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SCREENING OF SPORE FORMING D-LACTIC ACID PRODUCING BACTERIA AND ITS OPTIMIZATION

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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บุษบาทิพย์ ประเสริฐศักดิ์ : การคัดกรองแบคทีเรียสร้างสปอร์ที่ผลิตกรดดีแล็กติกและการหาภาวะเหมาะสม (SCREENING OF SPORE FORMING D-LACTIC ACID PRODUCING BACTERIA AND ITS OPTIMIZATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ณัฏฐา ทองจุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. สมบูรณ์ ธนาศุภวัฒน์, 119 หน้า.

งานวิจัยนี้มุ่งเน้นการคัดแยก คัดกรอง หาภาวะเหมาะสม รวมถึงการแสดงออกของเอนไซม์ที่สำคัญที่เกี่ยวกับการผลิตกรดดีแล็กติกสำหรับแบคทีเรียสายพันธุ์ใหม่ที่คัดเลือกได้โดยให้ค่า ผลผลิต อัตราการผลิต และค่าความบริสุทธิ์เชิงแสงของกรดดีแล็กติกที่สูง ผลจากการคัดกรองพบว่า NK26-11^{T} เป็นแบคทีเรียสายพันธุ์ใหม่ แบคทีเรียแกรมบวก สร้างคะตะเลส เจริญได้ทั้งภาวะมีและไม่มีอากาศ สร้างสปอร์ที่รูปร่างทรงแท่ง ที่คัดแยกได้จากดินในประเทศไทย สายพันธุ์นี้สามารถผลิตกรดดีแล็กติกจากกลูโคสแบบโฮโมเฟอร์เมนเททีฟ เจริญที่อุณหภูมิ 20-45 องศาเซลเซียส และพีเอช 5-8.5 ผนังเซลล์มี *meso*-diaminopimelic acid เป็นองค์ประกอบของเพปทิโดไกลแคน ในกระบวนการหายใจประกอบด้วยเมนาควิโนนชนิด 7 เป็นองค์ประกอบหลัก ดีเอนเอมีองค์ประกอบของก้วนีนกับไซโตซีนหลัก 42.6 เปอร์เซ็นต์โมล และองค์ประกอบกรดไขมันหลักแบบ anteiso-$C_{15:0}$ และ anteiso-$C_{17:0}$ ผลจากการวิเคราะห์ลำดับเบสในช่วง 16S rRNA gene พบว่าสายพันธุ์ NK26-11^{T} มีความสัมพันธ์ใกล้เคียง *Bacillus solimangrovi* JCM 18994^{T} (93.89 เปอร์เซ็นต์) *Pullulanibacillus naganoensis* LMG 12887^{T} (93.32 เปอร์เซ็นต์) *Sporolactobacillus inulinus* NRIC 1133^{T} (92.99 เปอร์เซ็นต์) *Tuberibacillus calidus* JCM 13397^{T} (92.98 เปอร์เซ็นต์) และ *Thalassobacillus devorans* DSM 16966^{T} (น้อยกว่า 90.93 เปอร์เซ็นต์) สายพันธุ์ NK26-11^{T} เป็นแบคทีเรียสกุลใหม่ที่เป็นตัวแทนระหว่างสกุล *Bacillus* และ *Sporolactobacillus* จึงได้เสนอ *Terrilactibacillus laevilacticus* เป็นแบคทีเรียสกุลใหม่โดยมี NK26-11^{T} เป็นตัวแทนของสกุลและสปีชีส์ (=LMG 27803^{T} =TISTR 2241^{T} =PCU 335^{T}) ผลจากการคัดกรองแบคทีเรียที่ผลิตกรดดีแล็กติกพบว่า *Terrilactibacillus laevilacticus* SK5-6 มีประสิทธิภาพในการผลิตกรดดีแล็กติก (กรดแล็กติกสุดท้าย 99.27 กรัมต่อลิตร ผลผลิต 0.90 กรัมของกรดดีแล็กติกต่อกรัมของกลูโคสที่ใช้ไป อัตราการผลิต 1.38 กรัมต่อลิตรต่อชั่วโมง และค่าความบริสุทธิ์เชิงแสงของกรดดีแล็กติก 99.00 เปอร์เซ็นต์) มากกว่าสปีชีส์ *Sporolactobacillus* และ *Terrilactibacillus* ไอโซเลทนี้สามารถผลิตกรดดีแล็กติกจากแหล่งน้ำตาลที่หลากหลาย ซึ่งตรงข้ามกับสายพันธุ์ *Sporolactobacillus* ซึ่งเป็นแบคทีเรียที่ผลิตกรดดีแล็กติกและไม่ผลิตคะตะเลส จากคุณลักษณะของ *T. laevilacticus* SK5-6 ที่ผลิตคะตะเลสได้จึงแบ่งการทดลองเป็นสองขั้นตอนสำหรับการผลิตกรดดีแล็กติก ขั้นตอนการเลี้ยงหัวเชื้อบ่มในภาวะมีอากาศส่งผลให้ได้มวลเซลล์ปริมาณมากในระยะเวลาที่สั้น การเตรียมหัวเชื้อในระยะที่เหมาะสม (อายุหัวเชื้อ) และความเข้มข้นมวลเซลล์ (ปริมาณหัวเชื้อ) ถ่ายเทสู่กระบวนการหมัก พบว่าสายพันธุ์ SK5-6 สามารถใช้กลูโคสเปลี่ยนเป็นกรดดีแล็กติกได้อย่างรวดเร็วภายใต้ภาวะ ไร้อากาศส่งผลให้ได้กรดดีแล็กติกสุดท้ายในปริมาณที่สูง (102.22 กรัมต่อลิตร) ผลผลิต (0.84 กรัมต่อกรัม) อัตราการผลิต (2.13 กรัมต่อลิตรต่อชั่วโมง) และไม่เกิดผลิตภัณฑ์ข้างเคียง นอกจากนี้ยังพบว่าสายพันธุ์ SK5-6 แสดงผลจลนพลศาสตร์ของการหมัก และการแสดงออกของเอนไซม์มากกว่า *S. laevilacticus* ซึ่งเป็นแบคทีเรียผลิตกรดดีแล็กติกที่ไม่สร้างคะตะเลส ฟอสโฟฟรุกโตไคเนสแสดงกิจกรรมของเอนไซม์ในปริมาณที่ต่ำซึ่งเกี่ยวข้องกับวิถีไกลโคไลซิสส่งผลต่ออัตราการผลิตกรดดีแล็กติกของสายพันธุ์ SK5-6 ในระหว่างกระบวนการหมักกรดแล็กติกพบว่าเมื่อพีเอชเพิ่มขึ้นส่งผลกระตุ้นการทำงานของดีแล็กเทตดีไฮโดรจีเนส มากกว่าแอลแล็กเทตดีไฮโดรจีเนส ส่งผลให้ค่าความบริสุทธิ์เชิงแสงของกรดดีแล็กติกสูงขึ้น ในขณะที่พีเอชค่าความเป็นกรดส่งผลต่อการทำงานของไคเนสในวิถีไกลโคไลซิส การเกิดไอโซเมอร์ไรเซชั่นจากการเปลี่ยนกรดแอลแล็กเทตเป็นกรดดีแล็กเทตโดยไอโซเมอเรส สามารถพบในระหว่างการหมักกรดแล็กติก จากการศึกษาครั้งนี้พบว่าคุณลักษณะที่โดดเด่นของสายพันธุ์ SK5-6 โดยเฉพาะผลผลิตที่สูง และไม่เกิดผลิตภัณฑ์ข้างเคียง จากคุณลักษณะที่สำคัญสามารถกล่าวได้ว่าเป็นแบคทีเรียที่เหมาะสมกับอุตสาหกรรมการหมักกรดดีแล็กติก

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5572876123 : MAJOR BIOTECHNOLOGY
KEYWORDS: D-LACTIC ACID BACTERIA / SCREENING / TERRILACTIBACILLUS LAEVILACTICUS / IDENTIFICATION / ENZYME EXPRESSION / 2-PHASE FERMENTATION / CATALASE POSITIVE / HOMOFERMENTATIVE

BUDSABATHIP PRASIRTSAK: SCREENING OF SPORE FORMING D-LACTIC ACID PRODUCING BACTERIA AND ITS OPTIMIZATION. ADVISOR: ASSOC. PROF. NUTTHA THONGCHUL, Ph.D., CO-ADVISOR: PROF. SOMBOON TANASUPAWAT, Ph.D., 119 pp.

In this study, isolation, screening, fermentation optimization, and determination of the expression of key enzymes involving in D-lactic acid production were conducted for selection of the novel D-lactic acid isolate with the high yield, productivity, and optical purity. From the screening experiment, the novel isolate, NK26-11^T, was obtained. The isolate NK26-11^T was a Gram-stain-positive, catalase-positive, facultatively anaerobic, spore-forming, rod-shaped bacterium isolated from soil sample in Thailand. This isolate homofermentatively fermented glucose for D-lactic acid and grew at the wide range of temperature between 20 and 45 °C and the pH of 5-8.5. The cell-wall peptidoglycan of NK26-11^T contained *meso*-diaminopimelic acid. The major respiratory quinone was menaquinone 7 (MK-7), the DNA G+C content was 42.6 mol%, and the major cellular fatty acids were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. On the basis of 16S rRNA gene sequences analysis, the isolate NK26-11^T was closely related to *Bacillus solimangrovi* JCM 18994^T (93.89% 16S rRNA gene sequence similarity), *Pullulanibacillus naganoensis* LMG 12887^T (93.32%), *Sporolactobacillus inulinus* NRIC 1133^T (92.99%), *Tuberibacillus calidus* JCM 13397^T (92.98%) and *Thalassobacillus devorans* DSM 16966^T (<90.93%). The isolate NK26-11^T represents a novel species of a new genus between *Bacillus* and *Sporolactobacillus* clusters, for which the name *Terrilactibacillus laevilacticus* gen. nov., sp. nov. was proposed. The type strain of the type species is NK26-11^T (=LMG 27803^T =TISTR 2241^T =PCU 335^T). From the fermentation screening of the selected D-lactic acid producing isolates, it was found that *Terrilactibacillus laevilacticus* SK5-6 exhibited a good D-lactate production performance (99.27 g/L final lactate titer with 0.90 g/g yield, 1.38 g/L.h, and 99.00% D-enantiomer equivalent) compared with other *Sporolactobacillus* sp. and *Terrilactibabacillus* sp. This isolate could ferment a wide range of sugars for D-lactic acid. Unlike the typical D-lactic acid producers, such as catalase negative *Sporolactobacillus* sp., *T. laevilacticus* SK5-6 acquired catalase activity; therefore, a 2-phase fermentation was simply employed for D-lactic acid production. Under an aerobic preculture stage, high-cell-density biomass was rapidly obtained as the result of aerobic respiration. At the correct physiological stage (inoculum age) and a proper concentration of biomass (inoculum size) transferred to the fermentation stage, SK5-6 rapidly converted glucose into D-lactic acid under anaerobic conditions resulting in a high final lactic acid titer (102.22 g/L), yield (0.84 g/g), and productivity (2.13 g/L.h) without byproduct formation. It was found that SK5-6 exhibited both fermentation kinetic and expression level of the key enzymes higher than those of *S. laevilacticus*, a catalase negative D-lactate producer. Low phosphofructokinase activity revealed that glycolysis controlled the apparent D-lactic acid productivity by SK5-6. Increasing the pH during fermentation phase activated the activity of D-LDH (D-lactate dehydrogenase) beyond that of L-LDH, resulting in the high optical purity of D-lactate, while the acidic pH promoted the activities of the kinases in glycolysis. The conversion of L-lactate to D-lactate by isomerase was also observed during fermentation. The findings in this study demonstrated the remarkable characteristics of SK5-6, in particular, a high product yield was obtained without byproduct formation. From the key characteristics of SK5-6, this isolate can be claimed as an industrial D-lactic acid producer.

Field of Study: Biotechnology
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I

INTRODUCTION

1.1 Significance of the study

Climate change and global warming become significantly impact on the environmental sustainability and lives. Many ways have been proposed to solve the global warming issue; those include using clean energy, green products, and smart energy solutions. One of the promising green products is bioplastics. Bioplastics made from biobased feedstocks via the biorefinery platform are considered as biocompatible, compostable, and renewable thus, helps minimize the environmental pollution unlike the petroleum based plastics widely available nowadays. Polylactic acid (PLA) is one of the bioplastics synthesized by direct condensation and polymerization of the optically pure lactic acid monomer. During the first generation of PLA product, it was produced via ring opening polymerization of L-lactide synthesized from dimerization of the optically pure L-isomer of lactic acid which has been widely available for food and pharmaceutical industries. Poly-L-lactic acid (PLLA) somehow has limited application compared to the petroleum based plastics due to its thermal and mechanical properties. Many attempts have been made to improve the properties of PLA so that it can substitute the conventional plastics currently available. Stereocomplex PLA tailored made from copolymerizing the pure L-isomer with the pure D-isomer of lactic acid gave the superior thermal and mechanical properties of the new generation of PLA products as it provided the increasing melting point (230 °C) compared with that of the 1st generation product (180 °C). This finding has generated the new market for D-lactic acid which has not been of commercial interest previously due to it was considered as toxic to the living systems when exposed at a high level.

Lactic acid commonly available in the market contains mainly the L-isomer with the optical purity approximately 85 %ee. This lactic acid product is produced via fermentation by the L-lactic acid producers such as *Bacillus* strains and *Lactobacillus* strains. Previous screening study reported that D-lactic acid producers scarcely existed in nature. Nonetheless, it was found that *Sporolactobacillus* strains and *Leuconostoc* strains are the natural D-lactic acid producers. Genetic modification was one tool that has been applied to the microbes of interest. Engineered *Escherichia coli* to produce D-lactic acid has also been reported. It should be noted that working with the engineered microbes has to bear with the chance of genetic instability after several passages and long term cultivation therefore, wild strain is preferable. In this work, the screening and identifying of D-lactic acid producers from the nature will be conducted. The first attempt is to obtain the isolates that produce an optically high D-lactic acid with high yield and productivity. The selected isolates will be used in D-lactic acid fermentation in flask to obtain the optimized condition.

It was claimed that lactate isomerization occurred during fermentation by *S. laevolacticus* and *S. inulinus* strains. Therefore, controlling the process conditions help maintain the optical purity percentage of lactic acid product. The expression of the key enzymes involved in lactate formation and isomerization including L-lactate dehydrogenase, D-lactate dehydrogenase, and lactate isomerase will be investigated during fermentation.

In addition, the fermentation process kinetics will be investigated under different operating conditions. In general, the naturally occurred lactic acid bacteria are facultative anaerobes which do not require oxygen for growth and metabolism. This explains the low productivity of lactic acid in the wild strains. Recently, some study reported the improved L-lactate productivity in the catalase producing L-lactate producers such as *B. coagulans* due to the ability to rapidly grow under aerobic conditions thus, resulting in the high cell concentration process. This is because cell growth under respiratory condition yields higher energy production per molecule of glucose consumed compared to that under fermentative condition. However, oxygen and its intermediates in an aqueous form are considered as toxic to cell when exposing at the critical level especially for anaerobes. In aerobes, on the other hand, they possess the enzymes that catalytically scavenge these aqueous oxygen intermediates thus, not harm to the cells. Catalase possessed in some lactic acid bacteria plays role against oxygen toxicity thus, allowing this group of bacteria to rapidly grow under aerobic condition resulting in high cell density process and enhanced productivity which is commonly required in industrial fermentation. Therefore, in this work, we screened and identified D-lactic acid bacteria that could achieve high yield, productivity, and optical purity from natural habitats and determined the optimal conditions for growth and D-lactic acid production. In order to achieve that, the mechanism and expression level of lactate dehydrogenase, lactate and isomerase were be determined. In addition, their roles to control the productivity and the optical purity during growth and fermentation were observed.

1.2 Objective and scope of research

In this study, isolation and screening of D-lactic acid producing bacteria were conducted. The goal is to seek for the novel D-lactic acid producer that can achieve high yield, productivity, and optical purity. In order to achieve that, further characterization on key enzymes involving in lactic acid production and growth (L-LDH, D-LDH, isomerase, PFK and PYK) was conducted.

CHAPTER II

THEORETICAL AND LITERATURE REVIEW

2.1 Lactic acid

Lactic acid, or 2-hydroxypropanoic acid, is an organic active molecule. Its chemical formula is $C_3H_6O_3$ and it contains a hydroxyl and carboxylic acid group. Lactic acid is a chiral carbon atom which has two optical isomers: L(+)-lactic acid and D(-)-lactic acid (the plus and minus signs demonstrate the direction of the rotation of plane polarized light). These two stereoisomers are enantiomers which are produced by L-lactate dehydrogenase and D-lactate dehydrogenase. The structures of L(+)-lactic acid and D(-)-lactic acid are shown in Figure 2.1. Regarding their properties as pure isomers, they have identical physical and chemical properties, while the other properties of lactic acid are given in Table 2.1 (Narayanan et al., 2004). L-lactic acid is naturally formed, while D-lactic acid, secreted by specific microorganisms, scarcely occurs naturally. Lactic acid is classified as generally recognized as safe (GRAS) for use as a general purpose food additive by the Food and Drug Administration (FDA), but an elevated level of D-lactic acid is harmful to human metabolism due to humans having only L-lactate dehydrogenase (L-LDH) to metabolize L-lactic acid. Thus, D-lactic acid is forbidden from use with food. Furthermore, the daily intake limit of D-lactic acid should be controlled to less than 100 mg kg^{-1} of body weight for adults (Reddy et al., 2008; Vaidya et al., 2005).

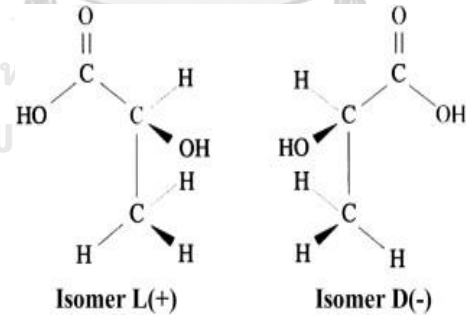

Figure 2.1 Structures of L(+)-lactic acid and D(-)-lactic acid (Martinez et al., 2013).

Table 2.1 Chemical and physical properties of lactic acid.

Chemical formula	$C_3H_6O_3$
Chemical name	2-Hydroxy-propanoic acid
Molecular weight	90.08
Physical appearance	Aqueous solution
Melting point	L:53 °C; D:53°C; DL: 16.8 °C
Boiling point	122 °C at 14 mm Hg
Dissociation Constant, Ka	$1.37x10^{-4}$
pKa	3.85
Physical state	Colorless to slightly yellow syrupy liquid
Toxicity	Oral rat LD50:3543 mg/kg
Specific gravity	1.2
Flash point	112 °C
Heat of combustion, H_c	1361 KJ/mole
Specific heat, C_p at 20 °C	190 J/mole / °C

(Narayanan et al., 2004; Vaidya et al., 2005).

2.2 Applications of lactic acid

Lactic acid has many commercial applications such as within the food, cosmetic, pharmaceutical and polymer industries.

Food industry: Lactic acid has long been used in the food industry, including in baked food, beverages, dairy products, and meat products. It is also used as a preservative or flavoring agent to prevent spoilage and inhibit pathogenic bacteria in food safety. In addition, it is used for acidification in food because it occurs naturally to improve flavor in food products.

Cosmetic industry: Lactic acid is used as a moisturizer, antimicrobial, acne preparation, and skin-lightening associated with the suppression of enzyme tyrosinase in the cosmetic industry.

Pharmaceutical and medical industry: Lactic acid is used as a supplement for pH regulation to adjust for a suitable pH in the synthesis of dermatologic drugs. Sodium lactate is used for injection solutions and kidney dialysis solutions.

Polymer industry: Its polymer and copolymers are used in applications such as sutures or bone screws in pharmaceuticals, food packaging such as cups, candy wrappers, bottles or food containers and for flexible film applications such as bags. It is added in the creations of plastic applications such as cases for computers and mobile phones, but PLA has limited uses because its its flexibility and heat stability (mechanical and thermal properties) need to be improved (John et al., 2007; Martinez et al., 2013; Wee et al., 2006).

2.3 The production of lactic acid

Lactic acid can be produced by chemical synthesis or microbial fermentation.

2.3.1 Chemical synthesis

Chemical synthesis makes a racemic lactic acid, L-lactic acid and D-lactic acid from petrochemical resources. This process uses high energy consumption at high atmospheric pressures and a limited supply of petrochemical resources. Thus, lactic acid production is low in efficiency and uneconomical. The following reactions are involved in this process (Ghaffar et al., 2014; Wee et al., 2006)

(a) Addition of hydrogen cyanide catalyst

CH_3CHO + HCN → $CH_3CHOHCN$

Acetaldehyde Hydrogencyanide Lactonitrile

(b) Hydrolysis by H_2SO_4

$CH_3CHOHCN + H_2O + 1/2H_2SO_4 \rightarrow CH_3CHOHCOOH + ½(NH_4)_2SO_4$

Lactonitrile Sulphuric acid Lactic acid Ammonium salt

(c) Esterification

$CH_3CHOHCOOH + CH_3OH \rightarrow CH_3CHOHCOOCH_3 + H_2O$

Lactic acid Methanol Methyl lactate

(d) Hydrolysis by H_2O

$CH_3CHOHCOOCH_3 + H_2O \rightarrow CH_3CHOHCOOH + CH_3OH$

Methyl lactate Lactic acid Methanol

2.3.2 Microbial fermentation

Three forms of lactic acid are produced via microbial fermentation: L-lactic acid, D-lactic acid and DL-lactic acid, depending on the microbes employed. This process produces lactic acid as it involves low cost from agricultural products or residues. For example, substrate material containing carbon 5 atom and 6 atom sugars can be used for microbial fermentation. Hence, it further offers an alternative to environmental pollution. Moreover, fermentation process is carried out under mild conditions with low energy consumption. Thus, microbial fermentation is an attractive option for lactic acid production (John et al., 2009; Wee et al., 2006; Xu et al., 2010).

Microbial fermentation can be classified into two groups: homofermentative and heterofermentative based (Xu et al., 2010) on the metabolic pathways. Homofermentative lactic acid bacteria metabolize glucose through the Embden-Meyerhof-Parnas (EMP) pathway. Aldolase is a key enzyme that divides the fructose-1,6-bisphosphate into 2 triose phosphate moieties: glyceroldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate. The glyceroldehyde-3-phosphate converts to pyruvate. Finally, pyruvate converts into lactic acid by lactate dehydrogenase (LDH). This pathway generates energy: 2 mole of ATP and 2 mole of lactic acid per 1 mole of glucose metabolized. Homofermentative bacteria are found in *Lactobacillus*, and

Sporolactobacillus produce more than 85 % lactic acid from glucose while heterofermentative lactic acid bacteria produce acid via the phosphoketolase pathway in which pentoses and hexoses are converted to xylulose-5-phosphate, which is then divided by the key enzyme phosphoketolase in the phosphoketolase pathway into GAP and acetyl phosphate. This pathway generates 1 mole of lactic acid, 1 mole of ethanol, 1 mole of CO_2 and 1 mole of ATP from 1 mole of glucose. Heterofermentative bacteria are observed in *Weisella*, *Leuconostoc* and *Lactobacillus*. They produce 50% of lactic acid and by-product compared to the glycolysis pathway; thus, glycolysis is more effective with high lactic acid yield (Fig. 2.2).

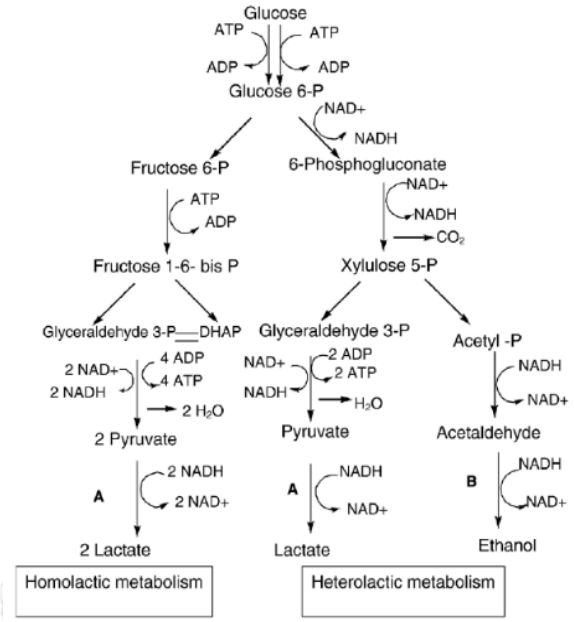

Figure 2.2 Metabolic pathways of homofermentative and heterofermentative lactic acid bacteria (Reddy et al., 2008).

2.4 Glucose metabolism of lactic acid

Lactic acid bacteria use the major pathway glycolysis for lactic acid biosynthesis. The bacteria flux into the glycolytic pathway mainly controlled and regulated by phosphofructokinase (PFK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) (Fig. 2.3).

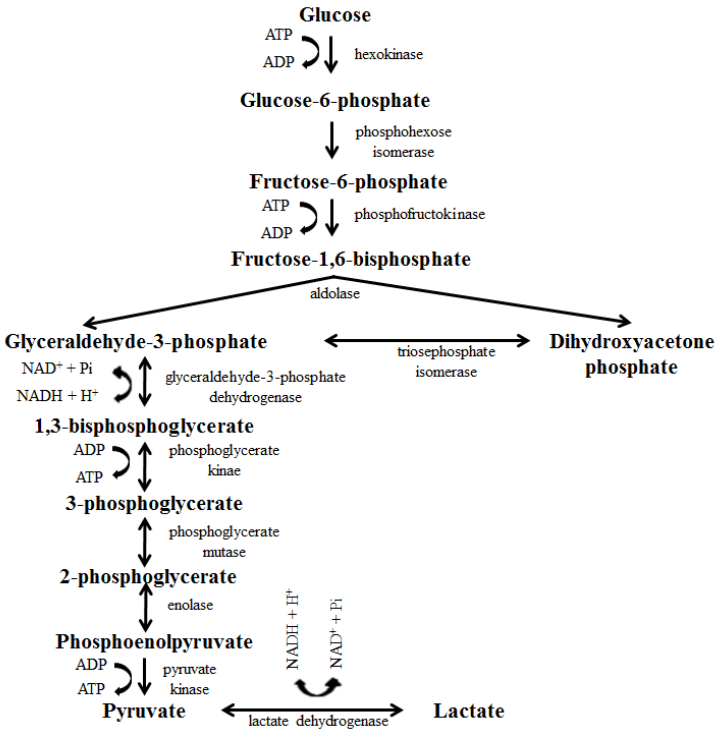

Figure 2.3 The glycolytic pathway of lactic acid fermentation.

2.4.1 Phosphofructokinase (PFK) (EC 2.7.1.11)

The PFK converts fructose-6-phosphate (F-6-P) to fructose-1,6-bisphosphate (F-1,6-P) in the presence of co-factor magnesium by transferring phosphate from ATP to F-6-P (Fig. 2.4). The F-1,6-P is obtained from the PFK activity that regulates the activity of LDH and PYK by allosteric activation; thus, the level of PFK activity is important for cell growth and D-lactate production (Fothergill-Gilmore and Michels, 1993; Zheng et al., 2017).

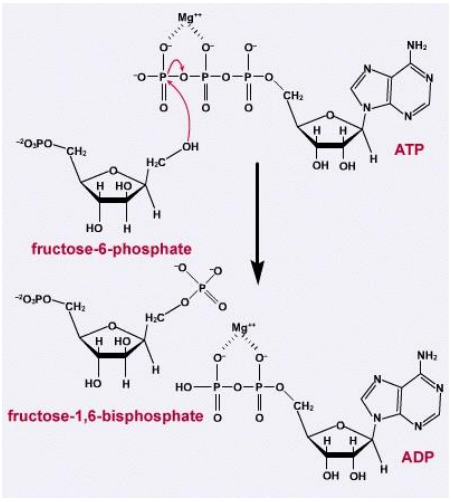

Figure 2.4 Phosphofructokinase mechanism (Pratt and Cornely, 2013).

The most common structure for PFK is the quaternary structure and homotetramer, with an arrangement beneficial for binding catalytic and allosteric effectors interfaces (Riley-Lovingshimer et al., 2002) such as *B. stearothermophilus*, *S. inulinus* and *L. bugalicus.* The molecular mass of 34.38 kDa from *S. inulinus* Y2-8, which shows a high degree of amino acid sequence identity with *B. stearothermophilus* 71% and *B. subtilis* 69% (Zheng et al., 2017). The amino acid sequences of PFK from *S. inulinus* compared with other LABs of PFK reveal that they have a homology conserved substrate and effector binding site in amino acid sequences (Paricharttanakul et al., 2005; Zheng et al., 2014). The active site lies between the domains of the subunit with the F-6-P binding site and the MgATP binding site of *L. delbrueckii* subspecies *bulgaricus*. All residues relative with F-6-P binding are strictly conserved in *L. delbrueckii* subspecies *bulgaricus* (Fig. 2.5), including the amino acid of Arg162, Arg243, His249 and Arg252 interacting with the 6-phosphate group of F-6-P and Asp127, Met169, and Glu222 interacting with the sugar moiety of F-6-P. The residues involved in MgATP binding are Gly104, Gly108 and Arg72 which interact, with the phosphate of the ATP conserved (Fig. 2.5). The side chains of Ala77, His107, Phe41 and Phe76 are conserved for the adenine ring of ATP (Paricharttanakul et al., 2005).

However, the activity of PFK is regulated by various metabolites that respond to change in the levels of metabolites and compete for binding at the allosteric site, which is a different site from the active site in the enzyme and changes catalytic properties in activation or inhibition. PFK is regulated by ATP, AMP and ADP. It has been claimed that the PFK of *S. inulinus* is inhibited by high ATP concentration that is abundant with phosphate and ATP pool of cell growth during the exponential phase (Bennett et al., 2009; Zheng et al., 2017). This inhibition may not only ATP but also, acid condition that was obtained from lactate production played a role in regulation of PFK activity due to pH optimum for activity of PFK was weakly alkaline (Tsuge et al., 2015; Zheng et al., 2017). Previous studies on key glycolytic enzymes that control cell growth and D-lactate production reported that the slight increase in the level of specific activity of PFK might affect product inhibition through LDH. Due to F-1,6-P being produced by PFK, the activate activity of LDH could be activated and thus result in high acid accumulation (Arai et al., 2002; Grabar et al., 2006). It was also reported in *Corynebacterium glutamicum* the product stopped producing when glucose consumption stopped due to the PFK encoding gene not being overexpressed from the product inhibition determined by D-lactate levels (Tsuge et al., 2015). Recently, Gong et al. (2016) demonstrated the activity of PFK and glucokinase (GK) were constructed with increasing PFK and GK activities from transgenic *L. casei*, resulting in enhanced final lactate production. Some bacterial, PFK could be activated by MgADP that increased the binding affinity for F-6-P in the active site while undergoing inhibition by PEP (Fothergill-Gilmore and Michels, 1993). It was also reported by Zheng et al. (2017) that PFK was activated by ADP but inhibited by phosphoenolpyruvate (PEP) from *S. inulinus*. Through cell growth under microaerobic conditions using *L. lactis*, it was shown that the specific activity of PFK increased, resulting in an increased specific rate of glucose consumption and lactate formation (Papagianni and Avramidis, 2011).

The enzyme activity of PFK depends on the divalent cation involved in the conformational change for the catalytic activity of PFK. The Mg^{2+} bridges between

the phosphate of F-1,6-P and the phosphate of ADP in closed subunits structure of PFK (Shirakihara and Evans, 1988). Mg^{2+} is the most efficient divalent to activate the activity of PFK, and other divalent cations such as Cu^{2+}, Ca^{2+}, Fe^{2+} and Ni^{2+} have no effect on catalysis. The PFK activity from *S. inulinus* can be activated by the addition of monovalent cations such as Na^{+}, K^{+} and NH_4^{+} in the presence of Mg^{2+}. The increasing Na^{+} can enhance the activity of PFK in the early growth stage of D-lactic acid production better than the two monovalent cations, K^{+} and NH_4^{+} (Zheng et al., 2017). It has been reported that the range of temperature and pH optimum for the activity of PFK are 28-30 °C and pH 8.2-8.5, respectively from *S. inulinus*, *B. stearothermophilus* and *L. bulgaricus.* The optimum pH of PFK activity is in a weakly alkaline condition; thus, the affinity for F-6-P and ATP decreases at acidic environment (Byrnes et al., 1994; Le Bras et al., 1991; Zheng et al., 2017). It has been indicated that the key glycolytic enzyme of PFK is controlled in the cell growth, glycolysis flux and D-lactic acid production (Zheng et al., 2017). Comparisons of the properties of PFK from different microorganisms are shown in Table 2.2.

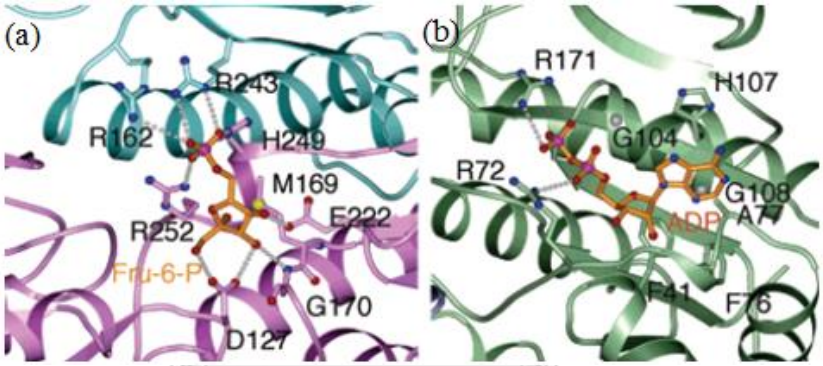

Figure 2.5 The active site of PFK from *L. delbrueckii* subspecies *bulgaricus*. (a) The F-6-P binding site and (b) the ADP binding site in the structure (Paricharttanakul et al., 2005).

Table 2.2 Comparison of the properties of PFK from microorganisms.

Parameters	*S. inulinus* Y2-8	*B. stearothermophilus*	*L. bulgaricus* B107
Optimum pH	8.5	8.2	8.2
Optimum temperature (°C)	30	-	28
Cation activator	Mg^{2+} Na^{+} K^{+} NH_4^{+}	K^{+} NH_4^{+}	NH_4^{+}
Activator	ADP GDP	ADP	-
Inhibitor	PEP ATP	PEP ATP	PEP ATP

2.4.2 Pyruvate kinase (PYK) (EC 2.7.1.40)

In glycolysis, one important allosterically regulated enzyme is PYK. PYK catalyses this step by transferring phosphate moiety from phosphoenolpyruvate (PEP) to ADP-producing ATP and pyruvate (Fig. 2.6). Pyruvate is important for many metabolic pathways that PYK links glycolysis to primary energy metabolism and cellular metabolism (Hess et al., 1966). The stimulation activity of PYK accelerates to pyruvate concomitant with ATP regeneration. Finally, pyruvate accumulation leads to increased biosynthesis of lactic acid.

