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Review

From transport to regulation: systems engineering for high-efficiency dicarboxylic acid biosynthesis

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Medium- and long-chain dicarboxylic acids (M/LCDAs) are key monomers for the synthesis of nylons and high-performance engineering plastics. Compared to traditional chemical methods, microbial synthesis offers advantages such as environmental friendliness and high regioselectivity. However, its industrial application remains limited by bottlenecks, including low mass transfer efficiency on hydrophobic substrates, instability of key oxidase systems, and cellular metabolic imbalances. This review summarizes recent strategies leveraging enzyme engineering, systems metabolic engineering, and diverse synthetic biology approaches to overcome current limitations in the biosynthesis of M/LCDAs. We specifically highlight mechanisms for enhancing the transmembrane transport of hydrophobic substrates and the mining of novel transporters. Furthermore, we elaborate on protein engineering efforts targeting key enzymes (e.g. cytochrome P450s), covering rational design, fusion expression, and novel dimerization techniques. At the systems level, we discuss metabolic network regulation achieved through the construction of the reverse β-oxidation cycle (r-BOX) and the reprogramming of cofactor regeneration and energy metabolism. Finally, future perspectives on integrating AI-aided design and waste valorization are proposed to provide theoretical guidance for the efficient and sustainable biomanufacturing of M/LCDAs.

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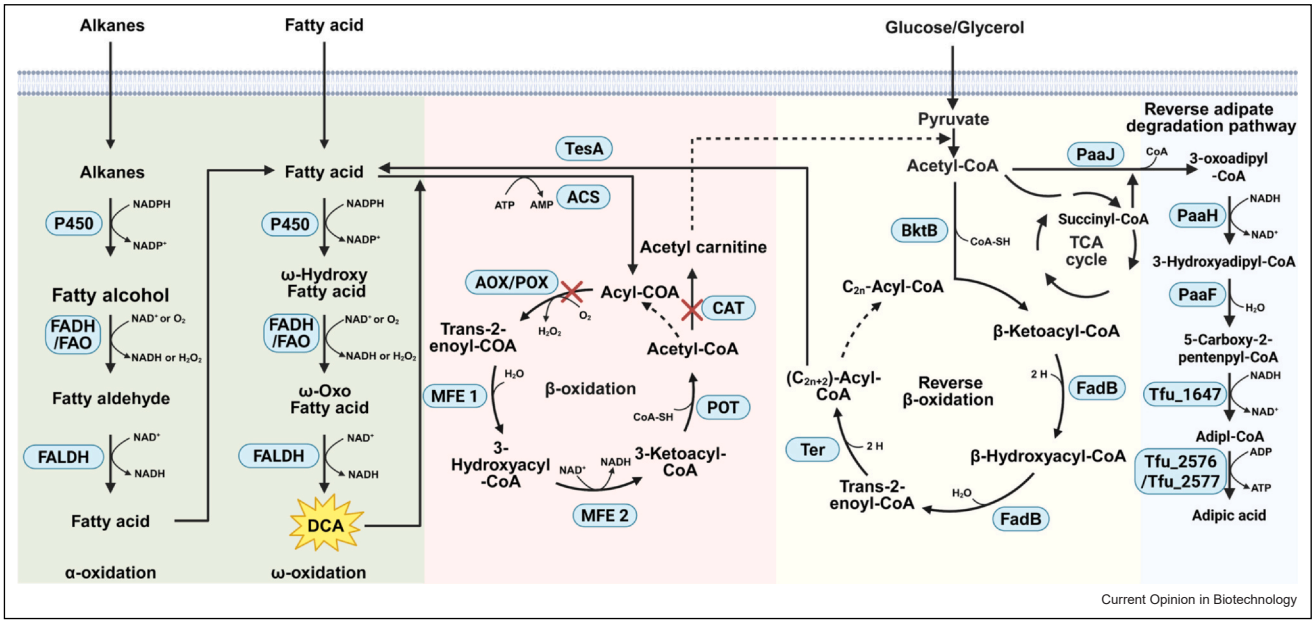
Introduction

Medium- and long-chain dicarboxylic acids (M/LCDAs), as a class of straight-chain aliphatic compounds containing two terminal carboxyl functional groups [1], serve not only as key monomers for the synthesis of nylons (such as nylon 6,6, nylon 6,12), polyesters, and high-performance engineering plastics, but also have wide applications in fragrances, plasticizers, and advanced lubricants [2,3]. Historically, industrial production relied on chemical oxidation methods, which face environmental challenges such as harsh reaction conditions and greenhouse gas emissions [4,5]. However, the landscape has shifted significantly with the successful commercialization of bio-based fermentation processes, exemplified by industry leaders like Cathay Industrial Biotech [6]. Recent breakthroughs in metabolic engineering have further pushed these boundaries; for instance, the engineering of *Candida viswanathii* (*C. viswanathii*) has achieved a dodecanedioic acid (DDA) titer of 224 g/L, demonstrating performance that rivals or exceeds traditional chemical processes [7].

