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Microbial production of chemicals driven by CRISPR-Cas systems

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Microorganisms have provided an attractive route for biosynthesis of various chemicals from renewable resources. CRISPR-Cas systems have served as powerful mechanisms for generating cell factories with desirable properties by manipulating nucleic acids quickly and efficiently. The CRISPR-Cas system provides a toolbox with excellent opportunities for identifying better biocatalysts, multiplexed fine-tuning of metabolic flux, efficient utilization of low-cost substrates, and improvement of metabolic robustness. The overall goal of this review highlights recent advances in the development of microbial cell factories for chemical production using various CRISPR-Cas systems. The perspectives for further development or applications of CRISPR-Cas systems for strain improvement are also discussed.

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Introduction

Microbial fermentation has been successfully introduced for the commercial production of many chemicals [1,2], such as citric acid and 1,3-propanediol. This strategy has drawn increasing attention because of its feasibility, convenient manipulation, outstanding cost performance and fewer greenhouse gas emissions. In this context the Design–Build–Test–Learn (DBTL) cycle is indispensable for the construction of an advanced cell factory [2,3], which always needs several rounds of engineering due to the complexity of cellular metabolism. Traditionally 'Build' has been seen as a rate-limiting step in the DBTL cycle, as it is generally time consuming and expensive to build engineered strains. Advances in strain engineering technologies in the last decade has, however, reduced time and costs associated with the construction of efficient cell factories.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems are progressing rapidly and have become the gold standard technology for genetic and metabolic engineering [4], thanks to easy-to-design, high specificity, and high functional genome-editing efficiency. Now CRISPR-based toolbox has been demonstrated to implement most of the genetic manipulations in high efficiency, and is therefore providing a remarkable solution for construction desirable cell factories (Figure 1).

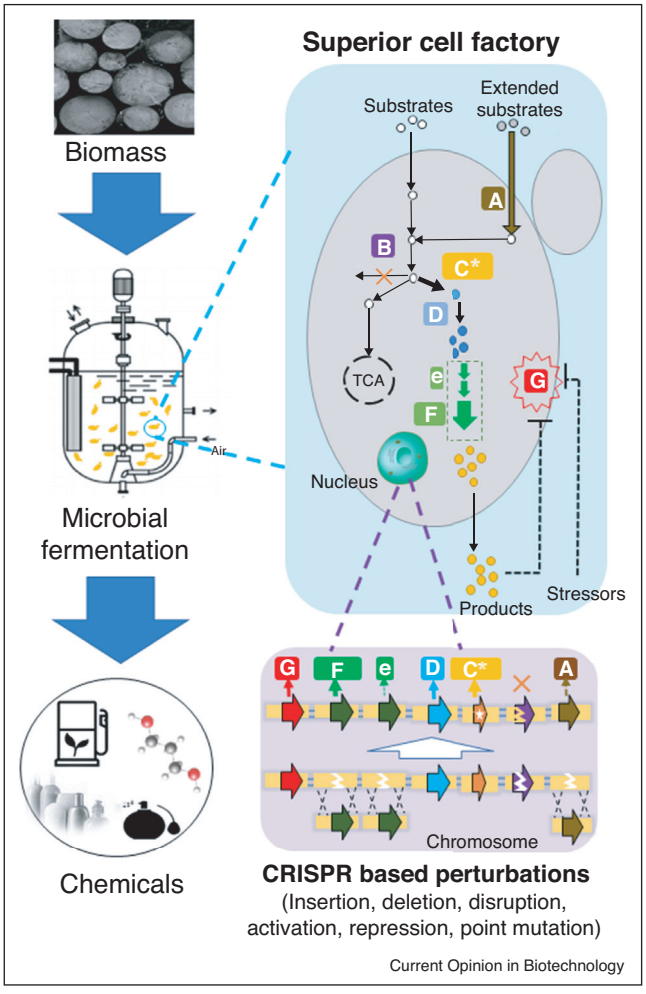
CRISPR-Cas systems originate as a heritable adaptive immunity reaction of bacteria and archaea, by introducing a double-strand break (DSB) at a specific target site complementary to crRNA or single-guide RNA (gRNA). As shown in Figure 2, the DSB will enable genetic insertions, deletions, or replacement through intrinsic DNA repair processes; what is more, dCas (nuclease-deficient Cas protein) can serve as a sequence recognizer without introducing a DSB, and flexible transcriptional regulation can be achieved by recruiting effector domains to the nuclease-deficient CRISPR complex [5]. More advanced functions for targeted modifications have been empowered by other functional effectors, such as methylation by fusion to methyltransferase [6] and base editing by fusion to deaminases [7••]. The establishment of a CRISPR-Cas system with orthogonal functions has emerged as a powerful and versatile tool to implement the combinatorial and multiplex modifications required for constructing a superior cell factory [8–10].