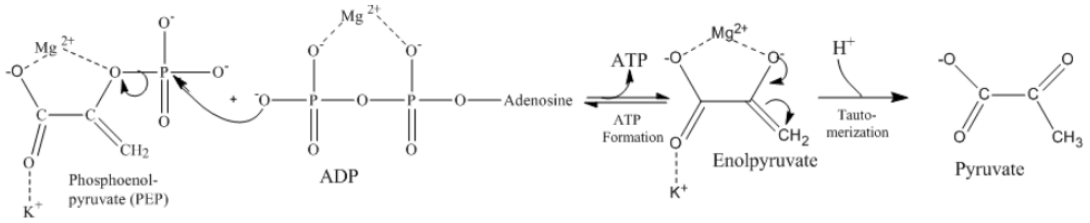

Figure 2.6 Pyruvate kinase mechanism (Robergs, 2009).

All organisms of PYK are homology observed in amino acid sequences in conserved function (Fothergill-Gilmore and Michels, 1993). The PYK of *S. inulinus* exists as a homotetramer of identical subunits with a subunit molecular mass of 51 kDa. The amino acid sequence of PYK contains 480 residues with a subunit molecular mass of 51 kDa. The molecular mass of native PYK is about 218±11 kDa (Zheng et al., 2014). The substrate binding site of PYK in the active site cleft between two domains is formed by residues Arg49, Asn51, Asp84, Ser213, Lys240, Glu242, Asp266 and Thr298. The phosphate of the substrate is in a favorable electrostatic coupling with Arg49 and Lys240 within the pocket. For the transfer of the phosphate group from substrate to ATP, using the amino acids of Arg49 and Lys240 and cation as shown in Figure 2.7 (Page and Di Cera, 2006). The allosteric site of PYK has a

high abundance of amino acids such as glycine, threonine, serine and alanine. It has conserved phosphate binding site in the allosteric binding site which is activated by some compounds composed of one sugar moiety and at least one phosphate moiety. The phosphate moieties play an important role in allosteric activation.

The allosteric activation of PYK by activators composed of sugar-phosphates such as F-1,6-P and glucose-6-phosphate (G-6-P) was found in *Lactococcus lactis*, *L. delbrueckii* and *Streptococcus mitis*. The F-1,6-P activator for *Enterococcus faecalis*, *L. plantarum*, *Lactococcus lactis*, *Streptococcus mutans* enhanced PYK activity. F-1,6-P, G-6-P, F-6-P, R-5-P, Gal-6-P and other sugar-phosphate compounds were activators for *Lactococcus lactis* (Abbe et al., 1983; Bourniquel, 2002; Collins and Thomas, 1974; Thomas, 1976; Veith et al., 2013). Mn^{2+} mediated allosteric activation of PYK from *S. inulinus* was enhanced by ribose-5-phosphate, adenosine monophosphate (AMP) but not F-1,6-P similar to PYK from *E. coli* (Malcovati and Valentini, 1982; Zheng et al., 2014). The result was also similar to *Bacillus licheniformis* and *Sporosarcina psychrophila* being activated by AMP and D-ribose 5-phosphate (Tanaka et al., 1995). With the increasing ADP concentration, the activity of PYK increased and then decreased whereas the PYK activity did not decrease when the PEP concentration was increased. Thus, a high ADP concentration might inhibit the PYK activity, while a high concentration of PEP will not (Zheng et al., 2014). The effective inhibitors were ATP, G6P, inorganic phosphate (Pi) and citrate which decreased V_{max} and low affinity for PEP, Pi and ATP were strong inhibition was enhanced with increased inhibitor concentrations found in *S. inulinus* (Zheng et al., 2014). The result was similar to *Bacillus licheniformis* and *Sporosarcina psychrophila* being inhibited by ATP (Tanaka et al., 1995).

PYK requires cations for optimal catalytic activity; for example, the activity of PYK from *S. inulinus* showed activity dependence on cations Mg^{2+}, Mn^{2+} and K^{+} (Zheng et al., 2014). The binding of the divalent cation to PYK probably triggered conformational change due to PYK using divalent cation for activity, such as Mg^{2+}, Mn^{2+} and Co^{2+} (Jakubovics and Jenkinson, 2001). Mg^{2+} is needed for the catalysis of GK and PYK in a glycolytic key enzyme that can increase the final D-lactic acid concentration (Zheng et al., 2012). The kinetics of PYK demonstrated that the Mn^{2+} probably mediates the allosteric activation of the *S. inulinus*. The interaction of Mn^{2+} in the active site with PYK enhances the interaction of the allosteric activator and PEP (Zheng et al., 2014). The optimum temperature of PYK from *S. inulinus* was 45 °C, while *B. licheniformis* and *B. stearothermophilus* were 45 and 65°C, respectively (Tanaka et al., 1995). It should be noted that the optimum pH of the glycolytic key enzyme of PYK was 7-7.2 from *B. licheniformis* and *B. stearothermophilus* (Tanaka et al., 1995), while *S. inulinus* showed the optimum pH of 7.5 with high activity between pH 6.5-8.5. Regarding the PYK activity at pH of 6.5 during *S. inulinus* D-lactic acid production, it was found that activity was less efficient; thus, inhibition of PYK by metabolite effectors during the D-lactic acid production (Zheng et al., 2012; Zheng et al., 2014).

The activation of PYK increases the conversion of PEP to pyruvate concomitant with ATP. Therefore, the accumulation of pyruvate enhances the biosynthesis of D-lactate. Comparisons of the properties of PYK from microorganisms are listed in Table 2.3.

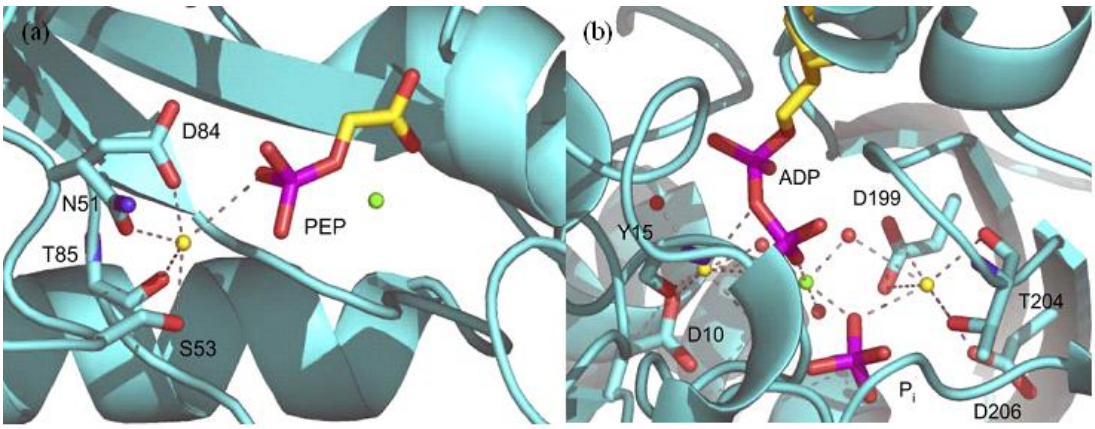

Figure 2.7 Structure of pyruvate kinase. (a) The substrate binding site (PEP) and (b) ADP binding site. It requires K^+ (yellow sphere) and Mn^{2+} (green sphere) for optimal catalytic activity (Page and Di Cera, 2006).

Table 2.3 Comparison of the properties of PYK from microorganisms.

Parameters	*S. inulinus* Y2-8	*B. licheniformis* ATCC 14580	*B. stearothermophilus* ATCC 12980	*L. delbrueckii* subsp. *lactis* NCC 88
Optimum pH	45	7-7.4	7.2	-
Optimum temperature (ºC)	7.5	46	> 65	-
Cation activator	Mn^{2+} Mg^{2+} Co^{2+} K^+	-	-	-
Activator	PEP R-5-P AMP	R-5-P AMP	AMP R-5-P	F-1,6-P F-6-P G-6-P
Inhibitor	ADP inorganic phosphate (Pi) ATP Citrate G-6-P	ATP	ATP	Pi ATP

2.4.3 Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) is one of the hydroxyacid dehydrogenases that act in the last step of the glycolytic pathway. It catalyzes the reduction of pyruvate into lactic acid, concomitant with the oxidation of NADH to NAD^+. A hydrogen ion is transferred from NADH to pyruvate during glycolytic reaction, while for the reverse reaction it is transferred to NAD^+ from lactate (Fig. 2.8).

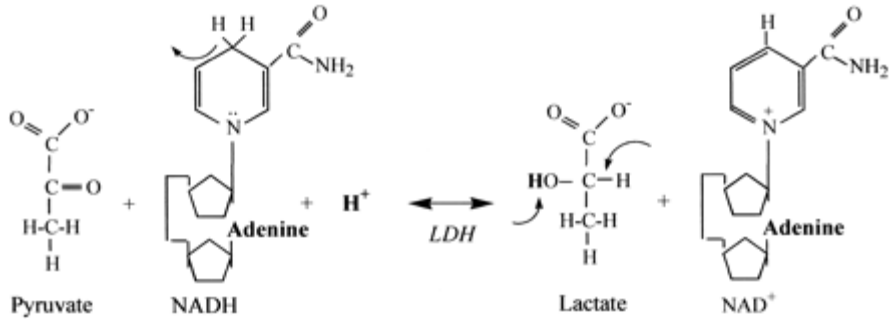

Figure 2.8 The lactatedehydrogenase mechanism (Robergs et al., 2004).

There are two forms of lactate dehydrogenase: L-lactate dehydrogenase (EC 1.1.1.27) and D-lactate dehydrogenase (EC 1.1.1.28), with D-Lactate dehydrogenase catalyzing virtually via the same reaction as L-Lactate dehydrogenase (Garvie, 1980). L-LDH is widely found throughout nature, while D-LDH is found in some bacteria such as *Lactobacillus* sp., *Sporolactobacillus* sp. and *Leuconostoc mesenteroides* subsp. *mesenteroides* (Garvie, 1980; Li et al., 2012; Long and Kaplan, 1968). However, lactate dehydrogenase is a highly diverged enzyme. It exhibits various in catalytic properties with different microorganisms (Taguchi, 2003).

Most structures of L-LDH are tetrameric enzymes composed of identical polypeptides (subunits) encoded into 323 amino acids (35.3 kDa) found in *L. helveticus* (Savijoki and Palva, 1997). The subunits comprise identically sized domains: NAD-binding and catalytic domains. Subunits are usually composed of 310-350 amino acids as well as D-LDH (332 amino acid). The NAD-binding domain is a Rossmann-folded structure located in the N-terminal half of the L-LDH primary structure. The structure of the catalytic domain resembles an NAD-binding domain which is located the C-terminal half appears unique in L-LDH (Li et al., 2012; Taguchi, 2003).

The allosteric types of L-LDH in bacterial cells usually activated by fructose-1,6-bisphosphate have been found in *L. casei*, *B. stearothermophilus* and *Bifidobacterium longum*, but their requirement has not been found in D-lactate dehydrogenase. Residues of Arg173 and His188 are involved in the allosteric binding site and it requires divalent metal ion such as Mn^{2+} for regulation by fructose-1,6-bisphosphate (Holland and Pritchard, 1975; Savijoki and Palva, 1997; Taguchi and Ohta, 1995).

Many D-LDH structures are composed of identical subunits in the quaternary structure. Some D-LDHs differ: *L. mesenteroides* subsp. *mesenteroides* ATCC 8293 have a tetramer structure and molecular weight of 123 kDa by gel permeation chromatography (GPC) (Li et al., 2012); *L. bulgaricus* is composed of 332 amino acid residues with a molecular weight of 36 kDa per subunit (Razeto et al., 2002); *L. jensenii* could form a homotetramer structure with molecular mass of 159 kDa by size-exclusion chromatography analysis (Jun et al., 2013). *Haemophilus influenzae* enzyme has a molecular weight of 35,000 and 135,000 by SDS-PAGE and gel filtration, respectively; thus, most D-LDH have a tetrameric structure. The subunits of most D-LDHs are composed of two domains: NAD-binding and catalytic domains (or substrate-binding). The NAD-binding domain of D-LDH is folded into a Rossmann fold similar to L-LDH. However, the catalytic domain of L-LDH is not folded into an

α/β structure, while D-LDH is folded in to an alpha/β structure that resembles the NAD-binding domain structure. The active site of D-LDH is located in the interdomain cleft (Razeto et al., 2002).

The kinetics of L-LDH are as follows: the coenzyme NADH (or NAD^+) is bound first, then the pyruvate as substrate (or L-lactate) is bound to the lactate dehydrogenase in the active site. In the case of pyruvate reduction, hydrogen is transferred from NADH onto the 2-carbon and the protonated imidazole of His195 (the numbering is based on the enzyme). The residue acts as an essential acid/base by donating a proton to the pyruvate carbonyl group. The carboxyl group of Asp168 is located near the imidazole group of His195 that promotes and stabilizes the catalytic function of His. The guanidine group of Arg171 interacts with the carboxyl group of substrate orientated in the catalytic site of the enzyme to bind the correct substrate. The guanidino group of Arg109 is located over the active site of the enzyme and near the carbonyl oxygen of pyruvate, and promotes the hydrogen transfer reaction by polarizing the substrate carbonyl group of pyruvate through the rearrangement (Clarke et al., 1986; Taguchi, 2003).

In the case of D-LDH, the amino acids of His296 and Glu264 perform the same function as His195 and Asp168, while Arg235 may take on the roles of Arg171 (Stoll et al., 1996). Glu264 interacts with His296 by H-bonds to stabilise His296, and Arg235 is stabilised by H-bonds to the sulphate ion located in front of the nicotinamide moiety. The methyl group of pyruvate will be close to Tyr101 and Asn77. The carboxylate group of pyruvate binds the chain nitrogen atoms of Val78 and Gly79 (Fig. 2.9) (Razeto et al., 2002). Thus, the carboxylate group of pyruvate will be close to the dihydronicotinamide group. Tyr101 and Met308 are conserved in D-specific dehydrogenases where tyrosine residues favour the binding of aromatic substrates for substrate binding (Kochhar et al., 1992). In the case of D-lactate dehydrogenase from *S. inulinus*, it was observed that Arg234 and Gly79, His295 and Phe298 residues are key residues in this enzyme activity. His295 is promoted to function as the acid-base catalyst in lactate dehydrogenase activity. Glu263 supports the stabilization of the protonated form of His295 and Arg234 forms H-bond with the nicotinamide ribose ring. Phe298 form H-bond with the carbonyl group of the nicotinamide ring. Gly79 is near where the substrate enters, while Tyr101 and Met307 stay inside the substrate entryway and might be specific for the substrate at the cleft between the substrate binding and cofactor binding domain. Phe298, Tyr299 and Trp135 might select the substrate specificity near the active site (Razeto et al., 2002; Zhu et al., 2015). Orientation of the substrate is very important for the enzyme reaction, thus Arginine and Glycine are essential for both L- and D-LDH (Antonyuk et al., 2009). Both L- and D-LDH are sensitive to chemical reagents such as diethylpyrocarbonate and 2,3-butandione for His imidazole and Arg guanidine groups, respectively (Taguchi, 2003).

F-1,6-P is required for L-lactate dehydrogenases of *Lactobacillus* sp. such as *L. casei* and *L. helveticus*, but its requirement has not been found in D-lactate dehydrogenase (Holland and Pritchard, 1975; Savijoki and Palva, 1997), whereas Triton X-100 and urea enhance the activity of *Thermoanaerobacter ethanolicus* JW200 (Zhou and Shao, 2010). AMP, ADP and ATP inhibit the catalytic reactions of LDH with competition with coenzymes at the NAD binding site. The LDH was

inhibited by *p*-hydroxymercuribenzoic acid (pHMB) from *L. helveticus* (Savijoki and Palva, 1997).

The LDH requires metal ions for enzyme activation which activate to bind activator due to reduce the mutual repulsive forces that lead to loss of affinity (Zhou and Shao, 2010). The enzyme is not inhibited by Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} and Na^{+} but is by Zn^{2+} from *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 (Li et al., 2012). The metal ions of Mn^{2+} stimulate the activity of the LDH enzyme for the allosteric binding site while Co^{2+}, Cd^{2+}, Cu^{2+} and Ni^{2+} effective like Mn^{2+} are found in *L. casei* (Holland and Pritchard, 1975). The enzyme is inhibited by Zn^{2+}, Ag^{+} and Cu^{2+} from *Thermoanaerobacter ethanolicus* JW200 (Zhou and Shao, 2010). Regarding the effect of metal ions on the LDH activity, Ca^{2+} and Zn^{2+} enhance the activity and enzyme activity is inhibited by Cd^{2+}, Co^{2+}, and $Cu^{2+,}$ while Mg^{2+} and Mn^{2+} do not affect the enzyme activity by *Lactobacillus helveticus* (Savijoki and Palva, 1997). It should be noted that L-LDH works at wide ranging temperature from 30-60 °C of *E.coli* FMJ379 and *Thermoanaerobacter ethanolicus* JW200 (Gaspar et al., 2007; Zhou and Shao, 2010), while D-LDH works at a lower range from 30-40 °C, as exhibited in *Leuconostoc mesenteroides* and *Staphylococcus* sp. (Isobe et al., 2002; Li et al., 2012) except for *L. jensenii* and *Thermoanaerobacter ethanolicus* JW200 (observed at 50-60 ºC). Other bacteria express D-lactate dehydrogenase less than at 50 ºC (Jun et al., 2013; Zhou and Shao, 2010). It has been reported that the enzyme expression for both D- and L-lactate dehydrogenases are influenced by change in pH during cultivation (Hofvendahl and Hahn-Hagerdal, 2000). Li et al. (2012) claimed that the optimal pH of the D-LDH was between 7.0 and 8.0, revealing this enzyme to be resistant to alkali conditions by *Leuconostoc mesenteroides* subsp. *mensenteroides* ATCC8293. This research concurs with previous reports by Gordon Doelle (1975) who found that production of racemic lactic acid in *Pediococcus cerevisiae* showed an optimum pH for D-lactate dehydrogenase of 8.0. However, the optimum pH for L-LDH is 5.5. This is in agreement with Garvie (1980) who reported the optimal pH values in which D-lactate dehydrogenase for pyruvate reduction and showed activity was wide-ranging at neutral to base conditions of pH 6.0-8.0. On the other hand, L-LDH in acidic conditions at pH 4.5-6.0 were found in *L. acidophilus*, *L. plantarum* and *Pediococcus pentosaceus*. For the isomeric form of L-lactic acid producing bacteria, *L. rhamnosus* decreased with increasing pH from 5.0-6.5 (98-97% optical purity of L-lactate) or when the composition of nutrients varied (Hofvendahl and Hahn-Hagerdal, 2000). Hence, the lactate dehydrogenase of strains D-lactate producing bacteria have greater activity at higher pH than L-lactate dehydrogenase. The characterization of lactate dehydrogenase is shown in Table 2.4.

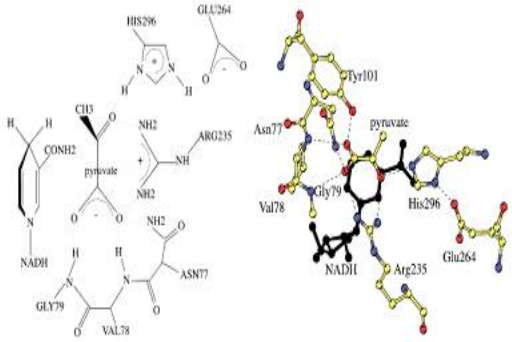

Figure 2.9 The active site in a subunit of D-LDH (Razeto et al., 2002).

Table 2.4 Comparison of the properties of LDH from microorganisms.

Parameters	D-Lactate dehydrogenase			L-Lactate dehydrogenase		
	Leuconostoc mesenteroides ATCC 8293	*L. jensenii* 1153	*L. brevis*	*L. helveticus* 53/7	*L. casei*	*Thermoanaerobacter ethanolicus* JW200
Optimum pH	8		9-10	5	4.5-5.5	5.8
Optimum temperature (ºC)	30	50	50-60	-	-	60
Cation activator	-	-	-	Ca^{2+} Zn^{2+}	Mn^{2+} Co^{2+} Cu^{2+} Cd^{2+} Ni^{2+}	-
Activator	-	-	-	F-1,6-P DTT Mercaptoethanol	F-1,6-P	F-1,6-P Triton X-100 Urea
Inhibitor	ADP ATP Iodoacetamide	2-Ketobutyric acid Sodium phenylpyruvate ADP ATP Oxamate, Iodoacetamide	ADP ATP Oxamate Iodoacetamide	*p*-Hydroxymercuribenzoic acid	Phosphate Citrate ADP ATP *p*-Hydroxymercuribenzoate	ATP EDTA DTT Mercaptoethanol

2.5 D-Lactic acid producing microorganisms

Microorganisms used for efficient D-lactic acid production have to meet several requirements. For industrial applications, a microorganism is necessary that produces high D-lactic acid production from low-cost substrates with a high yield, productivity and also, high optical purity of D-lactic acid from a short-time in fermentation. The bacteria can be separated into 2 groups: wild-type strains and mutants derived by many techniques such as random mutagenesis and genetically engineered strains which are investigated for biotechnological D-lactic acid production acid. Table 2.5 presents the most promising results in D-lactic acid production (Hofvendahl and Hahn-Hagerdal, 2000; Klotz et al., 2016).

2.5.1 Wild-type strains

The wild-type strains for D-lactic acid production belonged to the genera *Lactobacillus* and *Sporolactobacillus*.

Genus *Lactobacillus*

Cells are Gram-stain-positive, non-spore-forming, facultative anaerobic or microaerophilic rods and lack catalase or have weak catalase positive activity. It can grow in a temperature range of 32-47 °C and pH range of 3.5-7.8. D-lactic acid bacteria come from the genus *Lactobacillus* such as *L. coryniformis* and *L. delbrueckii*, while some microorganisms, *L. casei* and *L. rhamnosus* are L-lactic acid bacteria. A racemic mixture of L- and D-lactic acid is found in *L. plantarum*, *L. helveticus* and *L. brevis*. This utilizes various carbon sources such as hexose, pentose and disaccharides for lactic acid fermentation. *Lactobacillus* use homofermentative metabolism and some species use heterofermentative metabolism because of their ability to utilize the carbon source by different pathways. It can be found in a variety of sources such as dairy, animals, plants and milk. Many *Lactobacilli* are used as applications in the food industry and biotechnology because of their various metabolic activities and are generally recognized as safe (GRAS status), such as the use of *L. plantarum* for fermented foods (Gao et al., 2008; Garvie, 1980; Giraffa et al., 2010; Hofvendahl and Hahn-Hagerdal, 2000; Schepers et al., 2002).

Genus *Sporolactobacillus*

Cells are Gram-stain-positive, facultatively anaerobic rods, endospore-forming bacteria and lack catalase activity. The microorganism produces D- or DL-lactic acid homofermentatively. They contain *meso*-diaminopimelic acid in cell-wall peptidoglycan and have menaquinone with seven isoprene units (MK-7). They grow anaerobically in a temperature range of 20-45 °C and pH 4.5-9.0. Its differential characteristic of acid from sugars depends on the microorganism. Most acid is produced from galactose, glucose, D-mannitol, sucrose, D-tagatose and D-trehalose, but not from arabinose, cellobiose, ribose and xylose. The species *Sporolactobacillus inulinus* was isolated from chicken feed. Later, *S. nakayamae* subsp. *nakayamae*, *S. nakayamae* subsp. *racemicus*, *S. terrae*, *S. kofuensis*, *S. lactosu*s and *S. laevolacticus* were isolated from soil, and fermentation starters were proposed by Yanagida et al. (1997). In addition, *S. vineae*, *S. putidus* and *S. pectinivorans* were isolated from vineyard soil in Korea, spoiled orange juice and spoiled jelly, respectively (Chang et al., 2008; Fujita et al., 2010; Lan et al., 2016). *Sporolactobacillus spathodeae* and *Sporolactobacillus shoreae* were isolated from tree barks in Thailand (Thamacharoensuk et al., 2015). This genus can be classified as homofermentative, to generate more energy in the form of ATP and has effective lactic acid yield which sugar utilizes via the Embden-Meyerhof-Parnas (EMP) pathway; thus, this genus is more effective for the D-lactic acid microorganism.

2.5.2 Genetically engineered strains

D-Lactic acid production by genetically engineered microorganisms is generally based on major such as the enablement of pentose, xylose and lignocellulose biomass as substrates conversion via the homofermentative pentose

phosphate pathway for the efficient production of D-lactic acid. Modified L-LDH is inadequate in bacteria for only D-lactic acid production from xylose materials by inserting xylose assimilating genes encoding xylose isomerase and xylulokinase. In some cases, the deletion of some genes that disrupt the pathway leading to by products, results in higher production, yield and productivity. Thus, high efficiency in D-lactic acid production can be obtained (Klotz et al., 2016). The most promising results in D-lactic acid production are those from genetically engineered strains such as *C. glutamicum* holding a D-LDH from *L. delbrueckii* produced D-lactic acid of 195 g/L (Tsuge et al., 2015), *Klebsiella pneumoniae* was genetically manipulated and gave 142.1 g/L of D-lactic acid using glycerol as the substrate. Overexpression of lactate dehydrogenase and the knockout of genes associated with 1,3-PDO production improve D-lactic acid production (Feng et al., 2014). Insertion of D-LDH in *E. coli* using crude glycerol as substrate in fermentation was converted to D-lactic acid (Wang et al., 2015). However, the genetically modified microbes have limited industrial applications due to biosafety regulation limits and genetic instability; therefore, wild strains are still preferred in industrial D-lactate production facilities. D-Lactic acid production by wild-type and genetically engineered microorganisms are shown in Table 2.5.

Table 2.5 D-Lactic acid production of wild-type strains generated by random mutagenesis and genetically engineered strains.

Strain	Substrate	D-Lactic acid (g/L)	Yield (g/g)	Productivity (g/L.h)	Optical purity (%)	Reference
L.delbrueckii LD0025	Maltose	66.6	-	0.93	99.5	Fukushima et al., 2004
L. delbrueckii subsp. *lactis* QU 41	MRS medium/glucose	87.4	1.01	0.52	99.9	Tashiro et al., 2011
S. inulinus	Glucose/peanut meal	207.0	0.93	3.80	99.3	Wang et al., 2011
S. laevolacticus DSM 442	Glucose/cottonseed	144.4	0.96	4.13	99.3	Li et al., 2013
S. inulinus Y2–8	Corn flour hydrolysate/ yeast extract	145.8	0.965	1.62	>99	Zhao et al., 2014
S. inulinus NBRC 1359	Palmyra palm jiggery, whey protein hydrolysate	189.0	0.94	5.25	>98	Reddy Tadi et al., 2017
Sporolactobacillus sp. YBS1–5 [(a)]	Glucose/yeast extract, corn steep liquor, wheat bran	125.0	-	1.39	99	Sun et al., 2015
S. inulinus YBS1-5 [(a)]	Corn stover hydrolysate	70.7	83	0.65	-	Bai et al., 2016
Corynebacterium glutamicum LPglc267/pCRB2015 [(b)]	Glucose/ mineral salt medium	195.0	0.90	2.44	99.9	Tsuge et al., 2015
E. coli BLac-2106 [(b)]	Glycerol/NSB medium	105.0	0.87	2.63	99.9	Wang et al., 2015
E. coli HBUT-D (*DpflB Dpta DfrdABCD DadhE Dald DcscR*) [(b)]	Mineral salts medium	127	0.93	6.35	99.5	Liu et al., 2014
Klebsiella pneumoniae (*ΔdhaT ΔyqhD pBAD18-ldhA*) [(b)]	Glycerol/beef extract/peptone, yeast extract	142.1	0.82	2.96	~100	Feng et al., 2014

[(a)] Strains generated by random mutagenesis
[(b)] genetically engineered strains

2.6 The fermentation strategy to obtain efficient fermentative production of D-lactic acid

The fermentation strategy to obtain optimal D-lactic acid fermentation conditions depends on the microorganism, substrate, temperature and pH including fermentation mode.

2.6.1 Microorganisms

Lactic acid bacteria produce via microbial fermentation 3 forms of lactic acid: L-lactic acid, D-lactic acid and DL-lactic acid, depending on the microbes employed. It was found that D-lactic acid is only produced in some bacteria including *Lactobacillus* sp., *Leuconostoc* sp. and *Sporolactobacillus* sp. as a metabolic response to environmental stress (Bai et al., 2016; Mimitsuka et al., 2012; Tashiro et al., 2011; Zhao et al., 2014; Li et al., 2013). It has been posited that D-lactate production with the aforementioned strains usually results in poor fermentation performance for reasons such as temperature-tolerant strains, inability to convert pentoses using homofermentative lactic acid production and unfavourable utilization of renewable feedstocks. Genetic engineering has been employed for strain development to specifically produce optically pure D-lactate such as *Klebsiella pneumonia*, *Saccharomyces cerevisiae* and *C. glutamicum*. However, genetically modified microbes have limited industrial applications due to biosafety regulation limits and genetic instability. Therefore, wild strains are still preferred in industrial D-lactate production facilities (Baek et al., 2016; Feng et al., 2014; Tsuge et al., 2014). The potential D-lactic acid strain should be efficient homofermentative D-lactic acid bacteria from renewable feedstocks and high temperature-tolerant strains to give the highest D-lactic acid concentration, yield, productivity and optical purity of D-lactic acid.

2.6.2 Alternative substrates (carbon and nitrogen sources)

Carbon source

Normally, lactic acid bacteria use pure sugars-glucose as the substrate in fermentation. This is economically unfavourable because the substrate cost is expensive, but lactic acid is a cheap product and so the raw material cost has influence on the price of the lactic acid product (Tejayadi and Cheryan, 1995). Thus, alternative substrates from raw materials or waste products from agriculture that are cheap and abundant are desirable for lactic acid fermentation such as starch, cellulose, whey and molasses. Table 2.6 shows alternative substrates that replace refined glucose by D-lactic acid microorganisms.

An alternative substrate for lactic acid fermentative is whey. This is a waste product from cheese production which contains protein, lactose and salts. Whey hydrolyzate is hydrolysed, changing into glucose and galactose as nutrient source. Additionally, this substrate has been fermented with yeast extract, peptone or corn steep liquor and are found in *Lactobacillus helveticus* and *L. bulgaricus* for lactic acid fermentation (Cox and MacBean, 1977; Hofvendahl and Hahn-Hagerdal, 2000; Roy et al., 1986).

Molasses are a by-product, such as sugarcane molasses, sugarcane juice and sugar beet juice. This material is cheap, abundant, with a high concentration of sucrose, proteins, amino acids and vitamins which are all suitable for cell growth and lactic acid production. Thus, lactic acid fermentation is carried out using these substrates as alternative carbon sources. *L. delbrueckii* produces high D-lactic acid production using these sugar substrates that do not require treatment and which are cost effective for lactic acid fermentation (Calabia and Tokiwa, 2007; Hofvendahl and Hahn-Hagerdal, 2000).

Starch material such as wheat, corn, cassava, potato and rice which has to be hydrolysed to glucose and maltose to make lactic acid fermentable. In some cases, it was untreated or liquefied/gelatinized then fermented by an amylase-producing organism such as *L. fermentum*, *L. amylovorus* and *L. amylophilus.* Fermentation by some bacteria such as *Lactococcus lactis* of starch added the commercial enzymes α- and gluco-amylases and protease to hydrolyze the starch substrate for cell utilization by simultaneous saccharification and fermentation (SSF) (Hofvendahl and Hahn-Hagerdal, 2000). D-Lactic acid produced 79 g/L lactic acid, 80.6% yield and 3.59 g/L.h productivity using broken rice as the substrate by *L. delbrueckii* in the SSF method (Nakano et al., 2012). Starch material has been studied with *L. delbrueckii* LD 0028, *L. delbrueckii* IFO 3202 and *S. inulinus* Y2-8 fermented by rice saccharificate, rice bran and corn flour as alternative substrates, respectively (Fukushima et al., 2004; Tanaka et al., 2006; Zhao et al., 2014).

Lignocellulose contains hexoses (glucose, galactose and mannose) and pentoses (xylose and arabinose). Lignocellulosic materials are those such as plant materials, cardboard, filter paper and waste paper. It has to be treated to release sugars (monomers) by enzymatic treatment or hydrolysis of dilute acid-pretreated cellulose under mild hydrothermal conditions for lactic acid fermentation (Hofvendahl and Hahn-Hagerdal, 2000; Yanez et al., 2003). In a study with *L. coryniformis* subsp. *torquens*, it produced 24 g/L D-lactic acid and 0.5 g/L.h productivity from cellulose by simultaneous saccharification and fermentation (SSF) (Yanez et al., 2003). Singhvi et al. (2010) reported that D-lactic acid fermentation by *L. lactis* RM2-24 using SSF of cellulose obtained 73 g/L lactic acid and 1.52 g/L.h productivity.

Nitrogen source

The medium composition of enriched nutrient sources which have positive effect on lactic acid production is superior to yeast extract and better than peptone and malt extract. Lactic acid bacteria grow well in nutrient enrichment media because of the limited biosynthesis capacity of the cell. Yeast extract contains free amino acids, peptides, B vitamins, trace elements and nucleotides, which are all necessary nutrients. Many experiments use yeast extract substitutes that have negative effects on cell growth and D-lactic acid production because yeast extract contains vitamin B which is needed by the cell (Nancib et al., 2005). However, yeast extract is expensive and affects total production cost; thus, protein enriched agricultural raw materials can be supplied as alternative nutrient sources such as peanut meal, cottonseed and whey protein hydrolysate as shown in Table 2.6. Wang et al. (2011) used peanut meal and obtained a high D-lactic acid production of 207 g/L and productivity of 3.8 g/L.h from *S. inulinus* CASD. Li et al. (2013) used *S. laevolacticus* DSM 442 fermented in cottonseed as the nitrogen source and produced a high D-

lactic acid production of 144 g/L and productivity of 4.13 g/L.h. Both carbon and nitrogen sources as alternatives are replaced by palmyra palm jiggery and whey protein hydrolysate from *S. inulinus* NBRC 1359. High D-lactic acid production of 189.0 g/L and productivity of 5.25 g/L.h were achieved by Reddy Tadi et al. (2017) Corncob residue and cottonseed meal as alternative substrates were experimented with *S. inulinus* YBS1-5 giving a high production and productivity of 107.2 g/L and 1.19 g/L.h, respectively (Bai et al., 2016).

Table 2.6 D-lactic acid production from fermentation of alternative substrates.

