Trends in Microbiology

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REVIEWS

Review

Engineering Robust Production Microbes for Large-Scale Cultivation

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Systems biology and synthetic biology are increasingly used to examine and modulate complex biological systems. As such, many issues arising during scaling-up microbial production processes can be addressed using these approaches. We review differences between laboratory-scale cultures and larger-scale processes to provide a perspective on those strain characteristics that are especially important during scaling. Systems biology has been used to examine a range of microbial systems for their response in bioreactors to fluctuations in nutrients, dissolved gases, and other stresses. Synthetic biology has been used both to assess and modulate strain response, and to engineer strains to improve production. We discuss these approaches and tools in the context of their use in engineering robust microbes for applications in large-scale production.

Highlights

Strain engineering in the laboratory often does not consider process requirements in larger-scale bioreactors.

Systems and synthetic biology can be applied to design microbial strains that allow reliable and robust production on a large scale.

Commercial microbial platforms should be selected and developed based on their relevance to final process goals.

Challenges during Industrial Scale-up

It is well recognized that microbes can produce a vast range of compounds, from fuels and commodity chemicals to pharmaceuticals and fine chemicals [1–3]. Correspondingly, efforts to broaden the scope and demonstrate proof-of-concept pathways for new molecules are gaining in number and scope [2,4,5]. However, despite the development of microbial platforms to convert nearly any carbon source into any desired product, a rather modest number of these cases have seen successful transition to industrial-scale processes and marketed products. Economic competitiveness with established chemical or biosynthetic routes is an important factor. Low titers and yields in the laboratory setting also need to be overcome to proceed with the scale-up. However, at the level of core bioconversion technology, a dominant cause for this dearth of implementation is the challenge associated with predicting the strain performance in industrial-scale bioreactors. The environment of a commercial-scale bioreactor is drastically different from that of laboratory-scale cultures such as shake flasks. It has long been recognized that most strains do not perform the same way in the two scenarios [6]. Thus, strain development for industrial scale-up necessarily goes beyond pathway engineering [7,8]. A recent review places the cost of scaling-up a large-volume chemical in the range of $100 million to $1 billion USD [9]. The risk and cost of failure is high, and de-risking of the microbial chassis itself should be an important aspect of strain engineering.

Process engineering practices for industrial scale-up of a microbial production system are fairly well established, with multiple reviews written on the topic [10–12]. In contrast, the response of microbial phenotype to fluctuating bioreactor conditions, genotypic drift during long-term cultivation, and other physiological factors has been less explored, and the potential impacts of such factors on scale-up performance have only recently been addressed [8,13]. The best efforts in this area are in scale-down models, where large numbers of strains are generated and tested under conditions representative of large-scale growth and production, allowing better

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selection of strains that will scale up more predictably [14–18]. However, it remains difficult to develop strains that will maintain the desired production characteristics when scaled up.

Advances in systems and synthetic biology [8,19] may be able to provide solutions in this space. Beyond improvements such as the use of strains without plasmids or inducer requirement, there are some ambitious questions to be posed. Can we create genetically and phenotypically stable strains to prevent a phenotypic drift during subculturing of the seed cultures? Can we reliably increase the duration of the production phase to increase the profitability of the process? Can we track subpopulations and the causal parameters for phenotypic drift or heterogeneity within the culture? Can we dynamically regulate genes and pathways to respond to inhibitory byproducts and fluctuating stresses to reduce negative selections and enhance robust strain performance? There are examples of studies that address these questions individually and which, when presented collectively, reveal the full potential of the approach.

Most ambitiously, we envision microbial engineering approaches that can predict how a strain will behave in a commercial-scale bioreactor environment and that will enable us to pre-emptively design strains that meet those needs (Figure 1). In this review, we focus on systems and synthetic biology methods that may allow a better understanding of physiological changes

