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Strategies to increase tolerance and robustness of industrial microorganisms

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ABSTRACT

The development of a cost-competitive bioprocess requires that the cell factory converts the feedstock into the product of interest at high rates and yields. However, microbial cell factories are exposed to a variety of different stresses during the fermentation process. These stresses can be derived from feedstocks, metabolism, or industrial production processes, limiting production capacity and diminishing competitiveness. Improving stress tolerance and robustness allows for more efficient production and ultimately makes a process more economically viable. This review summarises general trends and updates the most recent developments in technologies to improve the stress tolerance of microorganisms. We first look at evolutionary, systems biology and computational methods as examples of non-rational approaches. Then we review the (semi-)rational approaches of membrane and transcription factor engineering for improving tolerance phenotypes. We further discuss challenges and perspectives associated with these different approaches.

1. Introduction

Industrially important microorganisms, including bacteria and yeast, have been widely applied to fermentation processes to produce pharmaceuticals, nutraceuticals, enzymes and food ingredients, fuels and biochemicals [1–4]. To meet commercial demands, microbial cells often need to be engineered to achieve specific metrics such as titer, yield, and productivity. The advancements in synthetic biology and metabolic engineering have significantly increased the ability to rapidly design and create cells with optimised pathways, such as genome editing by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) and CRISPR associated (Cas) proteins [5] and automated combinatorial gene assembly [6]. However, as living catalysts, microbial cells may change characteristics as they adapt to stressful conditions that arise during industrial production processes, which may lead to detrimental effects on the performance of these metabolically engineered cell factories.

Metabolic engineering is often necessary for producing novel chemicals or increasing native product titers. However, introducing new pathways and rewiring native metabolism can accumulate intermediates and toxic by-products that create cellular stress (Fig. 1). The presence and accumulation of these toxic compounds can adversely affect cell growth rates, which results in lower production capacity and ultimately diminish cost competitiveness. Although optimising the metabolic network may prevent the accumulation of such toxins, e.g., by introducing a dynamic enzyme regulation [7], the end-product itself can also reach toxic concentrations. In many cases, it is critical and necessary to reach high titers, which generally favours downstream processes. In some cases, product separation is possible (e.g. fatty alcohols via a dodecane overlay [8]) but often are complicated and expensive to implement in an industrial scale setup. Instead, the robustness of the host organisms can be engineered to tolerate higher product concentrations and allow for higher production.

One major cost factor in industrial-scale production is the carbon source, making up to 60% of the total process cost [9]. Therefore, lignocellulosic residues from agriculture and forestry have been explored as cheap and environmentally friendly carbon sources. However, these raw materials need to be pre-treated to make their sugars accessible for microorganisms, often releasing additional inhibitors deriving from lignin, cellulose and hemicellulose [10]. These inhibitors are usually phenolic compounds, weak acids, or furans [10], which can damage the plasma and cell membrane, inhibit key metabolic pathways (e.g. glycolysis), form reactive oxygen species (ROS) or cause ATP

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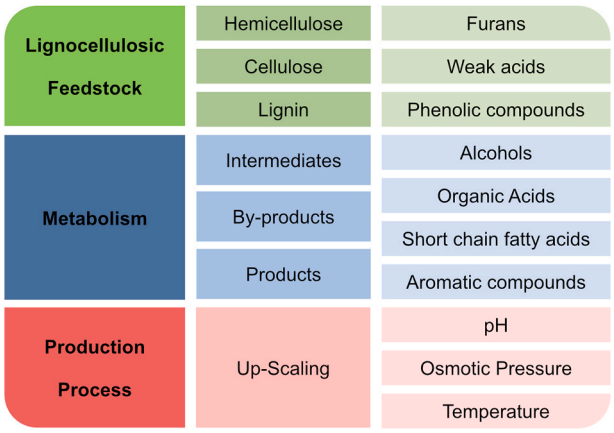

Fig. 1. Different causes of cellular stress (left), origin (middle) and examples (right). Lignocellulosic feedstock is composed of hemicellulose, cellulose, and lignin. During the pre-treatment, inhibitors such as furans, weak acids, or phenolic compounds can be formed. The natural and engineered metabolism can generate intermediates, by-products, or products that can generate cell stress, e.g. alcohols, organic acids, short chain fatty acids or aromatic compounds. Parameters such as pH, osmotic pressure, and temperature can further stress cells and often change during the up-scaling of a production process. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

depletion and anion accumulation [11,12]. Microorganisms need to be robust or engineered to tolerate these inhibitors (Fig. 1) and thus maintain high production capacity.

