การปรับตัวของ *Terrilactibacillus laevilacticus* SK5-6 เพื่อการเจริญและผลิตกรดดี-แลกติกในอาหารเลี้ยงเชื้อที่มีสารสกัดจากยีสต์ความเข้มข้นต่ำ

นายเศรษฐพัฒก์ ลิมสัมพันธ์เจริญ

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

ADAPTATION OF *Terrilactibacillus laevilacticus* SK5-6 FOR GROWTH AND D-LACTIC ACID PRODUCTION IN A LOW YEAST EXTRACT CONCENTRATIO N MEDIUM

Mr. Srettapat Limsampancharoen

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	ADAPTATION OF *Terrilactibacillus laevilacticus* SK5-6 FOR GROWTH AND D-LACTIC ACID PRODUCTION IN A LOW YEAST EXTRACT CONCENTRATION MEDIUM
By	Mr. Srettapat Limsampancharoen
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Nuttha Thongchul, Ph.D.
Thesis Co-Advisor	Associate Professor Kaemwich Jantama, 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
(Associate Professor Kaemwich Jantama, Ph.D.)

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

....Examiner
(Sitanan Thitiprasert, Ph.D.)

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

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

เศรษฐพัฒก์ ลิมสัมพันธ์เจริญ : การปรับตัวของ *Terrilactibacillus laevilacticus* SK5-6 เพื่อการเจริญและผลิตกรดดี-แลกติกในอาหารเลี้ยงเชื้อที่มีสารสกัดจากยีสต์ความเข้มข้นต่ำ (ADAPTATION OF *Terrilactibacillus laevilacticus* SK5-6 FOR GROWTH AND D-LACTIC ACID PRODUCTION IN A LOW YEAST EXTRACT CONCENTRATION MEDIUM) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ณัฏฐา ทองจุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. เขมวิทย์ จันต๊ะมา, 70 หน้า.

กรดแลกติกมีการใช้อย่างกว้างขวางในอาหาร เครื่องสำอาง และผลิตภัณฑ์ที่เกี่ยวกับยา นอกจากนี้กรดแลกติกสามารถใช้เป็นวัตถุดิบในการสังเคราะห์ พอลิแลกติกแอซิด (PLA) ซึ่งเป็นพลาสติกที่ย่อยสลายได้ โดยความต้องการของพลาสติกชีวภาพรวมทั้ง PLA เพิ่มขึ้นเนื่องจากผลกระทบด้านสิ่งแวดล้อมของพลาสติกที่ผลิตจากปิโตรเลียม ดังนั้นจึงเป็นการเร่งความต้องการของตลาดกรดแลกติก จากการสังเกตกรดแลกติกเกรดพอลิเมอร์จะแตกต่างจากที่ใช้ในเชิงพาณิชย์ทางด้านอาหาร และยา โดยในการพอลิเมอไรเซชั่น PLA ต้องการกรด ดี-แลกติก หรือ แอล-แลกติก ที่มีความบริสุทธิ์แสงสูง นอกจากนี้ต้นทุนในการผลิตจะต้องต่ำเพื่อให้สามารถแข่งขันกับพลาสติกชนิดอื่นที่ใช้กันอย่างแพร่หลายในปัจจุบัน โดยจะต้องพัฒนาแพลตฟอร์มในการหมักให้ง่ายและมีประสิทธิภาพสำหรับการผลิตกรดแลกติก ซึ่งสายพันธุ์จุลินทรีย์เป็นปัจจัยหนึ่งที่สำคัญ *Terrilactibacillus laevilacticus* SK5-6 เป็นแบคทีเรียสายพันธุ์ใหม่ที่ผลิตกรดแลกติกได้ คัดแยก และคัดกรองเบื้องต้นเพื่อผลิตกรดดี-แลกติก พบอัตราการผลิตสูงในการเพาะเลี้ยงขนาดเล็กโดยใช้อาหารเลี้ยงเชื้อที่อุดมไปด้วยสารสกัดจากยีสต์ ในอุตสาหกรรมการหมักกรดแลกติก สารสกัดจากยีสต์ถือเป็นส่วนประกอบสำคัญที่ช่วยในการเจริญเติบโตของเซลล์ และการผลิตกรดแลกติก และยังเป็นส่วนที่ทำให้ต้นทุนการผลิตสูง ในวิทยานิพนธ์ฉบับนี้ได้มีการนำเทคนิคการปรับตัวทางฟีโนไทป์ มาใช้ในสายพันธุ์ *Terrilactibacillus laevilacticus* SK5-6 เพื่อผลิตกรดดี-แลกติกในอาหารเลี้ยงเชื้อที่มีสารสกัดจากยีสต์ลดลง โดยสายพันธุ์ *Terrilactibacillus laevilacticus* SK5-6 ที่ผ่านการปรับตัว จะมีต้นทุนอาหารต่ำกว่าที่ต้องการในการผลิตกรดแลกติก ในช่วงการปรับตัวทางฟีโนไทป์ พบว่าช่วงแรกของการเพาะเลี้ยง (T4-T8) มีความเข้มข้นของเซลล์สุดท้ายต่ำ หลังจากนั้นความเข้มข้นของเซลล์สุดท้ายจะเพิ่มขึ้นและใกล้เคียงกับค่าทฤษฎี (อัตราผลผลิต 5% จากกลูโคส) ต่อมาได้นำสายพันธุ์ *Terrilactibacillus laevilacticus* SK5-6 ที่ผ่านการปรับตัว มาใช้ในการหมักกรดดี-แลกติก ความเข้มข้นผลผลิตสุดท้ายและอัตราผลผลิตในการผลิตที่คล้ายกับการหมักด้วยสายพันธุ์ปกติในอาหารปกติ จากผลที่พบในวิทยานิพนธ์นี้ การปรับตัวทางฟีโนไทป์ ได้รับการยืนยันว่าเป็นวิธีที่ดีในการปรับปรุงเพื่อเพิ่มประสิทธิภาพในกระบวนการหมัก

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

สาขาวิชา เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต ....
ปีการศึกษา 2560	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
	ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

5872116123 : MAJOR BIOTECHNOLOGY
KEYWORDS: D-LACTIC ACID / FERMENTATION / YEAST EXTRACT REDUCTION / METABOLIC EVOLUTION / METABOLIC ENGINEERING

SRETTAPAT LIMSAMPANCHAROEN: ADAPTATION OF *Terrilactibacillus laevilacticus* SK5-6 FOR GROWTH AND D-LACTIC ACID PRODUCTION IN A LOW YEAST EXTRACT CONCENTRATION MEDIUM. ADVISOR: ASSOC. PROF. NUTTHA THONGCHUL, Ph.D., CO-ADVISOR: ASSOC. PROF. KAEMWICH JANTAMA, Ph.D., 70 pp.

Lactic acid has been extensively used in food, personal care, and pharmaceutical products. Besides, lactic acid can be used as the sole feedstock in the synthesis of polylactic acid (PLA), the biodegradable plastic. The demand of the bioplastics including PLA has been increased due to the environmental impact of petroleum based plastic; therefore, this expedited the market demand of lactic acid. It should be noted that the polymer grade lactic acid is unlike those commercially available products for food and pharmaceutical applications. The high purity of stereoisomer either D-lactate or L-lactate is mandatory in PLA polymerization. In addition, the production cost should be low in order to be competitive with the plastic commodities widely used nowadays. To fill those production gaps, one needs to develop the simple yet effective fermentation platform for lactic acid production. Microbial strain is one of the crucial factors. *Terrilactibacillus laevilacticus* SK5-6, a novel lactic acid producing genus, was preliminary isolated and screened for the production of D-lactic acid at a remarkable high production rate in a small scale culture using the enriched culture medium containing yeast extract. In industrial lactate fermentation, yeast extract is considered as the key component that facilitate cell growth and lactate production yet mainly contribute to high production cost. In this thesis, phenotypic adaptation technique was implemented in *T. laevilacticus* SK5-6 to produce D-lactic acid in the production medium containing a lowering concentration of yeast extract. With the new adaptive isolate of *T. laevilacticus* SK5-6 to be obtained, a lower medium cost required in lactic acid production was acquired. During phenotypic adaptation, it was found that at the beginning of the preculture transfer (T4-T8), the low final cell biomass concentration was obtained. After that, the final cell biomass concentration was increased and became comparable to the theoretical value (5% biomass conversion yield from glucose). Later, the adapted isolate *T. laevilacticus* SK5-6 T20 was used in D-lactic acid fermentation. The similar final product concentration and yield with the prolonged production to that obtained in the fermentation by the wild type isolate in the original base case medium were obtained. From the findings in this thesis, phenotypic adaptation was claimed as an excellent tool in strain improvement for enhancing the fermentation process efficiency.

Field of Study: Biotechnology	Student's Signature ....
Academic Year: 2017	Advisor's Signature ....
	Co-Advisor's Signature ....

CHULALONGKORN UNIVERSITY

ACKNOWLEDGEMENTS

First of all, I would like to express my deeply appreciation to my thesis advisor, Assoc. Prof. Dr. Nuttha Thongchul, for expert guidance, support, understanding, patience, and kindheartedness during my Master Degree study. Her invaluable advice and proofreading make my thesis more complete.

Besides, I would like to express my sincerely gratitude to all members of thesis committee; Assoc. Prof. Nattaya Ngamrojanavanich, Assoc. Prof. Dr. Kaemwich Jantama, Assoc. Prof. Dr. Aphichart Karnchanatat, Dr. Sitanan Thitiprasert, and Asst. Prof. Dr. Suwattana Pruksasri for comments and suggestions. In addition, my sincere thanks also go to professors and researchers in the Institute of Biotechnology and Genetic Engineering.

Unforgettably, I would also like to extend my sincere thanks to Dr. Sitanan Thitiprasert, Miss Jirabhorn Piluk, Dr. Pajareeya Songserm, Dr. Budsabathip Prasirtsak, and Miss Tanapawarin Rampai various members of my laboratory in the Institute of Biotechnology and Genetic Engineering, for help and great atmosphere at work.

Furthermore, I greatly thankful to the Institute of Biotechnology and Genetic Engineering for all facilities and grant.

Most of all, I would like to express my deep appreciation to my beloved family and best friend for their greatest love, support, and encouragement.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CONTENTS

Page

THAI ABSTRACT ... iv
ENGLISH ABSTRACT ... v
ACKNOWLEDGEMENTS ... vi
CONTENTS ... vii
CHAPTER 1 INTRODUCTION ... 1
1.1 Background and Rationale ... 1
1.2 Research objectives and scope of work ... 5
1.3 Expected outcome ... 5
CHAPTER 2 THEORETICAL AND LITERATURE REVIEWS ... 6
2.1 Lactic acid ... 6
2.2 Application ... 8
2.2.1 Food and beverage industry ... 8
2.2.2 Cosmetic industry ... 9
2.2.3 Pharmaceutical industry ... 9
2.2.4 Polymer industry ... 9
2.3 Lactic acid production ... 10
2.3.1 Chemical synthesis ... 10
2.3.2 Microbial fermentation ... 12
2.3.2.1 Fungal fermentation ... 12
2.3.2.2 Bacterial fermentation ... 12
2.4 Genetic engineering ... 18
2.4.1 Metabolic Engineering ... 18
2.4.1.1 Gene deletion ... 19
2.4.1.2 Gene overexpression ... 20
2.4.1.3 Genetic and metabolic engineering in lactic acid production ... 20
CHAPTER 3 EXPERIMENTAL ... 23
3.1 Apparatus and Chemicals ... 23
3.1.1 Apparatus ... 23

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Page

3.1.2 Chemicals 24

3.2 Microorganism and inoculum preparation 25

3.3.1 Microorganism 25

3.3.2 Inoculum preparation 25

3.3 Medium composition 26

3.3.1 GYP agar medium (per liter) 26

3.3.2 GYP agar medium with reduced yeast extract (per liter) 26

3.3.3 Preculture medium for flask fermentation (per liter) 27

3.3.4 Preculture medium with reduced yeast extract for fleaker experiment (per liter) 27

3.3.5 Batch fermentation medium for fleaker experiment (per liter) 28

3.3.6 Salts solution (per 10 mL) 28

3.4 Methodology 29

3.4.1 Determining the growth profile of *T. laevilacticus* SK5-6 in the preculture step 29

3.4.2 Determining the initial yeast extraction concentration for adaptation 29

3.4.3 Phenotypic adaptation of *T. laevilacticus* SK5-6 in the fleaker 30

3.4.4 Testing D-lactate fermentation of the adapted isolate 31

3.5 Sample analyses 32

3.5.1 Measuring the OD and determining cell biomass concentration 32

3.5.2 Rapidly detecting glucose concentration by YSI glucose-lactate analyzer 32

3.5.1 Determining the remaining glucose and metabolites 33

3.5.2 Determining the optical purity of lactate product 33

CHAPTER 4 RESULTS AND DISCUSSION 34

4.1 Effect of inoculum size in preculture step in shake flask for the parental *T. laevilacticus* SK5-6 34

4.2 Growth of *T. laevilacticus* SK5-6 during preculture step with varied yeast extract concentrations 36

4.3 Adaptation of *T. laevilacticus* SK5-6 in the fleaker 42

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Page

4.4 D-lactic acid production by the adapted isolate *T. laevilacticus* SK5-6 T20 51

CHAPTER 5 CONCLUSION AND RECOMMENDATIONS 58

5.1 Summary... 58

5.2 Recommendations 59

REFERENCES 60

APPENDIX A ANALYTICAL METHOD 64

APPENDIX B CALCULATION.. 68

VITA 70

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF TABLE

Table 2.1 Identification and physical and chemical properties………………………..7

Table 2.2 Enhanced properties of lactic acid production via different engineering approaches……………………………………………………………………………21

Table 4.3 Growth profile determination during preculture step of *T. laevilacticus* SK5-6 in the shake flask with varied inoculum size…………………………...………35

LIST OF FIGURE

Figure 2.1 Isomer structure of Lactic acid……………………………………….….6

Figure 2.2 Homofermentative pathway for lactic acid production…………………...15

Figure 2.3 Heterofermentative pathway for lactic acid production…………………..16

Figure 2.4 A schematic of chemical synthesis and microbial fermentation…………..17

Figure 4.1 Effect of the inoculum size on the cell growth of *T. laevilacticus* SK5-6 during the preculture step in the flask culture. The culture was incubated at 37 °C, 200 rpm……………………………………………………………………………………35

Figure 4.2 Growth profile *T. laevilacticus* SK5-6 grown in the medium with varied yeast extract concentrations during the preculture step in the flask culture. The culture was incubated at 37 °C, 200 rpm…………………………………………………...36

Figure 4.3 Effect of yeast extract concentration on glucose consumption and lactate production of *T. laevilacticus* SK5-6 during the preculture step in the flask culture. The culture was incubated at 37 °C, 200 rpm. A. base case, B. 20% reduction, C. 50% reduction, D. 60% reduction, E. 70% reduction, F. 80% reduction……………….......39

Figure 4.4 Growth profiles of *T. laevilacticus* SK5-6 in the fleaker. The culture was incubated at 37 °C, 300 rpm. A. Base case, 80% and 90% reduction. B. 95%, 97% and 99% reduction and no add yeast extract……………………………….………..46

Figure 4.5 Effect of yeast extract concentration on glucose consumption and lactate production of *T. laevilacticus* SK5-6 during the preculture step in the fleaker. The culture was incubated at 37 °C, 300 rpm. A. 95% reduction, B. 97% reduction, and C. 99% reduction…………………………………………………………..………….47

Figure 4.6 Phenotypic adaptation of *T. laevilacticus* SK5-6 for improved growth and lactate production in the preculture medium with 97% reduced yeast extract

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

concentration A. cell density (OD_{600}), B. Glucose consumption and lactate production. The culture was incubated at 37 °C, 300 rpm in the fleaker…………………………...49

Figure 4.7 Micrographs of *T. laevilacticus* SK5-6. A. Wild type, B. Adapted strain..50

Figure 4.8 Effect of cultivation time of adapted strains *T. laevilacticus* SK5-6 in the preculture medium with 97% reduced yeast extract concentration. The culture was incubated at 37 °C, 200 rpm in the flask culture………………………………………51

Figure 4.9 Growth profile of the adapted *T. laevilacticus* SK5-6 T20 grown in the preculture medium with 97% reduced yeast extract concentration during the preculture step in the fleaker. The culture was incubated at 37 °C, 300 rpm……………….……..…52

Figure 4.10 Effect of inoculum age on growth of the adapted *T. laevilacticus* SK5-6 T20 during the fermentation step in fleaker. The culture was incubated at 37 °C, 300 rpm……………………………………………………………………………………....53

Figure 4.11 Effect of inoculum age on glucose consumption and lactate production of the adapted *T. laevilacticus* SK5-6 T20 during the fermentation step in fleaker. The culture was incubated at 37 °C, 300 rpm. A. Mid log phase, B. Late log phase……….54

Figure 4.12 Fermentation kinetics profiles A. cell density (OD_{600}), B. Glucose consumption and lactate production of the adapted *T. laevilacticus* SK5-6 T20 during the fermentation stage in the fleaker. The culture was incubated at 37 °C, 300 rpm…..57

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Nowadays, the plastic demand is increased significantly because they have been extensively used in many applications. For example, they can replace metals in manufacturing of automobile parts or digital devices according to their superior properties which are cheaper, lighter, safer, and easier to be recycled than metal components. Plastics currently available are produced from petrochemical feedstocks. While the plastic demand is kept rising up, the scarcity and the environmental impact of non degradable wastes should be aware of. Although landfilling and combustion facility have been claimed to properly manage plastic wastes, without the strict control regulation, high chances of contamination by the toxic derivatives after decomposition by the approaches mentioned above to the environments nearby can be existed (Goodall, 2011). Despite the profound environmental pollution generated by a high consumption of petrochemical based plastics, plastics are still widely available due to their versatile applications. This generated the motivation to develop the materials that are easily degraded biologically. Bioplastic is one of such products produced from biobased feedstocks via biorefinery platform. It is also considered as an environmental friendly product as it is biocompatible and compostable under the proper conditions (Müller et al., 2012). One of the promising bioplastic widely well-known is polylactic

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

acid (PLA). PLA is commonly synthesized by ring opening polymerization of the optically pure lactic acid (Zhao et al., 2010).

