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Review Article

Advances in stress-tolerance elements for microbial cell factories

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ABSTRACT

Microorganisms, particularly extremophiles, have evolved multiple adaptation mechanisms to address diverse stress conditions during survival in unique environments. Their responses to environmental coercion decide not only survival in severe conditions but are also an essential factor determining bioproduction performance. The design of robust cell factories should take the balance of their growing and bioproduction into account. Thus, mining and redesigning stress-tolerance elements to optimize the performance of cell factories under various extreme conditions is necessary. Here, we reviewed several stress-tolerance elements, including acid-tolerant elements, saline-alkali-resistant elements, thermotolerant elements, antioxidant elements, and so on, providing potential materials for the construction of cell factories and the development of synthetic biology. Strategies for mining and redesigning stress-tolerance elements were also discussed. Moreover, several applications of stress-tolerance elements were provided, and perspectives and discussions for potential strategies for screening stress-tolerance elements were made.

1. Introduction

Microorganisms have been employed as promising cell factories for bioconversion of various low-cost substrates such as lignocellulose [1], crude glycerol [2], and one-carbon sources including carbon dioxide [3], methane, and methanol [4] into high-valued bioproducts. The Organization for Economic Cooperation and Development (OECD) estimates that the microbial manufacturing industry accounts for approximately 40 % of the entire bioeconomy. By 2030, it is projected that 35 % of the chemical products will be replaced by microbial manufacturing in China [5]. The integration of synthetic biology with microbial fermentation technology forms the cornerstone of sustainable development industries [6].

However, during industrial bioprocesses, microbs often face multiple stresses, such as toxic inhibitors, solvents (*e.g.* from raw material pretreatment), extreme pH levels, high osmotic pressure, high-temperature stress, and oxidative stress [7,8]. Engineering microorganisms to withstand these stress conditions is crucial for maintaining production robustness [9]. Enhancing the stress tolerance of microbial cells through synthetic biology emerges as a direct and effective strategy to achieve high titer, yield, and productivity for industrial bioprocesses.

This review focus on the four major tpyes of stresses, including acid stress, saline-alkali stress (SAS), high-temperature stress, and oxidative stress. Acid stress, particularly concerning in the production of amino acids or organic acids [10,11], can cause DNA damage and enzyme inactivation due to undissociated weak acids present in acid-hydrolyzed lignocellulose liquor or as metabolic products or by-products [9,12,13]. Saline-alkali stress, occurring under high salt concentrations and elevated pH levels, significantly reduces bacterial community diversity, biomass and productivity [14,15]. High-temperature stress, often coused by increased solids, additional nitrogen, and more extensive ethanol production, detrimentally impacts yeast ethanol fermentation processes [16,17]. Oxidative stress, a universal threat during fermentation, usually accompanies acid, osmotic, and thermal stress [9,10,18,19], causing DNA damage and metabolic disorders that inhibit cell growth and productivity [20,21].

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To achieve robust tolerance to these stresses, we can learn from the nature and then design, engineer, and apply stress-resistant microorganisms. Extremophiles, which thrive in extreme environments like polar regions, volcanic areas, salt lakes, acidic rivers, mining sites, deep seas, and deserts, offer valuable insights for engineering stress-resistant microbial cell factories. These extremophiles, including acidophiles [22], alkaliphiles [23], halophiles [24], thermophiles [25], and psychrophiles [26] (Table 1 summarizes several stress-resistant microorganisms), have evolved complex mechanisms, providing potential sources of stress-tolerance elements [22,27–34].

Synthetic biology technologies offer another avenue for engineering microbial cells with robust production capabilities. These approaches involve modularizing and standardizing biological elements, modifying existing biological systems, designing and building new biological systems to achieve efficient bioproduction in industry, agriculture, and medicine [51,52]. Omics approaches, including metagenomics, comparative genomics, transcriptomics, and proteomics, facilitate the identification of stress-tolerant elements [12,52,53], which are then subjected to functional verification to elucidate their role in stress resistance mechanisms [29–31]. Furthermore, directed evolution and rational design methods, particularly those bolstered by artificial intelligence (AI), further refine stress-tolerant elements, optimizing their effectiveness [54,55].

Recent advances in synthetic biology, such as golden gate, gibson assembly, and yeast assembly, enable the rapid assembly and building of stress-tolerant elements, modules, and circuits, fostering the construction of robust microbial cell factories [9,56–58]. High-throughput screening strategies, like fluorescence-activated cell sorting (FACS), fluorescence-activated droplet sorting (FADS), and microplate assays using automated platforms, facilitate the efficient selection of stress-tolerant elements, modules, or circuits [59,60]. Furthermore, a stepwise evaluation strategy encompassing growth and fermentation assessments in laboratory and industrial settings ensures the suitability of stress-tolerance elements for real-world applications [9].

In this review, we summarize recent advances and developments in stress-tolerance elements. We also discuss strategies for mining, redesigning, and optimizing these stress-tolerance elements, and present their potentil applications for improving the performance of microbial cell factories in biomanufacturing under multi-stresses industrial settings.

2. Classification and mechanism of stress-tolerance elements

Recently, various stress-tolerance elements have been developed for synthetic biology to achieve efficient bioproduction at laboratory and industry scales [9,18,61]. According to different mechanisms for coping with diverse stresses, stress-tolerance elements in cell factories, stress-tolerance elements are divided into acid-tolerant elements, saline-alkali-resistant elements, thermotolerant elements, and antioxidant elements, and others (Table 2). In this section, several advances in various stress-tolerance elements are discussed (Fig. 1).

2.1. Acid-tolerant elements

Acid stress is usually caused by multiple complex factors such as acidic products in fermentation, significantly decreases the activity of intracellular enzymes and cell physiological activity, leading to low yields, low titer, and low productivity [84]. Microorganisms have evolved a range of tolerance mechanisms to maintain ion homeostasis under acid stress (Fig. 1A), including global transcriptional factor-mediated regulation [85], amino acid-dependent proton-consuming systems [57], ATPase-driven efflux pumping [86], alterations in membrane composition mediated by specific genes [87], macromolecular protection and repaire facilitated by chaperones and repaire enzymes [88]. These tolerance mechanisms provide a foundation for the subsequent exploration of acid-resistant elements to

Table 1
Summary of recent research on stress-resistant microorganisms.

Characteristics	Strains	Descriptions	Ref.
Acid-tolerant	*Rhizobium leguminosarum* bv. viciae SRDI969	an acid-tolerant, efficient nitrogen-fixing microorganism of *Vicia faba*	[35]
	Pseudomonas protegens CLP-6	an acid-tolerant strain (pH 5.5) producing volatile organic compounds	[36]
	Streptomyces albulus AAE89	an acid-tolerant strain (pH 3.0) producing ε-poly-l-lysine	[37]
	Gluconacetobacter entanii AV429	a highly acetic acid-tolerant bacterium from Vinegars	[38]
	Schizosaccharomyces pombe RF2	a yeast with high potential for acid tolerance to acetic acid at 400 mM, propionic acid at 75 mM, and lactic acid at 300 mM	[39]
	Saccharomyces cerevisiae TAMC	a yeast is tolerant at pH 2.3	[40]
	Zymomonas mobilis 3.5 M and 3.6 M	two strains exhibited 50–130 % enhancement on growth rate, 4–9 h reduction on fermentation time to consume glucose, and 20–63 % improvement on ethanol productivity than wild-type srain.	[41]
Saline-alkali-resistant	*Bacillus* sp. DYS211	a P-solubilizing bacteria isolated from bird droppings in saline-alkali regions with a good P-solubilizing effect at 1%–8% salinity	[42]
	Bacillus amyloliquefaciens CZ-B1	a saline-alkali resistant bacteria (the maximum NaCl tolerance concentration is 100–150 g/L, pH 9) screened from saline-alkali soil	[43]
	Halomonas TD01	a halophilic bacterium (NaCl concentration 200 g/L, pH 11.0) isolated from a salt lake in Xinjiang, China, which grew rapidly and accumulated high content of PHA	[44]
Thermotolerant	*Bacillus subtilis* TTP-06	a thermotolerant strain (55 °C) isolated from a hot spring of Tattapani able to produce lipases	[45]
	Cupriavidus sp. strain CB15	a newly thermotolerant polyhydroxyalkanoate (PHA) producing bacterium (45 °C) isolated from corncob compost	[46]
	Pyrolobus fumarii	a novel, irregular, coccoid-shaped archaeum was isolated from a hydrothermally heated black smoker wall can survive from 106 to 113 °C	[47]
	Methanopyrus kandleri strain 116	isolated from an in situ colonization system deployed in black smoker fluid of the Kairei hydrothermal field can grow up to 122 °C	[48]
Antioxidant	*Halococcus morrhuae*, *Halobacterium salinarium* and *Thermus filiformis*	extremophile microorganisms producing carotenoids, efficient scavengers of reactive oxygen species	[49]
	Lactobacillus plantarum IH14L, *Lactobacillus curvatus* GH5L and *Lactobacillus plantarum* IH16L	three lactic acid bacteria isolated from fermented Turkish Sucuk with different antioxidant activitiy	[50]

Table 2
Summary of diverse stress-tolerance elements discussed in this review.

