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

1. Introduction

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

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

55 Gonzalez, 2007). Table 1 compares the maximum theoretical yield of various metabolites,

56 ATP, and redox balances between glycerol and glucose.

57   Despite its great potential as a carbon source, glycerol is not efficiently metabolized

58 by many microorganisms under anaerobic or oxygen-limited conditions. The ability of *K.*

59 *pneumoniae* to assimilate glycerol under oxygen-limited conditions is outstanding and has

60 been studied extensively. Several review articles addressing this issue have also been

61 published (Clomberg and Gonzalez, 2013; Mattam et al., 2013; Yazdani and Gonzalez, 2007),

62 but the biotechnological potential and physiological aspects of this strain have not been fully

63 elucidated. This review explores and discusses glycerol metabolism in *K. pneumoniae* and the

64 production of value-added chemicals from glycerol. The potential and challenges of using *K.*

65 *pneumoniae* as a microbial cell factory are also discussed.

66 **2. Glycerol metabolism in *K. pneumoniae***

67   Many microorganisms can metabolize glycerol in the presence of external electron

68 acceptors (respiratory metabolism), but relatively few under non-respiratory conditions

69 (without exogenous electron acceptors). Among the latter, genera including *Citrobacter*,

70 *Enterobacter*, *Bacillus*, *Propionibacterium*, *Anaerobiospirillum*, *Klebsiella*, *Clostridium*, and

71 *Lactobacillus* are well documented (da Silva et al., 2009; Yazdani and Gonzalez, 2007).

72 Some *Lactobacillus* species including *L. reuteri*, *L. brevis*, and *L. bucheneri* convert glycerol

73 to PDO but only in the presence of a major carbon source for growth. Glycerol does not

74 support cell growth, limiting its biotechnological applications. In contrast, *Clostridium sp.*

75 grow well on glycerol as the sole carbon source. However, the use of *Clostridium* is limited

76 by its slow growth and requirement of strict anaerobic conditions. The lack of a convenient

77 genetic tool box for large-scale metabolic engineering is another serious drawback of

78 *Clostridium* (Branduardi et al., 2014; Lütke-Eversloh and Bahl, 2011).

From a biotechnological standpoint, *Klebsiella sp.*, especially *K. pneumoniae*, have

many advantages over the aforementioned microbes. They grow on glycerol as the sole

carbon source under both aerobic and anaerobic conditions (Arasu et al., 2011; Kumar et al.,

2012). Their growth is fast, typically growing at a rate >0.9/h, even on glycerol minimal

medium (Arasu et al., 2011). They naturally produce coenzyme B_{12}, an essential cofactor for

the synthesis of the commercially important chemicals PDO and 3-HP (Huang et al., 2013a;

Oh et al., 2012a). Furthermore, their evolutionary and biochemical proximity to *E. coli* allows

for the application of most of gene manipulation methods developed for *E. coli* without much

modification (Celińska, 2012; Kumar et al., 2013a).

2.1 Overview of glycerol metabolism in *K. pneumoniae*

In *K. pneumoniae*, glycerol is metabolized oxidatively and/or reductively (Ashok et

al., 2011, Kumar et al., 2013a). The oxidative pathway provides energy and carbon

constituents for the synthesis of cell biomass, while the reductive pathway reduces glycerol

and regenerates NAD^+, enabling the oxidative assimilation of glycerol under anaerobic

conditions.

Glycerol oxidation proceeds in either a respiratory or a fermentative manner.

Respiration requires an exogenous electron acceptor such as oxygen, nitrate, or fumarate

(Ashok et al., 2013a,b) and is carried out by enzymes encoded by the *glp* regulon. During

respiration, glycerol is first phosphorylated by glycerol kinase (*glpK*) to yield *sn*-glycerol-3-

phosphate using ATP or phosphoenolpyruvate (PEP) as a phosphate donor. Next, *sn*-

glycerol-3-phosphate is oxidized to dihydroxyacetone phosphate (DHAP) with electron

transfer to a quinone molecule, which is linked to the electron transport chain (ETC). This

transformation is catalyzed by glycerol-3-phosphate dehydrogenase, which is expressed

under both aerobic (*glpD*) and anaerobic (*glpABC*) conditions. In contrast, fermentation of

glycerol is conducted without a supply of external electron acceptors, and the reactions are

104   catalyzed by enzymes encoded by the *dha* regulon. Here, glycerol is oxidized to

105   dihydroxyacetone (DHA) by glycerol dehydrogenase (mainly encoded by *dhaD*) using NAD^+

106   as an electron acceptor. DHA then is phosphorylated by dihydroxyacetone kinase, an enzyme

107   encoded by the ATP-dependent *dhaK1/dhaK* and/or PEP-dependent

108   *dhaK2/dhaKLM/dhaK123*. DHAP obtained from respiration and/or fermentation is channeled

109   into the glycolytic pathway (Fig. 2). A variety of organic acids and alcohols, including acetic

110   acid, lactic acid, succinic acid, ethanol, BDO, and formic acid, are produced by the

111   downstream glycolytic pathway (Ashok et al., 2011; Kumar et al., 2013a).

112   The anaerobic growth capability of *K. pneumoniae* using glycerol as a carbon source

113   is attributed to its well-established reductive metabolism. In the reductive pathway, glycerol

114   first undergoes a difficult rearrangement reaction catalyzed by glycerol dehydratase (GDHt)

115   to yield 3-hydroxypropioaldehyde (3-HPA). Catalysis by GDHt requires coenzyme B_{12}, the

116   *de novo* synthesis of which is limited to only few genera of microorganisms. The 3-HPA is

117   subsequently reduced to PDO by at least two 1,3-propanediol oxidoreductases (1,3-PDORs),

118   including NADH-dependent DhaT (PDOR) and NADPH-dependent hypothetical

119   oxidoreductase (HOR), along with the regeneration of $NAD(P)^+$ (Fig. 3). *E. coli* and other

120   *enterobacter sp.* have the oxidative (respiratory) pathway of glycerol assimilation. However,

121   these organisms lack the reductive pathway and cannot grow on glycerol under anaerobic

122   conditions (Dharmadi et al., 2006; Kumar et al., 2013a).

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

124   The *dha* regulon, induced by the DHA molecule and expressed in the absence of

125   exogenous electron acceptors, encodes numerous genes needed for both the oxidative

126   (fermentative) and reductive pathways (Celińska, 2012; Forage and Foster, 1982) (Fig. 4A).

127   The *dha* regulon of *K. pneumoniae* contains the following genes arranged in the order as

128   appears: *Kpk_0615/dhaK*, *dhaM*, *dhaL*, *dhaK*, *dhaD*, *dhaR*, *orfW, CdAT, orfX* (*dhaG*), *dhaT*,

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129 *orfY, dhaB*, *dhaC*, *dhaE*, *orfZ* (*dhaF*), and *glpF*. Of these, *dhaB*, *dhaC*, *dhaE*, *orfX*, *orfZ*, and

130 *dhaT* are relatively well documented for their functions in the reductive metabolism of

131 glycerol. The *orfW* and *orfY* genes, although commonly found in the *dha* regulons of many

132 organisms, are not as well described. The proteins encoded by the *dha* regulon of *K.*

133 *pneumoniae* have high similarity (80%−95%) with those of *Citrobacter* species but not with

134 those of *Clostridium* species (30%−80%). Surprisingly, the sequence similarity of *dha*

135 proteins among *Clostridium* species is very low in some cases, even lower than their

136 similarity to the corresponding proteins in *K. pneumoniae* or *C. freundii* (Celińska, 2012; Sun

137 et al., 2003; Wei et al., 2014).

138   The complete *dha* regulon is found in only a few dozen species belonging to five

139 different taxonomic groups, including four bacterial species [Actinobacteria, Firmicutes,

140 Fusobacteria, and Proteobacteria (Gammaproteobacteria and Deltaproteobacteria)] and one

141 archaea species, *Halalkalicoccus jeotgali* (Martins-Pinheiro et al., 2016). Interestingly, an

142 incomplete *dha* regulon is present in more than 100 prokaryotes, suggesting that the enzymes

143 of the regulon have other functions not restricted to the anaerobic growth on glycerol. A well-

144 documented example is the one found in *Lactobacillus* species. *Lactobacillus* cannot grow on

145 glycerol as the sole carbon source because it lacks the enzymes for oxidative assimilation of

146 glycerol. However, the enzymes encoded in the incomplete *dha* regulon enable the

147 production of PDO from glycerol, which accelerates cell growth in *Lactobacillus* grown on

148 glucose (Årsköld et al., 2008; Martins-Pinheiro et al., 2016).

149   In the following sections, the major enzymes involved in fermentative glycerol

150 metabolism and their genes are described in detail.

151 **2.2.1 Glycerol dehydrogenase**

152   Encoded by the *dhaD* and/or *gldA* gene, glycerol dehydrogenase is an oxidoreductase

153 that converts glycerol to DHA. The enzyme extracts electrons from glycerol and transfers

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

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

2.2.2 Dihydroxyacetone kinases

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

179 *dhaL* and *dhaM,* which correspond to the medium, small, and large subunits of the enzyme,

180 respectively (Celińska, 2012; Sun et al., 2003; Wei et al., 2014). The names *dhaKLM* and

181 *dhaK123* are synonyms used without distinction (Martins-Pinheiro et al., 2016; Sun et al.,

182 2003; Wei et al., 2014). DhaK enzymes are found in many microorganisms. PEP-dependent

183 DhaK II is present in *E. coli*, *L. lactis*, and *C. butyricum*, whereas ATP-dependent DhaK I is

184 present in *Citrobacter freundii* (Garcia-Alles et al., 2004; Raynaud et al., 2011). The PEP-

185 dependent DhaK II of *E. coli*, encoded by *dhaKLM*, is homologous to *dhaK123* (*dhaKLM*) of

186 *K. pneumoniae* (Gutknecht et al., 2001). In a recent study, Wei et al. (2014) further elucidated

187 the physiological roles of DhaK I and DhaK II of the *dha* regulon of *K. pneumoniae*. They

188 found that disruption of ATP-dependent DhaK I had no significant effect on glycerol uptake,

189 indicating that PEP-dependent DhaK II is the major contributor to the conversion of DHA to

190 DHAP. As in *E. coli*, the subunits of DhaK II regulate expression of the *dha* regulon; *dha*

191 regulon expression was suppressed by the disruption of *dhaK1* (*dhaK*) and *dhaK2* (*dhaL*).

192 However, in contrast to *E. coli*, mutation of *dhaK3* (*dhaM*) in *K. pneumonia* did not

193 upregulate the *dha* regulon but rather decreased cell growth and glycerol uptake.

2.2.3 Glycerol dehydratase and reactivating factor

195 Glycerol dehydratase (GDHt) is the first enzyme in the reductive pathway and

196 catalyzes the dehydration of glycerol to 3-HPA. The same reaction can be carried out by diol

197 dehydratase (DDHt), although the latter has a higher activity on 1,2-propanediol than on

198 glycerol. GDHt requires coenzyme B_{12} or *S*-adenosyl methionine (SAM) as a cofactor. *K.*

199 *pneumoniae* has B_{12}-dependent GDHt only, while strict anaerobes such as *clostridia* sp. have

200 SAM-dependent GDHt. The three subunits of GDHt are encoded by the genes *dhaB1* (~1670

201 bp), *dhaB2* (~590 bp) and *dhaB3* (~430 bp), for which the gene products are the α (large,

202 60.7 kDa), β (medium, 21.3 kDa), and γ (small, 16.1 kDa) subunits, respectively (Xu et al.,

203 2009a). GDHt of *K. pneumoniae* is known to be present as a dimer of heterotrimers, $(αβγ)_2$.

204 The subunits of GDHt have high homology among *K. pneumoniae* strains; for example, *K.*

205 *pneumoniae* XJPD-Li and U30909 strains showed >99% identity.

206 Coenzyme B_{12} is present in the reaction center and plays an essential role in the

207 catalysis of GDHt. The catalytic cycle often results in the mechanism-based inactivation of

208 coenzyme B_{12} due to the irreversible breakage of the chemical bond between cobalt (Co) and

209 a carbon of the adenosyl moiety. Oxygen accelerates breakage of the Co–C bond. Oxygen is

210 also known to inactivate GDHt, even in the absence of the substrate glycerol. The gene

211 products of *orfZ/dhaG* (small subunit) and *orfX/dhaF* (large subunit) act as reactivating

212 factors of GDHt (GdrAB) (Celińska, 2012; Sun et al., 2003). They reactivate GDHt by

213 catalyzing the exchange of damaged for intact coenzyme B_{12} in the presence of ATP and

214 Mg^{2+}/Mn^{2+} (Celińska, 2012; Kumar et al., 2013a; Maervoet et al., 2011; Sun et al., 2003; Wei

215 et al., 2014). The reactivase is a molecular chaperone that functions as a heterotetramer

216 containing two elongated α subunits (63 kDa) and two globular β subunits (14 kDa).

217 Structurally, the α subunit resembles both GroEL and Hsp70 chaperones, while the β subunit

218 resembles that of the β subunit of glycerol dehydratase, except that it lacks some of the amino

219 acids responsible for coenzyme B_{12} binding (Liao et al., 2003).

220 In some organisms, the GDHt enzyme varies in terms of the number of subunits and

221 their copies (Fig. 4B) (Liu et al., 2010). For example, in *Mesorhizobium loti* and

222 *Mesorhizobium opportunistum*, the large and medium subunits (*dhaB1* and *dhaB2*) are fused

223 together and encoded by a single gene. The gene fusion may arise by frameshift mutations.

224 The active site of the fused enzyme differs slightly from that of unfused enzyme. Moreover,

225 these microorganisms lack the genes encoding the reactivation factor, indicating that the

226 reactivation process may not exist or is carried out by a different route. *Mycobacterium*

227 *smegmatis* has two subunits (fused *dhaB1* + *dhaB2* and *dhaB3*) that are similar to those of *M.*

228 *loti* and *M. opportunistum*; however, the reactivating factor differs in that it has one (large)

229 subunit. No small subunit has been identified in *M. smegmatis*. Fusobacterium *Ilyobacter*

230 *polytropus* is unique in that it has genes coding for both B_{12}-independent (SAM-dependent)

231 and B_{12}-dependent GDHt. The genes for B_{12}-independent GDHt are present in the genomic

232 DNA, while those encoding B_{12}-dependent GDHt along with its reactivation factor are

233 present in two copies, one in the chromosome and the other in a plasmid. Surprisingly, even

234 with this gene redundancy, no significant production of 3-HPA and/or PDO has been reported

235 in *I. polytropus* (Stieb and Schink, 1984). The B_{12}-independent proteins showed no homology

236 with B_{12}-dependent GDHt but significant similarity with the pyruvate formate lyases (PFL)

237 and PFL-activating enzymes and their homologues (Raynaud et al., 2003).

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

239   The 1,3-propanediol oxidoreductase (PDOR), responsible for the second step of the

240 reductive pathway of glycerol, catalyzes the conversion of 3-HPA to PDO with the oxidation

241 of NAD(P)H. 3-HPA is a toxic intermediate, and PDOR protects the cell from deleterious

242 damage by reducing 3-HPA to PDO (Celińska, 2012). In *K. pneumoniae*, PDOR is encoded

243 by the *dhaT* gene and is strictly NADH dependent. Active PDOR is a homo-octamer with a

244 monomeric molecular mass of 43 kDa. Despite its importance, the kinetic properties of DhaT

245 were elucidated only recently (Lama et al., 2015). DhaT has high specificity for 3-HPA

246 among various aldehydes. Under physiological conditions at pH 7.0 and 37°C, the catalytic

247 efficiency (k_{cat}/K_m) of the forward reaction (3-HPA to PDO) was found to be 77.2/s. mM,

248 which is 57-fold higher than that of the reverse reaction (1.3/s. mM; PDO to 3-HPA). The K_m

249 (mM) values for 3-HPA and NADH (forward reaction) are 0.77 and 0.03, respectively, while

250 those for PDO and NAD^+ (backward reaction) are 7.4 and 0.23, respectively. Furthermore,

251 the maximum activity of DhaT for the reverse reaction was obtained at pH 9.0 and 55°C.

252 These results strongly suggest that, under physiological conditions, DhaT catalyzes the

253 reduction of 3-HPA to PDO rather than the oxidation of PDO to 3-HPA. Nevertheless, the

254　reverse reaction (PDO→3-HPA) becomes significant as the PDO concentration increases.

255　This factor is considered a serious drawback of using DhaT for PDO production from

256　glycerol.

257　　　　*K. pneumoniae* has several oxidoreductases in addition to PDOR that can reduce 3-

258　HPA to PDO. One such enzyme, designated as hypothetical oxidoreductase (HOR), has been

259　studied extensively and shares 89% identity with YqhD of *E. coli* (Zhu et al., 2009; Zhuge et

260　al., 2010). Expression of *yqhD* in wild-type *K. pneumoniae* is not high, with an mRNA level

261　~20-fold lower than that of *dhaT*. However, deletion of *dhaT* increases the transcription of

262　*yqhD* by more than 10-fold (Ko et al., 2015). This observation suggests that in addition to its

263　wide-ranging activity on short-chain aldehydes, YqhD is a detoxification enzyme for many

264　aldehydes (Jarboe, 2011). YqhD exhibits negligible activity on PDO (backward reaction),

265　which is an important advantage over DhaT for use in the production of PDO from glycerol

266　(Celińska, 2012; Chen et al., 2011; Zheng et al., 2006). However, YqhD is kinetically inferior

267　to DhaT because of its low catalytic activity (k_{cat}/K_m) on 3-HPA and the requirement of

268　NADPH as a cofactor. The k_{cat}/K_m value of YqhD on 3-HPA (pH 7.0 and 37°C) is estimated

269　to be 2.1/s. mM, which is 36-fold lower than that of DhaT (unpublished data). The use of

270　NADPH as a cofactor prohibits this enzyme from being directly coupled to the reactions

271　catalyzed by DhaD and other enzymes in glycolysis and the TCA cycle, by which NADH is

272　mainly produced. Furthermore, excessive use of NADPH for PDO production can

273　significantly reduce the cellular NADPH level and this can disturb many anabolic reactions

274　requiring NADPH (Wang et al., 2003; Jarboe, 2011). NADH and NADPH differ in a

275　phosphate group only, and it has been suggested that electrostatic interaction of this

276　phosphate is the major factor distinguishing NADH from NADPH for enzymes using these

277　cofactors. Comparison of the amino acid sequences of DhaT and HOR from *K. pneumoniae*

278　and YqhD from *E. coli* has revealed that DhaT contains aspartic acid (Asp) at position 41,

279 while HOR and YqhD have valine (Val) at this position. The steric hindrance and

280 electrostatic repulsion between Asp in the active site and the phosphate group of NADPH

281 impede their binding. In contrast, the hydrophobic amino acid Val poses no such obstruction;

282 thus, YqhD uses NADPH as a cofactor (Ma et al., 2010).

283 Despite their difference in cofactor preference, DhaT and YqhD are similar (44%) in

284 amino acid sequences. *K. pneumoniae* has five enzymes that have at least 40% similarity in

285 amino acid sequence with DhaT and YqhD: putative alcohol dehydrogenase (PaldH), L-1,2-

286 propanediol oxidoreductase (FucO), ethanolamine-utilization enzyme (EutG), propanediol-

287 utilization propanol dehydrogenase (PduQ), and bifunctional alcohol dehydrogenase (AdhE)

288 (Ko et al., 2015). The roles of these enzymes in PDO production have been investigated.

289 **2.2.5 Regulatory protein**

290 The *dhaR* gene product is an important transcription factor that is responsible for

291 expression of the *dhaT* and *dhaB* genes (Sun et al., 2003). This gene is found in *K.*

292 *pneumoniae*, C. *freundii*, and *Clostridium botulinum* but not in every organism containing a

293 complete *dha* regulon (Martins-Pinheiro et al., 2016). The presence of DhaR is thought to

294 allow the *dha* operon to function as a separate regulatory system, independent of fumarate

295 nitrate reduction (FNR). Under anaerobic conditions, FNR acquires DNA-binding properties

296 and induces the expression of many anaerobiosis-related genes. DhaR contains domains for

297 GAF (52-199), PAS (203-267), σ^{54} factor interaction, and histidine HTH-8. The PAS senses

298 internal levels of energy charge, light, oxygen, and redox potential. The HTH-8 domain has a

299 helix-turn-helix conformation and acts as a DNA binding structure. The σ^{54} factor interaction

300 domain interacts with the σ^{54} factor of RNA polymerase and activates RNA transcription

301 from the σ^{54} promoters (Buck et al., 2000; Taylor and Zhulin, 1999). Thus, the DhaR protein

302 triggers the transcription of *dha* genes in response to intracellular levels of several important

303 physiological parameters (Celińska, 2012; Sun et al., 2003). Zheng et al. (2006) investigated

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304   the effect of overexpression of the putative regulatory gene *dhaR* in the *dha* regulon on

305   glycerol metabolism in *K. pneumoniae*. *dhaR* overexpression increased PDOR activity up to

306   6.7-fold over that of the control wild-type strain, confirming the role of DhaR as a positive

307   regulator of the *dhaT* gene. However, *dhaR* overexpression did not increase the activity of

308   glycerol dehydratase (DhaB). Furthermore, despite the significant increase in PDOR activity,

309   PDO production was unexpectedly low compared with that of the wild type (440 vs. 656.23

310   mM, respectively). More studies are required to determine the precise role of DhaR and its

311   regulatory function in the *dha* regulon.

312   DhaR in *E. coli* (70% identity to that of *K. pneumoniae*) has also been studied. This

313   protein stimulated transcription of the *dhaKLM* operon from a σ^{70} promoter. Interestingly, in

314   *E. coli*, DhaL was a positive regulator of the *dhaKLM* operon while phosphorylated DhaM

315   and DhaK were negative regulators of the same operon. DhaK contains the DHA-binding site.

316   DhaL uses ADP as a cofactor for the double displacement of phosphate from DhaM to DHA.

317   DHA is the oxidation product of glycerol metabolism; binding of DHA to DhaK reduces the

318   affinity of DhaK for DhaR. In the presence of DHA, DhaL::ATP is dephosphorylated,

319   leading to the displacement of DhaK by DhaL::ADP, which stimulates DhaR activity. In the

320   absence of DHA, DhaL::ADP is phosphorylated back to DhaL::ATP by the phototransferase

321   system (PTS), thereby inhibiting the binding of the phosphorylated complex (DhaL::ATP) to

322   DhaR. This phosphorylation is mediated by DhaM, providing a phospho-histidine relay

323   between the PTS and DhaL::ADP. This double-check mechanism of binding and turnover

324   increases the selectivity such that the binding of nonphosphorylated compounds does not

325   induce the *dha* operon (Bächler et al., 2005).

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

327   The *glp* regulon comprises the genes encoding proteins required for the conversion of

328   glycerol to DHAP via sn-glycerol-3-phosphate. The five operons that constitute the *glp*

329   regulon are *glpFK*, *glpD* (anaerobic glycerol-3-phosphate dehydrogenase), *glp ACB* (aerobic

330   glycerol-3-phosphate dehydrogenase), *glpTQ* (glycerol-phosphate

331   permease/glycerophosphodiesterase), and *glpEGR*. The *glpF* gene encodes a cytoplasmic

332   membrane protein that facilitates the diffusion of glycerol into the cell. The *glpE* gene

333   encodes an acidic, cytoplasmic protein of 108 amino acids with a molecular weight of

334   12,082. The *glpG* gene encodes a basic, cytoplasmic membrane-associated protein of 276

335   amino acids with a molecular weight of 31,278 (Lin, 1976; Yang and Larson, 1998; Zeng et

336   al., 1996). The biochemical functions of GlpE and GlpG are unknown. The *glpR* gene

337   encodes the GlpR repressor protein. These five operons are located at three different positions

338   on the chromosome. Transcription of these operons is subject to multiple controls, including

339   catabolite repression mediated by cAMP-CRP and respiratory control mediated by the FNR

340   and ArcA/ArcB systems. Moreover, each of the operons is negatively controlled by a

341   repressor specific for the regulon (Lin, 1987; Zeng et al., 1996).

