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Review Article

Advance of tolerance engineering on microbes for industrial production

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ABSTRACT

Industrial microbes have become the core of biological manufacturing, which utilized as the cell factory for production of plenty of chemicals, fuels and medicine. However, the challenge that the extreme stress conditions exist in production is unavoidable for cell factory. Consequently, to enhance robustness of the chassis cell lays the foundation for development of bio-manufacturing. Currently, the researches on cell tolerance covered various aspects, involving reshaping regulatory network, cell membrane modification and other stress response. In fact, the strategies employed to improve cell robustness could be summarized into two directions, irrational engineering and rational engineering. In this review, the metabolic engineering technologies on enhancement of microbe tolerance to industrial conditions are summarized. Meanwhile, the novel thoughts emerged with the development of biological instruments and synthetic biology are discussed.

1. Introduction

Facing the problems of climate warming, environmental degradation, and the exhaustion of petroleum and other petrochemical resources have been increasingly severe, a new development mode has become urgent for modern industry, which can reduce dependence on petroleum resources. Biological manufacturing is based on industrial microbial technology to produce energy, materials and chemicals by transforming existing manufacturing processes or using biomass, carbon dioxide and other renewable materials, which is a novel and environment-friendly industrial model. More and more microorganisms have been applied to bio-manufacture with the growing development of synthetic biology, like *E. coli*, *S. cerevisiae*, *Y. lipolytica* and other non-conventional strains [1].

However, the industrial environments used to be hostile to microbes, such as high temperature, high pressure, high osmotic pressure, and hyperacid/alkali, which inhibits microorganisms and limits the increase of titer, yield, and productivity (**TYP**) [2]. In terms of robustness of industrial microbe, the stress environments caused by stressors of basic or applied interest are the crucial limit factors, which results in cell growth restriction or even death, a decrease in production capacity and excessive industrial production costs. Therefore, improving the robustness of industrial microbes is an inevitable way to promote the development of biological manufacturing. Regarding the robustness of chassis cells, tolerance engineering could be divided into irrational and rational engineering (Fig. 1.). This review emphasizes on latest advances and current situation of tolerance engineering of industrial microbes.

1.1. Irrational engineering

The key of irrational engineering is the random mutation and directed screening, which is similar with directed evolution of enzymes. Essentially, it is to obtain cells of different genotypes of cells/enzymes and then to screen for mutants of interest in specific directions. Based on

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[1] Sadly, Prof. Pingkai Ouyang passed away in January, 2023. As this work was supervised by him, the rest of the authors decided to finish the review and to submit the paper with his name as coauthor. This is our tribute to our dear master.

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the logic, irrational engineering of tolerance could be operated without great comprehension of target strain, which is the most outstanding advantage compared with rational engineering. So, the efficiency of mutation of strain determines the quality of the strategy.

Applications of irrational engineering were not limited to tolerance. The engineered cells used to be accompanied by well-behaved phenotypes, such as high production and high yield [3]. The better tolerance of engineered cell was usually obtained with other results. Additionally, the difference of genotypes between engineered strain and parent strain could be identified through genome sequencing, transcriptome analysis and other analysis methods. Then, researchers could analyze the link between phenotype and genotype in order to explain the changes of phenotype. Accumulation of relationship between phenotype and genotype could contributie to our understanding of the systems biology of complex traits, which laid a foundation for the rational engineering.

This section summarizes technology applied in irrational engineering, including mutagenicity, adaptive laboratory evolution (ALE) and gene shuffling (GS). These are the most common and used irrational strategies.

1.1.1. *Mutagenicity*

Mutagenicity technique is efficient in artificially increasing the genetic mutation probability of microbes and changing the genetic structure and function of microbes through physical, chemical and biological methods. This technique can combine with efficient screening methods to obtain well-performance strains, such as high yield and excellent robustness. With the advantages of simple operation, low cost and high mutation rate, mutagenicity technique is widely employed in the field of microbial tolerance engineering and has achieved demonstrable results [4].

Currently, Atmospheric and Room-temperature Plasma (ARTP), one of the most widely used mutagenesis techniques, is a means of physical mutagenesis that acts on microbes via plasma and then induces the microbes to produce various types of mutations. Due to little demand for genetic information on target strain, the application of ARTP in microbial tolerance engineering has been reported for multiple chassis microorganisms (Table 1). Meanwhile, the kinds of stressors cannot make any restriction on application of ARTP, whatever specific compound such as ethanol or complex inhibitor and hydrolysates of lignocellulosic biomass.

Wu et al. reported that the mutation *Acetobacter pasteurianus* U1-1 by ARTP could grow in an 11 % ethanol medium with a titer of acetic acid improving of 385.7 %, compared with the parent strain [5]. In the research of Kong et al., the butanol-tolerant mutation *Clostridium beijerinckii* BT14 was obtained by ARTP, the production of butanol and ABE solvents was 25 % and 33 % higher than its parent strain respectively. In addition, the improved performance of the mutation BT14 was attributed to accurate and effective synthesis of intracellular NADH and high activity of NADH-dependent butanol dehydrogenase [6]. *Enterobacter cloacae* was engineered by ARTP to enhance tolerance to salt by Hua et al., and the mutation MU-1 exhibited regular growth in medium with 7.5 % NaCl. They found that the membrane permeability and the exopolysaccharide (EPS) level outside the membrane of MU-1 were changed, which could maintain the osmotic balance and be responsible for the NaCl-tolerant phenotype [7]. After the study of Hua, cadmium (Cd)-tolerant *E. cloacae* TU was obtained by ARTP in study of Xu et al. The engineered strain could grow in solution with 250 mg/L cadmium and its maximum Cd stabilization capacity was up to 67.0 mg/g dry cell weight [8]. *Clostridium beijerinckii* NCIMB 8052 was a high tolerance to ferulic acid from ARTP by Liu et al. Among mutation strains, the mutant M11 could produce most butanol under ferulic acid stress (0.5 g/L~0.9 g/L), which reflected that the high ferulic acid tolerance strain M11 could produce butanol with non-detoxified SAHHC [9].

