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Detoxification as metabolic rewiring: carbon and redox recovery from lignocellulosic inhibitors in biorefinery hosts

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The pretreatment process of lignocellulosic biomass results in the formation of inhibitory by-products such as furan aldehydes, phenolics, and weak acids that hinder the process of microbial fermentation. Historically, the detoxification process has been regarded as an exercise in waste management, resulting in carbon loss and reduced strength. The current review illustrates how detoxification can be viewed from a new perspective, making it possible to recover carbon and balance redox simultaneously. The results of our analysis indicate that oxidation-based detoxification pathways, and particularly the conversion of furfural into 2-furoic acid by aldehyde hydrogenases followed by entry into the TCA cycle, allow for the recovery of five carbon atoms from each molecule of inhibitor. Our findings revealed the importance of cofactor specificity in the detoxification process: NADPH-dependent reductases negatively affect biosynthesis while their NADH-dependent or bifunctional counterparts allow for the retention of the biosynthetic potential. The modification of aldo-keto reductases to favor NADH along with the combination of detoxification reactions with transhydrogenases and fusion proteins facilitate the process of detoxification without harming product yields. The integration of processes into host organisms is of critical significance acetyl-CoA pathway serves well for lipid synthesis by *Yarrowia lipolytica* while TCA pathway is beneficial for *Pseudomonas* spp. The co-utilization of inhibitors with sugars in conditions representative of real hydrolysates offers a win-win situation for carbon recovery when detoxification routes are implemented prior to key metabolic pathways. This approach allows us to regard the inhibitors as auxiliary substrates and develop biorefineries that benefit from the detoxification step in terms of increased product yield. It appears that industrial implementation will require systemic modeling and rational enzyme redesign.

KEYWORDS

biorefinery integration, carbon recovery, cofactor engineering, enzyme detoxification, host-specific metabolism, lignocellulosic inhibitors, metabolic rewiring, redox balance

1 Introduction

Lignocellulosic biomass is the most abundant renewable organic material on earth and is therefore an attractive, low-cost feedstock for sustainable production of biofuels and biochemical (Guo et al., 2022; Fernández-Sandoval et al., 2024). However, the complex structure of lignocellulose requires intensive pretreatment and hydrolysis to liberate fermentable sugars (Fernández-Sandoval et al., 2024), processes that inevitably generate inhibitory by-products. These include weak organic acids, furan derivatives, and various phenolic lignin fragments (Palmqvist and Hahn-Hägerdal, 2000; Fernández-Sandoval et al., 2024). Such lignocellulose-derived inhibitors are well known to impede microbial fermentations by imposing stress and causing metabolic bottlenecks, thereby reducing growth rates, product yields, and process productivity (Palmqvist and Hahn-Hägerdal, 2000).

In addition to their detrimental effects on microbial fermentation, pretreatment-derived inhibitors can also impair the enzymatic hydrolysis step. Among these compounds, lignin-derived phenolics are considered the major inhibitors of cellulases because they promote non-productive enzyme adsorption, reversible or irreversible enzyme deactivation, and reduced cellulose accessibility, ultimately lowering saccharification efficiency. In contrast, furan derivatives and weak acids generally exert a greater inhibitory effect on microbial metabolism than on hydrolytic enzymes, although elevated concentrations may also compromise enzymatic hydrolysis. Consequently, minimizing inhibitor formation or implementing appropriate detoxification strategies can improve both sugar release during saccharification and subsequent microbial fermentation. However, the present review focuses specifically on the downstream metabolic detoxification, carbon recovery, and redox balancing of these inhibitors after their generation during pretreatment (Zhai et al., 2022).

To mitigate inhibition, a variety of detoxification strategies have been developed. Traditionally, physical and chemical methods (such as washing, dilution, evaporation, alkali neutralization, over liming, activated carbon adsorption, ion exchange, and solvent extraction) have been applied to remove or transform inhibitors before fermentation (Guo et al., 2022). These methods can indeed reduce toxin levels but often at the cost of sugar loss, added process complexity, or chemical waste. In response, biological approaches have gained attention: specialized microbes or isolated enzymes can selectively degrade certain inhibitors while preserving fermentable carbon. For example, lignin-degrading enzymes (ligninolytic oxidoreductases such as laccases and peroxidases) have been shown to oxidize and polymerize phenolic inhibitors, thereby lowering their toxicity and enhancing hydrolysate fermentability (Fernández-Sandoval et al., 2024). The use of enzymes or microorganisms to detoxify biomass has received attention in recent reports; in particular, laccases may be applied in pretreatment or *in situ* detoxification steps to enhance biomass utilization as biofuels, showing results via the use of free enzymes or recombinant strains (Kuyper et al., 2004; Marsafari and Xu, 2020; Zhai et al., 2022). For instance, the research carried out by Moreno et al. (2013) showed that the particular laccase treatment applied *in situ* to steam-exploded wheat straw reduced total phenolic content by 50 to

80%, shortened the lag phase, improving the cell viability, and resulting in approximately 22 g/L ethanol production at significant substrate loadings. Fernández-Sandoval et al. (2026) indicated that the heterologous laccase applied for surface-display in *Saccharomyces cerevisiae* contributed to the simultaneous oxidation of phenolics during fermentation of teak wood hydrolysate and improving cell growth and ethanol production. Zeng et al. (2026) discovered the ancestral bacterial laccase (Anc44) used to degrade over 50% of four tested phenolic inhibitors, reducing the total phenolic shortfall by as much as 41% of wheat straw hydrolysate and consequently resulting in the production of microbial protein without the need for purification of enzymes.

Despite these advances, most prior work treats detoxification as an ancillary process separate from core metabolism. Current reviews tend to discuss inhibitor removal methods in isolation, without considering how inhibitor-derived carbon and electrons might be reused in the cell. Integrated analyses of detoxification in the context of the full biorefinery, including central carbon and redox metabolism, are rare. Building on this insight, we hypothesize a new paradigm: enzymes traditionally used only to neutralize inhibitors could be repurposed as active metabolic modules that reclaim inhibitor carbon and reducing power. In other words, rather than passively discarding inhibitors, engineered detox pathways could convert them into useful metabolites or feed into native pathways, improving carbon efficiency and redox balance.

In other words, this review asks how lignocellulosic inhibitor detoxification can be redesigned from a simple removal step into a host-specific metabolic strategy that recovers both carbon and reducing power. We focus on major production hosts, including yeast, bacteria, oleaginous microbes, and photosynthetic systems, and on the main inhibitor classes generated during pretreatment: furanics, weak acids, phenolics, and aldehydes while comparing reductive, oxidative, and assimilatory detox pathways that can be integrated into central metabolism.

Hence, the aim of this review is to articulate and substantiate this vision. We will survey the state of knowledge on lignocellulosic inhibitor chemistry and toxicity, existing detoxification methods, and relevant metabolic engineering strategies. In so doing, we seek to clarify how detox enzymes might be redesigned or regulated to channel inhibitor-derived substrates back into productive metabolism. This conceptual approach aspires to support a “closed-loop” biorefinery design, where inhibitor management not only alleviates toxicity but actively contributes to product yield. By citing recent primary research on lignocellulosic hydrolysate composition, microbial stress responses, detox enzymes, and metabolic flux engineering, this introduction provides a comprehensive background and frames the novel contribution of the review.

2 Carbon and redox bottlenecks in major biofuel hosts

The mechanistic basis for the central argument of this review is that detoxification should not be viewed only as inhibitor removal, but as a means to recover carbon and reducing power within the

host (Figure 1). Understanding where carbon and redox losses occur in different biofuel platforms is essential for identifying how detox enzymes can be integrated into native metabolism and improve overall process efficiency.

2.1 General concept of carbon-flux limitation in microbial fuel production

In microbial fermentation, the theoretical carbon yield of biofuel is often compromised by unavoidable losses. For example, converting pyruvate to ethanol in yeast emits CO₂ (one CO₂ per pyruvate), capping ethanol yield at 67% of sugar carbon. Likewise, many engineered pathways require CO₂-releasing steps (e.g. acetaldehyde dehydrogenase to acetate) that waste carbon (Kuyper et al., 2004; Marsafari and Xu, 2020). Mixed-sugar or pentose fermentations exacerbate this. For instance, *S. cerevisiae* redirected through the oxidative pentose phosphate pathway loses carbon as CO₂ (via glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) while also draining NADPH. In contrast, complete carbon retention would require redox-neutral steps, which seldom align with product pathways. Thus, excess carbon often ends up in byproducts or biomass rather than target fuels (Kuyper et al., 2004; Frick and Wittmann, 2005; He et al., 2007; Marsafari and Xu, 2020).

2.2 General concept of redox imbalance in bioconversion systems

Biofuel pathways also demand tight NADH/NADPH balance. In growth and production, cofactor pools must be re-oxidized or regenerated to sustain flux. When engineering microbes,

heterologous pathways frequently upset native cofactor balances. For instance, diverting glucose into a reductive product can create excess NADH, whereas anabolic reactions consume NADPH. Because many hosts lack efficient transhydrogenases, NADH and NADPH pools are only loosely connected. Redox imbalances manifest as stalled pathways or accumulation of reduced intermediates. Cells often respond by activating “overflow” routes or byproduct sinks such as glycerol in yeast or acetate in bacteria, to restore NAD(P) levels (Bakker et al., 2001; Wang et al., 2013). Maintaining redox homeostasis is therefore a key challenge: engineering must provide sinks or sources for NAD(P)H to match metabolic demands.

