RESEARCH

High yield 1,3-propanediol production by rational engineering of the 3-hydroxypropionaldehyde bottleneck in *Citrobacter werkmanii*

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Abstract

**Background:** Imbalance in cofactors causing the accumulation of intermediates in biosynthesis pathways is a frequently occurring problem in metabolic engineering when optimizing a production pathway in a microorganism. In our previous study, a single knock-out *Citrobacter werkmanii ΔdhaD* was constructed for improved 1,3-propanediol (PDO) production. Instead of an enhanced PDO concentration on this strain, the gene knock-out led to the accumulation of the toxic intermediate 3-hydroxypropionaldehyde (3-HPA). The hypothesis was emerged that the accumulation of this toxic intermediate, 3-HPA, is due to a cofactor imbalance, i.e. to the limited supply of reducing equivalents (NADH). Here, this bottleneck is alleviated by rationally engineering cell metabolism to balance the cofactor supply.

**Results:** By eliminating non-essential NADH consuming enzymes (such as lactate dehydrogenase coded by *ldhA*, and ethanol dehydrogenase coded by *adhE*) or by increasing NADH producing enzymes, the accumulation of 3-HPA is minimized. Combining the above modifications in *C. werkmanii ΔdhaD* resulted in the strain *C. werkmanii ΔdhaDΔldhAΔadhE::ChlFRT* which provided the maximum theoretical yield of 1.00 ± 0.03 mol PDO/mol glycerol when grown on glucose/glycerol (0.33 molar ratio) on flask scale under anaerobic conditions. On bioreactor scale, the yield decreased to 0.73 ± 0.01 mol PDO/mol glycerol although no 3-HPA could be measured, which indicates the existence of a sink of glycerol by a putative glycerol dehydrogenase, channeling glycerol to the central metabolism.

**Conclusions:** In this study, a multiple knock-out was created in *Citrobacter* species for the first time. As a result, the concentration of the toxic intermediate 3-HPA was reduced to below the detection limit and the maximal theoretical PDO yield on glycerol was reached.

**Keywords:** 1,3-propanediol, Glycerol, *Citrobacter werkmanii* DSM17579, NADH, Lactate dehydrogenase, Ethanol dehydrogenase, Multiple knock-out mutant, Rational engineering

Background

Nicotinamide adenine dinucleotide (NAD⁺) plays a central role in the cellular metabolism of microorganisms by functioning as a cofactor in over 300 oxidation-reduction reactions [1]. Moreover, the NADH/NAD⁺ ratio has a regulatory effect on the expression of some genes and the activity of certain enzymes. For example, a high NADH/NAD⁺ ratio results in an enhanced expression of the ethanol dehydrogenase AdhE [2], which catalyzes the formation of ethanol during fermentation, and increases the inhibition of the pyruvate dehydrogenase complex [3, 4]. Thus, reaching cofactor balance is important for a high titer, yield and rate in the biosynthesis of interesting metabolites.

During the production of 1,3-propanediol (PDO) from glycerol as sole carbon source in natural PDO producing
strains such as the opportunistic pathogen *Citrobacter werkmanii*, the cofactors are balanced via the two glycerol dissimilation pathways (Additional file 1: Figure S1). Via the oxidative branch, glycerol dehydrogenase (GDH, E.C. 1.1.1.6), encoded by *dhaD*, forms NADH which, in turn, is consumed by 1,3-propanediol dehydrogenase (PDODH, E.C. 1.1.1.202), coded by *dhaT* in the reductive branch [2]. Indeed, in our previous work an imbalance in the PDO production was observed in *C. werkmanii* DSM17579 due to the deletion of *dhaD* [5]. The cofactor/pathway imbalance not only resulted in the accumulation of the intermediate 3-hydroxypropionaldehyde (3-HPA) but also in growth inhibition and incomplete PDO production before substrate depletion.

