

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

## 30 **1. Introduction**

31 Crude glycerol is produced in large quantities as a byproduct of the biodiesel industry.  
32 Global biodiesel production was 22.7 million tons in 2012 and is forecasted to reach 36.9  
33 million tons by 2020. As a result, worldwide production of glycerol climbed from 1 million  
34 tons in 2000 to 3 million tons in 2011 and is expected to reach 6 million tons by 2025  
35 (Ciriminna et al., 2014; OECD, 2011; Katryniok et al., 2013; Sun et al. 2017). A significant  
36 amount of glycerol is also generated during bioethanol fermentation by yeast. The liquid  
37 fraction of the stillage obtained from ethanol separation contains up to 2% glycerol. Another  
38 source of glycerol is industrial waste generated from vegetable oils and animal fats. For  
39 example, the concentration of glycerol in waste streams from the oleochemical industry is  
40 55%–90% (da Silva et al., 2009; Yazdani and Gonzalez, 2007). The increased availability of  
41 glycerol has caused a substantial reduction in its cost. According to a recent report, the price  
42 of crude glycerol is \$0.24/kg and that of pure USP grade glycerol is \$0.9/kg (Kong et al.,  
43 2016). Extensive research has been conducted to investigate ways to utilize this surplus crude  
44 glycerol. The annual number of research articles addressing the use of glycerol has increased  
45 to >7000, doubling in number from the year 2000 to 2007. Numerous chemical conversions  
46 to valuable products have been reported (Ciriminna et al., 2014).

47 Glycerol can be used for the microbial production of 1,3-propanediol (PDO), ethanol,  
48 2,3-butanediol (BDO), butanol, lactic acid, 3-hydroxypropionic acid (3-HP), and succinic  
49 acid (Fig. 1) (Clomberg and Gonzalez, 2013; Mattam et al., 2013; Yazdani and Gonzalez,  
50 2007). Because of its reduced nature, glycerol generates twice the number of reducing  
51 equivalents per carbon than do traditional carbohydrates (glucose, xylose, and sucrose) when  
52 converted into the glycolytic intermediates phosphoenolpyruvate (PEP) or pyruvate  
53 (Dharmadi et al., 2006). As a consequence, glycerol gives higher yields of reduced  
54 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. buchneri* 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<sub>12</sub>, 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<sup>+</sup>, 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<sup>+</sup>  
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-hydroxypropionaldehyde (3-HPA). Catalysis by GDHt requires coenzyme B<sub>12</sub>, 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)<sup>+</sup> (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 *glpA* gene, glycerol dehydrogenase is an oxidoreductase  
153 that converts glycerol to DHA. The enzyme extracts electrons from glycerol and transfers

154 them to  $\text{NAD}^+$ . 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  $\text{NADH}$  disposal and recovery (Wang et al., 2014). The promiscuity of DhaD is responsible  
162 for BDO production in the  $\Delta budC$  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  $\text{NADH}$  in the presence of the *dhaD* gene.  $\text{NADH}$  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  $\text{NADH}$ , DhaD is able to switch its role  
168 from  $\text{NADH}$  production (glycerol oxidation) to  $\text{NADH}$  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  $\text{NADH}/\text{NAD}^+$  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. pneumoniae* 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<sub>12</sub> or S-adenosyl methionine (SAM) as a cofactor. *K.*  
199 *pneumoniae* has B<sub>12</sub>-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  $\alpha$  (large,  
202 60.7 kDa),  $\beta$  (medium, 21.3 kDa), and  $\gamma$  (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<sub>12</sub> 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<sub>12</sub> 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<sub>12</sub> in the presence of ATP and  
214 Mg<sup>2+</sup>/Mn<sup>2+</sup> (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  $\alpha$  subunits (63 kDa) and two globular  $\beta$  subunits (14 kDa).  
217 Structurally, the  $\alpha$  subunit resembles both GroEL and Hsp70 chaperones, while the  $\beta$  subunit  
218 resembles that of the  $\beta$  subunit of glycerol dehydratase, except that it lacks some of the amino  
219 acids responsible for coenzyme B<sub>12</sub> 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<sub>12</sub>-independent (SAM-dependent)  
231 and B<sub>12</sub>-dependent GDHt. The genes for B<sub>12</sub>-independent GDHt are present in the genomic  
232 DNA, while those encoding B<sub>12</sub>-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<sub>12</sub>-independent proteins showed no homology  
236 with B<sub>12</sub>-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<sup>+</sup> (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),  $\sigma^{54}$  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  $\sigma^{54}$  factor interaction  
300 domain interacts with the  $\sigma^{54}$  factor of RNA polymerase and activates RNA transcription  
301 from the  $\sigma^{54}$  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  $\sigma^{70}$  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 ( $K_d = 31 \mu\text{M}$ )  
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 ( $K_m = 10 \mu\text{M}$ ) 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 ( $K_m = 1 \text{ mM}$ )  
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<sup>+</sup> 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<sup>Glc</sup>, an  
412 intermediate in the phosphorylation cascade of the PTS, are key players in the CCR of  
413 enterobacteria. The EIIA<sup>Glc</sup>, 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<sup>Glc</sup> 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<sup>Glc</sup> 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<sup>Glc</sup>, and CRP. The growth on maltose of the  
434 mutants lacking EIIA<sup>Glc</sup> 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<sup>Glc</sup> 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<sub>12</sub> and NAD(P)H. *K. pneumoniae* has a *de novo* pathway for the biosynthesis of  
468 coenzyme B<sub>12</sub> 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. pneumoniae* (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 ( $\Delta ldhA\Delta adhE\Delta frdA$ ),  
510 PDO production was only marginally increased compared with that of the single  $\Delta ldhA$   
511 mutant. The failure of the triple mutant ( $\Delta ldhA\Delta adhE\Delta frdA$ ) 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. pneumoniae* (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<sup>+</sup>-dependent  $\gamma$ -glutamyl- $\gamma$ -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*  $\Delta dhaT \Delta yqhD$  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  $\text{NAD}^+$  is  
609 essential. Third, excessively high aeration decreases the expression of the Dha regulon and  
610 synthesis of coenzyme B<sub>12</sub>, 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  $\text{NAD}^+$  regeneration and coenzyme B<sub>12</sub>  
618 production, Ashok et al. (2013b) studied anaerobic respiration using nitrate as an external  
619 electron acceptor. They attempted to regenerate  $\text{NAD}^+$  from NADH by nitrate reduction  
620 while maintain anaerobic conditions to synthesize and stabilize the oxygen-sensitive  
621 coenzyme B<sub>12</sub>. 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<sup>+</sup> regeneration  
637 and coenzyme B<sub>12</sub> 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<sub>12</sub> 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  $\Delta$ *ackA-pta*  
671  $\Delta$ *yqhD\_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<sub>12</sub> 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<sub>600</sub>) 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<sub>600</sub>, <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<sub>12</sub>, 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  $\alpha$ -acetolactate and acetoin. The C5 intermediate,  $\alpha$ -  
696 acetolactate, is formed from two molecules of pyruvate by self-condensation catalyzed by  $\alpha$ -  
697 acetolactate synthase (*ALS*; *budB*).  $\alpha$ -Acetolactate is decarboxylated to acetoin by  $\alpha$ -  
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,  $\alpha$ -acetolactate is spontaneously decarboxylated to diacetyl, further reduced to acetoin

