ภาวะที่เหมาะสมของการหมักกรดดีแล็กติกโดย *Sporolactobacillus kofuensis* SB7-2 เพื่อเพิ่มอัตราผลผลิต

นางสาวอรชร ทองดอนง้าว

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
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OPTIMIZATION OF D-LACTIC ACID FERMENTATION BY *Sporolactobacillus kofuensis* SB7-2 TO INCREASE PRODUCTIVITY

Miss Orachorn Thongdonngaw

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
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อรชร ทองดอนง้าว : ภาวะที่เหมาะสมของการหมักกรดดีแล็กติกโดย *Sporolactobacillus kofuensis* SB7-2 เพื่อเพิ่มอัตราผลผลิต (OPTIMIZATION OF D-LACTIC ACID FERMENTATION BY *Sporolactobacillus kofuensis* SB7-2 TO INCREASE PRODUCTIVITY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ณัฏฐา ทองจุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. สมบูรณ์ ธนาศุภวัฒน์, 92 หน้า.

กรดดีแลกติกในปัจจุบันมีความน่าสนใจทั้งในระดับงานวิจัย และอุตสาหกรรม ตั้งแต่มีการค้นพบคุณสมบัติของเสตอริโอคอมเพลก-พอลีแลกติกแอซิด แต่อย่างไรก็ตาม ทางการผลิตเชิงพาณิชย์ของผลผลิตกรดดีแล็กติกยังคงมีข้อจำกัด เมื่อเปรียบเทียบกับผลผลิตกของกรดแอลแล็กติกที่ง่ายต่อการนำมาใช้งาน ซึ่งได้มีการนำมาใช้กันอย่างแพร่หลายในวงการอุตสาหกรรมอาหาร และเภสัช ดังนั้นงานวิจัยนี้มุ่งเน้นงานวิจัย และพัฒนาเพื่อการค้าผลผลิตภาคอุตสาหกรรมสำหรับอนาคต งานวิจัยล่าสุดที่มีการศึกษาการหมักกรดดีแล็กติกโดยสายพันธุ์ *Sporolactobacillus* sp. เช่น *S. laevolacticus* และ *S. nakayamae* ซึ่งในปัจจุบันยังคงไม่มีการศึกษาการหมักกรดดีแล็กติกจาก *S. kofuensis* ไอโซเลท SB7-2 สามารถคัดแยกได้จากเปลือกของต้นไม้ จากจังหวัดสระบุรี ผลจากการพิสูจน์เอกลักษณ์พบว่าเป็น *Sporolactobacillus kofuensis* (99.86%) เมื่อศึกษาผลการผลิตกรดดีแล็กติกในระดับขวดเขย่าเบื้องต้น พบว่าสามารถผลิตกรดดีแล็กติกได้สูงถึง 112 กรัมต่อลิตร ผลผลิต 0.93 กรัมของกรดดีแล็กติกที่ได้ต่อกรัมของกลูโคสที่ใช้ไป และอัตราการผลิต 1.6 กรัมต่อลิตรต่อชั่วโมง จากกลูโคสความเข้มข้น 120 กรัมต่อลิตร ในภาวะไร้อากาศ ทำการหาสภาวะที่เหมาะสมในการเลี้ยงเชื้อ คือเวลาในการเลี้ยงหัวเชื้อเริ่มต้นจากอาหารวุ้น ปริมาณหัวเชื้อเริ่มต้นที่ใช้ ความเข้มข้นกลูโคสเริ่มต้น ระยะเวลาของหัวเชื้อ และผลของการเขย่าและอากาศที่มีต่อการเจริญและผลิตกรดดีแล็กติกในระดับขวดเขย่า นอกจากนี้หาภาวะที่เหมาะสมของระยะเวลาและปริมาณของหัวเชื้อที่เหมาะสม ในการผลิตกรดดีแล็กติกในระดับถังปฏิกรณ์ชีวภาพแบบกวน 5 ลิตร ผลจากการทดลองพบว่าเมื่อเปรียบเทียบผลการผลิตกรดดีแล็กติกจากผลการทดลองเบื้องต้นเทียบกับภาวะที่เหมาะสมของการหมักกรดดีแล็กติกของ *S. kofuensis* SB7-2 พบว่าให้อัตราการผลิต และผลผลิต มากกว่า ซึ่งภาวะที่เหมาะสมของการผลิต คือ เซลล์อายุ 24 ชั่วโมงในอาหารวุ้นแข็ง GYP หัวเชื้อปริมาณ 3% ในอาหาร GYP ที่มีกลูโคสความเข้มข้นเริ่มต้น 10 กรัมต่อลิตร ที่มีอัตราการเขย่าที่ 200 รอบต่อนาที ในภาวะไร้อากาศ นาน 6 ชั่วโมง หลังจากนั้นถ่ายหัวเชื้อ5% เข้าสู่กระบวนการหมัก ที่มีอัตราการกวนที่ 200 รอบต่อนาที, ใช้แคลเซียมคาร์บอเนตในการควบคุมพีเอช ผลผลิตสูงสุดของกรดดีแล็กติกคือ 98.95 กรัมต่อลิตร ผลผลิต 0.96 กรัม/กรัมกลูโคส อัตราการผลิต 3.97 กรัมต่อลิตรต่อชั่วโมง ระยะเวลาการหมัก 24 ชั่วโมง ซึ่งภาวะนี้ให้ค่าอัตราการผลิตมากกว่าผลการทดลองเบื้องต้นประมาณ 2.5 เท่า

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5672139423 : MAJOR BIOTECHNOLOGY
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ORACHORN THONGDONNGAW: OPTIMIZATION OF D-LACTIC ACID FERMENTATION BY *Sporolactobacillus kofuensis* SB7-2 TO INCREASE PRODUCTIVITY. ADVISOR: ASSOC. PROF. NUTTHA THONGCHUL, Ph.D., CO-ADVISOR: PROF. SOMBOON TANASUPAWAT, Ph.D., 92 pp.

D-lactic acid now gains many interest in both research and industry levels since the discovery of the superior properties of stereocomplex polylactic acid. Nonetheless, the commercial production of D-lactic acid is still limited compared to the existing facilities for L-lactate to be widely used in food and pharmaceutical industries. Thus, this leaves the room for future research and development for pursuing industrial production. Recent studies reported fermentation of D-lactic acid by *Sporolactobacillus* sp. including *S. laevolacticus* and *S. nakayamae* but to date there is no D-lactic acid fermentation study of *S. kofuensis* found in the literatures. The in-house isolate, SB7-2, screened from the tree bark collected from the natural habitat in Saraburi was tested for its ability to produce D-lactic acid. From bacterial identification, SB7-2 acquired the high similarity percentage to *S. kofuensis* (99.86 %). From the preliminary results, *S. kofuensis* SB7-2 could produce D-lactic acid at the final titer up to 112 g/L with the remarkable yield of 0.93 g/g and productivity of 1.6 g/L·h from simple medium containing 120 g/L glucose during anaerobic cultivation in the shaken flask. To develop the valid fermentation platform for this isolate, several key successful fermentation factors were optimized both in the shake flask and the stirred fermentor. Those inclued the age of slant, inoculum size, initial glucose concentration, preculture time, mixing, and oxygen. With the optimized conditions determined in this study, the highest D-lactic acid of 98.95 g/L with the yield 0.96 g/g and the productivity of 3.97 g/L·h was obtained within 24 h. This productivity was 2.5 times higher than that from the preliminary result in the shake flask culture.

Field of Study: Biotechnology Student's Signature .....

Academic Year: 2016 Advisor's Signature .....

Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS AND SYMBOLS

℃	Degree celsius
C_x	Concentration of biomass
C_p	Concentration of product
DCW	Dry cell weight
DO	Dissolved oxygen
et al.	el alia (and others)
g	Gram
g/g	Gram per gram
g/g·h	Gram per gram per hour
g/L	Gram per liter
g/L·h	Gram per liter per hour
h	Hour
h^{-1}	Per hour
K	Dissociation constant
kg	Kilogram
L	Liter
L.	*Lactobacillus*
LA	Lactic acid
LDH	Lactate dehydrogenase
μ	Specific growth rate
µL	Microliter
M	Molar
min	Minute
ml	Milliliter
mM	Millimolar

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mol	Mole
N	Normal
OD	Optical density
PDLA	Poly(D-lactic acid)
pK_a	Acid dissociation constant
PLA	Poly(lactic acid)
PLLA	Poly(L-lactic acid)
ppm	Part per million
psi	Pounds per square inch
%ee	Percentage of enantiomer excess
Q_s	Concentration rate of substrate
Q_p	Production rate of product
q_s	Specific rate of substrate concentration
q_p	Specific rate of production concentration
rpm	Round per minute
SD	Standard deviation
sp.	Species
subsp.	Subspecies
UV	Ultraviolet
vvm	Air volume per volume of medium per minute
v/v	Volume by volume
w/v	Weight by volume
$Y_{p/s}$	Product yield from substrate

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CHAPTER 1

INTRODUCTION

1.1 Significance of the study

Polylactic acid (PLA) has attracted important attention in recent years as an environmentally friendly biodegradable polymer for substituting the petrochemical-based polymers, such as polystyrene or polyethylene (Source: Mitrus, Wojtowicz, & Moscicki, 2010; Piringer & Baner, 2008; Webb, Arnott, Crawford, & Ivanova, 2013). During the first PLA products generation, PLA was produced from optically pure L-lactic acid. Nonetheless, the poly L-lactic acid (PLLA) has low thermal and mechanical properties compared with those commercially thermoplastic products. In order to improve the thermal and mechanical properties of PLA, the stereocomplex structure was discovered. The stereocomplex structure of PLA comprising both the optically pure L-lactic acid and D-lactic acid gives the superior thermal and mechanical properties to PDLLA products; thus, expanding to the wide range applications similarly to the petroleum based plastics (Németh, Kiss, & Sevella, 2011; Varman, Yu, You, & Tang, 2013; Vijayakumar, Aravindan, & Viruthagiric, 2008).

Owing to the stereocomplex structure of PLA, D-lactic acid becomes considerably important as well as L-lactic acid. Microbial fermentation has the advantage that an optically pure D-lactic acid by choosing a specific microbial strain. Previous studies reported a few wide-type D-lactic acid producers that were isolated from soil and plant samples included *Lactobacillus delbrueckii*, *L. coryniformis* subsp. *torquens*, *Bacillus laevolacticus, Corynebacterium glutamicum* and *Sporolactobacillus* sp. (Joshi, Singhvi, Khire, & Gokhale, 2010; Tashiro et al., 2011; Q. Wang, Ingram, & Shanmugam, 2011). However, among D-lactic acid producers, *Sporolactobacillus* sp. is a promising wide-type strain due to an acceptable high productivity and optical purity. To our knowledge, very high D-lactic acid titer of 207 g/L with the productivity of 3.8 g/L·h and the optical purity of 99.3 %ee was

achieved from fermentation by *Sporolactobacillus* sp. CASD using peanut meal as the nitrogen source in a 30 L fermentor via fed-batch operation (L. Wang et al., 2011). In particular *S. laevolacticus*, several researchers claimed that this species had high potential production of D-lactic acid. According to study of Mimitsuka et al. (2012), *S. laevolacticus* produced D-lactic acid at the final titer of 87 g/L with the yield of 0.89 g/g and the production rate of 2.9 g/L·h in batch operation (Mimitsuka et al., 2012). Later in 2013, Li et al. reported that fed-batch fermentation of D-lactic acid by *S. laevolacticus* DSM442 gave the high final titer up to 144.4 g/L in 35 h with the yield of 0.96 g/g and the productivity of 4.13 g/L·h. The obtained optical purity of the D-lactate product was 99.3%. Our in house isolate, *S. laevolacticus* SK5-2 produced D-lactic acid in a 5 L stirred fermentor from glucose medium via batch operation. The final titer of 115 g/L with the yield of 0.92 g/g, the productivity of 3.83 g/L·h, and the optical purity approaching 100 %ee was achieved (Jantawon, 2013).

Another in house isolate, *S. kofuensis* SB7-2, screened from the tree bark collected in Saraburi Province is another promising D-lactate producer. From the first screening in flask fermentation, *S. kofuensis* SB7-2 produced D-lactic acid at the high final titer up to 112 g/L with the productivity of 1.55 g/L·h and the optical purity of 95.8 %ee. The merit in cultivating *S. kofuensis* SB7-2 for D-lactic acid is that this isolate is considered thermotolerant as it can grow at 20-50 $^{\circ}$C. This supports low energy consumption in a large scale fermentation process. Nonetheless, the productivity and optical purity of *S. kofuensis* seemed a bit low. To date, D-lactic acid production by *S. kofuensis* has yet been reported. Therefore, process optimization to achieve the improved productivity and optical purity as well as to generate another D-lactate producer platform is necessary.

Several methods have been used to enhance D-lactic acid production. The operation modes, i.e. batch, fed-batch, and continuous were applied to improve the process performance (Abdel-Rahman, Tashiro, & Sonomoto, 2013). Moreover, the process parameters including medium, pH, temperature, mixing, oxygen, and neutralizing agents were reported to affect lactic acid production. Jantawon (2013) studied the effects of inoculum size and age as well as the neutralizing agents on D-

lactic acid fermentation by *S. nakayamae* subsp. *nakayamae* CU72-1. Within 54 h, the high final titer of 110 g/L with the yield of 0.91 g/g glucose, the productivity of 3.68 g/L·h, and the optical purity approaching 100 %ee was obtained when inoculating the fermentor at 10% inoculum size and 24 h inoculum age with controlled pH by $CaCO_3$. Furthermore, some studies noted that initial glucose concentration is one of important factors affecting the lactic acid production. Y. Li, Wang, Ju, Yu, and Ma (2013) reported that the concentration of D-lactate increased in proportion with initial glucose concentration, reaching a maximum of 76.9 g/L with 100 g/L of glucose within 48 h by *S. laevolacticus* DSM442 but D-lactate production and cell growth were inhibited at higher glucose concentration. This inhibition may be affected by high osmotic pressure created by high substrate and end-product concentration. Owing to high osmotic pressure would disturb the fluid and electrolyte balance system of cells (Sochocka & Boratynski, 2011). Efforts were made to improve the productivity by decreasing the osmotic inhibition such as; screened osmotic-tolerant strains and modified strains were used for lactic acid fermentation; Fermentation strategies such as simultaneous saccharification and fermentation (SSF) and fed-batch fermentation were studied to obtain the higher productivity by a low level of initial substrate concentration (Choi, Al-Zahrani, & Lee, 2014; Ge, Yuan, Qin, & Zhang, 2011; S. Zhou, Grabar, Shanmugam, & Ingram, 2006; Zou et al., 2013). In addition, previous studies have reported that betaine can promote lactic acid fermentation as an effective osmoprotectant. In 2014, Xu and Xu (2014) reported that betaine has the positive effect on fermentation by *B. coagulans*. They found that the fermentation with betaine could produce 17.9% more lactic acid compared to the fermentation without betaine by protecting L-LDH activity and cell growth from osmotic inhibition, especially under high glucose concentrations and with poor organic nitrogen nutrients. Likewise, S. Zhou et al. (2006) reported that betaine can improve the growth (rate and yield), D-lactate productivity and D-lactate tolerance in *Escherichia coli* B strain SZ132 during batch fermentation in mineral salts medium with 10% (w/v) sugar . To date, effect of betaine on D-lactic acid production by *S.*

kofuensis has yet been reported. Therefore, this knowledge are interesting to apply in D-lactic acid fermentation by *S. kofuensis* SB7-2.

The above researches indicated that the efficiency of lactic acid fermentation associated with several factors. Not only the organism itself that provides the sufficiently high D-lactic acid production, but cultivation conditions that effectively manipulate the bacteria for high production rate is mandatory. The aim of this study is to optimize the cultivation conditions to achieve high D-lactate productivity and optical purity by *S. kofuensis* SB7-2.

1.2 Objective and scope of research

In this work, factors affecting the seed culture and fermentation process were investigated to achieve improved productivity and optical purity of D-lactic acid in 5 L stirred fermentor from glucose medium via batch operation. The effects of key controlling parameters including, mixing and oxygen, inoculum size, inoculum age and initial glucose concentration in preculture and fermentation process were elucidated. Moreover, the effect of betaine hydrochloride on D-lactic acid production by *S. kofuensis* was investigated. It believed that our study may have more general implications for industrial D-lactic acid fermentation process.

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CHAPTER 2

LITERATURE REVIEW

2.1 Lactic acid

Lactic acid, or 2-Hydroxy-propanoic acid, is an organic compound with the formula $C_3H_6O_3$ and a molecular weight of 90.08. It has two isomers – D-lactic acid and L-lactic acid. They are different in terms of the consequences of the hydroxyl group in the structure of chiral carbon as shown in Figure 2.1. The three types of lactic acid are L-lactic acid, D-lactic acid and DL-lactic acid. The boiling point of L-lactic acid is 82 – 85 $^{\circ}$C and its melting point 16.8 – 33 $^{\circ}$C while the boiling point of D-lactic acid is 103 $^{\circ}$C and its melting point 52.8 – 53.6 $^{\circ}$C. Lactic acid is miscible in water, ethanol and acetone but does not dissolve in chloroform, petroleum ether and carbon disulfide. The properties of lactic acid are shown in Table 2.1 (Castillo Martinez et al., 2013).

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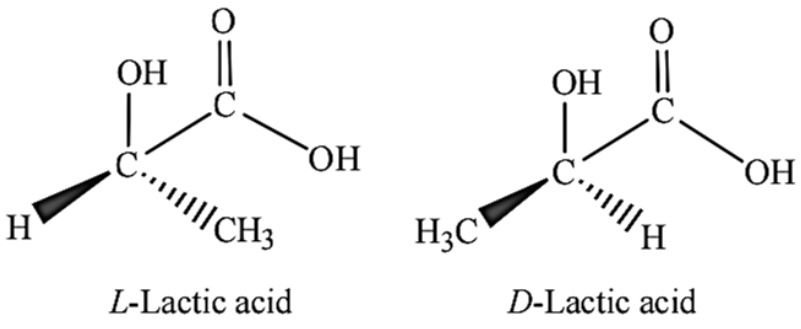


Figure 2.1 Structure of Lactic acid

L-lactic acid is common in nature – in fermented food, human and animal bodies – whereas D-lactic acid is found least in living organisms because a large

amount of D-lactic acid can adversely affect the metabolism of humans. L-lactic acid is the only form produced in human metabolism. The World Health Organization states that consumption of D-lactic acid should not exceed 100 milligrams per 1 kilogram of body weight per day (Fukushima, Sogo, Miura, & Kimura, 2004; Narayanan, Roychoudhury, & Srivastava, 2004).

Table 2.1 Physical and Chemical Properties of Lactic Acid

Chemical Abstracts Registration	CAS.-Reg.-No [79-33-4]
Chemical formula	$C_3H_6O_3$
Chemical name	2-Hydroxy-propanoic acid
Molecular weight	90.08
Physical characteristics	Aqueous solution
Taste	Mildly acidic
Melting point	53 °C / 127 °F
Boiling point	122 °C at 14 mmHg
Flash point	112 °C
Solubility in water (g/100 g H_2O)	Miscible
Acidity (pK_a)	3.86
pH (0.1% solution, 25°C)	2.90
pH (0.1 N solution, 25°C)	2.40
Heat of combustion, $\triangle H_c$	1361 KJ/mole
Specific Heat, C_p at 20°C	190 J/mole/°C
Specific gravity	1.2
Physical state	Colorless to slightly yellow syrupy liquid
Toxicity	Oral rat LD 50:3543 mg/kg
NFPA ratings	Health 3 Flammability 1 Reactivity 1

(Source: Adapted from Narayanan et al., 2004)

2.2 Benefits of lactic acid

This acid has versatile applications because it can be used in the food industry, pharmaceutical industry, plastics industry, solvent industry and cosmetics industry and its demand is increasing every year. Table 2.2 displays the potential applications of lactic acid.

