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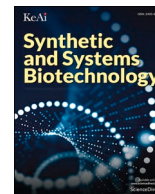
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Review Article

The biological principles and advanced applications of DSB repair in CRISPR-mediated yeast genome editing

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ABSTRACT

To improve the performance of yeast cell factories for industrial production, extensive CRISPR-mediated genome editing systems have been applied by artificially creating double-strand breaks (DSBs) to introduce mutations with the assistance of intracellular DSB repair. Diverse strategies of DSB repair are required to meet various demands, including precise editing or random editing with customized gRNAs or a gRNA library. Although most yeasts remodeling techniques have shown rewarding performance in laboratory verification, industrial yeast strain manipulation relies only on very limited strategies. Here, we comprehensively reviewed the molecular mechanisms underlying recent industrial applications to provide new insights into DSB cleavage and repair pathways in both *Saccharomyces cerevisiae* and other unconventional yeast species. The discussion of DSB repair covers the most frequently used homologous recombination (HR) and nonhomologous end joining (NHEJ) strategies to the less well-studied illegitimate recombination (IR) pathways, such as single-strand annealing (SSA) and microhomology-mediated end joining (MMEJ). Various CRISPR-based genome editing tools and corresponding gene editing efficiencies are described. Finally, we summarize recently developed CRISPR-based strategies that use optimized DSB repair for genome-scale editing, providing a direction for further development of yeast genome editing.

1. Introduction

Yeasts, such as *Saccharomyces cerevisiae*, *Scheffersomyces stipitis*, *Yarrowia lipolytica* and *Schizosaccharomyces pombe*, are considered important eukaryotic organisms in both molecular mechanism research and industrial production applications [1]. During the fermentation process, yeast genomes may undergo DSBs (double-strand breaks) when exposed to damaging agents. Unrepaired or erroneously repaired breaks can lead to the blockage of essential DNA transactions, genome instability and, potentially, cell death [2]. In response to these potential risks, yeasts have evolved multiple mechanisms to restore damaged genomes. End resection is generally believed to determine the outcome of DSB repair [3]. As shown in Fig. 1B, when end resection is abrogated, blunt-end DSBs are repaired by NHEJ (nonhomologous end joining) ligation without a homologous template [4]. Alternatively,

homology-based pathways, such as HR (homologous recombination) (Fig. 1C), SSA (single-strand annealing) (Fig. 1D) and MMEJ (microhomology-mediated end joining) (Fig. 1E), can be used [5,6].

With the development of genome editing technology, artificially introduced DSBs are being widely used in yeast systems to enhance the probability of on-target DSB repair [7]. In addition to ZFN (zinc finger nuclease) technology [8] and TALEN (transcription activator-like effector nuclease) technology [9], the most commonly used system is the type II CRISPR–Cas (clustered regularly interspaced short palindromic repeats CRISPR-associated genes) system originating from the immune defense mechanism of bacteria and archaea, which can effectively edit target genes in various genomic loci [10]. The cleavage protein Cas9 is guided by either one a single gRNA or multiple gRNAs to the target sites and then searches for the protospacer adjacent motif (PAM) sequence and creates double-stranded breaks (DSBs) [11] (Fig. 1A). After cleavage, the corresponding DSB repair machinery is

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Abbreviations

DSB	double-strand break
HR	homologous recombination
NHEJ	nonhomologous end joining
IR	illegitimate recombination
SSA	single-strand annealing
MMEJ	microhomology-mediated end joining
CRISPR	clustered regularly interspaced short palindromic repeats
ZFN	zinc finger nuclease
TALEN	transcription activator-like effector nuclease
PAM	protospacer adjacent motif

activated according to the end resection type and donor type. In addition to Cas9, some other cleavage proteins, such as Cas12a (Cpf1) [12], Cas12f (Cas14) [13], and an engineered Cas9 nickase [14], have also been developed as genome editing tools to meet a variety of demands.

This combination of CRISPR systems and DSB repair pathways offers numerous potential tactics for genome modification, wherein the CRISPR system can introduce DSBs at specific gene loci and the DSB repair pathways can facilitate varied genome modifications. The most commonly used combination of the CRISPR system with HR repair can lead to gene knockout or knock-in by donor substitution near the DSB site. The combination of the CRISPR system with NHEJ repair can introduce uncertain site mutations during the repair process, indicating the potential capability of this strategy to induce adaptive evolution.