701 by action of diacetyl reductase, and then reduced to BDO.  $\alpha$ -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<sup>+</sup> 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<sup>2+</sup>, an essential component of coenzyme B<sub>12</sub>.  
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  $\Delta\text{pH}$  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 ( $\Delta\text{pH} = 1.0$ ) 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 ( $\Delta mgsA$ ) 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 ( $\Delta pflB \Delta pta \Delta adhE \Delta frdA \Delta mgsA \Delta lldD \Delta ldhA::ldhA\_glpK-$   
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  $\gamma$ -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 Fertat-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 ( $\Delta glpK$ ) 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 dehydrogenase (*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.

1004 **Acknowledgement**

1005 This study was supported financially by the Advanced Biomass R&D Center (ABC) of  
1006 Global Frontier Project funded by the Korean Ministry of Science, ICT and Future planning  
1007 (ABC-2011-0031361).

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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

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

3

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17

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

## 30 **1. Introduction**

31 Crude glycerol is produced in large quantities as a byproduct of the biodiesel industry.  
32 Global biodiesel production was 22.7 million tons in 2012 and is forecasted to reach 36.9  
33 million tons by 2020. As a result, worldwide production of glycerol climbed from 1 million  
34 tons in 2000 to 3 million tons in 2011 and is expected to reach 6 million tons by 2025  
35 (Ciriminna et al., 2014; OECD, 2011; Katryniok et al., 2013; Sun et al. 2017). A significant  
36 amount of glycerol is also generated during bioethanol fermentation by yeast. The liquid  
37 fraction of the stillage obtained from ethanol separation contains up to 2% glycerol. Another  
38 source of glycerol is industrial waste generated from vegetable oils and animal fats. For  
39 example, the concentration of glycerol in waste streams from the oleochemical industry is  
40 55%–90% (da Silva et al., 2009; Yazdani and Gonzalez, 2007). The increased availability of  
41 glycerol has caused a substantial reduction in its cost. According to a recent report, the price  
42 of crude glycerol is \$0.24/kg and that of pure USP grade glycerol is \$0.9/kg (Kong et al.,  
43 2016). Extensive research has been conducted to investigate ways to utilize this surplus crude  
44 glycerol. The annual number of research articles addressing the use of glycerol has increased  
45 to >7000, doubling in number from the year 2000 to 2007. Numerous chemical conversions  
46 to valuable products have been reported (Ciriminna et al., 2014).

47 Glycerol can be used for the microbial production of 1,3-propanediol (PDO), ethanol,  
48 2,3-butanediol (BDO), butanol, lactic acid, 3-hydroxypropionic acid (3-HP), and succinic  
49 acid (Fig. 1) (Clomberg and Gonzalez, 2013; Mattam et al., 2013; Yazdani and Gonzalez,  
50 2007). Because of its reduced nature, glycerol generates twice the number of reducing  
51 equivalents per carbon than do traditional carbohydrates (glucose, xylose, and sucrose) when  
52 converted into the glycolytic intermediates phosphoenolpyruvate (PEP) or pyruvate  
53 (Dharmadi et al., 2006). As a consequence, glycerol gives higher yields of reduced  
54 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. buchneri* 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<sub>12</sub>, 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<sup>+</sup>, 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<sup>+</sup>  
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-hydroxypropionaldehyde (3-HPA). Catalysis by GDHt requires coenzyme B<sub>12</sub>, 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)<sup>+</sup> (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 *glpA* gene, glycerol dehydrogenase is an oxidoreductase  
153 that converts glycerol to DHA. The enzyme extracts electrons from glycerol and transfers

