Enabling anaerobic growth of *Escherichia coli* on glycerol in defined minimal medium using acetate as redox sink

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ABSTRACT

Glycerol has become an attractive substrate for bio-based production processes. However, *Escherichia coli*, an established production organism in the biotech industry, is not able to grow on glycerol under strictly anaerobic conditions in defined minimal medium due to redox imbalance. Despite extensive research efforts aiming to overcome these limitations, anaerobic growth of wild-type *E. coli* on glycerol always required either the addition of electron acceptors for anaerobic respiration (e.g. fumarate) or the supplementation with complex and relatively expensive additives (tryptone or yeast extract). In the present work, driven by model-based calculations, we propose and validate a novel and simple strategy to enable fermentative growth of *E. coli* on glycerol in defined minimal medium. We show that redox balance could be achieved by uptake of small amounts of acetate with subsequent reduction to ethanol via acetyl-CoA. Using a directed laboratory evolution approach, we were able to confirm this hypothesis and to generate an *E. coli* strain that shows, under anaerobic conditions with glycerol as the main substrate and acetate as co-substrate, robust growth ($\mu = 0.06\,\mathrm{h^{-1}}$), a high specific glycerol uptake rate (10.2 mmol/gDW/h) and an ethanol yield close to the theoretical maximum (0.92 mol per mol glycerol). Using further stoichiometric calculations, we also clarify why complex additives such as tryptone used in previous studies enable *E. coli* to achieve redox balance. Our results provide new biological insights regarding the fermentative metabolism of *E. coli* and offer new perspectives for sustainable production processes based on glycerol.

1. Introduction

In order to reduce the emission of the greenhouse gas CO_2, there has been an increasing demand to shift from a fossil-based to a sustainable production of chemicals and fuels. Biomass-derived fuels have come into focus over the last decades as one building block for this transformation (Yazdani and Gonzalez, 2007; Liao et al., 2016; Keasling et al., 2021). Next to the fermentative production of ethanol from carbohydrates, biodiesel, which is usually generated by transesterification from vegetable oil or waste animal fat, is the second major class of biofuels (Huber et al., 2006). However, large amounts of glycerol (10% w/w of produced biodiesel) accumulate during the production process. Thus, upcycling of this byproduct would make the overall production economically more feasible (Yang et al., 2012). Besides using glycerol as feedstock for chemical synthesis or as additive for animal feed, the microbial conversion to value-added products holds great potential for usage of the glycerol side stream (Yazdani and Gonzalez, 2007; da Silva et al., 2009; Dobson et al., 2012; Yang et al., 2012; Clomburg and Gonzalez, 2013). Due to its high degree of reduction, glycerol has advantages (higher maximal yield per C atom) for the microbial production of biofuels and other reduced chemicals compared to established substrates such as glucose (Clomburg and Gonzalez, 2013). At the same time, the high degree of reduction is the reason that only a few bacterial species (e.g. *Klebsiella pneumoniae* (Forage and Foster, 1982) and *Clostridium butyricum* (Raynaud et al., 2003)) are known to grow on glycerol under strictly anaerobic conditions without any external electron acceptor. Most of the time, the fermentative utilization of glycerol by these organisms is facilitated by the production of 1,3-propanediol (1,3-PDO), whose secretion is used as a redox sink (Saxena et al., 2009).

Escherichia coli is the most studied bacterium and one of the major work horses in the biotech industry. Over the last two decades, it has been extensively investigated whether and under which conditions *E. coli* can anaerobically grow on glycerol as sole substrate and convert it into ethanol or other valuable products (Dharmadi et al., 2006; Clomburg and Gonzalez, 2013; Clomburg et al., 2022). *E. coli* possesses an ATP-generating and redox-balanced pathway from glycerol to ethanol. However, biomass synthesis leads to net formation of NADH and with its standard metabolic repertoire *E. coli* is not able to balance this excess

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NADH and to replenish the NAD pool with glycerol as sole substrate under anaerobic conditions. Redox balance can be facilitated with the help of altered process conditions or targeted changes in metabolism. First, small amounts of molecular oxygen could be provided (Durnin et al., 2009; Trinh and Srienc, 2009; Nikel et al., 2010; Wong et al., 2014), however, such micro-aerobic conditions are difficult to control and adjust in large bioreactors. Second, external electron acceptors could be added to the medium (e.g. fumarate or nitrate) enabling the cell to use anaerobic respiration as electron sink (Unden and Bongaerts, 1997), but these components can be costly or/and result in other (un-desired) products. Another opportunity is to use an electric potential in combination with a chemical mediator (e.g. potassium ferricyanide). Here, the mediator acts as an external electron acceptor but can be recycled by oxidation at an electrode (Emde et al., 1989). However, those processes are still inefficient and the experimental setup is rather complex which makes this approach not feasible for industrial applications. Finally, introduction of the heterologous 1,3-PDO pathway from *K. pneumonia* into *E. coli* provides a direct mechanism to balance NADH (Tong et al., 1991; Tang et al., 2009), but processes relying on this approach used large amounts of yeast extract and may not be useful for the production of other products such as ethanol.

Recently, Gonzalez and co-workers proposed the existence of an endogenous pathway in *E. coli*, which generates 1,2-propanediol from glycerol via dihydroxyacetone, dihydroxyacetone phosphate, and methylglyoxal under net consumption of NADH, thus enabling redox-balanced growth (Gonzalez et al., 2008; Murarka et al., 2008; Clom-burg and Gonzalez, 2011, 2013; Clomburg et al., 2022). However, in all these studies, anaerobic growth of *E. coli* on glycerol could only be achieved if complex additives (e.g. tryptone) were supplemented to the medium. Similar results were also reported in studies from other groups focusing on the anaerobic conversion of glycerol into ethanol by *E. coli*, where tryptone or yeast extract needed to be added to the medium to facilitate cell growth (Cofré et al., 2012; Valle et al., 2015a, 2015b). However, the use of these additives in large-scale applications may significantly increase process costs and thus hamper economic viability.

