การปรับเปลี่ยนวิถีการสร้าง และสลายอีกครั้งของเชื้อ *Kelbsiella oxytoca* เพื่อการผลิต 2,3-บิวเทนไดออล

นายภัทรเศรษฐ์ พลเยี่ยม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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RE-ENGINEERING OF METABOLIC PATHWAY OF *KLEBSIELLA OXYTOCA* FOR 2,3-BUTANEDIOL PRODUCTION

Pattharasedthi Pholyiam

A Thesis Submitted in Partial Fulfillment of the Requirements for

the Degree of Master of Science in Biotechnology

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RE-ENGINEERING OF METABOLIC PATHWAY OF *KLEBSIELLA OXYTOCA* FOR 2,3-BUTANEDIOL PRODUCTION

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ภัทรเศรษฐ์ พลเยี่ยม : การปรับเปลี่ยนวิถีการสร้าง และสลายอีกครั้งของเชื้อ *Klebsiella oxytoca* เพื่อการผลิต 2,3-บิวเทนไดออล (RE-ENGINEERING OF METABOLIC PATHWAY OF *KLEBSIELLA OXYTOCA* FOR 2,3-BUTANEDIOL PRODUCTION) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.เขมวิทย์ จันต๊ะมา, 139 หน้า.

เชื้อสายพันธุ์ *Klebsiella oxytoca* ที่ได้ผ่านการปรับเปลี่ยนวิถีการสร้าง และสลายสำหรับการผลิต 2,3-บิวเทนไดออลในอาหารลี้ยงเชื้ออย่างง่ายภายใต้สภาวะที่มีอากาศน้อย (microaerobic conditions) จากเชื้อตั้งต้น *K. oxytoca* สายพันธุ์ KMS004 (Δ*adhE* Δ*pta-ackA*) เป็นสายพันธุ์ดั้งเดิมซึ่งผลิตกรดแลกติคชนิดดี (-) เป็นผลิตภัณฑ์หลัก ในการศึกษาครั้งนี้ได้ทำการตัดยีน *ldhA* ที่ควบคุมการสร้างเอนไซม์แลกเตต ดีไฮโดรจิเนส จากจีโนมของเชื้อสายพันธุ์ KMS004 ทำให้ได้เชื้อสายพันธุ์ใหม่ KMS005-76T (Δ*adhE* Δ*pta-ackA* Δ*ldhA′-cat-sacB-ldhA″*) ทั้งนี้เชื้อสายพันธุ์ KMS005-76T ได้รับการปรับปรุงสายพันธุ์ให้มีการสร้าง 2,3-บิวเทนไดออลที่สูงขึ้น หลังจากการทำวิวัฒนาการของวิถีการสร้าง และสลายแล้ว (metabolic evolution) พบว่าเชื้อสายพันธุ์ KMS005-76T ดังกล่าวมีอัตราการเจริญ การใช้น้ำตาล ตลอดจนการสร้าง 2,3-บิวเทนไดออลที่ดีขึ้น ทั้งนี้ในระหว่างกระบวนการหมักที่เกี่ยวข้องกับการสร้าง 2,3-บิวเทนไดออล พบว่าเชื้อสายพันธุ์ KMS-005-76T มีค่าการทำงานของเอนไซม์ เช่น แลกเตต ดีไฮโดรจิเนส (LDHA) แอลกอฮอล์ ดีไฮโดร-จิเนส (ADHE) และมาเลตดีไฮโดรจิเนส (MDH) ลดลง แต่พบว่ามีระดับการทำงานของเอนไซม์ 2,3-บิวเทนไดออลดีไฮโรจิเนส (BUDC) เพิ่มขึ้นเมื่อเทียบกับระดับของเอนไซม์ในกลุ่มที่กล่าวมา โดยที่เชื้อสายพันธุ์ดังกล่าวมีการสร้าง 2,3-บิวเทนไดออลที่ความเข้มข้น 23 กรัมต่อลิตร ได้ผล-ผลิต 0.46 กรัม ของ 2,3-บิวเทนไดออลต่อกรัม ของน้ำตาลกลูโคสที่ใช้ในอาหารเลี้ยงเชื้ออย่างง่าย ที่มีกลูโคสความเข้มข้น 50 กรัมต่อลิตร ในขวดรูปชมพู่ เมื่อเทียบกับผลผลิตทางทฤษฎีของ 2,3-บิวเทนไดออล 0.50 กรัมต่อกรัม สำหรับการผลิต 2,3-บิวเทนไดออลโดยใช้กากน้ำตาล-อ้อยนั้นได้ความเข้มข้นที่ 19 กรัมต่อลิตร เทียบเป็นผลได้ 0.42 กรัม ของ 2,3-บิวเทนไดออลต่อกรัม ของน้ำตาลทั้งหมดที่ใช้ ยิ่งไปกว่านั้นเชื้อสายพันธุ์ KMS005-76T ยังสามารถผลิต 2,3-บิวเทนไดออล จากไฮโดรไลซ์แป้งมันสำปะหลังที่ความเข้มข้น 19 กรัมต่อลิตร เทียบเป็นผลได้ 0.39 กรัม ของ 2,3-บิวเทนไดออลต่อกรัม ของน้ำตาลทั้งหมดที่ใช้ นอกจากนี้เชื้อดังกล่าวยังสามารถผลิต 2,3-บิวเทนไดออลจากมอลโตเดกซ์ทริน ที่ความเข้มข้น 9 กรัมต่อลิตร เทียบเป็นผลผลิตได้ 0.40 กรัม ของ2,3-บิวเทนไดออลต่อกรัม ของน้ำตาลทั้งหมดที่ใช้ ในการศึกษาครั้งนี้ได้ทำการศึกษาเกี่ยวกับผลของอัตราการให้อากาศต่อการผลิต 2,3-บิวเทนไดออลในถังหมักขนาด 2 ลิตร พบว่าเชื้อสายพันธุ์ KMS005-76T สามารถผลิต 2,3-บิวเทนไดออลที่ความเข้มข้น 20 กรัมต่อลิตร เทียบเป็น

อัตราผลผลิตสูงสุดที่ 0.67 กรัมต่อลิตรต่อชั่วโมง จากน้ำตาลกลูโคสความเข้มข้น 50 กรัมต่อลิตร ภายใต้สภาวะที่ให้อากาศในอัตรา 1.0 ของปริมาตรอากาศต่อปริมาตรน้ำหมักต่อนาที (vvm) ในระหว่างกระบวนการหมักแบบกึ่งกะต่อเนื่อง เชื้อสายพันธุ์ KMS005-76T สามารถผลิต 2,3-บิว-เทนไดออลที่ความเข้มข้น 117 กรัมต่อลิตร เทียบเป็นอัตราการผลิตได้ 1.20 กรัมต่อลิตรต่อชั่วโมง จากน้ำตาลกลูโคส และผลิต 2,3-บิวเทนไดออลที่ความเข้มข้น 93 กรัมต่อลิตร เทียบเป็นอัตราการผลิต 0.95 กรัมต่อลิตรต่อชั่วโมง จากมอลโตเดกซ์-ทรินตามลำดับ ดังนั้นจากผลการทดลองแสดงให้เห็นว่าเชื้อสายพันธุ์ KMS005-76T เป็นเชื้อที่มีศักยภาพสูงสำหรับการผลิต 2,3-บิวเทนไดออล จากแหล่งอาหารที่ไม่มีวันหมด

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

PATTHARASEDTHI PHOLYIAM : RE-ENGINEERING OF METABOLIC PATHWAY OF *KLEBSIELLA OXYTOCA* FOR 2,3-BUTANEDIOL PRODUCTION. THESIS ADVISOR : ASST. PROF. KAEMWICH JANTAMA, Ph.D. 139 PP.

2,3-BUTANEDIOL/*KLEBSIELLA OXYTOCA*/MICROAEROBIC CONDITIONS/ METABOLIC ENGINEERING

A metabolically engineered *Klebsiella oxytoca* was constructed for the production of 2,3-butanediol (2,3-BD) in mineral salts medium under microaerobic conditions. *K. oxytoca* KMS004 (Δ*adhE* Δ*pta-ackA*) parental strain exhibited D-(-)-lactic acid production as a major metabolite. In this study, lactate dehydrogenase (*ldhA*) gene was deleted from genomic DNA of the *K. oxytoca* KMS004. The mutant strain, KMS005 (Δ*adhE* Δ*pta-ackA* Δ*ldhA'-cat-sacB-ldhA"*) was constructed to improve 2,3-BD production yield. After metabolic evolution performance, KMS005-76T strain showed improvement in growth and sugar consumption rates with simultaneous production of 2,3-BD. During the fermentation process to produce 2,3-BD, KMS005-76T possessed lower specific enzymatic activities of lactate dehydrogenase (LDHA), alcohol dehydrogenase (ADHE) and malate dehydrogenase (MDH) but higher in the specific enzymatic activity of 2,3-butandiol dehydrogenase (BUDC) than those of the parental strain (KMS004). The mutant strain produced 2,3-BD at the concentration of 23 g/L with the yield of 0.46 g/g in the medium containing 50 g/L glucose in shake flask close to the theoretical 2,3-BD yield of 0.50 g/g. For sugarcane molasses, KMS005-76T produced 2,3-BD at the concentration of

19 g/L with the yield of 0.42 g/g total sugars consumed. Moreover, KMS005-76T showed high ability to produce 2,3-BD from hydrolyzed cassava starch at 19 g/L with the yield of 0.39 g/g total sugars consumed. This strain was also able to utilize maltodextrin and produced 2,3-BD at the concentration around 9 g/L with the yield of 0.40 g/g total sugars consumed. To study an effect of the aeration rate, 2,3-BD production was performed in a 2 L bioreactor. It was found that KMS005-76T could produce 2,3-BD concentration at 20 g/L with the highest productivity of 0.67 g/L.h from 50 g/L glucose under the aeration rate of 1.0 vvm. During the fed-batch fermentation, KMS005-76T could produce 2,3-BD at the concentration of 117 g/L with the productivity of 1.20 g/L.h from glucose and produce 2,3-BD at the concentration of 93 g/L with the productivity of 0.95 g/L.h from maltodextrin respectively. The results demonstrated that KMS005-76T would be a potential strain for economic bio-based 2,3-BD production from renewable substrates.

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LIST OF ABBREVIATIONS

A	=	Amplitude
ACKA	=	Acetate kinase
ADHE	=	Alcohol dehydrogenase
ATP	=	Adenosine 5'-tri-phosphate
α-ALD	=	α- Acetolactate decarboxylase
α-ALS	=	α- Acetolactate synthase
BDH	=	2,3 Butanediol dehydrogenase
BSA	=	Bovine Serum Albumin
bps	=	Base pairs
^{o}C	=	Degree Celsius
CFU	=	Colony forming unit
FDH	=	Formate dehydrogenase
FNR	=	Fumarate nitrate reduction regulatory
g	=	Gram (s)
g/L	=	Gram (s) per Liter
h	=	Hour (s)
HPLC	=	High performance liquid chromatography
K. oxytoca M5a1	=	*Klebsiella oxytaca* M5a1
Kb/min	=	Kilobase per Minute
L	=	Liter (s)
LDHA	=	Lactate dehydrogenase

LIST OF ABBREVIATIONS (Continued)

LB	=	Luria Bertani
M	=	Molar
mM	=	Milli-molar
mg	=	Milligram (s)
mg/L	=	Milligram (s) per Liter
min	=	Minute (s)
mL	=	Millilitre (s)
mm	=	Millimetre (s)
MW	=	Molecular weight
MDH	=	Malate dehydrogenase
NADH	=	Nicotinamide adenine dinucleotide (Reduced form)
NAD^+	=	Nicotinamide adenine dinucleotide (Oxidized form)
OD	=	Optical cell density
PCR	=	Polymerase chain reaction
PEP	=	Phosphoenolpyruvate
PFLB	=	Pyruvate formate-lyase
POXB	=	Pyruvate oxidase
PPC	=	Phosphoenolpyruvate carboxylase
PTA	=	Phosphotransacetylase
rpm	=	Revolution per minute
TBE	=	Tris-borate-EDTA
TDCD	=	anaerobic threonine dehydratase

LIST OF ABBREVIATIONS (Continued)

UV	=	Ultraviolet
V	=	Volt
v/v	=	Volume per volume
vvm	=	Gas volume flow per unit of liquid volume per minute
w/v	=	Weight per volume
X-gal	=	Bromo-chloro-indolyl-galactopyranoside
µL	=	Micro-liter

CHAPTER I

INTRODUCTION

1.1 Overview

2,3-butanediol (2,3-BD) is also known as 2,3-butylene glycol ordimethyl-ethylene glycol with the molecular weight of 90.121 g/mol. 2,3-BD can exist in 3 isomeric forms: D-(−)-, L-(+)- and meso. So far, 2,3-BD has mainly manufactured by microbial route and has acquired considerable progresses in the fermentation. 2,3-BD has extensive applications in varied industries including fuel, chemical, food, softening agents, explosives, and plasticizers as well as pharmaceutical agents. Furthermore, dehydration of 2,3-BD can be used in the industrial solvent such as methyl ethyl ketone (Khayati *et al.,* 2009; Xiao-Jun *et al.,* 2011). Several microorganisms (*Aeromonas*, *Bacillus*, *Klebsiella*, and *Paenibacillus*) can produce 2,3-BD from hexose and pentose sugars including cheaper carbon sources such as glycerol and molasses (Dien *et al.,* 2003). Over the past decade, some studies have attempted to change many microbial metabolite productions in these bacteria, via metabolic engineering in which the method improves the production of existing metabolites andproduces new metabolites. The design of rational approaches to metabolic engineering requires a proper understanding of pathways that are manipulated, their fluxes, control factors, and the genes involved as impart from other organic compounds for producing of 2,3-BD (Cameron *et al.,* 1997; Jacobsen and Khoslat, 1998; Ailong *et al*., 2010). Therefore, the varieties of genetic approaches

have been used to engineer many bacterial strains for 2,3-BD production. Maryam *et al*. (2009) engineered *Saccharomyces cerevisiae*strains overexpressing *GPD1*, encoding for glycerol-3-phosphatedehydrogenase, and lacking the acetaldehyde dehydrogenase *Ald6.* The deletion caused a carbonflux diversion from ethanol toward glycerol without converting acetate while accumulating acetoin to 2,3-BD. Similarly, Nielsen *et al*. (2010) developed *Escherichia coli* to produce 2,3-BD by deleting *ldhA* (lactate dehydrogenaseA) and *ilvC* (acetohydroxy acid isomeroreductase) genes and expressing *ilvBN* (acetolactate synthase) gene from *Lactobacillus lactis*.

K. oxytoca M5a1, an ideal microorganism for chemical production, has an ability to utilize such the broader range of carbon substratesthan those of *E. coli* or other microorganisms. It does not require any special or expensive source of nutrients during growth and has available techniques for genetic manipulations. It would be a next target microorganism to be engineered thus constructing the metabolic pathway for 2,3-BD production.

An engineered *K. oxytoca* KMS004 (Δ*adhE*Δ*pta-ackA*) strain (Sangproo *et al.,* 2012) rapidly and efficiently ferments sugars to D-(-)-lactate with high yields in mineral salts media. Therefore, this research was focused on 2,3-BD production by deleting a gene encoding *ldhA* (lactate dehydrogenase A) from KMS004. The greater amount of carbon flux through pyruvate route was expected rather than that through acetyl-CoA route during anaerobic and microanaerobic conditions. Further, acetyl-Co A was expected in channeling through α-acetolactate, thus reducing to 2,3-BD at high titer, yield and productivity in mineral salts medium.

1.2 Significance of the study

K. oxytoca KMS004 obtained from Sangproo *et al*. (2012) was used as a host strain to alter the metabolic pathway for producing 2,3-BD as a major fermentative product. Some possible native central metabolic genes that are responsible for producing primarily anaerobic fermentation products in *K. oxytoca* were inactivated by gene deletion techniques. The *ldhA* (lactate dehydrogenase A) was additionally eliminated from chromosomal DNA of the engineered *K. oxytoca* KMS004 (Δ*adhE*Δ*ackA-pta*) (Sangproo *et al.,* 2012). The mutant strains was constructed to develop an efficient microorganism for high production rates, yields, and productivity of 2,3-BD in minimal salts medium under microanaerobic conditions. The investigation of potentialfermentation for 2,3-BD production during batch and fed-batch fermentation was also carried out.

1.3 Research objectives

1. To investigate the effect of*ldhA* gene deletionto 2,3-BD production under microanaerobic conditions in *K. oxytoca* KMS005 (Δ*adhE,* Δ*ackA-pta,* Δ*ldhA'-cat-sacB-ldhA"*).

2. To select the mutant strains of *K. oxytoca* KMS005 that exhibited an efficient production of 2,3-BD using metabolic evolution technique.

3. To evaluate the production rate and yield of 2,3-BD of *K. oxytoca* KMS005 strain under microanaerobic conditions in the mineral salts medium (AM1) in batch fermentation.

4. To determine the activities of enzymes involved in metabolic pathway of 2,3-BD production in *K. oxytoca* KMS005 strains.

5. To perform 2,3-BD production by *K. oxytoca* KMS005-76T strain in batch and fed-batch fermentation using glucose and maltodextrin as carbon substrates.

CHAPTER II

REVIEW OF THE LITERATURES

2.1 The history of 2,3-Butanediol

2,3-butanediol (2,3-BD) is an example of bulk chemical. It has a history of more than 100 years. It was first started in 1906, when Harden and Walpole investigated the microbial production of 2,3-butanediol in *Aerobacter aerogenes* (formerly *Klebsiella pneuminiae*) strain. In 1926, Donker and co-workers was produced 2,3-BD by *Bacillus polymyxa*. Fulmer and co-workers were the first group proposed the 2,3-BD and diol production in industrial scale (Garg and Jain, 1995; Hatti-Kaul *et al.,* 2007). Attention of the strategic compound 1,3-butadiene during World War II focused a research effort on 2,3-BD fermentation. Therefore, development of cultivate pilot-scale for 1,3-butadiene and 2,3-BD was operated. Its production and conversion of 2,3-BD to 1,3-butadiene were used for synthetic rubber production. This work was discontinued in the encounter of competition from synthetic 1,3-butadiene because less expensive routes for chemically producing 1,3-butadiene from petroleum became available (Magee and Kosaric, 1987). Nevertheless, fundamental research studies still continued into physiology and biochemistry of the organisms capable of 2,3-BD production. In the year 1970, following the oil and petroleum price rises the interest was revived in the production of chemical feedstocks, fuels and biorefineries from bio-renewable resources, and this has included 2,3-BD, especially in the United states (Maddox,

1996). Recently, worldwide research of microbial 2,3-BD production has high attractive strategy. Fermentation of excess biomass or agricultural wastes and agro-industrial residues to produce chemicals or generate energy has high benefited considerable attention due to promising route for developing a low cost carbon sources as well as the need for more management of food and feed sources and keep a chance to a more sustainable future (Li *et al.,* 2010).

2.2 Physical and chemical properties of 2,3-Butanediol

2,3-BD is also known as 2,3-butylene glycol, dimethylene glycol or dimethyl-ethylene glycol and the IUPAC name is butane-2,3-diol. Its molecular weight is 90.121 g/mol, molecular formula: $C_4H_{10}O_2$. 2,3-BD can exist in three isomeric forms: D-(-)-, L-(+)- and meso- (Figure 2.1). Usually, a mixture of two types of 2,3-BD is formed from different microorganisms (Khayati *et al.,* 2009). Among the three isomeric forms, a meso- conformation in which the hydroxyl groups are gauche to each other (Figure 2.2) is favored because the hydrogen bond in this formation is gained energy. As a result, the methyl groups are gauche in the meso- form and D-(-)-, L-(+) (anti- form) in the optically active form. Therefore, the optically active form of the isomer is more stable than the meso- form and produced as major product (Voloch *et al.* 1985; Lopes Jesus *et al.,* 2006). The physical properties of 2,3-BD is a colorless and odorless liquid chemical with a very high boiling point and low freezing point. The boiling points of the three stereoisomers are slightly different, ranging from 177°C to 182°C. However, all of sterioisomeric forms are much higher than the boiling point of water. Thus, a large amount of water has to be evaporated due to distillation during recovery of 2,3-BD from fermentation broth (Voloch *et al.,*1985;

Celińska and Grajek, 2009). That is why alternative methods of 2,3-BD recovery are being developed (Sun *et al.,* 2009).

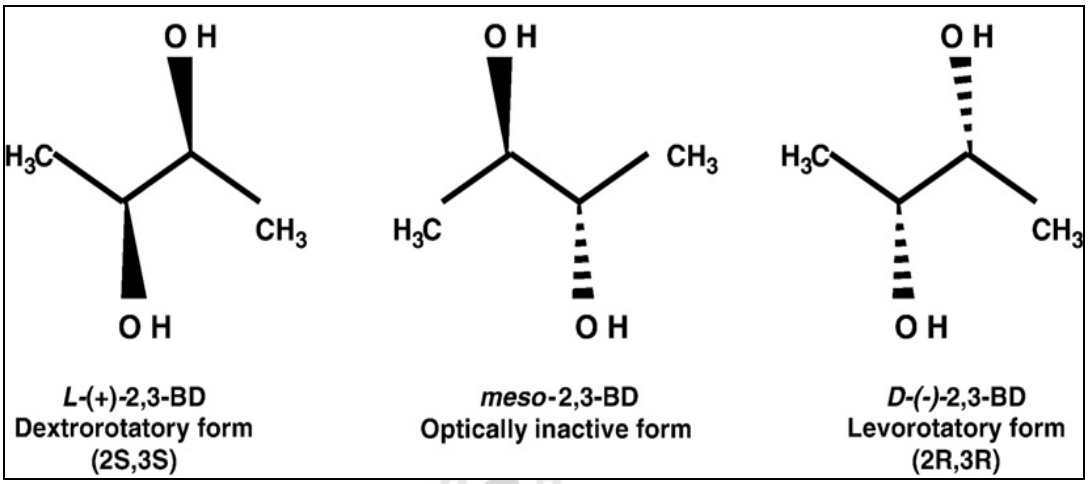

Figure 2.1 The stereoisomers of 2,3-butanediol (2,3-BD) (Celińska and Grajek, 2009).

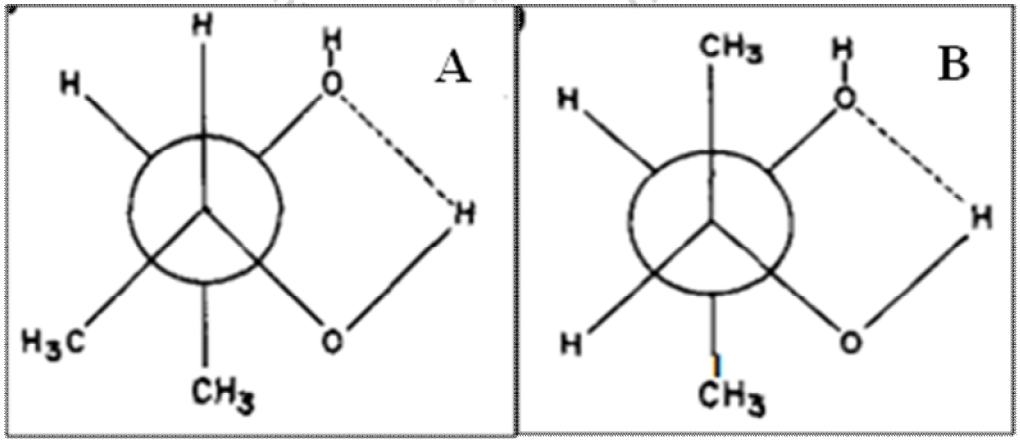

Figure 2.2 Newman projection Anti- and Gauche form of 2,3-butanediol (A) with meso- form OH group Gauche and (B) D-(-)-, L-(+) form OH group Anti (Adapted from Voloch *et al.,* 1985).

2.3 Derivatives of 2,3-Butanediol

2,3-BD is a high benefit chemical feedstock that can be produced by biotechnological route as fermentation of sugar from biomass. Recently, the need for renewable chemical feed stocksfrom biomass has increased due to increase petro-chemical derivatives price, and the finite supply of petroleum. Compound 2,3-BD can be further degraded into many other useful compounds when undergo different types of process, including esterification, ketalization, dehydration and dehydrogenation (Figure 2.3) (Xiao-Jun *et al.,* 2011). 2,3-BD with its derivatives has a wide range of potential application in the many parts of industry such as synthetic rubber, pharmaceutical cosmetics, food additive, solvents and precursors of plasticizers (Bartowsky and Henschke, 2004).

2.3.1 Esterification

The esterification of diesters 2,3-BD can be reacted with monobasic acids or their function equivalents. In the ester form, such as urethane foams, if can be prepared by esterification of 2,3-DB. It could be used as precursor for drugs and cosmetics. Moreover, the diesters group of 2,3-BD could be used as precursors of plasticizers for thermoplastic polymers such as cellulose nitrate, cellulose triacetate, cellulose acetate butyrate, polyvinyl choride, polyvinyl esters, polyacrylates, and polymethylacrylates (Voloch *et al.,* 1985).

2.3.2 Ketalization

The ketalization of 2,3-BD with acetone is to produce a tetramethyl compound. This compound is similar to methyl tert-butyl ether (MTBE) that

commonly used in potential gasoline blending agent. The production of MTBE and other new derived alkyl ether blending agents from other sources to replace petroleum stocks is essential strategy in extending gasoline supply (Stinson, 1979). Furthermore, the other new tetramethyl compound derived from ketalization of acetone and 2,3-BD has an advantage more than traditional method. Both of the substrates for the synthesis of this compound could be derived from fermentation processes (Voloch *et al.,* 1985; Ni and Sun, 2009).

2.3.3 Dehydration

The dehydration of 2,3-BD can be converted to methyl ethyl ketone (MEK; butan-2-one) which is an industrial solvent for resins and lacquer and an effective fuel additive having a higher heat of combustion than ethanol. The heating value of 2,3-BD is 27,198 $J.g^{-1}$ which is similar to other liquid fuels, such as methanol (22,081 $J.g^{-1}$) and ethanol (29,055 $J.g^{-1}$) (Flickinger, 1980; Van Haveren *et al.,* 2008). The dehydration of 2,3-BD reaction can be performed using catalyst such as alumina, or direct reaction with sulfuric acid. This reaction mechanism involves a hydride shift, in fact which could also be made possible by an enzyme catalyzing process. Speraza *et al*. (2001) reported the biosynthesis of MEK from 2,3-BD can be used as dioldehydratase (EC 4.2.1.28) extracted from *Lactobacillus brevis*. In the future, this method would be promising procedure for MEK biosynthesis (Xiao-Jun *et al*., 2011). Moreover, 2,3-BD can be converted to 1,3-butadiene. This product could lead to production of rubber synthesis and most important during World War II. In the past, industrial chemical 1,3-butadiene was derived from only cracked petroleum and then was optional converted 2,3-BD to compounds with ethylenic double bonds through

catalytic dehydration (Van Haveren *et al.,* 2008). Thus, the most successful process for production of 1,3-butadiene from 2,3-BD is using a pyrolysis of 2,3 diacetate or 2,3-BD dibromide. However, this method uses stoichiometric amount of either acetic acid or bromides. Therefore, the development of catalytic dehydration process of 2,3-BD to improve the production of 1,3-butadiene would be essential for commercial production opportunities (Van Haveren *et al.,* 2008; Xiao-Jun *et al.,*2011).

2.3.4 Dehydrogenation

The dehydrogenation reaction of 2,3-BD produces high valuable compounds such as acetoin and diacetyl. Acetoin is applied as an aroma carrier in flavors and perfume. Further, diacetyl is the most important for the organoleptic quality of dairy products, such as butter, cheese, fermented cream, and flavoring agent with a pungent butter aroma as well as also bacteriostatic food additive, since it inhibits growth of some microorganisms (Xiao and Xu, 2007). On the other hand, both acetoin and diacetyl could alternatively be produced by acetic acid bacteria in the oxidative fermentation of 2,3-BD by two sequential reaction enzymes such as membrane-bound alcohol dehydrogenase (EC 1.1.1.1) and aldehyde dehydrogenase (EC 1.2.1.3). Both of enzymes reactions are involved in respiratory chain located in the cytoplasmic membrane. Consequentially, they could catalyze D-(-)-, L-(+)-2,3-BD and meso-2,3-BD to produce chiral (R)-acetoin and (S)-acetoin (Romano *et al.,* 2002; Xiao *et al.,* 2010). However, acetoin can be oxidized to diacetyl by oxygen presenting in fermentation broth or reduced by enzyme reaction to 2,3-BD such as diacetylreductase (DAR) with NADH as a cofactor (Voloch *et al.,* 1985).

