RE-ENGINEERING OF *KLEBSIELLA OXYTOCA* KMS006 TO PRODUCE SUCCINIC ACID IN MINERAL SALTS MEDIUM

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A Thesis Submitted in Partial Fulfillment of the Requirements for the
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วิศวกรรมอีกครั้งของ *Klebsiella oxytoca* KMS006 เพื่อการผลิตกรดซักซินิกในอาหารเลี้ยงเชื้อเกลือแร่

นายชัชวาลย์ โพธิ์ศรีราน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ชัชวาลย์ โพธิ์ศรีราน: วิศวกรรมอีกครั้งของ *Klebsiella oxytoca* KMS006 เพื่อการผลิตกรดซักซินิกในอาหารเลี้ยงเชื้อเกลือแร่ (RE-ENGINEERING OF *KLEBSIELLA OXYTOCA* KMS006 TO PRODUCE SUCCINIC ACID IN MINERAL SALTS MEDIUM)
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สายพันธุ์ *Klebsiella oxytoca* KMS006 (Δ*adhE*Δ*pta-ackA*Δ*ldhA*) ถูกตัดต่อพันธุกรรมอีกครั้ง เพื่อเพิ่มคาร์บอนฟลักซ์ไหลไปสู่วิถีการสร้างซักซิเนต สายพันธุ์ที่ได้หลังการตัดต่อพันธุกรรม *K. oxytoca* KC004 (Δ*adhE*Δ*pta-ackA*Δ*ldhA*Δ*budAB*Δ*pflB*) แสดงการเจริญที่ไม่ดีและอัตราการใช้กลูโคสต่ำในอาหารเลี้ยงเชื้อเกลือแร่ (AM1) โดยที่ไม่มีการผลิตซักซิเนต เนื่องจากสายพันธุ์ดังกล่าวบกพร่องในการผลิต ATP และการรีออกซิเดชั่นของ NADH ภายใต้สภาวะไร้ออกซิเจน เพื่อแก้ปัญหาดังกล่าวได้มีการใช้วิธีการปรับตัวเชิงวิวัฒนาการมาจัดการกับสายพันธุ์นี้ หลังการทำวิธีการปรับตัวเชิงวิวัฒนาการพบว่า การใช้กลูโคสและการผลิตซักซิเนตของสายพันธุ์ที่ผ่านการปรับตัวเชิงวิวัฒนาการ (*K. oxytoca* KC004-TF160) ถูกปรับปรุงอย่างมีนัยสำคัญในอาหารเลี้ยงเชื้อ AM1 ที่มี 100 กรัมต่อลิตรกลูโคสพบว่า สายพันธุ์ KC004-TF160 ผลิตซักซิเนตที่ความเข้มข้น 84 กรัมต่อลิตร โดยให้ผลผลิตและผลิตผลสูงสุด 0.84 กรัมต่อกรัม และ 0.87 กรัมต่อลิตรต่อชั่วโมง ตามลำดับ และผลิตอะซิเตทที่ 14 กรัมต่อลิตร แต่ไม่พบผลิตภัณฑ์พลอยได้อื่น ๆ นอกเหนือจากนี้ พบว่าสายพันธุ์ KC004-TF160 มีความสารถผลิตซักซิเนตที่ได้ผลผลิตสูง 0.41 ถึง 0.87 กรัมต่อกรัม จากการใช้น้ำตาลชนิดต่างๆและกากน้ำตาลที่มีต้นทุนการผลิตที่ต่ำและเป็นแหล่งสารอาหารทางเลือกที่มีศักยภาพต่อการผลิตที่จะพัฒนาต่อไปได้ ในขณะที่การพัฒนาเพิ่มเติมของสายพันธุ์ KC004-TF160 เพื่อลดการผลิตอะซิเตทนั้นสามารถเพิ่มผลผลิตเพิ่มขึ้นเท่ากับ 0.88 กรัมต่อกรัม จากสายพันธุ์ *K. oxytoca* KP001-TF60 (Δ*adhE*Δ*pta-ackA*Δ*ldhA*Δ*budAB*Δ*pflB*Δ*tdcD*Δ*pmd*) และยังพบอะซิเตทที่ต่ำกว่า 1 กรัมต่อลิตร อย่างไรก็ตามสายพันธุ์นี้สามารถใช้น้ำตาลกลูโคสได้เพียงประมาณ 60 กรัมต่อลิตร และยังต้องการสารสกัดยีสต์เพื่อการเจริญของเซลล์ ถึงอย่างไรก็ตามทั้งสายพันธุ์ KC004-TF160 และ KP001-TF60 สามารถผลิตซักซิเนตเทียบเท่ากับสายพันธุ์ผู้ผลิตดั้งเดิมและสายพันธุ์พัฒนา *Escherichia coli* และเมื่อวิเคราะห์การเมตาบอลิซึมภายในพบว่ากิจกรรมของยีน *pck* ถูกเพิ่มขึ้นเพื่อตอบสนองการเพิ่มขึ้นของผลผลิตซักซิเนตและการผลิตพลังงาน ขณะที่กิจกรรมของยีน *pdh*, *tdcE* และ *tdcD* ตอบสนองการผลิตอะซิติลโคเอและอะซิเตทที่เป็นกระบวนการหลักสำหรับเป็นแหล่งพลังงานและความสมดุลของกระบวนการรีดอกซ์ และกิจกรรมของยีน *pck* ที่เพิ่มขึ้นอาจเกิดจากการเปลี่ยนแปลงของนิวคลีโอไทด์ในยีน *cyaA*, *ptsG*, *agaC*, และ *csrB* ซึ่งจาก

ผลการทดลองทั้งหมดแสดงให้เห็นว่า สายพันธุ์ KC004-TF160 ที่พัฒนาขึ้นใหม่อาจมีประโยชน์ในฐานะหนึ่งในแพลตฟอร์มจุลินทรีย์ที่มีศักยภาพสำหรับการผลิตซักซิเนตในเชิงพาณิชย์ ขณะที่สายพันธุ์ KP001-TF60 สามารถใช้เพื่อพัฒนาเป็นผู้ผลิตซักซิเนตต่อไปในอนาคต

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CHUTCHAWAN PHOSRIRAN: RE-ENGINEERING OF *KLEBSIELLA OXYTOCA* KMS006 TO PRODUCE SUCCINIC ACID IN MINERAL SALTS MEDIUM. THESIS ADVISOR: ASSOC. PROF. KAEMWICH JANTAMA, Ph.D., 127 PP.

Keyword: *Klebsiella oxytoca* KMS006/Metabolic engineering/Evolutionary adaptation/ Succinic acid

Klebsiella oxytoca KMS006 (Δ*adhE*Δ*pta-ack*AΔ*ldhA*) was re-engineered to enhance the carbon flux through the succinate-producing pathway. The resulting strain, KC004 (Δ*adhE*Δ*pta-ack*AΔ*ldhA*Δ*budAB*Δ*pflB*), exhibited a poor growth and low-glucose consumption rate in the mineral salts (AM1) medium without the succinate production due to its deficiencies in ATP production and NADH reoxidation under anaerobic conditions. To overcome these circumstances, evolutionary adaptation was conducted, resulting in spontaneous mutation in the developed strain named KC004-TF160. In the 100 g/L AM1 medium, KC004-TF160 produced succinate at a concentration of 84 g/L with a yield and productivity of 0.84 g/g and 0.87 g/L/h, respectively. Acetate was detected at 14 g/L, but no other byproducts were found. Additionally, KC004-TF160 was able to produce succinate at a yield of 0.41-0.87 g/g using a variety of sugars and sugarcane molasse for a potential low cost and alternative carbon sources. KC004-TF160 was further improved to the reduce acetate formation which resulted in enhancing the succinate yield up to 0.88 g/g by *K. oxytoca* KP001-TF60 (Δ*adhE*Δ*pta-ack*AΔ*ldhA*Δ*budAB*Δ*pflB*Δ*tdcD*Δ*pmd*). An acetate level of less than 1 g/L was detected. Unfortunately, this strain could consume 60 g/L of glucose and requires yeast extract for cell growth. Even though KC004-TF160 and KP001-TF60 can produce succinate as efficiently as previous native producers and developed *Escherichia coli* strains. Further analysis of internal metabolic revealed that the increased enzymatic activity of the *pck* gene was responsible for the increased succinate yield and ATP production, whereas the activities of *pdh*, *tdcE* and *tdcD* genes were responsible for acetyl-CoA and acetate formation which is the primary mechanism for energy sources and redox balance. An increased *pck* activity may cause nucleotide variations in genes *cyaA*, *ptsG*, *agaC*, and *csrB*. All results demonstrate that

the newly developed KC004-TF160 may be useful as one of the potential microbial platforms for the commercial production of succinate.

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Chutchawan Phosriran

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LIST OF ABBREVIATIONS

AM1	=	Mineral Salt Medium
AMG	=	Amyloglucosidase/Glucoamylase
ATP	=	Adenosines 5'-triphosphate
ADP	=	Adenosine diphosphate
Bps	=	Base pairs
°C	=	Degree celsius
cAMP	=	Cyclic adenosine monophosphate
cDNA	=	Complementary deoxyribonucleic Acid
CDW	=	Cell dried weight
CFU	=	Colony form unit
CO_2	=	Carbon dioxide
DNA	=	Deoxyribonucleic Acid
dNTP	=	Deoxynucleoside triphosphates
E. coli	=	*Escherichia coli*
EDTA	=	Ethylenediaminetetraacetic acid
Fig.	=	Figure
g	=	Gram
gDNA	=	Genomic deoxyribonucleic acid
g/L	=	Gram (s) per Liter
h	=	Hour
HPLC	=	High performance liquid chromatography
IPTG	=	Isopropyl ß- D-1-thiogalactopyranoside
IUPAC	=	International union of pure and applied chemistry
Kb	=	Kilo base pair
K. oxytoca	=	*Klebsiella oxytoca*
kg−1	=	Milligrams per kilogram
L	=	Liter (s)
LB	=	Luria Bertain

LIST OF ABBREVIATIONS (Continued)

M	=	Molar
mM	=	Milli-molar
mg	=	Milligram (s)
mg/L	=	Milligram (s) per Liter
min	=	Minutes (s)
mL	=	Milliliter (s)
mm	=	Millimeter (s)
mRNA	=	Messenger ribonucleic acid
MW	=	Molecular weight
NADH	=	Nicotinamide adenine dinucleotide (Reduced form)
NAD^+	=	Nicotinamide adenine dinucleotide (Oxidized form)
NCBI	=	National Center for Biotechnology Information
Ng	=	Nanogram
OD	=	Optical Density
PBS	=	Polybutyrate succinate
PCR	=	Polymerase chain reaction
PEP	=	Phosphoenolpyruvate
pH	=	Potential Hydrogen
RT-qPCR	=	Reverse transcription quantitative real-time PCR
PTS	=	Glucose phosphotransferase system
RNA	=	Ribonucleic acid
Rpm	=	Revolution per minute
s	=	Second
TBE	=	Tris-borate-EDTA
TCA	=	Tricarboxylic acid
U	=	Unit
UV	=	Ultraviolet
V	=	Volt
v/v	=	Volume per volume
w/v	=	Weight per volume

LIST OF ABBREVIATIONS (Continued)

μL	=	Micro-liter
μM	=	Micromolar

CHAPTER 1
INTRODUCTION

1.1 Overview

Bio-succinic acid is recognized by the U.S. Department of Energy as one of the top 12 building block chemicals that could be converted into intermediate compounds such as 1,4-butanediol and tetrahydrofuran (Werpy and Petersen, 2004; Bozell and Petersen, 2010; Takahashi et al., 2021). It is currently proposed as a potential suitable substitute for current petrochemical production, which is mainly used in a variety of applications including food ingredients, agriculture, green solvents, pharmaceutical products, and completely biodegradable plastics (Song et al., 2006; Okino et al., 2008; Cheng et al., 2012; Jiang et al., 2013, 2014). For this reason, its commercial demand is expected to increase at a compound annual growth rate of 27.4% (768 million metric tons) by the year 2025, up from succinic acid manufacturing in the year 1990 (18,000 metric ton) (Sharma et al., 2020). In general, most of the utilized succinic acid in the chemical industry is produced from petroleum-derived maleic anhydride, which is limited in natural feedstock, too expensive, and has negative environmental consequences for widespread use as a platform chemical (Chen et al., 2010; 2017). To overcome the mentioned problems, succinic acid can be produced through microbial fermentation using renewable feedstocks as a carbon source, with *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, and *Manheimia succiniciproducens* achieving the highest succinic acid titer (Carvalh et al., 2016; Guettler et al., 1996). Unfortunately, for optimal growth and high succinate production, these microorganisms require expensive nutrients as well as external supplies of carbon dioxide, hydrogen, or the mixture of gases, resulting in high costs in industrial-scale succinate production. Furthermore, these microorganisms are at the difficulty of metabolic engineering for eliminating by-products thus causing a trouble in succinate production and its purification (Kim et al., 2004).

Currently, a lot of efforts have been made to develop recombinant *Escherichia coli* strains capable of producing succinic acid efficiently. One of the most well-known *E. coli* strains developed, *E. coli* KJ122 strain was constructed by a combined metabolic engineering and metabolic evolution, resulting in high succinic acid at the titer of 80 g/L with the molar yield of 1.46 mol per mole of glucose consumed, 85% of the maximum theoretical yield (Jantama et al., 2008ab). However, this strain has a limited or an inefficient utilization for wide- range carbon sources due to its catabolic repression by glucose and natural incapability. Presently, *Klebsiella* is a good producer and has received a lot of attention for the production of commercially fermentable products such as 1,3-propanediol, 2,3-butanediol, and lactic acid due to its fast growth, undesirable any special or expensive source of nutrients during growth, availability of well-established methods for genetic tractability and an ability to utilize a broad substrate spectrum (Wojtusik et al., 2015; Cho et al., 2015a, b; 2012; Lawrence et al., 1996; Celinska, 2012). In recent years, a metabolically engineered *K. oxytoca* KMS006 (Δ*adhE* Δ*pta-ackA* Δ*ldhA*) strain, a derivative of *K. oxytoca* M5A1, has been demonstrated to be a biosafety strain as a production host for succinic acid production (Jantama et al., 2015). However, the concentration of succinic acid was not satisfactory due to its impaired growth during anaerobic growth, and it produced several by-products such as 2,3-butanediol, formic acid, and acetic acid under microaerobic conditions. Therefore, the goal of this study is to establish *K. oxytoca* KMS006 as a novel potential microbial platform for industrial-scale succinic acid production. *K. oxytoca* KMS006 was purposefully developed by combining metabolic engineering of the main gene associated with by-product generation and metabolic evolution to increase succinic acid yield.

1.2 Research objectives

The objectives of this study are to enhance succinic acid production in *K. oxytoca* KMS006 through a combination of metabolic engineering and metabolic evolution. Succinate fermentation was optimized for parameters affecting succinic acid final titer, yield, and productivity to achieve high succinic acid production. Thus, this work focused on:

1. Investigating effects of disrupting the 2,3-butanediol, acetyl-CoA, acetic acid, and lactic acid pathways on succinic acid production in mineral salts medium under anaerobic conditions in *K. oxytoca* KMS006.

2. Evolving the mutant strain under anaerobic conditions in mineral salt medium containing 5% and 10% w/w glucose to obtain an evolved strain exhibiting better growth and succinic acid production simultaneously.

3. Investigating transcription levels or mutations of any genes affecting succinic acid production after the combining genetic manipulation and evolutionary adaptation on the metabolites of engineered strain by comparing between the parental strain (*K. oxytoca* KMS006) and the evolved strain.

4. Investigating the ability of mutant strains to produce succinic acid from a wide range of carbon sources to provide global information for future strain development using alternative carbon sources.

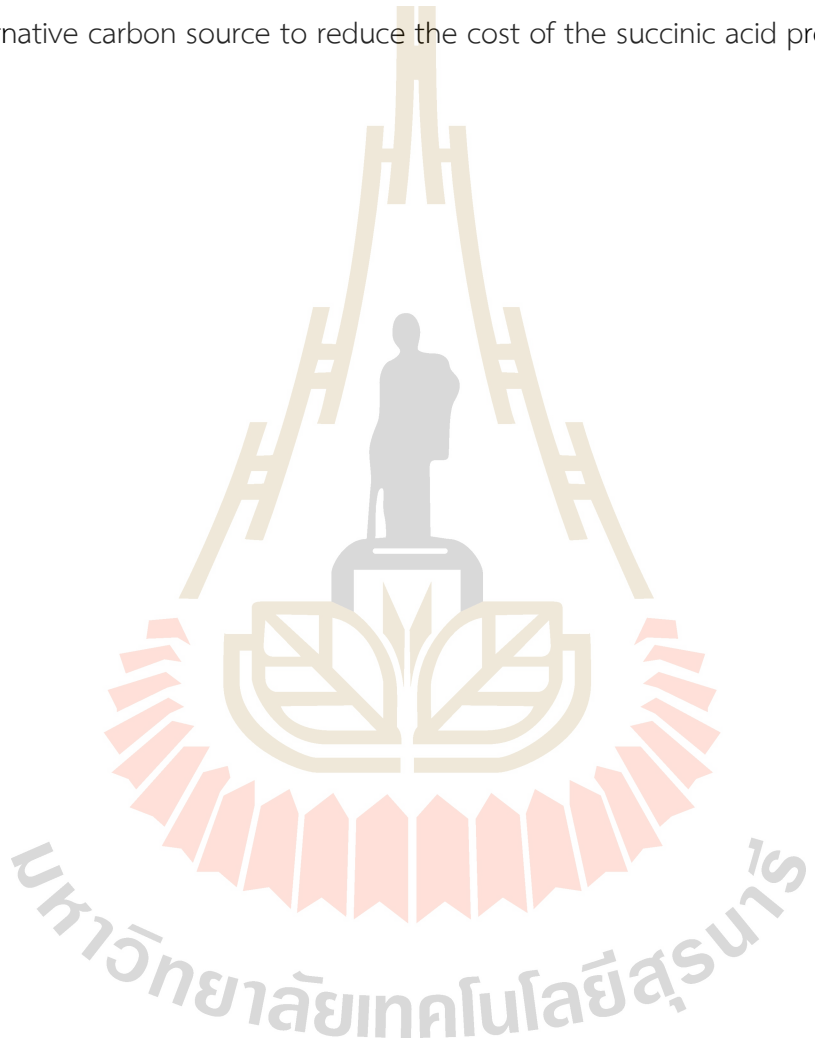
5. Improving the evolved strain's succinic acid production by optimizing the parameters such as initial OD_{550}, CO_2 supply, aeration and agitation rates affecting succinate fermentation in a 5 L bioreactor containing mineral salts medium and 100 g/L glucose.

6. Improving the production of succinic acid by applying batch fermentation using untreated sugarcane molasses as a carbon source.

1.3 Scope and limitations

K. oxytoca KMS006 was modified by deleting the main genes involves in the four biosynthetic pathways, which include the 2,3-butanediol pathway (catabolic **α**-acetolactate synthase (*budB*) and alpha-acetolactate decarboxylase (*budA*), on acetyl-CoA pathway (pyruvate formate-lyase (*pflB*), acetic acid pathway (propionate kinase (*tdcD*)), and lactic acid pathways (L-lactate dehydrogenase (*pmd*)). As a specific selective pressure, the engineered strain was evolved in AM1 medium containing 5-10 g/L of glucose and 100 mM potassium bicarbonate to obtain a newly strain with improved growth and high succinate production. The internal metabolic flux of newly strain was investigated by examining transcription levels of key enzyme activities in the primary three pathways, which include the reductive branch of the TCA cycle, the oxidative branch of the TCA cycle, and acetate synthesis. The new strain was tested

for their capacity to produce succinic acid from a variety of carbon sources by performing under anaerobic conditions in AM1 medium containing 50 g/L of glucose, sucrose, maltose, fructose, lactose, xylose, and glycerol. Batch fermentation was carried out in a 5 L bioreactor with AM1 medium containing 100 g/L glucose, and parameters affecting succinate fermentation, such as initial OD_{550}, CO_2 supply, and agitation rates, were optimized. In addition, untreated sugarcane molasses was used as an alternative carbon source to reduce the cost of the succinic acid process.



CHAPTER 2

LITERATURE REVIEW

2.1 Succinic acid and its applications

Succinic acid (IUPAC named: butanedioic acid) is one of the important carboxylic acid family with the chemical formula $HO_2C(CH_2)_2CO_2H$ as shown in Fig. 2.1.

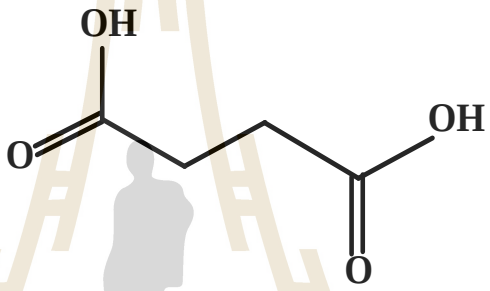


Figure 2.1 Structure of succinic acid.

Succinic acid, also referred to as amber acid, has traditionally been used in Europe as a natural antibiotic and general tonic (Saxena et al., 2017). Later, amber acid was purified using dry distillation by Georius Agricola, which are high volume specialty chemical and useful (Smyth et al., 1962). So, it has been recognized as a crucial propulsion of industrial chemical production as a sustainable alternative for bio-economy era. Today, succinic acid used as a precursor chemical to produce commodity and specialty derived succinate chemicals including adipic acid, 1,4-butanediol, tetrahydrofuran, *N*-methyl pyrrolidone, 2-pyrrolidinone, succinate salts, 4-amino butanoic acid, tetrahydrofuran, gamma-butyrolactone for many applications as shown in Fig. 2.2 (Beauprez et al., 2010).

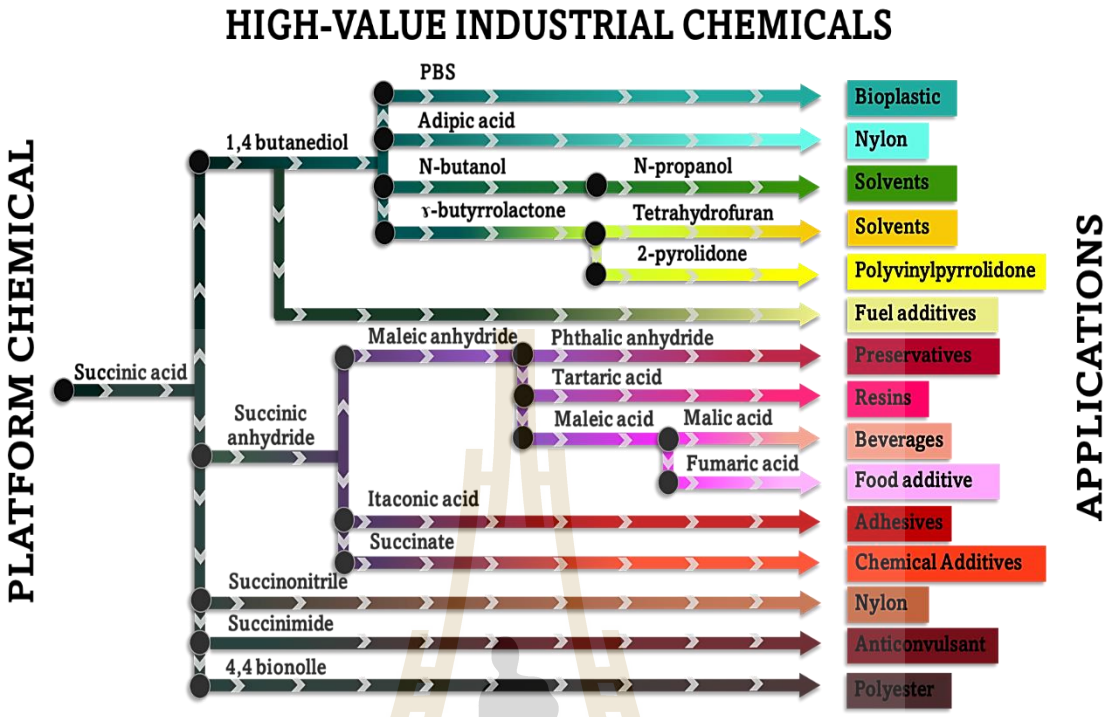


Figure 2.2 Possible production routes to succinate-based products as commodity and specialty chemicals (Zeikus et al., 1999).

Furthermore, according to Cheng et al. (2012), the increasing need for succinic acid is anticipated to encourage the synthesis of biodegradable polymers such as polybutyrate succinate (PBS), polyamides (Nylon®x,4), and a variety of green solvents. The succinic acid's properties are listed in Table 2.1 (Perry et al., 1997).

Table 2.1 Properties of succinic acid (Perry et al., 1997).

Succinic acid properties	Details	Value	Units
Acidity	pK_{a1}	4.21	-
	pK_{a2}	5.64	-
Boiling point		235	°C
Melting point		189	°C
Molar mass		118.09	$g.mol^{-1}$
Solubility	20 °C	58	g,l^{-1}
Specific gravity		1.57	$g.cm^{-3}$

2.2 Succinic acid production

Up to date, succinic acid can be produced in two ways that are biotechnological and chemical processes. Currently in chemical industry, most of succinate are produced by the chemical process involving the oxidation of n-butane to form maleic anhydride (Fig. 2.3) (Chen et al., 2010; Saxena et al., 2017). However, this process is high in manufacturing cost with the price of \$5.9 to 9.0 kg^{-1} depending on its purity (Lee et al., 1999; Song and Lee, 2006). Conversely, the manufacturing cost of the biotechnological process is inexpensive because this process can produce succinic acid from microbial fermentation using renewable feedstocks as carbon sources. Generally, microorganisms can produce succinic acid from three pathways including the reductive branch of tricarboxylic acid (TCA) cycle, the oxidative branch of the TCA cycle, and the glyoxylate cycle (Fig. 2.4) (Ahn et al., 2016; Saxena et al., 2017; Jantama et al., 2015).

In the reductive pathway, glucose is converted to 2 moles of phosphoenolpyruvate (PEP), and one mole of PEP is further reduced to oxaloacetate, malate, fumarate, and succinate by the actions of phosphoenolpyruvate carboxylase (*ppc*), malate dehydrogenase (*mdh*), fumarase (*fumABCD*), and fumarate reductase (*frd*), respectively. This pathway is absence of any enzymes for competitive converting succinic acid into other forms (Isar et al., 2006). While both the oxidative and glyoxylate pathways produce 1 mol of succinate from 1 mol of glucose under aerobic conditions, they are both primarily active in these conditions. The glyoxylate route can produce 2 moles of succinate from 2 moles of acetyl-CoA, 1 mole of oxaloacetate and 1 mole of NADH (Kornberg, 1966). However, both oxidative and glyoxylate pathway cannot be efficiently employed for only succinic acid production since during these cycles succinic acid can be converted into other forms including CO_2, glyoxylate, or oxaloacetate (Carel and Willie, 2013). So, the reductive pathway is preferred for succinate production in microorganisms because succinate is accumulated in the cell (Zubay, 1998).

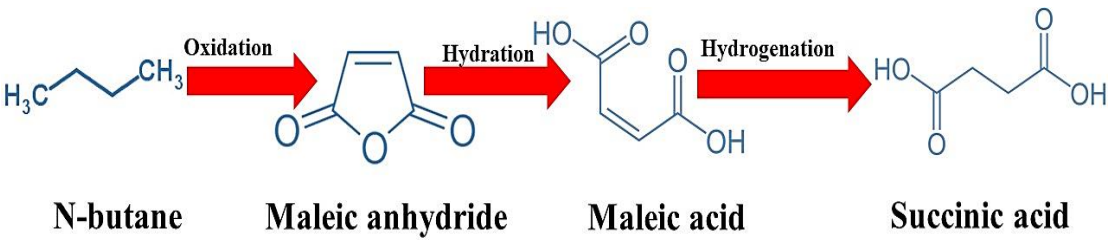


Figure 2.3 Succinic acid production from chemical synthesis (Saxena et al., 2017).

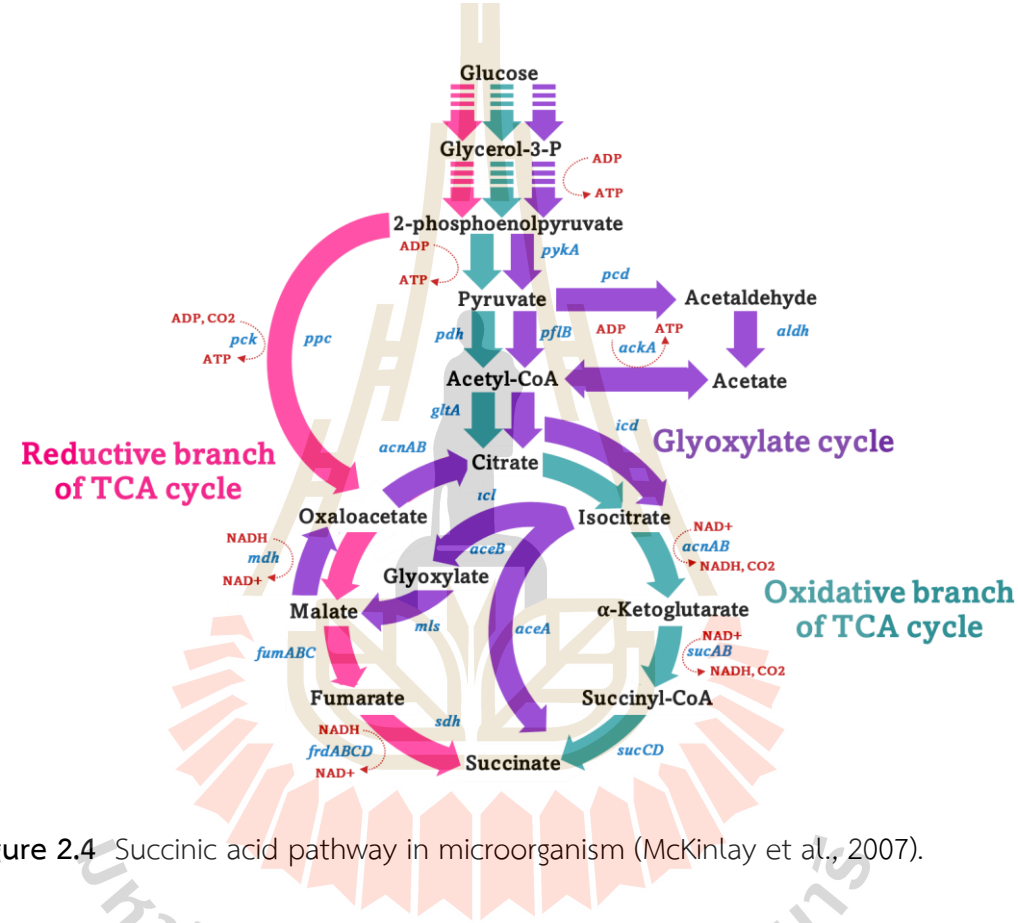


Figure 2.4 Succinic acid pathway in microorganism (McKinlay et al., 2007).

2.3 Microorganisms production of succinic acid

Succinic acid is known as an intermediate of the TCA cycle and can be accumulated as a fermentation end-product under anaerobic conditions. Thus, almost all microorganisms can be utilized to produce succinic acid, such as *E. coli*, *Pectinatus* sp., *Propionibacterium* species, *Corynebacterium crenatum*, *Corynebacterium glutamicum*, *Lactobacillus plantarum*, *Bacteroides* sp., rumen bacteria such as *Actinobacillus succinogens*, *Anaerobiospirillum succiniciproducens*, *Basfia succiniciproducens*, *Bacteroides amylophilus*, *Cytophaga succinicans*, *Prevotella ruminicola*, *Ruminococcus flavefaciens*, *Succinimonas amylolytica*, *Succinivibrio dextrinisolvens*, and *Wolineela succinogenes* (Becker et al., 2013; Bryant and Small,

1956; Bryant et al., 1958; Davis et al., 1976; Guettler et al., 1996a, b; Satohet et al., 2013; Jantama et al., 2015; Scheifinger and Wolin, 1973; Scholten et al., 2011, 2013; Van der Werf et al., 1997), fungi such as *Aspergillus niger*, *A. fumigatus*, *Byssochlamys nivea*, *Lentinus degener*, *Paecilomyces varioti*, *Penicillium viniferum* and yeast *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (Rossi et al., 1964; Ling et al., 1978; Zhiyong et al., 2017). The best of native producers for succinate production are summarized in Table 2.2. Fungi and bacteria are efficient producers of succinic acid. However, a use of fungi has been limited to the production of beverages and food owing to the difficulties in fermentation, separation, and purification as well as low productivities, whereas the bacteria isolated from the rumen, including *A. succinogenes* and *M. succiniciproducens*, are the best candidates for succinate production as they produce succinic acid as a major fermentation product and can ferment abroad substrate spectrum of carbon sources (Van der Werf et al., 1997; Guettler et al., 1999; Garrity et al., 2004). However, yields of succinate production of all microorganisms have not yet been satisfactory and several by-products were formed.

Currently, many microorganisms were engineered to improve succinate production and to reduce the production of by-products. For example, yeast *Y. lipolytica* was engineered for a production host of succinic acid. Wild-type *Y. lipolytica* produces succinate on glycerol of concentration 0.44 g/L with the yield of 0.02 g/g glucose. By deletion of succinate dehydrogenase gene, the mutant strain significantly increased succinate titer of 5.51 g/L, succinate yield of 0.24 g/g glucose and succinate productivity of 0.08 g/L/h, respectively (Gao et al., 2016). In bacteria, engineered *E. coli* stain KJ122 was constructed by deleting alcohol dehydrogenase (*adhE*), lactate dehydrogenase (*ldhA*), pyruvate formate-lyase (*pflB*), acetate kinase (*ackA*), threonine decarboxylase DE (*tdcDE*), pyruvate oxidase (*poxB*), methylglyoxal synthase (*mgsA*), citrate lyase (*citF*), aspartate aminotransferase (*aspC*) and NAD^+-linked malic enzyme (*sfcA*) to prevent formation of possible by-products. This mutant strain could produce highest succinic acid at levels of 88 g/L with the yield and productivity of 0.85 g/g glucose and 0.73 g/L/h in anaerobic batch fermentation.