The synthesis of M/LCDAs via microbial transformation represents a promising sustainable alternative, utilizing strategies ranging from precursor functionalization (via α- and ω-oxidation) to *de novo* biosynthesis from renewable feedstocks, all characterized by mild reaction conditions, superior regioselectivity, and environmental friendliness (Figure 1) [8–10]. Driven by the demand for efficiency, the field has shifted from early random

Figure 1



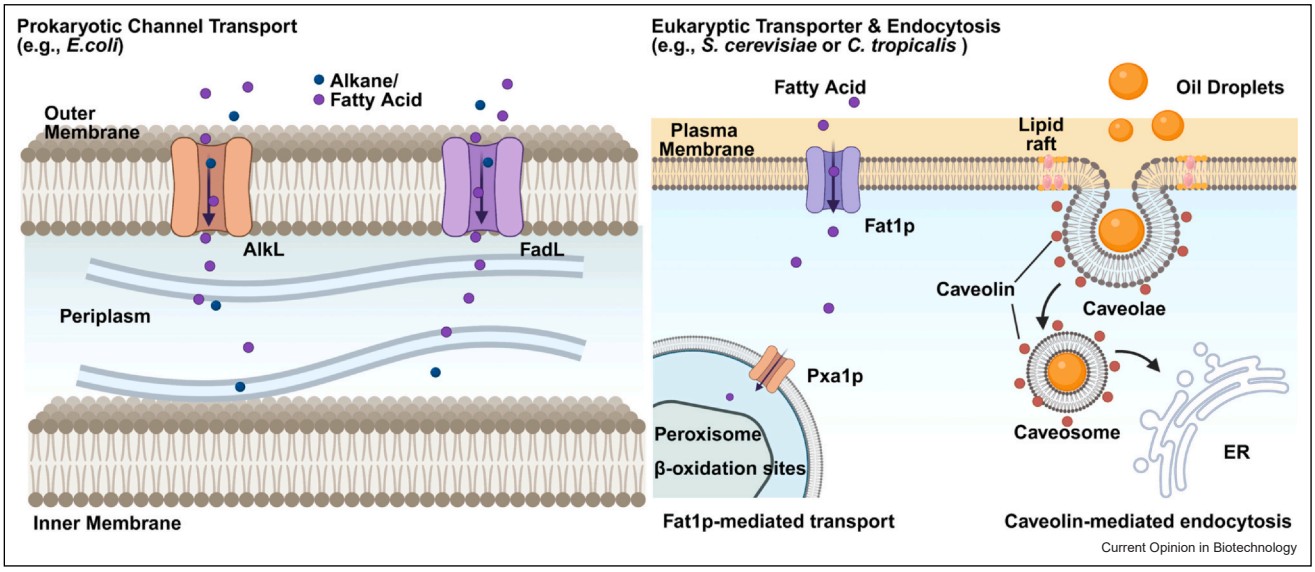
Schematic representation of the integrated metabolic pathway for the synthesis of M/LCDAs in microorganisms. Green area: The microbial fermentation pathway depicts the transformation process initiated with hydrophobic substrates (e.g. alkanes or fatty acids). Pink area: This region depicts the β-oxidation pathway that competes with the synthetic route. The red cross marks indicate key metabolic engineering strategies: disrupting the carbon skeleton degradation flux by knocking out acyl-CoA oxidase (AOX/POX) in the peroxisome or blocking carnitine acetyltransferase (CAT)-mediated transport, thereby redirecting metabolic flux towards the accumulation of M/LCDAs. Yellow area: This region illustrates the *de novo* biosynthetic pathway originating from glucose or glycerol. Blue area: This area depicts the reverse adipate degradation pathway (RADP) specifically for adipic acid production. Enzyme abbreviations are as follows: FAD, fatty alcohol dehydrogenase; FAO, fatty alcohol oxidase; FALDH, fatty aldehyde dehydrogenase; ACS, acyl-CoA synthase; AOX/POX, acyl-CoA oxidase; MFE 1, enoyl-CoA hydratase; MFE 2, 3-hydroxyacyl-CoA dehydrogenase; POT, 3-ketoacyl-CoA thiolase; CAT, carnitine acyltransferase; BktB, β-ketoacyl CoA thiolase; FadB, 3-hydroxyacyl-CoA dehydrogenase/dehydratase; Ter, trans-enoyl-CoA reductase; TesA, thioesterase; PaaJ, 3-oxoadipyl-CoA thiolase; PaaH, 3-hydroxyadipyl-CoA dehydrogenase; PaaF, 2,3-dehydroadipyl-CoA hydratase; Tfu_1647, trans-2-enoyl-CoA reductase; Tfu_2576-7, adipyl-CoA synthetase.

