Disruption of the oxidative Pentose Phosphate Pathway stimulates high-yield production using resting *Corynebacterium glutamicum* in the absence of external electron acceptors

Shen, Jing; Chen, Jun; Solem, Christian; Jensen, Peter Ruhdal; Liu, Jianming

Published in:
Applied and Environmental Microbiology

Link to article, DOI:
[10.1128/AEM.02114-20](https://doi.org/10.1128/AEM.02114-20)

Publication date:
2020

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Shen, J., Chen, J., Solem, C., Jensen, P. R., & Liu, J. (2020). Disruption of the oxidative Pentose Phosphate Pathway stimulates high-yield production using resting *Corynebacterium glutamicum* in the absence of external electron acceptors. *Applied and Environmental Microbiology*, 86(24), [e02114-20].
<https://doi.org/10.1128/AEM.02114-20>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1 **Disruption of the oxidative Pentose Phosphate Pathway stimulates high-yield production**
2 **using resting *Corynebacterium glutamicum* in the absence of external electron acceptors**

3 Jing Shen ^a, Jun Chen ^a, Christian Solem ^a, Peter Ruhdal Jensen ^{a*}, Jian-Ming Liu ^{a*}

4 ^a The National Food Institute, Technical University of Denmark, Kongens Lyngby 2800, Denmark

5

6 *Corresponding author:

7 Jian-Ming Liu

8 National Food Institute, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark;

9 telephone: +45 91922228; fax: +45 45932809; e-mail: jial@dtu.dk

10 Peter Ruhdal Jensen

11 National Food Institute, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark;

12 telephone: +45 20855601; fax: +45 45932809; e-mail: perj@food.dtu.dk

13

14

15

16

17

18

19

20 **Abstract**

21 Identifying and overcoming the limitations preventing efficient high-yield production of chemicals
22 remain to be an important task in metabolic engineering. In an attempt to rewire *Corynebacterium*
23 *glutamicum* into producing ethanol, we attained a low yield (63% of the theoretical), when using
24 resting cells on glucose, and large amounts of succinate and acetate were formed. To prevent the
25 by-products formation, we knocked out the malate dehydrogenase and replaced the native E3
26 subunit of the pyruvate dehydrogenase complex (PDHc) with the one from *Escherichia coli*, which
27 is only active under aerobic conditions. However, this tampering resulted in a 10 times reduced
28 glycolytic flux as well as a greatly increased NADH/NAD⁺ ratio. By substituting glucose with
29 fructose, we found that the glycolytic flux was greatly enhanced, which led us to speculate whether
30 the source of reducing power could be the the pentose phosphate pathway (PPP) that is bypassed
31 when fructose is metabolized. Indeed, after shutting down the PPP by deleting the *zwf* gene,
32 encoding glucose-6-phosphate dehydrogenase, the ethanol yield on glucose increased significantly
33 to 92% of the theoretical. Based on that, we managed to re-channel the metabolism of *C.*
34 *glutamicum* into D-lactate with high yield (98%), which is the highest that has been reported. It is
35 further demonstrated that the PPP-inactivated platform strain can offer high-yield production of
36 valuable chemicals using lactose contained in dairy waste as feedstock, which paves a promising
37 way for potentially turning dairy waste into value.

38

39 **Importance**

40 The widely used industrial workhorse *C. glutamicum* possesses a complex anaerobic metabolism
41 under non-growing conditions and we demonstrate here that the PPP in resting *C. glutamicum* is a
42 source of reducing power that can interfere with otherwise redox balanced metabolic pathways and

43 reduce yields of desired products. By harnessing this physiological insight, we employed the PPP-
44 inactivated platform strains to produce ethanol, D-lactate and alanine using the dairy waste – whey
45 permeate as the feedstock. The production yield is high and our results show that inactivation of the
46 PPP flux in resting cells is a promising strategy when the aim is to use non-growing *C. glutamicum*
47 cells for producing valuable compounds. Overall, we described the benefits to disrupt the oxidative
48 PPP in non-growing *C. glutamicum* and provide a feasible approach towards waste valorization.

49

50 **Introduction**

51 *Corynebacterium glutamicum* is widely employed for large-scale fermentative production of amino
52 acids, especially L-glutamate and L-lysine (1). In recent years, the fast development of tools for
53 genetic engineering as well as for studying the systems biology of *C. glutamicum* has accelerated
54 research on its metabolism and regulatory network (2–4). It is now apparent, that *C. glutamicum* has
55 a great potential for industrial production of a wide range of useful compounds, e.g. organic acids
56 and biofuels (5, 6). Whereas amino acids normally are produced under highly aerated conditions,
57 organic acids are generated under anaerobic conditions (7, 8). When oxygen and external electron
58 acceptors are absent, *C. glutamicum* displays limited growth in a medium devoid of rich
59 components, but retains an active fermentative metabolism. Glucose, for instance, is metabolized
60 via glycolysis to pyruvate that can be reduced into lactate. If carbon dioxide is available, anaplerotic
61 reactions can lead to formation of oxaloacetate, which normally is reduced into succinate (7, 9).
62 Sodium bicarbonate, as a source of carbon dioxide, was found to stimulate the formation of
63 succinate as well as the glycolytic flux (7, 10), and blocking the formation of either of these
64 compounds was found to hamper glycolysis. In one study, the NADH-sensitive glyceraldehyde-3-
65 phosphate dehydrogenase (GAPDH) was overexpressed, which improved the glucose consumption

66 rate (11), and it appears that the anaerobic glycolytic flux in *C. glutamicum* is sensitive to the
67 NADH/NAD⁺ ratio (9). Thus, when engineering *C. glutamicum* for the purpose of bio-production
68 under anaerobic conditions, it is imperative that the redox balance is considered carefully.

69 It has previously been reported that *C. glutamicum* can be engineered into producing ethanol under
70 anaerobic conditions by heterologous expression of the pyruvate decarboxylase (PDC) and the
71 alcohol dehydrogenase (ADHB) from *Zymomonas mobilis* (12–14). However, a low yield
72 production was achieved due to formation of by-products such as lactate, succinate and acetate (12).
73 Lactate production could be eliminated by disrupting the lactate dehydrogenase gene (*ldhA*), and
74 this resulted in a 3-fold higher ethanol yield compared to the wild-type strain expressing PDC and
75 ADHB (12). By further deleting the *ppc* gene, encoding PEP carboxylase, succinate formation was
76 reduced, but controlling the pH was still found to be necessary (12, 14). In principle, ethanol
77 formation is balanced redox-wise, as all the NADH generated in glycolysis is re-oxidized when 2
78 moles of ethanol are formed from 1 mole of glucose, and it is reasonable to expect that
79 simultaneous blocking of lactate and succinate formation should lead to a high-yield production of
80 ethanol.

81 In this study, we observed low yield production of ethanol from glucose under anoxic conditions in
82 the absence of an external electron acceptor. Characterization of strains blocked in NADH
83 consuming and forming steps prompted us to investigate if the PPP could be a source of reducing
84 power. We found this to be the case and based on our findings, we managed to divert most of the
85 carbon flux (glucose) towards either ethanol or D-lactate by inactivating the PPP. Furthermore, the
86 PPP disrupted platform strains were employed for high-yield production of ethanol, D-lactate and
87 alanine from lactose contained in dairy waste streams, which provided excellent examples of
88 turning waste into value.

