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Mitigation of host cell mutations and regime shift during microbial fermentation: a perspective from flux memory

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Microbial engineering forces flux redistribution to accommodate higher production rates, straining the cellular supply chain and leading to growth deficiency. Thus, there is a selective pressure to alleviate metabolic burden and revert towards the innate flux distribution ('flux memory') via mutations. Suboptimal fermentation exacerbates this phenomenon as increased number of generations prolong the selection window for the underlying flux memory to generate faster growing non-producers. New strategies to mitigate host genetic instability include laboratory evolution, high-resolution genome resequencing combined with phenotype screening, mismatch repair protein engineering, and advanced synthetic biology approaches (e.g. oscillators and biosensor regulators). Moreover, ^{13}C-metabolic flux analysis can quantify flux suboptimality driven by metabolic burdens and cultivation stresses. Elucidation of correlations between metabolic suboptimality and host mutation rates/spectra may lead to early stage risk assessments of culture-population's regime shift during process scale-up as well as strategies to boost bioproductions.

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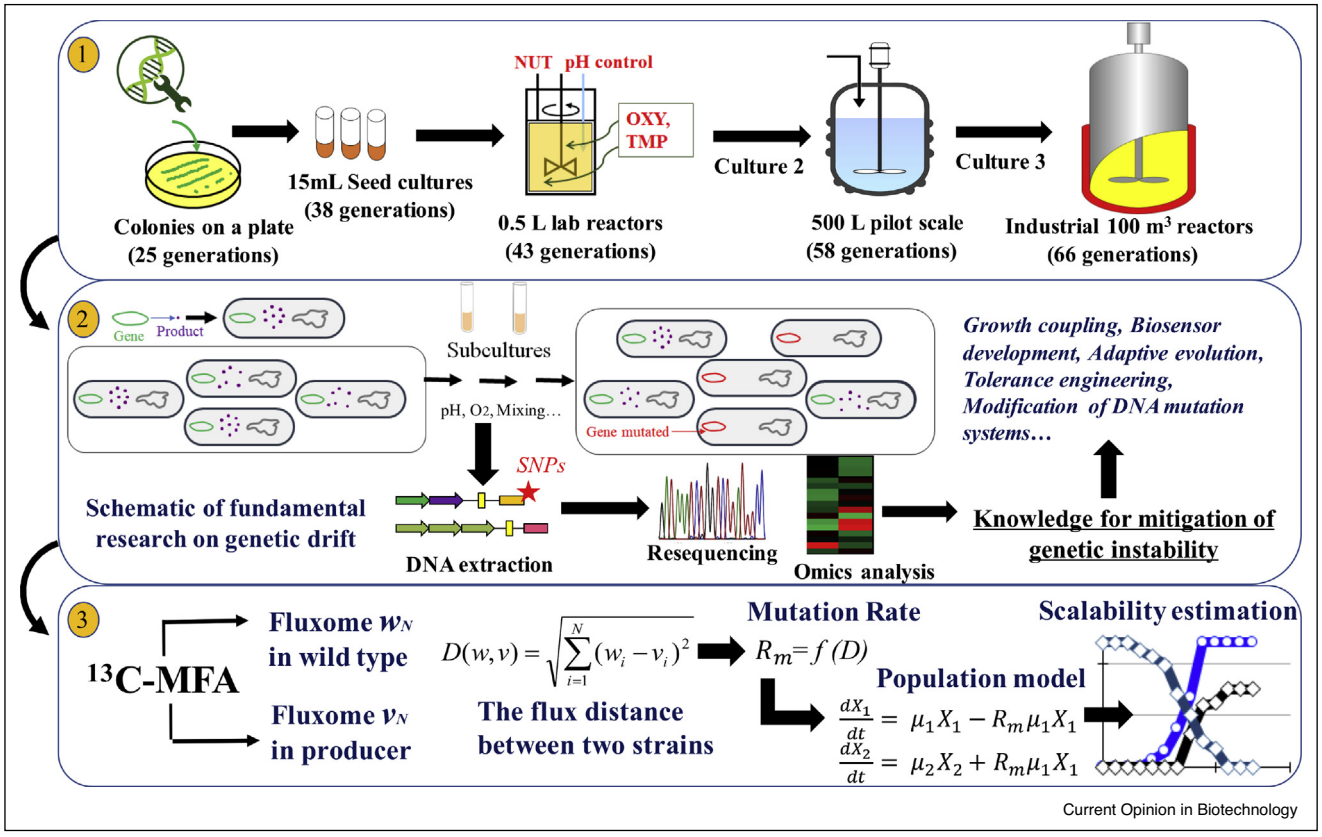
Introduction

