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Review Article

Bridging the Gap between Fluxomics and Industrial Biotechnology

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Metabolic flux analysis is a vital tool used to determine the ultimate output of cellular metabolism and thus detect biotechnologically relevant bottlenecks in productivity. ^{13}C-based metabolic flux analysis (^{13}C-MFA) and flux balance analysis (FBA) have many potential applications in biotechnology. However, noteworthy hurdles in fluxomics study are still present. First, several technical difficulties in both ^{13}C-MFA and FBA severely limit the scope of fluxomics findings and the applicability of obtained metabolic information. Second, the complexity of metabolic regulation poses a great challenge for precise prediction and analysis of metabolic networks, as there are gaps between fluxomics results and other omics studies. Third, despite identified metabolic bottlenecks or sources of host stress from product synthesis, it remains difficult to overcome inherent metabolic robustness or to efficiently import and express nonnative pathways. Fourth, product yields often decrease as the number of enzymatic steps increases. Such decrease in yield may not be caused by rate-limiting enzymes, but rather is accumulated through each enzymatic reaction. Fifth, a high-throughput fluxomics tool hasnot been developed for characterizing nonmodel microorganisms and maximizing their application in industrial biotechnology. Refining fluxomics tools and understanding these obstacles will improve our ability to engineer highlyefficient metabolic pathways in microbial hosts.

1. Introduction

Numerous chemical compounds, ranging from the antimalaria drug artemisinin [1] to the “biofuel” butanol [2, 3], have been produced with the aid of synthetic biology tools. The ability to efficiently synthesize natural or unnatural products requires a systems-level understanding of metabolism. Functional genomics tools such as genome sequencing, profiling of mRNA transcripts, and proteomics, are widely used to attain a comprehensive knowledge of how metabolic components (genes, proteins and metabolites) are regulated. In contrast to traditional omics tools, flux analysis (measurement of metabolite turnover rates) has become instrumental for physiological prediction and enzymatic rate quantification in metabolic networks [4]. This technology also allows for the identification of metabolic interactions and the knowledge-based design of cellular functions. As such, one can utilize this tool to rationally modify biological hosts and analyze global physiological changes resulting from genetic modifications.

Fluxomics, the cell-wide quantification of intracellular metabolite turnover rates, was first performed via flux balance analysis (FBA). This method uses the stoichiometry of the metabolic reactions in addition to a series of physical, chemical and biological characteristics (thermodynamics, energy balance, gene regulation, etc.) to constrain the feasible fluxes under a given objective function (e.g., maximal biomass production). FBA is an underdetermined model (the number of constraints is smaller than the number of reactions in the metabolic network), which may give unrealistic metabolic readout. In spite of this limitation, FBA provides a useful framework for predicting a wide variety of cellular metabolisms. A complementary approach, ^{13}C-based metabolic flux analysis (^{13}C-MFA) allows for precise determinations of metabolic status under a particular growth condition. The key to ^{13}C-MFA is isotopic labeling, whereby

microbes are cultured using a carbon source with a known distribution of ^{13}C. By tracing the transition path of the labeled atoms between metabolites in the biochemical network, one can quantitatively determine intracellular fluxes.

Flux analysis can not only provide genetic engineers with strategies for "rationally optimizing" a biological system, but also reveal novel enzymes useful for biotechnology applications [4]. However, flux analysis platforms are still not routinely established in biotechnology companies. This review paper addresses current developments and challenges in the field of fluxomics, which may guide future study to bridge the gap between systems analysis of cellular metabolism and application in biotechnology.

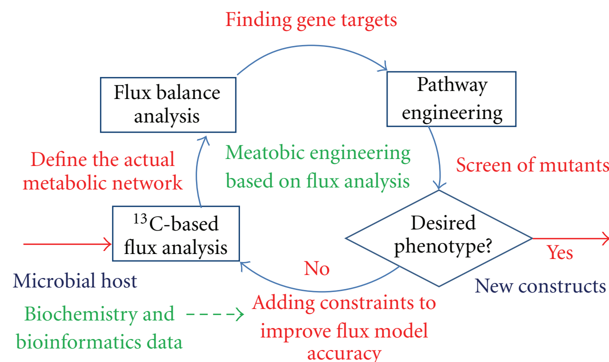

Figure 1: An iterative approach of fluxomic analysis and rational metabolic engineering.

2. Advances and Limitations in Metabolic Flux Analysis

2.1. Steady-State Flux Model. FBA and ^{13}C-MFA concentrate on the stoichiometric (rather than kinetic) properties of metabolic networks. FBA has been widely applied to predict cell growth rate, product yield using different feedstocks, lethality of gene knockouts, and advantageous pathway modifications [5]. Such a model provides general guidelines for metabolic engineering and thus is a viable first step towards improving biosynthetic yield [6]. The hallmark of large scale FBA is the constraint-based reconstruction and analysis toolbox (COBRA) [7], which provides a general platform for fluxomics studies.