Several strategies have been reported to balance the cofactors, to reduce the buildup of 3-HPA and thus to increase the yield of PDO on glycerol. Among them, the most important are cofactor regeneration, elimination of non-essential NADH-consuming enzymes, and promotion of the activity of NADH producing enzymes. Zhang et al. [6] and Luo et al. [7] have increased the yield of PDO on glycerol using an in vivo cofactor regeneration system which converts NAD$^+$ into NADH, while, respectively, CO$_2$ is produced from formate by formate dehydrogenase, and 3-HPA is changed into 3-hydroxypropionic acid by an aldehyde dehydrogenase AldH. For the deletion of the genes which products consume NADH, the focus was laid on the lactate dehydrogenase gene *ldhA* in *Klebsiella oxytoca* M5a1 [8] and *K. pneumoniae* HR526 [9] and on an aldehyde dehydrogenase gene *aldA* in *K. pneumoniae* YMU2 [10]. In both cases one NADH is consumed per reaction. Deletion of the genes resulted in a significantly enhanced NADH/NAD$^+$ ratio, and consequently an increased yield on glycerol of PDO and improved final PDO titer in *Klebsiella* sp. [8–11]. The last method to increase the intracellular NADH concentration, enhancement of NADH producing enzymes, is attained by manipulating the citric acid cycle (TCA cycle) and its regulation. Most NADH of the TCA cycle is produced under aerobic conditions: four NADH molecules are formed per molecule glucose. However, under anaerobic conditions, which are the circumstances of PDO production, only a reductive branched TCA cycle is active, which consumes, instead of produces, NADH (Additional file 1: Figure S2) [12]. Hence, to increase the production of NADH, the regulation of the TCA cycle should be altered to an aerobic arrangement under anaerobic conditions. To accomplish this, the phosphorylation of ArcA, one of the global regulators, should be avoided through deletion of *arcA* or *arcB*. This deletion has been used by several researchers, for example, to increase the activity of NADH-consuming reactions for the production of succinate and poly[(R)-3-hydroxybutyrate] [13–15]. However, the effect of this deletion on PDO production was never investigated before.

In this study, the last two approaches are tested (Additional file 1: Figure S1): deletion of the genes *ldhA* and *adhE*, coding for lactate dehydrogenase and ethanol dehydrogenase, respectively, and enhancement of the NADH production by deletion of *arcA*. The different single and multiple knock-out mutants of *C. werkmanii* DSM17579 are evaluated on the growth, PDO yield, final PDO concentration and NAD$^+/\text{NADH}$ ratio. The best performer is evaluated in batch fermentation at a higher concentration of glycerol. To the best of our knowledge, this is the first time the effect of *arcA* on PDO will be studied. Moreover, most research has been performed on improving PDO production using a single knock-out mutant in the pathogenic *Klebsiella* species. The (possible synergistic) effect(s) of combining a split of the glycerol metabolizing pathway and deletion of byproduct formation has barely been investigated.

**Results and discussion**

In order to test the hypothesis on cofactor improvement for minimizing the intermediate accumulation and maximizing PDO production in *C. werkmanii* ∆*dhaD* several single mutants were rationally designed and evaluated. Further, these mutants were recombined in a single strain, which significantly minimizes the 3-HPA accumulation and allows the strain to reach maximum PDO production yields.

**Construction and evaluation of single knock-out mutants**

Based upon previously reported strategies to improve cofactor availability in other microorganisms via metabolic engineering, three genes were selected to knock-out and to evaluate towards their effect on viability and production of 1,3-propanediol in *C. werkmanii* DSM17579. Two non-essential enzymes consuming NADH (LdhA, AdhE), and one global regulator, ArcA, which represses reactions producing NADH were eliminated, respectively resulting in the single gene knock-outs *C. werkmanii* ∆*dhaA*, *C. werkmanii* ∆*adhE::ChlFRT*, and *C. werkmanii* ∆*arcA*. After elucidation of the gene sequences, the mutants were constructed using an in-house gene deletion technique developed for *C. werkmanii* [5]. The sequence results of the different knock-outs are presented in Additional file 2.

To investigate the effect of the single knock-outs on the growth and metabolic profile of *C. werkmanii* DSM17579, the different mutants were grown in shake flasks with 163 mM glycerol as sole carbon source under anaerobic conditions. As depicted in Table 1, the growth
rate decreases for all mutants compared to the wild-type. For C. werkmanii ∆arcA the growth rate is halved. The decline is consistent with the findings of Zhang et al. [10] and Nizam and Shimizu [16], who deleted an adhE gene. These NADH molecules become available per glucose molecule after deleting the adhE gene. These NADH molecules can be used by PDODH to convert 3-HPA into pyruvate-formate lyse (E.C. 2.3.1.54). In our experiments, the ethanol concentration is also reduced, whereas the acetate concentration is enhanced. Therefore, we ascribe the metabolic redistribution rather to the altered NADH/NAD⁺ ratio than to the decreased flux through pyruvate-formate lyase. However, enzyme assays or metabolic flux analysis should be performed to confirm this.

