การหาจุดที่เหมาะสมของการผลิตกรดดี-แลคติกโดย *Terrilactibacillus laevilacticus* SK5-6 และการขยายส่วนกระบวนการหมักในถังปฏิกรณ์ชีวภาพขนาด 30 ลิตร

นายวรพจน์ โตเลี้ยง

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
เป็นแฟ้มข้อมูลของนิสิตเจ้าของวิทยานิพนธ์ ที่ส่งผ่านทางบัณฑิตวิทยาลัย
The abstract and full text of theses from the academic year 2011 in Chulalongkorn University Intellectual Repository (CUIR)
are the thesis authors' files submitted through the University Graduate School.

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

OPTIMIZATION OF D-LACTIC ACID PRODUCTION BY *Terrilactibacillus laevilacticus* SK5-6 AND FERMENTATION PROCESS SCALE UP IN 30 LITRE-FERMENTOR

Mr. Woraphot Toliang

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2017
Copyright of Chulalongkorn University

Thesis Title	OPTIMIZATION OF D-LACTIC ACID PRODUCTION BY *Terrilactibacillus laevilacticus* SK5-6 AND FERMENTATION PROCESS SCALE UP IN 30 LITRE-FERMENTOR
By	Mr. Woraphot Toliang
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Nuttha Thongchul, Ph.D.
Thesis Co-Advisor	Sitanan Thitiprasert, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Nattaya Ngamrojanavanich, Ph.D.)

.....Thesis Advisor
(Associate Professor Nuttha Thongchul, Ph.D.)

.....Thesis Co-Advisor
(Sitanan Thitiprasert, Ph.D.)

.....Examiner
(Associate Professor Aphichart Karnchanatat, Ph.D.)

.....Examiner
(Associate Professor Kittinan Komolpis, Ph.D.)

.....External Examiner
(Assistant Professor Suwattana Pruksasri, Ph.D.)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

วรพจน์ โตเลี้ยง : การหาจุดที่เหมาะสมของการผลิตกรดดี-แลคติกโดย *Terrilactibacillus laevilacticus* SK5-6 และการขยายส่วนกระบวนการหมักในถังปฏิกรณ์ชีวภาพขนาด 30 ลิตร (OPTIMIZATION OF D- LACTIC ACID PRODUCTION BY *Terrilactibacillus laevilacticus* SK5-6 AND FERMENTATION PROCESS SCALE UP IN 30 LITRE-FERMENTOR) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ณัฏฐา ทองจุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. สิตานัน ธิติประเสริฐ, 90 หน้า.

ในการผลิตพอลิแลคติกแอซิดซึ่งเป็นพลาสติกชีวภาพชนิดหนึ่งจะใช้กรดแอล-แลคติกและกรดดี-แลคติกที่มีความบริสุทธิ์สูงผ่านกระบวนการพอลิเมอไรเซชันเกิดเป็นโครงสร้างสเตอริโอบล็อก ซึ่งมีความสามารถในการทนความร้อนที่สูงขึ้น ดังนั้นกรดดี-แลคติกจึงมีความสำคัญต่ออุตสาหกรรมการผลิตพลาสติกชีวภาพ อย่างไรก็ตามงานวิจัยที่เกี่ยวกับกรดดี-แลคติกยังมีจำกัดเพราะโดยทั่วไปกรดแลคติกมักเป็นแอล-แลคติก ในงานก่อนหน้านี้ของกลุ่มวิจัยเองได้มีการคัดแยกเชื้อสายพันธุ์ใหม่ *Terrilactibacillus laevilacticus* SK5-6 โดยคัดแยกได้จากดินในประเทศไทยและสามารถผลิตกรดดี-แลคติกได้สูงจากกลูโคสและให้ค่าความบริสุทธิ์สูงด้วยในระดับขวดเขย่า ดังนั้นในงานวิจัยนี้จึงศึกษาการผลิตกรดดี-แลคติกจากการหมักด้วยเชื้อ *T. laevilacticus* SK5-6 ซึ่งจะหาภาวะที่เหมาะสมต่อการผลิตในถังปฏิกรณ์ชีวภาพขนาด 5 ลิตร โดยทำการทดลองหาภาวะที่เหมาะสมต่อการหมักโดยทำการทดสอบตัวแปรที่ส่งผล คือ ขนาดของหัวเชื้อเริ่มต้น, อายุของกล้าเชื้อ, และขนาดของกล้าเชื้อ ผลที่ได้คือ ที่ภาวะเหมาะสมนั่นคือใช้ขนาดหัวเชื้อ 1% และขนาดกล้าเชื้อ 10% เมื่อกล้าเชื้อเข้าสู่ช่วงกลางของระยะแบ่งตัวแบบทวีคูณ สภาวะดังกล่าวสามารถผลิตกรดดี-แลคติกได้ความเข้มข้น 88.0 กรัมต่อลิตร ด้วยอัตราผลผลิตต่อกลูโคส 0.87 กรัมต่อกรัม ให้อัตราการผลิต 1.83 กรัมต่อลิตรต่อชั่วโมง รวมไปถึงค่าความบริสุทธิ์ที่ 99.3% เมื่อได้ภาวะที่เหมาะสมแล้ว จึงทดสอบการขยายส่วนกระบวนการหมักต่อในถังปฏิกรณ์ชีวภาพขนาด 30 ลิตร ในขั้นนี้ได้ทดลองการพัฒนาการสร้างกล้าเชื้อโดยใช้ภาวะที่ได้ในก่อนหน้านี้ รวมไปถึงทดสอบการกำหนดค่าการปั่นกวนด้วยการยึดตัวแปรทางวิศวกรรมเมื่อขยายระดับเพื่อให้การถ่ายโอนมวลสารคงสภาวะเดิมจากระดับ 5 ลิตร จากการทดลองพบว่า ขนาดของกล้าเชื้อที่เลี้ยงในขวดเขย่า 250 มิลลิลิตร ที่เหมาะสม คือ 10% เพื่อใช้ถ่ายเชื้อไปยังขวดเขย่าขนาด 2 ลิตร และตัวแปรที่เหมาะสมต่อการกำหนดการปั่นกวนเพื่อทำการขยายส่วนกระบวนการหมัก คือ ค่ากำลังที่ใช้ต่อปริมาตร ภาวะดังกล่าวให้ความเข้มข้นกรดดี-แลคติกที่ 81.5 กรัมต่อลิตร ด้วยอัตราผลผลิตต่อกลูโคส 0.93 กรัมต่อกรัม ให้อัตราการผลิต 2.09 กรัมต่อลิตรต่อชั่วโมง ซึ่งสูงกว่าในถังปฏิกรณ์ชีวภาพขนาด 5 ลิตร เล็กน้อย รวมไปถึงค่าความบริสุทธิ์ที่ 99.3% ดังนั้น *T. laevialacticus* SK5-6 จึงมีความสามารถสูงในการผลิตกรดดี-แลคติก สามารถพัฒนาเพื่อใช้ในระดับอุตสาหกรรมต่อไปได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5872114923 : MAJOR BIOTECHNOLOGY

KEYWORDS: THE NOVEL BACTERIUM / D-LACTIC ACID / OPTIMIZATION / SCALE UP / FERMENTATION

WORAPHOT TOLIANG: OPTIMIZATION OF D-LACTIC ACID PRODUCTION BY *Terrilactibacillus laevilacticus* SK5-6 AND FERMENTATION PROCESS SCALE UP IN 30 LITRE-FERMENTOR. ADVISOR: ASSOC. PROF. NUTTHA THONGCHUL, Ph.D., CO-ADVISOR: SITANAN THITIPRASERT, Ph.D., 90 pp.

PLA, which is one of biodegradable plastic stereocomplex is made by block polymerization of optically pure L- and D-lactic acid. With stereoblock structure, heat and mechanical properties of PLA products can be improved. Thus, the demand of D-lactic acid has been increased. However, the market supply is still limited. In-house work reported that *Terrilactibacillus laevilacticus* SK5-6, the novel bacterium has been isolated from soil and later identified as the potent D-lactic acid producer. Moreover, it could produce high D-lactic acid from glucose with high optical purity in flask scale. To improve the fermentation performance, further process optimization was conducted in a 5 L stirred fermentor. The effects of inoculum size, seed age and volume of seed on D-lactic acid production were determined for establishing the platform. This work shows that the novel strain could produce high concentration of D-lactic acid with high yield and optical purity as well as the flask scale production. It was observed that 88.0 g/L of D-lactic acid with yield of 0.87 g/g glucose and productivity of 1.83 g/L/h were obtained with the mid log phase of 1% inoculum size and 10% seed transfer. Furthermore, the high optical purity of 99.3% could also be obtained from this isolate in the 5 L fermentor operation with above conditions. To develop the fermentative process scale up platform for D-lactic acid in the 30 L stirred fermentor. The seed train was developed for fermentation by used the conditions that had optimized in 5 L fermentor. In addition, the engineering parameters were tested as scale up factors by assuming constant mass transfer pattern in both scales. The results of testing scale up in 30 L fermentor show that *T. laevilacticus* SK5-6 could produce 81.5 g/L of D-lactic acid and productivity of 2.09 g/L/h, which was higher than 5 L scale slightly. They were obtained with 10% preculture seed transfer (seed in 250 mL flask) and using power input per volume (P/V) for agitation criteria. In addition, the high optical purity of 99.3% could also be obtained in the 30 L fermentor as well as production in 5 L scale. Therefore, the novel D-lactic acid producer, *T. laevilacticus* SK5-6 should be suggested as the potential strain for D-lactic acid production in the industrial scale.

Field of Study: Biotechnology
Academic Year: 2017

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

Firstly, I would like to express my deeply appreciation to Assoc. Prof. Dr. Nuttha Thongchul, my thesis advisor for expert guidance, support, understanding, patience, and kindheartedness during my thesis working. Importantly, I am most grateful for her teaching me, not only the research methodologies but also many other methodologies in my life. Besides, my grateful thanks Dr. Sitanan Thitiprasert, my thesis co-advisor for everything in my master student's life. I would not have achieved this far and this thesis would not have been completed without all the support that I have always received from her. In addition, I would also like to extend my sincere thanks Miss Vassana Tolieng for giving me the opportunity as master study. Moreover, I would like to express my sincerely gratitude to all members of thesis committee; Assoc. Prof. Nattaya Ngamrojanavanich, Assoc. Prof. Dr. .Aphichart Karnchanatat, Assoc. Prof. Dr. Kittinan Komolpis, and Asst. Prof. Dr. Suwattana Pruksasri for suggestions. Unforgettably, I greatly thankful to Mr. Kamonchai Mungkornrit for guidance and support during operating 30 L fermentor. Furthermore, I would also like to extend my sincere thanks to Miss Jirabhorn Piluk, Miss Budsabathip Prasirtsak, Miss Pajareeya Songserm, Miss Tanapawarin Rampai, Mr. Srettapat Limsampancharoen and various members of my laboratory in the Institute of Biotechnology and Genetic Engineering, for help and greatly support. I greatly thankful to the Institute of Biotechnology and Genetic Engineering for all facilities and grant. Finally, I would like to express my deep appreciation to my family and my friends for their greatest support, and encouragement.

CONTENTS

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Page

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF TABLES

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF FIGURES

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER 1

INTRODUCTION

1.1. Background and Rationale

Lactic acid or 2-hydroxypropionic acid is an organic acid that consists of the key functional hydroxyl and carboxyl groups. It is a chiral compound including 2 enantiomers; L-lactic acid and D-lactic acid. Lactic acid has long been utilized in various applications including food and non-food industries such as dairy products, pharmaceuticals, cosmetic and household products. Nowadays, the pollution problem from a big consumption of plastics becomes a crisis because the commodity plastics currently used require long term degradation. To solve this problem, one of the solutions is using biodegradable plastics such as polylactic acid (PLA) that is made from the optically pure L or D lactic acid. The first generation of PLA produced from synthesizing an optically pure L-lactic acid (PLLA). It has limited uses because of the low thermal and mechanical properties compared with the commercially available thermoplastic products made from petroleum feedstocks. Consequently, the tailored made PLA in stereocomplex structure by block polymerizing of L-lactic acid and D-lactic acid to obtain the precise chain structure in order to add up the thermotolerant property. Therefore, the optically pure stereoisomer of lactic acid is required. Most of the commercial lactic acid products are now available in the L-Lactic acid. Those have been extensively used in food and pharmaceutical applications. On the other hand, the

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

production of D-lactic acid is currently limited. Nonetheless, the stereocomplex PLA has driven the market demand of D-lactic acid. For this reason, the production of D-lactic acid is important (Okano et al., 2010)

The optically pure L or D-lactic acid. Lactic acid can be produced by chemical synthesis or microbial fermentation (Calabia and Tokiwa, 2007). Chemical synthesis requires petroleum feedstocks and a harsh operating condition to yield the racemic mixture while microbial fermentation utilizes the low cost biobased feedstocks under a suitable condition and low energy consumption. Lactate obtained from microbial fermentation can be either an optically pure isomer or the racemates depending on the microbial strains. Nevertheless, in PLA synthesis, the optically pure lactate is mandatory. Therefore, fermentation is preferable.

Bacteria, which produce lactic acid ferment glucose via glycolytic pathway toward the central metabolite pyruvate. Pyruvate is then reversibly converted to lactic acid by lactate dehydrogenase (LDH). NADH is required as an enzyme cofactor. Bacteria possess both L-LDH and D-LDH. The expression level of the two enzymes depends on the bacterial strain itself and the environmental factors such as temperature, pH, sugar and oxygen concentrations. For this reason, D-lactic acid can be produced by some bacteria. The typical bacteria used in D-lactic acid production include *Lactobacillus delbrueckii*, *Leuconostoc* sp. and *Sporolactobacillus* sp. as the reflection of stress response. Other than the wild type strains, the engineered microorganisms are now gaining more interest on industrial lactate production host due to their specificity in

Chulalongkorn University

producing the pure optical isomer at a high productivity. The examples include the engineered *Escherichia coli*, *Lactobacillus plantarum*, and *Saccharomyces cerevisiae*.

In 2016, Prasirtsak et al. reported that *Terrilactibacillus laevilacticus*, a novel D-lactic acid producing bacterium, was isolated from soil in Thailand (Prasirtsak et al., 2016). This isolate was preliminarily identified as the potent industrial strain. It is a gram positive, rod shaped, spore-forming and facultatively anaerobic bacteria. It able to be produced D-lactic acid from glucose homofermentatively, and grew at 20–45 °C and pH 5–8.5. In previous in-house work showed that this novel bacterial can be the potential D-lactic acid producer. The high optical purity as well as high production rate was obtained in the first screening fermentation by *T. laevilacticus* SK5-6 in the shaken flask culture. High product yield of 0.84 g/g and productivity of 2.13 g/L/h with high optical purity of 99.64% were acquired under an anaerobic condition at 48 h.

In industrial lactate production, one of the key success factors is to reduce the production cost; therefore, high fermentation performance (high yield and productivity) is mandatory. This can be successfully done by a scrutinized process optimization. Not only the high process performance, the viable production platform is also necessary to make scaling up simply and reproducible (Schmidt, 2005).

In this thesis, the fermentation platform of SK5-6 will be developed for an alternative D-lactate production. The optimization will be conducted in the 5 L stirred fermentor while the seed train platform will be developed for the 30 L stirred fermentor.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Process scale up using the constant mass transport assumption will be applied for predicting the condition in the large-scale operation.

1.2. Research objectives and scope of work

To determine the optimized fermentation protocol for D-lactic acid production by *Terrilactibacillus laevilacticus* SK5-6 in the 5 L stirred fermentor and develop the fermentative process's scale up platform for D-lactic acid in the 30 L stirred fermentor

1.3. Expected outcome

To obtain viable fermentation platform for industrial D-lactic acid production by *Terrilactibacillus laevilacticus* SK5-6

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

CHAPTER 2

THEORITICAL AND LITERATURE REVIEWS

2.1. Lactic acid

Lactic acid or 2-hydroxypropionic acid or 2-hydroxypropanoic acid, with the chemical formula $CH_3CH(OH)COOH$, is an organic acid that is widely used in daily life. The origin of lactic acid's history is being discovered in sour milk via C. W. Scheele, the Swedish chemist in 1780. The structure of lactic acid was identified by the German chemist J. Wislicenus in 1873. After that, the first commercial lactic acid was produced in 1881 via Charles E. Avery in USA.

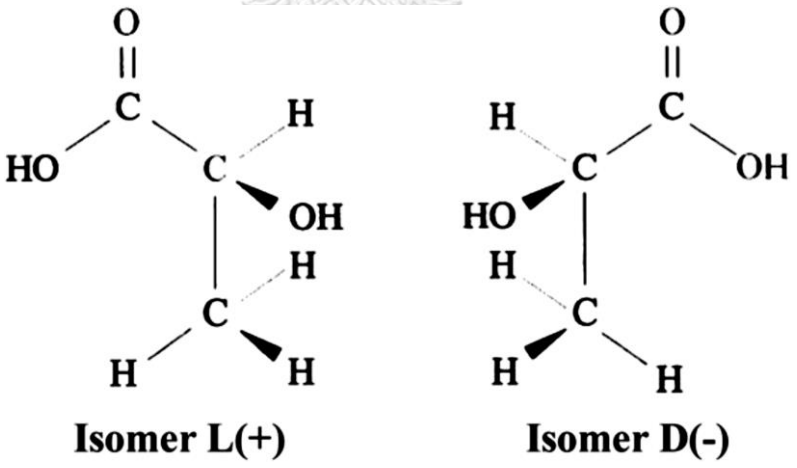


Figure 2.1 The structure of L(+) and D(-) lactic acid (Castillo Martinez et al., 2013)

The lactic acid molecule consists of 2 mainly functional groups, hydroxyl and carboxyl groups. It is a chiral compound that contains naturally in 2 enantiomeric structures: L(+)-lactic acid and D(-)-lactic acid (Figure 2.1). The difference of both

enantiomeric forms is the hydroxyl group that is on C2 atom. The chemical and physical properties of both forms are selfsame such as miscible solubility in water, boiling point. The summarization of lactic acid character is showed in table 2.1.

Table 2.1 Identification and physical and chemical properties

Parameter	**Details**
CAS number	D/L: 50-21-5
	L: 79-33-4
	D: 10326-41-7
EINECS number	200-018-0
H.S. code	2918.11
Formula	$CH_3CH(OH)COOH$
Molecular mass	90.08 g/mol
Specific gravity	1.2 g/mL
Melting point	L: 53 °C
	D: 53 °C
	D/L: 16.8 °C
Boiling point	122 °C (12 mmHg)
Flash point	112 °C
Physical state	Colorless to slinghtly yellow, syrupy liquid
Solubility in water	Miscible
Taste	Mild acid taste
Toxicity	Oral rat LD 50:3543 mg/kg
NFPA ratings	Health 3, Flammability 1, Reactivity 1
Stability	Stable under ordinary conditions

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.2. Application of lactic acid

Lactic acid could be applied in various application. Especially, L-lactic acid is widely used in food, beverages and pharmaceutical industries. L-isomer is popularly applied in many food products to increase acidity or to be the favoring agent such as daily-product, fermented foods, wine, among others. It can help in preservation because the acidity prevents the foods from pathogenic microorganisms. Moreover, L-lactic acid plays important role in pharmaceutical production to be a pH regulator, protecting the oxidative reaction, the natural body composition and metal sequestration agent's supporter. In addition, L-isomer is well-known as alpha hydroxy acid (AHA) in the cosmetic industry. It is also used as a pH regulator, antimicrobial agent and moisturizer by being an ingredient of skin lotion or cream. Above all, it has been shown that L-lactic acid with its wide range of applications but the D-lactic acid production is currently limited because it is toxicity to human cell (Li et al., 2016). However, D-lactic acid gained significantly in importance in the past 10 years because D-isomer is one of key-element to manufacture heat-resistant of polylactic acid (PLA) as bioplastics which can be popularly used, for example as packaging material, coatings, for textiles or in the automotive industry (Klotz et al., 2016).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.3. Polylactic acid (PLA)

Nowadays, a great consumption of plastics from petroleum feed-stock becomes a crisis because the it currently used to require long term degradation. One of the

solutions to solving this problem is using biodegradable plastics. The polylactic acid (PLA) is the most one of biodegradable plastic which can degrade easily. PLA can produce by synthesizing the high pure optically L or D isomers. The first generation of PLA is made from only L-lactic monomer (PLLA). The most problem of PLLA is thermotolerant property that is lower than the commodity plastic, which is made from chemical feedstock. In addition, the PLA that achieved from the racemic mixture of lactic acid results in amorphous materials (Klotz et al., 2016). To solve those problems, the next generation of polymers is the stereocomplex (scPLA) that is established from the enantiomerically pure PLLA, and PDLA by block-copolymerization (Figure 2.2). This structure has a melting temperature higher than PLLA and PDLA because of the sum of intermolecular hydrogen bridge bonds between the methyl group and the carbonyl group of the opposite homochiral polymer chains (Zhang et al., 2005, Tsuji, 2005). For this reason, scPLA has the potential to replace polymers from petroleum-based. Therefore, the optically pure stereoisomer of D-lactic acid is greatly required to PLA production other than L-lactic acid.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

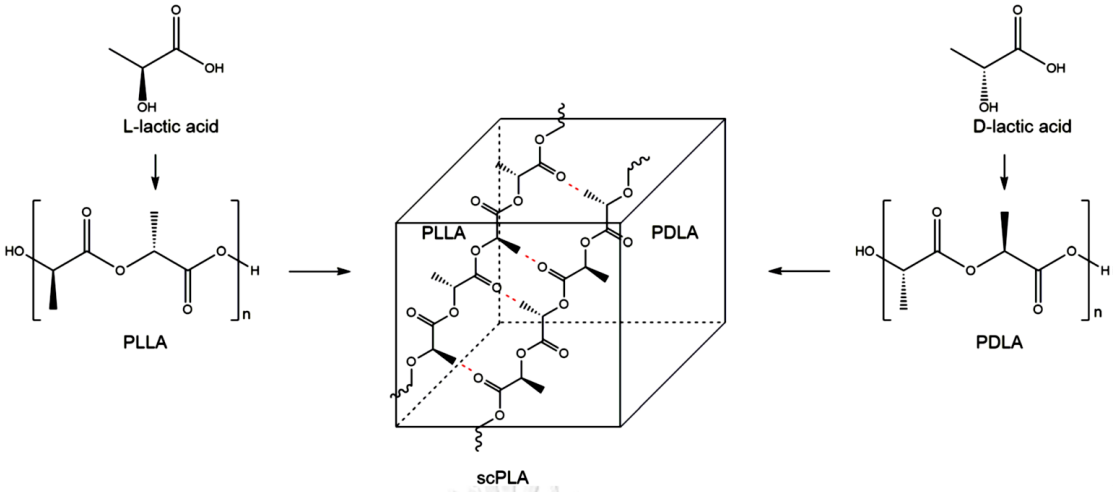


Figure 2.2 scPLA construction by block-copolymerization (Klotz et al., 2016)

2.4. Lactic acid production

The optically pure L or D-lactic acid. Lactic acid can be produced by chemical synthesis or microbial fermentation (Calabia and Tokiwa, 2007). Chemical synthesis requires petroleum feedstocks and a harsh operating condition to yield the racemic mixture while microbial fermentation utilizes the low cost biobased feedstocks under a suitable condition and low energy consumption.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.4.1. Chemical synthesis

For chemical synthesis, lactonitrile is produced firstly by reaction between acetaldehyde and hydrogen cyanide (HCN) under high pressure in liquid phase. Next step is purification. Lactonitrile is hydrolyzed to lactic acid via sulfuric acid (H_2SO_4) or hydrochloric acid (HCl), which has made methyl lactate by esterification with methanol, and this is recovered and purified by distillation (Castillo Martinez et al., 2013). However, lactic acid production by chemical synthesis has a few drawbacks including

that the reaction discharges the high atmospheric pressures, it is not environmentally friendly. In addition, petrochemical feedstocks are required as raw materials for chemical synthesis while price of petroleum oil are currently increasing. Moreover, chemical process obtains the racemic mixture of the L and D-lactic acid, which fails to be used in the food and beverage industries because D-lactic acid is harmful to the human cell.

2.4.2. Microbial fermentation

Microorganisms play important role in lactic acid production by fermentation. Some fungi or bacteria can convert the substrates to produce L or D-lactic acid as well as by-product by fermented process.

2.4.2.1. Fungal fermentation

Fungi is the one of microorganism that can produce lactic acid from agricultural materials by secreting the extracellular enzyme (amylase) for hydrolyzing raw materials such as starch into sugar. The example of most fungi that is widely used for lactic acid production is *Rhizopus* sp. However, the fungal fermentation may give high cost because it must require a lot of oxygen for cell-growth and producing lactic acid. Furthermore, fungal fermentation is heterofermentation that produce more by-products other than lactic acid such as ethanol, carbon dioxide, fumaric acid.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.4.2.2. Bacterial fermentation

Other than fungi, lactic acid bacteria (LAB) can produce lactic acid as well. It is more popularly than fungi because it must not require plenty oxygen as fungi and culture easily in a bioreactor. Moreover, bacterial fermentation is homofermentation that obtained only main product or a few by-product. In addition, LAB is essential that d-lactic acid be produced from low-cost materials with a high yield, high productivity and a high optical purity in a short time (Klotz et al., 2016). For these reasons, the cost of production via LAB is cheaper than fungal process.

2.5. Bacterial fermentation for D-lactic acid production

Bacteria, which produce lactic acid ferment glucose via glycolytic pathway toward the central metabolite pyruvate. Pyruvate is then reversibly converted to lactic acid by lactate dehydrogenase (LDH). NADH is required as an enzyme cofactor. Bacteria possess both L-LDH and D-LDH. The expression level of the two enzymes depends on the bacterial strain itself and the environmental factors such as temperature, pH, sugar and oxygen concentrations.

