RE-ENGINEERING *KLEBSIELLA OXYTOCA* KMS004 TO IMPROVE D-(-) LACTATE PRODUCTION YIELD

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2019

วิศวกรรมอีกครั้งของเชื้อ *Klebsiella oxytoca* KMS004 เพื่อเพิ่มผลผลิตแลคเตทชนิด D-(-)

นายสุกรา อิน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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ปีการศึกษา 2562

RE-ENGINEERING *KLEBSIELLA OXYTOCA* KMS004 TO IMPROVE D- (-) LACTATE PRODUCTION YIELD

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สุกรา อิน : วิศวกรรมอีกครั้งของเชื้อ *Klebsiella oxytoca* KMS004 เพื่อเพิ่มผลผลิตแลคเตทชนิด D-(-) (RE-ENGINEERING *KLEBSIELLA OXYTOCA* KMS004 TO IMPROVE D-(-) LACTATE PRODUCTION YIELD) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.เขมวิทย์ จันต๊ะมา, 94 หน้า.

Klebsiella oxytoca M5a1 สายพันธุ์ตั้งต้นได้ถูกดัดแปลงพันธุกรรมมาก่อนหน้านี้เพื่อให้สามารถผลิตแลคเตทชนิดดี (-) จากน้ำตาลกลูโคสในอาหารเลี้ยงเชื้ออย่างง่าย ยีน alcohol dehydrogenase (*adhE*) และ phospho transacetylase-acetate kinase (*pta-ackA*) ถูกตัดออกจากเชื้อสายพันธุ์ตั้งต้นเพื่อสร้างเชื้อสายพันธุ์ *K. oxytoca* KMS004 เชื้อสายพันธุ์ KMS004 สามารถผลิตแลคเตทชนิดดี (-) ในระดับความเข้มข้นที่สูงเมื่อเทียบกับเชื้อสายพันธุ์ตั้งต้น อย่างไรก็ตาม ผลผลิตอื่นๆ / ผลิตภัณฑ์อื่นๆ ยังคงเป็นปัญหาในการผลิตแลคเตท นอกจากนี้ เชื้อสายพันธุ์ KMS004 สามารถใช้น้ำตาลกลูโคสได้เพียง 50 กรัมต่อลิตร เพื่อการผลิตแลคเตทชนิดดี (-) ดังนั้น การศึกษาครั้งนี้ ยีน fumarate reductase ABCD (*frdABCD*) และ pyruvate formate lyase B (*pflB*) จึงถูกตัดออกเพื่อเพิ่มการผลิตแลคเตท โดยยีนทั้งสองนี้เป็นวิถีหลักในการสร้าง NAD^+ วิวัฒนาการของกระบวนการสร้างและสลายถูกนำมาใช้ โดยทำการถ่ายเชื้อสายพันธุ์ใหม่ *K. oxytoca* KIS004 ในอาหารเลี้ยงเชื้ออย่างง่ายชนิด AM1 ที่มีน้ำตาลกูลโคส 50 และ 100 กรัมต่อลิตร เพื่อเพิ่มการผลิตแลคเตทชนิดดี (-) สายพันธุ์ KIS004 ถูกนำมาทดสอบในอาหารเลี้ยงเชื้ออย่างง่ายชนิด AM1 ที่มีน้ำตาลกลูโคส 50 และ 100 กรัมต่อลิตร เพื่อการผลิต แลคเตทชนิดดี (-) ภายใต้ทั้งกระบวนการหมักแบบกะ และแบบกึ่งกะ ในสภาวะไร้ออกซิเจนที่มีการควบคุมความเป็นกรดด่าง ผลการศึกษาพบว่า ในระดับน้ำตาลกูลโคส 50 กรัมต่อลิตร สายพันธุ์ KIS004 ผลิตแลคเตทชนิดดี (-) ที่ความเข้มข้น 45.2±0.02 กรัมต่อลิตร โดยมีผลผลิตอยู่ที่ 0.96±0.05 กรัมต่อกรัม และอัตราการผลิตที่ 0.47±0.01 กรัมต่อลิตรต่อชั่วโมง หลังจากการทำวิวัฒนาการของกระบวนการสร้างและสลาย พบว่าสายพันธุ์ KIS004-91T ผลิตแลคเตทชนิดดี (-) ที่ความเข้มข้น 95.9±0.2 กรัมต่อลิตร มีค่าผลผลิต 0.95±0.01 กรัมต่อกรัม และอัตราการผลิต 1.00±0.01 กรัมต่อลิตรต่อชั่วโมง จากน้ำตาลกลูโคส 100 กรัมต่อลิตร ในขวดทดลองขนาด 500 มิลลิลิตร โดยใช้ปริมาตรน้ำหมัก 350 มิลลิลิตร เพื่อเพิ่มการผลิตแลคเตทชนิดดี (-) เชื้อสายพันธุ์ KIS004-91T ถูกนำมาทดสอบในถังปฏิกรณ์ชีวภาพขนาด 5 ลิตร ในกระบวนการหมักแบบกะ พบว่าแลคเตทชนิดดี (-) ถูกผลิตที่ความเข้มข้น 100±0.2 กรัมต่อลิตร มีค่าผลผลิต 0.96±0.02 กรัมต่อกรัม และอัตราการผลิต 2.01±0.01 กรัมต่อลิตรต่อชั่วโมง ในกระบวน การหมักแบบกึ่งกะ แลคเตทชนิดดี (-) ถูกผลิตขึ้นที่ความเข้มข้น 129±0.12 กรัมต่อลิตร มีค่าผลผลิต 0.95±0.12 กรัมต่อกรัม และอัตราการผลิต 1.9±0.02 กรัมต่อลิตรต่อชั่วโมง นอกเหนือจากนี้

กระบวนการหมักที่ทำควบคู่กับการย่อย (SHF) ถูกนำมาทดสอบใช้กับแป้งมันสำปะหลังที่เป็นสารตั้งต้น จากผลการศึกษาพบว่า แลคเตทชนิดดี (-) ถูกผลิตขึ้นที่ความเข้มข้น 98±0.8 กรัมต่อลิตร มีค่าผลผลิต 0.93±0.01 กรัมต่อกรัม และอัตราการผลิต 1.43±0.02 กรัมต่อลิตรต่อชั่วโมง ดังนั้นจึงสรุปได้ว่าสายพันธุ์ KIS004-91T เป็นสายพันธุ์ที่มีความสามารถต่อการผลิตแลคเตทชนิดดี (-) ได้สูง ยิ่งกว่าไปนั้น สายพันธุ์ KIS004-91T สามารถเป็นอีกหนึ่งทางเลือกสำหรับการผลิตแลคเตทชนิดดี (-) ในภาคอุตสาหกรรม

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2562 ลายมือชื่ออาจารย์ที่ปรึกษา_____

SOKRA IN : RE-ENGINEERING *KLEBSIELLA OXYTOCA* KMS004 TO IMPROVE D-(-) LACTATE PRODUCTION YIELD. THESIS ADVISOR : ASSOC. PROF. KAEMWICH JANTAMA, Ph.D., 94 PP.

RE-ENGINEERING/*KLEBSIELLA OXYTOCA* KMS004/D- (-) LACTATE

Klebsiella oxytoca M5a1 wild type strain was previously engineered to produce D-(-) lactate in the mineral salts medium containing glucose. Gene encoding, alcohol dehydrogenase (*adhE*) and phospho transacetylase- acetate kinase (*pta-ackA*) were removed from the wild type strain to construct *K. oxytoca* KMS004. KMS004 strain produced a high titer of D-(-) lactate compared to the *K. oxytoca* wild-type. However, other by-products are still a concern in the production of D-(-) lactate. Additionally, glucose at the concentration of 50 g/L could be only utilized in the experiment to produce D-lactate by KMS004. In this work, the fumarate reductase ABCD gene (*frdABCD*) and pyruvate formate lyase B (*pflB*) were removed to offer D-(-) lactate production as the key pathway to regenerate NAD^+. Metabolic evolution was also applied by repeatedly transferring the newly constructed strain, *K. oxytoca* KIS004 in the AM1 medium containing 50 and 100 g/L glucose to improve D-(-) lactate production. The KIS004 strain was tested in the mineral salt medium (AM1) containing 50 and 100 g/L glucose for D-(-) lactate production with both batch and fed-batch under anaerobic conditions with a pH control. The results indicated that in 50 g/L glucose, KIS004 produced D-(-) lactate at a concentration of 45.2±0.02 g/L, with a yield of 0.96±0.05 g/g and productivity of 0.47±0.01 g/L/h. After metabolic evolution, the evolved strain KIS004-91T produced D-(-) lactate at a concentration of 95.9±0.2 g/L, with a yield of 0.95±0.01 g/g and productivity of 1.00±0.01 g/L/h from 100 g/L glucose in 500 mL vessels with the working volume 350 mL. To improve D-(-) lactate production, KIS004-91T was applied in the 5 L bioreactor. In the batch process, the results showed that D-(-) lactate was

produced at a concentration of 100±0.2 g/L, with a yield of 0.96±0.02 g/g and productivity of 2.1±0.01 g/L/h. In the fed-batch process, D-(-) lactate at a concentration of 129±0.2 g/L, with a yield of 0.95±0.12 g/g and productivity of 1.9±0.02 g/L/h was produced. In addition, simultaneous hydrolysis and fermentation (SHF) was further performed using cassava starch as a substrate. The result revealed that a concentration of D-(-) lactate at 98.4±0.8 g/L, with a yield of 0.93±0.01 g/g and productivity of 1.43±0.02 g/L/h was produced. In conclusion, KIS004-91T is a high-capacity strain, which is able to produce high D-(-) lactate. Exclusively, KIS004-91T would be one of the feasible choices for D-(-) lactate production in industry.

School of Biotechnology Student's Signature _____

Academic Year 2019 Advisor's Signature _____

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and high appreciation to my advisor, Assoc. Prof. Dr. Kaemwich Jantama for supporting, guidance, construction comments, and encouragement during my study and especially for supporting a fully-financial fund under One Research One Graduation Scholarship (OROG).

I would like to say thank all of the faculty members in the School of Biotechnology in SUT. Thank would be extended to Dr. Panwana Khunnonkwao, Nonthaporn Wong, Pachamon Pichayajittipong, Weeranat Sima, Chutchawan Phosiran, Sokha Kory, Tassanon Chaleewong, and Saiparn Wannasiriwattana for their help in my research work.

Finally, I have to express my sincere thanks to my parents, Mr. Yean IN and Mrs. Khhea HOU as well as my brothers and sisters Mr. Sophat IN, Mrs. Sophen IN, Mrs.Sopha IN, Mr. Sokrat IN, and Mrs. Sreyrath IN for their grateful love and care, support and encouragement.

Sokra In

CONTENTS

Page

ABSTRACT IN THAI I

ABSTRACT IN ENGLISH III

ACKNOWLEDGEMENTS V

CONTENTS VI

LIST OF TABLES XIV

LIST OF FIGURES X

LIST OF ABBREVIATIONS XII

CHAPTER

I INTRODUCTION 1

1.1 Overview 1

1.2 Research objectives 2

1.3 Scope and limitations 3

II LITERATURE REVIEW 4

2.1 Lactic acid and its application 4

2.2 Microbial lactic acid producers 6

2.3 *K. oxytoca* KMS004 11

2.4 Factor affecting lactic acid production in fermentation 14

2.4.1 Effect of substrate concentration 15

2.4.2 Effect of pH 15

2.4.3 Effect of temperature 15

2.4.4 Effect of initial OD 16

CONTENTS (continued)

Page

2.4.5 Effect of enzyme loading 16

2.5 Cassava starch 17

2.5.1 Amylose 17

2.5.2 Amylopectin 18

2.5 Fermentation 19

III MATERIALS AND METHODS 22

3.1 Genetic engineering method 22

3.1.1 Strains, media, and growth condition 22

3.1.2 Amplify DNA fragment by PCR 25

3.1.3 Agarose gel electrophoresis of DNA 25

3.1.4 PCR clean-up method 26

3.1.5 PCR clean-up Gel Extraction 26

3.1.6 Preparation of *K. oxytoca* KMS004 competent cell 27

3.1.7 Transformation of *K. oxytoca* KMS004 by electroporation 27

3.1.8 Deletion of *frd* gene in *K. oxytoca* KMS004 27

3.1.9 Deletion of *pflb* gene in *K. oxytoca* KMS002 28

3.1.10 Metabolic evolution 29

3.2 Fermentation operation and conditions 30

3.2.1 Fermentation medium 30

3.2.2 Batch fermentation in 5 L Bioreactor 30

3.2.3 Fed-batch in 5 L bioreactor in initiate volume 2.5 L 30

CONTENTS (continued)

Page

3.2.4 Batch SHF in 5 L bioreactor of cassava starch 30

3.2.5 Analytical method 31

IV RESULTS AND DISCUSSION 32

4.1 Deletion of *frd* gene in *K. oxytoca* KMS004 32

4.2 Effect of *frd* gene deletion in KMS004 34

4.3 Deletion of a *pflB* gene in *K. oxytoca* KIS002 39

4.4 Effect of *frd* gene deletion in *K. oxytoca* KIS002 41

4.5 Metabolic evolution 42

4.6 Production of D-(-) lactate from 10% (w/v) glucose by KMS004, KIS002, KIS004, and KIS004-91T in fleaker 350 mL 45

4.7 Batch fermentation of D- lactic acid in AM1 medium containing 10 % (w/v) glucose in 5 L fermenter 48

4.8 Fed-batch fermentation of lactic acid in 5 L fermenter 49

4.9 Batch SHF in 5 L bioreactor of cassava starch 55

V CONCLUSION 58

REFFERENCES 59

APPENDIX 73

BIOGRAPHY 94

LIST OF TABLES

Table **Page**

2.1 Different microorganisms used for biotechnological production of L-(+) and D-(-) lactic acid 8

3.1 Strains, plasmids, and primers used in this study 21

3.2 Composition of AM1 mineral low salt medium (excluding carbon source) 24

3.3 PCR parameters for the amplification of specific genes 25

4.1 Fermentation profile of metabolically engineered KMS004, KIS002, and KIS004 strains in AM1 medium containing 5% (w/v) glucose 36

4.2 Fermentation profile of metabolically engineered KMS004, KIS002, KIS004 and KIS004-91T strains in AM1 medium containing 10% (w/v) glucose 46

4.3 Comparison of D-lactic acid production in various media and strains 52

LIST OF FIGURES

Figure **Page**

2.1 Stereoisomers form of L(+) lactic acid/(S)-lactic acid and D(-) lactic acid /(R)-lactic acid 5

2.2 Flowchart of commercial applications of lactic acid 6

2.3 Methods of lactate manufacturing by chemical synthesis and microbial fermentation 7

2.4 Fermentation pathway of *K. oxytoca* KMS004 under anaerobic conditions 13

2.5 Typical batch growth curve 14

2.6 Chemical structure of amylose α-(1-4)-glucan 18

2.7 Chemical structure of amylopectin α-(1-6)-glucan 18

2.8 Generalized schematic representation of a typical fermentation process 20

2.9 Growth of a typical microbial culture in batch condition 21

3.1 a) Process of frd gene deletion in *K. oxytoca* KMS004, (b). Process of pflB gene deletion in *K. oxytoca* KIS002 29

4.1 Plasmids pKJ1011 and pKJ1012 used for *frd* gene deletion 32

4.2 Gel electrophoresis confirmed the recombinant strain KIS002 33

4.3 Fermentation profile of K. oxytoca KMS004, KIS002, and KIS004 in AM1 medium containing 5% (w/v) glucose 37

4.4 Plasmids pKJ1014 and pKJ1015 used for *pflB* gene deletion 39

4.5 Gel electrophoresis confirmed the recombinant strain KIS004 40

LIST OF FIGURES (continued)

Figure **Page**

4.6 (a) Biomass formation and (b) D-lactic acid concentration during metabolic evolution of *K. oxytoca* KIS004 containing 10% glucose in AM1 medium in 500 mL fleaker with working volume 350 mL 44

4.7 Fermentation profile of *K. oxytoca* KMS004, KIS002, KIS004 and KIS004-91T in AM1 medium containing 10 % (w/v) glucose 47

4.8 Fermentation profile of batch fermentation profile of *K. oxytoca* KIS004-91T in AM1 medium containing 10 % (w/v) glucose in 5 L bioreactor with 2.5 L working volume 49

4.9 Fed-batch fermentation profile of *K. oxytoca* KIS004-91T in AM1 medium containing 10 % (w/v) glucose in 5 L bioreactor with 2.5 L working volume 51

4.10 Fermentation profile of separated hydrolysis and fermentation of KIS004-91T using cassava starch as substrate for D-lactic acid production 56

LIST OF ABBREVIATIONS

ACKA	=	Acetate kinase
ADHE	=	Alcohol dehydrogenase
AM1	=	Mineral Salt Medium
AMG	=	Amyloglucosidase/Glucoamylase
ATP	=	Adenosines 5’-triphosphate
Bps	=	Base pairs
BUD	=	2,3 butanediol dehydrogenase
°C	=	Degree Celsius
Camp	=	Cyclic adenosine monophosphate
CDW	=	Cell dried weight
CFU	=	Colony form unit
fdh	=	Formate dehydrogenase H
Fig.	=	Figure
frdABCD	=	Fumarate reductase ABCD
g	=	Gram
g/L	=	Gram (s) per Liter
GLC	=	Glucose
GRAS	=	Generally Recognized as Safe
h	=	Hour/Hours
HPLC	=	High performance liquid chromatography
IUPAC	=	International union of pure and applied chemistry
K. oxytoca	=	*Klebsiella oxytoca*
L	=	Liter (s)
LB	=	Luria Bertain
LDHA	=	Lactate dehydrogenase A
M	=	Molar
mM	=	Milli-molar

LIST OF ABBREVIATIONS (Continued)

mg	=	Milligram (s)
mg/L	=	Milligram (s) per Liter
min	=	Minutes (s)
mL	=	Milliliter (s)
mm	=	Millimeter (s)
MW	=	Molecular weight
NADH	=	Nicotinamide adenine dinucleotide (Reduced form)
NAD^+	=	Nicotinamide adenine dinucleotide (Oxidized form)
OD	=	Optical Density
PCR	=	Polymerase chain reaction
PEP	=	Phosphoenolpyruvate
pflB	=	Pyruvate formate-lyase B
pH	=	Potential Hydrogen
poxB	=	Pyruvate oxidase
pta	=	Phosphotransacetylase
rpm	=	Revolution per minute
SSF	=	Simultaneous Saccharification Fermentation
SHF	=	Separated Hydrolysis Fermentation
TBE	=	Tris-borate-EDTA
UV	=	Ultraviolet
V	=	Volt
v/v	=	Volume per volume
w/v	=	Weight per volume
µL	=	Micro-liter

CHAPTER I

INTRODUCTION

1.1 Overview

In recent years, researchers have been interested in biofuel and biochemical productions from biomass (Lee et al., 2017). Environmental pollution is increasing due to the use of petroleum and has become a major problem. Therefore, technologies to protect the environment are required (Ravikumar et al., 2017). Many essential chemicals including 2,3 butanediol (Jantama et al., 2015; Yamada et al., 2017a), ethanol (Xu et al., 2018), succinic acid (Jantama et al., 2008a; Khor et al., 2016; Khunnonkwao et al., 2018), acetic acid (Huo et al., 2015; Mas et al., 2014), and lactate (Sangproo et al., 2012; Thitiprasert et al., 2017; Thongchul et al., 2010) are produced from renewable sources. Among these, lactate has long been of interest as a biochemical product and precursor for several industrial chemicals including food additives, pharmaceuticals and fine chemicals (Valli et al., 2006). Furthermore, it has an excessive potential for producing polylactic acid (PLA) polymers known as production of the biocompatible and biodegradable chemical. PLA is currently driven the market expansion for the lactate application (Abdel-Rahman et al., 2013). Moreover, lactate has been producing by both of chemical synthesis and microbial fermentation. By the chemical synthesis, a mixture of DL-lactate is occasionally produced. On the other hand, fermentation offers advantages to the production of an optically pure D- or L-lactic acid from cheap renewable substrates (Abdel-Rahman et al., 2011; Abdel-Rahman et al., 2015). The production of stereoisomers of lactic acid depends on different kinds of microorganisms and fermentative conditions (Subramanian et al., 2015).