This article aims to improve the understanding of different types of DSB repair mechanisms that, in conjunction with CRISPR system-based editing systems, can be applied in industrial yeast genome modification. Furthermore, this review offers a summary and suggests prospects for the future development of yeast cell factory modification.

2. CRISPR-mediated genome editing through HR repair

As a representative yeast strain that predominantly undergoes HR repair, *S. cerevisiae* plays a vital role in mechanistic research. The investigation of different repair methods in *S. cerevisiae* offers ideas for genome editing in organisms in which HR repair is not the dominant mechanism. In this section, we review the advances in CRISPR-mediated genome editing through HR repair in *S. cerevisiae* and nonconventional yeast species (Table 1).

2.1. Molecular mechanism of HR

Due to its precise repair capacity, HR is the most frequently used DSB repair method for genome editing. During the HR process, a homologous fragment from the external or in vivo environment serves as a template for error-free repair. The process is mediated by members of the *RAD52* epistasis group, and these genes, including *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *MRE11*, and *XRS2*, are involved in the mechanisms of almost all HR subpathways. When DSB happens, an exonuclease is recruited to the break site for end resection, and this process is promoted by an essential complex called Mre11-Rad50-Xrs2 (MRX) [16]. Meanwhile, the hyperphosphorylated replication protein A (RPA) combines to the single-stranded DNA and prevents the formation of

