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Research Article

Metabolic response of *Pseudomonas putida* to increased NADH regeneration rates

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Pseudomonas putida efficiently utilizes many different carbon sources without the formation of byproducts even under conditions of stress. This implies a high degree of flexibility to cope with conditions that require a significantly altered distribution of carbon to either biomass or energy in the form of NADH. In the literature, co-feeding of the reduced C1 compound formate to *Escherichia coli* heterologously expressing the NAD⁺-dependent formate dehydrogenase of the yeast *Candida boidinii* was demonstrated to boost various NADH-demanding applications. *Pseudomonas putida* as emerging biotechnological workhorse is inherently equipped with an NAD⁺-dependent formate dehydrogenase encouraging us to investigate the use of formate and its effect on *P. putida*'s metabolism. Hence, this study provides a detailed insight into the co-utilization of formate and glucose by *P. putida*. Our results show that the addition of formate leads to a high increase in the NADH regeneration rate resulting in a very high biomass yield on glucose. Metabolic flux analysis revealed a significant flux rerouting from catabolism to anabolism. These metabolic insights argue further for *P. putida* as a host for redox cofactor demanding bioprocesses.

Keywords: Formate dehydrogenase / Glucose / NADH / *Pseudomonas putida* / Redox metabolism

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1 Introduction

The term “redox biocatalysis” can be defined as the biocatalytic production of chemicals, requiring the supply of reducing equivalents [1]. In enzymatic biocatalysis, these reducing equivalents are redox cofactors such as NADH and NADPH, which have to be added externally when isolated enzymes are applied. Since the requirement of stoichiometric amounts of NADH and NADPH can significantly influence the overall costs of such a process, redox biocatalysis with isolated enzymes is only profitable with an efficient artificial cofactor regeneration system [2, 3]. The use of whole cells as biocatalysts can be advantageous since the cofactors are synthesized and regenerated by the cellular metabolism. However, the pool of redox cofactors, mainly consisting of NADH and NADPH, is tightly regulated between the reduced and oxidized form and even between the pools of both

molecules in many organisms through transhydrogenases [1, 4]. If challenged with a redox-cofactor dependent biotransformation, the cell has to adjust the metabolism to maintain homeostasis. Indeed, high NADH regeneration rates do not automatically translate into corresponding biocatalytic activity [5]. This is especially relevant under biological stress, where an increased redox cofactor regeneration rate may additionally stabilize the viability of the cell being confronted with a redox biocatalytic reaction.

Since NADH is regenerated during catabolism of an energy source such as glucose, one could argue that by increasing the flux through the central carbon metabolism the NADH regeneration rate would be increased. However, recent studies have shown that increasing the uptake rate of a sugar is not trivial [6–8]. A biological alternative could be the use of higher reduced substrates than glucose such as sorbitol [9]. This strategy may require further genetic engineering if the bacterial host chosen for the redox biocatalytic reaction is not able to catabolize this alternative energy source. Moreover, sorbitol is more expensive

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Abbreviations: DCPIP, dichlorophenolindophenol; FDH, formate dehydrogenase; PMS, phenazine methosulfate

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(1500 €/t according to the Independent Chemical Information Service, ICIS 2010–2013) than glucose (< 400 €/t), imposing additional economic restraints, which consequently limits its applicability to high value products.

An applied common strategy to increase the NADH regeneration rate is a co-feed of an additional substrate, which delivers the electrons to regenerate NADH. Different approaches are reported involving the application of a heterologously expressed glucose- or formate dehydrogenase [10–15] and recently the application of an H₂-driven NADH regeneration system [16]. However, the use of heterologous genes may increase the metabolic burden, especially when these genes are expressed from plasmids requiring antibiotic selection. Although this could be circumvented by genomic integration, a host inherently equipped with an active NAD⁺-dependent FDH might be more suitable for an NADH demanding redox biocatalytic reaction when co-fed with formate.

Pseudomonas putida is a potential candidate for this kind of supported redox biocatalysis as it is capable of using C1 compounds as electron donors [17]. Moreover, it is naturally capable of regenerating NADH at a high rate [4] and it is endowed with active formate dehydrogenases [18] out of which at least one is expected to be NAD⁺-dependent (PP2183-2186) [19].

In this study, we investigated the use of formate by *P. putida* KT2440 in co-feed experiments with glucose, in which formate is oxidized to CO₂ mainly by an NAD⁺-dependent FDH. This set up was chosen, since glucose often serves as main carbon and energy source in industrial biocatalysis. With formate as an additional energy source *P. putida* KT2440 was able to drastically increase the NADH regeneration rate leading to a significant flux rerouting from catabolism to anabolism resulting in an increased biomass yield on glucose. Since the increased NADH regeneration rate highly exceeded cellular needs in the absence of an NADH consuming redox biocatalytic reaction, we argue that this surplus of NADH formation might be amenable for redox biocatalysis.