Various microorganisms have produced lactic acid such as *Lactobacillus rhamnosus* (Bernardo et al., 2016), *Rhizopus oryzae* (Thongchul et al., 2010), *Enterobacter aerogenes* (Thapa et al., 2017; Zhou et al., 2006) and metabolically engineered strains of *Escherichia coli* (Utrilla et al., 2009; Zhou et al., 2006) and *Klebsiella oxytoca* (Sangproo et al., 2012). A metabolically engineered *K. oxytoca* KMS004 was originally developed to produce an optically pure D-(-) lactate with impressive titers (Sangproo et al., 2012). However, Sangproo et al. (2012) reported that this strain produced succinate and formate as major by-product thus making lower D- (-) lactate production yield. Anyway, low concentration of glucose (5%) was utilized by this strain. Therefore, this study was focused on a combination of eliminating the formation of succinate and formate by re-engineering *K. oxytoca* KMS004 and performing metabolic evolution for the newly constructed strain in the medium containing 10% (w/v) glucose to improve the yield of D-lactic acid. Cassava starch is a cheaper source and readily available in tropical and sub-tropical areas, especially Thailand. The use of cassava starch as a carbon source may make the fermentation process of D-(-) lactate more economically competitive. Thus, cassava starch was used in this experiment as a potential source for D-lactate production by the strain.

1.2 Research objectives

The objectives of this study are to reduce by-products formation and to improve production yield of D-(-) lactate in *K. oxytoca* KMS004 by performing both of metabolic engineering and metabolic evolution. To achieve bio-based D-(-) lactate production at high yield. Thus, this work focused on:

1. To investigate the effect of fumarate reductase ABCD (*frdABCD*) and pyruvate formate lyase B (*pflB*) gene deletion on D-(-) lactate production under anaerobic condition in *K. oxytoca* KIS004.
2. To evolve the mutant strain in mineral salt medium containing 10% w/w glucose to simultaneously obtain a better growth and D-(-) lactate production

3. To improve the production of D-(-) lactate by applying a simple batch strategy in 5 L bioreactor by the mutant strain.
4. To improve the production of D-(-) lactate by applying a simple fed-batch strategy in 5 L bioreactor by the mutant strain.
5. To improve the production of D-(-) lactate by applying batch SHF using cassava starch as a carbon source

1.3 Scope and limitations

Gene fumarate reductase ABCD (*frdABCD*) and pyruvate formate lyase B (*pflB*) were eliminated from *K. oxytoca* KMS004. Metabolic evolution in AM1 medium containing 10% (w/v) glucose was applied to a mutant strain. Batch and fed-batch fermentation in anaerobic conditions were applied for the D-(-) lactate production by using AM1 medium containing 10% (w/v) glucose. Batch fermentation of SHF was also performed using cassava starch as substrate. Therefore, cost-effectiveness in D-lactate production is expected.

CHAPTER II

LITERATURE REVIEW

2.1 Lactic acid and its application

Lactate was discovered since 1780 by Scheele in sour milk. Lactate is a highly added-value biochemical, which is known as GRAS chemical, recognized as non-toxic by the US Food and Drug Administration (Martinez et al., 2013). Lactate is the 2-hydroxycarboxylic acid with a chiral carbon atom and known in two optically active stereoisomers include L(+) and D (-)-lactate (Razali et al., 2017). Lactic acid or milk acid is an organic compound with the formula $CH_3CH(OH)COOH$ or $C_3H_6O_3$. International Union of Pure and Applied Chemistry (IUPAC) name of lactic acid is 2-hydroxypropanoic acid. Lactic acid has a molecular weight of 90.08 g/mol, melting point of 53 °C, boiling point of 122 °C (252 °F, 395 K), acidity (pKa) of 3.86, Ka 1.38 x 10^{-4}, specific gravity/density of 1.2, standard enthalpy of combustion of 1361.9 k/mol (325.5 kcal/mol, 15.1 kJ/g, 3.61 kcal/g), aqueous solution, mild acid taste, solubility in water (100 mg/mL) miscible, color (white, light yellow solid, colorless, light yellow, clear liquid), and odorless (Martinez et al., 2013; Razali et al., 2017). Pure lactic acid is hydroscopic liquid. It is also known as a weak acid because of its partial dissociation in water as equation $H_3C\text{-}CH(OH)\text{-}COOH \leftrightarrow H^+ + H_3C\text{-}CH(OH)\text{-}COO^-$ (Ameen et al., 2017).

Lactic acid contains one terminal carbon atom, which is part of carboxyl group, another terminal carbon atom is in methyl or hydrocarbon group, and central carbon contains an alcohol carbon group (Narayaan et al., 2004). Additionally, two optically active stereoisomers of lactic acid exist: L (+) lactic acid form or named as (S)-lactic acid and D-(-) lactic acid or named as (R)-lactic acid (Fig. 2.1).

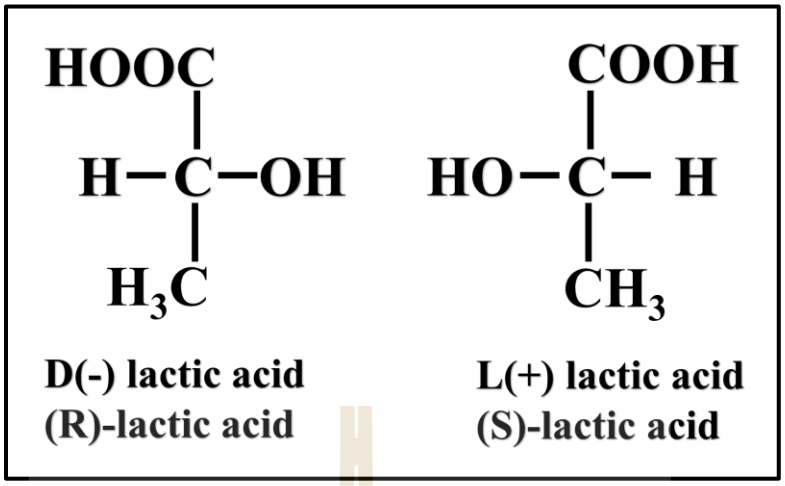


Figure 2.1 Stereoisomers form of L (+) lactic acid/(S)-lactic acid and D (-) lactic acid/(R)-lactic acid (Ameen et al., 2017).

For many years, lactic acid plays major roles in food and chemical industries because of its various functionals (Alsaheb et al., 2015). Furthermore, lactate is found in food and beverage including dairy products, fermented vegetable, wine, cider, and meat industry (Taskila et al., 2013). In the food industry, many products are applied, for instance, acidulates, preservatives, flavoring agent, pH regulators, microbial control, and mineral fortification. Application of lactate has been found in the cosmetic industry including skin-lightening agents, skin-rejuvenating agents, moisturizers, pH regulators, anti-acne agents, humectants, and anti-tartar agents. Application of chemical industry includes descaling agents, pH regulators, neutralizers, chiral intermediates, green solvents, cleaning agents, and a slow acid releasing agent. The application in chemical feedstock as a precursor for the production of propylene oxide, acetaldehyde, acrylic acid, propionic acid, 2,3-pentanedione, ethyl lactate, dilactide, and polylactic acid. The application in the pharmaceutical industry includes parenteral I.V. solution, dialysis solution, mineral preparations, tablet tings, prostheses, surgical sutures, and controlled drug delivery system Fig. 2.2 (Alsaheb et al., 2015; Wee et al., 2006). Besides, Alsaheb et al. (2015) reported that lactic acid is one of the major organic acids, which is being extensively applied around the world in the industrial and biotechnological application. A new optically effective application of lactic acid is used

as a chiral synthon. Therefore, application of polylactic acid is increased quickly in the pharmaceutical and medical fields.

Besides, Wee et al. (2006) reported that lactate produces two types of method include chemical synthesis and microbial fermentation (Fig. 2.3). However, production of lactate is considered a major amount of interest recently. Fermentative production of lactate could reduce the environmental pollution caused by the petrochemical industry in which a large supply of petroleum resources is usually utilized for lactate production by chemical synthesis.

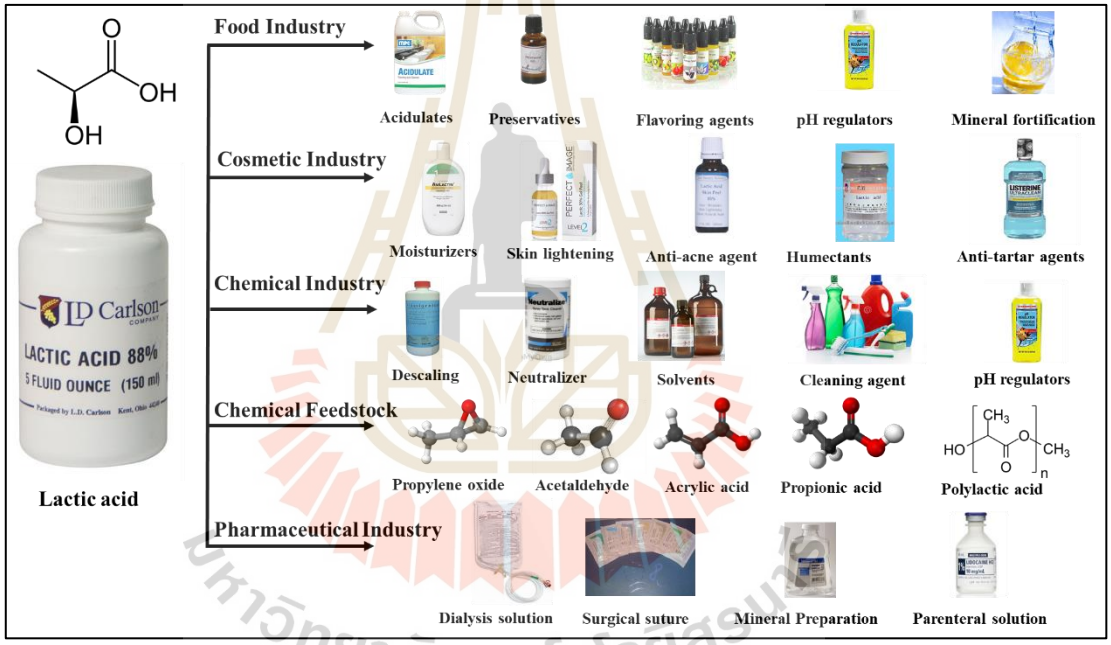


Figure 2.2 Flowchart of commercial applications of lactic acid (Alsaheb et al., 2015).

2.2 Microbial lactic acid producers

Lactic acid is a weak organic acid which is produced by various microorganisms. Table 2.1 shows lactate production form various microorganisms. For example, *Streptococcus mutans* (Dashper et al., 1996), *L. debrueckii* sp. *bulgaricus* (Dan et al., 2017), and *L. sakei* (Oguro et al., 2017) produced lactate as the key end-product of glycolysis under excess sugar or low pH. Meanwhile, almost all lactic acids are produced by lactic acid bacteria (LAB). LABs are the type of microbes which are found in plants, fermented foods, terrestrial marine animals and other related materials. LAB belongs to gram-positive bacteria.

Various genera of microorganisms produce lactic acid as primary or secondary fermentation products including *Lactobacillus, Carnobacterium, Lactococcus, Streptococcus, Enterococcus, Vagococcus, Leuconostoc, Oenococcus, Pediococcus, Tetragonococcus, Aerococcus,* and *Weissella* (Florou-Paneri et al., 2013). Litchfield (1996) reported that microorganisms producing lactate are separated into two categories, which are bacteria and fungi. Fungi strains produce only L (+) lactic acid in aerobic conditions using starch, glucose or sucrose (Zhou et al., 1999). However, bacteria strains can produce both D (-)-lactate and L (+) lactate or with a mixture of lactate by fermentation (Zhao et al., 2010). Until now, bacteria strains are the most favorite for researchers for lactate production because they could produce lactate with high yield (Oonkhanond et al., 2017). Similarly, to be industrially attractive microorganisms of lactic acid production, several essential features should be followed such as reduce time by improve the productivity, reduce substrate costs by improve yield, ability to use low cost medium or substrate to obtain high concentration, low amount of by-products to increase purification yield, and robust to contamination and infections (Auras et al., 2010).

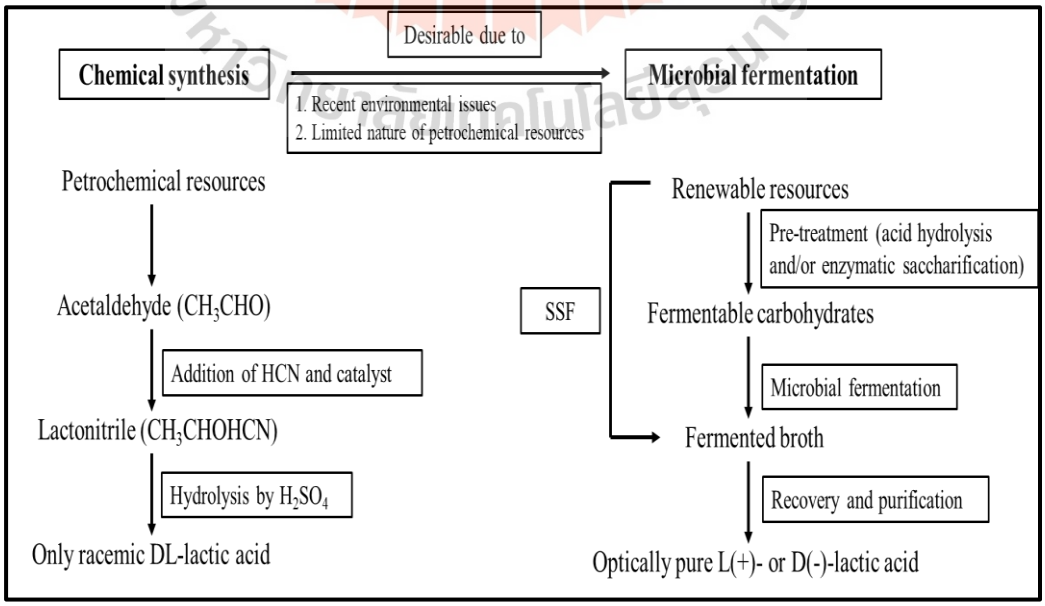


Figure 2.3 Methods of lactate manufacturing by chemical synthesis and microbial fermentation (Wee et al., 2006).

Table 2.1 Different microorganisms used for biotechnological production of L-(+) and D-(-) lactic acid**.**

Type of lactic acid	Microorganisms	Medium/Condition	Concentration (g/L)	Productivity (g/L/h)	Yield (g/g)	References
L(+)	*Rhizopus oryzae* ATTC 52311	YMP medium containing 95 g/L glucose, simple batch, pH controlled by $CaCO_3$, 60 h incubation time	83.0	2.6	0.88	Zhou et al. 1999
L(+)	*R. oryzae* NBRC 5378	The medium contained 25 g/l of sugar (Glucose and Xylose), pH-controlled by $CaCO_3$, Batch SSF, SHF, 96 h fermentation time	6.8	0.07	0.23	Saito et al., 2012
D-(-)	*L. delbrueckii*	400 g/L molasses, Batch fermentation, 40 h for fermentation time	190	4.15	0.95	Dumbepatil et al., 2008
D-(-)	*L. dlbrueckii* NCIM 2025	Pre-culture was prepared in 100 mL of MRS culture medium, pH-controlled by Batch, fermentation time 48 h	150	3.15	0.96	Bhatt et al., 2008
D-(-)	*Bifidobacterium* sp.	Simulated Fruit Vegetable Wastes (57% watermelon, 29% apple and 14% potato), batch, pH 4.0-5.0, Fermentation time 24 h	29.5	1.2	0.68	Wu et al., 2015
D-(-)	*Leuconostoc mesenteroides*	MRS medium, pH 6.3 was controlled by $CaCO_3$, batch, fermentation time 240 h	38.3	0.159	0.99	Mufidah et al., 2016

Table 2.1 (continued).

Type of lactic acid	Microorganisms	Medium/Condition	Concentration (g/L)	Productivity (g/L/h)	Yield (g/g)	References
D-(-)	*L. coryniformis* ssp. *torquens* ATCC 25600	30 g liquid/g solid SSF, pH controlled by 4 M NaOH, 50 h incubation time	24.0	0.5	0.89	Yanez et al., 2003
D-(-)	*Lactobacillus.* sp. RKY2	Wheat bran 200 g/L, simple batch, pH-controlled by 10 M NaOH, 18 h incubation time	47.0	2.6	0.92	Yum et al., 2004
L(+)	*Enterobacter aerogenes* ATCC 29007	112.32 g/L mannitol, LB medium (10 g/L peptone, 5 g/L yeast extract, and 5 g/L NaCl), Simple batch, fermentation time 84 h	46.02	0.5	0.41	Thapa et al., 2017
D-(-)	*K. oxytoca* KMS004	Cultures were grown in LB, pH 6 was controlled, simple batch, fermentation time 96 h	34.0	0.35	0.87	Sangproo et al., 2012
L(+)	*R. oryzae* NRRL 395	Cassava pulp as substrate, PDA medium, pH 6 was controlled in batch	21.8	0.43	0.31	Thongchul et al., 2010

		fermentation by adding 5 N NaOH, fermentation time 105 h				

Table 2.1 (continued).

