Systems and synthetic biology tools for advanced bioproduction hosts

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Introduction

The genomic revolution ushered in an era of discovery and characterization of enzymes from novel organisms that fueled engineering of microbes to produce commodity and high-value compounds. Over the past decade advances in synthetic biology tools in recent years contributed to significant progress in metabolic engineering efforts to produce both natural and non-natural biofuels and bioproducts [1,2] resulting in several bioproducts being brought to market [3*,4]. These successes represent a burgeoning bioeconomy, however, significant resources and time are still necessary to progress a system from proof-of-concept to market. In most cases, production of biofuels and bioproducts at economically-feasible titers, rates, and yields (TRY) rely on a systemic understanding of the metabolism of the organism and a suite of synthetic biology tools to

engineer them. Yet, examples of metabolic engineering efforts powered by a deep, systems-level knowledge of an organism and a toolbox filled with a diverse set of synthetic biology tools remain scattered. In order to fully utilize this potential, methods that examine biological systems in a comprehensive, systematic and high-throughput manner are essential [3*]. Recent success in metabolic engineering and synthetic biology efforts has coincided with the development of systems biology and analytical approaches that have kept pace and scaled with technology development. Here, we review a selection of systems biology methods and their potential use in synthetic biology approaches for developing microbial biotechnology platforms.

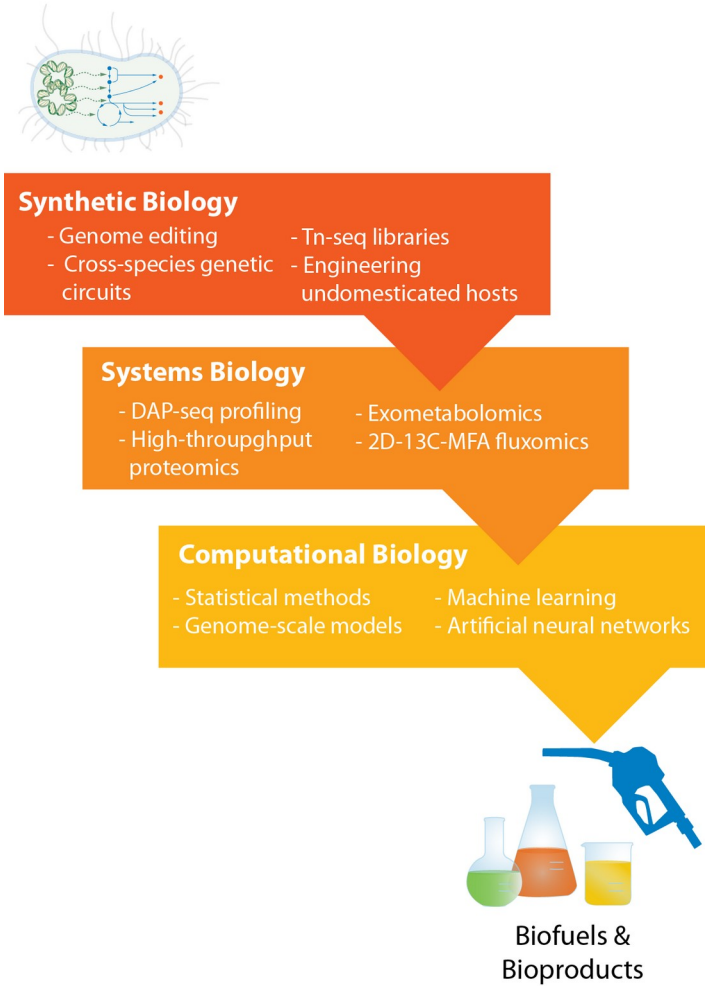
Recent developments in systems and synthetic biology

