Harnessing noncanonical redox cofactors to advance synthetic assimilation of one-carbon feedstocks

Orsi, Enrico; Hernández-Sancho, Javier M.; Remeijer, Maaike S.; Kruis, Aleksander J.; Volke, Daniel C.; Claassens, Nico J.; Paul, Caroline E.; Bruggeman, Frank J.; Weusthuis, Ruud A.; Nikel, Pablo I.

DOI
10.1016/j.copbio.2024.103195

Publication date
2024

Document Version
Final published version

Published in
Current Opinion in Biotechnology

Citation (APA)
Orsi, E., Hernández-Sancho, J. M., Remeijer, M. S., Kruis, A. J., Volke, D. C., Claassens, N. J., Paul, C. E., Bruggeman, F. J., Weusthuis, R. A., & Nikel, P. I. (2024). Harnessing noncanonical redox cofactors to advance synthetic assimilation of one-carbon feedstocks. *Current Opinion in Biotechnology*, *90*, Article 103195. https://doi.org/10.1016/j.copbio.2024.103195

Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Review

Harnessing noncanonical redox cofactors to advance synthetic assimilation of one-carbon feedstocks☆

Enrico Orsi[1,*], Javier M Hernández-Sancho[1,*],
Maaike S Remeijer[2,*], Aleksander J Kruis[3],
Daniel C Volke[1], Nico J Claassens[4],
Caroline E Paul[5], Frank J Bruggeman[2],
Ruud A Weusthuis[6] and Pablo I Nikel[1]

One-carbon (C1) feedstocks, such as carbon monoxide (CO), formate (HCO_2H), methanol (CH_3OH), and methane (CH_4), can be obtained either through stepwise electrochemical reduction of CO_2 with renewable electricity or via processing of organic side streams. These C1 substrates are increasingly investigated in biotechnology as they can contribute to a circular carbon economy. In recent years, noncanonical redox cofactors (NCRCs) emerged as a tool to generate synthetic electron circuits in cell factories to maximize electron transfer within a pathway of interest. Here, we argue that expanding the use of NCRCs in the context of C1-driven bioprocesses will boost product yields and facilitate challenging redox transactions that are typically out of the scope of natural cofactors due to inherent thermodynamic constraints.

Addresses
[1] The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kongens Lyngby, Denmark
[2] Amsterdam Institute for Life and Environment and Institute of Molecular and Life Sciences, Vrije Universiteit, Amsterdam, the Netherlands
[3] Acies Bio D.O.O., Ljubljana, Slovenia
[4] Microbiology, Wageningen University and Research, Wageningen, the Netherlands
[5] Department of Biotechnology, Delft University of Technology, the Netherlands
[6] Bioprocess Engineering, Wageningen University and Research, Wageningen, the Netherlands

Corresponding authors: Orsi, Enrico (enricoo@biosustain.dtu.dk), Nikel, PabloI (pabnik@biosustain.dtu.dk)
[*] These authors contributed equally and should be considered joint first-authors.

Current Opinion in Biotechnology 2024, **90**:103195

This review comes from a themed issue on **Chemical Biotechnology**

Edited by **Yong-Su Jin** and **Tomohisa Hasunuma**

Available online xxxx

https://doi.org/10.1016/j.copbio.2024.103195

Introduction

The transition from a fossil-based to a bio-based economy requires industrial bioprocesses utilizing biological systems for manufacturing [1,2]. In this framework, cheap, renewable feedstocks are converted into value-added compounds [3]. Efficient conversions can be achieved either by creating and implementing synthetic pathways that do not exist in nature or by optimizing production parameters for existing bioprocesses, including titer ($g_{product}\ l^{-1}$), volumetric productivity ($g_{product}\ l^{-1}\ h^{-1}$), and yield ($g_{product}\ g_{substrate}^{-1}$) [4,5]. However, the cost-efficient production of bulk chemicals, meeting large market demands (Gtons $year^{-1}$) at low production costs (< 1.0 USD kg^{-1}), remains a major challenge [6,7]. High substrate-to-product yields are essential for economic feasibility.

☆ Given the role as Editor in chief, Pablo I Nikel had no involvement in the peer review of the article and has no access to information regarding its peer-review. Full responsibility for the editorial process of this article was delegated to Guest editor, Yong-Su Jin.

Maximum theoretical yields are obtained when all electrons transfer from substrate to product [8]. Electron carriers, for example, nicotinamide adenine dinucleotide (in both its oxidized and reduced state, $NAD(P)^+/NAD(P)H$), are ubiquitous cofactors in metabolism and mediate a large number of biochemical reactions. This flow of redox currency causes inherent electron dissipation throughout the entire metabolic network, compromising product yields [9]. Introducing an independent, orthogonal electron circuit, with noncanonical redox cofactors (NCRCs), has been suggested as a strategy to approach maximum theoretical yields by confining electron flow within the target pathway [10••]. For example, a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and an alcohol dehydrogenase, each coupled to the same NCRC instead of NAD^+, would enforce ethanol biosynthesis and limit the formation of by-products from pyruvate while keeping the redox balance. The design, synthesis, and implementation of NCRCs in biotechnology are gaining momentum [11•,12], with successful examples of NCRC-supported *in vivo* catalysis [13,14••]. These studies exploited microbial growth as the proxy for intracellular NCRC reduction and oxidation, typically with sugars as the main carbon source. Selective product formation, on the other hand, could be increased from 2% to 80% by shifting from canonical redox cofactors to an NCRC [15•].

One-carbon (C1) substrates, such as methane (CH_4), methanol (CH_3OH), formate (HCO_2H), CO, and CO_2, are emerging feedstocks that can support efficient bioprocesses and contribute to a circular carbon economy [16–19]. These substrates, alone or with hydrogen (H_2), can support C1-trophic microbial growth, and utilizing CO_2-derived C1 feedstocks for bulk chemicals synthesis reduces reliance on agricultural feedstocks (e.g. sugars [20]). This is a key advantage of renewable electricity-based C1 feedstocks [21], as they avoid the competition with food production inherent in large-scale agricultural feedstock use, and they mitigate the shift in pressure from greenhouse gas emissions to other critical planetary boundaries [22]. Methane and CO_2 are the most concentrated C1 feedstock streams that are readily available [23]. These C1 substrates can be obtained from anaerobic digestion of organic matter [24]. Methanol, generated from hydrogenation of industrially emitted CO_2 [25], is an equally attractive substrate. Methanol and formate rank among the top C1 substrates for bioprocesses as they can be obtained from (concentrated) CO_2 through electrochemical reduction and are miscible in water, bypassing solubility issues related to gas–liquid transfer [26,27].

C1 feedstocks can be assimilated through a range of natural or synthetic pathways, differing in ATP demand and redox cofactor coupling. Both natural and synthetic C1-trophic organisms are considered for bioconversion and valorization of C1 feedstocks [28•–30]. Natural C1-trophs include model bacteria equipped with C1-assimilation pathways that are becoming tractable [31] as novel synthetic biology tools become available to the scientific community [32,33]. Synthetic C1-trophs [34], in contrast, could support the implementation of NCRC-based metabolism and NCRC-dependent enzymes for synthetic C1 assimilation. Although the growth performance of synthetic C1-trophs is typically below that of their natural counterparts [35•], the field of synthetic C1 assimilation is advancing rapidly and shows encouraging progress. In a recent example, a synthetic methylotroph achieved growth rates comparable to those of natural methylotrophs [36] — or even faster [37].

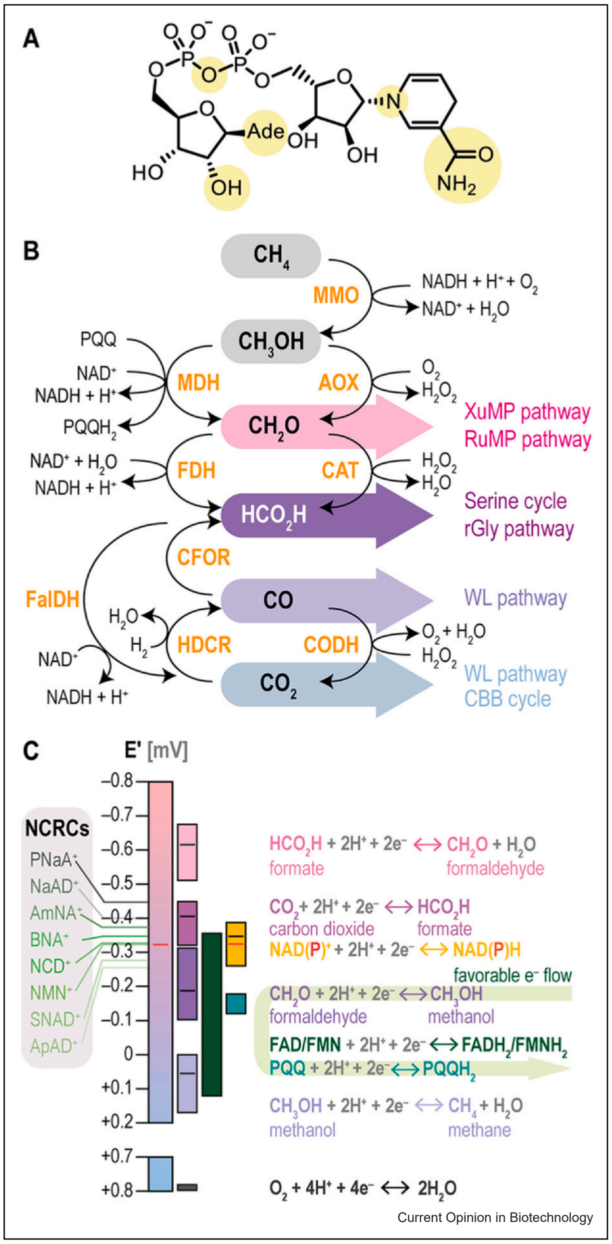
The expectations and motivating examples of synthetic C1 metabolism prompted us to explore strategies that could accelerate this expanding field. Here, we propose adopting NCRCs in metabolic engineering to optimize electron transfer from C1 feedstocks to target products. In this context, we review the state-of-the-art in NCRC engineering and discuss the opportunities and challenges in their implementation to support synthetic C1 metabolism.