89 Results

90 Deletion of the *mdh* gene represses glucose consumption by resting cells.

91 We have previously rewired *C. glutamicum* strain into producing ethanol. The rewired strain, JS122
92 (15), expresses pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis*, and lacks
93 lactate dehydrogenase and phosphoenolpyruvate (PEP) carboxylase activities (Fig. 1). When use
94 glucose as the carbon source for JS122, the yield for ethanol production only reached 63% of the
95 theoretical maximum, where considerable amounts of succinate and acetate were formed. In
96 principle it is redox-neutral for ethanol production and could lead to a much higher yield. In order to
97 increase the production yield of ethanol and elucidate the limitations in the anaerobic metabolism of
98 *C. glutamicum*, we decided to further investigate the anaerobic metabolic flux and its controlling
99 factors.

100 Since the method we applied for producing ethanol depends on non-growing cells and a high cell
101 density is required to achieve a good productivity, it is imperative that the cells can readily be
102 amplified aerobically to high cell densities on cheap mineral medium. Thus, the strain needs
103 functional anaplerotic pathways to replenish oxaloacetate, and it has previously been found that
104 either pyruvate carboxylase (PYC) or PEP carboxylase (PEPC) activity is needed (16). Thus,
105 inactivating both of these enzymes is not a viable solution for increasing ethanol yield. In an
106 attempt to reduce the formation of succinate, we inactivated the *mdh* gene encoding malate
107 dehydrogenase, resulting in strain JS124. JS124 generated 5-fold less succinate, and its ethanol
108 yield increased to 71.5% of the theoretical maximum. Unexpectedly, the glucose consumption rate
109 (glycolytic flux) dropped significantly from 2.02 mmol/gDCW/h in JS122 to only 0.19
110 mmol/gDCW/h in JS124 (Table S1). After a 15 h fermentation, 41.6 g/L glucose still remained
111 unmetabolized (Fig. 2).

112 We speculated that the drastic reduction in glucose consumption rate was due to an unbalanced
113 redox metabolism, since the regeneration of NAD^+ via the formation of succinic acid had been
114 blocked (17). We assayed the intracellular NADH/NAD^+ ratio of JS122 and JS124, and found that
115 the intracellular NADH/NAD^+ ratio for JS124 (1.85) was much higher than for JS122 (0.06) (Fig.
116 3A).

117 **The PDHc is not a major contributor to NADH in resting cells.**

118 Unlike PDHc from *E. coli* or *Lactococcus lactis*, which is solely functional under aerobic conditions
119 (18), the PDHc of *C. glutamicum* is also active under anaerobic conditions, which leads to
120 formation of acetate (19). As the oxidation of pyruvate to acetate via PDHc contributes one
121 additional NADH (Fig. 1), this could in principle perturb the redox balance and thus interfere with
122 glycolysis. To address this potential problem, we replaced the E3 subunit (*lpd*) of the *C.*
123 *glutamicum* PDHc with its *E. coli* counterpart.

124 The growth of the resultant strain JS125, which does not carry the ethanol plasmid, was slow on
125 glucose probably due to suboptimal expression of the *E. coli lpd*. After a 40 h aerobic cultivation,
126 the final cell density (OD_{600}) of JS125 only reached 0.36 in comparison to 11.7 for the parent strain
127 JS119. To improve the growth of JS125 on minimal medium with glucose, we performed adaptive
128 laboratory evolution for JS125 on the CGXII medium with 2% glucose. After a short-term
129 evolution (~106 generations), several fast-growing strains were isolated (Fig. 4A). We sequenced
130 the genome of the adapted strain JS125A, and identified only a single nucleotide substitution, C $\rightarrow$
131 T, 15 bases upstream the start codon of *lpd* in JS125.

132 Subsequently we introduced the ethanol plasmid pJS115 into this adapted strain and the outcome
133 was strain JS133, which was characterized. Fig. 2D shows that JS133 only generates small amounts
134 of acetate, and 0.3% of the consumed glucose ends up as acetate compared to 5% for JS124. The

135 ethanol yield for JS133 on glucose was 77.3%. However, the replacement of the E3 subunit of
136 PDHc only slightly decreased the intracellular NADH/NAD⁺ ratio in the resting cells (Fig. 3A), and
137 the glucose consumption rate of JS133 was not improved compared to that of JS124 (Fig. 2A).

138 **Deletion of the *zwf* gene in JS133 boosts the glycolytic flux.**

139 In JS133 we had blocked NAD⁺ regeneration by deleting *ldhA* and *mdh*, reconstituted PDHc and
140 subsequently introduced the ethanol plasmid, which in principle should restore the redox balance.
141 However, the outcome of our experiments indicated the presence of other pathways able to affect
142 the NADH/NAD⁺ ratio.

143 It has been reported that during anaerobic fermentation, *C. glutamicum* exhibits a higher sugar
144 consumption rate on fructose compared to glucose, which contrasts the observations made during
145 aerobic growth (20). Furthermore, it is known that the PPP is bypassed when fructose is
146 metabolized by *C. glutamicum* (21, 22). We decided to test whether the negative effects on
147 glycolysis could be alleviated by using fructose as a carbon source instead of glucose. Indeed,
148 JS133 could metabolize fructose faster than glucose and also the ethanol yield was improved on
149 fructose. Using the same initial sugar concentration (50 g/L), JS133 consumed 18.66 ± 4.01 g
150 fructose and produced 12.95 ± 2.85 g ethanol after 72 h of biocatalysis, whereas JS133 only
151 consumed 9.97 ± 1.16 g glucose and produced 6.56 ± 1.25 g ethanol. This raised the question of
152 whether the PPP could have an effect on glycolytic flux in JS133.

153 This prompted us to inactivate the first enzyme of the oxidative PPP, glucose-6-phosphate
154 dehydrogenase, which is encoded by *zwf*. The modification indeed boosted sugar consumption and
155 ethanol production for the resting cells. As shown in Fig. 2A, the glucose consumption rate of
156 JS134 was enhanced 2.6 fold when compared to JS133. The ethanol yield reached as high as 92%
157 of the theoretical maximum, a 29% increase compared to the starting strain JS122. These data

158 demonstrate that the PPP indeed is a source of reducing power for resting *C. glutamicum*. It is well-
159 known that the main role of the PPP is NADPH generation, however, for the resting cells we
160 detected a low transhydrogenase activity ($3.95 \pm 1.21 \mu\text{mol min}^{-1} \text{g}^{-1}$), which might explain the
161 formation of NADH.

162 Disrupting the PPP in JS134 prevented growth in minimal medium, and to restore growth we found
163 that it was necessary to add a nitrogen source such as yeast extract (YE) or corn steep liquor (CSL).
164 By adding 2% CSL the biomass density was able to reach OD_{600} 21.8 (Fig. 4B).

165 **Comparison of cofactor balance in different ethanol-producing strains.**

166 We measured the intracellular cofactor levels of different *C. glutamicum* constructs during ethanol
167 production under anoxic conditions. In Fig. 3A & B, the changes in NADH/NAD⁺,
168 NADPH/NADP⁺, and ATP/ADP ratios with different metabolic modifications are displayed. As we
169 mentioned earlier, deletion of *mdh* resulted in a sharp rise in the NADH/NAD⁺ ratio to 1.85 in
170 JS124 from 0.06 in JS122, and the *mdh* mutant had a greatly reduced glucose consumption rate (Fig.
171 2A). This modification drastically reduced the ATP/ADP ratio (Fig. 3B). Interestingly, the increase
172 in NADH/NAD⁺ ratio coincided with a considerable increase in the NADPH/NADP⁺ ratio. The
173 replacement of the E3 subunit of PDHc (JS133) only slightly reduced the NADH/NAD⁺ ratio, and
174 appeared not to release the hampering effect on glycolysis. However, the deletion of *zwf* (JS134)
175 reduced the NADPH/NADP⁺ ratio to 0.05, and was accompanied by a reduction of NADH/NAD⁺
176 ratio to 0.46. Blocking the PPP boosted glucose consumption in JS134, and the ATP/ADP ratio
177 recovered to 1.34.