Lactic acid (2-hydroxypropionic acid or $CH_3CHOHCOOH$) naturally exists in 3 forms, e.g. L-lactic acid, D-lactic acid, and DL-lactic acid while D-lactic acid is claimed to be toxic to the living organisms when exposed at high level (Reddy et al., 2008). It is noted that pure enantiomers of lactic acid are more valuable than the racemates due to their own specific industrial applications (Abdel-Rahman et al., 2011, Ali et al., 2010). Lactic acid can be produced via chemical synthesis or microbial fermentation. Chemical synthesis requires petroleum feedstocks and yields the racemic mixtures of lactic acid while microbial fermentation utilizes the renewable feedstocks which low cost substrate to produce an optically pure L-or D-lactic acid depending on the organism under mild conditions resulting in low energy consumption. Chemical synthesis requires petroleum feedstocks and drastic conditions while fermentation process utilizes the renewable feedstocks at mild conditions; thus, considered as the green process (Ren, 2011, Abdel-Rahman et al., 2013). Another advantage of fermentation process is that the optical purity of lactic acid product can be controlled at the desirable percentage by selecting a correct microbial strains whereas chemical synthesis always results in a racemic mixture of lactic acid product (Oh et al., 2005). Therefore, fermentation process is preferable. Via fermentation, lactic acid can be produced by bacteria or fungi. Lactic acid bacteria exhibit their ability to produce D, L, and DL-lactic acid, depending on their lactate dehydrogenases and racemases (John et al., 2009, Wee et al., 2006, Xu et al., 2010). The typical bacteria used in lactic acid production include *Bacillus* sp., *Lactobacillus* sp., *Streptococcus* sp. *Enterococcus* sp. *Rhizopus* sp., *Mucor* sp., and *Monilia* sp. Bacterial fermentation yields the high

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

productivity and yield while fungal fermentation gives the pure L-lactic acid enantiomer. Nonetheless, fungal fermentation is a complex process due to the morphological change during operation; thus, leads to the difficulty in operation and design. Therefore, to date bacterial fermentation is still preferable (Auras et al., 2010) in commercial production.

Currently, lactic acid produced via bacterial fermentation is mainly used in food and pharmaceutical applications as the additives in the racemic form (approximately 85%ee). On the other hand, PLA synthesis requires the pure enantiomer of lactic acid in order to achieve the desirable plastic properties similarly to those acquired from petroleum based plastics (John et al., 2009). Thus, this makes lactate production process for PLA different from those previously produced for food and pharmaceutical industries. Additionally, previously lactate consumption in those existing industries was in a small volume compared to the demand as the major feedstocks in PLA synthesis; thus, the production cost is another key factor. As a result, to supply lactate feedstocks for PLA industry, one needs the high production rate with the extremely high optical purity beyond 99.0%ee and the low production cost. There have been many attempts proposed in order to improve the production of lactic acid including fermentation and downstream process optimization to achieve the most plausible highest yield and productivity with the lowest cost. In addition, genetic and metabolic engineering were introduced to modify the microorganisms to perform fermentation as desired.

Although lactic acid bacteria produced lactic acid at high titer and yield, the strains typically exhibited slow growth and thus slightly low lactate productivity in

culture media without nutrient enrichment. Therefore, yeast extract and peptone have been simply used in lactic acid culture media to increase the production rate. The high nutritional requirements of lactic acid bacteria resulted in high production cost, difficulty in purification and waste disposal (Sangproo et al., 2012). Under inappropriate fermentation conditions, lactic acid bacteria were likely switching their metabolism towards mixed acid fermentation pathway instead of homofermentative pathway to solely produce lactic acid. As a result, end products such as acetic acid, acetaldehyde, ethanol, and diacetyl, were formed during fermentation. Furthermore, under inappropriate conditions, most of lactic acid bacteria rather produced the racemates resulted in the low percentage of optical purity less than 95%ee which did not meet the specification of polymer grade lactic acid (Zhou et al., 2006).

From the production gaps previously mentioned, the ideal lactic acid producing bacteria should exhibit high production rate with sufficiently rapid growth to support growth associated product formation kinetic model. In addition, low nutritional requirement is mandatory to reduce the production cost. Many attempts have been introduced to improve lactic acid bacterial fermentation. Metabolic engineering is one of the tools that has been successfully applied to several bacteria. It was also reported that the metabolic evolution by growth-based selection significantly improved the rate of D-lactate production from glucose and sucrose. The resulting strains would be expected to efficiently produce D-lactate equivalent to those of previously developed strains (Sangproo et al., 2012). An outstanding example of the evolved strains is the evolved strains of *Escherichia coli* KJ122 to highly produce succinic acid by the minimal medium (Jantama et al., 2015). While *Krebsilla oxytoca* KMS005 exhibited a

lower biomass generation and slow growth rate compared to its parental strain; thus, enhancing 2,3-butane diol production by the evolved strain (Jantama et al., 2015).

1.2 Research objectives and scope of work

In this work, strain evolution by phenotypic adaptation was applied in order to induce self-mutation in the fastidious lactic acid producing strain to become the low nutrient requirement ones. According to our previous in-house work, *Terrilactibacillus laevilacticus* SK5-6 was screened and fermentation optimization has been preliminarily conducted in a small-scale batch culture (Prasirtsak et al., 2017). The high yield and productivity were obtained at the comparable ranges to the commercial lactate production process. However, a medium supplemented with a relatively high concentration of yeast extract was used to culture this strain for lactate production. In order to make our strain to become more competitive, high final lactate titer with the reduced production cost is expected. To achieve that goal, phenotypic adaptation was introduced to *T. laevilacticus* SK5-6. Moreover, the fermentation platform of the adapted strain was proposed in this work.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

1.3 Expected outcome

The evolved D-lactate producing strain consumed a lower amount of yeast extract compared to its parental *T. leavilacticus* SK5-6.

CHAPTER 2

THEORETICAL AND LITERATURE REVIEWS

2.1 Lactic acid

Lactic acid, chemically known as 2-hydroxypropionic acid, or 2-hydroxypropanoic acid ($CH_3CHOHCOOH$), is an organic acid widely distributed throughout nature discovered in 1780 in sour milk by the Swedish chemist C.W. Scheele. It was first produced commercially in 1881 by Charles E. Avery in Massachusetts, USA. It exists naturally in two enantiomeric forms: L(+)- lactic acid and D(-)-lactic acid (Figure 2.1). The chemical synthesis results in a racemic mixture of two isomers.

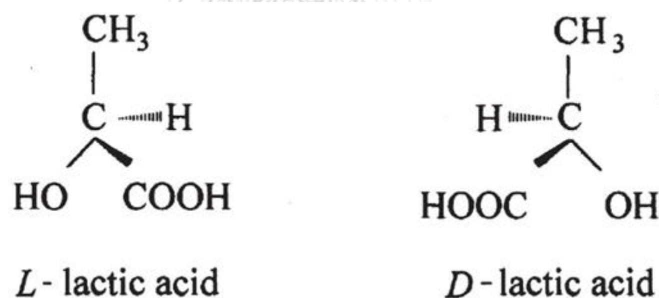


Figure 2.1 Isomer structure of Lactic acid

The difference between the two enantiomeric forms is an adjacent hydroxyl group at the chiral carbon atom. Because of this, they possess some different properties, such as their boiling and melting points. High optical purity lactic acid is colorless, miscible with water or ethanol, acetone and ether. However, it is not soluble in chloroform, petroleum ether or carbon disulfide. Other physical-chemical properties are summarized below in Table 2.1 (Castillo Martinez et al., 2013, Ren, 2011, Vaidya et al., 2005).

Table 2.1 Identification and physical and chemical properties

Parameter	
CAS number	D/L : 50-21-5 L : 79-33-4
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
Boiling point	122 °C (12 mmHg)
Flash point	112 °C
Physical state	Colorless to slightly yellow, syrupy liquid
Solubility in water	Miscible
Taste	Mild acid taste
Toxicity	Oral rat LD50 (3543 mg/kg)
NFPA ratings	Health 3, Flammability 1, Reactivity 1
Stability	Stable under ordinary conditions

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.2 Application

Lactic acid (LA) is a common part of common products because of its widespread application, mainly in pharmaceuticals, cosmetics and chemicals, as well as in the food industry. (Datta et al., 1995). About 70% of the lactic acid produced is utilized in the food production sector due to its crucial role in the manufacturing of yogurt and cheese (Castillo Martinez et al., 2013). Additionally, there is great potential for producing green biodegradable and biocompatible polylactic acid (PLA) polymers which are widely used as a raw material in packaging as well as fibers and foams. However, at the industrial scale, while the PLA is compared with petrochemical raw materials, it can actually be considered to be a relatively immature technology, mainly due to the high production cost of lactic acid as a basic raw material for PLA. Moreover, although the consumption of lactic acid as a substrate for the production of PLA has increased considerably, the amount of produced PLA from lactic acid is still lower than the total amount of petrochemical materials used for plastics production (Wang et al., 2016).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.2.1 Food and beverage industry

Lactic acid is widely applied in food products such as bread, meat, milk, beverages, and desserts, among others. Lactic acid is used as an acidulant to increase acidity and to serve as a pH regulator and flavoring agent. Furthermore, due to the acidity being able to prevent the growth of pathogens, it helps in preservation. Moreover, many fermented foods naturally produce lactic acid such as yogurt, cheese, milk, soy sauce, wine, meat products, and pickled vegetables (Callewaert and De Vuyst, 2000).

2.2.2 Cosmetic industry

In the cosmetics industry, lactic acid is popularly known as alpha hydroxy acid, or AHA. Lactic acid is used as a pH regulator, moisturizer and antimicrobial agent. It is a mixture of cream or lotion to hydrate and rejuvenate the skin. Additionally, by inhibition of the formation of tyrosinase, lactic acid is also used as a skin lightener.

2.2.3 Pharmaceutical industry

Lactic acid is used as a pH regulator that helps to adjust the pH. In addition, lactic acid can enhance drug solubility through use as an intermediary in assisting improvement of the drug combination. Moreover, lactic acid assists in preventing oxidation reactions in vitamin preparation, serves as metal sequestration agent, and a natural body constituent. Due to its descaling and antibacterial properties, lactic acid is also used in cleaning products. Furthermore, lactic acid derivatives are used to treat diseases such as calcium deficiency in bones and teeth and anemia.

2.2.4 Polymer industry

Currently, lactic acid is used as a monomer of polylactic acid or PLA production, a biodegradable plastic that is produced as a substitute for petrochemical plastic. PLA is classified into aliphatic polyester which is a high strength thermoplastic. These can be digested easily by hydrolysis of the ester bond (Abdel-Rahman et al., 2013, Vijayakumar et al., 2008).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.3 Lactic acid production

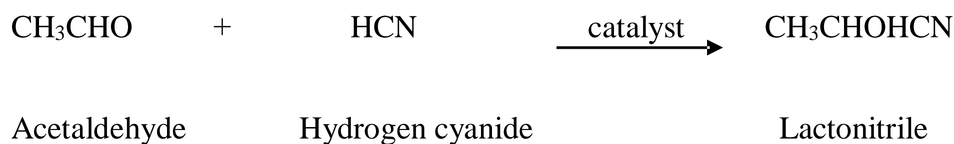
Lactic acid can be produced either by fermentative production routes (lactic acid fermentation) or by chemical synthesis routes. The latter results in a racemic mixture of D/L-lactic acid. While fermentative production routes can provide some advantages, such as utilization of cheap renewables as substrates, a lower amount of energy consumption and production temperatures. In addition, the production of optically pure D- or L-lactic acid by selection of appropriate microorganisms as lactic acid producers can be counted as further advantages (Eş et al., 2018).

2.3.1 Chemical synthesis

Industrially, the chemical synthesis process involves synthesis from lactonitrile. First, lactonitrile is produced by the reaction of hydrogen cyanide (HCN) and acetaldehyde occurring at high atmospheric pressure in the liquid phase. After that, purified lactonitrile is hydrolyzed to lactic acid by sulfuric acid or hydrochloric acid with lactic acid and ammonium salt being obtained. Before purification through distillation and hydrolysis by water, lactic acid is esterified by methanol to produce methyl lactate. This process is explained the following reaction.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

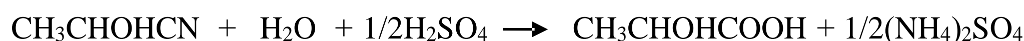
(a) Addition of Hydrogen Cyanide



$$CH_3CHO + HCN \xrightarrow{\text{catalyst}} CH_3CHOHCN$$

Acetaldehyde Hydrogen cyanide Lactonitrile

(b) Hydrolysis by H_2SO_4



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

Lactonitrile Sulfuric acid Lactic acid Ammonium salt

(c) Esterification

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

Lactic acid Methanol Methyl lactate

(d) Hydrolysis by H_2O

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

Methyl lactate Lactic acid Methanol

However, chemical synthesis has some disadvantages including the fact that the reaction has to occur at high atmospheric pressure. Additionally, chemical synthesis requires chemical derivatives from petrochemicals as raw materials which are currently rising in cost and are not environmentally friendly. Furthermore, the racemic mixture of the two isomers obtained by the chemical process cannot be used in the food and pharmaceutical industries due to the toxicity of D-lactic acid to the human body. Therefore, the fermentation process is preferable (Farooq et al., 2012).

CHULALONGKORN UNIVERSITY

2.3.2 Microbial fermentation

Lactic acid can be produced by several microorganisms, including bacteria, fungi, yeast, algae, and cyanobacteria. The selection of the strain is of great importance, especially regarding the reduced nutritional requirements, the ability for producing high optical purity lactic acid, and its ability to promote high yields and productivities. The fermentation process to lactic acid can be classified according to the type of LAB used (Rodrigues et al., 2017).

2.3.2.1 Fungal fermentation

Fungi are able to produce lactic acid from agricultural raw materials that are cheap and easy to find because of their ability to produce extracellular amylase to hydrolyze raw materials such as starch into sugars, a substrate of lactic acid production and cell growth. A widely available group of fungal strains with the ability to produce lactic acid is *Rhizopus* sp. The cost of fungal fermentation is high due to the requirement of a lot of oxygen for growth and lactic acid production. Moreover, fungal fermentation is hetero fermentation which can produce lactic acid and by-products such as fumaric acid, alcohol, and carbon dioxide.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

2.3.2.2 Bacterial fermentation

Lactic acid bacteria is fastidious; therefore, a wide range of growth factors including specific minerals, amino acids, vitamins, fatty acids, purines, and pyrimidines are required for their growth and biological activity. Additionally, lactic acid fermentation by bacteria requires an anaerobic condition. As a result, oxygen supply is not mandatory in the process. Compared with fungal fermentation that requires a large amount of oxygen, bacterial fermentation is considered as a simple operation that can

be done in the typical stirred tank bioreactor. Therefore, the production cost is cheaper than fungal fermentation.

There are four major steps in a typical bacterial fermentation and lactic acid recovery from the fermentation broth as follows:

(a) Fermentation and neutralization

$$C_6H_{12}O_6 + Ca(OH)_2 \xrightarrow{\text{fermentation}} 2(CH_3CHOHCOO^-)\,Ca^{2+} + 2H_2O$$

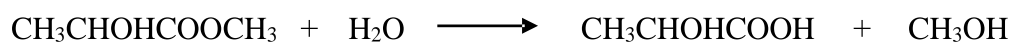
(b) Hydrolysis by H_2SO_4

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

(c) Esterification

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

(d) Hydrolysis by H_2O

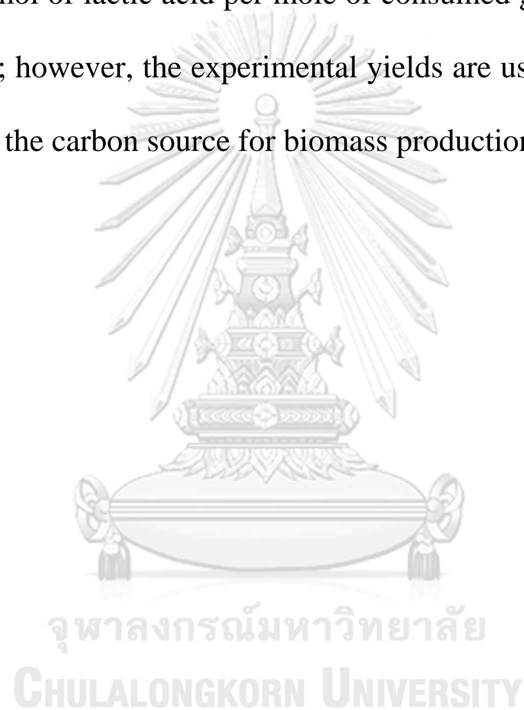


$$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH$$

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Lactic acid bacteria can ferment sugars to lactic acid through different pathways, including homofermentative, heterofermentative, and mixed-acid fermentation.

Homofermentative bacteria produce lactic acid as a major end product and use the Embden Meyerhof Parnas pathway (EMP). Glucose is subsequently converted into pyruvic acid and this is then reduced to lactic acid. Theoretically, the yield of fermentation is 2 mol of lactic acid per mole of consumed glucose (1 g of product per gram of substrate); however, the experimental yields are usually lower because of the use of a portion of the carbon source for biomass production.