A number of optimization algorithms and computational strategies for resolving *in silico* and *in vivo* inconsistencies have been proposed to improve the applicability of FBA [6, 8]. For example, incorporation of thermodynamic principles into FBA can constrain solution space (i.e., energy balance analysis) and obtain both stoichiometrically and thermodynamically feasible fluxes [9]. To describe the "nonoptimal" metabolic behaviors, FBA can use a bilevel optimization approach to estimate the potential trade-off between biomass accumulation and the yield of a desired product [10]. FBA can also relax the objective function for maximization of the biomass and apply a Minimization of Metabolic Adjustment Algorithm to solve fluxes in mutant strains [9]. Such an algorithm calculates fluxes by minimizing the difference between the wild-type flux distributions and the knockout-strain fluxes. Furthermore, FBA can be integrated with metabolic pathway analysis (MPA). MPA is a qualitative technique that examines functional routes existing in a metabolic network without requiring knowledge of reaction rates [11]. Combining MPA with FBA can quantitatively trace the plausible paths for optimal product synthesis, calculate cellular metabolism, and predict phenotypes under genetic manipulations or culture conditions [12]. One main advantage of FBA is its capability for genome-scale modeling (including thousands of reactions), which bridge genomic annotation and functional metabolic output. Accordingly, the number of FBA models has increased exponentially since 1999 [13].

^{13}C-MFA aims to rigorously quantify pathway activities in intracellular metabolism by using both the isotopic labeling approach and *in silico* computation. ^{13}C-MFA is accomplished by feeding microbes a ^{13}C-labeled carbon source, measuring the enrichment pattern of the isotopomer in metabolites (e.g., amino acids), and deciphering the fluxes via computational routines [14]. Since carbon fluxes through a metabolic network generate unique labeling patterns in metabolites, the overall flux distributions can be determined using isotopomer information. Advances in ^{13}C-MFA, including mass spectrometry-based metabolomics and isotopomer modeling approaches (such as novel algorithm using elementary metabolite units), have been discussed in recent papers [4, 15, 16].

Furthermore, open-source software has recently been published that facilitates *in silico* modelling. For example, WEbcoli is web-based software for flux balance analysis of *E.coli* [17]. In addition, OpenFLUX is computationally efficient software for ^{13}C-MFA [15], which incorporates the elementary metabolite unit (EMU) framework for calculation of isotopomer balances [18]. User-friendly software such as this allows biologists to perform fluxomics studies with little programming knowledge.

Methodologies for FBA and ^{13}C-MFA share two key characteristics: the use of a metabolic network and the assumption of a steady metabolic state (for internal metabolites). However, the two techniques have different purposes. FBA profiles the "optimal" metabolism for the desired performance; ^{13}C-MFA measures *in vivo* operation of a metabolic network. The two approaches to flux analysis are complementary when developing a rational metabolic engineering strategy. By comparing existent metabolic fluxes which were empirically determined via ^{13}C-MFA to the optimal metabolisms predicted by both FBA and other "omics" tools (such as transcription analysis), one can deduce gene targets for solving biotechnologically relevant productivity bottlenecks [19]. Figure 1 shows that iterative flux analysis and genetic engineering of microbial hosts can remove competitive pathways or toxic byproducts, amplify genes encoding key metabolites, and balance energy metabolism [6].

2.2. Metabolic Control and Dynamic Flux Analysis. FBA and ^{13}C-MFA disregard dynamic intracellular behavior. This

avoids the difficulties in developing kinetic models and performing intracellular experimental measurements. However, many biological systems may not maintain a meaningful metabolic (or isotopic) steady state during the fermentation process [20–22]. The description of metabolic perturbation and regulatory mechanisms requires kinetic modeling and control theories. For example, metabolic control analysis (MCA) couples local enzyme kinetics with systematic behavior to predict the control exerted on the targeted pathways by different components (e.g., transcription, enzymes) [23]. Although MCA is not a quantitative measurement of flux, MCA can pinpoint bottle-neck enzymes (enzymes having the largest effect on the desired flux) in a pathway and allow the analysis of steady-state metabolism in response to changes in the cellular environment [24]. In addition to MCA, the cybernetic approach (a model based on process dynamics and control) has been introduced for study of multienzyme systems and metabolic regulation [25]. By incorporating both the enzyme kinetics in pathways and the enzyme synthesis kinetics, the cybernetic approach emphasizes microbial process dynamics and control during complicated fermentations [26].

Both MCA and the cybernetics approach focus on a simplified pathway network. To perform cell-wide quantitative analysis of a dynamic system, it is necessary to integrate the kinetic modeling with FBA and ^{13}C-MFA. Dynamic FBA (dFBA) has been developed to illuminate changing global enzyme activities [27, 28]. To avoid ordinary differential equations and dynamic optimization for describing intracellular metabolism, dFBA can use the Static Optimization Approach (SOA) [29] which divides the time-course into numerous small intervals. At each time interval, a steady-state flux is calculated under the assumption of fast intracellular dynamics. By combining stoichiometric FBA for intracellular metabolism with dynamic mass balances on extracellular substrates and products, it is possible to reconstruct dFBA model for genome-scale analysis of microbial metabolisms in industrial fermentations, where product synthesis is often under dynamic control [30, 31].