Current advances in synthetic biology and automation have enabled the generation and screening of large microbial host libraries, and quite a few engineered microbial strains have reached industrial-scale biomanufacturing [1,2]; however, microbial cell factory development still faces economic challenges from relatively low strain-productivity and stability exacerbated by process variations from the design set points. The most straightforward strategy for improving fermentation scalability is to reduce bioreactor stresses (e.g. optimize O_2, pH and substrate gradients) [3•,4,5], and tools such as computational fluid-dynamics coupled with kinetic models have been developed to guide bioreactor operations [6]. However, even the most optimal large-scale reactor systems face severe heterogeneity in growth conditions as well as locally induced stresses. Systems biology may resolve production bottlenecks under stress conditions, complementing tolerance engineering [7] and adaptive laboratory evolution [8,9] to facilitate the moving of strains to industrial-scale applications. However, genetic divergence (random genetic alternations) is ubiquitous, leading to permanent loss of production during industrial fermentations. Genetic mutations are difficult to handle due to their stochastic nature. As a result, promising laboratory strains may exhibit poor productivity during commercialization. Because the risk for companies is considerable owing to the high capital costs (typically above $100 million) for implementing bioprocess development, host strains must be carefully designed and rigorously tested for robustness and compatibility with large-scale production [3•,4,5]. Scaled-down fermentations combined with systems biology methods gain insight into strain stability, but many aspects of how metabolic burden and environmental stresses drives genetic divergence remain elusive.

Genetic divergence in engineered strains

The process of DNA replication is inherently error prone and leads to spontaneous mutations during cell replication. A study examining mutation accumulation in wild type *Escherichia coli* over 20 years found that mutations occurred at a rate of 10^{-9} per base pair per generation [10]. At the typical *E. coli* production cell density, there are 2^{43} cells ($\sim 10^{12}$) in a 0.5 L bioreactor. At the basal mutation rate of 10^{-9} mutations per base and a 4.6 million bp genome, $\sim 5 \times 10^9$ cells in the next division will have one mutation (the so-called 'single nucleotide polymorphism' or SNP, denoted with a star, Figure 1). Considering an average sized bioproduction gene of 1 kb ($\sim$300 amino acids), $\sim 10^6$ cells will have at least one mutation across the coding region of that gene. For synthesis of products

Figure 1



Summary of mechanisms and modeling perspective of genetic drift in engineered strains.
For bioreactor production, it takes a single *E. coli* cell ~43 generations to reach typical production ODs in 0.5 L bioreactors and a further 23 generations for production in industry-scale reactors (100 000 L) [15••]. During this process, engineered hosts have numerous possibilities for genetic mutations [16] that lead to non-producing cells. Sequencing populations after exposure to a variety of stresses (e.g. pH, oxygen, and shear) can provide insights on likely mutational responses. Further combination of sequencing data with ^{13}C-MFA can lead to the development of models that can predict strain mutation rates.

involving multiple genes, the risks of mutations in the production pathway are accordingly increased (Table 1).

While mutation rates seem intrinsically low, mechanisms exist for mutations to occur at elevated frequencies during stressed conditions [11,12]. The increased rates can be mapped to specific pathways, such as stress response, DNA repair and respiration, as was shown for *Saccharomyces cerevisiae* growing under high ethanol levels [13]. Furthermore, species-specific factors can shape the mutational landscape, including chromosome size, GC content and mobile elements within the genome [14], causing some species to be more prone to genetic mutations. Quantifying these factors will help inform decisions on choices of microbial chassis.