154 them to  $\text{NAD}^+$ . 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  $\text{NADH}$  disposal and recovery (Wang et al., 2014). The promiscuity of DhaD is responsible  
162 for BDO production in the  $\Delta budC$  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  $\text{NADH}$  in the presence of the *dhaD* gene.  $\text{NADH}$  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  $\text{NADH}$ , DhaD is able to switch its role  
168 from  $\text{NADH}$  production (glycerol oxidation) to  $\text{NADH}$  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  $\text{NADH}/\text{NAD}^+$  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. pneumoniae* 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<sub>12</sub> or S-adenosyl methionine (SAM) as a cofactor. *K.*  
199 *pneumoniae* has B<sub>12</sub>-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  $\alpha$  (large,  
202 60.7 kDa),  $\beta$  (medium, 21.3 kDa), and  $\gamma$  (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<sub>12</sub> 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<sub>12</sub> 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<sub>12</sub> in the presence of ATP and  
214 Mg<sup>2+</sup>/Mn<sup>2+</sup> (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  $\alpha$  subunits (63 kDa) and two globular  $\beta$  subunits (14 kDa).  
217 Structurally, the  $\alpha$  subunit resembles both GroEL and Hsp70 chaperones, while the  $\beta$  subunit  
218 resembles that of the  $\beta$  subunit of glycerol dehydratase, except that it lacks some of the amino  
219 acids responsible for coenzyme B<sub>12</sub> 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<sub>12</sub>-independent (SAM-dependent)  
231 and B<sub>12</sub>-dependent GDHt. The genes for B<sub>12</sub>-independent GDHt are present in the genomic  
232 DNA, while those encoding B<sub>12</sub>-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<sub>12</sub>-independent proteins showed no homology  
236 with B<sub>12</sub>-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<sup>+</sup> (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),  $\sigma^{54}$  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  $\sigma^{54}$  factor interaction  
300 domain interacts with the  $\sigma^{54}$  factor of RNA polymerase and activates RNA transcription  
301 from the  $\sigma^{54}$  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  $\sigma^{70}$  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 ( $K_d = 31 \mu\text{M}$ )**  
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 ( $K_m = 10 \mu\text{M}$ ) 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 ( $K_m = 1 \text{mM}$ )  
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<sup>+</sup> 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<sup>Glc</sup>, an  
412 intermediate in the phosphorylation cascade of the PTS, are key players in the CCR of  
413 enterobacteria. The EIIA<sup>Glc</sup>, 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<sup>Glc</sup> 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<sup>Glc</sup> 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<sup>Glc</sup>, and CRP. The growth on maltose of the  
434 mutants lacking EIIA<sup>Glc</sup> 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<sup>Glc</sup> 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<sub>12</sub> and NAD(P)H. *K. pneumoniae* has a *de novo* pathway for the biosynthesis of  
468 coenzyme B<sub>12</sub> 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. pneumoniae* (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 ( $\Delta ldhA\Delta adhE\Delta frdA$ ),  
510 PDO production was only marginally increased compared with that of the single  $\Delta ldhA$   
511 mutant. The failure of the triple mutant ( $\Delta ldhA\Delta adhE\Delta frdA$ ) 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. pneumoniae* (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<sup>+</sup>-dependent  $\gamma$ -glutamyl- $\gamma$ -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*  $\Delta dhaT \Delta yqhD$  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  $\text{NAD}^+$  is  
609 essential. Third, excessively high aeration decreases the expression of the Dha regulon and  
610 synthesis of coenzyme B<sub>12</sub>, 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  $\text{NAD}^+$  regeneration and coenzyme B<sub>12</sub>  
618 production, Ashok et al. (2013b) studied anaerobic respiration using nitrate as an external  
619 electron acceptor. They attempted to regenerate  $\text{NAD}^+$  from NADH by nitrate reduction  
620 while maintain anaerobic conditions to synthesize and stabilize the oxygen-sensitive  
621 coenzyme B<sub>12</sub>. 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<sup>+</sup> regeneration  
637 and coenzyme B<sub>12</sub> 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<sub>12</sub> 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  $\Delta$ *ackA-pta*  
671  $\Delta$ *yqhD\_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<sub>12</sub> 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<sub>600</sub>) 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<sub>600</sub>, <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<sub>12</sub>, 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  $\alpha$ -acetolactate and acetoin. The C5 intermediate,  $\alpha$ -  
696 acetolactate, is formed from two molecules of pyruvate by self-condensation catalyzed by  $\alpha$ -  
697 acetolactate synthase (ALS; *budB*).  $\alpha$ -Acetolactate is decarboxylated to **acetoin by  $\alpha$ -**  
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,  $\alpha$ -acetolactate is spontaneously decarboxylated to diacetyl, further reduced to acetoin



701 by action of diacetyl reductase, and then reduced to BDO.  $\alpha$ -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<sup>+</sup> 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<sup>2+</sup>, an essential component of coenzyme B<sub>12</sub>.**  
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  $\Delta\text{pH}$  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 ( $\Delta\text{pH} = 1.0$ ) 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 ( $\Delta mgsA$ ) 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 ( $\Delta pflB \Delta pta \Delta adhE \Delta frdA \Delta mgsA \Delta lldD \Delta ldhA::ldhA\_glpK-$   
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  $\gamma$ -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 Fertat-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 ( $\Delta glpK$ ) 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 dehydrogenase (*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.

1004 **Acknowledgement**

1005 This study was supported financially by the Advanced Biomass R&D Center (ABC) of  
1006 Global Frontier Project funded by the Korean Ministry of Science, ICT and Future planning  
1007 (ABC-2011-0031361).

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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 1

