negative	0.752	3.64	1.62	Negative	17.96	0.16	99.1	0.9
CAR24	Negative	0.730	3.64	1.79	Negative	15.30	0.49	96.9	3.1
CAR126	Negative	0.329	4.02	0.79	Positive	5.62	0.21	96.3	3.7
CAR127	Negative	0.315	4.02	0.74	Positive	5.54	0.20	96.4	3.6
CAR128	Negative	0.506	3.99	0.76	Positive	7.52	0.00	100.0	0.0
CAR129	Negative	0.336	4.05	0.73	Positive	7.15	0.00	100.0	0.0
CAR130	Negative	0.113	4.26	0.62	Positive	5.75	0.00	100.0	0.0
CAR131	Negative	0.167	4.29	0.59	Positive	5.59	0.00	100.0	0.0
CAR132	Negative	0.338	4.49	0.48	Positive	5.28	0.13	97.6	2.4
CAR133	Negative	0.281	4.05	0.74	Positive	5.56	0.22	96.2	3.8
CAR134	Negative	0.435	4.00	0.76	Positive	7.89	0.00	100.0	0.0
CAR135	Negative	0.467	4.02	0.71	Positive	7.39	0.00	100.0	0.0
CAR136	Negative	0.180	4.22	0.67	Positive	5.94	0.00	100.0	0.0
CAR137	Negative	0.231	4.29	0.61	Positive	5.42	0.28	95.1	4.9
CAR138	Negative	0.131	4.53	0.51	Positive	4.95	0.00	100.0	0.0
CAR139	Negative	0.071	4.54	0.48	Positive	4.51	0.00	100.0	0.0

Table B1 (Continued) Screening of L-lactic acid-producing bacteria from isolates growing well in RAM medium, using MRS medium containing 2% glucose.

Isolate no.	Gas production	Growth (A600) (1:5, dilution)	pH	Total acidity (%)[a]	Starch hydrolysis	Lactic acid (LA) concentration (g/l)[b]		Optical purity of lactic acid (%)[c]	
						L-LA	D-LA	L-LA	D-LA
CAR140	Negative	0.309	4.15	0.66	Positive	6.65	0.00	100.0	0.0
CAR141	Negative	0.077	4.56	0.49	Positive	3.42	0.26	92.8	7.2
CAR142	Negative	0.066	4.53	0.52	Positive	3.46	0.25	93.3	6.7
CAR143	Negative	0.291	4.08	0.75	Positive	5.71	0.25	95.7	4.3
CAR144	Negative	0.232	4.15	0.68	Positive	4.87	0.00	100.0	0.0
CAR145	Negative	0.064	4.38	0.57	Positive	3.81	0.25	93.9	6.1
CWR1-17	Negative	0.111	4.88	1.68	Negative	1.90	0.15	92.6	7.4
CWR1-18	Negative	0.207	4.49	1.48	Positive	3.01	0.00	100.0	0.0
CWR1-19	Negative	0.344	4.53	1.34	Positive	3.81	0.00	100.0	0.0
CWR1-20	Negative	0.193	4.85	1.01	Positive	2.61	0.00	100.0	0.0
CWR1-21	Negative	0.194	4.93	1.58	Positive	1.96	0.12	94.1	5.9
CWR1-22	Negative	0.336	4.39	1.19	Negative	2.92	0.13	95.7	4.3
CWR1-24	Negative	0.697	3.32	1.92	Negative	16.34	0.32	98.1	1.9
CWR2-17	Negative	0.385	3.70	1.60	Positive	5.43	0.00	100.0	0.0
CWR2-18	Negative	0.266	4.38	1.31	Positive	3.29	0.00	100.0	0.0
CWR2-19	Negative	0.581	3.22	1.84	Negative	19.12	0.15	99.2	0.8
CWR2-20	Negative	0.398	4.21	1.38	Positive	4.84	0.00	100.0	0.0
CWR2-21	Negative	0.344	3.78	1.83	Positive	4.32	0.00	100.0	0.0
CWR2-22	Negative	0.457	4.25	1.42	Positive	5.16	0.00	100.0	0.0
CWR2-23	Negative	0.275	4.64	1.84	Positive	3.58	0.00	100.0	0.0
CSR1-17	Negative	0.135	4.85	0.97	Positive	2.81	0.00	100.0	0.0
CSR1-18	Negative	0.146	4.88	0.69	Positive	2.31	0.00	100.0	0.0
CSR1-19	Negative	0.221	4.72	1.03	Positive	3.30	0.00	100.0	0.0
CSR1-20	Negative	0.337	4.60	1.24	Positive	4.24	0.00	100.0	0.0
CSR1-21	Negative	0.601	3.21	1.73	Negative	17.86	0.63	96.6	3.4
CSR1-22	Negative	0.238	4.57	0.89	Negative	3.94	0.00	100.0	0.0
CSR1-23	Negative	0.028	5.65	0.49	Negative	0.91	0.00	100.0	0.0
CSR1-24	Negative	0.121	5.21	0.63	Positive	1.40	0.00	100.0	0.0

Table B1 (Continued) Screening of L-lactic acid-producing bacteria from isolates growing well in RAM medium, using MRS medium containing 2% glucose.

Isolate no.	Gas production	Growth (A600) (1:5, dilution)	pH	Total acidity (%)[a]	Starch hydrolysis	Lactic acid (LA) concentration (g/l)[b]		Optical purity of lactic acid (%)[c]	
						L-LA	D-LA	L-LA	D-LA
A5UV1	Negative	0.174	4.19	0.78	Positive	6.07	0.00	100.0	0.0
A5UV2	Negative	0.171	4.19	0.63	Positive	6.10	0.00	100.0	0.0
A5UV3	Negative	0.210	4.17	0.82	Positive	6.33	0.00	100.0	0.0
A5UV4	Negative	0.165	3.99	0.80	Positive	7.20	0.00	100.0	0.0
A5UV5	Negative	0.171	4.22	0.81	Positive	6.81	0.00	100.0	0.0
A5UV6	Negative	0.178	4.31	0.67	Positive	6.23	0.00	100.0	0.0
A5UVUN1	Negative	0.100	4.10	0.67	Positive	5.81	0.00	100.0	0.0
A5UVUN2	Negative	0.114	4.29	0.66	Positive	5.82	0.00	100.0	0.0
A5UVUN3	Negative	0.142	4.28	0.67	Positive	6.03	0.00	100.0	0.0
A5UVUU1	Negative	0.123	4.21	0.68	Positive	5.94	0.00	100.0	0.0
A5UVUU2	Negative	0.136	4.26	0.77	Positive	6.08	0.00	100.0	0.0
A5UVUU3	Negative	0.110	4.26	0.77	Positive	5.94	0.00	100.0	0.0
A5UVUU5	Negative	0.196	4.17	0.71	Positive	5.77	0.00	100.0	0.0
A5UND6	Negative	0.156	4.15	0.75	Positive	6.09	0.00	100.0	0.0
A5UND12	Negative	0.173	4.15	0.74	Positive	6.18	0.00	100.0	0.0
A5UND18	Negative	0.149	4.16	0.71	Positive	5.82	0.00	100.0	0.0
A5UND53	Negative	0.157	4.15	0.70	Positive	5.81	0.00	100.0	0.0
A5UNDU1	Negative	0.108	4.18	0.68	Positive	5.40	0.00	100.0	0.0
A5UNDU2	Negative	0.143	4.20	0.71	Positive	5.43	0.00	100.0	0.0
A5UNDU3	Negative	0.153	4.26	0.76	Positive	6.28	0.00	100.0	0.0
A5UNDU4	Negative	0.161	4.27	0.78	Positive	5.96	0.00	100.0	0.0
A5UNDU5	Negative	0.145	4.26	0.80	Positive	6.27	0.00	100.0	0.0
A5UNDU6	Negative	0.149	4.23	0.78	Positive	5.95	0.00	100.0	0.0
A5UNDU7	Negative	0.121	4.24	0.77	Positive	6.35	0.00	100.0	0.0
A5UNDU8	Negative	0.145	4.26	0.76	Positive	6.28	0.00	100.0	0.0
A5UNDU9	Negative	0.133	4.24	0.79	Positive	7.21	0.00	100.0	0.0
A5UNDU10	Negative	0.119	4.26	0.78	Positive	6.07	0.00	100.0	0.0
A5UNDU11	Negative	0.309	4.23	0.79	Positive	6.22	0.00	100.0	0.0

Table B1 (Continued) Screening of L-lactic acid-producing bacteria from isolates growing well in RAM medium, using MRS medium containing 2% glucose.

Isolate no.	Gas production	Growth (A600) (1:5, dilution)	pH	Total acidity (%)[a]	Starch hydrolysis	Lactic acid (LA) concentration (g/l)[b]		Optical purity of lactic acid (%)[c]	
						L-LA	D-LA	L-LA	D-LA
A5UNDN1	Negative	0.267	4.22	0.76	Positive	6.03	0.00	100.0	0.0
A5UNDN2	Negative	0.338	4.20	0.82	Positive	6.29	0.00	100.0	0.0
A5UNDN3	Negative	0.313	4.21	0.79	Positive	6.25	0.00	100.0	0.0
A5UNDN5	Negative	0.345	4.21	0.81	Positive	6.32	0.00	100.0	0.0
SUT501	Negative	0.429	4.20	0.82	Positive	6.66	0.00	100.0	0.0
SUT502	Negative	0.394	4.20	0.84	Positive	6.51	0.00	100.0	0.0
SUT503	Negative	0.302	4.20	0.84	Positive	6.34	0.00	100.0	0.0
SUT504	Negative	0.368	4.19	0.84	Positive	6.52	0.00	100.0	0.0
SUT505	Negative	0.357	4.19	0.82	Positive	6.85	0.00	100.0	0.0
SUT506	Negative	0.404	4.20	0.84	Positive	6.38	0.00	100.0	0.0
SUT507	Negative	0.398	4.19	0.82	Positive	6.56	0.00	100.0	0.0
SUT508	Negative	0.288	4.17	0.85	Positive	6.42	0.00	100.0	0.0
SUT509	Negative	0.210	4.34	0.72	Positive	5.37	0.00	100.0	0.0
SUT510	Negative	0.328	4.20	0.83	Positive	6.31	0.00	100.0	0.0
SUT511	Negative	0.394	4.17	0.87	Positive	6.47	0.00	100.0	0.0
SUT512	Negative	0.396	4.16	0.83	Positive	6.69	0.00	100.0	0.0
SUT513	Negative	0.380	4.20	0.81	Positive	8.60	0.00	100.0	0.0
SUT514	Negative	0.388	4.22	0.87	Positive	6.68	0.00	100.0	0.0
SUT515	Negative	0.392	4.18	0.86	Positive	6.28	0.00	100.0	0.0
SUT516	Negative	0.336	4.14	0.80	Positive	7.01	0.00	100.0	0.0
SUT518	Negative	0.385	4.18	0.87	Positive	6.23	0.00	100.0	0.0
SUT519	Negative	0.377	4.18	0.85	Positive	6.71	0.00	100.0	0.0
SUT520	Negative	0.414	4.17	0.81	Positive	6.68	0.00	100.0	0.0
SUT521	Negative	0.411	4.14	0.77	Positive	6.67	0.00	100.0	0.0
SUT522	Negative	0.469	4.15	0.81	Positive	6.75	0.00	100.0	0.0
SUT523	Negative	0.341	4.16	0.82	Positive	6.47	0.00	100.0	0.0
SUT524	Negative	0.351	4.17	0.93	Positive	6.51	0.00	100.0	0.0
SUT525	Negative	0.359	4.15	0.84	Positive	7.05	0.00	100.0	0.0
SUT526	Negative	0.361	4.13	0.91	Positive	6.76	0.00	100.0	0.0

Table B1 (Continued) Screening of L-lactic acid-producing bacteria from isolates growing well in RAM medium, using MRS medium containing 2% glucose.

Isolate no.	Gas production	Growth (A600) (1:5, dilution)	pH	Total acidity (%)[a]	Starch hydrolysis	Lactic acid (LA) concentration (g/l)[b]		Optical purity of lactic acid (%)[c]	
						L-LA	D-LA	L-LA	D-LA
SUT527	Negative	0.369	4.15	0.78	Positive	7.09	0.00	100.0	0.0
SUT528	Negative	0.377	4.16	0.78	Positive	6.90	0.00	100.0	0.0
SUT529	Negative	0.412	4.23	0.83	Positive	6.73	0.00	100.0	0.0
I5UVU1	Negative	0.341	4.23	0.73	Positive	6.59	0.00	100.0	0.0
I5UVU2	Negative	0.370	4.24	0.74	Positive	6.62	0.00	100.0	0.0
I5UVU3	Negative	0.303	4.23	0.78	Positive	6.91	0.00	100.0	0.0
I5UVU4	Negative	0.376	4.24	0.78	Positive	6.53	0.00	100.0	0.0
I5UVU5	Negative	0.382	4.25	0.75	Positive	6.81	0.00	100.0	0.0
I5UVU6	Negative	0.399	4.26	0.74	Positive	6.18	0.00	100.0	0.0
I5UVU7	Negative	0.388	4.22	0.75	Positive	6.52	0.00	100.0	0.0
I5UVU8	Negative	0.415	4.23	0.74	Positive	7.01	0.00	100.0	0.0
I5UVU9	Negative	0.387	4.21	0.75	Positive	7.31	0.00	100.0	0.0
I5UVU10	Negative	0.423	4.31	0.70	Positive	6.58	0.00	100.0	0.0
I5UVU11	Negative	0.390	4.22	0.72	Positive	7.09	0.00	100.0	0.0
I5UVU12	Negative	0.357	4.13	0.81	Positive	6.70	0.00	100.0	0.0
I5UVU13	Negative	0.351	4.14	0.74	Positive	6.01	0.00	100.0	0.0
I5UVU14	Negative	0.315	4.15	0.73	Positive	5.66	0.00	100.0	0.0
I5UVU16	Negative	0.170	4.27	0.68	Positive	5.28	0.00	100.0	0.0
I5UVU17	Negative	0.201	4.24	0.68	Positive	5.42	0.00	100.0	0.0
I5UVU18	Negative	0.239	4.22	0.70	Positive	5.22	0.00	100.0	0.0
I5UVU20	Negative	0.157	4.28	0.67	Positive	5.13	0.00	100.0	0.0
I5UND4	Negative	0.238	4.18	0.71	Positive	5.69	0.00	100.0	0.0
I5UND6	Negative	0.312	4.37	0.55	Positive	4.52	0.00	100.0	0.0
I5UND9	Negative	0.370	4.13	0.71	Positive	6.56	0.00	100.0	0.0
I5UND10	Negative	0.376	4.12	0.73	Positive	6.42	0.00	100.0	0.0
I5UND16	Negative	0.231	4.28	0.62	Positive	5.23	0.00	100.0	0.0
I5UVUN1	Negative	0.414	4.17	0.79	Positive	6.59	0.00	100.0	0.0
I5UVUN2	Negative	0.429	4.19	0.74	Positive	5.99	0.00	100.0	0.0
I5UVUN3	Negative	0.381	4.16	0.77	Positive	6.50	0.00	100.0	0.0

Table B1 (Continued) Screening of L-lactic acid-producing bacteria from isolates growing well in RAM medium, using MRS medium containing 2% glucose.

