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Perspective

Applying Non-canonical Redox Cofactors in Fermentation Processes

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SUMMARY

Fermentation processes are used to sustainably produce chemicals and as such contribute to the transition to a circular economy. The maximum theoretical yield of a conversion can only be approached if all electrons present in the substrate end up in the product. Control over the electrons is therefore crucial. However, electron transfer via redox cofactors results in a diffuse distribution of electrons over metabolism. To overcome this challenge, we propose to apply non-canonical redox cofactors (NRCs) in metabolic networks: cofactors that channel electrons exclusively from substrate to product, forming orthogonal circuits for electron transfer.

MICROBIAL CELL FACTORIES AS ENABLING TECHNOLOGIES

Microbial cell factories are one of the enabling technologies to produce chemicals efficiently and sustainably. The catalytic power of the whole metabolic network, consisting of thousands of enzymes, can be used to realize the desired conversion. Genome-editing techniques make it more and more possible to engineer the metabolism of microorganisms to optimize product formation (Brouns et al., 2008; Mohanraju et al., 2016; Wu et al., 2018; Mougiakos et al., 2018). Metabolism is, however, a very complex network of reactions, with a high degree of entanglement and tight regulation. Product formation is therefore prone to be affected by other metabolic processes and vice versa. This complicates the thorough understanding of metabolic processes and makes it difficult to realize an efficient design. A trend to reduce the entanglement of product formation with other metabolic processes is to use synthetic biology to establish orthogonal pathways: growth-independent pathways optimized for the production of a target molecule, characterized by the minimization of interactions between the chemical-producing pathways and the biomass-producing pathways (Martin et al., 2003; Hanai et al., 2007; Chinen et al., 2007; Prather and Martin, 2008).

MAXIMIZING YIELD

Microbial cell factories are designed to achieve high yield (g product/g substrate). This is especially important for the production of bulk chemicals, because they have to compete with chemicals derived from low-cost fossil-based resources. The yield shows how efficiently the substrate is converted into the product. The design target for yield is the maximal theoretical yield, which can be calculated using the degree of reduction of both substrate and product. This degree reflects the ability of molecules to donate or accept electrons in chemical reactions and therefore depends on their elemental composition. In organic molecules, C atoms can donate four electrons and H atoms one electron, whereas N atoms can accept three electrons and O atoms two electrons (Weusthuis et al., 2011). The degree of reduction of glucose ($C_6H_{12}O_6$) is 24 ($6 \times 4 + 12 \times 1 + 6 \times -2$), that of lactic acid ($C_3H_6O_3$) = 12 ($3 \times 4 + 6 \times 1 + 3 \times -2$), so the maximum theoretical yield of the conversion of glucose into lactic acid is 24/12 = 2 mol/mol.

The most straightforward approach for cell factory engineering to optimize the yield is therefore to design a metabolic network that enforces complete transfer of electrons present in the substrate to the final product. This requires strict control of electron transfer and redox reactions. However, redox couples that are spread over the metabolic network transfer electrons to each other, causing a diffuse distribution of electrons over metabolism. A good example is the utilization of glucose by *Escherichia coli*. The latter is able to transfer electrons via nicotinamide adenine dinucleotide NAD^+/NADH to terminal electron acceptors such as oxygen and nitrate—in a process called respiration—or to metabolic intermediates, forming products like ethanol, acetate, formate, succinate, and lactate—in a process called fermentation. *E. coli* also

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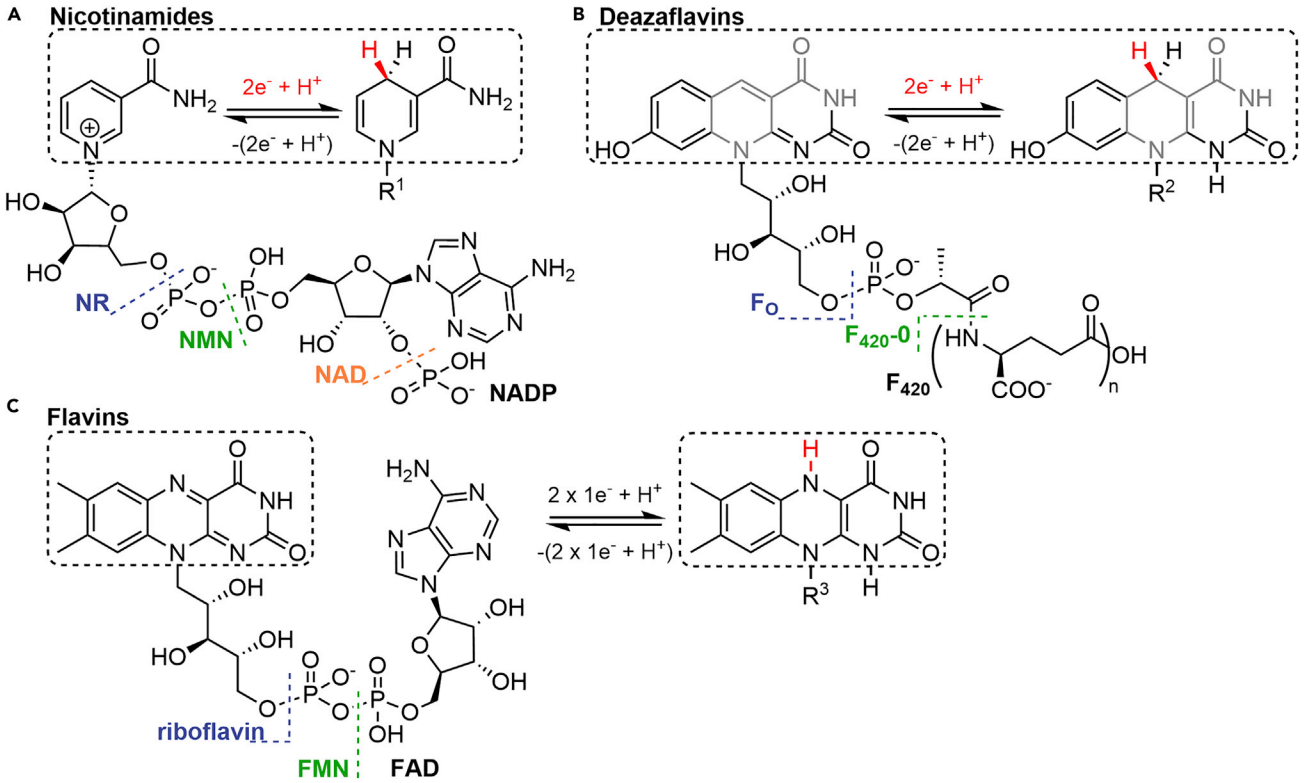

Figure 1. Chemical Structures of Selected Redox Cofactors

(A) Nicotinamide cofactors (reduced form) transfer two electrons in a one-step hydride transfer and become oxidized. NR, nicotinamide ribose; NMN, nicotinamide mononucleotide; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; AD, adenosine diphosphoribose.