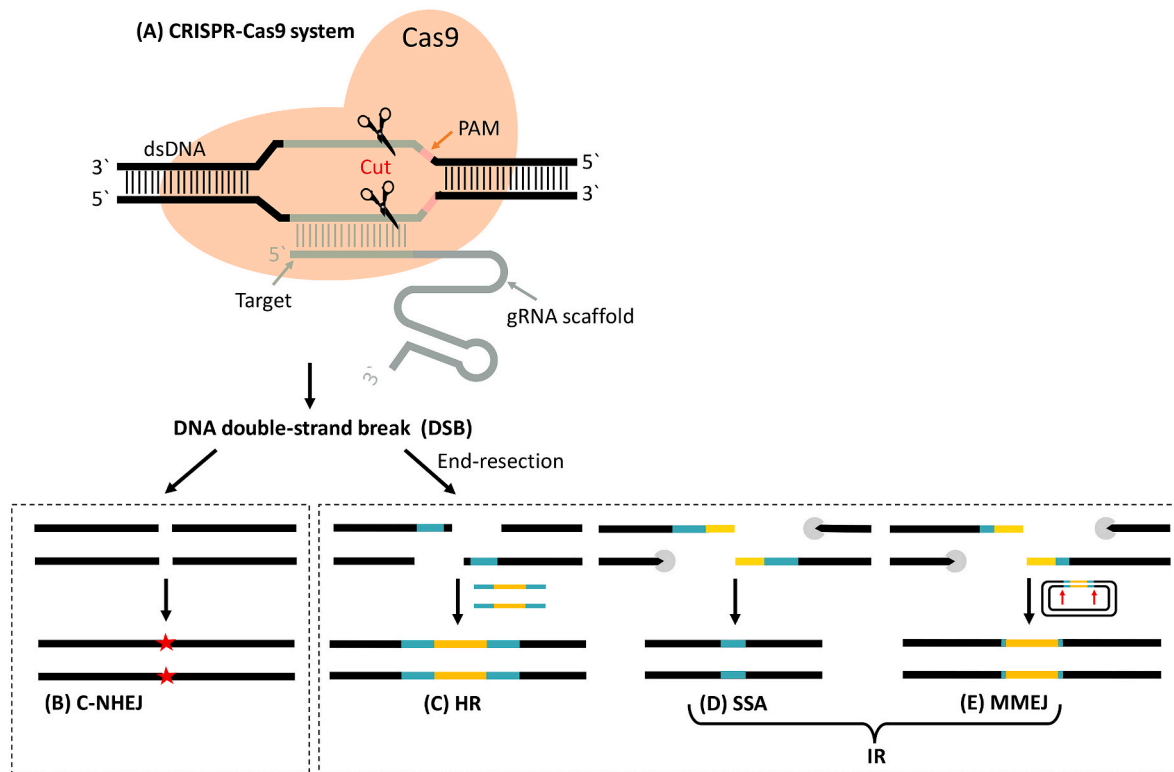


Fig. 1. Schematic diagram showing the CRISPR–Cas9 system and DSB repair strategies. **(A)** CRISPR–Cas9 system. The Cas9 protein is directed to the target site by a gRNA (guide RNA) and cleaves the double strand next to the PAM sequence to produce DSBs. **(B)** When end-resection is blocked, broken strands are restored through NHEJ without a donor fragment, and minor mutations or large indels may be introduced after repair. When end-resection occurs, broken strands could be repaired through **(C)** HR (homologous recombination) repair with a long-arm donor, which is a precise process; or **(D)** SSA (single-strand annealing) without a donor, a long homologous repeat (shown in blue) at the 3' end of a DSB tail is aligned, and then, the intervening sequence and 3' end are removed, no deletion or a large deletion may be introduced after repair; or **(E)** through MMEJ (microhomology-mediated end-joining) with a short-homologous donor, two shorter homologous sequences flanking the ends of DSB tails are aligned separately [15]. SSA and MMEJ are both considered one type of IR (illegitimate recombination).

Table 1
HR repair of double-strand break for genome editing.