Isolate no.	Gas production	Growth (A600) (1:5, dilution)	pH	Total acidity (%)[a]	Starch hydrolysis	Lactic acid (LA) concentration (g/l)[b]		Optical purity of lactic acid (%)[c]	
						L-LA	D-LA	L-LA	D-LA
I5UVUN4	Negative	0.398	4.15	0.78	Positive	6.44	0.00	100.0	0.0
I5UVUU9	Negative	0.351	4.18	0.79	Positive	6.01	0.00	100.0	0.0

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Optical purity (%) of lactic acid = (1-(D- or L-lactic acid / total lactic acid))×100.

Table B2 Screening of L-lactic acid-producing bacteria from isolates growing well in MRS medium, using MRS medium containing 2% glucose.

Isolate no.	Gas production	Growth (A600) (1:5, dilution)	pH	Total acidity (%)[a]	Starch hydrolysis	Lactic acid (LA) concentration (g/l)[b]		Optical purity of lactic acid (%)[c]	
						L-LA	D-LA	L-LA	D-LA
CAMR11	Negative	0.721	3.37	1.84	Negative	15.03	0.49	96.8	3.2
CAMR12	Negative	0.727	3.35	1.89	Negative	15.70	0.51	96.8	3.2
CAMR18	Negative	0.827	3.33	1.87	Negative	16.35	0.20	98.7	1.3
CAMR63	Positive	0.497	3.38	0.95	Negative	5.50	0.00	100.0	0.0
CAMR64	Positive	0.543	3.50	1.00	Negative	6.50	0.00	100.0	0.0
CAMR65	Positive	0.594	3.50	0.99	Negative	5.50	0.00	100.0	0.0
CAMR66	Positive	0.551	3.47	0.97	Negative	5.00	0.00	100.0	0.0
CAMR115	Positive	0.463	3.52	0.95	Negative	ND	ND	ND	ND
CAMR116	Positive	0.487	3.59	0.93	Negative	3.50	5.50	61.1	38.9
CAMR117	Negative	0.518	3.12	1.45	Negative	15.50	1.00	93.9	6.1
CAMR118	Negative	0.523	3.39	1.18	Negative	15.00	1.00	93.7	6.3
CAMR119	Positive	0.472	3.50	0.93	Negative	ND	ND	ND	ND
CAMR140	Positive	0.915	3.44	0.95	Negative	ND	ND	ND	ND
CAMR141	Positive	0.630	3.44	0.99	Negative	ND	ND	ND	ND
CAMR142	Positive	0.621	3.41	0.86	Negative	ND	ND	ND	ND
CAMR143	Positive	0.460	3.26	0.93	Negative	ND	ND	ND	ND

Table B2 (Continued) Screening of L-lactic acid-producing bacteria from isolates growing well in MRS medium, using MRS medium containing 2% glucose.

Isolate no.	Gas production	Growth (A600) (1:5, dilution)	pH	Total acidity (%)[a]	Starch hydrolysis	Lactic acid (LA) concentration (g/l)[b]		Optical purity of lactic acid (%)[c]	
						L-LA	D-LA	L-LA	D-LA
CAMR144	Positive	0.429	3.34	0.91	Negative	ND	ND	ND	ND
CAMR156	Positive	0.323	3.38	0.90	Negative	ND	ND	ND	ND
CAMR157	Positive	0.507	3.38	0.97	Negative	ND	ND	ND	ND
CAMR158	Positive	0.588	3.50	0.95	Negative	ND	ND	ND	ND
CAMR159	Positive	0.451	3.47	0.93	Negative	ND	ND	ND	ND
CAMR160	Positive	0.754	3.47	0.93	Negative	ND	ND	ND	ND
CAMR185	Positive	0.474	5.58	0.19	Negative	ND	ND	ND	ND
CAMR186	Positive	0.769	3.48	0.97	Negative	ND	ND	ND	ND
CAMR187	Positive	0.501	3.61	1.00	Negative	ND	ND	ND	ND
CAMR188	Positive	0.315	3.78	1.01	Negative	ND	ND	ND	ND
CAMR189	Positive	0.340	3.55	0.99	Negative	ND	ND	ND	ND
CAMR218	Negative	0.780	3.17	1.49	Negative	ND	ND	ND	ND
CAMR219	Positive	0.384	4.40	0.21	Negative	ND	ND	ND	ND
CAMR220	Negative	0.589	3.56	1.24	Negative	ND	ND	ND	ND
CAMR221	Negative	0.149	3.42	1.29	Negative	ND	ND	ND	ND
CAMR222	Negative	0.431	3.41	1.18	Negative	ND	ND	ND	ND
CWMR1-13	Negative	0.410	3.87	1.26	Negative	3.40	0.24	93.3	6.7
CWMR1-14	Negative	0.296	4.13	1.03	Negative	2.90	1.60	64.3	35.7
CWMR1-15	Negative	0.015	4.97	1.17	Negative	1.03	0.00	100.0	0.0
CWMR1-16	Negative	0.613	3.42	1.56	Negative	13.63	0.39	97.2	2.8
CWMR1-17	Negative	0.785	3.56	1.71	Negative	15.87	0.56	96.6	3.4
CSMR1-1	Negative	0.277	3.69	1.56	Negative	1.32	17.04	92.7	7.3
CSMR1-2-1	Positive	0.183	4.21	0.90	Negative	6.15	3.44	64.1	35.9
CSMR1-2-2	Positive	0.157	4.24	0.98	Negative	5.85	3.39	63.3	36.7
CSMR1-3	Positive	0.280	3.99	1.05	Negative	3.82	8.00	67.6	32.4
CSMR1-4	Negative	0.276	3.65	1.54	Negative	1.09	19.34	94.6	5.4
CSMR1-5	Negative	0.608	3.52	1.74	Negative	8.43	13.71	61.9	38.1
CSMR1-6	Positive	0.306	3.95	1.08	Negative	4.86	6.57	57.4	42.6
CSMR1-7	Positive	0.310	3.97	1.07	Negative	5.09	6.66	56.7	43.3

Table B2 (Continued) Screening of L-lactic acid-producing bacteria from isolates growing well in MRS medium, using MRS medium containing 2% glucose.

Isolate no.	Gas production	Growth (A600) (1:5, dilution)	pH	Total acidity (%)[a]	Starch hydrolysis	Lactic acid (LA) concentration (g/l)[b]		Optical purity of lactic acid (%)[c]	
						L-LA	D-LA	L-LA	D-LA
CSMR1-8	Positive	0.304	3.92	1.12	Negative	4.95	6.42	56.4	43.6
WMR1	Negative	0.241	3.05	1.39	Negative	17.00	1.00	94.4	5.6
WMR3	Positive	0.594	3.57	0.75	Negative	ND	ND	ND	ND
WMR4	Positive	0.389	3.40	0.84	Negative	ND	ND	ND	ND
WMR5	Positive	0.521	3.32	0.90	Negative	ND	ND	ND	ND
WMR32	Negative	0.010	6.24	0.23	Negative	0.50	1.00	66.6	33.4
WMR33	Negative	0.456	3.22	1.22	Negative	16.50	1.00	94.3	5.7
WMR34	Positive	0.325	3.42	0.86	Negative	ND	ND	ND	ND
WMR35	Positive	0.254	3.49	0.90	Negative	ND	ND	ND	ND
WMR36	Negative	0.486	3.17	1.26	Negative	16.00	1.50	91.4	8.6
WMR55	Positive	0.251	3.38	0.90	Negative	ND	ND	ND	ND
WMR56	Positive	0.318	3.56	0.92	Negative	ND	ND	ND	ND
WMR57	Positive	0.258	3.59	0.88	Negative	ND	ND	ND	ND
WMR58	Negative	0.881	3.01	1.58	Negative	7.00	12.50	64.1	35.9
WMR59	Positive	0.264	3.49	0.83	Negative	ND	ND	ND	ND
WMR77	Negative	0.153	4.10	0.59	Negative	6.00	0.0	100.0	0.0
WMR78	Negative	0.014	6.33	0.16	Negative	ND	ND	ND	ND
WMR79	Negative	0.169	4.15	0.58	Negative	5.50	0.0	100.0	0.0
WMR80	Negative	0.071	4.86	0.34	Positive	4.00	0.0	100.0	0.0
WMR81	Negative	0.139	3.91	0.56	Positive	5.50	0.0	100.0	0.0

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Optical purity (%) of lactic acid = (1-(D- or L-lactic acid / total lactic acid))×100.
ND = Not determined.

Table B3 Screening of L-lactic acid-producing bacteria from isolates growing well in MRS medium containing 0.5% calcium carbonate, using MRS medium containing 2% glucose.

Isolate no.	Gas production	Growth (A600) (1:5, dilution)	pH	Total acidity (%)[a]	Starch hydrolysis	Lactic acid (LA) concentration (g/l)[b]		Optical purity of lactic acid (%)[c]	
						L-LA	D-LA	L-LA	D-LA
CAMC13	Negative	0.767	3.31	1.61	Negative	14.35	0.83	94.5	5.5
CAMC15	Negative	0.641	3.30	1.69	Negative	15.59	2.39	86.7	13.3
CAMC18	Negative	0.857	3.22	1.79	Negative	14.82	0.50	96.8	3.2
CAMC91	Positive	0.098	3.64	0.87	Negative	ND	ND	ND	ND
CAMC92	Negative	0.124	3.32	1.12	Negative	14.00	0.00	100.0	0.0
CAMC93	Negative	0.503	3.12	1.28	Negative	17.50	1.00	94.6	5.4
CAMC94	Negative	0.076	3.88	0.59	Positive	7.00	0.00	100.0	0.0
CAMC95	Negative	0.049	4.10	0.53	Positive	ND	ND	ND	ND
CAMC125	Positive	0.502	3.39	0.99	Negative	ND	ND	ND	ND
CAMC126	Positive	0.189	3.38	0.99	Negative	7.50	9.50	55.9	44.1
CAMC127	Negative	0.383	3.20	1.24	Positive	13.50	1.00	93.1	6.9
CAMC128	Positive	0.462	3.31	1.15	Negative	7.50	9.00	54.5	45.5
CAMC129	Positive	0.377	3.36	0.86	Negative	ND	ND	ND	ND
CAMC160	Positive	0.459	3.36	1.01	Negative	7.00	9.00	56.3	43.7
CAMC161	Positive	0.419	3.40	0.96	Negative	ND	ND	ND	ND
CAMC162	Positive	0.509	3.42	0.94	Negative	ND	ND	ND	ND
CAMC163	Positive	0.388	3.39	1.03	Negative	6.50	8.50	56.7	43.3
CAMC164	Positive	0.492	3.41	0.94	Negative	ND	ND	ND	ND
CAMC169	Positive	0.230	3.40	1.01	Negative	6.50	8.50	56.7	43.3
CAMC170	Positive	0.401	3.39	0.92	Negative	ND	ND	ND	ND
CAMC171	Positive	0.237	4.45	0.43	Negative	ND	ND	ND	ND
CAMC172	Positive	0.434	3.56	0.99	Negative	ND	ND	ND	ND
CAMC173	Positive	0.400	3.42	1.01	Negative	4.50	6.50	59.1	40.9
CAMC203	Positive	0.422	3.92	0.67	Negative	ND	ND	ND	ND
CAMC204	Positive	0.308	3.34	0.99	Negative	5.50	8.00	59.3	40.7
CAMC205	Positive	0.357	3.32	0.83	Negative	ND	ND	ND	ND
CAMC206	Positive	0.352	3.34	0.76	Negative	ND	ND	ND	ND
CAMC207	Positive	0.111	3.35	0.97	Negative	ND	ND	ND	ND
CAMC265	Positive	0.353	3.33	1.06	Negative	7.00	8.50	54.8	45.2

Table B3 (Continued) Screening of L-lactic acid-producing bacteria from isolates growing well in MRS medium containing 0.5% calcium carbonate, using MRS medium containing 2% glucose.

Isolate no.	Gas production	Growth (A600) (1:5, dilution)	pH	Total acidity (%)[a]	Starch hydrolysis	Lactic acid (LA) concentration (g/l)[b]		Optical purity of lactic acid (%)[c]	
						L-LA	D-LA	L-LA	D-LA
CAMC266	Positive	0.359	3.37	1.01	Negative	5.00	8.50	63.0	37.0
CAMC267	Positive	0.509	3.42	0.94	Negative	ND	ND	ND	ND
CAMC268	Positive	0.388	3.39	1.03	Negative	6.50	8.50	56.7	43.3
CAMC269	Positive	0.492	3.41	0.94	Negative	ND	ND	ND	ND
WMC1	Positive	0.421	3.41	0.99	Negative	7.00	8.00	53.3	46.7
WMC2	Positive	0.151	4.04	0.56	Negative	ND	ND	ND	ND
WMC3	Positive	0.491	3.47	0.94	Negative	ND	ND	ND	ND
WMC5	Positive	0.242	3.39	0.94	Negative	6.50	7.50	53.6	46.4
WMC28	Positive	0.279	3.51	0.90	Negative	ND	ND	ND	ND
WMC29	Positive	0.277	3.48	0.95	Negative	ND	ND	ND	ND
WMC30	Negative	0.364	3.14	1.37	Negative	15.00	1.00	93.8	6.2
WMC31	Positive	0.350	3.50	0.86	Negative	ND	ND	ND	ND
WMC32	Positive	0.387	3.41	1.03	Negative	5.00	8.50	63.0	37.0
WMC85	Positive	0.350	6.14	0.00	Negative	ND	ND	ND	ND
WMC86	Negative	0.125	4.30	0.42	Negative	3.00	0.00	100.0	0.0
WMC87	Positive	0.561	5.36	0.20	Negative	ND	ND	ND	ND
WMC88	Negative	0.058	6.17	0.16	Negative	ND	ND	ND	ND
WMC89	Positive	0.219	3.39	0.99	Negative	ND	ND	ND	ND
WMC120	Negative	0.096	4.20	0.60	Positive	6.00	0.00	100.0	0.0
WMC121	Negative	0.131	4.00	0.59	Positive	5.00	1.00	83.3	16.7
WMC122	Negative	0.130	4.07	0.63	Positive	5.50	0.00	100.0	0.0
WMC123	Negative	0.143	4.15	0.56	Positive	5.00	1.50	76.9	23.1
WMC124	Negative	0.162	4.30	0.49	Positive	5.00	0.00	100.0	0.0

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Optical purity (%) of lactic acid = (1-(D- or L-lactic acid / total lactic acid))×100.
ND = Not determined

Table B4 Screening of L-lactic acid-producing bacteria from isolates growing well in M17 medium, using MRS medium containing 2% glucose.