During the introduction and optimisation of new synthetic pathways and the transition from lab-scale to large-scale fermentations, different stresses arise that can challenge the tolerance of the host organism and limit its productivity (Fig. 1). Additionally, the large-scale production process is often very different from the conditions used for small-scale proof-of-concept cultivations, leading to additional stress for the cells, e.g. pH, temperature or osmotic pressure [13].

Developing industrial microorganisms with enhanced robustness properties has been key to achieving economically sustainable fermentation processes for producing chemicals, materials, and fuels. Thus, several reviews have highlighted this topic [14–17], but new approaches are constantly being developed. Therefore, this review focuses on the most recent examples of established and emerging methods to improve stress tolerance and robustness of microorganisms, drawing examples mainly from *Escherichia coli* and yeast *Saccharomyces cerevisiae*. We first focus on examples of evolutionary, systems biology, and computations approaches (Fig. 2), which does not require prior knowledge of understanding toxicity and tolerance mechanisms. Afterwards, we discuss examples of semi-rational and rational approaches, such as membrane engineering and transcription factor engineering (Fig. 2), which are based on a partial understanding of toxicity and tolerance mechanisms. An overview of the discussed studies and their improvements is summarized in Table 1.

2. Non-rational approaches

Evolutionary, systems biology, and computation approaches can be used to identify genetic bases that can subsequently be used to improve strain robustness. Systems biology and computation approaches aim to deepen our understanding of the stress factor on cell physiology but require a rational engineering step to improve strain robustness. The evolutionary approach immediately results in an improved strain but requires further studies (e.g., genome sequencing) to identify targets for the engineering of other strains. All three methods are not limited to certain cell parts but can identify targets in any different part of the cell. In the following section, we discuss examples for each of the three approaches.

2.1. Adaptive laboratory evolution

Adaptive laboratory evolution (ALE) techniques consist of continuous cultivations over multiple generations under a constant or increasing selective pressure, such as temperature, pH, or toxin concentration. Often, this process relies on the basal rate of mutation (spontaneous) present in microorganisms, which is sufficient to generate enough mutants for selective pressure to act upon. However, in some cases, i.e., requiring more complex traits, other strategies such as random mutations (e.g., via UV-light) and transposon mutagenesis may

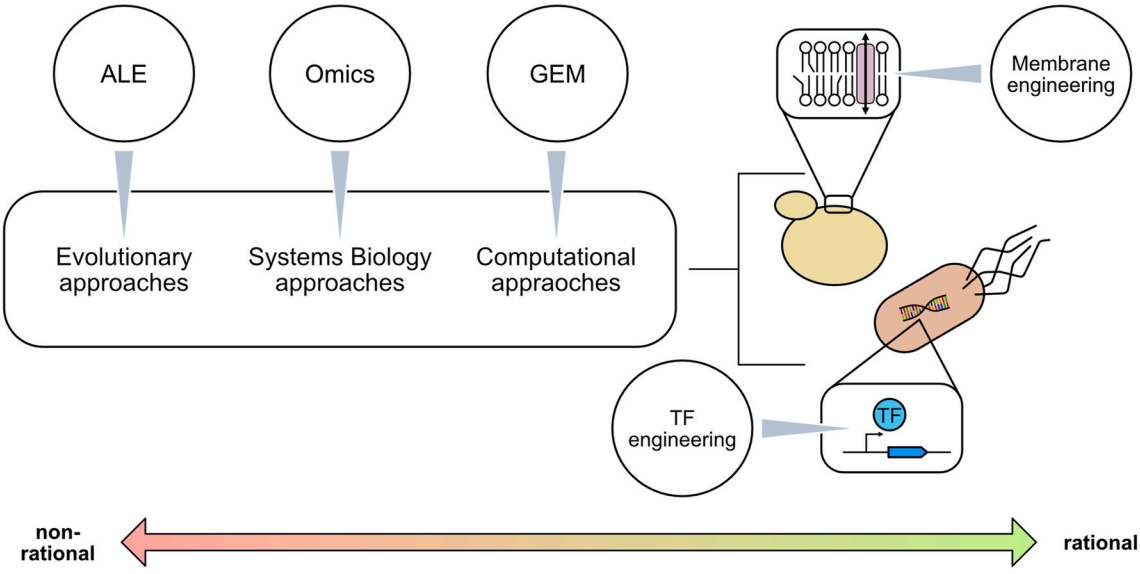

Fig. 2. Overview of approaches and methods to increase tolerance and robustness in microorganisms discussed in this review. Evolutionary, systems biology, and computational approaches are non-rational approaches that can identify genetic targets to increase stress tolerance in the host organism. While those approaches target the whole cell, more rational approaches can target specific parts of the cell, e.g., the membrane or transcription factors (TF). Circled terms represent exemplary technologies discussed in this review. ALE – Adapted Laboratory Evolution, GEM – Genome-scale Model, TF – transcription factor. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
List of studies with different approaches for increasing the tolerance and resistance of microorganisms and their improvements.

