PRODUCTION OF D-(-)-LACTIC ACID BY METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* M5a1 IN MINIMAL SALTS MEDIUM

Maytawadee Sangproo

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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การผลิตกรดแลคติกชนิดดี(-) โดยเชื้อ *Klebsiella oxytoca* M5a1 ที่ได้ผ่านการดัดแปลงวิถีการสร้างและสลายในอาหารเลี้ยงเชื้ออย่างง่าย

นางสาวเมธาวดี แสงพรู

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2555

PRODUCTION OF D-(-)-LACTIC ACID BY METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* M5a1 IN MINIMAL SALTS MEDIUM

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

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เมธาวดี แสงพรู : การผลิตกรดแลคติกชนิดดี(-) โดยเชื้อ *KLEBSIELLA OXYTOCA* M5a1 ที่ได้ผ่านการดัดแปลงวิถีการสล้างและสลายในอาหารเลี้ยงเชื้ออย่างง่าย (PRODUCTION OF D-(-)-LACTIC ACID BY METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* M5a1 IN MINIMAL SALTS MEDIUM) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.เขมวิทย์ จันต๊ะมา, 122 หน้า.

เชื้อสายพันธุ์ *Klebsiella oxytoca* M5a1 ถูกนำมาใช้เป็นสายพันธุ์ตั้งต้น เพื่อผลิตกรดแลคติกชนิดดี(-) ในอาหารเลี้ยงเชื้ออย่างง่ายจากการหมักแบบกะ และควบคุมความเป็นกรดด่าง ยีน alcohol dehydrogenase (*adhE*) และ phospho transacetylase-acetate kinase A (*pta-ackA*) ถูกตัดออกจากเชื้อสายพันธุ์ตั้งต้น มิวแตนท์ที่ได้ถูกตั้งชื่อว่า KMS002 (Δ*adhE*) และ KMS004 (Δ*adhE* Δ*pta-ackA*) โดยพบว่าเชื้อสองสายพันธุ์มีการสร้างกรดแลคติกชนิดดี(-) เป็นวิถีหลักเพื่อรีเจนเนอเรชั่น NADH เชื้อมิวแตนท์ทั้งสองสายพันธุ์สามารถผลิตกรดดีแลคติกชนิดดี(-) ประมาณ 11-13 กรัมต่อลิตร ในอาหารเลี้ยงเชื้อที่มีน้ำตาลกลูโคสที่ความเข้มข้น 20 กรัมต่อลิตร เทียบเป็นผลได้ประมาณ 0.64-0.71 กรัมของกรดแลคติกชนิดดี(-) ต่อกรัมของกลูโคสที่ถูกใช้ ในอาหารเลี้ยงเชื้อที่มีกากน้ำตาลอ้อยเชื้อสายพันธุ์ KMS002 และ KMS004 สามารถผลิตกรดแลคติกชนิดดี(-) ได้ 22-24 กรัมต่อลิตรเทียบเป็นผลได้ประมาณ 0.80-0.87 กรัมของกรดแลคติกชนิดดี(-) ต่อกรัมของกากน้ำตาลอ้อยที่ถูกใช้ นอกจากนี้เชื้อทั้งสองสายพันธุ์ยังสามารถใช้มอลโตเด็กซ์ตริน ที่ได้จากการย่อยแป้งมันสำปะหลัง และผลิตกรดแลคติกชนิดดี(-) ได้ 33-34 กรัมต่อลิตร เทียบเป็นผลได้ประมาณ 0.91-0.92 กรัมของกรดแลคติกชนิดดี(-) ต่อกรัมของน้ำตาลมอลโทสที่ถูกใช้โดยเซลล์ อุณภูมิที่เหมาะสมในการผลิตกรดแลคติกชนิดดี(-) คือ 40 องศาเซลเซียส สามารถผลิตกรดแลคติกชนิดดี(-) ได้ 37.28 ± 0.22 กรัมต่อลิตร เทียบเป็นอัตราผลผลิตสูงสุดประมาณ 0.38 กรัมต่อลิตรต่อชั่วโมง ภายใต้สภาวะไร้ออกซิเจนในขวดหมักขนาดปริมาตร 500 มิลลิลิตร ภายในเวลา 72 ชั่วโมง สำหรับผลกระทบของค่าความเป็นกรดด่างระหว่างการหมักพบว่า กรดแลคติกชนิดดี(-) สามารถผลิตได้สูงที่สุดเมื่อควบคุมค่าความเป็นกรดด่างที่ 6.5 นอกจากนี้ยังพบว่าค่าความขุ่นของเชื้อวัดที่ความยาวคลื่นแสง 550 นาโนเมตรเท่ากับ 0.5 มีความเหมาะสมในการผลิตกรดแลคติกชนิดดี(-) และมีอัตราผลผลิตประมาณ 0.50 กรัมต่อลิตรต่อชั่วโมง จากผลที่กล่าวมาข้างต้น เชื้อสายพันธุ์ KMS002 และ KMS004 จึงเป็นอีกหนึ่งทางเลือกที่มีความเป็นไปได้ในการนำไปใช้ผลิตกรดแลคติกชนิดดี(-) ในระดับอุตสาหกรรม

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา_____

MAYTAWADEE SANGPROO : PRODUCTION OF D-(-)-LACTIC ACID BY METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* M5a1 IN MINIMAL SALTS MEDIUM. THESIS ADVISOR : ASST. PROF. KAEMWICH JANTAMA, Ph.D., 122 PP.

D-(-)-LACTIC ACID/*KLEBSIELLA OXYTOCA* M5a1/ANAEROBIC CONDITION/ METABOLIC ENGINEERING

Klebsiella oxytoca M5a1 wild type strain was constructed to produce optically pure D-(-)-lactic acid by applying pH-controlled batch fermentation in mineral salts medium. The alcohol dehydrogenase gene, *adhE* and phospho transacetylase-acetate kinase A gene, *pta-ackA* were deleted from the wild type. The mutant strains were named as KMS002 (Δ*adhE*) and KMS004 (Δ*adhE* Δ*pta-ackA*), respectively. They exhibited D-(-)-lactic acid production as a primary pathway for the regeneration of NADH. Both strains produced D-(-)-lactic acid at concentrations of 11-13 g/L in the medium containing 20 g/L glucose with yields of 0.64-0.71 g/g glucose used. In sugarcane molasses, KMS002 and KMS004 produced D-(-)-lactic acid at concentrations of 22-24 g/L with yields of 0.80-0.87 g/g total sugars utilized. Both strains also utilized maltodextrin derived from cassava starch and produced D-(-)-lactic acid at concentrations of 33-34 g/L with yields of 0.91-0.92 g/g maltodextrin utilized. The optimal temperature at 40°C could produce D-(-)-lactic acid at concentrations of 36.28 ± 0.22 g/L with productivity of 0.38 g/L/h in 500 ml small anaerobic bottle within 72 h. For the effect of pH during fermentation, it was found that the highest D-(-)-lactic acid production was obtained with controlled pH at 6.5. In addition, the optimal initial cell concentration at OD_{550} on D-(-)-lactic

production was found to be suitable at 0.5, of which productivity of 0.50 g/L/h was obtained. These results demonstrated that KMS002 and KMS004 would be another feasible choice for D-(-)-lactic acid production in an industrial scale.

School of Biotechnology Student's Signature_____

Academic Year 2012 Advisor's Signature_____

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Maytawadee Sangproo

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LIST OF ABBREVIATIONS

ACKA	=	Acetate kinase
ADHE	=	Alcohol dehydrogenase
ATP	=	Adenosine 5'-tri-phosphate
bps	=	Base pairs
BUDB	=	2,3 butanediol dehydrogenase
°C	=	Degree Celsius
cAMP	=	Cyclic adenosine monophosphate
CFU	=	Colony form unit
FDH	=	Formate dehydrogenase
FRDABCD	=	Fumarate reductase
g	=	Gram (s)
g/L	=	Gram (s) per Liter
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
h	=	Hour (s)
HPLC	=	High performance liquid chromatography
K. oxytoca M5a1	=	*Klebsiella oxytaca* M5a1
L	=	Liter (s)
LB	=	Luria Bertain
LDHA	=	Lactate dehydrogenase

LIST OF ABBREVIATIONS (Continued)

M	=	Molar
MAL	=	Maltose/maltodextrin-utilizing system
mM	=	Milli-molar
mg	=	Milligram (s)
mg/L	=	Milligram (s) per Liter
min	=	Minute (s)
mL	=	Millilitre (s)
mm	=	Millimetre (s)
MW	=	Molecular weight
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
PTA	=	Phosphotransacetylase
rpm	=	Revolution per minute
SCRB	=	Sucrose-6-phosphate hydrolase
TBE	=	Tris-borate-EDTA

LIST OF ABBREVIATIONS (Continued)

TDCD	=	Propionate kinase
UV	=	Ultraviolet
V	=	Volt
v/v	=	Volume per volume
w/v	=	Weight per volume
µL	=	Micro-liter

CHAPTER I

INTRODUCTION

1.1 Overview

Technology for producing fuels and plastics from inexpensive, renewable, and abundantly available biomass resources is currently being developed in order to prevent the exhaustion of oil reveres and reduce global warming (Ohara *et al*., 2007). Lactic acid has a long history of use as a special chemical in food, pharmaceutical, cosmetic and chemical industries. Recently, polylactic acid (PLA) is considered as an important raw material for bioplastics as it can be produced from biomass. Stereocomplex PLA, which is composed of both poly L-(+) and D-(-)-lactic acid, made D-(-)-lactic acid more attractive by the improvement of its biodegradable plastic properties (Lu *et al*., 2009). As a result, the worldwide market for D-(-)-lactic acid will be increasing rapidly in the near future.

D-(-)-Lactic acid has been reported to be produced by several species of lactic acid bacteria, particularly *Lactobacillus delbrueckii* (Calabia *et al*., 2007; Manome *et al*., 1998; Tanaka *et al*., 2006), *Lactobacillus coryniformis* subsp. *torquens* (Manome *et al*., 1998; Yàñez *et al*., 2003), *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc carnosum*, *Leuconostoc fallax* (Manome *et al*., 1998), *Lactobacillus bulgaricus* (Benthin and Villadsen, 1995). However, under normal fermentation conditions, the main product of metabolism is D-(-)-lactic acid, while other products are formed as by-products, such as acetic acid, acetaldehyde, ethanol, and diacetyl. Hence the biosynthetic capacity of these

fermentive microorganisms is very limited and their physiology are relatively simple, these are important factors which make these organisms suitable objects for metabolic engineering. Over the past few decades numerous attempts have been made to change metabolite production from these bacteria, via metabolic engineering. It has been used to improve the production of existing metabolites, hence enabling the production of new metabolites. The design of rational approaches to metabolic engineering requires a proper understanding of the pathways that are use to manipulate their fluxes, control factors, and the genes involved as impart from other organic compounds to production of D-(-)-lactic acid. The varieties of genetic approaches have been used to engineer many bacterial strains to produce D-(-)-lactic acid.

Klebsiella oxytoca M5a1 has many advantageous characteristics as a production host. It dose not requirements any special or expensive source of nutrients during growth and has available techniques for genetic manipulations. Moreover, *K. oxytoca* M5a1 has an ability to utilize broader range of carbon substrates than these of *E. coli*. It would be a new target of microorganism to be engineered into metabolic pathway to produce D-(-)-lactic acid. This research focused on metabolic engineering of *K. oxytoca* M5a1 to produce optically pure D-(-)-lactic acid.

1.2 Scope of this study

The strategy of this study used in most instances was to remove *adhE* (alcohol dehydrogenase E), *pta* (phosphotransacetylase), and *ackA* (acetate kinase A). These were eliminated from chromosomal DNA of the parental host strain. The carbon flux through pyruvate route rather than through acetyl-CoA during anaerobic fermentation was expected in the *K. oxytoca* mutant strains, channeling the pyruvate through lactic

acid. In this thesis, I describe novel strains of *K. oxytoca* M5a1 that produce D-lactic acid at high titers and yields using different carbon sources in mineral salts medium during simple, pH-controlled, using cheap carbon substrates and batch fermentations without the addition of heterologous genes or plasmids. Therefore, the fermentative production of lactic acid with *K. oxytoca* would be one of the choices that are more feasible for the industrial scale.

1.3 Research objectives

1. To study the effect of *adhE* and *pta-ackA* genes deletion to D-(-)-lactate production under anaerobic conditions in *Klebsiella oxytoca* M5a1 comparing with the wild type strain.

2. To study the production rate and yield of D-(-)-lactate of the *K. oxytoca* mutant strains under anaerobic conditions in the mineral salts medium (AM1).

3. To study the effect of pH, temperature and inoculum size to the production of D-(-)-lactic acid under anaerobic conditions.

CHAPTER II

REVIEW OF THE LITERATURES

2.1 Lactic acid

Lactic acid has attracted a significant amount of attention as a chemical with many potential applications. There are four major categories for the current uses and applications of lactic acid: food, cosmetic, pharmaceutical, and chemical application (Fig. 2.1). Since lactic acid is classified as GRAS (generally recognized as safe) for use as a food additive by the USFDA (Food and Drug Administration) (Datta *et al*., 1995), it is widely used in almost every segment of the food industry, where it serves in a wide range of functions, such as flavouring, pH regulation, improved microbial quality, and mineral fortification. Moreover, lactic acid is used commercially in the processed meat and poultry industries, to provide products with an increased shelf life, enhanced flavour, and better control of food-born pathogens. Due to the mild acidic taste of lactic acid, it is also used as an acidulant in salads and dressings, baked goods, pickled vegetables, and beverages. Lactic acid is used in confectionery, not only for flavour, but also to bring the pH of the cooked mix to the correct point for setting. The advantages of adding lactic acid in confectionery include its low inversion rate, ease of handling, and ability to produce clear candies. Another potential application of lactic acid in the food industry is the mineral fortification of food products (Benninga, 1990).

Lactic acid offers natural ingredients for cosmetic applications. Although primarily used as moisturizers and pH regulators, they possess multiple other

properties such as antimicrobial activity, skin lightening, and skin hydration. The moisturizing effect is related directly to lactate's water retaining capacity, and the skin-lightening action of lactic acid is produced by the suppression of the formation of tyrosinase. Since they are natural ingredients of the human body, lactic acid and its salt fit perfectly into the modern trend towards natural and safer formulations, and they produce such effects as skin lightening and rejuvenation, which makes them very useful as active ingredients in cosmetics (Wee *et al*., 2006).

Lactic acid is also used in the pharmaceutical industry as an electrolyte in many parenteral/I.V. (intravenous) solutions that are intended to replenish the bodily fluids or electrolytes. Examples include Lactated Ringer's or Hartmann's solutions, CAPD (continuous ambulatory peritoneal dialysis) solution, and dialysis solution for conventional artificial kidney machines. Moreover, lactic acid is used in a wide variety of mineral preparations, which include tablets, prostheses, surgical sutures, and controlled drug delivery systems (Datta and Henry, 2006).

Lactic acid and its salt are used increasingly in various types of chemical products and processes. In this category of applications, lactic acid functions as a descaling agent, pH regulator, neutralizer, chiral intermediate, solvent, cleaning agent, slow acid-release agent, metal complexing agent, antimicrobial agent, and humectant. Natural lactic acid has an emerging use as an excellent and safe solvent, which is alternative in many fine mechanical cleaning applications. Due to the high solvency power and solubility of lactic acid, it is an excellent remover of polymer and resins. It is available with an isomeric purity greater than 98 %, and is suitable as a starting material in the production of herbicides or pharmaceuticals. Since lactic acid offers better descaling properties than conventional organic descalers do, it is often used in

many decalcification products, such as bathroom cleaners, coffee machines, and toilets. Ethyl lactate is used in many anti-acne preparations, because it combines excellent solvency power against oils and polymeric stains, with no environmental impact and toxicological effects (Wee *et al*., 2006).

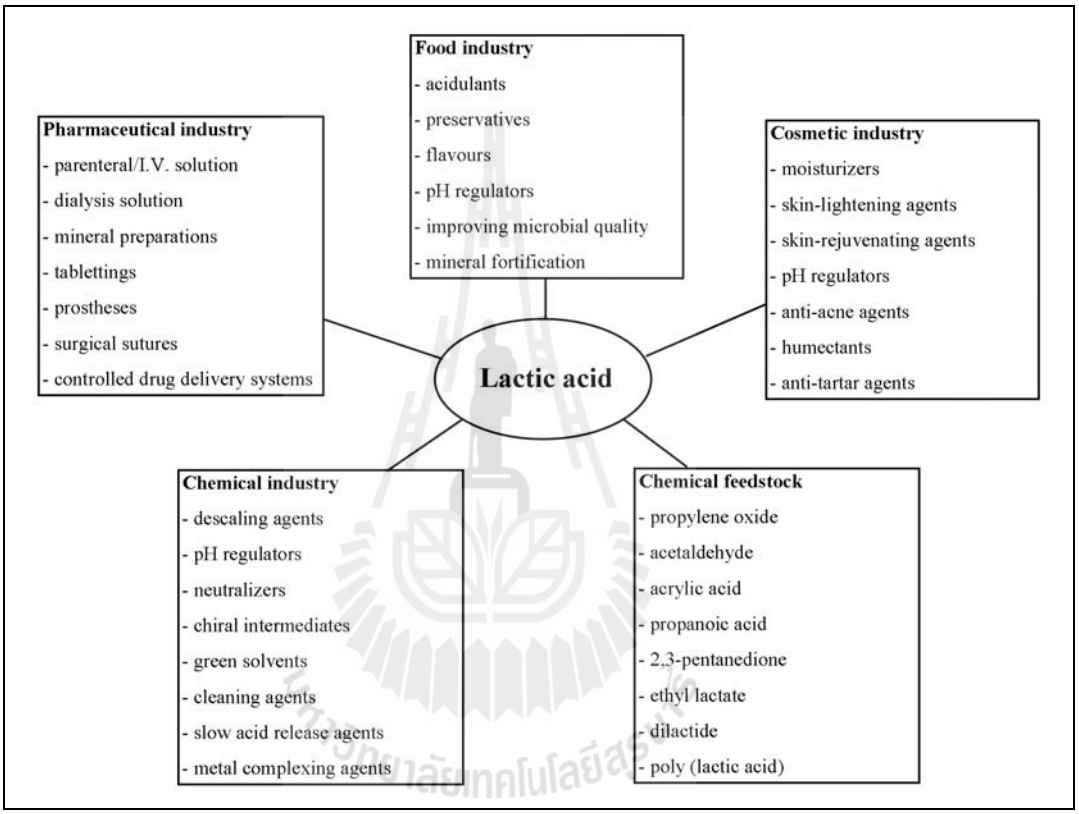

Figure 2.1 Diagram of commercial uses and applications of lactic acid and its salt. (Wee *et al*., 2006).

Currently, lactic acid is considered the most potential feedstock monomer for chemical conversions, because it contains two reactive functional groups, a carboxylic group and a hydroxyl group. Lactic acid can undergo a variety of chemical conversions into potentially useful chemicals, such as propylene oxide (*via*

hydrogenation), acetaldehyde (*via* decarboxylation), acrylic acid (*via* dehydration), propanoic acid (*via* reduction), 2,3-pentanedione (*via* condensation), and dilactide (*via* self-esterification) (Varadarajan and Miller, 1999). Lactic acid has recently received a great deal of attention as a feedstock monomer for the production of poly lactic acid (PLA), which serves as a biodegradable commodity plastic. The optically pure lactic acid can be polymerized into a high molecular mass PLA through the serial reactions of polycondensation, depolymerization, and ring-opening polymerization (Sodergard and Stolt, 2002). The resultant polymer, PLA, has numerous uses in a broad spectrum of applications, such as protective clothing, food packaging, mulch film, trash bags, rigid containers, shrink wrap, and short shelf-life trays (Drumright *et a*l., 2000 and Vink *et al*., 2003). The recent huge growth of the PLA market will stimulate future demands on lactic acid considerably (Datta *et al*., 1995).

2.1.1 Chemical structure and property

There are two optical isomer of lactic acid: L(+)-lactic and D(-)-lactic acid. D(-)-lactic acid, 2-hydroxypropionic acid, is a mirror image of L-lactic acid which could be soluble in water (Narayanan *et al*., 2004; John *et al*., 2007). It exhibits low volatility, and has chemical formula of $C_3H_6O_3$ (Fig. 2.2). The L-(+)-form diverge the D-(-)- form in its effect on polarized light. For L(+)-lactic acid, the plane is rotated in a clockwise (dextro) direction, whereas the D-form rotates the plane in a anticlockwise (laevo) direction. Since lactic acid has high reactivity due to containing both hydroxyl (-OH) and carboxyl (-COOH) groups, lactic acid can lose a proton from the acidic group, producing the lactate ion $CH_3CH(OH)COO^-$ in solution. The lactate ion could be precipitated with salts solution such as $MgCl_2$ (Benthin and Villadsen, 1995), $Ca(OH)_2$, and $CaCO_3$ (Narayanan *et al*., 2004). However, $MgCl_2$ is added to

the supernatant to neutralize the acid produced and to produce a magnesium salt of the acid which could improve purification method of lactic aid fermentation (Benthin and Villadsen, 1995).

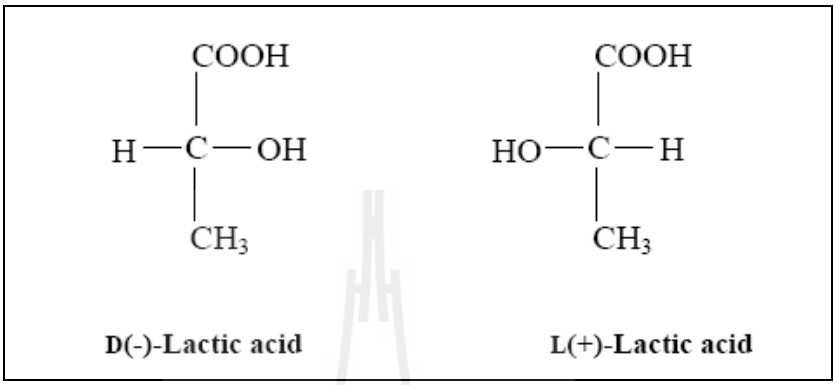

Figure 2.2 Enantiomers of lactic acid; D(-)-lactic acid and L(+)-lactic acid (Kharas *et al*., 1994).

Other properties of lactic acid and isomer of lactic acid are summarized in Table 2.1. Both enantiomers of lactic acid have the same physical properties

Table 2.1 Physical properties of lactic acid (Narayanan *et al*., 2004).

Property	**Value**
Molecular weight	0.08 g/mol
Melting point	L: 53°C
Boiling point	122°C at 14 mm Hg
Dissociation constant, K_a at 25°C	1.37×10^{4}
Acidity (pK_a)	3.85
Heat of combustion, ΔH_C	1361 KJ/mole
Specific heat, C_p at 20°C	190 J/mole/°C

2.1.2 Application of D-lactic acid

Recently, lactic acid consumption has increased considerably because of its role as a monomer in the production of biodegradable PLA, which is composed of both poly L-(+)- and D-(-)-lactic acid. The global consumption of lactic acid is expected to increase rapidly in the near future. NatureWorks LLC, a major PLA manufacturer established in the US, expects that the global PLA market may increase to 500,000 (metric) tones per year by 2012.

D-(-)-lactic acid is one of desirable monomers to be used for the production of poly D-(-)-lactic acid, PDLA, in bioplastics industry. PDLA is useful for improving thermostability of poly L-(+)-lactic acid, PLLA, which is the main component of PLA. PDLA is important to be applied to blend with PLLA in biopolymer production (Zhao *et al*., 2010). PLLA is a biodegradable polymer, and approved for use in food packaging in several countries, particularly United States of America (U.S.A.), European Union (EU) countries, and Japan (Narayanan *et al*., 2004). The polymer has increased in demand in recent years. Though L-(+)-lactic acid can be polymerized to give crystalline PLLA suited to commercial uses (Sodegard and Stolt, 2002), its application is limited by its low melting point. Thermal stability of PLA is not sufficiently high to some applications as an alternative of commercial polymers. Thermal processes such as melt molding and spinning causes thermal degradation of PLLA (Tsuji and Fukui, 2003). However, PLLA could form stereocomplex crystallites with PDLA in solution or during crystallization from the melt, which could enhance the mechanical performance, thermal stability, and hydrolysis-resistance of PLLA-based materials. Therefore its widen applications are an alternative of commercial polymers and drug delivery systems (Wang and Mano, 2008).

PDLA obtained by polymerization of D-(-)-lactic acid, was found to improve the thermostability of PLLA by the stereocomplex formation (Tsuji and Fukui, 2003). Also, Ikada *et al.* (1987) reported that the 1:1 blend of PLLA and PDLA produced a stereocomplex with *T*m around 230°C higher than melting temperature of PLLA (Tm around 175°C). Tsuji (2000, 2002) investigated the *in vitro* hydrolysis of well-stereo-complexed 1:1 blend and non-blended films from PLLA and PDLA to find in hydrolysis behaviors between the well-stereo-complexed 1:1 blend and non-blended films. For this investigation, the films were prepared from PLLA and PDLA both having a medium molecular weight MW = 1.5×105 by solvent evaporation method and their hydrolysis in phosphatebuffered solution (pH = 7.4) at 37°C. Hydrolysis of the 1:1 blend and non-blended films was performed up to 30 months, and the hydrolyzed films were studied using gel permeation chromatography (GPC), tensile tests, differential scanning calorimetry (DSC), scanning electron microscopy (SEM), optical polarizing microscopy, X-ray diffractometry, and gravimetry. It was found that the rate of reduction in molecular weight, tensile strength, Young's modulus, melting temperature, and mass remaining of the films in the course of hydrolysis was lower for the well-stereo-complexed 1:1 blend film than that for the non-blended films. The induction period until the start of decrease in tensile strength, Young's modulus, and mass remaining were longer for the wellstereo- complexed 1:1 blend film than that for the non-blended films. These findings strongly suggest that the well-stereo-complexed 1:1 blend film is more hydrolysisresistant than the non-blended. This is probably due to the peculiar strong interaction between L- and D-lactyl unit sequences in the amorphous state, resulting in the future decreased interaction of PLLA or PDLA chains and water molecules. However, the peculiar

strong interaction between PLLA and PDLA chains may have caused the retarded proteinase K-catalyzed hydrolysis of the PLLA/PDLA blend film compared with that expected from the hydrolysis rates of non-blended PLLA and PDLA films.