Recently, ^{13}C-dMFA (dynamic metabolic flux analysis) has been developed for isotopically nonstationary cultures. To profile the flux distributions for fed-batch cultures (slow dynamic metabolism), isotopic pseudosteady state was assumed and two dilution parameters were introduced to account for isotopic transients [92]. Another approach (Kinetic Flux Profiling) for solving intracellular fluxes is to create a sudden increase of the portion of ^{13}C in the substrate feed, then measure time-course samples as ^{13}C moves from the substrate into the metabolites [33]. The fluxes can be calculated based on the rates of isotopic enrichment multiplied by the intracellular metabolite concentrations. A similar principle has been proposed for the flux analysis of photoautotrophic microorganisms [34] and *E. coli* in an isotopic transient phase [35]. If the culture is under both metabolic and isotopic nonstationary state, exploratory and sophisticated ^{13}C-dMFA (dynamic ^{13}C-MFA) models have to be used to calculate both metabolic and isotopic kinetics [20, 36, 37]. To solve the ^{13}C-dMFA problem efficiently, a set of computational algorithms have been developed for tracing nonstationary isotopomer labeling in response to *in vivo* flux distributions [20, 36, 37]. The EMU (elementary metabolite unit) framework has also been applied in ^{13}C-dMFA [18, 38], because such algorithm can significantly improve computational times for tracing the labeling information [39]. To avoid extensive simulation of dynamic isotopomer patterns, the SOA has to be applied by dividing the growth period into small time intervals ($30 \sim 60$ min), then the "mini" quasi-steady state ^{13}C-MFA can be applied at each time interval based on constraints from simultaneous isotopomer analysis of the fast turnover metabolites [40]. By examining flux profiles over all time intervals, one can resolve the metabolic transients during the entire cultivation period.

2.3. Technical Limitations of Fluxomics. Cell-wide fluxomics tools (i.e., FBA and ^{13}C-MFA) have technical limitations. In genome-scale FBA models, the number of constraints (i.e., the availability of quantitative metabolite data) is much smaller than the number of reactions in the metabolic network. The calculation of such underdetermined systems depends on objective functions where one assumes that the metabolism optimizes its native "goals" (such as biomass or cofactor production) [41]. This optimization principle has been questioned for several reasons. First, biological systems (e.g., *Bacillus subtilis*) seem to display suboptimal growth performance [42]. Second, a previous study examined 11 objective functions in *E. coli* and found no single objective function that can perfectly describe flux states under various growth conditions [43]. For example, unlimited aerobic growth on glucose is best described by a nonlinear maximization of the ATP yield per flux unit, but nutrient-limited continuous cultures favor biomass yield as the objective function. Third, some native cellular processes cannot be simply described by FBA. For example, cyanobacterial species (i.e., *Cyanothece* 51142) maintain their circadian rhythms (e.g., nitrogen fixation and light dependent reaction activities) under nutrient-sufficient and continuous light conditions [44, 45].

The application of ^{13}C-MFA in industrial biotechnology also has several bottlenecks. The most prevalent constriction occurs because current techniques are insufficient for measuring large-scale metabolic networks. Obtaining labeling information of free metabolites rather than amino acids and solving large-scale nonlinear flux models pose two key challenges. As a result, most obtained flux information is limited to central metabolism. To date, only two large-scale ^{13}C-MFA (>300 reactions) have been reported, but many fluxes in their reports cannot be precisely determined due to insufficient constraints [46, 47]. The genome-scale ^{13}C-MFA is still in its infancy and requires further development of the relevant experimental techniques and computational tools [48]. A second issue is that ^{13}C-dMFA is still poorly developed for determining dynamic metabolic behavior. It is difficult for rapid sampling and precise measurements of metabolites at short time intervals throughout the entire culture period. For example, to measure absolute intracellular metabolite concentrations, one has to grow cell in fully ^{13}C-labeled medium,

then the labeled cells are extracted with quenching solvent containing known concentrations of unlabeled internal standards (the concentrations of metabolites are calculated using the isotope ratio-based approach) [49]. Such measurement requires extremely high cost of analytical efforts including quick sampling, rapid metabolite extraction, and a high-resolution LC-MS instrument. Furthermore, the time-dependent model includes ordinary differential equations and significantly increases the computational complexity [20, 35]. Third, flux determination assumes that enzymatic reactions are homogenous inside the cell and that there are no transport limitations between metabolite pools. However, eukaryotes have organelles (compartments) that may have diffusion limitations or metabolite channeling [14, 50]. Compartmentalization of amino acid biosynthesis further clouds the obtained amino acid-based labeling information [51]. Therefore, confident ^{13}C-MFA for eukaryotes not only requires the combination of different analytical tools (GC-MS, LC-MS and NMR) to obtain extensive labeling information [52], but also adequate sample processing and extraction methods (e.g., separation of compartments by ultracentrifugation). A fourth problem is that some industrial hosts and the great majority of environmental microbes resist cultivation in minimal media, and introducing other nutrient sources often significantly complicates metabolite labeling measurements and flux analyses [53]. Finally, a microbial community demonstrates complex metabolic interactions between species. To date, only a few FBA models have been developed for community studies [54, 55]. The exchange of metabolites among species is nearly impossible to unravel by ^{13}C-MFA because complete separation and measurement of metabolites from a single species in a microbial community is impossible [4]. These technical limitations in both FBA and MFA models are responsible for the gap between fluxomics and its applicability in biotechnology.