Type of lactic acid	Microorganisms	Medium/Condition	Concentration (g/L)	Productivity (g/L/h)	Yield (g/g)	References
L(+)	*Bacillus* sp. BC-001	GYP agar medium is used, pH was controlled at 6.5 by 7 M NH_4OH, and 10 M KOH, Fed-batch SSF was used, fermentation continued until glucose depletion (18h).	108.9	6.1	1.04	Thitiprasert et al., 2017
D-(-)	*L. delbrueckii spp. delbrueckii*	MRS broth was used, at 40 ^{0}C and 800 rpm, pH 5.8, 10 g/L biomass, 10 M NaOH was used to adjust pH, 30h fermentation	201	6.72	0.88	de la Torre et al., 2019
D-(-)	*E. coli*	10 g/L cellobiose, LB medium was used, 37 ^{0}C and 150 rpm, pH 7 was adjusted by 4M NaOH, 84h fermentation	5	0.25	0.5	Aso et al., 2019

2.3 *K. oxytoca* KMS004

K. oxytoca is a gram-negative bacterium. *K. oxytoca* was previously engineered to produce D-(-) lactate by fermentation in low nutrition medium. *AdhE,* and *pta-ackA* genes were knocked out from *K. oxytoca* M5a1. First, Sangproo et al. (2012) engineered *K. oxytoca* M5a1 by eliminating the alcohol dehydrogenase E gene (*adhE*) which is responsible for ethanol production. The mutant strain was named as KMS002. Second, phospho-transacetylase/acetate kinase A (*pta-ackA*) genes which are responsible for the production of acetyl-CoA and acetate, respectively were also deleted from the KMS002 strain. The strain was named as KMS004. After metabolic engineered *K. oxytoca* KMS004 was done, the productions rate of D-(-) lactate was increased rapidly using glucose and sugarcane molasse as substrates. In this case, the ethanol was not detected in the fermentation broth of KMS002 and KMS004 strain while other by-products including, succinate, formate, acetate, and butanediol were still produced. Formate and acetate were still produced as major by-products during fermentation of KMS004; even though the acetate kinase gene was eliminated in KMS004. The possible reason is that the strain KMS004 still possesses the *pflB* gene that is responsible for the utilization of pyruvate to acetyl-CoA and formate. Therefore, acetyl-CoA could be converted to acetate by any isoenzymes of acetate kinase. Hence, KMS004 could be further engineered by eliminating the gene responsible for production of formate and acetyl-CoA which is pyruvate formate-lyase B (*pflB*). KMS004 with *pflB* deletion will be improved the production yield of D-lactate because it may reduce the formation of other by-products including formate and acetate. By mean of fermentation, many products are produced by *K. oxytoca* using glucose as a substrate. *K. oxytoca* metabolic pathway occurs in both aerobic and anaerobic conditions (Fig. 2.4).

In the anaerobic pathway, *K. oxytoca* KMS004 could produce D-(-) lactate in higher titer and yield than that of the aerobic pathway compared to other by-products (Sangproo et al., 2012). In anaerobic condition, glucose is converted to pyruvate. Pyruvate can be

converted to D-(-) lactate by lactate dehydrogenase A in a reaction with the requirement of NADH. Pyruvate can be also converted to Acetyl-CoA. Acetyl-CoA can be converted either to ethanol and acetate in *K. oxytoca* wild type. On the other hand, *K. oxytoca* KMS004 can convert acetyl-CoA to the only acetate with a lower amount because alcohol dehydrogenase E and pyruvate transacetylase/acetate kinase A gene are already deleted as shown in (Fig. 2.4) indicated by the circled-crossed signs. Pyruvate can be converted to formate by pyruvate formate-lyase B, *pflB*. Carbon dioxide and hydrogen was produced from formate by formate-hydrogen lyase in a further step.

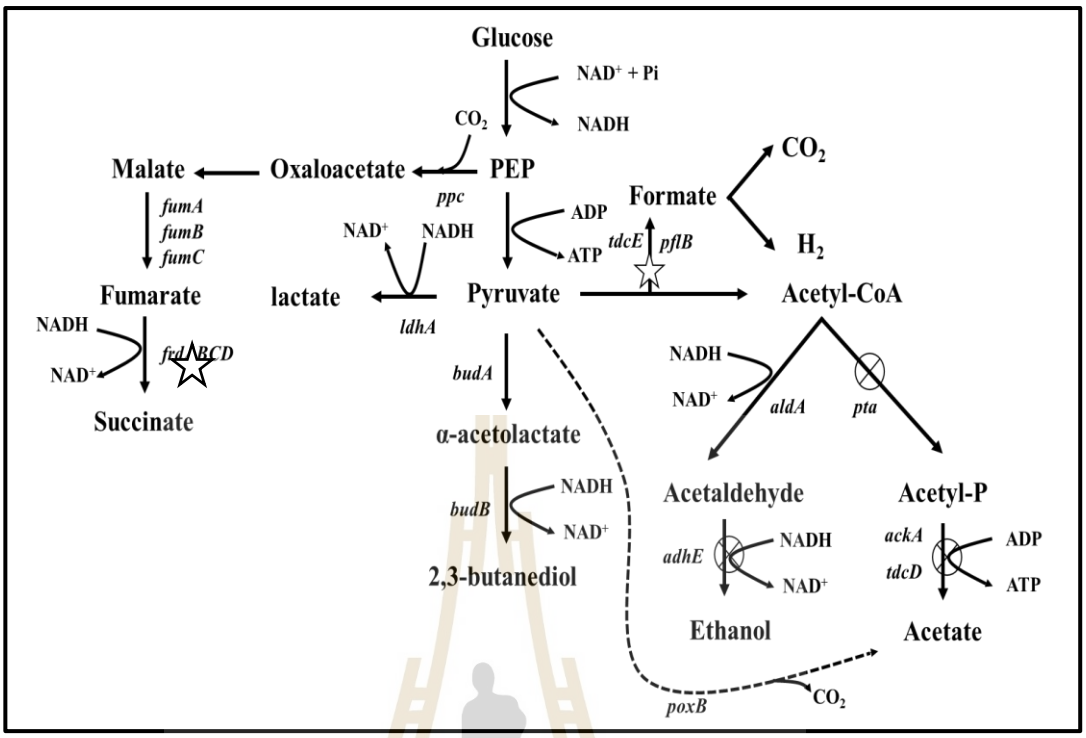


Figure 2.4 Fermentation pathway of *K. oxytoca* KMS004 under anaerobic conditions. Solid arrows represent central fermentative pathways. Dot arrow represents alternative acetate-producing pathway via pyruvate oxidase B (*poxB).* The cross sign represents the gene deletions performed to obtain KMS004 (*ΔadhE, ΔackA-pta).* The star sign represents the *frd* and *pflB* gene that were deleted in KMS004 (*ΔadhE, ΔackA-pta, Δfrd* and *ΔpflB).* Gene and enzymes: *ldhA*, lactate dehydrogenase*; pflB*, pyruvate formate-lyase; *pta*, phosphate acetyltransferase; *ackA*, acetate kinase; *tdcD*, propionate kinase; *tdcE*, threonine decarboxylase E; *adhE*, alcohol dehydrogenase; *ppc*, phosphoenolpyruvate carboxylase; *mdh*, malate dehydrogenase; *fumABC*, fumarase isozymes; *frdABCD*, fumarate reductase; *fdh*, formate dehydrogenase; *poxB*, pyruvate oxidase; *budA*, α-acetolactate decarboxylase; *budB*, α-acetolactate synthase, *budC*, butanediol dehydrogenase; *aldA*, aldehyde dehydrogenase; *dhaBCE*, glycerol dehydratase and *dhaT*, 1,3-propanediol oxidoreductase (Jantama et al., 2015).

2.4 Factor affecting lactic acid production in fermentation

Different systems and processes are applied such as a batch, a semi-batch, a fed-batch, and a continuous culture. The batch process is operated in a closed system, which is allowed to add all materials to the system at the start of the process. The products are allowed to remove when the process is completed. Moreover, several phases are observed in batch process including lag phase, growth phase or log phase, stationary phase, and death phase Fig. 2.5 (Doran, 1995). In this case, during the process, some factors are affected to the rate of D-(-) lactate production including, pH, temperature, types of microorganisms, carbon source, nitrogen source, and fermentation mode (Hofvendahl et al., 2000).

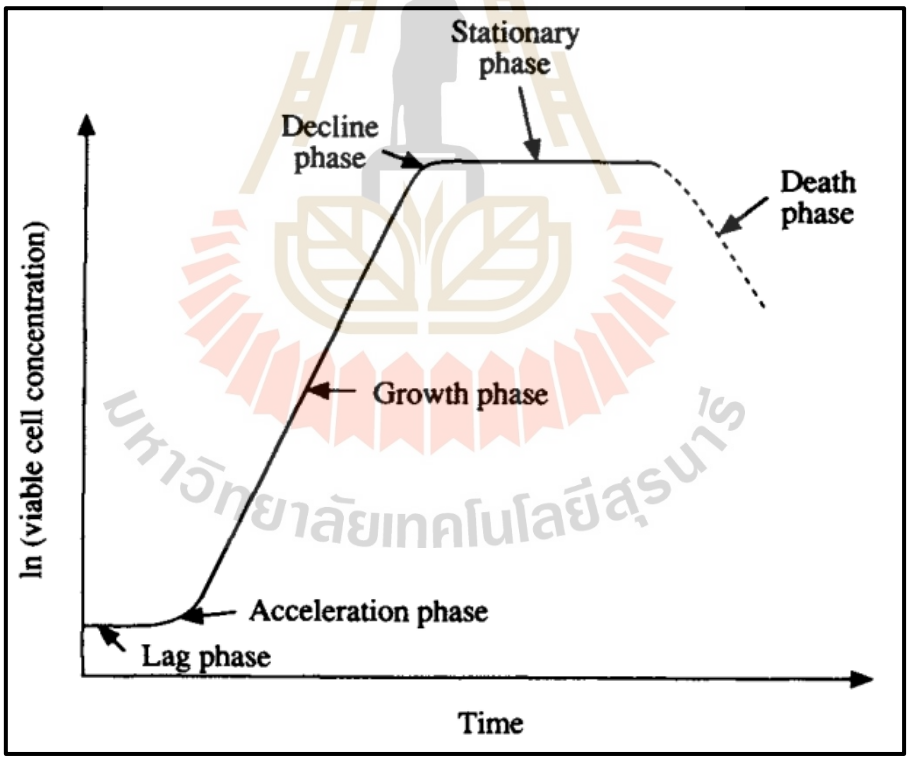


Figure 2.5 Typical batch growth curve (Doran, 1995).

2.4.1 Effect of substrate concentration

The substrate is just like the food for microorganisms but a high concentration of substrates can inhibit the enzyme activity thus causing inhibition of cell growth. Hence, the concentration of the initial substrate also drives an essential role in lactic acid fermentation (Ghaly et al., 2005). Substrate inhibition occurs when the high substrate concentration is supplied. For the reason, lower in specific growth rate and substrate utilization is usually observed at high substrate concentrations (Tango et al., 1999). In some works, the optimum initial substrate concentration for D-(-) lactate production by bacteria was observed at 15% (Ozen et al., 1992).

2.4.2 Effect of pH

pH is a strong parameter affecting cell metabolism. Thus, almost experiments have controlled the pH in the experiment. Wang et al. (2012) indicated that an optimum pH for lactate production using *Lactobacillus* spp. ranges between 6.0 and 6.5. In addition, pH 5 has also been demonstrated an optimal for lactic acid bacteria to obtain the high lactic acid yield (Tang et al., 2017). Likewise, *K. oxytoca* is able to grow at a pH of at least 5.0 (Ohta et al., 1991). However, the maximum production yield of lactate using *K. oxytoca* KMS004 was obtained at pH 7 compared with pH 5 (Sangproo et al., 2012).

2.4.3 Effect of temperature

Temperature is also the major factors affecting lactic acid production. Yuwono (2008) revealed that 39 °C was optimum temperature for lactate production from cassava root by *Streptococcus bovis.* Additionally, in batch fermentation by using immobilized *L. delbrueckii subsp. delbrueckii* ATCC 9646 under anaerobic conditions, the temperature at 37 °C is found optimum (Idris et al., 2004). However, in batch fermentation by using *K. oxytoca* KMS004, the optimal temperature for fermentation of lactate production was 37 °C (Sangproo et al., 2012).

2.4.4 Effect of initial OD

Optical densities (OD) commonly used to quantify various essential culture parameters, for instance, cell concentration, biomass and other changes in cell morphology (Sandnes et al., 2006). High cell density allowed offering high productivity and reducing the fermentation time. Moreover, a number of cells are significant in fermentation processes to complete substrate consumption and suitable for the yields (Agbogbo et al., 2007). Increasing the inoculum size also allowed to improve cell viability. In this case, the amount of cell in the initial inoculum maintains the amount of fermentation product. Furthermore, the cell concentration is able to control the fermentation rate, production rate, and the yield. However, high cell concentration resulted in the low specific productivity of D-(-) lactate (Souza et al., 2002). On the other hand, when initial cell inoculation is increased, the fermentation rate is also accelerated. Increasing initial cell densities are inoculated, decreasing the time for glucose utilization. Unfortunately, production yield does not significantly increase with high cell density (Palmqvist et al., 1996).

2.4.5 Effect of enzyme loading

Generally, the cost of enzymes is the matter existing in the lactate production from cellulosic and lignocellulosic materials. Therefore, many works of enzyme loading were studied to conduct fermentation by using a smaller number of enzymes, but to obtain a much higher yield of product (Absar et al., 2009). For example, the amount of glucoamylase used for saccharifying cassava powder was determined (Wang et al., 2012). Simultaneous saccharification and fermentation (SSF) of starch to lactate was studied to determine the optimum enzyme loading to produce D-(-) lactate by *L. plantarum* using cassava starch as substrate (Chookietwattana, 2014).

2.5 Cassava starch

Cassava is a tropical crop which is originated from the Amazon region (Demiate et al., 2011). It is a main source of energy in dietary food for people living in tropics and Central Africa. Moreover, the properties of cassava starch and flour are essential to produce other products (Oladunmoye et al., 2014). Zhu et al. (2015) revealed that the main component of cassava starch obtaining from roots are sustainable and cheap. Besides, it is considered a source of some nutrient includes riboflavin, thiamin, carbohydrate and nicotinic acid. Nowadays, cassava starch is grown all around the world and expected to reach up to 290 million tons in 2020 (Zhu et al., 2015). Starch granules are composed of 2 types of α-glucan includes amylose and amylopectin which contained about 98-99% of the dry weight (Tester et al., 2004). Starch functionality is affected by a ration of amylose and amylopectin and the amount of these components are depended on botanical origin and variety of starch (Oyeyinka et al., 2018). Starch is a fermentable sugar of polysaccharide consisting of a long chain of molecule glucose linked together known as glycosidic bonds (Visakh et al., 2012).

2.5.1 Amylose

Amylose is known as linear chain of D-glucose through α-(1-4)-linkage. It is one of the two components of starch, which is contained about 20-30% (Fig. 2.6) (Perez et al., 2010; Tester et al., 2005). Amylose is a long linear chain that is more readily crystallized than amylopectin. Thus, starch with high amylose is more resistant to enzymatic digestion (Birt et al., 2013). Amylose can exist in two different helical forms. Amylose in double helix form can bind with itself or with other hydrophobic molecules includes iodine, fatty acid, or aromatic compounds (Cohen et al., 2008). Helical amylose chain with loose form is a hydrophobic interior which can bind to hydrophobic molecules such as lipid, and aromatic compound (Chung et al., 2009).

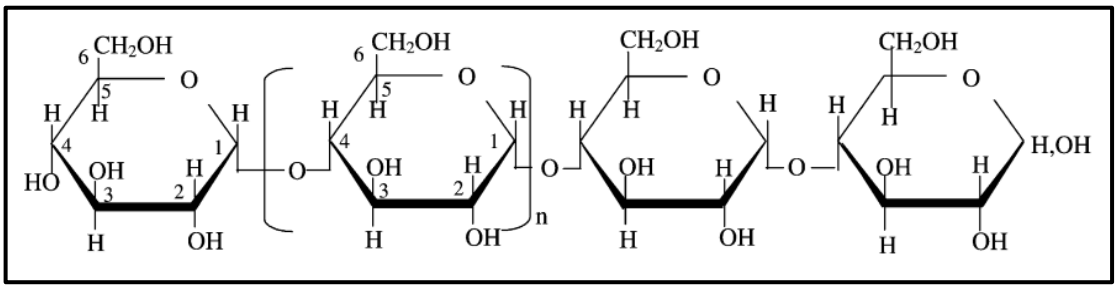


Figure 2.6 Chemical structure of amylose α-(1-4)-glucan (Perez et al., 2010).

2.5.2 Amylopectin

Amylopectin is also the main component of cassava starch and it has the ability to interact with iodine to produce reddish color in solution (Zhu et al., 2015). Glucose units are linked together by glycosidic bonds by α-(1-4)-linkage consisting of thousands of short linear chains. These short linear chains are linked to each other by α-(1-6)-linkage which is occurred in every 24 to 30 glucose units, known as amylopectin (Fig. 2.7). Furthermore, amylopectin is a large molecule with a molecular weight of 828.7 g/mole which is bigger than amylose 504.4 g/mole (National Center for Biotechnology Information, 2019). Amylopectin contained 95% of α-(1-4)-linkage and 5% of α-(1-6)-linkage (Tester et al., 2004). Jane (2009) revealed that normal starches contain 20-30% amylose and 70-80% amylopectin. The average chain length of amylopectin depends on origin and maturity of starch and varies with the location of molecules in the granule of starch.

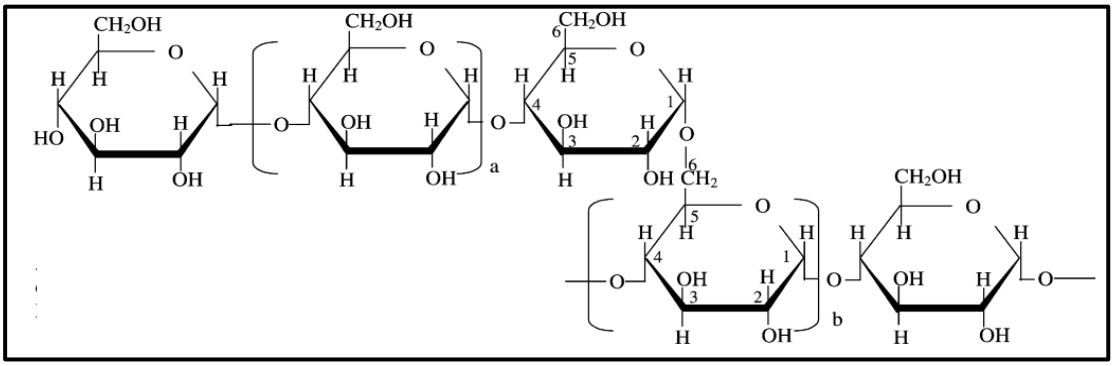


Figure 2.7 Chemical structure of amylopectin α-(1-6)-glucan (Tester et al., 2004).

2.6 Fermentation

Fermentation is the latin word indicates to boil. The word ‘fermentation’ is caused by the production of carbon dioxide bubbles in anaerobic catabolism of sugar. Though, different meanings of fermentation are meant by biochemists and microbiologists (Stanbury et al., 2003). Stanbury et al. (2003) reported that in commercially important fermentations are divided into five majors includes those that produce biomass, microbial enzymes, microbial metabolites, recombinant products as the products, and those that modify a compound which is added to the fermentation. In addition, six basic component parts are divided regardless of the type of fermentation. First, preparing the formulation of media to use in culturing the organism process during inoculum development and production of fermentation. Second, autoclave medium, fermenters, and all equipment to be used in this fermentation. Third, the production of an active, pure culture in sufficient quantity to inoculate the production vessel. Fourth, optimum conditions of organisms were used in fermentation. Fifth, products in the fermentation broth is extracted and purified. And sixth, disposal of effluents produced by the process should be performed (Fig. 2.8). Likewise, the types of culture used in fermentation depend on the type of products being produced. The fermentation may be carried out as a batch, fed-batch, and continuous processes (Doran, 1995).