The advanced CRISPR-Cas system has been shown to revolutionize strain construction faster and more reliably in many ways (Table 1), such as pathway construction and optimization, enzyme engineering and evolution, extending the substrate scope, and metabolic robustness improvement. This review focuses on the recent advances and immediate challenges for creating efficient cell factories for chemical production driven by CRISPR-Cas systems.

Assembly of biosynthetic pathways for target chemical production

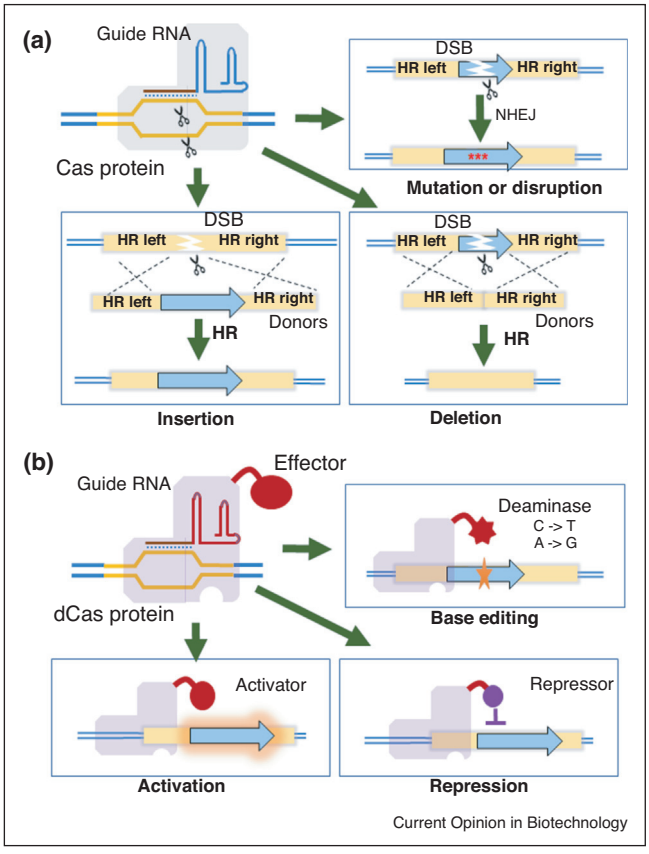
There are many chemicals that can be produced via microbes [11]. However, some chemicals are non-natural

Figure 1



CRISPR-based perturbations for the development of advanced microbial cell factories for production of various chemicals. The construction of a superior cell factory is the key for industrial biotechnology process, which required combinatorial and multiplex modifications in cell metabolisms, such as insertion, deletion, disruption, point mutation, activation, repression. CRISPR-Cas system has been demonstrated to implement most of the genetic manipulations from the enzyme to genome level, which was determined by the pool of gRNA used.