3. Integration of Fluxomics with Other "Omics"

It is desirable to integrate the concepts of systems biology (which combines the readouts from transcription as well as protein/metabolite profiling) with fluxomics (Figure 2) [48]. For example, ^{13}C-MFA, enzyme activity assays, and RT-PCR analysis can be used together to study *E.coli* mutants' metabolism [56]. Additionally, the responses of *E. coli* to genetic modification have been systematically examined by utilizing multiple high-throughput "omics" methods [57]. The results illuminate relatively small changes in mRNA and proteins in response to genetic disruptions, which allow the cell to maintain a stable metabolic state under changing growth conditions. A similar approach to the study of *Synechocystis* 6803 has shown that the regulation of some enzymes is sensitive to light conditions [58]. Many other regulatory mechanisms, however, still remain unknown. Furthermore, global regulators in industrial microorganisms have been successfully identified by correlating transcript/transduction levels and metabolic fluxes [59–62]. The discovery of functioning regulators provides insight to the entire regulation in metabolic network.

On the other hand, challenges in integrated "omics" studies are also present. The lack of understanding of metabolic regulation at different metabolic levels complicates the rational design of biological systems, which is a major barrier in industrial biotechnology. For example, posttranscriptional regulation poses a significant challenge in integrating fluxomics with other "omics" studies. It is well known that transcript and protein data correlate relatively well for specific pathways, yet this correlation can be poor in cell-wide analyses [76]. Furthermore, most mRNA expression studies insufficiently predict enzyme activities or flux changes in many *E. coli* pathways [77]. In studies on the adaptation of *E. coli* to environmental perturbations, the tricarboxylic acid cycle is found to correlate well with molecular changes at the transcriptional level, but flux alterations in other central metabolic pathways seem to be uncorrelated to changes in the transcriptional network [78]. Because of the complexity of regulatory mechanisms spanning multiple cellular processes, fluxomics and other "omics" studies may have inconsistent observations which complicate systems-level analyses.

4. Fluxomics of Microbes for Industrial Biotechnology

FBA allows *in silico* simulations of metabolism in "industrial workhorses," from which desired strains or targeted mutations can be identified. ^{13}C-MFA can assess *in vivo* metabolism of engineered strains under specific growth conditions and validate FBA results. Here, we summarize recent applications of FBA and ^{13}C-MFA for commonly-used industrial chassis (i.e., *E. coli*, *B. subtilis* and *S. cerevisiae*) and for nonmodel microorganisms (i.e., less-characterized or newly-discovered microorganisms).

4.1. Escherichia coli Model. E. coli is the most commonly utilized species in fermentation industry. *E. coli* flux models were reported as early as the 1990s [79, 80]. For biotechnology applications, the Liao group first applied metabolic pathway analysis (MPA) to guide the genetic manipulation of *E.coli* strains and channel the metabolic fluxes from carbohydrate to the aromatic amino acid pathway [81]. The Maranas group has integrated cell growth and product synthesis in the OptKnock toolbox [10] and applied it to construct high-performance mutants. The computer-aided designs have shown improved lactic acid, succinate, and 1,3-propanediol production [82]. FBA can predict lethality in a metabolic network where deletions of more than one nonessential gene mutants may trigger the death of the organism. For example, the Maranas group [83] analyzed the gene/reaction essentiality in a genome-scale model of *E. coli* and systemically identified possible pairs of synthetic lethals: nonessential genes whose simultaneous knockouts would have a potentially lethal effect. Incorporating information about synthetic lethality into the new model will curb the construction of ill-designed biological systems for biotechnology. Furthermore, FBA can be used to find rate-limiting steps for product synthesis. For example, FBA revealed gene