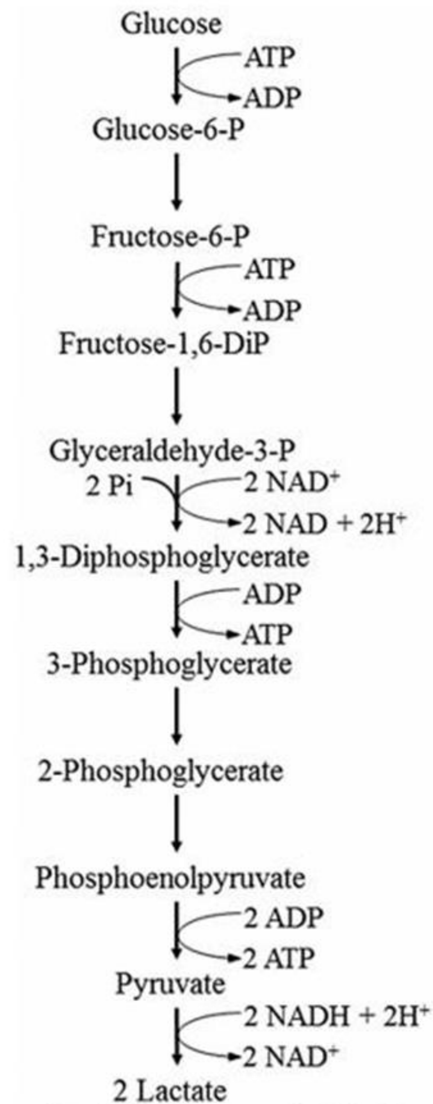


Figure 2.2 Homofermentative pathway for lactic acid production

The heterofermentation process is characterized by the formation of coproducts other than lactic acid such as CO_2, ethanol, and/or acetic acid. The maximal yield of lactic acid per glucose is 1.0 mol/mol or 0.5 g of lactic acid per gram of substrate. This process uses the alternate pentose monophosphate pathway converting 6-carbon sugars (hexoses) to 5-carbon sugars (pentoses) by phosphoketolase.

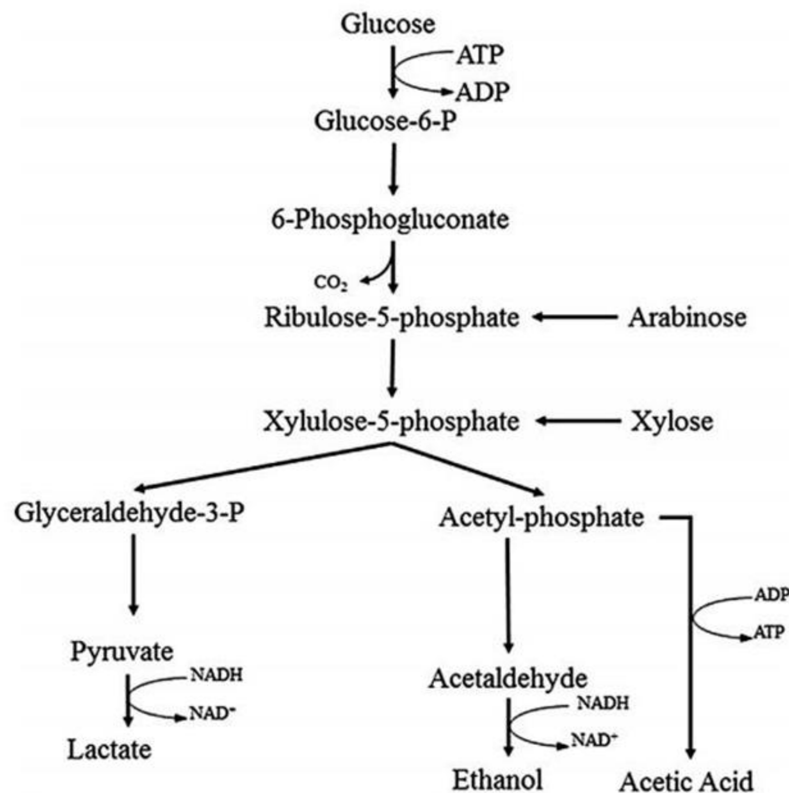


Figure 2.2 Heterofermentative pathway for lactic acid production

As shown above, chemical synthesis requires petroleum feedstock and drastic conditions, while the fermentation process utilizes renewable feedstock such as cellulose, starch, and molasses. Therefore, the latter is considered a green process which incurs low substrate costs. Moreover, bacterial fermentation yields high productivity while fungal fermentation provides 100% optical purity of L-lactic acid. Nonetheless, fungal fermentation is a complex process due to the morphological change during operation, which in turn leads to difficulties in operation and design. Therefore, to date, bacterial fermentation remains preferable options (Castillo Martinez et al., 2013, Datta and Henry, 2006, Thongchul, 2013, Rodrigues et al., 2017).

CHULALONGKORN UNIVERSITY

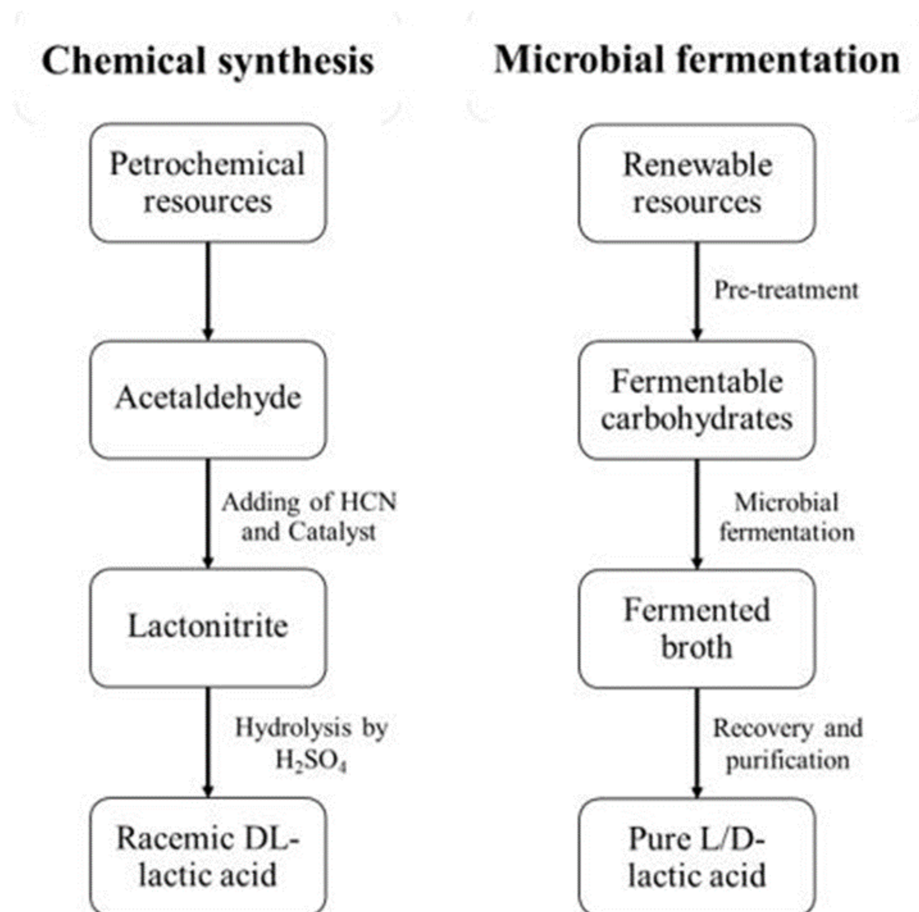


Figure 2.3 A schematic of chemical synthesis and microbial fermentation

There are many significant factors to lactic acid production by microbial fermentation such as microbial strains, nutritional requirements (e.g. carbon source, nitrogen source, mineral, and amino acid), neutralizing agents, substrates, aeration, and fermentation processes (Ghaffar et al., 2014).

Chulalongkorn University

2.4 Genetic engineering

Genetic engineering (also called genetic modification) is the deliberately controlled manipulation of an organism's genome using recombinant DNA technology. It therefore encompasses the use of a set of technologies to change the genetic makeup of cells including the transfer of genes within and across species boundaries with the objective of producing improved or novel organisms and/or unlimited amounts of otherwise unavailable or scarce biological products. Genetic engineering has revolutionized many scientific fields, from fundamental sciences to medicine and engineering, including biotechnology and bioengineering (Oliveira et al., 2017).

2.4.1 Metabolic Engineering

Metabolic engineering comprises the use of genetic engineering tools to modify the cellular metabolism of an organism, either through the optimization of existing biochemical pathways or through the introduction of new pathways or pathway components. The ultimate goal of metabolic engineering is to obtain high-yield production of specific (naturally or synthetically produced) metabolites and/or to provide organisms with the ability to efficiently utilize substrates that is not metabolized.

Metabolic engineering has come a long way since its early days when empirical evolutionary engineering approaches, such as random mutagenesis (natural or induced) followed by screening of mutants with the desired phenotype(s) under selective conditions were the only genetic engineering approaches available to generate microorganisms with enhanced metabolic performances. While fairly effective in the improvement of microorganisms, namely for industrial processes in which restrictions exist regarding the use of genetically modified organisms, random mutagenesis

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

approaches depend highly on suitable screening methods for the improved trait which limits their applications to only a few phenotypic improvements and leads to unknown genetic changes, thus hampering reproduction in other host cells. With the advent of recombinant DNA technology, several genetic engineering tools became available for the rational and targeted modification of metabolic pathways.

Adaptive evolution provides a complementary approach to improve the metabolic performance and robustness of rationally engineered organisms, depending on the design of an appropriate selection stress.

Metabolic evolution strategies still provide an excellent complementary approach for strain improvement in the present-day scenario by allowing the selection for improved performance of reactions/functions that are not currently predictable by existing models.

2.4.1.1 Gene deletion

In metabolic engineering, homologous recombination (HR) is the most frequently used strategy for gene deletion. In organisms that have very efficient HR systems (such as the yeast *S. cerevisiae* and the filamentous fungi *Ashbya gossypii*) genes/genomic regions can be directly disrupted by transformation with linear deletion modules amplified by PCR, containing a selectable marker cassette flanked by short DNA fragments (50–100 bp) homologous to the target gene/genomic region. In organisms with low recombination efficiency, plasmids are used to construct deletion modules with longer flanking homologous regions (500–1000 bp) which are then linearized before transformation. While very useful in allowing the removal and recycling of selectable markers (which are not abundant in variety and are undesirable

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

for industrial purposes), repeated use of these recombination systems for gene deletion may generate unintended chromosomal deletions, interfering with their stability.

2.4.1.2 Gene overexpression

Gene expression is the process by which the information encoded in a gene is transformed into a functional gene product, such as a protein or a functional RNA molecule (e.g., tRNA and rRNA). Protein-coding genes are expressed by being first transcribed into mRNA and then translated into proteins. Cells employ many different mechanisms to regulate gene expression and the understanding of these mechanisms is especially important for metabolic engineering, in which it is desirable to control the expression levels of desired genes (native and heterologous). Often the aim was to express the gene from one organism in another one (Oliveira et al., 2017).

2.4.1.3 Genetic and metabolic engineering in lactic acid production

Metabolic engineering in fermentation technology is an advanced tool to create compelling strains that produce LA with higher productivity and lower cost as well as reduce a by-product formation (Upadhyaya et al., 2014). Common problems in LA production are the low acid tolerance of strains and their ability to utilize a limited amount of substrates. LA is naturally produced by most of the LAB as a primary metabolite. However, chemicals, as well as optical purity of LA, can vary since they consume substrate via inefficient pathways (Eş et al., 2018). The main different genetic and metabolic engineering approaches that have been performed to enhance LA production are shown in Table 2.2.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 2.2 Enhanced properties of lactic acid production via different engineering approaches

Strain	Enhanced properties	Engineering approach	Reference
Synechocystis sp. PCC 6803	Optically pure D-lactic acid	Codon optimization and by balancing the cofactor (NADH) availability through the heterologous expression of a soluble transhydrogenase	(Varman et al., 2013)
Candida sonorensis ldhL strain	Increased productivity	Integration of two copies of the *ldhL* gene	(Koivuranta et al., 2014)
S. cerevisiae strain CEN.PK2-1D	Improved LA titer	Redox balance engineering and heterologous L-lactate dehydrogenase (LDH) gene replacement	(Lee Ju et al., 2014)
Pediococcus acidilactici TY112 and *P. acidilactici* ZP26	High titer L- and D-lactic acid production	*ldhD* or *ldh* gene disruption	(Yi et al., 2016)

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

S. cerevisiae strain CEN.PK2-1C	Increased glucose consumption and growth rate and increased productivity	Expressing *Escherichia coli*-derived acetaldehyde dehydrogenase (A-ALD) enzyme genes	(Song et al., 2016)
S. cerevisiae strain CEN.PK2-1C	Acid tolerance By-product elimination	Over-expression of the transcriptional activator Deletion of *GPD1* and *GPD2* genes	(Baek et al., 2016)
S. cerevisiae strain JHY5610	Acid tolerance Improved productivity By-product elimination Elimination of LA degradation	SUR1^{I245S} mutation Introduction of both SUR1^{I245S} and *erf*2Δ mutations Deletion of *ADH1*, *ADH2*, *ADH3*, *ADH4*, and *ADH5* for ethanol, *GPD1*, and *GPD2* for glycerol production Deletion of *DLD1*	(Baek et al., 2017)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER 3

EXPERIMENTAL

3.1 Apparatus and Chemicals

3.1.1 Apparatus

Apparatus	Model	Manufacturer	Country
Autoclave	KT-40L	ALP Co., Ltd.	Japan
Micro centrifuge	F-45-12-11	Eppendorf	Germany
Electronic balance	ML204/01	Mettler Toledo AG	Switzerland
Electronic balance	ML3002E/01	Mettler Toledo AG	Switzerland
Fleaker	MCL-2CM	B.E. Marubishi	Thailand
High Performance Liquid Chromatography	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
Spectrophotometer	UV-1280	Shimadzu	Japan

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.1.2 Chemicals

Chemicals	Manufacturer	Country
Agar	Patanasis Enterprise	Thailand
Ammonium chloride (NH_4Cl)	Riedel-de Haen	Germany
Calcium carbonate ($CaCO_3$)	Sigma	Germany
Copper sulfate ($CuSO_4 \cdot 5H_2O$)	Fluka	France
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)	Riedel-de Haen	Germany
Potassium phosphate dibasic (K_2HPO_4)	Riedel-de Haen	Germany
Sodium chloride (NaCl)	Sigma	Germany
Yeast extract	Bio springer	France

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.2 Microorganism and inoculum preparation

3.3.1 Microorganism

Novel *Terrilactibacillus laevilacticus* SK5-6 isolated from soil in Thailand, a lactic acid producing bacterium, was used in this study. The culture was grown under an aerobic condition at 37 ºC for 1 day on the Glucose-Yeast Extract-Peptone (GYP agar slant. To maintain the activity, the culture was transferred onto the freshly new GYP agar slant every week (Prasirtsak et al., 2017).

3.3.2 Inoculum preparation

one mL sterile DI water was transferred into 24-h fully-grown culture onto the GYP slant. The slant was thoroughly mixed to obtain the bacterial suspension. The optical density of the suspension was measured at the wavelength of 600 nm. The bacterial suspension was diluted with sterile DI water to the approximate OD_{600} of 30-40. The diluted suspension (0.5 mL) was transferred into the 250 mL Erlenmeyer flask containing 49.5 mL preculture medium. The culture was incubated at 37 ºC, 200 rpm.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.3 Medium composition

3.3.1 GYP agar medium (per liter)

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

3.3.2 GYP agar medium with reduced yeast extract (per liter)

Glucose	10	g
Yeast extract	0.45	g
NH_4Cl	4	g
KH_2PO_4	0.5	g
K_2HPO_4	0.5	g
$CaCO_3$	5	g
Agar	20	g
Salt solution	20	mL

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.3.3 Preculture medium for flask fermentation (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
Salt solution	20	mL

3.3.4 Preculture medium with reduced yeast extract for fleaker experiment (per liter)

Glucose	10	g
Yeast extract	0.45	g
NH_4Cl	4	g
KH_2PO_4	0.5	g
K_2HPO_4	0.5	g
$CaCO_3$	5	g
Salt solution	20	mL

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.3.5 Batch fermentation medium for fleaker experiment (per liter)

Initial glucose	100	g/L
$CaCO_3$	80	g
Yeast extract	0.225	g
NH_4Cl	2	g
KH_2PO_4	0.25	g
K_2HPO_4	0.25	g
Salt solution	10	mL

3.3.6 Salts solution (per 10 mL)

$MgSO_4 \cdot 7H_2O$	400	mg
$MnSO_4 \cdot 5H_2O$	20	mg
$FeSO_4 \cdot 7H_2O$	20	mg
NaCl	20	mg

All media were autoclaved at 121 ºC, 15 psig for 15 min.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

3.4 Methodology

3.4.1 Determining the growth profile of *T. laevilacticus* SK5-6 in the preculture step

T. laevilacticus SK5-6 was cultivated under aerobic conditions on the GYP agar slant at 37 °C for 1 day. 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 approximate 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. 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.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

3.4.2 Determining the initial yeast extraction concentration for adaptation

The preculture medium with reduced yeast extract concentrations (20%, 30%, 40%, 50%, and 80% reduction from the original initial concentration of 15 g/L) contained in the 250 mL flask was inoculated by the suspension previously prepared in 3.4.1 at the defined inoculum size. The preculture flask was incubated at 37 °C, 200 rpm. 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 proper initial yeast extract concentration was determined from the growth profiles.

3.4.3 Phenotypic adaptation of *T. laevilacticus* SK5-6 in the fleaker

The preculture flask (250 mL Erlenmeyer flask) was inoculated by the suspension previously prepared in 3.4.1 at the defined inoculum size. The preculture was cultivated at the conditions defined in 3.4.2 until approaching the correct physiological stage (mid log/late log phases). The preculture broth was then transferred into the 2 L fleaker containing the preculture medium with the reduced yeast extract concentration (80%, 90%, 95%, 97%, 99%, and 100% reduction from the original initial concentration of 15 g/L) to obtain the starting OD of 0.1. The fleaker set (B.E. Marubishi (Thailand)) was equipped with 6 of 2 L Erlenmeyer flask incubated in a temperature-controlled shaking water bath. The pH, temperature, and dissolved oxygen could be monitored and controlled by the sensors installed into the 2 L flask connected to the controller (Mettler Toledo). At the 2-L flask stopper, the gas vent, sampling line, and feeding ports were installed. The fleaker was incubated at 37 °C, 300 rpm. The culture was kept under an anaerobic condition. The sample was collected every 6 h for measuring OD and pH of the fermentation broth and analyzing the remaining glucose and metabolites. The fermentation kinetic profiles were plotted. The growth profiles and the substrate conversion were compared with the theoretical values (1 g glucose was converted to 0.05 g cell biomass under anaerobic conditions).