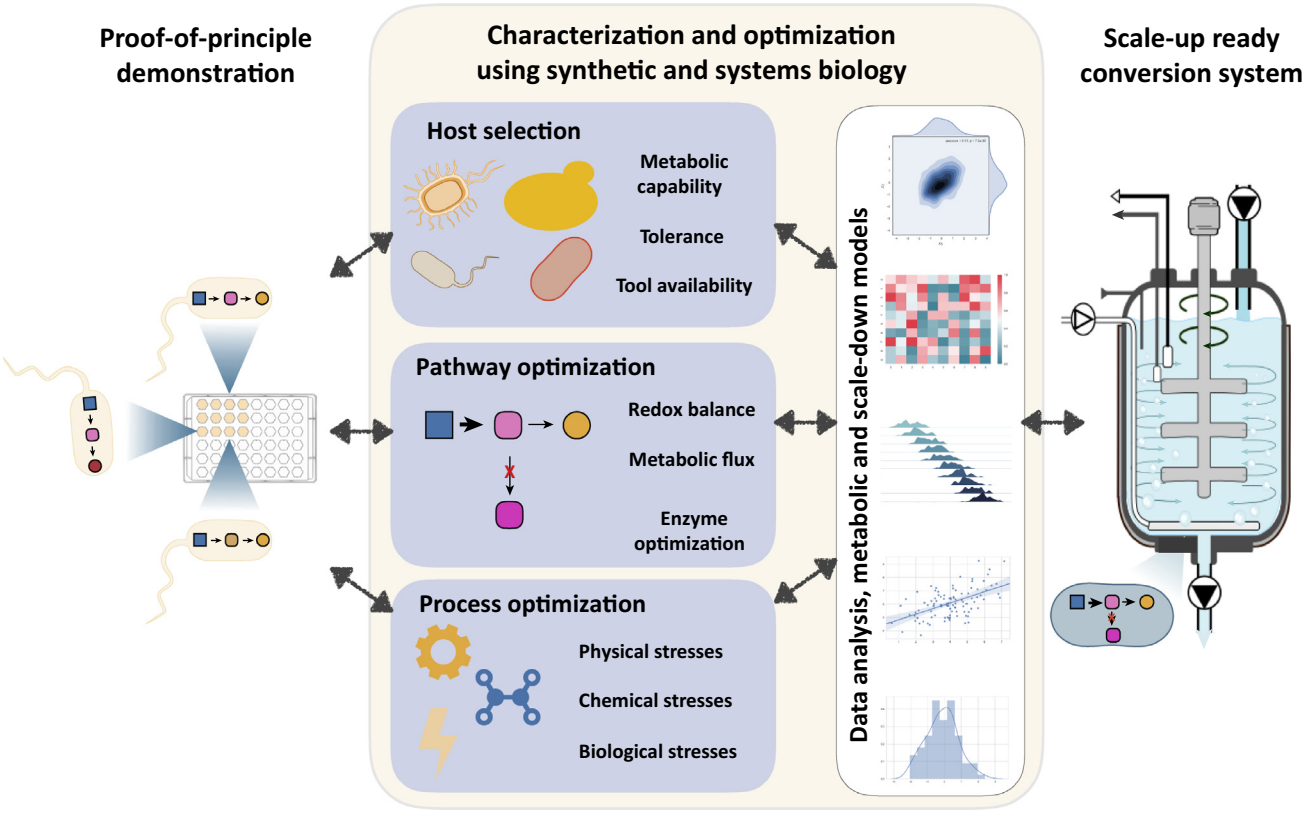

Figure 1. Workflow from Proof-of-Principle Strain Development to an Optimized Conversion System for Industrial Scale-up. Strains exhibiting the desired phenotype at laboratory scale can be further examined through –omics and other analytical approaches to compare biological states at higher scales. Host selection, pathway optimization, and process optimization can and should be geared towards titer, yield, and rates, and also robustness in commercial-scale performance.

during scale-up and under industrial conditions, and synthetic biology methods that may provide ways to mitigate or control these responses. Though these methodologies are as yet underutilized, there are some excellent examples that illustrate the potential of these approaches in predictive design of engineered strains.

Scale-up Parameters That Are Not Accounted for in Laboratory-Scale Strain Development

A wide range of chemical, physical, and biological factors can negatively impact microbial growth and product formation during bioprocess scale-up from microtiter plates, to shake flasks, to bench-scale bioreactors, and ultimately to commercial bioreactors. Compounding this issue is the fact that mimicking the environments encountered in these different stages of scale-up during high-throughput phenotypic screening in the initial strain engineering phase is not straightforward. Moreover, the associated challenges that need to be addressed during scale-up vary for different host microbes and cultivation products.

Furthermore, many measurements performed in microtiter plates and shake flasks are different from the analyses conducted during bioreactor studies (Figure 2). This exacerbates the risk that laboratory-scale strain optimization does not address or resolve challenges that will subsequently be seen as bioprocesses are scaled toward commercialization.

Physical and Mechanical Stresses

One conspicuous difference between laboratory-scale and large-scale cultivation is the operating pressure. As bioreactor volumes increase, the height of the water column in a bioreactor creates an increasing hydrostatic pressure gradient. In principle, pressure can influence biological properties, including enzyme activity and cell membrane permeability that are important to cell viability and metabolic flux [18]. The increased hydrostatic pressure at the bottom of a commercial-scale bioreactor raises the concentration of dissolved gases. For instance, dissolved CO_2 (dCO_2) is in equilibrium with bicarbonate and carbonate ions which contribute to medium osmolarity and affect broth pH. Thus, the physiological effect of dCO_2 could be a result of the accumulation of the dissolved gas itself, of osmotic pressure changes, of pH changes, or even a combination of all three factors [20].

Other colloidal or thermodynamic properties, such as broth bulk viscosity, emulsion stability, and product or biomass settling, could also be influenced by pressure, among other factors. Adverse effects on the cultivation may also persist during product harvest and impact the efficiency and quality of the downstream process (e.g., solid–liquid separation, chromatography, extraction, crystallization, distillation, chemical upgrading, and polymerization), resulting in higher production costs and reduced product quality [21].