Engineering and utilization of noncanonical redox cofactors

NCRCs retain the reactive nicotinamide moiety, as in $NAD(P)^+$ and deazaflavin cofactors, which serves as the hydride acceptor and donor enabling enzymes to catalyze redox reactions. However, other structural features of NCRCs differ from those of natural cofactors. Nonengineered enzymes use NCRCs less efficiently than the natural ones [11], leading to unwanted electron exchanges between engineered pathways and the native metabolism [38]. Strategies based on NCRCs developed thus far have adopted nicotinamide derivatives, where the pyridine ring is conserved, but different substituents decorate the nitrogen and C3 position, as well as the sugar and adenosine moieties (Figure 1a), determining both the redox potential and positioning of the NCRC in the enzyme active site.

When used *in vivo,* NCRCs act as mediators in orthogonal circuitry for electron transfer. This control over the fate of electrons helps overcoming intrinsic limitations of using natural cofactors in classical metabolic engineering, including electron and metabolic intermediate loss in central metabolism, undesired modification of target products due to enzymatic promiscuity, and thermodynamic constraints [11]. Only a few NCRCs have been engineered and adopted *in vivo*, such as nicotinamide cytosine dinucleotide (NCD^+) and nicotinamide mononucleotide (NMN^+). The standard redox potential of these carriers is comparable to that of natural cofactors [$NAD(P)^+$], limiting their range of application. Nevertheless, several interesting metabolic outcomes have resulted from their use, for example, as

Figure 1



NCRCs and relevant characteristics for synthetic C1 assimilation. **(a)** Chemical structure of NADH, highlighting the moieties and positions that can be modified to engineer NCRCs. Ade, adenine. **(b)** Overview of redox reactions involved in C1 substrate conversion. Methane (CH_4) and methanol (CH_3OH) cannot be directly assimilated (as they must be first transformed into other intermediates); C1 assimilation pathways for other compounds are displayed in colored arrows, with relevant enzymes indicated in orange. **(c)** Redox potential of redox cofactors and associated C1 reactions. The black lines indicate redox potentials under standard physiological conditions, while the width of the colored bars represents redox potentials under physiological conditions. Abbreviations: $APAD^+$, 3-acetylpyridine dinucleotide; $SNAD^+$, thionicotinamide adenine dinucleotide; BNA^+, 1-benzylnicotinamide; $AmNA^+$, 1-(2-carbamoylmethyl)-nicotinamide; $NaAD^+$, nicotinic acid adenine dinucleotide; $PNaA^+$, 1-propylnicotinic acid; $NAD(P)^+$, nicotinamide adenine dinucleotide (phosphate); FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; rGly pathway, reductive glycine pathway; WL pathway, Wood-Ljungdahl pathway, CBB cycle, Calvin–Benson–Bassham cycle; AOX, alcohol oxidase; FalDH, formate dehydrogenase; CFOR, CO:formate oxidoreductase; HDCR, H_2-dependent CO_2 reductase; CODH, carbon monoxide dehydrogenase; and CAT, catalase. Calculations and values for the figure are given in Tables S1 and S2 the Supplementary Data.

illustrated by the NCD^+-dependent circuit constructed by Wang et al. [39•]. The authors coupled an orthogonal phosphite (PO_3^{3-}) dehydrogenase to an engineered malic enzyme, thereby tightly controlling electron transfer between pyruvate and malate. A broader perspective of the biocatalytic potential of NCRCs has been demonstrated with the introduction of NMN^+ as a redox mediator. NCRCs can act as a thermodynamic driving force to displace redox reactions from equilibrium. Aspacio et al. [40] recently demonstrated an inverted NMN^+/NMNH ratio compared to natural cofactors to drive stereo-specific synthesis of *meso*-butanediol. In another example, Richardson et al. [41] achieved monoterpene aldehyde purity utilizing an orthogonal NMN^+/NMNH redox system that circumvented unspecific transformations of the aldehyde to alcohols.

Inspired by these possibilities, we propose that some of the challenges associated with engineering efficient assimilation of C1 feedstocks (constraints addressed in this review), can be partially overcome by implementing NCRC-dependent redox circuits. This overarching goal calls for NCRC-dependent reactions, which presents challenges in engineering enzymes to accept non-native cofactors. First, the native $NAD(P)^+$ cofactor should be excluded from binding, which can be achieved by steric (size) considerations or repulsive charges. Second, the NCRC should be accepted with high affinity, which can be supported by hydrogen bonding or ionic interactions. Since the Rossmann fold motif is highly conserved for cofactor binding via the adenosine moiety of $NAD(P)^+$, mutations in this protein domain might lead to misfolding or loss of activity. Nevertheless, several enzymes that convert C1 compounds or help balancing the NCRCs redox state in C1-related metabolic networks have already been modified to recognize these cofactor analogs. An overview of these enzymes is provided in Table 1.

Enzymes involved in the oxidation and reduction of C1 substrates

Relatively few enzymes are involved in the oxidation or reduction of C1 feedstocks. These enzyme covers all the different degrees of reduction of C1 molecules (Figure 1b), sequentially removing a pair of electrons from methane (CH_4, $\gamma = 8$) to CO_2 ($\gamma = 0$). This section provides an overview of these enzymes and the associated literature, focusing on engineering increased the affinity toward NCRCs.

Table 1

Redox enzymes engineered to accept NRCs that are potentially applicable to synthetic C1 assimilation.

Enzyme*	Source	Name and mutations	Redox cofactor	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M	Ref.
S-Butanediol dehydrogenase**	*Serratia* sp. AS13	*Ser S*-Bdh Ortho	NAD^+	1.6	0.019	0.012	[40]
			$NADP^+$	1.4	0.002	0.0014	
			NMN^+	4.3	0.38	0.086	
meso-Butanediol dehydrogenase**	*Klebsiella pneumoniae*	*Kp m*-Bdh Ortho	NAD^+	6.6	0.015	0.0022	[40]
			$NADP^+$	2.9	0.0067	0.0023	
			NMN^+	6.7	2.0	0.3	
Formaldehyde dehydrogenase	*Pseudomonas putida*	A192R/L218V/L236V/ R267V	NAD^+	4	0.5	0.125	[61]
			$NADP^+$	n.r.	n.r.	n.r.	
			NCD^+	1.5	14.6	9.73	
		A192R/L223V/L236V	NAD^+	2.5	0.8	0.32	
			$NADP^+$	n.r.	n.r.	n.r.	
			NCD^+	0.2	11.3	56.5	
FDH	*Pseudomonas* sp. 101	V198I/C256I/ P260S/E261P/ S381N/S383F	NAD^+	7.97	0.07	0.0088	[68]
			$NADP^+$	n.r.	n.r.	n.r.	
			NCD^+	0.1238	0.18	1.45	
GAPDH	*Streptococcus mutans*	GapN Penta	NAD^+	n.r.	n.r.	0.043	[38]
			$NADP^+$	2.6	0.26	0.1	
			NMN^+	12	0.82	0.068	
		GapN Ortho	NAD^+	2	0.021	0.011	
			$NADP^+$	8.3	0.016	0.002	
			NMN^+	7.7	0.081	0.01	
Glucose dehydrogenase	*Bacillus subtilis*	GDH Triple	NAD^+	3.7	0.41	0.11	[15]
			$NADP^+$	0.61	4.4	7.5	
			NMN^+	6.4	3.1	0.51	
		GDH Ortho	NAD^+	6.5	0.025	0.0038	
			$NADP^+$	2	0.022	0.011	
			NMN^+	5.9	1.2	0.21	
Glutathione reductase	*Escherichia coli*	Gor Ortho	NADH	n.r.	n.r.	0.06	[13]
			NADPH	n.r.	n.r.	0.1	
			NMNH	n.r.	n.r.	0.012	
LDH**	*Lactobacillus helveticus*	V152R/I177K/N213E	NAD^+	1.6	0.08	0.049	[95]
			$NADP^+$	n.r.	n.r.	n.r.	
			NCD^+	1.38	2.95	2.1	
		V152R/I177K/N213I	NAD^+	1.05	0.08	0.074	
			$NADP^+$	n.r.	n.r.	n.r.	
			NCD^+	0.66	2.02	3.1	
Malic enzyme	*Ascaris suum*	ME-L310R/ Q401C	NAD^+	10.4	3.8	0.36	[73]
			$NFCD^+$	1.7	162.4	96.7	
			NCD^+	1.02	158.2	154.6	
NADH oxidase	*Lactobacillus pentosus*	LP-7	NADH	1.7	47	27.65	[14]
			NADPH	0.89	27	30.34	
			NMNH	n.r.	n.r.	0.01	
		LP-3-EP	NADH	n.r.	n.r.	0.049	
			NADPH	n.r.	n.r.	0.051	
			NMNH	n.r.	n.r.	0.023	
NADH oxidase	*Lactobacillus lactis*	Nox Ortho	NADH	n.r.	n.r.	20	[40]
			NADPH	n.r.	n.r.	20	
			NMNH	n.r.	n.r.	55	

[*] The examples were selected based on the potential of the enzymes to support reduction or oxidation of C1 substrates and facilitate in vivo redox and carbon balances. Data adapted from Black et al. [11].

