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Recent advances in improving metabolic robustness of microbial cell factories

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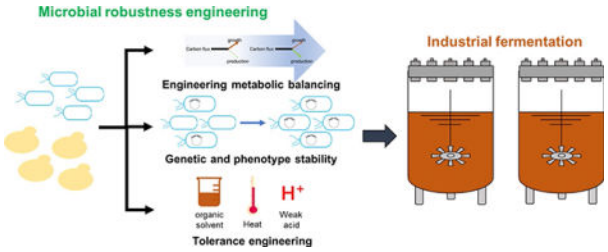
Abstract

Engineering microbial cell factories has been widely applied to produce compounds spanning from intricate natural products to bulk commodities. In each case, host robustness is essential to ensure the reliable and sustainable production of targeted metabolites. However, it can be negatively affected by metabolic burden, pathway toxicity, and harsh environment, resulting in a decreased titer and productivity. Enhanced robustness enables host to have better production performance under complicated growth circumstances. Here, we review current strategies for boosting host robustness, including metabolic balancing, genetic and phenotype stability enhancement, and tolerance engineering. In addition, we discuss the challenges and future perspectives on microbial host engineering for increased robustness.

Graphical abstract



Keywords

robustness; dynamic control; plasmids stability; tolerance engineering

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Conflict of interest statement

The authors declare no competing interest.

1. Introduction

Engineering microbial cell factories is a sustainable and promising approach for the synthesis of various compounds, such as natural products [1], biofuels [2], and bulk chemicals [3]. It can be achieved by combining heterologous or non-natural biosynthetic pathways into genetically advantageous microbial cell factories, such as *Escherichia coli* [4], *Saccharomyces cerevisiae* [5], and *Bacillus sublitis* [6]. To construct efficient microbial cell factories with improved titers, a number of metabolic engineering strategies have been developed, including enzyme selection/screening and engineering to overcome rate-limiting steps [7], pathway engineering for increased supply of precursors and cofactors [8], fine-tuning gene expression for optimal pathway performance [9], and host engineering to block competing pathways [10].

Despite the great progress achieved by these strategies, engineering industrial microbial cell factories remains a challenge. Engineered microbial cell factories constantly encounter disturbances caused by metabolic imbalance, genetic and phenotype instability, and harsh external environment in the process of large-scale fermentation [11]. The fluctuations of intracellular environment and concentrations of intermediates will affect the metabolic balance [12]. Besides, engineered strains usually contain recombinant pathways and tend to be more sensitive to stress factors than the wild type because of genetic and phenotype instability [13]. Moreover, harsh extracellular circumstances, such as high temperature, low pH, and metabolite toxicity, in laboratory- and commercial-scales require the host to be extremely robust to accomplish desired performance [14]. Therefore, increasing the host robustness against these perturbations becomes one of the major considerations in design and construction of microbial cell factories. We review the current research to increase robustness for high titer and productivity, focusing on engineering metabolic balancing, improving genetic and phenotype stability, and enhancing tolerance towards harsh environmental conditions. In addition, we discuss challenges and future perspectives on microbial host engineering for increased robustness.

2. Metabolic balancing

2.1 Engineering intermediates and cofactors balancing

The performance and robustness of industrial strains are often hindered by the accumulation of toxic metabolites and byproducts, imbalance of cofactors, and competition between production and cell growth. Traditionally, the accumulation of toxic intermediates can be addressed through modularized optimization to fine-tune the carbon fluxes (Fig. 1a) [15]. In a case of pyrogallol overproduction where the amino acid biosynthesis pathway were boosted, the relative expression levels of *aroL*, *ppsA*, *tktA* and $aroG^{fbr}$ (*APTA*), *entCBA* and $nahG^{opt}$ were fine adjusted to balance the carbon flux and successfully avoided the accumulation of harmful 2,3-dihydroxybenzoic acid in *E. coli*, resulting in a 2.44-fold improvement in pyrogallol production (893 mg/L) [16]. The balance of cofactors or redox homeostasis are usually achieved by the removal of cofactor competitive pathways or introduction of cofactor generating reactions [17]. However, rigid pathway engineering and control sometimes can exhibit undesired side effects and cause metabolic burdens when strains are exposed to fluctuated environmental perturbations [18]. Recent decades have