Classification	Stress-tolerance elements	Descriptions	Ref.
Acid-tolerant elements	cfa	an element coding cyclopropane fatty acid synthase (pH 3.5 and pH 3.2)	[7]
	CgMed2	overexpression of CgMed2 increased cell growth by 12.4 % and cell survival by 5.9 % compared to the wild-type *Candida glabrata*	[62]
	CpxRA	a two-component system CpxRA directly senses acidification through protonation of CpxA periplasmic histidine residues, and upregulates the fabA and fabB genes, leading to increased production of unsaturated fatty acids.,and improve intracellular pH homeostasis.	[63]
	mo-uvrA	an element coding an ATP-dependent DNA repair enzyme enable Escherichia coli survive in an acidic environment (pH = 3)	[64]
	gadE-hdeB-sodB-katE	a synthetic module could improve the robustness and productivity of industrial *E. coli* strain	[9]
	ter9-smo-idi	a heterologous pathway producing (S)-2,3-oxidosqualene to enhance the tolerance to 3-hydroxypropionate and fatty acids	[65]
	DsrA-Hfq (H4)	two best mutants of dsrA-Hfq, H4 and E11, could enhance the growth rate by 41–51 % than the wild-type strain at pH 4.5.	[66]
	HypB-HypC	an element could enhance the acid tolerance and d-lactic acid productivity of strain.	[67]
Saline-alkali-resistant elements	Salt-Tolerant Gene 1	an element coding endoplasmic reticulum localized protein, improving plant salt tolerance by maintaining high photosynthetic activity under salt stress conditions	[68]
	ZmGnTL	an element isolated from *Zoysia matrella* coding β-1,6-N-acetylglucosaminyltransferase like enzyme, improving the salt-tolerance of *Arabidopsis* through regulating ion homeostasis, reactive oxygen species scavenging, and osmotic adjustment	[69]
	SNAC1	an element coding transcription factor, improving salt tolerance in cotton and *Bambusa emeiensis*	[70]
	GsSAMS	an element isolated from soybean coding S-adenosyl-l-methionine synthetase could enhance the salt-alkaline tolerance of transgenic rice	[71]
Thermo-tolerant elements	Dsr11	a small noncoding RNA (ncRNA), targets genes of tRNA modification GTPase and arginase to enhance heat stress tolerance of organism.	[34,72]
	GroES/GroEL	two elements could rescue proteins from improper folding and aggregation through an ATP-driven mechanism.	[73–75]
	CspB/CspD	cold-shock proteins, playing a role in temperature regulation for organisms living in environments with fluctuating temperatures.	[74]
	DnaK/DnaJ	co-chaperones involved in the DnaK-GrpE interactions, play a crucial role in repairing heat-induced protein damage.	[74]
	ClpG	a standalone disaggregase, could enhance bacterial survival in extreme temperatures.	[76]
	Ctt1	an enzyme exhibits antioxidant properties, facilitating increasements of 30.95 % in the OD and 161 % in product yield of *Y. lipolytica* at 35 °C.	[77]
	HtpX	a membrane-associated protease known to be involved in the degradation of misfolded proteins under heat stress conditions.	[17,74]
	DR_2577	a surface-layer protein, could enhance ultraviolet radiation and heat stress resistance of *D. radiodurans*.	[18,33]
Antioxidant elements	OsSRO1c	an element coding a rice homologue of SRO (similar to RCD one) protein can regulate H_2O_2 homeostasis	[78]
	katG	an element coding catalase, regulating H_2O_2 homeostasis in *Escherichia coli*	[79]
	SigB	a general stress response sigma factor, contributes directly to the adaptations required for oxidative stress survival	[80]
Others	LPL1 and IZH3	Inactivation of LPL1 (encoding a putative lipase) and IZH3 (encoding a membrane protein related to zinc metabolism) increasing cell survival rates of yeast under methanol tolerance	[81]
	HAA1 and PRS3	elements to enhance acetic acid tolerance of *Saccharomyces cerevisiae*	[82]
	HtpG	an element improving the butyric acid tolerance of *Clostridium tyrobutyricum* ATCC 25755	[83]

alleviate the negative impact on microorganisms caused by acid stress.

Acid resistance (AR) in microorganisms involvs various mechanisms in a well-defined hierarchical transcriptional network pattern [85]. At the apex of the transcriptional network are the global regulators, which can simultaneously perturb the expression of hundreds of genes [85]. They can also directly or indirectly recognize specific sets of promoters, acting as crucial regulators in response to environmental changes and basal gene expression. Moreover, by mutating endogenous or heterogenous global regulators to fine-tune their binding affinity towards target promoters, it is possible to regulate the entire gene expression spectrum that confers stress-tolerant phenotype to microorganisms [89]. This approach is known as a global transcriptional machinery engineering (gTME)-based strategy [90].

Among these global regulators, RpoD, the sigma D factor, serves as the primary sigma factor responsible for transcribing housekeeping genes, while RpoS, the sigma S factor, acts as a general regulator of the response to different stresses [91]. These two regulators are promising targets for engineering acid-tolerant phenotypes [89,91]. It is worth noting that the engineering of RpoS usually uses a small RNA-mediated strategy, such as DsrA, RprA, and ArcZ, due to the tight regulation of RpoS at all levels [92]. Another famous regulator is irrE, derived from an extreme radiotolerance bacteria, *D. radiodurans*, which regulates multiple genome repair and protection pathways [52]. Heterologous expression of *irrE* has been successfully demonstrated to improve cell tolerance against multi-stress involved acid-stress, thermo-stress, and ethanol-stress in *E. coli*, *S. cerevisiae*, and *Zymomonas mobilis* [93–95]. Moreover, overexpression of gene *HAA1*, coding a weak acid stress transcriptional activator, and *PRS3*, coding a phosphoribosyl pyrophosphate synthetase, in industrial *S. cerevisiae*, resulted in a recombinant with superior growth in the presence of 4 g/L acetic acid and an enhanced adaptation to a non-detoxified hardwood hydrolysate with a high acetic acid content [82]. Furthermore, engineering TATA-binding protein Spt15, one of the components of the general factor RNA polymerase II (RNA Pol II) transcription factor D (TFIID), can improve the ethanol tolerance and production in *Kluyveromyces marxianus*, as well as the acid resistance and 3-hydroxypropionate production [96,97].

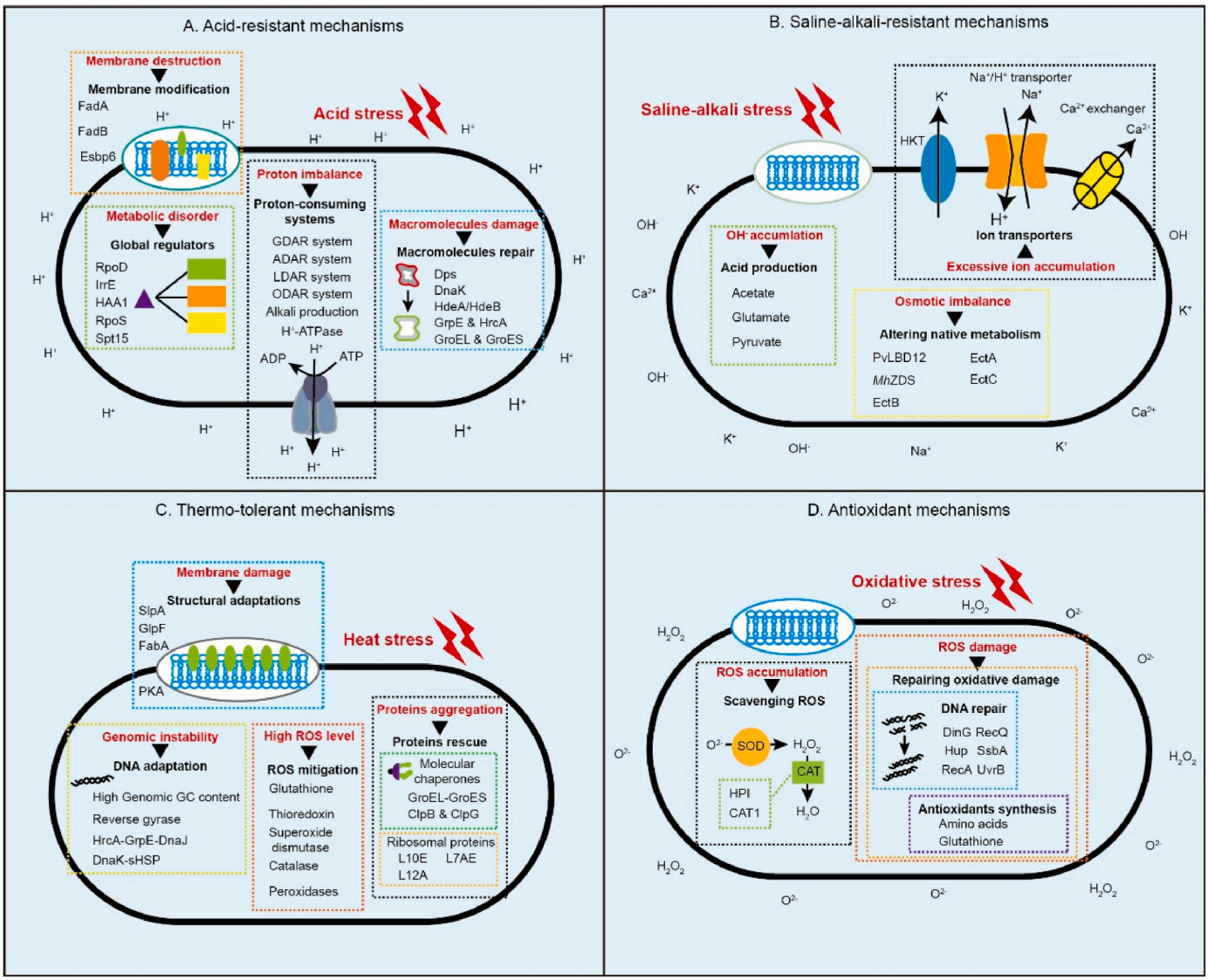

Fig. 1. Mechanisms of stress tolerance in microorganisms. A. Acid-resistant mechanisms in microorganisms under the acid stress. B. Saline-alkali-resistant mechanisms in microorganisms under the saline-alkali stress. C. Thermo-tolerant mechanisms in microorganisms under the heat stress. D. Antioxidant mechanisms in microorganisms under the oxidative stress.