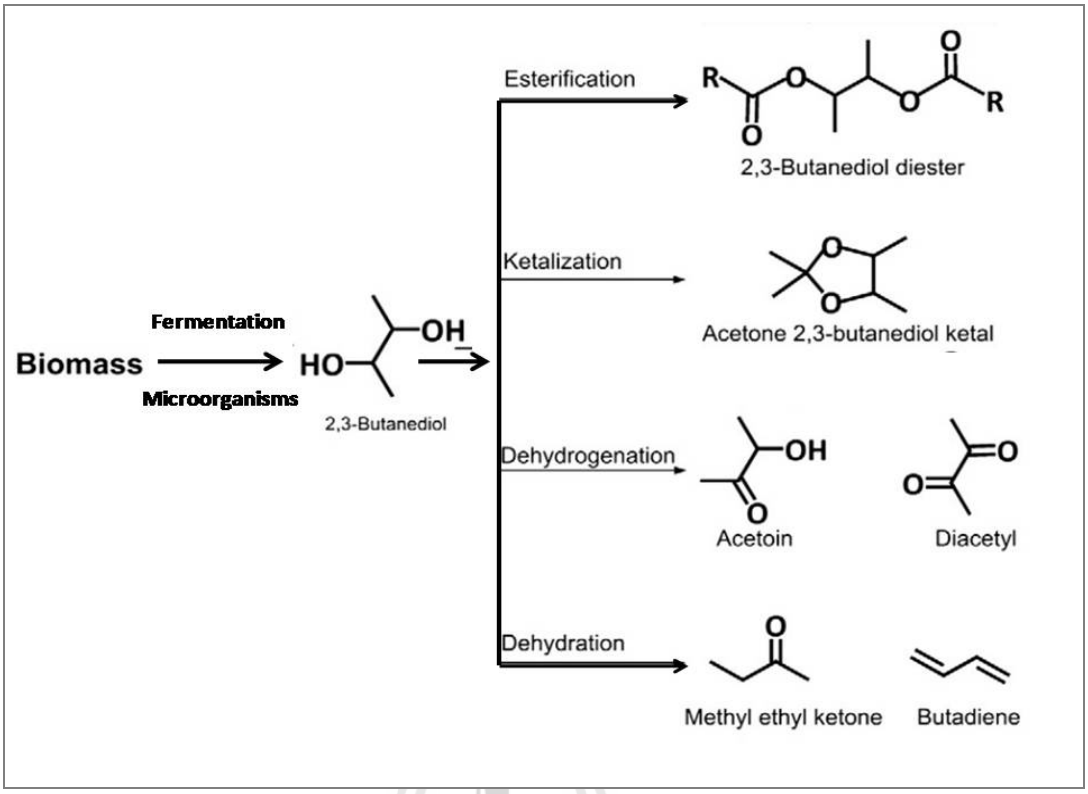

Figure 2.3 Derivatives of biologically produced 2,3-BD (Adapted from Xiao-Jun *et al.,* 2011).

2.4 Biochemistry and physiology of 2,3-BD production

2.4.1 2,3-BD Producing microorganisms

A number of microorganisms are able to produce 2,3-BD but only a few species which are noted for this ability include those belonging to the genera *Klebsiella, Enterobacter, Bacillus* and *Serratia,* which are robust for the industrial of 2,3-BD (Maddox, 1996). Moreover, the experimental data infer the presence of the 2,3-BD pathway in the following taxonomic groups for example, *Aeromonas hydrophila*, *B. subtilis*, *Brevibacillus brevis* S1, *Corynebacterium glutamicum*, *E. aerogenes*, *Aerobacter aeromonas* (also known as *K. pneumonia*), *L.bravis, L. casei*, *L. helveticus*, *L. plantarum*, *L. lactis, L. lactissub* sp. *lactis* bv. *diacetylactis*,

Leuconostoc lactis, *Leuconostoc mesenteroides* subsp. *cremoris*, *Oenococcus oeni, Pediococcus pentosaceus, Raoultella terrigena, Serratiam arcescens, B. polymixa, K. terrigena, Streptococcus faecalis, B. amyloliquefaciens*, (Hespell, 1996; Nakashimada *et al.,* 2000; Blomqvist *et al.,* 1993), *B. licheniformis* (Perego *et al.,* 2000), *Rhizobacterium chlororaphis* O6 (Cho *et al.,* 2008). Some strain of yeasts have the ability to produce 2,3-BD. Nevertheless, productivity of 2,3-BD that produced from yeast is very poor, and therefore bacteria are the most important organisms in the 2,3-BD fermentation (Grag and Jain, 1995). Previously described, 2,3-BD has three stereoisomeric forms, which are D-(-)-, L-(+)-2,3-BD and meso- form. The isomers produced dependent upon the different types of microorganism. Different microorganisms produced different stereoisomers, whereas a mixture of two stereoisomers is generally formed (Table 2.1). The productivity and product yields of 2,3-BD in different bacteria are also summarized in Table 2.1.

So far, *K. pneumonia*, *K. oxytoca*, and *P. polymyxa* have been invulnerable in the efficient production of 2,3-BD. *E. aerogenes* and *S. marcescens* are also considered promisingmicroorganisms for this application.These organisms are Gram-negative, facultatively anaerobic rods, and are members of the family Entero-bacteriaceae. Under strict anaerobic conditions, they produce preferentially hydrogen, but when performed under microaeration, they turn into a 2,3-BD producer, very similar to *K. oxytoca* (Perego *et al.,* 2000). In fact, it seems to be considering the advantages and disadvantages of each in somewhat greater detail. The advantages of *Klebsiella* sp. to produce 2,3-BD is that the species have broad substrate spectrum and present in hemicellose and cellulose hydrolysates, are wildly distributed in nature, and stable under a wide range of environmental condition (Chandel *et al.,* 2010).

However, the pathogenicity of opportunistic infection caused by the encapsulated *Klebsiella* sp. is a general problem obstructing the pilot scale 2,3-BD.

B. licheniformis is capable of 2,3-BD production and lactate as a byproduct. *B. subtilis* produces a mixture of D-(-)- and meso- stereoisomer (Table 2.1), as well as glycerol rather than ethanol as a byproduct (Maddox, 1996). Furthermore, *B. amyloliquefaciens* behaves similarly, but produces small amount of byproducts. In case of *K. oxytoca* and *B. polymyxa* can utilize pentose sugars and hexose sugars, which is the important property of both strains since hydrolysates of biomass materials can have a pentose glucose ratio of 1:1.5 (Tsao *et al.,* 1982). Thus, almost all of the sugars exist in hemicellulosic raw materials can be converted to 2,3-BD. Nevertheless, attention should be the presence and relative amounts of the byproducts, since they directly influence 2,3-BD yields and downstream procedures (Maddox, 1996).

Table 2.1 2,3-BD stereoisomers produced by different species (in parentheses: relative amount of the isomer is given).

Strains	2,3-Butanediol stereoisomer	Substrates	Productivity[a] [yield][b]	References
K. pneumonia	meso-, L-(+)-	Glucose	4.21 [0.43]	Ma *et al*., 2009
K. pneumonia	meso-, L-(+)-	Glucose	2.10 [0.49]	Qin *et al*., 2006
K. pneumonia	meso-, L-(+)-	Corncob molasses	1.35 [0.41]	Wang *et al*., 2010
K. pneumonia	meso-, L-(+)-	Glycerol	0.18 [0.39]	Petrov and Petrova, 2009
K. pneumonia	meso-, L-(+)-	Glycerol	0.47 [0.39]	Petrov and Petrova, 2010
K. pneumonia	meso-, L-(+)-	Jerusalem artichoke tuber	2.29 [0.32]	Sun *et al*., 2009b
K. pneumonia	meso-, L-(+)-	Jerusalem artichoke	1.18 [1.81]	Li *et al*., 2010a
K. oxytoca	meso-, L-(+)-	Corncob hydrolysate	0.59 [0.50]	Cheng *et al*., 2010
K. oxytoca	meso-, L-(+)-	Glucose	1.64 [0.68]	Ji *et al*., 2010
K. oxytoca	meso-, L-(+)-	Glucose	1.74 [0.49]	Ji *et al*., 2009
K. oxytoca	meso-, L-(+)-	Corncob molasses	2.40 [0.42]	Afschar *et al*., 1991

Table 2.1 (Continued) 2,3-BD stereoisomers produced by different species (in parentheses: relative amount of the isomer is given).

Strains	2,3-Butanediol stereoisomer	Substrates	Productivity[a] [yield][b]	References
K. oxytoca	meso-, L-(+)-	Cellulose	0.36 [0.31]	Cao *et al*., 1997
K. oxytoca	meso-, L-(+)-	Glucose	3.22 [0.50]	Qureshi and Cheryan, 1989
Enterobacter aerogenes	meso-, L-(+)-	Sucrose	2.67 [0.41]	Zeng *et al*., 1991
Serratia marcescens	meso-, D-(-)-	Sucrose	3.49 [0.47]	Zhang *et al*., 2010a
Serratia marcescens	D-(-)-, meso-	Sucrose	0.88 [0.50]	Zhang *et al*., 2010b
Paenibacillus polymyxa	D-(-)-, meso-	Sucrose	0.33 [0.38]	Gao *et al*., 2010
Bacillus subtilis	D-(-)-, meso-	Jerusalem artichoke tuber	0.47 [0.12]	Moes *et al*., 1985
Bacillus licheiformis	D-(-)-, meso-	Glucose	0.33 [0.38]	Nilegaonkar, *et al*., 1992
Bacillus amyloliquefaciens	D-(-)-, meso-	Glucose	- [0.12]	Alam *et al*., 1990

[a] 2,3-BD Productivity = 2,3-BD concentration produced divided by overall incubation time (g/L.h).

[b] 2,3-BD Yield = gram of 2,3-BD divided by gram substrate consumed (g/g).

2.4.2 Metabolic pathway of 2,3-BD production

A variety of monosaccharides such as hexoses and pentoses can be fermented to 2,3-BD (Syu, 2001). For the fermentation pathway, pyruvate produced during glycolysis is converted into mixed acids such as acetate, lactate, formate, succinate and ethanol and (Figure 2.4). In addition, 2,3-BD is produced from pyruvate in a mixed acid fermentation through several intermediate compounds, including α-acetolactate, acetoin and diacetyl (Figure 2.5) (Caspi, 2008).

Pyruvate can be converted either into lactate in a reaction with a requirement of NADH. This reaction is catalyzed by lactate dehydrogenase (LDH; EC 1.1.1.27) enzyme.Pyruvate is also decarboxylated to α-acetolactate. Then, it is catalyzed by α-acetolactate synthase (α-ALS; EC 4.1.3.18). α-Acetolactate is mostly produced under low availability of NADH, then α-acetolactate can be further converted to acetoin by α-acetolactate decarboxylase (α-ALD; EC 4.1.1.5), and this occurs under anaerobic conditions. Furthermore, during the fermentation of 2,3-BD, α-acetolactate can endure spontaneous decarboxylation thus generating diacetyl. Diacetylreductase or acetoin dehydrogenase (DAR; EC 1.1.1.303) can convert diacetyl to acetoin. At last, 2,3-butanediol dehydrogenase or acetoinreductase (BDH; EC 1.1.1.4) reduces acetoin to 2,3-BD.During under microaerobic conditions, LDH, pyruvate-formatelyase (PFL; EC 2.3.1.54), and α-ALS influence pyruvate to generate lactate, formate and 2,3-BD, respectively. Further, formate can be reduced to carbon dioxide and hydrogen by formate-hydrogen lyase complex, which can be suppressed under aerobic conditions or by presence of nitrate in anaerobic conditions. Alternatively, succinate generated from the flow of carbon through the phosphoenol pyruvate (PEP) pathway. PEP is carboxylated to oxaloacetate by PEP carboxylase

(PPC; EC 4.1.1.31), and thus generating succinate under strict anaerobic conditions (Figure 2.4) (Gottshalk, 1986; Magee and Kosaric, 1987).

Under slightly acid conditions (pH 6.0), α-ALS has an optimum selectivity condition and acetate is an effective inducer of α-ALS, α-ALD and BDH enzymes producing 2,3-BD from pyruvate (Gottshalk, 1989; Bryn *et al.,* 1973). Finally, all enzymes and metabolic compounds involved in 2,3-BD pathway are normally produced during the late log stationary phases of fermentation, under oxygen-limiting conditions (Malonee and speckman, 1988).

2.4.3 Genes and enzymes involved in 2,3-BD biosynthesis

The 2,3-BD operon of *K. terrigena* and *E. aerogenes* has been cloned and characterized by Blomqvist (1993). They show that the three enzymes involved in the bacterial synthesis of 2,3-BD from pyruvate are encoded by genes *budA*, *budB*, *budC* and *budR*, encoding catabolic α-acetolactate synthase (α-ALS; EC4.1.3.18), α-acetolactate decarboxylase (α-ALD; EC4.1.1.5), acetoin (diacetyl) reductase (AR) or 2,3-butanediol dehydrogenase (BDH; EC 1.1.1.4), and transcriptional activator gene, respectively (Figure 2.6). All three genes (*budA*, *budB*, *budC*) have potential ribosomal binding site 6 to 7 nucleotides in front of the translational start site. The site of this operon is 3.4 kb.

The catabolic α-ALS is involved in the formation of α-acetolactate from pyruvate. α-Acetolactate is decarboxylated by α-ALD into acetoin, which in turn it is reduced in a reversible into 2,3-BD by BDH. The 2,3-BD operon (*budABC*) appears to be regulated at the transcriptional level, as the highest amount of transcript was observed under conditions that favored 2,3-BD production. Furthermore, a putative

fumarate nitrate reduction regulatory (FNR) protein site was found at position -6. The FNR protein could activate genes at the transcription level of *budABC* operon under anaerobic processes (Blomqvist, 1993). In addition, the cloning of the*budABC* operon facilitated the study of gene regulation and improvement of the 2,3-BD biosynthesis (Blomqvist, 1993; Mayer *et al.,* 1995).

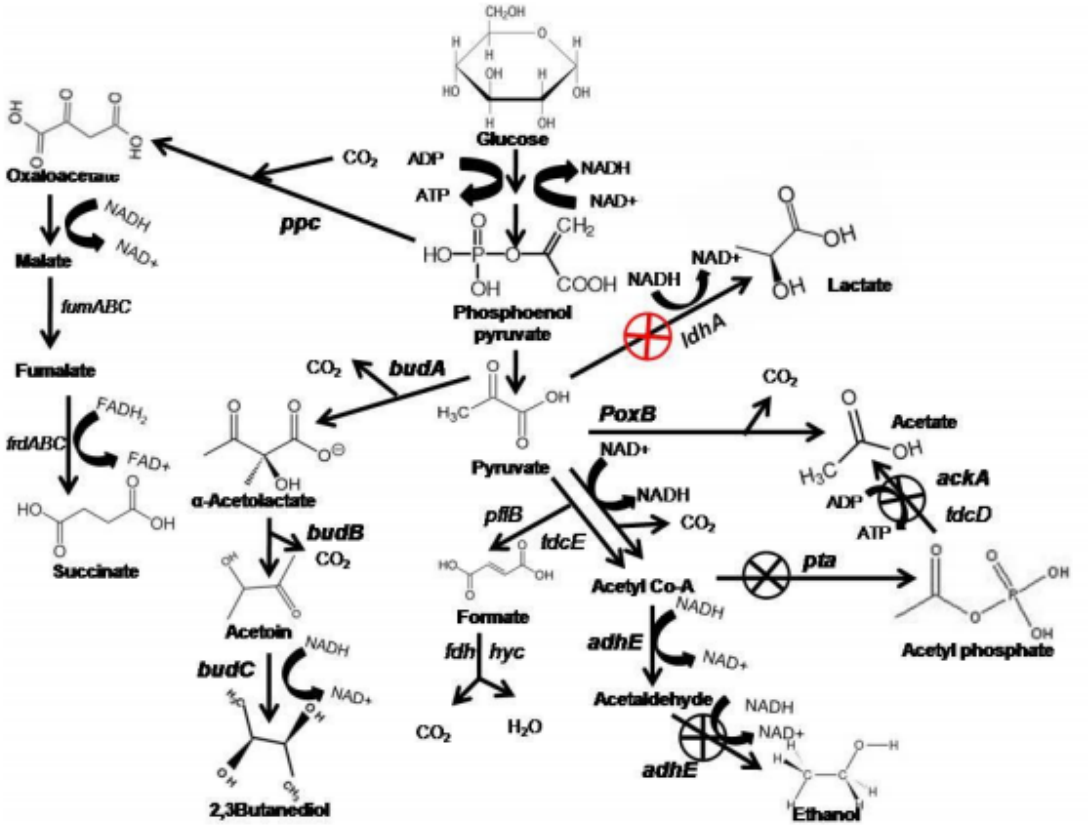

Figure 2.4 The fermentation pathway of 2,3-butanediol production in *K. oxytoca* under anaerobic conditions. Solid arrows represent central fermentative pathways. Black crosses represent the gene deletions performed in previous study to obtain KMS004 (Δ*adhE*, Δ*pta-ackA*). Red cross represent the gene deletion performed in this study to obtain KMS005 (Δ*adhE*, Δ*pta-ackA*, Δ*ldhÁ-cat-sacB-ldhÁ*˝) or KMS006 (Δ*adhE*, Δ*pta-*

ackA, Δ*ldhA*). Genes and enzymes: *ldhA*, lactate dehydrogenase; *pflB*, pyruvate formate-lyase; *pta*, phosphate acetyltransferase; *ackA*, acetate kinase; *adhE*, alcohol dehydrogenase; *ppc*, phosphoenolpyruvate carboxylase; *fumABC*, fumaraseisozymes; *frdABCD*, fumaratereductase; *fdh*, formate dehydrogenase; *poxB*, pyruvate oxidase; *budA*, acetolactate decarboxylase; *budB*, acetolactate synthase, *budC*, 2,3butanediol dehydrogenase; *aldA*, aldehyde dehydrogenase isozymes.

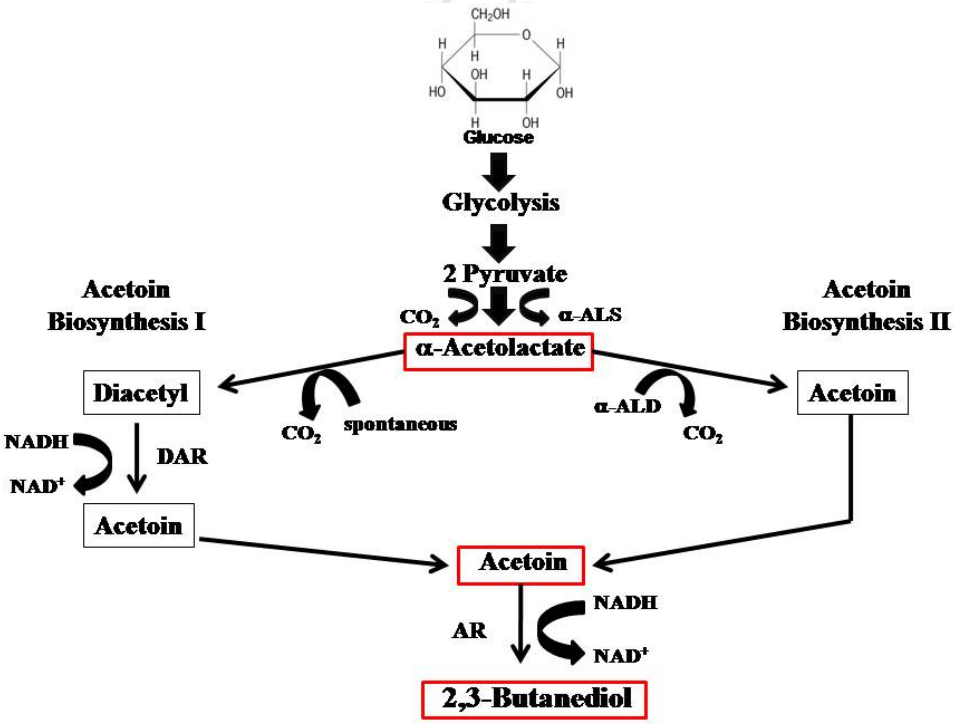

Figure 2.5 Metabolic pathways of 2,3-BD production from glucose. Two alternative pathways of acetoin synthesis (I and II) are pictured. α-ALS: α-acetolactatesynthase; α-ALD: α-acetolactate decarboxylase; DAR: diacetylreductase. AR: acetoinreductase. (Adapted from Celińska and Grajek, 2009).

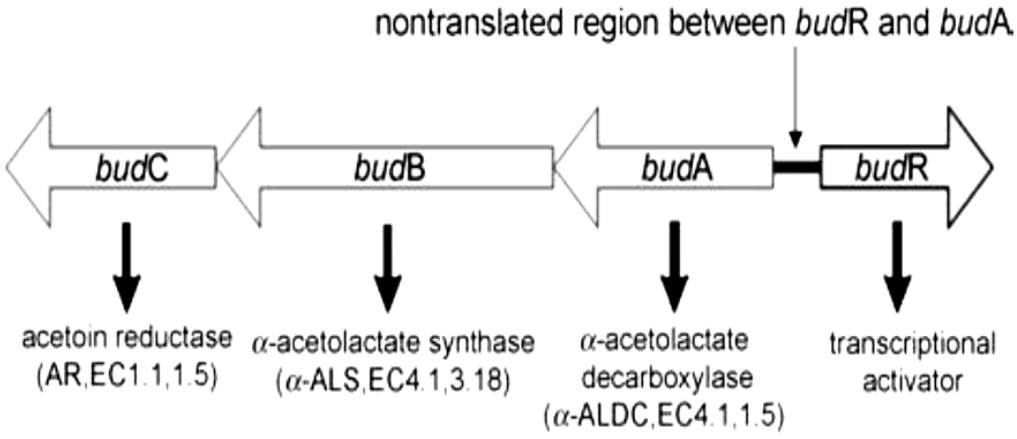

Figure 2.6 Genetic organization of *budABC* in *Klebsiella terrigena* (Xiao-Jun *et al*., 2010).

2.4.4 Physiological function of 2,3-BD in microbial metabolism

The function of metabolic 2,3-BD has not yet clarified. However, it plays an important role for preventing intracellular acidification by changing the acid metabolites to the formation of neutral compounds (Blomqvist, 1993; Van Houdt *et al.,* 2007). It is known that, 2,3-BD biosynthesis is induced under acid conditions. It suggests that 2,3-BD is a neutral metabolite then neutralized excessive acid in surrounding environment (Nakashimada *et al.,* 2000). In addition, it is known that acetate induces enzymes involved in 2,3-BD biosynthesis pathway (Barret *et al.,* 1983; Mayer *et al.,* 1995).

The possible second function of the 2,3-BD biosynthesis pathway is decreasing the excess reducing power associated with glycolysis pathway. Thus, the 2,3-BD pathway like other fermentative processes, participates in regulation ratio of the electron carrier such as $NADH/NAD^+$ in the cell (Magee and Kosaric, 1987).

Third, microorganisms can reutilize 2,3-BD during the stationary phase, when variety ofcarbon and energy sources have been depleted. Therefore, the

synthesis of 2,3-BD is regarded a carbon and energy storing-strategy (Xioa and Xu, 2007).

In summary, 2,3-BD is a product of fermentative metabolism whose production serves to maintain a redox balance within the cell during growth under oxygen limited conditions. In addition, its production is favored when the cell's requirement for energy (ATP) is low.

2.5 Substrates

2.5.1 Utilization of different carbon sources

A variety of carbohydrates can be used to produce 2,3-BD. 2,3-BD can be produced from microbial fermentation pathway, especially from bacterial utilizing hexose or pentose.

Recently, many studies have been focused on fermentation of sugars from lignocellulosic substrates, particularly those derived from the hemicelluloses fraction. Actually, all of these sugars can be utilized by *P. polymyxa* and *K. oxytoca* (Maddox, 1996). However, both strains require pre-treatment substrates, due to the lack of cellulases and hemicellulases. Unfortunately, some of the pre-treatment procedures consist cellular toxic or inhibitor derivatives produced during the fermentation process (Frazer and McCaskey, 1991).

Waste from starch hydrolysate was the most promising raw material, ensuring the highest product yield and productivity, which was similarly estimated from glucose. Furthermore, whey permeate, a byproduct from the dairy industry, has a interest as an alternative substrate for 2,3-BD production, since whey permeate contains high lactose content and is availability in many dairy farm countries.

However, comparing between whey permeates and starch or sugar substrates, whey permeate has relatively poor substrate considering overall reactor productivities in batch fermentation (Maddox, 1989). Sun *et al*. (2009) reported that the utilization of Jerusalem artichoke (topinumbur) tubers was performed by *K. pneumoniae*. The results showed that, process yields of 81.59 and 91.63 g/L were obtained in batch and fed-batch SSF (simultaneous saccharification and fermentation) processes, respectively. For the cheap substrate, glycerol was used with *Klebsiella* sp. especially in *K. pneumoniae* DSM2026 (Wu *et al.,* 2008) and *K. pneumonia*e GT1 under uncontrolled pH conditions, which favours the production of 2,3-BD (Menzel *et al.,* 1997). On the other hand, Biebl *et al*., (1998) found that when glycerol is used as carbon source, pH value below 7.0 (around 6.0) might be controlled in order to achieve a higher 2,3-BD production.

2.5.2 2,3-BD production from lignocellulosic substrates

Lignocellulose is the most abundant biomass on earth. It has attracted alternative feedstock because of its ready availability and renewable nature. The majority components of lignocellulosic substrates are lignin, cellulose and hemicelluloses. Normally, cellulose is a polymer of glucose, while hemicellolose is a polymer consisting of pentose sugars such as xylose, arabinose and ribose (Saha, 2003; Yan *et al.,* 2009). So far, the use of lignocellusic substrates as a low-cost and alternative substrate for 2,3-BD production to improve the economics of the process received considerable attention (Ragaukas *et al.,* 2006). Cao *et al*. (1997) reported the one kind of lignocellulosic substrate derived from corn process. Corn cob is a low-cost and wildly available agricultural residue. Pre-treatment process of corn cob was

performed with dilute ammonia and hydrochloric acid. After pretreatment 90% of cellulose in corn cob was converted to glucose, then it was used as the substrate for 2,3-BD fermentation. Cheng *et al.* (2010) treated the acid hydrolysates of corn cob by sequentially boiled, following withadsorbing the hydrolysate onto activated charcoal.Thus, pentose rich hydrolysate was used as substrate for 2,3-BD fermentation by *K. oxytoca* under optimal conditions. In addition, Wang *et al.* (2010) used corn cob molassesthat contained high concentrations of hemicelluloses to produce 2,3-BD by *K. pneuminiae*. The maximum 2,3-BD concentration was 78.9 g/L of fed-batch fermentation (Wang *et al.,* 2010). Using corn cob molasses for 2,3-BD production, not only reduce the feedstock cost, but also provided a method to utilize xylitol that is byproduct from industry thus reducing environmental pollution.

Jatropha hulls were successfully used as raw materials for the production of 2,3-BD via two step dilute sulfuric acid hydrolysis and fermentation with *K. oxytoca* (Jiang *et al.,*2012). After hydrolysis, maximum yield of 2,3-BD in shake flask from the first-and second step hydrolysis, equivalent to 71.2% and 82.8%, respectively, which is the first recorded amount obtained from plant seed hulls as a sole carbon source so far.

2.6 *Klebsiella oxytoca*: 2,3-BD producer

K. oxytoca M5a1 is a Gram-negative, rod-shaped and enteric bacterium which is found to grow in paper and pulp steams as well as around other sources of wood. This bacterium is in the Phylum of Proteobacteria, the class of Gamma Proteobacteria, the Order of Enterobacteriales, and the Family of Enterobacteriaceae (Mahon *et al.*, 2007). This microorganism is capable of growing at a pH at least 5.0 and temperature

as warm as 35°C in the minimal salts medium. In addition, this microorganism grows on a wide variety of sugars including hexoses and pentoses, without any requirements of expensive nutrients (Dien *et al.,* 2003). Furthermore, this microorganism has the ability to utilize glycerol as a sole carbon substrate. The wasted glycerol from biodiesel plant would be a potential carbon substrate for this microorganism used for production of chemicals. Thus, this microorganism would be applied as a model host strain for 2,3-BD production by using cheap and wasted substrates.

Moreover, many reports of 2,3-BD production from lignocellulosic biomass is an alternative approach in the conversion of biomass substrates to liquid fuels and chemical feedstocks (Johan *et al.,* 1975; Magee and Kosaric, 1987). The efficient utilization of all in the cellulose and hemicellulose would be economically attractive achievement (Yu and Saddler, 1983). *K. oxytoca* has been shown to utilize all of the major sugars including hexoses, pentoses, disaccharides and uronic acid derived from the hydrolysates of lignocellulosic material (Yu and Saddler, 1983).

2.7 Strategies for efficient 2,3-BD production in previous study

Many efforts have focused on improving strains to produce 2,3-BD with high yield, using low price substrates to lower the cost of feedstock and optimized the operation mode to make the process more efficiently. Table 2.2 shows 2,3-BD productivity and yield obtained from various 2,3-BD producers with various fermentation conditions.

Table 2.2 Previous study of 2,3-BD production from various microorganisms.