Host Strain	Tools	Editing efficiency	Donor	Target	Application	Ref.
<i>S. cerevisiae</i>	CRISPR–Cas9, single gRNA	Recombination rate as high as 100%	90 bp donor	<i>ADE2</i>	–	[22]
<i>K. marxianus</i>	CRISPR–Cas9, single gRNA	Integrate three genes with 60% efficiency	700 bp homology arm	EGFP, YFP, and DSRED at the ABZ1 locus	Production of aromatic alcohol 2-phenylethanol	[23]
<i>S. cerevisiae</i>	CRISPR–LbCpf1, crRNA array	Single integration: up to 89% efficiency Triple integration: up to 96% efficiency	50 bp homology arm	<i>crtE</i> , <i>crtYB</i> , <i>crtI</i> , and <i>YFP</i> <i>crtE</i> , <i>crtYB</i> , and <i>crtI</i> into <i>INT1</i> , <i>INT2</i> , and <i>INT3</i> loci	–	[24]
<i>Pichia pastoris</i>	CRISPR–Cpf1, crRNA	One, two or three genes with 90%, 80% and 30% efficiency	250 bp homology arm	<i>ADE2</i> , <i>CAN1</i> , <i>OCH1</i>	Introduction of lycopene biosynthetic pathway	[25]
<i>S. cerevisiae</i>	Prime Editing: reverse transcriptase fused SpCas9 nuclease, pegRNAs	Up to 37% efficiency	–	Plasmid with GFP–mCherry fusion reporter	–	[26]
<i>S. cerevisiae</i>	CRISPEY: CRISPR–Cas9, gRNA with bacterial retron element	Deletion of nearly 100% efficiency Insertion of 92% efficiency	50 bp ss DNA homology arm	<i>ADE2</i> <i>ADE1</i>	Reveal the relationship between genotype and phenotype at a single-base resolution	[28]
<i>S. cerevisiae</i>	GTR-CRISPR: CRISPR–Cas9, gRNA-tRNA arrays	Disruption of up to 8 genes with up to 87% efficiency	120 bp donors	<i>FAA1</i> , <i>FAA4</i> , <i>POX1</i> , <i>ARE2</i> , <i>ARE1</i> , <i>PAH1</i> , <i>LPP1</i> , and <i>DPP</i>	Production of free fatty acid	[30]
<i>S. cerevisiae</i>	CRISPR-AID: CRISPRa (A), CRISPRi (I), CRISPRd (D), gRNA library	5-fold activation (A), 10-fold interference (I), 98% for gene deletion (D)	100 bp donor for deletion	mCherry (A), mVenus(I), <i>ADE2(D)</i>	Production of β -carotene; increase the EGII display level	[32]
<i>S. cerevisiae</i>	CHANGE: CRISPR–Cas9, gRNA library,	Average editing efficiency of 90%	100 bp donor	<i>CAN1</i> and <i>UBC4</i>	Produce genome-wide yeast mutants with single-nucleotide precision.	[33]
<i>S. cerevisiae</i>	CRISPR–FnCpf1	Four genes deletion: up to 100% efficiency	60 bp homology arm	<i>ADE2</i> , <i>CAN1</i> , <i>HIS4</i> and <i>PDR12</i>	–	[34]
<i>Yarrowia lipolytica</i>	CRISPR–Cpf1, crRNA	Indel mutation higher than 93% Duplex editing: up to 83% Triplex editing: up to 41.7%	100 bp homology arm	<i>Can1</i> , <i>Ura3</i> , <i>Met25</i>	–	[36]
<i>Schizosaccharomyces pombe</i>	CRISPR–Cpf1, crRNA	Single deletion: up to 50% Double deletion: up to 17% Triple deletion: very low	800 bp homology arm	<i>Ade6</i> , <i>Leu1</i> , <i>His3</i>	–	[37]
<i>S. cerevisiae-nej1Δ</i>	CRISPR–Cas9, paired gRNA	Direct mutation rate of 95.5%–99.8%	–	<i>Ho</i> , <i>his2</i> , <i>mnd1</i> , <i>spo11</i> , <i>spo13</i> , <i>ste3</i> , <i>can1</i> <i>trp1</i>	–	[39]
<i>S. stipites-ku70Δku80Δ</i>	CRISPR–Cas9, gRNA	Up to 71%	500 bp homology arm	–	–	[41]
<i>S. stipitis</i>	CRISPR–Cas9	80%–100%	–	<i>ade2</i> , <i>trp1</i>	–	[42]
<i>P. pastoris-ku70Δ</i>	CRISPR–Cas9, gRNA	Single locus: 75%–97.9%. Double locus: 57.7%–70%. Triple locus 12.5%–32.1%. Up to 56%	1000 bp homology arm	eGFP and mCherry, BFP	Introduction of biosynthetic pathways of 6-methylsalicylic acid and 3-methylcatechol	[43]
<i>Y. lipolytica-ku70Δ</i>	–	Up to 56%	500 bp homology arm	<i>URA3</i> integration into <i>ADE2</i> locus	–	[44]
<i>Y. lipolytica</i>	CRISPR–Cas9, tRNA-sgRNA fusions	Up to 78% Up to 90%	1000 bp homology arm 50 bp homology arm	Integration of <i>URA3</i> into <i>PEX10</i> site Deletion of <i>XPR2</i>	–	[45]
<i>K. lactis</i> and <i>K. marxianus</i>	Golden Gate assembly	<i>K. lactis</i> CBS 2359 (31%) <i>K. marxianus</i> NBCR 1777 (24%)	480 bp homology arm	<i>ADE2</i>	–	[46]
<i>K. lactis-ku80Δ</i>	CRISPR–Cas9, gRNA	Triple integrations occurred at a rate of 2.1%	500 bp homology arm	Six genes in three groups into <i>DIT1</i> , <i>ADH1</i> , and <i>NDT80</i> loci <i>ADE12</i> , <i>PHO1</i> , <i>PHO11</i> , and <i>PHO84</i>	Integration of the muconic acid biosynthetic pathway	[47]
<i>Ogataea polymorpha</i>	CRISPR–Cas9, tRNA-sgRNA fusion	17%–71%	60 bp homology arm	<i>OpADE2</i> , <i>OpKU80</i>	Perturbation of phosphate-responsive signaling pathway	[48]
<i>O. polymorpha-ku80Δ</i>	CRISPR–Cas9, gRNA	93.4%–100%	1000 bp homology arm	–	Production of fatty alcohol	[49]
<i>Y. lipolytica</i> <i>K. marxianus</i> <i>H. polymorpha</i> <i>S. stipitis</i> <i>Y. lipolytica</i> <i>S. cerevisiae</i>	LINEAR, CRISPR–Cas9, gRNA	Deletion efficiency of 67–100%	500 bp homology arm	<i>PEX10</i> , <i>XYL2</i> , <i>ADE2</i> and <i>ARO10</i>	Introduction of the plant source (S)-norcoclaurine pathway	[51]
<i>S. pastoris</i> <i>K. lactis</i> <i>K. marxianus</i>	Cell cycle synchronization CRISPR–Cas9, gRNA	0%–15% 6%–17% 1.6%–5.4% 79%–97% Up to 97%	40 bp–50 bp homology arm 900 bp homology arm	<i>ADE2</i> <i>NatMX</i> into <i>URA3</i> site	– Enhancement of lipogenesis production	[53] [77]