Major carbon waste routes include: (1) Decarboxylation steps in central metabolism (e.g. pyruvate → acetyl-CoA + CO₂, or the oxidative pentose phosphate pathway) that release CO₂ (Martin et al., 2005). (2) Byproduct formation such as glycerol, acetate, or organic acids, which divert carbon away from the fuel. For example, *S. cerevisiae* produces glycerol (from DHAP) when NADH must be re-oxidized under anaerobic stress (Bakker et al., 2001; Guadalupe Medina et al., 2010). *E. coli* often secretes acetate at high flux (overflow metabolism) or lactate/ethanol under certain conditions, representing carbon loss (Gecse et al., 2024). (3) Incomplete substrate use: many hosts cannot consume all hydrolysate sugars. For instance, wild-type *S. cerevisiae* does not ferment xylose or arabinose, and even engineered strains may leave oligomers unused (Karhumaa et al., 2006). (4) Futile cycles: simultaneous activity of opposing pathways (e.g. glycolysis vs gluconeogenesis, or the cellodextrin futile cycle) wastes ATP and carbon, reducing net yield. Such metabolic “short circuits” can arise from regulatory imbalance in engineered strains. In sum, carbon yields are lowered by any flux that bypasses product formation, and each host has characteristic waste pathways.

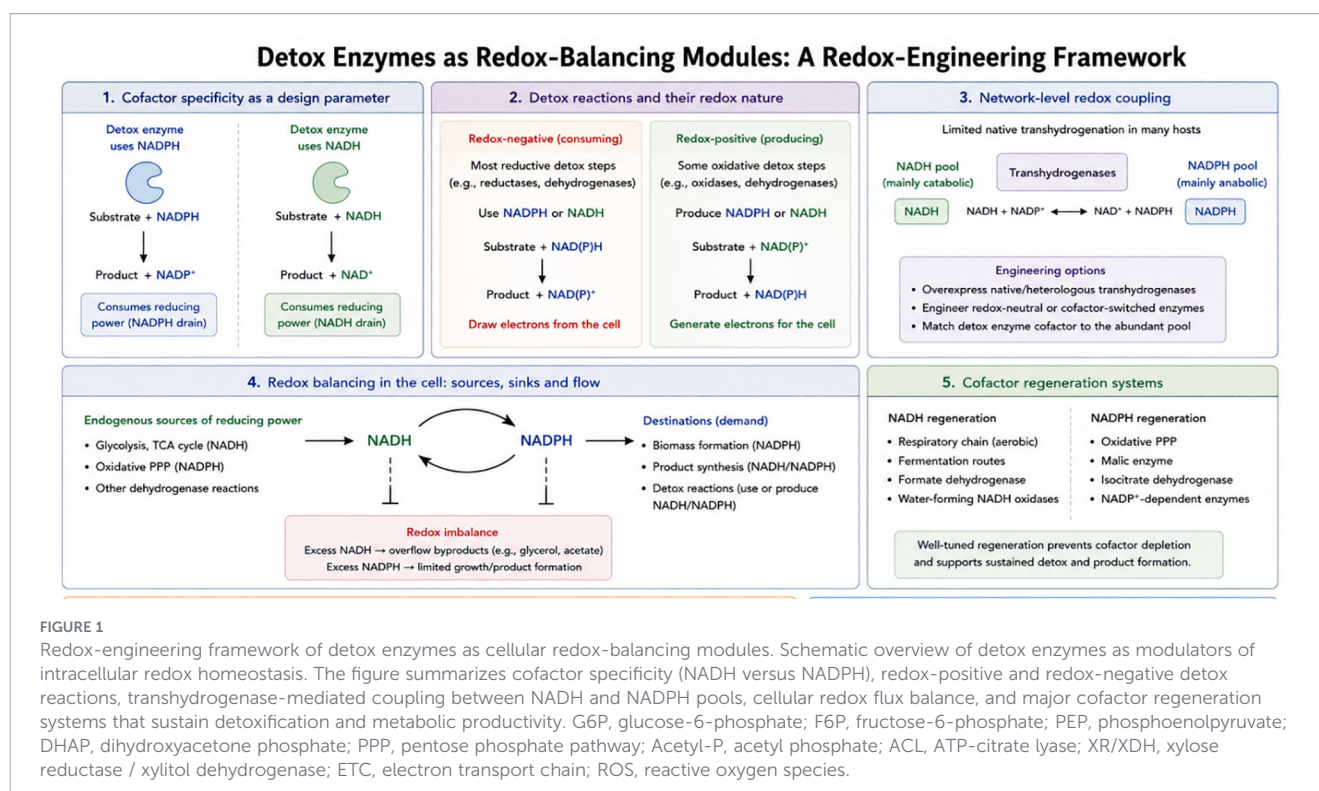


FIGURE 1

Redox-engineering framework of detox enzymes as cellular redox-balancing modules. Schematic overview of detox enzymes as modulators of intracellular redox homeostasis. The figure summarizes cofactor specificity (NADH versus NADPH), redox-positive and redox-negative detox reactions, transhydrogenase-mediated coupling between NADH and NADPH pools, cellular redox flux balance, and major cofactor regeneration systems that sustain detoxification and metabolic productivity. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; PEP, phosphoenolpyruvate; DHAP, dihydroxyacetone phosphate; PPP, pentose phosphate pathway; Acetyl-P, acetyl phosphate; ACL, ATP-citrate lyase; XR/XDH, xylose reductase / xylitol dehydrogenase; ETC, electron transport chain; ROS, reactive oxygen species.

Further, the key redox loss routes include several pathways. Initially throw excess NADH/NADPH drain. For instance, detoxification of inhibitors often consumes NADPH, as seen in *E. coli*'s reduction of furfural (Wang et al., 2013). This competes with biosynthesis and depletes reducing power. Secondly, throw cofactor competition. Heterologous pathways may compete with native ones for NADH vs NADPH. For instance, a synthetic NADPH-requiring pathway can starve biomass formation of NADPH, or vice versa (Qiu et al., 2024). Also, in aerobic hosts, the respiratory chain may be overloaded re-oxidizing excess NADH, especially if engineered pathways create more NADH than ATP demand. This can impose a metabolic burden and create reactive oxygen species (Xiao and Loscalzo, 2020). Electron leakage from redox reactions or respiratory chains generates ROS; cells must then expend reducing equivalents (e.g. via glutathione or thioredoxin systems) to quench ROS, indirectly wasting NADPH. For example, microalgae under high light or chemical stress accumulate ROS, damaging enzymes and consuming antioxidants (Koletti et al., 2025). Thus, redox imbalances trigger alternative fluxes (like glycerol or polyol formation in yeast) that re-oxidize cofactors but at the cost of product yield.

2.3 Why detoxification must be matched to host-specific metabolic architecture

Each host's carbon and redox network dictates how inhibitor-derived carbon and electrons can be recycled. In *S. cerevisiae*, ethanol fermentation is redox-balanced only when glycolysis-generated NADH is re-oxidized by ethanol production. However, pentose utilization disrupts this. Engineered xylose metabolism typically uses xylose reductase (XR) and xylitol dehydrogenase (XDH). If XR is NADPH-specific (common in many yeasts), it creates excess NADP⁺ and requires oxygen or external sinks to re-oxidize the NADH from XDH. *S. cerevisiae* has been used to develop xylose metabolic pathways, making use of the xylose reductase (XR) and the xylitol dehydrogenase (XDH) enzymes. When the XR is NADPH-specific, as is the case with several yeast types, NR produces NADP⁺ while the XDH produces NADH, leading to a cofactor imbalance that forces *S. cerevisiae* to secrete xylitol as a redox sink, thereby reducing ethanol production considerably (Guarnieri et al., 2017; Wang et al., 2024).

Even with an NADH-prefering XR, a significant fraction of carbon ends up as glycerol or xylitol. Glycerol formation is another hallmark redox sink: as Bakker et al. (2001) explain, anaerobic yeast produce glycerol (from DHAP) to re-oxidize excess NADH from biosynthesis (a closed redox loop) (Bakker et al., 2001). This glycerol path consumes ATP and carbon (glycerol carbon leaves the ethanol pathway), reducing product yield. Meanwhile, some carbon flows via the oxidative pentose phosphate pathway (PPP) (e.g. for NADPH supply), inherently releasing CO₂ (one CO₂ per two NADPH) and wasting carbon. In summary, *S. cerevisiae* often loses carbon to CO₂ (from pyruvate decarboxylation and PPP) and to glycerol or xylitol byproducts, primarily driven by the need to balance NADH/NADPH during anaerobic growth. In contrast, *E. coli* naturally ferments sugars to ethanol, acetate, and lactate, but its redox balancing differs from yeast. Under aerobic conditions it