When the growth profiles were obtained, and the substrate conversion was determined, the initial yeast extract concentration was theoretically determined. At this initial concentration, adaptation was performed by transferring the active culture in the fleaker to another one with the preculture medium containing the theoretically reduced yeast extract concentration at 10% inoculum size. The culture was repeatedly transferred until the growth pattern became stable (at least 5-10 transfers) and

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

approached the theoretical conversion yield. During the transfer, the broth sample was taken for plating on the GYP agar medium with the reduced yeast extract concentration for purification. Later, the pure single colony was selected and stored at -80 °C for further study.

3.4.4 Testing D-lactate fermentation of the adapted isolate

The 250 mL flask containing the preculture medium with the reduced yeast extract (97% reduction) was inoculated by the suspension adapted isolate in 3.4.3 at 1% varied cultivation time (24 h and 48 h). It should be noted that the GYP agar plate was prepared with the reduced yeast extract at 97% reduction. The preculture was cultivated at 37 °C, 200 rpm until reaching the mid log phase. The preculture broth was transferred into the fleaker containing the preculture medium with the reduced yeast extract (97% reduction) at 10% inoculum. The fleaker was incubated at 37 °C, 300 rpm. The culture was kept under an anaerobic condition. The sample was collected every hour for measuring OD and pH of the fermentation broth and analyzing the remaining glucose and metabolites. The preparation of seed 2 for inoculating the fermentation step was performed in the fleaker. Seed 2 with the varied inoculum size (10% and 50%) and inoculum age (mid log phase and late log phase) was transferred into the fermentation medium contained in another fleaker. The culture was incubated at the same operating conditions. The sample was collected every 12 h for measuring OD and pH of the fermentation broth and analyzing the remaining glucose and metabolites. The fermentation was proceeded until glucose depleted or lactate production stopped.

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 (Zhao et al., 2010).

$$optical\ purity = \frac{(D - Lactate) - (L - Lactate)}{(D - Lactate) + (L - Lactate)} \times 100\%$$

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 OD_{600} of 1 is equivalent to the cell concentration of 0.12 g/L.

3.5.2 Rapidly detecting glucose concentration by YSI glucose-lactate analyzer

Remaining glucose in the solution samples collected was analyzed by glucose-lactate analyzer YSI 2700 (Yellow Spring Instrument Co., Inc.). This analytical instrument is accurate within the ranges of glucose and L-lactic acid at concentration of 0-2 g/L. Before measurement, fermentation broth was centrifuged and diluted with distilled water.

3.5.1 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.

3.5.2 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 eluant 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

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Effect of inoculum size in preculture step in shake flask for the parental *T. laevilacticus* SK5-6

The operating condition during the preculture step of the catalase positive isolate *T. laevilacticus* SK5-6 was optimized in the shake flask cultivation. Several factors including temperature, pH, inoculum age, and inoculum size are considered as the keys in successful lactic acid production (Ghaffar et al., 2014). Growth profiles of *T. laevilacticus* SK5-6 in the preculture medium with the varied inoculum sizes are shown in Figure 4.1. From Table 4.1, it was found that a small inoculum size (1%) resulted in a longer lag phase. Increasing the inoculum size to 3% and 5% shortened the lag phase. Nevertheless, the specific growth rates during the log phase and final OD_{600} obtained from the cultures inoculated with those 3 inoculum sizes were similar. High inoculum size which is typically used for generating a high cell density culture is somewhat considered as the contribution to the high production cost due to the loss of nutrients dedicated to cells (Jantama et al., 2015). As a result, inoculum of 1% (w/v) was selected for further study due to a low quantity of bacterial suspension was required from the slant culture.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

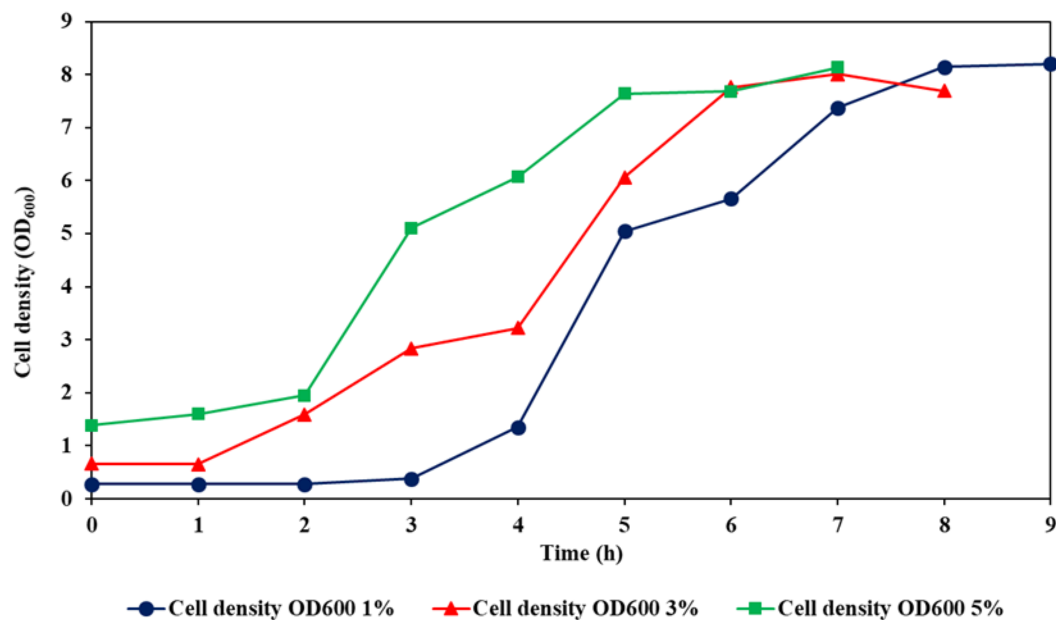


Figure 4.1 Effect of the inoculum size on the cell growth of *T. laevilacticus* SK5-6 during the preculture step in the flask culture. The culture was incubated at 37 °C, 200 rpm.

Table 4.1 Growth profile determination during preculture step of *T. laevilacticus* SK5-6 in the shake flask with varied inoculum size.

Inoculum size	**Mid Log Phase**	**Late Log Phase**	**Specific growth rates (μ, h^{-1})**
1 %	5 h	7-8 h	0.449
3 %	4 h	5-6 h	0.494
5 %	3 h	4-5 h	0.455

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4.2 Growth of *T. laevilacticus* SK5-6 during preculture step with varied yeast extract concentrations

The cost of nutrients is one of the main drawbacks for the competitive biotechnological production of lactic acid. Complex nitrogen source, especially yeast extract, is generally used for lactic acid fermentations because it offers rapid production and high productivity of lactic acid. Nevertheless, from an economical analysis of lactic acid fermentation, it was found that yeast extract supplementation represented 38% of the medium cost (Tejayadi and Cheryan, 1995). To lower the production cost in the production of lactic acid by *T. laevilacticus* SK5-6, we tested for the ability of this isolate to grow under reduced yeast extract concentration in the shake flask cultivation (Figure 4.2).

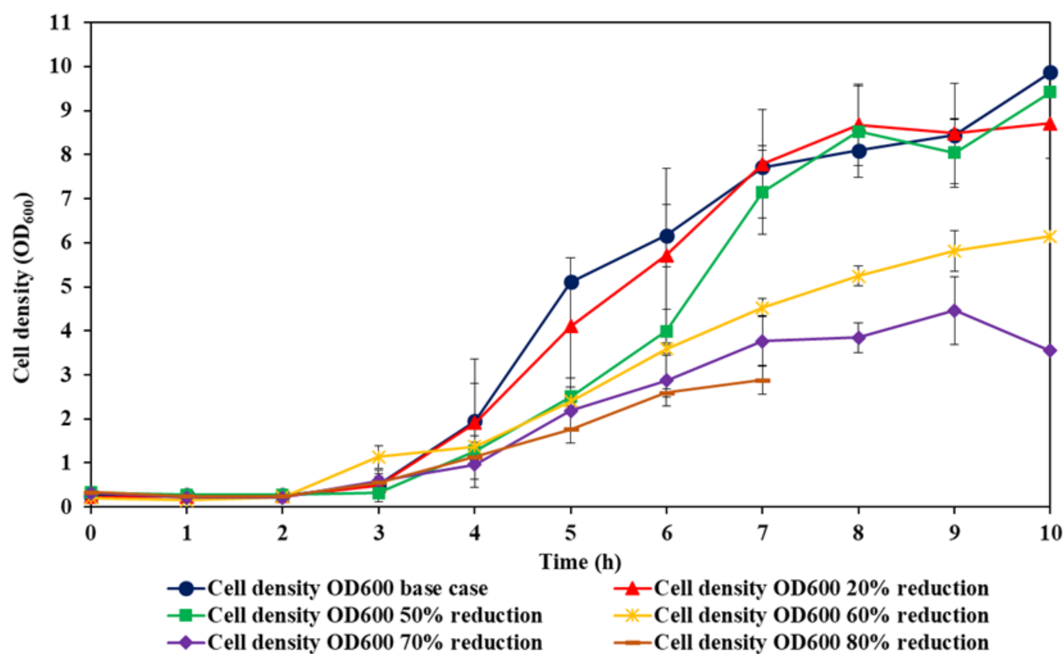


Figure 4.2 Growth profile *T. laevilacticus* SK5-6 grown in the medium with varied yeast extract concentrations during the preculture step in the flask culture. The culture was incubated at 37 °C, 200 rpm.

The similar lag phase of 3 h was observed from the growth profiles of *T. laevilacticus* SK5-6 grown in all yeast extract concentrations tested. The similar growth profiles to the original base case preculture profile (with 15 g/L yeast extract) were obtained in the culture with the reduced yeast extract concentration of 20% and 50% (Figure 4.2). The maximum cell density (OD_{600}) of approximately 8 was obtained. This indicated that the wild strain *T. laevilacticus* SK5-6 could grow and eventually produced lactic acid (Figures 4.2 and 4.3). From the findings in Figures 4.2 and 4.3, it can be presumably concluded that half of yeast extract can be cut off from the preculture medium in regardless of strain adaptation. Further reduction of yeast extract concentration in the preculture medium (60%, 70%, and 80% reduction from the original base case medium) resulted in the lower cell density (OD_{600}). This revealed the evidence of the limited nitrogen source for biosynthesis and growth of *T. laevilacticus* SK5-6. It should be noted that the fermentation efficiency should remain unchanged with the preculture seed obtained from the cultivation with the reduced yeast extract when compared with those observed in the base case cultivation.

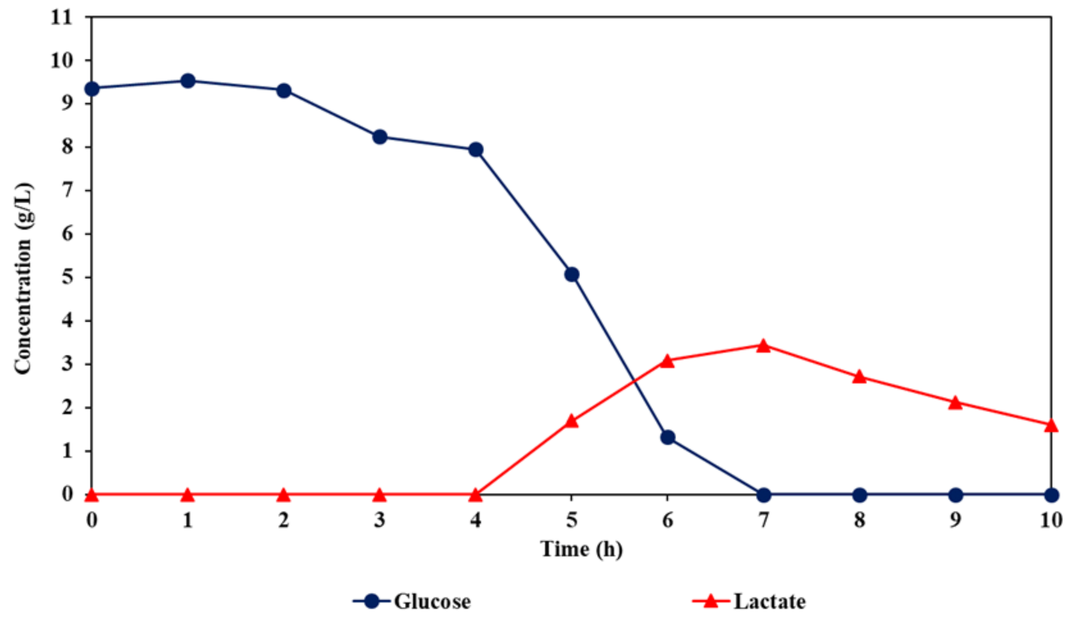
จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Figure 4.3 shows the effect of reduction of yeast extract in preculture seed on glucose consumption and lactic acid production. In base case preculture with 15 g/L yeast extract, glucose was completely consumed at 7 h with the maximum lactic acid concentration of 3.44 g/L (Figure 4.3A). Glucose consumption rate were obtained from the preculture seed grown in the medium with the reduced yeast extract concentration of 20%, 50%, and 60% compared to the consumption in the base case preculture seed (Figures 4.3B, 4.3C, and 4.3D). Further lowering yeast extract concentration (70% and 80%) resulted in the lower glucose consumption and lactic acid production (Figures 4.3E and 4.3F). The findings above indicated the ability of *T. laevilacticus* SK5-6 could

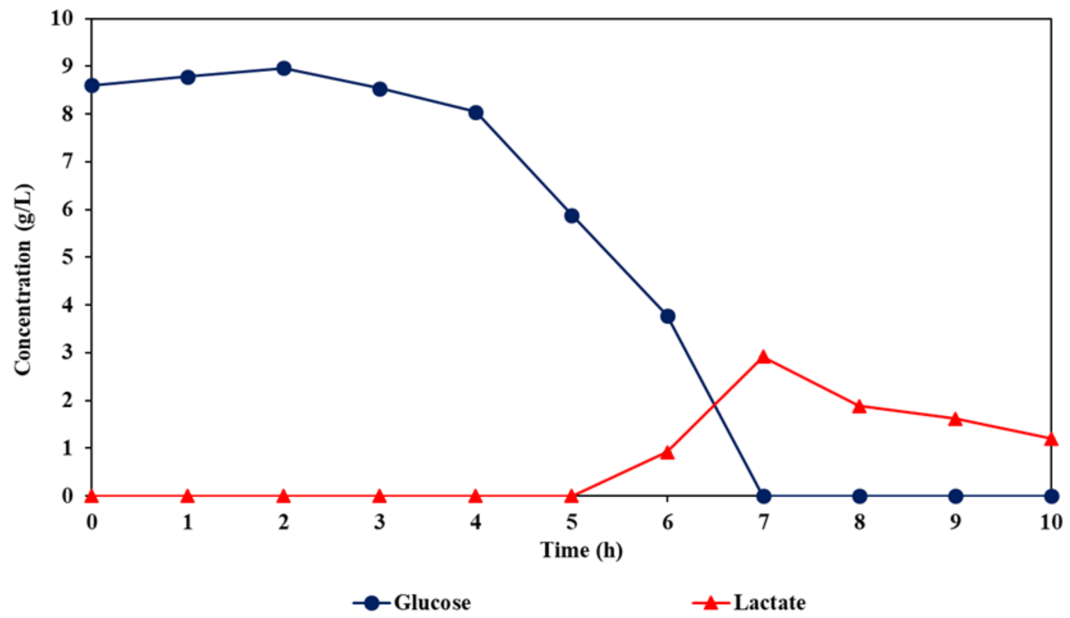
grow and produce lactic acid under the limited nitrogen source. It should be also noted that final cell biomass and lactic acid production increased significantly with the increasing initial yeast extract concentration up to 10 g/L (Nancib et al., 2005). On the other hand, the high cost of yeast extract was claimed as the negative impact on the process economic of its use in the industrial production. For this reason, much effort has been focused on reduction of yeast extract in the fermentation process with the sufficiently high production performance (Amrane and Prigent, 1999, Wee and Ryu, 2009).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