witnessed the rapid development of a more responsive method, namely the dynamic pathway regulation. Dynamic regulation is using biosensors to autonomously control the metabolic fluxes based on intracellular chemical signals or extracellular environmental status to avoid unbalanced co-factors or reduce the amounts of toxic metabolites [19–21]. The well-known example is the dynamic regulation of toxic intermediate farnesyl pyrophosphate (FPP) in isoprenoid production, resulting in a 2-fold increase in final titer of amorphadiene (1.6 g/L) [22]. Similar strategy has also been demonstrated in biosynthesis of fatty acid [23]. Bi-functional dynamic regulation was applied in *cis,cis*-muconic acid synthesis pathway. Through up-regulation of salicylic acid synthesis and down-regulation of competing pathway for the cosubstrate malonyl-CoA, a 4.72-fold increase in titer (1861.9 mg/L) was achieved compared with strains with only static control (394.5 mg/L) [12].

2.2 Decoupling cell growth and production

In addition to the burdens from toxic intermediates and unbalanced cofactors, some biosynthetic pathways also compete with cell growth. Conventionally, this can be resolved by blocking the competing pathway [10], but it may hamper the cell viability and thus decrease the productivity. Another option is to perform two-stage fermentation (growth stage and production stage), but this kind of fermentation requires manual stage change by adding inducers to activate the production pathway. Although researchers were given full control of stage change, it is often hard to determine the optimal switch time point. Autonomous dynamical pathway control, aided by metabolite biosensors or quorum sensing systems, becomes a more viable option for decoupling cell growth and production to avoid competition between product synthesis and cell growth (Fig. 1b) [24,25]. Applying a “nutrition” sensor responding to the concentration of glucose delayed the synthesis of vanillic acid and thus enabled decoupled cell growth and production in *E. coli*. The metabolic stress test revealed that the introduction of this nutrient-sensing module lowered the metabolic burden by 2.4-fold and achieved a robust growth rate during bioconversion to vanillic acid [26]. A layered dynamic pathway control strategy, combining a *myo*-inositol biosensor *ispA* and an AHL-responsive quorum sensing system, was employed in biosynthesis of glucaric acid. This strategy simultaneously balanced the production and consumption rates of the key intermediate *myo*-inositol and decoupled cell growth and production of glucaric acid to avoid competing with essential glycolysis, resulting in a 5-fold increase in glucaric acid titer (2 g/L) [27]. Biosensor-based dynamic pathway regulation allowed real-time monitoring of intracellular metabolism and environmental signals, and thus could provide more precise control and reduce the metabolic stress on microbial cell factories.

2.3 Growth-driven/product-addiction strategy

Instead of decoupling cell growth and production, another promising strategy to increase cell robustness is coupling the production of target compounds with cell growth. By manipulating and rewriting the microbial metabolism, the synthesis of target compounds become obligatory for cell growth, enabling a “growth-driven” phenotype and increased cell robustness (Fig.1c) [28]. A pyruvate-driven L-tryptophan strain was constructed by removing all major pyruvate generating steps but the one in the synthesis of L-tryptophan in *E. coli*. The strain with pyruvate-driven phenotype achieved 2.37-fold and 2.04-fold increase

of titer in L-tryptophan (1.73 g/L) and *cis-cis* muconic acid production (1.82 g/L), respectively [3]. A similar strategy was applied in butanone production driven by acetyl-CoA. By removing native acetate fermentation pathway (encoded by *poxB*, *ackA* and *pta*) and acetyl-CoA formation from acetate (encoded by *acs*), the only source of acetyl-CoA for *E. coli* was through the transfer of CoA moiety from 3-hydroxyvaleryl-CoA to acetate. The strain with acetyl-CoA driven phenotype produced 855 mg/L butanone, with complete deprivation of all supplied acetate [29]. However, establishing growth-driven phenotypes often requires that there are one or more essential components for cell growth can be generated from the pathway, which subsequently limits the generality of this method. A more general strategy is placing the essential genes *folP* and *glmM* under the control of a biosensor which only responds to the specific product, and thus forming a "product-addiction" phenotype (Fig. 1d). A synthetic product addiction system was constructed and enabled a stable mevalonate-overproducing strain, which can retain the performance over 95 generations [30]. The product addiction strategy holds greater generality than creating growth-driven phenotypes, but the application of product addiction could also suffer from limited biosensor availability.