Figure 2



Scheme of the CRISPR-Cas systems for genome editing and regulation.
(a) Basic applications of the CRISPR-Cas system for DSB-based genome editing, including gene deletion, mutation or disruption, insertion. In this system, a Cas nuclease (i.e. Cas9) binds to a specific site in the genome guided by gRNA, and generates a DSB. Then desired genome editing could be achieved by HR or NHEJ. Donors are required when precise editing is conducted by HR. **(b)** Advanced applications of the CRISPR-dCas system for non-DSB-based genome editing and regulation, including base editing, transcriptional activation (CRISPRa) and repression (CRISPRi). In this system, a dCas (dead Cas or nuclease-deficient Cas) is obtained by mutating a Cas nuclease, which can still recognize the binding sites but without the formation of DSB. Then, a customized function can be achieved by recruiting a desired effector (e.g. deaminase). DSB, double-strand break; HR, homology repair; NHEJ, non-homologous end joining.

to the host, and heterologous pathways must be established in the first step. The majority of previous endeavors employed non-integrative plasmids, but suffered from inherent problems of clonal variations, instability, and selection pressure requirements. In view of these limitations, as indicated in Figure 3a, the CRISPR-Cas system greatly facilitates the assembly, or knock-in, of heterologous pathways in chromosomes for stable expression of pathway genes [12,13]. It has been shown that the integration of large synthetic pathways into chromosomes of *Escherichia coli* for the production of 5-methylpyrazine-2-carboxylic acid (MPCA), and the integrated copy number of *xylM* and *xylA* could be adjusted by selecting different integration sites, improving the production of MPCA to 15.6 g/L [13].

Recently, six desirable intergenic loci were screened and engineering to build multiplex integration platform [14•]. Based on these pre-characterized sites, a simultaneous integration of 2-genes, 3-genes, 4-genes, 5-genes, or 6-genes was achieved to produce betalain or kauniolide. In parallel, successful examples for pathway integration could also be found in non-conventional microbes, such

Table 1

Selected CRISPR-Cas mediated metabolic engineering works for microbial chemical production

Host	Types of modifications	Products or desired traits	Achievements	Reference
Assembly of biosynthetic pathways for target chemicals				
E. coli	Pathway integration	5-Methylpyrazine-2-carboxylic acid (MPCA)	Improving the MPCA titer to 15.6 g/L	[13]
S. cerevisiae	Pathway integration	Betalain or kauniolide	A simultaneous integration of biosynthetic pathways for betalain (3-gene pathway) or kauniolide (6-gene pathway)	[14•]
P. kudriavzevii	Pathway integration	Itaconic acid	Improving the itaconic acid production to 401 mg/L	[15]
Z. mobilis	Pathway integration	Lactate	Improving the lactate production to 2.21 g/L	[16]
S. cerevisiae	Pathway integration	2, 3-Butanediol	A up to 25 copies of pathway integration, and improved 2, 3-butanediol production to 1.7 g/L	[18•]
Optimization of production pathways for target chemicals				
S. cerevisiae	Deletion	Free fatty acids (FFA)	Giving a 30-fold increase of FFA production	[19••]
E. coli	Repression	Isopentenol	Up to 98% enhancement in production of isopentenol	[20]
Synechocystis sp. PCC 6803	Repression	Fatty alcohols	Up to 3-fold enhancement in production of octadecanol and gave the highest specific titers (10.3 mg/g CDW)	[21]
M. xanthus	Activation	Epothilones	Improving the epothilones production to >20 mg/L	[23]
K. marxianus	Combinatorial integration	2-Phenylethanol	Improving the 2-phenylethanol production to 1943 mg/L	[24]
S. cerevisiae	Activation and repression	3-Hydroxypropionic acid (3-HP)	Giving a 36% increase of 3-HP production	[25]
S. cerevisiae	Activation and repression	Carotenoid and triacylglycerols (TAGs)	A modest increase in the carotenoid-associated phenotype; TAGs levels increased >2-fold over WT after 24 hours	[26]
S. cerevisiae	Activation, repression, and deletion	β-Carotene	Giving an improvement of β-carotene production by 3-fold	[10]
S. cerevisiae	Activation, repression, and deletion	α-Santalene	Giving an improvement of α-santalene production by 2.66-fold	[27•]
Corynebacterium glutamicum	Base editing	Lycopene	Achieving a lycopene yield of 2.9 mg/gCDW	[7••]
B. subtilis	Activation and repression	*N*-Acetylglucosamine	Achieving a *N*-acetylglucosamine production at 131.6 g/L	[28]
Yarrowia lipolytica	Repression	Naringenin	Giving a 74.8% increase of naringenin production	[29]
Screening or engineering of alternative enzymes				
S. cerevisiae	Integration	Carotenoids	An 11-fold improvement in carotenoid production by directed evolution of two essential enzymes	[30]
E. coli	Integration	Tryptophan (Trp)	Identification of variants of AroG with increased resistance to feedback inhibition and improved the Trp production by 38.5%	[31]
E. coli	Site targeted mutagenesis	Novel variant of *rpsE*	Identification of novel mutations in *rpsE* that conferred spectinomycin resistance	[32]
E. coli and *C. glutamicum*	Site targeted mutagenesis	Proline	Identification of a variant of ornithine aminotransferase that contributed a proline production of 38.4 g/L	[33•]
Diversifying the substrate scope				
E. coli	Integration	Ability for xylose-utilization	A 3-fold increase of the xylose-utilization rate	[34]
S. cerevisiae	Integration	Ability for xylose-utilization	Achievement of simultaneous co-fermentation of lignocellulosic hydrolysates (composed of glucose and xylose)	[35]
C. glutamicum and *Bacillus subtilis*	Base editing	Ability for utilization of glycerol and xylose	Improved glycerol utilization capability in *B. subtilis* and improved xylose utilization capability in *C. glutamicum*,	[7••]
E. coli	Integration, and deletion	Ability for simultaneous utilization of acetate and glucose	Improving the carbon molar yield from 0.67 to 0.75 mol/mol for glycolate production	[37]
E. coli	Deletion	Ability for utilization of ethanol	Production of 1.1 g/L polyhydroxybutyrate or 24 mg/L prenol from ethanol	[38]