Chemical Stresses

With increasing bioreactor volume, the mixing time increases from a few seconds (laboratory-scale) to several minutes (hundreds of cubic meters) [6,12]. Furthermore, other bioreactor internal components, such as spargers, baffles, and cooling coils, can create dead zones with poor mixing, heat transfer, and gas–liquid mass transfer. In large-scale production processes imperfect mixing results in microenvironments and inhomogeneities, resulting in gradients of bioprocess parameters such as pH, temperature, dissolved oxygen (DO), dCO_2, and the concentration of nutrients. Combined, these variations could lead to transient or permanent insults such as oxidative damage, nutrient limitations, and other stress responses that reduce microbial viability, stability, and productivity [22].

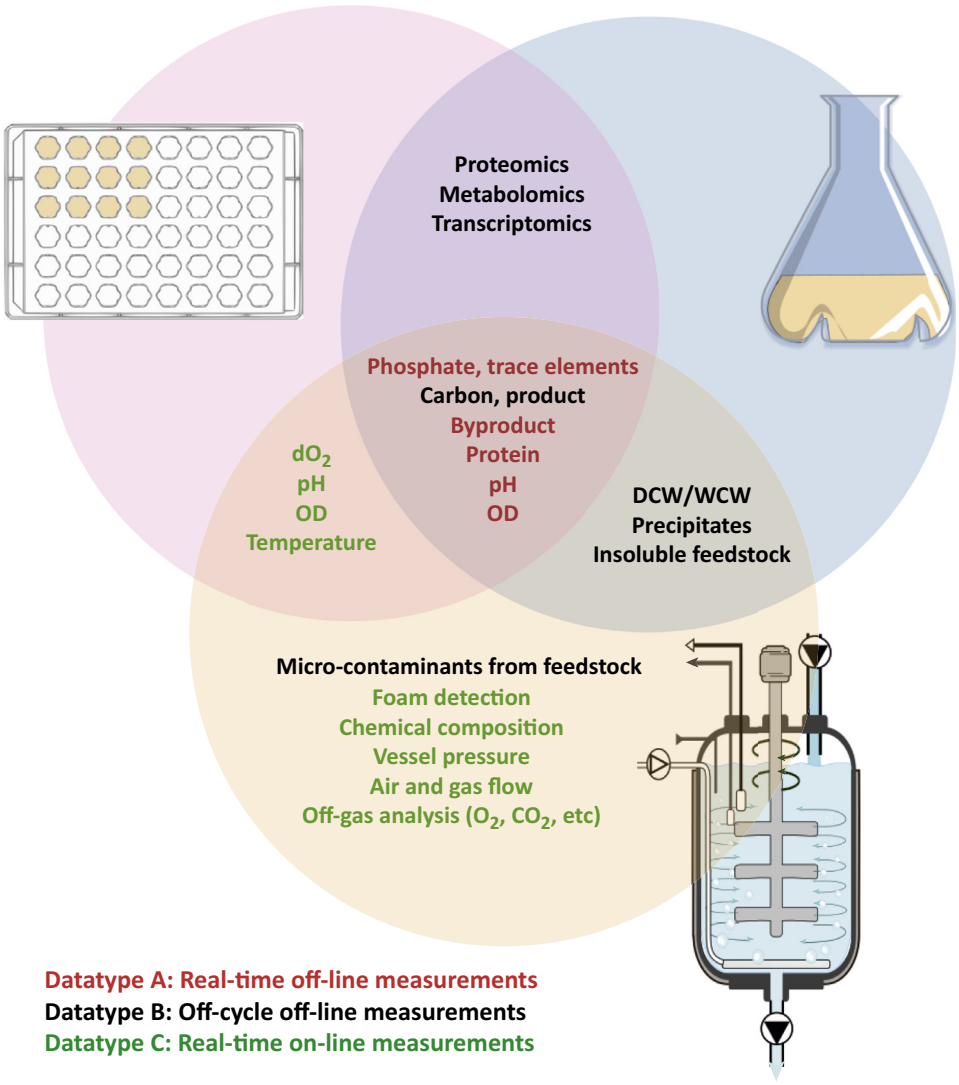

Figure 2. Measurements during Strain Development in Microtiter Well Plates, Shake-Flasks, and Bioreactors. The measurements are not only different, but they also vary in availability. Whereas high-throughput studies at laboratory scale, such as in microtiter well plates (e.g., flower plates from BioLector Pro®) and shake-flasks, enable testing of a large number of conditions and strains in parallel, bioreactors offer an opportunity for time-series testing but at very low relative throughput to compare strains and operating conditions. DCW, dry cell weight; OD, optical density; WCW, wet cell weight.