In this study, we present a novel and simple approach to balance redox equivalents enabling anaerobic growth of *E. coli* on glycerol with high-yield ethanol production in chemically defined minimal medium. In contrast to previous studies, our strategy does neither require complex medium supplements nor external electron acceptors for anaerobic respiration (such as fumarate or nitrate). Using a stoichiometric model of the metabolism of *E. coli* we found that the co-uptake of only small amounts of acetate could function as redox sink during growth: the uptake of one molecule of acetate and its conversion to one molecule of ethanol via acetyl-CoA leads to a net consumption of two molecules of NADH, which, in theory, should allow *E. coli* to grow anaerobically on glycerol (and acetate) without any further additives. As a positive side effect, glycerol and acetate are both being converted to the target product ethanol enhancing the overall process efficiency.

Based on these theoretical considerations, we cultivated the *E. coli* W wild-type strain in defined minimal medium with glycerol as carbon source and acetate as co-substrate and indeed, we observed exponential growth of *E. coli* under strictly anaerobic conditions after some time of adaption (while no growth was seen in a minimal glycerol medium where neither acetate nor tryptone were added). Following a laboratory evolution approach, we could generate an evolved strain with enhanced fitness, which showed robust anaerobic growth, a high glycerol uptake rate, and an excellent ethanol yield close to the theoretical maximum. Measured exchange rates for glycerol, acetate and ethanol are close to calculated values from the stoichiometric metabolic model confirming the validity of the predicted redox balancing mechanism. With further stoichiometric calculations, we also clarify why complex additives such as tryptone used in previous studies enabled *E. coli* to achieve redox balance. Our results offer new perspectives for anaerobic processes converting glycerol into value-added products.

2. Materials and methods

2.1. Strains, media, and culture conditions used in this study

All experiments were performed with the *E. coli* W wild-type strain (ATCC 9637) (Archer et al., 2011), which we obtained from the lab of Stefan Pflügl (TU Wien).

The minimal medium (MM) used for all main cultivations was adapted from (Tanaka et al., 1967) and contained 34 mM NaH_2PO_4, 64 mM K_2HPO_4, 20 mM $(NH_4)_2SO_4$, 1 μM $Fe(SO_4)_4$, 300 μM $MgSO_4$, 1 μM $ZnCl_2$, 10 μM $CaCl_2$, 10 mM sodium acetate, and 10 g/L ($\approx$109 mM) glycerol. When indicated, sodium acetate was left out of the medium, substituted with 2 g/L of tryptone, or added in larger amounts (100 mM) if glycerol was left out of the medium as carbon source.

For growth assays, 3 mL of LB_0 medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) were inoculated with a single colony of the respective strain or from a cryo stock and incubated for 5 h at 37 °C and 150 rpm in a test tube. The LB_0 culture was diluted 1:100 in 50 mL of MM in 100 mL flasks and incubated at 37 °C for 2 days without shaking to adapt to anaerobic conditions. Next, 25 mL of fresh MM were inoculated in 100 mL flasks from the non-shaking culture to an optical density at 420 nm (OD_{420}) of 0.5 and incubated at 37 °C for 2 days in an anaerobic workstation (Don Whitley Scientific Limited) with an oxygen-free atmosphere (80% N_2, 10% CO_2 10% H_2) to ensure strictly anaerobic conditions in the pre-culture. For the main culture, 20 mL of fresh MM were inoculated from the anaerobic pre-culture to an OD_{420} of 0.35 in 25 ml Schott bottles and incubated at 37 °C in the anaerobic workstation with stirring at 150 rpm.

Cell growth was monitored by measuring the OD_{420} and using a conversion factor of 0.22 gDW/OD_{420} to obtain the concentration of dry biomass in gDW/L (gDW: gram dry weight).

2.2. Laboratory evolution of E. coli W for faster anaerobic growth on glycerol and acetate

E. coli W was cultivated in MM with 4 g/L (44 mM) glycerol and 1 g/L (17 mM) acetate in an anaerobic workstation as described under 2.1. The initial culture was incubated until the optical density increased no further. An aliquot was diluted 1:10 into fresh medium keeping the cells under anaerobic conditions. Inoculation of grown cultures into fresh medium was repeated for about 20 times.

2.3. Sequencing of evolved strain

Chromosomal DNA of the wild-type *E. coli* W as well as of the evolved culture was prepared using the Puregene Yeast/Bact. Kit of Qiagen (158567) according to the manufacturer's instruction. Library preparation and sequencing was conducted by Genewiz Europe. The NGS reads were mapped onto the genome of *E. coli* W (GenBank entry CP002185)) and analyzed using the software Geneious (Biomatters).

2.4. Quantification of metabolites

The glycerol concentration in the medium was determined using the Enzytec Liquid Glycerol kit (E8360) and the ethanol concentration was measured using the Megazyme Ethanol Assay kit (K-ETOH) according to the manufacturer's instructions. Extracellular formate, acetate, and succinate was quantified by HPLC as described earlier (Boecker et al., 2019). Pyruvate, lactate, fumarate, and orotate were quantified by the same method but were not secreted in significant amounts.

2.5. Stoichiometric calculations and metabolic flux analysis

For all stoichiometric calculations and metabolic flux analysis we used the *EColiCore2* (ECC2) network model (Hädicke and Klamt, 2017) and the *CellNetAnalyzer* toolbox (Klamt et al., 2007; von Kamp et al.,

2017). Flux balance analysis (FBA) was used with different objective functions to analyze anaerobic metabolism of *E. coli* with glycerol as substrate (for details see Results section).

We used measurements of the biomass and of substrates and products to calculate the corresponding growth, uptake and excretion rates, respectively. The growth rate (μ) for the exponential phase was determined by plotting the natural logarithm of the biomass concentrations of each sampled time point (within the exponential growth period) against the cultivation time. The slope of the linear regression equals μ. Specific uptake and excretion rates for the exponential phase were determined with the formula

$$r_M = \mu \, (c_{M,e} - c_{M,s}) \, / \, (c_{X,e} - c_{X,s}) \; [mmol/gDW/h],$$

where μ is the growth rate, $c_{M,e}$ and $c_{M,s}$ represent the end and start concentrations of the respective metabolite M (mmol/L glycerol, ethanol, acetate, formate, or succinate) and $c_{X,e}$ and $c_{X,s}$ represent the end and start concentrations of the biomass (gDW/L).

