Biotechnology Advances 63 (2023) 108102

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Growth-coupled enzyme engineering through manipulation of redox cofactor regeneration

Jochem R. Nielsen [a], Ruud A. Weusthuis [b], Wei E. Huang [a,*]

[a] Department of Engineering Science, University of Oxford, Oxford OX1 3PJ, United Kingdom
[b] Department of Bioprocess Engineering, Wageningen University & Research, Wageningen 6700AA, the Netherlands

ARTICLE INFO

Keywords:
Growth-coupling
Metabolic engineering
Redox cofactor
Enzyme engineering
Redox cofactor auxotrophy
Non-canonical redox cofactor

ABSTRACT

Enzymes need to be efficient, robust, and highly specific for their effective use in commercial bioproduction. These properties can be introduced using various enzyme engineering techniques, with random mutagenesis and directed evolution (DE) often being chosen when there is a lack of structural information -or mechanistic understanding- of the enzyme. The screening or selection step of DE is the limiting part of this process, since it must ideally be (ultra)-high throughput, specifically target the catalytic activity of the enzyme and have an accurately quantifiable metric for said activity. Growth-coupling selection strategies involve coupling a desired enzyme activity to cellular metabolism and therefore growth, where growth (rate) becomes the output metric. Redox cofactors (NAD^+/NADH and $NADP^+$/NADPH) have recently been identified as promising target molecules for growth coupling, owing to their essentiality for cellular metabolism and ubiquitous nature. Redox cofactor oxidation or reduction can be disrupted through metabolic engineering and the use of specific culturing conditions, rendering the cell inviable unless a 'rescue' reaction complements the imposed metabolic deficiency. Using this principle, enzyme variants displaying improved cofactor oxidation or reduction rates can be selected for through an increased growth rate of the cell. In recent years, several *E. coli* strains have been developed that are deficient in the oxidation or reduction of NAD^+/NADH and $NADP^+$/NADPH pairs, and of non-canonical redox cofactor pairs NMN^+/NMNH and NCD^+/NCDH, which provides researchers with a versatile toolbox of enzyme engineering platforms. A range of redox cofactor dependent enzymes have since been engineered using a variety of these strains, demonstrating the power of using this growth-coupling technique for enzyme engineering. This review aims to summarize the metabolic engineering involved in creating strains auxotrophic for the reduced or oxidized state of redox cofactors, and the resulting successes in using them for enzyme engineering. Perspectives on the unique features and potential future applications of this technique are also presented.

1. Introduction

An increasingly wide range of chemicals are now being produced from renewable feedstocks through biotechnological means (Lee et al., 2019). Bioprocesses rely heavily on enzymatic catalysis for the efficient production of these compounds. These reactions can take place *in vitro* using purified enzymes, or through whole-cell *in vivo* catalysis (Claassens et al., 2019; Rasor et al., 2021). Ensuring that enzymes perform optimally in their required environment is therefore of high interest for sustainable production (Scown and Keasling, 2022).

When potentially useful enzymes are discovered, their native functionality and characteristics often align poorly with the requirements for their use in commercial bioprocesses. Properties such as substrate specificity, catalytic rate and (thermo)stability are among several critical factors that must be optimized for efficient enzyme-driven bioprocesses (Bornscheuer et al., 2012). This optimization can be time consuming, costly, and challenging, and therefore effective and cost-efficient ways for engineering enzymes with desirable characteristics are highly sought after.

To address these challenges, a wide variety of protein engineering approaches have been developed, which includes methodologies for identifying improved enzyme variants through screening and/or selection.

* Corresponding author.
E-mail addresses: jochem.nielsen@worc.ox.ac.uk (J.R. Nielsen), ruud.weusthuis@wur.nl (R.A. Weusthuis), wei.huang@eng.ox.ac.uk (W.E. Huang).

https://doi.org/10.1016/j.biotechadv.2023.108102
Received 1 September 2022; Received in revised form 11 January 2023; Accepted 15 January 2023
Available online 18 January 2023

1.1. Protein engineering

Protein engineering methods are now widely used to improve and optimize enzymatic properties. These methods can roughly be divided into rational-, semi-rational- and random approaches. The first requires full structural knowledge of the enzyme in question, allowing for structure-guided site-specific engineering (Brannigan and Wilkinson, 2002). Semi-rational approaches cover a spectrum of protein engineering methods, neither fully rational nor random. These methods usually involve predictions of enzyme structures, sometimes in combination with other data such as phylogenetic analyses or molecular dynamics simulations, to infer regions and/or single residues that may be suitable targets for engineering (Lutz, 2010). Computational predictions of protein structures, such as those made by AlphaFold, are further driving the use of (semi)-rational protein engineering approaches (Jumper et al., 2021). Recently, DeepMind has announced that their AlphaFold tool has now made computational predictions of protein structures for almost every known protein – some 200 million (Callaway, 2022). Protein structure prediction tools can also support *de novo* protein design, paving the way towards made-for-purpose proteins (Huang et al., 2016).

The final approach is collectively named random protein engineering, where diversity is introduced at random into the target sequence and vast populations of mutated enzymes are subsequently assessed through screening and/or selection methods (Packer and Liu, 2015). (Semi)-rational engineering approaches require at least some information about the relationship between sequence, structure and the contribution of specific amino acid residues to the function of interest of the target protein. This input information is not needed using random engineering approaches, making it a more accessible technique when faced with limited understanding of the relationship between protein sequence and performance (Arnold, 1998b).

1.2. Random mutagenesis methods

Several methods have been developed to introduce genetic diversity specifically into target sequences. A popular technique is *in vitro* mutagenesis *via* error-prone PCR, where a region of DNA is amplified using a mutagenic polymerase and/or under amplification conditions that give rise to a higher mutation rate (Mccullum et al., 2010). Other *in vitro* approaches include gene shuffling and (iterative) site-saturation mutagenesis (Christians et al., 1999; Coco et al., 2001; Reetz and Carballeira, 2007; Zheng et al., 2004). More recently developed continuous *in vivo* mutagenesis tools, such as EvolvR (Halperin et al., 2018), OrthoRep (Ravikumar et al., 2018), T7-DIVA (Álvarez et al., 2020) and eMuta (Park and Kim, 2021), allow researchers to introduce diversity into targeted sequences *in vivo*. Another popular technique is multiplex automated genome engineering (MAGE), which allows continuous *in vivo* mutagenesis of multiple targets simultaneously, making it possible to generate highly diverse combinatorial libraries (Wang et al., 2009). Using these and other methods (see Simon et al., 2019 for a comprehensive overview), diversity can now be introduced in a highly targeted -and often continuous- fashion. These continuous mutagenesis approaches also allow researchers to dynamically alter selection pressures by perturbing the conditions of the evolution process while it is ongoing.

1.3. Directed evolution

One of the most effective methods for exploiting random protein engineering is directed evolution (DE), where a chosen selective pressure is imposed on an enzyme mutant library to identify improved variants (Arnold, 1998a). Although DE has been successfully used to engineer a multitude of enzymes, in part due to advances mentioned in section 1.2, its efficacy is determined and limited by the screening or selection step. To fully harness the power of DE, this final step must be (ultra-)high throughput to ensure maximum sampling of the sequence space. In addition, the chosen method must be able to determine the activity of the target enzyme accurately, with limited false positives. This has been approached in a variety of ways since the inception of DE, ranging from petri dish or microtiter plate-based screening (10^4–10^6 variants per day) to fluorescence-activated cell-sorting (FACS) which can screen up to 10^7 variants per day (see Table 1) (Longwell et al., 2017). These methods can be used to measure production of the target product or consumption of the substrate, often *via* colorimetric or fluorescence detection methods.

1.4. Growth-coupled selection

Screening methods require the discrete observation of each enzyme variant in a library, whereas selection methods directly eliminate undesirable enzyme variants (Xiao et al., 2015). One form of enzyme selection is through growth complementation, which provides a cheap and intuitive method of selecting improved enzyme variants. When the catalytic activity of an enzyme is associated with the growth of a cell, it is said to be growth-coupled. The extent of growth-coupling can vary on a spectrum from weak to strong. Weak growth coupling is observed when a target compound is only produced in conditions where growth rates are above a certain threshold, an example being fermentative production of ethanol and acetate in *E. coli* and *S. cerevisiae* under anaerobic conditions, or during overflow metabolism (Alter and Ebert, 2019). Strong growth coupling refers to a state where the production of the target compound is essential for carbon metabolism and therefore growth. Examples of strong growth coupling are the essential secretion of oxidation byproduct CO_2, or lactate and acetate formation by acetogenic bacteria during growth on H_2 and CO_2 (Alter and Ebert, 2019).

When an enzyme's activity is strongly coupled to growth, cells with more active, stable, or highly expressed variants of this enzyme are expected to display an increased growth rate (given the growth rate is not yet at its maximum). Growth-coupling can be exploited as an ultra-high throughput form of selection since throughput is only limited by the transformation efficiency of the cell (Xiao et al., 2015).

Growth coupling can generally be achieved through two methods. The first of these is by ensuring growth is dependent on product synthesis by the target enzyme, for example by disrupting an essential biosynthetic pathway and allowing the target enzyme's product to 'rescue' the auxotrophic state caused by this disruption. This option lends itself well to the engineering of enzymes involved in native metabolite biosynthesis, as the production of almost any metabolite can theoretically be coupled to growth in several model organisms (Von Kamp and Klamt, 2017). An example of this approach includes the disruption of TCA cycle genes involved in succinate biosynthesis, rendering the cell inviable unless complemented by an alternative pathway that produces this essential metabolite (Theodosiou et al., 2022).

Although some growth coupling strategies may be designed holistically, the complexity of cell metabolism means that designing stringent growth coupling strategies often requires considerable knowledge of the cell's metabolic networks and their interactions. Untangling this metabolic complexity to achieve growth coupling has become increasingly accessible through the development of metabolic modelling tools designed for this specific purpose. For example, programs such as GCopt and GCfront allow users to specify a substrate, a desired reaction they wish to couple to growth and a maximum amount of gene deletions (Alter and Ebert, 2019; Legon et al., 2022). An algorithm then predicts a set of genetic modifications required to growth-couple the desired reaction. The GCfront program also outputs solutions with varying growth-coupling strengths, offering flexibility regarding the desired selection pressure stringency. If the target enzyme produces a non-native product, coupling growth to essential metabolite synthesis becomes more challenging, since it would require introducing additional reactions which link non-native product synthesis to host cell metabolism.

A second method of growth coupling involves linking enzyme

Table 1
Comparison of several widely used screening/selection techniques for enzyme engineering.

Technique	Throughput (variants/day)	Benefits	Limitations	References
In vitro				
Microchamber arrays	~10^6	Flexible platform, quantitative, measurements over time	Non-native cellular environment, expensive equipment, reporter-dependent	Chen et al., 2015; Longwell et al., 2017
Compartmentalization (*e.g.* oil/water) + FACS	~10^7	Flexible platform, quantitative	Non-native cellular environment, expensive equipment, reporter-dependent, endpoint measurement	Becker et al., 2004; Colin et al., 2015; Tawfik and Griffiths, 1998; Xiao et al., 2015
In vivo				
Agar plate-based (fluorescence/colorimetric)	10^4 - 10^5	Inexpensive, native cellular environment	Low throughput, qualitative, reporter-dependent, laborious	Longwell et al., 2017; Packer and Liu, 2015
Microtiter plate-based (fluorescence/colorimetric)	10^5 - 10^6	Quantitative, native cellular environment, measurements over time	Reporter-dependent, laborious	Dörr et al., 2016; Packer and Liu, 2015
Cell-as-compartment + FACS	~10^7	Quantitative, native cellular environment, iterative rounds of sorting possible	Expensive equipment, reporter-dependent	Aharoni et al., 2006; Longwell et al., 2017; Yang and Withers, 2009
Metabolite auxotrophy complementation	10^8 - 10^{10} (limited by transformation efficiency)	Inexpensive, growth-based, native cellular environment, continuous evolution possible	Requires metabolic engineering, semi-quantitative, platform limited in scope	Chen et al., 2022b; MacBeath et al., 1998; Orsi et al., 2021
Redox cofactor auxotrophy complementation	10^8 - 10^{10} (limited by transformation efficiency)	Inexpensive, growth-based, substrate/product independent, native cellular environment, versatile platform, continuous evolution possible	Requires metabolic engineering, reaction must involve cofactor, semi-quantitative, enzyme products not directly selected for	*Literature cited in this review*

activity to the global energy state of the cell, for example through ATP production and/or ensuring a cellular redox state conducive to growth (Alter and Ebert, 2019). This approach for growth-coupling may be more broadly applicable than complementing metabolite auxotrophy, as ATP availability and redox balancing are ubiquitous requirements for functional cell metabolism. In recent years, redox cofactors have been explored as growth-coupling targets for enzyme engineering strategies. The regeneration of these cofactors is deeply linked to the energy state of the cell and therefore to growth, making the coupling of this process to a target enzyme a viable selection strategy for stringent growth-coupling (Alter and Ebert, 2019).