Table 2.2 Summary of succinic acid production by microorganisms using glucose as the carbon source in fermentation.

Microorganism	Culture Condition	Titer (g/L)	Yield (g/g)	Reference
A. succinogenes 130Z	Batch, anaerobic	66.40	0.67	Guettlr et al. (1996)
A. succinogenes FZ53	Batch, anaerobic	105.80	0.80	Guettlr et al. (1996)
A. succiniciproducens	Batch, anaerobic	33.00	0.93	(Nghiem et al., 1997)
M. succiniciproducens	Batch, anaerobic	14.00	0.70	Lee et al. (2002)
M. succiniciproducens MBEL55E	Batch, anaerobic	10.50	0.45	Lee et al. (2006)
B. succiniciproducens DD1	Batch, anaerobic	20.00	0.49	Becker et al. (2013)
Mutant strain				
E. coli KJ122 (*aspC, citF, tdcDE, sfcA,*(*focA-pflB*), *ldhA*, *adhE*, *mgsA*, *ackA*, *poxB*)	Batch, anaerobic	88.00	0.85	Jantama et al. (2008b)
E. coli TG400 (KJ122; *galP* (G297D))	Batch, anaerobic	96.00	0.96	Grabar et al. (2012)
E. coli AFP111 (pTrc99-pyc)	Fed-batch, anaerobic	99.20	1.10	Lin et al. (2005)
M. succiniciproducens LPK7 (*ldhA, pfl, pta-ackA*)	Fed-batch, anaerobic	52.43	0.76	Lee et al. (2006)

2.4 *Klebsiella oxytoca*

Similar to *E. coli*, *K. oxytoca* is a gram-negative and facultative anaerobe bacterium (Fig. 2.5). It is in the Phylum of Proteobacteria, the class of Gamma Proteobacteria, the Order of Enterobacteriales and a genus of Enterobacteriaceae (Mahon et al., 2007). Outclassed *E. coli*, *K. oxytoca* has emerged as an excellent biocatalyst for a broad range of carbon substrates including glycerol, C5 and C6 monosaccharides and disaccharides. Wild-type *K. oxytoca* could produce succinate on glucose at concentrations 0.94 and 2.64 g/L under aerobic and anaerobic conditions, respectively. Whereas several engineered mutant *K. oxytoca* strains could produce succinic acid at highest succinate under aerobic and anaerobic conditions at levels of 0.24 g/L and 15.99 g/L, respectively (Table 2.3). The engineered *K. oxytoca* strain KMS005 was constructed by knocking out lactate dehydrogenase (*ldhA*), acetate kinase (*ackA*), phosphate acetyltransferase (*pta*), and aldehyde-alcohol dehydrogenase (*adhE*) genes to prevent formation of lactic acid, acetate, acetyl-CoA, and ethanol respectively (Fig. 2.6). The stain produced 2.36 g/L of succinic acid by anaerobic batch fermentation (Jantama et al., 2015). *K. oxytoca* GSC12206 was engineered through deleting *ldhA* gene to reduce lactic acid production. This stain showed the production of 15.99 g/L succinic acid by aerobic fed-batch fermentation (Kim et al., 2016). However, the yields of succinic acid production of engineered strains have not yet been satisfactory, and several by-products were formed. So, *K. oxytoca* should be further investigated for succinic acid production. It would be a potential microbial cell factory for the industrial chemical production in future perspective.

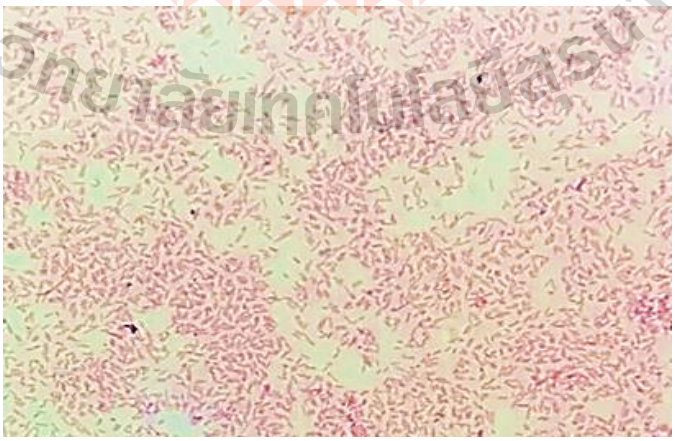


Figure 2.5 Gram strain of *K. oxytoca* M5A1.

Table 2.3 Summary of succinic acid production by *K. oxytoca* using glucose as the carbon source in fermentation.

Strain	Culture Condition	Titer (g/L)	Yield (g/g)	Reference
K. oxytoca M5A1	Batch, anaerobic	0.94	0.05	Jantama et al. (2015)
K. oxytoca GSC12206	Batch, aerobic	2.19	0.02	Kim et al. (2013)
Mutant strains				
K. oxytoca (Δ*ldhA*)	Batch, aerobic	6.25	0.03	Kim et al. (2016)
K. oxytoca (Δ*ldhA*)	Fed-batch, aerobic	14.1	0.02	Kim et al. (2016)
K. oxytoca KMS002 (Δ*adhE*)	Batch, anaerobic	0.24	0.01	Jantama et al. (2015)
K. oxytoca KMS004 (Δ*adhE*Δ*pta-ackA*)	Batch, anaerobic	0.48	0.02	Jantama et al. (2015)
K. oxytoca KMS005 (Δ*adhE*Δ*pta-ackA*Δ*ldhA*)	Batch, anaerobic	2.36	0.12	Jantama et al. (2015)
K. oxytoca GSC12206 (Δ*ldhA*)	Batch, aerobic	2.3	0.01	Kim et al. (2013)
K. oxytoca GSC12206 (Δ*ldhA*)	Fed-batch, aerobic	15.99	0.02	Kim et al. (2013)

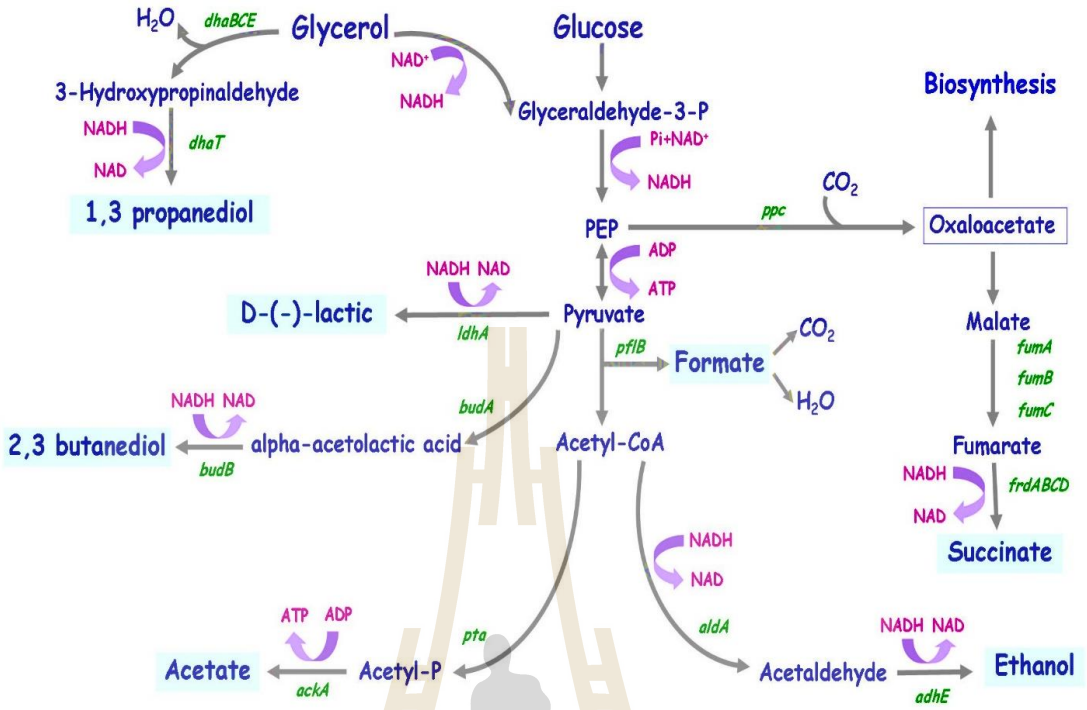


Figure 2.6 Standard pathway of glucose fermentation for *K. oxytoca* under anaerobic conditions.

2.5 Summary of major enzymes involving in anaerobic fermentations of *K. oxytoca* M5A1

Many microorganisms, including *K. oxytoca*, cannot perform oxidative phosphorylation in anaerobic environments. Instead, the cell generates energy through substrate-level phosphorylation, which is the process of breaking down the original substrate. The goal of the cell is to produce as much energy as possible. Because its supply is finite, the reducing power, NADH, that is created during substrate degradation must be reoxidized for the process to continue. It is essential to note that, at least in part, the pyruvate produced by the glycolytic pathway will enter the TCA pathway in order to supply crucial precursors for biosynthesis. Since there isn't enough oxygen in the cells to support oxidative phosphorylation, the NADH generated during the cycle cannot be converted to ATP. However, by reducing some carbon intermediates that amass under these circumstances, the cell can recycle NADH. In other words, the accumulating carbon intermediates are reduced in turn by the reducing equivalents (Fig. 2.6). Pyruvate is assimilated to re-oxidize NADH via *ldhA* and *adhE* activities, producing lactate and alcohol, respectively, in the central anaerobic metabolic

pathway. The simplest way to get rid of hydrogen involves reducing pyruvate to lactate at the expense of NADH. A cytoplasmic lactate dehydrogenase that is encoded by *ldhA* catalyzes the reaction. Together, anaerobiosis and an acid pH induce the enzyme. Dihydroxyacetone-phosphate (DHAP) can be used to make lactate. *MgsA* produces methylglyoxal from DHAP, which is then changed into lactate by *gloAB*-encoded glyoxalase activity (Wood, 1961).

Pyruvate formate-lyase, encoded by *pflB*, is post-translationally interconverted between active and inactive forms and is in charge of the anaerobic conversion of pyruvate to acetyl-CoA and formate. Anaerobiosis increases the production of enzymes, and pyruvate has the potential to increase it even more (Knappe & Sawers, 1990). When converted to acetate from pyruvate, acetyl-CoA can be used to either produce ATP from ADP or get rid of surplus reducing equivalents by turning them into ethanol. Phosphate acetyltransferase, likely encoded by *pta*, and acetate kinase, likely encoded by *ackA*, both acts sequentially in the first process. The respiratory state of the cell has no significant impact on the synthesis of these enzymes. Consequently, cells growing on glucose under aerobic conditions excrete the majority of the acetyl-CoA as acetate. The pathway catalyzed by acetyl-CoA synthethase, which is encoded by the *acs* gene, is primarily reversed in the absence of glucose to use external acetate (Kumari et al., 1995). Anaerobic fermentations also result in the conversion of acetyl-CoA to ethanol. At the expense of NADH, the acetyl group of acetyl-CoA is successively reduced to acetaldehyde and then to ethanol in this pathway. A single polypeptide, alcohol dehydrogenase, encoded by *adhE*, catalyzes the reactions. The proximity of the two reduction sites may prevent some of the chemically reactive acetaldehyde from escaping. Alcohol dehydrogenase and coenzyme A-linked acetaldehyde dehydrogenase are two of the enzyme activities of the ADHE protein. However, according to Clark (1989), alcohol dehydrogenase is more susceptible to inactivation by aerobic metabolism.

PEP is also assimilated through the process of carboxylation, which results in the production of succinic acid. Fumarate reductase is activated for PEP carboxylation and uses fumarate as an electron acceptor to reoxidize NADH. The enzyme phosphoenolpyruvate carboxylase, which is encoded by *ppc*, combines endogenous or exogenous carbon dioxide with PEP. Malate dehydrogenase, which is encoded by

mdh, converts the formed oxaloacetate to malate. The fumarase enzymes that *fumABC* codes for dehydrate malate to fumarate, and whose anaerobic induction depends on FNR regulation. Finally, fumarate reductase converts fumarate to succinate. In the end, four reducing equivalents (4H+ + 4e-) are disposed of. The menaquinone-encoded fumarate reductase can accept electrons from a variety of primary donor enzymes. According to Cecchini et al. (2002), fumarate induces fumarate reductase anaerobically while nitrate represses it either anaerobically or by oxygen.

Pyruvate oxidase, which is encoded by the poxB gene, is also in charge of producing C2 compounds from pyruvate when growth conditions alternate between strictly anaerobic and microaerobic. This enzyme decarboxylates pyruvate to produce carbon dioxide and acetate and couples the electron from pyruvate to ubiquinone (Abdel-Hamid et al., 2001). By using ubiquinone or menaquinone in the plasma membrane as a diffusible electron carrier or adaptor to connect a donor modular unit functionally to an acceptor modular unit, the electron transport system's versatility for generating proton motive force is made possible under both aerobic and anaerobic respirations. Depending on how genes are expressed in response to growth conditions, different electron carriers and donor modules are used for electron transport. The primary dehydrogenases of flavoproteins are the electron donor modular units in anaerobic environments. The terminal reductases in the acceptor modular units require a variety of materials, including Fe-S. In general, ubiquinone is used as a redox adaptor, such as in the case of pyruvate oxidase, when the terminal acceptor has a relatively high redox potential, such as oxygen. Menaquinone is used as an alternative when the terminal acceptor, such as fumarate, has a relatively low redox potential (Cecchini et al., 2002).

2.6 *Klebsiella oxytoca* KMS006

Previously *K. oxytoca* M5a1 was engineered to produce lactic acid and 2,3-butanediol in the mineral salt medium (Sangproo et al., 2012; Jantama et al., 2015). First, the wild type of *K. oxytoca* was deleted *adhE* and *pta-ackA* genes to eliminate ethanol and acetic acid formation under anaerobic conditions. The mutant strain was named KMS002 and KMS004 strains, respectively. However, the latest strain could produce lactic acid as a major production while ethanol was not detected during produce lactic acid as a major production while ethanol was not detected during fermentation in mineral salts media (Sangproo et al., 2012). KMS004 strain was further engineered to prevent lactic acid production by *ldhA* gene. The strain was named KMS006 (Jantama et al., 2015).

This strain produced formate, acetate and 2,3-butanediol as major by-products. Conversely, lactic acid was not detected in fermentation. Even though acetate kinase gene was eliminated, acetate was detected in the fermentation since *K. oxytoca* possesses *poxB* gene as a secondary gene to convert pyruvate into acetate during microaerobic conditions. Nonetheless, the strain KMS006 still possessed pyruvate formate lyase B, and butanediol dehydrogenase that are responsible for utilization of pyruvate to formate and 2,3 butanediol, respectively. So, the strain KMS006 may be further engineered to improve succinic acid production by eliminating gene of pyruvate formate lyase B and butanediol dehydrogenase to prevent production of formate and 2,3 butanediol, respectively (Fig. 2.7).

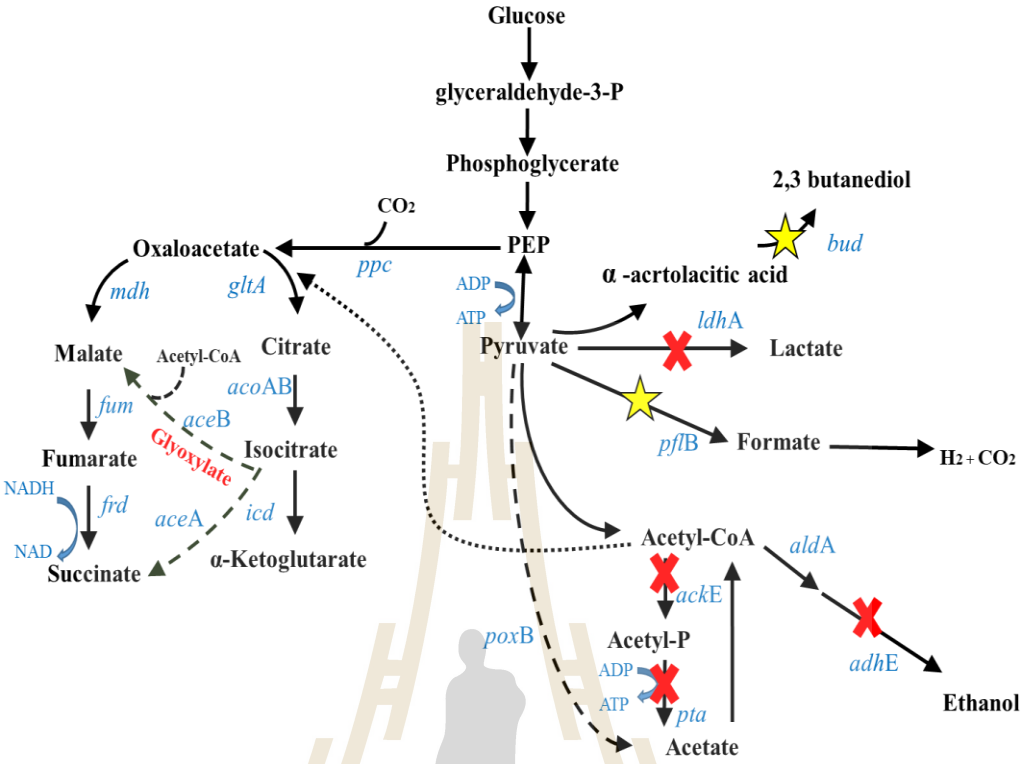


Figure 2.7 Metabolism pathway of *K. oxytoca* KMS006 in anaerobically fermentation. Solid arrows represent central fermentative pathways. Dashed arrow represents microaerophilic pathway to reserved acetate-production. Dotted arrows represent the glyoxylate pathway during aerobic metabolism. The crosses represent the gene deletions performed to obtain KMS006 (Δ*adhE*Δ*ackA*Δ*pta*Δ*ldhA*). The star represents both genes of *budAB* and *pflB* that will be deleted in further in *K. oxytoca* KMS006. Gene and enzymes: *pflB*, pyruvate formate-lyase; *budAB*, butanediol dehydrogenase; *adhE*, alcohol dehydrogenase; *ldhA*, lactate dehydrogenase; *pta*, phosphate acetyltransferase; *ackA*, acetate kinase; *aldA*, aldehyde dehydrogenase; *tdcD*, propionate kinase; *tdcE*, threonine decarboxylase E; acs, acetyl-CoA synthetase; *fdh*, formate dehydrogenase; *poxB*, pyruvate oxidase; *pdh*, pyruvate dehydrogenase; *ppc*, phosphoenolpyruvate carboxylase; *gltA*, citrate synthase; *acnA*, aconitase; *icd*, isocitrate dehydrogenase; *mdh*, malate dehydrogenase; *fum*, fumarase isozymes; *frd*, fumarate reductase, and *aceAB,* isocitrate lyase (Jantama et al., 2015).

CHAPTER 3

MATERIALS AND METHODS

3.1 Genetic engineering method

3.1.1 Bacterial strains, media, and growth conditions

Bacterial strains were engineered for succinate synthesis with the assistance of the Metabolic Engineering Research Unit (Jantama et al., 2015). Table 3.1 shows the bacterial strains, plasmids and primers used in this study. All strains and primary inoculum were cultured in Luria-Bertani (LB) broth containing 10 g/L peptone, 5 g/L yeast extract, and 10 g/L sodium chloride per 1 L of water at 37 °C and 200 rpm. For the screening of transformants, LB agar (20 g/L agar) with suitable antibiotics (50 g/mL kanamycin, apramycin, or chloramphenicol) was employed.

Table 3.1 Bacterial strains plasmids and primers used in this study.

Strains	Relevant characteristics	References
E. coli	TOP10F'	Invitrogen
M5Al	*K. oxytoca* wild type	Sangproo et al. (2012)
KMS005	*K. oxytoca ΔadhEΔpta-ackAΔldhA::cat-sacB*	Sangproo et al. (2012)
KMS006	*K. oxytoca ΔadhEΔpta-ackAΔldhA*	Sangproo et al. (2012)
KIS003	*K. oxytoca ΔadhEΔpta-ackAΔfrdΔpflB::cat-sacB*	In et al. (2020)
KIS004	*K. oxytoca ΔadhEΔpta-ackAΔfrdΔpflB*	In et al. (2020)
KC001	*K. oxytoca ΔadhEΔpta-ackAΔldhAΔbudAB::cat-sacB*	This study
KC002	*K. oxytoca ΔadhEΔpta-ackAΔldhAΔbudAB*	This study
KC003	*K. oxytoca ΔadhEΔpta-ackAΔldhAΔbudABΔpflB:: cat-sacB*	This study
KC004	*K. oxytoca ΔadhEΔpta-ackAΔldhAΔbudABΔpf*lB	This study
KC005	*K. oxytoca ΔadhEΔpta-ackAΔldhAΔbudABΔpflB ΔtdcD::cat-sacB*	This study
KC006	*K. oxytoca ΔadhEΔpta-ackAΔldhAΔbudABΔpflBΔtdcD*	This study

Table 3.1 (continued).

Strains	Relevant characteristics	References
KP001	*K. oxytoca ΔadhEΔpta-ackAΔldhAΔbudABΔpflBΔtdcD Δpmd::cat-sacB*	This study
KP002	*K. oxytoca ΔadhEΔpta-ackAΔldhAΔbudABΔpflBΔtdcD Δpmd*	This study
KC004-TF160	KC004 evolution with 160th transfer	This study
KP001-TF60	KP001 evolution with 60th transfer	This study
Plasmids		
pCR 2.1-TOPO	*bla kan,* TOPO TA cloning vector	Invitrogen
pLOI3420	*acc* ***γ β*** *exo* (Red recombinase), temperature-conditional replicon	(Wood et al., 2005)
pKC002	*bla kan; budAB* (PCR) from *K. oxytoca* KMS006 (using *budAB*-up/down) cloned into pCR2.1-TOPO	This study
pKC002.1	*cat-sacB* cassette (PCR) from *K. oxytoca* KMS005 (*ΔbudAB::cat-sacB*) into the PCR amplified inside-out product from pKC002 (using *budAB*-IO up/down)	This study
pKC002.2	PCR amplified inside-out product from pKC002 (using *budAB*-IO up/down) kinase treated then self-ligation	This study
pKC006	*bla kan*; *tdcD* (PCR) from *K. oxytoca* KC004-TF160 (using *tdcD*-up/down) cloned into pCR2.1-TOPO	This study
pKC006.1	*cat-sacB* cassette (PCR) from *K. oxytoca* KMS005 (*ΔtdcD::cat-sacB*) into the PCR amplified inside-out product from pKC006 (using *tdcD*-IO up/down)	This study
pKC006.2	PCR amplified inside-out product from pKC004 (using *tdcD*-IO up/down) kinase treated then self-ligation	This study
pKP002	*bla kan*; *tdcD* (PCR) from *K. oxytoca* KC006 (using *pmd*-up/down) cloned into pCR2.1-TOPO	This study
pKP002.1	(*Δpmd::cat-sacB*) into the PCR amplified inside-out product from pKP002 (using *tdcD*-IO up/down)	This study
pKP002.1	PCR amplified inside-out product from pKC004 (using pmd-IO up/down) kinase treated then self-ligation	This study

Table 3.1 (continued).

Primers	Sequence	References
budAB-	5’ AGCGCAGATAACGAAGTTCC 3’	This study
up/down	5’ GATCGCGTTGAGACACATCCA 3’	
pflB-	5’ GGATGCAAGGGAAGTATCAA 3’	This study
up/down	5’ CCAGCGGGTTTGAGCATAGT 3’	
tdcD-	5’ GCGAGGCGTCGATTATCGCT 3’	This study
up/down	5’ GGTGGTAATCGGTGGTCAGC 3’	
pmd-	5’ TAGTCGGCAATGATCAGCCGGGGAT 3’	This study
up/down	5’ TTTGTTCCTGATAAATGTCGTCAT 3’	
budAB-IO	5’ GGGTCGATAACGGCTACAAC 3’	This study
up/down	5’ TGCGGTACTGCGGCTGAAAC 3’	
tdcD-IO	5’ CGGAAACCTTAACCGTTTCAGCCTG 3’	This study
up/down	5’ GCTGGAATTTTAACGATGAGCTGCC 3’	
pmd-IO	5’ TAGTCGGCAATGATCAGCCGGGGAT 3’	This study
up/down	5’ TTTGTTCCTGATAAATGTCGTCAT 3’	
bud-cat-F	5’ GTTTCAGCCGCAGTATCGCAACACTGCTTCCGGTAGTCAA 3’	This study
	5’ GTTGTAGCCGTTATCGACCCCGGCACGTAAGAGGTTCCAA 3’	
cat-bud-F	5’ TTGGAACCTCTTACGTGCCGGGGTCGATAACGGCTACAAC 3’	This study
	5’ TTGACTACCGGAAGCAGTGTTGCGATACTGCGGCTGAAAC 3’	
tdcD-Hi	5’ CTTACAAAATAATGGCCATTAACGCCGGCAGCTCATCGTTAAAA	This study
up/down	TTCCAGCACACTGCTTCCGGTAGTCAATAAAC 3’	
	5’ CACCGCAACTTGCCGGGCGGCGAAATCAGGCTGAAACGGTTAA	
	GGTTTCCGCGGCACGTAAGAGGTTCCAACTTTC 3’	
pmd-Hi	5’ TAAAGTGGCAGTAATTTTGCGTCATGACGACATTTATCAGGAAC	This study
up/down	AAAACACTGCTTCCGGTAGTCAATA 3’	
	5’ TGAACTGCTGGGGCATATCCATCCCCGGCTGATCATTGCCGACT	
	ACGGCACGTAAGAGGTTCCAACT 3’	

3.1.2 The DNA amplification by polymerase chain reaction

The DNA amplification was accomplished by polymerase chain reaction (PCR) using 10x PCR master mix solution (Thermo Scientific). The PCR reaction was performed in automated Flexcyler PCR machine (Analytikjena, Germany), with working volume of 50 µL that constitutes of 25 µL of master mix (10x of PCR buffer, 2 mM of dNTP mix, and 1.25 U Dream Taq DNA Polymerase), 10 mM of each primer (forward and reverse primers), 100 ng of either plasmid or chromosomal DNA template and sterile distilled water in a final volume of 50 µL. The PCR condition is shown in Table 3.2.

Table 3.2 PCR parameters for the amplification of specific genes.

PCR profile to amplify gene				
Step	**Period**	**Temperature (°C)**	**Time**	**Number of cycles**
I	Pre-denaturing	95	5 min	1
	Denaturing	95	30 s	
II	Annealing	T_m-5	30 s	30
	Extension	72	3 min	
III	Extra-extension	72	5 min	1

3.1.3 Agarose gel electrophoresis of DNA

After the amplification reaction was completed, the PCR mixture was examined on 0.8% (w/v) agarose gel electrophoresis to determine the size of DNA fragments or PCR products. The 0.8% (w/v) agarose powder was dissolved homogeneously of the gel solution through boiling temperature with 0.5x TBE buffer (89 mM Tris-HC, 89 mM boric acid and 25 mM EDTA pH 8.0), and red safe nucleic acid staining solution for detecting DNA sample in agarose gels. Before loading the DNA sample into the wells of the solidified gel, it was mixed with 1 L of loading dye. The electrophoresis was performed at a constant voltage, 100 V, for 30 min. The solidified gel was examined under UV light and photographed by a gel documentation system to determine the size of the DNA sample.

3.1.4 PCR purification and Gel extraction

A PCR purification kit was used to remove impurities from PCR products before DNA ligation or gel extraction (Thermo scientific). One volume of PCR product and one volume of binding buffer were combined. The PCR mixture was transferred to the Gene JET purification column and centrifuged at 13,500 rpm for 1 min. A flow-through was removed. The Gene JET purification column was then filled with 700 µL of wash buffer and centrifuged at 13,500 rpm for 1 min. The flow-through was removed. The column was centrifuged again for cleaning. Finally, 30 µL of elution buffer were added and incubated for 10 min at room temperature before centrifuging at 13,500 rpm for 5 min. The purified DNA was kept at -20 °C. The PCR products were excised and purified from 0.8% agarose gel using the gel extraction kit's reagents and protocol after detecting the expected size of the DNA fragment (Thermo scientific). The DNA fragment was extracted from the gel slice and mixed with Binding Buffer in a 1:1 ratio (100 µL of Binding Buffer for every 100 mg of agarose gel). The gel mixture was incubated at 65 °C for 10 min to completely dissolve. After loading the solubilized gel solution into the Gene JET purification column, it was centrifuged at 13,500 rpm for 1 min. A flow-through was eliminated. To ensure thorough cleaning, the column was centrifuged again for 5 min at 13,500 rpm. Finally, 30 µL of elution buffer were added and incubated for 10 min at room temperature before centrifuging at 13,500 rpm for 5 min. The purified DNA was kept at -20 °C.

3.1.5 Preparation of *K. oxytoca* KMS006 competent cell

A single colony of *K. oxytoca* KMS006 was inoculated into 10 mL of LB broth and was incubated at 37 °C and at 200 rpm shaking until reaching the OD_{550} nm in the range of 0.3-0.5. The culture was centrifuged at 4,000 rpm, 4 °C for 10 min. The cell pellet was re-suspended and was washed with 20 mL of ice-cold de-ionized water for 2 times, and with 2 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 for used in transformation.

3.1.6 Transformation of *K. oxytoca* KMS006 by electroporation

Twenty microliters of chromosomal DNA or plasmid and 180 µL of competent cells were mixed and transferring to an ice-cold 0.4 cm electroporation cuvette. The cuvette was incubated on ice for 5 min. After incubating the mixture, the cells were pulsed by electroporator with the condition of 2500 V, pulse length 5 ms, and EcoR2 procedure. Immediately, 800 µL of ice-cold LB broth were added to the cuvette. The solution was transferred to a sterile 50 mL tube and incubated under the condition of 37 °C, 200 rpm and 2 h. Then, 100 µL of transformed cells were spread on LB agar plates containing appropriated antibiotics and incubated at 37 °C overnight. In case of pLOI3420 (helper vector) transformation, the cell was incubated at 30 °C overnight.

3.1.7 Deletion of *budAB* gene in *K. oxytoca* KMS006

The previous method published by Jantama et al. (2008a) was used to construct all plasmids and engineering strains. In this work, a previously modified *K. oxytoca* KMS006 strain by Sangproo et al. (2012) was utilized to develop a succinate producer. Clone Manager Program was used to design primers for gene deletion, verification as shown in Table 3.1. All plasmids in the experiment were maintained in *E. coli* TOP10F, that was used as a host . *K. oxytoca* KMS006 chromosomal *budAB* gene and its neighbour nucleotides were amplified using *budAB*-up/down primers set and cloned into PCR 2.1 TOPO plasmid (DNA linear) to produce the plasmid named pKC1001. Using *budAB*-IO, the plasmid pKC002 was inside-out amplified to remove the central part of the *budAB* gene. The flanking PCR product of the PCR 2.1 TOPO backbone comprises short *budAB*' and *budAB"* sequences on both sides. The obtained fragment was utilized to build two plasmids. To construct the first plasmid, flanking PCR product and DNA cassette containing a chloramphenicol resistance gene (*cat*) and a levansucrase gene (*sacB*) from *K. oxytoca* KMS005 gDNA were combined using *bud-cat*-F/*cat-bud*-F set, as described in Wong & Jantama, (2022). The resulting combination plasmid was designed namedd as pKC002.1 (‘*budAB-cat-sacB-budAB*”). While the flanking PCR product was kinase treated and self-ligated to produce pKC002.2 (‘*budAB-budAB*”). To remove the chromosomal *budAB* gene, fragments of ‘*budAB'-cat-sacB-budAB*” and ‘*budAB-budAB*” were amplified from pKC002.1 and pKC002.2 plasmids,

respectively, using *budAB*-up/down primers. Before conducting DNA transformation to induce homologous recombination by electroporation, a plasmid pLOI3420 was used as a helper plasmid and transferred into *K. oxytoca* KMS006. *K. oxytoca* KMS006 carrying pLOI3420 was grown on LB broth containing 2% arabinose at 30 °C, 200 rpm, and overnight to stimulate the red recombinase expression. The initial recombination replaced a part of the *budAB* gene in KMS006 with pLOI3420 with '*budAB-cat-sacB-budAB*" fragments. The chloramphenicol-resistant clone was known as *K. oxytoca* KC001. The *cat-sacB* cassette in the *K. oxytoca* KC001 strain carrying pLOI3420 was replaced with native sequences ('*budAB-budAB*"), omitting the region of deletion without antibiotic resistance genes on the *K. oxytoca* KC001 chromosome. The recombinants were evaluated for resistance loss on LB agar plates with suitable antibiotics such as 50 g/mL kanamycin, apramycin, and chloramphenicol. The construction of strain was further confirmed by PCR analysis and analysis of fermentation products. A clone without antibiotics' resistance gene was designed *K. oxytoca* KC002.

3.1.8 Deletion of *pflB* gene in *K. oxytoca* KC002

The *K. oxytoca* KC002 was used as a host strain to delete the *pflB* gene. The chromosomal *pflB* gene was deleted in the same manner as previously *budAB* gene deletion. The linear DNA fragments for integration steps I ("*pflB'-cat-sacB-pflB''*) and II ("*pflB'-pflB''*) were constructed from modifying *K. oxytoca* KIS003 and *K. oxytoca* KIS004, respectively strains (In et al., 2020). Both linear DNA fragments were amplified by using *pflB*-up/down primers set. To delete *pflB* gene, "*pflB'-cat-sacB-pflB''* fragments were transformed into *K. oxytoca* KC002 carrying pLOI3420, the chloramphenicol-resistant clone was known as the *K. oxytoca* KC003. The *cat-sacB* cassette in the *K. oxytoca* KC003 carrying pLOI3420 was replaced with native sequences ('*pflB-pflB*"), the accomplished clones were designated as *K. oxytoca* KC004.