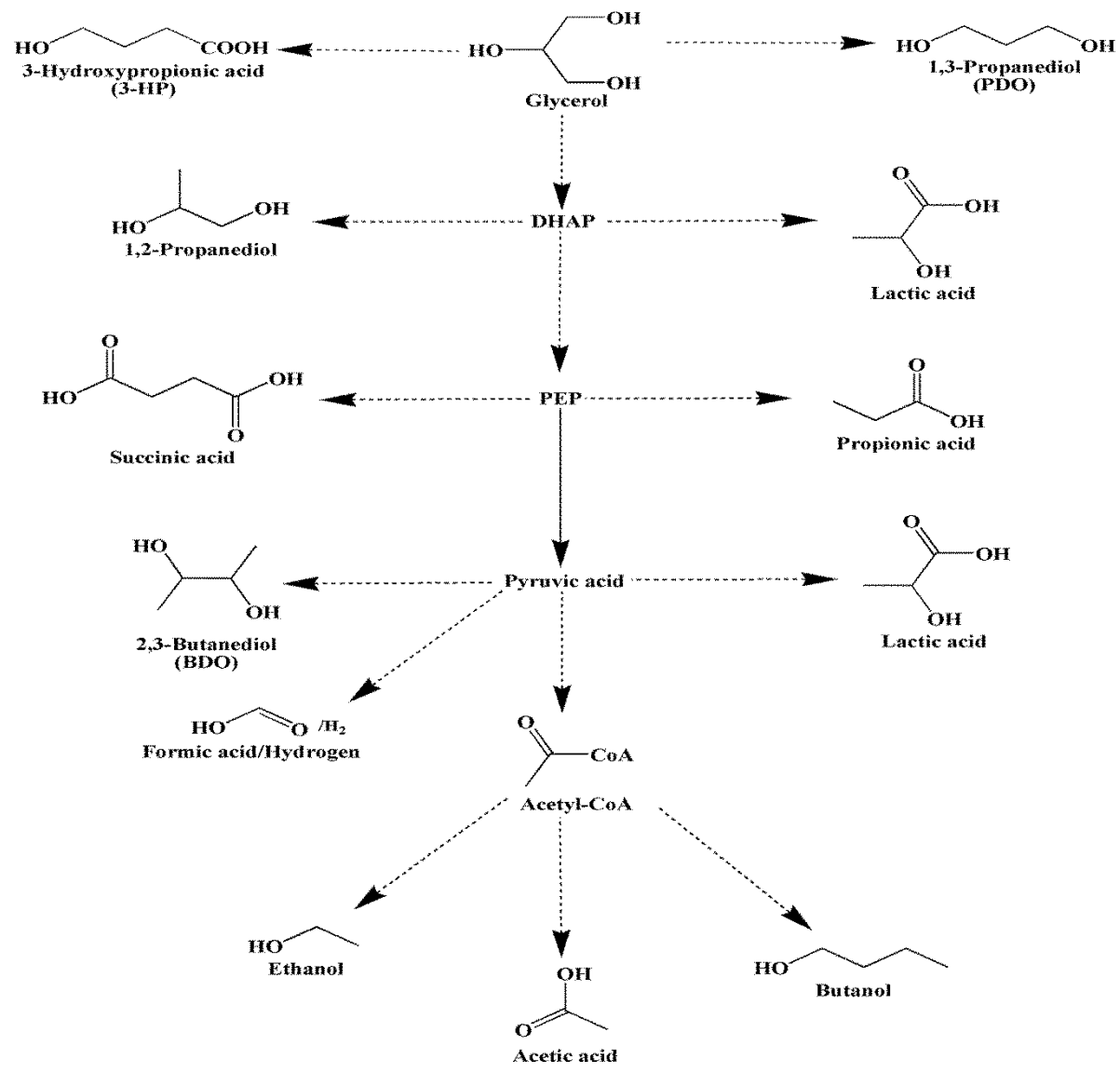
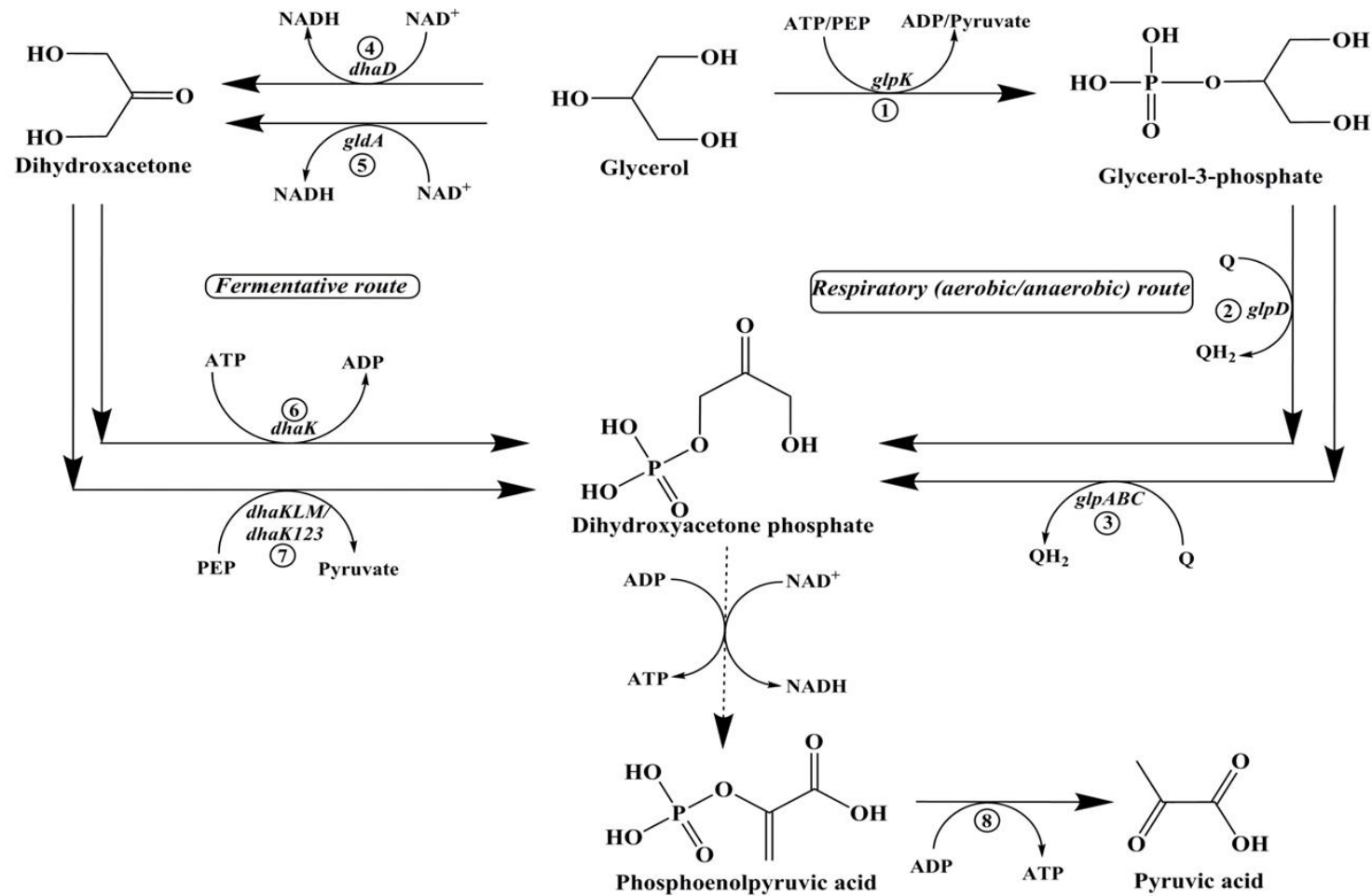