Recent reviews have covered several benefits and applications of using synthetic biology to create strains suitable for growth-coupled selection (Chen et al., 2022b; Orsi et al., 2021). This work aims to provide perspectives and summarize in greater detail the recent advances specifically in the engineering of strains deficient in the oxidized or reduced states of redox cofactor pairs, including the subsequent use of these strains as enzyme engineering platforms. The more widespread use of these *in vivo* enzyme engineering platforms may accelerate the development of improved biocatalysts and bioprocesses.

2. Redox cofactor oxidation or reduction rate as an enzyme selection strategy

2.1. Redox cofactors

Redox cofactors are essential redox couples that facilitate electron transfer for many biosynthetic and catabolic reactions (Wang et al., 2013). These molecules are ubiquitous to life and participate in around 25% of all metabolic reactions catalogued in the KEGG database (Opitz and Heiland, 2015). The most common cofactors are nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$) and their reduced counterparts NADH and NADPH. In general, the NAD^+/NADH cofactor pair is involved in oxidation reactions in both catabolism and anabolism, whilst $NADP^+$/NADPH is required for anabolic reduction reactions in *e.g.* DNA and fatty acid biosynthesis (Lin and Guarente, 2003; Spaans et al., 2015). The intracellular ratios of [NAD^+]/[NADH] and [$NADP^+$]/[NADPH] reflect their respective roles, as [NAD^+]/[NADH] ratios are usually high whilst [$NADP^+$]/[NADPH] ratios are low, although these ratios can vary significantly depending on growth state, environmental pressures, and nutrient availability (Bennett et al., 2009; De Graef et al., 1999; Spaans et al., 2015; Zhou et al., 2011). Overall, the intracellular concentration and ratio of the [NAD^+]/[NADH] redox cofactor couple thermodynamically favors the oxidation of substrates. In contrast, the ratio of [$NADP^+$]/[NADPH] reflects its role in providing reducing power for anabolic reactions required for cell growth and maintenance.

2.2. Redox cofactor auxotrophy

For the purposes of this review, the term ‘cofactor auxotrophy’ is used to refer to a cellular state where the redox state ratio of a cofactor pair is virtually entirely either reduced or oxidized. In this sense, the cell could be considered auxotrophic for a specific redox state of a certain cofactor pair. It is acknowledged that cells are not in fact auxotrophic for the biosynthesis of redox cofactors, however, an extreme redox state ratio of a cofactor pair manifests itself similarly to auxotrophy for any other essential metabolite; growth ceases until the extreme redox state ratio of the cofactor pair is relieved. Hence, ‘cofactor auxotrophy’ was considered an appropriate term in the context of growth-coupled selection.

In theory, the activity of virtually any reaction requiring redox cofactors can be coupled to growth by first inducing cofactor auxotrophy for either the oxidized or reduced state of a specific redox cofactor pair. When these mutated strains are subsequently complemented with a desired reaction that ‘rescues’ the imbalanced redox state ratio, the growth rate of the cell can be linked to enzyme activity through the cofactor reduction or oxidation rate (see Fig. 1A and B). Since redox cofactors are common reactants for a variety of enzymatic reactions, a single cofactor auxotrophic strain can be used to engineer a multitude of enzymes that are able to rescue the deficiency. This contrasts with other growth-coupling strategies where growth is coupled to the synthesis of a specific product, making these platforms less broadly employable.

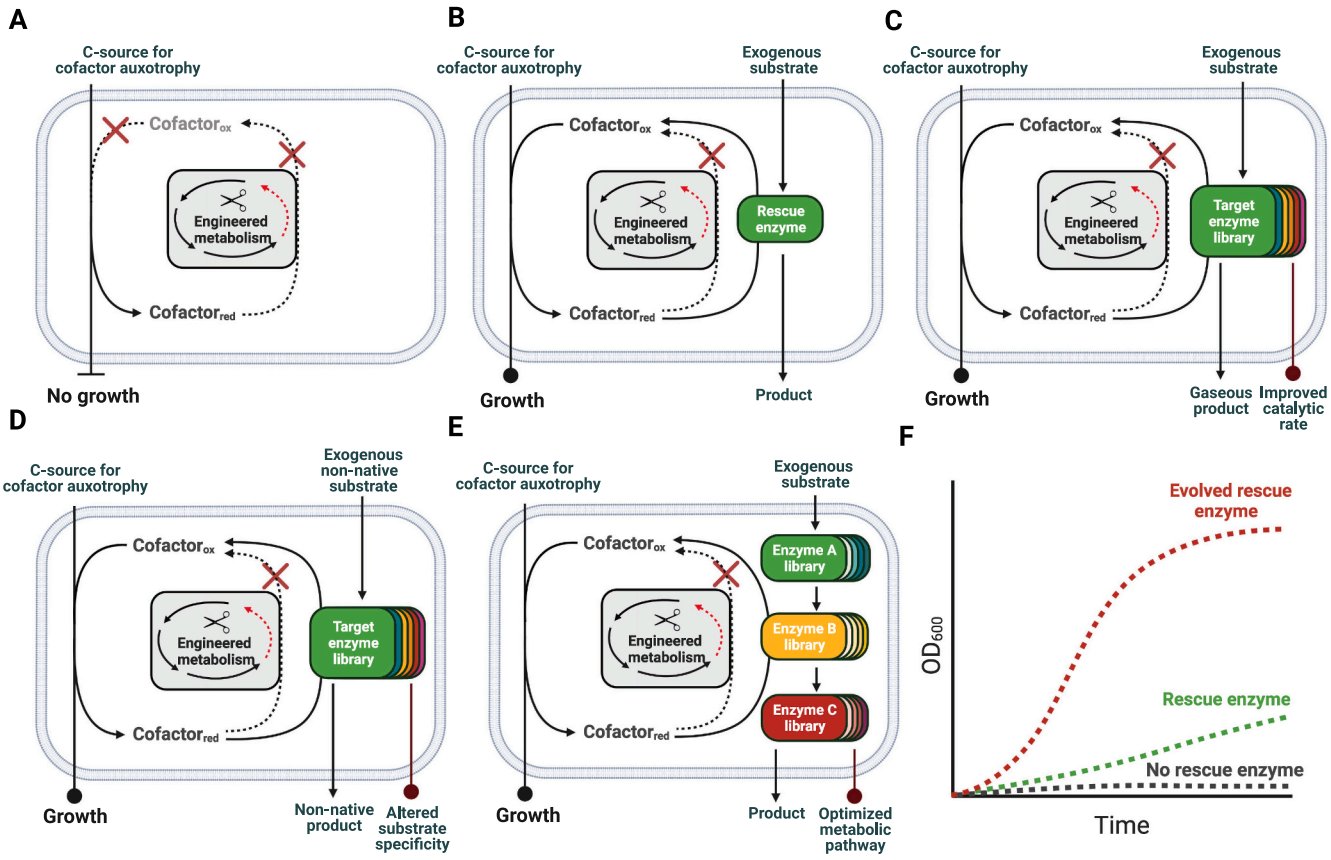

Fig. 1. Schematic representations of redox cofactor regeneration as a growth-coupling strategy, and several ways this can be employed for enzyme engineering. These figures use the example of a cell deficient in the oxidized state of a cofactor pair, for example NAD^+ or $NADP^+$, when grown on a certain carbon source. A) Engineered cells displaying redox cofactor auxotrophy, unable to grow without a functional cofactor regeneration pathway. B) Introduction of a 'rescue' enzyme and substrate to complement the deficient phenotype allows for cycling of the relevant cofactor and growth. C) Introduction of a randomly mutated enzyme library and a relevant substrate to select for highly active variants *via* the cofactor regeneration rate, even if the target product is gaseous and therefore not amenable for intracellular detection. D) Engineering enzyme substrate specificity by supplementing engineered cells with a non-native substrate for the target enzyme library. E) Multi-enzyme metabolic pathway targeted for mutagenesis, where more effective enzyme variant combinations will be selected for *via* the cofactor oxidation rate of the pathway, leading to selection for an optimal set of enzyme variants and an increased product synthesis. F) Schematic graph of the output metric -growth- in the absence and presence of a wildtype rescue enzyme, and a variant from a random mutagenesis library selected for using the redox cofactor growth-coupling approach. Created with BioRender.com.

2.3. Cofactor specificity engineering

One possible application of redox cofactor growth-coupling is to engineer the cofactor specificity of enzymes, which is especially relevant from a metabolic engineering perspective. For example, altering cofactor specificities of catabolic (*e.g.* glycolytic) enzymes from NAD^+/NADH to $NADP^+$/NADPH can result in higher yields of NADPH-dependent biosynthetic pathways. Conversely, engineering $NADP^+$/NADPH utilizing enzymes involved in substrate catabolism towards NAD^+/NADH dependence can increase yields of fermentation pathways (Aslan et al., 2017; Bengtsson et al., 2009; Liu et al., 2018; Shen et al., 2011; Tamakawa et al., 2011). Adapting the cofactor specificity of enzymes has become more accessible with tools such as CSR-SALAD (Cofactor Specificity Reversal–Structural Analysis and Library Design), which can predict amino acid residues likely to interact with NAD/NADP cofactors and suggests mutational libraries to screen for altering this specificity (Cahn et al., 2017).

2.4. Benefits of growth coupling via cofactor auxotrophy

Engineering cofactor specificity using cofactor auxotrophic strains directly selects for the desired specificity *via* the reduction or oxidation rate of the target cofactor pair. However, for many enzymes that require cofactors, other substrates or product(s) of the enzyme are of greater interest than the cofactor itself. In this case, when using cofactor auxotrophic strains, the cofactor oxidation or reduction rate acts as a proxy for the biosynthesis rate of the product of interest. This represents a unique benefit of using cofactor auxotrophy as a selection method: selection acts independently of substrates or products of the enzyme of interest. It is therefore particularly useful for engineering enzymes which require cofactors but catalyze the formation of desirable products that are otherwise difficult to detect, quantify or couple to growth *in vivo*. This could be due to these products being non-native to the cell which makes growth-coupling more challenging, or perhaps because the physical properties of the products make them inherently inaccessible *in vivo*. For example, coupling growth to products that are insoluble, gaseous, or rapidly secreted makes it challenging to identify an improved enzyme variant from a library due to a lack of compartmentalization and/or solubility of the product. This limits the engineering of enzymes involved in the biosynthesis of lipids, biofuels, gasses, organic solvents, or polymeric compounds, many of which are chemicals of significant commercial interest in the context of sustainable bioproduction (Jang et al., 2012; Liao et al., 2016; Zhou et al., 2018).

The substrate- and product independence of selection when using cofactor auxotrophs distinguishes this approach from other growth coupling strategies, offering several unique benefits. Firstly, by using the cofactor reduction or oxidation rate as a selection pressure, the desired product does not have to be amenable for detection, quantification, or

chemical alteration after it has been produced (Fig. 1C). Depending on the product, this may represent a significant benefit over using other high-throughput approaches requiring direct detection of the product for screening and selection. In addition, this property also makes redox cofactor growth-coupling a powerful method for engineering substrate specificity, since one platform strain can be used to engineer an enzyme towards specificity for a variety of substrates (Fig. 1D). Increased flux through metabolic pathways can also be selected for, as long as the pathway is cofactor dependent and the redox reaction is not rate-limiting (Fig. 1E). Growth coupling approaches also offer an easy readout for detecting improved enzymatic activity, although this is not unique to cofactor-based growth coupling approaches (Fig. 1F).

A final benefit of cofactor auxotrophs as growth coupling platforms stems from the ubiquitous nature of redox cofactors in microbial metabolism. Cofactor reduction and oxidation mainly occurs *via* central metabolic pathways, such as glycolysis (NAD^+-reducing), the pentose phosphate pathway ($NADP^+$-reducing), fermentation pathways (NADH-oxidizing) and the TCA cycle (NAD^+- and $NADP^+$-reducing) (De Graef et al., 1999; Spaans et al., 2015). Many of these metabolic pathways are central to microbial life, meaning that genetic engineering strategies for disrupting redox cofactor reduction or oxidation should mostly be laterally transferable to other microbes of interest. This is especially relevant for engineering enzymes for *in vivo* use, for example as part of a metabolic pathway. If engineering occurs *in vitro* or in a model organism not destined to be the final host of the enzyme, the environment the enzyme was engineered in may not adequately represent the cellular context it will ultimately be used in. Although various microbial hosts and expression systems have been developed specifically for heterologous protein expression, for example strains with decreased protease activity, several issues may still crop up when attempting to express non-native proteins (Kaur et al., 2018; Overton, 2014). These may include codon bias, erroneous disulfide bond formation and chaperone incompatibility (Rosano and Ceccarelli, 2014). In addition, eukaryotic protein expression in prokaryotic hosts may be problematic due to a lack of glycosylating activity, although developments are underway to establish glycosylating pathways in *E. coli* (Gupta and Shukla, 2015). If any of these issues occur, it may be necessary to optimize the enzyme again for *in vivo* use in the final host, costing time and resources (Golynskiy et al., 2013). Using strains modified for growth coupling, enzymes can be directly engineered within the environment of the desired microbial host, given that the host is genetically accessible.