Increasingly, a diverse set of host organisms, beyond *Saccharomyces cerevisiae* and *Escherichia coli*, are the focus of biomanufacturing research (Table 1). Development of genome editing tools, such as CRISPR-Cas9, make it possible to engineer organisms that historically have been intractable to metabolic engineering [5] despite having characteristics that are attractive to biomanufacturing applications. Complementing these tools are methods to transfer DNA into cells of undomesticated organisms based on the integrative and conjugative element from *Bacillus subtilis* (ICEBs1) [6], expression systems for cross-species genetic circuits [7–9], and modular design principles for metabolic pathways [10]. Likewise, a parts-based synthetic biology strategy for constructing mutant libraries enabled the genome-wide mutant fitness screening of 32 diverse bacteria across dozens of growth conditions [11,12**]. These synthetic biology developments give researchers new control of the organisms they want to engineer with respect to tunability, orthogonality and composability of the parts they use.

Similarly, there has been significant recent progress in systems biology tools. DNA affinity purification sequencing (DAP-Seq or DAP-chip) enables low-cost and high-throughput profiling genome-wide DNA-binding motifs of individual transcription factors (TFs) [13,14] to facilitate characterization of regulatory architecture of transcriptional networks [15,16]. Likewise, high-throughput proteomic analysis capable of analyzing hundreds of samples per day [17], tools to shorten method development time [18], and automated proteomic sample preparation procedures [19] make these types of experiments routine. As microorganisms uptake and secrete metabolites from and into their environment, exometabolomics analysis, supported by various high-throughput analytical methodologies, provides a rich and valuable

phenotypic data, and gains popularity in bioprocessing and biofuel development [20,21]. Additionally, systems biology approaches contributed to better understanding of metabolism and increased the quality of genome-scale metabolic model (GSM) in various organisms [22]. More development of systems biology tools and strategies for metabolic engineering efforts in model and non-model microorganisms could be found in recent reviews and the references cited therein [3*,23,24]. Applied in the context of biomanufacturing, these tools and workflows reduce the time and effort required to complete an iteration of a DBTL cycle, speeding progress toward economic biofuels and bioproducts.

Figure 1



Integration of synthetic and systems biology to improve biomanufacturing research

Rewiring cellular metabolism to produce valuable chemicals and materials at economically competitive level benefits from an integrated approach, where tools and strategies of synthetic, systems and computational biology are used to inform changes to cellular metabolism to optimize TRY of the desired product (Figure 1). The recent breakthroughs in omics technologies enable in-depth studies of cellular metabolic networks and regulation (Table 1). The data-driven systems biology approach provides valuable insights that lead researchers identifying metabolic pathway bottlenecks and conducting fine-tuned changes to meet industrial TRY requirements. One common bottleneck is the toxicity to the

Figure 1. Overview of Systems, Synthetic and Computational biology methods that allow the use of microbial hosts for the production of biofuels and bioproducts. Several selected approaches in each category are shown.

host organism of the final product or some of the intermediates present and/or generated during conversion. George *et al*. [25*] took an integrated multi-omics approach to understand the response of *E. coli* to the accumulation of isopentenyl pyrophosphate (IPP), a toxic intermediate, in isoprenoid-producing strains. Poudel *et al.* applied integrated omics analysis to reveal metabolic adaptations of *Clostridium thermocellum*'s specific response to cytotoxic compounds derived from hydrolysis of hemicellulose and lignin, which provided systems biological information to potentially improve *C. thermocellum*'s industrial efficacy [26]. Likewise, Ohtake *et al*. [27] took a metabolomic-driven approach to identify limitations in 1-butanol production in *E. coli*. The metabolomic analysis revealed an accumulation of butanoyl-CoA suggesting that aldehyde-alcohol dehydrogenase (Adhe2) was the cause of the bottleneck. Based on this knowledge they rationally engineered *E. coli* to remove the imbalance and increased the 1-butanol titer to 18.3 g/L [27]. Characterization of the effect of membrane viscosity on cellular respiratory metabolism was probed by an integrated systemic engineering approach to manipulate lipid composition in *E. coli* and *S. cerevisiae* [28]. Systematic engineering of industrial brewing yeast cell metabolism based on metabolomic and proteomic data was used to optimize the amount of hops flavors for beer [29], and in *Corynebacterium glutamicum* to develop a robust production system for the biogasoline target, isopentenol [30]. Lim *et al*. [31**] isolated *Vibrio sp. dhg*, a fast-growing bacterium, that is naturally capable of simultaneously assimilating mannitol and alginate, the most prominent sugars in brown macroalgae. Through genome analysis and the development of a synthetic biology toolbox for this strain the team engineered it to produce ethanol from these sugars at 25.7 g/L, 1.1 g/L/h and 64% of the theoretical maximum yield.

