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1 **Potential and Limitations of *Klebsiella pneumoniae* as a Microbial Cell Factory Utilizing**
2 **Glycerol as the Carbon Source**
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18 **Abstract**

19 *Klebsiella pneumoniae* is a Gram-negative facultative anaerobe that metabolizes
20 glycerol efficiently under both aerobic and anaerobic conditions. This microbe is considered
21 an outstanding biocatalyst for transforming glycerol into a variety of value-added products.
22 Crude glycerol is a cheap carbon source and can be converted by *K. pneumoniae* into useful
23 compounds such as lactic acid, 3-hydroxypropionic acid, ethanol, 1,3-propanediol, 2,3-
24 butanediol, and succinic acid. This review summarizes glycerol metabolism in *K.*
25 *pneumoniae* and its potential as a microbial cell factory for the production of commercially
26 important acids and alcohols. Although many challenges remain, *K. pneumoniae* is a
27 promising workhorse when glycerol is used as the carbon source.

28 **Key words**: *Klebsiella pneumoniae*; Glycerol; 3-Hydroxypropionic acid; 1,3-Propanediol;
29 2,3-Butanediol; Lactic acid; Ethanol

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1. Introduction

Crude glycerol is produced in large quantities as a byproduct of the biodiesel industry. Global biodiesel production was 22.7 million tons in 2012 and is forecasted to reach 36.9 million tons by 2020. As a result, worldwide production of glycerol climbed from 1 million tons in 2000 to 3 million tons in 2011 and is expected to reach 6 million tons by 2025 (Ciriminna et al., 2014; OECD, 2011; Katryniok et al., 2013; Sun et al. 2017). A significant amount of glycerol is also generated during bioethanol fermentation by yeast. The liquid fraction of the stillage obtained from ethanol separation contains up to 2% glycerol. Another source of glycerol is industrial waste generated from vegetable oils and animal fats. For example, the concentration of glycerol in waste streams from the oleochemical industry is 55%–90% (da Silva et al., 2009; Yazdani and Gonzalez, 2007). The increased availability of glycerol has caused a substantial reduction in its cost. According to a recent report, the price of crude glycerol is $0.24/kg and that of pure USP grade glycerol is $0.9/kg (Kong et al., 2016). Extensive research has been conducted to investigate ways to utilize this surplus crude glycerol. The annual number of research articles addressing the use of glycerol has increased to >7000, doubling in number from the year 2000 to 2007. Numerous chemical conversions to valuable products have been reported (Ciriminna et al., 2014).

Glycerol can be used for the microbial production of 1,3-propanediol (PDO), ethanol, 2,3-butanediol (BDO), butanol, lactic acid, 3-hydroxypropionic acid (3-HP), and succinic acid (Fig. 1) (Clomberg and Gonzalez, 2013; Mattam et al., 2013; Yazdani and Gonzalez, 2007). Because of its reduced nature, glycerol generates twice the number of reducing equivalents per carbon than do traditional carbohydrates (glucose, xylose, and sucrose) when converted into the glycolytic intermediates phosphoenolpyruvate (PEP) or pyruvate (Dharmadi et al., 2006). As a consequence, glycerol gives higher yields of reduced metabolites (e.g., succinate, ethanol, PDO, and butanol) than does glucose (Yazdani and

Gonzalez, 2007). Table 1 compares the maximum theoretical yield of various metabolites, ATP, and redox balances between glycerol and glucose.

Despite its great potential as a carbon source, glycerol is not efficiently metabolized by many microorganisms under anaerobic or oxygen-limited conditions. The ability of *K. pneumoniae* to assimilate glycerol under oxygen-limited conditions is outstanding and has been studied extensively. Several review articles addressing this issue have also been published (Clomberg and Gonzalez, 2013; Mattam et al., 2013; Yazdani and Gonzalez, 2007), but the biotechnological potential and physiological aspects of this strain have not been fully elucidated. This review explores and discusses glycerol metabolism in *K. pneumoniae* and the production of value-added chemicals from glycerol. The potential and challenges of using *K. pneumoniae* as a microbial cell factory are also discussed.

2. Glycerol metabolism in *K. pneumoniae*

Many microorganisms can metabolize glycerol in the presence of external electron acceptors (respiratory metabolism), but relatively few under non-respiratory conditions (without exogenous electron acceptors). Among the latter, genera including *Citrobacter*, *Enterobacter*, *Bacillus*, *Propionibacterium*, *Anaerobiospirillum*, *Klebsiella*, *Clostridium*, and *Lactobacillus* are well documented (da Silva et al., 2009; Yazdani and Gonzalez, 2007). Some *Lactobacillus* species including *L. reuteri*, *L. brevis*, and *L. bucheneri* convert glycerol to PDO but only in the presence of a major carbon source for growth. Glycerol does not support cell growth, limiting its biotechnological applications. In contrast, *Clostridium sp.* grow well on glycerol as the sole carbon source. However, the use of *Clostridium* is limited by its slow growth and requirement of strict anaerobic conditions. The lack of a convenient genetic tool box for large-scale metabolic engineering is another serious drawback of *Clostridium* (Branduardi et al., 2014; Lütke-Eversloh and Bahl, 2011).

From a biotechnological standpoint, *Klebsiella sp.*, especially *K. pneumoniae*, have many advantages over the aforementioned microbes. They grow on glycerol as the sole carbon source under both aerobic and anaerobic conditions (Arasu et al., 2011; Kumar et al., 2012). Their growth is fast, typically growing at a rate >0.9/h, even on glycerol minimal medium (Arasu et al., 2011). They naturally produce coenzyme B_{12}, an essential cofactor for the synthesis of the commercially important chemicals PDO and 3-HP (Huang et al., 2013a; Oh et al., 2012a). Furthermore, their evolutionary and biochemical proximity to *E. coli* allows for the application of most of gene manipulation methods developed for *E. coli* without much modification (Celińska, 2012; Kumar et al., 2013a).

2.1 Overview of glycerol metabolism in *K. pneumoniae*

In *K. pneumoniae*, glycerol is metabolized oxidatively and/or reductively (Ashok et al., 2011, Kumar et al., 2013a). The oxidative pathway provides energy and carbon constituents for the synthesis of cell biomass, while the reductive pathway reduces glycerol and regenerates NAD^+, enabling the oxidative assimilation of glycerol under anaerobic conditions.

Glycerol oxidation proceeds in either a respiratory or a fermentative manner. Respiration requires an exogenous electron acceptor such as oxygen, nitrate, or fumarate (Ashok et al., 2013a,b) and is carried out by enzymes encoded by the *glp* regulon. During respiration, glycerol is first phosphorylated by glycerol kinase (*glpK*) to yield *sn*-glycerol-3-phosphate using ATP or phosphoenolpyruvate (PEP) as a phosphate donor. Next, *sn*-glycerol-3-phosphate is oxidized to dihydroxyacetone phosphate (DHAP) with electron transfer to a quinone molecule, which is linked to the electron transport chain (ETC). This transformation is catalyzed by glycerol-3-phosphate dehydrogenase, which is expressed under both aerobic (*glpD*) and anaerobic (*glpABC*) conditions. In contrast, fermentation of glycerol is conducted without a supply of external electron acceptors, and the reactions are

104 catalyzed by enzymes encoded by the *dha* regulon. Here, glycerol is oxidized to
105 dihydroxyacetone (DHA) by glycerol dehydrogenase (mainly encoded by *dhaD*) using NAD^+
106 as an electron acceptor. DHA then is phosphorylated by dihydroxyacetone kinase, an enzyme
107 encoded by the ATP-dependent *dhaK1/dhaK* and/or PEP-dependent
108 *dhaK2/dhaKLM/dhaK123*. DHAP obtained from respiration and/or fermentation is channeled
109 into the glycolytic pathway (Fig. 2). A variety of organic acids and alcohols, including acetic
110 acid, lactic acid, succinic acid, ethanol, BDO, and formic acid, are produced by the
111 downstream glycolytic pathway (Ashok et al., 2011; Kumar et al., 2013a).

112 The anaerobic growth capability of *K. pneumoniae* using glycerol as a carbon source
113 is attributed to its well-established reductive metabolism. In the reductive pathway, glycerol
114 first undergoes a difficult rearrangement reaction catalyzed by glycerol dehydratase (GDHt)
115 to yield 3-hydroxypropioaldehyde (3-HPA). Catalysis by GDHt requires coenzyme B_{12}, the
116 *de novo* synthesis of which is limited to only few genera of microorganisms. The 3-HPA is
117 subsequently reduced to PDO by at least two 1,3-propanediol oxidoreductases (1,3-PDORs),
118 including NADH-dependent DhaT (PDOR) and NADPH-dependent hypothetical
119 oxidoreductase (HOR), along with the regeneration of $NAD(P)^+$ (Fig. 3). *E. coli* and other
120 *enterobacter sp*. have the oxidative (respiratory) pathway of glycerol assimilation. However,
121 these organisms lack the reductive pathway and cannot grow on glycerol under anaerobic
122 conditions (Dharmadi et al., 2006; Kumar et al., 2013a).

123 **2.2 Genes and enzymes involved in fermentative metabolism of glycerol**

124 The *dha* regulon, induced by the DHA molecule and expressed in the absence of
125 exogenous electron acceptors, encodes numerous genes needed for both the oxidative
126 (fermentative) and reductive pathways (Celińska, 2012; Forage and Foster, 1982) (Fig. 4A).
127 The *dha* regulon of *K. pneumoniae* contains the following genes arranged in the order as
128 appears: *Kpk_0615*/*dhaK*, *dhaM*, *dhaL*, *dhaK*, *dhaD*, *dhaR*, *orfW, CdAT*, *orfX* (*dhaG*), *dhaT*,

129 *orfY, dhaB*, *dhaC*, *dhaE*, *orfZ* (*dhaF*), and *glpF*. Of these, *dhaB*, *dhaC*, *dhaE*, *orfX*, *orfZ*, and
130 *dhaT* are relatively well documented for their functions in the reductive metabolism of
131 glycerol. The *orfW* and *orfY* genes, although commonly found in the *dha* regulons of many
132 organisms, are not as well described. The proteins encoded by the *dha* regulon of *K.*
133 *pneumoniae* have high similarity (80%–95%) with those of *Citrobacter* species but not with
134 those of *Clostridium* species (30%–80%). Surprisingly, the sequence similarity of *dha*
135 proteins among *Clostridium* species is very low in some cases, even lower than their
136 similarity to the corresponding proteins in *K. pneumoniae* or *C. freundii* (Celińska, 2012; Sun
137 et al., 2003; Wei et al., 2014).

138 The complete *dha* regulon is found in only a few dozen species belonging to five
139 different taxonomic groups, including four bacterial species [Actinobacteria, Firmicutes,
140 Fusobacteria, and Proteobacteria (Gammaproteobacteria and Deltaproteobacteria)] and one
141 archaea species, *Halalkalicoccus jeotgali* (Martins-Pinheiro et al., 2016). Interestingly, an
142 incomplete *dha* regulon is present in more than 100 prokaryotes, suggesting that the enzymes
143 of the regulon have other functions not restricted to the anaerobic growth on glycerol. A well-
144 documented example is the one found in *Lactobacillus* species. *Lactobacillus* cannot grow on
145 glycerol as the sole carbon source because it lacks the enzymes for oxidative assimilation of
146 glycerol. However, the enzymes encoded in the incomplete *dha* regulon enable the
147 production of PDO from glycerol, which accelerates cell growth in *Lactobacillus* grown on
148 glucose (Årsköld et al., 2008; Martins-Pinheiro et al., 2016).

149 In the following sections, the major enzymes involved in fermentative glycerol
150 metabolism and their genes are described in detail.

151 **2.2.1 Glycerol dehydrogenase**

152 Encoded by the *dhaD* and/or *gldA* gene, glycerol dehydrogenase is an oxidoreductase
153 that converts glycerol to DHA. The enzyme extracts electrons from glycerol and transfers

them to NAD^+. DhaD plays a major role during the anaerobic growth of *K. pneumoniae* on glycerol. Quantification of mRNA expression indicates that *dhaD* is highly induced when glycerol is used as carbon source. GldA expression is lower than that of DhaD and is thought to serve as a back up to DhaD during anaerobic growth on glycerol. However, DhaD and GldA exhibit a high level of similarity in amino acid sequence and have the same substrate-binding site (Wang et al., 2014).

Recently, DhaD was reported to catalyze the interconversion of acetoin and BDO for NADH disposal and recovery (Wang et al., 2014). The promiscuity of DhaD is responsible for BDO production in the Δ*budC* mutant of *K. pneumoniae*. The contribution by DhaD to or its participation in BDO production is also supported by the observed increase in BDO synthesis and increased concentration of NADH in the presence of the *dhaD* gene. NADH is generated by the oxidation of glycerol and consumed by the production of BDO from acetoin. The dual physiological functions of DhaD allow *K. pneumoniae* to control the intracellular redox level. In response to the intracellular level of NADH, DhaD is able to switch its role from NADH production (glycerol oxidation) to NADH consumption (BDO production) (Wang et al., 2014). The diversion of glycerol flux from organic acids toward alcohols such as BDO also helps the cells to counteract intracellular acidification (Petrov and Petrova, 2009). Thus, this promiscuity confers different physiological roles to the enzyme, which include regulation of the intracellular $NADH/NAD^+$ ratio, prevention of acidification, and storage of carbon and energy (Wang et al., 2014).

2.2.2 Dihydroxyacetone kinases

Dihydroxyacetone kinase (DHAK) phosphorylates DHA to DHAP, an intermediate of the glycolytic pathway. Sun et al. (2003) analyzed the genome of *K. pneumoniae* MGH 78578 and found, in addition to ATP-dependent DhaK I, a PEP-dependent DhaK II. DhaK I is encoded by the *dhaK* (*Kpk_0615*) gene, while DhaK II is encoded by three genes, *dhaK*,

179 *dhaL* and *dhaM,* which correspond to the medium, small, and large subunits of the enzyme,
180 respectively (Celińska, 2012; Sun et al., 2003; Wei et al., 2014). The names *dhaKLM* and
181 *dhaK123* are synonyms used without distinction (Martins-Pinheiro et al., 2016; Sun et al.,
182 2003; Wei et al., 2014). DhaK enzymes are found in many microorganisms. PEP-dependent
183 DhaK II is present in *E. coli*, *L. lactis*, and *C. butyricum*, whereas ATP-dependent DhaK I is
184 present in *Citrobacter freundii* (Garcia-Alles et al., 2004; Raynaud et al., 2011). The PEP-
185 dependent DhaK II of *E. coli*, encoded by *dhaKLM*, is homologous to *dhaK123* (*dhaKLM*) of
186 *K. pneumoniae* (Gutknecht et al., 2001). In a recent study, Wei et al. (2014) further elucidated
187 the physiological roles of DhaK I and DhaK II of the *dha* regulon of *K. pneumoniae*. They
188 found that disruption of ATP-dependent DhaK I had no significant effect on glycerol uptake,
189 indicating that PEP-dependent DhaK II is the major contributor to the conversion of DHA to
190 DHAP. As in *E. coli*, the subunits of DhaK II regulate expression of the *dha* regulon; *dha*
191 regulon expression was suppressed by the disruption of *dhaK1* (*dhaK*) and *dhaK2* (*dhaL*).
192 However, in contrast to *E. coli*, mutation of *dhaK3* (*dhaM*) in *K. pneumonia* did not
193 upregulate the *dha* regulon but rather decreased cell growth and glycerol uptake.

194 **2.2.3 Glycerol dehydratase and reactivating factor**

195 Glycerol dehydratase (GDHt) is the first enzyme in the reductive pathway and
196 catalyzes the dehydration of glycerol to 3-HPA. The same reaction can be carried out by diol
197 dehydratase (DDHt), although the latter has a higher activity on 1,2-propanediol than on
198 glycerol. GDHt requires coenzyme B_{12} or *S*-adenosyl methionine (SAM) as a cofactor. *K.*
199 *pneumoniae* has B_{12}-dependent GDHt only, while strict anaerobes such as *clostridia* sp. have
200 SAM-dependent GDHt. The three subunits of GDHt are encoded by the genes *dhaB1* (~1670
201 bp), *dhaB2* (~590 bp) and *dhaB3* (~430 bp), for which the gene products are the α (large,
202 60.7 kDa), β (medium, 21.3 kDa), and γ (small, 16.1 kDa) subunits, respectively (Xu et al.,
203 2009a). GDHt of *K. pneumoniae* is known to be present as a dimer of heterotrimers, $(\alpha\beta\gamma)_2$.

204 The subunits of GDHt have high homology among *K. pneumoniae* strains; for example, *K.*
205 *pneumoniae* XJPD-Li and U30909 strains showed >99% identity.
206 Coenzyme B_{12} is present in the reaction center and plays an essential role in the
207 catalysis of GDHt. The catalytic cycle often results in the mechanism-based inactivation of
208 coenzyme B_{12} due to the irreversible breakage of the chemical bond between cobalt (Co) and
209 a carbon of the adenosyl moiety. Oxygen accelerates breakage of the Co–C bond. Oxygen is
210 also known to inactivate GDHt, even in the absence of the substrate glycerol. The gene
211 products of *orfZ/dhaG* (small subunit) and *orfX/dhaF* (large subunit) act as reactivating
212 factors of GDHt (GdrAB) (Celińska, 2012; Sun et al., 2003). They reactivate GDHt by
213 catalyzing the exchange of damaged for intact coenzyme B_{12} in the presence of ATP and
214 Mg^{2+}/Mn^{2+} (Celińska, 2012; Kumar et al., 2013a; Maervoet et al., 2011; Sun et al., 2003; Wei
215 et al., 2014). The reactivase is a molecular chaperone that functions as a heterotetramer
216 containing two elongated α subunits (63 kDa) and two globular β subunits (14 kDa).
217 Structurally, the α subunit resembles both GroEL and Hsp70 chaperones, while the β subunit
218 resembles that of the β subunit of glycerol dehydratase, except that it lacks some of the amino
219 acids responsible for coenzyme B_{12} binding (Liao et al., 2003).
220 In some organisms, the GDHt enzyme varies in terms of the number of subunits and
221 their copies (Fig. 4B) (Liu et al., 2010). For example, in *Mesorhizobium loti* and
222 *Mesorhizobium opportunistum*, the large and medium subunits (*dhaB1* and *dhaB2*) are fused
223 together and encoded by a single gene. The gene fusion may arise by frameshift mutations.
224 The active site of the fused enzyme differs slightly from that of unfused enzyme. Moreover,
225 these microorganisms lack the genes encoding the reactivation factor, indicating that the
226 reactivation process may not exist or is carried out by a different route. *Mycobacterium*
227 *smegmatis* has two subunits (fused *dhaB1* + *dhaB2* and *dhaB3*) that are similar to those of *M.*
228 *loti* and *M. opportunistum*; however, the reactivating factor differs in that it has one (large)

229 subunit. No small subunit has been identified in *M. smegmatis*. Fusobacterium *Ilyobacter*
230 *polytropus* is unique in that it has genes coding for both B_{12}-independent (SAM-dependent)
231 and B_{12}-dependent GDHt. The genes for B_{12}-independent GDHt are present in the genomic
232 DNA, while those encoding B_{12}-dependent GDHt along with its reactivation factor are
233 present in two copies, one in the chromosome and the other in a plasmid. Surprisingly, even
234 with this gene redundancy, no significant production of 3-HPA and/or PDO has been reported
235 in *I. polytropus* (Stieb and Schink, 1984). The B_{12}-independent proteins showed no homology
236 with B_{12}-dependent GDHt but significant similarity with the pyruvate formate lyases (PFL)
237 and PFL-activating enzymes and their homologues (Raynaud et al., 2003).

238 **2.2.4 1,3-Propanediol oxidoreductase and hypothetical oxidoreductase**

239 The 1,3-propanediol oxidoreductase (PDOR), responsible for the second step of the
240 reductive pathway of glycerol, catalyzes the conversion of 3-HPA to PDO with the oxidation
241 of NAD(P)H. 3-HPA is a toxic intermediate, and PDOR protects the cell from deleterious
242 damage by reducing 3-HPA to PDO (Celińska, 2012). In *K. pneumoniae*, PDOR is encoded
243 by the *dhaT* gene and is strictly NADH dependent. Active PDOR is a homo-octamer with a
244 monomeric molecular mass of 43 kDa. Despite its importance, the kinetic properties of DhaT
245 were elucidated only recently (Lama et al., 2015). DhaT has high specificity for 3-HPA
246 among various aldehydes. Under physiological conditions at pH 7.0 and 37°C, the catalytic
247 efficiency (k_{cat}/K_m) of the forward reaction (3-HPA to PDO) was found to be 77.2/s. mM,
248 which is 57-fold higher than that of the reverse reaction (1.3/s. mM; PDO to 3-HPA). The K_m
249 (mM) values for 3-HPA and NADH (forward reaction) are 0.77 and 0.03, respectively, while
250 those for PDO and NAD^+ (backward reaction) are 7.4 and 0.23, respectively. Furthermore,
251 the maximum activity of DhaT for the reverse reaction was obtained at pH 9.0 and 55°C.
252 These results strongly suggest that, under physiological conditions, DhaT catalyzes the
253 reduction of 3-HPA to PDO rather than the oxidation of PDO to 3-HPA. Nevertheless, the

254 reverse reaction (PDO→3-HPA) becomes significant as the PDO concentration increases.
255 This factor is considered a serious drawback of using DhaT for PDO production from
256 glycerol.

257 *K. pneumoniae* has several oxidoreductases in addition to PDOR that can reduce 3-
258 HPA to PDO. One such enzyme, designated as hypothetical oxidoreductase (HOR), has been
259 studied extensively and shares 89% identity with YqhD of *E. coli* (Zhu et al., 2009; Zhuge et
260 al., 2010). Expression of *yqhD* in wild-type *K. pneumoniae* is not high, with an mRNA level
261 ~20-fold lower than that of *dhaT*. However, deletion of *dhaT* increases the transcription of
262 *yqhD* by more than 10-fold (Ko et al., 2015). This observation suggests that in addition to its
263 wide-ranging activity on short-chain aldehydes, YqhD is a detoxification enzyme for many
264 aldehydes (Jarboe, 2011). YqhD exhibits negligible activity on PDO (backward reaction),
265 which is an important advantage over DhaT for use in the production of PDO from glycerol
266 (Celińska, 2012; Chen et al., 2011; Zheng et al., 2006). However, YqhD is kinetically inferior
267 to DhaT because of its low catalytic activity (k_{cat}/K_m) on 3-HPA and the requirement of
268 NADPH as a cofactor. The k_{cat}/K_m value of YqhD on 3-HPA (pH 7.0 and 37°C) is estimated
269 to be 2.1/s. mM, which is 36-fold lower than that of DhaT (unpublished data). The use of
270 NADPH as a cofactor prohibits this enzyme from being directly coupled to the reactions
271 catalyzed by DhaD and other enzymes in glycolysis and the TCA cycle, by which NADH is
272 mainly produced. Furthermore, excessive use of NADPH for PDO production can
273 significantly reduce the cellular NADPH level and this can disturb many anabolic reactions
274 requiring NADPH (Wang et al., 2003; Jarboe, 2011). NADH and NADPH differ in a
275 phosphate group only, and it has been suggested that electrostatic interaction of this
276 phosphate is the major factor distinguishing NADH from NADPH for enzymes using these
277 cofactors. Comparison of the amino acid sequences of DhaT and HOR from *K. pneumoniae*
278 and YqhD from *E. coli* has revealed that DhaT contains aspartic acid (Asp) at position 41,

279 while HOR and YqhD have valine (Val) at this position. The steric hindrance and
280 electrostatic repulsion between Asp in the active site and the phosphate group of NADPH
281 impede their binding. In contrast, the hydrophobic amino acid Val poses no such obstruction;
282 thus, YqhD uses NADPH as a cofactor (Ma et al., 2010).

283 Despite their difference in cofactor preference, DhaT and YqhD are similar (44%) in
284 amino acid sequences. *K. pneumoniae* has five enzymes that have at least 40% similarity in
285 amino acid sequence with DhaT and YqhD: putative alcohol dehydrogenase (PaldH), L-1,2-
286 propanediol oxidoreductase (FucO), ethanolamine-utilization enzyme (EutG), propanediol-
287 utilization propanol dehydrogenase (PduQ), and bifunctional alcohol dehydrogenase (AdhE)
288 (Ko et al., 2015). The roles of these enzymes in PDO production have been investigated.

289 **2.2.5 Regulatory protein**

290 The *dhaR* gene product is an important transcription factor that is responsible for
291 expression of the *dhaT* and *dhaB* genes (Sun et al., 2003). This gene is found in *K.*
292 *pneumoniae*, C. *freundii*, and *Clostridium botulinum* but not in every organism containing a
293 complete *dha* regulon (Martins-Pinheiro et al., 2016). The presence of DhaR is thought to
294 allow the *dha* operon to function as a separate regulatory system, independent of fumarate
295 nitrate reduction (FNR). Under anaerobic conditions, FNR acquires DNA-binding properties
296 and induces the expression of many anaerobiosis-related genes. DhaR contains domains for
297 GAF (52-199), PAS (203-267), σ^{54} factor interaction, and histidine HTH-8. The PAS senses
298 internal levels of energy charge, light, oxygen, and redox potential. The HTH-8 domain has a
299 helix-turn-helix conformation and acts as a DNA binding structure. The σ^{54} factor interaction
300 domain interacts with the σ^{54} factor of RNA polymerase and activates RNA transcription
301 from the σ^{54} promoters (Buck et al., 2000; Taylor and Zhulin, 1999). Thus, the DhaR protein
302 triggers the transcription of *dha* genes in response to intracellular levels of several important
303 physiological parameters (Celińska, 2012; Sun et al., 2003). Zheng et al. (2006) investigated

the effect of overexpression of the putative regulatory gene *dhaR* in the *dha* regulon on glycerol metabolism in *K. pneumoniae*. *dhaR* overexpression increased PDOR activity up to 6.7-fold over that of the control wild-type strain, confirming the role of DhaR as a positive regulator of the *dhaT* gene. However, *dhaR* overexpression did not increase the activity of glycerol dehydratase (DhaB). Furthermore, despite the significant increase in PDOR activity, PDO production was unexpectedly low compared with that of the wild type (440 vs. 656.23 mM, respectively). More studies are required to determine the precise role of DhaR and its regulatory function in the *dha* regulon.