These rates were then fixed in the model and FBA was used to determine intracellular fluxes and to test the consistency of the measured rates as described in the Results section.

3. Results

3.1. Stoichiometric calculations

Using the ECC2 network model (Hädicke and Klamt, 2017) and the *CellNetAnalyzer* toolbox (Klamt et al., 2007; von Kamp et al., 2017) we started with some basic stoichiometric calculations related to anaerobic utilization of glycerol by *E. coli*. It has been shown earlier that anaerobic glycerol metabolization in *E. coli* involves the reactions of the glycerol dehydrogenase (GLD; converting glycerol to dihydroxyacetone (DHA) with generation of one NADH) and of the dihydroxyacetone kinase (DHAK; which uses PEP to phosphorylate DHA to dihydroxyacetone phosphate (DHAP)) (Clomburg and Gonzalez, 2013) (Fig. 1a). Since the DHAK reaction was not contained yet in the ECC2 network, we included it in the model, while we set reactions relevant for aerobic glycerol consumption (e.g. glycerol-3-phosphate dehydrogenase and glycerol kinase) to be inactive.

It is well-known that metabolization of glycerol along the GLD-DHAK-pathway and subsequent substrate phosphorylation in lower glycolysis and excretion of the fermentation products ethanol and formate (for the latter alternatively CO_2 and H_2 if the formate hydrogen lyase (FHL) is active; Fig. 1a) allows net synthesis of ATP with balanced reduction equivalents. The model indicates that 1 mol of glycerol is needed for net production of one mol of ATP (eq. (1) in Fig. 1b). This stoichiometry is exact for ethanol and CO_2+H_2 as end products. If formate is not decomposed to CO_2+H_2, depending on the export mechanism, one proton must be pumped from the cytoplasm to the periplasm per molecule of excreted formate to maintain charge balance. This would slightly reduce the ATP yield, however, in the following we assume an ATP yield of 1 which does not affect the main conclusions.

As expected, despite the fact that balanced ATP synthesis is possible, the model also reveals that growth with glycerol as sole substrate is not possible (maximal growth rate is zero) due to an imbalance of NADH. The surplus of NADH can be calculated as follows. We first introduce an artificial reaction that oxidizes NADH to NAD (NADH + H + X → NAD + XH_2, where X and XH_2 are pseudo compounds that need not be balanced in the model). For a fixed growth rate of 0.1 h^{-1} we then search for a flux distribution that minimizes the flux through the NADH oxidation pseudo reaction. Solving this optimization problem we found that ca. 1.50 mmol of NADH need to be oxidized in order to build 0.1 g of dry biomass and that 9.41 mmol of glycerol would be required for this operation (a large fraction of the glycerol is thereby converted to ethanol and formate / CO_2+H_2 to produce ATP; eq. (2) in Fig. 1b). It is this amount of NADH in excess that prevents anaerobic growth of *E. coli* on glycerol.

The key idea of this study is to show that co-utilization of acetate enables the cell to grow on glycerol because an electron acceptor becomes then available for balancing the NADH pool. While *E. coli* cannot grow on acetate as sole substrate under anaerobic conditions (as also confirmed by the model), it can be seen that uptake of acetate and its conversion to ethanol via acetyl-CoA would consume two NADH (Fig. 1a and eq. (3) in Fig. 1b). In addition, one ATP is needed for synthesis of acetyl-CoA from acetate via the reactions of the acetate kinase (ACK) and phosphotransacetylase (PTA) (two molecules of ATP would be needed if the acetate synthase (ACS) is used instead, however, this would not affect the main conclusions drawn below and the work in (Enjalbert et al., 2017) indicates the relevance of the ACK-PTA pathway for higher acetate concentrations). Substituting the requirement of ATP in eq. (3) by eq. (1) yields eq. (4) in Fig. 1b, which summarizes the overall stoichiometry to utilize acetate as redox sink. Finally, combining eqs. (2) and (4) we obtain the optimal stoichiometry for anaerobic biomass synthesis from glycerol with co-utilization of acetate (eq. (5) in Fig. 1b). The same result is returned by the model when FBA is used to find a flux distribution with minimal uptake of glycerol enabling synthesis of 0.1 g of dry biomass. Notably, the molar uptake ratio of acetate and glycerol is very low (0.074 mol$_{Ace}$/mol$_{Glyc}$) indicating that only small amounts of acetate are needed. On the other hand, approximately 90% of the glycerol are converted to ethanol and formate (CO_2+H_2).

These theoretical results clearly indicate that anaerobic growth of *E. coli* on glycerol as the main substrate could be enabled with small amounts of acetate as co-substrate.

3.2. Anaerobic cultivation of E. coli W on glycerol

To test the hypothesis, that *E. coli* is able to grow anaerobically in minimal medium on glycerol supplemented solely with acetate and without any further complex additives, *E. coli* W was cultivated in defined medium with different compositions: One batch contained 10 g/L ($\approx$109 mM) of glycerol and 2 g/L of tryptone (similar to previous studies (Dharmadi et al., 2006; Gonzalez et al., 2008; Yazdani and Gonzalez, 2008; Clomburg et al., 2022)), a second batch contained 10 g/L ($\approx$109 mM) of glycerol and 10 mM of acetate, a third only 10 g/L of glycerol without any additives and a fourth contained ca. 100 mM of acetate and no glycerol as a control (see section 2.1).

As shown in Fig. 2a and as reported previously (Dharmadi et al., 2006; Gonzalez et al., 2008; Yazdani and Gonzalez, 2008), *E. coli* grew on glycerol with the addition of tryptone with a relatively low growth rate of 0.01 h^{-1} and a biomass yield of 0.039 gDW/g$_{Glyc}$ (Table 1). The consumed glycerol (r_{Glyc} = 3.16 mmol/gDW/h) was converted in large parts to ethanol with a final yield of 0.76 mol$_{Eth}$/mol$_{Glyc}$ (Fig. 2b–c, Table 1). As a second fermentation product, succinate was formed in small amounts (r_{Suc} = 0.46 mmol/gDW/h, Y_{Suc} = 0.15 mol$_{Suc}$/mol$_{Glyc}$).