178 **RNA-seq analysis reveals the impact of *zwf* inactivation on energy metabolism.**

179 As the deletion of *zwf* had a great impact on the cofactor balance and cellular energy status of *C.*
180 *glutamicum* under anoxic conditions, we further investigated the impact on the transcriptional level

181 carrying out a transcriptome analysis for JS133 and JS134 (JS133 with *zwf* deletion). For each
182 strain three independent replicate experiments were included, and it was shown that JS133 and
183 JS134 had quite different transcriptomic profiles, where the differentially expressed genes in JS133
184 clustered separately from those of JS134 (Fig. 5A). When compared to JS133, a total of 592 and
185 334 genes had been down-regulated and up-regulated ($\log_2$ -fold-change > 1, p-value < 0.05) in
186 JS134, respectively (see all the differentially expressed genes in Table S3, S4). As expected, the *zwf*
187 gene was 9.0-fold ($\log_2$) down-regulated in JS134. Among the 592 down-regulated genes used for
188 Gene Ontology (GO) analysis, 13.7% genes could be allocated to the term “ATP binding” (Fig. 5B).
189 These genes were mainly involved in ATP-dependent process such as transport, helicase, kinase
190 and synthase. In terms of “proton-transporting ATP synthase activity”, seven genes encoded
191 subunits of the F₀F₁-ATP synthase, and they were down-regulated from 3.0 to 1.5 folds ($\log_2$) in
192 JS134. Interestingly, the term “nitrate reductase activity” was also enriched, even though nitrate was
193 not present in the medium used.

194 Of the up-regulated genes in JS134, most could be allocated to the term “DNA binding”, where
195 most of the genes were involved in cell division and transcriptional regulation. The *gapA* gene,
196 which encodes glyceraldehyde-3-phosphate dehydrogenase in glycolysis, appeared in the term
197 “NADP binding” and was also up-regulated.

198 **Expanding the repertoire - D-lactate production.**

199 After demonstrating that efficient high-yield ethanol production could be accomplished using JS134,
200 we decided to investigate whether other pyruvate-derived chemicals could be made using the strain
201 deficient in malate dehydrogenase and glucose-6-phosphate dehydrogenase. To achieve a high-yield
202 production of D-lactate in *C. glutamicum*, we overexpressed the D-lactate dehydrogenase from
203 *Lactobacillus delbruckii* in the strain JS132. The productivity of D-lactate in the resulting strain

204 JS138 was slightly lower than for the strain carrying only deletions in *ldhA* and *ppc* (JS137), but the
205 yield for JS138 reached 98% of the theoretical, which is 14% higher when compared to JS137 (Fig.
206 6). To the best of our knowledge, this is the highest yield of D-lactate reported this far for *C.*
207 *glutamicum*.

208 **Increasing resource utilization using dairy waste feedstock.**

209 We achieved extremely high-yield production of ethanol and D-lactate from glucose, which
210 stimulated us to explore the actual applications by harnessing this PPP - disrupted platform strain
211 JS132. Since we have previously engineered *C. glutamicum* into being able to metabolize lactose,
212 and this was done by introducing the transporter LacS, the β -glucosidase LacZ, and the Leloir
213 pathway (GalMKTE), which resulted in strain JS112 (Fig. 7A). Lactose is available in large
214 amounts in whey permeate (WP), which are abundantly generated as waste by dairy industry in
215 most parts of the world. We first optimized the growth of JS132 on WP with different types of
216 nitrogen source. The pure WP did not support its growth well, and the final cell density on this
217 substrate only reached 2.7 (OD₆₀₀) after 24 h. Adding the minimal medium salts improved the cell
218 density to 5.1 (OD₆₀₀). Adding organic nitrogen sources, either 1% YE or 5% CSL resulted in a
219 significant increase in biomass and the cell densities could reach 35 (OD₆₀₀) and 39 (OD₆₀₀),
220 respectively (Fig. S1).

221 **Production of chemicals from lactose - containing dairy wastes.**

222 Using the pure WP as the substrate, we achieved 48 g/L ethanol from 106 g/L lactose with a yield
223 of 85% (of the theoretical maximum) on JS134 compared with only 66% on JS122, which co-
224 produced 37 g/L ethanol and 23 g/L succinate (Fig. 7B). We also noticed the productivity was
225 reduced for JS134 due to the lower glycolytic flux, which was also the case in glucose medium.
226 Similarly, we got 92 g/L D-lactate with a yield of 86% on JS138. There was a combination of 79

g/L D-lactate and 12 g/L succinate on JS137 with a yield of 73% for D-lactate (Fig. 7C). The productivity, however, was reduced to 1.64 g/L/h in JS138 from 2.19 g/L/h in JS137.

We further constructed strain JM141 to produce alanine from lactose by overexpressing alanine dehydrogenase in our *zwf*-inactivated strain JS132. After 75 h, we achieved 89 g/L alanine from 110 g/L lactose in WP with a yield of 78% compared with 70 g/L (Yield: 61%) on JM140 (Fig. 7D). The productivity was reduced a little from 1.44 g/L/h to 1.37 g/L/h. The accumulation of galactose and succinate were observed for both strains (data not shown). These data demonstrate that inactivation of the PPP is a useful strategy for enhancing the yield for producing these valuable chemicals from WP. The yield improvement was 20% for ethanol, 13% for D-lactate and 17% for alanine, respectively.

237

238 Discussion

A major challenge when using resting *C. glutamicum* for producing fuels and chemicals is how to achieve a high yield. In previous studies where *C. glutamicum* was tailored into producing compounds via redox balanced reactions, lactate, acetate and succinate were three major by-products that affected the final product yield (11, 12, 17, 23). By deleting *ldhA*, the lactate formation could be eliminated, while aerobic growth remained unaffected. Under anaerobic conditions, the loss of lactate dehydrogenase was compensated by redirecting the flux to other compounds that require NADH for biosynthesis, typically succinate (9). Glycolysis and thus sugar consumption in *C. glutamicum* have been found to be negatively affected by a high intracellular NADH/NAD⁺ ratio, due to the inhibition of glyceraldehyde 3-phosphate dehydrogenase (24, 25). This is exactly what we observed in this study. When we knocked out the *mdh* gene in our ethanol producing strain JS122 to eliminate succinate formation, the intracellular NADH/NAD⁺ ratio

250 increased dramatically and the glycolytic flux was hampered (Fig. 2A & 3A) with a low cellular
251 energy status as the direct consequence of this (Fig. 3B). Other researchers have suggested that
252 unidentified metabolic pathways could contribute to the surplus NADH in *C. glutamicum* under
253 anoxic conditions (11). We first suspected that the formation of acetate under anoxic conditions
254 might be one reason, as the oxidation of pyruvate via pyruvate dehydrogenase generates NADH,
255 which could elevate the NADH/NAD⁺ ratio (Fig. 1 & 3A). Pyruvate dehydrogenase is a complex
256 enzyme composed of three subunits E1, E2, and E3 (19). Deletion of the E1 subunit can inactivate
257 PDHc and block the conversion of pyruvate to acetate (19). Without PDHc, the aerobic growth of *C.*
258 *glutamicum* requires exogenous acetate or ethanol as a precursor for synthesis of the essential
259 acetyl-CoA (26). It has a negative effect on glucose consumption with the co-metabolism of acetate
260 under aerobic conditions (27). In this study, we replaced the native E3 subunit with the one from *E.*
261 *coli* PDHc, which only functions under aerobic conditions (26, 36). By exchanging the *lpd* gene
262 encoding the E3 subunit, we successfully eliminated most of the acetate formation (maybe little can
263 still be formed by pyruvate:quinone oxidoreductase) (Fig. 2D). This modification increased the
264 ethanol yield, but did not improve the glucose consumption rate, and only a slight drop in
265 NADH/NAD⁺ ratio was achieved (Fig. 3A).