Strain	Substrate	D-Lactate (g/L)	$Y_{p/s}$	Productivity (g/L·h)	Optical purity (%)	References
L. delbrueckii JCM 1148	Sugarcane molasses	104.0	0.9	1.48	97.2	Calabia and Tokiwa, 2007
L. delbrueckii JCM 1148	Sugarcane juice	118.0	0.95	1.66	98.3	Calabia and Tokiwa, 2007
L. delbrueckii JCM 1148	Sugar beet juice	82.0	0.88	1.16	97.6	Calabia and Tokiwa, 2007
L. delbrueckii LD 0028	Rice saccharificate	62.6	0.73	-	98.4	Fukushima et al., 2004
L. delbrueckii IFO 3202	Rice bran	28.0	0.78	0.78	95	Tanaka et al., 2006
L. coryniformis subsp. *torquens* ATCC 25600	Filter paper	24.0	0.89	0.5	99.9	Yanez et al., 2003
L. lactis RM2–24	α-Cellulose	73.3	0.73	1.52	-	Singhvi et al., 2010
S. inulinus Y2–8	Corn flour	145.8	0.97	1.62	>99.0	Zhao et al., 2014
S. inulinus CASD	Peanut meal	207.0	0.93	3.8	99.3	Wang et al., 2011
S. laevolacticus DSM 442	Cottonseed	144.4	0.96	4.13	99.3	Li et al., 2013
S. inulinus NBRC 1359	Palmyra palm jiggery, whey protein hydrolysate	189.0	0.94	5.25	>98	Reddy Tadi et al., 2017
S. inulinus YBS1-5	Corncob residue, cottonseed meal	107.2	0.85	1.19	99.2	Bai et al., 2016

2.6.3 pH and temperature

pH

Neutralization reagents such as $CaCO_3$, $Ca(OH)_2$ and NH_4OH are used to maintain pH during lactic acid fermentation due to the decrease in pH from lactic acid production. The pH range has been reported to be between 5.0 and 7.0 for D-lactic acid production (Table 2.7). Effective neutralization of lactic acid fermentation should be easy to recover in the downstream process and of cheap cost compared to lactic acid production (Qin et al., 2010). Fermentation neutralized by $CaCO_3$ was used to control the pH in *Sporolactobacillus* sp. (Sun et al., 2015), but calcium lactate might inhibit the cell growth of bacteria under high calcium carbonate concentrations

and the solubility of calcium lactate increased under high temperature, especially at 80 ºC (Aran, 2001; Cao et al., 2001). Other neutralizing agents, NH_4OH and NaOH solutions using pH control were performed in *L. coryniformis*, *K. pneumonia*, *S. inulinus* Y2-8 and *L. debrueckii* (Feng et al., 2014; Nakano et al., 2012; Nguyen et al., 2013; Zhao et al., 2014). It was reported that the ammonium salts of D-lactate have high solubility during fermentation but the osmotic pressure is high and cost expensive compared to $CaCO_3$ and $Ca(OH)_2$ (Liu et al., 2014). The effects of neutralizing agents $Ca(OH)_2$, NH_4OH and NaOH on D-lactic acid production by *L. delbrueckii* revealed that $Ca(OH)_2$ was the best neutralizing agent for cell growth and production due to it reducing the lactate molarity in the fermentation broth and the lower osmotic pressure. However, it was clear that monovalent cations such as Na^+ and NH_3^+ were less effective in D-lactic acid production compared to the divalent cation Ca^{2+} (Nakano et al., 2012). It was found that a stable by neutralizing agent resulted in higher lactic acid production, yield and productivity compared with no neutralizing agent (Hofvendahl and Hahn-Hagerdal, 2000). Thus, effective neutralization and pH values are necessary for cell growth and lactic acid production during fermentation.

Temperature

D-Lactic acid can be produced by microbial fermentation at high titer, yield and productivity at temperatures between ~30-40 ºC, but not at 45°C (Feng et al., 2014; Nakano et al., 2012; Zheng et al., 2012). However, several D-lactic acid bacteria, such as *Sporolactobacillus* sp. and *Lactobacillus* sp. produce lower D-lactic acid and productivity than L-lactic acid fermentation using *Bacillus* sp. at 50-55 ºC. This might be because D-lactate dehydrogenase has lower thermostability than L-lactate dehydrogenase (Auerbach et al., 1998; Patel et al., 2006). Wang et al. (2011) studied *Bacillus* QZ19 engineered by deleting the native *ldh* (L-lactate dehydrogenase) and inserting *ldhA* (D-lactate dehydrogenase) and found it to produce high D-lactic acid at 50 ºC. It should be noted that high solubility of D-calcium lactate increases the temperature of the fermentation broth (Cao et al., 2001). However, D-lactic acid production requires high temperature-tolerant strains that will enhance the productivity and purification processes (Nakano et al., 2012). Thus, optimum temperatures are necessary for the productivity and economical production of D-lactic acid. The various temperatures for D-lactic acid production are given in Table 2.7.

Table 2.7 The pH value, neutralizing agent and temperature during D-lactic acid fermentation.

Strain	pH value	Neutralizing agent	T (ºC)	Lactate (g/L)	$Y_{p/s}$	Productivity (g/L·h)	Optical purity (%ee)	References
Sporolactobacillus sp. CASD	5.5-6.0	$CaCO_3$	42	207.0	0.93	3.80	99.3	Wang et al., 2011
S. laevolacticus DSM442	5.5	$CaCO_3$	37	144.2	0.96	4.13	99.3	Li et al., 2013
Sporolactobacillus sp. Y2-8	7.0	$CaCO_3$	37	125.0	-	1.39	99	Sun et al., 2015
S. inulinus NBRC13595	6.5	$CaCO_3$	37	189.0	0.94	5.25	>98	Reddy Tadi et al., 2017
S. inulinus YBS1-5	5.5	$CaCO_3$	37	107.2	0.85	1.19	99.2	Bai et al., 2016
S. laevolacticus JCM2513	6.0	$Ca(OH)_2$	37	67.3	0.98	11.20	-	Mimitsuka et al., 2012
L. debrueckii JCM1106	6.0	$Ca(OH)_2$	40	79.0	80.6	3.59	95-96	Nakano et al., 2012
E. coli HBUT-D	7.0	$Ca(OH)_2$	37	127	0.93	6.35	99.5	Liu et al., 2014
L. coryniformis	6.0	NH_4OH	34	186.4	0.85	3.11	-	Nguyen et al., 2013
K. pneumoniae ATCC25955	7.0	NH_4OH	30	142.1	0.82	2.96	100	Feng et al., 2014
S. inulinus Y2-8	6.5	NH_4OH	37	218.8	-	1.65	>99.0	Zhao et al., 2014
L. debrueckii JCM1106	6.0	NH_4OH	40	63.3	0.64	1.51	95-96	Nakano et al., 2012
L. debrueckii JCM1106	6.0	NaOH	40	64.1	65.4	1.40	95-96	Nakano et al., 2012

2.6.4 Fermentation mode

Batch and fed-batch fermentation

The batch fermentation technique is susceptible to less contamination in comparison to fed-batch fermentation with a high final lactic acid concentration and yield. However, this method might act as the substrate and produce inhibition; thus, the batch fermentation technique exhibits low productivities (Hofvendahl and Hahn-Hagerdal, 2000; Sun et al., 2015). The fed batch technique is more favourable when compared to the batch fermentation technique due to increased productivity with short-time fermentation. Moreover, this technique is advantageous because substrate concentrations are kept on a fermentation level that decreases substrate inhibition. Consequently, yield and productivity can be increased including

minimizing the lag-phase of cell growth (Hofvendahl and Hahn-Hagerdal, 2000; Klotz et al., 2016). For example, fed batch fermentation by *S. laevolacticus* DSM442 demonstrated a glucose concentration maintained at 60 g/L in the fermentation level, resulting in a high D-lactic acid concentration of 144.4 g/L with high productivity of 4.13 g/(L.h) (Li et al., 2013). Table 2.8 shows the different fermentation modes for D-lactic acid production.

Continuous fermentation and cell immobilization

The continuous technique generally results in higher productivity compared to other fermentation strategies for D-lactic acid production. This method is advantageous in reducing the time for fermentation and process costs because the dilution rate in continuous culture affects the substrate and nutrient concentrations that need nutrients for fermentation with a constant flow to maintain the optimal concentration. However, the critical factors using this technique are the efflux of non-utilized substrate, cell loss and decrease in lactic acid concentration by high dilution rate and possible risk of contamination (Mimitsuka et al., 2012; Tashiro et al., 2011). The combination of continuous fermentation with cell recycling or cell immobilization is an important and efficient method to achieve high cell densities, production and productivity. The cell is circulated or immobilized by solids to obtain high cell density. The separation of product from fermentation broth, diluting it with fresh medium, produces inhibition effects that can be avoided. For example, Tashiro et al. (2011) studied a continuous using microfiltration membrane to recycle flow-through cells from *L. delbrueckii* ssp. *lactis* QU 41 for D-lactic acid fermentation. This resulted in a productivity of 18 g/(L.h) with a low residual glucose concentration. Mimitsuka et al. (2012) used a strain of *S. laevolacticus* JCM 2513 by continuous fermentation process using a membrane-integrated system. The results showed a reduced amount of nutrients for culture fermentation, long stability of this process and high productivity of 11.2 g/(L.h). Zhao et al. (2014) reported that final D-lactic acid of 218.8 g/L and productivity of 1.65 can be obtained by fed-batch fermentation using a multi-pulse feeding strategy in a fibrous bed reactor (FBB) combined with immobilized cells. This method is reported to achieve higher productivity compared to other fermentation modes for D-lactic acid production (Table 2.8).

Table 2.8 Different fermentation modes for D-lactic acid fermentation.

Strain	Fermentation mode	Lactate (g/L)	$Y_{p/s}$	Productivity (g/L.h)	Optical purity (%ee)	References
Sporolactobacillus sp. CASD	Batch	80-90	0.86-0.97	1.77	-	Zhao et al., 2010
Sporolactobacillus sp. Y2-8	Batch	122	0.81	1.00	99.1	Zheng et al., 2012
Sporolactobacillus sp. Y2-8	Batch	125.0	-	1.39	99	Sun et al., 2015
S. inulinus NBRC13595	Batch	189.0	0.94	5.25	>98	Reddy Tadi et al., 2017
Sporolactobacillus sp. CASD	Fed-batch	207	0.93	3.80	99.3	Wang et al., 2011
S. laevolacticus DSM442	Fed-batch	144.2	0.96	4.13	99.3	Li et al., 2013
S. inulinus YBS1-5	Fed-batch	107.2	0.85	1.19	99.2	Bai et al., 2016
S. inulinus Y2-8	Fed-batch/ immobilized cells	218.8	-	1.65	>99.0	Zhao et al., 2014
S. inulinus JCM6014	Continuous	64	96	8.9	98.8	Sawai et al., 2011
L. delbrueckii ssp. *lactis* QU 41	Continuous/ microfiltration membrane	20.7	1.00	18.00	-	Tashiro et al., 2011
S. laevolacticus JCM2513	Continuous/ MFR	67.3	0.98	11.20	-	Mimitsuka et al., 2012
S. laevolacticus JCM 2513	Continuous/ MFR	67	96	12.2	99.8	Sawai et al., 2011

CHAPTER III

Terrilactibacillus laevilacticus gen. nov., sp. nov., isolated from soil

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Running title: *Terrilactibacillus laevilacticus* gen. nov., sp. nov.

Category: New Taxa (Firmicutes)

The DDBJ accession number for the 16S rRNA gene sequence of strain NK26-11^T is AB841307

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Abstract

A Gram-stain-positive, catalase-positive, facultatively anaerobic, spore-forming, rod-shaped bacterium, strain NK26-11^T, was isolated from soil in Thailand. This strain produced D-lactic acid from glucose homofermentatively, and grew at 20-45 °C and pH 5-8.5. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid. The major respiratory quinone was menaquinone 7 (MK-7), the DNA G+C content was 42.6 mol%, and the major cellular fatty acids were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. On the basis of 16S rRNA gene sequences analysis, strain NK26-11^T was closely related to *Bacillus solimangrovi* JCM 18994^T (93.89 %16S rRNA gene sequence similarity), *Pullulanibacillus naganoensis* LMG 12887^T (93.32 %), *Sporolactobacillus inulinus* NRIC 1133^T (92.99 %), *Tuberibacillus calidus* JCM 13397^T (92.98 %) and *Thalassobacillus devorans* DSM 16966^T (<90.93 %). Strain NK26-11^T could be clearly distinguished from the closely related genera based on phenotypic characteristics and DNA G+C content, and thus represents a novel species of a new genus between the *Bacillus* and *Sporolactobacillus* cluster, for which the name *Terrilactibacillus laevilacticus* gen. nov., sp. nov. is proposed. The type strain of the type species is NK26-11^T (=LMG 27803^T =TISTR 2241^T =PCU 335^T).

Introduction

The typical lactic acid bacteria are Gram-positive, nonspore-forming and catalase-negative, including the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*, which produced lactic acid as the major end product from sugar fermentation (Reddy et al., 2008). In addition, strains of the genera *Sporolactobacillus* and *Bacillus* were reported to produce lactic acid (Fujita et al., 2010; Jung et al., 2009; Kitahara and Suzuki, 1963; Nakamura et al., 1988). The genus *Sporolactobacillus* was catalase-negative, spore-forming, homofermentative lactic acid bacteria, while members of the genus *Bacillus* were Gram-positive, aerobic or facultatively anaerobic, catalase-positive, spore-forming, rodshaped bacteria (Yanagida et al., 1987). A comparison of physiological characteristics of the spore-forming rods in species of the genus *Sporolactobacillus* and *Bacillus coagulans* revealed that members of the genus *Sporolactobacillus* lacked catalase activity and required carbohydrate for growth (Chang et al., 2008; Jung et al., 2009; Kitahara and Lai, 1967; Yanagida et al., 1987). Recently, spore-forming strains of *Tuberibacillus calidus* from a compost pile and *Pullulanibacillus naganoensis* from soil have been reported (Hatayama et al., 2006). In our screening of lactic acid-producing bacteria from soils in Thailand, several bacterial strains have been isolated and characterized (Prasirtsak et al., 2013), and the Gram-stain-positive, catalase-positive and lactic acid-producing bacterium, strain NK26-11^T was also isolated. In this study, this strain is characterized based on polyphasic taxonomy and is proposed to represent a novel species of a new genus.

Material and methods

Bacterial isolation, phenotypic and chemotaxonomic characteristics

Strain NK26-11^T was isolated from soil samples collected from Nakhon Pathom Province, Thailand, using MRS agar (De Man et al., 1960) supplemented with 0.5 % $CaCO_3$ and incubated at 37 °C for 3 days. Cell form, cell size, cell arrangement and colonial appearance were examined using cells grown on MRS agar for 3 days. The Hucker-Conn modification was used for the Gram stain (Hucker and Conn, 1923). Spore formation was examined by a scanning electron microscope. Flagellum was stained by the method described by Forbes (1981). Catalase and oxidase activities, nitrate reduction and hydrolysis of arginine and starch were tested as previously reported (Tanasupawat et al., 1992, 1998). The effect of temperature (10, 20, 25, 30, 40, 45 and 50 °C), initial pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0, 8.5, 9.0 and 10) and NaCl concentration [1–10 (in 1 % increments), 20 and 21 %] were tested by using GYP broth (Prasirtsak et al., 2013). The pH values were achieved using the following buffers, each at a final concentration of 50 mM: acetate buffer (for pH 4-5), phosphate buffer (for pH 6.0-7.0), Tris buffer (for pH 8-9.0) and sodium carbonate (for pH 10) (Sorokin, 2005). Growth on marine agar (MA; Difco), TSA (Difco) with 1 % soluble starch and CYC medium (Hatayama et al., 2006) was also determined. All tests were carried out by incubating the cultures at 37 °C, except for the investigation of effects of temperature. Acid formation from carbohydrates was determined as reported previously (Tanasupawat et al., 1998). Additional biochemical characteristics were recorded after incubation for 2 days in API 50 CH strips (bioMe´rieux). Lactic acid concentration in the fermentation broth was determined by HPLC (Sumi Chiral OA-5000; Prasirtsak et al., 2013). Cell-wall peptidoglycan was analysed as described by Staneck Roberts (1974). Menaquinone was analysed by HPLC (Komagata and Suzuki, 1987). Fatty acid methyl esters were prepared from cells grown on GYP agar plates for strain NK26-11^T, cells grown on TSI (TSA supplemented with 1 % soluble starch and 1.5 % NaCl, and adjusted to pH 6) for strain NK26-11^T, *P. naganoensis* LMG 12887^T and *Tuberibacillus calidus* JCM 13397^T, and from cells grown on TSII (TSA supplemented with 1 % soluble starch and 4 % NaCl, and adjusted to pH 7) for *Bacillus solimangrovi* JCM 18994^T and *Thalassobacillus devorans* DSM 16966^T. All agar plates were incubated at 37 °C for 3 days. Cellular fatty acid profiles were analysed by GLC according to the instructions of the Microbial Identification System (MIDI) Sherlock version 6.0 (Sasser, 1990) with the RTSBA6 MIDI database. For polar lipids analysis, the lipids were extracted and determined by two-dimensional TLC following the procedure of Minnikin et al. (1984).

Genotypic characteristics

DNA was isolated from cells grown in GYP broth for 2 days and was purified by the method of Saito Miura (1963). DNA base composition was determined by reversed-phase HPLC (Tamaoka and Komagata, 1984). The 16S rRNA gene sequence of the novel strain was amplified and the PCR product was purified and sequenced as described previously (Tanasupawat et al., 2004). The sequence of strain NK26-11^T

was aligned with selected sequences obtained from the GenBank database by using CLUSTAL X version 1.81. The alignment was edited manually to remove gaps and ambiguous nucleotides prior to the reconstruction of phylogenetic trees. The phylogenetic trees based on the neighbour-joining (Saitou and Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods were reconstructed in MEGA software version 6 (Tamura et al., 2013). 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 (Kim et al., 2012).

Results and discussion

Strain NK26-11^T was Gram-stain-positive, catalase-positive, facultatively anaerobic, endospore-forming, rod-shaped (0.6-0.7 × 0.6-4.4 μm; Fig. Appendix B), and motile by means of peritrichous flagella (Fig. Appendix B). Colonies on GYP agar plates were circular, convex and ivory-white (1.0-1.4 mm in diameter). D-Lactic acid (96.4 % optical purity of D-lactic acid) was produced from glucose. The strain was positive for nitrate reduction. Strain NK26-11^T grew at 20-45 °C, at pH 5-8.5 and on GYP agar with 3 % NaCl, but not on GYP agar with 4 % NaCl. This is in contrast to *B. solimangrovi* JCM 18994^T and *P. naganoensis* LMG 12887^T, which could not grow on GYP agar plates. *Tuberibacillus calidus* JCM 13397^T could grow on GYP agar supplemented with 0.5 or 1.2 % (w/w) NaCl and *Thalassobacillus devorans* DSM 16966^T grew on GYP agar supplemented with 4 % (w/w) NaCl. Strain NK26-11^T grew on MA and CYC agar while *Sporolactobacillus inulinus* NRIC 1133^T grew on CYC agar but grew weakly on MA agar. The details of phenotypic characteristics are listed in the genus and species descriptions and in Table 3.1. *Meso*-diaminopimelic acid was contained in the cell-wall peptidoglycan. MK-7 was the major menaquinone component. Strain NK26-11^T contained major fatty acids of anteiso-$C_{17:0}$ (56.5 and 58.1 % on GYP agar and TSI, respectively) and anteiso-$C_{15:0}$ (23.8 and 19.2 %). This was different from *B. solimangrovi* JCM18994^T and *P. naganoensis* LMG 12887^T which contained iso-$C_{15:0}$ and/or iso-$C_{16:0}$ as major fatty acids. However, strain NK26-11^T showed the same profile as *Sporolactobacillus inulinus* NRIC 1133^T. The other fatty acids of strain NK26-11^T when cultured on GYP agar were $C_{12:0}$ (0.6 %), iso-$C_{14:0}$ (0.3 %), $C_{14:0}$ (1.0 %), iso-$C_{15:0}$ (2.5 %), iso-$C_{16:0}$ (8.3 %), $C_{16:0}$ (4.2 %), iso-$C_{17:0}$ (1.7 %), $C_{18:0}$ (0.3 %) and anteiso-$C_{19:0}$ (0.7 %) (Table 3.2). The DNA G+C content of strain was 42.6 mol% which was differentiated from type strains of species of related genera (Table 3.1). Strain NK26-11^T contained diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), two ninhydrin-positive glycolipids, two glycolipids and two unknown lipids (Fig. Appendix B). This strain contained DPG and PG like *B. solimangrovi* JCM 18994^T, which contained DPG, PG and three unknown phospholipids (Lee et al., 2014), while *Thalassobacillus devorans* DSM 16966^T had DPG, PG, an unidentified lipid and an unidentified phospholipid (Garcia et al., 2005), and *P. naganoensis* LMG 12887^T contained DPG, PG, an unidentified phospholipid, three glycolipids and one amino lipid (Hatayama et al., 2006).

The 16S rRNA gene sequence analysis indicated that strain NK26-11^T was located between the genera *Bacillus* and *Sporolactobacillus* (Figs 3.1 and Appendix B). Strain NK26-11^T was closely related to *B. solimangrovi* JCM 18994^T (93.89 % 16S rRNA gene sequence similarity), *Pullulanibacillus naganoensis* LMG 12887^T (93.32 %), *Sporolactobacillus inulinus* NRIC 1133^T (92.99 %), *Tuberibacillus calidus* JCM 13397^T (92.98 %) and *Thalassobacillus devorans* DSM 16966^T (<90.93 %). In addition, compared to the other phylogenetic cluster, it was closely related to *Salirhabdus euzebyi* LMG 22839^T (93.8 %) (Albuquerque et al., 2007), *Bacillus shackletonii* LMG 18435^T (93.6 %) (Logan et al., 2004) and *Thalassobacillus hwangdonensis* KCTC 13254^T (93.39 %) (Lee et al., 2010).

Strain NK26-11^T was differentiated from *B. solimangrovi* JCM 18994^T, *P. naganoensis* LMG 12887^T, *Sporolactobacillus inulinus* NRIC 1133^T, *Tuberibacillus calidus* JCM 13397^T and *Thalassobacillus devorans* DSM 16966^T based on growth at 3 % NaCl (w/v), nitrate reduction, production of acid from various carbohydrates, cellular fatty acids of $C_{16:1}\omega7c$ alcohol, iso-$C_{15:0}$, iso-$C_{17:1}\omega10c$ and anteiso-$C_{17:0}$, and DNA G+C content (Tables 3.1 and 3.2). In addition, it could be differentiated from other genera based on 16S rRNA gene sequence analyses. Therefore, strain NK26-11^T represents a novel species in a new genus of the *Bacillus* and *Sporolactobacillus* cluster, for which the name *Terrilactibacillus laevilacticus* gen. nov., sp. nov. is proposed.

Table 3.1 Differential characteristics of strain NK26-11^T and representatives of related genera.

Strains: 1, NK26-11^T; 2, *B. solimangrovi* JCM 18994^T; 3, *P. naganoensis* LMG 12887^T; 4, *S. inulinus* NRIC 1133^T; 5, *Tuberibacillus calidus* JCM 13397^T; 6, *Thalassobacillus devorans* DSM 16966^T. All data were obtained in this study unless otherwise indicated. T, Terminal; S (C), sub-terminal (central); +, positive; w, weakly positive; -, negative.

Characteristics	1	2	3	4	5	6
Spore position	T	T	S (C)	T	T	C
Growth temperature (°C)	20-45	10-40	30-35	20-40	40-60	15-45
Maximum NaCl for growth (%)	3	9	5	3	4	20
pH range for growth	5-8.5	5.5-12	4.5-6	4.5-9.0	5-7	6-10
Catalase	+	+	+	-	+	+
Nitrate reduction	+	-	-	-	+	+
Acid on GYP $CaCO_3$ agar	+	-	-	+	-	-
Acid production from:						
Aesculin	-	+	+	-	+	+
Amygdalin	-	+	+	-	-	w
Arbutin	-	+	+	-	-	w
Celliobiose	-	+	+	-	+	+
Erythritol	+	-	-	-	-	-
D-Fructose	+	+	+	+	w	+
D-Galactose	+	+	+	-	-	+
Glycerol	+	+	-	w	-	+
D-Lyxose	+	-	-	-	-	-
D-Maltose	+	+	+	-	-	+
D-Mannose	+	+	+	+	w	-
D-Melezitose	+	+	+	w	-	-
D-Melibiose	+	+	+	-	-	+
Methyl-α- D- glucopyranoside	+	+	+	+	-	-
Methyl-α-D- mannopyranoside	+	+	+	-	-	-
Potassium gluconate	+	-	-	-	-	-
Raffinose	+	+	+	w	-	+
Salicin	-	+	+	-	+	+
L-Sorbose	w	-	-	-	-	-
Sucrose	+	+	+	+	-	+
D-Tagatose	w	-	-	+	-	-

Table 3.1 (continue) Differential characteristics of strain NK26-11^T and representatives of related genera.

Strains: 1, NK26-11^T; 2, *B. solimangrovi* JCM 18994^T; 3, *P. naganoensis* LMG 12887^T; 4, *S. inulinus* NRIC 1133^T; 5, *Tuberibacillus calidus* JCM 13397^T; 6, *Thalassobacillus devorans* DSM 16966^T. All data were obtained in this study unless otherwise indicated. T, Terminal; S (C), sub-terminal (central); +, positive; w, weakly positive; -, negative.

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**
Trehalose	+	+	+	+	-	+
Turanose	+	+	-	w	-	+
Enzyme activity						
N-Acetyl- β -glucosaminidase	-	-	+	-	-	-
Acid phosphatase	w	-	w	w	w	+
Alkaline phosphatase	-	+	-	-	-	+
α-Chymotrysin	w	-	-	-	+	-
Cystine arylamidase	w	-	-	+	w	-
Esterase (C4)	w	-	w	-	+	-
Esterase Lipase (C8)	-	-	w	-	+	-
α-Galactosidase	-	-	+	-	-	-
β-Galactosidase	-	-	+	-	-	-
α-Glucosidase	+	-	+	-	-	+
β-Glucosidase	-	-	+	-	-	-
Leucine arylamidase	+	w	+	+	+	-
α-Mannosidase	-	-	+	-	w	-
Naphthol-AS-BI-phosphohydrolase	-	-	w	+	w	w
Valine arylamidase	+	-	-	+	-	-
DNA G+C content (mol%) *	42.6	35.5[a]	45±2[b]	47[c]	46[d]	42.4[e]

*Data from: *a*, Lee et al. (2014); *b*, Hatayama et al. (2006); *c*, Yanagida et al. (1987); *d*, Hatayama et al. (2006); *e*, Garcia et al. (2005)

Table 3.2 Cellular fatty acid contents (%) of strain NK26-11^T and representatives of related genera.

Strains: 1, NK26-11^T; 2, *B. solimangrovi* JCM 18994^T; 3, *P. naganoensis* LMG 12887^T; 4, *S. inulinus* NRIC 1133^T; 5, *T. calidus* JCM 13397^T; 6, *Thalassobacillus devorans* DSM 16966^T; -, Not detected.

Fatty acid	1		2	3	4	5	6
	GYP	TSI†	TSII	TSI	TSI	TSI	TSII
Straight-chain							
$C_{10:0}$	-	0.5	-	-	1.0	-	-
$C_{12:0}$	0.6	0.3	-	-	1.1	-	0.1
$C_{14:0}$	1.0	1.0	0.8	0.5	3.2	0.2	1.4
$C_{16:0}$	4.2	7.1	3.6	0.9	10.9	2.7	16.4
$C_{17:0}$	-	-	0.1	0.2	0.2	0.4	0.3
$C_{18:0}$	0.3	0.6	-	0.1	2.2	0.3	0.8
Unsaturated							
$C_{16:1}$ *ω7c* alcohol	-	-	2.0	2.5	-	-	-
$C_{16:1}$ *ω11c*	-	-	5.0	-	-	-	0.1
$C_{18:1}$ *ω9c*	-	-	0.2	-	3.2	-	0.3
Branched							
iso-$C_{14:0}$	0.3	0.2	1.2	7.0	0.8	0.2	1.5
iso-$C_{15:0}$	2.5	1.7	64.8	34.8	5.6	7.1	13.7
iso-$C_{16:0}$	8.3	8.1	2.0	26.8	9.9	18.5	8.2
iso-$C_{17:0}$	1.7	1.8	4.3	4.1	3.1	19.4	7.1
iso-$C_{18:0}$	-	0.2	-	0.1	0.3	0.4	0.2
iso-$C_{17:1}$ *ω10c*	-	-	5.2	0.2	-	-	-
anteiso-$C_{15:0}$	23.8	19.2	5.5	16.6	17.4	3.2	35.0
anteiso-$C_{17:0}$	56.5	58.1	1.3	5.9	37.8	42.2	11.0
anteiso-$C_{19:0}$	0.7	-	-	-	-	0.1	0.1
Summed features							
3	-	-	-	-	-	-	2.2
4	-	-	1.4	-	-	-	-
8	-	0.5	-	-	1.5	-	-

*Summed features are groups of two or more fatty acids that could not be separated using the MIDI system. Summed feature 3 contained $C_{16:1}$*ω7c* and/or $C_{16:1}$*ω6c*; summed feature 4 contained iso-$C_{17:1}$ and/or anteiso B; summed feature 8 contained $C_{18:1}$*ω7c*.

†TSI, TSA with 1 % soluble starch and 1.5 % (w/v) NaCl, pH 6; TSII, TSA with 1 % soluble starch and 4 % (w/v) NaCl, pH 7.

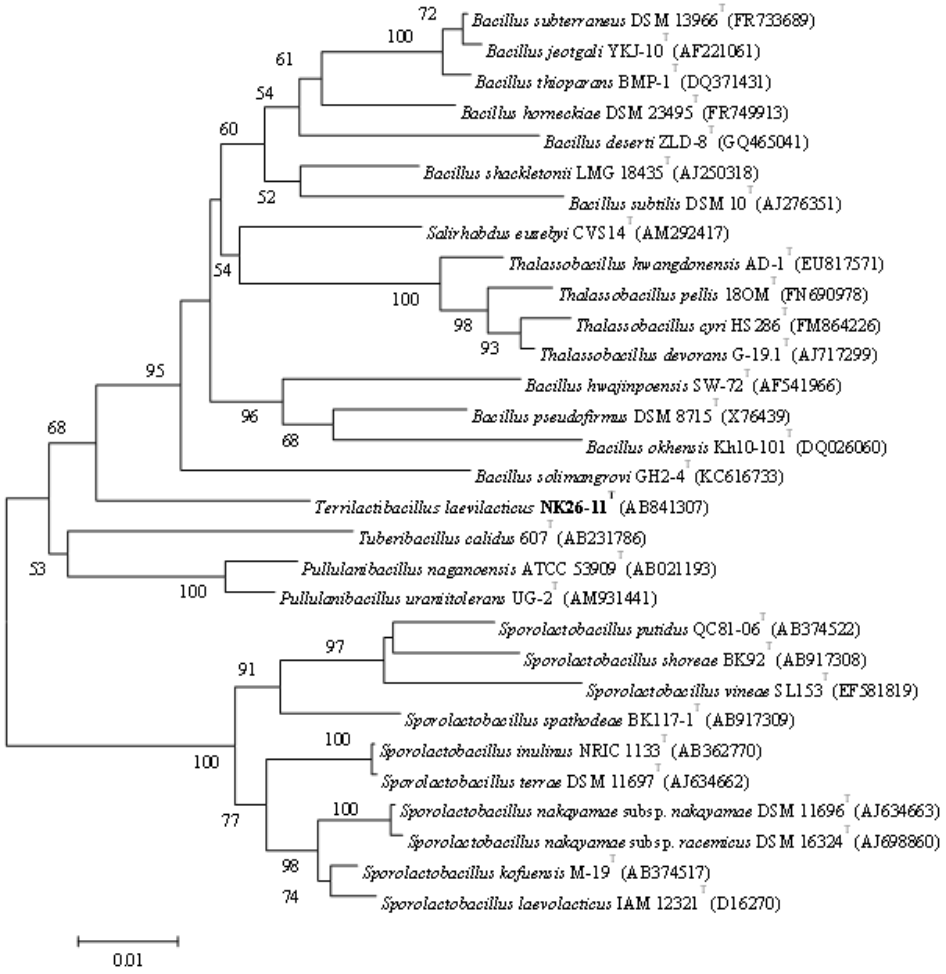

Figure 3.1 Neighbour-joining tree based on almost-complete 16S rRNA gene sequences showing relationships among strain NK26-11^T and related type species of the recognized representative members of family. Bootstrap values (>50 %) based on 1000 replications are given at branch nodes. Bar, 0.01 substitutions per nucleotide position.

Conclusion

Description of *Terrilactibacillus* gen. nov.

Terrilactibacillus (L. gen. n. *terra* of the earth; L. n. *lac lactis*, milk; L. masc. n. *bacillus* a small rod; N.L. masc. n.*Terrilactibacillus* soil lactic acid rodlet).

Cells are Gram-stain-positive, facultatively anaerobic, endospore-forming straight rods with rounded ends. The endospores are sub-terminal and oval with a swollen sporangium. Colonies on GYP agar plates are circular smooth ivory-white and non-pigmented. Produces D-lactic acid from glucose homofermentatively. Positive for catalase and nitrate reduction but negative for oxidase, hydrolysis of arginine and starch. Grows at 20-45 °C, at pH 5-8.5 and in the presence of 3 % (w/v) NaCl. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The major respiratory quinone is MK-7. Diphosphatidylglycerol and phosphatidylglycerol are the major polar lipids. The major fatty acids are anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$. The DNA G+C content of the type strain of the type species is 42.6 mol%.

The type species is *Terrilactibacillus laevilacticus*.

Description of *Terrilactibacillus laevilacticus* sp.nov.

Terrilactibacillus laevilacticus [lae.vi.lac'ti.cus. L. adj. *laevus* left; N.L. adj. *lacticus* referring to lactic acid; N.L. masc. adj. *laevilacticus* referring to D-(-)-lactic acid, the only lactic acid produced by the strain].