2 Materials and methods

2.1 Bacterial strains and cultivation conditions

The strain used in this study was *P. putida* KT2440 [20]. For solid media lysogeny broth (LB) [21] containing 5 g/l NaCl with 1.5% (w/v) agar-agar was used. In shake flask experiments, a minimal medium [22] with 20 mM glucose was used with a flask volume to liquid ratio of 1:10. The medium was adapted to achieve a higher buffer capacity through a fourfold increase of the phosphate buffer (final concentrations: 15.52 g/L K₂HPO₄ and 6.52 g/L NaH₂PO₄, pH = 7.0). Precultures were incubated in 100 mL Erlenmeyer flasks and main cultures in 500 mL Erlenmeyer flasks. The medium was supplemented with sodium formate using a 2 M stock solution (pH 7.0). Liquid cultures were incubated at 30°C, 200 rpm shaking speed with an amplitude of 50 mm in a Minitron shaker (INFORS, Bottmingen, Swiss). Bioreactor experiments were carried out in the Bioflow 115 systems from Eppendorf (Hamburg, Germany) with a working volume of 0.4 L. The stirring speed was set to 700 rpm, with 0.5 L/min head space aeration with air. The temperature was set to 30°C. The

pH was regulated to 7.0 by the addition of 4 M H₂SO₄ and 4 M NaOH. In the fermenter experiments, the standard buffer concentration was 3.88 g/L K₂HPO₄ and 1.63 g/L NaH₂PO₄ and formic acid was added instead of sodium formate. Continuous cultivations were performed at a dilution rate of D = 0.2 1/h. Physiological data were determined during steady state after at least five volume changes.

2.2 Analytical methods

Bacterial growth was monitored as optical density at a wavelength of 600 nm (OD₆₀₀) with an Ultrospec 10 Cell Density Meter (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The conversion factor of OD₆₀₀ to cell dry weight (CDW) in g/L was determined with continuously growing cells at a dilution rate of D = 0.2 1/h. Six 10 mL samples were collected in 15 mL dried pre-weighed falcon tubes during steady state. The samples were centrifuged at 6500 g and the supernatant was discarded. Subsequently, the samples were dried over night at 105°C and the CDW was determined from the difference between pre weighed tubes and tubes harboring the dried sample, resulting in an overall correlation factor of 0.505 ± 0.011 g_{Biomass}/OD_{unit}. Samples taken from liquid cultivation were centrifuged for 3 min at 17 000 g for the determination of extracellular metabolites. The supernatant was analyzed using an Ultimate 3000 HPLC system from Thermo Fisher Scientific (Waltham, MA, USA). Analytes were eluted at 60°C using a 300 × 8 mm organic acid resin column together with a 40 × 8 mm organic acid resin precolumn (both CS Chromatographie, Langerwehe, Germany) with 5 mM H₂SO₄ as mobile phase at a flow rate of 0.7 mL/min and detected with an UV detector, which was part of the HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 210 nm and a Shodex-101 RI detector at 35°C (Showa Denko, Tokyo, Japan).

[¹³C] labeling of proteinogenic amino acids was determined using a Trace GC Ultra Gas chromatography and mass spectrometry (GC-MS). Analytes were separated on a TG5 SilMs 30 m × 0.25 mm × 0.25 μm column with helium as carrier gas. Both GC-MS and column were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Samples taken during steady state of the chemostat and during exponential growth in shake flask and bioreactor batch fermentation experiments were processed according to Schmitz et al. [23].

Oxygen and carbon dioxide concentrations in the off-gas of bioreactor experiments were monitored using BlueInOne Ferm (BlueSens, Herten, Germany) off-gas sensors.

2.3 Calculation of extracellular fluxes

Growth and carbon uptake rate (CUR) of all experiments were calculated according to Heyland et al. [24] using SigmaPlot (V 12.5.0.38) in which “carbon” refers to the sum of residual glucose, gluconate and 2-ketogluconate at the specific time points as input for the calculations. Shake flask experiments presented in Section 3.1 were set up in duplicates, while the results presented in Sections 3.4 and 3.5 are dependent on triplicates. For shake flask experiments the formate uptake rate presented in Fig. 1B

Figure 1. Co-feed of formate and glucose in shake flask experiments; data represent the mean of two independent growth experiments; error bars indicate the SEM. (A) Concentration profile of C6 components (as the sum of glucose, gluconate, and 2-ketogluconate, open squares), formate (circles), and biomass (CDW, triangles) for the experiment with 60 mM initial formate concentration, error bars were so small that they are covered by the size of the symbols. (B) Uptake rates of C6 components (CUR, black bars) and formate (FUR, grey bars) of cultures with 20 mM glucose supplemented with different initial formate concentrations.

was calculated with Microsoft Excel 2010. For this, the formic acid concentration was plotted against the CDW in the same time frame as for the calculation of the growth and CUR with SigmaPlot. The data were fitted with a linear regression and the slope was multiplied with the growth rate determined with SigmaPlot. The presented error bars in Fig. 1B represent the standard error of the mean (SEM) of the calculated slopes while the error bars of the CURs depict the SEM of the values calculated with SigmaPlot.

