Chapter

Applications of CRISPR/Cas Technology to Research the Synthetic Genomics of Yeast

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Abstract

The whole genome projects open the prelude to the diversity and complexity of biological genome by generating immense data. For the sake of exploring the riddle of the genome, scientists around the world have dedicated themselves in annotating for these massive data. However, searching for the exact and valuable information is like looking for a needle in a haystack. Advances in gene editing technology have allowed researchers to precisely manipulate the targeted functional genes in the genome by the state-of-the-art gene-editing tools, so as to facilitate the studies involving the fields of biology, agriculture, food industry, medicine, environment and healthcare in a more convenient way. As a sort of pioneer editing devices, the CRISPR/Cas systems having various versatile homologs and variants, now are rapidly giving impetus to the development of synthetic genomics and synthetic biology. Firstly, in the chapter, we will present the classification, structural and functional diversity of CRISPR/Cas systems. Then we will emphasize the applications in synthetic genome of yeast (*Saccharomyces cerevisiae*) using CRISPR/Cas technology based on year order. Finally, the summary and prospection of synthetic genomics as well as synthetic biotechnology based on CRISPR/Cas systems and their further utilizations in yeast are narrated.

Keywords: applications, CRISPR/Cas, gene editing, *S. cerevisiae*, synthetic genomics, yeast

1. Introduction

Synthetic biology is a fundamentally interdiscipline. It has become an important methodology in biotechnology owing to its novel functions and regulation mechanisms. The scientific concept of synthetic biology can be traced back to the book "The Mechanism of Life" written by a French physical chemist Stéphane Leduc in 1911 [1]. It currently refers to the practical application discipline that integrating modern science and engineering technology to promote and accelerate the design, alteration and creation of bio-genetic materials in living organisms [2]. Briefly, synthetic biology can roughly be considered as the reverse process of analytical biology. Sustained advances in synthetic biology will depend on coordinated and paralleled developments within many different discipline areas, and cooperation of scientists from most countries. In China, scientists have made landmark contributions in this field with the success

of artificial synthesis of bovine insulin and yeast (*S. cerevisiae*) alanine transfer RNA. In USA, artemisinic acid, a precursor of antimalarial drug artemisinin, was synthesized in *S. cerevisiae* with a yield of 25 g/L, and has been industrialized in 2013 [3]. Nowadays, synthetic biology is rapidly penetrating into various fields including bio-science, gene engineering, agriculture, food industry and medicine, in which synthetic genomics plays a profound role in providing theoretical basis and technological support. Synthetic genomics is viewed as an important area of synthetic biology, which being engineered under a general genome scope, mainly refers to design and assembly of nucleotide fragments to generate functional living genomes [4], including recreated and recoded genomes as well as minimal genomes. Genetic manipulation is known as one of the central strategies to investigate the molecular basis of living things as well as their evolution and diversity, which advancing the understanding of biological systems at a micro level. Compared to conventional approaches in genetic manipulation, synthetic genomics has the characteristics of introducing large numbers and diversity of genetic modifications [5]. Additionally, synthetic genomics can theoretically create a synthetic genome to practically and feasibly build a simpler and more amenable genome-scale platform for biological system construction [6].

In a certain sense, the first synthetic gene synthesized in 1970s [7] marked the beginning of synthetic genomics. Then, viral chromosomes were the first to be synthesized in the early 2000's because of their comparatively small size [8]. Nowadays, as a first designer synthetic eukaryotic genome, Sc2.0 (*S. cerevisiae* 2.0) project has achieved significant progresses including real-world applications for industrial microbiology, and may create a big economic value in the future. Driven by rapid advances in gene assembly, genome editing and mathematical modeling techniques, synthetic genomics is developing quickly. As a young discipline, synthetic genomics has helped to promote our new understanding of genome structure and function. Recently, an important direction of synthetic genomics is to transform the natural biological systems through gene editing techniques. With emerging novel classes of programmable genetic tools, in particular, the establishment and optimization of CRISPR and associated technology platforms, synthetic biology and its vital field——synthetic genomics is entering a new era of more possibilities. Actually, early in 2014, the European Commission's synthetic biology summit has typically categorized synthetic biology tools as design, construction and diagnostic tools whereas synthetic biology methodology serves the study of DNA synthesis and synthetic genomics, engineering biology, xenobiology as well as protocell biology [9]. CRISPR/Cas nucleases have been extensively applied to manipulate the genomes of cultured and primary cells, animals and plants, vastly accelerating the pace of basic research and enabling breakthroughs in the field of synthetic biology and synthetic genomics [10]. It can be expect that, CRISPR toolkits are of particular importance to the future of synthetic genomics due to its great potential to open new pathways for manipulation and expression of genetic information, which will certainly transform synthetic genomics and synthetic biology greatly.