[**] Kinetic parameters were derived from literature and calculated for the oxidized form of the cofactors. n.r., not reported.

Methane oxidation

Methane monooxygenases (MMOs) oxidize CH_4, the most reduced C1 compound, to methanol (CH_3OH). This enzyme family activates the C—H bond in CH_4 and oxidizes it to CH_3OH. In this reaction, one NADH equivalent is oxidized, and O_2 acts as the electron acceptor. Both soluble MMO (sMMO) and membrane-bound particulate MMO exist in nature, although sMMO has been studied in more detail [42–44]. No reports have yet described MMOs engineered to accept other cofactor types.

Methanol oxidation

Three naturally occurring enzymes oxidize CH_3OH to formaldehyde [45•]: methanol oxidase (MOX), pyrroloquinoline-dependent methanol dehydrogenase (PQQ-MDH), and NAD^+-dependent methanol dehydrogenase (NAD-MDH). Since reduced cofactors are not generated in the MOX reaction, they are not a subject of this review.

Of the two cofactor-dependent MDHs, PQQ-dependent enzymes are more abundant in nature and have been described in at least 15 genera of Gram-negative bacteria. The PQQ-MDH complex, located in the bacterial periplasm, uses PQQ as a prosthetic group and initial electron recipient from CH_3OH oxidation to form reduced $PQQH_2$. $PQQH_2$ is then re-oxidized by passing electrons via specialized cytochromes to O_2 [46]. PQQ-MDHs are typically fast enzymes and can function at low CH_3OH concentrations due to the highly negative Gibbs free energy (ΔG) of the reaction [45], driven by the low E'^0 of PQQ reduction (Figure 1c and Table S1 in the Supplementary Material). Consequently, re-oxidation of $PQQH_2$ is limited to electron acceptors with even lower E'^0, mainly O_2. From an industrial perspective, these features translate into relatively low product yields on CH_3OH, increased need for aeration during the bioprocess, and higher operational costs.

The soluble, cytosolic NAD-MDH may be a better starting point for redirecting electrons from CH_3OH into central carbon metabolism. NAD-MDH enzymes have been described mainly in Gram-positive methylotrophs [46], but also in a few Gram-negative organisms, for example, *Cupriavidus necator* [47]. Thermodynamically, NAD-MDH is not efficient for CH_3OH assimilation ($\Delta G'^{\circ} = 30\ \mathrm{kJ\ mol^{-1}}$). However, its ability to process CH_3OH has been observed and demonstrated in synthetic methylotrophs [48,49]. The most studied natural methylotroph utilizing NAD-MDH is the thermophilic bacterium *Bacillus methanolicus*. This organism grows fast on a defined medium containing CH_3OH as the only carbon source, but it is unclear how *B. methanolicus* has overcome the thermodynamic and kinetic constraints of the NAD-MDH reaction. The high growth temperature (up to 60°C [50]) could be a key factor rendering the reaction thermodynamically feasible. Additionally, the presence of the ACT activator protein that interacts with the *B. methanolicus* NAD-MDH could also help improve its activity [51,52]. However, the characterization of this interaction has only been performed *in vitro*, not *in vivo*, and the connection between MDH and ACT remains unclear. To date, no NCRC-dependent CH_3OH dehydrogenases have been reported.

Formaldehyde oxidation

Formaldehyde is a highly reactive and toxic product of CH_3OH dehydrogenase, with a degree of reduction ($\gamma = 4$) comparable to microbial biomass ($\gamma = 4.2$, assuming an elemental biomass composition of $CH_{1.8}N_{0.2}O_{0.5}$ and the following degree of reductions: C = +4; H = +1; N = −3; and O = −2). Based on this parameter (γ), formaldehyde could theoretically be assimilated into carbon skeletons for biomass without additional reducing power. Natural routes for direct formaldehyde assimilation include the ribulose monophosphate (RuMP) pathway and the xylulose monophosphate (XuMP) pathway [53–55]. Another possible metabolic fate for formaldehyde is its stepwise oxidation to formate and eventually CO_2. This mechanism, once thought to be exclusively found in methylotrophs, has been recently identified in heterotrophic bacteria, for example, the metabolically versatile soil bacterium *Pseudomonas putida* [56–58]. This process involves a glutathione-dependent mechanism encoded in the *frmAC* operon, aided by the thiol-dependent enzymes FdhAB and AldB-II [59]. Identifying genes involved in formaldehyde detoxification is not a trivial task, as described by Berrios et al. [60] in their work with the methylotrophic yeast *Komagataella phaffii* (*Pichia pastoris*). Engineering formaldehyde dehydrogenases in the context of NCRCs has been attempted, with an engineered FalDH variant (FalDH*) from *P. putida* (Table 1) that displayed a > 150-fold preference for the NCRC NCD^+ over NAD^+ [61]. FalDH* displayed a $k_{cat}/K_M = 799 \pm 88$ for NCD^+ and 5.2 ± 0.6 for NAD^+, while the wild-type FalDH had a much higher k_{cat}/K_M for NAD^+ (26,100 ± 662) compared to NCD^+ (593 ± 296). FalDH* was generated through rational engineering, and the best-performing variant, mutant 9B2, had three amino acid substitutions (A192R/L223V/L236V) within the catalytic pocket [61••].

Formate oxidation

Oxidation of formate to CO_2 is catalyzed by formate dehydrogenase (FDH). FDHs can be classified as metal dependent and metal independent [62]. The reaction is reversible, allowing for CO_2 reduction to formate, especially with metal-dependent variants [62,63]. Several studies have explored alternative electron mediators for this enzyme. For instance, adding *N*,*N*′-dimethyl-4,4′-bipyridinium dichloride (methyl viologen or paraquat) improved the *in vitro* kinetics of an FDH in the reduction of CO_2 to formate [63]. FDHs have also been the subject of protein engineering programs, for example, the NAD^+-dependent enzyme from *Pseudomonas* sp. 101, which has been engineered to shift cofactor specificity toward $NADP^+$ [63]. The engineered FDH showed a catalytic efficiency (k_{cat}/K_M) for $NADP^+$ $> 100\ \mathrm{mM^{-1}\ s^{-1}}$, which is greater than threefold higher than natural NADP-FDHs ($k_{cat}/K_M \sim 30\ \mathrm{mM^{-1}\ s^{-1}}$ [64]). The engineered FDH variants have been adopted for metabolic engineering [65–67]. FDHs have also been engineered to accept NCRCs, including NCD^+ [68•,69••] and nicotinamide adenine dinucleotide 3′-phosphate (3′-$NADP^+$) [70]; these NCD-dependent FDHs achieved $k_{cat}/K_M = 1.45–1.5 \times 10^3$. Additionally, implementing an NCD-dependent lactate dehydrogenase (LDH) resulted in stoichiometric and stereospecific conversion of pyruvate into D-lactate [68]. Recent studies elucidated the structural architecture and mechanism of two O_2-tolerant FDHs from *Rhodobacter capsulatus* and *C. necator* [71,72], which exhibit potential for biotechnological applications. Consequently, an increase in studies exploring the engineering of these enzymes for NCRC acceptance is expected in the future.

CO_2 reduction and electron donors

The most oxidized C1 feedstock, CO_2, cannot be further oxidized, and its assimilation requires reducing power. This redox currency can be supplied within a CO_2 assimilation route; in some carboxylation reactions, for instance, CO_2 is directly activated with some cofactors, for example, NAD(P)H and ferredoxin [34]. Such carboxylation reactions are prime targets for engineering NCRC-dependent transformations. The only carboxylating enzymes engineered to accept NCRC are the malic enzyme [39,73] and FDH [68] that, as explained above, can act as a carboxylase in the reverse direction (CO_2 → formate).

An electron donor is needed to supply the reducing power needed for CO_2 assimilation. These mediators can be another, more reduced C1 molecule, or an inorganic donor, for example, H_2 or PO_3^{3-}. While H_2 can be efficiently generated from renewable electricity, technologies for PO_3^{3-} synthesis are not well developed. The strongly negative E' makes PO_3^{3-}, an attractive electron donor for thermodynamically challenging reductions. Hydrogenases, broadly classified as [NiFe]-hydrogenases, [FeFe]-hydrogenases, and [Fe]-hydrogenases, can harvest the electrons from H_2. The [NiFe]-hydrogenase enzyme from *Hydrogenophilus thermoluteolus* has been shown to accept NCRCs [74], with cofactor recycling supported by the addition of 1-benzyl-3-acetylpyridine (BAP^+). Phosphite dehydrogenases have also been engineered to accept NCD and NMN instead of their native cofactors [75,76].