2.5.1. Wild-type strains

D-lactic acid can be produced by some bacteria. The typical bacteria used in lactic acid production include *Lactobacillus delbrueckii*, *Leuconostoc* sp. and *Sporolactobacillus* sp. as the reflection of stress response. Fukushima et al., (2004)

Chulalongkorn University

reported that *L. delbrueckii* LD0028 can produce D-lactic acid at the higher optical purity from rice saccharificate as carbon and nitrogen sources in 5 L fermentor (Fukushima et al., 2004). The optical purity of 98.4% was obtained. Moreover, Wang et al. (2011) reported *Sporolactobacillus* sp. CASD could produce the high D-lactic acid from peanut meal and glucose that operated with 30 L fed-batch fermentation (Wang et al., 2011). Final D-lactate titer of 207 g/L was obtained with the productivity of 3.8 g/L/h and the optical purity of 99.3%. Furthermore, Tashiro et al. (2011) showed that the novel thermotolerant *L. delbrueckii* subsp. lactis QU41 could produce high D-lactic acid from glucose via the continuous process operated at 50 ^{o}C and pH 6.0 (Tashiro et al., 2011). This strain gave D-lactic acid concentration of 20.7 g/L with the productivity of 18.0 g/L/h and the optical purity higher than 99.9%. In addition, Sawai et al. (2011) reported the optical purity of D-Lactic acid approaching 99.8% enantiomeric excess by *S. laevolacticus* JCM2513 that operated with membrane-integrated fermentation system (MFR) (Sawai et al., 2011). Moreover, Mimitsuka et al. (2012) showed this strain can give 67.3 g/L D-lactic acid and yield of 0.98 g/g with productivity of 11.20 g/L/h, which operated by MFR as well (Mimitsuka et al., 2012). Furthermore, Zhang and Vadlani (2013) showed that *L. delbrueckii* could produce D-lactic acid from the pulp hydrolysate (Zhang and Vadlani, 2013). The final titer of 36.3 g/L of D-lactic acid with 99.8% optical purity was obtained in batch fermentation with the corresponding yield of 0.83 g/g and productivity of 1.01 g/L/h. Moreover, Zhao et al. (2014) reported that they used the fibrous bed bioreactor (FBB) to producing the D-lactic acid by *S. inulinus* Y2-8 that

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

consumed the derived sugars from corn flour (Zhao et al., 2014). The FBB system that operated via repeated-batch mode gived the average yield of 0.98 g/g with the average productivity of 1.62 g/L/h. In addition, Bai et al. (2016) reported the hydrolysate of corncob meal and cotton seed waste clould be utilized by *S. inulinus* YBS1-5 in 7 L fed-batch fermentation (Bai et al., 2016). The high D-Lactic acid of 107.2 g/L was obtained with productivity of 1.19 g/L/h and the optical purity was 99.2%.

2.5.2. Genetically engineered strain

Other than the wild type strains, the engineered microorganisms are now gaining more interest on industrial lactate production host due to their specificity in producing the pure optical isomer at a high productivity. The examples include the engineered *Escherichia coli*, *Lactobacillus plantarum*, and *Saccharomyces cerevisiae*. Liu et al. (2014) reported that the metabolically engineered *E. coli* HBUT-D of which D-lactate dehydrogenase gene was inserted (Wang et al., 2012) could produce the high yield of 0.93 g/g and the productivity of 6.35 g/L/h with the remarkably high optical purity of 99.5% in the pilot scale fermentation (6 tons fermentor) (Liu et al., 2014). Moreover, Hama et al. (2015) reported that the optically pure D-lactic acid was produced from cellulosic feedstocks (hardwood pulp) using the genetically modified *L. plantarum* by the simultaneous saccharification and fermentation (SSF) (Hama et al., 2015). It gived the maximum end-product of 84.6 g/L with the productivity of 2.61 g/L/h within 72 h. The optical purity was 99.2%.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.6. The novel D-lactic acid producing bacterium

In 2016, Prasirtsak et al. reported that *Terrilactibacillus laevilacticus* SK5-6, a novel D-lactic acid producing bacterium, was isolated from soil in Thailand (Prasirtsak et al., 2016). This isolate was preliminarily identified as the potent industrial strain. It is a gram positive, rod shaped, spore-forming and facultatively anaerobic bacteria. It able to be produced D-lactic acid from glucose homofermentatively, and grew at 20–45 oC and pH 5–8.5. In addition, the in-house study showed that this novel bacterial can be the potential D-lactic acid producer. The high optical purity as well as high production rate was obtained in the first screening fermentation by *T. laevilacticus* SK5-6 in the shaken flask culture. High product yield of 0.84 g/g and productivity of 2.13 g/L/h with high optical purity of 99.64% were acquired under an anaerobic condition at 48 h.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 2.2 D-lactic acid production by various strains that are reported in the literature and in this study

Strain	Operation	Substrate	Lactate (g/L)	Yield (g/g)	Productivity (g/L/h)	Optical purity (%ee)	References
Lactobacillus delbrueckii LD0028[a]	Batch	Rice saccharificate	62.6	0.70	0.652	98.4	(Fukushima et al., 2004)
Sporolactobacillus sp. CASD[a]	Fed-batch	Glucose, peanut meal	207	0.93	3.8	99.3	(Wang et al., 2011)
L. delbrueckii subsp. *Lactis* QU41[a]	Continuous	Glucose, Peptone	20.7	1.03	18.0	99.9	(Tashiro et al., 2011)
S. laevolacticus JCM2513[a]	Continuous (MFR)	Raw cane sugar, YE[d]	67.3	0.98	11.20	99.8	(Sawai et al., 2011), (Mimitsuka et al.,
L. delbrueckii ATCC9649[a]	Batch	Pulp hydrolysate	36.3	0.83	1.01	99.8	(Zhang and Vadlani, 2013)
S. inulinus Y2-8[a]	Fed-batch	Corn flour hydrolysate	218.8	-	1.65	>99.9	(Zhao et al., 2014)
S. inulinus YBS1-5[a]	Fed-batch	Corncob, cotton seed	107.2	0.85	1.19	99.2	(Bai et al., 2016)
Escherichia coli HBUT-D[b]	Batch	Glucose, YE[d]	127	0.93	6.35	99.5	(Liu et al., 2014)
L. plantarum NCIMB8826[b]	SSF[e]	Hardwood pulp	102.3	0.879	2.29	99.2	(Hama et al., 2015)
Terrilactibacillus laevilacticus SK5-6[c]	Shaken flask	Glucose, YE[d]	102.2	0.84	2.13	99.6	Previously in-house study

[a] Wild-type strains, [b] Genetically engineered strain, [c] This study, [d] Yeast extract, [e] Simultaneous saccharification and fermentation

CHAPTER 3

EXPERIMENTAL

3.1. Apparatus and Chemicals

3.1.1. Apparatus

Apparatus	Model	Manufacturer	Country
Autoclave	KT-40L	ALP Co., Ltd.	Japan
Centrifuge	MC-15A	Tomy Seiko Co., Ltd.	Japan
Electronic balance	ML204/01	Mettler Toledo AG	Switzerland
Electronic balance	ML3002E/01	Mettler Toledo AG	Switzerland
High Performance Liquid Chromatograph	Shimadzu LC-10A	Shimadzu Co., Ltd.	Japan
Laminar flow hood clean bench	NK system	International Scientific Supply	Thailand
Oven	UL-80	Memmert Co., Ltd.	Germany
pH meter	AB15	Fisher Scientific, Ltd.	Singapore
Rotary incubator shaker	G25	New Brunswick Scientific Co., Inc.	USA
Vortex mixer	K-550-GE	Scientific Industries, Inc.	USA
5 L stirred fermentor	MDL 300	B.E. Marubishi	Japan
30 L stirred fermentor	MSJ-J2	B.E. Marubishi	Japan
Spectrophotometer	UV-1280	Shimadzu	Japan

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.1.2. Chemicals

Chemicals	Manufacturer	Country
Agar	Patanasin Enterprise	Thailand
Ammonium chloride (NH_4Cl)	Merck	Germany
Ammonium sulfate ($(NH_4)_2SO_4$)	Merck	Germany
Calcium carbonate ($CaCO_3$)	Sigma	Germany
Copper sulfate ($CuSO_4 \cdot 5H_2O$)	Fluka	France
Ethanol (C_2H_5OH)	Merck	Germany
Glucose ($C_6H_{12}O_6$)	Siam Chai Chemical	Thailand
Hydrochloric acid (HCl)	Merck	Germany
Iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	Merck	Germany
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Riedel-de Haen	Germany
Manganese sulfate pentahydrate ($MnSO_4 \cdot 5H_2O$)	Merck	Germany
Peptone	Fluka	France
Potassium dihydrogen phosphate (KH_2PO_4)	Merck	Germany
Potassium phosphate dibasic (K_2HPO_4)	Riedel-de Haen	Germany
Sodium chloride ($NaCl$)	Sigma	Germany
Sodium hydroxide ($NaOH$)	Merck	Germany
Yeast extract	BioSpringer	France

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.2. Microorganism

The novel D-lactic acid producing bacterium, *Terrilactibacillus laevilacticus* SK5-6, screened from soil in Thailand, was used in this work. It was cultured onto glucose Yeast Extract Peptone (GYP) agar slant under an aerobic condition at 37 °C for 1 day. The stock culture was transferred onto the freshly new GYP agar slant every week for maintaining the activity.

3.3. Medium composition

3.3.1. Salts solution (per liter)

$MgSO_4 \cdot 7H_2O$	40	g
$MnSO_4 \cdot 5H_2O$	2	g
$FeSO_4 \cdot 7H_2O$	2	g
NaCl	2	g

Autoclaved at 121 °C, 15 psig for 15 min.

3.3.2. Liquid GY medium (per liter)

Glucose	10	g
Yeast extract	15	g
NH_4Cl	4	g
KH_2PO_4	0.5	g
K_2HPO_4	0.5	g
$CaCO_3$	5	g
Salts solution	20	mL

Autoclaved at 121 °C, 15 psig for 15 min.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.3.3. GYP agar medium (per liter)

Glucose	10	g
Yeast extract	5	g
Peptone	5	g
KH_2PO_4	0.5	g
K_2HPO_4	0.5	g
Agar	20	g
$CaCO_3$	5	g
Salts solution	20	mL

Autoclaved at 121 °C, 15 psig for 15 min.

3.3.4. Fermentation medium (per liter)

Glucose	120	g
Yeast extract	7.5	g
NH_4Cl	2	g
KH_2PO_4	0.25	g
K_2HPO_4	0.25	g
Salts solution	10	mL

Autoclaved at 121 °C, 15 psig for 15 min.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.4. Methodology

3.4.1. Optimizing process conditions in the 5 L stirred fermentor

3.4.1.1. Determining the growth profile in the preculture flask

T. laevilacticus SK5-6 was cultivated under the aerobic condition on the GYP agar slant at 37 °C for 24 h. The cell suspension was prepared by pipetting 1 mL sterile DI water into the agar slant before thoroughly mixing. The optical density (OD) of the the cell suspension was measured at the wavelength of 600 nm. The suspension was diluted with sterile DI water to the approximated OD of 30-40. The diluted suspension was transferred into the preculture medium contained in the 250 mL Erlenmeyer flask at 1%, 3%, and 5% inoculum size, respectively. The starting volume of the preculture flask was 50 mL. The preculture flask was incubated at 37 °C, 200 rpm under an aerobic condition. The sample was collected every hour for measuring OD and pH of the fermentation broth and analyzing the remaining glucose and metabolites. The fermentation kinetic profiles were plotted, and the growth cycle was defined. The proper inoculum size was selected from the growth profiles accordingly.

3.4.1.2. Determining the proper inoculum preculture for inoculating the fermentation stage in the 5 L stirred fermentor

The 5 L stirred fermentor containing the fermentation medium was autoclaved at 121 °C, 15 psig for 30 min. Before autoclave, the pH probe was calibrated with the technical buffers (pH 4.0 and 7.0). After autoclave, the fermentor was allowed to cool

down. The fermentor was controlled at 37 °C and 300 rpm before attempting dissolved oxygen (DO) probe calibration using sterile nitrogen and air. The inoculum prepared in 3.4.1.1 was transferred into the fermentor to bring the initial volume to 3.5 L. The age and the size of the inoculum in the preculture stage were varied (mid/late log phases and 3%, 5%, and 10%). The pH was controlled by $CaCO_3$ at the equivalent amount to the complete conversion of initial glucose added. Sample was collected every 3 h for OD reading, measuring pH, and analyzing for the remaining glucose and metabolites. The optical purity of lactate was also determined.

3.4.2. Calculating the operating conditions in the 30 L stirred fermentor

The agitation speed in the 30 L stirred fermentor was predicted from the optimized rate obtained from the experimental runs in the 5 L stirred fermentor by assuming constant mass transfer pattern in both fermentation scales. The 2 engineering parameters, e.g., power input per unit volume (P/V) and impeller tip speed (N_iD_i) were selected as the tested scale up factors (Table 3.1).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.4.2.1. Calculating the agitate speed to maintain constant P/V

Calculating Reynolds number (Re_i) to define the flow in a stirred tank (Figure 3.1)

$$Re_i = \frac{\rho N D_i^3}{\eta}$$

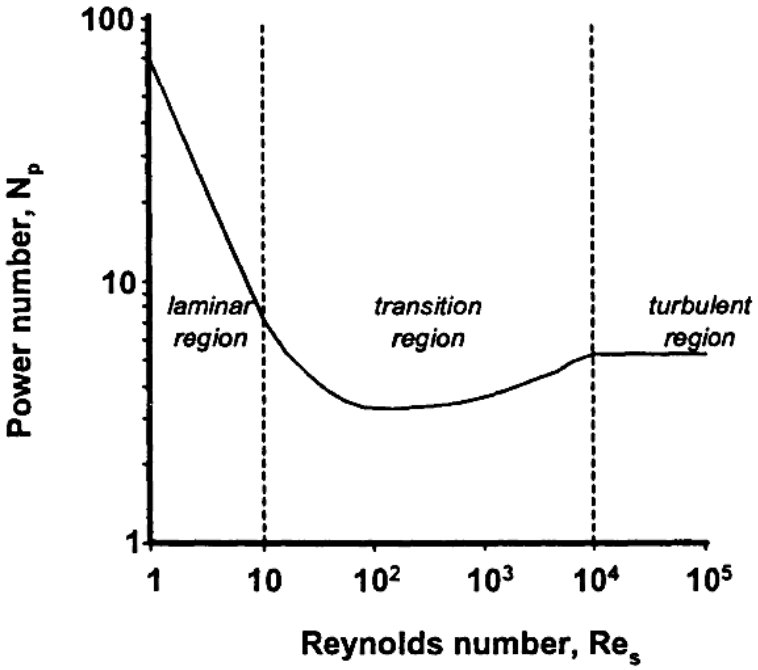


Figure 3.1 Schematically the variation of the power number with Reynolds number for a bioreactor equipped with Rushton turbine impeller and baffles (Nielsen et al., 2003)

At fully turbulent flow in tank ($Re_i \geq 10^4$)

$$P = N_P \rho N^3 D_i^5$$

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Thus

$$P/V = N_P \rho N^3 D_i^2$$

Scaling up with constant P/V;

$$N_1^3 D_{i_1}^2 = N_2^3 D_{i_2}^2$$

Where Re_i is Renolds number, ρ is density (kg/m^3), η is dynamic viscosity $(kg/m \cdot s)$, N_P is dimensionless power number, P/V is power input unit volume (W/m^3), N is agitated speed (rpm) and D_i is impeller diameter (m).

3.4.2.2. Calculating the agitate speed to maintain constant N_iD_i

$$u_i = \pi N D_i$$

Scaling up with constant N_iD_i;

$$N_1 D_{i_1} = N_2 D_{i_2}$$

Where u_i is impeller tip speed (m/s), N is agitated speed (rpm) and D_i is impeller diameter (m)

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 3.1 The parameters required to scale-up process and operating agitation speeds at different scale-up criteria to maintain the similarlity of mixing between 5 L and 30 L fermentor

5 L stirred fermentor		**30 L stirred fermentor**	
Tank inner diameter (mm)	147	Tank inner diameter (mm)	284
Impeller diameter (mm)	76	Impeller diameter (mm)	113
Number of impeller	1	Number of impeller	1
Type of impeller	4 blades Rushton turbine	Type of impeller	4 blades Rushton turbine
Speed range (rpm)	20-1,200	Speed range (rpm)	100-1,000
Motor (kW)	0.4	Motor (kW)	0.75
Agitate speed to maintain constant engineering scale-up parameter			
Constant P/V (rpm)	300	Constant P/V (rpm)	230
Constant N_iD_i (rpm)	300	Constant N_iD_i (rpm)	202

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

3.4.3. Developing the seed train for 30 L stirred fermentor

Following the protocols in 3.4.1 and 3.4.2, the preculture seed was developed in the 250 mL Erlenmeyer flask. This preculture seed (seed 1) was transferred into the 2 L Erlenmeyer flask containing preculture medium at varied inoculum sizes (3%, 5%, and 10%). After inoculation, the starting volume of the preculture flask (seed 2) was 500 mL. It was incubated at 37 °C, 200 rpm under the aerobic condition. Sample was taken every

1 h for OD reading, measuring pH, and analyzing for the remaining glucose and metabolites. The fermentation kinetic profiles were plotted, and the growth cycle was defined. The proper inoculum size was selected from the growth profiles accordingly.

3.4.4. Testing D-lactic acid fermentation in the 30 L stirred fermentor

The 30 L stirred fermentor containing the fermentation medium was sterilized in place by pressurized steam at 121 °C, 1.5 bar for 15 min. Before sterilization, the pH probe was calibrated with the technical buffers (pH 4.0 and 7.0). After sterilization, the fermentor was cool down by running process air and cooling water through the fermentor jacket. Later, the operating conditions were set up and the DO probe was calibrated. The temperature was controlled at 37 °C while the agitation was varied (Table 3.1). The fermentor was inoculated by the preculture broth in Seed 2 at 10% inoculum size. The initial volume of the fermentation was at 20 L. The pH during the fermentation was controlled by $CaCO_3$ at the equivalent amount of complete glucose conversion to lactate. Sample was taken every 3 h for OD reading, measuring pH, and analyzing for the remaining glucose and metabolites. The optical purity of lactate was also determined.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.5. Sample analyses

Fermentation broth sample (0.1 mL or 0.5 mL) collected during the experiment was acidified with 1 M HCl (0.9 mL or 0.5 mL). The acidified broth was centrifuged at 10000 g for 5 min. The supernatant and cell pellets were separated.

Product yield was determined from the ratio of the product formed to glucose consumed. Volumetric productivity was defined as the total amount of product formed per unit volume per time. The optical purity of D-lactate was defined from the peak areas of the chromatogram as follows according to the equation below (Zhao et al., 2014).

$$\text{Optical purity (\%ee)} = \frac{\text{D-lactic acid-L-lactic acid}}{\text{D-lactic acid+L-lactic acid}} \times 100$$

3.5.1. Measuring the OD and determining cell biomass concentration

The cell pellets were suspended in 1 mL DI water. The suspension was mixed thoroughly before spectrophotometrically measuring the OD at the wavelength of 600 nm. The conversion to cell biomass concentration was performed by the following basis. The reading of OD600 of 1 is equivalent to the cell concentration of 0.12 g/L.

3.5.2. Rapidly detecting glucose concentration by glucose-lactate analyzer

Remaining glucose in the samples that collected from fermentation was measured by glucose-lactate analyzer (YSI 2700). The supernatant of samples was diluted with DI water before analyzing by YSI 2700.

3.5.3. Determining the remaining glucose and metabolites

High Performance Liquid Chromatography was used to analyze the remaining glucose and metabolites in the fermentation broth. The acidified cell-free broth (1 mL) was diluted with DDI water then filtered through the hydrophilic PTFE membrane filter. The diluted sample (15 µL) was automatically injected into an Aminex HPX-87H ion exclusion organic acid column (Biorad) incubated at 45 °C. Sulfuric acid at the concentration of 0.005 M was used as a mobile phase at a flowrate of 0.6 mL/min. Glucose, lactate, and acetate were detected by a refractive index detector. The standards containing 0-2 g/L of each component were injected as references to determine sample concentration. The chromatogram peak area was used as the comparison basis in determining the concentration.

3.5.4. Determining the optical purity of lactate product

To determine the optical purity of lactic acid, 5 µL diluted cell-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-lactate were injected as references to determine the optical purity of the product.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.6. Statistical analysis

A factorial design was applied to this work. The three-way ANOVA was used to analyze the effect of inoculum size, seed age and volume of preculture seed in 5 L stirred fermentor. The two-way ANOVA was also used to analyze the effect of scale up criteria and volume of seed 1 (250 ml flask) for transferring into seed 2 (2 L flask) in 30 L stirred fermentor. Significance of differences was determined at confident intervals of 95% ($p \leq 0.05$). All statistical analyses were performed using the SPSS software, version 22 (IBM Inc., USA, 2013)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Optimizing process conditions in the 5 L stirred fermentor

4.1.1. Determining growth profiles of *Terrilactibacillus laevilacticus* SK5-6

The growth profiles of *T. laevilacticus* SK5-6 were observed in the preculture flask 50 mL preculture medium in the 250 mL flask cultivated under an aerobic condition. The preculture flask was inoculated with the bacterial suspension of OD_{600} of approximately 30-40 at varied inoculum sizes (1%, 3%, and 5%). Figure 4.1 shows the growth profiles of *T. laevilacticus* SK5-6. It was found that the lag phase was shorten when the preculture medium was inoculated with the bacterial suspension at the higher inoculum size. The shortest lag phase was obtained in the preculture seed inoculated with 5% cell suspension when compared with those inoculated with the suspension at 3% and 1% respectively (Figure 4.1). Similar finding was reported by Wardani et al. (2017) from the investigation on the growth kinetics of *Lactobacillus plantarum*. They claimed that increasing the inoculum size could shorten the lag phase.

During the log phase, it seemed that the growth rates of *T. laevilacticus* SK5-6 inoculated with the 3 inoculum sizes were similar. The maximum optical density (OD_{600}) achieved at each inoculum sizes were rather the same (8.0-8.2). In this work, it was found that the growth entered the mid log phase at 5 h and the late log phase at 7 h when the

preculture was inoculated with the suspension at 1% inoculum size, whilst increasing the inoculum size to 3% led to short the time. It reached the mid log phase at 4 h and the late lag phase at 6 h. Likewise, the growth of SK5-6 that was inoculated with 5% inoculum size entered the mid log phase at 3 h and late log phase at 5 h (Table 4.1).

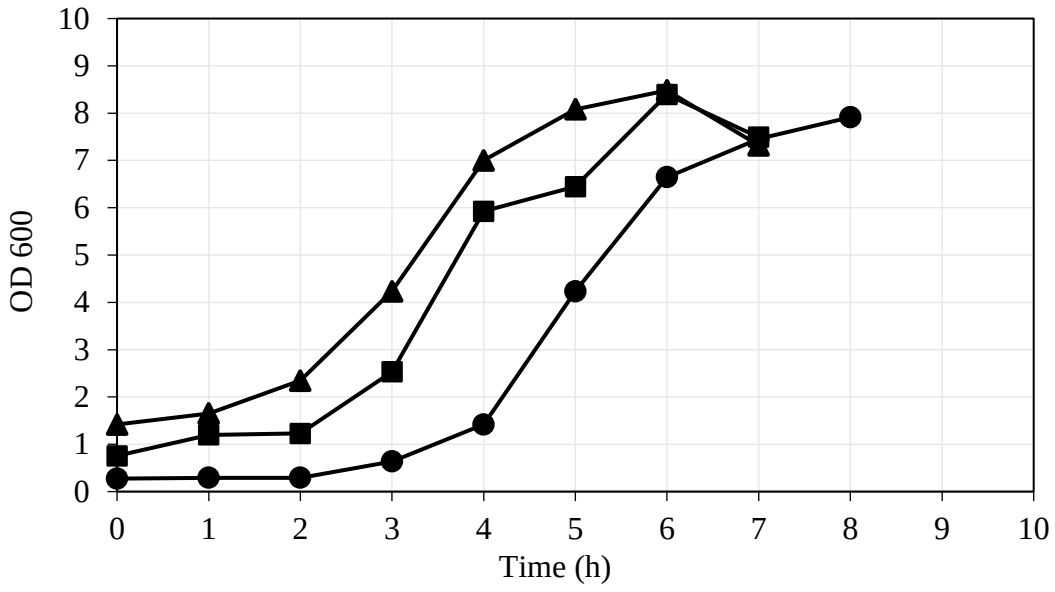


Figure 4.1 Growth profile of *T. laevilacticus* SK5-6 in the preculture medium inoculated with the bacterial suspension at varied inoculum sizes. Symbols: circle, 1% inoculum size; rectangle, 3% inoculum size; triangle, 5% inoculum size.

CHULALONGKORN UNIVERSITY

Table 4.1 Growth profile of *T. laevilacticus* SK5-6 in the preculture flask inoculated with the bacterial suspension at varied inoculum sizes.

Inoculum size (%)	**Mid log phase (h)**	**Late log phase (h)**
1	5	7
3	4	6
5	3	4

4.1.2. Defining the correct preculture seed for D-lactic acid production in the 5 L stirred fermentor

D-lactic acid production was observed in the 5 L stirred fermentor. The fermentor was inoculated with the preculture seed previously prepared in the preculture flask at varied inoculum sizes (3%, 5%, and 10%) and ages (mid log, late log).

Figures 4.2, 4.3, and 4.4 show the fermentation kinetics of *T. laevilacticus* SK5-6 during the fermentation stage. From the fermentation profiles at all conditions studied, cell growth continued with a subsequent increase in lactic acid concentration. From the results of HPLC chromatogram, all conditions showed that *T. laevilacticus* SK5-6 produce D-lactic acid without the formation of byproduct (Appendix C). The findings above (the increases in growth along with lactic acid formation) confirmed that lactic acid production followed growth associated product formation model (Berry et al., 1999). In addition, from the evidence of no byproduct formation, lactic acid production by *T. laevilacticus* SK5-6 under the operating conditions applied in this study was considered homofermentative (Wang et al., 2015).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 4.2 represents the fermentation performance of *T. laevilacticus* SK5-6 during the fermentation stage. Increasing the inoculum size transferred into the preculture flask for seed preparation in the 250 mL flask did not affect the performance of this isolate to grow and produce lactic acid because the growth rate at log phase of varied conditions were similar (Prasirtsak et al., 2017). Although the inoculum size showed the significant difference with the confident intervals of 95%, the analysis pointed out that the production performance was slightly impacted by the inoculum size (Appendix D). Thus, similar maximum OD600 and final lactic acid concentration were obtained at the end of the fermentations that were operated at the same conditions in both preculture and fermentation stages. To minimize the production cost during the initial stage of lactic acid production, we selected 1% inoculum size in transferring the bacterial suspension into the preculture stage for further study.