3. Development of $NAD(P)^+$/NAD(P)H growth-coupled selection platforms

Several studies have engineered and characterized strains of *E. coli, Pseudomonas putida* and *Corynebacterium glutamicum* unable to reduce or oxidize cofactors in certain conditions. These strains were created through a combination of rewiring central metabolism, supplying specific carbon sources that result in cofactor auxotrophy when consumed and through cultivation in either aerobic or anaerobic conditions (for elaboration and references see sections 3.1–3.4). These cofactor auxotrophic strains now exist for oxidized and reduced states of the NAD^+/NADH and $NADP^+$/NADPH redox cofactor pairs, and their use as enzyme engineering platforms has also been demonstrated.

3.1. Mutants deficient in NADH oxidation

The oxidation of NADH is achieved through two main routes in *E. coli*, depending on oxygen availability. In aerobic conditions, NADH is mainly oxidized by respiration, generating ATP. In the absence of oxygen, NADH can be oxidized through various fermentation pathways, generating products such as lactate and ethanol (De Graef et al., 1999). Blocking these mixed fermentation pathways in *E. coli* during anaerobic growth causes a deficiency in NADH oxidation, along with an accumulation of intermediate metabolites such as pyruvate and acetyl-CoA when grown on glycolytic substrates. This principle has been exploited to drive NADH oxidizing pathways for the anaerobic synthesis of 2-methylpropan-1-ol, 2,3-butanediol, 1-butanol and L-alanine (Bastian et al., 2011; Liang and Shen, 2017; Machado et al., 2012; Shen et al., 2011; Zhang et al., 2007). These studies focused on enhancing bioproduction of the mentioned chemicals but not on using the mutant strains for enzyme engineering.

One of the earliest examples of redox cofactor auxotrophic strains being used for enzyme engineering relied on exploiting NAD^+ auxotrophy as a selection pressure. Machado et al. used a strain previously engineered by Shen et al., comprising an *E. coli* with several deletions in mixed fermentation pathway genes, including lactate dehydrogenase (*ldhA*), alcohol dehydrogenase (*adhE*) and fumarate reductase (*frdBC*). This strain was unable to grown on glucose in anaerobic conditions, unless an NADH-oxidizing reaction was available. To achieve production of C6- and C8 alcohols, an NADH-dependent 3-hydroxy-acyl-CoA reductase from *Ralstonia eutropha* (PaaH1) was introduced. Expression of PaaH1 allowed anaerobic growth on glucose and improved hexanoic acid production. The enzyme was subsequently targeted for random mutagenesis by epPCR, and improved variants were identified by their faster anaerobic growth on glucose. This resulted in the discovery of several improved variants, finally allowing up to 67% higher n-hexanol titres (see Table 2) (Machado et al., 2012).

Other studies have engineering enzymes by exploiting similar redox principles. Sellés Vidal et al. also generated a strain unable to oxidize NADH anaerobically when supplying glucose as a carbon source, by deletion of *ldhA* and *adhE* (Sellés Vidal et al., 2021). The authors noted that the resulting *ΔldhA ΔadhE* strain was not completely unable to oxidize NADH when grown anaerobically, as growth was also observed when an NADPH-dependent alcohol dehydrogenase was expressed with supplementation of acetone. The authors hypothesized that transhydrogenase activity was responsible for this observed phenotype, and after additional deletion of both the soluble transhydrogenase (*sthA*) and the proton-driven pyridine nucleotide transhydrogenase (*pntAB*), growth of this strain in the same conditions was abolished. This highlighted the importance of separating the NAD^+/NADH and $NADP^+$/NADPH cofactor pools by removing transhydrogenase activity. Growth of strains depending on anaerobic conditions for selection can also be complemented by simply growing the mutant in aerobic conditions, providing a straightforward method for non-selective growth.

Using this anaerobic NAD^+-auxotrophic platform strain, the authors were able to revert the cofactor specificity of several enzymes, including two alcohol dehydrogenases, an imine reductase and a nitroreductase (see Table 2). Finally, the *ΔldhA ΔadhE* strain was used to engineer an isopropanol production pathway, where flux through the pathway alleviated the imposed cofactor redox state imbalance through the final enzymatic reaction which produced $NADP^+$ (transhydrogenase activity was left intact to also allow growth complementation through $NADP^+$ production). The promoters and RBSs of the isopropanol production pathway genes were randomized, and growth of cells transformed with this library were assessed in selective conditions. This resulted in a variant displaying the highest yield and titre of isopropanol from gluconate substrate to date. This strain may be applicable as a platform for engineering other NAD(P)H-dependent enzymes and pathways, especially those that are oxygen sensitive.

An NAD^+-auxotrophic *E. coli* strain for use in aerobic conditions required additional modifications since NAD^+ is regenerated through respiration. In addition to disrupting several fermentation pathways (*adhE, ldhA, frdBC*) and *pntAB*, Maxel et al. knocked out several respiration-associated genes including NADH-quinone oxidoreductase subunit (*nuoF*), type II NADH:quinone oxidoreductase (*ndh*) and chorismite pyruvate-lyase (*ubiC*) (Fig. 2). This strain displayed a greatly diminished capacity for NADH-oxidation, verified by observations of growth-rescue in cells expressing NADH-dependent oxidases, but not NADPH-dependent oxidases (Maxel et al., 2021). This platform was used to engineer a ~ 2900-fold relative cofactor specificity switch (NADPH to

Table 2
Engineered strains displaying cofactor regeneration deficiencies and any enzymes engineered using these platforms.

Regeneration deficiency	Strain and any genetic modifications	Carbon source(s) for auxotrophy	(An)aerobic cultivation	Enzyme target	Result	Reference
NADH → NAD$^+$	*E. coli* BW25113 *ΔldhA ΔadhE ΔfrdBC* + *Treponema denticola* and *Euglena gracilis* trans-enoyl-CoA reductases, +*Ralstonia eutropha* thiolase	Glucose	Anaerobic	*Ralstonia eutropha* 3-hydroxy-acyl-CoA reductase (NAD$^+$-dependent)	67% higher hexanoic acid titre	Machado et al., 2012
	E. coli BW25113 *ΔldhA ΔadhE*	Glucose	Anaerobic	*Clostridium beijerinckii* alcohol dehydrogenase (NADP$^+$-dependent)	Cofactor specificity reversal for NAD$^+$ (k_{cat}/K_M = 19 min^{-1} mM^{-1})	Sellés Vidal et al., 2021
				Thermoanaerobacter brockii alcohol dehydrogenase (NADP$^+$-dependent)	Cofactor specificity reversal and 7.1-fold increased k_{cat}/K_M for oxidation of isopropanol using NAD$^+$ compared to NADP$^+$	
	E. coli BW25113 *ΔldhA ΔadhE ΔpntAB ΔsthA*	Glucose	Anaerobic	*Myxococcus stipitatus* imine reductase (NADP$^+$-dependent)	Cofactor specificity reversal for NAD$^+$ (k_{cat}/K_M = 1.06 min^{-1} μM^{-1})	
				Enterobacter cloacae nitroreductase NfsB (NAD$^+$/NADP$^+$-dependent)	Evolved substrate specificity towards non-native substrate 2-nitrobenzoic acid. Improved efficiency for 4-nitrobenzyl alcohol	
NADH → NAD$^+$	*E. coli* BW25113 *ΔldhA ΔadhE ΔfrdBC ΔnuoF Δndh ΔubiC ΔpntAB*	Glucose	Aerobic	*Acinetobacter sp.* cyclohexanone monooxygenase (NADPH-dependent)	Cofactor specificity reversal, 2900-fold increased k_{cat}/K_M for NADH	Maxel et al., 2021
NAD$^+$ → NADH NADP$^+$ → NADPH	*E. coli* MG1655 *Δlpd*	Acetate	Aerobic	–	–	Wenk et al., 2020
NADPH → NADP$^+$	*E. coli* K12 (W3110) *ΔgapA* + *Streptococcus mutans gapN* (NADP$^+$-dependent)	Glucose	Anaerobic	*L. delbrueckii* lactate dehydrogenase (NADH-dependent)	Cofactor specificity reversal, 470-fold increased k_{cat}/K_M for NADPH	Zhang et al., 2018
NADPH → NADP$^+$	*E. coli* BW25113 *Δpgi Δedd ΔsthA Δqor*	Glucose	Aerobic	*Pseudomonas aeruginosa* 4-hydroxybenzoate hydroxylase (NADPH-dependent)	8-fold increased k_{cat}/K_M for 3,4-dihydroxybenzoic acid	Maxel et al., 2020a
				Bacillus megaterium P450 BM3 (NADPH-dependent)	2.5-fold improved total activity against acenaphthene substrate	Maxel et al., 2020b
				Acinetobacter sp. Cyclohexanone monooxygenase (NADPH-dependent)	Improved thermostability, 4.4-fold increased residual activity at 30 °C	Maxel et al., 2020c
NADPH → NADP$^+$	*E. coli* BW25113 *Δpgi ΔsthA*	Glucose	Aerobic	*Mycobacterium avium* carboxylic acid reductase (NADPH-dependent)	4.7-fold increase in k_{cat}/K_M for 2-methoxybenzoate, 17-fold increase in k_{cat}/K_M for adipate	Kramer et al., 2020
NADP$^+$ → NADPH	*E. coli* MG1655 *Δzwf ΔmaeB ΔsthA ΔpntAB Δicd*	Glucose, glycerol, pyruvate, acetate, xylose, succinate	Aerobic	*Pseudomonas sp.* 101 formate dehydrogenase (NADH-dependent)	Cofactor specificity reversal, 510-fold improved specificity ratio for NADPH	Calzadiaz-Ramirez et al., 2020; Lindner et al., 2018
NADP$^+$ → NADPH	*P. putida* KT2440 *ΔsthA, pntA*^{W238*}, *pntB*^{Q117*}, *pgi-I*$^{Q129*-W229*}$, *pgi-II*^{Q129*}, *ghrB*^{W124*}, *maeB*^{Q314*}, *icd*^{Q78*}	Pyruvate	Aerobic	–	–	Volke et al., 2022
NADP$^+$ → NADPH	*C. glutamicum* XL-1 *Δzwf ΔmalE* Δicd_{Cg}::icd_{Sm}	Glucose, acetate, succinate, pyruvate	Aerobic	–	–	Chen et al., 2022a
NMNH → NMN$^+$	*E. coli* BW25113 *Δpgi Δzwf Δgnd ΔpncC ΔnadR* + *B. subtilis gdh* (BsGDH Ortho, NMN$^+$-dependent), +*Z. mobilis glf.* +*R. eutropha gntK.* +*F. tularensis nadEV*	Glucose	Aerobic	–	–	Black et al., 2019
NMNH → NMN$^+$	*E. coli* BW25113 *ΔgapA ΔmgsA ΔpncC* + *S. mutans gapN* (GapN Ortho, NMN$^+$-dependent)	Glucose	Aerobic	–	–	King et al., 2022
NMN$^+$ → NMNH	*E. coli* T7 Shuffle Express (BL21 derivative harbouring *Δgor ΔtrxB*) + *E. coli gor* (Gor Ortho, NMNH-dependent)	Glucose, yeast extract	Aerobic	Thermostable *Pseudomonas stutzeri* phosphite dehydrogenase (TS-PTDH, NAD$^+$-dependent)	110-fold improved k_{cat}/K_M for NMN$^+$ compared to wildtype TS-PTDH	Zhang et al., 2022
NCDH → NCD$^+$	*E. coli* BW14329 + *E. coli* NcdS (engineered *nadD* for NCD$^+$ synthesis) + *Ralstonia sp.* 4506 PDH* (NCD$^+$-dependent)	Glycerol (+phosphite)	Aerobic	–	–	Liu et al., 2022

