Metabolic burden: cornerstones in synthetic biology and metabolic engineering applications

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Abstract

Engineering cell metabolism for bio-production not only consumes building blocks and energy molecules (e.g., ATP) but also triggers energetic inefficiency inside the cell. The metabolic burdens on microbial workhorses lead to undesirable physiological changes, placing hidden constraints on host productivity. We discuss cell physiological responses to metabolic burdens, as well as strategies to identify and resolve the carbon and energy burden problems, including metabolic balancing, enhancing respiration, dynamic regulatory systems, chromosomal engineering, decoupling cell growth with production phases, and co-utilization of nutrient resources. To design robust strains with high chances of success in industrial settings, novel genome-scale models (GSMs), ^{13}C -metabolic flux analysis (MFA), and machine learning approaches are needed for weighting, standardizing and predicting metabolic costs.

Key words: ^{13}C -MFA, genome-scale model, machine learning, chromosomal engineering

Opportunities and challenges in synthetic biology (SynBio) applications

Metabolic engineering has created diverse microbial cell factories for applications in food, pharmaceutical, biofuel, and commodity chemical synthesis. Initially, industrial microbes were developed by random mutations or by expressing only one or two new enzymes. In the past decade, new gene sequencing/synthesis/editing techniques have allowed for complex genetic manipulations to build new cellular functions, and the International Genetically Engineered Machine (iGEM) competition promoted the concept of using standard modules (e.g., synthetic pathway and genetic circuits) for facilitating bio-production [1,2]. Despite great technological advances, engineered microbial platforms cannot cheaply manufacture products due to poor production titer, rate and yield [3,4]. Moreover, engineered microbes often show unpredictable or unstable physiology, and thus iterative "build-design-test-learn" cycles have to be employed for strain improvement [5]. In March 2015, representatives from industry, academic institutions, and NIST addressed a key issue preventing synthetic biology from reaching its potential: “*Unlike silicon-based electronic devices, synthetic organisms assembled from genetic components do not always have predictable properties*” [6].

When a complex pathway is introduced into the host but shows low performance, one perspective is that the new pathway has "intermediate toxicity" or "low enzyme activity". Therefore, strain improvement often focuses on searching for "bottleneck" steps and tuning enzyme functions. Another possibility for low productivity in engineered strains is metabolic burden, a long-standing problem in biotechnology. **Metabolic burden** (see Glossary) is defined as the portion of a host cell’s resources — either energy molecules (e.g., NAD(P)H and ATP) or carbon building blocks— that is used to construct and operate engineered pathways [7]. Since intracellular carbon and energy resource distribution in healthy cells have been “optimized” by

natural evolution, hijacking cell resources for pathway overexpression, plasmid maintenance, and product synthesis may upset normal cellular processes.

Metabolic engineers first noticed metabolic burden. With the advance of recombinant DNA technology in the 1970s and 1980s, overexpression of proteins to produce desired products has been attempted, which often results in reduced cell growth and increased mutation rates [7-9]. Metabolic burden could also cause inefficiency in the cell ATP supply, affecting broad cellular functions. A typical example is that *Azotobacter vinelandii* transformed with plasmids gradually lost its ATP-dependent N₂ fixation ability when the plasmid copy number increased [7,10]. In yet another case, *Pseudomonas putida* completely lost its ability to synthesize siderophores after transformation with high copy plasmids [11]. Therefore, metabolic burden is a key factor leading to undesired physiological changes, and the cost/benefit of synthetic biology strategies needs to be evaluated under the light of metabolic burdens.