Organism	Medium/Condition	BDO (g/L) Productivity [g/L/h]	Yield [g/g]	References
K. oxytoca NBRF4 (chemical mutation)	- 44 g/L glucose, supplemented with YP medium, pH 4.3, aeration 10% dissolved oxygen, batch, 18 h incubation, 38°C, 200 rpm.	14.4 [0.78]	0.32	Han *et al.,* 2013
K. pneumonia KCTC2242 (Δ*wabG*)	- 70 g/L glucose, supplemented with minimal medium containing 5g/L yeast extract, trace elements solution, pH 5.5, batch, 72 h incubation, 37°C without shaking.	22.44 [0.31]	0.44	Jung *et al.,* 2013
K. oxytoca	- 90 g/L glucose, supplemented with a medium containing 5 g/L yeast extract, trace elements, pH 6.5, aeration 1.0 vvm, batch, 30 h incubation, 37°C without shaking.	30 [1.15]	0.33	Park *et al.,* 2013
K. pneumonia DSM 2026	- 72 g/L glucose, supplemented with minimal medium containing 5 g/L yeast extract, pH 6.5, aeration 3.0 L/min, batch, 10 h incubation, 37°C, 150 rpm.	17.6 [1.76]	0.27	Cho *et al.,* 2012

Table 2.2 (Continued) Previous study of 2,3-BD production from various microorganisms.

Organism	Medium/Condition	BDO (g/L) Productivity [g/L/h]	Yield [g/g]	References
E. coli BL21 (DE3) over expression of *bud* genes	- LB medium containing 40g/L glucose, 20 g/L diacetyl,100 μg/mL ampicillin, 1mM IPTG, pH 7.4, batch, 10 h incubation, 37°C, 180 rpm.	16.1 [2.70]	0.40	Li *et al.,* 2012
S. cerevisiae (Δ*adh1* Δ*adh3*Δ*adh5*Δ*gpd2*Δ*ald6*) and over-expressed *B. subtilisals S and E. aerogenesbudA* genes	- 20 g/L glucose, supplemented with 6.7 g/L yeast nitrogen base without amino acid, 0.42 g/L Tween 80 and 0.01 g/L cholesterol, without pH control, batch, 85 h incubation, 30°C, 250 rpm.	2.29 [0.26]	0.11	Ng *et al.,* 2012
Enterobacter cloacae subsp. *dissolvens* SDM	- 188 g/L cassava powder hydrolysate, supplemented with corn steep liquor (10 g/L) as nitrogen source, trace minerals, pH 6, batch (SSF), 24 h incubation, 30°C, 100 rpm.	78.3 [3.30]	0.42	Wang *et al.,* 2012
B. licheniformis (ΔldhA)	- 30 g/L glucose, supplemented with minimal medium, pH 5.0, aeration 0.5 L/min, batch fermentation, 12 h incubation, 50^{o}C, 250 rpm.	13.0 [0.07]	0.45	Wang *et al.,* 2012

Table 2.2 (Continued) Previous study of 2,3-BD production from various microorganisms.

Organism	Medium/Condition	BDO (g/L) Productivity [g/L/h]	Yield [g/g]	References
Geobacillus sp. XT15	- 220 g/L glucose, supplemented with GPY medium (80 g/L peptone, 10 g/L yeast extract), without pH control, batch, 48 h incubation, 55°C, 170 rpm.	14.5 [0.30]	0.22	Xiao *et al.,* 2012
E. coli W3110 over-expressed *K. pneumonia budAC*	- 60 g/L glucose, supplemented with a minimal medium, 100 μg/ ml ampicillin, pH 6.8, aeration 1.5 L/min, batch, 48 h incubation, 30°C, 200 rpm.	15.7 [0.33]	0.25	Lee *et al.,* 2012
Enterobacter aerogenes EMY-01*(ΔldhA)*	- 90 g/l glucose, supplemented with a medium containing 5 g/L yeast extract, pH 6, aeration 1.0 vvm, batch, 12 h incubation, 37°C, 150 rpm.	23.2 [1.93]	0.30	Jung *et al.,* 2012
K. pneumonia SGJSB04 over-expressed *budABC*	- 100 g/L glucose, supplemented with a minimal medium containing 5 g/L yeast extract, pH 5.5, aeration 0.75 vvm, fed-batch, 40 h incubation, 37°C, 300 rpm.	101.53 [2.54]	0.34	Borim *et al.,* 2012

Table 2.2 (Continued) Previous study of 2,3-BD production from various microorganisms.

Organism	Medium/Condition	BDO (g/L) Productivity [g/L/h]	Yield [g/g]	References
Syn. elongatus PCC7942	- BG-11 medium, containing 50 mM $NaHCO_3$, 10 mg/L thiamine, and 10 mg/L gentamicin with constant light (55 $\mu E \cdot s^{-1} \cdot m^{-2}$), pH 7.5, 72 h incubation, 30°C, 100 rpm.	2.38 [0.01]	NA	Oliver *et al.,* 2012
K. pneumonia CICC 10011	- 100 g/L glucose, supplemented with a minimal medium containing 0.5 g/L peptone, 1.5 g/L yeast extract, trace elements, pH 6.0, batch, 6 h incubation, 120 rpm, 37°C.	40.7 [6.70]	0.44	Liu *et al.,* 2011
K. pneumonia CICC 10011	- 100 g/L glucose, supplemented with a minimal medium containing 0.5 g/L peptone, 1.5 g/L yeast extract, trace elements, pH 6.0, batch, 6 h incubation, 120 rpm, 37°C.	40.7 [6.70]	0.44	Liu *et al.,* 2011
K. oxytoca ME-UD-3	- 220 g/L glucose, supplemented with a medium, pH 6.0, aeration 1.0 vvm, batch, 81 h incubation, 37°C, 200 rpm.	86.2 [1.06]	0.39	Nie *et al.,* 2011

Table 2.2 (Continued) Previous study of 2,3-BD production from various microorganisms.

Organism	Medium/Condition	BDO (g/L) Productivity [g/L/h]	Yield [g/g]	References
B. amyloliquefaciens B10-127	- 200 g/L glucose, supplemented with a medium containing 4 g/L K_2HPO_4, 10 g/L corn steep liquor, 10 g/L soybean meal without pH, batch, 72 h incubation, 37°C, 160 rpm.	74.3 [0.74]	0.37	Yang *et al.,* 2011
Clostidium ragsdalei, *C. autoethanogenum* and *C. ljungdalii*	- 0.5% (w/v) fructose for heterotrophic growth and steel mill waste gas containing 44% CO, 32% N_2, 22% CO_2, 2% H_2, supplemented with modified PETC medium for autotrophic growth. 30°C forv*C. ragsdalei*, 37°C for *C. autoethanogenum*and *C.ljungdalii*, without pH, batch (strict anaerobe), 200 h incubation, without shaking.	0.13-0.18 [NA]	0.26-0.36	Köpke *et al.,* 2011
C. acetobutylicum ATCC 824 over-expressed *budC*	- 60 g/L glucose, supplemented with MG medium containing 2.5 g/L yeast extract, 3.0 g/L *p*-aminobenzoic acid, 100 μg/mL ampicillin, 30 μg/mL chloramphenicol, 40 μg/mL erythromycin and 50 μg/mL IPTG, without pH, batch, 140 h incubation, 37°C, without shaking.	2.4 [0.02]	0.042	Siemerink *et al.,* 2011

Table 2.2 (Continued) Previous study of 2,3-BD production from various microorganisms.

Organism	Medium/Condition	BDO (g/L) Productivity [g/L/h]	Yield [g/g]	References
*K. pneumoniae subsp. pneumoniae*G31	- 30 g/L glycerol, supplemented with a medium, pH fluctuation, aeration varied between 1.1-2.2 vvm, fed-batch, 150 h incubation, 37°C, 200 rpm.	70.0 [0.47]	0.39	Petrov and Petrova, 2010
K. oxytoca ACCC 10370	- Corncob hemicellulose hydrolysate (39.5 g/L xylose, 9.9 g/L glucose, 1.5 g/L arabinose, and 1.8 g/L acetate), supplemented with a medium containing 1.5 g/L yeast extract, pH 6, aeration 0.3 vvm, batch, 48 h incubation, 37ºC, 300 rpm.	23.5 [0.49]	0.46	Cheng *et al.,* 2010
K. oxytoca ME-UD-3 (Δ*ald*A)	- 200 g/L glucose, supplemented with a medium containing EDTA 0.05 M, pH 6.5, aeration 1.0 vvm, fed-batch, 60 h incubation, 37°C, 200 rpm.	130 [1.63]	0.48	Ji*et al.,* 2010a
E. coli JM109 (Δ*ldhA*Δ*pta*Δ*adhE*Δ*pox B*) over-expressed of *alsSD B. subtilis*	- 60 g/L glucose, supplemented with LB medium containing 100 µg/mL ampicillin, pH 6.8, aeration 1.5 L/min, batch, 36 h incubation, 37°C, 200 rpm.	25.8 [0.71]	0.43	Li *et al.,* 2010

Table 2.2 (Continued) Previous study of 2,3-BD production from various microorganisms.

Organism	Medium/Condition	BDO (g/L) Productivity [g/L/h]	Yield [g/g]	References
Serratia marcescens H30 (Δ*swrW*)	- 200 g/L sucrose, supplemented with a medium containing 34 g/L yeast extract, pH 6.0, fed-batch, 57 h incubation, 30°C, aeration and agitation at 1.0 vvm, 600 rpm for first 15 h and at 0.5 vvm, 500 rpm for after 15 h.	152 [2.67]	0.92	Zhang *et al.*, 2010b

[a]The 2,3-BD productivity was calculated as 2,3-BD concentration produced divided by overall incubation time.

[a]2,3-BD Yield = gram of 2,3-BD divided by gram substrate consumed (g/g).

NA= Not available.

2.8 Factors effecting 2,3-BD production

Many literaturewere studied of the influence of different fermentation condition on the 2,3-BD production. Alam *et al*. (1990) reportedthe effect of various pH, carbon sources, initial substrate concentration, inoculums size and aeration on 2,3-BD production. They concluded that the initial substrate concentration and oxygen significantly influenced the product yield, productivity and byproducts formation. However, it should be noted that a variety of microorganisms and substrates were used, and the conditions of fermentation might not be optimized with regard to the discussed parameter.

2.8.1 Effect of pH

pH is a fundamental parameter for regulating a bacterial metabolism. The pH of the fermentation medium affects both cell biomass compositions and the bacterial metabolism involving enzymes activity and membrane transport in the cell. The optimum pH value for 2,3-BD production by *K. oxytoca* ranges from 5 to 6 (Voloch *et al.,* 1985). In *K. pneumoniae*, a pH above 6 causes a sharp decrease in the activity of α-acetolactate synthase (α-ALS), the one of the key enzymes in the 2,3-BD pathway (Stromer, 1968). For *E. aerogenes*, pH 6 is the optimal condition for 2,3-BD production (Couverti *et al.,* 2003). Pergo *et al*. (2003) also determined a pH value of 6 is the optimum condition for the 2,3-BD production by *E. aerogenes*. However, the production yield was nearly constant within a narrow range of pH 5.0 to 6.5, and it rapidly decreases either at lower or higher pH value. The lower pH value usually inhibits cell growth.

2.8.2 Effect of temperature

The efficiencies of bioprocesses are seriously temperature-dependent owing to the strong dependence of enzymatic activity and cellular maintenance upon temperature (Garg and Jain, 1995). In general, the appropriate temperature for the bacterial fermentationof 2,3-BD production is reported to be in the range of 30 to 35°C. Since, microbial production of 2,3-BD is generally accepted to be a growth-associated event, the maximum product formation must approximately be the same as that maximum biomass yield. In addition, Barret *et al*., (1983) determined the effects of temperature with *K. pneumoniae* and *E. aerogenes* in the range of 30 to 37°C on 2,3-BD production. The result showed the reduction of 2,3-BD production by 66%, but little effect was observed over the same temperature range with *E. aerogenes*. Bieblt *et al*. (1998) cultivated *K. pneumonia* at the lowering temperature in the range of 35 to 30°C. The result showed that significantconversion to ethanol biosynthesis favored of 2,3-BD formation. Therefore, the best condition for maximum 2,3-BD production by *K. pneumonia* would be the growth with temperature not more than 30°C. Accordingly, Petrov and Petrova (2010) set up fermentation with *K. pneumonia* subsp. *pneumonia* G31 in glycerol at 30°C. In addition, a temperature of 50°C has also been reported optimal for mutant *B. licheniformis* strain (Wang *et al.,* 2012). There are a number of literatures reported on 2,3-BD production using a variety of temperatures, for example, 30°C for *S. marcescens* H30 (Zhang *et al.,* 2010b), *Synechococcus elongates* PCC7942 (Oliver *et al.,* 2012), *E. coli* W3110 (Lee *et al.,* 2012), *E. cloacae* subsp. *dissolvens* SDM (Wang *et al.,* 2012), *S. cerevisiae* (Ng *et al.,* 2012), 37°C for *E. coli* JM109 (Li *et al.,* 2010), *K. oxytoca* ME-XJ-8 (Ji *et al.,* 2010), *K. oxytoca* ME-UD-3 (Ji *et al.,* 2010), *K. oxytoca* ACCC 10370 (Cheng *et al.,* 2010),

K. pneumonia SDM (Wang *et al.,* 2010), *C. acetobutylicum* ATCC (Siemerink *et al.,* 2011), *C. ragsdalei*, *C. autoethanogenum* and *C. ljungdalii* (Köpke *et al.,* 2011), *B. amyloliquefaciens* B10-127 (Yang *et al.,* 2011), *K. pneumonia* CICC 10011 (Liu *et al.,*2011), *K. pneumonia* SGJSB04 (Borim *et al.,* 2012), *E. coli* BL21 (DE3) (Li *et al.,* 2012), *K. pneumonia* DSM 2026 (Cho *et al.,* 2012), *K. pneumonia* KCTC2242 (Jung *et al.,* 2013), 38°C for *K.oxytoca* NBRF4 (Han *et al.,* 2013), and 55°C for *Geobacillus* sp. XT15 (Xiao *et al.,* 2012).

2.8.3 Effect of oxygen supply

Oxygen supply is the most important environmental parameter affecting the fermentation process of 2,3-BD including product yield, productivity, and byproduct formation (Jensen *et al.,* 1984). They found that high oxygen supply favored cell mass formation at the expense of 2,3-BD production. Once, the oxygen supply is decreased, it is lower the overall conversion rate due to lower cell concentrations (Converti *et al.,* 2003). It is essential to establish a proper oxygen supply control strategy to ensure efficient 2,3-BD production. The several tests have been tried to optimize 2,3-BD production. In one investigation, 2,3-BD is produced under low oxygen supply, for example, in the anaerobic fermentation in terms of maintaining an intracellular redox balance with respect to the pyridine nucleotide pool during glycolysis and biosynthesis (Converti *et al.,* 2003). Furthermore, NADH from glycolysis is regenerated by 2,3-BDH or AR in a reversible acetoin to 2,3-BD reaction. Therefore, the NAD^+/NADH balance is maintained by relative production of acetoin, and 2,3-BD (Blomqvist *et al.,* 1993). Accordingly, they found that under aerobic condition α-ALS is rapidly and irreversibly inactivated, thus preventing 2,3-

BD synthesis under high oxygen supply (Kosaric *et al.,* 1992; Nakashimada *et al.,* 2000; Converti *et al.,* 2003). Several microorganisms can obtain energy by two different pathways such as respiration and fermentation pathways. When the oxygen supply is limited, both pathways are active simultaneously. The yield of 2,3-BD depended on the relative activities of each pathway. On the other hand, the yield of 2,3-BD can be maximized by reducing the oxygen supply, because this limits the respiration. However, by lowering the availability of oxygen, biomass is also lowered, and thus 2,3-BD yield is reduced. Further, increase in oxygen supply resulted in production of acetate. Acetate is a byproduct involved in 2,3-BD formation.It could also act as inducer for the three enzymes involving in the conversion of pyruvate to 2,3-BD (Syu, 2001).

The influence of oxygen transfer rate (ORT), which is one parameter affecting the physiological state of aerobic culture was studied. The batch production of 2,3-BD by *K. oxytoca* was studied in which OTR was used to maintain growth rate and specific oxygen uptake rate. When OTR was set to maintain the culture at constant level of oxygen limitation, the final 2,3-BD concentration was similar to the experiment in which OTR was not controlled. However, the 2,3-BD productivity was 18% higher in OTR controlled fermentation (Beronio and Tsao, 1993).

Aeration is co-operative associated with agitation. It increases the efficiency of the fermentation by continuously exposing new substrate throughout the culture medium (Garg and Jain, 1995). Agitation significantly increases the 2,3-BD yield, such as in the culture of *E. aerogenes* and *K. pneumonia* with either glucose or lactose as substrate. The result showed that under agitation of 220 rpm, 2,3-BD production increased up to 5-fold with glucose, and up to 15-fold in lactose

fermentation (Barret *et al.,* 1983). In addition, aeration 300 ml/L.min in *E. aerogenes* culture led to a slightly reduction in 2,3-BD formation, although acetoin production significantly increased.

CHAPTER III

MATERIALS AND METHODS

3.1 Genetic methods

3.1.1 Strains, media and culture conditions

K. oxytoca KMS004 was constructed at Metabolic Engineering Research Unit, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, NakhonRatchasima, Thailand (Sangproo *et al.,* 2012). Bacterial strains, plasmids, and primers used in this study were summarized in Table 3.1. *K. oxytoca* wild type and mutant strains were grown at 37°C, 200 rpm in the modified Luria-Bertani (LB) broth containing the supplementary components per liter: 10 g peptone, 5 g yeast extract, 5 g sodium chloride and glucose as indicated. All of bacterial strains were also cultured on LB agar plates (20 g/L agar powder) containing appropriated antibiotics such as 50 µg/mL apramycin or ampicillin and 30 µg/mL chloramphenicol. The pCR2.1-TOPO plasmid was used as a cloning plasmid.

A low salts medium, AM1 (Martinez *et al.,* 2007) was used in anaerobic fermentations with *K. oxytoca* mutant and other strains (Table 3.2). This medium contains various concentrations of glucose as carbon source, supplemented with 1mM betaine HCl. The base used in the fermentation experiments for neutralization was 3N potassium hydroxide.

Table 3.1 The sequences of *ldh*A strain, plasmids and primers.

Material	Relevant characteristics		Source
Strain			
- KMS004	- *K. oxytoca* (Δ*adhE*Δ*ackA-pta*)		Sangproo*et al.,* 2012
- KMS005	- *K. oxytoca* (Δ*adhE* Δ*ackA-pta* Δ*ldhA'-cat-sacB-ldhA"*)		This study
- KMS005-76T	- *K. oxytoca* (Δ*adhE* Δ*ackA-pta* Δ*ldhA'-cat-sacB-ldhA"*)		This study
- KMS006	- *K. oxytoca* (Δ*adhE* Δ*ackA-pta* Δ*ldh A'-ldh A"*)		This study
Plasmids			
- pCR2.1-TOPO	- *bla, kan* TOPO TA cloning vecter		Invitrogen
- pLOI3420	- *acc*γ β *exo*(Red recombinase), temperature-conditional replicon		Wood *et al.,* 2005
- pLOI4162	- *cat-sacB* cassette		Jantama *et al.*, 2008
- pKJ1007	- *bla, kan ldh*A (PCR) from *K. oxytoca* M5a1 (using KO-*ldh*A-up/down cloned into pCR2.1-TOPO		This study
- pKJ1008	- *cat-sacB* cassette from pLOI4162 (digested with *sfoI-SmaI*) cloned into the PCR amplified inside-out product from pKJ1007 (using KO-*ldh*A-IO)		This study
- pKJ1009	- PCR amplified inside-out product from pKJ1007 (using KO-*ldh*A-IO) kinase treated then self-ligation		This study
Primers	**Forword**	**Reverse**	
- *ldhA*-up/down M5a1	- 5’ GGCGCCATCTGTATCGTATC 3’	5’ CAGCTCTTCAACCTTCAGCTC 3’	This study
- KO-*ldhA*-IO up/down	- 5’ CGTTCGATCCGTATCCAA GT 3’	5’ AGGCTGGAACTCGGACTACT 3’	This study

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

Component	**Concentration**
$(NH_4)_2HPO_4$	19.92 (mmol/L)
$NH_4H_2PO_4$	7.56 (mmol/L)
Total PO_4	27.48 (mmol/L)
Total N	47.93 (mmol/L)
[a]Total K	1.00 (mmol/L)
$MgSO_4.7H_2O$	1.50 (mmol/L)
Betaine-HCl	1.00 (mmol/L)
Trace Elements	(µmol/L) [b]
- $FeCl_3.6H_2O$	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
- $MnCl_2.4H_2O$	2.25
Total Salts	4.1 g/L

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

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

3.1.2 Genetic engineering techniques

3.1.2.1 *ldhA* gene amplified by Polymerase Chain Reaction (PCR) In this study, the PCR reaction was performed for amplification of*ldh*A DNA fragments from the *K. oxytoca* M5a1 wild type strain. The specific sequences of*ldh*A-up/down M5a1 primers were used for amplification (Table 3.1). The standard PCR reaction was performed using 10X PCR Master Mix solutions (Qiagen, Valencia, CA) in a PCR reaction of 50 μL. Twenty five microliters of master mix containing 10mM of each dNTP (dATP, dGTP, dCTP and dTTP), PCR reaction buffer (20mM Tris-HCl pH 8.8, 10mM KCl, 10mM $(NH_4)_2SO4$, 2mM $MgSO_4$, 1% (v/v) Triton® X-100, 1mg/mL nucleasefree BSA, and *Taq* polymerase enzyme), 40 pmole of each primer (forward and reverse primers), and 50 ng of chromosomal DNA template and distilled water were added. The reaction was performed in automated PCR thermocycler (Analytikjena, Germany) machine and the PCR condition was followed in Table 3.3. The PCR reaction mixture was investigated on 1.0% (w/v) agarose gel electrophoresis with Tris-HCl Borate EDTA (TBE) buffer.

3.1.2.2 Agarose gel electrophoresis of DNA The PCR reaction product and DNA fragment were analyzed by agarose gel electrophoresis. The appropriate concentration of agarose powder was dissolved in 0.5x TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 25 mM EDTA pH8.0) or 1x TAE buffer (40 mM Tris-HCl, 40 mM acetic acid, 25 mM EDTA pH8.0) then heat until agarose melting and ensure the homogeneity of the gel solution. Five microliters of loading dye [0.1% (w/v) bromophenol blue, 40% (w/v) Ficoll and 5 mM EDTA)] were added and gently mixed with the DNA samples before loading into the well of the solidified agarose gel. The

electrophoresis was performed under a constant voltage, 80 V, for 1 h. After the electrophoresis was finished, the gel was stained with 2 µg/mL ethidiumbromide for 2 to 4 min and de-stained in distilled water for 5 to 10 min. The DNA bands were visualized under photographed by gel documentation system (Bio-Rad, California, USA).

Table 3.3 PCR condition for amplification of *ldhA* gene, the extension time depends on the length of the genes (1 kb/min).

PCR profile to amplify genes				
Step	**Period**	**Temperature (°C)**	**Time (min)**	**Number of cycle(s)**
1	Pre-denaturing	95	5	1
2	Denaturing	95	1	
	Annealing	55	0.40	30
	Extension	72	4	
3	Extra-extension	72	10	1

3.1.2.3 PCR clean-up gel extraction The expected size of DNA fragments in PCR products were excised from 1% agarose gel. The reagents from the PCR clean-up gel extraction Kit (Macherey-Nagel) was used to purify the DNA fragments. The weight of gel slice was determined and transferred to a 1.5 mL clean microcentrifuge tube. The milligrams of agarose gel were added in 200 µL NT buffer. The sample was incubated at 50°C until the gel slices were dissolved (5-10 min).

During incubation, mixing by vortex or gently invert the tube was applied every 2-3 minutes until the gel slice were dissolved. A NucleoSpin® Extract II Column was placed into a collection tube (1.5 mL) and loaded the sample and then, centrifuged for 1 minute at 11,000 rpm. A flow-through was discarded. Then, 600 µL of buffer NT3 were added to the column and centrifuged for 1 min at 11,000 rpm. The flow-through was discarded. Then, the microcentrifuge tube was centrifuged for 2 min at 11,000 rpm to remove an excess buffer NT3. The column was placed into a clean 1.5 mL microcentrifuge tube. Finally, 15-50 µL of Elution buffer (NE) were added and incubated at room temperature for 1 min and then centrifuged for 1 min at 11,000 rpm.

3.1.2.4 Preparation of *K. oxytoca* KMS004 competent cells A single colony of *K. oxytoca* KMS004 was inoculated into 30 mL of LB broth and incubated at 37°C and shaking at 200 rpm until reaching the OD_{600} nm in the range 0.3-0.5. The culture was centrifuged at 4,000 x *g*, 4°C for 5 min. The cell pellet was re-suspended and washed in 30 mL of ice-cold de-ionized water for 2 times and 30 mL of ice-cold 10% glycerol for 2 times. After washing the cell, the white cell pellet was re-suspended in 1 mL of sterile ice-cold 10% glycerol and placed on ice for 10 min until used.

3.1.3 Metabolic engineering of *K. oxytoca* KMS004 for 2,3-BD production

3.1.3.1 Construction of plasmids used for *ldhA* gene deletion The target-deletion gene (*ldhA* gene) and neighboring sequences was amplified using specific primers. The *ldhA* fragment was cloned into the pCR2.1-TOPO-based vector

(Invitrogen, Carlbad, CA, USA). The resulting plasmid was used as a template in further PCR reactions in which another set of specific primers (KO-*ldhA*-IO up/down primers) was used for initiating the amplification of the template plasmid in the inside-out directions (Table 3.1). The resulting PCR product contained a vector-based backbone flanked by the part of region of target gene, missing the central part of the target-deletion gene. To facilitate the sequential deletion of chromosomal DNA, a removable *cat-sacB* cassette (from pLOI4162) with stop codons in all reading frames was ligated with the inside-out PCR product. The first resulting plasmid contains *cat-sacB* cassette flanked by some parts of the target gene to produce plasmid pKJ1008 (Table 3.1). The same inside-out PCR product was also kinase-treated and self-ligated for construction a second plasmid pKJ1009. The plasmid contains a part of the target-deletion gene without counter-selectable marker (Table 3.1). The PCR fragments amplified from the first and the second plasmid was used to replace the target-deletion genes by facilitation of λ *Red* recombinase enzyme expressing from a plasmid pLOI3420 (helper vector). The step for construction of plasmid for *ldhA* gene deletion was illustrated in Figure 3.1.

3.1.3.2 Transformation of pLOI3420 (helper vector) into *K. oxytoca* KMS004 by electroporation A single colony of *K. oxytoca* KMS004 was inoculated into 30 mL of LB broth and incubated at 37°C and shaking at 200 rpm until reaching the OD_{600} nm in the range 0.3-0.5. The culture was centrifuged at 4,000 x *g*, 4°C for 5 min. The cell pellet was re-suspended and washed in 30 mL of ice-cold de-ionized water for 2 times and 30 mL of ice-cold 10% glycerol for 2 times. After washing the cell, the cell pellet was re-suspended in 1 mL of sterile ice-cold 10% glycerol. One

microliter of 10 ng/µL pLOI3420 (helper vector) plasmid was mixed with electroporated *K. oxytoca* KMS004 competent cells prepared above. The mixture was transferred to an ice-cold 0.4 cm electroporation cuvette. The electroporation cuvette was incubated on ice for 5 min. The cells were pulsed by using electroporation under the conditions, 2,500 V, pulse 5 ms length. Then 1 mL of ice-cold LB broth was added to the cuvette immediately and the mixture was transferred to a sterile 15 mL tube. The tube was incubated at 30°C with 200 rpm shaking for 1 h. Transformed cells, 200 µL were spreaded on LB agar plates containing apramycin (50 µg/mL) and incubated at 30°C overnight.