Under transcriptional regulators, several functional systems confer acid tolerance, including a proton-consuming system, physiological adaptation, macromolecule protection or repair (genome repair and protection, protein quality control system), and reactive oxygen species (ROS) elimination system [32,98]. The basis for acid resistance systems is the direct consumption of intracellular protons or alkaline compounds to neutralize acid and counteract acid stress [57]. The proton-consuming system comprises a series of amino acid-dependent acid resistance systems, such as the glutamic acid-dependent acid resistance (GDAR) system, arginine-dependent acid resistance (ADAR) system, the lysine-dependent acid resistance (LDAR) system, ornithine-dependent acid resistance (ODAR) system [32]. Additionally, the GDAR and ADAR systems could protect cells from extreme acid stress, while the LDAR and ODAR predominantly operate under moderate acid conditions [32]. Among them, GDAR has the strongest activity in low pH environments, and it is more widely present in a variety of bacteria that can resist gastric acid shock. In GDAR, protons are consumed by decarboxylating glutamic acid to CO_2 and γ-aminobutyric acid (GABA) through GadA and GadB; the substrate glutamate is imported, and product GABA is exported via the glutamate/GABA antiporter GadC [32]. GDAR is regulated by the regulators GadE, GadX, and GadW, which exist in the acid fitness island (AFI) and comprise 14 genes contributing to acid resistance in *E. coli* [99]. Furthermore, activation of AFI has been successfully demonstrated to enhance cell growth robustness in low-pH fermentation [9]. Reconstruction of an artificial AFI in other industrial strains via synthetic biology approaches might be a promising strategy.

Some microorganisms also produce alkaline compounds, commonly in the form of ammonia, to counteract acid stress. This process is achieved through the catalytic activity of enzymes like urease, glutaminase YbaS, arginine deiminase (Adi), or the arginine dihydrolase system (Ads), which convert urea, glutamine, or arginine into ammonia [57]. Sulfur assimilation is also crucial for the synthesis and transportation of sulfur-containing amino acids, like glutathione, which can enhance acid tolerance [41,100]. Under anaerobic conditions, protons can be consumed to produce hydrogen via the hydrogenase with its accessory protein HypB-HypC [67].

Physiological adaptations to acid stress include membrane modification and biofilm formation to reduce proton influx [40,87]. Although biofilm formation exhibits strong resistance to various harmful environments, including low pH, it may not be suitable for engineering strains for fermentation purposes. Microbes adjust their membrane

composition against external acid stresses by increasing the presence of unsaturated fatty acids (UFA) through the upregulation of genes *fadA* and *fadB* [63,87]. Additionally, this leads to a decrease in membrane fluidity, maintaining the membrane integrity and improving intracellular pH homeostasis. Membrane microdomains, composed mainly of ergosterol, sphingomyelin, and scaffold proteins, provide a platform for forming H^+-ATPase complexes, facilitating intracellular H^+ homeostasis and improving cell tolerance [40]. Proton pumps situated in the cell membrane, such as F_0F_1-ATPase in *Lactobacillus*, *C. glutamicum*, and *E. coli*, as well as H^+-ATPase PMA1 in *S. cerevisiae*, actively pump protons out of the cell by hydrolyzing ATP to maintain intracellular pH homeostasis [12,40,63]. Furthermore, transporters like Esbp6 in *S. cerevisiae* mutants exhibited efficient export of aromatic acids, improving the acid tolerance of the cells [13].

One of the crucial reasons why *D. radiodurans* is able to survive against high-intensity radiation is its strong ability to protect or repair DNA and protein [52]. This suggests that genes involved in the protection or repair of macromolecules should be considered valuable stress-tolerant elements for any stress, including acid stress. This system is composed of DNA binding proteins (*e.g.*, Dps) [88], DNA repair enzymes (*e.g.*, RecA, DnaK, and UvrA) [52,101,102], protein chaperones (*e.g.*, HdeA/HdeB) [103], and protein degradation enzymes (*e.g.*, Clp protease). Importantly, some of these components, like Dps and DnaK, play a role in both DNA and protein protection [88,101]. Since the periplasmic space becomes the first line of defense and the periplasmic proteins become vulnerable, the protection afforded by chaperones is vital for acid tolerance. For example, HdeA/HdeB prevents the acid-induced aggregation of proteins in the periplasm by binding them to an acidic pH and releasing the proteins when pH returns to natural [103]. Moreover, other chaperones such as DnaK, GrpE and HrcA, GroEL and GroES, and Lo18 have been shown to protect proteins during acid stress.

Various stresses, including acid stress, significantly increase the level of intracellular ROS, leading to membrane destruction, macromolecular damage, and disturbance of redox homeostasis [9]. Superoxide dismutase converts superoxide radical to hydrogen peroxide, which is then further converted by catalase into water and oxygen. Thus, it is beneficial for superoxide dismutase and catalase to be over-expressed together. For example, co-overexpressing KatA and Dps in *C. glutamicum* or KatE and SodB in *E. coli* could enhance the acid tolerance of cells [9,100].

2.2. Saline-alkali-resistant elements

Saline-alkali stress typically induces excessive ion accumulation and osmotic imbalance in microorganisms during fermentation, leading to cell dehydration and even death [104,105]. Microorganisms have developed various strataegies to maintain intracellular osmotic and pH balance under SAS. These strategies include discharging excess ions into extracellular environment through ion transporters, secreting, or accumulating protective metabolites, and altering native metabolism (Fig. 1 B). Generally, microbies exchange substances with the external environment through ion transporters for normal metabolism during fermentation. Under SAS, ion transporters such as Na^+/H^+ antiporters, Ca^{2+}/H^+ exchangers, and high-K^+ affinity transporters (HKT) maintain intracellular ion homeostasis by transporting excess ions out of the cell by consuming energy, such as ATP, thereby reducing the damage caused by SAS [106,107]. To cope with high alkaline environments caused by SAS, microbies usually increase the production of acidic metabolites to neutralize excess alkalinity. Additionally, microorganisms upregulate relevant genes to secrete or accumulate osmoprotectants such as proline, betaine and trehalose for osmoregulation [108–110]. Altering native metabolism by activating transcriptional regulators is also an effective strategy for coping with SAS. These strategies pave the way for the mining of saline-alkali-resistant elements to improve the performance of redesigned cell factories.

Ion transporter engineering is one of the main approaches to maintaining intracellular environment homeostasis and can relieve the damage caused by SAS [111]. For instance, global transcriptome analysis on an extremely halophilic archaea *Halolamina* sp. YKT1 demonstrated that the genes related to membrane transporters were up-regulated under high salt concentrations [112]. In addition to improving the saline-alkali tolerance of microorganisms under environmental stimulation, ion transporters are applied to implement the efflux of harmful products to avoid the accumulation of toxicity. For example, studies have shown that several ion transporters belong to the main categories for microbial heavy-metal resistance. The cationic diffusion facilitator (CDF) family of transporters (*e.g.*, PbMTP8.1 originated from *Pyrusbretschneideri Rehd*, and GmMTP8.1 originated from *Stylosantheshamata*) play an essential role in the transport of heavy metal ions, significantly mitigating the danger of high concentrations of heavy metal salts to microorganisms [113]. Moreover, the P-type ATPase transporter, encoded by *zccE* from *Streptococcus mutans*, mediated the transport of zinc and three other metal ions; two other P-type pumps, encoded by *FgCrpA* from *Fusarium graminearum* and *PmtA* from *Streptococcus suis*, were responsible for copper ion and ferrous/cobalt efflux pump, respectively [86,114,115]. Therefore, enhancing the performance of plants and cell factories under SAS by introducing saline-alkali-resistant elements coding ion transporters is an effective strategy to improve the survival rate and productivity of crops and microbes. For instance, *AvHKT1*, a gene from *Actinidia valvata* encoding a high-K^+ affinity transporter, can improve the salinity tolerance of kiwifruit by facilitating ion transport under salt stress conditions [106]. Furthermore, a total of 16 HKT genes in *Spartina alterniflora* were discovered by deep learning-based methods, which are considered salt-tolerant elements for redesigning high salt-tolerant crops and microbes [107].

Fabricating protective substances directly or prompting a "neutralization reaction" to cancel the negative impact on cell growth is a universal tactic for the stress resistance of microorganisms. For example, one study indicates that alginate is an effective protectant against alkaline stress [116]. Considering the acid-base neutralization reaction, a logical idea is that the impact induced by acid or alkali can be eliminated by overexpressing the other [117]. Indeed, that's the contingent of microbial resistance to acid/alkali. For example, some microorganisms upregulate amino acid metabolism and increase the production of acidic metabolites (*e.g.*, acetate, glutamate, and pyruvate) to maintain cell growth and reproduction under alkaline stress [118]. Moreover, the overproduction of betaine and trehalose can improve salt stress tolerance by regulating osmoregulation [119].