Here in this chapter, we review the developments of CRISPR/Cas technology, the main types of CRISPR/Cas system, as well as the applicational research of synthetic genomics in yeast using CRISPR/Cas toolboxes. Finally, we also provide perspectives on future directions and applications of CRISPR/Cas-based methodology in the research of synthetic yeast genome.

2. CRISPR/Cas: a powerful and versatile toolkits for synthetic genomics

The survival battle between microbes and bacteriophage is the driving force behind the evolution and diversification of microbial adaptive immune system.

As part of the immune response in bacteria, CRISPR/Cas systems are responsible for tackling the invading phages or plasmids. These systems are of particular importance to the future of synthetic genomics owing to their great potential to open new doors for manipulation and expression of genetic sequences. Since the discovery of CRISPR in the *Escherichia coli* genome in 1987 [11], CRISPR/Cas systems now have been classified into two main classes (Class 1 and Class 2) and six types (type I, II, III, IV, V and VI) covering over 30 different subtypes [12]. The diversities of architecture and classification render CRISPR/Cas systems with broad functional versatilities. Of note, the diversity of protospacer adjacent motif (PAM) is also an important feature for CRISPR/Cas systems. Briefly, PAM refers to a short sequence resides in the exogenous nucleic acid elements (commonly at the 3′ end of the target DNA) but not the CRISPR array and its guide RNAs that support to discriminate self versus non-self of microbes' nucleic acid ingredients. The nucleotide sequences that can be edited by CRISPR/Cas systems have been limited by the PAM and gRNA sequences. Commonly, *Streptococcus pyogenes* Cas9 (SpCas9) recognizes 5'-NGG-3′, and *Staphylococcus aureus* Cas9 (SaCas9) recognizes 5'-NNGRRT-3′ as their respective functional PAM [13]. However, type V CRISPR/Cas system uses a T-rich PAM sequence such as "TTT", "TTA", "TTN" or "TTC" for target recognition. For example, Cas12a (Cpf1) uses the "TTN" PAM sequence to target dsDNA [14]. Cas proteins and their variants require different PAM sequences for efficiently and precisely manipulate and cleavage biological genomes, while type VI system relies on a PAM analogue termed protospacer flanking site (PFS) for RNA targeting [15]. Moreover, taking Cas9 protein as an example, a previous study has reported the important function of PAM recognition in the field of inducing target DNA unwinding, which underscored by the sequential variability of PAM recognition presented in engineered Cas variants [16].

In addition to the basic properties for antiviral roles, CRISPR/Cas systems have numerous potential applications for gene editing, transcriptional activation/inhibition, epigenetic modification, chromatin imaging, single base substitution, point-of-care diagnostics [17] and synthetic biology, among others [18]. However, taking account of off-target concerns, much more interrogations are needed to make these systems valuable and reliable toolboxes for utility research area.

