CHAPTER 2
LITERATURE REVIEW

2.1 Succinic acid and its applications

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

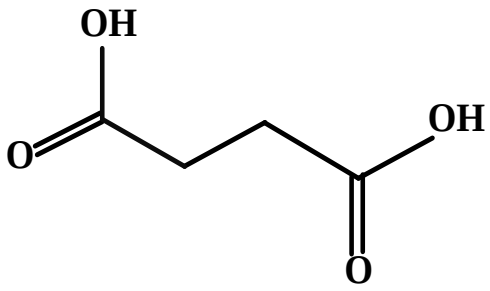

Figure 2.1 Structure of succinic acid.

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

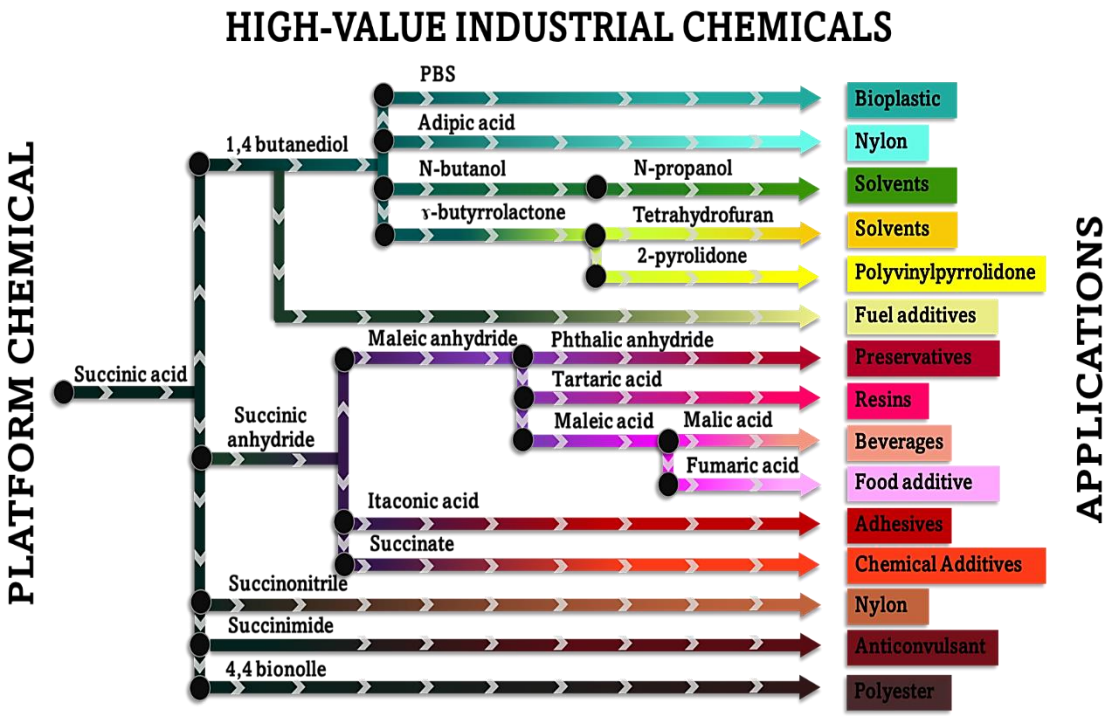

Figure 2.2 Possible production routes to succinate-based products as commodity and specialty chemicals (Zeikus et al., 1999).

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

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

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

2.2 Succinic acid production

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

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

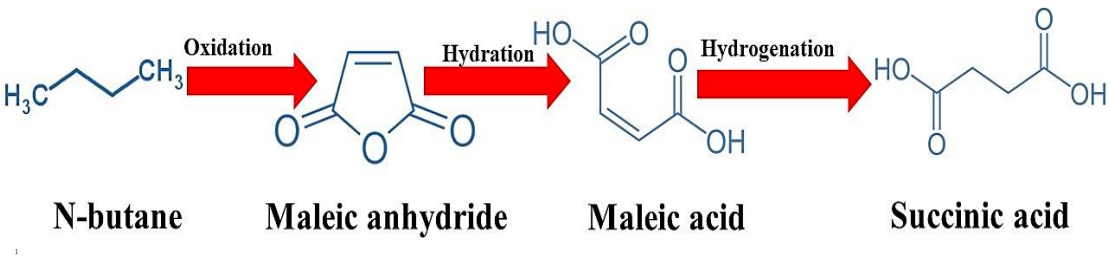

Figure 2.3 Succinic acid production from chemical synthesis (Saxena et al., 2017).