342   **2.3.1 GlpR and other regulatory proteins**

343   The GlpR repressor protein, predicted to contain 252 amino acids with a molecular

344   weight of 28,048, belongs to the DeoR bacterial regulatory factor super family. Although not

345   studied extensively, GlpR of *K. pneumonia* is expected to have the same biochemical

346   properties and regulatory functions as that of *E. coli*. GlpR negatively controls all the *glp*

347   operons by binding to operators that overlap or are close to the *glp* promoters. In case of *E.*

348   *coli*, glycerol-3-phosphate acts as an inducer and has a high affinity for GlpR ($K_d = 31$ μM)

349   (Lin, 1987; Weissenborn et al., 1992; Zeng et al., 1996). When complexed with glycerol-3-

350   phosphate, GlpR cannot bind to the operator sites, and repression is relieved. Glycerol-3-

351   phosphate is a product of GlpK and a substrate of GlpD/GlpABC; thus, the absence of GlpD

352   causes a substantial increase in the level of glycerol-3-phosphate, resulting in induction of the

353   *glp* regulon (Fig. 2). The GlpR protein exhibits differential binding affinity for the operators

354 of corresponding operons, as follows: *glpFK > glpD > glpTQ > glpACB*. The *glpFK* promoter

355 appears to be the strongest among the *glp* promoters. Therefore, the maximum strength of the

356 *glpFK* promoter combined with the high affinity of GlpK for glycerol (K_m = 10 μM) should

357 result in sufficient accumulation of the inducer, glycerol-3-phosphate, in the presence of

358 glycerol to relieve repression by GlpR. The relatively lower strength of the *glpD* promoter

359 along with the low affinity of the GlpD enzyme for glycerol-3-phosphate (K_m = 1 mM)

360 prevents excessive degradation of glycerol-3-phosphate that is needed for phospholipid

361 synthesis when the exogenous source of glycerol-3-phosphate becomes limiting, until after

362 accumulation of the inducer (Lin, 1987; Weissenborn et al., 1992). Recently, Jung et al.

363 (2014) investigated the role of GlpR in the aerobic production of 3-HP from glycerol in *E.*

364 *coli*. They found that elimination of this regulatory factor caused increased assimilation of

365 glycerol and higher production of 3-HP and suggested that the deletion of *glpR* led to the

366 upregulation of genes involved in glycerol transport and assimilation. *K. pneumoniae* has

367 several other regulatory genes in addition to *glpR*: the global regulatory genes *hdeB*, *hdeD,*

368 and *yfdX*, with unknown functions and *kvgS* and *kvgA*, encoding two proteins comprising a

369 two-component signal transduction system (Sun et al., 2003).

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

371 The glycerol facilitator GlpF is an integral membrane protein that forms aqueous

372 pores. The GlpF pores selectively allow passive transport of glycerol and other molecules

373 such as DHA and urea across the cytoplasmic membrane, but not water or ions (Heller et al.,

374 1980; Reizer et al., 1993). The GlpF protein contains six membrane-spanning helices that are

375 unique among prokaryotic transport proteins. These transmembrane helices are arranged in

376 two bundles. The GlpF protein may function as a homodimer with the two six-member

377 domains arranged in the membrane to form a channel (Weissenborn et al., 1992). *K.*

378 *pneumoniae* has two glycerol transporter genes, one next to *orfZ/dhaF* in the fermentative

379   *dha* regulon (see Fig. 4A) and the other in the respiratory *glpFK* operon. Both *glpF* genes

380   have a high similarity (85 %) to each other. When one but not both of the *glpF* genes is

381   disrupted, the *glpF* deletion mutants of *K. pneumoniae* can consume glycerol and convert it to

382   PDO at a high rate, similar to the wild type (unpublished data; Supplementary Fig. S1). This

383   observation indicates that both *glpF* genes encode active glycerol transporter proteins.

384   However, their presence is not essential for glycerol utilization in *K. pneumoniae* because the

385   double-deletion mutant for both facilitators can still uptake glycerol and convert it to PDO. In

386   *E. coli*, only one *glpF* gene is present in the *glpFK* operon; the GlpF protein shows a high

387   similarity (80.9%) to that (encoded from *glpFK*) of *K. pneumoniae* (Sun et al., 2003). The

388   importance of GlpF in *E. coli* also seems to be limited only at low levels of glycerol (<8

389   mM), as glycerol itself may pass through the membrane by diffusion at high concentrations

390   (Richey and Lin, 1972).

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

392   The presence of the *dha* and *glp* regulons affords *K. pneumoniae* metabolic flexibility.

393   The *dha* regulon enables fermentative utilization of glycerol, while the *glp* regulon enables

394   respiratory metabolism of glycerol. During fermentative metabolism of glycerol, redox

395   constraints force the production of PDO from glycerol, with a low ratio of carbon conversion

396   yield to cell biomass. Moreover, ATP is formed through substrate-level phosphorylation, and

397   NADH oxidation occurs through the formation of reduced metabolites. In the presence of

398   oxygen, the redox constraints forcing the production of PDO are relieved; therefore, the

399   carbon loss required for regeneration of NAD^+ is greatly reduced. As a consequence, glycerol

400   utilization, biomass synthesis, and even the formation of many metabolites increase (Durnin

401   et al., 2008). Interestingly, the fermentative route (*dha* regulon) is in operation under a wide

402   range of oxygen levels, from microaerobic to highly aerobic conditions, as evidenced by

403   PDO formation. For example, a high titer PDO (102.7 g/L) was produced under the condition

404   of 2.0 vvm aeration and even at 3.5 vvm, although the production was a little lowered in the

405   latter case (Oh et al., 2012a). These results suggest that the fermentative and respiratory

406   pathways might have evolved to complement each other for optimal control of cell growth

407   under a wide range of aeration conditions except the strictly anaerobic one.

408   **2.4 Carbon catabolite repression**

409   Carbon catabolite repression (CCR) refers to the suppression of the use of less-

410   preferred carbon sources, when a more preferred carbon, most prominently glucose, is

411   present. Cyclic AMP, cAMP receptor protein (CRP), and the enzyme $EIIA^{Glc}$, an

412   intermediate in the phosphorylation cascade of the PTS, are key players in the CCR of

413   enterobacteria. The $EIIA^{Glc}$, a cytosolic protein, exists in phosphorylated and

414   unphosphorylated forms. The phosphorylated form stimulates membrane-bound adenylate

415   cyclase, which catalyzes the formation of cAMP; the cAMP-CRP complex then induces

416   expression of the catabolic genes of less-preferred carbon sources. The unphosphorylated

417   form of $EIIA^{Glc}$ is also responsible for inhibiting the transport of less-preferred carbon

418   sources by inhibitory binding to permeases, causing so-called inducer exclusion (Eppler and

419   Boos, 1999; Eppler et al., 2002). Glycerol assimilation in *K. pneumoniae* is greatly inhibited

420   by CCR in the presence of glucose. This repression is related to not only low cAMP and

421   cAMP-CRP but also inhibition of GlpK (which functions mainly under aerobic conditions)

422   by fructose 1,6-bisphosphate, a metabolite produced from glucose. The unphosphorylated

423     $EIIA^{Glc}$ is also known to allosterically inhibit GlpK (Holtman et al., 2001). Anaerobic

424   glycerol metabolism mediated by the *dha* operons is also suppressed by the presence of

425   glucose. Enzyme-level inhibition similar to that of the aerobic GlpK has not been reported,

426   but we have observed that, in *K. pneumoniae*, transcription of the *dha* operons is greatly

427   reduced in the presence of glucose (Suman et al., 2017).

428   Interestingly, glycerol exerts catabolite repression on the assimilation of maltose in *E.*

429   *coli*. To exert catabolite repression, glycerol must be phosphorylated to glycerol-3-phosphate

430   (Hogema et al., 1998; Eppler et al. 2002). However, unlike other carbon sources, further

431   metabolism of glycerol is not required. According to Eppler and Boos (1999), glycerol-3-

432   phosphate reduces expression of MalT, a positive activator of all *mal* genes. The key players

433   in this repression are adenylate cyclase, EIIA^{Glc}, and CRP. The growth on maltose of the

434   mutants lacking EIIA^{Glc} or containing truncated adenylate cyclase was no longer repressed by

435   glycerol, and CRP-independent transcription of *malT* was also not influenced by glycerol

436   either. In addition, stimulation of adenylate cyclase by phosphorylated EIIA^{Glc} was controlled

437   by glycerol-3-phosphate. The mutation in adenylate cyclase relieved the repression caused by

438   glycerol-3-phosphate (Eppler et al., 2002). Although not investigated, we assume that the

439   glycerol repression on maltose catabolism also hold true in *K. pneumoniae*.

440   Glycerol also exerts catabolite repression on the assimilation of citrate in *K.*

441   *pneumoniae.* Citrate can be used as the sole carbon and energy source during anaerobic

442   growth of *K. pneumoniae*, but the presence of glycerol suppresses the expression of the

443   citrate fermentation genes. A reduced concentration of the cAMP–CRP complex has been

444   postulated to be the cause of this repression (Meyer et al., 2001). Further research is needed

445   to test the hypothesis.

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

447   During glycerol fermentation, *K. pneumoniae* produces many metabolites, including

448   PDO, BDO, and lactic acid. This section describes the biotechnological production of bulk

449   chemicals from glycerol in *K. pneumoniae*. 3-HP is not a natural product during the growth

450   of *K. pneumoniae* on glycerol. However, its production is possible using a minor

451   modification of the PDO synthetic pathway and is included here. The biochemical pathways

452   used for the synthesis of these chemicals are explained, and the metabolic engineering used to

453   improve their production is discussed.

454   **3.1 1,3-Propanediol**

455   PDO, also known as trimethylene glycol, is a promising platform chemical that has

456   two hydroxyl groups. With terephthalic acid, it can be co-polymerized to the novel co-

457   polymer polytrimethylene terephthalate (PTT). PDO also has applications in the food and

458   cosmetic industries. Currently, commercial PDO is mainly produced by recombinant *E. coli*

459   (Maervoet et al., 2011; Saxena et al., 2009). The global demand for PDO was 60.2 kilotons in

460   2012 and is predicted to reach approximately 150 kilotons by 2019. The market for PDO is

461   growing rapidly, from $157 million in 2012 with projections up to $560 million in 2019

462   (MarketsANDMarkets, 2012; Lee et al., 2015).

463   *K. pneumoniae* is one of the best native producers of PDO from glycerol. GDHt and

464   PDOR are two important enzymes for the conversion of glycerol to PDO (Fig. 3).

465   Physiologically, the synthesis of PDO from glycerol is essential for the anaerobic growth of

466   *K. pneumoniae*, as described previously. The production of PDO requires two cofactors,

467   coenzyme B_{12} and NAD(P)H. *K. pneumoniae* has a *de novo* pathway for the biosynthesis of

468   coenzyme B_{12} that comprises more than 20 genes. NAD(P)H is generated through the

469   oxidative metabolism of glycerol, during the production of cell biomass and/or oxidized

470   (pyruvate and acetate) or partially reduced (BDO, lactic acid) metabolites. The production

471   yield of PDO from glycerol varies depending on the cost of carbon for NAD(P)H production;

472   the lower the cost, the higher the yield of PDO from glycerol. NADPH can also be the

473   electron donor for the conversion of 3-HPA to PDO when YqhD is present. However, the

474   contribution of NADPH to PDO production in native *K. pneumoniae* seems to be limited

475   because the PP pathway, which is the main source of NADPH, is not active when glycerol is

476   the sole carbon source. Furthermore, *K. pneumoniae* has very low transhydrogenase activity

477   for the conversion of NADH to NADPH (Zhang and Xiu, 2009; Chen et al., 2011).

478   Several metabolic engineering strategies have been employed to improve PDO

479   production by *K. pneumonia* (Table 2). Amplification of the reductive pathway by

480   overexpressing DhaB, PDOR, or both has been attempted by many research groups. As

481   indicated previously, 3-HPA is highly toxic, and its accumulation is detrimental to cell

482   viability and toxic to PDO production. To reduce 3-HPA accumulation, overexpression of

483   *dhaT* has been attempted. In one study, 3-HPA accumulation was reduced by *dhaT*

484   overexpression, but no improvement in PDO production resulted (Hao et al., 2008).

485   Overexpression of both DhaT and DhaD increased PDO production (56.3%) in batch culture

486   but no increase in fed-batch culture (Chen et al., 2009). With another *K. pneumoniae* strain,

487   DSM 2026, the same experiments were repeated but also with no increase in PDO production

488   (Zheng et al., 2006). These results suggest that the reductive pathway in *K. pneumoniae* is

489   evolutionarily well established (perhaps in concert with the oxidative pathway) and that PDO

490   production is not limited by expression of the *dhaB* and *dhaT* genes of the reductive pathway.

491   However, in one study where the *E. coli yqhD* gene was highly overexpressed to give ~10-

492   fold higher PDOR activity (110 IU/mg total protein), a significant increase in PDO titer (25%;

493   67.6 g/L) and yield (from 0.53 to 0.62 mol/mol), along with less 3-HPA accumulation, was

494   achieved (Zhu et al., 2009). This observation suggests that in the recombinant strain, NADPH

495   can play an important role as a cofactor and that PDO production can be increased by

496   modifying the reductive pathway. It is also probable that the effect of DhaB and PDOR on

497   PDO production is highly dependent on the strain and/or culture conditions adopted.

498   Lactic acid, BDO, ethanol, and acetic acid are the major byproducts of PDO synthesis

499   in *K. pneumoniae*. Inactivation of the metabolic pathways that give rise to these products has

500   resulted in increased PDO synthesis. In particular, deletion of the *ldhA* gene encoding lactate

501   dehydrogenase has been highly beneficial. For example, by disrupting the *ldhA* gene, Xu et al.

502   (2009b) significantly increased PDO production without lactic acid production. They

503   observed increases in the PDO titer (95.4–102.1 g/L), conversion yield (0.48–0.52 mol/mol),

504   and productivity (1.98–2.13 g/L h; Table 2). Similarly, Oh et al. (2012a) achieved the high

505   PDO titer of 102.7 g/L by deleting *ldhA* in their own *K. pneumoniae* isolate. When lactic acid

506   production was eliminated, more ethanol and succinic acid were generated from the pyruvate

507   node. Reduction of these compounds has also been attempted by deleting *adhE* (encoding

508   alcohol dehydrogenase) and *frdA* (encoding fumarate reductase). Although the production of

509   ethanol and succinate was substantially reduced in the triple mutant (Δ*ldhA*Δ*adhE*Δ*frdA*),

510   PDO production was only marginally increased compared with that of the single Δ*ldhA*

511   mutant. The failure of the triple mutant (Δ*ldhA*Δ*adhE*Δ*frdA*) to increase PDO production was

512   mainly due to an increase in BDO production. Disruption of the BDO pathway encoded by

513   the *bud* operon (*budA*, *budB*, and *budC*) has also been studied (Fig. 5). Although partially

514   effective, individual inactivation of each of the three genes did not successfully decrease

515   BDO production (Oh et al., 2012a; Wu et al., 2013). Deletion of the entire *bud* operon

516   completely eliminated BDO synthesis (Kumar et al., 2016), but seriously hampered cell

517   growth and glycerol consumption. Consequently, no increase in PDO production was resulted.

518   Wu et al. (2013) attempted to decrease BDO production while increasing PDO production by

519   inserting the *fdh* gene from *Candida boidinii* (NADH-forming formate dehydrogenase) into

520   the *budC* locus. This protocol increased the PDO titer (62.3–72.2 g/L) and yield (0.47–0.57

521   mol/mol) and decreased the production of BDO by 52.2% and formic acid by 73.4%.

522   Acetic acid is one of the most toxic metabolites, accumulating in large quantities

523   during glycerol fermentation by *K. pneumonaie* (Celińska, 2012). The toxic effects of acetate

524   is realized even at the low concentration of 20 mM. Physiologically, acetate is a preferred

525   metabolite for many *Enterobacter* sp. because its production is accompanied by ATP

526 generation. In a typical bioreactor run for PDO production by *K. pneumoniae*, the

527 accumulation of acetic acid to >300 mM by the end is not uncommon, which often

528 completely stops glycerol fermentation. Acetate is produced by Pta-Ack and PoxB when

529 pyruvate formation is faster than its consumption, which is so-called 'overflow metabolism'

530 (Fig. 6) (De Mey et al., 2007). In *K. pneumoniae*, the contribution of the *poxB* gene is not

531 significant and its deletion does not much affect acetate production. In comparison, deletion

532 of the *pta-ack* genes greatly reduces cell growth and results in high accumulation of pyruvate

533 and pyruvate-based metabolites (Ko et al., 2017). Extensive studies to reduce or eliminate

534 acetate production have been conducted in *E. coli*. Compared to *poxB*, *ackA* and/or *pta* were

535 much more significant in reducing acetate production. However, deletion of the latter genes

536 was not beneficial because the glycolytic flux and cell yield were seriously reduced and the

537 accumulation of other byproducts such as pyruvate, lactate, and formate was greatly

538 increased (Causey et al., 2004; De Mey et al., 2007). Several indirect approaches to

539 decreasing pyruvate/acetate accumulation were investigated: stimulation of the anaplerotic

540 pathway by overexpression of pyruvate carboxylase, PEP carboxylase, or PEP carboxykinase

541 (Abdel-Hamid et al., 2001; Causey et al., 2004; Chao and Liao, 1993; Sanchez et al., 2005);

542 overexpression of acetyl-CoA synthetase (ACS); increase of the TCA cycle throughput by

543 deleting the transcriptional repressor *arcA* gene; and stimulation of the glyoxylate shunt by

544 deleting the transcriptional repressor *iclR* gene (De Mey et al., 2007; Jeong et al., 2004; Lin et

545 al., 2006). Moreover, the use of a mutant pyruvate dehydrogenase which is less sensitive to

546 inhibition by NADH has also been examined (Kim et al., 2008). In *E. coli*, these approaches

547 proved to be effective to varying extents under properly selected culture conditions. However,

548 they have not yet been fully investigated in *K. pneumoniae*.

549   To eliminate byproduct formation, reduction of glycerol flux through the oxidative

550 pathway at the glycerol node has also been attempted. The deletion of glycerol

551 dehydrogenase (*dhaD*) and/or dihydroxyacetone kinase (*dhaK*) resulted in decrease of the

552 oxidative flux and increase of the conversion yield of glycerol to PDO. In addition,

553 production of the byproducts lactate, ethanol, and succinate but not acetate decreased

554 significantly, even without blocking the pathways leading to these byproducts (Seo et al.

555 2009; Horng et al. 2010). However, cell growth and glycerol consumption rate were also

556 reduced. Interestingly, in these mutant strains, deletion of *dhaT* has almost no effect on PDO

557 production. In the absence of *dhaT*, expression of an HOR, highly homologous to YqhD, was

558 upregulated (see section 2.2.4).

559 The theoretical maximum yields of PDO on glycerol are 0.875 and 0.844 mol/mol

560 under aerobic and anaerobic conditions, respectively (Celińska, 2012). However,

561 experimentally, the yields are reported between 0.35 and 0.65 mol/mol (Saxena et al., 2009;

562 Sun et al., 2008). The low yield is mainly attributed to the accumulation of unnecessary

563 byproducts and inefficient metabolism for generating reducing equivalents (NADH) and ATP.

564 If acetyl-CoA is fully oxidized through the TCA cycle (without byproducts formation) and

565 the activity of the electron transport chain (ETC) oxidizing NADH is properly controlled, a

566 significant increase in PDO yield is expected. Attaining this goal requires extensive pathway

567 engineering of cellular metabolism. In bioreactor operation, the aeration rate should be

568 optimized. Oxygen regulates gene expression and the enzymatic reactions of glycerol

569 metabolism and determines the diversion of DHA to the PP pathway. Regeneration of NADH

570 by the TCA cycle and its oxidation by ETC are also controlled by oxygen levels.

571 **3.2 3-Hydroxypropionic acid**

572 As with PDO, 3-HP is an important platform chemical and has been selected by the

573 US Department of Energy (DOE) as one of the top 12 chemicals obtainable from biomass. 3-

574 HP is a C3 bifunctional molecule and a structural isomer of lactic acid (Werpy and Petersen,

575 2004). 3-HP can be converted into a variety of useful chemicals such as acrylic acid and acryl

576   amide (Pina et al., 2011; Kumar et al., 2013a). 3-HP can be synthesized by chemical routes

577   from acrylic acid, 3-propiolactone, 3-hydroxypropionitrile, allyl alcohol, vinyl acetate, and

578   PDO. However, none of the chemical processes is commercially feasible at present due to the

579   high cost of the starting materials, toxicity of intermediates, and/or the environmental

580   incompatibility of the processes (Jiang et al., 2009; Pina et al., 2011; Kumar et al., 2013a).

581   Many microorganisms, including both prokaryotes and eukaryotes, can naturally synthesize

582   3-HP as either an intermediate or end product through a range of metabolic pathways.

583   However, the production of 3-HP by these native microorganisms is too low to be

584   commercially meaningful (Jiang et al., 2009; Kumar et al., 2013a).

585   In studies pursuing commercial production of 3-HP, glycerol is first converted to 3-

586   HPA by GDHt. 3-HPA then is converted to 3-HP via the coenzyme A (CoA)-dependent or

587   CoA-independent pathway (Fig. 3). In the CoA-independent pathway, 3-HPA is oxidized to

588   3-HP by aldehyde dehydrogenase (ALDH); in the CoA-dependent pathway, 3-HPA is

589   converted to 3-HP via 3-HP-CoA and 3-HP-phosphate. In *K. pneumoniae*, both the CoA-

590   dependent and CoA-independent pathways are present. However, the titer of 3-HP produced

591   by the CoA-dependent pathway is low as <5.0 g/L (Luo et al., 2011 and 2012). High

592   production of 3-HP requires the disruption of oxidoreductases (e.g., DhaT, YqhD) and the

593   overexpression of a proper aldehyde dehydrogenase (ALDH). Ashok et al. (2013a) developed

594   a recombinant *K. pneumoniae* in which *dhaT* and *yqhD* were deleted, and the homologous

595   *puuC* gene encoding the NAD^+-dependent γ-glutamyl-γ-aminobutyraldehyde dehydrogenase

596   was overexpressed. The recombinant *K. pneumoniae* produced 3.8 g/L 3-HP in 12 h of flask

597   culture, but only under appropriate aeration (i.e., microaerobic) conditions. Under anaerobic

598   conditions, PDO (instead of 3-HP) was obtained as the main product, even though two

599   oxidoreductases were disrupted and *puuC* was highly overexpressed. In contrast, highly

600   aerobic conditions produced a high cell mass without much accumulation of either 3-HP or

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

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

626   h fed-batch bioreactor culture (Ashok et al., 2013b) (Table 3). However, disadvantages were

627   noted, including the requirement for large amounts of nitrate and the toxic effect of nitrite

628   generated as a reduced intermediate of nitrate. In a recent study, Li et al. (2016) optimized a

629   promoter for ALDH expression, culture conditions, and metabolic flux to achieve high-level

630   production of 3-HP in their *K. pneumoniae* isolate. One recombinant strain, *K. pneumoniae*

631   (pTAC-*puuC*) expressing *puuC* under the IPTG-inducible *tac* promoter, produced 73.4 g/L 3-

632   HP in a bioreactor with a molar yield of glycerol of 0.52 and productivity of 1.53 g/L h.

633   Further, elimination of the *ldh1*, *ldh2*, and *pta* genes elevated the titer and molar yield to 83.8

634   g/L 3-HP and 0.54 mol/mol, respectively, with decreased cell growth and productivity. This

635   titer of 3-HP is the highest produced by *K. pneumoniae* to date.