Although microbes can employ multiple approaches, such as efflux pump and production of protective matters, to diminish the impact caused by saline-alkali stresses, none of them can bypass the native metabolism [120]. As these approaches usually need substrates, extra energy, or both, a common occasion is altering the native metabolic pathways by activating transcriptional regulators. For instance, *Egicoccus halophilus* EGI 80432^T, a halotolerant bacterium isolated from saline-alkaline soil, upregulated the expression of genes involved in starch synthesis and the gene for the stress protector, trehalose synthase, under highly alkaline conditions (pH 10.0) [116]. Besides, *PvLBD12*, encoding a lateral organ boundaries domain protein as a plant-specific transcription factor, enhanced salt tolerance by increasing proline accumulation, improving K^+ accumulation, and reducing Na^+ absorption in switchgrass (*Panicum virgatum* L.) [110]. Moreover, ectoine, originally discovered in *Ectothiorhodospira halochloris* (*H. halochloris*) [121], is a vital compatible solute for osmotic balance in microorganisms. Notably, heterologous expression of ectoine synthesis genes, including L-diaminobutyric acid aminotransferase (EctB), L-diaminobutyric acid acetyltransferase (EctA), and ectoine synthase (EctC), can improve the hyperosmotic stress and alkali stress resistance of microbes and crops [122]. Furthermore, overexpression of the *Mh*ZDS gene (from *Malus halliana*), encoding a key enzyme (ζ-Carotene desaturase) in

the carotenoid biosynthesis pathway, has the potential to improve saline-alkali resistance by participating in the carotenoid synthesis pathway in *tobacco*, *Arabidopsis thaliana* and *apple calli*. Thus providing an excellent saline-alkali-resistant element for transgenic plants with strong saline-alkali resistance [123].

2.3. Thermotolerant elements

High-temperature stress can induce extensive damage, particularly to cell membranes and macromolecules [124]. Bacteria employ various mechanisms to protect cell membranes and macromolecules against high-temperature stress. These mechanisms broadly encompass molecular chaperones and protein repair systems, ROS mitigation and structural adaptations (Fig. 1C) [20]. Genetically, thermophiles exhibit features such as high GC content in their DNA, robust DNA repair systems, and frequent horizontal gene transfer, contributing to genome stability at high temperatures [125].

Molecular mechanisms involve the overexpression of specific genes that enhance thermal tolerance, such as those encoding molecular chaperones like GroEL-GroES and disaggregases like ClpB, which prevent protein misfolding and aggregation [75,76]. To combat the increase in ROS levels induced by high temperatures, bacteria upregulate antioxidant enzymes and molecules like glutathione and thioredoxin [124]. Additionally, they redirect metabolic pathways to boost NADPH production, essential for regenerating antioxidants and mitigating oxidative damage [126]. Structural adaptations include specialized surface layers and modifications in membrane lipid composition that maintain cellular integrity under heat stress [127]. Collectively, these mechanisms enable bacteria to survive in high-temperature environments by maintaining cellular and molecular integrity, ensuring protein stability, and protecting against oxidative stress.

In microorganisms, the expression of the aforementioned heat-tolerance modules is not consistently high at all times. Instead, their expression is regulated in response to the detection of environmental stress through various regulatory elements. A well-known example is the overexpression of prokaryotic regulator irrE from *D. radiodurans,* increasing thermal tolerance for yeast [93]. The noncoding RNA dsr11 from *D. radiodurans* also confers thermal stress by activating *trmE* encoding tRNA modification GTPase and dr_0651 encoding arginase [34]. Moreover, the *PDE2* gene, a cAMP phosphodiesterase gene in *S. cerevisiae*, reduces cAMP levels and subsequently decreases the activity of protein kinase A (PKA), maintaining cell wall integrity and enhancing the heat resistance of *S. cerevisiae* [18].

It has been discovered that unique characteristics are essential for maintaining a stable genome under high-temperature conditions [125]. These include a genome with high GC content, a strong DNA repair system, and high horizontal gene transfer ability. One specific DNA topoisomerase called reverse gyrase, which introduces positive supercoiling to increase the melting temperature of DNA, has been shown to play a critical role in thermophily in *T. kodakarensis* [128]. Moreover, the genome of *Thermotoga maritima* contains heat-shock operons hrcA-grpE-dnaJ, prasugrel, and dnaK-sHSP, which are DNA-binding proteins or molecular chaperones [129]. Intriguingly, genomic comparison studies have suggested that thermophiles tend to have smaller genomes compared to non-thermophiles, some of the genes involved in metabolism was lossed in thermopiles [125]. Another study also found that yeast undergoes duplication of chromosome III during adaptation to heat [130].

Molecular chaperones, like GroEL-GroES, rescue proteins from improper folding and aggregation, assisting in preventing protein misfolding, or aggregation through an ATP-driven mechanism [75]. Disaggregases like ClpB and ClpG also assist in extracting and reactivating misfolded proteins aggregated under high temperatures [76]. Additionally, Hsp90 not only assists in stabilizing the proteostasis of microorganisms but also serves as an indicator to evaluate their heat tolerance [131]. Consequently, misfolded or nonfunctional proteins are handed over to the protein degradation system. As such, they are recognized and ubiquitinated through the ubiquitin-proteasome system misfolded proteins, leading to their degradation into amino acids for reuse. Overexpressing ubiquitin ligase gene RSP5 in *S. cerevisiae* can significantly enhance the thermotolerance of yeast cells [132]. It is also noteworthy that the ubiquitin system plays a crucial role in DNA repair and replication, particularly in responding to DNA double-strand breaks, inter-strand crosslinks, and bypassing lesions during the replication process [133]. Another study on *Pyrococcus furiosus* found that ribosomal proteins, such as L10E, L12A, and L7AE, had obviously higher abundances at 90 °C than at 70 °C to maintain stable and efficient protein synthesis [134].

Elevated temperatures are typically accompanied by an increase in ROS levels, leading to damage in a variety of cellular components, including DNA, proteins, lipids, and other essential structures [124]. ROS are scavenged by nonenzymatic and enzymatic antioxidants such as glutathione (GSH), thioredoxin (TRX), superoxide dismutase, catalase, and peroxidases [124]. Therefore, thermophiles can achieve this by upregulating the expression of antioxidant enzymes, increasing the levels of molecules with antioxidant properties, and repairing oxidative damage, thereby mitigating the oxidative stress induced by high-temperature conditions [124]. Cells also activate the production of NADPH in the high-temperature condition that is required for the regeneration of GSH or a reduced form of TRX, which is mainly produced by the pentose phosphate pathway [126]. Furthermore, *K. marxianus* can enhance its heat tolerance by redirecting its metabolic pathway from glycolysis towards the pentose phosphate pathway, thereby increasing the production of NADPH [124].

D. radiodurans has a distinct surface (S)-layer with an ordered paracrystalline array of proteins enveloping the cell surface, exhibiting strong tolerance to heat stress. DR_2577, also known as SlpA, is a thermo-adapted protein maintaining the structural integrity and functional efficacy of the S-layer [127]. Additionally, the cell wall integrity pathway (CWIP) has demonstrated the enhancement of microbial heat resistance for *Aspergillus fumigatus* [135]. Moreover, mutations in GlpF, the glycerol uptake facilitator, increase osmic tolerance, and the mutation in *fabA* increases the degree of saturation in membrane lipids, which is a known adaptation to elevated temperatures.

2.4. Antioxidant elements

Oxidative stress disrupts DNA replication, transcription, and translation, resulting in DNA damage and metabolic disorders, which significantly affects microbial growth rates [20,21,136,137]. Microbes have developed a set of antioxidat mechanisms that can be broadly categorized into scavenging ROS and repairing oxidative damage. Generally, oxidative stress is caused by the accumulation of ROS including hydrogen peroxide (H_2O_2), superoxide anions (O^{2-}), hydroxyl radical (OH^-), and ozone (O_3). Indeed, nearly all stresses discussed above can result in oxidative stress after the accumulation of ROS. Thus, reducing ROS generation, through ROS scavengers is an effective strategy to cope with oxidative stress (Fig. 1 D). Moreover, in response to damage caused by oxidative stress such as DNA damage and metabolic disorders, microorganisms activate intracellular metabolic synthesis pathways and DNA damage repair systems to repair these damage (Fig. 1 D). These mechanisms collectively provide microbes with resistance to oxidative stress and inform the mining of antioxidant elements.

ROS scavengers including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) [138]. Specifically, SOD acts as the first defense line against oxidative stresses due to its function of converting O^{2-} to H_2O_2 and H_2O, playing an essential role in resisting oxidative damage. Then, CAT, such as catalase HPI from *E. coli*, encoded by the gene *kat*G, converts H_2O_2 into H_2O and O_2. Besides, other potential antioxidant elements, such as six elements, namely formate dehydrogenase, processes associated with iron ions, repair programs, multidrug resistance, antioxidant defense, and energy generation (*mqo*,

sdhC) might have contributed to oxidative stress tolerance in *Enterobacter* strain NRS-1 [139]. Additionally, the gene (*deipr_0871*) isolated from *Deinococcus proteolyticus* coding a response regulator can upregulate oxidative-stress-related genes such as *ahpC* and *sodA*, and acetyl-CoA-accumulation-associated genes via *soxS* regulon in *E. coli*. These not only enhance oxidative stress but also promote the growth of the recombinant *E. coli* and remarkably improve the productivity of PHB [137]. Furthermore, SigB, a general stress response sigma factor, contributes directly to the adaptations required for oxidative stress survival [136].