1.1.2. *Adaptive laboratory evolution (ALE)*

Adaptive laboratory evolution (ALE) is a straightforward and effective approach in biological research (Fig. 2). With a wide range of applications, ALE could improve cell capabilities like enhancing tolerance,

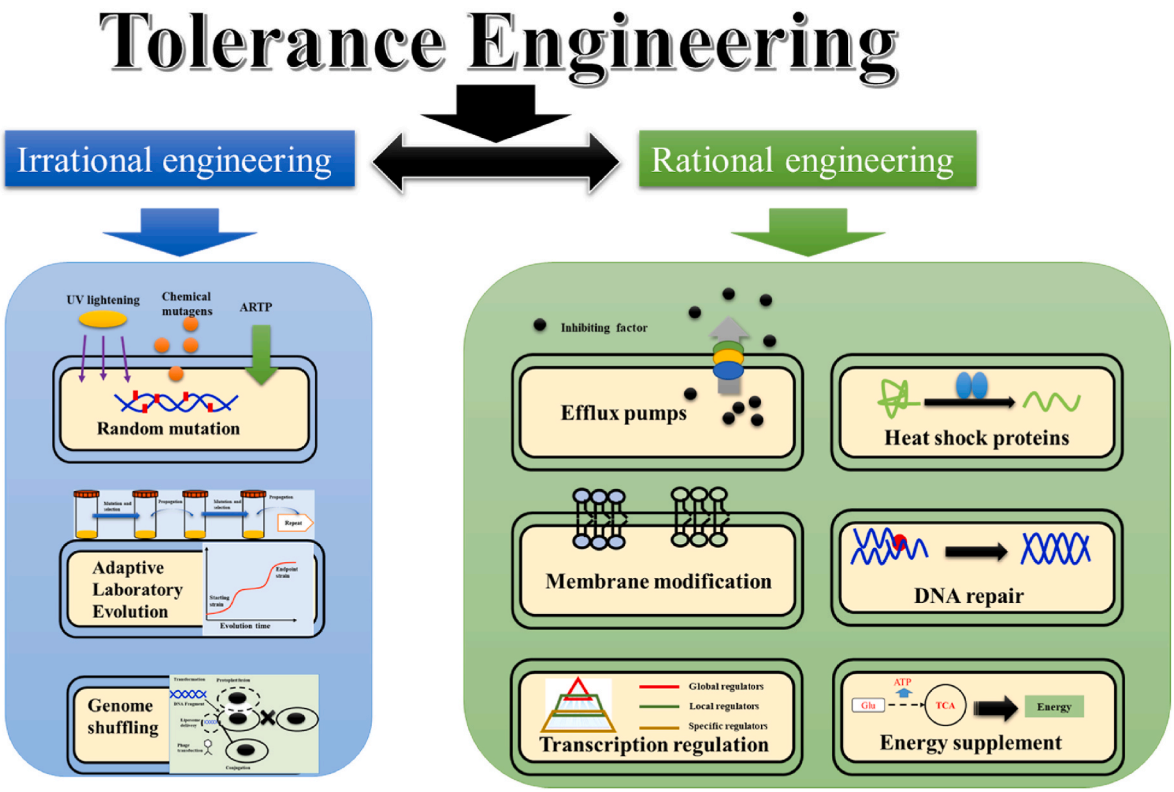

Fig. 1. Summary of tolerance engineering strategies.

Table 1
Summary of increased tolerance by ARTP.

Microbes	Stressors	Final results	Refs
Clostridium beijerinckii	ferulic acid	A mutation strain M11 possessed high tolerance to ferulic acid (0.9 g/L).	[9]
Acetobacter pasteurianus	ethanol	A mutation U1-1 could grow in medium with 11 % ethanol and the acetic acid production enhanced 385.7 % compared with the parent strain.	[5]
Aspergillus terreus	undetoxified enzymatic hydrolysate	A *Terreus* mutation AT-90 could grow and synthesize itaconic acid in the undetoxified enzymatic hydrolysate, where the highest titer of itaconic acid was 19.3 g/L with the yield of 36.01 %.	[109]
Clostridium beijerinckii	butanol	Tolerance to butanol of mutation BT14 was significantly improved. The titer of butanol and ABE solvents was enhanced by 25 % and 33 %, respectively.	[6]
Rhodosporidium toruloides	hydrolysates of lignocellulosic biomass	Three mutant strains feature good tolerance to vanillin, furfural, and acetic acid. Mutation M11 whose accumulated intracellular lipids up to 60 % of dry cell weight is notable.	[110]
Bacillus coagulans	hydrolysates of lignocellulosic biomass (5-hydroxymethylfurfural, vanillin, syringalde- hyde and *p*-hydroxybenzaldehyde)	An inhibitor-tolerant strain, GKN316, showed the ability of less toxic relevant alcohols biotransformation from those toxic inhibitors, which is evidence of the applicability of GKN316 to produce lactic acid from undetoxified lignin-lulosic hydrolysates.	[111]
Enterobacter cloacae	NacI	A mutation MU-1 can regularly grow in slurry cultivation with 7.5 % NaCl, which is 2.36 times of the wild-type strain.	[7]
Rhodosporidium toruloides	inhibitors in non-detoxification of lignocellulosic hydrolyzates	Mutant M18 has a lipid content close to 50 % and is highly tolerable to all inhibitors.	[112]

substrate biotransformation and general discovery, improving product yield or potency and growth rate optimization [10]. For candidate microbe, natural evolutionary efficiency of strain is accelerated by prolonging culturing of cells in a chosen environment. Then, the mutations with excellent performance will be selected for further study [11,12].