Table 1 (*Continued*)

Host	Types of modifications	Products or desired traits	Achievements	Reference
Improvement of metabolic robustness				
S. cerevisiae	Integration	Resistance to ethanol	Improving ethanol tolerance and giving an 2-fold improvement for ethanol production	[40]
E. coli	Integration	Resistance to styrene	Improving styrene tolerance and giving an 3.45-fold improvement for styrene production	[41••]
Synechocystis sp. PCC 6803	Repression	Resistance to lactate	Identification of a single repression of *bcp2* for a 49% improvement of growth rate in cultures with added L-lactate	[42]
S. cerevisiae	Integration, and deletion	Resistance to various environmental perturbations, including higher temperature	Identification of 68 small open reading frames which are vital for cell's robustness	[43]
S. cerevisiae	Activation	Thermotolerance	Achieving a faster growth rate in *OLE1*-overexpressing strain than the control strains at 42°C	[44]
S. cerevisiae	Integration	Resistance to furfural and acetic acid	Improving the tolerance to furfural and acetic acid by ~42-fold and ~20-fold	[45]
S. cerevisiae	Activation, repression, and deletion	Resistance to furfural	In the presence of 17.5 mM furfural, the screened strain can consume most of glucose in 2 days, while the control strain failed to grow after 6 days	[8]
S. cerevisiae	Activation and repression	Resistance to wheat straw hydrolysate	Giving a 2.3-fold increase in final biomass yield in medium with wheat straw hydrolysate	[46]

as the implementation of itaconic acid production in *Pichia kudriavzevii* [15] and lactate production in *Zymomonas mobilis* [16]. To further increase the copy number for a higher expression, DSB were generated at delta sites by the CRISPR-Cas system, and an up to 25-copy integration could be achieved in one step, resulting in engineered strains for efficient production of 2,3-butanediol [17,18•].