### Construction of double and triple mutants and evaluation of their synergetic effects

The single knock-out study revealed that only the adhE single knock-out has a positive effect on the yield of PDO on glycerol and that the titers of the fermentation products consuming NADH increase, especially the lactate titer in C. werkmanii ∆adhE. Therefore, combinations of the adhE and ldhA knock-outs with the dhaD knock-out were tested; the latter was proven beneficial in previous research [5]. Specifically, two double (C. werkmanii ∆dhaD∆adhE and C. werkmanii ∆dhaD∆adhE) and one triple (C. werkmanii ∆dhaD∆adhE) mutants were constructed to investigate synergistic effects. The mutant strains were grown anaerobically in shake flasks with 40 mM glucose and 120 mM glycerol, yielding a molar ratio of 0.33 glucose/glycerol. No double mutants containing the arcA knock-out were constructed as C. werkmanii ∆dhaD∆adhEarcA did not produce any 1,3-propanediol (data not shown).

Compared to the single knock-out mutant, C. werkmanii ∆dhaD, the newly constructed double mutant strains have a largely improved growth rate and yield a significantly higher final PDO concentration and PDO yield on glycerol due to a vastly reduced NAD⁺/NADH ratio (Tables 2, 3).

The final PDO titer is considerably increased, from 39.80 ± 2.67 mM in the C. werkmanii ∆dhaD knock-out to 62.90 ± 1.29 mM and 86.11 ± 7.19 mM in the C. werkmanii ∆dhaD∆adhE double knock-outs, respectively. As the residual glycerol concentration remains constant, the yield on glycerol of

### Table 1 Growth rate, PDO yield and metabolite profile of the wild-type (WT) and single knock-outs

| Strains                | Growth rate (h⁻¹) | PDO Yield (mol/mol) | Acetate (mM) | Ethanol (mM) | Lactate (mM) | Succinate (mM) |
|------------------------|-------------------|---------------------|--------------|--------------|--------------|---------------|
| WT                     | 0.33 ± 0.02       | 0.63 ± 0.01         | 38.20 ± 0.83 | 7.08 ± 0.37  | 1.14 ± 0.27  | 4.69 ± 0.14   |
| ∆adhE                  | 0.28 ± 0.02       | 0.59 ± 0.02         | 18.30 ± 1.07 | 0.21 ± 0.14  | BDL          | 4.72 ± 0.08   |
| ∆adhE:ChlFRT           | 0.26 ± 0.01       | 0.70 ± 0.01         | 44.30 ± 2.19 | BDL          | 4.67 ± 0.31  | 5.93 ± 0.09   |
| ∆arcA:ChlFRT           | 0.13 ± 0.00       | 0.65 ± 0.01         | 58.00 ± 1.30 | BDL          | 5.27 ± 0.84  | 7.09 ± 0.32   |

C. werkmanii DSM17579 (WT), C. werkmanii ∆ldhA, C. werkmanii ∆adhE:ChlFRT, and C. werkmanii ∆arcA:ChlFRT were cultivated under anaerobic conditions in shake flasks. The values are the average of two experiments with their corresponding standard deviations.

BDL below the detection limit

*Note: Prevalent research in the field has suggested that the elevated expression of the TCA cycle genes is an increased flux through pyruvate-formate lyase (E.C. 2.3.1.54). In our experiments, the ethanol concentration is also reduced, whereas the acetate concentration is enhanced. Therefore, we ascribe the metabolic redistribution rather to the altered NADH/NAD⁺ ratio than to the decreased flux through pyruvate-formate lyase. However, enzyme assays or metabolic flux analysis should be performed to confirm this.*
PDO increases to 0.84 ± 0.01 and 0.96 ± 0.01 mol PDO/mol glycerol using C. werkmanii ΔdhaDΔldhA and C. werkmanii ΔdhaDΔadhE, respectively. Furthermore, the final 3-HPA titer is reduced significantly comparing C. werkmanii ΔdhaD (Table 3): a 50 % reduction is observed in C. werkmanii ΔdhaDΔldhA, increasing to 80 % in C. werkmanii ΔdhaDΔadhE. The final 3-HPA concentration in the latter is far below the critical concentration [5]. These positive effects are most likely due to the rebalance of the NAD+/NADH ratio (Table 2). The ratio was decreased from 4.74 ± 0.14 for C. werkmanii ΔdhaD to 1.66 ± 0.03 and 2.20 ± 0.07 for C. werkmanii ΔdhaDΔldhA and C. werkmanii ΔdhaDΔadhE, respectively, which is much closer to the NAD+/NADH ratio of the wild-type C. werkmanii (1.58 ± 0.25).

