Insights into an alternative pathway for glycerol metabolism in a glycerol kinase deficient *Pseudomonas putida* KT2440

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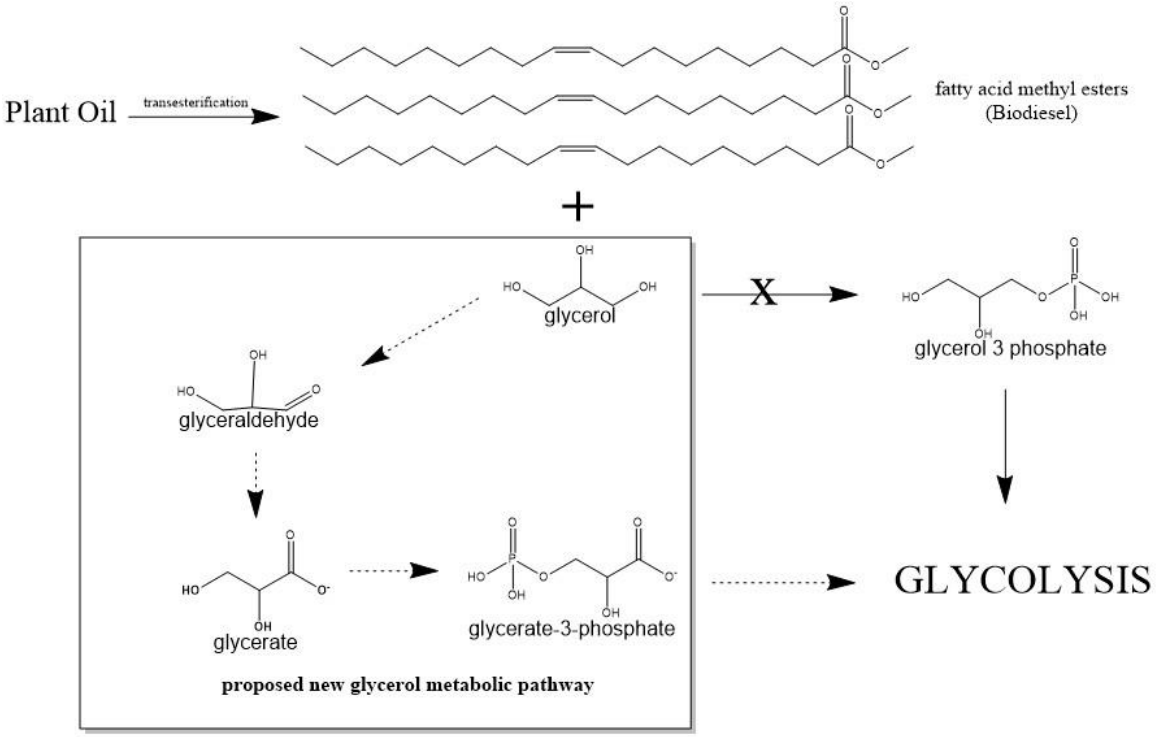
Abstract

Pseudomonas putida KT2440 is known to metabolise glycerol via glycerol-3-phosphate using glycerol kinase an enzyme previously described as critical for glycerol metabolism (1). However, when glycerol kinase was knocked out in *P. putida* KT2440 it retained the ability to use glycerol as the sole carbon source, albeit with a much-extended lag period and 2 fold lower final biomass compared to the wild type strain. A metabolomic study identified glycerate as a major and the most abundant intermediate in glycerol metabolism in this mutated strain with levels 21- fold higher than wild type. Erythrose-4-phosphate was detected in the mutant strain, but not in the wild type strain. Glyceraldehyde and glycraldehyde-3-phosphate were detected at similar levels in the mutant strain and the wild type. Transcriptomic studies identified 191 genes that were more than 2-fold upregulated in the mutant compared to the wild type and 175 that were down regulated. The genes involved in short chain length fatty acid metabolism were highly upregulated in the mutant strain. The genes encoding 3-hydroxybutyrate dehydrogenase were 5.8-fold upregulated and thus the gene was cloned, expressed and purified to reveal it can act on glyceraldehyde but not glycerol as a substrate.


Plant Oil
transesterification
fatty acid methyl esters
(Biodiesel)
+
glycerol
X
glycerol 3 phosphate
glyceraldehyde
glycerate
glycerate-3-phosphate
proposed new glycerol metabolic pathway
GLYCOLYSIS

31

32

33 **Introduction**

34 Glycerol is a major by-product of plant oil processing. It is also the major by-product of
35 biodiesel production (10 weight %). An increase in production of biodiesel (2) has led to an
36 increase in the availability of glycerol (3). Glycerol could be an interesting substrate for
37 fermentative production of value added products such as 1,3 propanediol, dihydroxyacetone
38 and polyhydroxyalkanoates (4,5). Polyhydroxyalkanoates (PHA) are polymers that are
39 naturally synthesized and stored in a range of microorganisms including *Pseudomonads* (6).
40 *Pseudomonas putida* KT2440 is a model organism for biocatalysis and synthetic biology.
41 While *P. putida* KT2440 can use glycerol as the sole carbon and energy source for growth,
42 glycerol metabolism in this strain has not been fully characterized. However, the pathway for
43 glycerol metabolism has been extensively studied in the opportunistic pathogen *Pseudomonas*
44 *aeruginosa* (7–9). As there is high sequence identity in the proposed glycerol metabolic
45 pathway genes in *P. putida* KT2440 with genes in *P. aeruginosa*, some information about
46 glycerol metabolism in *P. putida* KT2440 can be inferred from the research into *P. aeruginosa*
47 (10). In *P. aeruginosa*, the first step in glycerol metabolism is the facilitated diffusion across
48 the cytoplasmic membrane by a glycerol diffusion facilitator (GlpF). The glycerol is then
49 retained intracellularly through phosphorylation by a glycerol kinase (GlpK) to form glycerol-
50 3-phosphate, which cannot diffuse back through the cytoplasmic membrane. In *P. aeruginosa*,
51 the genes for GlpF and GlpK are organised together on one operon (8). The glycerol-3-
52 phosphate is converted to dihydroxyacetone-3-phosphate by a cytoplasmic-membrane
53 associated glycerol-3-phosphate dehydrogenase (GlpD) (11). The Glp operons (GlpFK and
54 GlpD) are negatively regulated by the transcriptional regulator GlpR in *P. aeruginosa* and *P.*
55 *putida* KT2440 (Schweizer and Po, 1996, Escapa *et al.*, 2013). Glycerol-3-phosphate interacts
56 with the GlpR protein, allowing transcription of the glycerol operon. Therefore glycerol-3-
57 phosphate is the true effector of the system. A non-specific kinase must be capable of acting

58 on glycerol or the repression must be leaky because some glycerol-3-phosphate is required to
59 stop repression of the glycerol operon. This repression by GlpR explains the long lag phase
60 when *P. putida* KT2440 is grown on glycerol as the sole carbon and energy source (10).