PDLA acted as a nucleating agent of PLLA, thereby increasing the crystallization rate (Yamane and Sasai, 2003). The role of stereocomplex as a nucleating agent and the crystallization of homo PLLA was initiated in instantaneous by homogeneous nucleation. When the blends were cooled from 200°C to 120°C, the size of the spherulites decreased and the number of the spherulites increased significantly with PDLA content. It is clear that the blends with higher PDLA content (5% PDLA) have a higher number of nucleation sites. These nucleation sites are stereocomplex crystallites with 3/1 helix in conformation and surrounded by PLLA crystalline phase.

2.2 The demands and concepts for developing bio-based D-(-)-lactic acid

Lactic acid production was around 5,000 kg per year (Inskeep *et al*., 1952), and in 1982, it was approximately 24,000-28,000 metric tones per year (Naveena, 2004). By 1999, the worldwide demand for lactic acid is estimated roughly to be 130,000 to 150,000 (metric) tones per year (Mirasol, 1999). As a result, the worldwide market for D-(-)-lactic acid will be increasing rapidly in the near future. Biotechnological processes for the production of D-(-)-lactic acid usually include D-(-)-lactic acid fermentation and product recovery and / or purification. There have been numerous investigations on the development of engineer the metabolic pathway for D-(-)-lactic acid, with the ultimate objectives to enable the process to be more efficient and economical.

2.3 Lactic acid producing route

Lactic acid can be produced by either microbial fermentation or chemical synthesis (Fig. 2.3). In the early 1960s, a method to synthesize lactic acid chemically was developed due to the need for heat-stable lactic acid in the baking industry (Vickroy, 1985). Only racemic DL-lactic acid is always produced by chemical synthesis from petrochemical resources, however, in contrast to an optically pure L-(+)- or D-(-)-lactic acid can be obtained by microbial fermentation or renewable resources when appropriate microorganism that can produce only one of the isomers is selected (Hofvendahl and Hahn-Hagerdal, 2000).

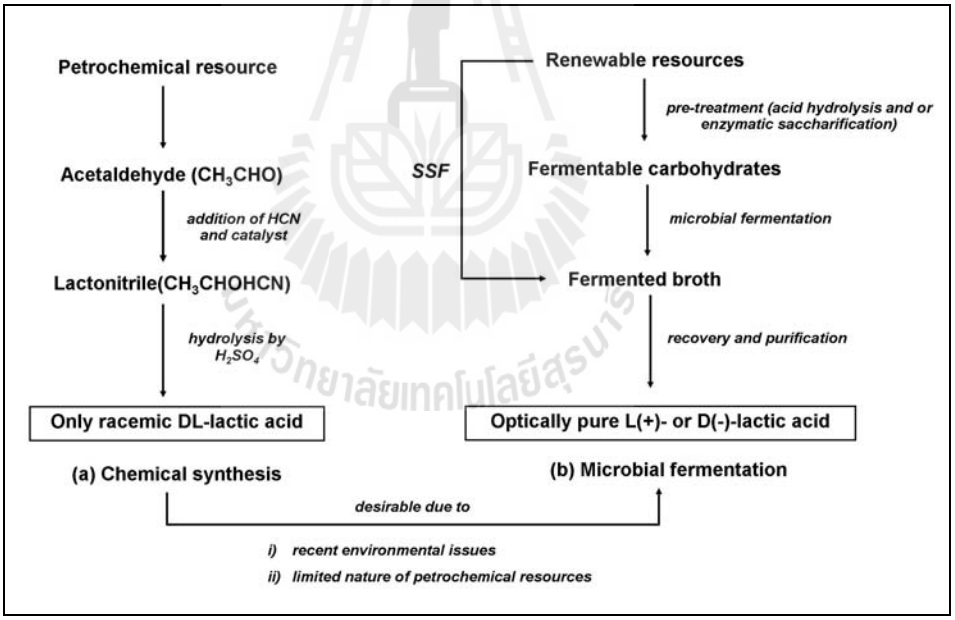

Figure 2.3 Overview of the two manufacturing methods of lactic acid; chemical synthesis (a) microbial fermentation (b). SSF represented simultaneous saccharification and fermentation (Ryu *et al*., 2006).

2.3.1 Chemical synthesis

The chemical synthesis always results in a racemic mixture of lactic acid, and is represented by reactions described in Fig. 2.4 (Narayanan *et al.*, 2004).

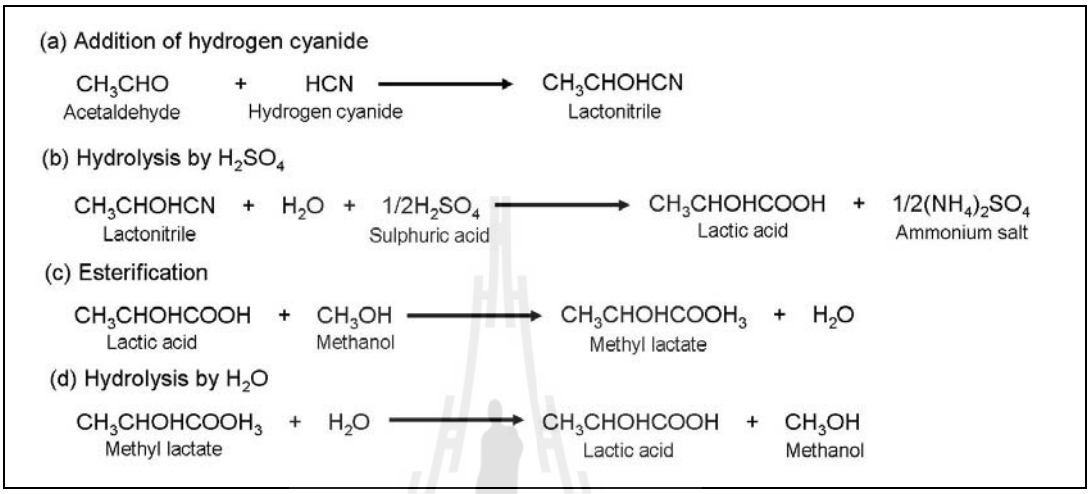

Figure 2.4 Lactic acid production process via chemical synthesis (Narayanan *et al*., 2004).

The commercial process for lactic acid synthesis is based on lactonitrile. Hydrogen cyanide is added to acetaldehyde in the presence of a base to produce lactonitrile. This reaction occurs in liquid phase at high atmospheric pressures. The crude lactonitrile is recovered and purified by distillation. It is hydrolyzed to lactic acid, either by concentrated HCl or by H_2SO_4 to produce the corresponding ammonium salt and lactic acid. Lactic acid is esterified with methanol to produce methyl lactate, which is removed and purified by distillation and hydrolyzed by water under acid catalyst to produce lactic acid and the methanol, which is recycled.

2.3.2 Microbial fermentation

Microbial fermentation processes are employed to produce a wide variety of bio-based. It is possible to push economically fermentative products by genetically modified microbial metabolism. Metabolic engineering is the rational alternation of metabolism in order for cell to generate a new product or to generate at a higher yield or rate.

Lactic acid producing microorganisms can be classified into two groups: bacteria and fungi (Litchfield, 1996). The microorganisms selected from recent studies of the biotechnological production of lactic acid are listed in Table 2.2. These fungal strains produced only L-(+)-lactic acid aerobically from glucose, sucrose or starch. Whereas, lactic acid bacteria could produce either D-(-)- or L-(+)-lactic acid or racemic mixture of lactic acid by fermentation depending on the species being used. Approximately, 90% of literatures reported on lactic acid production were focused on bacterial fermentation (Zhao *et al*., 2010). Lactic acid can be produced from sugars or sugar containing hydrolyzates or the single-step conversion of starchy or cellulosic wastes by direct conversion by amylolytic lactic acid-producing microorganisms or by the simultaneous hydrolysis and fermentation by adding enzymes and inoculum together. The configuration of lactic acid produced by fermentation is dependent upon the stereospecificity of the lactate dehydrogenase possessed by the organism.

Table 2.2 Microorganism used for recent investigations of the biotechnological production of lactic acid.

Organism	Medium/condition	Titer (g/L) Productivity [g/L/h][a]	Yield (g/g)	Reference
Rhizopus oryzae ATCC 52311	Glucose 95 g/L in YMP medium, simple batch, pH controlled by $CaCO_3$, 60 h incubation time.	83.0 [2.6]	0.88	Zhou *et al*., 1999
Lactobacillus delbrueckii NCIMB 8130	Beet molasses 200 ml molasses solution (equivalent to 100 g of sugar) , pH 7.0, 24 h incubation time.	90.0 [3.8]	0.96	Kotzanmanidis *et al.*, 2002
Enterococcus faecalis RKY1	Sugar molasses 200 g/L (equivalent to 102 g of sugar) and 15g of yeast extract, simple batch, pH controlled by 10 M NaOH, 60 h incubation time.	95.7 [4.0]	0.94	Wee *et al*., 2004
Enterococcus faecalis RKY1	Corn flour 200 g/L (equivalent to 67 g of glucose) , simple batch, pH controlled by 10 N NaOH,	63.0 [0.5]	0.94	Oh *et al*., 2005
Lactobacillus amylovorus ATCC 33620	Corn starch 10 g/L , pH controlled at 5.5, shake flask	10.1 [0.8]	NR	Xiaodong *et al*., 1997
Lactobacillus amylovorus ATCC 33620	Cassava starch 10 g/L , pH controlled at 5.5, shake flask	4.8 [0.2]	NR	Xiaodong *et al*., 1997
Lactobacillus sp. RKY2	Rice bran 200 g/L (equivalent to 60 g of glucose) simple batch, pH controlled by 10 M NaOH, 27 h incubation time.	55.0 [2.3]	0.92	Yun *et al*., 2004
Lactobacillus sp. RKY2	Wheat bran 200 g/L (equivalent to 51 g of glucose) simple batch, pH controlled by 10 M NaOH, 18 h incubation time.	47.0 [2.6]	0.92	Yun *et al*., 2004
Lactobacillus coryniformis ssp. *torquens* ATCC 25600	Filter paper (30 g liquid/g solid) SSF, pH controlled by 4 M NaOH, 50 h incubation time.	24.0 [0.5]	0.89	Yanez *et al*., 2003

D-(-)-lactic acid-producing bacteria contain D-(-)-lactate dehydrogenase (D-LDH), which is a key enzyme converting pyruvate to D-(-)-lactic acid. Some lactic acid bacterial strains have been studied for their D-(-)-lactic acid production capabilities. These strains include *Lactobacillus delbrueckii*, *Lactobacillus coryniformis* subsp. *torquens*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc carnosum*, and *Leuconostoc fallax* (Manome *et al*., 1998). In addition, Yáñez *et al.* (2003) reported that *Lactobacillus coryniformis* subsp. *torquens* could produce D-(-)-lactic acid from filter paper by simultaneous saccharification and fermentation (SSF) with adding enzymes for cellulose hydrolysis. *Lactobacills delbrueckii* JCM1148T was also reported to produce D-(-)-lactic acid from sugarcane molasses, sugarcane juice, and sugar beet juice (Calabia and Tokiwa, 2007). Recently, Okano *et al*. (2009a) reported that *Lactobacillus plantarum* NCIMB 8826T *ldhL1*: *amyA*, a strain replacing the *ldhL1* gene with an *amyA*-secreting expression cassette from *Streptococcus bovis* 148, could directly produce D-(-)-lactic acid from raw corn starch. Moreover, Okano *et al*. (2009b) reported that optically pure D-(-)-lactic acid fermentation from arabinose was achieved by using the *Lactobacillus plantarum* NCIMB 8826T *_ldhL1*. The mutant strain also had ability to direct fermentation of an optically pure D-lactic acid from cellulosic materials (Okano *et al*., 2010). Joshi *et al*. (2010) also reported the production of D(-)-lactic acid from sucrose, molasses and cellobiose using the mutant strain of *Lactobacillus lactis* NCIM 2368^{T}.

2.4 Lactic acid-producing by bacteria

There are two groups of bacteria classified in fermentative lactic acid production: homofermentative and heterofermentative. The homofermentative LAB convert glucose almost exclusively into lactic acid, however, the heterofermentative LAB catabolize glucose into ethanol and CO_2 as well as lactic acid (Fig. 2.5). The homofermentative LAB usually metabolize glucose *via* the Embden-Meyerhof pathway (*i.e.* glycolysis). Since glycolysis results only in lactic acid as a major end-product of glucose metabolism, two lactic acid molecules are produced from each molecule of glucose with a yield of more than 0.90 g/g (Thomas *et al*., 1979 and Smith *et al*., 1975). Only the homofermentative LAB are available for the commercial production of lactic acid (Yun *et al*., 2003).

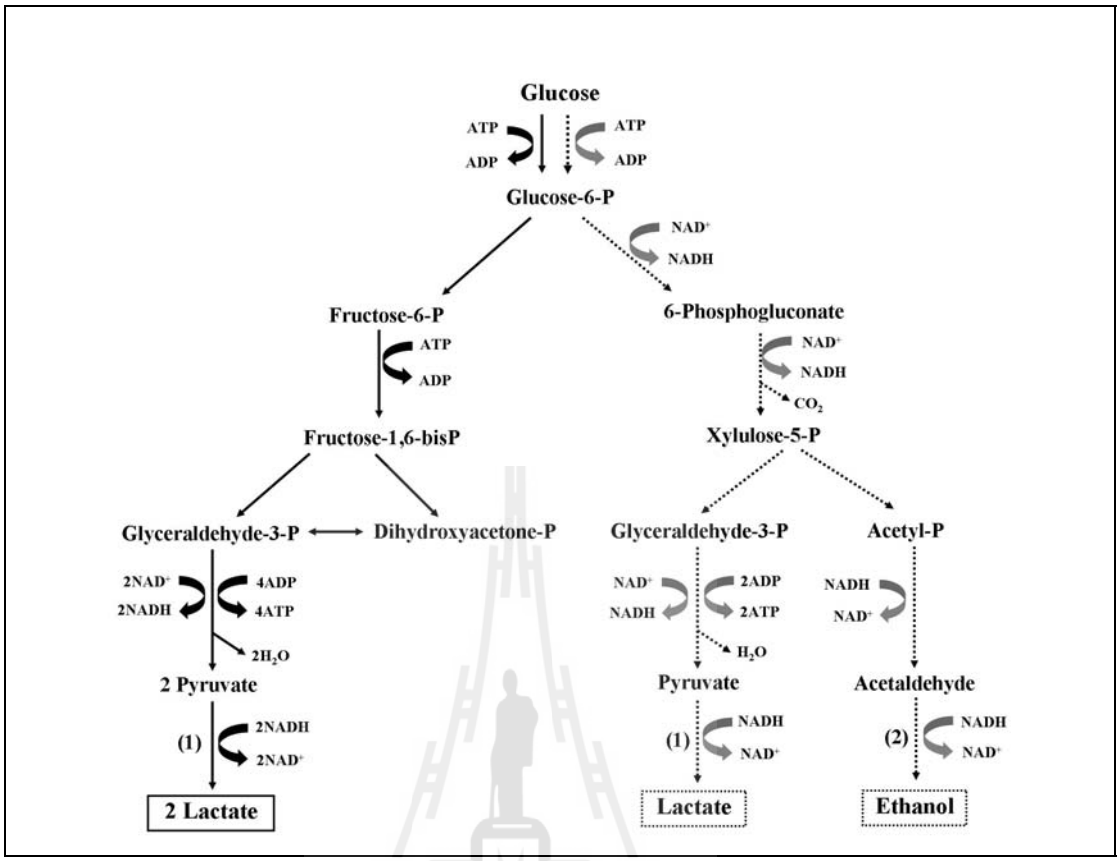

Figure 2.5 Metabolic pathways of homofermentative (solid line) and heterofermentative (dotted line) lactic acid bacteria: P, phosphate; ADP, adenosine 5'-diphosphate; NAD^+, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamode adenine dinucleotide (reduced form); (1), lactate dehydrogenase; (2), alcohol dehydrogenase (Wee *et al*., 2006).

The initial production technology of lactic acid fermentation was based on neutralization of produced acid with a suitable neutralizing agent. The major problems associated with lactic acid production by fermentation have been fastidious nutritional requirements of lactic acid bacteria, which add up to 35% of cost of production (Oh *et al*., 2005) and end product inhibition (Benninga, 1990).

The bacteria in the family of Enterobacteriaceae such as *E. coli* exhibit fast growth, ability to grow in minimal salts medium, and vailability of many genetic techniques. *E. coli* has been also used as a model microorganism and studied for practical lactic acid production in industry. In many past decades, the productions of lactic acid from *E. coli* and its metabolic–engineered derivatives have been published extensively (Table 2.3). The lactate production by fermentation have been performed and studied in rich media such a LB (Lurie-Bertani broth) medium, which contains sources of amino acids, proteins, and other chemicals from yeast extract and peptone. Contaminating proteins and cell byproducts would have to be removed from the final product. Thus, the separation process requires removal of the impurities including cells, proteins, organic acids, and other impurities. Antibiotics and IPTG used for maintaining plasmid and inducing gene expression in the lactate producing strains increase the cost of lactic acid production. Supplement of carbon dioxide and hydrogen gases to the reactor during the fermentation process also raise the production cost. The cost of inefficiencies related to media compositions, downstream processing has been very high and has made fermentation based lactic acid production impractical. However, *E. coli* strains have some limitations; the strains utilize a limited source of carbon substrates, commonly glucose. To become more feasible, the production of lactic acid should be performed in a minimal medium by any microorganisms that can utilize broader range of carbon substrates such as sucrose, cellobiose, cellotriose, and glycerol that are readily available inexpensive and abundant in Thailand.

Table 2.3 Comparison of lactic acid production from carbon sources with metabolically engineered strains.

Organism	Medium/condition	Titer (g/L) Productivity [g/L/h][a]	Yield (g/g)	Reference
E. coli SZ58 (*focA-pflB*, *frdBC* and *adhE*)	Glucose(50 g/L) in M9 medium, simple batch, pH controlled by 11.7 M KOH, 144 h incubation time.	50.80 [0.35]	0.96	Zhou *et al*., 2003
E. coli B SZ63 (pLOI3501)	Molasses(50 g/L) in modified LB, simple batch, pH controlled by 6 M KOH, 120 h incubation time.	48.73 [0.41]	0.90	Shukla *et al*., 2004
E. coli B SZ132 from SZ110, selected for vigorous fermentation	Glucose(50 g/L) in NBS medium, simple batch, pH controlled by 6 M KOH, 36 h incubation time.	44.13 [1.23]	0.88	Zhou *et al*., 2005
E. coli B SZ132 from SZ110, selected for vigorous fermentation	Glucose(100 g/L), 1mM betaine supplemented in NBS medium, simple batch, pH controlled by 6 M KOH, 72 h incubation time.	83.77 [2.20]	0.84	Zhou *et al*., 2006
E. coli SZ194 from SZ186, selected for rapid growth and fermentation in 10% glucose	Glucose(100 g/L), 1mM betaine supplemented in NBS medium, simple batch, pH controlled by 6 M KOH, 24 h incubation time.	74.22 [1.67]	0.96	Zhou *et al*., 2006
E. coli LA01 (*pflB* and *frdA*)	Glycerol(20 g/L), minimal medium designed by Neidhardt with Na_2HPO_4 + K_2HPO_4 , simple batch, pH controlled by MOPS, 36 h incubation time.	12.40 [0.34]	0.69	Mazumdar *et al*., 2010
E. coli JW2293-1 (*ackA*)	Glucose(20 g/L) in modified M9 medium contained 40g/l $CaCO_3$, shake flask	10.6 [0.25]	0.53	Zhou *et al*., 2011
E. coli JW2294-1 (*pta*)	Glucose(20 g/L) in modified M9 medium contained 40g/l $CaCO_3$, shake flask	11.8 [0.29]	0.59	Zhou *et al*., 2011

Another potential and feasible bacterium similar to *E. coli* in which it exhibits fast growth, no requirement of special and expensive source of nutrients and has available techniques for genetic manipulations, would be an ideal microorganism for chemical production. Since *K. oxytoca* M5a1 has more ability to utilize the variety of carbon substrates compared with *E. coli*, the bacterium would be a next target microorganism to be genetically engineered to alter the metabolic pathway for D-(-)-lactate production.

2.5 Enzymes involving in anaerobic fermentations of Enterobacteriaceae

In the family of Enterobacteriaceae including *K. oxytoca*, the process of oxidative phosphorylation cannot occur under anaerobic conditions and the cell produces energy from the process of degrading the original substrate known as substrate level phosphorylation. The cell is trying to maximize the formation of energy. The reducing power, NADH, which is produced during substrate degradation has to be reoxidized for the process to continue, as its supply is not limitless. It is crucial to note that pyruvate produced by the glycolytic pathway enters the tricarboxylic acid cycle (TCA), at least in part, to provide essential precursors for biosynthesis. The NADH, produced in the cycle cannot be converted to ATP as the cells have no supply of oxygen to drive oxidative phosphorylation. However, the cell can recycle NADH by means of reduction of some carbon intermediates that are accumulated under these conditions. In other words, the reducing equivalents react with the accumulated carbon intermediates to reduce them in their turn (Fig. 2.6).

In the central anaerobic metabolic pathway, pyruvate is assimilated to re-oxidize NADH via lactate dehydrogenase and alcohol dehydrogenase activities

resulting in lactate and alcohol productions, respectively. In the simplest method of hydrogen disposal, pyruvate is reduced to lactate at the expense of NADH. The reaction is catalyzed by a cytoplasmic lactate dehydrogenase encoded by *ldhA*. The enzyme is concomitantly induced by acid pH and anaerobiosis. Lactate can be produced from dihydroxyacetone-phosphate (DHAP). DHAP is converted to methylglyoxal by product of *mgsA* (methyglyoxal synthase) and is subsequently converted to lactate by glyoxalase activities encoded by *gloAB* (Wood *et al*., 1961).

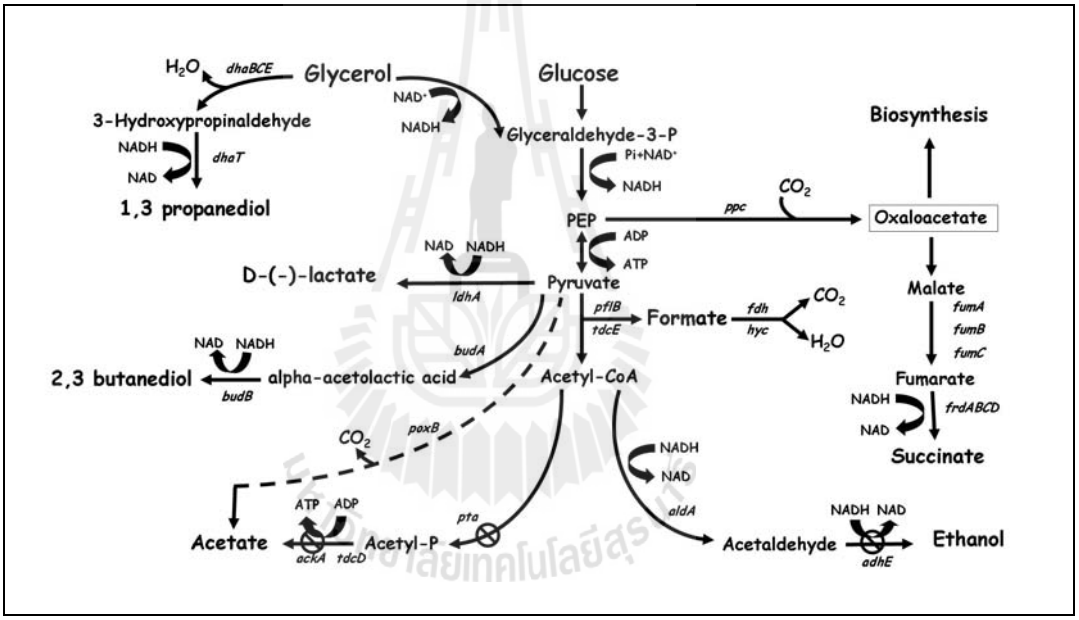

Figure 2.6 Standard pathway of glucose fermentation for *K. oxytoca* M5a1 under anaerobic conditions (Maytawadee *et al*., 2012).

Pyruvate formate-lyase encoded by *pflB*, which is responsible for anaerobic conversion of pyruvate to acetyl~CoA and formate, is posttranslationally interconverted between active and inactive forms. The enzyme synthesis is increased by anaerobiosis and can be raised further by pyruvate (Knappe and Sawers, 1990).

Acetyl-CoA produced from pyruvate can be used to generate ATP from ADP by conversion to acetate, or to dispose off extra reducing equivalents by conversion to ethanol. The first process depends on the consecutive action of phosphate acetyltransferase probably encoded by *pta* and acetate kinase encoded by *ackA*. Synthesis of these enzymes is not significantly changed by the respiratory condition of the cell. Consequently, most of the acetyl-CoA is excreted as acetate by cells growing on glucose under aerobic condition. In the absence of glucose, external acetate is mostly utilized by reversal of the pathway catalyzed by acetyl~CoA synthethase, encoded by *acs* (Kumari *et al*., 1995).

Acetyl-CoA is also converted to ethanol under anaerobic fermentation. The pathway involves a consecutive reduction of the acetyl group of acetyl-CoA to acetaldehyde, and acetaldehyde to ethanol at the expense of NADH. The reactions are catalyzed by a single polypeptide, which is alcohol dehydrogenase, encoded by *adhE*. The propinquity of the two sites of reduction might minimize escape of the acetaldehyde, which is chemically reactive. ADHE protein has dual enzyme activities, which are alcohol dehydrogenase and coenzyme-A-linked acetaldehyde dehydrogenase. However, alcohol dehydrogenase is more sensitive to inactivation by the aerobic metabolism (Clark, 1989).

The assimilation of PEP also occurs via carboxylation in which it generates succinic acid. For PEP carboxylation, fumarate reductase is activated and re-oxidizes NADH using fumarate as an electron acceptor. Endogenous or exogenous carbon dioxide is combined with PEP by phosphoenolpyruvate carboxylase encoded by *ppc*. The oxaloacetate formed is reduced to malate by the activity of malate dehydrogenase encoded by *mdh*. Malate is dehydrated to fumarate by fumarase enzymes encoded by

fumABC, whose anaerobic induction depends on FNR regulation. Fumarate is finally reduced to succinate by fumarate reductase. The net result is disposal of four reducing equivalents ($4H^+ + 4e^-$). Fumarate reductase encoded by *frdABCD* can accept electrons from various primary donor enzymes through menaquinone. Fumarate reductase is induced anaerobically by fumarate but is repressed by oxygen or anaerobically by nitrate (Cecchini *et al*., 2002).