The other metabolic fluxes are redistributed as well in the double knock-outs (Table 3). Comparing C. werkmanii ΔdhaD with C. werkmanii ΔdhaDΔldhA, the ethanol concentration decreases, while the acetalate concentration increases. This indicates that the flux from acetyl-CoA to acetate is increased, while the flux from acetyl-CoA to ethanol is reduced. Analogously, an enhanced acetate titer can be observed in C. werkmanii ΔdhaDΔadhE. Due to the adhE deletion, four NADH molecules become available per glucose, which are used in other NADH-consuming reactions. As a result, compared to the single ΔdhaD mutant, not only the PDO titer is elevated, but also the concentration of succinate and especially lactate, analogous to the single adhE knock-out compared to the wild-type.

In the triple knock-out mutant, the lactate concentration decreases till below the detection limit and the succinate titer stagnates. Moreover, the final PDO titer and yield on glycerol are further improved. The yield even reaches the maximum theoretical yield of 1.00 ± 0.03 mol PDO/mol glycerol with the use of a co-substrate. As such, at the end of this experiment, 3-HPA is no longer detected. The residual glycerol and glucose concentrations are increased in the triple knock-out mutant, compared to the single knock-out strain. The growth rate is reduced and the final pH is increased (Table 2), probably due to a metabolic burden.

Performance of C. werkmanii ΔdhaDΔldhAΔadhE::ChlFRT in bioreactors

The triple mutant C. werkmanii ΔdhaDΔldhAΔadhE::ChlFRT producing the maximum theoretical yield of 1 mol PDO/mol glycerol in minimal medium with glycerol and glucose in shake flasks, yielding a titer of 107.20 ± 4.22 mM PDO, was selected for batch fermentations on bioreactor scale. As the wild-type C. werkmanii DSM17579 showed the highest productivity at an initial concentration of 650 mM glycerol [18], this concentration was used for the batch fermentations on bioreactor
scale with the triple mutant. Analogous to the shake flask experiments, a ratio of 0.33 mol glucose/mol glycerol was used.

The maximum growth rate of the triple knock-out mutant obtained in the bioreactor is $0.11 \pm 0.01$ h$^{-1}$ with a lag phase of around 30 h. During the fermentation on bioreactor scale, glycerol and glucose are consumed simultaneously (Fig. 1a), but the consumption rate of glycerol is 10 times higher than that of glucose ($29.10 \pm 1.10$ mmol glycerol/h and $3.70 \pm 0.30$ mmol glucose/h). As a result, glycerol is depleted first, namely at the end of the exponential phase. The residual glucose is not used anymore for growth, but only for cell maintenance and production of acids and ethanol (Fig. 1b). These findings indicate that a reduced molar ratio glucose/glycerol may be desirable on bioreactor scale.

The PDO productivity during the growth, i.e. production phase, $14.07 \pm 0.65$ mM PDO/h, is in line with the results obtained with the wild-type strain in fermentation medium with glycerol as sole carbon source, during the same phase [18]. This indicates a recovery of the cofactor balance, which was lopsided in $C. werkmanii \Delta dhaD$.

The final titer of PDO is increased by 32 % comparing the cultivation of the triple knock-out