In addition to increased mutational frequencies in response to stress conditions, spontaneous mutations can occur at higher rates in overexpressed bioproduction genes [15••]. Bioproduction gene mutations can lead to

Table 1

Spontaneous mutagenesis rates based on genome sizes in bioproduction hosts

Organism	Genome size (bp)	Genes	Assumed mutation/bp	Mutation/genome	Mutation/gene
E. coli K12	4.6×10^{6}	4377	1.0×10^{-9}	4.6×10^{-3}	1.1×10^{-6}
S. cerevisiae	1.2×10^{7}	5770	5.0×10^{-10}	6.0×10^{-3}	1.0×10^{-6}
Y. lipolytica	2.1×10^{7}	7864	5.0×10^{-10}	1.1×10^{-2}	1.3×10^{-6}
CHO cells	5.2×10^{9}	24 383	5.0×10^{-9}	2.6×10^{1}	1.1×10^{-3}

non-producing (or lower-producing) populations with alleviated metabolic burden – hence faster growth. Such non-producer (or lower-producing) mutants do not simply impose a suboptimality problem by creating a zero-yield (or lower-yield) subpopulation but can also readily outcompete their producer ancestors after a number of cell generations (Figure 1). Stresses in bioreactors may create 'hypermutable' subpopulations [17], intensifying this outcome. Strains carrying complex engineered pathways will face more modes of deleterious mutations that favor the formation of non-producers. Population heterogeneity and stochasticity of cellular production can therefore be exacerbated via rapid accumulation of mutations during cell multiplication in suboptimal large bioreactors [18,19]. Thus, over the course of scale-up, the fermentation culture will eventually shift its population majority from producers towards non-producers. The daunting challenge of addressing these issues involves a more detailed understanding of the driving forces and mutational landscapes behind the rise of non-producers.

Perspective of metabolic flux memory

It is generally challenging to explore the underlying possibilities of a mutational take-over within a population holistically: metabolic burden, in addition to product toxicity, respiration impairment and stressed cultivation conditions, can all be drivers of increased mutation frequency with convoluted impacts. To capture these synergistic effects, we envision examining the driving force of genetic divergence through the lens of metabolic 'flux memory' (i.e. mutation of the host cells causes metabolic shifts towards the pre-engineered flux network). Metabolic fluxes represent the flow of material through enzymatic steps, while the fluxome covers the complete distribution of fluxes among the reaction network, which is functional output of the integrated response of all levels of cellular regulation systems [20]. Wild type cells have evolved flux configurations to maintain stable resource-allocation to provide the precursors, cofactors and energy molecules for growth. Such configurations are largely conserved by the cell via latent and redundant reactions in the presence of genetic and environmental variations [21–24]. Some flux modeling techniques, such as minimization of metabolic adjustment (MOMA), have adopted a form of the flux-memory assumption: '*metabolic fluxes undergo a minimal redistribution with respect to the flux configuration of the wild type*' [25]. However, engineering higher flux towards an endogenous or heterogeneous pathway forces a redistribution of the flux network, which strains the cellular supply chain and can cause growth stresses (e.g. toxicity). Although cell metabolism has elasticity and redundancies to deal with metabolic burden, high-producing strains often operate around a 'metabolic cliff', namely a breaking point for metabolic supply, as shown in one of our earlier papers [26]. At this unstable point, cell metabolism is vulnerable to bioreactor stresses. For example, decreased respiration efficiency can force cells to fall off the cliff, causing a significant loss of biosynthesis capability. Such perturbation is severe in large bioreactors and constantly affects high-producing strains. Therefore, mutations that restore the pre-engineered flux network are more likely to balance the metabolic usage for cell survival, leading to selective pressure to revert cell metabolism towards the native flux distribution.

Damping the effects of mutations in engineered cells

In plasmid-encoded bioproduction circuits, plasmid loss due to random segregation during cell division is a common problem. The plasmid-free cells can outcompete the producers due to their liberation from the fitness cost of plasmid maintenance. Incorporating active partitioning mechanisms (e.g. the *par* and the *cer* loci) in plasmids can ensure even plasmid distribution to minimize their loss [27]. Moreover, the use of antibiotics or auxotrophic mutants can further extend the stability of the bioproduction circuitry. However, plasmid loss still occur under such selection pressures, as plasmid-containing cells may protect plasmid-free cells from the selective agent, by either degrading antibiotics or producing public goods. Consistently, it was shown that a selective pressure on *E. coli* hosts was not sufficient to prevent their genetic circuit from failing in a 10 L bioreactor [28]. On the other hand, whereas chromosomally integrated synthetic circuits are more stable than plasmids, they still experience natural mutations and loss-of-function.

