Robustness: linking strain design to viable bioprocesses

Downloaded from: https://research.chalmers.se, 2024-04-23 23:44 UTC

Citation for the original published paper (version of record):
Olsson, L., Rugbjerg, P., Torello Pianale, L. et al (2022). Robustness: linking strain design to viable bioprocesses. Trends in Biotechnology, 40(8): 918-931.
http://dx.doi.org/10.1016/j.tibtech.2022.01.004

N.B. When citing this work, cite the original published paper.

research.chalmers.se offers the possibility of retrieving research publications produced at Chalmers University of Technology.
It covers all kind of research output: articles, dissertations, conference papers, reports etc. since 2004.
research.chalmers.se is administrated and maintained by Chalmers Library

(article starts on next page)

Trends in Biotechnology

CellPress OPEN ACCESS

Review

Robustness: linking strain design to viable bioprocesses

Lisbeth Olsson [1,3,*], Peter Rugbjerg [1,2,3,@], Luca Torello Pianale [1,3] and Cecilia Trivellin [1,3]

Microbial cell factories are becoming increasingly popular for the sustainable production of various chemicals. Metabolic engineering has led to the design of advanced cell factories; however, their long-term yield, titer, and productivity falter when scaled up and subjected to industrial conditions. This limitation arises from a lack of robustness – the ability to maintain a constant phenotype despite the perturbations of such processes. This review describes predictable and stochastic industrial perturbations as well as state-of-the-art technologies to counter process variability. Moreover, we distinguish robustness from tolerance and discuss the potential of single-cell studies for improving system robustness. Finally, we highlight ways of achieving consistent and comparable quantification of robustness that can guide the selection of strains for industrial bioprocesses.

Highlights

Microbial robustness is a complex multifaceted concept that is important for the predictability and efficiency of biological production. Robustness refers to the stability of specific phenotypic traits despite multiple perturbations.

Three simple principles that underlie the common features of robustness are applied in multiple fields.

Robustness must be distinguished from tolerance because the latter relates to cellular survival or growth in the face of a single perturbation.

Industrially relevant perturbations range broadly across chemical, biological, and physical factors that are often difficult to predict.

Single-cell analysis can elucidate the roles of subpopulations and population dynamics in microbial robustness.

Quantification of robustness is suggested as a tool for guiding strain and bioprocess development.

New cell factory designs are needed for robust industrial-scale bioprocesses

Over the past century microorganisms have been exploited in industrial bioproduction [1] of primary (e.g., ethanol and lactic acid) and secondary metabolites (e.g., terpenoids), cells (e.g., starter cultures and probiotics), and proteins (e.g., enzymes) [2–4]. Two different strategies have emerged to meet the increasing demand for bio-based products. In the first, inexpensive petrochemicals are replaced by sustainable microbially produced alternatives; however, abundant and complex raw materials, such as lignocellulose and side gas streams, remain a challenge for microbial fermentation [5]. In the second, a range of new bioproducts including agricultural probiotics, nutritional proteins, silk materials, and plastic-degrading bacteria are being developed, but their wider exploitation remains limited by process inefficiency and scaling-up issues [6]. Central to both strategies is microbial **robustness** (see Glossary), which describes the stability of a **phenotype** (e.g., titer, production rate, and yield) when challenged by different disturbances or **perturbations** [7–10]. Although strain engineering has improved tremendously since the mid-1990s [11], poor robustness limits industrial-scale microbial production. Bioindustries constantly strive for better strains, but their development in the laboratory often fails to consider the multiple perturbations encountered in industrial settings. This lack of strategic oversight results in poorly performing strains under large-scale conditions, leading to increased costs of commercializing new bioprocesses [12].

The present review defines and discusses microbial robustness from different perspectives, emphasizing the contribution of many fields of biology to the industrial applications of microbial robustness. We discuss the importance of subpopulations in strain performance and tools for understanding single-cell performance variations. We also discuss the various industrially relevant perturbations that demonstrate how robustness is a distinct and broader concept than **tolerance**. Finally, we propose principles for routinely quantifying robustness as a guide for engineering cell factories through mathematical models.

[1]Department of Biology and Biological Engineering, Division of Industrial Biotechnology, Chalmers University of Technology, Kemivägen 10, 412 96 Gothenburg, Sweden
[2]Enduro Genetics ApS, Copenhagen, Denmark
[3]Equal contributions

*Correspondence: lisbeth.olsson@chalmers.se (L. Olsson).

© 2022 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Three principles of robustness for strain and bioprocess improvement

Principles of robustness

The concept of robustness has gained increasing interest over the past 30 years across different fields [8]. Three ubiquitous principles underlie robustness, namely **modularity (M)**, **redundancy (R)**, and **dynamic control (DC)** (Figure 1) [13,14]. Modularity denotes the physical and/or functional compartmentalization of processes in modules to improve the efficiency of a system. Redundancy refers to processes in which different components or pathways perform the same task. Lastly, dynamic control describes the regulation of a process by its components [13]. For example, in a single-cell system, modularity can be represented by the organization of the genome into regulons of coregulated genes that govern specific pathways and phenotypic states [15]. Redundancy can be exemplified in yeast by the activation of stress-related genes by the transcription factors MSN2 and MSN4, whose functions have been shown to largely overlap [16]. Dynamic control is illustrated by the feedback inhibition of the amino acid tryptophan (Trp) on its biosynthetic pathway in bacteria [17]. These concepts manifest in different biological contexts. Genetic robustness denotes the ability of a genetic sequence to remain stable and avoid perturbing mutations through gene duplication (R) or alternative signaling pathways (M) [18]. In neurobiology, a neuronal activity for a learned task reconfigures (or 'drifts') over time, but its behavior remains the same (R) [19]. Ecosystem robustness summarizes the ability of an ecosystem to maintain a balance owing to the enrichment of different niches and complex environmental networks such as the feedback-regulated food chain (DC) [20,21]. Yeast metabolism uses the modular nature of central enzymatic pathways to achieve robustness against metabolic inhibitors. For example, when challenged by furaldehydes and phenolics released during pretreatment of lignocellulosic biomass, NAD(P)H is required for detoxification reactions. To maintain the redox balance, the metabolic fluxes enhance the pentose phosphate pathway over glycolysis (DC) [22].

Interdisciplinary strategies

The three principles – modularity, redundancy, and dynamic control – offer several approaches for improving industrial microbial robustness. A possible application of ecology and modularity in bioprocess design could involve the coculture of microbes that cooperate in substrate

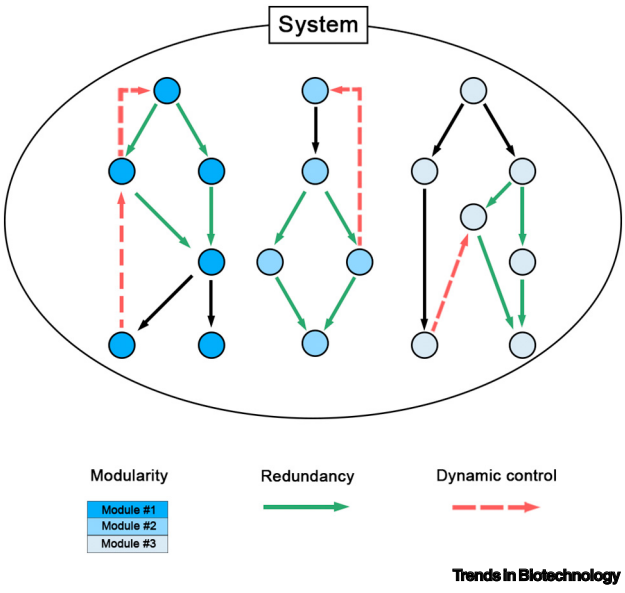

Figure 1. The three key principles of robustness. Schematic representation of a system (e.g., a cell) and its processes and component interactions. The components in the system are represented by circles (O), each of which is able to fulfill a different task represented by an arrow (→). The pathways connecting two different components are defined as processes (represented by O→ … →O). With this model it is possible to explain the three principles of robustness: modularity (e.g., sets of coexpressed genes involved in a process), redundancy (e.g., gene activation by multiple transcription factors), and dynamic control (e.g., feedback inhibition).