636   As another approach to addressing the problem associated with NAD^+ regeneration

637   and coenzyme B_{12} synthesis in 3-HP production, co-production of 3-HP and PDO has been

638   investigated. The simultaneous production of 3-HP and PDO can eliminate the dependency

639   on oxidative metabolism of glycerol (and electron transport chain), as the cofactor required

640   for 3-HP production is regenerated by PDO production or vice versa. Furthermore, the

641   problems associated with the expression of genes for the assimilation of vitamin B_{12} and

642   glycerol can be alleviated substantially because co-production can be performed under

643   anaerobic or microaerobic conditions (Kumar et al., 2012, 2013b). Ashok et al. (2011)

644   developed a recombinant strain of *K. pneumoniae* DSMZ by overexpressing ALDH and

645   deleting *dhaT*. With *dhaT* intact, PDOR activity was too high compared with that of ALDH,

646   even with overexpression of the latter enzyme from a multicopy plasmid. The recombinant *K.*

647   *pneumoniae* DSMZ accumulated 16.0 g/L 3-HP and 16.8 g/L PDO in 24 h, and the

648   cumulative yield of these two metabolites on glycerol was 51% (Table 3). Huang et al. (2012)

649   studied the same co-production using their *K. pneumoniae* isolate overexpressing ALDH and

650   obtained 24.4 3-HP and 49.9 g/L PDO, respectively, after 24 h under anaerobic conditions.

651　The cumulative molar yield of the two metabolites reached 0.61 (0.18 for 3-HP and 0.43 for

652　PDO). In a following study, they showed that the maximum titer of 48.9 g/L 3-HP (along

653　with 25.3 g/L PDO) was achieved at 1.5 vvm, while the highest concentration of 38.4 g/L

654　PDO (along with 16.6 g/L 3-HP) was obtained at 0.1 vvm (Huang et al., 2013a).

655　　　　During 3-HP production from glycerol by *K. pneumoniae*, the accumulation of such

656　byproducts as lactic acid, ethanol, acetic acid, formic acid, and 2,3-BDO is a serious

657　consideration, as in the case of 1,3-PDO production (Ashok et al., 2013a,b; Li et al., 2016).

658　Deletion of *ldhA* reduces lactic acid formation and increases the 3-HP yield. However,

659　deletion of other genes such as *adhE*, *pfl*, *budBAC*, and *pta-ack* had little desired effect and

660　seriously hampered cell growth and glycerol assimilation (unpublished data). Even with the

661　co-production of 3-HP and 1,3-PDO, where oxidative glycerol assimilation was greatly

662　reduced, accumulation of these byproducts was a serious drawback (Ko et al., 2017). To

663　prevent the formation of lactic acid along with other byproducts, Kumar et al. (2013b)

664　employed resting cells of recombinant *K. pneumoniae* J2B overexpressing ALDH and devoid

665　of lactate dehydrogenase (*ldhA*). For this strain, final titers of 3-HP and PDO of 22.7 and 23.5

666　g/L, respectively, were obtained without lactic acid accumulation. The cumulative product

667　yield increased to 0.77.

668　　　　In addition to *K. pneumoniae*, *E. coli* has been extensively studied for 3-HP

669　production from glycerol. Several successful results have been reported by Samsung Ltd. Co.

670　Korea. One study (Chu et al., 2015) used an engineered strain (*E. coli* W3110 *ΔackA-pta*

671　*ΔyqhD_dhaB_mutant gabD4*) harboring an active ALDH mutant (designated as GabD4) from

672　*Cupriavidus necator*, producing 71.9 g/L 3-HP with a productivity of 1.8 g/L h. However,

673　unlike *K. pneumoniae*, *E. coli* does not synthesize vitamin B_{12} naturally, and it was necessary

674　to add the coenzyme externally. Furthermore, a significant amount of glucose for cell growth

675　was required because for *E. coli*, glycerol is a less-preferred carbon source than glucose.

676   Consequently, the researchers at Samsung initially grew cells to a high density (40–300

677   OD_{600}) on glucose and then produced 3-HP by feeding glycerol (Chu et al., 2015; Kim et al.,

678   2014a). In comparison, *K. pneumoniae* can produce a high concentration of 3-HP growing

679   purely on glycerol at a much lower cell concentration (OD_{600}, <20) (Huang et al., 2013a,b; Li

680   et al., 2016). Together, the use of glucose for cell proliferation, exogenous addition of

681   expensive cofactor vitamin B_{12}, and low cumulative yield of 3-HP (on glucose plus glycerol)

682   increase the production cost. Thus, *K. pneumoniae* can be considered a better biocatalyst than

683   *E. coli*. However, for the commercial production of 3-HP, many other factors are important,

684   including biosafety, process stability, and downstream processing. Further studies to assess

685   the potential of these strains as hosts for 3-HP production are needed.

686   **3.3 2,3-Butanediol**

687   BDO has many applications in the pharmaceutical, biomedical, and other chemical

688   industries for the production of printing inks, perfumes, fumigants, spandex, moistening and

689   softening agents, and plasticizers (e.g., cellulose nitrate, polyvinyl chloride, polyacrylates)

690   (Celińska and Grajek, 2009; Ji et al., 2011). BDO can also be used as an antifreezing agent

691   and octane booster for petrol as is or can be converted to useful derivatives such as 1,3-

692   butadiene, and diacetyl and methyl ethyl ketone.

693   Unlike 3-HP and PDO, BDO is a product of oxidative metabolism. Of the three

694   stereoisomers of BDO, *K. pneumoniae* mainly produces the *meso* form (Ji et al., 2011). BDO

695   is synthesized from pyruvate via α-acetolactate and acetoin. The C5 intermediate, α-

696   acetolactate, is formed from two molecules of pyruvate by self-condensation catalyzed by α-

697   acetolactate synthase (ALS; *budB*). α-Acetolactate is decarboxylated to acetoin by α-

698   acetolactate decarboxylase (*budA*), and acetoin is reduced to BDO by 2,3-butanediol

699   dehydrogenase/acetoin reductase (*budC*) using NADH as a reductant. In the presence of

700   oxygen, α-acetolactate is spontaneously decarboxylated to diacetyl, further reduced to acetoin

by action of diacetyl reductase, and then reduced to BDO. α-Acetolactate is also the precursor

of branched-chain amino acids (valine, leucine, and isoleucine); thus, elimination of the *bud*

operon can arrest *K. pneumoniae* growth when cultured on glycerol minimal medium (Kumar

et al., 2016). In the anaerobic process, one NADH molecule is required for the conversion of

two pyruvates to one BDO, while the aerobic process requires two NADH (Fig. 5). The

enzyme DhaD may also contribute to BDO formation (see Section 2.2.1). In the BDO

pathway itself, the conversion of two pyruvates to BDO regulates or is regulated by the

intracellular $NADH/NAD^+$ ratio, similar to other fermentative pathways. Another important

role of the BDO pathway is to reduce intracellular acidification by converting acids to the

neutral metabolite, BDO (Celińska and Grajek, 2009; Ji et al., 2011).

Although BDO is one of the main byproducts of glycerol fermentation by *K.*

pneumoniae (Petrov and Petrova, 2009; Durgapal et al. 2014), its production from glycerol

has not been studied extensively. In few studies using glycerol as the carbon source, Petrov

and Petrova (2009) focused on the effects of pH and aeration with their *K. pneumoniae*

isolate G31. BDO production was greatest at alkaline initial pH when tested at a pH range of

5–8. Without pH control a sharp pH drop occurred initially due to the production of organic

acids, and this pH drop triggered the synthesis of BDO, which is considered the only non-

inhibitory compound produced by the oxidative metabolism of glycerol. Once neutral pH is

restored with the rising concentration of BDO, catabolism shifted toward the production of

ATP-generating acetic acid. The amount of BDO generated in each cycle (pH drop and

neutralization) was dependent on the extent of the pH drop. In one study, a BDO

concentration of 49.8 g/L was reached when the initial pH was set at 8.0 and no pH control

was given during the rest of fermentation. PDO was the major byproduct (11.6 g/L) (Table 4)

and its production decreased by exclusion of Co^{2+}, an essential component of coenzyme B_{12}.

They also found that intensive aeration led to a significant increase in BDO production; an

726 increase in aeration rate from 1.1 to 2.2 vvm enhanced the yield from 0.38 to 0.46 mol/mol

727 and productivity from 0.24 to 0.36 g/L h. BDO production was also increased by forced pH

728 fluctuations of the culture medium with discrete ΔpH values (1.0, 2.0, and 3.0) at

729 predetermined time intervals (6, 12, and 24 h). The highest BDO concentration (70.0 g/L)

730 was produced by increasing the pH by one unit (ΔpH = 1.0) every 12 h (Petrov and Petrova,

731 2010). The mechanism behind the effect of this peculiar pH change on BDO production has

732 not been elucidated. Although BDO is readily produced from glycerol by *K. pneumoniae*, the

733 volumetric productivity is low due to the long fermentation time (generally >150 h). Huang et

734 al. (2013b) isolated two *Klebsiella* strains that produce BDO plus acetoin with a total yield of

735 0.44–0.45 (g/g). One isolate utilized 64.5 g/L glycerol in 78 h and produced 24.9 g/L BDO,

736 3.9 g/L acetoin, and 3.2 g/L ethanol. Again, the volumetric productivity was not high.

737   Metabolic engineering efforts with *K. pneumoniae* to improve BDO production from

738 glycerol are limited. Using glucose as carbon source, Kim et al. (2014b) reported that

739 overexpression of the BDO pathway (*budA*, *budB*) in the *ldhA* deficient *K. pneumoniae* strain

740 KCTC2242 improved BDO production and resulted in 90 g/L BDO, with a productivity of

741 2.75 g/L. h. Because both glucose and glycerol are converted through the common

742 intermediate pyruvate, BDO production from glycerol is also expected to increase by

743 amplification of the *bud* operon. Further work with glycerol as carbon source is required to

744 prove this effect. Recently, co-production of BDO and PDO from glycerol has also been

745 attempted (Park et al., 2017). In glycerol fermentation, both alcohols always appear

746 simultaneously and co-production seems to be beneficial for cells to maintain redox balance

747 during anaerobic growth. Park et al. (2017) found that by using the *K. pneumoniae* mutant

748 deficient of *ldhA* and *mdh*, 50.1 g/L BDO was produced along with 63.8 g/L PDO in 75 h.

749 The ratio of the two alcohols varied according to aeration; with increasing aeration, the ratio

750 of [BDO]/[PDO] increased as observed by Petrov and Petrova (2010). In the 5,000 L pilot-

31

751 scale fed-batch fermentation using crude glycerol, they could produce 114 g/L diols (70 g/L

752 PDO and 44 g/L BDO) with the yield of 0.60 diols/glycerol (g/g) and a productivity of 2.2

753 g/L. h of diols. If separation is not an issue, co-production of the two diols can be a good

754 option.

755 **3.4 Lactic acid**

756 Lactic acid has applications in the food, chemical, cosmetic, and pharmaceutical

757 industries. Lactic acid is a monomer that can be polymerized to yield the biodegradable

758 plastics polylactic acid (PLA) and poly(3-hydroxybutyrate-*co*-lactate). Two optical isomers

759 of lactic acid, D- and L-lactic acid, are produced in racemic mixtures depending on the

760 chemical route used. Microbial fermentation can yield optically pure isomers. Currently,

761 nearly all commercial lactic acid comes from microbial fermentation (Abdel-Rahman et al.,

762 2013; Abdel-Rahman and Sonomoto, 2016).

763 Because of the availability of highly efficient lactic-acid–producing microbes derived

764 from *E. coli*, lactic acid bacteria, and even acid-tolerant yeasts (Abdel-Rahman et al., 2013),

765 *K pneumoniae* has received little attention as a lactic acid producer. However, as previously

766 described, lactic acid is a major byproduct of glycerol fermentation by *K. pneumoniae*, and its

767 potential as a producer is high. This bacterium contains metabolic pathways for the

768 production of both L- and D-isomers (Fig. 7). They are synthesized through the pyruvate

769 and/or methylglyoxal pathways in *K. pneumoniae* (Ashok et al., 2011). When produced from

770 pyruvate, lactate dehydrogenase (*ldhA*) reduces pyruvate using NADH as reductant. In the

771 methylglyoxal route, DHAP is converted to methylglyoxal, which can be transformed to both

772 D- and L-lactic acid. For the production of L-lactic acid, methylglyoxal is reduced to L-

773 lactaldehyde, which is subsequently oxidized to L-lactic acid. Using glutathione, D-lactic acid

774 is obtained through simultaneous aldehyde group oxidation and keto group reduction of

775 methylglyoxal (Booth et al., 2003; Booth, 2005). The methylglyoxal route has several

776   disadvantages. Methylglyoxal is highly toxic, and its accumulation can severely impair other

777   metabolisms and even lead to cell death. Second, the route is energetically inefficient, with an

778   ATP yield of −1. The L- or D-lactic acid product can be converted back into pyruvate by

779   respiratory lactate dehydrogenase (Mazumdar et al., 2010, 2013).

780   Several studies showing the potential of *K. pneumoniae* as a lactic acid producer are

781   available (Table 4). For example, *K. pneumoniae* DSMZ is reported to accumulate 22.7 g/L

782   lactic acid in 24 h during co-production of 3-HP and PDO from glycerol. The titer of lactic

783   acid was more than that of any of the targeted products, and the yield of lactic acid on

784   glycerol alone was 0.31 (Ashok et al., 2011). Similarly, fed-batch cultivation of *K.*

785   *pneumoniae* BLh-1 under oxygen-limited conditions gave lactic acid as the main product,

786   outcompeting PDO; 59 g/L lactic acid was produced in 40 h when crude glycerol from

787   biodiesel synthesis without any purification was used (Rossi et al., 2013). Durgapal et al.

788   (2014) also found that *K. pneumoniae* J2B and DSMZ strains produced lactic acid at 37.0 and

789   47.8 g/L, respectively (Table 4); in both strains, lactic acid levels surpassed those of the target

790   product PDO. Recently, Feng et al. (2014) engineered *K. pneumoniae* ATCC25955 by

791   overexpressing *ldhA* and deleting *dhaT* and *yqhD* (recall that these two genes are mainly

792   responsible for PDO production). They found that the recombinant produced 142.1 g/L of

793   optically pure D-lactic acid from glycerol in fed-batch cultivation under microaerobic

794   conditions (2.5 vvm and 400 rpm). This result is the highest lactic acid concentration

795   produced from glycerol reported to date. *E. coli* has also been engineered for the production

796   of D- and L-lactate from glycerol (Mazumdar et al., 2010, 2013). For diverting glycerol flux

797   toward L-lactic acid in *E. coli*, the chromosomal copy of D-lactate dehydrogenase was

798   replaced with *Streptococcus bovis* L-lactate dehydrogenase (Mazumdar et al., 2013).

799   Moreover, gene encoding *lldD* (respiratory L-lactate dehydrogenase) was inactivated to

800   prevent the consumption of L-lactic acid, and the methylglyoxal route (Δ*mgsA*) was blocked

801 to prevent production of toxic methylglyoxal and formation of racemic mixtures of D- and L-

802 lactic acid (Fig. 7). Furthermore, the respiratory route for glycerol assimilation was

803 overexpressed (*glpK* and *glpD*) to improve the ATP yield of the metabolic pathway through

804 transfer of electrons from glycerol-3-phosphate to oxygen (i.e., oxidative phosphorylation).

805 The engineered *E. coli* strain (Δ*pflB* Δ*pta* Δ*adhE* Δ*frdA* Δ*mgsA* Δ*lldD* Δ*ldhA*::*ldhA_glpK-*

806 *_glpD*) produced 50 g/L of L-lactate from 56 g/L of crude glycerol, at a yield 93% of the

807 theoretical maximum and with high optical (99.9%) and chemical (97%) purity (Mazumdar et

808 al., 2013). The approaches proven successful in *E. coli* should be applicable to *K.*

809 *pneumoniae*. These studies, along with the results by Feng et al. (2014), suggest that *K.*

810 *pneumoniae* is a good host for the production of D- and L-isomers of lactic acid, with high

811 optical purity, from glycerol.

812 **3.5 Ethanol and other metabolites**

813 *K. pneumoniae* has also been studied for the production of ethanol, succinic acid, 2-

814 ketogluconic acid, catechol, *cis,cis*-muconic acid and 2-butanol although its potential for such

815 production has not been fully explored (Cheng et al., 2013; Jung et al., 2015; Oh et al., 2014;

816 Wang et al. 2015; Wei et al., 2013). Of these chemicals, ethanol production has been

817 relatively well studied. Ethanol is a renewable energy source and is widely used as a fuel

818 additive for partial gasoline replacement. Currently, commercial ethanol is produced from the

819 fermentation of sugar and starch. However, glycerol is one of the cheapest substrates for

820 ethanol production, estimated as nearly 40% cheaper than sugar-based production (Yazdani

821 and Gonzalez, 2007). This factor has encouraged the development of microbial strains for

822 fermentative conversion of glycerol to ethanol. The metabolic route by which ethanol is

823 synthesized from glycerol is shown in Fig. 6. Oh et al. (2011, 2012b) constructed a mutant

824 strain of *K. pneumoniae* (termed GEM167) through γ-irradiation. PDO synthesis in this

825 mutant strain was lower than that of wild type (0.2 vs. 7.93 g/L, respectively), while ethanol

826   accumulation was higher (8.6 vs. 1.1 g/L, respectively). The mechanism underlying these

827   changes remains unclear. Introduction of the pyruvate decarboxylase (*pdc*) and aldehyde

828   dehydrogenase (*adhII*) genes into mutant strains lacking lactate dehydrogenase (*ldhA*)

829   resulted in the production of 31.0 g/L (673.9 mM) ethanol, with a yield of 0.89 (mol/mol) and

830   productivity of 1.2 g/L h (Oh et al., 2012b), the highest level of ethanol production from

831   glycerol reported to date. More extensive studies including estimation of the maximum

832   achievable titer and microbial tolerance to high-concentration ethanol, both of which are key

833   factors for commercialization, are required to assess the potential to produce ethanol from

834   glycerol by the use of *K. pneumoniae*.

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

836   The design of microbial cell factories is gaining unprecedented momentum as

837   metabolic engineering is progressively aided by advances in synthetic biology and multi-

838   omics analyses. Several bio-based chemicals have already been launched successfully in the

839   marketplace, and others are in the pipeline. For a bulk chemical to be considered for

840   commercial production, the production process should achieve a product concentration of 100

841   g/L, carbon yield of 0.50 (g/g), and volumetric productivity of 2.0 g/L/h. The aforementioned

842   products produced by *K. pneumoniae* from glycerol are no exception. Challenges to the use

843   of *K. pneumoniae* as a microbial cell factory using glycerol as a carbon source are described.

844   **4.1 Pathogenicity**

845   The pathogenicity of *K. pneumoniae* is one of the major obstacles to its commercial

846   application. *K. pneumoniae* is a Bio-safety Level 2 microorganism and causes nosocomial

847   and urinary tract infections. Moreover, capsulated *K. pneumoniae* cells are difficult to

848   separate from culture broth, complicating the downstream processing. The biotechnological

849   application of this bacterium will require attenuation of its pathogenicity and reduction of

850   biosafety concerns. *K. pneumoniae* produces a number of virulence factors that contribute to

851 its pathogenicity, including lipopolysaccharide (LPS), capsular antigens, fimbrial adhesins,

852 siderophores, and O antigens (Albertí et al., 1993; Fang et al., 2004; Podschun and Ullmann

853 1998). The mechanism by which this bacterium causes disease is still obscure because most

854 studies have been conducted on a limited number of virulence factors (El Fertas-Aissani et al.,

855 2013).

856   In the last decade, efforts have been made to overcome the pathogenicity of *K.*

857 *pneumoniae* (Shrivastav et al., 2013), suggesting hope for the generation of nonpathogenic *K.*

858 *pneumoniae*. The two major pathogenic determinants in *K. pneumoniae* are the capsular

859 polysaccharides (CPS), which cover the entire bacterial surface, and LPS, expressed on the

860 outer bacterial membrane. LPS consists of lipid A, a core oligosaccharide, and an O-antigenic

861 polysaccharide. In a highly virulent strain of *K. pneumoniae*, mutations in genes involved in

862 LPS biosynthesis (*waaC*, *waaF*, and *wabG*) resulted in a decrease in bacterial colonization

863 and virulence (Izquierdo et al., 2003; Jung et al., 2013). Mutant strains devoid of the outer

864 core LPS were also found to be deficient in the "cell-attached" capsular polysaccharides

865 covering the bacterial surface. These mutations abolished the highly virulent characteristics

866 of pathogenic *K. pneumoniae* when tested in different animal models. Furthermore, these

867 mutants were more sensitive to several hydrophobic compounds than were wild-type strains.

868 Reintroduction of the *waaC*, *waaF*, and *wabG* genes into *K. pneumoniae* rescued the

869 pathogenic properties, confirming the role of these genes in pathogenicity.

870   Another important study was conducted by Lin et al. (2012), in which eight highly

871 conserved residues of the MagA protein (G308, G310, G334, G337, R290, P305, H323, and

872 N324) encoded by the mucoviscosity-associated gene A (*magA*) of *K. pneumoniae* were

873 subjected to site-directed mutagenesis. The *magA* gene contributes to the biosynthesis of K1

874 capsular polysaccharide (CPS), which correlates with pathogenic phenotypes including

875 mucoviscosity, serum and phagocytosis resistance, and virulence. Alanine substitutions at

876 R290A or H323A abolished MagA function, with annihilation of CPS production, serum

877 resistance, and virulence. Some *K. pneumoniae* strains showing less pathogenicity were also

878 isolated. For example, *K. pneumoniae* strain J2B produced less LPS and demonstrated a high

879 sensitivity to many antibiotics and lower pathogenicity. Moreover, its biomass was readily

880 separated from fermentation broth by centrifugation (Arasu et al., 2011). Future studies

881 should include further engineering of these less pathogenic strains to make them completely

882 nonpathogenic, followed by careful evaluation of their toxicity.