As CRISPR systems continue to be discovered, a variety of programmable nucleases have joined the ranks of genome editing. Currently, three types of candidate nuclease systems including Cas9 and its homologs, DNA-targeting Cas12, as well as RNA-targeting Cas13 have together become the focus of gene-editing technology. Cas9 nuclease induces double strand breaks (DSB), and Cas9 nickase triggers single strand breaks (SSB); whereas dCas9 (dead Cas9) derived from Cas9 nuclease via mutating the HNH nuclease domain and RuvC-like domain, can still bind to the target region for transcriptional interventions, but without introducing a DSB. Based on similar mechanisms, dCas12a and dCas13 are capable to mediate CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) at the transcriptional levels. CRISPRi/a is a tremendously useful tool for transcriptional regulations which achieving by covalent modification of histones of Cas proteins as it allows to balance and optimize gene expression without genome editing. Besides, Cas9 and Cas12a (previously named as Cpf1) are RNA-guided endonucleases that can induce genome editing by triggering DSB repair at a specific site [19]. Cas9 and Cas13a can be used for targeted RNA interference. Cas13a fused to base editors can be used to modify nucleotides in RNA. Futhermore, the dCas nucleases have termed "discovery-based synthetic biology", constitute one part of toolbox to study synthetic biology and engineer biology, especially have functions in the field of characterizing the function of noncoding genes and regulatory elements of the genome, and strategies to design synthetic gene circuits [20]. Theoretically, CRISPR toolkits can be applied

to integrate synthetic sequences into the genome, to form genomic libraries, and to target on multiple functional loci conveniently for gene modifications with high efficiency in strains or cell lines. The increased knowledges of CRISPR classifications and their action mechanisms open up new scopes of applications in synthetic genome of an organism via these systems.

3. Synthetic genomics and the use of CRISPR technology in synthetic genomics of *S. cerevisiae*

Technical advances in chemical synthesis of poliovirus cDNA in the absence of a natural template have gained global attentions. The successful synthesis of the 7,740 bp poliovirus cDNA artificially not only indicated the feasibility of producing infectious virus using chemically synthesized oligonucleotides as initial materials but also showed the great potential of modifying and creating more complex genomes under laboratory conditions [21]. Then in 2003, the artificial synthesis of ΦX174 bacteriophage genome (5,386 bp) using synthetic oligonucleotides has paved the way for synthesizing bacterial genomes [22]. Since then, several other viral genomes and transposons have also been synthesized in their entirety. Bacterial genomes comprising millions of base pairs are very complex, which often regarded as advanced factories to synthetise biologically active chemical substances. Synthetic biologist Craig Venter and his research team have successively synthesized the 582,970 bp *Mycobacterium genitalium* genome and *Mycoplasma mycoides* genome JCVI-1.0, and the latter represented a milestone in the history of synthetic genomics [23, 24].

From historical perspective of human domestication, potentially the next best thing to fire is yeast [25]. In practical terms, yeast represents one of the simplest eukaryotic microorganisms, and as an attractive model organism has been widely used in the food industry [26]. The Synthetic Yeast Genome (Sc2.0) Project is ongoing and aims to explore yeast chromosome structure, minimal eukaryotic genome length and gene content, as well as to rewrite all 16 yeast chromosomes. As we known, DNA synthesis is an essential tool for synthetic genomics. Currently, with the aid of CRISPR/Cas technology, for instance, programmable Cas9 proteins can execute sequence-specific DSB depending on the target sites of the gRNA, and also genes in the genome of an organism can be programmed and are rewritable [27]. Generally, CRISPR/Cas systems have vastly simplified genome editing in yeasts via performing gene over-expression, knockin, knockout, mutations and deletions, and enabled easy-operation genetically engineering of products of fuel molecules, chemical components, food ingredients, and active pharmaceutical ingredients.

Early in 2013, DiCarlo and colleagues firstly piloted CRISPR/Cas9 system to engineer for site-specific mutagenesis and allelic replacement in the genome of *S. cerevisiae* using dsOligo (double-stranded 90-bp oligonucleotide) as a template with efficiency rates close to 100% [28]. A few months later, Farzadfard's research team reported a CRISPR/Cas-based eukaryotic transcriptional regulation system implemented in *S. cerevisiae*, which will open up new paths for drawing natural genetic circuits and their regulations regarding on cellular phenotypic mechanisms [29]. In the years following these initial works, several related research reports have been emerging.