DhaR in *E. coli* (70% identity to that of *K. pneumoniae*) has also been studied. This protein stimulated transcription of the *dhaKLM* operon from a σ^{70} promoter. Interestingly, in *E. coli*, DhaL was a positive regulator of the *dhaKLM* operon while phosphorylated DhaM and DhaK were negative regulators of the same operon. DhaK contains the DHA-binding site. DhaL uses ADP as a cofactor for the double displacement of phosphate from DhaM to DHA. DHA is the oxidation product of glycerol metabolism; binding of DHA to DhaK reduces the affinity of DhaK for DhaR. In the presence of DHA, DhaL::ATP is dephosphorylated, leading to the displacement of DhaK by DhaL::ADP, which stimulates DhaR activity. In the absence of DHA, DhaL::ADP is phosphorylated back to DhaL::ATP by the phototransferase system (PTS), thereby inhibiting the binding of the phosphorylated complex (DhaL::ATP) to DhaR. This phosphorylation is mediated by DhaM, providing a phospho-histidine relay between the PTS and DhaL::ADP. This double-check mechanism of binding and turnover increases the selectivity such that the binding of nonphosphorylated compounds does not induce the *dha* operon (Bächler et al., 2005).

2.3 Genes and enzymes involved in respiratory assimilation of glycerol

The *glp* regulon comprises the genes encoding proteins required for the conversion of glycerol to DHAP via sn-glycerol-3-phosphate. The five operons that constitute the *glp*

329 regulon are *glpFK*, *glpD* (anaerobic glycerol-3-phosphate dehydrogenase), *glp ACB* (aerobic
330 glycerol-3-phosphate dehydrogenase), *glpTQ* (glycerol-phosphate
331 permease/glycerophosphodiesterase), and *glpEGR*. The *glpF* gene encodes a cytoplasmic
332 membrane protein that facilitates the diffusion of glycerol into the cell. The *glpE* gene
333 encodes an acidic, cytoplasmic protein of 108 amino acids with a molecular weight of
334 12,082. The *glpG* gene encodes a basic, cytoplasmic membrane-associated protein of 276
335 amino acids with a molecular weight of 31,278 (Lin, 1976; Yang and Larson, 1998; Zeng et
336 al., 1996). The biochemical functions of GlpE and GlpG are unknown. The *glpR* gene
337 encodes the GlpR repressor protein. These five operons are located at three different positions
338 on the chromosome. Transcription of these operons is subject to multiple controls, including
339 catabolite repression mediated by cAMP-CRP and respiratory control mediated by the FNR
340 and ArcA/ArcB systems. Moreover, each of the operons is negatively controlled by a
341 repressor specific for the regulon (Lin, 1987; Zeng et al., 1996).

342 **2.3.1 GlpR and other regulatory proteins**

343 The GlpR repressor protein, predicted to contain 252 amino acids with a molecular
344 weight of 28,048, belongs to the DeoR bacterial regulatory factor super family. Although not
345 studied extensively, GlpR of *K. pneumonia* is expected to have the same biochemical
346 properties and regulatory functions as that of *E. coli*. GlpR negatively controls all the *glp*
347 operons by binding to operators that overlap or are close to the *glp* promoters. In case of *E.*
348 *coli*, glycerol-3-phosphate acts as an inducer and has a high affinity for GlpR (K_d = 31 μM)
349 (Lin, 1987; Weissenborn et al., 1992; Zeng et al., 1996). When complexed with glycerol-3-
350 phosphate, GlpR cannot bind to the operator sites, and repression is relieved. Glycerol-3-
351 phosphate is a product of GlpK and a substrate of GlpD/GlpABC; thus, the absence of GlpD
352 causes a substantial increase in the level of glycerol-3-phosphate, resulting in induction of the
353 *glp* regulon (Fig. 2). The GlpR protein exhibits differential binding affinity for the operators

354 of corresponding operons, as follows: *glpFK* > *glpD* > *glpTQ* > *glpACB*. The *glpFK* promoter
355 appears to be the strongest among the *glp* promoters. Therefore, the maximum strength of the
356 *glpFK* promoter combined with the high affinity of GlpK for glycerol (K_m = 10 μM) should
357 result in sufficient accumulation of the inducer, glycerol-3-phosphate, in the presence of
358 glycerol to relieve repression by GlpR. The relatively lower strength of the *glpD* promoter
359 along with the low affinity of the GlpD enzyme for glycerol-3-phosphate (K_m = 1 mM)
360 prevents excessive degradation of glycerol-3-phosphate that is needed for phospholipid
361 synthesis when the exogenous source of glycerol-3-phosphate becomes limiting, until after
362 accumulation of the inducer (Lin, 1987; Weissenborn et al., 1992). Recently, Jung et al.
363 (2014) investigated the role of GlpR in the aerobic production of 3-HP from glycerol in *E.*
364 *coli*. They found that elimination of this regulatory factor caused increased assimilation of
365 glycerol and higher production of 3-HP and suggested that the deletion of *glpR* led to the
366 upregulation of genes involved in glycerol transport and assimilation. *K. pneumoniae* has
367 several other regulatory genes in addition to *glpR*: the global regulatory genes *hdeB*, *hdeD,*
368 and *yfdX*, with unknown functions and *kvgS* and *kvgA*, encoding two proteins comprising a
369 two-component signal transduction system (Sun et al., 2003).

370 **2.3.2 Glycerol uptake/transport facilitator and other components**

371 The glycerol facilitator GlpF is an integral membrane protein that forms aqueous
372 pores. The GlpF pores selectively allow passive transport of glycerol and other molecules
373 such as DHA and urea across the cytoplasmic membrane, but not water or ions (Heller et al.,
374 1980; Reizer et al., 1993). The GlpF protein contains six membrane-spanning helices that are
375 unique among prokaryotic transport proteins. These transmembrane helices are arranged in
376 two bundles. The GlpF protein may function as a homodimer with the two six-member
377 domains arranged in the membrane to form a channel (Weissenborn et al., 1992). *K.*
378 *pneumoniae* has two glycerol transporter genes, one next to *orfZ/dhaF* in the fermentative

379 *dha* regulon (see Fig. 4A) and the other in the respiratory *glpFK* operon. Both *glpF* genes
380 have a high similarity (85 %) to each other. When one but not both of the *glpF* genes is
381 disrupted, the *glpF* deletion mutants of *K. pneumoniae* can consume glycerol and convert it to
382 PDO at a high rate, similar to the wild type (unpublished data; Supplementary Fig. S1). This
383 observation indicates that both *glpF* genes encode active glycerol transporter proteins.
384 However, their presence is not essential for glycerol utilization in *K. pneumoniae* because the
385 double-deletion mutant for both facilitators can still uptake glycerol and convert it to PDO. In
386 *E. coli*, only one *glpF* gene is present in the *glpFK* operon; the GlpF protein shows a high
387 similarity (80.9%) to that (encoded from *glpFK*) of *K. pneumoniae* (Sun et al., 2003). The
388 importance of GlpF in *E. coli* also seems to be limited only at low levels of glycerol (<8
389 mM), as glycerol itself may pass through the membrane by diffusion at high concentrations
390 (Richey and Lin, 1972).

391 **2.3.3 Simultaneous operation of fermentative and respiratory pathways**

392 The presence of the *dha* and *glp* regulons affords *K. pneumoniae* metabolic flexibility.
393 The *dha* regulon enables fermentative utilization of glycerol, while the *glp* regulon enables
394 respiratory metabolism of glycerol. During fermentative metabolism of glycerol, redox
395 constraints force the production of PDO from glycerol, with a low ratio of carbon conversion
396 yield to cell biomass. Moreover, ATP is formed through substrate-level phosphorylation, and
397 NADH oxidation occurs through the formation of reduced metabolites. In the presence of
398 oxygen, the redox constraints forcing the production of PDO are relieved; therefore, the
399 carbon loss required for regeneration of NAD^+ is greatly reduced. As a consequence, glycerol
400 utilization, biomass synthesis, and even the formation of many metabolites increase (Durnin
401 et al., 2008). Interestingly, the fermentative route (*dha* regulon) is in operation under a wide
402 range of oxygen levels, from microaerobic to highly aerobic conditions, as evidenced by
403 PDO formation. For example, a high titer PDO (102.7 g/L) was produced under the condition

404 of 2.0 vvm aeration and even at 3.5 vvm, although the production was a little lowered in the
405 latter case (Oh et al., 2012a). These results suggest that the fermentative and respiratory
406 pathways might have evolved to complement each other for optimal control of cell growth
407 under a wide range of aeration conditions except the strictly anaerobic one.

408 **2.4 Carbon catabolite repression**

409 Carbon catabolite repression (CCR) refers to the suppression of the use of less-
410 preferred carbon sources, when a more preferred carbon, most prominently glucose, is
411 present. Cyclic AMP, cAMP receptor protein (CRP), and the enzyme $EIIA^{Glc}$, an
412 intermediate in the phosphorylation cascade of the PTS, are key players in the CCR of
413 enterobacteria. The $EIIA^{Glc}$, a cytosolic protein, exists in phosphorylated and
414 unphosphorylated forms. The phosphorylated form stimulates membrane-bound adenylate
415 cyclase, which catalyzes the formation of cAMP; the cAMP-CRP complex then induces
416 expression of the catabolic genes of less-preferred carbon sources. The unphosphorylated
417 form of $EIIA^{Glc}$ is also responsible for inhibiting the transport of less-preferred carbon
418 sources by inhibitory binding to permeases, causing so-called inducer exclusion (Eppler and
419 Boos, 1999; Eppler et al., 2002). Glycerol assimilation in *K. pneumoniae* is greatly inhibited
420 by CCR in the presence of glucose. This repression is related to not only low cAMP and
421 cAMP-CRP but also inhibition of GlpK (which functions mainly under aerobic conditions)
422 by fructose 1,6-bisphosphate, a metabolite produced from glucose. The unphosphorylated
423 $EIIA^{Glc}$ is also known to allosterically inhibit GlpK (Holtman et al., 2001). Anaerobic
424 glycerol metabolism mediated by the *dha* operons is also suppressed by the presence of
425 glucose. Enzyme-level inhibition similar to that of the aerobic GlpK has not been reported,
426 but we have observed that, in *K. pneumoniae*, transcription of the *dha* operons is greatly
427 reduced in the presence of glucose (Suman et al., 2017).

428 Interestingly, glycerol exerts catabolite repression on the assimilation of maltose in *E.*
429 *coli*. To exert catabolite repression, glycerol must be phosphorylated to glycerol-3-phosphate
430 (Hogema et al., 1998; Eppler et al. 2002). However, unlike other carbon sources, further
431 metabolism of glycerol is not required. According to Eppler and Boos (1999), glycerol-3-
432 phosphate reduces expression of MalT, a positive activator of all *mal* genes. The key players
433 in this repression are adenylate cyclase, $EIIA^{Glc}$, and CRP. The growth on maltose of the
434 mutants lacking $EIIA^{Glc}$ or containing truncated adenylate cyclase was no longer repressed by
435 glycerol, and CRP-independent transcription of *malT* was also not influenced by glycerol
436 either. In addition, stimulation of adenylate cyclase by phosphorylated $EIIA^{Glc}$ was controlled
437 by glycerol-3-phosphate. The mutation in adenylate cyclase relieved the repression caused by
438 glycerol-3-phosphate (Eppler et al., 2002). Although not investigated, we assume that the
439 glycerol repression on maltose catabolism also hold true in *K. pneumoniae*.

440 Glycerol also exerts catabolite repression on the assimilation of citrate in *K.*
441 *pneumoniae.* Citrate can be used as the sole carbon and energy source during anaerobic
442 growth of *K. pneumoniae*, but the presence of glycerol suppresses the expression of the
443 citrate fermentation genes. A reduced concentration of the cAMP–CRP complex has been
444 postulated to be the cause of this repression (Meyer et al., 2001). Further research is needed
445 to test the hypothesis.

446 **3. *K. pneumoniae* as a microbial cell factory for the production of commodity chemicals**

447 During glycerol fermentation, *K. pneumoniae* produces many metabolites, including
448 PDO, BDO, and lactic acid. This section describes the biotechnological production of bulk
449 chemicals from glycerol in *K. pneumoniae*. 3-HP is not a natural product during the growth
450 of *K. pneumoniae* on glycerol. However, its production is possible using a minor
451 modification of the PDO synthetic pathway and is included here. The biochemical pathways

452 used for the synthesis of these chemicals are explained, and the metabolic engineering used to
453 improve their production is discussed.

454 **3.1 1,3-Propanediol**

455 PDO, also known as trimethylene glycol, is a promising platform chemical that has
456 two hydroxyl groups. With terephthalic acid, it can be co-polymerized to the novel co-
457 polymer polytrimethylene terephthalate (PTT). PDO also has applications in the food and
458 cosmetic industries. Currently, commercial PDO is mainly produced by recombinant *E. coli*
459 (Maervoet et al., 2011; Saxena et al., 2009). The global demand for PDO was 60.2 kilotons in
460 2012 and is predicted to reach approximately 150 kilotons by 2019. The market for PDO is
461 growing rapidly, from $157 million in 2012 with projections up to $560 million in 2019
462 (MarketsANDMarkets, 2012; Lee et al., 2015).

463 *K. pneumoniae* is one of the best native producers of PDO from glycerol. GDHt and
464 PDOR are two important enzymes for the conversion of glycerol to PDO (Fig. 3).
465 Physiologically, the synthesis of PDO from glycerol is essential for the anaerobic growth of
466 *K. pneumoniae*, as described previously. The production of PDO requires two cofactors,
467 coenzyme B_{12} and NAD(P)H. *K. pneumoniae* has a *de novo* pathway for the biosynthesis of
468 coenzyme B_{12} that comprises more than 20 genes. NAD(P)H is generated through the
469 oxidative metabolism of glycerol, during the production of cell biomass and/or oxidized
470 (pyruvate and acetate) or partially reduced (BDO, lactic acid) metabolites. The production
471 yield of PDO from glycerol varies depending on the cost of carbon for NAD(P)H production;
472 the lower the cost, the higher the yield of PDO from glycerol. NADPH can also be the
473 electron donor for the conversion of 3-HPA to PDO when YqhD is present. However, the
474 contribution of NADPH to PDO production in native *K. pneumoniae* seems to be limited
475 because the PP pathway, which is the main source of NADPH, is not active when glycerol is

476 the sole carbon source. Furthermore, *K. pneumoniae* has very low transhydrogenase activity
477 for the conversion of NADH to NADPH (Zhang and Xiu, 2009; Chen et al., 2011).
478 Several metabolic engineering strategies have been employed to improve PDO
479 production by *K. pneumonia* (Table 2). Amplification of the reductive pathway by
480 overexpressing DhaB, PDOR, or both has been attempted by many research groups. As
481 indicated previously, 3-HPA is highly toxic, and its accumulation is detrimental to cell
482 viability and toxic to PDO production. To reduce 3-HPA accumulation, overexpression of
483 *dhaT* has been attempted. In one study, 3-HPA accumulation was reduced by *dhaT*
484 overexpression, but no improvement in PDO production resulted (Hao et al., 2008).
485 Overexpression of both DhaT and DhaD increased PDO production (56.3%) in batch culture
486 but no increase in fed-batch culture (Chen et al., 2009). With another *K. pneumoniae* strain,
487 DSM 2026, the same experiments were repeated but also with no increase in PDO production
488 (Zheng et al., 2006). These results suggest that the reductive pathway in *K. pneumoniae* is
489 evolutionarily well established (perhaps in concert with the oxidative pathway) and that PDO
490 production is not limited by expression of the *dhaB* and *dhaT* genes of the reductive pathway.
491 However, in one study where the *E. coli yqhD* gene was highly overexpressed to give ~10-
492 fold higher PDOR activity (110 IU/mg total protein), a significant increase in PDO titer (25%;
493 67.6 g/L) and yield (from 0.53 to 0.62 mol/mol), along with less 3-HPA accumulation, was
494 achieved (Zhu et al., 2009). This observation suggests that in the recombinant strain, NADPH
495 can play an important role as a cofactor and that PDO production can be increased by
496 modifying the reductive pathway. It is also probable that the effect of DhaB and PDOR on
497 PDO production is highly dependent on the strain and/or culture conditions adopted.
498 Lactic acid, BDO, ethanol, and acetic acid are the major byproducts of PDO synthesis
499 in *K. pneumoniae*. Inactivation of the metabolic pathways that give rise to these products has
500 resulted in increased PDO synthesis. In particular, deletion of the *ldhA* gene encoding lactate

501 dehydrogenase has been highly beneficial. For example, by disrupting the *ldhA* gene, Xu et al.
502 (2009b) significantly increased PDO production without lactic acid production. They
503 observed increases in the PDO titer (95.4–102.1 g/L), conversion yield (0.48–0.52 mol/mol),
504 and productivity (1.98–2.13 g/L h; Table 2). Similarly, Oh et al. (2012a) achieved the high
505 PDO titer of 102.7 g/L by deleting *ldhA* in their own *K. pneumoniae* isolate. When lactic acid
506 production was eliminated, more ethanol and succinic acid were generated from the pyruvate
507 node. Reduction of these compounds has also been attempted by deleting *adhE* (encoding
508 alcohol dehydrogenase) and *frdA* (encoding fumarate reductase). Although the production of
509 ethanol and succinate was substantially reduced in the triple mutant (Δ*ldhA*Δ*adhE*Δ*frdA*),
510 PDO production was only marginally increased compared with that of the single Δ*ldhA*
511 mutant. The failure of the triple mutant (Δ*ldhA*Δ*adhE*Δ*frdA*) to increase PDO production was
512 mainly due to an increase in BDO production. Disruption of the BDO pathway encoded by
513 the *bud* operon (*budA*, *budB*, and *budC*) has also been studied (Fig. 5). Although partially
514 effective, individual inactivation of each of the three genes did not successfully decrease
515 BDO production (Oh et al., 2012a; Wu et al., 2013). Deletion of the entire *bud* operon
516 completely eliminated BDO synthesis (Kumar et al., 2016), but seriously hampered cell
517 growth and glycerol consumption. Consequently, no increase in PDO production was resulted.
518 Wu et al. (2013) attempted to decrease BDO production while increasing PDO production by
519 inserting the *fdh* gene from *Candida boidinii* (NADH-forming formate dehydrogenase) into
520 the *budC* locus. This protocol increased the PDO titer (62.3–72.2 g/L) and yield (0.47–0.57
521 mol/mol) and decreased the production of BDO by 52.2% and formic acid by 73.4%.
522 Acetic acid is one of the most toxic metabolites, accumulating in large quantities
523 during glycerol fermentation by *K. pneumonaie* (Celińska, 2012). The toxic effects of acetate
524 is realized even at the low concentration of 20 mM. Physiologically, acetate is a preferred
525 metabolite for many *Enterobacter* sp. because its production is accompanied by ATP

526 generation. In a typical bioreactor run for PDO production by *K. pneumoniae*, the
527 accumulation of acetic acid to >300 mM by the end is not uncommon, which often
528 completely stops glycerol fermentation. Acetate is produced by Pta-Ack and PoxB when
529 pyruvate formation is faster than its consumption, which is so-called ‘overflow metabolism’
530 (Fig. 6) (De Mey et al., 2007). In *K. pneumoniae*, the contribution of the *poxB* gene is not
531 significant and its deletion does not much affect acetate production. In comparison, deletion
532 of the *pta-ack* genes greatly reduces cell growth and results in high accumulation of pyruvate
533 and pyruvate-based metabolites (Ko et al., 2017). Extensive studies to reduce or eliminate
534 acetate production have been conducted in *E. coli*. Compared to *poxB*, *ackA* and/or *pta* were
535 much more significant in reducing acetate production. However, deletion of the latter genes
536 was not beneficial because the glycolytic flux and cell yield were seriously reduced and the
537 accumulation of other byproducts such as pyruvate, lactate, and formate was greatly
538 increased (Causey et al., 2004; De Mey et al., 2007). Several indirect approaches to
539 decreasing pyruvate/acetate accumulation were investigated: stimulation of the anaplerotic
540 pathway by overexpression of pyruvate carboxylase, PEP carboxylase, or PEP carboxykinase
541 (Abdel-Hamid et al., 2001; Causey et al., 2004; Chao and Liao, 1993; Sanchez et al., 2005);
542 overexpression of acetyl-CoA synthetase (ACS); increase of the TCA cycle throughput by
543 deleting the transcriptional repressor *arcA* gene; and stimulation of the glyoxylate shunt by
544 deleting the transcriptional repressor *iclR* gene (De Mey et al., 2007; Jeong et al., 2004; Lin et
545 al., 2006). Moreover, the use of a mutant pyruvate dehydrogenase which is less sensitive to
546 inhibition by NADH has also been examined (Kim et al., 2008). In *E. coli*, these approaches
547 proved to be effective to varying extents under properly selected culture conditions. However,
548 they have not yet been fully investigated in *K. pneumoniae*.
549 To eliminate byproduct formation, reduction of glycerol flux through the oxidative
550 pathway at the glycerol node has also been attempted. The deletion of glycerol

551 dehydrogenase (*dhaD*) and/or dihydroxyacetone kinase (*dhaK*) resulted in decrease of the
552 oxidative flux and increase of the conversion yield of glycerol to PDO. In addition,
553 production of the byproducts lactate, ethanol, and succinate but not acetate decreased
554 significantly, even without blocking the pathways leading to these byproducts (Seo et al.
555 2009; Horng et al. 2010). However, cell growth and glycerol consumption rate were also
556 reduced. Interestingly, in these mutant strains, deletion of *dhaT* has almost no effect on PDO
557 production. In the absence of *dhaT*, expression of an HOR, highly homologous to YqhD, was
558 upregulated (see section 2.2.4).

559 The theoretical maximum yields of PDO on glycerol are 0.875 and 0.844 mol/mol
560 under aerobic and anaerobic conditions, respectively (Celińska, 2012). However,
561 experimentally, the yields are reported between 0.35 and 0.65 mol/mol (Saxena et al., 2009;
562 Sun et al., 2008). The low yield is mainly attributed to the accumulation of unnecessary
563 byproducts and inefficient metabolism for generating reducing equivalents (NADH) and ATP.
564 If acetyl-CoA is fully oxidized through the TCA cycle (without byproducts formation) and
565 the activity of the electron transport chain (ETC) oxidizing NADH is properly controlled, a
566 significant increase in PDO yield is expected. Attaining this goal requires extensive pathway
567 engineering of cellular metabolism. In bioreactor operation, the aeration rate should be
568 optimized. Oxygen regulates gene expression and the enzymatic reactions of glycerol
569 metabolism and determines the diversion of DHA to the PP pathway. Regeneration of NADH
570 by the TCA cycle and its oxidation by ETC are also controlled by oxygen levels.

571 **3.2 3-Hydroxypropionic acid**

572 As with PDO, 3-HP is an important platform chemical and has been selected by the
573 US Department of Energy (DOE) as one of the top 12 chemicals obtainable from biomass. 3-
574 HP is a C3 bifunctional molecule and a structural isomer of lactic acid (Werpy and Petersen,
575 2004). 3-HP can be converted into a variety of useful chemicals such as acrylic acid and acryl

amide (Pina et al., 2011; Kumar et al., 2013a). 3-HP can be synthesized by chemical routes from acrylic acid, 3-propiolactone, 3-hydroxypropionitrile, allyl alcohol, vinyl acetate, and PDO. However, none of the chemical processes is commercially feasible at present due to the high cost of the starting materials, toxicity of intermediates, and/or the environmental incompatibility of the processes (Jiang et al., 2009; Pina et al., 2011; Kumar et al., 2013a). Many microorganisms, including both prokaryotes and eukaryotes, can naturally synthesize 3-HP as either an intermediate or end product through a range of metabolic pathways. However, the production of 3-HP by these native microorganisms is too low to be commercially meaningful (Jiang et al., 2009; Kumar et al., 2013a).