In fact, many kinds of stressful environments have been adopted ALE to enhance stress tolerance, such as extreme pH, inappropriate temperature, excessive osmotic pressure and a variety of inhibitors, which summarized in Table 2.

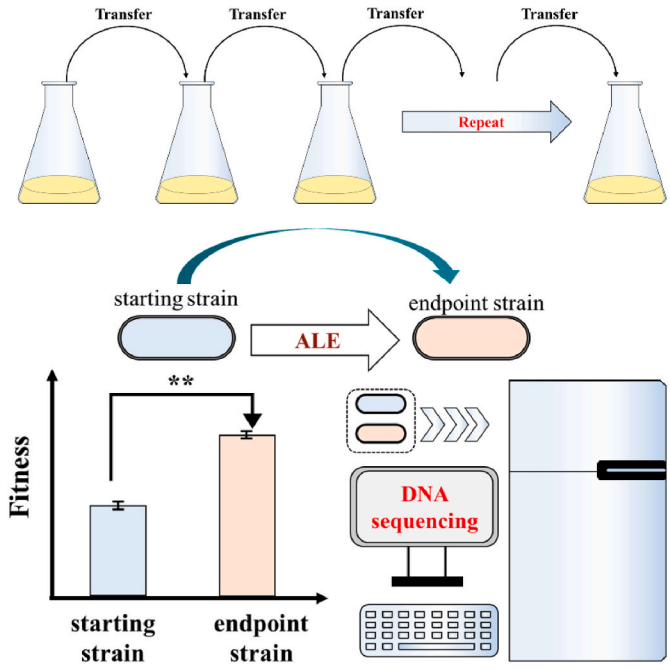

Fig. 2. Schematic of adaptive laboratory evolution.

The effects of extreme temperature were investigated by ALE in *E. coli to* enhance cell tolerance to high temperature [13–16], and the highest temperature was up to 48.5 °C [17]. In addition to temperature, there are a lot of reports that the cell tolerance to stressors, including pH [18], osmotic stress [18], isobutanol [19], ethanol [20,21], n-butanol [22,23], acetic acid [24,25] and mixed stressors [25–27]was enhanced by ALE. For example, Fletcher focused on yeast acid tolerance and employed ALR on *S. cerevisiae* to adapt to both organic (L-lactic acid) and inorganic (HCl) acid environments. The engineered strain was conferred with tolerance to HCl and to 0.3 mol/L L-lactic acid at pH 2.8. According to whole genome sequencing and RNA-seq analysis, HCl tolerance was based on the alteration of sterol composition and compromised iron acquisition and lactic acid tolerance was due to the development of a multicellular morphology and the metabolism of strong lactate [28]. In research of Kildegaard et al., a d 3-hydroxypropionic acid (3HP) tolerant *S. cerevisiae* was obtained by ALE. After genome sequencing, the toxicity of 3HP worked through 3-hydroxypropionic aldehyde, which was detoxicated by. glutathione-dependent reaction [29]. When Martin et al. studied on cross-stress protection, *E. coli* was evolved over 500 generations in five stress environments. Citing the results of genome sequencing, transcriptional profiling and functional gene analysis, they found that cross-stress protection is a ubiquitous phenomenon and bacterial populations possessed a high phenotypic plasticity [26].

Comparing to the fact that the specific stress tolerance of industrial strains fails to be improved by rational engineering due to the deficiency in requisite knowledge, ALE can improve hosts performance through simple, mechanical but effective manipulation. Thus, ALE is recommended as one of the most efficient tools for increasing the practicability and robustness of industrial host strains.

1.1.3. Gene shuffling

Genome shuffling (GS) is a classical laboratory evolution method that can be simply understood as iterative recombination on a genome scale [30]. The GS contains three key elements: obtaining genetically diverse population, screening or selecting to identify or enrich the best-performance mutants, and introducing them into a starting population of interest [31]. In addition, the process (Fig. 3) can repeatedly operate to find a satisfactory output [30].

GS was applied in many kinds of chemical products, such as

Table 2
Summary of ALE applied in tolerance engineering.

Microbes	Stressors	Final results	Refs
E. coli MG1655	High temperature	Maximum growth temperature was improved from 46 °C to 48 °C and Optimum growth temperature was arised from 37 °C to over 46 °C	[13]
E. coli	Temperature	30 groups of *Escherichia coli* evolved over 2000 generations	[14]
E. coli Bc251	Temperature	*Escherichia coli* evolved under high temperature condition (41.5 °C) for 2000 generations	[15]
E. coli MG1655	Temperature	*E. coli* evolved at 42 °C	[16]
E. coli MG1655	Temperature	*E. coli* could grow at max temperature at 48.5 °C	[17]
E. coli	pH, temperature or osmolarity.	By inserting evolved IS*10,* the expression of *otsBA* was shifted from RpoS-dependent to RpoS-independent, thus partially restoring the wild-type response to osmotic stress.	[18]
E. coli JCL260	isobutanol	The results showed distinction in the level of resistance to different inhibitors, with improved growth rates up to 57 %, 12 %, 22 %, and 24 % in hydrolysates, acetic acid, HMF and furfural, respectively.	[19]
E. coli	ethanol	Several of these pathways, in particular heat-shock stress response and osmoregulation, are familiar modification reagents of ethanol tolerance; while others, such as acid-stress response and fimbrial structures are emerging pathways.	[20]
E. coli	ethanol	Ethanol-induced inhibition, uncoupling of mRNA and protein synthesis through direct effects on ribosomal and RNA polymerase conformation are significant ethanol toxicity determinants in *E. coli*, and adaptive mutations in *metJ*, *rho* and *rpsQ* serve to safeguard the central dogma while the ethanol presents.	[21]
E. coli	*n*-butanol	Regarding studies on the potential role of iron-related genes in *n*-butanol tolerance, gene overexpression and deletion studies hypothesized that upregulation of iron-related genes indirectly leads to outer membrane modifications that enhance *n*-butanol tolerance.	[22]
E. coli	*n*-butanol	Additional membrane-associated and osmotic stress-related genes were identified in *E. coli* that confer *n*-butanol tolerance.	[23]
Saccharomyces cerevisiae	Acetic acid	The fraction of growing cells rose in all six mutants when transferred from a non-stressed environment to a medium with acetic acid.	[24]
Saccharomyces cerevisiae.	Acetic acid, furfural, and hydroxymethylfurfural	The results indicated differentiated degrees of resistance to different inhibitors, with increased growth rates of hydrolysates, acetic acid, HMF and furfural up to 57 %, 12 %, 22 %, and 24 %, respectively.	[25]
E. coli MG1655 and Δ*lacZ* strains	Five environments (osmotic, acidic, oxidative, *n*-butanol, and control; four biological replicates per environment)	Cross-stress dependencies are pervasive, strongly interrelated and can appear over short periods. Bacterial populations dominate a genotypic space allowing for a high degree of phenotypic plasticity as they adapt to fluctuating environments.	[26]
Saccharomyces cerevisiae	Oxidative, freezing–thawing, high-temperature and ethanol stress.	The optimal clones obtained from the populations were 102, 89, 62 and 1429 times more resistant to freezing–thawing, temperature, ethanol, and oxidative stress, respectively.	[27]