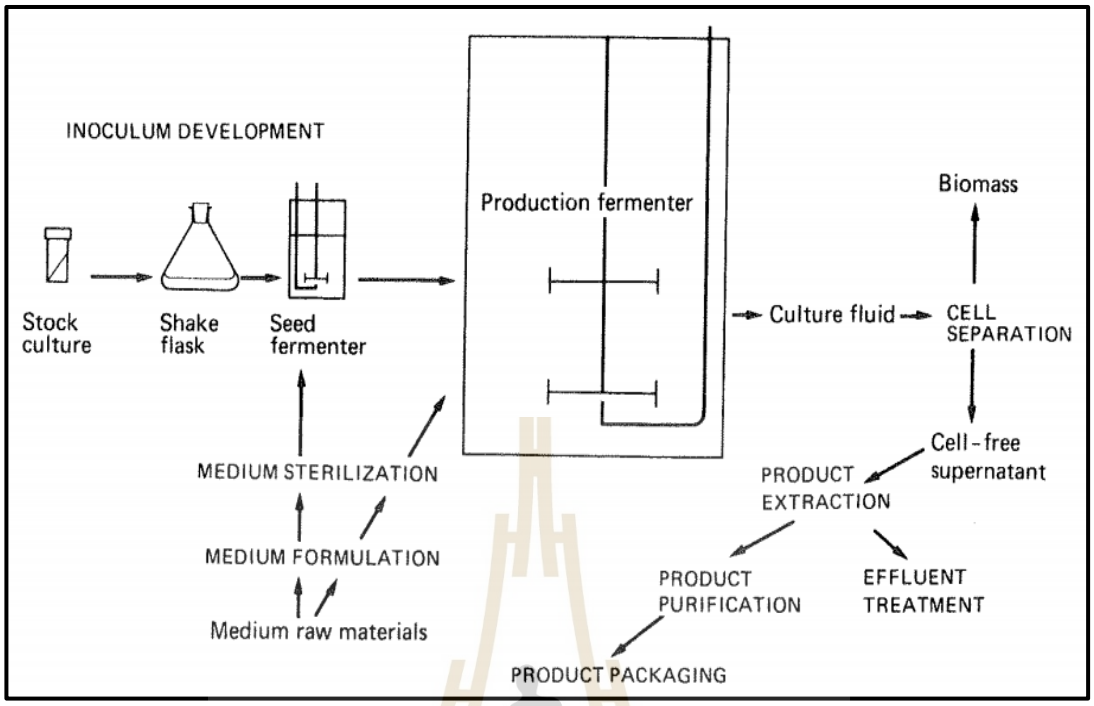


Figure 2.8 Generalized schematic representation of a typical fermentation process (Stanbury et al., 2003).

Batch culture is operated in a closed system, which is allowed to add all materials to the system at the start of the process. The inoculated culture passes through several phases (Fig. 2.9). In batch culture, the growth curve is shown by plotting the natural logarithm of viable cell concentration and time as shown in Fig. 2.9. Immediately after inoculation, the rate of growth is essentially zero ($\mu=0$) have known as a lag phase which the cell tries to adapt to their new environment. Then, the cells start to grow in the acceleration phase ($\mu< \mu_{max}$) and continue through the log phase ($\mu= \mu_{max}$) to the deceleration phase ($\mu< \mu_{max}$). After that, the stationary phase ($\mu=0$) is reached. During this phase, the cells will not further grow. The last phase is the death phase ($\mu< 0$) in which the cells are lost viability or destroyed by lysis (Doran, 1995; Stanbury et al., 2003).

Fed-batch culture is introduced by Yoshida et al. (1973) to describe the batch cultures that sequentially add medium to culture but did not remove the product till the end of fermenation. Normally, fed-batch fermentation has supplemented the medium to the

fermenter at a given time interval (Zhu et al., 2017). Continuous culture is a kind of process that is proposed to prolong the growth phase in batch culture by adding fresh medium to the bioreactor. In a similar way, continuous culture has been designed to provide the medium with substrate limitation but not toxin limitation. This exponential growth allows the growth until the substrate addition is exhausted or until the vessel is full (Stanbury et al., 2003).

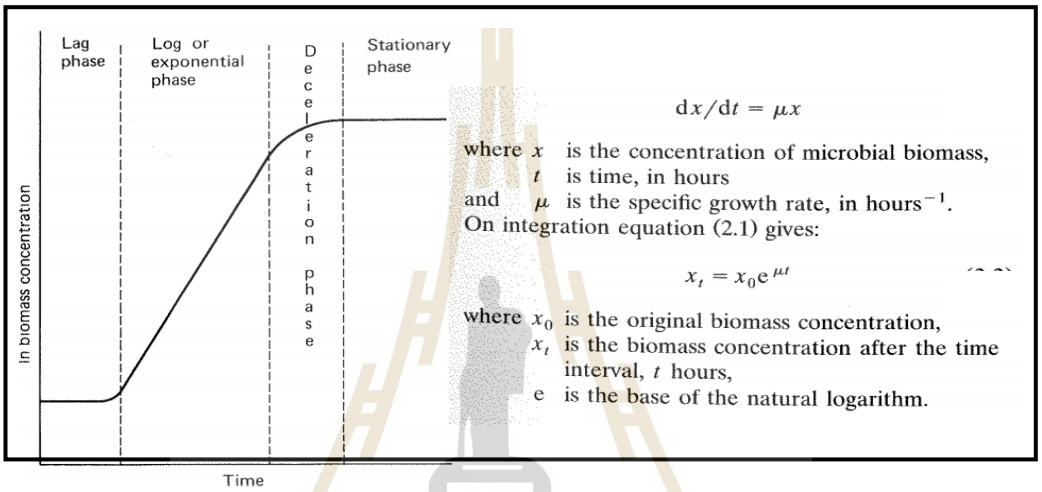


Figure 2.9 Growth of a typical microbial culture in batch condition (Stanbury et al., 2003).

CHAPTER III

MATERIALS AND METHODS

3.1 Genetic engineering method

3.1.1 Strains, media, and growth condition

K. oxytoca KMS004 was previously derived from Metabolic Engineering Research, SUT, Nakhon Ratchasima, Thailand. Bacterial strains, plasmids, and primers are listed in Table 3.1. The culture was grown at 37 °C, 200 rpm in LB broth containing 10 g of peptone, 5 g of yeast extract, and 5 g of sodium chloride per 1 liter of water. Cultures were maintained on LB agar (20 g/L agar), containing different antibiotics including 50 μg/mL kanamycin, chloramphenicol, and ampicillin. AM1 medium was used during fermentation. Its compositions as shown in Table 3.2.

Table 3.1 Strains, plasmids, and primers used in this study.

Strains	Relevant characteristics	References
E. coli	TOP 10 F'	Invitrogen
KMS004	*K. oxytoca* (*ΔadhE, Δpta-acka)*	Sangproo et al., 2012
KIS001	KMS004 (*Δfrd:cat-sacB*)	This study
KIS002	KMS004 (*Δfrd*)	This study
KIS003	KIS002 (*ΔpflB:cat-sacB*)	This study
KIS004	KIS002 (*ΔpflB*) Clone number 1	This study
KIS005	KIS002 (*ΔpflB*) Clone number 2	This study
KIS006	KIS002 (*ΔpflB*) Clone number 3	This study
KIS004-91T	KIS004 evolution with 91st transfers	This study

Table 3.1 (continued).

Plasmids	Relevant characteristics	References
pCR2.1-TOPO	*bla kan,* TOPO TA cloning vector	Invitrogen
pLOI4162	*cat-sacB cassette*	Jantama et al., 2008a
pLOI3420	*acc γ β exo* (*Red* recombinase), temperature-conditional replicon	Wood et al., 2005
pKJ1010	*frd* (PCR) from KMS004 (using *frd*-up/down) cloned into pCR2.1-TOPO	This study
pKJ1011	*cat-sacB* cassette from pSparkIII-'*frd-cat-sacB-frd*' (digested with *Sal*I) cloned into inside-out product amplified using *frd*-1/2	This study
pKJ1012	PCR amplified inside-out product (*frd*-1/2) kinase treated then self-ligation	This study
pKJ1013	*pflB* (PCR) from KIS002 (using *pflB*-up/down) ligated into pCR2.1-TOPO	This study
pKJ1014	*cat-sacB* cassette from pSparkIII-'*pflB-cat-sacB-pflB'* (digested with *Sal*I) ligated into inside-out product amplified using *pflB*-1/2	This study
pKJ1015	PCR amplified inside-out product (*pflB*-1/2) kinase treated then self-ligation	This study
Primers	**Sequence**	
frd-up	5' CCGGAGCTTATGCAGAAAAC 3'	This study
frd-down	5' CTGAGGTAAAAGCCGCGTA 3'	This study
frd-1	5' ACCGTGGTGATCCTGTTTGT 3'	This study
frd-2	5' ATCTCAGCCATTCGTCGTCT 3'	This study
pflB-up	5'ATGTCCGAGCTTAATGAAAAGTTAG 3'	This study
pflB-down	5' TTACATGGTCTGAGTGAAGGTACG 3'	This study
pflB-1	5' ACGCTATCCCGACTCAGTCC 3'	This study
pflB-2	5'AAATACGCTCAGTTCGTCTCTCTGCAA GAGAAACTGGAAAAC 3'	This study

Table 3.2 Composition of AM1 mineral low salt medium (excluding carbon source).

Component	**Concentration (mmol/L)**
$(NH_4)_2HPO_4$	19.92
$NH_4H_2PO_4$	7.56
Total PO_4	27.48
Total N	47.93
Total K	1.00
$MgSO_4$. $7H_2O$	1.50
Betaine-HCl	1.00
Trace Element	**(µmol/L)**
$FeCl_3$. $6H_2O$	8.88
$CoCl_2$. $6H_2O$	1.26
$CuCl_2$. $2H_2O$	0.88
$ZnCl_2$	2.20
Na2MoO4. 2H2O	1.24
H_2BO_3	1.21
$MnCl_2$. $4H_2O$	2.25
Total Salt	**4.1 g/L**

KOH will be used to neutralize betaine-HCl stock
Trace metal stock (1000x) was prepared in 120 mM HCl

3.1.2 Amplify DNA fragments by PCR

The 10x PCR master mix solution was used for Standard PCR in a PCR of 50 µL. Mastermix components was as Sangproo et al. (2012). The 40 pmole of forward and reverse primers, 50 ng of either plasmid or chromosomal DNA template and distilled water, were added to the mixture. Flexcyler PCR machine was used for the PCR reaction (Analytikjena, Germany). Agarose gel electrophoresis (1%) was examined after amplification reaction.

Table 3.3 PCR parameters for the amplification of specific genes.

PCR profile to amplify gene				
Step	**Period**	**Temperature (°C)**	**Time**	**Number of cycles**
1	Pre-denaturing	95	5 min	1
	Denaturing	95	30 s	
2	Annealing	55	30 s	35
	Extension	72	Vary	
3	Extra-extension	72	10 min	1

3.1.3 Agarose gel electrophoresis of DNA

PCR products, and DNA fragment were subjected to agarose gel electrophoresis. The limited amount of agarose powder was dissolved in 0.5x TBE buffer [89 mM Tris-HC, 89mM boric acid, 25 mM EDTA pH8.0] or 1x TAE buffer [40 mM Tris-HCl, 40 mM acetic acid, 25 mM EDTA pH 8.0] under 100 ^{o}C temperature to ensure the homogeneity of the gel solution. 6 µL of loading dye [0.1% (w/v) bromophenol blue, 40% (w/v) Ficoll and 5 mM EDTA)] were added and mixed well to the DNA samples before loading into the wells of the solidified gel. The constant voltage, 100 V, for 30 min was used. Gels were visualized under UV light and photographed by a gel documentation system to check the DNA bands.

3.1.4 PCR clean-up method

The Kit (Qiagen) were used for purification of PCR products. The two hundred microliters of PCR product were added in 1 mL Buffer PB. A QIA quick spin column was placed into 2 L tube and the sample was loaded into the column. The column was centrifuged for 1 minute at 13500 rpm. Then the flow-through was removed a flow-through. Then, 750 µL of PE buffer was added to the tube and spun down at 13,500 rpm for 1 minute. Then the flow-through was removed from the tube. The tube was additionally spun down for 1 minute at 13,500 rpm to remove an PE buffer. The tube was transferred into a clean 1.5 mL microcentrifuged. Finally, 50 µL of elution buffer was added into the column, and the mixture was incubated at 37 ºC for 1 minute and then the column was spun down at 13,500 rpm for 1 minute.

3.1.5 PCR clean-up Gel Extraction

PCR fragments after purification were removed from 0.7% agarose. Kit (Macherey-Nagel) were used for DNA purification. The 400 µL of Buffer QG and a hundred milligrams of agarose gel were mixed well. The sample was heated at 50 °C until the gel sliced were dissolved and vortex every 3 minutes. A NucleoSpin® Extract II column was placed into 2 mL collection tube and loaded the sample and then, spun down at 11 00 rpm for 1 minute. Then the flow-through was removed. Then, 750 µL of Buffer W1 was added to the column and centrifuged at 11000 rpm for 1 minute. Then the flow-through was removed. The tube was spun down at 11000 rpm for 2 minutes to remove buffer W1. The column was placed into a clean 1.5 mL micro-centrifuged. Finally, 20 µL of Elution buffer EB was added and incubated at 37 ºC for 1 minute and then spun down for 1 minute at 11000 rpm.

3.1.6 Preparation of *K. oxytoca* KMS004 competent cell

Colony of mutant strain was inoculated in LB broth of 3 mL at 37 °C, 200 rpm until reaching the OD550 nm in the range 0.3-0.5. The broth was spun down at 4000 rpm, 4°C for 10 min. The cell pellet was resuspended and washed well with 25 mL of ice-cold deionized water for 2 times, and in 4.5 mL of ice-cold 15% glycerol for 1 time. After washing the cell, the white cell pellet was resuspended in 1 mL of sterile ice-cold 15% glycerol. Two hundred microliters of aliquot were dispensed into an electroporation cuvette.

3.1.7 Transformation of *K. oxytoca* KMS004 by electroporation

pLOI3420 plasmid (1 microliter) was mixed gently with 200 µL of *K. oxytoca* KMS004 competent cells. Then, transferred the mixture to an ice-cold 0.4 cm cuvette. The cuvette was incubated on ice for 5 min. The cells were pulsed by using electroporator (Bio-Rad MicroPulser^{TM}, USA) under conditions used with *K. oxytoca* KMS004 (2500 V, pulse length 5 ms, *Eco*R2). Then 800 µL of LB broth was used in cuvette immediately and the solution was transferred to a sterile 1.5 mL tube. The tube was incubated at 30 °C with 200 rpm shaking for 2 h. 100 µL of transferred cells were spread on LB agar plates containing apramycin (50 µg/mL) and incubated at 30°C overnight.

3.1.8 Deletion of *frd* gene in *K. oxytoca* KMS004

To delete the *frd* gene, genomic DNA of KMS004 was served as templates to amplify the fragment using *frd*-up and *frd*-down primers set to produce DNA fragments for genome integration (*frd*) respectively. The PCR fragments were ligated into pCR2.1 TOPO to generate (pKJ1010). The plasmids were transformed into *E. coli* TOP 10F', incubated overnight at 37 °C. The plasmid pKJ1010 was extracted from *E. coli* TOP 10 F'. The plasmid pKJ1010 was served as templates to amplify the plasmid *frd'-frd''* in the inside-out deletion using *frd*-1 and *frd*-2 primers set to amplify the plasmid *frd'-frd''* in the inside out deletion. The PCR fragments were ligated with the *cat-sacB* gene in one reaction to yield pKJ101. PCR kinase treated and self-ligated in the other reaction to yield pKJ1012. Then, the *frd'*-

cat-sacB-frd'' fragments amplified using pKJ1011 as template and *frd*-1/2 primer set were transformed by electroporation into KMS004 harboring pLOI3420 (*Red* recombinase). The recombinants were spread on LB plates containing chloramphenicol (40 mg/L) at 42 °C overnight. KMS004 (*Δfrd:cat-sacB*) were obtained and were named KIS001. To remove *cat-sacB*, amplified fragments of *frd'-frd''* from pKJ1012 were transformed into KIS001 harboring pLOI3420. The mutant strains were incubated at 30 °C for 6 h and transferred into a 250-ml flask with working volume 100 ml of LB with 15% (w/v) sucrose. The clones were spread on LB plate containing 10% (w/v) sucrose (42 °C, 16 h). Clones were selected for loss of apramycin, ampicillin, and chloramphenicol resistance. Selected clone was further claimed by colony PCR. Clone of interest was selected and designated KIS002 (Fig. 3.1a).

3.1.9 Deletion of *pflB* gene in *K. oxytoca* KIS002

To delete the *pflB* gene, genomic DNA of KIS002 was served as templates for amplification using *pflB*-up and *pflB*-down primers set to produce linear DNA fragments for genome integration (*pflB*) respectively. The PCR fragments were ligated into pCR2.1 TOPO to generate (pKJ1013). The plasmids were transformed into *E. coli* TOP 10F', incubated overnight at 37 °C. The plasmid pKJ1013 was extracted from *E. coli* TOP 10 F'. The plasmid pKJ1013 was served as templates to amplify the plasmid *pflB'-pflB''* in the inside-out deletion using *pflB*-1 and *pflB*-2 primers set. The PCR fragments were ligated with the *cat-sacB* gene in one reaction to yield pKJ1014. PCR kinase treated and self-ligated in the other reaction to yield pKJ1015. Then, the *pflB'-cat-sacB-pflB''* fragments amplified using pKJ1014 as template and *pflB*-1/2 primer set were transformed by electroporation into KIS002 harboring pLOI3420 (*Red* recombinase). The recombinants were selected on LB plates containing chloramphenicol (40 mg/L) at 42 °C overnight. KMS004 (*ΔpflB:cat-sacB*) were obtained and were named KIS003. To remove *cat-sacB,* amplified fragments of *pflB'-pflB''* from pKJ1014 were transformed into KIS003 harboring pLOI3420. The mutant strains were incubated at 30 °C for 6 h and transferred into a 250 ml flask with working volume 100

ml of LB with 15% (w/v) sucrose. The clones were spread on LB plate containing 10% (w/v) sucrose (42 °C, 16 h). Clones were selected for loss of apramycin, ampicillin, and chloramphenicol resistance. Selected clone was further claimed by colony PCR. Clone of interest was selected and designated KIS002 (Fig. 3.1b).