respires NADH via the TCA cycle/ETC, but under stress or high flux (overflow), it secretes acetate (carbon loss). Critically, lignocellulosic inhibitors impose an NADPH burden. Wang et al. (2013) demonstrated that *E. coli*'s broad-specificity aldehyde reductase YqhD uses NADPH to reduce furfural to furfuryl alcohol, depleting NADPH and stalling sulfate assimilation and other biosynthesis (Wang et al., 2013). Deleting yqhD or overexpressing the transhydrogenase (pntAB) increased NADPH and rescued growth. Thus, *E. coli*'s NADPH drain for inhibitor reduction is a redox bottleneck. Carbon-wise, furfural detox produces byproducts (furan carboxylic acids) that can feed into the TCA, but often carbon is diverted to maintenance pathways or excreted. Moreover, product-specific pathways in *E. coli* have their own redox demands. For example, succinate production requires NADH whereas polyhydroxyalkanoate (PHA) formation needs NADPH. Mismatches between cofactor supply and pathway demand force use of alternative routes or shuttle systems. In sum, *E. coli* faces carbon loss via acetate overflow and potential incomplete sugar use, and redox stress mainly via NADPH depletion in detox and balancing multiple cofactor needs (Wang et al., 2013). On the other hand, *Y. lipolytica* is an oleaginous yeast that naturally directs carbon to lipids. It possesses an ATP-citrate lyase (ACL) to generate cytosolic acetyl-CoA, which is a carbon precursor for lipogenesis. However, acetyl-CoA supply can still be limiting for non-native products. It was found that for amorphadiene (a terpenoid) production in *Y. lipolytica*, boosting HMG-CoA flux (from acetyl-CoA) was essential: overexpression of HMG-CoA reductase and enhancing acetyl-CoA availability markedly raised yields (Marsafari and Xu, 2020). Similarly, a study increased β -farnesene by expressing mouse ACL (to further raise cytosolic acetyl-CoA) and by introducing a malic enzyme to regenerate NADPH (Marsafari et al., 2022). Thus, Yarrowia's carbon bottleneck is ensuring enough acetyl-CoA flows to the target pathway. Concurrently, lipid (and terpene) synthesis requires large amounts of NADPH. In *Y. lipolytica*, as in other lipogenic microbes, NADPH is often the true limiting cofactor; pathways like the pentose phosphate pathway and malic enzyme are harnessed to supply it. If NADPH is scarce, carbon either accumulates as citrate or is diverted to undesired sinks. Finally, photosynthetic hosts (green algae and cyanobacteria) have unique bottlenecks. Light reactions supply ATP and NADPH at fixed ratios. In *Synechocystis*, for example, linear electron flow produces an ATP/NADPH of 1.28 which is lower than the ~1.5 ratio required by the Calvin–Benson cycle and anabolic reactions. To balance this, cyanobacteria use cyclic electron flow or alternate pathways, but mismatches still occur. If NADPH accumulates (e.g. under low CO₂), the chain becomes over-reduced and generates ROS. Indeed, microalgae are highly sensitive to oxidative stress: environmental perturbations (light, nutrients, toxins) cause intracellular ROS surges that damage chloroplasts and consume antioxidant pools (Kugler and Stensjö, 2023; Koletti et al., 2025). On the carbon side, RuBisCO's low efficiency and oxygenase activity limit carbon fixation, so a large fraction of fixed carbon is often respired or excreted (photorespiration). Furthermore, most algae cannot efficiently metabolize lignocellulose-derived inhibitors at all, so these compounds mainly impose stress rather than serve as carbon. Thus, photosynthetic hosts suffer from energy/redox

imbalance (ATP/NADPH mismatch) and CO₂ fixation limits, along with severe sensitivity to oxidative inhibitors. Improving algal biofuels often requires engineering alternative electron sinks or CO₂-concentrating mechanisms to mitigate these bottlenecks. In summary, an effective detoxification strategy should not simply remove toxins; it should release the inhibitor's carbon and electrons in a form that complements the host's native pathways. By aligning detox enzymes with each host's metabolic "wiring" (e.g. linking an aldehyde dehydrogenase to the host's NADH pool or a phenol dioxygenase to the TCA), inhibitor-derived substrates become additional feedstock rather than wasted drain. This host-specific integration of detoxification can enhance carbon yield and redox balance, moving toward a closed-loop biorefinery where inhibitors become resources, not liabilities.

3 Detox enzymes as carbon-rewiring modules

Historically, microbial detoxification has been a carbon-discarding process: inhibitors are reduced or conjugated to harmless forms (e.g. reduction of furfural to alcohol) that cannot enter metabolism. While this alleviates toxicity, it irreversibly loses those carbons (Wang et al., 2024). By contrast, the "detox-to-metabolism" concept treats inhibitors as auxiliary substrates. Here, detox enzymes perform oxidative or ring-cleavage reactions that convert inhibitors into standard metabolites. For example, oxidative detoxification of furfural yields furoic acid instead of alcohol, preserving the carbon backbone. The key is to integrate these reactions into existing pathways: so-called carbon-rewiring enzymes funnel inhibitor-derived carbons into central metabolism rather than dumping them as waste. This shifts the role of detoxification from simple disposal to a carbon capture strategy, improving yield and reducing CO₂ release (Guarnieri et al., 2017; Wang et al., 2024).

3.1 Carbon recovery from furfural and HMF through oxidative catabolism

Furfural and 5-hydroxymethylfurfural (HMF) are important inhibitors formed in lignocellulose pretreatment. While most bacteria convert these aldehydes to their alcohols—causing irreversible loss of carbon—their method is based on using oxidation that keeps the carbon backbone intact. This method is the most thoroughly studied in *Cupriavidus basilensis* HMF14, which converts furfural to 2-furoic acid via a broad-spectrum aldehyde dehydrogenase (Zheng et al., 2025). The next step is to activate 2-furoic acid to its CoA ester and then to hydroxylate and cleave the ring to form 2-oxoglutarate (α -KG), an intermediate compound of the TCA cycle (Snoeck et al., 2024; Zheng et al., 2025). The process for HMF is very similar and involves oxidation of hydroxymethyl and aldehyde groups to form 2,5-furandicarboxylic acid (FDCA), which is further decarboxylated into 2-furoic acid to get into the same downstream path (Zheng et al., 2025). This process ensures that five carbon old

furfural and six carbon HMF skeletons are preserved and made available for the process so that the loss of carbon in the form of CO₂ is avoided in this way. The engineering of *P. putida* KT2440 by incorporation of the *hmf* gene cluster from *C. basilensis* HMF14 allows it to grow on furans as a sole carbon source, proving that it has synthesized the carbon present in the inhibitors and successfully used this carbon during the basic metabolic processes (Lipscomb, 2008). It should be noted that in these pathways, aldehyde dehydrogenases are typically found to utilize either NAD⁺ or NADP⁺ in order to produce NADH or NADPH instead of consuming them, which aids in the maintenance of redox homeostasis during detoxification (Zheng et al., 2025). However, it must be mentioned that these oxidation pathways are oxygen-dependent (which require dioxygenases for ring cleavage), making the process applicable for aerobic or microaerobic processes (Snoeck et al., 2024).

Similarly, HMF is routed via 2,5-furandicarboxylic acid (FDCA) to 2-furoic acid and then into central metabolism. Engineering *P. putida* KT2440 with a furfural/HMF catabolic gene cluster enabled growth on furans as sole carbon sources (Guarnieri et al., 2017). These oxidative routes preserve the five-carbon skeleton, recover carbon that would otherwise be lost as CO₂, and supply TCA intermediates with minimal NADPH drain (since aldehyde dehydrogenases often use NAD⁺/NADP⁺).

3.2 Conversion of inhibitor-derived intermediates into central metabolism

Successful carbon recovery requires that detoxification products be efficiently converted into central metabolites. For example, furoic acid (from furfural) is typically converted to succinyl-CoA or 2-oxoglutarate via CoA-activated intermediates and dihydroxylation steps. In engineered strains, 2-furoic acid is a metabolic bottleneck, so overexpressing its catabolic enzymes ensures flux into the TCA cycle (Guarnieri et al., 2017). Likewise, phenolic inhibitors (e.g. vanillic acid) are demethylated and dehydroxylated into aromatic diacids (protocatechuate or catechol), which are then cleaved by dioxygenases into succinate, acetyl-CoA or pyruvate. By coupling detox enzymes to downstream pathways, cells avoid accumulation of toxic intermediates. In practice, this means co-expressing detoxification and catabolic genes so that, for instance, furfural → furoate → 2-oxoglutarate occurs in a contiguous metabolic route (Sgro et al., 2023). This integration minimizes "carbon leak" and channelizes inhibitor carbon directly into biomass and products.