In summary, enzymes involved in redox transformations of formate, formaldehyde, and PO_3^{3-} have drawn significant attention for engineering NCRC dependence. The reactions catalyzed by these enzyme variants were proposed and sometimes demonstrated as alternative electron suppliers. The next challenge in using NCRCs in synthetic C1 metabolism is integrating NCRC-dependent enzymes within assimilation pathways (and potentially production pathways). These challenges are discussed in the following sections.

Implementing altered redox potentials for C1 processing with noncanonical redox cofactors

The redox potential of NCRCs can be altered by changing their reactive moiety. For instance, the reactive moiety of NAD^+ is conserved in NMN^+ and NCD^+; hence, these NCRCs have a comparable redox potential as their natural counterpart (−0.32 V). As indicated previously, structural modifications on the nitrogen and C3 position [11,77] can drastically change the redox potential (Figure 1a). In this section, we describe how this phenomenon could be exploited to streamline the intricate reactions involved in the oxidation and reduction of C1 feedstocks based on energetic and thermodynamic parameters (Tables S1 and S2 in the Supplementary Material).

The chemical oxidation of CH_4 to CH_3OH is particularly demanding due to its high activation energy and risk of substrate overoxidation [78]. In this transformation, CH_4 conversion via MMOs emerges as an attractive alternative to chemical oxidation (Figure 1b). MMOs are highly specific and produce almost no side products, and the cognate reaction is extremely exergonic with $\Delta G'^{\circ} = -374.4\ \mathrm{kJ\ mol^{-1}}$ [79]. NADH is used to supply the energy needed to activate this reaction. Given the large energy dissipation in CH_4 oxidation, NCRCs could provide electrons still matching the activation energy while wasting less energy and heat.

Among all the C1 substrates that can support bioprocesses, CH_3OH offers the most ΔG to aerobic organisms, functions as a liquid mediator under standard conditions, and displays relatively low toxicity. These advantages are, however, contrasted by the thermodynamic challenges surrounding enzymatic CH_3OH oxidation [45], which limit the industrial applications of production C1-trophic strains relying on NAD-MDHs. In an ideal scenario, all electrons should be extracted from CH_3OH and routed into central carbon metabolism. NCRCs could meet these criteria since both PQQ and NAD, the natural cofactors for CH_3OH oxidation, are limited in their capacity of transferring electrons. NCRCs with higher redox potential than NADH could support thermodynamically favorable, energy-conserving reactions (Figure 1c). The higher redox potential in these examples can be realized either by changing the standard redox potential or altering the ratio between oxidized and reduced cofactors. For NAD^+/NADH, the ratio is strictly regulated by cell physiology [80]. For NCRCs, the ratio is more relaxed and depends on the standard redox potential and the actual cofactor concentration.

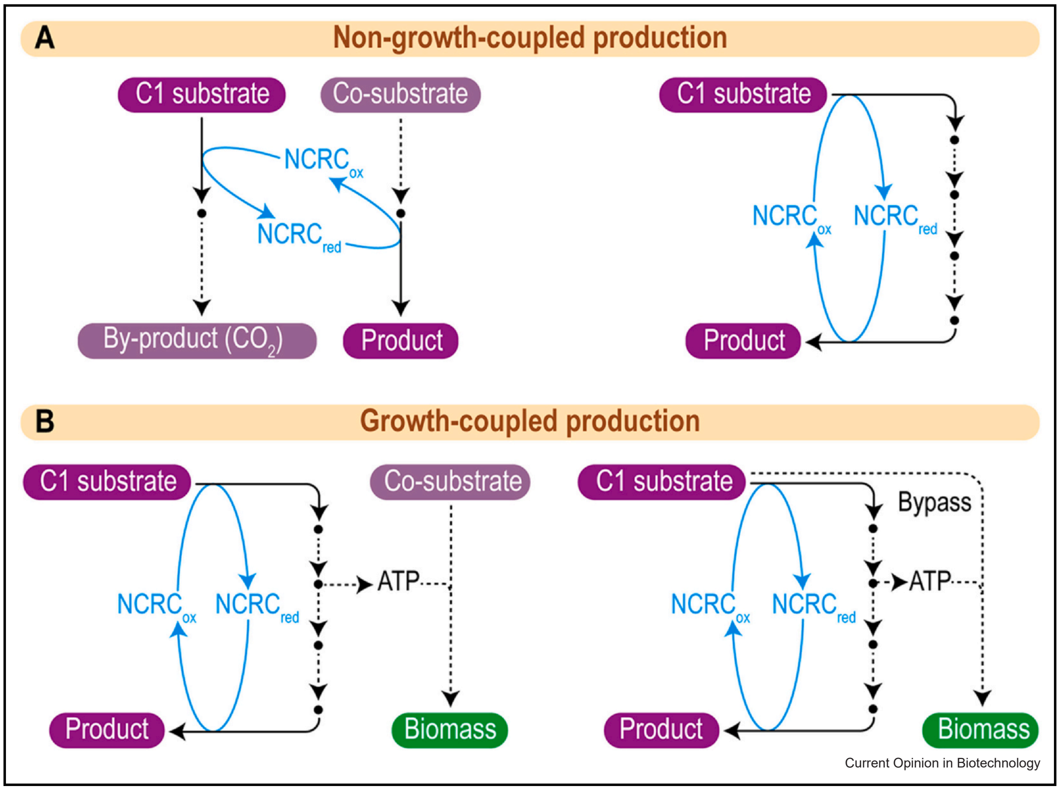
CO_2 is an equally attractive feedstock, although the industrial implementation of electrochemical CO_2 reduction faces several challenges, including a high energy demand, expensive catalysts, and challenging long-term operational stability and scalability [81,82]. The enzymatic reduction of CO_2 to formate, however, could potentially alleviate some hurdles associated with chemical reduction. Under physiological conditions, FDHs catalyze the oxidation of formate to CO_2 while reducing NAD^+; the equilibrium of the reaction is heavily shifted toward CO_2 and NADH formation (−14.4 kJ mol^{-1}). Accordingly, the reduction of CO_2 with NADH by FDH yields is not favored [83]. Establishing an NCRC with a redox potential < −0.42 V could tilt the equilibrium (Figure 1c). This possibility is intimated in the study by Zhang et al. [84], which employed methyl viologen (MV^{2+}), 1,1′-dicarboxymethyl-4,4′-bipyridinium bromine (DC^{2+}), and 1,1′-diaminoethyl-4,4′-bipyridinium bromine (DA^{2+}) instead of NADH. All these mediators have a redox potential between −0.39 and −0.48 V, which supported a catalytic efficiency of FDH > 500-fold higher than that observed with NAD^+. Such a drastic improvement was realized even without engineering FDH. Interestingly, the reaction proceeded the fastest with DA^{2+} even though this mediator has the highest redox potential (−0.39 V), attributed to the lower affinity of FDH for the other cofactors. Engineering FDH to increase the affinity toward NCRCs with stronger reducing power could significantly enhance conversion rates.

In summary, the examples in this section demonstrate how exploring the use of NCRCs with different reduction potentials can boost the conversion of C1 feedstocks. Implementing NCRCs that overcome some thermodynamic barriers associated inherently associated with the use of C1 substrates offers new solutions to long-standing problems in designing efficient C1 biorefineries.

Bringing electrons from C1 feedstocks into the metabolic circuit

We envision that once the electrons from C1 feedstocks are transferred to an NCRC, several applications become possible. As mentioned in the previous section, thermodynamically challenging conversions can be attempted by controlling the ratio between the reduced and oxidized pool of the NCRC in question by implementing metabolic bypasses [85]. Another option is to use NCRCs to directly couple C1 (or CO_2) conversion to product synthesis (Figure 2). This objective can be

Figure 2



Theoretical NCRC-dependent production schemes. Both **(a)** nongrowth-coupled and **(b)** growth-coupled production designs are contemplated. The production of a target molecule can either rely exclusively on C1 substrates (especially when they are sufficiently reduced) or on a combination of a C1 substrate and a co-substrate (e.g. a structurally complex product). Growth-coupled production can be achieved by having an NCRC-coupled route that is the only (or the most efficient) route in the cell to regenerate an essential metabolite (e.g. ATP). Therefore, growth can only occur if the production route is active. Other molecules essential for growth can be generated from a co-substrate utilization route or from a bypass that converts the C1 substrate into biomass building blocks. All schemes assume that a reduced C1 substrate is oxidized by the production pathway. Alternative designs apply to more oxidized C1 substrates (e.g. CO_2); in these cases, an electron carrier (H_2 or phosphite) supplies the reducing power and generates a by-product. The *red* and *ox* subindices identify the oxidation state of the NCRC.

achieved either in nongrowth-coupled or a growth-coupled scenarios.

In a nongrowth-coupled scenario, NCRC shuttles electrons between a substrate and product, but the operation of this coupling pathway is not essential for the growth of the cell factory. The stoichiometric coupling of formate consumption to the conversion of pyruvate (as a co-substrate) into malate fits this design. However, in such a setup, there is the risk that the pathway activity is lost during longer term cultivation. Often, such an NCRC-coupled production scheme is implemented in resting (nongrowing) cells. In contrast, in a growth-coupled scenario, the NCRC-coupled production pathway regenerates ATP (and, alternatively, other essential metabolites for growth that cannot be obtained or are produced less efficiently otherwise). Consequently, the cell factory becomes dependent ('addicted' [86•]) to the NCRC-coupled production pathway to support growth, allowing for stable operation (as it happens in continuous cultivations).