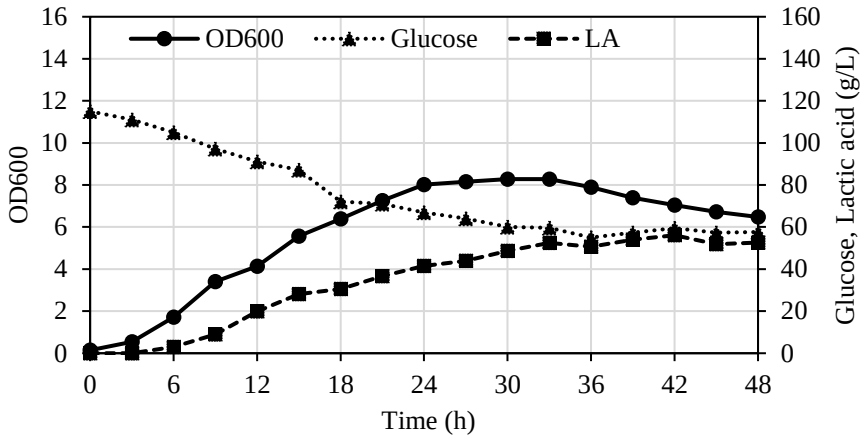
Kadam et al. (2006) reported the differential inoculum age of *Lactobacillus delbrueckii* NCIM 2365 could affect to lactic production significantly and also gave long lag phase. On the other hand, *T. laevilacticus* SK5-6 showed the performance better than them. The preculture seed at the different age to inoculate the fermentor with the same inoculum size could obtain similar lactic acid. Although the effect inoculum age was significant difference at confident intervals of 95% in final product and productivity, no significant difference in yield of D-lactic acid (Appendix D). Either mid log age or late log age gave the similar yield, and productivity of lactic acid at the end of

fermentation (Table 4.2). Besides, no lag phase was observed in the fermentation stage. For shorten the production process, mid log age transfer was selected for further study.

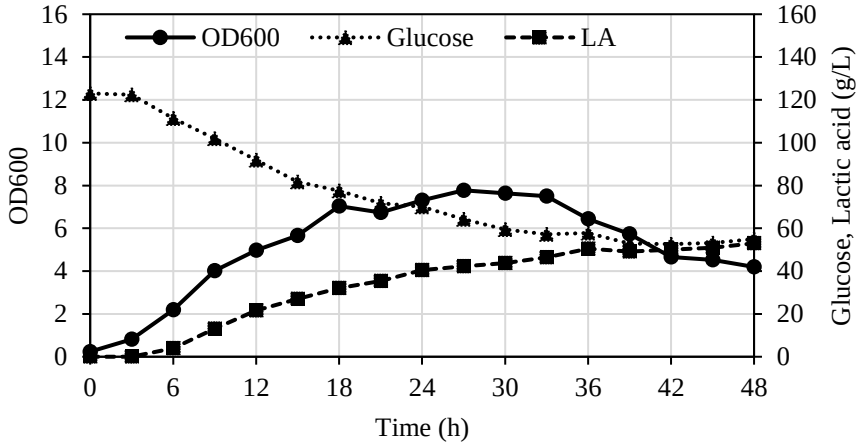
Unlike the inoculum size of the bacterial suspension and the age of the preculture seed, the percentage of preculture seed transferred into the fermentation stage strongly affected lactic acid production (Nagarjun, 2015). The effect of preculture seed transferred was difference significantly at confident intervals of 95% which was consistent to the fermentation kinetic data (Appendix D). The higher cell biomass and lactic acid concentration were obtained with the increasing inoculum size from 3% to 5% and 10%, respectively (Table 4.2). It was found that the maximum lactic acid production in the fermentation stage was obtained by transferring the mid log age preculture seed at 10% inoculum size into the fermentation stage. This condition was applied in further fermentation study in the pilot scale fermentation.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

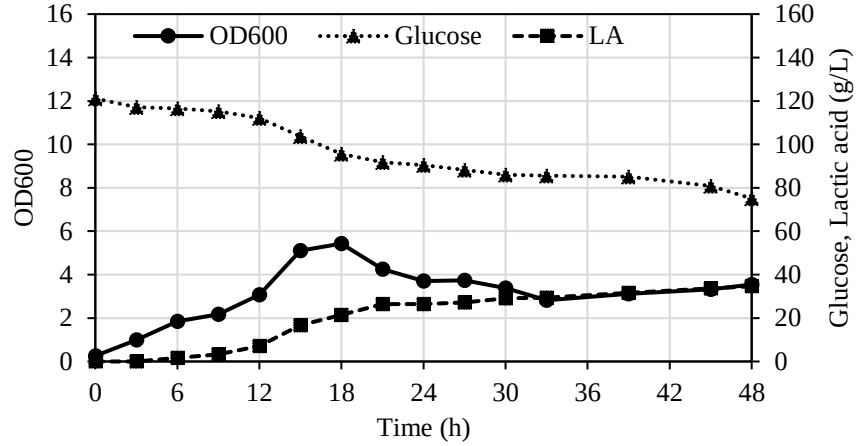
a


OD600
Glucose
LA
OD600
Glucose, Lactic acid (g/L)
Time (h)
0
2
4
6
8
10
12
14
16
0
20
40
60
80
100
120
140
160
0
6
12
18
24
30
36
42
48

b


OD600
Glucose
LA
OD600
Glucose, Lactic acid (g/L)
Time (h)
0
2
4
6
8
10
12
14
16
0
20
40
60
80
100
120
140
160
0
6
12
18
24
30
36
42
48

c


OD600
Glucose
LA
OD600
Glucose, Lactic acid (g/L)
Time (h)
0
2
4
6
8
10
12
14
16
0
20
40
60
80
100
120
140
160
0
6
12
18
24
30
36
42
48

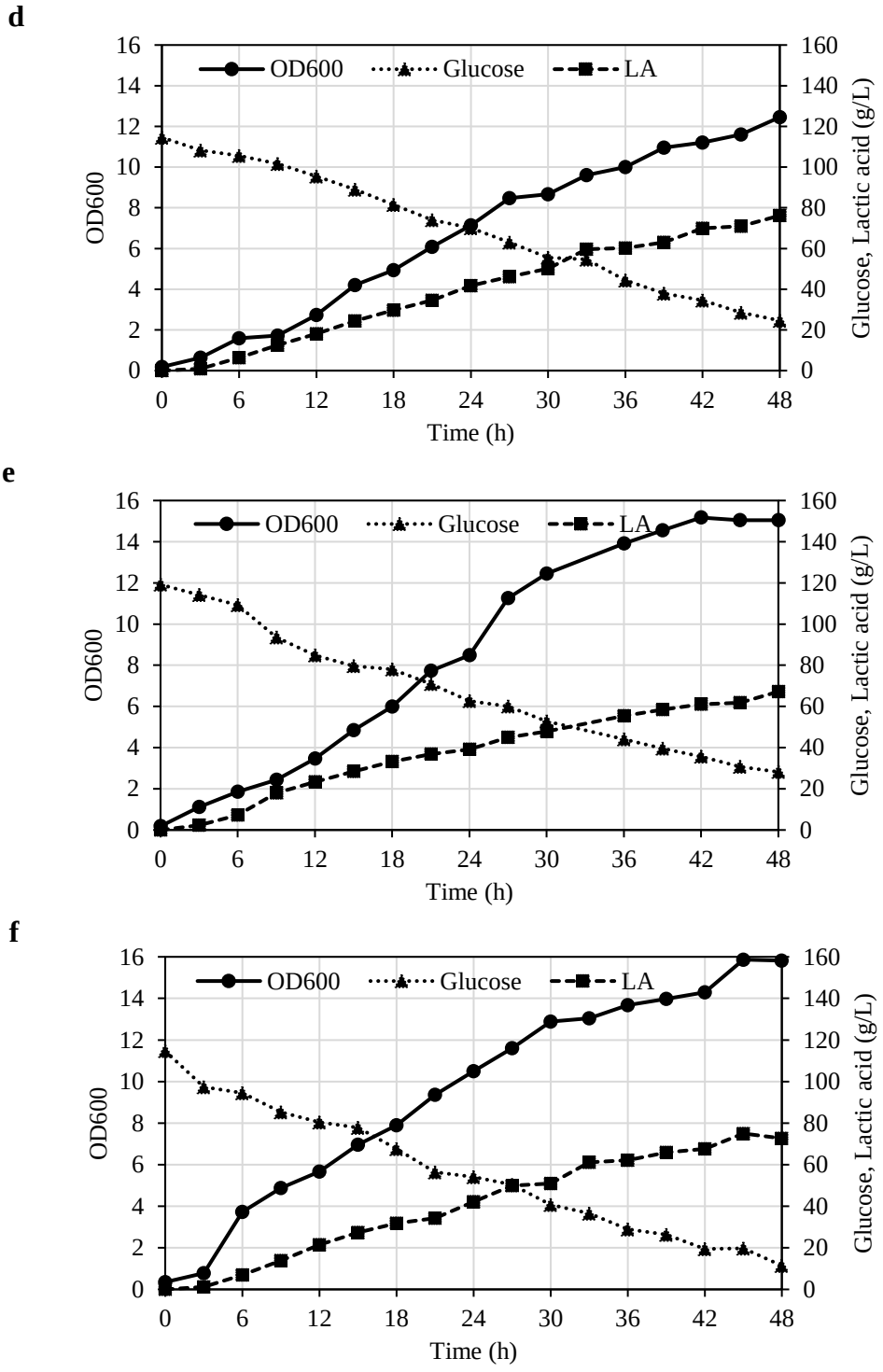
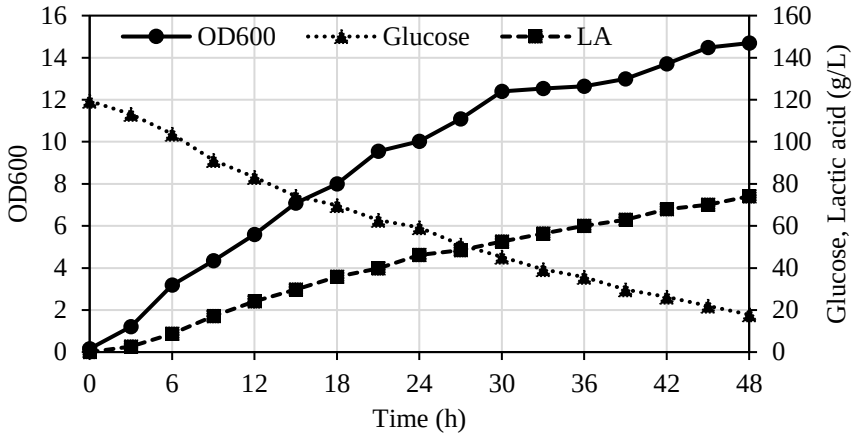
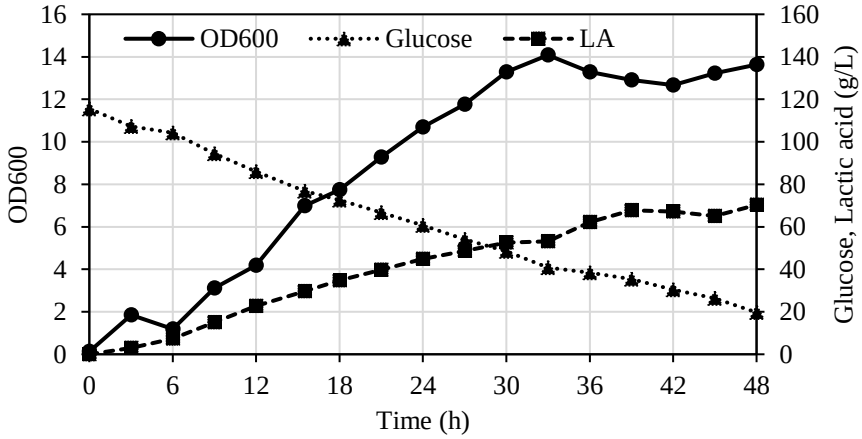


Figure 4.2 Fermentation kinetics profiles of *T. laevilacticus* SK5-6 during the fermentation stage. The 5 L stirred fermentor was inoculated by the preculture seed at 3% inoculum size with varied ages (mid log and late log phases). The preculture seed was prepared by inoculating the bacterial suspension at varied inoculum sizes (1%, 3%, and 5%). **a** mid log, 1% inoculum size; **b** mid log, 3% inoculum size; **c** mid log, 5% inoculum size; **d** late log, 1% inoculum size; **e** late log, 3% inoculum size; **f** late log, 5% inoculum size

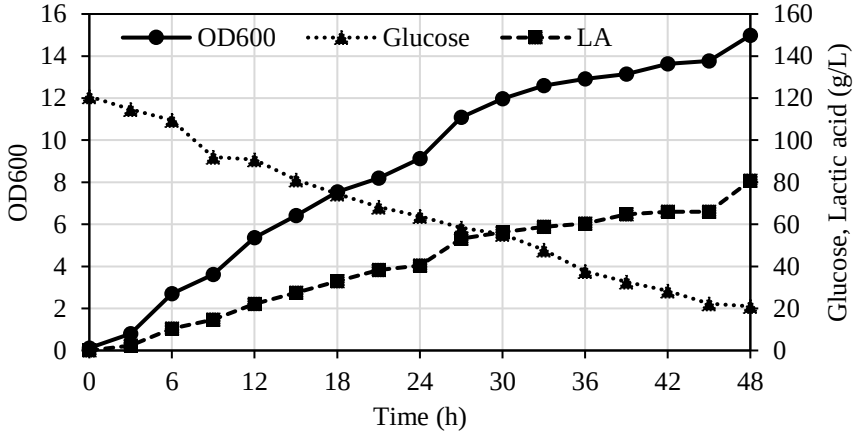
a


OD600
Glucose
LA
OD600
Glucose, Lactic acid (g/L)
Time (h)
0
2
4
6
8
10
12
14
16
0
20
40
60
80
100
120
140
160
0
6
12
18
24
30
36
42
48

b


OD600
Glucose
LA
OD600
Glucose, Lactic acid (g/L)
Time (h)
0
2
4
6
8
10
12
14
16
0
20
40
60
80
100
120
140
160
0
6
12
18
24
30
36
42
48

c


OD600
Glucose
LA
OD600
Glucose, Lactic acid (g/L)
Time (h)
0
2
4
6
8
10
12
14
16
0
20
40
60
80
100
120
140
160
0
6
12
18
24
30
36
42
48

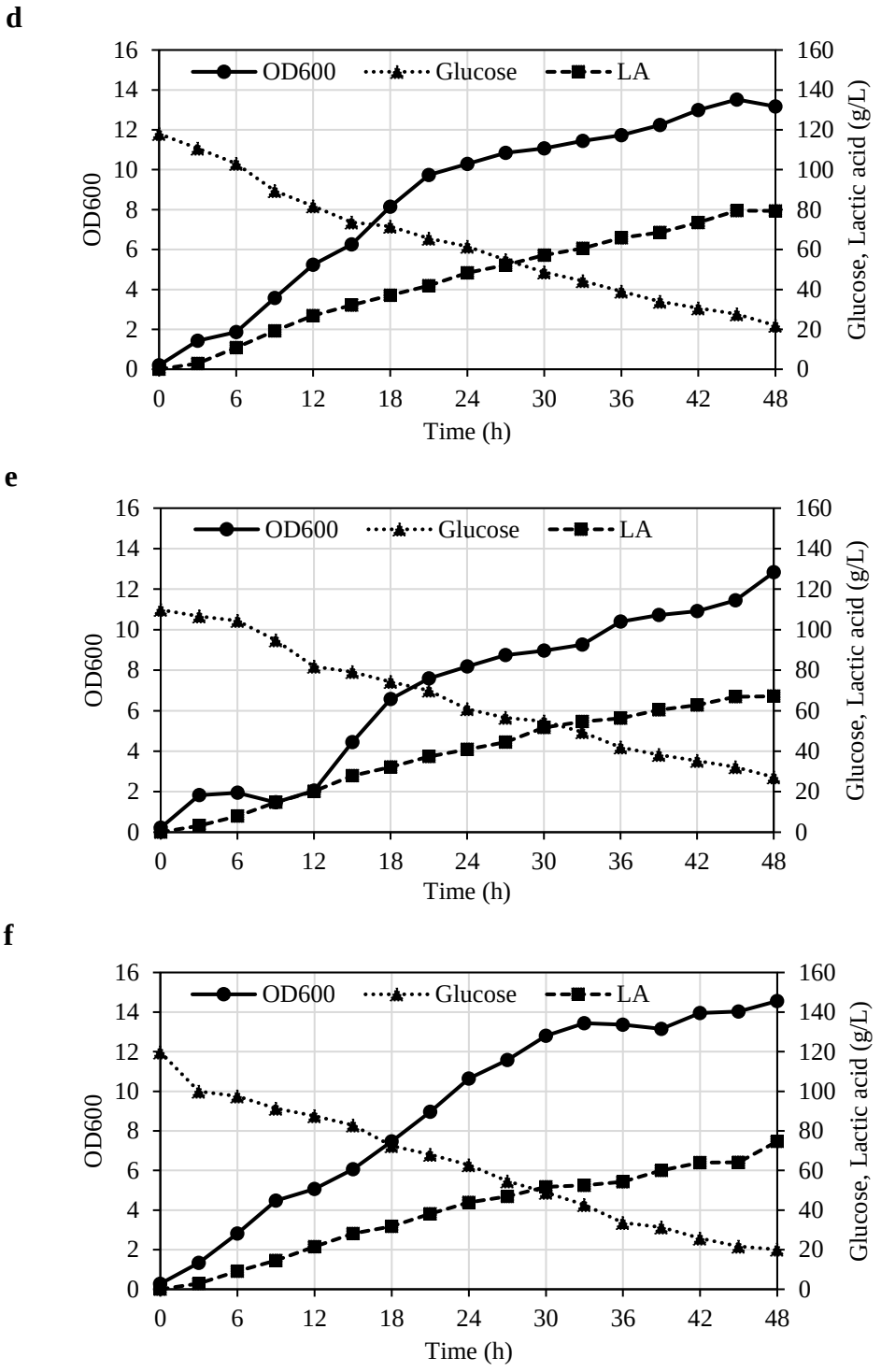
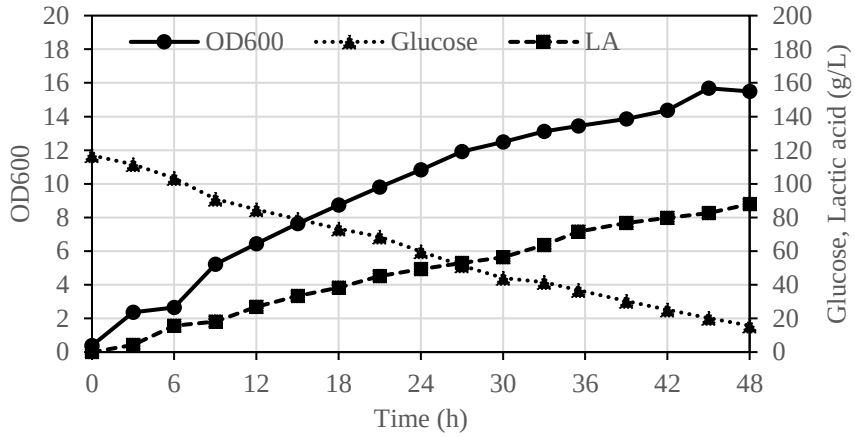
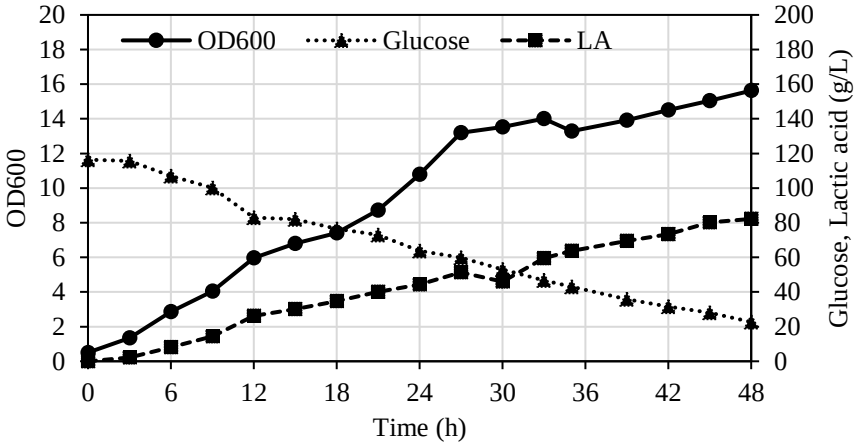


Figure 4.3 Fermentation kinetics profiles of *T. laevilacticus* SK5-6 during the fermentation stage. The 5 L stirred fermentor was inoculated by the preculture seed at 5% inoculum size with varied ages (mid log and late log phases). The preculture seed was prepared by inoculating the bacterial suspension at varied inoculum sizes (1%, 3%, and 5%). **a** mid log, 1% inoculum size; **b** mid log, 3% inoculum size; **c** mid log, 5% inoculum size; **d** late log, 1% inoculum size; **e** late log, 3% inoculum size; **f** late log, 5% inoculum size

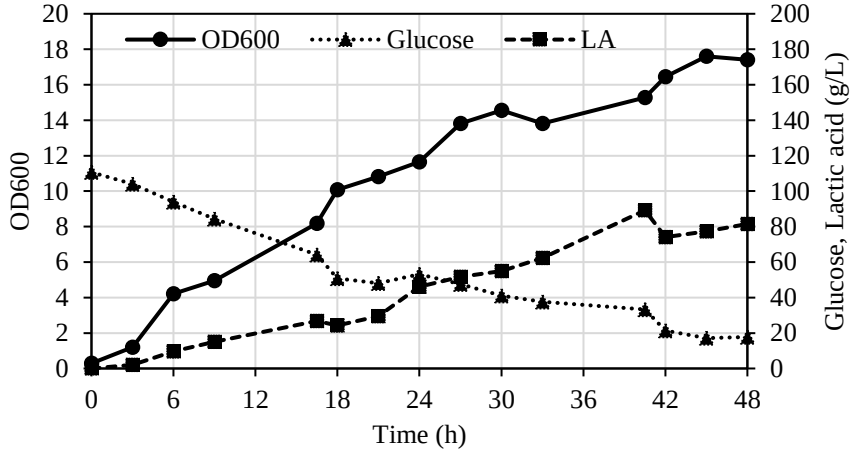
a


OD600
Glucose
LA
OD600
Glucose, Lactic acid (g/L)
Time (h)

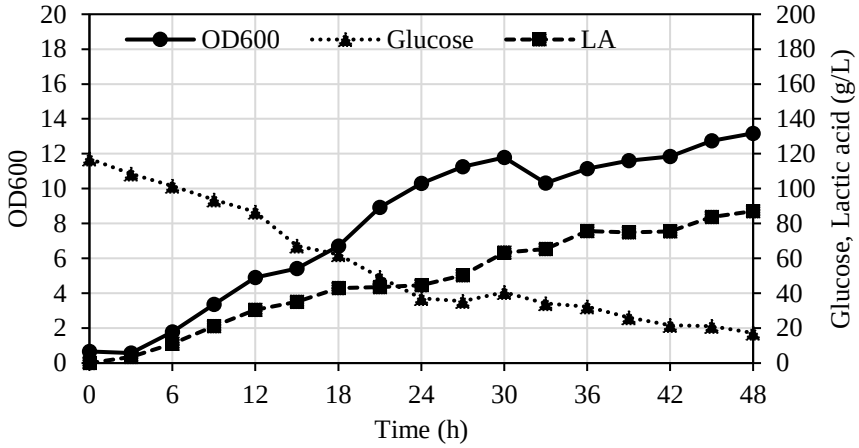
b


OD600
Glucose
LA
OD600
Glucose, Lactic acid (g/L)
Time (h)

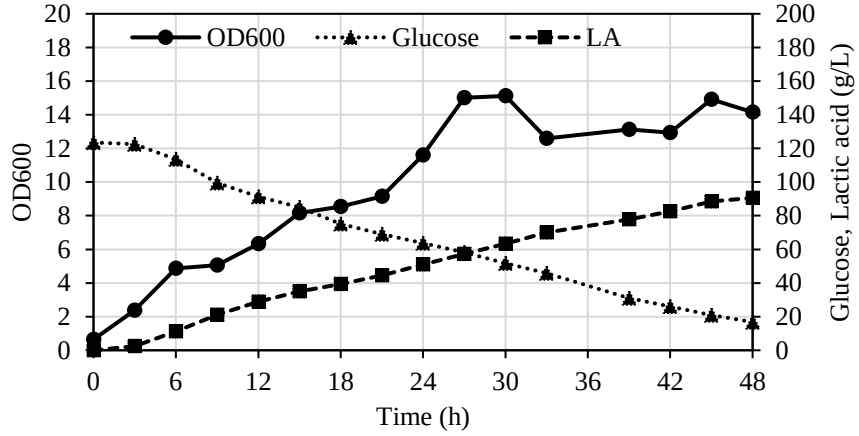
c


OD600
Glucose
LA
OD600
Glucose, Lactic acid (g/L)
Time (h)

d



e



f

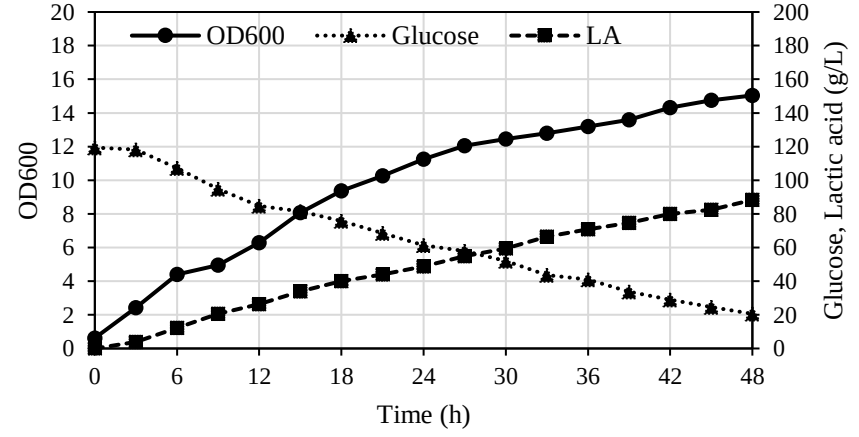


Figure 4.4 Fermentation kinetics profiles of *T. laevilacticus* SK5-6 during the fermentation stage. The 5 L stirred fermentor was inoculated by the preculture seed at 10% inoculum size with varied ages (mid log and late log phases). The preculture seed was prepared by inoculating the bacterial suspension at varied inoculum sizes (1%, 3%, and 5%). **a** mid log, 1% inoculum size; **b** mid log, 3% inoculum size; **c** mid log, 5% inoculum size; **d** late log, 1% inoculum size; **e** late log, 3% inoculum size; **f** late log, 5% inoculum size

Table 4.2 Fermentation kinetics of *T. laevilacticus* SK5-6 during the fermentation stage in 5 L stirred fermentor[a]

Inoculum size (%)	1	3	5	1	3	5	1	3	5	1	3	5	1	3	5	1	3	5
Age of seed	Mid log phase			Late log phase			Mid log phase			Late log phase			Mid log phase			Late log phase		
Volume of seed (%)	3						5						10					
Maximum OD600	8.26	7.77	5.43	12.45	15.17	15.87	14.72	14.08	14.99	13.52	12.83	14.58	15.68	15.63	17.41	13.17	15.12	15.04
Residual glucose (g/L)	57.7	54.9	75.1	24.6	28.2	19.7	17.8	19.7	21.0	21.9	27.2	20.1	15.7	18.5	17.8	17.3	16.8	20.6
Lactic acid (g/L)	52.8	53.0	34.6	76.2	67.2	72.7	77.9	70.3	80.7	79.4	67.1	74.7	88.0	82.2	81.4	87.1	90.6	88.3
Yield (g/g glucose)	0.92	0.78	0.75	0.85	0.74	0.70	0.73	0.73	0.81	0.78	0.81	0.75	0.87	0.88	0.88	0.87	0.85	0.89
Productivity (g/L/h)	1.10	1.10	0.72	1.59	1.40	1.51	1.54	1.46	1.68	1.65	1.40	1.56	1.83	1.71	1.70	1.81	1.89	1.84
Optical purity (%ee)	100	100	100	99.1	100	99.1	99.7	99.7	99.2	100	100	98.7	99.3	99.4	99.2	98.7	99.6	99.6

[a] End time at 48 h

4.2. Fermentation process scale up in the 30 L stirred fermentor

4.2.1. Determining seed train for lactic acid production in the 30 L stirred fermentor

Seed train was developed for the pilot scale lactic acid production by *T. laevilacticus* SK5-6 in the 30 L stirred fermentor. From the results obtained in 4.1, it was indicated that the correct seed physiology and concentration led to the good production process performance. For the 30 L stirred fermentor, another preculture seed after Seed 1 in the 250 mL flask was required for inoculating the production fermentor. Therefore, in this part, the growth profile of *T. laevilacticus* SK5-6 was determined in the 2 L preculture flask.