Displays the following characteristics in addition to those given in the genus description. Cells are 0.6-0.7 × 3.6-4.4 μm. In API 50 CH tests, acid is produced from glycerol, erythritol, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, maltose, melibiose, sucrose, L-sorbose (weak), trehalose, melezitose, raffinose, turanose, D-lyxose, D-tagatose (weak) and potassium gluconate, but not from D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl ß-D-xylopyranoside, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, amygdalin, arbutin, aesculin ferric citrate, salicin, cellobiose, lactose, inulin, starch, glycogen, xylitol, gentiobiose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate or potassium 5-ketogluconate. In the API ZYM system, positive for leucine arylamidase, valine arylamidase, and α-glucosidase activities, but negative for alkaline phosphatase, naphthol-AS-BI-phosphohydrolase, ß-galactosidase, ß-glucosidase, *N*-acetyl-ß-glucosaminidase, esterase lipase (C8), lipase (C4), trypsin, α-galactosidase, ß-glucuronidase, α-mannosidase and α-fucosidase activities. Weak enzymic activities are found for esterase (C4), cystine arylamidase, α-chymotrypsin and acid phosphatase. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, two ninhydrin-positive glycolipids, two glycolipids and two unknown lipids.

The type strain is NK26-11^T (=LMG 27803^T=TISTR 2241^T=PCU 335^T), isolated from soil in Thailand. The DNA G+C content of type strain is 42.6 mol%.

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CHAPTER IV

Characterization of D-lactic acid, spore-forming bacteria and *Terrilactibacillus laevilacticus* SK5-6 as potential industrial strain[☆]

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[☆] The GenBank/EMBL/DBBJ accession number for the 16S rRNA of strain SK5-6 is LC222555, for strain BRY67-1 is LC222556, for strain BRY67-2 is LC222557, for strain BRY67-3 is LC222558, for strain NK44-2 is LC222559, and for strain SP43-2 is LC222560.

Abstract

In this study, we screened and isolated D-lactic acid producing bacteria from soil and tree barks collected in Thailand. Among the obtained isolates, *Terrilactibacillus laevilacticus* SK5-6 exhibited a good D-lactate production performance in the primary screening fermentation (99.27 g/L final lactate titer with 0.90 g/g yield, 1.38 g/L·h, and 99.00% D-enantiomer equivalent). *T. laevilacticus* SK5-6 is a gram positive, endospore forming, homofermentative D-lactate producer. This isolate can ferment a wide range of sugars for D-lactate. Unlike the typical D-lactate producers, such as catalase negative *Sporolactobacillus* sp., *T. laevilacticus* SK5-6 acquired catalase activity; therefore, a 2-phase fermentation was simply employed for D-lactate production. Under an aerobic preculture stage, high-cell-density cell mass was rapidly obtained as the result of aerobic respiration. At the correct physiological stage (inoculum age) and a proper concentration of cell mass (inoculum size) transferred to the fermentation stage, *T. laevilacticus* rapidly converted glucose into D-lactate under anaerobic conditions resulting in a high final lactate titer (102.22 g/L), yield (0.84 g/g), and productivity (2.13 g/L·h). When shifting the process condition from an aerobic to an anaerobic environment, unlike other lactate producing bacteria, the mixed acid fermentation route was not activated in the culture of *T. laevilacticus* SK5-6 during the fermentation stage when some trace oxygen remained. This demonstrated the excellent characteristics of this isolate, in particular, a high product yield was obtained without byproduct formation. From the key characteristics of *T. laevilacticus* SK5-6, we claim this isolate as a novel D-lactate producer for the industrial fermentation.

Keywords: *Terrilactibacillus laevilacticus*; 2-phase fermentation; D-lactic acid; catalase positive; homofermentative

Introduction

Lactic acid has been widely used in many applications, for example, as an acidulant, a flavor enhancer, a food preservative, as well as in the pharmaceutical and chemical industries. Recently, the global awareness of the large consumption of non-renewable plastic products has driven the development of compostable plastics from the renewable resources as alternatives. Polylactic acid or PLA is one of the promising bio-based plastics drawing interest in research and development. During the early development stage, PLA was synthesized from the optically pure L-lactic acid owing to the availability of L-lactic acid in the market. The first generation of PLA products has limited uses since their mechanical and thermal properties were not competitive with the available commodity plastics. The mechanical and thermal properties of PLA can be improved with the stereoblock structure that yields an increasing melting temperature. To synthesize the stereoblock PLA, both optically pure L- and D-lactic acid are required. Nonetheless, unlike L-lactic acid with its wide range of applications, the production of D-lactic acid is currently limited (Li et al., 2013).

Lactic acid is currently produced via microbial fermentation under mild conditions and low energy consumption. Microbial fermentation utilizes low cost, renewable feedstocks to produce a racemic mixture or an optically pure isomer of lactic acid (L-lactic acid and D-lactic acid), depending on the microbes employed (John et al., 2009; Wee et al., 2006; Xu et al., 2010). It was found that D-lactic acid is only produced in some bacteria including *Lactobacillus delbrueckii*, *Leuconostoc* sp., and *Sporolactobacillus* sp. as a metabolic response to environmental stress (Bai et al., 2016; Li et al., 2013; Mimitsuka et al., 2012; Tashiro et al., 2011; Zhao et al., 2014). It has been mentioned that D-lactate production with the aforementioned strains usually results in poor fermentation performance. Genetic engineering has been employed for strain development to specifically produce optically pure D-lactate; however, the genetically modified microbes have limited industrial applications due to biosafety regulation limits and genetic instability (Baek et al., 2016; Feng et al., 2014; Tsuge et al., 2014). Therefore, wild strains are still preferred in industrial D-lactate production facilities.

Previous studies have revealed that novel D-lactate isolates have been screened from natural samples in Thailand. Recently, Thamacharoensuk et al. (2015) isolated 2 D-lactate producers from tree barks later identified as the novel species, *Sporolactobacillus spathodeae* and *Sporolactobacillus shoreae*. In addition, Prasirtsak et al. (2016) isolated the novel bacterial genus *Terrilactibacillus* from soil samples in Thailand. It was later identified as *Terrilactibacillus laevilacticus* NK26-11^{T}, a catalase positive D-lactate producer that attracted the researchers' attention due to its good fermentation performance (high yield, high productivity, and high optical purity). Previously mentioned findings have demonstrated that Thailand provides a good source for bacterial screening. Therefore, in this study, the bacterial screening, isolation, and identification for D-lactate producers was performed to obtain robust D-lactate producers. Among the D-lactate producers screened in this study, one is *T. laevilacticus* SK5-6, a novel genus, which exhibited a good fermentation performance in primary screening. Nonetheless, little is known about D-lactate production by this genus. Consequently, in this study we report the preliminary fermentation

optimization for D-lactate production by *T. laevilacticus* SK5-6 and propose that this novel genus is a promising D-lactate producing strain for use in industrial fermentation.

Material and methods

Sample collection

Seventy-six samples of soil and tree barks were collected from December 2014 to August 2015 in the northern, eastern, and central provinces of Thailand. Three samples from Chiangmai, 5 from Phitsanulok, 11 from Rayong, 4 from Suphanburi, 5 from Saraburi, 8 from Ayutthaya, 33 from Nakhonpathom, and 7 from Bangkok. The isolate SK5-6 was screened from a soil sample collected at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok.

Bacterial isolation

Natural samples (approximately 200-300 mg) were collected from soil and tree barks. To selectively revive the growth of bacteria, the collected sample (0.25 g) was transferred into the sterile enrichment medium (5 mL, pH 4.4) following by heat shock at 80 °C for 10 min. Later, the suspension was incubated at 37 °C until growth developed. The enrichment medium contained (per liter) 10 g glucose, 5 g yeast extract, 5 g peptone, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4 supplemented with 10 mL salt solution. The composition of salt solution consisted of (per 10 mL) 400 mg $MgSO_4 \cdot 7H_2O$, 20 mg $MnSO_4 \cdot 5H_2O$, 20 mg $FeSO_4 \cdot 7H_2O$, and 20 mg NaCl. After that, the suspension was transferred onto a Glucose - Yeast Extract - Peptone (GYP) agar plate containing $CaCO_3$ as the acid production indicator. The GYP agar medium contained (per liter) 10 g glucose, 5 g yeast extract, 5 g peptone, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4, 20 g agar, 5 g $CaCO_3$, and 10 mL salt solution. The pH of the GYP agar medium was adjusted to 6.80.

The GYP agar plate was incubated at 37 °C until colonies were developed. The colonies that formed in a large clear zone were transferred onto a freshly new GYP agar plate for single colony purification. A single colony was transferred into a new GYP agar slant. To maintain the activity of the selected colony, it was subcultured into the new GYP agar slant every 10 days. Subsequently, the single colony was transferred into the sterile skim milk solution and kept at -80 °C for long term storage.

Phenotypic characterization

The bacterial isolates were cultured in a phenotypic medium containing (per liter) 10 g glucose, 10 g yeast extract, 10 g peptone, 10 g CH_3COONa, and 5 mL salt solution at 37 °C in an anaerobic condition for phenotypic characterization. Cell form and cell size were examined under the microscope. The Hucker-Conn modification method was used for Gram staining (Tanasupawat et al., 1992; Tanasupawat et al., 1998). Spore formation was examined with a scanning electron microscopy. The effects of temperature, initial pH, and NaCl concentration on growth were observed in

the culture grown in the phenotypic medium. Catalase and oxidase activities, nitrate reduction, and hydrolysis of arginine and starch were tested following the method previously described in Tanasupawat et al. (1998). Additional biochemical characteristics of the isolates were determined after incubation for 2 days at 37 °C in an aerobic condition in API 50 CH strips (bioMerieux) according to the manufacturer's instructions.

Genotypic characterization

DNA was extracted from the cells grown in the GYP broth for 2 days and purified using the method described by Saito Miura (1963). The 16S rRNA gene sequences were amplified by polymerase chain reaction (PCR) with the following primers (Universal primers): 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 518F (5'-CCAGCAGCCGCGGTAATACG-3'), 800R (5'-TACCAGGGTATCTAATCC-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') and the PCR product was purified and sequenced as previously described (Saito and Miura, 1963; Tanasupawat et al., 2004). The sequence of the isolate was aligned with the selected sequences obtained from GenBank using CLUSTAL_X version 1.81. The alignment was manually edited to remove gaps and ambiguous nucleotides before constructing a phylogenetic tree with the neighbor-joining method and the maximum-likelihood method using MEGA version 6 (Felsenstein, 1981; Saitou and Nei, 1987; Tamura et al., 2013). The confidence values of the individual branches in the phylogenetic tree were determined using the bootstrap analysis of Felsenstein (1985) based on 1,000 replications. The values for the sequence similarity among the closest strains were determined by EzTaxon server (Kim et al., 2012).

Primary screening for D-lactate producing isolates

The bacterial isolates were categorized into 2 groups based on the catalase activity in the primary screening for D-lactate production. Each isolate was subcultured onto a fresh new GYP agar slant and incubated anaerobically at 37 °C for 48 h. To prepare the bacterial suspension, 2 mL of sterile preculture medium was transferred into the culture slant. The culture slant was then thoroughly mixed. The bacterial suspension was inoculated in the preculture flask containing 48 mL sterile preculture medium.

For the catalase negative isolate, 2 mL of the bacterial suspension was inoculated in the preculture medium (pH 6.8) containing (per 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 salt solution, and 5 g $CaCO_3$. The culture was incubated at 37 °C in an anaerobic condition for 26 h (in a flask plugged with a T-type silicone stopper and placed into a W-zip pouch containing AnaeroPack-Anaero from Mitsubishi Gas Chemical). After that 1 mL of the preculture broth was transferred into the sterile fermentation medium (49 mL) containing (per liter) 120 g glucose, 10 g yeast extract, 5 g peptone, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4, 10 mL salt solution, and 80 g $CaCO_3$. The culture was incubated at 37 °C in an anaerobic condition for 72 h (in a flask plugged with a T-type silicone stopper and placed into a W-zip pouch containing AnaeroPack-Anaero (Mitsubishi

Gas Chemical)). At the end of the fermentation, the sample was collected for analyses of the remaining glucose, D-lactate, byproducts, and optical purity of the D-lactate.

For the catalase positive isolate, the bacterial suspension was inoculated in preculture medium (pH 6.8) containing (per liter) 10 g glucose, 15 g yeast extract, 4 g NH_4Cl, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 20 mL salt solution, and 5 g $CaCO_3$. The culture was incubated at 37 °C, 200 rpm for 5 h in a flask plugged with a C-type silicone stopper. Later 25 mL of the preculture broth was transferred into 25 mL of the sterile fermentation medium containing (per liter) 240 g glucose and 80 g $CaCO_3$. The culture was then incubated at 37 °C for another 72 h in a flask plugged with a T-type silicone stopper and placed into a W-zip pouch containing AnaeroPack-Anaero (Mitsubishi Gas Chemical). At the end of the fermentation, the sample was collected for analyses of the remaining glucose, D-lactate, byproducts, and the optical purity of D-lactate.

Effects of inoculum size, oxygen, and mixing on growth of *T. laevilacticus* SK5-6 during the preculture stage

The operating conditions during both preculture and fermentation stages of the catalase positive isolate *T. laevilacticus* SK5-6 were further optimized in flask cultivation. The growth profile during the preculture stage was determined. *T. laevilacticus* SK5-6 was subcultured onto fresh new GYP agar slants and incubated at 37 °C for 24 h to prepare the bacterial suspension. The preculture medium was inoculated with the bacterial suspension (OD600 of 30-40 where OD600 of 1 is equivalent to 0.12 g/L cell dry weight) of *T. laevilacticus* SK5-6 at different inoculum sizes (0.5, 1, and 2%). The culture was incubated in the Erlenmeyer flask at 37 °C under different mixing (no mixing and mixing at 200 rpm) and gas permeability (to generate an aerobic/anaerobic culture environment) conditions. To manipulate air permeability of the culture flask in order to initiate an aerobic condition, the flask was plugged with a C-type silicone stopper to allow good air permeability. To generate an anaerobic condition, the flask was plugged with a T-type silicone stopper and placed into a W-zip pouch containing AnaeroPack-Anaero from Mitsubishi Gas Chemical. Samples were taken every hour for 8 h for analyses of cell mass, the remaining glucose, and product formation.

Effect of the preculture seed on D-lactate fermentation

T. laevilacticus SK5-6 was subcultured onto GYP agar slants and incubated at 37 °C. The bacterial suspension of OD600 of 30-40 was prepared for inoculation into the preculture medium at 1% inoculum size. The preculture flask was incubated at 37 °C under different mixing (no mixing and mixing at 200 rpm) and gas permeability (using a C-type silicone stopper or a T-type silicone stopper in a W-zip pouch containing AnaeroPack-Anaero) conditions. The preculture time was varied between the mid log phase (4 h) and the late log phase (5 h). After that, the preculture broth was transferred into the fermentation medium at 50% inoculum size. The fermentation culture was incubated at the same temperature for 48 h under varied mixing (no mixing and mixing at 150 rpm) and gas permeation (with either a C-type silicone stopper or a T-type silicone stopper in a W-zip pouch containing AnaeroPack-Anaero)

conditions. Samples were taken every 12 h for analyses of OD600 reading, the remaining glucose, and lactate and byproduct formation.

D-Lactate fermentation by *T. laevilacticus* SK5-6 in a 5 L stirred fermentor

The fermentation platform of *T. laevilacticus* SK5-6 was preliminarily determined in a 5 L stirred fermentor. A 24-h GYP agar slant was used to prepare the bacterial suspension. The bacterial suspension (1% inoculum size) was inoculated in a preculture flask plugged with a C-type silicone stopper. The preculture flask was incubated at 37 °C, 200 rpm for 4 h. After that, the preculture flask was transferred into a 5-L stirred fermentor containing 2.5 L of the sterile preculture medium at 10% inoculum size. The fermentor was operated at 37 °C, agitated at 300 rpm, with 1.0 vvm air. After 3 h, 0.5 L of the fermentation medium containing (per liter) 720 g glucose and 480 g $CaCO_3$ was added into the fermentor. Aeration was stopped and the agitation speed was varied at 200 and 300 rpm to obtain the optimal D-lactate production rate. Samples were taken every 6 h for 48 h for analyses of cell mass, remaining glucose, and lactate and byproduct formation.

Analytical methods

A sample of fermentation broth was centrifuged at 10,000 g for 5 min to separate the cell-free supernatant from the cell mass. The supernatant was collected for further analyses of the remaining glucose, lactic acid, byproducts, and the optical purity of D-lactate. The cell mass was acidified with 1 M HCl to remove insoluble $CaCO_3$ remaining in the sample. The acidified sample was centrifuged and particles were resuspended in deionized water for the OD reading. Spectrophotometry was used to determine the OD at the wavelength of 600 nm of the cell mass present in the fermentation broth. Cell mass concentration was then calculated from the correlation between OD600 and cell dry weight. (1 OD600 is equivalent to 0.12 g/L).

The concentration of products formed and glucose substrate remaining during the fermentation were analyzed using high performance liquid chromatography. Fermentation samples were centrifuged, filtered through a PTFE (hydrophilic) membrane, and diluted with double deionized water. For analyses of the remaining glucose, total lactic acid (both L- and D-lactic acid), and acetic acid, 15 μL diluted particle-free samples were automatically injected (Shimadzu-SIL-10A) into an organic acid analysis column (Biorad, Aminex HPX-87H ion exclusion organic acid column; 300 mm × 7.8 mm) and maintained at 45 °C in a column oven (Shimadzu-CTO-10A). A 0.005 M H_2SO_4 was pumped through the system at the flowrate 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, and acetate) were injected as references to determine sample concentration. The chromatogram peak area was used as the comparison basis in determining the concentration. To determine the optical purity of lactic acid, 5 μL diluted particle-free samples were automatically injected into a chiral column (Sumipack, Sumichiral OA5000) and maintained at 40 °C. A 0.001 M $CuSO_4$ was used as the eluent at the flowrate of 1.0 mL/min. The UV detector was used to detect

the lactate isomers at 254 nm. The standards containing 0-2 g/L of D- and L-lactic acid were injected as references to determine sample concentration.

Product yield ($Y_{p/s}$) was determined from the ratio of the product formed to carbon substrate consumed during the fermentation stage. Volumetric productivity was defined as the total amount of product formed per unit volume per time. The optical purity of the D-lactate was defined from the peak areas of the chromatogram as follows (Zhao et al., 2014).

$$\text{Optical purity} = \frac{\text{D} - \text{lactate} - \text{L} - \text{lactate}}{\text{D} - \text{lactate} + \text{L} - \text{lactate}} \times 100\%$$

Results and discussion

Isolation, identification, characterization, and screening of D-lactic acid producers

One hundred and sixteen bacterial isolates were obtained from 76 natural samples. From these isolates, there were only 6 isolates, including SK5-6, SP43-2, NK44-2, BRY67-1, BRY67-2, and BRY67-3, that produced D-lactic acid. From the neighbor-joining phylogenetic tree based on the 16S rRNA gene sequences, it was suggested that the isolate SK5-6 was 100% related to the novel *Terrilactibacillus laevilacticus* NK26-11^T (= LMG27803^T = TISTR2241^T = PCU335^T) previously reported in Prasirtsak et al. (2016). The isolate SP43-2 demonstrated a high similarity percentage in 16S rRNA sequence to *S. nakayamae* subsp. *nakayamae* DSM11696^T (99.6%) (Yanagida et al., 1997). The other isolates, including NK44-2, BRY67-1, BRY67-2, and BRY67-3, had similar percentages of 99.2–99.7% to *S. laevolacticus* DSM442^T (Hatayama et al., 2006) (Table 4.1 and Fig. 4.1).

From the morphological and biochemical characterization, it was found that all 6 isolates were gram positive, facultative anaerobic, spore forming, rod shaped bacteria. None were able to hydrolyze starch and arginine. They lacked oxidase and catalase except SK5-6 which exhibited a positive result on catalase activity. As a result, oxygen was not restricted in the cultivation of SK5-6 while the others required an anaerobic environment for growth. All 6 isolates produced D-lactate from glucose via a homofermentative route. They grew at 20-40 °C, with a pH of 5.5-8.5. They tolerated a salt concentration of up to 3%. These isolates utilized various substrates besides glucose (Table 4.1).

From the primary screening fermentation, it was found that all 6 isolates exhibited a high D-lactate production compared with the type strain *T. laevilacticus* NK26-11^T previously identified by our research group (Table 4.2). All 6 isolates also produced D-lactate from glucose at high yields of approximately 0.83-1.00 g/g glucose. Productivity in flask culture was in an acceptable range of 1.06-1.42 g/L·h with a sufficiently high final titer of 76.01-102.43 g/L from the initial glucose concentration of 120 g/L. While SK5-6, SP43-2, and NK44-2 yielded a high optical purity of D-lactate, BRY67-1, BRY67-2, and BRY67-3 produced an optical purity less than 99.0%ee. The lower optical purity of D-lactate resulted in a more complicated recovery and purification process to acquire a sufficiently higher purity

than 99.0% for the polymer-grade lactic acid. As a result, fermentation optimization by these 3 isolates was not continued. Comparison of SK5-6, SP43-2, and NK44-2 showed that SK5-6 exhibited strong catalase activity while the other 2 isolates were catalase negative bacteria. Therefore, it was possible to implement a 2-phase fermentation technique (aerobic preculture and subsequent anaerobic fermentation) in the fermentation of SK5-6. Via the 2-phase fermentation cultivation, the high-cell-density SK5-6 obtained during the aerobic preculture stage enabled the production of D-lactate during the anaerobic fermentation stage at a high production rate (Ma et al., 2014; Qin et al., 2010). In addition, *T. laevilacticus* is a novel bacterial genus capable of producing D-lactate previously identified by Prasirtsak et al. (2013) and Prasirtsak et al. (2016) but no report has been found on the process of fermentation optimization by this bacterial genus. Therefore, in this study, we attempted to determine the key process parameters that manipulated D-lactate fermentation by this novel genus.

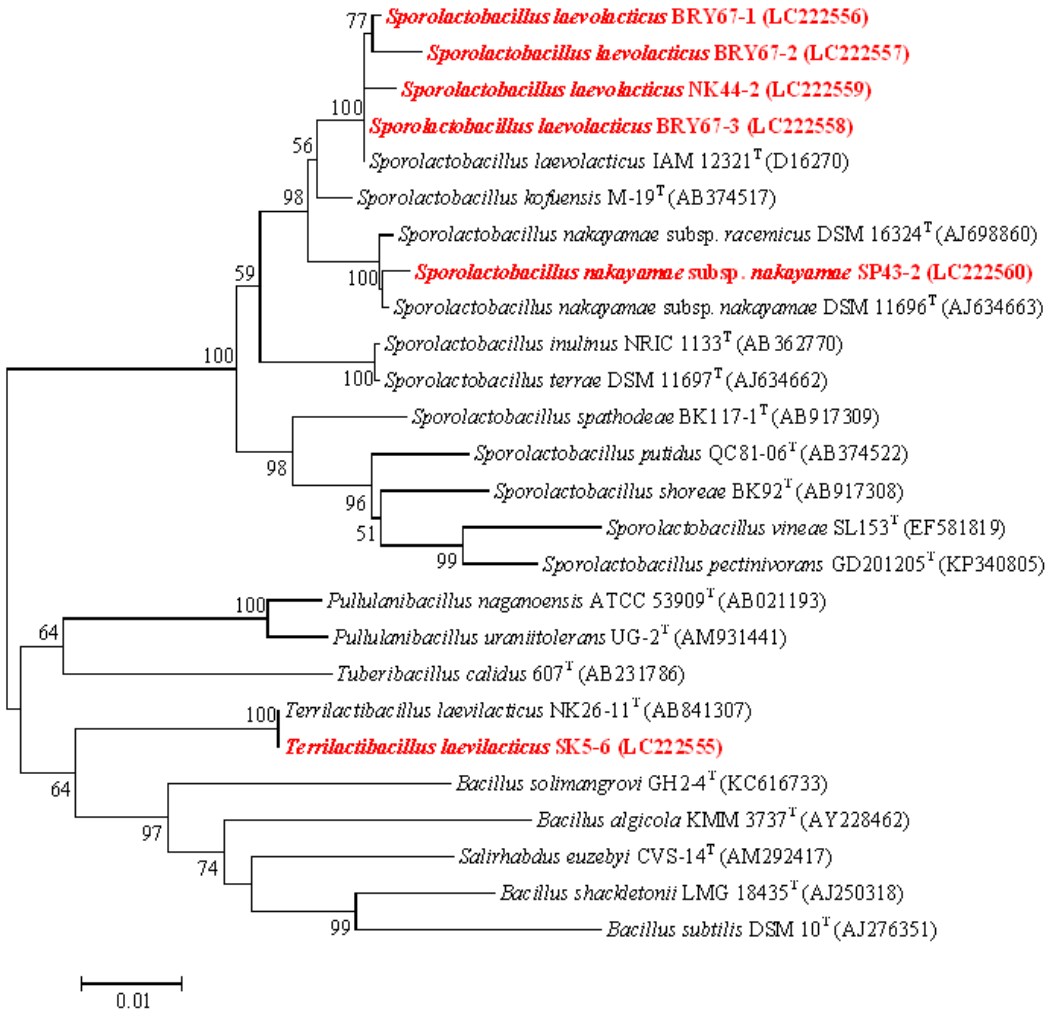


Figure 4.1 Neighbor-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic relationship of the strains SK5-6, SP43-2, NK44-2, BRY67-1, BRY67-2, BRY67-3, and related species. Bar 1% represents the sequence divergence.

Table 4.1 Selected characteristics of 6 D-lactate producing isolates and their closely related type strains.

Characteristic	SK5-6	*T. laevilacticus* NK26-11^T	SP43-2	*S. nakayamae* subsp. *nakyamae* DSM1196^T	NK44-2	BRY67-1	BRY67-2	BRY67-3	*S. laevolacticus* DSM442^T
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Spore formation	+	+	+	+	+	+	+	+	+
Temperature range (°C)	25-40	20-45	20-40	20-40	20-40	20-40	20-40	20-40	20-40
NaCl range (%)	0-3	0-3	0-3	0-3	0-3	0-3	0-3	0-3	0-2
pH range	5.5-8.5	5.0-8.5	4.0-9.0	4.5-8.5	4.0-8.5	4.0-9.0	4.0-9.0	4.0-9.0	4.0-9.0
Catalase activity	+	+	-	-	-	-	-	-	-
Lactate isomer produced	D	D	D	D	D	D	D	D	D
Acid production from									
Glycerol	+	+	-	-	-	-	-	-	-
Erythritol	+	+	-	-	-	-	-	-	-
D-Galactose	+	+	-	-	+	+	+	+	W
D-Glucose	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+
D-Lyxose	+	+	-	-	-	-	-	-	-
D-Mannose	+	+	+	+	+	+	+	+	+
D-Saccharose	+	+	+	+	+	+	+	+	+
D-Turanose	+	+	-	w	-	-	-	-	-
D-Ribose	-	-	-	-	-	-	-	-	-
D-Xylose	-	-	-	-	-	-	-	-	-
Origin	Soil	-	Bark	-	Bark	Bark	Bark	Bark	-

+ positive; - negative, w weakly positive

Table 4.2 D-Lactic acid production from 6 selected isolates in the primary screening compared with the type strain *T. laevilacticus* NK26-11^{T}. The fermentation was conducted in a shake flask culture with an initial glucose concentration of 120 g/L.

Isolate no.	D-lactate				Remaining glucose (g/L)
	Final conc. (g/L)	Optical purity (%ee)	Yield (g/g)	Productivity (g/L·h)	
NK26-11^{T}	60.52 ± 0.44	96.44 ± 0.68	0.50 ± 0.01	0.84 ± 0.02	0.00 ± 0.00
SK5-6	99.27 ± 0.71	99.00 ± 0.70	0.90 ± 0.00	1.38 ± 0.00	9.74 ± 0.14
SP43-2	102.43 ± 2.71	100.00 ± 0.00	0.85 ± 0.01	1.42 ± 0.02	0.00 ± 0.00
NK44-2	90.84 ± 2.57	100.00 ± 0.00	0.83 ± 0.01	1.26 ± 0.01	10.93 ± 0.08
BRY67-1	101.12 ± 0.72	98.76 ± 0.70	1.00 ± 0.01	1.40 ± 0.01	18.75 ± 0.13
BRY67-2	83.68 ± 0.59	98.30 ± 0.70	0.86 ± 0.01	1.16 ± 0.01	22.84 ± 0.16
BRY67-3	76.01 ± 1.07	98. 56 ± 1.39	0.85 ± 0.01	1.06 ± 0.01	30.41 ± 0.43

Determination of the correct physiological stage of preculture seed for D-lactate fermentation by *T. laevilacticus* SK5-6

In this study, we investigated the metabolic response of SK5-6 under both aerobic and anaerobic conditions. Also, the effect of mixing to allow a good gas diffusion and homogeneity in the culture was observed. Without mixing, a similar growth rate was obtained from the cultures incubated under anaerobic/aerobic conditions (Fig. 4.2). While mixing at 200 rpm, the culture incubated under the aerobic conditions yielded a slightly higher growth rate resulting in a higher final cell concentration when the growth reached stationary phase (5 h). With sufficient mixing, the lag phase was shortened and the growth rate was higher. Therefore, comparing the 2 parameters studied, mixing had a stronger effect on the growth of SK5-6. In addition, the higher growth rate and maximum cell dry weight achieved from the culture under the aerobic condition. With the presence of oxygen, SK5-6 generated more ATP via glycolysis, TCA, and ETC which eventually resulted in a higher growth rate. Nonetheless, the rate was not dramatically different.

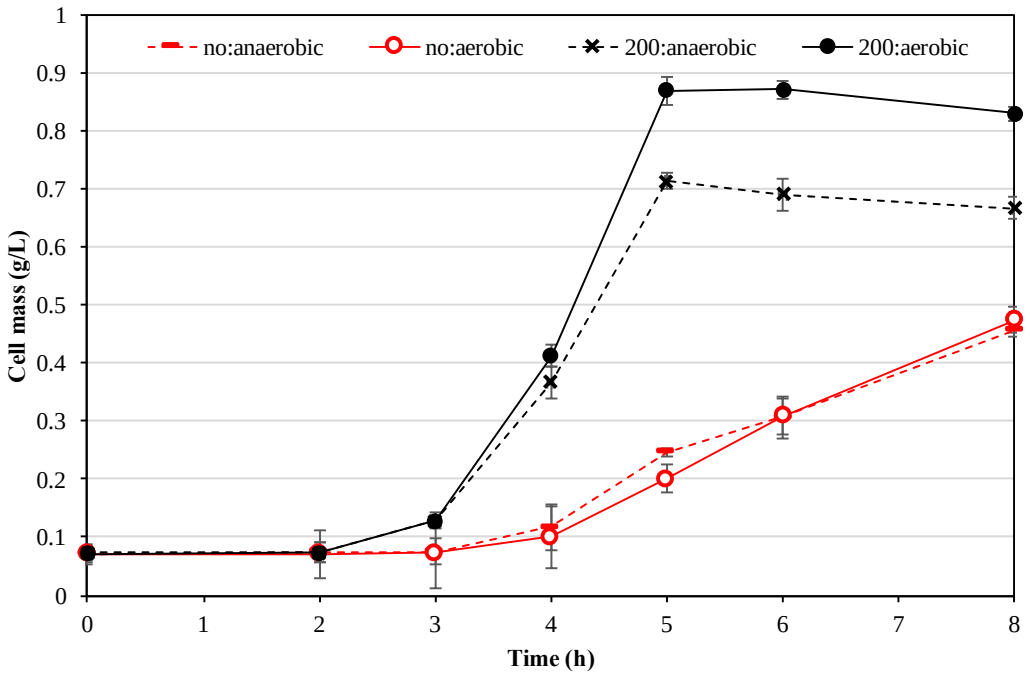


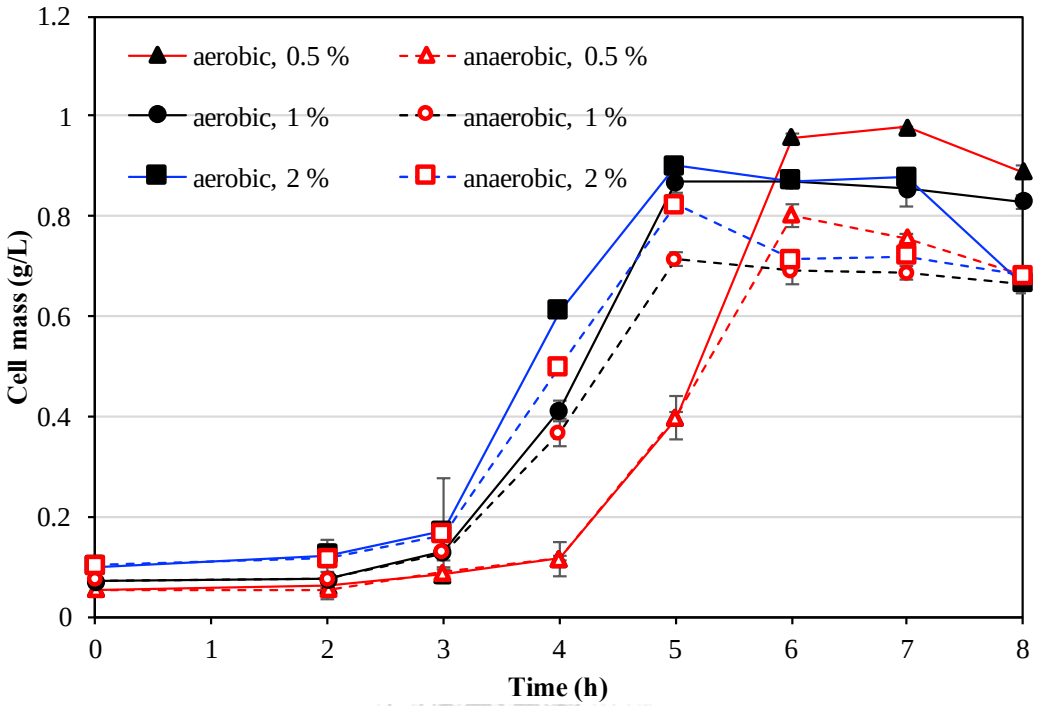
Figure 4.2 Effect of mixing and oxygen on growth of SK5-6 during the preculture stage in the flask culture. : no:anaerobic; : no:aerobic; : 200:anaerobic; : 200:aerobic.