3.1.9 Deletion of *tdcD* gene in *K. oxytoca* KC004-TF160

The *K. oxytoca* KC004-TF160 was used as a host strain to delete the *tdcD* gene. The plasmids and strains were constructed in the same manner as previously described in *budAB* gene deletion. *K. oxytoca* KC004-TF160 chromosomal *tdcD* gene

and its neighbour nucleotides were amplified using *tdcD*-up/down primers set and cloned into PCR 2.1 TOPO plasmid (DNA linear) to produce the plasmid named pKC006. The plasmid pKC006 was inside-out amplified to remove the central part of the *tdcD* gene by using *tdcD*-IO. The obtained fragment was utilized to build two plasmids, producing pKC006.1 by using *tdcD*-Hi-up/down (‘*tdcD-cat-sacB-tdcD*”) and pKC006.2 (‘*tdcD-tdcD*”) by using the same method as previously described in the *budAB* plasmids construction. To remove the chromosomal *tdcD* gene, inserts ‘*tdcD'-cat-sacB-tdcD*” and ‘*tdcD-tdcD*” were amplified from pKC006.1 and pKC006.2 plasmids, respectively, using *tdcD*-up/down primers. To delete *tdcD* gene, the initial recombination replaced a part of the *tdcD* gene in *K. oxytoca* KC004-TF160 carrying pLOI3420 with a ‘*tdcD-cat-sacB-tdcD*” fragment. The chloramphenicol-resistant clone was known as the *K. oxytoca* KC005. The *cat-sacB* cassette in the *K. oxytoca* KC005, carrying pLOI3420 was replaced with native sequences (‘*tdcD-tdcD*”). The accomplished clones were designated as *K. oxytoca* KC006.

3.1.10 Deletion of *pmd* genes in *K. oxytoca* KC006

The *K. oxytoca* KC006 was used as a host strain to delete the *pmd* gene. The plasmids and strains were constructed in the same manner as previously *budAB* gene deletion. *K. oxytoca* KC006 chromosomal *pmd* gene and its neighbour nucleotides were amplified using pmd-up/down primers set and cloned into PCR 2.1 TOPO plasmid (DNA linear) to produce the plasmid named pKP002. The plasmid pKP002 was inside-out amplified to remove the central part of the *pmd* gene by using *pmd*-IO primers. The obtained fragment was utilized to build two plasmids, producing pKP002.1 by using *pmd*-Hi-up/down (‘*pmd-cat-sacB-pmd*”) and pKP002.2 (‘*pmd-pmd*”) by using the same method as previously described in the *budAB* plasmids construction. To remove the chromosomal *pmd* gene, inserts ‘*pmd'-cat-sacB-pmd*” and ‘*pmd-pmd*” were amplified from pKP002.1 and pKP00.2 plasmids, respectively, using *pmd*-up/down primers. To delete *pmd* gene, the initial recombination replaced a part of the *pmd* gene in *K. oxytoca* KC006 carrying pLOI3420 with a ‘*pmd-cat-sacB-pmd*” fragment. the chloramphenicol-resistant clone was known as the *K. oxytoca* KP001. The *cat-sacB* cassette in the *K. oxytoca* KP001 carrying pLOI3420 was replaced with native sequences (‘*pmd-pmd*”). The accomplished clones were designated as *K. oxytoca* KP002.

3.2 Metabolic evolution

3.2.1 Metabolic adaptation of *K. oxytoca* KC004

The growth rate and succinic acid production of the *K. oxytoca* KC004 were enhanced simultaneously by metabolic evolution using a method previously described by Jantama et al. (2008). Metabolic evolution was performed in a 500 mL mini controlled-pH fermenter, and was performed at 37 °C, 200 rpm with a working volume of 350 mL AM1 mineral salts medium containing 50-100 g/L of glucose, 100 mM $KHCO_3$ and, 20 mM CH_3COONa under anaerobic conditions. Sodium acetate was used as a stimulator of the auxotrophic mutant by adding early transferring cells and gradually withdraw from the medium after sustainable growth of the cells. During the fermentation, a mixed solution of 6 M KOH and 3 M K_2CO_3 was automatically added to the culture to maintain the pH at 7.0 and supply CO_2. The culture was initially inoculated at OD_{550} of 0.01 and transferred into newly fresh AM1 medium when OD_{550} of previous cultures was equivalent to OD_{550} of 1.0. A series of cultivations were carried out until no further improvement in growth rate, substrate consumption, or succinic acid production could be achieved. After evolution was completed, the culture broth was spread on LB agar. Colonies of interest were chosen and screened for the engineered chromosomal conformation using PCR analysis. A confirmed clone was renamed *K. oxytoca* KC004-TF160.

3.2.2 Metabolic adaptation of *K. oxytoca* KP001

The growth rate and succinic acid production of the *K. oxytoca* KP001 were improved simultaneously in three steps of metabolic evolution. Firstly, the metabolic evolution was performed using a 250 mL flask with a working volume of 100 mL LB medium containing 20 g/L of glucose, 100 mM $KHCO_3$ and, 20 mM CH_3COONa under anaerobic conditions. The cells were cultured at 37 °C, 200 rpm (using magnetic stirring bar) without uncontrol pH. In second step, the adaptative strain was transferred to evolve with AM1 medium supplemented with 2 g/L of yeast extract and 20 g/L of glucose under the same conditions in first step. Finally, the adaptative strain was evolved in a 500 mL mini controlled-pH fermenter with a working volume of 350 mL AM1 mineral salts medium containing 50-100 g/L of glucose, 2 g/L of yeast- extract, 100 mM $KHCO_3$ and, 20 mM CH_3COONa under anaerobic conditions. Sodium acetate

was gradually withdrawn from the medium after sustainable growth of the cells. During the fermentation, a mixed solution of 6 M KOH and 3 M K_2CO_3 was automatically added to the culture to maintain the pH at 7.0 and supply CO_2. The culture was initially inoculated at OD_{550} of 0.1 and transferred into newly fresh AM1 medium when OD_{550} of previous cultures was equivalent to OD_{550} of 1.0. A series of cultivations were carried out until no further improvement in growth rate, substrate consumption, or succinic acid production could be achieved. After evolution was completed, the culture broth was spread on LB agar. Colonies of interest were chosen and screened for the engineered chromosomal conformation using PCR analysis. A confirmed clone was renamed *K. oxytoca* KP001-TF60.

3.3 Operation and conditions of fermentation

3.3.1 Fermentation medium

Alfredo Mertinez medium version 1 (AM1) (Martinez et al., 2007) was utilized as a fermentation medium for succinic acid production (component shown in Table 3.3). The AM1 medium was supplemented with 50-100 g/L of carbon source and 100 mM $KHCO_3$, while for KP001-TF60 strain cultivation, the AM1 medium supplemented with 2 g/L of yeast extract was applied. Glucose was used as a major carbon source. Sucrose, maltose, fructose, lactose, xylose, glycerol, and sugarcane molasse were used to investigate the capacity of sugars consumption for succinic acid production. Sugarcane molasse containing a glucose content of 19.87% (w/v), sucrose content of 17.70% (w/v) and fructose content of 18.67% (w/v), was obtained from Mitr Phol, Thailand. LB broth containing 2 g/L of carbon source was used to prepare the seed culture.

3.3.2 Batch fermentation in mini controlled-pH fermenter

A 500 mL mini controlled-pH fermenter with a working volume of 350 mL of AM1 broth with glucose and 100 mM $KHCO_3$ was used to investigate the capability of developed strain to consume carbon sources for succinic acid production. Succinate fermentation was carried out under anaerobic conditions at 37 °C, pH 7.0, and 200 rpm. During fermentation, a mixed solution of 6 M KOH and 3 M K_2CO_3 with 1:4 ratio was automatically added to the culture to maintain pH and supply CO_2. The end of succinic

acid fermentation was extended to 144 h, and the experiments were performed in duplicate. For seed culture, all strains were cultured on LB broth for overnight, and was transferred into AM1 medium containing 20 g/L of corresponding substrates for 24 h at 37 °C, 200 rpm.

Table 3.3 Composition of AM1 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	**47.39**
Total K	**1.00**
$MgSO_4$ $7H_2O$	1.50
Betaine-HCl[a]	1.00
Trace Elements	**Concentration (µmol/l)**
$FeCl_3$ $6H_2O$	8.88
$CoCl_2$ $6H_2O$	1.26
$CuCl_2$ $2H_2O$	0.88
$ZnCl_2$	2.20
Na_2MoO_4 $2H_2O$	1.24
H_3BO_3	1.21
$MnCl_2$ $4H_2O$	2.50
Total salts	4.1 g/l

[a]KOH was used to neutralize betaine-HCl stock.

[b]Trace metal stock (1000X) was prepared in 120 mM HCl.

3.3.3 Batch fermentation in 5 L bioreactor

A 5 L bioreactor with a 2.5 L working volume of AM1 broth supplemented with 100 g/L of glucose and 100 mM $KHCO_3$ was used for scale-up batch fermentation. Parameters of fermentation such as pH control, temperature, and agitation were performed with mini controlled-pH fermenter experiment for succinate production in

5 L bioreactor. To obtain high yield of succinic acid, initial OD_{550} (0.01, 0.1, and 0.3) and ratio of mixed solution (1:4 and 1:6 of 6 M KOH and 3 M K_2CO_3) were optimized to find the best condition. The end of succinic acid fermentation was extended to 144 h, and the experiments were performed in duplicate. Seed culture was carried out in the same manner as batch fermentation in the mini controlled-pH fermenter. After incubation, cells were harvested by centrifugation at 4,000 rpm for 10 min and resuspended with sterilized water. The cells solution was used in 5 L batch fermentation.

3.3.4 Batch fermentation in 5 L bioreactor of sugarcane molasse

Succinic acid fermentation by using *K. oxytoca* KC004-TF160 was carried out in a 5 L bioreactor with a 2.5 L working volume of AM1 broth supplemented with sugarcane molasse and 100 mM $KHCO_3$. The best parameters obtained from the batch fermentation in a 5 L bioreactor were used. Unpretreated sugarcane molasse was used and investigated the different initial concentrations (100 g/L, 150 g/L and 200 g/L) for succinic acid production. The AM1 medium buffering (content (%v/v) of 0, 25, 50) was optimized to increase succinate production efficiency. The colony-forming units per milliliter method (CFU/mL) was used to measure the number of viable KC004-TF160 cells during succinic acid fermentation. Numbers of *K. oxytoca* KC004-TF160 cells were counted on LB agar every 12 h during fermentations. The agar plate was incubated at 37 °C for 24 h.

3.3.5 Statistical analytical

The fermentation broths were collected every 12 or 24 hours for 144 hours of incubation to quantify cell mass, organic acids, and sugar. Cell growth was determined by measuring the optical density using a Bausch&Lomb Spectronic 70 spectrophotometer at OD_{550} nm and converted to biomass as cell dry weight (1 OD_{550} = 0.333 g CDW/L biomass). High-performance liquid chromatography (HPLC) with an Aminex 23 HPX-87H column (7.8300 mm, BIO-RAD, UAS) and refractive index detector was used to determine organic acid and sugars throughout fermentation (RI-150, Thermo Spectra System, USA). The mobile phase in the HPLC system was sulfuric acid (4 mM) at a flow rate of 0.4 mL/min. The fermentation culture was centrifuged to separate the cells and supernatant. To prepare the sample, the supernatant liquid was diluted with 20 mM H_2SO_4 and filtered through a 0.22 m nylon filter (In et al., 2020).

3.4 Analysis of genes expression level

3.4.1 RNA isolation

To isolate RNA, total mRNA was extracted using Gene JET RNA purification kit (Thermo Scientific). Total RNA was extracted from cells that were cultivated in succinate fermentation under anaerobic conditions for 24 h. The cells were harvested by centrifuged at 4000 rpm for 5 min at 4 ℃. The pellet was resuspended in 100 µL of freshly prepared TE buffer supplemented with 0.4 mg/mL lysozyme (final concentration). The resuspended cells were mixed by inverting and incubated at 25 ℃ for 5 min. Then, beads and 300 µL of lysis buffer supplement with 2 M of DTT were added and mixed thoroughly by vortexing for 15 min. The 180 µL of ethanol were added into the lysis solution and mixed by pipetting. Seven hundred microliters of lysate were transferred to the Gene JET RNA purification column placed in a collection tube. The column was centrifuged at 12,000 rpm for 60 s then the flow-through was discarded. The purification column was used to repeat the washing step with 600 µL of wash buffer 2. To complete RNA isolation, 100 µL of water nuclease-free was added and centrifuged at 12,000 rpm for 60 s. The purified RNA was used for downstream application or stored at -80 ℃.

3.4.2 Genomic DNA removal

DNase digestion was used to remove all genomic DNA using the DNase I (Thermo Scientific). The 1 µg of RNA, 1 µL of 10X reaction buffer with $MgCl_2$ and 1 µL of DNase I (1 u) were mixed, and the reaction volume was adjusted to 10 µL using water, nuclease-free. The reaction mixture was incubated at 37 ℃ for 30 min. Then, 1 µL of 50 mM EDTA was added and incubate at 65 ℃ for 10 min to inactivate the DNase I. Finally, the prepared RNA was repurified by protocol of RNA isolation using Gene JET RNA purification kit. The RNA purify was performed by starting the lysis step. The purified RNA was used for downstream application or stored at -80 ℃.

3.4.3 First strand cDNA synthesis

The first-strand cDNA was synthesized from total mRNA by using a reverse aid first stand cDNA synthesis kit (Thermo Scientific). Total RNA concentrations were determined by spectrophotometer (nanodrop). One hundred nanograms of total mRNA and 1 µL of random hexamer primer were mixed, and the reaction volume was

adjusted to 12 µL with nuclease-free waters. Then, 4 µL of 5x reaction buffer, 1 µL of riboLock RNase inhibitor (20 U/µL), 2 µL of 10 mM dNTP mix, and 1 µL of revert aid M-Mul V RT (200U/ µL) were added into the prepared reaction. The reaction was mixed and centrifuged briefly. The mixture was further incubated at 25 °C for 5 min, at 42 °C for 60 min respectively. The mixture was incubated at 70°C for inactivating the reaction. The reverse transcription reaction product was directly used in PCR application or stored at -20 °C.

3.4.4 Quantification of mRNA

To investigate the effect of combining metabolic engineering and metabolic adaptations in *K. oxytoca* KC004-TF160, we analysed transcription levels to compare intracellular metabolic flow between *K. oxytoca* KMS006, *K. oxytoca* KC004-TF160, *K. oxytoca* KC006, and *K. oxytoca* KP001-TF60. The transcription levels of *pck*, *mdh*, *fum*, and *frd* genes in the reductive branch of the TCA cycle, *gltA*, *acnAB*, and *icd* genes in oxidative TCA cycle, and *pdh*, *poxB*, *tdcD*, and *tdcE* related genes in acetate accumulation were analysed by using RT-qPCR. A 2X SYBR® Green PCR kit was used with a thermal cycler for RT-qPCR (Thermo Scientific). Primers as listed in Table 3.4. were used at final concentrations of 0.2 µM, 100 ng of cDNA, and 2X SYBR® Green PCR Master Mix were prepared in each 10 µL reaction. The threshold cycles for each sample were calculated based on fluorescence data using QuantStudio design & analysis software (Thermo Scientific). For relative quantification, the data were normalized to the *chaA* (Na-K/H+ antiporter gene) transcript band. The 2-ΔΔCt strategy was used to analyze the statistic as follow in equation (1) and quantified as band fluorescence intensity was quantified on a 2% agarose gel by dissolving with 0.5x TBE buffer that containing 40 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA pH 8.3. Band intensities were normalized to the *chaA* (Na-K/H+ antiporter gene) transcript band for relative quantification (Jarboe et al., 2008; Xiao et al., 2011; Zuo et al., 2014).

$2\text{-}\Delta\Delta Ct$ = Ct (target genes of K_C, K_{M1}, K_{M2} or K_{M3}) – Ct (*chaA* gene of K_C, K_{M1}, K_{M2} or K_{M3}) (1)

where, K_C: *K. oxytoca* M5A1 used serve as control.

K_{M1}: *K. oxytoca* KMS006

K_{M2}: *K. oxytoca* KC004-TF160

K_{M3}: *K. oxytoca* KP001-TF60

Table 3.4 Primers for analysis of the expression levels of all genes involving in succinic acid used in this study.

Genes	Primer Sequence	Product size (bp)	References
pykA	5' GCTGGCTGAGAAAGACAAAC 3'	257	This study
	5' CTTCAACCGGGATTTCAACG 3'		
ppc	5' GCGAAATGAACCGCATCGAG 3'	135	This study
	5' CGATAATCGCCGCATGACAG 3'		
pck	5' TGATCGGCGATGACGAGCAC 3'	189	This study
	5' TGCTGCCATCGGCGTAATCC 3'		
mdh	5' TGATTACCAACCCGGTGAAC 3'	173	This study
	5' CACCGGAACCTCAACATCTG 3'		
fum	5' GAAGTTAGAGCCCGGGATCG 3'	157	This study
	5' CATCATGCCGGGCCACATTC 3'		
frd	5' GCATCAGCGTCTGGACGAAG 3'	198	This study
	5' TCTCAGCCATTCGTCGTCTC 3'		
tdcD	5' CTACAGCGAGCTGGGCATTC 3'	193	This study
	5' CGGCGTAAAGCCCATTGAGG 3'		
tdcE	5' GCTGGAACTGATTGCTGAAC 3'	196	This study
	5' ATCGCCATTGACCAGATACC 3'		
acs	5' CACAGCTACCTGCTGTATGG 3'	181	This study
	5' CGATAGCCTTATCGCCTTCC 3'		
poxB	5' CCTGCGTCATCTGGAAATCG 3'	126	This study
	5' AGTAGCTGAAGCAGCGTGTC 3'		
pdh	5' AAGGCATCGCGGCTTATTAC 3'	150	This study
	5' TGACGGCGATTTGATACTGG 3'		
gltA	5' TATCCGCGCAACGATCTTTC 3'	104	This study
	5' TTCATCGCCTGCTCAATCAC 3'		
acnA	5' GTGGTGATTGCCGCCATTAC 3'	173	This study
	5' TTTGCGTGGGCCAGATAGTC 3'		
icd	5' ATTGCGGTATCGGCATCAAG 3'	109	This study
	5' TGCACCAGAGTCAGAGAATC 3'		
chaA	5' TGCTGGTTGCGCTGATCTCC 3'	144	This study
	5' TGCTATGGGCCGACGGTTTG 3'		

3.5 Whole-genome resequencing

The Gene JET Genomic DNA Purification Kit was used to prepare one microgram of KP001-TF60 genomic DNA according to the protocol for gram-negative bacteria genomic DNA purification (Thermo Scientific, USA). For genome analysis of *K. oxytoca* KP001-TF60, the original sequencing data was sequenced using Illumina NovaSeq 6000 sequencing technology and sequence reads by calling with the CASAVA software at Novogene AIT genomics, Singapore. To control the quality of the sequencing data, the Fastp software v0.20.0 was used. The effective sequencing data were aligned with the reference sequences through BWA software v0.7.17, and the mapping rate and coverage were performed by Sambamba software v0.67 and Samtools software v1. 8 (Cock et al. 2010). The duplicates were removed by Picard (Cock et al., 2010). GATK (v4.0.5.1), Breakdancer (v1.4.4), and CNVnator (v.0.3) softwares were used to detect genome variation in the KP001-TF60 strain, such as single nucleotide polymorphism (SNP), insertion or deletion (In Del), structural variants (SVs), and copy-number variation (CNV), respectively (Dwpristo et al. 2011; Chen et al. 2009; Abyzov et al. 2011). ANNOVAR software (v2015Mar22) was used to annotate all variation annotations (Wang et al. 2010). The whole genome sequences of *K. oxytoca* M5Al (*K. michiganensis*: No. NZ AP022547.1) from the National Center for Biotechnology Information (NCBI) were used as the reference for a comparative genomic and variative analysis. The nucleotide sequence variation in the *K. oxytoca* KP001-TF60 was investigated by searching into all important gene functions such as glucose metabolism, the reductive branch of the TCA cycle, the oxidative branch of the TCA cycle, and by-product formation. The online ClustalW2 program was used to align the nucleotide sequences of each gene in order to find point mutations (European Molecular Biology Laboratory, 2023.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Eliminating by-products and increasing succinic acid yield of *K. oxytoca* KMS006

4.1.1 Deletion of *budAB* gene in *K. oxytoca* KMS006

Plasmids pKC002.1 and pKC002.2 were used as templates to amplify linear DNA fragments of *budAB'-cat-sacB-budAB"* and *budAB'-budAB"* by using the *budAB*-up/down primers set to delete *budAB* gene, respectively (Fig 4.1).

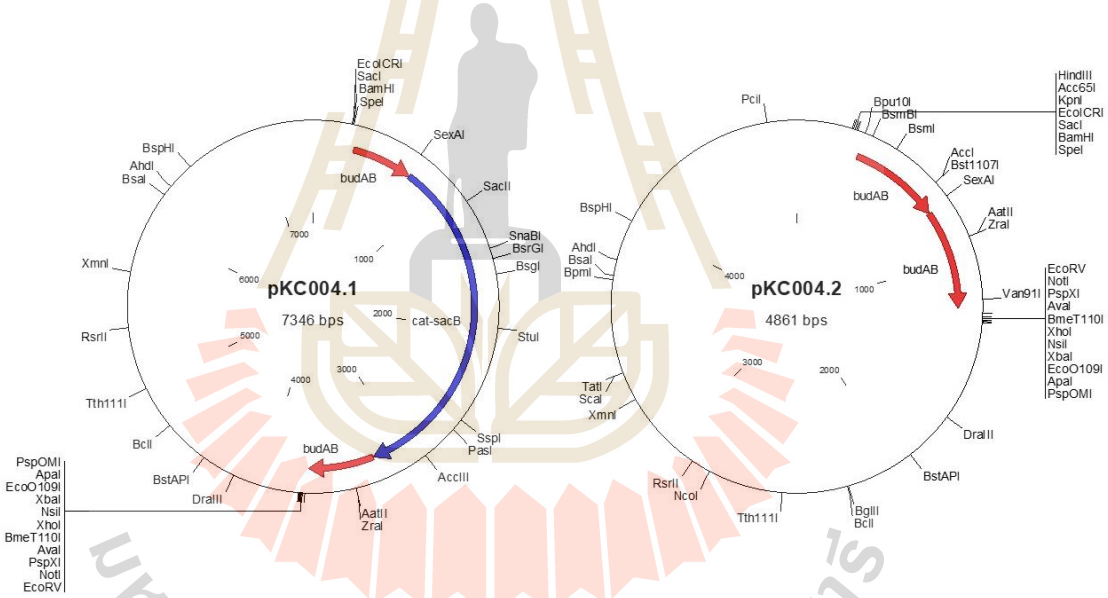


Figure 4.1 Plasmids pKC002.1 and pKC002.2 used for *budAB* gene deletion.

The *budAB'-cat-sacB-budAB"* fragment was electroporated into *K. oxytoca* KMS006 containing pLOI3420 (*Red* recombinase). The recombinant strains were selected on LB plates containing chloramphenicol (50 mg/L) and confirmed by PCR analysis using *budAB*-up/down primers set, resulting in a PCR product with the expected size of 3,504 bps (Fig 4.2). The clone containing chloramphenicol resistant gene was designated as *K. oxytoca* KC001.

To remove antibiotic resistance gene, the *cat-sacB* cassette in *K. oxytoca* KC001 strain containing pLOI3420 was replaced with native sequences (*budAB'-budAB"*) using the same method as in *budAB* deletion. The obtained clone was evaluated for the absence of chloramphenicol resistance gene by selecting on LB agar containing 150 g/L sucrose selected for loss of apramycin, ampicillin, kanamycin, chloramphenicol. Then, the construction of strain was clarified by PCR analysis using *budAB*-up/down primers set. The result showed that the PCR product was in size of 930 bps as expected. A clone without antibiotics resistance gene was designed as *K. oxytoca* KC002.

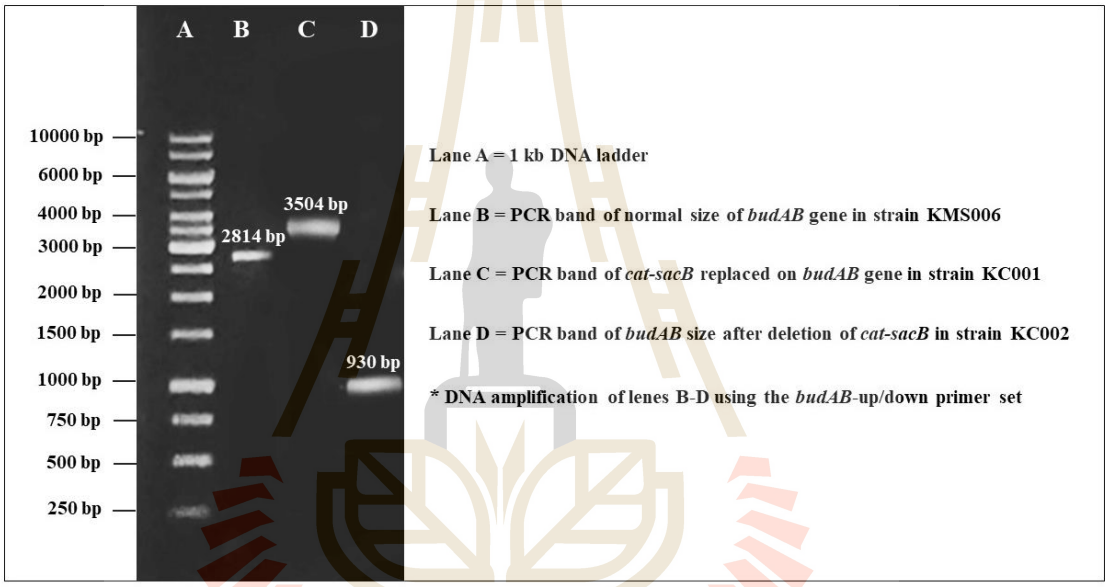


Figure 4.2 Gel electrophoresis confirmed the construction of KC001 and KC002 strains.

4.1.2 Effect of *budAB* gene deletion in *K. oxytoca* KMS006

In this study, we want to use the previously designed *K. oxytoca* KMS006 (*adhE pta-ackA ldhA*) strain (Sangproo et al. 2012) as a strong bio synthesizer for succinate production via a reductive branch of the TCA cycle, whose route produces the most succinate. Its process occurs in anaerobic environments and generates ATP synthesis and redox balance via substrate-level phosphorylation (SLP) and the creation or excretion of various fermentation products (Zhao et al., 2017). Meanwhile, their pathway produces several fermentation products in bacteria, as shown in figure 4.3 by the fermentation profiles of *K. oxytoca* M5A1 and *K. oxytoca* KMS006. Even though, the deletion of the *adhE*, *pta-ackA*, and *ldhA* genes in the KMS006 strain increased

succinate yield (0.08 g/g glucose) by 2.7-fold compared to the wild-type M5A1 strain in AM1 medium containing 50 g/L glucose (Table 4.1). The *K. oxytoca* KMS006, on the other hand, generated 2,3-butanediol (2.38 g/L) and acetate (11.61 g/L) as co-products for metabolic energy during anaerobic fermentation. Most carbon sources and NADH may lean toward the 2,3-butanediol route because raising the glucose level by 100 g/L could considerably increase the 2,3-butanediol production (8.66 g/L) by 3.6-fold while the acetate production did not improve (Table 4.1). The results indicated that the 2,3-butanediol pathway may have an influence on succinate production by producing byproducts, as 2 g of glucose can only provide 1 g of 2,3-butanediol with significant concomitant oxidation of NADH to NAD+ (Biswas et al. 2012); in contrast, succinate is formed by 1.12 g/g glucose in a reductive branch of the TCA cycle. However, the succinate route requires two moles of NADH, but glycolysis can only produce two moles of NADH from one mole of glucose. Additionally, Zhu et al. (2014) discovered that a deficiency of NADH is one of the constraints to succinate synthesis. *K. oxytoca* produces 2,3-butanediol naturally by converting pyruvate to alpha-acetolactate, acetoin, and, consequently, 2,3-butanediol via three important enzymes: *budB*, *budA*, and *budC* (Yang et al., 2014; Qi et al., 2014). Thus, the activity of the major enzymes the *budB* and *budA* genes were disrupted, causing overflowing of carbon and NADH into succinate production, and the resulting strain was designated *K. oxytoca* KC002. As expected, no 2,3-butanediol was detected during fermentation, but acetate levels increased significantly from 11.61 g/L in the *K. oxytoca* KMS006 to 18.67 g/L in the *K. oxytoca* KC002. In contrast, the maximum biomass was lowered by 43.7% and 35.11%, respectively, when compared to those of the parental and *K. oxytoca* KMS006. Simultaneously, as indicated in Table 4.1, succinate production in terms of concentration (2.10 g/L), yield (0.08 g/g), and productivity (0.02 g/L/h) did not improve when compared to the *K. oxytoca* KMS006 in AM1 medium containing 50 g/L glucose. These results suggest that knocking out of *budAB* genes in the *K. oxytoca* KC002 inhibited 2,3-butanediol synthesis made it possible to improve the yield of succinate but also impaired cell growth, which decreased succinate production rate. Decreasing growth may result in lack of NADH re-oxidation and an imbalanced metabolism. As previously mentioned, the synthesis of 2,3-butanediol has important physiological implications to cell bacteria, such as controlling the intracellular NADH/NAD+ ratio,

preventing intracellular acidification, and conserving energy and carbon for microbial growth (Ji et al. 2011). The same phenomenon was previously reported for disruption of the 2,3-butanediol pathway during the construction of *K. pneumoniae* biocatalysts to produce 1,3-propanediol, which revealed that mutation of the *budA*, *budB*, or *budC* gene not only decreased carbon flux to 2,3-butanediol and cell growth, but also increased the ratio of NADH to NAD+ by decreasing NADH consumption due to 2,3-butanediol production (Zhang et al. 2009; Zhous et al. 2019; Guo et al. 2013).

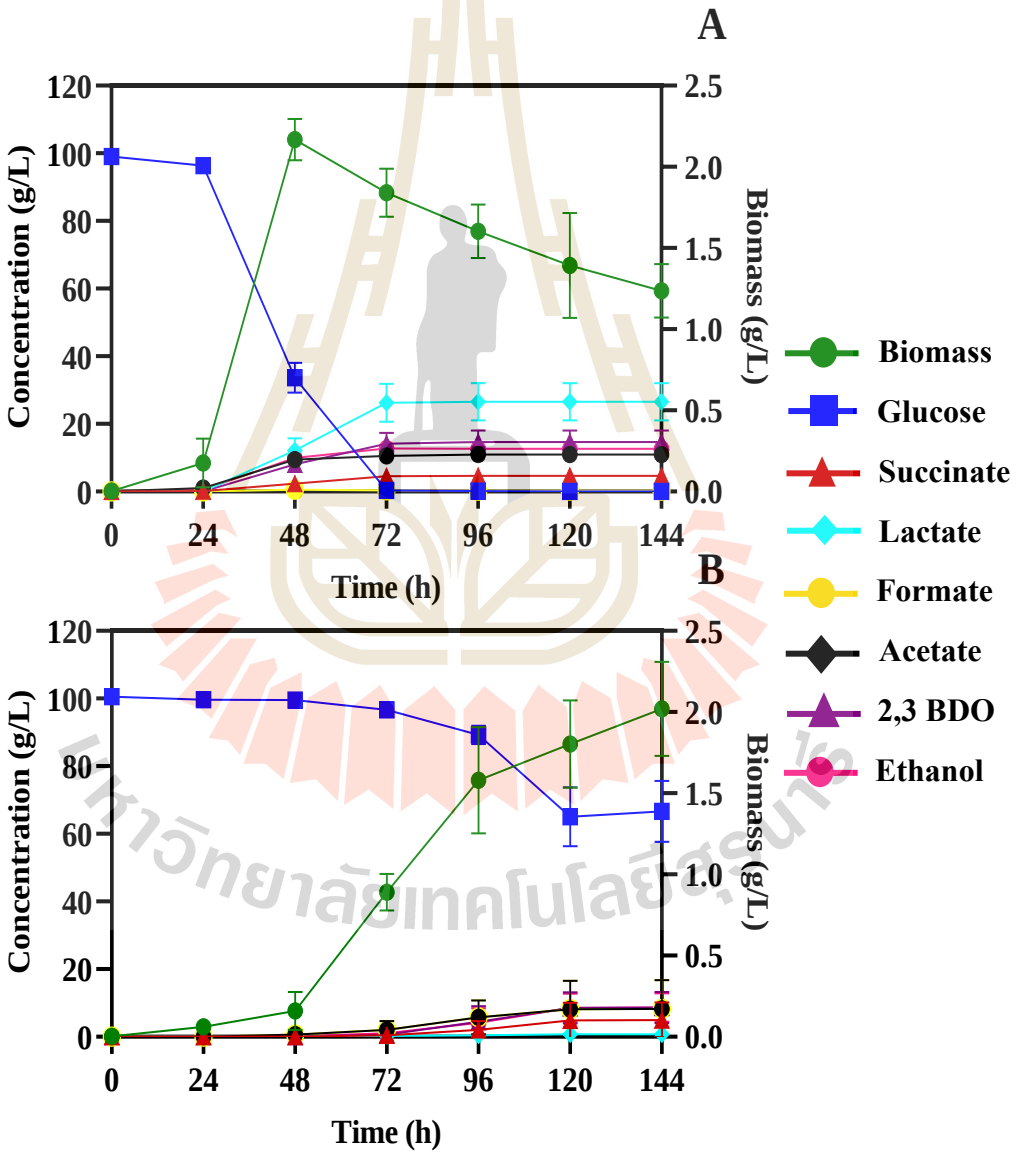


Figure 4.3 Fermentation profile of *K. oxytoca* in mini controlled-pH fermenter with a working volume of 350 mL AM1 medium containing 100 g/L glucose under anaerobic conditions. (A) *K. oxytoca* M5A1, (B) *K. oxytoca* KMS006.