Additional chemical stresses in large-scale industrial cultivations arise from raw material and microbial contaminants. To reduce production costs, many processes utilize crude raw materials which are utilized with little or no refinement and thus introduce impurities that accumulate in the broth to inhibitory/toxic levels. Some examples of these real-world feedstocks include corn slurry, corn steep liquor, sugar cane juice, molasses, sugar beet juice, agricultural residues, food-processing waste, municipal solid waste, and waste gases. Microbial contamination of the raw materials, harsh raw material pretreatment conditions, and microbial contamination of the cultivation can further increase the levels of deleterious

impurities and reduce product titer rate yield (TRY) metrics [22–26]. These factors can and should be addressed in host strain selection and strain-screening assays, but the priority may be underappreciated and thus the sensitivities understudied at the early stages of a strain/cultivation development program where sterile technique and very pure raw materials are commonly used during small-scale screening and optimization. If strain engineering and strain screening could account for these risks and mitigate them from the outset, process scaling would become more predictable, especially in the case of developing novel microbes as production platforms.

Systems Biology Approaches Can Generate Actionable Knowledge to Address the Effects of Scale-Related Factors

Exposure to heterogeneous conditions in large fermenters can trigger genetic and physiological responses in production microbes [18,27,28]. High-throughput analytics, such as automated DNA sequencing and –omics technologies (fluxomics, transcriptomics, proteomics, and metabolomics), as well as high-throughput screening platforms, enable these to be characterized by simulating industrial conditions in scaled-down laboratory experiments. Mathematical models of large-scale fermenters provide important guidance in designing the scaled-down conditions and in forecasting the impact of small-scale learnings in the large-scale environment. This approach increases the probability that key performance metrics, such as TRY, will translate fully and quickly from the laboratory to the manufacturing plant, thereby minimizing start-up costs [8,29]. Heterogeneity in gas mixing, in substrates and nutrient, as well as in the inherent heterogeneity in a microbial population have all been examined in this context.

Gas Mixing

Gas mixing, such as for CO_2 and O_2, is a key parameter resulting in measurable differences in microbial cell response in a bioreactor. In recombinant *Escherichia coli* cultures grown in batch cultivation, increasing dCO_2 in the broth resulted in a slower growth rate and higher by-product accumulation [30]. Another study found that fluctuating dCO_2 increased the time needed for *E. coli* to produce green fluorescent protein (GFP), and to observe a transcriptional response of glutamate decarboxylase and α-ketoglutarate dehydrogenase [15]. A comparative analysis of transcript levels revealed a fast transcriptional response of *Corynebacterium glutamicum* to alternating CO_2 and bicarbonate levels, which appeared to be strongly correlated with both strength and duration of the pCO_2 stimulus [31]. The authors observed increased expression of genes encoding enzymes necessary for oxaloacetate production and hypothesized this to be the result of rapid enhanced bicarbonate availability for PEP/pyruvate carboxylation. The first findings of the impact of oxygen supply were reported by Oosterhuis *et al.* [32]. In these early studies, *Gluconobacter oxydans* was cycled between zones with low and high oxygen availability to study the effect on gluconic acid production. More recent studies used similar approaches to study the effect of oxygen availability on intracellular processes in *E. coli* and *C. glutamicum* [18,33–36]. In these studies, transcriptional changes between aerobic and anaerobic environments explained observed alterations in stoichiometry and kinetics, as well as production of ethanol and organic acids, indicating that *E. coli* responds rapidly to dissolved oxygen gradients [33]. Fu *et al*. [37] carried out exometabolome measurements to study the physiological effects of scale-up of a high-density fed-batch process of *Saccharomyces cerevisiae* which revealed reduced oxygen availability at commercial scale, resulting in overflow metabolism. As one approach to mitigate the effects of oxygen availability in large bioreactors, Liu *et al.* introduced VHb (*Vitreoscilla* hemoglobin, a membrane protein facilitating O_2 transport) into a fatty-acid-producing strain of *E. coli* to promote oxygen supply and energy metabolism. The resulting strain yielded a 70% higher fatty acid titer as compared to its parental strain [38]. While host response to such parameters in large-scale production remains a challenge (see

Outstanding Questions), the selection of a host that is natively resilient to such fluctuations [35] and designing processes to overcome metabolic bottlenecks [39] or laboratory evolution [40] are a few approaches that may be used to address this issue.

Substrate and Nutrient Heterogeneity

Besides leading to suboptimal gas transfer effects, imperfect mixing in large-scale bioreactors can impose substrate supply gradients on bioreactor cultures. Recent findings have shown that *E. coli* can adopt both short- and long-term strategies to withstand stress conditions regarding changing nutrient availability [41–44].

In a scale-down study of penicillin production in *Penicillium chrysogenum*, the influence of substrate gradients on process performance and cell physiology was investigated by imposing an intermittent feeding regime on a laboratory-scale culture [17]. The authors observed that, while the biomass yield remained the same, the production of penicillin was reduced significantly in the intermittently fed cultures and based this observation on a reduction in flux of the pathway which was possibly caused by fluctuations in intracellular ATP and AMP levels. In *S. cerevisiae,* genome-wide analysis of transcriptional cross-regulation by different environmental parameters under different nutrient limitations (C,N,P,S) in both aerobic and anaerobic laboratory-scale chemostat cultures identified 155 oxygen-responsive genes and several other genes responsive to different macronutritional limitations [45,46].