Table 2.2 The commercial uses and application of lactic acid and its salt

Food industry	**Pharmaceutical industry**	**Cosmetic industry**	**Chemical industry**	**Chemical feedstock**
Acidulants	Dialysis solution	Moisturizers	Descaling agents	Propylene oxide
Preservatives	Mineral preparation	Skin-lightening agents	pH regulators	Acetaldehyde
Flavours	Tablettings		Neutralizers	Acrylic acid
pH regulators	Prostheses	Skin-rejuvenating-agents	Chiral intermediates	Propanoic acid
Improving microbial quality	Surgical sutures	pH regulators	Green solvents	-2,3-pentanedione
Mineral fortification	Controlled drug delivery systems	Anti-acne agents	Cleaning agents	Ethyl lactate
		Humectants	Slow acid release agents	Dilactide
		Anti-tartar gents	Metal complexing agents	Poly(lactic acid)

(Source: Wee et al., 2006)

2.2.1 Food industry

Lactic acid plays an important part in improving the acidic taste, preventing the spoilage of food and beverages such as salad, fermented vegetables and beer, and balancing the acidity in food because of its mid acidity. In addition, it is used as a preservative and it has a probiotic property so it can boost immunity. Food products containing lactic acid are shown in Table 2.3.

Table 2.3 Fermented foods and beverages and their associated lactic acid bacteria

Types of fermented products	Lactic acid bacteria
Dairy products	
Hard cheeses without eyes	*L. lactis subsp. lactis, L. lactis subsp. cremoris*
Cheeses with small eyes	*L. lactis subsp. cremoris, Leuc. mesenteroides subsp. cremoris*
Yoghurt	*L. delbrueckii subsp. bulgaricus, S. thermophiles*
Fermented, probiotic milk	*L. casei, L. acidophilus, L. rhamnosus, L. johnsonii, B. lactis, B. bifidum*
Kefir	*Lb. kefir, Lb. kefiranofacies, Lb. brevis*
Fermented meats	
Fermented sausage (Europe)	*Lb. sakei, Lb. curvatus*
Fermented sausage (USA)	*P. acidilactici, P. pentosaceus*
Fermented fish products	*L. alimentarius, C. piscicola*
Fermented vegetables	
Sauerkraut	*Leuc. Mesenteroides, L. plantarum, P. acidilactici*
Pickles	*Leuc. Mesenteroides, P. cerevisiae, Lb. brevis, Lb. plantarum*
Fermented soy sauce	*T. halophilus*
Fermented cereals	
Sourdough	*L. sanfransiscensis, L. farciminis, L. fermentum, L. brevis, L. plantarum, L. amylovorus, L. reuteri, L. pontis, L. panis, L. alimentarius, W. cibaria*
Alcholic beverages	
Wine (malolactic fermentation)	*O. oeni*
Rice wine	*L. sakei*

B: Bifidobacterium, C: Carnobacterium, L: Lactococcus, Lb: Lactobacillus, Leuc: Leuconostoc, O: Oenococcus, P: Pediococcus, S: Streptococcus, T: Tetragenococcus, W: Weissella

(Source: Adapted from Ali, 2010)

2.2.2 Pharmaceutical Industry

This includes drug preparation, drug delivery, and pH solution control. Currently, the acid is also made into implant materials such as catgut suture, orthosis and prosthesis, wound dressing, suture needle and bone tractor because polylactic acid is biodegradable and can fuse with tissue. It has been used in this area for more than two decades. It can also be used as a drug release material since it can effectively control the release of a drug.

2.2.3 Cosmetic industry

It is used to control the pH balance and since it can retain skin moisture, it is used as a component of skin cream and moisturizer in cosmetics. It, moreover, can inhibit the function of tyrosinase enzyme, which produces melanin skin pigment so the skin can stay fair. Combined with Ethyl lactate, it can be used as a base for acne-reducing medication (Bustos, Moldes, Alonso, & Vázquez, 2004; Chang, Jung, Park, & Oh, 2008; Fukushima et al., 2004; Wee, Kim, & Ryu, 2006).

2.2.4 Chemical industry

Lactic acid and its salt are used increasingly in a variety of chemical products and processes. It functions as a pH regulator, descaling agent, neutralizer, chiral intermediate, solvent, cleaning agent, antimicrobial agent, slow acid-release agent, metal complexing agent, and humectant. Currently, lactic acid is considered the most potential feedstock monomer for chemical conversions, because there are two reactive functional groups; a hydroxyl group and a carboxylic group. Lactic acid can undergo a variety of chemical conversions into potentially useful chemicals, such as propylene oxide, acetaldehyde, acrylic acid, propanoic acid, 2,3-pentanedione, and dilactide (Wee et al., 2006).

2.2.5 Plastic industry

It is used as a base substance called polylactic acid for producing bioplastics to replace plastics synthesized from petrochemicals. In order to produce bioplastics, the base substance has to be very pure with high temperature resistance. It does not dissolve in acid solutions and its color does not change at a temperature over 180 $^{\circ}$C. Bioplastics can be made into films, tree pots, bags, document files, food trays and candy coating. Polymerization can yield lactide with 99% purity.

Recently, PLA is one of the most promising biodegradable plastics to replace conventional polymer made from petrochemical feedstock. Pure L-lactic acid or D-lactic acid, or mixtures of both components are needed for the synthesis of PLA. The homopolymer of lactic acid is a white powder at room temperature with glass transition temperature (T_g) and melting temperature (T_m) values of about 55°C and 175°C, respectively. The High molecular weight PLA resulting in a colorless, glossy, rigid thermoplastic material with properties similar to polystyrene (Xiao, Wang, Yang, & Gauthier, 2012).

The synthesis of PLA can follow three main routes (Figure 2.2). The first route is direct polycondensation involves the removal of water by condensation and the use of solvent under high vacuum and temperature, results in low molecular weight (1,000-5,000 Daltons), brittle polymer, which, for the most part, is unusable, unless external coupling agents are employed to increase its chains length. The second route is azeotropic dehydrative condensation of lactic acid. It can be used to obtain high molecular weight PLA (M_w > 100,000 Daltons). The third and main process is ring opening polymerization to obtain high-molecular-weight PLA (average Mw > 100,000 Daltons). In the first step, the water is removed in under mild conditions the use of a solvent) to produce a low moleculer weight prepolymer. The second step, the prepolymer is catalytically converted to the cyclic dimer and then purified by distillation under vacuum (Avérous, 2008; Vink, Rábago, Glassner, & Gruber, 2003). Figure 2.3 presents the type of polylactides from lactide isomers: poly(L-lactide) (PLLA), which is hemicrystalline, and likewise with a regular chain structure; poly(D-lactide) (PDLA), a crystalline material with a regular chain structure; meso-PLA,

obtained by the polymerization of meso-lactide and a mixture of L-and D-lactides (PDLLA), called racemic lactide (rac-lactide) which is amorphous (Vink et al., 2003; Xiao et al., 2012). The changes of D-isomer content as a result impact the melt behavior, thermal, mechanical, optical properties, barrier properties and biological properties of PLA (Murariu & Dubois, 2016; Tizazu, Paolo, Hamdy, & David, 2013). Some of the PLA properties are summarized in Table 2.4.

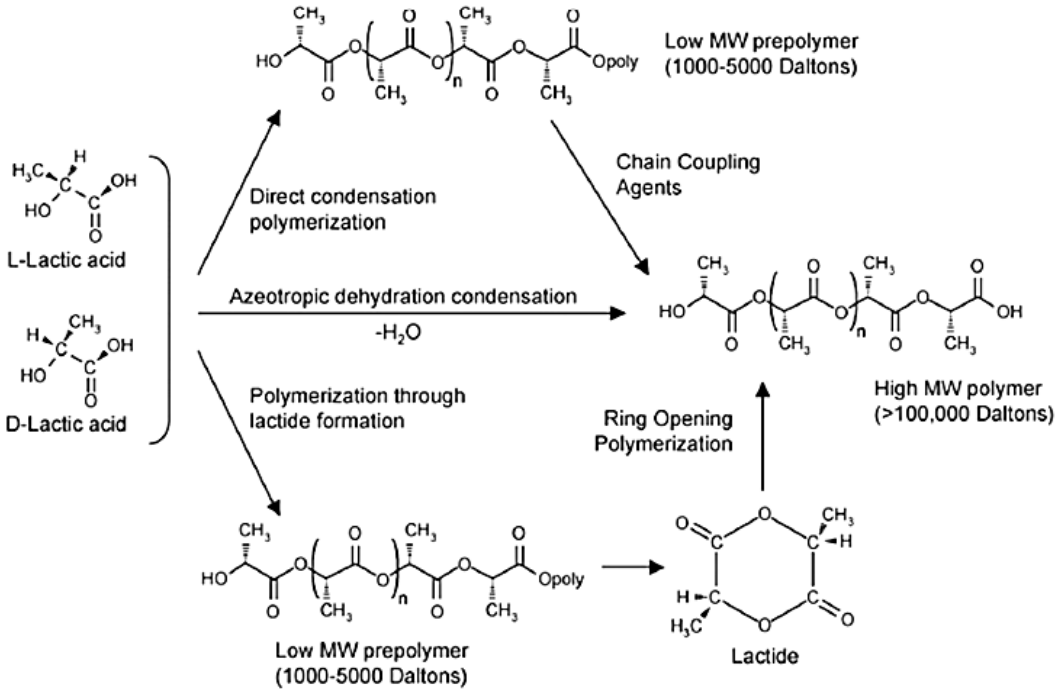


Figure 2.2 Polymerization routes to produce PLA (Murariu & Dubois, 2016)

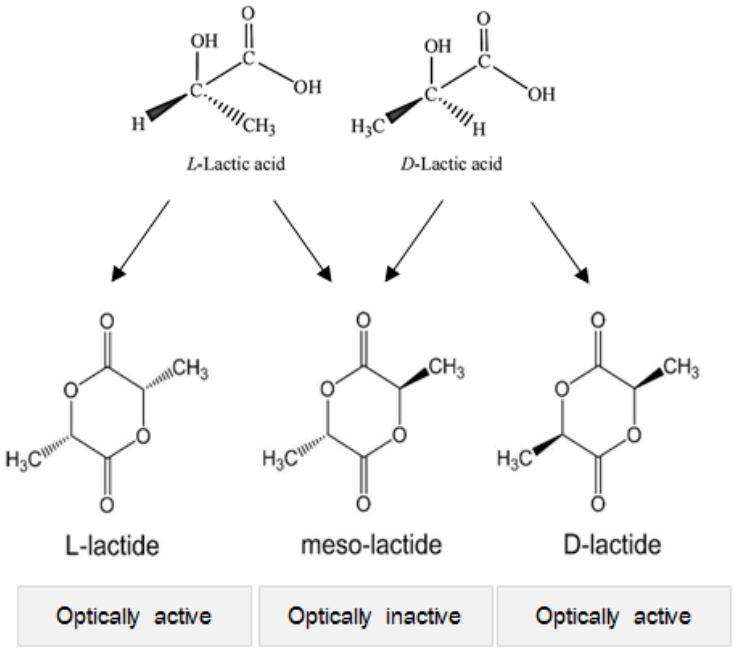


Figure 2.3 Chemical structures of cyclic dimer of lactic acid (Murariu & Dubois, 2016)

Table 2.4 The physical and chemical properties of PLA

Properties	PDLA	PLLA	PDLLA
Crystalline structure	Crystalline	Hemicrystalline	Amorphous
Melting temperature (T_m) °C	~180	~180	variable
Class transition temperature (T_g) °C	50-60	55-60	variable
Decomposition temperature °C	~200	~200	185-200
Elongation at break (%)	20-30	20-30	variable
Breaking strength (g/d)	4.0-5.0	5.0-6.0	variable
Half-life in 37 °C normal saline	4-6 months	4-6 months	2-3 months
Solubility	All are soluble in benzene, chloroform, acetonitrile, tetragydrofuran (THF), dioxane etc., but insoluble in ethanol, methanol, and aliphatic hydrocarbons		

(Source: Xiao et al., 2012)

Recently, it was found that blending PLLA with PDLA or the coexistence of PLLA or PDLA segments in a molecule as in stereo block copolymers result in stereocomplex or racemic crystallite formation (Figure 2.4) due to the strong interaction between L-lactic acid segments and D-lactic acid segments. The stereocomplex crystallites have a melting temperature (T_m) of 220–230 °C, which is 50 °C higher than that of PLLA or PDLA (170–180 °C). In addition, the strong interaction between L-lactic acid segments and D-lactic acid segments gives the superior mechanical properties, hydrolytic/thermal degradation-resistance, and gas barrier properties. Thus, expanding to the wide range applications similarly to the petroleum based plastics (Jamshidian, Tehrany, Imran, Jacquot, & Desobry, 2010; Masutani & Kimura, 2014; Singhvi & Gokhale, 2013; Tsuji, 2016).

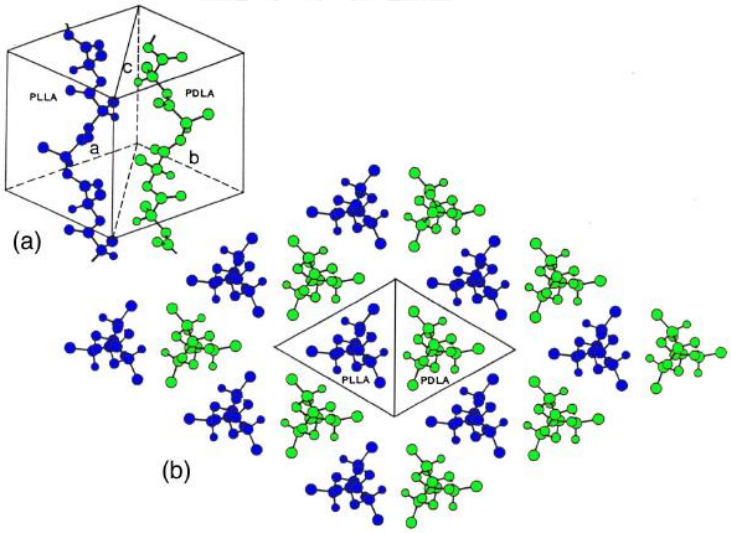


Figure 2.4 Crystal structure of stereocomplex of polylactic acid (Tsuji, 2016)

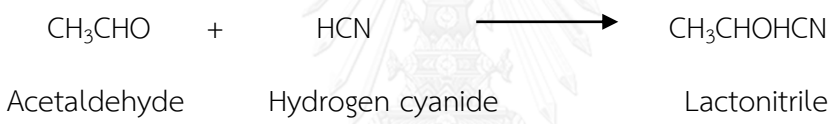
2.3 Process of synthesizing lactic acid

Lactic acid can be synthesized in two ways: the chemical process and the fermentation of micro-organism process.

2.3.1 Chemical process

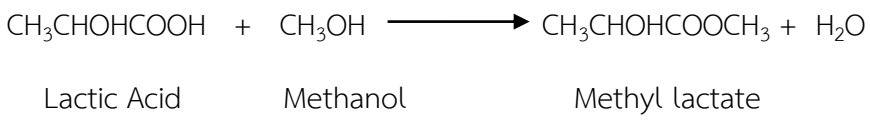
The chemical reaction for synthesizing lactic acid is based on lactonitrile. Hydrogen cyanide is added to the acetaldehyde in liquid phase at high atmospheric pressures to produce lactonitrile. Subsequently, the crude lactonitrile is recovered and purified by using a distillation technique. The purified lactonitrile is hydrolyzed with strong acid, i.e. hydrochloric acid (HCl) or sulfuric acid (H_2SO_4) to produce the resultant ammonium salt and lactic acid. Then lactic acid is esterified with methanol to produce methyl lactate; later the methyl lactate is removed and purified by distillation and hydrolyzed by water under acid catalyst to produce lactic acid and the methanol. The chemical process involves the following four steps:

Step 1: Acetaldehyde is combined with hydrogen cyanide. Lactonitrile is obtained.

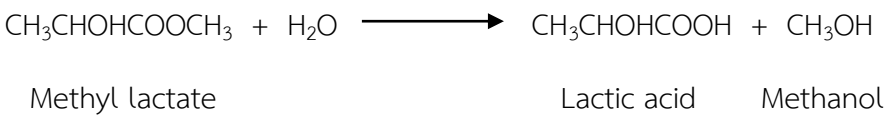


Step 2: Lactonitrile is combined with water and sulphuric acid. Lactic acid and ammonium salt are obtained. This process is called hydrolysis.

Step 3: Lactic acid is combined with methanol. Methyl lactate and water are obtained. This process is called esterification.



Step 4: Mehtyl lactate is combined with water. Lactic acid and methanol are obtained. This process is called hydrolysis.



The disadvantages of this chemical process are that the starter substance is expensive and is not environmentally friendly because it is a product of a petrochemical substance. Moreover, the end product, the lactic acid, is in the form of a racemic mixture of DL-lactic acid (Calabia & Tokiwa, 2007; Wee et al., 2006). In fact, lactic acid can be chemically produced by other processes such as sugar decomposition activated by alkaline, oxidation of propylene glycol, hydrolysis of acetaldehyde and carbon dioxide under pressure and hydrolysis of chloropropionic acid (John, G.S, Nampoothiri, & Pandey, 2009).

2.3.2 Fermentation by microbial

The microbial belong to the groups of fungi and bacteria.

2.3.2.1 Fermentation by fungi

The filamentous fungi as *Rhizopus oryzae* and *R. arrhizus* can yield pure L-lactic acid by hydrolyzing sugar and starch that are cheap raw materials. Filamentous fungi have amylolytic enzyme activity to convert agricultural residues as carbon sources to produce optical pure L-lactic acid. Furthermore, it can grow well and produce lactic acid in only a simple medium, leading to a simple and inexpensive downstream process. The best-known fungal source as a lactic acid producer is *Rhizopus oryzae*, *R. oryzae* NRRL 395 has been recognized as one of the most suitable fungi for lactic acid fermentation (Ruengruglikit & Hang, 2003). But, it also requires a lot of air in order to grow and produce lactic acid because *R. oryzae* is an obligate aerobe as a result of high production cost (John et al., 2009). The fungal

fermentation is attributed partially to the formation of by-products, such as fumaric acid, ethanol and carbon dioxide. In addition, the mycelia are not suitable enough for lactic acid as their morphology does not suit for fermentation because they increase the viscosity of the medium and cause in overflow lines as a result of low product yield due to the low reaction rate caused by mass transfer limitation (Jin, Yin, Ma, & Zhao, 2005; Jantawon, 2013; Narayanan et al., 2004; Wee et al., 2006).