Table 4.1 Production profile of *K. oxytoca* M5A1, *K. oxytoca* KMS006, *K. oxytoca* KC002, and *K. oxytoca* KC004 in AM1 medium containing 50 g/L glucose.

Medium	Strains	Maximum CDW[a] (g/L)	Glucose used[b] (g/L)	Succinate			By-products		
				Concentration[c] (g/L)	Yield[d] (g/g)	Productivity[e] (g/L/h)	Acetate	BDO	Formate
50 g/L glucose	M5A1	1.51 ± 0.26	50 ± 1.02	1.47 ± 0.31	0.03 ± 0.01	0.03 ± 0.01	8.90 ± 1.25	3.44 ± 0.95	0.5 ± 0.11
	KMS006	1.31 ± 0.65	50 ± 1.98	4.81 ± 0.16	0.08 ± 0.02	0.03 ± 0.01	11.61 ± 2.12	2.38 ± 0.32	0.83 ± 0.20
	KC002	0.85 ± 0.22	27 ± 3.31	2.10 ± 0.28	0.08 ± 0.01	0.02 ± 0.00	18.67 ± 7.28	ND	0.21 ± 0.05
	KC004	0.10 ± 0.03	3.3 ± 0.97	0.15 ± 0.01	0.05 ± 0.00	ND[f]	0.52 ± 0.07	ND	ND

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 grams per liter.

b Total glucose utilization was calculated by total glucose divided by glucose remain in gram per liter.

c Concentrations of succinate were calculated as gram per liter by weight succinate divided by the total volume of solution.

d Yields of succinate were calculated as a gram of succinate divided by a gram of glucose consumed.

e Productivities of succinate were calculated as succinate concentration was produced divided by overall incubation time.

f ND-Not Detected.

4.1.3 Deletion of *pflB* genes in *K. oxytoca* KC002

DNA fragments of *pflB'-cat-sacB-pflB"* and *'pflB-pflB"* were amplified from chromosomal *pflB* gene of *K. oxytoca* KIS003 and *K. oxytoca* KIS004 by using the *pflB*-up/down primers set to delete *pflB* gene, respectively (Fig 4.4) (In et al., 2020).

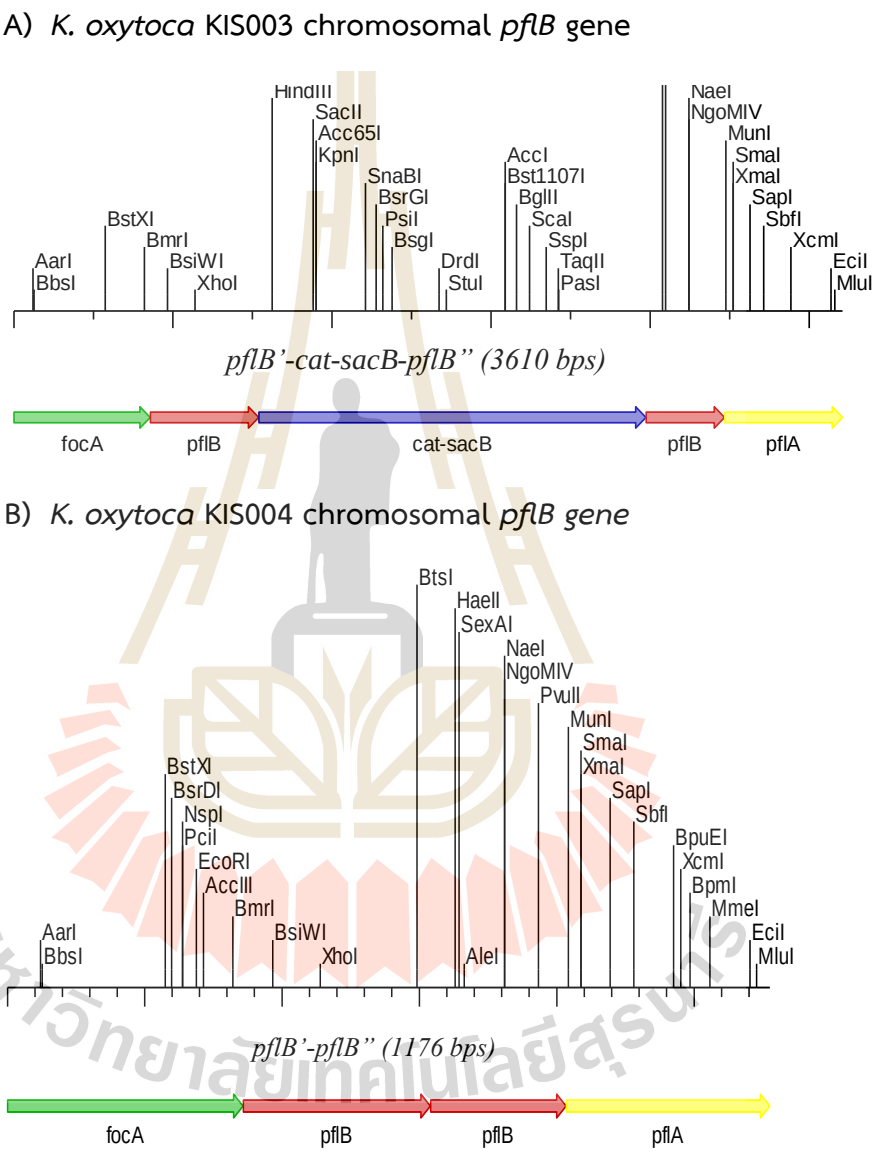


Figure 4.4 Chromosomal *pflB* gene of *K. oxytoca* KIS003 (A) and *K. oxytoca* KIS004 (B) used for *pflB* gene deletion.

The *pflB'-cat-sacB-pflB"* fragment was electroporated into *K. oxytoca* KC002 containing pLOI3420. The selections of recombinant strains were performed in a manner analogous to that used for the deletion of *budAB* gene and confirmed by PCR analysis using *pflB*-up/down primers set, resulting in a PCR product with the expected size of 3,698 bps (Fig 4.5). The clone containing chloramphenicol resistant gene was designated as *K. oxytoca* KC003.

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To remove antibiotic resistance gene, the *cat-sacB* cassette in *K. oxytoca* KC003 containing pLOI3420 was replaced with native sequences (*'pflB-pflB"*). The obtained clone was selected using the same method as in *budAB* deletion and clarified by PCR analysis using *pflB*-up/down primers set. The result showed that the PCR product was in size of 1,176 bps as expected. A clone without antibiotics resistance gene was designed *K. oxytoca* KC004.

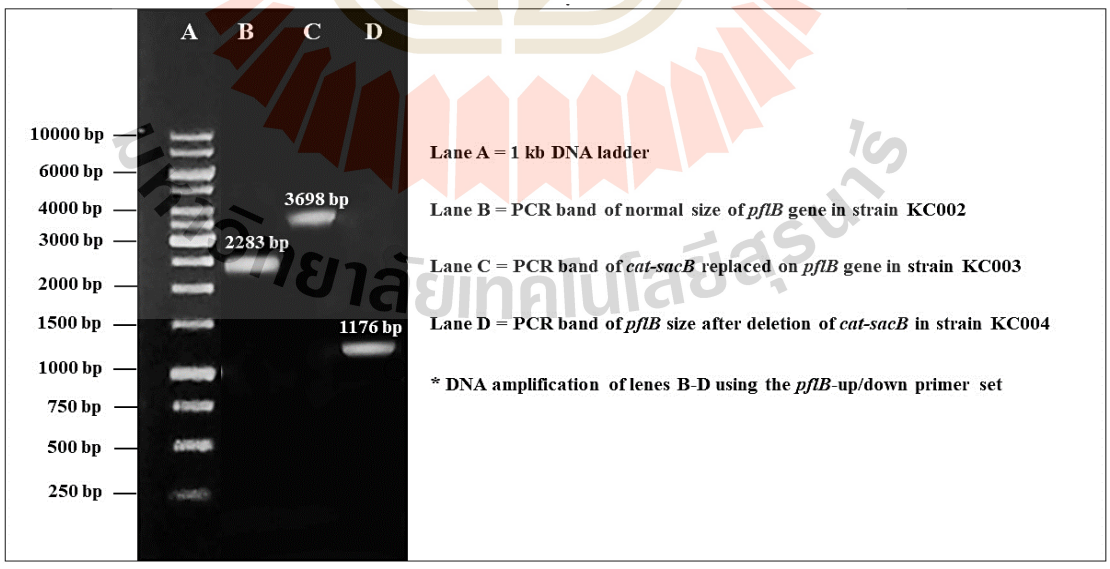

Figure 4.5 Gel electrophoresis confirmed the construction of *K. oxytoca* KC003 and *K. oxytoca* KC004.

4.1.4 Effect of *pflB* gene deletion in *K. oxytoca* KC002

Knocking out *budAB* genes in the *K. oxytoca* KC002 strain shifted the carbon flux away from the biosynthesis of 2,3-butanediol towards the production of acetate (18.67 g/L), which resulted in a primary byproduct in 50 g/L glucose fermentation that was significantly increased by 60.8%. Despite the fact that the *ackA* and *pta* genes had previously been deleted, acetate production by other enzyme activities might functionally replace *ackA* and *pta* enhancing ATP and acetate production in the *K. oxytoca* KC002. Acetate is produced from acetyl-CoA and serves as the primary product in various fermentation microorganisms; its synthesis is linked to improved ATP yield and NADH reoxidation via SLP (Rodriguez et al. 2006). Acetyl-CoA is generated as a byproduct of pyruvate catabolism together with formate by the enzymatic activity of pyruvate formate-lyase (*pflB*). Acetyl-CoA is converted to acetate or ethanol in pyruvate catabolism, whereas formate is catalyzed to CO_2 and H_2 by the enzyme activities of formate hydrogen-lyase (*hyc*) and formate dehydrogenase (*fdh*), respectively (Fig 2.5), resulting in several by-products in profiled fermentation of the *K. oxytoca* KMS006 and *K. oxytoca* KC002 (Table 4.1). In an anaerobic culture condition, inhibiting acetyl-CoA synthesis may practically enhance succinate yield. Therefore, the *pflB* gene in the *K. oxytoca* KC002 was removed to eliminate pyruvate catabolism into acetyl-CoA and formate synthesis in order to increase carbon flow toward succinate generation, and the resulting strain was designated as *K. oxytoca* KC004. As expected, eliminating the *pflB* gene resulted in reduced acetate production, with *K. oxytoca* KC004 exhibiting 95.5% and 97.2% decreases in acetate compared to the *K. oxytoca* KMS006 and *K. oxytoca* KC002, respectively. Moreover, other byproducts of pyruvate catabolism, such as formate and ethanol, were not detected during anaerobic fermentation. However, even after further disruption of the *pflB* gene in the *K. oxytoca* KC004, the yield of succinate (0.05 g/g glucose) remained slightly than that of the wild-type strain (Table 4.1). Furthermore, biomass production was reduced by more than 92.4% and 88.2%, respectively, as compared to the *K. oxytoca* KMS006 and *K. oxytoca* KC002. These results suggested that knocking out the *pflB* gene decreased acetate production while completely eliminated formate and ethanol generation during anaerobic fermentation. However, the *K. oxytoca* KC004's poor growth has been a serious impediment to using it for succinate production. The causes of the growth

defect include the lack of acetyl-CoA and a redox imbalance caused by cytosolic NADH accumulation (Kim et al., 2013; Van et al., 2004)

As suggested in previous reports, removing *pflB* gene resulted in decreased cell proliferation of the *K. oxytoca* KC004, which impacted the rate of succinate synthesis (Jantama et al., 2008a). Jung et al. (2014) demonstrated that deletion of the *pflB* gene in *K. pneumoniae* reduced acetate, ethanol, and cell mass production by 100%, 92%, and 49.2%, respectively, compared to its parental strain, while cell growth was reduced owing to inefficient NAD+ regeneration. Similarly, Lee et al., (2014) reported that the combining inactivation of the *aldA*, *ackA* and *pflB* genes nearly entirely prevented the production of lactate, acetate, and formate, resulting in a higher succinate yield of up to 1.42-fold by *M. succiniciproducens* LPK7 strain than the wild-type strain. However, cell growth was somewhat negatively affected probably due to a lack of ATP.

4.1.5 Evolutionary adaptation of *K. oxytoca* KC004 to achieve *K. oxytoca* KC004-TF160

In the interim, conventional removal of competing routes is a valuable technique for attenuating by-products and redirecting metabolic flux towards the targeted pathway (Liu et al. 2012). However, in most situations, gene deletion results in growth suppression or metabolic disruption due to an imbalance among enzymes or cofactors (Martin et al. 2003; Portnoy et al. 2010). To resolve these concerns, metabolic evolution has been carried out in order to alleviate the energy supply problem and enhance target products. Consequently, coupled genetic modification and metabolic evolution techniques have been extensively employed to construct microbial cell factories for bio-based products such as lactate, ethanol, malate, 2,3-butanediol, and succinate (Liu et al. 2012). As explained previously, the combined deletion of the *adhE*, *ackA-pta*, *ldhA*, *budAB*, and *pflB* genes resulted in the *K. oxytoca* KC004's growth deficiency, which limited the rate of succinate synthesis. As a result, the growth and succinate of the *K. oxytoca* KC004 were enhanced sequentially for multiple generations to improve for appealing performance by metabolic adaptation (Jantama et al., 2008). To begin, the *K. oxytoca* KC004 was grown and transferred to a fresh minimal medium containing 50 g/L glucose and a starting OD_{550} of 0.01. Due to the lack of pyruvate-formate lyase activity in the *K. oxytoca* KC004 after eliminating

the *pflB* gene, twenty millimolar sodium acetate were added to the medium until the transfer number 36 as a source acetyl-CoA. As previously reported, knocking out the *pflB* and *pta* genes in an engineered microbe results in acetate auxotrophs during anaerobic fermentation (Sawers and Bock, 1988; Jantama et al. 2008a; Jung et al. 2014; In et al. 2020). From the 1st transfer to the 33rd transfer, the culture's pH was left unregulated in order to avoid contamination during the alkaline solution connection. From the 1st transfer to 19th transfers, the *K. oxytoca* KC004 showed slower biomass generation and succinate production compared to those of later transfer (Fig 4.6A-B). Succinate productivity increased progressively from the 5th to the 33rd transfer, reaching 0.04, 0.08, 0.13, and 0.24 g/L/h, respectively. Simultaneously, acetate production increased from 0.38 to 2.22 g/L compared to the first transfer. Even though the highest level of biomass (≈ 1 g/L) and succinate production (≈6 g/L) of the developed strain were almost identical. However, biomass and succinate production significantly increased by 10-fold and 40-fold, respectively, when compared to the unevolved strain. The evolutionary adaptation improved biomass and succinate generation in the *K. oxytoca* KC004, as predicted, and the *K. oxytoca* KC004 required acetate for growth. Uncontrolled pH during culture, on the other hand, may restrict cell development, affecting succinate synthesis. Beginning with the 34th transfer, a solution of 6 M KOH and 3 M K_2CO_3 was automatically added to the serial transfers to avoid acidification and supply CO_2 during the fermentation. Furthermore, the concentration of sodium acetate was gradually reduced to 10 mM, 5 mM, 2.5 mM, and none during the 37th to 38th, 39th to 40th, 41st to 42nd, and 43rd to 44th transfers, respectively. The glucose concentrations were completely utilized from the 34th to the 51st transfer, and the maximum biomass and succinate production increased from 1 to 1.5 g/L and 6.53 to 43.21 g/L, respectively, when compared to transfers without the maintaining pH and supply of CO_2, whereas acetate production (≈8 g/L) did not change significantly. Interestingly, productivity increased from 0.58 to 0.90 g/L/h between the 43rd and 51st transfers, compared to the 34th and 36th transfers. The serial transfers were then continued to enhance the amount of industrial production by using greater glucose concentrations (100 g/L) from the 52nd transfer to the 160th transfer. During the transferring process, the culture from the 57th transfer had a greater concentration, yield, and productivity than the cultures from the 51st to the 56th transfers. However,

following the 57th transfer, glucose consumption and succinate synthesis steadily declined, but maximal biomass (2 g/L) and acetate (17.79 g/L) production increased 1.21-fold and 1.32-fold, respectively, from the 101st to the 160th transfers, compared to the 57th transfer. Furthermore, from the 101st to the 160th transfers, biomass reached the log phase within 24 h, then declined, whereas acetate reached more than 5 g/L within 48 h. This suggested that the biomass generation of the developed KC004 strain was dependent on acetate metabolism within the direction of converting acetate to acetyl-CoA by consuming 1 mol of ATP (Zhu et al. 2004). However, the acetate overflow might be a byproduct inhibitor that impacted the reduction of biomass after 24 h, resulting in a slower rate of glucose utilization. As a result, the strain at the 160th transfer was chosen as the best representative clone and designated *K. oxytoca* KC004-TF160. The resulting strain, *K. oxytoca* KC004-TF160, showed a significant increase in succinate production at a concentration of 82.88 g/L, with yield and productivity of 0.82 g/g and 0.69 g/L/h, respectively, which were approximately 12.32-fold, 27.67-fold, and 9.67-fold, respectively, higher than those of the *K. oxytoca* KMS006 (Fig4.6D). Acetate at a concentration of 15 g/L was only found as a by-product. This succinate synthesis yield was 73.74% of the theoretical maximum, or 1.26 mol/mol glucose used. These results revealed that combining metabolic engineering and metabolic evolution was a viable method for increasing succinate synthesis in the *K. oxytoca* KC004 in mineral salts medium. Similarly, Zhu et al. (2014) demonstrated that using metabolic engineering in combination with evolutionary adaptation increased succinate synthesis and yield in the HX024 strain by 350% and 9%, respectively, as compared to the parent NZ-037 strain. Using similar approaches, the *E. coli* KJ073 was produced after approximately 2,000 generations of growth-based selection, leading to the establishment of a stable phenotype with a significant increase in succinate synthesis and growth rate. Its succinate synthesis improved by 1,263% as compared to the beginning strain *E. coli* C (Jantama et al., 2008a).

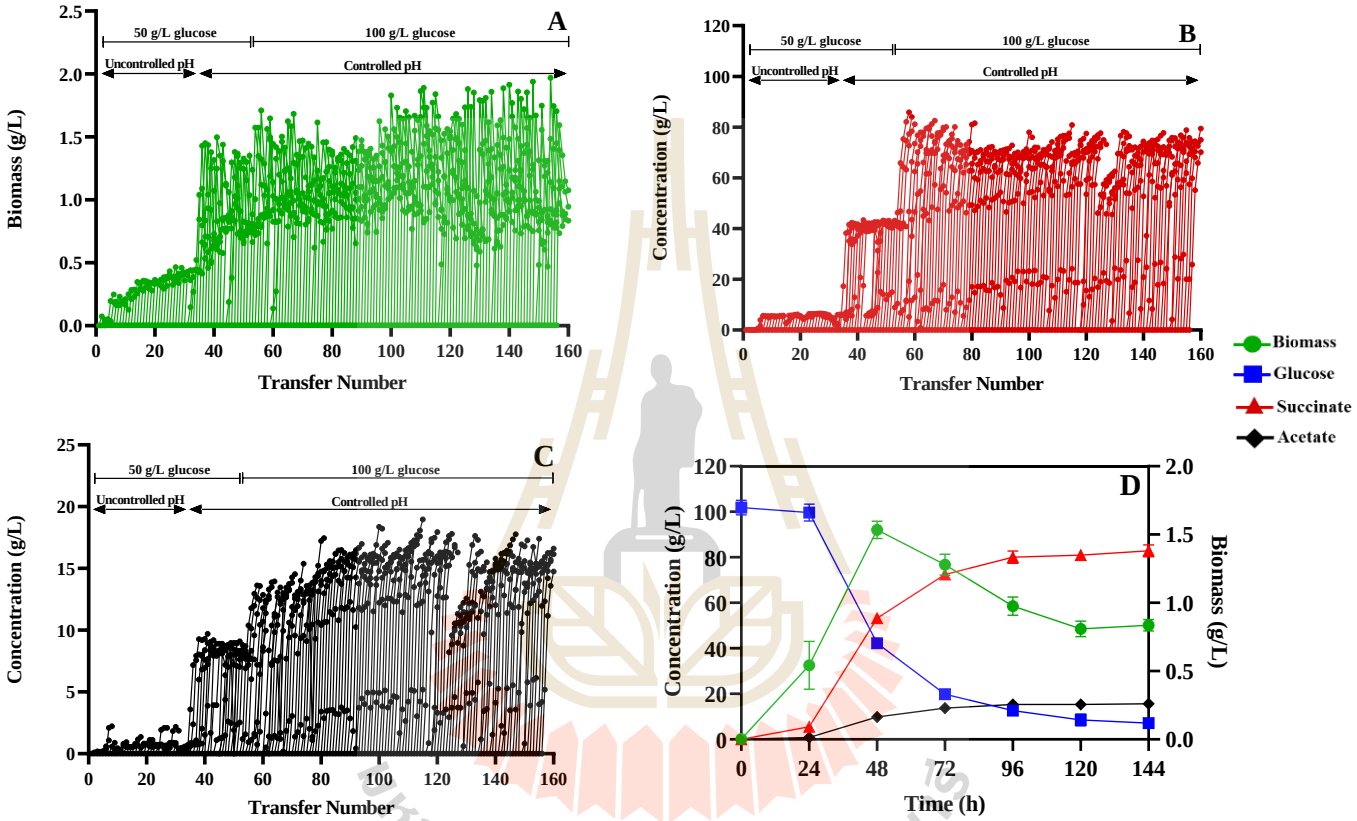

Figure 4.6 Metabolic evolution of *K. oxytoca* KC004 strain in AM1 medium containing glucose as a carbon source. Transfers 1st to 51st, fermentation with 50 g/L glucose; transfers 52nd to 156th, fermentation with 50 g/L glucose. Transfers 1st to 33rd, uncontrolled pH during fermentation; transfers 34th to 156th, controlled pH during fermentation. A-C Biomass, succinate, and acetate during metabolic evolution, respectively. D Fermentation profile of the evolved *K. oxytoca* KC004-TF160 strain.

4.1.6 Effect of the combining genetic manipulation and evolutionary adaptation on the metabolites production of *K. oxytoca* KC004-TF160

While genetic manipulation and metabolic adaptation were utilized in the establishment of the *K. oxytoca* KC004-TF160, they significantly enhanced succinate production. Spontaneous mutations that happened during metabolic evolution may have resulted in the strain with enhanced succinate production. Understanding the distribution of internal metabolic flux via metabolic networks in *K. oxytoca* KC004-TF160, on the other hand, was required to provide global information for problem-solving in future strain development. Transcriptome analysis is now being used in numerous studies to identify novel target genes for enhancing strain performance (Kim et al., 2015; Hirasawa et al., 2009; Olesen et al., 1987; Ookubo et al., 2008). Therefore, the internal metabolic flux of the *K. oxytoca* KC004-TF160 was studied by examining the transcription levels of important enzyme activities in the primary three pathways, which include the reductive branch of the TCA cycle, the oxidative branch of the TCA cycle, and acetate synthesis. Table 3.4 lists all genes that contribute to three pathways, and the relative transcription levels of the major enzymes that contribute to three pathways were analyzed using RT-qPCR. Transcript levels of all genes from the *K. oxytoca* KMS006 and *K. oxytoca* KC004-TF160 were measured during the exponential growth phase and normalized to *K. oxytoca* M5A1 values. As shown in Fig. 4.7. when compared to the *K. oxytoca* KMS006, the relative expression levels of *ppc* in the reductive branch of the *K. oxytoca* KC004-TF160 decreased significantly by 31.85-fold, while *mdh*, *fum*, and *frd* genes decreased slightly by 1.84-fold, 1.71-fold, and 1.61-fold. Surprisingly, the *pck* gene increased by up to 2.17-fold, when compared to the KMS006 strain. In oxidative branch of TCA cycle in *K. oxytoca* KC004-TF160 strain, the relative expression of *gltA*, *acnAB*, and *icd* genes decreased by 3.80-fold, 1.05-fold, and 3.86-fold, respectively, as compared to those of the *K. oxytoca* KMS006. Likewise, the relative expression of the genes encoding *pykA*, *pdh*, *poxB*, *tdcD*, and *acs* related genes in acetate accumulation decreased by 14.66-fold, 3.46-fold, 2.44-fold, 8.46-fold, and 18.98-fold, respectively, when compared to the *K. oxytoca* KMS006. Whereas the relative expression of the *tdcE* gene increased up to 20-fold, respectively, when compared to the *K. oxytoca* KMS006.

As above results, the transcript level of the *pck* gene was about 26.27-fold and 22.58-fold more than that of the *ppc* and *pykA* genes, respectively, in the KC004-TF160 strain. The result indicated that the function of *pck* gene was motivated and adapted as the major carboxylation activity in the *K. oxytoca* KC004-TF160, resulting in increased succinate and net ATP generation. This improved *K. oxytoca* KC004-TF160 pathway is similar to the pathway of native succinate producing rumen bacteria such as *A. succinogenes* and *M. succiniciproducens*, which plays the most important role in anaerobic growth of native producer and efficient succinate production (Van der et al., 1997; Kim et al., 2004). Furthermore, the *pck* gene function of *K. oxytoca* KC004-TF160 is similar to the overexpression of the *pck* gene in *E. coli*, resulting in higher succinate yield and net ATP generation for growth and maintenance (Li et al., 2016; Zhang et al., 2009). Unlike *K. oxytoca* KC004-TF160, the *K. oxytoca* KMS006's succinate production was dependent on *ppc* activity, which reduced succinate production in native *E. coli* due to energy loss associated with inorganic phosphate release (Zhang et al., 2009). Meanwhile, the decreasing *pykA* activity may confirm that the most carbon flux into carboxylation metabolism for increasing succinate production in *K. oxytoca* KC004-TF160. As previously reported, knocking out *pykA* gene from *E. coli* increases intracellular ATP levels and the yield of end products derived from PEP while not affecting glucose consumption (Zhoa et al., 2017). On the other hand, the decreasing *pykA* activity affected the decreasing the transcript level of *pdh* and *tdcE* genes in pyruvate catabolism resulting in low functioning for acetyl-CoA production, even though the *tdcE* gene activity was upregulated after multiple rounds of metabolic evolution. These spontaneous mutations may confirm that both genes are primarily enzyme activities for compensating the absence of the *pflB* gene in acetyl-CoA synthesis of the *K. oxytoca* KC004-TF160, but both the *pdh* and *tdcE* genes are insufficient to serve as functional enzymes. The *pdh* gene has approximately 5% residual activity under anaerobic conditions when compared to aerobic culture conditions (Zhou et al., 2008; Clark, 1989). However, *E. coli* and *B. subtilis* which lacks *pfl* gene, *pdh* has been shown to be capable of converting pyruvate to acetyl-CoA under anaerobic conditions (Kim et al., 2007; Nakano et al., 1997). Similarly, when the *pflB* gene is knocked out in the KJ071 strain, the *pdh* activity is activated to some extent to compensate for the lack of *pflB* activity. These previous results show the

capacity of *pdh* to carry out pyruvate to acetyl-CoA conversion, even in the absence of oxygen. However, the absence of *pflB* and *ackA* activities compensated for the loss in reducing power by accumulating pyruvate, resulting in a net production of 2 mol of NADH per mol of glucose. Furthermore, the carbon flux through *pdh* and *tdcE* activity was low, suggesting that the rate of NADH generation was not fast enough and/or available to efficiently intermedia conversion in succinate generation, resulting in a decrease in *mdh*, *fum*, and *frd* activity transcript levels in the *K. oxytoca* KC004-TF160 strain. The lack of NADH is also likely to have influenced the rate of conversion of malate to fumarate, which became the rate-limiting step in succinate pathway (Jantama et al., 2008). Aside from NADH imbalance, a decrease in *mdh* and *ppc* activity may be caused decreasing *pykA* activity. Normally, the high levels of pyruvate might result from high malic enzyme activity due to increased activation of *ppc* and mdh genes.

As seen in the data above, the low pyruvate catabolism after the lack of *pflB* activity may cause the decrease in transcript levels of all genes involved in the reductive branch of TCA, oxidative branch of TCA, and acetate accumulation. These spontaneous mutations could confirm that the *K. oxytoca* KC004-TF160's low biomass is due to deficiencies in acetyl-CoA intermedia and NADH reoxidation caused by reduced expression of the *acs*, *tdcD*, and *gltA* genes in the acetate and oxidative branch nodes respectively. As previously reported, most of the acetyl-CoA is excreted as acetate by cells growing on glucose under aerobic conditions. In the absence of glucose, external acetate is mostly utilized by reversal of the pathway catalysed by acetyl~CoA synthetase, encoded by *acs* gene (Kumari et al., 1995). In addition, the expression of the *tdcD* gene might functionally replace *ackA*, enhancing ATP and acetate production from acetyl-P and offering a competitive growth advantage (Jantama et al., 2008a). The carbon flow diversion to acetate could be a means to limit NADH in the cell since the flux through acetyl~CoA and acetate does not generate any reducing equivalents (El-Mansi et al., 1989). This product enzyme, like the *ackA* encoded acetate kinase is highly efficient (Reed et al., 2003). Coincidently, when acetate is utilized for anaerobic cell growth, the increased level of *gltA* gene expression in the oxidative branch node is thus largely related to cell biosynthetic needs, whereas together with *gltA* and *citDEF* gene expression could establish a futile cycle that wastes

the energy stored in acetyl-CoA and accumulates acetate (Jantama et al., 2008a). As seen in the data above, the metabolic engineering caused by reducing activities of the oxidative branch of TCA and acetate accumulation resulted in low cell growth and a reduction in the reductive branch of TCA activity in the *K. oxytoca* KC004-TF160. Obviously, acetate-linked ATP production may be activated as a mechanism responsible for anaerobic cell biosynthetic in the *K. oxytoca* KC004-TF160. Whereas an increase in succinate synthesis was associated with increased transcript levels of *pck* gene in the *K. oxytoca* KC004-TF160 as compared to the *K. oxytoca* KMS006. Overall, these results showed that the capacity of succinate production was improved due to the combining of two strategies of genetic manipulation and metabolic adaptation in *K. oxytoca* KC004-TF160 strain.

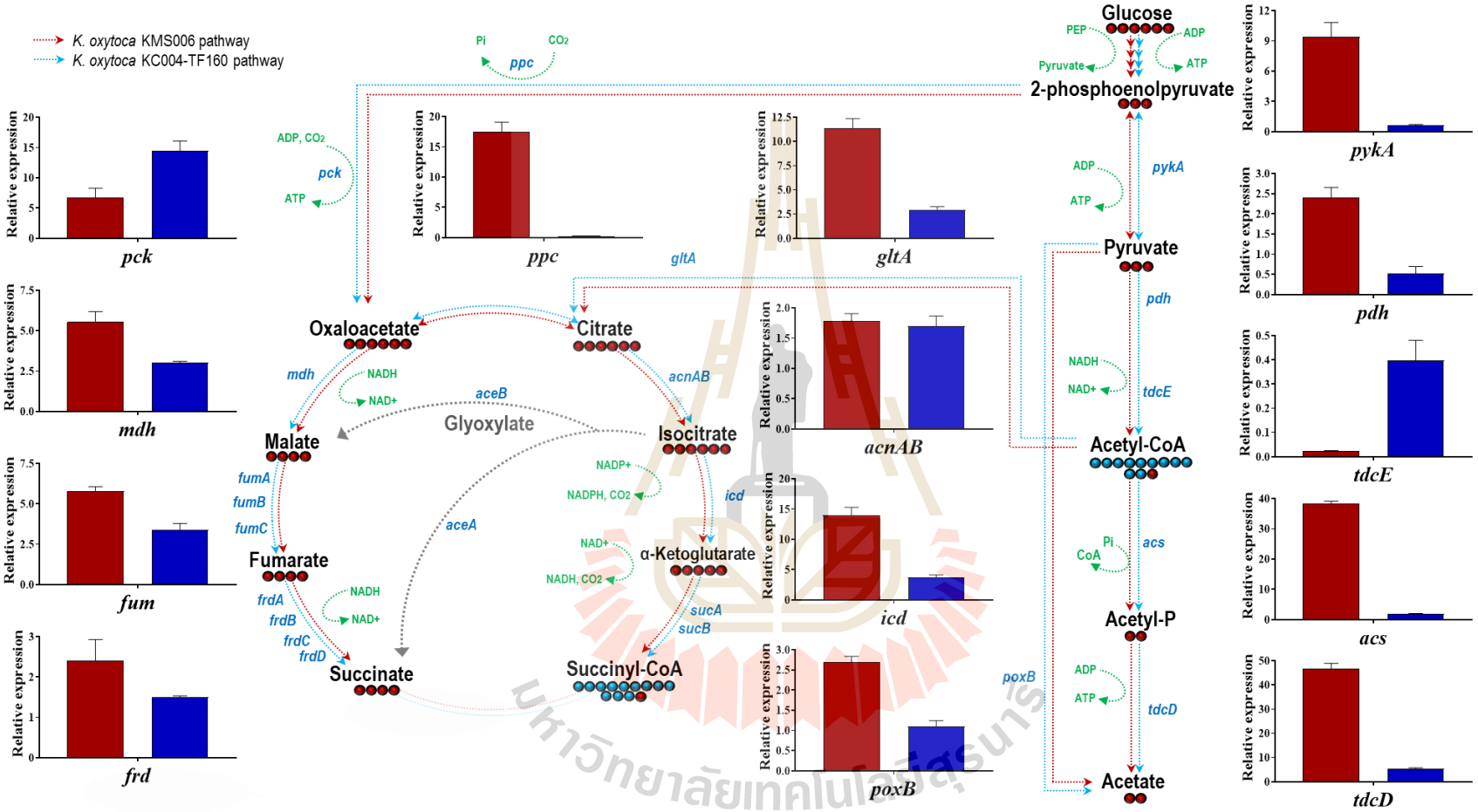

Figure 4.7 Relative expression levels of the major enzyme genes in *K. oxytoca* KMS006 and *K. oxytoca* KC004-TF160. Symbols: KMS006 (Red bars), and *K. oxytoca* KC004-TF160 (Blue bars).