A “cliff” of host productivity under metabolic burden

ATP powers all cellular functions and cells must produce sufficient ATP for both biosynthesis requirements and cellular maintenance. However, biological/physical factors restrict the capabilities of cellular energy metabolism [12]. For example, the ratio of cell membrane surface area to cell volume influences the overall mass exchange efficiency and the upper limit of nutrient and oxygen uptake rates [13]. For *E. coli* cultures [14], upper bound glucose uptake rates are ~18 mmol/gDW/h (gDW: grams of dry weight) in anaerobic conditions and 11 mmol/gDW/h in aerobic conditions with 5.3 ATP molecules per glucose consumed formed in aerobic conditions compared with 1.96 ATP per glucose in anaerobic conditions. The nutrient uptake places a hard constraint on cell catabolic rates. Moreover, ATP synthesis is a

thermodynamically inefficient process (part of the substrate energy will be lost as heat) [15]. Under optimal conditions, oxidation of one NADH generates 2.5 ATP (i.e., P/O=2.5) [15]. For engineered strains, proton gradients are dissipated through the cell membrane instead of charging ATP synthase, causing poor **P/O ratios** (<2) [16-18]. Therefore, cell physiology and bio-production are particularly sensitive to metabolic burden from ATP consumption.

To demonstrate the highly nonlinear impact of energy burden on biosynthesis, **flux balance analysis (FBA)** was used with a constraint-based model to simulate the adverse impacts of ATP metabolism on *E. coli* fatty acid, isobutanol and acetate yields (Figure 1) [19]. The simulations showed that native cell metabolism can withstand a certain amount of ATP loss without having apparent biosynthesis deficiency (i.e., forming a “plateau”). If cell metabolism demands more ATP, cells can utilize respiration (increase oxygen influx) to maintain their well-being. When catabolism and respiration become insufficient to accommodate the further increase of ATP expenditure, the biosynthesis yield will suddenly drop, forming a “cliff” towards the “death valley” (production yields drop to minimal levels). When a strain's metabolic status is on the “cliff”, it may show unstable performance and become highly sensitive to suboptimal growth conditions (such as low oxygen level). Moreover, three products show differences in the yield plateau phenomenon. Fatty acid synthesis consumes both NADPH and ATP. Cell productivity is sensitive to oxygen supply, maintenance loss, and P/O ratios (Figure 1A and 1B). Isobutanol synthesis only consumes NAD(P)H, and it can be produced in the non-growth phase due to competition with amino acid biosynthesis. Therefore, the area of the "death valley" in Figure 1C and 1D (showing isobutanol production) is reduced compared with fatty acid production, suggesting isobutanol has a higher chance of stable industrial production. Acetate synthesis has

net ATP generation; its production has the largest yield plateau and thus is more likely to achieve robust production.

Use of flux analysis to precisely measure metabolic burdens

Among systems biology tools, fluxomic measurements can provide direct knowledge of metabolic conversion of carbon sources into products. It is the only tool to understand the allocations of cell resources during biosynthesis. FBA uses the stoichiometry of biochemical reactions in addition to the measurement of inflow (uptake) fluxes to predict cellular phenotypes and biosynthesis yields. FBA can characterize cell energy metabolism by dividing maintenance costs into two categories [18]: **non-growth associated maintenance (NGAM)** by “resting” cells, and **growth associated maintenance (GAM)**. Due to the size and underdetermined nature of metabolic networks, FBA has a high degree of uncertainty and requires constraints to analyze the solution spaces. ^{13}C tracing can assist FBA to rigorously measure functional pathways throughout the metabolic network (which is called **^{13}C -metabolic flux analysis**, or ^{13}C -MFA). ^{13}C -MFA can validate the function of genes or genetic circuits, provide knowledge on bottleneck nutrient sources, identify metabolic engineering targets, and directly quantify energy flows (i.e., ATP and cofactor generations and consumptions). ^{13}C -MFA is widely used to identify specific flux changes in the network of such mutants.

During pathway engineering, cell resource over-expenditure for heterologous enzyme synthesis can trigger the re-organization of carbon fluxes and reduce cell well-being [20]. To identify “the straw that broke the camel’s back,” ^{13}C -MFA in combination with transcriptomics/proteomics and genome-scale modeling can provide a comprehensive understanding of cell responses to metabolic burdens at different cellular levels (from

transcriptome to fluxome). ^{13}C -MFA is particularly useful to link these synthetic biology components (such as transcriptional regulators) to cell functional outputs. In general, gene expression may have an unpredictable impact on the distribution of fluxes because central metabolic fluxes are rarely regulated at the expression level alone [21]. For example, ^{13}C -MFA and transcriptional analysis for fatty acid production in *E. coli* using overexpression of the free fatty acid pathway and fatty acid transcription regulator revealed complex fluxome responses to fatty acid overproduction, including high ATP maintenance loss, decreased acetate flux, enhanced NADPH producing pathway (pentose phosphate pathway and Entner-Doudoroff pathway), and strong transhydrogenation activity to achieve cofactor balance [22].