61 We constructed a knockout mutant of the glycerol kinase gene (*glpK*). Deletion of this gene
62 severely retarded growth on glycerol as the sole carbon source but did not remove it completely.
63 We therefore undertook transcriptomic and metabolomic studies to understand the metabolism
64 of glycerol in this strain in the absence of glycerol kinase activity.

65 **Methods**

66 **Strain Manipulation**

67 The Δ*glpK* mutant was constructed by replacing the coding region of the gene with a selective
68 gentamycin resistance cassette as previously described for polyphosphate kinase (*ppk*) gene
69 deletion (12). A complemented mutant strain was produced with an inducible copy of the wild-
70 type *glpK* harboured on a pJB861 expression vector. The resulting *glpK* gene was now under
71 the control of a P_m promoter which is stimulated by the XylR protein in the presence of an *m*-
72 toluic acid inducer.

73 **Growth and maintenance of strains**

74 *P. putida* KT2440 was maintained on LB agar supplemented with carbenicillin (50 mg/l). *P.*
75 *putida* KT2440 ΔglpK was maintained on LB agar supplemented with gentamycin (50 mg/ml).

76 Cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml of Minimal Salt Medium
77 (MSM) (13). For biomass determination, flasks were supplemented with 20 mM substrate and
78 incubated at 30°C, shaking at 200 rpm for 48 h. Cells were then harvested, lyophilised and
79 weighed for cell dry weight determination. For growth curves, flasks were supplemented with
80 50 mM glycerol and incubated at 30°C, shaking at 200 rpm for 72 h for the wild type cells and

81 300 h for *Δglpk* cells. Samples were taken periodically and OD_{540} measured in a
82 spectrophotometer to estimate growth.

83 Strains harbouring an exogenous copy of the *glpK* gene, located on the inducible expression
84 vector pJB861, were used to examine the effect of gene complementation. These strains were
85 grown in the same way as the wild type and *ΔglpK* mutant except for the addition of *m*-toluic
86 acid (as a sodium toluate salt). Transcription is initiated from the P_m promoter due to the
87 interaction of the aromatic *m*-toluic acid with the XylS protein, constitutively expressed by the
88 same plasmid expressing the *glpK* gene.

89 **Preparation of samples for metabolomics analysis**

90 *P. putida* KT2440 and Δglpk cells were grown as for growth curves until they reached an OD_{540}
91 between 0.5 and 1. 9 ml of 100 % methanol was aliquoted into polypropylene tubes, the tubes
92 were weighed and placed in a -50˚C ethanol bath. Cells (1.5ml) were added into the tubes and
93 the tubes vortexed. Tubes were weighed again to determine the weight of cells added and then
94 centrifuged at 5000 rpm for 5 minutes at -10˚C. The supernatant was discarded and the cell
95 pellets stored at -80˚C. To extract metabolites, 1 ml of 80 % (v/v) methanol was added to the
96 pellet and the pellet was redissolved completely. The resuspended cells were transferred to an
97 Eppendorf tube and incubated at 95˚C for 5 minutes with vigorous shaking. The tubes were
98 placed on ice for 5 minutes and then centrifuged at 5,000 rpm for 5 minutes at -10˚C.
99 Supernatants were transferred to a clean Eppendorf tube and stored at -80˚C before analysis.

100 **Metabolomics procedure**

101 200 µL cell-metabolite methanol extracts were added to Inno-Sil desactivated glass-vials (CS
102 Chromatography Service GmbH, Langerwehe, Germany). Subsequently 50 µL of 20 mg/mL
103 O-methoxyamine hydrochloride in pyridine to prevent degradation of ketone and aldehyde
104 groups during drying process *and* 4 µL of 1 mM m-erythritol (as internal standard) were added

105 to the metabolite extract and intensively mixed on a vortex shaker. Samples were dried for 6 h
106 using a speed vacuum concentrator system (1000 rpm, 30˚C) equipped with a freeze trap. Dried
107 samples were resuspended for about 3 min on a vortex in 50 µL freshly prepared O-
108 methoxyamine hydrochloride solution (20 mg/mL) in pyridine. For methoximation (1st step
109 derivatization) of carbonyl moieties into corresponding oximes with o-methoxyamine the
110 samples were incubated at 80°C for 30 minutes with vigorous shaking. After a brief
111 centrifugation at 1000 rpm, the procedure was proceeded by the 2nd derivatization step the
112 silylation of polar functional groups (incl. –COOH, -OH, -NH and –SH) to reduce polarity,
113 increase thermal stability and volatility giving TMS-MOX derivatives of the glycolytic
114 intermediates(14,15). 50 µL of N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) were
115 added and intensely mixed for 3 minutes. Subsequently, the samples were incubated at 80°C
116 for additional 30 minutes with vigorous shaking. After brief centrifugation at 1000 rpm the
117 samples were used for immediate GC-MS analysis or stored at 4˚C for maximal 3 d.

118 Samples were analyzed on a TSQ 8000 Triple-Quadrupole MS equipped with PTV-injector
119 (split 1:50; 2µl injection volume) and autosampler (Thermo-Fisher Scientific).
120 Chromatographic separation occurred on a CP-9013 capillary column VF-5ms (Agilent; 30m
121 x 0,25mm x 0,25µm + 10m EZ-guard; oven-program: initial 4 min at 60°C, increase 20°C/min
122 to 320°C, final hold 10 min; scan masses 50-800). The separated compounds were ionized by
123 electron ionization (EI) for reproducible and compound-specific spectra to confirm chemical
124 identities by comparing measured spectra with those of existing spectral libraries (e.g., Golm
125 metabolome database (16)) and standards. Generated ms-spectra were analyzed using Xcalibur
126 and AMDIS to differentiate and identify unknown / well-known metabolites and sort co-eluting
127 peaks. Chemical structures of compounds without reliable standards and retention times were
128 analyzed and identified using the commercial NIST-database.

129 **Preparation of RNA samples for transcriptomic analysis**

130 *P. putida* KT2440 wild type and Δglpk cells were grown as for growth determination and
131 harvested for RNA extraction at mid-log growth phase (approx. OD_{540} of 1). RNA was
132 extracted using a GeneJET RNA purification kit (Thermo Scientific, Dublin, Ireland) according
133 to the manufacturer's instructions. Samples were sent in duplicate to Baseclear (Leiden,
134 Netherlands) for transcriptomic analysis.

135 **Transcriptomics procedure**

136 This procedure was performed at Baseclear (Leiden, Netherlands). Ribosomal RNA molecules
137 were depleted from bacterial total RNA using the Epicentre bacteria Ribo-zero rRNA depletion
138 kit. The dUTP method was used to generate strand-specific mRNA-seq libraries (17,18). The
139 Illumina TruSeq stranded RNA-seq library preparation kit was used. The mRNA was
140 fragmented and converted to double-stranded cDNA. DNA adapters with sample-specific
141 barcodes were ligated and a PCR amplification performed. The library was size-selected using
142 magnetic beads, resulting in libraries with insert sizes in the rate of 100-400 bp. The libraries
143 were diluted, clustered and sequenced on an Illumina HiSeq 2500 instrument. The data
144 produced was processed by removing the sequence reads that are of too low quality and
145 demultiplexing based on sample specific barcodes. An additional filtering step was performed
146 using in house scripts to remove reads containing adaptor sequences of Illumina PhiX control
147 sequences.