The cultivation on glycerol or on acetate as sole substrates in minimal medium did not lead to any biomass formation (Fig. 2a). The strain cultivated on glycerol produced minor amounts of ethanol (Fig. 2b, Table 1) which enables formation of ATP but cannot resolve the redox imbalance under growth, as described in the previous section. In contrast, the strain cultivated on glycerol with the addition of 10 mM of acetate started to grow exponentially (μ = 0.032 h^{-1}) around 200 h after inoculation. At the same time, the cells started to take up glycerol (r_{Glyc} = 4.61 mmol/gDW/h) as well as small amounts of acetate (r_{Ace} = 0.29 mmol/gDW/h) and converted both substrates into the main product ethanol (r_{Eth} = 4.16 mmol/gDW/h, Fig. 2a–d, Table 1) with a yield of 0.9 mol$_{Eth}$/mol$_{Glyc}$. The ratio of acetate taken up and ethanol produced per consumed glycerol is in almost perfect agreement with the calculated net stoichiometries in eq. (5) in Fig. 1b (r_{Ace}/r_{Glyc}: measured 0.063 mmol/mmol, predicted 0.074 mmol/mmol; r_{Eth}/r_{Glyc}: measured 0.90 mmol/mmol, predicted 0.93 mmol/mmol). Formation of formate could be detected as well, but, after initial rise, the concentration in the medium decreased during cultivation, most likely due to FHL-mediated oxidation of formate to CO_2 and H_2 (a similar behavior was seen for
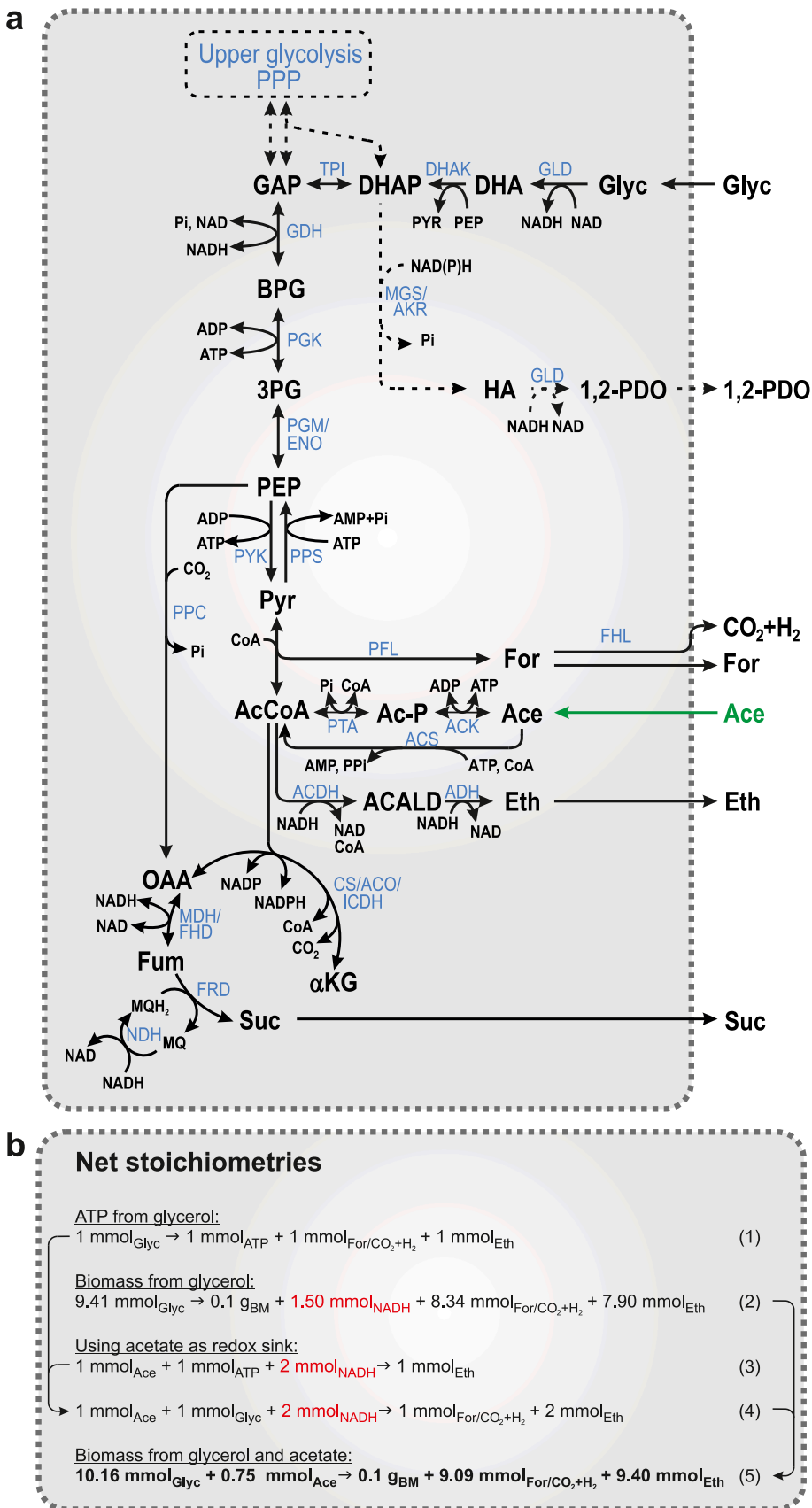
Fig. 1. Stoichiometric calculations with the *E. coli* network model for anaerobic consumption of glycerol as substrate. (a) Metabolic map showing the major pathways (reaction names in blue) and metabolites (black) of the central metabolism relevant for anaerobic consumption of glycerol. (b) Net stoichiometries for ATP and biomass synthesis with glycerol as substrate (with or without acetate as co-substrate). Abbreviations of metabolites: 1,2-PDO: 1,2-propanediol; 3PG: 3-phosphoglycerate; ACALD: acetaldehyde; AcCoA: acetyl coenzyme A; Ace: acetate; Ac–P: acetylphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate; ATP: adenosine