Glossary

Bet-hedging: a survival strategy based on phenotypic heterogeneity within a bulk isogenic population in which intrinsic cell-to-cell variation can play a beneficial role in the face of detrimental perturbations in the environment.
Dynamic control (DC): a robustness principle that refers to the feedback control that system components exercise on processes as a result of network interactions within the system itself. One example is the negative feedback regulation that glucose-1-P exerts on hexokinase 1 expression.
Modularity (M): a robustness principle that refers to the subdivision of a system into modules specialized for specific tasks with the aim of optimizing system processes. One example is the compartmentalization of a cell into different organelles.
Perturbation: environmental or genetic change that can alter the phenotype of a system. To maintain its functionality and performance, the system responds with phenotypic adjustments. Perturbations in bioprocesses can be divided into stochastic and predictable, based on their nature.
Phenotype: single or multiple observable cell characteristics (e.g., yield, specific growth rate, cell volume, etc.). Mathematically also referred to as functions.
Phenotypic heterogeneity: a concept of population heterogeneity that follows subpopulations with phenotypic and behavioral differences within the same bulk isogenic cell population. Such differences can be caused by both intrinsic factors (e.g., differential gene expression) and/or extrinsic factors (e.g., different physiochemical gradients of substrate).
Redundancy (R): a robustness principle that refers to the event in which the same process can be executed by different components within the system. For example, the conversion of furfural into furfuryl alcohol in yeast can be performed by both ADH1 and ADH5.
Robustness: the ability of a system to maintain unchanged performance when one or more perturbations occur. In an industrial environment, microbial robustness refers to the ability of the microbe to maintain constant production performance (defined as titers, yields, and rates) regardless of the different stochastic and unpredictable perturbations occurring in a bioprocess.

degradation and bioproduction, but do not compete for the same carbon source. For instance, coculture of *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum* for bioethanol production is a promising example [23]. *C. thermocellum* is able to metabolize cellulose and hemicellulose into ethanol and sugar monomers, which it is unable to consume. On the other hand, *T. saccharolyticum* is unable to degrade cellulose and hemicellulose, but can efficiently metabolize monomeric sugars into ethanol/weak acids. Process robustness in the face of a variety of perturbations in bioprocess streams may be achieved by cocultivation of multiple variants of the same strain, each specialized in tolerating a particular condition (R). In the treatment of dairy wastewater through high-rate anaerobic digestion, process robustness to physiochemical perturbations is maintained because of the functional redundancy of the microbial sludge community [24]. Alternatively, metabolic robustness may be strengthened by developing a microbial strain with switchable genetic modules that are activated depending on the type of stress encountered (DC and M). An increase of 17.5% in ethanol productivity was achieved by implementing in *S. cerevisiae* strain SyBE005 stress-driven modules aimed to improve of reactive oxygen species (ROS) detoxification and acetic acid degradation [25]. This study highlighted the importance of biosensors that can sense specific intracellular parameters (e.g., oxidative stress [26]) in the development of new strains and improvement of bioprocesses.

Tolerance: the capability of a microorganism to survive when exposed to a single or multiple perturbations. It is generally described only by growth-related parameters (such as viability or specific growth rate) and can also be referred to as resistance.

Cell factory design should incorporate robustness

Robustness goes beyond tolerance

In industrial microbial applications, the concepts of robustness and tolerance have sometimes been used interchangeably even though they refer to different phenomena. Tolerance (or resistance) represents the ability of a cell to grow (measured as viability or specific growth rate) in the presence of a single or multiple perturbations, such as the concentration of a chemical and/or a physical condition (e.g., temperature, acetic acid, etc.) (Figure 2A, left panel). Microbial robustness denotes the ability of a microorganism to maintain a stable industrial performance (quantified as titers, rates, and yields) when facing one or multiple challenges (Figure 2B) [10]. Therefore, although tolerance only considers viability and/or specific growth rate, microbial robustness encompasses the stability of the production and growth of a microbe in different conditions evaluated using different measures. Increased tolerance to one or multiple toxic compounds or conditions is usually tested by analyzing growth curves or viability (Figure 2A, right panel). Such studies are instrumental for understanding cell physiology, but are not sufficient for the development of industrially relevant strains because they do not include studies on production and performance stability [27,28]. New strains have often been developed under standard laboratory parameters using model organisms (e.g., *Escherichia coli* MG1655, 20 g/l glucose to fed-batch, pH = 5–7, 72 h); however, these do not accurately convey the complexity of industrial settings where robustness is crucial [29].

Trade-off between growth/production and robustness

In natural and engineered biological systems, higher robustness and tolerance to stresses is sometimes achieved at the expense of growth and/or production performance [30–32]. This trade-off generally results in slow-growing and/or slow-producing subpopulations that are often more tolerant and/or robust to perturbations (Figure 2C). Robust systems are often characterized by modularity, redundancy, and dynamic control, all of which are features that may be energy-demanding [14,33]. In parallel, during fast growth/production, microorganisms dedicate most of their resources for that purpose. This situation may underlie how biological systems trade-off robustness and high performance. For example, slow-growing bacteria are less sensitive to antibiotics [34], whereas slow-growing subpopulations of *Saccharomyces cerevisiae* exhibit higher tolerance to heat or acid stress [30]. It remains to be determined whether slow growth confers an inherent advantage by allowing the cells to balance metabolic networks, or