Figure 2



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

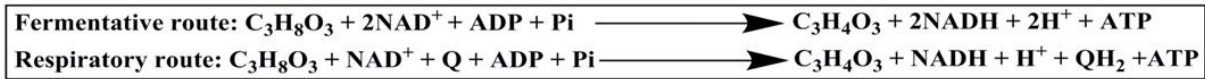
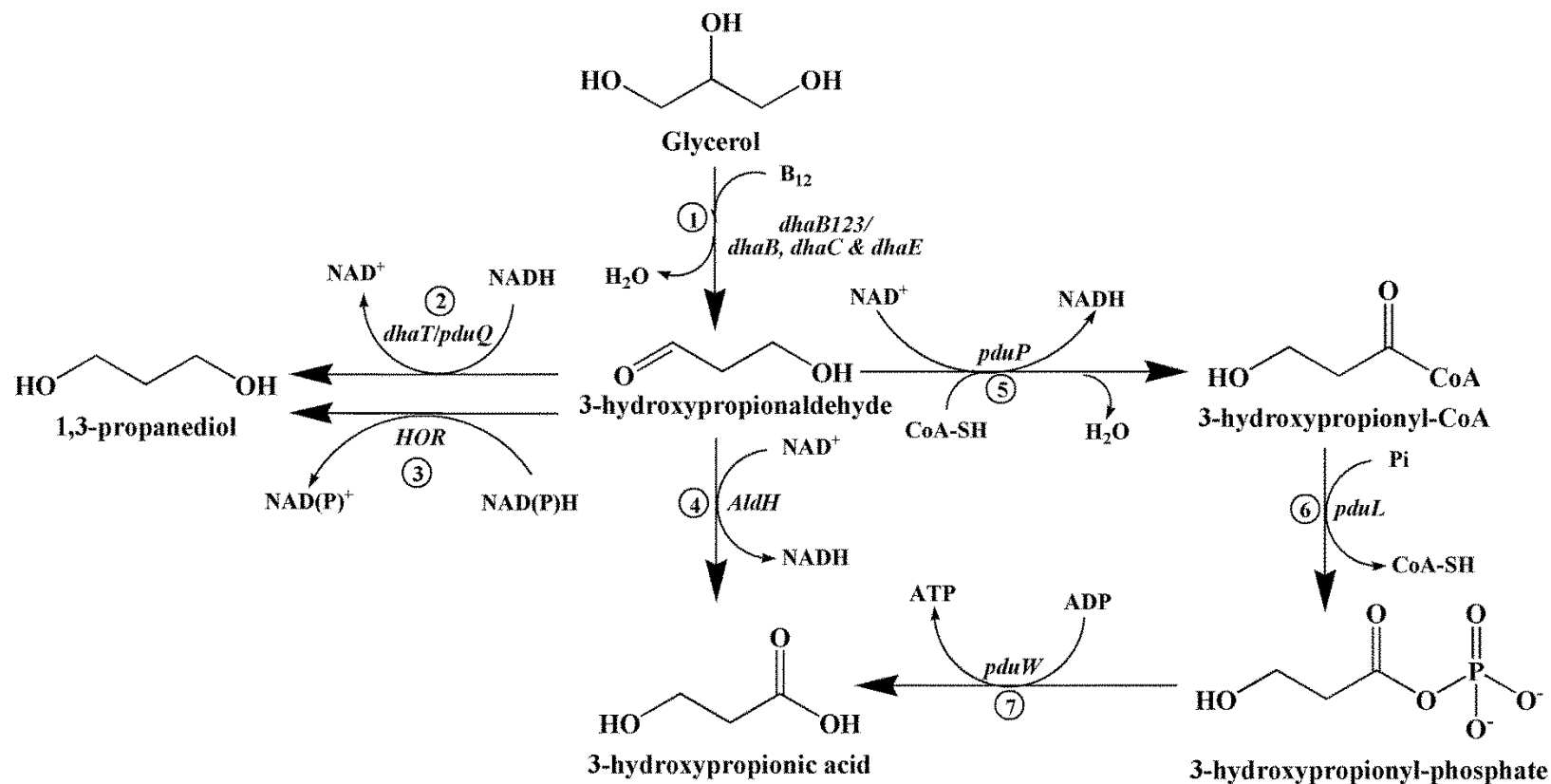




Figure 3



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

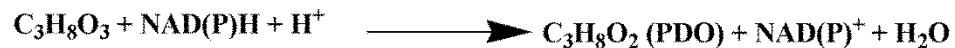


Figure 4

A)



B)

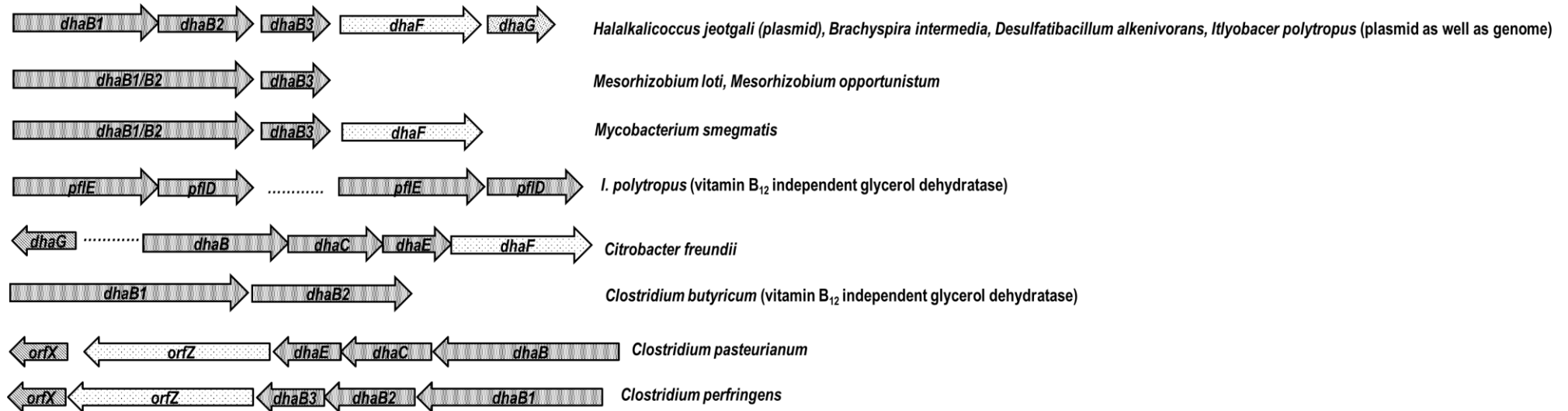
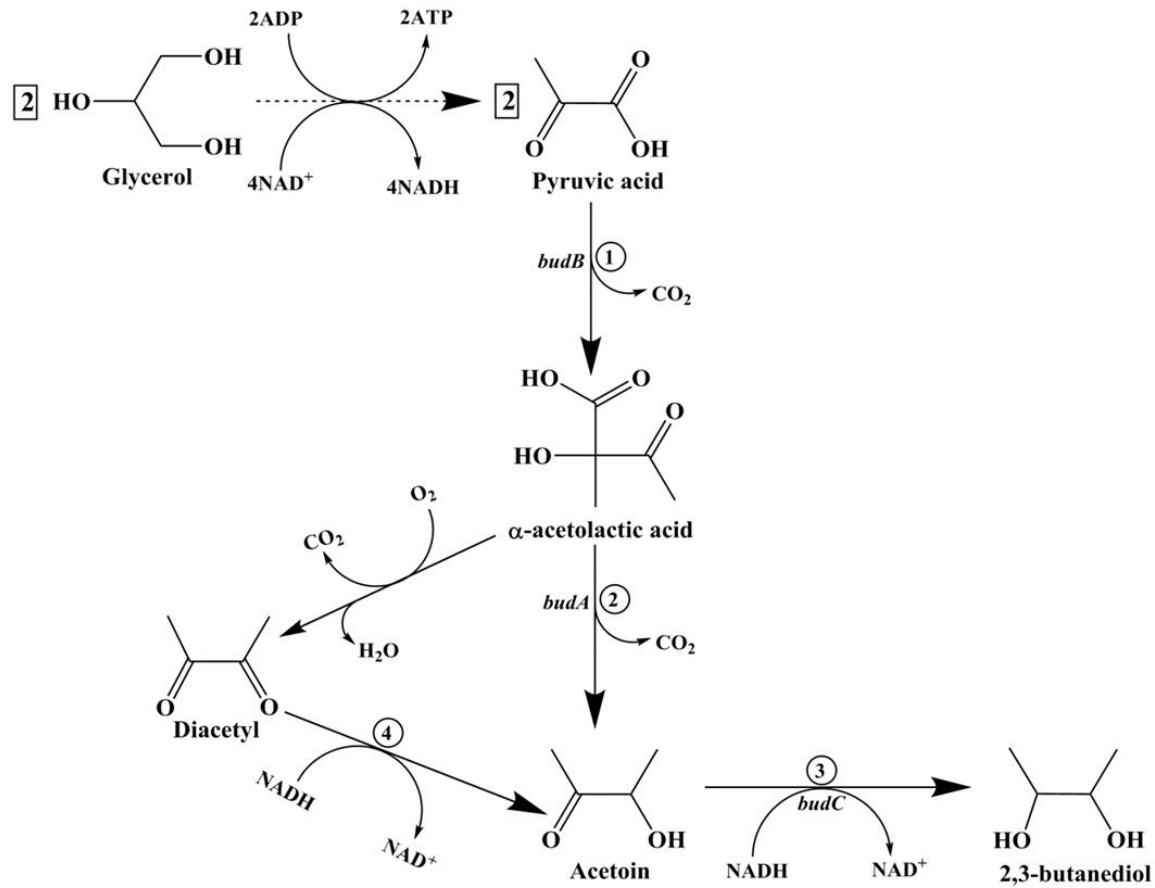