266 Wild-type *C. glutamicum* strain metabolizes glucose faster (5.25 mmol g⁻¹h⁻¹) than fructose (4.42
267 mmol g⁻¹h⁻¹) when growing aerobically (20), but the opposite behavior has been observed under
268 anoxic conditions (29), and this attracted our attention. When fructose was used as the sole carbon
269 source, four times less of the sugar taken up was channeled into the PPP when compared to glucose,
270 and as a consequence less NADPH was generated (18). We found that JS133 exhibited a
271 significantly higher ethanol production rate on fructose than on glucose, and we speculated that the
272 PPP somehow might be involved in regulating the glycolysis under anoxic conditions. After
273 inactivating glucose-6-phosphate dehydrogenase, a large reduction in the intracellular

274 NADPH/NADP⁺ ratio was achieved, and surprisingly the NADH/NAD⁺ ratio was also reduced (Fig.
275 3A). Blocking the PPP greatly increased the glycolytic flux as well as the ATP/ADP ratio in JS134
276 (Fig. 2A, 3B). Under anoxic conditions, 5% glucose was metabolized through the PPP compared to
277 95% through glycolysis in *C. glutamicum*, although the NADPH-consuming anabolism was halted
278 (30).

279 In this study, we also found the interconnections between NADPH and NADH during the anoxic
280 catabolism of sugars in *C. glutamicum*. When we blocked the formation of succinate, an increase in
281 the NADH/NAD⁺ ratio was accompanied by an increase in the NADPH/NADP⁺ (Fig. 3A). In the
282 opposite way, when we blocked the PPP to reduce the NADPH/NADP⁺ ratio, the NADH/NAD⁺
283 ratio concurrently decreased (Fig. 3A). A similar phenomenon was observed in Yamamoto *et al.*'s
284 study for alanine production in *C. glutamicum* (31). When they overexpressed the glycolytic genes
285 *gapA*, *pfk*, and *pgi*, individually, to enhance glucose consumption under oxygen deprivation
286 conditions, the NADPH/NADP⁺ ratio changed proportionally with the change of NADH/NAD⁺
287 ratio, even though the carbon flux into the PPP did not change significantly. There might be several
288 explanations for this. As we know, transhydrogenase participates in the interconversion of NADPH
289 and NADH in microbes to maintain the intracellular redox hemostasis (32). As it was shown in the
290 resting *Bacillus subtilis*, the PPP generated surplus NADPH and it was expected the
291 transhydrogenation activity was responsible for the conversion of NADPH to NADH in order to
292 maintain the intracellular redox hemostasis, though the responsible gene was not identified (33).
293 Although the gene encoding transhydrogenase is not found in *C. glutamicum* (34), we did detect a
294 minor transhydrogenase activity in the non-growing *C. glutamicum* cells under anoxic condition. It
295 might also be possible that the NADPH-dependent malate enzyme together with malate:quinone
296 oxidoreductase and oxaloacetate dehydrogenase could form a metabolic cycle and exhibit
297 transhydrogenase activities responsible for generating NADH from NADPH (35). Another

298 explanation might be due to the promiscuity of glucose-6-phosphate dehydrogenase (*zwf*-encoding),
299 which may accept NAD^+ for generating NADH directly under some conditions, although the
300 purified glucose-6-phosphate dehydrogenase did not display a NAD^+ dependent activity in vitro
301 (36). The glucose-6-phosphate dehydrogenase from both *Z. mobilis* and *Bacillus subtilis* are known
302 to be able to generate NADH (37, 38).

303 RNA-seq analysis also revealed a dramatic alteration of gene expression in response to the blockage
304 of PPP. When *zwf* was deleted, the expression of the F_1F_0 -ATP synthase was significantly down-
305 regulated in JS134 (Fig. 5), which indicated that the energy starvation caused by cofactor imbalance
306 could be mitigated by blocking PPP. Simultaneously, the expression of the nitrate reductase operon
307 was also regulated in JS134. It is known that *C. glutamicum* is capable of anaerobic respiration
308 using nitrate as a terminal electron acceptor (39). Even though, nitrate was not provided in the
309 fermentation broth, a down-regulation of nitrate reductase in the strain lacking *zwf* indicated that the
310 NADH/NAD^+ ratio could be involved in regulating expression of this gene. Of the up-regulated
311 genes, we found the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase was up-
312 regulated after deleting *zwf*. The observation was consistent to previous studies that have shown that
313 expression of glyceraldehyde-3-phosphate dehydrogenase is inhibited by high NADH/NAD^+ ratios
314 under anoxic conditions (40). Overall, the results from the transcriptional analysis were congruent
315 with the changes observed in the fermentation physiology, where deletion of *zwf* relieved energy
316 starvation and cofactor imbalance under anoxic conditions.

317 In this study, we successfully pinpointed the pathway responsible for the high NADH/NAD^+ ratio
318 observed in *C. glutamicum* under anoxic conditions. This knowledge can be harnessed to engineer
319 *C. glutamicum* mutants producing high yield of chemicals via redox-balanced reactions, which has
320 its intrinsic challenges (11). In a previous study, where a $\Delta\text{ppc}\Delta\text{ldhA}$ mutant of *C. glutamicum* was
321 used for producing D-lactate, only about 86% of the theoretical yield could be accomplished from

322 glucose (23). Here, by combining a modified pyruvate dehydrogenase and PPP inactivation, a yield
323 to 98% was attained, which is among the highest D-lactate yields achieved using microbial
324 fermentation (41).

325 By harnessing the fundamental discovery that the disruption of PPP strategy can benefit high-yield
326 production, we further demonstrate its applications on the conversion of lactose, which is rich in
327 dairy wastes, into valuable compounds. Growth of the PPP - disrupted strains could be restored on
328 the substrate WP with CSL. We finally achieved high-yield production of ethanol, D-lactate and
329 alanine on WP. Previously, we achieved a yield of 88% of the theoretical using JS122 on delactosed
330 WP (DWP) provided by MS Iceland Dairies (15) and the yield on WP provided by Arla Food
331 Ingredients (Denmark) in the present work was only 65%. As the biotin concentration is much
332 higher in WP than DWP and biotin is the cofactor for pyruvate carboxylase, which requires for the
333 production of succinate from pyruvate. So it might explain that we got a lower yield of ethanol on
334 WP using the same strain. We also noticed that the productivity was lowered in our PPP - disrupted
335 strains and this can be complemented in the future studies by overexpressing the glycolytic enzymes
336 or employing the ATP draining strategies through for instance, overexpression of the F1-ATPase
337 (42).

338 **Materials and methods**

339 **Bacterial strains, plasmids.**

340 All bacterial strains and plasmids used or constructed in this study are listed in Table 1.

341 **Growth medium and conditions.**

342 *E. coli* strains were grown aerobically in Luria-Bertani broth (LB) at 37°C, and *C. glutamicum*
343 strains were cultivated in Brain Heart Infusion (Oxoid) broth (BHI) at 30°C with 200 rpm shaking.