Pyruvate dehydrogenase multi-enzyme complex is composed of products of *aceEF* and *lpdA* genes. The reaction is the gateway to the TCA cycle, producing acetyl~CoA for the first reaction. The enzyme complex is composed of multiple copies of three enzymes: E1, E2 and E3, in stoichiometry of 24:24:12, respectively. The E1 dimers (encoded by *aceE*) catalyze acetylation of the lipoate moieties that are attached to the E2 subunits. The E2 subunits (encoded by *aceF*) are the core of pyruvate dehydrogenase complex and exhibit transacetylation. The E3 component is shared with 2-oxoglutarate dehydrogenase and glycine cleavage multi-enzyme complexes. Pyruvate is channeled through the catalytic reactions by attachment in thioester linkage to lipoyl groups carrying acetyl group to successive active sites. This enzyme complex is active under aerobic condition (CaJacob *et al*., 1985). Pyruvate can be converted to CO_2, Acetyl-CoA, and NADH via the enzyme complex.

Under micro-aerobic condition, pyruvate oxidase encoded by *poxB* is responsible for generating C_2 compounds from pyruvate during the transition between aerobic and strict anaerobic growth condition. This enzyme couples the electron from pyruvate to ubiquinone and decarboxylates pyruvate to generate carbon dioxide and acetate (Abdel-Hamid *et al*., 2001).

Under both aerobic and anaerobic respirations, the versatility of the electron transport system for generating proton motive force is made possible by employing ubiquinone or menaquinone in the plasma membrane as a diffusible electron carrier or adaptor to connect a donor modular unit functionally to an acceptor modular unit. The types of electron carrier and donor modulars used for electron transport depend on the pattern of gene expression in response to the growth conditions. In anaerobic conditions, the electron donor modular units are primary dehydrogenases of the flavoprotein kind. The acceptor modular units consist of terminal reductases requiring various components, such as Fe-S. In general, when the terminal acceptor has a relatively high redox potential such as oxygen, ubiquinone is used as the redox adaptor i.e. pyruvate oxidase case. When the terminal acceptor has a relatively low redox potential such as fumarate, menaquinone is used instead for example reduction of fumarate to succinate (Cecchini *et al*., 2002).

2.6 *Klebsiella oxytoca* M5a1 as a new microorganism to D-(-)-lactate production

K. oxytoca M5a1 is a gram-negative, rod-shaped and enteric bacterium growing in paper and pulp steams as well as around other sources of wood. The 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 as low as 5.0 and temperature as warm as 35 ˚C in the minimal salts medium (Ohta *et al*., 1991). Many genetic techniques can be applied in order to engineer or modify the metabolic pathway of this microorganism for chemical production in the industry.

2.7 The carbon source for *K. oxytoca* M5a1 production of D-(-)-lactic acid

The economics of production of lactic acid and its derivatives is dependent on many factors of which the cost of raw material is very significant (Nolasco-Hipolito *et al*., 2002). It is very expensive when purified sugars like glucose, sucrose etc. are given as the feedstock for lactic acid production. *K. oxytoca* M5a1 grows on a wide variety of sugars including hexoses and pentoses, as well as on cellobiose and cellotriose without any requirements of expensive nutrients (Dien *et al*., 2003). Also this microorganism has an ability to utilize glycerol as a sole carbon substrate. The wasted-glycerol from biodiesel plant will be a potential carbon substrate for chemical synthesis using the microorganism. Hence, this microorganism would play an important role as a model for D-(-)-lactate production from the cheap and wasted substrates.

2.8 Previous construction of plasmids for gene deletions in *K. oxytoca* M5a1

The gene knockout technique in *K. oxytoca* M5a1 has been developed recently in our lab (unpublished work). The method is adapted from the gene deletion techniques performed in *E. coli* (Jantama *et al*., 2008). The gene deletion is performed by once at a time to delete genes involving in anaerobic central metabolic pathway. The former deleted strain is serially used as a host for the following gene deletion steps. In other words, target genes was deleted from chromosomal DNA of *K. oxytoca* M5a1 wild type strain sequentially. The chloramphenical resistant-levan sucrase encoding gene (*cat-sacB* cassette) was used for gene deletions as a selectable marker. In this study, the PCR reaction was used performed to amplifications of *adhE* and *pta-ackA* deletion fragments using specific primers from the plasmids that were

constructed previously. The gene-deletion fragments harboring in the plasmids were constructed previously (personal communication). Briefly, the target-deletion gene and neighboring sequences were amplified using specific primers and the PCR product was cloned into the pCR2.1-TOPO-based vector. The resulted plasmid was used as a template in further PCR reactions in which another set of specific primers were used to initiate the amplification of the template plasmid in the inside-out directions. The resulted 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 with stop codons in all reading frames was ligated with the inside-out PCR product. The first successfully constructed plasmid contains *cat-sacB* cassette flanked by some part of target gene (Fig. 2.7).

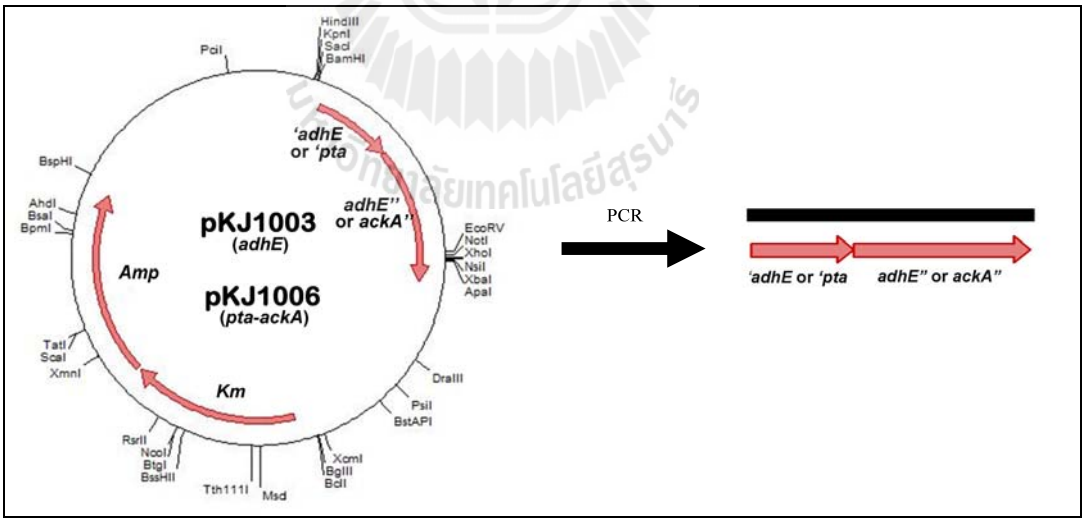

Figure 2.7 Selective/counterselective maker cassette of *adhE*, and *pta-ackA* gene deletion fragments or (*cat-sacB*) fragment.

The same inside-out PCR product was also kinase-treated and self-ligated for construction a second plasmid containing a part of the target-deletion gene without counter-selectable maker (Fig. 2.8).

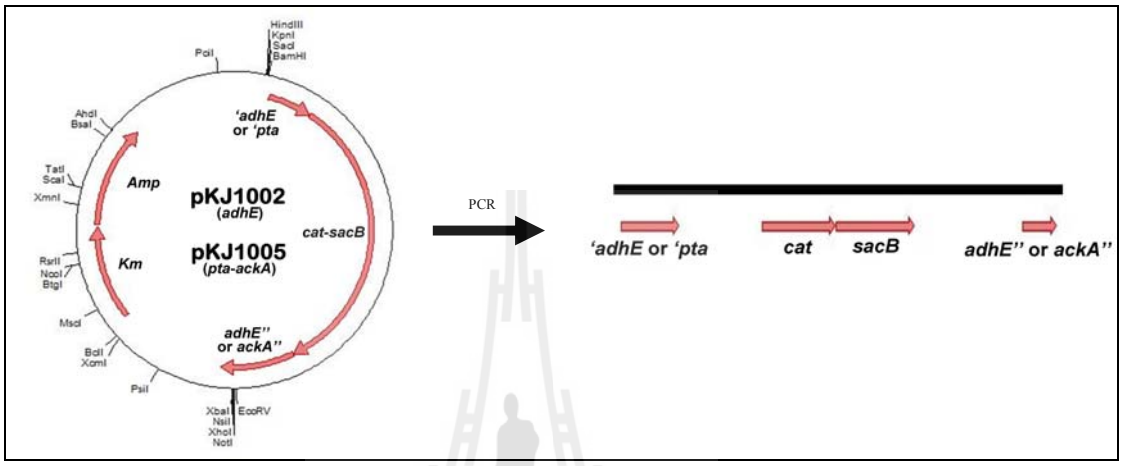

Figure 2.8 Self-ligation plasmid not containing a selective maker (*cat-sacB*) cassette.

The PCR fragments amplified from the first and the second plasmid (Fig. 2.7 and 2.8) were used to replace the target-deletion genes by facilitation of λ*Red* recombinase enzyme expressing from a special plasmid called pLOI3420 (helper vector). The λ*Red* recombinase system includes three genes in the same operon under controlling of *ara* promoter: γ, β, and *exo*, whose products are called Gam, Bet, and Exo, respectively. Gam inhibits the host RecBCD exonuclease V that prevents the degradation of double-stranded DNA ends or breaking during DNA repair mechanism. Therefore, Bet and Exo can gain access to DNA ends to promote recombination. With this plasmid, encoding λ*Red* enzyme helps the PCR-amplified fragments integrating into *K. oxytoca* M5a1 chromosomal DNA by double recombination.

2.9 Batch Culture

A batch-wise culture system where culture medium and inoculum are added into a culture vessel at the beginning of the fermentation process and then incubated at suitable temperature and gaseous environment for an appropriate period of time is called a batch culture. The microbial culture in batch culture system goes through a lag phase, accelerating growth phase, exponential growth phase, deceleration growth phase, stationary phase and sometimes the decline phase depends on the end product desired. The substrate concentration in the culture medium and growth parameters, such as pH, temperature, O.D. changes correspondingly throughout the growth phases. Thus, the physiology of microorganisms is always in transient stage, subjected to a continually changing culture conditions.

The batch culture system is still widely used in certain industrial processes, e.g. brewery industry, for its ease of operation; its less stringent sterilization; and its easy management of feed stock. Low level of microbial contamination in fermented products is at time tolerable, as long as the microbial contaminants are not pathogenic and do not alter the desired properties of the product, such as taste, color and texture.

2.9.1 Effect of pH

pH is an easily manipulated variable in the process and it has a very strong impact on the cell response and metabolism. From the production standpoint, pH control is absolutely required to achieve essential of lactic acid concentrations for an economical process. In general terms, *K. oxytoca* tolerates pH at least at low as 5.0 (Ohta *et al*., 1991). A pH range of 6.0-6.5 has been reported optimal for lactic acid production using *Lactobacillus casei* strain (Krischke *et al*., 1991). However, pH 5.5 has been used for lactic acid production using *Lactobacillus helveticus* by Ghaly *et al.* (2004). However, the optimum pH for D-lactic acid production generally ranges between 5.0 and 7.0.

2.9.2 Effect of temperature

The temperature is also one of the important factors, which influences the activity of metabolic/cell enzymes. Enzymes are most active at optimum temperature and enzymatic reaction proceeds at maximum rate. However, below and above optimal temperature, reaction rate is decreased, which causes the problems in cell metabolism (Panesar *et al*., 2010). Effects of temperature on D-lactic acid production are highly variable, and depend on the strain being used and the experimental conditions. The temperature for growth of *K. oxytoca* is at 35°C (Ohta *et al*., 1991). *Lactobacillus helveticus* and *Lactobacillus acidophilus* could use for lactate production in a temperature range of 37-45°C. Krischke *et al.* (1991) used 37°C temperature for lactic acid production using *Lactobacillus casei*. In addition, a temperature of 28°C has also been reported optimal for *Lactobacillus casei* (Nabi *et al*., 2004). There are a number of literatures reported on D-lactic acid production using a variety of temperatures, for example, 30°C for *Lactobacillus coryniformis* subsp. *torquens*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc carnosum*, and *Leuconostoc fallax* (Manome *et al*., 1998), 37°C for *Lactobacillus delbrueckii*, *Lactobacillus bulgaricus* (Benthin and Villadsen, 1995; Manome *et al*., 1998; Tanaka *et al*., 2006), 39°C for *Lactobacillus coryniformis* subsp. *torquens* (Yáñez *et al*., 2003), and 40°C for *Lactobacillus delbrueckii* (Calabia *et al*., 2007).

2.9.3 Effect of inoculum

The physiological stage (age and size) of the inoculum is an important factor in the determining the length of growth lag. The duration of lag period is prolonged when the inoculum added is at the initial stage of the exponential growth

phase. Nevertheless, the lag period could be eliminated by the addition of culture filtrate taken from a culture in the late exponential growth phase, as the intermediate products in the filtrate are readily available by increasing the inoculume size (Pirt, 1975).

Growth lag could represent priming of various essential metabolic pathways before substrates are metabolized at the maximum rates for conversion into biomass. Thus, a small inoculum may not be able to grow in a minimal medium containing only essential nutrient (Pirt, 1975).

The duration of the lag period is shortened when the growth of the inoculum added to the culture approaches the end of the exponential growth phase, as it consists mainly of young and active cells. The lag period increases when the inoculum is at the stationary growth phase. This could be attributed to the re-organization necessary in the cells to reverse the change caused by cessation of growth (Kolter *et al*., 1993).

CHAPTER III

MATERIALS AND METHODS

3.1 Genetic engineering methods

3.1.1 Strains, media and growth condition

Bacterial strains, plasmids, and primers were summarized in table 3.1. *K. oxytoca* M5a1 was provided by Microbiology and Cell Science Department, University of Florida, USA. Cultures were grown at 37^{o}C, 200 rpm in modified Luria-Bertani (LB) broth containing the following constituents per liter of broth: 10 g peptone and 5 g yeast extract with sugars as indicated. Cultures were also maintained on solid media (20 g/L agar). Ampicillin or apramycin (50 μg/mL) and chloramphenicol (34 μg/mL) were included as appropriate. The pCR2.1-TOPO plasmid was used as a cloning plasmid.

Table 3.1 Strains, plasmids and primer used in this study**.**

	Relevant characteristics	**Source**
Strains		
K. oxytoca	M5a1	Wild type
E. coli	TOP10F'	Invitrogen
KMS001	M5a1, Δ*adhE::cat-sacB*	This study
KMS002	KMS001, Δ*adhE*	This study
KMS003	KMS002, Δ*adhE* Δ*pta-ackA::cat-sacB*	This study
KMS004	KMS003, Δ*adhE* Δ*pta-ackA*	This study
Plasmids		
pCR2.1-TOPO	*bla kan*, TOPO TA cloning vector	Invitrogen
pLOI3420	*acc γ β exo* (Red recombinase), temperature-conditional replicon	Wood et al., 2005
pLOI4162	*cat-sacB* cassette	Jantama *et al*., 2008a
pKJ1001	*bla kan*; *adhE* (PCR) from *K. oxytoca* M5a1 (using KO-*adhE*-up/down) cloned into pCR2.1-TOPO	This study
pKJ1002	*cat-sacB* cassette from pLOI4162 (digested with *sfo*I-*Sma*I) cloned into the PCR amplified inside-out product from pKJ1001 (using KO-*adhE*-IO)	This study
pKJ1003	PCR amplified inside-out product from pKJ1001 (using KO-*adhE*-IO) kinase treated then self-ligation	This study
pKJ1004	*bla kan*; *pta-ackA* (PCR) from *K. oxytoca* M5a1 (using KO-*pta-ackA*-up/down) cloned into pCR2.1-TOPO	This study
pKJ1005	*cat-sacB* cassette from pLOI4162 (digested with *sfo*I-*Sma*I) cloned into the PCR amplified inside-out product from pKJ1004 (using KO-*pta-ackA*-IO)	This study
pKJ1006	PCR amplified inside-out product from pKJ1004 (using KO-*pta-ackA*-IO) kinase treated then self-ligation	This study
Primers		
KO-*adhE*-up/down	5'TTGTTTCCGCAATGCTATTT3' 5'ATTTTGCTGGCGTGGTCCGA3'	This study
KO-*adhE*-IO	5'CACCGGCTAAAGCAGAGAAG3' 5'GTGCGTTAAGTTCAGCGACA3'	This study
KO-*pta-ackA*-up/down	5'TCAGCACGTCTTTCTGGTTG3' 5'TTTGGGCAATGGCGCACTCA3'	This study
KO-*pta-ackA*-IO	5'GAGGAGCTACCGCAGTTCAG3' 5'ACCAACGAAGAGCTGGTCA3'	This study

A modified low salts medium, AM1 (Martinez et al., 2007) supplemented with 1 mM betaine (Table 3.2), was used in the fermentations. The neutralizing base used in the anaerobic fermentation experiments was a solution of 3 N KOH. Medium were sterilized by autoclave at 15 p.s.i for 15 minutes.

Table 3.2 Composition of AM1 medium supplemented with 1mM betaine (excluding carbon source).

Component	**Concentration (mmol/l)**
$(NH_4)_2HPO_4$	19.92
$NH_4H_2PO_4$	7.56
Total PO_4	**27.48**
Total N	**47.39**
[a]Total K	**1.00**
$MgSO_4$ $7H_2O$	1.50
Betaine-KCl	1.00
Trace metal	**(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
$MnCl_2$ $4H_2O$	2.50
Total salts	**4.1 g/l**

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

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

3.1.2 General genetic engineering techniques

3.1.2.1 DNA Amplification by Polymerase Chain Reaction (PCR)

The standard PCR reaction was performed using 10x PCR Master Mix solutions (Qiagen, Valencia, CA) in a PCR reaction of 50 μL. Twenty five micro-liters of master mix containing 10 mM of each dNTP (dATP, dGTP, dCTP and dTTP), PCR reaction buffer (20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgSO4, 1% (v/v) Triton® X-100, 1 mg/mL nucleasefree BSA, and *Taq* polymerase enzyme), 40 pmole of each primer (forward and reverse strand primers), and 50 ng of either plasmid or chromosomal DNA template and distilled water, were added to the mixture. The reaction was performed in automated Flexcycler PCR machine. The PCR condition was shown in Table 3.3. After the amplification reaction was finished, an aliquot of the PCR reaction mixture was examined on 1.0% (w/v) agarose gel electrophoresis.

Table 3.3 PCR parameters for the amplification of specific genes. The extension time is depended on the length of the genes (1 kb/min).

PCR profile to amplify genes				
Step	**Period**	**Temperature (°C)**	**Time**	**Number of cycles**
1	Pre-denaturing	95	5 min	1
2	Denaturing	95	30 sec	
	Annealing	55	30 sec	35
	Extension	72	3.30 min	
3	Extra-extension	72	10 min	1

3.1.2.2 Agarose Gel Electrophoresis of DNA

To analyze the size of DNA fragments, the PCR products and DNA fragments was subjected to agarose gel electrophoresis. The appropriate amount of agarose powder was dissolved in 0.5x TBE buffer [89 mM Tris-HCl, 89 mM boric acid, 25 mM EDTA pH 8.0] or 1x TAE buffer [40 mM Tris-HCl, 40 mM acetic acid, 25 mM EDTA pH 8.0] under boiling temperature to 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 mixed well to the DNA samples before loading into the wells of the solidified gel. The electrophoresis was performed at a constant voltage, 80 V, for 1 hour. After completion of electrophoresis, the gel was stained with 2 μg/mL ethidium bromide for 2 to 4 minutes and destained in distilled water for 10 minutes. The DNA bands were visualized under UV light and photographed by a gel documentation system.

3.1.2.3 PCR Clean-up Gel Extraction

PCR products were excised from 1% agarose gel (after detection of the expected size of DNA fragment). The reagents and protocol from the Kit (Macherey-Nagel) were used to purify the DNA fragments. The weight of gel slice was determined and transferred to a clean tube. The hundred milligrams of agarose gel were added in 200 μL Buffer NT. The sample was incubated at 50°C until the gel slices were dissolved (5 - 10 minutes). Vortex was applied every 2 - 3 minutes until the gel slices were dissolved. A NucleoSpin® Extract II Column was placed into a collection tube (2 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 minute at 11,000 rpm. The flow-through was

discarded. The tube was centrifuged for 2 minutes at 11,000 rpm to remove an excess buffer NT3. The column was placed into a clean 1.5 mL micro-centrifuged. Finally, 20 µL of Elution buffer NE were added and incubated at room temperature for 1 minute and then centrifuged for 1 minute at 11,000 rpm.

3.1.2.4 Preparation of *K. oxytoca* M5a1 Competent Cells

A single colony of *K. oxytoca* M5a1 was inoculated into 5 mL of LB broth and incubated at 37°C, 200 rpm overnight. Then, 0.5 mL of culture were inoculated into 50 mL LB broth and incubated at 37°C with shaking until the OD_{550} was 0.5. The culture was centrifuged at 4,000 rpm, 4°C for 10 minutes. The pellet was re-suspended and washed in 15 mL of ice-cold 10% (v/v) glycerol for 3 times. After washing the cell, the white cell pellet was re-suspended and placed on ice for 10 minutes. For long cell storage, 500 µL of GYT medium (10% (v/v) glycerol, 0.12% (w/v) yeast extract and 0.25% (w/v) tryptone) were added into the cell suspension then 100 µL aliquots were stored at -80°C.

3.1.3 Metabolic Engineering of *K. oxytoca* M5a1

3.1.3.1 Construction of plasmids used for deletion of *adhE* and *pta-ackA* genes

Chromosomal genes (*adhE* and *pta-ackA*) of *K. oxytoca* M5a1 were deleted without leaving any segment of foreign DNA as described previously (Jantama *et al*., 2008a). For the deletion of *adhE*, the *adhE* gene and neighboring regions (*ydhE''-adhE-tdk'*) were amplified using primer set KO-*adhE*-up/down and cloned into the pCR2.1-TOPO vector (Invitrogen) to produce plasmid pKJ1001. The plasmid DNA functioned as a template for inside-out amplification using the KO-*adhE*-IO primer set (both primers within the *adhE* gene and facing outward). The

resulted inside-out PCR product contained a vector-based backbone flanked *adhE* sequences without the central part of *adhE* gene. The obtainable fragment was ligated to the *SfoI/SmaI*-digested *cat-sacB* cassette from pLOI4162 to produce pKJ1002 (*ydhE''-adhE'-cat-sacB-adhE''-tdk'*). The inside-out PCR fragment was also kinase-treated and self-ligated to construct a plasmid pKJ1003. In pKJ1003, the central region of *adhE* was omitted (*ydhE''-adhE'-adhE''-tdk'*).

The *pta-ackA* deletions were conducted in an analogous manner as the *adhE* deletion. The required primer sets (KO-*pta-ackA*-up/down and KO-*pta-ackA*-IO) are shown in Table 3.1 together with the corresponding plasmids (pKJ1004, pKJ1005, and pKJ1006).

3.1.3.2 Transformation of pLOI3420 (helper vector) into *K. oxytoca* M5a1 by electoporation

A single colony of *K. oxytoca* M5a1 wild type was inoculated into 30 ml of LB broth and incubated at 37 °C with shaking at 200 rpm until the OD_{550} was 0.3-0.5. The culture was centrifuged at 4,000 rpm, 4 °C for 5 minutes. The pellet was re-suspended and washed in 10 ml of de-ionized water for 2 times. After washing the cell, the white cell pellet will be re-suspended in 1 ml of sterile ice-cold de-ionized water.

One micro-liter of pLOI3420 (helper vector) was mixed with electroporated competent cells which was prepared as shown in the above. The mixture was transferred to an ice-cold 0.4 cm electroporation cuvette. The cuvette was incubated on ice for 5 minutes. The cells were pulsed by using electroporation under the condition of 2,500 V, pulse 5 ms length. Then 1 ml of 1 M 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 hour. Transformed cells, 200 µl, were spreaded on LB agar plates containing apramycin (50 µg/ml) and incubated at 30 °C overnight.

3.1.3.3 Deletion of *adhE* and *pta-ackA* genes in *K. oxytoca* M5a1

To delete *adhE* gene, plasmids pKJ1002 and pKJ1003 served as templates for amplification using the KO-*adhE*-up/down primer set to produce linear DNA fragments for genome integration steps I (*ydhE''-adhE'-cat-sacB-adhE''-tdk'*) and II (*ydhE''-adhE'-adhE''-tdk'*) respectively. The step I fragment was transformed by electrophoresis into wild-type *K. oxytoca* harboring pLOI3420 (Red recombinase). The recombinants were selected on plates containing chloramphenicol (40 mg/L) at 39°C overnight. The chloramphenicol-resistant clone was designed as KMS001. Strain KMS001 harboring pLOI3420 was further subjected to electroporation with the step II fragment. Cells were incubated at 30°C for 6 h and transferred into a 250-ml flask containing a total volume of 100 ml of LB with 15% (w/v) sucrose. After overnight of incubation at 30°C, clones were selected on LB plates containing 10% (w/v) sucrose (39°C, 16 h). Resulting clones were tested for loss of apramycin, ampicillin, and chloramphenicol resistances. Construction was further confirmed by PCR analysis. Clone lacking in *adhE* gene and *cat-sacB* cassette was selected and designated KMS002.

For deletion of *pta-ackA* genes, plasmids pKJ1005 and pKJ1006 served as templates for amplification using the KO-*pta-ackA*-up/down primer set to produce the linear DNA fragments for integration steps I (*pta'-cat-sacB-ackA''*) and II (*pta'-ackA''*) respectively. Strain KMS002 was used to delete *pta-ackA* genes in a manner analogous to that of the deletion of *adhE*. The resulting strain was designated KMS004.

