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Research review paper

Relieving metabolic burden to improve robustness and bioproduction by industrial microorganisms

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ABSTRACT

Metabolic burden is defined by the influence of genetic manipulation and environmental perturbations on the distribution of cellular resources. The rewiring of microbial metabolism for bio-based chemical production often leads to a metabolic burden, followed by adverse physiological effects, such as impaired cell growth and low product yields. Alleviating the burden imposed by undesirable metabolic changes has become an increasingly attractive approach for constructing robust microbial cell factories. In this review, we provide a brief overview of metabolic burden engineering, focusing specifically on recent developments and strategies for diminishing the burden while improving robustness and yield. A variety of examples are presented to showcase the promise of metabolic burden engineering in facilitating the design and construction of robust microbial cell factories. Finally, challenges and limitations encountered in metabolic burden engineering are discussed.

1. Introduction

Over the past decade, advances in metabolic engineering and synthetic biology have provided a range of tools and strategies for the construction of efficient microbial cell factories (Choi et al., 2019; Kim et al., 2023; Ko et al., 2020). However, not even metabolically engineered microorganisms necessarily achieve the desired bioproduct titer, yield, and productivity. An important impediment is the role played by genetic or environmental changes in imposing a metabolic burden on host cells (Wu et al., 2016). Metabolic burden is generally defined as the influence of genetic manipulation and environmental perturbations on the allocation of host cellular resources, which results in subpar metabolism and growth (Glick, 1995; Snoeck et al., 2024; Tsoi et al., 2018). A common consequence of metabolic burden is the decline in growth rate of host cells (Lozano Terol et al., 2021). The draining of finite cellular resources, such as amino acids, nucleotides, ribosomes, polymerases, reducing equivalents, and energy, to adapt to such genetic manipulation and environmental perturbations inevitably affects basic physiological functions. This, in turn, has a profound impact on the production of desired chemicals in engineered cell factories.

Multiple internal factors, such as heterologous gene/pathway expression, plasmid maintenance, and endogenous genetic manipulation, as well as external factors, such as pH, temperature, light, salinity, osmotic pressure, dissolved oxygen, and nutrient availability, trigger metabolic burdens (Montaño López et al., 2022; Wu et al., 2016) (Fig. 1). Metabolic burden engineering aims to reduce the impact on genetically modified microorganisms and, consequently, improve both cell performance and metabolic efficiency. Prediction of burden-causing

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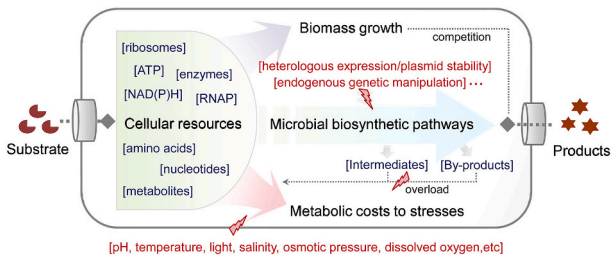

Fig. 1. Overview of metabolic burdens and the burden-causing stimuli on host cells.

bottlenecks by genome-scale modeling can point to ways for optimizing the synthesis of desired metabolites, thereby minimizing the unwanted influences of metabolic burdens (Kerkhoven, 2022). In addition to advancing genome-scale redesign of biological systems, efforts have also been made to develop potent tools and strategies for the direct alleviation of an existing metabolic burden. These include cellular reprograming by balancing flux distribution and redox homeostasis, the employment of dynamic control systems, the improvement of stress tolerance by industrial strains, and the design of microbial co-cultures or synthetic consortia (Brooks et al., 2023; Buerger et al., 2019; Chen et al., 2022a; Hartline et al., 2021; Liu et al., 2021). In brief, attempts to alleviate metabolic burdens by rewiring the host cell metabolism could provide a new perspective for developing robust cell factories.

In this review, we present a broad overview of metabolic burdens, with a focus on recent progress in metabolic burden engineering for improved bioproduction by industrial microorganisms. The challenges and perspectives for future advances in metabolic burden engineering are also discussed.

2. Predicting and determining metabolic burdens in biological systems

Metabolic burdens normally arise from the diversion of available resources in response to genetic and/or environmental perturbations. These changes can be investigated using genome-scale metabolic models (GEMs). The most useful tools are constraint-based models of metabolic network reconstructions, which incorporate the reaction stoichiometry and gene-protein-reaction associations (Kerkhoven, 2022; Sinha et al., 2021). Given that the resource can be formulated as a constraint in the linear programming problem, GEMs are suitable tools to compute resource allocation and predict metabolic burdens caused by perturbations. Experimental diagnosis and identification of metabolic burdens in cellular pathways can reveal the trade-offs between cell growth and bioproduction under diverse environmental conditions. Therefore, in silico mathematical prediction, along with experimental measurement of metabolic burden, could guide the construction of robust cell factories.

2.1. Investigation of metabolic burdens by metabolic modeling

Carbon sources (e.g., glucose) represent a key resource as their degradation (i.e., catabolism) produces building blocks (e.g., amino acids) and energy molecules (e.g., ATP) that are essential for cell growth and function. Thus, perturbations redirecting the carbon or energy flux away from basic activities could place a metabolic burden on the cell (Vogeleer et al., 2024). GEMs with constraint-based algorithms such as flux balance analysis have been widely used to simulate metabolic fluxes and, therefore, enable the estimation of metabolic burdens (Domenzain et al., 2022). Micronutrients, such as vitamins and minerals, are also vital resources for living cells, whose limited availability may impose a metabolic burden. Unlike macronutrients, vitamins and minerals are not catabolized further, but serve as cofactors for enzymes. Emerging modeling approaches enable prediction of metabolic burdens caused by reallocation of the limiting micronutrients (Chen et al., 2022b; Lloyd et al., 2021). An illustrative example is CofactorYeast, a yeast GEM integrated with enzyme cofactors, (e.g., metals) and thereby enabling simulation of burden of metal deficiency (Chen et al., 2021). By simulating the yeast cell factory producing *p*-coumaric acid, CofactorYeast identified the competition of iron between microbial biomass and *p*-coumaric acid synthesis. This highlighted the significance of iron deficiency as a burden and revealed potential strategies for improving the performance of the cell factory.

Besides external nutrients, internal resources are also affected by perturbations. Protein content within a cell is finite, which means that perturbations requiring additional proteome resources will drain those allocated to basic activities, imposing a substantial metabolic burden on the cell. It is thus necessary to estimate the cost in terms of proteome resources. Enzyme-constrained GEMs (ecGEMs) are genome-scale metabolic models integrated with enzyme information (e.g., enzyme kinetics and molecular weight) (Chen et al., 2024b; Domenzain et al., 2022), and able to calculate the protein cost for an enzymatic reaction or a metabolic pathway, namely protein mass required per flux through the reaction or pathway (Chen and Nielsen, 2019). Previous estimates of the costs involved in basic energy metabolism (Chen and Nielsen, 2019) and amino acid biosynthesis have confirmed the importance of the proteome resource (Chen and Nielsen, 2022). ecGEMs possess the capacity to calculate the cost of perturbations (e.g., expression of a heterologous pathway) and the resulting metabolic burdens imposed by metabolic engineering. Therefore, ecGEMs have been widely used to guide design and optimization of microbial cell factories. With an ecGEM of *Escherichia coli*, Ye et al. (2020) predicted that lysine production was mainly affected by the burden related to protein demand and synthesis, and thus improved lysine production by optimizing the expression of highly demanded proteins. A more systematic study was conducted with an ecGEM of *Saccharomyces cerevisiae*, which estimated protein and substrate costs for production of 102 chemicals in yeast and predicted knock-in, knock-out and overexpression targets for their overproduction (Iván et al., 2023).

Another example of internal resources that can be modeled is protein secretory capacity, which encompasses the limited secretory machinery and organelles within a cell. Intuitively, perturbations that occupy secretory resources are expected to prevent or retard proper process and transport of native proteins, leading to metabolic burdens (Ceroni et al., 2015; Glick, 1995). Recently, a yeast constraint-based modeling integrated with the protein secretory pathway, named pcSecYeast, calculated the costs and simulated the phenotypes caused by the competition between recombinant and native secretory proteins (Li et al., 2022a), demonstrating the potential of metabolic models as tools to estimate metabolic burdens. Furthermore, pcSecYeast can serve as a platform for the rational design of systems-level targets for improving production of recombinant proteins, and its predictions for enhancing α-amylase production were subsequently validated.

2.2. Determination of cellular metabolic burdens

Quantification of the metabolic burden, particularly in relation to growth rates, offers a useful guidance for the optimization of microbial hosts and fermentation conditions. However, existing approaches, such as DNA microarrays, two-dimensional polyacrylamide gel electrophoresis, and ^{13}C metabolic flux analysis, cannot effectively quantify the metabolic burden. These methods are often time-consuming, and the sample treatment is compatible only with a subsequent offline monitoring procedure (Antoniewicz, 2021; Chandran et al., 2020). Dissolved oxygen is a significant parameter during fermentation, and the oxygen transfer rate (OTR) can be regarded as an indicator of metabolic burden (Wollborn et al., 2022). The devices for sterile online measurement of the OTR, such as RAMOS, μTOM, allow to the real-time monitoring of metabolic state of industrial strains during small-scale cultivation processes (Dinger et al., 2022; Mühlmann et al., 2018). Recently, single-cell

metabolic analysis provides an unprecedented resolution for determining the metabolic burdens. Rouches et al. (2022) conducted single-cell timelapse measurements to explore the effects of plasmid copy number on cellular performance, and found that each plasmid imposed a 0.063% linear metabolic burden on their host strains. Moreover, the advances in mass spectrometry have been instrumental in driving the development of single-cell metabolite profiling, thus allowing an in-depth quantitative analysis of cell physiological states (Sun et al., 2023).

An additional highly precise and immediate in vivo monitoring system has been devised to ascertain and quantify the burden imposed by diverse plasmid constructs in *E. coli* (Borkowski et al., 2018; Ceroni et al., 2015). In this set-up, a constitutive reporter cassette was integrated into the bacterial genome, and its shifts in fluorescence intensity due to global gene expression reflected changes in cellular resource availability. As this observed depletion manifests across all dividing cells, this configuration holds promise for extension to a broad spectrum of microorganisms.