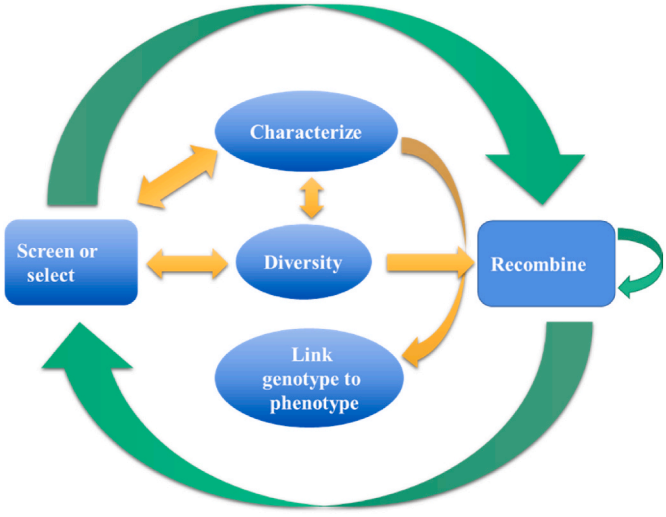

Fig. 3. Schematic of gene shuffling.

antimicrobial lipopeptide in *Bacillus amyloliquefaciens* [32], vitamin B12 in *Propionibacterium shermanii* [33] and daptomycin in *Streptomyces roseosporus* [34]. GS is also presented in tolerance engineering: salt of *Hansenula anomala* [35], acid tolerance of *Lactobacillus* [36], oxidative stress [37], glucose [38], thermos-tolerance of *Corynebacteria glutamicum* [39], acetic acid tolerance [40,41], multi-stress tolerance including osmotic, heat, and acid tolerances [42].

GS provides a powerful evolutionary method based on genetic diversity on the whole-genome scale. By continuous iterative recombination, the phenotype of host cells can tend to be perfect due to the theoretically unlimited GS process, where the process will not stop until an ideal phenotype is obtained [30]. However, the application scope and methods of genome shuffling are relatively narrow as so far. Nor does it rule out that under certain circumstances, the GS will excel.

2. Rational engineering

Rational design of chassis cells is a common method to improve tolerance of industrial microbes. The basis of rational engineering is that the specific stressor and how the stressor work has been determined. Meanwhile, it is necessary to know how to eliminate the effects of stressor. The link between genotype and phenotype mentioned before can be considered as the priori knowledge, which illustrate the how to improve cell tolerance to certain stressor by modification of genes. So, summarizing the tolerance engineering strategies is necessary and helpful for researchers and practitioners to solve related problem.

2.1. Regulator engineering

Microbes have intricate pyramid-shaped hierarchical regulatory networks of gene expression. Different regulators have auto-regulation, co-regulation and cross-talk regulation, complicating the metabolic regulatory network while allowing efficient and rapid adaptation to changes in the environment through this complex regulatory network. In particular, global regulators, which act as regulatory hubs in biological metabolic networks, can regulate gene expression in cells on a large scale, thereby allowing cells to adapt to new environments. Hence, regulators engineering is also an effective tool for tolerance engineering.

Global regulators are at the top of the regulatory network pyramid, which can regulate the transcription at the whole-gene level. Thus, the global transcriptional machinery engineering (gTME) taking the global regulators as targets exhibits a great advantage in improving complex tolerance phenotypes [43]. The related researches about gTME were summarized in Table 3. CRP is an important global regulator in *E. coli*, which is responsible for direct regulation of more than 490 genes. The research team of Rongrong Yang focused on researching the effects of CRP on tolerance in *E. coli*. In their studies, the engineering of CRP was employed to enhance *E. coli* tolerance to various stressors, such as osmotic pressure [44], toluene [45], oxidative stress [46], 1-butanol [47], acetate [48], low pH [49] and isobutanol [50]. Histone-like nucleoid structuring factor (H-NS) was engineered to potentiate the acid tolerance in *E. coli* by Xianxing Gao et al. When the recombinant *E. coli* strains overexpressing H-NS were cultured under pH 4.5 for 24 h, the final biomass was increased by 24 % than the control [51]. σ^{70} encoded by *rpoD* is another global regulator with a significant role in microbe robustness. Global transcription machinery engineering of sigma factor was reported by Alper et al. that the tolerance to ethanol was enhanced to 60 g/L and the SDS tolerance was improved as well [52]. According to Zhang et al., the organic-solvent tolerance was improved by global transcription machinery engineering on σ^{70}. The best *rpoD* mutant C9 exhibited tolerance to 69 % cyclohexane [53]. The engineering on sigma factor not only works in *E. coli*, but also in *Zymomonas mobilis*. In the research of Tan et al., four mutants showed elevated ethanol tolerance by random mutagenesis of *RpoD* protein. The ethanol production of the best strain ZM4-mrpoD4 were 13.0–14.1 g/L during the 30–54 h, which was about two times the initial strain [54].

