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Published in: Applied and Environmental Microbiology

Link to article, DOI: 10.1128/AEM.02114-20

Publication date: 2020

Document Version Peer reviewed version

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

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Abstract

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

Importance

The widely used industrial workhorse *C. glutamicum* possesses a complex anaerobic metabolism under non-growing conditions and we demonstrate here that the PPP in resting *C. glutamicum* is a source of reducing power that can interfere with otherwise redox balanced metabolic pathways and
reduce yields of desired products. By harnessing this physiological insight, we employed the PPP-
inactivated platform strains to produce ethanol, D-lactate and alanine using the dairy waste – whey permeate as the feedstock. The production yield is high and our results show that inactivation of the PPP flux in resting cells is a promising strategy when the aim is to use non-growing *C. glutamicum* cells for producing valuable compounds. Overall, we described the benefits to disrupt the oxidative PPP in non-growing *C. glutamicum* and provide a feasible approach towards waste valorization.

**Introduction**

*Corynebacterium glutamicum* is widely employed for large-scale fermentative production of amino acids, especially L-glutamate and L-lysine (1). In recent years, the fast development of tools for genetic engineering as well as for studying the systems biology of *C. glutamicum* has accelerated research on its metabolism and regulatory network (2–4). It is now apparent, that *C. glutamicum* has a great potential for industrial production of a wide range of useful compounds, e.g. organic acids and biofuels (5, 6). Whereas amino acids normally are produced under highly aerated conditions, organic acids are generated under anaerobic conditions (7, 8). When oxygen and external electron acceptors are absent, *C. glutamicum* displays limited growth in a medium devoid of rich components, but retains an active fermentative metabolism. Glucose, for instance, is metabolized via glycolysis to pyruvate that can be reduced into lactate. If carbon dioxide is available, anaplerotic reactions can lead to formation of oxaloacetate, which normally is reduced into succinate (7, 9). Sodium bicarbonate, as a source of carbon dioxide, was found to stimulate the formation of succinate as well as the glycolytic flux (7, 10), and blocking the formation of either of these compounds was found to hamper glycolysis. In one study, the NADH-sensitive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was overexpressed, which improved the glucose consumption
rate (11), and it appears that the anaerobic glycolytic flux in *C. glutamicum* is sensitive to the NADH/NAD$^+$ ratio (9). Thus, when engineering *C. glutamicum* for the purpose of bio-production under anaerobic conditions, it is imperative that the redox balance is considered carefully.

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

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

Deletion of the \textit{mdh} gene represses glucose consumption by resting cells.

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

Since the method we applied for producing ethanol depends on non-growing cells and a high cell density is required to achieve a good productivity, it is imperative that the cells can readily be amplified aerobically to high cell densities on cheap mineral medium. Thus, the strain needs functional anaplerotic pathways to replenish oxaloacetate, and it has previously been found that either pyruvate carboxylase (PYC) or PEP carboxylase (PEPC) activity is needed (16). Thus, inactivating both of these enzymes is not a viable solution for increasing ethanol yield. In an attempt to reduce the formation of succinate, we inactivated the \textit{mdh} gene encoding malate dehydrogenase, resulting in strain JS124. JS124 generated 5-fold less succinate, and its ethanol yield increased to 71.5\% of the theoretical maximum. Unexpectedly, the glucose consumption rate (glycolytic flux) dropped significantly from 2.02 mmol/gDCW/h in JS122 to only 0.19 mmol/gDCW/h in JS124 (Table S1). After a 15 h fermentation, 41.6 g/L glucose still remained unmetabolized (Fig. 2).
We speculated that the drastic reduction in glucose consumption rate was due to an unbalanced redox metabolism, since the regeneration of NAD\(^+\) via the formation of succinic acid had been blocked (17). We assayed the intracellular NADH/NAD\(^+\) ratio of JS122 and JS124, and found that the intracellular NADH/NAD\(^+\) ratio for JS124 (1.85) was much higher than for JS122 (0.06) (Fig. 3A).