secondary structures. When end resection is formed, Rad51 and Rad52 are recruited to the end tail and extended along ssDNA, and RPA is generally replaced with Rad55 and Rad57. Finally, the strand invasion happens with the help of Rad54 [17–19]. Notably, RAD52 unavailability leads to severe defects and radio sensitization. In addition to the RAD52 epistasis group, the DNA polymerase-encoding genes *POL1*, *POL2*, and *POL3* are required for DNA replication and HR repair [20] (Fig. 2). DNA polymerase models have been used to identify possible ways to enhance HR efficiency in different yeast species.

2.2. HR-based single-site genome editing with the CRISPR system

S. cerevisiae frequently serves as a cell factory for fermentation-based production of metabolites with significant industrial value [21]. Due to its profound ability to repair DSBs through the accurate HR repair method, numerous studies have been published on single-site or multiple-site DSB repair in this organism. The first record of genome editing in *S. cerevisiae* through CRISPR–Cas9 was published in 2013, which demonstrated three strategies for site-specific genome editing with both NHEJ and HR repair. When Cas9 was constitutively expressed with a transitory gRNA cassette, the direct mutagenesis of *CAN1* through NHEJ repair increased the mutation rate up to 140-fold. When a 90 bp double-stranded donor was added, the recombination rate of *ADE2* through HR repair increased up to 130-fold. When the gRNA was constitutively expressed from a plasmid, the recombination rate of donor DNA reached as high as 100% [22].

In addition to *S. cerevisiae*, the CRISPR–Cas9 mediated multigene integration tool is also employed in the stress-tolerant yeast *Kluyveromyces marxianus*. This system could successfully integrate a 5 kb insert consisting of three genes with 60% efficiency at the *ABZ1* locus. The engineered strain with high expression of *KmARO4*^{K221L}, *KmARO10* and *KmPHA2*, moderate expression of *KmARO7G141S*, and disruption of *KmEAT1* could produce as high as 766 ± 6 mg/L 2-phenylethanol after 5 days of fed-batch cultivation [23].

With the expansion of CRISPR–Cas family endonucleases, another

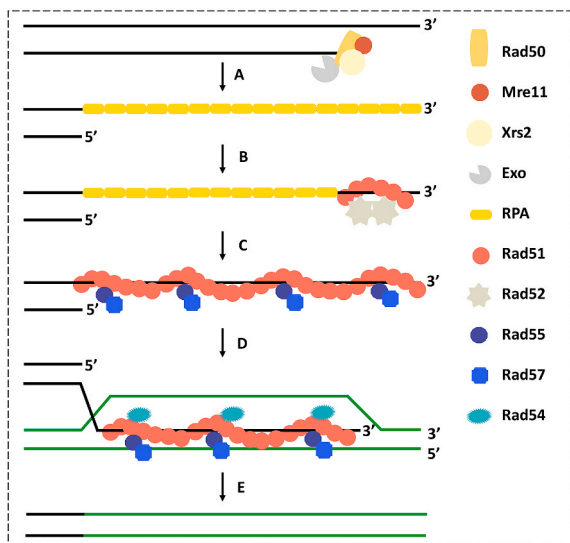


Fig. 2. Model showing strand invasion by Rad52 epistasis group proteins. (A) End resection is generated by an MRX (Mre11, Rad50, and Xrs2) complex and an exonuclease. RPA (replication protein A) coats the end tail to eliminate the formation of secondary structures. (B) Rad51 combines with the RPA-coated end tail through the recruitment of Rad52. (C) Rad51 is continuously extended along ssDNA (single-stranded DNA), RPA is generally replaced with Rad55 and Rad57. (D) The Rad51 nucleoprotein filament is located, pairs with a homologous DNA donor sequence and promotes chromatin remodeling and strand invasion by interacting with Rad54. (E) A DNA double-strand with the invading donor is formed.