3. Improving genetic and phenotype stability in engineered hosts

While genomic engineering is believed to be a stable approach for host engineering, plasmids-based systems are widely used due to its convenient modification and tunable expression levels. Selection and stable existence of plasmids are conventionally ensured by the resistance markers rescuing cells from antibiotic supplemented culture. However, cost, environmental and health concerns of antibiotic use have induced global discourage of such in food and biotech segments. To this end, strategies have been developed to maintain plasmids and strain phenotypes while minimizing antibiotics involvement. So far, developed plasmid maintenance approaches mainly include applying the toxin/antitoxin (TA) system, RNA-based interactions, operator-repressor titration (ORT), and auxotrophy complementation [13].

The TA system consists of a stable toxin protein interacting with a less stable antidote molecule (Fig. 2a). Such system has been adapted for use in plasmid-based production. For instance, a yefM/yoeB pair was introduced in *Streptomyces* by genome integration of the toxin gene and plasmid expression of the antitoxin gene. The resultant strain achieved stable production of proteins over 8-day incubation [31]. Meanwhile, RNA-based approaches and ORT were less explored recently. RNA-based approaches utilized the cis-acting of such oligonucleotides to interfere certain target genes (Fig.2b). ORT has been devised as a plasmid maintenance approach based on competitive binding of repressor on DNA-binding operator copies located between plasmids and the chromosome (Fig.2c) [32].

In addition to the above strategies, auxotrophy complementation appears to be a straightforward and effective way to confer symbiosis between the plasmids and cells, and thus has attracted great attention recently (Fig. 2d). Auxotrophy complementation sequesters cell growth-related genes to the plasmids so that cells with plasmids obtain a fitness advantage. Non-essential genes have been targeted due to ease of manipulation, however, in defined nutrient conditions. Knockout of triosephosphate isomerase (*tpiA*) was found to

significantly impair E. coli viability, while its complementation on the plasmid led to growth rescue and stable expression of β-glucanase [33]. On the other hand, sequestration of essential genes was also explored, but with strategies to bypass the strain fitness issue. Nonetheless, all the strategies have been focused on protein and plasmid expression. Not till recently, has the improved plasmid stability approaches been applied in cases of biochemical production. Kang et al. constructed a synthetic auxotrophic system based on the essential gene *infA* and further demonstrated the plasmid copy number can be controlled by varying its expression levels [34]. Although effective, deleting essential genes from chromosome requiring either complemented copy from plasmid or supplemented nutrient at all times to ensure cell viability, and thus extends the strain development timeframe. Zhang et al. introduced an intermediate plasmid to expedite the process, which harbors essential genes and can be easily cured and displaced by incoming production plasmids [13]. Sequestration of the essential genes *folP* from genome was found to largely improve plasmid maintenance and phenotype stability on strains producing salicylic acid for 80 generations [13].

While tremendous efforts have been put for engineering at individual cell level, cell-to-cell variation and heterogeneity pose great challenges for industrial applications [35]. Population quality control and heterogeneity mitigations were often used to tackle these challenges. For instance, by controlling a tetracycline efflux pump (TetA) through product-responsive biosensors, Xiao and colleagues linked cell fitness with production and demonstrated 3-fold enhanced production of both free fatty acid (FFA) and tyrosine [36].