mutagenesis (e.g. in *Candida* species) to rational design underpinned by systems metabolic engineering and other synthetic biology approaches. While viable tools (e.g. CRISPR/Cas9) have enabled precise modulation of metabolic fluxes [7,11,12], ongoing technical challenges must be addressed to further enhance bioconversion efficiency, expand the applicability of diverse renewable feedstocks, and optimize *de novo* biosynthetic pathways. Specifically, the poor mass transfer of hydrophobic substrates across cell membranes often restricts the initiation of biocatalysis [8,13,14], the limited catalytic activity and stability of key enzymes (e.g. CYP52 and CYP153) serve as major biochemical bottlenecks in terminal oxidation [8,15], and cellular metabolic imbalances, particularly regarding carbon flux and cofactor availability, significantly diminish overall productivity [16]. Thus, this review summarizes biosynthetic strategies for M/LCDAs reported over the past two years, focusing on four critical dimensions that are essential for achieving high-efficiency biomanufacturing: (i) optimizing transmembrane transport to enhance the bioavailability of hydrophobic substrates; (ii) mining and engineering key oxidase systems to improve catalytic turnover and stability; (iii) optimizing metabolic networks to redirect carbon flux toward M/LCDAs; and (iv) reprogramming cofactor regeneration and energy metabolism to satisfy the high reducing power and ATP demands of these pathways.

Enhancing substrate transport

Transmembrane transport often represents a major bottleneck in biosynthetic systems, particularly when hydrophobic, long-chain alkanes and fatty acids serve as substrates for dicarboxylic acid synthesis. In Gram-negative bacteria, the outer membrane presents a formidable barrier. However, the FadL family proteins circumvent this obstacle by translocating long-chain fatty acids directly into the phospholipid bilayer via a unique 'lateral diffusion' mechanism (Figure 2) [17]. Notably, the AlkL protein from *Pseudomonas putida* GPo1 functions as a highly efficient outer membrane transporter. Studies indicate that heterologous expression of AlkL in *Escherichia coli* (*E. coli*) serves as a universal 'plugin' that significantly accelerates the uptake of C_7–C_{16} alkanes and fatty acid methyl esters (Figure 2), consequently driving a substantial increase in whole-cell

Figure 2



Schematic diagram of transmembrane transport mechanisms for hydrophobic substrates in microbial cell factories. Prokaryotic Channel Transport (Left panel): Illustrates the uptake mechanism in Gram-negative bacteria (e.g. *E. coli*). Specific outer membrane proteins, AlkL and FadL, facilitate the translocation of alkanes and fatty acids from the extracellular environment into the periplasmic space. Eukaryotic Transporter & Endocytosis (Right panel): Depicts internalization strategies in yeasts (e.g. *S. cerevisiae* or *C. tropicalis*). This involves two distinct pathways: Fat1p-mediated transport, where fatty acids are imported across the plasma membrane by Fat1p and subsequently transported into peroxisomes via Pxa1p; and Caveolin-mediated endocytosis, a process where oil droplets interact with lipid rafts to induce the formation of caveolae. These invaginations pinch off to form caveosomes, which traffic substrates to the endoplasmic reticulum (ER).

biocatalytic efficiency [18,19]. Furthermore, the recently identified long-chain alkane transporter protein AltL from *Acinetobacter venetianus* RAG-1 addresses a critical knowledge gap regarding the transport mechanisms for ultralong-chain alkanes (C_{20}–C_{38}), a substrate range previously considered inaccessible to the well-characterized AlkL [14]. In *Candida tropicalis* (*C. tropicalis*), efficient internalization of hydrophobic substrates constitutes a primary bottleneck. Zhang et al. [13] characterized the plasma membrane transporter CtFat1p (Figure 2) and demonstrated that overexpression in *C. tropicalis* of this transporter effectively alleviates extracellular mass transfer limitations, resulting in a 30.10% increase in long-chain dicarboxylic acid production. Following cytosolic entry, fatty acids are translocated into peroxisomes via transporter CtPxa1p (Figure 2). Of note, a single-allele deletion of CtPxa1p restricted peroxisomal influx of fatty acids, thereby boosting long-chain dicarboxylic acid yield by 21.90% [12]. This strategy effectively mitigates substrate loss to the β-oxidation pathway, thereby diverting more carbon flux towards M/LCDA synthesis. Conversely, a complete blockade proved detrimental by depleting the acetyl-CoA pool, which is essential for central metabolism. This underscores the need to attenuate rather than completely eliminate β-oxidation to balance product accumulation with cellular fitness.