Figure 4.5 represents the growth profiles of *T. laevilacticus* SK5-6 in Seed 2. It was found that the larger inoculum size transferred from Seed 1 gave the shorter lag phase in Seed 2. Among the inoculum size studied, inoculating Seed 2 at 10% gave the most rapid growth during the log phase although the maximum OD_{600} achieved were similar. In addition, compared with the growth profiles in Seed 1 (Figure 4.1), the shorter lag phase was observed in Seed 2 in all inoculum sizes studied. This can be explained by the active mid log age preculture transferred into Seed 2 compared with the bacterial suspension from the agar slant transferred into Seed 1 that required adaptation from solid culture to liquid culture (Cheng et al., 2007). Table 4.3 defines the time reached mid log age of Seed 2. This evidence confirmed that a larger inoculum size was required for shorten the production time during seed development.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

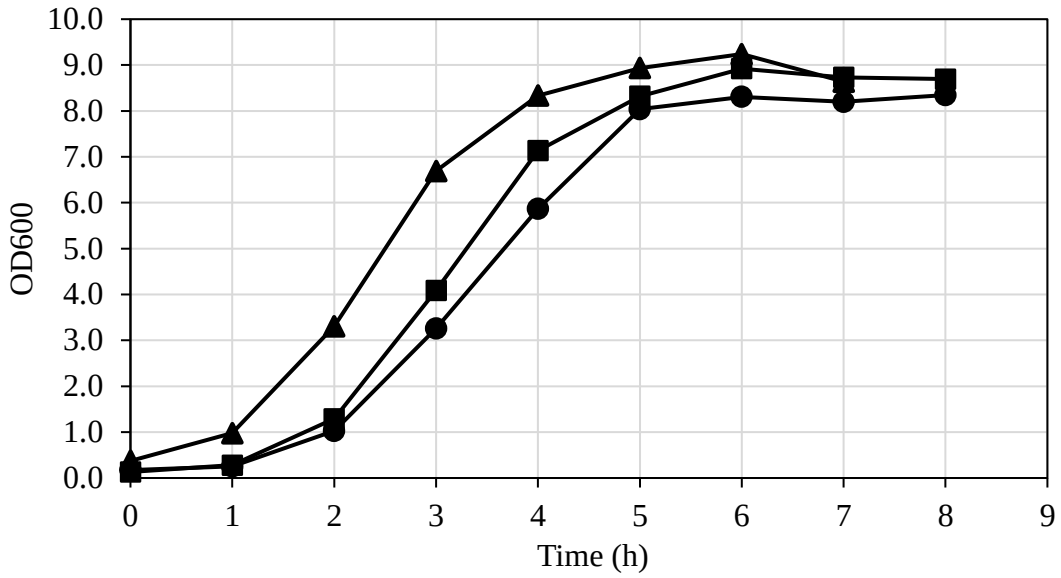


Figure 4.5 Growth profile of T. laevilacticus SK5-6 in Seed 2 inoculated with the preculture broth obtained from Seed 1 (250 mL flask) at varied inoculum sizes. Symbols: circle, 3% inoculum size; rectangle, 5% inoculum size; triangle, 10% inoculum size.

Table 4.3 Growth profile of *T. laevilacticus* SK5-6 in Seed 2 inoculated with the preculture seed from Seed 1 at varied inoculum sizes.

Preculture seed transferred from Seed 1 (%)	**Time reached mid log phase (h)**
3	3.5
5	3
10	2.5

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4.2.2. D-lactic acid fermentation in the 30 L stirred fermentor

Lactic acid production in the 30 L stirred fermentor was conducted after determining the growth profiles of preculture seed and developing the seed train. The seed train started from inoculating the 250 mL preculture flask (Seed 1) with the bacterial suspension at 1% inoculum size with the approximated OD_{600} of 30-40. Seed 1 at mid log age was used to transfer into the 2 L flask (Seed 2) at varied inoculum sizes. At mid log age, Seed 2 was then transferred into the production fermentor at 10% inoculum size. The fermentation was conducted using the operating conditions previously determined using 2 constant engineering parameters, i.e., Power input per volume (P/V) and Impeller tip speed (N_iD_i)

Figures 4.6 and 4.7 show the fermentation kinetics of *T. laevilacticus* SK5-6 during fermentation stage. The fermentation profiles of all condition show the cell growth was continued with a posterior increase in lactic acid concentration during the production stage. In addition, the chromatograms from HPLC showed this strain produce D-lactic acid without byproduct (Appendix C). Therefore, this confirmed that lactic acid fermentation by *T. laevilacticus* SK5-6 followed growth associated product formation model, which was homofermentative fermentation (Berry et al., 1999, Wang et al., 2015, Klotz et al., 2016). Consequently, the metabolic behavior of *T. laevilacticus* SK5-6 in 5 L and 30 L stirred fermentor were not different during fermentation stage.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The results in table 4.4 demonstrates the performance of fermentation by *T. laevilacticus* SK5-6 in 30 L stirred fermentor during the production stage. It was found that increasing the volume of preculture seed 1 (250 ml flask) for developing the preculture seed 2 in the 2 L flask obviously affect to the performance of D-lactic acid production by this strain in 30 L stirred fermentor that operated by using the power input per volume (P/V). The seed 1 transferred with 10% of inoculum size could gave the productivity higher than 5% and 3% respectivly at scale up by power input per volume (P/V). On the other hand, it could a few affect the performance when using the impeller tip speed (N_iD_i). Furthermore, although the statistical analysis by ANOVA explained that two factors were significant difference at confident level of 95%, the estimated marginal means confirmed that the scale up criteria affected kinetic values stronger than the proper seed effect (Appendix D). The graphs of main effect analysis referred that scale up by using power input per volume(P/V) was better than impeller tip speed (N_iD_i). Thus, it could be concluded that the power input per volume (P/V) exhibited the stronger influence on the fermentative performance by *T. laevilacticus* SK5-6 than operating with impeller tip speed (N_iD_i). Prasirtsak et al. (2017) reported that the novel D-lactic acid producer, *T. laevilacticus* SK5-6 possessed catalase activity. As a result, this isolate could better utilize oxygen to grow than other typical D-lactic acid bacteria. Moreover, power input per volume (P/V) often successfully applied as scale up parameter to biological production (Schmidt, 2005, Marques et al., 2010). Accordingly, power input

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

per volume (P/V) affects the mass rate transport as the stable size of flocks and gas bubbles even though the impeller tip speed (N_iD_i) determines the shear stress, which can reduce possible cell damage but the rate oxygen transport also decreases (Shukla et al., 2001, Garcia-Ochoa and Gomez, 2009, Kanjanachumpol et al., 2013).

Therefore, for the fermentation process scale up by *T. laevilacticus* SK5-6, the maximum lactic acid production in the fermentation stage was obtained when transferring the seed 1 at 10% inoculum size into seed 2 for preculture seed development before entering the fermentation stage. Furthermore, the appropriate scale up parameter was power input per volume (P/V) to agitation criteria in 30 L stirred fermentor.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

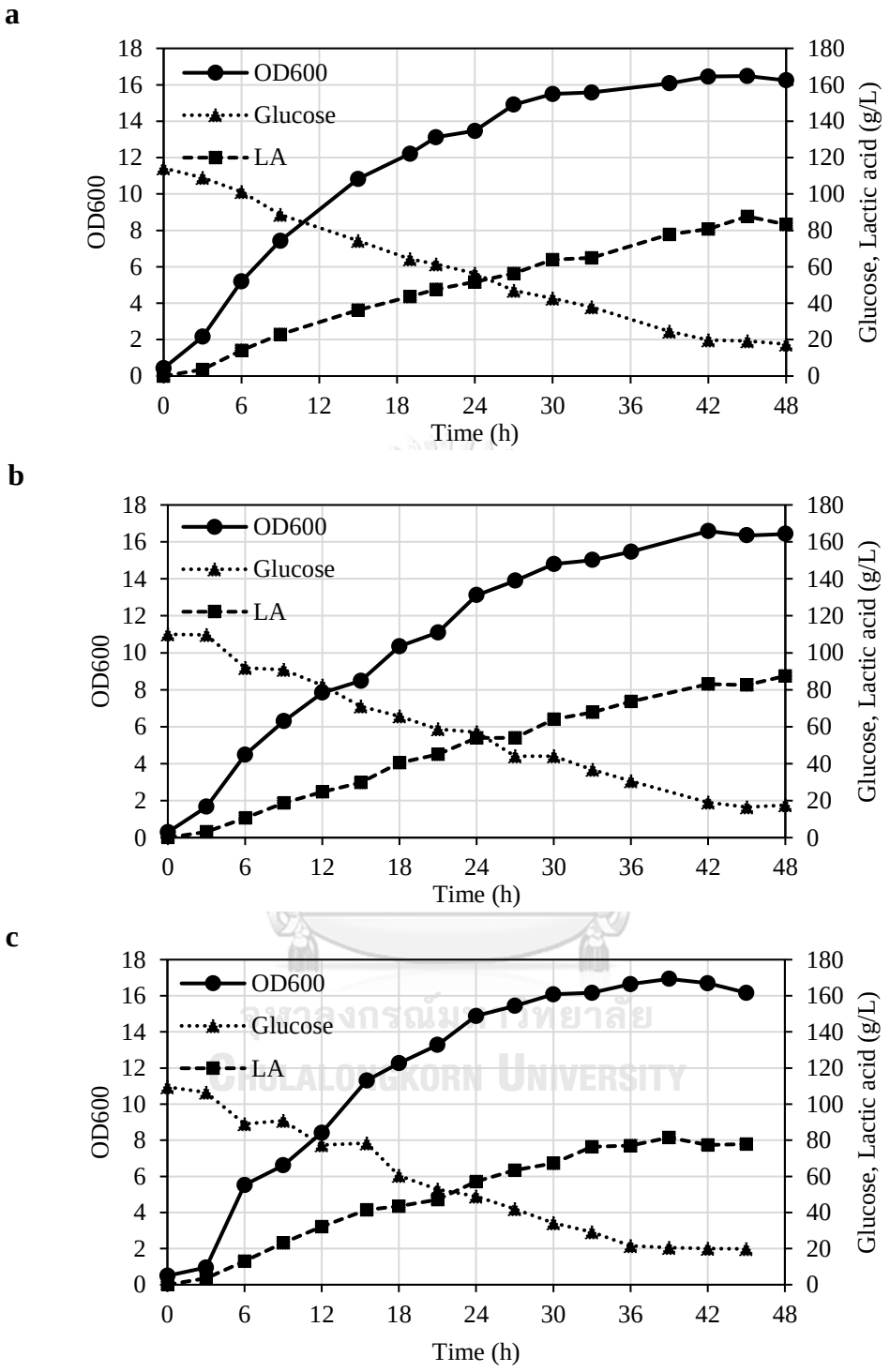


Figure 4.6 Fermentation kinetic profiles of *T. laevilacticus* SK5-6 during the testing fermentation. The 30 L stirred fermentor was inoculated by seed 2 (2 L flask) at 10% inoculum size with mid log phase. Seed 2 was prepared by inoculating with the preculture broth that obtained from Seed 1 (250 mL flask) at varied inoculum sizes (3%, 5%, and 10%). Power input per volume (P/V) was assigned to agitation criteria in 30 L scale up process. **a** 3% inoculum size; **b** 5% inoculum size; **c** 10% inoculum size

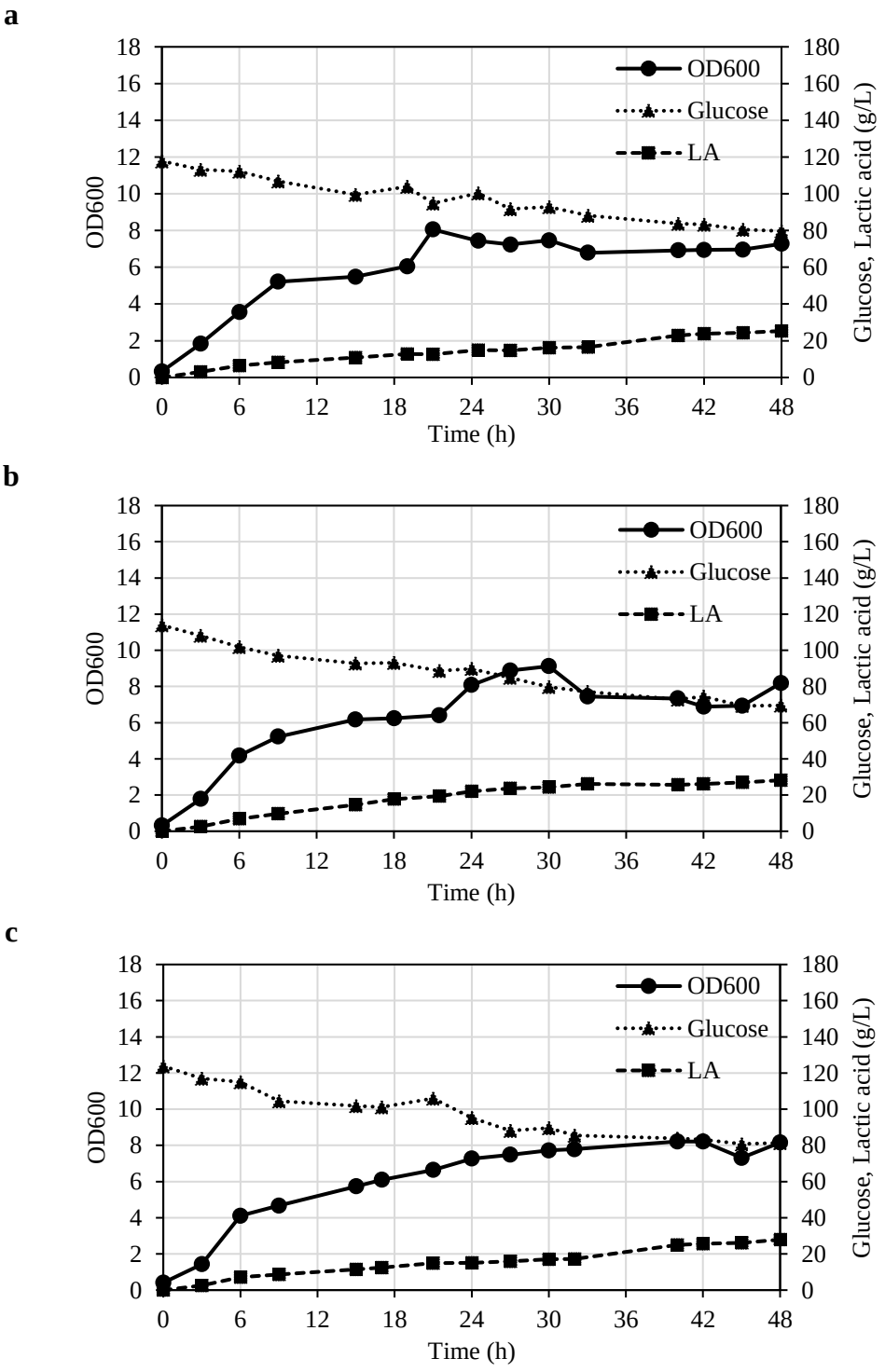


Figure 4.7 Fermentation kinetic profiles of *T. laevilacticus* SK5-6 during the testing fermentation. The 30 L stirred fermentor was inoculated by seed 2 (2 L flask) at 10% inoculum size with mid log phase. Seed 2 was prepared by inoculating with the preculture broth that obtained from Seed 1 (250 mL flask) at varied inoculum sizes (3%, 5%, and 10%). Impeller tip speed (N_iD_i) was assigned to agitation criteria in 30 L scale up process. **a** 3% inoculum size; **b** 5% inoculum size; **c** 10% inoculum size

Table 4.4 Fermentation kinetics of *T. laevilacticus* SK5-6 during the fermentation stage in 30 L stirred fermentor

Conditions		End time (h)	Maximum OD600	Residual glucose (g/L)	Lactic acid (g/L)	Yield (g/g glucose)	Productivity (g/L/h)	Optical purity (%ee)
Volume of seed 1 (%)	Scale-up criteria							
3	P/V	45	16.48	19.3	87.7	0.92	1.95	99.2
5	P/V	42	16.59	19.1	87.3	0.91	1.98	99.4
10	P/V	39	16.93	20.3	81.5	0.92	2.09	99.3
3	N_iD_i	48	8.05	79.7	25.3	0.66	0.53	100
5	N_iD_i	48	9.12	69.6	28.2	0.64	0.59	100
10	N_iD_i	48	8.21	81.4	27.9	0.66	0.58	100

Chulalongkorn University

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1. Summary

The novel bacterium, *Terrilactibacillus laevilacticus* SK5-6 was proven as the potent D-lactic acid producer. From previous in-house work, in the flask scale production results, *T. laevilacticus* SK5-6 could produce D-lactic acid at the high product yield and high productivity from 120 g/L glucose. Moreover, high optical purity of 99.6% could also be obtained from this isolate in the primary flask culture. To improve the production performance of this strain, further process optimization was conducted in a 5 L stirred fermentor. The effects of inoculum size, age and volume of seed on D-lactic acid production were determined for establishing the fermentation platform. All factors mentioned affected growth of *T. laevilacticus* SK5-6 and D-lactic acid production in the 5 L fermentor. This thesis work shows that the novel strain could produce high concentration of D-lactic acid with high yield and optical purity as well as the flask scale production. It was observed that 88.0 g/L of D-lactic acid and productivity of 1.83 g/L/h were obtained with the mid log phase of 1% inoculum size and 10% seed transfer. Furthermore, the high optical purity of 99.3% could also be obtained from this isolate in the 5 L fermentor operation with above conditions.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

To develop the fermentative process, scale up platform for D-lactic acid in the 30 L stirred fermentor, the viable production platform is necessary to make scaling up simply and reproducible. The preculture seed train was developed for fermentation by use the conditions that had optimized in 5 L stirred fermentor. In addition, the 2 engineering parameters including power input per unit volume (P/V) and impeller tip speed (N_iD_i) were selected as the tested scale up factors by assuming constant mass transfer pattern in both fermentation scales. The results of testing scale up in 30 L stirred fermentor show that *T. laevilacticus* SK5-6 could produce 81.5 g/L of D-lactic acid and productivity of 2.09 g/L/h, which was higher than 5 L scale slightly. They were obtained with 10% preculture seed transfer (seed in 250 mL flask) and using power input per volume (P/V) for agitation criteria. In addition, the high optical purity of 99.3% could also be obtained in the 30 L stirred fermentor as well as production in 5 L scale. Therefore, the novel D-lactic acid producer, *T. laevilacticus* SK5-6 should be suggested as the potential strain for D-lactic acid production in the industrial scale.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

5.2. Recommendations

The D-lactic acid fermentation platform by *T. laevilacticus* SK5-6 was established in this thesis work. However, this platform produces D-lactic acid from only glucose. Thus, this platform will be applied to using another substrate such as molasses or agricultural materials apart from being the base case for process scale up. Therefore, this fermentation platform is interesting for high pure D-lactic acid production in

industrial scale from low cost substrate or agricultural materials by *T. laevilacticus* SK5-6 in the future.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

REFERENCES

BAI, Z., GAO, Z., SUN, J., WU, B. & HE, B. 2016. D-Lactic acid production by *Sporolactobacillus inulinus* YBS1-5 with simultaneous utilization of cottonseed meal and corncob residue. *Bioresour Technol,* 207**,** 346-52.

BERRY, A. R., FRANCO, C. M. M., ZHANG, W. & MIDDELBERG, A. P. J. 1999. Growth and lactic acid production in batch culture of *Lactobacillus rhamnosus* in a defined medium. *Biotechnology Letters,* 21**,** 163-167.

CALABIA, B. P. & TOKIWA, Y. 2007. Production of D-lactic acid from sugarcane molasses, sugarcane juice and sugar beet juice by *Lactobacillus delbrueckii*. *Biotechnol Lett,* 29**,** 1329-32.

CASTILLO MARTINEZ, F. A., BALCIUNAS, E. M., SALGADO, J. M., DOMíNGUEZ GONZáLEZ, J. M., CONVERTI, A. & OLIVEIRA, R. P. D. S. 2013. Lactic acid properties, applications and production: A review. *Trends in Food Science & Technology,* 30**,** 70-83.

CHENG, K.-K., ZHANG, J.-A., LIU, D.-H., SUN, Y., LIU, H.-J., YANG, M.-D. & XU, J.-M. 2007. Pilot-scale production of 1,3-propanediol using *Klebsiella pneumoniae*. *Process Biochemistry,* 42**,** 740-744.

FUKUSHIMA, K., SOGO, K., MIURA, S. & KIMURA, Y. 2004. Production of D-lactic acid by bacterial fermentation of rice starch. *Macromol Biosci,* 4**,** 1021-7.

GARCIA-OCHOA, F. & GOMEZ, E. 2009. Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. *Biotechnol Adv,* 27**,** 153-76.

HAMA, S., MIZUNO, S., KIHARA, M., TANAKA, T., OGINO, C., NODA, H. & KONDO, A. 2015. Production of d-lactic acid from hardwood pulp by mechanical milling followed by simultaneous saccharification and fermentation using metabolically engineered *Lactobacillus plantarum*. *Bioresour Technol,* 187**,** 167-172.

KADAM, S. R., PATIL, S. S., BASTAWDE, K. B., KHIRE, J. M. & GOKHALE, D. V. 2006. Strain improvement of *Lactobacillus delbrueckii* NCIM 2365 for lactic acid production. *Process Biochemistry,* 41**,** 120-126.

KANJANACHUMPOL, P., KULPREECHA, S., TOLIENG, V. & THONGCHUL, N. 2013. Enhancing polyhydroxybutyrate production from high cell density fed-batch fermentation of *Bacillus megaterium* BA-019. *Bioprocess Biosyst Eng,* 36**,** 1463-74.

KLOTZ, S., KAUFMANN, N., KUENZ, A. & PRUSSE, U. 2016. Biotechnological production of enantiomerically pure d-lactic acid. *Appl Microbiol Biotechnol,* 100**,** 9423-9437.

LI, Q., HUDARI, M. S. & WU, J. C. 2016. Production of Optically Pure D-Lactic Acid by the Combined use of *Weissella* sp. S26 and *Bacillus* sp. ADS3. *Appl Biochem Biotechnol,* 178**,** 285-93.

LIU, Y., GAO, W., ZHAO, X., WANG, J., GARZA, E., MANOW, R. & ZHOU, S. 2014. Pilot scale demonstration of D-lactic acid fermentation facilitated by $Ca(OH)_2$ using a metabolically engineered *Escherichia coli*. *Bioresour Technol,* 169**,** 559-565.

MARQUES, M. P. C., CABRAL, J. M. S. & FERNANDES, P. 2010. Bioprocess scale-up: quest for the parameters to be used as criterion to move from microreactors to lab-scale. *Journal of Chemical Technology & Biotechnology,* 85**,** 1184-1198.

MIMITSUKA, T., NA, K., MORITA, K., SAWAI, H., MINEGISHI, S., HENMI, M., YAMADA, K., SHIMIZU, S. & YONEHARA, T. 2012. A membrane-integrated fermentation reactor system: its effects in reducing the amount of sub-raw materials for D-lactic acid continuous fermentation by *Sporolactobacillus laevolacticus*. *Biosci Biotechnol Biochem,* 76**,** 67-72.

NAGARJUN, P. A. 2015. Parametric optimization of lactic acid production and its scale up using free and immobilized cells of *Lactobacillus amylovorus* NRRL B-4542. *International Journal of Pure & Applied Bioscience,* 3**,** 159-168.

NIELSEN, J., VILLADSEN, J. & LIDEN, G. 2003. *Bioreaction Engineering Principles,* New York, Springer Science+Business Media, LLC.

OKANO, K., TANAKA, T., OGINO, C., FUKUDA, H. & KONDO, A. 2010. Biotechnological production of enantiomeric pure lactic acid from renewable resources: recent achievements, perspectives, and limits. *Appl Microbiol Biotechnol,* 85**,** 413-23.