![Graph](image-url)
While PDO is the main product, some byproducts are detected (Fig. 1b). The most important one is acetate (266.19 ± 11.50 mM), mainly produced during the exponential growth phase, and with a production profile similar to the one of glucose consumption. Succinate follows the same production profile, but to a much lower final titer (62.81 ± 2.76 mM). In the first half of the exponential growth phase, no lactate or ethanol is produced, but by the end of the exponential phase (period III) the triple knock-out does produce lactate and ethanol, albeit in minimal concentrations. It should be noted that the produced lactate may be L-lactate instead of D-lactate, since these metabolites cannot be separated with the used HPLC-method. Analogous results were obtained in a fed-batch fermentation on bioreactor scale with an idhA deficient K. pneumoniae strain, i.e. lactate started accumulating in the late-exponential phase [9]. Ethanol may be produced by an iron-containing alcohol dehydrogenase, EutG, which is present in Citrobacter sp. next to AdhE [24, 25]. A peculiar production profile is found for formate, which is produced early exponential, but consumed from the mid-exponential phase. According to Beauprez [13], this can be ascribed to the activity of the formate hydrogen lyase (FHL, E.C. 1.2.1.2), which converts formate and NAD⁺ in CO₂ and NADH. When enough NADH molecules are present in the cell, FHL is not active and formate accumulates. However, during shortage or surplus of NADH, the enzyme is active. In the former case, formate will be converted to CO₂ and NADH, while in the latter case, formate and NAD⁺ will be produced [13]. Based on the above observations, the experiment can be divided in three phases: (I) from 0 h to 36.5 h; (II) from 36.5 h to 40 h; and (III) from 40 h till the end of the fermentation. In period I enough NADH is present for the cell and formate accumulates. In period II, a surplus of NADH exists and CO₂ is converted to formate. This is reflected in a drop of the CO₂-curve and further increase of the formate concentration. Finally, in period III, the activity of FHL is reversed, formate is converted to CO₂ and NADH resulting in a lift of the CO₂ concentration and reduced formate concentration. The produced NADH is consumed by other enzymes, such as dehydrogenases, to form ethanol and lactate.

All glycerol and glucose is depleted by the end of the batch cultivation on bioreactor scale, but the PDO yield on glycerol is lower than expected, namely 0.73 ± 0.01 mol PDO/mol glycerol. Nevertheless, this is still one of the highest reported PDO yields on glycerol [11]. The closest results, 0.70 and 0.69 mol PDO/mol glycerol, were accomplished by, respectively, Zhang et al. [10] using the knock-out K. pneumoniae YMU2 DaldA in fed-batch mode and Seyfried et al. [26] with the thermophilic wild-type Caloramator viterbensis sp. nov. in Hungate tubes. Since the only intermediate in the conversion of glycerol to PDO is 3-HPA and neither 3-HPA was accumulated nor unknown peaks were visible in the HPLC chromatographs, glycerol must have been used for growth and maintenance of the cell. This hypothesis is strengthened by the calculated carbon and redox balances (Additional file 1: Table S1). When the conversion of glycerol to PDO is taken into account, 28.52 % of the carbon has ‘disappeared’, while when the conversion of glucose to the biomass and byproducts is considered, a surplus of 90.27 % carbon would be needed. However, when both pathways are considered together, a carbon and redox balance of, respectively, 95.56 % and 92.25 % are obtained, which confirms that glycerol is used not only for production of PDO, but also for cell growth and maintenance.

In order to confirm this hypothesis, a bioreactor with only 650 mM glycerol, thus without glucose, was inoculated with the triple knock-out strain. After a lag phase of 70 h, the strain started to grow, consumed glycerol and produced PDO (Table 4). So, although GDH, coded by dhaD and responsible for channeling glycerol to the central metabolism, is altered by gene deletion in this triple knock-out, the strain still uses glycerol as carbon source for cell growth and maintenance, indicating the presence of other enzymes which convert glycerol into central carbon metabolites supporting growth. The maximal cell dry weight of the triple mutant grown on glycerol only is 38.90 ± 2.07 mM, which is lower than on glucose and glycerol. This is as expected because in the former, glycerol is the only carbon source for cell growth and maintenance, while in the latter, glucose is also present in the medium. In both cases, the 3-HPA concentration is below the detection limit and the PDO yield on glycerol and the final PDO titer obtained are similar (Table 4). Furthermore, the metabolic profile is similar for both conditions, taking the difference in carbon concentration into account. The most important byproduct still is acetate and small amounts of succinate and lactate are formed. However, the production of ethanol is...
Enzyme activity (mU/mg protein) described in “Methods” section. The values are the average of two experiments with their corresponding standard deviations.

BDL not determined, below detection limit.

| Glucose 26.80 | Glycerol 72.70 |
|---------------|---------------|
| +             | ± 0.18        |

To check these theories, enzyme assays were performed using crude cell extract of the two bioreactor cultivations and those grown on glycerol alone and those grown on glycerol and glucose. Therefore, it is not likely that a GK enzyme is responsible for the dissimilation of glycerol via the oxidative pathway.