Table 3
Summary of gTME applied in tolerance engineering.

Regulators	Microbes	Stressors	Refs
CRP	*E. coli*	Isobutanol	[50]
CRP	*E. coli*	Acetate	[48]
CRP	*E. coli*	Low pH	[113]
CRP	*E. coli*	1-butanol	[47]
CRP	*E. coli*	Oxidative stress	[46]
CRP	*E. coli*	Toluene	[45]
CRP	*E. coli*	Osmotic pressure	[44]
σ70 encoded byrpoD	*E. coli*	Ethanol	[52]
σ70 encoded byrpoD	*E. coli*	Cyclohexane	[53]
σ70 encoded byrpoD	*Zymomonas mobilis*	Ethanol	[54]
IrrE	*E. coli*	Inhibitors from pretreatment of lignocellulosic biomass	[62]
H-NS	*E. coli*	Acid	[51]
SPT8	*Saccharomyces cerevisiae*	Ethanol	[59]
SPT15	*Saccharomyces cerevisiae*	Ethanol	[59]
SPT15	*Saccharomyces cerevisiae*	Ethanol	[60]
SPT15	*Kluyveromyces marxianus*	Ethanol	[61]
IrrE	*Saccharomyces cerevisiae*	Urfural, 5-hydroxymethylfurfural, formic and acetic acid),	[69]
IrrE	*Saccharomyces cerevisiae*	Furfural, acetic acid and phenol tolerance; thermal stress	[70]
IrrE	*E. coli*	Salt	[63]
IrrE	*E. coli*	Salt	[64]
IrrE	*E. coli*	Inhibitors of lignocellulosic hydrolysates	[62]
IrrE	*E. coli*	Osmotic stress	[67]
IrrE	*Lactococcus lactis*	Oxidative stress and high osmotic pressure	[66]
IrrE	*Zymomonas mobilis*	Ethanol, acid, osmotic, and thermal shocks	[68]
IrrE	*E. coli*	Salt stress	[65]

Similar tolerance modifications have been reported in yeast cells. CRZ1 from *S. cerevisiae* and TdCRZ1 from *Torulaspora delbrueckii* was overexpressed in *S. cerevisiae* HS13 to increase tolerance to salt and freeze. However, △cnb1 and △crz1 mutants exhibited no sensitivity to freezing, which indicated the effects that freeze tolerance from overexpression of CRZ1 seem to have nothing to do with known salt-responding pathways [55]. Additionally, the effect of CRZ1 was found in tolerance of Dongni Yan et al. reported that deletion of CgCRZ1 could severely inhibit cell growth and activity. According to genome-wide transcription analysis, the function of Crzlp mainly focuses on regulating genes involved in acid stress sensitivity. Overexpressing Crzlp contributed to increasing content of ergosterol and enhancing membrane integrity, fluidity and H^+-ATPase activity, ultimately improving the acid resistance of *C. glabrata* [56]. HAA1 is a widely-reported acetic acid-responsive transcriptional activator in *S. cerevisiae*. Sakihama et al. reported that overexpression of HAA1 was proved to effectively relieve acetic acid stress and improve cell growth and ethanol production [57]. Overexpression of HAA1 was also identified to positively affect yeast tolerance towards acetic acid compared with PRS3 overexpression. Under 4 g/L acetic acid, the recombinant *S. cerevisiae* exhibited superior growth [58].

The proteins interacting with the TATA-binding protein were the key to transcription regulation in *S. cerevisiae*. Alper et al. first operated gTME on *S. cerevisiae* to enhance ethanol tolerance. The SPT15 gene encoding the TATA-binding protein was selected and the spt15-300 mutant exhibited superior ethanol tolerance. The final DCW, specific productivity and volumetric productivity increased to/by 20 %, 14 % and 69 %, respectively, compared to the control [59]. A similar method was employed by Yang et al.: the strains iETS2 and iETS3 were

constructed and the ethanol yield and productivity were increased by 26 % and by 23 % compared to the control. According to the global transcriptional profiles of strains ETS2 and ETS3, 42 genes were identified with twofold change and deletion 18 genes resulted in ethanol sensitivity, among which eight genes were functionally unknown [60]. The SPT 15 was also applied to *K. marxianus* for ethanol tolerance, which improved ethanol tolerance and production [61]. Directed evolution is used for the SPT8 gene, suppressor of ty insertions 8, by Ting Xue et al., thus, the mutant strain showed 8.9 % ethanol tolerance higher than the control [59].