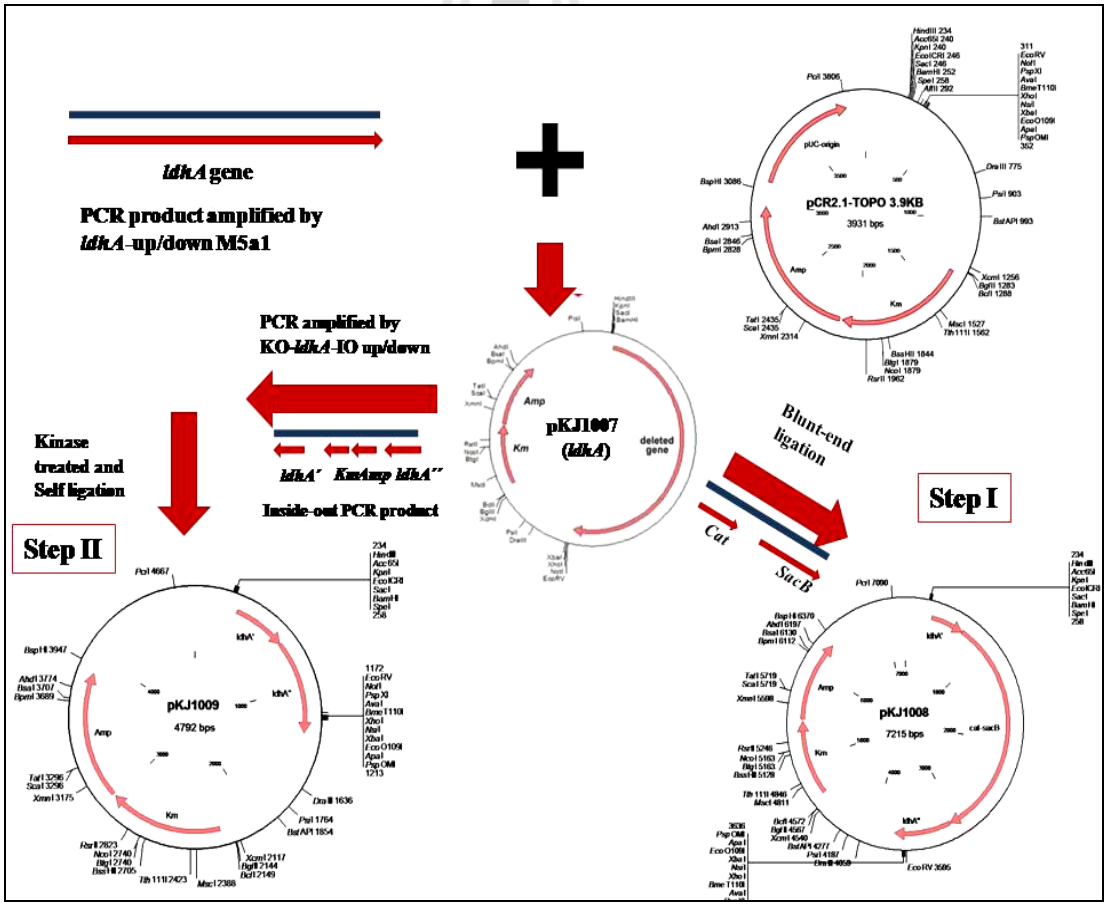

Figure 3.1 Construction of plasmids for deletion of *ldhA* gene in *K. oxytoca* KMS004.

3.1.3.3 Transformation of counter selective marker cassette (*cat-sacB*) for *ldhA* gene deletion in *K. oxytoca* KMS004 A single colony of *K. oxytoca* KMS004 harboring pLOI3420 plasmid was inoculated into 30 mL of LB broth supplemented with 50 µg/mL apramycin and 6% (w/v) L-arabinose, and incubated at 30°C with shaking at 200 rpm until the OD_{600} nm was 0.3-0.5. The culture was centrifuged at 4,000 x *g*, 4°C for 5 min. The cell pellet was re-suspended and washed in 30 mL of ice-cold de-ionized water for 2 times and ice-cold 10% glycerol for 2 times. After washing the cell, the cell pellet was re-suspended in 1 mL of double steriled ice-cold 10% glycerol. About 100 ng-10 µg of the PCR (*ldhA'-cat-sacB-ldhA"*) fragments (Figure 3.1) amplified from pKJ1008, was mixed with the competent cells. The mixture was transferred to an ice-cold 0.4 cm electroporating cuvette. The electroporating cuvette was incubated on ice for 5 min. The cells was pulsed under the conditions, 2,500 V, pulse length 5 ms. Then 1 mL of ice-cold LB broth was added to the cuvette immediately and the cell mixture was transferred to a sterile 15 mL conical tube. The tube was incubated at 30°C with 200 rpm shaking for 1 h. The transformed cells of 200 µL were spreaded on LB agar plates containing chloramphenicol (40 µg/mL) and incubated overnight at 39°C. The clones presented as chloramphenicol-resistant was designed as KMS005.

3.1.3.4 Transformation of the self-ligation fragment (without selectable marker cassette) for *ldhA* gene deletion A single colony of *K. oxytoca* KMS005 harboring pLOI3420 plasmid grown on LB agar containing 50 µg/mL apramycin was inoculated into 30 mL of LB broth supplemented with 50 µg/mL apramycin and 6% (w/v) L-arabinose, and incubated at 30°C with shaking at 200 rpm

until the OD_{600} nm was 0.3-0.5. The culture was centrifuged at 4,000 rpm, 4°C for 5 min. The pellet was re-suspended and washed in 30 mL of ice-cold de-ionized water for 2 times and ice-cold 10% glycerol for 2 times. After washing, the cell pellet was re-suspended in 1 mL of double steriled ice-cold 10% glycerol.

Linearized DNA, 0.1-10 μg, from PCR self-ligation (*ldhA'-ldhA"*) fragment amplified from pKJ1009, was mixed with competent cells. The mixture was transferred to an ice-cold 0.4 cm cuvette. The cells was pulsed under the conditions, 2,500 V, pulse length 5 ms. Then 1 mL of ice-cold LB broth was added to the cuvette immediately and the solution was transferred to a steriled 15 mL tube. The tube was incubated at 30°C with 200 rpm shaking for 3 h. The outgrowth culture was inoculated further for overnight in LB-broth containing 10% (w/v) sucrose at 30°C. The overnight culture was re-streaked on LB agar containing 10% (w/v) sucrose. Resulting clones were tested for loss of apramycin, and chloramphenicol resistances. The mutant strain was analyzed further to confirm the deletion of *ldhA* gene by using PCR technique. The clone lacking in *ldhA* gene and *cat-sacB* cassette was selected and designated KMS006.

3.2 Fermentation operation and conditions

3.2.1 Fermentation in shake flask using AM1 medium

K. oxytoca wild type, KMS004 and KMS005 inocula were prepared by inoculating seeds into AM1 medium containing 2% (w/v) glucose with 100 mL in 250 mL Erlenmeyer flask. Both of inocula were grown at 37°C, 200 rpm for 16-18 h. Fermentation were inoculated at OD_{550} of 0.1 into AM1 medium, supplemented with 1 mM betaine HCl, and carried out in a 250 mL Erlenmeyer flask with 150 mL

working volume at 37°C, 200 rpm, when 5% (w/v) concentration of glucose, sugarcane molasses, cassava hydrolysis starch and maltodextrin were used. The total incubation time was 96 h. No antibiotics were including during the growth of seed preparations or in fermentation broth and the experiments were performed in triplicate.

3.2.2 Batch fermentation in 2 liters bioreactor

The batch fermentation was performed at 37°C, 300 rpm, and 1.0 vvm aeration rate with 1.0 L working volume. The pH was controlled at 6.0 by automatically adding 3 N KOH and AM1 was also used as a basal medium. At initial OD_{550} 0.1, glucose concentration were varied on 50, 100 and 200 g/L to produce 2,3-BD by KMS005. The experiments were performed in triplicate.

3.2.3 Fed-batch fermentation in 2 liters bioreactor

The fed-batch fermentations were performed at 2 L stirred bioreactor with 1.0 L initial working volume. All fed-batch fermentations were conducted with an initial glucose and maltodextrin concentration at 100 g/L, and the feeding substrate was fed by intermittent addition of 800 g/L stock glucose concentration into the bioreactor. The residual sugar concentration in the culture was maintained between 10 and 40 g/L.The other cultivation conditions in fed-batch were applied the same way as in the batch experiments such as the controlled pH (6.0) by automatically adding 3 N KOH, temperature (37°C), agitation (300 rpm), and aeration (1.0 vvm). The experiments were performed in triplicate.

3.3 Metabolic evolution

Genetically modified *K. oxytoca* strain was subsequently selected for the best representative clone via metabolic adaptation or evolution. This is a selection of robust strongly genes within microorganism for survival under strong pressure of selection (Jantama *et al.,* 2008). The selection was conducted in mutant strain for production of high 2,3-BD from glucose. Starting at initial OD_{550} nm of 0.1, cells was grown in fresh AM1 medium. The culture was rapidly transferred in fresh AM1 medium when the OD_{550} nm of the culture approached in the range of 1.00 to 2.00. The transfers were performed until achievement of a clone, resulting in fast cell growth, rapid consumption of glucose, and high production and productivity of 2,3-BD, with less production of other organic acids.

3.4 Enzymatic activity assays

3.4.1 Lactate dehydrogenase (LDHA) enzymatic activity assay

Cells grown under 2,3-BD production conditions were collected at mid-exponential phase and washed twice with cold 30 mM KH_2P0_4 buffer pH 7.0. The cell pellets from continuous culture samples was re-suspended in cold 30 mM KH_2P0_4 buffer pH 7.0. The cell was disrupted using a Soniprobe (Dawe Instruments, London) at a current of 60 Amplitude (A) for 2 min, with alternate 30 sec on/off periods. Cell debris was removed by centrifugation (11,600 x *g*, 4°C, 15 min) and the supernatant liquid was used immediately. The activity measurement of lactate dehydrogenase in cell extracts with Lactate Dehydrogenase-P (LD-P) reagent was performed in the SYNCHRON CX® systems machine. LD-P reagent was used to measure lactate dehydrogenase activity by an enzymatic rate method described by Amador *et al*.,

(1963). In summary, the assay mixture consists of 5 µL of cell extraction, 242 µL of 1.4 mM sodium pyruvate in buffer solution (250 mM Tris-HCl pH 6.8), 8 µL of 0.23 mM NADH and total assay mixture is 255 µL. The system was monitored the change in absorbance at 340 nm. The change in absorbance was directly proportional to the activity of lactate dehydrogenase in the sample and was used by the system to calculate and express for the lactate dehydrogenase activity:

One unit of enzymatic activity for lactate dehydrogenase was defined as the amount of enzyme that catalyzed the formation of 1 mmol NADH per min at 30°C (SYNCHRON CX® 1992). The specific activity of LDH was defined as the enzyme unit (U) divided by the amount of total protein (mg).

3.4.2 Alcohol dehydrogenase (ADHE) enzymatic activity assay

Cells grown under 2,3-BD production conditions were collected and washed twice with cold 30 mM KH_2P0_4 buffer pH 7.0. The cell pellets from continuous culture samples was re-suspended in cold 30 mM KH_2P0_4 buffer pH 7.0. Disruption of cell pellets were performed using a Soniprobe (Dawe Instruments, London) at a current of 60 A for 2 min, with alternate 30 sec on/off periods. Cell debris was removed by centrifugation (11,600 x *g*, 4°C, 15 min) and the supernatant liquid was used immediately. Briefly, measurement of alcohol dehydrogenase activity was determined in the direction of ethanol oxidation contained 0.1 mL of 95% (v/v) ethanol and 1.5 mL of 15 mM β-NAD in 1.3 mL of 50 mM sodium pyrophosphate buffer, pH 9.8.The reaction was mixed by inversion and equilibrated to 25°C, then

monitor at the absorbance 340 nm until constant. The free cell extraction 0.1 mL was added to the reaction mixture to obtain final total volume of 3.0 mL (Sanchez *et al.,* 1998). The reactions were initiated by adding the supernatant sample.The reaction mixture was measured a change in absorbance at 340 nm for 6 min for both test and blank samples:

$$\text{Ethanol} + NAD^{+} \longrightarrow \text{Acetaldehyde} + NADH$$

The one unit of ADHE activity was defined as the amount of enzyme that catalyzed the formation of 1 pmol NADH per min per total protein at pH 8.8 at 25°C in supernatant fraction. NADH was used as a standard curve. The specific activity of ADHE was defined as the enzyme unit (U) divided by the amount of total protein (mg).

3.4.3 2,3-butanediol dehydrogenase (BUDC) enzymatic activity assay

Cells grown under 2,3-BD production conditions were collected and washed twice with cold sodium phosphate buffer pH 7.0. The cell pellets from continuous culture samples was re-suspended in cold sodium phosphate buffer pH 7.0. Disrupted using a Soniprobe (Dawe Instruments, London) at a current of 60 A for 2 min, with alternate 30 sec on/off periods. Cell debris was removed by centrifugation (11,600 x *g*, 4°C, 15 min) and the supernatant liquid was used immediately. The BUDC enzymatic activity was determined spectrophotometrically by measuring the change in 340 nm, 25°C corresponding to the oxidation of NADH or the reduction of NAD^{+} (González *et al*., 2000). Cell extractions were assayed for BUDC activity with 0.4 mL of 120 mM (2R,3R)-2,3-BD and 1.5 mL of 5 mM NAD^{+} in 1.0 mL of

33 mM sodium pyrophosphate pH 8.0, mixed by pipette then added 0.1 mL free-cell extraction. The total reaction volume was 3.0 mL. A quantitative assay for BUDC activity from cell extraction was developed using the reverse reaction:

$$\text{2,3-BD} + NAD^{+} \longrightarrow \text{acetoin} + NADH$$

Reduction reaction of NAD^{+} to NADH was monitored spectrophotometrically by the increasing rate of 2,3-butanediol-dependent absorbanceat 340 nm. Under these conditions, one unit of BUDC enzymatic activity corresponds to 1 mmol of NADH formed/min. The specific activity of BUDC was defined as the enzyme unit (U) divided by the amount of total protein (mg).

3.4.4 Malate dehydrogenase (MDH) enzymatic activity assay

Cells grown under 2,3-BD production conditions were collected and washed twice with cold 0.1 M phosphate buffer pH 7.4. The cell pellets from continuous culture samples was re-suspended in cold 0.1 M phosphate buffer pH 7.4. Disruption of cell pellets was performed using a Soniprobe (Dawe Instruments, London) at a current of 60 A for 2 min, with alternate 30 sec on/off periods. Cell debris was removed by centrifugation (11,600 x *g*, 4°C, 15 min) and the supernatant liquid was used immediately.The MDH enzyme assay was performed in double beam UV-VIS spectrophotometer (AnalytikJena, Germany). The MDH assay system for oxaloacetate reduction was assayed by measuring the decrease in absorbance at 340 nm due to NADH oxidation:

$$\text{oxaloacetate} + NADH \longrightarrow \text{malate} + NAD^{+}$$

The reaction mixture contained with 2.6 mL 0.1 M phosphate buffer pH 7.4, 0.2 mL 3.75 mM NADH (freshly prepared in 0.1 M potassium phosphate buffer, pH 7.4), 0.1 mL 6.0 mM oxaloacetate (freshly prepared in 0.1 M phosphate buffer pH 7.4. This reagent is unstable and should be stored in an ice bath during used) and 0.1 mL free-cell extraction in total reaction mixture 3.0 mL. Cuvettes of reaction mixture were incubated in spectrophotometer for 3-4 min to achieve temperature equilibration. MDH oxidation was deduced by measuring the increase in absorbance at 340 nm due to NAD^+ reduction, and record absorbance decreasing in absorbance 340 nm (ΔA_{340}) for 3-5 min. Calculate ΔA_{340} per min from the initial linear portion of the curve. One unit of enzyme activity, for the reduction of oxaloacetate was defined as the amount that catalyses the conversion of 1 μmol NADH to NAD^+ per min at 25°C (Nikolaos *et al*., 1997). The standard curve of NADH was prepared by dissolving NADH in distilled water and diluted to obtain various concentrations and measured the OD at 340 nm. Standard curve was made by plotting between the different concentrations of standard NADH against their absorbance values. The specific activity of MDH was defined as the enzyme unit (U) divided by the amount of total protein (mg) (Zeikus *et al.,* 1997).

3.4.5 Total protein assay

Total protein concentrationcell free extraction was determined by SYNCHRON® Systems micro total protein reagent. Protein in the sample reacts with the pyrogallol red (PR) molybdate (Mo) complex to form a purple color that has a maximum absorbance at 600 nm:

pyrogallol red (PR) + molybdate (Mo) + protein ⟶ PR-MO-protein complex

Bovine serum albumin (BSA) was used as a standard (0, 10, 20, 40, 60, 80 mg/L) (Watanabe *et al.,* 1986). The reaction mixture was carried on 300 µL total reaction volume contained with 10 µL free-cell extraction and gently mixed in 290 µL mixed reagent R (0.058 mmol/L pyrogallol red, 0.12 mmol/L sodium molybdate, 1.04 mmol/L sodium oxalate, 3.47 mmol/L sodium benzoate, 1.0 mmol/L ethanol and 46 mmol/L succinic acid sodium salt), incubated for 8 min at 37ºC. When the complex is combined with protein, a blue-purple color formed. The increase in absorbance at 600 nm is directly proportional to the protein concentration in the free-cell extraction and read the absorbance against reagent blank (Tietz, 2005).

3.5 Enzymatic hydrolysis of cassava starch

Two enzymes, α-amylase (Sigma-Aldrich) from *Bacillus subtilis* with an activity of 12,000 U/g and Glucoamylase (Sigma-Aldrich) from *Aspergillus niger* with an activity of 6,000 U/g were used. The method for hydrolysis of cassava hydrolysate was as follows: 90 g of cassava starch was suspended in waterwith a total volume 400 mL. The lique faction was carried out by adding 0.3% (v/v) of α-amylase to the slurry at pH 6.0 and incubated at 95°C for 2 h. Saccharification was carried out by adding 0.5% (v/v) of glucoamylase at pH 4.5 and maintained at 58°C for 15 h. The reducing sugar present in the enzymatic hydrolysate was measured using the 3, 5-dinitrosalicyclic acid (DNSA) method (Jesse *et al*., 2002). To measure the reducing sugar concentration in the hydrolysate cassava, 250 mg of hydrolysate were suspended in 15 mL of deionized water in a 50 mL beaker. The 0.5 mL of each samples were then added into 0.5 mL DNSA solution (ratio 1:1). The reaction mixture was boiled for 15 min and immediately cooled down. The four milliliters of DI water

were added into the reaction mixed. The reducing sugar contents in the samples were analyzed by measuring the optical density at 540 nm using anhydrous D-glucose solution (0, 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mg/mL in deionized water) and 0.5 mL of deionized water as a standard and blank, respectively. In order to prepare a standard curve, standard solution containing anhydrous D-glucose (0-2.0 mg/mL in deionized water) plot of glucose concentration (as reducing sugar) versus optical density relationship was established. The reducing sugar was read from the standard curve using least squares linear regression. However, reducing sugar (glucose and maltose) presenting in the enzymatic hydrolysate was also determined by the HPLC.

3.6 Dextrose equivalent (DE) test

Maltodextrin used in fermentation experiments was prepared from cassava starch. Cassava starch was obtained from the local market at Nakorn Ratchaseema province, Thailand. Gelatinized cassava starch was prepared by heating the starch slurry at 80°C. Lique faction and saccharification were carried out simultaneously by adding 0.3% (v/v) of α-amylase (Sigma-Aldrich, 12,000 U/g) to the slurry at pH 6.0 and incubating at 95°C for 2 h.

One gram of maltodextrin was dissolved with 100 mL DI water to the final concentration of 100 mg/mL. The samples were diluted at 2, 4, 6, 8 and 10 times. The 0.5 mL of each samples were then added into 0.5 mL 3, 5-dinitrosalicyclic acid (DNSA) solution (ratio 1:1). The reaction mixture was boiled for 15 min and immediately cooled down in cool water. The four milliliters of DI water were added into the reaction mixture. The reducing sugar contents in the samples were analyzed by measuring the optical density at 540 nm using glucose solution (0, 0.2, 0.4, 0.6,

0.8, 1.0 and 2.0 mg/mL) as a standard. The dextrose equivalent of maltodextrin obtained in this experiment was approximately 6.4 (w/w).

3.7 Analytical methods

Five milliliters of samples were collected during fermentation every 24 h for measurement of cell mass. Cell mass was estimated from the optimal density at 550 nm with a Spekol®1500 spectrophotometer. Organic acids and sugars were determined by using high performance liquid chromatography, HPLC, (Agilent Technology, Japan) equipped with anion exclusion column (Bio-RADAminex HPX-87H, USA) with column temperature of 45°C using 4 mM H_2SO_4 as mobile phase with flow rate of 0.4 mL/min. The cultures were centrifuged 13,500 rpm (Wisespin®) for 4 min to separate cells and supernatant. The supernatant was filtered through 0.2 µm filter membrane before injecting to HPLC.

3.8 Statistical analysis

Data were analyzed with the SPSS program (version 13.0). The comparison between mean was carried out using a Duncan's new multiple range test at $P<0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Construction of *K. oxytoca* KMS005 and KMS006 for 2,3-BD production

Homologous recombination technique was used to inactivate LDHA activity in *K. oxytoca* KMS004 (Δ*adhE,* Δ*pta-ackA*) with facilitation of λ *Red* recombinase enzyme system contained in pLOI3420 plasmid. The inside-out fragment containing *cat-sacB* cassette that encoding for chloramphenicol-resistant gene and levan sucrose B gene flanked by *ldhA* target gene of interest (*ldhA'-cat-sacB-ldhA"*) from plasmid pKJ1008 was introduced by electroporation into the host KMS004 genome (Figure 4.1).

The LB medium containing 40 µg/mL chloramphenicol was used to select mutants. The chloramphenicol-resistant colonies were screened and confirmed by colony PCR using a pair of *ldhA*-up/down M5a1 primers. The amplified 1850 bp and 3580 bp amplicons represented the parental strain and mutant strains, respectively (Figure 4.2). Therefore, the *ldhA* deleted mutant was developed with chloramphenicol-resistant gene marker (*ldhA'-cat-sacB-ldhA"*), and was designed as KMS005.

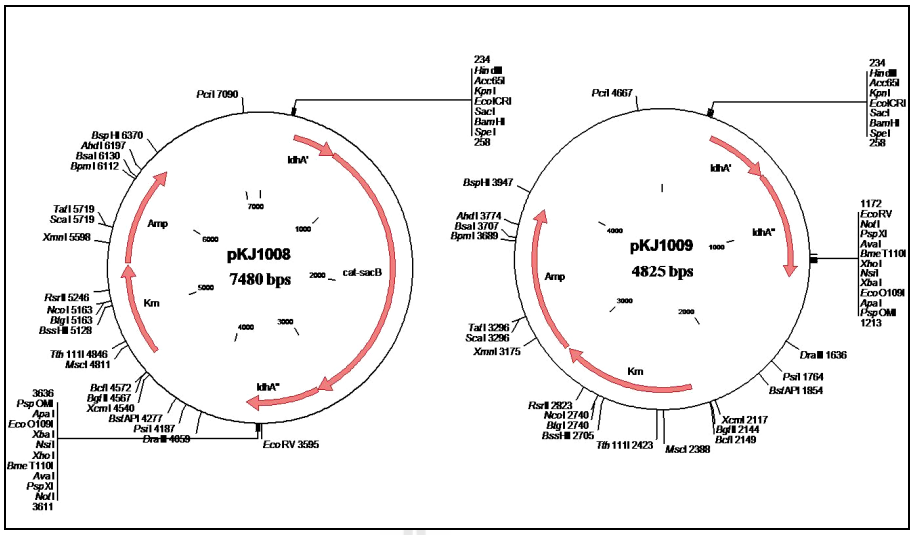

Figure 4.1 Plasmids pKJ1008 and pKJ1009 used for deletion of *ldhA* gene.

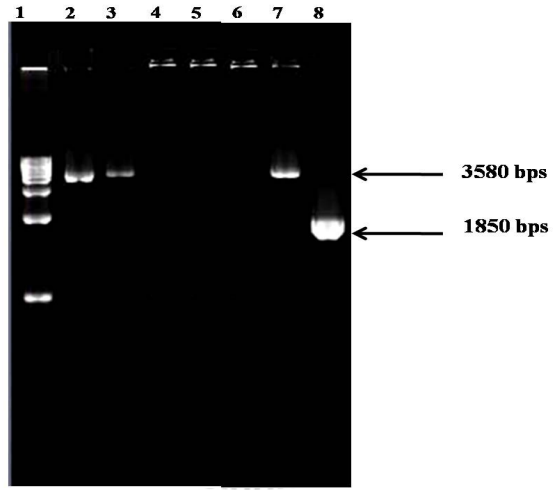

Figure 4.2 Gel electrophoresis performed to confirm the recombinant strain of KMS005 using the *ldhA*-up/down M5a1 primer set. Lane 1 represented marker 10,000 bps ladder. Lane 2 and 3 represented the PCR bands of integration of *ldhA'-cat-sacB-ldhA"* fragment in the genome of *K. oxytoca* KMS005 mutant strains, clone 1 and 2, respectively. Lane 4, 5 and 6 represented M13 primer set was used to confirm that the *K. oxytoca* M5a1 and the KMS005 (clone 1 and 2) did not carry the plasmid pKJ1008 inside the cell, respectively. Lane 7 represented the PCR product amplified from pKJ1008 using *ldhA*-up/down M5a1 primer set. Lane 8 represented the PCR band of *ldhA* gene amplified by *ldhA*-up/down M5a1 primer set in the *K. oxytoca* KMS004 strain.

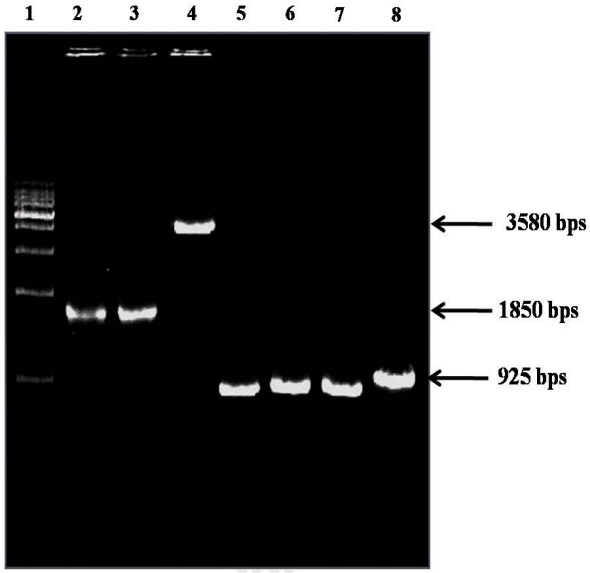

Figure 4.3 Gel electrophoresis preformed to confirm the recombinant strain of KMS006 using the *ldhA*-up/down M5a1 primer set. Lane 1 represented marker 10,000 bps ladder. Lane 2 and 3 represented the PCR band of *ldhA* gene in *K. oytoca* M5a1 and KMS004, respectively. Lane 4 represented the PCR band of *ldhA* gene (*ldhA'-cat-sacB-ldhA"*) in KMS005 strain. Lane 5 to 7 represented the PCR bands of integration fragment of *ldhA'-ldhA"* in the genome of KMS006 (clone 1, 2 and 3, respectively). Lane 8 was PCR product band (*ldhA'-ldhA"*) amplified from pKJ1009 plasmid.

Strain KMS005 harboring pLOI3420 was further introduced with the self-ligation (*ldhA'-ldhA"*) fragment amplified from pKJ1009 plasmid (Figure 4.1). The clones grown on LB medium containing sucrose were tested for loss of apramycin and chloramphenicol resistances. The positive mutants were further confirmed by PCR analysis. The clone lacking in *ldhA* gene and *cat-sacB* cassette was selected and designated as KMS006 (Figure 4.3).

Effect of *ldhA* disruption in *K. oxytoca* KMS005 strain

For *K. oxytoca* KMS004 (Δ*adhE,* Δ*pta-ackA*), lactic acid is a major fermentative product under anaerobic conditions. In contrast, *K. oxytoca* wild type produces mixed acids fermentative production including lactate, ethanol, 2.3-butanediol, succinate, acetate, and formate as metabolites during glucose fermentation (Table 4.1).

KMS004 was disrupted alcohol dehydrogenase, phosphotransacetylase and acetate kinase A genes. Thus, under anaerobic conditions, pyruvate is assimilated to re-oxidize NADH via lactate dehydrogenase activity resulting in lactate production. Pyruvate is reduced to lactate at the expense of NADH under anaerobic conditions. The reaction is catalyzed by a cytoplasmic lactate dehydrogenase encoded by *ldhA*. The enzyme is induced by lower pH and anaerobic conditions. Therefore, KMS004 produced D-lactate at a high concentration and mostly major product (11.46 ± 0.09 g/L) with a high yield and productivity of 0.64 ± 0.01 g/g and 0.36 ± 0.01 g/L/h, respectively carried out in small bottle at 37°C, 150 rpm under anaerobic conditions (Sangproo *et al.,* 2012). However, KMS004 is not the best lactate producer when performing the fermentation in shake flask. Because metabolic pathways exhibited the formation of 2,3-BD competing with lactic acid for biosynthesis and NADH reoxidation under microaerobic conditions (Fig 4.4 B). The results revealed that KMS004 produced 2,3-BD at higher concentration, yield and productivity of 17.56±0.43 g/L,0.40±0.01 g/g and 0.23±1.02 g/L.h, respectively in shake flask (Table 4.1) than those in anaerobic fermentation. Noticeably, pH and aeration were not controlled in shake flask. This could explain that fermentation in shake flask was not suitable for lactic acid production but was suitable for 2,3-BD in the strain KMS004

due to aeration. Furthermore, KMS004 has a potential as a parental strain for 2,3-BD production.

Lactate is the most plentiful by-product in *K. oxytoca* KMS004. Also, one mol of NADH is consumed when one mol of lactate is produced in which NADH also required to produce 2,3-BD from pyruvate. In addition, a higher affinity for pyruvate of BUDB encoding α-acetolactate synthase ($K_m^{pyruvate}$ = 8.0 mM) than that of LDHA ($K_m^{pyruvate}$ = 7.2 mM) might cause more efficient re-oxidation via the lactic acid production pathway in the strain KMS004 (Yang *et al.,* 2000; Sangproo *et al.,* 2012). It is also revealed that the high ratio of NADH to NAD^+ during an exponential growth phase activates LDHA activity while a low ratio of NADH to NAD^+ activates α-acetolactate (catalyzed by α-acetolactate synthase) thus converting pyruvate to 2,3-BD in *K. oxytoca* (Celinska, 2009; Sangproo *et al.,* 2012). Therefore, the deletion of *ldhA* gene in *K. oxytoca* KMS004 (Δ*adhE,* Δ*pta-ackA*) were expected to improve 2,3-BD production.