	Approach	Host	Engineering strategy	Study details	Improvement	Ref.
Non-rational approaches	ALE	*Yarrowia lipolytica*	ALE under ferulic acid stress	Upregulation of YALI0_E25201g, YALI0_F05984g, YALI0_B18854g, and YALI0_F16731g	+3-fold ferulic acid tolerance (from 0.5 g/L to 1.5 g/L)	[22]
		Pseudomonas putida	ALE under ferulic acid and *p*-coumaric acid stress	Deletion of *PP_3350* and *ttgB* contribute to aromatic acids tolerance	−37 h lag phase in 20 g/L *p*-coumaric acid +4-fold in growth rate in 30 g/L ferulic acid	[23]
		S. cerevisiae	ALE under dicarboxylic acids stress	Overexpression of *QDR3* (multidrug resistance transporter) confers resistance to dicarboxylic acid	+ 3-fold growth rate under 12 g/L adipic acid + 2-fold growth rate under 12 g/L pimelic acid	[24]
		S. cerevisiae	ALE under aromatic acid stress	Increased expression of the transporter gene ESBP6 helps to tolerate aromatic acids	+ ~12-fold change under 0.8 g/L *p*-coumaric acid enabled growth under 0.4 g/L ferulic acid	[25]
		E. coli	ALE under octanoic acid stress	Mutation of RNA polymerase subunit ($RpoC^{H419P}$)	+ 5-fold in carboxylic acid production +3.8-fold in growth rate under 10 mM octanoic acid	[27]
	Omics	*S. cerevisiae*	GWA	Identified deletion of *flo1* to convey hydrolysate toxin tolerance	+8-fold growth in synthetic hydrolysate	[30]
		S. cerevisiae	transcriptomics	Overexpressing *ADE1, ADE13, ADE17*	+39% biomass under acetic acid stress	[31]
		Clostridium acetobutylicum	metabolomics	Feeding of citric acid and ethylene glycol	+14.6% butanol production +21% growth	[32]
	GEM	*S. cerevisiae*	enzyme and temperature constrained GEM	Expression of thermostable squalene epoxidase (ERG1) following model prediction	~ +60% growth at 42 °C	[38]
		E. coli	combination of GEM with protein structures	Supplementing metabolites downstream of the identified growth limiting enzymes	+13% log-phase growth rate at 42 °C	[39]
(Semi-) rational approaches	Membrane engineering	*S. cerevisiae*	decrease saturation	Overexpression of OLE1 enhanced stress tolerance	improved tolerance in spot tests for various stresses	[45]
		E. coli	decrease membrane fluidity	Integration of trans unsaturated fatty acids by expression of *cis*-trans-isomerase from *Pseudomonas aeruginosa*	+29% octanoic acid production, and +15% growth rate and +25% biomass at 42 °C	[47]
		S. cerevisiae	increase fatty acid chain length	Expression of ACC1* increased oleic acid content	+84% growth rate in 0.7 mM octanoic acid	[48]
		E. coli	cyclopropane-fatty acid	Expression of cyclopropane-fatty acid-acyl-phospholipid synthase (cfa) from *Halomonas socia*	+50% polyhydroxyalkanoate production +58 growth in furfural +78% growth in 4-hydroxybenzaldehyde +230% growth in vanillin +119% growth in acetate	[49]
		S. cerevisiae	sphingolipid	Overexpression of sphingolipid acyl chain elongase ELO2	+21.9% cell growth in 1 M NaCl	[53]
		Candida glycerinogenes	altering membrane phospholipid composition	Increased 2-phenylethanol tolerance by overexpression of SLC1	+8.7% titer and +62.8% productivity of 2-phenylethanol production	[55]
		S. cerevisiae	transporter proteins	Expression of dicarboxylic acid transporter from *Schizosaccharomyces pombe* (SpMae1 (p)) and *Aspergillus carbonarius* (*Ac*Dct(p))	+ 3-fold succinic acid titer *Sp*Mae1 (p) + 5-fold fumaric acid titer *Sp*Mae1(p) + 8-fold/12-fold malic acid titer *Sp*Mae1(p)/(*Ac*Dct(p))	[57]
		E. coli	carotenoid treatment	Treatment of *E. coli* cells with polar carotenoids lutein and zeaxanthin	−30% butanol-induced membrane damage	[58]
	Transcription factor engineering	*S. cerevisiae*	gTME	Mutagenesis of the transcription factor Spt15p	+15% ethanol production + 13-fold in growth yield	[60]
		S. cerevisiae	specific TF engineering	Engineering of the transcription factor *HAA1*	reduced lag phase from 59 h to 37 h in 160 mM acetic acid	[61]
		S. cerevisiae	specific TF engineering	Altering one subunit of RNA polymerase II	+ 40% ethanol production	[62]
		S. cerevisiae	MINR	Identified a mutant with upregulated transcription factors (*HSP12, HSP30, SSA4 (HSP70), HSP82,* and *HSP104*)	+ 2-fold ethanol production	[64]
		S. cerevisiae	MINR	Identified *WAR1* and *K110 N* variants.	+ ~60% growth in isopropanol (50 g/L) + ~ 70% growth in isobutanol (14 g/L)	[66]