148 **Bioinformatics: transcriptome analysis**

149 Transcriptome analysis was also performed at Baseclear. Sequence reads were additionally
150 filtered and trimmed based on Phred quality scores. The filtered/trimmed reads were aligned
151 against the reference sequence AE015451.2 (*Pseudomonas putida* KT2440) using the CLCbio
152 "RNA-Seq" software. Normalised expression values were calculated and compared between

153 the samples. P-values were determined to assign the significance of expression differences
154 between samples. RPKM was the expression measure used. This is defined as the reads per
155 kilobase of exon model per million mapped reads (19). It seeks to normalize for the difference
156 in number of mapped reads between samples as well as transcript length. It is given by dividing
157 the total number of exon reads by the number of mapped reads (in millions) times the exon
158 length (in kilobases). Statistical analysis was performed using Baggerly et al's Beta-binomial
159 test (20). It compared the proportions of counts in a group of samples against those of another
160 group of samples and is suited to cases were replicates are available in the groups. The samples
161 were given different weights depending on their sizes (total counts). The weights are obtained
162 by assuming a beta distribution on the proportions in a group, and estimating these, along with
163 the proportion of a binomial distribution, by the method of moments. The result is a weighted
164 t-type test statistic.

165 **Generation of pET45b construct for His tag purification of *P. putida* KT2440 3-**
166 **hydroxybutyrate dehydrogenase (hbdH) protein**

167 Genomic DNA was isolated from 1 ml of *P. putida* KT2440 grown in LB at 30˚C, shaken at
168 200 rpm for 16 h using a GeneJET genomic DNA purification kit (Thermo Scientific, Dublin,
169 Ireland) according to the manufacturer's instructions. The *hbdH* gene was amplified from the
170 genomic DNA. The PCR product was gel purified from a 1% agarose gel using a GeneJET gel
171 extraction kit (Thermo Scientific, Dublin, Ireland) and ligated into pGEM-T Easy vector
172 (Promega, Madison, WI) using T4-DNA ligase. The ligation mixture was transformed in XL-
173 10 gold competent cells (Stratagene, Agilent Technologies, Santa Clara, CA) by heat shock
174 according to the manufacturer's instructions. Colonies were tested by PCR for the presence of
175 the hbdH gene. Positive colonies were inoculated into 2 ml of LB medium supplemented with
176 50 µg/l carbenicillin and grown for 16 h at 37˚C, shaking at 200 rpm. Plasmid DNA was
177 extracted from these cultures using a GeneJET plasmid miniprep kit (Thermo Scientific,

178 Dublin, Ireland) and sent to GATC (Konstanz, Germany) for sequencing. The *hbdH* gene was
179 excised from a sequence verified plasmid using restriction endonucleases (Promega, Madison,
180 WI). The expression vector pET45b (Novagen, Madison, WI) was also digested with the same
181 enzymes. The gene was ligated into the digested vector with T4 DNA ligase to generate the
182 pET45b_*hbdH* expression vector. The ligation mixture was transformed into BL-21 Gold
183 competent cells (Stratagene, Agilent Technologies, Santa Clara, CA) by heat-shock according
184 to the manufacturer's instructions. Positive transformants were selected by ampicillin
185 resistance and confirmed by PCR.

186 **Expression of his-tagged hbdH in *E.coli* BL21 cells**

187 *E. coli* BL21 cells containing the pET45b_*hbdH* plasmid were grown in 2 l shake flasks
188 containing 400 ml of LB medium supplemented with 50 µg/ml carbenicillin at 25˚C, shaking
189 at 200 rpm until the OD_{600} reached 0.4 (approximately 6 hours). Cultures were cooled on ice
190 for 30 minutes. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final
191 concentration of 0.5 mM and the cultures were grown at 25˚C, shaking at 200 rpm for 18 h.

192 **Purification of His-tagged 3-hydroxybutyrate dehydrogenase protein**

193 Cells were harvested by centrifugation at 3600 g for 12 minutes at 4˚C. The supernatant was
194 discarded, and cell pellets resuspended in lysis buffer (3ml Bugbuster mastermix (Merck
195 Millipore, Cork, Ireland) and 1.5 ml binding buffer (300 mM NaCl, 20mM imidazole, 50 mM
196 sodium phosphate) per 1 g of wet cell pellet. The resuspended pellets were incubated at 30˚C
197 for 30 minutes. Cell debris was removed by centrifuging for 30 minutes at 43,146g at 4˚C.
198 Cell lysate was filter using a sterile 0.45 µm filter. The cell lysate was then passed through a
199 1ml HisTrap column (GE healthcare, Little Chalfont, UK). For every 2 ml of cell lysate passed
200 though the column, 2 ml of binding buffer was also passed through the column. The HisTrap
201 column was then attached to an AKTA prime system (GE healthcare, Little Chalfont, UK).

202 The column was washed with 6ml binding buffer and then eluted using a gradient of elution
203 buffer (300 mM NaCl, 500 mM imidazole, 50mM sodium phosphate). 2 ml fractions were
204 collected. Fractions were analysed by SDS-PAGE under denaturing conditions. The resolving
205 gel contained 12 % and stacking gel 4 % acrylamide (w/v). Fractions containing the hbdH
206 protein were pooled and the protein concentration determined by BCA assay (21). 25 µl of
207 each fraction to be measured was added in duplicate to a 96 well microtitre plate. 200 µl of a
208 bicinchoninic acid solution containing 2 % (v/v) copper sulphate was added to each sample.
209 The plate was incubated at 40˚C for 30 minutes. The absorbance of each sample at 550 nm
210 was measured using a SPECTROstar Nano microplate reader (BMG Labtech, Ortenberg,
211 Germany).

212 **Assay for activity of 3-hydroxybutyrate dehydrogenase protein**

213 The activity of the purified protein was measured using an assay previously described (22).
214 NAD^+, the co-factor for this enzyme is converted to NADH during the reaction. NADH can be
215 measured spectrophotometrically at 340 nm. Assays were carried out in 200 µl volumes in a
216 96 well plate. 10mM substrate and 1.5 mM NAD+ were added to 50mM potassium phosphate
217 buffer, pH 8. Enzyme was added to start the reaction, the plate was placed in a plate reader at
218 23˚C and the change in absorbance at 340 nm was measured in a SPECTROstar Nano
219 microplate reader (BMG Labtech, Ortenberg, Germany). Values were converted to NADH
220 concentrations using an extinction coefficient of 6.3 $mM^{-1}cm^{-1}$. The natural substrate for the
221 enzyme, 3-hydroxybutyrate was used as the positive control. Negative control reactions
222 containing no enzyme, no NAD^+ and boiled enzyme were also carried out.