triphosphate; BPG: 1,3-bisphospho-D-glycerate; CO_2: carbon dioxide; CoA: coenzyme A; DHA: dihydroxyacetone; DHAP: dihydroxyacetone phosphate; Eth: ethanol; For: formate; Fum: fumarate; GAP: D-glyceraldehyde-3-phosphate; Glyc: glycerol; H_2: hydrogen; HA: hydroxyacetone; MQ: menaquinone; MQH_2: menaquinol; NAD: oxidized nicotin-amide adenine dinucleotide; NADH: reduced nicotin-amide adenine dinucleotide; NADP: oxidized nicotinamide adenine dinucleotide phosphate; NADPH: reduced nicotin-amide adenine dinucleotide phosphate; OAA: oxalo-acetate; PEP: phosphoenolpyruvate; Pi: phosphate; PPi: pyrophosphate; Pyr: pyruvate; Suc: succinate; αKG: α-ketoglutarate. Abbreviation of reaction names: ACDH: acetaldehyde dehydrogenase; ACK: acetate kinase; ACO: aconitate hydratase A/B; ACS: acetyl coenzyme A synthase; ADH: alcohol dehydrogenase; AKR: aldo-keto reductase; CS: citrate synthase; DHAK: dihydroxyacetone kinase; ENO: enolase; FHD: fumarase; FHL: formate hydrogenlyase; FRD: fumarate reductase; GHD: glyceraldehyde-3-phosphate dehydrogenase; GLD: glycerol dehydrogenase; ICDH: isocitrate dehydrogenase; MDH: malate dehydrogenase; MGS: methylglyoxal synthase; NDH: NADH dehydrogenase; PFL: pyruvate formate-lyase (also known as formate acetyltransferase); PGK: phosphoglycerate kinase; PGM: phosphoglycerate mutase; PPC: phosphoenolpyruvate carboxylase; PPP: pentose phosphate pathway; PPS: phosphoenolpyruvate synthetase; PTA: phosphate acetyltransferase; PYK: pyruvate kinase; TPI: triose-phosphate isomerase.
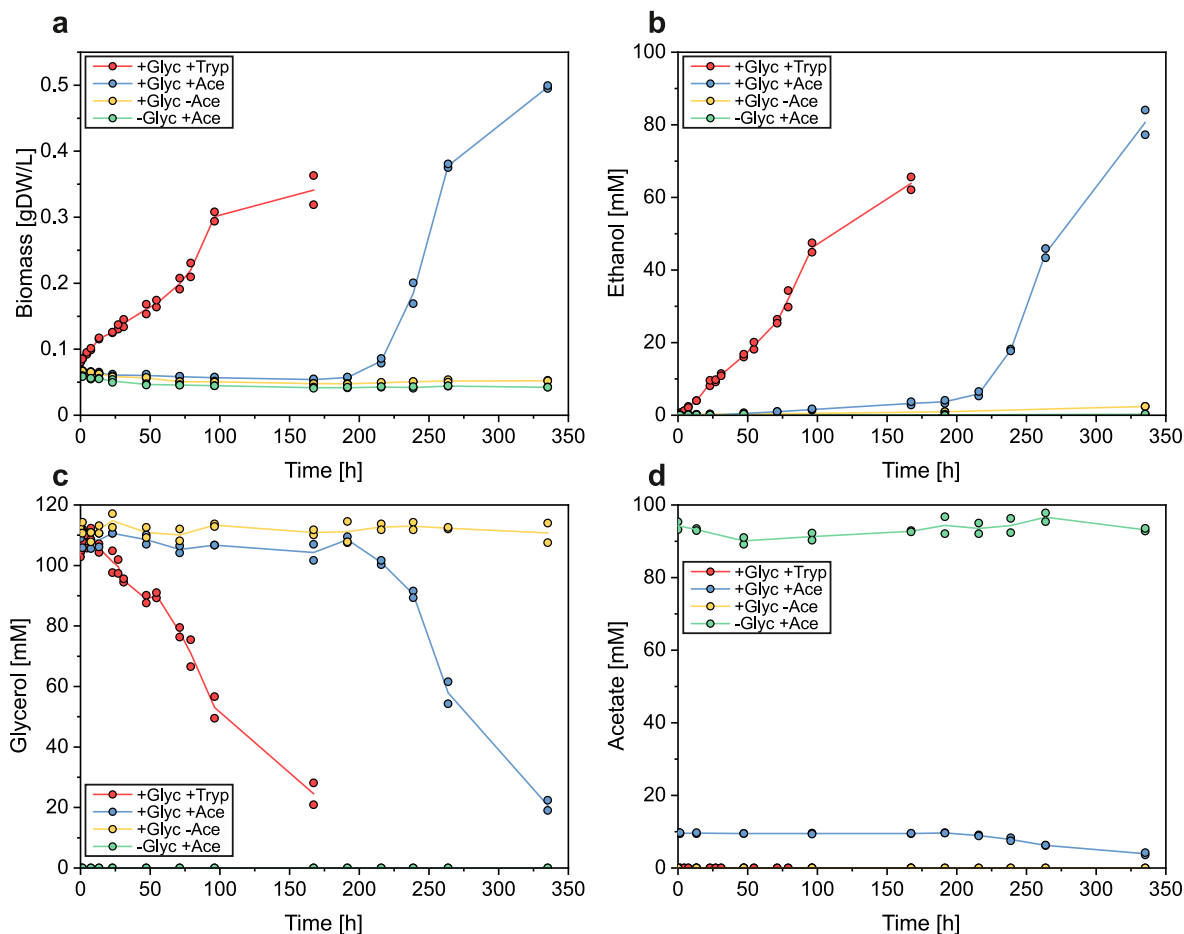
Fig. 2. Time course of concentrations of (a) biomass, (b) ethanol, (c) glycerol, and (d) acetate in an anaerobic culture of *E. coli* W in minimal medium with varying carbon sources and/or additives. +Glyc +Tryp: 10 g/L glycerol and 2 g/L tryptone; +Glyc +Ace: 10 g/L glycerol and 10 mM sodium acetate; +Glyc -Ace: 10 g/L glycerol; -Glyc +Ace: 100 mM sodium acetate. The means (solid lines) and individual data (dots) of $n = 2$ biologically independent samples are shown.