The respiration quotient (RQ) was calculated as CO₂ evolution rate (CER) divided by the O₂ uptake rate (OUR). Given errors were calculated according to Gaussian error propagation. The carbon balance depicted in Table 2 was calculated as CUR*6 (+FUR) = Y_{X/S}*CUR*6+CER. The electron balance was calculated as CO₂ from glucose releases 4 electrons, CO₂ from formate

Table 1. [¹³C] fraction of proteinogenic amino acids derived from cultures with 20 mM 100 % U-[¹³C]-labeled glucose co-fed with 30 mM unlabeled formate

Amino acid ^{a)}	Precursor ^{b)}	[¹³ C] Fraction ^{c)} (M-15) ⁺ /(M-57) ⁺
<u>Aspartate/Asparagine</u>	OAA	90 ± 0.0
<u>Isoleucine</u>	OAA + PYR	92 ± 0.0
<u>Lysine</u>	OAA + PYR	93 ± 0.5
<u>Methionine</u>	OAA	92 ± 0.5
<u>Threonine</u>	OAA	90 ± 0.3
<u>Glutamate/Glutamine</u>	OGA	92 ± 0.3
Serine	3PG	100 ± 0.3
Alanine	PYR	100 ± 0.5
Glycine	3PG	99 ± 0.3
Leucine	AcCoA + PYR	98 ± 0.0
Tyrosine	E4P + PEP	95 ± 3.4
Valine	PYR	100 ± 0.5
Phenylalanine	E4P + PEP	100 ± 0.5

^{a)}Underlined amino acids are derived from TCA cycle intermediates.

^{b)}OAA: Oxaloacetate, PYR: Pyruvate, OGA: 2-Oxoglutarate, 3-PG: 3-Phosphoglycerate, AcCoA: Acetyl-CoA, E4P: Erythrose-4-phosphate, PEP: Phosphoenolpyruvate

^{c)}Values were calculated for both presented fragments and afterwards averaged as fragments (M-15)⁺ and (M-57)⁺ represent the whole amino acid carbon backbone. Histidine and Proline were excluded as the (M-15)⁺ and (M-57)⁺ fragments were of low quality in this experiment. The pool of Asn and Asp is not distinguishable. The same is true for Gln and Glu.

releases 2 electrons while every oxygen molecule reduced to water accepts 4 electrons.

2.4 Enzyme assays

For the *in vitro* quantification of the formate dehydrogenase activities, a liquid culture (20 mM glucose, 60 mM formate) was cultivated to mid-exponential phase (OD₆₀₀ = 1.0–1.5) and stored on ice after the intended OD₆₀₀ was reached. Subsequently the cooled culture was centrifuged for 10 min at 6500 g and 4°C. The pellet was lysed using 2 mL of the BugBuster Protein Extraction Reagent from Merck Millipore (Darmstadt, Germany) in 15 min incubation time. Soluble and insoluble fractions were separated at room temperature using a table top centrifuge at 17 000 g for 2 min. The supernatant was used for the determination of the cytosolic, soluble NAD⁺-dependent FDH activity. The obtained pellet was considered to harbor the insoluble, membrane-bound protein fraction, which was mixed with 0.7 mL of BugBuster Protein Extraction Reagent. The total protein concentration in the soluble and insoluble fraction was determined according to Bradford [25]. Both fractions were kept on ice until further usage.

For the NAD⁺-dependent, cytosolic, soluble FDH assays [26], the soluble protein fraction and minimal medium without a carbon source were first added to a cuvette in different ratios in a total of 800 μL and heated to 30°C. After 5 min, 100 μL of 20 mM NAD⁺ were added for the quantification of the background activity. Subsequently, 100 μL of 500 mM sodium formate

(pH = 7.0) were added. Absorption was monitored at a wavelength of 340 nm. The activity is presented in units (U) referring to 1 μ mol of NADH formed or formate consumed per minute.

To investigate the NAD⁺-independent membrane bound FDH [27], the insoluble protein fraction suspension was diluted 1:10 with minimal medium without a carbon source. This solution was mixed with minimal medium without a carbon source in different ratios in a total of 760 μ L in a cuvette heated to 30°C. After 5 min, 100 μ L of 20 mM phenazine methosulfate (PMS) were added. Another 5 min later, 40 μ L of 50 mM dichlorophenolindophenol (DCPIP) were added to measure the background activity. NAD⁺-independent FDH activity was measured after 100 μ L of 500 mM sodium formate were added. Activity was monitored at a wavelength of 600 nm. Both assays were performed in a Cary 60 UV-Vis spectrophotometer from Agilent Technologies (Santa Clara, CA, USA).

2.5 Labeling experiments

In every labeling experiment, a ratio of 20:80 of U-[¹³C] labeled glucose (Euriso-top, Saint-Aubin Cedex, France) and naturally labeled glucose at a total concentration of 20 mM was used. The only exception was the labeling experiment in shake flasks with 30 mM of sodium formate to test incorporation of formate into the biomass, in which 100% of U-[¹³C] glucose and naturally labeled formate were used. The preculture was cultivated overnight on naturally labeled glucose. The main culture was inoculated with 3% of the estimated biomass concentration of the preculture at the harvesting point. During mid-exponential growth and after five generations of growth on the labeled substrate, samples of 3 mg CDW were taken for mass isotopomer distribution measurements of proteinogenic amino acids.

All cultivations for flux analysis were performed in bioreactors as described above using 20 mM glucose and 120 mM formic acid (where indicated). For the continuous cultivation, the shake flask preculture and the batch cultivation in the bioreactor (before switching to continuous cultivation conditions) were performed using naturally labeled glucose. When a metabolic steady state was reached (after five volume changes), the carbon source was changed from naturally labeled glucose to labeled glucose in the aforementioned ratio. Samples for the mass distribution measurements of proteinogenic amino acids were taken after five volume changes when the isotopic steady state on labeled glucose was reached. The formate co-feed was started by adding formic acid to the medium reservoir to a final concentration of 120 mM. After another five volume changes the steady state on glucose and formate was reached.