3. Balancing flux distribution and redox metabolism to alleviate metabolic burden

Biomass building blocks and energy-containing compounds [e.g., NAD(*P*)H, FADH, and ATP] play an important role in managing the trade-offs between cell growth and metabolite formation (Choi and Lee, 2023; Montaño López et al., 2022). Engineered strains recruit finite cellular resources, such as metabolic precursors, initiation factors, energy, ribosomes, and enzymes, to biosynthetic pathways for the production of desired compounds. This shift leads inevitably to a corresponding decrease of resources towards cell growth and overall metabolism. Therefore, a balanced resource allocation from biomass to production is a major determinant in minimizing host resource costs, with which to achieve both maximum growth and product biosynthesis (Basan, 2018; Liu et al., 2021; Toya and Shimizu, 2020). Engineering of metabolic flux distributions and redox balances helps optimize microbial metabolism (Montaño López et al., 2022), contributing to the fine-tuning of intracellular precursor and energy requirements, preserving strain robustness, and improving production outputs via decreased metabolic burden.

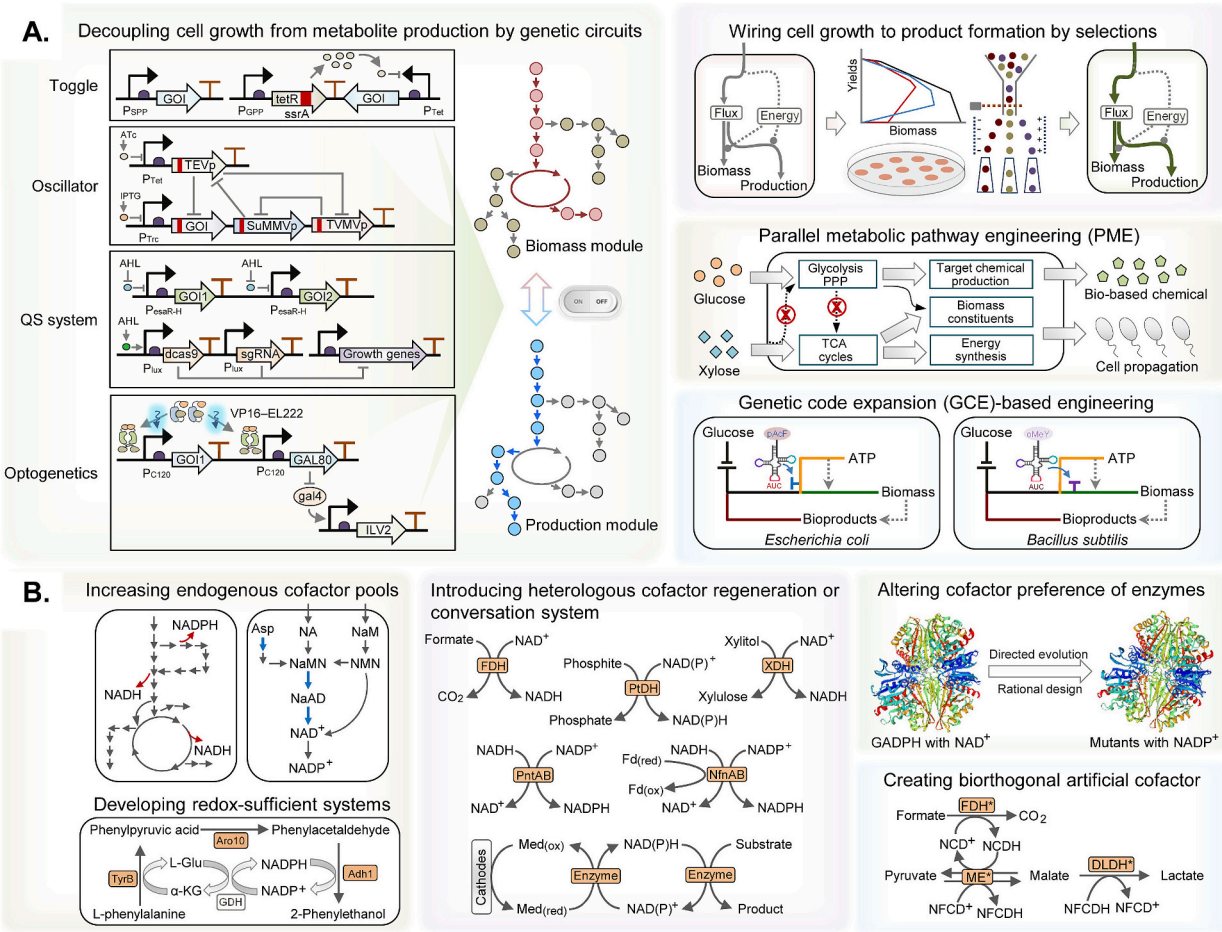

Fig. 2. Representative strategies of metabolic reprogramming for supporting cellular flux and redox balance. (A) Basic concepts for balancing flux distribution in metabolic pathways, including the employment of various gene circuits for decoupling growth from metabolite production, the rewiring of cell growth into product biosynthesis by selections, parallel metabolic pathway engineering for substrate co-utilization, and genetic code expansion-based orthogonal engineering. GOI, gene of interest; P_{SPP}, stationary phase promoter; ssrA, a C-terminal degradation tag (C-degron); P_{GPP}, growth phase promoter; PTet, tetracycline-responsive promoter; TEVp, tobacco etch virus protease; TVMVp, tobacco vein mottling virus protease; SuMMVp, sunflower mild mosaic virus protease; P_{lux} and P_{esaR}, quorum-sensing promoters, AHL, 3-oxohexanoyl homoserine lactone; VP16-EL222, light-sensitive EL222 with transcriptional activation domain of VP16 and a nuclear localization signal; P_{C120}, the EL222-regulated promoter; Gal4, transcription activator inhibited by Gal80; ILV2, acetolactate synthase; pAcF, *p*-acetyl-*L*-phenylalanine; oMeY, *O*-methyl-L-tyrosine. (B) The strategies for engineering cellular redox balance. The homeostasis of cellular redox metabolism can be realized based on a variety of tools, such as increasing intracellular cofactor pools by synthesis, developing redox-sufficient systems by an enzyme-dependent bridge, introducing the cofactor regeneration or conversation systems, altering cofactor preference by protein engineering, and creating biorthogonal artificial cofactors.

3.1. Balancing cell growth and product biosynthesis by redirecting metabolic flux

The biosynthesis of target metabolites generally competes with basic growth processes for the limited cellular resources present in industrial microorganisms. Thus, balancing cell growth and target product synthesis is an attractive and highly effective way to develop robust microbial chassis for biotechnological applications. In the past few years, many efforts have been made to minimize the trade-offs between cell growth and product biosynthesis (Fig. 2A). Among these strategies, genetic circuit tools for decoupling cell growth from metabolite production are extensively used to regulate the metabolic state and flux distribution within metabolic networks (Cui et al., 2021; Xu et al., 2024b). Utilizing genetic circuits responsive to metabolites, a feedback loop can be constructed to achieve real-time control of the cellular metabolic network. In contrast to open-loop circuits or unregulated pathways, the feedback loops hold the potential to markedly reduce the rise-time of metabolites and expedite the metabolic dynamics of pathways.

From the perspective of strain engineering, if metabolite production becomes independent from cell growth requirements by regulatory means, it will drive the re-allocation of limited resources based on process demand. Indeed, various gene circuits, such as biomolecular switches (e.g., toggle and oscillator), quorum sensing-based controllers, and optogenetic devices, have been designed to redistribute metabolic fluxes in engineered strains (Benisch et al., 2024; Dinh and Prather, 2019; Lu et al., 2009; Şimşek et al., 2023). For instance, Hou et al. (2020) developed several bifunctional molecular switches using growth phase-dependent promoters and degrons to redirect the partitioning of carbon fluxes. In this way, they increased the accumulation of shikimic acid (14.33 g/L) and D-glucaric acid (1.56 g/L), as well as growth in *E. coli* MG1655. Likewise, Wei et al. (2022) created a tunable growth phase-dependent autonomous bifunctional genetic switch (GABS) by coupling growth-responsive promoters and degrons to dynamically direct the carbon flux. They achieved an elevated titer of γ-aminobutyric acid from low-value glycerol, without any obvious growth retardation in *Corynebacterium glutamicum*. A similar study reported that D-xylonate productivity of 7.12 g/L·h with a titer of 199.4 g/L could be obtained without impairing cell viability by introducing an engineered protease-based oscillator (Gao et al., 2019a). Following a slightly different approach, Guo et al. (2020a) engineered the lifespan of *E. coli* cells using a two-output or multi-output synthetic recombinase-based state machine. They improved the production of poly (lactate-*co*-3-hydroxybutyrate) and butyrate by programming cell differentiation with gene circuits in fed-batch fermentation. Furthermore, *lux* and *esaR* quorum-sensing circuits have been evolved to dynamically regulate the expression of target genes in a cell density-dependent manner, resulting in significantly higher naringenin and salicylic acid production via better balance between product synthesis and cell growth (Dinh and Prather, 2019).

Recently, a light-inducible system independent from native cell metabolism has emerged as a new paradigm for modulating microbial physiology and biosynthesis (Chia et al., 2022). In one example, Bezold et al. (2023) discovered that optogenetics-triggered cell cycle interventions involve reallocating metabolic resources to enhance the synthesis of compounds in cell factories. They demonstrated that optogenetic interventions in the cell cycle of budding yeast can be utilized to increase the production of valuable chemical compounds such as terpenoid β-carotene and nucleoside analog cordycepin. As another example, Zhao et al. (2018) achieved optogenetic control of engineered pathways by incorporating a light-inducible growth phase and a darkness-inducible production phase. This system attained robust cell growth alongside enhanced production of isobutanol and 2-methyl-1-butanol in engineered *S. cerevisiae*. Likewise, Ding et al. (2020b) designed an elegant optogenetics-based cell division regulation strategy to control cell phenotypes (e.g., specific surface area and mean cell volume) by shortening or prolonging the C and D periods of cell division. They achieved higher efficiency of acetoin or poly (lactate-co-3-hydroxybutyrate) production in engineered *E. coli*. Moreover, Hu et al. (2021) developed the novel light-driven CO_2 fixation and CO_2 mitigation systems in *E. coli*. Their work led to significant advancements in L-malate and butyrate production, achieving the maximum theoretical yields of targeted chemicals without affecting normal *E. coli* growth.