novel endonuclease called Cpf1 (also known as Cas12a, Fig. 4) has been used for genome editing in *S. cerevisiae*. Cpf1 cleaves DNA distal from the PAM and generates staggered ends. This protein does not need a tracrRNA since it harbors a distinct endonuclease domain [24]. To date, three Cpf1 orthologs have been characterized for genome editing in *S. cerevisiae*. For single-site integration into the INT1 locus, the AsCpf1, LbCpf1, and FnCpf1 proteins could achieve up to 19%, 89%, and 87% efficiency in integrating the carotenoid pathway genes or up to 58%, 84%, and 88% efficiency in integrating the YFP gene, respectively. With a single crRNA array containing three guide sequences spaced with 20 bp direct repeats, the LbCpf1 protein achieved the highest genome editing efficiency of 91% ± 5% in integrating three carotenoid pathway genes, *crTE*, *crTYB*, and *crTI*, into three different integration loci, INT1, INT2, and INT3 [24]. In addition to *S. cerevisiae*, the first attempt to use Cpf1 in an unconventional yeast, *P. pastoris*, realized the deletion of DNA fragments as large as 20 kb and the integration of multiplexed gene fragments. The efficiencies of editing one, two or three genes through the optimized system reached 90%, 80% and 30%, respectively, which were higher than the documented editing efficiency of the Cas9 protein. This system successfully introduced a heterologous lycopene biosynthetic pathway by integrating the *CrtE*, *CrtB*, and *CrtI* expression cassettes into NTS loci [25].

Recently, a next-generation approach named prime editing was developed for precise genome editing. The cleavage protein was a fusion of a catalytically impaired Cas9 (H840A) nickase for single-strand cleavage and an M-MLV reverse transcriptase for DNA editing template synthesis. The prime editing guide RNA (pegRNA) contained a guide RNA for targeting and a primer editing sequence for introducing transversion, insertion, and deletion mutations (Fig. 4). This system was tested by restoring nonsense or frameshift mutations between GFP and mCherry in *S. cerevisiae*, which successfully restored the ability of 37% of yeast transformants to express both GFP and mCherry [26]. Subsequently, prime editing was modified for DSB repair based on a SpCas9 nuclease (PE_n), combining a reverse transcriptase with the wild-type SpCas9 nuclease, along with pegRNAs or modified Single PRimed INsertion gRNA (springRNAs) to realize both HR repair and precise NHEJ repair. This system was applied to promote small DNA insertions (6–18 bp) in HEK293T cells with efficiency ranging from 3% to 20% according to the target gene [27]. There have been no reports on the use of this strategy in yeast to date, though this would be a worthwhile and versatile genome editing toolbox to proceed with in the future.

A comparative study also focused on a single nucleotide resolution mutant named Cas9 Retron precISE Parallel Editing via homology (CRISPEY), which was developed soon after. A bacterial retron element was linked with gRNAs onto a low-copy-number plasmid and transformed into haploid *S. cerevisiae* to generate single-strand DNA molecules within the nuclease to enhance the HR repair efficiency (Fig. 4). CRISPEY was shown to have an editing efficiency of nearly 100% in single-locus editing targeting *ADE2*, as well as 92% efficiency in the insertion of a 765-base fragment into the genome [28]. This system can be applied to reveal the relationship between genotype and phenotype at a single-base resolution. A total of 16000 SNPs between the wild-type strain and variant strain were selected as targets, and 32000 guide-donor pairs were designed to introduce variant alleles into the wild-type strain. The edited colonies were then cultured and sampled at different time points to test the abundance of guide-donor oligos. The irreproducible discovery rate (IDR) was utilized to indicate the reliability of each variant caused by guide-donor pairs [29]. In this research, 572 variants were discovered with an IDR below 5%, indicating the fitness of specific variants between two strains [28].

As a preliminary test, single-site genome editing is frequently used when new strategies are tested, or existing strategies are tested in unconventional yeasts. It is highly possible that with the development of these strategies, multiple-site genome editing will be investigated for a wider range of applications.