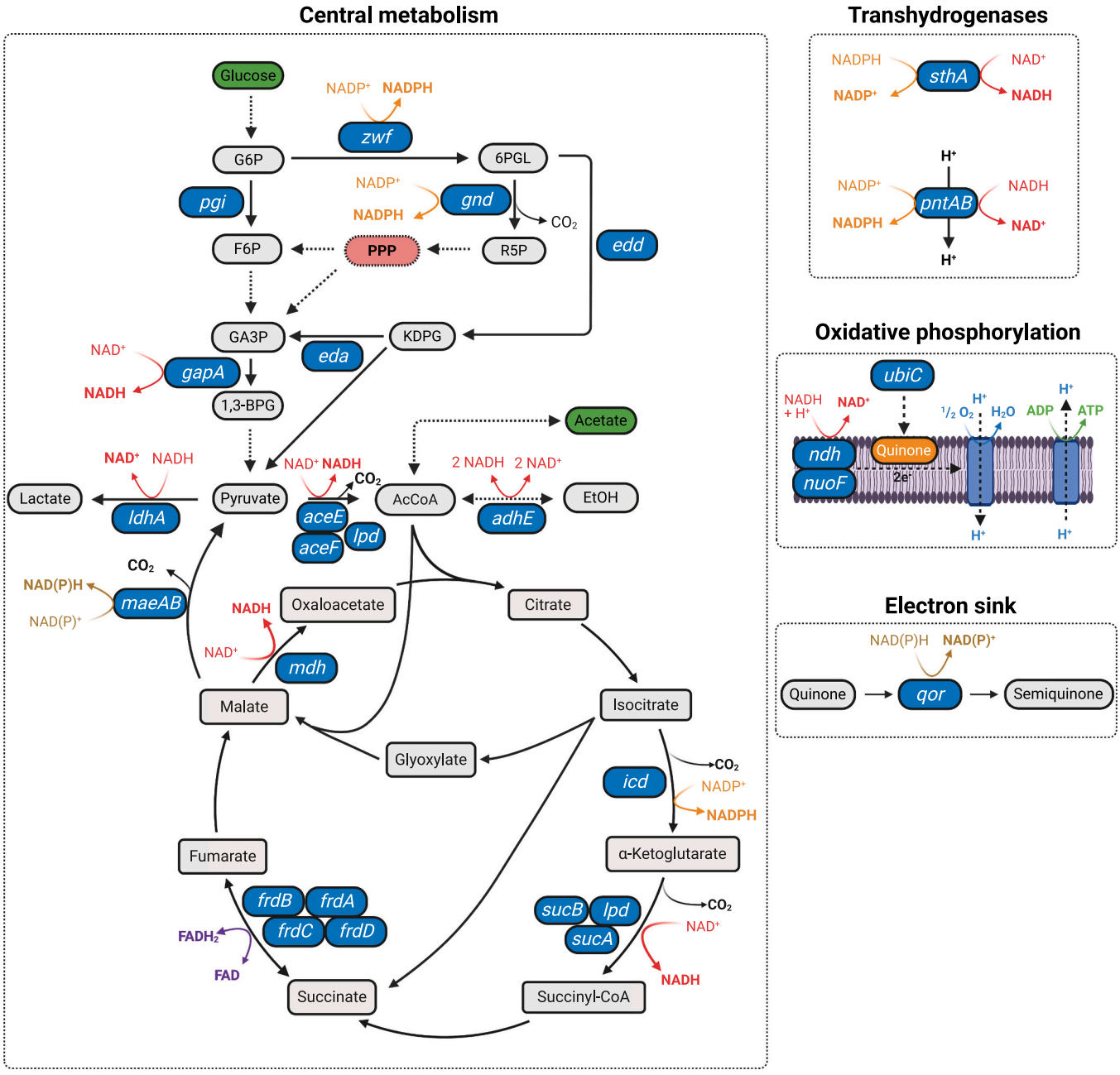

Fig. 2. *E. coli* central metabolic reactions involving redox cofactors. *E. coli* gene names (blue boxes) represent enzyme(s) (complexes) that have been targeted in various studies to disrupt redox cofactor regeneration. These reactions may involve NAD^+/NADH (red), $NADP^+$/NADPH (orange), $FAD/FADH_2$ (purple) or either NAD(P)H cofactor pair (gold). Abbreviations: *aceE* (pyruvate dehydrogenase); *aceF* (dihydrolipoamide acetyltransferase); *adhE* (alcohol dehydrogenase); *eda* (2-keto-3-deoxy-6-phosphogluconate aldolase); *edd* (phosphogluconate dehydratase); *frdA* (fumarate reductase subunit A); *frdB* (fumarate reductase subunit B); *frdC* (fumarate reductase subunit C); *frdD* (fumarate reductase subunit D); *gapA* (glyceraldehyde-3-phosphate dehydrogenase); *gnd* (6-phosphogluconate dehydrogenase); *icd* (isocitrate dehydrogenase); *ldhA* (lactate dehydrogenase); *lpd* (dihydrolipoyl dehydrogenase); *maeA* (NAD^+-dependent malic enzyme); *maeB* ($NADP^+$-dependent malic enzyme); *mdh* (malate dehydrogenase); *ndh* (NADH:quinone oxidoreductase); *nuoF* (NADH:quinone oxidoreductase); *pgi* (glucose-6-phosphate isomerase); *pntAB* (pyridine nucleotide transhydrogenase); *qor* (quinone oxidoreductase); *sthA* (soluble transhydrogenase); *sucA* (2-oxoglutarate dehydrogenase); *sucB* (dihydrolipoyllysine succinyltransferase); *ubiC* (chorismite pyruvate-lyase); *zwf* (glucose-6-phosphate 1-dehydrogenase). Created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NADH) for a cyclohexanone monooxygenase (CHMO), through use of a site-saturation mutagenesis library followed by one round of random mutagenesis and selection. This platform strain may be particularly useful for engineering other NADH-dependent oxygenases.

3.2. Mutants deficient in NAD^+ reduction

Through a single gene deletion, Wenk et al. engineered an *E. coli* strain that displayed auxotrophy for reducing power (NADH and NADPH) when aerobically grown on acetate. Dihydrolipoyl dehydrogenase (*lpd*) is a cofactor-binding subunit required for oxidative regeneration of lipoic acid, essential for the functionality of the pyruvate- and 2-ketoglutarate dehydrogenase complexes (PDHc and αKGDHc, respectively) and the glycine cleavage system (Wenk et al., 2020). Through deletion of *lpd*, carbon flux from glycolysis to the TCA cycle was blocked due to the absence of pyruvate dehydrogenase activity, although this is

not the case for strains displaying pyruvate oxidase activity (Li et al., 2006). The *Δlpd* strain was not able to grow on acetate as a sole carbon source due to a lack of reducing power generated through acetate metabolism. Acetate can still be activated to acetyl-CoA and assimilated *via* the glyoxylate shunt, however absence of αKGDHc activity makes generating reducing power from acetate impossible. In addition, the authors showed that growth on pyruvate or succinate as sole carbon sources, or when supplemented with acetate, was also not possible as generating reducing power from these substrates requires PDHc and/or αKGDHc activity. When acetate was supplemented with upper glycolytic substrates such as glucose or glycerol, growth was rescued due to the reducing power made available through glycolysis. Growth rescue of the *Δlpd* strain grown on acetate was also observed after expression of NAD^+-dependent formate-, ethanol- or methanol dehydrogenases, when the respective substrates for these enzymes were supplemented. This work demonstrated how redox cofactor auxotrophy could be made dependent on the supplied carbon source, providing an uncomplicated way to cultivate the auxotrophic strains simply by adding rescuing substrates until selection was required. The strain has not yet been used for enzyme engineering.

The *Δlpd* strain was only assessed for redox cofactor auxotrophy under aerobic conditions. A strain displaying NADH auxotrophy in anaerobic conditions would provide a useful platform for the engineering of oxygen-sensitive NADH-consuming enzymes.

3.3. Mutants deficient in NADPH oxidation

Two strategies have been described to induce $NADP^+$ auxotrophy, both relying on engineering the glycolytic pathway to overproduce NADPH but used in different conditions (anaerobic *versus* aerobic). Zhang et al. focused on the *E. coli gapA* gene (GAPDH: glyceraldehyde-3-phosphate dehydrogenase) to generate an $NADP^+$ auxotroph suitable for use as a selection platform for NADPH-dependent fermentative enzymes. The *gapA* enzyme is essential for glycolytic growth of *E. coli* and natively NAD^+-dependent (Seta et al., 1997; Yun et al., 2000). By expressing a heterologous phosphorylating or non-phosphorylating $NADP^+$-dependent GAPDH (*gapC* from *Clostridium acetobutylicum* or *gapN* from *Streptococcus mutans,* respectively) and knocking out the native *gapA* gene, glycolysis until the pyruvate node was engineered to be entirely NADPH-generating when grown on glucose. These GAPDH enzymes have previously been heterologously expressed in *E. coli* to increase the NADPH yield of glycolysis (Centeno-Leija et al., 2013; Martínez et al., 2008; Takeno et al., 2016). With glucose as the sole carbon source growth was not observed in *ΔgapA* strains, unless complemented with expression of either *gapC* or *gapN*. To prove NADP(H)-specific cofactor cycling was taking place, the authors grew the *gapN*- and *gapC*-complemented *ΔgapA* strains in anaerobic conditions in LB supplemented with glucose, with the strain additionally harbouring either an NADH-dependent alcohol dehydrogenase (ADH) from *Klebsiella pneumoniae* (*budC*) or an NADPH-dependent ADH from *Clostridium beijerinckii* (*adh*). Interestingly, the *ΔgapA* strain complemented with *gapC* displayed growth with expression of either ADH, and even in the absence of the ADH substrate acetoin, which the authors attributed to the less stringent cofactor specificity of *gapC* and NAD^+-reducing activity of native fermentation pathways. In contrast, growth of the *gapN*-complemented *ΔgapA* strain was only observed upon addition of acetoin and when the NADPH-dependent *adh* was expressed, but not with the NADH-dependent *budC*, indicating NADPH-specific cofactor cycling was taking place. The authors did not abolish transhydrogenase activity in this strain since no background growth was observed with acetoin supplementation and expression of *budC*, speculating that expression levels of *sthA* and *pntAB* were not sufficient to supply the required $NADP^+$ from transhydrogenase activity. This contrasts with other studies finding that removal of transhydrogenase activity was essential for ensuring specific cofactor auxotrophy and to avoid background growth (Lindner et al., 2018; Maxel et al., 2020a; Sellés Vidal et al., 2021). Regardless, the authors were able to use the *ΔgapA* + *gapN* strain to engineer a D-lactate dehydrogenase towards NADPH-specificity, improving the k_{cat}/K_M 470-fold in a single round of mutagenesis (Table 2) (Zhang et al., 2018).

Another study induced $NADP^+$ auxotrophy in *E. coli* and demonstrated its use in an aerobic environment. Glycolysis was also engineered, however in this work carbon flux was redirected through the pentose phosphate pathway (PPP) to force NADPH generation. This was achieved by knocking out the glucose-6-phosphate isomerase gene (*pgi*), an approach also used in several bioproduction studies aiming to increase NADPH production from glycolysis (Chemler et al., 2010; Chin and Cirino, 2011; Sekar et al., 2017). To ensure maximum NADPH was produced from the carbon flux, the Entner-Doudoroff pathway was also knocked-out by deletion of the *edd* gene. Finally, two NADPH sinks including the soluble transhydrogenase (*sthA*) and quinone oxidoreductase (*qor*) were knocked-out to avoid background NADPH oxidation. The resulting strain (Δ*pgi* Δ*edd* Δ*sthA* Δ*qor*) was unable to grow on glucose aerobically but displayed growth with glycerol substrate, since glycerol catabolism bypasses the glucose-6-phosphate isomerase and doesn't involve the pentose phosphate pathway. In addition, growth on glucose could also be rescued by expression of a heterologous NADPH-dependent oxidase, but not an NADH-dependent variant (Maxel et al., 2020a). This strain was subsequently used for engineering several enzyme properties in separate studies, including improving the catalytic activity of a 4-hydroxybenzoate hydroxylase (Maxel et al., 2020a), the substrate specificity of a P450 cytochrome (Maxel et al., 2020b) and the thermostability of a cyclohexanone monooxygenase (Maxel et al., 2020c) (see Table 2). This latter work represents the only study so far where thermostability was explicitly targeted for improvement using a cofactor auxotrophic strain. In these studies, enzyme diversity was generated using either site-saturation mutagenesis or random mutagenesis (Maxel et al., 2020a; Maxel et al., 2020b; Maxel et al., 2020c). The engineering successes achieved using this single platform strain showcased the flexibility of cofactor auxotrophs for engineering various properties of a range of enzymes.

A simpler version of the above strain has also been described, where the *edd* and *qor* genes were not deleted (Kramer et al., 2020). This mutant (*E. coli* BW25113 Δ*pgi* Δ*sthA*) cannot strictly be classified as an $NADP^+$-auxotrophic strain when grown on glucose, since after a ~ 24 h lag-phase in selective conditions the culture was found to resume growth. Nevertheless, this strain was used to engineer an NADPH-dependent carboxylic acid reductase (CAR) from *Mycobacterium avium*. The CAR enzyme was mutagenized by error-prone PCR and expressed with the supplementation of either 2-methoxybenzoate or adipate to the culture medium. In a single round of selection, variants with a 4.7-fold improvement in k_{cat}/K_M for 2-methoxybenzoate and a 17-fold improvement in k_{cat}/K_M for adipate were identified (Table 2).

3.4. Mutants deficient in $NADP^+$ reduction

The *E. coli* NADPH-auxotroph engineered by Lindner et al. was one of the first redox cofactor auxotrophs designed with its application as an enzyme engineering platform in mind (Lindner et al., 2018). Several central metabolic enzymes were knocked-out to avoid $NADP^+$ reduction when glucose was provided as a carbon source. Glycolysis and TCA cycle knockouts included glucose-6-phosphate dehydrogenase (*zwf*), isocitrate dehydrogenase (*icd*) and $NADP^+$-dependent malic enzyme (*maeB*) (Fig. 2). Strains with a deletion of *icd* required addition of 2-ketoglutarate to the medium as a precursor for glutamate and downstream amino acids biosynthesis. Upon deletion of the proton-driven transhydrogenase (*pntAB*) in addition to *zwf*, *icd* and *maeB*, growth was unexpectedly abolished on all carbon sources. This included gluconate, which is metabolized *via* NADPH-generating 6-phosphogluconate dehydrogenase (*gnd*) and was intended as a 'rescue' carbon source to allow growth in the auxotrophic strain. Upon additional deletion of the soluble transhydrogenase (*sthA*), growth on gluconate was restored, leading the

authors to conclude that *sthA* activity was sufficient to deplete the NADPH pool generated through gluconate assimilation. This implied that the *sthA* and *pntAB* transhydrogenase enzymes were depleting and regenerating cofactor pools in what seemed to be a futile cycle. As has been explored further in other work, this dual transhydrogenase activity may be beneficial for adapting to changes in substrate availability (Sauer et al., 2004). This NADPH-auxotrophic strain was used with the goal of engineering the cofactor specificity of an NAD^+-specific formate dehydrogenase (FDH) from *Pseudomonas sp.* 101 towards $NADP^+$-dependence. Using combinatorial saturation mutagenesis followed by selection using the NADPH-auxotrophic strain, a single round of mutagenesis yielded the most efficient and specific $NADP^+$-dependent FDH to date (Calzadiaz-Ramirez et al., 2020).