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 dehydrognease (*icd*), fumarase (*fumA*),

905   and malate dehydrogenase (*mdh*), in *K. pneumoniae* are significantly less transcribed than

906   those in *E. coli* (unpublished data). Furthermore, the activity of isocitrate dehydrogenase was

907   more than sevenfold lower in *K. pneumoniae* than in *E. coli*. If the TCA cycle is inefficient,

908   achieving a high cell density, which is essential for improving the productivity of target

909   metabolites, is difficult. Moreover, achieving high-yield generation of NADH with little

910   consumption of glycerol carbon is difficult. Furthermore, the overflow metabolism that

911   produces highly toxic acetate becomes more serious. These problems have been well

912   documented in PDO production. In a recent study, Kumar et al. (2016) attempted to increase

913   PDO production yield by blocking the lactate and BDO pathways, while diverting carbon

914   flux through the TCA cycle for efficient regeneration of NADH. However, this effort resulted

915   in the detrimental accumulation of pyruvate, acetate, and ethanol, causing low NADH

916   regeneration and low PDO production. Neither increased aeration nor the addition of good

917   nitrogen sources (complex nitrogen sources) alleviated the accumulation of these

918   intermediates or activated the TCA cycle. Furthermore, the accumulation of pyruvate quickly

919   terminated glycerol assimilation. *K. pneumoniae* has a well-established BDO production

920   pathway that likely compensates for its inefficient TCA cycle, avoiding the overflow

921   metabolism and carbon traffic at the pyruvate node. If this is the case, co-production of BDO

922   along with PDO or 3-HP, rather than single production of PDO or 3-HP, should be more

923   appropriate, which suits the nature of *K. pneumoniae*. In fact, when BDO and PDO were co-

924   produced, more PDO was generated than during PDO production alone. More studies are

925   required to gain a better understanding of glycerol metabolism, the slow operation of the

926 TCA cycle in *K. pneumoniae*, and its effect on the selection of target products and/or

927 development of suitable strains and processes. Otherwise, it will be difficult to exploit this

928 potential biocatalyst for commercial manufacturing of valuable chemicals.

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

930   A general but major challenge to the use of microbial cell factories for producing bulk

931 chemicals is the toxic effect of target products and metabolic intermediates at high

932 concentrations. In particular, during the production of PDO or 3-HP from glycerol, the highly

933 toxic intermediate 3-HPA is inevitably generated. Aldehydes generally damage DNA,

934 inactivate enzymes, and inhibit DNA synthesis. 3-HPA causes irreversible cessation of

935 metabolic activity and cellular growth, even at very low concentrations of 15–30 mM (Hao et

936 al., 2008; Zheng et al., 2008). Native *K. pneumoniae* does not accumulate 3-HPA at toxic

937 levels during PDO production, but in engineered strains that overexpress DhaB, high 3-HPA

938 accumulation is often experienced. To avoid 3-HPA accumulation, enzymes that consume 3-

939 HPA, ALDH (for 3-HP production), and PDOR (for PDO production) are expressed at

940 sufficiently (sometimes excessively) high levels compared with that of DhaB (Lim et al.,

941 2016). Intermediates such as methylglyoxal, DHA, DHAP, and glyceraldehyde-3-phosphate

942 are also known to be toxic.

943   Organic acids and alcohols are toxic at high concentrations. Generally, organic acids

944 are more toxic than their corresponding alcohols because they disturb the intracellular pH and

945 have anion-specific effects on metabolism (Chun et al., 2014; Warnecke and Gill, 2005).

946 When accumulated in cell membranes, organic compounds damage membrane integrity and

947 inhibit the activity of membrane-bound enzymes. In *K. pneumoniae*, cell growth and glycerol

948 assimilation are severely inhibited by high concentrations of PDO or 3-HP. Furthermore,

949 enzymes that play major roles in 3-HP and PDO production, such as DhaB, DhaT, and AldH,

950 are inhibited by 3-HP and PDO (Kumar et al. 2013b). Furthermore, *K. pneumoniae* is

951   sensitive to 24 g/L acetic acid, 26 g/L lactic acid, and 17 g/L ethanol under aerobic conditions

952   and 15, 19, and 26 g/L, respectively, under anaerobic condition (Celińska, 2012; Cheng et al.,

953   2005). The inhibition of pathways by target product(s) and/or unwanted byproducts often

954   places a limit on the maximum attainable titer of bulk chemicals.

955   Several studies have reported engineered improvements in microbial tolerance against

956   biochemicals and biofuels, especially in *E. coli*. Modifications of exporter proteins, heat

957   shock proteins, membrane composition, and stress responses have been attempted. Moreover,

958   *in situ* recovery of target products has been attempted for hydrophobic targets. Some tolerant

959   strains have been developed, but unfortunately, without appreciable increases in productivity

960   (Dunlop, 2011; Dunlop et al., 2011; Jarboe et al., 2011). Such efforts to improve tolerance to

961   chemicals have not been reported for *K. pneumoniae*.

962   **4.4 Availability of glycerol**

963   The global market for glycerol is known to be unpredictable and complex. Glycerol in

964   the current market mainly is from the biodiesel industry, and its supply is directly affected by

965   the use of biodiesel. In contrast, glycerol demand depends on many industries that use it as

966   feedstock. Between 2001 and 2011, the price varied significantly, from $0.70/kg to $1.70/kg

967   for pure glycerol and from $0.04/kg to $0.33/kg for crude glycerol (Ayoub and Abdullah,

968   2012; Ciriminna et al., 2014; Kong et al., 2016; Quispe et al., 2013). Nevertheless, glycerol

969   prices, for pure as well as crude, have come down significantly since 2004, when many

970   biodiesel production plants initiated operation. The countries producing biodiesel in large

971   quantities (in million liters) include Germany (2900), Brazil (2300), Argentina (2100), France

972   (2000), USA (1200), Spain (1100), Italy (800), Indonesia (700), and Thailand (600) (Kong et

973   al., 2016). The Asia-Pacific region overtook Europe as the largest glycerol market in the

974   world in 2009 and has remained so ever since. Market growth is driven by increases in the

975 uses for glycerol in such sectors as pharmaceuticals, personal hygiene, and food and beverage

976 production (Quispe et al., 2013).

977 The production of crude glycerol is expected to reach 6 million tons by 2025

978 (Ciriminna et al., 2014). At present, the surplus of glycerol from biodiesel and oleochemicals

979 is assumed to be sufficient to meet these new demands. However, the scenario may change in

980 the future with rapid growth of glycerol-based industries and a limited supply of crude

981 glycerol. For example, if 3-HP produced from glycerol were used as a substitute for the

982 chemical acrylic acid (global production, 5.85 million tons as of 2014), approximately 7

983 million tons of glycerol/year would be needed. If we include other platform chemicals such

984 as PDO, BDO, and ethanol, the demand will be even higher, much more than the expected

985 supply. The growth of glycerol-based biotechnology will eventually be constrained by the

986 supply and price of crude glycerol as a carbon source.

987 **5. Concluding remarks**

988 Glycerol is an excellent substrate for the production of biochemicals and biofuels.

989 Although the biodiesel industry has grown rather slowly in recent years, crude glycerol is still

990 cheap and generated in large quantities. *K. pneumoniae* has already been successfully

991 employed for the production of PDO, 3-HP, BDO, lactic acid, and ethanol from glycerol at

992 industrially attractive levels. Other metabolites such as acetic acid, pyruvic acid, malic acid,

993 fumaric acid, alanine, *n*-butanol, 1,2-propanediol, propionic acid, formic acid, and hydrogen

994 can also be produced from glycerol by *K. pneumoniae*, although these have not yet been

995 extensively explored. At present, sugar-based bioprocesses are prevalent, and no bioprocesses

996 employing *K. pneumoniae* and glycerol are commercially available. The pathogenicity of *K.*

997 *pneumoniae* and other technical issues are existing challenges to its use. However,

998 advancements in the fields of metabolic engineering, synthetic biology, systems biology, and

999 evolutionary engineering will enable us to further exploit the advantageous nature of *K.*

1000 *pneumoniae* as a biocatalyst and glycerol as a carbon source. A better understanding of

1001 glycerol metabolism in *K. pneumoniae* and improvements in its performance as a microbial

1002 cell factory should make this organism an attractive alternative to current sugar-based

1003 methods for the production of biochemicals and biofuels.

1004 **Acknowledgement**

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Figure captions

1462

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

1. Introduction

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

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

55   Gonzalez, 2007). Table 1 compares the maximum theoretical yield of various metabolites,

56   ATP, and redox balances between glycerol and glucose.

57   Despite its great potential as a carbon source, glycerol is not efficiently metabolized

58   by many microorganisms under anaerobic or oxygen-limited conditions. The ability of *K.*

59   *pneumoniae* to assimilate glycerol under oxygen-limited conditions is outstanding and has

60   been studied extensively. Several review articles addressing this issue have also been

61   published (Clomberg and Gonzalez, 2013; Mattam et al., 2013; Yazdani and Gonzalez, 2007),

62   but the biotechnological potential and physiological aspects of this strain have not been fully

63   elucidated. This review explores and discusses glycerol metabolism in *K. pneumoniae* and the

64   production of value-added chemicals from glycerol. The potential and challenges of using *K.*

65   *pneumoniae* as a microbial cell factory are also discussed.

66   **2. Glycerol metabolism in *K. pneumoniae***

67   Many microorganisms can metabolize glycerol in the presence of external electron

68   acceptors (respiratory metabolism), but relatively few under non-respiratory conditions

69   (without exogenous electron acceptors). Among the latter, genera including *Citrobacter*,

70   *Enterobacter*, *Bacillus*, *Propionibacterium*, *Anaerobiospirillum*, *Klebsiella*, *Clostridium*, and

71   *Lactobacillus* are well documented (da Silva et al., 2009; Yazdani and Gonzalez, 2007).

72   Some *Lactobacillus* species including *L. reuteri*, *L. brevis*, and *L. bucheneri* convert glycerol

73   to PDO but only in the presence of a major carbon source for growth. Glycerol does not

74   support cell growth, limiting its biotechnological applications. In contrast, *Clostridium sp.*

75   grow well on glycerol as the sole carbon source. However, the use of *Clostridium* is limited

76   by its slow growth and requirement of strict anaerobic conditions. The lack of a convenient

77   genetic tool box for large-scale metabolic engineering is another serious drawback of

78   *Clostridium* (Branduardi et al., 2014; Lütke-Eversloh and Bahl, 2011).

79   From a biotechnological standpoint, *Klebsiella sp.*, especially *K. pneumoniae*, have

80   many advantages over the aforementioned microbes. They grow on glycerol as the sole

81   carbon source under both aerobic and anaerobic conditions (Arasu et al., 2011; Kumar et al.,

82   2012). Their growth is fast, typically growing at a rate >0.9/h, even on glycerol minimal

83   medium (Arasu et al., 2011). They naturally produce coenzyme B_{12}, an essential cofactor for

84   the synthesis of the commercially important chemicals PDO and 3-HP (Huang et al., 2013a;

85   Oh et al., 2012a). Furthermore, their evolutionary and biochemical proximity to *E. coli* allows

86   for the application of most of gene manipulation methods developed for *E. coli* without much

87   modification (Celińska, 2012; Kumar et al., 2013a).

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

89   In *K. pneumoniae*, glycerol is metabolized oxidatively and/or reductively (Ashok et

90   al., 2011, Kumar et al., 2013a). The oxidative pathway provides energy and carbon

91   constituents for the synthesis of cell biomass, while the reductive pathway reduces glycerol

92   and regenerates NAD^+, enabling the oxidative assimilation of glycerol under anaerobic

93   conditions.

94   Glycerol oxidation proceeds in either a respiratory or a fermentative manner.

95   Respiration requires an exogenous electron acceptor such as oxygen, nitrate, or fumarate

96   (Ashok et al., 2013a,b) and is carried out by enzymes encoded by the *glp* regulon. During

97   respiration, glycerol is first phosphorylated by glycerol kinase (*glpK*) to yield *sn*-glycerol-3-

98   phosphate using ATP or phosphoenolpyruvate (PEP) as a phosphate donor. Next, *sn*-

99   glycerol-3-phosphate is oxidized to dihydroxyacetone phosphate (DHAP) with electron

100   transfer to a quinone molecule, which is linked to the electron transport chain (ETC). This

101   transformation is catalyzed by glycerol-3-phosphate dehydrogenase, which is expressed

102   under both aerobic (*glpD*) and anaerobic (*glpABC*) conditions. In contrast, fermentation of

103   glycerol is conducted without a supply of external electron acceptors, and the reactions are

catalyzed by enzymes encoded by the *dha* regulon. Here, glycerol is oxidized to

dihydroxyacetone (DHA) by glycerol dehydrogenase (mainly encoded by *dhaD*) using NAD^+

as an electron acceptor. DHA then is phosphorylated by dihydroxyacetone kinase, an enzyme

encoded by the ATP-dependent *dhaK1/dhaK* and/or PEP-dependent

dhaK2/dhaKLM/dhaK123. DHAP obtained from respiration and/or fermentation is channeled

into the glycolytic pathway (Fig. 2). A variety of organic acids and alcohols, including acetic

acid, lactic acid, succinic acid, ethanol, BDO, and formic acid, are produced by the

downstream glycolytic pathway (Ashok et al., 2011; Kumar et al., 2013a).

The anaerobic growth capability of *K. pneumoniae* using glycerol as a carbon source

is attributed to its well-established reductive metabolism. In the reductive pathway, glycerol

first undergoes a difficult rearrangement reaction catalyzed by glycerol dehydratase (GDHt)

to yield 3-hydroxypropioaldehyde (3-HPA). Catalysis by GDHt requires coenzyme B_{12}, the

de novo synthesis of which is limited to only few genera of microorganisms. The 3-HPA is

subsequently reduced to PDO by at least two 1,3-propanediol oxidoreductases (1,3-PDORs),

including NADH-dependent DhaT (PDOR) and NADPH-dependent hypothetical

oxidoreductase (HOR), along with the regeneration of $NAD(P)^+$ (Fig. 3). *E. coli* and other

enterobacter sp. have the oxidative (respiratory) pathway of glycerol assimilation. However,

these organisms lack the reductive pathway and cannot grow on glycerol under anaerobic

conditions (Dharmadi et al., 2006; Kumar et al., 2013a).

2.2 Genes and enzymes involved in fermentative metabolism of glycerol

The *dha* regulon, induced by the DHA molecule and expressed in the absence of

exogenous electron acceptors, encodes numerous genes needed for both the oxidative

(fermentative) and reductive pathways (Celińska, 2012; Forage and Foster, 1982) (Fig. 4A).

The *dha* regulon of *K. pneumoniae* contains the following genes arranged in the order as

appears: *Kpk_0615/dhaK*, *dhaM*, *dhaL*, *dhaK*, *dhaD*, *dhaR*, *orfW, CdAT*, *orfX* (*dhaG*), *dhaT*,

129   *orfY, dhaB*, *dhaC*, *dhaE*, *orfZ* (*dhaF*), and *glpF*. Of these, *dhaB*, *dhaC*, *dhaE*, *orfX*, *orfZ*, and

130   *dhaT* are relatively well documented for their functions in the reductive metabolism of

131   glycerol. The *orfW* and *orfY* genes, although commonly found in the *dha* regulons of many

132   organisms, are not as well described. The proteins encoded by the *dha* regulon of *K.*

133   *pneumoniae* have high similarity (80%–95%) with those of *Citrobacter* species but not with

134   those of *Clostridium* species (30%–80%). Surprisingly, the sequence similarity of *dha*

135   proteins among *Clostridium* species is very low in some cases, even lower than their

136   similarity to the corresponding proteins in *K. pneumoniae* or *C. freundii* (Celińska, 2012; Sun

137   et al., 2003; Wei et al., 2014).

138   The complete *dha* regulon is found in only a few dozen species belonging to five

139   different taxonomic groups, including four bacterial species [Actinobacteria, Firmicutes,

140   Fusobacteria, and Proteobacteria (Gammaproteobacteria and Deltaproteobacteria)] and one

141   archaea species, *Halalkalicoccus jeotgali* (Martins-Pinheiro et al., 2016). Interestingly, an

142   incomplete *dha* regulon is present in more than 100 prokaryotes, suggesting that the enzymes

143   of the regulon have other functions not restricted to the anaerobic growth on glycerol. A well-

144   documented example is the one found in *Lactobacillus* species. *Lactobacillus* cannot grow on

145   glycerol as the sole carbon source because it lacks the enzymes for oxidative assimilation of

146   glycerol. However, the enzymes encoded in the incomplete *dha* regulon enable the

147   production of PDO from glycerol, which accelerates cell growth in *Lactobacillus* grown on

148   glucose (Årsköld et al., 2008; Martins-Pinheiro et al., 2016).

149   In the following sections, the major enzymes involved in fermentative glycerol

150   metabolism and their genes are described in detail.

151   **2.2.1 Glycerol dehydrogenase**

152   Encoded by the *dhaD* and/or *gldA* gene, glycerol dehydrogenase is an oxidoreductase

153   that converts glycerol to DHA. The enzyme extracts electrons from glycerol and transfers

154 them to NAD$^+$. DhaD plays a major role during the anaerobic growth of *K. pneumoniae* on

155 glycerol. Quantification of mRNA expression indicates that *dhaD* is highly induced when

156 glycerol is used as carbon source. GldA expression is lower than that of DhaD and is thought

157 to serve as a back up to DhaD during anaerobic growth on glycerol. However, DhaD and

158 GldA exhibit a high level of similarity in amino acid sequence and have the same substrate-

159 binding site (Wang et al., 2014).

160 Recently, DhaD was reported to catalyze the interconversion of acetoin and BDO for

161 NADH disposal and recovery (Wang et al., 2014). The promiscuity of DhaD is responsible

162 for BDO production in the Δ*budC* mutant of *K. pneumoniae*. The contribution by DhaD to or

163 its participation in BDO production is also supported by the observed increase in BDO

164 synthesis and increased concentration of NADH in the presence of the *dhaD* gene. NADH is

165 generated by the oxidation of glycerol and consumed by the production of BDO from acetoin.

166 The dual physiological functions of DhaD allow *K. pneumoniae* to control the intracellular

167 redox level. In response to the intracellular level of NADH, DhaD is able to switch its role

168 from NADH production (glycerol oxidation) to NADH consumption (BDO production)

169 (Wang et al., 2014). The diversion of glycerol flux from organic acids toward alcohols such

170 as BDO also helps the cells to counteract intracellular acidification (Petrov and Petrova,

171 2009). Thus, this promiscuity confers different physiological roles to the enzyme, which

172 include regulation of the intracellular NADH/NAD$^+$ ratio, prevention of acidification, and

173 storage of carbon and energy (Wang et al., 2014).

174 **2.2.2 Dihydroxyacetone kinases**

175 Dihydroxyacetone kinase (DHAK) phosphorylates DHA to DHAP, an intermediate of

176 the glycolytic pathway. Sun et al. (2003) analyzed the genome of *K. pneumoniae* MGH

177 78578 and found, in addition to ATP-dependent DhaK I, a PEP-dependent DhaK II. DhaK I

178 is encoded by the *dhaK* (*Kpk_0615*) gene, while DhaK II is encoded by three genes, *dhaK*,

8

179 *dhaL* and *dhaM,* which correspond to the medium, small, and large subunits of the enzyme,

180 respectively (Celińska, 2012; Sun et al., 2003; Wei et al., 2014). The names *dhaKLM* and

181 *dhaK123* are synonyms used without distinction (Martins-Pinheiro et al., 2016; Sun et al.,

182 2003; Wei et al., 2014). DhaK enzymes are found in many microorganisms. PEP-dependent

183 DhaK II is present in *E. coli*, *L. lactis*, and *C. butyricum*, whereas ATP-dependent DhaK I is

184 present in *Citrobacter freundii* (Garcia-Alles et al., 2004; Raynaud et al., 2011). The PEP-

185 dependent DhaK II of *E. coli*, encoded by *dhaKLM*, is homologous to *dhaK123* (*dhaKLM*) of

186 *K. pneumoniae* (Gutknecht et al., 2001). In a recent study, Wei et al. (2014) further elucidated

187 the physiological roles of DhaK I and DhaK II of the *dha* regulon of *K. pneumoniae*. They

188 found that disruption of ATP-dependent DhaK I had no significant effect on glycerol uptake,

189 indicating that PEP-dependent DhaK II is the major contributor to the conversion of DHA to

190 DHAP. As in *E. coli*, the subunits of DhaK II regulate expression of the *dha* regulon; *dha*

191 regulon expression was suppressed by the disruption of *dhaK1* (*dhaK*) and *dhaK2* (*dhaL*).

192 However, in contrast to *E. coli*, mutation of *dhaK3* (*dhaM*) in *K. pneumonia* did not

193 upregulate the *dha* regulon but rather decreased cell growth and glycerol uptake.

194 **2.2.3 Glycerol dehydratase and reactivating factor**

195   Glycerol dehydratase (GDHt) is the first enzyme in the reductive pathway and

196 catalyzes the dehydration of glycerol to 3-HPA. The same reaction can be carried out by diol

197 dehydratase (DDHt), although the latter has a higher activity on 1,2-propanediol than on

198 glycerol. GDHt requires coenzyme B_{12} or *S*-adenosyl methionine (SAM) as a cofactor. *K.*

199 *pneumoniae* has B_{12}-dependent GDHt only, while strict anaerobes such as *clostridia* sp. have

200 SAM-dependent GDHt. The three subunits of GDHt are encoded by the genes *dhaB1* (~1670

201 bp), *dhaB2* (~590 bp) and *dhaB3* (~430 bp), for which the gene products are the α (large,

202 60.7 kDa), β (medium, 21.3 kDa), and γ (small, 16.1 kDa) subunits, respectively (Xu et al.,

203 2009a). GDHt of *K. pneumoniae* is known to be present as a dimer of heterotrimers, $(\alpha\beta\gamma)_2$.

9

204   The subunits of GDHt have high homology among *K. pneumoniae* strains; for example, *K.*

205   *pneumoniae* XJPD-Li and U30909 strains showed >99% identity.

206   Coenzyme B_{12} is present in the reaction center and plays an essential role in the

207   catalysis of GDHt. The catalytic cycle often results in the mechanism-based inactivation of

208   coenzyme B_{12} due to the irreversible breakage of the chemical bond between cobalt (Co) and

209   a carbon of the adenosyl moiety. Oxygen accelerates breakage of the Co–C bond. Oxygen is

210   also known to inactivate GDHt, even in the absence of the substrate glycerol. The gene

211   products of *orfZ/dhaG* (small subunit) and *orfX/dhaF* (large subunit) act as reactivating

212   factors of GDHt (GdrAB) (Celińska, 2012; Sun et al., 2003). They reactivate GDHt by

213   catalyzing the exchange of damaged for intact coenzyme B_{12} in the presence of ATP and

214   Mg^{2+}/Mn^{2+} (Celińska, 2012; Kumar et al., 2013a; Maervoet et al., 2011; Sun et al., 2003; Wei

215   et al., 2014). The reactivase is a molecular chaperone that functions as a heterotetramer

216   containing two elongated α subunits (63 kDa) and two globular β subunits (14 kDa).

217   Structurally, the α subunit resembles both GroEL and Hsp70 chaperones, while the β subunit

218   resembles that of the β subunit of glycerol dehydratase, except that it lacks some of the amino

219   acids responsible for coenzyme B_{12} binding (Liao et al., 2003).

220   In some organisms, the GDHt enzyme varies in terms of the number of subunits and

221   their copies (Fig. 4B) (Liu et al., 2010). For example, in *Mesorhizobium loti* and

222   *Mesorhizobium opportunistum*, the large and medium subunits (*dhaB1* and *dhaB2*) are fused

223   together and encoded by a single gene. The gene fusion may arise by frameshift mutations.