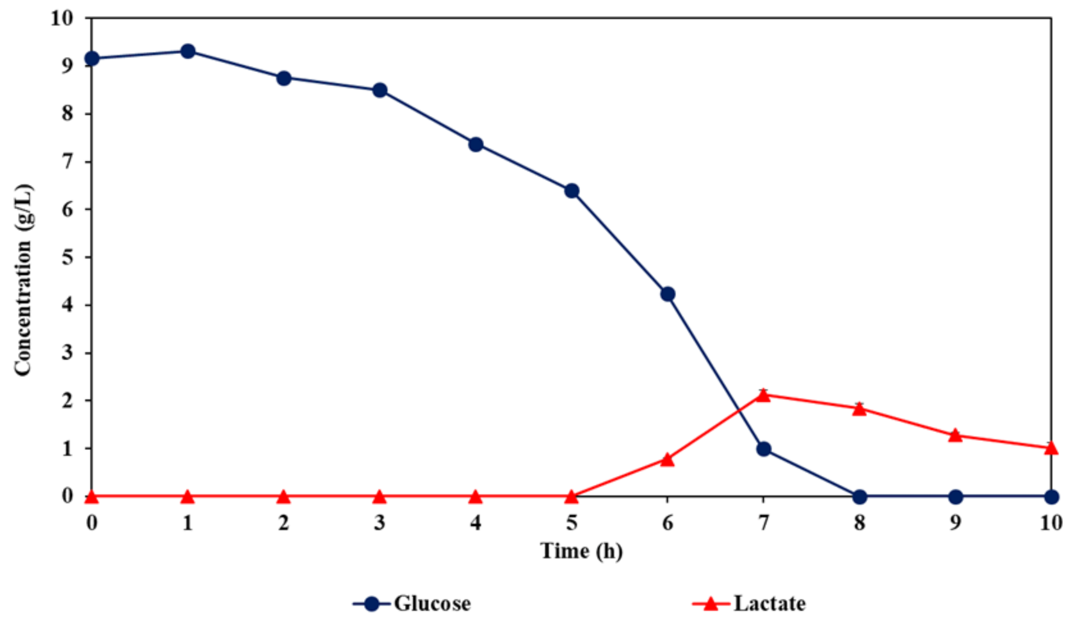
A Base Case



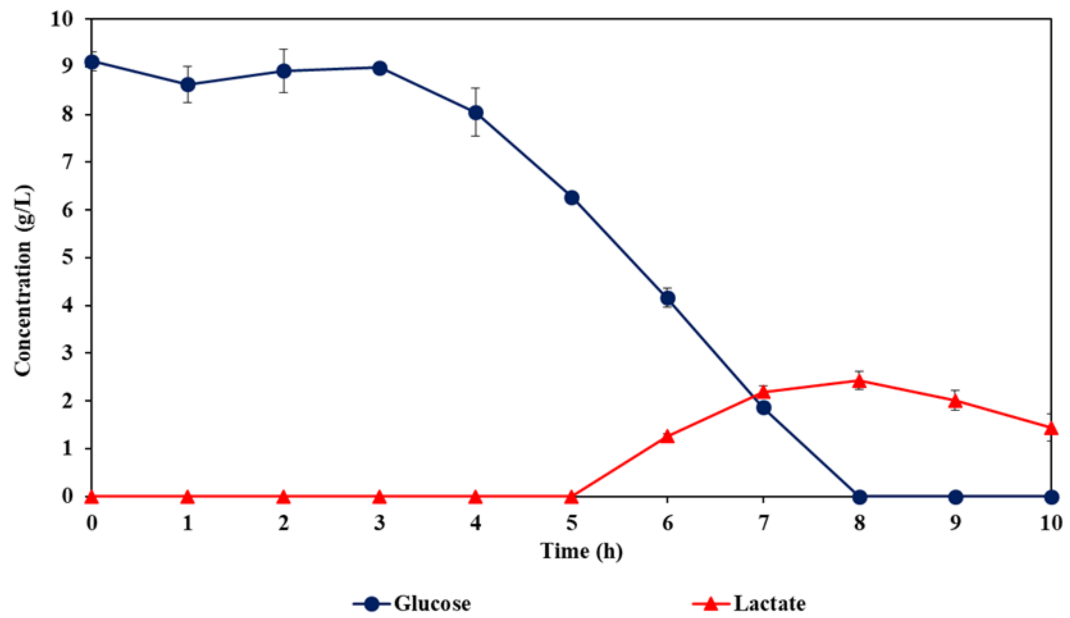
B 20% reduction



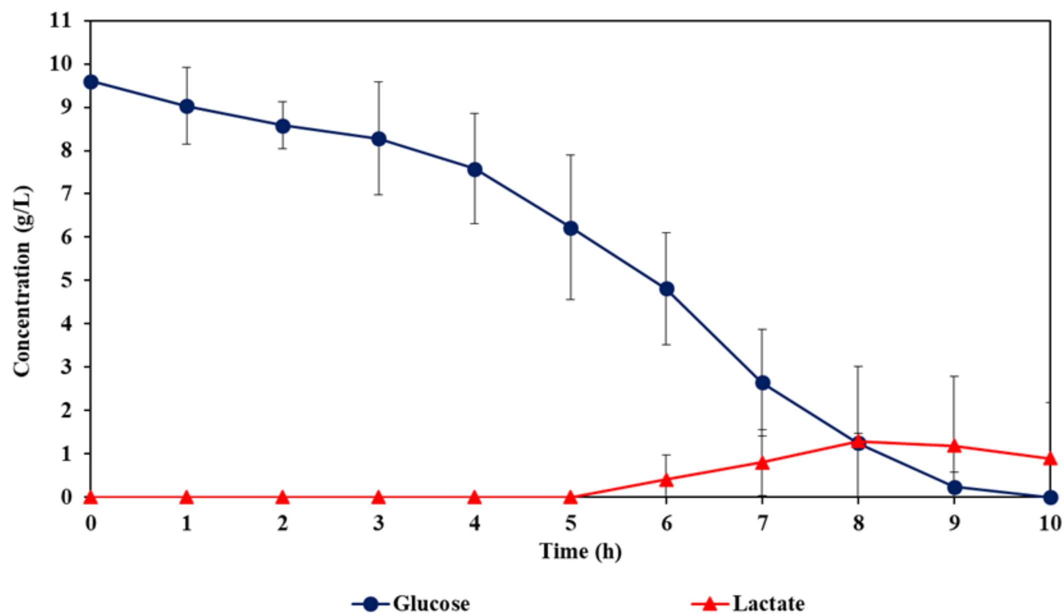
C 50% reduction



D 60% reduction



E 70% reduction



F 80% reduction

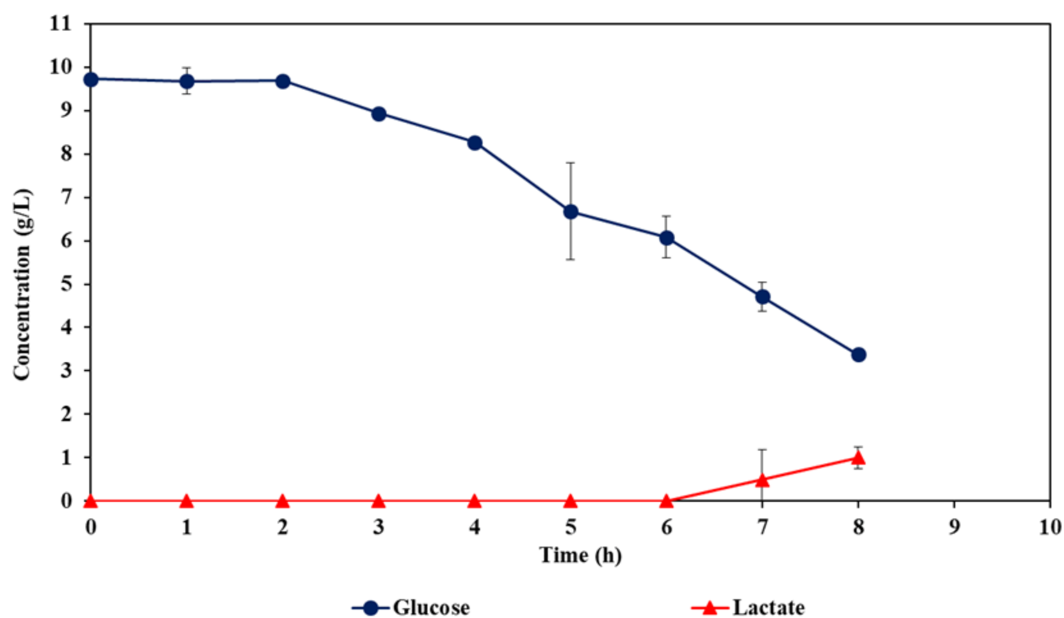


Figure 4.3 Effect of yeast extract concentration on glucose consumption and lactate production of *T. laevilacticus* SK5-6 during the preculture step in the flask culture. The culture was incubated at 37 °C, 200 rpm. A. base case, B. 20% reduction, C. 50% reduction, D. 60% reduction, E. 70% reduction, F. 80% reduction.

4.3 Adaptation of *T. laevilacticus* SK5-6 in the fleaker

T. laevilacticus SK5-6 was tested for the ability to grow in the preculture medium with the reduced yeast extract concentration in the fleaker for inducing phenotypic adaptation. By this cultivation method, it allowed the wild type microbes or even the engineered strains to cope with the physiological stresses and to thrive despite cellular and environmental perturbations (Cooper et al., 2003).

The wild type *T. laevilacticus* SK5-6 was subcultured in the preculture medium before transferring into the new preculture medium with the reduced yeast extract concentrations (80%, 90%, 95%, 97% and 99% reduction). Figure 4.4 shows the growth profiles of *T. laevilacticus* SK5-6 in the preculture medium with the reduced yeast extract concentration. From Figure 4.4A, it was found that the growth rate was still high even lowering the concentration of yeast extract by 80% and 90% (the conversion to cell biomass was still higher than the theoretical value at the critical carbon/nitrogen ratio). Nutrient availability guides the evolution of metabolic pathways, since the central role of metabolism is the conversion of small molecules to biomass and energy (Nam et al., 2011). Exponentially growing bacteria often have growth phenotypes (e.g., substrate uptake rate and secretion products) consistent with the predictions of flux balance analysis using genome-scale models of metabolism (Edwards et al., 2001). As a result, further reduction of yeast extract in the preculture medium was conducted (Figure 4.4B). From the fermentation kinetic profiles, it was found that at 97% reduction of yeast extract in the preculture medium, the biomass conversion (maximum OD_{600} of 1.85 (see OD_{600} trend line in Figure 4.4B)) as approaching the theoretical conversion (with completed consumption of glucose (10 g/L), 0.5 g/L cell biomass (approximately equivalent to 1.5 OD_{600}) would be obtained). Currently, many research

studies on genetic recombination microbes have reported on the use of xylose for ethanol production. *S. cerevisiae*, in use as the most common ethanol fermentation strain, had a higher ethanol yield and production rate under optimal conditions (0.45 g ethanol/g biomass, and 1.3 g ethanol/g cell·h) (Chen and Wang, 2017). This indicated that 97% reduction was suitable for an anaerobic growth of *T. laevilacticus* SK5-6 when completed conversion of both carbon and nitrogen sources was acquired. Figure 4.5 shows the effect of reduction of yeast extract in the preculture medium on glucose consumption and lactic acid production. It was found that at 95% reduction of yeast extract in the preculture medium, glucose was completely consumed at 18 h with the maximum lactic acid concentration of 8.02 g/L (Figure 4.5A). Similar glucose consumption profiles were obtained from the preculture medium with the reduced yeast extract concentration of 97% compared to the consumption in the reduced yeast extract concentration of 95%, glucose was completely consumed at 24 h with the maximum lactic acid concentration of 9.50 g/L (Figure 4.5B). Further lowering yeast extract concentration (99%) resulted in the lower glucose consumption and lactic acid production (Figure 4.5C). Therefore, the phenotypic adaptation was started with the medium with reduced yeast extract concentration by 97%. Further reduction of yeast extract concentration (99% reduction and the medium without yeast extract) in the preculture medium resulted in slow growth and low final OD_{600} (Figure 4.4B). This indicated the limited nitrogen source and the subsequent unbalanced carbon to nitrogen ratio. Previous study revealed the success in metabolic evolution of the parental microbes. One example is the evolved *Klebsiella oxytoca* KMS005 exhibited lower biomass generation and growth rate compared to its parental strains. Via metabolic evolution, its fermentation performance was improved. This procedure has been applied

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

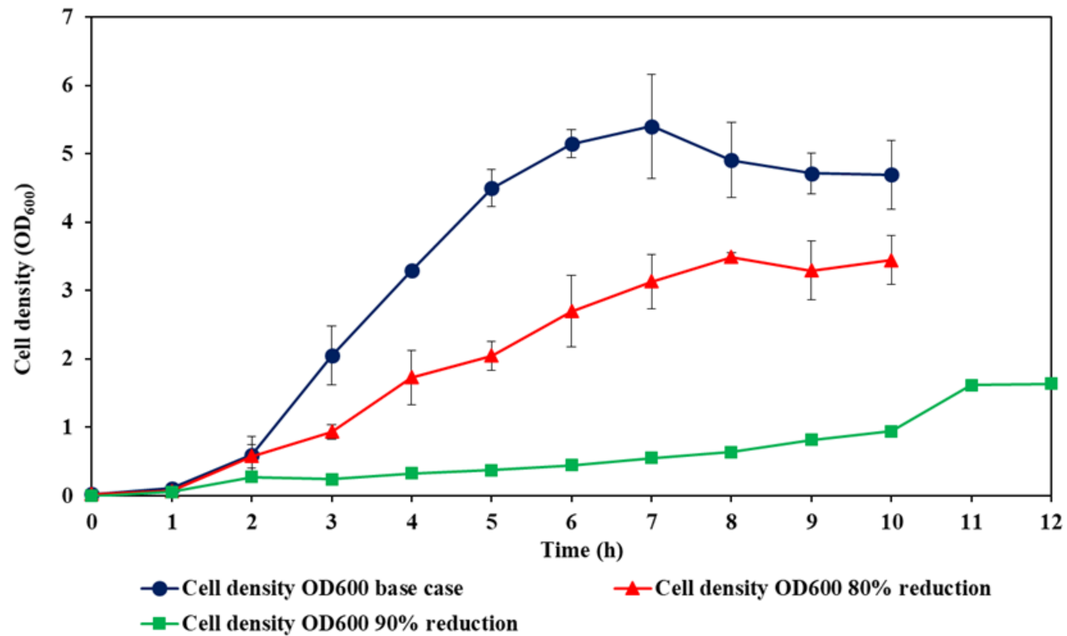
to microorganisms for many works (Wood et al., 2005, Woods et al., 2006, Jantama et al., 2008, Chou et al., 2009, Carroll and Marx, 2013).

Phenotypic adaptation of *T. laevilacticus* SK5-6 grown in the preculture medium with 97% reduced yeast extract concentration was observed in Figure 4.6. It was found that at the beginning of the preculture transfer (T4-T8), the final cell biomass concentration was obtained (low final OD_{600}) (Figure 4.6A). After that, the final OD_{600} was increased then remained constant at the value approaching the theoretical conversion ratio along with the consistency in glucose consumption and lactic acid production (Figures 4.6A and 4.6B). In addition, no byproduct was observed. This indicated the metabolic adaptation of *T. laevilacticus* SK5-6 after several passages in the medium with the reduced yeast extract concentration. The preliminary observation under the microscope revealed that the wild type *T. laevilacticus* SK5-6 were gram positive and rod shaped bacteria (Figure 4.7A). Nonetheless, the adapted strain of *T. laevilacticus* SK5-6 were gram positive, round and rod shaped bacteria (Figure 4.7B). This revealed the evidence of adaptation of *T. laevilacticus* SK5-6 at the correct physiological stage readily for transfer to fermentation phase later. Among the cultures collected at each transfer, it was found that *T. laevilacticus* SK5-6 T20 showed the stable growth on the agar plates after several passages. This adapted isolate was used later as a representative to conduct lactic acid fermentation. This similar concept was successfully applied in adaptation of *Klebsiella oxytoca* KMS005 to enhance butanediol (BDO) production in the AM1 medium with high initial glucose concentration. (Jantama et al., 2015) demonstrated that *K. oxytoca* KMS005 slowly grew in the AM1 medium containing 5% (w/v) glucose but it grew and produced BDO at the higher level after several passages in the fleaker (Jantama et al., 2015). From the results, the slight

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

increase in biomass production was observed from the beginning of the transfers (T1-T23) along with the increase in BDO production. Later, after T24 the initial glucose concentration in the AM1 medium was deviated from 5% to 10%. After passages to T73, the improved biomass and BDO production were obtained.