Carbon-13 metabolic flux analysis (^{13}C-MFA) is another approach that helps trace the relative contributions of different metabolic routes. ^{13}C-MFA-guided cofactor engineering, metabolomic analysis, and gene expression profiling were applied to direct *E. coli* strain development resulting in increased acetol titer from 0.91 g/L to 2.81 g/L [32]. Data from ^{13}C-MFA was used to identify a newly discovered fructokinase enzyme (FruA) in sucrose metabolism of *Basfia succiniciproducens* [33]. By knocking out the *fruA* gene, *B. succiniciproducens* produced 71 g/L of succinate with a yield of 2.5 mol/mol from sucrose under fed-batch conditions. ^{13}C-MFA can also be used to identify engineering targets that are unintuitive to most researchers. For example, ^{13}C-MFA was used to identify energy limitation in a strain of fatty acid producing *E. coli*. The limitation was removed by expression of

Vitreoscilla hemoglobin (VHb) a membrane protein facilitating O2 transport to promote cell respiration [34].

While these studies supplied information for rational engineering approaches, construction of a strain more tolerant to various stresses often requires non-rational engineering approaches. Adaptive laboratory evolution (ALE) coupled with systems biology analyses is an inverse engineering strategy for obtaining desirable host traits and characterizing the underlying molecular mechanisms of these traits. It has been applied successfully to gain novel insights into molecular mechanisms used by *E. coli* to overcome the metabolic burden or elevated cytotoxicity due to the deletion of major metabolic genes [35,36]. Laboratory evolution can also help identify non-intuitive gene deletion candidates to develop host organisms such as a *cydC* mutant in *E. coli* that not only conferred increased tolerance to inhibitory residual reagents in the carbon source, but also restored production of the bio-jetfuel target to optimal levels [37].

Metabolizing different nutrition sources are desirable features of cell factories to efficiently utilize lignocellulosic biomass. Quantitative metabolomics analysis identified pyruvate kinase as a major bottleneck of arabinose metabolism suppression by glucose in *C. glutamicum*, which directed rational engineering of manipulating only two genes to achieve simultaneous utilization of D-glucose and L-arabinose at the same rate [38]. A systems biology analysis of *Rhodococcus opacus* PD630 provided insight into lignocellulose hydrolysate utilization and subsequent evolved strains showed improved growth rate on phenol [39]. Multi-omic analysis *P. putida cultured* with glucose and benzoate simultaneously provided evidence that metabolic segregation represented an efficient strategy in *P. putida* to meet biosynthetic flux [40**]. Similarly, this approach was applied to study the molecular mechanisms that influence and regulate lipogenesis in the oleaginous yeast *Rhodosporidium toruloides* [41] and *Yarrowia lipolytica* [42], and identify bottlenecks in the bioconversion of glycerol into acetol in *E. coli* [32]. The non-targeted ALE approach is also a useful tool to engineer carbon source assimilation pathways and bypass catabolite repression regulatory systems [43*].

There are now several examples for improving biomanufacturing research using systems and synthetic biology via semi- or fully-automated metabolic engineering Design-Build-Test-Learn (DBTL) pipelines. For instance, Carbonell *et al*. [44**] developed an automated pipeline that uses a

retrobiosynthesis tool, RetroPath [45], to propose pathway selection and derives common design rules from machine learning (ML) techniques. Another computational retrobiosynthesis tool, BNICE.ch, was used to assess more than 3.6 million biosynthetic pathways from central carbon metabolites of *E. coli* to methyl ethyl ketone [46]. A Method for Metabolite Annotation and Gene Integration (MAGI) [47] is another workflow that integrates metabolomics data with genomic predictions to improve biological interpretations made by both. Discovery of novel enzymes is enabled by global mutant fitness data generated from RB-Tn-Seq experiments [48]. Likewise, literature evidence of more than one DBTL cycle utilizing omics data to inform subsequent designs in the field of biomanufacturing research is still rare [44**,49*,50]. Opgenorth *et al*. [49*] used their first DBTL cycle data to train machine learning algorithms that suggested protein profiles for the second DBTL cycle. It led to a 21% increase in dodecanol titer in cycle two.