4.2 Eliminating by-products and increasing succinic acid yield of *K. oxytoca* KC004-TF160

4.2.1 Deletion of *tdcD* gene in *K. oxytoca* KC004-TF160

Plasmids pKC006.1 and pKC006.2 were used as templates to amplify linear DNA fragments of *tdcD'-cat-sacB-tdcD"* and *tdcD'-tdcD"* by using the *tdcD*-up/down primers set to delete *tdcD* gene, respectively (Fig 4.8).

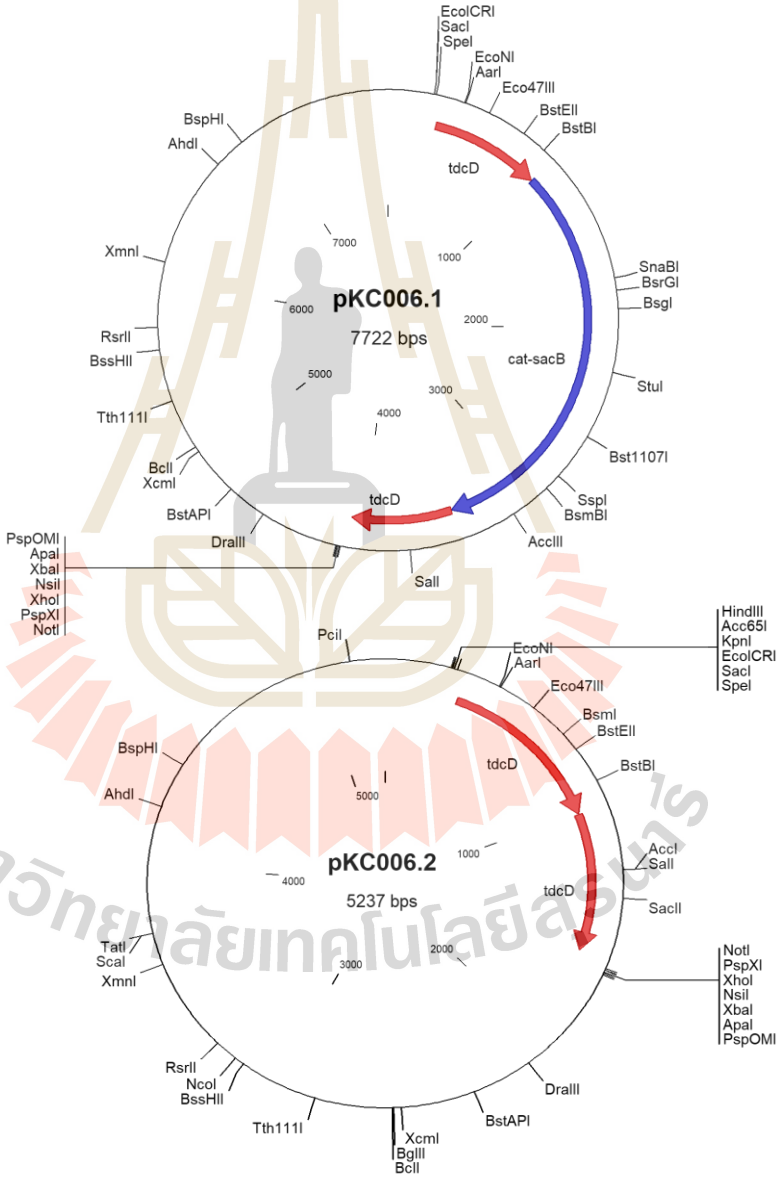

Figure 4.8 Plasmids pKC006.1 and pKC006.2 used for *tdcD* gene deletion.

The *tdcD'-cat-sacB-tdcD"* fragment was electroporated into the *K. oxytoca* KC004-TF160 containing pLOI3420. The recombinant strains were selected on LB plates containing chloramphenicol (50 mg/L) and confirmed by PCR analysis using *tdcD*-up/down primers set, resulting in a PCR product with the expected size of 3,880 bps (Fig 4.9). The clone containing chloramphenicol resistant gene was designated as the *K. oxytoca* KC005 strain.

To remove antibiotic resistance gene, the *cat-sacB* cassette in the *K. oxytoca* KC005 containing pLOI3420 was replaced with native sequences (*tdcD'-tdcD"*) The obtained clone was selected using the same method as in *tdcD* deletion and clarified by PCR analysis using *tdcD*-up/down primers set. The result showed that the PCR product was in size of 1,306 bps as expected. A clone without antibiotics resistance gene was designed as *K. oxytoca* KC006.

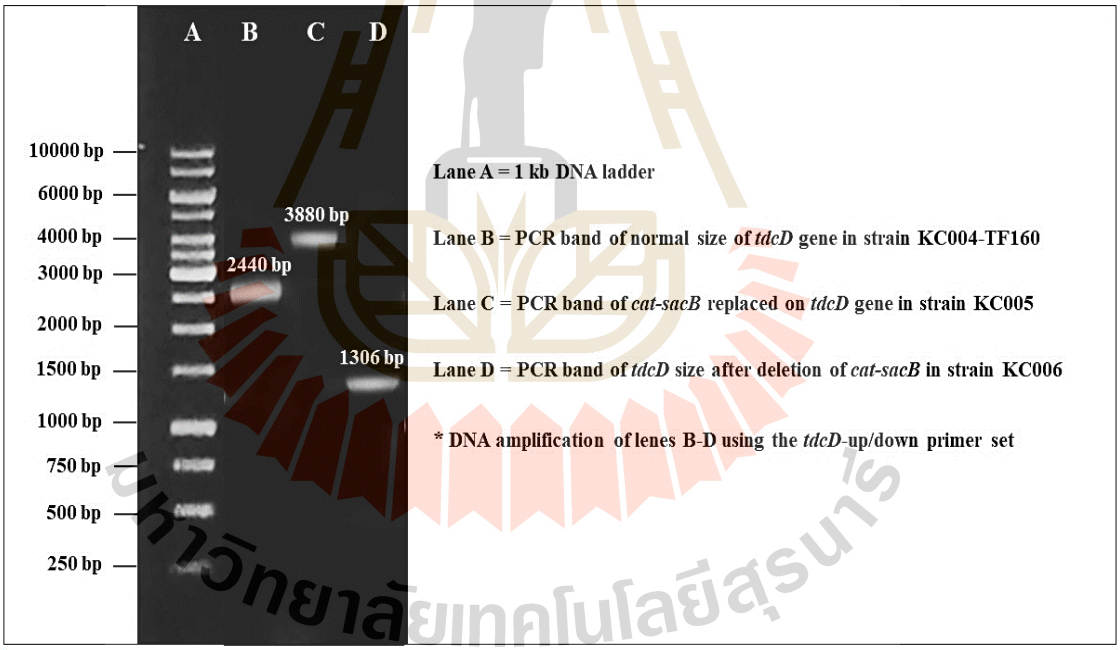

Figure 4.9 Gel electrophoresis confirmed the construction of *K. oxytoca* KC005 and *K. oxytoca* KC006.

4.2.2 Effect of *tdcD* gene deletion in *K. oxytoca* KC004-TF160

As previously reported, the *K. oxytoca* KC004-TF160 could be considered as a succinate host bacterium because it could produce succinate at high concentration and yield on mineral salts medium (AM1) under anaerobic conditions. Despite the fact that acetyl-CoA production was low in the *K. oxytoca* KC004-TF160 due to a lack of pyruvate formate-lyase activity, acetate production was high as a by-product during succinate fermentation. Coincidently, the transcription level analysis revealed that the *tdcD* gene responded as a main functional enzyme to replace *ackA-pta* activity for acetate and ATP production from acetyl-P and providing a competitive growth advantage. Even though this gene is one of the normal enzymes involved in the degradation of threonine, it exhibits *tdcD* activity and is an acetate kinase homologue (Hesslinger et al., 1998). High concentration of acetate could inhibit cell growth and resulted in a slow glucose consumption rate and succinate production after 72 h. As previously described, acetate excretion results not only in an inefficient carbon source utilization and inhibition of cellular growth and protein production, but also in the requirement for cofactor recycling in order to maintain balanced growth and cellular homeostasis (Chang et al., 1999). Therefore, the *tdcD* gene in the *K. oxytoca* KC004-TF160 was knocked out to disrupt the acetate production for improving downstream processing by simplifying the purification during succinate production. The *tdcD* mutant strain was designated as *K. oxytoca* KC006. As shown in Fig 4.10, acetate was not detected after *tdcD* gene deletion, but lactate was presented as a main product with a concentration of 15.75 g/L. Similar to *pflB* deletion, the biomass (0.42 g/L) and glucose consumption (21.75 g/L) of the *tdcD* mutant strain decreases significantly about 63.48% and 56.50% respectively, resulting that succinate (1.58 g/L) production was decreased by 96.22%, when compared with the *K. oxytoca* KC004-TF160 as shown in Fig 4.10. In addition, the yield and productivity of succinate significantly decreased by 91.66% and 98.85% respectively, when compared with the *K. oxytoca* KC004-TF160. However, the succinate yield of the *K. oxytoca* KC006 was 133.33% higher than that of the *K. oxytoca* M5A1. The results confirmed that the knocking out both of *tdcD* and *ackA* genes completely prevented acetate formation in the *K. oxytoca* KC004-TF160. In comparison to previous results, the *tdcDE* deletion in the KJ091 strain only reduced acetate accumulation during growth by only 50%.

(Jantama et al., 2008). In addition, the deletion of *ackA* and/or *tdcD* in *E. coli* could reduce acetate accumulation and production rate owing to reduced acetate kinase activity, and mutants with lesions in *ackA* and *tdcD* excreted less acetate than strains with a single deletion of *ackA* or *tdcD*. (Kumari et al., 1995; Zhao et al., 2015). Nevertheless, the *K. oxytoca* KC006's acetate and other byproducts pathway were blocked, the *K. oxytoca* KC006 adapted to maintain redox balance through lactate accumulation. According to San et al. (2002) and De Mey et al. (2007), reducing acetate by deleting the *pta* and/or *ackA* genes increases the production of other fermentation products such as pyruvate and lactate, whereas the mutants with deletions of *ackA* and/or *tdcD* accumulated higher concentrations of pyruvate and lactate (Zhou et al., 2011). This phenomenon occurs when the rate of glycolysis exceeds a critical value, which is caused by an imbalance between glucose uptake and the demand for energy and biosynthesis, resulting in pyruvate by-product formation (Aristidou et al., 1995; Farmer and Liao, 1997). At high glucose concentrations, the rate of NADH generated during glycolysis is greater than the rate of NADH oxidation. For this reason, another enzyme involved in lactate formation in the *K. oxytoca* KC006 by converting PEP to pyruvate and lactate, respectively, may be regulated to maintain the glycolytic metabolism for energy production and NADH oxidation balance. Lactate is produced to maintain stoichiometric and intracellular redox balances as glycolytic flow is promoted by oxygen limitation with a higher ATP requirement (Zhu & Shimizu, 2004). The presence of efficient NADH recycling pathways in *K. oxytoca* allowed for the maintenance of intracellular redox balance, suggesting that the lactate branch's response to NADH availability is highly sensitive and the NADH flux to lactate is flexible. Pyruvate is normally converted by different enzymes into L (+)- or D (-)-lactate. The deletion of one or two lactic acid pathways may not be lethal to *K. pneumoniae* due to a tailored compensation mechanism of lactic acid synthesis. Furthermore, *Klebsiella* has NAD-independent LDHs (*iLDH's*) enzyme like the lactic acid bacteria that function to convert lactate to pyruvate for cell growth (Garvie, 1990). According to previous research, when this enzyme was present, the cell could grow by using lactate as an energy source. So, lactate is a universal metabolite and energy source, yet the mode of lactate metabolism in many strictly anaerobic microorganisms. Aside from the *ldhA* gene, many enzymes potentially contribute to lactate synthesis in *Klebsiella* bacteria

such as L-lactate dehydrogenase (*pmd*), lactaldehyde dehydrogenase (*aldA*) and methylglyoxal (*mgsA*). Lactate production by *pmd* gene is in response to covert pyruvate, whereas lactate production by *aldA* and *mgsA* genes is dependent on methylglyoxal and lactaldehyde pathways, respectively, and lactate production through both may be minimal. Detoxification of methylglyoxal has little effect on lactate production and cell growth (Weng et al., 2018).

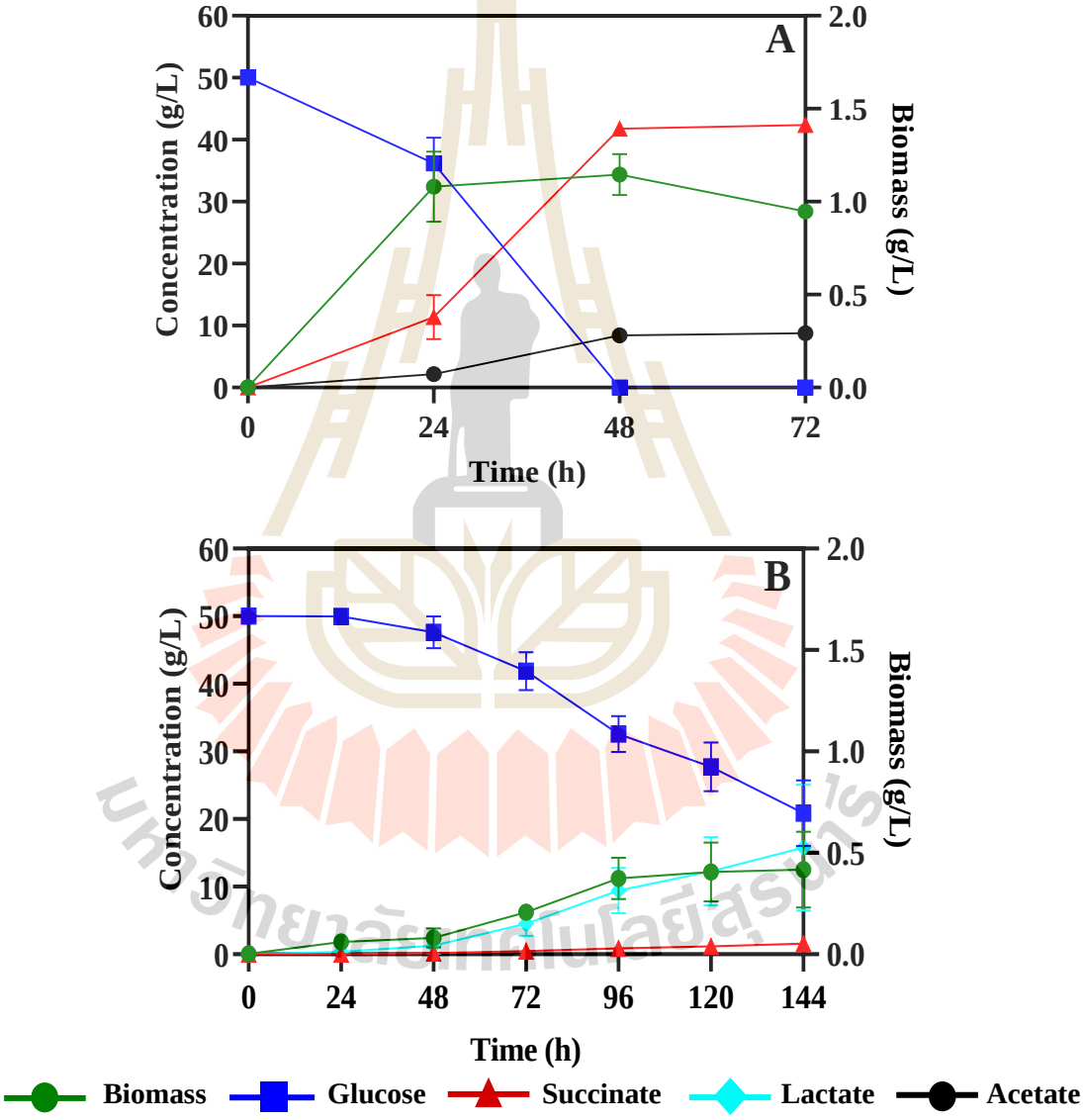

Figure 4.10 Fermentation profile of *K. oxytoca* in mini controlled-pH fermenter with a working volume of 350 mL AM1 medium containing 50 g/L glucose under anaerobic conditions. (A) *K. oxytoca* KC004-TF160, (B) *K. oxytoca* KC006.

4.2.3 Deletion of *pmd* gene in *K. oxytoca* KC006

Plasmids pKP002.1 and pKP002.2 were used as templates to amplify linear DNA fragments of *pmd'-cat-sacB-pmd"* and *pmd'-pmd"* by using the *pmd*-up/down primers set to delete *pmd* gene, respectively (Fig 4.11).

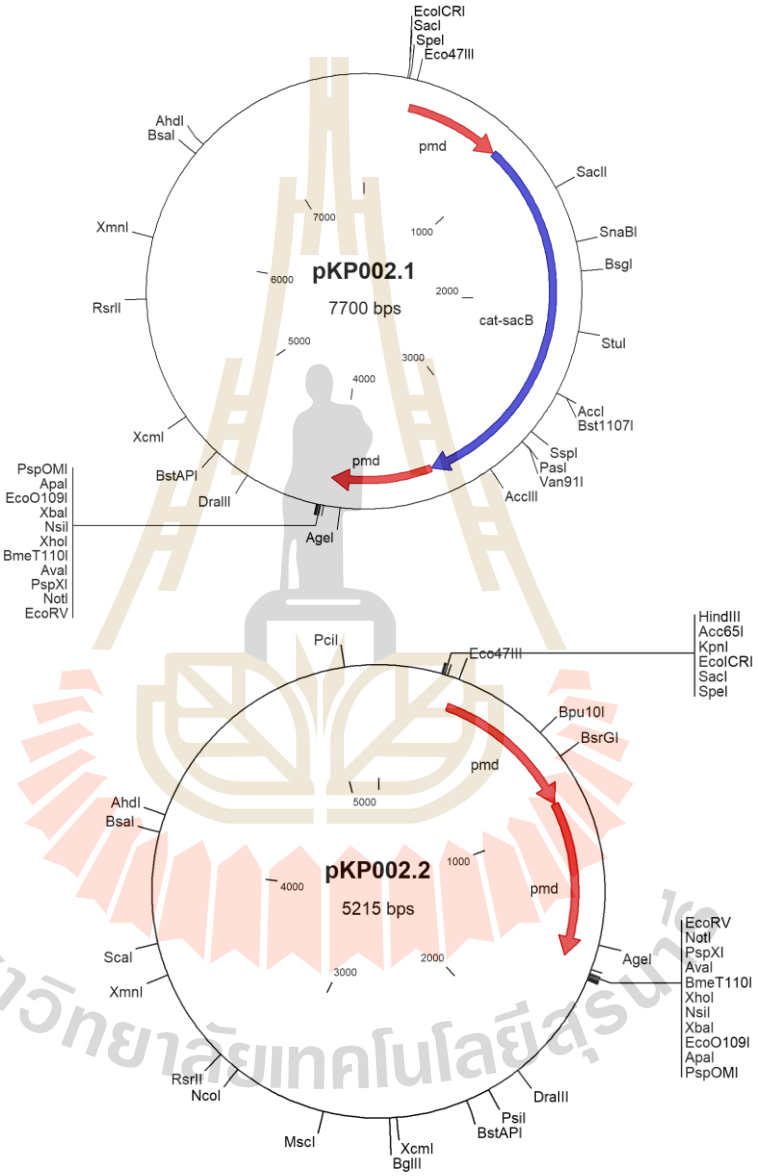

Figure 4.11 Plasmids pKP002.1 and pKP002.2 used for *pmd* gene deletion.

The *pmd'-cat-sacB-pmd"* fragment was electroporated into *K. oxytoca* KC006 containing pLOI3420. The recombinant strains were selected on LB plates containing chloramphenicol (50 mg/L) and confirmed by PCR analysis using *pmd*-up/down primers set, resulting in a PCR product with the expected size of 3,850 bps (Fig 4.12). The clone containing chloramphenicol resistant gene was designated as *K. oxytoca* KP001.

To remove antibiotic resistance gene, the *cat-sacB* cassette in KP001 strain containing pLOI3420 was replaced with native sequences (*pmd'-pmd"*). The obtained clone was selected using the same method as in *pmd* deletion and clarified by PCR analysis using *pmd* -up/down primers set. The result showed that the PCR product was in size of 1284 bps as expected. A clone without antibiotics resistance gene was designed *K. oxytoca* KP002.

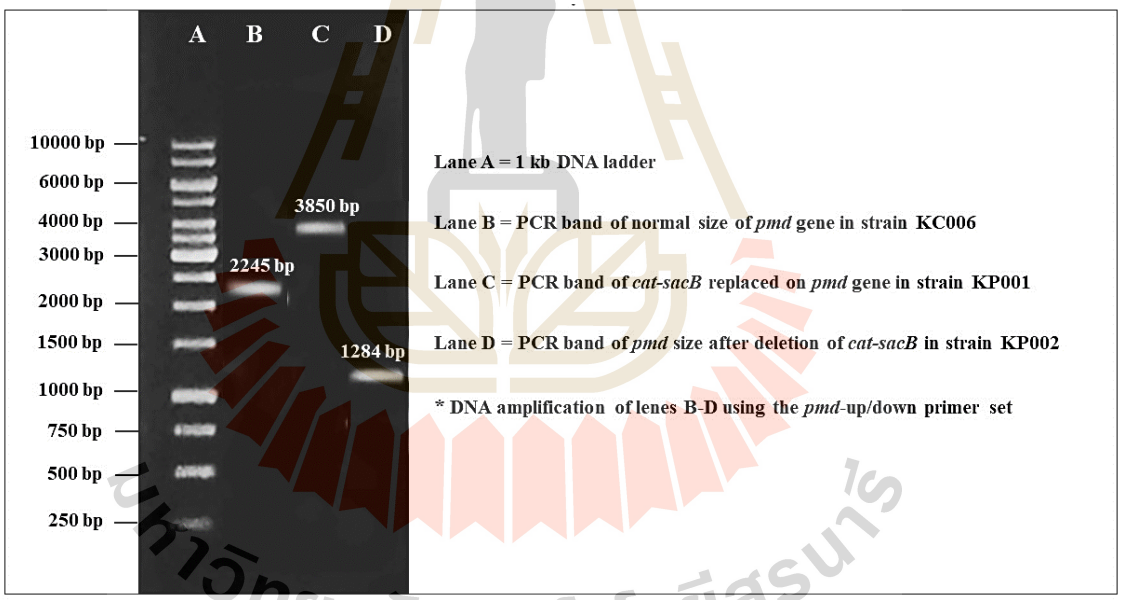

Figure 4.12 Gel electrophoresis confirmed the construction of *K. oxytoca* KP001 and *K. oxytoca* KP002.

4.2.4 Effect of *pmd* gene deletion in *K. oxytoca* KC006

As reported in *tdcD* deletion, lactate was produced as a main product during succinate fermentation after the *tdcD* gene was knocked out to disrupt acetate production in *K. oxytoca* KC006. The functional metabolism of lactate formation was present to maintain intracellular redox balance, which encourages the growth of *K. oxytoca* KC006 cells. However, the formation of lactate (1.0-0.8 mol from 1 mol of glucose) not only consumes carbon source but also entangles downstream separation (Li et al., 2015). Hence, the lactate formation was investigated to disrupt by deleting the *pmd* gene in the *K. oxytoca* KC006, and the *pmd* mutant was designated as *K. oxytoca* KP002 strain. Surprisingly, lactate and other byproducts were not detected during anaerobic fermentation on AM1 medium containing 20 g/L of glucose under anaerobic conditions. Unfortunately, this deleted strain did not grow in AM1 medium without lactate production. The knocking out the *pmd* gene further inhibited cell growth in the *K. oxytoca* KP002, resulting in a significant decrease in biomass (0.21 g/L) and succinate (1 g/L) of approximately 50% and 36.70%, respectively, when compared to that of the *K. oxytoca* KC006 strain. To dissolve a poor growth, the *K. oxytoca* KP002 was fermented on LB medium containing glucose as a carbon source under anaerobic conditions. As previously studied, a variety of genetic approaches resulted in engineered *E. coli* growth and sugar metabolism being very poor in mineral salts medium but very robust in complex LB medium. Because LB medium contains vitamins, amino acids, and other macromolecular precursors, potential regulatory problems in metabolism and biosynthesis caused by metabolic engineering may be hidden (Jantama et al., 2008). However, the biomass (0.21 g/L) was not significantly improved, whereas succinate (1.69 g/L) increased by approximately 69% and 6.96%, respectively, when compared to fermentation in AM1 medium and the KC006 strain. As shown in Fig 4.13, when compared to the *K. oxytoca* KC006, *K. oxytoca* KMS006, and *K. oxytoca* M5A1, the yield (0.57 g/g) of succinate production was increased by 691.66%, 612.50%, and 1,800%, respectively. However, the concentration and yield of succinate by *K. oxytoca* KP002 were reduced by 64.29% and 32.14%, respectively, when compared to the *K. oxytoca* KC004-TF160. These results indicate that the *pmd* activity was involved in lactate production, which is a competitive NADH pathway of the *K. oxytoca* KC006's succinate production. Therefore, the knocking out the *pmd*

gene could prevent lactate production while increasing succinate yield in the *K. oxytoca* KP002. However, the lack of *pmd* activity causes the *K. oxytoca* KP002 to grow slowly. As shown in Fig 4.14, the lactate production is caused by the activity of *pmd* gene in *K. oxytoca* KP002 for pyruvate catabolism. As a result of the causes of growth defects, the KP002 strain fails to ferment glucose efficiently under anaerobic conditions due to the loss of the maintaining pathway for energy production and NADH oxidation in glycolytic metabolism. It may be used for redox homeostasis to discard excess electrons from NADH by producing lactate under normal growth conditions (Drake et al., 2008).

Under enforced adaptation, bacteria may reverse its function, to facilitate using lactate as growth substrate (Rosenbaum et al., 2021). In this metabolism, 2 mol L-lactate are oxidized to 2 mol pyruvate and 2 mol NADH, then 2 mol pyruvate are decarboxylated to 2 mol acetyl-CoA, resulting in acetate biosynthesis. Thus, the ATP gain from substrate-level phosphorylation during lactate metabolism is 1 mol ATP for every 1 mol lactate oxidized. As previously reported, blocking acetyl-CoA and lactate formation by the *pflB* and *ldhA* double mutant limits NAD+ regeneration and, as consequence, disables proper anaerobic growth due to an imbalance between NADH formation and reoxidation (Singh et al., 2009). Furthermore, previously studies reported that the production of NADH-metabolism (consumption or regeneration)-related enzymes resulted in a dramatically altered ratio of NADH/NAD+ and a significantly altered spectrum of metabolic products when cofactor engineering approaches have been applied in *E. coli*, and *S. cerevisiae* (Felipe et al., 1998; Heux et al., 2006). An alteration of intracellular redox balance (represented by the ratio of NADH to NAD+) either reduced the glucose uptake efficiency (Berrios-Rivera et al., 2002a, b; Sanchez et al., 2005) or inhibited the cell growth (Heux et al., 2006).

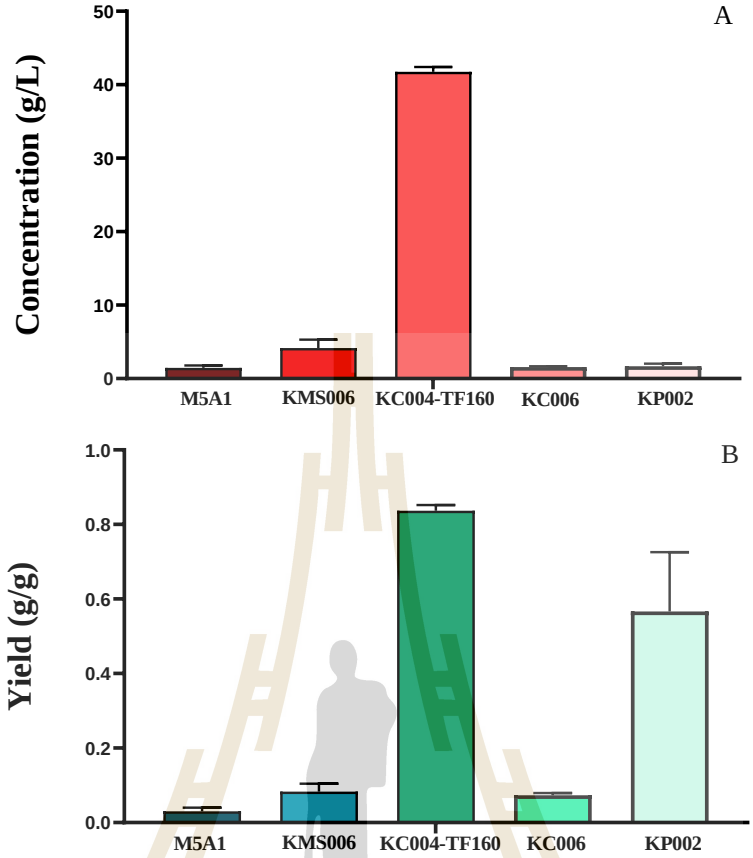

Figure 4.13 Comparison of succinate concentration and yield by different *K. oxytoca* strains.

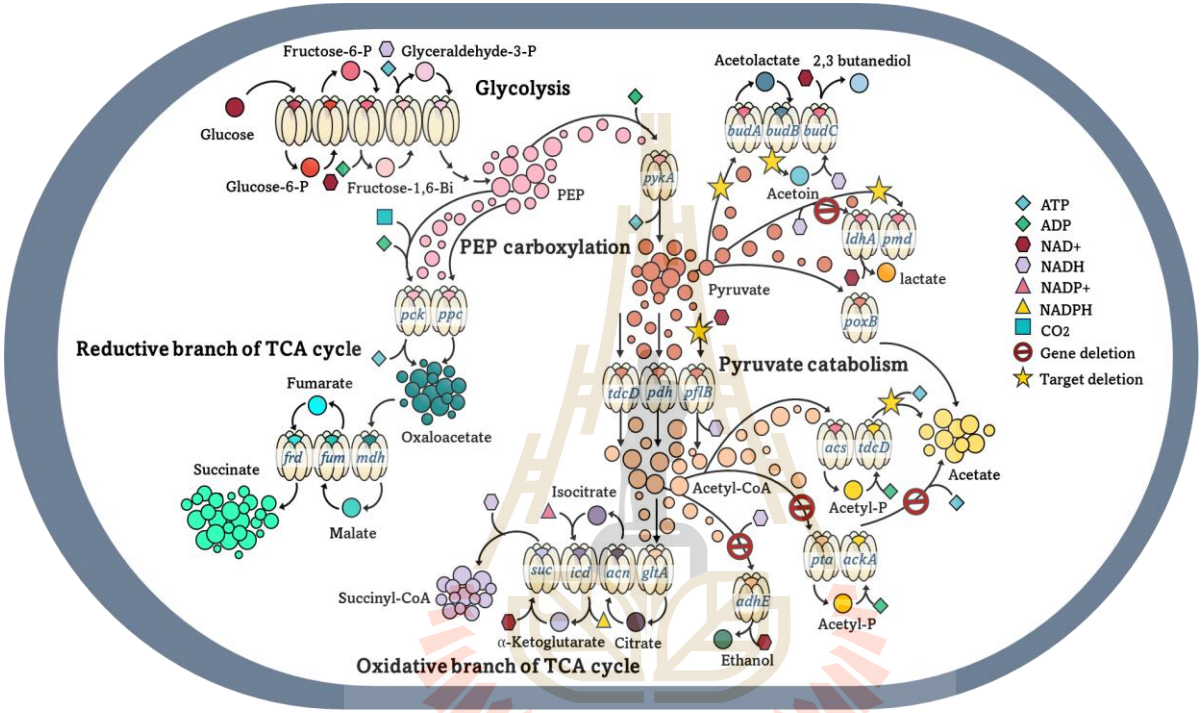

Figure 4.14 Metabolic pathway of *K. oxytoca* strains under anaerobic fermentation. Gene and enzymes: *pflB*, pyruvate formate-lyase; *budAB*, butanediol dehydrogenase; *adhE*, alcohol dehydrogenase; *ldhA*, lactate dehydrogenase; *pta*, phosphate acetyltransferase; *ackA*, acetate kinase; *aldA*, aldehyde dehydrogenase; *tdcD*, propionate kinase; *tdcE*, threonine decarboxylase E; *acs*, acetyl-CoA synthetase; *fdh*, formate dehydrogenase; *poxB*, pyruvate oxidase; *pdh*, pyruvate dehydrogenase; *ppc*, phosphoenolpyruvate carboxylase; *gltA*, citrate synthase; *acnA*, aconitase; *icd*, isocitrate dehydrogenase; *mdh*, malate dehydrogenase; *fum*, fumarase isozymes; *frd*, fumarate reductase, and *aceAB* isocitrate lyase; *pmd*, L-lactate dehydrogenase; *pykA*, pyruvate kinase II.