224   The active site of the fused enzyme differs slightly from that of unfused enzyme. Moreover,

225   these microorganisms lack the genes encoding the reactivation factor, indicating that the

226   reactivation process may not exist or is carried out by a different route. *Mycobacterium*

227   *smegmatis* has two subunits (fused *dhaB1* + *dhaB2* and *dhaB3*) that are similar to those of *M.*

228   *loti* and *M. opportunistum*; however, the reactivating factor differs in that it has one (large)

229   subunit. No small subunit has been identified in *M. smegmatis*. Fusobacterium *Ilyobacter*

230   *polytropus* is unique in that it has genes coding for both B_{12}-independent (SAM-dependent)

231   and B_{12}-dependent GDHt. The genes for B_{12}-independent GDHt are present in the genomic

232   DNA, while those encoding B_{12}-dependent GDHt along with its reactivation factor are

233   present in two copies, one in the chromosome and the other in a plasmid. Surprisingly, even

234   with this gene redundancy, no significant production of 3-HPA and/or PDO has been reported

235   in *I. polytropus* (Stieb and Schink, 1984). The B_{12}-independent proteins showed no homology

236   with B_{12}-dependent GDHt but significant similarity with the pyruvate formate lyases (PFL)

237   and PFL-activating enzymes and their homologues (Raynaud et al., 2003).

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

239   The 1,3-propanediol oxidoreductase (PDOR), responsible for the second step of the

240   reductive pathway of glycerol, catalyzes the conversion of 3-HPA to PDO with the oxidation

241   of NAD(P)H. 3-HPA is a toxic intermediate, and PDOR protects the cell from deleterious

242   damage by reducing 3-HPA to PDO (Celińska, 2012). In *K. pneumoniae*, PDOR is encoded

243   by the *dhaT* gene and is strictly NADH dependent. Active PDOR is a homo-octamer with a

244   monomeric molecular mass of 43 kDa. Despite its importance, the kinetic properties of DhaT

245   were elucidated only recently (Lama et al., 2015). DhaT has high specificity for 3-HPA

246   among various aldehydes. Under physiological conditions at pH 7.0 and 37°C, the catalytic

247   efficiency (k_{cat}/K_m) of the forward reaction (3-HPA to PDO) was found to be 77.2/s. mM,

248   which is 57-fold higher than that of the reverse reaction (1.3/s. mM; PDO to 3-HPA). The K_m

249   (mM) values for 3-HPA and NADH (forward reaction) are 0.77 and 0.03, respectively, while

250   those for PDO and NAD^+ (backward reaction) are 7.4 and 0.23, respectively. Furthermore,

251   the maximum activity of DhaT for the reverse reaction was obtained at pH 9.0 and 55°C.

252   These results strongly suggest that, under physiological conditions, DhaT catalyzes the

253   reduction of 3-HPA to PDO rather than the oxidation of PDO to 3-HPA. Nevertheless, the

254 reverse reaction (PDO→3-HPA) becomes significant as the PDO concentration increases.

255 This factor is considered a serious drawback of using DhaT for PDO production from

256 glycerol.

257   *K. pneumoniae* has several oxidoreductases in addition to PDOR that can reduce 3-

258 HPA to PDO. One such enzyme, designated as hypothetical oxidoreductase (HOR), has been

259 studied extensively and shares 89% identity with YqhD of *E. coli* (Zhu et al., 2009; Zhuge et

260 al., 2010). Expression of *yqhD* in wild-type *K. pneumoniae* is not high, with an mRNA level

261 ~20-fold lower than that of *dhaT*. However, deletion of *dhaT* increases the transcription of

262 *yqhD* by more than 10-fold (Ko et al., 2015). This observation suggests that in addition to its

263 wide-ranging activity on short-chain aldehydes, YqhD is a detoxification enzyme for many

264 aldehydes (Jarboe, 2011). YqhD exhibits negligible activity on PDO (backward reaction),

265 which is an important advantage over DhaT for use in the production of PDO from glycerol

266 (Celińska, 2012; Chen et al., 2011; Zheng et al., 2006). However, YqhD is kinetically inferior

267 to DhaT because of its low catalytic activity (k_{cat}/K_m) on 3-HPA and the requirement of

268 NADPH as a cofactor. The k_{cat}/K_m value of YqhD on 3-HPA (pH 7.0 and 37°C) is estimated

269 to be 2.1/s. mM, which is 36-fold lower than that of DhaT (unpublished data). The use of

270 NADPH as a cofactor prohibits this enzyme from being directly coupled to the reactions

271 catalyzed by DhaD and other enzymes in glycolysis and the TCA cycle, by which NADH is

272 mainly produced. Furthermore, excessive use of NADPH for PDO production can

273 significantly reduce the cellular NADPH level and this can disturb many anabolic reactions

274 requiring NADPH (Wang et al., 2003; Jarboe, 2011). NADH and NADPH differ in a

275 phosphate group only, and it has been suggested that electrostatic interaction of this

276 phosphate is the major factor distinguishing NADH from NADPH for enzymes using these

277 cofactors. Comparison of the amino acid sequences of DhaT and HOR from *K. pneumoniae*

278 and YqhD from *E. coli* has revealed that DhaT contains aspartic acid (Asp) at position 41,

279   while HOR and YqhD have valine (Val) at this position. The steric hindrance and

280   electrostatic repulsion between Asp in the active site and the phosphate group of NADPH

281   impede their binding. In contrast, the hydrophobic amino acid Val poses no such obstruction;

282   thus, YqhD uses NADPH as a cofactor (Ma et al., 2010).

283   Despite their difference in cofactor preference, DhaT and YqhD are similar (44%) in

284   amino acid sequences. *K. pneumoniae* has five enzymes that have at least 40% similarity in

285   amino acid sequence with DhaT and YqhD: putative alcohol dehydrogenase (PaldH), L-1,2-

286   propanediol oxidoreductase (FucO), ethanolamine-utilization enzyme (EutG), propanediol-

287   utilization propanol dehydrogenase (PduQ), and bifunctional alcohol dehydrogenase (AdhE)

288   (Ko et al., 2015). The roles of these enzymes in PDO production have been investigated.

289   **2.2.5 Regulatory protein**

290   The *dhaR* gene product is an important transcription factor that is responsible for

291   expression of the *dhaT* and *dhaB* genes (Sun et al., 2003). This gene is found in *K.*

292   *pneumoniae*, C. *freundii*, and *Clostridium botulinum* but not in every organism containing a

293   complete *dha* regulon (Martins-Pinheiro et al., 2016). The presence of DhaR is thought to

294   allow the *dha* operon to function as a separate regulatory system, independent of fumarate

295   nitrate reduction (FNR). Under anaerobic conditions, FNR acquires DNA-binding properties

296   and induces the expression of many anaerobiosis-related genes. DhaR contains domains for

297   GAF (52-199), PAS (203-267), σ^{54} factor interaction, and histidine HTH-8. The PAS senses

298   internal levels of energy charge, light, oxygen, and redox potential. The HTH-8 domain has a

299   helix-turn-helix conformation and acts as a DNA binding structure. The σ^{54} factor interaction

300   domain interacts with the σ^{54} factor of RNA polymerase and activates RNA transcription

301   from the σ^{54} promoters (Buck et al., 2000; Taylor and Zhulin, 1999). Thus, the DhaR protein

302   triggers the transcription of *dha* genes in response to intracellular levels of several important

303   physiological parameters (Celińska, 2012; Sun et al., 2003). Zheng et al. (2006) investigated

304   the effect of overexpression of the putative regulatory gene *dhaR* in the *dha* regulon on

305   glycerol metabolism in *K. pneumoniae*. *dhaR* overexpression increased PDOR activity up to

306   6.7-fold over that of the control wild-type strain, confirming the role of DhaR as a positive

307   regulator of the *dhaT* gene. However, *dhaR* overexpression did not increase the activity of

308   glycerol dehydratase (DhaB). Furthermore, despite the significant increase in PDOR activity,

309   PDO production was unexpectedly low compared with that of the wild type (440 vs. 656.23

310   mM, respectively). More studies are required to determine the precise role of DhaR and its

311   regulatory function in the *dha* regulon.

312   DhaR in *E. coli* (70% identity to that of *K. pneumoniae*) has also been studied. This

313   protein stimulated transcription of the *dhaKLM* operon from a σ^{70} promoter. Interestingly, in

314   *E. coli*, DhaL was a positive regulator of the *dhaKLM* operon while phosphorylated DhaM

315   and DhaK were negative regulators of the same operon. DhaK contains the DHA-binding site.

316   DhaL uses ADP as a cofactor for the double displacement of phosphate from DhaM to DHA.

317   DHA is the oxidation product of glycerol metabolism; binding of DHA to DhaK reduces the

318   affinity of DhaK for DhaR. In the presence of DHA, DhaL::ATP is dephosphorylated,

319   leading to the displacement of DhaK by DhaL::ADP, which stimulates DhaR activity. In the

320   absence of DHA, DhaL::ADP is phosphorylated back to DhaL::ATP by the phototransferase

321   system (PTS), thereby inhibiting the binding of the phosphorylated complex (DhaL::ATP) to

322   DhaR. This phosphorylation is mediated by DhaM, providing a phospho-histidine relay

323   between the PTS and DhaL::ADP. This double-check mechanism of binding and turnover

324   increases the selectivity such that the binding of nonphosphorylated compounds does not

325   induce the *dha* operon (Bächler et al., 2005).

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

327   The *glp* regulon comprises the genes encoding proteins required for the conversion of

328   glycerol to DHAP via sn-glycerol-3-phosphate. The five operons that constitute the *glp*

329 regulon are *glpFK*, *glpD* (anaerobic glycerol-3-phosphate dehydrogenase), *glp ACB* (aerobic

330 glycerol-3-phosphate dehydrogenase), *glpTQ* (glycerol-phosphate

331 permease/glycerophosphodiesterase), and *glpEGR*. The *glpF* gene encodes a cytoplasmic

332 membrane protein that facilitates the diffusion of glycerol into the cell. The *glpE* gene

333 encodes an acidic, cytoplasmic protein of 108 amino acids with a molecular weight of

334 12,082. The *glpG* gene encodes a basic, cytoplasmic membrane-associated protein of 276

335 amino acids with a molecular weight of 31,278 (Lin, 1976; Yang and Larson, 1998; Zeng et

336 al., 1996). The biochemical functions of GlpE and GlpG are unknown. The *glpR* gene

337 encodes the GlpR repressor protein. These five operons are located at three different positions

338 on the chromosome. Transcription of these operons is subject to multiple controls, including

339 catabolite repression mediated by cAMP-CRP and respiratory control mediated by the FNR

340 and ArcA/ArcB systems. Moreover, each of the operons is negatively controlled by a

341 repressor specific for the regulon (Lin, 1987; Zeng et al., 1996).

342 **2.3.1 GlpR and other regulatory proteins**

343   The GlpR repressor protein, predicted to contain 252 amino acids with a molecular

344 weight of 28,048, belongs to the DeoR bacterial regulatory factor super family. Although not

345 studied extensively, GlpR of *K. pneumonia* is expected to have the same biochemical

346 properties and regulatory functions as that of *E. coli*. GlpR negatively controls all the *glp*

347 operons by binding to operators that overlap or are close to the *glp* promoters. In case of *E.*

348 *coli*, glycerol-3-phosphate acts as an inducer and has a high affinity for GlpR ($K_d = 31$ μM)

349 (Lin, 1987; Weissenborn et al., 1992; Zeng et al., 1996). When complexed with glycerol-3-

350 phosphate, GlpR cannot bind to the operator sites, and repression is relieved. Glycerol-3-

351 phosphate is a product of GlpK and a substrate of GlpD/GlpABC; thus, the absence of GlpD

352 causes a substantial increase in the level of glycerol-3-phosphate, resulting in induction of the

353 *glp* regulon (Fig. 2). The GlpR protein exhibits differential binding affinity for the operators

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

2.3.2 Glycerol uptake/transport facilitator and other components

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

16

379   *dha* regulon (see Fig. 4A) and the other in the respiratory *glpFK* operon. Both *glpF* genes

380   have a high similarity (85 %) to each other. When one but not both of the *glpF* genes is

381   disrupted, the *glpF* deletion mutants of *K. pneumoniae* can consume glycerol and convert it to

382   PDO at a high rate, similar to the wild type (unpublished data; Supplementary Fig. S1). This

383   observation indicates that both *glpF* genes encode active glycerol transporter proteins.

384   However, their presence is not essential for glycerol utilization in *K. pneumoniae* because the

385   double-deletion mutant for both facilitators can still uptake glycerol and convert it to PDO. In

386   *E. coli*, only one *glpF* gene is present in the *glpFK* operon; the GlpF protein shows a high

387   similarity (80.9%) to that (encoded from *glpFK*) of *K. pneumoniae* (Sun et al., 2003). The

388   importance of GlpF in *E. coli* also seems to be limited only at low levels of glycerol (<8

389   mM), as glycerol itself may pass through the membrane by diffusion at high concentrations

390   (Richey and Lin, 1972).

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

392   The presence of the *dha* and *glp* regulons affords *K. pneumoniae* metabolic flexibility.

393   The *dha* regulon enables fermentative utilization of glycerol, while the *glp* regulon enables

394   respiratory metabolism of glycerol. During fermentative metabolism of glycerol, redox

395   constraints force the production of PDO from glycerol, with a low ratio of carbon conversion

396   yield to cell biomass. Moreover, ATP is formed through substrate-level phosphorylation, and

397   NADH oxidation occurs through the formation of reduced metabolites. In the presence of

398   oxygen, the redox constraints forcing the production of PDO are relieved; therefore, the

399   carbon loss required for regeneration of NAD^+ is greatly reduced. As a consequence, glycerol

400   utilization, biomass synthesis, and even the formation of many metabolites increase (Durnin

401   et al., 2008). Interestingly, the fermentative route (*dha* regulon) is in operation under a wide

402   range of oxygen levels, from microaerobic to highly aerobic conditions, as evidenced by

403   PDO formation. For example, a high titer PDO (102.7 g/L) was produced under the condition

404   of 2.0 vvm aeration and even at 3.5 vvm, although the production was a little lowered in the

405   latter case (Oh et al., 2012a). These results suggest that the fermentative and respiratory

406   pathways might have evolved to complement each other for optimal control of cell growth

407   under a wide range of aeration conditions except the strictly anaerobic one.

408   **2.4 Carbon catabolite repression**

409   Carbon catabolite repression (CCR) refers to the suppression of the use of less-

410   preferred carbon sources, when a more preferred carbon, most prominently glucose, is

411   present. Cyclic AMP, cAMP receptor protein (CRP), and the enzyme $EIIA^{Glc}$, an

412   intermediate in the phosphorylation cascade of the PTS, are key players in the CCR of

413   enterobacteria. The $EIIA^{Glc}$, a cytosolic protein, exists in phosphorylated and

414   unphosphorylated forms. The phosphorylated form stimulates membrane-bound adenylate

415   cyclase, which catalyzes the formation of cAMP; the cAMP-CRP complex then induces

416   expression of the catabolic genes of less-preferred carbon sources. The unphosphorylated

417   form of $EIIA^{Glc}$ is also responsible for inhibiting the transport of less-preferred carbon

418   sources by inhibitory binding to permeases, causing so-called inducer exclusion (Eppler and

419   Boos, 1999; Eppler et al., 2002). Glycerol assimilation in *K. pneumoniae* is greatly inhibited

420   by CCR in the presence of glucose. This repression is related to not only low cAMP and

421   cAMP-CRP but also inhibition of GlpK (which functions mainly under aerobic conditions)

422   by fructose 1,6-bisphosphate, a metabolite produced from glucose. The unphosphorylated

423    $EIIA^{Glc}$ is also known to allosterically inhibit GlpK (Holtman et al., 2001). Anaerobic

424   glycerol metabolism mediated by the *dha* operons is also suppressed by the presence of

425   glucose. Enzyme-level inhibition similar to that of the aerobic GlpK has not been reported,

426   but we have observed that, in *K. pneumoniae*, transcription of the *dha* operons is greatly

427   reduced in the presence of glucose (Suman et al., 2017).

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

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

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

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

452 used for the synthesis of these chemicals are explained, and the metabolic engineering used to

453 improve their production is discussed.

454 **3.1 1,3-Propanediol**

455 PDO, also known as trimethylene glycol, is a promising platform chemical that has

456 two hydroxyl groups. With terephthalic acid, it can be co-polymerized to the novel co-

457 polymer polytrimethylene terephthalate (PTT). PDO also has applications in the food and

458 cosmetic industries. Currently, commercial PDO is mainly produced by recombinant *E. coli*

459 (Maervoet et al., 2011; Saxena et al., 2009). The global demand for PDO was 60.2 kilotons in

460 2012 and is predicted to reach approximately 150 kilotons by 2019. The market for PDO is

461 growing rapidly, from $157 million in 2012 with projections up to $560 million in 2019

462 (MarketsANDMarkets, 2012; Lee et al., 2015).

463 *K. pneumoniae* is one of the best native producers of PDO from glycerol. GDHt and

464 PDOR are two important enzymes for the conversion of glycerol to PDO (Fig. 3).

465 Physiologically, the synthesis of PDO from glycerol is essential for the anaerobic growth of

466 *K. pneumoniae*, as described previously. The production of PDO requires two cofactors,

467 coenzyme B_{12} and NAD(P)H. *K. pneumoniae* has a *de novo* pathway for the biosynthesis of

468 coenzyme B_{12} that comprises more than 20 genes. NAD(P)H is generated through the

469 oxidative metabolism of glycerol, during the production of cell biomass and/or oxidized

470 (pyruvate and acetate) or partially reduced (BDO, lactic acid) metabolites. The production

471 yield of PDO from glycerol varies depending on the cost of carbon for NAD(P)H production;

472 the lower the cost, the higher the yield of PDO from glycerol. NADPH can also be the

473 electron donor for the conversion of 3-HPA to PDO when YqhD is present. However, the

474 contribution of NADPH to PDO production in native *K. pneumoniae* seems to be limited

475 because the PP pathway, which is the main source of NADPH, is not active when glycerol is

476   the sole carbon source. Furthermore, *K. pneumoniae* has very low transhydrogenase activity

477   for the conversion of NADH to NADPH (Zhang and Xiu, 2009; Chen et al., 2011).

478   Several metabolic engineering strategies have been employed to improve PDO

479   production by *K. pneumonia* (Table 2). Amplification of the reductive pathway by

480   overexpressing DhaB, PDOR, or both has been attempted by many research groups. As

481   indicated previously, 3-HPA is highly toxic, and its accumulation is detrimental to cell

482   viability and toxic to PDO production. To reduce 3-HPA accumulation, overexpression of

483   *dhaT* has been attempted. In one study, 3-HPA accumulation was reduced by *dhaT*

484   overexpression, but no improvement in PDO production resulted (Hao et al., 2008).

485   Overexpression of both DhaT and DhaD increased PDO production (56.3%) in batch culture

486   but no increase in fed-batch culture (Chen et al., 2009). With another *K. pneumoniae* strain,

487   DSM 2026, the same experiments were repeated but also with no increase in PDO production

488   (Zheng et al., 2006). These results suggest that the reductive pathway in *K. pneumoniae* is

489   evolutionarily well established (perhaps in concert with the oxidative pathway) and that PDO

490   production is not limited by expression of the *dhaB* and *dhaT* genes of the reductive pathway.

491   However, in one study where the *E. coli yqhD* gene was highly overexpressed to give ~10-

492   fold higher PDOR activity (110 IU/mg total protein), a significant increase in PDO titer (25%;

493   67.6 g/L) and yield (from 0.53 to 0.62 mol/mol), along with less 3-HPA accumulation, was

494   achieved (Zhu et al., 2009). This observation suggests that in the recombinant strain, NADPH

495   can play an important role as a cofactor and that PDO production can be increased by

496   modifying the reductive pathway. It is also probable that the effect of DhaB and PDOR on

497   PDO production is highly dependent on the strain and/or culture conditions adopted.

498   Lactic acid, BDO, ethanol, and acetic acid are the major byproducts of PDO synthesis

499   in *K. pneumoniae*. Inactivation of the metabolic pathways that give rise to these products has

500   resulted in increased PDO synthesis. In particular, deletion of the *ldhA* gene encoding lactate

501   dehydrogenase has been highly beneficial. For example, by disrupting the *ldhA* gene, Xu et al.

502   (2009b) significantly increased PDO production without lactic acid production. They

503   observed increases in the PDO titer (95.4–102.1 g/L), conversion yield (0.48–0.52 mol/mol),

504   and productivity (1.98–2.13 g/L h; Table 2). Similarly, Oh et al. (2012a) achieved the high

505   PDO titer of 102.7 g/L by deleting *ldhA* in their own *K. pneumoniae* isolate. When lactic acid

506   production was eliminated, more ethanol and succinic acid were generated from the pyruvate

507   node. Reduction of these compounds has also been attempted by deleting *adhE* (encoding

508   alcohol dehydrogenase) and *frdA* (encoding fumarate reductase). Although the production of

509   ethanol and succinate was substantially reduced in the triple mutant ($\Delta ldhA\Delta adhE\Delta frdA$),

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. pneumonaie* (Celińska, 2012). The toxic effects of acetate

524   is realized even at the low concentration of 20 mM. Physiologically, acetate is a preferred

525   metabolite for many *Enterobacter* sp. because its production is accompanied by ATP

526 generation. In a typical bioreactor run for PDO production by *K. pneumoniae*, the

527 accumulation of acetic acid to >300 mM by the end is not uncommon, which often

528 completely stops glycerol fermentation. Acetate is produced by Pta-Ack and PoxB when

529 pyruvate formation is faster than its consumption, which is so-called 'overflow metabolism'

530 (Fig. 6) (De Mey et al., 2007). In *K. pneumoniae*, the contribution of the *poxB* gene is not

531 significant and its deletion does not much affect acetate production. In comparison, deletion

532 of the *pta-ack* genes greatly reduces cell growth and results in high accumulation of pyruvate

533 and pyruvate-based metabolites (Ko et al., 2017). Extensive studies to reduce or eliminate

534 acetate production have been conducted in *E. coli*. Compared to *poxB*, *ackA* and/or *pta* were

535 much more significant in reducing acetate production. However, deletion of the latter genes

536 was not beneficial because the glycolytic flux and cell yield were seriously reduced and the

537 accumulation of other byproducts such as pyruvate, lactate, and formate was greatly

538 increased (Causey et al., 2004; De Mey et al., 2007). Several indirect approaches to

539 decreasing pyruvate/acetate accumulation were investigated: stimulation of the anaplerotic

540 pathway by overexpression of pyruvate carboxylase, PEP carboxylase, or PEP carboxykinase

541 (Abdel-Hamid et al., 2001; Causey et al., 2004; Chao and Liao, 1993; Sanchez et al., 2005);

542 overexpression of acetyl-CoA synthetase (ACS); increase of the TCA cycle throughput by

543 deleting the transcriptional repressor *arcA* gene; and stimulation of the glyoxylate shunt by

544 deleting the transcriptional repressor *iclR* gene (De Mey et al., 2007; Jeong et al., 2004; Lin et

545 al., 2006). Moreover, the use of a mutant pyruvate dehydrogenase which is less sensitive to

546 inhibition by NADH has also been examined (Kim et al., 2008). In *E. coli*, these approaches

547 proved to be effective to varying extents under properly selected culture conditions. However,

548 they have not yet been fully investigated in *K. pneumoniae*.

549   To eliminate byproduct formation, reduction of glycerol flux through the oxidative

550 pathway at the glycerol node has also been attempted. The deletion of glycerol

551 dehydrogenase (*dhaD*) and/or dihydroxyacetone kinase (*dhaK*) resulted in decrease of the

552 oxidative flux and increase of the conversion yield of glycerol to PDO. In addition,

553 production of the byproducts lactate, ethanol, and succinate but not acetate decreased

554 significantly, even without blocking the pathways leading to these byproducts (Seo et al.