3.3 Assimilation of aromatic inhibitors into protocatechuate, catechol, or β -ketoadipate routes

Aromatic inhibitors originating from lignin, including vanillin, syringaldehyde, ferulic acid, p-coumaric acid, and their corresponding products, are processed via a limited number of conserved catabolism pathways that exist in bacteria and fungi, with variations in pathways across different species (Costa et al., 2023; Zhu et al., 2025). The β -ketoadipate pathway is the most utilized pathway that

helps break down the aromatic ring and contribute to central carbon metabolism (Wang et al., 2025; Zou et al., 2025). In bacteria, the protocatechuate part of the β -ketoadipate pathway is studied in bacteria like *Pseudomonas putida*, *Acinetobacter* and *Rhodococcus* species (Yao et al., 2024; Zhu et al., 2025; Zou et al., 2025). In bacteria, the following general scheme applies: vanillin $\rightarrow$ vanillate (with the help of vanillin dehydrogenase) $\rightarrow$ protocatechuate (thanks to vanillate demethylase); p-coumarate $\rightarrow$ 4-hydroxybenzoate $\rightarrow$ protocatechuate; ferulic acid $\rightarrow$ vanillate $\rightarrow$ protocatechuate (Yao et al., 2024). Protocatechuate is converted into β -carboxymuconate with the help of protocatechuate 3,4-dioxygenase (or 4,5-dioxygenase) and then β -ketoadipate enol-lactone, β -ketoadipate, succinyl-CoA and acetyl-CoA (Wang et al., 2025; Zou et al., 2025). In addition, there is also a catechol branch in the process that involves catechol 1,2-dioxygenase, which transforms catechol into cis, cis-muconate and to β -ketoadipate (Zhu et al., 2025). Certain species of bacteria can use an alternate route for processing lignin-derived compounds, as demonstrated by the case of *Sphingobium* spp. and *Novosphingobium aromaticivorans*. Their lignin-induced enzymatic pathways include the lig pathway that assists in β -aryl ether cleavage and the des pathway which takes care of demethylation (Yao et al., 2024; Liu et al., 2026). As for fungi, their main species being *Aspergillus niger*, *Phanerochaete chrysosporium*, and *Saccharomyces cerevisiae*, which lack the innate capacity for aromatic degradation but can be engineered to process it artificially, they normally split aromatic compounds through the protocatechuate pathway of β -ketoadipate. The protocatechuate pathway that originates with the conversion of vanillic acid into protocatechuate, followed by β -ketoadipate, consists of certain stages (Kunjapur and Prather, 2015). White-rot fungi make use of certain enzymes that belong to the ligninolytic category and that work outside the cell in the process of lignin breaking and the release of monomers (Verma et al., 2021). Engineered systems have made it possible for *P. putida*, *A. niger* and *Rhodococcus* to take advantage of the conversion sites of lignin and convert them into co-substrates of lipids, polyhydroxyalkanoates and other bioproducts (Kunjapur and Prather, 2015; Tang et al., 2025; Wang et al., 2025).

3.4 Acetate conversion to acetyl-CoA as a carbon salvage strategy

Acetate is a common byproduct of pretreatment and overflow metabolism, and is often inhibitory at high concentrations. Rather than ignoring it, microbes can activate acetate to acetyl-CoA. In bacteria and yeast, acetyl-CoA synthetase (Acs) catalyzes acetate + ATP + CoA $\rightarrow$ acetyl-CoA + AMP + PP_i. This direct salvage route recovers carbon that would otherwise be excreted. By routing acetate into the acetyl-CoA pool, cells feed an abundant precursor for biosynthesis such as fatty acids, and isoprenoids (Sgro et al., 2023). In engineered biofuel strains where acetyl-CoA is a limiting node, upregulating Acs or alternative acetate-assimilation enzymes have improved conversion yields. Although Acs costs ATP (AMP), the carbon and reducing-equivalent gains can offset the energy input. In short, treating acetate not as waste but as a feedstock effectively closes the carbon loop in lignocellulosic fermentation.

3.5 Formate and other one-carbon inhibitor-derived compounds as carbon or energy contributors

Oxidative detoxification of lignin-derived aromatics can release C₁ products such as formate (Wu et al., 2014). While formate is often considered a waste product, it can contribute both carbon and energy. Cells with formate dehydrogenase can oxidize formate to CO₂, generating NADH and thus ATP, partially offsetting the energetic costs of detox (Gleizer et al., 2019). In synthetic biology, engineered formate-assimilation pathways via the reductive glycine or Calvin cycle modules, can even incorporate formate into biomass, although this is not yet common in lignocellulosic hosts. At minimum, formate oxidation provides extra reducing power to fuel detoxification. Other C₁ compounds such as methanol from demethoxylation, may similarly be oxidized or assimilated by native C₁ pathways, turning inhibitory by-products into useful metabolic inputs.

3.6 Advantages of integrating detox pathways upstream of the TCA cycle or acetyl-CoA pool

Positioning detox enzymes at strategic metabolic nodes maximizes their benefit. By feeding inhibitor carbon into acetyl-CoA or early TCA intermediates, cells gain flexibility to direct it into biomass or products. For instance, routing furfural into 2-oxoglutarate places that carbon at a key branch point for amino acid and cofactor synthesis. Similarly, generating acetyl-CoA from aromatics or acetate boosts the supply of precursors for lipids, polyketides, or isoprenoids. Upstream integration also helps balance redox: oxidative detox (using NAD⁺/O₂) can regenerate cofactors needed elsewhere. In practical terms, engineered strains that couple detox to central nodes show shorter lag phases and higher growth rates on real hydrolysate (Guarnieri et al., 2017). In contrast, pathways that dump carbon downstream (e.g. to ethanol or acetate) lose these advantages. Thus, steering inhibitor carbon into the acetyl-CoA/TCA hubs aligns detoxification with the cell's core metabolic economy. Further, when inhibitor carbon is successfully assimilated, it adds to the overall carbon yield. For example, converting vanillic acid into protocatechuate and then succinate/acetyl-CoA makes extra precursors available for product formation such as lipids or organic acids). In the engineered *P. putida* strain mentioned above, catabolism of furfural and HMF by a complete oxidative pathway improved carbon conversion compared to the wild type, which can only convert these to alcohols (Guarnieri et al., 2017). In essence, each mole of inhibitor “recovered” provides additional biosynthetic building blocks. At high inhibitor loadings, this can significantly boost product titers. Quantitatively, routing even a fraction of furan carbon into the TCA cycle can increase yields by several percent, a valuable gain in large-scale fermentations. Importantly, this approach can turn an inhibitor into an extra feedstock, enhancing the economy of the process. These benefits are not free. Many oxidative detox pathways require oxygen such as dioxygenases for ring cleavage, so they may not work anaerobically (Lipscomb, 2008). Acetate activation by acetyl-CoA synthetase

consumes ATP, imposing an energy cost (Zheng et al., 2025). Introducing entire catabolic routes adds metabolic burden, diverting resources to express non-native enzymes. If intermediate steps lag, toxic metabolites such as aldehydes, semialdehydes, or quinones can accumulate and stress the cell (Kunjapur and Prather, 2015; Snoeck et al., 2024). These trade-offs must be balanced: often, pathway expression is tuned or compartmentalized to avoid overloading the cell. Despite these costs, many engineered systems still show net gains because the extra carbon and redox flux outweigh the penalties.

4 Detox enzymes as redox-balancing modules

When detoxifying chemicals from cells, researchers will typically study the enzyme to determine which substrates the enzyme is selective toward and what products can be formed. In metabolic engineering or synthetic biology, however, there is an important inherent property of detoxifying enzymes: they are also redox balancing devices for the cell. Each detoxification reaction either uses or produces a certain cofactor (NADH, NADPH, FADH₂), thus directly linking the act of removing poisons from the cell with the main process of keeping electrons balanced within the cell. Therefore, when designing strong cell factories, cofactor specificity must be considered as a primary design consideration and not just as a secondary design consideration.

4.1 Cofactor specificity as a design parameter, not a secondary enzymatic feature

The increasing recognition of cofactor specificity as a tunable design variable in detoxifying enzymes, as opposed to being a fixed evolutionary endpoint, has accelerated the development of multiple routes to reduce the deleterious effects of cofactor maturity since 2020. The originally recognized problem of mono-NAD(P)H-dependent enzymes causing a redox bottleneck in organisms with limited *de novo* regeneration of mono-NAD(P)H has been addressed in directed evolution studies, including the redesign of an ADH from *Sphingobium* sp. SYK-6 to accept synthetic nicotinamide biomimetics as cofactors with a 6,750-fold increase in

cofactor-specificity (Zhu et al., 2025). However, progressive strategies for cofactor elimination from NSRs, such as the hybrid photocatalyst system developed by Wang et al. (2025), which utilizes reductive graphene quantum dots for AKR-catalyzed reductions in the absence of a nicotinamide cofactor through the photodraft of electrons from water using infrared light, illustrate a more significant departure from traditional approaches. Moreover, Zou et al. (2025) reported artificial metalloenzymes utilizing *de novo*-designed obligatory abiotic cofactor-binding proteins in the cytoplasm of *E. coli* that exhibited substantially stronger binding affinities to amorphous biogenic abiotic cofactors than from naturally occurring sources (KD ≤ 0.2 μM), indicating that alternate scaffolds could be utilized with this cofactor system.

4.2 NADPH-consuming detox enzymes versus NADH-consuming detox enzymes

The distinction between NADPH-consuming and NADH-consuming detox enzymes is fundamental to cellular redox compartmentalization, and recent researches have clarified the metabolic consequences of mismatching enzyme choice with host cofactor availability. As summarized in the Table 1 below.

4.3 Detox reactions that generate reducing power rather than only consume it

In accordance with the classic conception of detoxification as a process that consumes cofactors, it has been shown that certain enzymes are able to produce reducing equivalents as a result of inhibition. In effect, some enzymes may turn detoxification into a net energetically favorable event (Chen and Wan, 2017). An example of such an enzyme is aldehyde dehydrogenase (ALDH), which converts toxic aldehydes into harmless carboxylic acids while still reducing NAD(P)⁺ into NAD(P)H, thus making the process provide a certain amount of useful energy rather than consuming it. Genetics engineering techniques have been utilized to make use of the capability of ALDH to produce NADH while converting furfural into 2-furoic acid in microorganisms such as *C. basilensis* HMF14 or genetically modified *P. putida* (Lipscomb, 2008; Zheng et al., 2025). Similarly, redox-productive detoxification has been established in mammals, as in the case with ALDH7A1 that produces a membrane-bound pool of NADH that prevents damages caused by ferroptosis thanks to its involvement in lipid

TABLE 1 Comparison of NADPH-consuming versus NADH-consuming detoxification enzymes.