Recently, new methods have been reported to control the rate of naturally occurring mutations with varying success. One approach is to couple fitness and product production. For example, insertion of essential genes into the production-pathway operon can maintain the pathway components in the growing cells, leading to increases of stability (e.g. by additional 5–10 generations in an *E. coli* mevalonic acid producing strain), but this method does not prevent enzymatic loss-of-function via mutations that only affect enzyme activity rather than enzyme expression levels [15••]. Moreover, advanced feedback-genetic-circuits have been designed to automatically tune the expression of pathways or bypass resource sharing under metabolic burden or stresses [29••,30]. For example, Lv *et al.* achieved stability for over 300 generations in flavonoid-producing *Yarrowia lipolytica* strain via linking production to leucine biosynthesis by replacing the native leucine promoter with a flavonoid induced promoter (coupled with further strain optimization) [29••]. Despite the successful examples, biosensor-regulator circuits may introduce new cell stresses or burdens and often need extensive tuning [29••,31]. In addition, the engineered modularity can break down due to crosstalk between different regulatory systems or unexpected interactions with RNA, proteins or metabolites [28].

A different strategy involves de-coupling growth and bioproduction. Such de-coupling allows engineered systems to grow without burden from production, minimizing the selection pressure for deleterious mutations. Thereby, the production-phenotype is only triggered in the final large-scale reactor. For example, a quorum sensing based biosensor allows for autoinduction of production after cells reach the desired density [32•]. External signals can also be used as inducers to control the production phenotype. Recently, the addition of caffeine to activate protein kinase R can switch off most translation processes during the production phase of mammalian cell cultures and thus reduce their non-product proteins synthesis [33]. Moreover, copper inducible promoters have been built to improve dynamic regulations of *Yarrowia* bioproduction pathways [34]. However, the applicability of de-coupling strategy can still be limited by the quality and the availability of inducible promoters as well as the additional manufacturing costs associated with external inducers. Alternatively, ultraprecise synthetic oscillators [35••] can be used to drive expression of the engineered pathways, allowing for the periodic relaxation of metabolic burden for production, to avoid accumulation of toxic metabolites and deleterious runaway mutations, and to mitigate the effect of environmental perturbations on cell phenotypic states [36]. Simulations suggested that oscillators need further improvement for metabolite production, and their stability and scalability for industrial applications remain to be seen [37].

Another promising strategy is to create background strains that are less prone to mutations. For example, by the elimination of error-prone DNA polymerases and removal of transposable elements, an *E. coli* chassis can produce higher L-threonine and recombinant protein titers [38]. Modifying heat-shock chaperones (e.g. DnaK and GroEL) [16] or deleting the so-called 'evolvability factor' (i.e. *mfd* gene of *E. coli*) [39] has also proven promising to reduce strain mutational rates. Moreover, the DNA mismatch repair (MMR) system has been identified as a key mechanism for the formation of a hypermutator population in *E. coli* that exhibits increased mutation rates. The MMR system corrects erroneous insertions of bases and repairs various DNA damage that occurs during DNA replication and recombination. The levels of major MMR proteins (e.g. MutS, MutL and MutH) that are inherently low [40], are further repressed under stress conditions (such as nutrient limitation, extreme temperatures or pH) by the general stress regulon, namely RpoS in *E. coli* [41]. In the presence of stressors, the downregulation of the already limiting levels of MMR proteins thus creates an MMR-insufficient phenotype, which displays markedly increased mutational rates (stress-induced mutagenesis) [42,43]. Targeting the MMR system is therefore a promising approach to reduce mutability and increasing the MMR dosage has indeed been shown to prolong genome stability in *E. coli* [44]. However, this strategy must be carefully implemented since it is subject to the goldilocks principle: MMR levels cannot be arbitrarily high, as demonstrated in baker's yeast [45••]. Lastly, gene-specific analysis of mutational 'hot spots' can guide mitigation techniques, as illustrated in a recent investigation on mevalonic acid-producing *E. coli* over many generations [15••]. While ultra-deep time-lapse sequencing identified very few SNPs in the native genome, high-frequency of IS insertion appeared in the engineered-pathway genes. Selecting production-gene sequences without specific IS insertion sites increased stability while switching the host to a completely IS-free strain (MDS42) led to further improvement of pathway stability by ~20 additional generations.