The effects of inoculum size were also observed (Fig. 4.3). It was found that a small inoculum size (0.5%) resulted in a longer lag phase (Fig. 4.3a). Conversely, increasing the inoculum size to 1% and 2% shortened the lag phase. However, with respect to the log phase, it appears that the growth rate during the log phase of SK5-6 was similar for the 3 inoculum sizes studied. Diverting the culture from an aerobic to an anaerobic environment did not result in much difference on cell growth regardless of inoculum size. Glucose consumption (Fig. 4.3b) was consistent with the growth

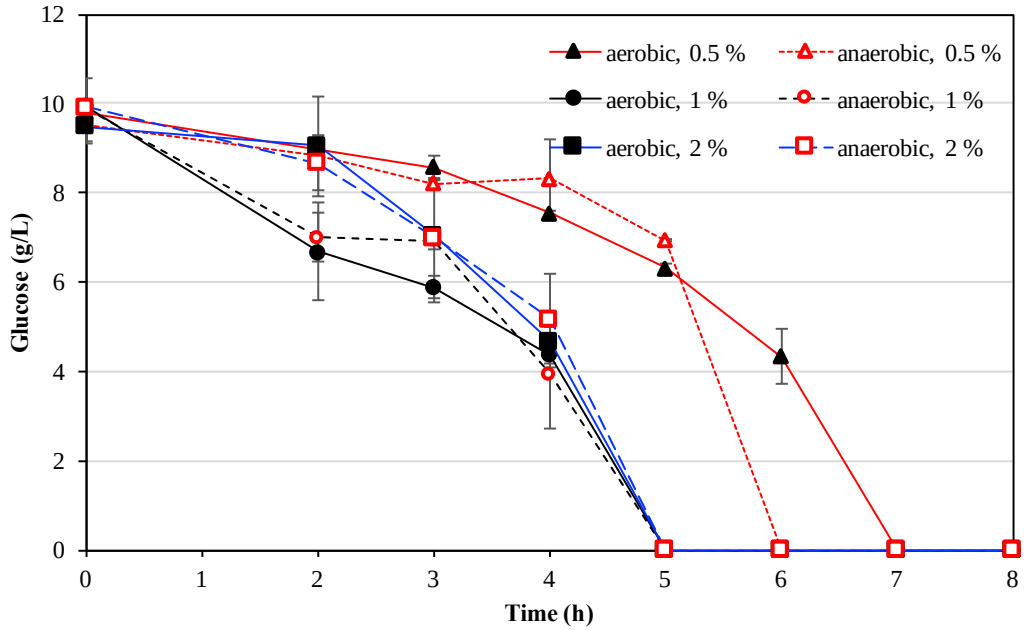
profile. Fig. 4.3c shows lactate formation appearing during the preculture stage. This indicates the expression of lactate dehydrogenase readily for lactate production when the metabolic flux shifted toward the anaerobic route. As shown in Figs. 4.3a and 4.3c, it was found that lactate started to accumulate when growth entered the log phase. At a small inoculum size (0.5%), lactate formation appeared when growth was in mid log phase. At a larger inoculum size (1% and 2%), lactate started to form since an early log phase. At 2% inoculum size, a high concentration of lactate (8.89-10.60 g/L) accumulated in the preculture broth, which created stress to the cells and later caused diverse effect on lactate formation during the fermentation stage. At 1% inoculum size used to inoculate the preculture flask, a shortened lag phase was observed with rapid growth, high cell mass concentration, and slight lactate formation. This indicates that proper cell density inoculated into the new medium induced high biosynthetic rates within a short time frame (Egervarn et al., 2007). Thus, 1% inoculum size was deemed suitable and selected for further optimization.

Not only did a sufficiently high concentration of the inoculum enhance the metabolic rate, the proper physiological stage also played a role in controlling the performance of SK5-6 during fermentation. It was reported that the inoculum promptly metabolized glucose to D-lactate at a high rate during the log phase (Zagari et al., 2013). In this study, we observed the effect on performance with respect to D-lactate production from inoculum during the mid log phase (4 h) and during the late log phase with higher cell concentration (5 h). The results revealed no difference in D-lactate production performance obtained from both inoculum seeds studied. This can be explained by fact that the specific growth rate of SK5-6 at the mid log phase was higher than that at the late log phase when cells were approaching the stationary phase, although the cell concentration at the mid log phase was about half that at the late log phase (Figs. 4.2 and 4.3a). To be more specific, the high growth rate could have compensated with high cell concentration. From this finding, it is suggested that the seed train development for SK5-6 was rather flexible and effective as this could occur at times between the mid log and the late log phases.

a


aerobic, 0.5 %
anaerobic, 0.5 %
aerobic, 1 %
anaerobic, 1 %
aerobic, 2 %
anaerobic, 2 %
Cell mass (g/L)
Time (h)
0
0.2
0.4
0.6
0.8
1
1.2
0
1
2
3
4
5
6
7
8

b


aerobic, 0.5 %
anaerobic, 0.5 %
aerobic, 1 %
anaerobic, 1 %
aerobic, 2 %
anaerobic, 2 %
Glucose (g/L)
Time (h)
0
2
4
6
8
10
12
0
1
2
3
4
5
6
7
8

c

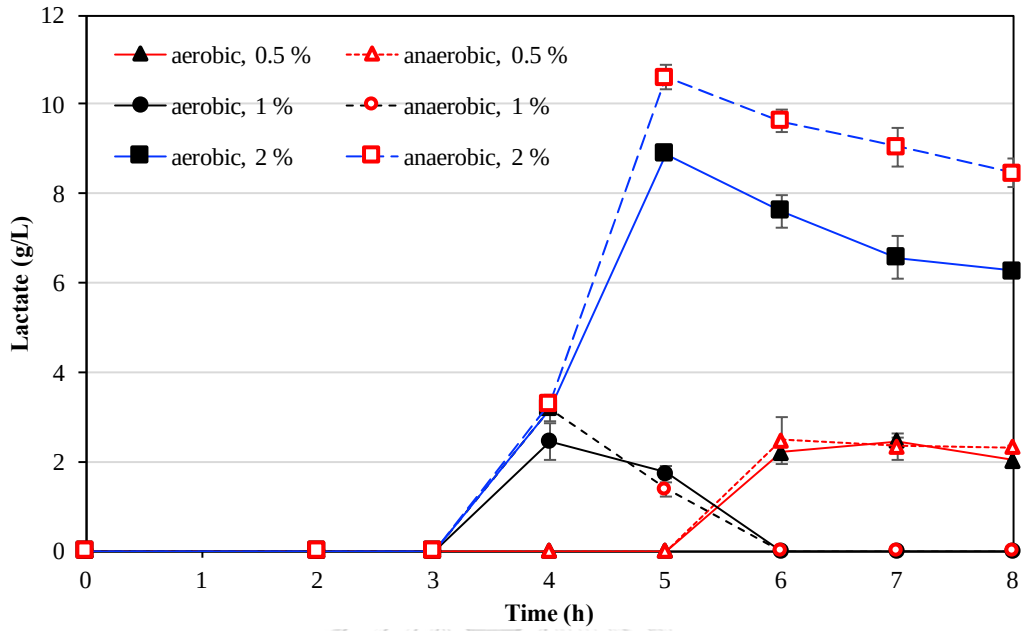


Figure 4.3 Effect of the inoculum size on growth, glucose consumption, and lactate formation of SK5-6 during the preculture stage in the flask culture. The culture was incubated at 37 °C, 200 rpm under either aerobic or anaerobic conditions. a Cell mass, b Glucose, and c Lactate. : aerobic, 0.5%; : aerobic, 1%; : aerobic, 2%; : anaerobic, 0.5%; : anaerobic, 1%; : anaerobic, 2%.

Investigation of the metabolic behavior of *T. laevilacticus* SK5-6 during the fermentation stage

The preculture seed obtained during the preculture stage under different conditions was used to inoculate into the fermentation medium for D-lactate production. Table 4.3 shows the metabolic response of SK5-6 under different cultivation conditions and the production performance during the fermentation stage. It is clear that mixing and an anaerobic condition were necessary for D-lactate production as indicated by the higher final lactate titer, yield, and productivity with the lower glucose remaining in the fermentation broth. No significant difference at $P \leq 0.05$ on D-lactic acid production when the fermentation was carried out at 150 rpm under anaerobic condition, although varied preculture seeds were used to transfer into the fermentation stage[a,b]. The high yield indicates that SK5-6 successfully utilized glucose for lactate during the anaerobic respiration process when oxygen was limited unlike those previously reported during the preculture stage where mixing played a significant role on promoting growth while aerobic/anaerobic conditions appeared to have less effect on growth. During the fermentation stage, the anaerobic condition

exhibited the positive effect on lactate production in regardless of mixing (Fig. 4.2 and Table 4.3).

When inoculating the fermentation medium with the aerobic preculture seed (both at the mid log and late log phases) and implementing the fermentation stage under the aerobic condition with mixing[c], cells grew rapidly and approached a stationary phase within 24 h (Table 4.3). Later cells entered the declining phase resulting in the lower cell dry weight compared with the other fermentation conditions (data not shown). As a result, low lactate production was obtained. It was observed that transferring the anaerobic preculture seed (both at the mid log and late log phases) into the fermentation stage with mixing and aeration[d] caused adverse effects on cell growth resulting subsequently in low lactate production (Table 4.3). Cell mass slowly increased (data not shown). This can be explained by anaerobic cells being transferred into the fermentation medium containing solely high concentration glucose and $CaCO_3$. The fermentation operated at 150 rpm under aerobic conditions, then the cells entered their lag phase as they required the expression of catalase activity to break down H_2O_2. As a result, the growth rate was low when shifting the metabolic pathway from the anaerobic preculture seed to the aerobic fermentation with sufficient mixing.

With respect to the effect of preculture seed being transferred into the fermentation stage, changing the inoculum age from the mid log to late log phases did not cause a dramatic change in the fermentation performance during the fermentation stage conducted under the same conditions[a] (Table 4.3). Also, the aerobic and anaerobic preculture seeds demonstrated similar performance[a] (Table 4.3). More interestingly, no byproduct was found during the fermentation stage indicating that SK5-6 converted glucose to D-lactate through homofermentation. From this preliminary finding, it was determined that high lactate production can be achieved when performing the fermentation at 150 rpm under anaerobic conditions. The preculture seed of SK5-6 transferred into the fermentation stage can be prepared under both aerobic and anaerobic conditions. Therefore, we can claim that the fermentation operation for SK5-6 is relatively simple compared with conventional lactate production which usually led to problems in terms of byproduct formation, low yield, and low productivity.

Table 4.3 D-Lactate production during the fermentation stage. The effects of preculture seed and cultivation condition during the fermentation stage on the production performance were investigated.

Preculture seeds[a]	Fermentation conditions[b]	At 48 h			Fermentation performance		
		Cell mass (g/L)	Glucose (g/L)	Lactate (g/L)	Yield (g/g)	Productivity (g/L·h)	Optical purity (%ee)
Mid log (4 h) Aerobic	No mixing, Aerobic	1.445 ± 0.012	32.97 ± 3.31	67.54 ± 0.94	0.75 ± 0.04	1.41 ± 0.02	98.76 ± 1.40
	No mixing, Anaerobic	1.798 ± 0.001	16.63 ± 0.92	85.46 ± 0.89	0.79 ± 0.03	1.78 ± 0.02	98.87 ± 0.35
	150 rpm, Aerobic	0.450 ± 0.004[c]	17.05 ± 2.09	66.84 ± 2.22	0.62 ± 0.03	1.39 ± 0.05	98.22 ± 0.14
	150 rpm, Anaerobic	1.475 ± 0.011	2.74 ± 3.88	102.22 ± 4.48	0.84 ± 0.00	2.13 ± 0.09	99.64 ± 0.07
Mid log (4 h) Anaerobic	No mixing, Aerobic	1.552± 0.001	14.57 ± 0.09	84.62 ± 3.26	0.78 ± 0.01	1.76 ± 0.07	98.68 ± 0.14
	No mixing, Anaerobic	1.546 ± 0.004	22.55 ± 3.56	81.05 ± 1.21	0.81 ± 0.05	1.69 ± 0.03	98.69 ± 0.28
	150 rpm, Aerobic	0.372 ± 0.004[d]	44.79 ± 3.74	35.43 ± 3.08	0.46 ± 0.06	0.74 ± 0.06	98.60 ± 0.35
	150 rpm, Anaerobic	1.289 ± 0.003	0.00 ± 0.00	101.76 ± 5.81	0.82 ± 0.06	2.12 ± 0.12	99.63 ± 0.07
Late log (5 h) Aerobic	No mixing, Aerobic	1.609 ± 0.001	22.63 ± 1.51	71.98 ± 1.75	0.71 ± 0.04	1.50 ± 0.04	99.32 ± 0.14
	No mixing, Anaerobic	1.756 ± 0.001	37.87 ± 1.17	71.88 ± 1.42	0.84 ± 0.01	1.50 ± 0.03	99.37 ± 0.21
	150 rpm, Aerobic	0.663 ± 0.003[c]	94.60 ± 2.10	18.98 ± 2.22	0.69 ± 0.04	0.40 ± 0.05	98.85 ± 0.35
	150 rpm, Anaerobic	1.356 ± 0.005	6.88 ± 0.44	99.22 ± 1.01	0.86 ± 0.01	2.07 ± 0.02	99.69 ± 0.28

Table 4.3 (Continue) D-Lactate production during the fermentation stage. The effects of preculture seed and cultivation condition during the fermentation stage on the production performance were investigated.

Preculture seeds[a]	Fermentation conditions[b]	At 48 h			Fermentation performance		
		Cell mass (g/L)	Glucose (g/L)	Lactate (g/L)	Yield (g/g)	Productivity (g/L·h)	Optical purity (%ee)
Late log (5 h) Anaerobic	No mixing, Aerobic	1.463 ± 0.002	12.12 ± 0.64	69.90 ± 0.45	0.62 ± 0.01	1.46 ± 0.01	99.35 ± 0.14
	No mixing, Anaerobic	1.498 ± 0.006	19.65 ± 0.49	79.67 ± 0.99	0.71 ± 0.03	1.66 ± 0.01	99.26 ± 0.07
	150 rpm, Aerobic	0.759 ± 0.018[d]	92.43 ± 1.83	18.68 ± 0.33	0.61 ± 0.07	0.39 ± 0.01	99.80 ± 0.58
	150 rpm, Anaerobic	1.262 ± 0.002	6.61 ± 0.48	101.02 ± 0.91	0.85 ± 0.02	2.10 ± 0.02	99.67 ± 0.25

[a]No significant difference at $P \leq 0.05$ on D-lactic acid production when preculture conditions were varied (age and oxygen) when the fermentation was carried out with 150 rpm under anaerobic condition.

[b]Changing the fermentation conditions (with varied mixing and oxygen), cell growth, glucose consumption, and D-lactic acid production were found significantly different at $P \leq 0.05$.

[c]Cell mass during the aerobic fermentation operated at 150 rpm reached the maximum concentration at 24 h (data not shown). Later, the concentration dropped, resulting in the low cell concentration.

[d]Cell mass slowly increased because of the long lag phase when shifting from the anaerobic preculture to the aerobic fermentation.

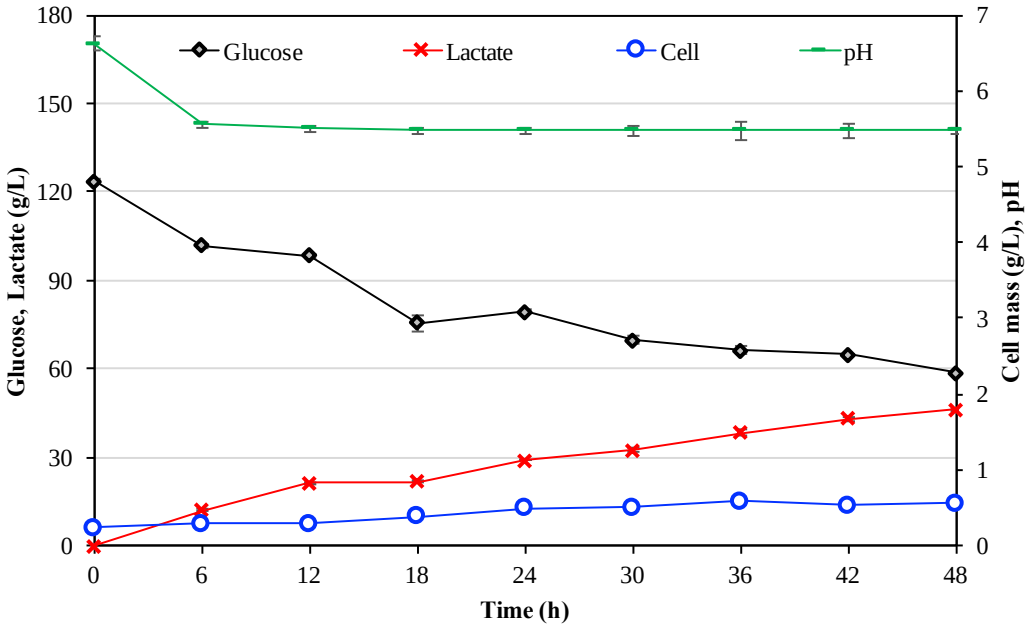
Effect of agitation on D-lactate fermentation by *T. laevilacticus* SK5-6 in the stirred fermentor

Fig. 4.4 shows the time course kinetics of SK5-6 during the fermentation stage. It was found that increasing the agitation speed from 200 rpm to 300 rpm significantly enhanced lactate production. With adequate mixing, glucose was rapidly consumed via the anaerobic fermentative pathway resulting in increasing cell mass and lactate as the growth associated product. The final cell mass obtained from the fermentation culture operated at 300 rpm (2.066 g/L) was almost 4 times higher than that in the culture that operated at 200 rpm (0.564 g/L). This subsequently resulted in high lactate production in the fermentation operated at 300 rpm (final titer of 92.60 g/L, yield of 0.94, and productivity of 1.93 g/L·h). On the other hand, low mixing rate (200 rpm) rather limited the bioconversion process; therefore, a lower glucose consumption rate resulted in a large amount of residual glucose (58.49 g/L, only half was consumed during fermentation) and subsequently low lactate production (final titer of 46.05 g/L with the yield of 0.71 and the productivity of 0.96 g/L·h). From the fermentation profiles in Fig. 4.4, it should be noted that the pH was successfully maintained during the fermentation by adding $CaCO_3$ at the beginning of the fermentation stage. The results suggested that with sufficient mixing, the homogeneous system generated the proper heat and mass transport which facilitated the bioconversion of glucose to D-lactate (Aguirre-Ezkauriatza et al., 2008; Ibrahim et al., 2010; Watanabe et al., 2012).

Mixing is another key parameter controlling the fermentation performance as it manipulates broth homogeneity as well as transport in the fermentation system. With sufficient mixing, it was found that glucose uptake rate was rapid. This resulted in a large pool of pyruvate being converted to D-lactate by lactate dehydrogenase (Hou et al., 2000). Mixing is typically employed for gas distribution in the culture. It was reported that the microaeration condition generated by sufficient mixing enhanced the cell growth of *Lactococcus lactis* MG1363 (Neves et al., 2005; Nordkvist et al., 2003). Combining mixing along with aeration, the growth of *L. lactis* was better than without mixing (Ishizaki and Ueda, 1995; Pedersen et al., 2008). In typical lactate fermentation, a calcium base is used in pH control. Without adequate mixing, heterogeneity is generated in the culture broth because of $CaCO_3$ and cell sedimentation. This eventually affects the overall process kinetics (Ma et al., 2014; Qin et al., 2010).

Comparing the preliminary fermentation optimization from the primary screening with the flask and fermentor culture, it was clear that under preliminary optimized process conditions, the catalase positive strain *T. laevilacticus* SK5-6, performed promisingly in terms of D-lactate production; therefore, this is considered a novel industrial producer besides the typical catalase negative *Sporalactobacillus* sp. (Table 4.4). It is believed that further process optimization can provide greater improvement in production performance of SK5-6 and the subsequent effective fermentation platform for large scale production operations.

a



b

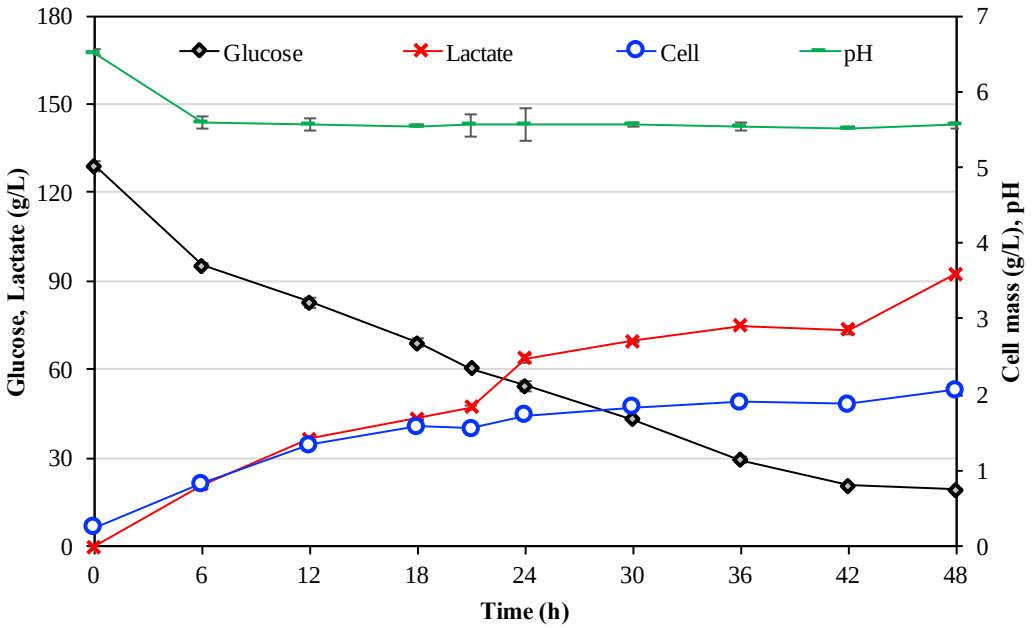


Figure 4.4 Effect of mixing during the fermentation stage of SK5-6 cultivated under anaerobic condition in the 5 L stirred fermentor. a. 200 rpm and b. 300 rpm. : Glucose; : Lactate; : Cell; : pH

Table 4.4 Preliminary optimization of fermentation condition for D-lactate production by SK5-6 resulted in the improved fermentation performance.

Optimized conditions	Time (h)	Residual glucose (g/L)	Final lactate (g/L)	Optical purity (%ee)	Yield (g/g)	Productivity (g/L·h)
Flask culture Primary screening	72	9.74 ± 0.14	99.27 ± 0.71	99.00 ± 0.70	0.90 ± 0.00	1.38 ± 0.02
Flask culture Aerobic preculture, Anaerobic fermentation	48	2.74 ± 3.88	102.22 ± 4.48	99.64 ± 0.07	0.84 ± 0.00	2.13 ± 0.09
Fermentor culture Aerobic preculture, Anaerobic fermentation agitated at 300 rpm	48	19.20 ± 0.03	92.60 ± 0.65	99.56 ± 0.07	0.84 ± 0.00	1.93 ± 0.01

Terrilactibacillus strain as a promising industrial D-lactate producing isolate

Table 4.5 shows the origin of the major D-lactate producers previously reported in literatures as well as those isolated in this study. It appears that D-lactate producers are distributed in limited sources and their preferred environment is a rather acidic pH (Sanders et al., 2003). Currently, *Sporolactobacillus* sp. is known as the key producer for D-lactate in industrial fermentation processes. *Sporolactobacillus* sp. can be found in soil, tree bark, and fermented food and feed. Previous studies reported that *S. inulinus* was isolated from chicken feed while *S. nakayamae* subsp. *nakayamae*, *S. nakayamae* subsp. *racemicus*, *S. lactosus*, *S. kofuensis*, and *S. terrae* were isolated from soil samples and fermentation starters (Yanagida et al., 1997). *S. vineae* and *S. putidas* were found in vineyard soil in Korea and in spoiled orange juice, respectively (Chang et al., 2008; Fujita et al., 2010). *Sporolactobacillus* sp. is a gram positive, facultatively anaerobic, motile, and endospore forming microbe. This genus lacks the activities of oxidase and catalase. In addition, it cannot reduce nitrate. The bacteria in this genus produce D-lactate from glucose and other carbon substrates depending on the sub characteristics of the type strains belonging in this genus (Fujita et al., 2010; Yanagida et al., 1997). Cultivation of *Sporolactobacillus* is restricted under anaerobic conditions when the oxygen is limited because of a lack of catalase activity and a cytochrome system (Holzapfel and Botha, 1988; Watanabe et al., 2012). This results in low ATP generation and a subsequently low metabolic rate compared to the aerobic culture.

Respiratory metabolism results in the conversion of glucose into ATP via the glycolysis, tricarboxylic acid cycle (TCA), and oxidative phosphorylation. The ATP is generated in this metabolic process because of the activity of the electron transport system. As the electrons are passed down through the electron transport chain (ETC), each molecule in the chain alternates between its reduced and oxidative forms. Cytochrome oxidase appearing at the end of the chain catalyzes the oxidation of the last cytochrome molecule by molecular oxygen. This final process results in the reduction of the molecular oxygen to water. Often, the incomplete reduction of oxygen leads to the production of hydrogen peroxide (H_2O_2) or the superoxide free radical (O_2^-), which are both toxic to the cell. Many aerobic or facultatively anaerobic cells produce the enzymes catalase and superdismutase that remove these toxic compounds, respectively, while strict anaerobes usually lack these enzymes and therefore are restricted to an oxygen free environment (Hassan and Fridovich, 1977; Pedersen et al., 2008).

From its biochemical characterization, novel *Terrilactibacillus* was found to exhibit the activity of catalase thus confirming that the bacteria in this genus can survive in the presence of oxygen. Unlike *Sporolactobacillus*, since *Terrilactibacillus* obtains catalase activity, the bacteria in this genus generate more ATP and grow rapidly in the presence of oxygen which subsequently results in high cell mass concentration and lactate productivity (Prasirtsak et al., 2016; Watanabe et al., 2012).

It should be noted that *Terrilactibacillus* sp. is also a gram positive and endospore forming D-lactate producing microbe; therefore, it can survive exposure to harsh conditions such as low pH (Sanders et al., 2003). As seen in Table 4.1, it was observed that *Terrilactibacillus* sp. consumed a wide range of carbon sugars, especially glycerol for D-lactate production while *Sporolactobacillus* demonstrated a

limited ability to convert several sugars. The ability of *Terrilactibacillus* strain to metabolize glycerol was the consequence of catalase activity acquired in the bacteria. It has been claimed that oxygen was involved in the consumption of polyhydroxy alcohols by some lactate producers as 1 mole of oxygen was required to convert 1 mole of glycerol substrate into 1 mole of lactate and 1 mole of H_2O_2 (Hassan and Fridovich, 1977).

From the taxonomical and biochemical characteristics previously mentioned, we can state that the novel *Terrilactibacillus* strain shows the promising performance as an industrial D-lactate producing strain.

Table 4.5 Origin of the major D-lactate producers reported in the literatures and those isolated in this study.

Source origin	Microorganism species	References
Chicken feed, soil, fermentation starter	*S. inulinus* *S. nakayamae* subsp. *nakayamae* *S. nakayamae* subsp. *racemicus* *S. lactosus* *S. kofuensis* *S. terrae*	Yanagida et al., 1997
Vineyard soil	*S. vineae*	Chang et al., 2008
Spoiled orange juice	*S. putidas*	Fujita et al., 2010
Bark of *Spathodea campanulata* P. *Beauv.*	*S. spathodeae*	Thamacharoensuk et al., 2015
Bark of *Shorea talura* Roxb	*S. shoreae*	Thamacharoensuk et al., 2015
Bark of *Lagerstroemia floribunda* Jack ex Blume.	*S. terrae* CU68-2	Prasirtsak et al., 2013
Bark of *Samanea saman*	*S. nakayamae* subsp. *nakayamae* SP43-2	This study
Bark of *Musa sapientum* Linn.	*S. laevolacticus* NK44-2	This study
Bark of *Sterculia foetida* L.	*S. laevolacticus* BRY67-1	This study
Bark of *Sterculia foetida* L.	*S. laevolacticus* BRY67-2	This study
Bark of *Sterculia foetida* L.	*S. laevolacticus* BRY67-3	This study
Soil	*T. laevilacticus* NK26-11^{T}	Prasirtsak et al., 2016
Soil	*T. laevilacticus* SK5-6	This study

Establishing D-lactate fermentation platform by *Terrilactibacillus* strain

Understanding the metabolic behavior of the microbe is mandatory in fermentation process optimization to achieve the targeted production performance. In this study, we have identified the key parameters that controlled the growth and synthesis of metabolites during the cultivation of SK5-6 and successfully introduced 2-phase fermentation to grow SK5-6 under aerobic conditions and ferment homo D-lactate under anaerobic conditions. The major advantage of 2-phase fermentation is that the high cell density obtained during the preculture stage accelerates the anaerobic conversion of glucose to lactate during the fermentation stage. However, previous studies reported drawbacks of this process when shifting the process condition from an aerobic to an anaerobic environment (Ma et al., 2014; Qin et al., 2010). Byproduct formation often appeared as the metabolic pathway shifted from aerobic respiration to the mixed acid fermentation when high glucose concentration was present with the remaining oxygen available at the initial stage of the fermentation while expelling the remaining oxygen from the preculture stage by flushing the fermentor with inert gas to obtain a strictly anaerobic condition is considered cumbersome. Nevertheless, this phenomenon did not exist in the 2-phase fermentation process with SK5-6 for D-lactate production. This strongly indicates the robustness of SK5-6 for a suitable industrial D-lactate producer.

Comparing D-lactate production performance by *Terrilactibacillus* isolate with other species

It was reported that D-lactate was produced in some bacteria including *S. inulinus*, *S. laevolacticus*, *L. delbrueckii*, and *Leuconostoc* sp. Among those genus, *Sporolactobacillus* sp. has been claimed as the promising candidate for industrial fermentation. Previous study has claimed that fermentation by wild type strains such as thermotolerant *L. delbrueckii* subsp. *lactis* QU41 yielded 87.4 g/L D-lactate with a high optical purity of 99.9%ee using MRS medium containing 100 g/L glucose (Tashiro et al., 2011). In 2012, Mimitsuka et al. (2012) conducted batch fermentation by *S. laevolacticus* JCM2513 from which D-lactate at the final titer of 55.7 g/L with the yield of 0.89 g/g and productivity of 0.25 g/L·h was obtained.

Comparing the production performance of D-lactate to that obtained from the well-established L-lactate production process, it was found that the final titer along with yield and productivity were still low, thus making the process become economically uncompetitive. Li et al. claimed that the poor efficiency of D-lactate fermentation was the result of the inhibition due to high substrate concentration (Li et al., 2013). In batch fermentation by *S. laevolacticus* DSM442 using high initial glucose concentration (>100 g/L) supplemented with cottonseed as the nitrogen source, lactic acid production was inhibited. Improved fermentation efficiency was obtained by a fed-batch process with an increasing final titer of 144.4 g/L, yield of 0.96 g/g, productivity of 4.13 g/L·h, and optical purity of 99.3%ee (Li et al., 2013). D-lactate production by *S. inulinus* Y2-8 was processed by batch fermentation in a fibrous bed bioreactor using corn flour hydrolysate. In that process, a high yield of 0.98 g/g with a productivity of 1.62 g/L·h and optical purity of 99.0%ee was obtained (Zhao et al., 2014). In addition, fed-batch D-lactate fermentation by *S. inulinus* YBS1-

5 with simultaneous utilization of cottonseed meal and corncob residue produced a high D-lactate concentration (107.2 g/L), productivity (1.19 g/L.h), and yield (0.85 g/g). An optical purity of D-lactate of 99.2%ee was obtained (Bai et al., 2016).

As seen in Table 4.6, it was observed that the wild strains, e.g. *Sporolactobacillus* sp. and *Lactobacillus* sp. exhibited slightly low productivity in batch and fed-batch processes (de Franca et al., 2009). Improved production performance was obtained during the continuous culture incorporated with the separation system (Koutinas et al., 2014). For example, by incorporating electrodialysis membrane unit with the fermentation system, the improved productivity of 8 g/L·h was obtained (Min-tian et al., 2005). Improved productivity was also obtained in the metabolically engineered strains such as *Escherichia coli* and *Klebsiella pneumoniae* (Baek et al., 2016; Feng et al., 2014; Tsuge et al., 2014). Nevertheless, the engineered strains usually have limited use especially in large scale operations due to biosafety regulation, high investment in production facilities, and genetic instability. As a result, the wild strain is still preferable. Comparing the production performance of SK5-6 to the other D-lactate producers, as reported in Table 4.6, it is believed that with further process optimization and fermentation platform development, SK5-6 can become a promising industrial strain for D-lactate fermentation.

Table 4.6 D-Lactate production by the potential strains reported in the literatures.

Strain	Operation	pH control	Lactate (g/L)	Yield (g/g)	Productivity (g/L·h)	Optical purity (%ee)	References
Sporolactobacillus sp. CASD	Batch	$CaCO_3$	80–90	0.86–0.97	1.77	-	Zhao et al., 2010
Sporolactobacillus sp. CASD	Fed-batch	$CaCO_3$	207	0.93	3.80	99.3	Wang et al., 2011
S. laevolacticus JCM2513	Continuous	$Ca(OH)_2$	67.3	0.98	11.20	-	Mimitsuka et al., 2012
Sporolactobacillus sp. Y2-8	Batch	$CaCO_3$	122	0.81	1.00	99.1	Zheng et al., 2012
S. laevolacticus DSM442	Fed-batch	$CaCO_3$	144.2	0.96	4.13	99.3	Li et al., 2013
L. coryniformis	Flask	NH_4OH	186.4	0.85	3.11	-	Nguyen et al., 2013
K. pneumoniae ATCC25955	Fed-batch	NH_4OH	142.1	0.82	2.96	100	Feng et al., 2014
E. coli HBUT-D	Pilot scale	$Ca(OH)_2$	127	0.93	6.35	99.5	Liu et al., 2014
S. inulinus Y2-8	Fed-batch	NH_4OH	218.8	-	1.65	>99.0	Zhao et al., 2014
Sporolactobacillus sp. Y2-8	Batch	$CaCO_3$	125.0	-	1.39	99	Sun et al., 2015
S. inulinus NBRC13595	Batch	$CaCO_3$	189.0	0.94	5.25	>98	Reddy Tadi et al. 2017
S. inulinus YBS1-5	Fed-batch	$CaCO_3$	107.2	0.85	1.19	99.2	Bai et al., 2016
T. laevilacticus SK5-6	Flask	$CaCO_3$	102.2	0.84	2.13	99.6	This study
T. laevilacticus SK5-6	Batch	$CaCO_3$	96.6	0.84	1.93	99.6	This study

Conclusion

In this study, D-lactate producers were isolated from natural samples in Thailand. Among 6 isolates obtained, *T. laevilacticus* SK5-6 showed good characteristics over other D-lactate producers previously reported in the literatures. SK5-6 demonstrated the ability to utilize several carbon substrates for D-lactate production. The optical purity of D-lactate was sufficiently high for polymer-grade specification (>99.5%ee). Unlike *Sporolactobacillus* sp., the common D-lactate strain, SK5-6 produced catalase; thus, oxygen was not restricted during the cultivation. By the simple 2-phase fermentation employed in this study, aerobic preculture was introduced to generate robust, high-cell-density cell mass of SK5-6 for D-lactate production during the anaerobic fermentation stage without byproducts. This resulted in the high yield and productivity. The results of this research therefore confirm the potential of using *Terrilactibacillus* sp. as a D-lactate producer.