2.3.2.2 Fermentation by bacteria

Bacteria for this purpose have to be more tolerant of low oxygen and low pH environments. The installment of its fermentation tank is not as complicated as that of fungi because bacteria are not fibrous and do not need oxygen for lactic acid fermentation; as a result, the installment of the tank costs less than that of the fungi. Fermentation using bacteria and downstream process involve the following four steps:

<u>Step 1</u>: Fermentation and neutralization

$$C_6H_{12}O_6 + Ca(OH)_2 \longrightarrow (2CH_3CHOHCOO^-)Ca^{2+} + 2H_2O$$

Carbohydrate Calcium hydroxide Calcium lactate

<u>Step 2</u>: Hydrolysis by H_2SO_4

$$(2CH_3CHOHCOO^-)Ca^{2+} + H_2SO_4 \longrightarrow 2CH_3CHOHCOOH + CaSO_4$$

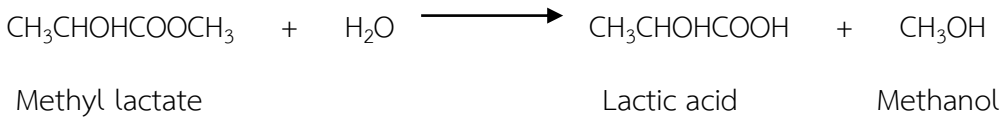
Calcium lactate Sulphuric acid Lactic acid Calcium sulphate

<u>Step 3</u>: Esterification

$$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O$$

Lactic acid Methanol Methyl lactate

Step 4: Hydrolysis by H_2O



Currently, approximate 90% of pure lactic acid can be synthesized by microbial fermentation of the following carbohydrates such as glucose, sucrose, lactose, and starch/maltose derived from feed-stocks such as beet sugar, molasses, whey, and barley malt. They can reduce the production cost in addition to using energy at a lower burning point. They can also produce purer L-lactic acid and D-lactic acid (John et al., 2009; Z. Li, Han, Ji, Wang, & Tan, 2010). However, most of the bacteria used to produce lactic acid cannot digest starch because they do not have such enzymes while fungi do. Certain bacteria such as *Lactobacillus fermentum, L. amylophilus, L. plantarum* and *L. amylovorus* possess α-amylases, enzyme that can convert starch into sugar for producing lactic acid.

Widely used method for the production of lactic acid is Batch fermentation. Conditions for fermentation are different for each industrial method. In fermentation process, batch culture is first grown in inoculums vessels than transferred to the fermentor. The fermentation is usually controlled at 35-45 $^{\circ}$C. During the growth and fermentation, bacteria continue to produce large amounts of lactic acid which decrease the pH value. The change of pH value is an important factor because cell growth requires appropriate pH condition that reflects metabolic activity of bacteria. Thus, pH is controlled at 5-6.5 by adding a suitable base, such as ammonium hydroxide, or calcium carbonate (Yen, Chen, Pan, & Wu, 2010). Other fermentations for lactic acid production are fed-batch, repeated batch and continuous batch. But the higher lactic acid concentration has obtained in batch and fed-batch fermentation than in others, whereas higher productivity has achieved by continuous fermentation (Ghaffar et al., 2014). To obtain lactic acid of the required purity, efficient downstream processing is necessary. The conventional fermentation process

produces calcium lactate precipitate which has to be acidified with a strong acid to get lactic acid and calcium sulphate. The insoluble calcium sulphate is removed by filtration. Then, the optical pure lactic acid is obtained by several techniques to reduce impurities in the final product, such as extraction, membrane separation, ion exchange, electrodialysis or distillation with chemical reaction (Castillo Martinez et al., 2013).

2.4 Lactic acid bacteria

Lactic acid-producing bacteria include wild-type and modified strains. These organisms can be divided into 4 main producers, namely, lactic acid bacteria, *Bacillus strains*, *Escherichia coli*, and *Corynebacterium glutamicum* (Abdel-Rahman, Tashiro, & Sonomoto, 2013). However, lactic acid bacteria are widely used as a producer for lactic acid fermentation in comparison with other bacteria.

Wild type of lactic acid bacteria are defined as Gram-positive, non-spore forming cocci, coccobacilli or rods with a DNA base composition of less than 53 mol% G+C. They generally are non-respiratory and lack catalase. They ferment glucose primarily to lactic acid, or to lactic acid, CO_2 and ethanol. They do not use oxygen for their energy production, but they are also able to grow in the presence of oxygen (aero tolerant anaerobe). Although they lack catalase, they possess superoxide dismutase and have alternative means to detoxify byproduct of oxygen (e.g. H_2O_2), generally through peroxidase enzymes. Lactic acid bacteria can grow at temperatures as low as 5 $^{\circ}$C or as high as 45 $^{\circ}$C and at pH 4.0-4.5, some also obtain at pH 3.2 or 9.6. Commonly, Lactic acid bacteria require complex growth factors, such as nitrogen, vitamins, and minerals for growth and lactic acid production (Y. Wang, Tashiro, & Sonomoto, 2015). Most are free-living or live in beneficial or harmless associations with animals, although some are opportunistic pathogens. They are found from various sources, such as milk and milk products, fermented food and in decaying plant materials. They are normal flora of humans in the oral cavity, the intestinal tract and the vagina, where they play a beneficial role. The term lactic acid

bacteria are conventionally reserved for genera *Lactobacillus, Carnobacterium, Streptococcus, Lactococcus, Pediococcus, Tetragenococcus, Aerococcus, Enterococcus, Vagococcus, Leuconostoc, Weisella and Oenococcus* (Y. Wang et al., 2015). The physiological characteristics of Lactic acid bacteria are compiled in Table 2.5.

2.4.1 *Lactobacillus*

Lactobacillus is the largest group of lactic acid bacteria, Gram-positive, non-spore forming rods, catalase-negative, usually non-motile, and occasionally nitrate reducers. The Bergey's Manual of Systematic Bacteriology, numerous *Lactobacillus* species were listed, and the nomenclature reorganized into three groups: group I (obligate homo-fermentative species), group II (facultative hetero-fermentative species) and group III (obligate hetero-fermentative species). This division suited the interests of food microbiologists; several species in groups I and II, and also some species in group III are used in fermented foods, although group III species are actually most commonly associated with food spoilage. In addition, *Lactobacillus* is considered to be used for commercial lactic acid production because of their safe strain and high acid tolerance (pH < 5), which confers them a competitive advantage over other Lactic acid bacteria (Castillo Martinez et al., 2013). Presently, 154 species from the genus *Lactobacillus* have been validly published isolated from different sources. Almost a third of those were isolated from human and animal intestinal tracts and faeces (Figure 2.5) (Hammes & Hertel, 2006; Liu et al., 2014).

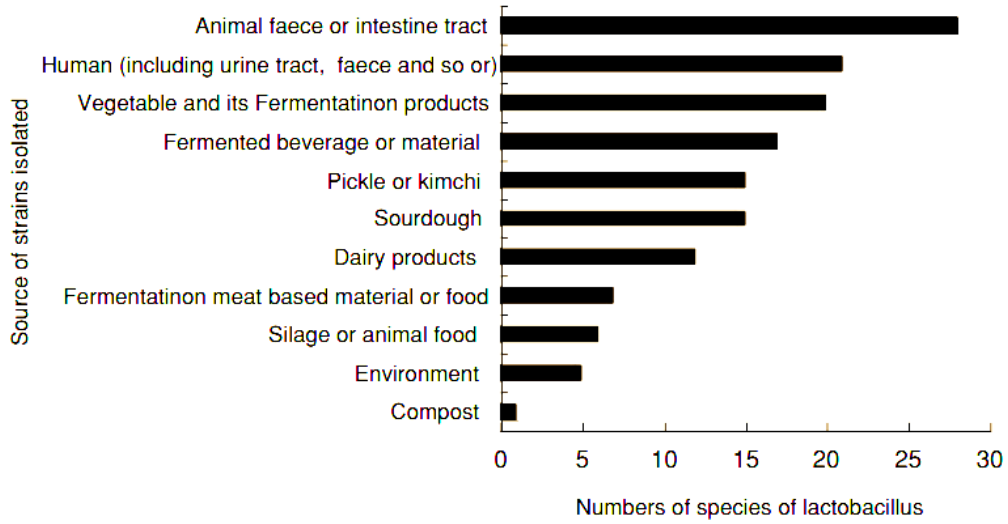


Figure 2.5 Source of the type specimens of *Lactobacillus* species

2.4.2 *Carnobacterium*

The genus *Carnobacterium* is created to accommodate heterofermentative, facultatively anaerobic, psychrotolerant, rod-shaped lactic acid bacteria that produce L-lactic acid from glucose (Snauwaert et al., 2013).

2.4.3 *Streptococcus*

The genus *Streptococcus* is Gram-positive bacteria in the shape of cocci. Individual bacteria are between 0.5 and 1.25 micrometers in diameter. Streptococci are strictly lactic acid homofermentative when glucose is utilized under anaerobic conditions (Smith & Sherman, 1942).

2.4.4 *Lactococcus*

The genus *Lactococcus* is Gram-positive, catalase-negative, non-motile cocci that are found singly, in pairs, or in chains. The lactococci comprise the species *Lactococcus lactis, L. garviae, L. plantarum, L. piscium* and *L. raffinolactis*. They have a homo-fermentative metabolism and produce exclusively L-lactic acid,

although some studies report that, D-lactic acid can be produced at low pH (Cock & Stouvenel, 2006).

2.4.5 *Pediococcus*

The genus *Pediococcus* is Gram-positive, catalase-negative, facultatively aerobic and homofermentative cocci which and produce lactic acid as major end product of glucose fermentation either to DL-lactic acid, or in the case of *Pediococcus claussenii*, to L-lactic acid. The cells are uniformly spherical and never ovoid or elongated, and they differ from all other lactic acid bacteria by alternate division in two perpendicular directions, resulting in tetrad formation (Franz et al., 2014).

2.4.6 *Tetragenococcus*

According to Bergey's Manual of Systematic Bacteriology, tetrad cocci which are tolerant of 18% NaCl are members of *Pediococcus halophilus*. Because *P. halophilus* is phylogenetically more closely related to enterococci and lactobacilli than to pediococci on the basis of 16s rRNA sequence data, thus *P. halophilus* should be reclassified in a new genus, the genus *Tetragenococcus* (Satomi, Kimura, Mizoi, Sato, & Fujii, 1997).

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2.4.7 *Aerococcus*

The genus *Aerococcus* was created to accommodate a group of Gram-positive, miroaerophilic, catalase-negative (although some display weak nonheme pseudocatalase activity); these coccoid organisms differed from *Streptococcus* species primarily by their characteristic tetrad cellular arrangement. *Aerococcus* species have been found from air, dust, vegetation and from the indigenous microbiota of human and animals and may be the causative agent in some infections (Lawson, 2014a).

2.4.8 *Enterococcus*

The genus Enterococci are Gram-positive diplococcic of intestinal origin, naturally associated with the gastrointestinal tract of humans and animals, however they may be isolated from food, especially from meat, dairy and plant food fermentations, as well as from the environment (Švec & Franz, 2014).

2.4.9 *Vagococcus*

Species of this genus are recovered from a wide range of habitats associated with animal, human and environmental sources. Vagococci are phylogenetically members of the *Enterococcaceae* sharing a close relationship with *Enterococcus, Melisococcus* and *Tetragenococcus* (Lawson, 2014b).

2.4.10 *Leuconostoc*

Gram-positive, coccoid to ovoid-like morphology, catalase negative, arginine negative, gas production from glucose, and production of primarily the D(–)-lactate isomer (Björkroth & Holzapfel, 2006).

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2.4.11 *Weisella*

The morphology of weissellas varies from spherical or lenticular cells to irregular rods. *W. hellenica* and *W. paramesenteroides*, produce the D(–)-lactate isomer during the fermentation of glucose, in contrast to the other *Weissella* species, which produce DL-lactate (Björkroth & Holzapfel, 2006).

2.4.12 *Oenococcus*

Oenococcus oeni is only one strain of the genus *Oenococcus*, acidophilic, growing at an initial pH of 4.8. Its habitat is wine and related habitats; therefore it tolerates ethanol and grows in media containing 10% ethanol (Björkroth & Holzapfel, 2006).

Table 2.5 Physiological characteristics of Lactic acid bacteria

Family	**Genera**	**Characteristics**								
		Shape	CO_2 from glucose	Growth at 10 C	Growth at 45 C	Growth in 6.5% NaCl	Growth in 18% NaCl	Growth at pH 4.4	Growth at pH 9.6	Type of lactic acid
Aerococcaceae	*Aerococcus*	Cocci (tetrads)	-	+	–	+	-	-	+	L
Carnobacteriaceae	*Carnobacterium*	Rods	-	+	-	ND	-	ND	-	L
Enterococcaceae	*Enterococcus*	Cocci	-	+	+	+	-	+	-	L
	Tetrageonococcus	Cocci (tetrads)		+	-	+	+	Variable	+	L
	Vagococcus	Cocci		+	-	-	-	ND	-	ND
	Pediococcus	Cocci (tetrads)	-	Variable	Variable	Variable	-	+	-	D, L, DL
Lactobacillaceae	*Lactobacillus*	Rods	Variable	Variable	Variable	Variable	-	ND	ND	D, L, DL
Leuconostocaecae	*Leuconostoc*	Cocci	+	+	-	Variable	-	Variable	-	D
	Oenococcus	Cocci	+	+	-	Variable	-	Variable	–	D
	Weissella	Cocci[a]	+	+	-	Variable	-	Variable	-	D, DL
Streptococcaceae	*Lactococcus*[b]	Cocci	-	+	-	-	-	Variable	-	L
	Streptococcus	Cocci	-	-	Variable	-	-	-	-	L

Note ND Not Determined

[a]Some *Weissella* strains are rod shaped

[b]In the old literature, *Lactococcus* species are referred to as Group *N Streptococci* (Björkroth & Holzapfel, 2006; Liu, Pang, Zhang, & Cai, 2014)

2.5 Metabolic pathways of lactic acid bacteria

Lactic acid ferment sugars such as hexose and pentose via different metabolic pathways that lead to homofermentation or heterofermentation. Lactic acid bacteria are grouped as either homo-fermentative or hetero-fermentative based on the fermentation end product as presented in Table 2.6.

2.5.1 Homofermentation

Homofermentation produces almost only lactic acid as the end product from hexose via the Embden-Meyerhof-Parnas pathway (EMP) pathway. The first steps of glycolysis are the phosphorylation of glucose to fructose 1,6-diphosphate (FDP) and its subsequent cleavage into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). The GAP is then converted to pyruvate via a route that includes 2 substrate-level phosphorylation steps. Finally, pyruvate is reduced to lactic acid by L-lactate dehydrogenase (L-LDH) or D-lactate dehydrogenase (D-LDH) with the oxidation of NADH to NAD^+ for the redox balancing. These bacteria can use glucose as a source of carbon but cannot use pentose or gluconate as a source of carbon. The principal by-product is lactic acid. One mole of glucose can produce two moles of lactic acid and two moles of energy in the form of ATP. With the glycolysis pathway, as much as 85 – 95% of lactic acid can be obtained as shown in Figure 2.6. Microorganisms that use only this route for the consumption of carbohydrates, include some species of *Lactobacillus* such as, *L. acidophilus, L. amylophilus, L. bulgaricus, L. helveticus* and *L. salivarius*, and most species of *Enterococcus, Lactococcus, Pediococcus* and *Streptococcus* (Abdel-Rahman et al., 2013; Castillo Martinez et al., 2013).

Recently, some studies report that some strains of lactic acid bacteria can metabolize pentose to lactic acid homofermentatively such as, *Enterococcus mundtii* QU 25 (Shiwa et al., 2014), engineered *Lactobacillus plantarum* (Okano et al., 2009) and *Lactococcus lactis* IO-1. According to research of Oshiro et al. (2009), they found that *L. lactis* IO-1 possesses not only metabolized xylose via the phosphoketolase

(PK) pathway but also via pentose phosphate (PP)/glycolic pathway. In this pathway, 3 mol of xylulose 5-phosphate (xylulose 5-P; 5 carbons), generated by the phosphorylation of pentose sugars such as xylose and arabinose, is converted to 5 mol of GAP (3 carbons) via 2 key enzymes: transketolase and transaldolase. The resulting GAP is converted to pyruvate and then to lactic acid (3 carbons) as the final product as displayed in Figure 2.6, thereby providing a theoretical yield of lactic acid from pentose of 1.0 g/g (1.67 mol/mol) (Oshiro et al., 2009; Tanaka et al., 2002).

2.5.2 Heterofermentation

These bacteria change one mole of glucose into one mole of lactic acid, one mole of ethanol, one mole of carbon dioxide and one mole of energy in the form of ATP; consequently, the growth rate of these bacteria is lower than that of the homofermentative bacteria. With the phosphoketolase pathway, as much as 50% of lactic acid is obtained (Reddy, Altaf, Naveena, Venkateshwar, & Kumar, 2008). Initially, glucose 6-phosphate (6 carbons) is converted to ribulose 5-phosphate (5 carbons) and carbon dioxide (1 carbon) by several enzymes. Then, pentose is cleaved to GAP and acetyl phosphate (acetyl-P) by phosphoketolase. The acetyl-P is reduced to ethanol (2 carbons) via acetyl-CoA and acetaldehyde intermediates, or converted to acetate via acetate kinase, while GAP further enters to EMP pathway to form lactic acid (3 carbons) as shown in Figure 2.6. Therefore, the theoretical yield of lactic acid from glucose reaches only 0.5 g/g (1.0 mol/mol). In addition, the relationship between the amounts of acetic acid and ethanol as a result of reducing the theoretical yield to 0.50 g/g is dependent on the redox potential in the cells. Some strains that use this metabolic pathway for the consumption of hexose are *Lactobacillus brevis, L. fermentum, L. parabuchneri* and *L. reuteri* (Abdel-Rahman et al., 2013; Y. Wang et al., 2015).