Table 1
Growth rates, substrate uptake and product excretion rates for *E. coli* W cultivated in minimal medium with varying carbon sources and/or additives.
+Glyc +Tryp: 10 g/L glycerol and 2 g/L tryptone; +Glyc +Ace: 10 g/L glycerol and 10 mM sodium acetate; +Glyc -Ace: 10 g/L glycerol; -Glyc +Ace: 100 mM sodium acetate. The means ± SD of $n = 2$ biologically independent samples are shown.

	Composition of medium			
	+Glyc +Tryp	+Glyc +Ace	+Glyc -Ace	-Glyc +Ace
μ [h^{-1}]	0.010 ± 0.001	0.032 ± 0.001	0	0
$r_{\text{Glyc\_up}}$ [mmol/gDW/h]	3.16 ± 0.16	4.61 ± 0.37	0.10 ± 0.08	0
$r_{\text{Ace\_up}}$ [mmol/gDW/h]	0	0.29 ± 0.03	0	0
$r_{\text{Eth\_ex}}$ [mmol/gDW/h]	2.36 ± 0.08	4.16 ± 0.02	0.14 ± 0.00	0
$r_{\text{Suc\_ex}}$ [mmol/gDW/h]	0.46 ± 0.06	0.36 ± 0.00	0	0

growth on glycerol with tryptone). Succinate was also formed but only in small amounts ($r_{\text{Suc}} = 0.36$ mmol/gDW/h).

3.3. Laboratory evolution improves fitness for anaerobic cultivation on glycerol and acetate

To further increase the fitness of *E. coli* for anaerobic growth on glycerol and acetate, the strain was subjected to laboratory evolution by re-inoculating fresh minimal medium with cells from grown cultures. After about 20 cycles, the cells showed an increased growth rate. A single colony of the evolved culture was purified and designated W$_{\text{Evo}}$. Next, we cultivated W$_{\text{Evo}}$ and found indeed superior performance compared to the initial cultivation on glycerol and acetate (Fig. 3, Table 2). The growth rate increased by 75% to 0.056 h^{-1} and the specific glycerol uptake even by 120% to 10.16 mmol/gDW/h. Again, acetate

was consumed in small amounts, while formate was only detected at the beginning after which most likely an FHL-mediated oxidation of formate to CO_2 and H_2 took place.

The measured rates of substrate uptake and product formation together with the respective yields of the evolved *E. coli* strain W$_{\text{Evo}}$ growing on minimal medium with glycerol and acetate are shown in Table 2 (second column). We used the ECC2 network model to test the consistency of the measured rates with stoichiometric predictions. Fixing all experimentally determined reaction rates (given in the second column in Table 2) to their measured value leads to an infeasible system due to slightly inconsistent redundancies in the measurements. We therefore left the values of acetate uptake and ethanol excretion unspecified which removes those redundancies. For the calculation of unknown rates we assumed that the substrate not used for growth was utilized to synthesize ATP for non-growth-associated maintenance,
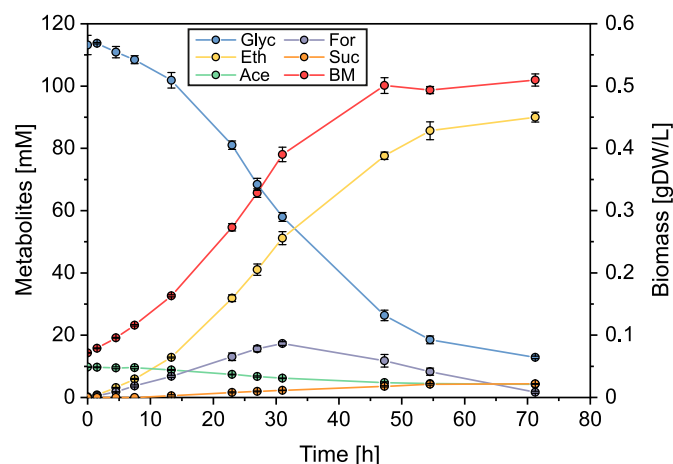
Fig. 3. Time course of biomass and metabolite concentrations in the culture of the evolved W_{Evo} strain grown in minimal medium with 10 g/L glycerol supplemented with 10 mM sodium acetate. Glyc: glycerol; Eth: ethanol; Ace: acetate; For: formate; Suc: succinate; BM: biomass. The means of $n = 3$ biologically independent samples are shown. The error bars represent SD.

Table 2
Growth rates, substrate uptake rates, product excretion rates and product yields for the evolved *E. coli* strain W_{Evo} growing on minimal medium with glycerol and acetate and comparison with calculated rates from metabolic flux analysis. The second column shows the measured values. The third column displays results from metabolic flux analysis, where the values in normal font were used as input for the calculation of rates shown in bold font. For the calculation of unknown rates it was assumed that the substrate not used for growth was utilized to synthesize ATP for non-growth-associated maintenance (r_{ATPM}). The means ± SD of $n = 3$ biologically independent samples are shown (second column). Note that the measured yields may deviate from the respective ratios of measured rates because yields were determined from the final product (or biomass) concentration at the end of the cultivation while rates were calculated for the exponential phase.