2.6 Flux analysis

Flux calculations were performed with OpenFLUX using standard settings and applying the gradient-based search algorithm for sensitivity analysis [28]. The stoichiometric model (provided in the supplemental material) was based on a previously published model of the central carbon metabolism of *P. putida* [4]. A partially cyclically operating Entner–Doudoroff

pathway has recently been reported for *P. putida* [29]. However, as the applied [¹³C] labeling strategy and mass distribution measurements of proteinogenic amino acids did not allow to resolve such a cyclic operation, we neglected this mode of operation and only allowed positive net flux values for the phosphoglucose isomerase catalyzed conversion of glucose-6-phosphate to fructose-6-phosphate. The initial model contained a phosphoenolpyruvate carboxykinase reaction, which decarboxylates oxaloacetate to phosphoenolpyruvate. The gene encoding this enzyme, however, has been annotated as a pseudogene due to an authentic frameshift [19, 29, 30]. Indeed, in all initial simulations the optimal phosphoenolpyruvate carboxykinase flux was zero, supporting this annotation. However, the fluxes were not resolved well and the simulations could not exclude the formation of a metabolic cycle (phosphoenolpyruvate > pyruvate > oxaloacetate > phosphoenolpyruvate). As the net reaction of this cycle is the hydrolysis of one ATP and its suppression does not alter the flux distribution, we excluded the phosphoenolpyruvate carboxykinase reaction in the final simulations.

To compute the intracellular fluxes, the model was constrained with the experimentally determined CUR (i.e., the sum of glucose, gluconate, and 2-ketogluconate), formate uptake rate, growth rate, and mass isotopomer distributions of proteinogenic amino acids (average data of independent bioreactor fermentations were used). Mass distribution vectors were corrected for unlabeled biomass introduced with the inoculum and natural abundance of heavy isotopes using iMS2Flux [31]. Simulated mass distribution vectors and experimental data are provided in the Supporting Information.

3 Results

The need of balancing of redox cofactors has an impact on the cellular fluxes and hence the physiology and performance of biocatalysts. One example is the Crabtree-positive yeast *Saccharomyces cerevisiae*, which re-oxidizes NADH at high glucose uptake rates by reducing acetaldehyde to ethanol resulting in carbon and energy loss. By contrast, *P. putida* KT2440 is known to direct carbon sources only to biomass, CO₂ and energy without the formation of byproducts [32]. Here, we investigated the effect of excess reducing equivalents on the central carbon metabolism of *P. putida* KT2440 through the addition of formate.

3.1 Formate is co-metabolized with glucose by *P. putida* KT2440

A cell has to simultaneously co-metabolize an additional energy source with the main carbon source in order to boost the rate of redox cofactor regeneration. Therefore, the ability of *P. putida* KT2440 to co-metabolize glucose and formate was investigated in shake-flask cultivations (Fig. 1A), which showed that indeed, glucose and formate were consumed simultaneously by *P. putida* KT2440 during growth.

Formate concentrations investigated for shake flask experiments were 30, 60, 80, and 100 mM. With 30 mM formate the

improvement in growth rate was comparable to 60 mM formate, however the formate uptake rate doubled in the experiments with 60 mM formate. With 80 and 100 mM formate, growth rates decreased due to a pH increase during the batch cultivation, since formate was added as sodium salt. Thus, 60 mM of formate and 20 mM of glucose were used as a standard condition in further shake flask experiments.

3.2 *Pseudomonas putida* KT2440's inherent formate dehydrogenases

For NADH-limited redox biocatalysis, a transfer of the electrons from formate to NAD^+ would be preferable since an increased NADH regeneration rate may directly lead to an increased redox biocatalytic performance [33]. *P. putida* KT2440 is equipped with at least two FDHs [18]. The genes *fmdE-fmdH* (PP'2183-2186) likely encode a soluble NAD^+ -dependent formate dehydrogenase since the FmdG protein contains a predicted NAD^+ binding domain (determined by SMART domain search [34]). However, the cofactor-dependency, if any, of the second FDH *fmdA-fmdD* (PP'0489-0492) in *P. putida* KT2440 is not clarified. These genes encode elements typical for membrane-bound FDHs and include binding sites for iron-sulfur clusters and molybdenum-cofactors, however, an NADH binding site is absent. The encoded proteins have high sequence identity (78–83%) to the nitrate-inducible formate dehydrogenase of *P. aeruginosa* [35, 36]. This enzyme complex transfers its electrons directly to nitrate reductase under anaerobic nitrate respiration. Since *P. putida* KT2440 is not able to respire nitrate, we hypothesize an alternative electron acceptor in this species. Possibly, electrons are transferred to the membrane-soluble quinone pool, as it is the case with the *E. coli* enzyme [37]. Indeed, *in vivo* mediation by a quinone can also not be excluded for the *P. aeruginosa* enzyme [38]. However, the *fmdA* gene contains a rare opal (TGA) codon encoding a selenocysteine [19, 39]. The latter is highly conserved [19], but likely often mis-annotated as a stop codon in databases. This selenocysteine is crucial for the activity of the enzyme, and relies on the addition of selenium to the medium. By omission of this element, possible unwanted side-activity of this NAD^+ -independent enzyme is likely suppressed, although some residual activity may be present [39].