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

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

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

Subsequently we introduced the ethanol plasmid pJS115 into this adapted strain and the outcome was strain JS133, which was characterized. Fig. 2D shows that JS133 only generates small amounts of acetate, and 0.3% of the consumed glucose ends up as acetate compared to 5% for JS124. The
ethanol yield for JS133 on glucose was 77.3%. However, the replacement of the E3 subunit of PDHc only slightly decreased the intracellular NADH/NAD\(^+\) ratio in the resting cells (Fig. 3A), and the glucose consumption rate of JS133 was not improved compared to that of JS124 (Fig. 2A).

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

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

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

This prompted us to inactivate the first enzyme of the oxidative PPP, glucose-6-phosphate dehydrogenase, which is encoded by *zwf*. The modification indeed boosted sugar consumption and ethanol production for the resting cells. As shown in Fig. 2A, the glucose consumption rate of JS134 was enhanced 2.6 fold when compared to JS133. The ethanol yield reached as high as 92% of the theoretical maximum, a 29% increase compared to the starting strain JS122. These data
demonstrate that the PPP indeed is a source of reducing power for resting *C. glutamicum*. It is well-known that the main role of the PPP is NADPH generation, however, for the resting cells we detected a low transhydrogenase activity (3.95 ± 1.21 μmol min⁻¹ g⁻¹), which might explain the formation of NADH.

Disrupting the PPP in JS134 prevented growth in minimal medium, and to restore growth we found that it was necessary to add a nitrogen source such as yeast extract (YE) or corn steep liquor (CSL). By adding 2% CSL the biomass density was able to reach OD₆₀₀ 21.8 (Fig. 4B).

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

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

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

As the deletion of *zwf* had a great impact on the cofactor balance and cellular energy status of *C. glutamicum* under anoxic conditions, we further investigated the impact on the transcriptional level
carrying out a transcriptome analysis for JS133 and JS134 (JS133 with zwf deletion). For each strain three independent replicate experiments were included, and it was shown that JS133 and JS134 had quite different transcriptomic profiles, where the differentially expressed genes in JS133 clustered separately from those of JS134 (Fig. 5A). When compared to JS133, a total of 592 and 334 genes had been down-regulated and up-regulated (log2-fold-change > 1, p-value < 0.05) in JS134, respectively (see all the differentially expressed genes in Table S3, S4). As expected, the zwf gene was 9.0-fold (log2) down-regulated in JS134. Among the 592 down-regulated genes used for Gene Ontology (GO) analysis, 13.7% genes could be allocated to the term “ATP binding” (Fig. 5B). These genes were mainly involved in ATP-dependent process such as transport, helicase, kinase and synthase. In terms of “proton-transporting ATP synthase activity”, seven genes encoded subunits of the F0F1-ATP synthase, and they were down-regulated from 3.0 to 1.5 folds (log2) in JS134. Interestingly, the term “nitrate reductase activity” was also enriched, even though nitrate was not present in the medium used.

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

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

After demonstrating that efficient high-yield ethanol production could be accomplished using JS134, we decided to investigate whether other pyruvate-derived chemicals could be made using the strain deficient in malate dehydrogenase and glucose-6-phosphate dehydrogenase. To achieve a high-yield production of D-lactate in *C. glutamicum*, we overexpressed the D-lactate dehydrogenase from *Lactobacillus delbruckii* in the strain JS132. The productivity of D-lactate in the resulting strain...
JS138 was slightly lower than for the strain carrying only deletions in \textit{ldhA} and \textit{ppc} (JS137), but the yield for JS138 reached 98\% of the theoretical, which is 14\% higher when compared to JS137 (Fig. 6). To the best of our knowledge, this is the highest yield of D-lactate reported this far for \textit{C. glutamicum}. 