Caveolae are plasma membrane invaginations in vertebrate cells that, generated by the CAV1-encoded protein caveolin, function primarily to form membrane buds and endocytic vesicles [20,21]. Leveraging this mechanism, Shin et al. [22] heterologously expressed caveolin in *E. coli*, thereby enhancing tolerance to toxic fatty acids and improving biotransformation efficiency by 1.6-fold. Furthermore, Zhang et al. [23] introduced caveolae-mediated endocytosis into *Saccharomyces cerevisiae* (*S. cerevisiae*) to facilitate soybean oil uptake, demonstrating their potential as an effective transport system for hydrophobic substrates (Figure 2). Collectively, engineering efficient transport systems is pivotal for overcoming bioavailability bottlenecks and enhancing bioconversions.

Engineering of key oxidases

The initiating step of the ω-oxidation pathway, catalyzed by cytochrome P450 monooxygenases, is typically rate-limiting due to inherent challenges, including poor enzyme stability, imprecise regioselectivity, and inefficient electron transfer. To address these limitations, diverse engineering strategies have been employed.

Rational design and genome mining: For bacterial $CYP153A_{Maq}$, Notonier et al. [24] remodeled the substrate-binding pocket via rational design, successfully

broadening the substrate range to include both short- (C_6–C_8) and long-chain (C_{16}–C_{18}) fatty acids. Similarly, exploiting the structural plasticity of the B/C-loop region in $CYP153A_{Maq}$, He et al. [25] introduced key mutations (e.g. Q129R/V141L) that significantly enhanced substrate binding affinity and catalytic efficiency through improved molecular anchoring. In the context of eukaryotic systems, Craft et al. [26] characterized the *C. tropicalis* CYP52 multigene family to identify chain-length-specific isozymes for targeted engineering. Through differential transcriptional profiling, they identified CYP52A13 and CYP52A14 as primary candidates, noting their selective induction by C_{18} fatty acids and alkanes, whereas other family members were transcriptionally silent. Extending beyond P450s, Seo et al. [27] engineered Baeyer-Villiger monooxygenases (BVMOs) via site-directed mutagenesis to enhance thermal stability and ring-opening activity, thereby facilitating the synthesis of odd-chain dicarboxylic acids like azelaic acid.

Construction of self-sufficient fusion enzymes: To further overcome diffusion-limited electron transfer and uncoupling, researchers have constructed chimeric enzymes to enforce spatial proximity. For instance, Scheps et al. [28] successfully fused *Marinobacter aquaeolei* CYP153A to the reductase domain of $P450_{BM3}$, demonstrating that optimized flexible linkers (e.g. 3xGGS) could significantly enhance coupling efficiency. Similarly, Fiorentini et al. [29] paired various CYP153 candidates with the eukaryotic-like reductase domain of CYP505 to create versatile biocatalytic tools. More recently, Pham et al. [7] developed a fusion strategy by genetically linking CYP52A19 with a truncated form of the reductase CPRb (residues 23–680). Utilizing a flexible linker (GSAG-SAAGSGEF) to connect the domains, the authors successfully created a functional self-sufficient enzyme complex. This engineered construct demonstrated a constructive role in DDA synthesis, validating the utility of physically coupling redox partners to support the ω-oxidation of dodecanoic acid. Beyond linear fusion, structural innovation has also been explored. Ge et al. [30] introduced a novel dimerization strategy by fusing the P450 catalytic domain to a dimerization domain (e.g. a leucine zipper), thereby inducing the formation of a stable P450 dimer. This structural organization enhanced interaction between P450 and its reductase partner, thereby improving ω-hydroxylation activity toward dodecanoic acid during the biosynthesis of the nylon-12 monomer, ω-aminododecanoic acid. Furthermore, fusion strategies extend beyond redox partners to enhance enzyme stability. Wang et al. [31] significantly enhanced the stability and catalytic efficiency of the BVMO by fusing a Flag-tag to its *N*-terminus. This optimization, combined with multi-dimensional engineering of the host, yielded a record-breaking adipic acid titer of 110 g/L from KA oil, demonstrating the efficacy of fusion-tag strategies in robust biocatalyst construction.

In summary, these studies demonstrate that rationally modulating enzyme structure and spatial conformation can significantly optimize electron transfer efficiency and stability, thereby overcoming the rate-limiting bottlenecks in the terminal oxidation step required for efficient dicarboxylic acid biosynthesis.