(B) Deazaflavin cofactors are nicotinamide cofactors (light gray highlights the nicotinamide structure, one-step hydride transfer) structurally disguised as flavins.

(C) Flavin cofactors can transfer two electrons in a two-step one-electron transfer mechanism. FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

transfers electrons via $NADP^+$/NADPH to reduce intermediates used in biomass production. If *E. coli* would be used as a microbial cell factory for the production of a certain chemical, all electrons from the substrate should end up in the product to approach the maximal theoretical yield and not be diffusely diverted to other reactions. A strict control over the fate of electrons is therefore evidently important.

REDOX COFACTORS

Electron transfer is mediated by specialized redox couples: redox cofactors. Three distinct families are operational in metabolism.

Nicotinamide Cofactors

Nicotinamide adenine dinucleotide cofactor NAD and its phosphorylated form NADP (Figure 1A) display an E_0' of −320 mV. Their actual redox potentials differ owing to different ratios of the reduced and oxidized pools. NAD/NADH is kept in the oxidized state by transferring electrons to the electron transport chain, alcohols/aldehydes, 2-oxo carboxylic acids/amino acids and the reduction of carbon-carbon double bonds. NAD/NADH is used in oxidation reactions in both assimilation and dissimilation, whereas NADP/NADPH is kept in its reduced form by accepting electrons from the oxidation of aldehydes to carboxylic acids and through oxidative decarboxylations.

Deazaflavin Cofactors

The deazaflavin cofactor 8-hydroxy-5-deazaflavin (F_{420}, Figure 1B) and its truncated versions (F_O, F_{420}-0) resemble the structure of flavins and reactivity of NAD (in gray Figure 1B versus 1A) and display a similar

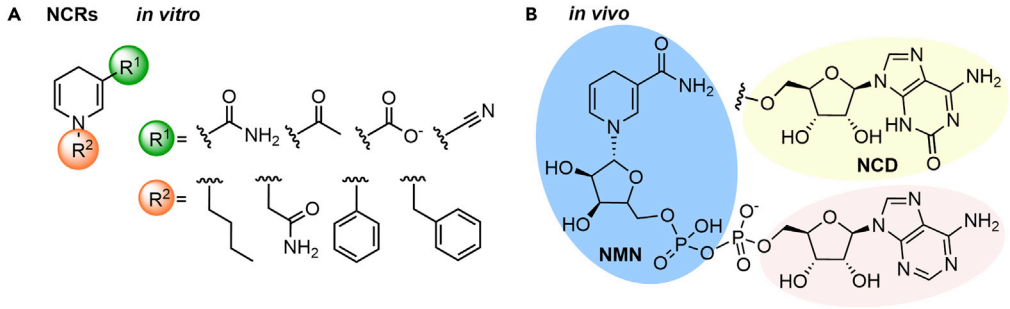

Figure 2. Chemical Structures of Selected NRCs

(A) NRCs used *in vitro*. These structures are examples of synthetic nicotinamide cofactor biomimetics successfully used with oxidoreductases.

(B) NRCs used *in vivo*. NADH structure, the moiety highlighted in pink, adenosine, is modifiable to cytosine, above in yellow. In blue NMN, nicotinamide mononucleotide; in yellow NCD, nicotinamide cytosine dinucleotide.

redox potential of −340 mV. This cofactor plays a role in primary and secondary metabolism in various archaea and bacteria. Its low-potential hydride transfer allows the mediation of various redox reactions involved in pathways such as antibiotic biosynthesis (Bashiri et al., 2019). As the natural biosynthesis of F_{420} does not exist in *E. coli*, recent efforts afforded its heterologous production by recombinant expression in *E. coli* (Bashiri et al., 2019), making this cofactor a good candidate for an orthogonal pathway.

Flavin Cofactors

Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD, Figure 1C) have a redox couple of −220 mV. Usually FAD and FMN are found as a prosthetic group in enzymes, shifting the E_0' to neutral values. They accept electrons from NAD(P)H and iron-sulfur clusters. Understanding electron flow through electron carrier proteins and their cofactors is key to engineer orthogonal pathways.

The redox cofactor NAD^+/NADH and its phosphorylated form $NADP^+$/NADPH are most relevant for product formation by microbial cell factories. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (www.genome.jp/kegg/) reports 983 NADH-dependent reactions and 1051 NADPH-dependent reactions, most of which are redox reactions. The iML1515 genome-scale constraint-based metabolic model (GSCBMM) of *E. coli* (Monk et al., 2017) lists 127 reactions using NAD^+/NADH and 113 reactions using $NADP^+$/NADPH. These values indicate that the electrons are diffusely distributed over the whole metabolic network. The current trend to design true orthogonal pathways for product formation to diminish entanglement and regulation is therefore futile, if these pathways are dependent on NAD^+/NADH and $NADP^+$/NADPH. True orthogonal pathways can only be realized when also the redox cofactor involved can be isolated as much as possible from other metabolic activities. The term "non-canonical redox cofactors" (NRCs) was used to describe such cofactors (Black et al., 2020). To date, NRCs have especially been studied *in vitro* as cofactor biomimetics for enzymatic reactions and are only beginning to be applied *in vivo*.