Recently, the redox metabolism of *Pseudomonas putida* was probed in work by Volke et al., leading to the construction of an NADPH-auxotrophic *P. putida* strain (Volke et al., 2022). The contribution of central metabolic enzymes to the maintenance of a redox balance in *P. putida* is less well understood than in *E. coli,* hence the authors decided to disrupt sets of target genes sequentially, to gain an understanding of their involvement in redox metabolism. CRISPR/nCas9-assisted engineering was used to disrupt genes of NADP(H)-dependent enzymes in modules based on their location in central metabolism, namely those involved in 'upper' metabolism (G6PDH and 6PGDH), 'lower' metabolism (Icd, MaeB and 2-ketoaldonate/hydroxypyruvate/glyoxylate reductase GhrB) and the two transhydrogenases (SthA and PntAB). The authors found that separate disruption of these modules had limited effects on the growth rate of strains using pyruvate as the sole carbon source. However, the combinatorial disruption of the upper metabolic enzymes (G6PDH and 6PGDH) and the transhydrogenases (SthA and PntAB), reduced the growth rate on pyruvate significantly. Upon additional disruption of the genes for the three lower metabolic enzymes, generating a strain designated 'NADPH-depleted *P. putida*', growth was not observed with pyruvate as the sole carbon source. The authors demonstrated that growth of this mutant could be rescued by supplementation with glutamate, the assimilation of which generated NADPH *via* glutamate dehydrogenase activity, or *via* supplementation with formate in conjunction with expression of an $NADP^+$-dependent formate dehydrogenase engineered in other work (Calzadiaz-Ramirez et al., 2020). Although no enzymes have been engineered using this strain yet, this NADPH-depleted *P. putida* represents the first *P. putida* strain that could be used for growth-coupled enzyme engineering based on cofactor auxotrophy.

Besides *E. coli* and *P. putida, Corynebacterium glutamicum* has recently also been engineered to exhibit NADPH-auxotrophy (Chen et al., 2022a). Chen et al. applied a strategy similar to that which Lindner et al. used to create an *E. coli* NADPH-auxotroph, as described earlier in section 3.4 (Lindner et al., 2018). Briefly, glucose assimilation through the NADPH-generating pentose phosphate pathway was blocked by deletion of *zwf*, in addition to deletion of the $NADP^+$-dependent malic enzyme *malE* and replacement of the $NADP^+$-dependent *icd* with an NAD^+-dependent *icd* from *Streptococcus mutans.* These changes yielded a *C. glutamicum* strain unable to grow on glucose, acetate, succinate, or pyruvate. Growth of this strain on gluconate was possible however, due to the activity of NADPH-generating 6-phosphogluconate dehydrogenase (*gnd*). In contrast to *E. coli*, *C. glutamicum* has not been found to possess either soluble or membrane-bound transhydrogenases, hence their activity did not have to be taken into account (Kabus et al., 2007; Yamauchi et al., 2014). The authors used this NADPH-auxotrophic strain as a starting point for optimizing NADPH/NADH ratios for L-leucine biosynthesis. First, the native NAD^+-dependent GAPDH was replaced with $NADP^+$-dependent GAPDH from *C. acetobutylicum*. Subsequently, *zwf* and the native NAD^+-dependent *icd* were both re-introduced with mutated promoters to alter their expression levels, and these strains were screened for improved L-leucine production. Using this approach, a strain with improved growth, L-leucine titre and productivity was identified. Although the authors did not use their strain as a selection platform for enzyme engineering, it demonstrated how cofactor auxotrophic strains can be used as a starting point for redox balance optimization in cell factories. In future work, the NADPH-auxotrophic version of this strain could be used as an enzyme selection platform for engineering $NADP^+$-dependent enzymes within the industrially relevant *C. glutamicum* chassis.

As described in section 3.2, the *E. coli* MG1655 *Δlpd* strain described by Wenk et al. displayed auxotrophy for reducing power when grown on acetate. Hence, this strain could also be used for engineering $NADP^+$-dependent enzymes, although growth rescue using $NADP^+$-dependent enzymes was not demonstrated explicitly in their initial study (Wenk et al., 2020). The NADPH-auxotrophic strains highlighted in this section were assessed in aerobic conditions, and an NADPH-auxotroph specifically for anaerobic use has yet to be described.

4. Development of non-canonical redox cofactor selection platforms

A relatively unexplored area of redox cofactor engineering is the use of 'non-canonical' redox cofactors. These molecules retain the nicotinamide ring found in NAD(P)-based cofactors but have alternative functional groups, for example nicotinamide mononucleotide (NMN^+/NMNH) and nicotinamide cytosine dinucleotide pairs (NCD^+/NCDH) (King et al., 2020). These compounds are amenable for use as redox cofactors, but their alternative structures usually cause them to display low interactions with native NAD(P)(H)-dependent enzymes. Even so, several enzymes have been engineered to accept non-canonical redox cofactors, as summarized in other work (King et al., 2020). Previous work has described the biosynthesis of NCD^+ (Wang et al., 2021) and NMN^+ (Black et al., 2020) cofactors *in vivo*, which allowed non-canonical cofactor cycling without exogenous supplementation of the cofactor. Besides their use *in vivo*, methods for employing non-canonical cofactor cycling in cell-free systems have also been developed (Black and Li, 2022). One of the potential benefits of applying these alternative cofactors is that it can offer greater control over the fate of electrons derived from substrates. This is due to limited cross-talk between native and non-canonical redox cofactor usage in cell metabolism, which may be exploited to improve product yields of fermentation processes (Weusthuis et al., 2020). Several strains have recently been engineered which display (partial) growth dependence on non-canonical cofactor cycling.

4.1. Mutants deficient in NMNH oxidation

One study developed an NMN^+-dependent glucose dehydrogenase ortholog from *Bacillus subtilis* (BsGDH Ortho), and used this enzyme to couple NMN^+/NMNH cofactor pair cycling to growth *via* the Entner-Doudoroff pathway (Black et al., 2019). To achieve this, knockouts of alternative glucose utilization routes (*Δzwf Δpgi Δgnd*) and potential NMN^+ degradation pathways (*ΔpncC ΔnadR*) were made. An NMNH-utilizing enoate reductase *xenA* from *P. putida* was overexpressed, in addition to glucose facilitator *glf* (*Zymomonas mobilis*), gluconate kinase *gntK* (*Ralstonia eutropha*) and the NMN^+-biosynthesis genes *nadE* and *nadV* (both from *Francisella tularensis*). The resulting strain only displayed growth when expressing both NMN^+-dependent BsGDH Ortho and NMNH-dependent XenA, implying that glucose utilization could only occur in strains displaying NMN^+/NMNH cofactor cycling. This strain, when lacking the NMNH-dependent XenA enzyme, could be used to engineer enzymes towards NMNH-dependency.

A second strain was engineered that utilized the NMN^+/NMNH cofactor pair in central metabolism (King et al., 2022). In this work, the $NADP^+$-dependent non-phosphorylating GAPDH from *Streptococcus mutans* (*gapN*) was engineered towards NMN^+ dependence through multiple rounds of rational mutagenesis, guided by *in silico* Rosetta modelling and molecular dynamics simulations. Two variants, GapN Penta and GapN Ortho, were highlighted in this study. The former

variant displayed a 45-fold improved k_{cat}/K_M for NMN^+ compared to the WT *gapN in vitro*, although this efficiency was still considerably lower than the WT enzyme for $NADP^+$. To assess the GapN variants' functionality *in vivo*, an *E. coli* BW25113 derivative (*ΔgapA ΔmgsA ΔpncC*) was used in conjunction with expression of the previously mentioned XenA enzyme, to allow cofactor cycling by oxidation of NMNH. In the presence of GapN Penta, the strain still displayed growth on glucose without NMN^+ supplementation, indicating non-specific ($NAD(P)^+$) cofactor reduction. The other variant, GapN Ortho, displayed significantly improved cofactor specificity *in vitro*, attaining a 3.4×10^6-fold cofactor specificity switch from $NADP^+$ to NMN^+. This was also demonstrated *in vivo*, as the GapN Ortho enzyme only supported minimal growth on glucose without NMN^+ supplementation (when supplemented with NMN^+, both GapN variants supported rapid growth). This strain may provide another platform for engineering NMNH-dependent enzymes, by replacing the currently used XenA enoate reductase with a target library. This work also reiterated the efficacy of computational approaches for rationally engineering cofactor specificity.

4.2. Mutants deficient in NMN^+ reduction

In recent work, a growth-coupled selection system was engineered based on a deficiency in NMN^+ reduction (Zhang et al., 2022). The production of reduced glutathione (GSH) was chosen for establishing coupling between cofactor cycling and growth. The *E. coli* SHuffle strain carries deletions in two genes involved in the production of GSH in *E. coli*, glutathione reductase (*gor*) and thioredoxin reductase (*trxB*) (Lobstein et al., 2012). Deletion of these genes resulted in a strain sensitive to oxidative stress, and supplementing the growth medium with 0.5 mM diamide was sufficient to inhibit cell growth. When native NADPH-dependent glutathione reductase was expressed on a plasmid in these conditions, growth was restored. To link this process to NMN^+/NMNH cycling, an NMNH-dependent glutathione reductase was developed in this work through several rounds of rational mutagenesis. This resulted in a variant (Gor Ortho) featuring a 6×10^4-fold cofactor specificity switch from NADPH to NMNH. As a proof-of-principle, an NMN^+-dependent glucose dehydrogenase, previously developed by the same group (BsGDH Ortho, see section 4.1), was used to support NMN^+/NMNH cofactor cycling and therefore growth in the SHuffle strain harbouring Gor Ortho. Supplementing the medium with NMN^+ was required to allow growth of strains dependent on NMN^+/NMNH-cycling. Growth was not observed when expressing NADPH-dependent BsGDH in the same conditions, demonstrating the stringency of the system.

This study also applied non-canonical redox cofactor auxotrophy for growth-coupled enzyme engineering for the first time (Zhang et al., 2022). A thermostable phosphite dehydrogenase from *Pseudomonas stutzeri* (TS-PTDH) (Johannes et al., 2005) was first rationally engineered to accept the NMN^+/NMNH cofactor, resulting in a variant with ~3-fold higher k_{cat}/K_M for NMN^+ compared to the wildtype (TS-PTDH A155N). This variant was used as the starting point for constructing a site-saturation mutagenesis library, which was subsequently transformed (~2.5×10^6 transformants) into the SHuffle strain harbouring Gor Ortho. After two rounds of growth-coupled selection, the authors identified a variant (LY-13) displaying 110- and 44-fold improved catalytic efficiency compared to TS-PTDH and TS-PTDH A155N, respectively. The evolved LY-13 enzyme also performed remarkably well regarding temporal stability *in vitro*, reaching total turnover numbers (TTN) of ~45,000 when coupled with NMNH-dependent XenA (see section 4.1), to produce levodione from ketoisophorone using phosphite as electron donor.

The work by Zhang et al. provided another useful strain for growth-coupled enzyme engineering, dependent on NMN^+/NMNH cycling. It represents the first platform strain auxotrophic for the reduced state of a non-canonical redox cofactor. The flexibility of this platform also allows it to be used for engineering other enzymes towards NMN^+-dependency.

4.3. Mutants deficient in NCDH oxidation

Another recent study established a growth-coupled NCD^+/NCDH cofactor cycling system, also involving phosphite dehydrogenases (Liu et al., 2022). However, their study revolved around rescuing phosphate auxotrophy in *E. coli via* NCD^+-dependent phosphite oxidation. The enzyme responsible for this, phosphite dehydrogenase (PDH, not to be confused with pyruvate dehydrogenase complex PDHc), is natively NAD^+-dependent, but the authors had previously engineered an NCD^+-dependent version (PDH*), which was used in this work (Liu et al., 2019). Further previous work described the development of a strain autotrophic for NCD^+, through reprogramming the nicotinic acid mononucleotide adenylyltransferase (NadD) to allow *in vivo* NCD^+ synthesis (Wang et al., 2021). These previously established elements were combined in a strain unable to metabolize phosphite (*E. coli* BW14329), due to inactivity of *phoA* and deletions in the *phn* operon (Metcalf et al., 1991). This resulted in a strain displaying growth defects in medium containing only phosphite as the phosphorus source, and use of the PDH* enzyme ensured a dependence on NCD^+/NCDH cycling in this context. On medium containing glycerol and phosphite, faster growth was observed when the NCD^+-dependent PDH* enzyme was expressed in conjunction with an NCDH-specific malic enzyme (ME*), in contrast to the native NADH-dependent variant (ME), implying that growth was (partially) dependent on NCD^+/NCDH cycling. This growth difference was not observed when using glucose instead of glycerol. The lack of full cofactor redox state auxotrophy observed in this system is likely due to low, but appreciable, specificity of PDH* and ME* towards the native NAD^+/NADH cofactor pair. Although there is room for improving the stringency of the system, this work represents a novel approach for growth-coupling NCDH-dependent enzymes in an aerobic environment.