In the synthetic biology field, ^{13}C -MFA has not become a routine tool because of two bottlenecks. First, ^{13}C -MFA based on amino acid labeling cannot probe cell metabolism in a rich medium, for non-growing cells, or through a genome-scale metabolic network. To resolve such problems, innovative genome-scale MFA and isotopically nonstationary MFA will assist synthetic biology to gain fast snapshots of cell metabolism for improving strain performance [23-25]. However, the time required to analyze the samples and process the data to calculate fluxes through complex metabolism is still long (often taking months for an experienced lab to quantify cell metabolism correctly), and this method cannot satisfy a fast turnover cycle during build-design-test-learn. Second, ^{13}C -MFA focuses on global fluxes rather than the activity of individual enzymes. Many synthetic biology hosts only achieve productivity at mg/L levels, and the priority is thought to improve the heterologous enzyme activities in the production pathway. From this aspect, ^{13}C -MFA is useful when the strain performance is close to industrial performance levels.

Synthetic biology methods to overcome metabolic burden

In traditional fermentation engineering, metabolic burden can be minimized through well-controlled bioreactor conditions (such as pH and oxygen) as well as the use of different media (including yeast extract or other intermediates to boost cell metabolism). Early metabolic engineers also relied on random mutations or adaptive evolution for improved synthesis of growth-associated products, leveraging natural selection for reducing metabolic burden. For example, *E. coli* strain can be "trained" to have fast growth using lactate: over 250 generations of evolution, its pathway capacity can be improved, especially for these flexible metabolic nodes (such as phosphoenolpyruvate, oxaloacetate, and acetyl-coenzyme A) [26]. In addition, co-cultures can also be a possible solution that divides a biosynthesis pathway into two sections then use two species to share the metabolic burdens.

When synthetic biology tools became available, they allowed more elegant strategies to control cell metabolism at different cellular levels to reduce metabolic burden. Transcriptional regulators have attracted interest since alteration of some regulators may alleviate metabolic repressions and unlock cell carbon and energy fluxes [27]. Catabolite repression is present in various microorganisms and influences carbon, energy and cofactor metabolism in response to carbon sources. For example, glucose is the preferred carbon source in *E. coli*. Deletion of catabolite repression regulators and overexpression of genes required for pentose metabolism allows cell fast growth using glucose and xylose simultaneously [28]. Moreover, Smolke and Keasling studied the effect of mRNA stability and DNA copy number on protein production and discovered the correlations among DNA, mRNA and protein levels are strongly nonlinear [29]. Their results indicate that optimally tuning the plasmid copy number and mRNA processing is essential for effective pathway construction. Currently, synthetic biology has already offered broad strategies to reduce energy burdens. Table 1 shows the common strategies to reduce

metabolic burden and balance cell bio-production, including copy number optimization, transcriptional optimization, translational optimization, post-translational optimization, dynamic balancing, compartmentalization, and co-culture strain ratio optimization.

In synthetic biology, transcriptional/protein-level regulation of cell production has become a focal point. Among these strategies, **dynamic sensors and regulatory systems (DSRSs)** employ a biosensor to detect the level of metabolite intermediate and control enzyme expressions, which may be useful to prevent the biosynthesis of unnecessary RNAs/proteins/metabolites and increase the efficiencies of energy and carbon usage [56,57,61]. Unlike static control, DSRSs can promote or repress pathways according to cell growth conditions or intermediate metabolite concentrations. Thereby, DSRSs may also be used to decouple cell biomass growth and production phases (e.g., quorum sensing) so that cell resources can be focused on one major task at a time. Recently, CRISPR systems have been implemented for both chromosomal engineering and systematic down-regulation of gene expression [62] (drawbacks of plasmid engineering include instability and burdens due to DNA/enzyme synthesis). Over the long term, synthetic biology may re-program the entire cell genome to create a “minimal or smart” cell of best energy fitness [63-65].