555 2009; Horng et al. 2010). However, cell growth and glycerol consumption rate were also

556 reduced. Interestingly, in these mutant strains, deletion of *dhaT* has almost no effect on PDO

557 production. In the absence of *dhaT*, expression of an HOR, highly homologous to YqhD, was

558 upregulated (see section 2.2.4).

559   The theoretical maximum yields of PDO on glycerol are 0.875 and 0.844 mol/mol

560 under aerobic and anaerobic conditions, respectively (Celińska, 2012). However,

561 experimentally, the yields are reported between 0.35 and 0.65 mol/mol (Saxena et al., 2009;

562 Sun et al., 2008). The low yield is mainly attributed to the accumulation of unnecessary

563 byproducts and inefficient metabolism for generating reducing equivalents (NADH) and ATP.

564 If acetyl-CoA is fully oxidized through the TCA cycle (without byproducts formation) and

565 the activity of the electron transport chain (ETC) oxidizing NADH is properly controlled, a

566 significant increase in PDO yield is expected. Attaining this goal requires extensive pathway

567 engineering of cellular metabolism. In bioreactor operation, the aeration rate should be

568 optimized. Oxygen regulates gene expression and the enzymatic reactions of glycerol

569 metabolism and determines the diversion of DHA to the PP pathway. Regeneration of NADH

570 by the TCA cycle and its oxidation by ETC are also controlled by oxygen levels.

571 **3.2 3-Hydroxypropionic acid**

572   As with PDO, 3-HP is an important platform chemical and has been selected by the

573 US Department of Energy (DOE) as one of the top 12 chemicals obtainable from biomass. 3-

574 HP is a C3 bifunctional molecule and a structural isomer of lactic acid (Werpy and Petersen,

575 2004). 3-HP can be converted into a variety of useful chemicals such as acrylic acid and acryl

24

amide (Pina et al., 2011; Kumar et al., 2013a). 3-HP can be synthesized by chemical routes

from acrylic acid, 3-propiolactone, 3-hydroxypropionitrile, allyl alcohol, vinyl acetate, and

PDO. However, none of the chemical processes is commercially feasible at present due to the

high cost of the starting materials, toxicity of intermediates, and/or the environmental

incompatibility of the processes (Jiang et al., 2009; Pina et al., 2011; Kumar et al., 2013a).

Many microorganisms, including both prokaryotes and eukaryotes, can naturally synthesize

3-HP as either an intermediate or end product through a range of metabolic pathways.

However, the production of 3-HP by these native microorganisms is too low to be

commercially meaningful (Jiang et al., 2009; Kumar et al., 2013a).

In studies pursuing commercial production of 3-HP, glycerol is first converted to 3-

HPA by GDHt. 3-HPA then is converted to 3-HP via the coenzyme A (CoA)-dependent or

CoA-independent pathway (Fig. 3). In the CoA-independent pathway, 3-HPA is oxidized to

3-HP by aldehyde dehydrogenase (ALDH); in the CoA-dependent pathway, 3-HPA is

converted to 3-HP via 3-HP-CoA and 3-HP-phosphate. In *K. pneumoniae*, both the CoA-

dependent and CoA-independent pathways are present. However, the titer of 3-HP produced

by the CoA-dependent pathway is low as <5.0 g/L (Luo et al., 2011 and 2012). High

production of 3-HP requires the disruption of oxidoreductases (e.g., DhaT, YqhD) and the

overexpression of a proper aldehyde dehydrogenase (ALDH). Ashok et al. (2013a) developed

a recombinant *K. pneumoniae* in which *dhaT* and *yqhD* were deleted, and the homologous

puuC gene encoding the NAD$^+$-dependent γ-glutamyl-γ-aminobutyraldehyde dehydrogenase

was overexpressed. The recombinant *K. pneumoniae* produced 3.8 g/L 3-HP in 12 h of flask

culture, but only under appropriate aeration (i.e., microaerobic) conditions. Under anaerobic

conditions, PDO (instead of 3-HP) was obtained as the main product, even though two

oxidoreductases were disrupted and *puuC* was highly overexpressed. In contrast, highly

aerobic conditions produced a high cell mass without much accumulation of either 3-HP or

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

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

626 h fed-batch bioreactor culture (Ashok et al., 2013b) (Table 3). However, disadvantages were

627 noted, including the requirement for large amounts of nitrate and the toxic effect of nitrite

628 generated as a reduced intermediate of nitrate. In a recent study, Li et al. (2016) optimized a

629 promoter for ALDH expression, culture conditions, and metabolic flux to achieve high-level

630 production of 3-HP in their *K. pneumoniae* isolate. One recombinant strain, *K. pneumoniae*

631 (pTAC-*puuC*) expressing *puuC* under the IPTG-inducible *tac* promoter, produced 73.4 g/L 3-

632 HP in a bioreactor with a molar yield of glycerol of 0.52 and productivity of 1.53 g/L h.

633 Further, elimination of the *ldh1*, *ldh2*, and *pta* genes elevated the titer and molar yield to 83.8

634 g/L 3-HP and 0.54 mol/mol, respectively, with decreased cell growth and productivity. This

635 titer of 3-HP is the highest produced by *K. pneumoniae* to date.

636   As another approach to addressing the problem associated with NAD^+ regeneration

637 and coenzyme B_{12} synthesis in 3-HP production, co-production of 3-HP and PDO has been

638 investigated. The simultaneous production of 3-HP and PDO can eliminate the dependency

639 on oxidative metabolism of glycerol (and electron transport chain), as the cofactor required

640 for 3-HP production is regenerated by PDO production or vice versa. Furthermore, the

641 problems associated with the expression of genes for the assimilation of vitamin B_{12} and

642 glycerol can be alleviated substantially because co-production can be performed under

643 anaerobic or microaerobic conditions (Kumar et al., 2012, 2013b). Ashok et al. (2011)

644 developed a recombinant strain of *K. pneumoniae* DSMZ by overexpressing ALDH and

645 deleting *dhaT*. With *dhaT* intact, PDOR activity was too high compared with that of ALDH,

646 even with overexpression of the latter enzyme from a multicopy plasmid. The recombinant *K.*

647 *pneumoniae* DSMZ accumulated 16.0 g/L 3-HP and 16.8 g/L PDO in 24 h, and the

648 cumulative yield of these two metabolites on glycerol was 51% (Table 3). Huang et al. (2012)

649 studied the same co-production using their *K. pneumoniae* isolate overexpressing ALDH and

650 obtained 24.4 3-HP and 49.9 g/L PDO, respectively, after 24 h under anaerobic conditions.

651   The cumulative molar yield of the two metabolites reached 0.61 (0.18 for 3-HP and 0.43 for

652   PDO). In a following study, they showed that the maximum titer of 48.9 g/L 3-HP (along

653   with 25.3 g/L PDO) was achieved at 1.5 vvm, while the highest concentration of 38.4 g/L

654   PDO (along with 16.6 g/L 3-HP) was obtained at 0.1 vvm (Huang et al., 2013a).

655   During 3-HP production from glycerol by *K. pneumoniae*, the accumulation of such

656   byproducts as lactic acid, ethanol, acetic acid, formic acid, and 2,3-BDO is a serious

657   consideration, as in the case of 1,3-PDO production (Ashok et al., 2013a,b; Li et al., 2016).

658   Deletion of *ldhA* reduces lactic acid formation and increases the 3-HP yield. However,

659   deletion of other genes such as *adhE*, *pfl*, *budBAC*, and *pta-ack* had little desired effect and

660   seriously hampered cell growth and glycerol assimilation (unpublished data). Even with the

661   co-production of 3-HP and 1,3-PDO, where oxidative glycerol assimilation was greatly

662   reduced, accumulation of these byproducts was a serious drawback (Ko et al., 2017). To

663   prevent the formation of lactic acid along with other byproducts, Kumar et al. (2013b)

664   employed resting cells of recombinant *K. pneumoniae* J2B overexpressing ALDH and devoid

665   of lactate dehydrogenase (*ldhA*). For this strain, final titers of 3-HP and PDO of 22.7 and 23.5

666   g/L, respectively, were obtained without lactic acid accumulation. The cumulative product

667   yield increased to 0.77.

668   In addition to *K. pneumoniae*, *E. coli* has been extensively studied for 3-HP

669   production from glycerol. Several successful results have been reported by Samsung Ltd. Co.

670   Korea. One study (Chu et al., 2015) used an engineered strain (*E. coli* W3110 *ΔackA-pta*

671   *ΔyqhD_dhaB_mutant gabD4*) harboring an active ALDH mutant (designated as GabD4) from

672   *Cupriavidus necator*, producing 71.9 g/L 3-HP with a productivity of 1.8 g/L h. However,

673   unlike *K. pneumoniae*, *E. coli* does not synthesize vitamin B_{12} naturally, and it was necessary

674   to add the coenzyme externally. Furthermore, a significant amount of glucose for cell growth

675   was required because for *E. coli*, glycerol is a less-preferred carbon source than glucose.

676   Consequently, the researchers at Samsung initially grew cells to a high density (40–300

677   OD_{600}) on glucose and then produced 3-HP by feeding glycerol (Chu et al., 2015; Kim et al.,

678   2014a). In comparison, *K. pneumoniae* can produce a high concentration of 3-HP growing

679   purely on glycerol at a much lower cell concentration (OD_{600}, <20) (Huang et al., 2013a,b; Li

680   et al., 2016). Together, the use of glucose for cell proliferation, exogenous addition of

681   expensive cofactor vitamin B_{12}, and low cumulative yield of 3-HP (on glucose plus glycerol)

682   increase the production cost. Thus, *K. pneumoniae* can be considered a better biocatalyst than

683   *E. coli*. However, for the commercial production of 3-HP, many other factors are important,

684   including biosafety, process stability, and downstream processing. Further studies to assess

685   the potential of these strains as hosts for 3-HP production are needed.

686   **3.3 2,3-Butanediol**

687   BDO has many applications in the pharmaceutical, biomedical, and other chemical

688   industries for the production of printing inks, perfumes, fumigants, spandex, moistening and

689   softening agents, and plasticizers (e.g., cellulose nitrate, polyvinyl chloride, polyacrylates)

690   (Celińska and Grajek, 2009; Ji et al., 2011). BDO can also be used as an antifreezing agent

691   and octane booster for petrol as is or can be converted to useful derivatives such as 1,3-

692   butadiene, and diacetyl and methyl ethyl ketone.

693   Unlike 3-HP and PDO, BDO is a product of oxidative metabolism. Of the three

694   stereoisomers of BDO, *K. pneumoniae* mainly produces the *meso* form (Ji et al., 2011). BDO

695   is synthesized from pyruvate via α-acetolactate and acetoin. The C5 intermediate, α-

696   acetolactate, is formed from two molecules of pyruvate by self-condensation catalyzed by α-

697   acetolactate synthase (ALS; *budB*). α-Acetolactate is decarboxylated to acetoin by α-

698   acetolactate decarboxylase (*budA*), and acetoin is reduced to BDO by 2,3-butanediol

699   dehydrogenase/acetoin reductase (*budC*) using NADH as a reductant. In the presence of

700   oxygen, α-acetolactate is spontaneously decarboxylated to diacetyl, further reduced to acetoin

701   by action of diacetyl reductase, and then reduced to BDO. α-Acetolactate is also the precursor

702   of branched-chain amino acids (valine, leucine, and isoleucine); thus, elimination of the *bud*

703   operon can arrest *K. pneumoniae* growth when cultured on glycerol minimal medium (Kumar

704   et al., 2016). In the anaerobic process, one NADH molecule is required for the conversion of

705   two pyruvates to one BDO, while the aerobic process requires two NADH (Fig. 5). The

706   enzyme DhaD may also contribute to BDO formation (see Section 2.2.1). In the BDO

707   pathway itself, the conversion of two pyruvates to BDO regulates or is regulated by the

708   intracellular $NADH/NAD^+$ ratio, similar to other fermentative pathways. Another important

709   role of the BDO pathway is to reduce intracellular acidification by converting acids to the

710   neutral metabolite, BDO (Celińska and Grajek, 2009; Ji et al., 2011).

711   Although BDO is one of the main byproducts of glycerol fermentation by *K.*

712   *pneumoniae* (Petrov and Petrova, 2009; Durgapal et al. 2014), its production from glycerol

713   has not been studied extensively. In few studies using glycerol as the carbon source, Petrov

714   and Petrova (2009) focused on the effects of pH and aeration with their *K. pneumoniae*

715   isolate G31. BDO production was greatest at alkaline initial pH when tested at a pH range of

716   5–8. Without pH control a sharp pH drop occurred initially due to the production of organic

717   acids, and this pH drop triggered the synthesis of BDO, which is considered the only non-

718   inhibitory compound produced by the oxidative metabolism of glycerol. Once neutral pH is

719   restored with the rising concentration of BDO, catabolism shifted toward the production of

720   ATP-generating acetic acid. The amount of BDO generated in each cycle (pH drop and

721   neutralization) was dependent on the extent of the pH drop. In one study, a BDO

722   concentration of 49.8 g/L was reached when the initial pH was set at 8.0 and no pH control

723   was given during the rest of fermentation. PDO was the major byproduct (11.6 g/L) (Table 4)

724   and its production decreased by exclusion of Co^{2+}, an essential component of coenzyme B_{12}.

725   They also found that intensive aeration led to a significant increase in BDO production; an

increase in aeration rate from 1.1 to 2.2 vvm enhanced the yield from 0.38 to 0.46 mol/mol

and productivity from 0.24 to 0.36 g/L h. BDO production was also increased by forced pH

fluctuations of the culture medium with discrete ΔpH values (1.0, 2.0, and 3.0) at

predetermined time intervals (6, 12, and 24 h). The highest BDO concentration (70.0 g/L)

was produced by increasing the pH by one unit (ΔpH = 1.0) every 12 h (Petrov and Petrova,

2010). The mechanism behind the effect of this peculiar pH change on BDO production has

not been elucidated. Although BDO is readily produced from glycerol by *K. pneumoniae*, the

volumetric productivity is low due to the long fermentation time (generally >150 h). Huang et

al. (2013b) isolated two *Klebsiella* strains that produce BDO plus acetoin with a total yield of

0.44–0.45 (g/g). One isolate utilized 64.5 g/L glycerol in 78 h and produced 24.9 g/L BDO,

3.9 g/L acetoin, and 3.2 g/L ethanol. Again, the volumetric productivity was not high.

Metabolic engineering efforts with *K. pneumoniae* to improve BDO production from

glycerol are limited. Using glucose as carbon source, Kim et al. (2014b) reported that

overexpression of the BDO pathway (*budA*, *budB*) in the *ldhA* deficient *K. pneumoniae* strain

KCTC2242 improved BDO production and resulted in 90 g/L BDO, with a productivity of

2.75 g/L. h. Because both glucose and glycerol are converted through the common

intermediate pyruvate, BDO production from glycerol is also expected to increase by

amplification of the *bud* operon. Further work with glycerol as carbon source is required to

prove this effect. Recently, co-production of BDO and PDO from glycerol has also been

attempted (Park et al., 2017). In glycerol fermentation, both alcohols always appear

simultaneously and co-production seems to be beneficial for cells to maintain redox balance

during anaerobic growth. Park et al. (2017) found that by using the *K. pneumoniae* mutant

deficient of *ldhA* and *mdh*, 50.1 g/L BDO was produced along with 63.8 g/L PDO in 75 h.

The ratio of the two alcohols varied according to aeration; with increasing aeration, the ratio

of [BDO]/[PDO] increased as observed by Petrov and Petrova (2010). In the 5,000 L pilot-

3.4 Lactic acid

Lactic acid has applications in the food, chemical, cosmetic, and pharmaceutical

industries. Lactic acid is a monomer that can be polymerized to yield the biodegradable

plastics polylactic acid (PLA) and poly(3-hydroxybutyrate-*co*-lactate). Two optical isomers

of lactic acid, D- and L-lactic acid, are produced in racemic mixtures depending on the

chemical route used. Microbial fermentation can yield optically pure isomers. Currently,

nearly all commercial lactic acid comes from microbial fermentation (Abdel-Rahman et al.,

2013; Abdel-Rahman and Sonomoto, 2016).

Because of the availability of highly efficient lactic-acid–producing microbes derived

from *E. coli*, lactic acid bacteria, and even acid-tolerant yeasts (Abdel-Rahman et al., 2013),

K pneumoniae has received little attention as a lactic acid producer. However, as previously

described, lactic acid is a major byproduct of glycerol fermentation by *K. pneumoniae*, and its

potential as a producer is high. This bacterium contains metabolic pathways for the

production of both L- and D-isomers (Fig. 7). They are synthesized through the pyruvate

and/or methylglyoxal pathways in *K. pneumoniae* (Ashok et al., 2011). When produced from

pyruvate, lactate dehydrogenase (*ldhA*) reduces pyruvate using NADH as reductant. In the

methylglyoxal route, DHAP is converted to methylglyoxal, which can be transformed to both

D- and L-lactic acid. For the production of L-lactic acid, methylglyoxal is reduced to L-

lactaldehyde, which is subsequently oxidized to L-lactic acid. Using glutathione, D-lactic acid

is obtained through simultaneous aldehyde group oxidation and keto group reduction of

methylglyoxal (Booth et al., 2003; Booth, 2005). The methylglyoxal route has several

776 disadvantages. Methylglyoxal is highly toxic, and its accumulation can severely impair other

777 metabolisms and even lead to cell death. Second, the route is energetically inefficient, with an

778 ATP yield of −1. The L- or D-lactic acid product can be converted back into pyruvate by

779 respiratory lactate dehydrogenase (Mazumdar et al., 2010, 2013).

780   Several studies showing the potential of *K. pneumoniae* as a lactic acid producer are

781 available (Table 4). For example, *K. pneumoniae* DSMZ is reported to accumulate 22.7 g/L

782 lactic acid in 24 h during co-production of 3-HP and PDO from glycerol. The titer of lactic

783 acid was more than that of any of the targeted products, and the yield of lactic acid on

784 glycerol alone was 0.31 (Ashok et al., 2011). Similarly, fed-batch cultivation of *K.*

785 *pneumoniae* BLh-1 under oxygen-limited conditions gave lactic acid as the main product,

786 outcompeting PDO; 59 g/L lactic acid was produced in 40 h when crude glycerol from

787 biodiesel synthesis without any purification was used (Rossi et al., 2013). Durgapal et al.

788 (2014) also found that *K. pneumoniae* J2B and DSMZ strains produced lactic acid at 37.0 and

789 47.8 g/L, respectively (Table 4); in both strains, lactic acid levels surpassed those of the target

790 product PDO. Recently, Feng et al. (2014) engineered *K. pneumoniae* ATCC25955 by

791 overexpressing *ldhA* and deleting *dhaT* and *yqhD* (recall that these two genes are mainly

792 responsible for PDO production). They found that the recombinant produced 142.1 g/L of

793 optically pure D-lactic acid from glycerol in fed-batch cultivation under microaerobic

794 conditions (2.5 vvm and 400 rpm). This result is the highest lactic acid concentration

795 produced from glycerol reported to date. *E. coli* has also been engineered for the production

796 of D- and L-lactate from glycerol (Mazumdar et al., 2010, 2013). For diverting glycerol flux

797 toward L-lactic acid in *E. coli*, the chromosomal copy of D-lactate dehydrogenase was

798 replaced with *Streptococcus bovis* L-lactate dehydrogenase (Mazumdar et al., 2013).

799 Moreover, gene encoding *lldD* (respiratory L-lactate dehydrogenase) was inactivated to

800 prevent the consumption of L-lactic acid, and the methylglyoxal route (Δ*mgsA*) was blocked

801   to prevent production of toxic methylglyoxal and formation of racemic mixtures of D- and L-

802   lactic acid (Fig. 7). Furthermore, the respiratory route for glycerol assimilation was

803   overexpressed (*glpK* and *glpD*) to improve the ATP yield of the metabolic pathway through

804   transfer of electrons from glycerol-3-phosphate to oxygen (i.e., oxidative phosphorylation).

805   The engineered *E. coli* strain (Δ*pflB* Δ*pta* Δ*adhE* Δ*frdA* Δ*mgsA* Δ*lldD* Δ*ldhA*::*ldhA_glpK-*

806   *_glpD*) produced 50 g/L of L-lactate from 56 g/L of crude glycerol, at a yield 93% of the

807   theoretical maximum and with high optical (99.9%) and chemical (97%) purity (Mazumdar et

808   al., 2013). The approaches proven successful in *E. coli* should be applicable to *K.*

809   *pneumoniae*. These studies, along with the results by Feng et al. (2014), suggest that *K.*

810   *pneumoniae* is a good host for the production of D- and L-isomers of lactic acid, with high

811   optical purity, from glycerol.

812   **3.5 Ethanol and other metabolites**

813   *K. pneumoniae* has also been studied for the production of ethanol, succinic acid, 2-

814   ketogluconic acid, catechol, *cis,cis*-muconic acid and 2-butanol although its potential for such

815   production has not been fully explored (Cheng et al., 2013; Jung et al., 2015; Oh et al., 2014;

816   Wang et al. 2015; Wei et al., 2013). Of these chemicals, ethanol production has been

817   relatively well studied. Ethanol is a renewable energy source and is widely used as a fuel

818   additive for partial gasoline replacement. Currently, commercial ethanol is produced from the

819   fermentation of sugar and starch. However, glycerol is one of the cheapest substrates for

820   ethanol production, estimated as nearly 40% cheaper than sugar-based production (Yazdani

821   and Gonzalez, 2007). This factor has encouraged the development of microbial strains for

822   fermentative conversion of glycerol to ethanol. The metabolic route by which ethanol is

823   synthesized from glycerol is shown in Fig. 6. Oh et al. (2011, 2012b) constructed a mutant

824   strain of *K. pneumoniae* (termed GEM167) through γ-irradiation. PDO synthesis in this

825   mutant strain was lower than that of wild type (0.2 vs. 7.93 g/L, respectively), while ethanol

826 accumulation was higher (8.6 vs. 1.1 g/L, respectively). The mechanism underlying these

827 changes remains unclear. Introduction of the pyruvate decarboxylase (*pdc*) and aldehyde

828 dehydrogenase (*adhII*) genes into mutant strains lacking lactate dehydrogenase (*ldhA*)

829 resulted in the production of 31.0 g/L (673.9 mM) ethanol, with a yield of 0.89 (mol/mol) and

830 productivity of 1.2 g/L h (Oh et al., 2012b), the highest level of ethanol production from

831 glycerol reported to date. More extensive studies including estimation of the maximum

832 achievable titer and microbial tolerance to high-concentration ethanol, both of which are key

833 factors for commercialization, are required to assess the potential to produce ethanol from

834 glycerol by the use of *K. pneumoniae*.

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

836   The design of microbial cell factories is gaining unprecedented momentum as

837 metabolic engineering is progressively aided by advances in synthetic biology and multi-

838 omics analyses. Several bio-based chemicals have already been launched successfully in the

839 marketplace, and others are in the pipeline. For a bulk chemical to be considered for

840 commercial production, the production process should achieve a product concentration of 100

841 g/L, carbon yield of 0.50 (g/g), and volumetric productivity of 2.0 g/L/h. The aforementioned

842 products produced by *K. pneumoniae* from glycerol are no exception. Challenges to the use

843 of *K. pneumoniae* as a microbial cell factory using glycerol as a carbon source are described.