Isolate no.	Gas production	Growth (A600) (1:5, dilution)	pH	Total acidity (%)[a]	Starch hydrolysis	Lactic acid (LA) concentration (g/l)[b]		Optical purity of lactic acid (%)[c]	
						L-LA	D-LA	L-LA	D-LA
CAM11	Positive	0.450	4.11	0.95	Negative	4.18	5.10	54.9	45.1
CAM12	Negative	0.686	3.65	1.65	Negative	5.82	9.08	60.9	39.1
CWM1-18	Negative	0.760	3.66	1.69	Negative	13.36	0.74	94.7	5.3
CWM2-12	Negative	0.439	3.58	1.40	Negative	17.11	1.83	90.3	9.7
CWM2-13	Negative	0.242	4.19	1.15	Negative	4.21	0.00	100.0	0.0
CWM2-14	Negative	0.285	3.63	1.47	Negative	14.62	1.30	91.8	8.2
CWM2-15	Positive	0.263	3.92	1.06	Negative	5.51	6.99	56.0	44.0
CWM2-16	Positive	0.256	3.86	1.00	Negative	5.46	6.93	56.0	44.0
CWM2-17	Positive	0.272	3.91	1.08	Negative	3.82	6.92	64.5	35.5
CWM2-18	Positive	0.228	4.20	0.87	Negative	4.54	4.78	51.3	48.7
CSM1-1	Negative	0.368	3.62	1.59	Negative	16.04	1.17	93.2	6.8
CSM1-2	Negative	0.410	3.59	1.53	Negative	15.72	0.98	94.1	5.9
CSM1-3	Negative	0.438	3.58	1.71	Negative	17.87	1.56	91.9	8.1
CSM1-4	Negative	0.415	3.68	1.44	Negative	15.04	1.64	90.1	9.9
CSM1-5	Positive	0.276	3.96	1.04	Negative	5.21	6.80	56.7	43.3
CSM1-6	Positive	0.211	3.96	1.05	Negative	3.54	3.94	52.7	47.3
CSM1-7	Negative	0.330	3.62	1.47	Negative	15.34	1.35	91.9	8.1
CSM1-8	Negative	0.508	4.82	0.72	Negative	0.24	0.14	63.6	36.4
CAM13	Negative	0.605	3.67	1.66	Negative	7.18	11.07	60.6	39.4
CAM14	Positive	0.248	3.99	0.98	Negative	4.19	5.48	56.6	43.3
CAM15	Positive	0.234	3.90	0.99	Negative	4.34	5.51	55.9	44.1
CAM16	Negative	0.221	3.92	0.99	Negative	4.39	5.65	56.3	43.7
CAM17	Positive	0.280	3.98	0.96	Negative	3.93	5.78	59.5	40.5
CAM18	Negative	0.410	3.65	1.44	Negative	12.73	0.53	95.9	4.1
CWM1-11	Negative	0.375	3.65	1.50	Negative	12.77	0.44	96.7	3.3
CWM1-12	Negative	0.667	3.65	1.63	Negative	12.78	0.80	94.1	5.9
CWM1-14	Negative	0.232	3.58	1.38	Negative	2.88	0.13	95.8	4.2
CWM1-17	Negative	0.703	3.68	1.48	Negative	12.86	0.64	95.2	4.8

[a] Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c] Optical purity (%) of lactic acid = (1-(D- or L-lactic acid / total lactic acid))×100.

Table B5 Comparison of lactic acid production by strain SUT513 when cultivated in MRS medium containing 2% glucose for 72 h.

Fermentation time (h)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
0	3.00×10^{5}	5.48	6.25	0.00±0.00	0.27±0.00	0.36±0.04	19.94±0.08
6	1.33×10^{8}	8.12	4.46	3.66±3.66	3.82±0.20	2.29±0.03	16.21±0.01
12	1.66×10^{9}	9.22	4.09	5.64±0.00	5.46±0.13	5.08±0.01	14.65±0.01
18	1.41×10^{9}	9.15	4.09	6.09±0.04	5.91±0.19	5.20±0.03	14.57±0.01
24	1.60×10^{9}	9.20	4.12	6.29±0.04	6.11±0.06	5.79±0.00	14.37±0.02
30	1.35×10^{9}	9.13	4.18	6.29±0.04	6.25±0.15	5.77±0.00	13.27±0.03
36	2.72×10^{9}	9.43	4.18	6.34±0.00	6.15±0.20	6.05±0.04	12.77±0.01
48	1.47×10^{9}	9.17	4.19	6.29±0.04	6.86±0.03	6.19±0.01	13.35±0.01
60	1.05×10^{9}	9.02	4.28	6.48±0.04	6.14±0.16	6.27±0.04	12.17±0.03
72	1.86×10^{9}	9.27	4.31	6.53±0.07	6.24±0.12	6.28±0.07	11.99±0.01

[a] to [d]: As mentioned in Table B6.

Table B6 Comparison of lactic acid production by strain SUT513 when cultivated in RAM medium containing 1% tapioca starch for 72 h.

Fermentation time (h)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
0	1.37×10^{5}	5.14	7.10	0.00±0.00	0.15±0.00	0.34±0.02	9.86±0.13
6	9.30×10^{7}	7.97	4.56	4.16±0.00	3.53±0.08	3.06±0.01	8.03±0.09
12	3.15×10^{9}	9.50	4.05	5.15±0.14	4.68±0.14	5.62±0.00	6.63±0.03
18	1.81×10^{10}	10.26	4.15	5.45±0.07	4.82±0.04	5.10±0.06	5.74±0.05
24	7.55×10^{9}	9.88	4.17	5.69±0.04	4.95±0.07	5.22±0.00	4.70±0.06
30	3.33×10^{9}	9.52	4.28	5.69±0.04	5.04±0.31	5.56±0.15	4.95±0.06
36	3.24×10^{9}	9.51	4.25	5.64±0.00	5.09±0.28	5.24±0.07	4.07±0.04
48	3.33×10^{9}	9.52	4.27	5.59±0.04	5.24±0.44	4.79±0.03	4.30±0.02
60	7.55×10^{8}	8.88	4.35	5.59±0.04	4.68±0.22	5.10±0.02	5.27±0.01
72	4.60×10^{8}	8.66	4.38	5.59±0.04	4.98±0.25	5.03±0.09	3.75±0.01

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table B7 Comparison of lactic acid production by strain SUT513 when cultivated in RAM medium containing 1% tapioca starch for 72 h.

Fermentation time (h)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
0	7.00×10^{6}	6.85	6.25	0.00±0.04	0.25±0.18	0.36±0.04	19.94±0.08
6	2.80×10^{9}	9.45	4.52	3.47±0.11	3.22±0.13	2.50±0.20	16.42±0.03
12	1.38×10^{9}	9.14	4.04	6.04±0.11	6.01±0.19	5.92±0.01	13.00±0.01
18	1.21×10^{9}	9.08	4.05	6.48±0.07	6.19±0.13	6.12±0.00	12.33±0.05
24	8.00×10^{8}	8.90	4.08	6.58±0.00	6.54±0.24	6.27±0.00	11.02±0.00
30	4.20×10^{8}	8.62	4.12	6.68±0.00	6.82±0.28	6.35±0.01	10.79±0.02
36	1.15×10^{8}	8.06	4.12	6.88±0.00	6.54±0.28	6.83±0.00	10.09±0.00
48	2.33×10^{7}	7.37	4.19	6.98±0.07	6.70±0.41	6.63±0.02	7.00±0.05
60	3.55×10^{6}	6.55	4.22	6.93±0.04	6.62±0.09	7.27±0.06	7.34±0.00
72	5.00×10^{5}	5.70	4.17	6.88±0.00	6.54±0.07	7.72±0.00	7.06±0.03

[a] to [d]: As mentioned in Table B8.

Table B8 Comparison of lactic acid production by strain CAR134 when cultivated in RAM medium containing 1% tapioca starch for 72 h.

Fermentation time (h)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
0	1.71×10^{7}	7.23	7.10	0.00±0.00	0.00±0.00	0.24±0.04	9.86±0.13
6	1.47×10^{9}	9.17	5.86	3.86±0.07	1.78±0.09	3.87±0.01	8.03±0.09
12	1.75×10^{9}	9.24	4.21	4.90±0.11	4.20±0.33	4.01±0.00	5.31±0.03
18	1.61×10^{9}	9.21	4.24	4.95±0.07	4.37±0.06	4.19±0.00	4.84±0.01
24	6.20×10^{8}	8.79	4.32	5.00±0.04	4.43±0.09	4.34±0.03	5.95±0.04
30	7.00×10^{7}	7.85	4.41	4.90±0.04	4.27±0.05	4.35±0.01	4.79±0.03
36	9.50×10^{6}	6.98	4.36	4.90±0.04	4.44±0.33	4.45±0.01	4.98±0.00
48	5.00×10^{5}	5.70	4.41	4.85±0.00	4.36±0.03	4.56±0.01	4.30±0.01
60	1.00×10^{5}	5.00	4.49	4.95±0.00	4.55±0.08	4.77±0.03	4.00±0.01
72	3.00×10^{4}	4.48	4.44	5.00±0.04	4.49±0.19	5.09±0.01	3.19±0.00

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

APPENDIX C

OPTIMIZATION AND PRODUCTION OF L-LACTIC ACID

Table C1 Comparison of bacterial growth and L-lactic acid production by strain SUT513 when cultivated in fermentation medium containing different concentrations (10-40 g/l) of tapioca starch for 48 h.

Tapioca starch (g/l)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
10	7.83×10^{7}	7.89	4.09	4.76±0.07	4.57±0.01	3.76±0.01	5.63±0.03
15	7.92×10^{7}	7.90	4.12	4.76±0.07	4.79±0.06	4.44±0.03	7.48±0.01
20	1.24×10^{8}	8.09	4.08	4.59±0.00	4.81±0.24	4.94±0.01	11.88±0.02
25	2.64×10^{8}	8.42	4.02	4.50±0.07	4.57±0.21	4.67±0.01	20.40±0.06
30	1.48×10^{8}	8.17	4.05	4.94±0.14	5.55±0.43	5.19±0.02	23.55±0.03
35	2.12×10^{8}	8.33	3.98	4.50±0.14	4.00±0.10	3.84±0.04	24.38±0.19
40	1.54×10^{8}	8.19	4.10	4.76±0.00	4.16±0.09	4.27±0.01	28.39±0.13

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table C2 Comparison of bacterial growth and L-lactic acid production by strain CAR134 when cultivated in fermentation medium containing different concentrations (10-40 g/l) of tapioca starch for 48 h.

Tapioca starch (g/l)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
10	5.50×10^{7}	4.85	4.24	4.33±0.00	4.36±0.09	3.73±0.03	5.60±0.03
15	5.99×10^{7}	6.27	4.22	4.16±0.07	4.78±0.12	4.80±0.02	10.34±0.01
20	1.38×10^{8}	5.20	4.22	4.33±0.07	4.83±0.16	6.35±0.08	14.76±0.04
25	5.58×10^{7}	6.00	4.17	4.55±0.04	4.66±0.12	4.71±0.03	19.88±0.06
30	6.02×10^{7}	7.52	4.22	4.20±0.11	5.22±0.18	6.46±0.01	24.08±0.01
35	7.21×10^{7}	5.04	4.13	3.98±0.07	4.41±0.31	4.50±0.04	28.57±0.01
40	4.64×10^{6}	6.48	4.13	4.07±0.14	4.84±0.29	5.47±0.06	32.46±0.11

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table C3 Effects of concentrations (2.0-5.0 g/l) of tryptone on bacterial growth and L-lactic production by strain SUT513 after cultivation for 48 h.

Tryptone (g/l)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
2.0	1.30×10^{4}	4.11	4.40	3.55±0.07	4.37±0.21	2.96±0.02	26.31±0.04
2.5	1.58×10^{5}	5.20	4.27	3.72±0.00	5.16±0.25	3.91±0.07	25.14±0.03
3.0	8.50×10^{4}	4.93	4.19	3.77±0.04	4.93±0.12	3.48±0.01	24.43±0.07
4.0	1.34×10^{5}	5.13	4.15	3.90±0.00	4.71±0.29	3.75±0.01	26.87±0.04
5.0	9.50×10^{3}	3.98	4.12	4.03±0.04	4.44±0.49	3.05±0.03	26.54±0.04

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table C4 Effects of concentrations (2-5 g/l) of tryptone on bacterial growth and L-lactic production by strain CAR134 after cultivation for 48 h.

Tryptone (g/l)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
2.0	1.52×10^{6}	6.18	4.16	3.55±0.00	4.72±0.02	3.34±0.03	25.51±0.00
2.5	1.95×10^{5}	5.29	4.25	3.55±0.00	4.59±0.15	3.34±0.08	24.11±0.00
3.0	2.05×10^{4}	4.31	4.19	3.72±0.00	4.77±0.18	3.36±0.01	24.39±0.00
4.0	2.61×10^{6}	6.42	4.11	3.98±0.00	5.12±0.22	4.83±0.01	25.20±0.00
5.0	1.82×10^{5}	5.26	4.11	4.29±0.04	4.70±0.38	3.82±0.02	25.56±0.01

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table C5 Comparison of bacterial growth and L-lactic acid production by strain SUT513 using different media (Table 4.1) and cultivation for 48 h.

Medium no. (Table)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
1	1.58×10^{5}	5.20	4.27	3.72±0.00	5.16±0.25	3.91±0.07	25.14±0.03
2	1.05×10^{4}	4.02	4.10	4.11±0.04	2.60±0.03	1.19±0.00	29.48±0.03
3	1.50×10^{3}	3.18	4.25	3.77±0.04	4.19±0.07	3.28±0.01	28.98±0.04
4	2.50×10^{3}	3.40	3.24	3.81±0.00	4.49±0.34	3.64±0.05	29.09±0.01
5	7.10×10^{4}	4.85	4.27	3.77±0.04	4.82±0.00	4.02±0.05	28.92±0.03
6	7.75×10^{4}	4.89	4.35	3.64±0.00	4.47±0.13	4.35±0.02	29.02±0.00

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table C6 Comparison of bacterial growth and L-lactic acid production by strain CAR134 using different media (Table 4.1) and cultivation for 48 h.

Medium no. (Table)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
1	2.61×10^{6}	6.42	4.11	3.98±0.00	5.12±0.22	4.83±0.01	25.20±0.00
2	5.35×10^{4}	4.73	4.09	4.76±0.07	3.92±0.13	2.98±0.03	30.11±0.00
3	2.00×10^{3}	3.30	4.24	4.33±0.00	4.51±0.08	3.74±0.00	29.69±0.00
4	3.00×10^{3}	3.48	4.28	4.29±0.04	5.34±0.00	5.56±0.02	28.69±0.00
5	5.00×10^{3}	3.70	4.33	4.29±0.04	5.03±0.20	4.70±0.05	28.84±0.07
6	4.00×10^{3}	3.60	4.37	4.29±0.04	4.89±0.64	4.13±0.09	32.41±0.01

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table C7 Effect of initial pH of fermentation medium containing 30 g/l (dry weight) of tapioca starch on growth and L-lactic acid production of strain SUT513 when cultivated under anaerobic condition for 48 h.

Initial pH	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
5.0	1.99×10^{4}	4.30	4.43	0.65±0.04	0.32±0.06	0.30±0.02	24.03±0.01
5.5	2.20×10^{7}	7.34	4.49	0.74±0.04	0.30±0.01	0.29±0.01	26.29±0.04
6.0	1.55×10^{7}	7.19	4.32	1.43±0.04	0.64±0.02	0.61±0.03	27.18±0.06
6.5	2.97×10^{5}	5.47	4.25	2.42±0.07	1.47±0.12	1.40±0.02	27.10±0.03
7.0	7.10×10^{4}	4.85	4.27	3.77±0.04	4.82±0.00	4.02±0.05	28.92±0.03
8.0	1.38×10^{5}	5.14	4.42	3.81±0.00	4.47±0.22	4.25±0.02	30.74±0.05

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table C8 Effect of initial pH of fermentation medium containing 30 g/l (dry weight) of tapioca starch on growth and L-lactic acid production of strain SUT134 when cultivated under anaerobic condition for 48 h.