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CHAPTER V

Enzyme expression and regulation in glycolysis pathway by *Terrilactibacillus laevilactius* SK5-6 during D-lactate fermentation

Manuscript of this chapter is under preparation

Enzyme expression and regulation in glycolysis pathway by *Terrilactibacillus laevilactius* SK5-6 during D-lactate fermentation

Abstract

T. laevilacticus SK5-6 is a novel D-lactate producer with a good fermentation performance for industrial purposes. D-Lactate biosynthesis is controlled by key responsible enzymes in glycolysis pathway such as phosphofructokinase (PFK), pyruvate kinase (PYK) and lactate dehydrogenase (LDH) to enhance lactate and optical purity of D-lactate. To date, there is no information of the enzyme regulation and expression by *T. laevilacticus* reported in the literatures. Therefore, in this research we aimed to investigate the regulatory mechanism and expression level of PFK, PYK, LDH and lactate isomerase to control the D-lactate biosynthesis. The fermentation was conducted and the factors affecting the expression of the key enzymes were observed. The results show that *T. laevilacticus* could produce D-lactate at the final titer of 102.5 g/L with yield and productivity of 0.84 g/g and 2.14 g/L·h, respectively. *T. laevilacticus* exhibited fermentation kinetic and expression level of the key enzymes were higher than *S. laevilacticus*, a catalase negative D-lactate producer. The kinetics data obtained were well correlated with the expression level of key enzymes in D-lactic acid production. It was observed that PFK activity was lower than other key enzymes indicating that the conversion of F-6-P to F-1,6-P was limited; thus, controlling the apparent rate of D-lactic acid production. Increasing the pH during fermentation phase would activate D-LDH expression higher than L-LDH activity, resulting in high optical purity of D-lactate. It was suggested that the acidic pH affected the activities of PFK and PYK that directly. The isomerization that convert L-lactic acid to D-lactic acid by isomerase was also observed during fermentation. In addition, lactate dehydrogenase expression resulting from various nitrogen compositions.

Keywords D-lactic acid • *Terrilactibacillus laevilacticus* • Fermentation • Phosphofructokinase • Pyruvate kinase • D-Lactate dehydrogenase • L-Lactate dehydrogenase • Isomerase

Introduction

Lactic acid is the common name given to 2-hydroxypropanoic acid that has many applications in diverse industries such as an acidulant, flavor enhancer, preservative in the food, pharmaceutical, leather, textile industries and a monomer building block of the biodegradable plastic, polylactic acid. During the early development stage, polylactic acid (PLA) was produced from the optically pure L-lactic acid because of the availability in market. The poly L-lactic acid product has limited uses because the mechanical and thermal properties could not be competitive with the commercially available petroleum based plastics. The properties of PLA products can be improved by the stereocomplex structure that yields the increasing melting point up to 230 °C which requires the optically pure isomers of both D- and L-lactic acid therefore, this finding has generated in D-lactic acid (Li et al., 2013). Lactic acid can be produced via microbial fermentation that utilize low cost from agricultural products or residues are converted to produce lactic acid with optically pure L(+)- or D(-)- lactic acid depending on the organisms used under mild conditions with low energy consumption. Microbial fermentation exhibit 3 forms of lactic acid, e.g., L-lactic acid, D-lactic acid, and DL-lactic acid. It depending on the microbes employed (John et al. 2009; Wee et al., 2006; Xu et al., 2010). It was found that D-lactic acid bacteria produced in some bacteria including *Lactobacillus* sp., *Sporolactobacillus* sp. and *Terrilactibacillus* sp. which response to environmental stress (Bai et al. 2016, Li et al., 2013; Mimitsuka et al., 2012; Prasirtsak et al., 2016; Tashiro et al., 2011; Zhao et al., 2014). It has been found that genus *Terrilactibacillus* is catalase positive bacteria that cultivate in the presence of oxygen; so, it generate higher biomass, production and productivity than D-lactate producer that grows under anaerobic condition. Thus, *T. laevilacticus* SK5-6 is a novel D-lactate producer with a good fermentation performance for industrial purposes.

D-Lactate biosynthesis is controlled by key responsible enzymes in glycolysis pathway such as phosphofructokinase (PFK), pyruvate kinase (PYK) and lactate dehydrogenase (LDH) to enhance lactate and optical purity of D-lactate (Fothergill-Gilmore and Michels 1993; Garvie, 1980). The first step in glycolysis is phosphorylation of glucose by hexokinases to form glucose 6-phosphate. This reaction consumes ATP. Latter, fructose 6-phosphate (F6P) change into fructose-1,6-bisphosphate (F-1,6-P) by PFK. The F-1,6-P could activate the activity of LDH and PYK by allosteric activation. Thus, the expression level of PFK is importance for cell growth and D-lactate production. This is also the rate-limiting step that it regulate metabolism for rapidly and efficiency in flux (Fothergill-Gilmore and Michels 1993; Zheng et al., 2017). The activity of PFK is controlled by various metabolites that respond to change in levels of metabolite in activation or inhibition. Divalent ion, Mg^{2+} is necessary for activity of this enzyme (Zheng et al., 2017). One important allosteric enzyme is PYK, it transfers of phosphate from phosphoenolpyruvate (PEP) to ADP producing ATP and pyruvate. The F-1,6-P is allosteric activator and require Mg^{2+} for the catalysis of PYK activity. PYK links glycolysis for the energy and cellular metabolism. The stimulation activity of PYK would accelerate to pyruvate concomitant with ATP regeneration. Finally, pyruvate accumulation lead to increase biosynthesis of lactic acid. So, pyruvate is important for metabolic pathways (Bourniquel et al., 2002; Hess et al., 1966; Zheng et al., 2014). The last key enzyme is

lactate dehydrogenase (LDH) which catalyzes the reduction of pyruvate into lactic acid, concomitant with the oxidation of NADH to NAD^+. There are two forms of lactate dehydrogenase and their expression depend on environmental factor such as medium composition, pH, temperature, growth conditions and also the nature of LDH in bacterial strains (Garvie, 1980; Klotz et al., 2016). It is suggested that the regulatory mechanism and expression level of PFK, PYK, LDH and lactate isomerase to control cell growth and the D-lactate biosynthesis could enhance efficient D-lactate production.

To date, there is no information of the enzyme regulation and expression by *T. laevilacticus* reported in the literatures. Therefore, in this research we aimed to investigate the regulatory mechanism and expression level of PFK, PYK, LDH and lactate isomerase to control the D-lactate biosynthesis. The fermentation will be conducted and investigate the factors affecting the expression of the key enzymes were observed. It is believed that the results from this work could provide a better understanding on the responses of enzyme regulation for D-lactate production by *T. laevilacticus*. Although, no information is available about it regulation of mechanism by novel genus.

Materials and methods

Microorganism and Inoculum Preparation

Terrilactibacillus laevilacticus SK5-6, a catalase positive producer was used for the D-lactate fermentation and determination of enzyme activity. The strain was subcultured onto GYP agar slants and incubated at 37 °C for 24 h to prepare the bacterial suspension. The pre-culture medium was inoculated with the bacterial suspension (OD600 of 30-40) at 1% inoculum size of *T. laevilacticus* SK5-6. The GYP agar medium contained (per liter) 10 g glucose per liter, 5 g yeast extract, 5 g peptone, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4, 20 g agar, 5 g $CaCO_3$, and 10 mL salt solution. The composition of the salt solution consisted of (per 10 mL) 400 mg $MgSO_4.7H_2O$, 20 mg $MnSO_4.5H_2O$, 20 mg $FeSO_4.7H_2O$, and 20 mg NaCl. The pH of the GYP agar medium was adjusted to 6.80.

For catalase negative producer, *S. laevolacticus* 0361^T is efficiency of D-lactate fermentation that was obtained from National Registration Identity Card (NRIC). Cell was subcultured onto a fresh new GYP agar slant and incubated anaerobically at 37 ºC for 48 h. The inoculum preparation was conducted under the same inoculum size and GYP agar medium of *T. laevilacticus* SK5-6.

Growing *T. laevilacticus* SK5-6 in the D-Lactate Fermentation Medium in Erlenmeyer Flasks

D-Lactate fermentation by *T. laevilacticus* SK5-6 consisted of two phases, i.e. growth phase for cell growth and production phase for producing D-lactate by cells obtained in the growth phase. The operating conditions during both pre-culture and fermentation stages of the *T. laevilacticus* SK5-6 was optimized in flask cultivation. *T. laevilacticus* SK5-6 was subcultured onto fresh new GYP agar slants and incubated

at 37 °C for 24 h to prepare the bacterial suspension (OD600 of 30-40) at a 1% inoculum sizes. The pre-culture medium (pH 6.8) containing (per liter) 10 g glucose, 15 g yeast extract, 4 g NH_4Cl, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 20 mL salt solution, and 5 g $CaCO_3$. The culture was incubated at 37 °C, mixing at 200 rpm for 4 h in a flask plugged with a C-type silicone stopper. Later, 25 mL of the pre-culture broth was transferred into 25 mL of the sterile fermentation medium containing (per liter) 240 g glucose and 80 g $CaCO_3$. The fermentation culture was incubated at the same temperature for 48 at mixing rate of 150 rpm with a T-type silicone stopper in a W-zip pouch containing AnaeroPack-Anaero conditions. The various nitrogen sources of organic (yeast extract) and inorganic (NH_4Cl and $(NH_4)_2HPO_4$) were operated condition in pre-culture medium and separated into 6 groups. Firstly, N1 (yeast extract 15 g/L and NH_4Cl 4 g/L), N2 (yeast extract 7.5 g/L and NH_4Cl 4 g/L), N3 (yeast extract 7.5 g/L and NH_4Cl 10 g/L), N4 (yeast extract 15 g/L and $(NH_4)_2HPO_4$ 4 g/L), N5 (yeast extract 7.5 g/L and $(NH_4)_2HPO_4$ 4 g/L) and the last, N6 (yeast extract 7.5 g/L and $(NH_4)_2HPO_4$ 10 g/L). The pH of medium was adjusted to 6.8 with 10 M NaOH. Samples were taken every 12 h for analyses of OD600 reading, remaining glucose, D-lactate, byproducts, optical purity of the D-lactate and enzyme assays.

Growing *S. laevolacticus* in the D-Lactate Fermentation Medium in Erlenmeyer Flasks

For the *S. laevolacticus* 0361^T isolate, an active 48 h GYP agar slant under anaerobic condition was used to prepare the bacterial suspension. The bacterial suspension at 1% inoculum size was inoculated in the pre-culture medium (pH 6.8) containing (per 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 salt solution, and 5 g $CaCO_3$. The culture was incubated at 37 °C in an anaerobic condition for 26 h (in a flask plugged with a T-type silicone stopper and placed into a W-zip pouch containing AnaeroPack-Anaero from Mitsubishi Gas Chemical). Later, 1 mL of the pre-culture broth was transferred into the sterile fermentation medium (49 mL) containing (per liter) 120 g glucose, 10 g yeast extract, 5 g peptone, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4, 10 mL salt solution and 80 g $CaCO_3$. The culture was incubated at 37 °C in an anaerobic condition for 72 h. At the end of the fermentation, the sample was collected every 12 h for analyses of OD600 reading, remaining glucose, D-lactate, byproducts, optical purity of the D-lactate and enzyme assays.

Cell Biomass OD600

A sample of fermentation broth was centrifuged at 10,000 g for 5 min to separate the cell-free supernatant from the biomass. The supernatant was collected for further analyses of the remaining glucose, lactic acid, byproducts, and the optical purity of D-lactate. The solid cell biomass was acidified with 1 M HCl to remove insoluble $CaCO_3$ remaining in the sample. The acidified sample was centrifuged and the solid particles were resuspended in deionized water for the OD reading. Spectrophotometry was used to determine the OD at 600 nm of the cell biomass present in the fermentation broth.

Substrate and Product Analyses

The concentration of the products formed and glucose substrate remaining during the fermentation were analyzed using high performance liquid chromatography (HPLC). Fermentation samples were centrifuged, filtered through a PTFE (hydrophilic) membrane, and diluted with double deionized water. For analyses of the remaining glucose, total lactic acid (both L- and D-lactic acid), and acetic acid, 15 μL diluted particle-free samples were automatically injected (Shimadzu-SIL-10A) into an organic acid analysis column (Biorad, Aminex HPX-87H ion exclusion organic acid column; 300 mm×7.8 mm) and maintained at 45 °C in a column oven (Shimadzu-CTO-10A). A 0.005 M H_2SO_4 was pumped through the system at the flowrate of 0.6 mL/min (Shimadzu-LC-10Avp). A refractive index detector (Shimadzu-RID-10A) was then used to detect the organic compounds. The standards containing 0-2 g/L of each component (glucose, lactate, and acetate) were injected as references to determine sample concentration. The peak area was used to determine the concentration. To determine the optical purity of lactic acid, 5 μL diluted particle-free samples were automatically injected into a chiral column (Sumipack, Sumichiral OA5000) and maintained at 40 °C. A 0.001 M $CuSO_4$ was used as the eluant at the flow rate of 1.0 mL/min. A UV detector was used to detect the lactate isomers at 254 nm. The standards containing 0-2 g/L of D- and L-lactic acid were injected as references to determine sample concentration.

Product Yields, Volumetric Productivities and Optical purity of D-lactate

Product yield ($Y_{p/s}$) was determined from the ratio of the product formed to carbon substrate consumed during the fermentation stage. Volumetric productivity was defined as the total amount of product formed per unit volume per time. The optical purity of the D-lactate was defined from the peak areas of the chromatogram

$$\text{Optical purity} = \frac{\text{D} - \text{lactate} - \text{L} - \text{lactate}}{\text{D} - \text{lactate} + \text{L} - \text{lactate}} \times 100\%$$

Cell Extraction, Partial Enzyme Purification, and Protein Determination

The sample from fermentation was harvested every 12 h until the end of fermentation. The samples of 5 mL were washed twice with cold 0.05 M phosphate buffer (pH 7.4) and centrifugation (10,000 rpm, 4 °C for 10 min). The cell pellets were resuspended in 5 mL of the phosphate buffer. Then they were homogenized in an ultrasonic disruptor with the glass beads (425-600 μm) for 5 min (active every 30 s for a 45 s duration) to obtain the cell lysate. Later, the glass beads were removed by centrifuged at 10,000 rpm, 4 °C for 10 min. Finally, the supernatant was used for enzyme assays and protein determination by the Lowry method with bovine serum albumin as the standard (Lowry et al., 1951).

Enzyme Assays

The crude enzyme obtained from the extraction of the fresh lactic acid bacteria collected during fermentation was analyzed for the activities of the enzymes of interest including phosphofructokinase, pyruvate kinase, lactate dehydrogenase (both L- and D-LDH) and isomerase to investigate the expression of enzyme activity in the living *T. laevilacticus* SK5-6 and *S. laevolacticus* 0361^T cultures.

Phosphofructokinase (PFK) activity was determined by phosphofructokinase colorimetric assay kit (Catalog number MAK093, Sigma). The enzyme activity was determined from a couple enzyme reaction, in which phosphofructokinase catalyzed the conversion of fructose-6-phosphate and ATP to fructose-1,6-bisphosphate and ADP. Then, the ADP was converted by the enzyme mix into NADH and AMP. The reaction mixture contained 42 µL PFK assay buffer, 2 µL PFK enzyme mix, 2 µL PFK developer, 2 µL ATP, and 2 µL PFK substrate was added into a 96-well plate. Then, 10 µL crude enzyme was added into each well. The resulting NADH reduced the colorless probe resulting in a colorimetric product at 450 nm proportionally to the PFK activity present in the enzyme mixture. Measured the absorbance at 450 nm of the reaction mixture was recorded every 57 s for 2 min. The average rate of the increasing absorbency at 450 nm per min was calculated. The activity of PFK was subsequently determined using the calibration plot of absorbency at 450 nm versus NADH concentration in micromoles. One unit of PFK was the amount of enzyme that generated 1.0 µmol of NADH per 1 min at 37 °C, pH 7.4.

The activity of pyruvate kinase (PYK) was assay by monitoring the changes in absorbance of NADH at 340 nm using L-lactate dehydrogenase as a coupling enzyme. The reaction mixture contained 50 mM imidazole-HCl (pH 7.5), 5 mM PEP, 2 mM ADP, 100 mM KCl, 10 mM $MgCl_2$, 0.6 mM NADH, 2 units L-LDH from rabbit muscle and 0.2 mL crude enzyme in a total volume of 3.15 mL. Enzyme reaction was monitored for 5 min at 37 °C (same temperature as the culture temperature). One unit of PYK activity is defined as the activity that converts 1.0 µmol of PEP to pyruvate per min (Zheng et al., 2014).

Lactate dehydrogenase (LDH) was assayed by following the oxidation of NADH at 340 nm in the reaction mixture of 3.15 mL containing 0.25 mL sodium pyruvate (0.10 M), 0.10 mL NADH (0.01 M), 2.5 mL phosphate buffer (0.10 M) pH 6.5 (for L-lactate dehydrogenase) or pH 7.3 (for D-lactate dehydrogenase) and 0.2 mL crude enzyme. The reaction was started with the addition of the crude enzyme into the reaction mixture. The activity was detected for 5 min at 37 °C (same temperature as the culture temperature) by following the change in absorbency at 340 nm which was later converted into µmol NADH oxidized using the calibration plot (absorbency at 340 nm versus NADH concentration in µmol). Following the method described in Thitiprasert et al. (2011). One unit of activity was defined as the amount of enzyme converting 1 µmol NADH per min.

The lactate isomerization activity was determined by assay kit (catalog number 11112821035, Boehringer Mannheim, R-biopharm). The enzyme activity was determined from a couple enzyme reaction, in which D-lactate dehydrogenase catalyzed the conversion of lithium D-lactate and NAD^+ to pyruvate and NADH. Pyruvate was later converted by glutamate-pyruvate transaminase in the presence of L-glutamate into L-alanine and 2-oxoglutarate. The reaction mixture was assays in

2.240 mL solution contained 1 mL glycylglycine assay buffer, 0.2 mL NAD^+, 0.020 mL glutamate-pyruvate transaminase and 0.1 mL crude enzyme. The reaction mixture was read absorbency at 340 nm after incubated for 5 min, added 0.020 mL lithium D-lactate substrate into reaction mixture then read absorbency at 340 nm after incubated for 30 min, added 0.020 mL lithium L-lactate substrate and incubated for 30 min and read absorbency at 340 nm. The activity was detected by following the amount of NADH formed in the reactions was stoichiometric to the amount of D-lactic acid and of L-lactic acid, respectively. The increase in NADH is determined by absorbance at 340 nm. One unit (µmol/min) of isomerization activity is defined as amount of activity required to transform 1 µmol/min of L-or D-lactic acid to the other isomer.

Results and discussion

Comparing Fermentation kinetics and Key Enzyme Responsible for D-Lactate Production by *T. laevilacticus* SK5-6 with *S. laevolacticus* 0361^T

The results of fermentation kinetic show that *T. laevilacticus* SK5-6, catalase positive producer consumed glucose via the anaerobic fermentative pathway, resulting in increasing cell biomass and lactate production as the growth associated product. This subsequently resulted in highest D-lactate at the final titer of 102.5 g/L with yield and productivity of 0.84 g/g and 2.14 g/L·h, respectively at 48 h (Fig. 5.1). The production performance of *S. laevolacticus* 0361^T, catalase negative producer demonstrated long lag phase and the highest D-lactate production and productivity were 97.70 and 1.36 g/L.h, respectively at 72 h (Fig. 5.2). Comparing the production performance of *T. laevilacticus* SK5-6 to *S. laevolacticus* 0361^T, it was suggested that *S. laevolacticus* 0361^T exhibited long lag phase with lower final titer and productivity than *T. laevilacticus* SK5-6; thus, *T. laevilacticus* SK5-6 can become a good fermentation performance for industrial purposes (Table 5.1 and Fig. 5.3).

Two strains, *T. laevilacticus* SK5-6 and *S. laevolacticus* 0361^T use glycolytic pathway to produce D-lactate, flux into glycolytic pathway for D-lactate synthesis correlate with specific activities of key glycolytic enzyme such as phosphofructokinase (PFK), pyruvate kinase (PYK) and lactate dehydrogenase (LDH) that control and regulatory role in cell growth and D-lactate production. Hence, the expression of key enzymes by both strains related to fermentation kinetic (Fig. 5.4-5.5).

The phosphorylation of fructose-6-phosphate (F-6-P) to fructose-1,6-bisphosphate (F-1,6-P) by PFK in the presence of cofactor magnesium and transfer phosphate from ATP to F-6-P that control growth and D-lactate biosynthesis (Fothergill-Gilmore and Michels, 1993; Zheng et al., 2017). *T. laevilacticus* SK5-6 showed slightly increased specific activity of PFK with increasing time. The low level of PFK activity, it was possible that ATP pool increased during exponential of cell growth and high energy source was obtained. Hence, cell grew well to respond change in the rate of increase in the size of the ATP pool. Therefore, specific activity of PFK was decreased by high ATP (Forrest, 1965) while *S. laevolacticus* 0361^T exhibited high PFK level at 12 h during lag phase to 24 h at early log phase of cell growth then decreased activity until declining phase. It was perhaps low of the ATP pool when cell entered at lag phase later decreased activity during declining phase.

This result was indicated that *T. laevilacticus* generate higher biomass, production and productivity than *S. laevolacticus* 0361^{T} by abundant of high ATP during exponential phase. It has been claimed that the PFK of *S. inulinus* was inhibited by high ATP concentration that abundant of phosphate and ATP pool of cell growth during exponential phase (Bennett et al., 2009; Zheng et al., 2017). However, both strains showed PFK activity was lower than other key enzymes indicating that the conversion of F-6-P to F-1,6-P was limited; thus, controlling the apparent rate of D-lactic acid production.less pyruvate and lactate formation. More explanation is that F-1,6-P obtained from the action of PFK regulated the activity of LDH and PYK by allosteric activation. Thus, the level of PFK activity is importance for cell growth and D-lactate production (Fothergill-Gilmore and Michels 1993; Zheng et al., 2017). This inhibition may not only ATP but also, acid condition that was obtained from lactate production played a role in regulation of PFK activity due to pH optimum for activity of PFK was weakly alkaline (Tsuge et al., 2015). Previous studies on key glycolytic enzyme that control in cell growth and D-lactate production reported that the specific activity of PFK was little increased level might affect product inhibition through LDH, due to F-1,6-P was produced by PFK, it could activate activity of LDH; thus, resulted in high acid accumulation (Arai et al., 2002; Grabar et al., 2006). It was also reported in *Corynebacterium glutamicum* was stopped producing product when glucose consumption stopped due to PFK encoding gene was not overexpressed from product inhibition determined by D-lactate levels (Tsuge et al., 2015). Recently, Gong et al. (2016) demonstrated the activity of PFK and glucokinase (GK) were constructed with increasing PFK and GK activities from transgenic *L. casei*, resulting in enhance final lactate production. Some bacterial, PFK could activate by MgADP that increasing binding affinity for F-6-P in active site while inhibition by PEP (Fothergill-Gilmore and Michels, 1993). It was also reported by Zheng et al. (2017) reported that PFK was activated by ADP but inhibited by phosphoenolpyruvate (PEP) from *S. inulinus*. Cell growth under microaerobic condition by *L. lactis*, it was shown that specific activity of PFK was increased, resulting increase specific rate of glucose consumption and lactate formation (Papagianni and Avramidis, 2011).

The pyruvate kinase adjusts the level glycolytic intermediate for degradation and synthesis of ATP for cell (Fothergill-Gilmore and Michels, 1993; Hess et al., 1966). The activation of PYK would increase the conversion of PEP to pyruvate concomitant with ATP. Thus, the accumulation of pyruvate would enhance biosynthesis of D-lactate (Zheng et al., 2014). *T. laevilacticus* SK5-6 showed PYK activity was increased during exponential phase which achieved the highest PYK activity at 24 h. In this experiment, the PYK activity exhibited low expression, it was possible that low ADP and F-1,6-P concentration from limited activity of PFK. Later, fermentation time at 36-48 h, the activity of PYK was decreased expression it might due to ADP inhibition from high level of PFK activity. At acidic condition would inhibit the regulation of PYK due to pH optimum of PYK was neutral-weak base condition (Zheng et al., 2014). It has been reported that high level of ATP at exponential of cell growth might be inhibitors that decreased and low affinity for substrate was found in *S. inulinus* (Zheng et al., 2014). The activity of PYK by *S. laevolacticus* 0361^{T} showed the highest PYK activity was obtained at 36 h, related to high specific activity of PFK was accumulated at 12-24 h. Later, the activity was dropped during declining phase. *T. laevilacticus* demonstrated enhancement of PYK

activity during exponential phase that similar pattern to *S. laevolacticus* 0361^T. Both strains exhibited low specific activity of PYK, it was possible that low ADP and F-1,6-P accumulation from limited activity of PFK. In previous researched, allosteric activation of PYK from *L. lactis* and *L. delbrueckii* were activated by F-1,6-P and G-6-P (Bourniquel et al., 2002). In D-lactate fermentation by *S. inulinus* require Mn^{2+}/Mg^{2+} that dependent PYK activity and it regulated by adenosine monophosphate (AMP) for activation and inorganic phosphate or ATP for inhibition. Thus, high pyruvate pool would accumulate high D-lactate production (Zheng et al., 2014).

The specific activities of LDH were observed between D-lactate dehydrogenase (D-LDH) and L-lactate dehydrogenase (L-LDH) that using different pH buffer 7.3 and 6.5, respectively. It was found that D-LDH expression higher than L-LDH activity. Some time it did not seem to be different between L- and D-lactate dehydrogenase activities. The specific activity of LDH by *T. laevilacticus* SK5-6 was enhancement during exponential phase, the highest specific activity of LDH was obtained at 24-36 h. Later, little decreased activity during the declining phase at 48 h. It was suggested that *T. laevilacticus* might obtained short F-1,6-P during exponential phase, after that PFK activity was increased so, enough F-1,6-P for sufficient LDH activity. Therefore, LDH was increased, also high D-lactate production. Observing specific activity of LDH by *S. laevolacticus* 0361^T, it was found that increased activity with increased time, the high activity was obtained at 36-72 h, correlated with PYK activity was accumulated high level at 36 h during exponential phase. Investigation of isomerase for D-lactate, it was observed that increased transformation of L-lactate, produced by L-LDH, to D-lactic acid by isomerization with increased time. Thus, the highest lactate production and optical purity of D-lactate were found at declining phase. This LDH expression might be both the presence of high level of D-lactate dehydrogenase with low level of L-LDH in the living *T. laevilacticus* SK5-6 and *S. laevolacticus* 0361^T. This result was presumably explained by the influence of the PFK and PYK activity that related to D-lactate synthesis because LDH is stimulated through the consumption of pyruvate, recycling of NAD^+ (Garvie, 1980) and the F-1,6-P from activity of PFK is importance due the amino acid of Arg and His are involved F-1,6-P binding site in allosteric enzyme of LDH for change in enzyme structure which active to bind substrate (Iwata and Ohta, 1993; Tsuge et al., 2015). It has been reported that LDH play key roles in D- and L-lactate fermentation depend on D- or L-LDH activity in cell (Garvie, 1980) such as *L. plantarum* ability to synthesize both isomer of lactic acid which showed activity of L-LDH was lower than D-LDH including lactate recemase activity (Sakai et al., 2006). However, some *Lactobacillus* and *Leuconostoc* sp. produced D-lactic acid production that lack L-LDH activity (Garvie, 1980) while *S. inulinus*, *S. laevolacticus* and *S. terrae* possessed L-LDH activity (Sawai et al., 2011). Batch fermentation of D-lactate by *S. inulinus* and *S. laevolacticus* produced low optical purity of D-lactate in early phase and after mid-log phase, it was increased again while *S. terrae* produced increasing optical purity throughout batch fermentation. Continuous fermentation demonstrated higher optical purity of D-lactate than batch fermentation by *S. inulinus* (98.8%) and *S. laevolacticus* (99.8%). The specific activity of D-LDH was increased during late log phase for *S. laevolacticus* and *S. terrae* but *S. inulinus* was decreased at late log phase. For specific activity of L-LDH by 3 *Sporolactobacillus* strains showed little activity and lower than D-LDH. The lactate isomerization activity for L-lactic acid to D-lactic acid

by *S. inulinus* and *S. laevolacticus* were enhanced from lag phase to late log phase and *S. terrae* but no activity of D-lactic acid to L-lactic acid (Sawai et al., 2011).

In this study was indicated that the kinetics data obtained were correlated with the expression level of the key enzymes in D-lactate production. It was observed that PFK activity was lower than other key enzymes indicating that the conversion of F-6-P to F-1,6-P was limited; thus, controlling the apparent rate of D-lactic acid production. Increasing the pH during fermentation phase would activate D-LDH expression higher than L-LDH activity, resulting in high optical purity of D-lactate and the acidic condition affected the activities of PFK and PYK that directly. The isomerization that convert L-lactic acid to D-lactic acid by isomerase was depend on fermentation factor.

From this results finding, *T. laevilacticus* SK5-6 is attractive for D-lactate fermentation due to its good fermentation performance that exhibit high specific activity of key glycolytic enzyme that control and regulatory role for cell growth and D-lactate production; therefore, resulting in increasing cell biomass and subsequently resulted in highest D-lactate production. In addition, *T. laevilacticus* as a catalase positive D-lactate producer and oxygen is not restricted in the cultivation while *S. laevolacticus* as catalase negative bacteria require an anaerobic environment for growth with low productivity of D-lactate. Therefore, this result confirms that *T. laevilacticus* SK5-6 is the potential D-lactate producing strain for the industrial fermentation.

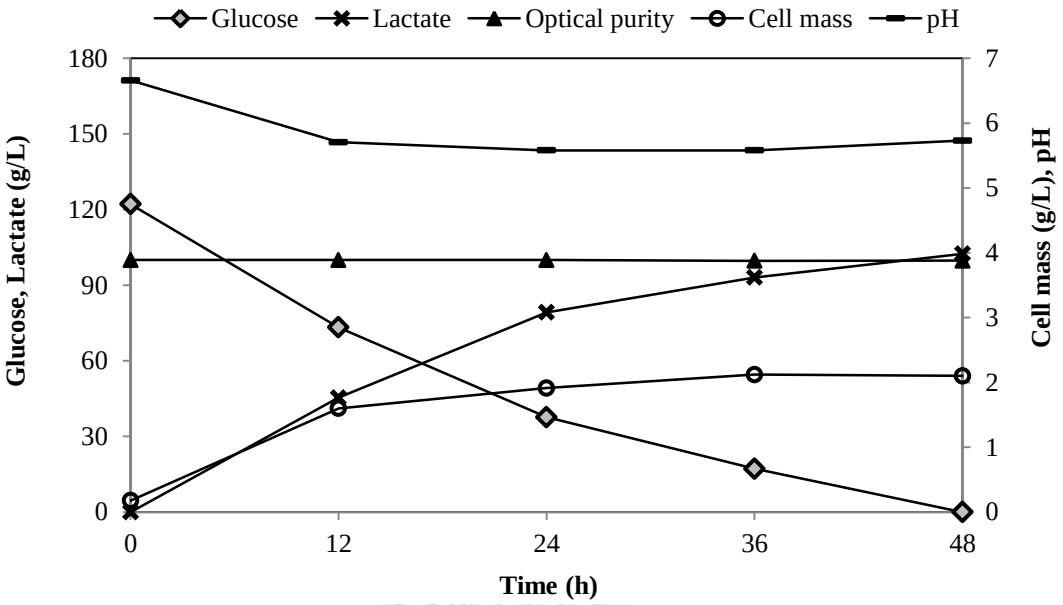


Figure 5.1 Fermentation kinetics of *T. laevilacticus* SK5-6 was inoculated in the pre-culture medium containing (per liter) 15 g yeast extract and 4 g NH_4Cl. The fermentation medium containing (per liter) 120 g glucose and 80 g $CaCO_3$. The culture was incubated in an Erlenmeyer flask at 37 °C for 48 h under anaerobic with mixing 150 rpm.