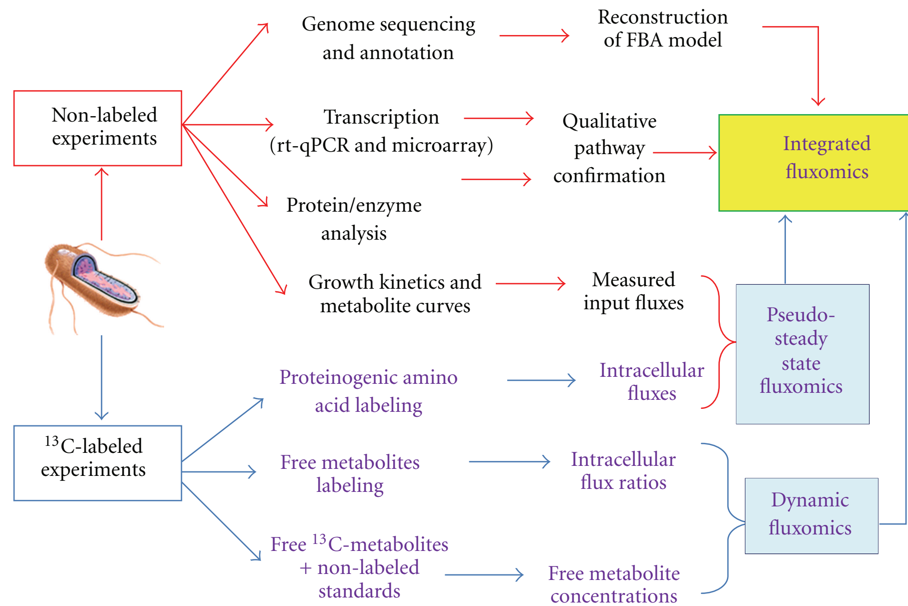

Figure 2: ^{13}C-assisted cellular metabolism analysis.

targets, and modification of those genes (i.e., knocking out the genes for pyruvate forming enzymes, overexpression of the glyoxylate shunt and glucose transport system) resulted in more than a ten-fold increase in succinate production [84–86]. FBA has also been used to improve genetic strategies for the overproduction of secondary metabolites, such as amino acids [87] and lycopene [88].

Besides genetic strategies, FBA can provide useful information for the design of optimal fermentation conditions. For example, an FBA model was used to identify nutrient limitations during recombinant interleukin-2 (IL-2) production in *E. coli.* By supplementing specific amino acids, IL-2 production increased two-fold in fed-batch fermentation [89]. Recently, a reactor-scale dFBA model was developed via a static optimization Approach to analyze *E. coli* metabolism for the production of a biopharmaceutical drug [27]. dFBA contains a steady state FBA model embedded within a dynamic kinetic model that describes the time evolution of fermentation process variables (e.g., biomass growth, glucose consumption and products synthesis). Such a model provided guidelines for the optimization of fermentations at the scale of a 1000L process.

The ^{13}C-MFA model was first used to investigate metabolic regulation in *E.coli* under different genetic and environmental conditions [90]. ^{13}C-MFA has also been used to examine various biotechnological processes involved in the production of pharmaceuticals, amino acids and polymers. A large scale ^{13}C-MFA with over 300 reactions was successfully developed for amorphadiene (a precursor of the antimalaria drug) producing *E. coli* strains [46]. Another study revealed a growth phase-dependent metabolic shift in a lysine-producing *E. coli* strain [91]. This work was performed in a fed-batch culture with rich medium (containing yeast extract), and metabolic fluxes in both exponential growth and stationary phases were estimated by measuring free metabolites. Metabolic analysis of the stationary phase is important since many products are synthesized during a nongrowth phase. In a third example, ^{13}C-MFA of a 1,3-propanediol producing *E. coli* strain was conducted in fed-batch fermentation [92]. The ^{13}C-MFA results showed a decrease in the split ratio between glycolysis and the pentose-phosphate pathway over the time-course of the culture in response to increasing 1,3-propanediol fluxes.

4.2. Bacillus subtilis Model. B. subtilis is the industrial organism of choice for the production of vitamins, antibiotics, enzymes, and nucleosides. The FBA model for *B. subtilis* was constructed based on a combination of genomic, biochemical, and physiological information [93]. The FBA model was iteratively corrected and improved using information from high-throughput phenotypic screens of mutants, substrate utilization, gene essentiality, and sequence analyses. The *B. subtilis* flux model is mostly studied for riboflavin production, focusing on four aspects: investigating phenotypes of wild type and knock-out strains, assessing production capacity, identifying the impact of different carbon sources on biosynthesis, and characterizing the cellular response to different culture conditions. The Sauer group has extensively investigated riboflavin-producing strains. They first used an FBA model to quantify growth maintenance coefficients, the maximum growth yield, and the specific riboflavin production rate in continuous cultivation [94]. Later on, they applied ^{13}C-MFA to the same strain and found that genetic manipulations should target the NADPH balance and riboflavin biosynthetic pathways [95]. In other studies on *B. subtilis*, they revealed several guidelines for high-yield riboflavin production (1) they compared the metabolic flux distributions and maintenance energy of eight

Bacillus strains and discovered that *B. licheniformis* was the most suitable for industrial biotechnology [96], (2) they found that using malate as a substrate resulted in a suppressed respiratory TCA cycle and an enhanced overflow metabolism [97], and (3) they found the pentose precursors of riboflavin were mainly synthesized via the nonoxidative pentose-phosphate pathway, so any suggested genetic modification should decrease the activity of the oxidative pentose-phosphate pathway [98]. Recently, they developed a ^{13}C-dMFA model for *B. subtilis* to identify the metabolic response of riboflavin overproduction under a glucose-limited fed-batch culture [40]. This dynamic flux analysis was obtained by recording changes in labeling patterns of intracellular amino acids under a metabolic pseudosteady state assumption.