Initial pH	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
5.0	3.97×10^{7}	7.60	4.27	0.43±0.07	0.48±0.11	0.45±0.04	30.05±0.03
5.5	8.97×10^{5}	5.95	4.20	1.34±0.04	0.82±0.10	0.77±0.05	29.94±0.02
6.0	1.70×10^{3}	3.23	3.86	1.39±0.00	1.36±0.04	1.29±0.02	29.20±0.09
6.5	2.95×10^{3}	3.47	3.95	1.77±0.04	2.20±0.02	2.09±0.02	29.16±0.04
7.0	3.00×10^{3}	3.48	4.28	4.29±0.04	5.34±0.00	5.56±0.02	28.69±0.00
8.0	9.70×10^{3}	3.99	4.22	4.03±0.04	4.40±0.02	4.18±0.02	29.12±0.06

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table C9 Effect of temperature on growth and L-lactic acid production of strain SUT513 when cultivated in the suitable fermentation medium containing 30 g/l (dry weight) of tapioca starch for 48 h.

Temp. (°C)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
25	5.53×10^{7}	7.74	4.28	3.96±0.07	4.60±0.04	4.37±0.01	24.21±0.03
30	1.67×10^{7}	7.22	4.25	4.06±0.07	4.75±0.17	4.51±0.02	26.73±0.00
35	7.10×10^{4}	4.85	4.27	3.77±0.04	4.82±0.00	4.02±0.05	28.92±0.03
37	4.65×10^{4}	4.67	4.39	3.76±0.00	4.57±0.20	4.35±0.02	27.77±0.07
40	1.36×10^{4}	4.13	4.41	3.71±0.04	4.14±0.23	3.93±0.02	27.00±0.08
45	8.65×10^{3}	3.94	4.49	3.47±0.00	4.11±0.06	3.91±0.01	28.02±0.04

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table C10 Effect of temperature on growth and L-lactic acid production of strain SUT134 when cultivated in the suitable fermentation medium containing 30 g/l (dry weight) of tapioca starch for 48 h.

Temp. (°C)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
25	5.00×10^{7}	7.70	4.34	3.76±0.00	3.92±0.05	3.72±0.02	29.23±0.01
30	3.06×10^{6}	6.49	4.29	4.16±0.00	4.02±0.06	3.82±0.02	29.15±0.02
35	3.00×10^{3}	3.48	4.28	4.29±0.04	5.34±0.00	5.56±0.02	28.69±0.00
37	1.74×10^{5}	5.24	4.39	3.96±0.00	4.68±0.26	4.43±0.03	28.30±0.02
40	4.25×10^{4}	4.63	4.41	3.66±0.00	4.02±0.03	3.82±0.01	29.08±0.03
45	1.59×10^{4}	4.20	4.58	3.37±0.07	3.63±0.11	3.45±0.01	28.20±0.03

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table C11 Effect of different inouculum sizes of strain SUT513 on L-lactic acid production when cultivated in the suitable fermentation medium containing 30 g/l (dry weight) of tapioca starch for 48 h.

Inoculum size (%)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
1	1.95×10^{5}	5.29	4.28	4.11±0.04	3.90±0.12	3.70±0.03	30.49±0.04
2	7.50×10^{5}	5.88	4.26	4.01±0.04	3.94±0.02	3.74±0.02	30.10±0.03
3	1.85×10^{5}	5.27	4.28	4.01±0.04	3.91±0.03	3.71±0.02	26.62±0.09
4	3.70×10^{5}	5.57	4.29	4.06±0.00	4.11±0.02	3.91±0.01	26.04±0.04
5	3.65×10^{5}	5.56	4.30	4.06±0.00	4.00±0.12	3.80±0.01	23.75±0.02

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table C12 Effect of different inouculum sizes of strain CAR134 on L-lactic acid production when cultivated in the suitable fermentation medium containing 30 g/l (dry weight) of tapioca starch for 48 h.

Inoculum size (%)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
1	3.30×10^4	4.52	4.29	4.16±0.07	4.69±0.43	4.74±0.02	28.70±0.03
2	1.60×10^4	4.20	4.28	4.16±0.07	4.67±0.05	4.43±0.02	28.66±0.04
3	1.50×10^4	4.18	4.30	4.06±0.00	4.65±0.12	4.42±0.02	28.53±0.04
4	5.50×10^4	4.74	4.32	4.11±0.04	4.94±0.12	4.69±0.03	28.45±0.03
5	5.00×10^3	3.70	4.30	4.16±0.07	4.55±0.03	4.32±0.02	28.42±0.04

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table C13 L-Lactic acid production by isolate SUT513 in 5 l optimized medium containing 30 g/l of tapioca starch in a 6.6 l bioreactor at 35°C and pH 7.0.

Fermentation time (h)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
0	1.21×10^{7}	7.08	7.07	0.00	0.00	0.00	36.05
2	1.35×10^{8}	8.13	7.04	0.00	0.04	0.04	29.16
4	2.02×10^{9}	9.30	6.94	0.63	0.94	0.90	32.26
6	2.36×10^{10}	10.37	6.90	4.14	5.96	5.66	21.30
8	1.05×10^{10}	10.02	6.91	8.56	11.69	11.11	22.69
10	9.45×10^{12}	12.98	6.92	12.16	16.19	15.38	19.73
12	1.58×10^{13}	13.20	6.94	15.40	20.20	19.19	10.76
14	7.75×10^{11}	11.89	6.92	17.74	24.94	23.70	10.06
16	3.20×10^{11}	11.51	6.93	19.91	26.75	25.41	6.56
18	7.50×10^{10}	10.88	6.95	21.89	28.66	27.23	4.09
20	2.62×10^{10}	10.42	6.93	23.60	32.68	31.04	1.64
22	1.80×10^{10}	10.26	6.94	24.86	37.74	35.85	1.97
24	9.00×10^{9}	9.95	6.95	25.85	37.39	35.52	1.74
26	8.65×10^{9}	9.94	6.94	25.94	37.48	35.60	1.51
28	9.10×10^{9}	9.96	6.94	26.21	38.93	36.98	0.95
30	8.15×10^{9}	9.91	6.94	26.48	33.56	31.88	0.80
32	1.74×10^{9}	9.24	6.94	26.75	38.04	36.14	1.01
34	2.05×10^{9}	9.31	6.94	26.93	34.30	32.59	1.15
36	1.02×10^{9}	9.01	6.94	27.20	37.14	35.28	1.07
38	1.71×10^{9}	9.23	6.94	27.47	37.32	35.45	0.82
40	1.39×10^{9}	9.14	6.94	27.65	34.77	33.03	1.43
42	1.34×10^{9}	9.13	6.95	27.74	37.02	35.17	1.45
44	1.97×10^{9}	9.29	6.96	27.83	35.59	33.81	1.35
46	8.06×10^{8}	8.91	6.98	27.92	34.03	32.32	1.64
48	6.70×10^{8}	8.83	6.95	27.92	31.88	30.28	1.65

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

Table C14 L-Lactic acid production by isolate CAR134 in 5-l optimized medium containing 30 g/l of tapioca starch in a 6.6 l bioreactor at 35°C and pH 7.0.

Fermentation time (h)	Growth		pH	Total acidity (%)[a]	L-Lactic acid (g/l)[b]	Total lactic acid (g/l)[c]	Total sugars (g/l)[d]
	CFU/ml	LogCFU/ml					
0	1.79×10^{7}	7.25	7.11	0.00	0.00	0.00	34.27
2	1.79×10^{9}	9.25	7.11	0.00	0.04	0.04	23.38
4	3.30×10^{9}	9.52	6.92	0.09	0.50	0.48	24.77
6	5.45×10^{9}	9.74	6.90	2.97	3.46	3.29	12.59
8	8.80×10^{10}	10.94	6.95	7.48	9.47	9.00	11.72
10	7.50×10^{10}	10.88	6.91	10.81	13.26	12.60	7.54
12	1.80×10^{11}	11.26	6.94	14.59	17.54	16.67	7.06
14	5.25×10^{11}	11.72	6.92	17.29	21.72	20.64	5.92
16	3.55×10^{10}	10.55	6.91	19.73	24.42	23.20	5.01
18	2.95×10^{10}	10.47	6.94	22.25	27.02	25.67	2.31
20	1.70×10^{10}	10.23	6.94	23.96	31.20	29.64	1.60
22	9.35×10^{9}	9.97	6.95	24.59	29.63	28.15	1.17
24	3.80×10^{9}	9.58	6.95	24.86	28.56	27.14	1.20
26	5.10×10^{9}	9.71	6.95	25.04	30.36	28.84	1.32
28	8.00×10^{9}	9.90	6.94	25.22	30.03	28.53	0.93
30	2.39×10^{9}	9.38	6.94	25.40	30.12	28.61	1.16
32	2.21×10^{9}	9.34	6.94	25.49	28.40	26.98	1.18
34	2.53×10^{9}	9.40	6.94	25.67	32.31	30.70	1.13
36	7.30×10^{9}	9.86	6.94	25.85	30.28	28.76	1.08
38	1.12×10^{9}	9.05	6.95	25.94	32.70	31.07	1.03
40	1.11×10^{9}	9.04	6.94	26.12	28.47	27.05	1.13
42	1.40×10^{9}	9.15	6.95	26.30	30.54	29.01	1.21
44	4.65×10^{8}	8.67	6.95	26.30	28.22	26.81	1.06
46	6.10×10^{8}	8.79	6.95	26.39	28.37	26.95	1.22
48	5.15×10^{8}	8.71	6.95	26.39	26.88	25.54	0.87

[a]: Titration method (AOAC International, 2000).
[b]: HPLC analysis (Yang and Chung, 2007).
[c]: Colorimetric assay (Kimberley and Taylor, 1996).
[d]: Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956).

APPENDIX D

STANDARD CURVES

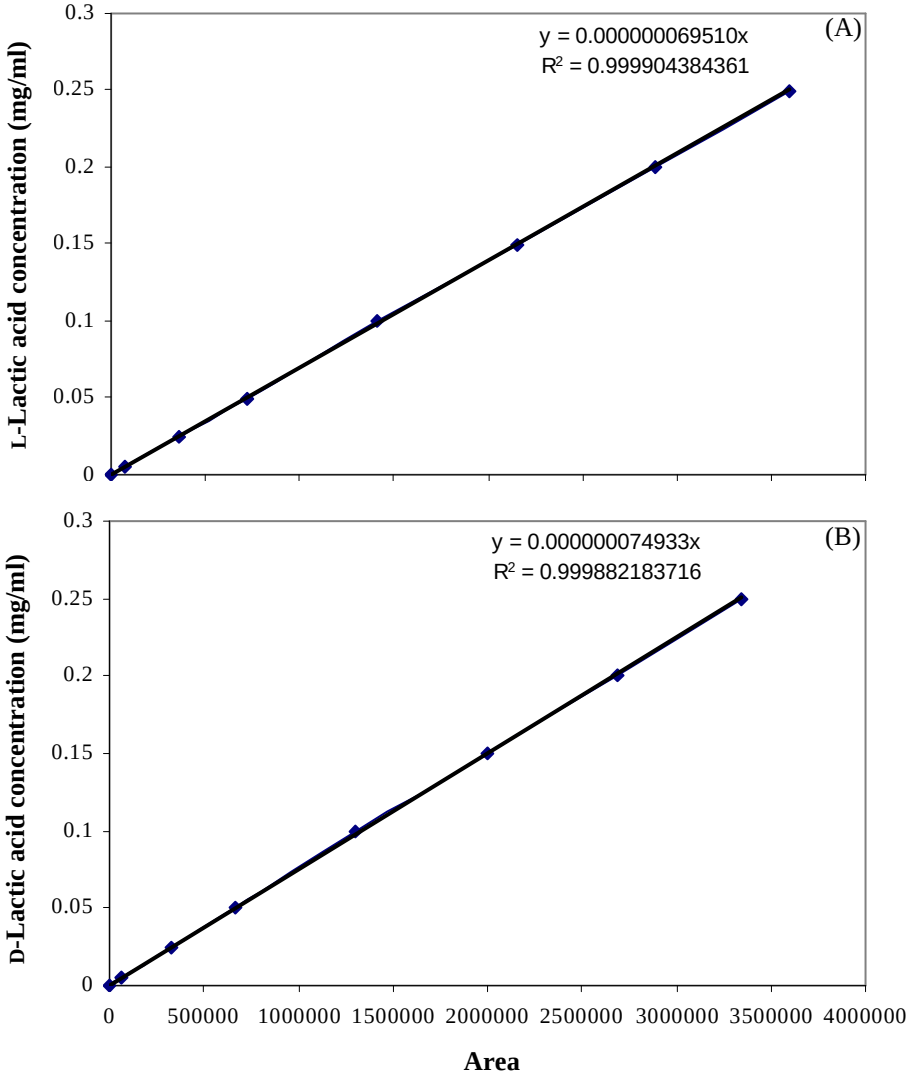

Figure 1D Standard curves of L-lactic acid (A), D-lactic acid (B), and peak area of acids determined using HPLC.

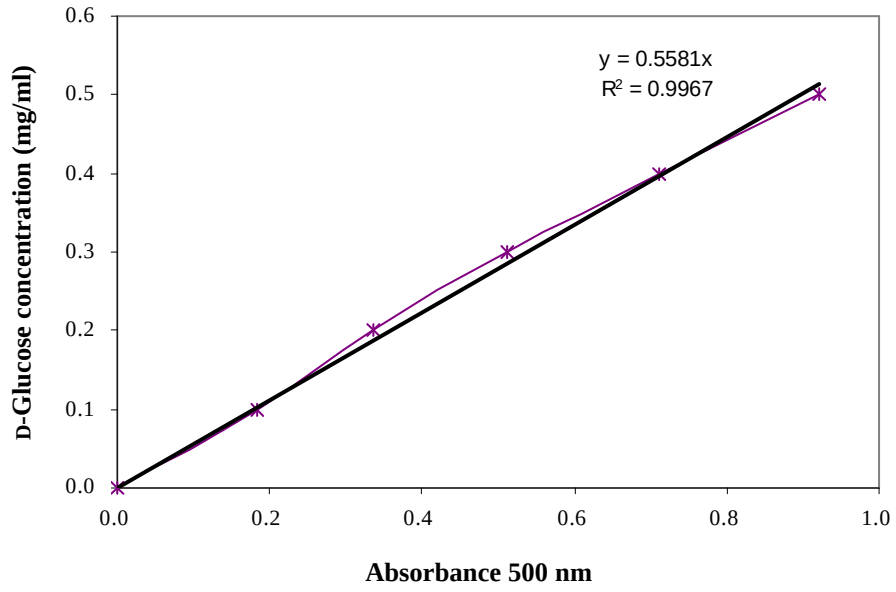

Figure 2D Standard curve of D-glucose determined by phenol-sulfuric acid method (Dubois *et al*., 1956).