ALE: Adapted Laboratory Evolution, GEM: Genome-scale Model, gTME: global Transcription Machinery Engineering, MINR: MultIplex Navigation of global Regulatory networks.

be combined to accelerate the mutation process. As one powerful approach, the topic of ALE has been the subject of multiple reviews [18–21]. More recently, ALE has been applied in several microbes to overcome a wide range of toxicities. In *Yarrowia lipolytica* and *Pseudomonas putida,* ALE was used to overcome aromatic acid toxicity [22,23] and in *S. cerevisiae* to overcome dicarboxylic acid and aromatic acid toxicity at low pH [24,25]. In *E. coli* an ALE was performed to evolve strains to tolerate octanoic acid, hexanoic acid, decanoic acid, n-butanol and isobutanol [26,27].

Despite the broad applications of this technique, some aspects must be considered. Although ALE is a straightforward process, discovering underlying mechanisms can sometimes be challenging. Typically, genomic sequencing of the evolved strains is required. Sometimes further analysis, such as multi-omics analysis, is required to characterise the genetic changes that lead to the evolved phenotype. If the ALE is performed in high throughput, these analyses can require handling big data knowledge and, consequently, increase the time and cost of the experiment. Additionally, an ALE is not always the best method for every application. For instance, if the tolerance of the strain for a product should be increased to allow higher product titers, an ALE can result in a strain degrading the product instead of tolerating it. Moreover, the fitness of host organisms will change to tolerate the applied selective pressure in ALE better. However, it is common for other traits to be comprised or even lost during this process. This so-called trade-off is difficult to predict and, in some cases, even unavoidable [28]. Therefore, ALE should be carefully designed to avoid generating strains with one improved trait at the cost of losing other important industrial traits, e.g. production titer, rate or yield.

2.2. Omics-based approach

Omics or multi-Omics analysis has been an integral part of strain development for detailed characterisation of cell physiology by measuring different cell components, including mRNA, protein and metabolites [29]. Omics based analysis has also been integrated with other methods to characterise and identify molecular targets responsible for tolerance phenotypes. However, this approach can be implemented alone to unveil underlying stress responses by comparing samples under stress and control conditions.

With a single omics analysis, it is already possible to find targets for tolerance improvement. One example is a genome-wide association (GWA) that aims to map thousands of single nucleotide polymorphisms (SNPs) of different samples to associate them with phenotypical traits. The analysis of 165 *S. cerevisiae* strains isolated from diverse geographical niches identified genetic variants underlying toxin tolerance. Results from GWA have further suggested that the strains genetic background greatly influences the mechanisms of hydrolysate tolerance [30]. A transcriptomic comparison was made to study how S. *cerevisiae* copes with acetic acid, revealing genes involved in purine biosynthesis showed significantly increased expression by zinc sulfate supplementation under acetate stress, and overexpression of these genes enhanced cell growth under various stress conditions [31]. In addition, omics can also be applied when looking into the metabolites present in a biological sample. In another example, in *Clostridium acetobutylicum*, a natural butanol production strain, intracellular metabolic profiling was performed to identify the critical intracellular metabolites as regulation nodes that influence strain growth and butanol production. With computational models, this method revealed a feeding strategy with yeast extract, citric acid, and ethylene glycol that could significantly increase cell growth and butanol production [32].

One can also imagine that multi-Omics approaches can be applied, especially when it is difficult to interpret the correlation between toxicity and cellular changes using only a single type of omics data. Although multi-omics has not been reported to map stress responses directly, this approach has been frequently used to understand tolerance mechanisms. An example is a genomic-proteomic integrated data analysis of ethanol adapted *S. cerevisiae* strains in which a key difference in the energy-producing metabolism was found. Ethanol adapted strains mainly used glycolysis and ethanol fermentation for energy production, whereas the non-adapted strains mainly used respiration when ethanol was present in the media [33].