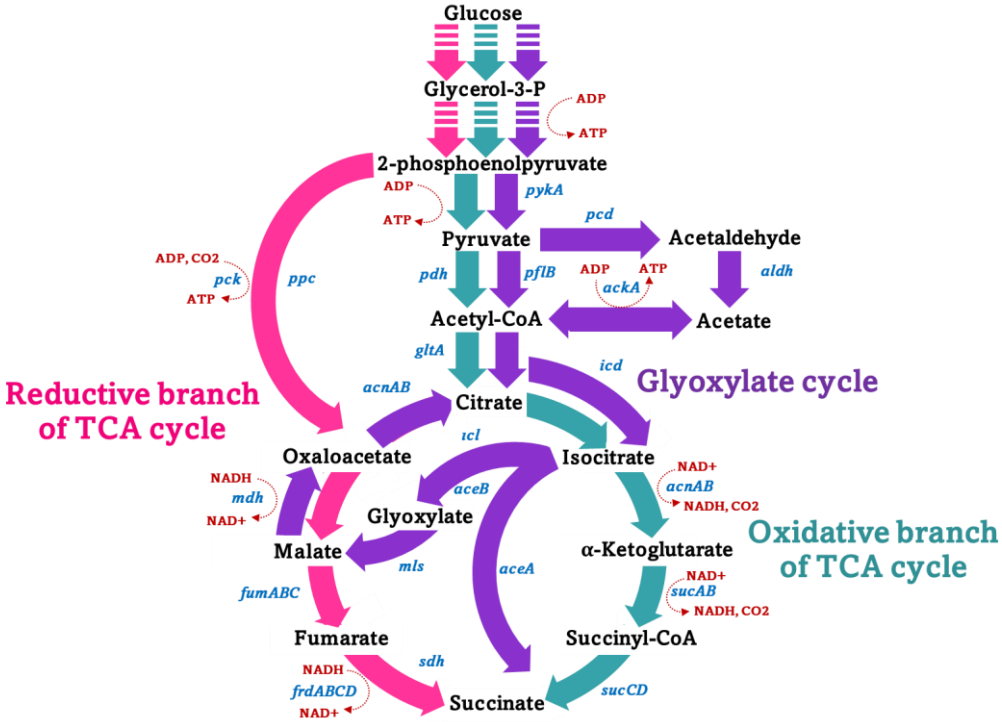

Figure 2.4 Succinic acid pathway in microorganism (McKinlay et al., 2007).

2.3 Microorganisms production of succinic acid

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

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

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

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

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

2.4 *Klebsiella oxytoca*

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

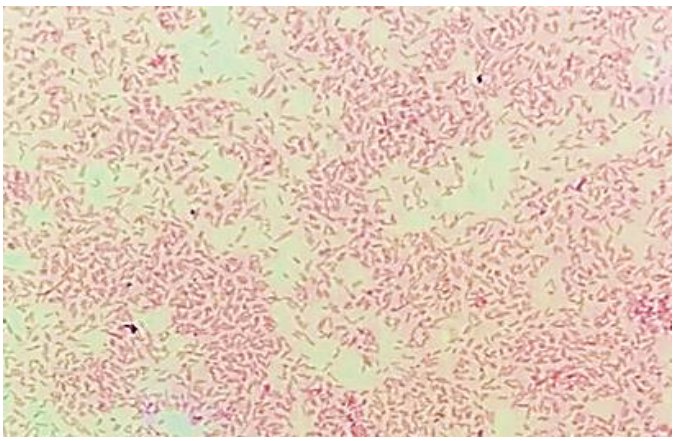

Figure 2.5 Gram strain of *K. oxytoca* M5A1.