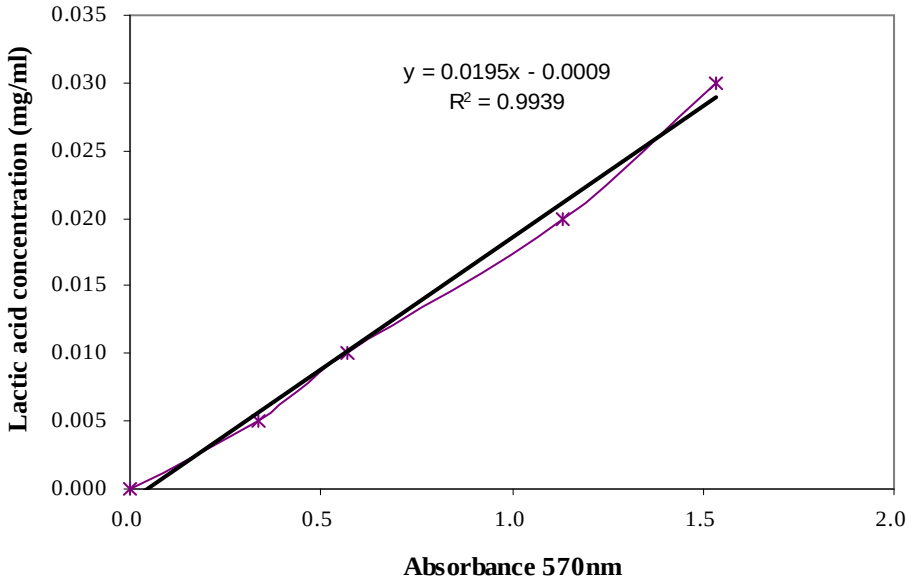

Figure 3D Standard curve of total lactic acid determined by colorimetric assay (Kimberley and Taylor, 1996).

APPENDIX E

HPLC CHROMATOGRAMS

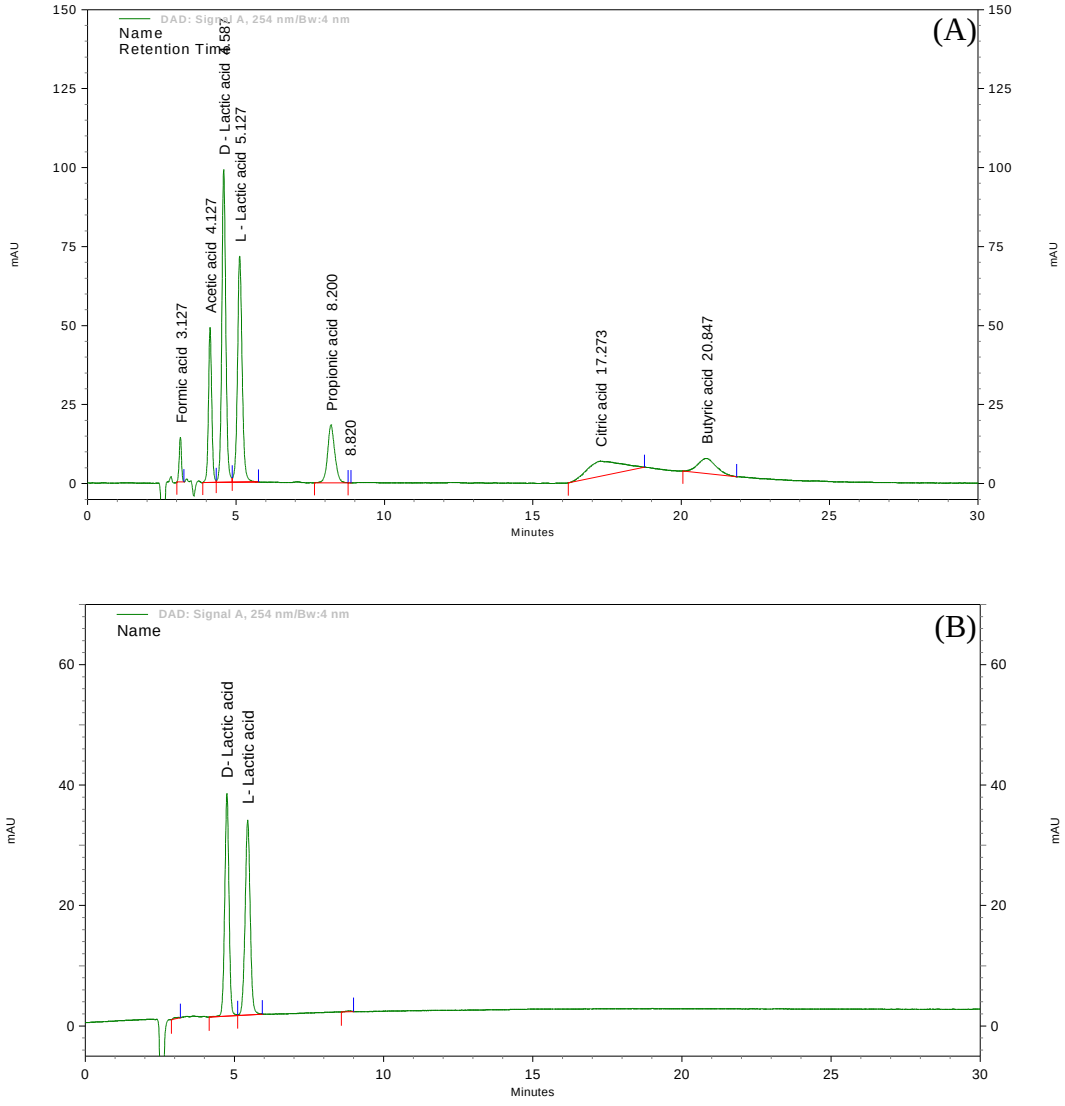

Figure 1E HPLC chromatograms of organic acid standards (0.1 mg/ml): formic, acetic, D-lactic, L-lactic, propionic, citric and butyric acids (A) and D(-)- and L(+)-lactic acids (0.05 mg/ml) (B) when analyzed using the chiral column (Astect CLC-L column, Sigma-Aldrich, Inc.).

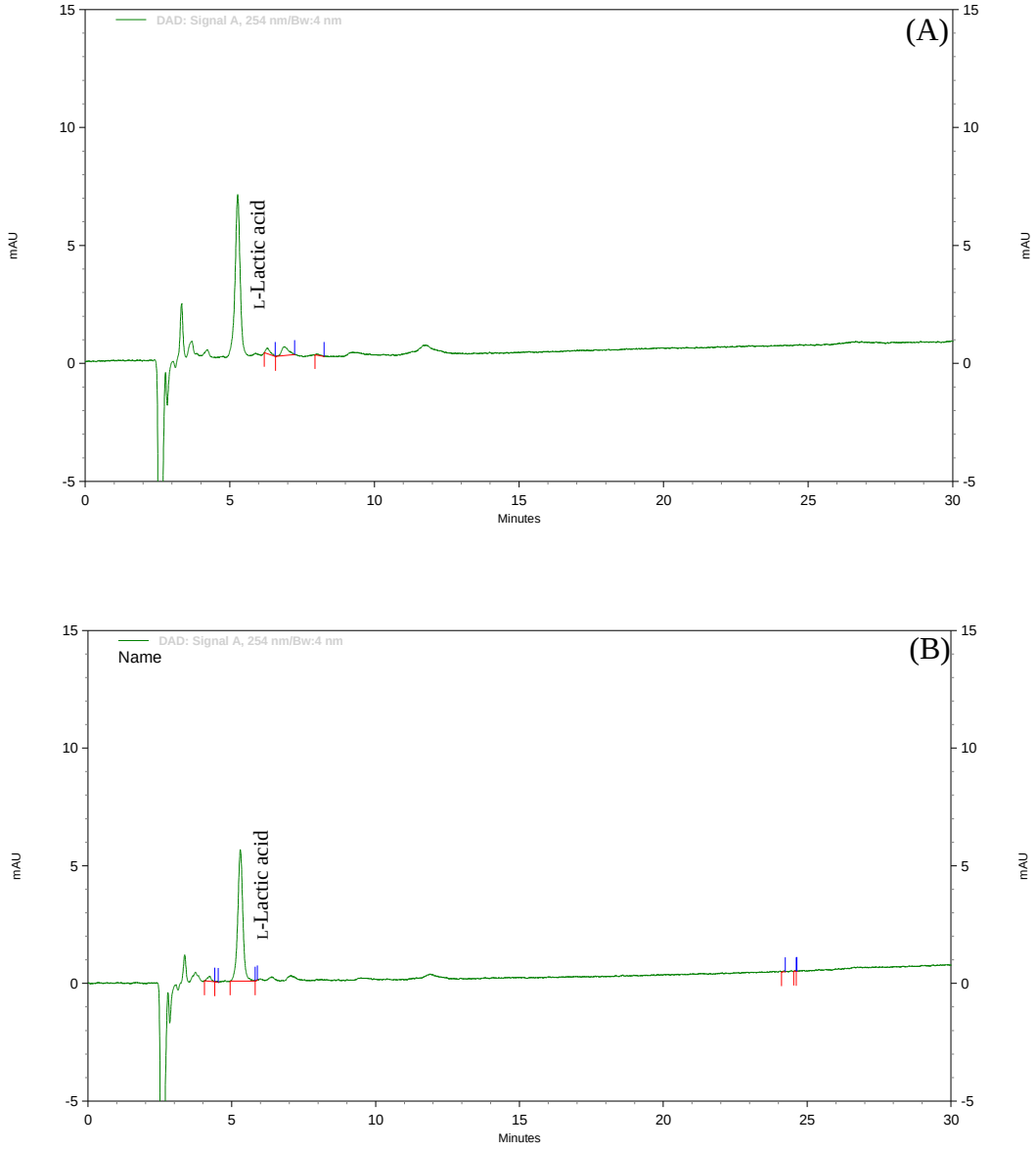

Figure 2E HPLC chromatograms of optimized fermentation media containing 30 g/l of tapioca starch (dry weight) after cultivating SUT513 (A) and CAR134 (B) at optimum conditions for 48 h (500× dilution), respectively; when analyzed using the chiral column (Astect CLC-L column, Sigma-Aldrich, Inc.).

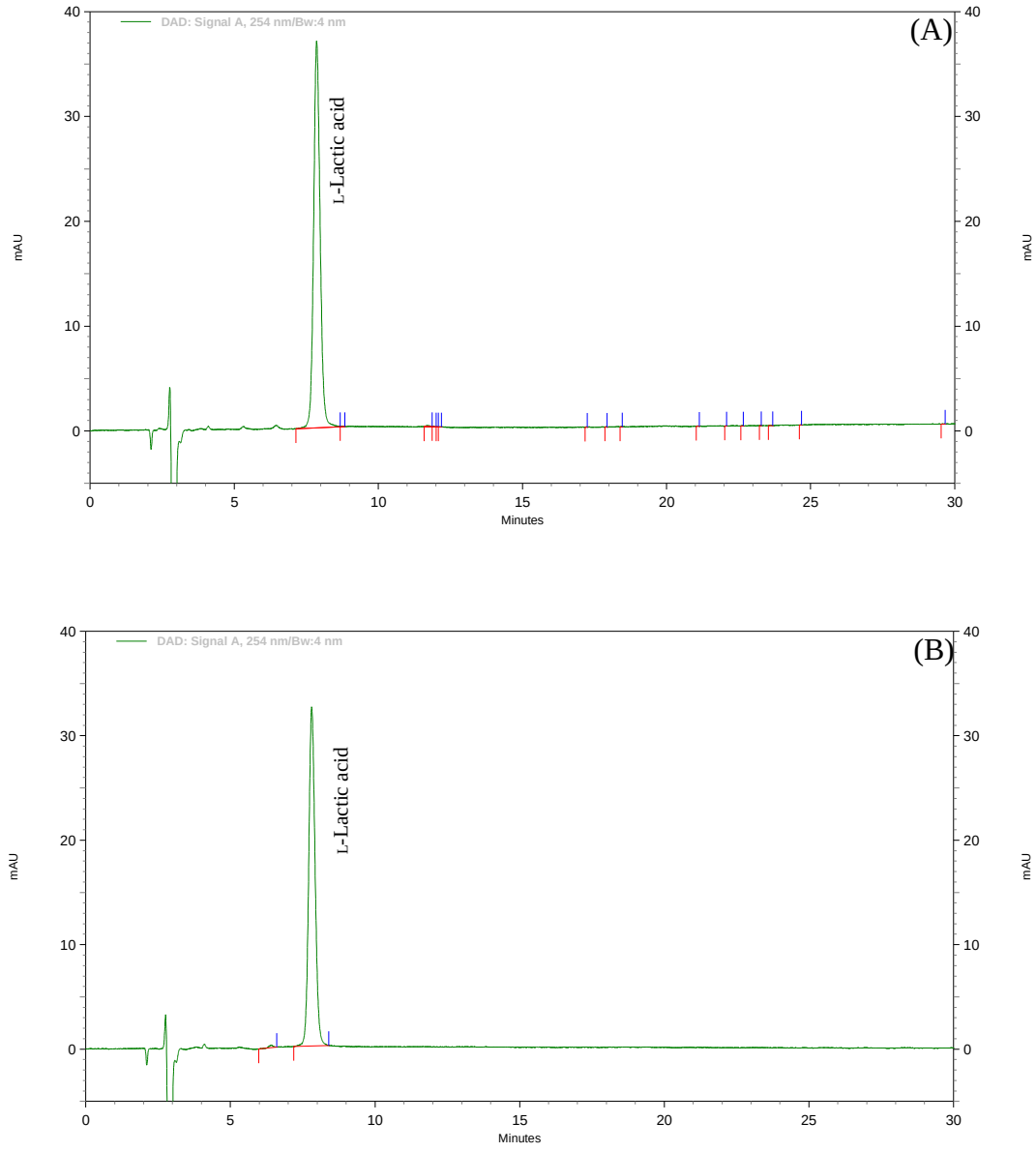

Figure 3E HPLC chromatograms of fermentation media after cultivating SUT513 (A) and CAR134 (B) for 48 h (500× dilution); when analyzed using the chiral column (Astect CLC-L column, Sigma-Aldrich, Inc.).

APPENDIX F

PHYSIOLOGICAL CHARACTERIZATION

F.1 Carbohydrate fermentation profile

Figure 1F Carbohydrate fermentation test using API 50 CH strips (bioMérieux, bioMérieux Industry, France) after 48 h incubation of bacterial isolates SUT513 (A); CAR134 (B); CAR128 (C); and CAR135 (D).