APPLICATION OF NON-CANONICAL REDOX COFACTORS IN *IN VITRO* BIOCATALYSIS

When performing *in vitro* biocatalytic processes, several factors such as cost, solubility, instability, or restricted activity of cofactors may impede further development of enzymes (Paul et al., 2014; Paul and Hollmann, 2016; Guarneri et al., 2019). Shorter versions of NADH, varying substituents on the dihydropyridine ring and nitrogen (Figure 2A), were synthesized and used as cofactor biomimetics with oxidoreductase enzymes to catalyze the reduction of carbon-carbon double bonds (Paul et al., 2013; Löw et al., 2016; Knaus et al., 2016; Falcone et al., 2019), the hydroxylation of benzoates (Ryan et al., 2008; Guarneri et al., 2020), or the oxidation of glucose (Nowak et al., 2017; Huang et al., 2019). These nicotinamide cofactor biomimetics allow for an orthogonal system when using enzymes as cell-free extracts for the reduction of carbon-carbon double bonds: activity by other oxidoreductases such as alcohol dehydrogenases was excluded, as these enzymes do not function with those truncated cofactors (Josa-Culleré et al., 2019; Paul et al., 2013).

#	Title	Description
Prerequisites for NRCs Application in Cell Factories		
1	Redox potential	The redox couple donating electrons should have a lower redox potential than the NRC, the redox couple accepting the electrons a higher redox potential.
2	Specificity	Strict control over the electrons necessitates that the NRC should only transfer electrons between the objective donating and receiving redox couple. Transfer of these electrons should therefore be catalyzed by enzymes with a high specificity for the NRC. Also, native enzymes should not be able to use the NRC and non-enzymatic transfer of electrons from the NRC to other redox cofactors should be avoided.
3	Location	Although many NRCs have been applied in enzymatic conversions, they are not all suitable in microbial cell factories. This namely requires the intracellular action of the NRC. Microbial cell factories should therefore be able to biosynthesize the NRC, either *de novo* or via salvation pathways, or be able to take it up from the medium.
4	Regulation	Many enzyme activities depend on regulation by NAD/NADH and/or NADP/NADPH concentration ratios. The NRC should not interfere with this regulation.
Additional Prerequisite for Application of NRCs in Fermentation Processes		
5	Dissimilation versus assimilation	The NRC should only be used for (ATP-generating) dissimilation and not for assimilation.

Table 1. Prerequisites Necessary for the Successful Application of NRCs in Cell Factories and Fermentation Processes

Stability and Activity

Modification of substituents on the NADH cofactor led to changes in redox properties as well as stability. The half-life of each cofactor depended on pH, temperature, and the presence of other chemical components such as phosphate buffer (Wu et al., 1986; Norris and Stewart, 1977). Further studies with synthetic cofactors displayed varying reaction rates depending on the substituted dihydropyridine ring for reduction and oxidation reactions (Knaus et al., 2016; Guarneri et al., 2020). These short synthetic analogs currently lack an efficient enzymatic recycling system (Zachos et al., 2018; Nowak et al., 2017). In parallel, NRCs recently used *in vivo*, NMN and nicotinamide cytosine dinucleotide (NCD, Figure 1B), rely on the natural structural backbone of NADH owing to the difficulty in engineering dehydrogenase enzyme toward the shorter truncated versions used *in vitro* (Huang et al., 2019; Nowak et al., 2017).

APPLICATION OF NON-CANONICAL REDOX COFACTORS *IN VIVO*

Applying these NRCs in microbial cell factories would provide an orthogonal circuit for electron transfer. It would increase the control over the fate of electrons and could therefore be used to improve bioprocesses. Although NRCs have been applied successfully in enzymatic conversions, additional prerequisites regarding redox potential, specificity, location, and regulation need to be fulfilled in order to function truly orthogonally *in vivo* (Table 1). Application of NRCs in microbial cell factories is a recent development; so far only two examples have been published. In the first example, nicotinamide cytosine dinucleotide (NCD^+/NCDH) (Figure 2B) was applied as an NRC for the production of malate by *E. coli* (Figure 3A) (Wang et al., 2017), where phosphite/phosphate was used as electron donor. The NAD^+/NADH-dependent phosphite dehydrogenase and malic enzyme were modified in such a way that they accepted NCD^+/NCDH as redox cofactor. NCD^+ was supplemented to the growth medium and taken up by *E. coli*. The low redox potential of phosphite/phosphate (E_0' = −650 mV [Claassens et al., 2018]) in combination with the high initial [phosphite]/[phosphate] ratio reversed the reaction catalyzed by malic enzyme, converting pyruvate and CO_2 into malate. This resulted in an overall increase in malate yield from 0.11 to 0.15 g/g.

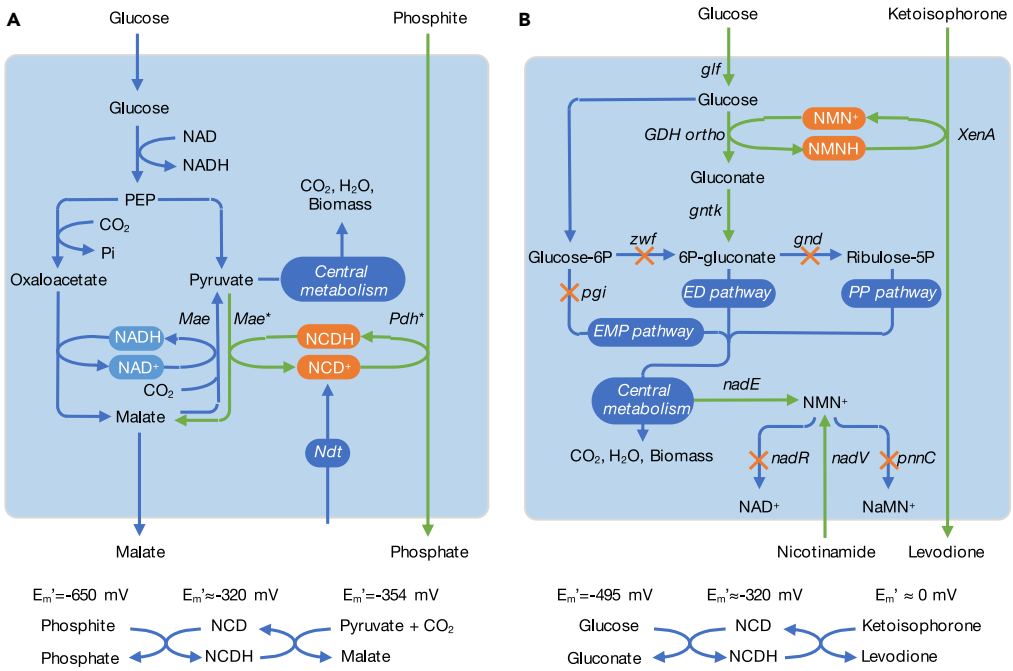

Figure 3. Examples of Applying Non-canonical Redox Cofactors in Microbial Cell Factories