2.3. HR-based multiple-site genome editing with the CRISPR system

Previous studies proposed future research directions for editing multiple genomic targets with an array of gRNAs cassette, which was subsequently achieved by the following strategies. One of them applied a gRNA-tRNA array (GTR) to realize high-efficiency disruption of multiple genes in *S. cerevisiae*. The gRNA cassette contained SNR52 promoters, and SNR52 terminators could disrupt eight genes with efficiencies of 36.5% and 86.7% via one and two promoters, respectively. Notably, the removal of tRNAs or adding additional promoters and terminators to the array could decrease gene disruption efficiency. This system was applied to improve the production of free fatty acids in *S. cerevisiae* by decreasing the endogenous production of the competition products sterol esters and triacylglycerols. Eight genes, *FAA1*, *FAA4*, *POX1*, *ARE2*, *ARE1*, *PAH1*, *LPP1*, and *DPP1*, were targeted for gene deletion with 120 bp donors. The engineered strain could produce 559.52 mg/L free fatty acid, which is a 30-fold increase compared with the wild-type strain [30]. This system was upgraded to GTR2.0 thereafter with modified RNA and Cas9 protein, exhibiting a lower off-target rate and higher accuracy [31].

Another strategy for multiple-gene editing named CRISPR-AID with modified CRISPR-Cas9 enzymes was developed to realize the combinatorial optimization of metabolic pathways. This system could realize triple functions, including transcriptional activation (CRISPRa), transcriptional interference (CRISPRi) and gene deletion (CRISPRd), simultaneously in *S. cerevisiae*. Through combination of overexpressed *HMG1*, downregulated *ERG9* and deleted *ROX1*, the production of β -carotene was increased by 2.8-fold, which was better than the yields of either single- or double-functional systems. In another application, several well-functioning genes that could affect the expression of *Trichoderma reesei* endoglucanase II (EGII), including 15 targets for CRISPRa, 18 targets for CRISPRi and 6 targets for CRISPRd, were selected to construct a gRNA library containing 1620 combinations. This library was expressed and screened for the best combination, which contained overexpressed *PD11*, downregulated *MNN9* and deleted *PMR1*, which increased the EGII display level and cellulose activity the most [32].

In another study, a strategy based on a genome-wide gRNA library and high-efficiency HR repair was developed to realize trackable precise editing within the whole yeast genome. A gRNA library containing 24,765 guide sequences targeting more than 97% ORFs annotated in the *S. cerevisiae* genome was designed to produce frameshift mutations, resulting in a pooled single mutant library with an 8 bp deletion in each gene. This mutant library was subsequently applied to improve furfural tolerance by single-nucleotide precision editing. Through a homologous donor with a 20 bp barcode flanked by two codons and two 40 bp homologous arms, the enriched nucleotide mutations D345 and T355 were validated and tracked [33]. The CHAnGE system offers a promising approach for precise genome editing; however, the complexity of library construction remains a major challenge.

The CRISPR-Cpf1 system was also applied for multiple-site gene editing. The LbCpf1 protein mentioned in the previous section was used to successfully integrate three carotenoid pathway genes into three different integration loci [24]. Another Cpf1 protein, FnCpf1, integrated into the genome with a crRNA expression cassette constructed onto a plasmid, could achieve simultaneous deletion of *ADE2*, *CAN1*, *HIS4* and *PDR12* with up to 100% efficiency in *S. cerevisiae*. Even though the PAM sequence of 5'-TTN-3' is functional during gene editing, FnCpf1 exhibited a strong preference for the PAM 5'-TTTV-3', and the absence of thymidine as the first base after the PAM could highly increase the genome editing efficiency [34].

In addition to *S. cerevisiae*, the CRISPR-Cpf1 system has also been employed in *Y. lipolytica*, *S. pombe*, and *P. pastoris*, greatly diversifying their impact in biotechnology-based production [35]. Single-gene editing for indel mutations in *Y. lipolytica* in both arginine permease gene *CAN1* and orotidine 5'-phosphate decarboxylase gene *URA3* achieved an efficiency higher than 93%. The editing efficiency for duplex genomic

targets (*CAN1-URA3* and *CAN1-MET25*) and triplex genomic targets (*CAN1-URA3-MET25*) reached 75%–83% and 41.7%, respectively [36]. In the fission yeast *S. pombe*, the phosphoribosyl aminoimidazole carboxylase gene *ade6* was successfully deleted with an efficiency of up to 50%. Double deletion among *ade6*, *leu1* and *his3* could achieve an efficiency of up to 17%. Although triple deletion of all these genes was achieved with a crRNA array driven by the *fbal1* promoter, the efficiency was still very low [37].