Data obtained from studies mirroring cellular responses to typical large-scale stimuli could be used to derive and validate adequate models for *in silico* predictions of commercial-scale performance. These predictions could be used to optimize the bioreactor hardware design configuration as well as the process operating conditions. This would mitigate potential reductions in performance and TRY metrics and could be used to derive design criteria to engineer production strains for improved robustness during commercial-scale manufacturing. Recently, a metabolically structured kinetic model developed for penicillin production by *P. chrysogenum* showed sufficient accuracy to enable the simulation of dynamic metabolic processes at relevant timescales for bioreactor mixing (which can range from seconds to minutes) in commercial-scale fed-batch cultivations [47].

Nitrogen-responsive regulation can be another source of culture heterogeneity or variation, as the C/N balance is known to impact metabolic output. One study in *E. coli* identified that *relA*, the key regulator responsible for the synthesis of signal molecule guanosine tetraphosphate (ppGpp) during the stringent response, is also activated during nitrogen starvation and thus the two major bacterial stress responses are coupled to manage conditions of nitrogen limitation [48]. Michalowski *et al.* combined mechanistic knowledge from this and several other studies to engineer a strain of *E. coli* which maintained a constant ppGpp pool independently of nutritional supply, thus allowing for increased intracellular pyruvate accumulation and greater metabolic flux towards a desired final product [49].

Phenotypic and Genetic Heterogeneity

Stochasticity of gene expression is commonly considered the main source of phenotypic heterogeneity in a microbial population, due to cell-to-cell variation in promoter expression, unequal transporter distribution, or bet-hedging after a diauxic shift [29,50]. For cells grown in large-scale bioreactors, this inherent heterogeneity may be exacerbated by emerging subpopulations associated with insufficient mixing [43,51]. Poor mixing or oscillations in nutrient levels can result in subpopulations of cells in different metabolic states, which in turn affects the activity of native metabolic pathways [52,53]. For instance, stationary-phase cultures

comprised of quiescent and nonquiescent cell populations may have different gene expression and respiratory profiles [52,54] and consequently different production levels relative to a homogeneous culture.

An early report which tried to differentiate the intrinsic heterogeneity of microbial populations from extracellular perturbations originating from bioreactor heterogeneity suggested that environmental fluctuations impact the level of biological 'noise' [55]. Tools for monitoring the physiology of subpopulations can be useful in this area. Recent advances in fluorescence-activated cell sorting (FACS) methods have enabled higher-throughput analysis [56], potentially enabling identification of cellular responses at the single-cell level. Heterogeneity and associated stresses in large-scale bioreactors may cause an increase in deleterious genetic mutations, thereby reducing TRY metrics over extended cultivations relative to laboratory-scale [13]. In addition to these aspects, the metabolic burden from engineered pathways is an important concern and is a well-reviewed topic [57]. The target compound and pathway may further impact phenotypic drift and loss of performance [58]. As a solution, a synthetic dependence on a nonconditional essential gene was used to generate *E. coli* strains with a reduced mutation rate and enhanced production stability [13]. Moreover, genome-scale models of microbial metabolism that take into account spatial and temporal variability in a bioreactor have also been reviewed as an approach to overcome the effects of the bioreactor environment heterogeneity [59].

Selection of the host microbe is an important consideration in developing robust bioconversion processes (Box 1). Some microbial hosts may have native physiological abilities to overcome the bioreactor environment heterogeneity. For example, performance loss during scale-up was explored in *C. glutamicum* producing 1,5-diaminopentane [34]. Systems biology approaches revealed that the central metabolism of *C. glutamicum* is flexibly rearranged under short-term oxygen depletion and carbon source excess to overcome a limitation in NAD^+ recycling. In two-compartment experiments, where dissolved oxygen could be swiftly varied, the authors found that, to balance the redox state, key enzymes for the non-oxygen-dependent fermentative NAD^+ regeneration were significantly upregulated while nonessential pathways were downregulated. Overall, *C. glutamicum* maintained carbon and redox equivalents, and these experiments indicated that it is a robust host that can withstand heterogeneity in large-scale processes.

Approaches to Engineer Strains for Stable and Reliable Production in Large-Scale Bioreactors

A systematic approach to engineering strains for scale-up first requires envisioning large-scale production conditions in order to simulate these conditions at a small scale and then screen for host/pathway combinations that will meet relevant requirements. Chemical inhibition (by media components, metabolites, and products) and the dependence of production on growth are two considerations that can be addressed via systems and synthetic biology approaches in small-scale screening environments.