Besides the native global regulators, the exogenous regulatory factors can greatly function in tolerance engineering. IrrE from *D. radiodurans* is the most widely-studied exogenous factor, which is identified as an essential regulator in DNA repair pathway [62]. Jie Pan et al. reported that expression of IrrE could enhance salt tolerance in *E. coli* and *B. napus*. They found that IrrE regulated various sets of proteins as a switch by comparative proteomic analysis [63]. In response to NaCl shock, whole-genome microarray assays were employed to analyze the function of IrrE expression in *E. coli* by Peng Zhao et al. IrrE increased the expression of the antiadaptors of RpoS which inhibit RpoS degradation. Therefore, expression of IrrE could improve *E. coli* tolerance to salt and other stress [64]. According to the salt resistance caused by overexpression of IrrE, Wenming Zhang et al. utilized seawater to produce succinic acid. The titer and succinic acid yield was 24.5 g/L and 0.88 g/g, respectively with Yellow Sea seawater, which illustrates that reasonable modification of chassis cells can reduce the production cost [65]. Besides salt resistance, expression of IrrE can confer host cells with tolerance to multiple stress like osmotic [66,67] ethanol, acid, osmotic, thermal shocks [68], inhibitors of lignocellulosic hydrolysates [62], furfural, 5-hydroxymethylfurfural, formic, acetic acid [69], furfural, acetic acid, phenol tolerance and thermal stress [70].

2.2. Cell membrane/wall engineering

The cell membrane/wall system is an important biological barrier separating microorganisms from their environment, which is the first prevention system when cells come into stress circumstance [71]. The existence of stressors attacks the cell membrane/wall with adverse effects on physiological function, consequently resulting in cell death [72]. Thus, engineering on cell membrane/wall has become necessary for industrial strains' tolerance to stressors.

2.2.1. Cell wall engineering

The cell wall is a significant part of the isolation of the cell from the external environment. Although rare, cell robustness can also be improved by changing the composition of the cell wall. Yuan et al. reported that overexpression of *murA2* gene, which is involved in peptidoglycan biosynthesis from *Lactobacillus plantrarum*, in *E. coli* to increase ethanol tolerance and production [73].

2.2.2. Cell membrane engineering

The cell membrane consists of phospholipids, sphingolipids, sterols and membrane proteins, which is a prominent target of attack in stressful surrounding. The integrity, fluidity and permeability of membrane are the critical parameters reflecting cell status under stress. Table 4 summarizes research on membrane engineering.

When *S. cerevisiae* was under attack of ethanol, the components of cell membrane would change to adapt to stress, where the fluidity of membrane is a key factor regulated by the proportion of saturated fatty acid (SFA) to unsaturated fatty acid (UFA) [74,75]. Overexpression of OLE1 in *S. cerevisiae* could enhance cell tolerance to various stressors including acetic acid, benzoic, propionic, sorbic acids, ethanol, NaCl, diamide, menadione and *tert*-butyl peroxide. This cross stress tolerance phenotype benefits from that Hog1 was constitutively active by overexpression of OLE1 through Ssk2 [76]. Expression of OLE1 was found to increase during the exponential phase in bioethanol fermentation of *S. cerevisiae*. Cell diameter and membrane integrity decrease while unsaturated fatty acids increase reflecting the increased membrane fluidity [74,77]. Additionally, ergosterol also played an important role in cell membrane, which contributed to maintaining the integrity, fluidity and permeability [77]. Thus, Kamthan et al. employed a heterologous ergosterol biosynthesis enzyme from an edible to express in fission yeast, resulting in the enhancement of tolerance to ethanol, low pH and high temperature [77]. Expression of the $Acc1^{S1157A}$ mutant of the native acetyl-CoA carboxylase was reported to increase oleic acid content and enhance the tolerance to octanoic acid and other stressors (hexanoic acid, 2-propanol, and n-butanol) as a result [78]. However, octanoic acid tolerance could be enhanced by improving integrity of cell membrane, and decreasing the fluidity of cell membrane. In the research of Tan et al., the Cti enzyme from *p. aeruginosa* was heterologously expressed in *E. coli* for better robustness by converting cis (CUFA)to trans unsaturated phospholipid fatty acids (TUFA) [79].

Table 4
Summary of membrane modification applied in tolerance engineering.

Microbes	Stressors	Final results	Refs
S. cerevisiae	Ethanol	The proportion of saturated fatty acids was replaced with the proportion of unsaturated fatty acids.	[74]
S. cerevisiae	Ethanol	The proportion of saturated fatty acid was changed to unsaturated fatty acid.	[75]
S. cerevisiae	Various stressors including acetic acid, benzoic, propionic, sorbic acids, ethanol, NaCl, diamide, menadione and *tert*-butyl peroxide	Hog1 was constitutively active by overexpression of OLE1 through Ssk2	[76]
S. cerevisiae	Ethanol and high temperature	Overexpression of OLE1 resulted in the increased membrane fluidity.	[77]
S. cerevisiae	Octanoic acid, hexanoic acid, 2-propanol, and *n*-butanol	The $Acc1^{S1157A}$ mutant of the native acetyl-CoA carboxylase was overexpressed.	[78]
E. coli	Octanoic acid	The *Cti* enzyme from *p. aeruginosa* was heterologously expressed to improve integrity of cell membrane.	[79]

2.2.3. Efflux pumps

Efflux pumps, one of the membrane transporters, consist of three proteins: an outer membrane channel, a periplasmic and an inner membrane protein that is responsible for substrate recognition and proton exchange (Table 5), which are capable of exporting toxic

Table 5
Summary of efflux pumps applied in tolerance engineering.

Microbes	Stressors	Methods	Refs
E. coli	limonene	A library of efflux pumps was constructed and a best efflux pumps to enhance tolerance to limonene was obtained by screening in the library.	[80]
E. coli	isopentenol	Genes up-regulated response to isopentenol was identified.	[81]
E. coli	1-hexene, 1-octene, and 1-nonene	Directed evolution of AcrB	[82]
E. coli	*n*-octane and α-pinene	Directed evolution of AcrB	[83]
E. coli	n-butanol	Directed evolution of AcrB	[84]
E. coli	n-butanol	The expression of efflux pumps was regulated by stress promoter.	[85]
S. cerevisiae	stressors from lignocellulosic ethanol fermentation	The tolerance genes was dynamically regulated by stress-driven promoters.	[114]

metabolites out of cell to relieve toxicity [72,80].