In 2014, Ryan and others improved the utilization of fiber disaccharide in diploid yeast by multiplex CRISPR system, which made the cellobiose fermentation rates increased by more than 10 times [30]. Similarly as DiCarlo's experimental methodology, Zhang et al. engineered the industrial polyploid strain ATCC4124, where URA3, TRP1, LEU2 and HIS3 were knocked out one-by-one with efficiencies varying from 15–60% to create an auxotrophic strain. And this method is likely to

be very valuable for yeast genome engineering due to having no need of selectable markers that labeled in the integrated DNA [31].

In 2015, Bao et al. and Mans et al. respectively, used a HI-CRISPR (homology-integrated CRISPR) or CRISPR/Cas9 strategy to successfully generate multiple gene modifications in yeast *S. cerevisiae* simultaneously [32, 33]. In the studies related to metabolic pathway engineering, Ronda et al. applied a new system called CrEdit (CRISPR/Cas9 mediated genome Editing) to enable simultaneous and highly efficient integration of three pathway genes (BTS1, crtYB and crtI) involved in the production of β-carotene at three different integration sites (X-3, XI-2, and XII-5 gene locus) in the genome of *S. cerevisiae* [34]. Jakočiūnas and collaborators applied CRISPR/Cas9 for multiplex gene knock-out to search for strains with improved production of mevalonate (a key intermediate for isoprenoid and sterol production) in yeast [35], followed by the utilization of CRISPR/Cas9 system to integrate crtYB, CrtI and crtE genes in three gene sites (ADE2, HIS3 and URA3) of *S. cerevisiae* for successfully constructing carotenoid biosynthesis pathway [36].

In 2016, more studies in relation to the use of CRISPR/Cas9 system for engineering in the genome of yeast have been reported [37–44]. Most notably, Tsarmpopoulos et al. reported the CRISPR/Cas9 adaptation for the engineering of bacterial genomes cloned in yeast. The result showed that applying 90 nt paired oligonucleotides as templates to promote recombination which achieved a seamless deletion of the mycoplasma glpO (glycerol-3-phosphate oxidase-encoding) gene without selection in one step. This work paves the way to high-throughput manipulation of natural or synthetic genomes in yeast *S. cerevisiae* [37].

In the year of 2017, Vanegas and her partners used a combination tool of Cas9 genome editing and dCas9 transcriptional regulation to engineer *S. cerevisiae* for production of flavonoid precursor naringenin and simultaneously restrainting formation of by-product phloretic acid [45]. Reider Apel et al. constructed a clone-free toolkit based on CRISPR/Cas9, which solved the problems of chromosome integration locus and promoter selection, protein localization and solubility in yeast metabolic engineering, and optimized the expression of taxadiene synthase by using the tool, which increased the yield of taxadiene by 25 times [46]. Contrary to the result of protein overexpression, Vigentini et al. employed the CRISPR/Cas9 system to successfully reduce urea production in *S. cerevisiae* wine yeasts via eliminating the CAN1 arginine permease pathway [47]. Interestingly, Mans et al. used CRISPR/Cas9 technology to explore the elusive mechanism for lactate export in *S. cerevisiae*. The 25-deletion strain in this experiment has taken the first step in building a yeast's 'minimal transportome' platform, which can be applicable to functional explanation of heterologous transport proteins and the assessment of metabolic engineering strategies [48]. The summary of CRISPR-based studies in yeast in the year of 2017 have showed in **Table 1** [45–59].

In the beginning of the year 2018, Verwaal et al. employed three gene-editing systems, Cpf1 orthologues (*Acidaminococcus spp*. BV3L6 (AsCpf1), *Lachnospiraceae bacterium* ND2006 (LbCpf1) and *Francisella novicida* U112 (FnCpf1)) for genome modification of *S. cerevisiae*. The result of this work demonstrated that Cpf1 can broaden application sphere of the genome-editing toolbox available for research of *S. cerevisiae* [69]. Li et al. firstly used the CRISPR/Cpf1 to delete large DNA fragment (the deletion of DNA fragment of ~38 kb between the two genes of TRM10 and REX4) in *S. cerevisiae*, which demonstrating that the CRISPR/Cpf1 system can be used for genome simplification of *S. cerevisiae*, and to facilitate the laboratory evolution of the genome of *S. cerevisiae* [70]. Later in the year, Dank et al. used CRISPR/Cas9 technology to construct *S. cerevisiae* mutants with lacking esterase IAH1 and/or TIP1. Very interestingly, not affecting by the double gene knockout of yeast mutant ΔIAH1ΔTIP1, a complex regulatory mechanism to compensate