(A) Transferring electrons from phosphite/phosphate to malate/pyruvate + CO_2 via NCD^+/NCDH. Modified malic enzyme (Mae*) and phosphite dehydrogenase (Pdh*) were introduced. Adapted from Wang et al. (2017).

(B) Transferring electrons from glucose/gluconate to ketoisophorone/levodione with NMN^+/NMNH as non-canonical cofactor. An NMN-dependent glucose dehydrogenase (GDH ortho) and ene reductase (XenA) were introduced. NMN^+ availability was enhanced by adding nicotinamide to the medium, knocking out the genes *nadR* and *pnnC* and overexpressing the genes *nadE* and *nadV*. The glycolytic flux was forced over glucose dehydrogenase by knocking out the genes *pgi*, *zwf*, and *gnd*. Blue lines: native pathways. Green lines: introduced or overexpressed gene/enzyme; ×: deleted gene/enzyme. Adapted from Black et al. (2020).

In the second example, nicotinamide mononucleotide (NMN^+/NMNH) (Figure 2B) was used as an NRC for the conversion of ketoisophorone, which was added to the medium, to levodione ((Black et al., 2020), Figure 3B). This process comprised an ene reductase enzyme to catalyze the reduction of the ketoisophorone carbon-carbon double bond, whose redox potential is near 0 V. Because the nicotinamide moiety of NAD(P) is responsible for electron transfer, the redox potential of NMN^+/NMNH is expected to be close to −320 mV. Its intracellular availability was enhanced by providing nicotinamide as nutrient, by overexpression of genes involved in NMN biosynthesis (*nadE* and *nadV*) and knocking out genes involved in NMN breakdown (*nadR* and *pnnC*). A glucose dehydrogenase (GDH), modified to accept NMN^+/NMNH as redox cofactor, and ene reductase (XenA) were introduced. These enzymes formed an orthogonal circuit for the transfer of electrons. Strains were created that had to rely on the activity of both enzymes for growth by knocking out key enzymes of the Embden-Meyerhoff-Parnas (EMP) pathway (*pgi*) and pentose phosphate (PP) pathway (*zwf* and *gnd*). Only strains that harbored both enzymes were able to grow, indicating that the two enzymes successfully established the new electrical circuit.

MODIFYING THE COFACTOR DEPENDENCY OF DEHYDROGENASES

To enable the application of NRCs it is required to change the cofactor specificity of oxidoreductases from the native redox cofactor to the NRC. In recent years, several methods have been developed to switch the cofactor dependency of dehydrogenases from NAD to NADP, and vice versa. In particular, a web tool named CSR-SALAD (Cofactor Specificity Reversal-Structural Analysis & Library Design) can assist, through a structure-guided and semi-rational approach, in the modification of the cofactor dependency of dehydrogenases (Cahn et al., 2017). Engineering this cofactor dependency is now a commonly used strategy in the optimization of redox metabolism and is starting to be applied for cofactor analogs, such as NRCs (Liu et al., 2018). For example, in the case of NCD, Liu et al. identified three residues in the cofactor-binding pocket of LDH from *Lactobacillus helveticus* that may be hotspots for cofactor preference

(Liu et al., 2020). Located in close proximity to the AD moiety of NAD, they are mostly conserved across NAD-dependent LDHs. Different combinations of the mutations V152R, I177K, N213E, and N213I enabled the switch from NAD to NCD dependency. Moreover, V152 is positioned at the N terminus of the conserved GX(X)GXXG sequence in the Rossmann fold motif and is present in other dehydrogenases, varying in some of them to L or I. With the malic enzyme (ME) from *E. coli*, the L310 residue (corresponding to V152 in LDH), was changed to an arginine to obtain an NCD-dependent ME (Ji et al., 2011), therefore confirming the potential of modifying cofactor specificity to NCD by reproducing the mutations of these hotspot residues.

Recently, Black et al. incorporated four mutations in the GDH from *Bacillus subtilis*, switching from NADP to NMN preference (Black et al., 2020). The mutations I195R and Y39Q were meant to form hydrogen bonds with the phosphate of NMN. Additionally, A93K was predicted to facilitate the interaction of Y39Q with NMN and S17E to promote the exclusion of NADP. The identification of these residues in other dehydrogenases can be challenging because protein-cofactor interactions are variable in NADP-dependent enzymes (Carugo and Argos, 1997a, 1997b) and might be unsuitable for NAD-dependent enzymes. Alternatively, introducing other mutations for the hotspot residues identified in LDH for NCD specificity could enable the switch to NMN.

In conclusion, cooperative mutations can change the cofactor dependency of dehydrogenases. Targeting residues scattered over a relatively broad range of gene sequence will make the reversal of all mutations unlikely. However, omitting only one of these mutations can already diminish the enzyme activity toward the NRC. The extent of this reduction and thereby the stability of the modifications will depend on the contribution of the reversed mutation to the switch of cofactor dependency.

APPLICATION OF NON-CANONICAL REDOX COFACTORS IN FERMENTATION PROCESSES

These results show that, in these examples, the four prerequisites for application in microbial cell factories (Table 1) were met, which opens up the possibility to use NRCs in fermentation applications.