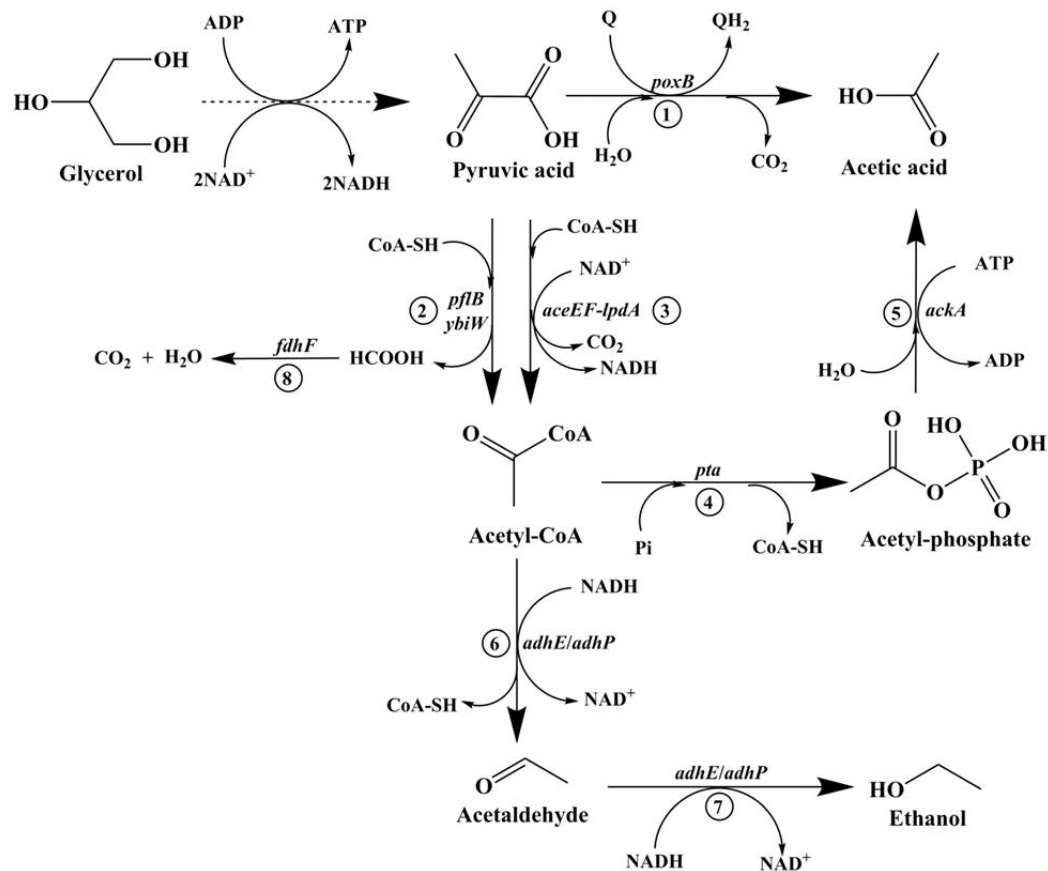
Figure 5



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



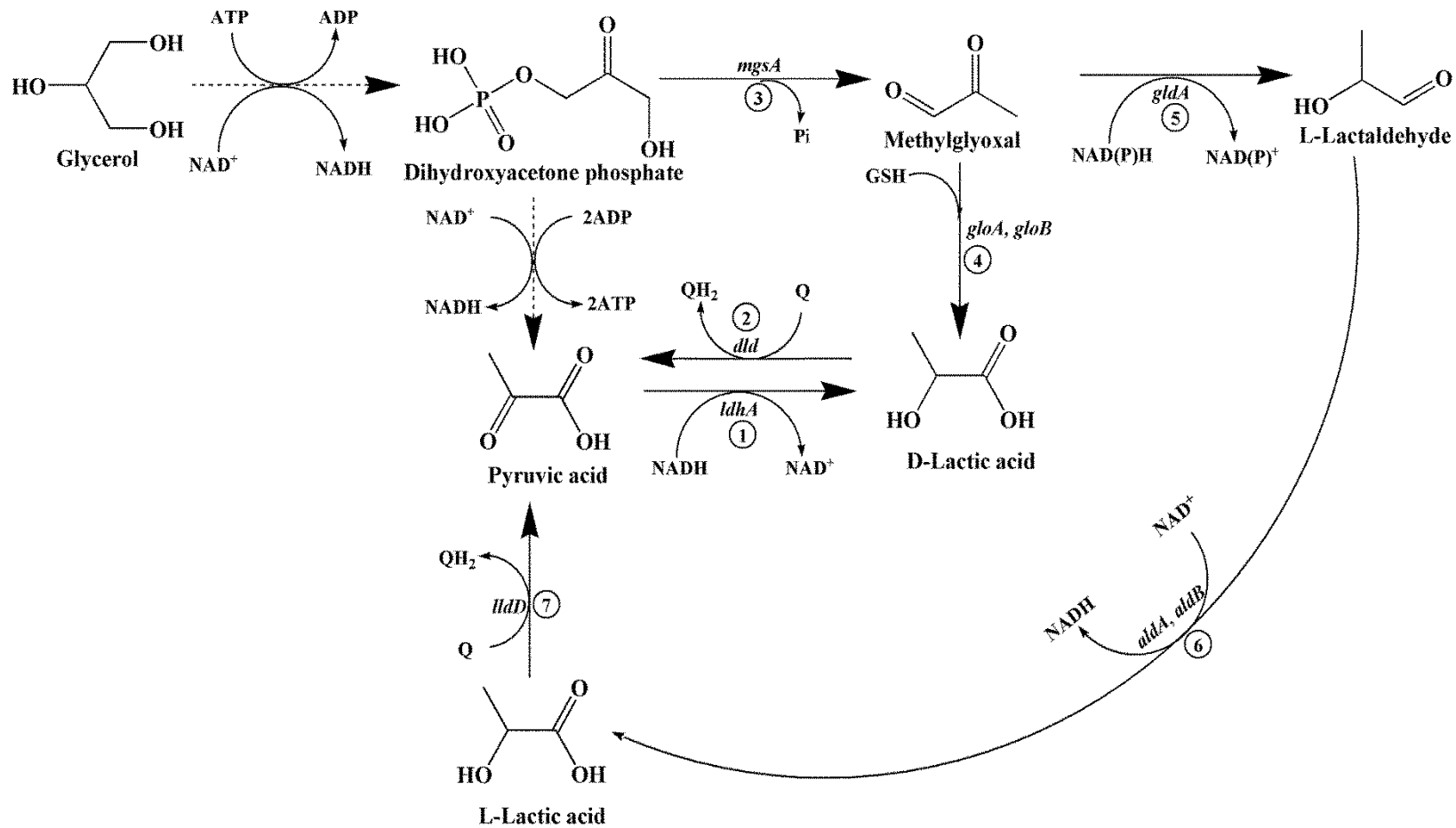
Figure 6



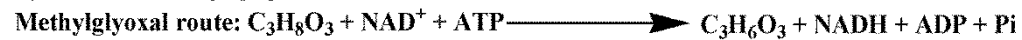
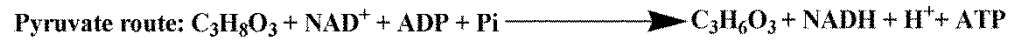
Key enzymes: 1- Pyruvate oxidase; 2-Pyruvate-formate lyase, putative formate acetyltransferase 3- Pyruvate dehydrogenase complex; 4- Phosphoacetyl transferase  