344 For ethanol and D-lactate production, *C. glutamicum* strains were aerobically cultivated in CGXII
345 medium supplemented with 5% glucose or fructose, and if necessary, 0.5% yeast extract (YE) was
346 added for the growth of *zwf* inactivated strains. When appropriate, kanamycin was added to a
347 concentration of 50 µg/mL for *E. coli* and 25 µg/mL for *C. glutamicum*, and spectinomycin was
348 used at a concentration of 100 µg/mL for *E. coli* and 50 µg/mL for *C. glutamicum*. For gene deletion
349 or replacement in *C. glutamicum*, 10% sucrose was used for counter-selection. Cell growth was
350 monitored by measuring the optical density at 600 nm (OD₆₀₀) of the culture broth using a UV1800
351 spectrophotometer (Shimadzu, Japan). The dry cell weight was calculated based on DCW (g/L) =
352 OD₆₀₀ × 0.3 (43).

353 **Construction of plasmids and strains.**

354 All molecular manipulations were performed according to the standard protocols (44) and
355 Handbook of *Corynebacterium glutamicum* (45). All plasmids/strains constructed in this study were
356 verified by Sanger Sequencing (Macrogen, Korea).

357 *C. glutamicum* mutants carrying gene deletions in *mdh* and *zwf* and mutants where the *E. coli lpd*
358 gene substituted the native *lpd*, were constructed via a two-step homologous recombination
359 procedure as described previously (45) using the vector pK18*mobsacB* (46). Plasmids were
360 transformed into *C. glutamicum* by electroporation.

361 For the construction of pK18-*mdh*, the regions up- and downstream (approximately 750-bp each) of
362 the *mdh* gene to be deleted were amplified (primers used can see Table S2). The resulting DNA
363 fragments were cloned into pK18*mobsacB* through Gibson assembly. The plasmid pK18-*zwf* was
364 constructed in a similar manner. A gBlock gene fragment containing the regions up- and
365 downstream of *zwf* was synthesized by Integrated DNA Technologies (sequences can see Fig. S3),
366 and then cloned into pK18*mobsacB*.

367 Plasmid pK18-lpd(E) was constructed in order to replace the chromosomal *lpd* gene from *C.*
368 *glutamicum*. For this purpose, a gBlock gene fragment containing the *lpd* gene of *E. coli* flanked by
369 the up- and downstream regions (approximately 750-bp each) of the *lpd* gene of *C. glutamicum*
370 was synthesis by Integrated DNA Technologies (sequences can see Fig. S2), in which the *lpd* gene
371 starts with GTG codon, and then cloned into pK18*mobsacB*.

372 For the construction of the expression plasmid pJS136 (D-lactate) and pJM140 (alanine), the 1.2-kb
373 *L. delbrueckii ldhA* gene coding D-LDH was amplified using *L. delbrueckii* chromosomal DNA as
374 the template, and the 1.1-kb *alaDH* encoding alanine dehydrogenase from *Lysinibacillus sphaericus*
375 was synthesized by Genscript (Piscataway, US). The resulting fragments were, individually,
376 inserted into the vector pEC-XX99E (47) through Gibson assembly.

377 **Conditions for ethanol and D-lactate production using the minimal medium.**

378 Ethanol production under oxygen deprivation was performed as previously described, with minor
379 alterations (12). *C. glutamicum* strains were aerobically cultivated at 30°C for 16-20 h in a 1 L flask
380 containing 200 mL of CGXII medium supplemented with 50 g/L glucose or fructose, if necessary,
381 0.5% YE was added. 0.1 mM IPTG was added in the medium for JS137 and JS138. Cells were
382 harvested by centrifugation (5000×g, 4°C, 10 min.) and then resuspended in fresh CGXII medium
383 with 50 g/L glucose or fructose to a concentration corresponding to 10 g DCW/L. Temperature was
384 maintained at 30°C. Oxygen deprivation was achieved with high cell density, no aeration, and
385 gentle agitation.

386 The procedure for D-lactate production was similar to ethanol production, however, to maintain pH
387 the medium was supplemented with 40 g/L CaCO₃.

388 **Procedure for adaptive laboratory evolution.**

389 The evolution was conducted using a serial-transfer regime with strain JS125, a *C. glutamicum*
390 derivative in which the genes *ldhA*, *ppc*, *mdh* were deleted and the native *lpd* gene was replaced
391 with the *lpd* gene from *E. coli*. A single colony of JS125 was inoculated into a test tube containing
392 5 mL CGXII medium with 2% glucose, and cultivated at 30°C with 200 rpm shaking. When the
393 culture entered the stationary phase, 0.05 mL of culture was transferred into a new test tube with
394 the same medium, which was equal to a 6.64-generation adaptation. Each week, a copy of the
395 culture was saved in 25% glycerol at -80°C. The growth performance of JS125 was regularly
396 checked. After a 100-generation adaptive evolution, culture from the final tube was streaked on the
397 CGXII glucose plate, and one fast-growing single colony was isolated, and was designated as
398 JS125A.

399 **Production of ethanol, D-lactate and alanine from whey permeate.**

400 Whey permeate (WP) is provided by the dairy company Arla Foods Ingredients Group P/S (Viby,
401 Denmark). It is the by-product of dairy industry for producing whey proteins from whey, which is
402 obtained in the cheese making process. WP has more than 10% (w/v) lactose. We tested the growth
403 of JS134 on 50% WP (diluted with water) with different types of nitrogen sources. Corn steep
404 liquor (CSL) with 50% solid content, which was purchased from Sigma-Aldrich (St. Louis, MO),
405 was centrifuged for 10 mins at 5,000 ×g and the supernatant was used. The minimal medium salts
406 contain (NH₄)₂SO₄ (7 g/L), FeSO₄ (20 mg/L), MnSO₄ (2 mg/L) and the trace element solution from
407 the CGXII medium. The pH for all the medium was adjusted to 7.0. A single colony of JS134 was
408 inoculated into 25 mL medium in 100 mL flask and cultivated at 30°C with 200 rpm shaking. The
409 cell density (OD₆₀₀) was measured after 24 h.

410 For the production of ethanol, D-lactate and alanine, we grew the relevant strains in 1 L flask
411 containing 200 mL 50% WP, MMS and 1% YE (for easy manipulation instead of CSL). The cells

412 were collected after 20 h and then resuspended in 50 mL pure WP medium to achieve a cell
413 concentration of 20 g DCW/L. The pH of the fermentation was maintained at 7.0 using the Biostat
414 A (Sartorius, Germany) pH-control system by supplementing 5 M ammonia solution.

415 **RNA sequencing.**

416 When we prepared the samples for transcriptomic analysis, we first grew the strains JS133 and
417 JS134 in exactly the same medium that is CGXII medium with 0.5% YE. Then we resuspended the
418 cells in fresh CGXII medium with 5% glucose. After 3 h incubation under anaerobic conditions, we
419 collected 200 µL culture that was spiked in 800 µL RNAlater solution (ThermoFisher Scientific).
420 After centrifugation at 12,000 g for 2 min, the supernatant was discarded, and the pellet was used
421 for extracting total RNA using the RNeasy Mini kit (Qiagen) according to the manufacture's
422 instruction. The integrity of RNA was determined by a 2100 BioAnalyzer (Agilent). Commercial
423 service of library preparation and sequencing were provided by BGI China. The sequencing was
424 performed on a BGISEQ 500 next-generation sequencer, from which datasets consisted of at least 20
425 M clean reads (single-paired) per sample were obtained. Afterwards, adaptor sequences,
426 contamination and low-quality reads were removed from the raw reads. Read trimming, mapping
427 and statistical analysis were performed on CLC Genomics Workbench. For the differentially
428 overexpressed genes, the heatmap for JS133 and JS134 (three independents each) was made using
429 the pheatmap package in R (49) and Go enrichment analysis was conducted using the DAVID tools
430 (49, 50). All the raw sequence read and processed data were deposited in Gene Expression
431 Omnibus (GEO) at NCBI under the accession NO. GSE143977 (token for reviewer access:
432 cdgjcuiqldwbtgl).