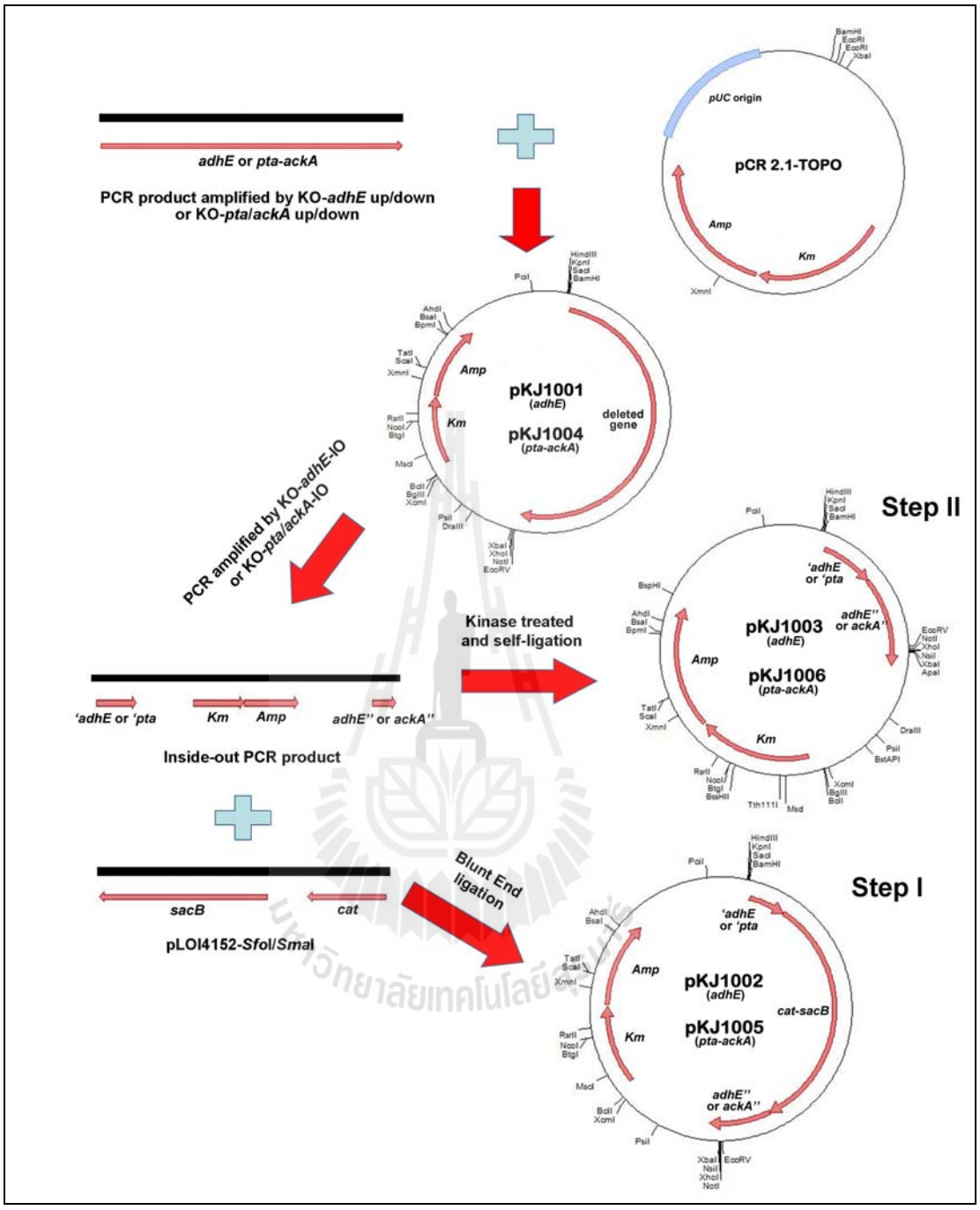

Figure 3.1 Construction of plasmids for deletion of *adhE* and *pta-ackA* genes in *K. oxytoca* M5a1.

3.2 Anaerobic fermentation

3.2.1 Anaerobic fermentation in small scale bottle using AM1 medium

KMS002 and KMS004 inocula were prepared by seeding into AM1 medium containing 2% (w/v) glucose. Fermentations were inoculated at an OD_{550} of 0.1 and carried out in a 500- mL bottle with a 350-mL working volume at 37^{o}C, 250 rpm. The pH was controlled at 7.0 by automatically adding a 3N KOH. The total incubation time was 96 hours. No antibiotic was included during the growth of seed cultures or in fermentation broth.

The production of D-(-)- lactic acid by KMS004 from various initial pH control (5.5, 6.0, 6.5, 7.0 and 7.5), temperature (30, 35, 37, 40and 42°C), initial OD_{550} (0.01, 0.1, 0.5, 1.0 and 2.0), incubation time (24, 48, 72, 96 and 120 h) and concentrations of glucose (10, 20, 50 and 100 g/L) were studied and optimized to find the best condition to obtain high yield of D-(-)-lactic acid.

3.3 Analytical methods

Fermentation samples were removed during fermentation every 24 hours for the analyses of cell mass, organic acids, and sugars. Cell growth was monitored at 550 nm and converted to biomass as cell dry weight (CDW) by an appropriate calibration curve. The fermentative products produced during anaerobic fermentations such as lactic acid, formic acid, ethanol, acetic acid, 1,3-propanediol, 2,3-butanediol, and residual sugars were determined using HPLC equipped with an ion exchange column (Aminex® HPX-87H, 7.8×300 mm, BioRad) and a refractive index detector (RI-150, Thermo Spectra System, USA). The mobile phase used in the HPLC system was 4 mM sulfuric acid at a flow rate of 0.4 mL/min. Culture collected from the

fermentation was centrifuged to discard the cells. The supernatant was further filtered through a 0.2 μm nylon filter prior to injecting into the HPLC. The lactate enantiomers were analyzed by using an Astec Chirobiotic™ R Chiral column, 5 μm, 15 cm × 2.1 mm (Sigma-Aldrich).

3.4 Dextrose equivalent (DE)

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. Liquefaction 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 obtain 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 Dinitrosalicylic (DNS) solution (raio 1:1). The reaction mixed was boiled for 15 min and immediately cool down in cool water. The 4mL DI water was added into the reaction mixed. The reducing sugars content in the samples were analyzed by measurement 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 was in the range of 16-17% (w/w).

3.5 Statistical

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 DISCUSSIONS

4.1 Deletion of *adhE* gene in *K. oxytoca* M5a1

To delete *adhE* gene, plasmids pKJ1002 and pKJ1003 (Fig. 4.1) are served as templates for amplification using the KO-*adhE*-up/down primer set to produce linear DNA fragments for genome integration steps I (*ydhE''-adhE'-cat-sacB-adhE''-tdk'*) and II (*ydhE''-adhE'-adhE''-tdk'*) respectively.

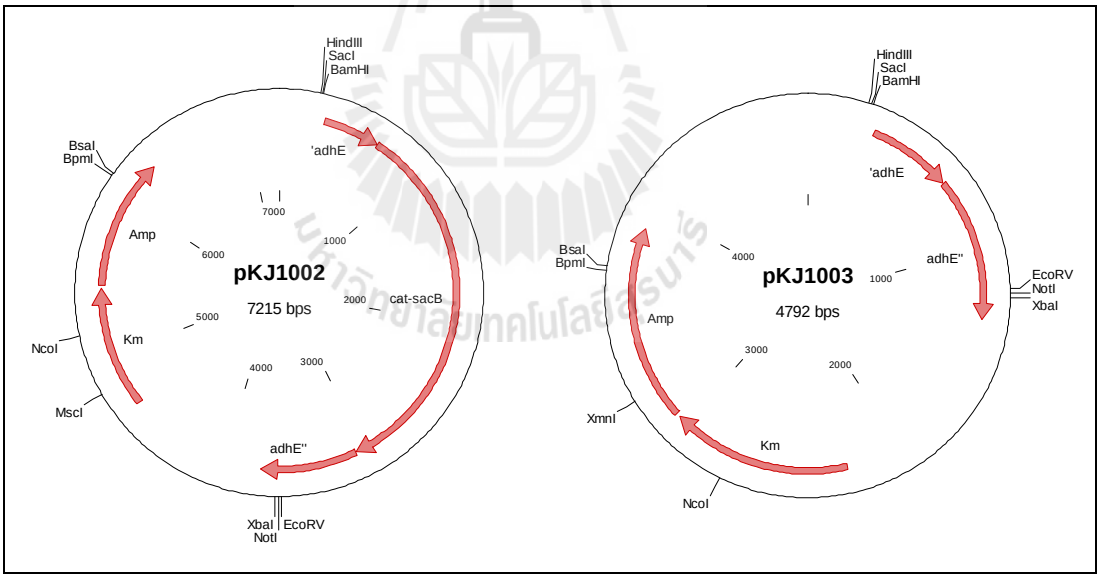

Figure 4.1 Plasmids pKJ1002 and pKJ1003 used for deletion of *adhE* gene.

The step I fragment was transformed by electrophoresis into wild-type *K. oxytoca* M5a1 harboring pLOI3420 (Red recombinase). The recombinants were selected on plates containing chloramphenicol (40 mg/L) at 39^{o}C overnight.

The chloramphenicol-resistant clone was designed as KMS001. Strain KMS001 was confirmed by the PCR analysis using the KO-*adhE*-up/down primer set and the M13 primer set. The result showed that the PCR product was in size of 4000 bps as expected (Fig. 4.2)

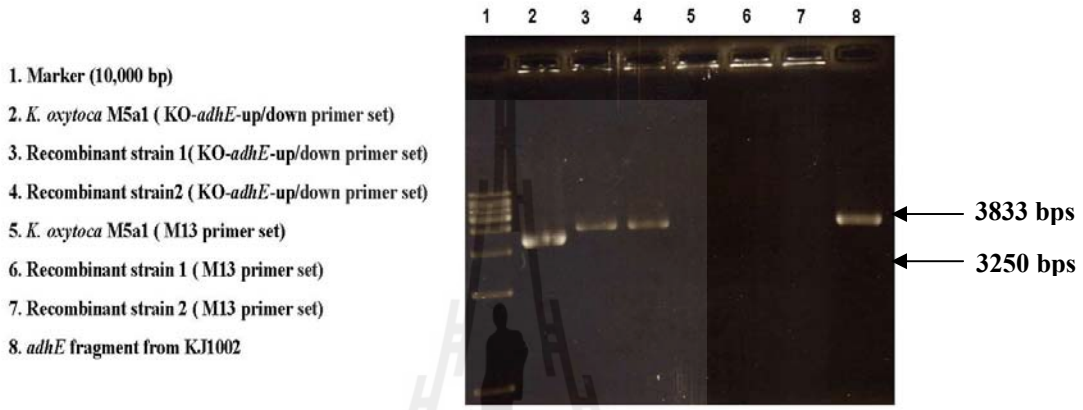

Figure 4.2 Gel electrophoresis preformed to confirm the recombinant strain of KMS001. Using the KO-*adhE*-up/down primer set, lane 2 represented the PCR band of *adhE* gene in the *K. oxytoca* M5a1 and lane 3, 4 represented the PCR bands of integration step I (*ydhE"-adhE'-cat-sacB-adhE"-tdk'*) in the genome of *K. oxytoca* M5a1. From lane 5 to 7, M13 primer set was used to confirm that the *K. oxytoca* M5a1 and the KMS001 did not carry the plasmid pKJ1002 inside the cell. Lane 8, PCR product from pKJ1002.

Strain KMS001 harboring pLOI3420 was further subjected to electroporation with the step II (*ydhE*"-*adhE*'-*adhE"-tdk'*) fragment. Resulting clones were tested for loss of apramycin, ampicillin, and chloramphenicol resistances. Construction was further confirmed by PCR analysis. The clone lacking in *adhE* gene and *cat-sacB* cassette was selected and designated KMS002 (Fig. 4.2)

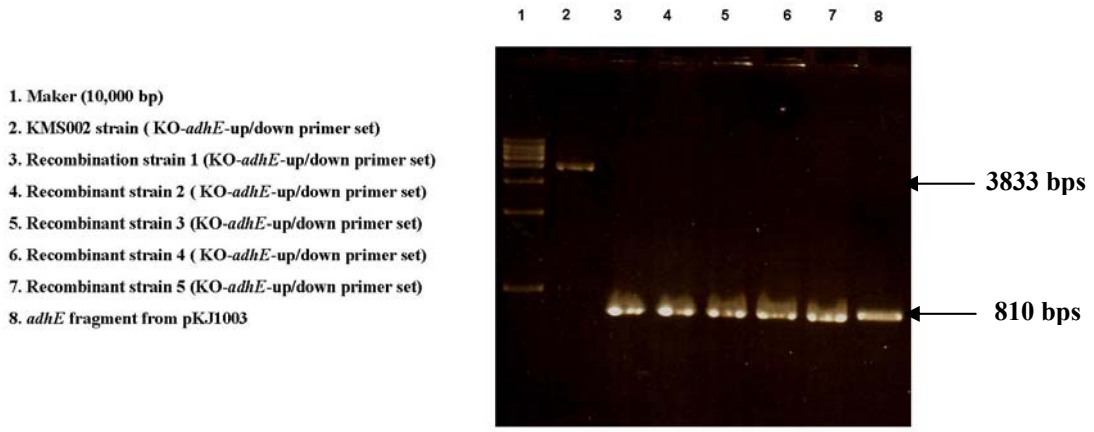

Figure 4.3 Gel electrophoresis preformed to confirm the recombinant strain of KMS002. Using the KO-*adhE*-up/down primer set, lane 2 represented the PCR band of *adhE* gene (*ydhE"-adhE'-cat-sacB-adhE"-tdk'*) in KMS001 strain and lane 3 to 7 represented the PCR bands of integration step II (*ydhE*"-*adhE*'-*adhE"-tdk'*) in the genome of KMS001. Lane 8, PCR product from pKJ1003.

4.1.1 Effect of *adhE* deletion in wild type *K. oxytoca* M5a1

In many microorganisms including *K. oxytoca* M5a1, the reducing power, NADH, which is produced during glycolysis, has to be re-oxidized for the process to continue under anaerobic conditions. In the central anaerobic metabolic pathway, pyruvate is assimilated mainly to re-oxidize NADH via lactate dehydrogenase (LdhA), and alcohol dehydrogenase (AdhE) activities resulting in lactate and alcohol productions respectively. Under anaerobic conditions, pyruvate is reduced to lactate at the expense of 1 mol of NADH. In addition, acetyl-CoA produced from pyruvate is also converted to ethanol under anaerobic conditions at the expenses of 2 mol of NADH (Gottschalk, 1985; Jantama *et al*., 2008b).

K. oxytoca M5a1 wild type produces a mixture of lactate, ethanol, 2.3-butanediol, succinate, acetate, and formate as metabolites during glucose fermentation (Fig. 4.4). Ethanol is produced as a major fermentative product in the wild-type strain (Table 4.1). Thus the ethanol formation pathway was eliminated from the wild type strain by deleting *adhE* to construct KMS002 (Δ*adhE*). KMS002 was tested for D-(-)-lactic acid production under anaerobic conditions in AM1 mineral salts medium containing 2% (w/v) glucose as a sole carbon source. This strain accumulated a significant amount of D-(-)-lactic acid as a major fermentative product at a concentration of 13.13±0.45 g/L with a yield and average productivity of 0.71±0.03 g/g and 0.38±0.03 g/L/h respectively after 72 h incubation. No ethanol was detected in the fermentation broth (Table 4.1). This result confirmed that ADHE activity was successfully abolished from KMS002. The average productivity of D-(-)-lactic acid was increased by 3-fold compared with that of the wild-type strain (Fig. 4.4a and 4.4b, Table 4.1). The results also indicated that the deletion of *adhE* redirected the carbon flux to the lactate production pathway.

Table 4.1 Fermentation profile of metabolically engineered *K. oxytoca* M5a1 strains in AM1 medium containing 2% (w/v) glucose

Strain	CDW (g/L)	Total glucose utilized (g/L)	D-(-)-lactic acid			Concentration of co-products[c] (g/L)				
			concentration (g/L)	Yield[a] (g/g)	Productivity[b] (g/L/h)	Succinate	Formate	Acetate	Ethanol	Butanediol
Wild type	0.84±0.01[d,€]	21.74±0.17[€]	2.70±0.29[€]	0.12±0.02[€]	0.11±0.01[€]	0.94±0.10[€]	3.61±0.18[€]	4.39±0.27[€]	3.44±0.28	3.06±0.27[€]
KMS002	0.82±0.21[€]	18.22±0.32[£]	13.13±0.45[£]	0.71±0.03[£]	0.38±0.03[£]	0.44±0.05[£]	0.14±0.01[£]	0.73±0.01[£]	ND[e]	0.38±0.02[£]
KMS004	1.21±0.37[£]	18.08±0.23[£]	11.46±0.09[¥]	0.64±0.01[¥]	0.36±0.01[£]	0.46±0.01[£]	1.99±0.08[¥]	2.36±0.08[¥]	ND	1.44±0.03[¥]

[a] The lactate yield was calculated as grams of D-(-)-lactic acid produced divided by grams of the glucose consumed.

[b] The lactate productivity was calculated as D-(-)-lactic acid concentration produced divided by overall incubation time.

The incubation time for wild type and KMS002 were 24 and 72h , respectively.

[c] No 1,3-propanediol was detected in the fermentation broth from all of the strains.

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

[e] ND = Not detected

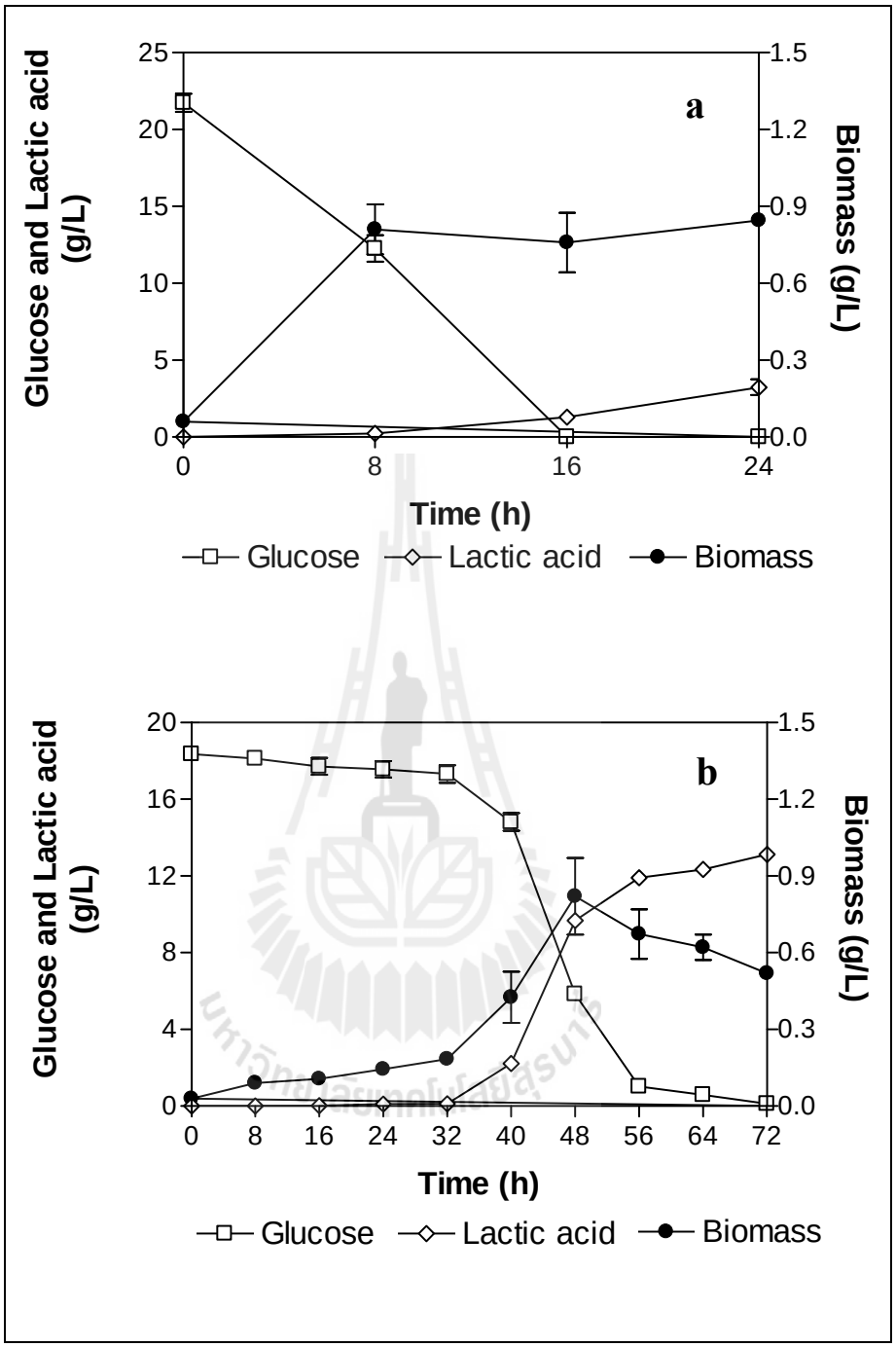

Figure 4.4 Fermentation profile of *K. oxytoca* wild type and mutant in AM1 medium containing 2% (w/v) glucose. (a) *K. oxytoca* wild type (b) KMS002 (c) KMS004. The symbols and error bar are average values and standard deviations of at least three measurements, respectively.

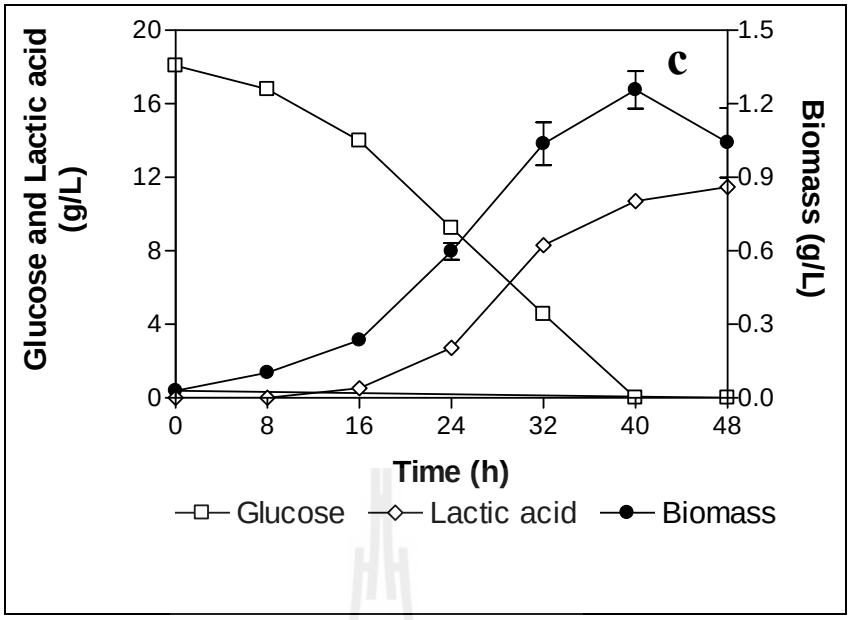

Figure 4.4 Fermentation profile of *K. oxytoca* wild type and mutant in AM1 medium containing 2% (w/v) glucose. (a) *K. oxytoca* wild type (b) KMS002 (c) KMS004. The symbols and error bar are average values and standard deviations of at least three measurements, respectively (Continued).

The levels of formate (0.14±0.01 g/L), acetate (0.73±0.01 g/L), succinate (0.44±0.05 g/L) and 2,3-butanediol (0.38±0.02 g/L) produced by KMS002 were dramatically decreased compared with those of wild type *K. oxytoca* M5a1 under anaerobic conditions (Table 4.1). The results suggested that the accumulated level of NADH generated during glycolysis was re-oxidized by means of LDHA to a greater extent than that of other pyruvate dissimilation routes in KMS002. This outcome was due to the carbon-flux partitioning through pyruvate dissimilation routes via pyruvate-formate lyase (PFLB) and acetate kinase (ACKA) not maintaining a balanced level of reducing powers, NADH and NAD^+ (Fig. 2.6). In addition, pyruvate dissimilation via 2,3-butanediol dehydrogenase (BUDB) did not competitively re-oxidize NADH in KMS002 since two-step reactions were required for 2,3-butanediol biosynthesis (Fig.

2.6). Also, a lower affinity for pyruvate of BUDB ($K_{m,}^{pyruvate}$ = 8.0 mM) than that of LDHA ($K_{m,}^{pyruvate}$ = 7.2 mM) (Yang *et al*., 2000) might cause more efficient re-oxidation via the lactic acid production pathway. Celinska (2009) revealed that the high ratio of NADH to NAD^+ generated from high glycolytic flux during an exponential growth phase activates LDHA activity while the low ratio of NADH to NAD^+ causes 2,3-butanediol production in *K. oxytoca* M5a1. This was consistent with the production of D-(-)-lactic acid during an exponential phase (Fig. 4.4b), but the production of 2,3-butanediol was only observed during the stationary phase (after 48 h incubation) in KMS002 (data not shown).

The deletion of *adhE* also significantly decreased the growth rate of KMS002 ($\mu = 0.06\ h^{-1}$) compared to that of wild-type *K. oxytoca* M5a1 ($\mu = 0.30\ h^{-1}$). The result indicated that the loss of ADHE activity in KMS002 caused a reduction in efficacy of NADH re-oxidation by 50% compared with that of the wild-type strain (Figure. 2.6). The level of NADH built up in KMS002 during glycolysis resulted in a higher ratio of NADH to NAD^+ which might affect the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) due to a lack of NAD^+ binding that hindered a conformational change of the enzymatic-activation state (Hillman, 1979). This phenomenon was reflected in the delay of the glucose consumption and growth in KMS002 (Fig. 4.4b). In addition, Zhou *et al* (2002; 2011) revealed that the low carbon flux through ACKA activity due to the high flux through LDHA also caused less ATP production. The delay in glucose consumption and reduced ATP production in KMS002 might result in a prolonged lag period. These findings are in accordance with those of Jantama *et al*. (2008b) that the deletion of alcohol dehydrogenase caused poor growth and glucose consumption in *E. coli* under anaerobic conditions, but not under aerobic conditions.

4.2 Deletion of *pta-ackA* genes in KMS002

For deletion of *pta-ackA* genes, plasmids pKJ1005 and pKJ1006 (Fig. 4.5) served as templates for amplification using the KO-*pta-ackA*-up/down primer set to produce the linear DNA fragments for integration steps I (*pta'-cat-sacB-ackA''*) and II (*pta'-ackA''*), respectively.