KMS005(Δ*adhE,* Δ*pta-ackA,* Δ*ldhA′-cat-sacB-ldhA″*) produced 2,3-BD as a major fermentative product at concentration of 23.52±0.47 g/L with a yield and productivity of 0.46±0.02 g/g and 0.20±0.01 g/L.h, respectively after 120 h incubation in AM1 medium containing 5% (w/v) glucose in shake flask (Table 4.1, Figure 4.4C). This result was consistent with the specific productivity of KMS005 (0.15±0.01 g/g.h) compared with that of wild type *K* (0.12±0.02 g/g.h) and KMS004 (0.21±0.02 g/g.h), respectively (Table 4.1). Moreover, the deletion of *adhE*, *pta-ackA* and *ldhA* also decreased the growth rate of KMS005 (μ= 0.05 h^{-1}) compared with that of wild type *K. oxytoca* (μ = 0.06 h^{-1}) and KMS004 (μ = 0.09 h^{-1}) (Table 4.1). Previous studies showed that the loss of ADHE activity in KMS004 caused a reduction in efficacy of

NADH re-oxidation by 50% compared with that of the wild-type strain (Sangproo *et al.,* 2012). In addition, Kabir *et al*. (2005) revealed that disruption of the *ldhA* in *E. coli* BW25113 had negative effect on cell growth and glucose consumption rate under anaerobic conditions. This phenomenon was occurred in microorganisms in the family of Enterobacteriaceae when culturing under anaerobic conditions, but not under aerobic conditions (Jantama *et al.,* 2008; Zhou *et al.,* 2011). It was likely that the different oxygen limiting conditions led to regulation of cell metabolism for differential intracellular oxidation states. For KMS005 strain, lactate production was completely blocked, while other by-products such as acetate (0.91±0.01 g/L) and succinate (0.20±0.09 g/L) were still presented with high 2,3-BD concentration. It indicated a conversion of carbon flux for generating NAD^+, which was sufficient for driving glycolysis and reductive branch of tricarboxylic acid pathway.

The acetate production could not be completely blocked by *pta-ackA* gene deletion in KMS005, probably due to the existence of other pathways or unexpected acetate-producing enzymes including pyruvate oxidase (*poxB*), and propionate kinase (*tdcD*). Especially, KMS004 produced acetate not only during exponential growth but also during the stationary phase (Sangproo *et al.,* 2012). Thus it implied the activation of *PoxB* activity in KMS004 and KMS005. Interestingly, POXB is necessary for a cell survival during the stationary phase by oxidation of pyruvate thus converting to acetate. The accumulation of pyruvate during glycolysis could activate POXB activity for decarboxylated pyruvate to acetate (Abdel-Hamid *et al.,* 2001).

The effect of *ldhA* deletion on KMS005 resulted in a few production of succinate. Phosphoenolpyruvate (PEP) is converted to malate via oxaloacetate (OAA) by phosphoenol pyruvate carboxylase (*ppc*) thus wasting the free energy. In contrast,

one ATP is produced by PEP converted to pyruvate with pyruvate kinase activity (*pyk*). Therefore, accumulated of PEP was more channeled through *pyk* pathway to supply the TCA cycle and conserving the free energy of PEP. However, this upregulation of pathway could not abolish the production rate of succinate (Figure 2.4, Table 4.1). Because, PEP is essential to supply the precursor metabolites for biomass production such as several amino acids. It may imply that the requirement of intracellular PEP in *ldhA* mutant is still maintained with small extent through re-directing the carbon flux from OAA (Kabir *et al.,* 2005). Li *et al*. (2010) revealed disruption of lactate, acetate and ethanol producing pathways in *E. coli* JM109 LPAP. The strain diverted more carbon flux into succinate production pathway to reduce the excess NADH to NAD^+, thus producing the small amount of succinate. However, more carbon fluxes were diverted through *pyk* and then BUDBC activities to conserve free energy and to reduce the ratio of NADH to NAD^+, respectively, in KMS005.

In KMS005, PEP dissimilation via phosphoenolpyruvate carboxylase (PPC) did not competitively re-oxidize NADH by succinate formation pathway in this strain since four-step reactions were required for succinate biosynthesis such as PEP carboxylated to OAA, OAA reduced to malate, malate hydrated to fumarate and fumarate reduced to succinate (Fig.2.4). Furthermore, a higher affinity for pyruvate of α-ALS (BUDB) (K_m^{pyruvate} = 8.0 mM) than that of PPC (K_m^{PEP} = 0.15 mM) and pyruvate kinase (PYK) (K_m^{PEP} = 6.6 mM) (Millard *et al.,* 1996; Yang *et al.,* 2000; Zoraghi *et al.,*) might cause more efficient NADH re-oxidation via the 2,3-BD production pathway, than that of succinate formation pathway.

Therefore, KMS005 strain lacking the key enzymes ADHE, PTA-ACKA and LDHA of the major ethanol, acetate and lactate synthesis pathways reduced ethanol,

acetate and lactate accumulation, respectively. KMS005 strain also exhibits an increased 2,3-BD synthesis rate. In this study, suggested that the majority of excessive carbon flux was redirected through the 2,3-BD formation pathway rather than the succinate synthesis pathway. This result indicated that α-ALS may be competitive at the pyruvate node.

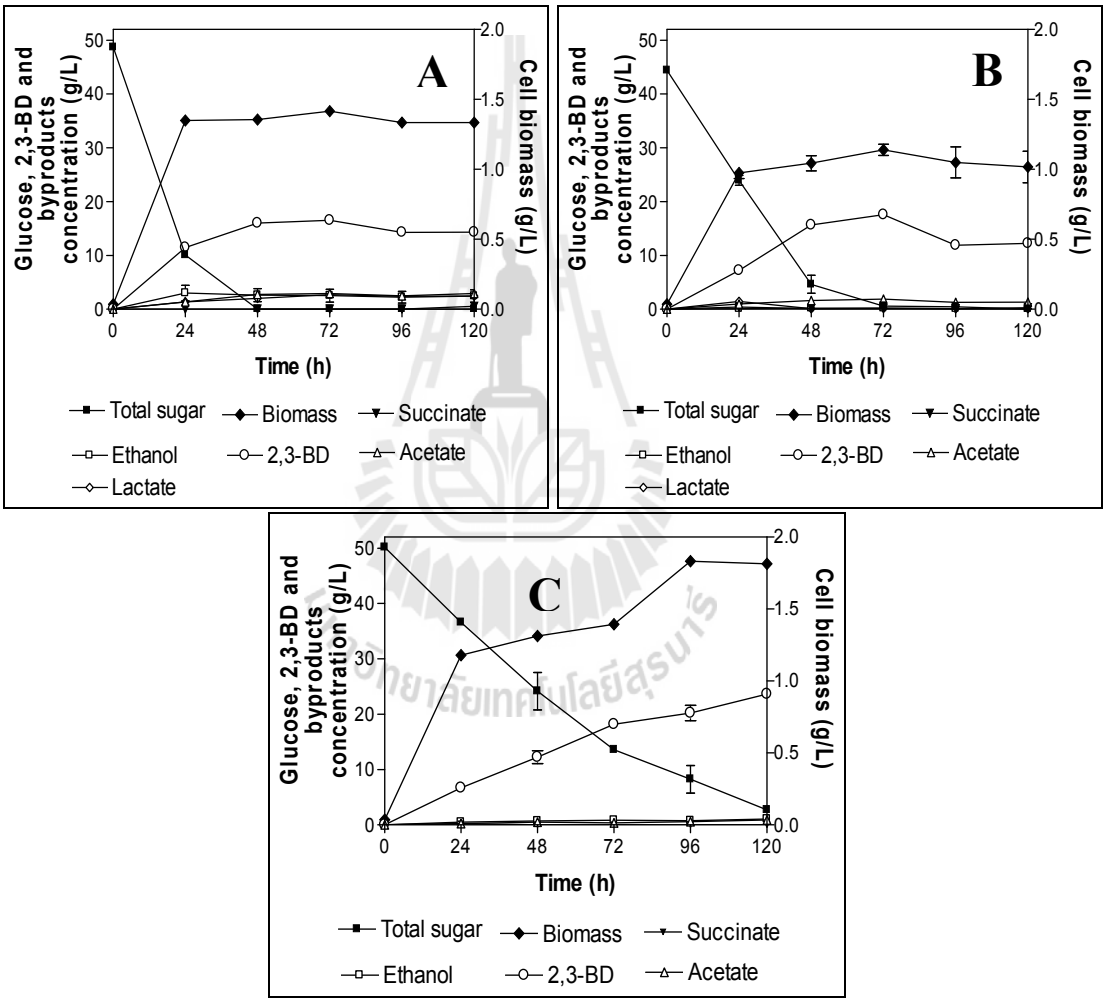

Figure 4.4 Fermentation profile of *K. oxytoca* M5a1 wild type and mutant strain in AM1 medium containing 5% (w/v) glucose in shake flask. (A) *K. oxytoca* wild type (B) KMS004 (C) KMS005.The symbols and error bar are average values and standard deviations of at least three measurements, respectively.

Table 4.1 Kinetic parameters of *K. oxytoca* wild type and mutant strains in AM1 medium containing 5% (w/v) glucose in shake flask experiment.

Strains	**Wildtype[h]**	**KMS004[h]**	**KMS005-76T[h]**
CDW (g/L)	1.32±0.05[f,€]	1.14±0.07[£]	1.82±0.02[¥]
μ (h^{-1})[a]	0.06±0.01[€]	0.09±0.02[£]	0.05±0.02[€]
Glucose consumption (g/L)	48.78±0.47[€]	43.38±1.14[£]	50.18±0.11[¥]
Specific glucose consumption (g/g.h)[b]	0.54±0.02[€]	0.37±0.07[£]	0.76±0.01[¥]
2,3-BD concentration (g/L)	14.72±1.92[€]	17.56±0.43[£]	23.52±0.47[¥]
2,3-BD yield (g/g)[c]	0.30±0.04[€]	0.40±0.01[£]	0.46±0.02[¥]
2,3-BD productivity (g/L.h)[d]	0.17±0.09[€]	0.23±1.02[£]	0.20±0.01[¥]
2,3-BD specific productivity (g/g.h)[e]	0.12±0.02[€]	0.21±0.02[£]	0.15±0.01[¥]
Succinate (g/L)	0.50±0.12[€]	0.29±0.11[£]	0.20±0.02[¥]
Lactate (g/L)	2.18±0.56[€]	1.45±0.02[£]	ND[g]
Formate (g/L)	ND	ND	ND
Acetate (g/L)	2.89±0.04[€]	1.90±0.26[£]	0.91±0.02[¥]
Ethanol (g/L)	3.69±0.35[€]	0.20±0.02[£]	1.04±0.01[¥]
Incubation time (h)	72	72	120

[a] The specific growth rate was calculated by slope of cell dry weigh concentration curve.

[b]The specific glucose consumption rate was calculated as glucose consumption multiplied by cell dry weight (CDW) and divided by overall incubation time.

[c]The 2,3-BD yield was calculated as grams of 2,3-BD produced divided by grams of the sugar consume.

[d]The 2,3-BD productivity was calculated as 2,3-BD concentration produced divided by overall incubation time.

[e]The 2,3-BDspecific productivity was calculated as 2,3-BD productivity divided by cell dry weight.

[f]All data represent the averages of three fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P<0.05$) among strains between column.

[g]ND, not detected.

[h]No 1,3-propanediol and acetoin were detected in the fermentation broth from all strains.

4.2 Metabolic evolution and growth based selection

K. oxytoca KMS005 strain was sub-sequentially selected for the best representative clone via metabolic adaptation or evolution. Serial transfers of the engineered strain were carried out in mineral salts medium containing 5% and 10% (w/v) glucose to select for strains with best representative clones for glucose utilization. This is a selection of strong genes within microorganisms for survival under strong pressure of selection (Jantama *et al.,* 2008). KMS005 was sub-culturing into the new freshly AM1 medium and transfered till the transfer number 23.The level of 2,3-BD was slightly increased along with slightly increased of the cell dry weight (CDW) (Figure 4.5 A, B and C). From the transfer number 24 to 73, the culture were improved in biomass and 2,3-BD production when other by-products were also observed. The clone from the transfer number 23 glucose could produce a high concentration of 2,3-BD (23.42 g/L) and CDW (0.60 g/L) within 24 h. Therefore, the representative clones from transfer number 23 indicated the improvement of population in the 5% (w/v) glucose concentration medium. The small amount of other by-product including ethanol, acetate and succinate was obtained during these sub-culture but lactate were not detectable.

At the end of the metabolic evolution, the result showed slightly increasing in 2,3-BD (27.70 g/L) and biomass comparing with the first 23 transfer (Figure 4.5Aand B).The culture of KMS005 produced higher levels of biomass and improved productivity of 2,3-BD, while small amount of other by-products were still observed. The formation of ethanol was elevated during metabolic evolution (Figure 4.5 C). The accumulation of ethanol might result from an activation of some enzymes by spontaneous mutation in the ethanol producing pathways, such as acetaldehyde

dehydrogenase 2 encoded by *mhpF* (Lee *et al.,* 2006), ethanol dehydrogenase P encoded by *adhP* and acetaldehyde dehydrogenase encoded by *aldh.* Three enzymes all involve the formation of ethanol competed with the biosynthesis of 2,3-BD from pyruvate. Zhang *et al.* (2011) suggested that 1,3-propanediol oxidoreductase encoded by *yqhD-1* in *K. oxytoca* was also able to oxidize broad range of alcohol derivatives such as ethanol, 1,3-propanediol, 1,2-propanediol, propanol, glycerol, 1-butanol, 1,3-butanediol and isopropanediol. In addition, the enzyme could reduce acetaldehyde and propaldehyde to their alcohols.

K. oxytoca KMS005 strain was further improved growth and cell biomass by several sub-culturing under strong pressure conditions or metabolic evolution. The clone passing through metabolic evolution process was selected and designated *K. oxytoca* KMS005-76T.

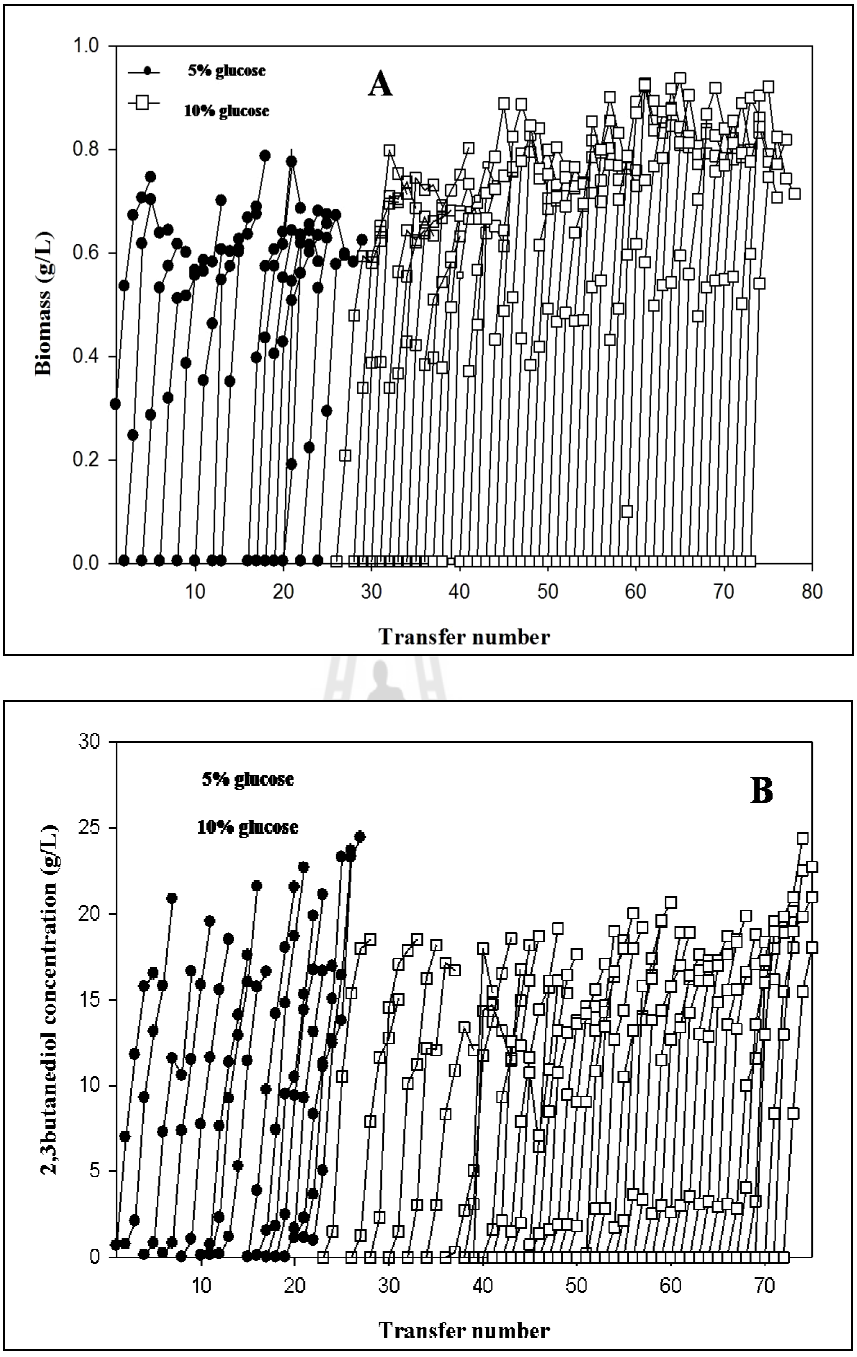

Figure 4.5 Fermentation products during the metabolic evolution of KMS005 containing 5% (w/v) and 10% (w/v) glucose in AM1 medium under 500 mL small bottle condition (A) Biomass (B) Maximum concentration of 2,3-BD and (C) fermentation products.

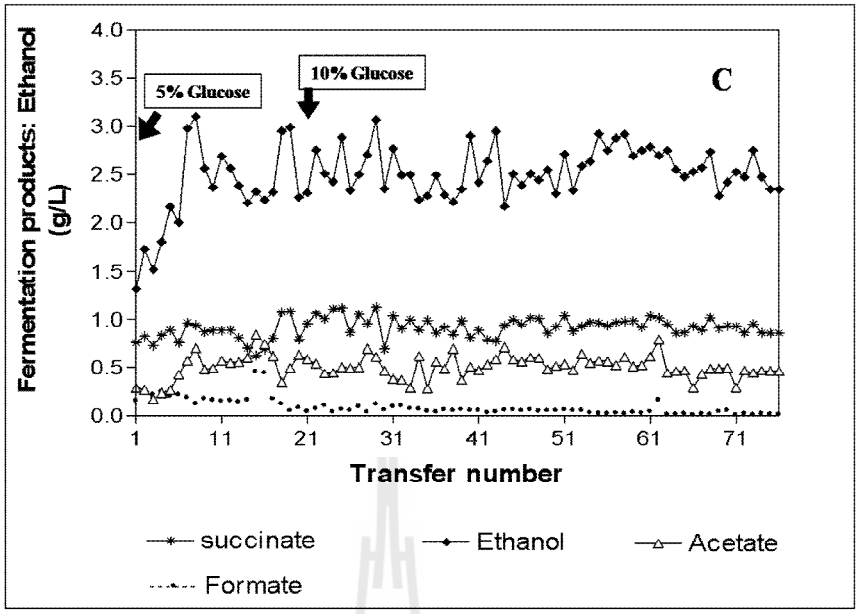

Figure 4.5 (Continued) Fermentation products during the metabolic evolution of KMS005 containing 5% (w/v) and 10% (w/v) glucose in AM1 medium under 500 mL small bottle condition (A) Biomass (B) Maximum concentration of 2,3-BD and (C) fermentation products.

4.3 Enzymatic activity assays

4.3.1 Alcohol dehydrogenase E (ADHE)

ADHE activities of wild type, KMS004 and KMS005-76T mutant strains were determined as show in Table 4.2 and Figure 4.6. The ADHE activities from wildtype, KMS004 and KMS005-76T were 8.43±0.53, 3.12±0.86 and 3.04±0.45 U/mg protein, respectively. The result confirmed that *adhE* gene was deleted from *K. oxytoca* wild type, resulting in a decrease of ADHE activity. However, the deletion of *adhE* in the mutant strain obviously decreased glucose consumption rate and also significantly decreased growth rate compared with wildtype strain and KMS004. Furthermore, the effect of triple genes deletion in KMS005-76T (*ΔadhE*, *Δpta-ackA*

Δ*ldhA'-cat-sacB-ldhA"*) not only decreased glucose consumption rate, but also impaired growth under anaerobic conditions of the strain. The result indicated that the loss of ADHE activity in *K. oxytoca* caused a reduction in efficacy of NADH re-oxidation by compared with that of the wildtype strain (Figure 4.4). Ji *et al.* (2010b) and Jantama *et al.* (2008) performed the deletion of alcohol dehydrogenase (*adhE*). They revealed that the *adhE* deletion caused poor growth and glucose consumption in *E. coli* under anaerobic conditions, but not under aerobic conditions. However, after metabolic evolution, KMS005-76T produced ethanol. Other enzymes, which have similar function, compensate ADHE activity in the ethanol producing pathway in both strains (Lee *et al.,* 2006; Ji *et al.,* 2010b; Zhang *et al.,* 2011).

Also, ADHE activity was detected in both KMS004 and KMS005-76T. It suggested that the accumulation of ethanol in KMS005-76T might result from spontaneous mutations of some iso-enzymes in the ethanol production pathway during metabolic evolution such as acetaldehyde dehydrogenase2 (encoded from *mhpF*) and 1,3-propanediol oxidoreductase (encoded by *yqhD-1*). However, this reaction requires NADH and the gene expression of this enzyme is induced by cAMP-CRP. Acetaldehyde from pyruvate is converted further to ethanol by ethanol dehydrogenase (encoded by *adhP)* in KMS004 and KMS005-76T. This reaction also requires NADH. Therefore, the activation of these enzymes might regenerate NAD^+ simultaneously with the accumulation of ethanol.

4.3.2 Lactate dehydrogenase A (LDHA)

KMS005-76T mutant strain was constructed from KMS004 (Δ*adhE*, Δ*pta-ackA*) by abolishing D-(-)-lactate dehydrogenase (LDHA) encoded by *ldhA* gene.

LDHA activities from wildtype, KMS004 and KMS005-76T were 0.21±0.02, 0.82±0.02 and 0.06±0.01 U/mg protein, respectively (Table 4.2 and Figure 4.6). The wildtype strain and KMS004 showed significantly 3-folds and 13-folds higher in LDHA activity than that of KMS005-76T mutant strain, respectively (Table 4.2). The LDHA activity was relatively lowest in KMS005-76T strain. It is in agreement with no deletion of lactate in KMS005-76T (Table 4.1). As expected, KMS004 showed the highest LDHA activity compared with wildtype and KMS005-76T strains, respectively. In *E. aerogenes* deletion of *ldhA* could enhance 2,3-BD production because lactate and 2,3-BD production pathways compete each other for pyruvate and NADH. Moreover, the decreased formation of ethanol and lactate due to *adhE* and *ldhA* inactivation in KMS005-76T strain would lead to a significantly increased $NADH/NAD^+$ ratio *in vivo*. The sufficient NADH supply could drive both of the reversible reaction from acetoin to 2,3-BD production and fulfill the redox balance *in vivo*. It resulted in lowering the accumulation of acetoin but increasing in a yield of 2,3-BD (Converti *et al.,* 2003; Ji *et al.,* 2009). It was confirmed that the yield of 2.3-BD was higher in KMS005-76T strain than those of KMS004 and even wildtype strains

4.3.3 2,3-Butanediol dehydrogenase (BUDC)

BUDC activities of the wildtype, KMS004 and KMS005-76T were determined. The specific activity of BUDC in mutant KMS005-76T strain was highest among those strains (Table 4.2). KMS005-76T (*ΔadhE*, *Δpta-ackA*, Δ*ldhA'-cat-sacB-ldhA''*) converted sufficient pyruvate to 2,3-BD. Meanwhile, KMS004 and wildtype strain (*ldhA*$^+$ gene) were not only produced 2,3-BD but also secreted lactate as

reduced fermentative production. These findings further supported that the deletion of *ldhA* gene provided additional NADH for 2,3-BD production via BUDC activity. It would result from that, the combined deletion of *adhE* and *ldhA* in KMS005-76T caused additionally lowering in growth and glucose consumption in minimal medium as a result of redox imbalance. In general, an expression of an appropriate NADH-oxidizing enzyme such as pyruvate dehydrogenase, BUDC, malate dehydrogenase (MDH) and succinate dehydrogenase (SDH) can restore the redox balance. In KMS005-76T, this redox imbalance could be restored by expression of BUDC activity. The dramatically decreased formation of ethanol and lactate due to *adhE* and *ldhA* inactivation in KMS005-76T led to a significantly increased in NADH/NAD^+ ratio. Consequently, sufficient pyruvate converted to acetoin and 2,3-BD by BUDC activity under microaerobic conditions for maintaining the intracellular redox balance. In addition, as shown in Table 4.1, the production of acetic acid by mutant strain was increased. Further, acetic acid can also act as an inducer for the BUDC in the conversion of pyruvate to 2,3-BD (Johansen *et al.,* 1975). Thus the carbon fluxes to 2,3-BD were increased in mutant strains (KMS004 and KMS005-76T).

4.3.4 Malate dehydrogenase (MDH)

The MDH activity in KMS004 were 3-folds and 5-folds higher (0.10± 0.04 U/mg protein) than those observed for wildtype (0.03±0.01U/mg protein) and KMS005-76T (0.02±0.01 U/mg protein), respectively (Table 4.2 and Figure 4.6). However, these experiments showed that MDH activity was low in all strains. This result was consistent with decreasing levels of malate and thus succinate production. Although, KMS005-76T were deleted genes encoding *adhE, pta-ackA* and *ldhA*. This

strain retained two branches for succinate and 2,3-BD pathways with MDH and BUDC as major routes for NADH oxidation, respectively. For strain KMS005-76T lacking LDHA, PTA, ACKA and ADHE, the flux towards succinate production was decreased but was preferred to 2,3-BD pathway branch (Table 4.1 and 4.2). For phosphoenolpyruvate-pyruvate-oxaloacetate node (PEP-PYR-OAA), flux distribution reveals that succinate produced by Enterobacteriaceae using the pathway generally regarded as the native fermentation pathway with phosphoenolpyruvate carboxylase (PPC). This pathway wastes the energy of phosphoenolpyruvate by producing inorganic phosphate. One ATP is lost per succinate produced by this pathway. Conserving this energy as ATP by using alternative enzyme systems represents an opportunity to increase cell growth. However, this node comprises a set of reactions that direct the carbon flux into appropriate directions and thus, it acts as a highly relevant switch point for carbon flux distribution within the central metabolism. Therefore, KMS004 and wildtype strain thus showed higher levels of MDH activity compared with strains KMS005-76T. It was likely that, the flux distribution through succinate production in the strain KMS005-76T was not preferred. Since, very low level of succinate was detected. It would also imply that, this would be an effect of gene deletions presenting for maintaining intracellular redox balance via 2,3-BD production instead of succinate production. Thus, the flow of carbon flux slightly flows through a non oxidative pathway to re-oxidize NADH accumulated during glycolysis. In addition, the fermentation condition performed in this study was not suitable for succinate formation via malate dehydrogenase activity because of microaerobic conditions and no external source of CO_2 were performed.

Table 4.2 Enzymatic activity of *K. oxytoca* wild type, KMS004 and KMS005-76T.

Enzyme activity (U/mg protein)	Wildtype[a]	KMS004	KMS005-76T
Lactate dehydrogenase (LDHA)	0.21±0.02[b,€]	0.82±0.02[¥]	0.06±0.01[£]
Alcohol dehydrogenase (ADHE)	8.43±0.53[€]	3.12±0.86[¢]	3.04±0.45[¢]
2,3-butanediol dehydrogenase (BUDC)	2.33±0.41[€]	3.42±0.33[¥]	4.24±0.30[£]
Malate dehydrogenase (MDH)	0.03±0.01[€]	0.10±0.04[¥]	0.02±0.01[€]

[a]Cells were grown in AM1 medium with 5% glucose, at 37 °C, pH 6.0, 300 rpm, 1.0 vvm aeration rate with 1.0 L working volume.

[b]Values bearing different Greek symbol are significantly different (P<0.05) between rows.