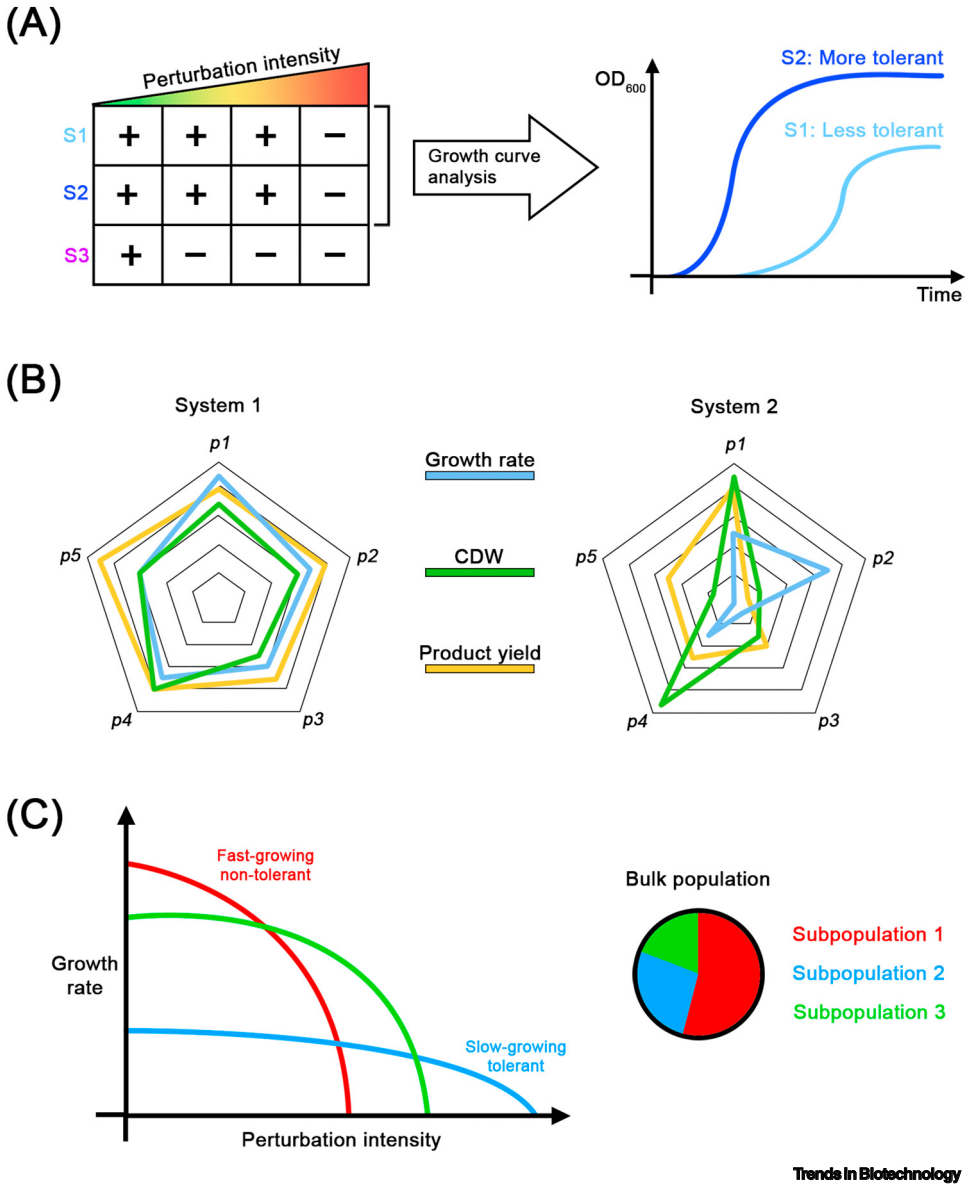

Figure 2. Tolerance, robustness, and trade-offs. (A) Tolerance can be tested by growing different microorganisms (S1, S2, and S3) under increasing intensities of perturbation and assessing their ability to grow (+) or not (−). In the example, S1 and S2 are more tolerant than S3 (left panel). Detailed analysis of growth curves can discriminate between microorganisms with the same growth threshold and apparent tolerance level. In the example, S2 is more tolerant than S1 (right panel). (B) Microbial robustness takes into consideration the phenotype stability of microorganisms. In the example, system 1 and system 2 represent two different microorganisms compared in terms of specific growth rate, cell dry weight (CDW), and production yield. System 1 shows more stability across different perturbations (*p1*, …, *p5*) than system 2 and can be considered to be more robust. (C) Trade-off denotes a situation in which a quality or property is diminished in favor of another quality or property. In the example, a system constituted by a bulk population of cells includes multiple subpopulations, each of which might be subjected to a trade-off with respect to specific growth rate and perturbation intensity.

whether it reflects the metabolic burden of producing stress-protecting molecules such as the sugar trehalose, which accumulates in slower-growing older cells [30]. Population dynamics and single-cell studies could help to select slow-growing or slow-producing strains/subpopulations

that are capable of withstanding a broader range of perturbations and conditions, as opposed to faster-growing and -producing but less robust microbial mutants [35].

Population dynamics at single-cell resolution provides insights into robustness mechanisms

Phenotypic heterogeneity and bet-hedging

Growing evidence shows significant single-cell variation in bioprocesses, which influences overall productivity [36]. This has led to the concept of **phenotypic heterogeneity**, which refers to phenotypically diverse subpopulations within an isogenic bulk population. Such non-genetic phenotypic cell-to-cell variation may underlie the observed response to perturbations (Figure 3). Phenotypic heterogeneity may occur owing to intrinsic factors (e.g., individual physiological state, stochastic gene expression/noise, and cell-cycle phases) and/or heterogeneous extrinsic factors (e.g., chemical mixing gradients and variation in cell density) [37]. Phenotypic heterogeneity may thus be reversible, whereas the emergence of genetic heterogeneity represents intrinsic one-way

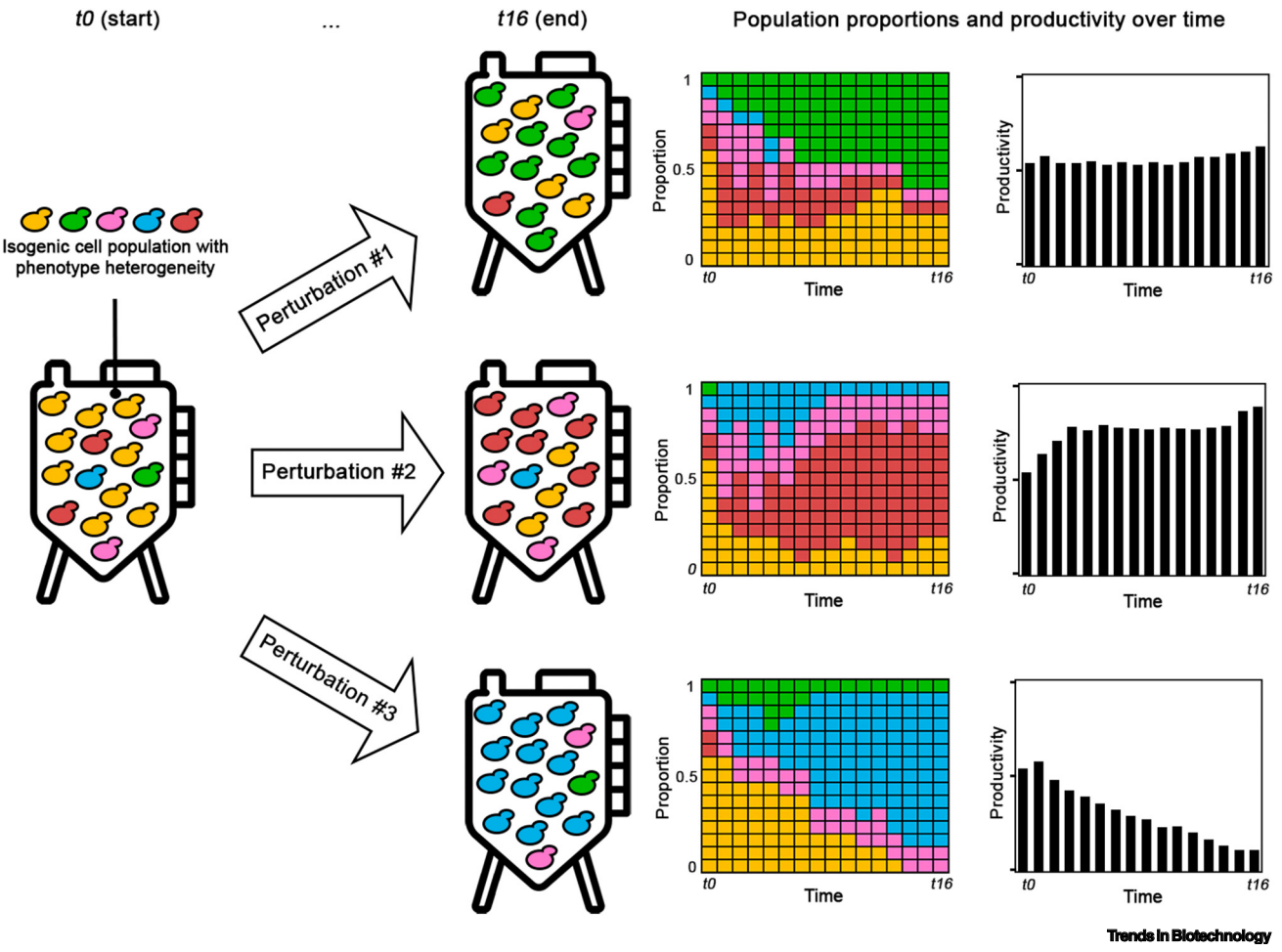

Figure 3. Population heterogeneity affects robustness in industrial processes. Schematic representation of population dynamics for an isogenic population together with the productivity of a desired industrial process at timepoints *t0* (start) to *t16* (end). Even though all cells share the same genome, there are subpopulations with different phenotypes (cells with different colors). Over time and upon the occurrence of different stresses, subpopulations with greater fitness against a specific perturbation take over (left graphs), even though productivity might not be constant over the entire process (right graphs). In the example, perturbation #3 results in lower productivity compared to perturbation 1 because it favors the blue subpopulation, whereas perturbation 2 leads to higher productivity with respect to perturbation 1.

degeneration of the high-production phenotype [38]. Intrinsic causes of heterogeneous proliferation and stress tolerance might be linked, for example, to differences in mitochondrial membrane potential within cell populations [39], whereas extrinsic factors include physicochemical bioreactor concentration gradients [36]. A biosensor for L-valine in bacteria highlighted how only a proportion of the cells were actually involved in L-valine production, confirming the presence of different growth and production patterns within the same population [40].