433 **Analytical techniques.**

434 **I) Product formation and sugar consumption.** Culture samples were withdrawn, centrifuged
435 (15,000×g, 4°C, 5 min.), and the supernatant saved at -20°C for later analysis. Sugar, ethanol, D-
436 lactate, succinate, acetate and glycerol were quantified using an Ultimate 3000 high-pressure liquid
437 chromatography system (Dionex, Sunnyvale, USA) equipped with an Aminex HPX-87H column
438 (Bio-Rad, Hercules, USA) and a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan). The
439 column oven temperature was set at 60°C, the mobile phase was at 5 mM H₂SO₄, and the flow rate
440 was 0.5 mL/min. The alanine concentration was measured using the alanine colorimetric kit (VWR
441 No: BIOVK652-100).

442 **II) NAD(P)H/NAD(P)⁺ ratio.** To measure intracellular NAD⁺, NADH, NADP⁺, NADPH
443 concentrations, cell extraction was performed as follows. NAD(P)H and NAD(P)⁺ concentrations
444 were determined at 3 h during ethanol production. The collected samples were immediately
445 quenched by mixing with 1.0 mL cold methanol (-80°C), and the resultant cell suspension (0.5 mL)
446 was mixed vigorously with 0.5 mL chloroform and 0.5 mL H₂O (-20°C) to disrupt cells. After
447 incubation for 60 min at -20°C, the sample solution was centrifuged (20,000×g, 4°C, 5 min.), and
448 an aliquot of the upper layer (50 µL) was mixed with 50 µL H₂O or standard mixture solutions. The
449 mixtures were further centrifuged (20,000×g, 4 °C, 5 min), and the resultant supernatant was
450 analyzed using an NAD(P)/NAD(P)H Assay Kit (Sigma-Aldrich, St. Louis, MO, USA).

451 **III) ATP/ADP.** Samples were collected at 3 h during ethanol production. Cell extraction was
452 performed as previously described (51). Cultures (5 mL) were quenched with 1 volume 80°C
453 phenol. After being centrifuged (4,000×g, 10 min.), the water phase was transferred into a new tube
454 and extracted with 1 volume of chloroform twice. ATP was measured using a BioThema ATP kit
455 HS, and ADP was converted into ATP by 1 mM PEP and 1 U pyruvate kinase. The luminescence
456 was subsequently measured on a Tecan Infinite M200 Pro microplate reader.

457 **IV) Measurement of transhydrogenase activity.** Samples were collected at 3 h during ethanol
458 production under oxygen deprivation conditions. Cell suspensions were disrupted by bead beating
459 using a FastPrep system (MP Biomedicals) with acid-washed glass beads (106 μm , Sigma-Aldrich
460 prod. No.: G4649). The transhydrogenase activity was measured according to the procedure
461 described by Sauer *et al.* (32) . Briefly, the absorbance at 375 nm was monitored using a Tecan
462 Infinite M200 Pro microplate reader at 25°C in a mixture containing 50 mM Tris·HCl (pH 7.6), 2
463 mM MgCl_2 , 500 μM NADPH, 1mM 3-acetylpyridine adenine dinucleotide, and 10-100 μL cell
464 extract. The specific activity was obtained through dividing the measured slope by the protein
465 concentration. Protein concentrations were determined using Bradford according to the manual
466 (Sigma-Aldrich).

467 **Supplementary material**

468 Supplementary File 1

469 **Acknowledgements**

470 This work was supported by the DTU PoC Fund (“Sweet as Sugar” project), the Danish Dairy
471 Research Foundation (Optimering af smagsdannelse i hårde oste), and Innovation Fund Denmark
472 (Grant No. 6150-00036B).

473

474

475

476

477 **References**

- 478 1. Becker J, Wittmann C. 2012. Systems and synthetic metabolic engineering for amino acid production
479 - the heartbeat of industrial strain development. *Curr Opin Biotechnol* 23:718–26.
- 480 2. Becker J, Giebelmann G, Hoffmann S, Wittmann C. 2018. *Corynebacterium glutamicum* for
481 sustainable bioproduction: from metabolic physiology to systems metabolic engineering. *Adv*
482 *Biochem Eng Biotechnol* 162:217–263.
- 483 3. Inui M, Toyoda K. 2013. *Corynebacterium glutamicum*: Biology and Biotechnology.
- 484 4. Becker J, Wittmann C. 2017. Industrial microorganisms: *Corynebacterium glutamicum*. *Ind*
485 *Biotechnol* 183–220.
- 486 5. Becker J, Wittmann C. 2012. Bio-based production of chemicals, materials and fuels -
487 *Corynebacterium glutamicum* as versatile cell factory. *Curr Opin Biotechnol* 23:631–40.
- 488 6. Wieschalka S, Blombach B, Bott M, Eikmanns BJ. 2013. Bio-based production of organic acids with
489 *Corynebacterium glutamicum*. *Microb Biotechnol* 6:87–102.
- 490 7. Okino S, Inui M, Yukawa H. 2005. Production of organic acids by *Corynebacterium glutamicum*
491 under oxygen deprivation. *Appl Environ Microbiol* 68:475–480.
- 492 8. Yamamoto S, Sakai M, Inui M, Yukawa H. 2011. Diversity of metabolic shift in response to oxygen
493 deprivation in *Corynebacterium glutamicum* and its close relatives. *Appl Microbiol Biotechnol*
494 90:1051–61.
- 495 9. Inui M, Murakami S, Okino S, Kawaguchi H, Vertès A a, Yukawa H. 2004. Metabolic analysis of
496 *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation
497 conditions. *J Mol Microbiol Biotechnol* 7:182–96.
- 498 10. Okino S, Noburyu R, Suda M, Jojima T, Inui M, Yukawa H. 2008. An efficient succinic acid
499 production process in a metabolically engineered *Corynebacterium glutamicum* strain. *Appl*
500 *Microbiol Biotechnol* 81:459–464.
- 501 11. Jojima T, Fujii M, Mori E, Inui M, Yukawa H. 2010. Engineering of sugar metabolism of
502 *Corynebacterium glutamicum* for production of amino acid L-alanine under oxygen deprivation. *Appl*
503 *Microbiol Biotechnol* 87:159–65.
- 504 12. Inui M, Kawaguchi H, Murakami S, Vertès AA, Yukawa H. 2004. Metabolic engineering of
505 *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. *J Mol*