Elucidation of mutations in engineered cells

The common technique for utilizing genetic divergence is adaptive laboratory engineering and subsequent phenotype screening that reveals evolutional trends to enforce cell memory of desired fluxome configurations [46,47]. In one example, adaptive laboratory evolution led to improvement of microbial growth with minimal intracellular flux rewiring [48••]. In another example, adaptive evolution balanced the metabolic tradeoffs between microbial growth rate and biosynthesis yield by fine-tuning flux ratios in a few reaction nodes (e.g. between the glycolysis and the pentose phosphate pathway) [49]. In this context, the MMR genes can be purposefully deleted (e.g. *ΔmutS* in *E. coli*) to accelerate the mutational rate and the exploration of the mutational landscape during adaptive evolution [50•]. This allows for the rapid generation of a wide range of genotypes and the evolution of bioproduction workhorses towards increased yields [51].

High throughput micro-bioreactor apparatuses, genetic diversification methods (random mutagenesis by error-prone PCR, genome shuffling, MAGE, CRISPR-Cas, etc.) and fluorescence-activated cell sorting (FACS) are enabling approaches towards rapid strain generation as well as screening and identification of enhanced production phenotypes [1]. Advancements are also allowing for real time visualization of mutations, following their effects on growth directly, and quantifying mutation rates for single cells [52•]. Furthermore, it is possible to isolate single cells of *E. coli* growing in well-defined trenches on a similar microfluidic device, while monitoring dynamic gene expression profiles and growth properties of individual strains in a complex library with high temporal resolution [35••]. As opposed to FACS, which relies on single-dimensional data for screening, this platform can enable isolation of the best performing mutants yielding high bioproduction with minimally compromised growth rates with high precision.

The aforementioned state-of-the-art approaches for high-throughput generation and screening of strains will undoubtedly synergize with advances in building of better models for deciphering genotype-phenotype linkages. For example, a recent deep-learning model can predict

effects of genomic alterations on physiological phenotypes such as growth rates, thus providing an invaluable tool for assessing the genotype-phenotype associations [53]. Recent computational strain design algorithms have also made strides to elucidate regulatory-metabolic networks from data, allowing for the pinpointing of gene targets that contribute to strain enhancement and mutation landscape [54]. While these *in silico* techniques provide hypotheses for addressing strain production metrics, there are still knowledge gaps between the degree of genome alternations, metabolic burdens and flux re-organizations under stressed bioreactor conditions. The process of scaling up, which is usually empirical and fraught with failure, begs for approaches that possess predictive power to reliably gauge strain performance *a priori*.

Authors' perspective: explore flux memory to predict genetic drift rates and pseudoreversions

Starting with the perspective of flux-network rigidity as a driving force behind strain mutations a comparative analysis of the flux differences between an engineered or adapted strain versus the wild-type configuration can reveal keystone reaction fluxes associated with dynamics in commercial-scale bioreactors, while genomics approaches can help identify pathway-specific mutations. Such analyses (namely, ^{13}C-MFA) have been employed to discover that the adaptive evolution of a *pgi* knockout *E. coli* mutant changed cofactor metabolism and glucose transport by mutations in the trans-hydrogenase genes and phosphotransferase system [55]. Similar studies identified three SNPs relevant to energy pathways that enabled the relatively fast photosynthesis and growth in the cyanobacterial *Synechococcus elongatus* 2973 [56•,57]. Reverse engineering these SNPs in the slower growing *S. elongatus* 7942 led to a doubling in growth rate, putting it on par with that of *S. elongatus* 2973. These examples illustrate that adaptive evolution, genome resequencing and ^{13}C-MFA provide an effective framework to reveal the linkage between mutations and cellular outcomes under specified growth conditions.