Feature	NADPH-consuming enzymes	NADH-consuming enzymes
Typical examples	Glutathione reductase, CYP450 reductases, aldo-keto reductases (AKRs), some alcohol dehydrogenases (e.g., GldA in <i>E. coli</i>) (Costa et al., 2023)	NADH-dependent alcohol dehydrogenases (ADHs), lactate dehydrogenase, aldehyde dehydrogenases (ALDHs) (Yao et al., 2024)
Primary role in detox	Reduction of oxidized glutathione, peroxides, and electrophilic carbonyls (Costa et al., 2023)	Reduction of aldehydes to alcohols (e.g., ethanol → acetaldehyde oxidation requires NAD ⁺ , but reduction of toxic aldehydes such as furfural often uses NADH) (Yao et al., 2024)
Cellular context	Anabolic, antioxidant defense, often in cytosol and peroxisomes (Costa et al., 2023)	Catabolic, fermentative, mostly cytosol and mitochondria
Consequence of overuse	Depletes NADPH, cripples antioxidant systems and biosynthesis (fatty acids, nucleotides) (Verma et al., 2021; Costa et al., 2023)	Depletes NADH, shifts fermentation balance (e.g., accumulates pyruvate or acetate) (Yao et al., 2024)

antioxidant FSP1 activity, as well as by utilizing engineered enzymes, such as LOXCAT (fusion enzyme lactate oxidase-catalase), as a mean to reduce reductive stress (Wierckx et al., 2011; Du et al., 2024). Although these mammalian interventions cannot be applied to industrial bioprocesses, they can still show that detoxification can be engineered in a way to bring measurable benefits in terms of energy produced. The applicability of this idea is of particular importance for biorefinery operators since the costs of detoxification of inhibitors may often decrease the output of the products. If detoxification enzymes can be selected or designed that can produce NAD(P)H instead of consuming it, strains will be able to help recover the energy needed for detoxification. For instance, the use of ALDHs instead of NADPH-dependent furfural reductases has a benefit of conserving carbon and producing reducing power because ALDHs oxidize furfural into furoic acid (Lipscomb, 2008; Zheng et al., 2025). Thus, the “redox-positive” detoxification module can be viewed as a novel design concept for metabolic engineering applied to lignocellulosic biorefineries.

4.4 Replacement of NADPH-draining reductases with NADH-dependent alternatives

NADH-dependent replacements of cofactor-dependent detoxification enzymes have emerged as a strong approach for reducing cofactor burden; however, engineering will need to be done to address the kinetic limitations imposed by the variant's higher K_m and lower k_{cat} value (Tang et al., 2025). Tang and others recently reported the first successful engineering of carbonyl reductase from strain F42 of *Exiguobacterium* sp. (the M30 enzyme) to switch its substrate preference from NADPH to NADH using a computationally assisted cavity engineering strategy, creating hexa-mutant M36 with > 1000-fold greater substrate selectivity for NADH and able to convert 99% of the labeled chloramphenicol substrate (Ujor and Okonkwo, 2022). This represents the first instance of cofactor preference reversal in carbonyl reductases using a dynamic reductive kinetic resolution strategy (Tang et al., 2025). Economic analysis of this engineered module indicates a 42% reduction in cost for single-batch production and a 75% reduction in waste management cost due to the price difference between the two cofactors, with NADPH costing approximately 10 times as much as NADH (Tang et al., 2025). Liu et al.'s recent work (Liu et al., 2026) focused on the kinetic limit imposed by shikimate dehydrogenase by using a structure-guided saturation mutagenesis approach. Specifically, they targeted the binding pocket of the cofactor to create an ideal mutant SDHNAD-S193H, which displayed a remarkable 25-fold increase in its NAD-dependent efficiency (k_{cat}/K_m) due to a large decrease in K_m for NAD, and they showed that SDHNAD-S193H performed well in both *in vitro* assays and via *in vivo* complementation of shikimate auxotrophic *E. coli* (Liu et al., 2026). Although engineering cytochrome P450 reductases toward being NADH-dependent continues to be a significant challenge, the engineering efforts validated on both carbonyl reductases and shikimate dehydrogenase (i.e., rational

cavity design, structure-guided saturation mutagenesis and high-throughput screening) represent a transferable approach for the mitigation of the NADH-associated kinetic penalty (Liu et al., 2026). Collectively, these studies illustrate that systematic protein engineering can be used to overcome the kinetic constraints of NADH-dependent variants, with the reported improvements in recent literature being in the range of 25–1000 fold.

4.5 Detox pathways as redox sinks that relieve stress and prevent cofactor depletion

Paradoxically, a detox pathway can prevent cofactor depletion even when it consumes a cofactor, a phenomenon observed when the toxin itself would otherwise trigger a futile cycle that wastes more reducing equivalents. A classic example is methylglyoxal (MG) stress: MG spontaneously reacts with glutathione (GSH), depleting the GSH pool, after which the cell spends NADPH to regenerate GSH via glutathione reductase (Kant et al., 2023). The glyoxalase system, the primary detoxification pathway for MG consumes GSH and produces D-lactate while utilizing NAD⁺/NADH cycling far more efficiently than the spontaneous damage pathway (Black et al., 2023; Kant et al., 2023). By preventing GSH depletion, this detox module paradoxically “saves” NADPH rather than merely consuming it (Kant et al., 2023). Alternative glutathione-independent mechanisms exist, such as glyoxalase III (GLY III), which detoxifies MG in a single step without GSH consumption (Coukos et al., 2023). Overexpression of OsDJ-1C in rice regulated MG levels under stress conditions, improving root architecture, photosynthesis, and yield while demonstrating an improved GSH/GSSG ratio and increased antioxidant capacity (Rathore et al., 2024). The human PARK7/DJ-1 protein has also been shown to act as a stereospecific enzyme to detoxify methylglyoxal to L-lactate via a glutathione-independent mechanism, with no cofactor drain (Coukos et al., 2023). Cancer cells utilize this mechanism in human lung tumors to reduce the detoxification of methylglyoxal due to glyoxalase oxidation but do not allow for the accumulation of advanced glycation end-products in tumors. Tumors accomplish this by increasing the activation of GAPDH to directly decrease the production of MG at its source (Tomin et al., 2025). The intersection of oxidative stress and the methylglyoxal pathway shows another important aspect of the metabolism of MGO; specifically, that metabolites of MGO, such as S-D-lactoylglutathione and D-lactate, can be taken up and metabolized by mitochondria, which together allow for the establishment of cytosol-mitochondrial crosstalk, preservation of cellular GSH pools, production of energy, as well as the restoration of the GSH/GSSG ratio (de Bari et al., 2021). Therefore, a detoxification enzyme can also function as a “redox sink”, thereby maintaining the integrity of the overall cofactor landscape. So, rather than measuring net cofactor consumption as an overall design metric, measuring the amount of wasted cellular resources from cycle of redox reaction would provide a better metric for the effectiveness of the enzyme to prevent wasteful depletion of cellular resources.

4.6 Coupling detox reactions to transhydrogenases or cofactor regeneration systems

Three auxiliary modules can separate detoxification from overall redox pools if a detoxifying enzyme has an “incorrect” cofactor specificity: transhydrogenases (e.g. PntAB) transfer reducing equivalents (NADH and NADPH) at the cost of dissipating proton motive force, cofactor regeneration systems utilize sacrificial substrates (e.g. glucose-6-phosphate for G6PDH) but add processing costs, and fusions of detoxifying enzymes (e.g. AKR-G6PDH) create self-sufficient redox modules (Celebi et al., 2025). Using peptide bridges to channel electrostatic cofactors has substantially improved the performance of fusion enzymes; for instance, the addition of a decapeptide (R10) increased conversions by 2.1-fold versus mixing free enzymes, while a five-arginine linkage (R5) reduced NADPH input by two orders of magnitude (1/150) (Guo and Sun, 2025). Fusion enzymes of cyclohexanone monooxygenase and alcohol dehydrogenase using optimized linkers facilitate the recycling of cofactors through shorter distances of diffusion particularly in aqueous-organic biphasic systems, and crosslinked enzyme aggregates of the fusions exhibit operational stability through five cycles in micro aqueous media (Vernet et al., 2023; Ma et al., 2025). The strategy applied will depend on the major constraint between energy dissipation (transhydrogenases), substrate expense (regeneration systems), or engineering effort (fusions).