In studies pursuing commercial production of 3-HP, glycerol is first converted to 3-HPA by GDHt. 3-HPA then is converted to 3-HP via the coenzyme A (CoA)-dependent or CoA-independent pathway (Fig. 3). In the CoA-independent pathway, 3-HPA is oxidized to 3-HP by aldehyde dehydrogenase (ALDH); in the CoA-dependent pathway, 3-HPA is converted to 3-HP via 3-HP-CoA and 3-HP-phosphate. In *K. pneumoniae*, both the CoA-dependent and CoA-independent pathways are present. However, the titer of 3-HP produced by the CoA-dependent pathway is low as <5.0 g/L (Luo et al., 2011 and 2012). High production of 3-HP requires the disruption of oxidoreductases (e.g., DhaT, YqhD) and the overexpression of a proper aldehyde dehydrogenase (ALDH). Ashok et al. (2013a) developed a recombinant *K. pneumoniae* in which *dhaT* and *yqhD* were deleted, and the homologous *puuC* gene encoding the NAD^+-dependent γ-glutamyl-γ-aminobutyraldehyde dehydrogenase was overexpressed. The recombinant *K. pneumoniae* produced 3.8 g/L 3-HP in 12 h of flask culture, but only under appropriate aeration (i.e., microaerobic) conditions. Under anaerobic conditions, PDO (instead of 3-HP) was obtained as the main product, even though two oxidoreductases were disrupted and *puuC* was highly overexpressed. In contrast, highly aerobic conditions produced a high cell mass without much accumulation of either 3-HP or

601 1,3-PDO. In a glycerol-fed batch bioreactor experiment under a constant dissolved oxygen
602 (DO) concentration of 5% (considered "proper" microaerobic condition), the recombinant *K.*
603 *pneumoniae* Δ*dhaT* Δ*yqhD* overexpressing both PuuC and DhaB produced >28 g/L 3-HP in
604 48 h, with a yield of >40%. In contrast, the same strain produced only 10.5 g/L 3-HP when
605 cultivated under the higher 10% DO conditions. Several important conclusions regarding 3-
606 HP production by *K. pneumoniae* were made, as follows. First, in addition to DhaT and
607 YqhD, *K. pneumoniae* has more unidentified oxidoreductases that can produce PDO from 3-
608 HPA. Second, to produce 3-HP rather than PDO, proper aeration to regenerate NAD^+ is
609 essential. Third, excessively high aeration decreases the expression of the Dha regulon and
610 synthesis of coenzyme B_{12}, an essential cofactor for GDHt catalysis.
611 To eliminate PDO production even under limited aeration conditions, Ko et al.
612 attempted to identify and disrupt other potential PDORs from *K. pneumoniae* (Ko et al.,
613 2015). A mutant strain devoid of *dhaT*, *yqhD*, *ahpF*, and *adhE* genes was developed;
614 however, the mutant neither eliminated 1,3-PDO production nor increased 3-HP production.
615 Again, this result suggests that *K. pneumoniae* has more unidentified oxidoreductases, and
616 thus, the complete elimination of 1,3-PDO production during 3-HP production is highly
617 challenging. To address the important problem, of NAD^+ regeneration and coenzyme B_{12}
618 production, Ashok et al. (2013b) studied anaerobic respiration using nitrate as an external
619 electron acceptor. They attempted to regenerate NAD^+ from NADH by nitrate reduction
620 while maintain anaerobic conditions to synthesize and stabilize the oxygen-sensitive
621 coenzyme B_{12}. Disruption of the *glpK* gene (encoding for glycerol kinase) was also necessary
622 because with *glpK* intact, the rate of anaerobic respiration was too fast, and most of the
623 glycerol was metabolized through GlpK to produce biomass, as in the case of highly aerobic
624 cultivation. The anaerobic glycerol fermentation in the presence of nitrate was successful:
625 1.74 g/L 3-HP was obtained from a 12-h flask culture and 22 g/L 3-HP was produced in a 48-

626 h fed-batch bioreactor culture (Ashok et al., 2013b) (Table 3). However, disadvantages were
627 noted, including the requirement for large amounts of nitrate and the toxic effect of nitrite
628 generated as a reduced intermediate of nitrate. In a recent study, Li et al. (2016) optimized a
629 promoter for ALDH expression, culture conditions, and metabolic flux to achieve high-level
630 production of 3-HP in their *K. pneumoniae* isolate. One recombinant strain, *K. pneumoniae*
631 (pTAC-*puuC*) expressing *puuC* under the IPTG-inducible *tac* promoter, produced 73.4 g/L 3-
632 HP in a bioreactor with a molar yield of glycerol of 0.52 and productivity of 1.53 g/L h.
633 Further, elimination of the *ldh1*, *ldh2*, and *pta* genes elevated the titer and molar yield to 83.8
634 g/L 3-HP and 0.54 mol/mol, respectively, with decreased cell growth and productivity. This
635 titer of 3-HP is the highest produced by *K. pneumoniae* to date.
636 As another approach to addressing the problem associated with NAD^+ regeneration
637 and coenzyme B_{12} synthesis in 3-HP production, co-production of 3-HP and PDO has been
638 investigated. The simultaneous production of 3-HP and PDO can eliminate the dependency
639 on oxidative metabolism of glycerol (and electron transport chain), as the cofactor required
640 for 3-HP production is regenerated by PDO production or vice versa. Furthermore, the
641 problems associated with the expression of genes for the assimilation of vitamin B_{12} and
642 glycerol can be alleviated substantially because co-production can be performed under
643 anaerobic or microaerobic conditions (Kumar et al., 2012, 2013b). Ashok et al. (2011)
644 developed a recombinant strain of *K. pneumoniae* DSMZ by overexpressing ALDH and
645 deleting *dhaT*. With *dhaT* intact, PDOR activity was too high compared with that of ALDH,
646 even with overexpression of the latter enzyme from a multicopy plasmid. The recombinant *K.*
647 *pneumoniae* DSMZ accumulated 16.0 g/L 3-HP and 16.8 g/L PDO in 24 h, and the
648 cumulative yield of these two metabolites on glycerol was 51% (Table 3). Huang et al. (2012)
649 studied the same co-production using their *K. pneumoniae* isolate overexpressing ALDH and
650 obtained 24.4 3-HP and 49.9 g/L PDO, respectively, after 24 h under anaerobic conditions.

651 The cumulative molar yield of the two metabolites reached 0.61 (0.18 for 3-HP and 0.43 for
652 PDO). In a following study, they showed that the maximum titer of 48.9 g/L 3-HP (along
653 with 25.3 g/L PDO) was achieved at 1.5 vvm, while the highest concentration of 38.4 g/L
654 PDO (along with 16.6 g/L 3-HP) was obtained at 0.1 vvm (Huang et al., 2013a).

655 During 3-HP production from glycerol by *K. pneumoniae*, the accumulation of such
656 byproducts as lactic acid, ethanol, acetic acid, formic acid, and 2,3-BDO is a serious
657 consideration, as in the case of 1,3-PDO production (Ashok et al., 2013a,b; Li et al., 2016).
658 Deletion of *ldhA* reduces lactic acid formation and increases the 3-HP yield. However,
659 deletion of other genes such as *adhE*, *pfl*, *budBAC*, and *pta-ack* had little desired effect and
660 seriously hampered cell growth and glycerol assimilation (unpublished data). Even with the
661 co-production of 3-HP and 1,3-PDO, where oxidative glycerol assimilation was greatly
662 reduced, accumulation of these byproducts was a serious drawback (Ko et al., 2017). To
663 prevent the formation of lactic acid along with other byproducts, Kumar et al. (2013b)
664 employed resting cells of recombinant *K. pneumoniae* J2B overexpressing ALDH and devoid
665 of lactate dehydrogenase (*ldhA*). For this strain, final titers of 3-HP and PDO of 22.7 and 23.5
666 g/L, respectively, were obtained without lactic acid accumulation. The cumulative product
667 yield increased to 0.77.

668 In addition to *K. pneumoniae*, *E. coli* has been extensively studied for 3-HP
669 production from glycerol. Several successful results have been reported by Samsung Ltd. Co.
670 Korea. One study (Chu et al., 2015) used an engineered strain (*E. coli* W3110 *ΔackA-pta*
671 *ΔyqhD_dhaB_mutant gabD4*) harboring an active ALDH mutant (designated as GabD4) from
672 *Cupriavidus necator*, producing 71.9 g/L 3-HP with a productivity of 1.8 g/L h. However,
673 unlike *K. pneumoniae*, *E. coli* does not synthesize vitamin B_{12} naturally, and it was necessary
674 to add the coenzyme externally. Furthermore, a significant amount of glucose for cell growth
675 was required because for *E. coli*, glycerol is a less-preferred carbon source than glucose.

676 Consequently, the researchers at Samsung initially grew cells to a high density (40–300
677 OD_{600}) on glucose and then produced 3-HP by feeding glycerol (Chu et al., 2015; Kim et al.,
678 2014a). In comparison, *K. pneumoniae* can produce a high concentration of 3-HP growing
679 purely on glycerol at a much lower cell concentration (OD_{600}, <20) (Huang et al., 2013a,b; Li
680 et al., 2016). Together, the use of glucose for cell proliferation, exogenous addition of
681 expensive cofactor vitamin B_{12}, and low cumulative yield of 3-HP (on glucose plus glycerol)
682 increase the production cost. Thus, *K. pneumoniae* can be considered a better biocatalyst than
683 *E. coli*. However, for the commercial production of 3-HP, many other factors are important,
684 including biosafety, process stability, and downstream processing. Further studies to assess
685 the potential of these strains as hosts for 3-HP production are needed.

686 **3.3 2,3-Butanediol**

687 BDO has many applications in the pharmaceutical, biomedical, and other chemical
688 industries for the production of printing inks, perfumes, fumigants, spandex, moistening and
689 softening agents, and plasticizers (e.g., cellulose nitrate, polyvinyl chloride, polyacrylates)
690 (Celińska and Grajek, 2009; Ji et al., 2011). BDO can also be used as an antifreezing agent
691 and octane booster for petrol as is or can be converted to useful derivatives such as 1,3-
692 butadiene, and diacetyl and methyl ethyl ketone.

693 Unlike 3-HP and PDO, BDO is a product of oxidative metabolism. Of the three
694 stereoisomers of BDO, *K. pneumoniae* mainly produces the *meso* form (Ji et al., 2011). BDO
695 is synthesized from pyruvate via α-acetolactate and acetoin. The C5 intermediate, α-
696 acetolactate, is formed from two molecules of pyruvate by self-condensation catalyzed by α-
697 acetolactate synthase (ALS; *budB*). α-Acetolactate is decarboxylated to acetoin by α-
698 acetolactate decarboxylase (*budA*), and acetoin is reduced to BDO by 2,3-butanediol
699 dehydrogenase/acetoin reductase (*budC*) using NADH as a reductant. In the presence of
700 oxygen, α-acetolactate is spontaneously decarboxylated to diacetyl, further reduced to acetoin

701 by action of diacetyl reductase, and then reduced to BDO. α-Acetolactate is also the precursor
702 of branched-chain amino acids (valine, leucine, and isoleucine); thus, elimination of the *bud*
703 operon can arrest *K. pneumoniae* growth when cultured on glycerol minimal medium (Kumar
704 et al., 2016). In the anaerobic process, one NADH molecule is required for the conversion of
705 two pyruvates to one BDO, while the aerobic process requires two NADH (Fig. 5). The
706 enzyme DhaD may also contribute to BDO formation (see Section 2.2.1). In the BDO
707 pathway itself, the conversion of two pyruvates to BDO regulates or is regulated by the
708 intracellular NADH/NAD^+ ratio, similar to other fermentative pathways. Another important
709 role of the BDO pathway is to reduce intracellular acidification by converting acids to the
710 neutral metabolite, BDO (Celińska and Grajek, 2009; Ji et al., 2011).
711 Although BDO is one of the main byproducts of glycerol fermentation by *K.*
712 *pneumoniae* (Petrov and Petrova, 2009; Durgapal et al. 2014), its production from glycerol
713 has not been studied extensively. In few studies using glycerol as the carbon source, Petrov
714 and Petrova (2009) focused on the effects of pH and aeration with their *K. pneumoniae*
715 isolate G31. BDO production was greatest at alkaline initial pH when tested at a pH range of
716 5–8. Without pH control a sharp pH drop occurred initially due to the production of organic
717 acids, and this pH drop triggered the synthesis of BDO, which is considered the only non-
718 inhibitory compound produced by the oxidative metabolism of glycerol. Once neutral pH is
719 restored with the rising concentration of BDO, catabolism shifted toward the production of
720 ATP-generating acetic acid. The amount of BDO generated in each cycle (pH drop and
721 neutralization) was dependent on the extent of the pH drop. In one study, a BDO
722 concentration of 49.8 g/L was reached when the initial pH was set at 8.0 and no pH control
723 was given during the rest of fermentation. PDO was the major byproduct (11.6 g/L) (Table 4)
724 and its production decreased by exclusion of Co^{2+}, an essential component of coenzyme B_{12}.
725 They also found that intensive aeration led to a significant increase in BDO production; an

726 increase in aeration rate from 1.1 to 2.2 vvm enhanced the yield from 0.38 to 0.46 mol/mol
727 and productivity from 0.24 to 0.36 g/L h. BDO production was also increased by forced pH
728 fluctuations of the culture medium with discrete ΔpH values (1.0, 2.0, and 3.0) at
729 predetermined time intervals (6, 12, and 24 h). The highest BDO concentration (70.0 g/L)
730 was produced by increasing the pH by one unit (ΔpH = 1.0) every 12 h (Petrov and Petrova,
731 2010). The mechanism behind the effect of this peculiar pH change on BDO production has
732 not been elucidated. Although BDO is readily produced from glycerol by *K. pneumoniae*, the
733 volumetric productivity is low due to the long fermentation time (generally >150 h). Huang et
734 al. (2013b) isolated two *Klebsiella* strains that produce BDO plus acetoin with a total yield of
735 0.44–0.45 (g/g). One isolate utilized 64.5 g/L glycerol in 78 h and produced 24.9 g/L BDO,
736 3.9 g/L acetoin, and 3.2 g/L ethanol. Again, the volumetric productivity was not high.
737 Metabolic engineering efforts with *K. pneumoniae* to improve BDO production from
738 glycerol are limited. Using glucose as carbon source, Kim et al. (2014b) reported that
739 overexpression of the BDO pathway (*budA*, *budB*) in the *ldhA* deficient *K. pneumoniae* strain
740 KCTC2242 improved BDO production and resulted in 90 g/L BDO, with a productivity of
741 2.75 g/L. h. Because both glucose and glycerol are converted through the common
742 intermediate pyruvate, BDO production from glycerol is also expected to increase by
743 amplification of the *bud* operon. Further work with glycerol as carbon source is required to
744 prove this effect. Recently, co-production of BDO and PDO from glycerol has also been
745 attempted (Park et al., 2017). In glycerol fermentation, both alcohols always appear
746 simultaneously and co-production seems to be beneficial for cells to maintain redox balance
747 during anaerobic growth. Park et al. (2017) found that by using the *K. pneumoniae* mutant
748 deficient of *ldhA* and *mdh*, 50.1 g/L BDO was produced along with 63.8 g/L PDO in 75 h.
749 The ratio of the two alcohols varied according to aeration; with increasing aeration, the ratio
750 of [BDO]/[PDO] increased as observed by Petrov and Petrova (2010). In the 5,000 L pilot-

751 scale fed-batch fermentation using crude glycerol, they could produce 114 g/L diols (70 g/L
752 PDO and 44 g/L BDO) with the yield of 0.60 diols/glycerol (g/g) and a productivity of 2.2
753 g/L. h of diols. If separation is not an issue, co-production of the two diols can be a good
754 option.

755 **3.4 Lactic acid**

756 Lactic acid has applications in the food, chemical, cosmetic, and pharmaceutical
757 industries. Lactic acid is a monomer that can be polymerized to yield the biodegradable
758 plastics polylactic acid (PLA) and poly(3-hydroxybutyrate-*co*-lactate). Two optical isomers
759 of lactic acid, D- and L-lactic acid, are produced in racemic mixtures depending on the
760 chemical route used. Microbial fermentation can yield optically pure isomers. Currently,
761 nearly all commercial lactic acid comes from microbial fermentation (Abdel-Rahman et al.,
762 2013; Abdel-Rahman and Sonomoto, 2016).

763 Because of the availability of highly efficient lactic-acid–producing microbes derived
764 from *E. coli*, lactic acid bacteria, and even acid-tolerant yeasts (Abdel-Rahman et al., 2013),
765 *K pneumoniae* has received little attention as a lactic acid producer. However, as previously
766 described, lactic acid is a major byproduct of glycerol fermentation by *K. pneumoniae*, and its
767 potential as a producer is high. This bacterium contains metabolic pathways for the
768 production of both L- and D-isomers (Fig. 7). They are synthesized through the pyruvate
769 and/or methylglyoxal pathways in *K. pneumoniae* (Ashok et al., 2011). When produced from
770 pyruvate, lactate dehydrogenase (*ldhA*) reduces pyruvate using NADH as reductant. In the
771 methylglyoxal route, DHAP is converted to methylglyoxal, which can be transformed to both
772 D- and L-lactic acid. For the production of L-lactic acid, methylglyoxal is reduced to L-
773 lactaldehyde, which is subsequently oxidized to L-lactic acid. Using glutathione, D-lactic acid
774 is obtained through simultaneous aldehyde group oxidation and keto group reduction of
775 methylglyoxal (Booth et al., 2003; Booth, 2005). The methylglyoxal route has several

776 disadvantages. Methylglyoxal is highly toxic, and its accumulation can severely impair other
777 metabolisms and even lead to cell death. Second, the route is energetically inefficient, with an
778 ATP yield of −1. The L- or D-lactic acid product can be converted back into pyruvate by
779 respiratory lactate dehydrogenase (Mazumdar et al., 2010, 2013).
780 Several studies showing the potential of *K. pneumoniae* as a lactic acid producer are
781 available (Table 4). For example, *K. pneumoniae* DSMZ is reported to accumulate 22.7 g/L
782 lactic acid in 24 h during co-production of 3-HP and PDO from glycerol. The titer of lactic
783 acid was more than that of any of the targeted products, and the yield of lactic acid on
784 glycerol alone was 0.31 (Ashok et al., 2011). Similarly, fed-batch cultivation of *K.*
785 *pneumoniae* BLh-1 under oxygen-limited conditions gave lactic acid as the main product,
786 outcompeting PDO; 59 g/L lactic acid was produced in 40 h when crude glycerol from
787 biodiesel synthesis without any purification was used (Rossi et al., 2013). Durgapal et al.
788 (2014) also found that *K. pneumoniae* J2B and DSMZ strains produced lactic acid at 37.0 and
789 47.8 g/L, respectively (Table 4); in both strains, lactic acid levels surpassed those of the target
790 product PDO. Recently, Feng et al. (2014) engineered *K. pneumoniae* ATCC25955 by
791 overexpressing *ldhA* and deleting *dhaT* and *yqhD* (recall that these two genes are mainly
792 responsible for PDO production). They found that the recombinant produced 142.1 g/L of
793 optically pure D-lactic acid from glycerol in fed-batch cultivation under microaerobic
794 conditions (2.5 vvm and 400 rpm). This result is the highest lactic acid concentration
795 produced from glycerol reported to date. *E. coli* has also been engineered for the production
796 of D- and L-lactate from glycerol (Mazumdar et al., 2010, 2013). For diverting glycerol flux
797 toward L-lactic acid in *E. coli*, the chromosomal copy of D-lactate dehydrogenase was
798 replaced with *Streptococcus bovis* L-lactate dehydrogenase (Mazumdar et al., 2013).
799 Moreover, gene encoding *lldD* (respiratory L-lactate dehydrogenase) was inactivated to
800 prevent the consumption of L-lactic acid, and the methylglyoxal route (Δ*mgsA*) was blocked

801 to prevent production of toxic methylglyoxal and formation of racemic mixtures of D- and L-
802 lactic acid (Fig. 7). Furthermore, the respiratory route for glycerol assimilation was
803 overexpressed (*glpK* and *glpD*) to improve the ATP yield of the metabolic pathway through
804 transfer of electrons from glycerol-3-phosphate to oxygen (i.e., oxidative phosphorylation).
805 The engineered *E. coli* strain (Δ*pflB* Δ*pta* Δ*adhE* Δ*frdA* Δ*mgsA* Δ*lldD* Δ*ldhA*::*ldhA_glpK*-
806 *_glpD*) produced 50 g/L of L-lactate from 56 g/L of crude glycerol, at a yield 93% of the
807 theoretical maximum and with high optical (99.9%) and chemical (97%) purity (Mazumdar et
808 al., 2013). The approaches proven successful in *E. coli* should be applicable to *K.*
809 *pneumoniae*. These studies, along with the results by Feng et al. (2014), suggest that *K.*
810 *pneumoniae* is a good host for the production of D- and L-isomers of lactic acid, with high
811 optical purity, from glycerol.

812 **3.5 Ethanol and other metabolites**

813 *K. pneumoniae* has also been studied for the production of ethanol, succinic acid, 2-
814 ketogluconic acid, catechol, *cis,cis*-muconic acid and 2-butanol although its potential for such
815 production has not been fully explored (Cheng et al., 2013; Jung et al., 2015; Oh et al., 2014;
816 Wang et al. 2015; Wei et al., 2013). Of these chemicals, ethanol production has been
817 relatively well studied. Ethanol is a renewable energy source and is widely used as a fuel
818 additive for partial gasoline replacement. Currently, commercial ethanol is produced from the
819 fermentation of sugar and starch. However, glycerol is one of the cheapest substrates for
820 ethanol production, estimated as nearly 40% cheaper than sugar-based production (Yazdani
821 and Gonzalez, 2007). This factor has encouraged the development of microbial strains for
822 fermentative conversion of glycerol to ethanol. The metabolic route by which ethanol is
823 synthesized from glycerol is shown in Fig. 6. Oh et al. (2011, 2012b) constructed a mutant
824 strain of *K. pneumoniae* (termed GEM167) through γ-irradiation. PDO synthesis in this
825 mutant strain was lower than that of wild type (0.2 vs. 7.93 g/L, respectively), while ethanol

826 accumulation was higher (8.6 vs. 1.1 g/L, respectively). The mechanism underlying these
827 changes remains unclear. Introduction of the pyruvate decarboxylase (*pdc*) and aldehyde
828 dehydrogenase (*adhII*) genes into mutant strains lacking lactate dehydrogenase (*ldhA*)
829 resulted in the production of 31.0 g/L (673.9 mM) ethanol, with a yield of 0.89 (mol/mol) and
830 productivity of 1.2 g/L h (Oh et al., 2012b), the highest level of ethanol production from
831 glycerol reported to date. More extensive studies including estimation of the maximum
832 achievable titer and microbial tolerance to high-concentration ethanol, both of which are key
833 factors for commercialization, are required to assess the potential to produce ethanol from
834 glycerol by the use of *K. pneumoniae*.

835 **4. Challenges to bioconversion of glycerol using *K. pneumoniae* as a biocatalyst**

836 The design of microbial cell factories is gaining unprecedented momentum as
837 metabolic engineering is progressively aided by advances in synthetic biology and multi-
838 omics analyses. Several bio-based chemicals have already been launched successfully in the
839 marketplace, and others are in the pipeline. For a bulk chemical to be considered for
840 commercial production, the production process should achieve a product concentration of 100
841 g/L, carbon yield of 0.50 (g/g), and volumetric productivity of 2.0 g/L/h. The aforementioned
842 products produced by *K. pneumoniae* from glycerol are no exception. Challenges to the use
843 of *K. pneumoniae* as a microbial cell factory using glycerol as a carbon source are described.

844 **4.1 Pathogenicity**

845 The pathogenicity of *K. pneumoniae* is one of the major obstacles to its commercial
846 application. *K. pneumoniae* is a Bio-safety Level 2 microorganism and causes nosocomial
847 and urinary tract infections. Moreover, capsulated *K. pneumoniae* cells are difficult to
848 separate from culture broth, complicating the downstream processing. The biotechnological
849 application of this bacterium will require attenuation of its pathogenicity and reduction of
850 biosafety concerns. *K. pneumoniae* produces a number of virulence factors that contribute to

851 its pathogenicity, including lipopolysaccharide (LPS), capsular antigens, fimbrial adhesins,
852 siderophores, and O antigens (Albertí et al., 1993; Fang et al., 2004; Podschun and Ullmann
853 1998). The mechanism by which this bacterium causes disease is still obscure because most
854 studies have been conducted on a limited number of virulence factors (El Fertas-Aissani et al.,
855 2013).

856 In the last decade, efforts have been made to overcome the pathogenicity of *K.*
857 *pneumoniae* (Shrivastav et al., 2013), suggesting hope for the generation of nonpathogenic *K.*
858 *pneumoniae*. The two major pathogenic determinants in *K. pneumoniae* are the capsular
859 polysaccharides (CPS), which cover the entire bacterial surface, and LPS, expressed on the
860 outer bacterial membrane. LPS consists of lipid A, a core oligosaccharide, and an O-antigenic
861 polysaccharide. In a highly virulent strain of *K. pneumoniae*, mutations in genes involved in
862 LPS biosynthesis (*waaC*, *waaF*, and *wabG*) resulted in a decrease in bacterial colonization
863 and virulence (Izquierdo et al., 2003; Jung et al., 2013). Mutant strains devoid of the outer
864 core LPS were also found to be deficient in the "cell-attached" capsular polysaccharides
865 covering the bacterial surface. These mutations abolished the highly virulent characteristics
866 of pathogenic *K. pneumoniae* when tested in different animal models. Furthermore, these
867 mutants were more sensitive to several hydrophobic compounds than were wild-type strains.
868 Reintroduction of the *waaC*, *waaF*, and *wabG* genes into *K. pneumoniae* rescued the
869 pathogenic properties, confirming the role of these genes in pathogenicity.

870 Another important study was conducted by Lin et al. (2012), in which eight highly
871 conserved residues of the MagA protein (G308, G310, G334, G337, R290, P305, H323, and
872 N324) encoded by the mucoviscosity-associated gene A (*magA*) of *K. pneumoniae* were
873 subjected to site-directed mutagenesis. The *magA* gene contributes to the biosynthesis of K1
874 capsular polysaccharide (CPS), which correlates with pathogenic phenotypes including
875 mucoviscosity, serum and phagocytosis resistance, and virulence. Alanine substitutions at

876 R290A or H323A abolished MagA function, with annihilation of CPS production, serum
877 resistance, and virulence. Some *K. pneumoniae* strains showing less pathogenicity were also
878 isolated. For example, *K. pneumoniae* strain J2B produced less LPS and demonstrated a high
879 sensitivity to many antibiotics and lower pathogenicity. Moreover, its biomass was readily
880 separated from fermentation broth by centrifugation (Arasu et al., 2011). Future studies
881 should include further engineering of these less pathogenic strains to make them completely
882 nonpathogenic, followed by careful evaluation of their toxicity.

883 **4.2 Complex glycerol metabolism**

884 Although glycerol metabolism has been extensively studied, the mechanism and
885 regulation of its gene expression in *K. pneumoniae* have not been fully elucidated. The
886 diversity of glycerol metabolism should be a great advantage for *K. pneumoniae* survival in
887 different environments and for its biotechnological utilization as well. However, a high level
888 of complexity poses difficulty in the engineering of a strain to suit our purposes. Metabolic
889 complexity is likely responsible in part for the fact that, despite serious attempts, the molar
890 yield of many important products such as PDO and 3-HP is still far below the theoretical
891 maximum. Efforts based on our current knowledge, such as the overexpression of DhaB,
892 DhaT, YqhD and AldH and inactivation/deletion of GlpK, DhaD, DhaK and DhaKLM, were
893 not satisfactory (Maervoet et al. 2011; Kumar et al. 2013a; Kumar et al., 2016). Another
894 challenge is to understand how the ratio of glycerol distribution between respiratory and
895 fermentative routes is controlled. Because the K_M of glycerol kinase toward glycerol is low, it
896 has been speculated that a major fraction of glycerol flows through the respiratory route when
897 oxygen is present. However, some experimental results do not support this hypothesis. For
898 example, deletion of *glpK* does not decrease the glycerol flow through the oxidative pathway
899 in the presence of oxygen (Ashok et al. 2013a). In fact, wild-type and mutant (Δ*glpK*) strains
900 of *K. pneumoniae* demonstrate similar cell growth, glycerol consumption, and PDO

901 production profiles under (micro)aerobic conditions. These observations suggest the presence
902 and/or involvement of other kinases.