Challenges in Fermentation Processes

In fermentation processes, the substrate is converted into oxidized intermediates, which are reduced to final products with concomitant ATP production. The two most efficient bioprocesses are based on this principle: ethanol and lactate production. The yields obtained for these two processes approach the maximal theoretical yield (for recent reviews see Aditiya et al., 2016 and de Oliveira et al., 2018). With respect to the production of bulk chemicals, this is the mode of choice. The design of metabolic networks for new fermentation processes is, however, challenging. By-product formation is difficult to prevent, and even if the $\Delta G'$ of the conversions is sufficiently negative, it often proves difficult to realize net ATP production. Application of NRCs in fermentation processes could remedy this matter. NRCs have not been applied in fermentation processes yet. Recently, the development of an NCD-dependent lactate dehydrogenase (LDH) was described, which could be a prelude to such a development aiming at the production of lactate (Liu et al., 2020).

Metabolic Engineering with NRCs

The redox potential of redox couples and the chemical structure of NRCs are leading factors in the design of NRC-dependent metabolic networks for product formation, because they determine the thermodynamic feasibility of the conversion. To remain close to wild-type situations it seems reasonable to focus initially on NRCs with redox potentials close to those of NAD^+/NADH and $NADP^+$/NADPH. $F_{420}/F_{420}H_2$ and nicotinamide derivatives such as NCD^+/NCDH and NMN^+/NMNH are in the suitable range.

Application of an NRC requires a change in cofactor dependency of at least two enzymes: one reducing the NRC, the other oxidizing it. Figure 4 gives an overview of the redox reactions and their potentials involved in the dissimilation of glucose.

Only few reactions transfer electrons to NAD^+/NADH and $NADP^+$/NADPH. These reactions can be categorized into two groups: those involving the conversion of aldehydes into organic acids and oxidative decarboxylations (Figure 4). The associated redox couples have a redox potential lower than that of $NAD(P)^+$/NAD(P)H. These redox couples are good candidates to reduce NRC^+/NRCH. GAPDH, both

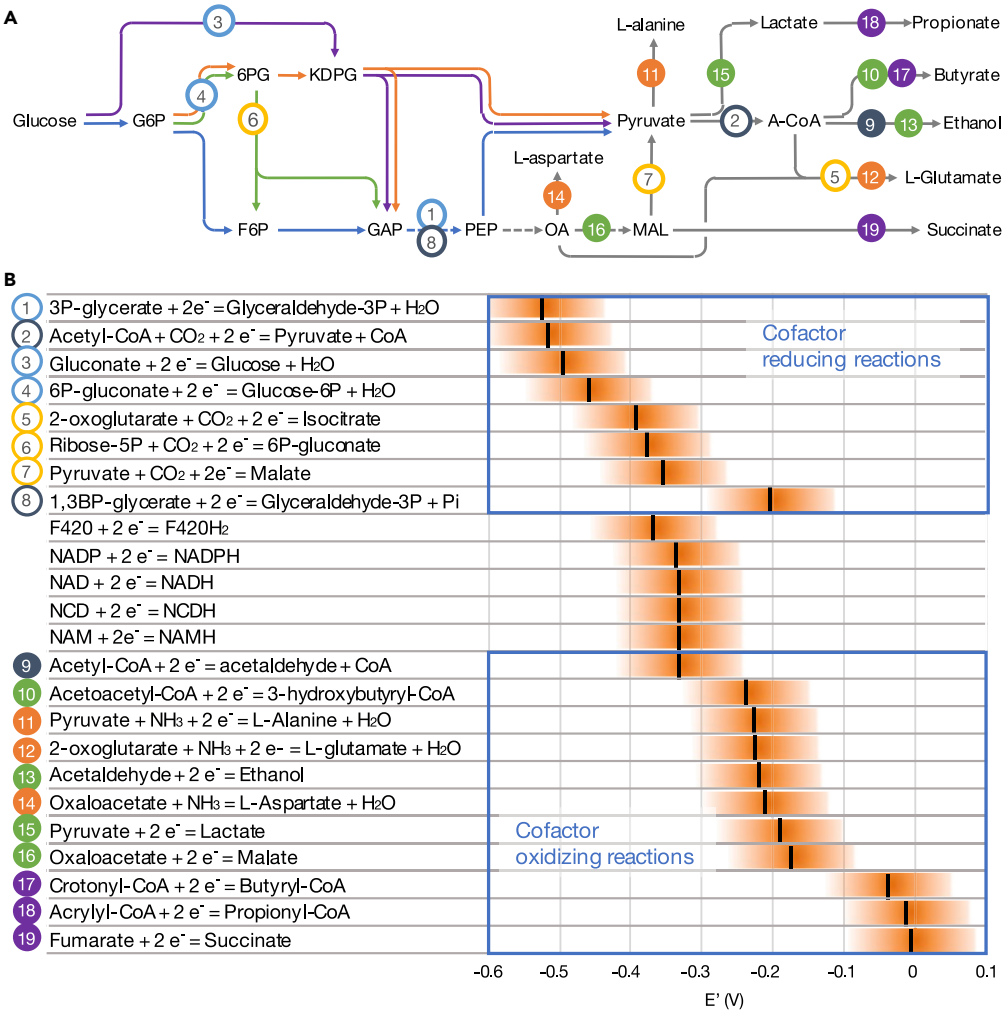

Figure 4. An Overview of the Redox Reactions and Their Potentials Involved in the Dissimilation of Glucose

(A) The Glycolytic Pathways and the Redox Reactions Involved. Embden-Meyerhof-Parnas pathway (blue arrows), Entner-Doudoroff pathway (orange arrows), semi-phosphorylating Entner-Doudoroff pathway (purple arrows), pentose phosphate pathway (green arrows), fermentation pathways (gray arrows).

(B) The redox potentials of relevant redox couples. | = E_m', the redox potential at pH 7, at 1 mM concentrations of the redox couple components (Flamholz et al., 2012). The orange band indicates the range of the E' between 1% and 99% oxidation. The redox reactions in the glycolytic pathways have a low redox potential, the redox reaction in the fermentation pathway a high redox potential. Aldehydes/organic acids (blue circles); oxidative decarboxylations (orange circles); alcohols/aldehydes (green circles); reduction of carbon-carbon double bonds (purple circles); other (gray circles). Interrupted lines depict a number of reactions. G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; KDPG, 2-dehydro-3-deoxy-phosphogluconate; F6P, fructose-6-phosphate; PEP, phosphoenolpyruvate; GAP, glyceraldehyde-3-phosphate; OA, oxaloacetate; MAL, malate; A-CoA, acetyl-CoA.

phosphorylating and non-phosphorylating, is the only enzyme involved in all pathways and therefore an obvious candidate.