Phenotypic heterogeneity arising from intrinsic factors sometimes offers a natural survival strategy to unknown future challenges, a phenomenon known as **bet-hedging** [41]. Accordingly, isogenic populations can increase their chances of survival upon sudden environmental changes by distributing risks (i.e., different phenotypes) among the bulk population. In an isogenic yeast bulk population, a slower-growing small subpopulation was found to overexpress *TSL1*, a gene involved in the synthesis of trehalose [30]. Following temperature stress, the *TSL1*-overexpressing subpopulation immediately became the predominant subpopulation. Studies using microfluidic devices showed how, in the presence of the antibiotic cycloheximide, a subpopulation expressing higher levels of *PDR5*, an ATP-dependent membrane transporter, exhibited a higher specific growth rate compared to a subpopulation with low *PDR5* expression [42]. Another example is the diauxic shift in *Lactococcus lactis* and *S. cerevisiae*, whereupon growth in a medium with two different carbon sources allows a subpopulation to prepare for subsequent growth on a less preferable carbon source [43,44]. Similarly, exponentially growing *S. cerevisiae* cultures respiring on glucose consist of two distinct subpopulations, of which the slowest appears to be more prepared for sudden starvation [4]. Therefore, bet-hedging subpopulations may enjoy an advantage in an unstable environment (e.g., different raw material streams), allowing better-suited cells to quickly dominate a new condition, as a degree of robustness [45].

Single-cell analysis allows the discovery of new robustness traits

The link between robustness/tolerance and population dynamics is gaining more relevance in bioprocesses. Over recent years several single-cell technologies have provided new insights into different biological events. For example, cellular barcoding represents an efficient way to monitor how different subpopulations evolve during the desired process and for identifying the mutational order driving this change [46–48]. Single-cell RNA-seq has been used to study the dynamics of isogenic yeast populations growing on a shifting carbon source, highlighting the accompanying heterogeneity and key molecular processes [49]. Flow cytometry was used to identify early stationary-phase subpopulations with increased lignocellulosic inhibitor tolerance [50], as well as growth-phase heterogeneity in a bioreactor [51]. Cell-sorting techniques together with fluorescent biosensors of intracellular fluxes (e.g., glycolytic flux [52]), products (e.g., octanoic acid [53]), or concentrations (e.g., ATP [54]) can be used for the identification of more active subpopulations and for their selection during adaptive laboratory evolution experiments [55], as in the case of improved L-valine production in bacteria [56]. A toolbox of biosensors able to sense different aspects of the intracellular environment might be used for unveiling such dynamics and features [57]. Unlike flow cytometry, mass cytometry (CyTOF) enables the detection of >40 surface and intracellular markers per cell among millions of single cells [58]. Although CyTOF has been applied mainly in immunological studies [59], it could be adapted to microorganisms in industrial applications. Single-cell analysis and population dynamics could link intracellular parameters with strain performance, allowing the design of more robust strains.

Predictable and stochastic perturbations challenge microbial performance

When scaling up a process from laboratory to industrial settings, not all perturbations are taken into account, even though they may crucially affect the success of a newly developed strain [27]. Challenges related to industrial conditions range from predictable and reproducible to stochastic, occasionally leading to lost batches [60,61] (Figure 4).

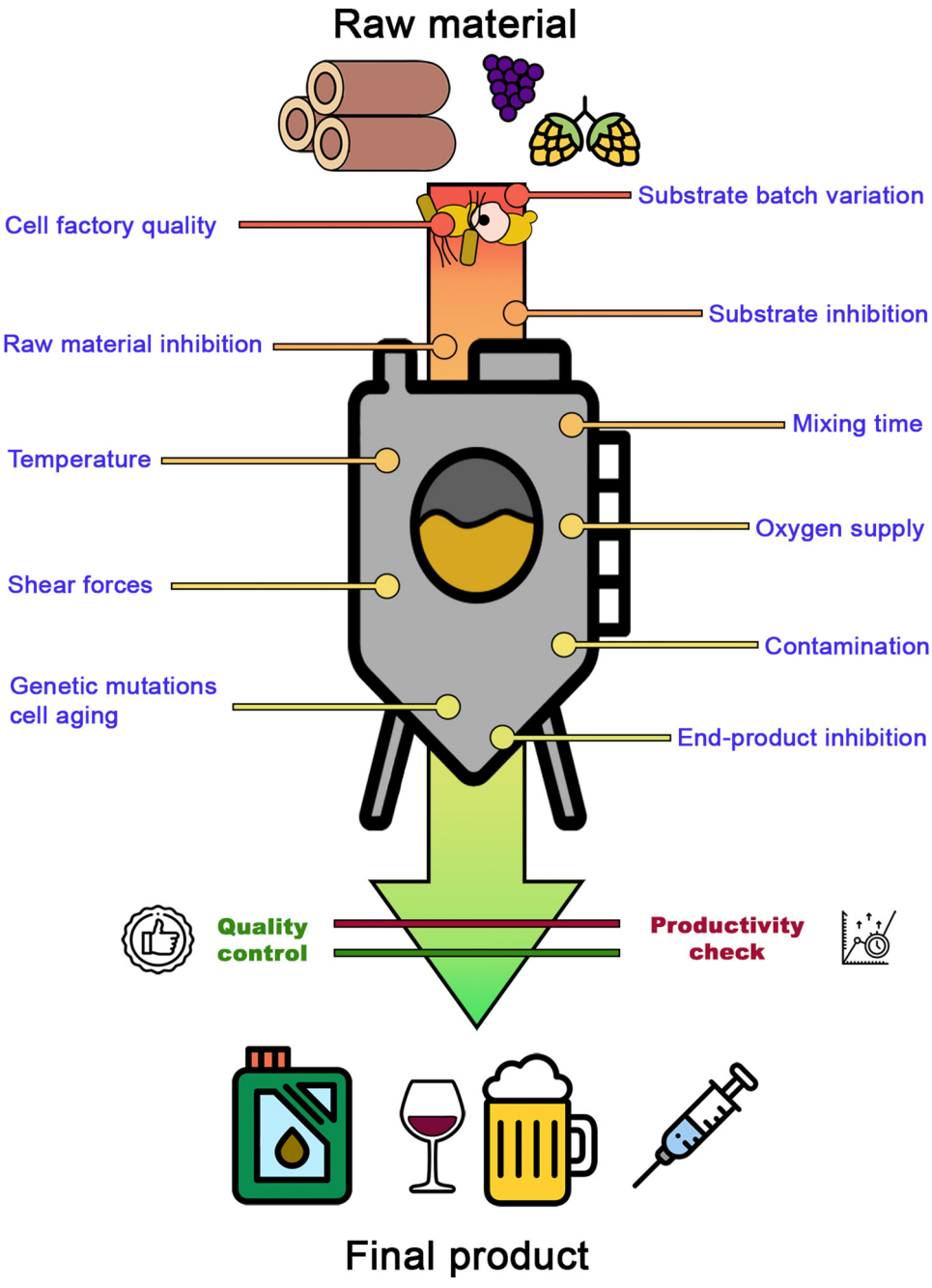

Figure 4. Perturbations in industrial processes. Relevant perturbations in industrial processes are shown along the process pipeline. Substrate batch variation (differences in the chemical composition of raw materials) and the initial quality of the cell factory are the first problems an industrial process might face. Pretreatments of the raw material can release compounds that interfere with the growth and productivity of microorganisms. Once the reaction starts, both predictable and stochastic perturbations can arise. The former includes physical (temperature, mixing time, oxygen supply, and shear force) as well as chemical (substrate and end-product inhibition) perturbations, whereas the latter may occur stochastically as a result of contaminations or mutations that lead to compromised product quality.