Apart from genetic circuits, another effective means of improving productivity is by wiring cell growth to product formation via growth-coupled selection based on auxotrophic and antimetabolite-resistant mutants (Buerger et al., 2019). The high-throughput selection of robust evolved mutants offers a "win-win" synergy between cell growth and product biosynthesis. For example, the OptKnock and AERITH algorithms have been shown to predict promising targets capable of stimulating production of compounds of interest while maximizing cell growth (Shirai and Kondo, 2022; Wehrs et al., 2019). However, this strategy is laborious and inefficient, and is usually applied to increase titers rather than yields. Another promising example of metabolite balancing is parallel metabolic pathway engineering for co-utilizing diverse carbon substrates without decreasing growth. Fujiwara et al. (2020) developed an engineered *E. coli* strain to utilize xylose for generating the building blocks for growth and glucose mainly for synthesizing the target product. This strategy avoids substrate competition and relieves metabolic burdens during production of shikimate pathway derivatives. In a more impressive example, Tian et al. (2020) demonstrated a genetic code expansion (GCE)-based orthogonal translation system for incorporating the non-canonical amino acids (ncAAs) into proteins via amber stop codon insertion. By constructing the ncAA-dependent expression patterns, the precise control of expression levels for genes determining growth and biosynthesis could be achieved through the titration of different ncAAs. Proof-of-concept applications demonstrated that the GCE-based strategy for cell growth and biosynthesis balance resulted in the enhancements in the production of *N*-acetylglucosamine and *N*-acetylneuraminic acid, with respective increases in titers of 4.54-fold and 2.34-fold. Collectively, this GCE-based orthogonal system, which incorporates ncAAs into proteins by reassigning non-canonical codons to ncAAs, qualifies for balancing cellular metabolism by minimizing the connections between cell growth and product biosynthesis.

3.2. Cofactor engineering for cellular redox balance

Redox couples, such as NAD^+/NADH, $NADP^+$/NADPH, and FAD^+/$FADH_2$, are critical for cellular electron transfer, because they maintain a reduced redox state and support an efficient energy metabolism (Montaño López et al., 2022; Wang et al., 2017a; Xiao et al., 2018). NAD^+ is an essential electron acceptor responsible for many cellular processes including glycolysis; whereas NADH is typically used as an electron donor for the electron transport system to generate ATP. Likewise, the cofactor NADPH is an electron donor during the reductive biosynthesis of fatty acids, steroids, amino acids, and nucleotides. Thus, tight control of redox homeostasis is essential for obtaining robust microbial strains capable of elevated production efficiency (Jiang et al., 2020; Ko et al., 2020; Liu et al., 2018c; Mu et al., 2021). To balance the intracellular redox state, multiple tools and strategies have been proposed (Fig. 2B), including enhancing the biosynthesis of endogenous cofactor pools, improving the cellular redox self-balance systems, introducing heterologous cofactor regeneration or conversion systems for the dynamic balance of overall NAD(*P*)H/NAD(P)$^+$ ratios, altering the cofactor preference of certain metabolic enzymes, as well as creating novel artificial cofactor systems.

Among these strategies, a cofactor self-sufficient system was demonstrated by coupling regeneration of the co-substrate with the cofactor supply, thereby removing the cost burden for provision of expensive cofactors (Nielsen et al., 2023; Zou et al., 2024). Explicitly, a bridge between 2-phenylethanol and its precursor *L*-phenylalanine was constructed by coupling glutamate dehydrogenase with aromatic

transaminase and alcohol dehydrogenase. This enabled the simultaneous regeneration of co-substrate and redox equivalents for the efficient production of 2-phenylethanol in *E. coli*. However, its application requires such pair system available in bioconversion processes. Given that NADH is more abundant than NADPH in most heterotrophic microorganisms, switching cofactor specificity of enzymes from NADPH to NADH promotes microbial growth and bioproduction (Wang et al., 2017a). For example, when glyceraldehyde 3-phosphate dehydrogenase was engineered to obtain dual NAD^+/$NADP^+$ cofactor specificity, amino acid production was increased without negatively affecting cell growth (Slivinskaya et al., 2022). However, the changes in intracellular NADH and NADPH pools often affect all cofactor-dependent reactions, thereby leading to unintended and unpredictable pleiotropic effects on cell metabolism. Bio-orthogonal redox systems represent a new avenue for overcoming these limitations, as they exploit the independence between metabolic pathways in host cells. For example, nicotinamide flucytosine dinucleotide ($NFCD^+$) emerges as an artificial analog of NAD^+ with great promise in NAD^+-dependent enzymes (Guo et al., 2020b). Moreover, a variety of synthetic cofactors incorporating nicotinamide were reported, including Hantzsch ester, 9,10-dihydrophenanthridine, and nicotinamide cofactor mimics (Liu et al., 2018c). Overall, the integration of artificial cofactor analogs presents a promising strategy for preserving cellular redox equilibrium while minimizing interference with native host cell metabolism.

Glutathione and thioredoxin serve as additional sources of reducing equivalents to prevent excessive oxidative stress and maintain a favorable redox environment (Xiao and Loscalzo, 2020). Improving the antioxidant properties of lactic acid bacteria has been suggested to protect host cells from various environmental stresses and support the creation of robust strains with desired production characteristics (Bryukhanov et al., 2022). Moreover, cytochrome P450s are extremely versatile redox enzymes, which catalyze a vast variety of oxidative metabolic reactions with potential applications in industrial biotechnology (Li et al., 2020c; Urlacher and Girhard, 2019). Genome mining and protein engineering of promiscuous P450s offers compelling avenues for bolstering microbial cell factories against metabolic burdens arising from accumulated intermediates.

4. Engineering metabolic control systems to alleviate the metabolic burden

Discrepancies in the expression of pathway genes and enzyme activity can result in the accumulation of toxic intermediates or byproducts. This subsequent metabolic burden imposed on host cells leads to suboptimal growth and biosynthesis. Ordinarily, on-demand expression of hub or rate-limiting enzymes coupled with the elimination of competing pathways can effectively reduce metabolic burdens. Several metabolic control strategies have attempted to resolve these bottlenecks (Choi et al., 2019; Li et al., 2020b; Xu et al., 2020a) (Fig. 3A). They include improving expression of rate-limiting enzyme nodes by gene overexpression, impairing metabolic shunting of the competing pathways by gene deletion, promoter and ribosome-binding site engineering,