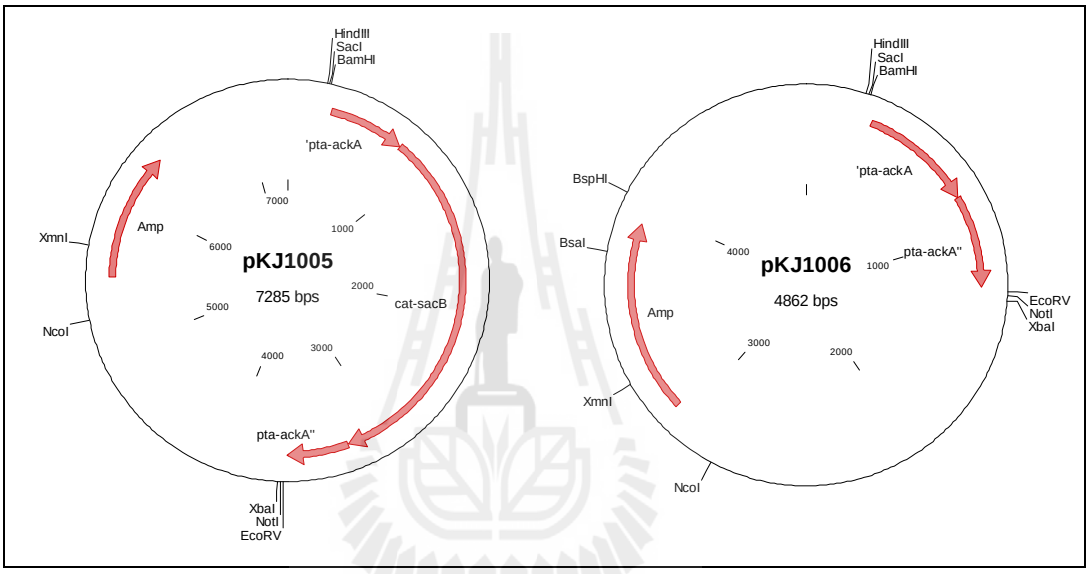

Figure 4.5 Plasmids pKJ1005 and pKJ1006 used for deletion of *pta-ackA* genes.

The step I (*pta'-cat-sacB-ackA''*) fragment was transformed by electrophoresis into KMS002 harboring pLOI3420 (Red recombinase). The recombinants were selected on plates containing chloramphenicol (40 mg/L) at 39^{o}C overnight. The chloramphenicol-resistant clone was designed as KMS003. Strain KMS003 was confirmed by the PCR analysis using the KO-*pta-ackA*-up/down primer set and M13 primer set. The result showed that the PCR product was in size of 3753 bps as expected (Fig. 4.6).

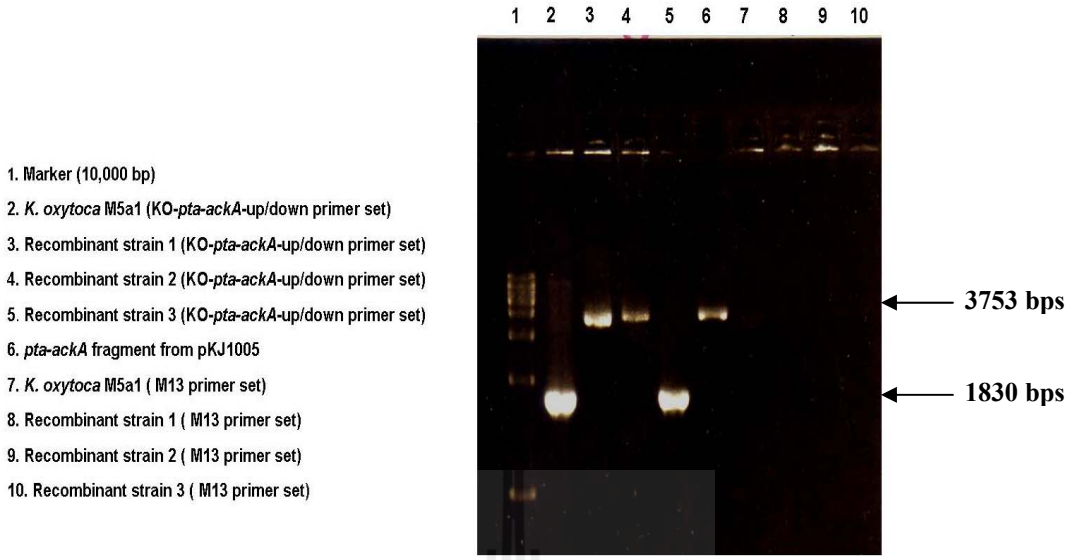

Figure 4.6 Gel electrophoresis preformed to confirm the recombinant strain of KMS003. Using the KO-*pta-ackA*-up/down primer set, lane 2 represented the PCR band of *pta-ackA* gene in the *K. oxytoca* M5a1 and lane 3 to 5 represented the PCR bands of integration step I (*pta'-cat-sacB-ackA"*) in the genome of KMS002. Lane 6, PCR product from pKJ1005. From lane 7 to 10, the M13 primer set was used to confirm that the *K. oxytoca* M5a1 and the KMS003 did not carry the plasmid pKJ1005 inside the cell.

The strain KMS003 harboring pLOI3420 was further subjected to electroporation with the step II (*pta'-ackA''*) fragment. Resulting clones were tested for lost of apramycin, ampicillin, and chloramphenicol resistances. Construction was further confirmed by PCR analysis (Fig. 4.7). The clone lacking in *pta-ackA* genes and *cat-sacB* cassette was selected and designated KMS004 (Fig 4.6).

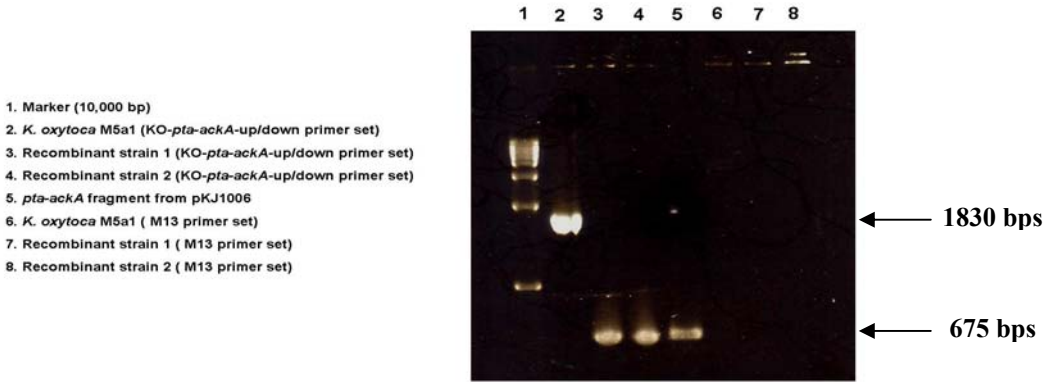

Figure 4.7 Gel electrophoresis preformed to confirm the recombinant strain of KMS004. Using the KO-*pta-ackA*-up/down primer set, lane 2 represented the PCR band of *pta-ackA* genes in *K. oytoca* M5a1 strain and lane 3, 4 represented the PCR bands of integration stepII (*pta'-ackA"*) in the genome of KMS003. Lane 5, PCR product from pKJ1006. From lane 6 to 8, the M13 primer set was used to confirm that the *K. oxytoca* M5a1 and the KMS004 did not carry the plasmid pKJ1006 inside the cell.

4.2.1 Effect of *pta-ackA* deletion in KMS002

KMS002 produced the highest amounts of acetate during glucose fermentation (Table 4.1). A decrease in acetate production may improve the yield of lactate, and the percentage of carbon recovery. Also, downstream processes including purification steps can be simplified due to less by-product contamination. Under anaerobic conditions, wild-type *K. oxytoca* M5a1 produces acetyl-CoA that is converted to acetyl-P by means of phosphotransacetylase (PTA) activity. Acetyl-P is further converted to acetate primarily by ACKA activity (Fig. 2.6). Therefore, the deletion of *pta-ackA* in KMS002 was simultaneously performed to construct KMS004 (Δ*adhE*, Δ*pta-ackA*) to reduce acetate formation.

KMS004 produced D-(-)-lactic acid at a concentration of 11.46±0.09 g/L with a yield and productivity of 0.64±0.01 g/g and 0.36±0.01 g/L/h, respectively after 48 h incubation in AM1 medium containing 2% (w/v) glucose under anaerobic conditions (Table 5.1). The titer, yield, and average productivity of D-(-)-lactic acid produced by KMS002 and KMS004 were not greatly different. It is likely that the deletions of *pta-ackA* did not affect the D-lactate production in KMS004 much. However, it was surprising to observe a higher level of acetate for the strain KMS004 (2.36±0.08 g/L) than for KMS002 (0.73±0.01 g/L) even though KMS004 contained deletions of both *pta* and *ackA*. The result implied that an increased level of acetate production in KMS004 might stem from the activation of other acetate-producing pathways such as pyruvate oxidase (PoxB), and propionate kinase (TDCD).

PoxB is responsible for a cell survival during the stationary phase by oxidation of pyruvate resulting in acetate accumulation. An elevated intracellular pyruvate level accumulating during glycolysis usually activates PoxB activity, which directs pyruvate to acetate via decarboxylation (Abdel-Hamid *et al*., 2001). In KMS004, PoxB activity likely functioned since acetate was also produced not only during exponential growth but also during the stationary phase. Jantama *et al*. (2008b) demonstrated that PoxB activity was activated when the deletion of *ackA* was introduced in *E. coli*. They also showed that the additional deletion of *poxB* gene in the strain decreased acetate production during anaerobic fermentation. Another enzyme which is suspected to compensate AckA activity in KMS004 is a propionate kinase (TdcD) encoded by *tdcD* (Fig. 2.6). Based on the protein sequence, TdcD is highly similar to AckA (Reed *et al*., 2003). The expression of *tdcD* might be activated and TdcD activity might replace. AckA activity thus increasing the production of ATP

and acetate from acetyl-P and providing a competitive growth advantage. KMS004 also produced higher biomass and finished glucose fermentation within 48 h while KMS002 exhibited a long lag with lower cell yield and finished fermentation after 72 h incubation (Fig. 4.4b and 4.4c). This phenomenon confirmed the TdcD activation compensating the AckA activity, and was also consistent with an impaired growth observed in *E. coli* containing both deletions of *ackA*, and *tdcD*, but not *ackA* or *tdcD* alone. Also the deletion of *tdcD* resulted in a significant reduction in the acetate level during anaerobic fermentation by *E. coli* (Jantama *et al*., 2008a).

The low level of formate (0.14±0.01 g/L) detected in the fermentation broth of KMS002 (Table 4.1) suggested that the strain might utilize formate to produce CO_2 and H_2 via formate hydrogen-lyase (encoded by *fdh* and *hyc*) to support growth. The electron produced by the formate hydrogen-lyase reaction generates ATP by proton motive force (Thauer *et al*., 1977). Axley *et al*. (1990) revealed that formate serves as a growth substrate in many microorganisms when most the pyruvate serves as an electron acceptor to produce lactate. Therefore, formate produced during fermentation was consumed to compensate for capability deficit in an energy production due to low flux through AckA activity in KMS002. However, this phenomenon likely did not occurre in KMS004 since the strain accumulated formate at a significant level (Table 4.1). The results might also imply that KMS004 did not need to utilize formate for the promotion of growth produced energy via PoxB and TdcD activities instead. Besides, *tdcE*, located in the same operon as *tdcD* encodes an alpha-ketobutyrate/pyruvate formate-lyase (Reed *et al*., 2003) exhibiting PflB-like activity. It is likely that the expression of *pflB* and *tdcDE* allowed KMS004 to dissimilate more pyruvate to acetyl-CoA rather than acetate as compared with

KMS002. These results were confirmed with the increased productions of acetate (2.36±0.08 g/L) and formate (1.99±0.08 g/L) by KMS004 (Table 4.1).

KMS004 produced a higher amount of 2,3-butanediol (1.44±0.03 g/L) than KMS002 (0.38±0.02 g/L) from 2% (w/v) glucose. The significant increase in 2,3-butanediol compared with KMS002 might result from the induction of 2,3-butanediol biosynthesis in KMS004. Nakashimada *et al*. (2000) revealed that the enzymes involved in 2,3-BD biosynthesis were usually induced under acidic conditions. The higher levels of acetate and formate accumulating in the fermentation broth suggested the induction of 2,3-butanediol biosynthesis in KMS004. Van Houdt *et al*. (2007) showed that the metabolic pathway of 2,3-butanediol played a role in preventing intracellular acidification by changing the metabolism from acid production to the formation of neutral compounds. In addition, 2,3-butanediol biosynthesis together with lactate production have also been regarded as participating in the regulation of the $NADH/NAD^+$ ratio in KMS004. Thus, a slightly lower in yield of lactate observed in KMS004 was caused by higher carbon fluxes through *pflB* and *budB* activities.

Although the D-(-)-lactic acid yield and productivity of lactic acid bacteria were better than those from the engineered strains in the current study, the optical purity of D- lactate produced by KMS002 and KMS004 was 99.5%. This makes KMS002 and KMS004 gaining benefits over lactic acid bacteria that produce D-(-) or L-(+)-lactic acid with optical purities of less than 95%.

4.3 Production of D-(-)-lactic acid from sugarcane molasses by KMS002 and KMS004

Sugarcane molasses is a waste material from the sugar production industry, and is considered as an inexpensive carbon substrate. The sugars in sugarcane molasses (sucrose, glucose, and fructose) can be utilized by *K. oxytoca* M5a1 to produce low-cost D-(-)-lactic acid. At 96 hours, KMS002 and KMS004 produced D-(-)- lactic acid at comparable levels at concentrations of 24.34±0.45 g/L and 21.86±0.27 g/L, respectively from a sugarcane molasses concentration of 50 g/L (about 25 g/L total sugars equivalent) in AM1 medium (Fig. 4.8). Co-products were produced at low levels in both strains (Table 4.2). Shukla *et al*. (2004) produced D-(-)-lactic acid from sugarcane molasses by metabolically engineered *E. coli* SZ63 harboring sucrose-utilizing genes (*cscKBA*) with a comparable yield to that of KMS002 and KMS004. However, the fermentation by SZ63 strain required antibiotics to maintain the expression of *cscKBA*. Even though high concentrations and production yields of D-(-)-lactic acid were obtained, the requirement of antibiotics means that it is not economical on a large-scale to produce D-(-)-lactic acid by SZ63. Since *K. oxytoca* M5a1 naturally utilizes sucrose, KMS002 and KMS004 are alternative strains for D-(-)-lactic acid production from sugarcane molasses.

Table 4.2 Fermentation profile of metabolically engineered *K. oxytoca* M5a1 strains in AM1 medium containing sugarcane molasses and maltodextrin.

Substrate	Strain	Total sugar utilized (g/L)	D-(-)-lactic acid			Concentration of coproducts[c] (g/L)				
			concentration (g/L)	Yield[a] (g/g)	Productivity[b] (g/L/h)	Succinate	Formate	Acetate	Ethanol	Butanediol
Cane Molasses	Wild type	26.93±0.29[d,€]	1.79±0.43[€]	0.07±0.01[€]	0.02±0.01[€]	1.90±0.66[€]	4.77±0.23	4.65±0.91[€]	4.59±0.67	3.01±1.21[€]
	KMS002	27.98±0.30[€]	24.34±0.45[£]	0.87±0.02[£]	0.25±0.03[£]	1.51±0.75[£]	ND[e]	1.59±0.98[£]	ND	0.87±0.02[£]
	KMS004	27.33±0.69[€]	21.86±0.27[£]	0.80±0.03[£]	0.23±0.02[£]	2.16±0.52[€]	ND	2.74±0.68[¥]	ND	0.91±0.57[£]
Maltodextrin	Wild type	45.49±1.23[€]	4.11±0.33[€]	0.09±0.03[€]	0.04±0.02[€]	1.54±0.75[€]	6.15±2.94[€]	6.59±1.01[€]	9.38±1.93	8.84±1.43[€]
	KMS002	36.50±0.36[£]	33.63±0.65[£]	0.92±0.06[£]	0.35±0.05[£]	1.45±0.06[€]	ND	0.58±0.09[£]	ND	ND
	KMS004	36.35±0.18[£]	32.95±0.65[£]	0.91±0.06[£]	0.34±0.07[£]	2.57±0.32[£]	0.61±0.11[£]	1.34±0.23[£]	ND	0.94±0.47[£]

[a] The lactate yield was calculated as grams of D-(-)-lactic acid produced divided by grams of the total sugars consumed.

[b] The lactate productivity was calculated as lactate concentration produced divided by overall incubation time (96 h)

[c] No 1,3-propanediol was detected in the fermentation broth from all of the strains.

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

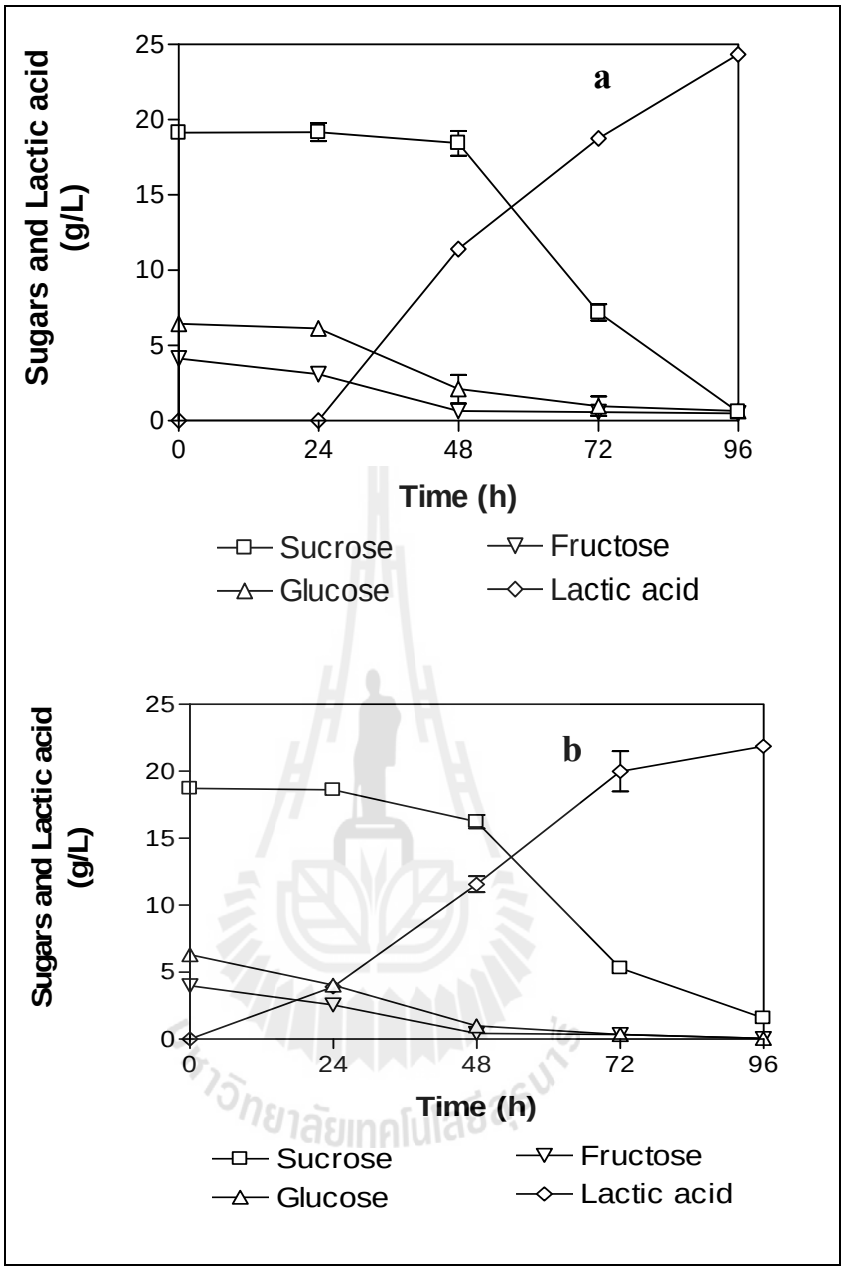

Figure 4.8 Fermentation profile of *K. oxytoca* M5a1 mutants in AM1 medium containing 50 g/L of sugarcane molasses. (a) KMS002 (b) KMS004. The symbols and error bar are average values and standard deviations of at least three measurements, respectively.

Sugar analyses during fermentation indicated that different sugars were fermented at different rates (Fig. 4.8). This result suggested that the presence of glucose and fructose in sugarcane molasses affected sucrose utilization in both KMS002 and KMS004 strains. *Klebsiella* sp. possess the sucrose utilization genes, *scrKYABR*, and sucrose-6-phosphate hydrolase (encoded by *scrB*) enables this microorganism to metabolize sucrose. A LacI-like sucrose regulator (encoded by *scrR*) controls the sucrose utilization in the presence of glucose. The *scrKYABR* operon contains putative cAMP-CrpA binding sites presenting in the -35 regions that could serve as regulatory sites for glucose-repression (Reid and Abratt, 2005). Engels *et al*. (2008) also revealed that fructose greatly inhibited sucrose-6-phosphate hydrolase activity. These findings explain why the rate of sucrose metabolism dramatically increased after the exhaustion of glucose and fructose at 48 h incubation (Fig. 4.8).

Incubation for 96 h was required to complete sugar utilization for D-(-)-lactic acid with a yield of 0.80 to 0.87 g/g sugars consumed (Table 4.2). The growth of both strains was also delayed at the first 24 h of incubation (Fig. 4.8). The production of D-(-)-lactic acid from sugarcane molasses by both strains occurred with an average productivity of 0.23 to 0.25 g/L/h. The average productivity observed from molasses was significantly lower compared with that from glucose (Table 4.1 and 4.2). Shukla *et al*. (2004) revealed that the production of D-(-)-lactic acid from sugarcane molasses by metabolically engineered *E. coli* was delayed compared to glucose alone. This can be attributed to inhibitors present in molasses as well as additional inhibitors that may be produced during sterilization (Chan *et al*., 2012; Liu *et al*., 2008).

4.4 Production of D-(-)- lactic acid from maltodextrin by KMS002 and KMS004

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. The price of maltodextrin is approximately $0.60 per kg, a price that is lower than that of glucose (Sriroth *et al*., 2000).

KMS002 and KMS004 produced D-(-)-lactic acid at concentrations of 33.63±0.65 g/L and 32.95±0.68 g/L respectively after 96 h incubation (Fig. 4.9). The average productivity of D-(-)-lactic acid from maltodextrin observed in both strains was similar to that obtained from glucose. It was surprising that the yield of D-(-)-lactic acid from maltodextrin by both strains was higher (0.90 g/g maltodextrin utilized) than that from glucose alone (Table 4.1 and 4.2). Most of the carbon fluxes were directed to the D-(-)-lactic acid production pathway when maltodextrin was supplied to KMS002 and KMS004, indicating that KMS002 and KMS004 responded with a different the carbon flux partitioning when different substrates were used for cultivation.

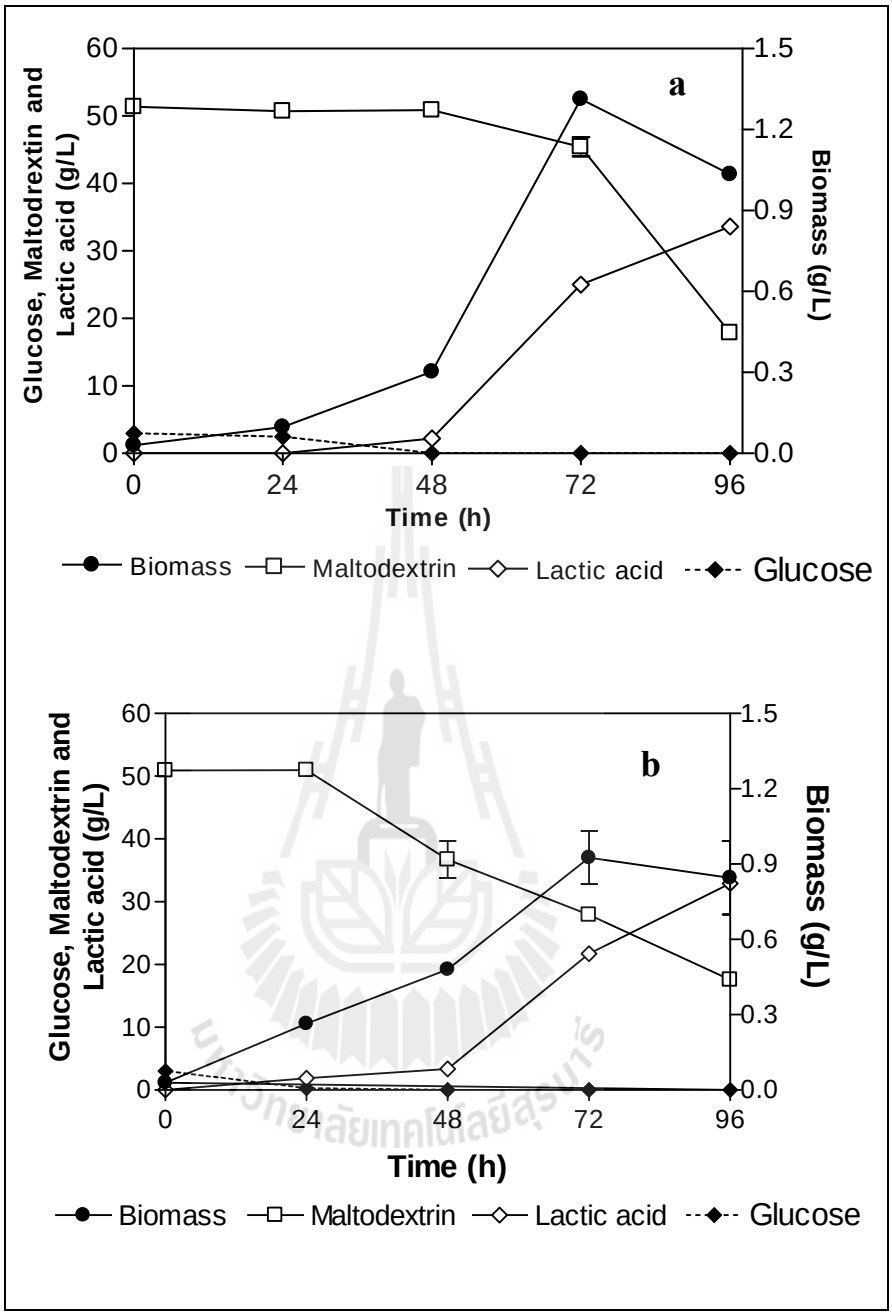

Figure 4.9 Fermentation profile of *K. oxytoca* M5a1 mutants in AM1 medium containing 50 g/L of maltodextrin. (a) KMS002 (b) KMS004. The symbols and error bar are average values and standard deviations of at least three measurements, respectively.