The above studies highlight the readiness of non-canonical redox cofactor cycling to be used for growth-coupled enzyme engineering. With the recent surge in interest in non-canonical redox cofactor cycling systems, it is likely that more industrially relevant enzymes will soon be engineered towards, and optimized for, non-canonical redox cofactor use. This could allow stricter control over electron flux from substrate to product leading to better yields in industrial processes.

5. Limitations of growth coupling *via* cofactor auxotrophy

As is the case with all high-throughput enzyme engineering strategies, one must carefully consider the first law of random mutagenesis; "*you get what you screen for*" (Arnold, 1998a), which also applies to selection. If using the cofactor reduction or oxidation rate as a proxy for the synthesis of an enzyme product, synthesis of the desired product is in fact not being directly selected for. Furthermore, in all strategies using cofactor reduction or oxidation as a selection method there is evolutionary pressure not just on the target enzyme, but on all enzymes present in the cell reasonably capable of evolving towards alleviating the cofactor redox state deficiency. The implications of this problem were recently experimentally demonstrated by cultivation of an *E. coli* strain unable to reduce $NADP^+$ (Lindner et al., 2018). The strain was continuously cultured for 500–1100 generations in selective conditions, after which it had evolved to circumvent the imposed limitation through reversion of the cofactor specificity of either malic enzyme (*maeA*) or dihydrolipoyl dehydrogenase (*lpd*) towards $NADP^+$ (Bouzon et al., 2021). Naturally, the diversity introduced into an error-prone PCR library or *via in vivo* targeted hypermutagenesis should far exceed the diversity generation rate through natural evolution, but long-term continuous evolution experiments as described above may result in 'cheating' cells. To avoid this situation, it may be necessary to monitor the population's genotype or to develop a counter-selection method which can remove cheating cells from the population.

The evolution of known enzymes or pathways towards complementing metabolic deficiencies such as cofactor auxotrophy could be

monitored with relative ease. More challenging however is taking into account the contribution of 'dark matter' metabolism in complementing engineered auxotrophies. These 'dark' metabolic pathways represent parts of cellular metabolism that are poorly characterized, for example 25% of proteins in *E. coli* have uncharacterized functions despite being one of the best characterized model organisms (MohammadiPeyhani et al., 2022). In the context of growth coupled selection, these metabolic 'blind spots' could prove problematic as they could alleviate imposed auxotrophies, either directly or *via* evolutionary adaptation as described earlier. This limitation may be more pronounced when attempting to introduce metabolic auxotrophies into poorly characterized organisms, as opposed to using model organisms with generally well-understood metabolisms.

Besides cells potentially circumventing the imposed cofactor auxotrophy *via* evolutionary adaptation or 'dark matter' metabolic pathways, there are also practical limitations to using growth coupling approaches. Most of the cofactor auxotrophic systems described in section 4 rely on supplementation of the cell medium with the substrate for the enzyme of interest, for example formate (Calzadiaz-Ramirez et al., 2020), phosphite (Liu et al., 2022), cyclohexanone (Maxel et al., 2020c), carboxylic acids (Kramer et al., 2020), acetone or 2-methyl-1-pyrroline (Sellés Vidal et al., 2021). The efficacy of enzyme selection will depend in part on how well this substrate is taken up by the cell and whether or not it is toxic, as low uptake may affect enzyme kinetics and the concomitant growth rate of the cell, while toxicity will similarly affect cell growth. Limited substrate uptake can be circumvented by using a native metabolite as the substrate for the enzyme of interest, but in cases where exogenous addition of substrate is required the uptake and toxicity of the substrate will both have to be assessed.

6. Perspectives and outlook

Despite the considerable successes achieved so far, several avenues of research for redox cofactor-based growth-coupling remain unexplored. One of these includes the more widespread engineering of cofactor auxotrophic strains of organisms besides *E. coli*. The generation of NADPH-auxotrophic *Corynebacterium glutamicum* and *Pseudomonas putida* strains has recently been reported, which represent the first cofactor auxotrophic strains of these species. Although they have not yet been used for enzyme engineering, these strains can be employed for this purpose, opening up the possibility for growth-coupled enzyme engineering within two widely used chassis. However, other industrially relevant hosts, such as *Ralstonia eutropha, Clostridium sp., Yarrowia lipolytica* and *Saccharomyces cerevisiae* could be suitable candidates for adapting into similar enzyme engineering platforms. Considering that cofactor auxotrophy can already be induced through introduction of a single knockout in *E. coli* MG1655 (Δ*lpd*) with growth on acetate (Wenk et al., 2020), the construction of orthogonal strains from different species may be relatively easily achieved. In addition, one functional platform strain can be used to engineer a multitude of enzymes, which may make development of these platforms a worthwhile investment for researchers.

Growth-coupling *via* cofactor oxidation or reduction rates represents a major opportunity for engineering the biocatalysis of products which can't satisfy the requirements for other high-throughput enzyme engineering approaches. This includes the need for compartmentalization of products and their fast and accurate quantification, usually essential conditions to ensure that the sequence of an enzyme variant from a library can be linked to its performance. Considerable interest exists for the sustainable production of gasses, aliphatic hydrocarbons, and organic solvents, particularly in the context of decarbonisation and the production of sustainable platform chemicals. However, most of these products do not satisfy the above requirements, due to their inherent physical properties or simply a lack of suitable available methods. Cofactor-based growth coupling can remove significant roadblocks for engineering metabolic pathways or enzymes involved in producing these chemicals, by providing *in vivo* ultra-high throughput screening capacity where it may not have been possible using other techniques. Application of these growth coupling systems is not limited to engineering these specific types of enzymes however. Any cofactor-utilizing enzyme or pathway can in theory be targeted for engineering using these platform strains, which could provide an accelerated pathway towards altering the properties of a wide variety of enzymes. Applications of these growth coupling platforms may include altering the cofactor specificity of relevant enzymes to optimize cofactor availability in cell factories, screening cofactor-utilizing enzymes with libraries of substrates to uncover novel catalytic capabilities, and optimizing the expression levels of heterologous metabolic pathway enzymes by coupling pathway flux to growth.

Besides their use as enzyme engineering platforms, strains displaying growth dependence may prove useful as bioproduction hosts. For example, the use of antibiotics in bioproduction can be costly and lead to antibiotic resistant strains. Strains dependent on enzyme activity and/or exogenous substrate addition for growth will be selected for without requiring antibiotic selection pressure. In addition, the use of cofactor auxotrophic hosts could alleviate biosafety concerns, if growth were made dependent on specific culturing conditions and/or the supplementation of substrates only present in the bioproduction environment.

Finally, the use of non-canonical redox cofactors could enhance the efficient biosynthesis of products and remains a largely unexplored area of metabolic engineering. Yields and productivities of bioprocesses are often insufficient for commercial exploitation, in part because cell factories tend to divert considerable amounts of energy and carbon from substrates towards growth. High yield bioproduction may be achievable through tighter control over electron flux from substrate to product, which is challenging when using $NAD(P)^+$/NAD(P)H cofactors involved in many reactions. The development of non-canonical redox cofactor cycling within cell factories could allow for a closed-loop and controllable system of electron transfer (King et al., 2020, 2022; Weusthuis et al., 2020). Engineering relevant bioproduction enzymes towards non-canonical redox cofactor utilization can be accelerated through using strains auxotrophic for these cofactors, in conjunction with growth-coupling approaches.

7. Conclusion

The recent flurry of interest in redox cofactor auxotrophic strains showcases their broad applicability and potential for enzyme engineering. Growth-coupling is a powerful selection method, since it is ultra-high throughput, provides an affordable and simple method of detecting improved enzyme variants and allows for enzyme engineering directly within desired cellular environments. At least one auxotrophic *E. coli* strain exists for every redox state of the canonical $NAD(P)^+$/NAD(P)H cofactor pairs, and considerable progress has been made in the generation of strains auxotrophic for redox states of non-canonical redox cofactor pairs NMN^+/NMNH and NCD^+/NCDH. Taken together, these represent a set of highly versatile platforms for engineering redox cofactor-utilizing enzymes. Several strains have been used for engineering a range of redox cofactor-dependent enzymes, often with considerable success after only one round of mutagenesis. Substrate specificity, catalytic rate and (thermo)stability are important properties for enzyme use in commercial applications, and redox cofactor auxotrophic strains have now been successfully used to enhance all three of these properties in a variety of enzymes. Further development of (non-canonical) redox cofactor auxotrophic strains is of great interest for accelerating the engineering of robust and efficient biocatalysts. This in turn is important for the overarching goal of developing highly efficient cell factories which can achieve commercially viable titres, yields and productivities for sustainable bioproduction.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

This work was supported by funding from the Biotechnology and Biological Sciences Research Council (BBSRC) [grant number BB/M011224/1] and the Oxford Interdisciplinary Doctoral Training Partnership. We also thank the Engineering and Physical Sciences Research Council (EPSRC) for their support [grant number EP/M002403/1 and EP/N009746/1].

References

Aharoni, A., Thieme, K., Chiu, C.P.C., Buchini, S., Lairson, L.L., Chen, H., Strynadka, N.C. J., Wakarchuk, W.W., Withers, S.G., 2006. High-throughput screening methodology for the directed evolution of glycosyltransferases. Nature Methods 3 (8), 609–614. https://doi.org/10.1038/nmeth899.

Alter, T.B., Ebert, B.E., 2019. Determination of growth-coupling strategies and their underlying principles. BMC Bioinformatics 20 (1), 447. https://doi.org/10.1186/s12859-019-2946-7.

Álvarez, B., Mencía, M., de Lorenzo, V., Fernández, L.Á., 2020. In vivo diversification of target genomic sites using processive base deaminase fusions blocked by dCas9. Nature Communications 11 (1), 1–14. https://doi.org/10.1038/s41467-020-20230-z.

Arnold, F.H., 1998a. Design by Directed Evolution. http://www.che.caltech.edu/groups/fha. https://doi.org/10.1021/ar960017f.

Arnold, F.H., 1998b. When Blind is Better: Protein Design by Evolution". Commentary in Nature Biotechnology 16, 617–618.

Aslan, S., Noor, E., Bar-Even, A., 2017. Holistic bioengineering: rewiring central metabolism for enhanced bioproduction. In: *Biochemical Journal* (Vol. 474, Issue 23, pp. 3935–3950). Portland Press Ltd. https://doi.org/10.1042/BCJ20170377.

Bastian, S., Liu, X., Meyerowitz, J.T., Snow, C.D., Chen, M.M.Y., Arnold, F.H., 2011. Engineered ketol-acid reductoisomerase and alcohol dehydrogenase enable anaerobic 2-methylpropan-1-ol production at theoretical yield in Escherichia coli. Metab. Eng. 13 (3), 345–352. https://doi.org/10.1016/J.YMBEN.2011.02.004.

Becker, S., Schmoldt, H.U., Adams, T.M., Wilhelm, S., Kolmar, H., 2004. Ultra-high-throughput screening based on cell-surface display and fluorescence-activated cell sorting for the identification of novel biocatalysts. Curr. Opin. Biotechnol. 15 (4), 323–329. https://doi.org/10.1016/J.COPBIO.2004.06.001.

Bengtsson, O., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F., 2009. Xylose reductase from Pichia stipitis with altered coenzyme preference improves ethanolic xylose fermentation by recombinant Saccharomyces cerevisiae. Biotechnology for Biofuels 2 (1), 1–10. https://doi.org/10.1186/1754-6834-2-9.

Bennett, B.D., Kimball, E.H., Gao, M., Osterhout, R., Van Dien, S.J., Rabinowitz, J.D., 2009. Absolute metabolite concentrations and implied enzyme active site occupancy in Escherichia coli. Nature Chemical Biology 2009 5:8 5 (8), 593–599. https://doi.org/10.1038/nchembio.186.

Black, W.B., Li, H., 2022. Cell-free noncanonical redox cofactor systems. Methods Mol. Biol. 2433, 185–198. https://doi.org/10.1007/978-1-0716-1998-8_11.

Black, W.B., Zhang, L., Mak, W.S., Maxel, S., Cui, Y., King, E., Fong, B., Sanchez Martinez, A., Siegel, J.B., Li, H., 2019. Engineering a nicotinamide mononucleotide redox cofactor system for biocatalysis. Nature Chemical Biology 2019 16:1 16 (1), 87–94. https://doi.org/10.1038/s41589-019-0402-7.

Black, W.B., Aspacio, D., Bever, D., King, E., Zhang, L., Li, H., 2020. Metabolic engineering of Escherichia coli for optimized biosynthesis of nicotinamide mononucleotide, a noncanonical redox cofactor. Microb. Cell Factories 19 (1), 1–10. https://doi.org/10.1186/S12934-020-01415-Z.

Bornscheuer, U.T., Huisman, G.W., Kazlauskas, R.J., Lutz, S., Moore, J.C., Robins, K., 2012. Engineering the third wave of biocatalysis. Nature 485 (7397), 185–194. https://doi.org/10.1038/nature11117.