Promotion of metabolic capability in microbial chassis

Synthetic biology has attempted to reduce metabolic burden by removing unnecessary genes from microbial chassis. However, such efforts did not show significant benefits. For example, genome reduction of 332 dispensable genes in *Bacillus subtilis* does not affect the cell's fluxome [66]. This result is consistent with the fact that the DNA synthesis accounts for a very small cell resource usage. In contrast, cell energy metabolism has shown thermodynamic

constraints to both biomass growth and bio-productivity, which are often limited and difficult to improve. For heterotrophic microbes, ATP generation is coupled with cell catabolism and the outputs from ATP synthase is limited by the cell membrane space. In addition, synthetic biology components may interfere with the proton motive force and reduce ATP generation capabilities [67]. Therefore, improving intracellular energy generation and catabolic metabolism allow the hosts to carry more synthetic biology modules. Three directions can be pursued.

First, ATP is mainly synthesized through oxidative phosphorylation in aerobic metabolism. However, respiration rates in many strains are far below theoretical maxima in bioreactors. The respiration efficiency can be successfully increased using the *Vitreoscilla* hemoglobin, which was first recombinantly expressed in *E. coli* [68]. This enzyme can improve oxygen membrane transfer and the P/O ratio and reduce waste byproducts. Moreover, Zamboni and Sauer enhanced riboflavin production in *Bacillus subtilis* by knockout of cytochrome *bd* oxidase to reduce cell ATP maintenance costs [69]. Engineering of respiration may apply to species with several sets of respiratory chains with different efficiencies [70,71] and successful application of this strategy is also closely related to other factors, such as oxygen concentration and medium composition [72].

Second, photosynthetic microorganisms convert CO₂ to useful products with light as the energy source [73]. However, the photoautotrophic process has been hindered by CO₂ and light availability inside of photobioreactors, which leads to low cell density and cost-inefficient harvesting. To circumvent these problems, a photomixotrophic strategy is advantageous [74]. Under conditions of sufficient light and glucose, microalgae can consume both CO₂ and glucose for biomass production, which potentially leads to higher biomass density. The ability to utilize light as an energy source can be enabled for non-photosynthetic species through heterologous

expression of proteorhodopsins [75-77]. Proteorhodopsins are a class of membrane proteins distributed in a considerable number of species ranging from bacteria to archaea and fungi [78]. They can absorb energy from light of different wavelengths and generate a proton gradient used for ATP synthesis. Compared with photosystems, a distinctive advantage for proteorhodopsin is its simplicity for heterologous photosynthesis [79]: the function of proteorhodopsin relies on a single protein with 249 amino acids. Hence, the metabolic costs associated with heterologous expression of proteorhodopsins will be much smaller than intact photosystems. An added potential benefit is that species with proteorhodopsin have demonstrated increased tolerance to environmental stress [75,77].

Third, H₂ can be utilized by a broad range of microbes. However, using hydrogen as the energy supplier in industrial fermentations is not preferred. This is due to various undesirable properties of H₂ such as its low solubility, volatility, and explosiveness. Compared with H₂, formate is a better source of energy supply in terms of uptake efficiency. The utilization of formate by microbes as an extra energy source was reported for *Candida utilis* that can uptake formate in the presence of glucose to increase biomass yield [80]. Meanwhile, similar phenomena were observed in *Hansenula polymorpha* and *Pichia pastoris* [81,82]. The strategy of employing formate as an extra energy source has been extended to other species that have a formate dehydrogenase (*fdh*), such as oleaginous yeasts for improving lipid production [83], *Penicillium chrysogenum* for enhancing penicillin productivity [84], and *Bacillus thuringiensis* for promoting thuringiensin yield [85]. Further, formate can be generated through an electrochemical process to feed engineered *Ralstonia eutropha* to produce isobutanol and 3-methyl-1-butanol [86].