Optimization of metabolic flux for target chemical production

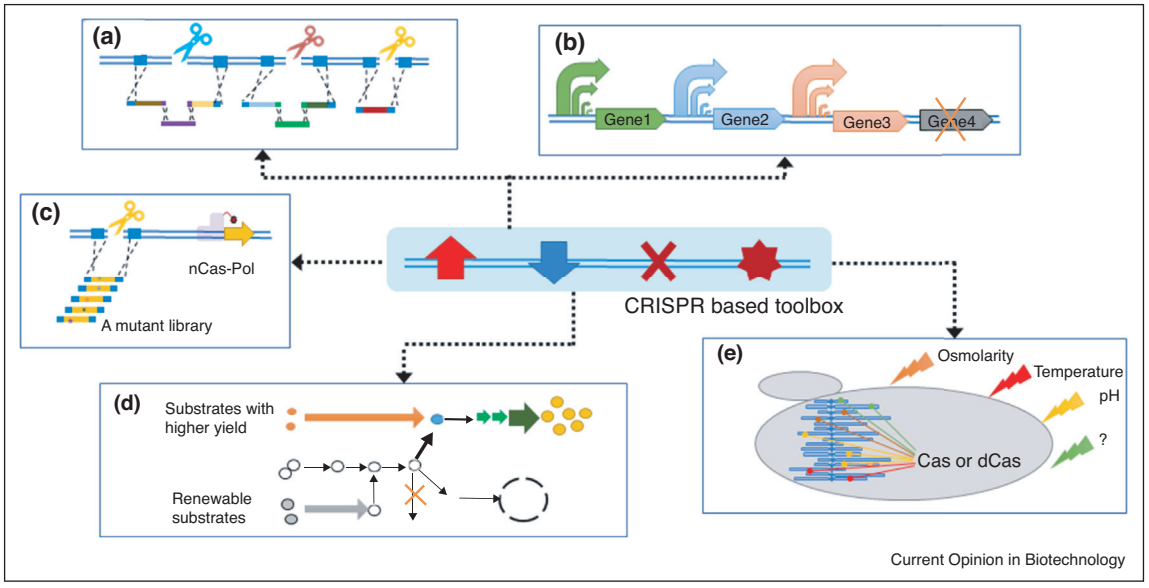
On the other hand, cells have been not evolved for chemical production. Thus, optimization of metabolic flux is required for maximizing the production, such as deletion, knock-down or overexpression of target genes, which has been enabled with the assistance of CRISPR technology in a markerless and efficient manner (Figure 3b). For example, to achieve high production of free fatty acids (FFAs), CRISPR-Cas was used to simplify the lipid network of yeast by deleting eight non-essential FA utilization genes in ten days, giving a 30-fold increase in FFA production [19••]. Similarly, the competing pathway could also be knocked down by CRISPR interference (CRISPRi), where the inactive Cas enzyme (dCas) was used. CRISPRi was demonstrated to improve the production of isopentenol in *E. coli* [20] or fatty alcohols in *Synechocystis* [21] at varying strengths of repression for combinations of several target genes.

Overexpressing genes of interest is another widespread genetic manipulation, and CRISPR-Cas has been repurposed to activate gene expression (CRISPRa) when a transcriptional activator was coupled to dCas. Initially, CRISPRa was actively employed in eukaryotes since there are many activation domains reported [10]. Now, a generalizable platform was available to screen and select transcriptional activators that can be used in different bacterial species [22]. Recently, CRISPRa was shown to upregulate the expression of biosynthetic genes for the production of epothilones in *Myxococcus xanthus* [23]. In this study, the activation effects of each gene in the pathway were also clarified by using different sgRNAs and activator proteins. Generally, CRISPRi or CRISPRa is more suitable for multiplex engineering, as multiplex deletion/integration would be more harmful to the host.