844 **4.1 Pathogenicity**

845   The pathogenicity of *K. pneumoniae* is one of the major obstacles to its commercial

846 application. *K. pneumoniae* is a Bio-safety Level 2 microorganism and causes nosocomial

847 and urinary tract infections. Moreover, capsulated *K. pneumoniae* cells are difficult to

848 separate from culture broth, complicating the downstream processing. The biotechnological

849 application of this bacterium will require attenuation of its pathogenicity and reduction of

850 biosafety concerns. *K. pneumoniae* produces a number of virulence factors that contribute to

35

851   its pathogenicity, including lipopolysaccharide (LPS), capsular antigens, fimbrial adhesins,

852   siderophores, and O antigens (Albertí et al., 1993; Fang et al., 2004; Podschun and Ullmann

853   1998). The mechanism by which this bacterium causes disease is still obscure because most

854   studies have been conducted on a limited number of virulence factors (El Fertas-Aissani et al.,

855   2013).

856   In the last decade, efforts have been made to overcome the pathogenicity of *K.*

857   *pneumoniae* (Shrivastav et al., 2013), suggesting hope for the generation of nonpathogenic *K.*

858   *pneumoniae*. The two major pathogenic determinants in *K. pneumoniae* are the capsular

859   polysaccharides (CPS), which cover the entire bacterial surface, and LPS, expressed on the

860   outer bacterial membrane. LPS consists of lipid A, a core oligosaccharide, and an O-antigenic

861   polysaccharide. In a highly virulent strain of *K. pneumoniae*, mutations in genes involved in

862   LPS biosynthesis (*waaC*, *waaF*, and *wabG*) resulted in a decrease in bacterial colonization

863   and virulence (Izquierdo et al., 2003; Jung et al., 2013). Mutant strains devoid of the outer

864   core LPS were also found to be deficient in the "cell-attached" capsular polysaccharides

865   covering the bacterial surface. These mutations abolished the highly virulent characteristics

866   of pathogenic *K. pneumoniae* when tested in different animal models. Furthermore, these

867   mutants were more sensitive to several hydrophobic compounds than were wild-type strains.

868   Reintroduction of the *waaC*, *waaF*, and *wabG* genes into *K. pneumoniae* rescued the

869   pathogenic properties, confirming the role of these genes in pathogenicity.

870   Another important study was conducted by Lin et al. (2012), in which eight highly

871   conserved residues of the MagA protein (G308, G310, G334, G337, R290, P305, H323, and

872   N324) encoded by the mucoviscosity-associated gene A (*magA*) of *K. pneumoniae* were

873   subjected to site-directed mutagenesis. The *magA* gene contributes to the biosynthesis of K1

874   capsular polysaccharide (CPS), which correlates with pathogenic phenotypes including

875   mucoviscosity, serum and phagocytosis resistance, and virulence. Alanine substitutions at

876   R290A or H323A abolished MagA function, with annihilation of CPS production, serum

877   resistance, and virulence. Some *K. pneumoniae* strains showing less pathogenicity were also

878   isolated. For example, *K. pneumoniae* strain J2B produced less LPS and demonstrated a high

879   sensitivity to many antibiotics and lower pathogenicity. Moreover, its biomass was readily

880   separated from fermentation broth by centrifugation (Arasu et al., 2011). Future studies

881   should include further engineering of these less pathogenic strains to make them completely

882   nonpathogenic, followed by careful evaluation of their toxicity.

883   **4.2 Complex glycerol metabolism**

884   Although glycerol metabolism has been extensively studied, the mechanism and

885   regulation of its gene expression in *K. pneumoniae* have not been fully elucidated. The

886   diversity of glycerol metabolism should be a great advantage for *K. pneumoniae* survival in

887   different environments and for its biotechnological utilization as well. However, a high level

888   of complexity poses difficulty in the engineering of a strain to suit our purposes. Metabolic

889   complexity is likely responsible in part for the fact that, despite serious attempts, the molar

890   yield of many important products such as PDO and 3-HP is still far below the theoretical

891   maximum. Efforts based on our current knowledge, such as the overexpression of DhaB,

892   DhaT, YqhD and AldH and inactivation/deletion of GlpK, DhaD, DhaK and DhaKLM, were

893   not satisfactory (Maervoet et al. 2011; Kumar et al. 2013a; Kumar et al., 2016). Another

894   challenge is to understand how the ratio of glycerol distribution between respiratory and

895   fermentative routes is controlled. Because the K_M of glycerol kinase toward glycerol is low, it

896   has been speculated that a major fraction of glycerol flows through the respiratory route when

897   oxygen is present. However, some experimental results do not support this hypothesis. For

898   example, deletion of *glpK* does not decrease the glycerol flow through the oxidative pathway

899   in the presence of oxygen (Ashok et al. 2013a). In fact, wild-type and mutant (Δ*glpK*) strains

900   of *K. pneumoniae* demonstrate similar cell growth, glycerol consumption, and PDO

901 production profiles under (micro)aerobic conditions. These observations suggest the presence

902 and/or involvement of other kinases.

903   Another challenging issue for the use of *K. pneumoniae* is its inefficient TCA cycle

904 (Cabelli, 1955). Several genes, including isocitrate dehydrognease (*icd*), fumarase (*fumA*),

905 and malate dehydrogenase (*mdh*), in *K. pneumoniae* are significantly less transcribed than

906 those in *E. coli* (unpublished data). Furthermore, the activity of isocitrate dehydrogenase was

907 more than sevenfold lower in *K. pneumoniae* than in *E. coli*. If the TCA cycle is inefficient,

908 achieving a high cell density, which is essential for improving the productivity of target

909 metabolites, is difficult. Moreover, achieving high-yield generation of NADH with little

910 consumption of glycerol carbon is difficult. Furthermore, the overflow metabolism that

911 produces highly toxic acetate becomes more serious. These problems have been well

912 documented in PDO production. In a recent study, Kumar et al. (2016) attempted to increase

913 PDO production yield by blocking the lactate and BDO pathways, while diverting carbon

914 flux through the TCA cycle for efficient regeneration of NADH. However, this effort resulted

915 in the detrimental accumulation of pyruvate, acetate, and ethanol, causing low NADH

916 regeneration and low PDO production. Neither increased aeration nor the addition of good

917 nitrogen sources (complex nitrogen sources) alleviated the accumulation of these

918 intermediates or activated the TCA cycle. Furthermore, the accumulation of pyruvate quickly

919 terminated glycerol assimilation. *K. pneumoniae* has a well-established BDO production

920 pathway that likely compensates for its inefficient TCA cycle, avoiding the overflow

921 metabolism and carbon traffic at the pyruvate node. If this is the case, co-production of BDO

922 along with PDO or 3-HP, rather than single production of PDO or 3-HP, should be more

923 appropriate, which suits the nature of *K. pneumoniae*. In fact, when BDO and PDO were co-

924 produced, more PDO was generated than during PDO production alone. More studies are

925 required to gain a better understanding of glycerol metabolism, the slow operation of the

926   TCA cycle in *K. pneumoniae*, and its effect on the selection of target products and/or

927   development of suitable strains and processes. Otherwise, it will be difficult to exploit this

928   potential biocatalyst for commercial manufacturing of valuable chemicals.

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

930   A general but major challenge to the use of microbial cell factories for producing bulk

931   chemicals is the toxic effect of target products and metabolic intermediates at high

932   concentrations. In particular, during the production of PDO or 3-HP from glycerol, the highly

933   toxic intermediate 3-HPA is inevitably generated. Aldehydes generally damage DNA,

934   inactivate enzymes, and inhibit DNA synthesis. 3-HPA causes irreversible cessation of

935   metabolic activity and cellular growth, even at very low concentrations of 15–30 mM (Hao et

936   al., 2008; Zheng et al., 2008). Native *K. pneumoniae* does not accumulate 3-HPA at toxic

937   levels during PDO production, but in engineered strains that overexpress DhaB, high 3-HPA

938   accumulation is often experienced. To avoid 3-HPA accumulation, enzymes that consume 3-

939   HPA, ALDH (for 3-HP production), and PDOR (for PDO production) are expressed at

940   sufficiently (sometimes excessively) high levels compared with that of DhaB (Lim et al.,

941   2016). Intermediates such as methylglyoxal, DHA, DHAP, and glyceraldehyde-3-phosphate

942   are also known to be toxic.

943   Organic acids and alcohols are toxic at high concentrations. Generally, organic acids

944   are more toxic than their corresponding alcohols because they disturb the intracellular pH and

945   have anion-specific effects on metabolism (Chun et al., 2014; Warnecke and Gill, 2005).

946   When accumulated in cell membranes, organic compounds damage membrane integrity and

947   inhibit the activity of membrane-bound enzymes. In *K. pneumoniae*, cell growth and glycerol

948   assimilation are severely inhibited by high concentrations of PDO or 3-HP. Furthermore,

949   enzymes that play major roles in 3-HP and PDO production, such as DhaB, DhaT, and AldH,

950   are inhibited by 3-HP and PDO (Kumar et al. 2013b). Furthermore, *K. pneumoniae* is

951　sensitive to 24 g/L acetic acid, 26 g/L lactic acid, and 17 g/L ethanol under aerobic conditions

952　and 15, 19, and 26 g/L, respectively, under anaerobic condition (Celińska, 2012; Cheng et al.,

953　2005). The inhibition of pathways by target product(s) and/or unwanted byproducts often

954　places a limit on the maximum attainable titer of bulk chemicals.

955　　　　Several studies have reported engineered improvements in microbial tolerance against

956　biochemicals and biofuels, especially in *E. coli*. Modifications of exporter proteins, heat

957　shock proteins, membrane composition, and stress responses have been attempted. Moreover,

958　*in situ* recovery of target products has been attempted for hydrophobic targets. Some tolerant

959　strains have been developed, but unfortunately, without appreciable increases in productivity

960　(Dunlop, 2011; Dunlop et al., 2011; Jarboe et al., 2011). Such efforts to improve tolerance to

961　chemicals have not been reported for *K. pneumoniae*.

962　**4.4 Availability of glycerol**

963　　　　The global market for glycerol is known to be unpredictable and complex. Glycerol in

964　the current market mainly is from the biodiesel industry, and its supply is directly affected by

965　the use of biodiesel. In contrast, glycerol demand depends on many industries that use it as

966　feedstock. Between 2001 and 2011, the price varied significantly, from $0.70/kg to $1.70/kg

967　for pure glycerol and from $0.04/kg to $0.33/kg for crude glycerol (Ayoub and Abdullah,

968　2012; Ciriminna et al., 2014; Kong et al., 2016; Quispe et al., 2013). Nevertheless, glycerol

969　prices, for pure as well as crude, have come down significantly since 2004, when many

970　biodiesel production plants initiated operation. The countries producing biodiesel in large

971　quantities (in million liters) include Germany (2900), Brazil (2300), Argentina (2100), France

972　(2000), USA (1200), Spain (1100), Italy (800), Indonesia (700), and Thailand (600) (Kong et

973　al., 2016). The Asia-Pacific region overtook Europe as the largest glycerol market in the

974　world in 2009 and has remained so ever since. Market growth is driven by increases in the

975 uses for glycerol in such sectors as pharmaceuticals, personal hygiene, and food and beverage

976 production (Quispe et al., 2013).

977 The production of crude glycerol is expected to reach 6 million tons by 2025

978 (Ciriminna et al., 2014). At present, the surplus of glycerol from biodiesel and oleochemicals

979 is assumed to be sufficient to meet these new demands. However, the scenario may change in

980 the future with rapid growth of glycerol-based industries and a limited supply of crude

981 glycerol. For example, if 3-HP produced from glycerol were used as a substitute for the

982 chemical acrylic acid (global production, 5.85 million tons as of 2014), approximately 7

983 million tons of glycerol/year would be needed. If we include other platform chemicals such

984 as PDO, BDO, and ethanol, the demand will be even higher, much more than the expected

985 supply. The growth of glycerol-based biotechnology will eventually be constrained by the

986 supply and price of crude glycerol as a carbon source.

987 **5. Concluding remarks**

988 Glycerol is an excellent substrate for the production of biochemicals and biofuels.

989 Although the biodiesel industry has grown rather slowly in recent years, crude glycerol is still

990 cheap and generated in large quantities. *K. pneumoniae* has already been successfully

991 employed for the production of PDO, 3-HP, BDO, lactic acid, and ethanol from glycerol at

992 industrially attractive levels. Other metabolites such as acetic acid, pyruvic acid, malic acid,

993 fumaric acid, alanine, *n*-butanol, 1,2-propanediol, propionic acid, formic acid, and hydrogen

994 can also be produced from glycerol by *K. pneumoniae*, although these have not yet been

995 extensively explored. At present, sugar-based bioprocesses are prevalent, and no bioprocesses

996 employing *K. pneumoniae* and glycerol are commercially available. The pathogenicity of *K.*

997 *pneumoniae* and other technical issues are existing challenges to its use. However,

998 advancements in the fields of metabolic engineering, synthetic biology, systems biology, and

999 evolutionary engineering will enable us to further exploit the advantageous nature of *K.*

1000 *pneumoniae* as a biocatalyst and glycerol as a carbon source. A better understanding of

1001 glycerol metabolism in *K. pneumoniae* and improvements in its performance as a microbial

1002 cell factory should make this organism an attractive alternative to current sugar-based

1003 methods for the production of biochemicals and biofuels.

1004 **Acknowledgement**

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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

3-Hydroxypropionic acid (3-HP) ← Glycerol → 1,3-Propanediol (PDO)

1,2-Propanediol ← DHAP → Lactic acid

Succinic acid ← PEP → Propionic acid

2,3-Butanediol (BDO) ← Pyruvic acid → Lactic acid

Formic acid/Hydrogen

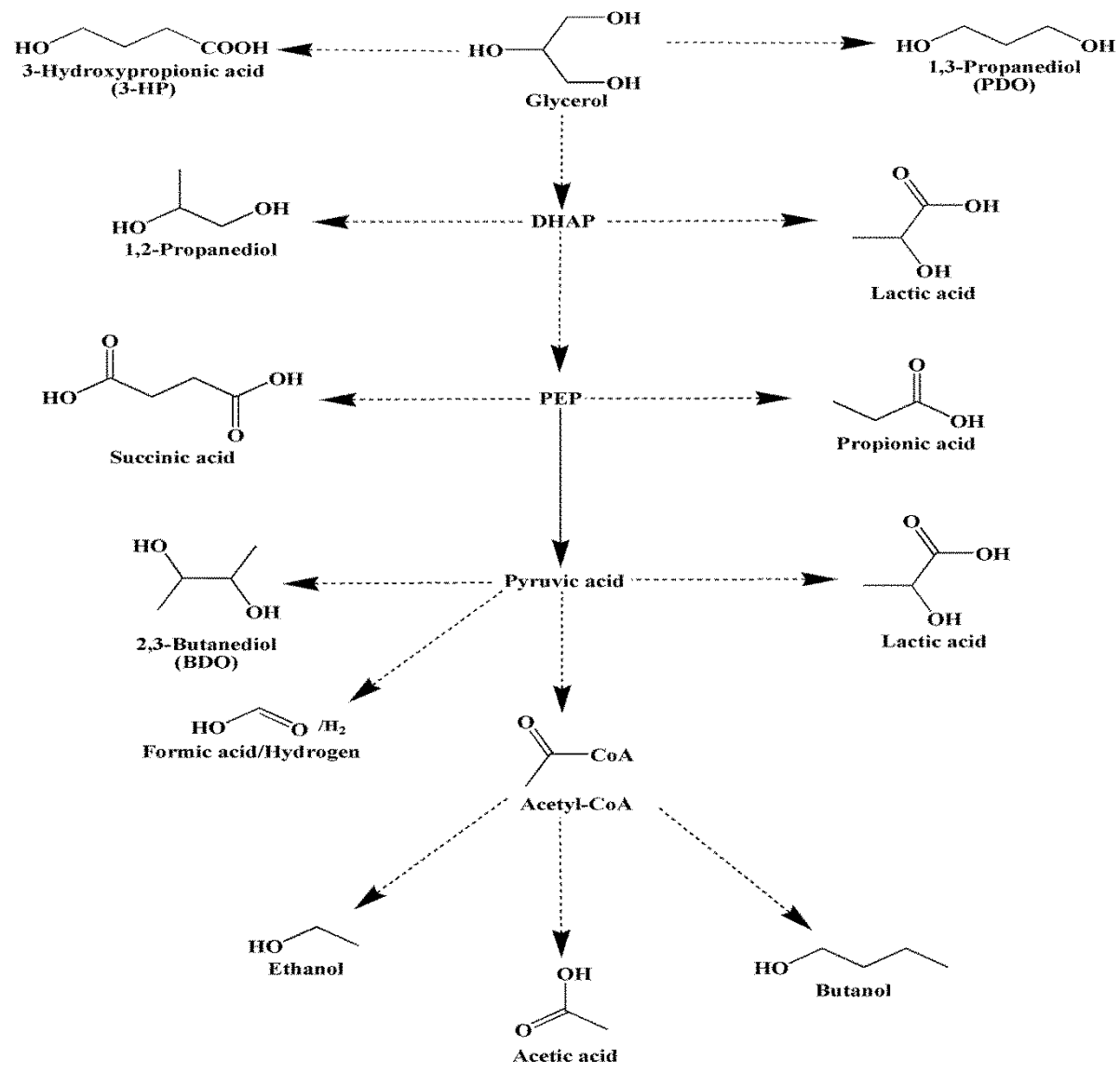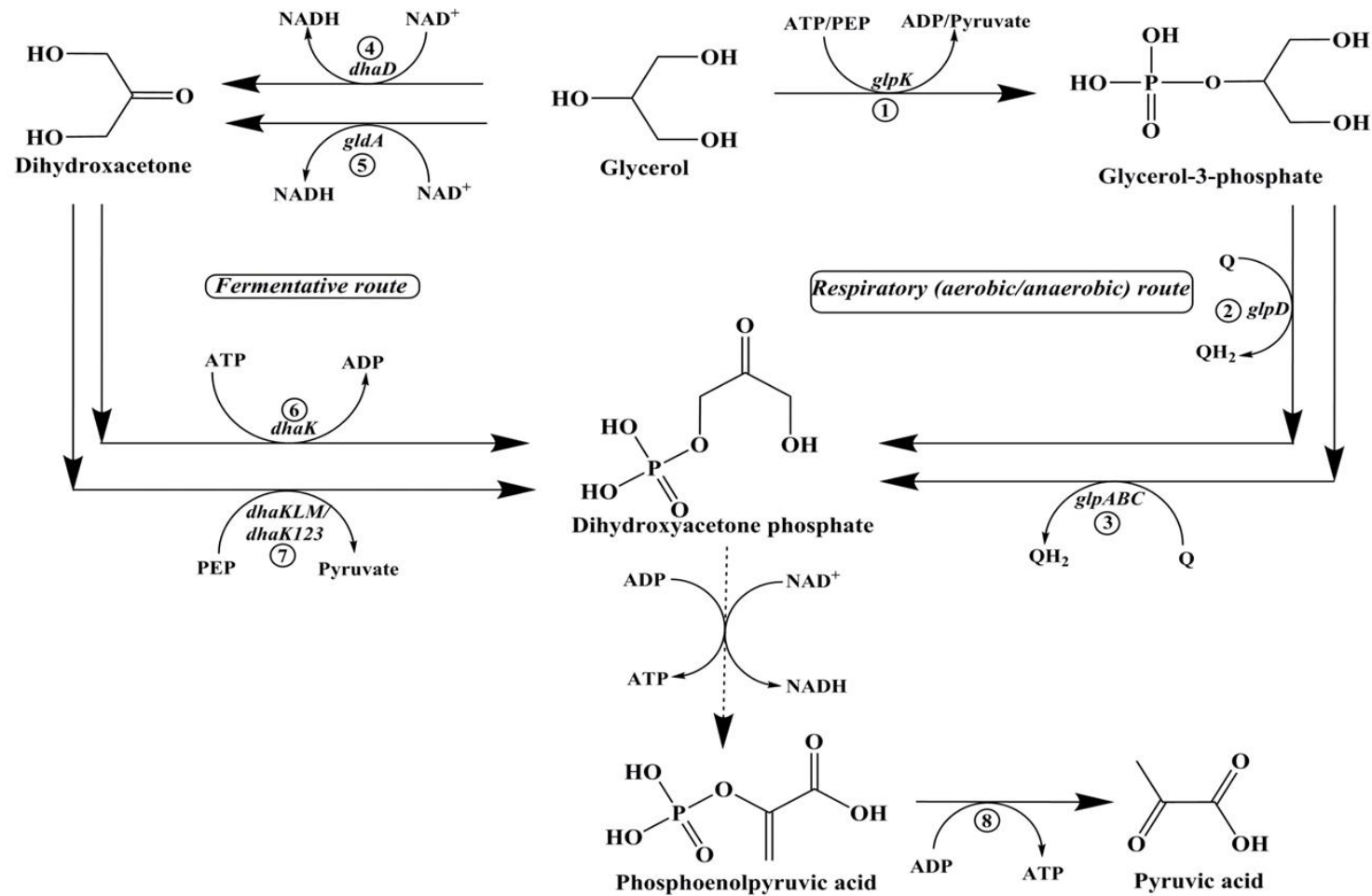Acetyl-CoA → Ethanol, Acetic acid, Butanol

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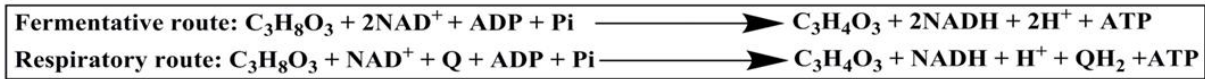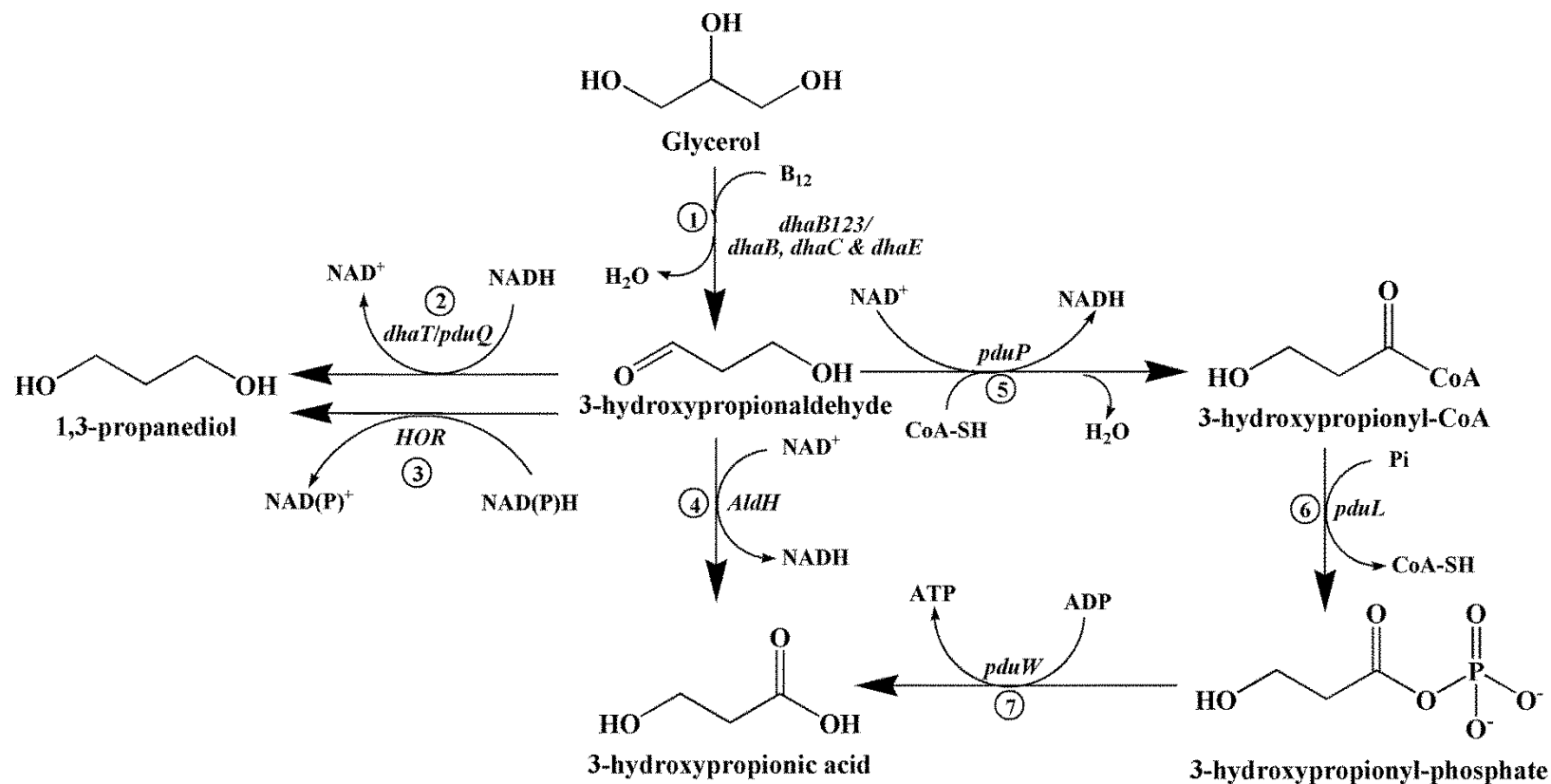
Fermentative route: $C_3H_8O_3 + 2NAD^+ + ADP + Pi \longrightarrow C_3H_4O_3 + 2NADH + 2H^+ + ATP$