Tolerance Engineering and Growth-Coupled Production

Engineering microbial strains for improved tolerance to raw materials, metabolic intermediates, by-products, and final products has been undertaken in a large number of cases using both targeted and combinatorial methods. Protein targets for tolerance engineering range from chaperones to transporters and may be identified by screening natural diversity or by directed evolution experiments. Since tolerance phenotypes require consolidation with production phenotypes, semitargeted approaches have also been developed to obtain mixed phenotypes such as via transcription factors and global regulators. Tolerance engineering has been

Box 1. Choice of the Host Microbe

The choice of the microbial host has a critical bearing on the robustness of process scale-up. Model microbes, *Escherichia coli* and *Saccharomyces cerevisiae*, are often the default hosts for laboratory synthetic biology studies. However, microbial systems used in the industry for large-scale production, and for demonstration at pilot-scale, reveal a larger set of host choices based on diverse metabolic capabilities and phenotypes (Table 1). An excellent example for host selection based on process requirements is the CB1 yeast strain developed by Cargill for lactic acid production. Though the initial process used lactobacilli [89], issues of contamination (including phage), the need for expensive broth neutralization, and associated salt waste motivated the isolation and engineering of the organic-acid-tolerant CB1 yeast, which bypassed both of these issues[ii].

Selection and engineering of the host strains is an essential aspect to ultimately ensure not only commercial relevance but also the optimum economic outcome [9]. This involves many considerations beyond the engineered pathway, as discussed in this review. If the microbial host lacks one or more of these phenotypes natively, it necessitates process accommodations and/or results in performance deterioration that increase production costs. Even when the ultimate commercial consequences are recognized, the choice of host microbe often tracks with microbes for which we have the most genetic tools and knowledge. This bias holds especially true when the top priority is the discovery and development of the metabolic conversion pathway in highly controlled laboratory conditions. Evaluating and optimizing an engineered strain for performance during scale-up and in industrially relevant conditions are too often deferred or even ignored altogether.

Postgenomic era approaches now allow us to evaluate the suitability of a microbe for a particular scale-up process upfront and may neutralize the current bias towards model microbes. As examples, retrosynthetic-based pathway predictor algorithms that reveal the metabolic potential of a microbial host are invaluable to guide this choice [5,90]. Another example is an evaluation of growth couple-able metabolites in a given microbe [65], which would reveal critical caveats in selecting a host, such as its ability to maintain growth-coupled production under different respiratory modes. These resources will become increasingly available in a larger set of microbes as they are adopted for genome-scale modeling [91] and functional genomics evaluations (RB-TnSeq, [92]), and allow de-risking of the host microbe itself, separate from the considerations for product pathways.

It is worth recognizing that, as we attempt to convert increasingly complex and crude carbon sources to larger groups of final products, no one microbe will natively provide all the desired properties for an optimum process. But with many more well-developed, well-understood hosts, including systems biology data on how strains will behave in a scaled-up format, the options improve and the choices become better informed.

reviewed recently [8,26]. Genome-wide approaches for tolerance engineering include laboratory evolutions [60], and a sophisticated version of this emerging approach is automated laboratory evolution (ALE). ALE shows potential in a wide range of applications such as improving and broadening catabolic potential and tolerance to different cultivation conditions and metabolites [25,40,61]. In a recent example, ALE was applied to improve strain tolerance to catechol, a toxic intermediate arising during the conversion of renewable plant biomass and known to negatively affect product titers. Correspondingly, the adapted strain, with increased tolerance to catechol, showed improved production in the presence of this inhibitor [62]. However, these strains have yet to be tested under large-scale conditions.

Product tolerance and the growth dependence of production are related concepts in strain engineering. If production is independent of growth requirement, growth inhibition due to expression of a burdensome pathway and/or production of an inhibitory compound can cause strains to drift from their optimal production phenotype due to selective advantage. Therefore, engineering strains with increased tolerance to stresses and inhibitors is critical where production is independent of growth requirement. However, if applicable, coupling growth with production is a powerful strategy for evolving strains to maintain high titers, rates, and yield. For example, the Optknock® tool uses metabolic models to identify gene deletion targets that couple growth and product biosynthesis, making the production of the target compound obligatory. The Optknock® approach to predict gene deletions in *E. coli* that successfully coupled succinate overproduction with growth [63] is an example of systems-biology-aided strain improvement (Table 1). Using similar tools, 1,4-butanediol production is an example of overcoming final product toxicity to meet commercial TRY goals using *E. coli* as a production

Table 1. Examples of Production Scale-up[a] for a Range of Engineered Microbial Strains

Microbe	Scale (l)	Titer rates and yields and source[b]	Product	Company	Refs/resources
Escherichia coli	13 000[c]	99 and 2.1 g l^{-1} 0.35 g/g (glucose)	1,4-Butanediol	Genomatica	[63,64]
Saccharomyces cerevisiae	300[d]	900 g l^{-1} Cane Syrup	β-Farnesene	Amyris	[iii]
CB1 yeast	1 000 000	Glucose	Lactic acid	Cargill	[ii]
Corynebacterium glutamicum	1500	92.5 and 81.2 g l^{-1} 0.35 g/g glucose	l-Arginine		[93]
Pseudomonas putida	500	52% (w/w) (octanoic acid and levulinic acid)	Polyhydroxy alkanoate		[94]
Rhodosporidium toruloides	20	2.2 g l^{-1} (sorghum hydrolysate)	Bisabolene		[87]
Mannheimia succiniciproducens	7	15.4 g l^{-1} and 0.86 g/g glucose	Succinate		[95]