Predictable perturbations in bioprocesses

One of the requirements for greener bioproduction is the use of low-cost, renewable raw materials such as lignocellulosic biomass. Different batches and pretreatments can significantly affect the composition of the substrate used in the bioprocess and add variability [62]. Alongside substrate inhibition (e.g., high sugar content [29]), pre-treatment of raw materials may release inhibitors of microbial metabolism: furans, weak acids, and phenolics [63]. Short-term lignocellulose-adapted precultures, whereby microorganisms are challenged with diluted substrate during the propagation phase, may help to overcome this variability [64]. A recent study highlighted the importance of the physiological state of the cells before exposure to lethal ethanol levels, and of their long-term transcriptional survival strategies after contact with the stressor [65]. Nevertheless, the increasing concentration of end-product following substrate bioconversion will inevitably cause inhibition. During high-gravity ethanol production, elevated ethanol concentrations can lead to a sudden drop in cell viability and a consequent reduction in ethanol productivity [66,67]. A possible solution to this problem comes from studies on aneuploidy, which suggest that aneuploidy of chromosome III enhances ethanol tolerance [68]. Industrial fermenters add several physicochemical variables that challenge microbial growth. These include low pH, as in the case of succinic acid production [69], the nutrient and oxygen gradients formed as a result of prolonged mixing times and elevated shear pressure [62,70], and high temperatures (above 30–37°C), which hinder competing microorganisms and invading bacteriophages or offer a compromise between substrate saccharification and fermentation temperatures [71].

Stochastic perturbations in bioprocesses

Being able to predict stochastic perturbations, that often lead to cost increases, would greatly streamline strain and fermentation development. Starter seed cultures are stored in freezers, a condition that might cause cell viability and vitality to vary between batches and vials [72]. Therefore, both the dry weight and activity of cells in the initial bioprocess inoculum should be considered [73]. Viral, bacteriophage, and bacterial contamination can severely impact on both mammalian and microbial bioproduction (e.g., leading to premature shutdown of bioprocesses); this can be avoided through rigorous PCR-based testing of raw materials [74], the use of antibiotics [75], or by engineering strains which efficiently assimilate xenobiotic media compounds and thus outcompete contaminants [76]. It is possible to use specific testing regimes or bacteriophage-resistant mutant strains to avoid phage contamination in microbial bioreactors containing *Lactobacillus* ssp. [77]. The harsh conditions within industrial processes put selective pressure on microorganisms, and thus promote genetic heterogeneity within the population, whereby subpopulations with different genetic compositions arise as a result of spontaneous mutations [78]. Although mutations are stochastic, the constantly high selective pressure and the presence of a mutation already in a cell bank could lead to recurring enrichments of such faster-growing subpopulations, thus limiting the volumetric scale of a fermentation process, as captured through deep DNA sequencing [38]. Moreover, age-driven modifications in physiology, morphology, and gene expression lower the performance of microorganisms [79]. All the above-mentioned stochastic events highlight the central role that robust microorganisms play in overcoming various perturbations and ensuring stable performance.

Robustness quantification for accurate microbial performance predictions

Robustness is an abstract and relative term that can be difficult to quantify in a standardized manner. Its quantification would help industrial strain engineers to identify robust strains for more efficient and cost-effective processes, or to elucidate complex cellular functions such as protein production and cancer proliferation [80]. A high-throughput study on *E. coli* with disrupted central carbon metabolism showed that, despite numerous genetic and environmental perturbations, the bacteria were able to redirect fluxes with only minor changes in transcriptome and protein

expression [81]. This example confirms the tight link between robustness quantification and the biochemical, metabolic, and genetic aspects determining cellular functions (e.g., the manifestation and quantification of relevant phenotypes) [82]. Robustness has been quantified with biomarkers that measure the advantage against lethal stresses conferred by exposure to mild stresses. The index of robustness was expressed as the number of microorganisms *N* surviving the stress after a time *t* compared to *N* at time zero [83] (according to our Glossary, the latter method would quantify tolerance). Taking the quantification further, considering robustness *R* as a property of a system *s* to maintain a function *a* under internal and external perturbations *P* [14], robustness can be explained by Equation 1 introduced by Kitano [9]:

$$R_{a,P}^{S} = \int_{P} \psi(p) D_{a}^{s}(p) dp \quad [1]$$

Robustness is defined here as the integration across the space *P* of the probability function $\psi(p)$ (the probability of occurrence of a single perturbation, *p*) multiplied by an evaluation function $D_a(p)$. The latter describes to what degree a specific function *a* is perturbed when subjected to *p* compared to a non-perturbed state p_0 (Figure 5A). If the function (e.g., yields, specific growth rate) of a system is not altered across several perturbations, the system is more robust than one whose function is highly affected over the same range of perturbations (Figure 5B). Equation 1 could describe the robustness of a specific microorganism (the system, e.g., *S. cerevisiae* CEN.PK113-7D) with respect to the ethanol production titer (the function) under industrial growth conditions (the perturbation space, e.g., temperature, oxygenation, inhibitors) weighted by the probability to encounter the condition itself (e.g., 30°C, very probable; 50°C, rarely probable). To attain a broader estimate of robustness, the concept can be expanded by considering multiple desired functions *a*, such as other performance and growth indicators. When multiple parameters are combined, the robustness of each function can then be weighted differently based on the desired outcome of the process: maximum specific growth rate and biomass yield are more important than ethanol yield when *S. cerevisiae* is used in baking. Equation 1 has been used in recent years to perform conditional robustness analysis on ordinary differential equation (ODE) models describing pathways or biochemical interaction networks of lung cancer [84]. The analysis identified nodes in the pathway that are crucial for cancer cell proliferation and represent potential new drug targets [84].

Conditional robustness analysis, as used for lung cancer networks, could be applied in industrial biotechnology for the identification of network nodes that can act as robustness markers [80,84]. By identifying metabolic pathways linked to the robustness of specific functions (e.g., through the use of genome-scale metabolic models), one could simulate how enzymes levels change when kinetic parameters are perturbed by environmental or intracellular perturbations [80,84]. In addition to Equation 1, another commonly used measure of robustness is the coefficient of variation (CV), which is expressed as the standard deviation σ of the mean μ of a measured quantity (e.g., product yield, gene expression profile, etc.) divided by the mean μ. Measurements have included the size of transcriptional mRNA bursts [85] to determine expression noise, as well as temporal stability evaluations in ecology [86]. CV can be substituted by the Fano factor, in which the variance of a measured quantity is divided by its mean. The Fano factor is often preferred over the CV because it allows more reliable and standardized quantification [85–87]. In metabolic engineering, the CV has been used to calculate the robustness of metabolic pathway fluxes [88] as described in Equation 2:

$$R = 1 - \frac{\sigma}{\mu} \quad [2]$$

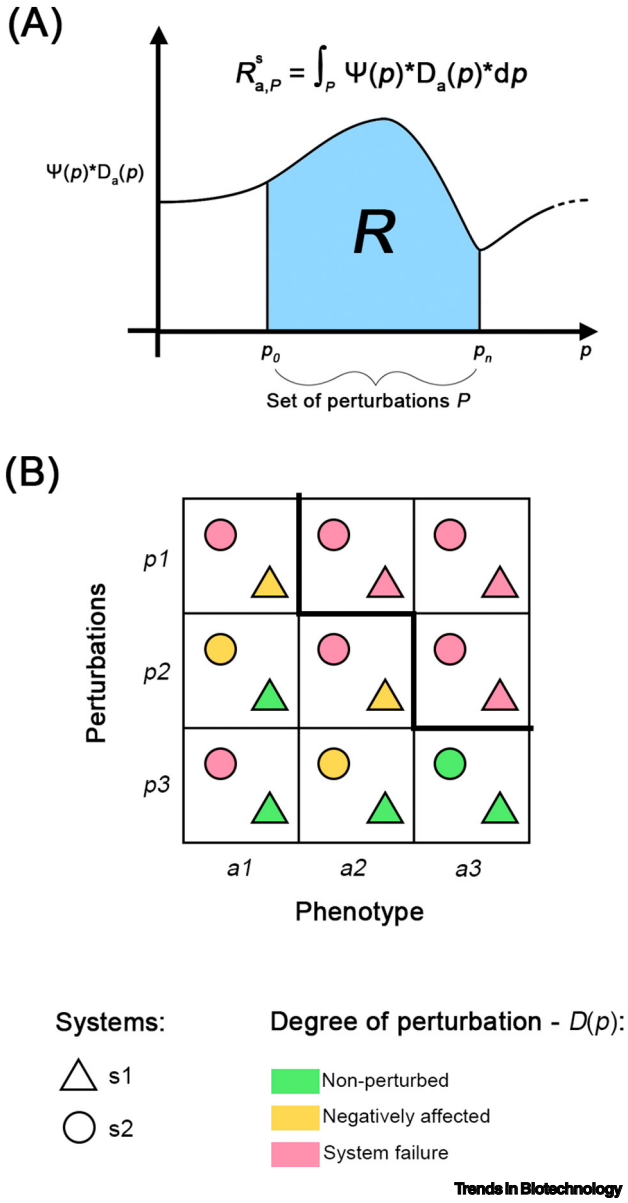

Figure 5. Robustness quantification. (A) Representation of robustness according to Equation 1. Robustness (*R*) is described as the integration across the space *P* (set of multiple perturbations, p_n) of the probability function $\psi(p)$ multiplied by an evaluation function $D_a(p)$ (ratio between the function *a* in the selected perturbation *p* and the same function *a* in the control condition p_0). (B) Perturbations (*p1*, *p2*, and *p3*) and phenotypes (*a1*, *a2*, and *a3*) of two systems (s1 and s2) are shown in the grid. In the example, system 1 comprises more non-perturbed states than system 2 and can be considered to be more robust. The thick line across the graph represents a threshold above which all phenotypes fail for all systems.

Kitano's formula (Equation 1), the CV (Equation 2), and the Fano factor all determine the stability of a given function in a perturbed space (i.e., robustness). However, whereas CV describes the variability of the measured quantity across various conditions with respect to the mean, Kitano measures how the quantity changes when exposed to different perturbations compared to a control condition. Therefore, Kitano requires a control condition, and attributes to robustness the ability to maintain all functions equal to the control condition. By contrast, CV focuses on the variability of the functions, regardless of whether they perform differently from a control condition. Furthermore, Kitano does not consider all the perturbations to be equally relevant, but directs the robustness index towards the most probable perturbations.

CV can also be applied for single-cell measurements to evaluate population heterogeneity. Indeed, some subpopulations might exhibit greater robustness with respect to the averaged *R*, causing an imprecise classification of the selected strain [89].

Quantification of microbial robustness will be a fundamental measurement to integrate in big-data analytics to optimize bioprocesses [90]. Large datasets can be used as basis for emerging artificial intelligence to predict and control bioprocesses. In cream cheese production, an artificial neuronal network together with a mechanistic model have been developed to predict overall fermentation time using only the initial biomass, lactose, and lactic acid concentrations as variables [73]. A hybrid framework integrating data-driven methods with a genome-scale metabolic model demonstrated promising accuracy when predicting *E. coli* performance under typical bioprocess conditions and available pathways [91]. Big data gathered in fermentation processes (for example from *in situ* biosensors [92], spectroscopic sensors [93], and free-floating wireless sensors [94]), potentially stored in phenomic datasets, could be used to calculate robustness for many strains *in silico*, and have subsequently been adopted in strain design and large-scale fermentations. Data-driven and mechanistic models, such as partial least-square regression, could predict how robustness changes as a function of internal and external perturbations, for example by measuring possible robustness markers. Therefore, mathematical quantification of robustness, together with machine learning and big data, could become significant players in guiding strain design.

Concluding remarks and future perspectives

Better integration and understanding of robustness in strain and process design are central to resolving the bottleneck faced by bio-based industries in getting their products to the market. The broad application of robustness in biotechnology will likely inspire novel approaches such as the utilization of microbial consortia with dynamic interactions among their members (see Outstanding questions) [95,96]. In contrast to tolerance, microbial robustness is a multidimensional concept. Robustness covers the stability of multiple phenotypes (e.g., production-related), whereas tolerance only covers on growth-related phenotype. Both tolerance and robustness might concern either single or multiple perturbations. However, for a bioprocess to be robust, the selection and design of strains should consider the collective set of industrial perturbations. A common strategy to improve the tolerance and/or production of microorganisms for industrial applications is through adaptive laboratory evolution, whereby natural selection and mutations act in synergy under selective pressure [97,98]. A computational evolution system showed that mutations acquired during fluctuating conditions made a new strain more prone to adapt to previously encountered conditions and devise future adaptations [99]. This highlights the importance of varying the nature and composition of perturbations during adaptive laboratory evolution to achieve robust strains capable of facing multiple challenges, rather than only increasing the harshness of a single perturbation and improving tolerance.

Ongoing efforts in modeling the complex networks of interactions defining microbial robustness will eventually provide a solid and reliable instrument for the characterization and selection of industrially relevant cell factories. Recent single-cell techniques unveiling population dynamics are offering new insights into bioprocesses, as well as key features and indicators of cellular and/or process status. Finally, robustness quantification, used both as a tool to find intracellular robustness markers and as a measure of phenotype stability, could lead the choice towards a highly stable and performing industrial microorganism. Although we are only now beginning to understand the complex mechanisms behind robustness, a full comprehension will pave the way for successful bioprocess and strain design.

Outstanding questions

Will the new single-cell analysis tools be able to identify new features/characteristics/genes related to robustness to improve industrial processes?

How can robustness quantification methods be used for industrial purposes?

Are some perturbations more relevant than others in predicting strain robustness?

What role do subpopulations play in robust processes?

To what extent do production phenotypes trade-off with robustness?

Can robustness be engineered with a combination of modularity, redundancy, and dynamic control?

How can transfer to larger scales with ensured robustness take place?

Acknowledgments

Financial support by Novo Nordisk Foundation grant DISTINGUISHED INVESTIGATOR 2019 – Research within biotechnology-based synthesis and production (#0055044) is gratefully acknowledged. We thank the late Professor Stefan Hohmann for fruitful discussions about robustness.

Declaration of interests

The authors declare no conflicts of interest.