4.2.5 Evolutionary adaptation of *K. oxytoca* KP001 to achieve *K. oxytoca* KP001-TF60

As explained previously in the report, the combined knocking out of the *tdcD* and *pmd* genes with the deletion of *adhE*, *ackA-pta*, *ldhA*, *budAB*, and *pflB* genes strain results in an unbalanced metabolism in the *K. oxytoca* KP002, limiting growth and succinate production. So, the previously described method of evolutionary *K. oxytoca* KC004 was used to recover the biomass and succinate production of the *K. oxytoca* KP002. Unfortunately, the *K. oxytoca* KP002 found it difficult to control other bacterial contamination during metabolic evolution, and selection was difficult when contamination occurred. As a result, the *K. oxytoca* KP001 was used to replace the *K. oxytoca* KP002 due to its antibiotic resistance gene. To avoid contamination during the alkaline solution connection, the culture was performed in a shake flask with an unregulated pH from the 1st transfer to the 45th. At beginning transfer, the *K. oxytoca* KP002 was grown and transferred to a fresh LB medium containing 20 g/L glucose with a starting OD_{550} of 0.05. As shown in Fig. 4.15, the *K. oxytoca* KP002 consumed only 2.39 g/L of glucose to produce the biomass and succinate at 0.21 g/L and 0.83 g/L respectively, in the first transfer. From the 2nd transfer to 11st transfer, the glucose consumption (≈ 7 g/L), biomass (≈ 0.30 g/L), and succinate production (≈ 2 g/L) were increased by 2.93-fold, 1.43-fold, and 2.41-fold, respectively when compared to the first transfer. On the 12th transfer, twenty mM sodium acetate was added to the medium as an acetyl-CoA source due to the *K. oxytoca* KP001's low ability in acetyl-CoA synthesis and lack of acetate production after removing the *pflB*, *ackA*, and *tdcD* genes. Succinate and biomass increased progressively from the 12th transfer to 21st transfer reaching 5 and 0.50 g/L, respectively. As a result, the growth and succinate production by *K. oxytoca* KC006 strain was restored by the addition of 20 mM sodium acetate. When transferring until 30th, biomass (≈ 0.63 g/L), and succinate production (≈ 5.61 g/L) were increased by 2.1-fold, and 2.81-fold, respectively when compared to the 11th transfer while acetate was detected at only 0.90 g/L as shown in Fig. 4.13-D. However, the glucose consumption was identical. So, the strain at the 30th transfer was chosen as the best representative clone for AM1 transferring and designated *K. oxytoca* KP001-TF30.

Finally, we aimed to increase succinate production by using AM1 medium in our study. As a result, the 30th transfer cell was sub-cultured on AM1 medium containing 20 g/L glucose. However, yeast extract (2 g/L) and sodium acetate 20 (mM) was still added to AM1 medium because the mutant strain could not grow and showed the slow sugar consumption rate in the absence of yeast extract and sodium acetate respectively. In minimal salts medium, their glucose consumption rate (0.20 g/L/h), biomass (0.06 g/L), and succinate production (2.93 g/L) decreased 2.5-fold, 10.83-fold, and 1.92-fold, respectively, compared to the *K. oxytoca* KP001-TF30. From the 3rd to the 15th transfer, the glucose consumption rate, biomass, and succinate production all increased gradually, reaching 0.05 g/L/h, 0.40 g/L, and 6.50 g/L, respectively (Fig. 4.16). However, due to the uncontrolled pH during culture, the sugar consumption rate and succinate productions were identical. To avoid acidification and supply CO_2 during the fermentation, a solution of 6 M KOH and 3 M K_2CO_3 was automatically added to the serial transfers beginning with the 16th transfer. As expected, the glucose concentrations were completely utilized from the 16th to the 30th transfer. Moreover, the maximum biomass and succinate production increased from 0.40 to 1.2 g/L and 6.50 to 16 g/L, respectively, when compared to transfers without the maintaining pH and supply of CO_2, whereas acetate was detected only 1.45 g/L.

Interestingly, succinate productivity increased from 0.09 to 0.23 g/L/h between the 16th and 20th transfers, compared to the 3rd and 15th transfers. The serial transfers were then continued to enhance the succinate production by using 50 g/L of glucose from the 21st transfer to the 30th transfer. The maximum succinate and biomass production reached 37 g/L and 1.68 g/L during the transferring process, while the succinate production and glucose consumption rates were identical. So, the strain from the 30th transfer was chosen as the best representative clone and designated *K. oxytoca* KP001-TF60. When fermentation on 100 g/L of glucose, the *K. oxytoca* KP001-TF60 showed a significant increase in succinate production at a concentration of 53.51 g/L, with yield and productivity of 0.88 g/g and 0.45 g/L/h, respectively, as shown in Fig. 4.16. The concentration, yield and productivity were approximately 26.49-fold, 29-fold, and 7.50-fold, respectively, higher than those of the *K. oxytoca* KMS006. Acetate at a concentration of 1 g/L was only found as a by-product. However, only 60 g/L of glucose was consumed, indicating that this strain is limited. Unexpectedly, the succinate

synthesis yield was 77.40% of the theoretical maximum, or 1.32 mol/mol glucose used, and was 1.05-fold higher than that of the *K. oxytoca* KC004-TF160. These results revealed that metabolic evolution was a viable method for increasing succinate and biomass synthesis in the *K. oxytoca* KP001. However, the limitation of glucose uptake and poor growth when cultured on AM1 medium may be affected in a variety of genetic approaches, requiring *K. oxytoca* KP001-TF60 to supplement with yeast extract during anaerobic fermentation. The yeast extract is an essential component and a source of numerous cofactors and nutrients but also provides oligopeptides and amino acids. These nutrients are fundamental substrates feeding many metabolic pathways, including TCA cycle (Olga et al., 2017). Surprisingly, the fermentation of the *K. oxytoca* KP001-TF60 required only 2 g/L of yeast extract, which was less than the 5 g/L yeast extract supplement required by native succinate bacteria. Therefore, the *K. oxytoca* KP001-TF60 could be used in future research to increase a glucose uptake rate, resulting in tolerance in succinate production.

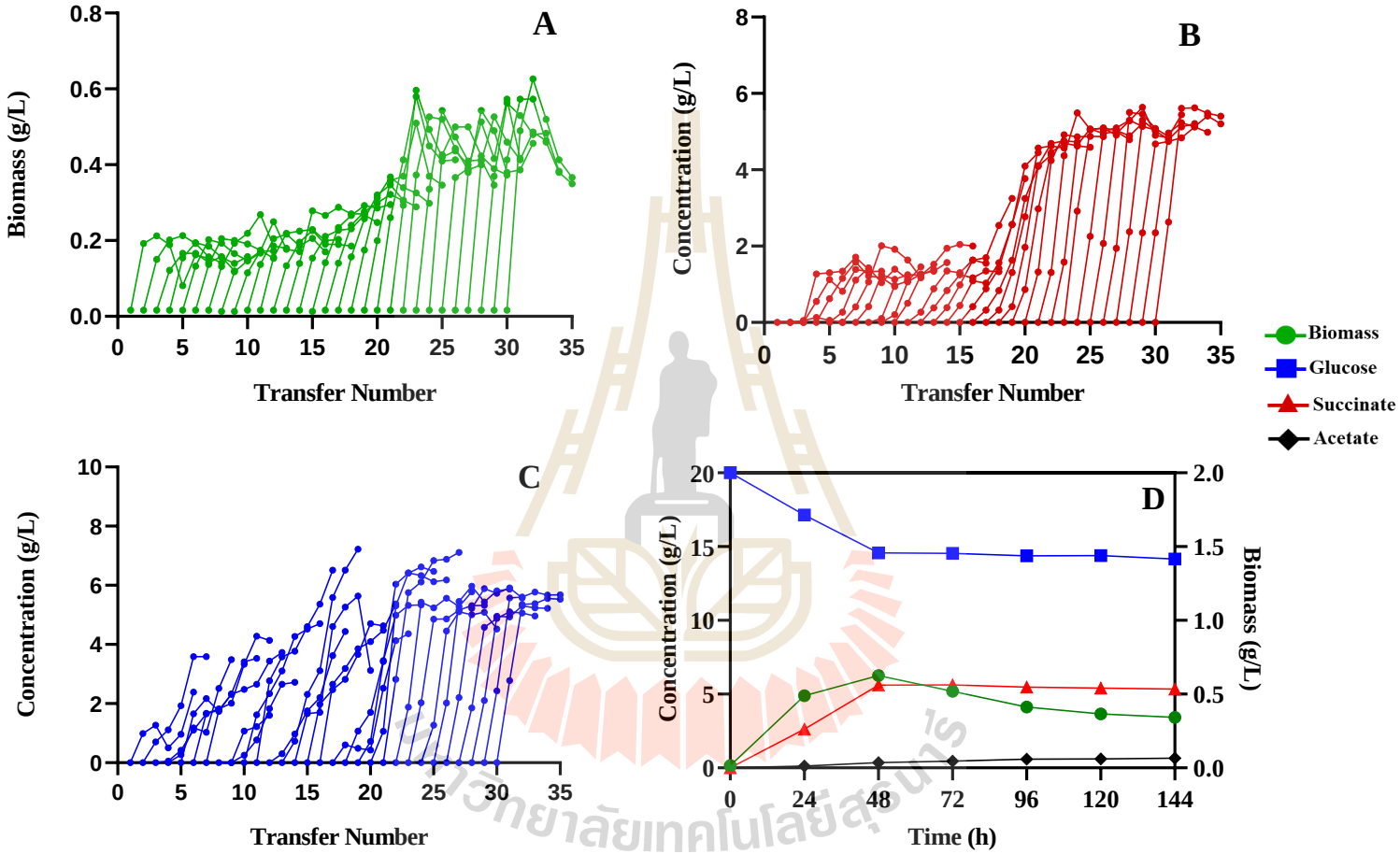

Figure 4.15 Metabolic evolution of *K. oxytoca* KP001 in LB medium containing glucose as a carbon source. A-C Biomass, succinate, and glucose during metabolic evolution, respectively. D Fermentation profile of the evolved *K. oxytoca* KP001-TF30.

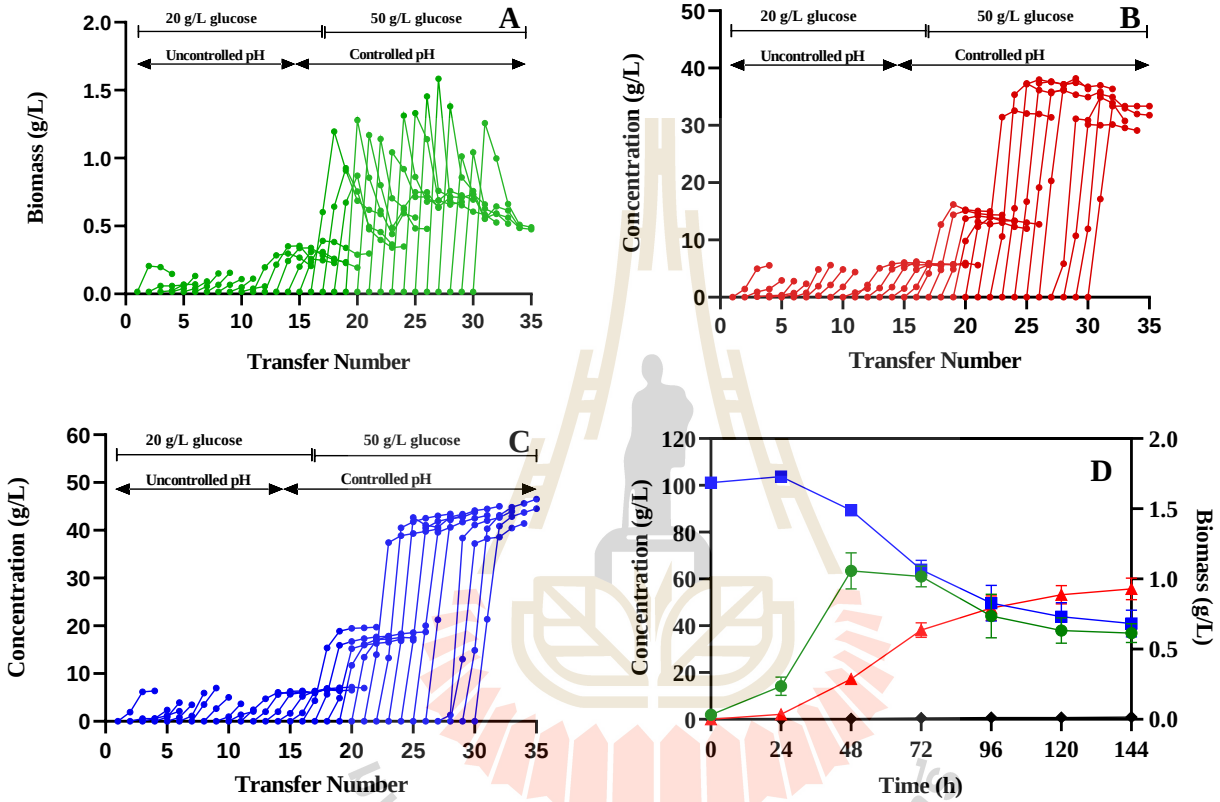

Figure 4.16 Metabolic evolution of *K. oxytoca* KP001 in AM1 medium containing glucose as a carbon source. Transfers 1st to 20th, fermentation with 20 g/L glucose; transfers 21st to 30th, fermentation with 50 g/L glucose. Transfers 1st to 15th, uncontrolled pH during fermentation; transfers 16th to 30th, controlled pH during fermentation. A-C Biomass, succinate, and glucose during metabolic evolution, respectively. D Fermentation profile of the evolved *K. oxytoca* KP001-TF60.

4.2.6 Effect of the combining genetic manipulation and evolutionary adaptation on the metabolites of *K. oxytoca* KP001-TF60

As result of combining metabolic engineering and metabolic evolution, we can improve the yield of succinate production in *K. oxytoca* KP001-TF60. However, the disruption of all genes involved in byproducts synthesis may affect the pyruvate mechanism in glycolysis pathway, resulting in low growth and glucose uptake. So, the internal metabolic flux of the *K. oxytoca* KP001-TF60 was investigated by examining the transcription levels of important enzyme activities in the primary three pathways using the same method used in the *K. oxytoca* KC004-TF160 analysis for conforming and providing global information for future strain development. Figure 4.17 shows the transcription levels of all mutant strains in the primary three pathways. In reductive pathway, when compared to the *K. oxytoca* KC004-TF160, the relative expression levels of the *pck*, *ppc*, *fum*, and *frd* genes in the KP001-TF60 were 3.53-fold, 2.37-fold, 1.36-fold, and 3.95-fold higher, respectively, while the *mdh* gene was about 1.02-fold lower. Surprisingly, the *mdh* gene was up-regulated 1.47-fold, while the *pck* gene was down-regulated by 1.46-fold in the *K. oxytoca* KC006 when compared to the *K. oxytoca* KC004-TF160. In the oxidative branch of TCA cycle, the relative expression levels of the *gltA*, *acnA*, and *icd* genes in *K. oxytoca* KP001-TF60 increased by 3.28-fold, 1.41-fold, and 1.54-fold respectively when compared to the *K. oxytoca* KC004-TF160. When compared to *K. oxytoca* KC006, the *gltA*, and *acnA* genes increased by 2.98-fold and 1.69-fold respectively, whereas the *icd* gene decreased by 1.20-fold. Similarly, when compared to the *K. oxytoca* KC004-TF160, the relative expression levels of the *pykA*, *tdcE*, and *poxB* genes in the *K. oxytoca* KP001-TF60 increased 7.73-fold, 1.58-fold, and 1.52-fold respectively, while the *pdh* and *acs* genes decreased 2.67-fold and 1.35-fold, respectively. Overall result, the relative expression levels of *pck* and *tdcE* in the *K. oxytoca* KP001-TF60 were up-regulated by 7.66-fold and 31.5-fold, respectively, when compared to the *K. oxytoca* KMS006.

As shown above, the transcript level of the *pck* gene in the *K. oxytoca* KP001-TF60 was about 39.89-fold and 10.32-fold higher than that of the *ppc* and *pykA* genes, respectively, even though both genes were up regulated after *tdcD* and *pmd* mutants. Similarly, the *fum* and *frd* genes were up regulated. However, inhibiting both *tdcD* and *pmd* genes reduced *mdh* gene activity, whereas inhibiting only *tdcD* activity increased

mdh gene transcript levels. Simultaneously, inhibiting *tdcD* and *pmd* genes also increased the *gltA*, *acnA*, and *icd* activities, while decreasing *pdh* and *acs* activities in both the *K. oxytoca* KC006 and *K. oxytoca* KP001-TF60. These results show that the combination of metabolic engineering and evolutionary adaptation results in adaptation of the *pck* gene as the main functional enzyme which led to greater improvement of succinate production in improved strains than in the *K. oxytoca* M5A1 and *K. oxytoca* KMS006. The *K. oxytoca* KP001-TF60 is dependent on the *pck* activity in the reductive TCA cycle pathway after ATP imbalance when glycolytic and acetate metabolisms were low in activity. As previously reported, the reaction catalyzed by *pck* gene is accompanied by ATP generation, which discovered that *E. coli* can increase the specific enzyme activity of *pck* gene to obtain more ATP supply (Wu et al., 2007). Furthermore, the reducing metabolic flux to pyruvate is also critical for improving succinate production as evidenced by a decrease in *pykA* activity in the *K. oxytoca* KC004-TF160 to *K. oxytoca* KP001-TF60. As previously study reported, the blocking pyruvate formation via triple deletion of *ptsG*, *pykF* and *pykA* genes produced succinate at a concentration of 2.05 g/L, which was more than 7-fold higher than the *K. oxytoca* M5A1 (0.29 g/L) (Lee et al., 2005). While the blocking of acetate synthesis via *tdcD* activity may increase the flux of acetyl-CoA into the oxidative pathway, resulting in increasing of transcript level of genes associated with the oxidative branch of the TCA cycle such as *gltA*, *acnAB* and *icd* genes in the *K. oxytoca* KP001-TF60. However, under anaerobic conditions, *gltA* activity contributes significantly to cellular biosynthesis rather than energy generation (Park et al., 1994). Therefore, the ATP availability of the *K. oxytoca* KP001-TF60 was limited in the system due to lacking the formation of ATP by *ackA* or *tdcD* activities. Furthermore, even though the inactivating NADH competing pathway increases NADH availability and redirects carbon into the carboxylation pathway to increase succinate yield. However, a variety of genetic approaches had a greater impact on decrease in *pykA and tdcE* activities while the high NADH levels inhibited *pdh* activity in *K. oxytoca* KP001-TF60 (Leibeke et al., 2011). These findings suggest that the *K. oxytoca* KP001-TF60 had low glycolytic and acetyl-CoA activity, resulting in ATP deficiency and redox imbalances, even though ATP could be generated via *pck* activity. These suggested that cellular fitness of the succinate production strains appears to be limited by sub-optimal acetyl-CoA metabolism, while the formation of

acetyl-CoA from pyruvate is required for biosynthesis, subsequent formation of acetate reduces the overall yield. While NADH or NAD+ is one of the most important cofactors, which serves as a cofactor in over 300-oxido-reduction reactions and regulates a variety of enzyme activities and genetic processes (Foster et al., 1990). When the intracellular redox balance was altered (represented by the ratio of NADH to NAD+) either reduced the glucose uptake efficiency of *E. coli* (Berrios-Rivera et al., 2002a, b; Sanchez et al., 2005) or inhibited the cell growth of *S. cerevisiae* (Heux et al., 2006). Therefore, redox and energy balance are important factors in metabolic engineering and strain development. Moreover, the bacterial cell requires more ATP as a transporter to remove succinate out of the cells and keep it steady during fermentation in order to maintain high succinate productivity (Warnecke et al., 2005). These spontaneous mutations could explain why glucose uptake and cell growth were limited in the *K. oxytoca* KP001-TF60. This information could be used to guide us in future genetic modifications to enhance redox and energy balance in order to increase glucose uptake and cell growth in the *K. oxytoca* KP001-TF60.

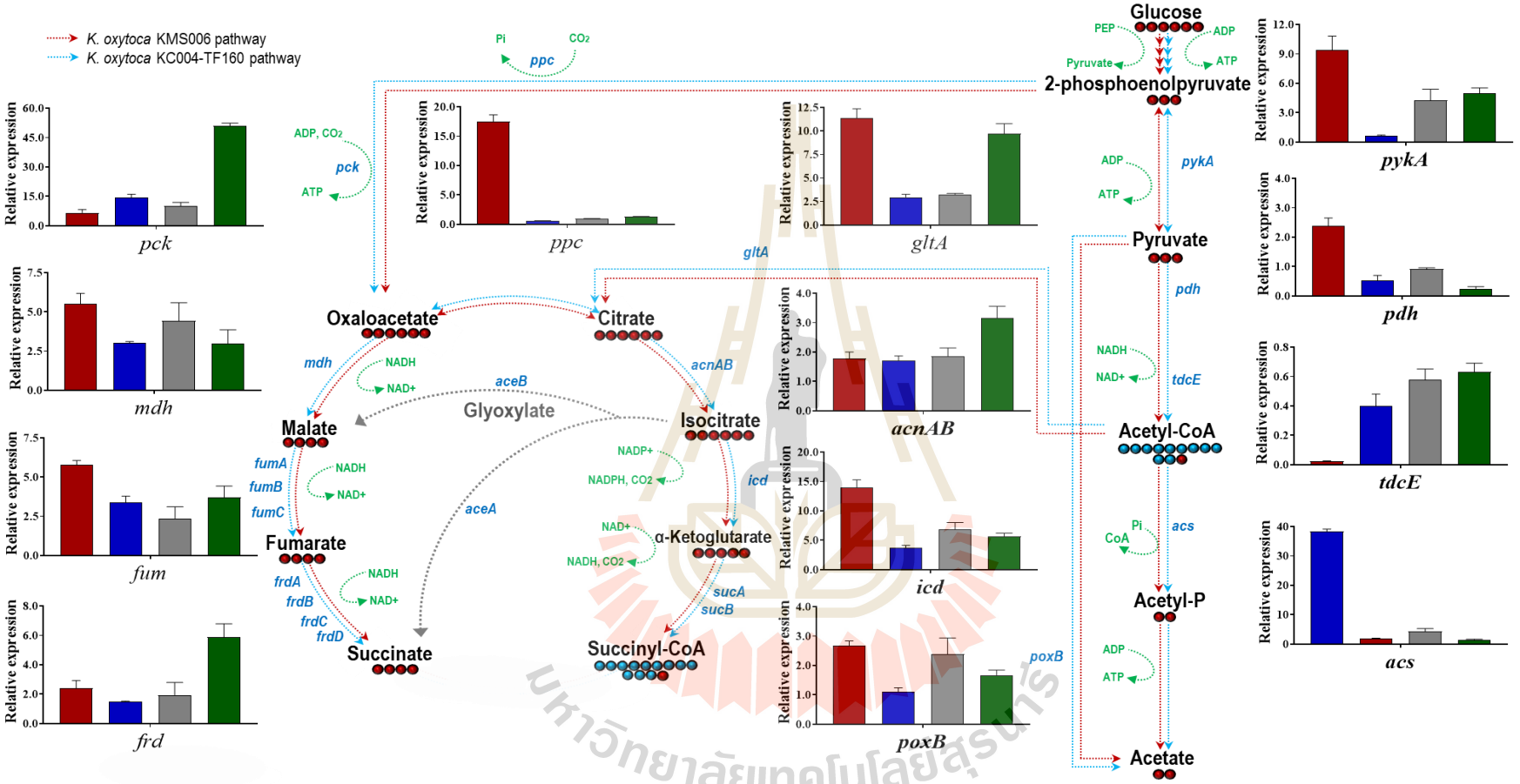

Figure 4.17 Relative expression levels of the major enzyme genes in *K. oxytoca* KMS006, *K. oxytoca* KC004-TF160, *K. oxytoca* KC006 and *K. oxytoca* KP001-TF60. Symbols: *K. oxytoca* KMS006 (Red bars), and *K. oxytoca* KC004-TF160 (Blue bars), *K. oxytoca* KC006 (Gray bars), and *K. oxytoca* KP001-TF60 (Green bars).

4.3 Effect of the combining genetic manipulation and evolutionary adaptation on the DNA sequence gene of *K. oxytoca* KP001-TF60

4.3.1 Genome sequencing

Genomic information was required to gain a clear understanding of the mechanism's activity in the *K. oxytoca* KC004-TF160 and *K. oxytoca* KP001-TF60. After genome sequencing analysis, the genome assembly revealed that *K. oxytoca* KP001-TF60 genome consisted of 42 contigs with a total size of 5,670,048 bps, an N50 contig length of 5,935,402, and a GC content (55.74%). The annotation produced 5,421 genes, of which 5,215 were coding sequences, 127 miscellaneous RNAs (non-categorised non-coding RNA), 3 ribosomal RNAs, 1 transfer-messenger RNA and 75 transfer RNA. According to Table 4.20, the ANNOVAR predicted that the nucleotide, structure, and copy-number of genomic *K. oxytoca* KP001-TF60 were mutated by total variation at approximately 256,464, 251, and 380 positions, respectively. On the genomic *K. oxytoca* KP001-TF60, the nucleotide sequence discovered an insertion and deletion of frameshift about 1,781 and 1,800 positions. InDels are additions or deletions of one or more nucleotides in DNA sequence frameshift (Lin et al., 2017). This results in a significant importance for the molecular evolution of proteins and their coding genes, resulting in an altered coding sequence of a protein that may be useless or a completely new protein (Wang et al., 2022). As above resulted, succinate, biomass product formation, and glucose consumption of the *K. oxytoca* KC004-TF160 and *K. oxytoca* KP001-TF60 were significantly different from the *K. oxytoca* M5A1 after improving the combining metabolic engineering and metabolic evolution techniques. Consequently, a whole genome analysis was performed to decipher the complete set of genes involved in carboxylation, glucose metabolism, catabolite repression, glucose uptake, reductive branch of the TCA cycle and oxidative branch of the TCA cycle.

4.3.2 DNA sequence variation on genes involved in glucose metabolism, catabolite repression and glucose uptake of *K. oxytoca* KP001-TF60

Genes associated with glucose uptake and catabolite repression and glucose uptake by the phosphotransferase system were sequenced (Table 4.2). Only one gene in the glucose phosphotransferase system (PTS) was discovered to have a

non-frameshift mutation. Interestingly, eight hundred forty-nine nucleotides of the glucose-specific PTS enzyme IIBC component (*ptsG* gene) were lost. The *ptsG* gene encodes the enzyme IIBC, a member of the phosphoenolpyruvate (PEP)-dependent functional superfamily that mediates glucose uptake and phosphorylation. In addition, the expression of *ptsG* is regulated at the post-transcriptional level in response to glycolytic flux. With transcriptional regulation, *ptsG* expression is regulated post-transcriptionally via modulation of *ptsG* mRNA stability in response to glycolytic flux in the cells. (Kimata et al., 2001). These findings indicate that the expression of the *ptsG* gene in the PTS system is regulated in a highly complex and dynamic manner in response to various growth environments and carbon source availability. Therefore, the absence of *ptsG* activity in the efficient glucose transporter led to limited cell growth and glucose consumption in *K. oxytoca* KC004-TF160 and *K. oxytoca* KP001-TF60. As previously reported, the destabilization of *ptsG–crp* mRNA was largely eliminated by frameshift mutations in the transmembrane region in *E. coli* (Kawamoto et al., 2015). When the glucose-specific PTS is disrupted, *E. coli* C strain cannot uptake glucose and grows very slowly under anaerobic conditions (Kim et al., 2022). In addition, the N-acetyl-galactosamine permease IIC component 1 or galactosamine-specific PTS enzyme IIC component (*agaC* gene) was discovered in a non-frameshift insertion of about 24 nucleotides in *K. oxytoca* KP001-TF60. It is one of the gene activities in the Aga/Gam system that performs a PTS function (Hu et al., 2013). This is a major carbohydrate active-transport system that catalyzes the phosphorylation of incoming sugar substrates as they pass through the cell membrane. However, this enzyme activity may not be functional due to mutations. Meanwhile, a non-frameshift insertion of about 78 nucleotides was also found in adenylate cyclase (*cyaA* gene). The *cyaA* gene function catalyzes the synthesis of cyclic adenosine 3’, 5’-monophosphate (cAMP) through an intramolecular transfer of ATP's adenylyl group to the 3'-hydroxy group. cAMP is an important signaling molecule that acts as a positive regulator of catabolite-regulated gene expression. Additionally, the cooperative action of cAMP bind and activates CRP can be used to activate operons participating in the use of alternative carbon sources such as lactose and maltose in the absence of glucose (Joseph et al., 1982; Franchini et al., 2015; Kilicaslan et al., 2021).

Therefore, changes in nucleotide sequences of *cyaA* gene may repress its adenylate cyclase activity thus resulting in a decreased level of cAMP-CRP complex. Consequently, this may activate other operons involving in the transporting glucose without requiring PEP utilization. As a result, *K. oxytoca* KC004-TF160 and *K. oxytoca* KP001-TF60 may be able to uptake glucose through non-PTS system including ABC transporters such as Mal ABC transporter, Mgl ABC transporter, ExuT and GalP. When glucose is transported via non-PTS system, the succinic acid production increases due to an increase in the PEP pool, similar to *ptsG* deficient *E. coli* (Chatterjee et al., 2001).

Table 4.2 Nucleotide variations in genes involved in glucose metabolism, catabolite repression and glucose uptake in *K. oxytoca* KP001-TF60.

Gene	Protein	Function	Nucleotides (bp)		Mutation	
			M5Al	KP001-TF60	Type	Amount (bp)
pgi	Glucose-6-phosphate isomerase	Catalyzes the reversible isomerization of glucose-6-phosphate to fructose-6-phosphate	1,650	1,650	NM	NM
pfkA	ATP-dependent 6-phosphofructokinase isozyme 1	Catalyzes the phosphorylation of D-fructose 6-phosphate to fructose 1,6-bisphosphate by ATP	963	963	NM	NM
pfkB	ATP-dependent 6-phosphofructokinase isozyme 2	Catalyzes the phosphorylation of D-fructose 6-phosphate to fructose 1,6-bisphosphate by ATP	930	930	NM	NM
fbaA	Fructose-bisphosphate aldolase class 2	Catalyzes glyceraldehyde 3-phosphate to fructose 1,6-bisphosphate	1,080	1,080	NM	NM
fbaB	Fructose-bisphosphate aldolase class 1	Catalyzes the reversible conversion of glyceraldehyde 3-phosphate to fructose 1,6-bisphosphate	1,053	1,053	NM	NM
tpiA	Triosephosphate isomerase	Catalyzes the conversion of dihydroxyacetone phosphate to D-glyceraldehyde-3-phosphate	768	768	NM	NM
gapA	Glyceraldehyde-3-phosphate dehydrogenase A	Catalyzes the glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate using the cofactor NAD	996	996	NM	NM
pgk	Phosphoglycerate kinase 1	Catalyzes one of the two ATP producing reactions in the glycolytic pathway	1,164	1,164	NM	NM

Table 4.2 (continued).

Gene	Protein	Function	Nucleotides (bp)		Mutation	
			M5Al	KP001-TF60	Type	Amount (bp)
ppsA	Phosphoenolpyruvate synthase	Catalyzes the phosphorylation of pyruvate to phosphoenolpyruvate	2,379	2,379	NM	NM
crr	PTS system glucose specific EIIA component	Major carbohydrate active transport system	510	510	NM	NM
cyaA	Adenylate cyclase	Catalyzes the formation of the second messenger cAMP from ATP	2,550	2,628	Non-frameshift insertion	78
crp	CRP-cyclic-AMP DNA-binding transcriptional dual regulator	Regulates the expression of over 180 genes which is involved in cAMP and glucose uptake	633	633	NM	NM
ptsG	PTS system glucose specific EIICB component	Mediates the uptake and concomitant phosphorylation of glucose.	1,434	585	Non-frameshift deletion	849
ptsI	Phosphoenolpyruvate-protein phosphotransferase	Mediates carbohydrate uptake and often serves in carbon control.	1,728	1,728	NM	NM
ptsH	Phosphocarrier protein HPr	Major carbohydrate active-transport system of PTS system	258	258	NM	NM
agaC	N-acetyl galactosamine permease IIC component 1	Major carbohydrate active-transport system of PTS system	780	804	Non-frameshift insertion	24

NM = No mutations.

4.3.3 DNA sequence variation on genes involved in PEP carboxylation and the reductive branch of the TCA cycle of *K. oxytoca* KP001-TF60

The increased *pck* activity or decreased *ppc* activity in carboxylation of the developed strain did not result from a DNA sequence mutation on genomic DNA as shown in Table 4.3. Furthermore, no mutations were found in the *Cra* protein, which has been reported to be involved in the expression of the *pck* gene (Saler et al., 1996). Unexpectedly, a frameshift mutation in the carbon storage regulator B gene (*CsrB*) (15-bp deletion of position 1,673 bps) was discovered after completing *K. oxytoca* KP001-TF60 strain construction. *CsrB* activity is one of two small regulatory RNAs that antagonize the activity of the carbon storage regulator A gene (*CsrA*) (Liu et al., 1997; Weilbacher et al., 2003). The *CsrA* system has been reported to regulate the expression of *pck* gene and other genes involved in glucose metabolism by influencing mRNA stability (Babitzke et al., 2007; Suzuki et al., 2002). The *CsrB* mutation may increase *CsrA* activity, resulting in higher levels of *pck* transcripts in the KC004-TF160 and KP001-TF60. Meanwhile, the expression levels of *CsrB* activity are positively regulated in amino acid levels, changes in metabolite and protein levels in glycolysis and the TCA cycle (McKee et al., 2012). Then, this may postulate that *CsrB* mutation may affect the biomass production in the *K. oxytoca* KP001-TF60. Among genes in the reductive branch of TCA pathway, only fumarate reductase flavoprotein subunit (*frdA* gene) was found to be a non-frameshift mutation in *K. oxytoca* KP001-TF60. However, the non-frameshift mutation had no effect on *frdA* activity for functional fumarate-to-succinate conversion in the developed strains. As previously described, non-frameshifting insertion/deletion variants result in the gain or loss of several nucleotides divisible by three, with no disruption to the mRNA's reading frame (Pagel et al., 2019).