Because of the complex regulatory metabolic network of living cells, combinatorial and multiplex pathway editing is needed to alter cell metabolism. For example, a multigene integration system mediated by CRISPR-Cas9 was developed for pathway refactoring in *Kluyveromyces marxianus* [24]. The system was demonstrated to create a 3^3 combinatorial library to optimize the expression of three key shikimate pathway genes, *ARO4*, *ARO7* and *PHA2*. Later, simultaneous transcriptional activation or repression was reported for different strategies that improved the production of target chemicals. Recently, a platform tool was established to upregulate or downregulate gene expression using dCas9 coupled to the VP64-p65-Rta (VPR) [25]. This tool was used to allow transcriptional perturbation of the 168 selected genes, and increased the production yield of 3-hydroxypropionic acid by 36% through targeting the gene encoding adenylate kinase 1. Another work has also shown the use of RNA scaffold systems for repression or activation of target gene expression to enhance the production of carotenoid and triacylglycerols [26].

In a recent study, three orthogonal Cas proteins were used to construct a tri-functional CRISPR system for

Figure 3



Applications of CRISPR-Cas toolbox in development of microbial cell factories for chemical production.
(a) Assembly of biosynthetic pathways for target chemical production. Several biosynthetic enzymes are assembled into functional metabolic structures, and the integrated copy number could be adjusted at the same time by selecting desirable intergenic loci. **(b)** Optimization of metabolic flux for target chemical production. Combinatorial and multiplexed genetic manipulation is established to optimize or balance metabolic flux to target chemicals. **(c)** Identification of alternative enzymes for biocatalyst. Enzymes with altered or enhanced activities could be created by direct integration of protein variants or continuously targeted *in vivo* mutagenesis. Protein variants could be generated by error-prone PCR, or (semi-) rational design. The *in vivo* mutagenesis could be achieved by using engineered DNA polymerases (nCas-Pol) targeted to loci via CRISPR-guided nickases. **(d)** Diversification of the substrate scope. Considerable efforts have been expended to efficiently use renewable substrates or alternative substrates with a higher theoretical yield. **(e)** Improvement of metabolic robustness. Metabolic robustness or tolerance have been improved to various cell stressors via screening of functional genes.

transcriptional activation, interference, and gene deletion, which was applied to enhance the production of β-carotene by threefold in a single step in *Saccharomyces cerevisiae* [10]. Later, it also demonstrated that a single Cas9-VPR protein can accomplish these tri-combinatorial manipulations and improve α-santalene production 2.66-fold [27•]. Following this, a CRISPR-guided base editor was designed for combinatorial optimization of ten endogenous genes for lycopene biosynthesis by diversifying tailored ribosome binding sites (RBSs) [7••]. In this method (named BETTER), the library was generated in situ, and the theoretical maximum library can be up to 2.7×10^{33}, providing a significant advantage for diversifying multigene expression.

Dynamic control of metabolic flux is highly effective in optimizing biological systems. Recently, the production of *N*-acetylglucosamine was increased to 131.6 g/L by *Bacillus subtilis* using CRISPRi-based NOT gates with the biosensor of intermediate glucosamine-6-phosphate [28]; similarly, the production of naringenin was increased by 74.8%, enabled by the combined use of CRISPRi and a fatty acid sensor [29]. Both dynamically self-adjust the expression of the pathway genes using an autoregulatory genetic circuit for a balanced flux to the biosynthesis of the target product, which can be used as a generalized tool for pathway optimization. However, these proposed programmable circuits are limited to the availability of biosensors.

Screening or engineering of alternative enzymes for biocatalyst

Enzymes are the basic units for the design and construction of efficient chemical-producing pathways. However, natural enzymes may have limitations, such as lower catalytic efficiency or feedback inhibition. Recently, as shown in Figure 3c, the CRISPR-Cas system was shown to directly and effectively integrate protein variants of interest into the yeast genome with efficiencies reaching 98–99% [30]. Using this method, two key enzyme variants in the mevalonate pathway were identified with improved activity from millions of mutants by colony color, which resulted in an 11-fold improvement in carotenoid production. Similarly, when the integrated library was coupled with growth, several Phe-resistant AroG variants were found that can be used to facilitate the biosynthesis of aromatic amino acids [31].