4.3. Saccharomyces cerevisiae Model. *S. cerevisiae* is a robust eukaryotic chassis used for the expression of a wide range of products. For example, flux analysis revealed target genes in two native pathways for the overexpression of succinate: the TCA and glyoxylate cycles [99]. Another study showed the enhancement of sesquiterpene production via *in silico* driven metabolic engineering [100]. Additionally, flux analysis has been extensively applied for improving ethanol production. First, a number of strategies were developed for the metabolic engineering of redox processes in *S. cerevisiae*, resulting in a decrease in the yield of glycerol by 40% and an increase in ethanol production under both glucose and xylose/glucose growth conditions [101]. Second, Dikicioglu et al. [102] applied a genome-scale FBA model to analyze respiration-deficient mutants of *S. cerevisiae* for ethanol production. They found that many genetic manipulation strategies (e.g., the overexpression of the glutamate synthase gene) were unnecessary in a respiration-deficient metabolic background. This indicates that the rate limiting steps for ethanol production can change after the initial genetic manipulations of targeted genes. Third, a ^{13}C-MFA model was used to screen ethanol production in 14 hemiascomycetous yeast strains [51]. This study suggests that *S. cerevisiae* is the ideal ethanol production candidate due to a strong NADPH-driven pentose-phosphate pathway. Other ^{13}C-MFA studies characterized the metabolic shift between oxidative growth and fermentative growth with ethanol production [103], investigated alternative carbon substrate (xylose) metabolisms [104], revealed key factors influencing biomass growth on xylose [32], and examined the consumption of ethanol and other storage carbohydrates in a glucose-limited chemostat culture [105].

Furthermore, a genome-scale FBA indicates an apparent enzyme dispensability, that is, 80% of yeast genes seem to be nonessential for viability under laboratory conditions [106]. The FBA illustrated the influence of nonessential genes on metabolic robustness and environmental fitness due to genetic buffering through alternative genes, while a ^{13}C-MFA (consisting of over 700 reactions) revealed a similar effect of metabolic network robustness on null mutations [47]. Understanding the role of these redundant genes is important for a valid and efficient genetic modification.

4.4. Nonmodel Microorganisms. Fluxomics is an important tool for the rigorous study of metabolism in less-characterized microbes that provides novel insights for application of these species to biotechnology. However, fluxomics have not been sufficiently applied to nonmodel microorganisms as compared to model microbial hosts. Table 1 summarizes some milestone papers in fluxomics studies on nonmodel species that are potentially useful for synthetic biology. Compared to the work done in the field of fluxomics for industrial workhorses, far fewer studies have been performed on nonmodel microorganisms. This is due to the complicated growth conditions, poorly-understood metabolic networks, and significant lack of genetic and molecular biology tools. However, nonmodel environmental microorganisms are also important for industrial biotechnology because they often possess native biochemical pathways for chemical synthesis or the ability to utilize cheap substrates [120]. Furthermore, flux analysis can be used to discover novel enzymes that can be cloned into industrial microbes to improve their capacity for product synthesis. For example, ^{13}C-MFA revealed a citramalate pathway for isoleucine biosynthesis (independent of the common threonine ammonia-lyase pathway) [121, 122]. Citramalate synthase, which has also been detected in some environmental bacteria [123–125], can be engineered into *E. coli* for 1-propanol and 1-butanol production. The new pathway bypasses threonine biosynthesis and represents the shortest keto-acid-mediated pathway; as such, it improved biofuel yield 9 to 22-fold [126]. Currently, high-throughput genome sequencing methods are mapping genomes in novel microbes at a pace that far exceeds the pace of functional characterization of these species. Therefore, a high throughput ^{13}C-MFA technique is required for screening nonmodel microorganisms for new enzymes and maximizing their application in industrial biotechnology [4].

5. Finding Bottlenecks for Industrial Biotechnology

One of the main goals of fluxomics is to indentify bottlenecks for industrial biotechnology and thereby assist in the creation of rational engineering strategies. Simple measurements of metabolism, however, are not enough to overcome unpredictable challenges in industrial biotechnology. Metabolic regulation is very complex, and systems biology tools are incapable of revealing a general strategy for synthetic biology [127].

Bottlenecks in industrial biotechnology can be explained from the view of fluxomics. First, metabolic robustness (the ability to maintain metabolic performance under genetic or environmental perturbations) is a long-recognized key property of microbial systems [128]. This basic mechanism is often responsible for the gap between computationally aided design and final experimental outcomes. For example, a ^{13}C-MFA study indicates that *E. coli* shows remarkable robustness in the central carbon metabolism in the presence of genetic variation, and is even more flexible in response to altered

Table 1: Recent application of fluxomics of nonmodel microbes to bioproduct synthesis.