Despite the advances in omics technologies and their broad application, the biological interpretation of data remains a challenge. When performing omics to determine tolerance responses in microbes, it is likely to produce an extensive array of targets. In some cases, not a single target manipulation but a combination of several targets could confer the improved phenotype, which makes it difficult to validate by reverse engineering. Another limitation of the omics-based approach is the often missing linear correlation between gene expression, the level of protein abundance, and the *in vivo* catalytic activity due to various layers of cellular regulation, such as post-transcriptional mechanisms. Consequently, results can have different trends among the different layers of data. To prevent this, one can integrate multi-omics data to combine multiple layers of information [34,35].

2.3. Genome-scale models

Genome-scale models (GEMs) are a mathematical representation of a cell or an organism [36]. GEMs allow simulations of a large number of combinatorial gene changes to be tested *in silico* and offer predictions of bottlenecks in metabolic pathways and thus have been used to improve the production of various products [37]. Recently they have also been used to identify bottlenecks to improve microbial robustness. By constructing an enzyme and temperature constrained GEM (etcGEM), it was possible to identify enzymes in *S. cerevisiae* that are growth rate-limiting at high temperatures. As validation, the predicted target gene ERG1 (squalene epoxidase) was replaced by the homologue of the thermotolerant yeast *Kluyveromyces marxianus*, which resulted in significantly better growth at 40 °C [38]. In *E. coli*, combining a GEM with protein structures allowed predicting growth-limiting target genes at high temperatures. Instead of exchanging the proteins for thermostable homologues for their validation, Chang et al. chose to do a supplementation experiment. Supplementing metabolites downstream of the identified growth limiting enzymes increased the log-phase growth rate by 13% at 42 °C [39]. A further improvement of this model, FoldME, additionally includes the protein-folding network to predict the cellular response to unfolding stress [40]. Furthermore, OxidizeME (https://github.com/SBRG/oxidizeme) [41] includes the impact of ROS and AcidifyME (https://github.com/bdu91/acidify-ME) [42], the mechanisms of acid stress mitigation to predict the acid stress tolerance of *E. coli*. As illustrated in these examples, expansion of GEMs allows for the simulation of different gene expression levels across different environmental changes. Systematic and mechanistic understanding of stress response is expected to result in the identification of further targets.

Despite their great success in identifying bottlenecks in metabolic pathways, the usage of GEMs to increase the robustness of microorganisms is limited by our understanding of toxicity and tolerance mechanisms. Computational models can only reflect and simulate known reactions and interactions. Therefore, fundamental science to improve our understanding of molecular stress reactions will remain vital for improving computational models.

3. (Semi-) rational approaches

As an alternative to above mentioned non rational approaches, rational and semi-rational engineering can be a practical approach to improve tolerance and robustness of industrial microorganisms. These approaches are especially useful in circumstances where the mechanisms behind the toxicity are at least partially known. In the following section, we will first discuss membrane engineering and transcription factor engineering to illustrate the usefulness of this strategy.

3.1. Membrane engineering

The microbial membrane defines the insides of cells and separates them from the extracellular environment. As most industrial organisms are mesotrophiles, the membrane consists of a phospholipid bilayer with integrated proteins of different functions such as signalling, transport, and energy metabolism. To fulfil these functions, the membrane has to keep three key parameters at balance: the integrity, which describes the quality of the intact cell membrane; the fluidity, which is defined by the viscosity of the lipid bilayer; and the selective permeability, which controls the uptake of molecules for growth and removal of waste products [43,44]. Various factors from toxic environmental and stress conditions can disrupt these key parameters, which leads to slower growth or cell damage. Therefore, membrane engineering is a feasible and efficient way to increase stress tolerance by maintaining membrane hemostasis.

Most common strategies for membrane engineering have been focused on modulation of lipid composition, which is implicated in improving membrane integrity and regulating fluidity (Fig. 3A). One way of altering the lipid composition is a change in the degree of lipid saturation. While saturated fatty acids are more packed and result in a stiff membrane, *cis*-unsaturated fatty acids occupy more space because of the nick at the double bond of their carbon chain, which increases the permeability and fluidity of the membrane. For instance, In *S. cerevisiae*, the overexpression of the Δ9 desaturase Ole1 increased the ratio of unsaturated to saturated fatty acids and increased the stress tolerance to various stresses (e.g. weak acids and ethanol) [45]. While in another example, a *Geobacillus* acyl-ACP thioesterase was employed to reduce the unsaturated fatty acid content in the membrane and improved the viability of a free fatty acid (FFA)-producing *E. coli* strain [46]. Unlike *cis*-unsaturated fatty acids, *trans*-unsaturated fatty acids are more linear in form. Thus, it can increase lipid density and consequently decrease membrane fluidity. As one example, a *cis-trans* isomerase (Cti) from *Pseudomonas aeruginosa* was expressed in *E. coli*, resulting in enhanced exogenous octanoic acid tolerance and production [47]. The study demonstrates that the increase in straight-chain unsaturated fatty acids increased membrane rigidity and improved stress tolerance.