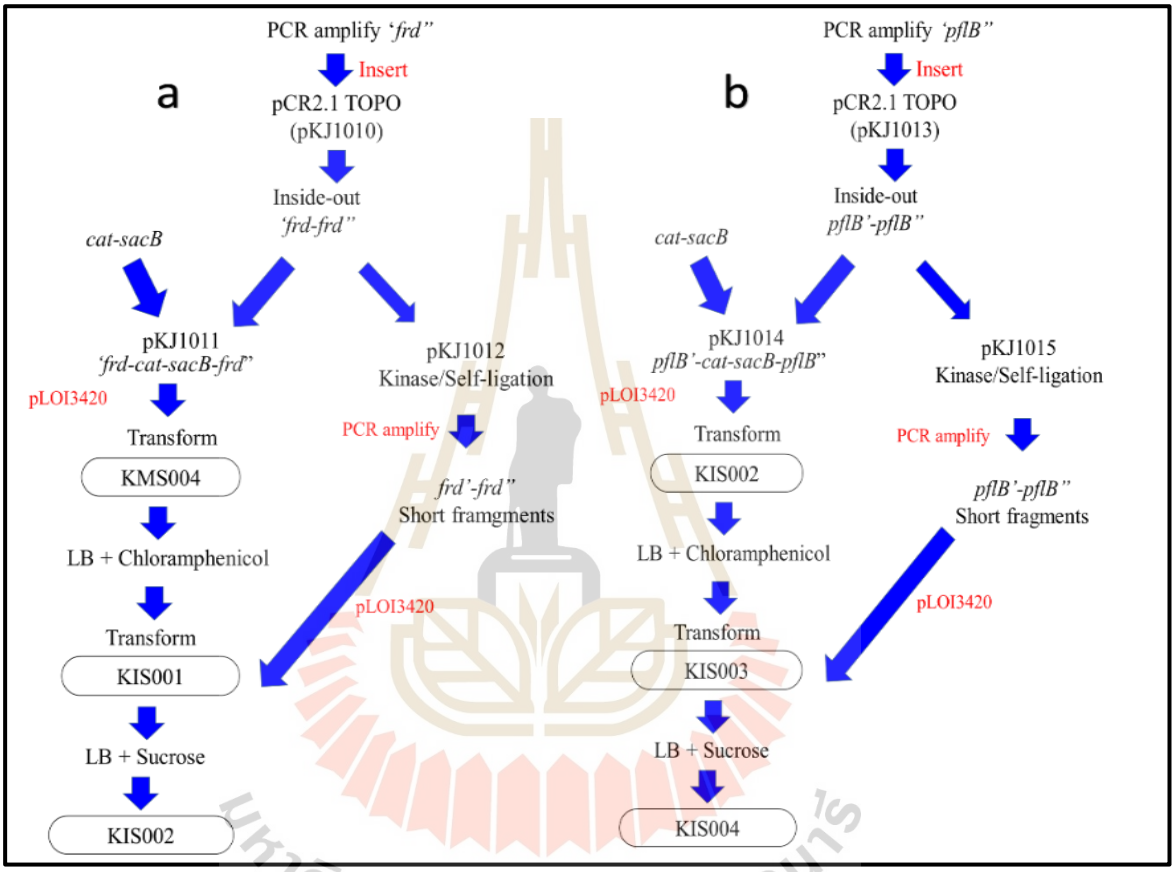


Figure 3.1 (a) Process of *frd* gene deletion in *K. oxytoca* KMS004, (b). Process of *pflB* gene deletion in *K. oxytoca* KIS002.

3.1.10 Metabolic evolution

Serial transfers in the pH-controlled fleakers were used for metabolic evolution (Jantama et al., 2008a). *K. oxytoca* KIS004 was repeatedly sub-cultured in AM1 medium containing 10% (w/v) glucose. The cells were incubated at 37 °C, pH 7, 200 rpm with the starting OD_{550} of 0.01. The culture was transferred into AM1 medium every 24 h. The transfers were continued until no further improvement in the growth rate and D-lactic acid production was observed. After completion of transfers, the broth was spread on LB agar with 1% glucose. Colonies of mutant strains were selected.

3.2 Fermentation operation and conditions

3.2.1 Fermentation medium

AM1 (Martinez et al., 2007) was applied under anaerobic fermentation with *K. oxytoca* mutants (Table 3.2). This medium contains 100 g/L of carbon source (glucose or cassava starch), with 1mM betaine HCl. The 6M of potassium hydroxide (6M KOH) was used in the fermentation experiments for neutralization.

3.2.2 Batch fermentation in 5 L Bioreactor

Batch process to produce D-(-) lactate, 5 L bioreactor with 2.5 L working volume was used (New Brunswick BioFlo®/CelliGen® 115 Benchtip Fermentor & Bioreactor, Operating Manual M1369-0050 Revision E, Germany). Parameters obtained from the previous metabolic evolution experiments were used for D-(-) lactate production in 5 L bioreactor. The AM1 medium containing 10% (w/v) glucose was added to the bioreactor and autoclaved at 121 °C for 15 min. Seed culture was prepared in LB medium containing 2% (w/v) glucose then the culture medium was incubated at 37 ºC, 200 rpm to reach semi-log phase. The culture was initiated by inoculation with a seed culture. The pH was controlled by automatic addition of 6M KOH.

3.2.3 Fed-batch in 5 L bioreactor in initiate volume 2.5 L

Fed-batch was performed with the same procedure as a batch process. Intermittent feeding strategy was applied as 100 g/L glucose was the initial substrate. Glucose solution was added when the residual glucose in the broth reached below 30 g/L. The fermentations were stopped while having no more increasing of D-lactic acid concentration.

3.2.4 Batch SHF in 5 L bioreactor of cassava starch

Batch process to produce D-(-) lactate, 5 L bioreactor with 2.5 L working volume was used (New Brunswick BioFlo®/CelliGen® 115 Benchtip Fermentor & Bioreactor, Operating Manual M1369-0050 Revision E, Germany). The optimized parameters were the

same as previous batch experiments. A hundred g/L of cassava starch was added to the bioreactor and autoclaved at 121 °C for 20 min. Alpha-amylase and glucoamylases (AMG) with activities of 42 169 U/g and 213 000 U/mL were used to hydrolyze the cassava starch, respectively. To completely hydrolyze of cassava starch, 2% (v/v) of alpha-amylase and 0.5% (v/v) of AMG were added to a bioreactor. AM1 medium was added into fermenter containing above-mentioned components after the hydrolysis was complete. The experiment was initiated by inoculating with the seed culture. Automatic addition of 6M KOH was used to adjust and control pH.

3.2.5 Analytical method

The sample was taken from fermenters every 12 h to analyze cell mass, biochemical, and sugar. Measured the cells growth at OD_{550} nm and calculated to biomass as cell dry weight (1 OD550 = 0.33 g CDW/L biomass). All products obtained from fermentation were analyzed by using HPLC equipped with an ion change column (Aminex® HPX-87H, 7.8×300 mm, BioRad) and refractive index detector (RI-150, Thermo Spectra System, USA). 4 mM sulfuric acid at a flow rate of 0.4 mL/min was used in the HPLC system. Culture collects from the experiment was spun down to remove the pellets. The flow-through was filtrated by using 0.2 µm nylon filter prior to injecting into the HPLC.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Deletion of *frd* gene in *K. oxytoca* KMS004

Plasmids pKJ1011 and pKJ1012 (Fig. 4.1) were used as templates to amplify linear DNA fragments of (*frd'-cat-sacB-frd*'') and (*frd'-frd*'') using the *frd*-up/down primers set to delete *frd* gene, respectively.

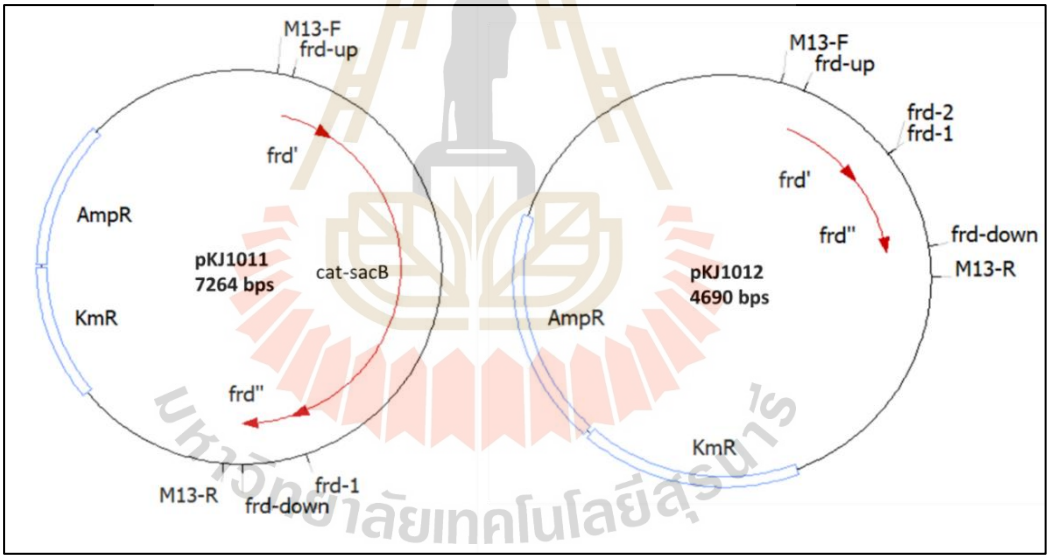


Figure 4.1 Plasmids pKJ1011 and pKJ1012 used for *frd* gene deletion.

The fragment (*frd'-cat-sacB-frd*'') was transformed by electroporation into KMS004 containing pLOI3420 (*Red* recombinase). The recombinant strains were spread on LB plates containing chloramphenicol (40 mg/L) at 42 °C overnight. The clone containing chloramphenicol resistant gene was designated as KIS001. Strain KIS001 was confirmed by PCR analysis using *frd*-up/down primers set. The results showed that the PCR product was at the expected sizes (Fig. 4.2).

Strain KIS001 containing pLOI3420 was further used to be transformed by electroporation with the *frd'-frd''* short fragment to remove *frd'-cat-sacB-frd''* fragment in the genome of KIS001. Then, obtained clones were selected for loss of apramycin, ampicillin, kanamycin, and chloramphenicol resistance. Colony was clarified by PCR. The clones were knocked-out *frd* gene and *cat-sacB* cassette was selected and designed as KIS002.

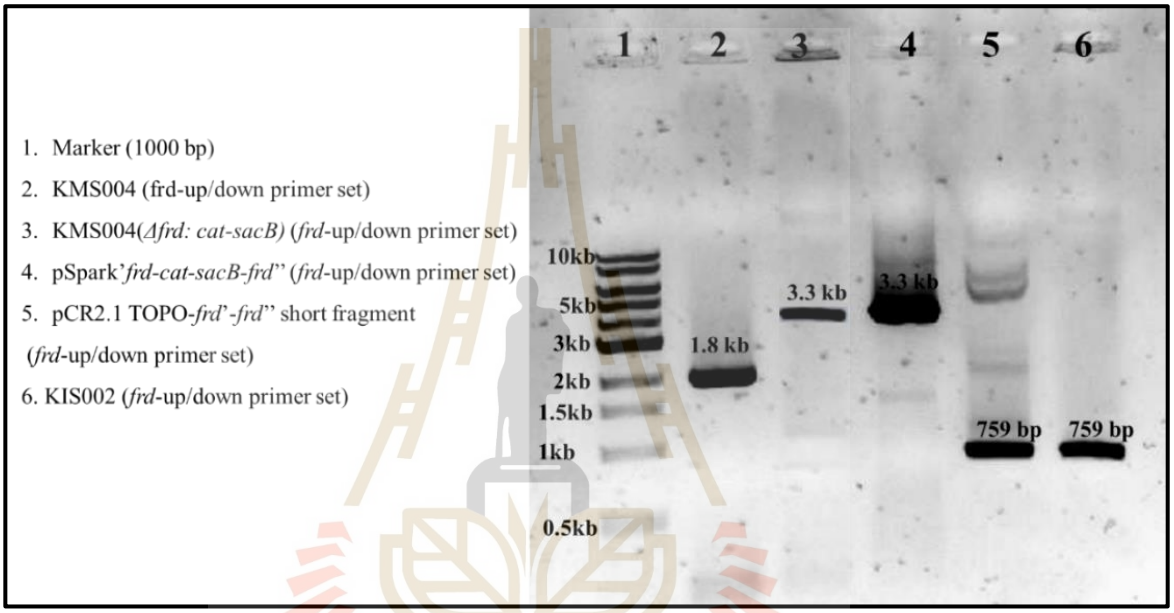


Figure 4.2 Gel electrophoresis confirmed the recombinant strain KIS002. Using *frd*-up/down primer set. Lane 1 represented the marker, lane 2 represented the PCR band of *frd* gene in KMS004, lane 3 represented the PCR band of integration '*frd-cat-sacB-frd''* gene in genomic of KMS004, lane 4 represented the PCR band of '*frd-cat-sacB-frd''* fragment using *pSpark'frd-cat-sacB-frd''* as template to amplify and integrate into KMS004 genome, lane 5 represented the PCR band of *frd'-frd''* short fragment gene using plasmid pCR2.1-TOPO-'*frd-frd*'' short fragment as template to amplify and remove *cat-sacB* gene from genome of KMS004, and lane 6 represented the PCR band of *frd'-frd''* short fragment which was removed the *cat-sacB* gene from the genome of KMS004 to obtain KIS002.

4.2 Effect of *frd* gene deletion in KMS004

Generally, in the glycolysis pathway, glucose is metabolized into two types of the pathway, known as Embden-Meyerhof-Parnas (EMP) and hexose monophosphate (HMP). From this metabolism, pyruvate is decarboxylated oxidatively to acetyl-CoA to be oxidized in the Kreb cycle (Kim et al., 2008). Glycolysis is the process of glucose converts to pyruvate and pyruvate is oxidized in the citric acid cycle (Gerhard et al., 2012). Gottschalk (1985) and Jantama (2008b) said that in the anaerobic metabolic pathway, glucose metabolism generates ATP, NADH, and pyruvate. Pyruvate is ingested to re-oxidize NADH through *ldhA*, and *adhE*. In this case, KMS004 (*ΔadhE*, *Δpta-ackA*) produced only D-lactic acid (Sangproo et al., 2012). Furthermore, fumarate reductase (*frd*) is a key enzyme for anaerobic functioning of almost microorganisms. Fumarate is functioned as the terminal electron acceptor to catalyze the reduction of fumarate to succinate. Moreover, the *frd* (fumarate reductase) gene is described mainly in microorganisms including *E. coli* and *K. oxytoca*. FRD membrane-bound comprises of non-identical subunits such as *frdA*, *frdB,* and *frdD* (Jaap et al., 1994). Additionally, three primary routes for NADH oxidation are indicated includes lactate (via lactate dehydrogenase), succinate (via fumarate reductase), and ethanol (via alcohol dehydrogenase) in the mixed-acid pathway (Zhang et al., 2009). Thus, deletion of the *frd* gene could allow NADH oxidation flown directly to lactic acid, not succinic acid in KMS004 strain.

K. oxytoca KMS004 produced a mixture of lactate, succinate, formate, acetic acid, and 2,3-butanediol as metabolites during fermentation of glucose. Sangproo et al., (2012) engineered KMS004 to disrupt *adhE* and *pta-ackA* genes. Therefore, pyruvate is ingested to re-oxidize NADH via LDA activity resulting in D-(-) lactate production under anaerobic conditions. Pyruvate is a precursor to produce D-(-) lactate by reoxidizing NADH under anaerobic conditions. Hence, KMS004 produced D-lactate at high titer 11.5 ± 0.01 g/L with the yield of 0.64 g/g, and productivity of 0.36 g/L/h in 2% (w/v) glucose, respectively

(Sangproo et al., 2012). Similarly, the results of *K. oxytoca* KMS004 fermentation in 5% glucose were obtained lactic acid, acetic acid, and 2,3-butanediol as the metabolite (Table 4.1). Since, *K. oxytoca* KMS004 produced lactate and succinate under anaerobic conditions, the succinate producing pathway was knocked out from the mutant strain KMS004 by deleting *frd* gene to construct the KIS002 strain, KIS002 strain was performed for D-(-) lactate production in AM1 medium containing 5% (w/v) glucose as a sole carbon source under anaerobic conditions. KIS002 produced with higher amount of D-(-) lactate as a major product with a concentration of 35.4 g/L, yield of 0.75 g/g and productivity of 0.59 g/L/h. respectively after 96 h incubation. No succinate was produced in the broth. This result claimed that FRD activity was successfully removed from KIS002. However, the D-(-) lactate by KMS004 was obtained higher concentration than KIS002 strain. The average productivity of D-(-) lactate of KIS002 strain was not increased compared with KMS004 strain (Table 4.1). These caused by that the growth of KIS002 strain became slower after re-engineering since KIS002 due to partial loss of NADH re-oxidation capability thus exhibiting a lower growth rate compared to what of KMS004 strain during the fermentation (Fig. 4.3).

Table 4.1 Fermentation profile of metabolically engineered KMS004, KIS002, and KIS004 strains in AM1 medium containing 5%(w/v) glucose.

Strains	CDW [a] (g/L)	Total [b] Glucose Utilized (g/L)	D-(-) Lactate			Concentration of by-product	
			Concentration [c] (g/L)	Yield [d] (g/g)	Productivity [e] (g/L/h)	Acetate (g/L)	2,3-Butanediol (g/L)
KMS004	0.49±0.13	51.4±0.32	36.3±0.2	0.71±0.01	0.38±0.01	0.75±0.3	0.67±0.38
KIS002	0.29±0.11	47.5±0.2	35.4±0.12	0.74±0.02	0.37±0.01	0.86±0.03	0.12±0.06
KIS004	0.32±0.02	47.2±0.21	45.2±0.02	0.96±0.05	0.47±0.01	ND [f]	0.13±0.08

All data represent the averages of three fermentations with standard deviations.

[a] Cell Dry Weight was calculated by the optical density of cells divided by 3 in gram per liter.

[b] Total glucose utilization were calculated by total glucose divided by glucose remain in gram per liter.

[c] Concentrations of lactate were calculated as gram per liter by weight D-(-) lactate divided by the total volume of solution.

[d] Yields of lactate were calculated as a gram of D-(-) lactate divided by a gram of glucose consumed.

[e] Productivities of lactate were calculated as D-(-) lactate concentration was produced divided by overall incubation time (96 h). The incubation time of all strains was 96 h, respectively.