Carbon flux redirection

Specifically for dicarboxylic acid biosynthesis, metabolic flux redirection aims to maximize the intracellular pool of fatty acyl chains, preserving them as essential substrates for the terminal oxidation process that generates the final dicarboxylic acid products. To achieve this, carbon flux redirection is implemented primarily through two synergistic strategies: blocking the native β-oxidation pathway to prevent precursor degradation, and reconstruction of the reverse β-oxidation cycle to facilitate *de novo* synthesis from simple carbon sources.

Blocking the β-oxidation pathway: Fatty acids and dicarboxylic acids inherently undergo degradation via the β-oxidation pathway, resulting in substrate depletion and carbon chain shortening (Figure 1) [26,32]. Disrupting peroxisomal acyl-CoA oxidase (POX/AOX) genes represents a canonical strategy to arrest this catabolic flux (Figure 2). However, complete inactivation often impairs cell growth and induces toxicity due to the accumulation of toxic acyl-CoAs. To balance production and survival, Ju et al. [32] demonstrated that knocking out specific isoenzymes (AOX4/AOX5) in *C. tropicalis* effectively accumulates adipic acid while retaining essential metabolic functions. Similarly, the decisive role of POX deletion for dicarboxylic acid accumulation has also been confirmed in *Yarrowia lipolytica* [33,34]. Furthermore, disrupting carnitine acetyltransferase (CAT) presents another strategy to enhance production. However, since CAT mediates the transport of acetyl-CoA into mitochondria for the tricarboxylic acid cycle, deleting both alleles compromises cellular energy homeostasis, thereby severely inhibiting cell growth [35].

Reconstruction of the reverse β-oxidation cycle: Distinct from bioconversion strategies that rely on exogenous precursors such as alkanes or fatty acids, the *de novo* synthesis of dicarboxylic acid aims to use simple carbon sources such as glucose. To overcome the high ATP consumption and carbon loss associated with traditional fatty acid synthesis, Clomburg et al. [36] reconstructed the reverse β-oxidation cycle (r-BOX) in *E. coli* (Figure 1). This non-decarboxylative pathway offers superior theoretical carbon yields by employing a four-step cycle driven by the irreversible reduction of trans-enoyl-CoA reductase (Ter), which acts as a thermodynamic valve to

enforce chain elongation (Figure 1). Building on this modular platform, significant progress has been made in dicarboxylic acid (DCA) synthesis. Zhao et al. [37] assembled the 'Reverse adipate degradation pathway' (RADP) using genes (*paaJ*, *paaH*, *paaF*, *Tfu_1647*, and *Tfu_2576*/*Tfu_2577*) from *Thermobifida fusca*, achieving high-titer adipic acid production from glucose by strictly limiting the cycle to the C_6 length (Figure 1). Demonstrating the pathway's iterability, Cheong et al. [38] applied a 'programmable' approach by coupling r-BOX with specific thioesterases. This allowed precise control over chain lengths (C_6–C_{10}) and enabled the synthesis of odd-chain DCAs, such as pimelic acid, by altering the primer to propionyl-CoA. Furthermore, Sathesh-Prabu et al. [39] extended this pathway to synthesize functionalized intermediates, such as β-ketoadipic acid, by intercepting the cycle before full reduction. In summary, r-BOX reconstruction represents a paradigm shift toward 'designing metabolism', providing a thermodynamically superior and highly tunable platform for the *de novo* production of diverse M/LCDAs.

Cofactor regeneration engineering

The ω-oxidation pathway of fatty acids imposes a substantial metabolic burden due to the high NADPH demand of P450 monooxygenases (2 moles per mole of substrate), which contrasts with the NADH-generating downstream steps. To resolve this stoichiometric mismatch, strategies have evolved from enzyme engineering to global metabolic regulation (Figure 3). Lu et al. [40] achieved cofactor self-balancing by engineering alcohol dehydrogenase (ADH) to utilize $NADP^+$, creating a closed-loop cycle that recycles NADPH and significantly boosts sebacic acid yields. At the global metabolic level, the membrane-bound transhydrogenase (PntAB) functions as a primary NADPH generator. Sauer et al. [41] fundamentally demonstrated that PntAB catalyzes the proton-motive-force-driven transfer of hydride equivalents from NADH to NADPH, supplying 35–45% of the anabolic reducing power in *E. coli*. Building on this mechanism, Jan et al. [42] proved that modulating PntAB expression alongside NAD kinase significantly increases intracellular NADPH availability to support high-yield isobutanol synthesis. Ge et al. [30] recently overexpressed PntAB to rebalance the cofactor pool during biosynthesis. This modification successfully converted the excess NADH generated by upstream glycolysis into NADPH, thereby satisfying the high reducing power demand of the rate-limiting P450 hydroxylation step. These findings offer a compelling blueprint for M/LCDA biosynthesis: since the P450-mediated step is a major NADPH sink, integrating global redox engineering strategies can effectively alleviate the cofactor bottleneck and maximize catalytic turnover at the rate-limiting hydroxylation step.