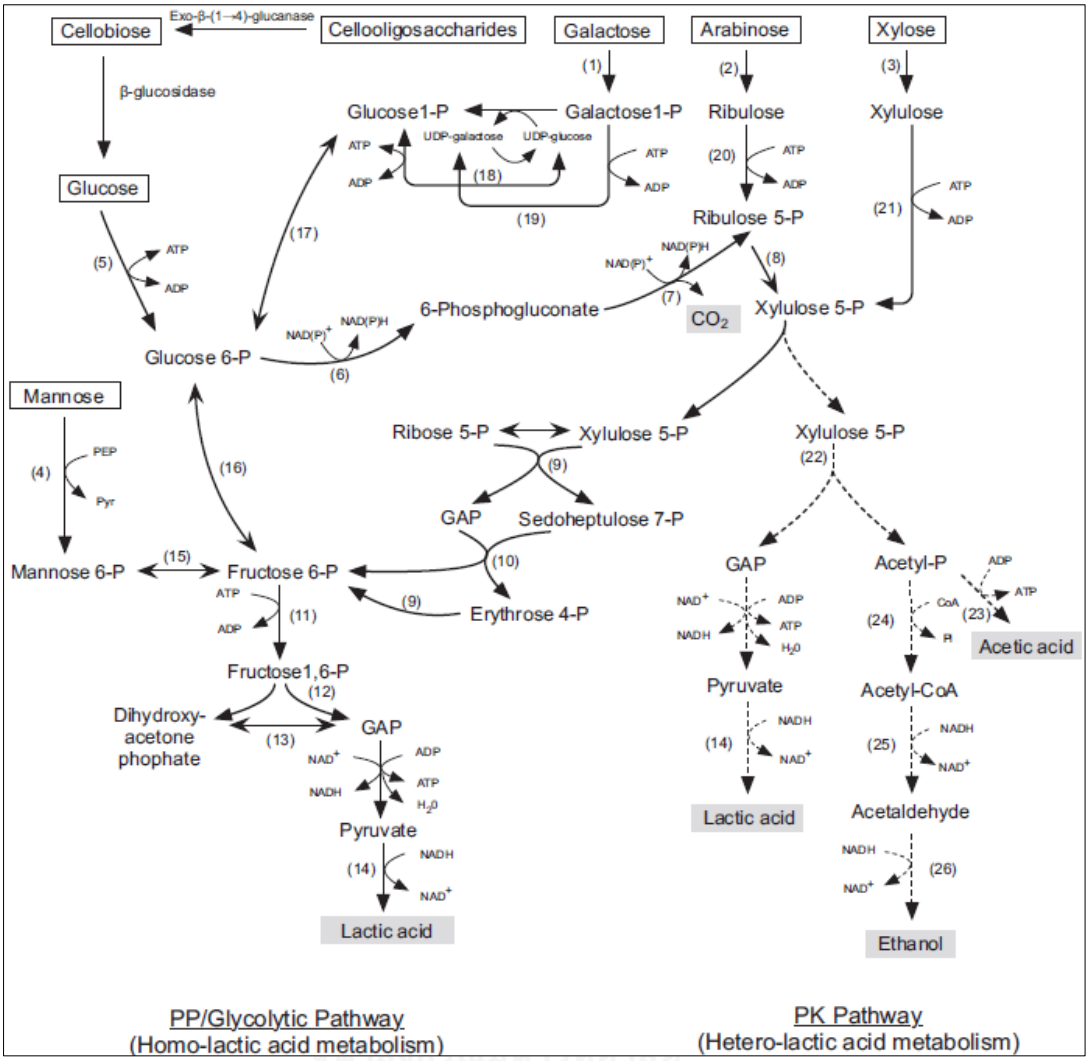

Figure 2.6 Pathways for lactic acid production by lactic acid bacteria.

GAP, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate.

Enzymes: (1), galactokinase; (2), arabinose isomerase; (3), xylose isomerase; (4), mannose phosphotransferase system; (5), hexokinase; (6), glucose-6-phosphate dehydrogenase; (7), 6-phosphogluconate dehydrogenase; (8), ribulose-5-phosphate 3-epimerase; (9), transketolase; (10), transaldolase; (11), 6-phosphofructokinase; (12), fructose-bisphosphate aldolase; (13), triosephosphate isomerase; (14), lactate dehydrogenase; (15), phosphomannose isomerase; (16), phosphoglucose isomerase; (17), phosphoglucomutase; (18), galactose-1-phosphate uridyl transferase; (19), glucosyltransferase; (20), ribulokinase; (21), xylulokinase; (22), phosphoketolase; (23), acetate kinase; (24), phosphotransacetylase; (25), aldehyde dehydrogenase; (26), alcohol dehydrogenase (Y. Wang et al., 2015).

Most heterofermentative lactic acid bacteria strains convert the pentose sugars to lactic acid and byproducts (e.g. acetic acid, ethanol) via PK pathway with a maximum lactic acid yield at 0.6 g lactic acid per gram of pentose (1.0 mol/mol). The first steps of metabolism of pentose sugars (e.g. xylose, arabinose) are phosphorylation of pentose sugars to xylulose 5-P (5 carbons) as a common intermediate, which is then cleaved to GAP and acetyl-P. The resulting acetyl-P is metabolized to synthesize acetic acid or ethanol (both 2 carbons), whereas the GAP is converted to pyruvic acid and then to end product of lactic acid (3 carbons) as shown in Figure 2.6. There are some species of lactic acid bacteria that use this pathway for the consumption of pentose such as, *Lactobacillus alimentarius*, *L. plantarum, L. casei, L. rhamnosus, L. pentosus, L. xylosus* and *Lactococcus lactis* (Castillo Martinez et al., 2013; Y. Wang et al., 2015).

Table 2.6 Homo-ferementation and hetero-fermentation of lactic acid bacteria

Characterization	Homoferementation	Heterofermentation
Products	Lactic acid	Lactic acid, ethanol, diacetyl, formate, acetoin or acetic acid, and carbon dioxide
Metabolic pathways	Hexose: Embden–Meyerhof pathway Pentose:pentose phosphate pathway	Hexose: phosphogluconate and phosphoketolase pathway Pentose: phosphoketolase pathway
Theoretical yield of lactic acid from sugars	Hexose: 1.0 g/g (2.0 mol/mol) Pentose: 1.0 g/g (1.67 mol/mol)	Hexose: 0.5 g/g (1.0 mol/mol) Pentose: 0.6 g/g (1.0 mol/mol)
Genera	*Lactococcus, Streptococcus, Pediococcus, Enterococcus*, some *Lactobacillus* species	*Leuconostoc, Oenococcus*, some *Lactobacillus* species
Commercial lactic acid production	Available due to high selectivity	Not available due to high by-product formation

(Source: Abdel-Rahman et al., 2013)

Table 2.7 Bacteria with different isomers

D-lactic acid	L-lactic acid	DL-lactic acid
Lactobacillus	***Carnobacterium***	***Lactobacillus***
Lb. bulgaricus	*C. divergens*	*Lb. helveticus*
Lb. coryniformis	*C. piscicola*	*Lb. plantarum*
Lb. delbrueckii spp. *Bulgaricus*	*C. alterfunditum*	*Lb. acidophilus*
Lb. jensenii	***Enterococcus***	***Pediococcus***
Lb. lactis	*E. faecium*	*P. cerevisiae*
Leuconostoc	*E. mundtii*	*P. hennebergi*
Leuc. cremoris	***Lactobacillus***	*P. lindneri*
Leuc. dextranicum	*Lb. amilophylus*	***Weissella***
Leuc. lactis	*Lb. brevis*	*W. beninensis*
Oenococcus	*Lb. buchneri*	
O. oeni	*Lb. delbrueckii*	
Weissella	*Lb. rhamnosus*	
W. paramesenteroides	*Lb. casei*	
Sporolactobacillus	*Lb. bavaricus*	
S. laevolacticus	*Lb. maltaromicus*	
S. kofuensis	***Lactococcus***	
S. spathodeae	*L. garviae*	
S. spathodeae	*L. lactis*	
	L. piscium	
	L. plantarum	
	L. raffinolactis	
	Pediococcus	
	P. claussenii	
	Streptococcus	
	S. bovis	
	S. lactis	
	Tetrageonococcus	
	T. halophilus	

(Source: Bai, Gao, Sun, Wu, & He, 2016; Castillo Martinez et al., 2013; Garvie, 1969; Gordon & Doelle, 1975; Narita, Nakahara, Fukuda, & Kondo, 2004; Prasirtsak, 2011)

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2.6 Three types of bacteria producing lactic acid based on the different types of lactate dehydrogenase

The lactic acid producing bacteria can produce two types of isomers: D-lactic and L-lactic and depending on the lactate dehydrogenase in the bacteria cells, three types of lactic acid can be obtained: D-lactic acid, L-lactic acid and DL-lactic acid. Bacteria with L-lactate dehydrogenase produce isomers with L-lactic. Likewise, the bacteria with D-lactate dehydrogenase produce isomers with D-lactic but certain bacteria can produce both types of isomers: DL-lactic. Bacteria with different isomers are shown in Table 2.7 (Hofvendahl & Hahn–Hägerdal, 2000).

2.7 Spore-forming lactic acid producting bacteria

Owing to the stereocomplex structure of PLA, D-lactic acid becomes considerably important as well as L-lactic acid. Historically, D-lactic acid producers are composed of lactic acid bacteria in genus *Lactobacillus, Leuconostoc, Oenococcus, Weissella*. Recently, *Sporolactobacillus* strains have been explored and are promising wide-type strain of D-lactic acid producer due to acceptable high productivity and optical purity. In 1963, *Sporolactobacillus* was discovered by Kitahara and Suzuki. They isolated an unusual strain of a lactic acid bacterium in assorted chicken feed and found that some characters of bacteria possess intermediate between the genera of *Bacillus* and *Lactobacillus*; a catalase-negative, Gram-positive, microaerophilic, motile, sporeforming, homofermentative metabolism and lactic acid-producing. Later, the researchers created the new subgenus *Sporolactobacillus* within the family Lactobacillaceae in order to accommodate this unusual bacterium (Kitahara & Suzuki, 1963). In 1972, Kitahara & Toyota transferred the genus *Sporolactobacillus* to the family Bacillaceae comprising firstly bacterial species, *Sporolactobacillus inulinus* (Kitahara & Toyota, 1972). More recently, in *Bergey's Manual of Systematic Bacteriology*, all members representing sporolactobacilli were grouped in the family 'Sporolactobacillaceae'(Garrity & Holt, 2001)

The cell wall of *Sporolactobacillus* contains *meso*-diaminopimelic acid, MK-7 was the predominant isoprenoid quinone, and the cellular fatty acids were anteiso- and iso-branched-chain fatty acids. The DNA base compositions ranged from 43 to 50 mol% G+C. The species have been reported in the genus *Sporolactobacillus* containing *S. inulinus*, *S. kofuensis, S. lactosus, S. laevolacticus, S. nakayamae, S. nakayamae* subsp. *nakayamae*, *S. nakayamae* subsp. *racemicus*, *S. putidus, S. spathodeae*, *S. shoreae S. terrae* and *S. vineae*.(Chang et al., 2008; Claus, Fritze, & Kocur, 2006)

2.7.1 *Sporolactobacillus inulinus*

The original isolate, *S. inulinus*, grows between 15–40°C with an optimum temperature at around 30°C and pH as low as 5.0. Acid is produced from glucose, fructose, and mannose. The DNA base compositions ranged 46.8–50.2 mol% G+C. *S. inulinus* has been shown to ferment hexoses exclusively to D-lactic acid (homolactic acid fermentation) producing less than 1% of volatile acids or ethanol (Garrity & Holt, 2001; Yanagida, Suzuki, Kozaki, & Komagata, 1997).

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2.7.2 *Sporolactobacillus kofuensis*

The strain grows at a temperature between 25 and 40°C with the concentration of 3-7% NaCl. The acid is produced from galactose, maltose, sucrose, raffinos, mannitol and inulin but not from arabinose, ribose, xylose, rhamnose, cellobiose, lactose and inulin. Its %G+C mole is 43 %. It can be extracted from soil (Yanagida et al., 1997).

2.7.3 *Sporolactobacillus lactosus*

The strain grows at a temperature between 15 and 45°C with the concentration of 3-4% NaCl. Lactic acid is produced from galactose, lactose, melibiose and trehalose. This strain produces lactic acid from maltose, sucrose,

raffinose and inulin. Its %G+C mole is between 43 and 47%. It can be extracted from soil (Yanagida et al., 1997)

2.7.4 *Sporolactobacillus laevolacticus*

laevolacticus, referring to D-lactic acid, the only lactic acid produced by the organisms. Glucose or other carbohydrates are required for growth. Lactic acid is produced from glucose and mannitol but not from arabinose or xylose. The G+C content of the DNA is 43 to 45 mol% (Andersch, Pianka, Fritze, & Claus, 1994; Hatayama, Shoun, Ueda, & Nakamura, 2006).

2.7.5 *Sporolactobacillus nakayamae* subsp. *nakayamae*

It grows at a temperature between 15 and 40°C with the concentration of 3-7% NaCl. Lactic acid is produced from maltose, galactose, sucrose and trehalose but not from arabinose, ribose, xylose, rhamnose, lactose, cellobiose, melibiose, salicin and starch. Its %G+C mole is between 43 and 47%. It can be extracted from soil (Yanagida, Suzuki, Kozaki, & Komagata, 1997)

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2.7.6 *Sporolactobacillus nakayamae* subsp. *racemicus*

DL-Lactic acid is produced by *S. nakayamae* subsp. *racemicus*. Litmus milk is acidified. Lactic acid is produced from galactose, maltose, melibiose, sucrose, raffinose, trehalose, and inulin, but not from arabinose, ribose, and xylose. The DNA G+C content ranges from 43 to 46 mol% (Yanagida et al., 1997).

2.7.7 *Sporolactobacillus putidus*

The size of this round colony is 1 – 2 millimeters, growing at a temperature between 30 - 45°C whose pH ranges from 3.5 – 5.5; growth does not occur at pH 3.0 or 6.0. It does not produce oxidase, catalase or reduce nitrate. Acid is produced

from galactose, D-glucose, D-fructose, D-mannose, mannitol, maltose, sucrose and trehalose. DL-lactic acid is produced. The main quinone is menaquinone 7. The G+C content is 47.5 mol%. It can be extracted from rancid vinegar (Fujita, Mochida, Kato, & Goto, 2010).

2.7.8 *Sporolactobacillus spathodeae*

Cells are Gram-stain-positive, facultatively anaerobic, motile, endospore-forming straight rods with rounded ends (0.3–0.461.5–6.1 mm). D-lactic acid is produced from glucose. Catalase, oxidase and nitrate reduction are negative. It grows at 20–40 $^{\circ}$C (optimally at 30 $^{\circ}$C), pH 4.5–8.0 (optimally at pH 6.0–7.0) and with 1% NaCl (Thamacharoensuk, Kitahara, Ohkuma, Thongchul, & Tanasupawat, 2015).

2.7.9 *Sporolactobacillus shoreae*

Cells are Gram-stain-positive, facultatively anaerobic, motile, endospore-forming straight rods with rounded ends (0.4–0.661.5–6.6 mm). D-lactic acid is produced from glucose. Catalase, oxidase and nitrate reduction are negative. It grows at 25–40 $^{\circ}$C (optimally at 30 $^{\circ}$C), at pH 4.5–8.0 (optimally at pH 6.0) and with 1% NaCl (Thamacharoensuk et al., 2015).

2.7.10 *Sporolactobacillus terrae*

It grows at a temperature between 15 and 40°C with a concentration of 3-5% NaCl. Lactic acid is produced from trehalose, galactose, sucrose and inulin but not from ribose, xylose, rhamnose, lactose, sorbitol, arabinose, melibiose and starch. Its %G+C mole is between 43 and 46%. It can be extracted from soil (Yanagida et al., 1997).

2.7.11 *Sporolactobacillus vineae*

It grows in the pH range of 6 – 7 at a temperature between 25 and 40°C with the concentration of 7% NaCl. Lactic acid is produced from fructose, mannose and sorbitol. It cannot reduce nitrate or produce catalase or oxidase. DNA G+C content is 50.6–51.6 mol%. It can be extracted from the soil in a vineyard in Korea (Chang et al., 2008).

2.8 Factors affecting the production of lactic acid

In microbial fermentation, the bacteria are allowed to grow under controlled conditions to ensure high amounts of product and high production rate. The factors affecting the production are the strains of bacteria, temperature, pH balance and composition of medium.

2.8.1 Strains of bacteria

Lactic acid bacteria are well known as efficient lactic acid producers, especially in the genus *Lactobacillus* and *Streptococcus* which were interesting more than other microorganisms. They metabolized glucose via EMP pathway or phosphoketolase pathway to produce lactic acid as mentioned earlier and so called homofermentative and heterofermentative LAB, respectively. In industries, lactic acid production is favorable used homofermentative LAB because they produce lactic acid as the major end product. Table 2.8 shows Bacteria producing lactic acid with isomers and different types of fermentation.

Table 2.8 Bacteria producing lactic acid with isomers and different types of fermentation

Genus	Type of fermentation	Principal product	Type of lactic acid
Lactococcus	Homofermentative	Lactate	L
Enterococcus	Homofermentative	Lactate	L
Pediococcus	Homofermentative	Lactate	DL and L
Thermobacterium	Homofermentative	Lactate	D,L and DL
Streptobacterium	Heterofermentative	Lactate : Acetate (1:1)	D,L and DL
Betabacterium	Heterofermentative	Lactate : Acetate : CO_2 (1:1:1)	DL
Leuconostoc	Heterofermentative	Lactate : Acetate : CO_2 (1:1:1)	D
Bifidobacterium	Heterofermentative	Lactate : Acetate (2:3)	L

(Source: Adapted from Bai et al., 2016; Narita et al., 2004; Prasirtsak, 2011)

2.8.2 Temperature

Temperature affects the growth of bacteria and the production of lactic acid. The bacteria belonging to the mesophile group grows well at a temperature between 28 – 45°C while those belonging to thermophile at a temperature between 45 – 62°C. Therefore, the application of an inappropriate temperature means low yields. In general, the right temperature for bacteria to produce high yields of lactic acid is between 37 – 45°C. For example, *Lactobacillus casei* can produce the highest yield at 37°C and the higher the temperature, the lower the yield (Hujanin and Linko, 1996). According to the study of Tashiro et al. (2011), *L. delbrueckii* subsp. *lactis* QU 41 could produce D-lactic acid at 50°C. The tolerance to high temperature is useful for installing a fermentation tank because this could reduce the risk of contamination by other bacteria (Vaidya et al., 2005).

2.8.3 pH balance

Most living organisms are sensitive to change in pH balance. The fermentation causes a change in the pH balance. During the fermentation, lactic acid is produced and the acidity level in the tank is too high. The bacteria that produce lactic acid generally stop growing if the pH balance is lower than 4.5; therefore, the pH balance has to be controlled at 5.5 – 6.5 by addition of NH_4OH, $CaCO_3$ or NaOH. The right and stable pH balance is essential for the yield, resulting in a high and stable productivity level. The control of pH balance produces more lactic acid than the uncontrolled pH balance (Hofvendahl and Hagerdal, 2000; Vaidya et al., 2005).

2.8.4 Nutrient requirement

Whether bacteria can grow well and produce more lactic acid depends on nutrient. Such quality food contains vitamins, amino acid, peptide, minerals, nucleic acid and fatty acid (Reddy et al., 2008) because these bacteria cannot produce their own vitamins and certain substances are necessary for their growth. Fundamental food sources are carbon in glucose and nitrogen in organic substances such as peptone-yeast extract, corn steep liquor or nitrogen in inorganic substances such as ammonium sulfate, and ammonium phosphate. In addition, they require such minerals as magnesium, manganese and iron.

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2.8.4.1 Sources of carbon

Most complex nutrients are expensive; consequently, to reduce the production cost, inexpensive but quality food is essential. To obtain such food, simple pretreatment of raw materials is performed. In Thailand, agricultural wastes include sucrose from sugar production, whey from milk production, flour from cassava or wheat factories or from Lignocellulose.

Whey

Most lactic acid is made from whey obtained from cheese production. Whey contains protein, salt and lactose. After hydrolysis, glucose and lactose are derived. Whey is usually combined with peptone-yeast extract or corn steep liquor as a source of nitrogen; as a result, the fermentation is a source of carbon and nitrogen. Popular strains are *Lactobacillus bulgaricus* and *L. helveticus* (Hofvendahl and Hagerdal, 2000).

Molasses

Molasses are full of sucrose and cheap. It is commonly used as animal feed in the production of ethanol or cheese. *L. delbrueckii* was used to produce lactic acid (Hofvendahl and Hagerdal, 2000; Vaidya et al., 2005).