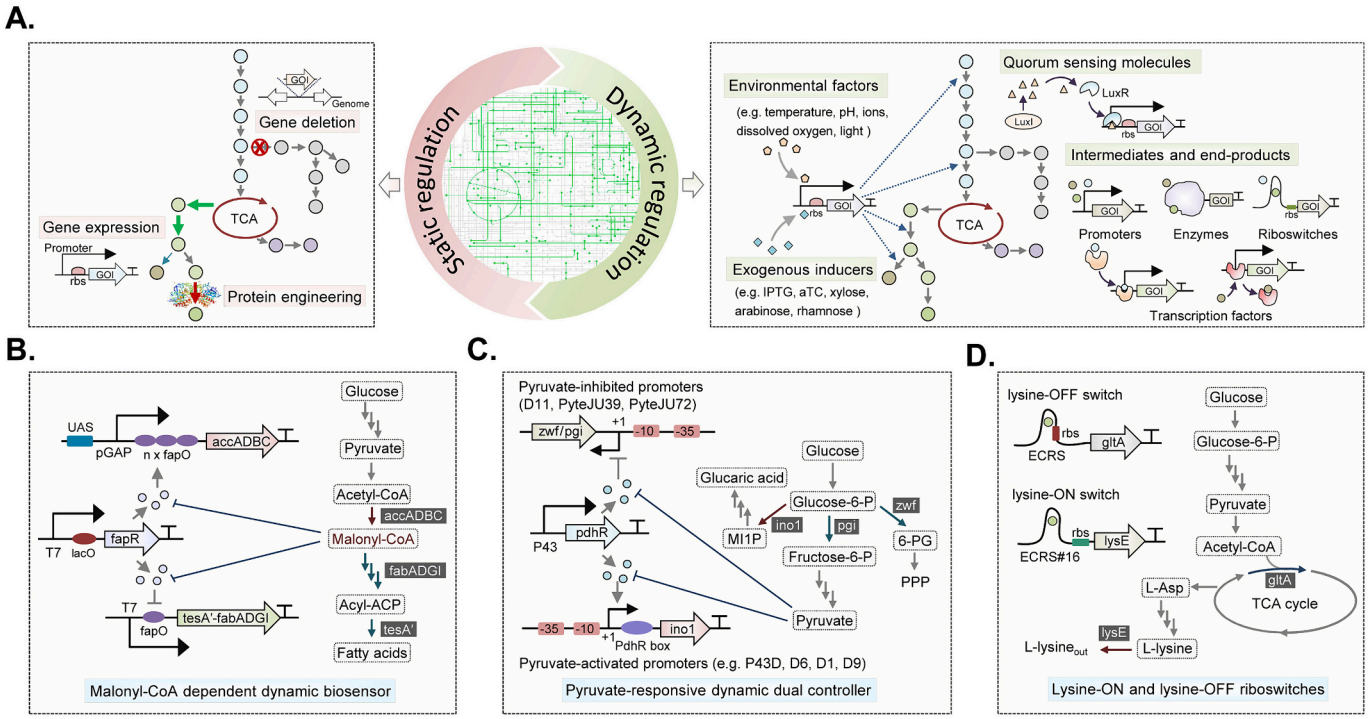

Fig. 3. Representative strategies and examples for overcoming pathway bottlenecks. (A) Static and dynamic control of microbial metabolic pathways. The static regulation strategies have implicated in genetic changes such as gene disruptions, uncontrollable gene expression, and enzyme engineering. The dynamic control of cellular metabolism can be realized base on changes in stress-responsive signals, including environmental factors, exogenous inducers, quorum sensing molecules, and cellular metabolites. GOI, gene of interest; rbs, ribosome-binding site; TCA, tricarboxylic acid cycle. (B) Schematic representation of FapR-based malonyl-CoA biosensor and its application in improving fatty acid production. The transcriptional regulator FapR from *B. subtilis* can specifically recognize malonyl-CoA to dynamically modulate the expression of pGAP promoter-driven malonyl-CoA source pathway (acetyl-CoA carboxylase, encoded by *accADBC*) and T7 promoter-driven malonyl-CoA sink pathway (fatty acids synthase, encoded by *fabADGI* and *tesA'*). fapO, FapR repressor-binding site; lacO, LacI repressor-binding site; UAS, upstream activation sequence. (C) Schematic representation of PdhR-based pyruvate-responsive dual biosensor and its application in improving the production of glucaric acid. The pyruvate-activated promoters were used to enhance the expression of *ino1* gene involved in glucaric acid biosynthetic pathway, and the antisense pyruvate-responsive promoters were used to repress the expression of *pgi* and *zwf* genes involved in the competitive pathways. PdhR box, PdhR-binding site. (D) Schematic representation of lysine riboswitch and its application in improving the production of L-lysine. A natural lysine-OFF riboswitch from *E. coli* (ECRS) was used to regress the expression of *gltA* gene that is essential but undesirable for lysine production, and a synthetic lysine-ON riboswitch (ECRS#16) was used to upregulate the expression of lysE gene responsible for the export of L-lysine.

sRNA and CRISPR/Cas9 interference systems, as well as developing advanced or novel enzymes with desired properties via enzyme mining, directed evolution, and rational design. However, due to the complexity and diversity of metabolic networks, traditional genetic approaches including deletion, attenuation or overexpression of specific genes, are just static adjustments to metabolic networks that tend to cause other unpredictable imbalances in the host metabolism.

Apart from traditional static regulation strategies, dynamic metabolic control has recently emerged as a powerful strategy for precision pathway engineering (Hartline et al., 2021; Liu et al., 2018b; Ream and Prather, 2023; Shen et al., 2019) (Fig. 3A). Compared to static regulation, dynamic control achieves the real-time sensing of a pathway's metabolic status, thereby dynamically regulating its cellular carbon and energy fluxes following changes in responsive signals. The stimuli most often used in dynamic pathway regulation include environmental factors, exogenous inducers, cell density, cellular intermediates, and the levels of end-products (Gao et al., 2019b; Shen et al., 2019; Xiao et al., 2023). Owing to the benefits of dynamic metabolic control on biosynthesis, this strategy has been widely applied and has substantially increased bio-based production of fatty acids, amino acids, lycopene, isopropanol and glucaric acid (Han et al., 2023; Hartline et al., 2021).

Dynamic pathway engineering aims to construct metabolic production systems embedded with intracellular regulatory mechanisms to enhance efficiency (Ko et al., 2020). However, pathway design involves assembling multiple biological parts into appropriate circuit architectures and fine-tuning each component's function precisely. This leads to a large design space that is prohibitively expensive to explore solely through experimentation. Artificial intelligence (AI) and machine learning methods are increasingly recognized as valuable tools to accelerate the design process in bioengineered systems, owing to their capacity to discern hidden patterns in data and rapidly screen through extensive design libraries (Merzbacher and Oyarzún, 2023; Patra et al., 2023). As an example, Kotopka and Smolke (2020) developed the highly accurate predictive models to predict promoter activity by sequence-based deep learning, and enabled rapid generation of artificial yeast promoters with advantageous properties for synthetic biology applications.

4.1. Genetically-encoded biosensors for dynamic pathway control

Living microorganisms require a variety of biochemical reactions to maintain their metabolic and physiological functions. Enzymes facilitate chemical reactions by lowering activation energy barriers. Thus, hub or rate-limiting enzymes in metabolic pathways have long been regarded as critical nodes for efficient cell growth and bioproduction (McIntosh and Owens, 2021; Xu et al., 2020a). Hindering rate-limiting metabolic nodes or the overexpression of upstream enzymes in metabolic pathways often leads to the accumulation of metabolic intermediates, which increases the metabolic burden and slows growth. Thus, fine-tuning expression timing and the levels of rate-limiting steps in given metabolic pathways represents an effective approach for alleviating metabolic bottlenecks and minimizing the burdens on robust bioproduction (Li et al., 2020b; Mao et al., 2023; Wu et al., 2016).

To overcome the trade-off between resource availability and accumulation of toxic metabolites, biosensors capable of continuous and real-time monitoring of target metabolites have gained growing attention (Qin et al., 2022; Teng et al., 2022; Yu et al., 2023c). The architecture of metabolite biosensors is based on sensing elements (e.g., transcription factors, riboswitches, enzymes, and promoters) that can interact with the analyte of interest to generate a dose-dependent "ON" or "OFF" signal for precisely regulating the expression of the actuator module (e.g., fluorescent proteins, selection markers, pathway enzymes, or genetic circuits) (Fig. 3A). Metabolite-responsive biosensors have thus emerged as powerful tools that allow for dynamic pathway control, high-throughput strain evolution, and single-cell analysis (Shi et al., 2018; Shi et al., 2022; Teng et al., 2022). In most cases, metabolites-based biosensors effectively fine-tune pathway fluxes in response to changes in intracellular metabolite pools and alleviate the accumulation of toxic intermediates. This, in turn, reduces the physiological burden on host cells and augments product biosynthesis. An outstanding example is the design and application of genetically-encoded malonyl-CoA biosensors (Johnson et al., 2017; Qiu et al., 2019; Zhou et al., 2022) (Fig. 3B). Malonyl-CoA is a central precursor for many value-added compounds, including fatty acids, polyketides, flavonoids, and biopolymers. As the rate-limiting substrate for fatty acid biosynthesis, malonyl-CoA is crucial for cell metabolism and membrane structure. Based on the dual transcriptional regulator FapR, Xu et al. (2014) designed two dynamic malonyl-CoA biosensors to control the expression of genes involved in the supply and consumption of malonyl-CoA. Using this synthetic malonyl-CoA switch, the production of fatty acids in engineered cells was increased 2.1-fold compared to a static control strategy. This example inspired a bifunctional pyruvate-responsive genetic circuit based on the pyruvate-responsive transcription factor PdhR (Fig. 3C), in which a dynamic feedback loop relied on pyruvate concentration to control metabolic fluxes (Xu et al., 2020b). This strategy achieved a 154% increase in glucaric acid titers in engineered *Bacillus subtilis*; whereas a conventional static control method achieved an increase of 35%. LysG and Lrp from *C. glutamicum* are two additional examples of transcription factors used as amino acids biosensors (Mahr and Frunzke, 2016; Wang et al., 2017b) (Fig. 3D). They have been widely applied in high-throughput screening of hyper-producing mutant strains for monitoring L-lysine, L-methionine, and branched-chain amino acids levels (Huang et al., 2023; Liu et al., 2022b; Zhang et al., 2022). As a cis-acting regulatory mRNA element, riboswitch can bind and sense specific metabolites, thereby mediating the expression of downstream target genes (Manna et al., 2021; Wu et al., 2023). Hence, riboswitch-based biosensors are employed to optimize metabolic biosynthetic pathways. Accordingly, several lysine-responsive riboswitches were found to redirect fluxes towards L-lysine biosynthesis, resulting in a significant improvement of L-lysine production in *C. glutamicum* (Liu et al., 2022c; Zhou and Zeng, 2015). Another example is the design of a synthetic naringenin riboswitch, Hwang et al. (2021) utilized this tool to screen the optimized strains with multi-level rebalancing at both transcription and translation stages. They achieved a 3-fold higher naringenin production from glycerol than the parental strain, which carried only the statically controlled metabolic pathways.

Besides the examples mentioned above, metabolites-based biosensors have also been used extensively to improve robustness and bioproduction of many value-added compounds in engineered cells (Table 1). However, although substantial progress has been made in identifying metabolite-responsive modules to date, naturally-occurring transcription factors, riboswitches or promoters that specifically recognize and bind a metabolite of interest remain poorly exploited. Therefore, discovering metabolite-responsive components for novel biosensor design, or engineering the sensitivity and specificity of an existing regulator or riboswitch to generate custom-made biosensors for desired molecules will greatly promote the use of biosensors in metabolic pathway control and strain improvement.

4.2. Metabolic burden-driven feedback control of gene expression

Generally, the expression and maintenance of synthetic constructs are essential for developing microbial cell factories capable of high titers, yields, and productivity (Choi and Lee, 2023; Ko et al., 2020). However, robust expression of heterologous or native genes in engineered constructs often leads to unintended host–construct interactions and substantial burdens to host cells. To overcome this challenge, Ceroni et al. (2018) developed a universal, modular, and tunable burden-responsive feedback control system that regulated the expression of synthetic constructs based on an in vivo fluorescence reporter in *E. coli* (Fig. 4A and B). The authors performed RNA-sequencing and in vivo assays to identify significant gene expression changes in host strains

Table 1
Representative examples of metabolites-based biosensors applied in strain development.