The examples above provide a qualitative understanding of the potential for employing NCRCs in C1-trophic production platforms. However, their implementation will depend on the specific host metabolic network and cellular homeostasis. To illustrate these points, we adopted a genome-scale metabolic model (GSMM) to explore the possibility of coupling NCRC reduction to the oxidation of a C1 feedstock under growing conditions. We have previously shown that the production of lactate could be stoichiometrically coupled to glucose consumption via NCRCs, in an ATP-producing route that supports microbial growth [10]. As a case study, we assessed NCRC-dependent growth of *Escherichia coli* engineered to assimilate CH_3OH via the RuMP pathway and producing lactate. CH_3OH is an interesting case study as it has a higher degree of reduction ($\gamma = 6$) than lactate ($\gamma = 4$) and other value-added products. We examined if the excess of reducing power could be used to fix additional CO_2 and increase product yield while ideally coupling the production pathway to bacterial growth. To test whether this synthetic metabolism is feasible, we posed the following questions: (1) can CH_3OH be used as only carbon and energy source if its oxidation is exclusively mediated by NCRCs? and (2) would the growth rate be negatively affected in such a scenario?

We used the most updated GSMM for *E. coli*, *i*ML1515 [87], to simulate optimal lactate production coupled to bacterial growth with NCRC circuits consisting of three dehydrogenases: (1) CH_3OH dehydrogenase (MDH, for NCRC reduction and assimilation of CH_3OH), (2) GAPDH (for NCRC reduction or oxidation in different CH_3OH assimilation routes), and (3) LDH (for NCRC oxidation and lactate synthesis). Details on the reactions modified in the *i*ML1515 GSMM are presented in Table S3 in the Supplementary Material. Next, we simulated four different NCRC circuits (Figure 3) as follows: (i) MDH reducing and LDH oxidizing, (ii) MDH reducing and GAPDH oxidizing, (iii) MDH reducing, GAPDH, and LDH oxidizing, and (iv) GAPDH reducing, LDH oxidizing.

A stoichiometric imbalance exists between the three CH_3OH molecules that need to be oxidized (thus generating three reduced NCRCs); this is not matched by synthesis of one lactate molecule (which can re-oxidize two NCRCs). This imbalance can be resolved by (partially) using an MDH coupled to ubiquinol to release some electrons, by co-fixing CO_2 during CH_3OH assimilation ($\gamma = 0$) via ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO shunt [88]), or by a combination of the two.

When these options were considered, we could model four circuits in which lactate production can be directly coupled to CH_3OH assimilation (Figure 3). In most of these scenarios, however, the cell has a more efficient route to generate ATP from CH_3OH than the NCRC-coupled route toward lactate (e.g. by fully oxidizing CH_3OH via PQQ-dependent MDH, formaldehyde dehydrogenase, and FDH and using the electrons from quinones and NADH to drive ATP generation via respiration). In our modeling calculations, the cell generates a net amount of ATP within the lactate production route only in circuit (iv), while the more ATP-efficient route via formaldehyde dehydrogenase and FDH could be eliminated. In other words, this last circuit is the only one that can be strictly coupled to growth. Although this synthetic circuit has the lowest theoretical lactate yield on CH_3OH, it offers the advantage of providing growth-coupling, generating an NCRC-dependent route that could still result in stable, C1-driven production of a bulk chemical.

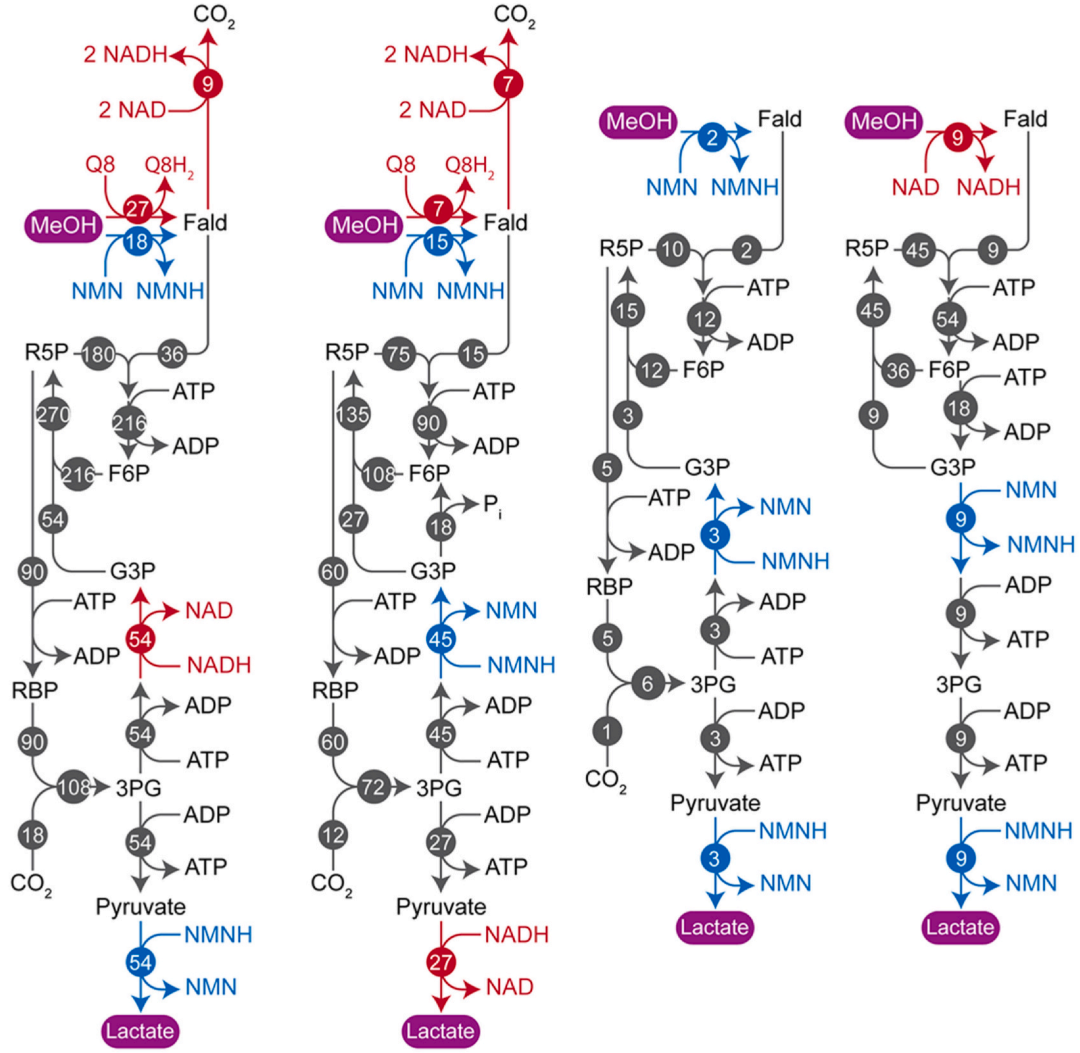
Outlook

In this review, we explored the possibility of combining NCRCs with synthetic C1 assimilation by engineered cell factories. We envision that intersecting these approaches will open avenues for C1-based biomanufacturing. For example, as illustrated in some of the examples, using NCRCs to support assimilation of C1 feedstocks could alleviate thermodynamic limitations inherent to processing these substrates. Additionally, coupling C1 oxidation to product formation in a mixotrophic or resting cell setup could increase the stoichiometric yield of the overall process. *In vivo* implementation of these orthogonal circuits will determine if the modeling data reported here is supported by experimental results. Another interesting case that

Figure 3

Synthetic circuit	(i) MDH and LDH	(ii) MDH and GAPDH	(iii) MDH,GAPDH and LDH	(iv) GAPDH and LDH

Graphical representation



	(i) MDH and LDH	(ii) MDH and GAPDH	(iii) MDH,GAPDH and LDH	(iv) GAPDH and LDH
Net conversion (catabolic)	45 MeOH + 9 CO_2 + 13.5 O_2 → 18 Lactate 1 MeOH + 0.2 CO_2 + 0.3 O_2 → 0.4 Lactate	22 MeOH + 5 CO_2 + 6 O_2 → 9 Lactate 1 MeOH + 0.23 CO_2 + 0.27 O_2 → 0.41 Lactate	2 MeOH + 1 CO_2 → 1 Lactate 1 MeOH + 0.5 CO_2 → 0.5 Lactate	9 MeOH + 4.5 O_2 → 3 Lactate 1 MeOH + 0.5 O_2 → 0.33 Lactate
ATP yield [ATP/MeOH]	0.5	0	–0.5	2.1
Growth-coupling potential	No	No	No	Yes

Current Opinion in Biotechnology

Metabolic pathways to convert methanol (MeOH) into lactate in engineered *E. coli*. The simulations used four different oxido-reductase couples via the synthetic RuMP pathway with possible additional CO_2 fixation. The numbers in the flux distribution represent the flux of the reaction relative to the carbon atoms. The ATP yield was calculated assuming a P/O ratio of 1.5. The growth coupling potential was assessed in an *E. coli* GSMM where the catabolic pathway with the highest yield is active; coupling potential is possible when a set of knock-outs within the native metabolic network render the pathway of interest as the one with the highest ATP yield. Modifications introduced in the *i*ML1515 GSMM of *E. coli* are listed in Table S3 in the Supplementary Material.

deserves exploration is using NCRCs to enable growth-coupled, stable production from C1 feedstocks.