903 Another challenging issue for the use of *K. pneumoniae* is its inefficient TCA cycle
904 (Cabelli, 1955). Several genes, including isocitrate dehydrognease (*icd*), fumarase (*fumA*),
905 and malate dehydrogenase (*mdh*), in *K. pneumoniae* are significantly less transcribed than
906 those in *E. coli* (unpublished data). Furthermore, the activity of isocitrate dehydrogenase was
907 more than sevenfold lower in *K. pneumoniae* than in *E. coli*. If the TCA cycle is inefficient,
908 achieving a high cell density, which is essential for improving the productivity of target
909 metabolites, is difficult. Moreover, achieving high-yield generation of NADH with little
910 consumption of glycerol carbon is difficult. Furthermore, the overflow metabolism that
911 produces highly toxic acetate becomes more serious. These problems have been well
912 documented in PDO production. In a recent study, Kumar et al. (2016) attempted to increase
913 PDO production yield by blocking the lactate and BDO pathways, while diverting carbon
914 flux through the TCA cycle for efficient regeneration of NADH. However, this effort resulted
915 in the detrimental accumulation of pyruvate, acetate, and ethanol, causing low NADH
916 regeneration and low PDO production. Neither increased aeration nor the addition of good
917 nitrogen sources (complex nitrogen sources) alleviated the accumulation of these
918 intermediates or activated the TCA cycle. Furthermore, the accumulation of pyruvate quickly
919 terminated glycerol assimilation. *K. pneumoniae* has a well-established BDO production
920 pathway that likely compensates for its inefficient TCA cycle, avoiding the overflow
921 metabolism and carbon traffic at the pyruvate node. If this is the case, co-production of BDO
922 along with PDO or 3-HP, rather than single production of PDO or 3-HP, should be more
923 appropriate, which suits the nature of *K. pneumoniae*. In fact, when BDO and PDO were co-
924 produced, more PDO was generated than during PDO production alone. More studies are
925 required to gain a better understanding of glycerol metabolism, the slow operation of the

926 TCA cycle in *K. pneumoniae*, and its effect on the selection of target products and/or
927 development of suitable strains and processes. Otherwise, it will be difficult to exploit this
928 potential biocatalyst for commercial manufacturing of valuable chemicals.

929 **4.3 Toxicity of intermediate metabolites and end-products**

930 A general but major challenge to the use of microbial cell factories for producing bulk
931 chemicals is the toxic effect of target products and metabolic intermediates at high
932 concentrations. In particular, during the production of PDO or 3-HP from glycerol, the highly
933 toxic intermediate 3-HPA is inevitably generated. Aldehydes generally damage DNA,
934 inactivate enzymes, and inhibit DNA synthesis. 3-HPA causes irreversible cessation of
935 metabolic activity and cellular growth, even at very low concentrations of 15–30 mM (Hao et
936 al., 2008; Zheng et al., 2008). Native *K. pneumoniae* does not accumulate 3-HPA at toxic
937 levels during PDO production, but in engineered strains that overexpress DhaB, high 3-HPA
938 accumulation is often experienced. To avoid 3-HPA accumulation, enzymes that consume 3-
939 HPA, ALDH (for 3-HP production), and PDOR (for PDO production) are expressed at
940 sufficiently (sometimes excessively) high levels compared with that of DhaB (Lim et al.,
941 2016). Intermediates such as methylglyoxal, DHA, DHAP, and glyceraldehyde-3-phosphate
942 are also known to be toxic.

943 Organic acids and alcohols are toxic at high concentrations. Generally, organic acids
944 are more toxic than their corresponding alcohols because they disturb the intracellular pH and
945 have anion-specific effects on metabolism (Chun et al., 2014; Warnecke and Gill, 2005).
946 When accumulated in cell membranes, organic compounds damage membrane integrity and
947 inhibit the activity of membrane-bound enzymes. In *K. pneumoniae*, cell growth and glycerol
948 assimilation are severely inhibited by high concentrations of PDO or 3-HP. Furthermore,
949 enzymes that play major roles in 3-HP and PDO production, such as DhaB, DhaT, and AldH,
950 are inhibited by 3-HP and PDO (Kumar et al. 2013b). Furthermore, *K. pneumoniae* is

951 sensitive to 24 g/L acetic acid, 26 g/L lactic acid, and 17 g/L ethanol under aerobic conditions
952 and 15, 19, and 26 g/L, respectively, under anaerobic condition (Celińska, 2012; Cheng et al.,
953 2005). The inhibition of pathways by target product(s) and/or unwanted byproducts often
954 places a limit on the maximum attainable titer of bulk chemicals.

955 Several studies have reported engineered improvements in microbial tolerance against
956 biochemicals and biofuels, especially in *E. coli*. Modifications of exporter proteins, heat
957 shock proteins, membrane composition, and stress responses have been attempted. Moreover,
958 *in situ* recovery of target products has been attempted for hydrophobic targets. Some tolerant
959 strains have been developed, but unfortunately, without appreciable increases in productivity
960 (Dunlop, 2011; Dunlop et al., 2011; Jarboe et al., 2011). Such efforts to improve tolerance to
961 chemicals have not been reported for *K. pneumoniae*.

962 **4.4 Availability of glycerol**

963 The global market for glycerol is known to be unpredictable and complex. Glycerol in
964 the current market mainly is from the biodiesel industry, and its supply is directly affected by
965 the use of biodiesel. In contrast, glycerol demand depends on many industries that use it as
966 feedstock. Between 2001 and 2011, the price varied significantly, from \$0.70/kg to \$1.70/kg
967 for pure glycerol and from \$0.04/kg to \$0.33/kg for crude glycerol (Ayoub and Abdullah,
968 2012; Ciriminna et al., 2014; Kong et al., 2016; Quispe et al., 2013). Nevertheless, glycerol
969 prices, for pure as well as crude, have come down significantly since 2004, when many
970 biodiesel production plants initiated operation. The countries producing biodiesel in large
971 quantities (in million liters) include Germany (2900), Brazil (2300), Argentina (2100), France
972 (2000), USA (1200), Spain (1100), Italy (800), Indonesia (700), and Thailand (600) (Kong et
973 al., 2016). The Asia-Pacific region overtook Europe as the largest glycerol market in the
974 world in 2009 and has remained so ever since. Market growth is driven by increases in the

975 uses for glycerol in such sectors as pharmaceuticals, personal hygiene, and food and beverage
976 production (Quispe et al., 2013).
977 The production of crude glycerol is expected to reach 6 million tons by 2025
978 (Ciriminna et al., 2014). At present, the surplus of glycerol from biodiesel and oleochemicals
979 is assumed to be sufficient to meet these new demands. However, the scenario may change in
980 the future with rapid growth of glycerol-based industries and a limited supply of crude
981 glycerol. For example, if 3-HP produced from glycerol were used as a substitute for the
982 chemical acrylic acid (global production, 5.85 million tons as of 2014), approximately 7
983 million tons of glycerol/year would be needed. If we include other platform chemicals such
984 as PDO, BDO, and ethanol, the demand will be even higher, much more than the expected
985 supply. The growth of glycerol-based biotechnology will eventually be constrained by the
986 supply and price of crude glycerol as a carbon source.

987 **5. Concluding remarks**

988 Glycerol is an excellent substrate for the production of biochemicals and biofuels.
989 Although the biodiesel industry has grown rather slowly in recent years, crude glycerol is still
990 cheap and generated in large quantities. *K. pneumoniae* has already been successfully
991 employed for the production of PDO, 3-HP, BDO, lactic acid, and ethanol from glycerol at
992 industrially attractive levels. Other metabolites such as acetic acid, pyruvic acid, malic acid,
993 fumaric acid, alanine, *n*-butanol, 1,2-propanediol, propionic acid, formic acid, and hydrogen
994 can also be produced from glycerol by *K. pneumoniae*, although these have not yet been
995 extensively explored. At present, sugar-based bioprocesses are prevalent, and no bioprocesses
996 employing *K. pneumoniae* and glycerol are commercially available. The pathogenicity of *K.*
997 *pneumoniae* and other technical issues are existing challenges to its use. However,
998 advancements in the fields of metabolic engineering, synthetic biology, systems biology, and
999 evolutionary engineering will enable us to further exploit the advantageous nature of *K.*

pneumoniae as a biocatalyst and glycerol as a carbon source. A better understanding of glycerol metabolism in *K. pneumoniae* and improvements in its performance as a microbial cell factory should make this organism an attractive alternative to current sugar-based methods for the production of biochemicals and biofuels.

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1462 **Figure captions**

1463 **Figure 1:** Transformation of glycerol into a number of valuable chemicals. The solid line
1464 represent single step while broken one indicate multiple steps.

1465 **Figure 2:** Oxidative metabolism of glycerol in *K. pneumoniae* (Ashok et al., 2011; Kumar et
1466 al., 2013a). Gene names are shown in italics on arrows. Solid lines indicate single steps;
1467 broken lines indicate multiple steps.

1468 **Figure 3:** Metabolic pathways for the production of PDO and 3-HP from glycerol (Ashok et
1469 al., 2011; Kumar et al., 2013a). Gene names are shown in italics on arrows.

1470 **Figure 4 (A):** Arrangement of genes in the *dha* regulon of *K. pneumoniae.* 1, *dhaK* (ATP-
1471 dependent dihydroxyacetone kinase); 2, *dhaK3*/*dhaM* (large subunit of ATP-dependent
1472 dihydroxyacetone kinase); 3, *dhaK2*/*dhaL* (small subunit of ATP-dependent
1473 dihydroxyacetone kinase); 4, *dhaK1*/*dhaK* (medium subunits of ATP-dependent
1474 dihydroxyacetone kinase); 5, *orfW* (hypothetical protein); 6, *dhaD* (glycerol dehydrogenase);
1475 7, *dhaR* (transcription regulator); 8, *orfW*/CdAT [cob(I)yrinic acid a,c-diamide
1476 adenosyltransferase]; 9, *orfX/dhaG* (small subunit of reactivating factor of glycerol
1477 dehydratase); 10, *dhaT* (1,3-propanediol oxidoreductase); 11, *orfY* (hypothetical protein); 12,
1478 *dhaB/B1* (large subunits of glycerol dehydratase); 13, *dhaC/B2* (medium subunit of glycerol
1479 dehydratase); 14, *dhaE/B3* (small subunit of glycerol dehydratase); 15, *orfZ*/*dhaF* (large
1480 subunit of reactivating factor of glycerol dehydratase); 16, *glpF* (glycerol uptake facilitator);
1481 17, 22- *hdeB*; 18, *hdeD* (*hdeBD*-genes related to global regulation); 19, *yfdX*; 20, *kvgS*; and
1482 21, *kvgA* (*kvgS* and *kvgA* encode for a two-component signal transduction system) (Celińska,
1483 2012; Sun et al., 2003; Wei et al., 2014). **(B):** Glycerol dehydratase gene arrangements and
1484 reactivation factors in different organisms (Martins-Pinheiro et al., 2016).

1485 **Figure 5:** Metabolic pathway for the synthesis of BDO from glycerol (Ashok et al., 2011;
1486 Celińska and Grajek, 2009; Ji et al., 2011). Gene names are shown in italics on arrows. Solid
1487 lines indicate single steps; broken lines indicate multiple steps.

1488 **Figure 6:** Biochemical routes for ethanol and acetic acid production from glycerol (Ashok et
1489 al., 2011; Kumar et al., 2013a). Gene names are shown in italics on arrow. Solid lines
1490 indicate single steps; broken lines indicate multiple steps.

1491 **Figure 7:** Lactic acid production from glycerol via methylglyoxal and pyruvate routes
1492 (Ashok et al., 2011; Mazumdar et al., 2010; 2013). Gene names are shown in italics on arrow.
1493 Solid lines indicate single steps; broken lines indicate multiple steps.

1494 **Fig. S1** Effect of deletion of glycerol transporters, *glpF1* and *glpF2*, on glycerol consumption
1495 and PDO production in *K. pneumoniae* J2B3.

1496

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1 **Potential and Limitations of *Klebsiella pneumoniae* as a Microbial Cell Factory Utilizing**
2 **Glycerol as the Carbon Source**
3
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18 **Abstract**

19 *Klebsiella pneumoniae* is a Gram-negative facultative anaerobe that metabolizes
20 glycerol efficiently under both aerobic and anaerobic conditions. This microbe is considered
21 an outstanding biocatalyst for transforming glycerol into a variety of value-added products.
22 Crude glycerol is a cheap carbon source and can be converted by *K. pneumoniae* into useful
23 compounds such as lactic acid, 3-hydroxypropionic acid, ethanol, 1,3-propanediol, 2,3-
24 butanediol, and succinic acid. This review summarizes glycerol metabolism in *K.*
25 *pneumoniae* and its potential as a microbial cell factory for the production of commercially
26 important acids and alcohols. Although many challenges remain, *K. pneumoniae* is a
27 promising workhorse when glycerol is used as the carbon source.

28 **Key words**: *Klebsiella pneumoniae*; Glycerol; 3-Hydroxypropionic acid; 1,3-Propanediol;
29 2,3-Butanediol; Lactic acid; Ethanol

1. Introduction

Crude glycerol is produced in large quantities as a byproduct of the biodiesel industry. Global biodiesel production was 22.7 million tons in 2012 and is forecasted to reach 36.9 million tons by 2020. As a result, worldwide production of glycerol climbed from 1 million tons in 2000 to 3 million tons in 2011 and is expected to reach 6 million tons by 2025 (Ciriminna et al., 2014; OECD, 2011; Katryniok et al., 2013; Sun et al. 2017). A significant amount of glycerol is also generated during bioethanol fermentation by yeast. The liquid fraction of the stillage obtained from ethanol separation contains up to 2% glycerol. Another source of glycerol is industrial waste generated from vegetable oils and animal fats. For example, the concentration of glycerol in waste streams from the oleochemical industry is 55%–90% (da Silva et al., 2009; Yazdani and Gonzalez, 2007). The increased availability of glycerol has caused a substantial reduction in its cost. According to a recent report, the price of crude glycerol is $0.24/kg and that of pure USP grade glycerol is $0.9/kg (Kong et al., 2016). Extensive research has been conducted to investigate ways to utilize this surplus crude glycerol. The annual number of research articles addressing the use of glycerol has increased to >7000, doubling in number from the year 2000 to 2007. Numerous chemical conversions to valuable products have been reported (Ciriminna et al., 2014).

Glycerol can be used for the microbial production of 1,3-propanediol (PDO), ethanol, 2,3-butanediol (BDO), butanol, lactic acid, 3-hydroxypropionic acid (3-HP), and succinic acid (Fig. 1) (Clomberg and Gonzalez, 2013; Mattam et al., 2013; Yazdani and Gonzalez, 2007). Because of its reduced nature, glycerol generates twice the number of reducing equivalents per carbon than do traditional carbohydrates (glucose, xylose, and sucrose) when converted into the glycolytic intermediates phosphoenolpyruvate (PEP) or pyruvate (Dharmadi et al., 2006). As a consequence, glycerol gives higher yields of reduced metabolites (e.g., succinate, ethanol, PDO, and butanol) than does glucose (Yazdani and

55 Gonzalez, 2007). Table 1 compares the maximum theoretical yield of various metabolites,
56 ATP, and redox balances between glycerol and glucose.

57 Despite its great potential as a carbon source, glycerol is not efficiently metabolized
58 by many microorganisms under anaerobic or oxygen-limited conditions. The ability of *K.*
59 *pneumoniae* to assimilate glycerol under oxygen-limited conditions is outstanding and has
60 been studied extensively. Several review articles addressing this issue have also been
61 published (Clomberg and Gonzalez, 2013; Mattam et al., 2013; Yazdani and Gonzalez, 2007),
62 but the biotechnological potential and physiological aspects of this strain have not been fully
63 elucidated. This review explores and discusses glycerol metabolism in *K. pneumoniae* and the
64 production of value-added chemicals from glycerol. The potential and challenges of using *K.*
65 *pneumoniae* as a microbial cell factory are also discussed.

66 2. Glycerol metabolism in *K. pneumoniae*

67 Many microorganisms can metabolize glycerol in the presence of external electron
68 acceptors (respiratory metabolism), but relatively few under non-respiratory conditions
69 (without exogenous electron acceptors). Among the latter, genera including *Citrobacter*,
70 *Enterobacter*, *Bacillus*, *Propionibacterium*, *Anaerobiospirillum*, *Klebsiella*, *Clostridium*, and
71 *Lactobacillus* are well documented (da Silva et al., 2009; Yazdani and Gonzalez, 2007).
72 Some *Lactobacillus* species including *L. reuteri*, *L. brevis*, and *L. bucheneri* convert glycerol
73 to PDO but only in the presence of a major carbon source for growth. Glycerol does not
74 support cell growth, limiting its biotechnological applications. In contrast, *Clostridium sp.*
75 grow well on glycerol as the sole carbon source. However, the use of *Clostridium* is limited
76 by its slow growth and requirement of strict anaerobic conditions. The lack of a convenient
77 genetic tool box for large-scale metabolic engineering is another serious drawback of
78 *Clostridium* (Branduardi et al., 2014; Lütke-Eversloh and Bahl, 2011).

79 From a biotechnological standpoint, *Klebsiella sp.*, especially *K. pneumoniae*, have
80 many advantages over the aforementioned microbes. They grow on glycerol as the sole
81 carbon source under both aerobic and anaerobic conditions (Arasu et al., 2011; Kumar et al.,
82 2012). Their growth is fast, typically growing at a rate >0.9/h, even on glycerol minimal
83 medium (Arasu et al., 2011). They naturally produce coenzyme B_{12}, an essential cofactor for
84 the synthesis of the commercially important chemicals PDO and 3-HP (Huang et al., 2013a;
85 Oh et al., 2012a). Furthermore, their evolutionary and biochemical proximity to *E. coli* allows
86 for the application of most of gene manipulation methods developed for *E. coli* without much
87 modification (Celińska, 2012; Kumar et al., 2013a).

88 **2.1 Overview of glycerol metabolism in *K. pneumoniae***

89 In *K. pneumoniae*, glycerol is metabolized oxidatively and/or reductively (Ashok et
90 al., 2011, Kumar et al., 2013a). The oxidative pathway provides energy and carbon
91 constituents for the synthesis of cell biomass, while the reductive pathway reduces glycerol
92 and regenerates NAD^+, enabling the oxidative assimilation of glycerol under anaerobic
93 conditions.

94 Glycerol oxidation proceeds in either a respiratory or a fermentative manner.
95 Respiration requires an exogenous electron acceptor such as oxygen, nitrate, or fumarate
96 (Ashok et al., 2013a,b) and is carried out by enzymes encoded by the *glp* regulon. During
97 respiration, glycerol is first phosphorylated by glycerol kinase (*glpK*) to yield *sn*-glycerol-3-
98 phosphate using ATP or phosphoenolpyruvate (PEP) as a phosphate donor. Next, *sn*-
99 glycerol-3-phosphate is oxidized to dihydroxyacetone phosphate (DHAP) with electron
100 transfer to a quinone molecule, which is linked to the electron transport chain (ETC). This
101 transformation is catalyzed by glycerol-3-phosphate dehydrogenase, which is expressed
102 under both aerobic (*glpD*) and anaerobic (*glpABC*) conditions. In contrast, fermentation of
103 glycerol is conducted without a supply of external electron acceptors, and the reactions are

104 catalyzed by enzymes encoded by the *dha* regulon. Here, glycerol is oxidized to
105 dihydroxyacetone (DHA) by glycerol dehydrogenase (mainly encoded by *dhaD*) using NAD^+
106 as an electron acceptor. DHA then is phosphorylated by dihydroxyacetone kinase, an enzyme
107 encoded by the ATP-dependent *dhaK1/dhaK* and/or PEP-dependent
108 *dhaK2/dhaKLM/dhaK123*. DHAP obtained from respiration and/or fermentation is channeled
109 into the glycolytic pathway (Fig. 2). A variety of organic acids and alcohols, including acetic
110 acid, lactic acid, succinic acid, ethanol, BDO, and formic acid, are produced by the
111 downstream glycolytic pathway (Ashok et al., 2011; Kumar et al., 2013a).

112 The anaerobic growth capability of *K. pneumoniae* using glycerol as a carbon source
113 is attributed to its well-established reductive metabolism. In the reductive pathway, glycerol
114 first undergoes a difficult rearrangement reaction catalyzed by glycerol dehydratase (GDHt)
115 to yield 3-hydroxypropioaldehyde (3-HPA). Catalysis by GDHt requires coenzyme B_{12}, the
116 *de novo* synthesis of which is limited to only few genera of microorganisms. The 3-HPA is
117 subsequently reduced to PDO by at least two 1,3-propanediol oxidoreductases (1,3-PDORs),
118 including NADH-dependent DhaT (PDOR) and NADPH-dependent hypothetical
119 oxidoreductase (HOR), along with the regeneration of $NAD(P)^+$ (Fig. 3). *E. coli* and other
120 *enterobacter sp*. have the oxidative (respiratory) pathway of glycerol assimilation. However,
121 these organisms lack the reductive pathway and cannot grow on glycerol under anaerobic
122 conditions (Dharmadi et al., 2006; Kumar et al., 2013a).

123 **2.2 Genes and enzymes involved in fermentative metabolism of glycerol**

124 The *dha* regulon, induced by the DHA molecule and expressed in the absence of
125 exogenous electron acceptors, encodes numerous genes needed for both the oxidative
126 (fermentative) and reductive pathways (Celińska, 2012; Forage and Foster, 1982) (Fig. 4A).
127 The *dha* regulon of *K. pneumoniae* contains the following genes arranged in the order as
128 appears: *Kpk_0615/dhaK*, *dhaM*, *dhaL*, *dhaK*, *dhaD*, *dhaR*, *orfW, CdAT*, *orfX* (*dhaG*), *dhaT*,

129 *orfY, dhaB*, *dhaC*, *dhaE*, *orfZ* (*dhaF*), and *glpF*. Of these, *dhaB*, *dhaC*, *dhaE*, *orfX*, *orfZ*, and
130 *dhaT* are relatively well documented for their functions in the reductive metabolism of
131 glycerol. The *orfW* and *orfY* genes, although commonly found in the *dha* regulons of many
132 organisms, are not as well described. The proteins encoded by the *dha* regulon of *K.*
133 *pneumoniae* have high similarity (80%–95%) with those of *Citrobacter* species but not with
134 those of *Clostridium* species (30%–80%). Surprisingly, the sequence similarity of *dha*
135 proteins among *Clostridium* species is very low in some cases, even lower than their
136 similarity to the corresponding proteins in *K. pneumoniae* or *C. freundii* (Celińska, 2012; Sun
137 et al., 2003; Wei et al., 2014).

138 The complete *dha* regulon is found in only a few dozen species belonging to five
139 different taxonomic groups, including four bacterial species [Actinobacteria, Firmicutes,
140 Fusobacteria, and Proteobacteria (Gammaproteobacteria and Deltaproteobacteria)] and one
141 archaea species, *Halalkalicoccus jeotgali* (Martins-Pinheiro et al., 2016). Interestingly, an
142 incomplete *dha* regulon is present in more than 100 prokaryotes, suggesting that the enzymes
143 of the regulon have other functions not restricted to the anaerobic growth on glycerol. A well-
144 documented example is the one found in *Lactobacillus* species. *Lactobacillus* cannot grow on
145 glycerol as the sole carbon source because it lacks the enzymes for oxidative assimilation of
146 glycerol. However, the enzymes encoded in the incomplete *dha* regulon enable the
147 production of PDO from glycerol, which accelerates cell growth in *Lactobacillus* grown on
148 glucose (Årsköld et al., 2008; Martins-Pinheiro et al., 2016).

149 In the following sections, the major enzymes involved in fermentative glycerol
150 metabolism and their genes are described in detail.

151 **2.2.1 Glycerol dehydrogenase**

152 Encoded by the *dhaD* and/or *gldA* gene, glycerol dehydrogenase is an oxidoreductase
153 that converts glycerol to DHA. The enzyme extracts electrons from glycerol and transfers

154 them to NAD^+. DhaD plays a major role during the anaerobic growth of *K. pneumoniae* on
155 glycerol. Quantification of mRNA expression indicates that *dhaD* is highly induced when
156 glycerol is used as carbon source. GldA expression is lower than that of DhaD and is thought
157 to serve as a back up to DhaD during anaerobic growth on glycerol. However, DhaD and
158 GldA exhibit a high level of similarity in amino acid sequence and have the same substrate-
159 binding site (Wang et al., 2014).

160 Recently, DhaD was reported to catalyze the interconversion of acetoin and BDO for
161 NADH disposal and recovery (Wang et al., 2014). The promiscuity of DhaD is responsible
162 for BDO production in the Δ*budC* mutant of *K. pneumoniae*. The contribution by DhaD to or
163 its participation in BDO production is also supported by the observed increase in BDO
164 synthesis and increased concentration of NADH in the presence of the *dhaD* gene. NADH is
165 generated by the oxidation of glycerol and consumed by the production of BDO from acetoin.
166 The dual physiological functions of DhaD allow *K. pneumoniae* to control the intracellular
167 redox level. In response to the intracellular level of NADH, DhaD is able to switch its role
168 from NADH production (glycerol oxidation) to NADH consumption (BDO production)
169 (Wang et al., 2014). The diversion of glycerol flux from organic acids toward alcohols such
170 as BDO also helps the cells to counteract intracellular acidification (Petrov and Petrova,
171 2009). Thus, this promiscuity confers different physiological roles to the enzyme, which
172 include regulation of the intracellular $NADH/NAD^+$ ratio, prevention of acidification, and
173 storage of carbon and energy (Wang et al., 2014).

174 **2.2.2 Dihydroxyacetone kinases**

175 Dihydroxyacetone kinase (DHAK) phosphorylates DHA to DHAP, an intermediate of
176 the glycolytic pathway. Sun et al. (2003) analyzed the genome of *K. pneumoniae* MGH
177 78578 and found, in addition to ATP-dependent DhaK I, a PEP-dependent DhaK II. DhaK I
178 is encoded by the *dhaK* (*Kpk_0615*) gene, while DhaK II is encoded by three genes, *dhaK*,

179 *dhaL* and *dhaM,* which correspond to the medium, small, and large subunits of the enzyme,
180 respectively (Celińska, 2012; Sun et al., 2003; Wei et al., 2014). The names *dhaKLM* and
181 *dhaK123* are synonyms used without distinction (Martins-Pinheiro et al., 2016; Sun et al.,
182 2003; Wei et al., 2014). DhaK enzymes are found in many microorganisms. PEP-dependent
183 DhaK II is present in *E. coli*, *L. lactis*, and *C. butyricum*, whereas ATP-dependent DhaK I is
184 present in *Citrobacter freundii* (Garcia-Alles et al., 2004; Raynaud et al., 2011). The PEP-
185 dependent DhaK II of *E. coli*, encoded by *dhaKLM*, is homologous to *dhaK123* (*dhaKLM*) of
186 *K. pneumoniae* (Gutknecht et al., 2001). In a recent study, Wei et al. (2014) further elucidated
187 the physiological roles of DhaK I and DhaK II of the *dha* regulon of *K. pneumoniae*. They
188 found that disruption of ATP-dependent DhaK I had no significant effect on glycerol uptake,
189 indicating that PEP-dependent DhaK II is the major contributor to the conversion of DHA to
190 DHAP. As in *E. coli*, the subunits of DhaK II regulate expression of the *dha* regulon; *dha*
191 regulon expression was suppressed by the disruption of *dhaK1* (*dhaK*) and *dhaK2* (*dhaL*).
192 However, in contrast to *E. coli*, mutation of *dhaK3* (*dhaM*) in *K. pneumonia* did not
193 upregulate the *dha* regulon but rather decreased cell growth and glycerol uptake.