- 506 Microbiol Biotechnol 8:243–54.
- 507 13. Sakai S, Tsuchida Y, Okino S, Ichihashi O, Kawaguchi H, Watanabe T, Inui M, Yukawa H. 2007.
508 Effect of lignocellulose-derived inhibitors on growth of and ethanol production by growth-arrested
509 *Corynebacterium glutamicum* R. Appl Environ Microbiol 73:2349–2353.
- 510 14. Jojima T, Noburyu R, Sasaki M, Tajima T, Suda M, Yukawa H, Inui M. 2015. Metabolic engineering
511 for improved production of ethanol by *Corynebacterium glutamicum*. Appl Microbiol Biotechnol
512 99:1165–1172.
- 513 15. Shen J, Chen J, Jensen PR, Solem C. 2019. Development of a novel, robust and cost-efficient process
514 for valorizing dairy waste exemplified by ethanol production. Microb Cell Fact 18:51.
- 515 16. Peters-Wendisch PG, Kreutzer C, Kalinowski J, Pátek M, Sahm H, Eikmanns BJ. 1998. Pyruvate
516 carboxylase from *Corynebacterium glutamicum*: characterization, expression and inactivation of the
517 pyc gene. Microbiology 144:915–927.
- 518 17. Wieschalka S. 2012. Engineering *Corynebacterium glutamicum* as a designer-bug for the bio-based
519 production of chemical building blocks and biofuel. Open Access Repositorium der Universität Ulm.
520 Dissertation.
- 521 18. Snoep JL, de Graef MR, Westphal a H, de Kok A, Teixeira de Mattos MJ, Neijssel OM. 1993.
522 Differences in sensitivity to NADH of purified pyruvate dehydrogenase complexes of *Enterococcus*
523 *faecalis*, *Lactococcus lactis*, *Azotobacter vinelandii* and *Escherichia coli*: implications for their
524 activity in vivo. FEMS Microbiol Lett 114:279–83.
- 525 19. Yasuda K, Jojima T, Suda M, Okino S, Inui M, Yukawa H. 2007. Analyses of the acetate-producing
526 pathways in *Corynebacterium glutamicum* under oxygen-deprived conditions. Appl Microbiol
527 Biotechnol 77:853–60.
- 528 20. Dominguez H, Coccagn-Bousquet M, Lindley ND. 1997. Simultaneous consumption of glucose and
529 fructose from sugar mixtures during batch growth of *Corynebacterium glutamicum*. Appl Microbiol
530 Biotechnol 47:600–603.
- 531 21. Dominguez H, Rollin C, Guyonvarch A, Guerquin-Kern J, Bousquet M, D.Lindley N. 1998. Carbon-
532 flux distribution in the central metabolic pathways of *Corynebacterium glutamicum* during growth on
533 fructose. Eur J Biochem 254:96–102.
- 534 22. Kiefer P, Heinze E, Zelder O, Wittmann C. 2004. Comparative metabolic flux analysis of lysine-
535 producing *Corynebacterium glutamicum* cultured on glucose or fructose. Appl Environ Microbiol

- 536 70:229–239.
- 537 23. Okino S, Suda M, Fujikura K, Inui M, Yukawa H. 2008. Production of D-lactic acid by
538 *Corynebacterium glutamicum* under oxygen deprivation. Appl Microbiol Biotechnol 78:449–454.
- 539 24. Danshina P V, Schmalhausen E V, Avetisyan A V, Muronetz VI. 2001. Mildly oxidized
540 glyceraldehyde-3-phosphate dehydrogenase as a possible regulator of glycolysis. IUBMB Life
541 51:309–314.
- 542 25. Tsuge Y, Uematsu K, Yamamoto S, Suda M, Yukawa H, Inui M. 2015. Glucose consumption rate
543 critically depends on redox state in *Corynebacterium glutamicum* under oxygen deprivation. Appl
544 Microbiol Biotechnol 99:5573–5582.
- 545 26. Schreiner ME, Fiur D, Holátko J, Pátek M, Eikmanns BJ. 2005. E1 enzyme of the pyruvate
546 dehydrogenase complex in *Corynebacterium glutamicum*: molecular analysis of the gene and
547 phylogenetic aspects. J Bacteriol 187:6005–6018.
- 548 27. Blombach B, Schreiner ME, Holátko J, Bartek T, Oldiges M, Eikmanns BJ. 2007. L-valine
549 production with pyruvate dehydrogenase complex-deficient *Corynebacterium glutamicum*. Appl
550 Environ Microbiol 73:2079–84.
- 551 28. Kim Y, Ingram LO, Shanmugam KT. 2008. Dihydrolipoamide dehydrogenase mutation alters the
552 NADH sensitivity of pyruvate dehydrogenase complex of *Escherichia coli* K-12. J Bacteriol
553 190:3851–3858.
- 554 29. Michel A, Koch-Koerfges A, Krumbach K, Brocker M, Bott M. 2015. Anaerobic growth of
555 *Corynebacterium glutamicum* via mixed-acid fermentation. Appl Environ Microbiol 81:7496–7508.
- 556 30. Radoš D, Turner DL, Fonseca LL, Carvalho L, Blombach B, Eikmanns BJ, Neves AR, Santos H.
557 2014. Carbon flux analysis by ¹³C nuclear magnetic resonance to determine the effect of CO₂ on
558 anaerobic succinate production by *Corynebacterium glutamicum*. Appl Environ Microbiol 80:3015–
559 3024.
- 560 31. Yamamoto S, Gunji W, Suzuki H, Toda H, Suda M, Jojima T, Inui M, Yukawa H. 2012.
561 Overexpression of genes encoding glycolytic enzymes in *Corynebacterium glutamicum* enhances
562 glucose metabolism and alanine production under oxygen deprivation conditions. Appl Environ
563 Microbiol 78:4447–57.
- 564 32. Sauer U, Canonaco F, Heri S, Perrenoud A, Fischer E. 2004. The soluble and membrane-bound
565 transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of *Escherichia*

- 566 *coli*. J Biol Chem 279:6613–6619.
- 567 33. Rühl M, Le Coq D, Aymerich S, Sauer U. 2012. ¹³C-flux analysis reveals NADPH-balancing
568 transhydrogenation cycles in stationary phase of nitrogen-starving *Bacillus subtilis*. J Biol Chem
569 287:27959–70.
- 570 34. Kabus A, Georgi T, Wendisch VF, Bott. 2007. Expression of the *Escherichia coli* pntAB genes
571 encoding a membrane-bound transhydrogenase in *Corynebacterium glutamicum* improves L-lysine
572 formation. Appl Microbiol Biotechnol 75:47–53.
- 573 35. Sauer U, Eikmanns BJ. 2005. The PEP-pyruvate-oxaloacetate node as the switch point for carbon
574 flux distribution in bacteria. FEMS Microbiol Rev 29:765–794.
- 575 36. Moritz B, Striegel K, De Graaf AA, Sahm H. 2000. Kinetic properties of the glucose-6-phosphate and
576 6-phosphogluconate dehydrogenases from *Corynebacterium glutamicum* and their application for
577 predicting pentose phosphate pathway flux in vivo. Eur J Biochem 267:3442–3452.
- 578 37. Scopes RK. 1997. Allosteric control of *Zymomonas mobilis* glucose-6-phosphate dehydrogenase by
579 phosphoenolpyruvate. Biochem J 326:731–5.
- 580 38. Black WB, Zhang L, Mak WS, Maxel S, Cui Y, King E, Fong B, Sanchez Martinez A, Siegel JB, Li
581 H. 2020. Engineering a nicotinamide mononucleotide redox cofactor system for biocatalysis. Nat
582 Chem Biol 16:87–94.
- 583 39. Nishimura T, Vertès AA, Shinoda Y, Inui M, Yukawa H. 2007. Anaerobic growth of
584 *Corynebacterium glutamicum* using nitrate as a terminal electron acceptor. Appl Microbiol
585 Biotechnol 75:889–897.
- 586 40. Tsuge Y, Uematsu K, Yamamoto S, Suda M, Yukawa H, Inui M. 2015. Glucose consumption rate
587 critically depends on redox state in *Corynebacterium glutamicum* under oxygen deprivation. Appl
588 Microbiol Biotechnol 99:5573–5582.
- 589 41. Juturu V, Wu JC. 2016. Microbial production of lactic acid: the latest development. Crit Rev
590 Biotechnol 36:967–977.
- 591 42. Liu J, Kandasamy V, Würtz A, Jensen PR, Solem C. 2016. Stimulation of acetoin production in
592 metabolically engineered *Lactococcus lactis* by increasing ATP demand. Appl Microbiol Biotechnol
593 100:9509–17.
- 594 43. Wang Z, Liu J, Chen L, Zeng AP, Solem C, Jensen PR. 2018. Alterations in the transcription factors