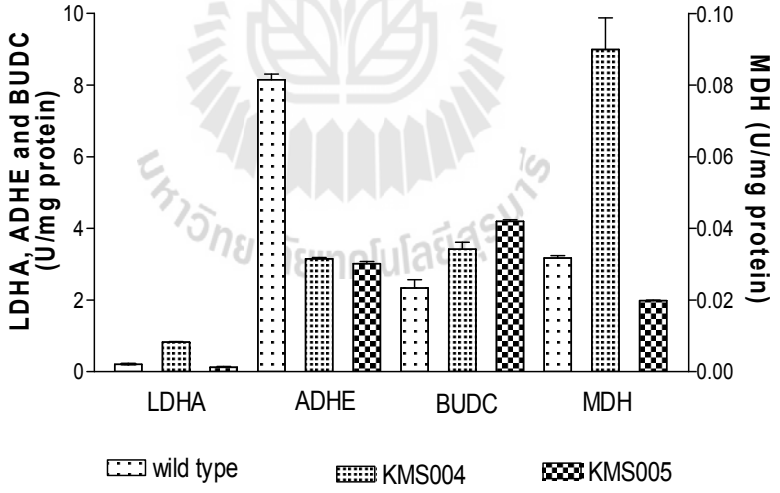

Figure 4.6 LDHA, ADHE, BUDC and MDH activity determination in *K. oxytoca* wild type; KMS004 and KMS005 strains. The cell were grown in AM1 medium with 5% glucose, at 37°C, pH 6.0, 300 rpm, 1.0 vvm aeration rate with 1.0 L working volume. The symbols and error bar are average values and standard deviations of at least three measurements, respectively.

4.4 Production of 2,3-BD from various carbon sources

4.4.1 Production of 2,3-BD from sugarcane molasses in shake flask

Availability of inexpensive carbon sources or carbohydrate raw material is essential for developing an economical fermentation for the cost efficient production of 2,3-BD. Sugarcane molasses are one of the agricultural wastes from sugar manufacturing operating process and also are the low cost carbon substrate. The sugarcane molasses have major three sugars which are sucrose, glucose, and fructose. *K. oxytoca* KMS005-76T could utilize sugarcane molasses (50 g/L) and produced 2,3-BD at a concentration of 19.21±0.27 g/L in AM1 medium after 120 h incubation. Other by-products such as succinate, lactate, acetate and ethanol were also observed but at low concentration (Table 4.3 and Figure 4.7). The 2,3-BD yield obtained was higher in KMS005-76T than those of the wild type and KMS004 strains (Table 4.3).

Jung *et al*. (2013a) reported that 2,3-BD (22.51±0.36 g/L), acetoin (0.82±0.55 g/L), ethanol (12.76±0.48 g/L), lactate (0.01±0.01 g/L) and succinate (1.29±0.18 g/L) were obtained from invert sugarcane molasses (60 g/L) supplemented in the medium containing 5 g/L yeast extract, 10 g/L casamino acid and 0.5g/L citric acid in flask culture by engineered *E. aerogenes* EMY-68 (Δ*ldhA*, Δ*scrR*). However, *E. aerogenes* EMY-68 strain produced significant high amount of ethanol (12.76±0.48 g/L). In addition, *E. aerogenes* EMY-68 strain produced 2,3-BD at a low yield (0.37±0.02 g/g) compared with those of KMS005-76T (0.42±0.02 g/g), and KMS005-76T did not also require special nutrients for growth such as yeast extract and casamino acid.

In this study, the utilizations of main sugars in sugarcane molasses such as glucose, fructose, and sucrose were observed (Figure 4.7). Glucose was a preferred carbon source for all three strains. After glucose was depleted, wildtype, KMS004 and

KMS005-76T strains utilized fructose and sucrose at similar rates (Figure 4.7). However, wildtype utilized sucrose much faster than that of KMS004 and KMS005-76T strains (Figure 4.7A). This result suggested that the presence of glucose and fructose in sugarcane molasses affected sucrose utilization in KMS004 and KMS005-76T. In addition, the use of preferred carbon sources repressesor activates the activity of catabolic mechanisms that enable the utilization of secondary carbon sources (Görke and Stülke, 2008). In Enterbacteriaceae, all three sugars (glucose, fructose and sucrose) from sugarcane molasses are transferred across the cytoplasmic membrane by PTS. Therefore, a few possible mechanisms can be suggested for the change in carbon source preference (Ji *et al.,* 2010a; Jung *et al.,* 2013b). Recently, the *Cra* (encoding catabolite repressor/activator protein) binding site was found on the promoter region of the *csc* operon encoding the genes for sucrose catabolism in *E. coli* (Sabri *et al.,* 2013). Similarly, it is likely that *Cra* is involved in the regulation of the *scr* operon in *Klebsiella* sp. Reid and Abratt (2005) revealed that sucrose-6-phosphate hydrolase (encoded by *scrB*) enables this microorganism to metabolize sucrose. The *scrKYABR* operon contains putative cAMP-CrpA (encoding cAMP receptor protein) binding sites presenting in the -35 regions that could serve as regulatory sites for glucose-repression. Moreover, the expression of these PTS mediated-sugar utilization operons are often modulated by transcription regulators that contain duplicated PTS-regulatory domains (PRDs). The phosphorylated level of PRDs by the PTS-mediated sugar transporter EIIB or HPr has been known to enable the sequential utilization of PTS-mediated sugar (Van Tilbeurgh and Declerck, 2001; Graille *et al.,* 2005). Jung and co-workers (2013) revealed that the changed in carbon source preference by the deletion of the *ldhA* and *scrA* gene in *E. aerogenes* might be triggered by PRDs mechanism in 2,3-BD production with sugarcane molasses fermentation.

Table 4.3 Kinetic parameters of *K. oxytoca* wild type and mutant strains in AM1 medium containing 5% (w/v) sugarcane molasses in shake flask experiment.

Strains	**Wild type**	**KMS004**	**KMS005-76T**
Total sugar concentration (g/L)	45.57±1.00[c,€]	40.10±2.25[£]	46.06±0.82[€]
2,3-BD concentration (g/L)	14.60±0.09[€]	15.93±1.01[£]	19.21±0.27[¥]
2,3-BD yield (g/g)[a]	0.35±0.02[€]	0.39±0.07[£]	0.42±0.01[¥]
2,3-BD productivity (g/L.h)[b]	0.30±0.02[€]	0.13±0.01[£]	0.27±0.03[¥]
Succinate (g/L)	1.33±0.06[€]	0.78±0.06[£]	2.42±0.03[¥]
Lactate (g/L)	1.52±0.14[€]	1.78±0.06[£]	ND[d]
Formate (g/L)	ND	ND	ND
Acetate (g/L)	4.68±0.85[€]	2.90±0.07[£]	4.64±0.19[€]
Ethanol (g/L)	4.06±0.84[€]	ND	ND
Incubation time (h)	72	96	120

[a]The 2,3-BD yield was calculated as grams of 2,3-BD produced divided by grams of the sugar consumed.

[b]The 2,3-BD productivity was calculated as 2,3-BD concentration produced divided by overall incubation time.

[c]No 1,3-propanediol and acetoin were detected in the fermentation broth from all strains.

[d]ND, not detected.

[e]All data represent the averages of three fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P<0.05$) between columns.

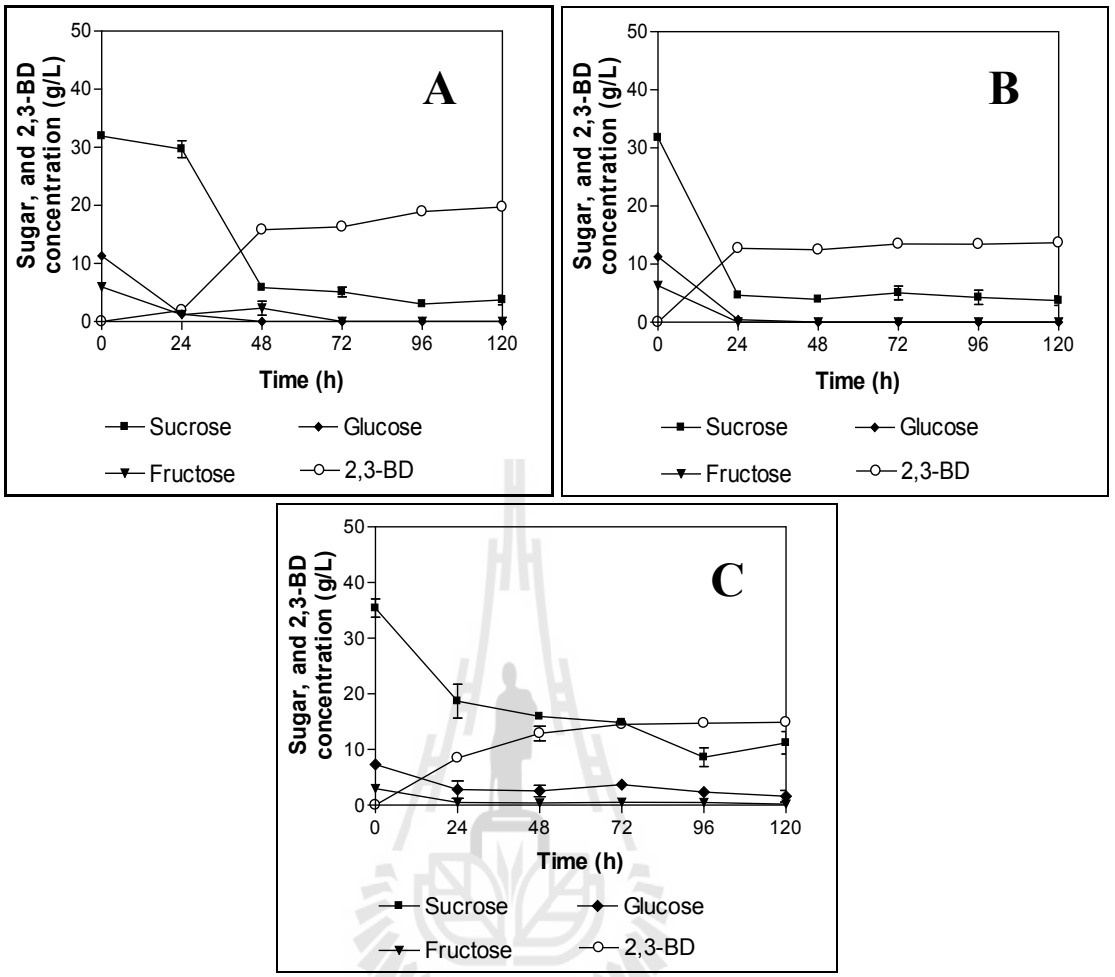

Figure 4.7 Fermentation profile of *K. oxytoca* M5a1 wild type and mutant in AM1 media containing 5% (w/v) sugarcane molasses. (A) *K. oxytoca* wild type (B) KMS004 (C) KMS005-76T. The symbols and error bar are average values and standard deviations of at least three measurements, respectively.

4.4.2 Production of 2,3-BD from hydrolyzed cassava starch in shake flask

Hydrolyzed cassava starch used in this study was prepared from cassava starch, treated with α-amylase and glucoamylase enzymes. Reducing sugar was presented in the enzymatic hydrolysate including glucose and maltose. KMS005-76T, KMS004 and wildtype strains produced 2,3-BD from cassava hydrolyzed starch in

AM1 medium at concentration of 19.11±0.35 g/L, 12.76±1.07 g/L and 14.31±0.07 g/L, respectively after 96 h incubation time (Table 4.4 and Figure 4.8). KMS005-76T, KMS004 and wildtype strains utilized hydrolyzed cassava starch for 2,3-BD production with yields of 0.39±0.02 g/g, 0.36±0.03 g/g and 0.32±0.01 g/g, respectively and the productivities of 0.19±0.01 g/L.h, 0.17±0.01 g/L.h and 0.20±0.02 g/L.h, respectively (Table 4.4). In addition, the specific productivity of 2,3-BD production from these strains were KMS005 (0.16±0.01 g/g.h) compared with that of wildtype (0.15±0.01 g/g.h) and KMS004 (0.24±0.02 g/g.h), respectively (Table 4.4). The growth rate of KMS005-76T and KMS004 strains in hydrolyzed cassava starch was similar to what had been observed in glucose fermentation. This result was consistent with the specific growth rate of KMS005 (μ= 0.04±0.01 h^{-1}) compared with that of wildtype *K. oxytoca* (μ = 0.05±0.01 h^{-1}) and KMS004 (μ = 0.04±0.01 h^{-1}) (Table 4.1 and Figure 4.8). Although, KMS005-76T was delayed in growth for the first 24 h. It might be result from that KMS005-76T produced higher level of acetate from hydrolyzed cassava starch during 2,3-BD fermentation. However, acetate was produced not only an inhibitor but also some of acetate in the medium might be converted to 2,3-BD through conversion to acetaldehyde, condensation to form acetoin, and subsequent reduction to 2,3-BD (Ji *et al.,* 2008). Maddox (1996) explained that an induced pathway of 2,3-BD was affected by accumulation of acidic products in the medium rather than the internal pH. Therefore, transmembrane pH gradient causes accumulation of acetate, thus inducing the activity of α-acetolactate synthase resulting in conversion of acetate to 2,3-BD (Stromer *et al.,* 1968).

Cheng *et al.* (2010) reported that 2,3-BD production from corncob acid hydrolysate by *K. oxytoca* ATCC 10370. The maximal 2,3-BD concentration of 35.7

g/L was observed. Moreover, it was found that high level of acetic acid in the hydrolysate could inhibit the growth of *K. oxytoca*. Similarly, KMS004 was produced high acetic acid and lactic acid. It would result in decreasing pH of the culture broth, thus affecting glucose consumption (Table 4.4). Moreover, Wang *et al.* (2012) produced that 2,3-BD (78.3 g/L) from crude cassava powder (90 g/L) supplemented with 5 g/L yeast extract, 10 g/L peptone in SSF 5L fermentation by non-engineered *E. cloacae* subsp. *dissolvens* SDMA.

Cassava contains toxic chemical cyanogen, which may inhibit the growth and metabolism of bacteria. This may be a reason that the yield of 2,3-BD from cassava powder is lower than that from glucose. However, it was shown from this study that a high concentration of 2,3-BDwas obtained from KMS005-76T using hydrolyzed cassava starch. Therefore, KMS005-76T would be a good candidate for the 2,3-BD production and hydrolyzed cassava starch could be used as a low-cost substrate for the economical production of 2,3-BD.

Table 4.4 Kinetic parameters of *K. oxytoca* wild type and mutant strains in AM1 medium containing 5% (w/v) hydrolyzed cassava starch in shake flask experiment.

Strains	**Wild type[h]**	**KMS004[h]**	**KMS005-76T[h]**
CDW (g/L)	1.33±0.05[f,€]	0.50±0.12[£]	1.27±0.03[¥]
μ (h^{-1})[a]	0.05±0.01[€]	0.04±0.02[£]	0.04±0.01[£]
Total sugar consumption (g/L)	45.98±0.87[€]	30.94±1.67[£]	47.87±0.20[¥]
Specific glucose consumption (g/g.h)[b]	0.51±0.01[€]	0.14±0.03[£]	0.51±0.01[€]
2,3-BD concentration (g/L)	14.31±0.07[€]	12.76±1.07[£]	19.11±0.35[¥]
2,3-BD yield (g/g)[c]	0.32±0.01[€]	0.36±0.03[£]	0.39±0.02[¥]
2,3-BD productivity (g/L.h)[d]	0.20±0.02[€]	0.17±0.01[£]	0.19±0.01[€]
2,3-BD specific productivity (g/g.h)[e]	0.15±0.01[€]	0.24±0.02[£]	0.16±0.01[¥]
Succinate (g/L)	0.84±0.09[€]	0.74±0.51[£]	1.68±0.05[¥]
Lactate (g/L)	2.58±0.56[€]	3.61±0.09[£]	ND[g]
Formate (g/L)	ND	ND	ND
Acetate (g/L)	2.79±0.11[€]	5.72±0.10[£]	3.26±0.02[¥]
Ethanol (g/L)	5.67±0.02[€]	0.95±0.32[£]	ND
Incubation time (h)	72	72	96

[a] The specific growth rate was calculated by slope of cell dry weigh concentration curve.

[b]The specific glucose consumption rate was calculated as glucose consumption multiplied by cell dry weight (CDW) and divided by overall incubation time.

[c]The 2,3-BD yield was calculated as grams of 2,3-BD produced divided by grams of the sugar consume.

[d]The 2,3-BD productivity was calculated as 2,3-BD concentration produced divided by overall incubation time.

[e]The 2,3-BDspecific productivity was calculated as 2,3-BD productivity divided by cell dry weight.

[f]All data represent the averages of three fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P<0.05$) among strains between columns.

[g]ND, not detected.

[h]No 1,3-propanediol and acetoin were detected in the fermentation broth from all strains.

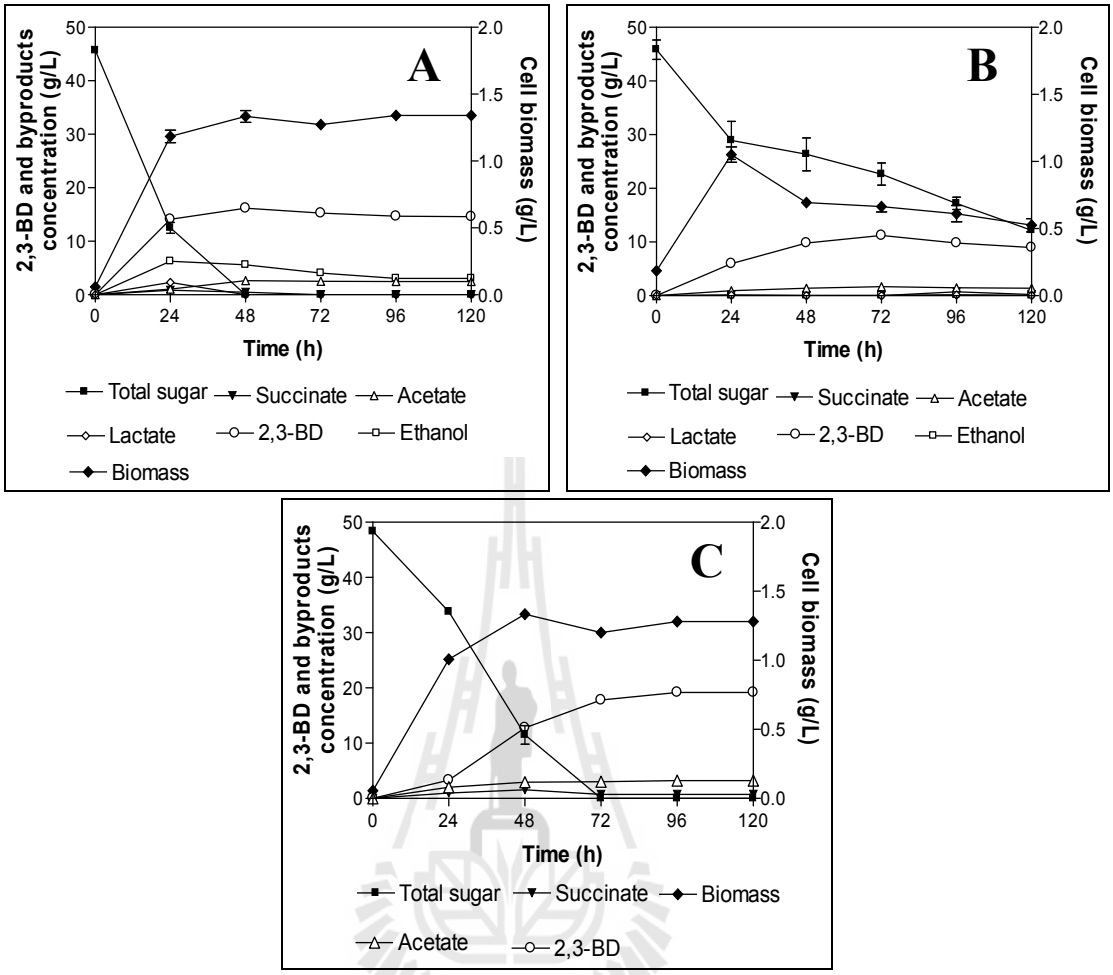

Figure 4.8 Fermentation profile of *K. oxytoca* M5a1 wild type and mutant in AM1 medium containing 5% (w/v) hydrolyzed cassava starch. (A) *K. oxytoca* wild type (B) KMS004 (C) KMS005-76T. The symbols and error bar are average values and standard deviations of at least three measurements, respectively.

4.4.3 Production of 2,3-BD from maltodextrin in shake flask

Maltodextrin is an oligosaccharide that is used as a food additive. It is produced from variety of starch by partial enzymatic hydrolysis. Maltodextrin consists of D-glucose units connected in chains of variable length. The glucose units are primarily linked with α (1→4) glycosidic bond. Maltodextrin is typically

composed of a mixture of chains that vary from three to seventeen glucose units. Moreover, the price of maltodextrin in Thailand market is much lower than glucose around US $600-1000 per ton (Cepeda *et al.,* 2001). KMS005-76T, KMS004 and wild type produced 2,3-BD at concentrations of 9.02±1.26g/L, 10.01±0.50 g/L and 10.24±1.32 g/L, respectively from maltodextrin at 120 h in shake flasks (Table 4.5 and Figure 4.9). The mutant strain KMS004 showed the highest productivity of 2,3-BD (0.18±0.02 g/L.h) from maltodextrin. In contrast, KMS005-76T produced the lowest productivity (0.12±0.01 g/L.h) compared with both strains. Furthermore, this result was consistent with the lower specific productivity of KMS005 (0.10±0.01 g/g.h) compared with that of wild type (0.12±0.01 g/g.h) and KMS004 (0.38±0.08 g/g.h), respectively (Table 4.5). Sangproo *et al.* (2012) reported that KMS004 produced D-(-)-lactic acid (32.95±0.68 g/L) but less production of 2,3-BD (0.94±0.47 g/L) from maltodextrin (50 g/L sugar mixture) in anaerobic bottles, pH 7.0. It was surprising that different culture conditions led to different by-products. KMS004 responded with a different the carbon flux partitioning when different substrates and conditions were used for cultivation. Most of the carbon fluxes were directed towards the D-(-)-lactic acid production pathway when maltodextrin was supplied in anaerobic conditions (Sangproo *et al.,* 2012). In contrast, wild type produced higher specific growth rate (μ= 0.05 h^{-1}) and increased in sugar consumption rate than those of KMS005-76T (μ= 0.02 h^{-1}) and KMS004 (μ= 0.04 h^{-1}) during maltodextrin fermentation (Table 4.5) under microaerobic conditions (shake flask). Shin *et al.* (2010a) reported that engineered strain *E. coli* UT5600 produced 2,3-BD from 10 g/L cellodextrin derived from cellulose at the concentration of 4.2 g/L under anaerobic conditions.

The concentrations of maltodextrin in fermentation broth of KMS005-76T, KMS004 and wild type strain remained about 35 g/L, 27 g/L and 25 g/L, respectively, and were not completely utilized after 120 h incubation (Figure 4.9). The delay in maltodextrin consumption of *K. oxytoca* might result from carbon catabolite repression (CCR) due to a presence of glucose moiety in maltodextrin used in this study. CCR has been studied extensively in enteric bacteria with ability of glucose to block induction of genes for other substrate utilization. In enteric bacteria, when glucose is absent from the medium, the IIA component of glucose-specific PTS ($EIIA^{Glc}$) is mainly in its phosphorylated state. $EIIA^{Glc}$ is bound to an enzyme adenylatecyclase and activating its cAMP biosynthesis, leading to increase cAMP concentration. Thus, cAMP binds to a cAMP receptor protein (CRP) causing the induction of carbon catabolite repressed genes (Görke and Stülke, 2008). The maltose/maltodextrin-utilizing (*mal*) system is subjected to catabolite repression in *Klebsiella* spp. The expressions of malT (encoding *MalT*, a central activator of mal regulon) and genes involved in the *mal* system are controlled by the cyclic AMP/catabolite gene activator protein system (Sangproo *et al.,* 2012). The interaction not only inhibited MalT enzymatic activity as a transcriptional activator but also activated Mlc as a transcriptional repressor of malT.

Table 4.5 Kinetic parameters of *K. oxytoca* wild type and mutant strains in AM1 medium containing 5% (w/v) maltodextrin in shake flask experiment.

Strains	**Wildtype[h]**	**KMS004[h]**	**KMS005-76T[h]**
CDW (g/L)	1.42±0.01[f,€]	0.60±0.06[€]	1.16±0.05[¥]
μ (h^{-1})[a]	0.05±0.01[€]	0.04±0.01[€]	0.02±0.01[¥]
Total sugar consumption (g/L)	24.83±1.94[€]	23.18±1.90[£]	22.03±2.03[¥]
Specific glucose consumption(g/g.h)[b]	0.31±0.01[€]	0.10±0.15[£]	0.22±0.02[¥]
2,3-BD concentration (g/L)	10.24±1.32[€]	10.01±0.50[€]	9.02±1.26[¥]
2,3-BD yield (g/g)[c]	0.33±0.03[€]	0.35±0.04[£]	0.40±0.08[¥]
2,3-BD productivity (g/L.h)[d]	0.17±0.02[€]	0.18±0.02[€]	0.12±0.01[¥]
2,3-BD specific productivity(g/g.h)[e]	0.12±0.01[€]	0.38±0.08[£]	0.10±0.01[€]
Succinate (g/L)	ND	0.72±0.05[£]	ND
Lactate (g/L)	0.04±0.01[€]	0.16±0.10[£]	ND[g]
Formate (g/L)	ND	ND	ND
Acetate (g/L)	0.31±0.03[€]	1.70±0.47[£]	1.03±0.01[¥]
Ethanol (g/L)	1.32±0.24[€]	0.15±0.01[£]	0.09±0.02[¥]
Incubation time (h)	120	120	120

[a] The specific growth rate was calculated by slope of cell dry weigh concentration curve.

[b]The specific glucose consumption rate was calculated as glucose consumption multiplied by cell dry weight (CDW) and divided by overall incubation time.

[c]The 2,3-BD yield was calculated as grams of 2,3-BD produced divided by grams of the sugar consume.

[d]The 2,3-BD productivity was calculated as 2,3-BD concentration produced divided by overall incubation time.

[e]The 2,3-BD specific productivity was calculated as 2,3-BD productivity divided by cell dry weight.

[f]All data represent the averages of three fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P<0.05$) among strains between column.

[g]ND, not detected.

[h]No 1,3-propanediol and acetoin were detected in the fermentation broth from all strains.

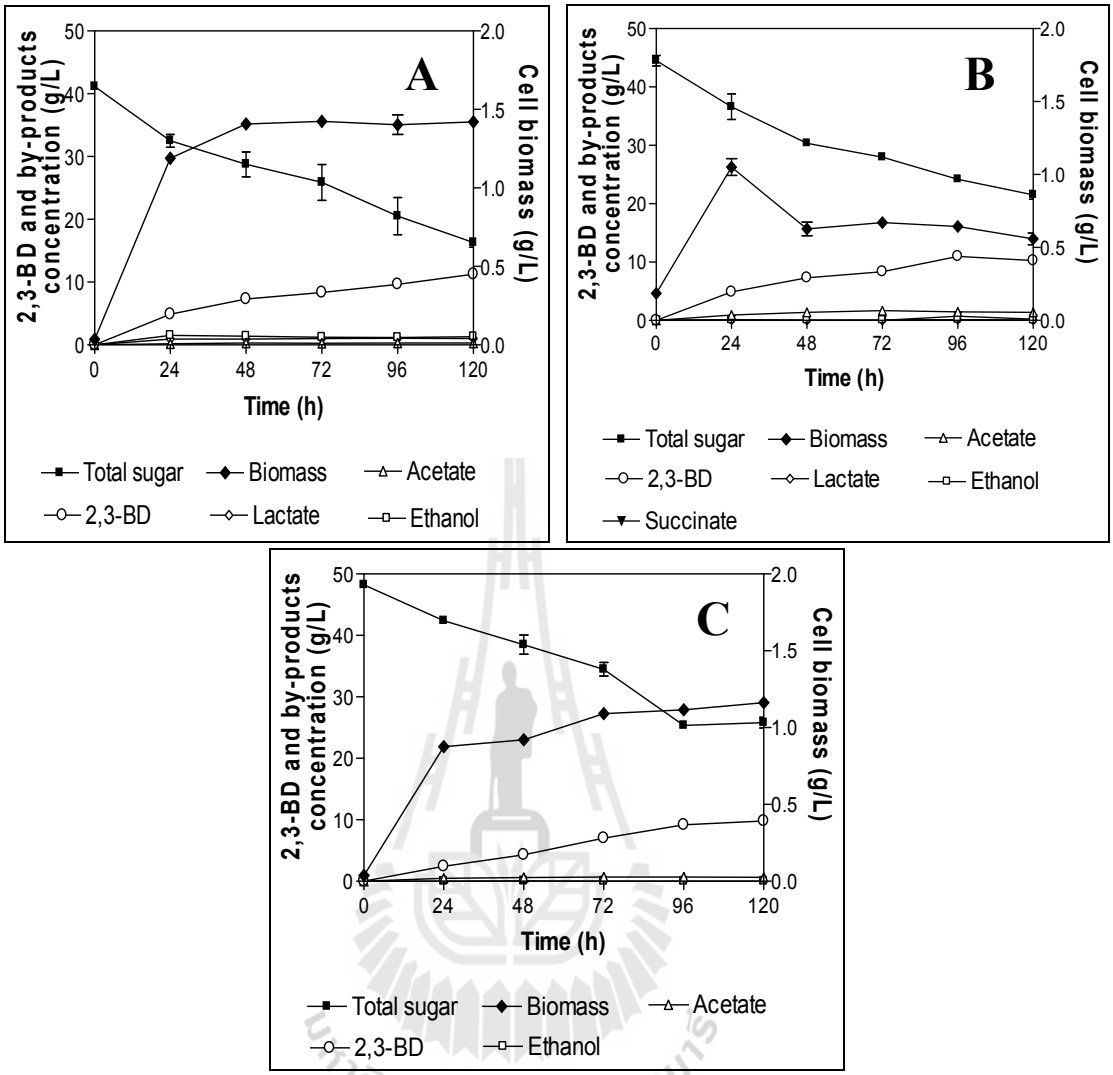

Figure 4.9 Fermentation profile of *K. oxytoca* M5a1 wild type and mutant in AM1 medium were containing 5% (w/v) maltodextrin. (A) *K. oxytoca* wild type (B) KMS004 (C) KMS005-76T. The symbols and error bar are average values and standard deviations of at least three measurements, respectively.