Computational approaches to systems biology data analysis

Integrative multi-omics analyses attempt to draw connections between disparate omics data sources, either with or without biological knowledge. Yet, extracting actionable information from omics datasets is quite challenging. Consequently, there has been significant effort directed into the development of computational tools that integrate different omics datasets acquired during the Test step of a typical engineering DBTL cycle to assist with the Learning step [51]. Several toolboxes exist to integrate transcriptomics, proteomics, fluxomics and metabolomics data. Tn-Core [52] is a recent example that can automate integration of Tn-Seq data in addition to RNA-seq data to generate context specific models that may provide a “systems-level” view for metabolic engineering purposes. Traditionally, statistical methods have been used to evaluate the significance of different design parameters (promoters and plasmid copy numbers) on product titers [44**]. Principal component analysis (PCA) was recently utilized to evaluate different engineered strains for metabolites that contribute to acetol production using metabolomics datasets [32] that helped identify NADPH regeneration as the bottleneck for efficient acetol biosynthesis. PCA has also been used on extracellular metabolomics datasets to identify metabolites that are the main drivers of variation in IPP production engineered strains [25*].

Machine learning approaches

Machine learning has the capability to extract patterns from high throughput biological data and shows great potential in systems metabolic engineering [53]. It has been used to propose pathway selection [44**,54] as well as build kinetic models using proteomic and metabolomic time-series data from isopentenol-producing and limonene-producing *E. coli* strains [55]. These models qualitatively predict pathway dynamics and outperformed a classical Michaelis-Menten kinetic model. Artificial Neural Networks (ANN) are another tool used for integration of omics datasets independently or as a component of a ML workflow. Recently, improvement in β-carotene production was assessed in *S. cerevisiae* through the use of an ANN model in conjunction with a YeastFab Assembly strategy (MiYA) [56]. Variable expression levels of three heterologous β-carotene genes and product titers were used to train an ANN model. This model was then used to predict the optimal level of expression for these enzymes in the next iteration of strain construction. After two cycles of ANN training and evaluation, it was found that 7 out of 10 top predicted engineered strains had improved product titers compared to the highest-producing strain of the initial library. This workflow was extended to improve violacein production (five enzyme pathway) that led to successful prediction of a strain that showed a 2.42-fold titer improvement in violacein production among 3125 possible promoter combinatorial designs. Going forward, ML algorithms need high-quality experimental data, larger training data sets, more pathway independent data, and more libraries to improve their prediction power.

Biology-informed approaches

Genome-scale metabolic models (GSM) and constraint-based modeling (CBM) methods [57*] assist in integrating disparate omics data onto the metabolic map of the host and improve phenotype predictions. Yet, constructing GSMs requires comprehensive networks of enzymatic reactions and metabolism of the host organism. Recently omics data has been integrated with a GSM for *E. coli* to identify key factors for bioproduction and prediction of cell factory performance [58*]. GSMs are also important inputs to computational strain optimization methods (CSOM) (*e.g.*, Optknock [59], constrained minimal cut sets (cMCS) [60]) that are used to identify genetic knockouts, over/under expression of genes for strain optimization efforts [2]. A multi-omics integrative analysis based on GSM of *A. niger* was performed to understand the mechanisms supporting a high yield of glucoamylase production and global metabolic regulation under oxygen limitation, an