A



B

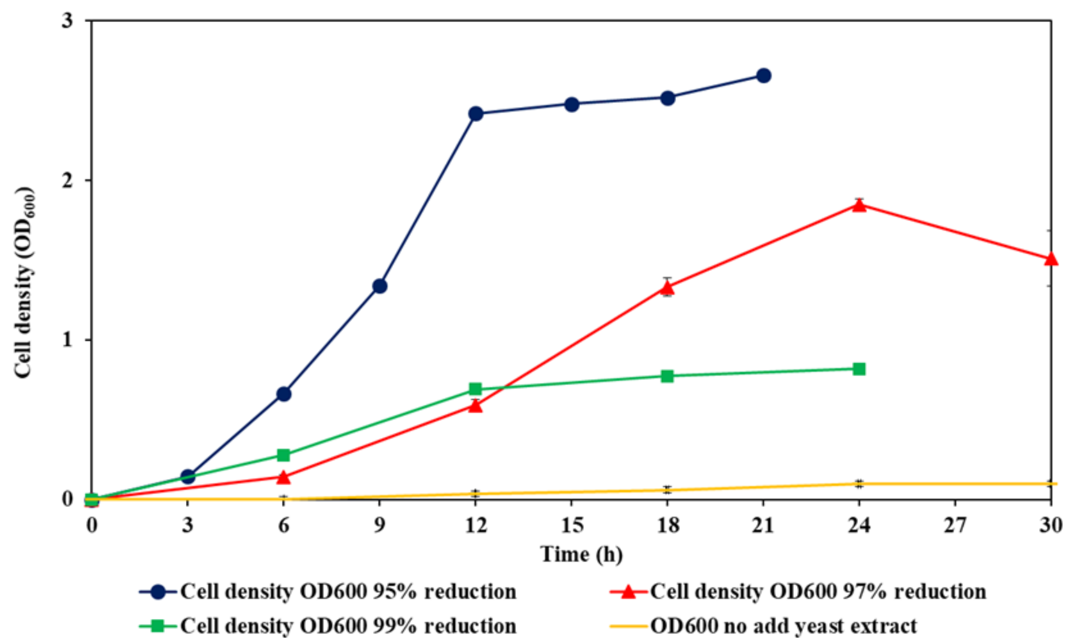
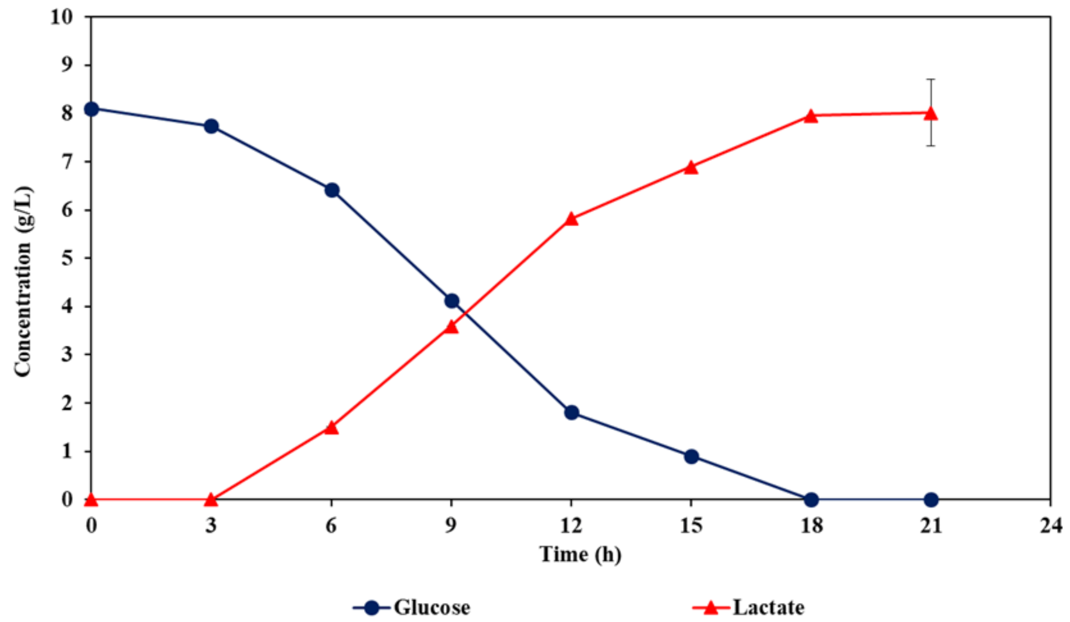
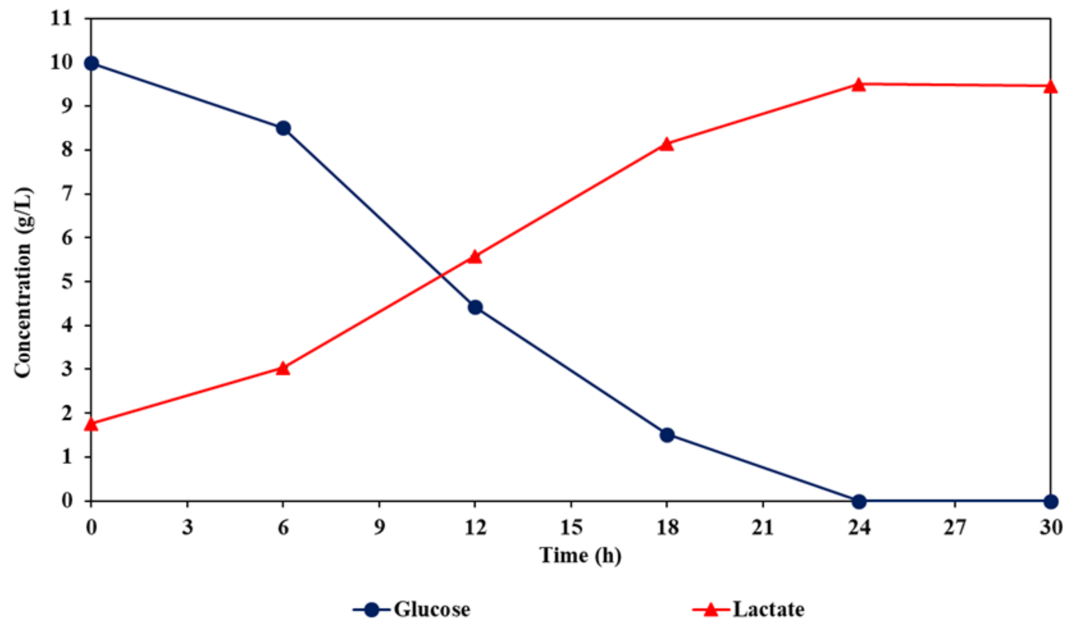


Figure 4.4 Growth profiles of *T. laevilacticus* SK5-6 in the fleaker. The culture was incubated at 37 °C, 300 rpm. A. Base case, 80% and 90% reduction. B. 95%, 97% and 99% reduction and no add yeast extract.

A 95% reduction



B 97% reduction



C 99% reduction

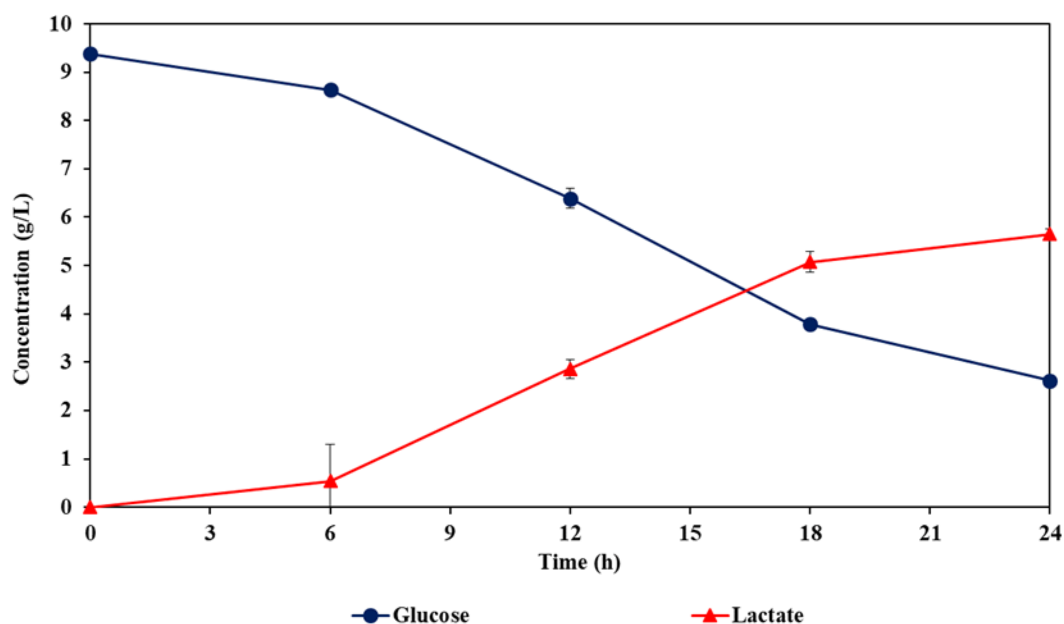
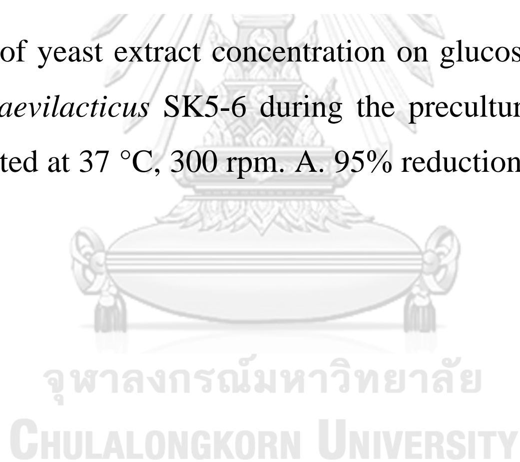
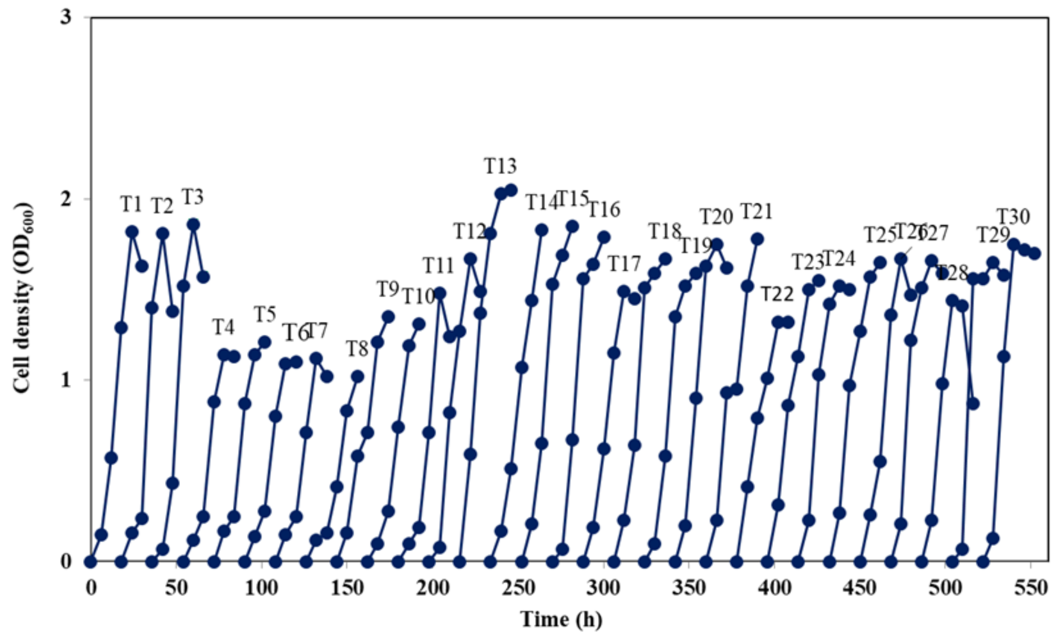


Figure 4.5 Effect of yeast extract concentration on glucose consumption and lactate production of *T. laevilacticus* SK5-6 during the preculture step in the fleaker. The culture was incubated at 37 °C, 300 rpm. A. 95% reduction, B. 97% reduction, and C. 99% reduction.



จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

A Cell density (OD_{600})



B Glucose consumption and lactate production

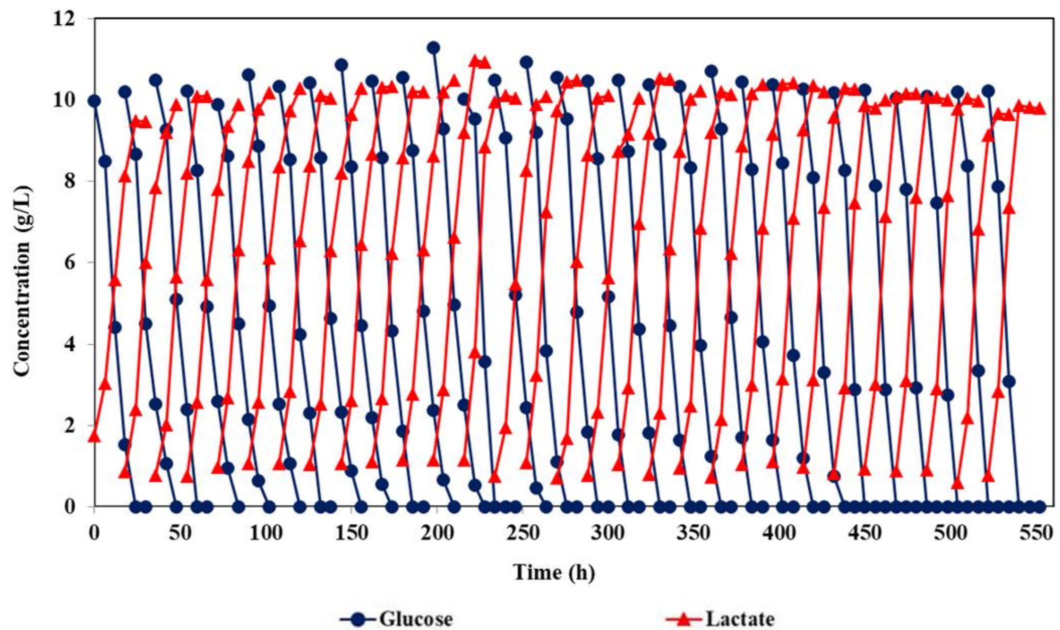
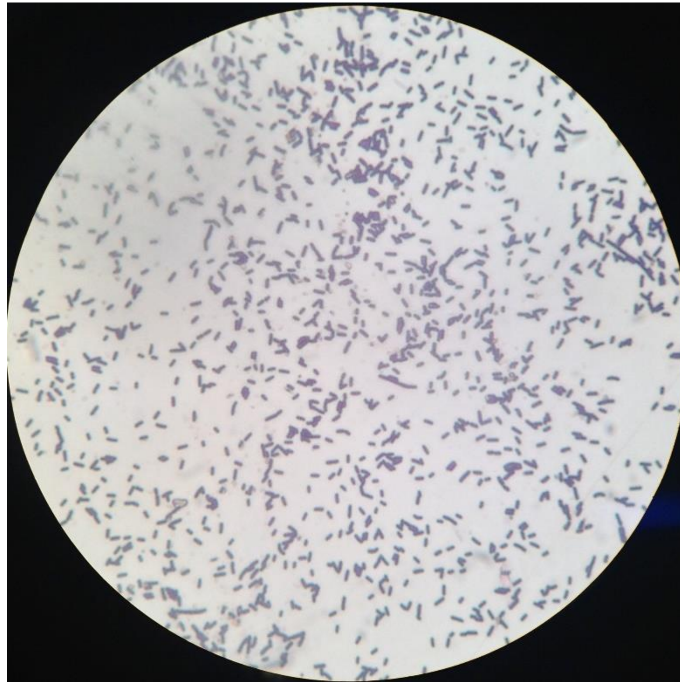


Figure 4.6 Phenotypic adaptation of *T. laevilacticus* SK5-6 for improved growth and lactate production in the preculture medium with 97% reduced yeast extract concentration A. cell density (OD_{600}), B. Glucose consumption and lactate production. The culture was incubated at 37 °C, 300 rpm in the fleaker.

A Wild type



B Adapted strain

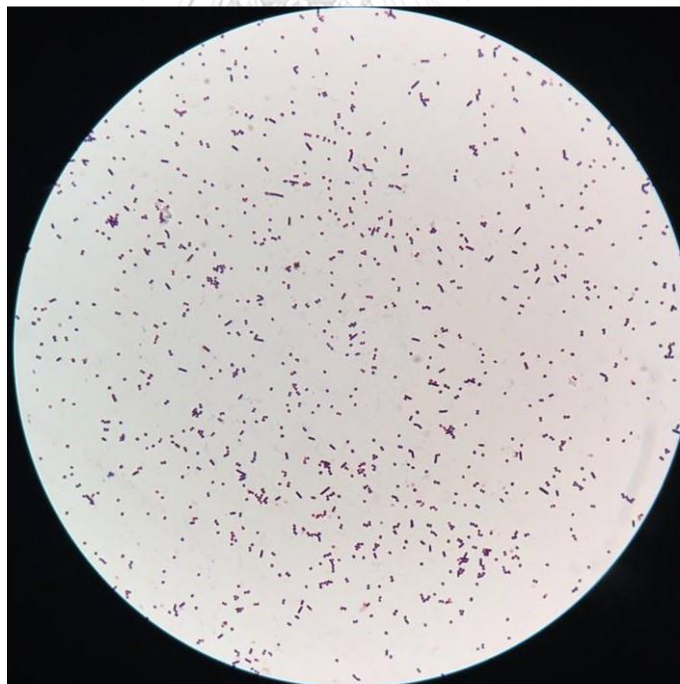


Figure 4.7 Micrographs of *T. laevilacticus* SK5-6. A. Wild type, B. Adapted strain.

4.4 D-lactic acid production by the adapted isolate *T. laevilacticus* SK5-6 T20

The fermentation platform of the adapted *T. laevilacticus* SK5-6 T20 was defined in this study. Initially, the proper incubation time of the stock culture in the agar slant was determined from the preculture growth in the medium with 97% reduced yeast extract concentration in the shaken flask culture (250 mL flask) (Figure 4.8).

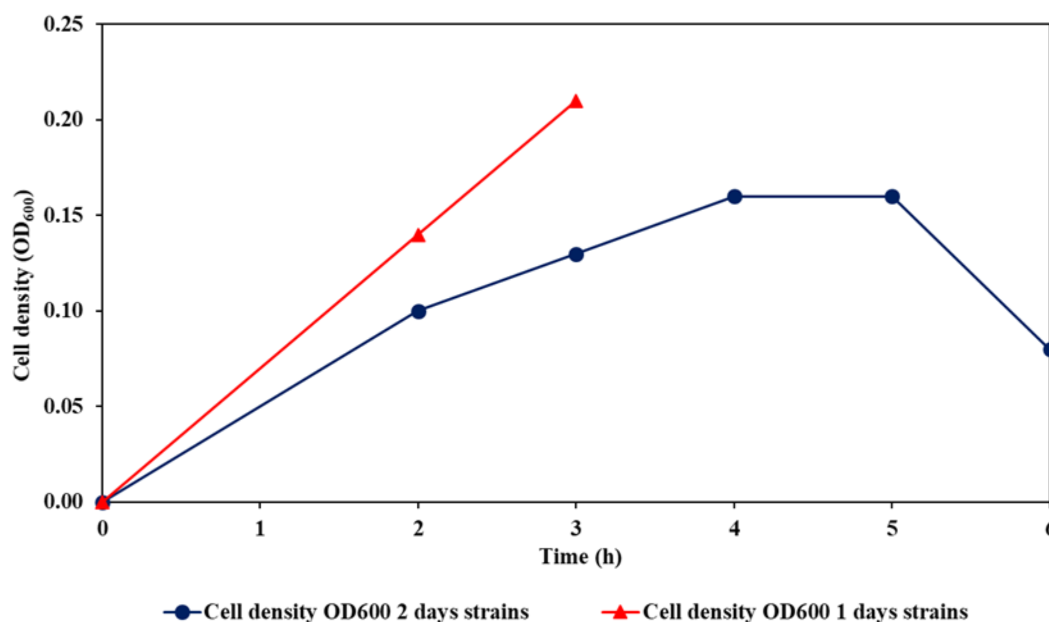


Figure 4.8 Effect of cultivation time of adapted strains *T. laevilacticus* SK5-6 in the preculture medium with 97% reduced yeast extract concentration. The culture was incubated at 37 °C, 200 rpm in the flask culture.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

From Figure 4.8, using the 1-day culture grown on the GYP agar slant to prepare the inoculum suspension gave the better growth in the preculture step. The higher growth rate and final OD_{600} were obtained. As a result, the 1-day culture grown on the GYP agar slant was selected for further experiment.