In wild-type fermentation situations the electrons are transferred from $NAD(P)^+$/NAD(P)H to redox couples with higher redox potentials. These reactions can be categorized into three groups: the reduction of aldehydes to alcohols, the reductive amination of 2-oxoacids with NH_3 to amino acids, and the reduction of carbon-carbon double bounds (Figure 4). When designing an NRC-dependent network suitable for fermentative product formation it is therefore necessary to include such an NRC-dependent reaction.

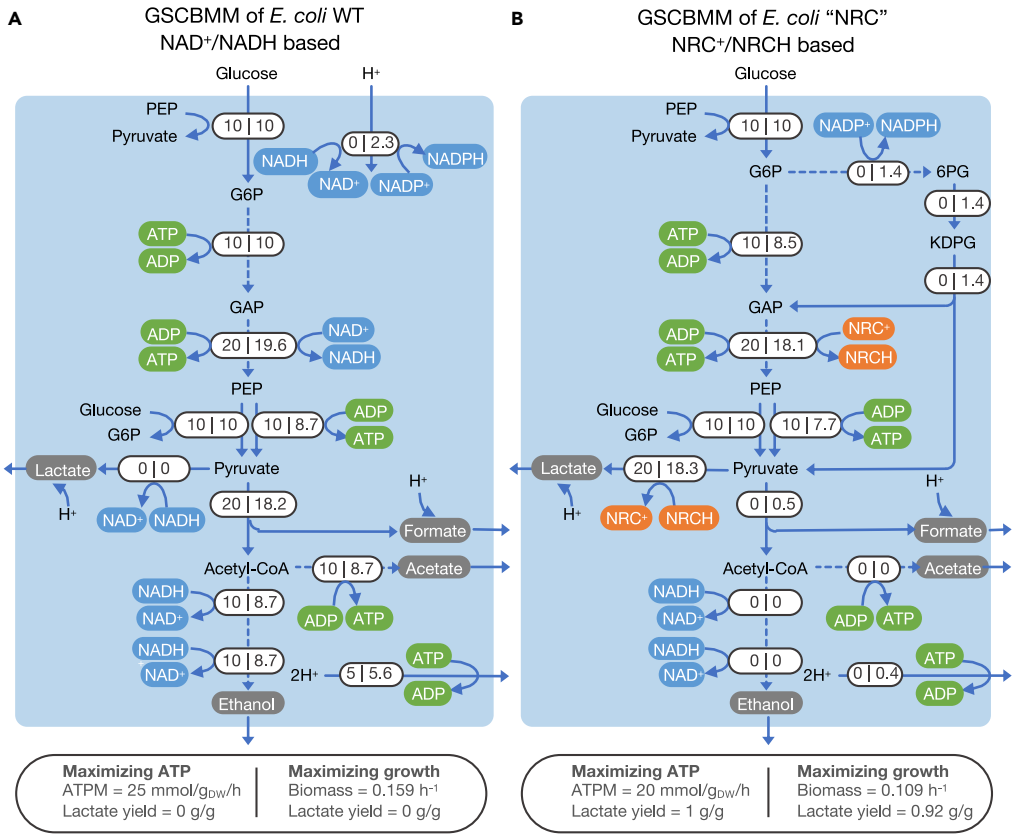

Figure 5. Carbon Flux Distributions Predicted by a Modified Version of GSCBMM *i*ML1515

Carbon flux distribution in (A) wild-type *E. coli* and (B) *E. coli* "NRC" carrying non-canonical redox cofactor "NRC"-dependent glyceraldehyde-3-phosphate dehydrogenase (NRC-GAPDH) and lactate dehydrogenase (NRC-LDH) under anaerobic conditions. A modified iML1515 genome-scale metabolic model of *E. coli* was used to predict the effect of introducing NRC-dependent enzymes in *E. coli* under anaerobic conditions on ATP maintenance requirement (ATPM) and biomass formation (growth rate). For more information about the model and the modifications introduced, see Supplemental Information.

The redox potentials of both NAD^+/NADH and $NADP^+$/NADPH are fixed within certain boundaries by cellular regulation, NAD^+/NADH more oxidized and $NADP^+$/NADPH more reduced. The NRCs offer more flexibility to fine-tune their redox potentials, by varying the ratio of the NRC^+ and NRCH concentrations, between those of the cofactor reducing and oxidizing reactions because they only depend on two enzyme activities. This may have benefits, especially for the functioning of GAPDH, which operates on the edge of thermodynamic feasibility with NAD^+/NADH as cofactor (Figure 4).

The synthesis of some compounds requires more than one oxidation and reduction reaction. Examples are the production of succinate (Meng et al., 2016), L-glutamate (Chinen et al., 2007), and 1,4-butanediol (Burgard et al., 2016). In these cases, it would be interesting to investigate whether it is required to make all involved dehydrogenases NRC dependent, or if it is possible to restrict this system to GAPDH as cofactor reducing enzyme, and a limited number of cofactor-oxidizing dehydrogenases able to regenerate NRCs.

GSCBMM Model Predictions

We used GSCBMM iML1515 of *E. coli* to illustrate how NRCs can be applied and which challenges will be encountered, aiming at lactate production using NRC-dependent GAPDH and LDH (Figure 5).

GAPDH is applied for both dissimilation and assimilation purposes. In wild-type situations (Figure 5A) GAPDH transfers electrons via NAD^+/NADH. The NAD^+ reduced in dissimilation is completely regenerated by the synthesis of fermentation products. This also generates the ATP required for growth and

maintenance purposes. The model, when maximizing ATP production, predicts a mixture of ethanol, acetate, and formate. The NAD^+ used in assimilation cannot be regenerated by producing the same dissimilatory fermentation products. Instead, it can be regenerated by a transhydrogenase transferring electrons from NAD^+/NADH to $NADP^+$/NADPH, which is required for assimilation, or by producing a by-product whose formation requires net NADH input (e.g., succinate or glycerol). Indeed, the model, when maximizing growth, predicts that the NAD^+ used by GAPDH for assimilation purposes is regenerated by action of the transhydrogenase STH.