4. Tolerance engineering

4.1 Thermal stability

Cooling system is required to maintain the optimal temperature (usually ≤ 37 °C) for fermentation since cell growth is an exothermic process releasing up to half of the energy stored in substrates as heat [37]. Tolerance engineering to maintain host performance at high temperature saves the expensive cooling cost [37]. In addition, fermentation at higher temperature also helps prevent bacteria contamination and reduces the cost of cooling the fermenter or heating for product separation [37]. Heat shock causes multiple cell damages including intracellular protein aggregation, cell structure destruction, and nuclear processing interference (Fig. 3a). Heat shock proteins (HSPs) can protect cells against these damages. Heterogenous expression of HSPs in a riboflavin-producing strain *Bacillus subtilis* 446 improved cell survival by up to 5 folds after the 10h heat shock treatment at 50 °C, and increased the riboflavin titers by 38–59% under 39 °C fermentation [38].

Adaptive laboratory evolution (ALE) is another approach to improve microbial heat tolerance (Fig. 3e). Heat shock response has complicated mechanism, and ALE has the advantage of letting many nonintuitive beneficial mutations occur in parallel. In previous research, thermotolerant yeast strains were obtained through ALE, with ~1.5 times [39] faster growth at 40°C. Correspondingly, ethanol productivity of the evolved strains was 1.6 times faster [40]. The T_{max} of *C. glutamicum* strain GLY3 was also improved from 37 to 40.5 °C in the minimal medium [41].

4.2 Weak acid

The accumulation of acidic intermediates and by-products often occurs during the microbial fermentation. In these processes, the excessive proton (H^+) disrupts transmembrane components, negatively affects internal pH homeostasis, and damages the conformation of structural proteins, leading to defected host growth and decreased final titer and productivity. The maintenance of intracellular pH requires the exportation of protons, which will consume a large amount of ATP. The addition of neutralizing reagents, such as $NH_3 \bullet H_2O$ and $CaCO_3$, has been used in industrial fermentation, but this can increase the cost and introduce unnecessary elements [42].

Thus, it is necessary to equip the microbial cells with increased robustness in acidic environment. Researchers have already characterized some genes effective for acid resistance (Fig. 3b). Overexpression of WHI2 gene, encoding a cytoplasmatic globular scaffold protein, in *S. cerevisiae* can increase the acetic acid tolerance and the pH. The final titer of ethanol was 5-times higher than the control in glucose fermentation with acetic acid [43]. Overexpression of HAA1, encoding a transcriptional activator involved in weak acid stress response, resulted in a 10% increase of final lactic acid titer without neutralization [44]. Some other genes related to acid resistance are also identified, such as *EUG1*, *SAC6*, *ASG1*, *ADH3*, *SKS1*, *GIS4* [45]. In addition to rational engineering of specific genes, some random strategies were also applied for screening of acid tolerance strains. Genome shuffling (Fig. 3d) was used in *Actinobacillus succinogenes* for improved acid tolerance leading to a 2.6-fold succinic acid titer increase [46].

4.3 Organic solvents

Many biochemicals, such as biofuels and biopolymers, are competitive replacements for gasoline and diesel. With high industrial values, however, they are solvent-like hydrocarbons and thus toxic to microorganisms [47]. Therefore, engineering strains to improve their tolerance on organic solvents is necessary. Altering membrane lipid composition can be an effective approach (Fig. 3c). It is previously suggested that increasing acyl chain tail length of membrane lipids is beneficial to host tolerance [48]. *E. coli* evolved under octanoic acid pressure was proved to have increased average lipid length and demonstrated around 15% higher n-butanol and isobutanol tolerance [49].

Another more straightforward strategy is introducing pumps to export the toxic solvents from cells. Recently, Baslet et al. reported that controlled expression of the native resistance-nodulation-cell division (RND)-type efflux pump *TtgABC* effectively improved the survival rate of *Pseudomonas putida* exposing to multiple short-chain alcohols by at least 10 folds [50]. Zhang et al. evaluated native efflux pumps of *E. coli* and recognized four major transporters (MdtJ, Bcr, MdtH and YdeA) able to improve short and medium carbon chain alcohol tolerance by 35% – 50% [51]. Considering the similarities between damaging mechanisms of heat shock and organic solvents, HSPs are also capable of improving solvent tolerance. Co-overexpressing GrpE, GroESL, and ClpB increased cell growth by 200%, 390%, and 78% respectively in 7% ethanol, 1% n-butanol, or 25% 1,2,4-butanetriol [52].