Flour

Flour is another good choice because after being digested, it yields glucose and maltose. The flour is obtained from wheat, corn, cassava, potato, rice, sorghum and barley. *L. fermentum* and *L. amylovorus* are examples of bacteria used to digest flour but most bacteria do not have an enzyme for such digestion. To produce lactic acid from flour, therefore, alpha amylase and glucose amylase are added to help the bacteria digest flour (Hofvendahl and Hagerdal, 2000).

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Lignocellulose

The principal components of lignocellulose are 6-atom carbon sugars – glucose, galactose and mannose – and 5-atom carbon sugars – xylose and arabinose, which can be found in paper and wood (Hofvendahl and Hagerdal, 2000). Its good point is that it is a complete and cheap carbon source, but most bacteria cannot readily use this carbon source whose main component is cellulose. It has to go

through pretreatment processes physically and chemically in addition to enzymes such as endoglucanase cellobiohydrolase and β-glucosidase (Okano et al., 2009)

2.8.4.2 Sources of nitrogen

Nitrogen is a component of nutrient for producing lactic acid. The amount of nitrogen and amino acid varies according to the source of nitrogen. Nitrogen obtained from organic substances such as peptone-yeast extract is expensive. Substitutes are from corn steep liquor, hydrolysate from wheat bran, protein hydrolysate from soybean, ammonium sulfate and ammonium phosphate (Hofvendahl and Hagerdal, 2000).

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CHAPTER 3

EXPERIMENTAL

3.1 Materials

3.1.1 Equipment and instruments

Equipment/Instrument	**Model**	**Company and country**
Air compressor	OS-25	PUMA Industrial Co., Ltd., Taiwan
Autoclave	KT-40L	ALP Co., Ltd., Japan
Centrifuge	KR-20000T	Kubota Corporation, Japan
Centrifuge	MC-15A	Tomy Seiko Co., Ltd., Japan
Dissolved oxygen electrode	O_2 sensor InPro	Metter Toledo, Switzerland
Electronic balance	ML 204/01	Metter Toledo, Switzerland
Electronic balance	ML 3002E/01	Metter Toledo, Switzerland
Fermentor 5 Liters	MDFT-N-5L	B.E. Marubishi, Ltd, Thai
Glucose lactate analyzer	YSI 7100	Yellow Spring Instrument, U.S.A.
High Performance Liquid Chromatography (HPLC)	Shimadzu-LC-10Avp	Shimadzu Co., Ltd., Japan
Incubator	-	Sanyo, Japan
Laminar flow hood	NK system Clean bench	International Scientific Supply, Japan
Microscope	Alphaphot-2 YS2-H	Nikon, Japan

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Equipment/Instrument	Model	Company and country
Oven	UL-80	Memmert Co., Ltd., Germany
pH combination electrode	405-DPAS-SC-K85/325	Mettler Tobedo, Switzerland
pH meter	AB15	Fisher Scientific, Ltd. Singapore
Recirculation chiller	IL-008-05	STIK Instrument Equipment (Shanghai) Co., Ltd, China
Rotary incubator shaker	G25	New Brunswick Scientific Co., Inc., U.S.A.
Spectrophotometer	UVmini-1240	Shimadzu Co., Ltd., Japan
Ultrasonic sonicator	UD-201	Tomy, Japan
Vortex mixer	K-550-GE	Scientific Industries, Inc, U.S.A.

3.1.2 Chemicals

Chemical	Company	Country
Agar	Patanasin Enterprise	Thai
Ammonium choride (NH_4Cl)	Riedel-de Haen	Germany
Antifoam Y-30 Emulsion	Sigma	U.S.A.
Betaine hydrochloride	Sigma	U.S.A.
Calcium carbonate ($CaCO_3$)	Sigma	Germany
Copper sulfate ($CuSO_4.5H_2O$)	Fluka	France
Ethanol (C_2H_6O)	Merck	Germany
Glucose monohydrate ($C_6H_{12}O_6.H_2O$)	Siamchai Chemical	Thai
Hydrochloric acid (HCl)	Merck	Germany
Hydrogenperoxide (H_2O_2)	Merck	Germany

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Chemical	Company	Country
Iron sulfate heptahydrate ($FeSO_4.7H_2O$)	Merk	Germany
Magnesium sulfate ($MgSO_4.7H_2O$)	Merck	Germany
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	Riedel-de Haen	Germany
Peptone	Fluka	France
Potassium phosphate monobasic (KH_2PO_4)	Riedel-de Haen	Germany
Potassium phosphate dibasic (K_2HPO_4)	Riedel-de Haen	Germany
Sodium acetate (CH_3COONa)	Carlo Erba	Italy
Sodium choride (NaCl)	Sigma	Germany
Sodium hydroxide (NaOH)	Grand Chemical	Thai
Sulfuric acid (H_2SO_4)	Merck	Germany
Yeast extract	Bio springer	France

3.2 Methodology

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3.2.1 Bacterial identification

3.2.1.1 Phenotypic characterization

Cell form, cell size, cell arrangement, and colonial appearance were examined on the cell grown on A medium agar (Appendix A) incubated under anaerobic conditions for 3 days. Hucker-Conn modification was used for Gram stain (Hucker & Conn, 1923) Spore formation was examined by scanning electron microscope. Catalase, oxidase, nitrate reduction, hydrolysis of arginine and starch were tested as previously reported. The effect of temperature (10, 20, 25, 30, 40, 45 and 50 °C), different starting pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0 and 8.5) [each at a final concentration of 50 mM of acetate buffer (pH 4-5), phosphate buffer (pH 6.0-

7.0) and Tris buffer (pH 8-8.5) Sorokin, 2005], different concentrations of NaCl (1, 2, 3, 4, 5, 6, and 7%) were tested by using A medium broth (Appendix A). All tests were carried out by incubating the cultures at 30 °C, except for the investigation of effects of temperature. Acid formation from carbohydrates was determined as reported previously (Tanasupawat, Okada, & Komagata, 1998). Additional biochemical characteristics were recorded after incubation for 2 days in API 50 CH strips (bioMe´rieux).

3.2.1.2 16S rRNA gene sequence analysis

DNA was isolated from cells grown in GYP broth after incubating for 2 days and was purified by the method of Saito and Miura (1963). The 16S rRNA gene of the strain was amplifed and PCR product was purified and sequenced as described previously (Tanasupawat et al., 2004). The sequences of strain were aligned with selected sequences obtained from GenBank by using CLUSTAL_X version 1.81. The alignment was edited manually to remove gaps and ambiguous nucleotides prior to the construction of phylogenetic trees. A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) with the program MEGA version 5.05. The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein (1985) based on 1000 replications. The values for sequence similarity among the closest strains were determined using the EzTaxon server (Chun et al., 2007).

3.2.2 Microorganism and culture medium preparation

Sporolactobacillus kofuensis SB7-2 was used in this study. The culture was maintained on GYP slant containing (in one liter) 6 g glucose, 5 g yeast extract, 5 g peptone, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4, 10 mL salts solution (comprising of 0.40 g $MgSO_4 \cdot 7H_2O$, 0.02 g $MnSO_4 \cdot 5H_2O$, 0.02 g $FeSO_4 \cdot 7H_2O$ and 0.02 g NaCl dissolved in 10

mL DI water), 5 g $CaCO_3$, and 20 g agar (Appendix A). The initial pH was 6.8. The stock slant was incubated at 37 °C under anaerobic conditions and stored at 4 ^{o}C.

3.2.3 Optimizing seed culture of *S. kofuensis* SB7-2 for D-lactate production

3.2.3.1 Effect of glucose concentrations in GYP slant on cell growth during stock culture

The stock was subcultured on the fresh GYP agar medium at different glucose concentrations (3, 4, 5, 6, 8 and 10 g/L). The culture slant was incubated at 37 °C for 24 h under anaerobic conditions. Later, 200 µL of sterilized 0.85% NaCl was added into culture slant for getting suspended cell of each concentration of glucose. The cell suspensions were diluted to 100-fold and measured by determining the optical density at 600 nm (OD_{600}). It is noted that proper glucose concentration should result in the sufficiently high cell growth in term of OD_{600}.

3.2.3.2 Determining stock culture incubation time to enhance growth of *S. kofuensis* SB7-2 during preculture

The culture slant was incubated at 37 °C for 24, 32, and 40 h, respectively. The fresh working culture slant incubated at these 4 different ages was used to transfer into the preculture medium containing (in one liter) 10 g glucose, 5 g yeast extract, 5 g peptone, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4, 10 mL salts solution (comprising of 0.40 g $MgSO_4{\cdot}7H_2O$, 0.02 g $MnSO_4{\cdot}5H_2O$, 0.02 g $FeSO_4{\cdot}7H_2O$, and 0.02 g NaCl dissolved in 10 mL DI water), and 5 g $CaCO_3$ (Appendix A). The culture suspension of 0.5 ml (OD_{600} of 30-40) was inoculated into 50 mL preculture medium in the 250 mL Erlenmeyer flask. The culture was incubated at 37 °C and 200 rpm under anaerobic conditions. Sample was taken every 3 h for analyses of the remaining glucose, growth, and lactate formation until glucose repletion or constant growth. It is noted

that proper working culture age should result in the short lag time in preculture with sufficiently high growth rate.

3.2.3.3 Effect of mixing on growth of *S. kofuensis* SB7-2 during preculture

After obtaining the suitable working culture age to be transferred into the preculture medium, further optimization was conducted during preculture step to obtain the proper seed for D-lactate fermentation step later on. From the preliminary screening study, *S. kofuensis* SB7-2 lacked catalase; thus, fermentation required no oxygen. Nonetheless, mixing was claimed to be one of the key factors facilitating growth. Thus in this study, the growth rate of *S. kofuensis* SB7-2 was observed under static and rotational mixing. The 24-h culture slant was used to prepare the stock culture at the OD_{600} of 30-40. The stock culture was inoculated into 50 mL preculture medium at 1% inoculum size. For static condition, the culture was anaerobically incubated at 37 °C without mixing (WO/A) while the rotational mixing condition, the culture was anaerobically incubated at the same temperature with the mixing rate of 200 rpm (W/A). Sample was collected every 3 h for analyses of the remaining glucose, growth, and lactate formation.

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3.2.3.4 Effect of initial glucose concentration on growth of *S. kofuensis* SB7-2 during preculture

High cell concentration is normally required in fermentation as cells are considered as the machinery to generate the desired product. In this study, initial glucose concentration in the preculture medium was varied in order to achieve high cell concentration for passage into the fermentation stage. The stock culture prepared from the 24-h culture slant at the OD_{600} of 30-40 was inoculated into 50 mL preculture medium at 1% inoculum size. The glucose concentration in the

preculture medium in this part was varied (5, 10, and 15 g/L). The culture was anaerobically incubated at 37 °C and 200 rpm. Sample was collected every 3 h for analyses of the remaining glucose, growth, and lactate formation.

3.2.3.5 Effect of inoculum sizes on growth of *S. kofuensis* SB7-2 during preculture

After obtaining glucose concentration to be suitable for the preculture medium, further optimization was conducted during preculture step to obtain the proper inoculum size for D-lactate fermentation step later on. The inoculum size plays crucial role in controlling both high cell concentration and short lag phase. The stock culture prepared from the 24-h culture slant at the OD_{600} of 30-40 was inoculated into 50 mL preculture medium at different inoculum size (1, 2, and 3%). The culture was anaerobically incubated at 37 $^{\circ}C$ and 200 rpm. Sample was collected every 3 h for analyses of the remaining glucose, growth, and lactate formation.

3.2.4 D-lactic acid fermentation optimization in a 5-L stirred fermentor

3.2.4.1 Determining the proper preculture time for enhancing D-lactic acid production during fermentation by *S. kofuensis* SB7-2

D-lactic acid fermentation was investigated in the 5-L stirred fermentor. The 24-h stock culture at the OD_{600} of 30-40 was transferred into the preculture medium at 3% inoculum size. The preculture flask was anaerobically incubated at 37 $^{\circ}C$ and 200 rpm. The incubation time was varied at 3, 6, and 9 h. Later, the preculture flask was used to inoculate the fermentor containing (per liter) 100 g glucose, 10 g yeast extract, 5 g peptone, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4, and 10 mL salts solution at 5 % inoculum size. The starting fermentation volume was 3 L. The initial pH of the fermentation medium was adjusted to 6.8. Later, the pH was controlled by $CaCO_3$.

The fermentor was controlled at 37 ^{o}C and 200 rpm. Sample was collected every 3 h until glucose depletion.

3.2.4.2 Effect of inoculum sizes on D-lactic acid fermentation by *S. kofuensis* SB7-2

After obtaining the suitable conditions to be used into the seed culture preparation, the 24-h stock culture at the OD_{600} of 30-40 was transferred into the preculture medium at 3% inoculum size with anaerobically incubated at 37 ^{o}C and 200 rpm for 6 h of preculture time. Further optimization was conducted during fermentation step to obtain the proper inoculum size for D-lactate fermentation step later on. D-lactic acid fermentation was investigated in the 5-L stirred fermentor. Later, the inoculum size in the fermentation medium in this part was varied (2, 5 and 10%). The starting fermentation volume was 3 L containing 100 g/L glucose concentration. The initial pH of the fermentation medium was adjusted to 6.8 and controlled pH by $CaCO_3$. The fermentor was controlled at 37 ^{o}C and 200 rpm. Sample was collected every 3 h until glucose depletion.

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3.2.5 Analytical methods

Fermentation broth sample was centrifuged at 10,000 g for 5 min. The supernatant was collected for further analyses of the remaining carbon substrates, lactic acid and byproduct formation, and the optical purity of lactate products. Whilst solid cell biomass was acidified with 1 M HCl to remove insoluble calcium bases remained in the sample. The acidified sample was centrifuged and the obtained solid particles were resuspended in DI water for optical density reading. Spectrophotometry was used to determine the OD at 600 nm of the cell biomass present in the fermentation broth. The OD at 600 was converted to dry cell weight (DCW) according to the relationship between OD_{600} and DCW (g/L) by a linear regression line with OD600 = 2.1201(DCW) - 3.5403.

Amounts of products formed and carbon substrates remained during the fermentation were analyzed by high performance liquid chromatography (HPLC). Fermentation samples were centrifuged, filtered through PTFE (hydrophilic) membrane, and diluted with DDI water. For analyses of glucose remained, total lactic acid (both L- and D-lactate), and acetic acid produced, 15 µL diluted particle-free samples were automatically injected (Shimadzu) into an organic acid analysis column (Biorad, Aminex HPX-87H ion exclusion organic acid column; 300mm×7.8mm) maintained at 45°C in a column oven (Shimadzu-CTO-10A). 0.005 M H_2SO_4, was pumped through the system at the flow rate of 0.6 mL/min (Shimadzu-LC-10Avp). A refractive index detector (Shimadzu-RID-10A) was used to detect the organic compounds. The standards containing 0-2 g/L of each component (glucose, lactate, acetate and ethanol) were injected as the references to determine the sample concentration. The peak area was used in determining the concentration.

Lactic acid isomeric purity is measured by HPLC using a chiral column (Sumipack, Sumichiral OA5000) at 40 °C, eluted with 1 mM $CuSO_4$ at flow rate of 1.0 mL/min and detected at 254-nm with a UV detector. The standards containing 0-2 g/L of each component (D-lactate and L-lactate) were injected as the references to determine the sample concentration. The peak area was used in determining the concentration. The optical purity of D-lactic acid was defined as follows: D-lactic acid/(L-lactic acid + D-lactic acid)x100%.

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Screening, identification, and prilimimary characterization of isolate SB7-2

Isolate SB7-2 was Gram-stain-positive, facultatively anaerobic, spore-forming and rod shaped (Figure 4.1). The results from the biochemical test show that this isolate was negative for oxidase, catalase, nitrate reduction. It was also unable to hydrolyze arginine and starch. Isolate SB7-2 could produce D-lactic acid from glucose homofermentatively. The growth temperature fell in between 20 °C and 40 °C while the pH range was in between 4.5 and 8.0. This isolate could grow in the medium containing up to 3% NaCl. It was found that SB7-2 could utilized several carbohydrates for acid production. Those include N-acetylglucosamine, D-fructose, D-glucose, D-mannose, D-mannoitol, D-raffinose, L-rhamnose, D-saccharose (sucrose), D-Tagatose, and D-trehalose. There are some carbohydrates that this isolate could not utilize for acid production, e.g., D-adonitol, amygdalin, arabinose, arabitol, arbutin, D-celiobiose, dulcitol, erythritol, fucose, D-galactose, glycerol, inulin, D-lactose, D-maltose, D-melibiose, melezitose, D-ribose, sorbose, starch, and D-xylose. All biochemical characteristics of SB7-2 compared with type strains are shown in Table 4.1.

Isolate SB7-2 screened from the tree bark collected in Saraburi Province is considered as a promising D-lactate producer. From bacterial identification, it was suggested that SB7-2 acquired the high similarity percentage to *Sporolactobacillus kofuensis* DSM 11701^{T} of 99.86 % (Yanagida et al., 1997) (Table 4.2). The evidence shown by the neighbor joining phylogenetic tree based on 16S rRNA gene sequence

in Figure 4.2 confirms that isolate SB7-2 is closely related to *S. kofuensis* DSM 11701^{T} (99.86 %) (Yanagida et al., 1997).

Previous studies reported a few wild-type D-lactic acid producers that were isolated from soil and plant samples including *Lactobacillus delbrueckii*, *Lactobacillus coryniformis* subsp. *torquens*, *Bacillus laevolacticus, Corynebacterium glutamicum,* and *Sporolactobacillus* strain (Joshi et al., 2010; Tashiro et al., 2011; L. Wang et al., 2011). However, among D-lactic acid producers, *Sporolactobacillus* strain is a promising wild-type strain due to an acceptable high productivity and optical purity. Genus *Sporolactobacillus* comprises 10 species and 2 subspecies, e.g., *S. nakayamae* subsp. *nakayamae*, *S. nakayamae* subsp. *racemicus*, *S. nakayamae, S. inulinus*, *S. terrae*, *S. kofuensis*, *S. lactosus*, *S. laevolacticus*, *S. putidus, S. vineae*, *S. shoreae* and *S. spathodeae* (Chang et al., 2008; Fujita et al., 2010; Thamacharoensuk, 2015; Yanagida et al., 1997). It should be noted that originally, genus *Sporolactobacillus* was Gram-stain-positive, facultatively anaerobic, motile, endospore-forming, catalase, oxidase, and nitrate reductase negative. The bacterium in this genus produces D-lactic acid from glucose or other sugars depending on the subcharacteristics of the type strains (Yanagida et al., 1997).

From the first production screening in flask fermentation, *S. kofuensis* SB7-2 produced D-lactic acid at the high final titer up to 112 g/L with the productivity of 1.55 g/L·h and the optical purity of 95.8 %ee. Nonetheless, the productivity and optical purity of *S. kofuensis* seemed a bit low. To date, D-lactic acid production by *S. kofuensis* has yet been reported. Therefore, process optimization to achieve the improved productivity and optical purity as well as to generate another D-lactate producer platform is necessary.