On the other side, heterologous expression of an *fdh* gene enables formate usage in hosts that lack the ability to oxidize formate. For instance, after chromosomal insertion of the *fdh* gene, *C. glutamicum* was able to utilize formate and produce 20% more succinate anaerobically in the presence of glucose (formate was used as both NADH and carbon donor) [87]. In yet another case, *fdh* was introduced into a succinate-producing *E. coli* strain, leading to reduced formate and improved succinate yield [88].

Current metabolic modeling approach for rational metabolic engineering

Metabolic burden has a direct affect on the biochemical productivity of engineered strains and thus, it will be important to explicitly consider metabolic burden during the design process. Numerous good review papers are available to cover different tools and conceptual approaches to model-driven metabolic engineering design (Box 1) including a review by Medema *et al.* [89] that presents *de novo* design as involving six steps. The steps include: 1) Pathway prediction (*de novo* discovery of possible biosynthetic routes), 2) Pathway prioritization (ranking of possible pathways), 3) Metabolic modeling (modeling biochemical function in network context), 4) Pathway selection, 5) Pathway refactoring and integration (molecular-level design specification of necessary DNA constructs), and 6) Product synthesis. These design approaches primarily focus on specifying biochemical pathway usage necessary to produce theoretical yields of a desired product, but experimental strains fall short of theoretical yields due to unaccounted *in vivo* considerations such as overall metabolic burden.

Cell-wide genome-scale models for complex cell systems

Genome-scale models (GSMs) are stoichiometric representations of the biochemical capabilities of a metabolic network based upon the contents of an organism's genome. A key to this approach is establishing gene-protein-reaction (GPR) relationships where identifying a gene in the genome implies the possibility of the associated protein and biochemical reaction. However, if details associated with the molecular aspects of moving from DNA to mRNA and mature protein are absent (as is the case with basic GSMs) then these models allow for any/all reactions to be used as much as needed to fulfill an objective with no restriction meaning that there is no explicit cost to expressing a gene.

A substantial amount of work to improve GSMs has been undertaken recently to account for this shortcoming allowing for computational consideration of metabolic costs/burden. Beginning with a careful curation of literature associated with *E. coli*, an expression matrix (E-Matrix) was developed that included 13,694 reactions associated with all facets of transcription and translation [95] to account for synthesis reactions for DNA, mRNA, proteins, and protein complexes in a sequence-specific manner. The E-Matrix formalism was directly integrated with metabolic networks to create ME (metabolism and expression) models of *Thermotoga gammatima* [96] and *Escherichia coli* [97]. The ME models involved extensive attention to molecular detail and formulation of new objective functions for simulations and resulted in improved predictive capabilities including the ability to predict *in silico* transcriptomes and proteomes [98].

In addition to GSMs, a whole-cell model of *Mycoplasma genitalium* has been generated using 28 modularized subsystems [99]. The approach of this work differed from the development of ME models: in the case of the whole cell *Mycoplasma* model, different modeling approaches (e.g. ordinary differential equations, Boolean statements, probabilistic, constraint-based) were implemented for the different modules to allow for flexibility to dynamically model each process

in an appropriate way. By developing and integrating modules, it was possible to simulate dynamic whole cell function at a high level of detail. Subsequent to this initial publication, the WholeCellKB database has been established to facilitate development of additional whole cell models using the modularized approach implemented for *Mycoplasma* [100].