The activity of GDH on the other side is almost double the glycerol kinase activity (Table 5). Furthermore, when the triple mutant was grown under anaerobic conditions in medium with glycerol as sole carbon source, the specific GDH activity is three times higher than when the strain is grown under the same conditions in medium with glycerol and glucose. These results indicate that a promiscuous dehydrogenase is responsible for the utilization of glycerol for cell growth and maintenance, or that a second glycerol dehydrogenase coding gene is present in the genome of C. werkmanii DSM17579.

An NCBI-BLAST of the C. werkmanii DSM17579 glycerol dehydrogenase to the protein sequences of Citrobacter species indeed reveals a second, putative glycerol dehydrogenase enzyme [27]. This putative glycerol dehydrogenase shows 32 % amino acid identity to the glycerol dehydrogenase enzyme coded by dhaD of Citrobacter sp. (GenBank: WP_042998939.1, E-value = 1e−39, bitscore = 147). In Fig. 2 a comparison is made between the amino acid sequences of GDHs coded by dhaD of Citrobacter sp. (GenBank: WP_042998939.1) C. werkmanii DSM17579 (GenBank: AFX65883.1) and C. freundii (GenBank: AAB48844.1), and putative glycerol dehydrogenases found in Citrobacter sp. (GenBank: ABV13669.1, EFE08361.1, EHL83381.1) and E. coli (GenBank: NP_41532.1). Glycerol dehydrogenases belong to the family of iron-dependent alcohol dehydrogenases [28]. Surprisingly, the conserved regions of GDHs, as described in Maervoet et al. [29], can all be found in this putative glycerol dehydrogenase. The NAD⁺- (marked as boxes) and Manganese- (orange) binding sites are well conserved suggesting that these two elements also are the cofactors for this second, putative enzyme, as they are for the GDH coded by dhaD. Even the glycerol binding sites

Table 4 Comparison of batch cultivations [glycerol (Gly), or glycerol and glucose (Glu + Gly)] with C. werkmanii ΔdhaDΔldhAΔdhaE::ChlFRT

| Strain | Yield (mol PDO/mol glycerol) | Residual concentration (mM) | Metabolite concentration (mM) | CDW |
|--------|-----------------------------|----------------------------|-------------------------------|-----|
|        | Gly | Glucose | PDO | Acetate | Ethanol | Lactate | Succinate |
| Gly    | 0.72 ± 0.05 | 93.14 ± 1.97 | ND | 404.80 ± 8.51 | 166.76 ± 3.49 | BDL | 5.86 ± 0.01 | 25.74 ± 0.44 | 38.83 ± 2.07 |
| Glu + Gly | 0.73 ± 0.01 | BDL | 165.1 ± 0.03 | 422.01 ± 19.37 | 266.19 ± 11.50 | 33.11 ± 4.74 | 10.60 ± 0.38 | 62.81 ± 2.76 | 64.82 ± 2.84 |

The strain was grown on bioreactor scale in fermentation medium under anaerobic conditions. The cell dry weight is estimated from OD600nm measurements as described in “Methods” section. The values are the average of two experiments with their corresponding standard deviations.

ND not determined, BDL below detection limit.

Table 5 The glycerol dehydrogenase and glycerol kinase activity (mU/mg protein) of C. werkmanii ΔdhaDΔldhAΔdhaE::ChlFRT

| Carbon source | Enzyme activity (mU/mg protein) |
|---------------|---------------------------------|
|               | Glycerol dehydrogenase | Glycerol kinase |
| Glycerol      | 72.70 ± 1.76              | 10.67 ± 1.34 |
| Glycerol + Glucose | 26.80 ± 0.18          | 13.85 ± 0.20 |

The strain was grown under anaerobic conditions in fermentation medium with glycerol only or with 0.33 molar ratio glucose to glycerol in batch cultivations on bioreactor scale. The values are the averages of two experiments with their corresponding standard deviations.
(marked with an arrow) are conserved, except for Asp121 in GDH coded by \textit{dhaD}, which was replaced by Cys123 in the putative glycerol dehydrogenase. As such, we presume that this putative glycerol dehydrogenase replaces the GDH activity coded by \textit{dhaD} in the triple knock-out mutant and that this enzyme is responsible for the cell growth and maintenance. However, a knock-out mutant of this putative glycerol dehydrogenase gene should be created to confirm the hypothesis.