By adopting lactate production from CH_3OH as a modeling case, we demonstrate that growth-coupled production from this C1 feedstock is possible and favorable. However, this analysis also reveals some limitations in identifying growth-coupled circuits due to imbalanced stoichiometries between NCRC reduction and oxidation and restrictions in the ATP yields from some NCRC-coupled circuits. Achieving growth-coupled production requires that the synthetic circuit offers the highest ATP yield. Following this reasoning, a similar analysis can be performed to identify such circuits for other C1 substrates and products.

Although *in vivo* NCRC-dependent pathways have been realized [89•], the successful implementation of alternative NCRCs for C1 assimilation has yet to occur. Therefore, more enzymes involved in C1 or H_2 oxidation need to be engineered to become NCRC-reducing enzymes. Similarly, downstream enzymes involved in reducing reactions for CO_2 fixation or product formation need to be converted to NCRC-oxidizing enzymes. This can be achieved either through rational protein engineering or, in some cases, growth-coupled *in vivo* selection, especially when an existing NCRC-reducing or NCRC-oxidizing enzyme can be coupled to the functionality of the enzyme being improved.

In addition, the increasing amount of data on NCRC-dependent enzymes could be used to train machine learning algorithms to predict the modifications required for expanding the use of NCRCs in other redox enzymes. Developing high-throughput platforms to increase the enzymatic data set for these algorithms will be equally important. We propose that the combined use of machine learning approaches [90] and high-throughput screening systems [91–93] will accelerate the development of new NCRC-dependent enzymes, leading to the engineering and implementation of truly orthogonal redox systems that can either improve C1 feedstock utilization or enable completely new assimilation pathways. In this scenario, libraries of rationally generated mutant enzymes that can be coupled to growth could be easily tested. Growth fitness associated with specific mutations can be used to inform the machine learning pipeline and strengthen our ability to predict beneficial mutations for NCRCs utilization. This exciting prospect holds the potential of revolutionizing biomanufacturing and promoting a true circular carbon economy [94].

CRediT authorship contribution statement

E.O., D.C.V., R.A.W., and P.I.N.: Conceptualization; **J.M.H.S., M.S.R., A.J.K., D.C.V., C.E.P., and F.J.B.:** Methodology, Validation, Formal analysis, Resources; **N.J.C., C.E.P., F.J.B., R.A.W., and P.I.N.:** Project administration; **R.A.W., and P.I.N.:** Funding acquisition; **E.O., J.M.H.S., M.S.R., A.J.K., D.C.V., N.J.C., C.E.P., F.J.B., R.A.W., and P.I.N.:** Writing – original draft; **E.O., J.M.H.S., D.C.V., and P.I.N.:** Writing – review & editing.

Data Availability

Data will be made available on request.

Declaration of Competing Interest

Nothing declared.

Acknowledgements

The seminal ideas that led to this article originated from fruitful discussions in the context of the *Taking control of metabolism in microbial cell factories by applying noncanonical redox cofactors* consortium. E.O. was supported by the European Union's Horizon 2020 Research and Innovation Program under the Marie Skłodowska-Curie grant agreement 101065339 (ROAD). M.S.R., N.J.C., C.E.P., F.J.B., and R.A.W. were supported by the Netherlands Science Organization (NWO) through the OCENW.XL21. XL21.007 grant (*Taking control of metabolism in microbial cell factories by applying noncanonical redox cofactors*). The financial support from the Novo Nordisk Foundation (NNF10CC1016517, NNF18CC0033664, and NNF23OC0083631) to P.I.N. is gratefully acknowledged.

Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.copbio.2024.103195.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Gargalo CL, Udugama I, Pontius K, López PC, Nielsen RF, Hasanzadeh A, Mansouri SS, Bayer C, Junicke H, Gernaey KV: **Towards smart biomanufacturing: a perspective on recent developments in industrial measurement and monitoring technologies for bio-based production processes**. *J Ind Microbiol Biotechnol* 2020, **47**:947-964.
2. Konzock O, Nielsen J: **TRYing to evaluate production costs in microbial biotechnology**. *Trends Biotechnol* 2024, https://doi.org/10.1016/j.tibtech.2024.04.007

3. Czajka J, Wang Q, Wang Y, Tang YJ: **Synthetic biology for manufacturing chemicals: constraints drive the use of non-conventional microbial platforms**. *Appl Microbiol Biotechnol* 2017, **101**:7427-7434.

4. van Dien S: **From the first drop to the first truckload: commercialization of microbial processes for renewable chemicals**. *Curr Opin Biotechnol* 2013, **24**:1061-1068.

5. Nielsen J, Keasling JD: **Engineering cellular metabolism**. *Cell* 2016, **164**:1185-1197.

6. Ögmundarson O, Herrgård MJ, Forster J, Hauschild MZ, Fantke P: **Addressing environmental sustainability of biochemicals**. *Nat Sustain* 2020, **3**:167-174.

7. Fu R, Kang L, Zhang C, Fei Q: **Application and progress of techno-economic analysis and life cycle assessment in biomanufacturing of fuels and chemicals**. *Green Chem Eng* 2023, **4**:189-198.

8. Kracke F, Lai B, Yu S, Krömer JO: **Balancing cellular redox metabolism in microbial electrosynthesis and electro fermentation — a chance for metabolic engineering**. *Metab Eng* 2018, **45**:109-120.

9. Folch PL, Bisschops MMM, Weusthuis RA: **Metabolic energy conservation for fermentative product formation**. *Microb Biotechnol* 2021, **14**:829-858.

10. •• Weusthuis RA, Folch PL, Pozo-Rodríguez A, Paul CE: **Applying non-canonical redox cofactors in fermentation processes**. *iScience* 2020, **23**:101471.

A perspective article proposing the use of NCRCs to achieve maximum theoretical yields of substrate-to-product conversions.

11. • Black WB, Perea S, Li H: **Design, construction, and application of noncanonical redox cofactor infrastructures**. *Curr Opin Biotechnol* 2023, **84**:103019.

A recent review article discussing the state-of-the-art use of NCRCs in biological systems.

12. King E, Maxel S, Li H: **Engineering natural and noncanonical nicotinamide cofactor-dependent enzymes: design principles and technology development**. *Curr Opin Biotechnol* 2020, **66**:217-226.

13. Zhang L, King E, Black WB, Heckmann CM, Wolder A, Cui Y, Nicklen F, Siegel JB, Luo R, Paul CE, Li H: **Directed evolution of phosphite dehydrogenase to cycle noncanonical redox cofactors via universal growth selection platform**. *Nat Commun* 2022, **13**:5021.

14. •• King E, Maxel S, Zhang Y, Kenney KC, Cui Y, Luu E, Siegel JB, Weiss GA, Luo R, Li H: **Orthogonal glycolytic pathway enables directed evolution of noncanonical cofactor oxidase**. *Nat Commun* 2022, **13**:7282.

Seminal work describing the establishment of a growth-coupled design using NMNH-based reducing power to support microbial growth through a synthetic glycolysis.

15. • Black WB, Zhang L, Mak WS, Maxel S, Cui Y, King E, Fong B, Sanchez Martinez A, Siegel JB, Li H: **Engineering a nicotinamide mononucleotide redox cofactor system for biocatalysis**. *Nat Chem Biol* 2020, **16**:87-94.

An elegant demonstration of the use of the NCRC NMN$^+$/(H) pair *in vivo* for generating a value-added compound with high product specificity.

16. Bachleitner S, Ata Ö, Mattanovich D: **The potential of CO_2-based production cycles in biotechnology to fight the climate crisis**. *Nat Commun* 2023, **14**:6978.

17. Claassens NJ, Cotton CAR, Kopljar D, Bar-Even A: **Making quantitative sense of electromicrobial production**. *Nat Catal* 2019, **2**:437-447.

18. Lv X, Yu W, Zhang C, Ning P, Li J, Liu Y, Du G, Liu L: **C1-based biomanufacturing: advances, challenges and perspectives**. *Bioresour Technol* 2023, **367**:128259.

19. Zhan C, Li X, Yang Y, Nielsen J, Bai Z, Chen Y: **Strategies and challenges with the microbial conversion of methanol to high-value chemicals**. *Biotechnol Bioeng* 2021, **118**:3655-3668.

20. Lips D: **Fuelling the future of sustainable sugar fermentation across generations**. *Eng Biol* 2022, **6**:3-16.

21. Claassens NJ, Sánchez-Andrea I, Sousa DZ, Bar-Even A: **Towards sustainable feedstocks: a guide to electron donors for microbial carbon fixation**. *Curr Opin Biotechnol* 2018, **50**:195-205.

22. Scown CD: **Prospects for carbon-negative biomanufacturing**. *Trends Biotechnol* 2022, **40**:1415-1424.

23. Nguyen AD, Lee EY: **Engineered methanotrophy: a sustainable solution for methane-based industrial biomanufacturing**. *Trends Biotechnol* 2021, **39**:381-396.