Crude cell extracts of glucose and formate grown cells in shake flasks were used to reveal the cofactor dependence of *P. putida* KT2440's FDHs.

3.3 Metabolic fate of formate in *P. putida*

The distribution of formate between the cytosolic and membrane bound FDHs of *P. putida* KT2440 was elucidated in *in vitro* enzyme assays. The cytosolic FDH was tested with NAD^+ as a cofactor. The membrane-bound FDH was very unlikely considered to be NAD^+ dependent. Due to this we assayed the membrane-bound FDH with PMS-DCPIP as a typical cofactor of membrane-bound enzymes [1]. As a control the insoluble, membrane-bound fraction was tested with the addition of glucose in the assay. The insoluble fraction was considered to

harbor the cytoplasmic membrane-bound glucose dehydrogenase, a PQQ-dependent enzyme [40]. With PMS/DCPIP as a suitable redox pair for the detection of PQQ-dependent enzymes [41], an activity of approx. $250 \text{ U/g}_{\text{Protein}}$ of the PQQ-dependent glucose dehydrogenase was measured, suggesting an active membrane fraction.

Most of the formate was converted by the cytosolic NAD^+ -dependent FDH. An activity of approx. $247 \pm 20 \text{ U/g}_{\text{Protein}}$ was determined, which is nearly seven fold higher than the PMS/DCPIP-dependent FDH activity of $37 \pm 7 \text{ U/g}_{\text{Protein}}$ of the insoluble, membrane-bound FDH. No activity was observed when NAD^+ was replaced with NADP^+ as a cofactor in the enzymatic assays of the cytosolic, soluble FDH. By intentionally omitting selenium from the medium, expression of *fmdA-fmdD* was suppressed to guarantee that formate was consumed by an NAD^+ -dependent FDH.

These results indicate that under the investigated conditions *P. putida* KT2440 metabolizes formate mostly through an NAD^+ -dependent FDH. However, certain Pseudomonads, such as species AM1, are known to incorporate C1 compounds into biomass [42]. Therefore, we investigated whether *P. putida* KT2440 is equipped with known pathways incorporating formate into biomass, which would lower the NADH yield from formate.

Three common pathways for C1 compound assimilation are the ribulose biphosphate (RuBP) pathway, the ribulose monophosphate (RuMP) pathway, and the serine pathway. The RuBP pathway is a CO_2 assimilation pathway, which is unique to autotrophic organisms such as “knallgas” bacteria of the *Pseudomonas* type like *Hydrogenomonas facilis* [43] and is thus not present in the heterotrophic organism *P. putida* KT2440. A C1 compound enters the RuMP pathway via formaldehyde [17], which is more strongly reduced than formate. Hence, *P. putida* KT2440 would have to reduce formate first before incorporating it as formaldehyde into biomass via fusion to ribulose 5-phosphate through a hexulose phosphate synthase, resulting in glucose-6-phosphate. Assimilation via the serine pathway, which is mainly present in methylotrophic microorganisms [44], would attach formate as C1 compound to the C2 carbon backbone of glycine, resulting in serine [42, 45].

In order to ascertain whether formate is incorporated into the biomass through any of these pathways, *P. putida* KT2440 was cultured in mineral medium with 100% ^{13}C -labeled glucose with the addition of unlabeled formate. The concentration of formate was reduced to 30 mM in these experiments to make sure that the culture was not affected by pH effects as observed at an increased formate concentration (see above). If the unlabeled formate is incorporated into biomass, the labeling of the proteinogenic amino acids is diluted. The results of the labeling experiments are summarized in Table 1 and indicate that only those amino acids originating from intermediates of the TCA cycle [46] (see underlined amino acids) show a dilution of the ^{13}C fraction. For all amino acids which are not derived from TCA cycle intermediates the labeling is equal or close to 100% indicating no incorporation of formate derived carbon atoms. This labeling pattern is consistent with the direct oxidation of formate to CO_2 . The unlabeled carbon observed, e.g., in oxaloacetate and 2-oxoglutarate,

Table 2. Physiology of pH-controlled batch and chemostat cultures grown in bioreactors with and without a formate co-feed^{a)}

	Batch	Batch + FA	Chemostat	Chemostat + FA
μ [1/h]* ^{b)}	0.61 ± 0.01	0.61 ± 0.02	0.2 ± 0.01	0.2 ± 0.01
$Y_{X/S}$ [g _{CDW} /g _{glucose}]*	0.56 ± 0.05	0.70 ± 0.00	0.55 ± 0.02	0.69 ± 0.02
CUR [mmol/g _{CDW} /h]	6.0 ± 0.5	4.9 ± 0.0	2.0 ± 0.1	1.6 ± 0.1
FUR [mmol/g _{CDW} /h]	–	64.1 ± 9.2	–	7.6 ± 0.9
CER [mmol/g _{CDW} /h]	11.8 ± 2.7	68.7 ± 9.3	4.1 ± 0.4	9.4 ± 1.2
OUR [mmol/g _{CDW} /h]	10.6 ± 2.9	35.4 ± 4.8	3.7 ± 0.4	5.2 ± 0.8
RQ [CER/OUR]	1.1 ± 0.4	1.9 ± 0.4	1.1 ± 0.2	1.8 ± 0.4
Recovered carbon [%]	89	96	89	93
Recovered electrons [%]	90	96	180	93

^{a)} Data represent the mean of at least two independent bioreactor experiments with SEM for measured values (asterisk) and half of error range for estimated values (see supplemental material); 20 mM of glucose was used and formate (FA) was added where indicated at a concentration of 120 mM; μ : growth rate, $Y_{X/S}$: biomass yield on glucose; CUR: uptake rate of C6 components (as the sum of glucose, gluconate and 2-ketogluconate); FUR: formate uptake rate; OUR: O₂ uptake rate; CER: CO₂ evolution rate.