Antioxidant metabolites synthesis and DNA damage repair play significant roles in the ROS damage repair system (Fig. 1 D). Microorganisms usually regulate metabolic pathways to synthesize several antioxidants such as amino acids and glutathione for repairing the damage caused by the overaccumulation of ROS under oxidative stress [11]. Notably, DNA damage often occurs under several environmental stresses during high-cell-density fermentation. For example, the ROS-oxidized nucleotide bases (*e.g.*, 8-oxo-deoxyguanosine) incorporated into the genome appear frequently after the single-strand and double-strand breaks under the accumulation of ROS [140]. For some solvents, like ethanol, the situation is more complicated. Ethanol will result in replication fork stalling and recruit translesion polymerases, which cause a higher mutation rate and further inhibit cell cycle progression [141]. In addition to sabotaging DNA and disrupting its replication directly, it impacted transcription and translation processes due to increased ribosome stalling and Rho-dependent termination for RNA polymerase activity, resulting in a decrease induced by ethanol [142]. Thus, repairing DNA damage caused by high levels of ROS is also crucial to increase survival rate during high-cell-density fermentation [80]. Generally, microorganisms have precise and efficient DNA repair mechanisms to cope with DNA damage caused by oxidative stress [143]. As such, studies revealed what has occurred to the microbial genome and uncovered part of the whole picture of the repairing systems. For example, *Promicromonospora* PT9T, a strain separated from irradiated roots of plants, proves that genes *dinG*, *hup*, *recA*, *recQ*, and *ssbA* are responsible for repairing double-strand breaks (DSB) after exposure to infrared ray (IR) and are included in its genome [144]. Furhtermore, DNA repairing was also found in UV-exposed *Nesterenkonia* sp. strains, consistent with the observation of overexpression of proteins involved in the DNA-repair process [145]. UvrB proteins also play an important role in nucleotide excision repair in *E. coli* [146].

3. Mining and redesign of stress-tolerance elements

While synthetic biology is rapidly developing, it is also facing many challenges, including undefined and incompatible parts, complexity, difficulty in handle, unpredictable circuitry, and variability that can crash the system [147]. Therefore, the excavation, modification, and standardization of new biological elements are the current focus in synthetic biology. To date, various strategies have been developed to mine stress-tolerance elements for effective bioproduction. Generally, these excavated elements are challenging to be effectively applied in synthetic biology without further modification. In this section, we review the progress of effective mining and rational redesign of stress-tolerance elements.

3.1. Mining of stress-tolerance elements

With the rapid advancements in sequencing technology, we have entered the era of big data, where omics data such as genome, transcriptome, proteome, and metabolome can be efficiently obtained. To identify resistance regulators or resistance genes, several typical methods can be employed: (1) Direct discovery through Blast or similar alignment tools based on research interests; (2) Direct analysis of genomes and transcriptomes of extremophiles, or comparison with omics data from non-extremophiles; (3) Integrative multiomics analysis of mutants or adaptive laboratory evolution (ALE) obtained strains under different culture conditions or growth stages. The strains with overexpression or deletion candidate genes are subjected to phenotypic analysis under stress conditions in the original strains or in the model chassis cells such as *E. coli*, *C. glutamicum*, and *S. cerevisiae*. In addition to these approaches, we introduce several state-of-the-art techniques, including synthetic biology tools and powerful strategies enabled by deep learning and automation platforms.

Collection and construction of a genomic library of naturally stress-tolerance strains and further verification performed in cell factories for potential candidate genes after omics analysis is an effective strategy for mining stress-tolerance elements (Fig. 2). Initially, samples were collected from several extreme environments for screening of natural stress-tolerance microorganisms [22]. For instance, the Special Environmental Microbial Database (DSEMR), a comprehensive database dedicated to unique environment microorganisms, including 5268 strains from 620 genera, was developed for stress-tolerance elements excavation [148]. Subsequently, genomic libraries of naturally stress-tolerance strains were constructed, and candidate genes were excavated based on sequence or function alignment and bioinformatics analysis. For example, *Egicoccus halophilus* EGI 80432^{T} was sequenced, and physiological analysis and comparative transcriptomics were performed to screen salt tolerance elements [149]. Finally, candidate genes were verified as stress-tolerance elements through metabolic engineering, including knockout, overexpression, and others. Two stress-tolerance elements from *Halomonas zhaodongensis* were discovered and designated UmpA and UmpB (encode paired unknown homologous membrane proteins belonging to DUF1538 family) by genomic DNA screening, and co-expression of two elements in *E. coli* KNabc achieved the tolerance to 0.4 M NaCl and 30 mM LiCl, and an alkaline pH resistance at 8.0 [150].

Another strategy is to obtain evolved strains by ALE, physical and chemical mutagenesis, or other approaches. Then, omics analysis, including transcriptomics analysis, proteomics analysis, and metabolomics analysis, was performed for wild-type and evolved strains to screen potential stress-tolerance elements [108–110]. The potential stress-tolerance elements were further identified under different conditions such as acid stress, saline-alkali stress, and others (Fig. 2). For example, ALE was performed for *S. cerevisiae* to screen the dicarboxylic acids (glutaric acid, adipic acid, and pimelic acid) tolerance elements and explore its tolerance mechanism. Whole-genome sequencing of tolerant mutants was performed to find the critical tolerance elements, in which a new stress-tolerance element *QDR3* (coding a multidrug transporter) was discovered. Notably, overexpression of *QDR3* improved the tolerance of *S. cerevisiae* to all three dicarboxylic acids tested and two additional ones (muconic and glutaconic acid), resulting in muconic acid final concentration from 0.25 g/L to 0.41 g/L [98]. Moreover, ALE was performed for *Bacillus siamensis* A72 to screen saline-resistant elements and improve the production of macrolactins (MLNs), a type of macrolide antibiotic toxic to the producer strains. From this investigation, *his*D^{D41Y} was found to be a saline-resistant element via RNA sequencing, metabolomics analysis, and genome sequencing of a saline-resistant mutant strain *B. siamensis* IMD4001 and the parental strain *B. siamensis* A72. Furthermore, MLN production was 3.42 times higher than the control in the overexpression *his*D^{D41Y} strain [151].

In addition, the combination of the CRISPR/Cas9 gene-editing tool with massively parallel oligomer synthesis enabled trackable genome engineering (CREATE) to link each guide RNA to homologous repair cassettes that both edit loci and function as barcodes to track genotype–phenotype relationships [152]. The CREATE strategy enables editing around 10^4 to 10^5 loci in a population and allows for the parallel mapping of each edit to a targeted trait using conventional sequencing. Thus, CREATE has been powerful in the identification of stress tolerance relative genes, not only screening the target genes from the genome but also introducing mutations in the target genes to be available for stress-tolerance. Based on this CREATE strategy, 34 thousand mutations

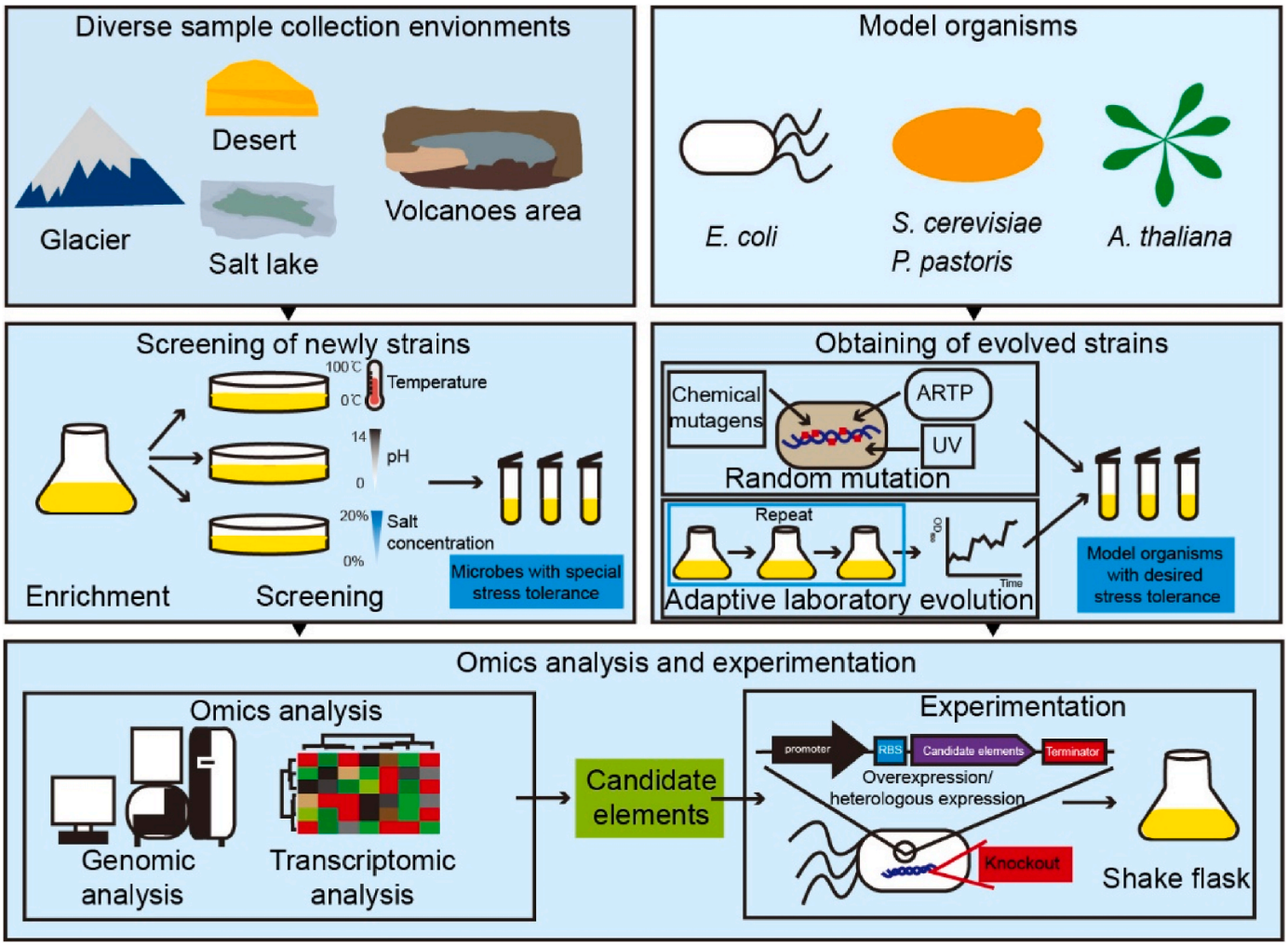

Fig. 2. Schematic of stress-tolerance elements mining strategies. Obtaining candidate strains with desired tolerance characteristics from diverse extreme environments or evolution. Then, screening and verification of stress-tolerance elements from candidate strains by omics analysis and experimentation.