Gene Action Modes	CRISPR System	Expression Products	Gene Sites (or gene number)	Authors and Year	References
Downregulation	Cas9/dCas9 based system	Naringenin	*TSC*13	Vanegas *et al*. (2017)	[45]
Gene integration	CRISPR/Cas9	Taxadiene	23 genomic loci	Reider Apel *et al*. (2017)	[46]
Genetic modification	CRISPR/Cas9	Urea	*CAN*1	Vigentini *et al*. (2017)	[47]
Gene deletion	CRISPR/Cas9	Lactate	25 genomic loci	Mans *et al*. (2017)	[48]
Gene regulation	dCas9-VPR	Naringenin	*NDE*2, *CYC*1, *GPD*1, *TDH*1	Vanegas *et al*. (2017)	[49]
Gen regulation and integration	CRISPR/Cas9	Cellulase; isobutanol; glycerol	δ-regions	Si *et al*. (2017)	[50]
Graded expression	CRISPR/dCas9	Pathway enzymes	Multi-genes (e.g., *ZWF*1, *TAL*1, *TKL*1)	Deaner *et al*. (2017)	[51]
Gene modulation	dCas9-VPR	-	Up to 4 native genes	Deaner *et al*. (2017)	[52]
Transcriptional reprogramming	dCas9 systems	Isoprenoid; TAG	-	Jensen *et al*. (2017)	[53]
Logic circuits	dCas9-Mxi1	-	-	Gander *et al*. (2017)	[54]
Gene regulation	CRISPR-AID	Beta-carotene	*HMG*1; *ERG*9; *ROX*1	Lian *et al*. (2017)	[55]
Gene editing	CRISPR/Cas9	Glutathione	*ADE*2, *URA*3, *LEU*2, *TRP*1, *HIS*3	Zhou *et al*. (2017)	[56]
Construction of mutants	"CRISPR Nickase system"	-	*CAN*1, *CDC*25	Satomura *et al*. (2017)	[57]
Strain Generation; Gene drive	CRISPR/Cas9	Strain mutants	-	Roggenkamp *et al*. (2017)	[58]
Genome editing; point mutation	FnCpf1	-	*ADE*2, *HIS*4, *PDR*12, *CAN*1	Swiat *et al*. (2017)	[59]
Multiplexed engineering	GTR-CRISPR	-	8 genes (e.g., *CAN*1, *ADE*2, *LYP*1, etc.)	Ferreira *et al*. (2018)	[60]
Gene activation, interference, and deletion	dLbCpf1, dSpCas9, SaCas9	Recombinant *Trichoderma reesei endoglucanase* II	Unspecified target genes	Schultz *et al*. (2018)	[61]
Chromosome fusion	CRISPR/Cas9	-	Multiple-chromosome	Shao *et al*. (2018)	[62]
Genomic integration	CRISPR/Cas9	Natural genetic variants	*SEC*14 gene	Roy *et al*. (2018)	[63]
Gene disruption	CRISPR/Cas9	Bioethanol	*ADH*2	Xue *et al*. (2018)	[64]

Gene Action Modes	CRISPR System	Expression Products	Gene Sites (or gene number)	Authors and Year	References
Cocktail integration	CRISPR/Cas9 combination	Target products: beta-carotene	*ADE*2, *URA*3, *CAN*1	Hou *et al*. (2018)	[65]
Genetic manipulations	CRISPR/Cas9	Mating-types, diploids and polyploids	*MAT* locus	Xie *et al*. (2018)	[66]
Single-nucleotide genome-editing	CRISPR/Cas9 combination	Genetic variants	*CAN*1, *ADE*2, *LYP*1, etc.	Bao *et al*. (2018)	[67]
Genomic integration	FnCpf1	Beta-carotene	*Gal*1-7 locus, *Gal*80 locus, *HO* locus, etc.	Li *et al*. (2018)	[68]

Table 1.
The summary table of CRISPR-based studies in yeast in the year of 2017 and 2018.

multiple genomic defects in aroma metabolism is generated and activated to show an aroma composition comparable to wild type levels [71]. Using CRISPR technology, the related studies in yeasts for creating genome mutations and integrations have also been respectively carried out by Guo et al. [72] and Jakociunas et al. [73]. And much more related works have listed in **Table 1** [60–68, 74].