Another strategy is to change the length of lipid in the membrane. The longer the carbon chain of the lipid is, the more van der Waals interactions form between the lipids, which increases the stability of the membrane. For example, in *S. cerevisiae,* overexpression of an activity enhanced acetyl-CoA carboxylase mutant (ACC1*) increased the oleic acid content and the average lipid chain length. These membrane changes led to increased membrane integrity and cell viability at increasing concentrations of octanoic acid [48].

Additionally, the membrane lipid composition can be further modified by inserting cyclopropanted or branched fatty acids (Fig. 3B). For example, introducing a cyclopropane-fatty acid-acyl phospholipid synthase from *Halomonas socia* in *E. coli* resulted in increased cyclopropane fatty acids, leading to increased salt tolerance [49]. Furthermore, the lipid composition can be modulated by changing the polar head groups of phospholipids, altering the sterol or sphingolipid content [44,50–52]. For example, the salt tolerance of *S. cerevisiae* can be increased by increasing the content of complex sphingolipids in the membrane [53] and by engineering the phospholipid composition [54]. In the yeast *Candida glycerinogenes,* the overexpression of SLC1 (1-acyl-sn-glycerol-3-phosphate acyltransferase), which catalyses the first step of the phospholipid synthesis, lead to increased tolerance and production of 2-phenylethanol [55].

Besides membrane lipid composition, membrane proteins include integral membrane proteins and transport proteins, which are also important to maintain membrane hemostasis (Fig. 3C). Thereby, reconstructing the native membrane proteins and expressing heterologous transport proteins have been widely used to improve stress tolerance and microbial robustness [14,43]. Especially efflux transporter proteins can reduce the intracellular concentration of harmful substances and thereby prevent cell damage. In the case of toxic products, alleviating toxicity via efflux proteins would decrease the intracellular accumulation of final products and act as a driving force for further increase in yield and productivity. However, efflux transporters either

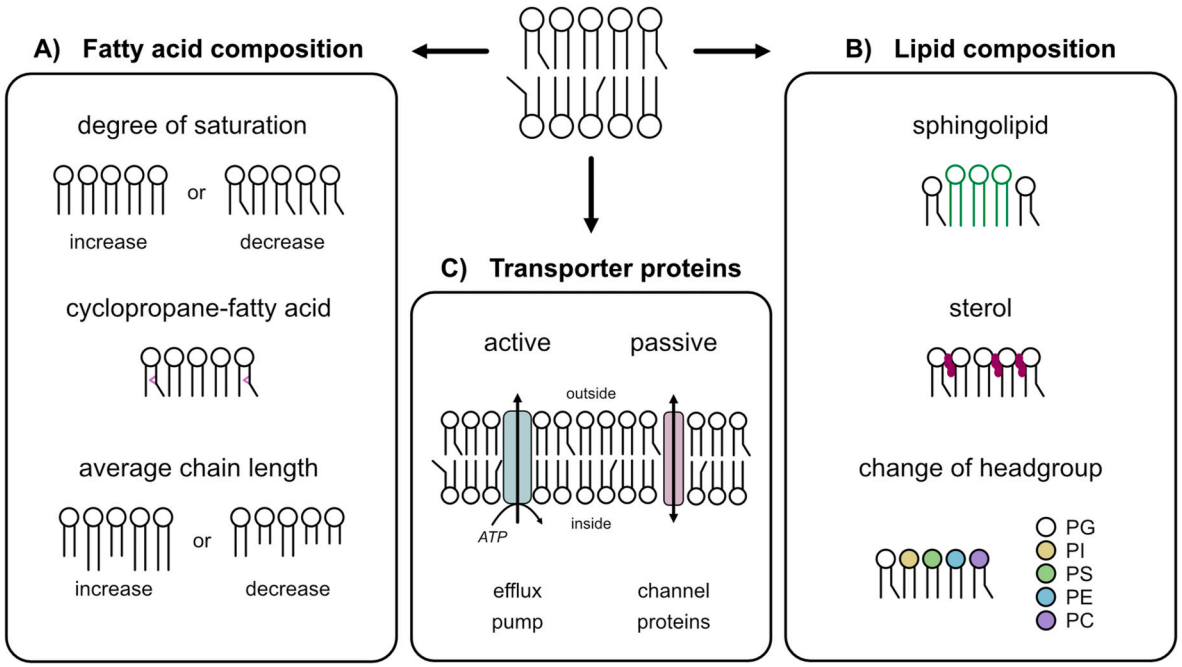

Fig. 3. Overview of different membrane engineering strategies to increase stress tolerance and resistance. **A)** Engineering of the fatty acid composition of the membrane by altering the degree of saturation, the average chain length, or integrating cyclopropane-fatty acids. **B)** Engineering lipid composition by altering the sphingolipid or sterol content or changing the phospholipid headgroup (PG Phosphatidylglycerol, PI Phosphatidylinositol, PS Phosphatidylserine, PE Phosphatidylethanolamine, PC Phosphatidylcholine). **C)** Integrating transporter proteins into the membrane, which can either be passive channel proteins or active energy-consuming efflux pumps. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