223 **Results**

224 **Generation of *P. putida* KT2440 Δ*glpK* deletion mutant**

225 The *glpK* gene was successfully replaced with a gentamycin cassette on the chromosome to
226 yield a *P. putida* KT2440 Δ*glpK* strain. The mutation was confirmed by gentamycin resistance
227 screening, Southern blot technique and by DNA sequencing of the mutant chromosome.

228 **Growth of *P. putida* KT2440 wild type and Δglpk on glucose, sodium octanoate and**
229 **glycerol**

230 Growth of the wild type and Δglpk mutant was determined after 48h of cultivation in minimal
231 salt medium containing 20 mM of the carbon source at 30°C and shaking at 200 rpm. All
232 conditions were tested in a minimum of three separate shake flasks. Growth levels of the strains
233 was comparable when grown on glucose or sodium octanoate as the sole carbon source (Figure
234 1). Minimal growth of the Δglpk mutant was detected when grown on glycerol for 48 hours
235 (figure 1). When incubation was extended the Δglpk mutant achieved a final OD_{540} of 3.1
236 which is 1.6-fold lower than that observed for the wild type strain (Figure 2).

237 **Complementation of *P. putida* KT2440 *ΔglpK* mutant**

238 To show conclusively that the observed phenotype was as a direct result of the deletion of the
239 gene and not by other polar effects caused by the mutagenesis process, a selectively inducible
240 copy of the gene, harboured in the pJB8621 expression vector, was introduced into the mutant
241 and wild-type strain and the ability of the strains to grown on glycerol was analysed.

242 The complemented mutant *P. putida* KT2440 *ΔglpK* pJB861/*glpK* reached near wild-type
243 levels of biomass (96 % recovery). *P. putida* KT2440 pJB861/*glpK,* which is the wild type
244 strain harbouring an induced extra copy of the *glpK* gene, grew to very similar levels to that of
245 the wild-type strain (95 % of wild-type CDW) (Figure 3).

246 **Transcriptomics of *P. putida* KT2440 wild type and ΔglpK grown on glycerol as the sole**
247 **carbon source**

248 RNA was isolated from *P. putida* KT2440 wild type and Δglpk cells in mid logarithmic phase
249 of growth and it was sent to Baseclear (Leiden, Netherlands) for transcriptomic analysis. There
250 were statistically significant differences in the expression levels of 2962 transcripts. 1757 of
251 these are upregulated in Δglpk cells and 1205 are downregulated. Of these 2962 differentially
252 expressed transcripts, 191 were more than 2-fold upregulated and 175 more than 2-fold down
253 regulated.

254 The transcription of enzymes in the glycerol metabolic pathway were all down-regulated,
255 ranging from 32-fold for glpK to 1.2-fold for glpR. As the *glpK* was knocked out by inserting
256 a gentamicin resistance cassette in place of the gene, small parts of the gene remained on either
257 side of the gentamicin resistance cassette. This resulted in a small number of short transcripts
258 being assigned to glpK rather than 0 as may be expected. Other enzymes that are potentially
259 involved in glycerol metabolism show small differences in transcription in wild type cells
260 compared with ΔglpK cells. Metabolites from glycerol metabolism enter central metabolism
261 through the glycolysis. All the enzymes in this pathway are slightly down regulated (1.2 - 1.6-
262 fold) except for pyruvate kinase which is 1.5-fold upregulated. There were no significant
263 differences in transcription of any genes involved in fatty acid β-oxidation or PHA synthesis.
264 However, transcription of genes involved in short chain length fatty acid metabolism were
265 highly upregulated in ΔglpK cells versus wild type cells (Table 1). 54 genes whose functions
266 are unknown were more than 2-fold upregulated in the ΔglpK mutant compared to the wild
267 type and 27 transcriptional regulators are also more than 2-fold upregulated in the ΔglpK
268 mutant compared to the wild type. The change in expression of relevant genes is shown in
269 Table 1.

270 **Metabolomic analysis of *P. putida* KT2440 wild type and ΔglpK cells grown on glycerol**
271 **as the sole carbon source**

272 Metabolites were extracted from *P. putida* KT2440 wild type and ΔglpK cells in mid
273 logarithmic phase of growth. Metabolites potentially produced in glycerol catabolism as well
274 as common metabolites from central metabolism were analysed in cell extracts and levels
275 compared in wild type and ΔglpK cells (Figure 4). Production of glycerate is highly
276 upregulated (21 fold) in the ΔglpK mutant. Furthermore erythrose-4-phosphate is detected in
277 ΔglpK mutant cell extracts, but not in wild type cells. Interestingly levels of glyceraldehyde
278 and 3-phosphoglycerate are similar in both strains. As expected, levels of glycerol-3-phosphate
279 are much higher in the wild type strain, however some glycerol-3-phosphate is produced in the
280 ΔglpK strain, suggesting that there may be a non-specific kinase acting on glycerol in the
281 mutant strain. Dihydroxyacetone is detected only in the wild type strain. The upregulation of
282 glycerate levels in cell extracts suggests that glycerol may be metabolised via this intermediate.
283 This could be achieved through the action of a dehydrogenase, converting glycerol to
284 glyceraldehyde and then to glycerate.

285 **Cloning, expression and purification of 3-hydroxybutyrate dehydrogenase.**

286 Analysis of the transcriptome revealed that 3-hydroxybutyrate dehydrogenase (3HBDH)
287 transcription was 5.8-fold up regulated in the ΔglpK mutant. To investigate if this enzyme
288 could act on glycerol, the 770 bp gene was cloned into pET45b, which has an N-terminal 6
289 histidine tag.

290 The protein was expressed in *E. coli* BL21 cells and purified using a nickel affinity column on
291 an Akta basic protein purification system. The protein was eluted from the column using a
292 gradient of 500 mM imidazole. 2 ml fractions were collected, and the fractions run on a 12%
293 SDS PAGE gel to determine which fractions contained the 3HBDH. Fractions containing the
294 3HBDH protein were pooled and assayed for activity. Protein concentration was determined
295 by BCA assay.

296 **Assay for 3-hydroxybutyrate dehydrogenase activity**

297 The purified protein was assayed for its activity towards 3-hydroxybutyrate, glycerol and
298 glyceraldehyde. As NAD^+ is a cofactor for the enzyme, NADH production was used as a
299 measure of enzyme activity. Activity was detected when 3 hydroxybutyrate and
300 glyceraldehyde were used as substrates. The rate of NADH production was much higher for
301 3-hydroxybutyrate than for glyceraldehyde (Figure 5). No production of NADH was observed
302 when glycerol was used as the substrate. There was no production of NADH in any of the
303 negative controls (using no enzyme, no NAD^+ or enzyme that had been boiled at 100˚C for 10
304 minutes).