4.5 Production of 2,3-butanediol in 2 liters batch bioreactor

4.5.1 Effect of aeration rate on 2,3-BD production

Many research have been devoted to the study of different fermentation conditions for 2,3-BD production. It was found that oxygen supply is a critical factor affecting product yield, productivity and by-product formation. The effect of oxygen was expected to enhance cell growth and 2,3-BD production in KMS005-76T. To study this effect, 2,3-BD production was performed in 2 L bioreactor with and without aeration (1.0 vvm). At the end of fermentation, 2,3-BD concentration were obtained at 20.27±0.60 g/L under aeration conditions and 8.05±0.08 g/L under non-aeration conditions, respectively (Table 4.6 and Figure 4.10). Moreover, under aeration conditions, KMS005-76T strain produced an impressive yield and productivity of 2,3-BD at 0.47±0.03 g/g and 0.67±0.11 g/L.h, respectively at 36 h. The concentrations of succinate, acetate and ethanol were 0.94±0.38 g/L, 1.50±0.52 g/L and 1.28±0.49 g/L, respectively (Figure 4.10A). While at 96 h with non-aeration conditions, KMS005-76T strain produced lower yield and productivity of 2,3-BD at 0.31±0.02 g/g and 0.13±0.01 g/L.h, respectively. The small amount of by-products such as succinate, acetate, formate, ethanol and glucose residue were also observed at the levels of 0.80±0.04 g/L, 1.30±0.03 g/L, 0.85±0.05 g/L, 2.06±0.67 g/L and 24.33±1.64 g/L, respectively (Table 4.6 and Figure 4.10B). Moreover, aeration also increased the specific growth rate of KMS005-76T (μ= 0.15±0.01 h^{-1}) compared with non-aeration conditions of KMS005-76T (μ= 0.02±0.01 h^{-1}) (Table 4.6). Therefore, the result confirmed that aeration could improve glucose consumption rate, cell growth and 2,3-BD production by KMS005-76T.

Table 4.6 Kinetic parameters of *K. oxytoca* KMS005-76T in AM1 medium containing 5% (w/v) glucose in 2L bioreactor.

Treatment conditions	Aeration[f]	Non-aeration[g]
CDW (g/L)	2.97±0.02[d,£]	0.81±0.02[€]
μ (h^{-1})[a]	0.15±0.01[£]	0.02±0.01[€]
Glucose consumption (g/L)	50.84±0.74[£]	25.67±1.64[€]
2,3-BD concentration (g/L)	20.27±0.60[£]	8.05±0.08[€]
2,3-BD yield (g/g)[b]	0.47±0.03[£]	0.31±0.02[€]
2,3-BD productivity (g/L.h)[c]	0.67±0.11[£]	0.13±0.01[€]
Succinate (g/L)	0.94±0.38[£]	0.80±0.04[€]
Lactate (g/L)	ND[c]	ND[e]
Formate (g/L)	ND	0.85±0.05[€]
Acetate (g/L)	1.50±0.52[£]	1.30±0.03[€]
Ethanol (g/L)	1.28±0.49[£]	2.06±0.67[€]
Incubation time (h)	36	96

[a] The specific growth rate was calculated by slope of cell dry weigh concentration curve.

[b]The 2,3butanediol yield was calculated as grams of 2,3-BD produced divided by grams of the sugar consumed.

[c]The 2,3butanediol productivity was calculated as 2,3-BD concentration produced divided by overall incubation time.

[d]All data represent the averages of three fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P<0.05$) among strains between columns.

[e] ND, not detected.

[f] Fermentation in AM1 medium containing 5% (w/v) glucose, pH 6.0, in 2L bioreactor without aeration.

[g] Fermentation in AM1 medium containing 5% (w/v) glucose, pH 6.0, 1.0 vvm aeration rate in 2L bioreactor.

The above results could be explained that 2,3-BD is produced under low oxygen supply or microaerobic fermentation. Microaerobic fermentation is described in terms of maintaining aninternal redox balance with respect to the NADH/NAD^+ pool during glycolysis and biosynthesis (Converti *et al.,* 2003). NAD^+ from glycolysis is regenerated by BUDC activity in a reversible reaction from acetoin to 2,3-BD. The NAD^+/NADH balance is maintained by relative production of acetoin to 2,3-BD (Blomqvist *et al.,* 1993). An increasing the oxygen supply rate leads to a higher cell density and to higher 2,3-BD production yield. Moreover, the ratio of oxygen demand and supply can control the proportions of metabolites produced (Voloch *et al.,* 1985). The shift in metabolism from production of biomass to production of mixed acid with 2,3-BD is the maintenance of NAD+/NADH balance. Under aerobic conditions, NADH from glycolysis is regenerated via respiration. Under low oxygen supply, reduced fermentative products such as ethanol, lactate, formate and 2,3-BD are usually formed (Voloch *et al.,*1985). This study demonstrated that KMS005-76T produced high amount of 2,3-BD but less amount of either ethanol or lactate (Table 4.6). This resulted from the deletion of *adhE* and *ldhA* genes in KMS005-76T strain.

Kosaric *et al.* (1992) also found that under aerobic conditions, oxygen was rapidly inactivated α-ALS enzyme, thus preventing 2,3-BD synthesis under high oxygen supply. On the other hand, an increased in α-ALD enzymatic activity resulted in conversion of acetolactate to acetoin under microaeration conditions. In addition, acetolactate acts as an oxygen sensitive molecule which spontaneously decarboxylates to diacetyl under even microaerophilic conditions (Monnet *et al.,* 2000). Thus, in both cases, oxygen does not directly influence genes expression but acts upon maintenances, by adjusting the accumulation of an inducing metabolite. Li (2010)

also revealed that when oxygen supply was increased biomass and 2,3-BD with small amounts of succinate, acetate, and ethanol were obtained by *E. coli* JM109 harboring pEnBD plasmid.

In conclusion, this study revealed the potential 2,3-BD production by engineered *K. oxytoca* KMS005-76T under microaerobic conditions. Under oxygen supply conditions, KMS005-76T strain improved glucose consumption and cell growth rate accompanying with enhanced 2,3-BD production. In contrast, without aeration KMS005-76T still accumulated a major 2,3-BD as fermentative product, but decreased in glucose uptake and cell growth rate resulting in lower yield and productivity of 2,3-BD production. Furthermore, an optimized control of aeration rate should be further studied and adjusted to achieve high level of 2.3-BD production.

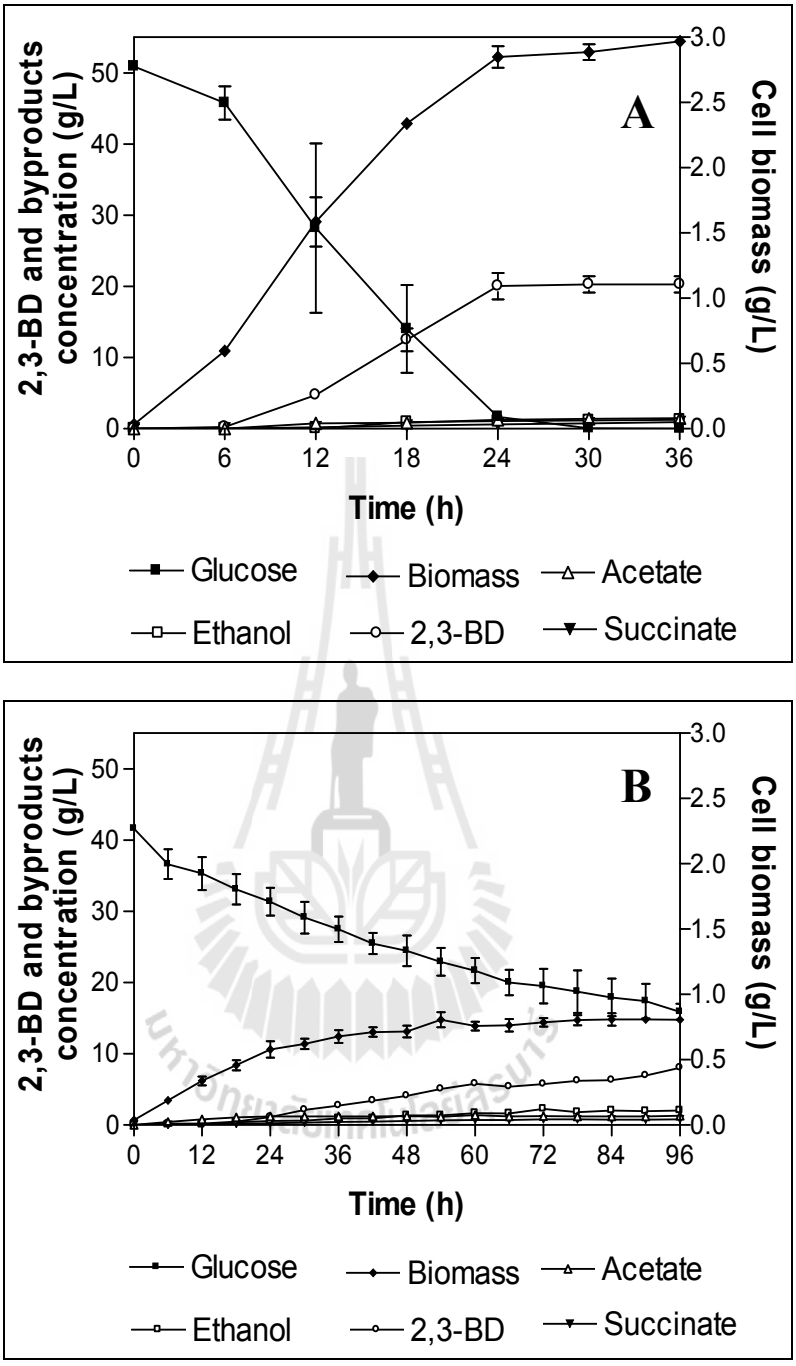

Figure 4.10 The effect of aeration rate of *K. oxytoca* KMS005-76T in AM1 medium contains 5% (w/v) glucose in 2L bioreactor. (A) Fermentation performed with aeration (B) Fermentation performed without aeration. The symbols and error bar are average values and standard deviations of at least three measurements, respectively.

4.5.2 Effect of glucose concentration on 2,3-BD production

Glucose is the most important simple sugar in all living organisms. In addition, glucose is an efficient substrate for all microorganisms as well as *K. oxytoca.* Therefore, the effect of glucose concentrations on 2,3-BD production was determined for 2,3-BD production. KMS005-76T was cultivated in a 2L bioreactor under batch process. Initial glucose concentration at 100 g/L could produce 2,3-BD at the concentration of 41.54±2.23 g/L with productivity of 0.95±0.02 g/L.h and yield of 0.42±0.04 g/g after 72 h. Furthermore, KMS005-76T produced 2,3-BD at the concentration of 86.36±1.65 g/L with productivity of 1.18±0.33 g/L.h and yield of 0.42±0.02 g/g from 200 g/L glucose after 96 h (Table 4.7 and Figure 4.11). In addition, this result was consistent with the specific productivity of KMS005 in both 100 g/L glucose (0.22±0.01 g/g.h) and 200 g/L glucose (0.32±0.02 g/g.h), respectively (Table 4.7). At the end of fermentation of 100 g/L glucose concentration, KMS005-76T produced 2.56±1.27 g/L succinate accompanied with small amounts of acetate (1.85±0.58 g/L) and ethanol (1.23±0.55 g/L) (Table 4.7). However, glucose concentration at 100 g/L produced slightly increase of 2,3-BD at 60 h until 72 h (Figure 4.11A). For 200 g/L glucose concentration, KMS005-76T produced 7.12±0.25 g/L succinate, 1.65±0.42 g/L acetate and 1.22±0.94 g/L ethanol at 96 h incubation (Figure 4.11B).

Table 4.7 Kinetic parameters of *K. oxytoca* KMS005-76T in AM1 medium containing 10% (w/v) and 20% (w/v) glucose, pH 6.0, 1.0 vvm in 2L bioreactor.

Glucose concentrations	**100 g/L**	**200 g/L**
CDW (g/L)	4.42±0.06[e,€]	3.68±0.25[£]
μ (h^{-1})[a]	0.15±0.01[€]	0.11±0.01[£]
Glucose consumption (g/L)	97.12±2.31[€]	200.34±1.13[£]
2,3-BD concentration (g/L)	41.54±2.23[€]	86.36±1.65[£]
2,3-BD yield (g/g)[b]	0.42±0.04[€]	0.42±0.02[€]
2,3-BD productivity (g/L.h)[c]	0.95±0.02[€]	1.18±0.33[£]
2,3-BD specific productivity (g/g.h)[d]	0.22±0.01[€]	0.32±0.02[£]
Succinate (g/L)	2.56±1.27[€]	7.12±0.25[£]
Lactate (g/L)	ND	ND[f]
Formate (g/L)	ND	ND
Acetate (g/L)	1.85±0.58[€]	1.65±0.42[£]
Ethanol (g/L)	1.23±0.55[€]	1.22±0.94[€]
Incubation time (h)	54	96

[a] The specific growth rate was calculated by slope of cell dry weigh concentration curve.

[b]The 2,3butanediol yield was calculated as grams of 2,3-BD produced divided by grams of the sugar consumed.

[c]The 2,3butanediol productivity was calculated as 2,3-BD concentration produced divided by overall incubation time.

[d]The 2,3-BD specific productivity was calculated as 2,3-BD productivity divided by cell dry weight.

[e]All data represent the averages of three fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P<0.05$) between columns.

[f] ND, not detected.

KMS005-76T cultivated in 100 g/L glucose concentration showed faster cell growth rate compared with cultivation in 200 g/L glucose. Moreover, the specific growth rate of KMS005-76T cultivated in 200 g/L glucose (μ= 0.11±0.01 h^{-1}) higher than that cultivated in 100 g/L glucose (μ= 0.15±0.01 h^{-1}) (Table 4.7). Further, glucose was exhausted in both conditions after fermentation (Table 4.7 and Figure 4.11). In this study, we found that KMS005-76T strain increased in 2,3-BD titer and prolonged log phase as well as higher cell density during the fermentation of 200 g/L glucose. This finding was contrasted to Wang *et al.* (2012) while they reported that cell density and 2,3-BD production were sharply decreased when high concentration of hydrolyzed cassava starch was used. This result showed that the high initial substrate concentration would affect the metabolism of strain *Enterobacter cloacae* subsp. *dissolvens* SDM. The inhibition of high sugar concentration on the cell growth and 2,3-BD production probably resulted from a fall in water activity that affected the metabolic rates (Wang *et al.,* 2012). Silveira *et al.* (1998) also reported that *K. pneumoniae* growth and 2,3-butanediol/acetoin formation were increasingly inhibited by initial sucrose concentrations of 204 g/L sucrose. The decreased in level of a mixture of 2,3-butanediol/acetoin at concentration of 84.3 g/L with a lower yield of 0.41 g/g and a productivity of 1.06 g/L/h were observed.

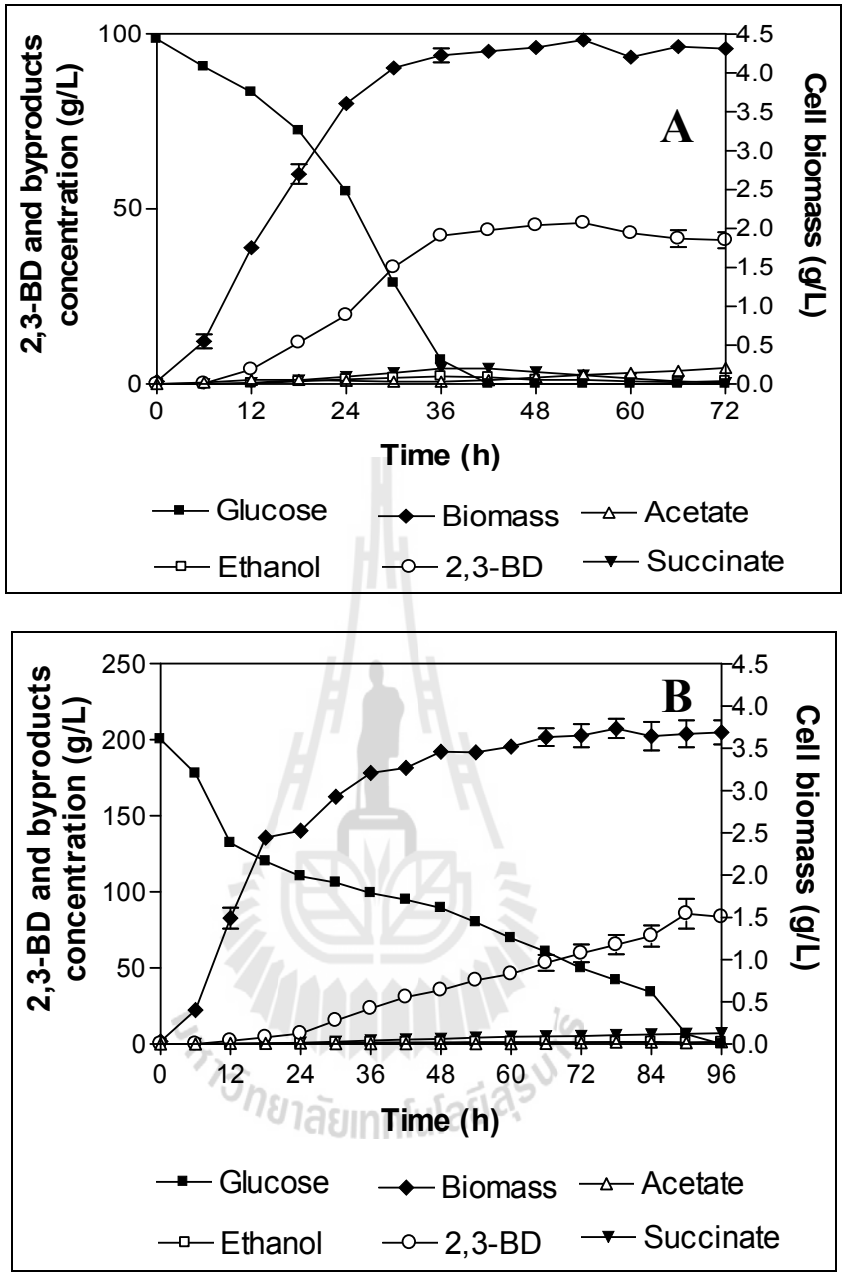

Figure 4.11 The effect of glucose concentration of *K. oxytoca* KMS005-76T in AM1 medium in 2L bioreactor. (A) 100 g/L glucose concentration (B) 200 g/L glucose concentration. The symbols and error bar are average values and standard deviations of at least three measurements, respectively.

4.6 2,3-butanediol production in fed-batch operation

4.6.1 2,3-BD production using glucose in fed-batch operation

The effects of reactor operation mode on 2,3-BD formation is one of critical factors in the establishment of an optimal process design. For efficient 2,3-BD production, a series of reactor operation modes were adopted previously, including batch and fed-batch, continuous culture, cell recycle, and immobilized cell systems (Celìnska and Grajek, 2009). Fed-batch fermentation is a batch process fed continuously or sequentially with substrate. Fed-batch processes were introduced in order to avoid substrate inhibition resulting in high osmotic pressure at high sugar concentration (Ozmichi and Kargi, 2007). Fed-batch operation offer special advantages over batch and continuous operation by eliminating substrate inhibition as a result of slow feeding of highly concentrated substrate solution. Fermentation is started with relatively low substrate concentration at a low volume. As the substrate is consumed, it is replaced by addition of a concentrated substrate solution at a low rate. In addition, fed-batch process is keeping the substrate concentration in the reaction below the toxic level (Qureshi and Blaschek, 2001).

Fed-batch fermentation for 2,3-BD production was carried out in 2L bioreactor with an initial volume of 1.0 L. KMS005-76T cell was initial grown in batch mode. At the late exponential phase of batch fermentation, highly concentrated glucose solution (800 g/L) was intermittently fed into the bioreactor. Glucose was added during 27-84h to maintain glucose concentration in the range of 15-40 g/L. The result showed that, during the first 24 h, the glucose concentration dramatically utilized from 100 g/L to 25 g/L by KMS005-76T and the cell dry weigh was rapidly increased (4.05±0.10 g/L) (Figure 4.12). At the same time, 2,3-BD began to accumulate.

Table 4.8 Kinetic parameters of *K. oxytoca* KMS005-76T in AM1 medium containing 10% (w/v) glucose and maltodextrin, pH 6.0, 1.0 vvm aeration rate in 2L fed-batch operation.

Substrate types	**Glucose**	**Maltodextrin**
CDW (g/L)	4.32±0.10[d,€]	5.21±0.38[£]
μ (h^{-1})[a]	0.10±0.07[€]	0.12±0.01[£]
Glucose consumption (g/L)	238.82±5.74[€]	230.14±2.49[£]
2,3-BD concentration (g/L)	117.36±4.48[€]	93.21±0.71[£]
2,3-BD yield (g/g)[b]	0.49±0.02[€]	0.41±0.01[£]
2,3-BD productivity (g/L.h)[c]	1.20±0.05[€]	0.95±0.04[£]
Succinate (g/L)	0.57±0.06[€]	1.04±0.24[£]
Lactate (g/L)	ND	ND[e]
Formate (g/L)	ND	ND
Acetate (g/L)	1.53±0.26[€]	1.96±1.94[£]
Ethanol (g/L)	0.41±0.04[€]	0.45±0.10[£]
Incubation time (h)	126	126

[a] The specific growth rate was calculated by slope of cell dry weigh concentration curve.

[b]The 2,3butanediol yield was calculated as grams of 2,3-BD produced divided by grams of the sugar consumed.

[c]The 2,3-butanediol productivity was calculated by slope of 2,3butanediol concentration curve.

[d]All data represent the averages of three fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P<0.05$) between columns.

[e] ND, not detected.

After 24 h, glucose reservoir was added at different concentration in each interval. The cell growth slightly decreased when 2,3-BD concentration reached 70.66±3.07 g/L at 72 h, after which the cell enter the stationary phase. The 2,3-BD production continued to increase117.36±4.48 g/L at 126 h (Figure 4.12). This indicates that 2,3-BD is a mixed-growth-associated product. The final cell dry weight (4.32±0.10 g/L) and specific growth rate (0.10±0.07 h^{-1}) were about similar in batch fermentation. The overall productivity and yield of 2,3-BD production were 1.20±0.05 g/L.h and 0.49±0.02 g/g, respectively (Table 4.8). At the end of fermentation, succinate, acetate and ethanol were obtained at concentration of 0.57±0.06 g/L, 1.53±0.26 g/L and 0.41±0.04 g/L, respectively (Table 4.8).

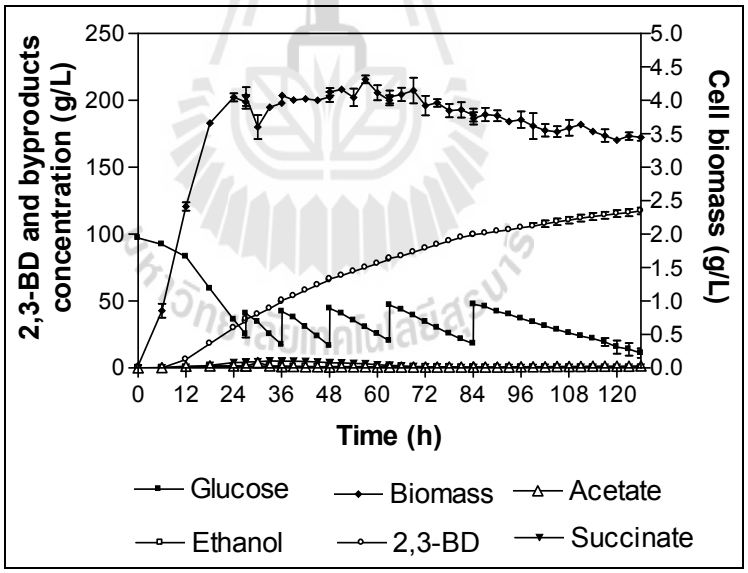

Figure 4.12 Fed-batch fermentation profile using initial 100 g/L glucose of *K. oxytoca* KMS005-76T in AM1 medium in 2L bioreactor. The symbols and error bar are average values and standard deviations of at least three measurements, respectively.

Further, the fermentation by-products accumulated at very low levels in the fermentation broth throughout the fermentation process. Meanwhile, the by-products were participated in the NADH oxidation pathway and ATP generation pathway. However, compared with batch fermentation, 2,3-BD concentration, yield and productivity obtained by KMS005-76T were significantly improved during fed-batch operation. Nie *et al.* (2011) reported that fed-batch fermentations for the production of 2,3-BD with *Klebsiella oxytoca* ME-UD-3 were investigated in 3-L-fermenters. The highest 2,3-BD concentration was up to 127.9 g/L with a productivity and yield were 1.78 g/L.h and 0.48 g/g, respectively at 72 h with 80 g/L initial glucose concentration and maintained 20-40 g/L glucose concentration with controlled pH 6.0. In this report, glucose feeding stock and sodium base were fed at the same time and this method can be applied for the fermentation which exist a constant relationship between substrate consumption and acid formation. However, the feeding occasion is not so easy to determine due to the lack of glucose online mornitoring equipment, and this system is not sufficiently robust to control glucose concentrations consistently at the desired levels. Also, the feeding amount of glucose was according to the glucose consumption before interval, and thus could not respond to the real information. In some cases, this would cause the glucose not to be timely fed and 2,3-BD would be reused as the carbon source for cell growth thus lowering 2,3-BD titer (Nie *et al.,*2011).

4.6.2 2,3-BD production using maltodextrin in fed-batch operation

Raw materials are a large part of microbial 2,3-BD production cost. A variety of works with biomass shows promise in helping to reduce the costs of

fermentation. In the past, the relatively high cost of conventional substrates such as starch or sugar has been identified as a mainly cost affecting the economic viability of 2,3-BD fermentation (Ji *et al.,* 2011). Thus, cheaper alternative biomass-derived sugars for 2,3-BD production was pursued. Maltodextrin is traditionally produced from cassava starch and consists of D-glucose units linked in chains of variable lengths which can be metabolized by *K. oxytoca* M5a1. Fed-batch fermentation with maltodextrin feeding was also performed. The result of this study showed that, 230.14±2.49g/L of maltodextrin were consumed and the highest 2,3-BD production reached 93.21±0.71 g/L at 126 h of cultivation time. The 2,3-BD yield and productivity were obtained at 0.41±0.01 g/g sugar and 0.95±0.04 g/L.h, respectively (Table 4.8 and Figure 4.13). The final cell density of fed-batch fermentation (5.21±0.38 g/L) and specific growth rate (0.12±0.01 h^{-1}) were higher than those of fed-batch using glucose (4.32±0.10 g/L and 0.10±0.07 h^{-1}) fermentation. Furthermore, succinate (1.04±0.24 g/L), acetate (1.96±1.94 g/L) and ethanol (0.45±0.10 g/L) were accumulated at very low levels in the fermentation broth throughout the fermentation process. Lactate was not detected. Meanwhile, ethanol was steadily produced until 42 h of cultivation, after which its concentration was slightly decreased at 126 h (Figure 4.13). Similarly, Jung *et al.* (2013) reported that 11.10 g/L ethanol was steadily produced until 10 h incubation. At 36 h, ethanol concentration was slightly decreased to 3.51 g/L. As a primary metabolite, ethanol was produced during the logarithmic growth phase and then was evaporated by relatively high temperature of fermentation (37°C). In addition, the fed-batch fermentation of KMS005-76T with maltodextrin was as efficient as that with glucose. This high maltodextrin concentration did not affect cell growth and metabolism of this strain.