KMS002 and KMS004 did not complete fermentation after 96 h incubation. The concentration of maltodextrin in the fermentation broth of both strains of about 20 g/L remained after 96 h (Fig. 4.9). Like sugarcane molasses, the delay in maltodextrin utilization might result from catabolite repression due to a presence of glucose (approximately 3 g/L) in the maltodextrin used in this study. In *Klebsiella* spp., the maltose/maltodextrin-utilizing (*mal*) system is subjected to catabolite repression since 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. Eppler *et al*. (2002) also revealed that MalK, the ATP-hydrolyzing subunit of the maltose/maltodextrin ABC transporter, interacted with unphosphorylated $EIIA^{Glc}$ of the glucose-specific phosphotransferase system (PTS) to limit transport activity. The interaction not only inhibited MalT activity as a transcriptional activator but also activated Mlc as a transcriptional repressor of *malT*. Thus, maltodextrin consumption started after glucose was depleted by both strains (Fig. 4.9).

Oh *et al*. (2005) revealed that *Enterococcus faecalis* produced lactate from maltodextrin derived from corn flour with yields and titers comparable to those from KMS002 and KMS004. Xiaodong *et al*. (1997) also reported that lactate could be produced from maltodextrin derived from corn starch and cassava starch by *Lb. amylovorous* ATCC 33620 but low titers (4-10 g/L) and yields (0.1-0.4 g/g maltodextrin utilized) were obtained. However, the requirement of yeast extract and peptone means that it is not economical on a large-scale to produce D-(-)-lactic acid by these strains.

4.5 Effect of temperature

The various temperatures were examined in order to find the optimum temperature for D-(-)-lactic acid production. The incubation temperatures were varied at 30, 35, 37, 40 and 42°C. The production of D-(-)-lactic acid was performed in AM1 medium containing 50 g/L glucose. The D-(-)-lactic acid concentrations obtained were 23.53 ± 0.30 g/L, 34.68 ± 0.65 g/L, 37.56 ± 1.09 g/L, 36.28 ± 0.22 g/L and 0.58 ± 0.04 g/L individually at 120 h, respectively (Fig. 4.10).

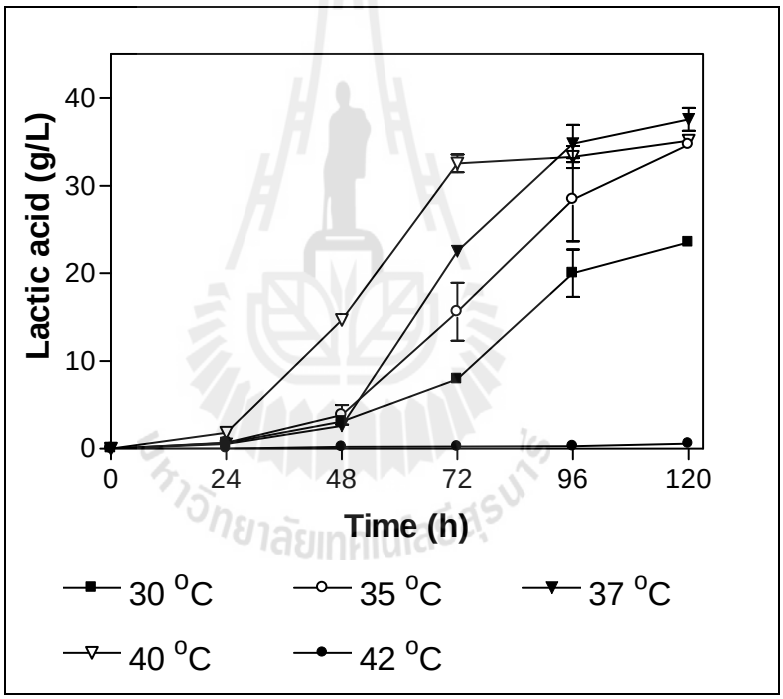

Figure 4.10 Production of D-(-)-lactic acid under various temperature at 30, 35, 37, 40 and 42°C in AM1 medium containing 50 g/L glucose.

At 120 h, the D-(-)-lactic acid concentration at 35, 37 and 40°C were not significantly different in AM1 medium (Table 4.3). In addition, the highest D-(-)-lactic acid concentration was 32.29 ± 0.81 g/L when the temperature was at 40°C.

Here it can be assumed that temperature 37°C was the optimum temperature for cell growth but the optimum temperature was 40°C for D-(-)-lactic acid production. This could be explained that the temperature is also one of the important factors, which influences the activity of metabolic/cell enzymes. However, below and above optimal temperature, the metabolic reaction rate is decreased, which causes the problems in cell metabolism. The optimal temperature for growth of lactic acid bacteria varies between genera from 20 to 45°C (Wood and Holzapte, 1995). Buchta (1983) reported that temperature of 45°C was suitable for lactic acid fermentation by *Lactobacillus delbrueckii* and *Lactobacillus bulgaricus*. *Lactobacillus helveticus* and *Lactobacillus acidophilus* could grow at temperatures ranging from 37-45°C (Wood and Holzapte, 1995). Krischke *et al.* (1991) reported a temperature at 37°C was used for lactic acid production using *Lactobacillus casei*. However, a temperature at 28°C has also been reported to be optimal for *Lactobacillus casei* in a separate study (Nabi *et al*., 2004). A variety of temperatures were reported to be suitable for on D-(-)-lactic acid production, for example, 30°C for *Lactobacillus coryniformis* subsp. *torquens*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc carnosum*, and *Leuconostoc fallax* (Manome *et al*., 1998), 37°C for *Lactobacillus delbrueckii* and *Lactobacillus bulgaricus* (Benthin and Villadsen, 1995; Manome *et al*., 1998; Tanaka *et al*., 2006), 39°C for *Lactobacillus coryniformis* subsp. *torquens* (Yáñez *et al*., 2003) and 40°C for *Lactobacillus delbrueckii* (Calabia *et al*., 2007). According to our results, the temperature at 40°C was suitable for D-(-)-lactic acid production by KMS004 strain.

Table 4.3 Effect of temperature for D-(-)-lactic acid production in AM1 medium containing 5% (w/v) glucose by KMS004 strain.

Temperature (°C)	CDW (g/L)	Total glucose utilized (g/L)	D-(-)-lactic acid			Concentration of co-products[c] (g/L)				
			concentration (g/L)	Yield[a] (g/g)	Productivity[b] (g/L/h)	Succinate	Formate	Acetate	Ethanol	Butanediol
30	0.53± 0.22[d, €]	37.12±0.43[€]	23.53 ± 0.30[€]	0.63±0.08[€]	0.19±0.06[€]	3.70±0.31[€]	ND[e]	1.46±0.03[€]	ND	7.43±0.44[€]
35	0.82± 0.15[£]	50.49±0.78[£]	34.68±0.65[£]	0.68±0.04[£]	0.28±0.03[£]	2.12±0.10[£]	ND	2.64±0.20[£]	ND	6.93±0.12[€]
37	1.19± 0.34[£]	50.12±0.30[£]	37.56±1.09[£]	0.74±0.03[¥]	0.39±0.03[¥]	2.27±0.16[£]	ND	2.41±0.23[£]	ND	5.75±0.31[£]
40	1.04± 0.25[£]	50.31±0.88[£]	36.28±0.22[£]	0.72±0.05[¥]	0.38±0.04[¥]	1.50±0.48[¥]	ND	2.59±0.13[£]	ND	5.56±0.26[£]
42	0.04±0 .09[¥]	1.32±0.13[¥]	0.58±0.04[¥]	0.43±0.03[¢]	0.005±0.03[¢]	0.03±0.06[¢]	ND	0.18±0.08[¥]	ND	0.23±0.01[¥]

[a] The lactate yield was calculated as grams of D-(-)-lactic acid produced divided by grams of the glucose consumed.

[b] The lactate productivity was calculated as lactate concentration produced divided by overall incubation time (120 h)

[c] No 1,3-propanediol was detected in the fermentation broth from all of all strains.

[d] All data represent the averages of two fermentations with standard deviations. Value bearing different Greek symbol are significantly different (P<0.05).

[e] ND = Not detected

4.6 Effect of initial pH

The effect of pH for D-(-)-lactic acid production was evaluated by using the initial pH in the range of 5.5 to 7.5 (5.5, 6.0, 6.5, 7.0 and 7.5). The D-(-)-lactic acid concentrations obtained were at 10.95 ± 0.20 g/L, 22.17 ± 0.37 g/L, 36.30 ± 0.60 g/L, 37.56 ± 0.23 g/L and 36.34 ± 0.69 g/L individually at 120 h, respectively (Fig 4.11). For 96h incubation, the concentration of D-(-)-lactic acid at pH 6.0, 7.0 and 7.5 were not significantly different. Moreover, the concentration of D-(-)-lactic acid at pH 6.5 (36.30 ± 0.60 g/L) was higher than pH 7.0 and 7.5 at 72 h. Wee *et al*. (2005) reported that *Lactobacillus* sp. RKY2 seemed to prefer the acidic conditions for lactic acid production. From our results, the initial pH of D-(-)-lactic acid production at 6.5 for KMS004 was considered to be the optimum pH for the maximum D-(-)-lactic acid production because it utilized base less than pH 7.0 (22.53 ± 0.68 g/L) and 7.5 (34.64 ± 0.90 g/L) in KMS004 strain (Table 4.4). Fu *et al*. (1999) reported that the pH affected at least two aspects of microbial cell such as functioning of its enzymes and the transport of nutrients into the cell. It limits the synthesis of metabolic enzymes responsible for the synthesis of new protoplasm. The pH value also affect RNA and protein synthesis. When microorganisms were grown on either side of their optimum pH range, their lag phases might be increased.

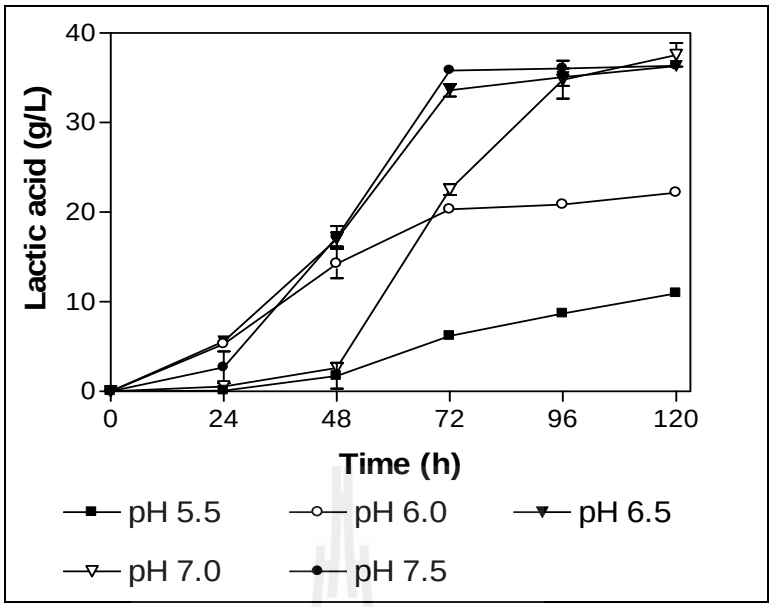

Figure 4.11 Production of D-(-)-lactic acid under various pH at 5.5, 6.0, 6.5, 7.0 and 7.5 into AM1 medium containing 50 g/L glucose.

Manome *et al*. (1998) showed that the optimal conditions for D-(-)-lactic acid production by *Lactobacillus delbrueckii* was at 37°C and pH 6.8. In general, lactic acid bacteria would tolerate pH values between 3.4 and 8.0, but growth and lactic acid production mostly occur between pH 5.4 and 6.4, with the optimum pH being strain-dependent (Kharas *et al*., 1994).

Table 4.4 Effect of pH for D-(-)-lactic acid production in AM1 medium containing 5% (w/v) glucose by KMS004 strain.

pH	CDW (g/L)	Total glucose utilized (g/L)	D-(-)-lactic acid			Concentration of co-products[c] (g/L)				
			concentration (g/L)	Yield[a] (g/g)	Productivity[b] (g/L/h)	Succinate	Formate	Acetate	Ethanol	Butanediol
5.5	0.22±0.32[d, €]	15.59±0.98[€]	10.95 ± 0.20[€]	0.70±0.05[€]	0.08±0.03[€]	1.04±0.14[€]	ND[e]	0.85±0.18[€]	ND	2.54±0.26[€]
6.0	0.45±0.27[£]	27.51±0.33[£]	22.17±0.37[£]	0.80±0.03[£]	0.17±0.02[£]	1.29±0.09[£]	ND	1.14±0.22[€]	ND	2.41±0.23[€]
6.5	0.67±0.24[¥]	48.87±0.26[¥]	36.30±0.60[¥]	0.74±0.07[¥]	0.36±0.05[¥]	2.24±0.03[¥]	ND	2.05±0.28[£]	ND	4.81±0.13[£]
7.0	1.19 ±0.34[¥]	50.12±0.30[¥]	37.56±0.23[¥]	0.74±0.03[¥]	0.39±0.03[¥]	2.27±0.16[¥]	ND	2.41±0.23[£]	ND	5.75±0.31[¥]
7.5	0.86±0.21[¥]	50.59±0.45[¥]	36.34±0.69[¥]	0.71±0.02[€]	0.37±0.02[¥]	1.51±0.16[£]	ND	1.85±0.11[¥]	ND	5.85±0.19[¥]

[a] The lactate yield was calculated as grams of D-(-)-lactic acid produced divided by grams of the glucose consumed.

[b] The lactate productivity was calculated as lactate concentration produced divided by overall incubation time (120 h).

[c] No 1,3-propanediol was detected in the fermentation broth from all of the strains.

[d] All data represent the averages of two fermentations with standard deviations. . Value bearing different Greek symbol are significantly different (P<0.05).

[e] ND = Not detected

4.7 Effect of initial OD_{550}

Initial inoculum sizes were also studied in order to investigate the effect of cell growth on the D-(-)-lactic acid. Inocula at OD_{550} of 0.01, 0.1, 0.5, 1.0 and 2.0 were inoculated into AM1 medium containing 50 g/L glucose for D-(-)-lactic acid production. The D-(-)-lactic acid concentrations obtained were at 33.38 ± 0.82 g/L, 37.56 ± 1.09 g/L, 37.28 ± 1.25 g/L, 38.07 ± 1.17 g/L and 38.73 ± 1.54 g/L individually at 120 hours, respectively (Fig. 4.12 and Table 4.5).

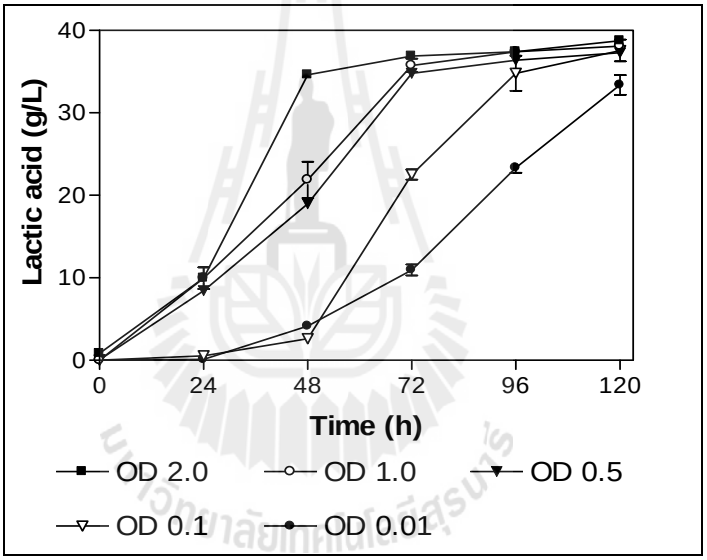

Figure 4.12 Production of D-(-)-lactic acid under various initial OD550 at 0.01, 0.1, 0.5, 1.0 and 2.0 in AM1 medium containing 50 g/L glucose.

At 72 hours, initial OD_{550} at 2.0 and 1.0 could produce D-(-)-lactic acid as 36.88 ± 0.44 g/L and 35.76 ± 1.31 g/L, respectively, while the initial OD_{550} at 0.5, 0.1 and 0.01 produced D-(-)-lactic acid at 34.78 ± 0.63, 22.23 ± 1.37 and 10.94 ± 0.95 g/L, respectively. Since D-(-)-lactic acid is growth associated product, increasing inoculum size always improves productivity. For 72 h incubation, initial OD_{550} at 0.5,

1.0 and 2.0 were not significantly different for D-(-)-lactic acid production. Small inoculum size at 0.01 caused lowest D-(-)-lactic acid production (33.38 ± 0.82 g/L) in term of titer. For 48h incubation time, initial OD_{550} at 2.0 was highest in D-(-)-lactic acid productivity (0.79 ± 0.05 g/L/h) even though high inoculum size was required. Considering to the cost of inoculums preparation and D-(-)-lactic acid titer, the initial OD_{550} at 0.5 could be the optimal initial cell concentration or inoculum for the D-(-)-lactic acid production.

Table 4.5 Effect of initial OD_{550} for D-(-)-lactic acid production in AM1 medium containing 5% (w/v) glucose by KMS004 strain.

OD_{550}	CDW (g/L)	Total glucose utilized (g/L)	D-(-)-lactic acid			Concentration of co-products[c] (g/L)				
			concentration (g/L)	Yield[a] (g/g)	Productivity[b] (g/L/h)	Succinate	Formate	Acetate	Ethanol	Butanediol
0.01	0.76±0.37[d, €]	39.83±0.27[€]	33.38±0.82[€]	0.83±0.04[€]	0.28±0.04[€]	1.05±0.08[€]	ND[e]	1.03±0.21[€]	ND	4.68±0.10[€]
0.1	1.19±0.34[£]	50.12±0.30[£]	37.56±0.23[£]	0.74±0.03[£]	0.39±0.03[£]	2.27±0.16[£]	ND	2.41±0.23[£]	ND	5.75±0.31[£]
0.5	1.22±0.21[£]	50.30±0.32[£]	37.28±1.25[£]	0.74±0.06[£]	0.50±0.04[¥]	2.29±0.09[£]	ND	0.15±0.22[¥]	ND	5.64±0.14[£]
1.0	1.32±0.38[¥]	50.19±0.37[£]	38.07±1.17[£]	0.75±0.09[£]	0.52±0.06[¥]	2.31±0.10[£]	ND	2.57±0.12[£]	ND	6.03±0.35[£]
2.0	1.43±0.33[¢]	50.73±0.17[£]	38.73±1.54[£]	0.76±0.06[£]	0.79±0.05[¢]	2.45±0.31[¥]	ND	2.59±0.12[£]	ND	6.61±0.1[¥]1

[a] The lactate yield was calculated as grams of D-(-)-lactic acid produced divided by grams of the glucose consumed.

[b] The lactate productivity was calculated as lactate concentration produced divided by overall incubation time (120 h).

[c] No 1,3-propanediol was detected in the fermentation broth from all of the strains.

[d] All data represent the averages of two fermentations with standard deviations. Value bearing different Greek symbol are significantly different (P<0.05).

[e] ND = Not detected

CHAPTER V

CONCLUSION

In this study, genes including *adhE* (alcohol dehydrogenase E), *pta* (phosphotransacetylase), and *ackA* (acetate kinase A) were deleted from *K. oxytoca* M5a1 wild type. KMS002 (Δ*adhE*) and KMS004 (Δ*adhE* Δ*pta-ackA*) exhibited D-(-)-lactic acid production as a primary pathway for the regeneration of NAD^+. The outcome of this study was focused on the cost effectiveness of bio-based D-(-)-lactic acid production from cheap medium as well as simple batch operation. Both strains produced 11-13 g/L of D-(-)-lactic acid in the medium containing 20 g/L glucose with yields of 0.64-0.71 g/g glucose used. In sugarcane molasses, KMS002 and KMS004 produced 22-24 g/L of D-(-)-lactic acid with the yields of 0.80-0.87 g/g total sugars utilized. Both strains also utilized maltodextrin derived from cassava starch and produced D-(-)-lactic acid at a concentration of 33-34 g/L with yields of 0.91-0.92 g/g maltodextrin utilized. The highest D-(-)-lactic acid yield (0.72 g/g) was obtained when cultivating KMS004 strain in AM1 medium at 40°C. The effect of various pH, it was found that the highest D-(-)-lactic acid production was obtained with a controlled pH at 6.5. In addition, the initial OD_{550} at 0.5 could be the optimal inoculum for D-(-)-lactic acid production which resulted in 37.28 ±1.25 g/L with productivity of 0.50 g/L/h.

KMS002 (Δ*adhE*) and KMS004(Δ*adhE* Δ*pta-ackA*) strains were able to produce impressive titers and yields of optical pure D-(-)-lactatic acid in a low-cost medium. Fewer by-products were observed in both strains. However, growth-based

selection should be performed to select strains with improved D-(-)-lactatic acid productivity. Other genes involved in NADH re-oxidation under anaerobic fermentation from both strains should be further deleted to direct more carbon flux towards D-lactate. Moreover, Zhou et al. (2005) suggested that the deletions of *frdABCD* and *pflB* in combination with *ackA* and *adhE* deletions could considerably improve lactate yield in *E. coli* SZ63. Zhou et al. (2006) also reported that the metabolic evolution by growth-based selection significantly improved the rate of D-(-)-lactic acid production from glucose and sucrose. Therefore, further works for genetic modifications including deletions of *pflB, frdABCD*, *tdcD*, and *poxB*, and metabolic evolution could be performed in KMS004. The resulting strains would be expected to efficiently produce D-(-)-lactic acid equivalent to those of previously developed *E. coli* strains. The development of *K. oxytoca* M5a1 as a microbial platform for D-(-)-lactic acid production would also be beneficial when cellulose or sugars derived from cellulosic materials such as cellobiose and cellotriose are used as substrates. *E. coli* strains do not usually utilize cellobiose and cellotriose due to mutations in either of the two cryptic operons, *chb* or *asc* (Vinuselvi and Lee, 2011). Therefore, KMS002 and KMS004 would be alternative strains for the development of economic D-(-)-lactatic acid production from renewable substrates.

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APPENDICES

APPENDIX 1

Sequence of plasmid pKJ1002 used for deletion of *adhE* gene

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAA

TGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAA

CGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACT

TTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTC

ACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTC

GGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGCCCTTTTGTTTCC

GCAATGCTATTTAATCCTTTAATTAGCATACCTAATCATTACACTGATCAA

ACAGGTCAAAAAACGACCTGCTGAAAGGTATCAGCTTGGCTGATTATTGA

TTTAGATCACATAAATTGTACCCAGAAGTGAGTAACCTTGTTTACATCCAG

CGAGGAGGCAAAACAGCGGTACCTGAAAGAATAAAGCAAAGCTTTTTTAG

TCAATCGGTGAAGTCAGAAGCACAAAATTCTTTACGTTAACATTTACATGC

AAGCCGTTAACACAATGTCTATACTGTCGAATCGAGCTACTGATTGACTAA

AAAAGTTTAACATTATCAGGAGAGCATTATGGCTGTTACTAATGTCGCTGA

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ATCCCAATGGCATCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCTCA

ATGTACCTATAACCAGACCGTTCAGCTGGATATTACGGCCTTTTTAAAGAC

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CCGCCTGATGAATGCTCATCCGGAATTCCGTATGGCAATGAAAGACGGTG

AGCTGGTGATATGGGATAGTGTTCACCCTTGTTACACCGTTTTCCATGAGC

AAACTGAAACGTTTTCATCGCTCTGGAGTGAATACCACGACGATTTCCGGC

AGTTTCTACACATATATTCGCAAGATGTGGCGTGTTACGGTGAAAACCTGG

CCTATTTCCCTAAAGGGTTTATTGAGAATATGTTTTTCGTCTCAGCCAATCC

CTGG**GTGAGTTTCACCAGTTTTGATTTAAACGTGGCCAATATGGACAA**

CTTCTTCGCCCCCGTTTTCACCATGGGCAAATATTATACGCAAGGCGA

CAAGGTGCTGATGCCGCTGGCGATTCAGGTTCATCATGCCGTTTGTGA

TGGCTTCCATGTCGGCAGAATGCTTAATGAATTACAACAGTACTGCGA

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GGTCGTTAAATAGCCGCTAGATCTAAGTAAATCGCGCGGGTTTGTTAC

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TGACTCCCGCGGATCAAAAATGACGATTGACGGCATTACGTCTAACGA

TATTTACATGCTTGGTTATGTTTCTAATTCTTTAACTGGCCCATACAAG

CCGCTGAACAAAACTGGCCTTGTGTTAAAAATGGATCTTGATCCTAAC

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ACAAACAATCAACGTTTGCGCCAAGCTTCCTGCTGAACATCAAAGGCA

AGAAAACATCTGTTGTCAAAGACAGCATCCTTGAACAAGGACAATTAA

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CGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAAC

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TCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAG

CGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTC

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CAAATACTGTTCTTCTAGTGTACCGTAGTTAGGCCACCACTTCAAGAACTC

TGTAGCACCGCCTACATACCTCCTCTGCTAATCCTGTTACCAGTGGCTGCT

GCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTT

ACCGGATAAGGCGCAGCGGTCGGCTGAACGGGGGGTTCGTGCACACAGCC

CAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGC

TATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCC

GGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGG

GGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTT

GAGCGTCGATTTTTGTGATGCTCGAGGGGGGCGGAGCCTATGGAAAAACG

CCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCA
CATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCC
TTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGA
GTCAGTGAGCGAGGAAGCGGAAG

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

Sequence of plasmid pKJ1003 used for deletion of *adhE* gene

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAA

TGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAA

CGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACT

TTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTC

ACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTC

GGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGC*CCTTTTGTTTCCG*

CAATGCTATTTAATCCTTTAATTAGCATACCTAATCATTACACTGATCAAACAGGT

CAAAAAACGACCTGCTGAAAGGTATCAGCTTGGCTGATTATTGATTTAGATCACA

TAAATTGTACCCAGAAGTGAGTAACCTTGTTTACATCCAGCGAGGAGGCAAAAC

AGCGGTACCTGAAAGAATAAAGCAAAGCTTTTTTAGTCAATCGGTGAAGTCAGA

AGCACAAAATTCTTTACGTTAACATTTACATGCAAGCCGTTAACACAATGTCTATA

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*TATGGCTGTTACTAATGTCGCTGAACTTAACGCACCATGGAGAAAA*AGTTAACA

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TAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCC

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TCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGCTCCCTTTAGGGTTCCG

ATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGG

TTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTT

GGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACT

CAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCG

GCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTT

AACAAAATTCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACACGTAGAA

AGCCAGTCCGCAGAAACGGTGCTGACCCCGGATGAATGTCAGCTACTGGG

CTATCTGGACAAGGGAAAACGCAAGCGCAAGAGAAAGCAGGTAGCTTGC

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GCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGG

GGATCAAGATCTGATCAAGAGACAGGAGAGGATCGTTTCGCATGATTGAA

CAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATT

CGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGT

TCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGT

CCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTG

GCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCG

GGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTC

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GATTTTTGTGATGCTCGAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAA

CGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTC

TTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGT

GAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTG

AGCGAGGAAGCGGAAG

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

Sequence of plasmid pKJ1005 used for deletion of *pta-ackA* genes

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TTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTC

ACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTC

GGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGCCCTTGCCGCCGC

TA*TCGGCGCCATCTGTATCGTATCCGCTGGCTCAATGCCGAGCGCCTCGCTCG*

CGGCCAGCTGTTTCTGTGCCGCCACGGCGCGTGGGCGCTCTTTTTTAGCCGCT

TTCTTTCTCCGCTGCGCGCCACCGTGCCGCTGGTAACCGGCGCCAGCGGCAC

CTCTTTCTGGCATTTTCAGCTCGCCAACGTGCTCCGGGCTGCTCTGGCCGCTGA

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GCTTAATGAATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGT

AATTTTTTTAAGGCAGTTATTGGTGCCCTTAACGCCTGGTGCTACGCC

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TTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCG

CAGCCGAACGACCGAGCGCAGCGAGTCAGTGA GCGAGGAAGCGGAAG

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

Sequence of plasmid pKJ1006 used for deletion of *pta-ackA* genes

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAA

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CAGTGA GCGAGGAAGCGGAAG

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

APPENDIX 2

TECHNICAL PUBLICATION

Sangproo, M., Polyiam, P., Jantama, S.S., Kanchanatawee, S. and Jantama, K. (2012). **Improved D-(-)-Lactate Production by Genetically Modified *Klebsiella axytoca***., Proceedings of the 19th Tri-University International Joint Seminar and Symposium 2012 on October 21-26th, 2012, Bogor, Indonesia

Sangproo, M., Polyiam, P., Jantama, S.S., Kanchanatawee, S. and Jantama, K. (2012). **Metabolic Engineering of *Klebsiella oxytoca* M5a1 to Produce Optically Pure D-Lactate in Mineral Salts Medium**., Bioresource Technology, Vol. 119. pp. 191-198.