	E. coli W_{Evo} (experimental)	Metabolic flux analysis
μ [h^{-1}]	0.056 ± 0.001	0.056 (fixed)
r_{Glyc} [mmol/gDW/h]	10.16 ± 0.46	10.16 (fixed)
r_{Ace} [mmol/gDW/h]	0.60 ± 0.04	**0.42 (calculated)**
r_{Eth} [mmol/gDW/h]	9.01 ± 0.45	**9.29 (calculated)**
r_{PFL} ($= r_{For} + r_{FHL}$) [mmol/gDW/h]	not determined	**9.11 (calculated)**
r_{ATPM} [mmol/gDW/h]	not determined	**3.59 (calculated)**
r_{Suc} [mmol/gDW/h]	0.44 ± 0.04	0.44 (fixed)
$Y_{BM/Glyc}$ [gDW/mol]	5.97 ± 0.26	5.51 (fixed by given rates)
$Y_{Eth/Glyc}$ [mmol/mmol]	0.92 ± 0.04	**0.91 (calculated)**
$Y_{Suc/Glyc}$ [mmol/mmol]	0.042 ± 0.00	0.043 (fixed by given rates)

hence, we maximized the rate of the r_{ATPM} reaction quantifying that demand (note that the growth rate was fixed, i.e. maximization of growth as usually done in FBA would not be meaningful here and maximization of ATP yield appears then most reasonable). The third column in Table 2 shows that the calculated rates of acetate uptake and ethanol production are very close to the measured values. Furthermore, it can be seen that a maximum of 3.59 mmol ATP can be produced per gDW and hour with the given constraints, a value that is close to generally accepted values in the literature (Orth et al., 2011). Overall, metabolic flux analysis indicated a very good consistency of the measured data with model predictions, also with respect to the stoichiometries given in Fig. 1b.

For the sake of completeness we also tested whether the evolved strain could now grow without acetate in the medium, but found that

acetate is still required for anaerobic growth on minimal medium with glycerol.

To determine mutations that occurred during the evolution process, whole genome sequencing of the evolved culture was performed. As whole genome sequencing was intended as an initial screen, only one sequencing run was performed. gDNA for sequencing was prepared directly from the evolved culture, hence, mutations that are present in only a subset of the cells might not be detected. Sequences were compared to the *E. coli* W genome sequence (GenBank entry CP002185). We observed several sequence deviations of our strain compared to the published sequence (Supplementary Table 1). However, most of these deviations relate to Rhs and phage proteins or were present also in our parent strain. From the genes with clear metabolic functions, mutations were detected only in *dhaK* and *gldA*, which are both related to glycerol metabolism. For *gldA*, the data clearly show two populations, the wild-type and a mutated allele in the culture. The detection of a mutation in *dhaK* and the occurrence of mutations in *gldA* fit nicely to the data of (Gonzalez et al., 2008), who identified the pathway using GLD and DHAK as being important for anaerobic glycerol metabolism. Later they could also show that overexpression of *dhaKLM* and *gldA* improved anaerobic growth on glycerol (Cintolesi et al., 2012). The identified *dhaK* and gldA mutations have not been described before and hence their effect on enzyme activity is unknown. With respect to the phenotype one might speculate that the mutations improve the activity or expression of the enzymes during anaerobic growth with glycerol. Interestingly, none of the found mutations seem to be related to acetate utilization.

4. Discussion and conclusion

Anaerobic fermentation of glycerol by *E. coli* has been investigated in detail over the last 15 years (Dharmadi et al., 2006; Gonzalez et al., 2008; Murarka et al., 2008; Cofré et al., 2012; Clomburg and Gonzalez, 2013; Valle et al., 2015a, 2015b; Clomburg et al., 2022). So far, anaerobic growth of wild type *E. coli* with glycerol as carbon substrate was only possible either with the addition of tryptone (or/and yeast extract) or by adding electron acceptors (such as fumarate) to the medium enabling the cell to balance redox by anaerobic respiration (Unden and Bongaerts, 1997; Unden et al., 2014). While the mechanisms of anaerobic respiration are well understood, it is not yet clear why the addition of tryptone enables *E. coli* to utilize glycerol under anoxic conditions. Again, stoichiometric modeling helps to unravel this phenomenon. Tryptone is an assortment of peptides and provides a source of amino acids. In fact, it can be shown that uptake of certain amino acids as co-substrates for glycerol may resolve the redox imbalance (eq. (2) in Fig. 1b) during growth. As example we chose alanine (Ala), which is synthesized from pyruvate (Pyr) by a transamination reaction with glutamate (Glu) (Fig. 4):

$$Pyr + Glu \leftrightarrow Ala + \alpha KG \tag{1}$$

(αKG stands for α-ketoglutarate). Glutamate can be recycled from αKG, ammonium and NADPH by the action of the glutamate dehydrogenase. The overall reaction stoichiometry then reads (for simplicity, water, protons etc. are not shown):

$$Pyr + NADPH + NH_4^+ \leftrightarrow Ala + NADP \tag{2}$$

Given the fact that synthesis of pyruvate from glycerol leads to co-production of two NADH, it can be concluded that synthesis of alanine from glycerol leads to a net production of one reduction equivalent. Hence, taking up alanine and using it as amino acid for protein and peptidoglycan synthesis already reduces the excess of reduction equivalents (namely one reduction equivalent per uptaken alanine molecule). Moreover, alanine can also be used as redox sink similar as acetate: using the alanine transaminase reaction (eq. (1)) in reverse direction (see e.g. (Katsube et al., 2019)) and recycling afterwards αKG
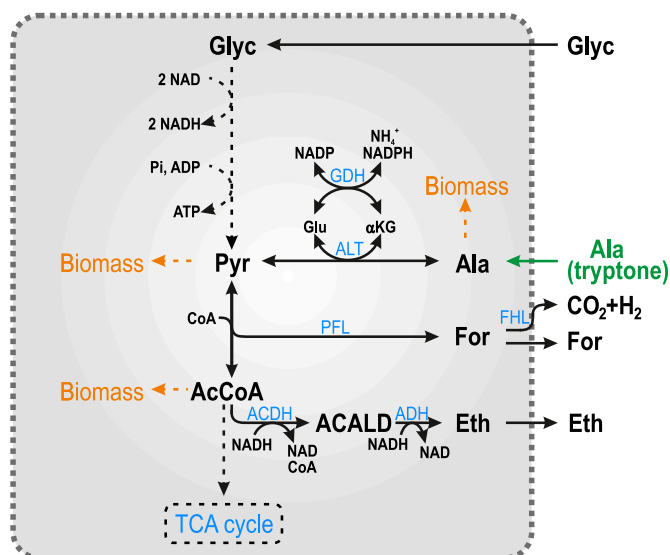
Fig. 4. Relevant reactions and metabolites involved in the metabolization of alanine (obtained from tryptone) supporting redox balancing under anaerobic growth of *E. coli* on glycerol. Abbreviations of metabolites: ACALD: acetaldehyde; AcCoA: acetyl coenzyme A; ADP: adenosine diphosphate; Ala: L-alanine; ATP: adenosine triphosphate; CO_2: carbon dioxide; CoA: coenzyme A; Eth: ethanol; For: formate; Glu: L-glutamate; Glyc: glycerol; H_2: hydrogen; NAD: oxidized nicotin-amide adenine dinucleotide; NADH: reduced nicotin-amide adenine dinucleotide; NADP: oxidized nicotinamide adenine dinucleotide phosphate; NADPH: reduced nicotinamide adenine dinucleotide phosphate; NH_4^+: ammonium; Pi: phosphate; Pyr: pyruvate; αKG: α-ketoglutarate. Abbreviations of reaction names: ACDH: acetaldehyde dehydrogenase; ADH: alcohol dehydrogenase; ALT: alanine transaminase; FHL: formate hydrogenlyase; GDH: glutamate dehydrogenase; PFL: pyruvate formate-lyase (also known as formate acetyltransferase); TCA cycle: tricarboxylic acid cycle.