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

We achieved extremely high-yield production of ethanol and D-lactate from glucose, which stimulated us to explore the actual applications by harnessing this PPP-disrupted platform strain JS132. Since we have previously engineered \textit{C. glutamicum} into being able to metabolize lactose, and this was done by introducing the transporter LacS, the $\beta$-glucosidase LacZ, and the Leloir pathway (GalMKTE), which resulted in strain JS112 (Fig. 7A). Lactose is available in large amounts in whey permeate (WP), which are abundantly generated as waste by dairy industry in most parts of the world. We first optimized the growth of JS132 on WP with different types of nitrogen source. The pure WP did not support its growth well, and the final cell density on this substrate only reached 2.7 (OD$_{600}$) after 24 h. Adding the minimal medium salts improved the cell density to 5.1 (OD$_{600}$). Adding organic nitrogen sources, either 1\% YE or 5\% CSL resulted in a significant increase in biomass and the cell densities could reach 35 (OD$_{600}$) and 39 (OD$_{600}$), respectively (Fig. S1).

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

Using the pure WP as the substrate, we achieved 48 g/L ethanol from 106 g/L lactose with a yield of 85\% (of the theoretical maximum) on JS134 compared with only 66\% on JS122, which co-produced 37 g/L ethanol and 23 g/L succinate (Fig. 7B). We also noticed the productivity was reduced for JS134 due to the lower glycolytic flux, which was also the case in glucose medium. 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.

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

Wild-type *C. glutamicum* strain metabolizes glucose faster (5.25 mmol g⁻¹ h⁻¹) than fructose (4.42 mmol g⁻¹ h⁻¹) when growing aerobically (20), but the opposite behavior has been observed under anoxic conditions (29), and this attracted our attention. When fructose was used as the sole carbon source, four times less of the sugar taken up was channeled into the PPP when compared to glucose, and as a consequence less NAPDH was generated (18). We found that JS133 exhibited a significantly higher ethanol production rate on fructose than on glucose, and we speculated that the PPP somehow might be involved in regulating the glycolysis under anoxic conditions. After inactivating glucose-6-phosphate dehydrogenase, a large reduction in the intracellular
NADPH/NADP⁺ ratio was achieved, and surprisingly the NADH/NAD⁺ ratio was also reduced (Fig. 3A). Blocking the PPP greatly increased the glycolytic flux as well as the ATP/ADP ratio in JS134 (Fig. 2A, 3B). Under anoxic conditions, 5% glucose was metabolized through the PPP compared to 95% through glycolysis in *C. glutamicum*, although the NAPDH-consuming anabolism was halted (30).

In this study, we also found the interconnections between NADPH and NADH during the anoxic catabolism of sugars in *C. glutamicum*. When we blocked the formation of succinate, an increase in the NADH/NAD⁺ ratio was accompanied by an increase in the NADPH/NADP⁺ (Fig. 3A). In the opposite way, when we blocked the PPP to reduce the NADPH/NADP⁺ ratio, the NADH/NAD⁺ ratio concurrently decreased (Fig. 3A). A similar phenomenon was observed in Yamamoto et al.’s study for alanine production in *C. glutamicum* (31). When they overexpressed the glycolytic genes *gapA, pfk*, and *pgi*, individually, to enhance glucose consumption under oxygen deprivation conditions, the NADPH/NADP⁺ ratio changed proportionally with the change of NADH/NAD⁺ ratio, even though the carbon flux into the PPP did not change significantly. There might be several explanations for this. As we know, transhydrogenase participates in the interconversion of NADPH and NADH in microbes to maintain the intracellular redox hemostasis (32). As it was shown in the resting *Bacillus subtilis*, the PPP generated surplus NADPH and it was expected the transhydrogenation activity was responsible for the conversion of NADPH to NADH in order to maintain the intracellular redox hemostasis, though the responsible gene was not identified (33).