It was indicated that physiological stage and cell density of the seed culture played a critical role in improving fermentation production performance (Zhao et al.,

2010). In this work, to generate the robust fermentation platform of *T. laevilacticus* SK5-6 T20 for D-lactic acid production, correct seed production step was mandatory. Growth profile of the adapted *T. laevilacticus* SK5-6 T20 grown in the preculture medium with 97% reduced yeast extract concentration was determined in the fleaker (Figure 4.9). From the growth profile, it was found that *T. laevilacticus* SK5-6 T20 immediately grew after inoculation (no evidence of lag phase). The culture reached the mid log phase at 9 h then approached to the late log phase at 15 h. At 21 h, maximum biomass production was obtained at the OD_{600} of 1.2.

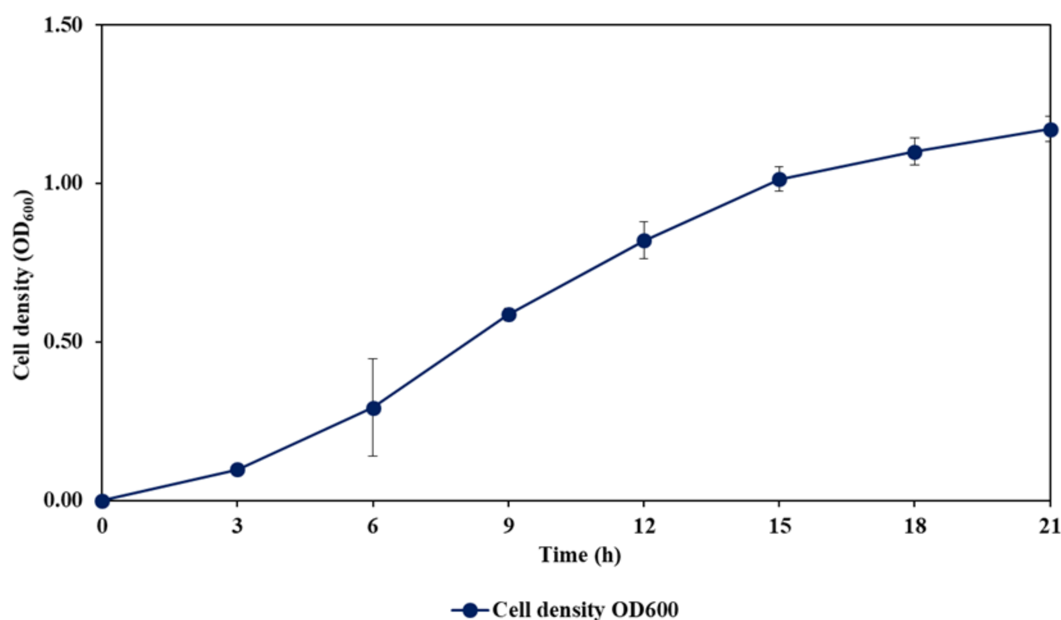


Figure 4.9 Growth profile of the adapted *T. laevilacticus* SK5-6 T20 grown in the preculture medium with 97% reduced yeast extract concentration during the preculture step in the fleaker. The culture was incubated at 37 °C, 300 rpm.

The preculture seed obtained in the fleaker was further transferred to the fermentation fleaker at varied inoculum age (mid log and late log preculture seed) (Figure 4.10). Similar growth patterns were obtained from the 2 seed ages studied.

While the transfer was performed during the mid log stage, samples were kept collecting until reaching the stationary phase (24 h). In addition, both could produce lactic acid (Figure 4.11). With respect to the effect of preculture seed being transferred into the fermentation stage, changing the inoculum age from the mid log to late log phases did cause a dramatic change in the fermentation performance during the fermentation stage conducted under the same conditions. As a result, mid log was selected for further study due to higher cell density (OD_{600}) achieved.

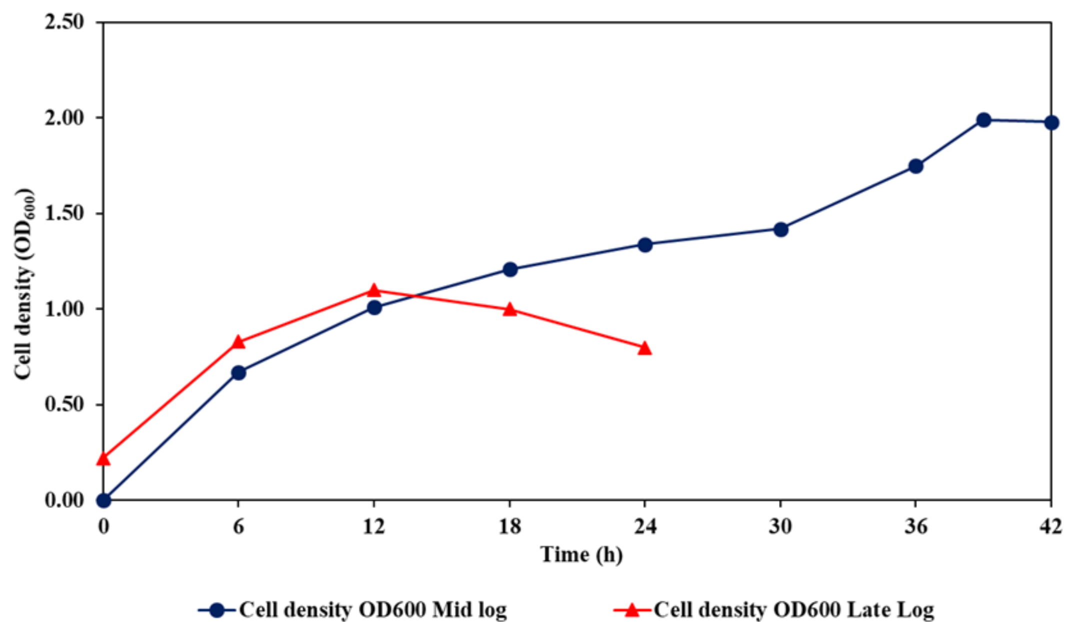
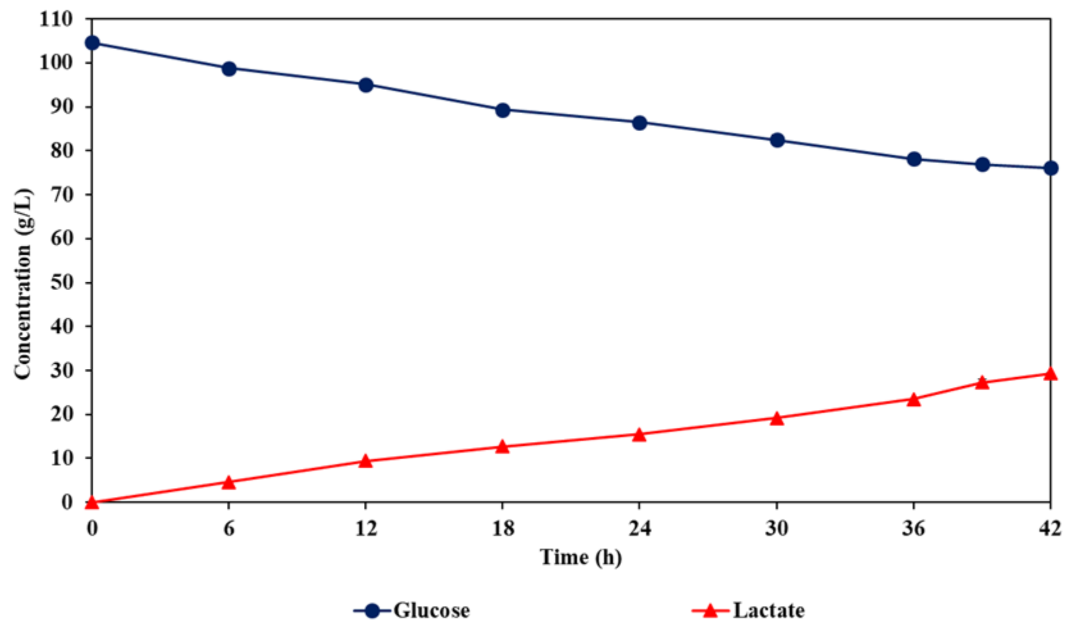


Figure 4.10 Effect of inoculum age on growth of the adapted *T. laevilacticus* SK5-6 T20 during the fermentation step in fleaker. The culture was incubated at 37 °C, 300 rpm.

A



B

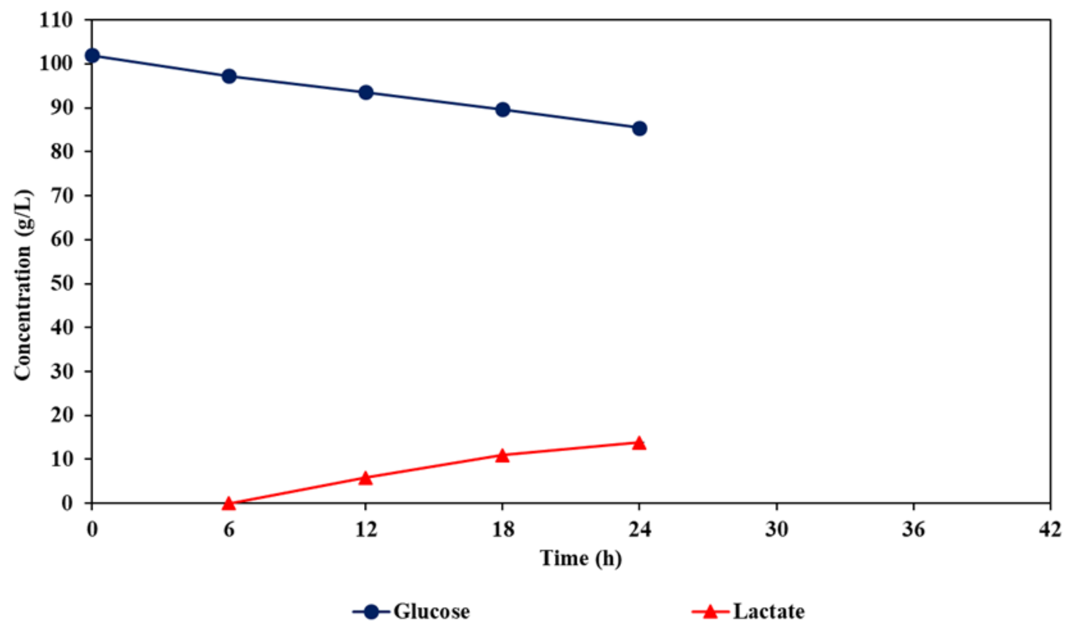


Figure 4.11 Effect of inoculum age on glucose consumption and lactate production of the adapted *T. laevilacticus* SK5-6 T20 during the fermentation step in fleaker. The culture was incubated at 37 °C, 300 rpm. A. Mid log phase, B. Late log phase.

Figure 4.12 shows the fermentation kinetics profiles of the adapted *T. laevilacticus* SK5-6 T20 during the fermentation stage in the fleaker. With the initial glucose concentration of 100 g/L, the maximum lactic acid concentration of approximately 75 g/L (yield of 0.87 g/g and productivity of 0.42 g/L·h) and the maximum OD_{600} of approximately of 2.6 were obtained at 180 h. The maximum OD_{600} was achieved at 108 h and remained unchanged while lactic acid production continued accumulation until the end of the fermentation (180 h). The results obtained in this study indicated the sufficiently high lactic acid conversion yield from glucose; however, the productivity and the optical purity of D-lactic acid (67%ee) were considered low compared with the wild type *T. laevilacticus* SK5-6. In 2017, Preasirtsak et al. demonstrated that the wild type *T. laevilacticus* SK5-6 exhibited the good characteristics as the D-lactic acid producer. It provided a sufficiently high optical purity of D-lactic acid for the polymer-grade specification (>99.5%ee). The optical purity of lactic acid product rather depended on the C/N ratio and the availability of the N-source which were responsible to the stress during the cultivation. Typically, D-lactic acid was primarily produced in the wild strain of *T. laevilacticus* SK5-6 under the optimized C/N weight ratio (100:15). After adaptation in the reduced yeast extract medium (only 0.45 g/L yeast extract available in the preculture medium), the adapted *T. laevilacticus* SK5-6 T20 was capable to rapidly grow. This indicated the changes of the adapted strain to survive under high stress condition. The new stability in the phenotypic change was claimed to be responsible by the genomic change of the adapted strain. As a result, when growing under the same operating condition as that previously used in the fermentation of the wild type strain resulted in a high glucose flux in glycolysis that was dramatically channeled through an L-lactate producing pathway

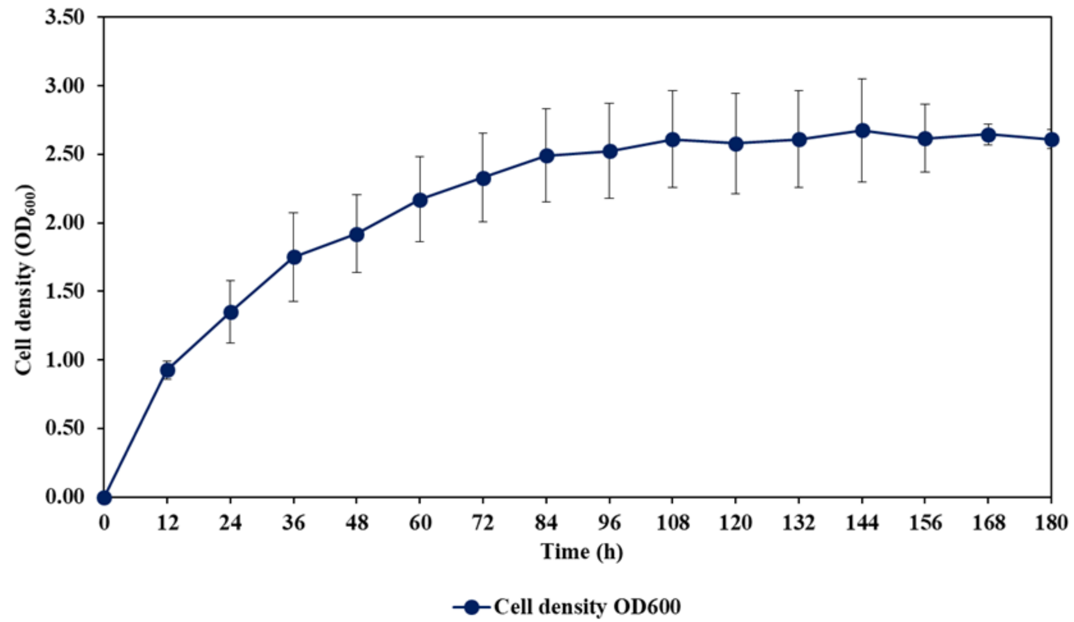
จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

(Jantama et al., 2015). This can be further explained by the capability of the adapted strain to utilize the limited nitrogen source; therefore, a high pool of pyruvate could have been generated during the fermentation. This eventually resulted in the conversion of pyruvate to the plausible metabolites such as acetate and L-lactate instead of D-lactate which was commonly produced when the cells signaled the stress conditions.

The previous study reported the fermentation of the wild type strain which gave the high yield (0.84 g/g) and productivity (2.14 g/L·h). Nonetheless, the fermentation platform earlier developed required a high amount of yeast extract. From the findings in this work and those Prasirtsak et al. previously determined, this indicated the potential of using *Terrilactibacillus* sp. as the D-lactic acid producer (Prasirtsak et al., 2017). The major challenges associated with lactic acid production that can be addressed by employing genetic engineering strategies are isomer purity, acid tolerance, carbon source consumption, and industrial parameters. Also, the adapted isolate can provide lactic acid production with the low cost if further improvement in productivity and optical purity can be acquired.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

A



B

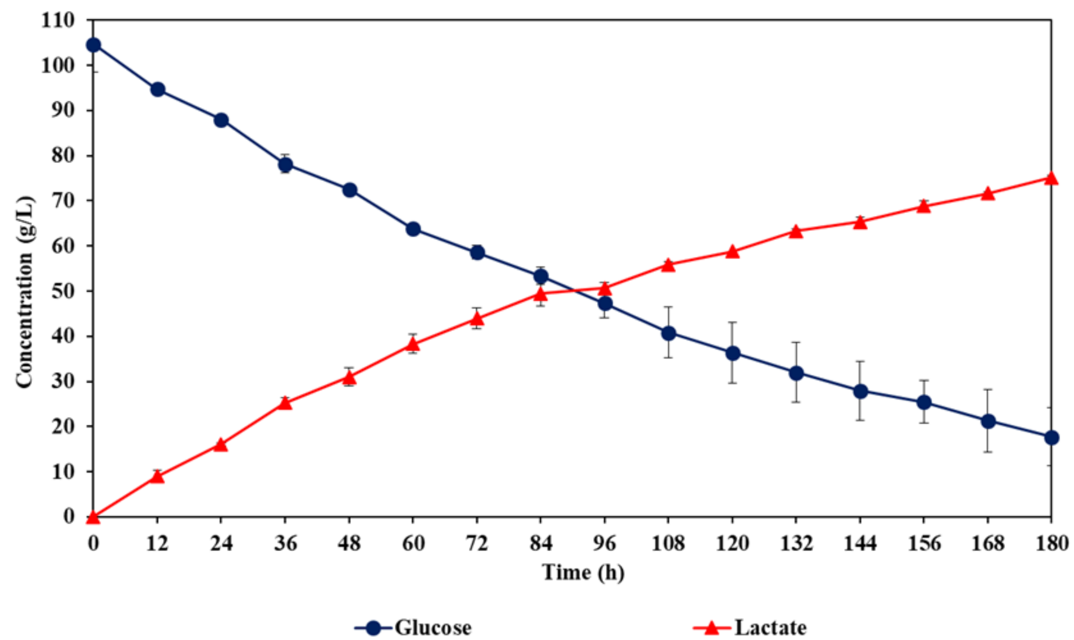


Figure 4.12 Fermentation kinetics profiles A. cell density (OD_{600}), B. Glucose consumption and lactate production of the adapted *T. laevilacticus* SK5-6 T20 during the fermentation stage in the fleaker. The culture was incubated at 37 °C, 300 rpm.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Summary

T. laevilacticus SK5-6 was proven as the potential industrial strain for D-lactic acid fermentation. Previous study reported that not only solely producing an optically pure D-lactic acid which is essential in PLA synthesis, but it was also found that this isolate could grow and ferment lactic acid at the acceptably high yield and productivity. Further increasing final lactic acid concentration up to 102.22 g/L with the acceptable yield of 0.84 g/g and productivity of 2.13 g/L·h from the preliminary fermentation results conducted in the shake flask cultivation.