PRASIRTSAK, B., THITIPRASERT, S., TOLIENG, V., ASSABUMRUNGRAT, S., TANASUPAWAT, S. & THONGCHUL, N. 2017. Characterization of D-lactic acid, spore-forming bacteria and *Terrilactibacillus laevilacticus* SK5-6 as potential industrial strains. *Annals of Microbiology,* 67**,** 763-778.

PRASIRTSAK, B., THONGCHUL, N., TOLIENG, V. & TANASUPAWAT, S. 2016. *Terrilactibacillus laevilacticus* gen. nov., sp. nov., isolated from soil. *Int J Syst Evol Microbiol,* 66**,** 1311-1316.

SAWAI, H., NA, K., SASAKI, N., MIMITSUKA, T., MINEGISHI, S., HENMI, M., YAMADA, K., SHIMIZU, S. & YONEHARA, T. 2011. Membrane-integrated fermentation system for improving the optical purity of D-lactic acid produced during continuous fermentation. *Biosci Biotechnol Biochem,* 75**,** 2326-32.

SCHMIDT, F. R. 2005. Optimization and scale up of industrial fermentation processes. *Appl Microbiol Biotechnol,* 68**,** 425-35.

SHUKLA, V. B., VEERA, U. P., KULKARNI, P. R. & PANDIT, A. B. 2001. Scale-up of biotransformation process in stirred tank reactor using dual impeller bioreactor. *Biochemical Engineering Journal,* 8**,** 19-29.

TASHIRO, Y., KANEKO, W., SUN, Y., SHIBATA, K., INOKUMA, K., ZENDO, T. & SONOMOTO, K. 2011. Continuous D-lactic acid production by a novel thermotolerant *Lactobacillus delbrueckii* subsp. lactis QU 41. *Appl Microbiol Biotechnol,* 89**,** 1741-50.

TSUJI, H. 2005. Poly(lactide) stereocomplexes: formation, structure, properties, degradation, and applications. *Macromol Biosci,* 5**,** 569-97.

WANG, L., ZHAO, B., LI, F., XU, K., MA, C., TAO, F., LI, Q. & XU, P. 2011. Highly efficient production of D-lactate by *Sporolactobacillus* sp. CASD with simultaneous enzymatic hydrolysis of peanut meal. *Appl Microbiol Biotechnol,* 89**,** 1009-17.

WANG, Y., TASHIRO, Y. & SONOMOTO, K. 2015. Fermentative production of lactic acid from renewable materials: recent achievements, prospects, and limits. *J Biosci Bioeng,* 119**,** 10-8.

WANG, Y., TIAN, T., ZHAO, J., WANG, J., YAN, T., XU, L., LIU, Z., GARZA, E., IVERSON, A., MANOW, R., FINAN, C. & ZHOU, S. 2012. Homofermentative production of D-lactic acid from sucrose by a metabolically engineered *Escherichia coli*. *Biotechnol Lett,* 34**,** 2069-75.

WARDANI, S. K., CAHYANTO, M. N., RAHAYU, E. S. & UTAMI, T. 2017. The effect of inoculum size and incubation temperature on cell growth, acid production and curd formation during milk fermentation by *Lactobacillus plantarum* Dad 13. *International Food Research Journal,* 24**,** 921-926.

ZHANG, J., SATO, H., TSUJI, H., NODA, I. & OZAKI, Y. 2005. Infrared spectroscopic study of CH3 center dot center dot center dot O = C interaction during poly(L-lactide)/poly(D-lactide) stereocomplex formation. *Macromolecules,* 38**,** 1822–1828.

ZHANG, Y. & VADLANI, P. V. 2013. D-Lactic acid biosynthesis from biomass-derived sugars via *Lactobacillus delbrueckii* fermentation. *Bioprocess Biosyst Eng,* 36**,** 1897-904.

ZHAO, T., LIU, D., REN, H., SHI, X., ZHAO, N., CHEN, Y. & YING, H. 2014. D-lactic acid production by *Sporolactobacillus inulinus* Y2-8 immobilized in fibrous bed bioreactor using corn flour hydrolyzate. *J Microbiol Biotechnol,* 24**,** 1664-72.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

APPENDIX A

ANALYTICAL METHOD

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

A1 High Performance Liquid Chromatography for determining the remaining glucose and metabolites

High performance liquid chromatography (HPLC) was used to analyze the composition of fermentative broth including glucose, lactic acid, acetic acid acid, and ethanol. Samples were diluted with double distilled water (DDI water). After that diluted particle-free sample (15 µL) were automatically injected into an exclusion organic acid column (Biorad, Aminex HPX-87H ion exclusion organic acid column; 300mm x 7.8mm) maintained at 45ºC in a column oven (Shimadzu-CTO-6A). Sulfuric acid at the concentration of 0.005 M was used as a mobile phase at a flowrate of 0.6 mL/min. An RI detector (Shimadzu-RID-6A) was set at the range of 200 to detect the organic compounds. A standard containing 2 g/L of each component was injected as a reference to determine the sample concentration in rage of 0 to 2 g/L (preparation follow below).

Concentration (g/L)	**Standard 2 g/L (µL)**	**DDI (µL)**
0.25	125	875
0.5	250	750
1.0	500	500
1.5	750	250
2.0	1000	-

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Retention time of glucose, lactic acid, acetic acid, and ethanol is 8.9, 12.9, 15.3, and 21.6 respectively.

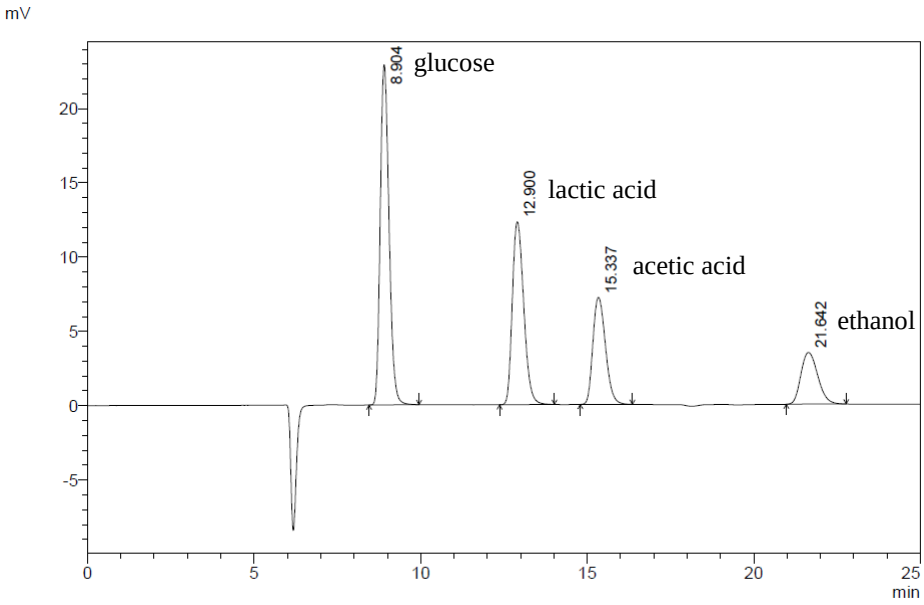


Figure A1 chromatogram of the standard solution (2 g/L) for analyzing the remaining glucose and metabolites

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

A2 High Performance Liquid Chromatography for determining the optical purity

High performance liquid chromatography (HPLC) was also used to analyze the optical purity of product. 5 µL diluted cell-free samples were automatically injected into a chiral column (Sumipack, Sumichiral OA5000) and maintained at 40 °C in a column oven (Shimadzu-CTO-6A). 0.001 M $CuSO_4$ was used as the eluent at the flowrate of 1.0 mL/min. The UV detector (Shimadzu-UV-VIS-6A) was used to detect the lactate isomers at 254 nm. The standards containing 0-2 g/L of D- and L-lactate were injected as references to determine the optical purity of the product (preparation follow below).

Concentration (g/L)	**Standard 2 g/L (µL)**	**DDI (µL)**
0.25	125	875
0.5	250	750
1.0	500	500
1.5	750	250
2.0	1000	-

Retention time of L-lactic acid and D-lactic acid is 15.7 and 19.0 respectively.

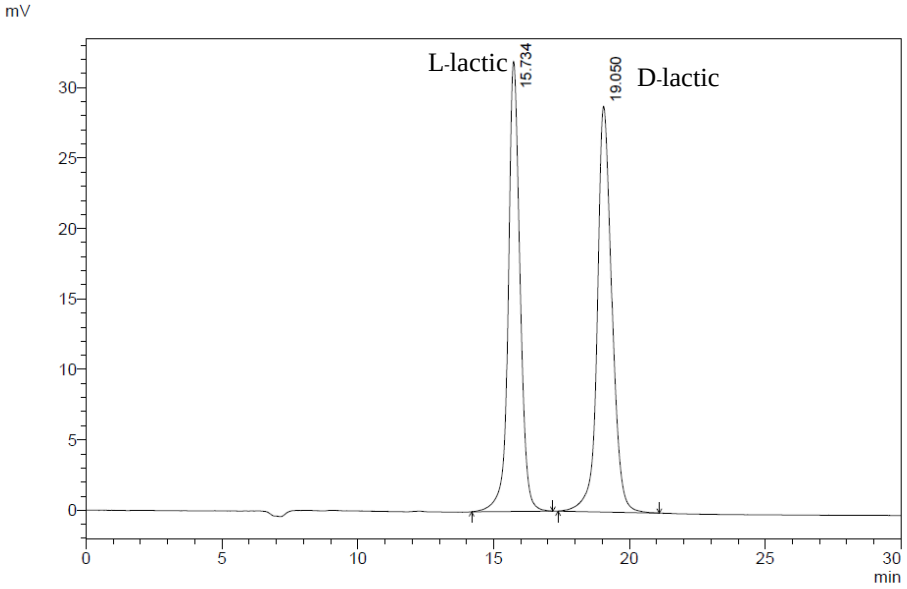


Figure A2 chromatogram of the standard solution (0.5 g/L) for analyzing the optical purity of D-lactic acid

A3 Glucose Analyzer

Remaining glucose in the samples that collected from fermentation was measured by YSI 2700 glucose-lactate analyzer (Yellow Spring Instrument Co., Inc.). This analytical instrument is accurate within the range of 0-2 g/L glucose. The supernatant of samples was diluted with DI water before analyzing.

The calibrator standard contained

L(+)-lactic acid	0.5 g/L
Glucose	2.5 g/L
Benzoic acid	1.0 g/L
NaEDTA	2.0 g/L

The buffer powder used in this equipment was prepared by mixing

K_2H_2EDTA	4.4 g
kanamycin sulfate	0.05 g
sodium benzoate	7.3 g
NaH_2PO_4	12.0 g
Na_2PO_4	54.7 g
NaCl	21.5 g

The buffer solution was prepared by dissolving 12.7 g buffer powder in 900 mL distilled water.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

APPENDIX B

CALCULATION OF KINETIC VALUES

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

B1 Yield (g/g glucose)

$$\text{Yield (g/g)} = \frac{\text{Final lactic acid (g/L)}}{\text{(Initial glucose-Remaining glucose (g/L))}}$$

B2 Productivity (g/L/h)

$$\text{Productivity (g/L/h)} = \frac{\text{Final lactic acid (g/L)}}{\text{Fermentation time (h)}}$$

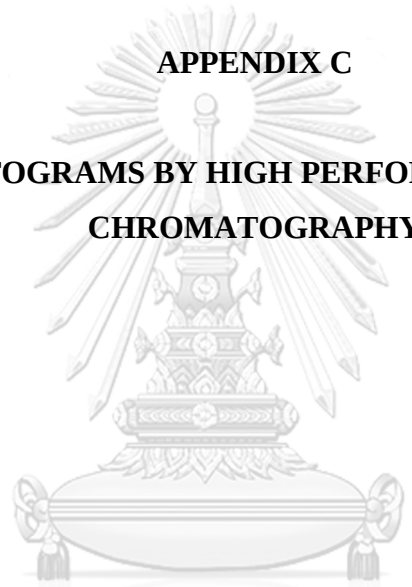
B3 Optical purity (%ee)

$$\text{Optical purity (\%ee)} = \frac{\text{D-lactic acid-L-lactic acid}}{\text{D-lactic acid+L-lactic acid}}$$

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

APPENDIX C

CHROMATOGRAMS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY



จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

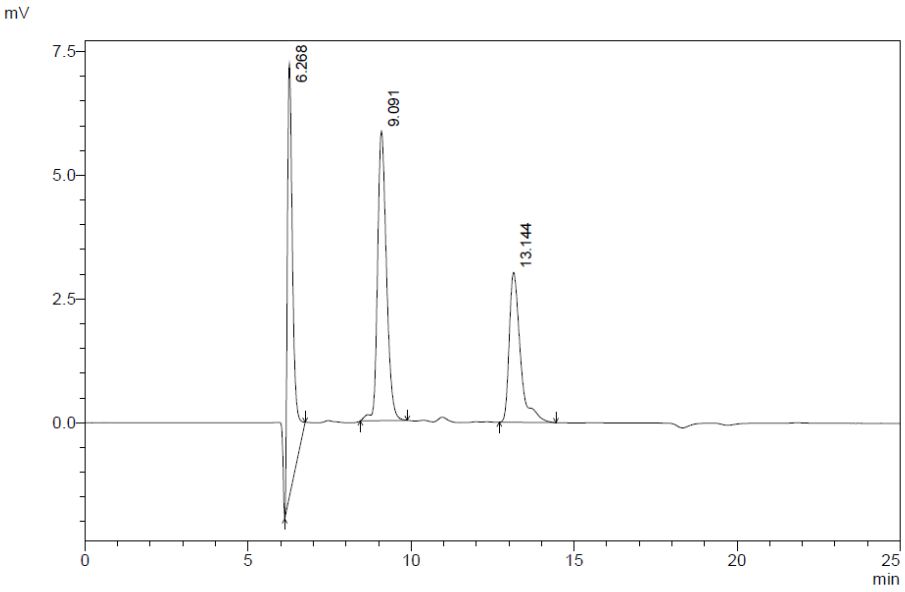


Figure C1 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 1% inoculum size, mid log phase and 3% preculture size

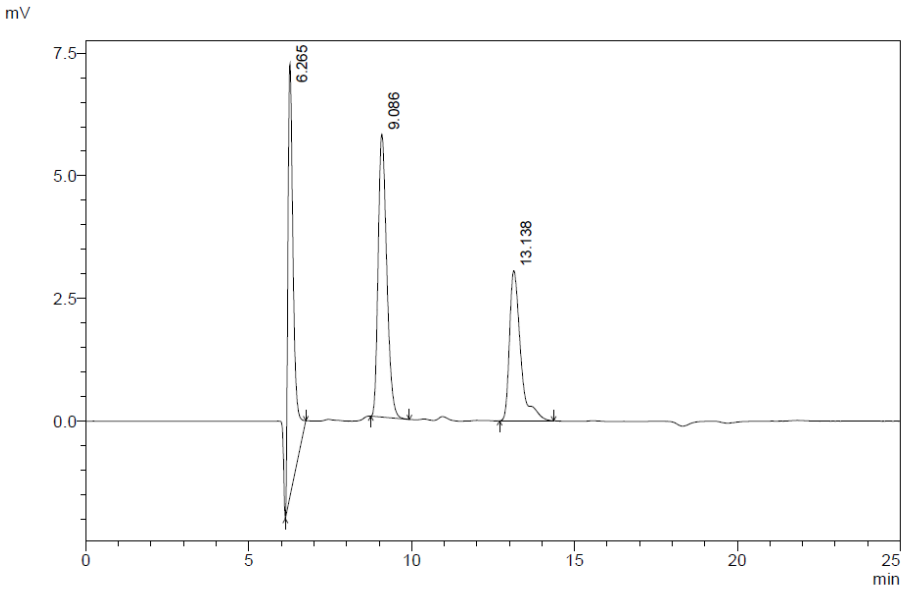


Figure C2 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 3% inoculum size, mid log phase and 3% preculture size

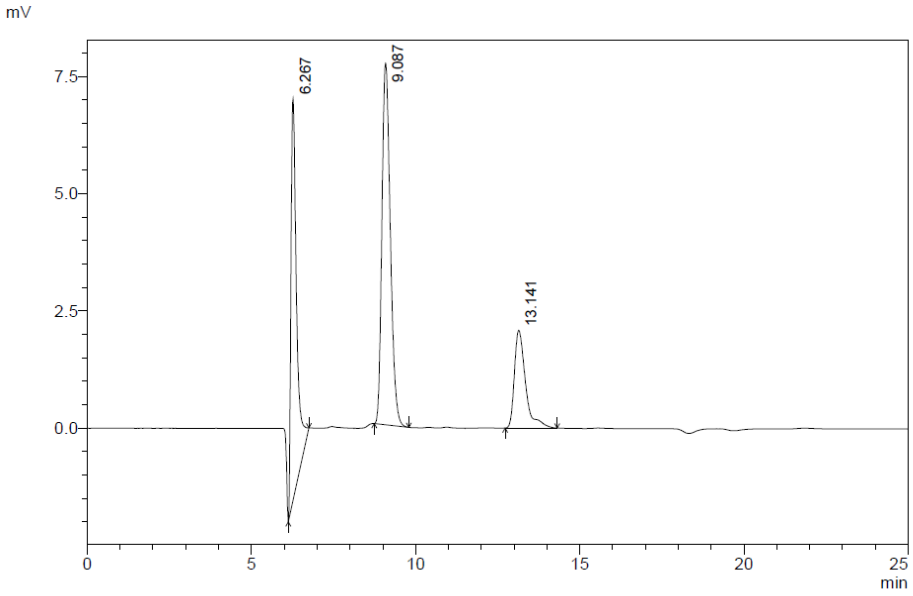


Figure C3 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 5% inoculum size, mid log phase and 3% preculture size

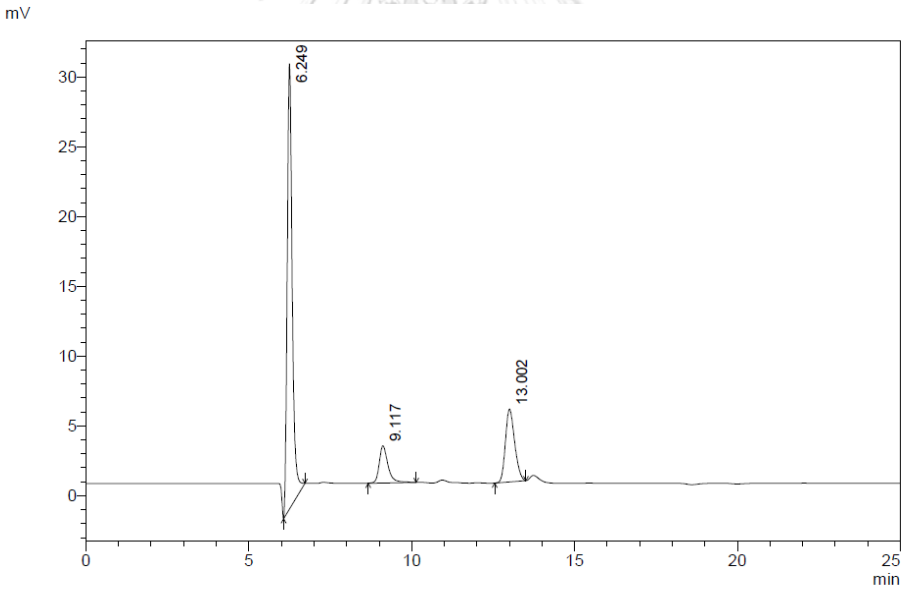


Figure C4 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 1% inoculum size, late log phase and 3% preculture size

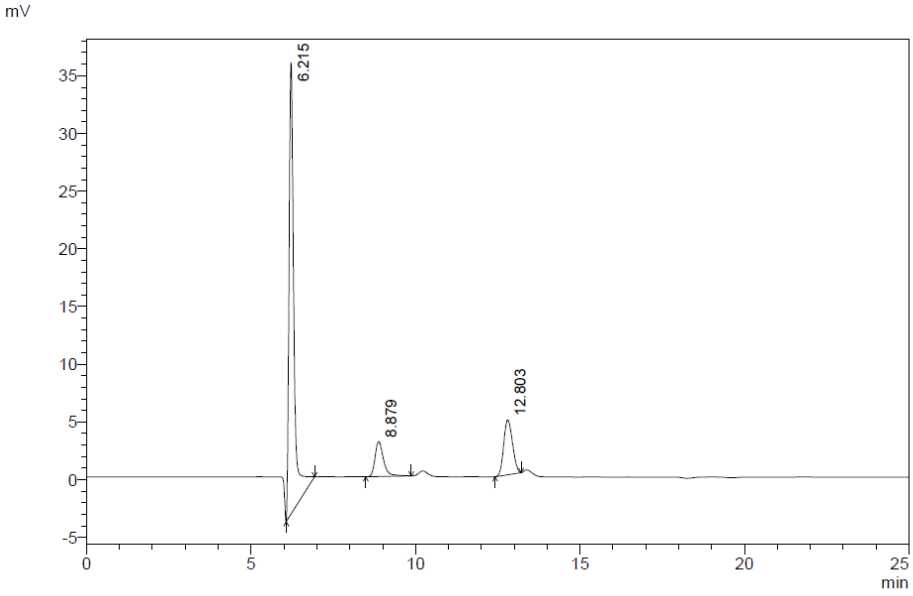


Figure C5 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 3% inoculum size, late log phase and 3% preculture size

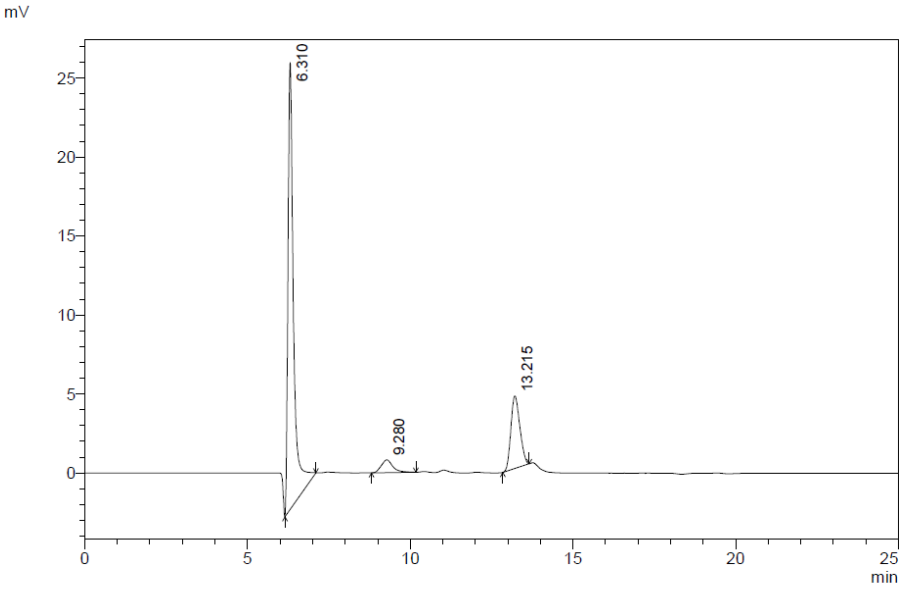


Figure C6 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 5% inoculum size, late log phase and 3% preculture size

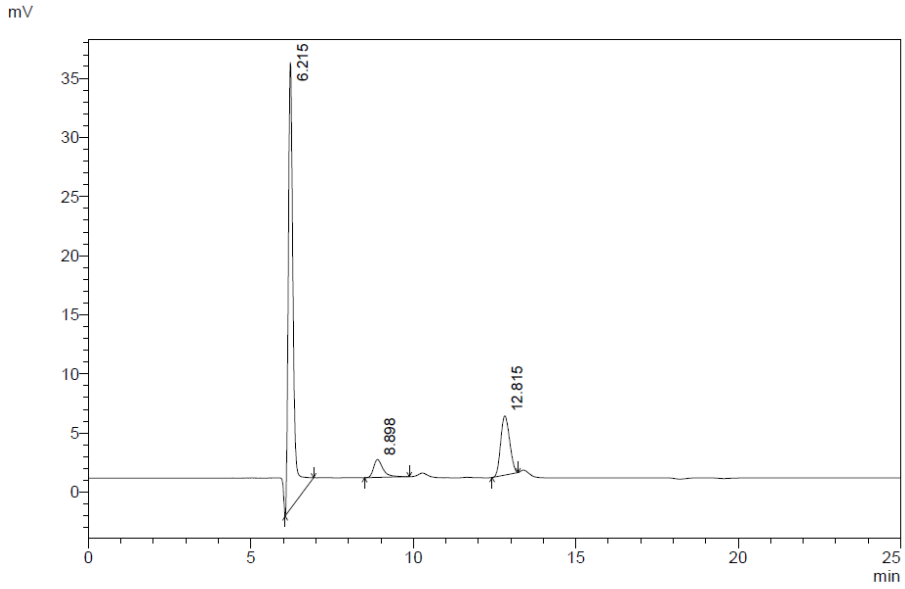


Figure C7 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 1% inoculum size, mid log phase and 5% preculture size

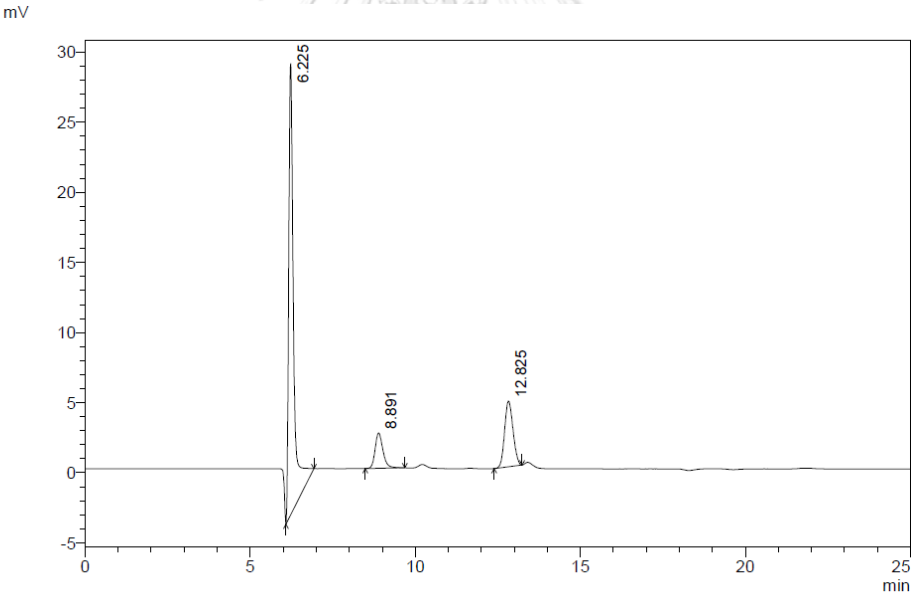


Figure C8 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 3% inoculum size, mid log phase and 5% preculture size