across 23 global regulators were efficiently identified against multiple inhibitors in *E. coli* [153]. The study further found that upregulation of *ilvA* and *nadA-pnuC*, deletion of *potF*, or the small RNA sgrS increased the tolerance to acetate. By combining ALE and CREATE, we found that the knockout of sRNA sgrS and the overexpression of sRNA arrS significantly increased furfural tolerance [154]. Moreover, Bao et al. developed another version, called the CRISPR/Cas9-and homology-directed-repair (HDR)-assisted genome-scale engineering (CHAnGE) method for *S. cerevisiae* [155].

More recently, based on the development of deep learning and big data, artificial intelligence methods have significantly contributed to the task of protein function prediction. For identifying stress-tolerant elements from virous strains, the Contrastive learning–enabled enzyme annotation (CLEAN) approach to annotate enzymes with better accuracy, reliability, and sensitivity compared with BLASTp tool [156]. Notably, CLEAN, using a contrastive learning framework, can be applied to annotate understudied enzymes, correct mislabeled enzymes, and identify promiscuous enzymes in silico.

Though methods based on big data have contribution in mining stress-tolerant elements, the multiomics data are complex, highly dimensional, and heterogeneous, which poses an important challenge, and can generate the curse of dimensionality during data mining and reduce the generalization ability of the model [157]. Deep learning methods have also emerged to integrate multiomics data, which can be utilized as an efficient framework to process a large number of multiomics, high-dimensional, and complex data. Construction and training of a multiple natural language processing neural network model, including LSTM, Attention, and BERT, were developed to form a unified pipeline to autonomous learning of sequence features, and microbiome data resources can be used to discover specific functional genes [158]. As such, 83.8 % (181/126) of the predicted sequence by this large-scale method (4409 genomes) of human metagenomic data demonstrated antimicrobial activities. Additionally, an automated platform for the plasmid construction process and cell growth and production assays accelerated the identification process for the candidate genes mined by the AI scheme [159].

3.2. *Stress-tolerance elements redesigning for construction of cell factory*

Although several stress-tolerance elements have been excavated, there are still many challenges, including fitness, activity, and controllability of the newly excavated stress-tolerance elements, that need to be addressed for follow-up application. For example, several stress-tolerance elements were discovered in prokaryotic organisms, which might not be applicable to eukaryotic organisms. A recombinant *S. cerevisiae* with NAD-dependent methanol dehydrogenase from *Bacillus methanolicus* MGA3 and D-6-phospho-3-hexuloisomerase and hexulose 6-phosphate synthase in ribulose monophosphate pathway cycle, a methanol assimilation pathway in prokaryotic organisms, from *B. subtilis* 168 were integrated into the chromosome could not growth in the

defined medium using methanol as the sole carbon source [160]. In addition to the complex environments faced by microorganisms, it is beneficial to assemble multiple stress-tolerant elements into a powerful stress-tolerant module capable of handling various stressful environments. Therefore, rational or semi-rational engineering of natural stress-tolerance elements is commonly necessary to adapt them to target strains and the specific stresses they encounter. There are two main strategies for this, which are directed evolution and computer-aided rational design (Fig. 3).

Directed evolution has become a standard practice in molecular biology as it allows for the rapid selection of biomolecule variants with properties that make them more suitable for stress-tolerance applications [54] (Fig. 3 A). Various techniques have been developed to address the two main steps of directed evolution: genetic diversification (i.e., library generation) and selection or screening of desired variants. There are several highly recommended reviews available on the development of these two steps [161]. Unlike general protein-directed evolution, stress element-directed evolution can be screened in a specific stress, often proving to be more efficient. Several global regulators, such as RpoD, H-NS, and CRP, have been engineered through directed evolution strategies to improve further their ability to confer acid tolerance in cells [89,162,163]. Recently, advancements in genome editing tools have allowed for the construction of mutation libraries directly on the genomic DNA. Notably, these strategies demonstrate a strong ability to identify stress elements and engineer them, as well as construct desirable cell factories. For instance, CRISPR/Cas9-mediated directed evolution of the sRNA DsrA, along with its chaperone Hfq (DsrA-Hfq module), in the genomic context has significantly enhanced acid tolerance [66]. The best mutants exhibited a 51–72 % increase in growth performance at pH 4.5 compared to the original strain. Although the CREATE or CREATE-based strategies do not employ directed evolution, they follow a similar scheme [153]. Additionally, stress-tolerant elements can be rapidly assembled into multifunctional stress-tolerant modules. For example, by combining the acid-responsive promoter Pasr with different strengths with four genes, including the proton-consuming system regulator *gadE*, periplasmic chaperone *hdeB*, and ROS scavengers *sodB* and *katE*, significant improvements in growth and production robustness of industrial *E. coli* strains have been achieved at low pH [9].

Continuous advancements in computer technology have sparked a keen interest in leveraging computer-aided rational design for elements (Fig. 3 B). When compared to directed evolution, computer-aided rational design exhibit significant advantages in terms of the speed and efficiency of synthesizing biological components [164]. Through the utilization of computational methods for analyzing biological data, we acquire fresh insights into the microbial systems, thereby propelling the techniques of designing elements. For instance, notable examples such as iEnhancer-CNN [165] and DeepSTARR [166] effectively showcase the application of computer techniques in designing of enhancer. Concurrently, algorithms rooted in minimum free energy principles, such as NUPACK [167], have validated the efficacy of computer-aided rational design in fabricating RNA regulatory elements. Furthermore, through thorough exploration of the characteristics of natural promoter sequences, we can employ computer-aided rational design to meticulously craft entirely novel synthetic promoters from scratch [164]. Meanwhile, computer-aided methods have emerged as powerful tools for protein mining and design. Frances Arnold introduced the concept of using machine learning models to delineate protein functional space and