use ATP or an ion gradient as an energy source to transport specific molecules or molecule classes across the membrane out of the cell (as extensively reviewed in Ref. [56]). In a recent study, transporter *Sp*Mae1 from *Schizosaccharomyces pombe*, belonging to the voltage-dependent slow-anion channel transporter (SLAC1), have been shown to increase titers of succinic, malic and fumaric acids by 3-, 8- and 5-fold in *S. cerevisiae* without affecting growth. Because these voltage-dependent SLAC1 transporters do not use proton, sodium or ATP motive force to export organic acids, they have great promise for industrial production of these chemicals with a higher overall product yield [57].

Additionally, the supplementation of molecules that can be integrated into the membrane and increase tolerance poses a promising alternative to genetic engineering approaches. A recent study in *E. coli* has shown that carotenoids (lutein and zeaxanthin) can integrate into the membrane to increase butanol tolerance. The authors suggest using carotene-rich waste (e.g. tomato pomace) as a cheap carbon and carotene source, making this approach even more interesting for the sustainable production of chemicals [58].

Membrane engineering has proven to be a valuable tool for increasing microbial stress tolerance. However, membrane homeostasis is a highly complex and regulated process [59], and further studies are required to increase our understanding of this process. Furthermore, there is a need for improved genetic regulatory networks to allow microbial cell factories to adapt their membrane composition to a wide range of changing environmental stress factors.

3.2. Transcription factor engineering

Cells have evolved and optimised their cellular functions through coordinated regulation of a multitude of enzymes and pathways via different transcriptional factors in response to different environmental conditions. Therefore, engineering transcription factors can be a feasible strategy to increase the tolerance and robustness of industrial microorganisms. Transcription factor engineering is particularly useful for building complex traits that a monogenic modification approach cannot achieve. With newly developed technologies for the efficient and quick construction of mutation libraries, transcription factor engineering can be easily applied to many stresses and microorganisms by setting different selective pressures. This so-called global transcription machinery engineering (gTME) was firstly developed by Alper et al. to build *S. cerevisiae* strains resistant to high concentrations of ethanol and glucose [60]. Both are complex traits that are essential for the ethanol industry. Two target proteins Spt15 and Taf25, involved in TATA-binding events, were selected, as TATA-binding regulates SAGA-dependent and TATA-containing genes, comprising more than 15% of all genes in yeast and controlling many different metabolic processes. Two mutant libraries were constructed through an error-prone polymerase chain reaction (PCR) and screened with increased ethanol and glucose concentrations. The best outcome, *spt15*-300, showed significant growth improvement in the presence of 6% (v/v) ethanol and 100 g/L of glucose. Thereafter, a few key regulators have been successfully engineered to reprogram global transcription profile and to select for tolerance improved phenotypes, such as sigma factor δ70, cyclic AMP receptor protein (CRP) in *E. coli,* and zinc finger-containing artificial transcription factor in yeast [14].

Besides global transcription factors, it is also possible to target more specific transcription factors with specific applications. Recently, the regulon-specific transcription factor Haa1 was engineered to improve acetic acid tolerance in *S. cerevisiae* due to its involvement in the activation of approximately 80% of the acetic acid-responsive genes [61]. In addition to focusing directly on transcription factors, the RNA polymerase II has also been a target for altering the global transcription profile and improving tolerance phenotypes. One subunit of RNA polymerase II, Rpb7, was mutated by error-prone PCR and screened for improved ethanol tolerance, leading to a 40% improvement in ethanol production [62].

CRISPR/Cas methods have significantly enhanced the efficiency of genome engineering at various scales. While it can be used to include single genetic changes at a time, it can also be combined with high throughput methods. One of these methods is the CRISPR EnAbled Trackable genome Engineering (CREATE), which consists of a plasmid library containing three variable but covalently coupled components: a gRNA expression region, a barcode, and a replacement cassette [63]. This allows mutating multiple loci in parallel and tracking the desired phenotype mutations. CREATE can be used to target multiple transcription factors simultaneously with an approach named MultIplex Navigation of global Regulatory networks (MINR) [64].