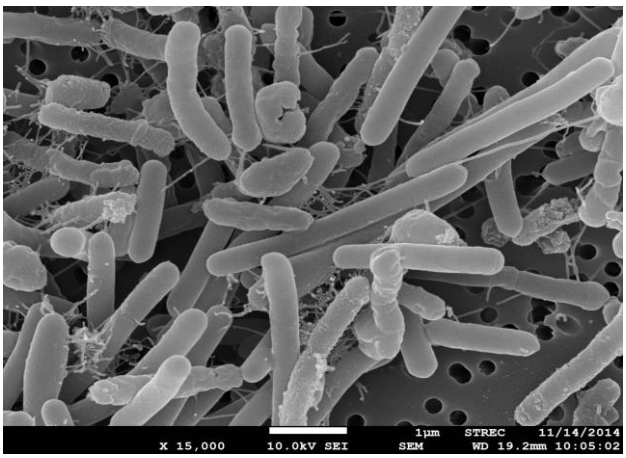

Figure 4.1 Scanning electron micrograph of *S. kofuensis* SB7-2 grown on glucose-yeast extract-peptone (GYP) agar containing $CaCO_3$

Table 4.1 Characteristics of *S. kofuensis* SB7-2 and representative of related type strain

Characteristics	**SB7-2**	*S. kofuensis* DSM 11701^T
Cell form	Rods	Rods
Spore formation	+	+
Temperature range for growth (°C)	20-40	20-40
NaCl (%) range for growth	3	3
pH range for growth	4.5-8.0	4.0-8.5
Catalase activity	-	-
Nitrate reduction	-	-
Oxidase	-	-
Hydrolysis of starch	-	-
Hydrolysis of L-arginine	-	-

Characteristics	**SB7-2**	*S. kofuensis* DSM 11701^T
Acid from:		
Amygdalin	-	w
D-Celiobiose	-	-
D-fructose	+	+
D-Fucose	-	w
D-Galactose	-	w
D-Glucose	+	+
Inulin	-	w
D-Lyxose	-	w
D-Mannose	+	+
D-Mannoitol	+	+
D-Melibiose	-	w
Methyl-α-D-glucopyranoside	-	w
D-Raffinose	w	-
L-Rhamnose	+	w
D-Ribose	-	-
D-Saccharose (sucrose)	+	+
D-Sorbitol	-	-
L-Sorbose	-	w
D-Tagatose	+	+
D-Trehalose	+	+
D-Xylose	-	-
Isomer of lactic acid	D	D

Symbols: +: Positive; -: Negative; w: Weakly positive; D: D-lactic acid.

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Table 4.2 Bacterial isolate, natural sample, location, sequence similarity (%) and closest species

Bacterial isolate	Sample	Province	Similarity (%)	Closest species
SB7-2	Tree bark	Saraburi	99.86	*S. kofuensis* DSM 11701^T

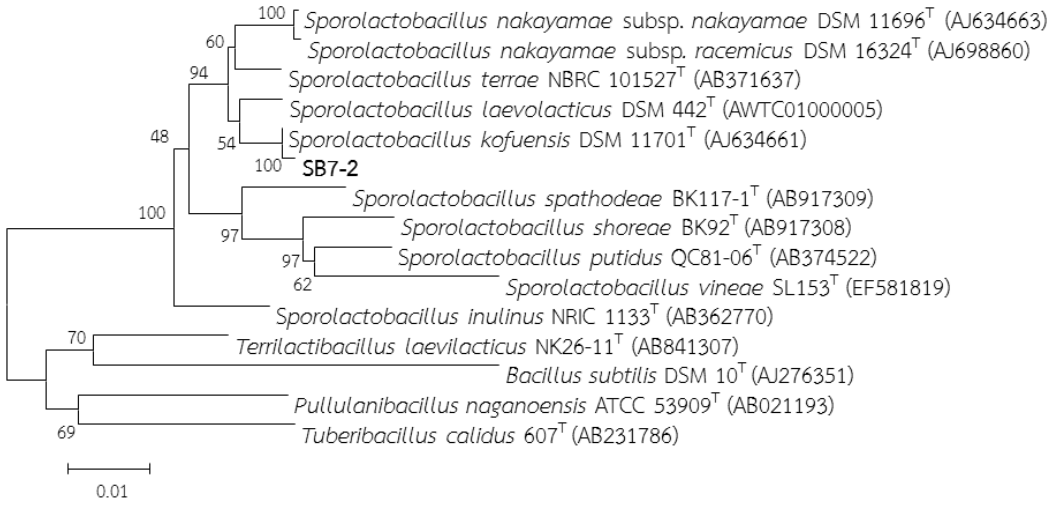

Figure 4.2 Phylogenetic tree constructed using the neighbour-joining method showing the position of isolate SB7-2 and related species based on 16S rRNA gene sequences

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4.2 Optimizing seed culture of *S. kofuensis* SB7-2 for D-lactate production

4.2.1 Effect of mixing and oxygen on preculture seed of SB7-2

Sporolactobacillus strains were classified into the micro-anerophilic group, therefore, growth can be only where a low concentration of oxygen is diffused into the fermentation medium. From Bergey's Manual, the absence of catalase and cytochrome system in *Sporolactobacillus* was mentioned (Holzapfel & Botha, 1988).This strongly confirms that in the presence of oxygen during the cultivation, hydrogen peroxide (H_2O_2), the derivative oxygen species to be formed, exists in the fermentation broth and being toxic to the cells due to the bacteria lack catalase. Therefore, the presence of oxygen during cultivation should be carefully taken into consideration. There are 2 major factors contributing on oxygen level maintained in the fermentation broth, e.g., mixing and aeration. To study the effect of mixing on growth of SB7-2, the culture was controlled at reduced oxygen level by being placed into a CO_2 generating packet. The optimization of growth of *S. kofuensis* SB7-2 was carried out using conventional method of one-factor-at-a-time.

Figure 4.3 represents the fermentation kinetics during preculture period of *S. kofuensis* SB7-2 under anaerobic conditions. The effect of mixing on metabolism was investigated. It was found that maximum OD approximately at OD600 of 5.0 was achieved within 12-15 h cultivation time (Figure 4.3A) with the complete glucose consumption (Figure 4.3B). During preculture period, D-lactate was slightly produced without any other byproducts indicating homofermentative, and growth associated product kinetics of SB7-2. Comparing the preculture seed incubated under anaerobic conditions without shaking (WO/A) with that controlled under anaerobic condition with 200 rpm shaking (W/A), slightly higher rates of glucose uptake, growth, and lactate production were observed from the kinetics profiles. By this, it was revealed that mixing was necessary during preculture stage in order to maintain broth homogeneity. Without shaking, cell sedimentation at the bottom of the flask was

observed. By this, it led to limited glucose uptake and $CaCO_3$ added would not have been sufficiently working for pH control (Boontawan, 2010). Therefore, preculture under anaerobic conditions with sufficient shaking at 200 rpm was chosen for the subsequent experiments.

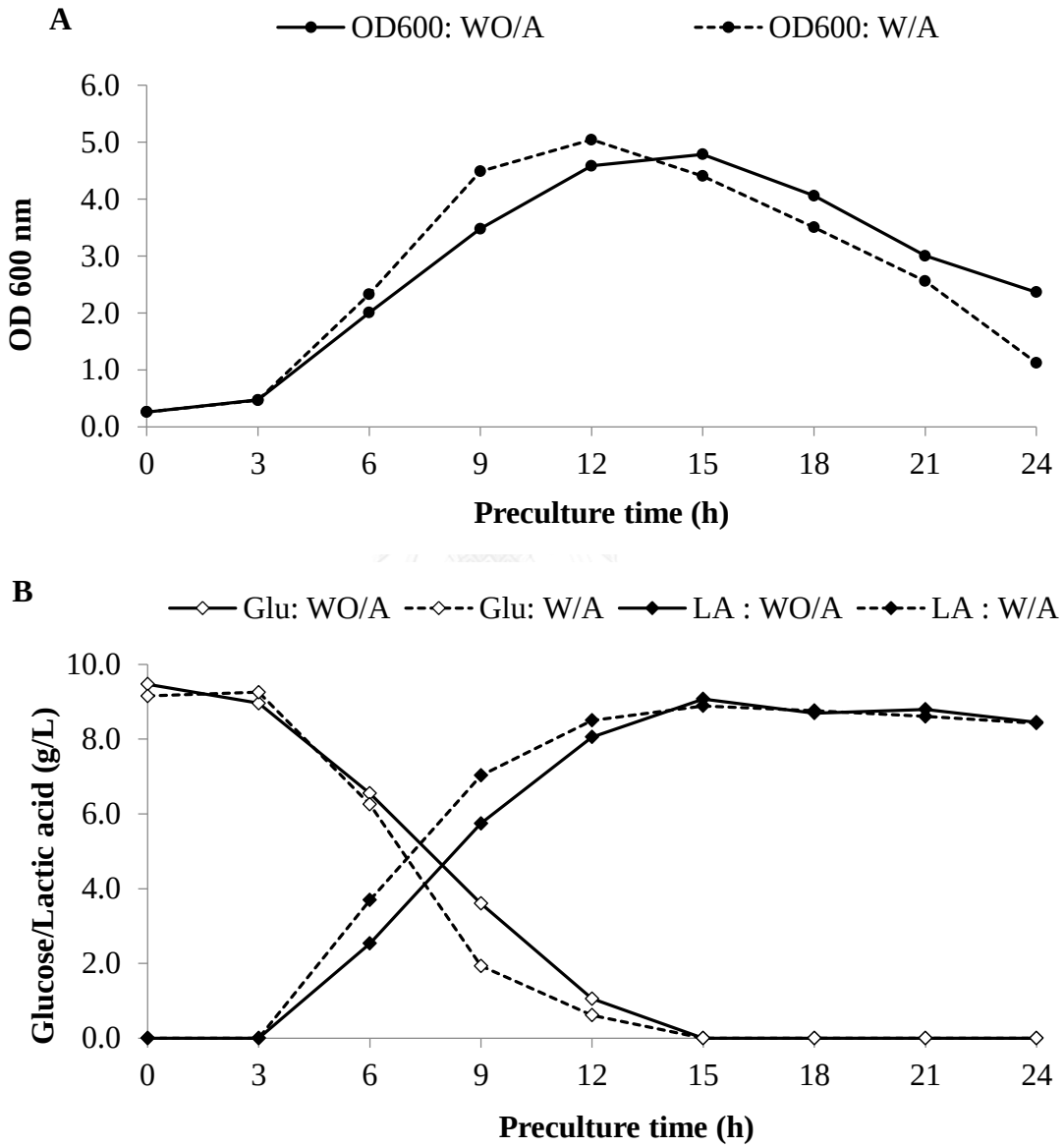

Figure 4.3 Fermentation profiles of *S. kofuensis* SB7-2 grown in the shake flask. A. Growth profiles of SB7-2 under anaerobic condition without shaking (WO/A) and with shaking at 200 rpm (W/A). B. Residual glucose and D-lactic acid formation.

4.2.2 Effect of transfer slant age on growth and metabolism of SB7-2 during preculture

Preparation of the active slant is the first step that determines the fermentation performance. To understand the role of this step and to be able to develop the robust fermentation process for SB7-2, in this part the effect of transfer slant age was investigated. *S. kofuensis* SB7-2 was transferred onto the GYP agar slants and incubated at 24, 32, and 40 h before being inoculated at 1% inoculum size into the preculture medium.

As shown in Figure 4.4, the slant age appeared to have a noticeable effect on cell growth. Longer slant incubation resulted in slower rates of cell biomass production (Figure 4.4A), glucose uptake (Figure 4.4B), and lactate production (Figure 4.4C). Too long incubation at 40 h caused a very long lag phase during preculture due to cells in the slant entering dormant stage. Therefore, when changing the medium, longer adaptation time was required for cells to return to the active stage. From the results obtained in this part, 24-h slant was selected for further uses in this work.

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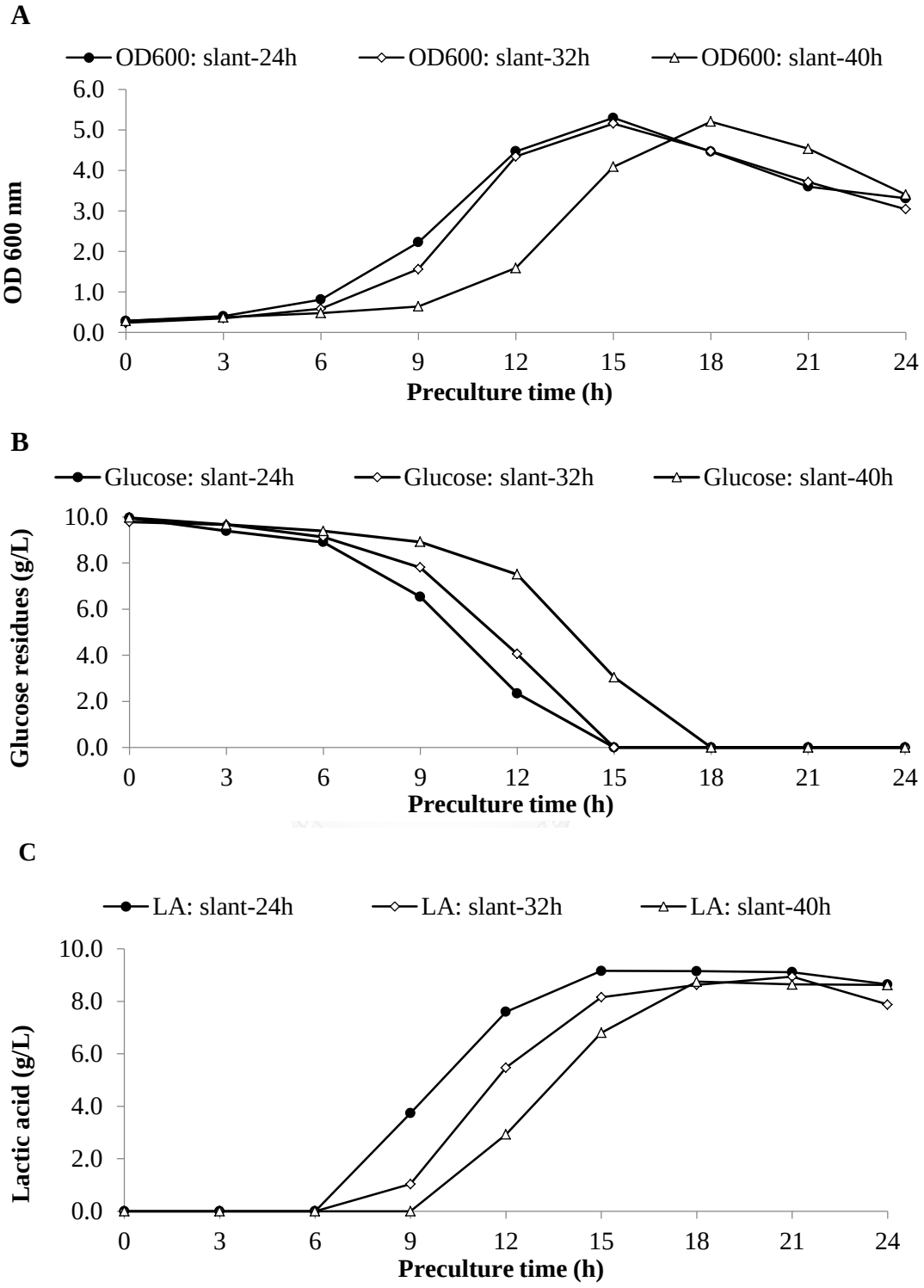

Figure 4.4 Preculture profiles of *S. kofuensis* SB7-2 in a shake flask culture using the inoculum at different incubation ages. A. growth curves, B. Residual glucose, and C. D-lactic acid formation.

4.2.3 Effect of initial glucose concentration in preculture medium on preculture seed

Figure 4.5 shows the kinetics profiles of SB7-2 cultivated in the preculture medium with different initial glucose concentrations. There was no evident of glucose repression observed among 3 concentrations studied. This resulted in similar rates of cell biomass production, glucose uptake, and lactate formation at the early stage. Nontheless, the final cell concentration and lactate titer increased with an increasing glucose concentration. For cell biomass production, the maximum OD reached ~2.8 with the initial glucose of 5 g/L while the maximum OD of ~4.8-4.9 was obtained with the initial glucose of 10 and 15 g/L. On the other hand, lactate formation kept increasing with the increasing glucose concentration used in this study. This could be presumably due to the increasing C/N ratio when initial glucose was increased with the same amount of nitrogen source added. At 10 g/L initial glucose concentration, the C/N ratio was proper for promoting growth with sufficient lactate production. Further increasing C/N ratio at 15 g/L initial glucose concentration drove more glucose flux towards lactate production resulted in lowering pH (data not shown) that was unfavorable for growth (Meng, Xue, Yu, Gao, & Ma, 2012). This somehow could generate metabolic stress of the preculture seed to be transferred to the fermentation stage. Therefore, 10 g/L initial glucose concentration was considered as the optimal glucose concentration in the preculture medium.

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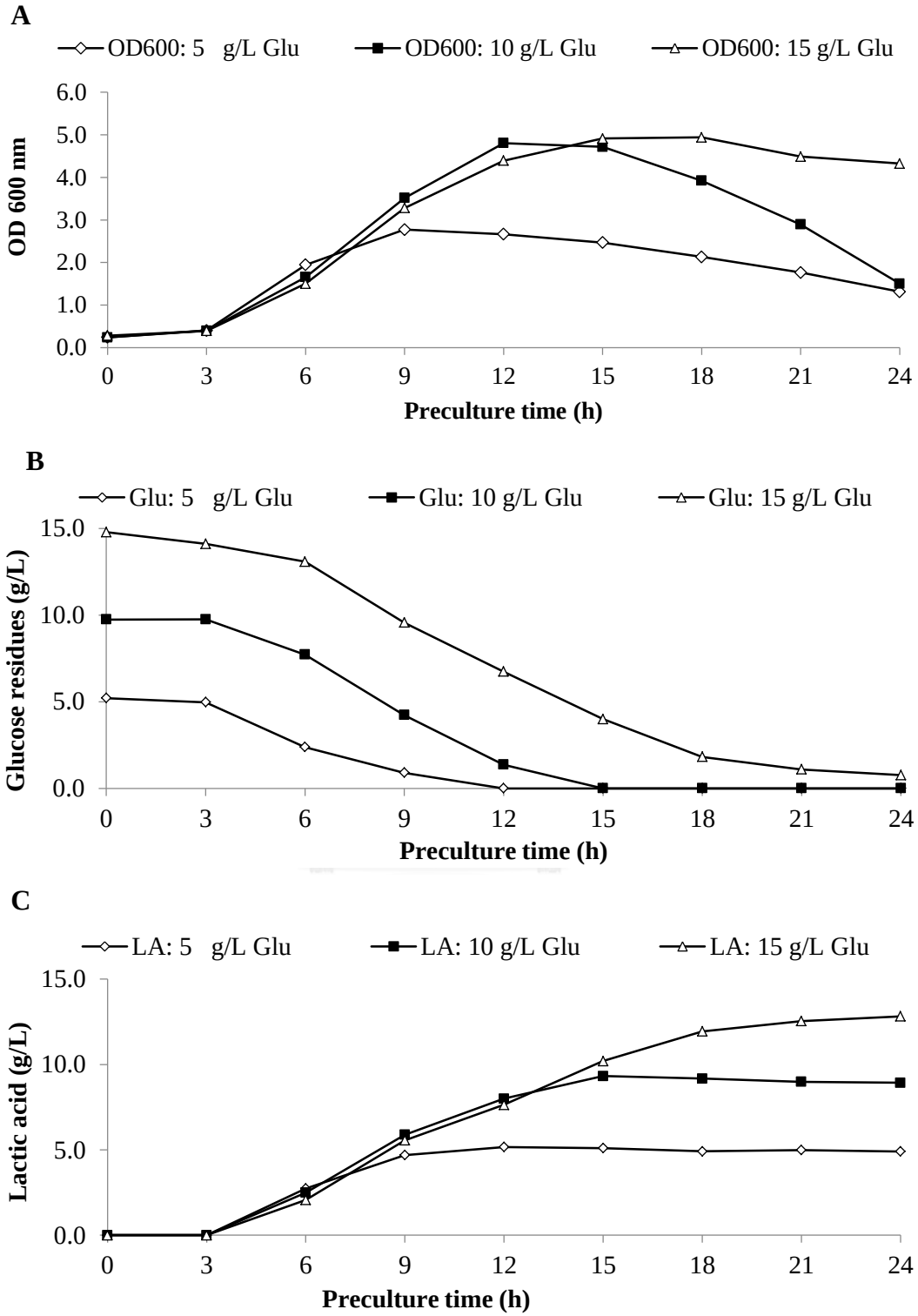

Figure 4.5 Preculture profile of *S. kofuensis* SB7-2 grown in preculture medium with different initial glucose concentration during preculture stage. A. Growth curves, B. Residual glucose, and C. D-lactic acid formation.