In our perspective, the knowledge of the mutational landscape and rates in the context of bioproduction-induced flux reorganization may identify escape mechanisms and offer guidelines for preemptive strain designs. Such correlations will also allow for risk analysis when moving cells from laboratories to large-scale industrial applications so that scale-up fermentation would become more predictable and that strains could be developed with an eye towards 'biomanufacturability'. In the authors' opinion, the flux-memory concept can therefore be valuable for predicting strain scalability and performance in large reactors. Quantifying the distance between the parent non-producer configuration and the post-engineering flux network can provide an estimate for the speed of metabolic shifts the production strain may experience. To determine how the flux network will be redistributed, ^{13}C-MFA can capture the degree of flux suboptimality (i.e. the distance between a parent strain's flux distribution and an engineered strain). To this end, several mathematical expressions exist to determine flux differences. For example, for a given engineered strain, the Euclidean distance [25] from a non-producer strain can be calculated by Eq. (1) (assuming all flux differences are equally weighted):

$$D(w,v) = \sqrt{\sum_{i=1}^{N} (w_i - v_i)^2} \tag{1}$$

where the parent non-producer strain has the flux vector w_i and the engineered strain has the flux vector v_i. Obtaining D values and genomic information from many different strains can help elucidate a generalization of mutation rates as a function of D.

$$R_m = f(D) \tag{2}$$

where R_m is the deleterious mutation rate (namely, the mutation rate that generates non-producing cells). The distance index D is expected to be in a nonlinear function (Eq. (2)) due to the complex outputs of engineered cells (likely stemming from intricate networks with feedback loops as well as interactions exhibiting various cooperativities and stoichiometries). To verify this hypothesis, ^{13}C-MFA of a large number of engineered mutants along with evolutional experiments to determine the mutational frequency of these mutants can be a necessary direction to help elucidate the correlation function (Eq. (2)) [58]. Simple models can then be built to predict the number of producing cells during scale-up processes. For example, a simple model with two ordinary differential equations (ODEs) shows that increased rates of mutations and larger metabolic burden lead to more rapid loss of stability. The model includes: mutation rates R_m, which generate non-producing cells; growth rates of producing and non-producing cells (μ_1 and μ_2, respectively) and the number of producing and nonproducing cells (X_1 and X_2, respectively). The ODEs describing the cell subpopulation growth are as follows:

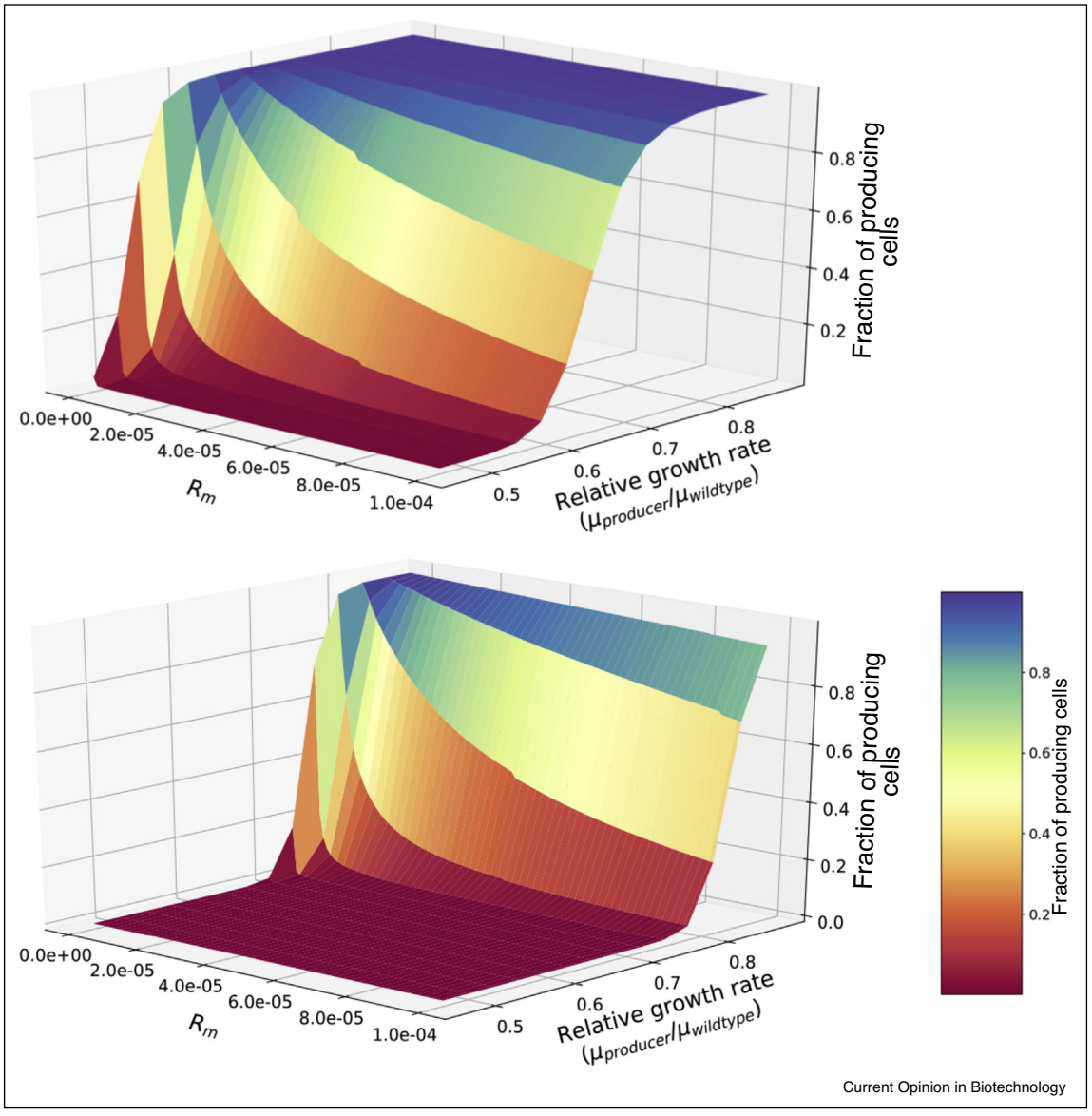
$$\frac{dX_1}{dt} = \mu_1 X_1 - R_m \mu_1 X_1 \tag{3}$$