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

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

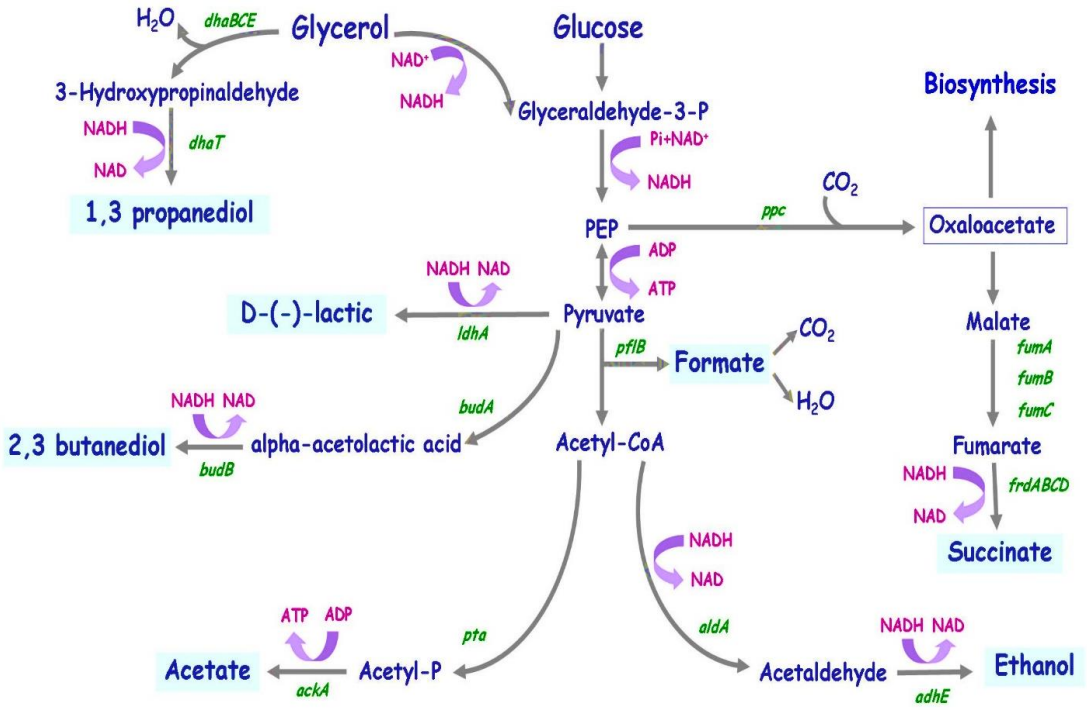

Figure 2.6 Standard pathway of glucose fermentation for *K. oxytoca* under anaerobic conditions.

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

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

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

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

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

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

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

2.6 *Klebsiella oxytoca* KMS006

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

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

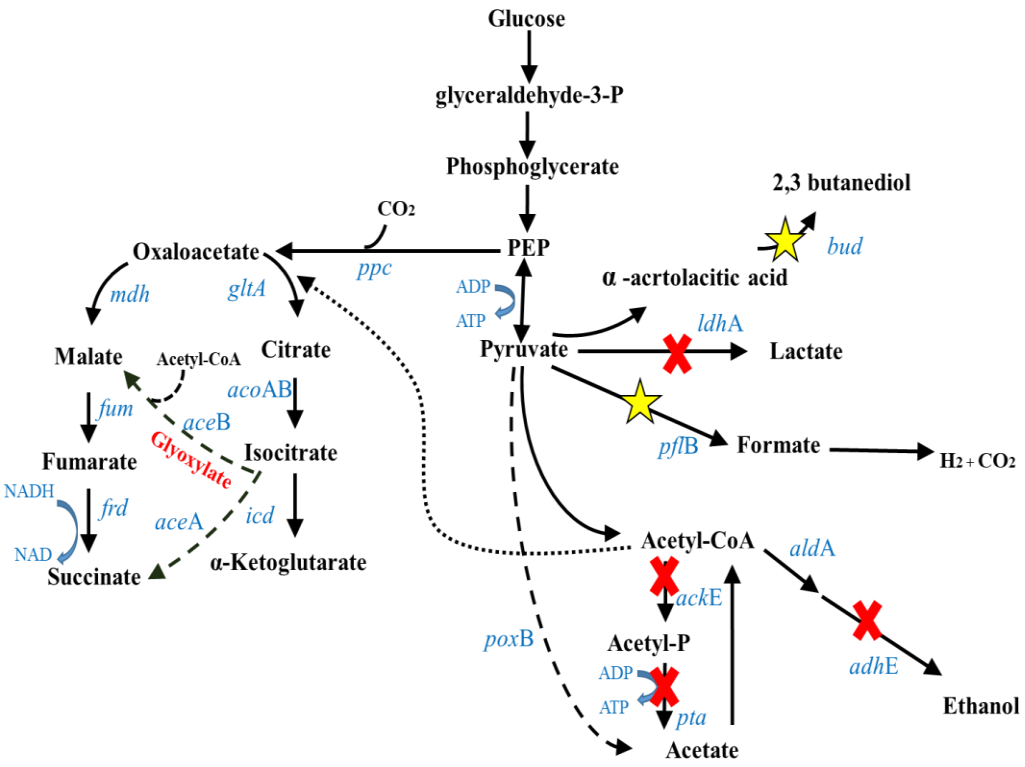

Figure 2.7 Metabolism pathway of *K. oxytoca* KMS006 in anaerobically fermentation. Solid arrows represent central fermentative pathways. Dashed arrow represents microaerophilic pathway to reserved acetate-production. Dotted arrows represent the glyoxylate pathway during aerobic metabolism. The crosses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