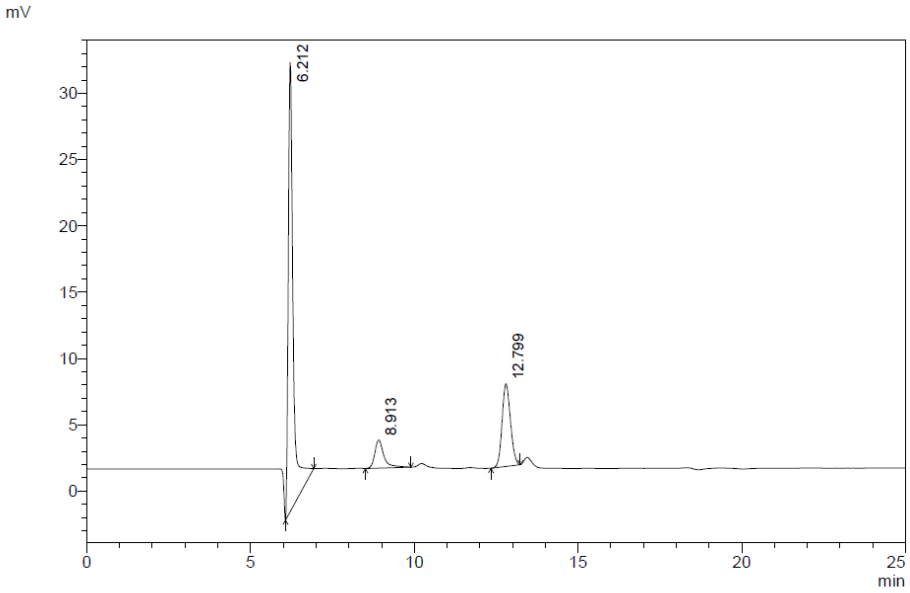


Figure C9 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 5% inoculum size, mid log phase and 5% preculture size

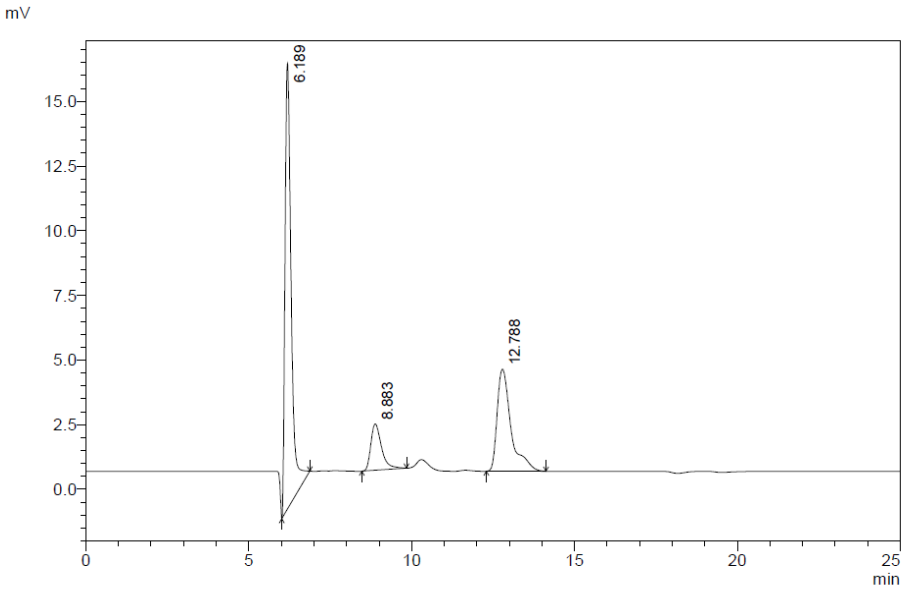


Figure C10 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 1% inoculum size, late log phase and 5% preculture size

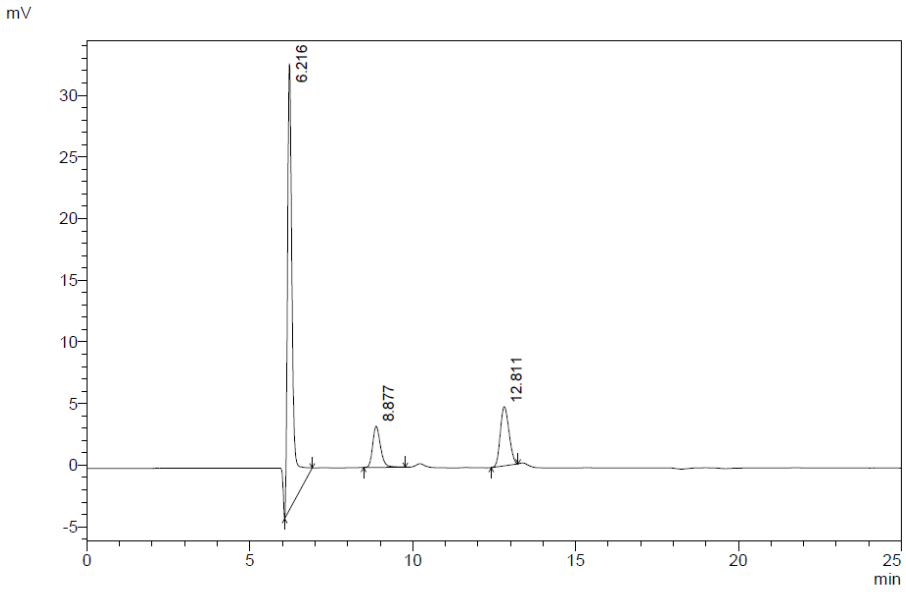


Figure C11 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 3% inoculum size, late log phase and 5% preculture size

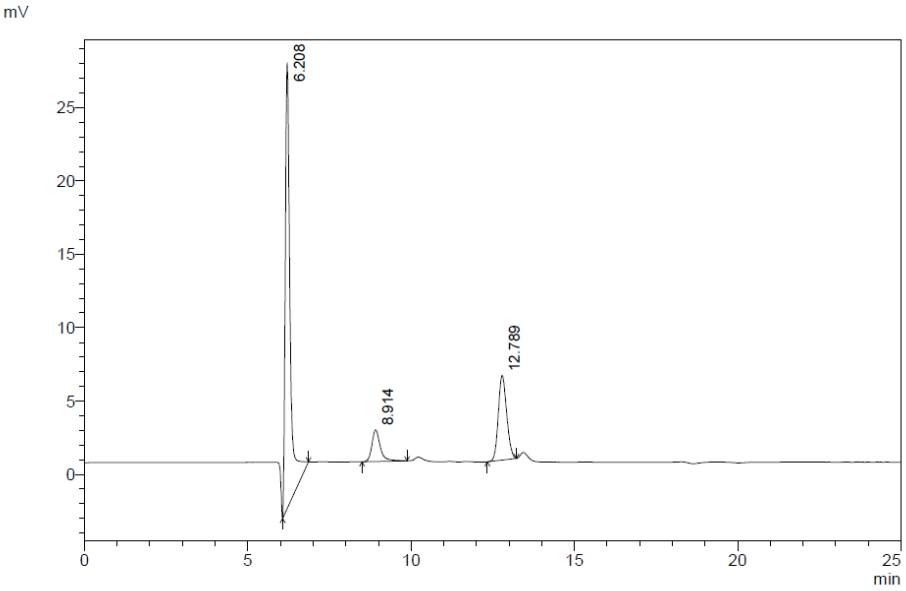


Figure C12 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 5% inoculum size, late log phase and 5% preculture size

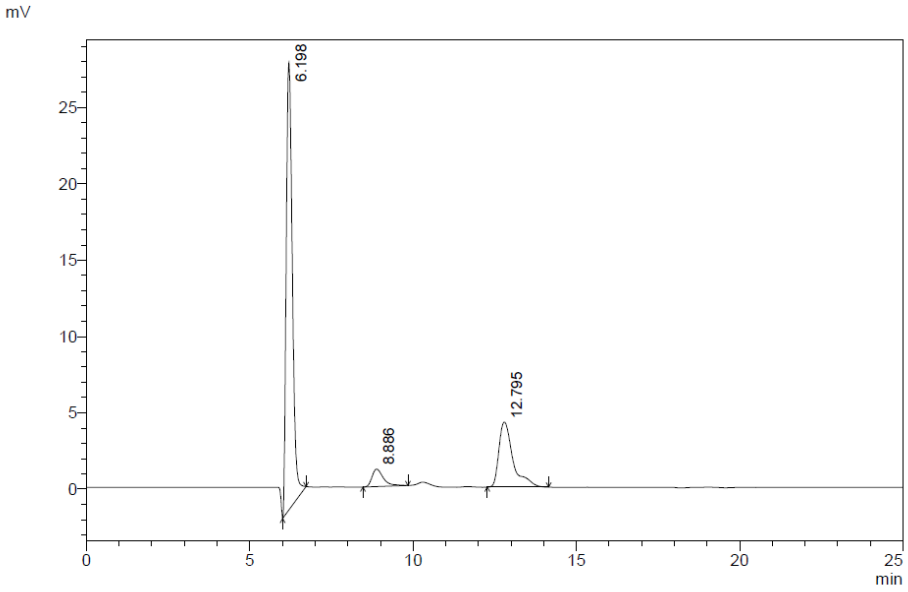


Figure C13 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 1% inoculum size, mid log phase and 10% preculture size

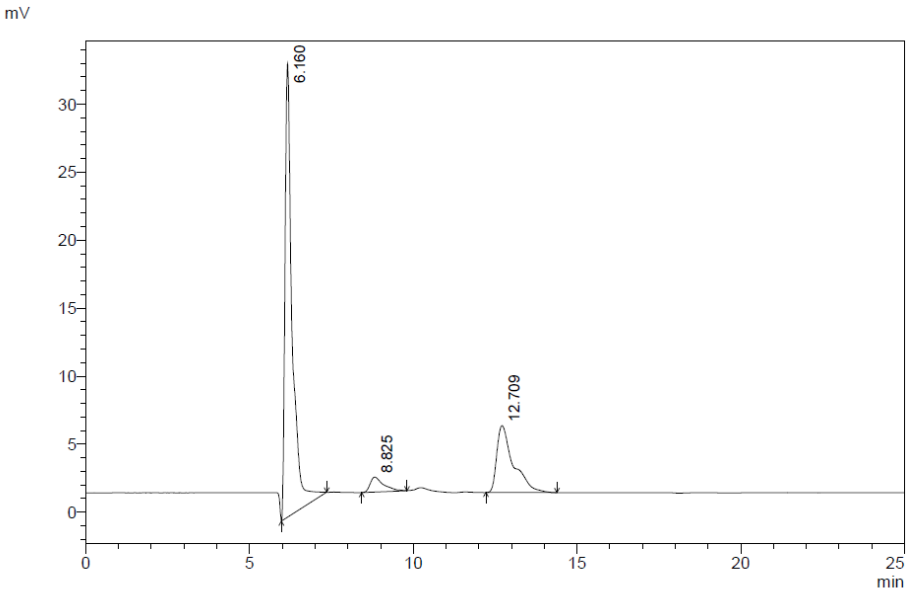


Figure C14 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 3% inoculum size, mid log phase and 10% preculture size

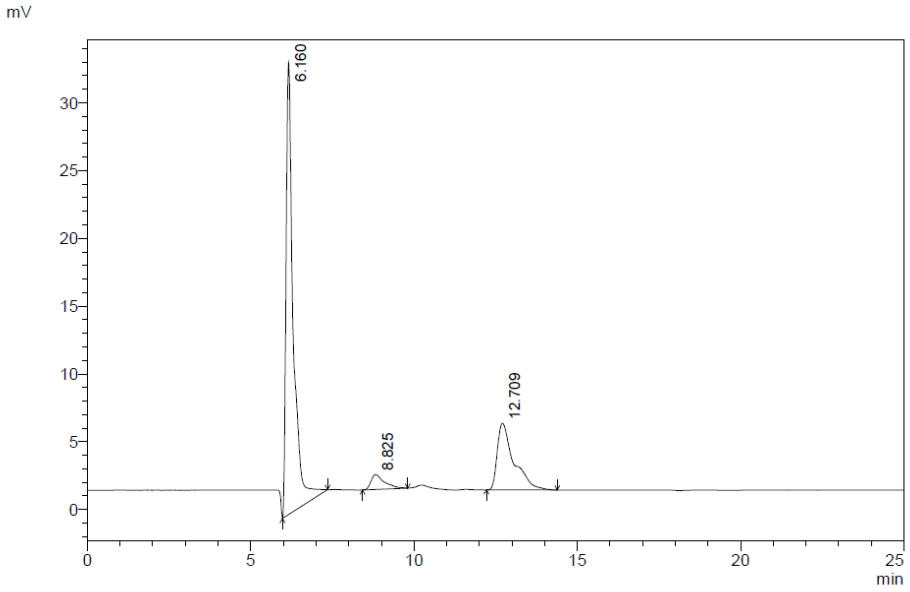


Figure C15 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 5% inoculum size, mid log phase and 10% preculture size

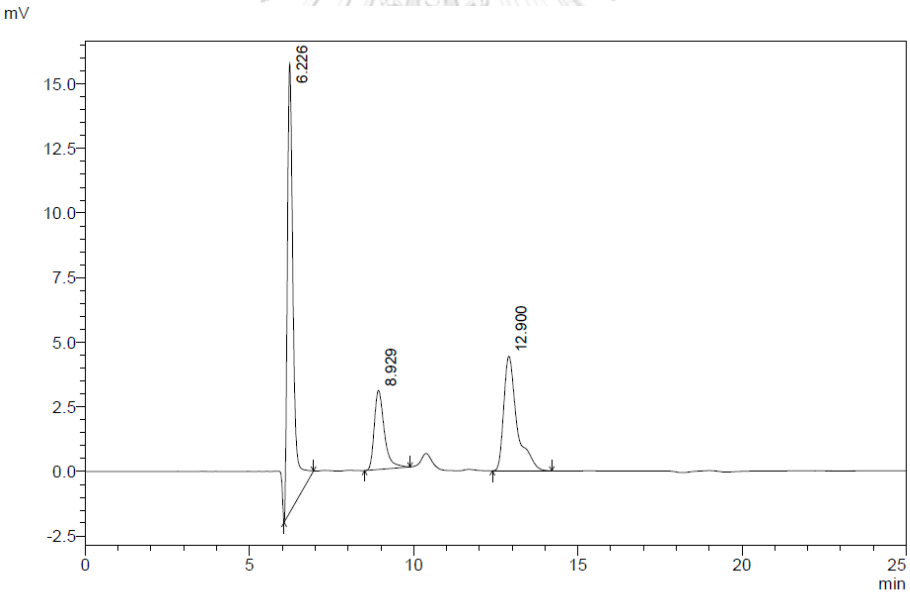


Figure C16 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 1% inoculum size, late log phase and 10% preculture size

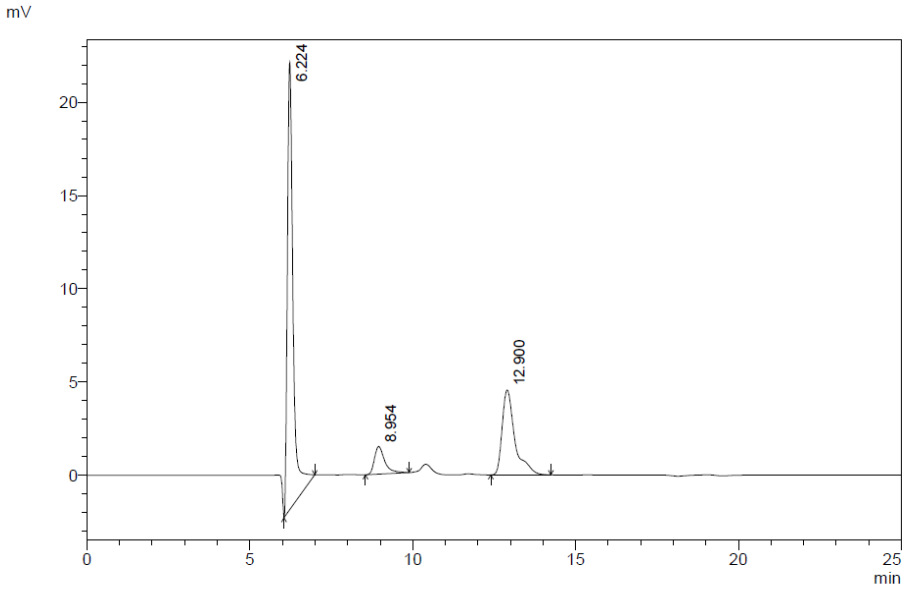


Figure C17 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 3% inoculum size, late log phase and 10% preculture size

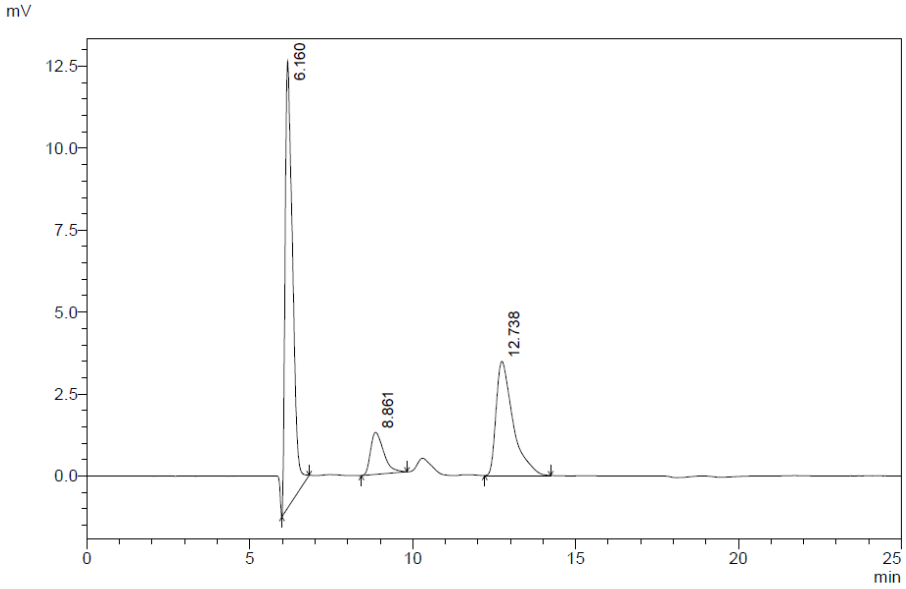


Figure C18 chromatogram of D-lactic acid fermentation in 5 L fermentor at 48 h by using 5% inoculum size, late log phase and 10% preculture size

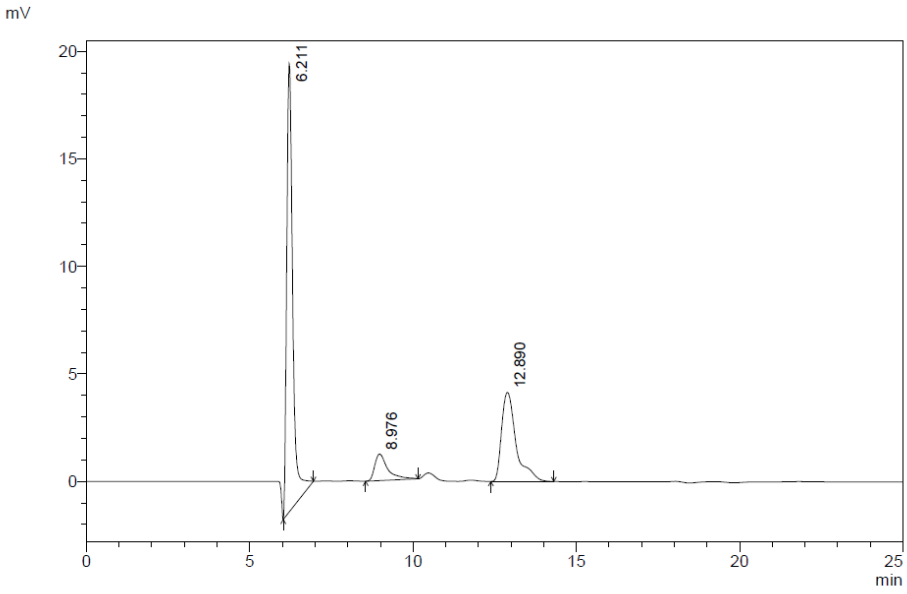


Figure C19 chromatogram of D-lactic acid fermentation in 30 L fermentor at 45 h by using 3% transfer size of seed 1 (250 flask) and scale up with P/V

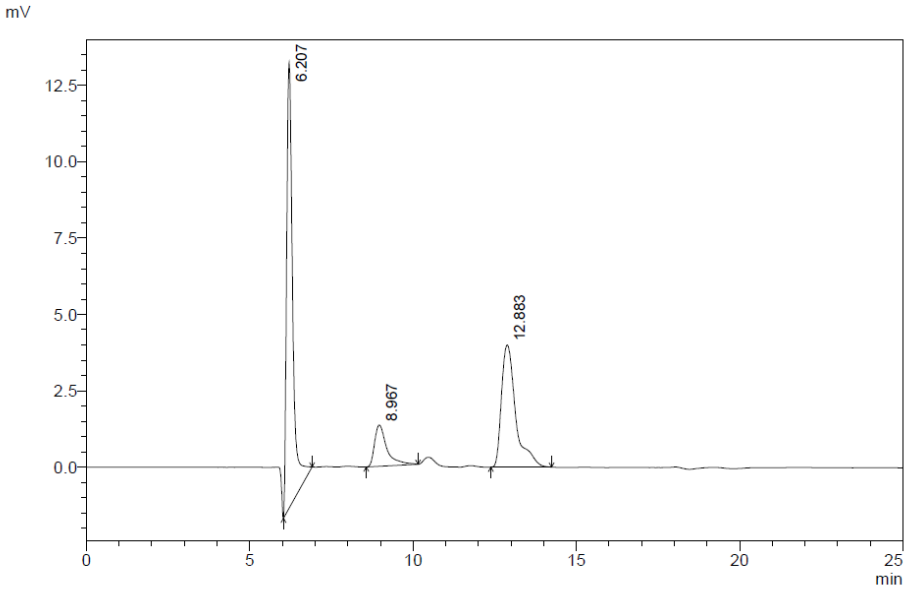


Figure C20 chromatogram of D-lactic acid fermentation in 30 L fermentor at 42 h by using 5% transfer size of seed 1 (250 flask) and scale up with P/V

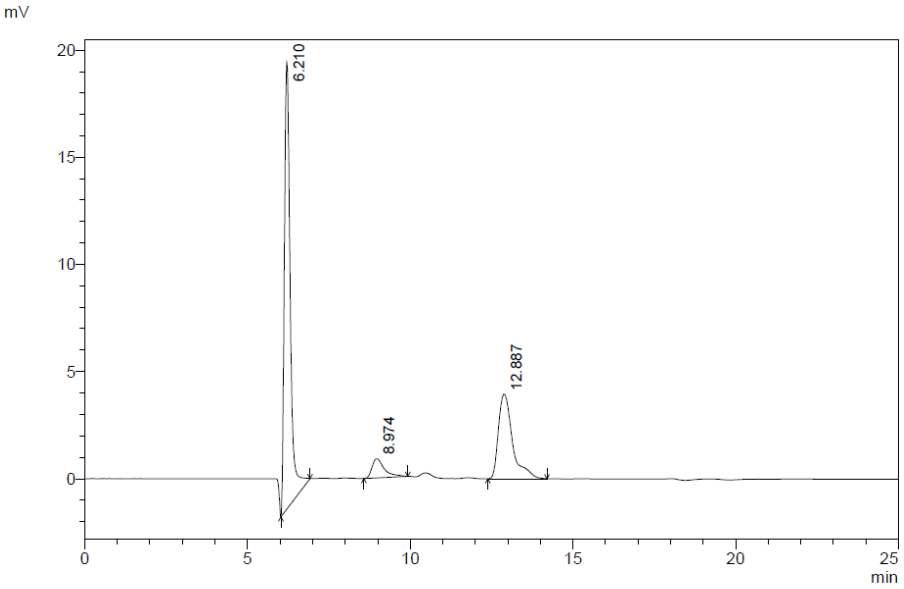


Figure C21 chromatogram of D-lactic acid fermentation in 30 L fermentor at 39 h by using 10% transfer size of seed 1 (250 flask) and scale up with P/V

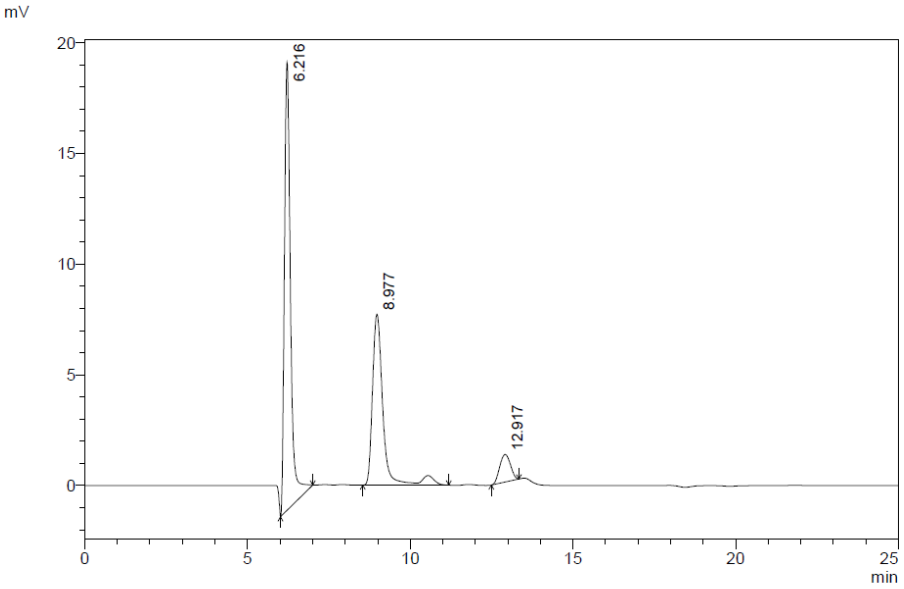


Figure C22 chromatogram of D-lactic acid fermentation in 30 L fermentor at 48 h by using 3% transfer size of seed 1 (250 flask) and scale up with N_iD_i