24. Li Y, Alaimo CP, Kim M, Kado NY, Peppers J, Xue J, Wan C, Green PG, Zhang R, Jenkins BM, *et al.*: **Composition and toxicity of biogas produced from different feedstocks in California**. *Environ Sci Technol* 2019, **53**:11569-11579.

25. Zang G, Sun P, Elgowainy A, Wang M: **Technoeconomic and life cycle analysis of synthetic methanol production from hydrogen and industrial byproduct CO_2**. *Environ Sci Technol* 2021, **55**:5248-5257.

26. Cotton CA, Claassens NJ, Benito-Vaquerizo S, Bar-Even A: **Renewable methanol and formate as microbial feedstocks**. *Curr Opin Biotechnol* 2020, **62**:168-180.

27. Stöckl M, Claassens NJ, Lindner SN, Klemm E, Holtmann D: **Coupling electrochemical CO_2 reduction to microbial product generation — identification of the gaps and opportunities**. *Curr Opin Biotechnol* 2022, **74**:154-163.

28. • Orsi E, Nikel PI, Nielsen LK, Donati S: **Synergistic investigation of natural and synthetic C1-trophic microorganisms to foster a circular carbon economy**. *Nat Commun* 2023, **14**:6673.

This perspective article proposes a synergistic approach to studying C1 assimilation in natural and synthetic hosts to advance C1-based biomanufacturing.

29. Schulz-Mirbach H, Dronsella B, He H, Erb TJ: **Creating new-to-nature carbon fixation: a guide**. *Metab Eng* 2024, **82**:12-28.

30. Gassler T, Sauer M, Gasser B, Egermeier M, Troyer C, Causon T, Hann S, Mattanovich D, Steiger MG: **The industrial yeast *Pichia pastoris* is converted from a heterotroph into an autotroph capable of growth on CO_2**. *Nat Biotechnol* 2020, **38**:210-216.

31. Sauvageau D, Stein LY, Arenas E, Das S, Iacobelli M, Lawley M, Lazic M, Rondón FL, Weiblen C: **Industrializing methanotrophs and other methylotrophic bacteria: from bioengineering to product recovery**. *Curr Opin Biotechnol* 2024, **88**:103167.

32. Pfeifenschneider J, Brautaset T, Wendisch VF: **Methanol as carbon substrate in the bio-economy: metabolic engineering of aerobic methylotrophic bacteria for production of value-added chemicals**. *Biofuels Bioprod Bioref* 2017, **11**:719-731.

33. Volke DC, Orsi E, Nikel PI: **Emergent CRISPR-Cas-based technologies for engineering non-model bacteria**. *Curr Opin Microbiol* 2023, **75**:102353.

34. Bierbaumer S, Nattermann M, Schulz L, Zschoche R, Erb TJ, Winkler CK, Tinzl M, Glueck SM: **Enzymatic conversion of CO_2: from natural to artificial utilization**. *Chem Rev* 2023, **123**:5702-5754.

35. • Sanford PA, Woolston BM: **Synthetic or natural? Metabolic engineering for assimilation and valorization of methanol**. *Curr Opin Biotechnol* 2022, **74**:171-179.

Thought-provoking discussion of the pros and cons of engineering methylotrophy in natural or synthetic microbial hosts.

36. Reiter MA, Bradley T, Büchel LA, Keller P, Hegedis E, Gassler T, Vorholt JA: **A synthetic methylotrophic *Escherichia coli* as a *chassis* for bioproduction from methanol**. *Nat Catal* 2024, **7**:560-573.

37. Nieh LY, Chen FYH, Jung HW, Su KY, Tsuei CY, Lin CT, Lee YQ, Liao JC: **Evolution engineering of methylotrophic *E. coli* enables faster growth than native methylotrophs**: *bioRxiv,* 2024, 〈doi:10.1101/2024.04.18.589993〉.

38. King E, Cui Y, Aspacio D, Nicklen F, Zhang L, Maxel S, Luo R, Siegel JB, Aitchison E, Li H: **Engineering Embden–Meyerhof–Parnas glycolysis to generate noncanonical reducing power**. *ACS Catal* 2022, **12**:8582-8592.

39. • Wang X, Feng Y, Guo X, Wang Q, Ning S, Li Q, Wang J, Wang L, Zhao ZK: **Creating enzymes and self-sufficient cells for biosynthesis of the non-natural cofactor nicotinamide cytosine dinucleotide**. *Nat Commun* 2021, **12**:2116.

Demonstration of how a synthetic NCD$^+$/(H) circuit can support the conversion of malate into lactate *in vivo*.

40. Aspacio D, Zhang Y, Cui Y, King E, Black WB, Perea S, Luu E, Siegel JB, Li H: **Shifting redox reaction equilibria on demand using an orthogonal redox cofactor**; *bioRxiv*, 2023, ⟨doi:10.1101/2023.1108.1129.555398⟩.

41. Richardson KN, Black WB, Li H: **Aldehyde production in crude lysate- and whole cell-based biotransformation using a noncanonical redox cofactor system**. *ACS Catal* 2020, **10**:8898-8903.

42. Lipscomb JD: **Biochemistry of the soluble methane monooxygenase**. *Annu Rev Microbiol* 1994, **48**:371-399.

43. Murrell JC, Gilbert B, McDonald IR: **Molecular biology and regulation of methane monooxygenase**. *Arch Microbiol* 2000, **173**:325-332.

44. Khider MLK, Brautaset T, Irla M: **Methane monooxygenases: central enzymes in methanotrophy with promising biotechnological applications**. *World J Microbiol Biotechnol* 2021, **37**:72.

45. • Krüsemann JL, Rainaldi V, Cotton CA, Claassens NJ, Lindner SN: **The cofactor challenge in synthetic methylotrophy: bioengineering and industrial applications**. *Curr Opin Biotechnol* 2023, **82**:102953.

A comprehensive survey of the challenges in engineering efficient microbial methylotrophy using naturally occurring redox cofactors.

46. Le TK, Lee YJ, Han GH, Yeom SJ: **Methanol dehydrogenases as a key biocatalysts for synthetic methylotrophy**. *Front Bioeng Biotechnol* 2021, **9**:787791.

47. Wu TY, Chen CT, Liu JT, Bogorad IW, Damoiseaux R, Liao JC: **Characterization and evolution of an activator-independent methanol dehydrogenase from *Cupriavidus necator* N-1**. *Appl Microbiol Biotechnol* 2016, **100**:4969-4983.

48. Chen FY, Jung HW, Tsuei CY, Liao JC: **Converting *Escherichia coli* to a synthetic methylotroph growing solely on methanol**. *Cell* 2020, **182**:933-946.

49. Keller P, Reiter MA, Kiefer P, Gassler T, Hemmerle L, Christen P, Noor E, Vorholt JA: **Generation of an *Escherichia coli* strain growing on methanol via the ribulose monophosphate cycle**. *Nat Commun* 2022, **13**:5243.

50. Schendel FJ, Bremmon CE, Flickinger MC, Guettler M, Hanson RS: **L-lysine production at 50°C by mutants of a newly isolated and characterized methylotrophic *Bacillus* sp.** *Appl Environ Microbiol* 1990, **56**:963-970.

51. Arfman N, Van Beeumen J, De Vries GE, Harder W, Dijkhuizen L: **Purification and characterization of an activator protein for methanol dehydrogenase from thermotolerant *Bacillus* spp.** *J Biol Chem* 1991, **266**:3955-3960.

52. Ochsner AM, Müller JE, Mora CA, Vorholt JA: ***In vitro* activation of NAD-dependent alcohol dehydrogenases by Nudix hydrolases is more widespread than assumed**. *FEBS Lett* 2014, **588**:2993-2999.

53. Gregory GJ, Bennett RK, Papoutsakis ET: **Recent advances toward the bioconversion of methane and methanol in synthetic methylotrophs**. *Metab Eng* 2022, **71**:99-116.

54. Federici F, Orsi E, Nikel PI: **From rags to riches: exploiting the Calvin-Benson-Bassham cycle for biomanufacturing**. *ChemCatChem* 2023, **15**:e202300746.

55. Zhang W, Zhang T, Wu S, Wu M, Xin F, Dong W, Ma J, Zhang M, Jiang M: **Guidance for engineering of synthetic methylotrophy based on methanol metabolism in methylotrophy**. *RSC Adv* 2017, **7**:4083-4091.

56. de Lorenzo V, Pérez-Pantoja D, Nikel PI: ***Pseudomonas putida* KT2440: the long journey of a soil-dweller to become a synthetic biology *chassis***. *J Bacteriol* 2024, **206**:e00136-24.

57. Volke DC, Calero P, Nikel PI: **Pseudomonas putida**. *Trends Microbiol* 2020, **28**:512-513.

58. Weimer A, Kohlstedt M, Volke DC, Nikel PI, Wittmann C: **Industrial biotechnology of *Pseudomonas putida*: advances and prospects**. *Appl Microbiol Biotechnol* 2020, **104**:7745-7766.

59. Turlin J, Puiggené Ò, Donati S, Wirth NT, Nikel PI: **Core and auxiliary functions of one-carbon metabolism in *Pseudomonas putida* exposed by a systems-level analysis of transcriptional and physiological responses**. *mSystems* 2023, **8**:e00004-23.

60. Berrios J, Theron CW, Steels S, Ponce B, Velastegui E, Bustos C, Altamirano C, Fickers P: **Role of dissimilative pathway of *Komagataella phaffii* (*Pichia pastoris*): formaldehyde toxicity and energy metabolism**. *Microorganisms* 2022, **10**:1466.