Bouzon, M., Döring, V., Dubois, I., Berger, A., Stoffel, G.M.M., Ramirez, L.C., Meyer, S.N., Fouré, M., Roche, D., Perret, A., Erb, T.J., Bar-Even, A., Lindner, S.N., 2021. Change in cofactor specificity of oxidoreductases by adaptive evolution of an escherichia coli nadph-auxotrophic strain. MBio 12 (4). https://doi.org/10.1128/MBIO.00329-21.

Brannigan, J.A., Wilkinson, A.J., 2002. Protein engineering 20 years on. Nature Reviews Molecular Cell Biology 3 (12), 964–970. https://doi.org/10.1038/nrm975.

Cahn, J.K.B., Werlang, C.A., Baumschlager, A., Brinkmann-Chen, S., Mayo, S.L., Arnold, F.H., 2017. A general tool for engineering the NAD/NADP cofactor preference of oxidoreductases. ACS Synth. Biol. 6 (2), 326–333. https://doi.org/10.1021/acssynbio.6b00188.

Callaway, E., 2022. 'The entire protein universe': AI predicts shape of nearly every known protein. Nature 608 (7921), 15–16. https://doi.org/10.1038/D41586-022-02083-2.

Calzadiaz-Ramirez, L., Calvó-Tusell, C., Stoffel, G.M.M., Lindner, S.N., Osuna, S., Erb, T. J., Garcia-Borràs, M., Bar-Even, A., Acevedo-Rocha, C.G., 2020. In vivo selection for Formate dehydrogenases with high efficiency and specificity toward NADP +. ACS Catal. 10 (14), 7512–7525. https://doi.org/10.1021/acscatal.0c01487.

Centeno-Leija, S., Utrilla, J., Flores, N., Rodriguez, A., Gosset, G., Martinez, A., 2013. Metabolic and transcriptional response of Escherichia coli with a NADP(+)-dependent glyceraldehyde 3-phosphate dehydrogenase from Streptococcus mutans. Antonie Van Leeuwenhoek 104 (6), 913–924. https://doi.org/10.1007/S10482-013-0010-6.

Chemler, J.A., Fowler, Z.L., McHugh, K.P., Koffas, M.A.G., 2010. Improving NADPH availability for natural product biosynthesis in Escherichia coli by metabolic engineering. Metab. Eng. 12 (2), 96–104. https://doi.org/10.1016/J.YMBEN.2009.07.003.

Chen, B., Lim, S., Kannan, A., Alford, S.C., Sunden, F., Herschlag, D., Dimov, I.K., Baer, T. M., Cochran, J.R., 2015. High-throughput analysis and protein engineering using microcapillary arrays. Nature Chemical Biology 12 (2), 76–81. https://doi.org/10.1038/nchembio.1978.

Chen, J., Wang, Y., Zheng, P., Sun, J., 2022b. Engineering synthetic auxotrophs for growth-coupled directed protein evolution. Trends Biotechnol. https://doi.org/10.1016/J.TIBTECH.2022.01.010.

Chen, S.L., Liu, T.S., Zhang, W.G., Xu, J.Z., 2022a. An NADPH-auxotrophic Corynebacterium glutamicum recombinant strain and used it to construct L-leucine high-yielding strain. Int. Microbiol. 1–14 https://doi.org/10.1007/S10123-022-00270-9.

Chin, J.W., Cirino, P.C., 2011. Improved NADPH supply for xylitol production by engineered Escherichia coli with glycolytic mutations. Biotechnol. Prog. 27 (2), 333–341. https://doi.org/10.1002/BTPR.559.

Christians, F.C., Scapozza, L., Crameri, A., Folkers, G., Stemmer, W.P.C., 1999. Directed evolution of thymidine kinase for AZT phosphorylation using DNA family shuffling. Nature Biotechnology 17 (3), 259–264. https://doi.org/10.1038/7003.

Claassens, N.J., Burgener, S., Vögeli, B., Erb, T.J., Bar-Even, A., 2019. A critical comparison of cellular and cell-free bioproduction systems. Curr. Opin. Biotechnol. 60, 221–229. https://doi.org/10.1016/J.COPBIO.2019.05.003.

Coco, W.M., Levinson, W.E., Crist, M.J., Hektor, H.J., Darzins, A., Pienkos, P.T., Squires, C.H., Monticello, D.J., 2001. DNA shuffling method for generating highly recombined genes and evolved enzymes. Nature Biotechnology 19 (4), 354–359. https://doi.org/10.1038/86744.

Colin, P.Y., Zinchenko, A., Hollfelder, F., 2015. Enzyme engineering in biomimetic compartments. Curr. Opin. Struct. Biol. 33, 42–51. https://doi.org/10.1016/J.SBI.2015.06.001.

De Graef, M.R., Alexeeva, S., Snoep, J.L., Teixeira De Mattos, M.J., 1999. The steady-state internal redox state (NADH/NAD) reflects the external redox state and is correlated with catabolic adaptation in Escherichia coli. J. Bacteriol. 181 (8), 2351–2357. https://doi.org/10.1128/JB.181.8.2351-2357.1999.

Dörr, M., Fibinger, M.P.C., Last, D., Schmidt, S., Santos-Aberturas, J., Böttcher, D., Hummel, A., Vickers, C., Voss, M., Bornscheuer, U.T., 2016. Fully automatized high-throughput enzyme library screening using a robotic platform. Biotechnol. Bioeng. 113 (7), 1421–1432. https://doi.org/10.1002/BIT.25925.

Golynskiy, M.V., Haugner, J.C., Morelli, A., Morrone, D., Seelig, B., 2013. In vitro evolution of enzymes. Methods in Molecular Biology (Clifton, N.J.) 978, 73. https://doi.org/10.1007/978-1-62703-293-3_6.

Gupta, S.K., Shukla, P., 2015. Advanced technologies for improved expression of recombinant proteins in bacteria: perspectives and applications. Crit. Rev. Biotechnol 36 (6), 1089–1098. https://doi.org/10.3109/07388551.2015.1084264.

Halperin, S.O., Tou, C.J., Wong, E.B., Modavi, C., Schaffer, D.V., Dueber, J.E., 2018. CRISPR-guided DNA polymerases enable diversification of all nucleotides in a tunable window. Nature 560 (7717), 248–252. https://doi.org/10.1038/s41586-018-0384-8.

Huang, P.S., Boyken, S.E., Baker, D., 2016. The coming of age of de novo protein design. Nature 537 (7620), 320–327. https://doi.org/10.1038/nature19946.

Jang, Y.S., Kim, B., Shin, J.H., Choi, Y.J., Choi, S., Song, C.W., Lee, J., Park, H.G., Lee, S. Y., 2012. Bio-based production of C2-C6 platform chemicals. Biotechnol. Bioeng. 109 (10), 2437–2459. https://doi.org/10.1002/BIT.24599.

Johannes, T.W., Woodyer, R.D., Zhao, H., 2005. Directed evolution of a thermostable phosphite dehydrogenase for NAD(P)H regeneration. Appl. Environ. Microbiol. 71 (10), 5728–5734. https://doi.org/10.1128/AEM.71.10.5728-5734.2005.

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S.A.A., Ballard, A.J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Hassabis, D., 2021. Highly accurate protein structure prediction with AlphaFold. Nature 596 (7873), 583–589. https://doi.org/10.1038/s41586-021-03819-2.

Kabus, A., Georgi, T., Wendisch, V.F., Bott, M., 2007. Expression of the Escherichia coli pntAB genes encoding a membrane-bound transhydrogenase in Corynebacterium glutamicum improves L-lysine formation. Appl. Microbiol. Biotechnol. 75 (1), 47–53. https://doi.org/10.1007/S00253-006-0804-9.

Kaur, J., Kumar, A., Kaur, J., 2018. Strategies for optimization of heterologous protein expression in E. coli: roadblocks and reinforcements. Int. J. Biol. Macromol. 106, 803–822. https://doi.org/10.1016/J.IJBIOMAC.2017.08.080.

King, E., Maxel, S., Li, H., 2020. Engineering natural and noncanonical nicotinamide cofactor-dependent enzymes: design principles and technology development. Curr. Opin. Biotechnol. 66, 217–226. https://doi.org/10.1016/J.COPBIO.2020.08.005.

King, E., Cui, Y., Aspacio, D., Nicklen, F., Zhang, L., Maxel, S., Luo, R., Siegel, J.B., Aitchison, E., Li, H., 2022. Engineering Embden–Meyerhof–Parnas glycolysis to generate noncanonical reducing power. ACS Catal. 8582–8592 https://doi.org/10.1021/ACSCATAL.2C01837.

Kramer, L., Le, X., Rodriguez, M., Wilson, M.A., Guo, J., Niu, W., 2020. Engineering carboxylic acid reductase (CAR) through a whole-cell growth-coupled NADPH recycling strategy. ACS Synth. Biol. 9 (7), 1632–1637. https://doi.org/10.1021/ACSSYNBIO.0C00290.

Lee, S.Y., Kim, H.U., Chae, T.U., Cho, J.S., Kim, J.W., Shin, J.H., Kim, D.I., Ko, Y.S., Jang, W.D., Jang, Y.S., 2019. A comprehensive metabolic map for production of bio-based chemicals. Nature Catalysis 2 (1), 18–33. https://doi.org/10.1038/s41929-018-0212-4.

Legon, L., Corre, C., Bates, D.G., Mannan, A.A., 2022. gcFront: a tool for determining a Pareto front of growth-coupled cell factory designs. Bioinformatics. https://doi.org/10.1093/BIOINFORMATICS/BTAC376.

Li, M., Ho, P.Y., Yao, S., Shimizu, K., 2006. Effect of lpdA gene knockout on the metabolism in Escherichia coli based on enzyme activities, intracellular metabolite concentrations and metabolic flux analysis by 13C-labeling experiments. J. Biotechnol. 122 (2), 254–266. https://doi.org/10.1016/J.JBIOTEC.2005.09.016.

Liang, K., Shen, C.R., 2017. Selection of an endogenous 2,3-butanediol pathway in Escherichia coli by fermentative redox balance. Metab. Eng. 39, 181–191. https://doi.org/10.1016/J.YMBEN.2016.11.012.

Liao, J.C., Mi, L., Pontrelli, S., Luo, S., 2016. Fuelling the future: microbial engineering for the production of sustainable biofuels. Nature Reviews Microbiology 14 (5), 288–304. https://doi.org/10.1038/nrmicro.2016.32.

Lin, S.-J., Guarente, L., 2003. Nicotinamide adenine dinucleotide, a metabolic regulator of transcription, longevity and disease. Curr. Opin. Cell Biol. 15, 241–246. https://doi.org/10.1016/S0955-0674(03)00006-1.

Lindner, S.N., Calzadiaz Ramirez, L., Krü, J.L., Yishai, O., Belkhelfa, S., He, H., Bouzon, M., Dö, V., Bar-Even, A., 2018. NADPH-Auxotrophic E. coli: A Sensor Strain for Testing In vivo Regeneration of NADPH. https://doi.org/10.1021/acssynbio.8b00313.

Liu, J., Li, H., Zhao, G., Caiyin, Q., Qiao, J., 2018. Redox cofactor engineering in industrial microorganisms: strategies, recent applications and future directions. J. Ind. Microbiol. Biotechnol. 45 (5), 313–327. https://doi.org/10.1007/S10295-018-2031-7.

Liu, Y., Feng, Y., Wang, L., Guo, X., Liu, W., Li, Q., Wang, X., Xue, S., Zhao, Z.K., 2019. Structural insights into Phosphite dehydrogenase variants favoring a non-natural redox cofactor. ACS Catal. 9 (3), 1883–1887. https://doi.org/10.1021/ACSCATAL.8B04822.

Liu, Y., Li, Z., Guo, X., Wang, X., Zhao, Z.K., Chloramphenicol, C., Kanamycin, K., 2022. A phosphite-based screening platform for identification of enzymes favoring nonnatural cofactors. Scientific Reports 12 (1), 1–8. https://doi.org/10.1038/s41598-022-16599-0.

Lobstein, J., Emrich, C.A., Jeans, C., Faulkner, M., Riggs, P., Berkmen, M., 2012. SHuffle, a novel Escherichia coli protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm. Microb. Cell Factories 11 (1), 1–16. https://doi.org/10.1186/1475-2859-11-56.

Longwell, C.K., Labanieh, L., Cochran, J.R., 2017. High-throughput screening technologies for enzyme engineering. Curr. Opin. Biotechnol. 48, 196–202. https://doi.org/10.1016/J.COPBIO.2017.05.012.

Lutz, S., 2010. Beyond directed evolution - semi-rational protein engineering and design. Curr. Opin. Biotechnol. 21 (6), 734. https://doi.org/10.1016/J.COPBIO.2010.08.011.

MacBeath, G., Kast, P., Hilvert, D., 1998. Redesigning enzyme topology by directed evolution. Science 279 (5358), 1958–1961. https://doi.org/10.1126/SCIENCE.279.5358.1958.

Machado, H.B., Dekishima, Y., Luo, H., Lan, E.I., Liao, J.C., 2012. A selection platform for carbon chain elongation using the CoA-dependent pathway to produce linear higher alcohols. Metab. Eng. 14 (5), 504–511. https://doi.org/10.1016/J.YMBEN.2012.07.002.