industrially relevant scenario [61]. Recently a workflow to improve the accuracy of predictions of large scale kinetic models in metabolic engineering studies was proposed [62]. Likewise, a kinetic model of metabolism at a genome scale containing 457 model reactions, 337 metabolites and 295 substrate-level regulatory interactions was developed and applied to multiple mutant strains [63]. Omics data of optimally grown *E. coli* was integrated into a stoichiometric model and used to construct populations of non-linear large-scale kinetic models consistent with the physiology of the *E. coli* aerobic metabolism [62]. Genome-scale ^{13}C-MFA is computationally expensive method that requires knowledge of all of the carbon transitions in the network. Two-scale ^{13}C-MFA (2S-^{13}C-MFA) is an alternative that overcomes the problem associated with genome scale ^{13}C-MFA by constraining fluxes in the genome-scale model simultaneously using two resolution scales: for core reactions, both stoichiometric and ^{13}C labeling constraints are used, whereas, for non-core reactions, only stoichiometric constraints are used [64].

Concluding remarks

To date, most of the successes we see in the biomanufacturing space are the result of an Edisonian 'trial-and-error' approach which are rarely generalizable across products or microbial platforms. Current designs continue to rely on historical knowledge to design pathways and engineer microbial hosts in an artisanal manner. However, the studies outlined in this review indicate that the increasing availability of high-throughput synthetic biology methods, coupled with lowering costs of systems biology experiments to inform computational biology enable predictive approaches to aid our biofuel and bioproduct research and development. Further, there is an emerging need for open source (e.g. Python based) frameworks such as IMPACT (Integrated Microbial Physiology: Analysis, Characterization and Translation) for bioengineers working with big biological data to interpret, model, and visualize data [65]. Using such pipelines, metabolic engineers can opt for such scalable data integration and analysis workflows, alleviating a bottleneck limiting the throughput of bioengineering research. As these efforts mature, they can be extended to improve automation and real-time learning capabilities of global biofoundries [66,67] in academic, research and commercial institutions in the biomanufacturing research and application space.

Conflict of interest statement

Nothing declared.

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Figure and Table Captions

Table 1. Selected examples of tools to integrate systems biology data for biomanufacturing research

Figure 1. Overview of Systems, Synthetic and Computational biology methods that allow the use of microbial hosts for the production of biofuels and bioproducts. Several selected approaches in each category are shown.