^{b)} SEM of the growth rate for the chemostat experiments was assumed to be 0.01 as it is depending on the feeding pump rates which exhibit low deviations.

originates from unlabeled CO₂ (from air and oxidized formate) through anaplerotic reactions such as phosphoenolpyruvate or pyruvate carboxylase. The C4 amino acids aspartate, methionine, and threonine originate from oxaloacetate which labeling is close to 90% and not 75%. Labeled CO₂ from glucose originating from the oxidative pentose phosphate pathway and the TCA cycle might explain why the labeling of these amino acids is not 75%, which could be assumed from one molecule CO₂ being attached to the C3 carbon backbone of pyruvate (for a detailed explanation the reader is referred to the supplemental material). Hence, formate can be considered as an energy source, providing reduction equivalents, primarily NADH, by its oxidation to CO₂, which is emitted from the medium.

3.4 Addition of formate increases the biomass yield on glucose

To further investigate how a culture of *P. putida* KT2440 benefits from a co-feed of formate and glucose, pH-controlled bioreactor experiments were performed containing 120 mM of formate. With the control of the pH unwanted side effects of an increased pH like in shake flasks with formate concentrations above 60 mM were circumvented. A formate concentration of 120 mM was chosen for continuous bioreactor experiments at $D = 0.2$ 1/h with 20 mM glucose. This concentration was chosen from a pilot experiment in which formate addition was raised stepwise from one steady state to the other resulted in the highest biomass yield at this formate concentration (data not shown).

With formate as additional energy source, the biomass yield on glucose increased considerably by $25 \pm 11\%$ in batch experiments and by $24 \pm 6\%$ in continuous cultures, while the growth rate remained unaffected (Table 2). The CUR decreased by approximately $19 \pm 5\%$ in both cultivation conditions when formate was added.

An increased CER was expected in the cultures with a formate co-feed. In the case of the batch cultivation, the CER increased 5.8 ± 1.6 fold compared to the culture without formate, while in the C-limited chemostat culture the rate only

increased 2.3 ± 0.4 fold upon the addition of formate. The addition of formate also affected the OUR, which increased approximately 3.4 ± 1.0 fold in batch and 1.4 ± 0.3 fold in chemostat cultivations.

3.5 Flux analysis reveals a decreased flux of carbon through the TCA cycle upon formate addition

[¹³C]-based metabolic flux analysis allows the determination of intracellular reaction rates and was applied here to elucidate the impact of a formate co-feed on the activity of the central carbon metabolism. A small scale metabolic model of *P. putida* was used to compute the fluxes (Supporting Information [4]). The addition of formate resulted in a significant flux rerouting from catabolism to anabolism as indicated by the decreased flux through the TCA cycle (Fig. 2). The pentose phosphate pathway only played a minor role, independent of the investigated cultivation conditions supporting the observations of previous investigations [29, 32, 47]. The optimal values for the glyoxylate shunt were zero, but a minor activity of the glyoxylate shunt cannot be completely ruled out. Interestingly, the pool of oxaloacetate is almost completely replenished from pyruvate rather than from malate in all conditions investigated since malate was mostly converted to pyruvate by the malic enzyme.

The effect of formate oxidation on the NAD(P)H regeneration rate is illustrated in Fig. 3. The addition of 120 mM formate resulted in a total NAD(P)H regeneration rate increase from 28 ± 7 to approximately 80 ± 10 mmol/g_{CDW}/h during batch cultivation. During C-limited growth the total NAD(P)H regeneration rate increased from 9.5 ± 2.7 to 13.2 ± 2.0 mmol/g_{CDW}/h. Assuming that all formate is converted to CO₂ by the NAD⁺-dependent FDH, NADH from formate oxidation accounts for approximately $80 \pm 15\%$ of the total NAD(P)H regeneration rate during batch cultivation and for $58 \pm 11\%$ percent during C-limited growth. In both cases the regeneration rate of NADH from glucose oxidation decreased by approximately $58 \pm 15\%$ when *P. putida* KT2440 was supplied with formate (Table 3). NAD(P)H available for redox biocatalytic reactions increased by

Figure 2. Flux distribution of *P. putida* KT2440 with 20 mM glucose and 120 mM formate (FA, where indicated); the illustrated direction indicates the estimated net flux (forward minus reverse flux) the glucose uptake rate was determined like presented in the materials and methods section and was set to 100%, all other values are related to this value; values represent from top to bottom batch, batch with FA, chemostat, and chemostat with FA. Abbreviations: GLC: Glucose (as sum of glucose, gluconate and 2-ketogluconate), G6P: Glucose-6-phosphate, 6PG: 6-phosphogluconate, R5P: Ribose-5-phosphate, S7P: Seduheptulose-7-phosphate, T3P: Triose-3-phosphate, E4P: Erythrose-4-phosphate, F6P: Fructose-6-phosphate, FBP: Fructose bisphosphate, PEP: Phosphoenolpyruvate, PYR: Pyruvate, AcCoA: Acetyl-CoA, ICT: Isocitrate, OGA: 2-oxoglutarate, SUC: Succinate, MAL: Malate, OAA: Oxaloacetate, FA: Formate

3.9 ± 1.6 fold during batch cultivation and by 1.6 ± 0.7 fold during limited growth (Fig. 3).