305 **Discussion**

306 The first step in the proposed pathway for glycerol catabolism in *P. putida* KT2440 is
307 phosphorylation by the glycerol kinase (PP_1075). We knocked out this gene and found that
308 the knockout mutant retained the ability to grow on glycerol as the sole carbon source, though
309 it had a much-extended lag period and achieved 1.6-fold less biomass than the wild type
310 strain. The ΔglpK mutant grew similarly to the wild type when grown on glucose or sodium
311 octanoate indicating the *glpK* gene is affecting early stage metabolism of glycerol and not
312 affecting central metabolism (23). This is consistent with the literature where the *glpK* gene
313 product is only induced in the presence of glycerol and is only be expressed at basal levels
314 during incubation with glucose or sodium octanoate.(9,24)

315 It has previously been established in *E. coli* and *P. aeruginosa* that this *glpK* gene product is
316 vital for the production of glycerol-3-phosphate and hence for the up-regulation of all major
317 glycerol metabolic genes (1,25). However, we have found that the ΔglpK mutant still grows
318 albeit with a lag period and a lower growth yield but an alternative glycerol metabolic pathway
319 must exist for growth of *P. putida* KT2440 in the absence of the glycerol kinase.

320 In bacteria, there are two major pathways involved in glycerol metabolism. The
321 phosphorylation pathway and the oxidation pathway (26). As previously outlined,
322 *Pseudomonas putida* KT2440 uses the most common biological pathway namely the
323 phosphorylation pathway. Other microorganisms such as *Klebsiella pneumoniae* use the
324 oxidation pathway in which a glycerol dehydrogenase converts glycerol into dihydroxyacetone
325 (27). Some facultative anaerobic bacteria such as *Klebsiella aerogenes* use the phosphorylation
326 pathway under aerobic conditions, but can employ the oxidation pathway in the absence of
327 oxygen (28). In some bacteria, it is also possible for glycerol to be oxidised to glyceraldehyde
328 (29), which may then be converted to glycerate (30).

329 As expected, enzymes in the glycerol kinase pathway were down regulated in the ΔglpK
330 mutant. However *glpR*, the regulator of expression of glycerol kinase is only 1.2-fold
331 downregulated. This is in keeping with previous studies that show the levels of *glpR* remain
332 the same regardless of carbon source used (31). There is a report of a glycerol kinase deletion
333 mutant of *E. coli* K12 which could use glycerol as the sole carbon source for growth by using
334 an NAD^+ linked glycerol dehydrogenase to metabolise glycerol. That enzyme showed much
335 higher activity towards dihydroxyacetone compared with glyceraldehyde (32). Metabolite
336 analysis showed that no dihydroxyacetone was produced by our mutant strain when grown on
337 glycerol, therefore this pathway is not likely to be employed for metabolism of glycerol in the
338 absence of the glycerol kinase in *P. putida* KT2440.

339 Levels of glyceraldehyde and glycerol-3-phosphate are approximately the same in both wild
340 type and mutant. However, levels of glycerate are highly upregulated in the mutant, suggesting
341 that glycerol may be converted to glyceraldehyde and then to glycerate in the absence of the
342 glycerol kinase. While transcription of glycerate kinase is 1.5-fold upregulated in the ΔglpK
343 mutant strain compare to the wildtype, levels of 3-phospho-glycerate are similar in both strains.

344 Erythrose-4-phosphate is also detected in the mutant strain but not in the wildtype.
345 Furthermore higher levels of glyceraldehyde-3-phosphate are also present in the mutant
346 compared to the wild type KT2440. Transaldolase, the enzyme that converts glyceraldehyde-
347 3-phosphate + sedoheptulose-7-phosphate to erythrose-4-phosphate + fructose-6-phosphate is
348 1.4-fold upregulated in the mutant. However, neither sedoheptulose-7-phosphate nor fructose-
349 6-phosphate are detected in the mutant. As glycerate is upregulated in the mutant strain, it is
350 possible that excess glyceraldehyde is also formed in the mutant strain, which may be easily
351 converted to glyceraldehyde-3-phosphate, which can be converted to erythrose-4-phosphate.
352 Glyceraldehyde-3-phosphate can enter the Embden-Meyerhof-Parnas pathway (33). Enzymes
353 in this pathway are slightly downregulated in the mutant strain compared to the wildtype
354 indicating that EMP is still used for glycerol metabolism by the ΔglpK mutant but the lower
355 expression may contribute to the slower growth rate of the mutant compared to the wild type.

356 If glycerol is converted to glyceraldehyde in the mutant strain, there must be a dehydrogenase
357 enzyme acting non-specifically in this strain. Transcription of the enzyme 3-hydroxybutyrate
358 dehydrogenase was highly upregulated in the ΔglpK mutant compared to the wild type. 3-
359 hydroxybutyrate dehydrogenase converts 3-hydroxybutyrate to acetoacetate in a reversible
360 reaction with NAD^+ as a cofactor. As it was upregulated in the mutant, it was hypothesized
361 that it may also be able to non-specifically catalyse the conversion of glycerol to
362 glyceraldehyde or glyceraldehyde to glycerate.

363 3-hydroxybutyrate dehydrogenase from *P. putida* KT2440 was expressed in *E. coli*, purified
364 and tested for activity towards glyceraldehyde and glycerol. Activity was detected towards
365 glyceraldehyde, albeit at a slower rate than towards 3-hydroxybutyrate, the natural substrate
366 for the enzyme. However, no activity was detected towards glycerol. Analysis of the KT2440
367 genome revealed 5 further dehydrogenases that could be responsible for glycerate production
368 in the mutant strain, these enzymes were between 2 and 2.7 fold upregulated in the

369 transcriptomic analysis. A companion study to this work, which investigated the physiological
370 responses of *P. putida* KT2440 towards rare earth elements during growth with different
371 growth substrates, identified that two periplasmic PQQ-dependent alcohol dehydrogenases that
372 are essential to initiate an alternative glycerol pathway via the oxidation of glycerol to
373 glyceraldehyde (34). No significant difference in the expression of these PQQ-dependent
374 dehydrogenases was observed in the transcriptomic analysis. Now that an alternative glycerol
375 pathway in *P. putida* KT2440 has been discovered it opens up the possibilities for studies into
376 the regulation of both pathways in which could increase the efficiency of glycerol consumption
377 in this strain and open up new biotechnological possibilities.

378 In conclusion a glycerol kinase negative mutant of *P. putida* KT2440 is capable of growth
379 using glycerol as a sole carbon and energy source with a very long lag phase. Transcriptome
380 and metabolome analysis suggest glycerate and erythrose-4-phosphate as major metabolites in
381 the mutant strain. Glyceraldehyde and glyceraldehyde-3-phosphate are also present in the
382 mutant but at similar levels to the wild type strain. Short chain fatty acid metabolism genes are
383 upregulated in the Δglpk mutant. One of these enzymes, 3-hydroxybutyrate dehydrogenase,
384 has activity towards glyceraldehyde which could explain the increase in the concentration of
385 glycerate in the ΔglpK mutant.