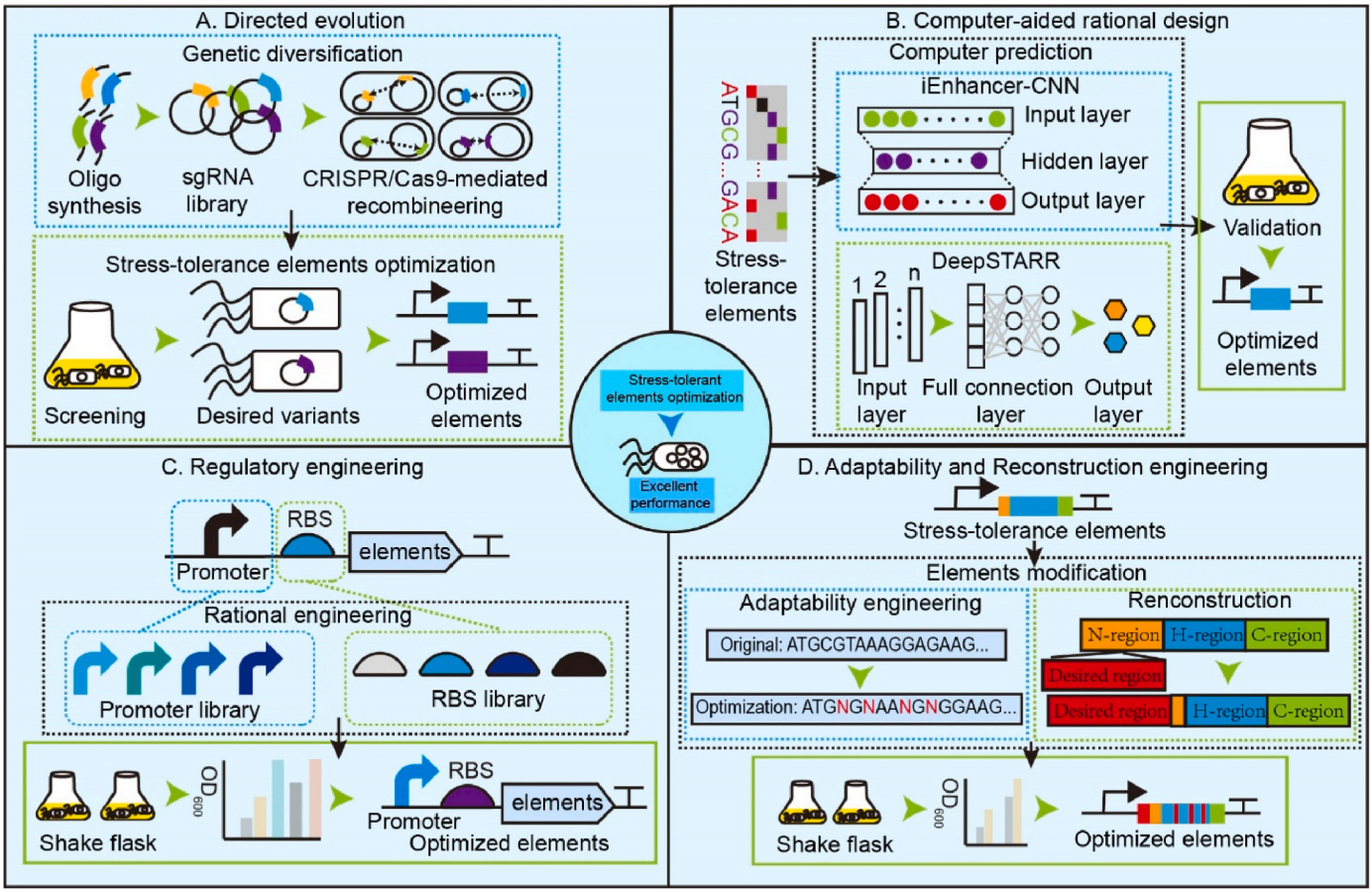

Fig. 3. Strategies to redesign stress-tolerance elements. A. Directed evolution for optimization of stress-tolerance elements. B. Computer-aided rational design for optimization of stress-tolerance elements. C. Regulatory engineering for redesign of stress-tolerance elements. D. Codon and structure optimization for redesign of stress-tolerance elements.

guide evolutionary processes [168]. Wu Bian's team leveraged artificial intelligence protein design techniques to conduct molecular redesign of aspartase derived from Bacillus, resulting in the successful generation of a range of artificial β-amino acid synthetases exhibiting precise location selectivity and stereo selectivity [169]. Similarly, David Baker's team harnessed deep neural networks to achieve de novo protein design tailored to specific functionalities [55]. Furthermore, machine learning-based approaches have demonstrated efficacy in swiftly and effectively identifying antimicrobial peptide candidates from metagenomic datasets [158]. Collectively, these studies suggests the potential for employing computer-aided rational design in de novo genetic element design.

Some stress-tolerance elements also have risks of being unable to regulate following heterologous expression. Therefore, many efforts have been made to improve the regulatability of elements in cell factories through computer-aided rational design, which also is a suitable strategy for stress-tolerance elements redesigning (Fig. 3 C). Notably, promoter engineering is an alternative approach to control the transcript production of elements intracellular through devise strengths of synthetic promoters [9,170]. For example, a porin promoter library was constructed and charactered in *E. coli* and *H. bluephagenesis* TD01, and the PHA production was improved after promoter optimization [171]. Subsequently, this promoter library was also used to enhance the 3HV content in PHBV synthesized by *H. bluephagenesis* TY19 [172]. Using acid-responsive promoters to fine-tune the expression of the ghsk module could enhance the final OD_{600} of strains of 43–51 % and maintain the productivity of industrial *E. coli* strains upon mildly acidic conditions [9]. Moreover, through comprehensive transcriptome analysis, a series of promoters responsive to the combined stresses of 36 °C high temperature and 10 % high glucose concentration were discovered [173]. These stress-responsive promoters were utilized to fortify the glutathione biosynthesis pathway and the acetic acid degradation pathway, thereby enhancing yeast tolerance to reactive oxygen species and acetic acid stress induced by high temperatures. This enhancement also significantly improved the robustness and productivity of yeast in lignocellulosic ethanol fermentation. Besides, ribosomal binding sites (RBS) are also critical for elements to achieve effective performance. *E. coli* accumulated 0%–92 % poly(3-hydroxybutyrate) contents in cell dry weight, which was achieved by rationally designing RBS libraries with defined strengths to regulate three genes, respectively [174].

In addition, modifying the N-terminal tail of elements is another effective strategy to achieve better performance in bioproduction (Fig. 3 D). A study demonstrated that redesigning the N-region of α-factor, a secretory signal peptide, could significantly enhance the secretion of human lactoferrin in *Phichia pastoris* [175]. Moreover, replacing the N-terminal tail of Hxt2 (a high-affinity glucose transporter) with the corresponding region of Hxt11 (a sugar transporter that is stably expressed at the membrane) resulted in Hxt11/2 transporters, which improves the growth of *S. cerevisiae* under high glucose concentration (8 %) and the tolerance of acetic acid [176].

4. Application of stress-tolerance elements

Numerous chemicals and materials such as biofuels, bio-rubber, and natural products have been produced by microbial cell factories, which are considered promising implements to cope with severe threats from the environment and resources [177]. The conventional model microbes, such as *E. coli* and *S. cerevisiae*, and the non-model microbes, exemplified by *B. subtilis*, *Streptomyces* spp., *Pseudomonas* spp., *Aspergillus* spp., and *Y. lipolytica*, have been developed and applied in the production of high-value biochemicals and proteins as industrially used chassis [178]. However, industrial cell factories usually need to address complex environmental stresses, including toxic inhibitors (brought by raw material pretreatment), temperature, acid, oxidative, osmotic stress, and solvents, during the process of industrial bioprocess, which have a significant negative impact on microbial growth and inhibit the production of metabolites [65,179]. Fortunately, with the advancement of systems and synthetic biology technologies, improving microbial robustness through the introduction of effective stress-tolerance elements provides an alternative approach to enhance the performance of cell factories, such as maintaining the phenotype of stability and improving titer and productivity of desired bioproducts under various harsh industrial conditions [178]. Importantly, such stress-tolerance elements have potential applications in multiple fields, such as biomanufacturing (Fig. 4).

The poor tolerance of microorganisms to toxic substrates or products is a major challenge in biomanufacturing [65]. Some stress-tolerance elements can also enhance the tolerance of substrates or bioproducts to improve the productivity or titer of cell factories during the process of fermentation (Fig. 4 A). For instance, methanol is an ideal and renewable feedstock for biomanufacturing. However, the toxicity of methanol limits the effective bioconversion of methanol toward high-valued bioproducts. Inactivation of *LPL1* (encoding a putative lipase) and *IZH3* (encoding a membrane protein related to zinc metabolism) not only improves the methanol tolerance of methylotrophic yeast *Ogataea polymorpha* by restoring phospholipid metabolism but also results in high-level production of free fatty acids from sole methanol [81]. Overexpression of *QDR3* in *S. cerevisiae* can improve the tolerance of the target product (muconic acid) and the production of muconic acid [98]. Moreover, the accumulation of high ethanol concentration was the main factor affecting cell growth and vitality, inhibiting the activity of certain key enzymes, interfering with various cell metabolism, and resulting in poor ethanol yield during bioethanol production [180]. Overexpression of the element *murA2*, an alcohol-tolerant element from the alcohol-tolerant organism *Lactobacillus plantarum*, in the ethanologenic *E. coli* KO11 significantly improved ethanol tolerance and ethanol production (52.4 g/L, control 40.2 g/L) [181]. Furthermore, the overexpression of Tryptophan biosynthesis elements (*Trp2* and *Trp5*) and tryptophan permease element (*TAT2*) can effectively endow *S. cerevisiae* with higher ethanol tolerance [182].

Generally, introducing a single stress-tolerance-related element into a cell factory is capable of improving the performance of cell factories under desired environmental stress (Fig. 4 B). For instance, overexpression of *CgMed2*, an element encoding Mediator tail subunit, in *Candida glabrata* increased cell growth by 12.4 % and cell survival by 5.9 % compared to the wild-type strain under pH 2, which significantly enhanced the performance of *C. glabrata* during the process of fermentation under acid condition [62]. Overexpression of HypB/HypC could also enhance the acid tolerance and D-Lactic acid production of *E. coli* at pH 5.5 in 5-L bioreactors. The atmospheric and room temperature plasma (ARTP) and ALE strategies were conducted in *E. coli*, and the mutant BER208 showed increased growth rate, glucose utilization rate, and succinic acid productivity of 3.2-fold, 3.7-fold, and 2.5-fold [183]. Moreover, overexpression of Esbp6 enhanced the acid tolerance of *S. cerevisiae*, with improvements in cell growth reaching up to 17 % and increased coumaric acid production of 38 %–47 % [13].