5- Acetate kinase; 6 and 7- Bifunctional (acetaldehyde/alcohol) dehydrogenase/putative alcohol dehydrogenase; 8-Formate dehydrogenase, part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



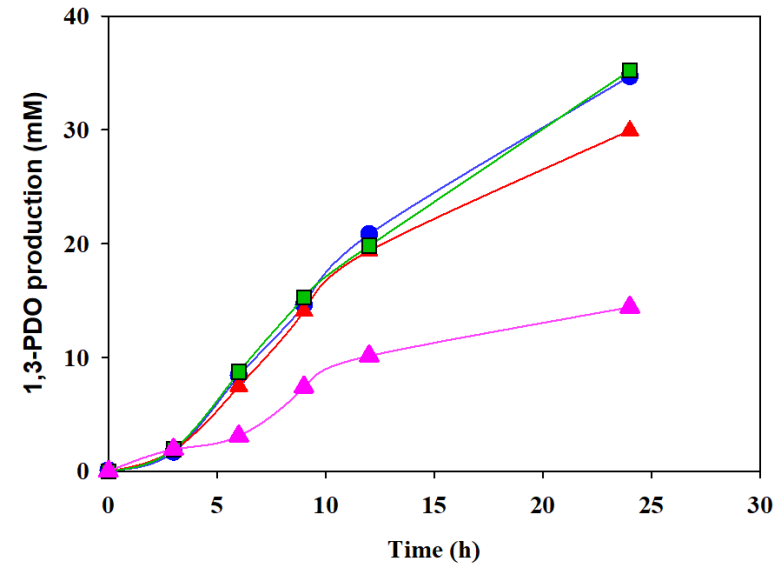
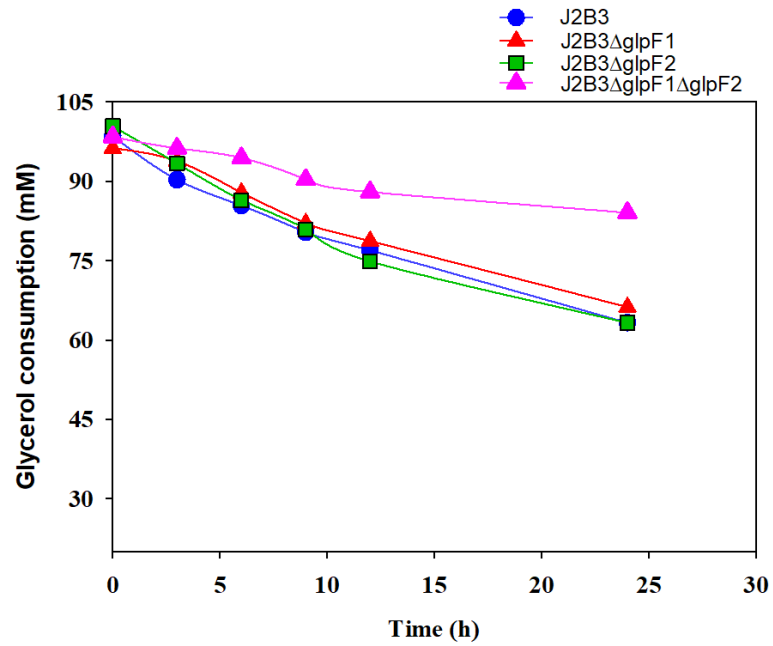
Figure 7



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), glucose was added at 100 mM because the strain cannot grow on glycerol as the sole carbon source (Lama et al., 2017)