[f] ND= Not Detected

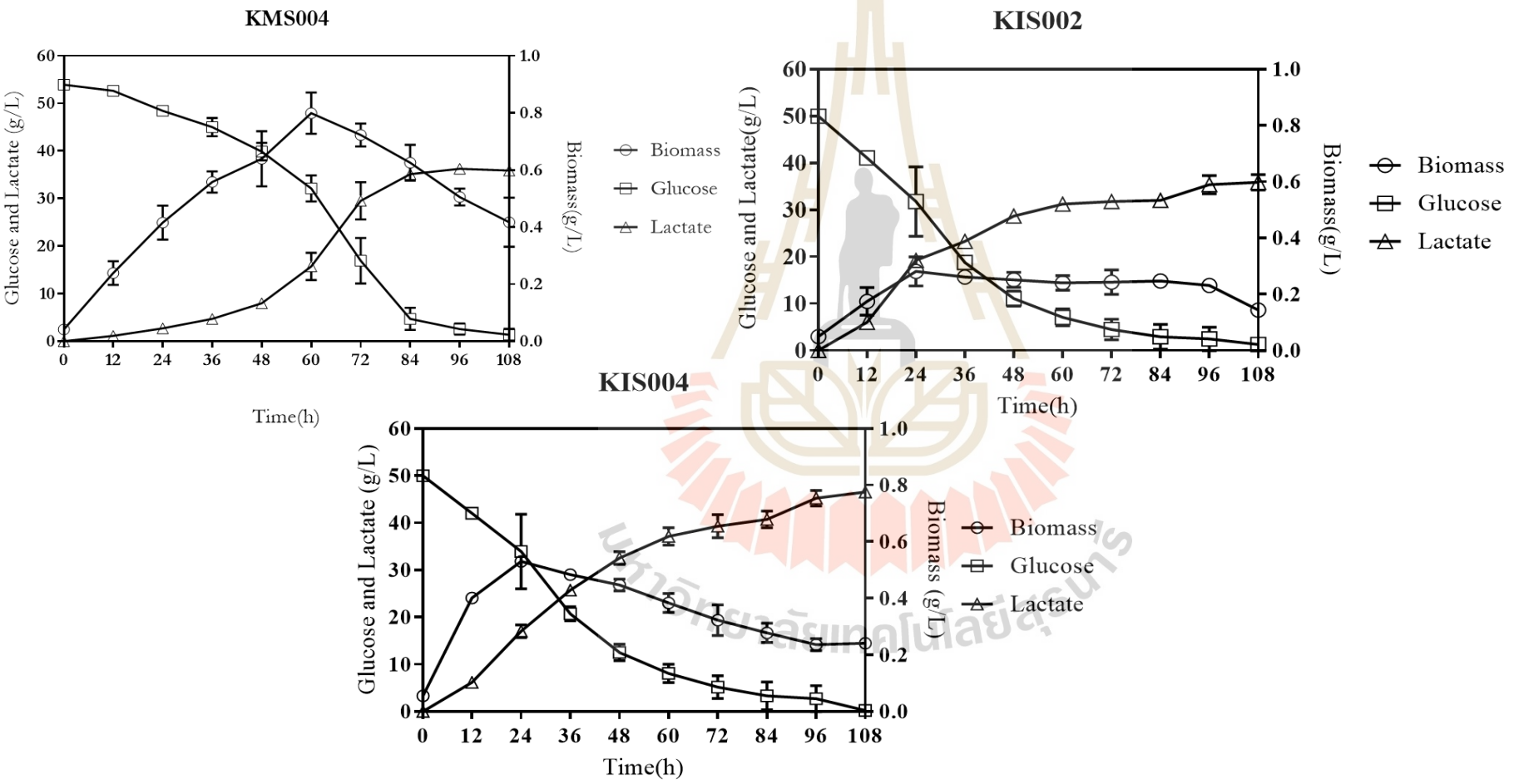
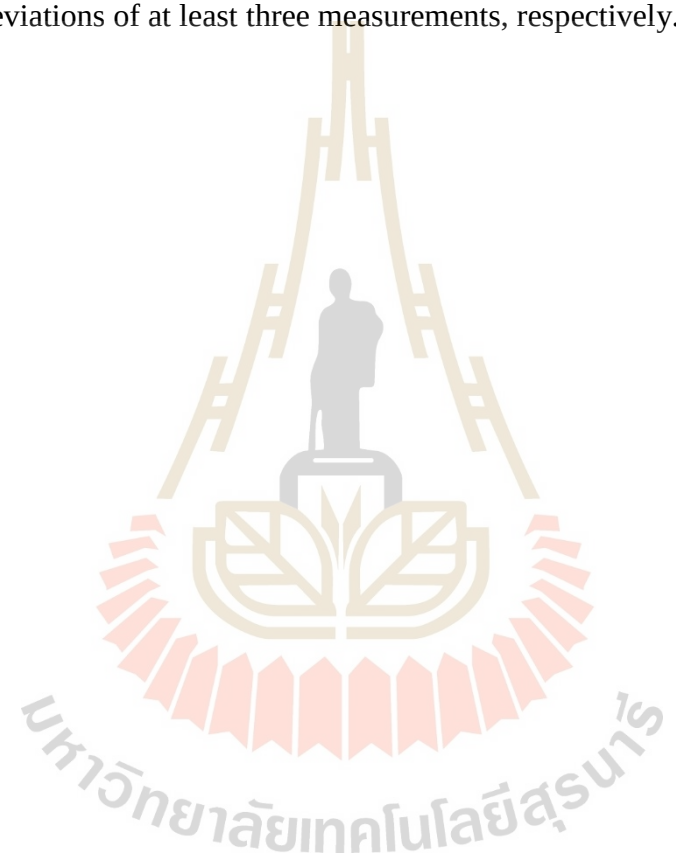


Figure 4.3 Fermentation profile of *K. oxytoca* KMS004, KIS002, and KIS004 in AM1 medium containing 5% (w/v) glucose. The symbols and errors bar are average values and standard deviations of at least three measurements, respectively.



The level of acetate (0.865 g/L) was produced by KIS002 is not significantly different compared with KMS004. On the other hand, the level of 2,3-butanediol (0.12 g/L) produced by KIS002 is affectedly decreased compared with KMS004 under anaerobic conditions (Table 4.1). The production of D-(-) lactate by KIS002 (35.4 g/L) was not significantly improved compared with KMS004. The results suggested that the removal of fumarate reductase (*frd*) gene was affected to the cell growth even though NADH oxidation in KIS002 strain still occurred via lactic acid production pathway. For this reason, the level of D-(-) lactate (35.4 g/L) was not significantly different compared to that of KMS004. Additionally, PEP dissimilation via *frd* gene did not competitively re-oxidize NADH in KIS002 because four-step reactions were required for succinic acid production (Fig. 2.4), and the carbon dioxide source was not provided during fermentation. FRD is activated and re-oxidized NADH by using electron acceptor known as fumarate for PEP carboxylation. PEP and carbon dioxide are combined by phosphoenolpyruvate carboxylase encoded by *ppc*. The oxaloacetate is reduced to produce malate by malate dehydrogenase activity encoded by *mdh.* Then, malate is dehydrated by fumarase enzyme to produce fumarate encoded by *fumABC*. Finally, succinate is produced by fumarate reductase from fumarate with NADH re-oxidation (Cecchini e al., 2002; Sangproo et al., 2012; Simon et al., 1998). However, fumarate reductase was removed in the KIS002 strain, NADH re-oxidation by the succinate production pathway was reduced. Consequently, NADH level was increased for the D-(-) lactate production pathway. Therefore, D-lactate production pathway was expected to only be utilized for re-oxidation of NADH in KIS002 strain under anaerobic conditions. However, the deletion of *frd* gene affected its growth rate and glucose consumption.

4.3 Deletion of a *pflB* gene in *K. oxytoca* KIS002

Plasmids pKJ1014 and pKJ1015 (Fig. 4.4) were used to amplify linear DNA fragments of (*pflB'-cat-sacB-pflB''*) and (*pflB'-pflB''*) using the *pflB*-up/down primers set to delete *pflB* gene, respectively.

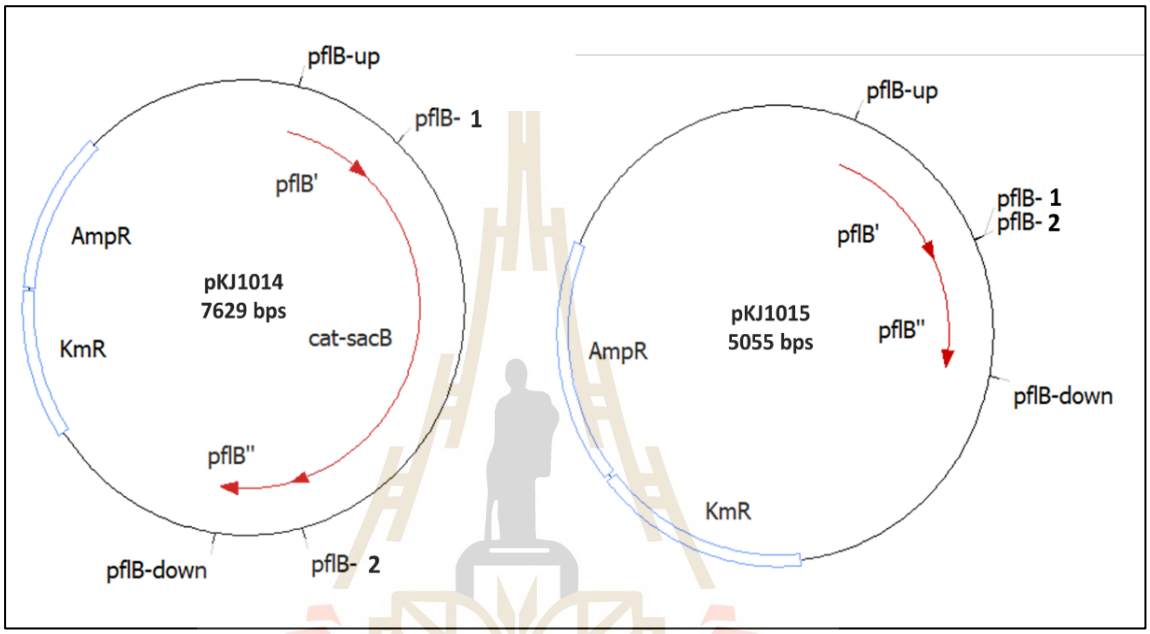


Figure 4.4 Plasmids pKJ1014 and pKJ1015 used for *pflB* gene deletion.

The fragment (*pflB'-cat-sacB-pflB''*) was transformed by electroporation into KIS002 containing pLOI3420 (*Red* recombinase). The recombinant strains were spread on LB plates containing chloramphenicol (40 mg/L) at 42 °C overnight. The clone containing chloramphenicol resistant gene was designed as KIS003. Strain KIS003 was claimed by PCR analysis using *pflB*-up/down primers set. The results showed that the PCR product was at the expected sizes (Fig. 4.5).

Strain KIS003 containing plasmid pLOI3420 was further used to be transformed electroporation with the *pflB'-pflB''* short fragment to remove *pflB'-cat-sacB-pflB''* fragment from the genome of KIS003. Then, obtained clones were selected for loss of apramycin, ampicillin, kanamycin, and chloramphenicol resistance. Colony was clarified by PCR. The clones were knocked out *pflB* gene and *cat-sacB* cassette was selected and designed as KIS004.

Figure 4.5 Gel electrophoresis confirmed the recombinant strain KIS004. Using *frd*-up/down primer set for *frd* gene deletion, and *pflB*-up/dwon primer set for *pflB* gene deletion. Lane M represented the PCR band of marker, lane A represented the PCR band of normal size of *frdABCD* of 1853 bp, lane B represented PCR band of normal size of *pflB* of 2283 bp, lane C represented PCR band of *cat-sacB* replaced on *frdABCD* resulting 3333 bp in strain (Δ*frd: cat-sacB*), lane D represented PCR band of normal size of *pflB* of 2283 bp in strain KMS004 (Δ*frd:: cat-sacB*), lane E represented PCR band of frd'-frd'' fragment in KIS002, lane F represented PCR band of wild type size of pflB of 2283 bp in strain KIS002, lane G represented PCR band of *frd* size after deletion of *cat-sacB* in strain KIS002 (Δ*pflB:cat-sacB*), lane H represented PCR band of *cat-sacB* replaced on *pflB*, resulting 3698 bp in strain KIS002 (Δ*pflB:cat-sacB*), lane I represented PCR band of *frd* size after deletion of *cat-sacB* in stain KIS004, resulting 759 bp, and lane J represented PCR band of *pflB'-pflB''* in KIS004.

4.4 Effect of *pflB* gene deletion in *K. oxytoca* KIS002

Acetate was not detected in the KIS004 strain while KMS004 and KIS002 strains produced acetate at levels of 0.75 g/L and 0.86 g/L, respectively in 5% (w/v) glucose in AM1 medium during fermentation (Table 4.1). Decreasing production of acetate may essential to improve the production of D-(-) lactate and carbon recovery. In addition, it could be simplified for the downstream processes including a purification step to reduce by-product contamination (Sangproo et al., 2012). In pathway of *K. oxytoca* wild-type, acetyl-CoA is produced and transformed to acetyl-P by the activity of phosphotransacetylase (*pta*). Then, Acetyl-P is transformed to acetate by mean of *ackA* activity. After that, KMS004 was engineered to knock out these genes (*pta* and *ackA*). Consequently, acetate production was reduced. Likewise, pyruvate formate lyase activity is a potential source of acetate in which it produces formate as an excessive source of acetyl-CoA under anaerobic conditions (Jantama et al., 2008b).

KIS004 produced D-(-) lactate at concentration of 45.2 g/L, with the yield of 0.96 g/g, and productivity of 0.47 g/L/h, respectively, after 96 h incubation in AM1 medium containing 5% (w/v) glucose under anaerobic conditions (Table 4.1). Concentration, yield, and productivity of D-lactic acid produced by KMS004 and KIS002 were not significantly different. On the other hand, KIS004 produced with higher performances compared to KMS004 and KIS002 under anaerobic conditions. It suggests that the deletion of *pflB* extremely affected the D-(-) lactic acid production in KIS004. Alternatively, the concentration of acetate in KMS004 and KIS002 were not quite different, while acetate production in KIS004 (not detected) was reduced significantly (Table 4.1).

Besides, deletion of *pflB* led a lower level of 2,3-butanediol for the strain KIS004 (0.13 g/L) compared to what of KMS004 (0.67 g/L). The result implied that deletion of *pflB* enhanced the lactic acid production and reduced 2,3-butanediol formation. Once, Yang et al.

(2000) have suggested that dissimilation route of pyruvate to 2,3-butanediol needs 2 step reactions while lactic acid production is used only one-step reaction. Affinity for pyruvate of *ldhA* ($K_m^{pyruvate}$ = 7.2 mM) is higher than *budB* ($K_m^{pyruvate}$ = 8 mM). This reason shows that the pathway to produce 2,3-butanediol may be affected by D-(-) lactate production. Additionally, Sangproo et al. (2012) indicated that D-(-) lactate production and 2,3-butanediol participated together in the regulation of the NADH/NAD^+ ratio in KMS004. Therefore, lower yields of acetate and 2,3-butanediol in KIS004 were caused by *pflB* gene deletion, when carbon fluxes through *pflB* and *budB* activities were reduced. Similarly, Park et al. (2013) reported that knock out of *pflB* gene decreased the formation rate of by-products dramatically in *K. oxytoca*. The remaining metabolic fluxes are redirected into the primary product (lactic acid in KIS004).

4.5 Metabolic evolution

After deletions of *frdABCD* and *pflB* gene, *K. oxytoca* KIS004 strain was reduced the capacity to grow and to utilize glucose. KIS004 was selected the best clone for the best in growth and substrate utilization by metabolic evolution to resolve the issue. The serial transfer was applied to this engineered strain in which the metabolic evolution was performed in mineral salt medium (AM1) containing 10% (w/v) glucose. The engineered strain that utilized all glucose and produced D-(-) lactate with the highest concentration was selected as the representative strain. Jantama et al. (2008b) reported that metabolic evolution is the method of selecting microorganisms for survival under the strong pressure. KIS004 was inoculated in LB medium containing 2% (w/v) glucose as a seed culture and transferred to a new fresh AM1 medium till the 18th transfer. The level of biomass was increased slowly. Since, KIS004 was removed pyruvate formate lyase which acetate was not produced anymore, the strain becomes auxotroph to acetate. Hence, metabolic evolution by adding sodium acetate was performed. Acetate concentration was reduced until the strain grew well

without acetate. From 19th till 28th transfers, 20 mM of sodium acetate were added to the medium, the biomass was increased significantly from 0.3 g/L to 0.6 g/L compared to the transfers without adding sodium acetate (Fig. 4.6a). In addition, the amount of lactate was also increased from 35 g/L to 45 g/L (Fig. 4.6b) with an initial OD_{550} of 0.01. The serial transfers were continuously applied with reducing sodium acetate concentration from 20 mM to 10 mM in transfer 29th to 37th in which the biomass was not decreased. This result showed that the KIS004 was adapted to the medium by using a small amount of acetate. The sodium acetate additions were reduced gradually to levels of 5 mM, 1.5 mM, 0.7 mM, 0.35 mM, and none in 38th till 43rd, 44th till 61st, 62nd till 68th, 69th till 72nd, and 73rd till 79th transfers, respectively. As shown in Fig. 4.6a and 4.6b, KIS004 was adapted to the AM1 medium without adding sodium acetate. KIS004-79T reached biomass 0.73 g/L and produced D-(-) lactate at the concentration of 71.9 g/L with initial OD_{550} of 0.01. After serial transfer from 0 to 79th, the results showed that the engineered strain could grow well and reached the log phase within 24h. After that, the biomass was decreased after 48 h which was similar to the growth of KMS004 (Sangproo et al., 2012). The D-(-) lactate production from this condition was obtained only at 71.9 g/L but glucose was still remained. However, D-lactic acid is usually a growth-associated product. Thus, to force the cell to utilize all glucose, serial transfer was continued by using higher initial inoculation (OD_{550} of 0.1) while some components in AM1 medium were increased in concentrations (Betaine-KCl (0.5M): 3.5 mL, $MgSO_4.7H_2O$ (1.5M): 1.75, Trace Metal (1000x): 1.75 mL) from the 80th to 92nd transfers. The results indicated that biomass was increased significantly by 1.6 g/L in the 91st transfer. All glucose concentrations were utilized and D-(-) lactic acid at concentration of 98.6 g/L was obtained while other by-products were not produced (Fig. 4.6a and 4.6b). Therefore, culture of the 91st transfer was selected and spread on the LB plate containing 1% glucose. The clone was renamed as KIS004-91T.

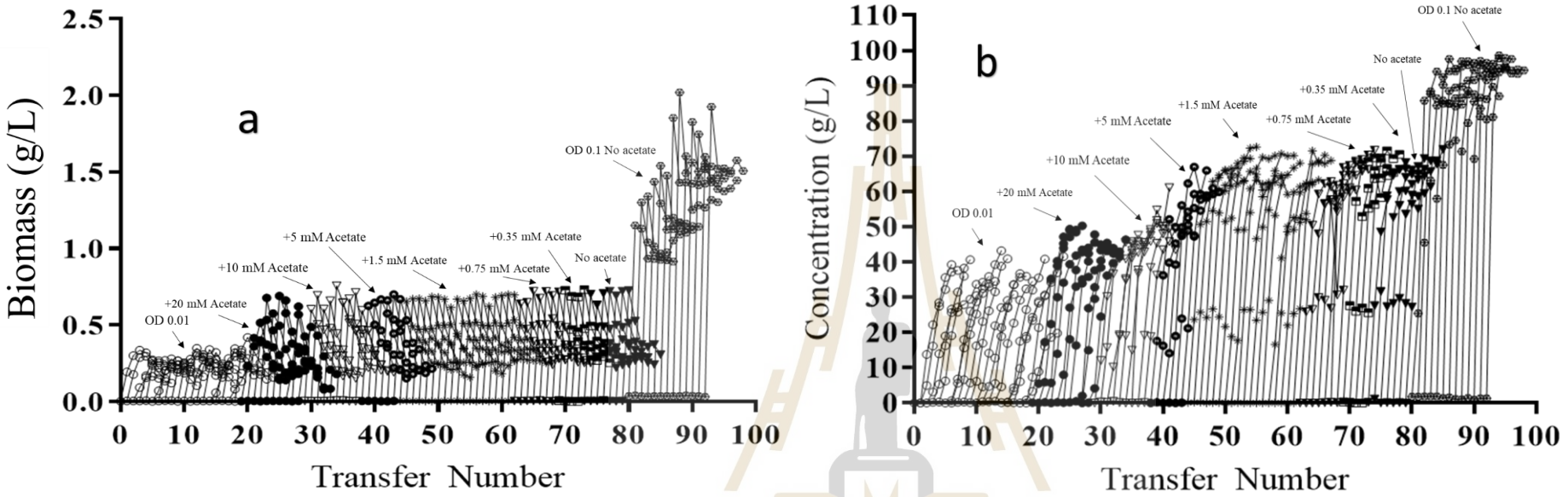


Figure 4.6 (a) Biomass formation and (b) D-lactic acid concentration during metabolic evolution of *K. oxytoca* KIS004 containing 10% glucose in AM1 medium in 500 mL fleaker with working volume 350 mL. Transfers 0 to 79; starting OD550 = 0.01, transfers 80 to 92; starting OD550 = 0.1, transfers 1 to 18; without adding sodium acetate, transfers 19 to 28; adding 20 mM sodium acetate, transfers 29 to 37; adding 10 mM sodium acetate, transfers 38 to 43; adding 5 mM sodium acetate, transfer 44 to 61; adding 1.5 mM sodium acetate, transfers 62 to 68; adding 0.75 mM sodium acetate, transfers 69 to 72; adding 0.35 mM sodium acetate, transfers 73 to 79; stop adding sodium acetate and OD550=0.1, and transfers 80 to 92; increased Betaine-KCl (0.5M): 3.5 mL, MgSO4.7H2O(1.5M): 1.75, Trace Metal (1000x): 1.75 mL.

