CHAPTER 3
MATERIALS AND METHODS

3.1 Genetic engineering method

3.1.1 Bacterial strains, media, and growth conditions

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

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

Strains	Relevant characteristics	References
E. coli	TOP10F'	Invitrogen
M5Al	*K. oxytoca* wild type	Sangproo et al. (2012)
KMS005	*K. oxytoca* Δ*adhE*Δ*pta-ackA*Δ*ldhA::cat-sacB*	Sangproo et al. (2012)
KMS006	*K. oxytoca* Δ*adhE*Δ*pta-ackA*Δ*ldhA*	Sangproo et al. (2012)
KIS003	*K. oxytoca* Δ*adhE*Δ*pta-ackA*Δ*frd*Δ*pflB::cat-sacB*	In et al. (2020)
KIS004	*K. oxytoca* Δ*adhE*Δ*pta-ackA*Δ*frd*Δ*pflB*	In et al. (2020)
KC001	*K. oxytoca* Δ*adhE*Δ*pta-ackA*Δ*ldhA*Δ*budAB*::*cat-sacB*	This study
KC002	*K. oxytoca* Δ*adhE*Δ*pta-ackA*Δ*ldhA*Δ*budAB*	This study
KC003	*K. oxytoca* Δ*adhE*Δ*pta-ackA*Δ*ldhA*Δ*budAB*Δ*pflB:: cat-sacB*	This study
KC004	*K. oxytoca* Δ*adhE*Δ*pta-ackA*Δ*ldhA*Δ*budAB*Δ*pf*lB	This study
KC005	*K. oxytoca* Δ*adhE*Δ*pta-ackA*Δ*ldhA*Δ*budAB*Δ*pflB* Δ*tdcD::cat-sacB*	This study
KC006	*K. oxytoca* Δ*adhE*Δ*pta-ackA*Δ*ldhA*Δ*budAB*Δ*pflB*Δ*tdcD*	This study

Table 3.1 (continued).

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

Table 3.1 (continued).

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

3.1.2 The DNA amplification by polymerase chain reaction

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

Table 3.2 PCR parameters for the amplification of specific genes.

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

3.1.3 Agarose gel electrophoresis of DNA

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

3.1.4 PCR purification and Gel extraction

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

3.1.5 Preparation of *K. oxytoca* KMS006 competent cell

A single colony of *K. oxytoca* KMS006 was inoculated into 10 mL of LB broth and was incubated at 37 °C and at 200 rpm shaking until reaching the OD_{550} nm in the range of 0.3-0.5. The culture was centrifuged at 4,000 rpm, 4 °C for 10 min. The cell pellet was re-suspended and was washed with 20 mL of ice-cold de-ionized water for 2 times, and with 2 mL of ice-cold 15% glycerol for 1 time. After washing the cell, the white cell pellet was resuspended in 1 mL of sterile ice-cold 15% glycerol. Two hundred microliters of aliquot were dispensed into an electroporation cuvette for used in transformation.

3.1.6 Transformation of *K. oxytoca* KMS006 by electroporation

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

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

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

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

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

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

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

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

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

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

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

3.2 Metabolic evolution

3.2.1 Metabolic adaptation of *K. oxytoca* KC004

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

3.2.2 Metabolic adaptation of *K. oxytoca* KP001

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

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

3.3 Operation and conditions of fermentation

3.3.1 Fermentation medium

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

3.3.2 Batch fermentation in mini controlled-pH fermenter

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

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

Table 3.3 Composition of AM1 medium (excluding carbon source).

Component	**Concentration (mmol/l)**
$(NH_4)_2HPO_4$	19.92
$NH_4H_2PO_4$	7.56
Total PO_4	**27.48**
Total N	**47.39**
Total K	**1.00**
$MgSO_4$ $7H_2O$	1.50
Betaine-HCl[a]	1.00
Trace Elements	**Concentration (µmol/l)**
$FeCl_3$ $6H_2O$	8.88
$CoCl_2$ $6H_2O$	1.26
$CuCl_2$ $2H_2O$	0.88
$ZnCl_2$	2.20
Na_2MoO_4 $2H_2O$	1.24
H_3BO_3	1.21
$MnCl_2$ $4H_2O$	2.50
Total salts	4.1 g/l

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

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

3.3.3 Batch fermentation in 5 L bioreactor

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

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

3.3.4 Batch fermentation in 5 L bioreactor of sugarcane molasse

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

3.3.5 Statistical analytical

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

3.4 Analysis of genes expression level

3.4.1 RNA isolation

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

3.4.2 Genomic DNA removal

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

3.4.3 First strand cDNA synthesis

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

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

3.4.4 Quantification of mRNA

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

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

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

K_{M1}: *K. oxytoca* KMS006

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

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

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

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

3.5 Whole-genome resequencing

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