In the conventional lactic acid fermentation, a large amount of yeast extract was required in both preculture and fermentation stages by *T. laevilacticus* SK5-6. However, the high cost of yeast extract has a negative impact on the economics of its use in an industrial scale process. For this reason, much effort has been focused on trying to reduce yeast extract concentration to minimizing costs without loss in product yield. Therefore, metabolic evolution was implemented in the strain SK5-6. It was found that at the beginning of the preculture transfer (T4-T8), the low final cell biomass concentration was obtained. After that, the final cell biomass concentration was increased then remained constant at the value approaching the theoretical conversion ratio along with the consistency in glucose consumption and lactic acid production.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Metabolic evolution was claimed as an excellent tool in strain improvement for enhancing the fermentation process efficiency.

After successfully adapted *T. laevilacticus* SK5-6 to grow in the preculture medium with the reduced yeast extract. The adapted isolate T20 was used to prepare the preculture seed to inoculate into the fermentation stage. From the results obtained, this adapted isolate could produce lactic acid at the similarly high concentration to that obtained in the cultivation using the wild type isolate. The conversion yield from glucose was also comparable to what from wild type. However, the productivity and the optical purity of D-lactic acid were lower. Nonetheless, this can be improved by further adaptation in the fermentation stage.

5.2 Recommendations

Further experiment to improve the fermentation performance of the adapted isolate of *T. laevilacticus* SK5-6 can be conducted by continuing phenotypic adaptation during the fermentation stage using "Chemostat concept". The factors influencing the optical purity should be also further investigated.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

REFERENCES

ABDEL-RAHMAN, M. A., TASHIRO, Y. & SONOMOTO, K. 2011. Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: Overview and limits. Journal of Biotechnology, 156, 286-301.

ABDEL-RAHMAN, M. A., TASHIRO, Y. & SONOMOTO, K. 2013. Recent advances in lactic acid production by microbial fermentation processes. Biotechnology Advances, 31, 877-902.

ALI, Z., ANJUM, F. & ZAHOOR, T. 2010. Production of lactic acid from corn cobs hydrolysate through fermentation by *Lactobaccillus delbrukii*. African Journal of Biotechnology, 8, 4175-4178.

AMRANE, A. & PRIGENT, Y. 1999. Analysis of growth and production coupling for batch cultures of *Lactobacillus helveticus* with the help of an unstructured model. Process Biochemistry, 34, 1-10.

AURAS, R., LIM, L.-T., SELKE, S. & TSUJI, H. 2010. Poly (Lactic Acid): Synthesis, Structures, Properties, Processing, and Applications.

BAEK, S.-H., JEONG, D. & PRIYA, S. 2017. Improvement of D-lactic acid production in *Saccharomyces cerevisiae* under acidic conditions by evolutionary and rational metabolic engineering. Bioresource Technology, 12, 1-26.

BAEK, S.-H., KWON, E. Y., KIM, Y. H. & HAHN, J.-S. 2016. Metabolic engineering and adaptive evolution for efficient production of D-lactic acid in *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology, 100, 2737-2748.

CALLEWAERT, R. & DE VUYST, L. 2000. Bacteriocin production with *Lactobacillus amylovorus* DCE 471 is improved and stabilized by fed-batch fermentation. Applied and Environmental Microbiology, 66, 606-13.

CARROLL, S. M. & MARX, C. J. 2013. Evolution after introduction of a novel metabolic pathway consistently leads to restoration of wild-type physiology. PLOS Genetics, 9, e1003427.

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.

CHEN, H. & WANG, L. 2017. Chapter 7 - Microbial Fermentation Strategies for Biomass Conversion. In: CHEN, H. & WANG, L. (eds.) Technologies for Biochemical Conversion of Biomass. Oxford: Academic Press.

CHOU, H. H., BERTHET, J. & MARX, C. J. 2009. Fast growth increases the selective advantage of a mutation arising recurrently during evolution under metal limitation. PLOS Genetics, 5, e1000652.

COOPER, T., ROZEN, D. & LENSKI, R. 2003. Parallel changes in gene expression after 20,000 generations of evolution in *Escherichia coli*. Microbial Ecology, 100, 1072-1077.

DATTA, R. & HENRY, M. 2006. Lactic acid: recent advances in products, processes and technologies — a review. Journal of Chemical Technology & Biotechnology, 81, 1119-1129.

DATTA, R., TSAI, S.-P., BONSIGNORE, P., MOON, S.-H. & FRANK, J. R. 1995. Technological and economic potential of poly(lactic acid) and lactic acid derivatives. FEMS Microbiology Reviews, 16**,** 221-231.

EDWARDS, J. S., IBARRA, R. U. & PALSSON, B. O. 2001. In silico predictions of *Escherichia coli* metabolic capabilities are consistent with experimental data. Nature Biotechnology, 19**,** 125-130.

EŞ, I., MOUSAVI KHANEGHAH, A., BARBA, F. J., SARAIVA, J. A., SANT'ANA, A. S. & HASHEMI, S. M. B. 2018. Recent advancements in lactic acid production - a review. Food Research International, 107**,** 763-770.

FAROOQ, U., ANJUM, F., ZAHOOR, T., RAHMAN, S., RANDHAWA, M., AHMED, A. & AKRAM, K. 2012. Optimization of lactic acid production from cheap raw material: Sugarcane molasses. Pakistan Journal of Botany, 44**,** 333-338.

GHAFFAR, T., IRSHAD, M., ANWAR, Z., AQIL, T., ZULIFQAR, Z., TARIQ, A., KAMRAN, M., EHSAN, N. & MEHMOOD, S. 2014. Recent trends in lactic acid biotechnology: A brief review on production to purification. Journal of Radiation Research and Applied Sciences, 7**,** 222-229.

GOODALL, C. 2011. Bioplastics: an important component of global sustainability [Online]. Available: https://www.carboncommentary.com/blog/2011/09/02/bioplastics-an-important-component-of-global-sustainability [Accessed].

JANTAMA, K., POLYIAM, P., KHUNNONKWAO, P., CHAN, S., SANGPROO, M., KHOR, K., JANTAMA, S. S. & KANCHANATAWEE, S. 2015. Efficient reduction of the formation of by-products and improvement of production yield of 2,3-butanediol by a combined deletion of alcohol dehydrogenase, acetate kinase-phosphotransacetylase, and lactate dehydrogenase genes in metabolically engineered *Klebsiella oxytoca* in mineral salts medium. Metabolic Engineering, 30**,** 16-26.

JANTAMA, K., ZHANG, X., MOORE, J. C., SHANMUGAM, K. T., SVORONOS, S. A. & INGRAM, L. O. 2008. Eliminating side products and increasing succinate yields in engineered strains of *Escherichia coli* C. Biotechnol Bioeng, 101**,** 881-93.

JOHN, R. P., G.S, A., NAMPOOTHIRI, K. M. & PANDEY, A. 2009. Direct lactic acid fermentation: Focus on simultaneous saccharification and lactic acid production. Biotechnology Advances, 27**,** 145-152.

KOIVURANTA, K. T., ILMEN, M., WIEBE, M. G., RUOHONEN, L., SUOMINEN, P. & PENTTILA, M. 2014. L-lactic acid production from D-xylose with Candida sonorensis expressing a heterologous lactate dehydrogenase encoding gene. Microb Cell Fact, 13**,** 107.

LEE JU, Y., KANG CHANG, D., LEE SEUNG, H., PARK YOUNG, K. & CHO KWANG, M. 2014. Engineering cellular redox balance in *Saccharomyces cerevisiae* for improved production of L-lactic acid. Biotechnology and Bioengineering, 112**,** 751-758.

MüLLER, C., TOWNSEND, K. & MATSCHULLAT, J. 2012. Experimental degradation of polymer shopping bags (standard and degradable plastic, and biodegradable) in the gastrointestinal fluids of sea turtles. Science of The Total Environment, 416**,** 464-467.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

NAM, H., CONRAD, T. M. & LEWIS, N. E. 2011. The role of cellular objectives and selective pressures in metabolic pathway evolution. Current Opinion in Biotechnology, 22**,** 595-600.

NANCIB, A., NANCIB, N., MEZIANE-CHERIF, D., BOUBENDIR, A., FICK, M. & BOUDRANT, J. 2005. Joint effect of nitrogen sources and B vitamin supplementation of date juice on lactic acid production by *Lactobacillus casei subsp*. *rhamnosus*. Bioresource Technology, 96**,** 63-67.

OH, H., WEE, Y.-J., YUN, J.-S., HO HAN, S., JUNG, S. & RYU, H.-W. 2005. Lactic acid production from agricultural resources as cheap raw materials. Bioresource Technology, 96**,** 1492-1498.

OLIVEIRA, C., AGUIAR, T. Q. & DOMINGUES, L. 2017. Principles of Genetic Engineering. Current Developments in Biotechnology and Bioengineering. Elsevier.

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

REDDY, G., ALTAF, M., NAVEENA, B. J., VENKATESHWAR, M. & KUMAR, E. V. 2008. Amylolytic bacterial lactic acid fermentation — A review. Biotechnology Advances, 26**,** 22-34.

REN, J. 2011. Biodegradable Poly (Lactic Acid): Synthesis, Modification, Processing and Applications.

RODRIGUES, C., VANDENBERGHE, L. P. S., WOICIECHOWSKI, A. L., DE OLIVEIRA, J., LETTI, L. A. J. & SOCCOL, C. R. 2017. Production and Application of Lactic Acid. Current Developments in Biotechnology and Bioengineering. Elsevier.

SANGPROO, M., POLYIAM, P., JANTAMA, S. S., KANCHANATAWEE, S. & JANTAMA, K. 2012. Metabolic engineering of *Klebsiella oxytoca* M5a1 to produce optically pure D-lactate in mineral salts medium. Bioresource Technology, 119**,** 191-198.

SONG, J.-Y., PARK, J.-S., KANG, C. D., CHO, H.-Y., YANG, D., LEE, S. & CHO, K. M. 2016. Introduction of a bacterial acetyl-CoA synthesis pathway improves lactic acid production in *Saccharomyces cerevisiae*. Metabolic Engineering, 35**,** 38-45.

TEJAYADI, S. & CHERYAN, M. 1995. Lactic acid from cheese whey permeate. Productivity and economics of a continuous membrane bioreactor. Applied Microbiology and Biotechnology, 43**,** 242-248.

THONGCHUL, N. 2013. Production of Lactic Acid and Polylactic Acid for Industrial Applications. Bioprocessing Technologies in Biorefinery for Sustainable Production of Fuels, Chemicals, and Polymers.

UPADHYAYA, B. P., DEVEAUX, L. C. & CHRISTOPHER, L. P. 2014. Metabolic engineering as a tool for enhanced lactic acid production. Trends in Biotechnology, 32**,** 637-644.

VAIDYA, A. N., PANDEY, R. A., MUDLIAR, S., KUMAR, M. S., CHAKRABARTI, T. & DEVOTTA, S. 2005. Production and Recovery of Lactic Acid for

Polylactide—An Overview. Critical Reviews in Environmental Science and Technology, 35, 429-467.

VARMAN, A. M., YU, Y., YOU, L. & TANG, Y. J. 2013. Photoautotrophic production of D-lactic acid in an engineered cyanobacterium. Microbial Cell Factories, 12, 117.

VIJAYAKUMAR, J., THANGAVELU, V. & RAJENDRAN, A. 2008. Recent Trends in the Production, Purification and Application of Lactic Acid. Chemical and Biochemical Engineering Quarterly, 22, 245-264.

WANG, Y., MENG, H., CAI, D., WANG, B., QIN, P., WANG, Z. & TAN, T. 2016. Improvement of L-lactic acid productivity from sweet sorghum juice by repeated batch fermentation coupled with membrane separation. Bioresource Technology, 211, 291-297.

WEE, Y.-J., KIM, J.-N. & RYU, H.-W. 2006. Biotechnological Production of Lactic Acid and Its Recent Applications. Food Technol. Biotechnol, 44, 163-172.

WEE, Y.-J. & RYU, H.-W. 2009. Lactic acid production by *Lactobacillus sp*. RKY2 in a cell-recycle continuous fermentation using lignocellulosic hydrolyzates as inexpensive raw materials. Bioresource Technology, 100, 4262-4270.

WOOD, B. E., YOMANO, L. P., YORK, S. W. & INGRAM, L. O. 2005. Development of industrial medium required elimination of the 2,3-butanediol fermentation pathway to maintain ethanol yield in an ethanologenic strain of *Klebsiella oxytoca*. Biotechnol Prog, 21, 1366-72.

WOODS, R., SCHNEIDER, D., WINKWORTH, C. L., RILEY, M. A. & LENSKI, R. E. 2006. Tests of parallel molecular evolution in a long-term experiment with *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America, 103, 9107-9112.

XU, T. T., BAI, Z. Z., WANG, L. J. & HE, B. F. 2010. Breeding of D(-)-lactic acid high producing strain by low-energy ion implantation and preliminary analysis of related metabolism. Appl Biochem Biotechnol, 160, 314-21.

YI, X., ZHANG, P., SUN, J., TU, Y., GAO, Q., ZHANG, J. & BAO, J. 2016. Engineering wild-type robust *Pediococcus acidilactici* strain for high titer l- and D-lactic acid production from corn stover feedstock. Journal of Biotechnology, 217, 112-121.

ZHAO, B., WANG, L., MA, C., YANG, C., XU, P. & MA, Y. 2010. Repeated open fermentative production of optically pure L-lactic acid using a thermophilic *Bacillus sp*. strain. Bioresource Technology, 101, 6494-6498.

ZHOU, S., SHANMUGAM, K. T., YOMANO, L. P., GRABAR, T. B. & INGRAM, L. O. 2006. Fermentation of 12% (w/v) glucose to 1.2 M lactate by *Escherichia coli* strain SZ194 using mineral salts medium. Biotechnol Lett, 28, 663-70.

CHULALONGKORN UNIVERSITY

APPENDIX A

ANALYTICAL METHOD

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

A1 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was used to analyze the organic compounds (glucose, lactic acid, fumaric acid, and ethanol) present in the fermentation broth. Samples from this studies were diluted with double distilled water (DDI water). After that diluted particle-free sample (15 µL) were injected into an organic acid analysis column (Biorad, Aminex HPX-87H ion exclusion organic acid column; 300mm x 7.8mm) maintained at 45ºC in a column oven (Shimadzu-CTO-6A). 0.005 N H2SO4 was used as an eluent at 0.6 mL/min flow rate. 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 water (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 9.525, 13.386, 15.117, and 22.442 respectively.

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	-

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Retention time of L-lactic acid and D-lactic acid is 13 and 15 respectively.

A3 Glucose Analyzer

Glucose and L(+)-lactic acid were analyzed by YSI 7100 glucose analyzer (Yellow Spring Instrument Co., Inc.). This analytical instrument is accurate within the range of 0-2 g/L glucose and L-lactic acid. Before measurement, fermentation broth was centrifuged and diluted with distilled water.

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

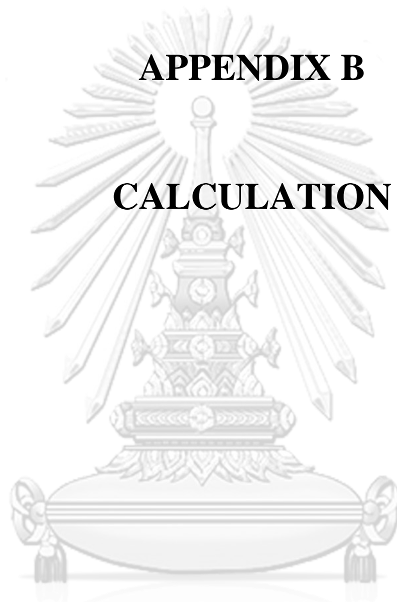
K_2H_2EDTA	4.4	g
Kanamycin sulfate	0.05	g
Sodium benzoate	7.3	g
NaH_2PO_4	12.0	g
Na_sPO_4	54.7	g
NaCl	21.5	g

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

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

APPENDIX B

CALCULATION



จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1. **Yield (g/g)**

$$Yield = \frac{(final\ lactic\ acid\ (g/L))}{(initial\ glucose - remaining\ glucose\ (g/L))}$$

2. **Productivity (g/L.h)**

$$Productivity = \frac{(final\ lactic\ acid\ (g/L))}{(Fermantation\ time\ (h))}$$

3. **Optical purity (%)**

$$optical\ purity = \frac{(D - Lactate) - (L - Lactate)}{(D - Lactate) + (L - Lactate)} \times 100\%$$

4. **Specific growth rate (µ)**

$$specific\ growth\ rate = \frac{\ln \frac{OD_{600_{t2}}}{OD_{600_{t1}}}}{t_2 - t_1}$$

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

VITA

Mr. Srettapat Limsampancharoen was born on Thursday 30th December, 1993, in Bangkok, Thailand. In 2015, he graduated with a Bachelor's degree of Biotechnology, Faculty of Engineering and Industrial Technology, Silpakorn University. 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

Limsampancharoen, S., Jantanma, K., Thitiprasert, S., Thongchul, N. Adaptation of Terrilactibacillus laevilacticus SK5-6 in a reduced nitrogen based medium for growth and D-lactate production. Poster presentation and proceedings. The 7th International Conference on Fermentation Technology for Value Added Agricultural Products, The 12th Asian Biohydrogen & Biorefinery Symposium. 25-28 July 2017. Pullman Khon Kaen Raja Orchid Hotel, Khon Kaen, Thailand.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University