Beyond redox balance, energy homeostasis is equally critical for maintaining the high flux of the reverse β-oxidation cycle (e.g. RADP). Although the core condensation steps are ATP-independent, the robust supply of precursors and cofactors imposes a heavy systemic ATP burden. To address this issue, Moon et al. [43] reprogrammed the intracellular ATP metabolism in *E. coli* by fine-tuning pantothenate kinase (PanK) and acetyl-CoA synthetase (Acs) expression. This coordinated regulation resolved the ATP bottleneck in the RADP cycle, significantly enhancing adipic acid production. Furthermore, Yuan et al. [44] developed a plasmid-free strain by integrating precursor engineering with cofactor regeneration modules directly into the genome. This strategy eliminated the metabolic burden and instability associated with plasmid maintenance, thereby enabling sustained, high-yield adipic acid synthesis throughout the fermentation process. These findings offer a compelling blueprint: integrating redox and energy engineering is essential to drive the energy-intensive condensation and reduction cycles required for high-titer dicarboxylic acid production.

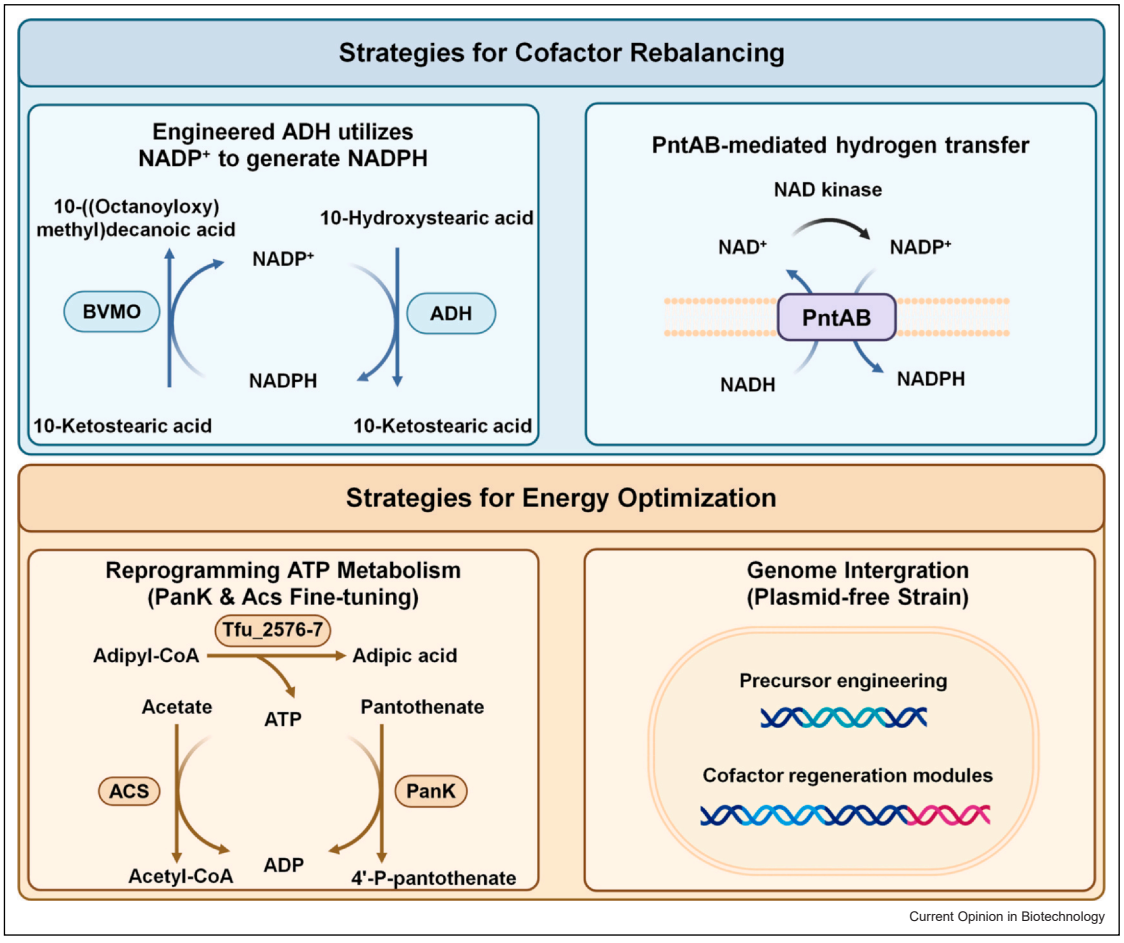
Future outlook

Despite the milestone progress made in the microbial synthesis of M/LCDAs in elucidating the enzymatic mechanisms and constructing chassis cells, substantial breakthroughs are still needed in atom economy, system robustness, and production cost to achieve the leap from laboratory scale to industrial mass production and ultimately replace mature petrochemical processes. Future research should not be limited to optimizing single strains but shift towards a multidimensional green biomanufacturing paradigm (Figure 4).