Taken together, the most promising process is the one where glycerol and glucose are both used as co-substrates, as the productivity is the highest ($4.35 \pm 0.20 \text{ mM/h}$ for both substrates and $3.15 \pm 0.07 \text{ mM/h}$ for glycerol only), and the residual glycerol concentration is below the detection limit.

Conclusions
In this study, genes were deleted coding for non-essential NADH-consuming enzymes to examine the reduction of the 3-HPA concentration and the related enhancement of the final PDO titer and yield on glycerol. The triple mutant \textit{C. werkmanii} \textit{\Delta dhaD\Delta ldhA\Delta adhE} reached the maximum theoretical yield of $1.00 \pm 0.03 \text{ mol PDO/mol glycerol}$, and a final titer of $107.20 \pm 4.22 \text{ mM PDO}$ when grown in shake flasks on glucose and glycerol as carbon sources under anaerobic conditions. When the strain was grown on glycerol and glucose on bioreactor scale, glycerol was depleted, the toxic intermediate 3-HPA was below the detection limit and $422.01 \pm 19.37 \text{ mM PDO}$ was produced.

Methods
All chemicals were obtained from Sigma-Aldrich (Belgium), unless otherwise stated.

Strains and plasmids
The strains used in this work are summarized in Table 6. The different strains were preserved in a (1:1) glycerol (70 % v/v):LB (Luria Broth)-medium solution. The plasmids used are described in Maervoet et al. [5].

Genetic methods
The primers (Additional file 1: Table S2) to unravel a part of or the complete sequence of \textit{adhE}, \textit{ldhA} and \textit{arcA} of \textit{C. werkmanii}.
**Table 6  Bacterial strains used in this work**

| Strains             | Reference                  |
|---------------------|----------------------------|
| C. werkmanii DSM17579 | DSMZ, Braunschweig, Germany |
| C. werkmanii DSM17579 Δadha | Maervoet et al. [5]          |
| C. werkmanii DSM17579 Δdhah | This study                 |
| C. werkmanii DSM17579 ΔadhE::ChlFRT | This study               |
| C. werkmanii DSM17579 ΔarcA::ChlFRT | This study                |
| C. werkmanii DSM17579 ΔdhaΔdihA::ChlFRT | This study                |
| C. werkmanii DSM17579 ΔdhaΔdihA::ChlFRT | This study                |
| C. werkmanii DSM17579 ΔdhaΔdihAΔadhe::ChlFRT | This study                |
| C. werkmanii DSM17579 ΔdhaΔdihAΔadhe::ChlFRT | This study                |

*ChlFRT* chloramphenicol cassette flanked by FRT sites

**wercmanii** DSM17579 were developed by comparing the homologous regions of the genes from *C. koseri* ATCC BAA-895, *C. rodentium* ICC168, and *Citrobacter* sp. 30.2. The genes were picked up by PCR with Taq DNA polymerase (New England Biolabs, Belgium), cloned in the pGEM-T vector (Promega, Belgium), and transformed in chemically competent *E. coli* DH5α cells [30]. The genes were sequenced (LG Genomics, Germany) using the primers SPbis and T7bis (Additional file 1: Table S2). The sequence data of *idhA* and *arcA* were submitted to the Genbank database and assigned accession numbers KJ957079 and KJ957080, respectively.

The primers used for the knock-out protocol were developed using the unraveled gene sequences, as summarized in (Additional file 1: Table S2). The method to create the knock-out mutants is described in Maervoet et al. [5] and in Additional file 3. All knock-out mutants were confirmed by colony PCR and sequencing using the control primers. The sequences of the knock-out strains are presented in Additional file 2. When multiple genes were knocked-out in *C. werkmanii* DSM17579, all deletions were double checked to see whether no other chromosomal rearrangements had occurred due to the flippase recognition target scars. The chloramphenicol resistance cassette was not removed from the knock-out strains *C. werkmanii ΔadhE::ChlFRT, C. werkmanii ΔdhaDΔadhe::ChlFRT*, and *C. werkmanii ΔdhaDΔdihAΔadhe::ChlFRT*.