In parallel, the mutation library of a target enzyme was also demonstrated as being capable of generation *in vivo*

by the CRISPR-Cas system for the continuous evolution of user-defined genes (Figure 3c), called EvolvR [32]. In EvolvR, nucleotides within user-defined regions were targeted for mutagenesis using CRISPR-guided engineered DNA polymerases, offering a mutation rate 7 770 000-fold greater than wild-type, and novel mutations of *rpsE* gene were identified that conferred spectinomycin resistance. Later, EvolvR was used to identify a variant of ornithine aminotransferase that showed 2.85-fold improvement in catalytic efficiency [33•]. Now, facilitated by the CRISPR-Cas system, innovative strategies have been developed that accelerate the generation and expression of mutated variants. However, further optimization of the screening method for desired mutants would be required via growth-coupled or sensor-guided strategies.

Diversifying the substrate scope of natural hosts

There is growing interest in producing chemicals from waste or inexpensive substrates to reach economic viability and avoid competition with foodstuffs (Figure 3d). For example, facilitated by CRISPR, a one-step integration and optimization of the xylose utilization pathway has been reported in *E. coli*, which gave a threefold increase in the xylose utilization rate [34]. Similarly, a xylose/glucose co-fermenting yeast was also developed by rational engineering using markerless CRISPR tools and evolutionary engineering, as well as overexpression of selected genes from the pentose phosphate pathway [35]. As there is no marker left, the co-fermenting yeast would be an excellent platform strain to produce various chemicals. In parallel, the previously mentioned BETTER method was successfully used to reprogram xylose and glycerol utilization to replace tailored RBSs in the original genes [7••]. The combinatorial re-programmed strains showed a much higher growth rate on these two substrates, and at the same time optimal RBSs were screened for efficient gene expression based on the change of growth rate. The use of current renewable substrates offers economical and sustainable production of chemicals. Furthermore, the emerging 3G biorefinery that uses CO_2 as the carbon source represents an attractive alternative for microbial chemical production in a carbon-neutral manner [36].

In addition, it has been known that the adoption of approximate substrates may give a higher theoretical yield (Figure 3d). Recent advances in computational biology and *in silico* modeling have facilitated the design of efficient pathways for the desired chemical. Guided by this, a synergetic biosynthetic pathway was designed and constructed by the CRISPR system to enable simultaneous utilization of acetate and glucose, which increased the carbon molar yield from 0.67 to 0.75 mol/mol for glycolate production [37]. With a followed systematic engineering, the final strain can produce 73.3 g/L glycolate in fed-batch fermentation, reaching a carbon yield of 0.6 mol/mol. In another work, facilitated by the CRISPR system, *E. coli* was engineered to grow on ethanol for producing acetyl-CoA derived compounds [38]. The engineered strain was shown to produce 1.1 g/L of polyhydroxybutyrate or 24 mg/L of prenol from ethanol. Compared with glucose or other substrates, this study showed that ethanol provided building blocks for the synthesis of acetyl-CoA derived compounds in a shorter and more carbon-efficient pathway. As shown above, the balance use of selected substrates will make cells to efficiently utilize the feedstock and maximize the production yield and rate of end products. This strategy has been used to further improve the theoretical maximum yield of a wide range of products by redesigning cell metabolism to assimilate alternative feedstocks such as fatty acids, glycerol, methanol, and formaldehyde [39].

Improvement of metabolic robustness

Following efficient chemical production, the accumulated chemical products can sometimes be toxic to the cells. Consequently, it is necessary to improve metabolic robustness or tolerance of the host cell for maximized productivity (Figure 3e). In a recent study, a combinatorial library targeting 25 genes was constructed based on CRISPR and massively parallel oligomer synthesis for improving ethanol resistance [40]. Several identified mutants screened from the library not only increased ethanol tolerance but also gave a twofold higher ethanol production. Later, the same group was extended using this method to improve the tolerance of styrene [41••]. Similarly, the identified new mutations improved tolerance and the production of styrene at the same time. In another report, it was found that stress tolerance mutants could be screened by CRISPRi in the presence of 0.1 M L-lactate [42]. Of the screened targets, a single repression of the *bcp2* gene made a 49% improvement in growth rate.

In the process of industrial fermentation, environmental perturbations can affect the performance of a cell factory, such as inhomogeneities caused by insufficient mixing. Therefore, it is highly desirable to develop cells with increased robustness in harsh conditions. Recently, a Cas9-based approach was used to generate a genome-wide library containing sequence deletions, substitutions, and replacements [43]. Yeast mutants were screened with improved resistance to various environmental perturbations, including higher temperature, in the presence of hydroxyurea or fluconazole. Finally, 68 small open reading frames were found to be vital for the cell's robustness. A focused CRISPRa library was created to screen functional genes for thermotolerance [44], and upregulation of *OLE1* was identified as a key factor in obtaining thermotolerant yeast.

Feedstocks for actual fermentation utilize complex substrates, which may include growth inhibitors, such as furfural and acetic acid from lignocellulosic hydrolysate.

The robustness of these inhibitory compounds is highly desirable to alleviate the inhibition of growth and metabolism. A recent method called CHAnGE was reported that conducts CRISPR-Cas9 and homology-directed repair-assisted (HDR) genome-scale engineering [45]. The method can generate a genome-scale yeast mutant library at single-nucleotide precision, and was applied to improve the tolerance to furfural and acetic acid by ~42-fold and ~20-fold, respectively. Meanwhile, the same group combined oligo pools and the tri-functional CRISPR for transcriptional activation/interference, and gene deletion in genome-wide, which was applied to identify genetic determinants for furfural tolerance [8]. Notably, several targets were only reported in this study. Furthermore, this method also identified synergistic interactions between chosen targets for enhanced furfural tolerance. In another study, with dCas9-based CRISPRi and CRISPRa, the tolerance towards wheat straw hydrolysate was successfully improved by tuning the expression of a previous known key gene, *SSK2* [46]. The strain with modified expression of *SSK2* gave a 2.3-fold increase in final biomass yield when grown in wheat straw hydrolysate.

Perspectives and conclusions

The CRISPR-Cas system is becoming an essential tool to accelerate the creation of cell factories for chemical production. In particular, with the features of orthogonality and simplicity, CRISPR-Cas can easily be implemented in both model and non-model organisms. Furthermore, the capabilities of the CRISPR-Cas system have been extended with the incorporation of novel functional proteins [4], such as DNA polymerases, reverse transcriptase, deaminases acting on RNA, or transposase. However, challenges still exist for some applications of the CRISPR-Cas system.

The design and effectiveness of gRNA are sensitive to many factors. There are now several software applications, websites, and rules that can predict the targeting ability of gRNA [47]. However, their accuracy still needs improvement. In CRISPRa and CRISPRi, there is also a strict position effect determined by the chosen gRNA. Recently, two studies showed a good understanding of the rules for designing effective positions of gRNA target sites [48,49]. There is also a growing interest in giving gRNA design more flexibility by searching protospacer adjacent motif (PAM) free nucleases [50].

To obtain a high-performing strain, it has been usual to conduct combinatorial and multiplex editing/regulation for tens or even hundreds of metabolic engineering targets. Now, up to 12 sgRNAs can be assembled and expressed with a co-expression of Csy4 [51]. Therefore, we should pay more attention to developing strategies for the expression of a greater number of gRNAs in one step.

At present, most examples carried in genome-scale or multiplex engineering by CRISPR-Cas are limited to a growth-associated phenotype. However, it is highly demanding to evaluate a large library of strains efficiently. The integration of chemical-responsive sensors or high-throughput small-scale culture and detection techniques could change screening for new traits [52].

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Shuobo Shi: Conceptualization, Writing - original draft, Writing - review & editing. **Nailing Qi:** Writing - review & editing. **Jens Nielsen:** Conceptualization, Supervision, Writing - review & editing.

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- •• of outstanding interest

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