The primary task is to drive a revolutionary shift in the use of raw materials, moving from traditional glyco-based fermentation to 'carbon-negative' manufacturing and a circular bioeconomy. Given that raw material costs dominate biomanufacturing [45], future chassis cells must be able to utilize non-grain, low-carbon raw materials. This includes not only achieving a closed loop of high-value utilization of waste, such as kitchen waste oil, through adaptive evolution strategies, but also developing new pathways for utilizing C_1 compounds, such as CO_2 [46] and methanol [47]. By constructing a hybrid system that couples electrochemistry and biofermentation, future cell factories are expected to directly convert greenhouse gases into long-chain dicarboxylic acids, thereby making the production of M/LCDAs a highly efficient carbon capture and utilization technology, and completely decoupling chemical manufacturing from food supply.

At the same time, the design logic of cell factories is undergoing a qualitative leap from 'trial and error' to 'generative' biology. With the deep integration of artificial intelligence and big data, the traditional DBTL [48] cycle will be reshaped. Utilizing protein language

Figure 3

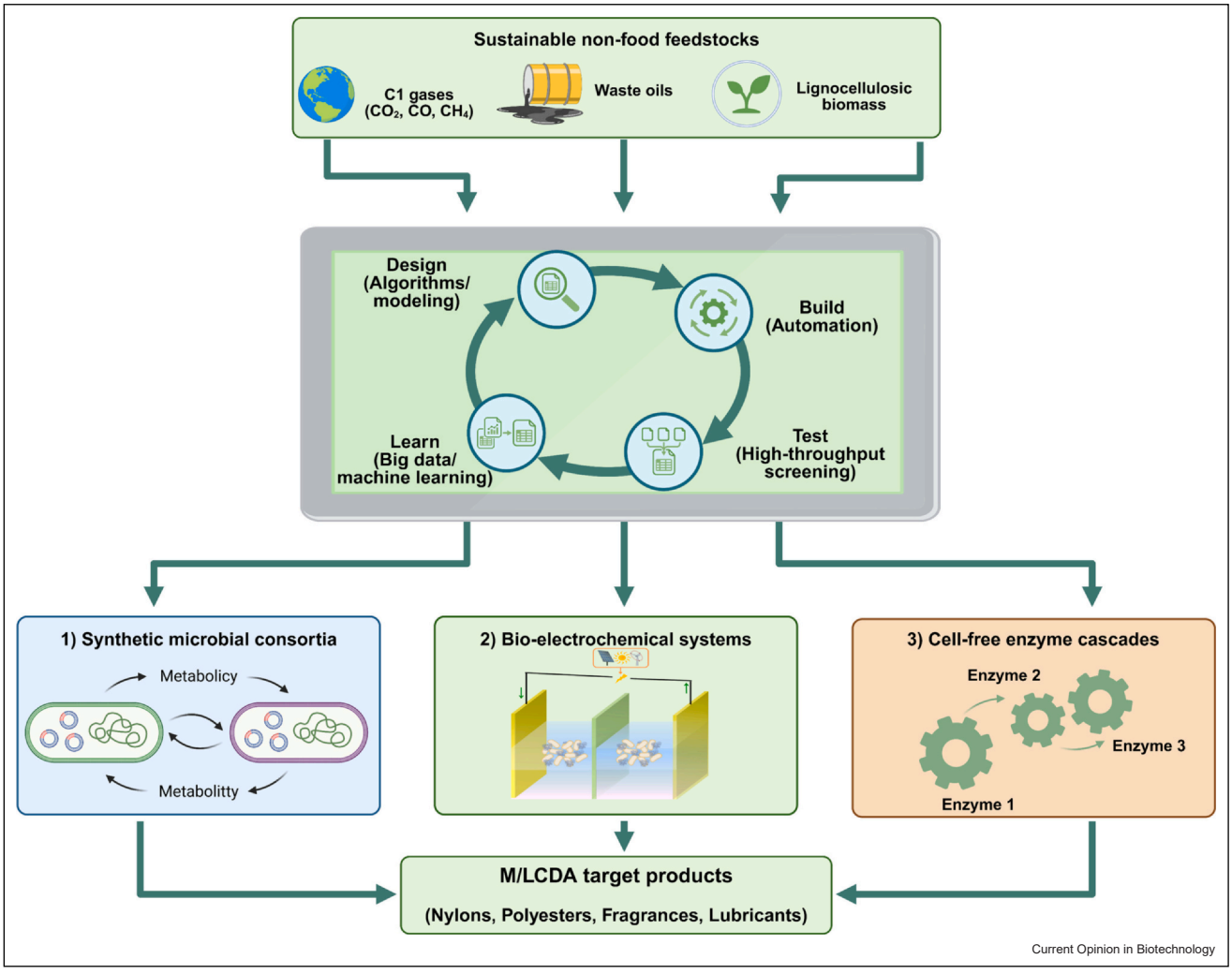


Engineering strategies for cofactor rebalancing and energy optimization in M/LCDAs biosynthesis. Strategies for Cofactor Rebalancing (Top panel). Engineered ADH: Illustrates the construction of a self-balancing redox cycle by engineering alcohol dehydrogenase (ADH) to utilize $NADP^+$, thereby recycling NADPH to support the high demand of the rate-limiting P450 hydroxylation step. Transhydrogenase (PntAB)-mediated hydrogen transfer: Depicts the global regulation of the redox pool through the overexpression of membrane-bound PntAB and NAD kinase, which facilitates the proton-motive-force-driven conversion of excess NADH into NADPH. Strategies for Energy Optimization (Bottom panel). Reprogramming ATP Metabolism: Shows the fine-tuning of pantothenate kinase (PanK) and acetyl-CoA synthetase (Acs) to resolve the ATP bottleneck, ensuring a robust supply of energy for energy-intensive condensation and reduction cycles (e.g. adipic acid production via Tfu_2576-7). Genome Integration: Illustrates the construction of a stable, plasmid-free strain by integrating precursor engineering and cofactor regeneration modules directly into the genome to eliminate metabolic burden and enhance system robustness.

models [49,50] and generative AI [51] for *de novo* design and functional prediction of key enzymes (such as P450 enzymes) will solve the bottlenecks of enzyme activity and specificity by surpassing the speed of natural evolution. Digital twin technology, which combines genome-scale metabolic models, will allow researchers to conduct global simulations and stress tests of metabolic flows in a virtual environment [52]. This data-driven rational design model will significantly shorten the development cycle of high-efficiency strains and enable precise regulation of complex metabolic networks.

