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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-

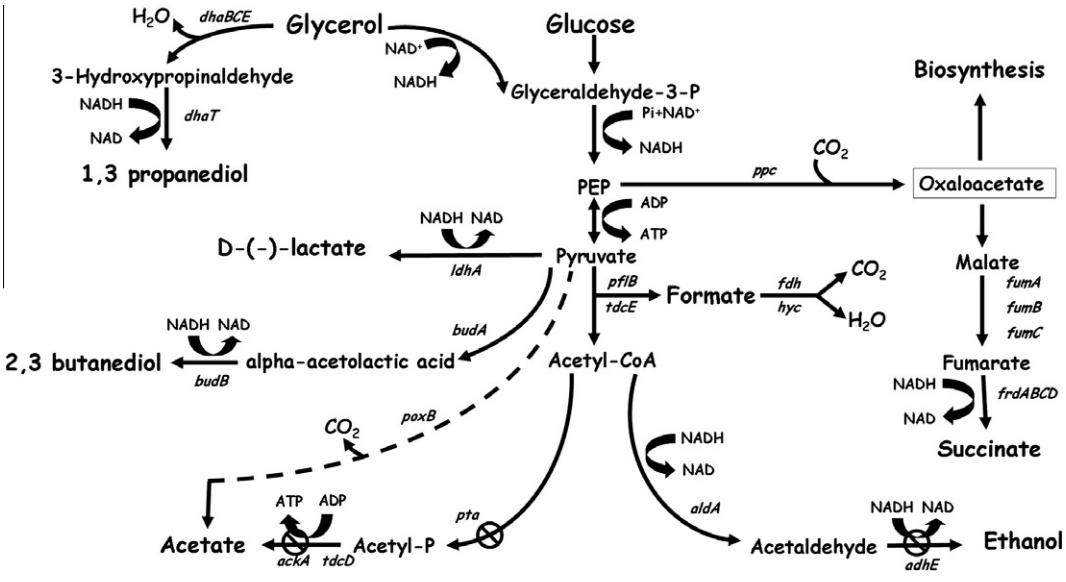

Fig. 1. Fermentation pathway in *K. oxytoca* under anaerobic conditions. Central metabolism indicating genes deleted in constructs engineered for D-lactate production. Solid arrows represent central fermentative pathways. Dashed arrow represents microaerobic pathway (*poxB*). Crosses represent the gene 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 *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 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,€]	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-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