References

1. Demain, A.L. (2000) Microbial biotechnology. *Trends Biotechnol.* 18, 26–31
2. Rahmat, E. and Kang, Y. (2020) Yeast metabolic engineering for the production of pharmaceutically important secondary metabolites. *Appl. Microbiol. Biotechnol.* 104, 4659–4674
3. Fenster, K. *et al.* (2019) The production and delivery of probiotics: a review of a practical approach. *Microorganisms* 7, 83
4. Bagamery, L.E. *et al.* (2020) A putative bet-hedging strategy buffers budding yeast against environmental instability. *Curr. Biol.* 30, 4563–4578
5. Zhang, Y. *et al.* (2009) The importance of engineering physiological functionality into microbes. *Trends Biotechnol.* 27, 664–672
6. Wehrs, M. *et al.* (2019) Engineering robust production microbes for large-scale cultivation. *Trends Microbiol.* 27, 524–537
7. Stelling, J. *et al.* (2004) Robustness of cellular functions. *Cell* 118, 675–685
8. Félix, M.A. and Barkoulas, M. (2015) Pervasive robustness in biological systems. *Nat. Rev. Genet.* 16, 483–496
9. Kitano, H. (2007) Towards a theory of biological robustness. *Mol. Syst. Biol.* 3, 137
10. Gong, Z. *et al.* (2017) Engineering robustness of microbial cell factories. *Biotechnol. J.* 12, 1700014
11. Bailey, J.E. (1996) Metabolic engineering. *Adv. Mol. Cell Biol.* 15, 289–296
12. Dheskali, E. *et al.* (2020) Risk assessment modeling of bio-based chemicals economics based on Monte-Carlo simulations. *Chem. Eng. Res. Des.* 163, 273–280
13. Zhu, L. *et al.* Engineering the robustness of industrial microbes through synthetic biology. Trends Microbiol. 20, 94–101
14. Kitano, H. (2004) Biological robustness. *Nat. Rev. Genet.* 5, 826–837
15. Segal, E. *et al.* (2003) Module networks: identifying regulatory modules and their condition-specific regulators from gene expression data. *Nat. Genet.* 34, 166–176
16. Estruch, F. and Carlson, M. (1993) Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 13, 3872–3881
17. Santillán, M. and Mackey, M.C. (2001) Dynamic regulation of the tryptophan operon: a modeling study and comparison with experimental data. *Proc. Natl. Acad. Sci.* 98, 1364–1369
18. Plata, G. and Vitkup, D. (2014) Genetic robustness and functional evolution of gene duplicates. *Nucleic Acids Res.* 42, 2405–2414
19. Rule, M.E. *et al.* (2020) Stable task information from an unstable neural population. *eLife* 9, e51121
20. Mumby, P.J. *et al.* (2014) Ecological resilience, robustness and vulnerability: how do these concepts benefit ecosystem management? *Curr. Opin. Environ. Sustain.* 7, 22–27
21. Cai, Q. and Liu, J. (2016) The robustness of ecosystems to the species loss of community. *Sci. Rep.* 6, 35904
22. Larochelle, M. *et al.* (2006) Oxidative stress-activated zinc cluster protein Stb5 has dual activator/repressor functions required for pentose phosphate pathway regulation and NADPH production. *Mol. Cell. Biol.* 26, 6690–6701
23. Lynd, L.R. *et al.* (2016) Advances in consolidated bioprocessing using *Clostridium thermocellum* and *Thermoanaerobacter saccharolyticum*. In *Industrial Biotechnology: Microorganisms* (Wittmann, C. and Liao, J.C., eds), pp. 365–394, Wiley-VCH Verlag
24. Paulo, L.M. *et al.* (2020) Microbial community redundancy and resilience underpins high-rate anaerobic treatment of dairy-processing wastewater at ambient temperatures. *Front. Bioeng. Biotechnol.* 8, 192
25. Qin, L. *et al.* (2020) Stress-driven dynamic regulation of multiple tolerance genes improves robustness and productive capacity of *Saccharomyces cerevisiae* in industrial lignocellulose fermentation. *Metab. Eng.* 61, 160–170
26. Zhang, J. *et al.* (2016) Engineering an NADPH/$NADP^+$ redox biosensor in yeast. *ACS Synth. Biol.* 5, 1546–1556
27. Van Dien, S. (2013) From the first drop to the first truckload: commercialization of microbial processes for renewable chemicals. *Curr. Opin. Biotechnol.* 24, 1061–1068
28. Lang, G.I. *et al.* (2009) The cost of gene expression underlies a fitness trade-off in yeast. *Proc. Natl. Acad. Sci. U. S. A.* 106, 5755–5760
29. Deparis, Q. *et al.* (2017) Engineering tolerance to industrially relevant stress factors in yeast cell factories. *FEMS Yeast Res.* 17, fox036
30. Levy, S.F. *et al.* (2012) Bet hedging in yeast by heterogeneous, age-correlated expression of a stress protectant. *PLoS Biol.* 10, e1001325
31. Rugbjerg, P. *et al.* (2021) Short and long-read ultra-deep sequencing profiles emerging heterogeneity across five platform *Escherichia coli* strains. *Metab. Eng.* 65, 197–206
32. Sleight, S.C. and Sauro, H.M. (2013) Visualization of evolutionary stability dynamics and competitive fitness of *Escherichia coli* engineered with randomized multigene circuits. *ACS Synth. Biol.* 2, 519–528
33. Kitano, H. (2010) Violations of robustness trade-offs. *Mol. Syst. Biol.* 6, 384
34. Pontes, M.H. and Groisman, E.A. (2019) Slow growth determines nonheritable antibiotic resistance in *Salmonella enterica*. *Sci. Signal.* 12, eaax3938
35. Johnson, M.S. *et al.* (2019) Higher-fitness yeast genotypes are less robust to deleterious mutations. *Science* 366, 490–493
36. Heins, A.L. and Weuster-Botz, D. (2018) Population heterogeneity in microbial bioprocesses: origin, analysis, mechanisms, and future perspectives. *Bioprocess Biosyst. Eng.* 41, 889–916
37. Gasperotti, A. *et al.* (2020) Phenotypic heterogeneity of microbial populations under nutrient limitation. *Curr. Opin. Biotechnol.* 62, 160–167
38. Rugbjerg, P. and Sommer, M.O.A. (2019) Overcoming genetic heterogeneity in industrial fermentations. *Nat. Biotechnol.* 37, 869–876
39. Dhar, R. *et al.* (2019) Single cell functional genomics reveals the importance of mitochondria in cell-to-cell phenotypic variation. *eLife* 8, e38904
40. Mustafi, N. *et al.* (2014) Application of a genetically encoded biosensor for live cell imaging of l-valine production in pyruvate dehydrogenase complex-deficient *Corynebacterium glutamicum* strains. *PLoS One* 9, e85731
41. De Jong, I.G. *et al.* (2011) Bet hedging or not? A guide to proper classification of microbial survival strategies. *Bioessays* 33, 215–223
42. Jing, W. *et al.* (2018) Measuring single-cell phenotypic growth heterogeneity using a microfluidic cell volume sensor. *Sci. Rep.* 8, 17809
43. Venturelli, O.S. *et al.* (2015) Population diversification in a yeast metabolic program promotes anticipation of environmental shifts. *PLoS Biol.* 13, e1002042
44. Solopova, A. *et al.* (2014) Bet-hedging during bacterial diauxic shift. *Proc. Natl. Acad. Sci. U. S. A.* 111, 7427–7432