Researches about efflux pumps in tolerance engineering could be categorized into several sections: screening and expressing efflux pumps, directed evolution of the inner membrane protein and dynamic regulating expression of efflux pumps. In the research of Dunlop et al., a list of efflux pumps was obtained by bioinformatics and a library of 43 efflux pumps was constructed in *E. coli* depending on the result generated. To screen the effect of efflux pumps, a novel competitive growth assay based on the mechanism that tolerant strains will dominate the growth was established. Through the screen method, an uncharacterized pump from *Alcanivorax borkumensis* was found to contribute to enhancing tolerance to limonene and increase yield even under a concentration which would not cause inhibition [80]. Additionally, Transcriptome analysis can also contribute to identifying key genes. The genes upregulated in response to isopentenol were overexpressed in *E. coli* to confirm the performance of tolerance. Some genes, including oxidative stress response, general stress response, heat shock-related response, and transport groups genes, could confer isopentenol tolerance onto host cells. In these tolerance genes, MdlB belonging to ABC transporter could efficiently improve isopentenol production with a 12 % improvement, which was the first native efflux pumps that could promote the production of short-chain alcohols and increase tolerance [81].

Applying enzyme engineering into tolerance engineering is a clever idea. Due to plenty of kinds of toxic metabolites, the specific efflux pumps need to be modified to enhance efficiency of effluent. The AcrAB-TolC, a RND efflux pump of *E. coli*, was focused on by Mingardon et al. In their study, the tolerance to three compounds, 1-hexene, 1-octene, and 1-nonene, was found to increase by overexpression of AcrAB. For further enhancing tolerance to 1-hexene, directed evolution of AcrB was employed and a random mutagenesis library of inner membrane protein AcrAB was constructed. Six mutations were obtained, which conferred an improved 1-hexene-tolerance to host cell. In addition, the recombinant strains harboring the combination of mutations showed an increase of both tolerance and production of 1-hexene [82]. The same strategy was reported by Leong et al. The inner membrane transporter, AcrB, was modified by directed evolution to enhance tolerance to *n*-octane and α-pinene. The best mutations showed an increase of 47 % and 400 % in efflux efficiency [83]. In addition, the directed evolution of AcrB was conducted by Fisher et al. to n-butanol tolerance in *E. coli* and the growth rate was increased by 25 % in the presence of stressor as a result [84].

However, the inhibition on cell growth caused by overexpressing efflux pumps has been proposed [85]. The regulation of efflux pumps expression has become an important issue. Sergey et al. reported that a novel system combing stress promoter and efflux pumps could efficient improve *E. coli* tolerance to n-butanol and increase titer of n-butanol. This system ingeniously balances the adverse effects caused by overexpressing efflux pumps and the tolerance advantage brought by efflux pumps [85]. The method combining dynamic regulation and expressing genes for enhancing tolerance gradually became a trend in tolerance engineering. The stress-driven dynamic regulation of tolerance genes was applied to ethanol production to enhance the robustness of *S. cerevisiae* in lignocellulosic ethanol fermentation; hence, cell growth, xylose utilization and ethanol production were improved [90].

2.3. Chaperones

Molecular chaperone, heat shock proteins (HSPs), is a significant component of general stress response system, which is essential in protein folding and transportation, as well as in repairing damaged or misfolded proteins [86]. When cells were subjected to a stress environment, the expression of HSPs showed an upward trend according to genomic studies [87]. Several genes related to HSPs were reported significant change, including *rpoH* [88], *dnaJ*, *htpG*, *ibpAB* [89], *groESL* [90], *GroL* [91], *dnaK*, and *hsp90* [92]. These studies that applied HSPs to tolerance engineering have been summarized in Table 6. The GroESL

Table 6
Summary of HSPs applied in tolerance engineering.

HSPs	Microbes	Methods	Final results	Refs
groESL	*Clostridium tyrobutyricum*	overexpressing native groESL	Tolerance to butyric acid was markedly improved.	[93]
groESL	*Clostridium tyrobutyricum*	overexpressing native groESL	Tolerance to lignocellulosic hydrolysate-derived inhibitors was noticeably improved, in particular to phenolic compounds	[115]
groESL	*Escherichia coli*	overexpressing native groESL	Adequate expression level of GroESL is pivotal in improving phloroglucinol tolerance.	[116]
groESL	*Escherichia coli*	heterologously overexpressing native groESL from *Pseudomonas putida*	The thermo-tolerance and ethanol-tolerance were significantly increased.	[117]
groESL	*Clostridium acetobutylicum*	heterologously overexpressing native groESL from *Thermoanaerobacter tengcongensis*	Tolerance to corn cob hydrolysates was improved.	[117]
groESL	*C. acetobutylicum* ATCC824	overexpressing native groESL	Tolerances to a variety of stresses including butanol, furfural, oxidation and acid was improved.	[98]
groESL	*C. acetobutylicum* ATCC824	heterologously overexpressing native groESL from *Deinococcus wulumuqiensis* R12	Tolerances to a number of stresses including butanol, furfural, oxidation and acid were improved.	[98]
groESL	*Clostridium tyrobutyricum*	overexpressing native groESL	Tolerance of *C. tyrobutyricum* to furfural was improved	[97]
htpG	*Clostridium tyrobutyricum*	overexpressing native groESL	The tolerance to butyric acid was significantly improved.	[93]
DnaK	*C. acetobutylicum ATCC824*	heterologously overexpressing native DnaK from *Deinococcus wulumuqiensis* R12	Tolerances to some stress conditions including butanol, furfural, oxidation and acid were improved.	[98]
DnaK	*Escherichia coli*	heterologously overexpressing native DnaK from *Bacillus halodurans*	The salt resistance and high pH (9.5) tolerance were improved.	[118]
DnaK	*Lactococcus lactis NZ9000*	overexpressing native Dnak	The engineered strain exhibited greater heat resistance at 40 °C.	[99]
DnaK	*Lactococcus lactis* NZ9000	heterologously overexpressing	The engineered strain exhibited	[99]