Integration of metabolic burdens of pathway engineering in GSMs

As metabolic engineering design strategies become more sophisticated and involve implementation of larger DNA constructs, it has become increasingly important to consider the implications of the metabolic burden imposed by expression of synthetic constructs. The approaches discussed above are possible frameworks for implementing a new level of modeling detail that can account for metabolic burden associated with expression and activity of proteins. Improvements to computational modeling frameworks to explicitly include metabolic burden considerations should greatly facilitate metabolic engineering design strategies and help avoid strategies that impose a large non-native metabolic burden. For example, mutants may have decreased productivity due to ATP loss associated with heterologous gene expression. Previous studies have shown that protein synthesis consumes >60% of the ATP from cell energy metabolism [101], while protein overexpression is a major metabolic burden in engineered cells [7]. Based upon experimental proteomic studies in *E. coli* [102], the energetic costs for a single protein synthesis can be calculated to range from about 400 – 50000 in terms of high-energy phosphate bond equivalents. The average cost per amino acid ranged from 18 – 38 high-energy phosphate bond equivalents. The unit of phosphate bond equivalents can be related to cellular energy as stored in the phosphate bonds of ATP. Considering protein synthesis costs in terms of phosphate bonds enables a new level of energetic-associated analysis within the framework of GSMs that can predict overall metabolic burden. By explicitly considering the protein production

cost and amino acid composition of individual proteins, metabolic burden associated with enzymes (and their affiliated biochemical reaction) can be analyzed.

The use of phosphate bond costs associated with each amino acid provides the means for connecting computational simulations with experimental ^{13}C -MFA results related to ATP generation and consumption. In addition, FBA may be used to calculate metabolic burdens from synthesis of plasmid DNA (based on sizes and copy numbers). Moreover, ^{13}C -MFA can be combined to offer comprehensive insights into the intracellular activities responding to the increase in corresponding metabolic reactions [103]. Traditional FBA models lack a precise determination of ATP loss, while ^{13}C -MFA can provide a quantitative measurement of ATP losses: the ATP maintenance is a sum of the all of the burden in the cell for all the different metabolic functions (protein expression, energy consumption associated with individual reactions, etc.), and thus is a cumulative result of explicitly accounting for burden in individual components of the FBA model. Lastly, RNA profiles can be used with the genome-scale FBA modeling components to provide weighting to expression costs. The integration of high throughput transcriptomics with ^{13}C -MFA and metabolic models can also be used for analysis of regulatory networks (such as synthetic biology circuits).

Innovative models for assisting synthetic biology applications

First, integrating FBA with process models is important to study scale-up fermentations because large-bioreactors are often dynamic and heterogeneous. These multi-scale models can offer knowledge of intracellular function and overall fermentation performance. For example, dynamic FBA (a combination of kinetic models and FBA) simulates the industrial fermentation of *E. coli* to produce recombinant proteins [19]. The hybrid of constrained FBA models with agent-based model successfully simulate highly heterogeneous systems (e.g., biofilm) [104].

Integration of FBA with other information or models will be widely adopted to strengthen the capability of modeling tools and to provide guidance when moving laboratory strains to industrial applications [105].

Second, industrial standards play fundamental roles in research & development and communication within their respective discipline. In the case of synthetic biology, lacking a standard for journal publications hinders fast communication among synthetic biology researchers as well as reproducibility of published work. Thereby, we want to emphasize standardization of publications. For example, the published papers need to clarify their reports on: 1) genetic methodologies; 2) fermentation conditions such as substrate type, incubation mode, nutrient condition, etc.; 3) production titer, rate and yield. For these high profile papers, tracer experiments should be employed to make sure the engineered pathways are actually functional.

Third, data-driven models using published synthetic biology papers can provide complementary information to mechanistic based models. For example, a linear empirical model with numerical and categorical variables was generated to predict production yields based on dozens of published papers associated to *E. coli* and *S. cerevisiae* [8,19]. That study identified key factors controlling production yields, including biosynthesis steps, metabolic engineering methods, nutrient supplementation, and fermentation conditions. The large number of papers published on metabolic engineering provides a rich database for performing **machine learning** studies and capture the microbial outputs (production titer, rate, yield) in response to genetic and fermentation conditions. Lessons from the past can allow us to better evaluate the synthetic biology projects. Those published papers can be stored as structural data so that machine learning methods could be applied to extract knowledge for future rational strain development.

Machine learning evolved from pattern recognition, statistics, and optimization, which makes data-driven predictions for outcomes of mutant physiologies [92]. It can assist mechanistic-based GSMs for rational metabolic engineering (Figure 2).