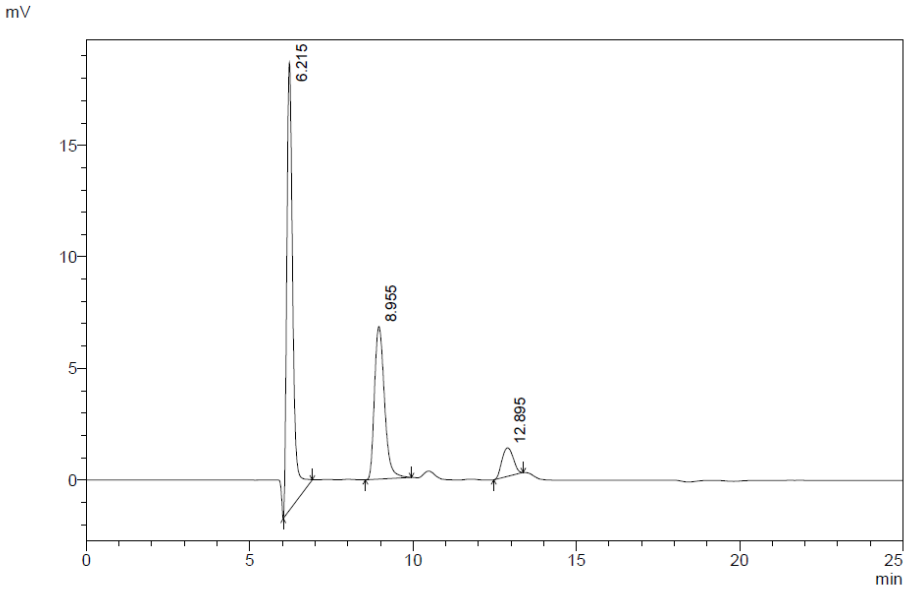


Figure C23 chromatogram of D-lactic acid fermentation in 30 L fermentor at 48 h by using 5% transfer size of seed 1 (250 flask) and scale up with N_iD_i

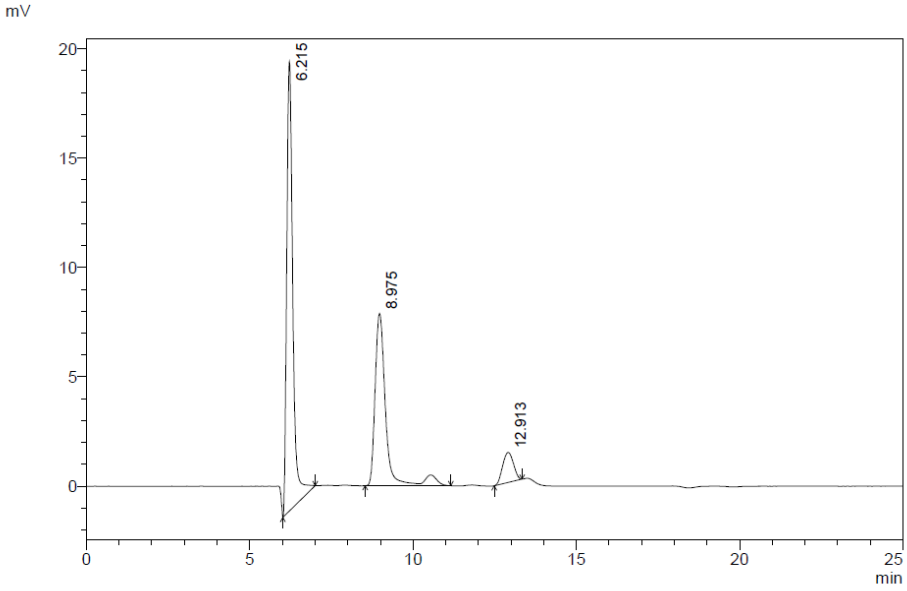


Figure C24 chromatogram of D-lactic acid fermentation in 30 L fermentor at 48 h by using 10% transfer size of seed 1 (250 flask) and scale up with N_iD_i

APPENDIX D

STATISTICAL ANALISIS

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

D1 Optimizing process conditions in the 5 L stirred fermentor

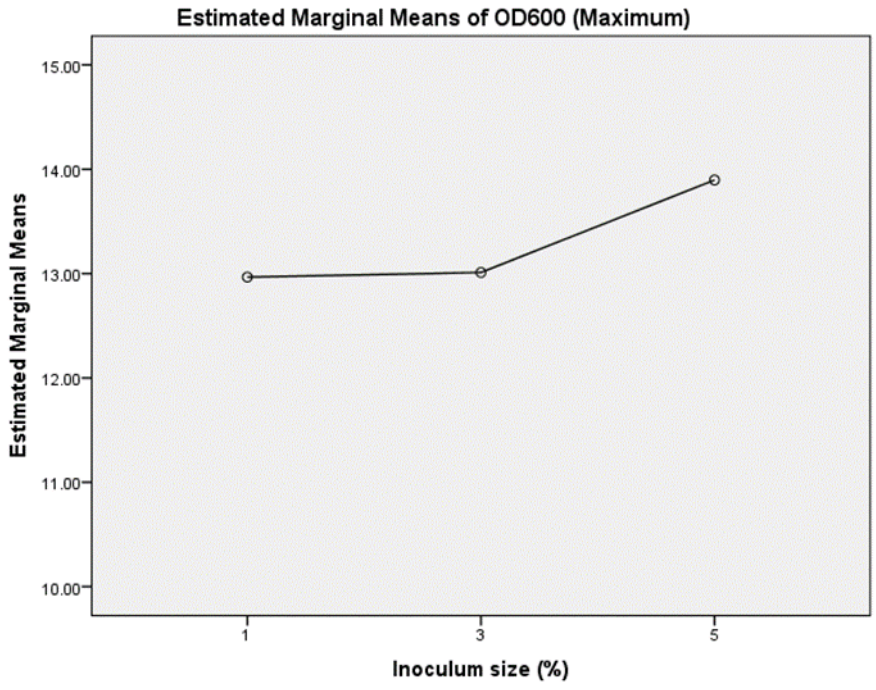
D1.1. OD_{600} (maximum)

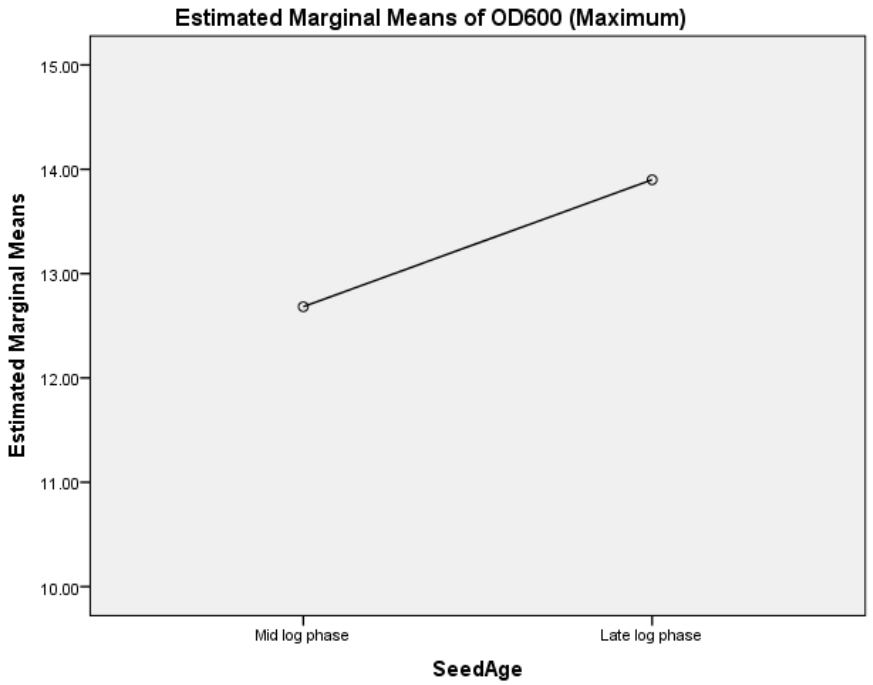
Dependent Variable: OD600 (Maximum)

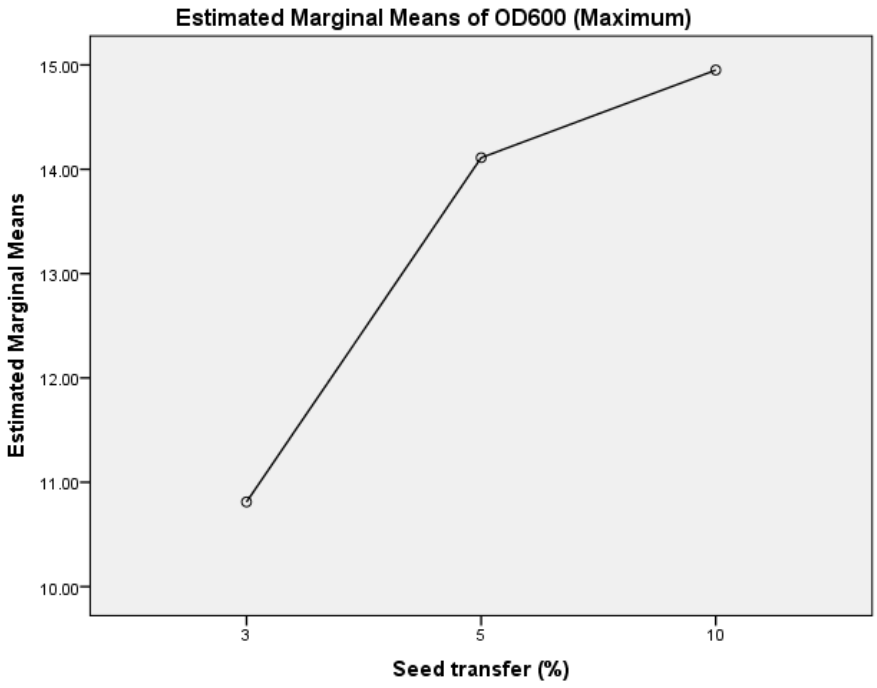
Source	Type III Sum of Squares	df	Mean Square	F	Sig.[b]
Corrected Model	505.217[a]	17	29.719	391.340	.000
Intercept	9539.562	1	9539.562	125618.502	.000
InoculumSize	9.912	2	4.956	65.262	.000
SeedAge	19.984	1	19.984	263.149	.000
SeedVolume	172.362	2	86.181	1134.843	.000
InoculumSize * SeedAge	11.989	2	5.995	78.937	.000
InoculumSize * SeedVolume	16.566	4	4.142	54.537	.000
SeedAge * SeedVolume	256.970	2	128.485	1691.912	.000
InoculumSize * SeedAge * SeedVolume	17.434	4	4.358	57.393	.000
Error	2.734	36	.076		
Total	10047.513	54			
Corrected Total	507.951	53			

a. R Squared = .995 (Adjusted R Squared = .992)

b. Significant level = 0.05 (confident intervals of 95%)




Estimated Marginal Means of OD600 (Maximum)
Estimated Marginal Means
15.00
14.00
13.00
12.00
11.00
10.00
Mid log phase
Late log phase
SeedAge


Estimated Marginal Means of OD600 (Maximum)
Estimated Marginal Means
15.00
14.00
13.00
12.00
11.00
10.00
3
5
10
Seed transfer (%)

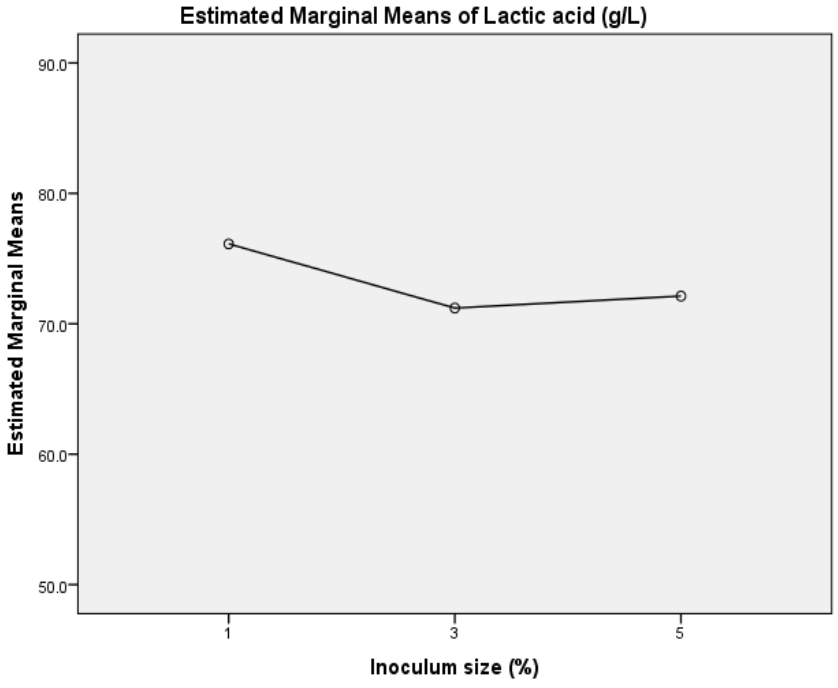
D1.2. Final concentration of D-lactic acid (g/L)

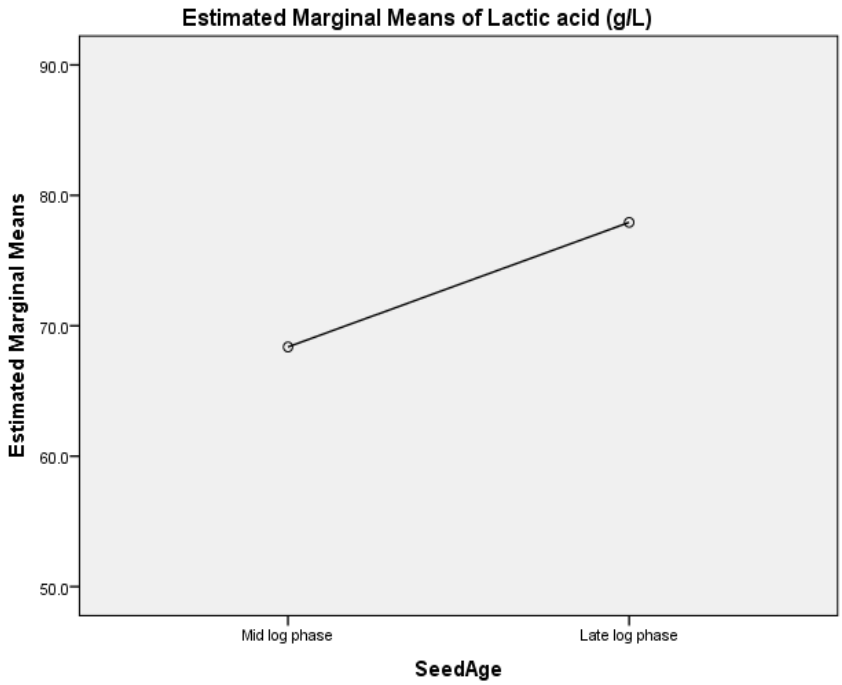
Dependent Variable: Lactic acid (g/L)

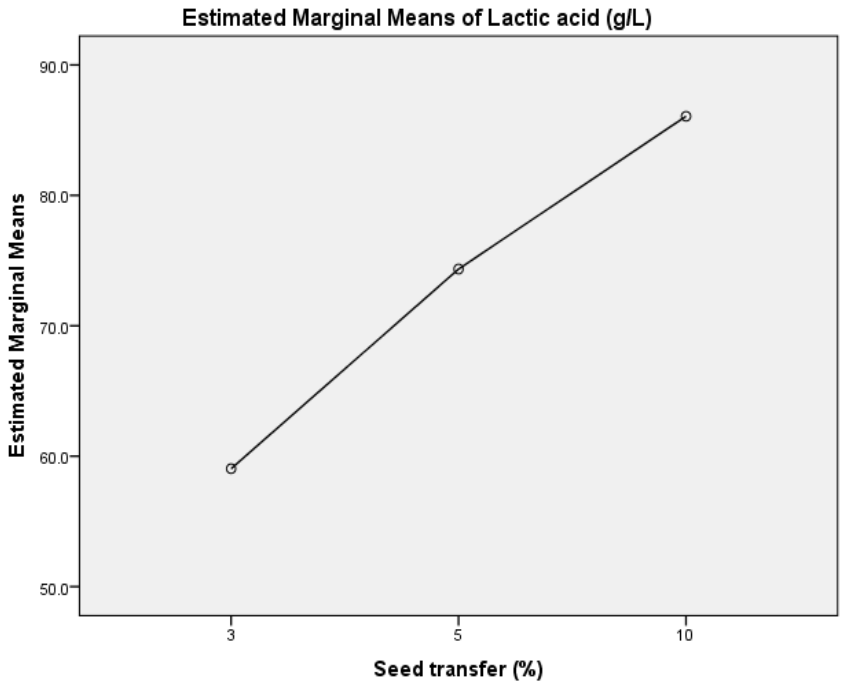
Source	Type III Sum of Squares	df	Mean Square	F	Sig.[b]
Corrected Model	7261.235[a]	17	427.131	251.375	.000
Intercept	192644.914	1	192644.914	113375.373	.000
InoculumSize	164.097	2	82.048	48.287	.000
SeedAge	821.014	1	821.014	483.183	.000
SeedVolume	4407.387	2	2203.694	1296.918	.000
InoculumSize * SeedAge	47.309	2	23.654	13.921	.000
InoculumSize * SeedVolume	354.843	4	88.711	52.208	.000
SeedAge * SeedVolume	1147.872	2	573.936	337.773	.000
InoculumSize * SeedAge * SeedVolume	318.713	4	79.678	46.892	.000
Error	30.585	18	1.699		
Total	199936.734	36			
Corrected Total	7291.820	35			

a. R Squared = .996 (Adjusted R Squared = .992)

b. Significant level = 0.05 (confident intervals of 95%)




Estimated Marginal Means of Lactic acid (g/L)
Estimated Marginal Means
90.0
80.0
70.0
60.0
50.0
Mid log phase
Late log phase
SeedAge


Estimated Marginal Means of Lactic acid (g/L)
Estimated Marginal Means
90.0
80.0
70.0
60.0
50.0
3
5
10
Seed transfer (%)

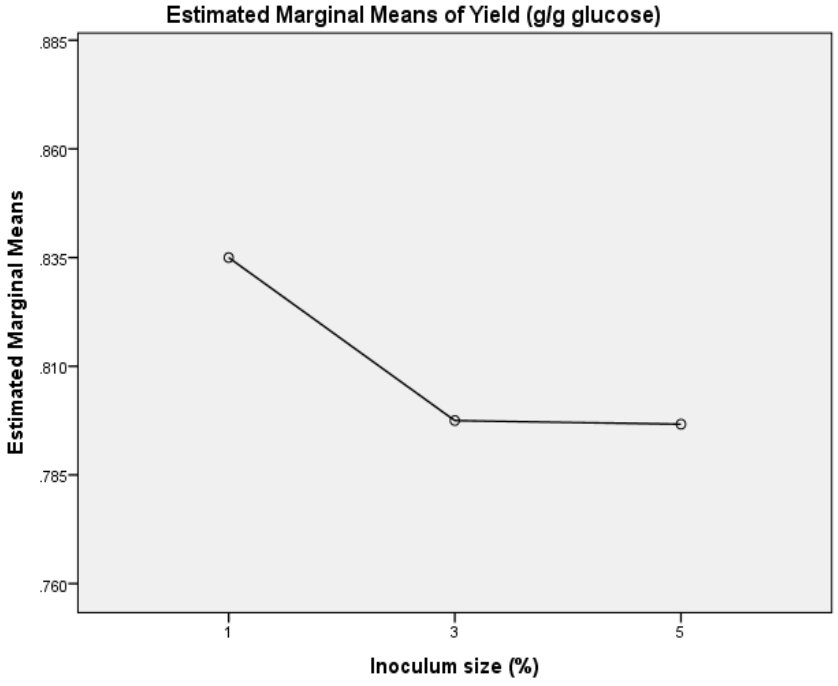
D1.3. Yield (g/g glucose)

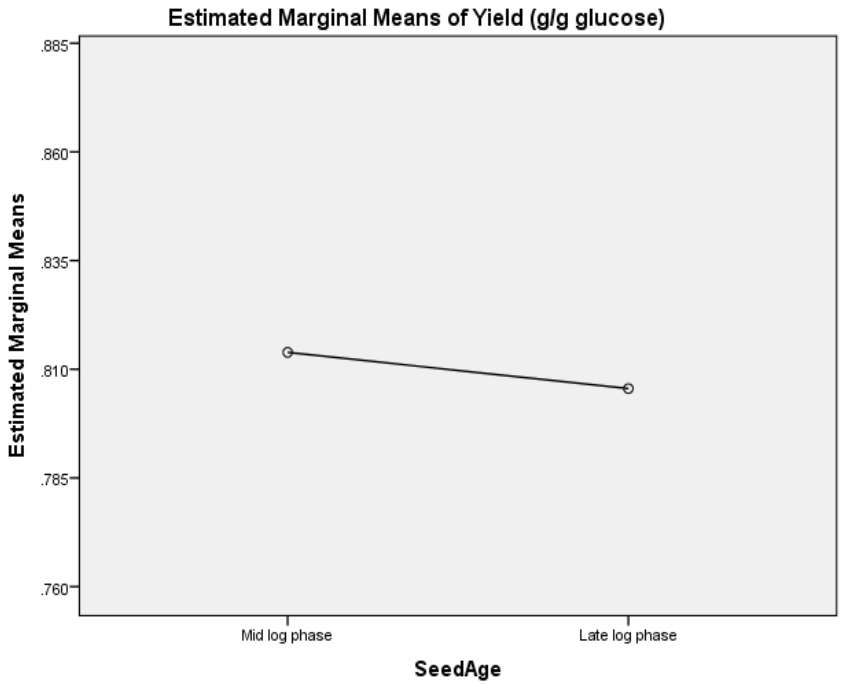
Dependent Variable: Yield (g/g glucose)

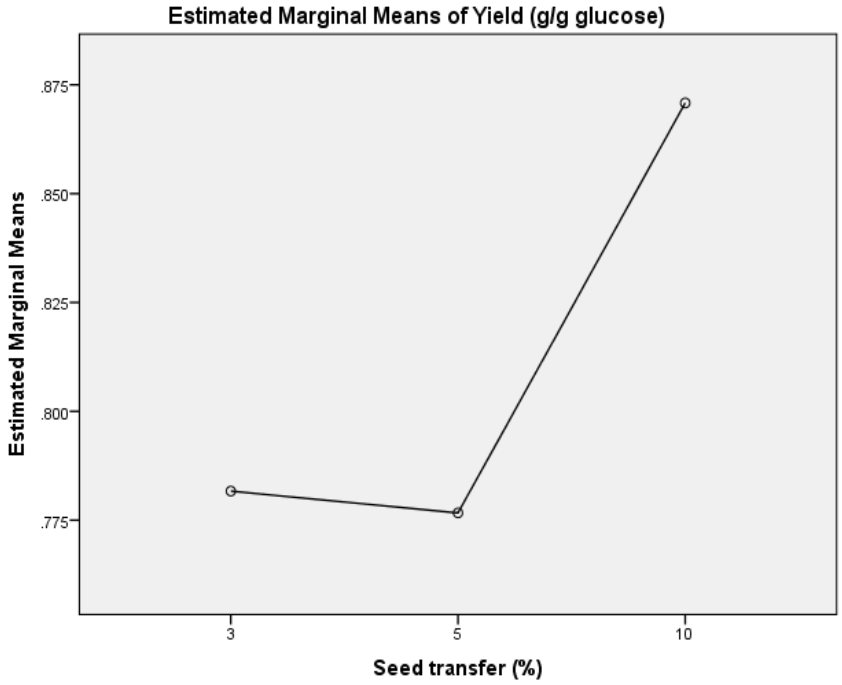
Source	Type III Sum of Squares	df	Mean Square	F	Sig.[b]
Corrected Model	.135[a]	17	.008	33.595	.000
Intercept	23.603	1	23.603	99967.353	.000
InoculumSize	.012	2	.006	24.365	.000
SeedAge	.001	1	.001	2.647	.121
SeedVolume	.067	2	.034	142.671	.000
InoculumSize * SeedAge	.003	2	.001	6.035	.010
InoculumSize * SeedVolume	.035	4	.009	37.106	.000
SeedAge * SeedVolume	.008	2	.004	17.188	.000
InoculumSize * SeedAge * SeedVolume	.009	4	.002	9.882	.000
Error	.004	18	.000		
Total	23.743	36			
Corrected Total	.139	35			

a. R Squared = .969 (Adjusted R Squared = .941)

b. Significant level = 0.05 (confident intervals of 95%)




Estimated Marginal Means of Yield (g/g glucose)
Estimated Marginal Means
.885
.860
.835
.810
.785
.760
Mid log phase
Late log phase
SeedAge


Estimated Marginal Means of Yield (g/g glucose)
Estimated Marginal Means
.875
.850
.825
.800
.775
3
5
10
Seed transfer (%)

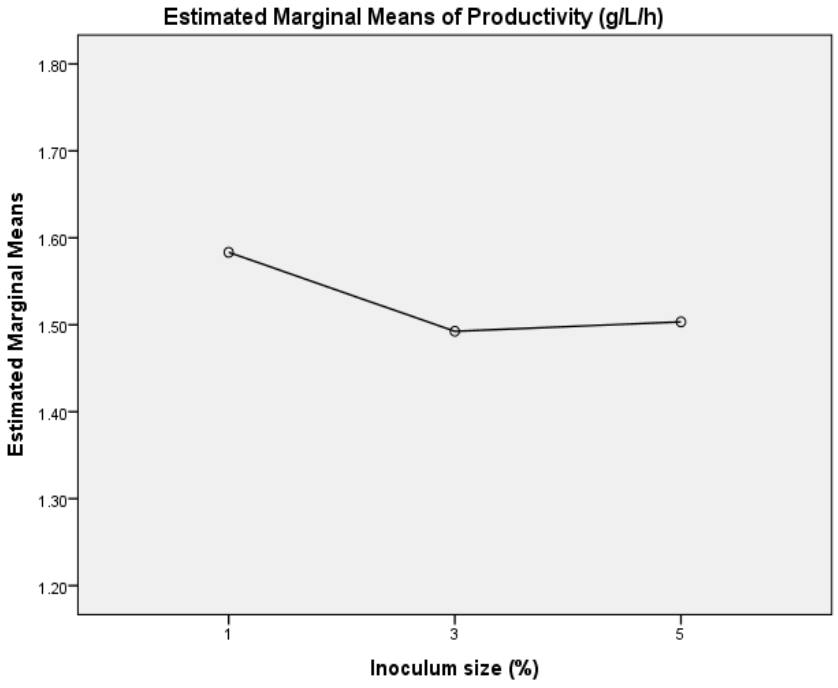
D1.4. Productivity (g/L/h)