4.7 Balance between redox relief and pathway burden

A detoxification module provides reduced toxicity (a benefit), but it incurs some costs such as: cofactor drain, and the burden of proteins. The optimal balance between these two effects can be expressed mathematically using the following equation: Net Benefit = (Amount of Redox Relief) - (Cofactor Drain + Protein Burden) (Coukos et al., 2023; Jin et al., 2025). One way to detoxify methylglyoxal (MG) using the glyoxalase system (GLO1/GLO2) is through glutathione (GSH); however, extended periods of elevated blood glucose levels can inhibit GLO1 activity due to dephosphorylation, causing an increase in levels of MG (Campos et al., 2025). Tomin et al. (2025) demonstrated that human lung tumors have adapted a mechanism of detoxification from MG without glyoxalases; instead, they use the enzyme GAPDH to decrease the formation of MG at its source thereby, indicating that reducing the production of a toxin prevents the incurrance of cost associated with its detoxification. Coukos et al. (2023) identified PARK7/DJ-1 as an alternative method of detoxifying MG by converting it into L-lactate without the use of GSH. Jin et al. engineered an *E. coli* strain containing an excess of NADPH (an RIFD-based approach) to produce L-threonine at high yields (117.65 g/L); however, the original growth inhibition observed with this mutant indicated that excessive driving force can be harmful to cellular growth; therefore, dynamic balance must be instituted. At low levels of toxins, constant detoxification creates a burden on resources; at high levels of toxins, insufficient

detoxification will result in death. Dynamic control (e.g., inducible promoters) maintains the availability of cofactors for continual detoxifying activity during normal cellular growth, while providing rapid detoxification activities when cellular toxins accumulate.

4.8 Validation approaches: intracellular NADH/NADPH quantification, redox reporters, and yield measurements

An integrated workflow combining LC-MS/MS, fluorescent biosensors, and yield measurements is needed for validating a redox-balanced detox module (Klinke et al., 2004; Aliyah et al., 2026). However, validation approaches must be tailored to the specific host and application:

For yeast (*S. cerevisiae*, *Y. lipolytica*):

- LC-MS/MS is suitable for absolute quantification of NAD⁺/NADH and NADP⁺/NADPH ratios in cell extracts (Klinke et al., 2004).
- Fluorescent biosensors (e.g., Peredox for NADH/NAD⁺ ratio, Apollo-NADP⁺ for NADPH) can monitor real-time cofactor dynamics (Pandey et al., 2026).
- Key metrics: ethanol yield, lipid/terpenoid titer, growth rate in hydrolysate

For bacteria (*E. coli*, *P. putida*, *C. glutamicum*):

- LC-MS with deuterated internal standards provides detection limits as low as 0.055 nmol (Klinke et al., 2004).
- NADH/NADPH ratios are particularly critical (Aliyah et al., 2026); developed calibration constants for calculating redox ratios without authentic standards
- Key metrics: acetate overflow, product yield (e.g., succinate, 3-HP, PHA), furfural/HMF consumption rate

For oleaginous microbes (*Y. lipolytica*, *Rhodococcus*):

- NADPH availability is the primary validation metric, as NADPH drives fatty acid synthesis
- Two-photon FLIM of NAD(P)H can distinguish free vs. protein-bound NAD(P)H, indicating whether glycolysis or oxidative phosphorylation dominates (Pandey et al., 2026)
- Key metrics: lipid accumulation (FAME analysis), TAG content, acetyl-CoA pool size

For photosynthetic hosts (cyanobacteria, microalgae):

- Real-time NADPH monitoring via biosensors is challenging due to chloroplast compartmentalization; extraction-based LC-MS is preferred.
- ATP/NADPH ratio is critical due to light-driven constraints (Gleizer et al., 2019).
- Key metrics: ROS levels, photorespiration rate, biomass yield in inhibitor-containing media

5 Co-utilization of inhibitory compounds

Lignocellulose-derived inhibitors such as furans, weak acids, aromatics, can be reconceived not just as wastes to remove but as co-substrates that supplement carbon and reducing power. Many microbes naturally co-metabolize sugars with inhibitors under the right conditions. For instance, *Rhodococcus jostii* RHA1 can grow on mixtures of glucose plus acetate and aromatic monomers such as vanillate, p-coumarate, and ferulate, using them to produce triacylglycerol (Chen and Wan, 2017). Chen et al. showed that wild-type *Rhodococcus jostii* utilized lignin monomers in varying degrees when co-fed with glucose, and evolved strains had shortened lag phases on such mixtures (Chen and Wan, 2017). Similarly, engineered yeast or bacteria can reduce furfural/HMF while fermenting sugars, yielding the less-toxic furfuryl/furan dicarboxylic acids (Wierckx et al., 2011). In one example, a thermotolerant *Kluyveromyces marxianus* strain co-fermented glucose, xylose and arabinose simultaneously with acetate, formate, furfural and vanillin to produce ethanol at 0.46 g/g yield (Du et al., 2024). Co-utilization yields depend on context. For instance, mixed sugars and inhibitors often inhibit growth more than each alone due to synergistic toxicity (Chen and Wan, 2017). However, in other cases co-substrates enhance cofactor supply. For example, adding a reduced co-substrate like glycerol to *Clostridium beijerinckii* boosted NAD(P)H supply and increased furfural detoxification (Ujor and Okonkwo, 2022). Thus, whether co-feeding helps or hurts depends on concentration and microbial capacity. Low to moderate inhibitor loads may be metabolized or tolerated, whereas high loads can overwhelm cells (Table 2).

5.1 Host requirement

Successful co-utilization requires the organism to efficiently take up, detoxify, and assimilate inhibitory compounds while

maintaining cellular redox and energy balance. Weak acids such as acetic acid and formic acid can cross biological membranes in their undissociated forms by passive diffusion, leading to intracellular acidification and metabolic stress under low-pH conditions (Casal et al., 1996; Deprez et al., 2021). Microorganisms with robust acid-tolerance systems are nevertheless able to reutilize acetate by converting it into acetyl-CoA through acetyl-CoA synthetase-dependent pathways, thereby integrating inhibitor-derived carbon into central metabolism even under oxygen-limited or anaerobic conditions (Deprez et al., 2021). Furan aldehydes such as furfural and 5-HMF are commonly taken up through passive diffusion or nonspecific transport systems and are subsequently detoxified by NAD(P)H-dependent oxidoreductases that reduce the aldehydes to their corresponding alcohols or oxidize them into less toxic acids (Chung et al., 2015). In *Saccharomyces cerevisiae*, multiple alcohol dehydrogenases and aldehyde reductases participate in the NADPH-dependent conversion of furfural and HMF into furfuryl alcohol and related derivatives, which alleviates growth inhibition during lignocellulosic fermentation (Heer et al., 2009). Similarly, the Zn-dependent alcohol dehydrogenase FurX from *Cupriavidus necator* JMP134 catalyzes efficient furfural reduction using NADH generated from ethanol oxidation, demonstrating how specialized detoxification enzymes can contribute to redox balancing during inhibitor conversion (Li et al., 2011). These enzymes such as native alcohol dehydrogenases, and aldehyde reductases, must be sufficiently expressed and cofactor balanced. Aromatic inhibitors including vanillin, ferulate, syringaldehyde, and related phenolics generally require more specialized uptake and assimilation systems because of their structural complexity and toxicity. These compounds are frequently transported through ATP-binding cassette (ABC) transporters or major facilitator superfamily (MFS) transporters before entering oxidative catabolic pathways (Yazaki, 2006). Once internalized, aromatic compounds are funneled through pathways such as the β -ketoadipate route, in which

TABLE 2 Key properties of common lignocellulose inhibitors.

Inhibitor class	Representative compounds	Typical concentration in hydrolysates	Toxicity profile	Assimilation	O ₂ -requirement	Metabolic entry
Furanics	Furfural, 5-HMF	0.5–10 g/L (varies with pretreatment)	High: IC ₅₀ 0.5–9 mM in yeast/bacteria; furfural generally more toxic than HMF; synergistic toxicity with weak acids	Partial: often reduced to alcohol/acid; only select bacteria (e.g., <i>C. basillensis</i>) fully degrade	Usually aerobic	Via 2-furoic acid → TCA (2-oxoglutarate) in fully oxidative pathways
Weak Acids	Acetic acid, Formic acid, Levulinic acid	Acetic: 1–10 g/L; Formic: 0.1–1 g/L	Moderate: IC ₅₀ 5–50 mM depending on pH and host; toxic as undissociated acid crosses membrane and acidifies cytosol	Yes — converted to acetyl-CoA (or formyl-CoA) via Acs or similar	Low (can be fermentatively assimilated)	Acetyl-CoA (glyoxylate or TCA) via acetyl-CoA synthetase
Aromatics	Vanillin, Syringaldehyde, Ferulic acid, p-Coumaric acid	0.1–1 g/L (highly variable with lignin content and pretreatment)	Host- and concentration-dependent: IC ₅₀ ranges from <0.5 mM (vanillin in <i>S. cerevisiae</i>) to >10 mM (some phenolics in tolerant <i>P. putida</i>); can be stimulatory at very low concentrations but inhibitory at high	Yes — via β -ketoadipate or other catabolic pathways	Aerobic (requires dioxygenases)	Acetyl-CoA + succinyl-CoA (via catechol/protocatechuate cleavage)

aromatic rings are converted into catechol or protocatechuate intermediates prior to ring cleavage and assimilation into TCA cycle metabolism (Wells and Ragauskas, 2012). In short, hosts need transport and enzymatic pathways for the inhibitors of interest, as well as regulatory and redox adaptations such as sufficient NADH/NADPH regeneration, to support these auxiliary metabolisms.