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Improved D-(-)-Lactate Production by Genetically Modified *Klebsiella oxytoca*

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Abstract Technology for production of fuels and bioplastics from inexpensive, renewable, and abundantly available biomass resources are currently being developed in order to prevent the exhaustion of oil reveres and reduce global warming. Lactic acid has a long history of use as a specialty chemical in food, pharmaceutical, cosmetic and chemical industries. Recently, polylactic acid (PLA) is considered as an important raw material for bioplastics that can be produced from biomass. Stereocomplex PLA, which is composed of both poly L- and D-lactic acid, made D-lactic acid more attractive by the improvement of its biodegradable plastic properties. As a result, the worldwide market for D-lactic acid is increasing rapidly in the near future. A metabolically engineered *Klebsiella oxytoca* was constructed to produce D-(-)-lactic acid in minimal salts medium under simple batch fermentation. In this study, genes including *adhE* (alcohol dehydrogenase E), *pta* (phosphotransacetylase), and *ackA* (acetate kinase A) were deleted from *K. oxytoca* wild type. The mutant strain exhibited D-(-)-lactic acid as a major product in low-cost medium containing glucose without complex nutrients. Production of D-(-)-lactic acid of mutant strain is approximately six fold higher than that of wild type strain. The results proved that inactivation of *adhE*, *ackA* and *pta* genes led to a significantly improved D-(-)-lactic acid production.

Keywords: D-(-)-lactic acid, metabolic engineering, *Klebsiella oxytoca*

1. Introduction

Lactic acid is an important end product of industrial importance[1]. In the present day, petroleum sources have been decreasing, some microorganisms may represent an alternative for producing lactic acid by fermentation. Fermentation of sugars by lactic acid bacteria is responsible for 90% of current commercial lactic acid, most of which is used in food, agricultural, and medical products[2]. However lactic acid bacteria have complex and expensive nutritional requirements such as yeast extract and peptone that increase costs associated with production, purification, and waste disposal and they may be infected with bacteriophage, resulting in the strain' instability during large scale and continuous production[3]. A potential bacterium used for lactic acid production would exhibit fast growth and no requirements for expensive nutrients.

K. oxytoca is an enteric bacterium found growing in paper and pulp streams as well as around other source of wood. The microorganism is capable of growing at a pH at least as low as 5.0 and temperatures as warm as 35°C. *K. oxytoca* has many advantageous as a production host, such as no requirements of special or expensive source of nutrients during growth and grow on a wide variety of sugars[4] . Moreover, *K. oxytoca* has available techniques for genetic manipulation. Therefore, it would be a new target microorganism to be engineered to alter the metabolic pathway to produce lactic acid.

The strategy of used in most instances was to remove *adhE* (alcohol dehydrogenase E), *pta* (phosphotransacetylase), and *ackA* (acetate kinase A). These genes were eliminated from chromosomal DNA of *K. oxytoca* wild type. In this study, we described the mutant strain that produced D-(-)-lactic acid at high titers in mineral salts medium during simple pH-controlled and batch fermentations without the addition of heterologous genes or plasmids. Therefore, the fermentative production of D-(-)-lactic acid with the mutant strain will be one of choices that are feasible for the industrial scale.

2. Materials and Methods

2.1 Strains, media and growth condition

K. oxytoca was provided by the Microbiology and Cell Science Department, University of Florida, USA. Cultures were grown at 37 °C, 200 rpm in modified Luria-Bertani (LB) broth containing 10 g/L peptone, 5 g/L yeast extract and 5 g/L NaCl. Ampicillin or apramycin (50 μg/ml) and chloramphenicol (40 μg/ml) were added as appropriated.

2.2 Genetic methods

Chromosomal genes (*ahdE* and *pta-ackA*) of wild type strain were deleted without segments of foreign DNA as described previously[5]. *Red* recombinase technology was used to facilitate chromosomal integration[6-7].

2.3 Anaerobic fermentation

The seed inoculums were in AM1 medium containing 2% (w/v) glucose. Fermentations were inoculated at an OD_{550} of seed inoculums. Fermentations were carried out in a 500 ml bottle with a 350 ml working volume at 37 °C, 200 rpm. Broth was maintained at pH 7.0 by automatic addition of 3M KOH. No antibiotics were included during the growth of seed cultures or in fermentation broths.

2.4 Analytical methods

Fermentation samples were removed during fermentation every 24 hours for the analyses of cell mass, organic acid and sugars. Optical density was measured at 550 nm and used as an estimate of cell mass (1 OD_{550} = 0.34 g dry weight/liter)[8]. The fermentative products produced during anaerobic fermentation such as lactic acid, acetic acid, ethanol, formic acid, 2,3-butanediol, and sugars were determined using HPLC (high-performance liquid chromatography) equipped with an ion exchange column (Aminex® HPX-87H, 7.8 × 300 mm, Biorad) and a refractive index detector (RI-150, Thermo Spectra System, USA). The mobile phase used in the HPLC system is 4 mM sulfuric acid at a 0.4 mL/min flow rate was used. A culture collected from the fermentation was centrifuged to discard cell pellet. The supernatant was further passed through a 0.2 µm filter prior to injecting to the HPLC. The 10 µl injection volumes were automatically analyzed. Organic acids and sugars are analyzed according to their molecular weight and structure. The lactic acid enantiomers were analyzed using an Astec Chirobiotic™ R Chiral column, 5 µm, 15 cm × 2.1 mm (Sigma-Aldrich).

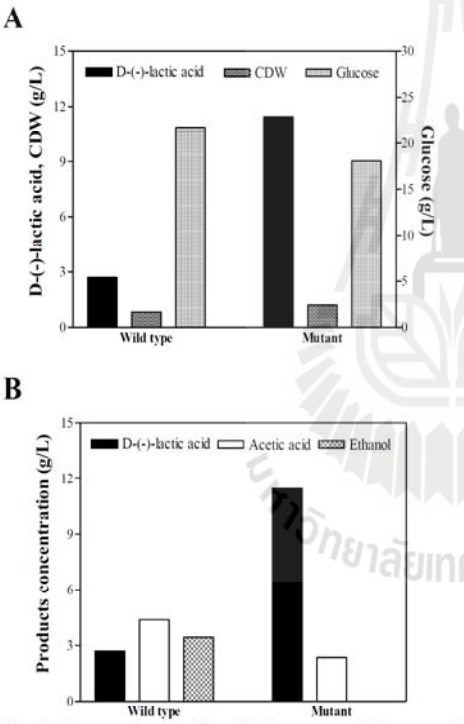

Fig. 1 Fermentation profile of *K. oxytoca* wild type and mutant (Δ*adhE*, Δ*pta-ackA*) strain in AM1 medium containing 2% (w/v) glucose. (A) D-(-)-lactic acid production, cell growth, and glucose consumption in a 500 ml bottle with a 350 ml working volume at 37 °C. (B) Fermentative products

3. Results and Discussion

During glucose fermentation, glucose is converted to organic compounds such as ethanol, lactic acid and acetic acid by *K. oxytoca* wild type strain. The fermentative lactate dehydrogenase (*ldhA*) is a soluble NAD-linked enzyme that converts pyruvate to D-(-)-lactic acid[9]. This reaction consumes one NADH per pyruvate, so recycling the NADH generated during glycolysis[10]. The mutant strain (Δ*adhE*, Δ*pta-ackA*) was tested for D-(-)-lactic acid production under anaerobic conditions in AM1 mineral salts medium containing 2% (w/v) glucose as a sole carbon source. This mutant strain accumulated a significant amount of D-(-)-lactic acid as a major fermentation product. Production of D-(-)-lactic acid of mutant strain was approximately six fold higher than that of wild type strain after 48 h incubation in AM1 medium containing 2% (w/v) glucose under anaerobic condition (Fig. 1A). This result demonstrated that high level of D-(-)-lactic acid could be produced from glucose by mutant strain. In mutant strain, the pathway for D-(-)-lactic acid production remained as the primary route for the regeneration of NAD as compared to the wild type. Under anaerobic conditions, ATP production for growth was obligately coupled to pyruvate kinase by requirement for NADH oxidation through D-(-)-lactic acid production pathway.

In the mutant strain, no ethanol was detected in the fermentation broth (Fig. 1B). This result confirmed that *adhE* activity was successfully abolished from mutant strain. After the deletion of *pta-ackA* in mutant strain, acetic acid production was estimated two fold lower than that of wild type strain (Fig. 1B). The result advised that a level of acetic acid production in mutant strain might stem from the activation of other acetic acid-producing pathways.

4. Conclusions

K. oxytoca was metabolically engineered to produce D-(-)-lactic acid. The mutant strain (Δ*adhE*, Δ*pta-ackA*) produced impressive titers in low-cost medium containing glucose without complex nutrients under simple-batch fermentation. The mutant strain would be an alternative strain for the development of an economic D-(-)-lactic acid production.

Acknowledgements

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Metabolic engineering of *Klebsiella oxytoca* M5a1 to produce optically pure D-lactate in mineral salts medium

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HIGHLIGHTS

- *Klebsiella oxytoca* was engineered to produce optical pure D-lactate with fewer by-products.
- Strains produced D-lactate from sucrose and maltodextrin with impressive yields.
- The fermentation was performed in low salt medium under simple anaerobic conditions.
- The fermentation conditions are expected to lower cost of D-lactate production.
- The costs of downstream processing and waste disposal would be also decreased.

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ABSTRACT

Klebsiella oxytoca strains were constructed to produce optical pure D-lactate by pH-controlled batch fermentation in mineral salts medium. The alcohol dehydrogenase gene, *adhE*, and the phospho-transacetylase/acetate kinase A genes, *pta-ackA*, were deleted from the wild type. KMS002 (Δ*adhE*) and KMS004 (Δ*adhE* Δ*pta-ackA*) exhibited D-lactate production as a primary pathway for the regeneration of NAD^+. Both strains produced 11–13 g/L of D-lactate in medium containing 2% (w/v) glucose with yields of 0.64–0.71 g/g glucose used. In sugarcane molasses, KMS002 and KMS004 produced 22–24 g/L of D-lactate with yields of 0.80–0.87 g/g total sugars utilized. Both strains also utilized maltodextrin derived from cassava starch and produced D-lactate at a concentration of 33–34 g/L with yields of 0.91–0.92 g/g maltodextrin utilized. These D-lactate yields are higher than those reported for engineered *E. coli* strains.

1. Introduction

Lactic acid is one of many chemicals with many potential applications in the food, pharmaceutical, polymer and chemical industries. Lactic acid can be produced by chemical synthesis from petrochemical resources which usually yields the racemic mixture of DL-lactate or by fermentation from renewable feedstocks which can produce D- or L-lactate as major fermentation products depending on the microorganism used (Singh et al., 2006; Wee et al., 2006). Even though lactic acid bacteria produce lactic acid with high titers and productivities, the strains exhibit low growth rates in culture or synthetic media without rich or complex nutrients. Other by-products, such as acetic acid, acetaldehyde, ethanol, and diacetyl, are also formed during fermentation. Lactic acid bacteria have complex and expensive nutritional requirements such as yeast extract and peptone that increase costs associated with production, purification, and waste disposal, and they may be infected with bacteriophage, resulting in the strains' instability during large scale and continuous production. In addition, most lactic acid bacteria produce lactic acid that is less than 95% pure because they possess L- and D-lactate dehydrogenases (Zhou et al., 2006). An ideal bacterium used for lactate production would exhibit fast growth and no requirements for expensive nutrients.

Many attempts have been made to change *Escherichia coli* via metabolic engineering and serial transfer evolution to produce desired fermentative products and a variety of genetic approaches have been used to engineer strains for the production of the optically pure D- or L-lactate. For example, genes encoding fumarate reductase (*frdABCD*), alcohol/aldehyde dehydrogenase (*adhE*), pyruvate formate lyase (*pflB*), and acetate kinase (*ackA*) were inactivated

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in *E. coli*, and the resulting strain, SZ63, produced D-lactate at the concentration of 48 g/L with a yield of 0.96 g/g glucose used in minimal salts medium (Zhou et al., 2003). Zhou et al. (2006) performed metabolic evolution with SZ63 to develop SZ194 that exhibited an improved lactate production of 110 g/L of D-lactate from 12% (w/v) glucose with a yield of 0.95 g/g glucose utilized within 72 h. In addition, Mazumdar et al. (2010) reported that *E. coli* strain LA02 (*frdA*$^-$, *pta*$^-$, *adhE*$^-$) with the additional deletion of the respiratory D-lactate dehydrogenase (*dld*) gene and over-expression of glycerol-utilizing genes produced 32 g/L of D-lactate from 40 g/L of glycerol at a yield of 0.85 g/g glycerol utilized under microaerobic conditions. This strain exhibited maximum volumetric productivity for D-lactate production of 1.5 g/L/h.

The inexpensive production of sugars from cellulosic substrates is an essential step for the fermentative production of renewable chemicals including D-lactate (Aristidou and Penttila, 2000). A mixture of endoglucanase, exoglucanase, and cellobiase (β-glucosidase) is required to convert cellulosic substrates to fermentable sugars. Endoglucanase and exoglucanase enzymes function together to make crystalline and amorphous cellulose soluble. Cellobiase cleaves cellobiose and cellotriose into monomers (Zhou and Ingram, 2001). However, the cost of these enzymes represents a major difficulty for the commercialization of a cellulose-to-chemicals process. Although most *E. coli* strains can utilize a variety of carbon substrates, they cannot metabolize cellobiose and cellotriose (Vinuselvi and Lee, 2011). Unlike *E. coli*, *Klebsiella oxytoca* M5a1 has the ability to utilize a broader range of carbon substrates including cellobiose and cellotriose, thus avoiding the need for cellobiase when converting cellulose into utilizable sugars. *K. oxytoca* M5a1 is also used as a production host for many valued chemicals such as 1,3 propanediol (Huang et al., 2012) and 2,3 butanediol (Jiang et al., 2012). The strains exhibit fast growth under aerobic and anaerobic conditions, and have no special nutritional requirements.

In the present study, the metabolic pathway of wild type *K. oxytoca* M5a1 was altered by the elimination of the chromosomal genes, *adhE* (alcohol dehydrogenase E) and *pta-ackA* (phosphotransacetylase-acetate kinase A) under anaerobic conditions (Fig. 1). The mutant strains produced D-lactate with high titers and yields in AM1 mineral salts medium containing sugarcane molasses and maltodextrin derived from cassava starch during simple, pH-controlled batch fermentations.

2. Methods

2.1. Strains, media and growth condition

Bacterial strains, plasmids, and primers are listed in Table 1. *K. oxytoca* M5a1 was provided by the Microbiology and Cell Science Department, University of Florida, USA. Cultures were grown at 37 °C, 200 rpm in modified Luria–Bertani (LB) broth containing the following constituents per liter of broth: 10 g peptone and 5 g yeast extract with sugars as indicated. Cultures were also maintained on solid media (20 g/L agar). Ampicillin or apramycin (50 μg/mL) and chloramphenicol (34 μg/mL) were included as appropriate. The pCR2.1-TOPO plasmid was used as a cloning plasmid. A modified low salt medium, AM1 (Martinez et al., 2007) supplemented with 1 mM betaine, was used in the fermentations. The neutralizing base used in the anaerobic fermentation experiments was a solution of 3 N KOH.

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. Liquefaction 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. The dextrose equivalent of maltodextrin obtained was in the range of 16–17% (w/w).

2.2. Metabolic engineering of *K. oxytoca*

2.2.1. Construction of plasmids used for deletion of *adhE* and *pta-ackA* genes

Chromosomal genes (*adhE* and *pta-ackA*) of *K. oxytoca* M5a1 were deleted without leaving segments of foreign DNA as de-

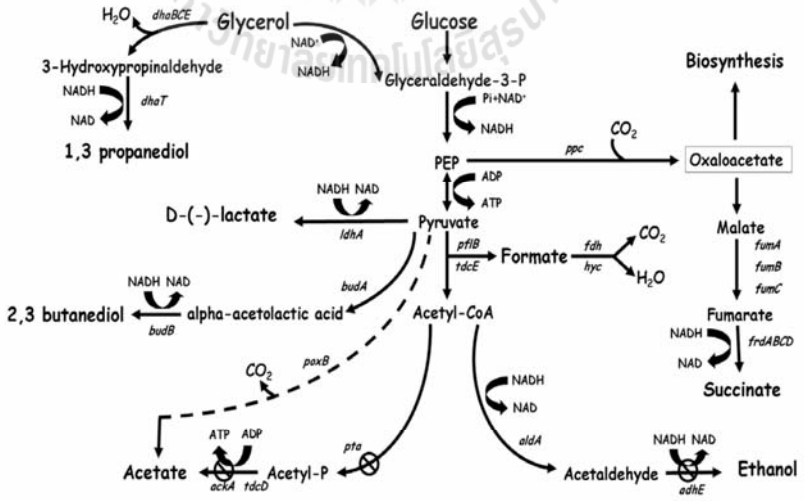

Fig. 1. Fermentation pathway in *K. oxytoca* under anaerobic conditions. Central metabolism indicating genes deleted in constructs engineered for D-lactate production. Solid arrows represent central fermentative pathways. Dashed arrow represents microaerobic pathway (*poxB*). Crosses represent the gene deletions performed in this study to obtain KMS002 (*adhE*) and KMS004 (*adhE*, *pta-ackA*). Genes and enzymes: *ldhA*, lactate dehydrogenase; *pflB*, pyruvate formate-lyase; *pta*, phosphate acetyltransferase; *ackA*, acetate kinase; *adhE*, alcohol dehydrogenase; *ppc*, phosphoenolpyruvate carboxylase; *fumABC*, fumarase isozymes; *frdABCD*, fumarate reductase; *fdh*, formate dehydrogenase; *poxB*, pyruvate oxidase; *budA*, acetolactate synthase, *budB*, butanediol dehydrogenase; *aldA*, aldehyde dehydrogenase isozymes; *dhaBCE*, glycerol dehydratase and *dhaT*, 1,3-propanediol oxidoreductase.

Table 1
Strains, plasmids and primers used in this study.

	Relevant characteristics	Source
Strains		
K. oxytoca	M5a1	Wild-type
E. coli	TOP10F'	Invitrogen
KMS001	M5a1, Δ*adhE*::*cat-sacB*	This study
KMS002	KMS001, Δ*adhE*	This study
KMS003	KMS002, Δ*adhE* Δ*pta-ackA*::*cat-sacB*	This study
KMS004	KMS003, Δ*adhE* Δ*pta-ackA*	This study
Plasmids		
pCR2.1-TOPO	*bla kan*, TOPO TA cloning vector	Invitrogen
pLOI3420	*acc γ β exo* (Red recombinase), temperature-conditional replicon	Wood et al. (2005)
pLOI4162	*cat-sacB* cassette	Jantama et al. (2008a)
pKJ1001	*bla kan*; *adhE* (PCR) from *K. oxytoca* M5a1 (using KO-*adhE*-up/down) cloned into pCR2.1-TOPO	This study
pKJ1002	*cat-sacB* cassette from pLOI4162 (digested with *sfo*I-*Sma*I) cloned into the PCR amplified inside-out product from pKJ1001 (using KO-*adhE*-IO)	This study
pKJ1003	PCR amplified inside-out product from pKJ1001 (using KO-*adhE*-IO) kinase treated then self-ligation	This study
pKJ1004	*bla kan*; *pta-ackA* (PCR) from *K. oxytoca* M5a1 (using KO-*pta-ackA*-up/down) cloned into pCR2.1-TOPO	This study
pKJ1005	*cat-sacB* cassette from pLOI4162 (digested with *sfo*I-*Sma*I) cloned into the PCR amplified inside-out product from pKJ1004 (using KO-*pta-ackA*-IO)	This study
pKJ1006	PCR amplified inside-out product from pKJ1004 (using KO-*pta-ackA*-IO) kinase treated then self-ligation	This study
Primers		
KO-*adhE*-up/down	5′TTGTTTCCGCAATGCTATTT3′ 5′ATTTTGCTGGCGTGGTCCGA3′	This study
KO-*adhE*-IO	5′CACCGGCTAAAGCAGAGAAG3′ 5′GTGCGTTAAGTTCAGCGACA3′	This study
KO-*pta-ackA*-up/down	5′TCAGCACGTCTTTCTGGTTG3′ 5′TTTGGGCAATGGCGCACTCA3′	This study
KO-*pta-ackA*-IO	5′GAGGAGCTACCGCAGTTCAG3′ 5′ACCAACGAAGAGCTGGTCA3′	This study

scribed previously (Jantama et al., 2008a). For the deletion of *adhE*, the *adhE* gene and neighboring regions (*ydhE''-adhE-tdk'*) were amplified using primer set KO-*adhE*-up/down and cloned into the pCR2.1-TOPO vector (Invitrogen) to produce plasmid pKJ1001. The plasmid DNA served as a template for inside-out amplification using the KO-*adhE*-IO primer set (both primers within the *adhE* gene and facing outward). The resulting inside-out PCR product contained a vector-based backbone flanked *adhE* sequences without the central part of *adhE* gene. The resulting fragment was ligated to the *Sfo*I/*Sma*I-digested *cat-sacB* cassette from pLOI4162 to produce pKJ1002 (*ydhE''-adhE'-cat-sacB-adhE''-tdk'*). The inside-out PCR fragment was also kinase-treated and self-ligated to construct a plasmid pKJ1003. In pKJ1003, the central region of *adhE* was omitted (*ydhE''-adhE'-adhE''-tdk'*).

The *pta-ackA* deletions were generated in an analogous manner as the *adhE* deletion. The required primer sets (KO-*pta-ackA*-up/down and KO-*pta-ackA*-IO) are included in Table 1 together with the corresponding plasmids (pKJ1004, pKJ1005, and pKJ1006).

2.2.2. Deletion of adhE and pta-ackA genes in K. oxytoca

To delete *adhE* gene, plasmids pKJ1002 and pKJ1003 served as templates for amplification using the KO-*adhE*-up/down primer set to produce linear DNA fragments for genome integration steps I (*ydhE''-adhE'-cat-sacB-adhE''-tdk'*) and II (*ydhE''-adhE'-adhE''-tdk'*) respectively. The step I fragment was transformed by electrophoresis into wild-type *K. oxytoca* harboring pLOI3420 (Red recombinase). The recombinants were selected on plates containing chloramphenicol (40 mg/L) at 39 °C overnight. The chloramphenicol-resistant clone was designed as KMS001. Strain KMS001 harboring pLOI3420 was further subjected to electroporation with the step II fragment. Cells were incubated at 30 °C for 6 h and transferred into a 250-ml flask containing 100 ml of LB containing 15% (w/v) sucrose. After overnight incubation (30 °C), clones were selected on LB plates containing 10% (w/v) sucrose (39 °C, 16 h). Resulting clones were tested for loss of apramycin, ampicillin, and chloramphenicol resistances. Construction was further confirmed by PCR analysis. A clone lacking the *adhE* gene and *cat-sacB* cassette was selected and designated KMS002.

For deletion of *pta-ackA* genes, plasmids pKJ1005 and pKJ1006 served as templates for amplification using the KO-*pta-ackA*-up/down primer set to produce the linear DNA fragments for integration steps I (*pta'-cat-sacB-ackA''*) and II (*pta'-ackA''*) respectively. Strain KMS002 was used to delete *pta-ackA* genes in a manner analogous to that of the deletion of *adhE*. The resulting strain was designated KMS004.

2.3. Anaerobic fermentations

The seed inoculums were prepared in AM1 medium containing 2% (w/v) glucose. Fermentations were inoculated at an OD_{550} of 0.1 and carried out in a 500- mL bottle with a 350-mL working volume at 37 °C, 150 rpm. The production of D-lactate by KMS002 and KMS004 from various carbon substrates was studied. The pH was controlled at 7.0. The total incubation time was 96 h. No antibiotics were included during the growth of seed cultures or in fermentation broths.