5. Conclusion and future perspectives

Engineering host robustness is essential to maintain production performance of microbial cell factories. A stable host is more economically feasible to be scaled-up to industrial fermentation. In this review, we summarized the current approaches to improve host robustness, including engineering metabolic balancing, enhancing genetic and phenotype stability, and tolerance engineering. In addition to the strategies we reviewed, there are other methods to enhance the host robustness, such as organelle engineering, omics technology, global transcription machinery engineering, functional redundancy, or mathematical model [53–55]. The strategies summarized above effectively increased the microbial hosts robustness and expanded their applications in industrial biotechnology. However, there are still some challenges in engineering microbial hosts robustness. First, dynamic regulation application could be hindered by the limitations of biosensors, such as low sensitivity, narrow dynamic range, and limited spectrum of effectors. The sensitivity of the biosensor determines when to switch to production and the dynamic range affects the productivity of the biosynthesis pathway. Systematic optimization is required to modify the sensitivity and alter the dynamic range of biosensors to facilitate their application in dynamic regulation. In terms of sensor engineering, computational strategies can be effective to engineer robust biosensor systems [56,57]. Meanwhile, developing new biosensors is an effective approach to broaden the spectrum of detectable metabolites. Novel biosensors can be possibly mined and characterized based on sequence similarity with existing ones. Besides, existing biosensors can also be modified to sense various compounds by either directed evolution or rational protein engineering [58].

Second, conventional microbial hosts are usually mesophilic and have limited capabilities to overcome harsh environmental stresses even with certain tolerance enhancement. Some non-conventional hosts, such as thermophilic and acidophilic strains, can possibly remedy the limitations. As for tolerance engineering, elucidating the key resistance mechanism is challenging, but it is important to better guide the engineering direction for enhanced microbial tolerance. A series of potential tools can be used to reveal the mechanism, including global metabolite profiling, adaptive lab evolution combined with sequence analysis and omics analysis [59]. Besides, CRISPR-Cas9 as an unprecedentedly flexible tool for gene knockout or knockdown has also been adopted for microbial tolerance mechanism elucidation [60]. Further engineering and development of non-conventional hosts may be a future direction for building robust microbial cell factories.

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Highlights

- Robustness engineering is essential to increase the production performance of microbial cell factories.
- Typical strategies for enhancing the microbial host robustness were reviewed and summarized, including engineering metabolic balancing, enhancing plasmid and phenotype stability, and tolerance engineering.
- The challenges and future perspectives of robustness engineering were discussed.