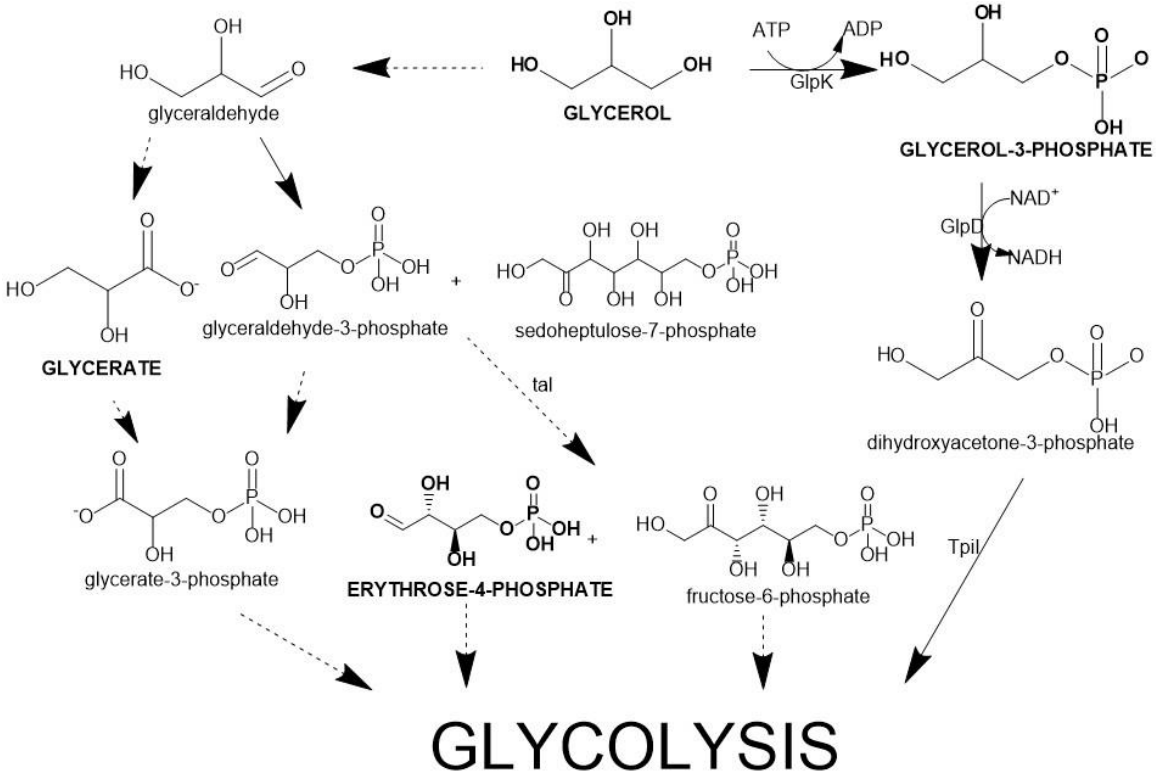


Figure 1 Glycerol metabolic pathway in *P. putida* KT2440 (solid arrows) and proposed metabolic pathway in the absence of glycerol kinase (dotted arrows). Metabolites detected at high levels (>1M counts) in the ΔglpK mutant are highlighted in bold and uppercase.

Author statements

Conflicts of interest

The authors declare that there are no conflicts of interest.

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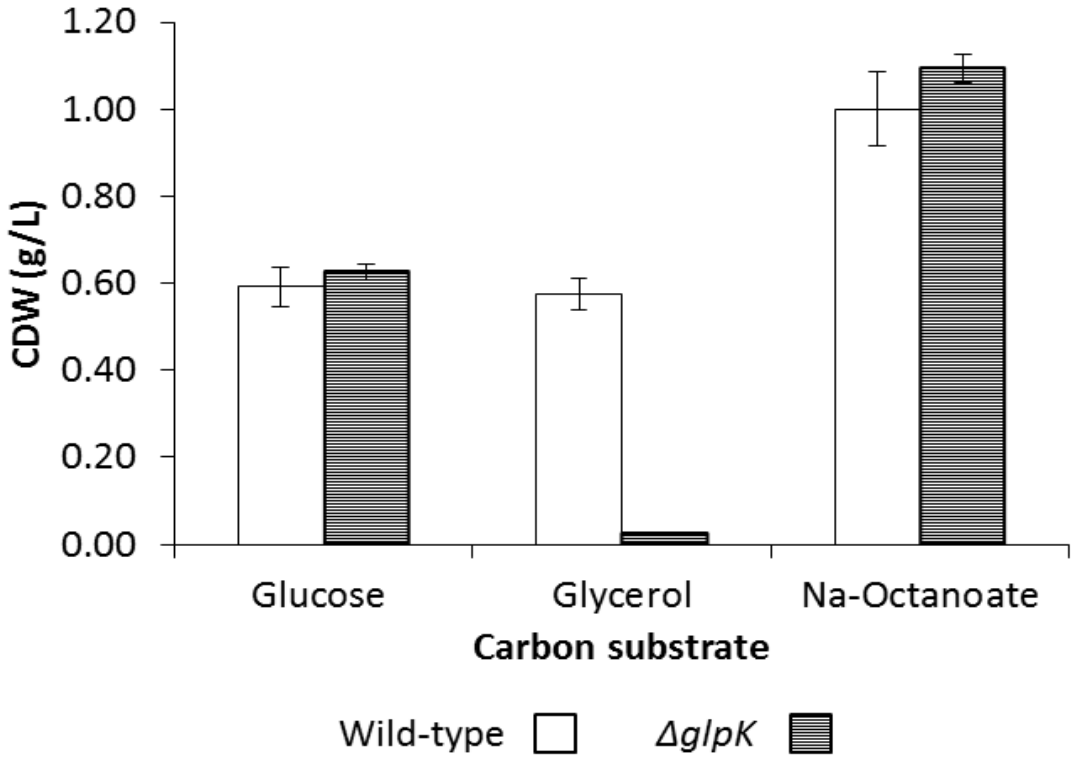
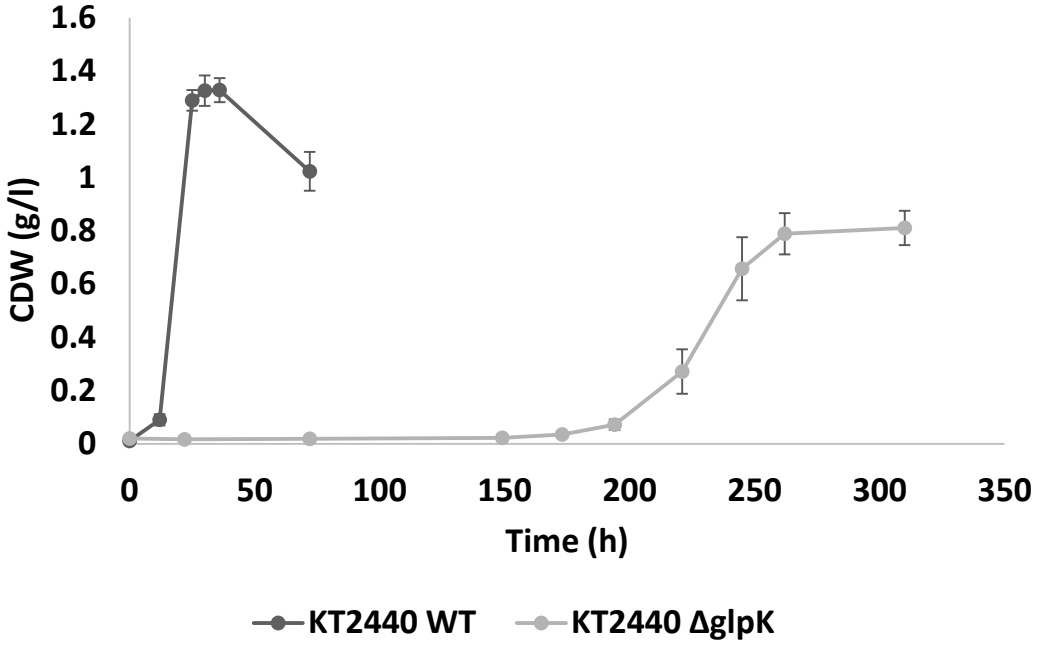
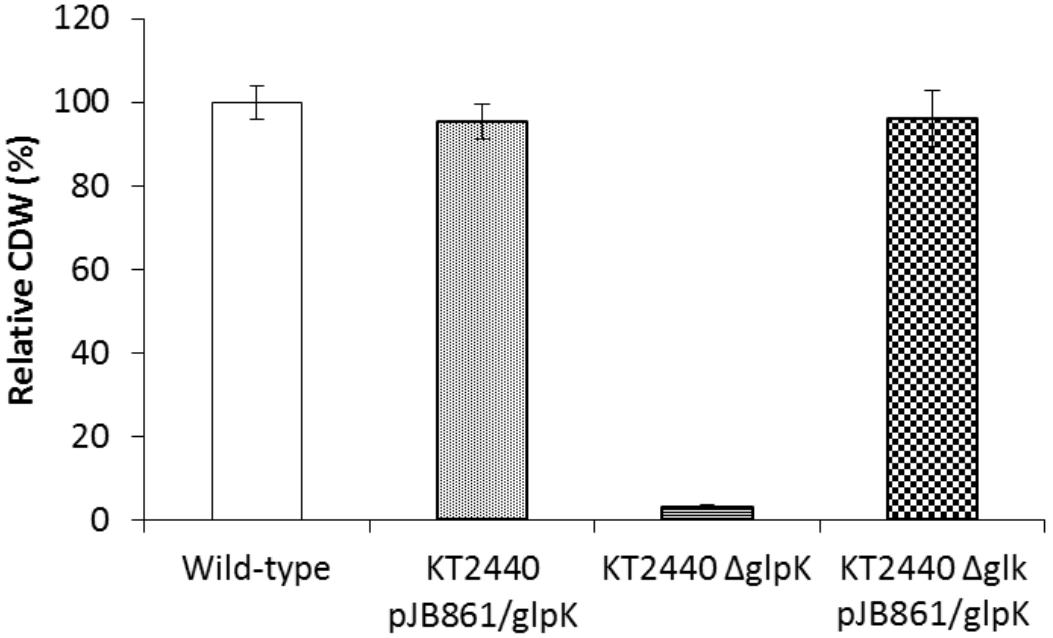