* of special interest
** of outstanding interest

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Table 1: Selected examples of tools to integrate systems biology data for biomanufacturing research					
Type of Data	**Analytical Technique(s)**	**Research Interest**	**Computational Tool(s) and Database(s)**	**Host**	**Reference**
Single-Omics					
Genome-scale phenotyping	RB-TnSeq, High-throughput phenotyping	Functional annotation of unknown genes in diverse bacteria	Pfam, TIGRFam, SEED/RAST and KEGG	32 different bacteria	[12**]
Genome-scale phenotyping	RB-TnSeq, High-throughput phenotyping	Gap Filling in Amino acid metabolic pathways	Pfam, TIGRFam, SEED/RAST and KEGG	10 different bacteria	[48]
Transcriptomics	RNA-Seq	Host engineering of a fast growing bacterium that utilizes non traditional carbon sources	Machine learning	*Vibrio sp. dhg*	[31**]
Proteomics	LC-MS/MS	Chassis-independent recombinase-assisted genome engineering	Diffusion-coupled reaction modeling	*E. coli*	[28]
Proteomics	LC-MS/MS	Tolerance and Metabolic engineering for improved production	-	*C. glutamicum*	[30]
Metabolomics	LC-MS/MS, GC-FID	Metabolic engineerin for improved production	Principal component analysis	*E. coli*	[27]
Metabolomics	LC-MS/MS,	Metabolic engineering for improved production	openBIS software	*E. coli*	[44**]
Metabolomics	LC-MS/MS	Identifying bottlenecks limiting carbon source utilization	-	*C. glutamicum*	[38]
Fluxomics	13C-MFA, GC-MS	Metabolic engineering for improved production	Constraint-based modeling and Metabolic flux analysis using OPENFLUX	*B. succiniciproducens*	[33]
Phenomics	LC-UV/VIS/DAD/RID	Optimization of heterologous pathway	Machine learning using artificial neural network	*S. cerevesiae*	[55]
Multi-Omics					
Genomics + Proteomics	NGS, LC-MS/MS	Toxicity Tolerance and Metabolic engineering for improved production	EggNOG database	*E. coli*	[37]
Genomics + Metabolomics	NGS, LC-MS/MS	Metabolite identification, gene annotation and biochemical predictions for poorly annotated genes	Metabolite Annotation and Gene Integration (MAGI) database	*S. coelicolor*	[47]
Transcriptomics + Metabolomics	LC-UV, RT-qPCR, GC-MS	Metabolic engineering for improved production	-	*E. coli*	[50]
Proteomics + Metabolomics	LC-MS/MS, GC-MS, NIMS, MSI	Metabolic engineering for improved production	Machine learning, OpenMSI Arrayed Analysis Toolkit	*E. coli*	[49**]
Proteomics + Metabolomics	LC-MS/MS, LC-RID, GC-MS	Metabolic engineering for improved hoppy flavored beer	Machine learning	*S. cerevesiae*	[29]
Fluxomics + Metabolomics +Transcriptomics	LC-MS/MS, LC-RID, 13C-MFA, RT-qPCR	Metabolic engineering for improved production	Principal component analysis, Constraint-based modeling and Metabolic flux analysis	*E. coli*	[32]
Trancriptomics + Proteomics + Metabolomics	LC-MS/MS, DNA Microarray, GC-MS	Host response to toxicity during deconstruction and utilization of bioenergy relevant feedstock	Functional enrichment using PIANO package and Cytoscape	*C. thermocellum*	[27]
Proteomics + Metabolomics + Fluxomics	LC-MS/MS, 13C-MFA	Metabolic segregation during co-utilization of different carbon sources	Constraint-based modeling and Metabolic flux analysis using 13CFLUX2	*P. putida*	[40**]
Transcriptomics + Proteomics + Metabolomics	LC-MS/MS, RNA-Seq	Toxicity tolerance	Principal component analysis, Functional enrichment analysis using clusterProfiler and Cytoscape	*E. coli*	[25*]
Transcriptomics + Metabolomics + Phenomics	LC-MS/MS, GC-MS, RNA-Seq	Global Metabolic Regulation under industrially relevant condition for enzyme production	Principal component analysis, Functional enrichment, Constraint-based modeling using COBRA Toolbox	*A. niger*	[60]
Transcriptomics + Proteomics + Metabolomics	LC-MS/MS, RNA-Seq, RT-qPCR	Effect of phosphate limitation on lipogenesis	Differential gene expression analysis, Enrichment analysis using WEGO software, Principal component analysis	*R. toruloides*	[41]
Transcriptomics + Proteomics + Metabolomics	LC-MS/MS, RNA-Seq, GC-MS	Effect of carbon or nitrogen limitation on lipogenesis	Gene set analysis using PIANO package, Constraint-based modeling	*Y. lipolytica*	[42]
Genomics + Transcriptomics + Proteomics + Metabolomics	NGS, LC-MS/MS, RNA-Seq, GC-MS	Metabolic trade-offs of glycerol utilization	Differential expression analysis using DEseq package, HTSeq python library, Constraint-based modeling	*S. cerevesiae*	[43*]
Genomics + Transcriptomics + Metabolomics + Fluxomics + Phenomics	NGS, LC-MS/MS,13C-MFA, RNA-Seq	Mechanism to overcome cytotoxicity due to loss of a major metabolic gene	Constraint-based modeling and Metabolic flux analysis using INCA	*E. coli*	[35]
Genomics + Transcriptomics + Metabolomics + Fluxomics + Phenomics	NGS, LC-MS/MS,13C-MFA, RNA-Seq	Mechanism to overcome metabolic imbalance due to loss of a major metabolic gene	Constraint-based modeling and Metabolic flux analysis using INCA, Biopython	*E. coli*	[36]

652