Although the gene encoding transhydrogenase is not found in *C. glutamicum* (34), we did detect a minor transhydrogenase activity in the non-growing *C. glutamicum* cells under anoxic condition. It might also be possible that the NADPH-dependent malate enzyme together with malate:quinone oxidoreductase and oxaloacetate dehydrogenase could form a metabolic cycle and exhibit transhydrogenase activities responsible for generating NADH from NADPH (35). Another
explanation might be due to the promiscuity of glucose-6-phosphate dehydrogenase (zwf-encoding), which may accept NAD$^+$ for generating NADH directly under some conditions, although the purified glucose-6-phosphate dehydrogenase did not display a NAD$^+$ dependent activity in vitro (36). The glucose-6-phosphate dehydrogenase from both Z. mobilis and Bacillus subtilis are known to be able to generate NADH (37, 38).

RNA-seq analysis also revealed a dramatic alteration of gene expression in response to the blockage of PPP. When zwf was deleted, the expression of the F$_1$F$_0$-ATP synthase was significantly down-regulated in JS134 (Fig. 5), which indicated that the energy starvation caused by cofactor imbalance could be mitigated by blocking PPP. Simultaneously, the expression of the nitrate reductase operon was also regulated in JS134. It is known that C. glutamicum is capable of anaerobic respiration using nitrate as a terminal electron acceptor (39). Even though, nitrate was not provided in the fermentation broth, a down-regulation of nitrate reductase in the strain lacking zwf indicated that the NADH/NAD$^+$ ratio could be involved in regulating expression of this gene. Of the up-regulated genes, we found the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase was up-regulated after deleting zwf. The observation was consistent to previous studies that have shown that expression of glyceraldehyde-3-phosphate dehydrogenase is inhibited by high NADH/NAD$^+$ ratios under anoxic conditions (40). Overall, the results from the transcriptional analysis were congruent with the changes observed in the fermentation physiology, where deletion of zwf relieved energy starvation and cofactor imbalance under anoxic conditions.

In this study, we successfully pinpointed the pathway responsible for the high NADH/NAD$^+$ ratio observed in C. glutamicum under anoxic conditions. This knowledge can be harnessed to engineer C. glutamicum mutants producing high yield of chemicals via redox-balanced reactions, which has its intrinsic challenges (11). In a previous study, where a ΔppcΔldhA mutant of C. glutamicum was used for producing D-lactate, only about 86% of the theoretical yield could be accomplished from
glucose (23). Here, by combining a modified pyruvate dehydrogenase and PPP inactivation, a yield to 98% was attained, which is among the highest D-lactate yields achieved using microbial fermentation (41).

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

**Materials and methods**

**Bacterial strains, plasmids.**

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

**Growth medium and conditions.**

*E. coli* strains were grown aerobically in Luria-Bertani broth (LB) at 37°C, and *C. glutamicum* strains were cultivated in Brain Heart Infusion (Oxoid) broth (BHI) at 30°C with 200 rpm shaking.
For ethanol and D-lactate production, *C. glutamicum* strains were aerobically cultivated in CGXII medium supplemented with 5% glucose or fructose, and if necessary, 0.5% yeast extract (YE) was added for the growth of *zwf* inactivated strains. When appropriate, kanamycin was added to a concentration of 50 μg/mL for *E. coli* and 25 μg/mL for *C. glutamicum*, and spectinomycin was used at a concentration of 100 μg/mL for *E. coli* and 50 μg/mL for *C. glutamicum*. For gene deletion or replacement in *C. glutamicum*, 10% sucrose was used for counter-selection. Cell growth was monitored by measuring the optical density at 600 nm (OD<sub>600</sub>) of the culture broth using a UV1800 spectrophotometer (Shimadzu, Japan). The dry cell weight was calculated based on DCW (g/L) = OD<sub>600</sub> × 0.3 (43).