5.2 Partial versus complete assimilation

Inhibitors may be only partially metabolized. Yeasts and many bacteria often merely reduce furan aldehydes to alcohols using up NADH or NADPH, without further breakdown. For example, under anaerobic conditions *Corynebacterium glutamicum* converts furfural only to furfuryl alcohol, consuming NAD(P)H (Ujor and Okonkwo, 2022). By contrast, a few aerobic bacteria such as *Cupriavidus basilensis*, and *Pseudomonas*, can fully oxidize furfural/HMF to central intermediates: furfural $\rightarrow$ 2-furoic acid $\rightarrow$ 2-oxoglutarate that enter the TCA cycle (Wierckx et al., 2011). Likewise, some acetogenic or *Clostridial* strains can assimilate acetic/formic acids into acetyl-CoA and onward albeit often at energy cost (Ljungdahl et al., 1989). Aromatic monomers often require O₂ to be broken to succinate/acetate via catechol- or protocatechuate-pathways. Thus, co-feeding may only yield detoxified by-products unless the host harbors complete degradation routes. Complete assimilation yields more reducing power and carbon for product synthesis, while partial reduction mainly relieves toxicity. The trade-off between partial detoxification and carbon recovery is a key design consideration.

5.3 Product-specific impacts

The utility of inhibitor co-substrates depends on the product. For ethanol or other biofuels that undergo anaerobic fermentation, co-metabolizing inhibitors can supply extra reducing equivalents. For example, reducing furfural to furfuryl alcohol uses NADH, potentially rebalancing redox and supporting continued fermentation. However, in strictly anaerobic processes such as butanol, and solvent fermentation, inhibitors mostly get stalled as alcohols – e.g. in *Clostridium cultures* furfural is reduced to furfuryl alcohol, which still impairs butanol synthesis (Ujor and Okonkwo, 2022). In contrast, aerobic bio-productions such as lipids, polyketides, and isoprenoids, can fully oxidize or incorporate inhibitor carbon. Oleaginous microbes readily use acetate (a primary inhibitor) as a cheap C₂ feedstock; for instance, *Yarrowia lipolytica* and *Cupriavidus necator* convert acetate into lipids, polyketides and isoprenoids by feeding it into acetyl-CoA and the mevalonate/MVA pathways (Lee et al., 2025; Aliyah et al., 2026). Similarly, *Rhodococcus* and engineered *Pseudomonas* can channel lignin monomers into fatty acids or aromatic-derived products (Chen and Wan, 2017; Kumar et al., 2021). Thus, co-utilization can directly boost target yields: acetate-derived acetyl-CoA feeds isoprenoid and fatty acid routes and aromatic ring carbon can augment succinyl-CoA pools for lipid synthesis (Chen and Wan, 2017; Du et al., 2024).

6 Enzyme catalogue and design rules

The enzymatic detoxification of lignocellulosic hydrolysates involves a diverse array of enzymes, each operating via distinct chemical mechanisms to neutralize inhibitory compounds such as furfural, hydroxymethylfurfural (HMF), and acetic acid.

6.1 Classification of detox enzymes by reaction type: reductases, dehydrogenases, oxidases, hydrolases, conjugating enzymes, and laccases/peroxidases

Detoxifying lignocellulosics through enzyme activity has been classified into six categories of detoxification enzymes based on their mechanism of action. The reductase family of enzymes (*Kodamaea ohmeri* SSK, ADH/AKR) use NAD(P)H to reduce furfural and 5-hydroxymethyl furfural (HMF) as shown by Yang et al. (2025). Dehydrogenases such as aldehyde dehydrogenases (ALDHs) perform NAD(P)⁺-dependent oxidation, converting aldehydes to carboxylic acids that feed into central metabolism (Singh et al., 2025). The hydrolases (ferulic acid esterases) of this family also work to degrade lignin via the cleavage of ester bonds (Leontakianakou et al., 2025). The conjugating enzymes (Nu-class GSTs) also enable lignin degradation by cleaving the β -aryl ether lignin backbone using a glutathione lyase mechanism as shown by Kontur et al. (2018). The laccases/peroxidases (LiP, MnP) of the laccase/peroxidase family have phenol derivatives as a substrate and require the assistance of a laccase/peroxidase and/or other auxiliary enzymes to achieve reaction. All of these detoxifying enzyme classes provide the components necessary for the design of a rational detoxification module.

6.2 Substrate classes: furanics, aromatics, weak acids, aldehydes, and phenolic derivatives

The major substrate classes of lignocellulosic-derived inhibitors encompass furanics, aromatics, weak acids, aldehydes, and phenolic derivatives, each originating from distinct chemical pathways during biomass pretreatment (Klinke et al., 2004). Furanics, including furfural (from pentose dehydration) and 5-hydroxymethylfurfural (HMF, from hexose dehydration), are among the most potent inhibitors, disrupting glycolytic enzymes and causing redox imbalance (Pandey et al., 2026). Weak acids such as acetic acid (from hemicellulose acetyls), formic acid, and levulinic acid accumulate at low pH, diffuse across membranes, and dissociate intracellularly to lower cytosolic pH, impairing ATP synthesis and substrate uptake. Phenolic derivatives constitute a diverse class of aromatic lignin breakdown products including vanillin, syringaldehyde, coniferyl aldehyde, and ferulic acid, which inhibit microbial growth and ethanol productivity by partitioning into lipid membranes and disrupting proton motive force (Kumar et al., 2025). Within the same phenol functional group (aldehyde, ketone, or acid), inhibition of volumetric ethanol

productivity correlates with methoxyl substituent number and hydrophobicity (log P), with more hydrophobic phenols exhibiting stronger toxicity (Tian et al., 2025). Aldehydes such as syringaldehyde, vanillin, and 4-hydroxybenzaldehyde represent a subclass of aromatic inhibitors that are particularly toxic due to their electrophilic reactivity with cellular nucleophiles, inhibiting key dehydrogenases and glycolysis (Zhang et al., 2025). These five substrate classes act synergistically in lignocellulosic hydrolysates, with combinations such as furfural/HMF and furans/acetic acid showing enhanced toxicity compared to individual compounds, necessitating multi-target detoxification strategies (Pandey et al., 2026). Rational engineering of detoxification enzymes must consider substrate specificity across these classes, with aldo-keto reductases targeting furanic aldehydes, laccases and peroxidases addressing phenolic aromatics, and hydrolases managing acetate release.

6.3 Cofactor preference of each enzyme class, Reaction directionality and reversibility, and Kinetic behavior and substrate promiscuity

The functional classification of detoxification enzymes is defined by their cofactor preference, reaction directionality, and kinetic behavior. Aldo-keto reductases (AKRs) are NADPH-dependent oxidoreductases that reversibly reduce carbonyl compounds to alcohols (Di Luccio et al., 2006). While most AKRs prefer NADPH, the NADH-specific AKR11B2 from *E. coli* represents the first reported enzyme with exclusive NADH preference; mutation of a key aspartate residue converts it to dual specificity, enabling engineering of cofactor utilization (Di Luccio et al., 2006) (Table 3). The AKR7-2-1 mutant Y53F increased coenzyme

specificity for NADH by 875-fold (Zhang et al., 2023). For AKR13B3 from *Devosia*, the double mutant R134F/D217A enhanced catalytic efficiency (kcat/Km) by 3.15-fold and improved thermal stability, with molecular dynamics revealing that enhanced catalysis stems from a shortened nucleophilic attack distance and reduced binding free energy (Liu et al., 2026). Glutathione transferases (GSTs), such as *S. cerevisiae* ECM4, operate without nicotinamide cofactors, utilizing glutathione (GSH) as a nucleophilic cosubstrate in irreversible conjugation reactions via a Ping Pong mechanism. ECM4 expression is upregulated upon genotoxic stress, and the enzyme localizes to the cytoplasm, providing a detoxification strategy that bypasses cellular redox constraints entirely (Xun et al., 2010). This diversity AKRs offering engineerable NAD(P)H-dependent reduction and GSTs providing cofactor-independent conjugation constitutes a versatile toolkit for metabolic rewiring in lignocellulosic biorefineries.

6.4 Host compatibility and expression considerations

The successful deployment of detoxification enzymes as metabolic rewiring modules requires careful consideration of host compatibility, expression strategies, and enzyme selection criteria that prioritize carbon recovery, redox relief, and co-utilization (Figure 2). Host selection must balance intrinsic tolerance to lignocellulosic hydrolysates with genetic tractability; *Saccharomyces cerevisiae* remains a preferred host for bioethanol production, with engineered strains demonstrating enhanced inhibitor conversion (Wu et al., 2025), while extreme thermophiles such as *Caldicellulosiruptor bescii* and *Pyrococcus furiosus* offer unique advantages including reduced contamination risk and consolidated bioprocessing (Tanwee et al., 2024). Expression strategy design must address host cytotoxicity of active detoxification enzymes

TABLE 3 Enzyme detoxification modules: substrates, cofactors, products, and engineering value.