[a]Ranging from demonstration-scale bioreactors to commercial-scale.
[b]As available.
[c]Current scales for 1,4-butanediol production are at 200 000–600 000 l.
[d]Results from pilot-scale in a report[iii]. Commercial scales for β-farnesene production are at 200 000 l.

host [64]. Applying genome-scale metabolic models, studies have shown that coupling of growth and production is feasible under appropriate conditions for almost all metabolites in five major production organisms [65]. Emerging applications seek to combine growth-coupled production with laboratory evolution experiments, leveraging these two powerful approaches to rapidly generate high producing strains that maintain the desired phenotype.

Growth-Decoupled Production

For some metabolic targets, it is not possible to biochemically couple the target metabolism with a growth dependence. In other cases, efficient growth coupling may be restricted to certain cultivation conditions [65]. Consequently, selective pressure is needed to maintain the production pathway and to avoid selecting for strains with improved growth at the expense of production [8,66]. In these cases, decoupling growth from production is seen as an alternative strategy to maintain stable strain performance during production.

One approach to decouple growth from production is to activate production pathways only after a culture reaches a cell density threshold or stationary phase [67]. An example of the former is the development of an *E. coli* strain engineered to induce sustained glucose conversion to fatty alcohols during nitrogen-limited stationary phase [68]. In another recent example of growth-decoupled production [69], vanillin production from ferulic acid was limited to the stationary phase by coupling vanillin production to glucose starvation, after a high biomass had been reached. Starvation-inducible promoters, such as *csiD*, used in this case, are easily adapted to constrain activity of a heterologous gene pathway to stationary phase. However, as *csiD* is expressed only in response to glucose starvation [70], such a sensor would have to be further modified for use in fluctuating levels of glucose present in large-scale bioreactors. In this regard, dynamically activated pathways in response to intracellular inhibitors [71], cell density [72], and nutrients [73] have also been used for decoupled approaches. Most recently, a synthetic biology approach that combined a quorum sensing-based and biosensor-based system was used for mutually independent and tunable dynamic regulation of two different enzymes to improve heterologous production of d-glucaric acid in *E. coli* [74], resulting in the highest reported titers for this compound.

Biosensors/Biocontrollers

Biosensors, such as RNA aptamers or proteins which bind to small molecules and elicit a transcriptional or allosteric response, are key synthetic biology tools and have been comprehensively reviewed [75–77]. As noted in earlier sections, applications of biosensors range from reporting metabolic states or heterogeneity in cellular response to being coupled to regulation, enabling a dynamic response to stresses. Multiple biosensors have been discovered and successfully developed that regulate genes for optimal expression and reduce inhibitory intermediates or toxic proteins, without the aid of inducers [78,79]. There are biosensors and corresponding regulators that control pathways by responding to metabolic switches [80]. Biosensors have also been used to consolidate multiple signals. These complex biosensor circuits can be deployed for sophisticated feedback regulation to maintain the response of an engineered system in a heterogeneous bioreactor environment. An example of such a circuit is a 4-input AND gate with 3 circuits that controls 4 inducible systems, thus integrating the response from up to four different signals [81]; it could be used to report bioreactor stresses or developing strains for scale-up. The efficacy of such genetic circuits has been evaluated for their applicability in bioreactors [82]. Biosensors that can report on the target metabolite production, especially those that can otherwise be detected only via lower-throughput analytical methods, are also of value – for example, yeast G-protein display systems [83] and evolved sensors [84]. A potential pitfall in the use of these designed systems is that circuits using biosensors to regulate pathways or to develop reporter systems suffer from interference from cellular physiology and cross talk with other synthetic circuits. These shortcomings have begun to be addressed via automated gene circuit design platforms [82,85] to enable construction of circuits that are insulated from each other, presenting a new and challenging area of investigation.

Tools That Provide the Data for Systems and Synthetic Biology Approaches

Unlike microtiter plates and shake flasks, well-instrumented bioreactors provide real-time measurements of DO and gas metabolism that are used to calculate oxygen transfer rate (OTR), oxygen uptake rate (OUR), carbon dioxide evolution rate (CER), and respiratory quotient (RQ). These parameters are representative of metabolic activity and can be controlled by adjusting air flow and nutrient feed rates. However, these measurements are based on the liquid and gas phases of the bioreactor and do not adequately represent the various cellular states of the potentially heterogeneous culture. As such, multi-omics comparison of laboratory-scale and bioreactor cultures can help to identify additional indicators of scale-up performance, for example genetic drift, differences in metabolite levels, protein stability changes, oxidative damage, and gene-expression perturbation. In turn, these indicators can be applied in laboratory-scale engineering of strains for more predictable scale-up performance. Bioreactors offer an opportunity for time-series testing, due to a higher level of process measurement and control. However, since bioreactor operation can be an expensive endeavor, the scope of testing process conditions can be very limited when compared to laboratory-scale strain performance screening in plate- or flask-based assays. Coordinated analysis of real-time measurements from a bioreactor together with multi-omics approaches can provide substantial information on growth and production performance sensitivities across a range of cultivation scales.