Martínez, I., Zhu, J., Lin, H., Bennett, G.N., San, K.Y., 2008. Replacing Escherichia coli NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with a NADP-dependent enzyme from Clostridium acetobutylicum facilitates NADPH dependent pathways. Metab. Eng. 10 (6), 352–359. https://doi.org/10.1016/j.ymben.2008.09.001.

Maxel, S., Aspacio, D., King, E., Zhang, L., Acosta, A.P., Li, H., 2020a. A growth-based, high-throughput selection platform enables remodeling of 4-Hydroxybenzoate hydroxylase active site. ACS Catal. 10 (12), 6969–6974. https://doi.org/10.1021/ACSCATAL.0C01892.

Maxel, S., King, E., Zhang, Y., Luo, R., Li, H., 2020b. Leveraging oxidative stress to regulate redox balance-based, in vivo growth selections for oxygenase engineering. Cite This: ACS Synth. Biol 2020, 3133. https://doi.org/10.1021/acssynbio.0c00380.

Maxel, S., Zhang, L., King, E., Acosta, A.P., Luo, R., Li, H., 2020c. In vivo, high-throughput selection of thermostable cyclohexanone monooxygenase (CHMO). Catalysts 10 (8), 935. https://doi.org/10.3390/catal10080935.

Maxel, S., Saleh, S., King, E., Aspacio, D., Zhang, L., Luo, R., Li, H., 2021. Growth-based, high-throughput selection for NADH preference in an oxygen-dependent biocatalyst. ACS Synth. Biol. 10 https://doi.org/10.1021/ACSSYNBIO.1C00258.

Mccullum, E.O., Williams, B.A.R., Zhang, J., Chaput, J.C., 2010. Error based PCR mutagenesis protocols. Methods Mol. Biol. 634 (3), 103–109. http://link.springer.com/10.1007/978-1-60761-652-8.

Metcalf, W.W., Wanner, B.L., Chen, C.-M., Ye, Q.-Z., Zhu, Z., Wanner, B.L., Walsh, C.T., Biol, J., 1991. Involvement of the Escherichia coli phn (psiD) gene cluster in assimilation of phosphorus in the form of phosphonates, Phosphite, pi esters, and pi. J. Bacteriol. 173 (2), 587–600.

MohammadiPeyhani, H., Hafner, J., Sveshnikova, A., Viterbo, V., Hatzimanikatis, V., 2022. Expanding biochemical knowledge and illuminating metabolic dark matter with ATLASx. Nature Communications 13 (1), 1–12. https://doi.org/10.1038/s41467-022-29238-z.

Opitz, C.A., Heiland, I., 2015. Dynamics of NAD-metabolism: everything but constant. Biochem. Soc. Trans. 43 (6), 1127–1132. https://doi.org/10.1042/BST20150133.

Orsi, E., Claassens, N.J., Nikel, P.I., Lindner, S.N., 2021. Growth-coupled selection of synthetic modules to accelerate cell factory development. Nature Communications 12 (1), 1–5. https://doi.org/10.1038/s41467-021-25665-6.

Overton, T.W., 2014. Recombinant protein production in bacterial hosts. Drug Discov. Today 19 (5), 590–601. https://doi.org/10.1016/J.DRUDIS.2013.11.008.

Packer, M.S., Liu, D.R., 2015. Methods for the directed evolution of proteins. Nature Reviews Genetics 16 (7), 379–394. https://doi.org/10.1038/nrg3927.

Park, H., Kim, S., 2021. Gene-specific mutagenesis enables rapid continuous evolution of enzymes in vivo. Nucleic Acids Res. 49 (6), e32. https://doi.org/10.1093/NAR/GKAA1231.

Rasor, B.J., Vögeli, B., Landwehr, G.M., Bogart, J.W., Karim, A.S., Jewett, M.C., 2021. Toward sustainable, cell-free biomanufacturing. Curr. Opin. Biotechnol. 69, 136–144. https://doi.org/10.1016/J.COPBIO.2020.12.012.

Ravikumar, A., Arzumanyan, G.A., Obadi, M.K.A., Javanpour, A.A., Liu, C.C., 2018. Scalable, continuous evolution of genes at mutation rates above genomic error thresholds. Cell 175 (7), 1946. https://doi.org/10.1016/J.CELL.2018.10.021.

Reetz, M.T., Carballeira, J.D., 2007. Iterative saturation mutagenesis (ISM) for rapid directed evolution of functional enzymes. Nature Protocols 2 (4), 891–903. https://doi.org/10.1038/nprot.2007.72.

Rosano, G.L., Ceccarelli, E.A., 2014. Recombinant protein expression in Escherichia coli: advances and challenges. Front. Microbiol. 5 (APR), 172. https://doi.org/10.3389/FMICB.2014.00172.

Sauer, U., Canonaco, F., Heri, S., Perrenoud, A., Fischer, E., 2004. The soluble and membrane-bound Transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of Escherichia coli*. J. Biol. Chem. 279 (8), 6613–6619. https://doi.org/10.1074/JBC.M311657200.

Scown, C.D., Keasling, J.D., 2022. Sustainable manufacturing with synthetic biology. Nature Biotechnology 40 (3), 304–307. https://doi.org/10.1038/s41587-022-01248-8.

Sekar, B.S., Seol, E., Park, S., 2017. Co-production of hydrogen and ethanol from glucose in Escherichia coli by activation of pentose-phosphate pathway through deletion of phosphoglucose isomerase (pgi) and overexpression of glucose-6-phosphate dehydrogenase (zwf) and 6-phosphogluconate dehyd. Biotechnology for Biofuels 10 (1), 1–12. https://doi.org/10.1186/S13068-017-0768-2.

Sellés Vidal, L., Murray, J.W., Heap, J.T., 2021. Versatile selective evolutionary pressure using synthetic defect in universal metabolism. Nature Communications 12 (1), 1–15. https://doi.org/10.1038/s41467-021-27266-9.

Seta, F. Della, Boschi-Muller, S., Vignais, M.L., Branlant, G., 1997. Characterization of *Escherichia coli* Strains with gapA and gapB Genes Deleted. In: Journal of Bacteriology (Vol. 179, Issue 16).

Shen, C.R., Lan, E.I., Dekishima, Y., Baez, A., Cho, K.M., Liao, J.C., 2011. Driving forces enable high-titer anaerobic 1-butanol synthesis in Escherichia coli. Appl. Environ. Microbiol. 77 (9), 2905–2915. https://doi.org/10.1128/AEM.03034-10.

Simon, A.J., d'Oelsnitz, S., Ellington, A.D., 2019. Synthetic evolution. Nature Biotechnology 37 (7), 730–743. https://doi.org/10.1038/s41587-019-0157-4.

Spaans, S.K., Weusthuis, R.A., van der Oost, J., Kengen, S.W.M., 2015. NADPH-generating systems in bacteria and archaea. In: Frontiers in Microbiology (Vol. 6, Issue JUL, p. 742). Frontiers Research Foundation. https://doi.org/10.3389/fmicb.2015.00742.

Takeno, S., Hori, K., Ohtani, S., Mimura, A., Mitsuhashi, S., Ikeda, M., 2016. L-lysine production independent of the oxidative pentose phosphate pathway by Corynebacterium glutamicum with the Streptococcus mutans gapN gene. Metab. Eng. 37, 1–10. https://doi.org/10.1016/J.YMBEN.2016.03.007.

Tamakawa, H., Ikushima, S., Yoshida, S., 2011. Ethanol production from xylose by a recombinant Candida utilis strain expressing protein-engineered xylose reductase and xylitol dehydrogenase. Biosci. Biotechnol. Biochem. 75 (10), 1994–2000. https://doi.org/10.1271/BBB.110426.

Tawfik, D.S., Griffiths, A.D., 1998. Man-made cell-like compartments for molecular evolution. Nat. Biotechnol. 16. https://doi.org/10.1038/nbt0798-652.

Theodosiou, E., Tüllinghoff, A., Toepel, J., Bühler, B., 2022. Exploitation of hetero- and phototrophic metabolic modules for redox-intensive whole-cell biocatalysis. Frontiers in Bioengineering and Biotechnology 10, 462. https://doi.org/10.3389/FBIOE.2022.855715.

Volke, D.C., Martino, R.A., Kozaeva, E., Smania, A.M., Nikel, P.I., 2022. Modular (de) construction of complex bacterial phenotypes by CRISPR/nCas9-assisted, multiplex cytidine base-editing. Nature Communications 13 (1), 1–14. https://doi.org/10.1038/s41467-022-30780-z.

Von Kamp, A., Klamt, S., 2017. Growth-coupled overproduction is feasible for almost all metabolites in five major production organisms. Nature Communications 8 (1), 1–10. https://doi.org/10.1038/ncomms15956.

Wang, H.H., Isaacs, F.J., Carr, P.A., Sun, Z.Z., Xu, G., Forest, C.R., Church, G.M., 2009. Programming cells by multiplex genome engineering and accelerated evolution. Nature 460 (7257), 894–898. https://doi.org/10.1038/nature08187.

Wang, X., Feng, Y., Guo, X., Wang, Q., Ning, S., Li, Q., Wang, J., Wang, L., Zhao, Z.K., 2021. Creating enzymes and self-sufficient cells for biosynthesis of the non-natural cofactor nicotinamide cytosine dinucleotide. Nature Communications 12 (1), 1–9. https://doi.org/10.1038/s41467-021-22357-z.

Wang, Y., San, K.Y., Bennett, G.N., 2013. Cofactor engineering for advancing chemical biotechnology. Curr. Opin. Biotechnol. 24 (6), 994–999. https://doi.org/10.1016/J.COPBIO.2013.03.022.

Wenk, S., Schann, K., He, H., Rainaldi, V., Kim, S., Lindner, S.N., Bar-Even, A., 2020. An "energy-auxotroph" *Escherichia coli* provides an in vivo platform for assessing NADH regeneration systems. Biotechnol. Bioeng. 117 (11), 3422–3434. https://doi.org/10.1002/bit.27490.

Weusthuis, R.A., Folch, P.L., Pozo-Rodríguez, A., Paul, C.E., 2020. Applying non-canonical redox cofactors in fermentation processes. In: *iScience* (Vol. 23, Issue 9, p. 101471). Elsevier Inc. https://doi.org/10.1016/j.isci.2020.101471

Xiao, H., Bao, Z., Zhao, H., 2015. High throughput screening and selection methods for directed enzyme evolution. Ind. Eng. Chem. Res. 54 (16), 4011–4020. https://doi.org/10.1021/IE503060A.

Yamauchi, Y., Hirasawa, T., Nishii, M., Furusawa, C., Shimizu, H., 2014. Enhanced acetic acid and succinic acid production under microaerobic conditions by Corynebacterium glutamicum harboring Escherichia coli transhydrogenase gene pntAB. J. Gen. Appl. Microbiol. 60 (3), 112–118. https://doi.org/10.2323/JGAM.60.112.

Yang, G., Withers, S.G., 2009. Ultrahigh-throughput FACS-based screening for directed enzyme evolution. ChemBioChem 10 (17), 2704–2715. https://doi.org/10.1002/cbic.200900384.

Yun, M., Park, C.G., Kim, J.Y., Park, H.W., 2000. Structural analysis of glyceraldehyde 3-phosphate dehydrogenase from Escherichia coli: direct evidence of substrate binding and cofactor-induced conformational changes†,‡. Biochemistry 39 (35), 10702–10710. https://doi.org/10.1021/BI9927080.

Zhang, L., King, E., Luo, R., Li, H., 2018. Development of a high-throughput, in vivo selection platform for NADPH-dependent reactions based on redox balance principles. ACS Synth. Biol. 7 (7), 1715–1721. https://doi.org/10.1021/acssynbio.8b00179.

Zhang, L., King, E., Black, W.B., Heckmann, C.M., Wolder, A., Cui, Y., Nicklen, F., Siegel, J.B., Luo, R., Paul, C.E., Li, H., 2022. Directed evolution of phosphite dehydrogenase to cycle noncanonical redox cofactors via universal growth selection platform. Nature Communications 13 (1), 1–12. https://doi.org/10.1038/s41467-022-32727-w.

Zhang, X., Jantama, K., Moore, J.C., Shanmugam, K.T., Ingram, L.O., 2007. Production of L-alanine by metabolically engineered Escherichia coli. Appl. Microbiol. Biotechnol. 77 (2), 355–366. https://doi.org/10.1007/S00253-007-1170-Y.

Zheng, L., Baumann, U., Reymond, J.L., 2004. An efficient one-step site-directed and site-saturation mutagenesis protocol. Nucleic Acids Res. 32 (14), e115. https://doi.org/10.1093/NAR/GNH110.

Zhou, Y., Wang, L., Yang, F., Lin, X., Zhang, S., Zhao, Z.K., 2011. Determining the extremes of the cellular NAD(H) level by using an Escherichia coli NAD +-auxotrophic mutant. Appl. Environ. Microbiol. 77 (17), 6133–6140. https://doi.org/10.1128/AEM.00630-11.

Zhou, Y.J., Kerkhoven, E.J., Nielsen, J., 2018. Barriers and opportunities in bio-based production of hydrocarbons. Nature Energy 3 (11), 925–935. https://doi.org/10.1038/s41560-018-0197-x.