F.2 Physiological characterization

F2.1 Temperature

Medium	Bacterial isolate code	Temperature (°C)											
		5	10	15	20	30	35	37	40	42	45	50	55
RAM	SUT513	-	-	-	++++	++++	++++	++++	++++	++++	++++	-	-
	CAR128	-	-	-	++++	++++	++++	++++	++++	++++	-	-	-
	CAR134	-	-	-	++++	++++	++++	++++	++++	++++	++++	-	-
	CAR135	-	-	-	++++	++++	++++	++++	++++	++++	+	-	-
MRS	SUT513	-	-	-	++++	++++	++++	++++	++++	++++	++++	-	-
	CAR128	-	-	-	++++	++++	++++	++++	++++	++++	-	-	-
	CAR134	-	-	-	+	++	++++	++++	++++	++++	+	-	-
	CAR135	-	-	-	+	++	++++	++++	++++	++++	++++	-	-

F2.2 pH (initial pH of fermentation medium)

Medium	Bacterial isolate code	pH								
		4.0	4.4	4.8	5.0	6.0	6.5	7.0	8.0	9.6
RAM	SUT513	-	-	-	++	+++	+++++	+++++	+++++	-
	CAR128	-	-	-	-	+++	+++++	+++++	+++++	-
	CAR134	-	-	+	++	+++	+++++	+++++	+++++	-
	CAR135	-	-	+	+++	+++	+++++	+++++	+++++	-
MRS	SUT513	-	-	-	+	+++++	+++++	+++++	+++++	-
	CAR128	-	-	-	-	+++++	+++++	+++++	+++++	-
	CAR134	-	-	+++++	+++++	+++++	+++++	+++++	+++++	-
	CAR135	-	-	+++++	++++	+++++	+++++	+++++	+++++	-

F2.3 NaCl added to fermentation medium

Medium	Bacterial isolate code	% NaCl						
		0	3.0	4.0	6.5	8.0	10.0	18.0
RAM	SUT513	+++	+	-	-	-	-	-
	CAR128	+++	++	+	-	-	-	-
	CAR134	+++	++	+	-	-	-	-
	CAR135	+++	++	+	-	-	-	-
MRS	SUT513	++++	+++	-	-	-	-	-
	CAR128	++++	++++	++++	-	-	-	-
	CAR134	++++	+++	++	-	-	-	-
	CAR135	++++	++++	++	-	-	-	-

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

APPENDIX G

NUCLEOTIDE SEQUENCE DATA

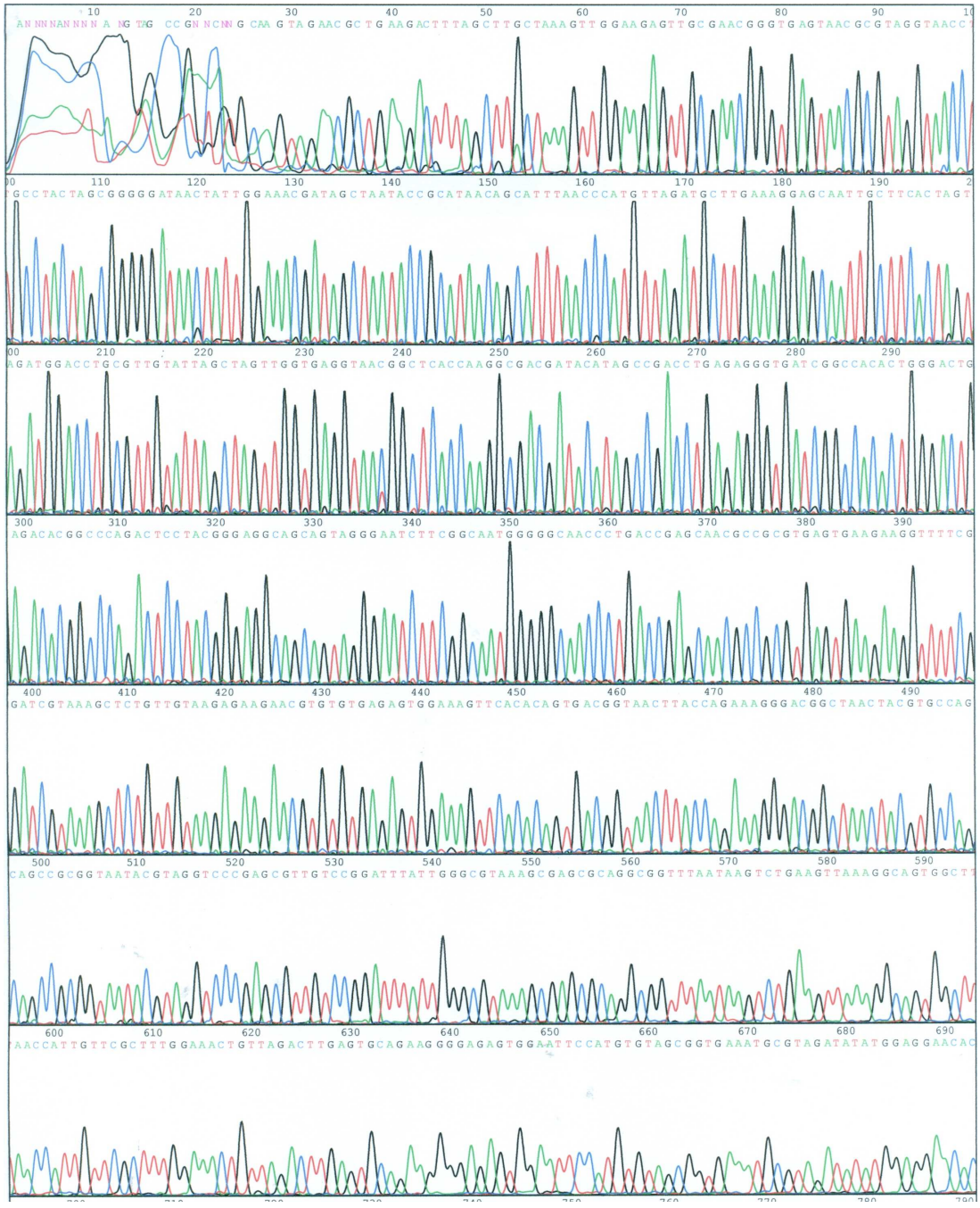

Figure 1G Sequence electrophenogram of 16S rRNA gene of bacterial isolate SUT513 using fD1 primer as the sequencing primer (Macrogen Inc., Korea).

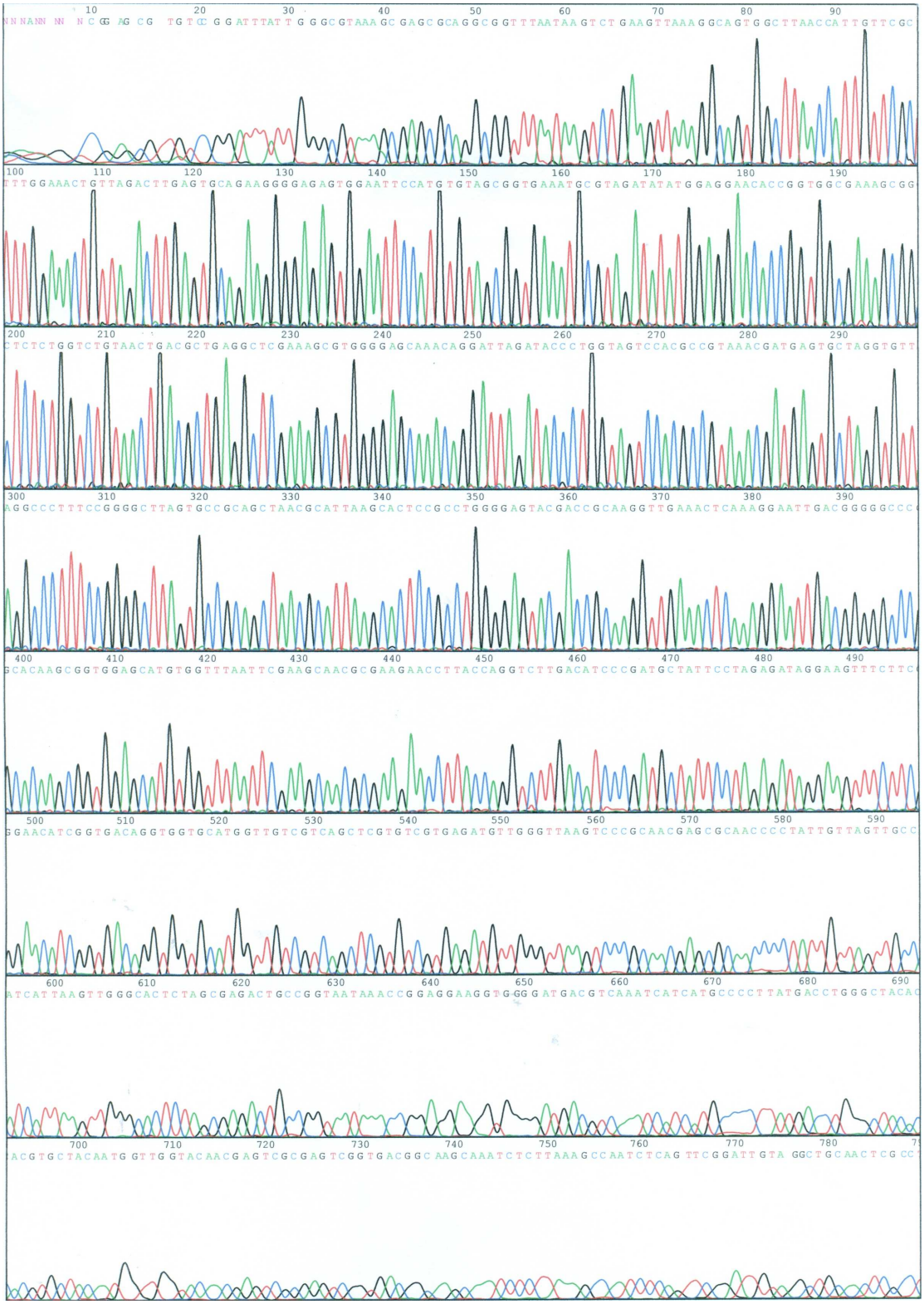

Figure 2G Sequence electrophenogram of 16S rRNA gene of bacterial isolate SUT513 using rP2 primer as the sequencing primer (Macrogen Inc.).

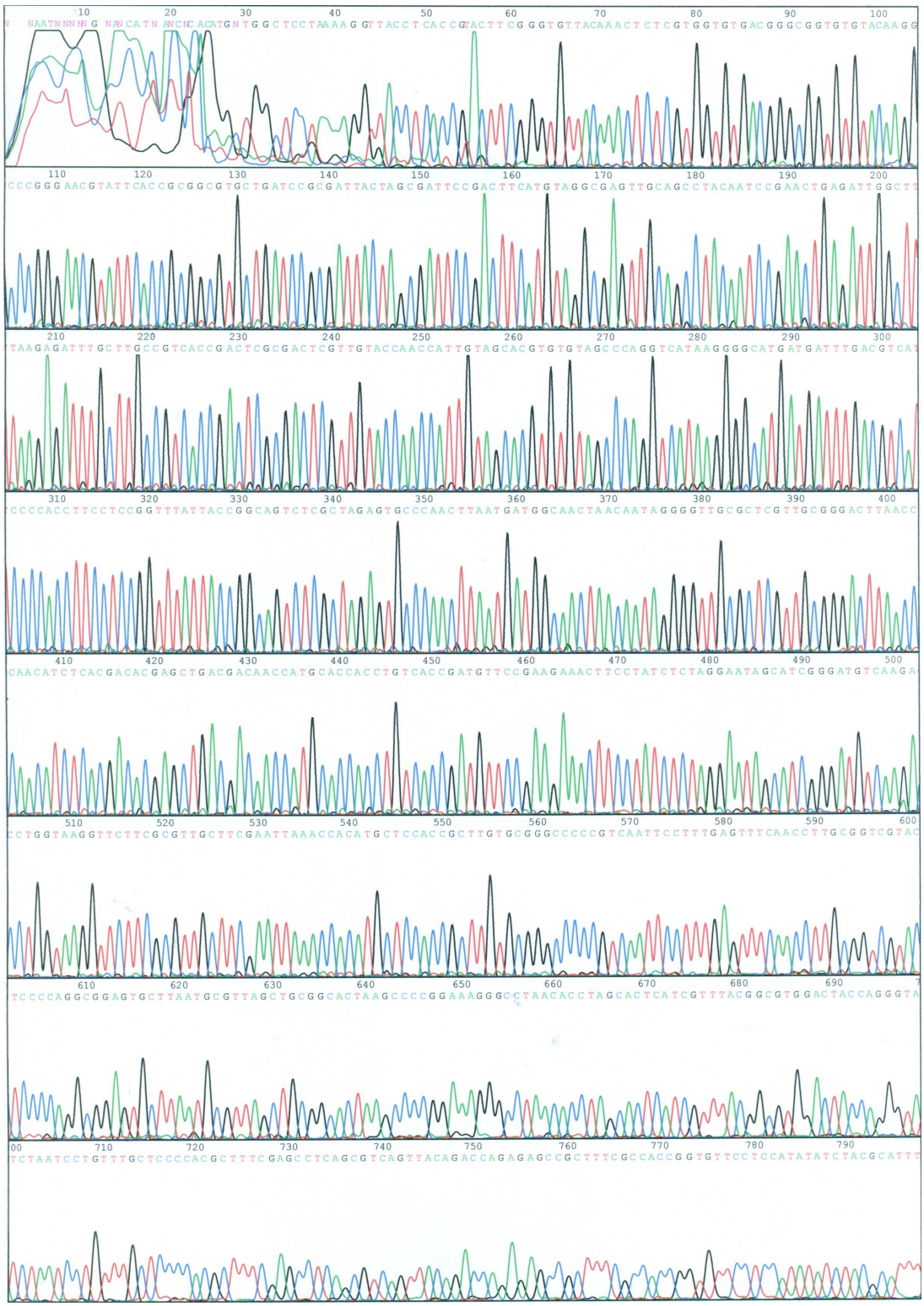

Figure 3G Sequence electrophenogram of 16S rRNA gene of bacterial isolate SUT513 using walking forward primer (at 500 bp position) as the sequencing primer (Macrogen Inc.).

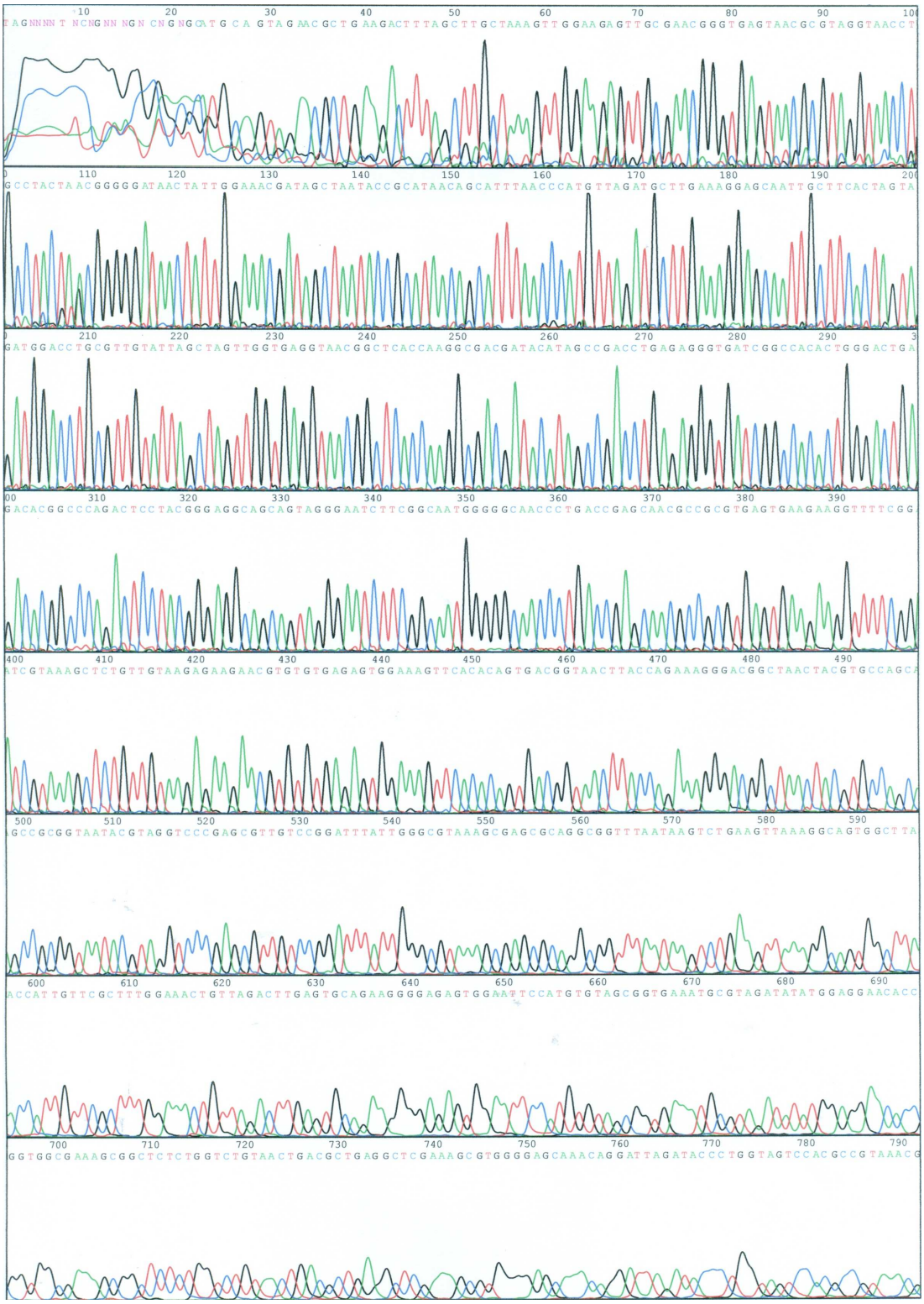

Figure 4G Sequence electrophenogram of 16S rRNA gene of bacterial isolate CAR134 using fD1 primer as the sequencing primer (Macrogen Inc.).