In addition, cell factories have to cope with complex environments during the process of bioproduction. Assembling multiple stress-tolerance elements into more efficient stress-tolerance modules is a feasible strategy to enhance the performance of microorganisms (Fig. 4 C). For instance, ROS is usually generated by the accumulation of the damages caused by stress conditions such as acid stress, heat, and so on. Co-expression of *katE* (an antioxidant element coding CAT) and *sodB* (an antioxidant element coding SOD) in *E. coli* recombinant improved 5-Aminolevulinic acid (ALA), which is a value-added bioproduct with several applications and can cause severe cell damage and morphology change of *E. coli* through generating ROS, tolerance and its production levels, achieving a 117 % (11.5 g/L) increase of ALA titer in a 5 L bioreactor [11]. Moreover, there are also some stress-tolerance elements that can enhance multiple stress tolerance of cell factories. For example, overexpression of *OLE1*, encoding the sole and essential Δ-9 desaturase, in *S. cerevisiae* achieved the improvement of multiple stress tolerance,

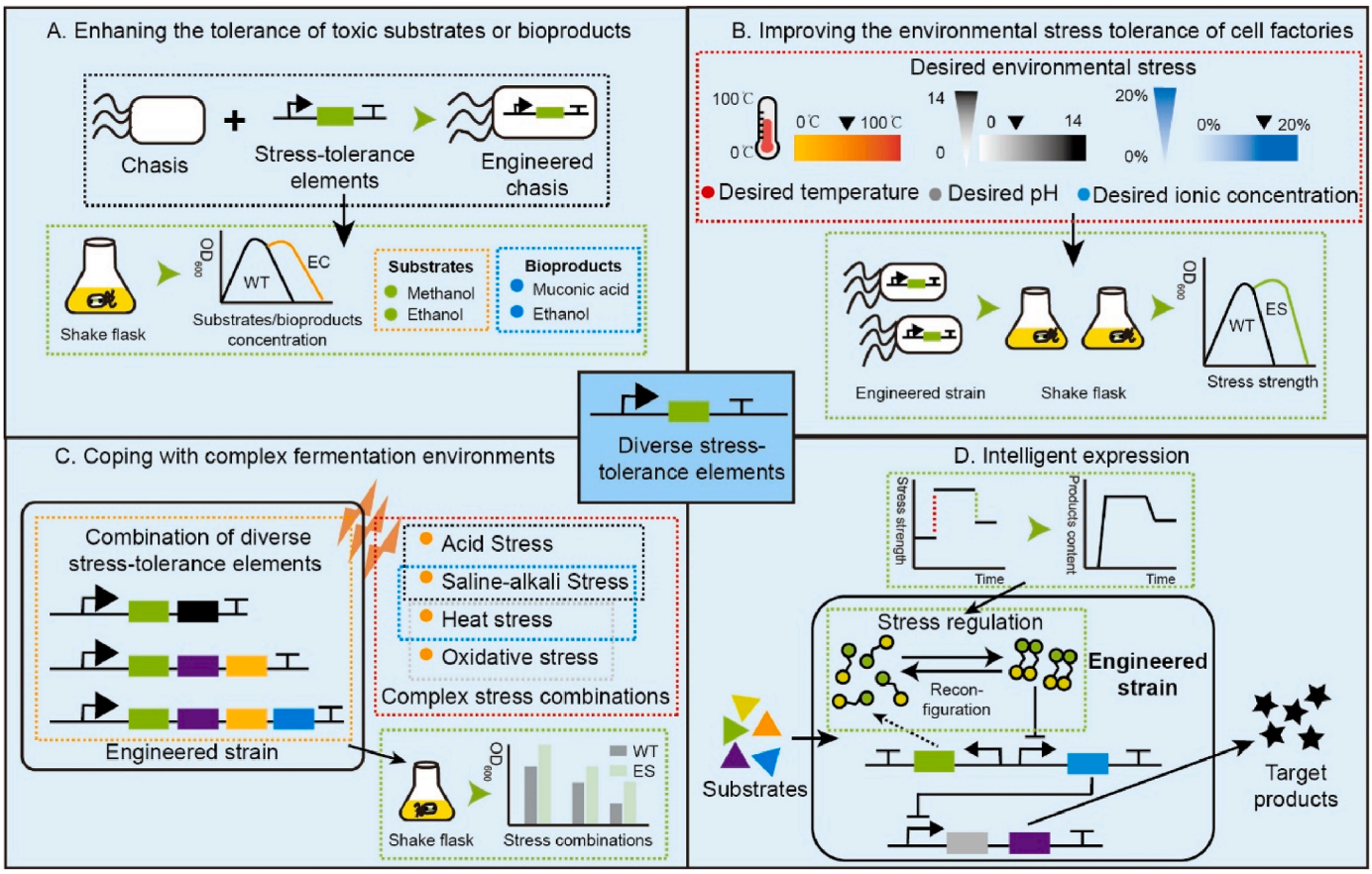

Fig. 4. Biomanufacturing applications of stress-tolerance elements. A. Stress-tolerance elements are used to increase the tolerance of chasis to toxic substrates or bioproducts. B. Stress-tolerance elements are used for improving the performance of cell factory under desired environmental stress. C. Stress-tolerance elements are combined into stress-tolerance modules for cell factory to cope with complex fermentation environments. D. Stress-tolerant elements are used to intelligent expression. WT: wide type; EC: engineered stress-tolerance chasis; ES: engineered stress-tolerance strain.

including weak acids, ROS, ethanol, and so on [184].

In the process of enhancing the tolerance of engineered microbial strains, it is common practice to manipulate the strain's endogenous stress-resistance genes or introduce exogenous stress-resistance genes [9,18]. However, this approach carries the risk of increasing the metabolic burden on the host cell, potentially leading to imbalances in the overall cellular metabolism or uncontrolled cell growth [179]. Our ultimate goal in engineering microbial strains is to produce the desired products more efficiently and cost-effectively for human needs. Therefore, the judicious and timely expression of stress-resistant modules is a reasonable requirement (Fig. 4 D). The Intelligent Microbial Heat-Regulating Engine (IMHeRE) system, integrated with a quorum sensing mechanism and employing various heat shock proteins and RNA thermometers, intelligently regulates the expression of heat-resistant genes in *E. coli* [73]. Notably, this system enhances the thermal resilience and bioconversion efficiency of organisms by intelligently responding to abiotic stress through the activation of adaptive modules. This results in significantly improved microbial growth and productivity under high-temperature conditions, highlighting the importance of intelligent, stress-responsive gene expression strategies.

5. Conclusions and perspectives

The development and application of stress-tolerance elements are practical approaches to enhance the stress tolerance of cell factories and thereby enhance their performance of bioproduction and reduce their consumption under multiple stress fermentation conditions. Having long-term adaption to natural stress conditions, extremophiles have developed unique and efficient stress response mechanisms, providing numerous resources for screening stress-tolerance elements. Though engineered extremophiles are attractive for sustainable manufacturing, the tolerance engineering of model microbes, such as *E. coli* and *S. cerevisiae*, is still of great value for their unrivaled superiority of clear genetic background, well-developed genetic tools, and wide application [185]. Furthermore, the understanding of stress response mechanisms in extremophiles is not yet comprehensive. Large-scale molecular modification and synthesis technologies for their genomes and intracellular metabolic networks are still maturing, significantly limiting the screening of stress-tolerance elements in extremophiles.

To date, the lack of efficient and stable genetic manipulation systems for a large number of non-model microorganisms or industrial production strains, especially molecular tools for precise and dynamic regulation of gene expression levels or synchronous manipulation of multiple genes/large fragments, is an urgent issue for achieving effective development and application of stress-tolerance elements. Therefore, combining numerous omics techniques, metabolic engineering operations, and high-throughput screening techniques to mine stress-tolerance elements with significant application potential for the construction of robustness modal microorganisms is a promising alternative. The expression of stress-tolerant elements usually needs strict regulation, requiring specific timing and quantity to balance cellular stress tolerance and product production. Notably, synthetic biology offers many tools to realize dynamic and precise regulation, in which gene circuits are one of the most important. Likewise, pulse-generators could realize the expression pattern of target genes from ON to OFF, which means just-in time and just-enough [51,186]. Integrating the sensing

elements like stress-responsive promoters or specific metabolites-responsive riboswitches, along with the stress-tolerant elements into logic gates, could realize the intelligent expression [9,58]. Moreover, it is suggested that characterizations and optimizations of gene circuits be conducted under variable contexts, especially the application seniors, to maintain robustness for further application.

Strategies based on AI, particularly large language models (LLM), have been employed for biotechnologies, including protein sequence generation, drug discovery, and computational biology. Additionally, universal LLM (*e.g.*, ChatGPT4) also shows great potential in biology research [187], which is also used for effective recognition, prediction, and design of biological elements [188]. Prediction models primarily focus on determining the properties and characteristics of unknown biological elements. This encompasses methods like contrastive learning [156], transfer learning [189], multi-track systems [190], and multi-modal techniques [191]. For example, the Promoter calculator [170] has been designed to predict site-specific transcription initiation rates across any RpoD promoter sequence. Pattern recognition models, utilizing tools such as message-passing neural networks [192], convolutional neural networks [193], and recurrent neural networks [194], are adept at identifying patterns within extensive biological datasets. DeepSNR, with three convolution layers, has been developed to mine DNA or RNA motifs from original DNA or RNA sequences. Moreover, design models use complex algorithms to synthesize new biological structures or modify existing one biological elements, using approaches such as generative model [195], reinforcement learning [196], and deep network hallucination [55]. Deepseed [197], with expert systems, has also demonstrated improvements in the properties of *E. coli*, IPTG-inducible, and mammalian cell doxycycline (Dox)-inducible promoters. Moreover, deep-learning methods could offer significant capacity for the discovery and design of biological elements, as well as the construction of cell factories [107]. Furthermore, it could be a possible approach for generalizing and standardizing stress-tolerance elements to promote the development of synthetic biology. However, the efficacy of AI techniques in biology largely hinges on the quality of the available data [188]. Likewise, existing databases often contain redundant and erroneous data, which can significantly impede the efficiency of AI techniques [198]. Therefore, the establishment and maintenance of high-quality databases are crucial for enhancing the performance and accuracy of AI techniques in biological research. Moreover, establishing automated platforms and comprehensive evaluation systems combined with technologies powered by synthetic biology for non-labor-intensive and effectively excavating stress-tolerance elements is the future trend.

Credit author statement

The listed authors participated in the creation of this study in the following ways: Zheyi Kuang and Xiaofang Yan wrote the manuscript and drafted the tables and figures. Xiaofeng Yang, Haitao Yue and Jianwen Ye proposed the idea and revised the manuscript. Yanfei Yuan, Ruiqi Wang, Haifan Zhu, Youyang Wang and Jianfeng Li drafted and revised the tables. All authors read and approved the manuscript. Zheyi Kuang and Xiaofang Yan contributed equally to this paper.

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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