**Media and cultivation conditions**

The cultivation medium [163 mM glycerol as sole carbon source or 40 mM glucose and 120 mM glycerol (0.33 mol/mol glucose/glycerol)] and conditions as described in Maervoet et al. [18] were used for the shake flask experiments.

The growth medium and cultivation conditions for the reactor experiments are described in Maervoet et al. [18]. 220 mM glucose and 650 mM glycerol were used as C-source. The CO₂ was measured with an EL3020 off-gas analyzer (ABB Automation GnbH, Germany) and the data were logged with the Sartorius MFCS/win v3.0 system (Sartorius Stedim Biotech, Germany).

**Analytical methods**

The biomass concentration was measured as absorbance at 600 nm. During the fermentation experiments, the value of the optical density was converted to cell dry weight by an appropriate calibration curve. A molecular weight of 25.73 g/mol was used to convert the cell dry weight from g/L to M. Glyceraldehyde, PDO, lactate, acetate, succinate, formate and ethanol were quantified with an HPLC system (Varian, Belgium) coupled with a Refractive Index Detector and a dual UV Detector (wavelength of 210 and 265 nm). The compounds were separated by using an Aminex 300 × 7.8 mm HPX-87H Organic Acid Analysis Column (Bio-Rad Laboratories, Belgium) and eluted at 600 µL/min isocratically in 5 mM H₂SO₄ at 65 °C. Glucose was analyzed using the YSI 2700 SELECT Biochemistry Analyzer (YSI Life Sciences, Ankersmid Scientific, Belgium). 3-hydroxypropionaldehyde (3-HPA) was determined by an HPLC system with a Rezex ROA Organic Acid Analysis column (Phenomenex, Belgium) using a dual Ultraviolet Detector with a wavelength of 210 and 265 nm. The metabolite was eluted at 500 µL/min isocratically in 10 mM H₂SO₄ at 40 °C.

**Determination of enzyme activities**

The preparation of the cell free extract and the enzyme test for glycerol dehydrogenase are described in Maervoet et al. [5].

The assay mixture to determine the glycerol kinase activity, with a total volume of 3 mL, contained 0.7 mL reagent solution, 0.28 M glycine with 30 mM potassium carbonate (pH 8.9), and 0.033 M glycerol. The reagent solution contained 8.5 mM ATP, 1.22 mM NADH, 2 mM phosphoenol pyruvate, 15.3 μM lactate dehydrogenase, 7 μM pyruvate kinase, 28 mM MgSO₄,7H₂O, and 26 mM reduced glutathione (pH 7.4). The reaction was started by the addition of crude cell extract diluted in 0.1 M triethanolamine buffer (pH 7.4) to the assay mixture. The reaction velocity was measured in a coupled system with pyruvate kinase and lactate dehydrogenase. One unit is defined as the oxidation of 1.0 μmol of NADH per min at 25 °C and pH 8.9. Protein concentrations were measured using the BCA Protein Assay Kit from Thermo Scientific (Belgium).

**Quantification of NADH and NAD⁺ concentrations**

NADH and NAD⁺ concentrations were determined using Enzychrom NAD⁺/NADH assay kit (Gentaur, Belgium) following the manufacturer’s protocol. The assay...
utilizes alcohol dehydrogenase for NAD(H) quantification. Colorimetric changes in the samples were measured at 565 nm.

Additional files

Additional file 1: Figure S1. Simplified scheme of glycerol metabolizing pathways and rational engineering strategy. Figure S2. A comparison of the carbon metabolism in E. coli under (A) aerobic and (B) anaerobic conditions [12]. Table S1. Carbon and redox balances. Table S2. Primers used in the study.

Additional file 2: Sequence data confirming different knock-out strains. Green = sequence of P1 primer; blue = sequence of P2 primer; red = FRT scar; purple = chloramphenicol resistance gene.

Additional file 3: Detailed, optimized protocol for the creation of a knock-out in Citrobacter werkmanii DSM17579.

Abbreviations

3-HPA: 3-hydroxypropionaldehyde; FHL: formate hydrogen lyase; GDH: glycerol dehydrogenase; GK: glycerol kinase; PDO: 1,3-propanediol; PDODH: 1,3-propanediol dehydrogenase.

Authors' contributions

VM designed and carried out this work, and drafted the manuscript. SDM supervised the research and helped to draft the manuscript. JB, WS and MDM supervised the research and edited the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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