from glutamate yields the inverse net stoichiometry of eq. (2):

$$Ala + NADP \leftrightarrow Pyr + NADPH + NH_4^+ \qquad (3)$$

Further conversion of pyruvate to ethanol leads to consumption of two NADH, hence, in total one reduction equivalent NAD(P)H would be consumed in the synthesis of ethanol from alanine:

$$Ala + NAD(P)H \leftrightarrow Eth + NH_4^+ + NAD(P) + For / CO_2 + H_2 \qquad (4)$$

(NAD(P)H denotes the lumped pool of NADH and NADPH). Eq. (4) with alanine could thus replace eq. (3) with acetate in Fig. 1b. In addition, note that pyruvate obtained in eq. (3) could also be used as precursor or as starting point to synthesize other precursors (e.g., acetyl-CoA), which would again save one NAD(P)H since pyruvate synthesis from alanine produces only one NAD(P)H while two are synthesized when using glycerol. Hence, uptake and metabolization of alanine saves effectively one NAD(P)H (in all three directions: for synthesizing biomass, for generating other precursors, or in the conversion to ethanol) and, therefore, exactly the double amount of alanine (1.5 mmol) would be needed compared to acetate (0.75 mmol) for synthesis of 0.1 g of dry biomass (eq. (5) in Fig. 1b). This is also confirmed by the metabolic model when allowing uptake of alanine (instead of acetate). As detailed in the Supplementary Text 1, further model analysis revealed that 8 of the 20 amino acids could, in a similar manner to alanine, facilitate this kind of redox balancing. This explains the finding that *E. coli*, in the absence of anaerobic respiration, could only grow on glycerol under anaerobic conditions if tryptone was provided (Clomburg et al., 2022) and that the observed formation of other products (e.g., 1, 2-propanediol) could not resolve the redox balance alone (Gonzalez et al., 2008). Similar effects can be expected when using yeast extract instead of (or in combination with) tryptone.

We are not aware of any previous study where acetate, which is normally a fermentation product of *E. coli*, is used as co-substrate for strictly anaerobic cultivation of *E. coli*, but the application of acetate as electron acceptor in fermentations is not completely new. Guadalupe Medina et al. (Guadalupe Medina et al., 2010) used acetic acid as co-substrate together with glucose to minimize glycerol production and thereby to increase ethanol yield during fermentative growth of *Saccharomyces cerevisiae*. However, we could not find any study where acetate served as electron sink to enable conversion of glycerol (as the only carbon source) to ethanol. The opportunity to ferment glycerol with *E. coli* in minimal medium with only small amounts of acetate has considerable potential for biotechnological applications. Complex medium supplements, which could become a cost factor in large-scale applications, are not required anymore allowing for a more economic conversion of glycerol to ethanol. A particular advantage of using acetate as co-substrate, also in comparison to electron acceptors for anaerobic respiration, is that its use as redox sink leads to the same end product ethanol further enhancing process efficiency. In fact, if formate is decomposed to CO_2 and H_2 and if synthesis of the small amount of succinate is completely repressed by knockout of the fumarate reductase genes, ethanol would remain as the only fermentation product in the medium. Acetate is an inexpensive substrate and although most of the acetate used in industry today is currently synthesized from petroleum-derived feedstock, sustainable production of acetate comes into reach. "Green acetate" could, for example, be produced as a side-stream from lignocellulosic biomass in biorefineries or from C1 gases by anaerobic gas fermentation using acetogenic microorganisms (Kiefer et al., 2021).

Anaerobic conversion of glycerol with co-substrate acetate could also become a viable option for production of other chemicals, including also more oxidized products. For example, this strategy could allow knockout of the pyruvate-formate lyase to avoid synthesis of formate and to redirect metabolic fluxes towards other products than ethanol. Here, acetate would not only function as redox sink (by which, depending on the reduction degree of the target product, some amounts of ethanol would still be produced), but also as source for acetyl-CoA required for biomass synthesis. This could be a particularly interesting strategy for products derived from pyruvate. For other target products (e.g., succinate), this might require to implement an alternative route for anaerobic glycerol utilization: since DHAK uses PEP as phosphate donor, pyruvate is produced. Using the PEP synthetase, pyruvate could be cycled back to PEP. However, this is energetically rather expensive. Here it would be more efficient to use heterologous enzymes (e.g. DHAK from *Citrobacter freudii* or *Schizosaccharomyces pombe*) that utilize ATP for phosphorylation of DHA instead of PEP (Daniel et al., 1995; Itoh et al., 1999). Finally, using acetate as co-substrate, functioning as redox sink or/and as source for the acetyl-CoA pool (if synthesis of acetyl-CoA from pyruvate is intentionally blocked), could be a promising strategy also for many other combinations of substrates (including glucose) and target products. One prominent example in this direction is the synthesis of isobutyl acetate from glucose and acetate (Tashiro et al., 2015).

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2022.05.006.

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