Conclusion

The long-held assumption of never-ending rapid growth in biotechnology in general and synthetic biology in particular has been recently questioned due to lack of substantial return of investment [107]. One of the main reasons of failures in synthetic biology is the largely unaccounted for metabolic burden that may offset synthetic biology benefits and thus careful consideration is necessary at the stage of strain design (see Outstanding Questions). To avoid wrong commercial decisions or investments in those “perpetual-motion-machine” types of projects, ^{13}C -MFA provides excellent measures and standards for synthetic biology that defines the *limitation of synthetic biology*, leading to rational metabolic or bioprocess engineering strategies to bypass metabolic burdens (e.g., use of co-cultures or integration of biological conversion with chemical conversions). More importantly, constructing new genome-scale modeling analyses and novel machine learning methods can give broad guidelines for strain development and resolve metabolic unbalancing from synthetic biology applications.

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387 **GLOSSARY**

388 **Cipher of Evolutionary Design (CiED)**: an FBA approach that relies on a genetic algorithm in
389 order to predict gene knockouts for improving fluxes through a metabolic pathway of interest.

390 **Dynamic sensor and regulatory system (DSRS)**: an approach that uses biosensors for
391 intracellular metabolites to regulate metabolic pathways.

392 **Flux balance analysis (FBA)**: a mathematical approach for deriving possible metabolic fluxes in
393 metabolic networks.

394 **Genome scale model (GSM)**: a metabolic network reconstructed based on genome-scale
395 annotations.

396 **Growth associated maintenance (GAM)**: the amount of ATP consumption associated with
397 biosynthesis.

398 **Machine learning**: a field of study that gives computers the ability to learn from a large amount
399 of experimental data without being explicitly programmed.

400 **Metabolic burden**: the portion of a host cell's resources — either energy molecules (e.g.,
401 NAD(P)H and ATP) or carbon building blocks — that is used to construct and operate
402 engineered pathways

403 **Metabolic flux analysis (MFA)**: an analytical method that uses isotopomers to measure in vivo
404 enzyme reaction rates.

405 **Non-growth associated maintenance (NGAM)**: the amount of ATP consumption that does not
406 contribute to biomass synthesis.

407 **OptForce, OptStrain series**: computational procedures that identify all possible engineering
408 interventions in the metabolic model depending upon whether their flux values must increase,
409 decrease or become equal to zero to meet a pre-specified overproduction target.

410 **Phosphate/Oxygen ratio (P/O ratio):** the amount of ATP produced by reduction of an oxygen
411 atom.

412

FIGURE CAPTIONS

Figure 1. Biosynthesis yields (g product/g substrate) in engineered *E. coli* in responses to P/O ratio, ATP burden from maintenance loss (mmol ATP /g dry cell weight/h), and O₂ uptake fluxes (mmol O₂ /g dry cell weight/h). A genome-scale metabolic model (iJO1366) is used for simulations [19].

Figure 2. A new modeling paradigm for synthetic biology.

TABLE

Table 1. Summary of experimental metabolic balancing efforts.

Host	Product	Titer	Type of Pathway Optimization	Reference
<i>E. coli</i>	amorphadiene	293 mg/L/OD600	CN, TS	[30]
<i>E. coli</i>	amorphadiene	n.r.	CN, TS	[31]
<i>E. coli</i>	amorphadiene	1.6 g/L	PTL	[32]
<i>E. coli</i>	amorphadiene	3.6 g/L	TL	[33]
<i>S. cerevisiae</i>	amorphadiene	20 mg/L	CP	[34]
<i>S. cerevisiae</i>	valencene	1.5 mg/L	CP	[34]
<i>E. coli</i>	poly-3-hydroxybutyrate	70 % DCW	CN	[35]
<i>E. coli</i>	lycopene	11,000 p.p.m.	CN	[35]
<i>E. coli</i>	neurosporene	4.2 mg/gDCW	TS, TL	[36]
<i>E. coli</i>	mevalonate	740 mg/L	PTL	[37]
<i>E. coli</i>	glucaric acid	1.7 g/L	PTL	[37]
<i>E. coli</i>	glucaric acid	2.5 g/L	PTL	[38]
<i>E. coli</i>	glucaric acid	1.2 g/L	DB	[39]
<i>E. coli</i>	<i>cis,cis</i> muconic acid	2 g/L	SR	[40]
<i>E. coli</i>	<i>cis,cis</i> muconic acid	4.7 g/L	SR	[41]
<i>E. coli</i>	4-hydroxybenzoic acid	2.3 g/L	SR	[41]
<i>E. coli</i>	myo-inositol	1.31 g/L	DB	[42]
<i>E. coli</i>	riboflavin	2.70 g/L	TL	[43]
<i>A. nidulans</i>	penicillin	n.r.	CP	[44]