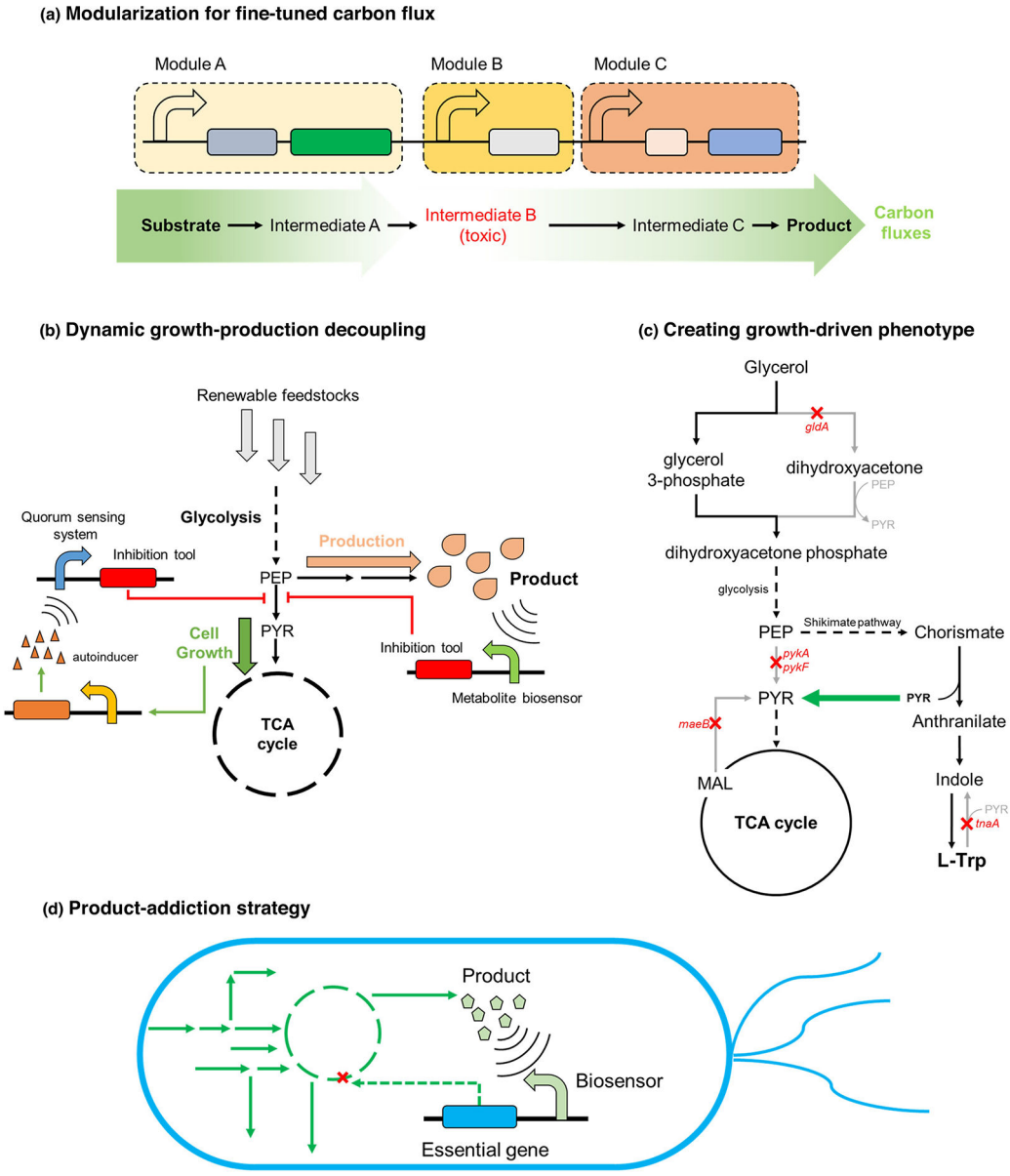

Figure 1.

(a) Fine-tuning the carbon flux through modularization of gene expression. Module A, B and C are responsible for the production of intermediate A, B, and C, respectively. The accumulation of toxic intermediate B can be minimized by tuning the expression level of every module. (b) Dynamic regulation for decoupling cell growth and production. At initial stage, the cell can utilize most of resources to grow, with only a limited concentration of product accumulating. As fermentation proceeds, the accumulation of product will activate the inhibition on competing pathways and cell growth, redirecting carbon fluxes and other sources to synthesis of target compounds. (c) Growth-driven production. For example, all major source of pyruvate is removed except the one in L-Trp synthesis. Thus, the L-Trp production will become obligatory for cell growth and biomass generation based on pyruvate. (d) Product-addiction strategy. An essential gene was removed in genome and

another copy is placed under the control of a product-inducible biosensor, so only the cells that are able to synthesize the target product will be able express the essential gene and survive.