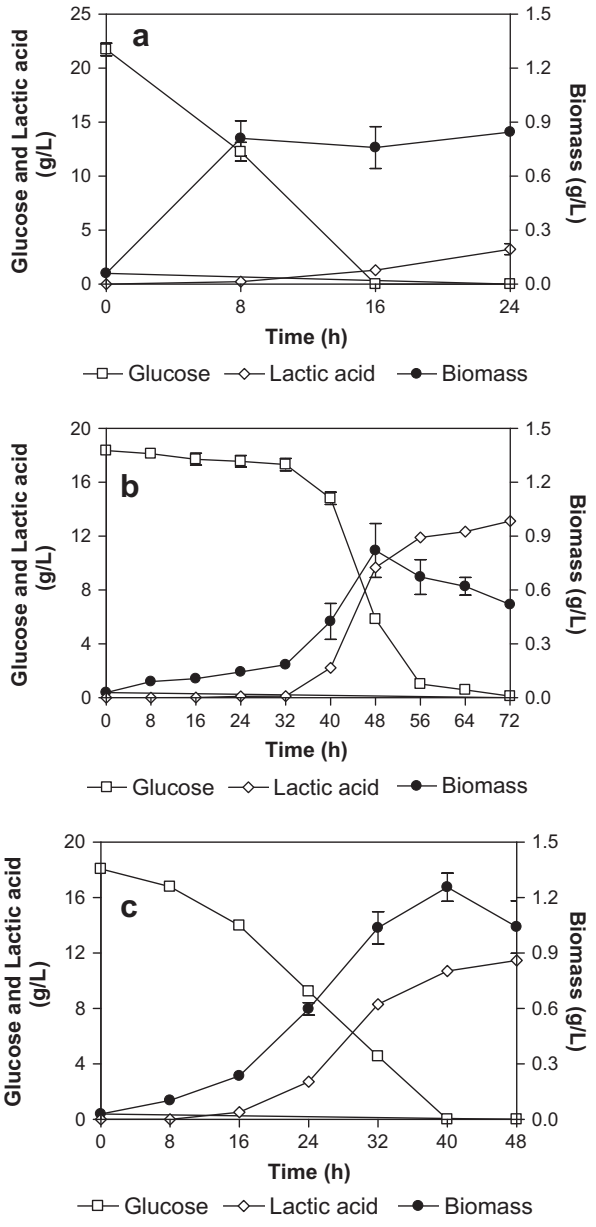

Fig. 2. Fermentation profile of *K. oxytoca* wild type and mutants in AM1 medium containing 2% (w/v) glucose. (a) *K. oxytoca* wild type (b) KMS002 (c) KMS004. The symbols and error bars are average values and standard deviations of at least three measurements, respectively.

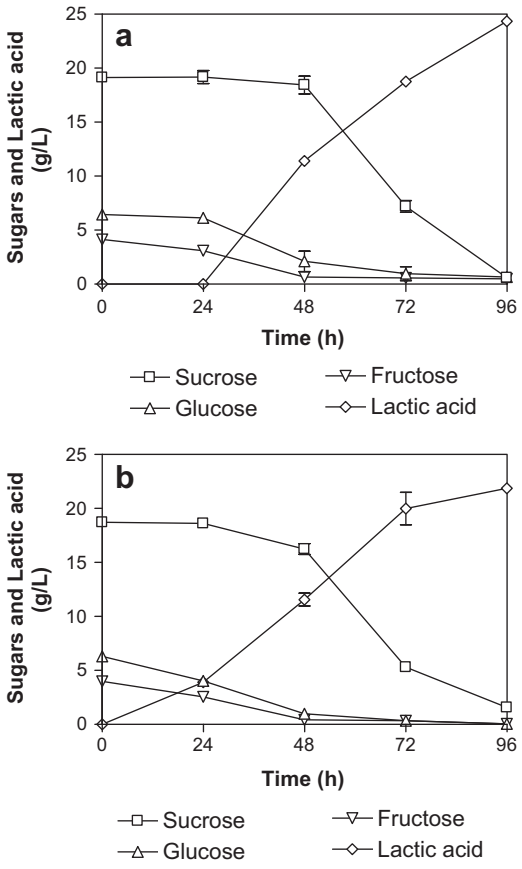

Fig. 3. Fermentation profile of *K. oxytoca* mutants in AM1 medium containing 50 g/L of sugarcane molasses. (a) KMS002 (b) KMS004. The symbols and error bars are average values and standard deviations of at least three measurements, respectively.

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.

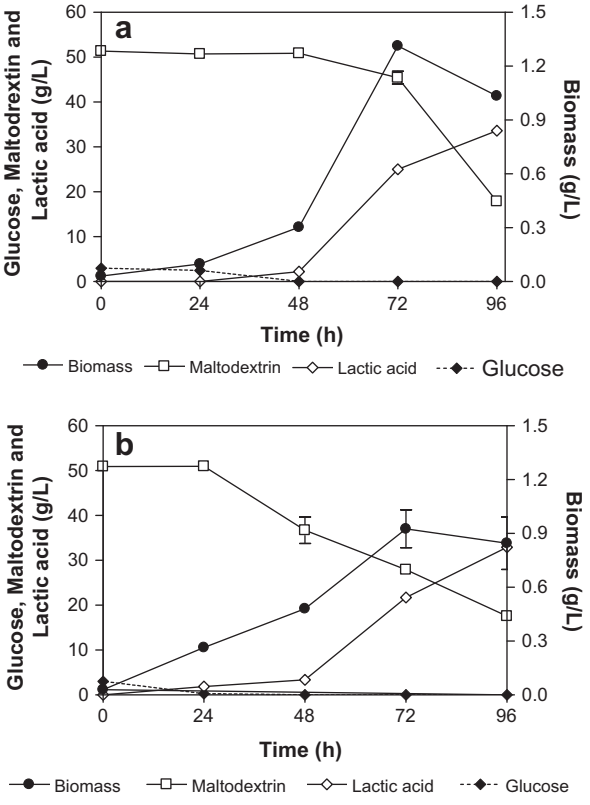

Fig. 4. Fermentation profile of *K. oxytoca* mutants in AM1 medium containing 50 g/L of maltodextrin. (a) KMS002 (b) KMS004. The symbols and error bars are average values and standard deviations of at least three measurements, respectively.

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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