**Construction of plasmids and strains.**

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

*C. glutamicum* mutants carrying gene deletions in *mdh* and *zwf* and mutants where the *E. coli lpd* gene substituted the native *lpd*, were constructed via a two-step homologous recombination procedure as described previously (45) using the vector pK18mobsacB (46). Plasmids were transformed into *C. glutamicum* by electroporation.

For the construction of pK18-mdh, the regions up- and downstream (approximately 750-bp each) of the *mdh* gene to be deleted were amplified (primers used can see Table S2). The resulting DNA fragments were cloned into pK18mobsacB through Gibson assembly. The plasmid pK18-zwf was constructed in a similar manner. A gBlock gene fragment containing the regions up- and downstream of *zwf* was synthesized by Integrated DNA Technologies (sequences can see Fig. S3), and then cloned into pK18mobsacB.
Plasmid pK18-lpd(E) was constructed in order to replace the chromosomal lpd gene from *C. glutamicum*. For this purpose, a gBlock gene fragment containing the *lpd* gene of *E. coli* flanked by the up- and downstream regions (approximately 750-bp each) of the *lpd* gene of *C. glutamicum* was synthesized by Integrated DNA Technologies (sequences can be seen in Fig. S2), in which the *lpd* gene starts with GTG codon, and then cloned into pK18mobsacB.

For the construction of the expression plasmid pJS136 (D-lactate) and pJM140 (alanine), the 1.2-kb *L. delbrueckii* ldhA gene coding D-LDH was amplified using *L. delbrueckii* chromosomal DNA as the template, and the 1.1-kb *alaDH* encoding alanine dehydrogenase from *Lysinibacillus sphaericus* was synthesized by Genscript (Piscataway, US). The resulting fragments were, individually, inserted into the vector pEC-XK99E (47) through Gibson assembly.

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

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

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

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

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

Whey permeate (WP) is provided by the dairy company Arla Foods Ingredients Group P/S (Viby, Denmark). It is the by-product of dairy industry for producing whey proteins from whey, which is obtained in the cheese making process. WP has more than 10% (w/v) lactose. We tested the growth of JS134 on 50% WP (diluted with water) with different types of nitrogen sources. Corn steep liquor (CSL) with 50% solid content, which was purchased from Sigma-Aldrich (St. Louis, MO), was centrifuged for 10 mins at 5,000 xg and the supernatant was used. The minimal medium salts contain (NH$_4$)$_2$SO$_4$ (7 g/L), FeSO$_4$ (20 mg/L), MnSO$_4$ (2 mg/L) and the trace element solution from the CGXII medium. The pH for all the medium was adjusted to 7.0. A single colony of JS134 was inoculated into 25 mL medium in 100 mL flask and cultivated at 30°C with 200 rpm shaking. The cell density (OD$_{600}$) was measured after 24 h.

For the production of ethanol, D-lactate and alanine, we grew the relevant strains in 1 L flask containing 200 mL 50% WP, MMS and 1% YE (for easy manipulation instead of CSL). The cells
were collected after 20 h and then resuspended in 50 mL pure WP medium to achieve a cell concentration of 20 g DCW/L. The pH of the fermentation was maintained at 7.0 using the Biostat A (Sartorius, Germany) pH-control system by supplementing 5 M ammonia solution.

**RNA sequencing.**

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

**Analytical techniques.**
I) Product formation and sugar consumption. Culture samples were withdrawn, centrifuged (15,000×g, 4°C, 5 min.), and the supernatant saved at -20°C for later analysis. Sugar, ethanol, D-lactate, succinate, acetate and glycerol were quantified using an Ultimate 3000 high-pressure liquid chromatography system (Dionex, Sunnyvale, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, USA) and a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan). The column oven temperature was set at 60°C, the mobile phase was at 5 mM H₂SO₄, and the flow rate was 0.5 mL/min. The alanine concentration was measured using the alanine colorimetric kit (VWR No: BIOV652-100).