4 Discussion

This study revealed that a co-feed of formate increases the regeneration rate of redox cofactors in *P. putida* KT2440, most likely leading mainly to the intended NADH. This increased redox cofactor regeneration rate resulted in a significant flux rerouting towards anabolism resulting in an increased biomass yield on glucose.

The strategy of increasing the NADH regeneration rate through the addition of formate was also reported for the indus-

trially well-established microbes *S. cerevisiae* [11] and *E. coli* [13]. However, *E. coli* is equipped with three FDHs, none of which is NAD⁺ dependent [48] and therefore needs to be genetically manipulated. The *in vitro* determined *P. putida* KT2440 FDH activity of approximately 136 U/g_{CDW} (assuming that 55% of the CDW is composed of proteins) is lower than the heterologous FDH activity in *E. coli* of approx. 250 U/g_{CDW} expressed from a multi-copy plasmid [13]. However, the use of *P. putida*'s native NAD⁺-dependent FDH has certain advantages, such as the omission of plasmid-encoded heterologous FDH constructs, which need to be maintained by antibiotic selection. Stable genomic integration into an FDH negative host of, e.g., the widely used FDH from *C. boidinii* might circumvent such drawbacks of plasmid encoded expression systems. A promising approach for such

Table 3. Calculation of the NAD(P)H yield on glucose derived from metabolic flux analysis

	Batch	Batch + FA	Chemostat	Chemostat + FA
CUR [mmol _{glucose} /g _{CDW} /h] ^{a)}	6.0 ± 0.5	4.9 ± 0.0	2.0 ± 0.1	1.6 ± 0.1
NAD(P)H [mmol _{NAD(P)H glucose} /g _{CDW} /h] ^{b)}	27.8 ± 7.2	15.9 ± 1.2	9.5 ± 2.7	5.6 ± 1.0
NAD(P)H yield [mmol _{NAD(P)H} /mmol _{glucose}]	4.6 ± 1.2	3.3 ± 0.2	4.7 ± 1.3	3.5 ± 0.6

^{a)}CUR: total glucose-derived C6 CUR as indicated in Table 2.

^{b)}Sum of all NAD(P)H regenerating reactions only from glucose (see Table S1 in the Supporting Information).

Figure 3. NAD(P)H regeneration rate determined by flux analysis for the different cultivation conditions; it was assumed that the formate uptake rate presented in Table 2 corresponds equally to an NADH regeneration rate; the bar height represents the total NAD(P)H regeneration rate, black bars represent NAD(P)H used for the formation of biomass, while grey bars represent the NAD(P)H available for miscellaneous processes, white bars indicate the proportion of total NADH originating from formate.

a genomic integration is presented by Zobel et al. [49] with the use of the mini Tn7 technique, which is applicable to a broad host range. *S. cerevisiae* possesses two isoenzymes of the NAD⁺-dependent FDH [50] and the reported activities in carbon limited cultures are comparable to the FDH activity determined here in *P. putida* KT2440.

The enzyme activity determined in the *in vitro* assay (247 U/g_{protein}) is comparable to that derived from the estimates of the continuous cultivation (127 U/g_{CDW} $\triangleq$ 231 U/g_{protein}). However, during batch cultivation the FDH activity was increased to approximately 1000 U/g_{CDW} (1800 U/g_{protein}). Assuming that the specific activity of the FDH enzyme of *P. putida* KT2440 is comparable to that of *Pseudomonas* sp. 101 [51] of 2430 U/g_{protein}, as an indirect and rough estimate, this would mean that *P. putida* increases the amount of the FDH from approximately 10 to 74% of the total protein content. This amount seems rather unrealistic suggesting that the specific activity of *P. putida* KT2440's FDH is higher and that other physiological effects are likely involved [18]. The lower *in vivo* activity of the chemostat conditions compared to the batch cultivation condition in bioreactors can likely be explained by substrate excess.

However, it might be that not all of the formate is channeled through the NAD⁺-dependent FDH in *P. putida* KT2440.

Both annotated FDHs oxidize formate to CO₂ with either the regeneration of NADH (PP'2183-2186) or possibly a quinol, which can feed electrons into the electron transport chain (PP'0489-0492). As indicated above, most of the formate is likely converted by the NAD⁺-dependent FDH in the exponential phase of growth, as demonstrated by *in vitro* enzyme assays. However, since *P. putida* KT2440 is endowed with more than the two annotated FDHs [18] a transfer of the electrons from formate to NAD⁺ with growing cells can only be ensured, if the genes encoding the potential NAD⁺-independent FDH(s) and the unspecific dehydrogenases are identified and disrupted.