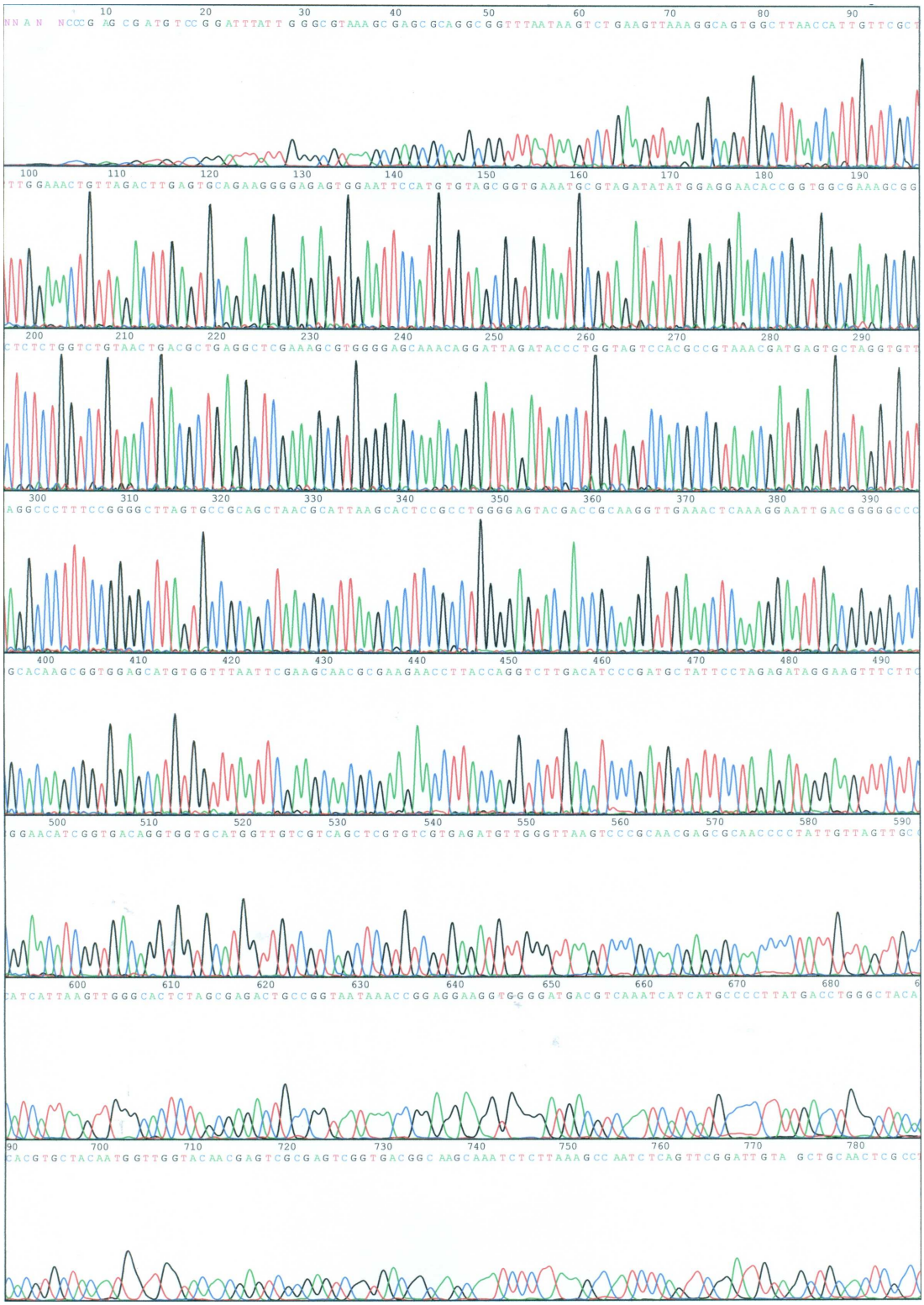

Figure 5G Sequence electrophenogram of 16S rRNA gene of bacterial isolate CAR134 using rP2 primer as the sequencing primer (Macrogen Inc.).

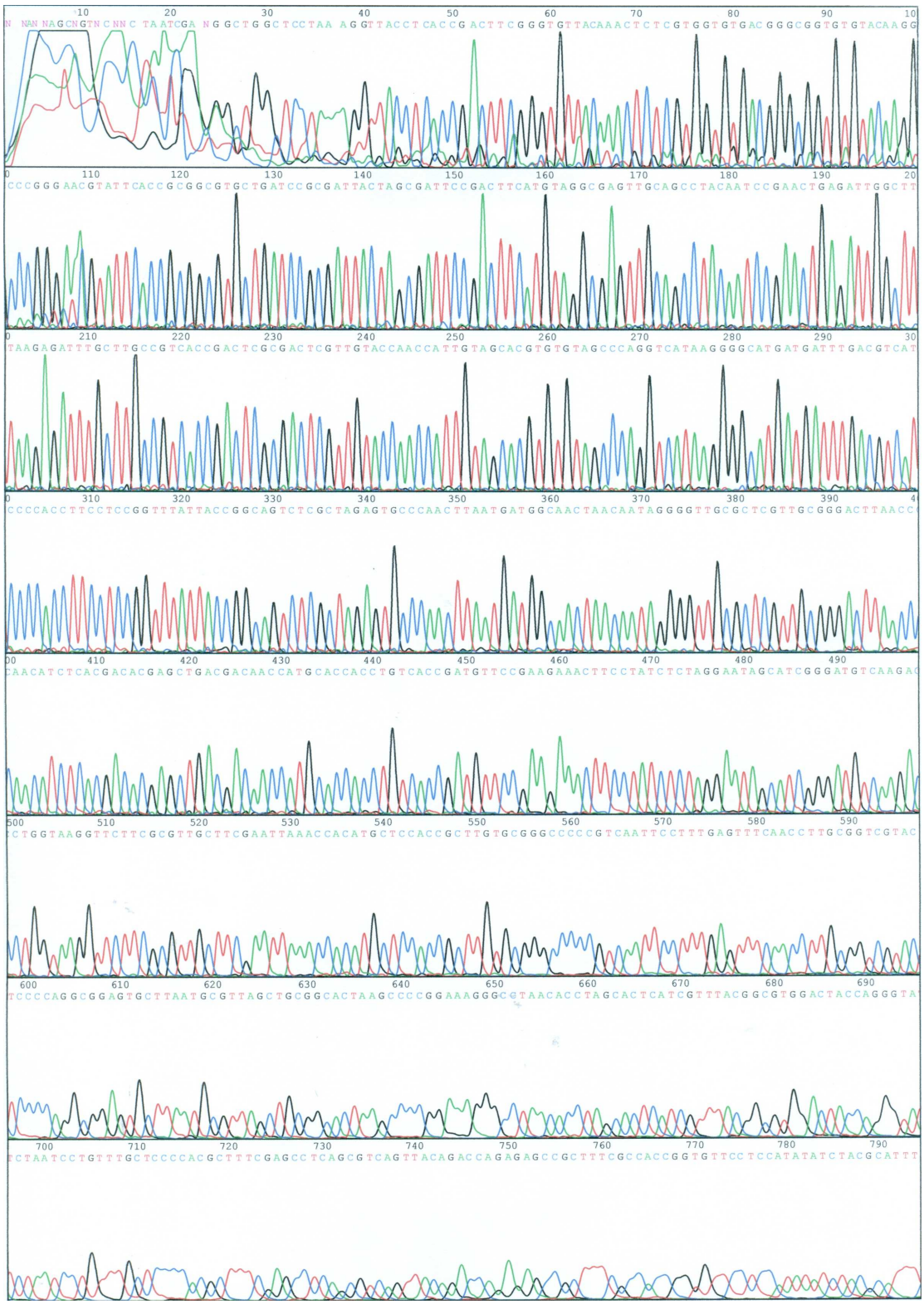

Figure 6G Sequence electrophenogram of 16S rRNA gene of bacterial isolate CAR134 using walking forward primer (at 500 bp position) as the sequencing primer (Macrogen Inc.).

APPENDIX H

LIST OF PRESENTATIONS

Poster presentation

Rodtong, S. and Pikul-ngoen, Y. (2010). **Protein profiles from lectin extract as an aid for detection and identification of lectin-producing mushrooms.** The 9th International Mycological Congress (IMC9), 1-6 August 2010, Edinburgh, U.K.

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INVESTIGATION OF L-LACTIC ACID PRODUCTION BY LACTIC ACID BACTERIA ISOLATED FROM TAPIOCA STARCH PRODUCTION WASTE

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L-Lactic acid is one of the useful compounds utilized in food, pharmaceutical and chemical industries. Stereo-selective two isomers exist for having a chiral carbon. It can be polymerized to biodegradable plastics, i.e. poly (L-lactic acid), which has great potential for replacing petrochemical plastics. In the polymerization process, the stereospecificity of lactic acid is very important, and selective production of stereospecific lactic acid has been carried out by lactic acid bacteria. In this study, lactic acid bacterial strains were isolated from tapioca starch production waste samples produced in Thailand. A total of 134 lactic acid bacterium isolates were cultivated using MRS medium supplemented with 2% glucose and incubated under anaerobic condition at 30°C for 24 h. Low pH values and high titratable acidity contents were used to screen potential lactic acid-producing strains. The isomer type of lactic acid was analyzed by high performance liquid chromatography. One hundred and eleven isolates were able to produced L-lactic acid. They were homofermentative and produced L-lactic acid as a main end-product from D-glucose. L-Lactic acid-producing strains gave the maximum L-lactic acid yield of 19.12 g/l. Potential lactic acid-producing isolates for L-lactic acid production from tapioca starch were selected for further investigation.

Keywords: L-Lactic acid, Lactic acid bacteria, Production, Tapioca starch, Waste

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Protein profiles from lectin extracts as an aid for detection and identification of lectin-producing mushrooms

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Some certain mushroom species or strains can produce lectins, di- or multi-valent carbohydrate-binding proteins or glycoproteins of non-immune origin. In this study, protein profiles from lectin extracts were investigated to apply as an aid for the detection and identification of lectin-producing mushrooms. A total of 330 mushroom specimens (fruit bodies) were collected from their natural habitats and local markets in Thailand. The specimens were identified by conventional methods. Forty four genera of 24 families were recorded. Lectins were extracted from these fruit bodies, determined for their protein profiles according to molecular masses using SDS-PAGE, and detected for their lectin accumulation by hemagglutination assay using human and animal (goose, guinea pig, mouse, rabbit, rat and sheep) erythrocytes. Approximately 60% of the extracts were found to predominantly perform hemagglutinating for rabbit and rat erythrocytes. The high incidence of lectin accumulation was observed in specimens belonging to genera Amanita, Boletus, Cantharellus, Lentinus, Lycoperdon, Macrolepiota, Marasmius, Russula, Schizophyllum, Termitomyces, and Volvariella. Different mushroom strains contained different protein profiles having the average molecular weights ranging from 15 to 150 kilodaltons, corresponding to their different lectin properties. Protein profile analysis could be potentially applied for the detection and preliminary identification of specific species/strains of lectin-producing mushrooms.

Keywords: Lectin-producing mushrooms, Mushroom identification, Protein profiles, Lectins

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Optically pure L-lactic acid production from tapioca starch supplemented with spent brewer's yeast by a starch-utilizing lactic acid bacterium

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Abstract

The production of optically pure L-lactic acid were investigated among 250 lactic acid bacterial strains isolated from their natural habitats. Isolate SUT 513 was then selected for the acid production from tapioca starch, a low cost substrate compared to sugars, the common raw material. The strain SUT 513 was identified as belonging to the genus *Streptococcus* according to its morphological and biochemical characteristics, and 16S rDNA sequence. Optimization of growth and acid production conditions emphasizing on tapioca starch supplemented with spent brewer's yeast were performed. It was found that the suitable medium composed of main ingredients as follows: 30 g/l (dry weight) of tapioca starch supplemented with 5 g/l of spent brewer's yeast, and 2.5 g/l of tryptone at initial pH 7.0. When lactic acid fermentation was performed in a bioreactor containing 5 l of the optimized medium under optimal temperature at 35°C for 48 h, the strain could produce the maximum L-lactic acid concentration of 38.9 g/l with >99% optical purity after cultivation for 28 h. The L-lactic acid yield ($Y_{LA/S}$) of 0.9974 g/g (99.74%), productivity of 1.61 g/l.h., and the specific growth rate (μ_{max}) of 0.51 h^{-1} were achieved. The high optical purity of L-lactic acid produced could be potentially applied for biodegradable plastics production.

Keywords: L-Lactic acid, Lactic acid bacteria, Tapioca starch, Spent brewer's yeast, Biopolymer

Introduction

L-Lactic acid is considered to be one of the most useful chemicals used in food (as a preservative, acidulant, and flavouring agent), chemical, textile, and pharmaceutical industries (Åkerberg and Zacchi, 2000). It also functions as the main monomer for the production of biodegradable poly(L-lactic acid) (PLLA or PLA) (Datta *et al.*, 1995). The optical purity of lactic acid is very important for the biopolymer production. Two enantiomers of lactic acid, D- and L-forms, have been naturally found. L-Lactic acid can be produced by either chemical synthesis or microbial fermentation but the biological method has the advantage that an optically pure L-lactic acid can be obtained by choosing a specific strain of microorganism. Most widely used substrates for the production of lactic acid by fermentation are refined sugars, which are expensive. Lactic acid is also produced from abundant and

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renewable substances such as whey, molasses, beet and cane sugar, starch (Vishnu *et al*., 2002). Tapioca starch, a cheap agricultural product in Thailand, has also been reported to be used for the production of lactic acid without pretreatment by enzymic saccharification to glucose (Rodtong and Ishizaki, 2003). Several bacterial strains have been studied for lactic acid fermentation from starchy materials such as *Lactobacillus plantarum* (Panda and Ray, 2008), *L. manihotivorans* (Ohkouchi and Inoue, 2006), *L. amylophilus* (Altaf *et al*., 2007), *L. amylovorus* (Nagarjun *et al*., 2005), *Lactococcus lactis* subsp. *lactis* (Petrov *et al*., 2008), *Enterococcus faecium* (Shibata *et al*., 2007), and *Streptococcus bovis* (Narita *et al*., 2004). The present work studies the production of high optical purity of L-lactic acid from tapioca starch supplemented with spent brewer's yeast by a starch-utilizing lactic acid bacterium.