Bioreactor-based process development studies are resource intensive, limiting our ability to replicate multiple experiments. Recent high-throughput growth measurement plate designs cater to both laboratory-scale requirements of low volume and high-throughput testing while providing several bioreactor-scale datatypes. The flower plate-based BioLector Pro® system from m2p Labs Inc. is equipped with pH, fluorescence (for optical density), and DO optical

probes (optodes) to measure and control these parameters that are otherwise unavailable in shake-flask culturing [86]. Mixing and off-gas data available in 250 ml Ambr® high-throughput, multiparallel bioreactors systems[i] can further bridge the gap by introducing shear effects and OUR-based control of the cultivation process that mimic pilot-scale bioreactors. HEL's Bio-Xplorers can be used to conduct fed-batch cultivations at pressures up to 5 bar. By working with these 1–250 ml size reactors, researchers can perform reasonably high-throughput cultivations in more commercially relevant conditions.

Miniaturizing commercial-scale culture conditions and the associated cost-effective, high-throughput capabilities can significantly increase the quantity of parameters tested at laboratory-scale. This has allowed testing new hosts for performance at different scales [87] (Table 1). However, the traditional statistical design-of-experiments (DOE) approach to process development is often limited by researchers' understanding of commercial-scale conditions. The quality of parameters selected for testing should also be improved along with the quantity of parameters being tested. For example, one study used computational fluid dynamics to simulate 54 000 l cultivation with *P. chrysogenum* to predict gradients of glucose, dissolved oxygen, etc. to select parameters and their levels needed to test a pool of mutants for production robustness [88]. By 'beginning with end in mind', strain developers can identify relevant parameters (stresses from thermal, mechanical, and chemical sources; gradients of oxygen, CO_2, glucose, etc.; toxicity from water, substrates, intermediates, products, etc.) and develop strategies (dynamically regulated pathways, growth coupling/decoupling) and analytics (sensors and biosensors) needed to develop strains for bioprocessing with high titer, rates, yields, and robustness in production.

Concluding Remarks

Many research efforts are now focused on developing strains that address challenges specific to industrial scale-up [22,96]. Genomatica's announcement that production of biobutanediol using its technology exceeded 10 000 tons[iv] is a key milestone in demonstrating both systematic strain engineering and the application of interdisciplinary tools to achieve industrial biobased production.

In the current state of the art, synthetic and systems biology tools are increasingly enabling characterization of strain physiology under industrially relevant conditions in a bioreactor. Various methods already exist to engineer or evolve strains that are robust under these conditions, including methods to couple production phenotypes with growth. Miniaturization and automation of strain design, strain construction, and high-throughput measurements provide the data and data-driven approaches to translate production performance from laboratory to pilot and commercial scales. Implementation of advanced statistical methods, such as machine learning, can expedite strain-engineering cycles [80,97,98] and are being adopted by the biotech industry focused on high-throughput strain engineering [99,100]. The state of computational approaches and modeling has not been a focus of this review but has been discussed recently in several comprehensive articles [8,101].

The recent establishment of microbiological platforms for classes of bioproducts provides a promising outlook for more rapid introduction of additional new products with lower R&D investment and risk. Synthetic and systems biology tools could be used as the starting point to assess physiology of the strain for suitability of performance in the industrially relevant conditions of a bioreactor (see Outstanding Questions). As our mechanistic understanding of the biological processes grows stronger, and our ability to use large systematically collected data sets becomes more sophisticated, microbial conversion can provide powerful implementable solutions for large-scale biomanufacturing of commodities, materials, food, pharmaceuticals, and fuels.

Outstanding Questions

What are the best measures to ensure that process performance goals drive the design of the microbial conversion systems that are being developed for commercial applications?

How can laboratory experiments be conducted to better reflect key aspects of growth and metabolism in larger scales and regimes? While some approaches exist, this topic remains challenging.

What key phenotypic requirements, other than the development of a bioconversion pathway, should drive our selection or engineering of a host microbe? Examples may include product and inhibitor tolerance, feedstock utilization, genetic stability, and metabolic robustness with respect to heterogeneous conditions and process fluctuations.

What types of data sets and tools will allow us to better assess host requirements for a given process and facilitate the use of alternative production hosts?

Some parameters in large commercial bioreactors remain especially challenging as factors that negatively impact productivity. These include fluctuations in oxygen transfer rate due to heterogeneous mixing and vertical pressure gradients. Can we identify or engineer microbial strains to overcome these impediments?

Can microbes be engineered not only to improve titers, rates, and yields at the laboratory scale, but also scalability?

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Resources

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