Considering the metabolic network of *P. putida* KT2440, ten molecules of NAD(P)H can theoretically be regenerated per molecule of glucose being completely oxidized with non-growing but metabolically active cells. This study demonstrates that this maximal theoretical yield can only be achieved by approximately 50% with growing cells independent of carbon-excess or -limited conditions (Table 3). As a result of the formate co-feeding the cells even decreased the NADH yield per consumed molecule of glucose to one third of the maximal yield demonstrating that the cell changed the distribution of carbon to the benefit of biomass synthesis.

The central aim of this study was to investigate the fundamental effects of formate as an additional energy source on the metabolism of *P. putida* KT2440. While co-catabolism of formate increased the biomass yield on glucose indicating energy-limited growth during glucose as sole carbon and energy source, the growth rate remained unaffected under excess energy conditions demonstrating that the growth rate is rather limited by the capacity of the biomass synthesis machinery than by the energy available. 0.7 g_{CDW}/g_s was the maximum achievable biomass yield on glucose with formate as additional energy source under both cultivation conditions. Considering an average biomass composition of CH_{1.8}O_{0.5}N_{0.2} with a molecular weight of $M = 24.6$ g/Cmole_{CDW} and a molecular weight of $M = 30$ g/Cmole_{glucose} a maximum theoretical biomass yield on glucose of 0.82 Cmole_{CDW}/Cmole_{glucose} can be achieved [52]. Since 0.7 g_{CDW}/g_{glucose} translates into 0.85 Cmole_{CDW}/Cmole_{glucose} *P. putida* KT2440 was able to achieve this threshold upon formate addition. This observation is supported by a nearly closed carbon balance especially regarding the experiments with the addition of formate (Table 2). *P. putida* decreased the glucose uptake rate when formate was co-fed to the culture demonstrating that the carbon uptake of this organism is driven by demand as mentioned previously [32].

Conversely, the uptake of formate was more subject to a passive diffusion mechanism rather than driven by demand since the formate uptake rate and thus the NADH regeneration rate highly exceeded cellular demands demonstrated by a maximum achievable biomass yield accompanied by a severely increased OUR. The surplus NADH regeneration rate from the FDH activity must be balanced in order to maintain cellular homeostasis. Since we did not detect any reduced extracellular metabolites the electron transport chain remains as the only fate of NADH with oxygen as the final electron acceptor. Indeed, with glucose and formate releasing 4 and 2 moles of electrons per mole of CO₂ these electrons are nearly completely recovered with the estimated oxygen uptake rate demanding 4 moles of electrons for each mole of oxygen being reduced to water with the exception of the chemostat (Table 2). One reason for the unclosed electron balance especially in the chemostat without formate is likely an overestimated oxygen uptake rate. The surplus energy is likely 'burned' with a less efficient branch of the electron transport chain [53,54], which was included in our model containing alternative reactions differing in the number of protons transferred over the membrane during oxidation of ubiquinol (although these flux estimates could only be poorly resolved). However, even the less efficient electron transport chain resulted in an increased ATP regeneration, which was likely balanced in futile reactions.

These observations have clear implications for possible applications of a formate co-feed in redox biocatalysis. Our results indicate that unlimited growth conditions might be favorable due to the substantially increased NADH regeneration rate available for miscellaneous processes compared to a carbon-limited condition. This increased NADH availability would be supported if the cells operate an efficient electron transport chain during redox biocatalysis. If and to which extent the surplus of NADH can be consumed for redox-cofactor driven product synthesis depends on the cellular demand for redox cofactors and energy, which often is increased during biocatalysis due to protein overproduction, product or substrate toxicity issues or uncoupling reactions [32].

Altogether, a co-feed of formate in addition to a carbon source seems to be a promising strategy to supply *P. putida* KT2440 with an additional energy source, e.g., to support a redox biocatalytic reaction. Indeed, the price of formate is comparable to that of glucose (see Section 1) but since NADH regeneration rates comparable to those presented in Fig. 3 with an increased glucose uptake rate are only obtainable under conditions of stress [32] formate is also an economically interesting co-substrate, especially if formate is delivered from biotechnological production processes [55]. The application of cathodic bioelectrochemical systems, i.e., is likely of high potential. Unfortunately, such a biological mechanism is not well understood [56]. Therefore, formate as an electron donor in a well-defined system, as shown here, allows already today rates that are of industrial interest. Therefore, *P. putida* KT2440, as emerging biotechnological workhorse, may constitute an alternative to long-established cell factories such as *S. cerevisiae* and *E. coli* in terms of NADH supplied redox biocatalytic reactions, such as monooxygenase-based systems.

Practical application

Numerous cellular and biotechnological processes are dependent on the efficient supply of NADH, especially during redox biocatalysis. This constitutes a severe drain on NADH potentially leading to a tradeoff between cellular viability and biocatalysis. Therefore, this study presents an approach of supplying the biocatalyst with formate as an additional electron donor for increasing the rate of NADH regeneration. A prerequisite for a co-feed of an additional energy source is the profound knowledge of its metabolic fate. Non-genetically engineered *P. putida* co-oxidized formate at high rate, thereby substantially increasing the NADH regeneration rate. This native ability opens the door for *P. putida* being applied as a hyper active redox biocatalyst.

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