In the first published MINR study, a library was designed and constructed with 43000 mutations by targeting 25 regulatory genes. With growth competition in increased ethanol and/or glucose concentrations, several regulatory genes, such as SMP1, which is involved in regulating Hog1-dependent osmo-responsive genes, were newly identified to confer improved ethanol and/or glucose tolerance and also improved ethanol production in *S. cerevisiae* [64]. A more extensive library containing over 83000 mutations was constructed in a follow-up study by performing saturation mutagenesis [65] of the active sites of 47 transcriptional regulators that interact with more than half of the yeast genome. A variant of WAR1, a transcription factor in response to weak acid, was identified to confer tolerance to isopropanol and isobutanol [66].

Transcription factor engineering, such as gTME, focuses on one transcription factor target at a time. Although this affects the regulation of numerous genes, they represent only a small fraction of the whole genome. Additionally, it is challenging to study how different regulators interact in a cell. Especially for eukaryotic cells, in which the transcription network is more complex than in prokaryotic cells. Thanks to the CRISPR technique, it is possible to study global regulatory network interactions with the MINR approach. Furthermore, with tractable design, the evolved phenotypes can be traced to the corresponding mutation. Nevertheless, MINR is still challenged by SNPs on oligo cassettes which can affect the spacer region of the MINR cassettes and decrease gRNA activity.

4. Conclusions and perspectives

In this review, we discussed non-rational and (semi-)rational approaches to improve tolerance when designing microbial cell factories. From the reviewed methods, the non-rational approaches do not require knowledge of any underlying genetic mechanisms and instead allow for identifying genetic targets for a particular phenotype. Depending on the applications and technical availabilities, ALE or omics-based analysis can be applied to improve stress phenotypes. This is becoming more achievable with recent technology advancements that enable cost-effective sequencing and functional genomics. Furthermore, these technologies can complement each other and be combined. For example, an evolutionary approach and an omics-based approach can be used first to evolve a phenotype and then map the genome of the evolved strain to identify its genetic basis. Also, computational approaches can be used to predict targets and then validate with omics-based profiling approaches. Still, these targets need to be experimentally verified by subsequent genetic manipulation to endow beneficial phenotypes on other industrial production strains.

With prior knowledge of toxicity and tolerance mechanisms available, the (semi-)rational approaches often target specific cell parts. For instance, due to the gatekeeper function of microbial membranes, engineering plasma membranes has been a feasible strategy for improving stress tolerance. Alternatively, cellular regulation can be manipulated by changing a few transcription factors to fine-tune a multitude of enzymes and proteins to cope with different environmental changes or stresses. Although the regulation network is complex, and we still do not entirely understand it, even for model microorganisms, the situation is going to change. For example, the MINR method has allowed the

manipulation of many transcription factors to study the correlation with different stresses.

Moreover, with the rapid development of Artificial Intelligence (AI) and automation, it is becoming more affordable to screen big mutant libraries with robots. Similarly, sequencing platforms are also cheaper and more competitive, and in combination with automated cultivation systems, it becomes more feasible to perform multi-omics analysis. It is expected that software and big data analytics such as AI will be the key technological drivers of the 2020s.

Nevertheless, choosing a method to engineer microbial cell factories is determined by multiple factors: the available knowledge, genetic tool kits for the studied organism, and the desired outcome. If the available knowledge is scarce, the application of (semi-) rational approaches is limited, while non-rational approaches can still be used. Additionally, even if knowledge is available, genetic tool kits and protocols must exist for the chosen organism to implement some of the discussed methods. For example, the introduction of transporter proteins requires basic toolkits to genetically engineer organisms that might not be available for unconventional hosts. Finally, the chosen approach depends on the desired outcome. For instance, if the aim is to generate an improved host, evolutionary approaches are suitable. If the aim is to understand further the biological process underlying the improvement, the combination with other methods, e.g. omics approaches, is needed. However, it is notable that all discussed methods have shown to be very efficient in improving stress tolerance in microbial cell factories (Table 1).

Microbial cell factories have been developed primarily on model microorganisms such as *E. coli* and *S. cerevisiae*. However, non-model organisms may be more suitable hosts for target products, as they can cope better with toxic products or intermediates than traditional model organisms [67]. In recent years, the knowledge of genetic engineering tools has increased, and it is becoming easier to work with non-conventional host organisms, which may become an important direction for future cell factory development.

Declaration of competing interest

None.

CRediT authorship contribution statement

Marta Tous Mohedano: wrote, revised, and edited the draft. approved the manuscript. **Oliver Konzock:** wrote, revised, and edited the draft. approved the manuscript. **Yun Chen:** wrote, revised, and edited the draft. approved the manuscript.

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