- 595 GntR1 and RamA enhance the growth and central metabolism of *Corynebacterium glutamicum*.
596 Metab Eng 48:1–12.
- 597 44. Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring
598 Harbor laboratory press.
- 599 45. Eggeling L, Bott M. 2005. Handbook of *Corynebacterium glutamicum*. Taylor & Francis.
- 600 46. Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A. 1994. Small mobilizable multi-
601 purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of
602 defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69–73.
- 603 47. Kirchner O, Tauch A. 2003. Tools for genetic engineering in the amino acid-producing bacterium
604 *Corynebacterium glutamicum*. J Biotechnol 104:287–299.
- 605 48. Liu J, Dantoft SH, Würtz A, Jensen PR, Solem C. 2016. A novel cell factory for efficient production
606 of ethanol from dairy waste. Biotechnol Biofuels 9:33.
- 607 49. Huang DW, Sherman BT, Lempicki RA. 2008. Bioinformatics enrichment tools: paths toward the
608 comprehensive functional analysis of large gene lists. Nucleic Acids Res 37:1–13.
- 609 50. Huang DW, Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene lists
610 using DAVID bioinformatics resources. Nat Protoc 4:44–57.
- 611 51. Chen J, Shen J, Solem C, Jensen PR. 2013. Oxidative stress at high temperatures in *Lactococcus*
612 *lactis* due to an insufficient supply of riboflavin. Appl Environ Microbiol 79:6140–6147.
- 613 52. Kurosawa K, Wewetzer SJ, Sinskey AJ. 2013. Engineering xylose metabolism in triacylglycerol-
614 producing *Rhodococcus opacus* for lignocellulosic fuel production. Biotechnol Biofuels 6:134.

615

616

617

618

619

620

621

622 **Table 1.** Strains and plasmids used in the study.

Strain/Plasmid	Description/Function	Reference
Strains		
JS112	<i>C. glutamicum</i> :: <i>lacSZ::galMKTE</i> , Δ <i>ldh</i> , Δ <i>ppc</i>	(15)
JS119	JS112, Δ <i>mdh</i>	This study
JS122	JS112 harboring pJS115 (ethanol-producing plasmid)	(15)
JS124	JS119 harboring pJS115	This study
JS125	<i>C. glutamicum</i> Δ <i>ldh</i> , Δ <i>ppc</i> , Δ <i>mdh</i> , replacement of the <i>lpd</i> gene with <i>E.coli</i> 's	This study
JS125A	Isolation from evolved JS125	This study
JS132	JS125A, Δ <i>zwf</i>	This study
JS133	JS125A harboring pJS115	This study
JS134	JS132 harboring pJS115	This study
JS137	JS112 harboring pJS136 (D-lactate-producing plasmid)	This study
JS138	JS132 harboring pJS136	This study
JM140	JS112 harboring pJM140 (Alanine-producing plasmid)	This study
JM141	JS132 harboring pJM140	This study
Plasmids		
pAL347	<i>E. coli</i> - <i>C. glutamicum</i> expression shutter vector, Spec ^R	(52)
pEC-XK99E	<i>E. coli</i> - <i>C. glutamicum</i> expression shutter vector,	(47)
pK18mobsacB	used for the construction of gene insertion and deletion <i>C.glutamicum</i> mutants, Kan ^R	(46)
pJS115	pAL347 derivative with the <i>pdh</i> and <i>adhB</i> genes from <i>Z. mobilis</i> , Spec ^R	This study
pJS136	pEC-XK99E derivative with the <i>D-ldh</i> gene from <i>Lactobacillus delbrueckii</i>	This study
pJM140	pEC-XK99E derivative with the <i>alaDH</i> gene from <i>Lysinibacillus sphaericus</i>	This study
pK18-mdh	Deletion of <i>mdh</i> gene in <i>C. glutamicum</i>	This study
pK18-lpd	Replacement of <i>lpd</i> gene with <i>E. coli</i> 's	This study
pK18-zwf	Deletion of <i>zwf</i> gene in <i>C. glutamicum</i>	This study

623

624

625

626

627

628

629 **Figure 1.** Schematic overview of sugar metabolism in *C. glutamicum* under anoxic conditions. The
 630 grey boxes indicate the relevant enzymes. LDH, lactate dehydrogenase (*ldhA* gene); PEPC,
 631 phosphoenolpyruvate carboxylase (*ppc* gene); PYC, pyruvate carboxylase (*pyc* gene); MDH,
 632 malate dehydrogenase (*mdh* gene); FUM, fumarase; SDH, succinate dehydrogenase; PDH, pyruvate
 633 carboxylase; G6PDH, glucose-6-phosphate dehydrogenase (*zwf* gene). The *Z. mobilis* PDC
 634 (pyruvate decarboxylase (*pdc* gene)) and ADH (alcohol dehydrogenase (*adhB* gene)) enzymes,
 635 which expressed in *C. glutamicum* in this study, are shown in the red boxes. Red Xes indicate the
 636 deletion of the relevant genes.

637

Figure 2. Determination of glucose consumption and metabolites accumulation in different strains under anoxic conditions. A, glucose; B, ethanol; C, succinate; D, acetate. The cell concentration was 10 g DCW/L. Two biologically independent experiments were performed to calculate the standard deviation.

646

647

648 **Figure 3.** Measurement of NADH/NAD⁺, NADPH/NADP⁺ and ATP/ADP in different strains under
649 anoxic conditions. Two biologically independent experiments were performed to calculate the
650 standard deviation.

651

652

653

654

655

656

657

658

659

660

661 **Figure 4.** Characterization of strain growth. A. Comparison of the final cell density (OD₆₀₀) after 40
 662 h aerobic growth in minimal medium containing glucose, the growth experiments were carried out
 663 in 100 mL shake flask with 20 mL medium. The standard deviations were calculated from three
 664 independent experiments. B. Cell growth was monitored on Biolector (M2p-labs, Germany) with
 665 1500 rpm shaking and the OD₆₀₀ after 30 h was measured on a UV1800 spectrophotometer.

666

667

668

669

670

671

672

673

674

675

676

677 **Figure 5.** Transcriptomics for JS134 and JS133 (three independent replicates for each). A. The
678 heatmap for the differentially expressed genes was made using pheatmap in R. B. Enriched GO
679 terms for the up-regulated and down-regulated gene in JS134 when using JS133 as the reference.

680

681

682

683

684 **Figure 6.** Comparison of glucose consumption and D-lactate production between JS137 and JS138.

685 The cell concentration was 10 g/L. Two biologically independent experiments were performed to

686 calculate the standard deviation.

687

688

689

690

691

692

Figure 7. Transformation of lactose in dairy wastes into valuable chemicals. A. The lactose
metabolism pathway. B. Comparison of lactose consumption and ethanol production between JS122
and JS134. C. Comparison of lactose consumption and D-lactate production between JS137 and
JS138. D. Comparison of lactose consumption and alanine production between JM140 and JM141.
The cell concentration was 20 g/L. Two biologically independent experiments were performed to
calculate the standard deviation.

699