Metabolite	Type	Sensor	Actuator module	Host strain	Bottleneck application	Products	Titer/Yield	References
malonyl-CoA	TF	FapR	P_{GAP} promoter-based acetyl-CoA carboxylase ACC; P_{T7} promoter-based fatty acids synthase FAS	*E. coli*	Dynamically control gene expressions involved in the supply and consumption of malonyl-CoA	fatty acids	3.86 g/L	(Xu et al., 2014)
malonyl-CoA	TF	FapR	pGPM1 promoter-based tdTomato fluorescent reporter	*S. cerevisiae*	Genome-wide screening gene targets that improved intracellular malonyl-CoA concentration	3-HP	~1.2 g/L	(Li et al., 2015)
malonyl-CoA	TF	FapR	pTEF1 promoter-based malonyl-CoA reductase MCR_{Ca}	*S. cerevisiae*	Continuous pathway control by self-adjusting target gene expression for 3-HP production	3-HP	1 g/L	(David et al., 2016)
malonyl-CoA	TF	FapR/LacI	P_{FR1} promoter-based LacI repressor; P_{T7} promoter-based acetyl-CoA carboxylase ACC	*E. coli*	Constructing a negative feedback circuit to improve fatty acid production and cell growth	fatty acids	2.03 g/L	(Liu et al., 2015)
p-coumaroyl-CoA	TF	CouR	P_{CCW12}/P_{TEF1} promoter-based indirect control of 4CL expression	*S. cerevisiae*	Dynamic regulation of 4CL in the naringenin synthetic pathway to reduce *p*-coumaroyl-CoA accumulation and toxicity	naringenin	47.3 mg/L	(Liu et al., 2022a)
acyl-CoA	TF	FadR	Synthetic promoter P_{AR}-based tetracycline efflux protein TetA	*E. coli*	Designing a in vivo population quality control system to exploit the non-genetic variants for enhanced biosynthesis	fatty acids	21.5 g/L	(Xiao et al., 2016)
acyl-CoA	TF	FadR-NLS	Modified pTEF1 promoter-based GFP fluorescent reporter	*S. cerevisiae*	Screening for gene targets enhancing acyl-CoA levels for production of fatty acids and derived products.	fatty alcohol	~11 mg/g DCW	(Dabirian et al., 2019)
mevalonate	TF	AraC-mev	P_{BAD} promoter-based sfGFP fluorescent reporter	*M. extorquens*	Screening the mutated QscR to increase acetyl-CoA carbon flux for downstream synthesis of metabolites	mevalonate	2.67 g/L	(Liang et al., 2017)
ectoine	TF	AraC-ect	P_{BAD} promoter-based eGFP fluorescent reporter	*E. coli*	Engineering of the rate-limiting aminotransferase EctB for ectoine hyper-producing strains	ectoine	~10 mg/g DCW	(Chen et al., 2015a)
salicylate	TF	AraC-SA	P_{BAD} promoter-based LacZ reporter	*E. coli*	identification of optimal gene expression patterns in pathways for improved salicylate production	salicylate	17 mM	(Qian et al., 2019)
FBP	TF	Cra	P_{par} promoter-based 6-phosphofructokinase PfkA	*E. coli*	Dynamic control of the glycolysis flux in the EP-bifido pathway for high MVA production	mevalonate	111.3 g/L	(Zhu et al., 2021)
FBP	TF	Cra	P_{pam} promoter-based 6-phosphofructokinase PfkA; $P_{R23107\text{-}cra}$ F6P aminotransferase GlmS	*E. coli*	Dynamic and dual control of glycolysis flux and GlcNAc biosynthesis	GlcNAc	8.63 g/L	(Zhu et al., 2021)
pyruvate	TF	PdhR	Phase-dependent promoter-based *ino1* gene; antisense promoter-based *pgi* and *zwf* genes	*B. subtilis*	Designing a feedback loop control system to fine-tune metabolic fluxes towards products of interest	glucaric acid	527 mg/L	(Xu et al., 2020b)
tyrosine	TF	TyrR	P_{aroF} promoter-based proofreading exonuclease of DNA polymerase III MutD5 and mCherry reporter	*E. coli*	Constructing an adaptive control system for evolving increased tyrosine production	tyrosine	~15 mg/L	(Chou and Keasling, 2013)
tryptophan	TF	TnaC	Maltose hydrolase MalQ fused to the leader peptide of the *tnaCAB* operon	*E. coli*	Enabling maltose growth-based selection for the microbes with	tryptophan	~25 mg/L/OD	(Liu et al., 2017a)

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Table 1 (*continued*)

Metabolite	Type	Sensor	Actuator module	Host strain	Bottleneck application	Products	Titer/ Yield	References
					enhanced tryptophan production			
kaempferol	TF	QdoR	P_{qdoI} promoter-based anti-σ molecule; P_{20} promoter-based naringenin chalcone synthase CHS	*E. coli*	Establishing a feedback controller to regulate the naringenin pathway against malonyl-CoA availability	naringenin	~1 g/L	(Boada et al., 2020)
p-coumaric acid, naringenin	TF	PadR, FadeR	P_{padC} promoter-based expression of 4CL, CHS and anti-sense RNA of fabD, P_{fdeA} promoter-based additional expression of CHS	*E. coli*	Both relieved metabolic burdens and increased enzyme availability to enhance the naringenin biosynthesis by the tri-function circuits	naringenin	277.2 mg/L	(Jiang et al., 2022)
vanillate	TF	VanR	P_{vanO} promoter-based mYFP fluorescent reporter	*E. coli*	Rapid bioprospecting of the rate-limiting catechol *O*-methyltransferases in vanillin pathway	vanillin	NA	(Kunjapur and Prather, 2019)
shikimate	TF	ShiR	P_{shiA} promoter-based eGFP fluorescent reporter	*C. glutamicum*	High-throughput screening of high-yield shikimate-producing strains	shikimate	NA	(Liu et al., 2018a)
inosinic acid	TF	GlxR	P_{cg3195} promoter-based eGFP fluorescent reporter	*C. station*	High-throughput screening of inosinic acid-producing strains	Inosinic acid	NA	(Jiang et al., 2024)
L-methionine; L-valine; L-isoleucine L-leucine	TF	Lrp	P_{brnFE} promoter-based mYFP fluorescent reporter	*C. glutamicum*	High-throughput screening of improved rate-limiting enzymes or high-performance strains	L-methionine; L-valine; L-isoleucine; L-leucine	NA	(Han et al., 2020; Mustafi et al., 2012)
L-isoleucine	TF	Lrp	Modified P_{BrnFE}N promoter-based odhI, vgb and ido genes	*C. glutamicum*	Dynamic control of multiple pathway genes for efficient 4-Hydroxyi-soleucine biosynthesis	4-HIL	135.3 mM	(Tan et al., 2020)
L-lysine; L-arginine; L-histidine	TF	LysG	P_{LysE} promoter-based eYFP fluorescent reporter	*C. glutamicum*	High-throughput screening of improved rate-limiting enzymes or high-performance strains	L-lysine; L-arginine; L-histidine	NA	(Della Corte et al., 2020; Schendzielorz et al., 2014)
diamine	TF	CgmR	P_{cgmA} promoter-based sfGFP fluorescent reporter	*C. glutamicum*	Contributing to rewiring metabolic circuits and facilitating directed evolution of recombinant strains	diamine	NA	(Zhao et al., 2021)
erythritol	TF	EryD	Hybrid T7 system consisting of EryD operon and eGFP reporter	*Y. lipolytica*	High-throughput screening of mutant strain libraries	erythritol	148 g/L	(Qiu et al., 2020)
L-cysteine	TF	CcdR	P_{ccdA} promoter-based GFP fluorescent reporter	*C. glutamicum*	Direct evolution of pathway key enzyme and high L-cysteine-producing strains	L-cysteine	195.7 mg/L	(Gao et al., 2022)
alkane	TF	AlkS	P_{alkB} promoter-based eGFP fluorescent reporter	*E. coli*	High-throughput screening of alkane-producing strains from the key enzyme mutant library	long-chain alkanes	40.1 mg/L	(Chen et al., 2023)
L-lysine	Riboswitch	NA	Natural lysine riboswitches-based citrate synthase GltA	*C. glutamicum*	Dynamic control of metabolic fluxes in lysine biosynthetic pathways	lysine	0.227 mol/ mol	(Zhou and Zeng, 2015)
naringenin	Riboswitch	NA	A synthetic aptamer, ribosome-binding site, and a translational fusion of *tetA* and *sfgfp* genes as a reporter	*E. coli*	High-throughput screening of high-performance strains for naringenin production	naringenin	260.2 mg/L	(Hwang et al., 2021)
NeuAc	Riboswitch	NA	Naturally occurred ligand-responsive aptazyme fused to sfGFP fluorescent reporter	*E. coli*	High-throughput screening of improved rate-limiting *N*-acetylneuraminate synthase or high-performance strains	NeuAc	8.31 g/ L	(Yang et al., 2017)
theophylline	Riboswitch	NA	A riboswitch that binds to theophylline, and a GFP gen Theophylline riboswitch D	*E. coli*	Selecting the optimal genotype for the sustained conversion of caffeine to theophylline	theophylline	NA	(Eckdahl et al., 2015)

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Table 1 (*continued*)

Metabolite	Type	Sensor	Actuator module	Host strain	Bottleneck application	Products	Titer/ Yield	References
			fused to fluorescent reporter or the *tetA* tetracycline gene					
adenosylcobalamin	Riboswich	NA	A RNA riboswitch responds to elevated levels of adenosylcobalamin and downregulates transcriptional repressor transcription to express GFP fluorescent as reporter	*S. meliloti*	High-throughput screening of mutant strain libraries for adenosylcobalamin biosynthesis	adenosylcobalamin	150 mg/L	(Yang et al., 2024)
PRPP, E4P, 3PG	Enzyme	NA	HGPRT ligand binding domain fused to GFP fluorescent reporter	*E. coli*	Dynamic control of central metabolic pathways for improved yield of target products	phenylalanine	10.72 g/L	(Ding et al., 2020a)
L-DOPA	Enzyme	NA	DOPA dioxygenase-based betaxanthin fluorescence	*S. cerevisiae*	High-throughput screening of improved rate-limiting tyrosine hydroxylases or high-performance strains	(*S*)-reticuline	80.6 μg/L	(DeLoache et al., 2015)
lactate	Enzyme	NA	Lactate oxidase-based resorufin fluorescence	*E. coli*	Microfluidic high-throughput culturing of single cells for l-lactate producer	lactate	~ 3 g/L	(Wang et al., 2014)
glucose	DNA	NA	pHXT1-controlled squalene synthase gene *ERG9*	*S. cerevisiae*	Dynamic change of squalene synthetic pathway in response to the change of glucose concentration	carotenoid	1156 mg/L	(Xie et al., 2015)
L-lysine	DNA	NA	pA-controlled eGFP fluorescent reporter	*E. coli*	High-throughput screening of lysine-hyperproducing strain	lysine	136.51 g/L	(Wang et al., 2016)
DHS	DNA	NA	P_{CusR}-controlled GFP fluorescent reporter	*E. coli*	High-throughput screening of 3-dehy-droshikimate-producing strain	DHS	36.72 g/L	(Li et al., 2019)
cAMP	DNA	NA	CRP-regulated promoters and GFP reporter	*E. coli*	Directed Evolution of HemA and screening of ALA over-producing strains	5-ALA	58.54 g/L	(Wang et al., 2024b)

harboring different synthetic constructs. To this end, they employed the burden-responsive P_{htpG1} promoter to build a tunable CRISPR–dCas9-based feedback circuit. This device can automatically sense any cellular burden in host cells and, in turn, adjust the expression of the corresponding genes, regardless of synthetic construct content or growth conditions. The above feedback controller can be extended to other strains and could effectively protect against highly sophisticated metabolic stresses.