Table 4.3 Nucleotide variations in genes involved in carboxylation and the reductive branch of the TCA cycle in *K. oxytoca* KP001-TF60.

Gene	Protein	Function	Nucleotides (bp)		Mutation	
			M5Al	KP001-TF60	Type	Amount (bp)
pck	Phosphoenolpyruvate carboxykinase (ATP)	Catalyzes the conversion of phosphoenolpyruvate to oxaloacetate	1,641	1,641	NM	NM
ppc	Phosphoenolpyruvate carboxylase	Catalyzes the conversion of phosphoenolpyruvate to oxaloacetate	2,652	2,652	NM	NM
CsrA	Carbon storage regulator A	Regulates the translation initiation and/or mRNA stability for its effects on central carbon metabolism	186	186	NM	NM
CsrB	Carbon storage regulator B	One of two *CsrA*-binding small regulatory RNAs	369	362	Frameshift Deletion	7
CsrC	Carbon storage regulator C	One of two *CsrA*-binding small regulatory RNAs	255	255	NM	NM
uvry	Response regulator UvrY	Regulates carbon metabolism through the *CsrA/CsrB* regulatory system	657	657	NM	NM
barA	Signal transduction histidine-protein kinase BarA	Regulates carbon metabolism through the *CsrA/CsrB* regulatory system	2,754	2,754	NM	NM
mdh	Malate dehydrogenase	Catalyzes the reversible oxidation of malate to oxaloacetate	939	939	NM	NM
fumA	Fumarate hydratase class I, aerobic	Catalyzes the reversible hydration of fumarate to (S)-malate	1,647	1,647	NM	NM

Table 4.3 (continued).

Gene	Protein	Function	Nucleotides (bp)		Mutation	
			M5Al	KP001-TF60	Type	Amount (bp)
fumB	Fumarate hydratase class I, anaerobic	Catalyzes the reversible hydration of fumarate to (S)-malate	1,656	1,656	NM	NM
fumC	Fumarate hydratase class II	Catalyzes the stereospecific interconversion of fumarate to L-malate	1,401	1,401	NM	NM
fumD	Fumarase D	In vitro catalyzes the addition of water to fumarate, forming malate	213	213	NM	NM
frdA	Fumarate reductase flavoprotein subunit	Responses the catalysis of fumarate and succinate interconversion	1,791	1,668	Non-frameshift deletion	123
frdB	Fumarate reductase iron-sulfur subunit	Responses the catalysis of fumarate and succinate interconversion	735	735	NM	NM
frdC	Fumarate reductase subunit C	Responses the catalysis of fumarate and succinate interconversion	396	396	NM	NM
frdD	Fumarate reductase subunit D	Responses the catalysis of fumarate and succinate interconversion	360	360	NM	NM

NM = No mutations.

4.3.4 DNA sequence variation on genes involved in the oxidative branch of the TCA cycle, glycolysis pathway, and pyruvate catabolism of *K. oxytoca* KP001-TF60

As expected, the left-over nucleotide sequences of *adhE*, *ldhA*, *ackA*, *budAB*, *pflB*, *tdcD* and *pmd* genes were found to be the correct size, as designed in genetic engineering experiments (Table 4.4). Unexpectedly, the nucleotide of the *pta* gene did not mutate, despite previously reported double deletion with the *ackA* gene. The *pta* gene may have been returned due to a DNA repair process during metabolic evolution while improving the *K. oxytoca* KC004 strain. This can even be beneficial. However, *pta* activity had no effect in acetate production when the *tdcD* gene in the *K. oxytoca* KC006 was deleted. The results demonstrated that pyruvate was mostly fluxed to acetyl-CoA synthesis as an intermediate for biosynthesis in the TCA cycle's oxidative branch. While the decreasing or increasing of transcript level of all genes involved in pyruvate metabolism (*pykA*, *pykF, tdcE*, *poxB*, and *acs* genes) and oxidative branch of TCA cycle (*gltA*, *acnAB*, and *icd* genes) did not result from a nucleotide sequence mutation. Interestingly, pyruvate dehydrogenase complex 2 (*aceF* gene) was found 15 nucleotides non-frameshift insertion. This gene, one of three pyruvate dehydrogenase complex enzyme (*pdh*) is synthesizing acetyl-CoA from pyruvate which involved in energy production and conversion (Tohsato et al., 2010; Moxley et al., 2021). This mutation may cause the 5% residual activity of *pdh* gene in developed strain for converting pyruvate into acetyl-CoA under oxygen limitation. In addition, the pyruvate formate lyase activating enzyme (*pflA* gene) was found 90 nucleotides non-frameshift insertion. Under anaerobic conditions, *pflA* gene usually acts as an activator for the *pflB* gene, which converts pyruvate to acetyl-CoA in bacteria (Zhang et al., 2018). Therefore, this has no impact on the KP001-TF60 strain's deficient *pflB*'s ability to convert pyruvate. Furthermore, 56 nucleotide sequence frameshift insertions in the L-lactate dehydrogenase were discovered (*lldD* gene). It functions in aerobic respiration and has a role in anaerobic nitrate respiration to interconvert pyruvate and L-lactate (Nishimura et al., 1983; Luchi et al., 1994). Since, *lldD* insertion mutant has lost the ability to grow on L-lactate as the sole source of carbon and energy but can still utilize D-lactate (Dong et al., 1993). This spontaneous mutation may exist when *pmd* was activated to produce lactate and used as energy source and redox balance in

K. oxytoca KC006. However, *lldD* gene may be unable to function in *K. oxytoca* KP001-TF60 because L-lactate and D-lactate productions were not observed. Besides a non-frameshift insertion was also discovered on the *mgsA* gene by about 15 nucleotides. However, this gene has low activity in response to lactate production, despite previous reports of lactate production (Weng et al., 2018).

Table 4.4 Nucleotide variations in genes involved in the oxidative branch of the TCA cycle, glycolysis pathway, and pyruvate catabolism in *K. oxytoca* KP001-TF60.

Gene	Protein	Function	Nucleotides (bp)		Mutation	
			M5Al	KP001-TF60	Type	Amount (bp)
pykA	Pyruvate kinase II	Catalyzes the conversion of PEP into pyruvate under anaerobic	1,443	1,443	NM	NM
pykF	Pyruvate kinase I	Catalyzes the conversion of PEP into pyruvate under aerobic	1,413	1,413	NM	NM
tdcE	2-ketobutyrate formate-lyase	Catalyzes the conversion of pyruvate into acetyl-CoA	2,295	2,295	NM	NM
poxB	Pyruvate dehydrogenase	Catalyzes the conversion of pyruvate into acetate	1,719	1,719	NM	NM
acs	Acetyl-coenzyme A synthetase	Catalyzes the conversion of acetate into acetyl-CoA	1,959	1,959	NM	NM
pflA	Pyruvate formate-lyase 1-activating enzyme	Activation of *pflB* under anaerobic conditions	741	831	Non-frameshift insertion	90
focA	Formate channel FocA	Involved in the bidirectional transport of formate	858	816	Non-frameshift insertion	42
aceE	Pyruvate dehydrogenase complex 1	Component of *pdh* complex, that catalyzes the pyruvate to acetyl-CoA and CO_2	2,664	2,664	NM	NM

Table 4.4 (continued).

Gene	Protein	Function	Nucleotides (bp)		Mutation	
			M5Al	KP001-TF60	Type	Amount (bp)
aceF	Acetyltransferase	Component of *pdh* complex, that catalyzes the pyruvate to acetyl-CoA and CO_2	1,875	1,890	Non-frameshift insertion	15
pdh	Pyruvate dehydrogenase complex repressor	Catalyzes the conversion of pyruvate into acetyl-CoA	780	780	NM	NM
lldD	L-lactate dehydrogenase	Catalyzes the conversion of L-lactate to pyruvate	1,236	1,287	Frameshift insertion	56
mgsA	Methylglyoxal synthase	Catalyzes the formation of methylglyoxal from dihydroxyacetone phosphate	459	474	Non-frameshift insertion	15
gltA	Citrate synthase	Catalyzes the formation of citrate from acetyl-CoA and/or oxalacetate	1,284	1,284	NM	NM
acnA	Aconitate hydratase A	Catalyzes the reversible isomerization of citrate to isocitrate via cis-aconitate	2,673	2,673	NM	NM
acnB	Aconitate hydratase B	Catalyzes the reversible isomerization of citrate to isocitrate via cis-aconitate	2,598	2,598	NM	NM
icd	Isocitrate dehydrogenase	Catalyzes the formation of citrate from acetyl-CoA and/or oxalacetate	1,251	1,251	NM	NM
adhE	Bifunctional aldehyde-alcohol dehydrogenase	Catalyzes the acetyl-CoA to acetaldehyde and then to ethanol	2,676	1,287	Non-frameshift deletion	1,389

Table 4.4 (continued).

Gene	Protein	Function	Nucleotides (bp)		Mutation	
			M5Al	KP001-TF60	Type	Amount (bp)
ackA	Acetate kinase	Catalyzes the formation of acetyl phosphate from acetate and ATP	1,203	0	Non-frameshift deletion	1,203
pta	Phosphate acetyltransferase	Catalyzes the reversible interconversion of acetyl-CoA and acetyl phosphate	1,440	1,440	NM	NM
ldhA	D-lactate dehydrogenase	Catalyzes the formation of D-lactate from pyruvate	1,743	114	Frameshift deletion	1,630
budA	Alpha-acetolactate decarboxylase	Catalyzes the formation of acetoin from acetolactate	459	0	Non-frameshift deletion	459
budB	Catabolic -acetolactate synthase	Catalyzes the formation of acetolactate from pyruvate	1,284	0	Non-frameshift deletion	0
pflB	Pyruvate formate-lyase	Catalyzes the formation of acetyl-CoA and formate from pyruvate	2,283	1,158	Non-frameshift deletion	1,125
tdcD	Propionate kinase	Catalyzes the conversion of propionyl phosphate and ADP to propionate or acetate and ATP	1,215	0	Non-frameshift deletion	1,215
pmd	D-lactate dehydrogenase	Catalyzes the conversion of L-lactate to pyruvate	945	0	Non-frameshift deletion	945

NM = No mutations.

4.4 Succinate fermentation

4.4.1 Effect of different carbon sources on the succinate production

So far, developing a bio-based industry for succinate production has necessitated the capacity of producing organisms to consume a diverse variety of sugar feedstocks and generate succinate in high yields in order to use the least expensive raw material (Andersson et al., 2007). Recently, low-cost sugar feedstocks derived from agricultural and industrial wastes such as lignocellulosic biomass, sugarcane molasses, and glycerol have been used to lower the fermentation process's production cost. Therefore, the capability of the developed strains to produce succinate acid from diverse carbon sources was investigated in order to provide global information for future strain development using alternative carbon sources. The developed strains were fermented in AM1 medium with pure 50 g/L glucose, sucrose, maltose, fructose, lactose, xylose, and glycerol. As expected, the highest titer (41.77 g/L), yield (0.84 g/g), and productivity (0.87 g/L/h) of succinate production by the *K. oxytoca* KC004-TF160 was obtained from glucose fermentation (Figure 4.18a). While the *K. oxytoca* KP001-TF60 produced succinate at concentration of 36.92 g/L with a yield and productivity of 0.80 g/g and 0.45 g/L/h, respectively (Data not shown). Unfortunately, the resulting *K. oxytoca* KP001-TF60 possesses low glucose utilization and yeast extract dependence, which were not suitable for industrial production. Therefore, this stain was not evaluated in other sugar fermentations. As showed in Fig. 4.19b-e, the succinate titer from maltose fermentation (40.9 g/L) was comparable, while fermentation with sucrose, fructose and xylose resulted in succinate production of 35.43, 37.62, 17.62, and 14.24 g/L, respectively, which were lower than with glucose as a carbon source. The yield of succinate by maltose, sucrose, fructose, and xylose resulted in a yield 0.81, 0.82, 0.80, and 0.62 g/g, respectively. The lowest yield of succinate production was observed from lactose (0.41 g/g), which was lower than with glucose as a carbon source. In comparison to glucose, the productivity of succinate fermentation from maltose, sucrose, and fructose resulted in 0.56, 0.37, and 0.39 g/L/h, respectively, while lactose and xylose fermentation had a succinate productivity of less than 0.20 g/L/h (Fig. 4.18a-e).

As expected, the *K. oxytoca* KC004-TF160 and *K. oxytoca* KP001-TF60 showed succinate production from glucose, higher than those of the *K. oxytoca* M5A1 and *K. oxytoca* KMS006 as shown in Fig. 4.19. While succinate production by *K. oxytoca* KMS006 was constant or increased slightly when compared to those of the *K. oxytoca* M5A1. Obviously, the succinate titer, yield, and productivity from glucose by *K. oxytoca* KC004-TF160 increased 8.68-fold, 10.50-fold, and 29-fold, respectively, when compared to those of the *K. oxytoca* KMS006. Similarly, succinate titer, yield, and productivity of *K. oxytoca* KP001-TF60 increased 7.615-fold 7, 1015-fold and 15-fold, respectively, when compared to those of the *K. oxytoca* KMS006. However, the biomass of *K. oxytoca* KC004-TF160 and *K. oxytoca* KP001-TF60 was reduced by 1.31-fold and 1.14-fold, respectively, compared to those of the *K. oxytoca* M5A1, while the biomass of the *K. oxytoca* KMS006 was constant or decreased slightly. For other sugar fermentation, the *K. oxytoca* KC004-TF160 produced more succinate from maltose, sucrose, fructose, lactose, and xylose than the *K. oxytoca* M5A1 and *K. oxytoca* KMS006. In contrast to the *K. oxytoca* KMS006, the succinate titer from maltose, sucrose, fructose, lactose, and xylose by the *K. oxytoca* KC004-TF160 increased 11.57-fold, 12.47-fold, 81.78-fold, 8.80-fold, and 24.13-fold, respectively, while the yield increased 10.25-fold, 13.42-fold, 6.83-fold, and 12.4-fold, accordingly.

Furthermore, succinate productivity increased up to14-fold, 12-fold, 13-fold, 9-fold, and 15-fold, respectively (Fig. 4.18a-c). Acetate (≥ 8 g/L) was only detected as by-products, which was lower than in the *K. oxytoca* M5A1 and *K. oxytoca* KMS006. When cultured in glucose, maltose, sucrose, fructose, lactose, and xylose, the biomass of the *K. oxytoca* KC004-TF160 was reduced by 1.21-fold, 1.06-fold, 1.16-fold, 1.11-fold, and 1.56-fold, respectively, compared to those of the *K. oxytoca* M5A1, while the biomass of KMS006 strain was constant or decreased slightly. As shown in Table 4.4, a comparison of recently published succinate producers by different carbon sources, the *K. oxytoca* KC004-TF160 produced slightly more succinate (0.82 g/g) from sucrose fermentation than engineered native producers such as *A. succinogenes* NJ113 (0.77 g/g) and *M. succinogenes* PALFK (0.74 g/g) (Jiang et. al., 2013; Lee et. al., 2016). In addition, the succinate yield by *K. oxytoca* KC004-TF160 was nearly the developed *E. coli* KJ122-pKJUC-24T strain. The final succinate concentration and yield were 53.40 g/L and 0.90 g/g respectively, (Chan et al., 2012). Unlike sucrose,

the *K. oxytoca* KC004-TF160 produced comparable succinate yield (0.80 g/g) from fructose fermentation, comparing with SBS550MG (0.82 g/g) strains (Wang et al., 2011). Unexpectedly, the *K. oxytoca* KC004-TF160 produced more succinate than the established *E. coli* BA305 strain due to a more over-expressed *pck* gene (Liu et al., 2013). However, succinate synthesis from xylose in our strain was lower than in previously engineered *E. coli* KJ12201 strain, due to the combination of XylFGH transporter deletion and evolutionary adaptability (Khunnonkwao et al., 2018). As previously stated, when fermented on maltose, sucrose, and fructose, the *K. oxytoca* KC004-TF160 exhibited succinate production yields and productivity in the ranges of 0.50-0.94 g/g substrates and 0.3 and 1.56 g/ L h for a wide range of substrates. As previously mentioned, the transport mechanisms for different sugars were different, which may explain the disparities in yield and productivities (Almqvist et. al., 2016). Nonetheless, the *K. oxytoca* KC004-TF160 showed that it was able to produce succinic acid from a variety of carbon sources with minimal byproduct formation. Therefore, the *K. oxytoca* KC004-TF160 could be future strain development using alternative carbon sources for the commercial production of succinate.

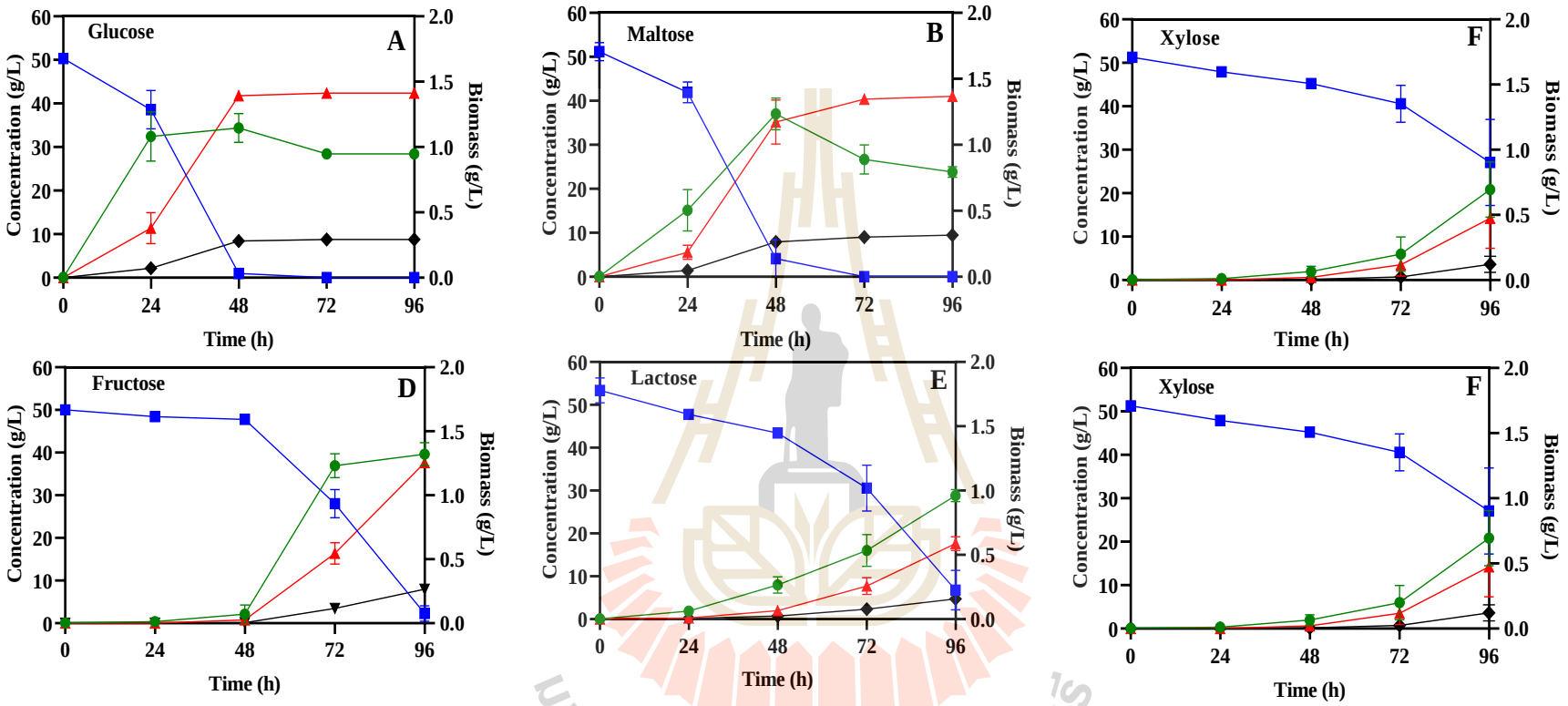

Figure 4.18 Fermentation profile of *K. oxytoca* KC004-TF160 for succinate production in AM1 medium with different carbon source.

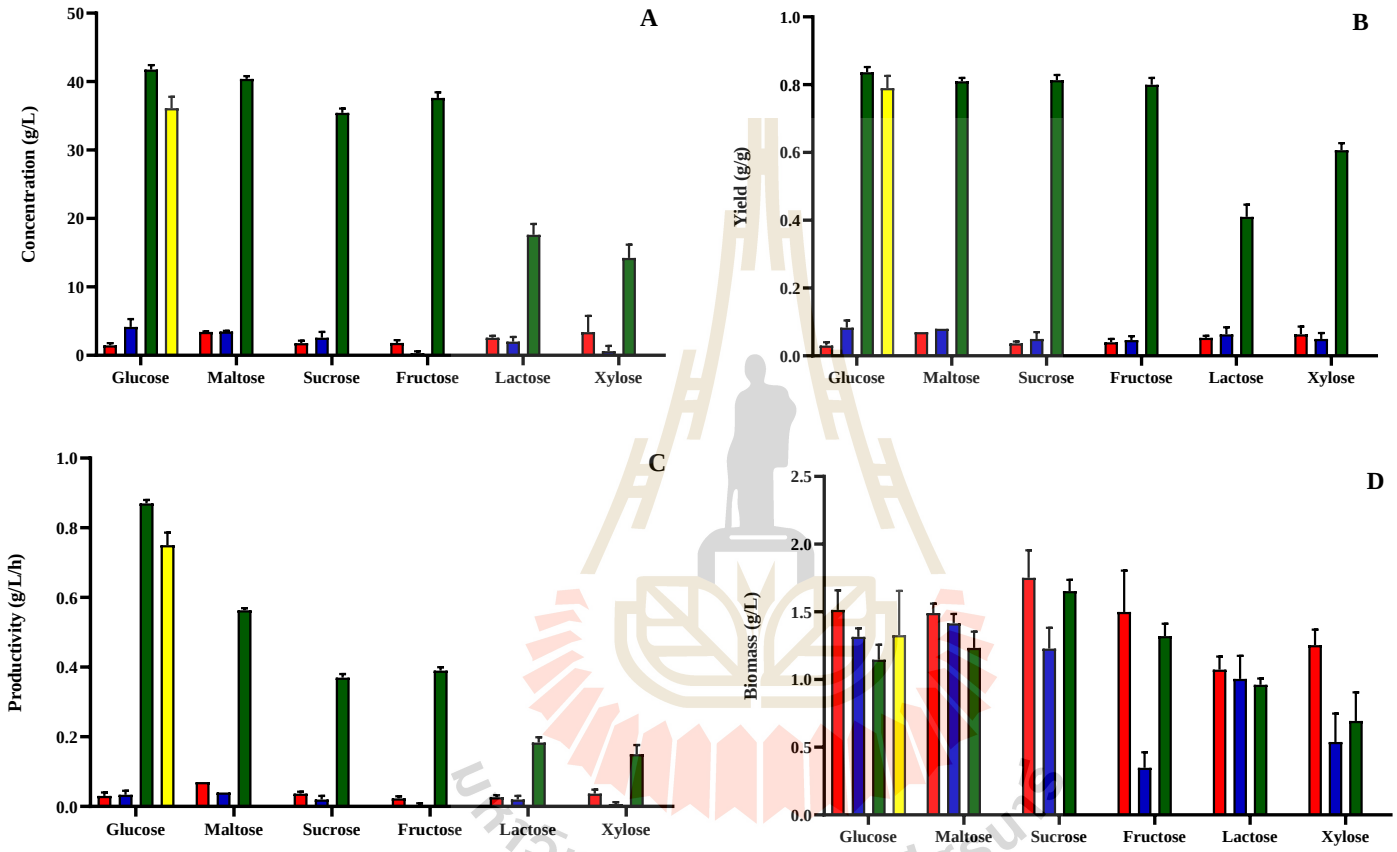

Figure 4.19 Comparison of succinate production by different *K. oxytoca* strains in AM1 medium with different carbon sources. Symbols: MA51 (red bars), KMS006 (blue bars), KC004-TF160 (green bars) and KP001-TF60 strains (yellow bars). Maximum concentration (A), yield (B), and productivity (C) succinate production (D) Maximum biomass.

4.4.2 The succinate production in a 5-L bioreactor from glucose by *K. oxytoca* KC004-TF160

Batch fermentation was performed in 5-L stirred bioreactors with a 2.5 L cultured volume of AM1 broth supplemented with 100 g/L of glucose and 100 mM $KHCO_3$ at 37 °C, 200 rpm. The *K. oxytoca* KC004-TF160 was preliminarily fermented by inoculating at the initial OD_{550} of 0.01 and produced succinate at the concentration of 77.70 g/L with the yield and productivity of 0.78 g/g and 0.59 g/L/h, respectively. The yield of succinate dropped by 6% when compared to the small-scale fermentation. To enhance succinate production, a large-scale batch experiment was carried out with a higher starting OD_{550} of 0.1. Only productivity was significantly increased by about 10%, while the concentration and yield of succinate production remained unchanged. Previously, increasing succinate production was achieved by optimizing CO_2 supply (jiang et al., 2017). The main advantage of succinate production in anaerobic fermentation is that CO_2 could be fixed when PEP is decarboxylated to oxaloacetate. Theoretically, 1 mol of CO_2 is required to form 1 mol of succinic acid. Higher CO_2 level (100 mol CO_2/100 mol glucose) resulted in increased succinic acid production at the expense of ethanol and formic acid (Vaswani, 2010). Therefore, the mixed alkali and carbonate solution ratio was optimized in order to enhance succinate production in scale-up batch fermentation. To optimize the condition of the CO_2 supply, the mixing solution ratio of 6:1 (3 M K_2CO_3 and 6 M KOH) was automatically added to the culture during succinate fermentation. Results showed that succinate at 82.31 g/L concentration with yield and productivity of 0.82 g/g and 0.62 g/L/h, respectively, resulted in a 5.12% increase in yield when compared to the mixing solution ratio of 4:1, but productivity did not improve. A higher initial OD_{550} of 0.5 was assessed for succinate production. the non-labeled inoculum with subsequent labeling measurements (Wittmann, 2007). The succinate yield (0.84 g/g) and productivity (0.87 g/L/h) of the *K. oxytoca* KC004-TF160 were significantly improved by about 7.69% and 33.85% when compared to the small-scale fermentation. Acetate at 14 g/L was only detected as a by-product. Meanwhile, the *K. oxytoca* KP001-TF60 was fermented by the best parameter from the *K. oxytoca* KC004-TF160 and produced succinate at a concentration of 36.74 g/L with a yield and productivity of 0.86 g/g and 0.26 g/L/h, respectively. The yield of succinate increased by 2.38% when compared to the

K. oxytoca KC004-TF160. Acetate at >1 g/L was only detected as a by-product. However, glucose remained at around 47 g/L after fermentation was completed. To enhance succinate production, a large-scale batch experiment was carried out with a higher agitation (400 rpm) and produced succinate at the concentration of 44.41 g/L with a yield and productivity of 0.88 g/g and 0.31 g/L/h, respectively was observed. While acetate (>1 g/L) did not increase. When compared to 200 rpm operation, the concentration, yield, and productivity increased by about 20.88%, 2.33%, and 19.23%, respectively. In addition, biomass also increased by about 15.56% when compared to 200 rpm operation. This result may suggest that microaerobic fermentation occurred at 400 rpm, resulting in the *K. oxytoca* KP001-TF60 producing succinate via combining anaerobic fermentation and glyoxylate route. Because NADH production and consumption in *K. oxytoca* KP001-TF60 need to be balanced, appropriate flux proportioning among succinate routes is required. As previously demonstrated, combined pathways resulted in the most efficient conversion of glucose to succinate with the highest yield, requiring only 1.25 moles of NADH per mole of succinate, as opposed to the sole fermentative pathway, which requires 2 moles of NADH per mole of succinate (Sanchez et al., 2005). In conclusion, 2 moles of succinate can be formed from 2 moles of acetyl-CoA, 1 mole of OAA, and 1 mole of NADH. Hence, this result could confirm that the KP001-TF60 strain can synthesize succinate using a combination of the two pathways, resulting in the highest succinate yield under fully anaerobic conditions. However, when compared to the *K. oxytoca* KC004-TF160, the ability of the *K. oxytoca* KP001-TF60 to consume glucose decreased by about 50%, making it unsuitable for industrial-scale succinate production. While the *K. oxytoca* KC004-TF160 showed higher performance as a candidate for succinate production on an industrial scale.

As shown in Table 4.5, a comparison of recently published succinate producers, the *K. oxytoca* KC004-TF160 and *K. oxytoca* KP001-TF60 demonstrated higher capabilities in succinate synthesis in terms of concentration, yield, and specific productivity, when compared to other previously reported engineered *Klebsiella* sp. strains. Native succinate producers, such as *M. succiniciproducens* MBEL55E (wild type) produced succinate at concentration of 10.49 g/L with a yield of 0.45 g/g glucose and when were modified to disrupt acetate, formate, and lactate accumulation by deleting

the *ldhA*, *pflB*, *pta* and *ackA* genes, resulting LPK7 strain (Lee et al., 2006). An engineered strain LPK7 was developed and produced 52.4 g/L succinate with a yield of 0.76 g/g glucose and a productivity of 1.8 g/L in fed-batch fermentation. Other than native producer, *C. acetoacidophilum* R was engineered by disrupting lactate formation and overexpressing the pyruvate carboxylase gene, the final succinate concentration and yield were 107 g/L and 0.88 g/g respectively (Liu et al., 2015). *Basfia succiniciproducens* was expressed to activate glyoxylate shunt and malate synthase resulting in increased succinate yield (0.87 g/g glucose) by about 77.55% when compared to with *K. oxytoca* M5A1 (0.49 g/g glucose). Similarly, *A. succinogenes* variant FZ 53 produced over 94 g/1 in 34.25 h under fermentation used a total of 95 g of $MgCO_3$, representing a 54% improvement over the parent 130Z. Although they could produce succinate with a high productivity, the strain's complicated nutritional requirements resulted in a high fermentation cost, limiting the potential of commercialization (Jiang et al., 2017). *E. coli* has also been utilized to produce succinate due to plentiful of genetic tools available, the simplicity of the culture media, and the rapid cell growth. So far, *E. coli* can be classified into four major strategies: improvement of substrate or product transportation, augmentation of pathways directly engaged in succinate synthesis, deletion of pathways implicated in by-product accumulation, and combinations of these methods (Cheng et al., 2013). *E. coli* KJ060 was constructed by a combination of gene deletions on *E. coli* ATCC8739 and metabolic evolution, producing 86.6 g/L of succinate with a yields of 0.92 g/g glucose and a productivity of 0.9 g /L/ h in batch fermentations using mineral salts medium under anaerobic environment. However, acetate was produced reach to 15 g/L as by-product during fermentation (Jantama et al., 2008a). Analogously, a new generation KJ134 strain was developed to decrease acetate accumulation. The yield of KJ134 was up to 1 g/g glucose, and the by-product acetate was decreased by 85 % when compared with KJ060 strain (Jantama et al., 2008b). Currently, *E. coli* HX024 strain was modified to improve succinate synthesis by deactivating competing fermentation pathways and boosting energy and precursor supplies. suggesting that they exhibited a synergistic effect for improving succinate yield (Zhu et al., 2014). This strategy resulted in a succinate production of 0.88 g/g (88% of theoretical maximum), comparable to the KJ134 strain. Besides from decreased by-product formation, *E. coli* has been

developed for high succinate synthesis by collaborative control of CO_2 supply and fixation such as *E. coli* Tang1527 and AFP111 strains (Yu et al., 2016; Li et al., 2017). This strategy increased productivity to 1.24 g/L/h, but the yield of succinate production remained approximately the same as in the previously engineered strain. Furthermore, expensive nutrients such as yeast extract and peptone, antibiotics, and IPTG in the medium were required for maintaining expression plasmids during fermentation, resulting in higher production costs for these strains. As reported above, the *K. oxytoca* KC004-TF160 may be comparable or superior compared to other previously reported engineered strains.

Table 4.5 Comparison of succinate production by various strains from different carbon sources.

Strain	Media/mode of process	Sucinate				References
		Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	Max specific productivity (g/g CDW/h)	
K. oxytoca KC004-TF160	43 g/L sucrose/AM1/batch	35.43 ± 1.90	0.82 ± 0.03	0.37 ± 0.02	0.22	This study
(Δ*adhE*Δ*pta-*	50 g/L maltose/AM1/batch	40.39 ± 0.40	0.81 ± 0.01	0.56 ± 0.01	0.46	This study
*ackA*Δ*ldhA*Δ*budAB*Δ*pflB*)	47 g/L fructose/AM1/batch	37.62 ± 1.90	0.80 ± 0.03	0.39 ± 0.02	0.30	This study
	22 g/L xylose/AM1/batch	22.90 ± 1.90	0.62 ± 0.03	0.15 ± 0.02	0.19	This study
A. succinogenes NJ113	75 g/L sucrose// YE:CSL /batch	57.50	0.77	1.60	ND	Jiang et. al. (2013)
M. succinogenes PALFK	86.6 g/L sucrose/CDM /fed-batch	63.90	0.74	3.19	ND	Lee et. al. (2016)
E. coli KJ122-pKJUC-24T	70 g/L sucrose/AM1 /batch	53.40	0.90	0.74	0.41	Chan et al. (2012)
E. coli M6PM (pTrchisA-pyc)	12.20 g/L fructose/M9/ dual-phase	2.00	0.16	0.04	0.09	Olaiuyin et al. (2019)
E. coli SBS550MG (pHL413)	44.24 g/L fructose/M9/ dual-phase	36.24	0.82	0.82	0.16	Wang et al. (2011)
E. coli M6PM (pTrchisA-pyc)	1.02 g/L fructose/M9/ dual-phase	2.00	0.16	0.04	0.09	Olaiuyin et al. (2019)
E. coli BA305	20 g/L xylose/YE/ batch	5.20	0.26	0.05	0.09	Liu et al. (2013)
(Δ*ldhA*Δ*pflB*Δ*ppc*Δ*ptsG*)						
and over-expressed of *pck*						
E. coli KJ12201 *(*KJ122,	100 g/L xylose/AM1/ batch	70.8	0.87	0.58	0.19	Khunnonkwao et al.
Δ*xylFGH*)						(2018)

ND = No data.