$$\frac{dX_2}{dt} = \mu_2 X_2 + R_m \mu_1 X_1 \tag{4}$$

The two equations can estimate the remaining fraction of producing cells within the population after certain number of generations during batch culture.

Figure 2 illustrates an example for a range of mutation (R_m) and relative growth (μ_1/μ_2) rates. Interestingly, the figure shows a population production cliff that results in the regime shift. In brief, when cell generation numbers are low and the growth rate of the producer is close to its non-producer counterpart, the non-producers may not have an apparent impact on the fermentation performance. The increase of initial non-producer subpopulation, mutation rates, number of cellular generations, or differences in growth rates (i.e. μ_1 and μ_2) can push fermentation cultures to a regime where cell production can rapidly drop due to the emergence of non-producers. The location, where the population regime shift takes place, determines the baseline of the scalability of the engineered strains, which helps engineers make better informed decisions on microbial chassis for industrial applications.

Figure 2



Estimating the scalability of engineered strains in a batch culture.
Fraction of the cell population that generates the bioproduct at 25 generations (top) and 65 generations (bottom) as a function of rate of generation of non-producer cells and metabolic burden (relative growth rate). Simulation is based on Eqs. (3) and (4).

Conclusion

Elucidation of the causes of heterogeneity in engineered microbial hosts may facilitate developing new approaches to control the rate and landscape of mutations during industrial fermentation processes. There are still critical knowledge gaps in our understanding of the synergistic effects between introduced genetic parts and the metabolic alterations encountered in multiple stages of scale-up. Employing fluxomics and genomics will help identify the linkage between mutation rates and metabolic stresses of engineered strains, leading to strategies to mitigate deleterious mutations and enhance production stability. The acquired knowledge on dynamics and spectra of mutations (e.g. SNPs, Indels, and structural rearrangements) as well as their fitness effects can lead to preemptive engineering of gene targets and background strains that have low frequency mutations for increased stability. Finally, we hypothesize that ^{13}C-MFA and the concept of flux memory together bolster a robust opportunity for the prediction towards laboratory strain scalability. For validation, new experiments involving measurements of flux as well as rates of mutations and growth should be designed and carefully performed.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Jeffrey J Czajka: Writing - original draft, Writing - review & editing. **Burak Okumuş:** Conceptualization, Writing - review & editing. **Mattheos AG Koffas:** Writing - review & editing. **Mark Blenner:** Writing - original draft, Writing - review & editing. **Yinjie J Tang:** Conceptualization, Writing - original draft, Writing - review & editing.

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