Figure 2 Growth of *P. putida* KT2440 and *glpK* on glucose, glycerol and sodium octanoate in minimal salts medium. Optical density (OD) was measured at 540 nm. When harvested? seems to not be in line with figure 3. Can figure 1 and 2 be presented as a and b.



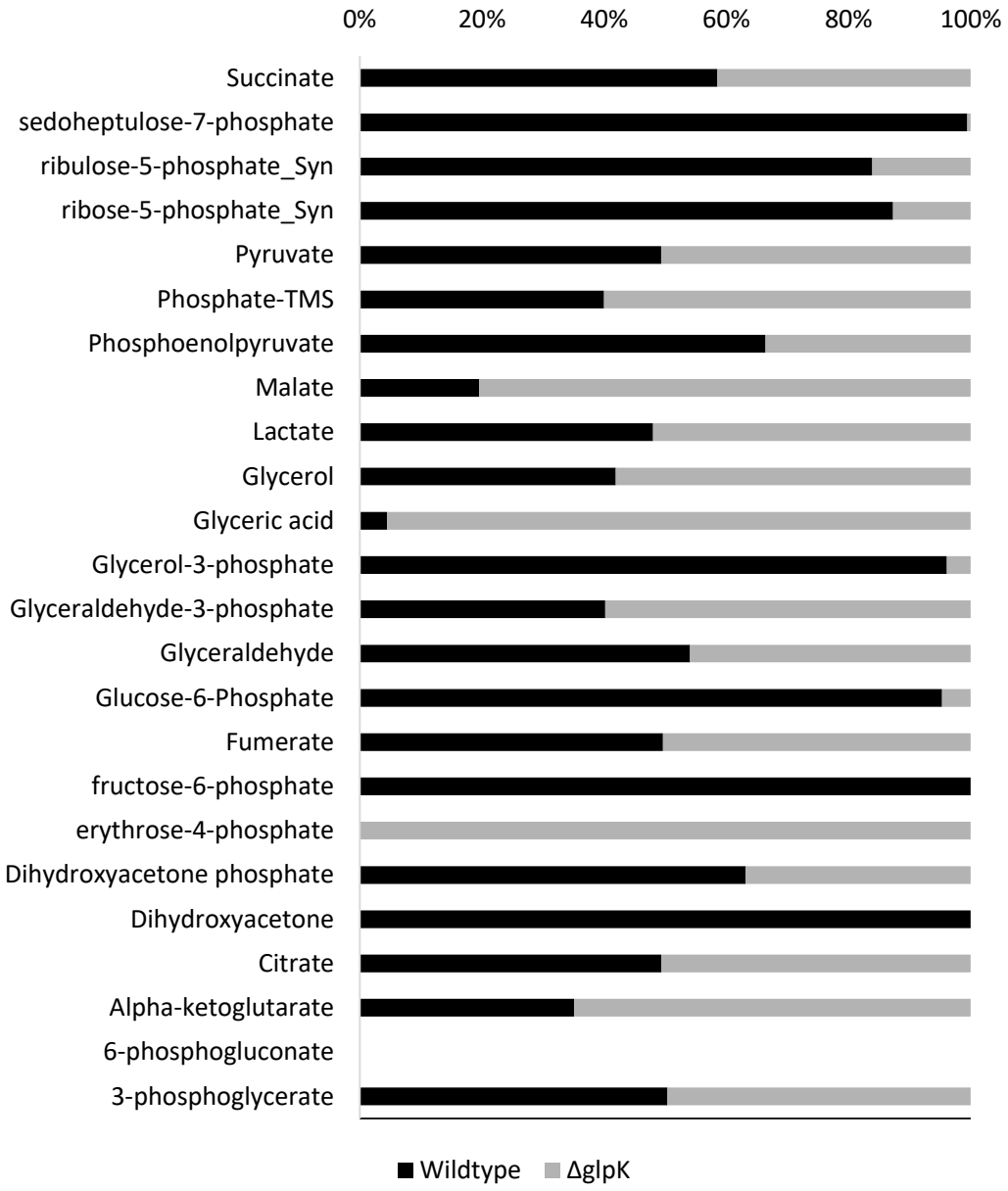
505

506 **Figure 3** Growth of *P. putida* KT2440 wildtype and ΔglpK on glycerol as the sole carbon source.
507 Optical density (OD) was measured at 540 nm.