194 **2.2.3 Glycerol dehydratase and reactivating factor**

195 Glycerol dehydratase (GDHt) is the first enzyme in the reductive pathway and
196 catalyzes the dehydration of glycerol to 3-HPA. The same reaction can be carried out by diol
197 dehydratase (DDHt), although the latter has a higher activity on 1,2-propanediol than on
198 glycerol. GDHt requires coenzyme B_{12} or *S*-adenosyl methionine (SAM) as a cofactor. *K.*
199 *pneumoniae* has B_{12}-dependent GDHt only, while strict anaerobes such as *clostridia* sp. have
200 SAM-dependent GDHt. The three subunits of GDHt are encoded by the genes *dhaB1* (~1670
201 bp), *dhaB2* (~590 bp) and *dhaB3* (~430 bp), for which the gene products are the α (large,
202 60.7 kDa), β (medium, 21.3 kDa), and γ (small, 16.1 kDa) subunits, respectively (Xu et al.,
203 2009a). GDHt of *K. pneumoniae* is known to be present as a dimer of heterotrimers, $(\alpha\beta\gamma)_2$.

204 The subunits of GDHt have high homology among *K. pneumoniae* strains; for example, *K.*
205 *pneumoniae* XJPD-Li and U30909 strains showed >99% identity.

206 Coenzyme B_{12} is present in the reaction center and plays an essential role in the
207 catalysis of GDHt. The catalytic cycle often results in the mechanism-based inactivation of
208 coenzyme B_{12} due to the irreversible breakage of the chemical bond between cobalt (Co) and
209 a carbon of the adenosyl moiety. Oxygen accelerates breakage of the Co–C bond. Oxygen is
210 also known to inactivate GDHt, even in the absence of the substrate glycerol. The gene
211 products of *orfZ/dhaG* (small subunit) and *orfX/dhaF* (large subunit) act as reactivating
212 factors of GDHt (GdrAB) (Celińska, 2012; Sun et al., 2003). They reactivate GDHt by
213 catalyzing the exchange of damaged for intact coenzyme B_{12} in the presence of ATP and
214 Mg^{2+}/Mn^{2+} (Celińska, 2012; Kumar et al., 2013a; Maervoet et al., 2011; Sun et al., 2003; Wei
215 et al., 2014). The reactivase is a molecular chaperone that functions as a heterotetramer
216 containing two elongated α subunits (63 kDa) and two globular β subunits (14 kDa).
217 Structurally, the α subunit resembles both GroEL and Hsp70 chaperones, while the β subunit
218 resembles that of the β subunit of glycerol dehydratase, except that it lacks some of the amino
219 acids responsible for coenzyme B_{12} binding (Liao et al., 2003).

220 In some organisms, the GDHt enzyme varies in terms of the number of subunits and
221 their copies (Fig. 4B) (Liu et al., 2010). For example, in *Mesorhizobium loti* and
222 *Mesorhizobium opportunistum*, the large and medium subunits (*dhaB1* and *dhaB2*) are fused
223 together and encoded by a single gene. The gene fusion may arise by frameshift mutations.
224 The active site of the fused enzyme differs slightly from that of unfused enzyme. Moreover,
225 these microorganisms lack the genes encoding the reactivation factor, indicating that the
226 reactivation process may not exist or is carried out by a different route. *Mycobacterium*
227 *smegmatis* has two subunits (fused *dhaB1* + *dhaB2* and *dhaB3*) that are similar to those of *M.*
228 *loti* and *M. opportunistum*; however, the reactivating factor differs in that it has one (large)

229 subunit. No small subunit has been identified in *M. smegmatis*. Fusobacterium *Ilyobacter*
230 *polytropus* is unique in that it has genes coding for both B_{12}-independent (SAM-dependent)
231 and B_{12}-dependent GDHt. The genes for B_{12}-independent GDHt are present in the genomic
232 DNA, while those encoding B_{12}-dependent GDHt along with its reactivation factor are
233 present in two copies, one in the chromosome and the other in a plasmid. Surprisingly, even
234 with this gene redundancy, no significant production of 3-HPA and/or PDO has been reported
235 in *I. polytropus* (Stieb and Schink, 1984). The B_{12}-independent proteins showed no homology
236 with B_{12}-dependent GDHt but significant similarity with the pyruvate formate lyases (PFL)
237 and PFL-activating enzymes and their homologues (Raynaud et al., 2003).

238 **2.2.4 1,3-Propanediol oxidoreductase and hypothetical oxidoreductase**

239 The 1,3-propanediol oxidoreductase (PDOR), responsible for the second step of the
240 reductive pathway of glycerol, catalyzes the conversion of 3-HPA to PDO with the oxidation
241 of NAD(P)H. 3-HPA is a toxic intermediate, and PDOR protects the cell from deleterious
242 damage by reducing 3-HPA to PDO (Celińska, 2012). In *K. pneumoniae*, PDOR is encoded
243 by the *dhaT* gene and is strictly NADH dependent. Active PDOR is a homo-octamer with a
244 monomeric molecular mass of 43 kDa. Despite its importance, the kinetic properties of DhaT
245 were elucidated only recently (Lama et al., 2015). DhaT has high specificity for 3-HPA
246 among various aldehydes. Under physiological conditions at pH 7.0 and 37°C, the catalytic
247 efficiency (k_{cat}/K_m) of the forward reaction (3-HPA to PDO) was found to be 77.2/s. mM,
248 which is 57-fold higher than that of the reverse reaction (1.3/s. mM; PDO to 3-HPA). The K_m
249 (mM) values for 3-HPA and NADH (forward reaction) are 0.77 and 0.03, respectively, while
250 those for PDO and NAD^+ (backward reaction) are 7.4 and 0.23, respectively. Furthermore,
251 the maximum activity of DhaT for the reverse reaction was obtained at pH 9.0 and 55°C.
252 These results strongly suggest that, under physiological conditions, DhaT catalyzes the
253 reduction of 3-HPA to PDO rather than the oxidation of PDO to 3-HPA. Nevertheless, the

254 reverse reaction (PDO→3-HPA) becomes significant as the PDO concentration increases.
255 This factor is considered a serious drawback of using DhaT for PDO production from
256 glycerol.

257 *K. pneumoniae* has several oxidoreductases in addition to PDOR that can reduce 3-
258 HPA to PDO. One such enzyme, designated as hypothetical oxidoreductase (HOR), has been
259 studied extensively and shares 89% identity with YqhD of *E. coli* (Zhu et al., 2009; Zhuge et
260 al., 2010). Expression of *yqhD* in wild-type *K. pneumoniae* is not high, with an mRNA level
261 ~20-fold lower than that of *dhaT*. However, deletion of *dhaT* increases the transcription of
262 *yqhD* by more than 10-fold (Ko et al., 2015). This observation suggests that in addition to its
263 wide-ranging activity on short-chain aldehydes, YqhD is a detoxification enzyme for many
264 aldehydes (Jarboe, 2011). YqhD exhibits negligible activity on PDO (backward reaction),
265 which is an important advantage over DhaT for use in the production of PDO from glycerol
266 (Celińska, 2012; Chen et al., 2011; Zheng et al., 2006). However, YqhD is kinetically inferior
267 to DhaT because of its low catalytic activity (k_{cat}/K_m) on 3-HPA and the requirement of
268 NADPH as a cofactor. The k_{cat}/K_m value of YqhD on 3-HPA (pH 7.0 and 37°C) is estimated
269 to be 2.1/s. mM, which is 36-fold lower than that of DhaT (unpublished data). The use of
270 NADPH as a cofactor prohibits this enzyme from being directly coupled to the reactions
271 catalyzed by DhaD and other enzymes in glycolysis and the TCA cycle, by which NADH is
272 mainly produced. Furthermore, excessive use of NADPH for PDO production can
273 significantly reduce the cellular NADPH level and this can disturb many anabolic reactions
274 requiring NADPH (Wang et al., 2003; Jarboe, 2011). NADH and NADPH differ in a
275 phosphate group only, and it has been suggested that electrostatic interaction of this
276 phosphate is the major factor distinguishing NADH from NADPH for enzymes using these
277 cofactors. Comparison of the amino acid sequences of DhaT and HOR from *K. pneumoniae*
278 and YqhD from *E. coli* has revealed that DhaT contains aspartic acid (Asp) at position 41,

279 while HOR and YqhD have valine (Val) at this position. The steric hindrance and
280 electrostatic repulsion between Asp in the active site and the phosphate group of NADPH
281 impede their binding. In contrast, the hydrophobic amino acid Val poses no such obstruction;
282 thus, YqhD uses NADPH as a cofactor (Ma et al., 2010).

283 Despite their difference in cofactor preference, DhaT and YqhD are similar (44%) in
284 amino acid sequences. *K. pneumoniae* has five enzymes that have at least 40% similarity in
285 amino acid sequence with DhaT and YqhD: putative alcohol dehydrogenase (PaldH), L-1,2-
286 propanediol oxidoreductase (FucO), ethanolamine-utilization enzyme (EutG), propanediol-
287 utilization propanol dehydrogenase (PduQ), and bifunctional alcohol dehydrogenase (AdhE)
288 (Ko et al., 2015). The roles of these enzymes in PDO production have been investigated.

289 **2.2.5 Regulatory protein**

290 The *dhaR* gene product is an important transcription factor that is responsible for
291 expression of the *dhaT* and *dhaB* genes (Sun et al., 2003). This gene is found in *K.*
292 *pneumoniae*, C. *freundii*, and *Clostridium botulinum* but not in every organism containing a
293 complete *dha* regulon (Martins-Pinheiro et al., 2016). The presence of DhaR is thought to
294 allow the *dha* operon to function as a separate regulatory system, independent of fumarate
295 nitrate reduction (FNR). Under anaerobic conditions, FNR acquires DNA-binding properties
296 and induces the expression of many anaerobiosis-related genes. DhaR contains domains for
297 GAF (52-199), PAS (203-267), σ^{54} factor interaction, and histidine HTH-8. The PAS senses
298 internal levels of energy charge, light, oxygen, and redox potential. The HTH-8 domain has a
299 helix-turn-helix conformation and acts as a DNA binding structure. The σ^{54} factor interaction
300 domain interacts with the σ^{54} factor of RNA polymerase and activates RNA transcription
301 from the σ^{54} promoters (Buck et al., 2000; Taylor and Zhulin, 1999). Thus, the DhaR protein
302 triggers the transcription of *dha* genes in response to intracellular levels of several important
303 physiological parameters (Celińska, 2012; Sun et al., 2003). Zheng et al. (2006) investigated

304 the effect of overexpression of the putative regulatory gene *dhaR* in the *dha* regulon on
305 glycerol metabolism in *K. pneumoniae*. *dhaR* overexpression increased PDOR activity up to
306 6.7-fold over that of the control wild-type strain, confirming the role of DhaR as a positive
307 regulator of the *dhaT* gene. However, *dhaR* overexpression did not increase the activity of
308 glycerol dehydratase (DhaB). Furthermore, despite the significant increase in PDOR activity,
309 PDO production was unexpectedly low compared with that of the wild type (440 vs. 656.23
310 mM, respectively). More studies are required to determine the precise role of DhaR and its
311 regulatory function in the *dha* regulon.

312 DhaR in *E. coli* (70% identity to that of *K. pneumoniae*) has also been studied. This
313 protein stimulated transcription of the *dhaKLM* operon from a σ^{70} promoter. Interestingly, in
314 *E. coli*, DhaL was a positive regulator of the *dhaKLM* operon while phosphorylated DhaM
315 and DhaK were negative regulators of the same operon. DhaK contains the DHA-binding site.
316 DhaL uses ADP as a cofactor for the double displacement of phosphate from DhaM to DHA.
317 DHA is the oxidation product of glycerol metabolism; binding of DHA to DhaK reduces the
318 affinity of DhaK for DhaR. In the presence of DHA, DhaL::ATP is dephosphorylated,
319 leading to the displacement of DhaK by DhaL::ADP, which stimulates DhaR activity. In the
320 absence of DHA, DhaL::ADP is phosphorylated back to DhaL::ATP by the phototransferase
321 system (PTS), thereby inhibiting the binding of the phosphorylated complex (DhaL::ATP) to
322 DhaR. This phosphorylation is mediated by DhaM, providing a phospho-histidine relay
323 between the PTS and DhaL::ADP. This double-check mechanism of binding and turnover
324 increases the selectivity such that the binding of nonphosphorylated compounds does not
325 induce the *dha* operon (Bächler et al., 2005).

326 **2.3 Genes and enzymes involved in respiratory assimilation of glycerol**

327 The *glp* regulon comprises the genes encoding proteins required for the conversion of
328 glycerol to DHAP via sn-glycerol-3-phosphate. The five operons that constitute the *glp*

329 regulon are *glpFK*, *glpD* (anaerobic glycerol-3-phosphate dehydrogenase), *glp ACB* (aerobic
330 glycerol-3-phosphate dehydrogenase), *glpTQ* (glycerol-phosphate
331 permease/glycerophosphodiesterase), and *glpEGR*. The *glpF* gene encodes a cytoplasmic
332 membrane protein that facilitates the diffusion of glycerol into the cell. The *glpE* gene
333 encodes an acidic, cytoplasmic protein of 108 amino acids with a molecular weight of
334 12,082. The *glpG* gene encodes a basic, cytoplasmic membrane-associated protein of 276
335 amino acids with a molecular weight of 31,278 (Lin, 1976; Yang and Larson, 1998; Zeng et
336 al., 1996). The biochemical functions of GlpE and GlpG are unknown. The *glpR* gene
337 encodes the GlpR repressor protein. These five operons are located at three different positions
338 on the chromosome. Transcription of these operons is subject to multiple controls, including
339 catabolite repression mediated by cAMP-CRP and respiratory control mediated by the FNR
340 and ArcA/ArcB systems. Moreover, each of the operons is negatively controlled by a
341 repressor specific for the regulon (Lin, 1987; Zeng et al., 1996).

342 **2.3.1 GlpR and other regulatory proteins**

343 The GlpR repressor protein, predicted to contain 252 amino acids with a molecular
344 weight of 28,048, belongs to the DeoR bacterial regulatory factor super family. Although not
345 studied extensively, GlpR of *K. pneumonia* is expected to have the same biochemical
346 properties and regulatory functions as that of *E. coli*. GlpR negatively controls all the *glp*
347 operons by binding to operators that overlap or are close to the *glp* promoters. In case of *E.*
348 *coli*, glycerol-3-phosphate acts as an inducer and has a high affinity for GlpR (K_d = 31 μM)
349 (Lin, 1987; Weissenborn et al., 1992; Zeng et al., 1996). When complexed with glycerol-3-
350 phosphate, GlpR cannot bind to the operator sites, and repression is relieved. Glycerol-3-
351 phosphate is a product of GlpK and a substrate of GlpD/GlpABC; thus, the absence of GlpD
352 causes a substantial increase in the level of glycerol-3-phosphate, resulting in induction of the
353 *glp* regulon (Fig. 2). The GlpR protein exhibits differential binding affinity for the operators

354 of corresponding operons, as follows: *glpFK* > *glpD* > *glpTQ* > *glpACB*. The *glpFK* promoter
355 appears to be the strongest among the *glp* promoters. Therefore, the maximum strength of the
356 *glpFK* promoter combined with the high affinity of GlpK for glycerol (K_m = 10 μM) should
357 result in sufficient accumulation of the inducer, glycerol-3-phosphate, in the presence of
358 glycerol to relieve repression by GlpR. The relatively lower strength of the *glpD* promoter
359 along with the low affinity of the GlpD enzyme for glycerol-3-phosphate (K_m = 1 mM)
360 prevents excessive degradation of glycerol-3-phosphate that is needed for phospholipid
361 synthesis when the exogenous source of glycerol-3-phosphate becomes limiting, until after
362 accumulation of the inducer (Lin, 1987; Weissenborn et al., 1992). Recently, Jung et al.
363 (2014) investigated the role of GlpR in the aerobic production of 3-HP from glycerol in *E.*
364 *coli*. They found that elimination of this regulatory factor caused increased assimilation of
365 glycerol and higher production of 3-HP and suggested that the deletion of *glpR* led to the
366 upregulation of genes involved in glycerol transport and assimilation. *K. pneumoniae* has
367 several other regulatory genes in addition to *glpR*: the global regulatory genes *hdeB*, *hdeD,*
368 and *yfdX*, with unknown functions and *kvgS* and *kvgA*, encoding two proteins comprising a
369 two-component signal transduction system (Sun et al., 2003).

370 **2.3.2 Glycerol uptake/transport facilitator and other components**

371 The glycerol facilitator GlpF is an integral membrane protein that forms aqueous
372 pores. The GlpF pores selectively allow passive transport of glycerol and other molecules
373 such as DHA and urea across the cytoplasmic membrane, but not water or ions (Heller et al.,
374 1980; Reizer et al., 1993). The GlpF protein contains six membrane-spanning helices that are
375 unique among prokaryotic transport proteins. These transmembrane helices are arranged in
376 two bundles. The GlpF protein may function as a homodimer with the two six-member
377 domains arranged in the membrane to form a channel (Weissenborn et al., 1992). *K.*
378 *pneumoniae* has two glycerol transporter genes, one next to *orfZ/dhaF* in the fermentative

379 *dha* regulon (see Fig. 4A) and the other in the respiratory *glpFK* operon. Both *glpF* genes
380 have a high similarity (85 %) to each other. When one but not both of the *glpF* genes is
381 disrupted, the *glpF* deletion mutants of *K. pneumoniae* can consume glycerol and convert it to
382 PDO at a high rate, similar to the wild type (unpublished data; Supplementary Fig. S1). This
383 observation indicates that both *glpF* genes encode active glycerol transporter proteins.
384 However, their presence is not essential for glycerol utilization in *K. pneumoniae* because the
385 double-deletion mutant for both facilitators can still uptake glycerol and convert it to PDO. In
386 *E. coli*, only one *glpF* gene is present in the *glpFK* operon; the GlpF protein shows a high
387 similarity (80.9%) to that (encoded from *glpFK*) of *K. pneumoniae* (Sun et al., 2003). The
388 importance of GlpF in *E. coli* also seems to be limited only at low levels of glycerol (<8
389 mM), as glycerol itself may pass through the membrane by diffusion at high concentrations
390 (Richey and Lin, 1972).

391 **2.3.3 Simultaneous operation of fermentative and respiratory pathways**

392 The presence of the *dha* and *glp* regulons affords *K. pneumoniae* metabolic flexibility.
393 The *dha* regulon enables fermentative utilization of glycerol, while the *glp* regulon enables
394 respiratory metabolism of glycerol. During fermentative metabolism of glycerol, redox
395 constraints force the production of PDO from glycerol, with a low ratio of carbon conversion
396 yield to cell biomass. Moreover, ATP is formed through substrate-level phosphorylation, and
397 NADH oxidation occurs through the formation of reduced metabolites. In the presence of
398 oxygen, the redox constraints forcing the production of PDO are relieved; therefore, the
399 carbon loss required for regeneration of NAD^+ is greatly reduced. As a consequence, glycerol
400 utilization, biomass synthesis, and even the formation of many metabolites increase (Durnin
401 et al., 2008). Interestingly, the fermentative route (*dha* regulon) is in operation under a wide
402 range of oxygen levels, from microaerobic to highly aerobic conditions, as evidenced by
403 PDO formation. For example, a high titer PDO (102.7 g/L) was produced under the condition

404 of 2.0 vvm aeration and even at 3.5 vvm, although the production was a little lowered in the
405 latter case (Oh et al., 2012a). These results suggest that the fermentative and respiratory
406 pathways might have evolved to complement each other for optimal control of cell growth
407 under a wide range of aeration conditions except the strictly anaerobic one.

408 **2.4 Carbon catabolite repression**

409 Carbon catabolite repression (CCR) refers to the suppression of the use of less-
410 preferred carbon sources, when a more preferred carbon, most prominently glucose, is
411 present. Cyclic AMP, cAMP receptor protein (CRP), and the enzyme $EIIA^{Glc}$, an
412 intermediate in the phosphorylation cascade of the PTS, are key players in the CCR of
413 enterobacteria. The $EIIA^{Glc}$, a cytosolic protein, exists in phosphorylated and
414 unphosphorylated forms. The phosphorylated form stimulates membrane-bound adenylate
415 cyclase, which catalyzes the formation of cAMP; the cAMP-CRP complex then induces
416 expression of the catabolic genes of less-preferred carbon sources. The unphosphorylated
417 form of $EIIA^{Glc}$ is also responsible for inhibiting the transport of less-preferred carbon
418 sources by inhibitory binding to permeases, causing so-called inducer exclusion (Eppler and
419 Boos, 1999; Eppler et al., 2002). Glycerol assimilation in *K. pneumoniae* is greatly inhibited
420 by CCR in the presence of glucose. This repression is related to not only low cAMP and
421 cAMP-CRP but also inhibition of GlpK (which functions mainly under aerobic conditions)
422 by fructose 1,6-bisphosphate, a metabolite produced from glucose. The unphosphorylated
423 $EIIA^{Glc}$ is also known to allosterically inhibit GlpK (Holtman et al., 2001). Anaerobic
424 glycerol metabolism mediated by the *dha* operons is also suppressed by the presence of
425 glucose. Enzyme-level inhibition similar to that of the aerobic GlpK has not been reported,
426 but we have observed that, in *K. pneumoniae*, transcription of the *dha* operons is greatly
427 reduced in the presence of glucose (Suman et al., 2017).

428 Interestingly, glycerol exerts catabolite repression on the assimilation of maltose in *E. coli*. To exert catabolite repression, glycerol must be phosphorylated to glycerol-3-phosphate (Hogema et al., 1998; Eppler et al. 2002). However, unlike other carbon sources, further metabolism of glycerol is not required. According to Eppler and Boos (1999), glycerol-3-phosphate reduces expression of MalT, a positive activator of all *mal* genes. The key players in this repression are adenylate cyclase, $EIIA^{Glc}$, and CRP. The growth on maltose of the mutants lacking $EIIA^{Glc}$ or containing truncated adenylate cyclase was no longer repressed by glycerol, and CRP-independent transcription of *malT* was also not influenced by glycerol either. In addition, stimulation of adenylate cyclase by phosphorylated $EIIA^{Glc}$ was controlled by glycerol-3-phosphate. The mutation in adenylate cyclase relieved the repression caused by glycerol-3-phosphate (Eppler et al., 2002). Although not investigated, we assume that the glycerol repression on maltose catabolism also hold true in *K. pneumoniae*.

440 Glycerol also exerts catabolite repression on the assimilation of citrate in *K. pneumoniae.* Citrate can be used as the sole carbon and energy source during anaerobic growth of *K. pneumoniae*, but the presence of glycerol suppresses the expression of the citrate fermentation genes. A reduced concentration of the cAMP–CRP complex has been postulated to be the cause of this repression (Meyer et al., 2001). Further research is needed to test the hypothesis.

446 **3. *K. pneumoniae* as a microbial cell factory for the production of commodity chemicals**

447 During glycerol fermentation, *K. pneumoniae* produces many metabolites, including PDO, BDO, and lactic acid. This section describes the biotechnological production of bulk chemicals from glycerol in *K. pneumoniae*. 3-HP is not a natural product during the growth of *K. pneumoniae* on glycerol. However, its production is possible using a minor modification of the PDO synthetic pathway and is included here. The biochemical pathways

452 used for the synthesis of these chemicals are explained, and the metabolic engineering used to
453 improve their production is discussed.

454 **3.1 1,3-Propanediol**

455 PDO, also known as trimethylene glycol, is a promising platform chemical that has
456 two hydroxyl groups. With terephthalic acid, it can be co-polymerized to the novel co-
457 polymer polytrimethylene terephthalate (PTT). PDO also has applications in the food and
458 cosmetic industries. Currently, commercial PDO is mainly produced by recombinant *E. coli*
459 (Maervoet et al., 2011; Saxena et al., 2009). The global demand for PDO was 60.2 kilotons in
460 2012 and is predicted to reach approximately 150 kilotons by 2019. The market for PDO is
461 growing rapidly, from $157 million in 2012 with projections up to $560 million in 2019
462 (MarketsANDMarkets, 2012; Lee et al., 2015).

463 *K. pneumoniae* is one of the best native producers of PDO from glycerol. GDHt and
464 PDOR are two important enzymes for the conversion of glycerol to PDO (Fig. 3).
465 Physiologically, the synthesis of PDO from glycerol is essential for the anaerobic growth of
466 *K. pneumoniae*, as described previously. The production of PDO requires two cofactors,
467 coenzyme B_{12} and NAD(P)H. *K. pneumoniae* has a *de novo* pathway for the biosynthesis of
468 coenzyme B_{12} that comprises more than 20 genes. NAD(P)H is generated through the
469 oxidative metabolism of glycerol, during the production of cell biomass and/or oxidized
470 (pyruvate and acetate) or partially reduced (BDO, lactic acid) metabolites. The production
471 yield of PDO from glycerol varies depending on the cost of carbon for NAD(P)H production;
472 the lower the cost, the higher the yield of PDO from glycerol. NADPH can also be the
473 electron donor for the conversion of 3-HPA to PDO when YqhD is present. However, the
474 contribution of NADPH to PDO production in native *K. pneumoniae* seems to be limited
475 because the PP pathway, which is the main source of NADPH, is not active when glycerol is

476 the sole carbon source. Furthermore, *K. pneumoniae* has very low transhydrogenase activity
477 for the conversion of NADH to NADPH (Zhang and Xiu, 2009; Chen et al., 2011).

478 Several metabolic engineering strategies have been employed to improve PDO
479 production by *K. pneumonia* (Table 2). Amplification of the reductive pathway by
480 overexpressing DhaB, PDOR, or both has been attempted by many research groups. As
481 indicated previously, 3-HPA is highly toxic, and its accumulation is detrimental to cell
482 viability and toxic to PDO production. To reduce 3-HPA accumulation, overexpression of
483 *dhaT* has been attempted. In one study, 3-HPA accumulation was reduced by *dhaT*
484 overexpression, but no improvement in PDO production resulted (Hao et al., 2008).
485 Overexpression of both DhaT and DhaD increased PDO production (56.3%) in batch culture
486 but no increase in fed-batch culture (Chen et al., 2009). With another *K. pneumoniae* strain,
487 DSM 2026, the same experiments were repeated but also with no increase in PDO production
488 (Zheng et al., 2006). These results suggest that the reductive pathway in *K. pneumoniae* is
489 evolutionarily well established (perhaps in concert with the oxidative pathway) and that PDO
490 production is not limited by expression of the *dhaB* and *dhaT* genes of the reductive pathway.
491 However, in one study where the *E. coli yqhD* gene was highly overexpressed to give ~10-
492 fold higher PDOR activity (110 IU/mg total protein), a significant increase in PDO titer (25%;
493 67.6 g/L) and yield (from 0.53 to 0.62 mol/mol), along with less 3-HPA accumulation, was
494 achieved (Zhu et al., 2009). This observation suggests that in the recombinant strain, NADPH
495 can play an important role as a cofactor and that PDO production can be increased by
496 modifying the reductive pathway. It is also probable that the effect of DhaB and PDOR on
497 PDO production is highly dependent on the strain and/or culture conditions adopted.