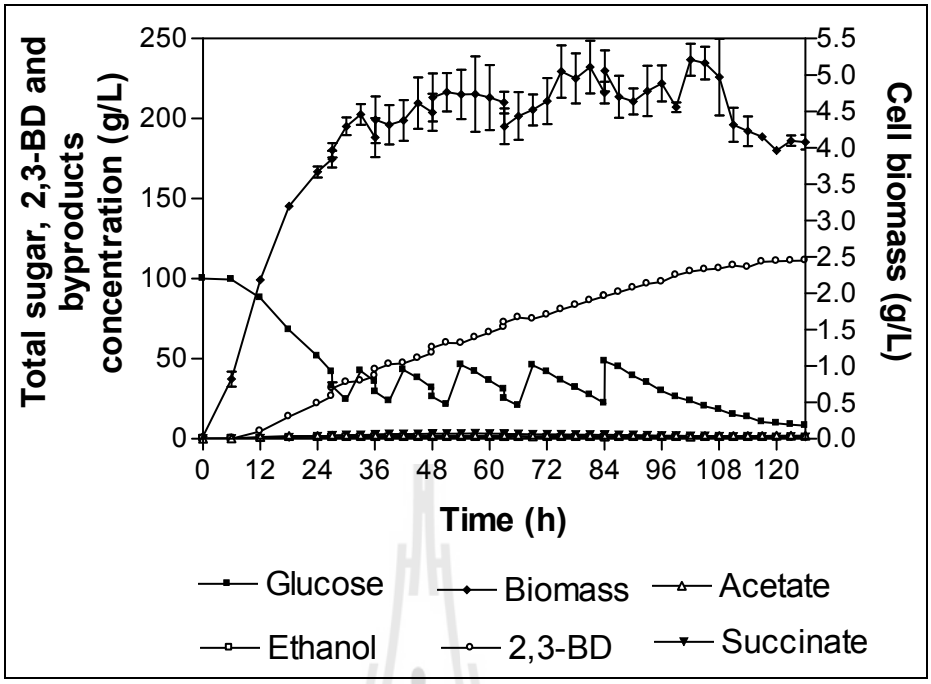

Figure 4.13 Fed-batch fermentation profiles using initial 100 g/L maltodextrin of *K. oxytoca* KMS005-76T in AM1 medium in 2L bioreactor. The symbols and error bar are average values and standard deviations of at least three measurements, respectively.

Wang *et al.* (2012) also reported that fed-batch fermentations for the production of 2,3-BD with *E. cloacae* subsp. *dissolvens* SDM. The experiments were carried out in 5L bioreactor with cassava powder. The liquefied cassava powder 500 g/L was added when residual glucose concentration was reduced to 30 g/L. The 2,3-BD and acetoin concentrations were 93.9 g/L and 5.3 g/L after 47 h in which the yield and titer similar to those produced by KMS005-76T were obtained. Although, cassava powder corresponds to a theoretical yield of 94% from glucose on the calculation of 1.0 g starch producing 1.1 g glucose. In addition, cassava consists of toxic chemical such as cyanogen, which may inhibit the metabolism of bacteria. This may be a

reason that the yield of 2,3-BD from cassava powder was lower than that of 2,3-BD from glucose (Wang *et al.,* 2012).

In conclusion, fed-batch fermentation can greatly enhance 2,3-BD production and has advantages over batch process. This process permitted effective control of residual glucose concentration level. In this study, maltodextrin was used as a carbon source for 2.3-BD production with metabolically engineered *K. oxytoca* KMS005-76T. The maximum concentration of 2,3-BD reached 93.21±0.71 g/L with the small amount of by-products under microaerobic conditions (Table 4.8). This method was proved to be an effective method for efficient 2,3-BD production. Furthermore, it would be feasible for industrial 2.3-BD production employed in the future and could be applied for other similar fermentation processes.

4.7 Comparison of *K. oxytoca* KMS005-76T and KMS006 for 2,3-BD production

The ultimate goal of developing new strains by means of metabolic engineering that new strain would not be contained foreign genes or parts of genes from other organisms that inserted into the genome of the new mutant strain. Therefore, in this study KMS005-76T (Δ*adhE,* Δ*pta-ackA,* Δ*ldhA'-cat-sacB-ldhA"*) strain with foreign genes (*cat-sac*B) within the genome was further developed by removing foreign genes out from KMS005-76T genome. As a result, causing generated new strain KMS006 (Δ*adhE* Δ*ackA-pta* Δ*ldhA'-ldhA"*). Therefore, batch fermentation of *K. oxytoca* KMS005-76T and KMS006 was performed at a 50 g/L glucose concentration in 2L bioreactor as shown in Table 4.9 and Figure 4.13. The fermentation profile of both strains showed that KMS005-76T and KMS006

produced 2,3-BD as a major fermentative product at concentration of 22.65± 0.49 g/L and 21.75±1.11 g/L respectively. In addition, KMS005-76T and KMS006 produced 2,3-BD with yields of 0.43±0.12 g/g and 0.43±0.03 g/g, respectively and productivities of 0.94±0.02 g/L.h and 0.92±0.05 g/L.h, respectively after 24h. Furthermore, the specific productivity of KMS005-76T and KMS006 was 0.33±0.01 g/g.h and 0.32±0.02 g/g.h (Table 4.9, Figure 4.13). KMS005-76T and KMS006 attained high 2,3-BD yield and productivity under microaeration conditions. No lactate was detected in the fermentation broth (Table 4.9). This result confirmed that LDHA activity was successfully abolished from KMS005-76T and KMS006. However, both strains showed that the levels of acetate (1.50±0.52 g/L and 2.02±0.46 g/L), succinate (0.94±0.38 g/L and 0.85±0.58 g/L) and ethanol (1.28±0.49 g/L and 1.00±0.38 g/L) were not significant different under microaerobic conditions (Table 4.9). Moreover, the growth rate of KMS005-76T and KMS006 (μ= 0.13 h^{-1}) were not significant different (Table 4.9). In conclusion, both strains, KMS005-76T and KMS006 were very slightly different in genotype but similar phenotypic expression.

Table 4.9 Kinetic parameters of *K. oxytoca* KMS005-76T and KMS006 in AM1 medium containing 5% (w/v) glucose in 2L bioreactor.

Strains	**KMS005-76T[g]**	**KMS006[g]**
CDW (g/L)	2.97±0.02[e,€]	2.83±0.09[€]
μ (h^{-1})[a]	0.13±0.01[€]	0.13±0.01[€]
Glucose consumption (g/L)	50.84±0.74[€]	49.32±3.59[€]
2,3-BD concentration (g/L)	22.65±0.49[€]	21.75±1.11[€]
2,3-BD yield (g/g)[b]	0.43±0.12[€]	0.43±0.03[€]
2,3-BD productivity (g/L.h)[c]	0.94±0.02[€]	0.92±0.05[£]
2,3-BD specific productivity(g/g.h)[d]	0.33±0.01[€]	0.32±0.02[€]
Succinate (g/L)	0.94±0.38[€]	0.85±0.58[£]
Lactate (g/L)	ND[c]	ND[f]
Formate (g/L)	ND	ND
Acetate (g/L)	1.50±0.52[€]	2.02±0.46[£]
Ethanol (g/L)	1.28±0.49[€]	0.99±0.38[£]
Incubation time (h)	24	24

[a] The specific growth rate was calculated by slope of cell dry weigh concentration curve.

[b]The 2,3butanediol yield was calculated as grams of 2,3-BD produced divided by grams of the sugar consumed.

[c]The 2,3butanediol productivity was calculated as 2,3-BD concentration produced divided by overall incubation time.

[d]The 2,3-BD specific productivity was calculated as 2,3-BD productivity divided by cell dry weight.

[e]All data represent the averages of three fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P<0.05$) between columns.

[f] ND, not detected.

[g] Fermentation in AM1 medium containing 5% (w/v) glucose, pH 6.0, air sparged 1.0 vvm in 2L bioreactor.

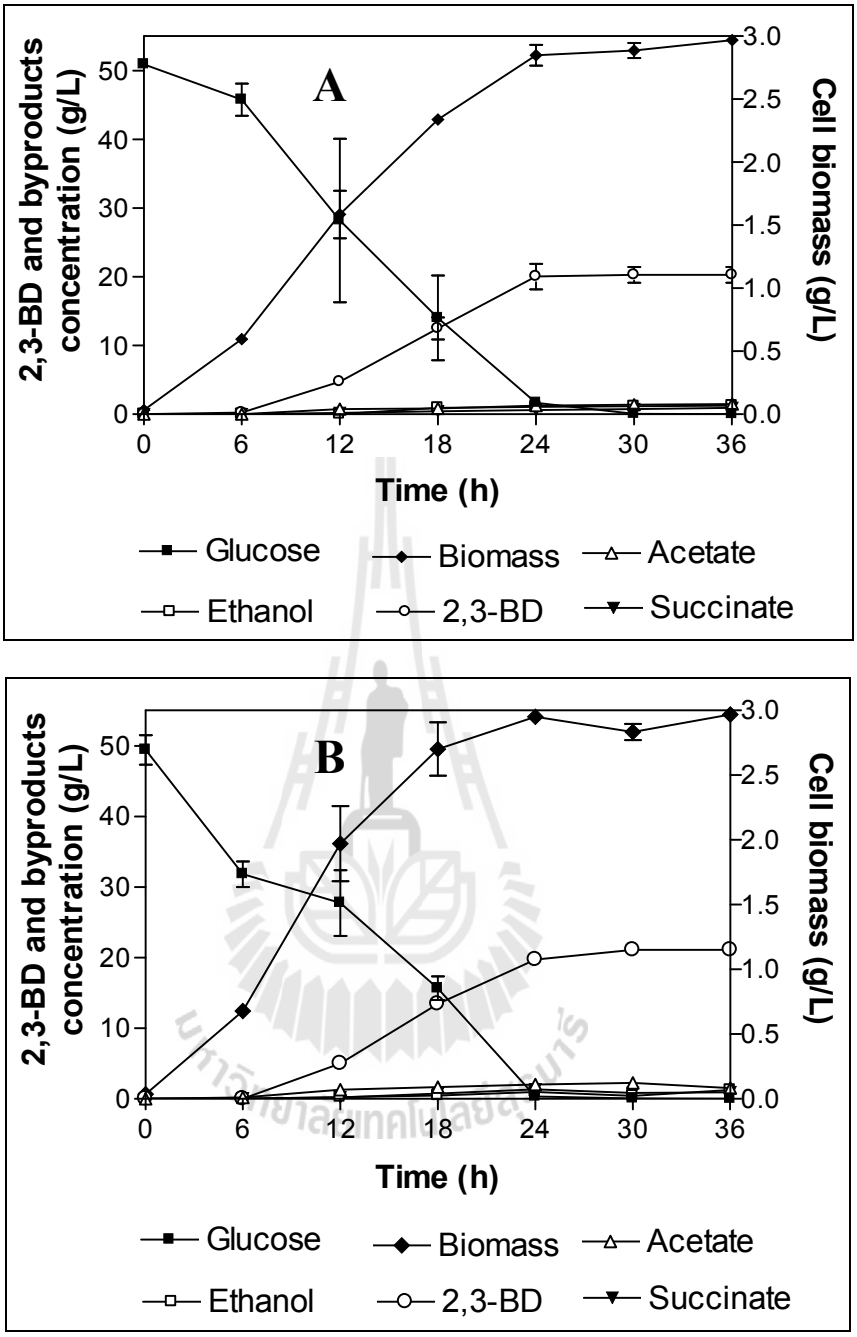

Figure 4.14 Comparison of *K. oxytoca* KMS005-76T and KMS006 produced 2,3-BD in glucose concentration 50 g/L in AM1 medium in 2L bioreactor. (A) *K. oxytoca* KMS005-76T (B) *K. oxytoca* KMS006. The symbols and error bar are average values and standard deviations of at least three measurements, respectively.

CHAPTER V

CONCLUSION

2,3-BD obtained by biotechnological process is preferred for industrial applications especially, fuel plasticizers and pharmaceutical agents. *K. oxytoca* are robust organisms for 2,3-BD fermentation.

In this study, *ldhA* (lactate dehydrogenase A) gene was additionally eliminated from chromosomal DNA of the metabolically engineered *K. oxytoca* KMS004 (Δ*adhE* Δ*ackA-pta*) (Sangproo *et al.,* 2012) and generated as *K. oxytoca* KMS005 (Δ*adhE* Δ*ackA-pta* Δ*ldhA'-cat-sacB-ldhA''*). KMS005 was engineered strain to achieve high production of 2,3-BD comparing with the theoretical yield (0.5 g/g). This strain was further improved growth, cell biomass and 2,3-BD production by metabolic evolution. The clone passing through metabolic evolution process was selected and designated as *K. oxytoca* KMS005-76T.

KMS005-76T demonstrated 2,3-BD production as a pathway for regulation of the intracellular NADH/NAD^+ ratio, carbon storage and energy for microbial growth. The growth and 2,3-BD production by KMS005-76T were carried out in the simple mineral salts medium AM1 containing glucose and other cheap carbon substrates such as cassava starch, sugarcane molasses and maltodextrin under simple-batch and fed-batch microaerobic conditions. KMS005-76T produced 2,3-BD concentration at 23 g/L with the highest yield 0.46 g/g in the medium containing 50 g/L glucose in shake flask. The use of inexpensive medium is essential for the economic production of bio-

base 2,3-BD production. It was also found that KMS005-76T produced 19.21 g/L of 2,3-BD with the yield of 0.42 g/g total sugars consumed from sugarcane molasses. Moreover, KMS005-76T showed a high ability to produce 2,3-BD from hydrolyzed cassava starch at 19 g/L with the yield of 0.39 g/g total sugars consumed. This strain also utilized maltodextrin to produce 2,3-BD at a concentration of around 9 g/L with the yield of 0.40 g/g total sugars consumed in shake flask. However, results from the fermentation of glucose was found to be effective in the production of 2,3-BD higher when compared with other carbon sources.

Among the metabolic enzymes involved under anaerobic fermentation, KMS005-76T possessed lower specific activities of lactate dehydrogenase (LDHA), alcohol dehydrogenase (ADHE) and malate dehydrogenase (MDH) but higher in specific activity of 2,3-butandiol dehydrogenase (BUDC) than those of wild type and KMS004. Therefore KMS005-76T, exhibited a significantly improvement in 2,3-BD production with low level of by-products (Table 4.5). Further, NADH from glycolysis is regenerated by BUDC activity in a reversible reaction from acetoin to 2,3-BD. Thus, pyruvate is likely to be available for 2,3-BD conversion. Moreover, it was found that KMS005-76T produced high 2,3-BD concentration at 20 g/L with productivity of 0.67 g/L.h from 50 g/L glucose under aeration conditions. In addition, fermentation of 200 g/L glucose concentration could be the maximal substrate concentration for 2,3-BD production in batch process, which resulted in 86 g/L with productivity of 1.18 g/L.h in 2 L bioreactor.

Considering the conventional batch processes for 2,3-BD production commonly suffered from reduced cell growth and productivity due to substrate inhibition. Fed-batch fermentation for 2,3-BD production by KMS005-76T provided

a better advantage over batch fermentation in term of alleviated substrate inhibition effect. The substrate concentration was maintained at optimum level of 15-40 g/L during cultivation. The result showed that KMS005-76T produced 2,3-BD concentration at 117 g/L with the highest productivity of 1.20 g/L.h from initial 100 g/L glucose concentration. Furthermore, the production of 2,3-BD using maltodextrin in fed-batch fermentation by KMS005-76T produced 93 g/L with the productivity of 0.41 g/L.h.

KMS005-76T (Δ*adhE* Δ*ackA-pta* Δ*ldhA'-cat-sacB-ldhA"*) strain could be a potential strain to produce high titers and yields of 2,3-BD in low-cost medium, using abundant and feasible renewable substrates such as sugarcane molasses, hydrolyzed cassava starch and maltodextrin. Fewer by-products were observed in this strain. However, other genes involved in NADH re-oxidation fermentation from KMS005-76T strains should be further deleted to direct more carbon flux towards 2,3-BD. Moreover, Li *et al.* (2010) suggested that the deletions of *ldhA*, *pta*, *adhE*, and *poxB* which functioned for the mixed acid fermentation pathways could considerably improve 2,3-BD production yield and productivity in *E. coli* JM109. Jung *et al.* (2013) reported that the deletion of sucrose regulator (*ScrR*) gene from the genome of engineered *E. aerogenes* KCTC 2190 (Δ*ldhA*) could also improve 2,3-BD production. Thus, the knockout of *scrR* gene was expected to improve sucrose or sugarcane molasses utilization. Therefore, further recommendation works for genetic manipulation including deletions of *poxB*, *tdcD* and *scrR* should be performed in KMS005-76T for improving 2,3-BD production. In conclusion, KMS005-76T would be an alternative strain for the development of 2,3-BD production from renewable substrates and fulfills the requirements for industrial application.

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APPENDICES

APPENDIX A

NUCLEOTIDE SEQUENCES

1. Sequence of plasmid pKJ1008 used for deletion of *ldhA* gene

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCT
GGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGA
GTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGT
TGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATT
ACGCCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAA
TTCGC*GCCGCCGCTATCGGCGCCATCTGTATCGTATCCGCTGGCTCAATGCCGAGCG*
CCTCGCTCGCGGCCAGCTGTTTCTGTGCCGCCACGGCGCGTGGGCGCTCTTTTTTAG
CCGCTTTCTTTCTCCGCTGCGCGCCACCGTGCCGCTGGTAACCGGCGCCAGCGGCAC
CTCTTTCTGGCATTTTCAGCTCGCCAACGTCAGCTCCGGGCTGCTCTGGCCGCTGAT
CCTGCTGGCGCCAGGGGCGTTAAGCCTCAGCTTTTGATGAAAGGTATTGTCTTTTAA
AGAGATTTCTTAACACCGCGATATGCTCTAGAATTATTACTATAACCTGCTGATTAA
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TATCTGCAGCATGTTAATGATGCATATGGCTTTGAACTGGAATTTTTTGATTTCCTG
*CTAACCGAAAAGACCG***GTGAGTTTCACCAGTTTTGATTTAAACGTGGCCAATATGGA**
CAACTTCTTCGCCCCCGTTTTCACCATGGGCAAATATTATACGCAAGGCGACAAGGT
GCTGATGCCGCTGGCGATTCAGGTTCATCATGCCGTTTGTGATGGCTTCCATGTCGG
CAGAATGCTTAATGAATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGTAATT
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Note: Normal letters are pCR2.1-TOPO-based vector, italic letters are parts of upstream sequences of *ldhA*(*ldhÁ*) gene (incomplete gene), bold letters are *cat-sacB*gene, underline letters are parts of downstream sequences of *ldhA*(*ldhÁ̋*) gene(incomplete gene.

2. Sequence of plasmid pKJ1009 used for deletion of *ldhA* gene

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GAGTCAGTGAGCGAGGAAGCGGAAG

Note: Normal letters are pCR2.1-TOPO-based vector, italic letters are parts of upstream sequences of *ldhA*(*ldhÁ*) gene (incomplete gene), underlineletters are parts of downstream sequences of *ldhA*(*ldhÁ*) gene(incomplete gene).

APPENDIX B

STANDARD CALIBRATION CURVE

1. NADH Standard calibration curve

The standard curve of NADH was prepared by dissolving NADH in distilled water and diluted to obtain various concentrations (Table 1B.) and measured the OD at 340 nm. Standard curve was made by plotting between different concentrations of standard NADH against their absorbance values as shown as in Figure 1B.

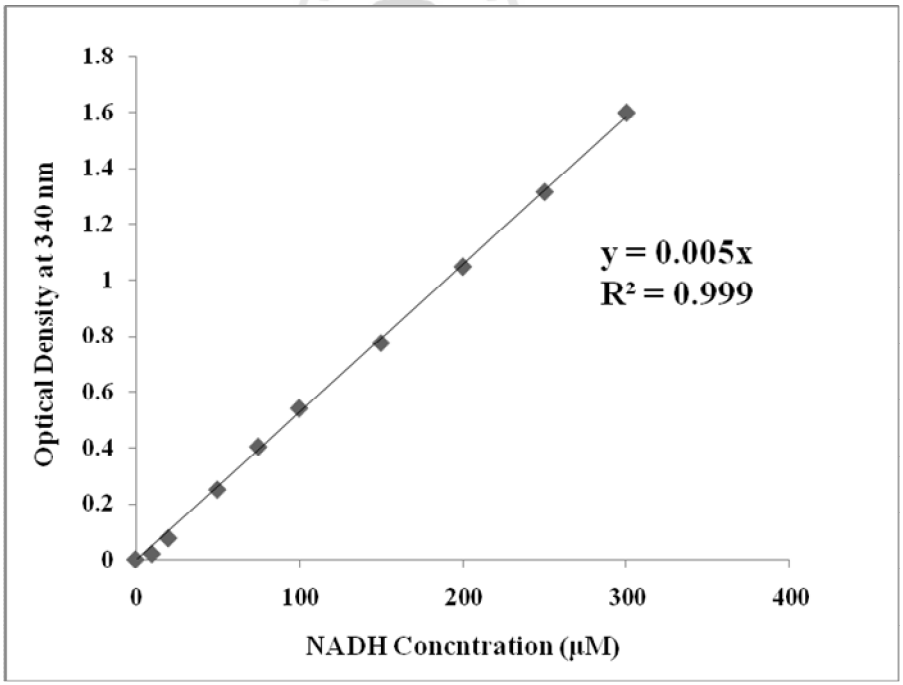

Figure 6.1 Correlation lines between optical density (OD_{340}) and NADH concentration.

Table 6.1 The correlation between final concentration of standard NADH and their absorbance value at 340 nm.

NADH concentration (μM)	**OD_{340} (Average)**
0	0.000
10	0.020
20	0.076
50	0.251
75	0.406
100	0.541
150	0.775
200	1.049
250	1.346
300	1.600

2. Total protein Standard calibration curve

The standard curve of total protein was prepared by dissolving BSA in distilled water and diluted to obtain various concentrations (Table 2B) and measured the OD at 600 nm. Standard curve was made by plotting between different concentrations of standard BSA against their absorbance values as shown as in Figure 2B.

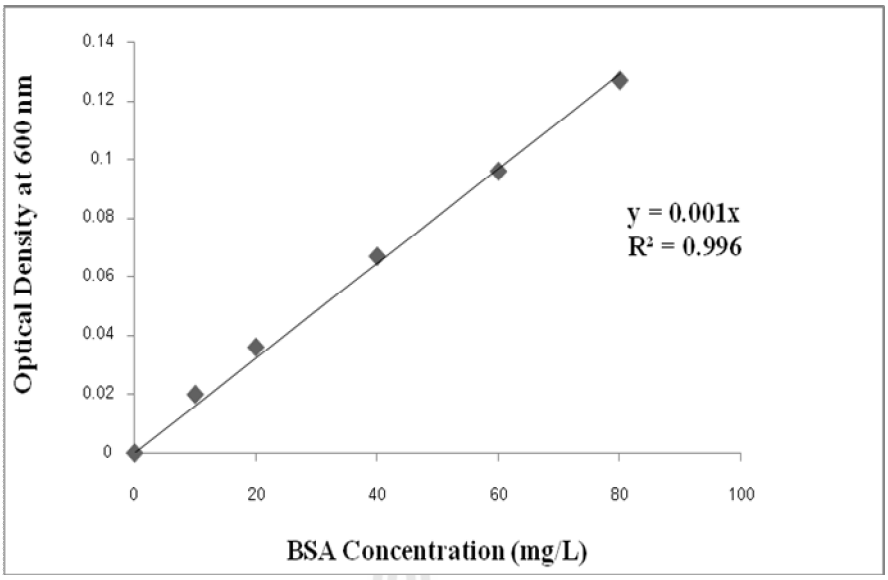

Figure 6.2 Correlation lines between optical density (OD_{600}) and BSA concentration.

Table 6.2 The correlation between final concentration of standard BSA and their absorbance value at 600 nm.

BSA concentration (mg/L)	**OD_{600} (Average)**
0	0.000
10	0.020
20	0.036
40	0.067
60	0.096
80	0.127

3. Glucose Standard calibration curve for Dextrose Equipvalent test

The standard curve of glucose was prepared by dissolving glucose in distilled water and diluted to obtain various concentrations (Table 3B). Take the sample take each sample for 0.5 mL into test tube then add Dinitrosalicylic (DNSA) solution 0.5 mL (raio 1:1) gently mixed. Take the reaction mixed into water bath 100°C for 15 min then cool down in cool water immediately, add DI water 4 mL into reaction mixed then gently mixed. Take the sample for measurement at OD 540 nm and standard curve was made by plotting between different concentrations of standard glucose against their absorbance values as shown as in figure 3B.

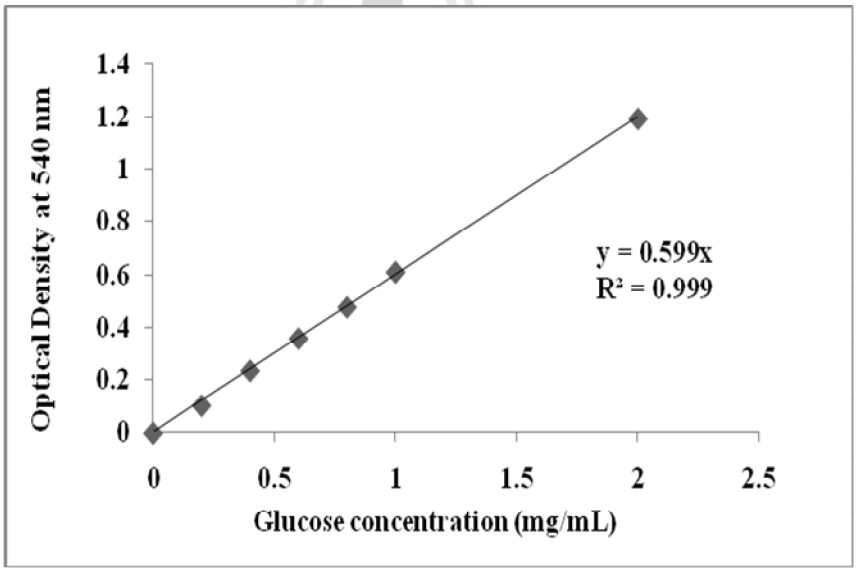

Figure 6.3 Standard calibration curve of sugar concentration by measurement density using a spectrophotometer at 540 nm by DNSA method.

Table 6.3 The correlation between concentration of standard glucose and their absorbance value at 540 nm.

Glucose concentration (mg/mL)	**OD_{540} (Average)**
0	0.000
0.2	0.103
0.4	0.236
0.6	0.359
0.8	0.479
1.0	0.612
2.0	1.195

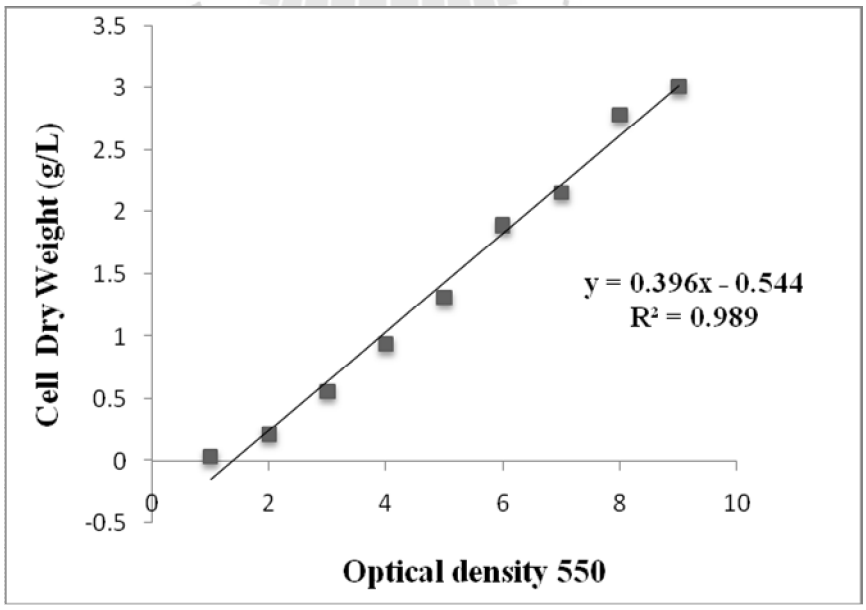

Figure 6.4 Correlation lines between optical density (OD_{550}) and biomass concentration (dry cell weight).

BIOGRAPHY

Mr. Pattharasedthi Pholyiam was born in Roi-Et, Thailand. In 1997, he studied in Medical Technology, Faculty of Associated Medical Science, Khonkaen University, Khonkaen. He graduated with bachelor degree of Science in Medical Technology in 2000. After graduation, he had an experience on medical laboratory work for two years at Department of Clinical pathology, Weerawatyothin Military Hospital, Surin. For eight years at Department of Clinical pathology, Maharat Nakhorn Ratchasima Hospital, Nakorn Ratchasima. In 2010, he decided to further study for master degree in the field of Bioprocess engineering at school of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakorn Ratchasima, Thailand. His research topic was entitled of "Re-engineering of metabolic pathway of *Klebsiella oxytoca* for 2,3-butanediol production". After he graduates from Suranaree University of Technology, he plans as a lecturer at Clinical hematology Unit, Department of Clinical microscopy, Faculty of Medical Technology, Nakhorn Ratchsima College, Nakhorn Ratchsima Province, Thailand.