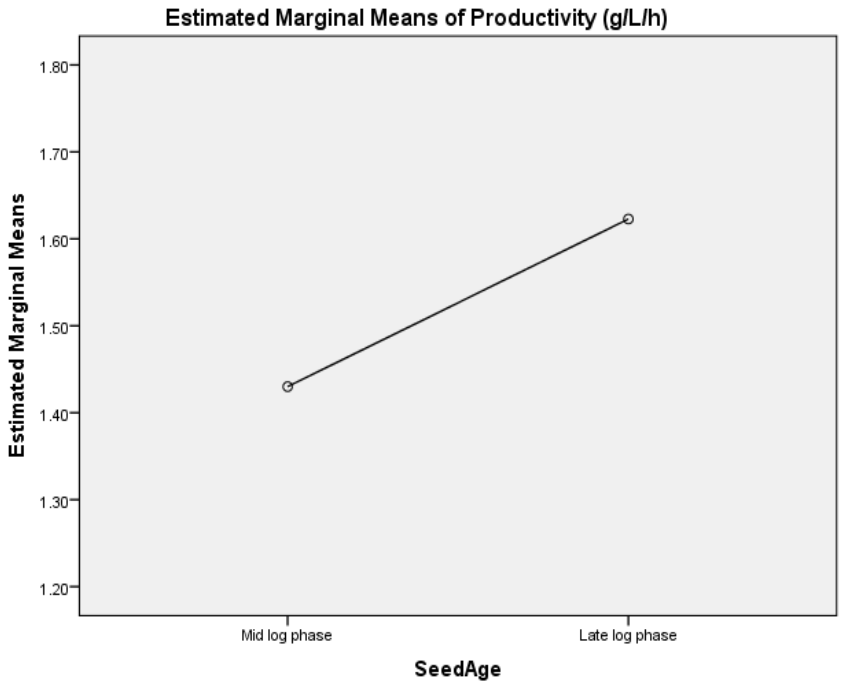
Dependent Variable: Productivity (g/L/h)

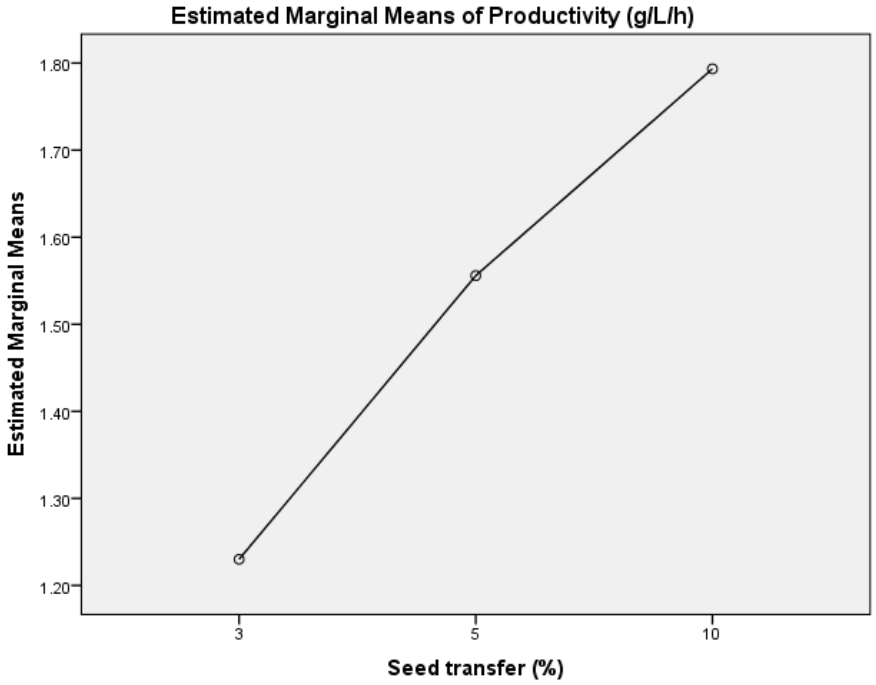
Source	Type III Sum of Squares	df	Mean Square	F	Sig.[b]
Corrected Model	3.126[a]	17	.184	585.779	.000
Intercept	83.875	1	83.875	267212.611	.000
InoculumSize	.059	2	.030	94.097	.000
SeedAge	.334	1	.334	1065.566	.000
SeedVolume	1.920	2	.960	3057.885	.000
InoculumSize * SeedAge	.024	2	.012	37.973	.000
InoculumSize * SeedVolume	.137	4	.034	109.004	.000
SeedAge * SeedVolume	.518	2	.259	824.664	.000
InoculumSize * SeedAge * SeedVolume	.134	4	.034	106.854	.000
Error	.006	18	.000		
Total	87.007	36			
Corrected Total	3.131	35			

a. R Squared = .998 (Adjusted R Squared = .996)

b. Significant level = 0.05 (confident intervals of 95%)




Estimated Marginal Means of Productivity (g/L/h)
Estimated Marginal Means
1.80
1.70
1.60
1.50
1.40
1.30
1.20
Mid log phase
Late log phase
SeedAge


Estimated Marginal Means of Productivity (g/L/h)
Estimated Marginal Means
1.80
1.70
1.60
1.50
1.40
1.30
1.20
3
5
10
Seed transfer (%)

D2 Fermentation process scale up in 30 L stirred fermentor

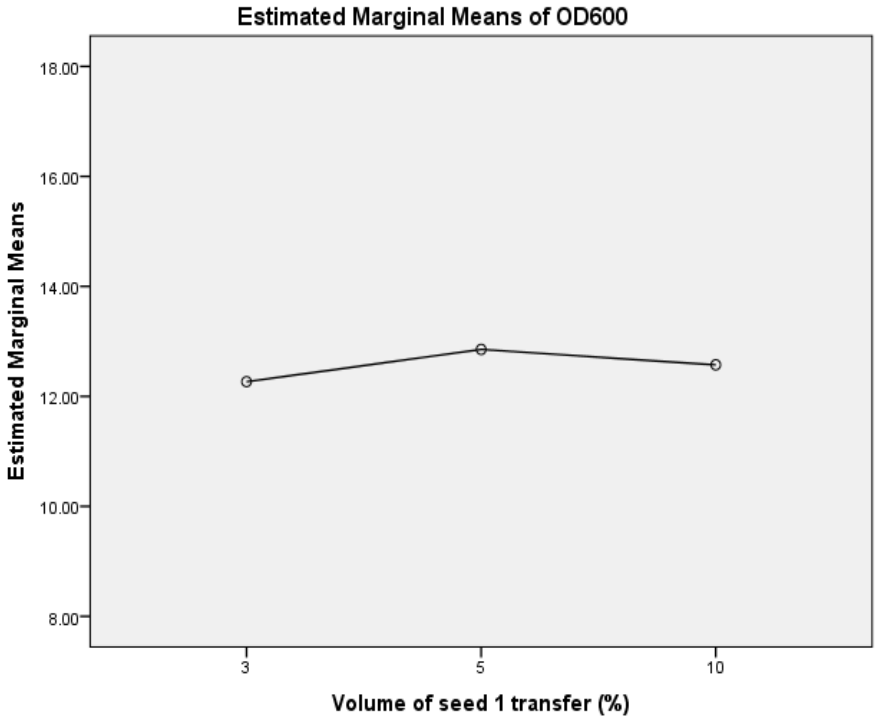
D2.1. OD_{600} (maximum)

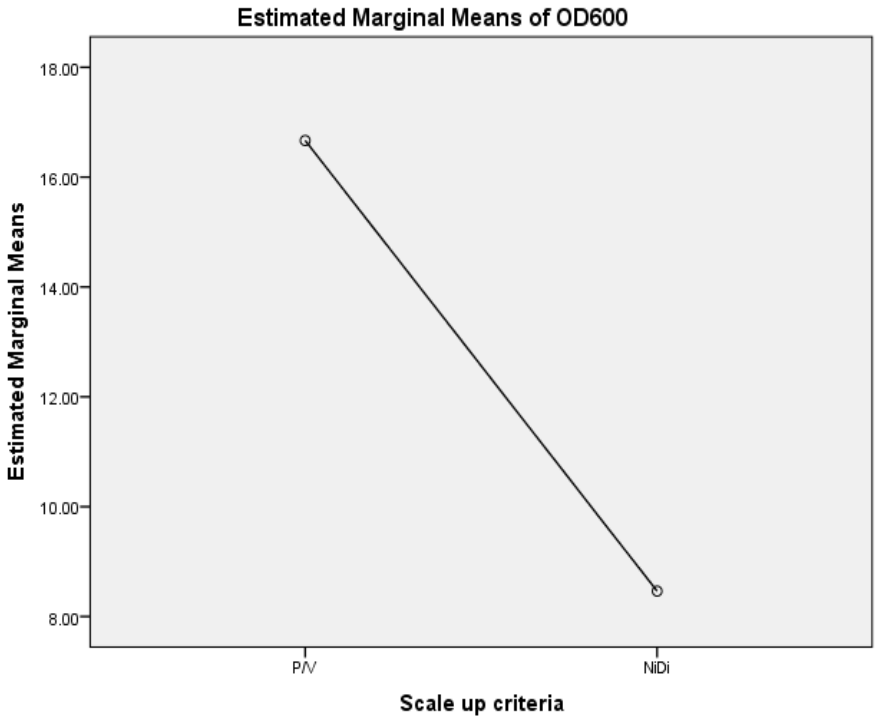
Dependent Variable: OD600

Source	Type III Sum of Squares	df	Mean Square	F	Sig.[b]
Corrected Model	305.231[a]	5	61.046	944.661	.000
Intercept	2841.575	1	2841.575	43972.099	.000
Seed1Volume	1.033	2	.517	7.994	.006
Mixing	302.908	1	302.908	4687.367	.000
Seed1Volume * Mixing	1.289	2	.645	9.975	.003
Error	.775	12	.065		
Total	3147.581	18			
Corrected Total	306.006	17			

a. R Squared = .997 (Adjusted R Squared = .996)

b. Significant level = 0.05 (confident intervals of 95%)





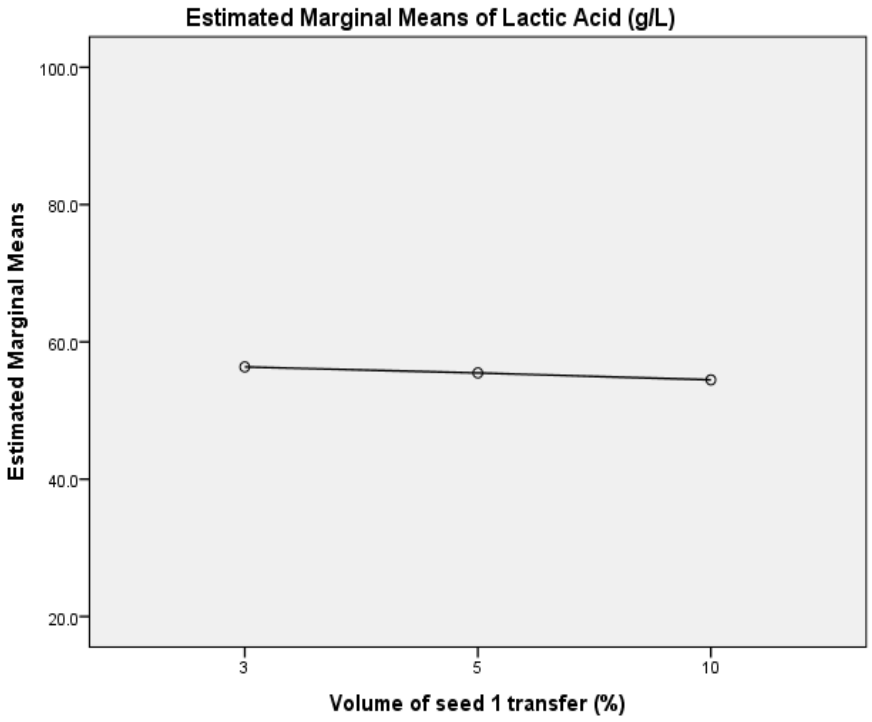
D2.2. Final concentration of D-lactic acid (g/L)

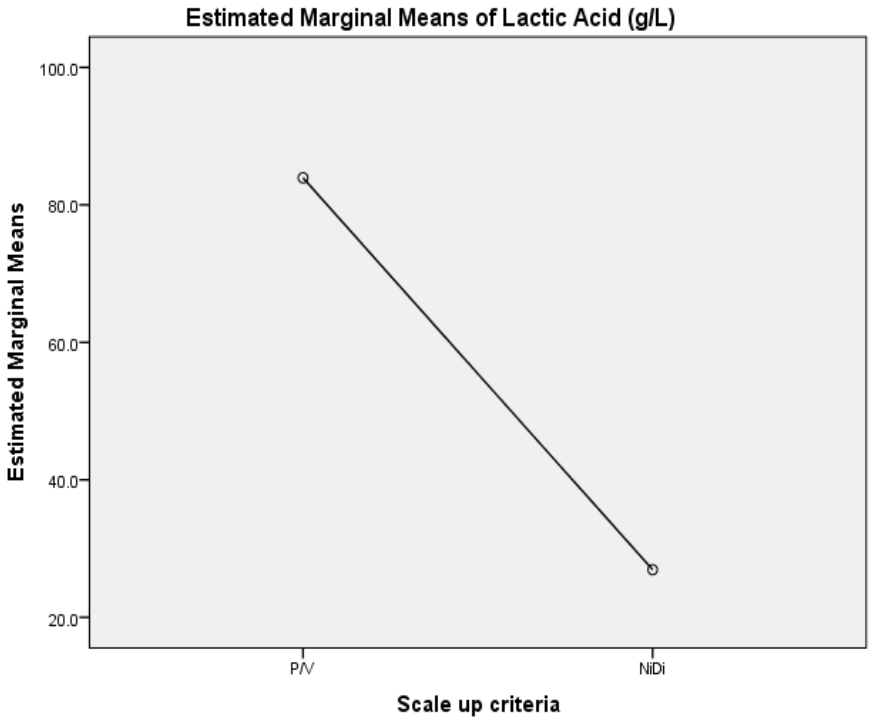
Dependent Variable: Lactic Acid (g/L)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.[b]
Corrected Model	9798.387[a]	5	1959.677	26722.873	.000
Intercept	36874.253	1	36874.253	502830.727	.000
Seed1Volume	7.042	2	3.521	48.011	.000
Mixing	9747.000	1	9747.000	132913.636	.000
Seed1Volume * Mixing	44.345	2	22.173	302.352	.000
Error	.440	6	.073		
Total	46673.080	12			
Corrected Total	9798.827	11			

a. R Squared = 1.000 (Adjusted R Squared = 1.000)

b. Significant level = 0.05 (confident intervals of 95%)


Estimated Marginal Means of Lactic Acid (g/L)
Estimated Marginal Means
100.0
80.0
60.0
40.0
20.0
3
5
10
Volume of seed 1 transfer (%)


Estimated Marginal Means of Lactic Acid (g/L)
Estimated Marginal Means
100.0
80.0
60.0
40.0
20.0
P/V
NiDi
Scale up criteria

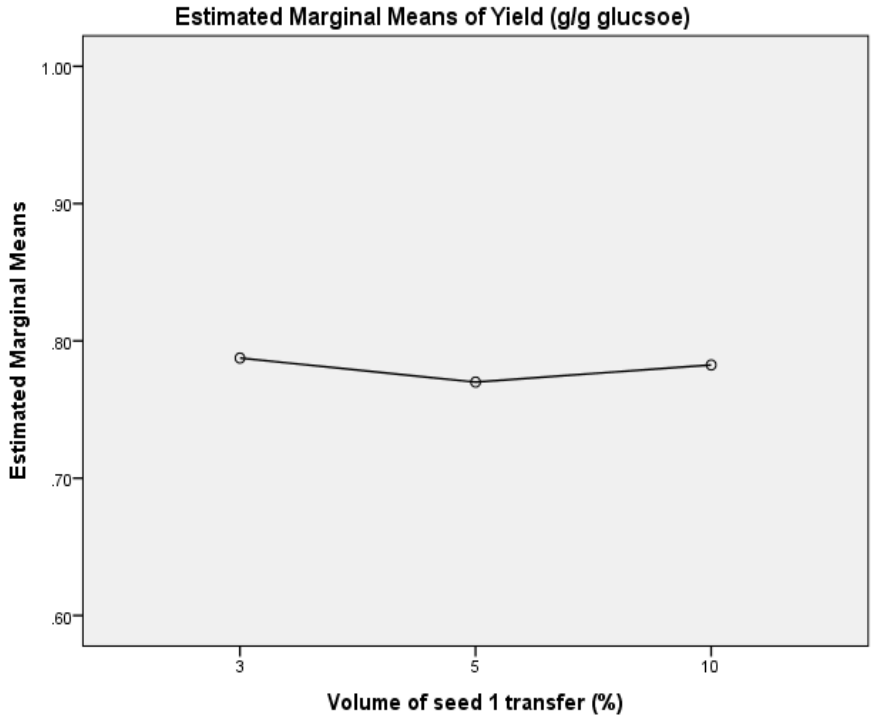
D2.3. Yield (g/g glucose)

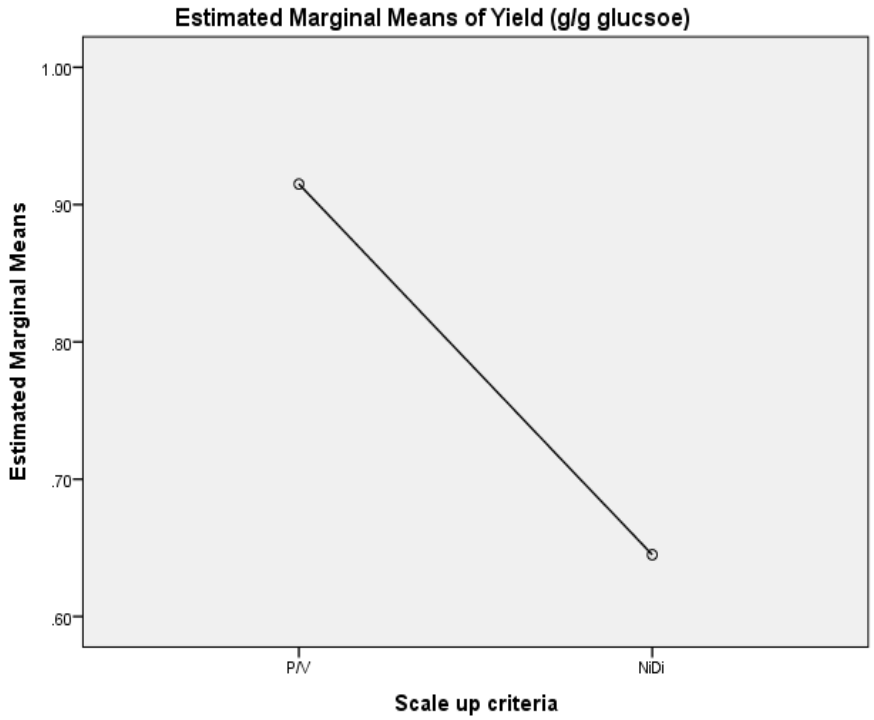
Dependent Variable: Yield (g/g glucsoe)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.[b]
Corrected Model	.220[a]	5	.044	526.800	.000
Intercept	7.301	1	7.301	87609.600	.000
Seed1Volume	.001	2	.000	3.900	.082
Mixing	.219	1	.219	2624.400	.000
Seed1Volume * Mixing	.000	2	7.500E-05	.900	.455
Error	.001	6	8.333E-05		
Total	7.521	12			
Corrected Total	.220	11			

a. R Squared = .998 (Adjusted R Squared = .996)

b. Significant level = 0.05 (confident intervals of 95%)





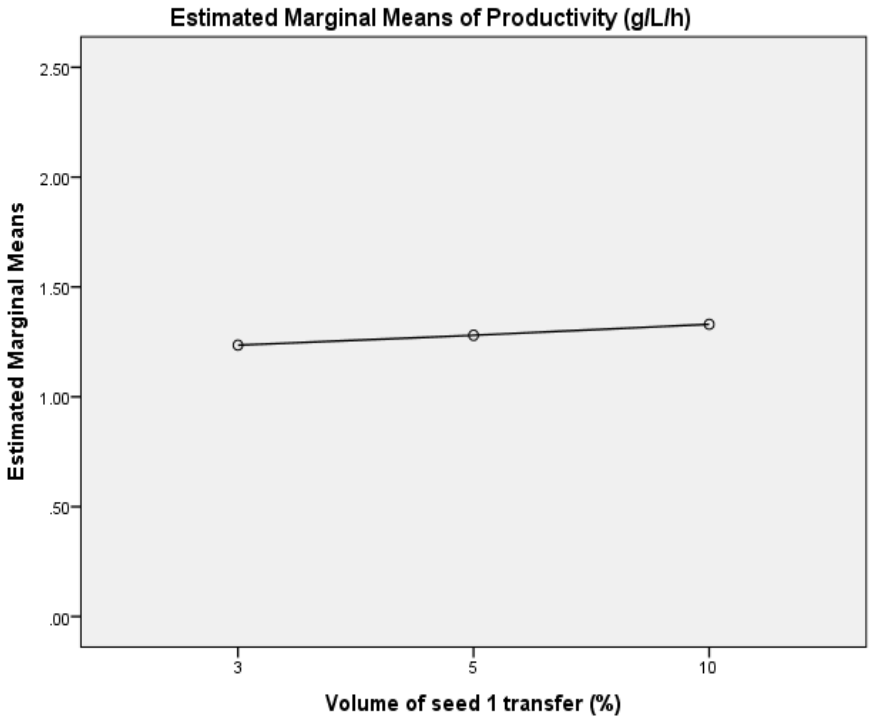
D2.4. Productivity (g/L/h)

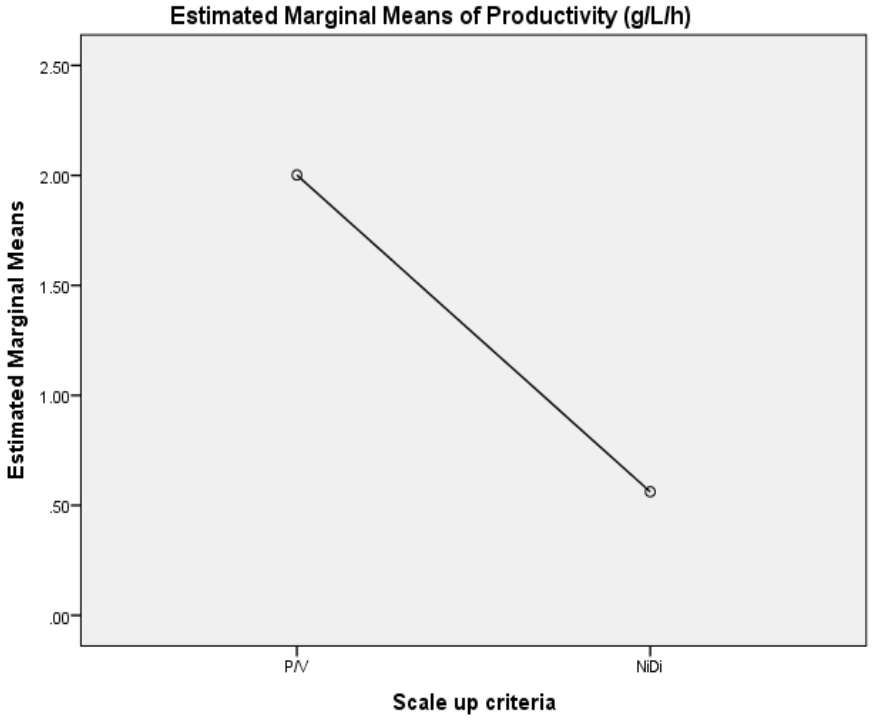
Dependent Variable: Productivity (g/L/h)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.[b]
Corrected Model	6.247[a]	5	1.249	24986.667	.000
Intercept	19.712	1	19.712	394240.667	.000
Seed1Volume	.018	2	.009	180.667	.000
Mixing	6.221	1	6.221	124416.000	.000
Seed1Volume * Mixing	.008	2	.004	78.000	.000
Error	.000	6	5.000E-05		
Total	25.959	12			
Corrected Total	6.247	11			

a. R Squared = 1.000 (Adjusted R Squared = 1.000)

b. Significant level = 0.05 (confident intervals of 95%)


Estimated Marginal Means of Productivity (g/L/h)
Estimated Marginal Means
2.50
2.00
1.50
1.00
.50
.00
3
5
10
Volume of seed 1 transfer (%)


Estimated Marginal Means of Productivity (g/L/h)
Estimated Marginal Means
2.50
2.00
1.50
1.00
.50
.00
P/V
NiDi
Scale up criteria

VITA

Mr. Woraphot Toliang was born on Monday, January 6th, 1992, in Suphanburi, Thailand. He graduated High School in Mathematics-Science program from Suankularb Wittayalai School in 2010. Then, he graduated with a Bachelor's degree of Science in Microbiology , from Chulalongkorn University in 2014. After that, he has been studied for a Master's degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University, and completed the program in 2017.

Research presentation experience

Toliang, W., Thitiprasert, S., Thongchul, N. A novel in-house isolate Terrilactibacillus laevilacticus SK5-6 for D-lactic acid production. Poster presentation and proceedings. The 7th International Conference on Fermentation Technology for Value Added Agricultural Products. July 25th–28th, 2017. Pullman Khon Kaen Raja Orchid Hotel, Khon Kaen, Thailand.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY