Bioresource Technology 332 (2021) 125071

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Design and construction of novel biocatalyst for bioprocessing: Recent advances and future outlook

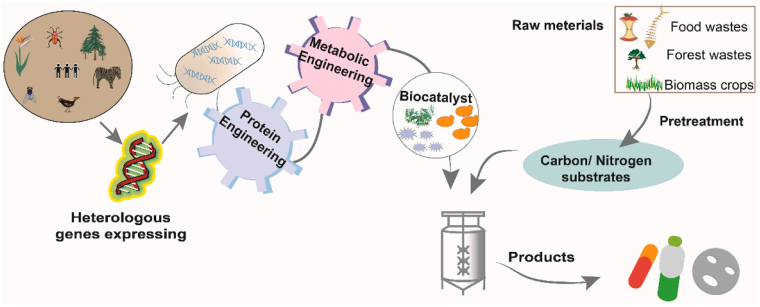
Yameng Xu [a,b], Yaokang Wu [a,b], Xueqin Lv [a,b], Guoyun Sun [a,b], Hongzhi Zhang [c], Taichi Chen [a,b], Guocheng Du [a,b], Jianghua Li [a,b], Long Liu [a,b,*]

[a] Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, PR China
[b] Science Center for Future Foods, Jiangnan University, Wuxi 214122, PR China
[c] Shandong Runde Biotechnology Co., Ltd., Tai'an 271000, PR China

HIGHLIGHTS

- The strategies of designing and screening novel enzymes provide possibilities for ideal biocatalysts.
- Protein engineering strategies can be used to improve the activities of biocatalysts.
- High-efficient metabolic engineering tools facilitate the development of microbial cell factories, which can be applied as catalysts in bioprocessing.
- The combined application of protein engineering and metabolic engineering effectively enhances the production levels of value-added products.
- The genome-scale model, fermentation model, big data technique, and artificial intelligence are needed for exploiting biocatalysts.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Biocatalyst
Bioprocessing
Fermentation
Biosensor
Dynamic regulation

ABSTRACT

Bioprocess, a biocatalysis-based technology, is becoming popular in many research fields and widely applied in industrial manufacturing. However, low bioconversion, low productivity, and high costs during industrial processes are usually the limitation in bioprocess. Therefore, many biocatalyst strategies have been developed to meet these challenges in recent years. In this review, we firstly discuss protein engineering strategies, which are emerged for improving the biocatalysis activity of biocatalysts. Then, we summarize metabolic engineering strategies that are promoting the development of microbial cell factories. Next, we illustrate the necessity of using the combining strategy of protein engineering and metabolic engineering for efficient biocatalysts. Lastly, future perspectives about the development and application of novel biocatalyst strategies are discussed. This review provides theoretical guidance for the development of efficient, sustainable, and economical bioprocesses mediated by novel biocatalysts.

* Corresponding author at: Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, PR China.
E-mail address: longliu@jiangnan.edu.cn (L. Liu).

https://doi.org/10.1016/j.biortech.2021.125071
Received 17 February 2021; Received in revised form 19 March 2021; Accepted 23 March 2021
Available online 31 March 2021

1. Introduction

Biocatalysis is one of the important solutions for global challenges, such as climate change, fossil replacement, and food shortage, due to its ability to improves resource efficiency in food, feed, and non-food industries (Rangel et al., 2020). Different from traditional chemical manufacturing, bioprocess is using living cells or their components (*e.g.*, bacteria, fungi, enzymes) to fabricate desired products by biocatalysts (Patel et al., 2020). However, many shortcomings, such as stability issue, presence of inhibitors, side products, enzyme specificity/turn over capacity, and low substrate specificity, limit the bioconversion efficiency of natural biocatalysts (Barragán-Ocaña et al., 2020).

To enhance the conversion efficiency of biocatalysts in bioprocess, many novel strategies have been exploited to remold biocatalysts in recent years. For instance, in the protein level, site-directed mutagenesis was used to increase the stability of retroviral reverse transcriptase [EC 2.7.7.49] (Konishi et al., 2014); fusion partners were proved to increase the expression and solubility of proteins (Singha et al., 2017); and promoter engineering was efficiently used to enhance and optimize the expression of enzymes (Gong et al., 2020a). After solving the above obstacles in protein levels, many other problems may appear. For example, overexpression of enzymes can lead to the metabolic burden, imbalance of metabolic flux, and disruption of complex metabolic networks (Wu et al., 2020; Hohenschuh et al., 2021). To address these issues, metabolic engineering strategies at the cellular level have been developed. For example, quorum sensing mediated dynamic regulation systems have been used to enhance the production of menaquinone-7 (Cui et al., 2019). In another study, a multi-output recombinase-based state machine has been applied to modulated the chronological lifespan of *Escherichia coli* (Guo et al., 2020). Thus, the rapid development of metabolic engineering strategies is meaningful for future bioprocesses.

In this review, we firstly summarize these novel strategies and the theoretical basis for the activities and expression improving of enzymes at the level of proteins. Next, we summarize the methods for analyzing and regulating metabolic networks of cells. Then, we list the combining applications of the innovative tools for the titer enhancing of value-added products in bioprocess that mediated by biocatalysts. Finally, the prospects of development and applications of novel biocatalysts for higher efficient bioprocess are provided.

2. Protein engineering for improving activities of biocatalyst

Many natural enzymes have been directly used as biocatalysts in the fermentation processes. However, the catalytic activity of most natural biocatalysts is limited by the instability of enzyme itself, poor performance under certain reacting condition, or low solubility of some substrates (Musil et al., 2018). As shown in Table 1, various methods have been exploited to overcome these limitations recently.

2.1. Design and screening of ideal biocatalysts

Directed evolution technology is creating mutants manually and select the mutants with required properties. Compared with natural evolution, directed evolution is much faster and has greatly contributed to the biocatalyst process in the past few decades (Wang et al., 2012). The procedure for directed evolution can be mainly classified into three steps: building mutant libraries, selecting mutants, and isolating the improved genes (Gargiulo & Soumillion, 2021). At present, many researchers put their efforts into the development of directed evolution and have made significant progress. To satisfy the demands of industry, artificial intelligence has been introduced as a facilitating device and applied to design promoters and enzymes (Nanda & Koder, 2009).

2.1.1. Random mutagenesis

Random mutagenesis is a powerful tool to change the properties of enzymes. The structures and functions of the objective proteins are not

Table 1
protein engineering strategies for the improving of biocatalysts.

Improvement strategy	Related enzyme	Application example	Reference
Random mutagenesis	Esterase	Sto-EST from *Sulfolobus tokodaii* has been random mutagenesis with 9-fold higher activity than the wild-type.	(Kurahashi et al., 2020)
	Xylanase	X11 xylanase (accession number AGN70389) was engineered through error-prone PCR with 15.9-fold greater catalytic efficiency, high thermal stability, and pH tolerance (temperature 80℃ and pH 6.0).	(Boonyapakron et al., 2020)
Directed evolution	4-Hydroxyphenyl pyruvate dioxygenase	*BkHPPD* from *Burkholderia* sp. BW-1 resistance to topramezone was improved by mutation.	(Sheng et al., 2020)
	D-Threonine Aldolase	The catalytic performance and selectivity of AxDTA from *Alcaligenes xylosoxidans* CGMCC 1.4257 was enhanced in an aldol reaction.	(Gong et al., 2020b)
De novo design	Syn-F4	A de novo protein allows *E. coli* to grow in the iron-limited medium.	(Donnelly et al., 2018)
	Peptide P1	P1 was designed to inhibit biofilms of gram-positive bacteria.	(Espeche et al., 2020)
Fusion partners	Glycosyltransferase	By co-expression UGT76G1 with protein tag Smt3M, the titer of rebaudioside was enhanced to 1.97 g/L.	(Shu et al., 2020)
	6-Hydroxy-L-Nicotine oxidase	MBP tag added to the N-terminus of HLNO improving the thermal stability and extending the half-life from ~ 2 h to ~ 22 h at 37 °C.	(Deay et al., 2020)
Extracellular expression	L-Aspartate-α-Decarboxylase	Extracellular expression resulted in enhanced L-aspartate-α-decarboxylase production.	(Feng et al., 2019)
	Feruloyl esterase	*E. coli* was used to secret expression the feruloyl esterase, which was from *Lactobacillus crispatus S524*.	(Xu et al., 2019b)
Promoter engineering	Keratinases	The *aprE* promoter was used to promote the expression of keratinase, which titer was enhanced from 165 U/mL to 2605 U/mL in *B. subtilis*.	(Gong et al., 2020a)

(*continued on next page*)

Table 1 (*continued*)

Improvement strategy	Related enzyme	Application example	Reference
	Squalene	Replaced the native promoter of ERG9 with P_{MET3} to regulate its expression by methionine.	(Asadollahi et al., 2008)

necessary to be known, which permits us to simulate natural selection at the laboratory scale (Xu et al., 2020a). Lately, various mutagenesis technologies have been used to create high-quality mutant libraries for directed evolution, such as "error-prone" PCR (Polymerase Chain Reaction), rolling circle error-prone PCR, atmospheric and room temperature plasma (ARTP), nitrous acid, and DNA shuffling (Su'etsugu et al., 2017; Zhu et al., 2019). These tools can construct the mutant libraries efficiently (Fig. 1A), and have been applied to enhance the activities of the low-efficient enzymes (Tachioka et al., 2016). For instance, random mutagenesis was applied to improve the catalytic efficiency of 11 *endo*-β-1,4-xylanase (X11) [EC 3.2.1.8] from a thermophilic glycosyl hydrolase family, which can produce xylooligosaccharides at high temperature (Boonyapakron et al., 2020). To select a nisin dehydratase NisB with the high substrate specificity, Zhao *et al.* constructed a full mutant library with about 100 000 variants by error-prone PCR and the mutants with dehydration ability were screened out (Zhao et al., 2020). Although random mutagenesis is widely applied to hunt for ideal enzymes, but most mutants are unimproved mutants. Therefore, a huge mutant library is always necessary, which promotes the application of random mutagenesis.

2.1.2. *Site-directed mutagenesis*

The functional properties of proteins are mainly determined by the types, amounts, sequences of amino acids, and the spatial structures of proteins. One or more of these characteristics can be precisely changed by mutagenesis at the specific sites such as the active center and the substrate pocket of enzymes (Wang et al., 2011). As a precise tool, site-directed mutagenesis has been served to enhance the biotransformation efficiency by improving the stability, solubility, specificity, and activity of the enzymes (Noh et al., 2020). For example, with the help of computational modeling and the redox balance-based growth selection method, site-directed mutagenesis was applied to modify the cytochrome P450-BM3, and a mutant with improved catalytic efficiency and activity of acenaphthene degradation was selected (Maxel et al., 2020). Isopentenyl diphosphate isomerase (IDI) [EC 5.3.3.2] is one of the rate-limiting enzymes in the mevalonate pathway and plays an important role in industrial valuable secondary metabolites. Chen *et al.* improved the IDI activity by the combination of random and site-directed evolution strategies (Chen et al., 2018). In addition to the traditional methods, the CRISPR-Cas9 system is a powerful genome editing approach, and has been developed to be a site-specific mutagenesis tool (Fig. 1B) (Tan et al., 2019). This creative tool can satisfy the single-base or multiple-bases editing by expressing or providing multiple gRNAs, which has been adopted to enhance the biocatalysis activity of enzyme. Wang *et al.* applied the CRISPR-Cas9 system to generate site-directed mutants efficiently and successfully, and obtained yeast colonies with improved resveratrol production (Wang et al., 2018). As a special device for biocatalysts, site-directed mutagenesis has been widely used in bioprocess; it can maximize the possibility of generating beneficial mutants, therefore can decrease the size of mutant library. However, site-directed mutagenesis strategies may lead to the loss of protein function or reduce their stability.

2.1.3. *De novo design of bioactive enzymes*

As a rational design strategy, *de novo* protein design relies on the computer simulation of protein evolutionary trajectory in nature (Fig. 1C). Meanwhile, *de novo* design can generate new proteins as ideal biocatalysts, which are developed by physical principles and different from the existing natural proteins (Huang et al., 2016). Normally, to *de novo* design a novel protein, the following steps need to be performed (Xu et al., 2020a). Firstly, an active center for the enzyme model is needed to be constructed. Secondly, an optimal backbone scaffold is needed to be selected. Thirdly, the neighboring residues need to be refined to stabilize those interactions. Then, the designed enzymes are evaluated and ranked. Finally, the characterization of new proteins is tested by the experimental testing. With the fast development of *de novo* design techniques, many metabolic engineering designs (Madhavan et al., 2021), including bioactive protein switches, enzymes, spatial scaffolds, and fusion partners, have been proposed for bioprocess improvements. As demonstrated by Donnelly *et al.*, the Syn-F4, a *de novo* protein with the catalyzed activities *in vitro*, can be used to catalyze a life-sustaining reaction in *E. coli*. The expression of Syn-F4 allowed the

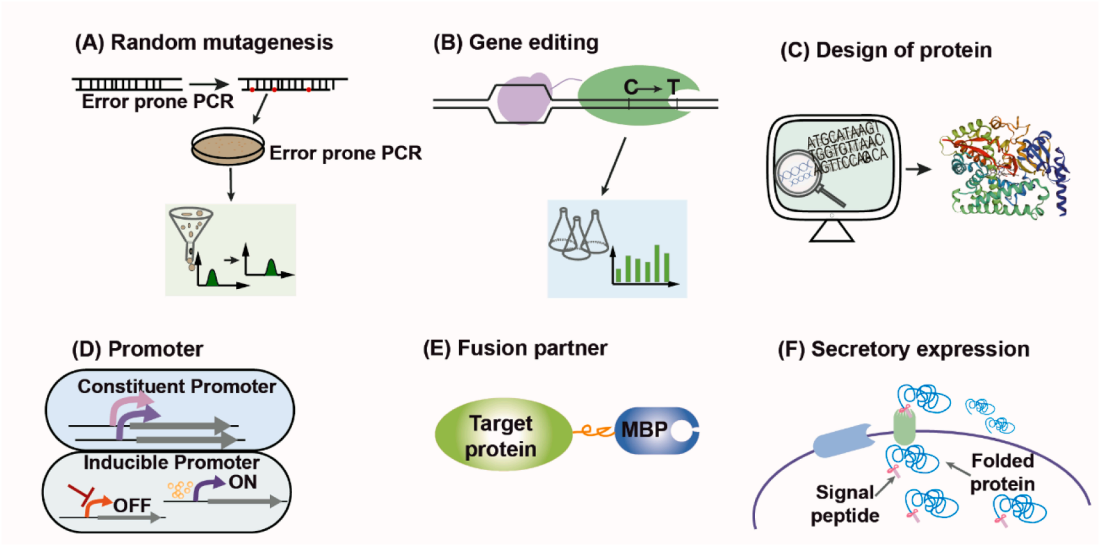


Fig. 1. Strategies for improving the activity of enzymes. (A) Screening new enzymes from the mutagenesis library. (B) dCas9 was linked to site-directed change the base(C-T). (C) Novel enzymes are designed by artificial intelligence. (D) Inducible promoters and constituent promoters are applied in metabolic engineering. (E) The direct fusion of MBP tag to a target protein. (F) The signal peptide is used to increase secretory expression.

mutant *E. coli* strain to grow in the iron-limited medium (Donnelly et al., 2018). Surprisingly, Langan *et al.* applied this method to design the bioactive protein switches (Langan et al., 2019). They provided the orthogonal cage–key proteins (LOCKR) systems, which are functional with up to 40-fold activation *in vitro,* in yeast, and in mammalian cells. Besides, this strategy has been used to control metabolic pathways. Based on the LOCKR technology, Ng *et al.* constructed a designer protein-degron LOCKR, and exploited the modular and tunable feedback regulation system in yeast (Ng et al., 2019). In their study, the degron LOCKR was fused to endogenous signaling molecules to obtain the positive and negative feedback sensors, which were used *in vivo* to tune control the biosynthetic circuit and the mating pathway. The ability to *de novo* design functional and switchable proteins is considered as a milestone for the *de novo* protein design technology, which has become an interesting strategy to obtain an ideal biocatalyst. *De novo* design of protein can be identified to build "a small but smart library"; however, in many cases, the designed proteins are inactive *in vivo*. Therefore, it is necessary to increase our understanding of natural design principles for designing more effective *de novo* components.

2.2. Enhancing and optimizing the expression of enzymes

To produce value-added products, genes encoding proteins from various sources have been introduced into industrial microbes to build microbial cell factories. However, these proteins are often in low expression, insoluble, or toxic to cell, which are limiting their application for large-scale production. To overcome these limitations, researchers are developing efficient tools, such as promoter optimization, fusion partner, and secretory expression signal peptide.

2.2.1. Enhancing the expression of enzymes

Formerly, codon preference has been considered as the main restricting factor for the expression of heterologous proteins, therefore, codon optimization has been used as a basic approach to enhance the expression of heterologous proteins (Mauro, 2018). However, promoters, as the important element for gene expression regulation, have been neglected previously. Recently, different types of promoters are developed to satisfy the demand for building complex regulation systems *in vivo* (Fig. 1D). Huang *et al.* enhanced the expression of Leech hyaluronidase (LHyal) [EC 3.2.1.36] by optimizing the combination of native promoters P_{GAP}, $P_{GAP(m)}$, and P_{TEF1} (Huang et al., 2020). However, recent studies showed that most native promoters are unsuitable to express the heterologous proteins for industrial production, because they are activated and regulated by complex metabolic regulation networks (Zhou et al., 2020). To solve these problems, researchers have proposed new approaches to optimize the activities of native promoters and replace native promoters with endogenous promoters with desired transcriptional strength (Xu et al., 2019a). Zarzhitsky *et al.* exploited a series of *De novo* Expression Enhancer Proteins (DEEPs), which showed excellent solubility and can be applied to promote high-level expression (Zarzhitsky et al., 2020). Moreover, they found that the artificial fusion partner was superior to the natural protein SUMO-tag to enhance the expression of various heterologous proteins.

Expression space is another important influence factor for biocatalyst. Most natural heterologous proteins are expressed *in vivo*, however, many of them may lead to metabolic stress against the host cells due to their accumulations and harmful actions (Feng et al., 2019). Therefore, the production of those proteins will not satisfy the need for industrial manufacturing. To enhance the production of these proteins and decrease their damage to cells, several signal peptides have been developed to accelerate the secretion of target protein, including alpha-factor mating signal, native signal peptide (nsB), and amphipathic peptides (Fig. 1F). Kang *et al.* enhanced the production of LHyal to 1.68 $\times$ 10^6 U/mL by increasing its secretion expression level (Kang et al., 2015). In another study, the alpha-factor mating signal sequence was used to enhance the secretion of length IgG1 in *Saccharomyces cerevisiae*, and increased the production by 180-fold than the wild-type. In another study, to improve the secretion of *Candida antarctica* lipase B (CalB) [EC 3.1.1.3] in *Pichia pastoris*, Vadhana *et al.* replaced the alpha-factor mating signal peptide with the native signal peptide (nsB) from *C. antarctica* lipase B, and showed about a 3-fold increase of CalB production (Vadhana et al., 2013).

In general, a suitable promotor can promote the expression of proteins, and control the time and space of protein expression. The fusion of the secretion signal peptides can improve the extracellular secretion of proteins to enhance the protein production, and reduce the separation difficulty. However, currently, signal peptides are difficult to satisfy with all kinds of proteins, and most of them are in low secretion efficiency (Ng & Sarkar, 2013). Therefore, a universal, high-efficiency, and optimal signal peptide is expected to be developed in the future.

2.2.2. Optimizing the character of enzymes

Usually, the insoluble and poor performance of the heterologous enzymes are a common phenomenon. Many appropriate tactics have been exploited to modify the catalyst characters and substrate specificity. For instance, to build a thermostable amylomaltase, Mehboob *et al.* applied site-directed mutagenesis at the 5′-end of the enzyme gene by analyzing the mRNA with bioinformatics (Mehboob et al., 2020). The fabricated protein achieved high thermostability with a half-life of 6 h at 100 °C and high specific activity of 690 U/mg. In another study, the catalyst pocket of the sodium–alanine symporter AgcS was changed by site-directed mutagenesis and achieved a mutant with the ability to the uptake of larger amino acids (Ma et al. 2019a). Moreover, fusion partners are usually co-expressed as fusion proteins by DNA recombination techniques *in vitro* (Fig. 1E), which can enhance solubility, increase thermal stability, and extend the half-life of proteins. There are several successful fusion partners exploited from nature, including mannan-binding protein (MBP), thioredoxin (Trx), calmodulin-binding peptide (CBP), and small ubiquitin-like modifier (SUMO) (Roca-Pinilla et al., 2020). As a traditional and effective strategy for production improvement, fusion partners have been widely applied and developed with the *de novo* protein design. For example, Shu *et al.* improved the conversion of the glucosyltransferase UGT76G1 by 60% via applying a fusion partner of Smt3 (homologous sequence of SUMO in *S. cerevisiae*) (Shu et al., 2020). MBP tag was also applied to increase the thermal stability of 6-Hydroxy-L-Nicotine oxidase (HLNO), an enzyme worked in the pyridine pathway in several bacteria, and extended the protein half-life from 2 h to 22 h at 37 °C (Deay et al., 2020). SUMO-tag was applied to increase the expression of human leukemia inhibitory factor (hLIF) in *Nicotiana benthamiana*, and the yield of hLIF reached 32.49 mg/kg (Islam et al., 2020). In short, fusion partners are excellent tools to enhance the expression of the low-solubility and insolubility proteins, but may affect the activities of the enzymes. Therefore, it is urgent to develop a better and universal connexin to eliminate the influence on the activity of the fusion partners.

3. Metabolic engineering strategies for promoting the development of microbial cell factories

In microbial factories, the high-efficient expression of the enzymes is one of the prerequisite factors for developing ideal biocatalysts. However, the native metabolic networks are tightly regulated by complex mechanisms in cells; the expression times and spaces of natural enzymes are controlled by the concentration of metabolites (Kang et al., 2019). Thus, the introduction of heterology pathways may change the balance of native metabolic networks; the metabolic regulation system may also be interfered or even broken in the engineering strains. For instance, overexpression of the mevalonate (MVA) pathway can enhance the production of terpenoids, but the accumulation of toxic intermediates dimethylallyl pyrophosphate (DMAPP) and the imbalance of coenzyme NADPH will appear, which is limiting the growth of engineering cells (Dudley et al., 2016). To overcome these barriers, genome-scale models,

dynamic regulation elements, multi-enzymatic cascade reactions, and subcellular compartmentalization, have been invented to analyze and reconstruct the metabolic networks, boost the precursors for target products, and balance the cell growth and product synthesis (Table 2) (Deng et al., 2019a).

3.1. Genome-scale models

Stoichiometric metabolic models, especially the genome-scale models (GEMs), are becoming indispensable tools for bioprocesses. Different from the traditional methods, GEMs can be applied to predict the distribution of metabolic flux (Fig. 2A). The optimal metabolic network can be calculated by computational approaches according to the gene-protein-reaction links and the nutritional requirement for cell growth. GEMs can help researchers to understand the ways to modify the native metabolic networks by the prediction and analysis tools, including OptForce, OptKnock, and flux balance analysis (Schroeder & Saha, 2020). GEMs have been applied in industrial bioprocesses to enhance production. Yang *et al.* used GEMs to predict the metabolic fluxes for the expressing of *Cupriavidus neactor* 3-ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB) in *E. coli* (Yang et al., 2018). Based on the flux modulation, they selected five promoters with different strengths to modulate the expression of PhaAB, and obtained the strains to produce D-phenyllactate from glucose. To enhance the production of poly(γ-glutamic acid) (γ-PGA) in *B. subtilis*, Massaiu *et al.* built a GEM with Enzymatic Constraints by using the Kinetic and Omics data (GECKO) model, and obtained an engineered strain with higher than the 2-fold yield of the original strain γ-PGA (Massaiu et al., 2019). In another example, Zhang *et al.* constructed a GEM for *Corynebacterium. glutamicum* ATCC 13,032 to predict metabolic fluxes, and rebuilt an engineered strain with the L-proline production of 66.43 g/L in 60 h (Zhang et al., 2017). Recently, GEMs have become a popular computing platform for accommodating data sets. Various algorithms have been developed to integrate the ever-increasing mass of omics data with GEMs (Yu et al., 2019).

In addition, the network coverage of GEMs has been expanded to handle biological processes of genome-scale model of metabolism and macromolecule expression, including gene expression of the metabolism pathway, all annotated gene functions, and the whole-cell model. These models are expected to facilitate innovative systematic metabolic engineering. However, current databases cannot fully realize the prediction and analysis of all pathways for all microbial species, and current modeling technologies are only used by part researchers. Therefore, a more comprehensive and opening database is needed for most researchers.

3.2. Multi-enzymatic cascade reactions

In the bioprocesses, the low reaction efficiency has always been a limitation of bioconversion, which is more obvious in the constructed pathways that related with heterologous enzyme. Multi-enzymes complexes can enhance the reaction efficiency and cascade enzymatic activity (Fu et al., 2014). As a traditional method, direct fusion proteins with a short protein linker are used to increase the reaction efficiency. However, this method leads to the decreasing or losing of activities for most biocatalysts. To avoid this negative effect, many novel multi-enzyme complex strategies have been developed to mimic the natural process of multi-enzymatic cascade reactions.

Creating a synthetic metabolic channel by the assemble of enzymes can significantly improve the reaction efficiency. Various types of spatial scaffolds and scaffold-free enzyme assembly methods have been employed in bioprocesses recently. For example, the functional membrane microdomain, as a spatial scaffold, was selected to construct a multi-enzyme complex assembly system, which improved the titer of N-acetylglucosamine in *B. subtilis* successfully (Lv et al., 2020). The enzyme complexes can be applied as efficient strategies to enhance the reaction efficiency; meanwhile, as regulation tools, they can fine control metabolic fluxes by changing sequence-to-structure relationships to balance metabolic networks. Consequently, the titers of value-added products can be increased. Besides, as shown in Fig. 2B, a pair of short peptide tags (RIAD and RIDD) were applied to create scaffold-free enzyme assemblies. Kang *et al.* showed that cascade biocatalysis and metabolic flux were optimized to increase the production of lycopene (Kang et al., 2019). In another example, Dueber *et al.* applied the synthetic protein scaffolds GBD-SH3-PDZ to optimize the stoichiometry of three mevalonate biosynthesis enzymes, including the acetoacetyl-CoA thiolase (AtoB), hydroxy-methylglutaryl-CoA synthase (HMGS), and hydroxymethylglutaryl-CoA reductase (HMGR). By employing this method, the metabolic load was decreased and the product titer was increased by 77-fold. Moreover, the scaffolds have also been used to enhance the yield of glucaric acid and a 3-fold improvement was achieved (Dueber et al., 2009). All the above experiments proved that multi-

Table 2
Metabolic engineering strategies for predicting and balancing metabolic fluxes.

Methods	Related enzymes	Functions	Application examples	Refs
Genome-scale models (GEMs)	Tryptophan	Predicting the optimization of metabolic flux.	Firstly, constraint-based modeling was used to predict single gene targets for perturbating tryptophan production. Then, the 192 genes which showed considerable changes with the production of tryptophan were analyzed. Thirdly, according to the analysis for statistical overrepresentation of genome-scale modeled metabolic pathways, the pentose phosphate pathway and glycolysis were modified for improving tryptophan titer and productivity.	(Zhang et al., 2020b)
Dynamic regulation	2′-Fucosyllactose	Balancing the production and cell growth; decreasing the metabolic burden.	The aptamer-based synthetic regulatory circuit was built to dynamically upregulate and downregulate the expression of target genes by the ligand thrombin at transcriptional and translational levels, and the system was applied to regulate the production of 2′-fucosyllactose.	(Deng et al., 2019b)
Multienzymes Cascade Reactions	Amorpha-4,11-diene	Modular control over metabolic fluxes.	Three terpene biosynthetic enzymes were assembled to particle protein scaffold that likes the Tobacco mosaic virus and is linked by orthogonal reactive protein pairs (SpyCatcher/SpyTag and SnoopCatcher/SnoopTag), and the assembly was regulated by the production of amorpha-4,11-diene.	(Wei et al., 2020)
Compartmentalization	Muconic acid	Modular control over metabolic fluxes; providing special reaction conditions, substrate, and space.	The maize storage protein gamma-Zein with N-terminal self-assembling domain was composed of the signal peptide of the ER, which was utilized to assemble the heterologous enzymes of CCM production into vesicles.	(Reifenrath et al., 2020)
Adjustment of physiological parameters	α-Amylase	Optimizing reaction space for biocatalysts.	Engineering a protein secretory pathway of endosome-to-Golgi trafficking to enhance the production of α-amylase.	(Huang et al., 2018)

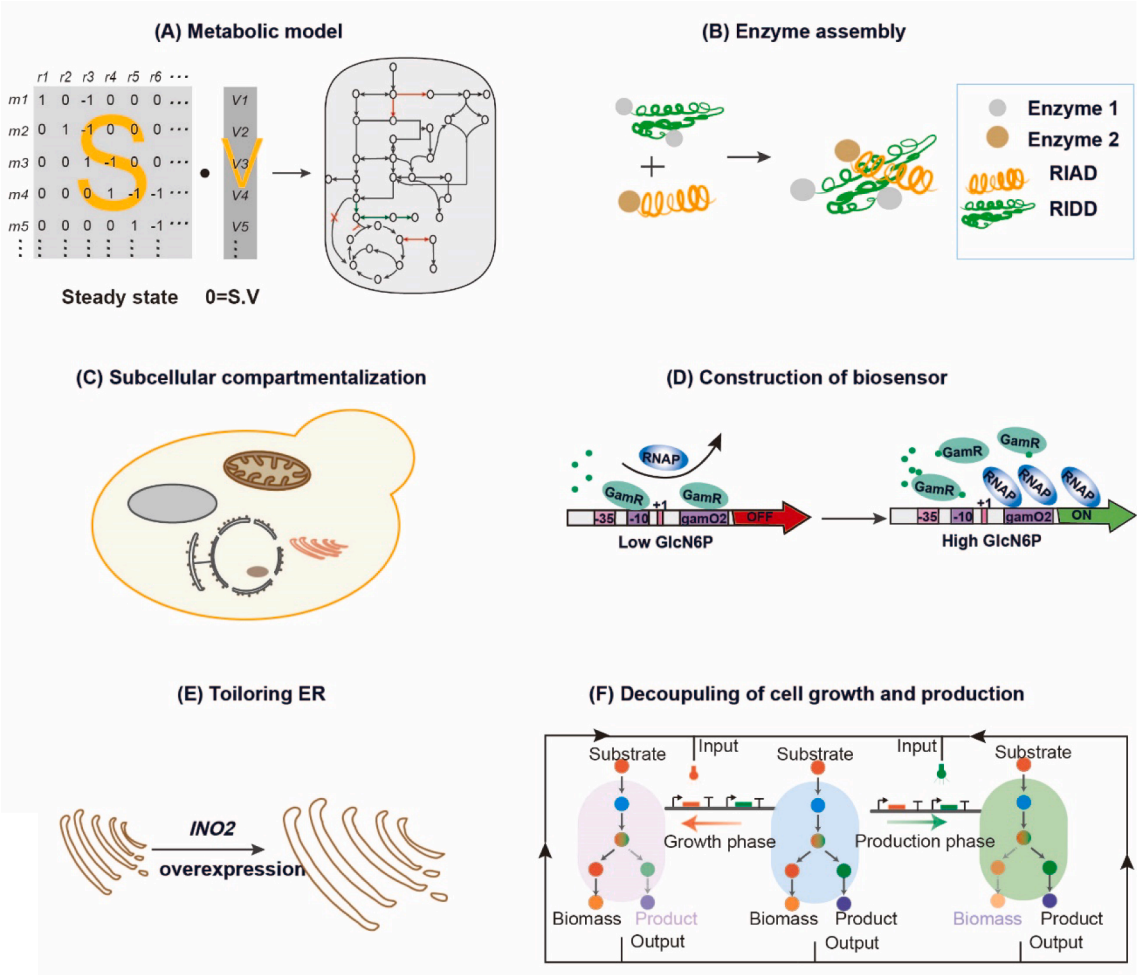


Fig. 2. Methods to analyze and regulate the metabolic networks. (A) Prediction of the optimization metabolic models by artificial intelligence. (B) A pair of short peptide tags (RIAD and RIDD) applied to create a multi-enzymatic cascade reaction. (C) Using yeast organelles to improve the titer of value-added products. (D) A biosensor was constructed as a biosensor to sense the concentration of GlcN6P. (E) The size of the ER was enlarged by overexpressed of INO2, which was used for functional assembly of the terpene synthesis pathway. (F) Dynamic regulation is applied to decouple cell growth and production.

enzymatic cascade reaction can help to combine different modules and optimize metabolite flux, which will become a powerful tool to fine-tune pathway flux. However, the applications of protein scaffolds may cause the loss of enzyme activity. Therefore, the universal and more efficient scaffold systems should be further developed.

3.3. *Compartmentalization of the biosynthesis pathway*

Compartmentalization is a powerful strategy to enhance the production of many value-added products by microbial cell factories (Trantidou et al., 2017). As a typical compartmentalization in bacteria, bacterial microcompartments (BMCs) represent proteinaceous macromolecular nano-bioreactors. They provide enhanced flux and protection against toxic intermediates by enclosing the central metabolic enzyme or pathway in a semi-permeable shell (Lee et al., 2019). In eukaryotes, several complex organelle systems have been developed into workshops or suppliers to produce value-added products in microbial cell factories, such as mitochondria, endoplasmic reticulum, and peroxisome (Fig. 2C). Due to the abundant existence of acetyl-CoA, peroxisomes have been used as compartmentalization for the biosynthesis pathways of value-added products in yeast. For example, the metabolic of poly(3-hydroxybutyrate) (PHB) was compartmentalized by peroxisomes, which was achieved via fusing the enhanced Peroxisome Targeting Sequences (ePTS1) to the PHB synthetic genes, including *phaA*, *phaB*, and *phaC* (Huttanus & Senger, 2020). Besides, Yuan *et al.* applied mitochondria as subcellular metabolic space to produce the terpenoid amorpha-4,11-diene from the acetyl-CoA pool (Yuan & Ching, 2016). Additionally, a plasma membrane system with natural lipid bilayers has also been developed as a workshop for the production and storage of fat-soluble products such as menaquinone-7 (Cui et al., 2020), α-farnesene (Yang et al., 2016), and ent-kaurene (Geiselman et al., 2020). Each organelle and its multiple essential enzymes are regulated by complex mechanisms. Therefore, the strategies of subcellular compartmentalization and membrane-bound enzyme re-localization may disturb the delicate metabolic system and lead to metabolic stress (Guirimand et al., 2020).

Accordingly, some important physiological parameters are adjustable by the organelle metabolic engineering to satisfy the demands of biocatalysts. For instance, the size of the endoplasmic reticulum (ER) has been enlarged to increase the production of the P450 enzyme by the overexpression of the regulatory factor gene INO2 (Fig. 2E). This modification, which is associated with global regulation systems, improved both enzyme expression and terpene production (Kim et al., 2019). Sometimes, organelles can be used as the space for storing enzymes or products, appropriately adjusting their physiological parameters can enhance the yield, and decrease the metabolic burden and toxicity. In a recent study, peroxisomes were rewired to improve alkaloid production by regulating three peroxisome proliferation-related transcription factors, ADR1, OAF1, and PIP2 (Grewal et al., 2020). Compartmentalization can provide an independent environment for

metabolic fluxes. Since a part of proteins may be recognized as defective or aging proteins after fusing with peptides, and then transported into the vacuole or proteasome for proteolysis, this strategy cannot meet the needs of all the products.

3.4. Dynamic regulation

With the development of metabolic engineering, dynamic regulating strategies have been applied to separate the growth and producing phase by sensing the change of the intermediary metabolites or environment (Adeniran et al., 2015). In this way, the damage on the host cell is minimized by enhancing the synthetic pathway of the target product and inhibiting the competing pathways dynamically.

Now, a series of genetic regulatory elements have been developed to make the cells sensible by the changing of specific metabolites. These regulatory elements can be classified into three levels: transcriptional, post-transcriptional, and translational (Robert & Bucheton, 2004). At the transcriptional level, transcription factors can respond to the special signaling factors inside and outside of the cells directly or respond to the change of the fermentation environment indirectly. The regulation of the post-transcriptional level in prokaryotic organisms is achieved by the *cis*-acting elements of mRNA, such as riboswitch and non-coding RNA. In eukaryotes, the balancing of metabolic systems is regulated by the specific small interfering RNA and microRNA (Cerutti et al., 2011). In the dynamic regulation systems, the target pathways are positively regulated, while the branch pathways are negatively controlled or degraded by the regulation elements at the special time and space. Many successful examples have been reported in the past few years. For example, Xu *et al.* regulated central metabolism by pyruvate-responsive genetic circuits, and increased the titer of glucaric acid from 207 to 527 mg/L (Xu et al., 2020b). As shown in Fig. 2D, Wu *et al.* constructed an intermediate glucosamine-6-phosphate biosensor to balance the metabolic flux of N-acetylglucosamine (GlcNAc), and the production of GlcNAc was enhanced to 131.6 g/L (Wu et al., 2020). Tian *et al.* employed N-terminal coding sequences as a regulatory tool to fine-tune gene expression dynamically to improve the production of N-acetyl neuraminic acid by 3.21-fold (Tian et al., 2019).

Recently, several regulatory elements have been constructed to control metabolic flux by sensing the change of environment. As an example, Lo *et al.* built a balancing system that can decouple cell growth with metabolite production in *E. coli*, and constructed an auto-regulated enzymatic pathway worked by sensing the concentration change of glucose and substrate (hydroxycinnamic acid or oleic acid) (Lo et al., 2016). After using the system, the cell metabolic stress decreased 2-fold in the fermentation process; the growth rate and productivity, which were controlled by the substrate, hydroxycinnamic acid, increased 2-fold and 5-fold, respectively. Except the hydroxycinnamic acid controller, oleic acid increased the growth rate by 1.3-fold and the productivity by 2.4-fold. In another example, a light-inducible genetic (optogenetic) switch was applied to control the central metabolic flux of *E. coli* dynamically; the metabolic flux of Embden-Meyerhof-Parnas (EMP) and oxidative pentose phosphate (oxPP) pathways were redistributed by the light-inducible metabolic switch (Tandar et al., 2019). The flux ratio of EMP:oxPP was controlled to 50:49 and 0.5:99 (of total glycolytic flux) by exposing to green and red light, respectively. In addition, a cold-shock-triggered temperature controlling system was developed to control a two-stage fermentation in *S. cerevisiae*, and the titer of tocotrienols was increased to 320 mg/L (Shen et al., 2020).

Generally, the dynamic regulation strategy can effectively avoid the problems caused by the traditional engineering methods, including the imbalance of co-factors, the accumulation of intermediate metabolites, and the damage of cell growth. This strategy is also suitable for multiple microorganisms, including *E. coli*, *B. subtilis*, *S. cerevisiae*, *Y. lipolytica*, *C. glutamicum*, and so on. However, this strategy is full of uncertainty, any modification may lead to a negative impact on the engineering strains. With the assistance of artificial intelligence, which can predict the optimal regulating method, dynamic regulation strategy can provide more possibilities for the construction of high-performance engineered strains.

4. Applications of combining protein engineering and metabolic engineering methods

Many properties of a biocatalyst are not only limited by the enzyme concentration or their characteristics, but also limited by the metabolic fluxes. Therefore, in many situations, adopting protein engineering alone cannot achieve the desired goal. For example, in the synthesis pathway of terpenoids, the terpene synthases are usually weaker than catalytic performance, and limiting the yield improvement of desire products (Daletos et al., 2020). To strengthen the precursor pathway, protein engineering strategies are applied. However, after the accumulation of intermediate metabolites, a stress response appears, which is harmful to cell growth. In this case, the metabolic engineering strategies are necessary to re-balance the metabolic fluxes. To date, the combination of multiple strategies is easier to obtain the outstanding biocatalysts with high catalytic performance. As listed in Table 3, many value-added products were successfully produced, including GlcNAc, menaquinone-7, and β-carotene, in which the metabolic pathways were re-balanced by the combining of dynamic regulation, compartmentalization, or fusion partners methods.

The combining strategies can also synthesize the products that are previously difficult to obtain from the cheap substrates (*e.g.,* glucose, xylose). For example, Yuan *et al.* built a co-culture system of *E. coli* - *S. cerevisiae* to *de novo* produce resveratrol from glucose, and enhanced the production to 36 mg/L (Yuan et al., 2020). Besides, the maximum transformation rate of by-products and industrial wastes into object products are expected in bioprocessing (Fig. 2F). To meet the industrial needs, more and more strategies have been introduced, such as optimization of the fermentation process, knockout, or inhibition of the by-product synthesis genes (Xu et al., 2020c). Therefore, the combination of protein engineering with metabolic engineering strategies to tune a heterologous pathway at regulatory nodes would be a valuable approach to achieve both conferring an overproduction phenotype and minimizing toxicity in microorganisms.

Usually, the combining strategy is better than single strategy. However, finding the best combination is a heavy workload due to the complex non-linear relationships of the products and conditions. For example, when combining the expression of heterologous enzymes with a co-culture system of two strains, several factors should be optimized, including the strength of promoter, expression time of the enzymes, inoculation ratio, inoculation time, culturing medium, temperature, pH, and so on. If five gradients are tested for each variable, there are 5^n kinds of combinations to be optimized for n variables, which cannot be achieved by the traditional methods. However, if these variables can be optimized by a modeling algorithm, the combining strategy would be easier to applied and enhance the synthesis of products.

5. Application of artificial intelligence techniques in bioprocessing

Designation of microbial cells and fermentation scale-up are the two main parts in bioprocesses. However, the large-scale processes are usually nonlinear, multidimensional, and dynamic (Udugama et al., 2021). Therefore, it is difficult to find the best conditions by the traditional methods. Many artificial intelligence (AI) technologies, including artificial neural network (ANN), fuzzy logic, and genetic algorithm, have been carried out to optimize and control the bioprocesses (Venkatasubramanian, 2019). However, the lack of online-data and self-defects of algorithms have limited their applications.

Recently, with the technology accumulation of big data in bioprocesses, machine learning (ML), a subset of AI, has gradually appeared in the fermentation process. ML focuses on the analyzing and

Table 3
Applications of combining protein engineering and metabolic engineering strategies for improving biocatalysts.

Product	Strain	Strategies	Substrate	Concentration (g/L)	Refs
N-acetylglucosamine (GlcNAc)	*B. subtilis*	**1. Protein engineering** (Strong promoters *P43* was used to overexpress the expression of the enzymes for producing GlcNAc, such as the glucose-6-phosphate isomerase (Pgi) and PTS system glucose-specific EIICB component (PtsG).) **2. Metabolic engineering** (The dynamic regulation system works by sensing the level of glucosamine-6-phosphate (GlcN6P), and autonomously adjust the expression levels according to the GlcN6P sensor.) **3. Blocking by-product acetoin formation** (The gene of *alsSD* was Knocked out to eliminate the formation of acetoin.)	Glucose	131.6	(Wu et al., 2020)
Menaquinone–7 (MK-7)	*B. subtilis*	**1. Protein engineering** (The key enzymes in the synthesis pathway of MK-7 were overexpressed, such as farnesyl diphosphate synthase (IspA), 1,4-dihydroxy-2-naphthoate heptaprenyltransferase (MenA), and 1-deoxyxylulose-5-phosphate synthase (Dxs).) **2. Metabolic engineering** (Phr60-rap60-spo0A quorum-sensing system was constructed to balance the production of MK-7 and cell growth.) **3. Blocking by-products formation** (The isochorismatase (DhbB) was knocked out and the prenyltransferase (UppS) was inhibited to boost the synthesis of MK-7 precursors.)	Glucose and glycerol	360	(Cui et al., 2019)
Glucaric acid	*B. subtilis*	**1. Construction of d*e novo* synthetic pathway for producing glucaric acid** (Three heterologous were integrated into *B. subtilis,* including *ino1* (GenBank ID: CAA89448.1), *miox52* and gene *udh* (GenBank ID: SPD80607.1).) **2. Protein engineering** (To strengthen the synthesis of glucaric acid, the P43 promoter was used to replace the native promoter of the gene *suhB*.) **3. Dynamic regulation system** (Pyruvate-responsive genetic circuits were developed to dynamic control of central metabolism and used to enhance the production of glucaric acid.) **4. Blocking by-products formation** (To reduce the metabolic overflow and maximize the accumulation of glucaric acid., the four genes were knocked out, including *uxaC* (encoding galacturonate isomerase), *gudD* (encoding glucarate dehydratase), *yrbE* (encoding inositol-related oxidoreductase), and *iolG* (encoding inositol 2-dehydrogenase).)	Glucose	0.802	(Xu et al., 2020a)
Astaxanthin	*E. coli*	**1. Protein engineering** (Enzyme strategies were applied to enhance biocatalysts activity, including fusion tag, and truncated of single peptide.) **2. Metabolic engineering** (The balance of metabolic networks was controlled by IPTG (isopropyl-beta-$_D$-thiogalactopyranoside).) **3. Optimization of fermentation parameters** (Carbon sources and cultivation temperatures were optimized to enhance the production of astaxanthin.)	Glucose	0.43282	(Yang et al., 2018)
α-tocopherol	*S. cerevisiae*	**1. Construction of d*e novo* synthetic pathway for producing α-tocopherol** (The relative gene for the biosynthesis of α-tocopherol from plants was integrated into S. cer*evisiae*, including HPPD (4-hydroxyphenylpyruvate dioxygenase), SyHPT (homogentisate phytyltransferase), MPBQMT (2-methyl-6-phytylbenzoquinol methyltransferase, TC tocopherol cyclase), and γ-TMT (γ-tocopherol methyltransferase).) **2. Protein engineering** (The precursor was enhanced by overexpressing of relative genes, such as tHMG1 (truncated 3-hydroxy-3-methyl-glutaryl reductase), Aro4 (3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase), and Aro7 (chorismate mutase); Elimination of the rate-limiting steps was eliminated by overexpression endogenous expression of enzymes and, truncated of the single peptide.) **3. Metabolic engineering** (Balancing Metabolic networks were rebalanced by comprehensive pathway optimization; The dynamic regulation was triggered by cold-shock temperature.)	Glucose	0.32	(Shen et al., 2020)
Squalene	*S. cerevisiae*	**1. Protein engineering** (The key enzymes in MVA pathway were overexpressed, including ERG10, ERG13, tHMG1, ERG12, ERG8, MVD1, IDI1, ERG20, and ERG9.) **2. Metabolic engineering** (Compartmentalization of squalene in peroxisomes can ensure sufficient precursor acetyl-CoA supply, and reduce the metabolic burden for the engineered strain.)	Glucose and ethanol	11	(Liu et al., 2020)
Free fatty acids	*S. cerevisiae*	1. **Enhancing the supply of acetyl-CoA (**altered subcellular metabolic trafficking)0.**2. Cofactors regeneration** (fine-tuned NADPH and ATP supply). **3. Metabolic engineering** (The metabolic network was rebuilt to balance the cell growth and production, by increasing the supply of precursor and decreased carbon flux to biomass.)	Glucose	33.4	(Yu et al., 2018)
4-Hydroxybenzoic Acid (4-HBA)	*C. glutamicum*	1. **Protein engineering (**High tolerance strain and enzyme to 4-HBA was selected for enhancing the biocatalysis activity.) **2. Metabolic engineering** (Metabolic flux was rebalanced by increasing the supply of precursor and avoiding the accumulation of intermediate metabolite.) **3. Blocking by-product production** (To reduce the formation of by-products, the two genes *hdpA* (haloacid dehalogenase) and *pyk* (pyruvate kinase) were knocked out.)	Glucose	36.6	(Kitade et al., 2018)
Hyaluronic acid	*C. glutamicum*	1. **Protein engineering (**Several strategies were used to improving the activities of biocatalysts, including site-directed mutant, combinatorial overexpression, extracellular expression, and screening enzymes from different sources.) 2. **Metabolic engineering** (The metabolic flux was	Glucose	74.1	(Wang et al., 2020b)

(*continued on next page*)

Table 3 (*continued*)

Product	Strain	Strategies	Substrate	Concentration (g/L)	Refs
		rebalanced by increasing the supply of precursor and avoiding the accumulation of intermediate metabolite.)			
5-Aminolevulinic acid	*C. glutamicum*	**1. Metabolic engineering (**Dynamic regulation was constructed by temperature-dependent control strategy, and growth-regulated promoter co-regulate *odh A* transcription.) **2. Modulation of the cofactor (**ATP, NADPH, and PLP were modulated to enhance biocatalysis of enzymes.) **3. Dynamic efflux** (A two-component system was used to regulate the expression of exporter-encoding *rhtA*.) **4. Two-stage fermentation (**Engineered cells were cultured at 28 °C for 12 h followed by 37 °C for another 52 h.)	Glucose	3.16	(Zhang et al., 2020a)
2–Phenylethanol	*Y. lipolytica*	**1. Enhance biocatalysts activity** (optimal catalytic modules, enzymes assembly). **2. Modulation, and eliminating mitochondrial compartmentalization** (co-expression enzymes in cytosolic). **3. Optimization of central carbon metabolism** (blocking the precursor-competing pathways and mitigating fatty acid synthesis). **4. Substrate co-feeding** (Glucose and L-phenylalanine as carbon sources).	Glucose and L-phenylalanine	2.66954	(Gu et al., 2020)
β-carotene	*Y. lipolytica*	**1. Enhance biocatalysts activity** (promoter engineering, golden gate DNA assembly, the addition of an extra copy). **2. Co-production with lipid. 3. Optimization of bioreactor control conditions** (media, glucose concentration, fermentation times).	Glucose	6.5	(Larroude et al., 2018)
Triacetin acid lactone	*Y. lipolytica*	**1. Metabolic flux optimality** (engineering acetyl-*CoA* metabolic shortcut, boosting precursor malonyl-CoA, recycling cytosolic NADPH). **2. Balance metabolic flux** (increase supply of precursor and dehydrogenase of pyruvate). **3. Optimization of fermentation parameters** (pH, carbon source, acetic acid concentration).	Acetic acid	4.76	(Liu et al., 2019)
Daptomycin	*Streptomyces*	**1. Enhance biocatalysts activity** (removal of the inhibitory effects, random mutagenesis combined with a reporter-guided screening strategy). 2. **Dynamic regulation** (*PhaR* is positively auto-regulated by directly binding to its promoter, promoter engineering).	Glucose	960	(Luo et al., 2018)

interpreting of both patterns and structures of data to enable learning, reasoning, and decision making, without human interaction (Kim et al., 2020). ML can be used to discover the pattern from the complex non-linear relationships by the in-depth mining of data (Kim et al., 2020). By combining traditional mechanistic models with ML technique, many high-efficient AI technologies have been successful exploited in several levels for bio-manufacturing.

Firstly, the AI technologies can be applied to construct intelligent engineering strains in the cell level. For example, based on 100 ^{13}C metabolic flux analyzing papers, Wu *et.al* developed a web-based platform MFlux (http://mflux.org) by combining ML and metabolic models (Wu et al., 2016). The platform can predict the distribution of intracellular metabolic flow according to the microbial species, carbon source types, oxygen dissolution conditions, cell growth rates, etc. Oyetunde et al. (2019) proposed a data-driven method that combined with a genome-scale metabolic model to evaluate microbial biological yield (yield, potency, and rate), which is based on more than 100 metabolic engineering papers of *E. coli*. Besides, model-based AI process allowed easier optimization and design of experiments (DOE). Traditional DOE is covering the designing space, generating response surfaces, and finding the best operating conditions to achieve a certain goal by a set of experiments. With the develop of modeling techniques, experiments can be designed and optimized according to the simulation results. For instants, Rodriguez-Granrose *et.al* showed that the hybrid approach of ANN-DOE was significantly better to improve bioprocess outputs than the existing linear regression model, including standard least squares (SLS), and one factor at a time (OFAT) experimentation (Rodriguez-Granrose et al., 2021). Their results proved ANN-DOE could find optimal bioprocessing conditions with higher efficiency and lower workload.

After constructing the high-efficient strains and optimizing the fermentation parameters in laboratory-scale reactors, bioprocess scale-up is necessary for the industrialization of biological manufacturing. However, the process scale-up is still the major problem. The difference between laboratory-scale and industrial-scale reaction is the main external barrier; and the non-linear characteristics of microbial response to the complex metabolic environmental is the main internal factors. To overcome the above barriers, researchers developed AI technologies to monitor and control the bioprocesses in real-time. Here, the design of intelligent bioreactors is important. Traditionally, bioreactors were imperfectly designed based on a single measurement or the adaptation of an established bioreactor. Currently, computational fluid dynamics (CFD) combined with powerful stimulus-response metabolic models has been applied in bioprocess to enable faster scale-up and lower wastage; this technology can also predict and evaluate the fermentation process accurately and efficiently (Wang et al., 2020a). The first step is building metabolic models based on the metabolic-hydrodynamic pathways. Then, the industrial-scale parameters of fermentation can be estimated according to the predictive models. Recently, bioprocess model prediction has been successfully applied in *E. coli*, *S. cerevisiae*, and *Penicillium chrysogenum* for vaccine high-producing (Zhang et al., 2020c), glucose uptake (Haringa, 2017), penicillin production (Pigou & Morchain, 2015), and so on. The design of these intelligent bioreactors facilitates the transition from lab-scale fermentations to industrial-scale systems.

In summary, the ML has been applied to obtain deep knowledge from data and realize the intelligent perception of bioprocess. By combining ML with other strategies, researchers can build high-performance intelligent engineering strains in the microbial level, and develop monitorable and controllable systems in the scale-up bioprocess. However, the online data of bioprocess is still not enough to meet the requirements for training ML; meanwhile, the on-line detection technology for biological processes is still insufficient. Therefore, the on-line detection technology should easier to be applied and enhance the development of intelligent biological manufacturing.

6. Future perspectives for developing biocatalysts in bioprocess

Bioprocesses can convert industrial waste and by-products into value-added products in the industry; this biocatalysis-based technology has gained increased attention recently. Additionally, comparing with the traditional chemical synthesis processes, bioprocess shows many advantages: mild reaction conditions, environmental friendliness, and a broad range of substrates (Zhang et al., 2019). Nevertheless, there are still some disadvantages in the processes, such as instability and lower

expression of enzymes, poor performance under certain reaction conditions, high cost due to the complex downstream processing, and limited knowledge in microbiology and the designing of bioprocesses. Those obstacles are often hindering the scale-up from the laboratory scale to the manufacturing scale. Therefore, biotechnologies are in demand for novel bioprocesses to develop efficient, sustainable, and economical bioprocesses.

To obtain stable and high-efficient biocatalysts, over the past five years, protein engineering strategies, including *de novo* design of enzymes, and directed evolution, have made a great contribution. In the process, the powerful high throughput screening methods are demanded to sort the ideal enzymes. Most existing screening strategies are based on the microtiter plate culturing process, which is expensive, complex, time-consuming, and have many limitations (van Rossum et al., 2013). Droplet microfluidics, an exquisite method with higher throughput, lower assay volumes, and faster screening speed, can be used to select biocatalysts to overcome the above difficulties. Ma *et al.* used droplet microfluidics to screen xylanase mutants and achieved a higher than 1.3-fold enhancement of xylanase activity (Ma et al., 2019b). In the future, the combining strategy of droplet microfluidics and protein engineering will be a powerful screening tool in bioprocessing.

To enhance and optimize the protein expression, many methods have been exploited, such as promoter engineering, fusion partner, and secretory expression. Besides, many protein engineering strategies are developed for biocatalysts, but it is still difficult to obtain the crystal structures of complex proteins or membrane proteins (Li et al., 2020). Therefore, structural biology, biochemistry, and computational biology are important for the development of protein engineering, which may contribute to the exploration of original reaction mechanisms and further modification for ideal enzymes.

In addition, overexpression of enzymes will lead to the destroying of metabolic fluxes and may break the sophisticated regulatory systems of engineering strains. To maintain the metabolic balance by redistributing it, the metabolic engineering device for predicting and re-building the metabolic fluxes has been developed, including metabolic network models, dynamic regulation, genetic and metabolic regulation cycles (Lo et al., 2016). These methods can minimize the damage of cells through separating the growth and production phase by space or time. Furthermore, the combination of these strategies is more efficient for the development of innovative and high-efficient biocatalysts to produce value-added products from low-cost substrates. However, many challenges are still existed underlying the design and construction of novel biocatalysts for bioprocessing. Continuous efforts are necessary to exploit and develop high-performance strategies for designing and screening ideal enzymes. To balance the expression of genes and the production of products, designing genetic biosensors with prospective functions, such as sensitivity, substrate specificity, dynamic range, and complex genetic circuit, are demand (Hossain et al., 2020).

As currently envisaged, a future challenge is how to combine those strategies efficiently to increase the possibility for designing and constructing prospective biocatalysts and to develop microbial hosts with high performance to produce value-added products in bioprocessing. Therefore, the future development of metagenomics technology will explore the complete microbial population dynamics by integrating different methods, such as controlling fermentation conditions, nucleic acid analysis, and the metabolic and fermentation dynamic models (Kumar Awasthi et al., 2020). Metagenomics technology will give comprehensive knowledge about the natural sequences that exist in human, animals, and microorganisms. Big data analysis will become a better tool to predict new models of structures and substructures, and also provide better understanding of broader biological systems. Artificial intelligence can assist the designing of model building and optimization of metabolic pathways. The genome-scale metabolic model can simulate cellular behaviors and metabolic states, and predict the optimal metabolism fluxes of engineering strains under various environments. Importantly, most of the strategies and tools are universal. Therefore, high-efficient combination and application of those methods will facilitate the development of industrial biological treatment and provide commercial opportunities for those high-value-added but low-produced products.

7. Conclusion

With the exploitation and development of high-efficient biocatalysts, it is promising to transfer the biosynthesis of value-added products from proof-of-concept to industrial scale. To enhance bioconversion and productivity, various efficient protein engineering strategies for biocatalyst have been developed. Meanwhile, to avoid the imbalance of metabolic fluxes that caused by the overexpression of enzymes, different metabolic regulation strategies have been exploited and applied successfully. The future trend is combining the powerful artificial intelligence technology with the developed bioprocess strategies to facilitate industrial manufacturing.

CRediT authorship contribution statement

Yameng Xu: Conceptualization, Investigation, Writing - original draft. **Yaokang Wu:** Conceptualization, Supervision, Project administration, Writing - original draft. **Xueqin Lv:** Supervision, Project administration, Writing - review & editing. **Guoyun Sun:** Supervision, Project administration, Writing - review & editing. **Hongzhi Zhang:** Supervision, Writing - review & editing. **Taichi Chen:** Supervision, Writing - review & editing. **Guocheng Du:** Writing - review & editing. **Jianghua Li:** Writing - review & editing. **Long Liu:** Funding acquisition, Writing - review & editing.

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.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31930085, 31870069, 31871784); the Key Research and Development Program of China (2018YFA0900300 and 2020YFA0908300); the Shandong Province Key R & D Program (Major Science and Technology Innovation Project) Project (2019JZZY011002); and the Fundamental Research Funds for the Central Universities (JUSRP52019A).

References

Asadollahi, M.A., Maury, J., Møller, K., Nielsen, K.F., Schalk, M., Clark, A., Nielsen, J., 2008. Production of plant sesquiterpenes in *Saccharomyces cerevisiae*: Effect of ERG9 repression on sesquiterpene biosynthesis. Biotechnol. Bioeng. 99 (3), 666–677. https://doi.org/10.1002/bit.21581.

Adeniran, A., Sherer, M., Tyo, K.E.J., 2015. Yeast-based biosensors: design and applications. FEMS Yeast Res. 15 (1), 1–15. https://doi.org/10.1111/1567-1364.12203.

Barragán-Ocaña, A., Silva-Borjas, P., Olmos-Peña, S., Polanco-Olguín, M., 2020. Biotechnology and Bioprocesses: Their Contribution to Sustainability. Processes 8 (4). https://doi.org/10.3390/pr8040436.

Boonyapakron, K., Chitnumsub, P., Kanokratana, P., Champreda, V., 2020. Enhancement of catalytic performance of a metagenome-derived thermophilic oligosaccharide-specific xylanase by binding module removal and random mutagenesis. J. Biosci. Bioeng. https://doi.org/10.1016/j.jbiosc.2020.09.008.

Cerutti, H., Ma, X., Msanne, J., Repas, T., 2011. RNA-mediated silencing in algae: biological roles and tools for analysis of gene function. Eukaryot. Cell 10 (9), 1164–1172. https://doi.org/10.1128/EC.05106-11.

Chen, H., Li, M., Liu, C., Zhang, H., Liu, H., 2018. Enhancement of the catalytic activity of Isopentenyl diphosphate isomerase (IDI) from *Saccharomyces cerevisiae* through random and site-directed mutagenesis. Microb. Cell Fact. 17 (1) https://doi.org/10.1186/s12934-018-0913-z.

Cui, S., Lv, X., Wu, Y., Li, J., Du, G., Ledesma-Amaro, R., Liu, L., 2019. Engineering a Bifunctional Phr60-Rap60-Spo0A quorum-sensing molecular switch for dynamic

fine-tuning of menaquinone-7 synthesis in *Bacillus subtilis*. ACS Synth. Biol. 8 (8), 1826–1837. https://doi.org/10.1021/acssynbio.9b00140.
Cui, S., Xia, H., Chen, T., Gu, Y., Lv, X., Liu, Y., Du, G., Liu, L., 2020. Cell membrane and electron transfer engineering for improved synthesis of menaquinone-7 in *Bacillus subtilis*. iScience 23 (3). https://doi.org/10.1016/j.isci.2020.100918.
Daletos, G., Katsimpouras, C., Stephanopoulos, G., 2020. Novel strategies and platforms for industrial isoprenoid engineering. Trends Biotechnol. 38 (7), 811–822. https://doi.org/10.1016/j.tibtech.2020.03.009.
Deay 3rd, D.O., Colvert, K.K., Gao, F., Seibold, S., Goyal, P., Richter, M.L., 2020. An active site mutation in 6-hydroxy-l-Nicotine oxidase from *Arthrobacter nicotinovorans* changes the substrate specificity in favor of (S)-nicotine. Arch. Biochem. Biophys. 692, 108520 https://doi.org/10.1016/j.abb.2020.108520.
Deng, C., Lv, X., Liu, Y., Li, J., Lu, W., Du, G., Liu, L., 2019a. Metabolic engineering of *Corynebacterium glutamicum* S9114 based on whole-genome sequencing for efficient N-acetylglucosamine synthesis. Synth. Syst. Biotechnol. 4 (3), 120–129. https://doi.org/10.1016/j.synbio.2019.05.002n.
Deng, J., Chen, C., Gu, Y., Lv, X., Liu, Y., Li, J., Ledesma-Amaro, R., Du, G., Liu, L., 2019b. Creating an *in vivo* bifunctional gene expression circuit through an aptamer-based regulatory mechanism for dynamic metabolic engineering in *Bacillus subtilis*. Metab. Eng. 55, 179–190. https://doi.org/10.1016/j.ymben.2019.07.008.
Donnelly, A.E., Murphy, G.S., Digianantonio, K.M., Hecht, M.H., 2018. A de novo enzyme catalyzes a life-sustaining reaction in *Escherichia coli*. Nat. Chem. Biol. 14 (3), 253–255. https://doi.org/10.1038/nchembio.2550.
Dudley, Q.M., Anderson, K.C., Jewett, M.C., 2016. Cell-Free mixing of *Escherichia coli* crude extracts to prototype and rationally engineer high-titer mevalonate synthesis. ACS Synth. Biol. 5 (12), 1578–1588. https://doi.org/10.1021/acssynbio.6b00154.
Dueber, J.E., Wu, G.C., Malmirchegini, G.R., Moon, T.S., Petzold, C.J., Ullal, A.V., Prather, K.L.J., Keasling, J.D., 2009. Synthetic protein scaffolds provide modular control over metabolic flux. Nat. Biotechnol. 27 (8), 753–759. https://doi.org/10.1038/nbt.1557.
Espeche, J.C., Martínez, M., Maturana, P., Cutró, A., Semorile, L., Maffia, P.C., Hollmann, A. 2020. Unravelling the mechanism of action of "de novo" designed peptide P1 with model membranes and gram-positive and gram-negative bacteria. Archives of Biochemistry and Biophysics, 693 10.1016/j.abb.2020.108549.
Feng, Z., Zhang, J., Chen, G., Ge, Y., Zhang, X., Zhu, H., 2019. Extracellular expression of L-aspartate-α-decarboxylase from *Bacillus tequilensis* and its application in the biosynthesis of β-alanine. Appl. Biochem. Biotechnol. 189 (1), 273–283. https://doi.org/10.1007/s12010-019-03013-1.
Fu, J., Yang, Y.R., Johnson-Buck, A., Liu, M., Liu, Y., Walter, N.G., Woodbury, N.W., Yan, H., 2014. Multi-enzyme complexes on DNA scaffolds capable of substrate channelling with an artificial swinging arm. Nat. Nanotechnol. 9 (7), 531–536. https://doi.org/10.1038/nnano.2014.100.
Gargiulo, S., Soumillion, P., 2021. Directed evolution for enzyme development in biocatalysis. Curr. Opin. Chem. Biol. 61, 107–113. https://doi.org/10.1016/j.cbpa.2020.11.006.
Geiselman, G.M., Zhuang, X., Kirby, J., Tran-Gyamfi, M.B., Prahl, J.P., Sundstrom, E.R., Gao, Y., Munoz Munoz, N., Nicora, C.D., Clay, D.M., Papa, G., Burnum-Johnson, K. E., Magnuson, J.K., Tanjore, D., Skerker, J.M., Gladden, J.M., 2020. Production of ent-kaurene from lignocellulosic hydrolysate in *Rhodosporidium toruloides*. Microb. Cell Fact. 19 (1), 24. https://doi.org/10.1186/s12934-020-1293-8.
Gong, J.-S., Ye, J.-P., Tao, L.-Y., Su, C., Qin, J., Zhang, Y.-Y., Li, H., Li, H., Xu, Z.-H., Shi, J.-S., 2020a. Efficient keratinase expression via promoter engineering strategies for degradation of feather wastes. Enzyme Microb. Technol. 137 https://doi.org/10.1016/j.enzmictec.2020.109550.
Gong, L., Xu, G., Cao, X., Han, R., Dong, J., Ni, Y., 2020b. High-Throughput screening method for directed evolution and characterization of aldol activity of D-Threonine Aldolase. Appl. Biochem. Biotechnol. https://doi.org/10.1007/s12010-020-03447-y.
Grewal, P.S., Samson, J.A., Baker, J.J., Choi, B., Dueber, J.E., 2020. Peroxisome compartmentalization of a toxic enzyme improves alkaloid production. Nat. Chem. Biol. https://doi.org/10.1038/s41589-020-00668-4.
Gu, Y., Ma, J., Zhu, Y., Xu, P., 2020. Refactoring ehrlich pathway for high-yield 2-phenylethanol production in *Yarrowia lipolytica*. ACS Synth. Biol. 9 (3), 623–633. https://doi.org/10.1021/acssynbio.9b00468.
Guirimand, G., Kulagina, N., Papon, N., Hasunuma, T., Courdavault, V., 2020. Innovative tools and strategies for optimizing yeast cell factories. Trends Biotechnol. https://doi.org/10.1016/j.tibtech.2020.08.010.
Guo, L., Diao, W., Gao, C., Hu, G., Ding, Q., Ye, C., Chen, X., Liu, J., Liu, L., 2020. Engineering *Escherichia coli* lifespan for enhancing chemical production. Nat. Catal. 3 (3), 307–318. https://doi.org/10.1021/jf500355p.
Haringa, C., 2017. Euler-Lagrange analysis towards representative down-scaling of a 22 m3 aerobic *S. cerevisiae* fermentation. Chem. Eng. Sci. 170, 653–669. https://doi.org/10.1016/j.ces.2017.01.014.
Hohenschuh, W., Hector, R.E., Chaplen, F., Murthy, G.S., 2021. Using high-throughput data and dynamic flux balance modeling techniques to identify points of constraint in xylose utilization in *Saccharomyces cerevisiae*. Systems Microbiology and Biomanufacturing 1, 58–75. https://doi.org/10.1007/s43393-020-00003-x.
Hossain, G.S., Saini, M., Miyake, R., Ling, H., Chang, M.W., 2020. Genetic biosensor design for natural product biosynthesis in microorganisms. Trends Biotechnol. 38 (7), 797–810. https://doi.org/10.1016/j.tibtech.2020.03.013.
Huang, H., Liang, Q., Wang, Y., Chen, J., Kang, Z., 2020. High-level constitutive expression of leech hyaluronidase with combined strategies in recombinant *Pichia pastoris*. Appl. Microbiol. Biotechnol. 104 (4), 1621–1632. https://doi.org/10.1007/s00253-019-10282-7.
Huang, M., Wang, G., Qin, J., Petranovic, D., Nielsen, J., 2018. Engineering the protein secretory pathway of *Saccharomyces cerevisiae* enables improved protein production. Proc. Natl. Acad. Sci. 115 (47), E11025–E11032. https://doi.org/10.1073/pnas.1809921115.
Huang, P.-S., Boyken, S.E., Baker, D., 2016. The coming of age of *de novo* protein design. Nature 537 (7620), 320–327. https://doi.org/10.1038/nature19946.
Huttanus, H.M., Senger, R.S., 2020. A synthetic biosensor to detect peroxisomal acetyl-CoA concentration for compartmentalized metabolic engineering. PeerJ 8. https://doi.org/10.7717/peerj.9805.
Islam, M.R., Choi, S., Muthamilselvan, T., Shin, K., Hwang, I., 2020. *In vivo* removal of N-terminal fusion domains from recombinant target proteins produced in *Nicotiana benthamiana*. Front. Plant Sci. 11, 440. https://doi.org/10.3389/fpls.2020.00440.
Kang, W., Ma, T., Liu, M., Qu, J., Liu, Z., Zhang, H., Shi, B., Fu, S., Ma, J., Lai, L.T.F., He, S., Qu, J., Wing-Ngor Au, S., Ho Kang, B., Yu Lau, W.C., Deng, Z., Xia, J., Liu, T. 2019. Modular enzyme assembly for enhanced cascade biocatalysis and metabolic flux. Nature Communications, 10 (1) 10.1038/s41467-019-12247-w.
Kang, Z., Zhang, N., Zhang, Y., 2015. Enhanced production of leech hyaluronidase by optimizing secretion and cultivation in *Pichia pastoris*. Appl. Microbiol. Biotechnol. 100 (2), 707–717. https://doi.org/10.1007/s00253-015-7056-5.
Kim, G.B., Kim, W.J., Kim, H.U., Lee, S.Y., 2020. Machine learning applications in systems metabolic engineering. Curr. Opin. Biotechnol. 64, 1–9. https://doi.org/10.1016/j.copbio.2019.08.010.
Kim, J.-E., Jang, I.-S., Son, S.-H., Ko, Y.-J., Cho, B.-K., Kim, S.C., Lee, J.Y., 2019. Tailoring the *Saccharomyces cerevisiae* endoplasmic reticulum for functional assembly of terpene synthesis pathway. Metab. Eng. 56, 50–59. https://doi.org/10.1016/j.ymben.2019.08.013.
Kitade, Y., Hashimoto, R., Suda, M., Hiraga, K., Inui, M., Kivisaar, M., 2018. Production of 4-hydroxybenzoic acid by an aerobic growth-arrested bioprocess using metabolically engineered coryne bacterium glutamicum. Appl. Environ. Microbiol. 84 (6) https://doi.org/10.1128/AEM.02587-17.
Konishi, A., Hisayoshi, T., Yokokawa, K., Barrioluengo, V., Menéndez-Arias, L., Yasukawa, K., 2014. Amino acid substitutions away from the RNase H catalytic site increase the thermal stability of *Moloney murine leukemia virus* reverse transcriptase through RNase H inactivation. Biochem. Biophys. Res. Commun. 454 (2), 269–274. https://doi.org/10.1016/j.bbrc.2014.10.044.
Kumar Awasthi, M., Ravindran, B., Sarsaiya, S., Chen, H., Wainaina, S., Singh, E., Liu, T., Kumar, S., Pandey, A., Singh, L., Zhang, Z. 2020. Metagenomics for taxonomy profiling: tools and approaches. Bioengineered, 11 (1), 356–374 10.1080/21655979.2020.1736238.
Kurahashi, R., Tanaka, S.-I., Takano, K., 2020. Highly active enzymes produced by directed evolution with stability-based selection. Enzyme Microb. Technol. 140 https://doi.org/10.1016/j.enzmictec.2020.109626.
Langan, R.A., Boyken, S.E., Ng, A.H., Samson, J.A., Dods, G., Westbrook, A.M., Nguyen, T.H., Lajoie, M.J., Chen, Z., Berger, S., Mulligan, V.K., Dueber, J.E., Novak, W.R.P., El-Samad, H., Baker, D., 2019. *De novo* design of bioactive protein switches. Nature 572 (7768), 205–210. https://doi.org/10.1038/s41586-019-1432-8.
Larroude, M., Celinska, E., Back, A., Thomas, S., Nicaud, J.-M., Ledesma-Amaro, R., 2018. A synthetic biology approach to transform *Yarrowia lipolytica* into a competitive biotechnological producer of *β*-carotene. Biotechnol. Bioeng. 115 (2), 464–472. https://doi.org/10.1002/bit.26473.
Lee, M.J., Palmer, D.J., Warren, M.J., 2019. Biotechnological advances in bacterial microcompartment technology. Trends Biotechnol. 37 (3), 325–336. https://doi.org/10.1016/j.tibtech.2018.08.006.
Li, C., Zhang, R., Wang, J., Wilson, L.M., Yan, Y., 2020. Protein engineering for improving and diversifying natural product biosynthesis. Trends Biotechnol. 38 (7), 729–744. https://doi.org/10.1016/j.tibtech.2019.12.008.
Liu, G.-S., Li, T., Zhou, W., Jiang, M., Tao, X.-Y., Liu, M., Zhao, M., Ren, Y.-H., Gao, B., Wang, F.-Q., Wei, D.-Z., 2020. The yeast peroxisome: A dynamic storage depot and subcellular factory for squalene overproduction. Metab. Eng. 57, 151–161. https://doi.org/10.1016/j.ymben.2019.11.001.
Liu, H., Marsafari, M., Wang, F., Deng, L., Xu, P., 2019. Engineering acetyl-CoA metabolic shortcut for eco-friendly production of polyketides triacetic acid lactone in *Yarrowia lipolytica*. Metab. Eng. 56, 60–68. https://doi.org/10.1016/j.ymben.2019.08.017.
Lo, T.-M., Chng, S.H., Teo, W.S., Cho, H.-S., Chang, M.W., 2016. A two-layer gene circuit for decoupling cell growth from metabolite production. Cell Systems 3 (2), 133–143. https://doi.org/10.1016/j.cels.2016.07.012.
Luo, S., Chen, X.-A., Mao, X.-M., Li, Y.-Q., 2018. Transposon-based identification of a negative regulator for the antibiotic hyper-production in *Streptomyces*. Appl. Microbiol. Biotechnol. 102 (15), 6581–6592. https://doi.org/10.1007/s00253-018-9103-5.
Lv, X., Wu, Y., Tian, R., Gu, Y., Liu, Y., Li, J., Du, G., Ledesma-Amaro, R., Liu, L., 2020. Synthetic metabolic channel by functional membrane microdomains for compartmentalized flux control. Metab. Eng. 59, 106–118. https://doi.org/10.1016/j.ymben.2020.02.003.
Ma, J., Lei, H.-T., Reyes, F.E., Sanchez-Martinez, S., Sarhan, M.F., Hattne, J., Gonen, T., 2019a. Structural basis for substrate binding and specificity of a sodium–alanine symporter AgcS. PNAS 116, 2086–2090. https://doi.org/10.1073/pnas.1806206116.
Ma, C., Tan, Z.L., Lin, Y., Han, S., Xing, X., Zhang, C., 2019b. Gel microdroplet–based high-throughput screening for directed evolution of xylanase-producing *Pichia pastoris*. J. Biosci. Bioeng. 128 (6), 662–668. https://doi.org/10.1016/j.jbiosc.2019.05.008.
Madhavan, A., Arun, K.B., Binod, P., Sirohi, R., Reshmy, R., Kumar Awasthi, M., Sindhu, R., 2021. Design of novel enzyme biocatalysts for industrial bioprocess: Harnessing the power of protein engineering, high throughput screening and

synthetic biology. Bioresour. Technol. 325 https://doi.org/10.1016/j.biortech.2020.124617.
Massaiu, I., Pasotti, L., Sonnenschein, N., Rama, E., Cavaletti, M., Magni, P., Calvio, C., Herrgård, M.J., 2019. Integration of enzymatic data in *Bacillus subtilis* genome-scale metabolic model improves phenotype predictions and enables *in silico* design of poly-γ-glutamic acid production strains. Microb. Cell Fact. 18 (1) https://doi.org/10.1186/s12934-018-1052-2.
Mauro, V.P., 2018. Codon optimization in the production of recombinant biotherapeutics: potential risks and considerations. BioDrugs 32 (1), 69–81. https://doi.org/10.1007/s40259-018-0261-x.
Maxel, S., King, E., Zhang, Y., Luo, R., Li, H., 2020. Leveraging oxidative stress to regulate redox balance-based, *in vivo* growth selections for oxygenase engineering. ACS Synth. Biol. https://doi.org/10.1021/acssynbio.0c00380.
Mehboob, S., Ahmad, N., Munir, S., Ali, R., 2020. Gene cloning, expression enhancement in *Escherichia coli* and biochemical characterization of a highly thermostable amylomaltase from *Pyrobaculum calidifontis*. Int. J. Biol. Macromol. https://doi.org/10.1016/j.ijbiomac.2020.09.071.
Musil, M., Konegger, H., Hon, J., Bednar, D., Damborsky, J., 2018. Computational design of stable and soluble biocatalysts. ACS Catal. 9 (2), 1033–1054. https://doi.org/10.1021/acscatal.8b03613.
Nanda, V., Koder, R.L., 2009. Designing artificial enzymes by intuition and computation. Nat. Chem. 2 (1), 15–24. https://doi.org/10.1038/nchem.473.
Ng, A.H., Nguyen, T.H., Gómez-Schiavon, M., Dods, G., Langan, R.A., Boyken, S.E., Samson, J.A., Waldburger, L.M., Dueber, J.E., Baker, D., El-Samad, H., 2019. Modular and tunable biological feedback control using a *de novo* protein switch. Nature 572 (7768), 265–269. https://doi.org/10.1038/s41586-019-1425-7.
Ng, D.T.W., Sarkar, C.A., 2013. Engineering signal peptides for enhanced protein secretion from *Lactococcus lactis*. Appl. Environ. Microbiol. 79 (1), 347–356. https://doi.org/10.1128/AEM.02667-12.
Noh, H., Lee, S.J., Jo, H.-J., Choi, H.W., Hong, S., Kong, K.-H. 2020. Histidine residues at the copper-binding site in human tyrosinase are essential for its catalytic activities. Journal of Enzyme Inhibition and Medicinal Chemistry, 35(1), 726-732 10.1080/14756366.2020.1740691.
Oyetunde, T., Liu, D., Martin, H.G., Tang, Y.J., 2019. Machine learning framework for assessment of microbial factory performance. PLoS ONE 14 (1), e0210558. https://doi.org/10.1371/journal.pone.0210558.
Patel, A.K., Choi, Y.Y., Sim, S.J., 2020. Emerging prospects of mixotrophic microalgae: Way forward to sustainable bioprocess for environmental remediation and cost-effective biofuels. Bioresour. Technol. 300 https://doi.org/10.1016/j.biortech.2020.122741.
Pigou, M., Morchain, J., 2015. Investigating the interactions between physical and biological heterogeneities in bioreactors using compartment, population balance and metabolic models. Chemical Engineering Science 126, 267–282. https://doi.org/10.1016/j.ces.2014.11.035.
Rangel, A.E.T., Gómez Ramírez, J.M., González Barrios, A.F., 2020. From industrial by-products to value-added compounds: the design of efficient microbial cell factories by coupling systems metabolic engineering and bioprocesses. Biofuels, Bioprod. Biorefin. https://doi.org/10.1002/bbb.2127.
Reifenrath, M., Oreb, M., Boles, E., Tripp, J., 2020. Artificial ER-derived vesicles as synthetic organelles for *in vivo* compartmentalization of biochemical pathways. ACS Synth. Biol. https://doi.org/10.1021/acssynbio.0c00241.
Robert, V., Bucheton, A., 2004. Regulation of the expression of repeated sequences and RNA interference. Medical Science (Paris) 20 (8–9), 767–772. https://doi.org/10.1051/medsci/2004208-9767.
Roca-Pinilla, R., López-Cano, A., Saubi, C., Garcia-Fruitós, E., Arís, A., 2020. A new generation of recombinant polypeptides combines multiple protein domains for effective antimicrobial activity. Microb. Cell Fact. 19 (1) https://doi.org/10.1186/s12934-020-01380-7.
Rodriguez-Granrose, D., Jones, A., Loftus, H., Tandeski, T., Heaton, W., Foley, K.T., Silverman, L., 2021. Design of experiment (DOE) applied to artificial neural network architecture enables rapid bioprocess improvement. Bioprocess Biosyst. Eng. 27 (2) https://doi.org/10.1007/s00449-021-02529-3.
Schroeder, W.L., Saha, R., 2020. OptFill: A tool for infeasible cycle-free gapfilling of stoichiometric metabolic models. iScience 23 (1). https://doi.org/10.1016/j.isci.2019.100783.
Shen, B., Zhou, P., Jiao, X., 2020. Fermentative production of Vitamin E tocotrienols in *Saccharomyces cerevisiae* under cold-shock-triggered temperature control. *Nature*. Communications 11 (1). https://doi.org/10.1038/s41467-020-18958-9.
Sheng, M., Liu, B., Xu, J., Peng, Q., Zhang, L., Chen, K., He, J., 2020. Cloning of a novel topramezone-resistant 4-hydroxyphenylpyruvate dioxygenase gene and improvement of its resistance through pressure acclimation. Enzyme Microb. Technol. 140 https://doi.org/10.1016/j.enzmictec.2020.109642.
Shu, W., Zheng, H., Fu, X., Zhen, J., Tan, M., Xu, J., Zhao, X., Yang, S., Song, H., Ma, Y., 2020. Enhanced heterologous production of glycosyltransferase ugt76g1 by co-expression of endogenous *prpD* and *malK* in *Escherichia coli* and its transglycosylation application in production of rebaudioside. Int. J. Mol. Sci. 21 (16) https://doi.org/10.3390/ijms21165752.
Singha, T.K., Gulati, P., Mohanty, A., Khasa, Y.P., Kapoor, R.K., Kumar, S., 2017. Efficient genetic approaches for improvement of plasmid based expression of recombinant protein in *Escherichia coli*: A review. Process Biochem. 55, 17–31. https://doi.org/10.1016/j.procbio.2017.01.026.
Su'etsugu, M., Takada, T., Tsujimoto, H., 2017. Exponential propagation of large circular DNA by reconstitution of a chromosome-replication cycle. Nucleic Acids Res. 45 (20), 11525–11534. https://doi.org/10.1093/nar/gkx822.
Tachioka, M., Sugimoto, N., Nakamura, A., Sunagawa, N., Ishida, T., Uchiyama, T., Igarashi, K., Samejima, M., 2016. Development of simple random mutagenesis protocol for the protein expression system in *Pichia pastoris*. Biotechnol. Biofuels 9 (1). https://doi.org/10.1186/s13068-016-0613-z.
Tandar, S.T., Senoo, S., Toya, Y., Shimizu, H., 2019. Optogenetic switch for controlling the central metabolic flux of *Escherichia coli*. Metab. Eng. 55, 68–75. https://doi.org/10.1016/j.ymben.2019.06.002.
Tan, J., Zhang, F., Karcher, D., Bock, R., 2019. Engineering of high-precision base editors for site-specific single nucleotide replacement. *Nature*. Communications 10 (1). https://doi.org/10.1038/s41467-018-08034-8.
Tian, R., Liu, Y., Chen, J., Li, J., Liu, L., Du, G., Chen, J., 2019. Synthetic N-terminal coding sequences for fine-tuning gene expression and metabolic engineering in *Bacillus subtilis*. Metab. Eng. 55, 131–141. https://doi.org/10.1016/j.ymben.2019.07.001.
Trantidou, T., Friddin, M., Elani, Y., Brooks, N.J., Law, R.V., Seddon, J.M., Ces, O., 2017. Engineering compartmentalized biomimetic micro- and nanocontainers. ACS Nano 11 (7), 6549–6565. https://doi.org/10.1021/acsnano.7b03245.
Udugama, I.A., Lopez, P.C., Gargalo, C.L., Li, X., Bayer, C., Gernaey, K.V., 2021. Digital Twin in biomanufacturing: challenges and opportunities towards its implementation. Syst. Microbiol. Biomanufactur. https://doi.org/10.1007/s43393-021-00024-0.
Vadhana, A.K.P., Samuel, P., Berin, R.M., Krishna, J., Kamatchi, K., Meenakshisundaram, S., 2013. Improved secretion of *Candida antarctica* lipase B with its native signal peptide in *Pichia pastoris*. Enzyme Microb. Technol. 52 (3), 177–183. https://doi.org/10.1016/j.enzmictec.2013.01.001.
van Rossum, T., Kengen, S.W.M., van der Oost, J., 2013. Reporter-based screening and selection of enzymes. FEBS J. 280 (13), 2979–2996. https://doi.org/10.1111/febs.12281.
Wang, L., Deng, A., Zhang, Y., Liu, S., Liang, Y., Bai, H., Cui, D., Qiu, Q., Shang, X., Yang, Z., He, X., Wen, T., 2018. Efficient CRISPR–Cas9 mediated multiplex genome editing in yeasts. Biotechnol. Biofuels 11 (1). https://doi.org/10.1186/s13068-018-1271-0.
Wang, M., Si, T., Zhao, H., 2012. Biocatalyst development by directed evolution. Bioresour. Technol. 115, 117–125. https://doi.org/10.1016/j.biortech.2012.01.054.
Wang, G., Haringa, C., Noorman, H., Chu, J., Zhuang, Y., 2020a. Developing a computational framework to advance bioprocess scale-up. Trends Biotechnol. 38 (8), 846–856. https://doi.org/10.1016/j.tibtech.2020.01.009.
Wang, Y., Hu, L., Huang, H., Wang, H., Zhang, T., Chen, J., Du, G., Kang, Z., 2020b. Eliminating the capsule-like layer to promote glucose uptake for hyaluronan production by engineered *Corynebacterium glutamicum*. *Nature*. Communications 11 (1). https://doi.org/10.1038/s41467-020-16962-7.
Wang, Z., Chen, T., Ma, X., Shen, Z., Zhao, X., 2011. Enhancement of riboflavin production with *Bacillus subtilis* by expression and site-directed mutagenesis of *zwf* and *gnd* gene from *Corynebacterium glutamicum*. Bioresour. Technol. 102 (4), 3934–3940. https://doi.org/10.1016/j.biortech.2010.11.120.
Wei, Q., He, S., Qu, J., Xia, J., 2020. Synthetic multienzyme complexes assembled on virus-like particles for cascade biosynthesis in cellulo. Bioconjug. Chem. 31 (10), 2413–2420. https://doi.org/10.1021/acs.bioconjchem.0c00476.
Venkatasubramanian, V., 2019. The promise of artificial intelligence in chemical engineering: Is it here, finally? AIChE J. 65, 466–478. https://doi.org/10.1002/aic.16489.
Wu, S.G., Wang, Y., Jiang, W., Oyetunde, T., Yao, R., Zhang, X., Shimizu, K., Tang, Y.J., Bao, F.S., 2016. Rapid prediction of bacterial heterotrophic fluxomics using machine learning and constraint programming. PLoS Comput. Biol. 12, e1004838 https://doi.org/10.1371/journal.pcbi.1004838.
Wu, Y., Chen, T., Liu, Y., Tian, R., Lv, X., Li, J., Du, G., Chen, J., Ledesma-Amaro, R., Liu, L., 2020. Design of a programmable biosensor-CRISPR*i* genetic circuits for dynamic and autonomous dual-control of metabolic flux in *Bacillus subtilis*. Nucleic Acids Res. 48 (2), 996–1009. https://doi.org/10.1093/nar/gkz1123.
Xu, N., Wei, L., Liu, J., 2019a. Recent advances in the applications of promoter engineering for the optimization of metabolite biosynthesis. World J. Microbiol. Biotechnol. 35 (2) https://doi.org/10.1007/s11274-019-2606-0.
Xu, N., Liu, Y., Jiang, H., Liu, J., Ma, Y., 2020a. Combining protein and metabolic engineering to construct efficient microbial cell factories. Curr. Opin. Biotechnol. 66, 27–35. https://doi.org/10.1016/j.copbio.2020.06.001.
Xu, X., Li, X., Liu, Y., Zhu, Y., Li, J., Du, G., Chen, J., Ledesma-Amaro, R., Liu, L., 2020b. Pyruvate-responsive genetic circuits for dynamic control of central metabolism. Nat. Chem. Biol.
Xu, X., Liu, Y., Du, G., Ledesma-Amaro, R., Liu, L., 2020c. Microbial chassis development for natural product biosynthesis. Trends Biotechnol. 38 (7), 779–796. https://doi.org/10.1016/j.tibtech.2020.01.002.
Xu, Z., Wang, T., Zhang, S., 2019b. Extracellular secretion of feruloyl esterase derived from *Lactobacillus crispatus* in *Escherichia coli* and its application for ferulic acid production. Bioresour. Technol. 288 https://doi.org/10.1016/j.biortech.2019.121526.
Yang, J.E., Park, S.J., Kim, W.J., Kim, H.J., Kim, B.J., Lee, H., Shin, J., Lee, S.Y., 2018. One-step fermentative production of aromatic polyesters from glucose by metabolically engineered *Escherichia coli* strains. Nat. Commun. 9 (1) https://doi.org/10.1038/s41467-017-02498-w.
Yang, X., Nambou, K., Wei, L., Hua, Q., 2016. Heterologous production of α-farnesene in metabolically engineered strains of *Yarrowia lipolytica*. Bioresour. Technol. 216, 1040–1048. https://doi.org/10.1016/j.biortech.2016.06.028.
Yu, J., Peng, J., Chi, H., 2019. Systems immunology: Integrating multi-omics data to infer regulatory networks and hidden drivers of immunity. Current Opinion in Systems Biology 15, 19–29. https://doi.org/10.1016/j.coisb.2019.03.003.
Yu, T., Zhou, Y.J., Huang, M., Liu, Q., Pereira, R., David, F., Nielsen, J., 2018. Reprogramming yeast metabolism from alcoholic fermentation to lipogenesis. Cell 174 (6), 1549–1558.e14. https://doi.org/10.1016/j.cell.2018.07.013.

Yuan, S.-F., Yi, X., Johnston, T.G., Alper, H.S., 2020. *De novo* resveratrol production through modular engineering of an *Escherichia coli–Saccharomyces cerevisiae* co-culture. Microb. Cell Fact. 19 (1) https://doi.org/10.1186/s12934-020-01401-5.

Yuan, J., Ching, C.-B., 2016. Mitochondrial acetyl-CoA utilization pathway for terpenoid productions. Metab. Eng. 38, 303–309. https://doi.org/10.1016/j.ymben.2016.07.008.

Zarzhitsky, S., Jiang, A., Stanley, E., Hecht, M., 2020. Harnessing synthetic biology to enhance heterologous protein expression. Protein Sci. 29 (8), 1698–1706. https://doi.org/10.1002/pro.3907.

Zhang, C., Li, Y., Zhu, F., Li, Z., Lu, N., Li, Y., Xu, Q., Chen, N., 2020a. Metabolic engineering of an auto-regulated *Corynebacterium glutamicum* chassis for biosynthesis of 5-aminolevulinic acid. Bioresour. Technol. 318 https://doi.org/10.1016/j.biortech.2020.124064.

Zhang, J., Petersen, S.D., Radivojevic, T., Ramirez, A., Pérez-Manríquez, A., Abeliuk, E., Sánchez, B.J., Costello, Z., Chen, Y., Fero, M.J., Martin, H.G., Nielsen, J., Keasling, J. D., Jensen, M.K., 2020b. Combining mechanistic and machine learning models for predictive engineering and optimization of tryptophan metabolism. *Nature*. Communications 11 (1). https://doi.org/10.1038/s41467-020-17910-1.

Zhang, L., Jiang, C., Chen, X., Gu, J., Song, Q., Zhong, H., Xiong, S., Dong, Q., Yu, J.C., Deng, N., 2020c. Large-scale production, purification, and function of a tumor multi-epitope vaccine: Peptibody with bFGF/VEGFA. Eng. Life Sci. 20 (9–10), 422–436. https://doi.org/10.1002/elsc.202000020.

Zhao, X., Cebrián, R., Fu, Y., Rink, R., 2020. High-Throughput screening for substrate specificity-adapted mutants of the nisin dehydratase NisB. ACS Synthetic Biology 9 (6), 1468–1478. https://doi.org/10.1021/acssynbio.0c00130.

Zhang, X., Liu, Y., Liu, L., Li, J., 2019. Microbial production of sialic acid and sialylated human milk oligosaccharides: Advances and perspectives. Biotechnol. Adv. 37 (5), 787–800. https://doi.org/10.1016/j.biotechadv.2019.04.011.

Zhang, Y., Cai, J., Shang, X., Wang, B., Liu, S., Chai, X., Tan, T., Zhang, Y., Wen, T., 2017. A new genome-scale metabolic model of *Corynebacterium glutamicum* and its application. Biotechnology for Biofuels 10 (1). https://doi.org/10.1186/s13068-017-0856-3.

Zhou, Q., Ning, S., Luo, Y., 2020. Coordinated regulation for nature products discovery and overproduction in Streptomyces. Synth. Syst. Biotechnol. 5 (2), 49–58. https://doi.org/10.1016/j.synbio.2020.04.002.

Zhu, L., Wu, D., Zhang, H., Li, Q., Zhang, Z., Liu, Y., Zhou, S., Wang, W., Li, Z., 2019. Effects of atmospheric and room temperature plasma (artp) mutagenesis on physicochemical characteristics and immune activity *in vitro* of hericium erinaceus polysaccharides. Molecules 24 (2). https://doi.org/10.3390/molecules24020262.