We introduced a new redox couple in the model named NRC^+/NRCH and changed the cofactor specificity of GAPDH and LDH accordingly. The predictions are shown in Figure 5B. The dissimilatory flux was redirected from ethanol/acetate/formate to lactate only. Lactate synthesis was responsible for ATP production and resulted in complete cofactor regeneration when the model maximized for ATP production. Using the EMP pathway as single glycolytic pathway for assimilation would result in incomplete regeneration of NRC^+. The model used the Entner-Doudoroff (ED) pathway instead. This pathway converts glucose into GAP and pyruvate, partially bypassing GAPDH. This resulted in two glycolytic fluxes: a dissimilatory one using the NRC via a combination of the EMP and ED pathways and an assimilatory one using NAD(P) exclusively via the ED pathway.

This indicates that a fifth prerequisite for the successful usage of NRCs in fermentation processes applies: the NRC should only be used for (ATP-generating) dissimilation and not for assimilation (Table 1). In the case of an NRC-dependent GAPDH, this can be realized by using the ED pathway.

Adaptive Laboratory Evolution

Adaptive laboratory evolution (ALE) is a powerful technique for the improvement of microbial traits that are advantageous for growth or survival of the microorganism (Dragosits and Mattanovich, 2013). We did not ask the model to redirect the fluxes to lactate, or to implement the NRC-dependent enzymes, but to optimize for either ATP or biomass production, with high lactate production as result. This indicates that ALE can be applied to improve the flux over the NRC-dependent enzymes provided they are able to carry sufficient flux to fulfill maintenance requirements. It cannot be excluded, however, that the application of ALE will result in reversion of the NRC-dependent enzymes to their original cofactor dependency.

Aerobic NRC Application

As indicated above, when respiration is applied, it is impossible to reach the maximum theoretical yield. This problem is significantly reduced when dissimilation depends on NRC-depending enzymes because reduced NRC is not a substrate for NADH dehydrogenase. This indicates that NRCs can also be applied for the synthesis of fermentation products under aerobic conditions. This opens the opportunity to use NRCs in strict aerobic microorganisms that rely on the availability of oxygen for the synthesis of biomass buildings blocks. It would also enable NAD^+ used in assimilatory pathways (approximately 8% of the total flux in the lactate case) to be regenerated by respiration, at the expense of a somewhat lower yield. An additional advantage could be additional ATP synthesis via respiration that could support metabolic networks that generate too little ATP. We tried to simulate aerobic application in the GSCBMM by making oxygen available. We expected that the NRC would support the dissimilatory flux to lactate and that the $NAD(P)^+$ used by assimilation would be regenerated by respiration. The model indeed produced lactate using the NRC-dependent enzymes but subsequently converted lactate by an ubiquinol-dependent LDH back to pyruvate, which was used for further cellular processes. Knocking out this enzyme resulted in a situation in which a circular EMP/ED pathway was used, as described for *P. putida* (Nikel et al., 2015), in which the NRC-dependent GAPDH was completely bypassed. The ED produced NADPH instead of NADH. The model used STH to convert NADPH into NADH, which was subsequently used in respiration. Knocking out the gene encoding STH (*sthA*) could prevent this, forcing the flux over the NRC-dependent GAPDH, but the model kept finding new combinations of reactions that together acted as a transhydrogenase. These predictions are largely possible owing to the exceptional flexibility of GSCBMMs. In real life many of the reactions required to bypass NRC-dependent GAPDH are not switched on owing to regulation, e.g., by catabolite repression in presence of glucose. Aerobic application of NRCs could therefore be successful. Nevertheless, these model predictions indicate that mutations that open up alternative routes, bypassing the application of the NRCs, will be very advantageous for growth under aerobic

conditions. This may trigger the appearance of mutated strains that do not rely on the NRC for product formation.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

NRCs have successfully been applied in microbial cell factories, providing an orthogonal circuit for electron transfer, making it possible to have full control over the fate of electrons.

Fermentation processes are being developed to enable the sustainable production of chemicals. To approach the maximum theoretical yield, it is required to transfer all electrons from the substrate to the product. This is not straightforward as electrons are diffusely distributed over metabolism by redox cofactors.

Highlights

In this paper, we have shown that application of NRCs could prevent this. Using NRCs in fermentation processes has the potential to tremendously impact the design and application of fermentation processes. It will maximize the transfer of electrons from substrate to product, and as such, make it possible to approach the maximal theoretical yield. Consequently, it will minimize by-product formation. Product formation will also result in ATP production. The whole metabolic network can therefore be optimized using ALE. Moreover, it may be applied under aerobic conditions, broadening the scope of application to aerobic microorganisms.

Remaining Questions

NRCs have not been applied in fermentation processes yet. Several questions remain to be addressed before this system can be applied successfully.

- Making dissimilation dependent on an NRC is a tremendous interference in cellular metabolism. How will this affect cellular functioning?
- NRC application may often result in less ATP synthesis, being detrimental for growth and maintenance. Will NRC-independent mutant strains appear and outcompete NRC-dependent strains? Will these mutants revert to the original cofactor dependency, or will they bypass the NRC-dependent enzymes? What can be done to prevent the appearance of such strains?
- In this paper, the application of NRC for the production of lactate was worked out as an example, using NRC-dependent GAPDH and LDH. Other options for electron-donating and electron-receiving reactions are possible and have to be investigated.
- Using NRCs should be limited to dissimilatory fluxes, indicating that NRC-independent assimilation pathways have to be constructed. We have identified the ED pathway as option in combination with GAPDH. What other options are possible?
- The cofactor dependency of a few enzymes has been changed from NAD(P) to NCD and NMN by changing three and four amino acids, respectively. Is it possible to apply this strategy to other oxidoreductases as well?
- Other redox cofactors are applied in fermentation 8processes (Box 1), such as ferredoxin and F_{420}. Will ferredoxin- and F_{420}-based NRCs also be beneficial for product formation, and, if so, how can this be realized?
- Microbial cell factories have been constructed that are able to synthesize sufficient NMN^+/NMNH and NCD^+/NCDH *de novo* or by salvation pathways. Is this also sufficient to sustain the high fluxes required for fermentation processes?