4.3. Orthogonal genetic constructs for bypassing host cellular resources

Orthogonality is expected to reduce metabolic interference from host resources and minimize cross-talk interactions with cell metabolism (Costello and Badran, 2021; Van Brempt et al., 2022). Advances in synthetic biology enable the design of orthogonal genetic systems to minimize interference from foreign parts and modules on host expression profiles, thereby facilitating the simulation of predictable biological functions (Borkowski et al., 2016; Zhang et al., 2021). In one such example, Cameron and Collins created an orthogonal protein degradation machinery in *E. coli* based on the Lon protease from *Mesoplasma florum*. This system targets and degrades only the tagged proteins expressed from synthetic constructs, without overloading the host degradation machinery (Cameron and Collins, 2014). Segall-Shapiro et al. (2014) designed a resource allocator based on a highly fragmented phage T7 RNA polymerase to decouple transcription from the host machinery. This inherent decoupling property reduced the reliance on host resources necessary for growth and maintenance. Notably, even if orthogonal strategies can bypass some of the shared resources in host cells, these attempts cannot fully eliminate metabolic burdens. This limitation may arise from unavoidable competitions between synthetic constructs and host cells for the finite cellular resources, including transcriptional-translational machinery, enzymes, ATP and so on.

5. Improving strain robustness and tolerance to alleviate metabolic burden

Apart from stress arising from the accumulation of toxic intermediates and by-products caused by introducing heterologous pathways and/or rewiring native cellular metabolism, industrially important microorganisms are often exposed to a variety of external stressors. During fermentation, pH, temperature, osmolarity, salinity, oxygen supply, nutrient availability, and shear force may affect strain performance (Guan et al., 2017; Olsson et al., 2022). These stress factors may damage lipids, proteins, and DNA, thereby leading to detrimental effects on host cell growth and production capacity. Thus, strategies aimed at strengthening robustness and tolerance of industrial microorganisms by stabilizing growth and production traits despite multiple stress perturbations, will allow host cells to cope with the overall metabolic burden imposed on them (Gong et al., 2017; Jiang et al., 2020; Mohedano et al., 2022) (Fig. 5).

5.1. Evolutionary engineering aimed at obtaining robust cells with desired traits

Evolutionary engineering offers a powerful strategy for strain improvement (Castle et al., 2021; Chen et al., 2024a; Zhu et al., 2018).

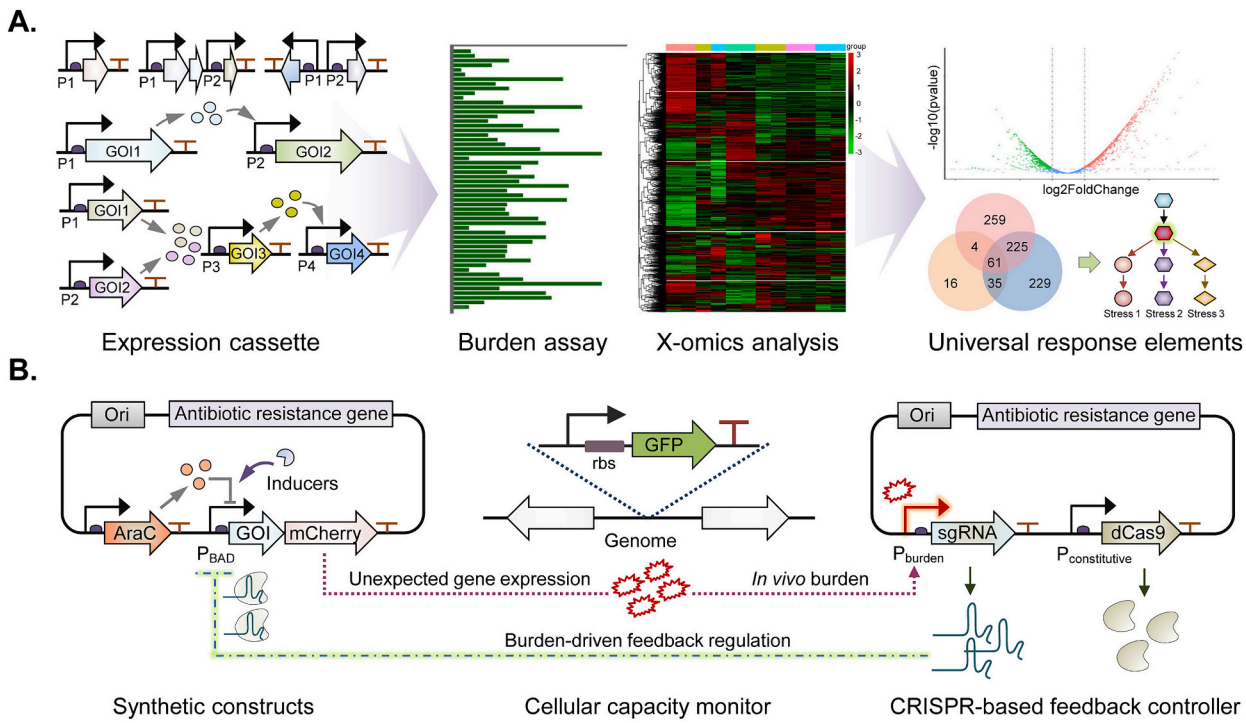

Fig. 4. Burden-driven feedback control system. (A) The general workflow for mining the shared genes in response to metabolic burdens imposed by different expression constructs. (B) A schematic of the burden-responsive feedback device. The expression of CRISPR sgRNA is controlled by the burden-inducible promoter, and it directs binding of dCas9 to the targeted promoter region for automatically regulating gene expression. P: promoter; GOI: gene of interest; P_{BAD}: arabinose-inducible promoter; P_{burden}: burden-inducible promoter; $P_{constitutive}$: constitutive promoter.

Over the past decades, adaptive laboratory evolution (ALE) has emerged as an effective tool for generating microbial phenotypes with desired traits, despite it being a time-consuming and laborious task (Lee and Kim, 2020; Wu et al., 2022). This method has been widely used to enhance stress tolerance of industrial microorganisms, resulting in improved growth rates and metabolic performance under specific stress conditions. To maximize its multigenic effects, ALE is sometimes combined with whole-genome engineering procedures, such as random overexpression/knockout mutant libraries, genome shuffling, global transcription machinery engineering (gTME), and genome replication engineering-assisted continuous evolution (GREACE) to increase genetic variance (Fernández-Cabezón et al., 2019). Moreover, advances in synthetic biology and high-throughput screening have sped up strain evolution by applying in vivo mutagenesis coupled-with selection (Ju et al., 2023; Yang et al., 2019). In recent years, various CRISPR-based methods have emerged to facilitate efficient mutation and selection for genome-wide-directed evolution of stress-resistant strains (Simon et al., 2019; Wei and Li, 2023). These strategies encompass CRISPR/Cas9 and λ Red recombineering-based MAGE technology (CRMAGE), CRISPR-enabled trackable genome engineering (CREATE), Cas9-mediated protein evolution reaction (CasPER), and EvolvR. In one notable example, Liu et al. (2018d) performed 13 rounds of genome-scale editing using the iterative CREATE strategy, targeting ~162,000 mutations across 115 genes involved in 3-hydroxypropionic acid (3-HP) biosynthesis and tolerance. The combinatorial mutants, paired with genome modifications conferring 3-HP tolerance, yielded a 60-fold increase in 3-HP titer compared with the wild-type.

Although evolution and genome-scale engineering proven to be powerful to improve strain tolerance and robustness, challenges remain as these approaches reply on high-throughput screening methods, such as cell sorting-based methods or growth-based selection. It is often labor and resource-intensive, and only a limited number of metabolites or products can be screened by using dedicated biosensors or specific growth conditions. Laboratory automation has emerged as a solution to overcome these constraints. The integration of robotics has been shown to speed up ALE experiments and phenotypic characterization (Lennen et al., 2023). In a recent study, an Automated Scientist called Lila was reported to automate the entire process of microbial strain engineering and to accelerate the biosynthesis of a total of 242 molecules with minimal human intervention (Amoolya et al., 2023). Despite this is not reachable by academic setting, in the future, automation approach and high-throughput methods will lead to more effective construction of robust microbial strains.