4.2.4 Effect of inoculum size on preculture seed of SB7-2

To establish the proper seed train for fermentation process, there are many important steps being involved including the inoculum size for transferring into the next process step. The cell density of the starter culture is strongly relevant to the performance of metabolic rates. Proper starter density leads to adequately high rates of biosynthesis within the shortest possible time (França, Jesus, & Oliveira, 2009).

In this part, the inoculum size being transferred into the preculture flask was varied. The results are shown in Figure 4.6. It was observed that increasing the inoculum size accelerated growth (Figure 4.6A). The maximum cell growth (OD600 of ~5.7) reached at 9 h with 3% (v/v) inoculum size with the short lag phase. Rapid glucose consumption (Figure 4.6B) was also found with high growth rate and lowest concentration of lactic acid (Figure 4.6C). The following criteria are nowadays recognized as necessary to obtain a good inoculum quality (Stanbury and Whitaker, 1984; Atkinson and Mavituna, 1992): (i) the inoculum must be in an active and healthy status to minimize the duration of the lag phase in the subsequent fermentation, (ii) it must be available in sufficiently large volume to provide an inoculum of optimum size (3–10% of the medium volume), (iii) the inoculum must be in a suitable morphological form, (iv) it must be free of contamination and (v) the inoculated biomass must retain its product-forming capabilities (Neves et al., 2000). From the results therefore 3% inoculum size was selected for the subsequent studies in this work.

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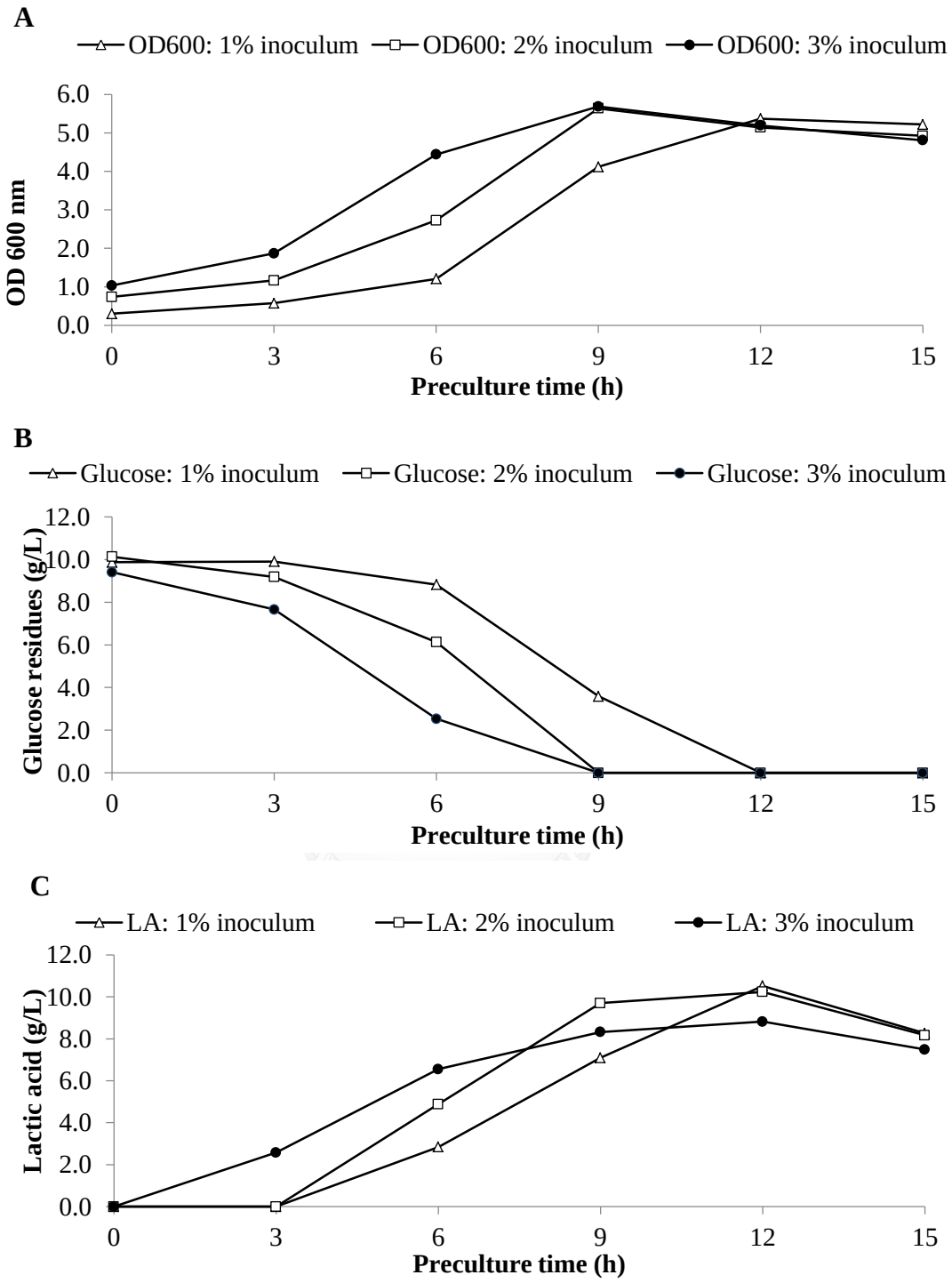

Figure 4.6 Preculture profile of *S. kofuensis* SB7-2grown in preculture medium in the shake flask. The preculture stage was inoculated with the 24-h slant inoculum at the different inoculum size by. A. Growth curves, B. Residual glucose, and C. D-lactic acid formation.

4.3 optimization of D-lactic acid fermentation in a 5-L stirred fermentor

4.3.1 Effect of the seed age during preculture stage on D-lactate ferementation by SB7-2

This part of works compared the effect of seed age transferred from the preculture flask on lactate production by *S. kofuensis* SB7-2 during fermentation stage. The seed ages tested were selected from the growth profiles shown in Figure 4.6 including the seed during early log (~3 h), mid log (~6 h), and late log (~9 h). The seed at different ages was transferred into the fermentation medium at 5% (v/v) inoculum size. Starting with the initial glucose concentration of 100 g/L during fermentation, the change in seed age showed the different lag state of the culture during fermentation while the final cell biomass and D-lactate production remained unchanged (Figure 4.7 and Table 4.3). The seed age of 3 h obviously caused the longer lag phase compared with other 2 conditions. Apparently, the seed at 12 h was required to enter log phase when lactate production started. The production was continued until 30 h when the highest lactate concentration of 91.28 g/L with the productivity of 3.04 g/L·h was acquired. When using the 6-h seed from preculture flask to be transferred into the fermentation stage resulted in the shorter lag phase (6 h) and the improved lactate productivity (3.97 g/L·h) (Figure 4.7B and Table 4.3). Further increasing the seed age from 6 h to 9 h did not show the significant change of lag phase and lactate production. The higher growth rate and lactate productivity obtained during fermentation stage when using the preculture seed at 6 and 9 h could be explained by the sufficiently high cell density being transferred from the preculture flask (OD transfer* in Table 4.3). In addition, the cells being transferred were in the active stage; therefore, when entering the new medium in the proper environmental control, they were prompt to metabolize substrates into end products. The longer lag phase observed during fermentation using 3-h seed could

be attributed to the fact that cells have yet entered rapid growth stage (mid log phase). Therefore, with the lower cell numbers and low metabolic rate, this resulted in longer lag phase during fermentation after transfer (Narita et al., 2004).

From the preliminary optimization for pursuing lactate production by SB7-2 in the fermentor, it could be summarized that higher yield and productivity were achieved when culturing SB7-2 in the fermentor as compared to that obtained in the shake flask culture. The optimized process steps included transferring the 24-h active GYP slant at 3% inoculum size into the preculture flask containing 10 g/L initial glucose. The preculture flask was incubated at 37 °C and 200 rpm for 6 h before being transferred (5% inoculum size) into the 5 L stirred fermentor operated at 37 °C and 200 rpm where the fermentation stage began. $CaCO_3$ was used for pH control during fermentation stage. At the end of the fermentation, the final lactate titer of 95.34 g/L with the yield 0.96 g/g and the productivity of 3.97 g/L·h were obtained within 24 h. The increasing productivity by 2.5 times compared to that obtained in the shake flask study was noticeable (Table 4.4). Nonetheless, further fermentation optimization would be conducted in the stirred fermentor in order to reduce the lag phase of SB7-2 during fermentation for further improved productivity to be comparable to other lactate producing strains being reported in previous published.

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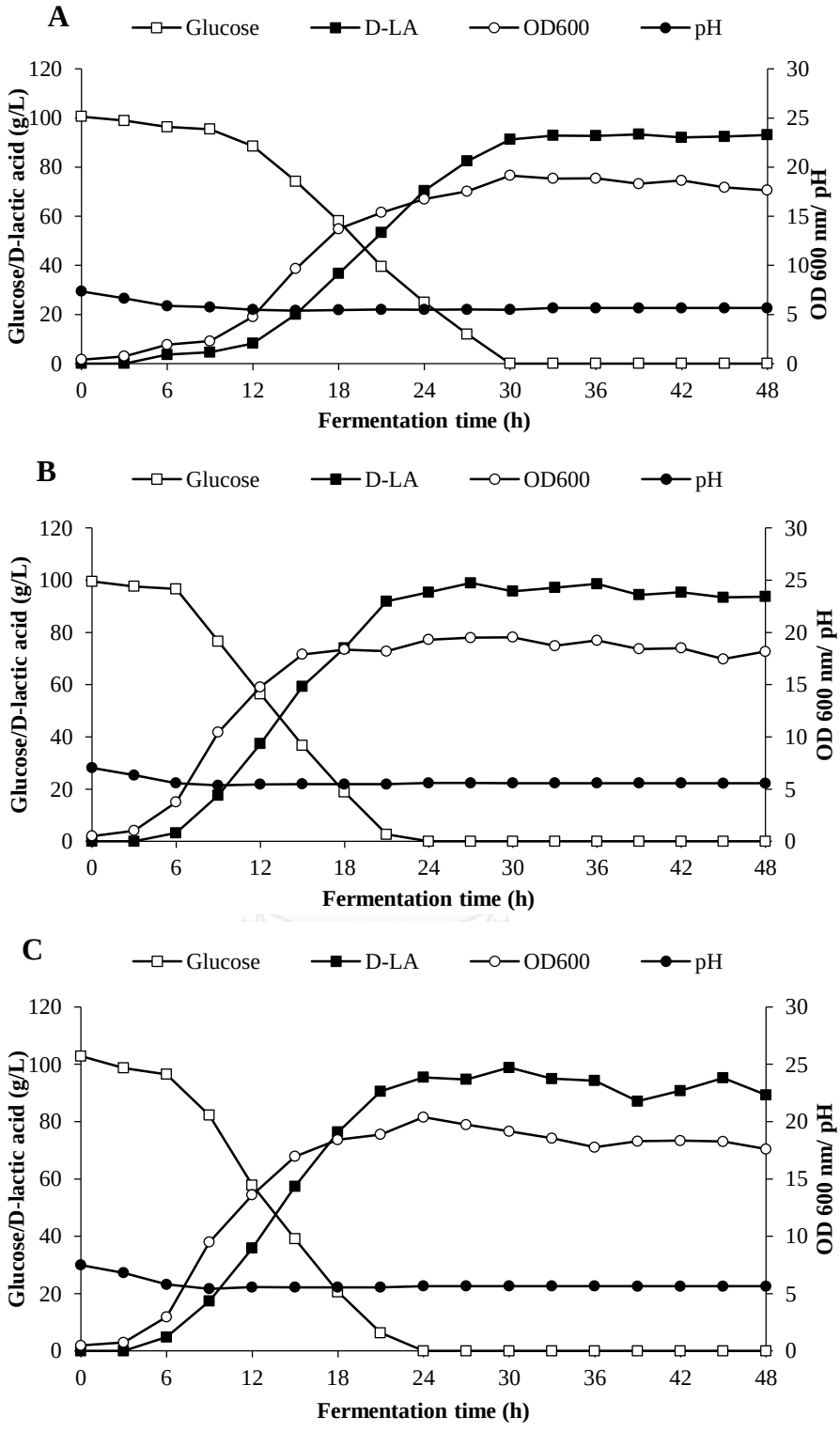

Figure 4.7 Fermentation profile of *S. kofuensis* SB7-2 during the fermentation stage. The culture was inoculated by the preculture broth with the different preculture ages. A. 3 h inoculum age, B. 6 h inoculum age and C. 9 h inoculum age.

Table 4.3 Fermentation kinetics data of *S. kofuensis* SB7-2 during the fermentation stage in the 5 L stirred fermentor. The culture was inoculated by the preculture seed at different ages in a shake flask culture at 5% inoculum size.

Inoculum age	OD transfer*	Fermentation time (h)	Maximum OD_{600}	LA (g/L)	Yield (g/g)	Productivity (g/L·h)
3 h	2.85	30	19.15	91.28	0.91	3.04
6 h	4.00	24	19.30	95.34	0.96	3.97
9 h	4.47	24	20.38	95.48	0.93	3.98

Remark: OD transfer* was the OD reading of the preculture seed to be transferred into the fermentor

Table 4.4 Comparison on D-lactate production by *S. kofuensis* SB7-2 in the shaken flask culture previously conducted with the developed fermentation protocol in the 5 L stirred fermentor (this study).

Operation	Initial glucose (g/L)	Final lactate (g/L)	Yield (g/g)	Productivity (g/L·h)	Optical purity (%ee)
Batch, Flask	120	112	0.93	1.60	95.8
Batch, Fermentor	100	95	0.96	3.97	97.6

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4.3.2 Determination of the proper inoculum size for lactate production by SB7-2

As aforementioned in 4.3.1, in this part, the length of the lag phase during fermentation stage was investigated at the same fermentation protocol using the different inoculum size. From Figure 4.8, it was obvious that increasing the inoculum size could reduce the lag phase. Transferring only 2 % (v/v) preculture seed into the fermentor caused longer lag phase of 6 h while using 5 % and 10 % (v/v) to inoculate the fermentor could shorten the lag phase to 3 h (Figure 4.8A). The maximum cell biomass production (OD600 of 21.58 at 24 h) was obtained from the culture inoculated with 10 % (v/v) preculture seed. This was consistent with lactate production and glucose uptake rate (Figures 4.8B and 4.8C). Increasing the inoculum size from 5 to 10 % (v/v) did not show the significant improvement on cell growth and lactate production. The findings in this part confirmed that the inoculum size besides the age had a strong impact on cell growth, substrate unilization, and product formation (Ding, Tian, Cheng, & Yuan, 2009).

The fermentation kinetics of SB7-2 grown in the fermentor inoculated with the preculture seed at different inoculm sizes shown in Table 4.5 also confirmed that 5% inoculum size was suitable for lactate fermention by SB7-2 (Argun & Dao, 2016).

Previous studies reported a few wild-type D-lactic acid producers that were isolated from soil and plant samples include *Lactobacillus delbrueckii*, *L. coryniformis* subsp. *torquens*, *Bacillus laevolacticus, Corynebacterium glutamicum* and *Sporolactobacillus* sp. (Joshi et al., 2010; Tashiro et al., 2011; L. Wang et al., 2011). However, among D-lactic acid producers, *Sporolactobacillus* sp. is a promising wild-type strain due to an acceptable high productivity and optical purity. To our knowledge, very high D-lactic acid titer of 207 g/L with the productivity of 3.8 g/L•h and the optical purity of 99.3 %ee was achieved from fermentation by *Sporolactobacillus* sp. CASD using peanut meal as the nitrogen source in a 30 L

fermentor via fed-batch operation (L. Wang et al., 2011). In particular *S. laevolacticus*, several researchers claimed that this species had high potential production of D-lactic acid. According to study of Mimitsuka et al. (2012), *S. laevolacticus* produced D-lactic acid at the final titer of 87 g/L with the yield of 0.89 g/g and the production rate of 2.9 g/L·h in batch operation. Later in 2013, Li et al. reported that fed-batch fermentation of D-lactic acid by *S. laevolacticus* DSM442 provided the high final titer up to 144.4 g/L in 35 h with the yield of 0.96 g/g and the productivity of 4.13 g/L·h. The obtained optical purity of the D-lactate product was 99.3%ee. Our in house isolate, *S. laevolacticus* SK5-2 produced D-lactic acid in a 5 L stirred fermentor from glucose medium via batch operation. The final titer of 115 g/L with the yield of 0.92 g/g, the productivity of 3.83 g/L·h, and the optical purity approaching 100 %ee was achieved (Jantawon, 2013).

Moreover, the process parameters including medium, pH, temperature, mixing, oxygen, and neutralizing agents were reported to affect lactic acid production. Jantawon (2013) studied the effects of inoculum size and age as well as the neutralizing agents on D-lactic acid fermentation by *S. nakayamae* subsp. *nakayamae* CU72-1. Within 54 h, the high final titer of 110 g/L with the yield of 0.91 g/g glucose, the productivity of 3.68 g/L·h, and the optical purity approaching 100 %ee was obtained when inoculating the fermentor at 10% inoculum size and 24 h inoculum age with controlled pH by $CaCO_3$. Furthermore, some studies noted that initial glucose concentration is one of important factors affecting the lactic acid production. Y. Li et al. (2013) reported that the concentration of D-lactate increased in proportion with initial glucose concentration, reaching a maximum of 76.9 g/L with 100 g/L of glucose within 48 h by *S. laevolacticus* DSM442 but D-lactate production and cell growth were inhibited at higher glucose concentration. This inhibition may be affected by high osmotic pressure created by high substrate and end-product

concentration. Owing to high osmotic pressure would disturb the fluid and electrolyte balance system of cells (Sochocka & Boratynski, 2011). Efforts were made to improve the productivity by decreasing the osmotic inhibition such as; screened osmotic-tolerant strains and modified strains were used for lactic acid fermentation; Fermentation strategies such as simultaneous saccharification and fermentation (SSF) and fed-batch fermentation were studied to obtain the higher productivity by a low level of initial substrate concentration (Choi et al., 2014; Ge et al., 2011; Zhang & Vadlani, 2013; L. Zhou et al., 2012).