498 Lactic acid, BDO, ethanol, and acetic acid are the major byproducts of PDO synthesis
499 in *K. pneumoniae*. Inactivation of the metabolic pathways that give rise to these products has
500 resulted in increased PDO synthesis. In particular, deletion of the *ldhA* gene encoding lactate

501 dehydrogenase has been highly beneficial. For example, by disrupting the *ldhA* gene, Xu et al.
502 (2009b) significantly increased PDO production without lactic acid production. They
503 observed increases in the PDO titer (95.4–102.1 g/L), conversion yield (0.48–0.52 mol/mol),
504 and productivity (1.98–2.13 g/L h; Table 2). Similarly, Oh et al. (2012a) achieved the high
505 PDO titer of 102.7 g/L by deleting *ldhA* in their own *K. pneumoniae* isolate. When lactic acid
506 production was eliminated, more ethanol and succinic acid were generated from the pyruvate
507 node. Reduction of these compounds has also been attempted by deleting *adhE* (encoding
508 alcohol dehydrogenase) and *frdA* (encoding fumarate reductase). Although the production of
509 ethanol and succinate was substantially reduced in the triple mutant (Δ*ldhA*Δ*adhE*Δ*frdA*),
510 PDO production was only marginally increased compared with that of the single Δ*ldhA*
511 mutant. The failure of the triple mutant (Δ*ldhA*Δ*adhE*Δ*frdA*) to increase PDO production was
512 mainly due to an increase in BDO production. Disruption of the BDO pathway encoded by
513 the *bud* operon (*budA*, *budB*, and *budC*) has also been studied (Fig. 5). Although partially
514 effective, individual inactivation of each of the three genes did not successfully decrease
515 BDO production (Oh et al., 2012a; Wu et al., 2013). Deletion of the entire *bud* operon
516 completely eliminated BDO synthesis (Kumar et al., 2016), but seriously hampered cell
517 growth and glycerol consumption. Consequently, no increase in PDO production was resulted.
518 Wu et al. (2013) attempted to decrease BDO production while increasing PDO production by
519 inserting the *fdh* gene from *Candida boidinii* (NADH-forming formate dehydrogenase) into
520 the *budC* locus. This protocol increased the PDO titer (62.3–72.2 g/L) and yield (0.47–0.57
521 mol/mol) and decreased the production of BDO by 52.2% and formic acid by 73.4%.
522 Acetic acid is one of the most toxic metabolites, accumulating in large quantities
523 during glycerol fermentation by *K. pneumonaie* (Celińska, 2012). The toxic effects of acetate
524 is realized even at the low concentration of 20 mM. Physiologically, acetate is a preferred
525 metabolite for many *Enterobacter* sp. because its production is accompanied by ATP

526 generation. In a typical bioreactor run for PDO production by *K. pneumoniae*, the
527 accumulation of acetic acid to >300 mM by the end is not uncommon, which often
528 completely stops glycerol fermentation. Acetate is produced by Pta-Ack and PoxB when
529 pyruvate formation is faster than its consumption, which is so-called 'overflow metabolism'
530 (Fig. 6) (De Mey et al., 2007). In *K. pneumoniae*, the contribution of the *poxB* gene is not
531 significant and its deletion does not much affect acetate production. In comparison, deletion
532 of the *pta-ack* genes greatly reduces cell growth and results in high accumulation of pyruvate
533 and pyruvate-based metabolites (Ko et al., 2017). Extensive studies to reduce or eliminate
534 acetate production have been conducted in *E. coli*. Compared to *poxB*, *ackA* and/or *pta* were
535 much more significant in reducing acetate production. However, deletion of the latter genes
536 was not beneficial because the glycolytic flux and cell yield were seriously reduced and the
537 accumulation of other byproducts such as pyruvate, lactate, and formate was greatly
538 increased (Causey et al., 2004; De Mey et al., 2007). Several indirect approaches to
539 decreasing pyruvate/acetate accumulation were investigated: stimulation of the anaplerotic
540 pathway by overexpression of pyruvate carboxylase, PEP carboxylase, or PEP carboxykinase
541 (Abdel-Hamid et al., 2001; Causey et al., 2004; Chao and Liao, 1993; Sanchez et al., 2005);
542 overexpression of acetyl-CoA synthetase (ACS); increase of the TCA cycle throughput by
543 deleting the transcriptional repressor *arcA* gene; and stimulation of the glyoxylate shunt by
544 deleting the transcriptional repressor *iclR* gene (De Mey et al., 2007; Jeong et al., 2004; Lin et
545 al., 2006). Moreover, the use of a mutant pyruvate dehydrogenase which is less sensitive to
546 inhibition by NADH has also been examined (Kim et al., 2008). In *E. coli*, these approaches
547 proved to be effective to varying extents under properly selected culture conditions. However,
548 they have not yet been fully investigated in *K. pneumoniae*.
549 To eliminate byproduct formation, reduction of glycerol flux through the oxidative
550 pathway at the glycerol node has also been attempted. The deletion of glycerol

551 dehydrogenase (*dhaD*) and/or dihydroxyacetone kinase (*dhaK*) resulted in decrease of the
552 oxidative flux and increase of the conversion yield of glycerol to PDO. In addition,
553 production of the byproducts lactate, ethanol, and succinate but not acetate decreased
554 significantly, even without blocking the pathways leading to these byproducts (Seo et al.
555 2009; Horng et al. 2010). However, cell growth and glycerol consumption rate were also
556 reduced. Interestingly, in these mutant strains, deletion of *dhaT* has almost no effect on PDO
557 production. In the absence of *dhaT*, expression of an HOR, highly homologous to YqhD, was
558 upregulated (see section 2.2.4).

559 The theoretical maximum yields of PDO on glycerol are 0.875 and 0.844 mol/mol
560 under aerobic and anaerobic conditions, respectively (Celińska, 2012). However,
561 experimentally, the yields are reported between 0.35 and 0.65 mol/mol (Saxena et al., 2009;
562 Sun et al., 2008). The low yield is mainly attributed to the accumulation of unnecessary
563 byproducts and inefficient metabolism for generating reducing equivalents (NADH) and ATP.
564 If acetyl-CoA is fully oxidized through the TCA cycle (without byproducts formation) and
565 the activity of the electron transport chain (ETC) oxidizing NADH is properly controlled, a
566 significant increase in PDO yield is expected. Attaining this goal requires extensive pathway
567 engineering of cellular metabolism. In bioreactor operation, the aeration rate should be
568 optimized. Oxygen regulates gene expression and the enzymatic reactions of glycerol
569 metabolism and determines the diversion of DHA to the PP pathway. Regeneration of NADH
570 by the TCA cycle and its oxidation by ETC are also controlled by oxygen levels.

571 **3.2 3-Hydroxypropionic acid**

572 As with PDO, 3-HP is an important platform chemical and has been selected by the
573 US Department of Energy (DOE) as one of the top 12 chemicals obtainable from biomass. 3-
574 HP is a C3 bifunctional molecule and a structural isomer of lactic acid (Werpy and Petersen,
575 2004). 3-HP can be converted into a variety of useful chemicals such as acrylic acid and acryl

576 amide (Pina et al., 2011; Kumar et al., 2013a). 3-HP can be synthesized by chemical routes
577 from acrylic acid, 3-propiolactone, 3-hydroxypropionitrile, allyl alcohol, vinyl acetate, and
578 PDO. However, none of the chemical processes is commercially feasible at present due to the
579 high cost of the starting materials, toxicity of intermediates, and/or the environmental
580 incompatibility of the processes (Jiang et al., 2009; Pina et al., 2011; Kumar et al., 2013a).
581 Many microorganisms, including both prokaryotes and eukaryotes, can naturally synthesize
582 3-HP as either an intermediate or end product through a range of metabolic pathways.
583 However, the production of 3-HP by these native microorganisms is too low to be
584 commercially meaningful (Jiang et al., 2009; Kumar et al., 2013a).
585 In studies pursuing commercial production of 3-HP, glycerol is first converted to 3-
586 HPA by GDHt. 3-HPA then is converted to 3-HP via the coenzyme A (CoA)-dependent or
587 CoA-independent pathway (Fig. 3). In the CoA-independent pathway, 3-HPA is oxidized to
588 3-HP by aldehyde dehydrogenase (ALDH); in the CoA-dependent pathway, 3-HPA is
589 converted to 3-HP via 3-HP-CoA and 3-HP-phosphate. In *K. pneumoniae*, both the CoA-
590 dependent and CoA-independent pathways are present. However, the titer of 3-HP produced
591 by the CoA-dependent pathway is low as <5.0 g/L (Luo et al., 2011 and 2012). High
592 production of 3-HP requires the disruption of oxidoreductases (e.g., DhaT, YqhD) and the
593 overexpression of a proper aldehyde dehydrogenase (ALDH). Ashok et al. (2013a) developed
594 a recombinant *K. pneumoniae* in which *dhaT* and *yqhD* were deleted, and the homologous
595 *puuC* gene encoding the NAD^+-dependent γ-glutamyl-γ-aminobutyraldehyde dehydrogenase
596 was overexpressed. The recombinant *K. pneumoniae* produced 3.8 g/L 3-HP in 12 h of flask
597 culture, but only under appropriate aeration (i.e., microaerobic) conditions. Under anaerobic
598 conditions, PDO (instead of 3-HP) was obtained as the main product, even though two
599 oxidoreductases were disrupted and *puuC* was highly overexpressed. In contrast, highly
600 aerobic conditions produced a high cell mass without much accumulation of either 3-HP or

601 1,3-PDO. In a glycerol-fed batch bioreactor experiment under a constant dissolved oxygen
602 (DO) concentration of 5% (considered “proper” microaerobic condition), the recombinant *K.*
603 *pneumoniae* Δ*dhaT* Δ*yqhD* overexpressing both PuuC and DhaB produced >28 g/L 3-HP in
604 48 h, with a yield of >40%. In contrast, the same strain produced only 10.5 g/L 3-HP when
605 cultivated under the higher 10% DO conditions. Several important conclusions regarding 3-
606 HP production by *K. pneumoniae* were made, as follows. First, in addition to DhaT and
607 YqhD, *K. pneumoniae* has more unidentified oxidoreductases that can produce PDO from 3-
608 HPA. Second, to produce 3-HP rather than PDO, proper aeration to regenerate NAD^+ is
609 essential. Third, excessively high aeration decreases the expression of the Dha regulon and
610 synthesis of coenzyme B_{12}, an essential cofactor for GDHt catalysis.

611 To eliminate PDO production even under limited aeration conditions, Ko et al.
612 attempted to identify and disrupt other potential PDORs from *K. pneumoniae* (Ko et al.,
613 2015). A mutant strain devoid of *dhaT*, *yqhD*, *ahpF*, and *adhE* genes was developed;
614 however, the mutant neither eliminated 1,3-PDO production nor increased 3-HP production.
615 Again, this result suggests that *K. pneumoniae* has more unidentified oxidoreductases, and
616 thus, the complete elimination of 1,3-PDO production during 3-HP production is highly
617 challenging. To address the important problem, of NAD^+ regeneration and coenzyme B_{12}
618 production, Ashok et al. (2013b) studied anaerobic respiration using nitrate as an external
619 electron acceptor. They attempted to regenerate NAD^+ from NADH by nitrate reduction
620 while maintain anaerobic conditions to synthesize and stabilize the oxygen-sensitive
621 coenzyme B_{12}. Disruption of the *glpK* gene (encoding for glycerol kinase) was also necessary
622 because with *glpK* intact, the rate of anaerobic respiration was too fast, and most of the
623 glycerol was metabolized through GlpK to produce biomass, as in the case of highly aerobic
624 cultivation. The anaerobic glycerol fermentation in the presence of nitrate was successful:
625 1.74 g/L 3-HP was obtained from a 12-h flask culture and 22 g/L 3-HP was produced in a 48-

626 h fed-batch bioreactor culture (Ashok et al., 2013b) (Table 3). However, disadvantages were
627 noted, including the requirement for large amounts of nitrate and the toxic effect of nitrite
628 generated as a reduced intermediate of nitrate. In a recent study, Li et al. (2016) optimized a
629 promoter for ALDH expression, culture conditions, and metabolic flux to achieve high-level
630 production of 3-HP in their *K. pneumoniae* isolate. One recombinant strain, *K. pneumoniae*
631 (pTAC-*puuC*) expressing *puuC* under the IPTG-inducible *tac* promoter, produced 73.4 g/L 3-
632 HP in a bioreactor with a molar yield of glycerol of 0.52 and productivity of 1.53 g/L h.
633 Further, elimination of the *ldh1*, *ldh2*, and *pta* genes elevated the titer and molar yield to 83.8
634 g/L 3-HP and 0.54 mol/mol, respectively, with decreased cell growth and productivity. This
635 titer of 3-HP is the highest produced by *K. pneumoniae* to date.
636 As another approach to addressing the problem associated with NAD^+ regeneration
637 and coenzyme B_{12} synthesis in 3-HP production, co-production of 3-HP and PDO has been
638 investigated. The simultaneous production of 3-HP and PDO can eliminate the dependency
639 on oxidative metabolism of glycerol (and electron transport chain), as the cofactor required
640 for 3-HP production is regenerated by PDO production or vice versa. Furthermore, the
641 problems associated with the expression of genes for the assimilation of vitamin B_{12} and
642 glycerol can be alleviated substantially because co-production can be performed under
643 anaerobic or microaerobic conditions (Kumar et al., 2012, 2013b). Ashok et al. (2011)
644 developed a recombinant strain of *K. pneumoniae* DSMZ by overexpressing ALDH and
645 deleting *dhaT*. With *dhaT* intact, PDOR activity was too high compared with that of ALDH,
646 even with overexpression of the latter enzyme from a multicopy plasmid. The recombinant *K.*
647 *pneumoniae* DSMZ accumulated 16.0 g/L 3-HP and 16.8 g/L PDO in 24 h, and the
648 cumulative yield of these two metabolites on glycerol was 51% (Table 3). Huang et al. (2012)
649 studied the same co-production using their *K. pneumoniae* isolate overexpressing ALDH and
650 obtained 24.4 3-HP and 49.9 g/L PDO, respectively, after 24 h under anaerobic conditions.

651 The cumulative molar yield of the two metabolites reached 0.61 (0.18 for 3-HP and 0.43 for
652 PDO). In a following study, they showed that the maximum titer of 48.9 g/L 3-HP (along
653 with 25.3 g/L PDO) was achieved at 1.5 vvm, while the highest concentration of 38.4 g/L
654 PDO (along with 16.6 g/L 3-HP) was obtained at 0.1 vvm (Huang et al., 2013a).

655 During 3-HP production from glycerol by *K. pneumoniae*, the accumulation of such
656 byproducts as lactic acid, ethanol, acetic acid, formic acid, and 2,3-BDO is a serious
657 consideration, as in the case of 1,3-PDO production (Ashok et al., 2013a,b; Li et al., 2016).
658 Deletion of *ldhA* reduces lactic acid formation and increases the 3-HP yield. However,
659 deletion of other genes such as *adhE*, *pfl*, *budBAC*, and *pta-ack* had little desired effect and
660 seriously hampered cell growth and glycerol assimilation (unpublished data). Even with the
661 co-production of 3-HP and 1,3-PDO, where oxidative glycerol assimilation was greatly
662 reduced, accumulation of these byproducts was a serious drawback (Ko et al., 2017). To
663 prevent the formation of lactic acid along with other byproducts, Kumar et al. (2013b)
664 employed resting cells of recombinant *K. pneumoniae* J2B overexpressing ALDH and devoid
665 of lactate dehydrogenase (*ldhA*). For this strain, final titers of 3-HP and PDO of 22.7 and 23.5
666 g/L, respectively, were obtained without lactic acid accumulation. The cumulative product
667 yield increased to 0.77.

668 In addition to *K. pneumoniae*, *E. coli* has been extensively studied for 3-HP
669 production from glycerol. Several successful results have been reported by Samsung Ltd. Co.
670 Korea. One study (Chu et al., 2015) used an engineered strain (*E. coli* W3110 *ΔackA-pta*
671 *ΔyqhD_dhaB_mutant gabD4*) harboring an active ALDH mutant (designated as GabD4) from
672 *Cupriavidus necator*, producing 71.9 g/L 3-HP with a productivity of 1.8 g/L h. However,
673 unlike *K. pneumoniae*, *E. coli* does not synthesize vitamin B_{12} naturally, and it was necessary
674 to add the coenzyme externally. Furthermore, a significant amount of glucose for cell growth
675 was required because for *E. coli*, glycerol is a less-preferred carbon source than glucose.

676 Consequently, the researchers at Samsung initially grew cells to a high density (40–300
677 OD_{600}) on glucose and then produced 3-HP by feeding glycerol (Chu et al., 2015; Kim et al.,
678 2014a). In comparison, *K. pneumoniae* can produce a high concentration of 3-HP growing
679 purely on glycerol at a much lower cell concentration (OD_{600}, <20) (Huang et al., 2013a,b; Li
680 et al., 2016). Together, the use of glucose for cell proliferation, exogenous addition of
681 expensive cofactor vitamin B_{12}, and low cumulative yield of 3-HP (on glucose plus glycerol)
682 increase the production cost. Thus, *K. pneumoniae* can be considered a better biocatalyst than
683 *E. coli*. However, for the commercial production of 3-HP, many other factors are important,
684 including biosafety, process stability, and downstream processing. Further studies to assess
685 the potential of these strains as hosts for 3-HP production are needed.

686 **3.3 2,3-Butanediol**

687 BDO has many applications in the pharmaceutical, biomedical, and other chemical
688 industries for the production of printing inks, perfumes, fumigants, spandex, moistening and
689 softening agents, and plasticizers (e.g., cellulose nitrate, polyvinyl chloride, polyacrylates)
690 (Celińska and Grajek, 2009; Ji et al., 2011). BDO can also be used as an antifreezing agent
691 and octane booster for petrol as is or can be converted to useful derivatives such as 1,3-
692 butadiene, and diacetyl and methyl ethyl ketone.

693 Unlike 3-HP and PDO, BDO is a product of oxidative metabolism. Of the three
694 stereoisomers of BDO, *K. pneumoniae* mainly produces the *meso* form (Ji et al., 2011). BDO
695 is synthesized from pyruvate via α-acetolactate and acetoin. The C5 intermediate, α-
696 acetolactate, is formed from two molecules of pyruvate by self-condensation catalyzed by α-
697 acetolactate synthase (ALS; *budB*). α-Acetolactate is decarboxylated to acetoin by α-
698 acetolactate decarboxylase (*budA*), and acetoin is reduced to BDO by 2,3-butanediol
699 dehydrogenase/acetoin reductase (*budC*) using NADH as a reductant. In the presence of
700 oxygen, α-acetolactate is spontaneously decarboxylated to diacetyl, further reduced to acetoin

701 by action of diacetyl reductase, and then reduced to BDO. α-Acetolactate is also the precursor
702 of branched-chain amino acids (valine, leucine, and isoleucine); thus, elimination of the *bud*
703 operon can arrest *K. pneumoniae* growth when cultured on glycerol minimal medium (Kumar
704 et al., 2016). In the anaerobic process, one NADH molecule is required for the conversion of
705 two pyruvates to one BDO, while the aerobic process requires two NADH (Fig. 5). The
706 enzyme DhaD may also contribute to BDO formation (see Section 2.2.1). In the BDO
707 pathway itself, the conversion of two pyruvates to BDO regulates or is regulated by the
708 intracellular NADH/NAD^+ ratio, similar to other fermentative pathways. Another important
709 role of the BDO pathway is to reduce intracellular acidification by converting acids to the
710 neutral metabolite, BDO (Celińska and Grajek, 2009; Ji et al., 2011).

711 Although BDO is one of the main byproducts of glycerol fermentation by *K.*
712 *pneumoniae* (Petrov and Petrova, 2009; Durgapal et al. 2014), its production from glycerol
713 has not been studied extensively. In few studies using glycerol as the carbon source, Petrov
714 and Petrova (2009) focused on the effects of pH and aeration with their *K. pneumoniae*
715 isolate G31. BDO production was greatest at alkaline initial pH when tested at a pH range of
716 5–8. Without pH control a sharp pH drop occurred initially due to the production of organic
717 acids, and this pH drop triggered the synthesis of BDO, which is considered the only non-
718 inhibitory compound produced by the oxidative metabolism of glycerol. Once neutral pH is
719 restored with the rising concentration of BDO, catabolism shifted toward the production of
720 ATP-generating acetic acid. The amount of BDO generated in each cycle (pH drop and
721 neutralization) was dependent on the extent of the pH drop. In one study, a BDO
722 concentration of 49.8 g/L was reached when the initial pH was set at 8.0 and no pH control
723 was given during the rest of fermentation. PDO was the major byproduct (11.6 g/L) (Table 4)
724 and its production decreased by exclusion of Co^{2+}, an essential component of coenzyme B_{12}.
725 They also found that intensive aeration led to a significant increase in BDO production; an

726 increase in aeration rate from 1.1 to 2.2 vvm enhanced the yield from 0.38 to 0.46 mol/mol
727 and productivity from 0.24 to 0.36 g/L h. BDO production was also increased by forced pH
728 fluctuations of the culture medium with discrete ΔpH values (1.0, 2.0, and 3.0) at
729 predetermined time intervals (6, 12, and 24 h). The highest BDO concentration (70.0 g/L)
730 was produced by increasing the pH by one unit (ΔpH = 1.0) every 12 h (Petrov and Petrova,
731 2010). The mechanism behind the effect of this peculiar pH change on BDO production has
732 not been elucidated. Although BDO is readily produced from glycerol by *K. pneumoniae*, the
733 volumetric productivity is low due to the long fermentation time (generally >150 h). Huang et
734 al. (2013b) isolated two *Klebsiella* strains that produce BDO plus acetoin with a total yield of
735 0.44–0.45 (g/g). One isolate utilized 64.5 g/L glycerol in 78 h and produced 24.9 g/L BDO,
736 3.9 g/L acetoin, and 3.2 g/L ethanol. Again, the volumetric productivity was not high.
737 Metabolic engineering efforts with *K. pneumoniae* to improve BDO production from
738 glycerol are limited. Using glucose as carbon source, Kim et al. (2014b) reported that
739 overexpression of the BDO pathway (*budA*, *budB*) in the *ldhA* deficient *K. pneumoniae* strain
740 KCTC2242 improved BDO production and resulted in 90 g/L BDO, with a productivity of
741 2.75 g/L. h. Because both glucose and glycerol are converted through the common
742 intermediate pyruvate, BDO production from glycerol is also expected to increase by
743 amplification of the *bud* operon. Further work with glycerol as carbon source is required to
744 prove this effect. Recently, co-production of BDO and PDO from glycerol has also been
745 attempted (Park et al., 2017). In glycerol fermentation, both alcohols always appear
746 simultaneously and co-production seems to be beneficial for cells to maintain redox balance
747 during anaerobic growth. Park et al. (2017) found that by using the *K. pneumoniae* mutant
748 deficient of *ldhA* and *mdh*, 50.1 g/L BDO was produced along with 63.8 g/L PDO in 75 h.
749 The ratio of the two alcohols varied according to aeration; with increasing aeration, the ratio
750 of [BDO]/[PDO] increased as observed by Petrov and Petrova (2010). In the 5,000 L pilot-

751 scale fed-batch fermentation using crude glycerol, they could produce 114 g/L diols (70 g/L
752 PDO and 44 g/L BDO) with the yield of 0.60 diols/glycerol (g/g) and a productivity of 2.2
753 g/L. h of diols. If separation is not an issue, co-production of the two diols can be a good
754 option.

755 **3.4 Lactic acid**

756 Lactic acid has applications in the food, chemical, cosmetic, and pharmaceutical
757 industries. Lactic acid is a monomer that can be polymerized to yield the biodegradable
758 plastics polylactic acid (PLA) and poly(3-hydroxybutyrate-*co*-lactate). Two optical isomers
759 of lactic acid, D- and L-lactic acid, are produced in racemic mixtures depending on the
760 chemical route used. Microbial fermentation can yield optically pure isomers. Currently,
761 nearly all commercial lactic acid comes from microbial fermentation (Abdel-Rahman et al.,
762 2013; Abdel-Rahman and Sonomoto, 2016).

763 Because of the availability of highly efficient lactic-acid–producing microbes derived
764 from *E. coli*, lactic acid bacteria, and even acid-tolerant yeasts (Abdel-Rahman et al., 2013),
765 *K pneumoniae* has received little attention as a lactic acid producer. However, as previously
766 described, lactic acid is a major byproduct of glycerol fermentation by *K. pneumoniae*, and its
767 potential as a producer is high. This bacterium contains metabolic pathways for the
768 production of both L- and D-isomers (Fig. 7). They are synthesized through the pyruvate
769 and/or methylglyoxal pathways in *K. pneumoniae* (Ashok et al., 2011). When produced from
770 pyruvate, lactate dehydrogenase (*ldhA*) reduces pyruvate using NADH as reductant. In the
771 methylglyoxal route, DHAP is converted to methylglyoxal, which can be transformed to both
772 D- and L-lactic acid. For the production of L-lactic acid, methylglyoxal is reduced to L-
773 lactaldehyde, which is subsequently oxidized to L-lactic acid. Using glutathione, D-lactic acid
774 is obtained through simultaneous aldehyde group oxidation and keto group reduction of
775 methylglyoxal (Booth et al., 2003; Booth, 2005). The methylglyoxal route has several

776 disadvantages. Methylglyoxal is highly toxic, and its accumulation can severely impair other
777 metabolisms and even lead to cell death. Second, the route is energetically inefficient, with an
778 ATP yield of −1. The L- or D-lactic acid product can be converted back into pyruvate by
779 respiratory lactate dehydrogenase (Mazumdar et al., 2010, 2013).