5.2. Rational strain design aimed at improving physiological functions

Consolidating external barriers and improving repair capacity effectively augments microbial stress tolerance (Liu et al., 2021). When microorganisms are exposed to various external stresses, the cell wall and membrane represent the first-line of defense against these stimuli. Hence, remodeling cell wall architecture to maintain cell integrity (Mueller et al., 2019; Ribeiro et al., 2022), ameliorating the diversity and fluidity of the plasma membrane (Qi et al., 2019), and enhancing membrane transfer of toxic metabolites by transporter engineering (Zhu et al., 2020), represent potent tools for generating more robust microbes. Moreover, engineering stress-responsive transcriptional regulators including sigma factors offers another avenue for modulating the transcriptional regulatory network in response to multiple stress conditions (Wang et al., 2020b). For example, the introduction of an engineered exogenous global regulator IrrE from *Deinococcus radiodurans* confers increased resistance to numerous abiotic stresses by orchestrating diverse defense systems in both *E. coli* and yeast (Wang et al., 2024a). The heightened expression of stress-related proteins, such as regulators, heat shock proteins or molecular chaperones, facilitates the repair of damaged cells and enhances microbial robustness (Alagar Boopathy et al., 2022; Wang et al., 2019). In one such example, Yao et al. (2022) designed a synthetic acid stress-tolerance module consisting of the acid resistance regulator GadE, periplasmic chaperone HdeB, and

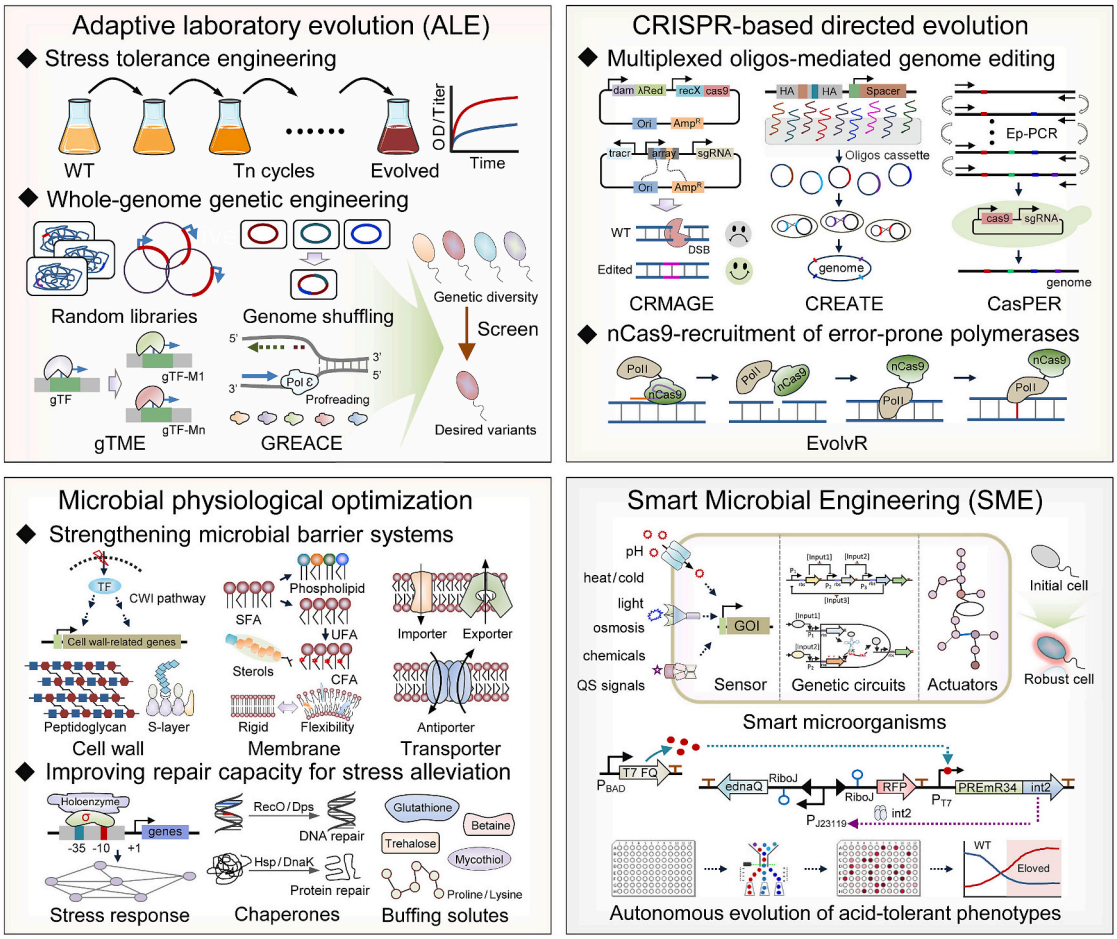

Fig. 5. Representative strategies for improving the robustness of industrial microbes. The robust strains with superior stress tolerance can be obtained by several different techniques, including adaptive laboratory evolution, CRISPR-based directed evolution, microbial physiological optimization, and genetic circuit-assisted smart microbial engineering. gTF, global transcription factor; gTF-Mn, global transcription factor mutant; gTME, global transcription machinery engineering; DSB, DNA double-strand break; GREACE, genome replication engineering assisted continuous evolution; CRMAGE, CRISPR/Cas9 and λ Red recombineering based MAGE technology; CREATE, CRISPR-enabled trackable genome engineering; CasPER, Cas9-mediated protein evolution reaction; TF, transcription factor; CWI, cell wall integrity; SFA, saturated fatty acid; UFA, unsaturated fatty acid; CFA, cyclopropane fatty acid. σ, sigma factor; GOI, gene of interest. T7 TQ, T7 RNA polymerase; PREmR24, pH-responsive RNA element; int2, integrases; RiboJ, ribozyme-based insulator; ednaQ, dnaQ mutant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reactive oxygen species scavengers SodB/katE to improve growth robustness and lysine productivity of *E. coli* at low fermentation pH. The resulting strain grew best and produced the highest L-lysine titers at pH 6.0, instead of neutral pH as for the parent strain. Besides, the modulation of intracellular accumulation of stress-buffering metabolites such as proline, glutathione, betaine, trehalose, and mycothiol, is also found to facilitate the repair of damaged cells and contributes to the performance of industrial strains under stressful conditions (Liu et al., 2021).

5.3. Strain improvement via intelligent microbial cell factories

Advances in synthetic biology offer innovative approaches for constructing robust microbial strains with higher tolerance and responsiveness to external stress factors (Han et al., 2023; Meng and Ellis, 2020). The concept of smart microbial engineering, integrating high-quality biosensors and complex genetic circuits, has been proposed for sensing different stress signals and automatically adjusting unfavorable metabolic states to designed characteristics without artificial interventions (Gao et al., 2019b). To address this, Pham et al. (2017) developed an engineered pH-sensitive riboswitch, which autonomously regulated the expression of an error-prone mutant of *dnaQ* encoding the exonuclease proofreading subunit of the DNA polymerase III holoenzyme. This pH-sensing system enabled self-directed evolution and enrichment of acid-tolerant phenotypes in *E. coli*, and two acid-tolerant mutants with improved growth rates were obtained after only three rounds of experimental evolution. Overall, despite challenges still persist in designing and constructing intelligent microorganisms, their capacity for intelligent response and adaptation to environmental stresses shows promise in alleviating cellular burdens and improving host fitness.

In addition to employing synthetic biology tools for the construction of intelligent microbial strains, recent years have seen a significant rise in interest in artificial intelligence (AI)-centered data-driven techniques to aid in the design of these desired intelligent microbial strains for bioproduction (Cho et al., 2022; Jang et al., 2022). AI techniques broaden the exploration of design possibilities while narrowing down potential variants for experimentation, thus increasing the likelihood of identifying optimal designs. For example, Yu et al. (2023b) utilized contrastive learning to enhance Enzyme Commission (EC) number prediction, while Li et al. (2022b) and Yu et al. (2023a) employed deep learning to predict enzyme kinetic parameters. These AI approaches could facilitate enzyme selection and pathway design, paving a way for innovative smart cell factory design. Furthermore, by leveraging mathematical frameworks such as machine learning or deep learning, adjustments to microbial robustness may be made swiftly and precisely, circumventing the necessity to consider a mechanistic understanding of

the biological system (Xu et al., 2024a). For example, machine learning analysis of multi-omics data could yield valuable insights into cellular functions and facilitate the development of predictive models for cell behaviors. Radivojević et al. (2020) introduced a machine learning Automated Recommendation Tool, named ART, for guiding the bioengineering process, which allows for the prediction of microbial strain performance and providing recommendations for the next engineering cycle.

6. Harnessing microbial consortia to reduce the metabolic burden

Although substantial progresses have been achieved in the engineering of single microbial species for the efficient production of value-added compounds, inherent limitations persist within these single cultures (Ibrahim et al., 2021). For instance, the incorporation of long or complex metabolic pathways into a single strain and the utilization of mixed or complex substrates (e.g., lignocellulosic feedstock) remain challenging. The cellular microenvironment of a mono-culture system cannot ensure the expression of all metabolite-producing enzymes, and there is mutual interference among different biological modules. As the fields of synthetic biology have advanced, microbial consortia with well-defined interactions holds great promise for overcoming the constraints of the mono-cultures owing to the division of labor and the exchange of cellular resources (Li et al., 2022c; Wang et al., 2020a; Zhao et al., 2023) (Fig. 6). It has been demonstrated to possess advantageous properties, such as modularity, high robustness against perturbations, efficient task allocation, all of which contribute to more effective metabolic tasks in bioprocessing when compared to mono-cultures.

6.1. *Metabolic division of labor reduces the burden on a single host strain*

Division of labor is an important feature of microbial consortia, whereby distinct populations perform different but complementary metabolic tasks. This feature offers a promising alternative for reducing the metabolic burden at a single-cell level (Roell et al., 2019; Tsoi et al., 2019; Tsoi et al., 2018). On the one hand, it allows different microorganisms to specialize and perform advanced functions that are difficult or potentially impossible for monocultures, such as the effective utilization of lignocellulosic biomass. On the other hand, it lowers overall complexity by dividing the entire metabolic process across multiple populations, thus alleviating the metabolic burden imposed on each individual strain.

Tsoi et al. (2018) formulated general criteria defining the conditions favoring the division of labor. They demonstrated that engineered constructs with high enzyme expression, toxic intermediates/products, complicated pathways or large amounts of extracellular steps were more suitable for microbial consortia. Exploiting design principles of burden division, microbial consortia have been applied extensively in biotechnology (McCarty and Ledesma-Amaro, 2019; Qian et al., 2020). An example is the design of a three-species microbial consortium for power generation (Liu et al., 2017b), in which *E. coli* was engineered to convert glucose to lactate for *Shewanella oneidensis* as a carbon source and an electron donor, *B. subtilis* was engineered to convert glucose to riboflavin as an electron shuttle, and *S. oneidensis* was selected as the exoelectrogen to generate electricity and provide another potential carbon source for *E. coli* and *B. subtilis* along with the oxidation of lactate to acetate. The three-species consortium displayed impressive collective performance, demonstrating high levels of electricity generation and exceptional functional robustness. Taken together, the division-of-labor-based cooperation and mutualistic interactions within consortia significantly improved the performance by considerably reduced burdens that would otherwise will be imposed on a single chassis.