As CRISPR systems continue to be extensively used, dozens of articles (more than 60 scientific papers in PubMed) in relation to CRISPR-based yeast have been published in 2019. Based on PubMed database, 2019 is the year of the most articles published in recent years in the field of CRISPR-edited yeasts. Among them, Zhang et al. deleted 6 genes in the yeast genomes in 3 days through developing a multiplexed gene-editing platform termed GTR-CRISPR (a gRNA-tRNA array for CRISPR/Cas9) with 60% efficiency using reported gRNAs and 23% using unimproved gRNAs. They further concluded that GTR-CRISPR may be suggested to the most valuable complement for the toolkit of synthetic biology and auto-operation [75]. Notably, Laughery et al. found that the cause of dCas9 targeting boosted mutagenesis in yeast is likely affected mutationally by dCas9-mediated R-loop formation. These findings not only showed important implications for the applications of additional mutagenesis in dCas9 (and Cas9), but also offered a novel method for interrogating the mechanism of targeted R-loop formation induces eukaryotic genome variability and/or mutagenesis [76]. As excellent as above two work, another two investigations respectively described the CRISPR/Cas9 based functional chromosome fusions [77] and CRISPR/dCas9 based AND gate in yeast [78]. Additionally, as typical research examples, this review only list 5 valuable articles published in 2019 (**Table 2**) [79–83].

Good works are also seen in 2020. Wu and his team found that specific cleavage via CRISPR/Cas9 near the centromere of a *S. cerevisiae* chromosome can lead to elimination of the whole chromosome and initiate chromosome drive [89]. van Wyk et al. utilized CRISPR/Cas9 technology to generate a self-cloned wine yeast strain that over-expresses two genes of oenological relevance, GPD1 (glycerol-3-phosphate dehydrogenase 1) and ATF1 (alcohol acetyltransferase 1), which directly implicated in glycerol and acetate ester production respectively. The result provided an alternative strategy to obtain increased glycerol and lower acetic acid levels, without disrupting the aldehyde dehydrogenase activity [90]. In addition to the above two, here enumerate 5 representative literatures on CRISPR-based yeasts in this year used for references (**Table 2**) [84–88].

References	Authors/year	Gene regulation	Cas modes	Target loci	Descriptions
[79]	Ai et al./2019	Deletion	Cas9	gal80	Engineered yeasts that can produce artemisinic acid without galactose induction.
[80]	Chen et al./2019	down-regulation or knock-out	Cas9	erg9; rox1; ypl062w; yjl064w	Achieved high production of valencene through CRISPR/Cas9-mediated metabolic engineering.
[81]	Laughery et al./2019	Genome editing	Cas9	RNR1 gene	Describe a simple protocol for constructing Cas9-expressing plasmids and protocols for genome editing in yeast.
[82]	Ciurkot et al./2019	Genome editing	Cas12a	INT1, INT2 and INT3 genomic sites	Demonstrated Cas12a-mediated multiplex genome editing in yeasts, and created yeast pixel art with an acoustic liquid handler using yeast strains that producing differently colored carotenoid.
[83]	Yan & Finnigan/2019	Gene drive	Cas9	HIS3 locus	Developed a gene drive in yeasts that allowing for the examination of alternative drive designs and control mechanisms.
[84]	Cámara et al./2020	Gene expression	CRISPR/Cas	PDR12 locus	Constructed a toolkit for CRISPRa/i for a polyploid industrial yeast strain.
[85]	Levi & Arava/2020	Gene modification	Cas9	HTS1; OM14; FRS1	Provided a facile alternative to manipulate the yeast genome.
[86]	Li et al./2020	Genome engineering	Cas9	UPC2 gene; Gal1-7, Gal80 sites	Cas-3P allowed single-, double- and triple-loci gene targeting in yeasts.
[87]	Mitsui et al./2020	Genome evolution	GMES /CRISPR	13 genes: HXT7, HXK2, PGI1, etc.	Aimed to construct a lactic acid-tolerant yeast to reduce the neutralization cost in LA production.
[88]	Yang et al./2020	Gene targeted mutation	Cas9	ERG20	Facilitate construction of genomic mutations of essential genes for functional genomic analysis and metabolic flux regulation in yeasts.