<i>S. cerevisiae</i>	xylose utilization	n.a.	TS	[45]
<i>E. coli</i>	chondroitin	2.4 g/L	TS	[46]
<i>E. coli</i>	violacein	1.83 g/L	TS	[47]
<i>S. cerevisiae</i>	violacein and derivatives	n.r.	TS	[48]
<i>E. coli</i>	(+)-catechin	911 mg/L	CN, PTL	[49]
<i>E. coli</i>	caffeic acid	3.8 g/L	TS	[50]
<i>E. coli</i>	caffeic acid	106 mg/L	CN	[51]
<i>E. coli</i>	resveratrol	35 mg/L	CN, TS	[52]
<i>E. coli and S. cerevisiae</i>	oxygenated taxanes	33 mg/L	SR	[53]
<i>E. coli</i>	taxadiene	1.02±0.08 g/L	CN, TS	[54]
<i>E. coli</i>	fatty acids	8.6 g/L	CN, TL	[55]
<i>E. coli</i>	fatty acids	3.9 g/L	DB	[56]
<i>E. coli</i>	fatty acid ethyl esters	1.5 g/L	DB	[57]
<i>E. coli</i>	butyrate	7.2 g/L	PTL	[58]
<i>E. coli</i>	butanol	6.2 g/L	SR	[59]
<i>S. cerevisiae</i>	isobutanol	635 mg/L	CP	[60]
<i>S. cerevisiae</i>	isopentanol	95 mg/L	CP	[60]
<i>S. cerevisiae</i>	2-methyl-1-butanol	118 mg/L	CP	[60]

CN, DNA copy number optimization; TS, transcriptional optimization; TL, translational optimization; PTL, post-translational optimization; DB, dynamic balancing; CP, compartmentalization; SR, co-culture strain ratio optimization; n.r., not reported; n.a., not applicable.

Box 1. Rational algorithmic design

In this overall framework, some of the most heavily developed work has focused on the metabolic modeling component that is directly tied to metabolic burden. Metabolic control analysis (MCA) is the earliest approach that quantified the correlations between the flux through a pathway and the kinetics of the constituent enzymes. MCA can describe the different levels of flux regulation through a single pathway, but not yet for the distribution of flux through the entire network [21]. On the other hand, genome-scale models (GSMs) used in conjunction with flux balance analysis constitute the most commonly used approach to estimate optimal growth rates and product yields from different feedstocks and the lethality of gene knockouts. Such GSMs can identify the best biosynthesis pathways through elementary mode analysis [90] or systematic pathway modifications (e.g. OptKnock [23,24] and **OptStrain** [25]).

A methodology for predicting optimal metabolic landscapes for the production of a desired metabolite termed Cipher of Evolutionary Design, **CiED** [91] was initially developed by integrating an evolutionary algorithm around constraint-based modeling, allowing one to simultaneously search an unlimited number of gene targets for modifications. Furthermore, another constraint-based modeling approach termed **OptForce** [91-94] was utilized to identify genetic perturbations leading to increases in intracellular malonyl-CoA. OptForce suggested the up-regulation of glycolytic reactions, namely glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase as well as up-regulation of pyruvate dehydrogenase and acetyl-CoA carboxylase. It also suggested down-regulation of reactions in the citric acid cycle, namely malate dehydrogenase, fumarase and aconitase in order to reduce the drain of carbon towards TCA cycle products. OptForce also suggested reducing the activity of TCA reactions instead of completely eliminating them.

451

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Figure 1

Figure 2