4.6 Production of D-(-) lactate from 10% (w/v) glucose by KMS004, KIS002, KIS004, and KIS004-91T in fleaker 350 mL

High substrate concentrations cause an impaired growth in many microorganisms. The high substrate concentration can inhibit the enzyme activity of the cells thus causing inhibition of cell growth (Ghaly et al., 2005). Cosio et al., (1995) reported that a high concentration of glucose inhibits and affects cell proliferation. Hence, the high substrate concentration is rarely applied in fermentation in an industrial scale. The fermentation with 10% (w/v) glucose showed that, KMS004, KIS002, and KIS004 produced D-(-) lactate at concentration of 35.6±0.15 g/L, 13.5±0.2 g/L, and 26.0±0.2 g/L, respectively, with biomass needs of 0.40±0.13 g/L, 0.28±0.13 g/L, and 0.33±0.15 g/L, respectively (Table 4.2). The results proofed that a high concentration of glucose could inhibit the cell growth and was also reduced D-(-) lactate production significantly (Fig. 4.7). KIS004-91T was also tested the D-lactate production with 10% (w/v) glucose in 500 mL containers with the working volume of 350 mL. The results showed that 95.9±0.2 g/L of D-(-) lactate were produced with the biomass of 0.91±0.14 g/L. Therefore, after the serial transfers, the mutant strain was able to utilize all 10% glucose and produced a high concentration of D-lactate. Thus, KIS004-91T is a new strain that could be used to solve the problem dealing with high substrate inhibition mentioned above to produce a high production of D-lactate.

Table 4.2 Fermentation profile of metabolically engineered KMS004, KIS002, KIS004 and KIS004-91T strains in AM1 medium containing 10%(w/v) glucose.

Strain	CDW[a] (g/L)	Total[b] Glucose Utilized (g/L)	D-(-)Lactate			Concentration of by-product	
			Concentration[c] (g/L)	Yield[d] (g/g)	Productivity[e] (g/L/h)	Acetate (g/L)	2,3-Butanediol (g/L)
KMS004	0.40±0.13	42.9±0.24	35.6±0.15	0.71±0.14	0.37±0.012	0.48±0.14	0.59±0.26
KIS002	0.28±0.13	24.1±0.2	13.5±0.2	0.55±0.04	0.14±0.04	0.29±0.16	0.37±0.28
KIS004	0.33±0.15	55.2±0.2	26.0±0.2	0.50±0.01	0.27±0.01	ND[f]	0.12±0.05
KIS004-91T	0.91±0.14	101±0.05	95.9±0.2	0.95±0.01	1±0.01	0.06±0.02	0.62±0.05

All data represent the averages of three fermentations with standard deviations.

[a] Cell Dry Weight was calculated by the optical density of cells divided by 3 in gram per liter.

[b] Total Glucose Utilized were calculated by total glucose divided by glucose remain in gram per liter.

[c] Concentrations of Lactate were calculated as gram per liter by weight D-(-) lactate divided by the total volume of solution.

[d] Yields of lactate were calculated as a gram of D-(-) lactate divided by a gram of glucose consumed.

[e] Productivities of lactate were calculated as D-(-) lactate concentration produced divided by overall incubation time (96 h). The incubation time of all strains were 96 h, respectively.

[f] ND= Not Detected.

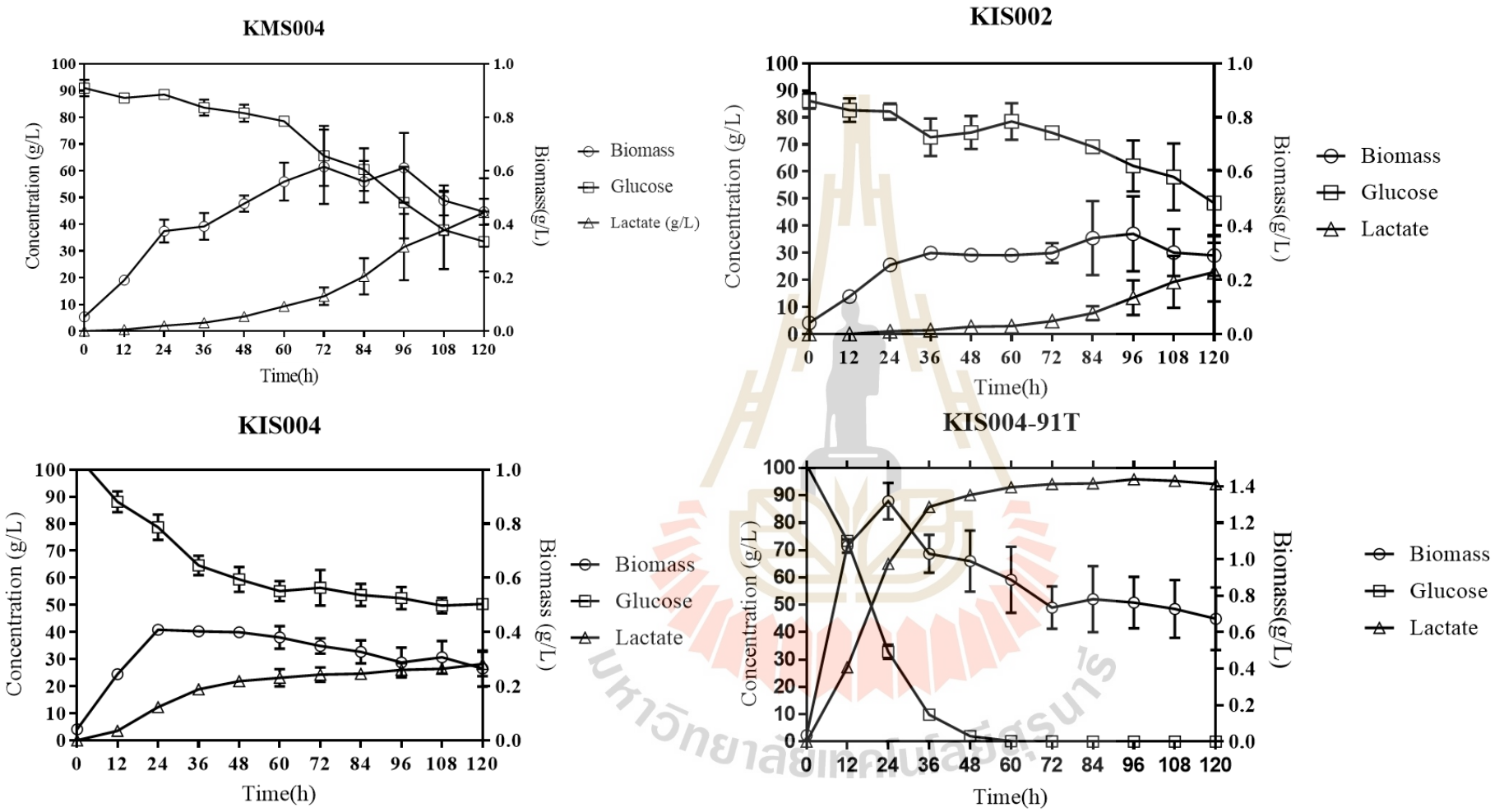


Figure 4.7 Fermentation profile of *K. oxytoca* KMS004, KIS002, KIS004 and KIS004-91T in AM1 medium containing 10% (w/v) glucose. The symbols and errors bar are average values and standard deviations of at least three measurements, respectively.

4.7 Batch fermentation of D-lactic acid in AM1 medium containing 10% (w/v) glucose in 5 L fermenter

Batch fermentation provides advantages to the experiment including less microbial contaminations, lower capital investment compared to a continuous process, and more flexibility for the varying product. Batch experiments for D-lactate production by KIS004-91T were performed with the working volume of 2.5 L in 5 L bioreactor. Initial OD_{550} of 0.3 was performed. After 48 h of fermentation, the concentration of D-lactate, acetate, and 2,3-butanediol were 100±0.2 g/L, 0.01±0.01 g/L, and 0.46±0.02 g/L from 106±0.3 g/L glucose, respectively (Fig. 4.8). As well, the results showed that KIS004-91T could utilize all 10 % glucose. In addition, higher D-(-) lactate production was observed. Similarly, Martinez et al. (2011) demonstrated that increasing of cell density results in high production of D-(-) lactate. Moreover, Sangproo et al. (2012) pointed out that *K. oxytoca* KMS004 produced D-(-) lactate with OD_{550} at 0.5 for 48 h was the optimal condition. However, considering the cost of inoculums preparation, time, and D-lactate titer, the initial inoculum for OD_{550} of 0.3 could be suggested as the starting condition for producing high D-lactic acid by KIS004-91T strain. In summary, increasing the initial optical density of KIS004-91T decreased fermentation time. The results were in an accordance with Kirin et al. (2016). Thus, high inoculum should be applied to offer high rate of glucose utilization thus reflecting high productivity of D-lactic acid by the strain.

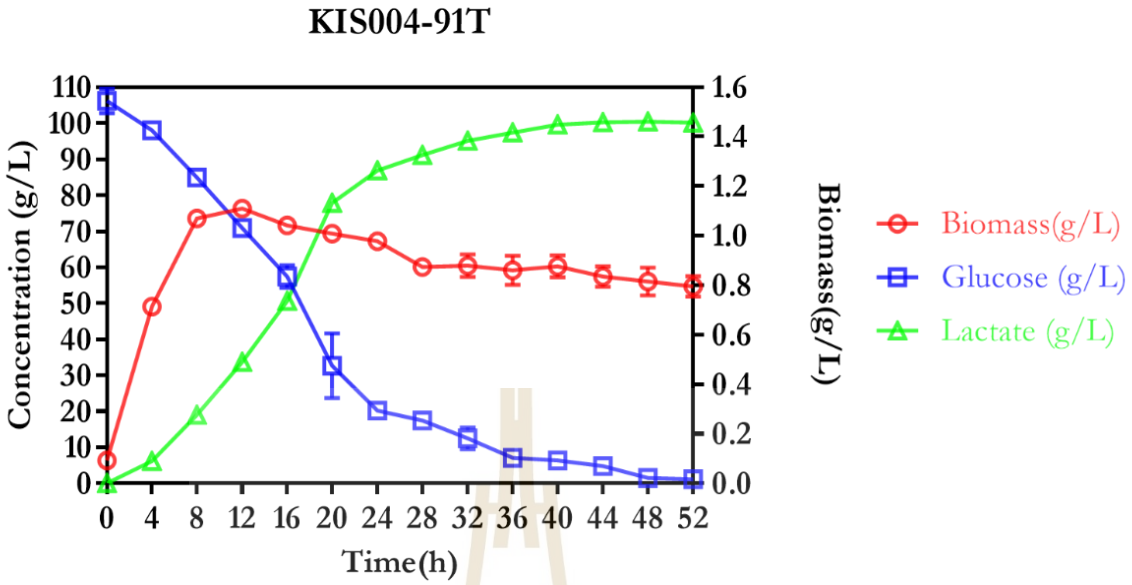


Figure 4.8 Fermentation profile of batch fermentation profile of *K. oxytoca* KIS004-91T in AM1 medium containing 10% (w/v) glucose in 5 L bioreactor with 2.5 L working volume.

4.8 Fed-batch fermentation of lactic acid in 5 L fermenter

Batch cultures that sequentially add substrate to culture without removal of culture from the medium are usually called as a fed-batch culture. Normally, fed-batch fermentation has supplemented the medium at a given time interval to the bioreactor (Zhu et al., 2017). A purpose of fed-batch is performed to avoid inhibition of substrate. The high concentration of substrate causes high osmotic pressure to the medium. Glucose utilization was decreased when high sugar concentration was increased (Ozmihci et al., 2009). Recently, fed-batch fermentation has gained attention to improve the productivity of bioprocesses and increased the economy by enhancing the production yield (Mohd et al., 2018). Furthermore, substrate concentration was fed in the reaction below the toxic level by the fed-batch process (Queshi et al., 2001). Besides, fed-batch culture is used in order to obtain higher yield and increases the chance for optimizing conditions of cells during log phase. Additionally, the main advantages of fed-batch over batch cultures can prolong the period of product synthesis (Kuila et al., 2018).

Fed-batch of D-lactate production was carried out in 5 L fermenter with the working volume of 2.5 L. Glucose was added at 16 and 24 h to maintain level of glucose concentration in the range of 30 to 70 g/L. After 16 h, the results reported that the cell grew and utilized glucose well about 74 g/L with the cell dry weight increased from 0.1 to 1.24 g/L. At 24 h, second feeding was employed while the cell continued consuming glucose and produced D-lactic acid. However, after 28 h, cells dry weight was decreased dramatically (Fig. 4.9). In this case, glucose feeding was stopped after 24 h and the fermentation time was continued until 76 hours. As a result, at the end of the fed-batch process, D- lactic acid concentration of 129±0.2 g/L, with the yield of 0.95±0.12 g/L, and productivity of 1.9±0.02 g/L/h was produced, respectively. Cells were slightly decreased when D-lactic acid reached 129±0.2 g/L at 68 h. D-lactic acid was produced with high concentration while other by-products almost diminished (Fig. 4.9).

In recent years, many publications were published to improve D-lactate production and reduce the cost of this biochemical in industry. For instance, Sangproo et al., (2012) and Mazumdar et al., (2010) produced D-lactate with concentration of 34 g/L and 32 g/L, respectively. Then, D-lactic acid was improved by Lu et al., (2016) and Yamada et al., (2017b) to produce D-lactate with concentration of 83 g/L and 60.3 g/L, respectively. Additionally, Zheng et al., (2014), Zhou et al., (2012), and Feng et al., (2017), Okano et al., (2017) produced high concentration of D-lactic acid of 115 g/L, 122 g/L, 125 g/L, and 117 g/L, respectively. The results demonstrated that higher titer of D-lactate was obtained by using various strains and media. However, KIS004-91T still produced higher titer compared to those of researches (Table 4.2). Zheng et al., (2017), and Park et al., (2018) realized the highest concentration of D-lactic acid at 145 g/L and 154 g/L, respectively. Nevertheless, high-cost medium was used to produce D-lactic acid. Anyway, high productivities were also obtained at 4.16 g/L/h (Park et al., 2018), 4.32 g/L/h (Zhou et al., 2012), and 3.48 g/L/h (Feng et al., 2017). The results indicated that large inoculums were added to the media of the

experiments to obtain high productivities. Considering the cost of inoculum, even high concentration of D-lactic acid was produced but large inoculum was required and paid out for the experiment. High biomass in the broth was also affected to downstream process. Comparing the specific productivity of KIS004-91T to all researches in (Table 4.3), results showed that KIS004-91T produced D-lactic acid at the highest level of 2.60 g/gCDW/h compare to those ever reported before. High specific productivity of KIS004-91T described that the ability of cells could produce high production of D-lactic acid. In summary, KIS004-91T has a great potential to produce productivity of D-lactic acid with low-cost medium that makes more advantages compared to current other research groups.

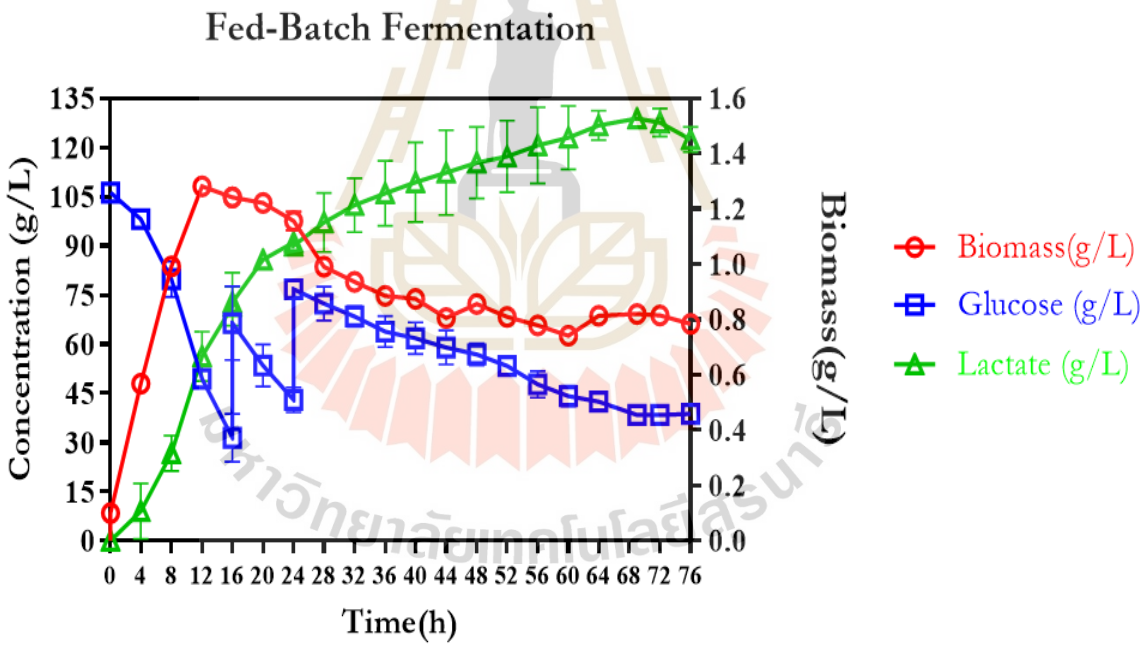


Figure 4.9 Fed-batch fermentation profile of *K. oxytoca* KIS004-91T in AM1 medium containing 10% (w/v) glucose in 5 L bioreactor with 2.5 L working volume. During 24 h of fermentation, glucose was fed every 4 h to the medium.

Table 4.3 Comparison of D-lactic acid production in various media and strains.