Species	Product	Substrate	Model description	Results from study	Reference
Corynebacterium glutamicum	Lysine	Glucose (sucrose, fructose)	^{13}C-MFA	MFA models (combining transcriptome, metabolome analysis) have been developed to study fluxes under different cultivation modes (minibioreactor, batch, fed-batch) using various carbon sources.	[107]
Corynebacterium glutamicum	Methionine	Glucose	^{13}C-MFA only focuses on flux distribution in the methionine pathway.	The *C. glutamicum* mutant (mcbR) showed no overproduction of methionine, but accumulation of homolanthionine.	[108]
Corynebacterium glutamicum	Glutamate	Glucose	^{13}C-MFA (focus on anaplerotic pathways)	The flux from phosphoenolpyruvate to oxaloacetate catalyzed by phosphoenolpyruvate carboxylase (PEPc) was active in the growth phase, whereas pyruvate carboxylase was inactive.	[109]
Actinobacillus succinogenes	Succinate formate and acetate	Glucose $NaHCO_3$	^{13}C-MFA (via NMR and GC-MS) and enzyme assay	The model indicated (1) NADPH was produced primarily by transhydrogenase and/or by NADP-dependent malic enzyme (2) oxaloacetate and malate were converted to pyruvate (3) the effects of $NaHCO_3$ and H_2 on metabolic fluxes were quantified.	[110, 111]
Geobacillus thermoglucosidasius	Ethanol	Glucose	FBA and ^{13}C-MFA	The model characterized the ethanol production under three oxygen conditions. The FBA analysis pointed out several gene targets for improving ethanol production.	[19]
Clostridium acetobutylicum	Butanol	Glucose	Genome-scale-FBA	The engineered strain was able to produce 154 mM butanol with 9.9 mM acetone at pH 5.5, resulting in a butanol selectivity (a molar ratio of butanol to total solvents) of 0.84.	[112]
Penicillium chrysogenum	Penicillin	Gluconate/glucose	^{13}C -MFA (focus on pentose phose phase pathway and glycolysis)	The model determined the pentose-phosphate pathway split ratio and estimated NADPH metabolism.	[113]
Synechocystis sp. PCC6803	Hydrogen	CO_2	FBA	The results included H_2 photoproduction, strategies to avoid oxygen inhibition, and analysis of hetero-, auto-, and mixotrophic metabolisms.	[114, 115]
Synechocystis sp. PCC6803	Light energy & Biomass	Glucose/CO_2	^{13}C-MFA and dynamic ^{13}C -MFA	The model analyzed heterotrophic, autotrophic and mixotrophic metabolisms.	[34, 58]
Chlamydomonas reinhardtii	Light energy & Biomass	CO_2	FBA model including three metabolically active compartments	The model indicated that heterotrophic growth had a low biomass yield on carbon, while mixotrophical and autotrophical growth had higher carbon utilization efficiency.	[116]
Zymomonas mobilis	Ethanol	Glucose/xylose	FBA with various biological objectives	Model analyzed the metabolic boundaries of *Z. mobilis*. The study indicated that ethanol and biomass production depend on anaerobic respiration stoichiometry and activity.	[117]
Zymomonas mobilis	Ethanol	Glucose/fructose/ xylose	^{13}C–MFA via 1H-NMR 31P-NMR spectroscopy	The model characterized the intracellular metabolic state during growth on glucose, fructose and xylose in defined continuous cultures.	[118]

Table 1: Continued.

Species	Product	Substrate	Model description	Results from study	Reference
Coculture (Desulfovibrio vulgaris and Methanococcus maripaludis)	CH_4	Lactate	FBA analysis of microbial consortia	The model predicted the ratio of *D. vulgaris* to *M. maripaludis* cells during growth. It was possible to eliminate formate as an interspecies electron shuttle, but H_2 transfer was essential for syntrophic growth.	[55]
Community (oxygenic phototrophs, filamentous anoxygenic phototrophs, and sulfate-reducing bacteria).	Biomass and nitrogen fixation	CO_2	FBA and elementary mode analysis	The model predicted and described relative abundances of species, by-products, and the metabolic interactions.	[54]
Phaffia rhodozyma and Haematococcus pluvialis	Astaxanthin	Glucose with (peptone & yeast extract)	FBA analysis of mix culture	The two major astaxanthin-producing microorganisms exhibited elevated yields (2.8-fold) under mixed culture conditions compared to pure culture.	[119]

environmental conditions (e.g., different nutrients or oxygen levels) [90]. Analyses of *E. coli* components at multiple "omics" levels also reveal unexpectedly small changes in messenger RNA, proteins and metabolite levels for most genetic disruptions. This is because *E. coli* actively regulates enzyme levels to maintain a stable metabolic state in the presence of perturbations [57, 78]. Similarly, *B. subtilis* shows rigidity and suboptimal performance for its flux regulation in over 137 genetic backgrounds [42]. Furthermore, gene essentiality and pairwise genetic interactions have been investigated in *S. cerevisiae* [106, 129]. It has been found that a gene's function is buffered by duplication in *S. cerevisiae* genomic DNA or by an alternative biochemical pathway. Although only 13% of genes were suggested to be essential by single knockout experiments, simultaneous deletion of pairs of nonessential genes (>70% of the total metabolic genes) were found to inhibit growth. Invariability of metabolic flux under mutagenic genotypes seems to be an important feature in many biological systems, and thus successful metabolic strategies highly depend on an understanding of robust cellular nature [130–132].