45. Levy, S.F. and Siegal, M.L. (2012) The robustness continuum. *Adv. Exp. Med. Biol.* 751, 431–452
46. Jasinska, W. *et al.* (2020) Chromosomal barcoding of *E. coli* populations reveals lineage diversity dynamics at high resolution. *Nat. Ecol. Evol.* 4, 437–452
47. Roy, K.R. *et al.* (2018) Multiplexed precision genome editing with trackable genomic barcodes in yeast. *Nat. Biotechnol.* 36, 512–520
48. Nguyen Ba, A.N. *et al.* (2019) High-resolution lineage tracking reveals travelling wave of adaptation in laboratory yeast. *Nature* 575, 494–499
49. Jariani, A. *et al.* (2020) A new protocol for single-cell RNA-seq reveals stochastic gene expression during lag phase in budding yeast. *eLife* 9, e55320
50. Narayanan, V. *et al.* (2017) Increased lignocellulosic inhibitor tolerance of *Saccharomyces cerevisiae* cell populations in early stationary phase. *Biotechnol. Biofuels* 10, 114
51. Delvigne, F. *et al.* (2015) Dynamic single-cell analysis of *Saccharomyces cerevisiae* under process perturbation: comparison of different methods for monitoring the intensity of population heterogeneity. *J. Chem. Technol. Biotechnol.* 90, 314–323
52. Ortega, A.D. *et al.* (2021) A synthetic RNA-based biosensor for fructose-1,6-bisphosphate that reports glycolytic flux. *Cell Chem. Biol.* 28, 1554–1568
53. Baumann, L. *et al.* (2018) A yeast-based biosensor for screening of short- and medium-chain fatty acid production. *ACS Synth. Biol.* 7, 2640–2646
54. Botman, D. *et al.* (2020) An improved ATP FRET sensor for yeast shows heterogeneity during nutrient transitions. *ACS Sensors* 5, 814–822
55. Delvigne, F. and Goffin, P. (2014) Microbial heterogeneity affects bioprocess robustness: dynamic single-cell analysis contributes to understanding of microbial populations. *Biotechnol. J.* 9, 61–72
56. Mahr, R. *et al.* (2015) Biosensor-driven adaptive laboratory evolution of L-valine production in *Corynebacterium glutamicum*. *Metab. Eng.* 32, 184–194
57. Torello Pianale, L. *et al.* (2022) Real-time monitoring of yeast intracellular state during bioprocesses with a toolbox of biosensors. *Front. Microbiol.* 12, 802169
58. Bandura, D.R. *et al.* (2009) Mass cytometry: technique for real time single cell multitarget immunoassay based on inductively coupled plasma time-of-flight mass spectrometry. *Anal. Chem.* 81, 6813–6822
59. Gadalla, R. *et al.* (2019) Validation of CyTOF against flow cytometry for immunological studies and monitoring of human cancer clinical trials. *Front. Oncol.* 9, 415
60. Brexó, R.P. and Sant'Ana, A.S. (2017) Impact and significance of microbial contamination during fermentation for bioethanol production. *Renew. Sust. Energ. Rev.* 73, 423–434
61. Baltz, R.H. (2018) Bacteriophage-resistant industrial fermentation strains: from the cradle to CRISPR/Cas9. *J. Ind. Microbiol. Biotechnol.* 45, 1003–1006
62. Attfield, Paul V. (1997) Stress tolerance: the key to effective strains of industrial baker's yeast. *Nat. Biotechnol.* 15, 1351–1357
63. Koppram, R. *et al.* (2014) Lignocellulosic ethanol production at high-gravity: challenges and perspectives. *Trends Biotechnol.* 32, 46–53
64. van Dijk, M. *et al.* (2019) Strain-dependent variance in short-term adaptation effects of two xylose-fermenting strains of *Saccharomyces cerevisiae*. *Bioresour. Technol.* 292, 121922
65. Yang, J. and Tavazoie, S. (2020) Regulatory and evolutionary adaptation of yeast to acute lethal ethanol stress. *PLoS One* 15, e0239528
66. Devantier, R. *et al.* (2005) Metabolite profiling for analysis of yeast stress response during very high gravity ethanol fermentations. *Biotechnol. Bioeng.* 90, 703–714
67. Pereira, F.B. *et al.* (2011) Robust industrial *Saccharomyces cerevisiae* strains for very high gravity bio-ethanol fermentations. *J. Biosci. Bioeng.* 112, 130–136
68. Morard, M. *et al.* (2019) Aneuploidy and ethanol tolerance in *Saccharomyces cerevisiae*. *Front. Genet.* 10, 82
69. Hakkaart, X. *et al.* (2020) Physiological responses of *Saccharomyces cerevisiae* to industrially relevant conditions: slow growth, low pH and high CO_2 levels. *Biotechnol. Bioeng.* 117, 721–735
70. Takors, R. (2012) Scale-up of microbial processes: impacts, tools and open questions. *J. Biotechnol.* 160, 3–9
71. Olofsson, K. *et al.* (2008) A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnol. Biofuels* 1, 7
72. Kapoore, R.V. *et al.* (2019) Effects of cryopreservation on viability and functional stability of an industrially relevant alga. *Sci. Rep.* 9, 2093
73. Li, B. *et al.* (2021) Application of mechanistic modelling and machine learning for cream cheese fermentation pH prediction. *J. Chem. Technol. Biotechnol.* 96, 125–133
74. Barone, P.W. *et al.* (2020) Viral contamination in biologic manufacture and implications for emerging therapies. *Nat. Biotechnol.* 38, 563–572
75. Olendorff, S.A. *et al.* (2021) Survey of antibiotics residues in DDGS from 14 different states by LCMS. *Cereal Chem.* 98, 81–88
76. Shaw, A.J. *et al.* (2016) Metabolic engineering of microbial competitive advantage for industrial fermentation processes. *Science* 353, 583–586
77. Callanan, M.J. and Klaenhammer, T. (2008) *Bacteriophages in industry*. *In Encyclopedia of Life Sciences*. Published online September 15, 2008. https://doi.org/10.1002/9780470015902.a0000776.pub2
78. Rugbjerg, P. *et al.* (2018) Diverse genetic error modes constrain large-scale bio-based production. *Nat. Commun.* 9, 787
79. Powell, C.D. *et al.* (2003) The impact of brewing yeast cell age on fermentation performance, attenuation and flocculation. *FEMS Yeast Res.* 3, 149–157
80. Bianconi, F. *et al.* (2015) Conditional robustness analysis for fragility discovery and target identification in biochemical networks and in cancer systems biology. *BMC Syst. Biol.* 9, 70
81. Ishii, N. *et al.* (2007) Multiple high-throughput analyses monitor the response of *E. coli* to perturbations. *Science* 316, 593–597
82. Mielenz, J.R. and Hogsett, D.A. (2014) Improving microbial robustness using systems biology. In *Manual of Industrial Microbiology and Biotechnology* ((3rd edn)) (Bull, A.T. *et al.*, eds), pp. 605–620, ASM Press
83. den Besten, H.M.W. *et al.* (2010) Short- and long-term biomarkers for bacterial robustness: a framework for quantifying correlations between cellular indicators and adaptive behavior. *PLoS One* 5, e13746
84. Bianconi, F. *et al.* (2019) CRA toolbox: software package for conditional robustness analysis of cancer systems biology models in MATLAB. *BMC Bioinforma.* 20, 385
85. Wang, Y. *et al.* (2020) Signaling mechanism of transcriptional bursting: a technical resolution-independent study. *Biology (Basel)* 9, 339
86. Ma, Z. *et al.* (2017) Climate warming reduces the temporal stability of plant community biomass production. *Nat. Commun.* 8, 15378
87. Trivellin, C. *et al.* (2021) Quantification of microbial robustness. *BioRxiv* Published online December 10, 2021. https://doi.org/10.1101/2021.12.09.471918
88. Yang, L. *et al.* (2015) Characterizing metabolic pathway diversification in the context of perturbation size. *Metab. Eng.* 28, 114–122
89. Vasdekis, A.E. *et al.* (2019) Eliciting the impacts of cellular noise on metabolic trade-offs by quantitative mass imaging. *Nat. Commun.* 10, 848
90. Gargalo, C.L. *et al.* (2020) Towards smart biomanufacturing: a perspective on recent developments in industrial measurement and monitoring technologies for bio-based production processes. *J. Ind. Microbiol. Biotechnol.* 47, 947–964
91. Oyetunde, T. *et al.* (2019) Machine learning framework for assessment of microbial factory performance. *PLoS One* 14, e0210558
92. Yakovleva, M. *et al.* (2013) The enzyme thermistor – a realistic biosensor concept. A critical review. *Anal. Chim. Acta* 766, 1–12
93. Samorski, M. *et al.* (2005) Quasi-continuous combined scattered light and fluorescence measurements: a novel measurement technique for shaken microtiter plates. *Biotechnol. Bioeng.* 92, 61–68
94. Busse, C. *et al.* (2017) Sensors for disposable bioreactors. *Eng. Life Sci.* 17, 940–952
95. Kehe, J. *et al.* (2019) Massively parallel screening of synthetic microbial communities. *Proc. Natl. Acad. Sci. U. S. A.* 116, 12804–12809

96. Campbell, K. *et al.* (2015) Self-establishing communities enable cooperative metabolite exchange in a eukaryote. *eLife* 4, e09943
97. Sandberg, T.E. *et al.* (2019) The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. *Metab. Eng.* 56, 1–16
98. Mans, R. *et al.* Under pressure: evolutionary engineering of yeast strains for improved performance in fuels and chemicals production. Curr. Opin. Biotechnol. 50, 47–56
99. Canino-Koning, R. *et al.* (2019) Fluctuating environments select for shortterm phenotypic variation leading to longterm exploration. *PLoS Comput. Biol.* 15, e1006445