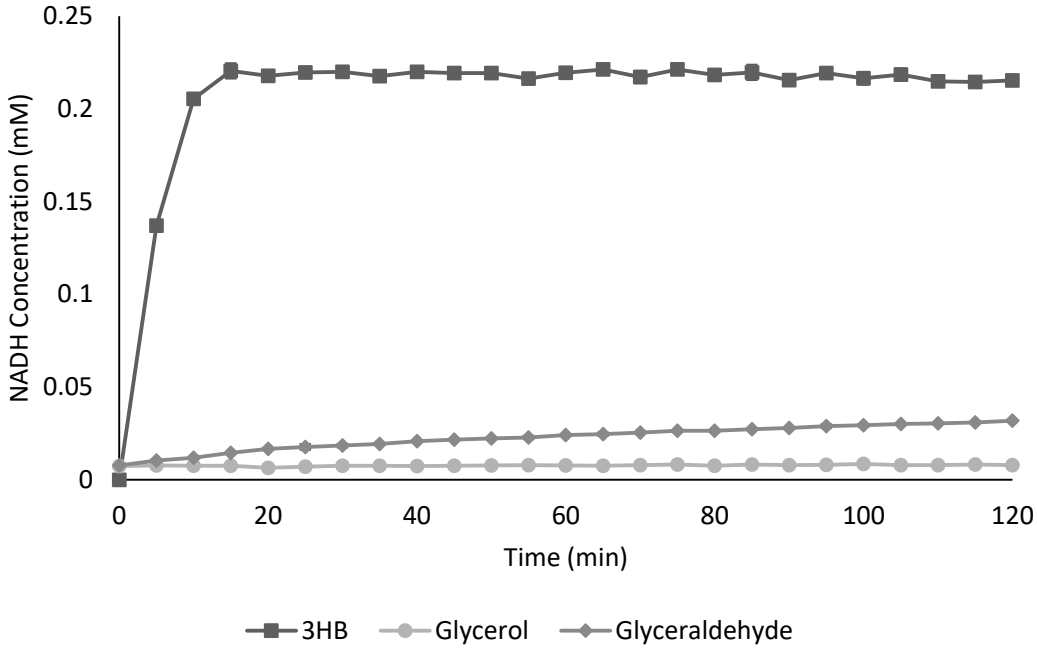
508

509 **Figure 4** Growth of *P. putida* KT2440 wild type and ΔglpK cells, with a complemented copy of glpK
510 relative to growth of the wild type *P. putida* KT2440. The glpk gene was expressed on pJB861
511 plasmid. Glycerol was supplied as the sole carbon and energy source.



512

513 **Figure 5** Metabolites identified in extracts of *P. putida* KT2440 wildtype and ΔglpK cells grown on
514 glycerol as the sole carbon and energy source. Cells were harvested at the mid log phase of growth.
515 Values expressed as a percentage of total metabolite detected in both samples.



516

517 **Figure 6** NADH production when 3 hydroxybutyrate (3HB), glycerol or glyceraldehyde was used as
518 the substrate for recombinantly produced 3-hydroxybutyrate dehydrogenase from *P. putida* KT2440.

519

520 **Table 1** Change in expression of genes in ΔglpK versus wild type *P. putida* KT2440 cells in mid
521 logarithmic phase grown on glycerol as the sole carbon and energy source.

Gene Name	Locus Tag	Description	Fold Change
Glycerol metabolism			
glpK	PP_1075	glycerol kinase	-32.1
glpD	PP_1073	glycerol-3-phosphate dehydrogenase	-6.0
glpF	PP_1076	glycerol uptake facilitator protein	-3.6
glpR	PP_1074	glycerol-3-phosphate regulon repressor	-1.2
plsY	PP_0391	Glycerol-3-phosphate acyltransferase	1.3
plsB	PP_1520	glycerol-3-phosphate O-acyltransferase	-1.1
plsX	PP_1912	glycerol-3-phosphate acyltransferase PlsX	-1.4
PP_0058	PP_0058	1-acyl-sn-glycerol-3-phosphate acyltransferase	-1.2
PP_0923	PP_0923	1-acyl-sn-glycerol-3-phosphate acyltransferase	no change
plsC	PP_1844	1-acyl-sn-glycerol-3-phosphate acyltransferase	1.2
dgkA-I	PP_1636	diacylglycerol kinase (ATP)	1.3
dgkA-II	PP_2973	diacylglycerol kinase (ATP)	no change
lip	PP_4854	triacylglycerol lipase	1.3
PP_2694	PP_2694	aldehyde dehydrogenase (NAD+)	1.5
amaB	PP_5258	aldehyde dehydrogenase (NAD+)	1.1
garK	PP_3178	glycerate-2-kinase	1.5
ttuD	PP_4300	glycerate-2-kinase	1.5
Embden-Meyerhof-Parnas pathway			
glk	PP_1011	glucokinase	-1.4
gltR-II	PP_1012	transcriptional regulator	-1.5
	PP_1013	integral membrane sensor	-1.5
pgi-I	PP_1808	glucose-6-phosphate isomerase	-1.3
fbp	PP_5040	fructose-1,6-bisphosphatase	-1.4
fba	PP_4960	fructose-1,6-bisphosphate aldolase	-1.2
tpiA	PP_4715	triosephosphate isomerase	-1.6
gapA	PP_1009	glyceraldehyde-3-phosphate dehydrogenase, type I	-1.6
gapB	PP_2149	glyceraldehyde-3-phosphate dehydrogenase, type II	-1.3
pgk	PP_4963	phosphoglycerate kinase	-1.4
pgm	PP_5056	phosphoglyceromutase	-1.2
eno	PP_1612	phosphopyruvate hydratase	-1.2
pyk	PP_1362	pyruvate kinase	1.5
Short chain length fatty acid metabolism			
atoB	PP_3123	3-oxoacid CoA-transferase subunit B	26.8
atoA	PP_3122	3-oxoacid CoA-transferase subunit A	28.5
hbdH	PP_3073	3-hydroxybutyrate dehydrogenase	5.8
yqeF	PP_4636	acetyl-CoA C-acetyltransferase	28.9
bhbP	PP_3074	D-beta-hydroxybutyrate permease	16.1
-	PP_3075	Transcriptional regulator	1.1