Enzyme class	Substrate(s)	Cofactor(s)	Product(s)	Engineering value (with Reference)
Aldo-keto reductases (AKRs)	Furfural, HMF, vanillin, other aldehydes	NADPH (preferred) or NADH	Furfuryl alcohol, HMF alcohol, vanillyl alcohol	Enables cofactor switching (NADPH→NADH) to relieve redox bottlenecks; NADH-specific AKR11B2 from <i>E. coli</i> is first reported exclusive NADH-preferring AKR; Y53F mutation in AKR7-2-1 increased NADH specificity 875-fold (Di Luccio et al., 2006; Zhang et al., 2023).
Alcohol dehydrogenases (ADHs)	Furfural, HMF, aliphatic aldehydes	NADPH or NADH	Furfuryl alcohol, HMF alcohol, ethanol	Can be engineered for dual cofactor specificity; bifunctional ADH-AKR from <i>Kodamaea ohmeri</i> SSK exhibits both activities with strong NADPH preference (kcat/Km = $1.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for NADPH vs 2.1×10^3 for NADH) (Yang et al., 2025).
Aldehyde dehydrogenases (ALDHs)	Furfural, HMF, acetaldehyde, other aldehydes	NAD ⁺ or NADP ⁺	Furoic acid, HMFA (5-hydroxymethyl-2-furancarboxylic acid), acetate	Enables carbon recovery by directing inhibitors into TCA cycle via CoA ligation; NAD ⁺ -dependent ALDH from <i>E. coli</i> (AldB) shows 3.2-fold increased kcat under acetic acid stress (Ho and Weiner, 2005).
HMF/furfural oxidases (HmfH)	HMF, furfural	O ₂ (no nicotinamide cofactor)	FDCA (2,5-furandicarboxylic acid), furoic acid	Cofactor-independent detoxification bypasses cellular redox constraints; directed evolution improved thermostability 4.5-fold (T ₅₀ increased to 62 °C) enabling simultaneous saccharification and detoxification (Koopman et al., 2010).
Laccases	Phenolic compounds (vanillin, syringaldehyde, guaiacol, ferulic acid, caffeic acid, p-coumaric acid)	O ₂ (four-electron reduction), copper centers	Phenoxy radicals → dimers or oxidative ring-cleavage products	Broad substrate promiscuity; Anc44 ancestral laccase degrades >50% of four phenolic inhibitors and reduces total phenolic content in wheat straw hydrolysate by 41% without purification; bifurcated radical pathway enables syringyl/guaiacyl ring cleavage (Zeng et al., 2026).

Carbon and Redox Bottlenecks in Major Biofuel Hosts

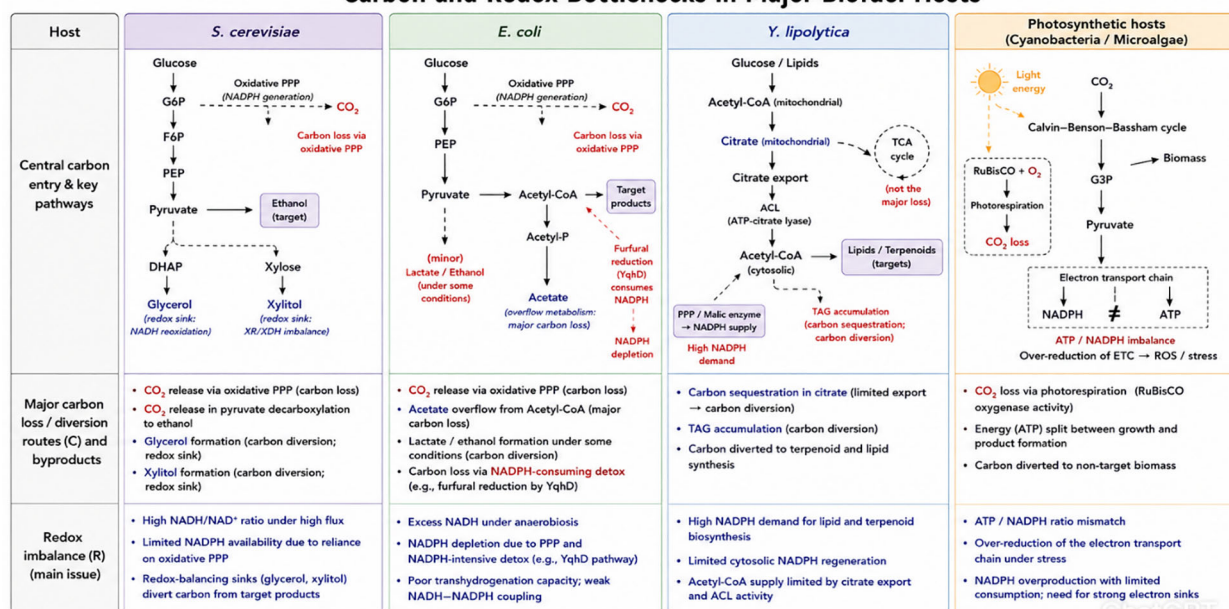


FIGURE 2

Carbon and redox bottlenecks in major biofuel hosts: host-specific carbon diversion, cofactor imbalance, and metabolic constraints during biofuel production. The schematic compares the main pathways of carbon entry, product formation, carbon loss/diversion, and redox imbalance in four host groups: *Saccharomyces cerevisiae*, *Escherichia coli*, *Yarrowia lipolytica*, and photosynthetic hosts (cyanobacteria/microalgae). In *S. cerevisiae*, glucose is funneled through glycolysis to pyruvate and ethanol, but carbon is lost through the oxidative pentose phosphate pathway (PPP; CO₂ release) and diverted to glycerol and xylitol, which act as redox sinks to re-oxidize NADH or reflect XR/XDH cofactor imbalance during xylose metabolism. In *E. coli*, glucose is metabolized via glycolysis to pyruvate and acetyl-CoA for target products, while acetate overflow causes major carbon loss; in parallel, furfural detoxification by YqhD consumes NADPH, creating a redox burden, and minor lactate/ethanol formation may occur under some conditions. In *Y. lipolytica*, glucose/lipids are converted to mitochondrial acetyl-CoA and citrate, which is exported and cleaved by ATP-citrate lyase (ACL) to supply cytosolic acetyl-CoA for lipid/terpenoid biosynthesis; however, carbon can be sequestered in citrate or diverted to triacylglycerol (TAG) accumulation, and high NADPH demand for lipid synthesis is a key constraint. In photosynthetic hosts, light-driven electron transport supports CO₂ fixation through the Calvin–Benson–Bassham cycle, but RuBisCO oxygenase activity drives photorespiration and CO₂ loss, while mismatch between ATP and NADPH generation and consumption can over-reduce the electron transport chain and promote ROS formation/stress. Solid black arrows indicate the principal metabolic flow; dashed arrows indicate secondary, auxiliary, or compartmental fluxes; red text/arrows denote carbon loss, carbon diversion, or NADPH drain; and blue text highlights major redox sinks or host-specific bottlenecks. The dashed circle in the *Y. lipolytica* panel indicates the TCA cycle, shown here as a connected but not major loss route. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; PEP, phosphoenolpyruvate; DHAP, dihydroxyacetone phosphate; PPP, pentose phosphate pathway; XR/XDH, xylose reductase/xylitol dehydrogenase; ACL, ATP-citrate lyase; TAG, triacylglycerol; ETC, electron transport chain; ROS, reactive oxygen species; CBB, Calvin–Benson–Bassham.

through tightly regulated inducible promoters, fusion partners (GST, MBP, SUMO tags), compartmentalized expression, or cell-free systems that completely decouple enzyme synthesis from host viability. Codon optimization and chaperone co-expression further enhance soluble yields, as demonstrated by *S. cerevisiae* constitutively expressing *FjMiox* for myo-inositol conversion.

7 Future research directions

Future work should prioritize host-specific systems models that connect inhibitor chemistry with carbon flux, cofactor availability, oxygen demand, and product formation. Genome-scale modeling should be integrated with transcriptomics, metabolomics, and ¹³C flux analysis to identify where inhibitor-derived carbon can enter central metabolism without creating new energy or redox bottlenecks. These models must be tested in compositionally realistic hydrolysates rather than single-inhibitor media.

A second priority is enzyme and pathway redesign. Cofactor preference, reaction directionality, substrate range, and intermediate toxicity should be engineered together rather than independently. Dynamic promoters, enzyme scaffolds or fusions, transporter engineering, and compartmentalization may help match detoxification capacity to inhibitor load while limiting protein burden. Oxidative routes that conserve carbon are especially promising for aerobic hosts, whereas anaerobic processes will require alternative electron-balancing strategies and careful control of ATP cost.

Finally, the field needs standardized evidence for carbon-positive detoxification. Future studies should report inhibitor conversion, labeled-carbon fate, intracellular cofactor ratios, product yield, biomass yield, CO₂ evolution, and overall carbon closure. Scale-up experiments should also assess pathway stability, oxygen transfer, enzyme expression burden, and performance across variable hydrolysate batches. These data will determine whether inhibitor co-utilization provides a genuine process advantage rather than only improved tolerance.

8 Conclusion

Rather than just a process of cleaning of lignocellulosic inhibitors, detoxification should be seen as a chance for acquiring carbon and reducing power that helps to perform better as a host. When detoxification pathways are incorporated with main metabolic pathways, one can transform toxic compounds like furfural, HMF, acetate, formate, and phenolics into acetyl-CoA and intermediate compounds of TCA cycle, which allows getting rid of toxicity of substances and increases efficiency in respect of carbon and redox state. However, detoxification process faces big difficulties in terms of cofactor mismatch, energy requirements, dependence on oxygen and other factors. Moreover, designing pathways for a particular host, working on specific enzymes, redox control and verification in real hydrolysate conditions are of great importance for getting positive results in the process.

Author contributions

BF: Writing – original draft, Conceptualization, Writing – review & editing. AR: Writing – original draft. AA: Writing – review & editing, Resources, Conceptualization. SN: Writing – original draft, Writing – review & editing.

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