Table 4.5 Comparative evaluation of major data obtained using various inoculum sizes in D-lactic acid fermentation by *S. kofuensis* SB7-2

Inoculum size	Fermentation time (h)	Maximum OD_{600}	Glucose consumption rate (g/L˙h)	LA (g/L)	Yield (g/g)	Productivity (g/L˙h)
2%	27	19.35	3.70	96.29	0.90	3.57
5%	24	19.30	4.17	98.95	0.96	3.97
10%	24	21.58	4.17	99.22	0.97	4.10

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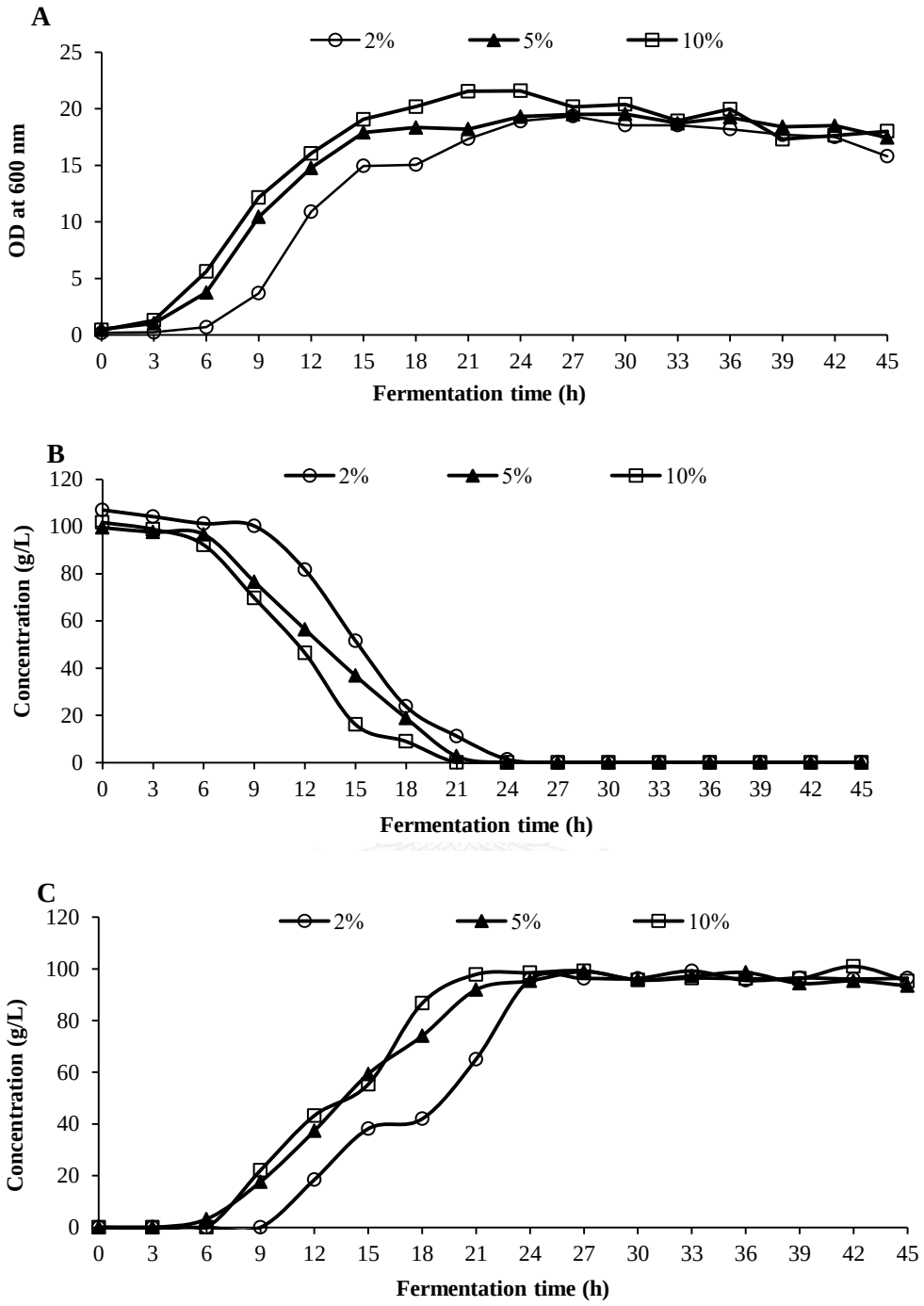

Figure 4.8 Comparison of cell growth, glucose consumption, and D-lactic acid production during fermentation stage when the culture was inoculated by diferrent inoculum sizes of *S. kofuensis* SB7-2 prepared in preculture flask. A. cell growth, B. glucose consumption, and C. D-lactic acid production.

CHAPTER 5

CONCLUSION AND SUGGESTION

5.1 Conclusion

Isolated SB7-2 was a promising D-lactic acid producer which was screened from tree bark at Saraburi province, Thailand. This isolated was closely related to *S. kufuensis* DSM 11701^{T} at the similarity percentage of 99.86%. Isolate SB7-2 was a rod shaped, gram positive, facultative anaerobe, and spore-forming bacterium. From the biochemical test, this isolated showed negative effect on oxidase, catalase, and nitrate reduction. SB7-2 could grow at the temperature in between 20 °C and 40 °C. The growth pH was in between 4.5 and 8.0. Other than glucose, several carbon sources could be uptaken for lactic acid production by this isolate, e.g. N-acetylglucosamine, D-fructose, D-glucose, D-mannose, D-mannoitol, D-raffinose, L-rhamnose, D-saccharose (sucrose), D-Tagatose, and D-trehalose. However, there are some carbohydrates that could nost be utilized by SB7-2, including sugar alcohol (e.g., arabitol, dulcitol, and erythritol), reducing sugar (e.g., D-cellobiose, lactose, and maltose), pentose sugar (e.g., ribose, arabinose, and xylose), polymeric carbohydrates (e.g., starch), glycerol, and fructose.

The preliminary results showed that *S. kofuensis* SB7-2 could produce D-lactic acid at the final titer up to 112 g/L with the remarkable yield of 0.93 g/g and productivity of 1.6 g/L·h from the simple medium containing 120 g/L glucose during anaerobic cultivation in the shaken flask. So this isolate was considered as the promising D-lactic acid producer. To develop pattern of D-lactic acid fermentation in *S. kofuensis* SB7-2, process optimization to determine the preculture conditions was studied in both the shaken flask and the stirred fermentor. It was found that to

obtain high lactate production in the shaken flask culture, the 24-h culture slant was used to prepare the stock culture. The stock culture was inoculated into the preculture medium containing 10 g/L glucose at 3% inoculum size. The seed culture was anaerobically cultivated at 200 rpm. Later, batch fermentation of D-lactic acid was also studied in the 5-L stirred fermentor. The seed culture for the 5-L stirred fermentor was prepared in the shaken flask. To produce D-lactic acid in the stirred fermentor, optimization of inoculum age and size was also conducted. It was found that higher productivity and product yield were obtained by the following optimized conditions, e.g., using 5% inoculum size at 6-h incubation in the shaken flask being inoculated into the fermentor. The seed culture in the shaken flask was prior prepared by transferring 3% inoculum size of the 24-h active slant into the preculture medium containing 10 g/L glucose. Using the optimized conditions and process steps determined in this thesis, the highest D-lactic acid of 98.95 g/L with the yield 0.96 g/g and the productivity of 3.97 g/L·h was obtained within 24 h. This productivity was 2.5 times higher than that from the preliminary result in the shake flask culture.

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5.2 Suggestion

This study attempted to describe several factors that could impact on the development of D-lactic acid production by SB7-2. With the optimized conditions determined here, SB7-2 exhibited the high potential on D-lactic acid production. However, to further increase the production of D-lactate by this isolate, higher initial substrate concentration or other inexpensive substrates such as lignocellulosic materials should be tested for D-lactate production. Increasing substrate concentration should be determined. Moreover, from the results in both shake flask and fermentor, the optical purity of D-lactic acid (%ee) in SB7-2 was still lower

compared to other strains; therefore, improving on optical purity in this isolated would be of further study.

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APPENDIX A

CULTURE MEDIUM AND REAGENTS

1. GYP agar

Glucose	10	g/L
Yeast extract	5	g/L
Peptone	5	g/L
KH_2PO_4	0.25	g/L
K_2HPO_4	0.25	g/L
Salt solution	10	ml/L
$CaCO_3$	5	g/L
Agar	20	g/L

The ingredients were dissolved and final volume was adjusted to 1,000 mL with deionized water. The pH was adjusted to 6.8 with 1 M NaOH. Then, the solution was sterilized by autoclaving for 15 min at 121 ºC.

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2. Salt solution

$MgSO_4.7H_2O$	400	mg
$MnSO_4.5H_2O$	20	mg
$FeSO_4.7H_2O$	20	mg
NaCl	20	mg

The ingredients were dissolved and final volume was adjusted to 10 mL.

3. 10 % Skim milk

Skim milk	10	g

The ingredient was dissolved and final volume was adjusted to 100 ml with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 115 ºC.

4. Preculture broth (GYP medium)

Glucose	10	g/L
Yeast extract	5	g/L
Peptone	5	g/L
KH_2PO_4	0.25	g/L
K_2HPO_4	0.25	g/L
Salt solution	10	ml/L
$CaCO_3$	5	g/L

The ingredients were dissolved and final volume was adjusted to 1,000 ml with deionized water. The pH was adjusted to 6.8 with 1 M NaOH. Then, the solution was sterilized by autoclaving for 15 min at 121 ºC.

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5. Fermentation broth

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

The ingredients were dissolved and final volume was adjusted to 1,000 ml with deionized water. The pH was adjusted to 6.8 with 1 M NaOH. Then, the solution was sterilized by autoclaving for 15 min at 121 ºC.

6. A medium agar

Glucose	10	g/L
Yeast extract	5	g/L
Peptone	5	g/L
CH_3COONa	10	g/L
Salt solution	10	ml/L
$CaCO_3$	5	g/L
Agar	20	g/L

The ingredients were dissolved and final volume was adjusted to 1,000 ml with deionized water. The pH was adjusted to 6.8 with 1 M NaOH. Then, the solution was sterilized by autoclaving for 15 min at 121 ºC.

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7. Nitrate reduction

Yeast extract	5	g/L
Peptone	10	g/L
NaCl	10	g/L
KNO_3	1	g/L

The ingredients were dissolved and final volume was adjusted to 1,000 ml with deionized water. The pH was adjusted to 6.8 with 1 M NaOH. Then, the solution was sterilized by autoclaving for 15 min at 121 ºC.

8. Spore formation

Glucose	1	g/L
Yeast extract	10	g/L
Peptone	10	g/L
CH_3COONa	10	g/L
Salt solution	10	ml/L
$CaCO_3$	5	g/L
Agar	20	g/L

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The ingredients were dissolved and final volume was adjusted to 1,000 ml with deionized water. The pH was adjusted to 6.8 with 1 M NaOH. Then, the solution was sterilized by autoclaving for 15 min at 121 ºC.

9. Acid production

Carbon source	0.5	g
Yeast extract	0.5	g
Peptone	0.5	g
Salt solution	0.25	ml

The ingredients were dissolved and final volume was adjusted to 1,000 ml with deionized water. The pH was adjusted to 6.8 with 1 M NaOH. Then, the solution was sterilized by autoclaving for 10 min at 110 ºC.

10. Starch hydrolysis

Soluble starch	20	g/L
Yeast extract	10	g/L
Peptone	10	g/L
CH_3COONa	10	g/L
Salt solution	5	g/L
Agar	20	g/L

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The ingredients were dissolved and final volume was adjusted to 1,000 ml with deionized water. The pH was adjusted to 6.8 with 1 M NaOH. Then, the solution was sterilized by autoclaving for 15 min at 121 ºC.

11. Arginine hydrolysis

Peptone	1	g/L
NaCl	5	g/L
K_2HPO_4	0.3	g/L
L(+) arginine HCl	10	g/L
Agar	15	g/L

The ingredients were dissolved and final volume was adjusted to 1,000 ml with deionized water. The pH was adjusted to 6.8 with 1 M NaOH and added phenol red indicator. Then, the solution was sterilized by autoclaving for 15 min at 121 °C.

12. Standard of lactic acid, glucose, acetic acid and ethanol

Glucose	0.1	g
Lactate.$5H_2O$	0.1712	g
Ethanol	105.3	µl
Acetic acid	95	µl

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The ingredients were dissolved and final volume was adjusted to 50 ml with ultrapure water.

13. Ammonium oxalate crystal violet

A solution

Crystal violet	2	g
Ethyl alcohol	20	ml

B solution

Ammonium oxalate	0.8	g
Deionized water	80	ml

14. Lugol's solution

Iodine	1	g
KI	2	g
Deionized water	300	ml

15. Counterstain solution

Safranin O	10	ml
Deionized water	100	ml

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16. Nitrate solution

A solution:

Sulfanilic acid	0.8	g
Acetic acid (5N)	100	ml

B solution:

Dimethyl-naphthylamine	0.06	g
Acetic acid (5N)	100	ml

17. Mixed indicator

Bromthymol blue	0.2	g
Neutral red	0.1	g
Ethanol	300	ml

18. N,N,N',N'-Tetramethyl-1,4-phenylene diamine dihydrochloride

N,N,N',N'-Tetramethyl-1,4-phenylene diamine dihydrochloride 1 loop was dissolved in 3 ml deionized water.


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APPENDIX B

PRODUCT DETERMINATION

1. Product determination

Amounts of products formed and carbon substrates remained during the fermentation were analyzed by high performance liquid chromatography (HPLC). Fermentation samples were centrifuged, filtered through PTFE (hydrophilic) membrane, and diluted with DDI water. For analyses of glucose remained, total lactic acid (both L- and D-lactate), and acetic acid produced, 15 µL diluted particle-free samples were automatically injected (Shimadzu) into an organic acid analysis column (Biorad, Aminex HPX-87H ion exclusion organic acid column; 300mm × 7.8mm) maintained at 45°C in a column oven (Shimadzu-CTO-10A). 0.005 M H_2SO_4, was pumped through the system at the flow rate of 0.6 mL/min (Shimadzu-LC-10Avp). A refractive index detector (Shimadzu-RID-10A) was used to detect the organic compounds. The standards containing 0-2 g/L of each component (glucose, lactate, acetate and ethanol) were injected as the references to determine the sample concentration. The peak area was used in determining the concentration.

Lactic acid isomeric purity is measured by HPLC using a chiral column (Sumipack, Sumichiral OA5000) at 40 °C, eluted with 1 mM $CuSO_4$ at flow rate of 1.0 mL/min and detected at 254-nm with a UV detector. The standards containing 0-2 g/L of each component (D-lactate and L-lactate) were injected as the references to determine the sample concentration. The peak area was used in determining the concentration. The optical purity of D-lactic acid was defined as follows: D-lactic acid/(L-lactic acid + D-lactic acid) x 100%.

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2. Product yield ($Y_{p/s}$)

Product yield was determined from the ratio of product formed to glucose consumed during fermentation.

Example D-lactic acid fermentation in 5-L stirred fermentor by *S. kofuensis* SB7-2

Table B-1 Product yields in production phase

Time (h)	Glucose (g/L)		Lactic acid (g/L)	Yield
	Initial	Residual		(g/g glucose)
0	100.57	100.57	0.00	0.00
27	100.57	11.95	82.46	0.93
30	100.57	0.00	91.28	0.91
33	100.57	0.00	92.89	0.92

The lactic acid yield in fermentation at 33 h of 0.92 g/g glucose.

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3. Productivity (g/L.h)

Volumetric productivity was defined as the total amount of product formed per volume per time.

Example D-lactic acid fermentation in 5-L stirred fermentor by *S. kofuensis* SB7-2

Table B-2 Volumetric productivities in production phase

Time (h)	Glucose (g/L)		Lactic acid (g/L)	Productivity (g/L.h)
	Initial	Residual		
0	100.57	100.57	0.00	0.00
27	100.57	11.95	82.46	3.05
30	100.57	0.00	91.28	3.04
33	100.57	0.00	92.89	2.81

The lactic acid productivity in fermentation at 33 h of 2.81 g/L.h.

4. Optical purity of D-lactic acid (%ee.)

The optical purity of D-lactic acid was defined as follows: D-lactic acid/(D-lactic acid + L-lactic acid) × 100%.

Example D-lactic acid fermentation in 5-L stirred fermentor by *S. kofuensis* SB7-2

Table B-3 Optical purity of D-lactic acid by *S. kofuensis* SB7-2 in production phase

Time (h)	Area of D-Lactic acid	Area of L-Lactic acid	D-Optical purity (%ee.)
0	ND	ND	ND
27	1327570	29872	95.60
30	1510659	32247	95.82
33	1473142	27532	96.33

ND, not determined

The D-lactic acid optical purity in fermentation at 33 h of 96.33 %ee.

APPENDIX C

NECLEOTIDE SEQUENCING

16S rRNA gene sequence of *S. kofuensis* SB7-2

GGAGCTTGCGTCCCTGAGGTGAGCGGCGGATGGGTGAGTAACACGTGGGCAACCTGCCTGTCAGACTGGGAT
AACTGTGGGAAACCGCAGCTAATACCGGATAATCCTCTGCACCGCATGGTGCAGGGTTGAAAGATGGTTTCGG
CCATCAATGACAGATGGGCCCGCGGTGCATTAGTTAGTTGGCGGGGTAACGGCCCACCAAGACCGCGATGCAT
AGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGCGAAGAAGGTTTTCGGATCGTAAA
GCTCTGTTGCTGGAGAAGAACGAGTGCTAGAGGAAATGCTAGTGCTGTGACGGTATCCAGCCAGAAAGCCACG
GCTAAATACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGC
GCGCAGGCGGCTTCTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAACGGTCATTGGAAACTGGGAAGC
TTGAGTGCAGAAGAGGAGAGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCAGTG
GCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGGTCCAACCCTTAGTGCTGAAGTTACACATT
AAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGT
GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAAGCCTAGAGA
TAGGCCGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATCTGGGCTACACACGTGC
TACAATGGGTGGTACAAAGGGCAGCGAAACCGCGAGGTCGAGCTAATCCCATAAAGCCACCCCCAGTTCGGAT
TGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATCCGT
TCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGCGAGAACCTTTA
TGGACTCAGCCGCCGAAGGTGG

VITA

Miss Orachorn Thongdonngaw was born on Friday 17th May, 1991, in Bangkok, Thailand. In 2013, she graduated with a Bachelor's degree of Science (First Class Honors) in Biotechnology, from King Mongkut's Institute of Technology Ladkrabang. After that, she has been studied for a Master's degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University, and completed the program in 2016.

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Thongdonngaw, O., Tolieng, V., Thitiprasert, S., Piluk, J., and Thongchul, N. Effects of preculturing conditions on growth of Sporolactobacillus kofuensis SB7-2 to increase productivity of D-lactic acid fermentation. Poster presentation and proceedings. The 27th Annual Meeting of the Thai Society for Biotechnology and International Conference "TSB 2015: Innovative Biotechnology". 17-20 November 2015. Mandarin Hotel Bangkok managed by Centre Point, Bangkok, Thailand.

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