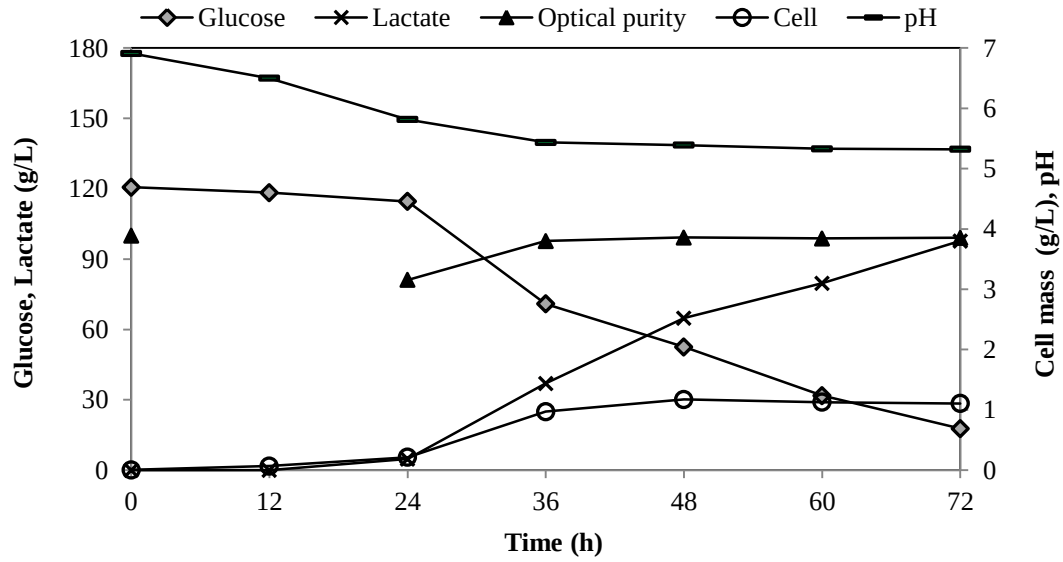
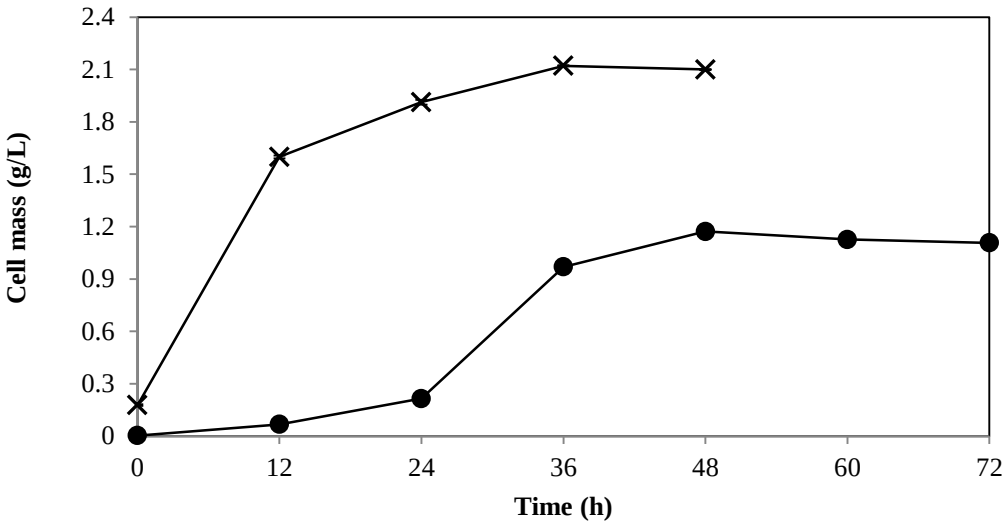
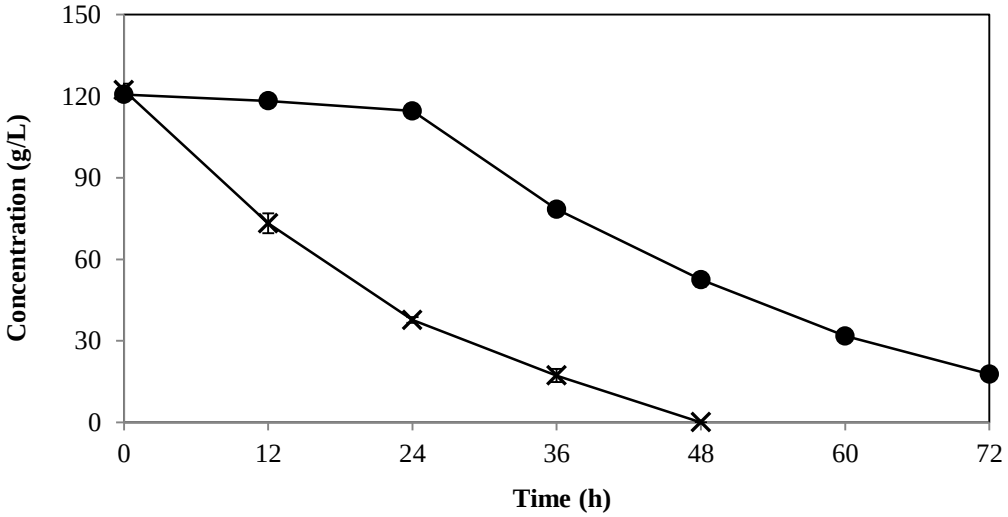


Figure 5.2 Fermentation kinetics of *S. laevolacticus* 0361^T was inoculated in the pre-culture medium containing (per liter) 10 g yeast extract and 5 g peptone. The fermentation medium containing (per liter) 120 g glucose, 80 g $CaCO_3$ and nutrient solution. The culture was incubated in an Erlenmeyer flask at 37 °C for 72 h under anaerobic without mixing.

(a)


Cell mass (g/L)
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
0
12
24
36
48
60
72
Time (h)

(b)


Concentration (g/L)
150
120
90
60
30
0
0
12
24
36
48
60
72
Time (h)

(c)

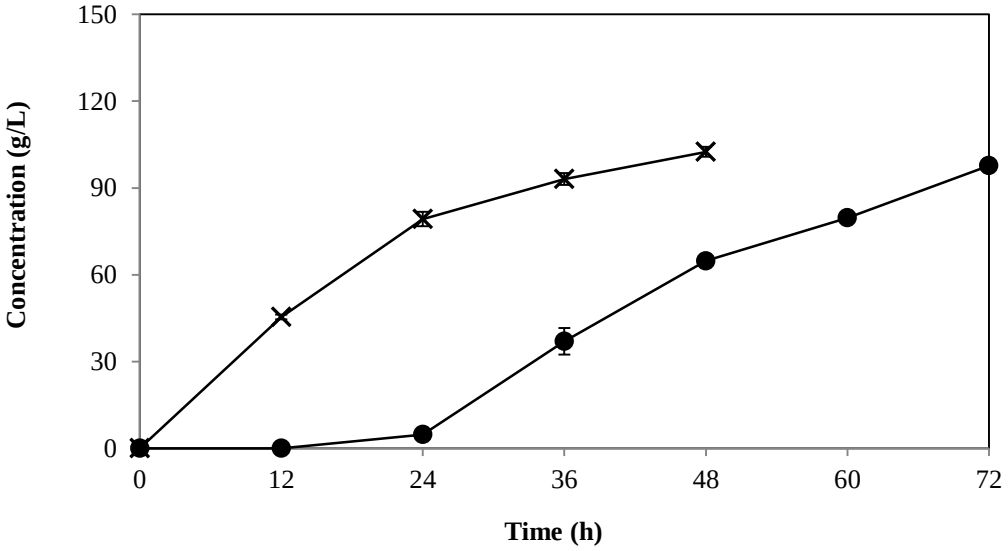


Figure 5.3 Fermentation kinetics of *T. laevilacticus* SK5-6 compared to *S. laevolacticus* 0361^T during the fermentation stage. (a) Cell, (b) Glucose (g/L) and (c) Lactate (g/L). ⟶✕⟵: *T. laevilacticus* SK5-6; ⟶●⟵ : *S. laevolacticus* 0361^T

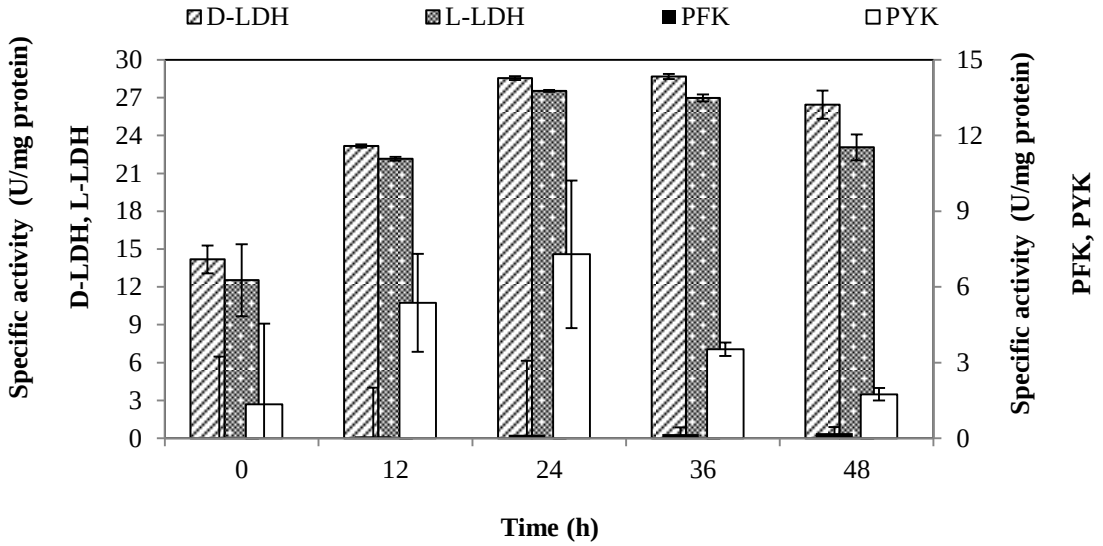
Table 5.1 Fermentation performance of *T. laevilacticus* SK5-6 and *S. laevolacticus* 0361^T during fermentations stage.

Strain	Fermentation time (h)	Cell mass (g/L)	Fermentation performance				Remaining glucose (g/L)	pH
			Final conc. (g/L)	$Y_{p/s}$	Productivity (g/L·h)	Optical purity (%ee)		
SK5-6*	0	0.178±0.004	0	0	0	100.00	122.15±2.48	6.66±0.01
	12	1.599±0.009	45.4±0.85	0.93	3.78	100.00	73.25±3.61	5.71±0.01
	24	1.913±0.014	79.25±2.48	0.94	3.30	100.00	37.65±1.06	5.58 ±0.03
	36	2.121±0.003	93.05±2.05	0.89	2.58	99.67	17.2±2.40	5.58 ±0.03
	48	2.100±0.003	102.5±1.70	0.84	2.14	99.74	0	5.73 ±0.04
0361^T**	0	0.004±0.002	0	0	0	100.00	120.65±0.50	6.91±0.01
	12	0.068±0.011	0	0	0	DL	118.30±0.42	6.50 ±0.01
	24	0.215±0.005	4.75 ±0.21	0.78	0.20	81.10	114.55±2.33	5.82± 0.04
	36	0.970±0.007	36.95±4.60	0.74	1.03	97.73	78.40±0.42	5.44±0.02
	48	1.172±0.006	64.80±1.84	0.95	1.35	99.24	52.50±2.40	5.39±0.01
	60	1.127±0.008	79.7±0.99	0.90	1.33	98.79	31.75±2.05	5.33±0.04
	72	1.107±0.013	97.70±0.99	0.95	1.36	99.09	17.75±0.21	5.32±0.03

* *T. laevilacticus* SK5-6 was inoculated in the pre-culture medium containing (per liter) 15 g yeast extract and 4 g NH_4Cl. The fermentation medium containing (per liter) 120 g glucose and 80 g $CaCO_3$. The culture was incubated in an Erlenmeyer flask at 37 °C for 48 h under anaerobic with mixing.

** *S. laevolacticus* 0361^T was inoculated in the pre-culture medium containing (per liter) 10 g yeast extract and 5 g peptone. The fermentation medium containing (per liter) 120 g glucose, 80 g $CaCO_3$ and nutrient solution. The culture was incubated in an Erlenmeyer flask at 37 °C for 72 h under anaerobic without mixing.

(a)



(b)

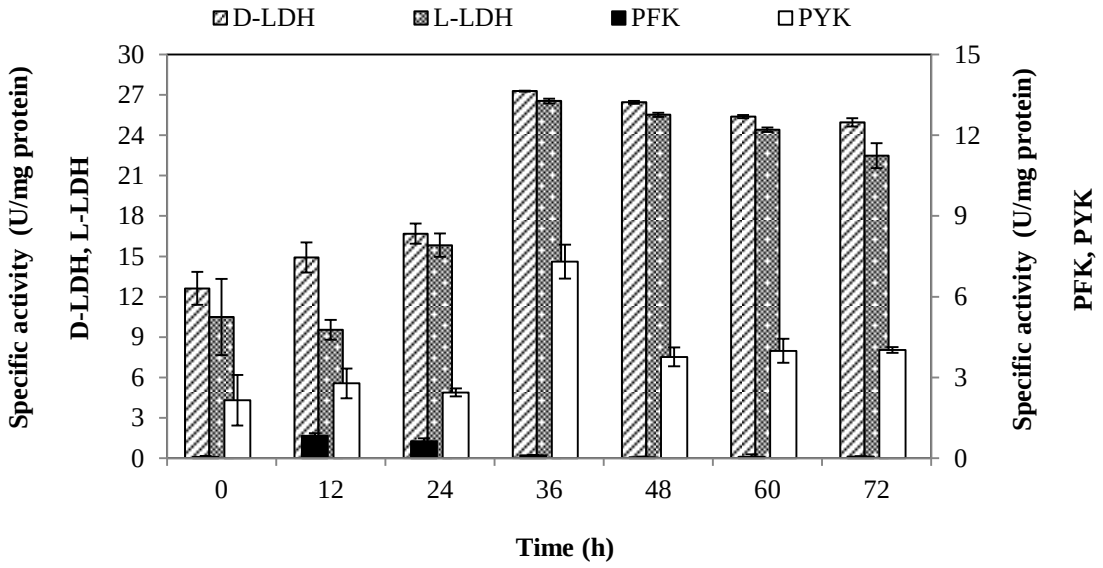
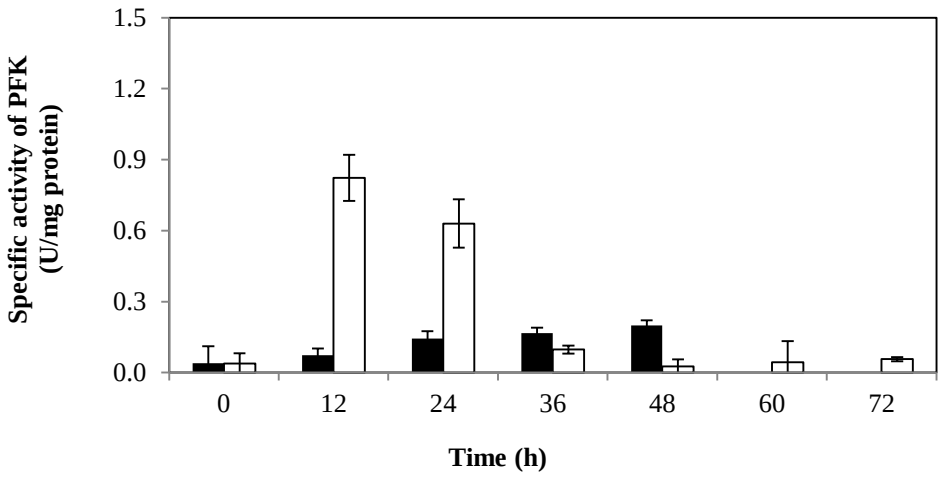
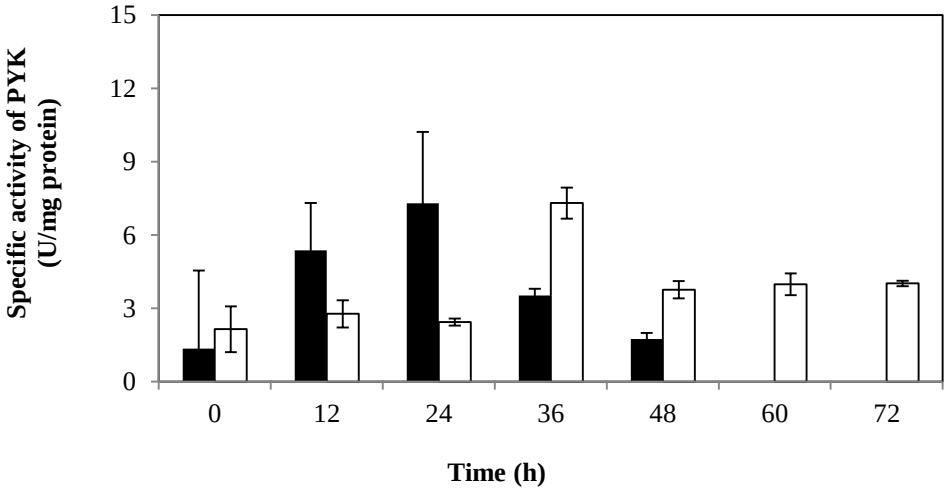


Figure 5.4 Specific activity of key responsible enzymes in D-lactic acid production was observed from fermentation stage. (a) *T. laevilacticus* SK5-6 and (b) *S. laevolacticus* 0361^{T}.

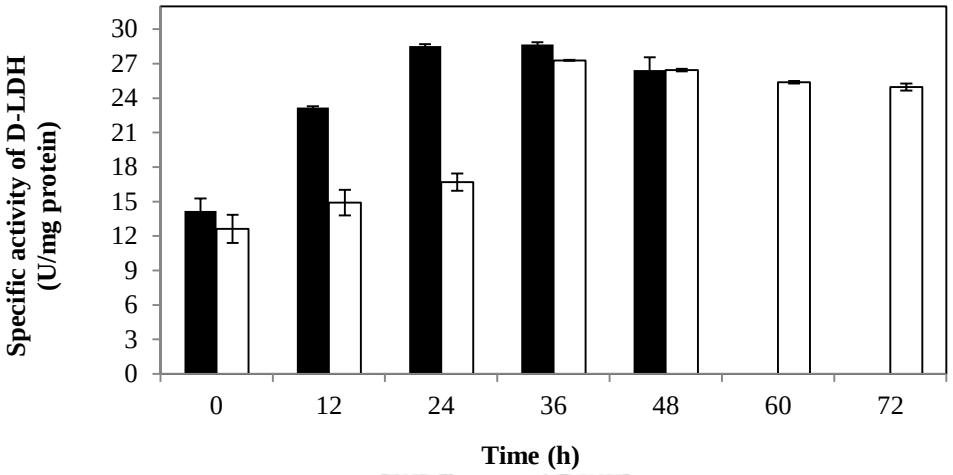
(a)


Specific activity of PFK (U/mg protein)
1.5
1.2
0.9
0.6
0.3
0.0
0
12
24
36
48
60
72
Time (h)

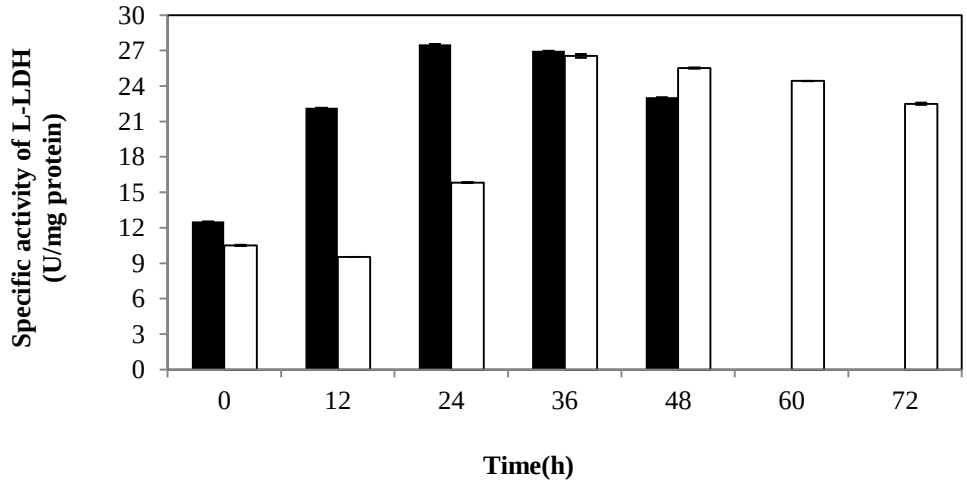
(b)


Specific activity of PYK (U/mg protein)
15
12
9
6
3
0
0
12
24
36
48
60
72
Time (h)

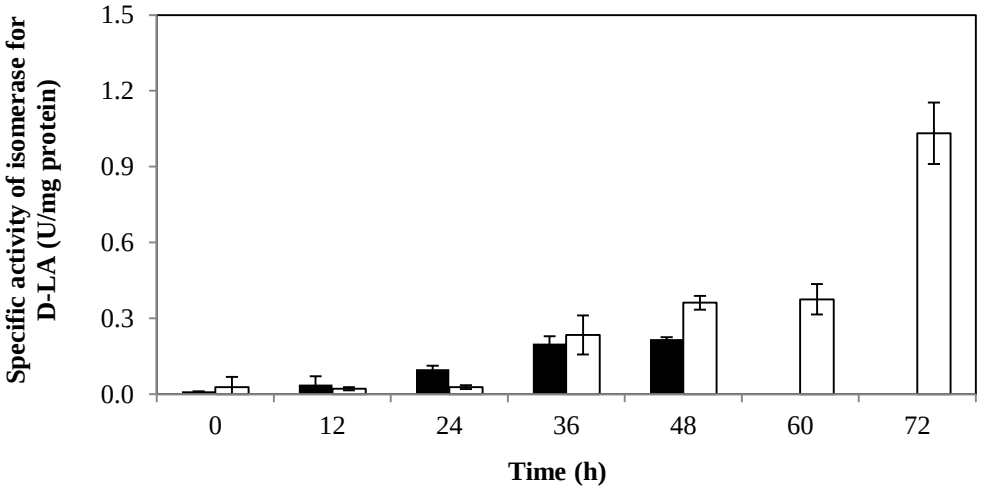
(c)


Specific activity of D-LDH (U/mg protein)
30
27
24
21
18
15
12
9
6
3
0
0
12
24
36
48
60
72
Time (h)

(d)


Specific activity of L-LDH (U/mg protein)
30
27
24
21
18
15
12
9
6
3
0
0
12
24
36
48
60
72
Time(h)

(e)



(f)

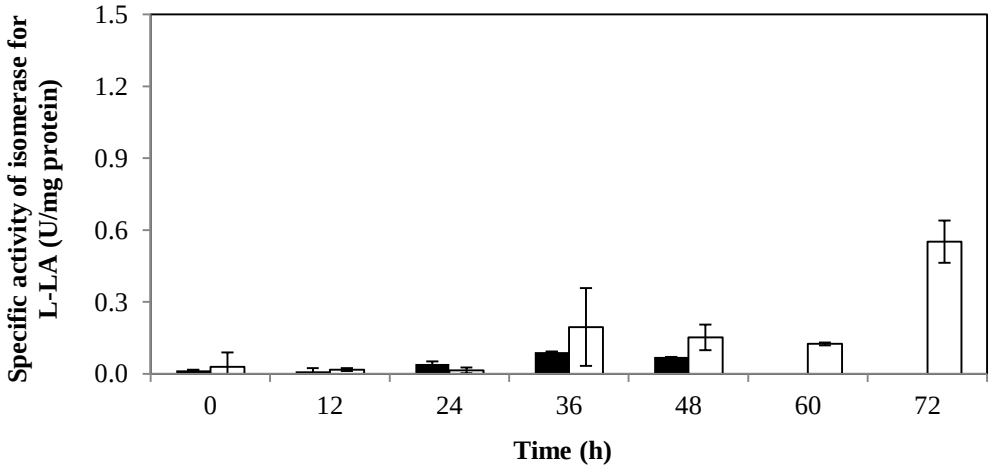


Figure 5.5 Specific activities of PFK, PYK, LDH and isomerase in D-lactate production were compared between *T. laevilacticus* SK5-6 and S. *laevolacticus* 0361^{T}. (a) PFK, (b) PYK, (c) D-LDH, (d) L-LDH, (e) isomerase for D-lactate and (f) isomerase for L-lactate. ■, *T. laevilacticus* SK5-6; □, *S. laevolacticus* 0361^{T}.

Influence of Diammonium Hydrogen Phosphate on Lactate Production and Optical Purity of D-Lactate in *T. laevilacticus* SK5-6

Previous result, we observed that the specific activity of PFK in glycolysis pathway by *T. laevilacticus* SK5-6 was lower than other key enzymes indicating that the conversion of F-6-P to F-1,6-P was lower. Thus, this present study we aimed to investigate the effect of diammonium hydrogen phosphate in medium on D-lactate production to enhance cell biomass, final lactate and optical purity of D-lactate. It was believed that supplementation of medium with diammonium hydrogen phosphate would enhance enzyme activity of PFK, PYK and LDH that metabolites respond by allosteric activation. Moreover, monovalent ion of NH_4^+ stimulate activity of PFK for catalytic reaction in *T. laevilacticus* SK5-6 (Fothergill-Gilmore and Michels, 1993; Singhvi et al., 2013; Zheng et al., 2017). Therefore, this experiment would add diammonium hydrogen phosphate replacement NH_4Cl as nitrogen source in the medium was investigated for D-lactate fermentation. This experimentation, the pre-culture seed was obtained during the pre-culture stage under aerobic condition with mixing at 200 rpm for 4 h was used to inoculate into the fermentation medium under anaerobic with mixing rate of 150 rpm for D-lactate production. Table 5.2 shows the metabolic response of *T. laevilacticus* SK5-6 in medium containing different nitrogen sources by combination of the three most promising the nitrogen sources such as yeast extract, ammonium chloride and diammonium hydrogen phosphate during the fermentation stage. Cell no growth was observed in fermentation at low amount of yeast extract (7.5 g/L) supplemented with $(NH_4)_2HPO_4$ or NH_4Cl were added into medium. Except for the medium containing yeast extract 15 g/L and $(NH_4)_2HPO_4$ 4 g/L, at 48 h culture gave high D-lactate of 92.00 g/L, productivity of 1.92 g/L.h and optical purity of 99.09 %ee while the highest cell growth, lactate formation and optical purity of D-lactate were obtained in medium with the presence of 15 g/L yeast extract and 4 g/L NH_4Cl during fermentation phase, cell biomass 2.1 g/L, lactate of 102.5 g/L, yield of 0.84 g/g glucose, productivity of 2.14 g/L.h and optical purity of D-lactate of 99.74 %ee (Fig. 5.6 and Table 5.2). As a result, higher optical purity of D-lactate and final product were obtained from NH_4Cl higher than $(NH_4)_2HPO_4$. It was indicated that expression level of the key enzymes in glycolysis especially, D-lactate dehydrogenase in D-lactate production might be activated by NH_4Cl better than $(NH_4)_2HPO_4$. The result of kinetic fermentation by *T. leavilacticus* SK5-6 showed final D-lactate was directly proportional to the increase of yeast extract, this result was quite similar to those of a previous report by Hujanen and Linko (1996), who reported that lactate formation and cell biomass were increased when increasing yeast extract due to it consist of amino acid and essential vitamins such as purine and pyrimidine bases and vitamins B essential for the cell growth and production of lactic acid bacteria. The previous studies claimed that the influence of types of nitrogen sources effect on lactate dehydrogenase expression such as *L. delbrueckii* NBRC 3202 grew in MRS medium, it was found that yeast extract exhibited high titer 3.16 g/L and optical purity of D-lactate 97.33% while peptone, whey protein hydrolysate and ammonium citrate gave lower optical purity of D-lactate 96.65, 94.19 and 95.87 %ee, respectively (Cingadi et al., 2015). A change in lactate production and optical purity of D-lactate were obtained when media containing different nitrogen sources resulted in lower or higher of productivity and optical purity of D-lactate (Feng et al., 2014).

Nevertheless, the negative effect of $(NH_4)_2HPO_4$ from this experiment, it might inhibit cell growth because there was high osmotic pressure and alkaline of the medium resulting from the addition of $(NH_4)_2HPO_4$ that effect on bacterial growth (Yaganza et al., 2009). It has been reported that the glucose utilization and final lactate were decreased with different anions and their concentrations. The same anion concentration, chloride ion resulted in higher lactate production than phosphate ions by *L. plantarum* (Lu et al., 2002). Cl^- is also a component of cell growth, flagellum, endospore forming and chloride could induce genes in lactic acid bacteria was involved in acid stress response in *Halobacillus halophilus* (Roessler et al., 2003). Cotter and Hill (2003) proposed induction by chloride could induce genes in lactic acid bacteria for survival during lactic acid fermentation that the microorganism encounters a highly acidic. Therefore, $(NH_4)_2HPO_4$ did not enhance productivity and optical purity of D-lactate compared to NH_4Cl by *T. laevilacticus* SK5-6. The result was different from *L. lactis* RM2-24 exhibited high D-lactate production due to activate D-LDH activity by adding $(NH_4)_2HPO_4$ (Singhvi et al., 2013). In 2016, Sun and colleagues claimed that $(NH_4)_2HPO_4$ could stimulate L-LDH to enhance L-lactic acid fermentation by *B. coagulans* 2-6 similar result was obtained from *Streptococcus faecal* which produced high titer and optical purity of L-lactate was improved by diammonium hydrogen phosphate from 97.1 to 99.8 % optical purity of L-lactate (Ohara and Yoshida, 1993). From this finding, it was evident that the production and optical purity of D-lactate were influenced by composition of nitrogen sources that correlated to lactate production and enantiomeric ratio that lactate dehydrogenase expression during fermentation (Table 5.3).

Table 5.2 Lactate production, yields, productivities and optical purity of D-lactate during fermentations stage by *T. laevilacticus* SK5-6. The medium containing yeast extract with NH_4Cl or $(NH_4)_2HPO_4$ at different concentration.

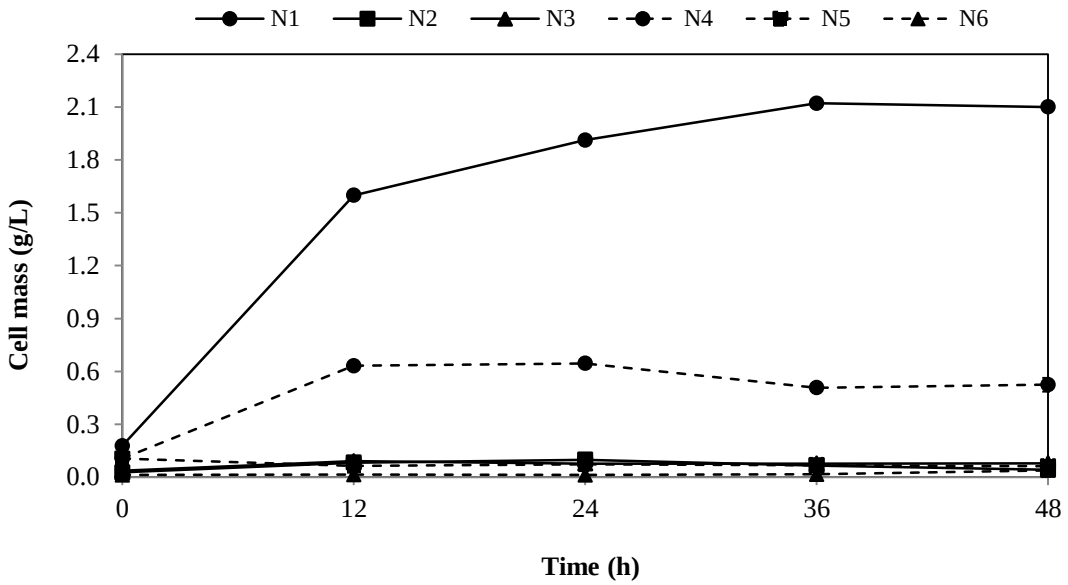
Nitrogen condition	At 48 h			Fermentation performance		
	Cell mass (g/L)	Residual glucose (g/L)	Lactate (g/L)	$Y_{p/s}$	Productivity (g/L·h)	Optical purity (%ee)
N1) YE = 15 g/L NH_4Cl = 4 g/L	2.10±0.003	0.00±0.00	102.50±1.70	0.84	2.14	99.74 ±0.05
N2) YE = 7.5 g/L NH_4Cl = 4 g/L	0.042±0.052	60.40±0.68	27.90±1.2	0.44	0.58	99.00 ±0.52
N3) YE = 7.5 g/L NH_4Cl = 10 g/L	0.079±0.006	99.91±2.65	23.04±1.92	0.89	0.480	99.21 ±1.11
N4) YE = 15 g/L $(NH_4)_2HPO_4$ = 4 g/L	0.524±0.039	32.37±2.61	92.00±2.15	0.98	1.92	99.09 ±0.47
N5) YE = 7.5 g/L $(NH_4)_2HPO_4$ = 4 g/L	0.061±0.022	108.08±1.19	0.00	0.00	0.00	100.00±0.00
N6) YE = 7.5 g/L $(NH_4)_2HPO_4$= 10 g/L	0.039±0.014	109.95±0.78	6.60±0.14	0.55	0.138	100.00±0.00

YE; yeast extract

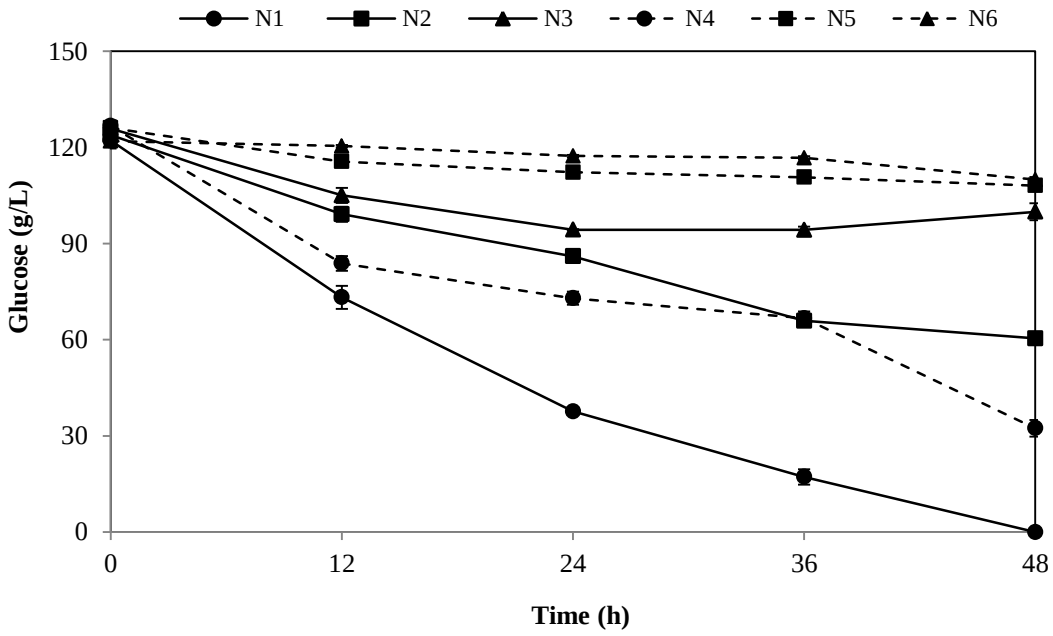
Table 5.3 Comparison of D-lactate production from different nitrogen sources by microorganisms.