Materials and Methods

Bacterial strains and media

Two hundred and fifty lactic acid bacterial strains obtained from stock cultures of the Microbial Culture Collection Laboratory, Suranaree University of Technology, were used to screen for their capability to produce optically pure L-lactic acid. The bacteria were maintained at -80°C with the addition of skim milk to 5% (v/v) final concentration. The medium used for cultivation of these strains was 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.

Selection of starch-utilizing bacterial strain producing optically pure L-lactic acid

Two hundred and fifty lactic acid bacterial strains as mentioned above were in RAM medium containing 1% tapioca starch rather than 2% glucose. The starch (Sanguanwongse industries Co., Ltd., Thailand) contained carbohydrate (85.53%), moisture content (11.95%), fiber (0.18%), total nitrogen (0.35%), fat (1.64%), and ash (0.35%). The strain produced high yield of optically pure L-lactic acid was selected for further study.

Identification of the selected lactic acid bacterium

The selected bacterial isolate was identified using morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Biochemical tests were also carried out using API50 CH/CHL kit (Biomerieux, RCS Lyon, France). And its 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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Analytical methods

Viable cell counts (CFU/ml) were estimated by plating serial dilutions of bacterial suspension on RAM agar containing 1% tapioca starch. L-Lactic acid concentration in the supernatant was measured after centrifugation at 4,500 rpm for 20 min at 4°C to separate bacterial cells. Acid production was examined by measurement of pH values, and determined titratable acidity (AOAC International, 2000). The optical purity of L-lactic acid was detected using high performance liquid chromatography (HP 1200, Agilent Technology Inc., USA), equipped with a tunable UV detector set at 254 nm and a chiral Astec CLC-L column (5 μm, 4.6 mm×15 cm, Sigma Chemical Co., USA), which was eluted with 0.005 M $CuSO_4$ as a mobile phase at a flow rate of 0.7 ml/min. The column temperature was maintained at 35°C. The total sugars content was also determined by the phenol-sulfuric acid method (Dubois *et al*., 1956), using glucose as a standard.

Optimization of L-lactic acid production conditions

The suitable condition for growth and acid production of the selected strain was investigated. Suitable concentrations of tapioca starch, tryptone and yeast extract were determined. Erlenmeyer flask containing 100 ml-working volume of RAM-based medium were inoculated with 2% (v/v) of culture (approximately 10^6 CFU/ml) and incubated at 35°C under anaerobic condition for 48 h. Tapioca concentrations of 10, 15, 20, 25, 30, 35 and 40 g/l were applied to achieve the optimal concentration. The starch concentration that gave the maximal L-lactic acid yield was chosen for investigating the effect of nitrogen source and concentration on lactic acid production. Tryptone concentrations of 2, 2.5, 3, 4 and 5 g/l were applied. Also concentrations of 1, 2 and 3 g/l of yeast extract were optimized and replaced by spent brewer's yeast of 3, 5, and 10 g/l. The spent brewery's yeast (Boonrawd brewery Co., Ltd., Thailand) contained carbohydrate (75.53%), moisture content (10.62%), fiber (5.21 %), total nitrogen (6.21%), fat (2.37%), and ash (5.18%). The nitrogen source concentration that gave the maximal L-lactic acid yield was chosen for this optimized medium. The initial pH of the optimized medium for L-lactic acid production was studied. The medium pH was adjusted to 5.0, 5.5, 6.0, 6.5, 7.0 and 8.0 using 1 N HCl and 1 N NaOH. The suitable temperatures for L-lactic acid production were also investigated. Bacterial strains were cultivated in the suitable medium at various incubation temperatures at 25, 30, 35, 37, 40 and 45°C.

Production of optically pure L-lactic acid

Batch fermentation for L-lactic acid by the selected bacterial strain was carried out in a 6.6-l controlled fermentor containing 5.0-l of the optimized fermentation medium. An inoculum size (1%, v/v) containing approximately 10^6 CFU/ml was added into the suitable medium. The fermentation temperature was conducted for 48 h at the optimum temperature and at the optimum pH by automatic addition of 5 N NaOH. Changes in L-lactic acid concentration, starch concentration, and bacterial growth were measured at each time interval during 48 h cultivation.

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Results and Discussion

Selection and identification of lactic acid bacterium

Among 250 lactic acid bacterial strains tested for L-lactic acid production from tapioca starch, strain SUT 513 was selected. The strain was recognized as Gram-positive, catalase-negative, non-spore forming and non-motile cocci. Cells were 0.38-0.51 μm in size, occurring singly, in pairs or in chains. Its colonies on RAM agar plate were circular, white, low convex with entire margin (1.0-2.0 mm diameter). Clear zones of starch hydrolysis were observed on exposure of colonies growth on RAM agar containing 1% tapioca starch to iodine solution (Figure 1C).

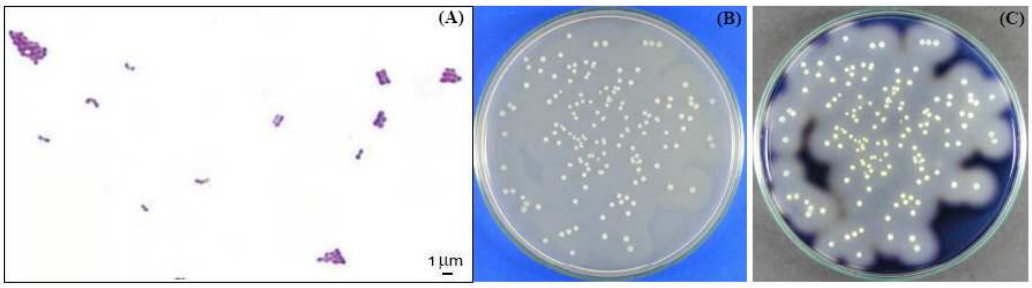

Figure 1. Cell morphology (A) and colony morphology (B) of bacterial strain SUT513 on RAM agar at 35°C for 48 h. This colony produced enzyme to hydrolyze tapioca starch as shown by the reaction of iodine (C)

Bacterial strain SUT 513 had its growth temperature range of 20-45°C and pH range of 5.0-8.0. It could grow in 3.0% (w/v) NaCl. The bacterial strain did not exhibit oxidase and nitrate reducing activities, produced only L-lactic acid. No gas was formed from glucose, produced acid from D-glucose, D-galactose, D-fructose, D-mannose, D-maltose, D-lactose, D-saccharose (sucrose) and D-rafinose, and hydrolyzed *N*-acetylglucosamine, esculin, salicin, amidon (starch) and glycogen. The strain could be identified as belonging to the genus *Streptococcus* with percentages of 16S rRNA gene sequence similarity of 99% compared to *Streptococcus bovis*, *S. equinus*, *S. infantarius*, and *S. luteiensis* (Table 1).

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Table 1. 16S rRNA gene sequence similarity of strain SUT513 and closely related species

Bacterial isolates	SUT513	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SUT513	100																
1	99	100															
2	99	99	100														
3	99	99	99	100													
4	98	98	98	98	100												
5	99	99	99	99	98	100											
6	96	96	96	96	95	96	100										
7	98	98	98	98	97	98	98	100									
8	98	99	98	98	97	99	95	97	100								
9	95	96	96	96	95	96	93	95	96	100							
10	96	96	96	96	95	96	93	94	96	97	100						
11	93	94	94	94	93	94	91	92	94	93	93	100					
12	93	93	93	93	92	93	91	92	94	93	93	97	100				
13	93	94	94	93	93	94	91	92	94	93	95	93	94	100			
14	92	92	92	92	91	92	90	91	92	92	92	93	93	93	100		
15	93	94	94	94	92	94	91	92	93	91	91	93	93	94	93	100	
16	91	91	91	91	90	91	89	90	91	90	90	90	91	91	92	91	100

SUT513: *Streptococcus* sp. SUT513, 1: *Streptococcus infantarius* strain CIP 106106 (DQ232529), 2: *Streptococcus lutetiensis* strain CIP 106849 (DQ232532), 3: *Streptococcus bovis* strain ATCC 27960 (AB002481), 4: *Streptococcus bovis* strain ATCC 33317 (AB002482), 5: *Streptococcus equinus* strain NRIC 1535 (AB362710), 6: *Streptococcus equinus* strain ATCC 9812 (AJ301607) 7: *Streptococcus luteciae* strain NEM 760 (AJ297214), 8: *Streptococcus gallolyticus* subsp. *gallolyticus* strain ATCC 43143 (AF104114), 9: *Streptococcus intestinalis* strain ACTT 43492 (AB002519), 10: *Streptococcus alactolyticus* strain ATCC 43077 (AF201899), 11: *Streptococcus downei* strain ATCC 33748 (AY188350), 12: *Streptococcus sobrinus* strain ATCC 33478 (AY188349), 13: *Streptococcus ratti* strain ATCC 19645 (NR_025516), 14: *Streptococcus ferus* strain ATCC 33477 (AY584479), 15: *Streptococcus mutans* strain ATCC 25175 (AY188348), 16: *Streptococcus macacae* strain ATCC 35911 (AY188351).

Optimization of L-lactic acid production conditions

For the efficient production of L-lactic acid, cultivation conditions involving with medium composition, pH of the medium, growth and lactic acid production temperature, for the selected strain SUT 513 were investigated. The highest L-lactic acid concentration was achieved with 30 g/l of starch. Total L-lactic acid was significantly increased with the increase the initial tapioca starch concentration from 10 g/l to 30 g/l. When the medium supplemented with tapioca starch concentrations of 35 and 40 g/l, the produced L-lactic acid amount did not change. The effect of nitrogen source on lactic acid production was investigated. Since two components, tryptone and yeast extract, of RAM medium could be served as nitrogen source. It was found that the strain SUT 513 could produce L-lactic acid and the maximum production was achieved when using 2.5 g/l of tryptone. Yeast extract is used in most

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of the fermentation studies as supplement, but high price hinders its use in large quantities. To obtain the nitrogen source based on a cheap and abundant raw material, spent brewer's yeast was used to replace yeast extract. Result showed that yeast extract was completely replaced by 5 g/l of spent brewer's yeast in the medium. The suitable pH range for the bacterial growth was 5.0-8.0. The maximum concentration of L-lactic acid was obtained when the medium was adjusted to initial pH of 7.0 while the maximum cell count was at pH 5.5. In addition, the concentration of L-lactic acid produced, increased when the cultivation temperature was increased from 25°C to 35°C but at 37, 40 and 45°C, the production rate was reduced. Thus, the optimum temperature for the production of L-lactic acid was 35°C. Under the optimized cultivation conditions, strain SUT 513 could produce the maximum L-lactic acid concentration of 4.82 g/l and L-lactic acid yield ($Y_{LA/S}$) of 0.7779 g/g (77.79%). In all experiment, the optical purity of L-lactic acid was found to be >99%.

Production of optically pure L-lactic acid

L-Lactic acid fermentation by strain SUT513 was performed in 5-l optimized medium containing 30 g/l (dry weight) of tapioca starch, 2.5 g/l of tryptone, 5 g/l of spent brewer's yeast, 6 g/l of K_2HPO_4, 0.57 g/l of $MgSO_4.7H_2O$, 0.12 g/l of $MnSO_4.4H_2O$ and 0.03 g/l of $FeSO_4.7H_2O$. The cultivation temperature and pH were automatically controlled at 35°C and pH 7.0 for 48 h with agitation speed at 200 rpm. As show in figure 2A, L-lactic acid production started at 4 h after inoculation and continuously increased until 28 h which reached the maximum L-lactic acid yield of 38.9 g/l. The profile of cell growth and pH value during the lactic acid fermentation was showed in Figure 2A. The maximum cell growth was obtained as 1.58×10^{13} CFU/ml for 12 h. Tapioca starch was completely consumed within 48 h. The strain could produce L-lactic acid yield ($Y_{LA/S}$) of 0.9974 g/g (99.74%) at production rate of 1.61 g/l.h. It had specific growth rate (μ_{max}) of 0.51 h^{-1}. The acid produced was only L-lactic acid isomer (Figure 2B).

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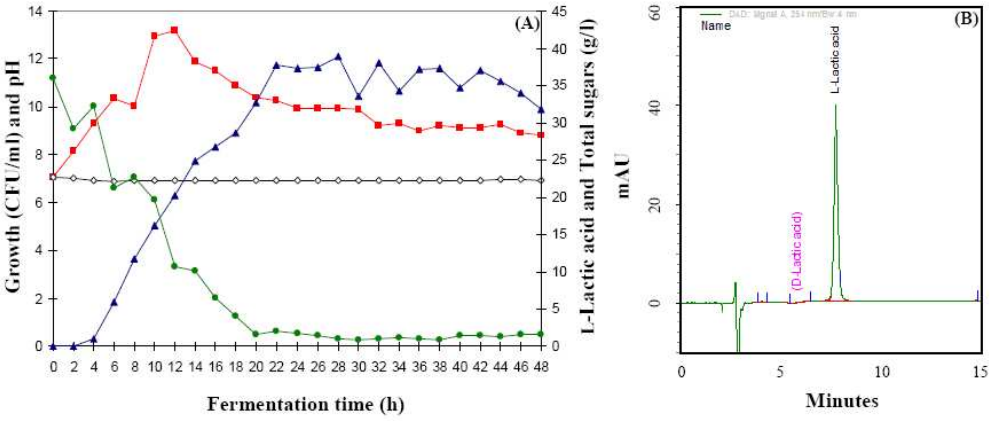

Figure 2. L-Lactic acid production by bacterial strain SUT513 in 5-l optimized medium containing 30 g/l of tapioca starch supplemented with 5 g/l of spent brewer's yeast at 35°C and pH 7.0 for 48 h (A); ■ Growth; ◇ pH; ▲ L-lactic acid; ● total sugar. HPLC Chromatograms of L-lactic acid after cultivating isolate SUT513 for 28 h (500× dilution) (B)

Conclusion

Optically pure L-lactic acid could be produced from tapioca starch supplemented with spent brewer's yeast by the bacterial strain SUT513, which was identified as belonging to the genus *Streptococcus*. The suitable lactic acid production medium for the bacterium composed of 30 g/l (dry weight) of tapioca starch, 5 g/l of spent brewer's yeast, and 2.5 g/l of tryptone as main ingredients, at the initial pH of 7.0. When cultivated strain SUT 513 in a bioreactor containing 5-l of the suitable medium at 35°C for 48 h, the bacterium had its specific growth rate (μ_{max}) of 0.51 h^{-1}. And the maximum L-lactic acid concentration of 38.9 g/l with >99% optical purity corresponding to L-lactic acid yield ($Y_{LA/S}$) of 0.9974 g/g (99.74%) and productivity of 1.61 g/l.h, were achieved. The high optical purity of L-lactic acid for manufacturing of biodegradable poly(L-lactic acid) (PLA) could be produced from tapioca starch, a low cost substrate, by *Streptococcus* sp. SUT513.

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