780 Several studies showing the potential of *K. pneumoniae* as a lactic acid producer are
781 available (Table 4). For example, *K. pneumoniae* DSMZ is reported to accumulate 22.7 g/L
782 lactic acid in 24 h during co-production of 3-HP and PDO from glycerol. The titer of lactic
783 acid was more than that of any of the targeted products, and the yield of lactic acid on
784 glycerol alone was 0.31 (Ashok et al., 2011). Similarly, fed-batch cultivation of *K.*
785 *pneumoniae* BLh-1 under oxygen-limited conditions gave lactic acid as the main product,
786 outcompeting PDO; 59 g/L lactic acid was produced in 40 h when crude glycerol from
787 biodiesel synthesis without any purification was used (Rossi et al., 2013). Durgapal et al.
788 (2014) also found that *K. pneumoniae* J2B and DSMZ strains produced lactic acid at 37.0 and
789 47.8 g/L, respectively (Table 4); in both strains, lactic acid levels surpassed those of the target
790 product PDO. Recently, Feng et al. (2014) engineered *K. pneumoniae* ATCC25955 by
791 overexpressing *ldhA* and deleting *dhaT* and *yqhD* (recall that these two genes are mainly
792 responsible for PDO production). They found that the recombinant produced 142.1 g/L of
793 optically pure D-lactic acid from glycerol in fed-batch cultivation under microaerobic
794 conditions (2.5 vvm and 400 rpm). This result is the highest lactic acid concentration
795 produced from glycerol reported to date. *E. coli* has also been engineered for the production
796 of D- and L-lactate from glycerol (Mazumdar et al., 2010, 2013). For diverting glycerol flux
797 toward L-lactic acid in *E. coli*, the chromosomal copy of D-lactate dehydrogenase was
798 replaced with *Streptococcus bovis* L-lactate dehydrogenase (Mazumdar et al., 2013).
799 Moreover, gene encoding *lldD* (respiratory L-lactate dehydrogenase) was inactivated to
800 prevent the consumption of L-lactic acid, and the methylglyoxal route (Δ*mgsA*) was blocked

801 to prevent production of toxic methylglyoxal and formation of racemic mixtures of D- and L-
802 lactic acid (Fig. 7). Furthermore, the respiratory route for glycerol assimilation was
803 overexpressed (*glpK* and *glpD*) to improve the ATP yield of the metabolic pathway through
804 transfer of electrons from glycerol-3-phosphate to oxygen (i.e., oxidative phosphorylation).
805 The engineered *E. coli* strain (Δ*pflB* Δ*pta* Δ*adhE* Δ*frdA* Δ*mgsA* Δ*lldD* Δ*ldhA*::*ldhA_glpK-*
806 *_glpD*) produced 50 g/L of L-lactate from 56 g/L of crude glycerol, at a yield 93% of the
807 theoretical maximum and with high optical (99.9%) and chemical (97%) purity (Mazumdar et
808 al., 2013). The approaches proven successful in *E. coli* should be applicable to *K.*
809 *pneumoniae*. These studies, along with the results by Feng et al. (2014), suggest that *K.*
810 *pneumoniae* is a good host for the production of D- and L-isomers of lactic acid, with high
811 optical purity, from glycerol.

812 **3.5 Ethanol and other metabolites**

813 *K. pneumoniae* has also been studied for the production of ethanol, succinic acid, 2-
814 ketogluconic acid, catechol, *cis,cis*-muconic acid and 2-butanol although its potential for such
815 production has not been fully explored (Cheng et al., 2013; Jung et al., 2015; Oh et al., 2014;
816 Wang et al. 2015; Wei et al., 2013). Of these chemicals, ethanol production has been
817 relatively well studied. Ethanol is a renewable energy source and is widely used as a fuel
818 additive for partial gasoline replacement. Currently, commercial ethanol is produced from the
819 fermentation of sugar and starch. However, glycerol is one of the cheapest substrates for
820 ethanol production, estimated as nearly 40% cheaper than sugar-based production (Yazdani
821 and Gonzalez, 2007). This factor has encouraged the development of microbial strains for
822 fermentative conversion of glycerol to ethanol. The metabolic route by which ethanol is
823 synthesized from glycerol is shown in Fig. 6. Oh et al. (2011, 2012b) constructed a mutant
824 strain of *K. pneumoniae* (termed GEM167) through γ-irradiation. PDO synthesis in this
825 mutant strain was lower than that of wild type (0.2 vs. 7.93 g/L, respectively), while ethanol

826 accumulation was higher (8.6 vs. 1.1 g/L, respectively). The mechanism underlying these
827 changes remains unclear. Introduction of the pyruvate decarboxylase (*pdc*) and aldehyde
828 dehydrogenase (*adhII*) genes into mutant strains lacking lactate dehydrogenase (*ldhA*)
829 resulted in the production of 31.0 g/L (673.9 mM) ethanol, with a yield of 0.89 (mol/mol) and
830 productivity of 1.2 g/L h (Oh et al., 2012b), the highest level of ethanol production from
831 glycerol reported to date. More extensive studies including estimation of the maximum
832 achievable titer and microbial tolerance to high-concentration ethanol, both of which are key
833 factors for commercialization, are required to assess the potential to produce ethanol from
834 glycerol by the use of *K. pneumoniae*.

835 **4. Challenges to bioconversion of glycerol using *K. pneumoniae* as a biocatalyst**

836 The design of microbial cell factories is gaining unprecedented momentum as
837 metabolic engineering is progressively aided by advances in synthetic biology and multi-
838 omics analyses. Several bio-based chemicals have already been launched successfully in the
839 marketplace, and others are in the pipeline. For a bulk chemical to be considered for
840 commercial production, the production process should achieve a product concentration of 100
841 g/L, carbon yield of 0.50 (g/g), and volumetric productivity of 2.0 g/L/h. The aforementioned
842 products produced by *K. pneumoniae* from glycerol are no exception. Challenges to the use
843 of *K. pneumoniae* as a microbial cell factory using glycerol as a carbon source are described.

844 **4.1 Pathogenicity**

845 The pathogenicity of *K. pneumoniae* is one of the major obstacles to its commercial
846 application. *K. pneumoniae* is a Bio-safety Level 2 microorganism and causes nosocomial
847 and urinary tract infections. Moreover, capsulated *K. pneumoniae* cells are difficult to
848 separate from culture broth, complicating the downstream processing. The biotechnological
849 application of this bacterium will require attenuation of its pathogenicity and reduction of
850 biosafety concerns. *K. pneumoniae* produces a number of virulence factors that contribute to

851 its pathogenicity, including lipopolysaccharide (LPS), capsular antigens, fimbrial adhesins,
852 siderophores, and O antigens (Albertí et al., 1993; Fang et al., 2004; Podschun and Ullmann
853 1998). The mechanism by which this bacterium causes disease is still obscure because most
854 studies have been conducted on a limited number of virulence factors (El Fertas-Aissani et al.,
855 2013).

856 In the last decade, efforts have been made to overcome the pathogenicity of *K.*
857 *pneumoniae* (Shrivastav et al., 2013), suggesting hope for the generation of nonpathogenic *K.*
858 *pneumoniae*. The two major pathogenic determinants in *K. pneumoniae* are the capsular
859 polysaccharides (CPS), which cover the entire bacterial surface, and LPS, expressed on the
860 outer bacterial membrane. LPS consists of lipid A, a core oligosaccharide, and an O-antigenic
861 polysaccharide. In a highly virulent strain of *K. pneumoniae*, mutations in genes involved in
862 LPS biosynthesis (*waaC*, *waaF*, and *wabG*) resulted in a decrease in bacterial colonization
863 and virulence (Izquierdo et al., 2003; Jung et al., 2013). Mutant strains devoid of the outer
864 core LPS were also found to be deficient in the "cell-attached" capsular polysaccharides
865 covering the bacterial surface. These mutations abolished the highly virulent characteristics
866 of pathogenic *K. pneumoniae* when tested in different animal models. Furthermore, these
867 mutants were more sensitive to several hydrophobic compounds than were wild-type strains.
868 Reintroduction of the *waaC*, *waaF*, and *wabG* genes into *K. pneumoniae* rescued the
869 pathogenic properties, confirming the role of these genes in pathogenicity.

870 Another important study was conducted by Lin et al. (2012), in which eight highly
871 conserved residues of the MagA protein (G308, G310, G334, G337, R290, P305, H323, and
872 N324) encoded by the mucoviscosity-associated gene A (*magA*) of *K. pneumoniae* were
873 subjected to site-directed mutagenesis. The *magA* gene contributes to the biosynthesis of K1
874 capsular polysaccharide (CPS), which correlates with pathogenic phenotypes including
875 mucoviscosity, serum and phagocytosis resistance, and virulence. Alanine substitutions at

876 R290A or H323A abolished MagA function, with annihilation of CPS production, serum
877 resistance, and virulence. Some *K. pneumoniae* strains showing less pathogenicity were also
878 isolated. For example, *K. pneumoniae* strain J2B produced less LPS and demonstrated a high
879 sensitivity to many antibiotics and lower pathogenicity. Moreover, its biomass was readily
880 separated from fermentation broth by centrifugation (Arasu et al., 2011). Future studies
881 should include further engineering of these less pathogenic strains to make them completely
882 nonpathogenic, followed by careful evaluation of their toxicity.

883 **4.2 Complex glycerol metabolism**

884 Although glycerol metabolism has been extensively studied, the mechanism and
885 regulation of its gene expression in *K. pneumoniae* have not been fully elucidated. The
886 diversity of glycerol metabolism should be a great advantage for *K. pneumoniae* survival in
887 different environments and for its biotechnological utilization as well. However, a high level
888 of complexity poses difficulty in the engineering of a strain to suit our purposes. Metabolic
889 complexity is likely responsible in part for the fact that, despite serious attempts, the molar
890 yield of many important products such as PDO and 3-HP is still far below the theoretical
891 maximum. Efforts based on our current knowledge, such as the overexpression of DhaB,
892 DhaT, YqhD and AldH and inactivation/deletion of GlpK, DhaD, DhaK and DhaKLM, were
893 not satisfactory (Maervoet et al. 2011; Kumar et al. 2013a; Kumar et al., 2016). Another
894 challenge is to understand how the ratio of glycerol distribution between respiratory and
895 fermentative routes is controlled. Because the K_M of glycerol kinase toward glycerol is low, it
896 has been speculated that a major fraction of glycerol flows through the respiratory route when
897 oxygen is present. However, some experimental results do not support this hypothesis. For
898 example, deletion of *glpK* does not decrease the glycerol flow through the oxidative pathway
899 in the presence of oxygen (Ashok et al. 2013a). In fact, wild-type and mutant (Δ*glpK*) strains
900 of *K. pneumoniae* demonstrate similar cell growth, glycerol consumption, and PDO

901 production profiles under (micro)aerobic conditions. These observations suggest the presence
902 and/or involvement of other kinases.

903 Another challenging issue for the use of *K. pneumoniae* is its inefficient TCA cycle
904 (Cabelli, 1955). Several genes, including isocitrate dehydrognease (*icd*), fumarase (*fumA*),
905 and malate dehydrogenase (*mdh*), in *K. pneumoniae* are significantly less transcribed than
906 those in *E. coli* (unpublished data). Furthermore, the activity of isocitrate dehydrogenase was
907 more than sevenfold lower in *K. pneumoniae* than in *E. coli*. If the TCA cycle is inefficient,
908 achieving a high cell density, which is essential for improving the productivity of target
909 metabolites, is difficult. Moreover, achieving high-yield generation of NADH with little
910 consumption of glycerol carbon is difficult. Furthermore, the overflow metabolism that
911 produces highly toxic acetate becomes more serious. These problems have been well
912 documented in PDO production. In a recent study, Kumar et al. (2016) attempted to increase
913 PDO production yield by blocking the lactate and BDO pathways, while diverting carbon
914 flux through the TCA cycle for efficient regeneration of NADH. However, this effort resulted
915 in the detrimental accumulation of pyruvate, acetate, and ethanol, causing low NADH
916 regeneration and low PDO production. Neither increased aeration nor the addition of good
917 nitrogen sources (complex nitrogen sources) alleviated the accumulation of these
918 intermediates or activated the TCA cycle. Furthermore, the accumulation of pyruvate quickly
919 terminated glycerol assimilation. *K. pneumoniae* has a well-established BDO production
920 pathway that likely compensates for its inefficient TCA cycle, avoiding the overflow
921 metabolism and carbon traffic at the pyruvate node. If this is the case, co-production of BDO
922 along with PDO or 3-HP, rather than single production of PDO or 3-HP, should be more
923 appropriate, which suits the nature of *K. pneumoniae*. In fact, when BDO and PDO were co-
924 produced, more PDO was generated than during PDO production alone. More studies are
925 required to gain a better understanding of glycerol metabolism, the slow operation of the

926 TCA cycle in *K. pneumoniae*, and its effect on the selection of target products and/or
927 development of suitable strains and processes. Otherwise, it will be difficult to exploit this
928 potential biocatalyst for commercial manufacturing of valuable chemicals.

929 **4.3 Toxicity of intermediate metabolites and end-products**

930 A general but major challenge to the use of microbial cell factories for producing bulk
931 chemicals is the toxic effect of target products and metabolic intermediates at high
932 concentrations. In particular, during the production of PDO or 3-HP from glycerol, the highly
933 toxic intermediate 3-HPA is inevitably generated. Aldehydes generally damage DNA,
934 inactivate enzymes, and inhibit DNA synthesis. 3-HPA causes irreversible cessation of
935 metabolic activity and cellular growth, even at very low concentrations of 15–30 mM (Hao et
936 al., 2008; Zheng et al., 2008). Native *K. pneumoniae* does not accumulate 3-HPA at toxic
937 levels during PDO production, but in engineered strains that overexpress DhaB, high 3-HPA
938 accumulation is often experienced. To avoid 3-HPA accumulation, enzymes that consume 3-
939 HPA, ALDH (for 3-HP production), and PDOR (for PDO production) are expressed at
940 sufficiently (sometimes excessively) high levels compared with that of DhaB (Lim et al.,
941 2016). Intermediates such as methylglyoxal, DHA, DHAP, and glyceraldehyde-3-phosphate
942 are also known to be toxic.

943 Organic acids and alcohols are toxic at high concentrations. Generally, organic acids
944 are more toxic than their corresponding alcohols because they disturb the intracellular pH and
945 have anion-specific effects on metabolism (Chun et al., 2014; Warnecke and Gill, 2005).
946 When accumulated in cell membranes, organic compounds damage membrane integrity and
947 inhibit the activity of membrane-bound enzymes. In *K. pneumoniae*, cell growth and glycerol
948 assimilation are severely inhibited by high concentrations of PDO or 3-HP. Furthermore,
949 enzymes that play major roles in 3-HP and PDO production, such as DhaB, DhaT, and AldH,
950 are inhibited by 3-HP and PDO (Kumar et al. 2013b). Furthermore, *K. pneumoniae* is

951 sensitive to 24 g/L acetic acid, 26 g/L lactic acid, and 17 g/L ethanol under aerobic conditions
952 and 15, 19, and 26 g/L, respectively, under anaerobic condition (Celińska, 2012; Cheng et al.,
953 2005). The inhibition of pathways by target product(s) and/or unwanted byproducts often
954 places a limit on the maximum attainable titer of bulk chemicals.

955 Several studies have reported engineered improvements in microbial tolerance against
956 biochemicals and biofuels, especially in *E. coli*. Modifications of exporter proteins, heat
957 shock proteins, membrane composition, and stress responses have been attempted. Moreover,
958 *in situ* recovery of target products has been attempted for hydrophobic targets. Some tolerant
959 strains have been developed, but unfortunately, without appreciable increases in productivity
960 (Dunlop, 2011; Dunlop et al., 2011; Jarboe et al., 2011). Such efforts to improve tolerance to
961 chemicals have not been reported for *K. pneumoniae*.

962 **4.4 Availability of glycerol**

963 The global market for glycerol is known to be unpredictable and complex. Glycerol in
964 the current market mainly is from the biodiesel industry, and its supply is directly affected by
965 the use of biodiesel. In contrast, glycerol demand depends on many industries that use it as
966 feedstock. Between 2001 and 2011, the price varied significantly, from \$0.70/kg to \$1.70/kg
967 for pure glycerol and from \$0.04/kg to \$0.33/kg for crude glycerol (Ayoub and Abdullah,
968 2012; Ciriminna et al., 2014; Kong et al., 2016; Quispe et al., 2013). Nevertheless, glycerol
969 prices, for pure as well as crude, have come down significantly since 2004, when many
970 biodiesel production plants initiated operation. The countries producing biodiesel in large
971 quantities (in million liters) include Germany (2900), Brazil (2300), Argentina (2100), France
972 (2000), USA (1200), Spain (1100), Italy (800), Indonesia (700), and Thailand (600) (Kong et
973 al., 2016). The Asia-Pacific region overtook Europe as the largest glycerol market in the
974 world in 2009 and has remained so ever since. Market growth is driven by increases in the

975 uses for glycerol in such sectors as pharmaceuticals, personal hygiene, and food and beverage
976 production (Quispe et al., 2013).

977 The production of crude glycerol is expected to reach 6 million tons by 2025
978 (Ciriminna et al., 2014). At present, the surplus of glycerol from biodiesel and oleochemicals
979 is assumed to be sufficient to meet these new demands. However, the scenario may change in
980 the future with rapid growth of glycerol-based industries and a limited supply of crude
981 glycerol. For example, if 3-HP produced from glycerol were used as a substitute for the
982 chemical acrylic acid (global production, 5.85 million tons as of 2014), approximately 7
983 million tons of glycerol/year would be needed. If we include other platform chemicals such
984 as PDO, BDO, and ethanol, the demand will be even higher, much more than the expected
985 supply. The growth of glycerol-based biotechnology will eventually be constrained by the
986 supply and price of crude glycerol as a carbon source.

987 **5. Concluding remarks**

988 Glycerol is an excellent substrate for the production of biochemicals and biofuels.
989 Although the biodiesel industry has grown rather slowly in recent years, crude glycerol is still
990 cheap and generated in large quantities. *K. pneumoniae* has already been successfully
991 employed for the production of PDO, 3-HP, BDO, lactic acid, and ethanol from glycerol at
992 industrially attractive levels. Other metabolites such as acetic acid, pyruvic acid, malic acid,
993 fumaric acid, alanine, *n*-butanol, 1,2-propanediol, propionic acid, formic acid, and hydrogen
994 can also be produced from glycerol by *K. pneumoniae*, although these have not yet been
995 extensively explored. At present, sugar-based bioprocesses are prevalent, and no bioprocesses
996 employing *K. pneumoniae* and glycerol are commercially available. The pathogenicity of *K.*
997 *pneumoniae* and other technical issues are existing challenges to its use. However,
998 advancements in the fields of metabolic engineering, synthetic biology, systems biology, and
999 evolutionary engineering will enable us to further exploit the advantageous nature of *K.*

1000 *pneumoniae* as a biocatalyst and glycerol as a carbon source. A better understanding of
1001 glycerol metabolism in *K. pneumoniae* and improvements in its performance as a microbial
1002 cell factory should make this organism an attractive alternative to current sugar-based
1003 methods for the production of biochemicals and biofuels.

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1462 **Figure captions**

1463 **Figure 1:** Transformation of glycerol into a number of valuable chemicals. The solid line
1464 represent single step while broken one indicate multiple steps.

1465 **Figure 2:** Oxidative metabolism of glycerol in *K. pneumoniae* (Ashok et al., 2011; Kumar et
1466 al., 2013a). Gene names are shown in italics on arrows. Solid lines indicate single steps;
1467 broken lines indicate multiple steps.

1468 **Figure 3:** Metabolic pathways for the production of PDO and 3-HP from glycerol (Ashok et
1469 al., 2011; Kumar et al., 2013a). Gene names are shown in italics on arrows.

1470 **Figure 4 (A):** Arrangement of genes in the *dha* regulon of *K. pneumoniae.* 1, *dhaK* (ATP-
1471 dependent dihydroxyacetone kinase); 2, *dhaK3*/*dhaM* (large subunit of ATP-dependent
1472 dihydroxyacetone kinase); 3, *dhaK2*/*dhaL* (small subunit of ATP-dependent
1473 dihydroxyacetone kinase); 4, *dhaK1*/*dhaK* (medium subunits of ATP-dependent
1474 dihydroxyacetone kinase); 5, *orfW* (hypothetical protein); 6, *dhaD* (glycerol dehydrogenase);
1475 7, *dhaR* (transcription regulator); 8, *orfW*/CdAT [cob(I)yrinic acid a,c-diamide
1476 adenosyltransferase]; 9, *orfX/dhaG* (small subunit of reactivating factor of glycerol
1477 dehydratase); 10, *dhaT* (1,3-propanediol oxidoreductase); 11, *orfY* (hypothetical protein); 12,
1478 *dhaB/B1* (large subunits of glycerol dehydratase); 13, *dhaC/B2* (medium subunit of glycerol
1479 dehydratase); 14, *dhaE/B3* (small subunit of glycerol dehydratase); 15, *orfZ*/*dhaF* (large
1480 subunit of reactivating factor of glycerol dehydratase); 16, *glpF* (glycerol uptake facilitator);
1481 17, 22- *hdeB*; 18, *hdeD* (*hdeBD*-genes related to global regulation); 19, *yfdX*; 20, *kvgS*; and
1482 21, *kvgA* (*kvgS* and *kvgA* encode for a two-component signal transduction system) (Celińska,
1483 2012; Sun et al., 2003; Wei et al., 2014). **(B):** Glycerol dehydratase gene arrangements and
1484 reactivation factors in different organisms (Martins-Pinheiro et al., 2016).

1485 **Figure 5:** Metabolic pathway for the synthesis of BDO from glycerol (Ashok et al., 2011;
1486 Celińska and Grajek, 2009; Ji et al., 2011). Gene names are shown in italics on arrows. Solid
1487 lines indicate single steps; broken lines indicate multiple steps.

1488 **Figure 6:** Biochemical routes for ethanol and acetic acid production from glycerol (Ashok et
1489 al., 2011; Kumar et al., 2013a). Gene names are shown in italics on arrow. Solid lines
1490 indicate single steps; broken lines indicate multiple steps.

1491 **Figure 7:** Lactic acid production from glycerol via methylglyoxal and pyruvate routes
1492 (Ashok et al., 2011; Mazumdar et al., 2010; 2013). Gene names are shown in italics on arrow.
1493 Solid lines indicate single steps; broken lines indicate multiple steps.

1494 **Fig. S1** Effect of deletion of glycerol transporters, *glpF1* and *glpF2*, on glycerol consumption
1495 and PDO production in *K. pneumoniae* J2B3.

1496

Figure

Figure 1

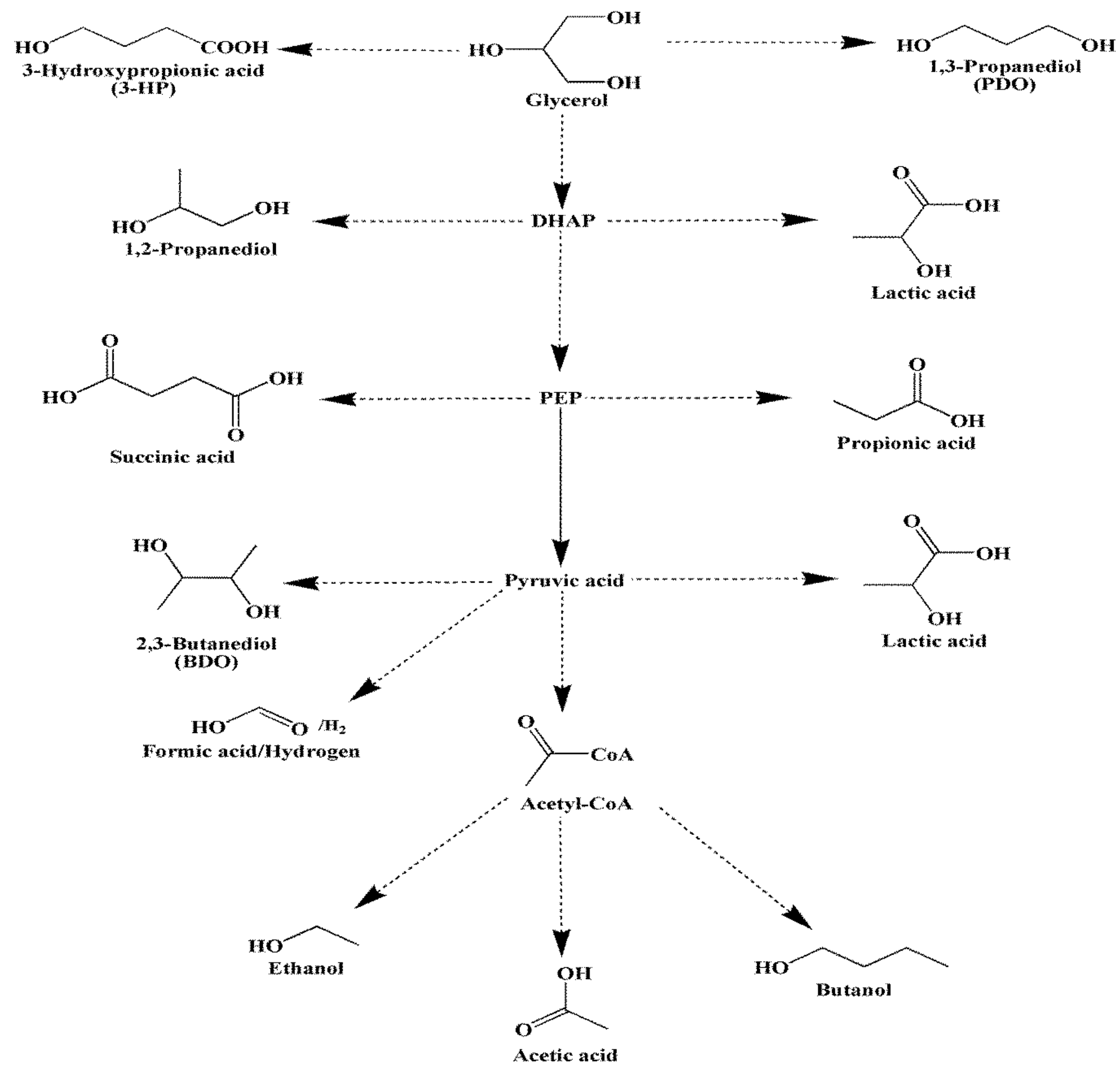


Figure 2

NADH NAD$^+$ ④ *dhaD*

HO HO O Dihydroxacetone

gldA ⑤ NADH NAD$^+$

HO OH OH Glycerol

ATP/PEP ADP/Pyruvate *glpK* ①

OH HO P O O OH OH Glycerol-3-phosphate

Fermentative route

Respiratory (aerobic/anaerobic) route

Q ② *glpD* QH_2

ATP ADP ⑥ *dhaK*

dhaKLM/ dhaK123 ⑦ PEP Pyruvate

O HO P O OH HO O Dihydroxyacetone phosphate

glpABC ③ QH_2 Q

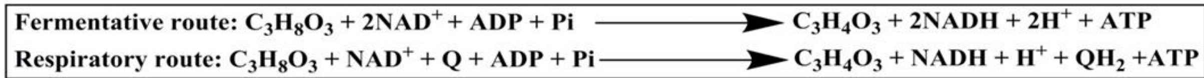
ADP NAD$^+$ ATP NADH

O HO O HO P O OH Phosphoenolpyruvic acid

⑧ ADP ATP

O O OH Pyruvic acid

Key enzymes: 1- Glycerol kinase; 2- Glycerol-3-phosphate dehydrogenase (aerobic); 3- Glycerol-3-phosphate dehydrogenase (anaerobic); 4- Glycerol dehydrogenase; 5- Putative glycerol dehydrogenase; 6- Dihydroxyacetone kinase I; 7- Dihydroxyacetone kinase II; 8- Pyruvate kinase