Limitations of the Study

To predict the effect of applying NRCs in fermentation processes, especially with respect to assimilation, we have used a genome-scale constraint-based metabolic model of *E. coli* (iML1515). More elaborate metabolic models are available (e.g., enzyme-constrained models), whose application may give different results.

Box 1. Glossary

Adaptive Laboratory Evolution Adaptive laboratory evolution is a frequent method in biological studies to gain insights into the basic mechanisms of molecular evolution and adaptive changes that accumulate in microbial populations during long-term selection under specified growth conditions (Dragosits and Mattanovich, 2013).

Assimilation and Dissimilation Assimilation is the energy-requiring metabolic process that converts a substrate into biomass. Dissimilation is the metabolic process that converts a substrate into products, concomitantly producing the energy required for assimilation and maintenance.

Biocatalysis Use of biological systems such as whole cells (*in vivo*) or isolated enzymes (*in vitro*) to catalyze reactions. Enzymes can catalyze chemical reactions with exquisite regio-, stereo-, and/or chemoselectivity, with high rates of reactions depending on the type of reaction and the substrates used.

Cofactors Non-protein organic molecules (coenzymes) or metal ions required by an enzyme to assist during a catalytic reaction. Most common coenzymes for oxidoreductases are redox cofactors: nicotinamide adenine dinucleotide NAD(P), flavin adenine dinucleotide FAD and flavin mononucleotide FMN, and heme. Coenzymes can be covalently or tightly bound as a prosthetic group to a protein or only transiently bound and used as cosubstrate. Inorganic cofactors, such as Zn, Mg, Co, Mo, and iron-sulfur (Fe-S) clusters, can play both functional and structural roles. An enzyme without its cofactor is an inactive apoenzyme.

Fermentation Process A metabolic process that releases energy from a sugar or other organic compound without requiring an external electron acceptor.

Genome Editing The modification of the genome within a living cell through the insertion, deletion, or replacement of one or more segments of DNA (Fang et al., 2019).

Genome-Scale Constraint-Based Metabolic Model (GSCBMM) A stoichiometric model of metabolism of a certain (micro)organism, including all enzymes annotated based on its genome and associated reactions and metabolites. The model can predict fluxes through the metabolic model within certain preset flux constraints.

Metabolic Engineering The practice of optimizing genetic and regulatory processes within cells to increase the cells' production of a certain substance. These processes are chemical networks that use a series of biochemical reactions and enzymes that allow cells to convert raw materials into molecules necessary for the cell's survival. Metabolic engineering specifically seeks to mathematically model these networks, calculates a yield of useful products, and pinpoints parts of the network that constrain the formation of these products (Yang et al., 1998).

Microbial Cell Factories Use of microbial cells as a means to produce targeted chemicals. Optimization of the process relies mostly on metabolic engineering.

Oxidoreductases One of the seven classes of enzymes, they catalyze reduction (gain of electrons) and oxidation (loss of electrons) reactions. Approximately 80% of known oxidoreductases require the nicotinamide adenine dinucleotide cofactor.

Orthogonal Pathways Growth-independent pathways optimized for the production of a target chemical. These pathways are characterized by the minimization of interactions between the chemical-producing pathways and the biomass-producing pathways (Pandit et al., 2017).

Synthetic Biology Applying the engineering paradigm of systems design to biological systems in order to produce predictable and robust systems with novel functionalities that do not exist in nature.

Data and Code Availability

All data and code required to repeat the model predictions are available in the Supplemental Information section.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101471.

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AUTHOR CONTRIBUTIONS

All authors wrote the paper. P.L.F. performed the model predictions.

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Supplemental Information

Applying Non-canonical Redox Cofactors in Fermentation Processes

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Supplemental information

Transparent Methods

The iML1515 genome-scale metabolic model from Monk et al. (2017) was modified to predict the effect of non-canonical redox cofactor "NRC"-dependent glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase on the anaerobic metabolism of *E. coli*. The models can be found in SBML format/scripts in the supplementary files "E. coli WT.ipynb", "E. coli WT.xml", "E. coli NRC-GAPDH and NRC-LDH.ipynb" and "E. coli NRC-GAPDH and NRC-LDH. xml".

To introduce the NRC-dependent enzymes, the native reactions catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GAPD) and D-lactate dehydrogenase (LDH_D) were first knocked out by setting the upper and lower bounds of the reactions to 0. The two new cofactors – non-canonical redox cofactor oxidized (NRCox) and reduced (NRCred) – were added to the model as new metabolites. Then, the reactions catalyzed by the NRC-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPD_NRC) and NRC-dependent D-lactate dehydrogenase (LDH_D_NRC) were added to the model. The upper and lower bounds of the reactions were set at the same values than the native enzyme ([-1000 ; 1000]).

Additional modifications were performed in the models for both *E. coli* WT and *E. coli* carrying NRC-dependent glyceraldehyde-3-phosphate dehydrogenase and D-lactate dehydrogenase regardless of the objective to maximize used.

The reaction catalyzed by the membrane-bound transhydrogenase PntAB (THD2pp) was modified to allow the translocation of one proton instead of two. The reactions catalyzed by dihydroxyacetone phosphotransferase (DHAPT) and pyruvate dehydrogenase complex (PDH) were knocked out by setting the upper and lower bounds of the reactions to [0; 0].

The direction of the reaction catalyzed by acyl-CoA dehydrogenase (butanoyl-CoA) (ACOAD1fr) was reversed by modifying the bounds of the reaction from [0; 1000] to [-1000; 0].

Finally, anaerobic conditions were enabled by setting the upper and lower bounds of the oxygen exchange (EX_o2_e) reaction to [0; 0].

The models were run using 10 mmol/g_{DW}/h glucose as carbon source. Biomass (BIOMASS_Ec_iML1515_core_75p37M) and ATP maintenance requirement (ATPM) were used as objectives to maximize in the modified iML1515 genome-scale metabolic models. Gurobi was used as solver.

Supplemental References

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