Many pathway enzymes exhibit promiscuous activities towards substrates with closely similar structures (Singla and Bhardwaj, 2020; Xu et al., 2020a). It is challenging to improve the catalytic performance and substrate specificity of industrial enzymes by rational protein engineering (Li et al., 2020a). However, the co-culture strategy offers an effective method to addressing this issue by assembling promiscuous reactions to construct multi-step metabolic pathways across different engineered strains. For instance, Brooks et al. (2023) proposed a tripartite microbial co-culture system to alleviate the high metabolic burden and enzyme promiscuity associated with the phenylpropene pathway. This enabled the efficient production of diverse

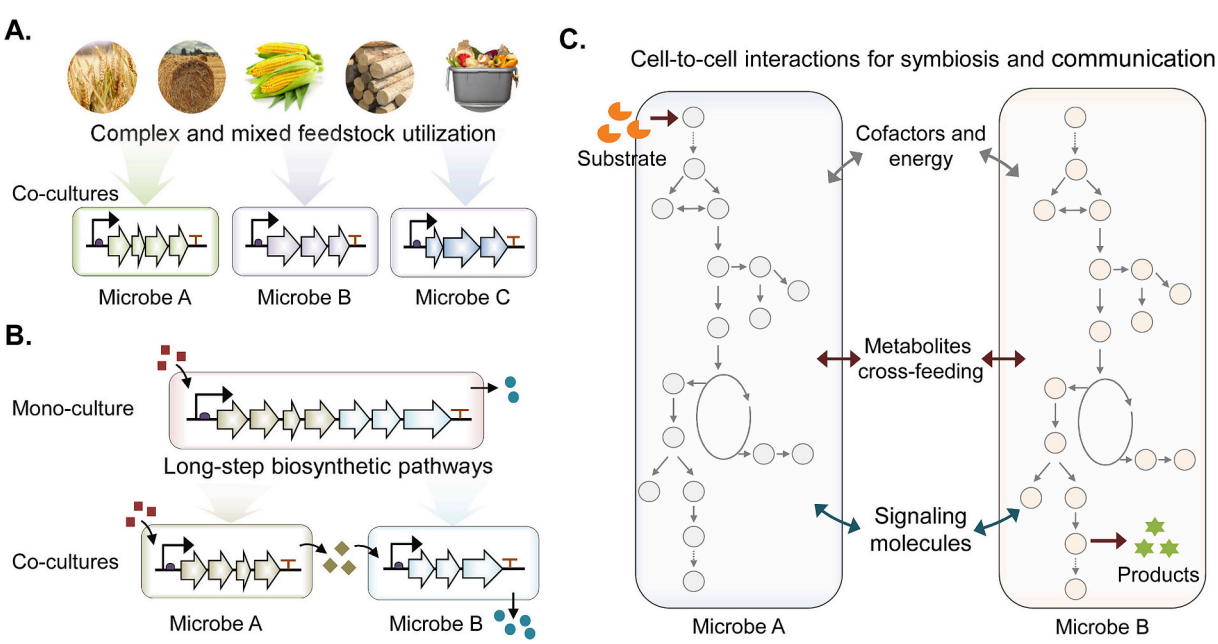

Fig. 6. Microbial consortia-based division of labor for reducing metabolic burdens. (A) Engineering co-cultures enables the utilization of complex and mixed substrates for bioproduction via functional specialization among community members. (B) Division of long-step biosynthesis pathways into multiple strains is conducive to the reduction of metabolic burdens imposed on each constituent strain. (C) Cell-to-cell interactions contribute to minimize metabolic burdens by the exchange of host cell resources, such as metabolites, cofactors, energy and signal molecules.

phenylpropenes by strategically dividing the pathway into distinct modules, thereby bypassing promiscuous enzymes and allowing the seamless interchangeability of upstream enzymes.

6.2. Engineering and modulation of robust microbial consortia

As an emerging technology, creating microbial consortia with defined behaviors still face challenges, such as strain stability, species compatibility, and population ratios. To obtain robust microbial consortia, many important principles have been proposed for modulating intercellular interactions and coordination (Gupta et al., 2020; Li et al., 2022c; Sgobba and Wendisch, 2020). For one example, the improved resource partitioning and functional flexibility have been shown to facilitate the maintenance of microbial diversity by reducing interspecific competition for resources. Designing metabolic symbiosis and cell-to-cell communication are valuable ways to ensure and stabilize coexistence among different populations. Programming spatial organization enables three-dimensional multicellular morphologies, which can physically separate populations into specific micro-environments.

Of the above strategies, quorum sensing modules for population-level control have been used extensively to construct microbial consortia with defined behaviors (McCarty and Ledesma-Amaro, 2019; Tsoi et al., 2019). Chen et al. (2015b) designed a synthetic microbial consortium consisting of activator and repressor strains, in which two orthogonal intercellular signaling molecules regulated gene expression within synthetic circuits spanning both strains. Upon co-cultivation, the two strains engendered coupled feedback loops at the population level, manifesting heightened robustness to perturbations compared to a single-strain oscillator. In addition, several computational models, including dynamic, stoichiometric, and agent-based models, can be very useful for designing and predicting behaviors of microbial consortia (Qian et al., 2020). By using these models, engineers can obtain a better understanding of expression patterns, cellular morphologies, and physical interactions between each consortium member.

Although there have been some advancements in engineering microbial consortia for bioprocessing, the industrial application of these consortia still face numerous unknowns and challenges (Duncker et al., 2021; Qian et al., 2020; Roell et al., 2019). These challenges include ensuring the stability of co-cultures, controlling population dynamics, managing the limited time-window for stable production, optimizing the growth of microbial communities, addressing intermediate metabolite dilution, and other related issues. Enhancing our understanding of how various environmental factors influence the composition and stability of microbial communities is the key to construct and control microbial consortia. In this regard, a toolkit has been developed to study the critical interactions for stability and dynamic control of synthetic yeast communities (Peng et al., 2024). With the development of more toolkits and the accumulation of knowledge from these endeavors, it is expected microbial consortia can be harnessed to create more efficient bioprocesses.

7. Conclusions and perspectives

Metabolic burden is considered an important determinant of the decline in both growth rate and product formation by engineered microorganisms. The vigorous development of synthetic biology and systems metabolic engineering has opened a new avenue for overcoming metabolic burdens, allowing the construction of microbial cell factories with superior efficiency and predictability. Nevertheless, the complete elimination of metabolic burden remains an extensive challenge. To achieve this goal, researchers should fully consider the implications of diverse metabolic burdens at the initial stage of rational strain design. The approaches encompassing genome-scale modeling frameworks and in silico simulation algorithms offer promise in bypassing metabolic burdens and achieving outstanding performances (Faure et al., 2023; Hasibi et al., 2024). Additionally, the advancements in artificial intelligence such as machine learning pave a novel road to precisely predict the outcomes of microbial metabolism by utilizing an amount of "stress-output" training datasets (Patra et al., 2023).

Cell-to-cell variability poses a considerable challenge for developing efficient cell factories with improved robustness and productivity (Mu and Zhang, 2023; Olsson et al., 2022; Wehrs et al., 2019). Cell-to-cell variations are presumed to cause lower yields and reduce bioprocess stability, due to the existence of low-producing subpopulations. However, this heterogeneity also confers adaptive advantages, enabling the populations with diverse phenotypes to thrive in rapidly changing environments within natural habitats. Accordingly, minimizing phenotypic heterogeneity promises to achieve robust and high-yield bioprocesses. Many strategies and tools have been found to be effective in developing microbial cells with uniform behavior, for example strain engineering via promoter, plasmid, and transporter modifications, cell lysis, or physiological manipulations (Binder et al., 2017; Diao et al., 2021). However, efficiently shifting heterogeneous populations towards homogeneity remains a daunting task. Hence, future efforts should be dedicated to uncovering the underlying mechanisms that contribute to phenotypic heterogeneity, and further expanding the existing toolkit to mitigate cell-to-cell variations. In many instances, the engineering of phenotypic homogeneity will foster stable and predictable bioprocesses, ultimately enhancing overall robustness and productivity.

The design of a universal burden-driven feedback controller is also of increasing interest for the development of robust cell factories (Barajas and Del Vecchio, 2022; Ceroni et al., 2018). This innovative approach can empower the engineered cells to dynamically respond to a diverse array of burden-causing stimuli, thereby enhancing their adaptability and overall performance. Through integrative analysis of multi-omics data coupled with machine-learning techniques, it will allow synthetic biologists to find more standard biological parts that show a universal and striking response to diverse metabolic burdens across various biological systems. Undoubtedly, this burden-compensating system offers a novel alternative for dynamically ameliorating cellular fitness, especially in the context of fluctuating environmental conditions commonly encountered in scaled-up microbial fermentation.

The integration of artificial intelligence (AI) into synthetic biology presents great opportunities to improve metabolic robustness of microbial cell factories (Goshisht, 2024; Kim et al., 2020; Patra et al., 2023; Zhou et al., 2022). By employing AI-driven metabolic modeling and prediction techniques, researchers can pinpoint potential bottlenecks and optimize pathways to alleviate metabolic burdens. AI algorithms can explore vast design spaces to develop genetic circuits and pathways that are more efficient and robust across varying conditions. Furthermore, AI can be harnessed to design better synthetic biology tools, such as CRISPR-based gene editing systems or high-throughput screening platforms, thereby augmenting the precision and efficiency in genetic engineering efforts (Dixit et al., 2024). Additionally, AI integration with multi-omics furnishes a comprehensive understanding of cellular metabolism, facilitating the identification and mitigation of metabolic bottlenecks (Patra et al., 2023). Overall, the attempts of combining synthetic biology and AI promises to overcome challenges associated with metabolic burden engineering, ultimately leading to more predictable and efficient biosystems design in the future.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Data availability

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

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