Note: CRISPRa/i, CRISPR activation and interference; Cas-3P, Cas9 and three marked plasmid backbones; GMES/CRISPR, global metabolic engineering strategy/CRISPR.

Table 2.
CRISPR-based applications in yeast in the year of 2019.

Stepping into 2021, innovations remain advancing. Gong et al. reported a gRNA-tRNA array and SpCas9-NG (GTR 2.0) for the applications of highly efficient genome damage and base editing. In this study, they achieved gene cleavages with almost 100 efficiencies in the cells of *S. cerevisiae*. During the process, gene editing includes all 16 possible NGN PAMs and all 12 possible single-nucleotide mutations (N to N). Further, they employed GTR 2.0 system for multiplexed single-nucleotide mutations to simultaneously produce 4 single-nucleotide conversions in single gene with 66.67% mutation efficiency, and to create simultaneously 2 single-nucleotide mutations in two different genes with 100% mutation efficiency [91].

McGlincy et al. showed a comprehensive yeast CRISPRi library, based on empirical design rules, containing 10 distinct guides for most genes, which providing a strategy for genome-wide CRISPR interference screening in budding yeast [92]. Furthermore, a short communication introduced a GDi-CRISPR system (gene drive delta site integration system by the CRISPR system) for multi-copy integration in *S. cerevisiae*, which holds great promising for advancing the development of *S. cerevisiae* multi-copy integration tools [93].

4. Future challenges and prospections in the applications

As an emerging field, synthetic biology has high potential applications in drug-discovery, development of medical therapeutics, diagnostic tools and improvement of bioproducts. And its emerging applications include vaccine development, cancer treatment, prevention and treatment of infection, microbiome engineering, cell therapy and regenerative medicine, biofuels as well as genome engineering [94]. New technologies, such as CRISPR/Cas-mediated genome editing, will enable synthetic biologists to take a more holistic engineering approach, modifying synthetic circuits and the host genome with relative ease [95]. Nowadays, the CRISPR/Cas system is only 8 years old. With great progress in gene editing technology, CRISPR/Cas systems surely will greatly boost the development of gene therapy, basic biological research, and synthetic biology, let alone in the research field of *S. cerevisiae*. However, the applications of CRISPR/Cas systems have still encountered several major challenges including off-target effects, delivery modalities, Cas9 cleavage activities and immune responses.

Off-target effects of CRISPR/Cas systems usually result from mismatches between the guide RNAs and their target gene sequences [96], and may result in targeting to unexpected sequences of nucleic acids. Many efforts have been done to lower unwanted off-target effects of CRISPR/Cas. Previous studies reported that the systems could effectively maximize on-target activity and minimize off-target effects for genome engineering either by modifying guide RNA or Cas9 nuclease [97, 98].

5. Conclusion

Thanks to the eximious predominance of yeast genetics, the organism *S. cerevisiae* has enjoyed gene regulation technology for decades. In spite of some limitations needed to be conquered, the advent of the CRISPR techniques have undoubtedly created a new era for genomic engineering in yeasts. CRISPR/Cas system as one of the most cutting-edge biotechnology will continue to not only improve our insight into the complexity of cells but also help us make better utilization of living systems. Taking together, the use of CRISPR/Cas systems for various synthetic biology applications, specially in the synthetic genome of yeast *S. cerevisiae*, has greatly accelerate food industry, biomedical study and agricultural research.

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Conflict of interest

The authors declare no conflict of interest.

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