2.4. Analytical methods

Fermentation samples were removed during fermentation every 24 h for the analyses of cell mass, organic acids, and sugars. Cell growth was monitored at 650 nm and converted to biomass as cell dry weight (CDW) by an appropriate calibration curve. The fermentative products produced during anaerobic fermentations such as lactic acid, formic acid, ethanol, acetic acid, 1,3-propanediol, 2,3-butanediol, and sugars were determined using HPLC equipped with an ion exchange column (Aminex® HPX-87H, 7.8 × 300 mm, Bio-Rad) and a refractive index detector (RI-150, Thermo Spectra System, USA). The mobile phase used in the HPLC system was 4 mM sulfuric acid at a flow rate of 0.4 mL/min. A culture collected from

the fermentation was centrifuged to discard the cells. The supernatant was further passed through a 0.2 μm nylon filter prior to injecting into the HPLC. The lactate enantiomers were analyzed using an Astec Chirobiotic™ R Chiral column, 5 μm, 15 cm × 2.1 mm (Sigma–Aldrich).

2.5. Statistical

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 $P < 0.05$.

3. Results and discussion

3.1. Effect of adhE deletion in wild type K. oxytoca M5a1

In many microorganisms including *K. oxytoca*, the reducing power, NADH, which is produced during glycolysis, has to be re-oxidized for the process to continue under anaerobic conditions. In the central anaerobic metabolic pathway, pyruvate is assimilated mainly to re-oxidize NADH via lactate dehydrogenase (LdhA), and alcohol dehydrogenase (AdhE) activities resulting in lactate and alcohol productions respectively. Under anaerobic conditions, pyruvate is reduced to lactate at the expense of 1 mol of NADH. In addition, acetyl-CoA produced from pyruvate is also converted to ethanol under anaerobic conditions at the expenses of 2 mol of NADH (Gottschalk, 1985; Jantama et al., 2008b).

K. oxytoca wild type produces a mixture of lactate, ethanol, 2.3-butanediol, succinate, acetate, and formate as metabolites during glucose fermentation (Fig. 1). Ethanol is produced as a major fermentative product in the wild-type strain (Table 2). Thus the ethanol formation pathway was eliminated from wild type strain by deleting *adhE* to construct KMS002 (Δ*adhE*). KMS002 was tested for D- lactate production under anaerobic conditions in AM1 mineral salts medium containing 2% (w/v) glucose as a sole carbon source. This strain accumulated a significant amount of D-lactate as a major fermentative product at a concentration of 13.13 ± 0.45 g/L with a yield and average productivity of 0.71 ± 0.03 g/g and 0.38 ± 0.03 g/L/h respectively after 72 h incubation. No ethanol was detected in the fermentation broth (Table 2). This result confirmed that AdhE activity was successfully abolished from KMS002. The average productivity of D-lactate increased 3-fold compared to that of the wild-type strain (Fig. 2a and b, Table 2). The results also indicated that the deletion of *adhE* redirected the carbon flux to the lactate production pathway.

The levels of formate (0.14 ± 0.01 g/L), acetate (0.73 ± 0.01 g/L), succinate (0.44 ± 0.05 g/L) and 2,3-butanediol (0.38 ± 0.02 g/L) produced by KMS002 were dramatically decreased compared with those of wild type *K. oxytoca* under anaerobic conditions (Table 2). The results suggested that the accumulated level of NADH generated during glycolysis was re-oxidized by means of LDHA to a greater extent than that of other pyruvate dissimilation routes in KMS002. This outcome was due to the carbon-flux partitioning through pyruvate dissimilation routes via pyruvate-formate lyase (PflB) and acetate kinase (AckA) not maintaining a balanced level of reducing powers, NADH and NAD^+ (Fig. 1). In addition, pyruvate dissimilation via 2,3-butanediol dehydrogenase (BudB) did not competitively re-oxidize NADH in KMS002 since two-step reactions were required for 2,3-butanediol biosynthesis (Fig. 1). Also, a higher affinity for pyruvate of BudB ($K_m^{pyruvate}$ = 8.0 mM) than that of LdhA ($K_m^{pyruvate}$ = 7.2 mM) (Yang et al., 2000) might cause more efficient re-oxidation via the lactic acid production pathway. Celinska (2009) revealed that the high ratio of NADH to NAD^+ generated from high glycolytic flux during an exponential growth phase activates LdhA activity while a low ratio of NADH to NAD^+ causes 2,3-butanediol production in *K. oxytoca*. This was consistent with the production of D-lactate during an exponential phase (Fig. 2b), but the production of 2,3-butanediol was only observed during the stationary phase (after 48 h incubation) in KMS002 (data not shown).

The deletion of *adhE* also significantly decreased the growth rate of KMS002 ($\mu = 0.06\ h^{-1}$) compared to that of wild-type *K. oxytoca* ($\mu = 0.30\ h^{-1}$). The result indicated that the loss of AdhE activity in KMS002 caused a reduction in efficacy of NADH re-oxidation by 50% compared with that of the wild-type strain (Fig. 1). The level of NADH built up in KMS002 during glycolysis resulted in a higher ratio of NADH to NAD^+ which might affect the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) due to a lack of NAD^+ binding that hindered a conformational change of the enzymatic-activation state (Hillman, 1979). This phenomenon was reflected in the delay of the glucose consumption and growth in KMS002 (Fig. 2b). In addition, Zhou et al. (2011) revealed that the low carbon flux through AckA activity due to the high flux through LDHA also caused less ATP production. The delay in glucose consumption and reduced ATP production in KMS002 might result in a prolonged lag period. These findings are in accordance with those of Jantama et al. (2008b) that the deletion of alcohol dehydrogenase caused poor growth and glucose consumption in *E. coli* under anaerobic conditions, but not under aerobic conditions.

3.2. Effect of pta-ackA deletion in KMS002

KMS002 produced the highest amounts of acetate during glucose fermentation (Table 2). A decrease in acetate production may improve the yield of lactate, and the percentage of carbon recovery. Also, downstream processes including purification steps can be simplified due to less by-product contamination. Under anaerobic conditions, wild-type *K. oxytoca* produces acetyl-CoA that is converted to acetyl-P by means of phosphotransacetylase (Pta) activity. Acetyl-P is further converted to acetate primarily by ACKA activity (Fig. 1). Therefore, the deletion of *pta-ackA* in KMS002 was simultaneously performed to construct KMS004 (Δ*adhE*, Δ*pta-ackA*) to reduce acetate formation.

KMS004 produced D-lactate at a concentration of 11.46 ± 0.09 g/L with a yield and productivity of 0.64 ± 0.01 g/g and 0.36 ± 0.01 g/L/h respectively after 48 h incubation in AM1 medium containing 2% (w/v) glucose under anaerobic conditions (Table 2). The titer, yield, and average productivity of D-lactate produced by KMS002 and KMS004 were not greatly different. It is likely that the deletions of *pta-ackA* did not affect the D-lactate production in KMS004 much. However, it was surprising to observe a higher level of acetate for the strain KMS004 (2.36 ± 0.08 g/L) than for KMS002 (0.73 ± 0.01 g/L) even though KMS004 contained deletions of both *pta* and *ackA*. The result implied that an increased level of acetate production in KMS004 might stem from the activation of other acetate-producing pathways such as pyruvate oxidase (PoxB), and propionate kinase (TdcD).

PoxB is responsible for a cell survival during the stationary phase by oxidation of pyruvate resulting in acetate accumulation. An elevated intracellular pyruvate level accumulating during glycolysis usually activates PoxB activity, which directs pyruvate to acetate via decarboxylation (Abdel-Hamid et al., 2001). In KMS004, PoxB activity likely functioned since acetate was also produced not only during exponential growth but also during the stationary phase. Jantama et al. (2008b) demonstrated that PoxB activity was activated when the deletion of *ackA* was introduced in *E. coli*. They also showed that the additional deletion of *poxB* gene in the strain decreased acetate production during anaerobic fermentation. Another enzyme which is suspected to compensate AckA activity in KMS004 is a propionate kinase (TdcD) encoded by *tdcD* (Fig. 1). Based on the protein sequence, TdcD is highly

Table 2
Fermentation profile of metabolically engineered *K. oxytoca* strains in AM1 medium containing 2% (w/v) glucose.

Strain	CDW (g/L)	Total glucose utilized (g/L)	D-lactate			Concentration of co-products[c] (g/L)				
			Concentration (g/L)	Yield[a] (g/g)	Productivity[b] (g/L/h)	Succinate	Formate	Acetate	Ethanol	Butanediol
Wild type	0.84 ± 0.01[d,ε]	21.74 ± 0.17[ε]	2.70 ± 0.29[ε]	0.12 ± 0.02[ε]	0.11 ± 0.01[ε]	0.94 ± 0.10[ε]	3.61 ± 0.18[ε]	4.39 ± 0.27[ε]	3.44 ± 0.28	3.06 ± 0.27[ε]
KMS002	0.82 ± 0.21[ε]	18.22 ± 0.32[ε]	13.13 ± 0.45[ε]	0.71 ± 0.03[ε]	0.38 ± 0.03[ε]	0.44 ± 0.05[ε]	0.14 ± 0.01[ε]	0.73 ± 0.01[ε]	ND[ε]	0.38 ± 0.02[ε]
KMS004	1.21 ± 0.37[ε]	18.08 ± 0.23[ε]	11.46 ± 0.09[¥]	0.64 ± 0.01[¥]	0.36 ± 0.01[ε]	0.46 ± 0.01[ε]	1.99 ± 0.08[¥]	2.36 ± 0.08[¥]	ND	1.44 ± 0.03[¥]

[a] The lactate yield was calculated as grams of D-lactate produced divided by grams of the sugar consumed.
[b] The lactate productivity was calculated as D-lactate concentration produced divided by overall incubation time. The incubation time for wild type, KMS002, and KMS004 were 24, 72, and 48 h, respectively.
[c] No 1,3-propanediol was detected in the fermentation broth from all strains.
[d] All data represent the averages of three fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P < 0.05$).
[e] ND, not detected.

similar to AckA (Reed et al., 2003). The expression of *tdcD* might be activated and TdcD activity might replace AckA activity thus increasing the production of ATP and acetate from acetyl-P and providing a competitive growth advantage. KMS004 also produced higher biomass and finished glucose fermentation within 48 h while KMS002 exhibited a long lag with lower cell yield and finished fermentation after 72 h incubation (Fig. 2b and Fig. 2c). This phenomenon confirmed the TdcD activation compensating the AckA activity, and was also consistent with an impaired growth observed in *E. coli* containing both deletions of *ackA*, and *tdcD*, but not *ackA* or *tdcD* alone. Also the deletion of *tdcD* resulted in a significant reduction in the acetate level during anaerobic fermentation by *E. coli* (Jantama et al., 2008a).

The low level of formate (0.14 ± 0.01 g/L) detected in the fermentation broth of KMS002 (Table 2) suggested that the strain might utilize formate to produce CO_2 and H_2 via formate hydrogen-lyase (encoded by *fdh* and *hyc*) to support growth. The electron produced by the formate hydrogen-lyase reaction generates ATP by proton motive force (Thauer et al., 1977). Axley et al. (1990) revealed that formate serves as a growth substrate in many microorganisms when most the pyruvate serves as an electron acceptor to produce lactate. Therefore, formate produced during fermentation was consumed to compensate for capability deficit in energy production due to low flux through AckA in KMS002. However, this phenomenon likely did not occur in KMS004 since the strain accumulated formate at a significant level (Table 2). The results might also imply that KMS004 did not need to utilize formate for the promotion of growth but produced energy via PoxB and TdcD activities instead. Besides, *tdcE*, located in the same operon as *tdcD* encodes an alpha-ketobutyrate/pyruvate formate-lyase (Reed et al., 2003) exhibiting PflB-like activity. It is likely that the expression of *pflB* and *tdcDE* allowed KMS004 to dissimilate more pyruvate to acetyl-CoA rather than acetate as compared with KMS002. These results were confirmed with the increased productions of acetate (2.36 ± 0.08 g/L) and formate (1.99 ± 0.08 g/L) by KMS004 (Table 2).

KMS004 produced a higher amount of 2,3-butanediol (1.44 ± 0.03 g/L) than KMS002 (0.38 ± 0.02 g/L) from 2% (w/v) glucose. The significant increase in 2,3-butanediol compared with KMS002 might result from the induction of 2,3-butanediol biosynthesis in KMS004. Nakashimada et al. (2000) revealed that the enzymes involved in 2,3-BD biosynthesis were usually induced under acidic conditions. The higher levels of acetate and formate accumulating in the fermentation broth suggested the induction of 2,3-butanediol biosynthesis in KMS004. Van Houdt et al. (2007) showed that the metabolic pathway of 2,3-butanediol played a role in preventing intracellular acidification by changing the metabolism from acid production to the formation of neutral compounds. In addition, 2,3-butanediol biosynthesis together with lactate production have also been regarded as participating in the regulation of the NADH/NAD^+ ratio in KMS004. Thus, a slightly lower in yield of lactate observed in KMS004 was caused by higher carbon fluxes through PflB and BudB activities.

Although the D-lactate yield and productivity of lactic acid bacteria were better than those from the engineered strains in the current study, the optical purity of D-lactate produced by KMS002 and KMS004 was 99.5%. This makes KMS002 and KMS004 gaining benefits over lactic acid bacteria that produce D- or L- lactate with optical purities of less than 95%. Moreover, Zhou et al. (2003) suggested that the deletions of *frdABCD* and *pflB* in combination with *ackA* and *adhE* deletions could considerably improve lactate yield in *E. coli* SZ63. Zhou et al. (2006) also reported that the metabolic evolution by growth-based selection significantly improved the rate of D-lactate production from glucose and sucrose. Therefore, further genetic modifications including deletions of *pflB*, *frdABCD*, *tdcD*, and *poxB*, and metabolic evolution could be performed in KMS004. The resulting strains would be expected to efficiently produce D-lactate equivalent to those of previously developed *E. coli* strains. The development of *K. oxytoca* as a microbial platform for D-lactate production would also be beneficial when cellulose or sugars derived from cellulosic materials such as cellobiose and cellotriose are used as substrates. *E. coli* strains do not usually utilize cellobiose and cellotriose due to mutations in either of the two cryptic operons, *chb* or *asc* (Vinuselvi and Lee, 2011).

3.3. Production of D-lactate from molasses by KMS002 and KMS004

Sugarcane molasses is a waste material from the sugar production industry and is considered as an inexpensive carbon substrate. The sugars in sugarcane molasses (sucrose, glucose, and fructose) can be utilized by *K. oxytoca* to produce low-cost D-lactate. At 96 h, KMS002 and KMS004 produced D-lactate at comparable levels at concentrations of 24.34 ± 0.45 g/L and 21.86 ± 0.27 g/L, respectively from a sugarcane molasses concentration of 50 g/L (about 25 g/L total sugars equivalent) in AM1 medium (Fig. 3a and b). Co-products were produced at low levels in both strains (Table 3). Shukla et al. (2004) produced D-lactate from sugarcane molasses by metabolically engineered *E. coli* SZ63 harboring sucrose-utilizing genes (*cscKBA*) with a comparable yield to that of KMS002 and KMS004. However, the fermentation by SZ63 strain required antibiotics to maintain the expression of *cscKBA*. Even though high concentrations and production yields of D-lactate were obtained, the requirement of antibiotics means that it is not economical on a large-scale to produce D-lactate by SZ63. Since *K. oxytoca* naturally utilizes sucrose, KMS002 and KMS004 are alternative strains for D-lactate production from sugarcane molasses.

Sugar analyses during fermentation indicated that different sugars were fermented at different rates (Fig. 3a and b). This result

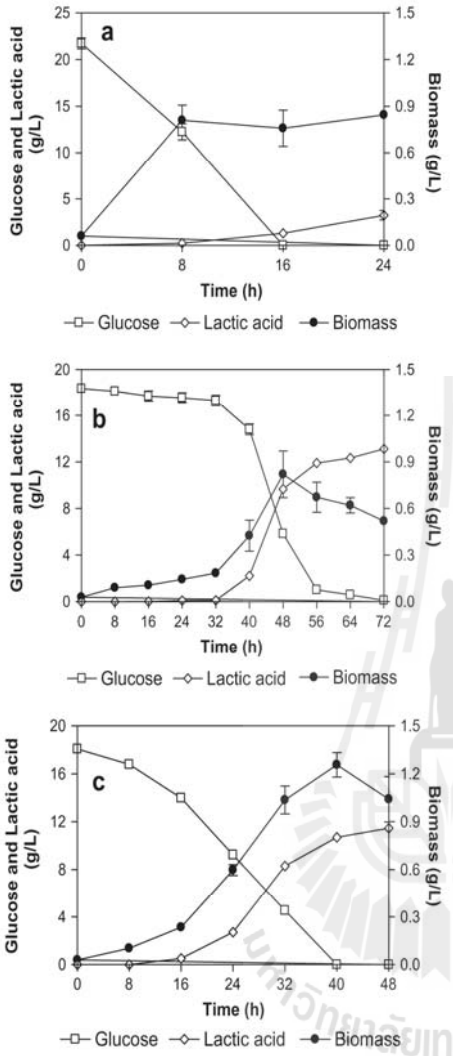

Fig. 2. Fermentation profile of *K. oxytoca* wild type and mutants in AM1 medium containing 2% (w/v) glucose. (a) *K. oxytoca* wild type (b) KMS002 (c) KMS004. The symbols and error bars are average values and standard deviations of at least three measurements, respectively.

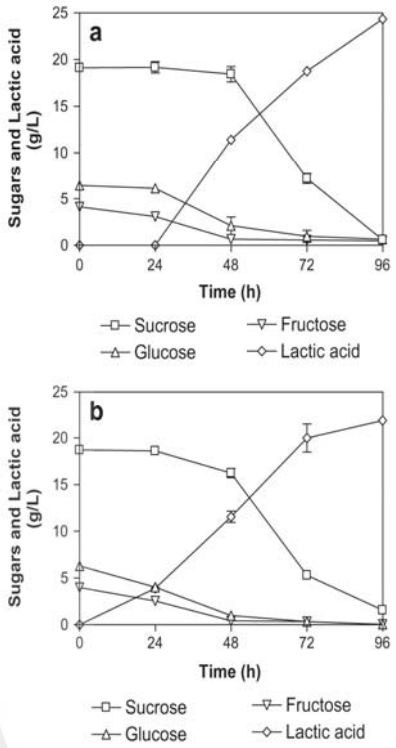

Fig. 3. Fermentation profile of *K. oxytoca* mutants in AM1 medium containing 50 g/L of sugarcane molasses. (a) KMS002 (b) KMS004. The symbols and error bars are average values and standard deviations of at least three measurements, respectively.

suggested that the presence of glucose and fructose in sugarcane molasses affected sucrose utilization in both KMS002 and KMS004 strains. *Klebsiella* sp. possess the sucrose-utilization genes, *scrKYABR*, and sucrose-6-phosphate hydrolase (encoded by *scrB*) enables this microorganism to metabolize sucrose. A LacI-like sucrose regulator (encoded by *scrR*) controls the sucrose utilization in the presence of glucose. The *scrKYABR* operon contains putative cAMP-CrpA binding sites presenting in the −35 regions that could serve as regulatory sites for glucose-repression (Reid and Abratt, 2005). Engels et al. (2008) also revealed that fructose greatly inhibited sucrose-6-phosphate hydrolase activity. These findings explain why the rate of sucrose metabolism dramatically increased after the exhaustion of glucose and fructose at 48 h incubation (Fig. 3a and b).

Incubation for 96 h was required to complete sugar utilization for D-lactate with a yield of 0.80 to 0.87 g/g sugars consumed (Table 3). The growth of both strains was also delayed at the first 24 h of incubation (Fig. 3a and b). The production of D- lactate from sugarcane molasses by both strains occurred with an average productivity of 0.23 to 0.25 g/L/h. The average productivity observed from molasses was significantly lower compared with that from glucose (Tables 2 and 3). Shukla et al. (2004) revealed that the production of D-lactate from sugarcane molasses by metabolically engineered *E. coli* was delayed compared to glucose alone. This can be attributed to inhibitors present in molasses as well as additional inhibitors that may be produced during sterilization (Chan et al., 2012; Liu et al., 2008).

3.4. Production of D-lactate from maltodextrin by KMS002 and KMS004

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*. KMS002 and KMS004 produced D-lactate at concentrations of 33.63 ± 0.65 g/L and 32.95 ± 0.68 g/L, respectively after 96 h incubation (Fig 4a and b). The average productivity of D-lactate from maltodextrin observed in both strains was similar to that obtained from glucose. It was surprising that the yield of D-lactate from maltodextrin by both strains was higher (0.90 g/g maltodextrin utilized) than that from glucose alone (Table 3). Most of the carbon fluxes were directed to the D-lactate production pathway when maltodextrin was supplied to KMS002 and KMS004, indicating that KMS002 and KMS004 responded with a different the carbon flux partitioning when different substrates were used for cultivation.

Table 3
Fermentation profile of metabolically engineered *K. oxytoca* strains in AM1 medium containing sugarcane molasses and maltodextrin.

Substrate	Strain	Total sugar utilized (g/L)	D-lactate			Concentration of coproducts[c] (g/L)				
			Concentration (g/L)	Yield[a] (g/g)	Productivity[b] (g/L/h)	Succinate	Formate	Acetate	Ethanol	Butanediol
Sugarcane Molasses	Wild type	26.93 ± 0.29 [d]	1.79 ± 0.43	0.07 ± 0.01	0.02 ± 0.01	1.90 ± 0.66	4.77 ± 0.23	4.65 ± 0.91	4.59 ± 0.67	3.01 ± 1.21
	KMS002	27.98 ± 0.30	24.34 ± 0.45	0.87 ± 0.02	0.25 ± 0.03	1.51 ± 0.75	ND[e]	1.59 ± 0.98	ND	0.87 ± 0.02
	KMS004	27.33 ± 0.69	21.86 ± 0.27	0.80 ± 0.03	0.23 ± 0.02	2.16 ± 0.52	ND	2.74 ± 0.68	ND	0.91 ± 0.57
Maltodextrin	Wild type	45.49 ± 1.23	4.11 ± 0.33	0.09 ± 0.03	0.04 ± 0.02	1.54 ± 0.75	6.15 ± 2.94	6.59 ± 1.01	9.38 ± 1.93	8.84 ± 1.43
	KMS002	36.50 ± 0.36	33.63 ± 0.65	0.92 ± 0.06	0.35 ± 0.05	1.45 ± 0.06	ND	0.58 ± 0.09	ND	ND
	KMS004	36.35 ± 0.18	32.95 ± 0.65	0.91 ± 0.06	0.34 ± 0.07	2.57 ± 0.32	0.61 ± 0.11	1.34 ± 0.23	ND	0.94 ± 0.47

[a] The lactate yield was calculated as grams of D-lactate produced divided by grams of the sugar consumed.
[b] The lactate productivity was calculated as lactate concentration produced divided by overall incubation time (96 h).
[c] No 1,3-propanediol was detected in the fermentation broth from all strains.
[d] All data represent the averages of three fermentations with standard deviations.
[e] ND, Not detected.

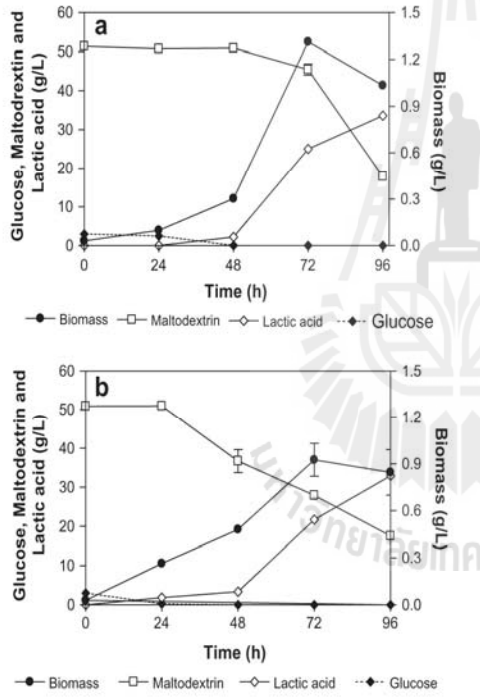

Fig. 4. Fermentation profile of *K. oxytoca* mutants in AM1 medium containing 50 g/L of maltodextrin. (a) KMS002 (b) KMS004. The symbols and error bars are average values and standard deviations of at least three measurements, respectively.

KMS002 and KMS004 did not complete fermentation after 96 h incubation. The concentration of maltodextrin in the fermentation broth of both strains of about 20 g/L remained after 96 h (Fig 4a and b). Like sugarcane molasses, the delay in maltodextrin utilization might result from catabolite repression due to a presence of glucose (approximately 3 g/L) in the maltodextrin used in this study. In *Klebsiella* spp., the maltose/maltodextrin-utilizing (*mal*) system is subjected to catabolite repression since 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. Eppler et al. (2002) also revealed that MalK, the ATP-hydrolyzing subunit of the maltose/maltodextrin ABC transporter, interacted with unphosphorylated $EIIA^{Glc}$ of the glucose-specific phosphotransferase system (PTS) to limit transport activity. The interaction not only inhibited MalT activity as a transcriptional activator but also activated Mlc as a transcriptional repressor of *malT*. Thus, maltodextrin consumption started after glucose was depleted by both strains (Fig. 4a and b).

Oh et al. (2005) revealed that *Enterococcus faecalis* produced lactate from maltodextrin derived from corn flour with yields and titers comparable to those from KMS002 and KMS004. Xiaodong et al. (1997) also reported that lactate could be produced from maltodextrin derived from corn starch and cassava starch by *Lb. amylovorous* ATCC 33620 but low titers (4–10 g/L) and yields (0.1–0.4 g/g maltodextrin utilized) were obtained. However, the requirement of yeast extract and peptone means that it is not economical on a large-scale to produce D-lactate by these strains.

4. Conclusions

K. oxytoca was metabolically engineered to produce D-lactate. KMS002 (Δ*adhE*) and KMS004 (Δ*adhE* Δ*pta-ackA*) strains produced impressive titers and yields of optically pure D-lactate in a low-cost medium containing glucose, sugarcane molasses, and maltodextrin without complex nutrients under simple-batch fermentations. Fewer by-products were observed in both strains. However, growth-based selection should be performed to select strains with improved D-lactate productivity. Other genes involved in NADH re-oxidation under anaerobic fermentation from both strains should be further deleted to direct more carbon flux towards D-lactate. KMS002 and KMS004 would be alternative strains for the development of economic D-lactate production from renewable substrates.

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