Strains	Media/mode of process	D-Lactate				References
		Concentration (g/L)	Yield (g/g)	Overall Productivity (g/L/h)	Specific productivity (g/gCDW/h)	
K. oxytoca KIS004-*91T (ΔadhE, Δpta-ackA, Δfrd, ΔpflB)*	100 g/L Glucose/AM1/Batch fermentation	100	0.96	2.1	2.60	This study
	100 g/L Glucose/AM1/Fed-batch fermentation	129	0.95	1.9	2.32	
	100 g/L Cassava starch/AM1/SHF	98.4	0.93	1.43	1.24	
K. pneumoniae (ΔbudB, ΔackA, ΔadhE)	20 g/L of (Xylose, arabinose, glucose) /LB/Fed-batch fermentation	125	0.91	3.48	0.83	Feng et al., 2017
K. oxytoca KMS004 *(ΔadhE, Δpta-ackA)*	50 g/L Glucose/AM1/Batch fermentation	34	0.92	0.34	0.34	Sangproo et al., 2012
E. coli (Δack,Δpps, ΔfrdA ΔpflB, Δdld,ΔpoxB, ΔadhE, ldhA)	500 g/L Glucose/LB/Fed-batch fermentation	122	0.89	4.32	0.82	Zhou et al., 2012

Table 4.3 (continued).

Strains	Media/mode of process	D-Lactate				References
		Concentration (g/L)	Yield (g/g)	Overall Productivity (g/L/h)	Specific productivity (g/gCDW/h)	
Sporolactobacillus inulinus(PYK)	100 g/L Glucose/LB/Batch fermentation	115	0.81	0.68	0.22	Zheng et al., 2014
Saccharomyces cerevisiae (Δdpdc1, Δdadh1)	20 g/L Glucose/LB/ Batch fermentation	60.3	0.646	2.8	0.5	Yamada et al., 2017b
Pichia kudriavzevii (pdc1 to D-ldh)	20 g/L Glucose/LB/ Fed-batch fermentation	154	0.72	4.16	0.57	Park et al., 2018
E. coli (ΔfrdBC, ΔldhA ΔackA, ΔpflB ΔpdhR::pflBp6-acEF-lpd, ΔmgsA ΔadhE,ΔldhA::ldhL)	100 g/L Glucose or Xylose/NBS/Batch fermentation	83	0.83	0.86	0.35	Lu et al., 2016

Table 4.3 (continued).

Strains	Media/mode of process	D-Lactate				References
		Concentration (g/L)	Yield (g/g)	Overall Productivity (g/L/h)	Specific productivity (g/gCDW/h)	
Bacillus coagulans (ΔldhL, ΔldhL2, LdldhD)	50 g/L Sucrose/LB/Fed-batch fermentation	145	0.98	1.5	NA	Zhang et al., 2017
E. coli (ΔpflB, ΔfrdA, ΔadhE)	40 g/L Glycerol/LB/Batch fermentation	32	0.85	1.5	0.75	Mazumdar et al., 2010
Lactobacillus plantarum (ldhL1::amyA)	20 g/L Brown rice/CSL/Batch fermentation	117	0.93	0.8	NA	Okano et al., 2017

The D-(-) lactate yield was calculated as gram(s) of D-(-) lactate divided by gram(s) of glucose consumed. The overall productivity was calculated as D-(-) lactate concentration produced divided by overall incubation time. The specific D-(-) lactate productivity was calculated as D-(-) lactate productivity divided by cell dry weight.

4.9 Batch SHF in 5L bioreactor of cassava starch

To make this research more interesting, cassava starch was used for D-lactate production in 5L bioreactor with separated hydrolysis and fermentation (SHF). A 100 g/L of cassava starch was added to fermenter and sterilized at 121 ^{o}C for 20 min. 2% of α-amylase (42 169 U/g) was added to the fermenter after the temperature was decreased to 70 ^{o}C. Hydrolysis by α-amylase was performed 4 h at temperature ranging from 45-50^{o}C at pH 6. Then, 0.5% (213 000 U/mL) of glucoamylase (AMG) was put on and the hydrolysis was kept running for 1 h. After hydrolysis, seed culture and AM1 medium were added to bioreactor. After 68 h incubation, D-lactic acid at concentration of 98.4±0.8 g/L, with the yield of 0.93±0.01 g/g, and productivity of 1.45±0.02 g/L/h was produced (Fig. 4.10). The result indicated that KIS004-91T could produce a high yield of D-lactate from cassava starch. However, Berlowska et al. (2018) revealed that simultaneous saccharification and fermentation (SSF) has advantages over SHF. Additionally, the SSF process is currently performed to produce organic acids because it makes lower enzyme inhibition by glucose during fermentation (Dahnum et al., 2015). Therefore, SSF would be further employed to reduce the time of fermentation and also improve the concentration, yield, and productivity of D-lactate by the strain.

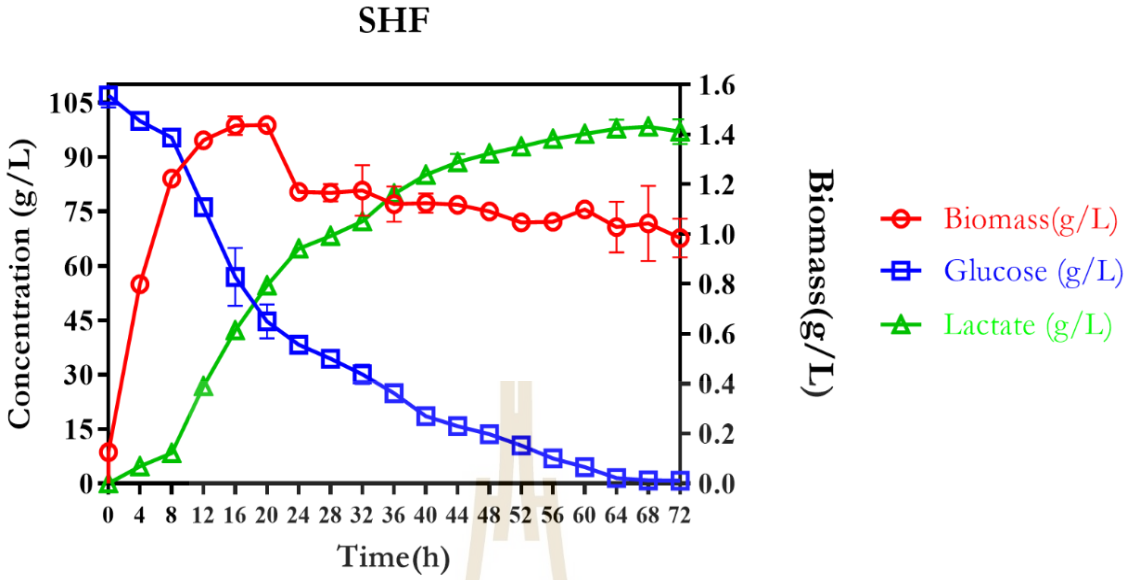


Figure 4.10 Fermentation profile of separated hydrolysis and fermentation of KIS004-91T using cassava starch as substrate for D-lactic acid production.

CHAPTER V

CONCLUSION

K. oxytoca M5a1 was previously metabolically engineered by Sangproo et al. (2012) to produce D-lactate. This strain was named as KMS004 (*ΔadhE*, and *Δpta-ackA*). After that, KMS004 was further engineered to remove fumarate reductase and pyruvate formate lyase genes (*frd* and *pflB*) named as KIS004. KIS004 (*ΔadhE, Δpta-ackA, ΔfrdABCD, and ΔpflB*) strain produced high production of D-(-) lactic acid in low mineral salt medium (AM1) containing 5% (w/v) glucose. In addition, to improve the capacity of KIS004 strain for utilization of substrate, metabolic evolution was applied to the strain performing in AM1 medium containing 10% (w/v) glucose. After the 91st transfer, KIS004-91T consumed all 10% (w/v) glucose and produced D-lactic acid with high concentration. KIS004-91T has the ability to produce high titer, productivity, and yield of lactate while other by-products were almost diminished. After the re-engineering KIS004 exhibited D-lactate as a key pathway to regenerate NAD^+. KIS004 produced 45.2 g/L of D-lactate from AM1 medium containing 5% (w/v) glucose while KIS004-91T produced 95.9 g/L of D-lactate from AM1 medium containing 10% (w/v) glucose. To improve the yield and productivity, 5 L fermenter with working volume 2.5 liters was performed to produce D-lactate production. In the batch process, KIS004-91T produced D-lactic acid with the concentration of 100 g/L, yield of 0.96 g/g and productivity of 2.1 g/L/h, respectively. In the fed-batch process, KIS004-91T produced D-lactate at concentration of 129 g/L, yield of 0.95 g/g, and productivity of 1.9 g/L/h. SHF process of cassava starch was also performed and result showed that KIS004-91T produced concentration, yield, and productivity of D- lactic acid at 97 g/L, 0.93 g/g, and 1.43 g/L/h, respectively. Therefore, KIS004-91T is a potential strain for high D-lactic acid production.

However, batch and fed-batch simultaneous saccharification and fermentation (SSF) using cassava starch as substrate for D-lactic acid by KIS004-91T are recommended for further experiments to increase the D-(-) lactic acid productivity. In conclusion, KIS004-91T could be the marvelous strain to develop the economics of lactic acid production from the low-cost substrate.

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APPENDIX

APPENDIX

Sequences of plasmid pKJ1011 used for deletion of *frd* gene

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCA

GCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATT

AATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCG

GCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAG

CTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGG

CCGCCAGTGTGCTGGAATTCGCCCTT*CCGGAGCTTATGCAGAAAACGGTCGATAA*

GCTCGCCGAGCTGCAGGAGCGCTTCAAGCGCGTGCGGATCACCGACACGTCCAGC

GTGTTCAATACCGACCTGCTGTACACCATCGAACTGGGCCATGGTCTGAACGTCGCC

GAATGTATGGCGCATTCCGCCCTCGCGCGTAAAGAGTCCCGCGGCGCGCATCAGCG

CCTGGATGAAGGCTGCACCGAGCGCGACGACGTCAACTTCCTCAAGCATACCCTCG

CTTTCCGCGATGCCGATGGTCACACCAGTCTGGAGTACAGTGATGTGAAAATCACCA

CTCTGCCGCCGGCAAAACGCGTGTACGGTGCGGAAGCGGAAGCAGCCGAGAAGAA

*GGAGACGACGAATGGCTGAGAA***CACTGCTTCCGGTAGTCAATAAACCGGNNAT**

CGGCATTTTCTTTTGCGTTTTTATTTGTTAACTGTTAATTGTCCTTGTTCAA

GGATGCTGTCTTTGACAACAGATGTTTTCTTGCCTTTGATGTTCAGCAGGA

AGCTTGGCGCAAACGTTGATTGTTTGTCTGCGTAGAATCCTCTGTTTGTCA

TATAGCTTGTAATCACGACATTGTTTCCTTTCGCTTGAGGTACAGCGAAGT

GTGAGTAAGTAAAGGTTACATCGTTAGGATCAAGATCCATTTTTAACACAA

GGCCAGTTTTGTTCAGCGGCTTGTATGGGCCAGTTAAAGAATTAGAAACAT

AACCAAGCATGTAAATATCGTTAGACGTAATGCCGTCAATCGTCATTTTTG

ATCCGCGGGAGTCAGTGAACAGGTACCATTTGCCGTTCATTTTAAAGACGT

TCGCGCGTTCAATTTCATCTGTTACTGTGTTAGATGCAATCAGCGGTTTCA

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GAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCT
GTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGG
GGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGC
CTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGG
ATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGA
CCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAG

Note: Normal letter are pCR2.1-TOPO-based vector, italic letters are *frd'* gene (incomplete gene), bold letters are *cat-sacB* gene, underline letters are *frd''*-(incomplete gene).

Sequences of pKJ1012 used for deletion of *frd* gene

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCA

GCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATT

AATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCG

GCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAG

CTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGG

CCGCCAGTGTGCTGGAATTCGCCCTT*CCGGAGCTTATGCAGAAAACGGTCGATAA*

GCTCGCCGAGCTGCAGGAGCGCTTCAAGCGCGTGCGGATCACCGACACGTCCAGC

GTGTTCAATACCGACCTGCTGTACACCATCGAACTGGGCCATGGTCTGAACGTCGCC

GAATGTATGGCGCATTCCGCCCTCGCGCGTAAAGAGTCCCGCGGCGCGCATCAGCG

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CTTTCCGCGATGCCGATGGTCACACCAGTCTGGAGTACAGTGATGTGAAAATCACCA

CTCTGCCGCCGGCAAAACGCGTGTACGGTGCGGAAGCGGAAGCAGCCGAGAAGAA

*GGAGACGACGAATGGCTGAGAT*ACCGTGGTGATCCTGTTTGTCGCGCTGTTCTGG

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TCAGCTACGAACGCGTGCTGGCTTTCGCCCATAGCTTTATTGGCCGCGCATTCA

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CACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTA

CCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGA
CGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCAC
ACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTG
AGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCC
GGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGA
AACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTC
GATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAAC
GCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCC
TGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGA
TACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAA
GCGGAAG

Note: Normal letters are pCR2.1-TOPO-based vector, italic letters are *frd'* gene (incomplete gene), underline letters are *frd'* (incomplete gene).

Sequences of pKJ1014 for deletion of *pflB* gene

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCA

GCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATT

AATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCG

GCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAG

CTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGG

CCGCCAGTGTGCTGGAATTCGCCCT*TATGTCCGAGCTTAATGAAAAGTTAGCCACA*

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CATCCAGAAAAACTATACCCCGTACGAAGGTGACGAGTCCTTCCTGGCTGGCGCAAC

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CAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCT

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CGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCT

TTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAG

CGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACG

TTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAG

GATCTCCTGTCATCCCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGAT

GCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAA

GCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGA

TCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCG

CCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGC

GATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATC

GACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTAC

CCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCT

TTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGAC

GAGTTCTTCTGAATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCG

CCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAAC

GCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACA

TCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAA

CGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCC

GTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAAT

GACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGAC

AGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCA

ACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACA

ACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAA

GCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAAC

GTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATT

AATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCC

TTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTC

GCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTA

TCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCT

GAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCA

TATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGA

AGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCA

CTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTT
TCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGG
TTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCA
GCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACC
ACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTAC
CAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGAC
GATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACA
CAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGA
GCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCG
GTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAA
ACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCG
ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACG
CGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCT
GCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGAT
ACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAG
CGGAAG

Note: Normal letter are pCR2.1-TOPO-based vector, italic letters are *pflB'* gene (incomplete gene), bold letters are *cat-sacB* gene, underline letters are *pflB''*-(incomplete gene).

Sequences of plasmid pKJ1015 for deletion of *pflB* gene

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCA

GCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATT

AATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCG

GCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAG

CTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGG

CCGCCAGTGTGCTGGAATTCGCCCTT*ATGTCCGAGCTTAATGAAAAGTTAGCCACA*

GCCTGGGAAGGTTTTGCGAAAGGTGACTGGCAGAACGAAGTCAACGTCCGCGACTT

CATCCAGAAAAACTATACCCCGTACGAAGGTGACGAGTCCTTCCTGGCTGGCGCAAC

TGACGCGACCACCAAGCTGTGGGACACCGTAATGGAAGGCGTTAAACAGGAAAACC

GCACTCACGCGCCTGTTGATTTTGATACTTCCCTTGCATCCACCATCACTTCTCATGA

CGCTGGCTACATCGAGAAAGGTCTCGAGAAAATCGTTGGTCTGCAGACTGAAGCTCC

GCTGAAACGCGCGATTATCCCGTTCGGCGGCATCAAAATGGTAGAAGGTTCCTGCAA

AGCGTACAATCGCGAGCTGGACCCGATGCTGAAGAAAATCTTCACTGAATACCGCAA

AACTCACAACCAGGGCGTGTTTGACGTTTACACCAAAGACATCCTGAACTGCCGTAA

ATCTGGCGTTCTGACCGGTCTGCCGGATGCCTATGGCCGTGGTCGTATCATCGGTG

ACTACCGTCGCGTTGCGCTGTACGGTATCGACTTCCTGATGAAAGACAAATACGCTC

*AGTTCGTCTCTCTGCAAGAGAAACTGGAAAAC*ACGCTATCCCGACTCAGTCCGTTC

TGACCATCACCTCTAACGTTGTGTATGGTAAGAAAACCGGTAACACCCCTGACG

GTCGTCGCGCTGGCGCTCCGTTCGGACCAGGTGCTAACCCGATGCACGGCCGTG

ACCAGAAAGGTGCTGTTGCCTCTCTGACCTCCGTTGCGAAACTGCCGTTTGCTT

ACGCGAAAGATGGTATTTCTTACACCTTCTCTATCGTGCCGAACGCGCTGGGTA

AAGACGACGAAGTTCGTAAAACTAACCTCGCCGGCCTGATGGATGGTTACTTC

CACCACGAAGCGTCCATCGAAGGCGGTCAGCATCTGAACGTCAACGTTATGAA

CCGCGAAATGCTGCTCGACGCGATGGAAAACCCGGAAAAATATCCGCAGCTGA

CCATCCGCGTATCCGGCTACGCAGTACGTTTTAACTCCCTGACGAAAGAACAGC

AGCAGGACGTTATTACTCGTACCTTCACTCAGACCATGTAAAAGGGCGAATTCT
GCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAAT
TCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGT
GACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCT
TTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACA
GTTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAGCGC
GGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAG
CGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCC
CGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGG
CACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATC
GCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGT
GGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTG
ATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTT
AACAAAAATTTAACGCGAATTTTAACAAAATTCAGGGCGCAAGGGCTGCTAAA
GGAAGCGGAACACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCCGGAT
GAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAAAGAGA
AAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTT
ATGGACAGCAAGCGAACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTG
GGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGG
CGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGAT
TGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTAT
TCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCC
GGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTG
CCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACG
GGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTG
GCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCCCACCTTGCTCC

TGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTG

ATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCA

CGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCA

TCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCG

ACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGG

TGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGG

ACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCG

GCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGC

AGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAATTGAAAAAGGAA

GAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTT

TGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAA

GATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAA

GATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAA

AGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACT

CGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCAC

AGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCA

TAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGA

CCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTT

GATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACAC

CACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAAC

TACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAA

GTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGAT

AAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCC

AGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAA

CTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAG

CATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAA

CTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGA

CCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAA

AGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCA

AACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTAC

CAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTG

TTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGC

CTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATA

AGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAG

CGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGAC

CTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTC

CCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGG

AGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTG

TCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGG

GGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCC

TTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGA

TAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGAC

CGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAG

Note: Normal letter are pCR2.1-TOPO-based vector, italic letters are *pflB'* gene (incomplete gene), underline letters are *pflB''* (incomplete gene)

BIOGRAPHY

Mr. Sokra In was born on July 25, 1992 at Kampong Cham province, Cambodia. He obtained his Bachelor degree of Science in Food Processing from Faculty of Agriculture and Food Processing, University of Battambang, Battambang, Cambodia, in 2016. After graduation, he decided to further study for Master degree in the field of Biotechnology at school of Biotechnology, Institute of Agricultural of Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. His research topic is re-engineering of *Klebsiella oxytoca* KMS004 to improve D-lactic acid production yield. After he graduates from Suranaree University of Technology, he plans to work in industry or as a lecturer in Cambodia.