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

Figure 3

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
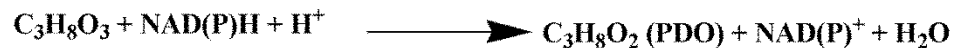
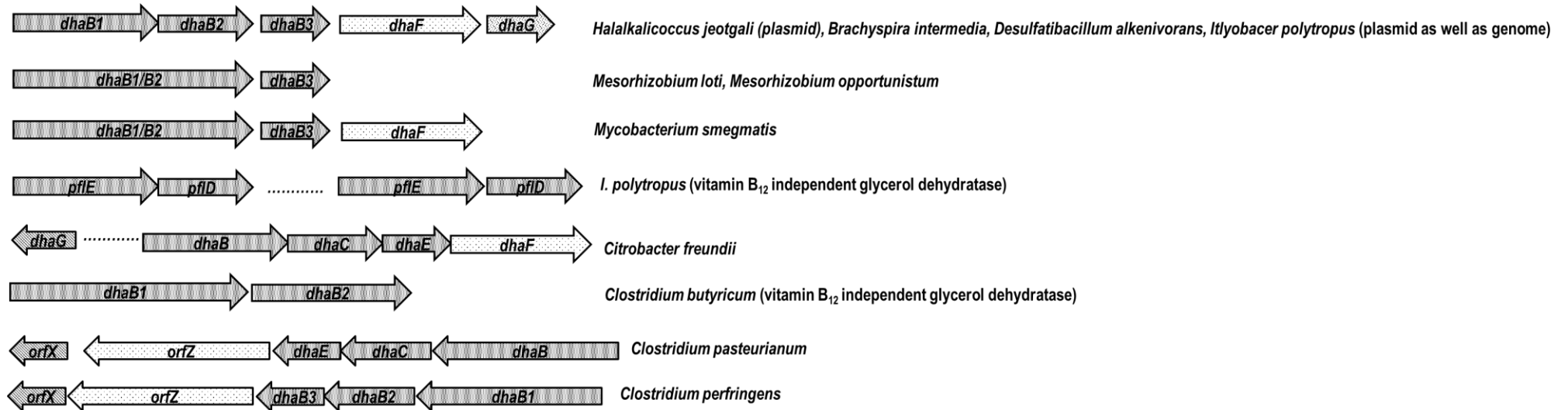CoA-independent route: $C_3H_8O_3 + NAD^+ \longrightarrow C_3H_6O_3$ (3-HP) $+ NADH + H^+$

CoA-dependent route: $C_3H_8O_3 + NAD^+ + ADP + Pi \longrightarrow C_3H_6O_3$ (3-HP) $+ NADH + H^+ + ATP$

$C_3H_8O_3 + NAD(P)H + H^+ \longrightarrow C_3H_8O_2$ (PDO) $+ NAD(P)^+ + H_2O$

3

Figure 4

A)

B)

dhaB1 — dhaB2 — dhaB3 — dhaF — dhaG   *Halalkalicoccus jeotgali (plasmid), Brachyspira intermedia, Desulfatibacillum alkenivorans, Itlyobacer polytropus (plasmid as well as genome)*

dhaB1/B2 — dhaB3   *Mesorhizobium loti, Mesorhizobium opportunistum*

dhaB1/B2 — dhaB3 — dhaF   *Mycobacterium smegmatis*

pflE — pflD .... pflE — pflD   *I. polytropus (vitamin B_{12} independent glycerol dehydratase)*

dhaG .... dhaB — dhaC — dhaE — dhaF   *Citrobacter freundii*

dhaB1 — dhaB2   *Clostridium butyricum (vitamin B_{12} independent glycerol dehydratase)*

orfX — orfZ — dhaE — dhaC — dhaB   *Clostridium pasteurianum*

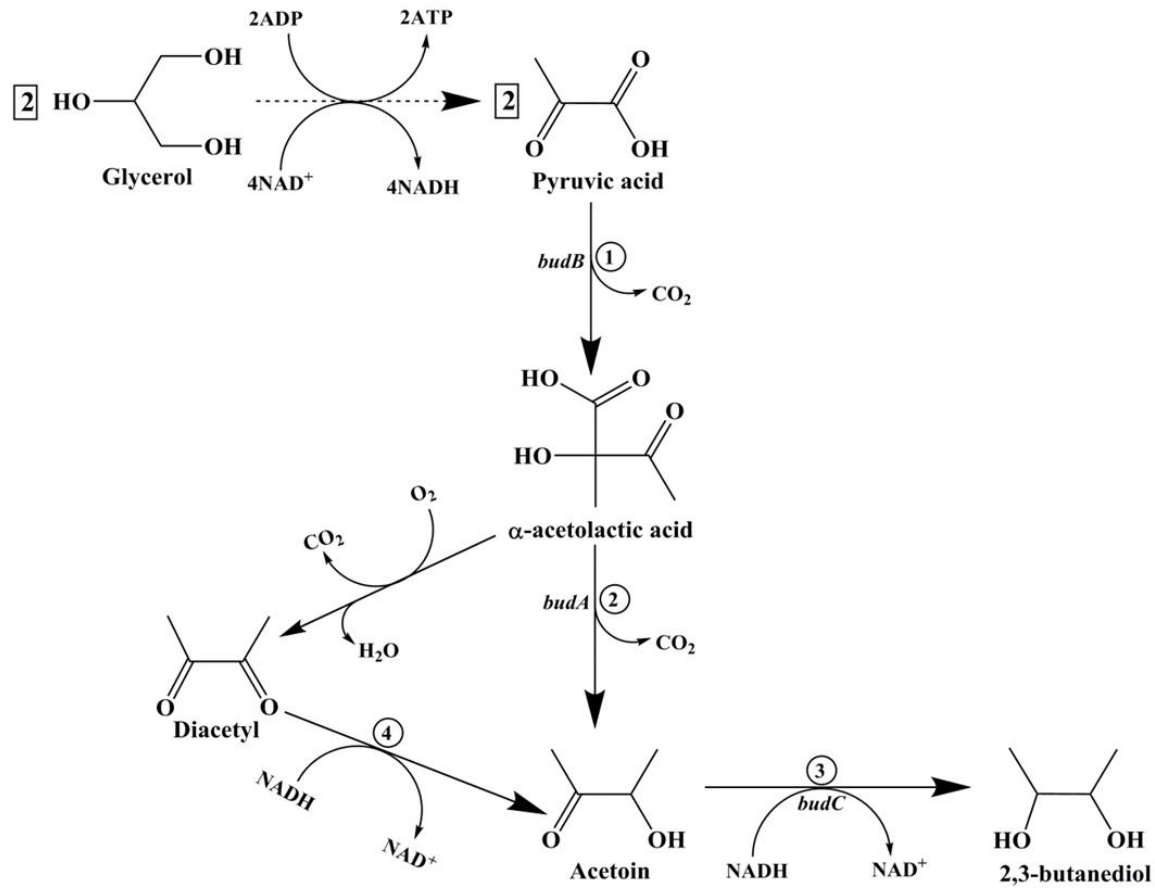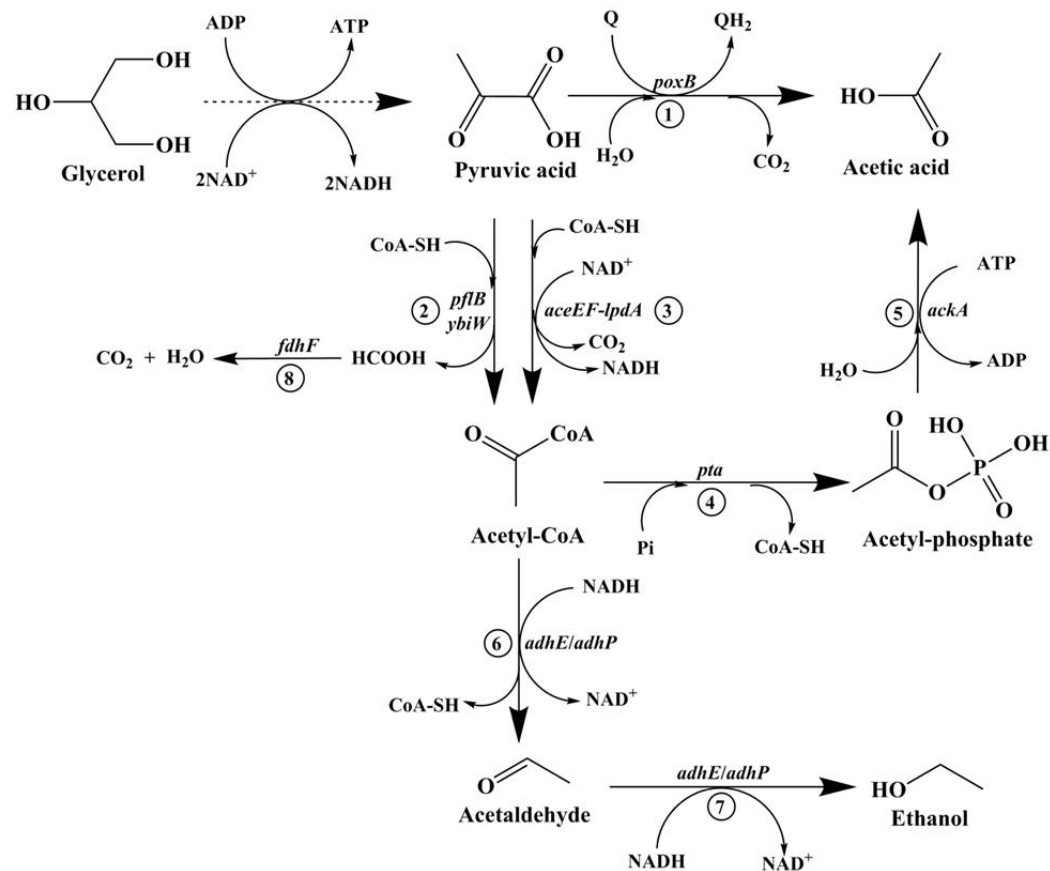orfX — orfZ — dhaB3 — dhaB2 — dhaB1   *Clostridium perfringens*

Figure 5

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

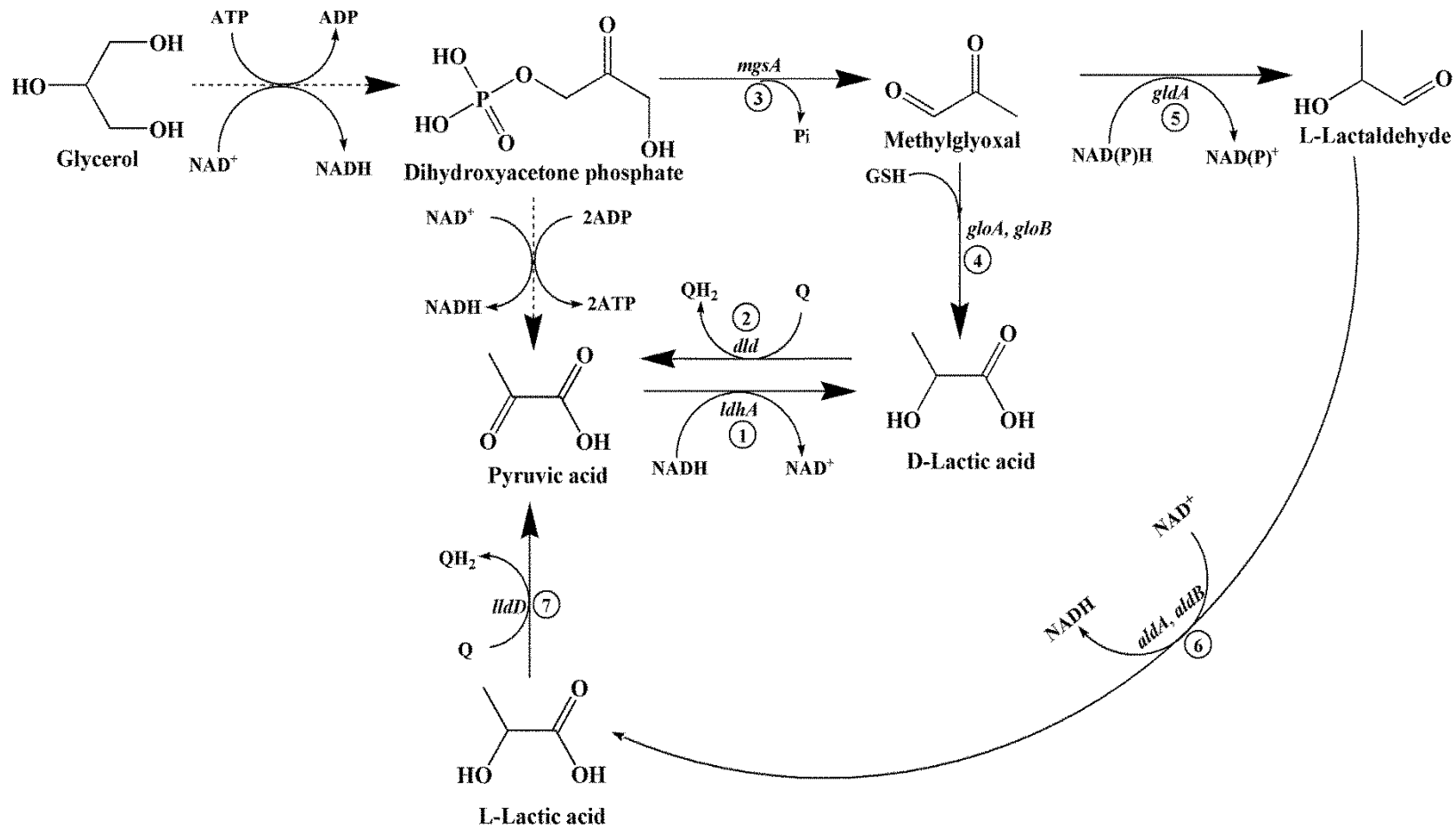Anaerobic route: $2C_3H_8O_3 + 3NAD^+ + 2ADP + 2Pi \longrightarrow C_4H_{10}O_2 + 3NADH + 3H^+ + 2CO_2 + 2ATP$

Aerobic route: $2C_3H_8O_3 + 2NAD^+ + 0.5O_2 + 2ADP + 2Pi \longrightarrow C_4H_{10}O_2 + 2NADH + 2H^+ + 2CO_2 + H_2O + 2ATP$
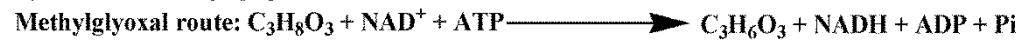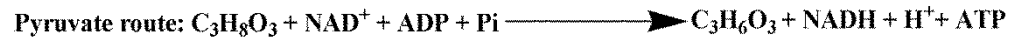
Figure 6

Key enzymes: 1- Pyruvate oxidase; 2-Pyruvate-formate lyase, putatative formate acetyltransferanse 3- Pyruvate dehydrogenase complex; 4- Phosphoacetyl transferase
5- Acetate kinase; 6 and 7- Bifunctional (acetaldehyde/alcohol) dehydrogenase/putative alcohol dehydrogenase; 8-Formate dehydrogenase, part of the formate hydrogenlyase complex
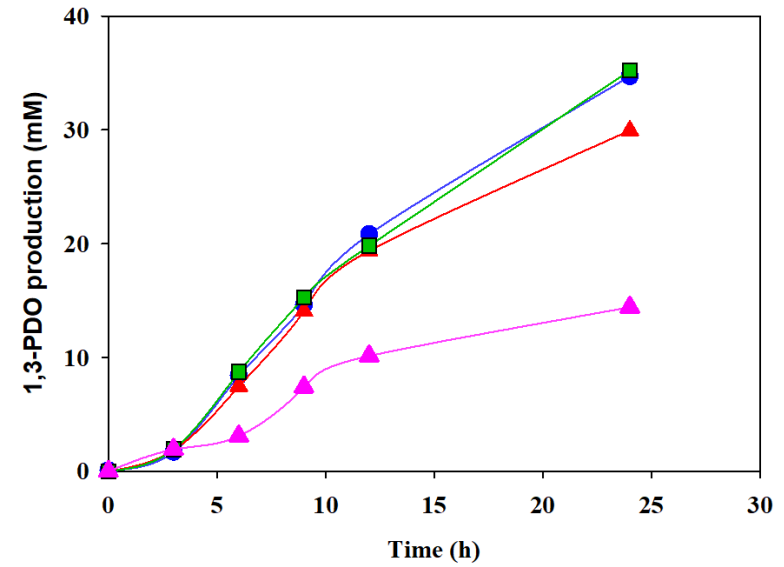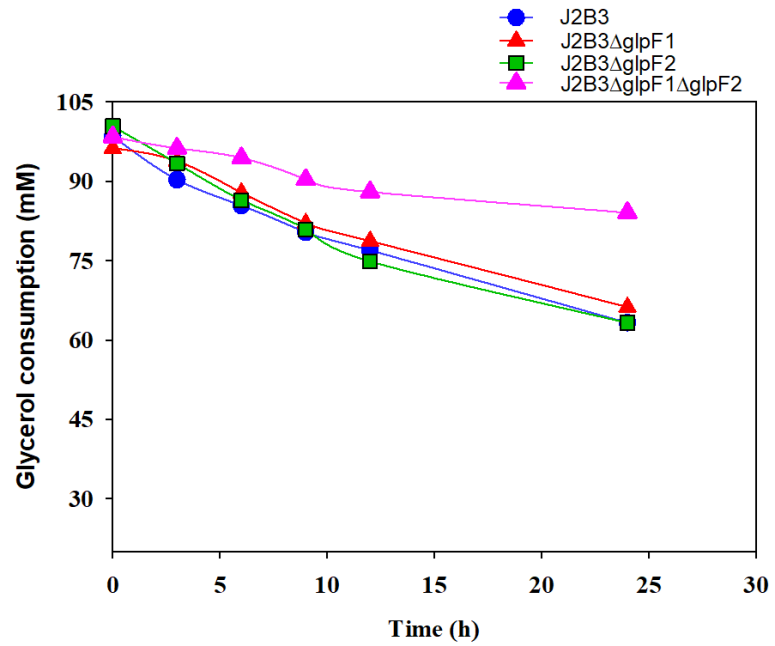
PFL route: $C_3H_8O_3 + ADP + Pi \longrightarrow C_2H_6O + HCOOH + ATP$

PDHC route: $C_3H_8O_3 + NAD^+ + ADP + Pi \longrightarrow C_2H_6O + NADH + H^+ + CO_2 + ATP$

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).

Pyruvate route: $C_3H_8O_3 + NAD^+ + ADP + Pi \longrightarrow C_3H_6O_3 + NADH + H^+ + ATP$

Methylglyoxal route: $C_3H_8O_3 + NAD^+ + ATP \longrightarrow C_3H_6O_3 + NADH + ADP + Pi$

7

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*

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

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

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

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

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

ΔbudC_fdh

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

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

Strain	Carbon source(s)	Aeration condition	Titer* (g/L)	3-HP yield on glycerol (mol/mol)	Productivity (g/L. h)	Reference
K. pneumoniae DSM 2026 Δ *dhaT_ puuC*	Glycerol	Microerobic	16.0 (16.8)	0.23	0.67	Ashok et al. 2011
K. pneumoniae WM3 *pUC18kan_aldHec*	Glycerol	Anaerobic	24.4 (49.3)	0.18	1.02	Huang et al. 2012
K. pneumoniae J2B _ *KGSADH*	Glycerol	Anaerobic	11.3 (15.9)	0.27	0.94	Kumar et al. 2012
K. pneumoniae Δ*glpK*Δ*dhaT_puuC*	Glycerol	Anaerobic	22.0 (5.9)	0.30	0.46	Ashok et al. 2013a
K. pneumoniae Δ*dhaT*Δ*yqhD_dhaB_puuC*	Glycerol	Aerobic	28.1 (3.3)	0.40	0.58	Ashok et al. 2013b
K. pneumoniae WM3 *pUC18kan_aldHec*	Glycerol	Microaerobic	48.9 (25.3)	0.41	1.75	Huang et al. 2013a
K. pneumoniae J2B Δ *ldhA _ KGSADH*	Glycerol	Microaerobic	22.7 (23.4)	0.35	0.38	Kumar et al. 2013b
E. coli W3110 *ΔackA-pta ΔyqhD_dhaB_mutant gabD4*	Glucose & glycerol	Aerobic	71.9	-	1.8	Chu et al. 2015
K. pneumoniae DSM 2026 Δ *ldh1*Δ*ldh2*Δ*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

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

Δ ldhA _ KGSADH							
K. pneumoniae BLh-1	Fed-batch	Glycerol	Microaerobic	59.0	0.48	1.48	Rossi et al. 2013
K. pneumoniae J2B	Fed-batch	Glycerol	Microaerobic	37.0	0.32	0.77	Durgapal et al. 2014
K. pneumoniae DSMZ	Fed-batch	Glycerol	Microaerobic	47.8	0.39	1.00	Durgapal et al. 2014
K. pneumoniae ATCC25955 *ΔdhaT_ ΔyqhD_ ldhA*	Fed-batch	Glycerol	Microaerobic	142.1 (D-isomer)	0.84	2.96	Feng et al. 2014
E. coli MG1655 *Δpta_ΔadhE_ΔfrdA-Δdld_glpK_glpD*	Fed-batch	Glycerol	Microaerobic	32.0 (D-isomer)	0.82	0.54	Mazumdar et al. 2010
E. coli MG1655 *ΔpflB_Δpta_ΔadhE_ΔfrdA _ΔmgsA_ΔlldD_ΔldhA::ldhA_glpK-_glpD*	Fed-batch	Glycerol	Microaerobic	50.0 (L-isomer)	0.92	0.60	Mazumdar et al. 2013

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

Kumar, Vinod