(*continued on next page*)

Table 6 (*continued*)

HSPs	Microbes	Methods	Final results	Refs
		native DnaK from *Escherichia coli* JM109	excellent tolerance to multiple stresses, such as 3 % NaCl, 5 % ethanol and 0.5 % lactic acid.	
DnaK	*Escherichia coli* BL21 (DE3)	heterologously overexpressing native DnaK from *Bacillus pumilus strain* B3	The engineered strain was imparted tolerance to host cells under high temperature.	[119]
DnaK	*Escherichia coli* BL21 (DE3)	heterologously overexpressing native DnaK from *Alicyclobacillus acidoterrestris*	The resistance against heat (54–58 °C) and acid (5.07–7.0) stresses was significantly enhanced.	[120]
DnaK	*Pseudomonas putida*	mutation in dnaK	Tolerance to toluene was improved.	[100]
SecB	*E. coli*	screen HSPs library and directed evolution on secB	Tolerance to n-butanol and the growth increased by 3.2-fold compared to the control strain in 1.2 % (v/v) butanol. Mutation secBt10a could greatly grew from 9.14 to 14.4 % at 1.2 % butanol, which was 5.3 times higher than the control strain	[101]

belongs to Hsp60 chaperone family, which was one of the most reported HSPs in enhancement of cell tolerance. Overexpression of autologous or heterologous GroESL worked in multiple strains, including *Clostridium tyrobutyricum* [93,94], *Clostridium acetobutylicum* [95,96] and *E. coli* [95]. Suo et al. reported that overexpression of GroESL could improve tolerance to furfural and enhance titer of butyrate in combination with short-chain dehydrogenase/reductase (SDR) expressed in *Clostridium beijerinckii* NCIMB 8052 [97]. In addition to GroESL, DnaK was another important HSP in tolerance engineering [98,99]. Kobayashi et al. isolated a mutation *Pseudomonas putida* KT2442 that an Arg445 was mutated to Pro in DnaK and designated R2. The R2 exhibited increased toluene tolerance through upregulating expression of other HSPs genes [100]. Notably, it could be a better and more complete strategy that combines screening of endogenous and heterologous HSPs with modification of target HSP in application of HSPs in tolerance engineering. Xu et al. reported that overexpression of secB, which was obtained from the mining of 30 HSPs library, displayed significant tolerance to n-butanol and the growth increased by 3.2-fold compared to the control strain in 1.2 % (v/v) butanol. Then, they obtained 2800 mutant strains after directed evolution of secB, and successfully screened out $secB^{t10a}$, whose growth greatly improved from 9.14 to 14.4 % at 1.2 % butanol, which was 5.3 times higher than the control strain [101].

2.4. Other methods

The inhibitions from environment are quietly various, resulting in multiple anti-stress mechanisms in the microorganism. Besides the methods mentioned above, there are many other approaches to enhance host cell robustness. Under environmental stress, DNA damage would occur in cells with concentration-dependent relationship, presenting as double-strand breaks, frameshift mutation, etc. DNA repair protein RecO from *Lactobaciilus casei* was a markedly up-regulation in acid stress [102]. Thus, Wu et al. overexpressed RecO from *L. casei* in *Lactococcus lactis* NZ9000 to improve tolerance to acid, salt and oxidative stress [103]. Additionally, antioxidant defense system was employed to address oxidative stress. Superoxide dismutase SOD and catalase KatG were overexpressed in *Arthrobacter simplex* to enhance organic solvent tolerance by Luo et al. [104]. However, oxidative stress was common stress, which could be relieved by multiple approaches, like overexpressing HSPs or engineering on global regulator. Similarly, the energy-related genes works in circumstances of ATP deficiency, which could be replaced by other engineering strategies [104,105].

3. Conclusion and discussion

Bio-manufacturing has become increasingly attractive for the less pollution and environment-friendly. However, industrial production conditions of tended to be severe, which was detrimental for cell production. Therefore, improving the robustness of industrial microorganisms was a permanent and inevitable demand in the development of cell factories. Due to the various stress from industrial production, cell damage also varies, including cell membrane damage, DNA damage, oxidative stress and other problems, resulting in diverse methods of tolerance engineering. In this review, the engineering methods of cell to enhance tolerance were summarized and summed up a demand irrational engineering and rational engineering. Irrational engineering focused on random mutation of DNA and screening for target phenotype without requirement of host cell genetic information. Although the study of irrational engineering was time-consuming and heavy workload, each gene could not be covered, which explained why the results of irrational engineering are the faster rather than the best. Nowadays, high throughput screening technology was rapid, such as bio-foundry, making it possible that illustrates the effects of single gene knockout/overexpression on cell performance [106].

In contrast, ration engineering aimed at specific engineering methods like membrane modification or overexpression of efflux pumps, which was based on a clear genetic background of host cell. However, there are some matters that need attention. For example, plenty of research reported that the effect on tolerance of overexpression/deletion of single gene was more efficient than combination of multiple genes modification while not absolutely. Additionally, much attention was required to pay to regulation of expressing tolerance genes, especially expression of efflux pumps which could arouse inhibition on cell growth. Through dynamic regulation strategy, the expression of tolerance gene elements could be more effective in enhancing the tolerance of cells to stressors [107,108]. Furthermore, the problem of environment stress could be solved by multiple aspects. The tolerance engineering on organic solvent stress, including expression chaperones, gTME, AEL and other methods, was outstanding representative. This phenomenon reflects complexity of cellular resistance to stress. In all, both rational and irrational engineering studies contributed to summarizing the relationship between genotype and phenotype and promoting the development of cell tolerance engineering.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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