Metabolic engineering of industrial chassis is based on the premise that the yield of a desired product can be increased by identifying and overexpressing the enzymes that catalyze the rate-limiting steps in a given metabolic pathway. However, a method based on overexpressing rate-limiting enzymes will only work if these rate-limiting enzymes exist and remain rate-limiting when their activities are increased. Previous studies have shown that the commonly-believed "rate-limiting" enzymes may not exist in some industrial microbes and an increase in productivity has to be achieved by coordinated expression of entire pathways [133]. Furthermore, rate-limiting steps in a metabolic network often shift after initial targets have been engineered. For example, phenotypic data in *S. cerevisiae* mutants revealed that some FBA-predicted gene targets for ethanol production are invalid if the cell's respiratory genes have been knocked-out [102]. Another example of this phenomenon is highlighted by the metabolic consequences of the deletion of the methionine and cysteine biosynthesis repressor protein (McbR) in *Corynebacterium glutamicum*, which yielded no overproduction of methionine but drastic accumulation of homolanthionine [108]. The above evidence indicates that rate-limiting steps often shift after initial targets have been engineered. Additionally, simultaneous importation and expression of a few heterologous genes to improve the rate-limiting pathway may fail if the nonnative pathway is incompatible with the host. These efforts often lead to metabolic imbalance and accumulation of toxic metabolites [2, 3].

Based on the recent publications, we have constructed a linear regression model which shows that the yield of biosynthetic products decreases exponentially as a function of the steps away from central metabolism in *S. cerevisiae* (Figure 3). It is easier to achieve high carbon fluxes to the central metabolites, possibly because enzyme efficiency in central metabolism is usually high [134]. However, the yields of secondary metabolites are smaller because each additional enzymatic step may not be perfectly efficient (model regression shows an average of ~67% efficiency in each enzymatic step in secondary metabolisms). This loss of yield is unavoidable due to the metabolism channeling the intermediates away from the desired product. Potential solutions to this problem include (1) designing host-compatible enzymes with high product specificity [135], (2) feeding intermediates to the cell to reduce the number of enzymatic steps to final product [136], and (3) creating synthetic protein scaffolds, which significantly improve intermediate conversion efficiency and overall biosynthetic yield [137].

In conclusion, fluxomics studies enable the quantification of intracellular metabolism. However, this tool is not fully developed, and it remains difficult to deduce cell-wide pathway bottlenecks and to provide effective strategies

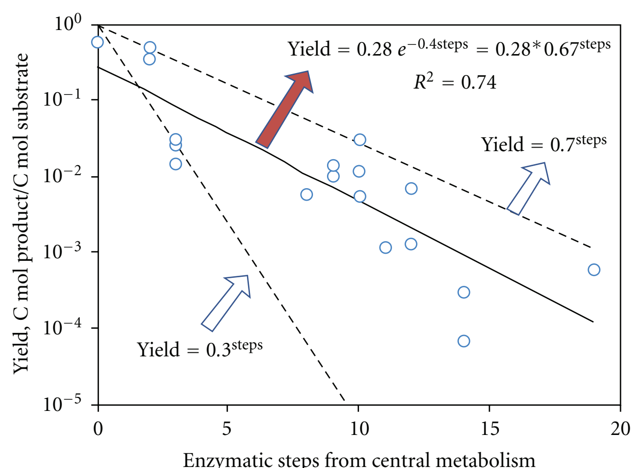

Figure 3: Product yields as a function of enzymatic steps from central metabolism. The solid line is the regression of published product yields by *S. cerevisiae* as a function of reaction steps from intermediate metabolites in central metabolism (including glycolysis, TCA cycle and pentose-phosphate pathways). The yield declines exponentially as the number of reaction steps increases. The dotted lines are boundary curves with yield efficiencies of 30% and 70% respectively. All yield data from initial carbon sources are estimated from recent papers using our best judgment. The synthesized products and reaction steps are: Poly(R-3-hydroxybutyrate) [63] (steps = 3); Glycerol [64] (steps = 2); Artemisinic acid [1] (steps = 10); Amorphadiene [65] (steps = 9); Pyruvate [66] (steps = 0); Geranylgeraniol [67] (steps = 10); Hydrocortisone [68] (steps = 19); Squalene [69] (steps = 9); β-carotene [70] (steps = 12); Lycopene [70] (steps = 11); Phytoene [70] (steps = 10); p-hydroxycinnamic acid [71] (steps = 12); Naringenin [72] (steps = 14); Pinocembrin [72] (steps = 14); Xylitol and Ribitol [73] (steps = 3); Ethanol [74] (steps = 2); L-ascorbic acid [75] (steps = 8).

for biotechnology applications. Numerous technical difficulties in developing flux analysis methods and complicated metabolic regulatory mechanisms have severely limited the scope of fluxomics in industrial biotechnology. It is necessary for the future development of flux analysis to combine other advanced "omics" analysis and molecular biology techniques to resolve challenges in the fluxomics fields.

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