4.4.3 The succinate production in a 5-L bioreactor from sugarcane molasses by *K. oxytoca* KC004-TF160

The developed *K. oxytoca* KC004-TF160 could produce succinic acid from a diversity of carbon sources, particularly succinate production from glucose, maltose, sucrose, and fructose. As a result, to achieve industrial succinate production via fermentation, the inexpensive carbon sources and media were investigated. In recent years, sugarcane molasses is often used to be the potential substrate for biogas production, biohydrogen, and organic acids to lower manufacturing costs (Kongjan et al., 2011). Sugarcane molasses is a by-product of sugar manufacture that is a mixture of the disaccharide sucrose and its monomers glucose and fructose, as well as nutrients (Chacon et al., 2020). According to the cooperatives of source agricultural produce, the approximate price of US sugarcane molasses was less than $0.40/ kg (Selina Wamucii, 2022). Therefore, the potential of the *K. oxytoca* KC004-TF160 to utilize untreated sugarcane molasses as a carbon source with different sugarcane molasses concentrations (100, 150, and 200 g/L sugarcane molasses) for succinate production was investigated. The *K. oxytoca* KC004-TF160 was fermented in a 2.5 L working volume with 100% AM1 medium (4.2 g/L salts), 100 mM $KHCO_3$, and 100 g/L sugarcane molasses. As shown in Table 4.6, a concentration of 37.36 g/L succinate, with a yield of 0.80 and productivity of 0.31 g/L/h were obtained using 100 g/L sugarcane molasses fermentation, and succinate was produced up to 43.27 g/L when the sugarcane molasses concentration was increased to 150 g/L. The consumption rates of glucose, sucrose, and fructose in molasse were 0.42, 0.26 and 0.12 g/L.h respectively, with 4 g/L of fructose remaining after 120 h. As previously reported, when multiple sugars were fermented, glucose was used first or preferentially. When the sugarcane molasses concentration was increased to 150 g/L, glucose. The consumption rates of glucose (0.24 g/L.h), sucrose (0.26 g/L.h), and fructose (0.05 g/L.h) in molasse reduced to 42.86%, 3.85% and 58.33% respectively, when compared to the 100 g/L molasse fermentation. Unexpectedly, the *K. oxytoca* KC004-TF160 could tolerate high sugarcane molasses concentrations up to 200 g/L, but when the sugarcane molasses concentration was increased to 200 g/L, sugar consumption was severely inhibited resulting in low cell growth and succinate production. As previously reported, sugarcane molasses normally contains some hazardous substances such as

5-hydroxymethylfurfural and excessive metal ions (calcium, sodium, iron, magnesium, copper, etc.) and suspended colloids are generated during sugar manufacturing process, which are toxic to cell growth, causing low conversion yield and productivity (Bicker et al., 2003). For instance, when the concentration of sodium ion was greater than 4 g/L, which could inhibit the glucose transport and cell growth (Lee et al., 1999). Thus, to avoid high cost of pretreatment, fermentation parameters such as sugarcane molasses concentration and AM1 medium buffering were optimized to increase succinate production efficiency. The KC004-TF160 strain was fermented in AM1 content (%) of 0, 25, 50 respectively.

As expected, reducing the AM1 content increased succinate yield and productivity, however fructose remained at the end of fermentation. Acetate at 8 g/L was only detected as byproducts. As shown in Table 4.6, the reducing AM1 content when 100 g/L molasse fermentation, the succinate concentration (40-44 g/L), and productivity (0.33-0.38 g/L/h) were increased by about 7.25-18.40%, and 6.45-22.58% respectively, and the yield remained constant when compared to the 100% AM1 content fermentation. Moreover, reducing 25% AM1 content in 100 g/L molasse fermentation, the consumption rate of glucose (0.68 g/L.h) and sucrose (0.57 g/L.h) in increased to 61.90% and 119.23%, respectively, while fructose consumption rate remained constant, when compared to the 100% AM1 content fermentation. The sugar consumption rates and reducing AM1 content by 25% to 50% had no discernible effect. As a result, reducing AM1 content by 50% was disused in 150-200 g/L sugarcane molasses fermentation. Similarly, when compared to the 100% AM1 content fermentation, reducing AM1 content in 150 g/L molasse fermentation resulted in 17.40-28.93%, 8.75%, and 22.22-33.33% increases in concentration (50.80-55.79 g/L), yield (0.87 g/g), and productivity (0.44-0.48 g/L/h), respectively. When the AM1 content was reduced by 25%, the sugar consumption rates of glucose (0.49 g/L.h), sucrose (0.29 g/L.h), and fructose (0.14 g/L.h) increased by approximately 104.1%, 26.1%, and 180%, respectively. Surprisingly, when reducing the AM1 content with the 200 g/L molasse fermentation, the succinate concentration (50-55 g/L), yield (0.77-0.84 g/g) and productivity (0.31-0.48 g/L/h) were significantly increased by approximately 242.83-461.26%, 10-20% and 244.44-433.33% respectively, when compared to the 100% AM1 content fermentation. Especially, fermentation with 200 g/L molasse and no AM1

content resulted in increased glucose (0.28 g/L.h), sucrose (0.17 g/L.h), and fructose (0.12 g/L.h) consumption rate of approximately 104.1%, 26.1%, and 180% respectively, when compared to the 100% AM1 content fermentation. The sugar consumption rates, on the other hand, were found to be slower, and all sugar remained after 120 hours. This result was consistent with the findings of Shukla et al. (2004), who discovered that inhibitors present in sugarcane molasses caused slow fermentation. Although the *K. oxytoca* KC004-TF160 has an ability to produce succinate in high concentration and yield when fermented without the use of pre-treated sugarcane molasse and additional nutrient or plasmid supplement. Normally, sugarcane molasse was generally pre-treated to reduce the level of various inhibitors before application in fermentation, and nutrient supplementation was required, which increased the cost of fermentation and workload in previously reported (Xu & Xu., 2014). For example, native *A. succinogenes* CGMCC1593 produced succinate at the concentration of 44.2 g/L from the pretreated sugarcane molasse supplemented with 15 g/L of yeast extract in anaerobic bottles (Liu et al., 2008). Chan et al. (2012) reported that succinate (55.8 g/L) was obtained from sugarcane molasse by *E. coli* KJ122 harboring pKJSUC. These results suggest that sugarcane molasses could be used as one application for production of succinate by *K. oxytoca* KC004-TF160, and *K. oxytoca* KC004-TF160 can be compared to native *A. succinogenes* and other developed strains for production of succinate from sugarcane molasse as shown in Table 4.7.

Table 4.6 Comparison of succinate production in various media containing glucose by different engineered strains.

Strain	Media/mode of process	Succinate				References
		Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	Max specific productivity (g/g CDW/h)	
K. oxytoca KC004-TF160	100 g/L glucose/AM1/batch	84.00 ± 1.90	0.84 ± 0.03	0.87 ± 0.02	0.54 ± 0.01	This study
K. oxytoca KP001-TF60	50 g/L glucose/AM1+YE/batch	44.41 ± 2.86	0.88 ± 0.03	0.33 ± 0.03	0.30 ± 0.00	This study
K. oxytoca KMS006	100 g/L glucose/AM1/batch	6.73 ± 0.77	0.03 ± 0.02	0.06 ± 0.01	0.03 ± 0.01	This study
K. pneumoniae CICC10011	86 g/L glucose/complex medium/fed-batch	28.70	0.33	0.46	0.12	Cheng et al. (2013)
E. coli KJ060	96 g/L glucose /AM1 /batch	86.50	0.90	0.93	0.46	Jantama et al. (2008a)
E. coli KJ134	94 g/L glucose/AM1/ batch	71.60	1.00	0.75	0.32	Jantama et al. (2008b)
E. coli AFP111	86 g/L glucose/serum bottles/ dual-phase	68.66	0.79	0.66	0.10	Li et al. (2017)
E. coli HX024	108 g/L glucose/AM1/ batch	95.90	0.88	0.96	0.35	Zhu et al. (2014)
A. succinogenes 130Z wild type	100 g/L glucose/AM1/ batch	69	0.68	1.20	0.32	Guettler et al. (1996)

Table 4.6 (continued).

Strain	Media/mode of process	Succinate				References
		Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	Max specific productivity (g/g CDW/h)	
A. succinogenes FZ53	120 g/L dextrose/ complex medium/batch	94	0.78	2	ND	Guettler et al. (1996)
B. succiniproducens wild type	108 g/L glucose/AM1/ batch	20	0.49	ND	ND	Becker et al. (2013)
B. succiniproducens LU15224	41 g/L glucose / complex medium/batch	46.30	0.87	ND	ND	Scholten et al. (2014)
M. succiniciproducens MBEL55E	22 g/L glucose/MMH3/fed-batch	10.49	0.45	ND	ND	Lee et al. (2006)
M. succiniciproducens LPK7	69 g/L glucose/MMH3/fed-batch	52.40	0.76	1.80	2.61	Lee et al. (2006)
C. acetoacidophilum R	40 g/L glucose/ A-medium/fed-batch	107	0.88	1.12	ND	Liu et al. (2015)

Table 4.7 Fermentation of succinic acid from sugarcane molasse by *K. oxytoca* KC004-TF160.

Total initial molasses (g/L)	AM1 content (%)	Sugar consumption (g/L)	Sugar remained (g/L)	Maximum cell viability (10^8 CFU/mL)	Succinate			Acetate (g/L)
					Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	
100	0	54.00 ± 1.80	8.04 ± 0.99	2.51 ± 0.35	44.24 ± 3.81	0.85 ± 0.07	0.38 ± 0.03	7.95 ± 0.28
	25	50.13 ± 3.14	3.52 ± 1.01	2.68 ± 0.05	41.32 ± 7.58	0.82 ± 0.08	0.34 ± 0.00	8.63 ± 0.07
	50	49.90 ± 2.61	5.79 ± 0.50	1.96 ± 0.11	40.07 ± 3.68	0.80 ± 0.01	0.33 ± 0.03	8.12 ± 0.34
	100	44.73 ± 0.60	4.04 ± 0.68	5.18 ± 0.32	37.36 ± 4.65	0.81 ± 0.16	0.31 ± 0.04	7.34 ± 0.74
150	0	66.16 ± 1.24	18.94 ± 3.59	2.06 ± 0.48	55.79 ± 1.43	0.87 ± 0.01	0.48 ± 0.01	8.63 ± 1.05
	25	60.30 ± 0.43	12.17 ± 0.14	3.63 ± 0.45	50.80 ± 0.75	0.87 ± 0.02	0.44 ± 0.01	8.00 ± 0.74
	100	54.10 ± 0.96	22.73 ± 2.03	5.40 ± 0.42	43.27 ± 1.10	0.80 ± 0.01	0.36 ± 0.01	8.72 ± 2.71
200	0	68.75 ± 0.17	41.26 ± 0.45	0.62 ± 0.70	57.53 ± 2.31	0.84 ± 0.03	0.48 ± 0.02	8.91 ± 1.06
	25	45.19 ± 3.58	63.81 ± 3.66	1.13 ± 5.79	35.14 ± 4.47	0.83 ± 0.03	0.31 ± 0.02	6.29 ± 1.00
	100	14.66 ± 2.40	92.44 ± 2.10	1.57 ± 0.76	10.25 ± 1.66	0.62 ± 0.05	0.09 ± 0.01	1.02 ± 0.14

Table 4.8 Comparison of succinate production from sugarcane molasse by different bacteria strains.

Strain	Medium/mode of process	Succinate				Reference
		Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	Max Specific productivity (g/g CDW/h)	
K. oxytoca KC004-TF160	150 g/L Sugarcane molasses/AM1/batch	55.79	0.87	0.48	ND	This study
E. coli isolated from the remen of buffalo	10% v/v Sugarcane molasses/supplemented with 10% CSL/batch	17	0.34	0.56	0.29	Agarwal et al. (2006)
E. coli KJ122-pKJSUC-24T	150 g/L Sugarcane molasses/AM1/batch	55.80	0.96	0.77	0.33	Chan et al. (2012)
A. succinogenes CGMCC1593	150 g/L Sugarcane molasses/ AS medium supplemented to 15 g/L yeast actract/fed batch	55.20	ND	1.15	0.49	Liu et al. (2008)
A. succinogenes CGMCC1593	150 g/L Sugarcane molasses/ AS medium supplemented to 15 g/L yeast actract/batch	45.20	0.79	0.75	ND	Liu et al. (2008)

ND = No data.

CHAPTER 5
CONCLUSION

In this study, the *K. oxytoca* KMS006 was developed as a novel potential microbial platform for industrial-scale succinic acid production. The combining metabolic engineering and metabolic evolution strategy was successfully employed for improving the succinate production in both *K. oxytoca* KC004 (Δ*adhE*Δ*pta-ackA*Δ*ldhA* Δ*budAB* Δ*pflB*) in mineral salts medium. The evolved *K. oxytoca* KC004-TF160 significantly enhanced the succinate concentration, yield, and productivity up to 84 g/L, 0.84 g/g, and 0.87 g/L/h, respectively, during anaerobic conditions. No byproducts including ethanol, lactate, formate and 2,3-butanediol were detected by the *K. oxytoca* KC004-TF160. Only acetate at 14 g/L was detected. Additionally, *K. oxytoca* KC004-TF160 could produce succinate with the yield in the range of 0.41, 0.67, 0.80, 0.81, and 0.82 g/g from lactose, xylose, fructose, sucrose, and maltose respectively. Furthermore, *K. oxytoca* KC004-TF160 has the ability to produce succinate with the yield of 0.87 g/g from non-pretreated sugarcane molasse without the addition of any nutrients or plasmids. While the further reducing acetate formation of *K. oxytoca* KC004-TF60 resulted in *K. oxytoca* KP001-TF60 (Δ*adhE*Δ*pta-ackA*Δ*ldhA*Δ*budAB*Δ*pflB* Δ*tdcD*Δ*pmd*), which improved succinate yield by approximately 4.76%. The *K. oxytoca* KP001-TF60 significantly enhanced the succinate yield up to 0.88 g/g of glucose, whereas acetate levels of less than 1 g/L were detected in batch fermentation. Unfortunately, this strain could only consume 60 g/L of glucose, which is a limitation in industrial-scale production. Unlike *K. oxytoca* KMS006, the increased activity of the *pck* gene was responsible for the increased succinate yield and ATP production, whereas the activities of the *pdh*, *tdcE*, *tdcD* genes were responsible for acetyl-CoA and acetate formation which is the primary mechanism for energy sources and redox balance. Increased *pck* activity may cause nucleotide variations in the genes *cyaA*, *ptsG*, *agaC*, and *CsrB* genes. While these variations may affect glucose metabolism when deficient acetate mechanism, resulting in decreased glucose consumption and yeast extract requirement for *K. oxytoca* KP001-TF60. Even though, *K. oxytoca* KC004-TF160

and *K. oxytoca* KP001-TF60 were able to efficiently produce succinate equivalent those of previously native producer and developed *E. coli* strains. Therefore, the newly developed *K. oxytoca* KC004-TF160 strain may serve as one of the potential microbial platforms for the commercial production of succinate, while *K. oxytoca* KP001-TF60 may further improve for succinate producers in the future.

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APPENDIX

APPENDIX

A.1 Sequences of *cat-sacB* gene

ACACTGCTTCCGGTAGTCAATAAACCGGNNATCGGCATTTTCTTTTGCGTTTTTATTTGT
TAACTGTTAATTGTCCTTGTTCAAGGATGCTGTCTTTGACAACAGATGTTTTCTTGCCTTTGATG
TTCAGCAGGAAGCTTGGCGCAAACGTTGATTGTTTGTCTGCGTAGAATCCTCTGTTTGTCATAT
AGCTTGTAATCACGACATTGTTTCCTTTCGCTTGAGGTACAGCGAAGTGTGAGTAAGTAAAGGT
TACATCGTTAGGATCAAGATCCATTTTTAACACAAGGCCAGTTTTGTTCAGCGGCTTGTATGGG
CCAGTTAAAGAATTAGAAACATAACCAAGCATGTAAATATCGTTAGACGTAATGCCGTCAATCG
TCATTTTTGATCCGCGGGAGTCAGTGAACAGGTACCATTTGCCGTTCATTTTAAAGACGTTCGC
GCGTTCAATTTCATCTGTTACTGTGTTAGATGCAATCAGCGGTTTCATCACTTTTTTCAGTGTGT
AATCATCGTTTAGCTCAATCATACCGAGAGCGCCGTTTGCTAACTCAGCCGTGCGTTTTTTATC
GCTTTGCAGAAGTTTTTGACTTTCTTGACGGAAGAATGATGTGCTTTTGCCATAGTATGCTTTGT
TAAATAAAGATTCTTCGCCTTGGTAGCCATCTTCAGTTCCAGTGTTTGCTTCAAATACTAAGTAT
TTGTGGCCTTTATCTTCTACGTAGTGAGGATCTCTCAGCGTATGGTTGTCGCCTGAGCTGTAGT
TGCCTTCATCGATGAACTGCTGTACATTTTGATACGTTTTTCCGTCACCGTCAAAGATTGATTTA
TAATCCTCTACACCGTTGATGTTCAAAGAGCTGTCTGATGCTGATACGTTAACTTGTGCAGTTGT
CAGTGTTTGTTTGCCGTAATGTTTACCGGAGAAATCAGTGTAGAATAAACGGATTTTTCCGTCA
GATGTAAATGTGGCTGAACCTGACCATTCTTGTGTTTGGTCTTTTAGGATAGAATCATTTGCATC
GAATTTGTCGCTGTCTTTAAAGACGCGGCCAGCGTTTTTCCAGCTGTCAATAGAAGTTTCGCCG
ACTTTTTGATAGAACATGTAAATCGATGTGTCATCCGCATTTTTAGGATCTCCGGCTAATGCAAA
GACGATGTGGTAGCCGTGATAGTTTGCGACAGTGCCGTCAGCGTTTTGTAATGGCCAGCTGTCC
CAAACGTCCAGGCCTTTTGCAGAAGAGATATTTTTAATTGTGGACGAATCGAATTCAGGAACTT
GATATTTTTCATTTTTTTGCTGTTCAGGGATTTGCAGCATATCATGGCGTGTAATATGGGAAATG
CCGTATGTTTCCTTATATGGCTTTTGGTTCGTTTCTTTCGCAAACGCTTGAGTTGCGCCTCCTGC
CAGCAGTGCGGTAGTAAAGGTTAATACTGTTGCTTGTTTTGCAAACTTTTTGATGTTCATCGTTC
ATGTCTCCTTTTTTATGTACTGTGTTAGCGGTCTGCTTCTTCCAGCCCTCCTGTTTGAAGATGGC
AAGTTAGTTACGCACAATAAAAAAAGACCTAAAATATGTAAGGGGTGACGCCAAAGTATACACT
TTGCCCTTTACACATTTTAGGTCTTGCCTGCTTTATCAGTAACAAACCCGCGCGATTTACTTAGA
TCTAGCGGCTATTTAACGACCCTGCCCTGAACCGACGACCGGGTCGAATTTGCTTTCGAATTTC

TGCCATTCATCCGCTTATTATCACTTATTCAGGCGTAGCACCAGGCGTTTAAGGGCACCAATAAC
TGCCTTAAAAAAATTACGCCCCGCCCTGCCACTCATCGCAGTACTGTTGTAATTCATTAAGCATT
CTGCCGACATGGAAGCCATCACAAACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCT
TGTCGCCTTGCGTATAATATTTGCCCATGGTGAAAACGGGGGCGAAGAAGTTGTCCATATTGGC
CACGTTTAAATCAAAACTGGTGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTCTCA
ATAAACCCTTTAGGGAAATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGT
GTAGAAACTGCCGGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTC
ATGGAAAACGGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCGTCTTTCATTGCC
ATACGGAATTCCGGATGAGCATTCATCAGGCGGGCAAGAATGTGAATAAAGGCCGGATAAAACT
TGTGCTTATTTTTCTTTACGGTCTTTAAAAAGGCCGTAATATCCAGCTGAACGGTCTGGTTATAG
GTACATTGAGCAACTGACTGAAATGCCTCAAAATGTTCTTTACGATGCCATTGGGATATATCAAC
GGTGGTATATCCAGTGATTTTTTTCTCCATTTTAGCTTCCTTAGCTCCTGAAAATCTCGATAACT
CAAAAAATACGCCCGGTAGTGATCTTATTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCG

A.2 Sequences of *budAB* gene (*budAB'-budAB''*)

AGCGCAGATAACGAAGTTCCATATCGAAAACGTCTCAAACAAGCATAGATTCTATATTGG
AACTGTGAGCTGAATCGCGTCAACATTTATTTAACCTTTCTGATATTCGTTGAACGAGGAAGCGG
GTAATGAACCATTCTGCTGAATGCTCTTGTGAAGAGAGCCTGTGTGAAACTCTACGAGGGTTTT
CCGCGCAACATCCCGATAGCGTCATCTACCAGACCTCTCTGATGAGCGCGCTGTTGAGCGGCGT
TTATGAAGGCAATACCACCATCGCTGATTTACTCACCCACGGCGATTTTGGCCTGGGAACCTTT
AATGAACTGGACGGCGAGCTGATCGCGTTTAGCAGCGAGGTATACCAGCTGCGCGCCGACGGC
AGCGCCCGTAAAGCCCGAATGGAACAGCGCACGCCGTTTGCGGTGATGACCTGGTTTCAGCCGC
AGTAGGGTCGATAACGGCTACAACATGGTGGCAATTCAGGAAGAGAAAAAATATCAGCGGCTAT
CCGGCGTTGAGTTCGGCCCGGTGGATTTTAAAGCCTATGCCGAAGCCTTCGGCGCCAGAGGGTT
CGCGGTCGAGAGCGCCGCCGCCCTTGAGCCGACGCTGCGGGCGGCGATGGACGTCGATGGCCC
CGCCGTGGTCGCCATCCCCGTCGATTACAGCGACAACCCGCTGCTGATGGGCCAGCTTCATCTC
AGTCAACTACTTTGAGTCACTACAGAAGGAATCCAGCAATGAAAAAAGTCGCACTCGTGACCGG
CGCAGGCCAGGGTATCGGTAAAGCTATCGCCCTTCGCCTGGTTCAAGATGGCTTTGCCGTGGCC
ATCGCCGATTATAACGATGCCACCGCACAGGCGGTCGCTGACGAAATTAACCAGCACGGCGGCC
AGGCGCTGGCGGTGAAGGTCGATGTCTCGAAACGCGATC

Note: Normal letters are *budAB'* gene (incomplete gene), underline letters are *budAB"* (incomplete gene).

A.3 Sequences of *budAB* gene *pflB* (*pflB'-pflB''*)

TATGTCCGAGCTTAATGAAAAGTTAGCCACAGCCTGGGAAGGTTTTGCGAAAGGTGACTG
GCAGAACGAAGTCAACGTCCGCGACTTCATCCAGAAAAACTATACCCCGTACGAAGGTGACGAG
TCCTTCCTGGCTGGCGCAACTGACGCGACCACCAAGCTGTGGGACACCGTAATGGAAGGCGTTA
AACAGGAAAACCGCACTCACGCGCCTGTTGATTTTGATACTTCCCTTGCATCCACCATCACTTCT
CATGACGCTGGCTACATCGAGAAAGGTCTCGAGAAAATCGTTGGTCTGCAGACTGAAGCTCCGC
TGAAACGCGCGATTATCCCGTTCGGCGGCATCAAAATGGTAGAAGGTTCCTGCAAAGCGTACAA
TCGCGAGCTGGACCCGATGCTGAAGAAAATCTTCACTGAATACCGCAAAACTCACAACCAGGGC
GTGTTTGACGTTTACACCAAAGACATCCTGAACTGCCGTAAATCTGGCGTTCTGACCGGTCTGC
CGGATGCCTATGGCCGTGGTCGTATCATCGGTGACTACCGTCGCGTTGCGCTGTACGGTATCGA
CTTCCTGATGAAAGACAAATACGCTCAGTTCGTCTCTCTGCAAGAGAAACTGGAAAACACGCTA
TCCCGACTCAGTCCGTTCTGACCATCACCTCTAACGTTGTGTATGGTAAGAAAACCGGTAACAC
CCCTGACGGTCGTCGCGCTGGCGCTCCGTTCGGACCAGGTGCTAACCCGATGCACGGCCGTGAC
CAGAAAGGTGCTGTTGCCTCTCTGACCTCCGTTGCGAAACTGCCGTTTGCTTACGCGAAAGATG
GTATTTCTTACACCTTCTCTATCGTGCCGAACGCGCTGGGTAAAGACGACGAAGTTCGTAAAAC
TAACCTCGCCGGCCTGATGGATGGTTACTTCCACCACGAAGCGTCCATCGAAGGCGGTCAGCAT
CTGAACGTCAACGTTATGAACCGCGAAATGCTGCTCGACGCGATGGAAAACCCGGAAAAATATC
CGCAGCTGACCATCCGCGTATCCGGCTACGCAGTACGTTTTAACTCCCTGACGAAAGAACAGCA
GCAGGACGTTATTACTCGTACCTTCACTCAGACCATGTAA

Note: Normal letters are *pflB'* gene (incomplete gene), underline letters are *pflB"* (incomplete gene).

A.4 Sequences of *tdcD* gene (*tdcD'-tdcD''*)

GCGAGGCGTCGATTATCGCTTGCGATATCGCCACCAAATCCGGCGCGGTGGAGATCGGT
TTTCTCGACCGTTTTACCGGCGCCGTGGTGCTGACCGGCGACGTTTCCGCCGTGGAATATGCCC
TCAAGCAGGTGACGCGCACCCTGGGCGAGATGATGCAGTTTACTACCTGCTCAATAACCCGGAC
GTAGCGCGATGAAGCGTCTGATGTTTATCGGCCCGAGTCAGTGCGGCAAGACCTCGCTAACCCA
GGGCCTGCGCGGCGAAGCGCTCCATTATAAGAAAACCCAGGCCATTGAATGGTCGCCGATGGCC
ATCGATACGCCGGGGGAATATCTGGAGAACCGCTGCCTGTATAGCGCCCTGCTGACCAGCGCCT
GCGAGGCTGACGTTATCGCGCTGGTACTGAATGCCGACGCCCAGTGGTCGCCGTTTTCTCCGGG
GTTTACCGCCCCGATGAATCGCCCGACGATTGGCCTGGTCACCAAAGCCGACCTCGCCGACCCT
CAGCGGATATCGCTCATCGCGGAGTGGCTTACGCAGGCTGGCGCCGGGCAGATTTTTGTTACCA

GCGCGTTGAACAATTCGGGTCTCGACGCGGTGCTGGATTTTCTGAATTCGAAGGAACCCTTATG
TCTTACAAAATAATGGCCATTAACGCCGGCAGCTCATCGTTAAAATTCCAGCCGGAAACCTTAA
CCGTTTCAGCCTGATTTCGCCGCCCGGCAAGTTGCGGTGAATCATGGTAATAGCGGGATCGGCA
TGACACTATGCGCCCTGGAAACCTGAGCAATAGGTGAATATGTGGCCGTAGCGCAATGCCCCGC
CTCCTGTGGGGAACTCATCCAGGGCTGGATCCTCGGCAGCGAGAAGCTGGTCTCCTGCCCCGTT
GACTGGTACAGCACCGTCGAGGTGGACTACGGTTCGCCTCGCGCAGATGAACGCCCGCTGTCGC
GGGCGATGGTCGACCGGCTGCTGGGCTACTGGCGCTATCCGGCGGAGCTAGGCAAAGAGATTC
GGATCGATATTTGCTCCACGATTCCGGTCGCCAAAGGGATGGCCAGCAGCACCGCGGATATCGC
CGCGACGGCGGTGGCCACCGCGCATCATCTCGGCCATCCGCTGGATGAAACCACCCTCGCCCG
GCTCTGCGTTTCCCTGGAGCCCACCGACAGCACCCTGTTCCGCCAGTTGACGCTATTCGATCAC
AATACCGCCGCCACGCAAATTACCTGCGGCTGCCAGCCTCAGCTCGATCTGCTGGTGCTGGAAA
GCCCCGCCACGCTGCTGACCACCGATTACCACC

Note: Normal letters are *tdcD'* gene (incomplete gene), underline letters are *tdcD"* (incomplete gene).

A.5 Sequences of *tdcD* gene (*pmd'-pmd''*)

GTTTATAGCGCTGCTGAACGAAGATCGGCAAGCAGCATCTTCTCCATAAAGATGCTTAAC
GGATCCGGCTGATACGGCGCAAAGGCGCTACGGGTCTGGTAGCCATTGCGCGTATATAACGCAA
CGGCGGCGTGCTGATGGATGCCCGTTTCCAGGCGCACGGTATGGCATTCGCGATTTATCGCCTC
TTCCTCCAGCGCGGCCAGCAGTCGTTCTCCCAGCTGCTGCCCGCGATGGGCCGGGTGAATAAAC
ACCCTTTTCATCTCGCCAACCCCCTCTTCGCCGAGCACGATCGCCCCGCAGCCGACGGCCTGGC
GCTGCGTATCGCGAATAAGAAGCGCAATAACCGACTCCGCCGGTAGCGCGCTCAGGTCGATCAA
ATGGTTGCTCTCAGCCGGGTAGAGCGTCTGCTGATACGCGTCCAGTTCGGCAATTAAGGCCATC
AGATCCGGGTGGTTTTGCGGGGTTGGGGTGATTGTGTACATAAAGAAGCTCCTTTTTGACCATA
CTGTAGCGCGGAGCGTCTCGCAGGCGGCCATAGCATTAACTTATCGCTTCACACCTCGCTTGCG
CTTCCCCCTAAAGTGGCAGTAATTTTGCGTCATGACGACATTTATCAGGAACAAACCATGAATAC
CAAAGCGCGCAATATCGCCCGCCTACCGTAACCCGCTTTCCCGGCTAGTCGGCAATGATCAGCC
GGGGATGGATATGCCCCAGCAGTTCACGCGTCTCTTCCGGCAGATGATTATCGGTGATGATGTC
GGTGAGCGAGTCGAGATGGGTGATGTTAAACAGCGACCAGGCGCCGTATTTTGAGCTGTCCGCC
AGCAGAATGCGCCGCCGGGCGTTCGCGATTAAATCCCGCTTCAGCGCCGCTTTCTCTTCCGTTG
GCGAGGTTACGCCCTTCTCCAGATCCCAGCCGTTGCAGCTGACGAACGCCAGATCGGGCCAGAT
ATTTTGCAGCAGACGGCGTCCGTGATCGCCGATGCAGGACTGGCTGCTGTCGTCGATGCGTCCG

CCGATGATGGTAACTTCAATTTGCTTAAATTCCGAGAGAAACAGGGCGATATGCAGATCGCTGG
TGATCACCCGCAGCGGCAAGTGGGTTATCTGCCGGGCAAGTTCGATCATCGTGGTTCCGGCATC
GAGGACGATGGCGTCCCCGGCCTTGACTAACGCCGCCGCCGCGCTGGCGATGGCGTATTTTTCC
TGCAGACTGCGCTGGGACTTCTCCTGGGTGGTGGGCTGTGAAGGAATAAACCGGTTGAGCGTGA
CGCCGCCGTGGGTACGGCTGATGACCCCTTCCTGATCGAGCTTAATCAGGTCGCGGCGAATT

Note: Normal letters are *pmd'* gene (incomplete gene), underline letters are *pmd"* (incomplete gene).

VITAE

Mr. Chutchawan Phosriran was born on September 27th, 1994, at Nakhon Sawan province, Thailand. He obtained his bachelor's degree of Science in Biology from Faculty of Science and Technology, Nakhon Sawan Rajabhat University, Nakhon Sawan, Thailand. In 2017. During bachelor's degree, his research interest was entitled "Study of adding a lotus root extracted as prebiotic in fermented rice noodle for the production of lactic acid by *Lactobacillus acidophilus* LA-5". In addition, during his intern at the Center of Medical Biotechnol, Department of Medical Sciences Ministry of Public Health, Nonthaburi, Thailand, he also researched cancer cells, HaCaT cells, HaCaT cell culture optimization, and herbal extract as a wound healing stimulant test in HaCaT cells. After graduation, he decided to further study for Doctorate degree in the field of Biotechnology at school of Biotechnology, Institute of Agricultural of Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. During his attendance, he was fully financial funded under the Royal Golden Jubilee scholarships from the National Research Council of Thailand. He had the opportunity to broaden his international research experience, which in his case is to conduct research under the supervision of Professor Dr. Maobing Tu, University of Cincinnati, United States of America. His research topic is re-engineering of *Klebsiella oxytoca* KMS006 to produce succinic acid in mineral salts medium. He Some parts of results from this study had been presented as an oral presentation in the 2021 National RGJ and RRI Conferences, Thailand. After he graduates from Suranaree University of Technology, he plans to work in industry or as a lecturer in Thailand.