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

III) ATP/ADP. Samples were collected at 3 h during ethanol production. Cell extraction was performed as previously described (51). Cultures (5 mL) were quenched with 1 volume 80°C phenol. After being centrifuged (4,000×g, 10 min.), the water phase was transferred into a new tube and extracted with 1 volume of chloroform twice. ATP was measured using a BioThema ATP kit HS, and ADP was converted into ATP by 1 mM PEP and 1 U pyruvate kinase. The luminescence was subsequently measured on a Tecan Infinite M200 Pro microplate reader.
IV) Measurement of transhydrogenase activity. Samples were collected at 3 h during ethanol production under oxygen deprivation conditions. Cell suspensions were disrupted by bead beating using a FastPrep system (MP Biomedicals) with acid-washed glass beads (106 μm, Sigma-Aldrich prod. No.: G4649). The transhydrogenase activity was measured according to the procedure described by Sauer et al. (32). Briefly, the absorbance at 375 nm was monitored using a Tecan Infinite M200 Pro microplate reader at 25°C in a mixture containing 50 mM Tris·HCl (pH 7.6), 2 mM MgCl₂, 500 μM NADPH, 1 mM 3-acetylpyridine adenine dinucleotide, and 10-100 μL cell extract. The specific activity was obtained through dividing the measured slope by the protein concentration. Protein concentrations were determined using Bradford according to the manual (Sigma-Aldrich).

Supplementary material

Supplementary File 1

Acknowledgements

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


GntR1 and RamA enhance the growth and central metabolism of *Corynebacterium glutamicum*. Metab Eng 48:1–12.


Table 1. Strains and plasmids used in the study.

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Description/Function</th>
<th>Reference</th>
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<td>JS119</td>
<td>JS112, Δndh</td>
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<td>JS122</td>
<td>JS112 harboring pJS115 (ethanol-producing plasmid)</td>
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<td>JS124</td>
<td>JS119 harboring pJS115</td>
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<td>JS132 harboring pJS136</td>
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</tr>
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<td>JM141</td>
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Figure 1. Schematic overview of sugar metabolism in C. glutamicum under anoxic conditions. The grey boxes indicate the relevant enzymes. LDH, lactate dehydrogenase (ldhA gene); PEPC, phosphoenolpyruvate carboxylase (ppc gene); PYC, pyruvate carboxylase (pyc gene); MDH, malate dehydrogenase (mdh gene); FUM, fumarase; SDH, succinate dehydrogenase; PDH, pyruvate carboxylase; G6PDH, glucose-6-phosphate dehydrogenase (zwf gene). The Z. mobilis PDC (pyruvate decarboxylase (pdc gene)) and ADH (alcohol dehydrogenase (adhB gene)) enzymes, which expressed in C. glutamicum in this study, are shown in the red boxes. Red Xes indicate the deletion of the relevant genes.
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.
Figure 3. Measurement of NADH/NAD⁺, NADPH/NADP⁺ and ATP/ADP in different strains under anoxic conditions. Two biologically independent experiments were performed to calculate the standard deviation.
Figure 4. Characterization of strain growth. A. Comparison of the final cell density (OD$_{600}$) after 40 h aerobic growth in minimal medium containing glucose, the growth experiments were carried out in 100 mL shake flask with 20 mL medium. The standard deviations were calculated from three independent experiments. B. Cell growth was monitored on Biolector (M2p-labs, Germany) with 1500 rpm shaking and the OD$_{600}$ after 30 h was measured on a UV1800 spectrophotometer.
Figure 5. Transcriptomics for JS134 and JS133 (three independent replicates for each). A. The heatmap for the differentially expressed genes was made using pheatmap in R. B. Enriched GO terms for the up-regulated and down-regulated gene in JS134 when using JS133 as the reference.
Figure 6. Comparison of glucose consumption and D-lactate production between JS137 and JS138. The cell concentration was 10 g/L. Two biologically independent experiments were performed to calculate the standard deviation.
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.