Finally, to overcome the metabolic limits of natural biological systems, the manufacturing paradigm will evolve towards modularization and the integration of non-biological elements. Artificial microbial community technology [53] effectively addresses the metabolic burden of single cells by breaking down complex long-chain synthetic pathways and distributing them across different cellular modules, and by utilizing quorum-sensing mechanisms to achieve metabolic specialization. A more cutting-edge trend is to break biological boundaries by using cell-free enzyme cascades or bio-abiotic hybrid systems [54] to bypass cell membrane transport limitations and thermodynamic barriers, and to build efficient and robust semi-artificial photosynthesis [55] or electro-driven manufacturing platforms [54]. In conclusion, by integrating cutting-edge technologies in

Figure 4



Next-generation M/LCDAs biomanufacturing paradigm. Upstream (Feedstocks): Emphasizes the shift to sustainable non-food feedstocks, including C_1 gases (CO_2, CO, CH_4), waste oils, and lignocellulosic biomass. Midstream (AI Core): Showcased an intelligent design platform centered on the AI-driven DBTL (Design-Build-Test-Learn) cycle, which accelerates strain development through algorithmic modeling and big data analysis. Downstream (New Platforms): (1) Synthetic Microbial Consortia for metabolic specialization; (2) Bio-electrochemical Systems for biosynthesis driven by electrical energy; and (3) Cell-Free Enzyme Cascades for eliminating cell membrane limitations. These platforms collectively aim at the efficient green manufacturing of M/LCDA target products such as nylon and polyester.

synthetic biology, artificial intelligence, and process engineering, next-generation M/LCDAs biomanufacturing will reshape the global nylon and fragrance industry supply chain, providing strong technological support for the green transformation of the chemical industry.

CRediT authorship contribution statement

Zhijun Kong: Writing – original draft, Validation, Visualization. **Xu Zhao**: Writing – review & editing, Funding acquisition. **Li Ma**: Funding acquisition. **Hui Chen**: Writing – review & editing, Funding acquisition, Supervision, Conceptualization. **Shengying Li**: Writing – review & editing, Funding acquisition, Supervision, Conceptualization.

Data Availability

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

Declaration of Competing Interest

The authors declare no conflict of interest.

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- •• of outstanding interest

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