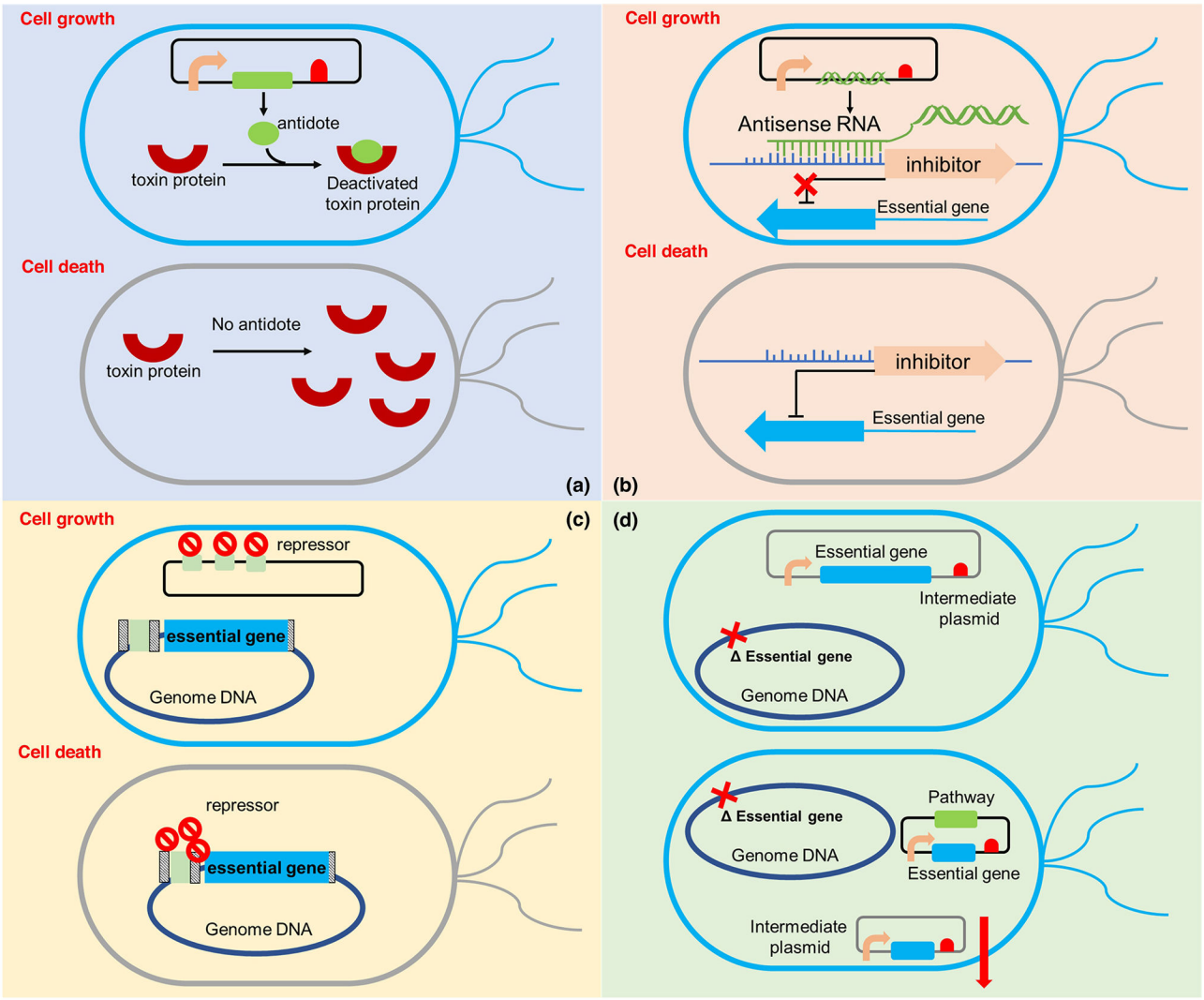

Figure 2.

Strategies have been developed to maintain plasmids and strain phenotypes stability. (a) TA system; it consists of a stable toxin protein in genome interacting with a less stable antidote molecule in plasmid. If losing the antidote-containing plasmid, the toxin protein cannot be repressed by antidote and the host will be killed by the toxin. (b) RNA-based interactions; the essential gene is under the control of a regulatory promoter, which can be repressed by a corresponding inhibitor. A plasmid-transcribed antisense RNA (asRNA) can interact with the promoter sequence of this inhibitor. When the plasmid is present, the transcribed asRNA can bind with the promoter sequence of inhibitor and repress the expression of the inhibitor. Then the inhibition on the essential gene will be relieved. With the successfully expressed essential gene, the cells can grow well. However, when the plasmid is absent, the inhibitor cannot the repressed by asRNA and the expression of this inhibitor will repress the essential gene, which will cause growth defects on cells. (c) operator-repressor titration (ORT); it has been devised as a plasmid maintenance approach based on competitive binding of repressor on DNA-binding operator copies between plasmids and the chromosome. In the existence of a plasmid containing the same operator, most of the repressors will bind to the operators in