Fermentative route: $C_3H_8O_3 + 2NAD^+ + ADP + Pi \longrightarrow C_3H_4O_3 + 2NADH + 2H^+ + ATP$

Respiratory route: $C_3H_8O_3 + NAD^+ + Q + ADP + Pi \longrightarrow C_3H_4O_3 + NADH + H^+ + QH_2 + ATP$

Figure 3

Glycerol

B_{12}

① *dhaB123/ dhaB, dhaC & dhaE*

H_2O

3-hydroxypropionaldehyde

NAD^+ ② *dhaT/pduQ* NADH

1,3-propanediol

HOR ③ $NAD(P)^+$ NAD(P)H

NAD^+ *pduP* ⑤ NADH CoA-SH H_2O

3-hydroxypropionyl-CoA

④ *AldH* NAD^+ NADH

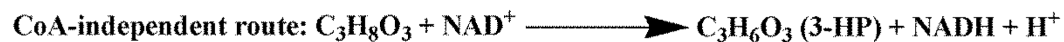
3-hydroxypropionic acid

⑥ *pduL* Pi CoA-SH

3-hydroxypropionyl-phosphate

ATP *pduW* ⑦ ADP

Key enzymes: 1- Glycerol dehydratase; 2- NADH-dependent PDOR/Propanol dehydrogenase; 3- Hypothetical oxidoreductase; 4- Aldehyde dehydrogenase; 5- Propionaldehyde dehydrogenase; 6- Phosphotransacylase; 7- Propionate kinase



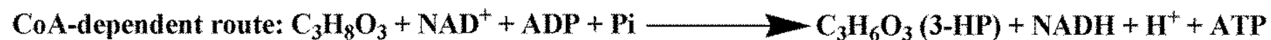


Figure 4

A)

B)

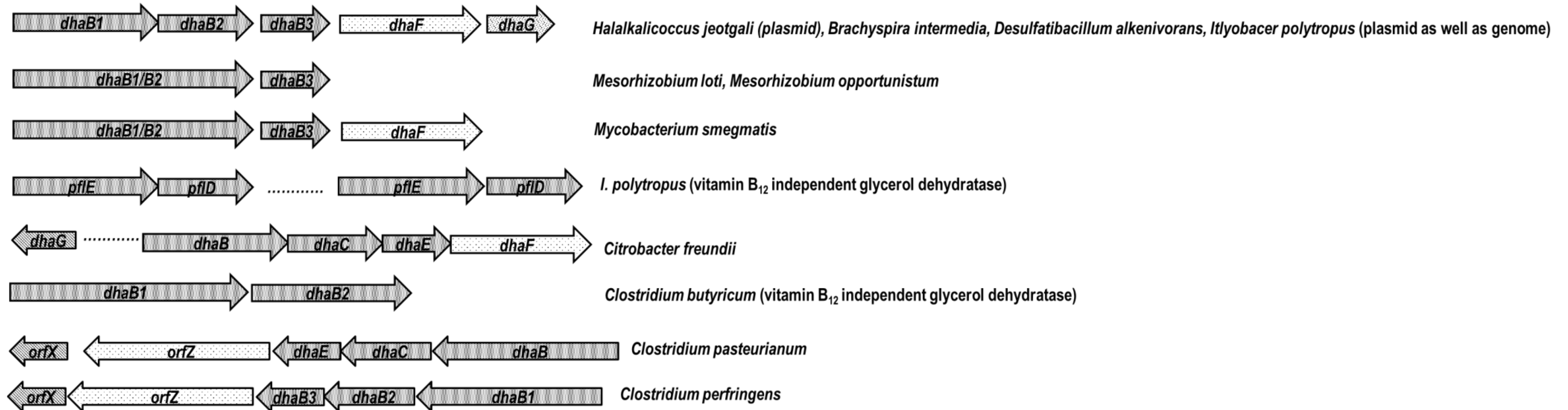
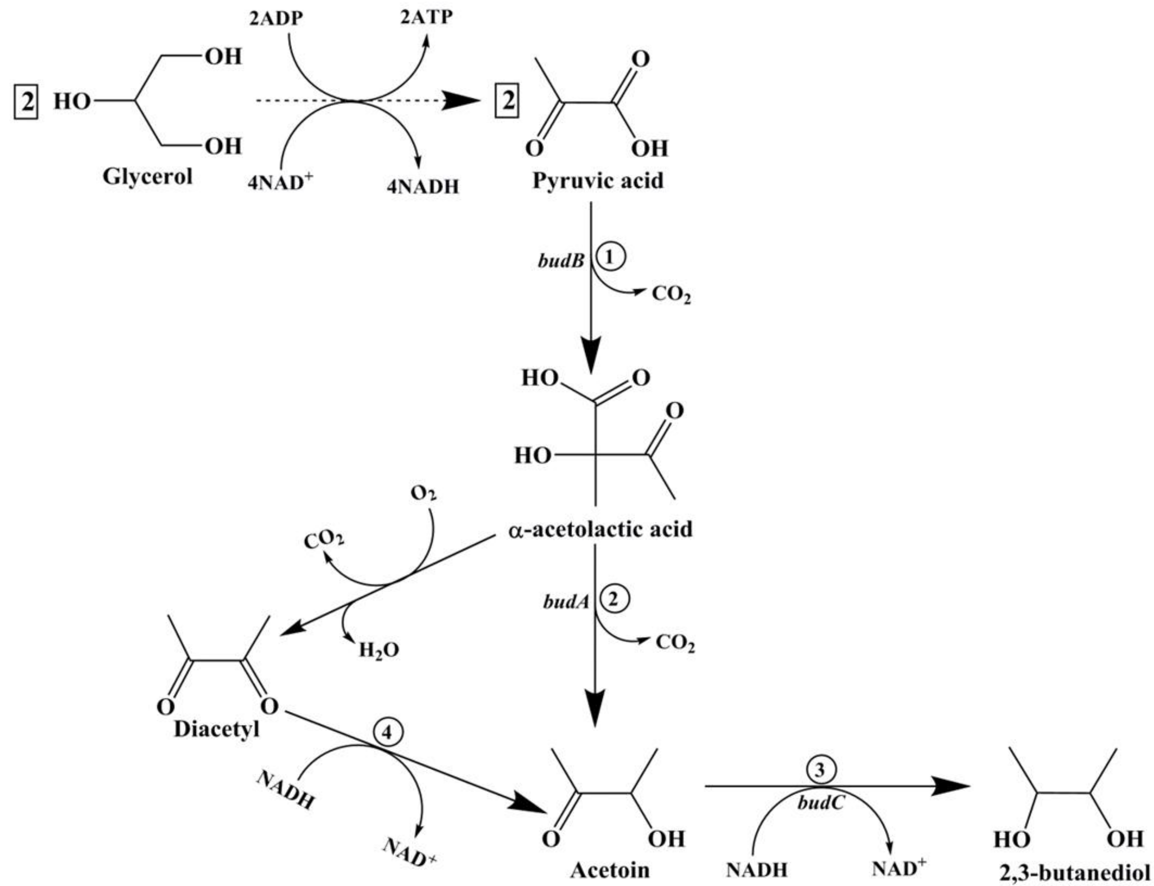


Figure 5

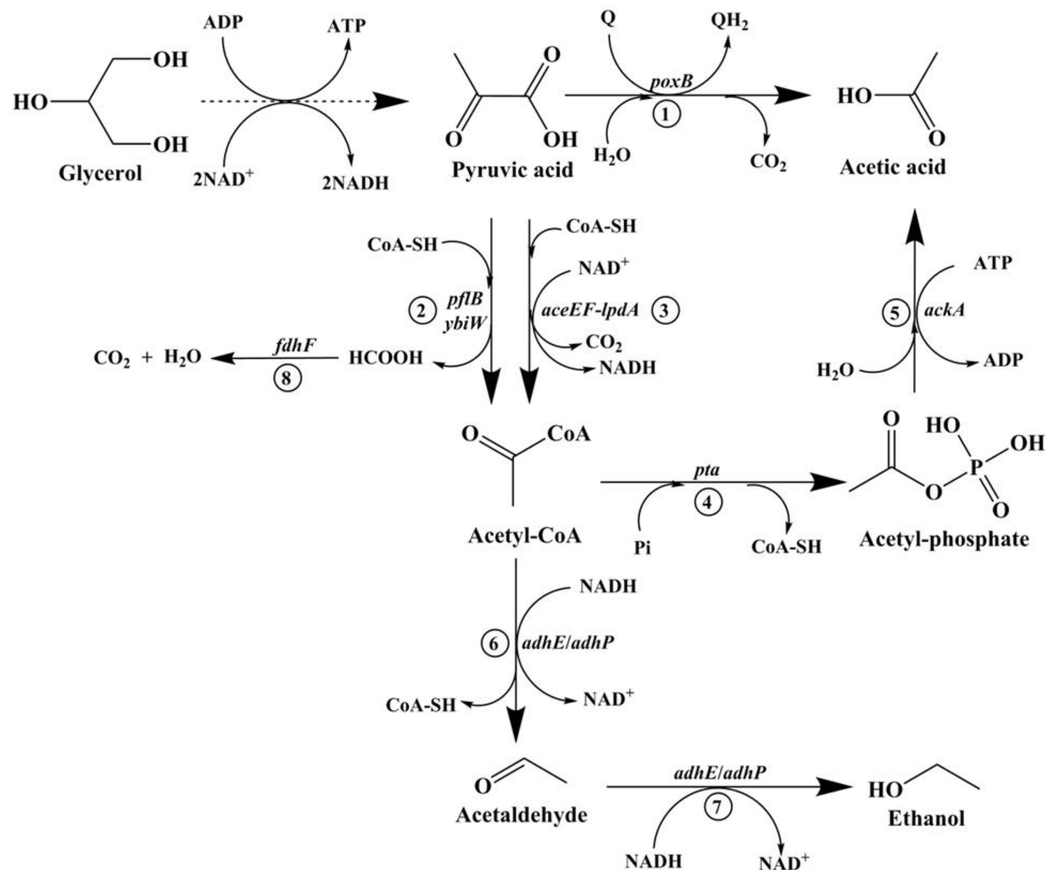


Key enzymes: 1- α-Acetolactate synthase; 2- α-Acetolactate decarboxylase; 3- Acetoin reductase ; 4-Diacetyl reductase

Anaerobic route: $2C_3H_8O_3 + 3NAD^+ + 2ADP + 2Pi \longrightarrow C_4H_{10}O_2 + 3NADH + 3H^+ + 2CO_2 + 2ATP$

Aerobic route: $2C_3H_8O_3 + 2NAD^+ + 0.5O_2 + 2ADP + 2Pi \longrightarrow C_4H_{10}O_2 + 2NADH + 2H^+ + 2CO_2 + H_2O + 2ATP$

Figure 6

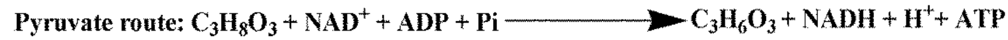


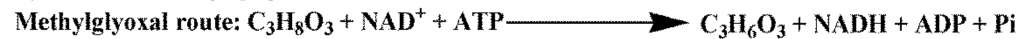
Key enzymes: 1- Pyruvate oxidase; 2-Pyruvate-formate lyase, putatative formate acetyltransferanse 3- Pyruvate dehydrogenase complex; 4- Phosphoacetyl transferase 5- Acetate kinase; 6 and 7- Bifunctional (acetaldehyde/alcohol) dehydrogenase/putative alcohol dehydrogenase; 8-Formate dehydrogenase, part of the formate hydrogenlyase complex

Figure 7

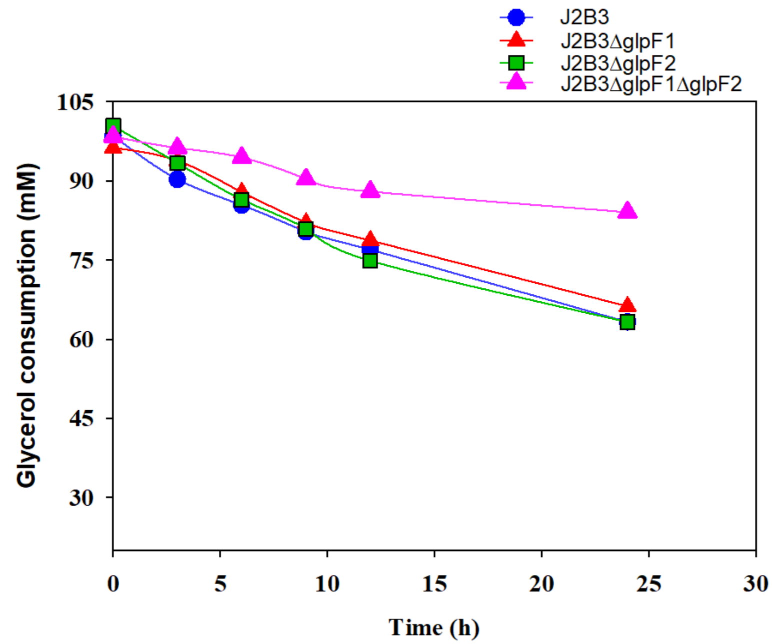
Glycerol
ATP
ADP
NAD+
NADH
Dihydroxyacetone phosphate
mgsA
③
Pi
Methylglyoxal
gldA
⑤
NAD(P)H
NAD(P)+
L-Lactaldehyde
NAD+
2ADP
NADH
2ATP
GSH
gloA, gloB
④
QH_2
Q
②
dld
ldhA
①
NADH
NAD+
Pyruvic acid
D-Lactic acid
NAD+
NADH
aldA, aldB
⑥
QH_2
lldD
⑦
Q
L-Lactic acid

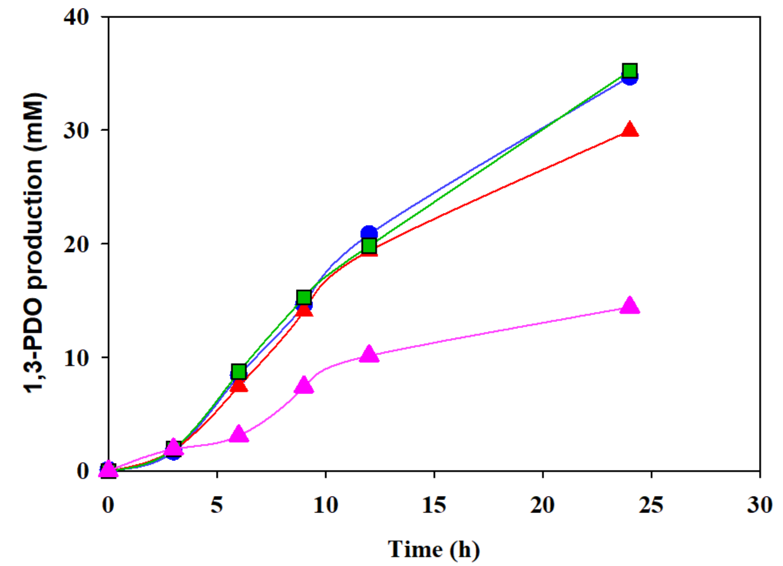
Key enzymes :1-D-Lactate dehydrogenase (fermentative); 2-D-Lactate dehydrogenase (respiratory); 3-Methylglyoxal synthase; 4- Glyoxalase I and II; 5-Glycerol dehydrogenase; 6-Aldehyde dehydrogenase isozymes; 7-L-Lactate dehydrogenase (respiratory).





Supplementary Figure S1





glpF1 **designates the gene located at the respiratory *glpFK* operon, while *glpF2* indicates a separate operon in the fermentative *dha* regulon located between *orfZ*/*dhaF* operon and *hdeB* operon (see Fig. 4A). For the growth of *K. pneumoniae* J2B3 which is deleted for *glpK*, *glpD*, *glpA* and *dhaD* (see Fig. 2 for their roles), gluco se was added at 100 mM because the strain cannot grow on glycerol as the sole carbon source (Lama et al., 2017)**

Table

Table 1: Comparison of maximum theoretical yield, ATP yield and redox balance during microbiological production of different metabolites from glycerol and glucose*

Metabolite	Maximum theoretical yield**		ATP generated/Metabolite (mol/mol)		Redox constrained [NAD(P)H generated & consumed/ Metabolite]	
	Glucose (mol/0.5 mol)	Glycerol (mol/mol)	Glucose	Glycerol	Glucose	Glycerol
1,3-propanediol $C_3H_8O_2$	0.75	0.88	-1	0	Yes (0 & 2)	Yes (0 & 1)
3-hydroxypropionic acid $C_3H_6O_3$	1.0	1.17	1/0/-1/-0.33	0/1	No (1 & 1;2 & 2)	Yes (1 & 0)
2,3-butanediol $C_4H_{10}O_2$	0.55	0.64	2	2	Yes/No (2 & 1/2)	Yes (4 & 1/2)
Ethanol C_2H_6O	1.0	1.17	1	1	Yes/No (1/2 & 2)	Yes/No (2/3 & 2)
Succinic acid $C_4H_6O_4$	0.86	1.0	0/1	0/1	Yes (1 & 2)	No (2 & 2)
Lactic acid $C_3H_6O_3$	1.0	1.17	1/-1	1/-1	No (1 & 1; 0 & 0)	Yes (2 & 0; 1 & 0)
Pyruvic acid $C_3H_4O_3$	1.2	1.4	1	1	Yes (1/2 & 0)	Yes (2/3 & 0)
Acetic acid $C_2H_4O_2$	1.5	1.75	1/2	1/2	Yes (1/2 & 0)	Yes (2/3 & 0)
1-Butanol $C_4H_{10}O$	0.5	0.58	2	2	No (4 & 4)	Yes (6 & 4)

***The variation in ATP yield, NADH generated and consumed is due to formation of metabolite through different routes.**

****The theoretical yields of metabolites were calculated on the basis of degree of reduction (Dugar and Stephanopoulos, 2011).**

Table 2: Summary of PDO production from glycerol using different *K. pneumoniae* strains

Strain	Mode of operation	Carbon source(s)	Aeration condition	Titer (g/L)	Yield (mol/mol)	Productivity (g/L. h)	Reference
Wild type strains							
K. pneumoniae M5al	Batch	Glycerol	Anaerobic	58.8	0.53	0.92	Cheng et al. 2007
K. pneumoniae XJPD-Li	Fed-batch	Glycerol	Aerobic	65.3	0.56	3.16	Ma et al. 2009
K. pneumoniae AC 15	Fed-batch	Glycerol	Aerobic	74.1	0.62	3.08	Zheng et al. 2008
K. pneumoniae DSM 2026	Fed-batch	Glycerol	Anaerobic	81.0	0.64	3.00	Liu et al. 2007
K. pneumoniae DSM 2026	Fed-batch	Glycerol	Microaerobic	77.0	0.62	2.70	Liu et al. 2007
K. pneumoniae HR526	Fed-batch	Glycerol	Aerobic	95.4	0.48	1.98	Xu et al. 2009b
K. pneumoniae KG1	Fed-batch	Glycerol	Aerobic	98.8	0.51	3.29	Zhao et al. 2009
Mutant strains							
K. pneumoniae HR526 Δ*ldhA*	Fed-batch	Glycerol	Aerobic	102.1	0.52	2.13	Xu et al. 2009b
K. pneumoniae Cu Δ*ldhA*	Fed-batch	Glycerol	Aerobic	102.7	0.50	1.53	Oh et al. 2012a
K. pneumoniae YMU2 Δ*aldA*	Fed-batch	Glycerol	Anaerobic	70.6	0.70	1.1	Zhang et al. 2006
K. pneumoniae YMU2	Fed-batch	Glycerol	Anaerobic	72.2	0.57	2.41	Wu et al. 2013

Δ*budC_fdh*							
Recombinant strains (Overexpression of Genes from the Reductive Branch)							
K. pneumoniae TUAC01_ *dhaT*	Batch	Glycerol	Aerobic	15.0	0.61	1.36	Hao et al. 2008
K. pneumoniae KG1_ *dhaT*	Fed-batch	Glycerol	Aerobic	90.9	0.64	2.16	Zhao et al. 2009
K. pneumoniae ME-308_*yqhD*	Fed-batch	Glycerol	Microaerobic	67.6	0.62	1.69	Zhu et al. 2009
K. pneumoniae Δ*dhaD* Δ*dhaK* Δ*dhaT_yqhD*	Batch	Glycerol	Aerobic	7.7	0.53	0.26	Seo et al. 2010

Table 3: Different strategies carried out for 3-HP production through fed-batch mode

Strain	Carbon source(s)	Aeration condition	Titer* (g/L)	3-HP yield on glycerol (mol/mol)	Productivity (g/L. h)	Reference
K. pneumoniae DSM 2026 Δ *dhaT_ puuC*	Glycerol	Microerobic	16.0 (16.8)	0.23	0.67	Ashok et al. 2011
K. pneumoniae WM3 *pUC18kan_aldHec*	Glycerol	Anaerobic	24.4 (49.3)	0.18	1.02	Huang et al. 2012
K. pneumoniae J2B _ *KGSADH*	Glycerol	Anaerobic	11.3 (15.9)	0.27	0.94	Kumar et al. 2012
K. pneumoniae ΔglpKΔdhaT_puuC	Glycerol	Anaerobic	22.0 (5.9)	0.30	0.46	Ashok et al. 2013a
K. pneumoniae ΔdhaTΔyqhD_dhaB_puuC	Glycerol	Aerobic	28.1 (3.3)	0.40	0.58	Ashok et al. 2013b
K. pneumoniae WM3 *pUC18kan_aldHec*	Glycerol	Microaerobic	48.9 (25.3)	0.41	1.75	Huang et al. 2013a
K. pneumoniae J2B Δ *ldhA* _ *KGSADH*	Glycerol	Microaerobic	22.7 (23.4)	0.35	0.38	Kumar et al. 2013b
E. coli W3110 *ΔackA-pta ΔyqhD_dhaB_mutant gabD4*	Glucose & glycerol	Aerobic	71.9	-	1.8	Chu et al. 2015
K. pneumoniae DSM 2026 Δ *ldh1Δldh2Δpta_puuC* (pTAC)	Glycerol	Microaerobic	83.8 (22.1)	0.54	1.16	Li et al. 2016

***The values shown in the bracket are the PDO concentration obtained along with 3-HP.**

Table 4: Comparison of BDO, ethanol and lactic acid production by different strains of *K. pneumoniae* and other organisms

Strain	Mode of operation	Carbon source(s)	Aeration condition	Titer (g/L)	Yield (mol/mol)	Productivity (g/L. h)	Reference
BDO							
K. pneumoniae G31	Fed-batch	Glycerol	Microaerobic	49.2	0.37	0.17	Petrov and Petrova, 2009
K. pneumoniae G31	Fed-batch	Glycerol	Aerobic	70.0	0.40	0.47	Petrov and Petrova, 2010
Klebsiella sp. 2	Fed-batch	Glycerol	Aerobic	24.9	0.40	0.34	Huang et al. 2013b
K. pneumoniae SDM	Fed-batch	Glucose	Aerobic	150.0	0.84	3.93	Ma et al. 2009
K. pneumoniae KCTC2242 *ΔldhA_budA_budB*	Fed-batch	Glucose	Aerobic	90.0	0.76	2.75	Kim et al. 2014b
Ethanol							
K. pneumoniae GEM167 mutant	Fed-batch	Glycerol	Microaerobic	21.5	0.62	0.93	Oh et al. 2011
K. pneumoniae GEM167 mutant *ΔldhA_pdc_adhII*	Fed-batch	Glycerol	Microaerobic	31.0	0.89	1.20	Oh et al. 2012b
E. coli (nine gene knockout strain)	Batch	Glycerol	Microaerobic	18.0	0.90	0.38	Trinh and Srienc, 2009
Lactic acid							
K. pneumoniae DSM 2026 *Δ dhaT_ puuC*	Fed-batch	Glycerol	Microaerobic	22.7	0.32	0.95	Ashok et al. 2011
K. pneumoniae J2B	Fed-batch	Glycerol	Microaerobic	28.2	0.28	0.39	Kumar et al. 2013b

Δ *ldhA* _ *KGSADH*							
K. pneumoniae BLh-1	Fed-batch	Glycerol	Microaerobic	59.0	0.48	1.48	Rossi et al. 2013
K. pneumoniae J2B	Fed-batch	Glycerol	Microaerobic	37.0	0.32	0.77	Durgapal et al. 2014
K. pneumoniae DSMZ	Fed-batch	Glycerol	Microaerobic	47.8	0.39	1.00	Durgapal et al. 2014
K. pneumoniae ATCC25955 Δ*dhaT*_ Δ*yqhD*_ *ldhA*	Fed-batch	Glycerol	Microaerobic	142.1 (D-isomer)	0.84	2.96	Feng et al. 2014
E. coli MG1655 Δ*pta*_Δ*adhE*_Δ*frdA*-Δ*dld*_*glpK*_*glpD*	Fed-batch	Glycerol	Microaerobic	32.0 (D-isomer)	0.82	0.54	Mazumdar et al. 2010
E. coli MG1655 Δ*pflB*_Δ*pta*_Δ*adhE*_Δ*frdA* _Δ*mgsA*_Δ*lldD*_Δ*ldhA*::*ldhA*_*glpK*-_*glpD*	Fed-batch	Glycerol	Microaerobic	50.0 (L-isomer)	0.92	0.60	Mazumdar et al. 2013

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Potential and limitations of Klebsiella pneumoniae as a microbial cell factory utilizing glycerol as the carbon source

Kumar, Vinod

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