61. •• Wang J, Guo X, Wan L, Liu Y, Xue H, Zhao ZK: **Engineering formaldehyde dehydrogenase from *Pseudomonas putida* to favor nicotinamide cytosine dinucleotide**. *ChemBioChem* 2022, **23**:e202100697.

This research article presents the engineering of a formaldehyde dehydrogenase to increase its preference for the NCRC NCD$^+$.

62. Nielsen CF, Lange L, Meyer AS: **Classification and enzyme kinetics of formate dehydrogenases for biomanufacturing via CO_2 utilization**. *Biotechnol Adv* 2019, **37**:107408.

63. Calzadiaz-Ramírez L, Meyer AS: **Formate dehydrogenases for CO_2 utilization**. *Curr Opin Biotechnol* 2021, **73**:95-100.

64. Hatrongjit R, Packdibamrung K: **A novel NADP$^+$-dependent formate dehydrogenase from *Burkholderia stabilis* 15516: screening, purification and characterization**. *Enzym Microb Technol* 2010, **46**:557-561.

65. Kim S, Lindner SN, Aslan S, Yishai O, Wenk S, Schann K, Bar-Even A: **Growth of *E. coli* on formate and methanol via the reductive glycine pathway**. *Nat Chem Biol* 2020, **16**:538-545.

66. Volke DC, Martino RA, Kozaeva E, Smania AM, Nikel PI: **Modular (de)construction of complex bacterial phenotypes by CRISPR/nCas9-assisted, multiplex cytidine base-editing**. *Nat Commun* 2022, **13**:3026.

67. Turlin J, Dronsella B, De Maria A, Lindner SN, Nikel PI: **Integrated rational and evolutionary engineering of genome-reduced *Pseudomonas putida* strains promotes synthetic formate assimilation**. *Metab Eng* 2022, **74**:191-205.

68. • Guo X, Wang X, Liu Y, Li Q, Wang J, Liu W, Zhao ZK: **Structure-guided design of formate dehydrogenase for regeneration of a non-natural redox cofactor**. *Chemistry* 2020, **26**:16611-16615.

A formate dehydrogenase engineered to contribute to an orthogonal electron circuit mediated by the NCD$^+$/(H) pair.

69. •• Guo X, Liu Y, Wang Q, Wang X, Li Q, Liu W, Zhao ZK: **Non-natural cofactor and formate-driven reductive carboxylation of pyruvate**. *Angew Chem Int Ed Engl* 2020, **59**:3143-3146.

Smart coupling of an FDH and an NCRC-dependent malic enzyme for formate assimilation, both as a C1 source and electron donor.

70. Vainstein S, Banta S: **Engineering *Candida boidinii* formate dehydrogenase for activity with the non-canonical cofactor 3′-NADP(H)**. *Protein Eng Des Sel* 2023, **36**:gzad009.

71. Radon C, Mittelstädt G, Duffus BR, Bürger J, Hartmann T, Mielke T, Teutloff C, Leimkühler S, Wendler P: **Cryo-EM structures reveal intricate Fe-S cluster arrangement and charging in *Rhodobacter capsulatus* formate dehydrogenase**. *Nat Commun* 2020, **11**:1912.

72. Harmer JR, Hakopian S, Niks D, Hille R, Bernhardt PV: **Redox characterization of the complex molybdenum enzyme formate dehydrogenase from *Cupriavidus necator***. *J Am Chem Soc* 2023, **145**:25850-25863.

73. Ji D, Wang L, Hou S, Liu W, Wang J, Wang Q, Zhao ZK: **Creation of bioorthogonal redox systems depending on nicotinamide flucytosine dinucleotide**. *J Am Chem Soc* 2011, **133**:20857-20862.

74. Reeve HA, Nicholson J, Altaf F, Lonsdale TH, Preissler J, Lauterbach L, Lenz O, Leimkühler S, Hollmann F, Paul CE, Vincent KA: **A hydrogen-driven biocatalytic approach to recycling**

synthetic analogues of NAD(P)H. *Chem Commun* 2022, **58**:10540-10543.

75. Liu Y, Feng Y, Wang L, Guo X, Liu W, Li Q, Wang X, Xue S, Zhao ZK: **Structural insights into phosphite dehydrogenase variants favoring a non-natural redox cofactor**. *ACS Catal* 2019, **9**:1883-1887.

76. Wang L, Ji D, Liu Y, Wang Q, Wang X, Zhou YJ, Zhang Y, Liu W, Zhao ZK: **Synthetic cofactor-linked metabolic circuits for selective energy transfer**. *ACS Catal* 2017, **7**:1977-1983.

77. Paul CE, Arends IWCE, Hollmann F: **Is simpler better? Synthetic nicotinamide cofactor analogues for redox chemistry**. *ACS Catal* 2014, **4**:788-797.

78. Dummer NF, Willock DJ, He Q, Howard MJ, Lewis RJ, Qi G, Taylor SH, Xu J, Bethell D, Kiely CJ, Hutchings GJ: **Methane oxidation to methanol**. *Chem Rev* 2023, **123**:6359-6411.

79. Beber ME, Gollub MG, Mozaffari D, Shebek KM, Flamholz AI, Milo R, Noor E: ***eQuilibrator* 3.0: a database solution for thermodynamic constant estimation**. *Nucleic Acids Res* 2022, **50**:D603-D609.

80. Spaans SK, Weusthuis RA, van der Oost J, Kengen SW: **NADPH-generating systems in bacteria and archaea**. *Front Microbiol* 2015, **6**:742.

81. Garg S, Li M, Weber AZ, Ge L, Li L, Rudolph V, Wang G, Rufford TE: **Advances and challenges in electrochemical CO_2 reduction processes: an engineering and design perspective looking beyond new catalyst materials**. *J Mat Chem* 2020, **8**:1511-1544.

82. Yuan L, Zeng S, Zhang X, Ji X, Zhang S: **Advances and challenges of electrolyzers for large-scale CO_2 electroreduction**. *Mater Rep* 2023, **3**:100177.

83. Zhang Z, Muschiol J, Huang Y, Sigurdardóttir SB, von Solms N, Daugaard AE, Wei J, Luo J, Xu BH, Zhang S, Pinelo M: **Efficient ionic liquid-based platform for multi-enzymatic conversion of carbon dioxide to methanol**. *Green Chem* 2018, **20**:4339-4348.

84. Zhang Z, Vasiliu T, Li F, Laaksonen A, Mocci F, Ji X: **Electrochemically driven efficient enzymatic conversion of CO_2 to formic acid with artificial cofactors**. *J CO_2 Util* 2021, **52**:101679.

85. Orsi E, Claassens NJ, Nikel PI, Lindner SN: **Optimizing microbial networks through metabolic bypasses**. *Biotechnol Adv* 2022, **60**:108035.

86. • Orsi E, Claassens NJ, Nikel PI, Lindner SN: **Growth-coupled selection of synthetic modules to accelerate cell factory development**. *Nat Commun* 2021, **12**:5295.

A thorough description of growth-coupled designs to streamline and facilitate the DBTL cycle of synthetic biology when applied to microbial cell factories.

87. Monk JM, Lloyd CJ, Brunk E, Mih N, Sastry A, King Z, Takeuchi R, Nomura W, Zhang Z, Mori H, *et al*.: ***i*ML1515, a knowledgebase that computes *Escherichia coli* traits**. *Nat Biotechnol* 2017, **35**:904-908.

88. Westenberg R, Peralta-Yahya P: **Toward implementation of carbon-conservation networks in nonmodel organisms**. *Curr Opin Biotechnol* 2023, **81**:102949.

89. • Nielsen JR, Weusthuis RA, Huang WE: **Growth-coupled enzyme engineering through manipulation of redox cofactor regeneration**. *Biotechnol Adv* 2023, **63**:108102.

Detailed review article illustrating growth-coupling strategies for efficient regeneration of redox cofactors, including NCRCs.

90. Mazurenko S, Prokop Z, Damborsky J: **Machine learning in enzyme engineering**. *ACS Catal* 2020, **10**:1210-1223.

91. Gurdo N, Volke DC, McCloskey D, Nikel PI: **Automating the design-build-test-learn cycle towards next-generation bacterial cell factories**. *New Biotechnol* 2023, **74**:1-15.

92. Gurdo N, Volke DC, Nikel PI: **Merging automation and fundamental discovery into the design–build–test–learn cycle of nontraditional microbes**. *Trends Biotechnol* 2022, **40**:1148-1159.

93. Kozaeva E, Volkova S, Matos MRA, Mezzina MP, Wulff T, Volke DC, Nielsen LK, Nikel PI: **Model-guided dynamic control of essential metabolic nodes boosts acetyl-coenzyme A–dependent bioproduction in rewired *Pseudomonas putida***. *Metab Eng* 2021, **67**:373-386.

94. Reardon S: **How synthetic biologists are building better biofactories**. *Nature* 2024, **628**:224-226.

95. Liu Y, Li Q, Wang L, Guo X, Wang J, Wang Q, Zhao ZK: **Engineering D-lactate dehydrogenase to favor an non-natural cofactor nicotinamide cytosine dinucleotide**. *ChemBioChem* 2020, **21**:1972-1975.