the plasmid and only a small fraction of repressors will bind to the operators in the genome, because of the competitive binding between repressors and operators. Thus, the inhibition efficiency on the essential gene will decrease and it can be expressed to help the cell growth. However, if no plasmid is present, all the repressors will bind to the operator located in the genome and inhibit the expression of the essential gene. Without the essential gene, the cells will be unbale to grow. (d) auxotrophy complementation; this system is to optimize the process of traditional auxotrophic strain construction. In the optimized process, first, the essential gene was expressed in a temperature-sensitive intermediate plasmid and the essential gene in genome was deleted. Then, the production plasmid containing pathway gene and the deleted essential gene were transferred into host with the intermediate plasmid. When increasing the incubating temperature to 42 °C, the intermediate plasmid will be degraded and only the production plasmid was kept in the cell, resulting an auxotrophic strain. In this strain, if the plasmid is lost or mutated during fermentation, the cells will be unbale to grow due to the loss of essential gene.

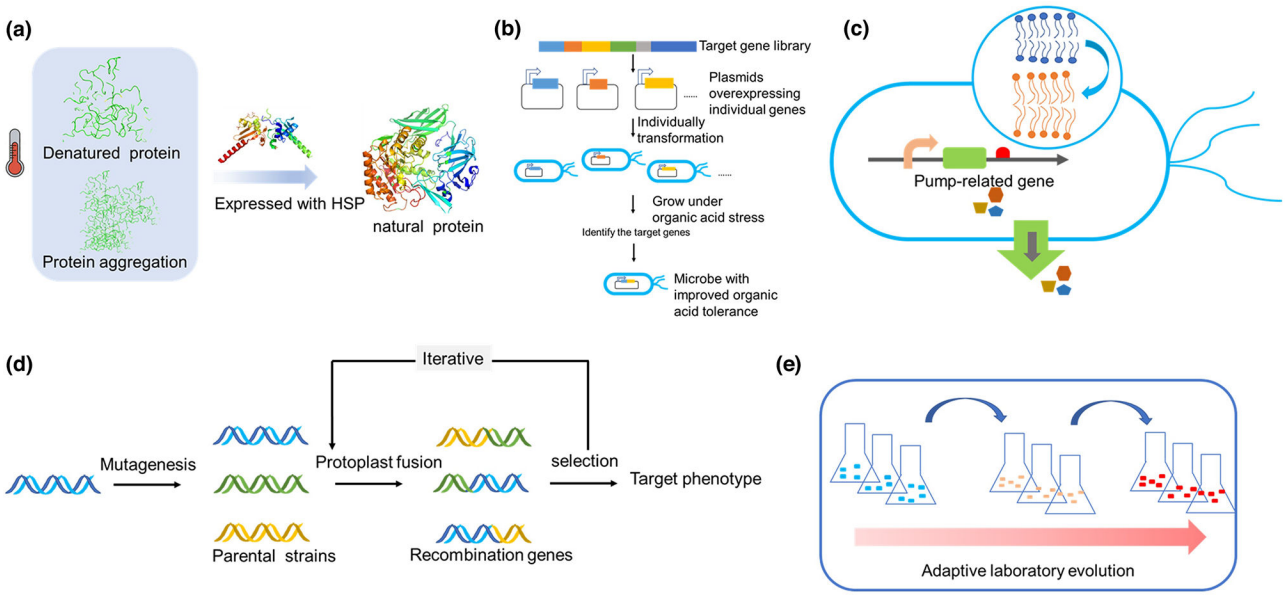

Figure 3.

(a) Thermal stability; HSP reduces denatured protein and protein aggregation by promoting the re-folding or degradation of unfolded proteins. HSP, heat shock protein. (b) Acid tolerance engineering. (c) organic solvent tolerance; Increasing acyl chain tail length of membrane lipids and overexpressing efflux pumps can improve microbial tolerance against organic solvents. (d) Genome shuffling; phenotype can be rapidly improved by recursive genomic recombination and screening of generated strains. (e) Adaptive laboratory evolution; strain tolerance can be improved after adaptive evolution under severe selection pressure.