Strain	Substrate	D-Lactate (g/L)	Yield (g/g)	Productivity (g/L.h)	Optical purity (%ee)	References
Sporolactobacillus sp. CASD	Glucose; peanut meal	207	0.93	3.8	99.3	Wang et al., 2011
Lactobacillus delbrueckii subsp. *lactis* QU 41	Glucose; yeast extract, peptone and meat extract	20.1	1.01	1.67	>99.9	Tashiro et al., 2011
Sporolactobacillus laevolacticus DSM442	Glucose; cotton seed hydrolysate	144.4	0.96	4	99.3	Li et al., 2013
Escherichia coli HBUT-D	Glucose; yeast extract	127	0.93	6.35	99.5	Liu et al., 2014
Sporolactobacillus inulinus Y2–8	Cornflour hydrolysate; yeast extract	145.8	1.62	1.62	>99	Zhao et al., 2014
Sporolactobacillus spp. Y 2–8	Glucose; yeast extract, corn steep liquor and wheatbran	127.0	-	1.72	99	Sun et al., 2015
S. inulinus NBRC13595	Palmyra palm jaggeric sugar; whey protein hydrolysate	189.0	0.94	5.25	>98	Reddy Tadi et al., 2017
S. inulinus YBS1-5	Corn cob hydrolysate; cottonseed meal	107.2	0.85	1.19	99.2	Bai et al., 2016
T. laevilacticus SK5-6	Glucose; yeast extract and NH_4Cl	102.5	0.84	2.14	99.74	This study
T. laevilacticus SK5-6	Glucose; yeast extract and $(NH_4)_2HPO_4$	92.00	0.98	1.92	99.09	This study

(a)


N1
N2
N3
N4
N5
N6
Cell mass (g/L)
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
0
12
24
36
48
Time (h)

(b)


N1
N2
N3
N4
N5
N6
Glucose (g/L)
150
120
90
60
30
0
0
12
24
36
48
Time (h)

(c)

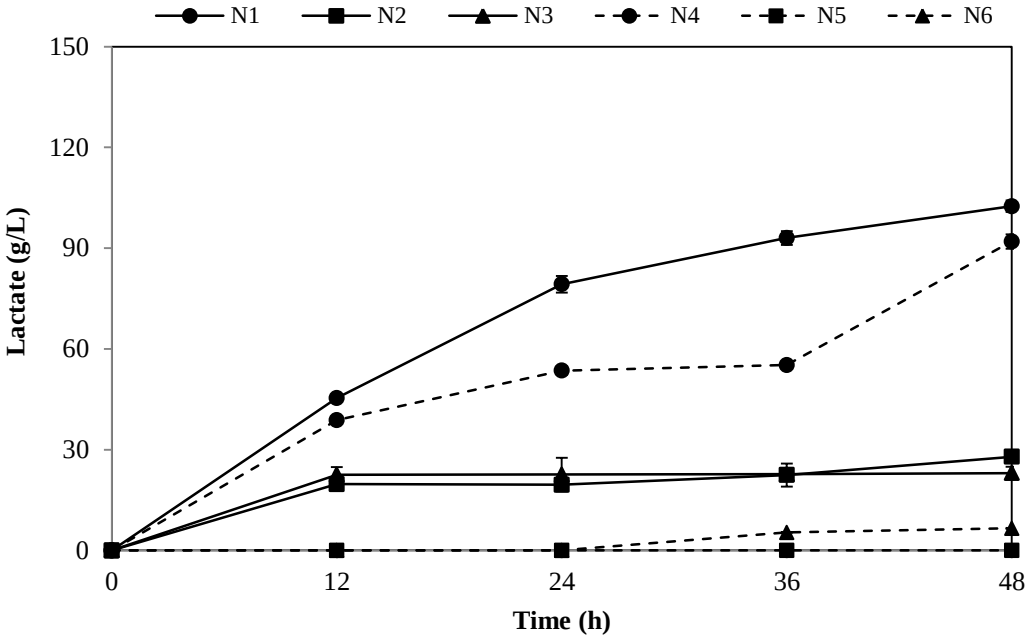


Figure 5.6 Effect of nitrogen source on growth, glucose consumption, lactate formation and optical purity of D-lactate by SK5-6 during fermentation stage in the flask culture. All the experiments were carried out at 37 °C, 200 rpm in preculture step, later fermentation step were carried out at the same temperature, 150 rpm under anaerobic condition. (a) cell, (b) glucose and (c) lactate.

N1: Yeast Extract 15 g/L+NH_4Cl 4 g/L;

N2: Yeast Extract 7.5 g/L+NH_4Cl 4 g/L;

N3: Yeast Extract 7.5 g/L+NH_4Cl 10 g/L;

N4: Yeast Extract 15 g/L+$(NH_4)_2HPO_4$ 4 g/L;

N5: Yeast Extract 7.5 g/L+$(NH_4)_2HPO_4$ 4 g/L;

N6: Yeast Extract 7.5g/L+$(NH_4)_2HPO_4$ 10 g/L.

Influence of pH on lactate dehydrogenase expression during the fermentation stage by *T. laevilacticus* SK5-6

In this study, we investigated the parameter of pH on lactate dehydrogenase expression from medium containing 15 g/L yeast extract was supplemented with 4 g/L NH_4Cl or $(NH_4)_2HPO_4$. The other nitrogen conditions, pH did not seem to affect the optical purity due to low lactate production during fermentation (data not showed).

It was found that when cell grew and approached a stationary phase within 36 h. Later cells entered the declining phase at 48 h resulting in the lower OD600 and higher pH. As a result, the optical purity of D-lactate was increased from 99.67 to 99.74 %ee with increased pH from 5.58 to 5.73 using NH_4Cl as nitrogen source. Also, $(NH_4)_2HPO_4$ demonstrated similar performance that produced high optical purity of D-lactate from 98.57 to 99.09 %ee with increased pH from 5.69 to 6.09 (Fig. 5.7). It was indicated that increasing pH during declining phase affected to D-LDH expression, resulting in high optical purity of D-lactate in *T. laevilacticus* SK5-6. This study agree with previous reports by Li et al. (2012) claimed that the optimal pH of the D-LDH was between 7.0-8.0, revealing this enzyme was resistant to alkali condition by *Leuconostoc mesenteroides* subsp. *mensenteroides* ATCC8293. Gordon and Doelle (1975) have been reported that production of racemic lactic acid in *Pediococcus cerevisiae* showed optimum pH for D-lactate dehydrogenase was pH 8.0. It agreement with Garvie (1980) reported that the optimal pH values in which D-lactate dehydrogenase for pyruvate reduction shown activity was wide range at neutral to base conditions, pH 6.0-8.0. On the other hand, L-LDH in acidic conditions at pH 4.5-6.0 was found in *L. acidophilus*, *L. plantarum*, *Pediococcus cerevisiae* and *P. pentosaceus*. For isomeric form of L-lactic acid producing bacteria, *L. rhamnosus* was decreased optical purity of L-lactate from 98 to 97%ee when increasing pH from 5.0 to 6.5 or when composition of nutrients were varied (Gordon and Doelle, 1975; Hofvendahl and Hahn-Hagerdal, 2000). The D-lactate fermentation in the presence of calcium carbonate was used to prevent acid-forming during fermentation. In this experiment, the calcium carbonate reacts with the lactic acid to produce calcium lactate; thus, the pH of the fermentation broth is maintained at about 5.0-5.6 (Liu et al., 2012; Yao and Toda, 1990). However, some strains were grown under medium without controller pH. It was found that *Pediococcus pentosaceus*, *P. cerevisae*, *L. corniformis* NCDC 369 and *L. delbrueckii* NBRC 3202 exhibited the ratio of the isomers changed and unstable proportion of L-lactate and D-lactate when the medium without $CaCO_3$ (Garvie, 1980; Reddy Tadi et al., 2017). Both L- and D-LDH were sensitive to pH in environment for His imidazole and Arg guanidino groups in their catalytic site. It has been reported that two forms of lactate dehydrogenase and their expression depend on environmental factor such as pH (Hofvendahl and Hahn-Hagerdal, 2000; Iwata and Ohta, 1993). This result suggested that D-LDH of strains D-lactate producing bacteria showed greater activity at higher pH than L-lactate dehydrogenase that showed similar result of *T. laevilacticus* SK5-6.

The decreasing enzyme activity of PFK and PYK during fermentation is explained by acid condition that maintained by $CaCO_3$ as neutralizing agent. It has been reported that optimum pH of glycolytic key enzyme from PFK was 8.5 and PYK was 7.5 in D-lactic acid bacteria. The optimum pH of PFK activity for weakly alkaline condition; thus, the affinity for F-6-P decreased at neutral and acidic

environment. It might protonation of critical residue that activity was weakened. The F-1,6-P from PFK activity that as activator for PYK might relate to environment. The acidification of environment by high lactate that negative affect to metabolism enzyme in cell (Even et al., 2002; Hofvendahl and Hahn-Hagerdal, 2000; Le Bras et al., 1991; Paricharttanakul et al., 2005; Zheng et al., 2012; Zheng et al., 2014; Zheng et al., 2017). This result finding, acidic condition was not suitable for D-lactate production due to decrease enzyme activity of PFK, PYK and D-LDH expression in glycolysis pathway. Therefore, the increased pH in environment would relate with intracellular pH, resulting in high activities of key enzymes that directly affect growth and D-lactate production (Sun et al., 2015).

Expecting the enhancement of lactate productivity and optical purity of D-lactate that not affect by neutralizer, the choice of neutralizer such as NH_4OH that contain ammonium ion would stimulate the activity of PFK that is a rate limiting-step to achieve high efficient D-lactate production by *T. laevilacticus* SK5-6.

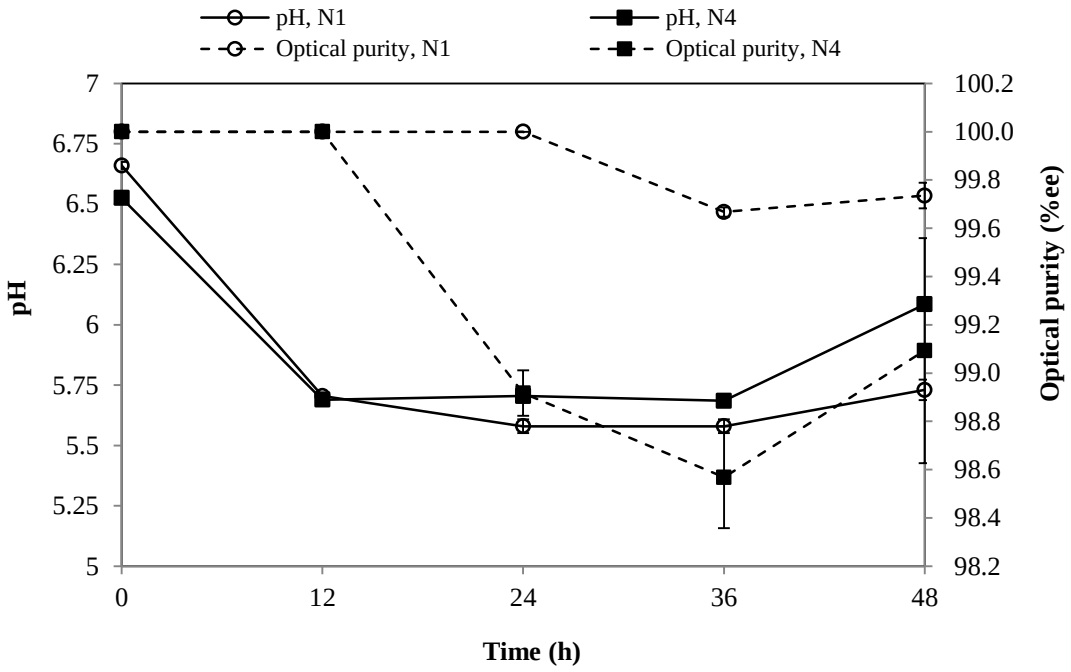


Figure 5.7 Relation between pH and optical purity of D-lactate during fermentation stage in *T. laevilacticus* SK5-6. The experiments from medium using yeast extract of 15 g/L was supplemented with NH_4Cl or $(NH_4)_2HPO_4$ of 4 g/L.

Conclusion

This study reveals the mechanism and expression level of phosphofructokinase, pyruvate kinase, lactate dehydrogenase and their isomerization to enhance the D-lactate production and the optical purity during fermentation by *T. laevilacticus* SK5-6. It was observed that PFK activity was lower than other key enzymes indicating that the conversion of of F-6-P to F-1,6-P was limited; thus, less pyruvate shifts toward D-lactate production that directly affect to cell biomass and lactate production. Comparing the production performance D-lactate and specific activity of key enzyme in glycolysis by *T. laevilacticus* SK5-6 (as catalase positive D-lactate producer) to *S. laevolacticus* (as catalase negative D-lactate producer), the result was found that a catalase positive D-lactate producer was attractive for D-lactate fermentation due to its good fermentation performance with high D-lactate, yield and productivity were observed. The kinetics data obtained were well correlated with the expression level of the key enzymes in D-lactate production. Investigation of diammonium hydrogen phosphate was added in culture medium to improve performance of D-lactate, resulting in slower growth, lower lactate productivity and optical purity of D-lactate than NH_4Cl as nitrogen source. From this result finding, lactate dehydrogenase expression was influenced by composition of nitrogen sources that correlated with lactate production and enantiomeric ratio that lactate dehydrogenase expression during fermentation. Increasing the pH during fermentation phase would activate D-LDH expression higher than L-LDH activity, resulting in high optical purity of D-lactate. It was suggested that the acidic pH affected the activities of PFK and PYK that directly. The isomerization that convert L-lactic acid to D-lactic acid by isomerase was also observed during fermentation, this result could be explained that strain possess both L-LDH and D-LDH that expression level of the D- or L-LDH depend on fermentation factors such as pH and medium composition. This somewhat implies the possibility to improve the lactate production and optical purity of D-lactate during fermentation by effector of pH on the key enzyme in glycolysis.

CHAPTER VI

CONCLUSION AND SUGGESTION

6.1 Conclusion

Strain NK26-11^T, firstly isolated and identified in this study, was Gram-stain-positive, facultatively anaerobic, endospore-forming straight rods with rounded ends bacteria. The endospores were sub-terminal and oval with a swollen sporangium. Colonies on GYP agar plates were circular smooth ivory-white and non-pigmented. This strain produced D-lactic acid from glucose homofermentatively. It showed the positive results for the activities of catalase and nitrate reduction but negative results for oxidase, starch and arginine hydrolysis. This strain could grow at 20-45 °C, at pH 5-8.5 and in the presence of 3 % (w/v) NaCl. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid. The major respiratory quinone was MK-7. Diphosphatidylglycerol and phosphatidylglycerol were the major polar lipids. The major fatty acids were anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$. The DNA G+C content of the type strain of the type species was 42.6 mol%. The strain was proposed to be novel species, namely *Terrilactibacillus laevilacticus*.

D-lactate producers were isolated from natural samples in Thailand. Among 6 isolates obtained, the isolate SK5-6, identified later as *T. laevilacticus* SK5-6, exhibited the good characteristics over other D-lactate producers previously reported in the literatures. SK5-6 demonstrated the ability to utilize several carbon substrates for D-lactate production. The optical purity of D-lactate was sufficiently high for polymer-grade specification (>99.5%ee). Unlike *Sporolactobacillus* sp., the common D-lactate strain, SK5-6 produced catalase; thus, oxygen was not restricted during the cultivation. By the simple 2-phase fermentation employed in this study, aerobic preculture was introduced to generate robust, high-cell-density cell mass of SK5-6 for D-lactate production during the anaerobic fermentation stage without byproducts. This resulted in the high yield and productivity. The results of this research therefore confirm the potential of using *Terrilactibacillus* sp. as a D-lactate producer.

Due to the excellent fermentation performance of SK5-6 and the attempt to introduce *Terrilactibacillus* strain as the alternated commercial D-lactate producing strain, further study on the metabolic rates of this isolate in glucose utilization for D-lactate was investigated. The mechanism and expression level of phosphofructokinase, pyruvate kinase, lactate dehydrogenase and their isomerization to enhance D-lactate production and the optical purity during fermentation by SK5-6 were observed. The results show that PFK activity was lower than the activities of other key enzymes indicating that the conversion of of F-6-P to F-1,6-P was less activity; thus, less pyruvate shifted toward D-lactate production that directly affected cell biomass and lactate production. Comparing the production performance of D-lactate and specific activity of key enzymes in glycolysis by *T. laevilacticus* SK5-6 (as the catalase positive D-lactate producer) to *S. laevolacticus* (as the catalase

negative D-lactate producer), it was found that the catalase positive D-lactate producer was attractive for D-lactate fermentation due to its good fermentation performance with high D-lactate, yield and productivity. The kinetics data obtained were well correlated with the expression level of the key enzymes in D-lactate production.

Investigation on changes of nitrogen source in the fermentation medium from NH_4Cl to $(NH_4)_2HPO_4$ revealed the slower growth, lower lactate productivity and optical purity of D-isomer. This was presumably due to the expression of D-LDH was influenced by the composition of nitrogen source that directly correlated with lactate production and control of the enantiomeric ratio during the fermentation. It was also observed that increasing the pH during the fermentation phase stimulated the expression of D-LDH resulting the high optical purity of D-lactate. The results also suggested that the acidic pH enhanced the activities of PFK and PYK. In addition, the isomerase activity was observed during the fermentation. The expression level of isomerase to convert L-lactate to D-lactate was dependent on the environmental factors such as the medium compositions and pH. From the findings obtained in this part of the study, it is believed that the productivity and optical purity of D-lactate can be manipulated by the effective control of the operating conditions.

6.2 Suggestion

This study attempted to describe several environmental factors that impact the development of D-lactic acid production by SK5-6. It was found that a higher cell biomass, final lactate titer, yield, and productivity were achieved with a high agitation rate during the fermentation stage in the 5 L stirred fermentor. Thus, further process optimization and fermentation platform development by increasing agitation speed and aeration should be determined in the stirred fermentor. In addition, for easy operation, other alkali solution such as NaOH, NH_4OH, and KOH should be tested for D-lactate fermentation in the stirred fermentor. From the expression level of key glycolytic enzymes, PFK activity was low indicating the rate limiting step; therefore, it was expected that the enhancement of PFK activity that correlate with efficient D-lactate production and glycolytic flux should be further conducted. From the literatures, it was suggested that the activity of PFK was activated by the increased glucose-6-phosphate and Mg^{2+}concentration. To achieve the improved PFK activity, it can be done by medium optimization.

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APPENDIX

APPENDIX A

16S rRNA gene sequences of strains

16S rRNA gene sequence of NK26-11

AGGAAGCAAAACAATCCTTCGGGTGCGTTTTGTGGAATGAGCGGCGGACG
GGTGAGTAACACGTGGGCAACCTGCCTGTAAGACGGGGATAACTTCGGGA
AACCGGGGCTAATACCGGGTAATCTTTTGCATCGCATGATGCAAGGGTAA
AAGATGGTTCTGCCATCGCTTACAGATGGGCCCGCGGTGTATTAGCTAGTT
GGTGAGGTAATGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGG
GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCC
GCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGAGAAGA
ATAGGTATCATAGGAAATGATGGTACTGTGACGGTATCTAACCAGAAAGC
CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTG
ATGTGAAAGCCCCCAGCTCAACTGGGGAGGGTCATTGGAAACTGGGAAAC
TTGAGTACAGAAGAGGAGAGTAGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAATACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACT
GACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGG
TAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGGTCCAACCCTT
AGTGCTGAAGTTAACACATTAAGCACTCCGCCTGGGGAGTACGACCGCAA
GGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGCAT
GTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCT
CTGACAAGCCTAGAGATAGGCCGTTCCCCTTCGGGGGACAGAGTGACAGG
TGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTA
AGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATC
ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACA
AAGGGCAGCGAAGCCGCGAGGCCGAGCCAATCCCATAAAGCCATTCTCA
GTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATTGCTAGTA
ATCGCGGATCAGCATGCCGCGGTGAATCCGTTCCCGGGCCTTGTACACAC
CGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACC
TTTTGGGGCCAGCCGCCGAAGGTGGGACAAATGATTGGGGGTGAAGTCGT
AACAAGGTA

16S rRNA gene sequence of SK5-6

AACGCGGGCGGCGTGCCTAATACATACAAGTCGAGCGCAGGAAGCAAAA
CAATCCTTCGGGTGCGTTTTGTGGAATGAGCGGCGGACGGGTGAGTAACA
CGTGGGCAACCTGCCTGTAAGACGGGGATAACTTCGGGAAACCGGGGCTA
ATACCGGGTAATCTTTTGCATCGCATGATGCAAGGGTAAAAGATGGTTCT
GCCATCGCTTACAGATGGGCCCGCGGTGTATTAGCTAGTTGGTGAGGTAA
TGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG
AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAT

GAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGAGAAGAATAGGTATCAT
AGGAAATGATGGTACTGTGACGGTATCTAACCAGAAAGCCACGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATT
ATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCC
CCCAGCTCAACTGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTACAGA
AGAGGAGAGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGG
AGGAATACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGG
CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGAGTGCTAGGTGTTAGGGGGTCCAACCCTTAGTGCTGAAGT
TAACACATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTC
AAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAAGCCTA
GAGATAGGCCGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT
ATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAA
GCCGCGAGGCCGAGCCAATCCCATAAAGCCATTCTCAGTTCGGATTGCAG
GCTGCAACTCGCCTGCATGAAGCCGGAATTGCTAGTAATCGCGGATCAGC
ATGCCGCGGTGAATCCGTTCCCGGGCCTTGTACACACCGCCCGTCACACC
ACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGGGCCAGC
CGCCGAAAGTGGGACAAATGATTGGGGGTGAAGTCGTAACAAGGTAGCC
GTA

16S rRNA gene sequence of SP43-2

CCCGGATGTGAGCGGCGGATGGGTGAGTAACACGTGGGTAACCTGCCTGT
CAGATTGGGATAACTGTGGGAAACCGCAGCTAATACCGAATGATCCCCTG
CACCACATGGTGCAGGGTTGAAAGATGGTTTCGGCCATCACTGACAGATG
GGCCCGCGGTGCATTAGTTAGTTGGCGGGGTAACGGCCCACCAAGACAGC
GATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACAC
GGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACG
AAAGTCTGATGGAGCAACGCCGCGTGAGCGAAGAAGGTTTTCGGATCGTA
AAGCTCTGTTGCCGGAGAAGAATGAGTATGAGAGGAAATGCTTGTACTGT
GACGGTATCCGGCCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCG
CGCAGGCGGCTTCTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAA
TGGTCATTGGAAACTGGGAAGCTTGAGTGCAGAAGAGGAGAGTAGAATTC
CACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAATACCAGTGGCGAA
GGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGC
AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAG
GTGTTAGGGGGTCCAACCCTTAGTGCTGAAGTTAACACATTAAGCATTCC
GCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCAGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC
CTTACCAGGTCTTGACATCCTCTGACAAGCCTAGAGATAGGCCGTTCCCCT
TCGGGGGACAAAATGACAGGTGGTGCAGGGTTGTCGTCAGCTCGTGTCCT
GAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGC
CAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGA

AAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATCTGGGCTACAC
ACGTGCTACAATGGGTGGTACAAAGGGCAGCGAAACCGCGAGGTCAAGC
GAATCCCATAAAGCCACCCCCAGTTCGGATTGCAGGCTGCAACTCGCCTG
CATGAAGCCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATC
CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTG

16S rRNA gene sequence of NK44-2

GCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCAT
CGATGGGAGCTTGCTCCCTGAAGTGAGCGGCGGATGGGTGAGTAACACGT
GGGTAACCTGCCTGTCAGATCGGGATAACTGTGGGAAACCGCAGCTAATA
CCGGATAATCCTTCGCACCGCATGGTGCGGAGTTGAAAGATGGTTTCGGC
CATCACTGACAGATGGGCCCGCGGTGCATTAGTTAGTTGGCGGGGTAACG
GCCCACCAAGACTGCGATGCATAGCCGACCTGAGAGGGTGATCGGCCACA
TTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAA
TCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGCGAAGA
AGGTTTTCGGATCGTAAAGCTCTGTTGCTGGAGAAGAACGAGTGCGAGAG
GAAATGCTCGTACTAGTGACGGTATCCAGCCAGAAAGCCACGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTA
TTGGGCGTAAAGCGCGCGCAGGCGGCTTCTTAAGTCTGATGTGAAATCTT
GCGGCTCAACCGCAAACGGTCATTGGAAACTGGGAAGCTTGAGTGCAGAA
GAGGAGAGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAG
GAATACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGAATGCTAGGTGTTAGGGGGGTCCAACCCCTTAGTGCTGAAGT
TAACACATTAAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTC
AAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGCATGTGGTTTAATTC
GAAGCAACGCAAAGAACCTTACCAGGTCGTGACATCCTCTGACAAGCCTA
GAGATAGGCCGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACATCAAATCATCATGCCCCTT
ATGATCTGGGATACACACGTGCTACAATGGGTGGTACAAAGGGCAGCAAA
CCGCGAGGTCGAGCTAATCCCATAAAGCCACCCCCAGTTCGGATTGCAGG
CTGCAACTCGCCTGCATGAAGCCGGAATTGCTAGTAATCGCGGATCAGCA
TGCCGCGGTGAATCCGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
CGAGAGTTTGTAACACCCGAAGTCGGTG

16S rRNA gene sequence of BRY67-1

TCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCATCG
ATGGGAGCTTGCTCCCTGAAGTGAGCGGCGGATGGGTGAGTAACACGTGG
GTAACCTGCCTGTCAGATCGGGATAACTGTGGGAAACCGCAGCTAATACC
GGATAATCCTTCGCACCGCATGGTGCGGAGTTGAAAGATGGTTTCGGCCA
TCACTGACAGATGGGCCCGCGGTGCATTAGTTAGTTGGCGGGGTAACGGC
CCACCAAGACAGCGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATT
GGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGCGAAGAA
GGTTTTCGGATCGTAAAGCTCTGTTGCTGGAGAAGAACGAGTGCGAGAGG

AAATGCTCGTATGTGACGGTATCCAGCCAGAAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTG
GGCGTAAAGCGCGCGCAGGCGGCTTCTTAAGTCTGATGTGAAATCTTGCG
GCTCAACCGCAAACGGTCATTGGAAACTGGGAACTTGAGTGCAGAAGAG
GAGAGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAA
TACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCGCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGAATGCTAGGTGTTAGGGGGTCCAACCCTTAGTGCTGAAATTAACA
CATTAAGCATTCCCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGA
ATTGACAGGGGCCCGCACAAGCACTGGAGCATGTGGTTTAATTCGAACAA
CGCGAAGAACTTACCAGGTCTTGACATCCTCTGACAAGCCTAGAGATAGG
CCGTTCCCCTTCGGGGACAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTT
AGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATCTGGGC
TACACACGTGCTACAATGGGTGGTACAAAGGGCAGCGAAACCGCGAGGT
CGAGCTAATCCCATAAAGCCACCCCCAGTTCGGATTGCAGGCTGCAACTC
GCCTGCATGAAGCCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGT
GAATCCGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTT
GTAACACCCGAAGTCGGTGCGAGAACCTTTATG

16S rRNA gene sequence of BRY67-2

CGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCATCGATGGGA
GCTTGCTCCCTGAAGTGAGCGGCGGATGGGTGAGTAACACGTGGGTAACC
TGCCTGTCAGATCGGGATAACTGTGGGAAACCGCAGCTAATACCGGATAA
TCCTTCGCACCGCATGGTGCGGAGTTGAAAGATGGTTTCGGCCATCACTG
ACAGATGGGCCCGCGGTGCATTAGTTAGTTGGCGGGGTAACGGCCCACCA
AGACAGCGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACT
GAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCAC
AATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGCGAAGAAGGTTTTC
GGATCGTAAAGCTCTGTTGCTGGAGAAGAACGAGTGCGAGAGGAAATGCT
CGTATGTGACGGTATCCAGCCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAA
AGCGCGCGCAGGCGGCTTCTTAAGTCTGATGTGAAATCTTGCGGCTCAAC
CGCAAACGGTCATTGGAAACTGGGAAGCTTGAGTGCAGAAGAGGAGAGT
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GGGACACAGTGACACGTGGTGCATGGGTGTCGTCACTCGTGTCTGAGATT
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C

16S rRNA gene sequence of BRY67-3

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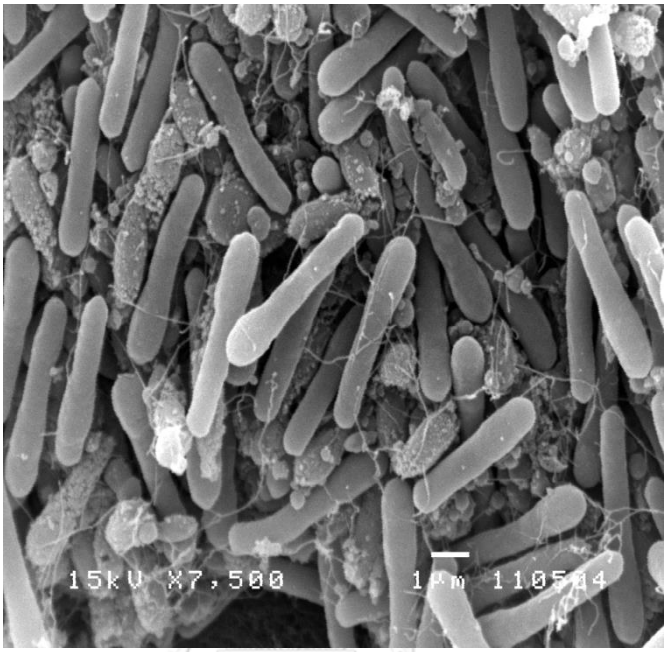
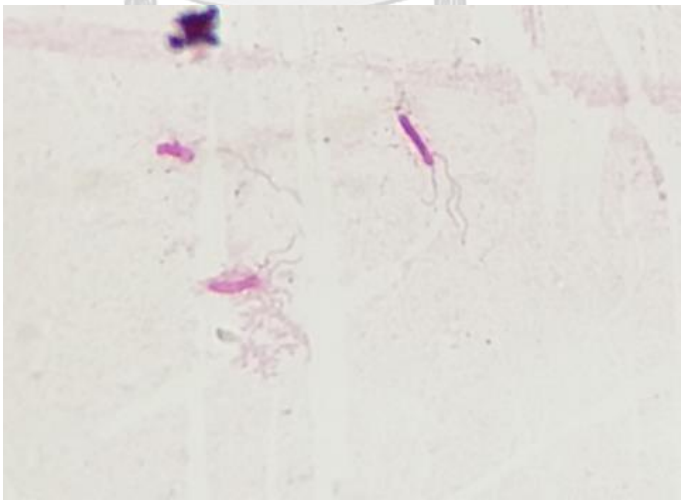
APPENDIX B

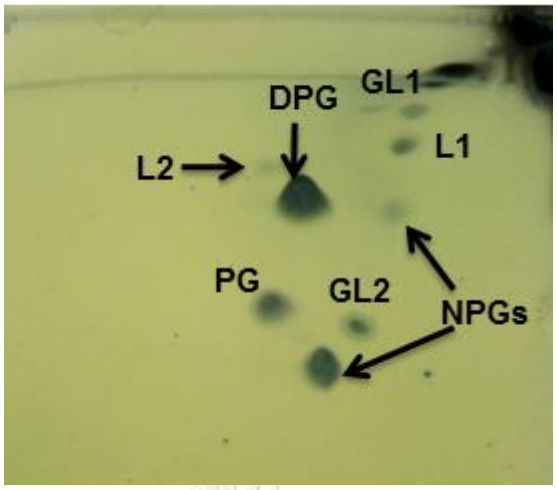
Characteristics of *Terrilactibacillus laevilacticus* NK26-11^{T}



Scanning electron micrograph of strain NK26-11^{T} grown on GYP agar for 7 days.

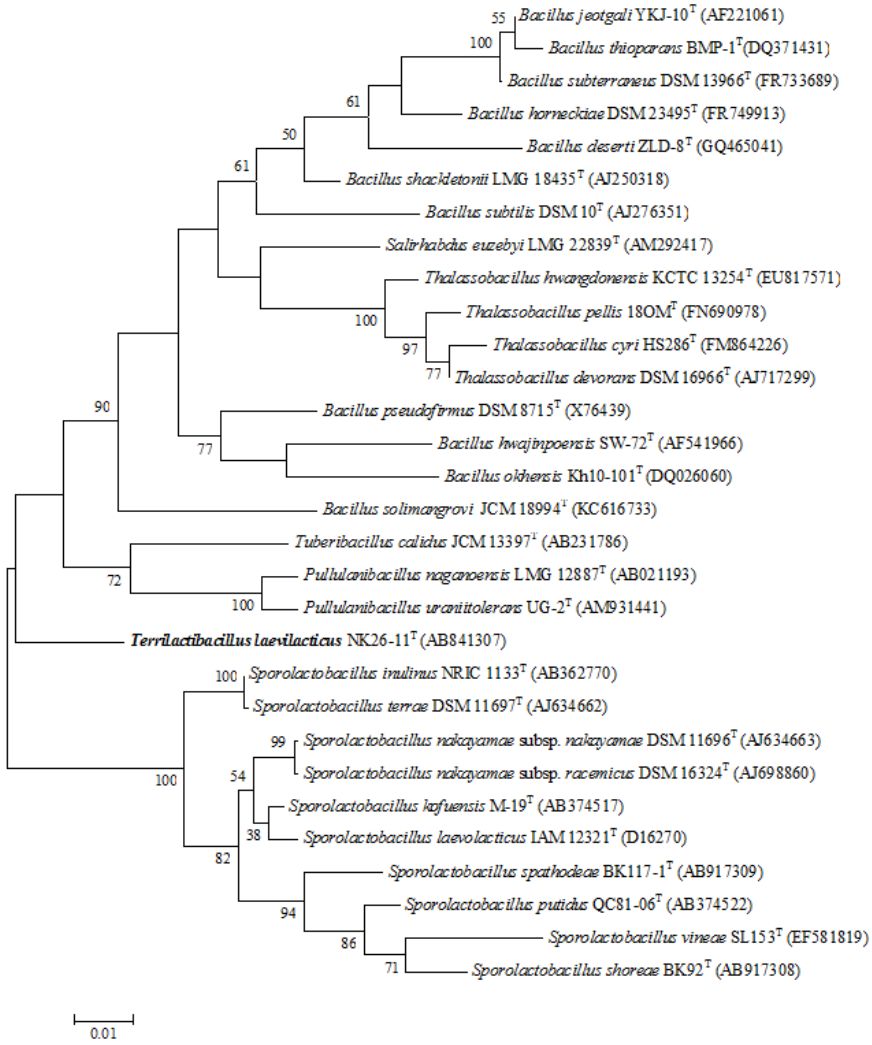


Photomicrograph of flagella of strain NK26-11^{T} grown on GYP agar for 3 days.



Polar lipid profile of strain NK26-11^{T} on a two dimensional thin layer chromatogram that was detected with phosphomolybdic acid.

DPG, diphosphatidylglycerol; PG, phosphatidglycerol; GL1 and GL2, unknown glycolipids; NPGs, unknown ninhydrin positive glycolipids; L1 and L2, unknown lipids.



Maximum-likelihood tree based on almost complete 16S rRNA gene sequences showing relationships among strain NK26-11^T and related type species of the recognized representative members of family. Bootstrap values (>50 %) based on 1000 replications are given at branch nodes. Bar, 0.01 substitutions per nucleotide position.

VITA

Miss Budsabathip Prasirtsak was born on September 22, 1985 in Nakhonpathom, Thailand. She was obtained a Bachelor Degree of Science in Biotechnology from Department of Biotechnology, Faculty of Engineering and Industrial Technology, Silpakorn University, Thailand in 2008 and obtained a Master Degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University, Thailand in 2012. She received a Doctoral Degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University, Thailand in 2017.

Academic publications

Prasirtsak, B., Thongchul, N., Tolieng, V. and Tanasupawat. S. 2016. Terrilactibacillus laevilacticus gen. nov., sp. nov., isolated from soil. International Journal of Systematic and Evolutionary Microbiology. 66: 1311-1316.

Prasirtsak, B., Thitiprasert, S., Tolieng, V., Assabumrungrat, S., Tanasupawat, S. and Thongchul, N. 2017. Characterization of D-lactic acid, spore-forming bacteria and Terrilactibacillus laevilacticus SK5-6 as potential industrial strain. Annals of Microbiology. 67: 763-778.