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

<b>Metabolite</b>	<b>Maximum theoretical yield**</b>		<b>ATP generated/Metabolite (mol/mol)</b>		<b>Redox constrained [NAD(P)H generated &amp; consumed/ Metabolite]</b>	
	<b>Glucose (mol/0.5 mol)</b>	<b>Glycerol (mol/mol)</b>	<b>Glucose</b>	<b>Glycerol</b>	<b>Glucose</b>	<b>Glycerol</b>
1,3-propanediol C <sub>3</sub> H <sub>8</sub> O <sub>2</sub>	0.75	0.88	-1	0	Yes (0 & 2)	Yes (0 & 1)
3-hydroxypropionic acid C <sub>3</sub> H <sub>6</sub> O <sub>3</sub>	1.0	1.17	1/0/-1/-0.33	0/1	No (1 & 1; 2 & 2)	Yes (1 & 0)
2,3-butanediol C <sub>4</sub> H <sub>10</sub> O <sub>2</sub>	0.55	0.64	2	2	Yes/No (2 & 1/2)	Yes (4 & 1/2)
Ethanol C <sub>2</sub> H <sub>6</sub> O	1.0	1.17	1	1	Yes/No (1/2 & 2)	Yes/No (2/3 & 2)
Succinic acid C <sub>4</sub> H <sub>6</sub> O <sub>4</sub>	0.86	1.0	0/1	0/1	Yes (1 & 2)	No (2 & 2)
Lactic acid C <sub>3</sub> H <sub>6</sub> O <sub>3</sub>	1.0	1.17	1/-1	1/-1	No (1 & 1; 0 & 0)	Yes (2 & 0; 1 & 0)
Pyruvic acid C <sub>3</sub> H <sub>4</sub> O <sub>3</sub>	1.2	1.4	1	1	Yes (1/2 & 0)	Yes (2/3 & 0)
Acetic acid C <sub>2</sub> H <sub>4</sub> O <sub>2</sub>	1.5	1.75	1/2	1/2	Yes (1/2 & 0)	Yes (2/3 & 0)
1-Butanol C <sub>4</sub> H <sub>10</sub> 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**

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



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*ΔbudC\_fdh*

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**Recombinant strains (Overexpression of Genes from the Reductive Branch)**

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<i>K. pneumoniae</i> TUAC01_ <i>dhaT</i>	Batch	Glycerol	Aerobic	15.0	0.61	1.36	Hao et al. 2008
<i>K. pneumoniae</i> KG1_ <i>dhaT</i>	Fed-batch	Glycerol	Aerobic	90.9	0.64	2.16	Zhao et al. 2009
<i>K. pneumoniae</i> ME- 308_ <i>yqhD</i>	Fed-batch	Glycerol	Microaerobic	67.6	0.62	1.69	Zhu et al. 2009
<i>K. pneumoniae</i> $\Delta$ <i>dhaD</i> $\Delta$ <i>dhaK</i> $\Delta$ <i>dhaT</i> _yqhD	Batch	Glycerol	Aerobic	7.7	0.53	0.26	Seo et al. 2010

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**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
<i>K. pneumoniae</i> DSM 2026 $\Delta dhaT\_puuC$	Glycerol	Microaerobic	16.0 (16.8)	0.23	0.67	Ashok et al. 2011
<i>K. pneumoniae</i> WM3 <i>pUC18kan\_aldHec</i>	Glycerol	Anaerobic	24.4 (49.3)	0.18	1.02	Huang et al. 2012
<i>K. pneumoniae</i> J2B _ <i>KGSADH</i>	Glycerol	Anaerobic	11.3 (15.9)	0.27	0.94	Kumar et al. 2012
<i>K. pneumoniae</i> $\Delta glpK\Delta dhaT\_puuC$	Glycerol	Anaerobic	22.0 (5.9)	0.30	0.46	Ashok et al. 2013a
<i>K. pneumoniae</i> $\Delta dhaT\Delta yqhD\_dhaB\_puuC$	Glycerol	Aerobic	28.1 (3.3)	0.40	0.58	Ashok et al. 2013b
<i>K. pneumoniae</i> WM3 <i>pUC18kan\_aldHec</i>	Glycerol	Microaerobic	48.9 (25.3)	0.41	1.75	Huang et al. 2013a
<i>K. pneumoniae</i> J2B $\Delta ldhA\_KGSADH$	Glycerol	Microaerobic	22.7 (23.4)	0.35	0.38	Kumar et al. 2013b
<i>E. coli</i> W3110 $\Delta ackA\text{-}pta$ $\Delta yqhD\_dhaB\_mutant\ gabD4$	Glucose & glycerol	Aerobic	71.9	-	1.8	Chu et al. 2015
<i>K. pneumoniae</i> DSM 2026 $\Delta ldh1\Delta ldh2\Delta pta\_puuC$ (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**

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

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<i>Δ ldhA _ KGSADH</i>							
<i>K. pneumoniae</i> BLh-1	Fed-batch	Glycerol	Microaerobic	59.0	0.48	1.48	Rossi et al. 2013
<i>K. pneumoniae</i> J2B	Fed-batch	Glycerol	Microaerobic	37.0	0.32	0.77	Durgapal et al. 2014
<i>K. pneumoniae</i> DSMZ	Fed-batch	Glycerol	Microaerobic	47.8	0.39	1.00	Durgapal et al. 2014
<i>K. pneumoniae</i> ATCC25955	Fed-batch	Glycerol	Microaerobic	142.1	0.84	2.96	Feng et al. 2014
<i>ΔdhaT_ ΔyqhD_ ldhA</i>				(D-isomer)			
<i>E. coli</i> MG1655	Fed-batch	Glycerol	Microaerobic	32.0	0.82	0.54	Mazumdar et al. 2010
<i>Δpta_ ΔadhE_ ΔfrdA_ Δlld_ glpK_ glpD</i>				(D-isomer)			
<i>E. coli</i> MG1655	Fed-batch	Glycerol	Microaerobic	50.0	0.92	0.60	Mazumdar et al. 2013
<i>ΔpflB_ Δpta_ ΔadhE_ ΔfrdA_ ΔmgsA_ ΔlldD_ ΔldhA::ldhA_ glpK_ glpD</i>				(L-isomer)			

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

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2017-10-19

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Vinod Kumar, Sunghoon Park, Potential and limitations of *Klebsiella pneumoniae* as a microbial cell factory utilizing glycerol as the carbon source, *Biotechnology Advances*, Volume 36, Issue 1, January–February 2018, Pages 150-167

<https://doi.org/10.1016/j.biotechadv.2017.10.004>

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