The metabolic pathways relevant for industrial production in *S. cerevisiae* often require the collaboration of multiple genes and many regulatory factors; thus, multi-locus editing techniques are essential for the industrial application of genome editing technologies. The optimization of existing strategies to meet the demands of higher product yield is currently an important research direction.

2.4. Optimization of HR-based genome editing by knocking out the NHEJ pathway

In most unconventional yeast species, gene introduction at target sites shows a limited HR rate with equivalent homology templates, and this rate dramatically decreases when a large expression cassette or multiple blocking sequences are inserted [38]. To prevent unnecessary repair other than HR, essential genes associated with NHEJ repair, including *KU70*, *KU80* and *NEJ1* [39], could be deleted. Knocking out the *ku70* and *ku80* genes in the Ku complex is a popular strategy for artificially enhancing the HR rate. This strategy shows better performance when applied to unconventional yeasts with reduced HR efficiency.

The unconventional yeast strain *S. stipitis* shows an outstanding ability to consume xylose and can potentially produce natural product derivatives [40]. However, this species tends to repair DSBs through the less precise NHEJ repair [41]. By applying the CRISPR-Cas9 system, the gene knockout efficiency of *S. stipitis* targeting *ade2* could be improved from <1% to more than 80% [42]. With this CRISPR-Cas9 system, the *ku70* and *ku80* genes were deleted through two rounds of knockout assays. The engineered strain exhibited HR-based genome editing dominance and enhanced accurate gene knockout efficiency targeting *trp1* by up to 4.5-fold compared with the parent strains. Notably, NHEJ was not totally useless here, as one of the Ku complex genes was knocked out through NHEJ repair without a donor [41].

Apart from *S. stipitis*, the corresponding systems were also developed in several other frequently used unconventional yeast hosts, including *Y. lipolytica*, *P. pastoris*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, and *Ogataea polymorpha* (*Hansenula polymorpha*). An attempt in *P. pastoris* with *KU70* deletion was first constructed using the CRISPR-Cas9 based gene deficiency approach. The resulting strain could repair DSBs through HR with increased efficiency from less than 20% to greater than 70% [43]. The verification of the *Y. lipolytica* $\Delta ku70$ mutant for *URA3* gene replacement at the *ADE2* locus has been described, which showing 43% and 56% efficiency with 50 bp and 500 bp homologous arms, respectively. However, deletion of *KU80* could not realize HR regardless of the length of the flanking fragments and even decreased NHEJ activity by 60% compared with the wild-type strain [44]. Based on tRNA-sgRNA fusion, the integration of *URA3* at the *PEX10* site showed HR rates of 78% and 29% with 1 kb and 0.5 kb homology arms, respectively. Deletion of *XPR2* showed as high as 90% efficiency with 50-bp-long homology arms [45].

When constitutively expressing Cas9 along with a plasmid containing ribozyme-flanked gRNAs, two *Kluyveromyces* species, *K. lactis* and *K. marxianus*, exhibited at least 24% efficiency for HR targeting *ADE2* [46]. Another system with an efficiency as high as 64% for triple deletion in *K. lactis* was used for integration of the muconic acid biosynthetic pathway, which contained 6 DNA fragments. The Cas9 protein was integrated into the *GAL80* locus in the genome and *YKU80* was deleted to eliminate the effects of NHEJ. The engineered strain appeared to produce the precursor PCA at 0.9 g/l and could fully convert all available catechol to

muconic acid when fed with catechol [47]. However, a similar strategy in the *ku80* mutant of the methylotrophic yeast *Ogataea parapolymorpha* only achieved an efficiency of lower than 1% for *ADE2* deletion [46]. When tRNA-sgRNA fusion was applied, the editing efficiency of another strain of *O. polymorpha* ranged from 17% to 71%, targeting *ADE12*, *PHO1*, *PHO11*, and *PHO84* [48]. A comparative study modified *O. polymorpha* by overexpression of HR-related proteins and downregulation of *KU80*, which led to an increase in the HR rate from 20%–30% to 60%–70%. This system was applied for homologous integration of the fatty alcohol production pathway. The strain with triple deletion of *HFD1*, *POX1*, and *FAA1*, overexpression of MmCAR and its cofactor *npgA*, and alcohol dehydrogenase *ADH5* originating from *S. cerevisiae* achieved the highest fatty alcohol production of 3.26 G 0.51 mg/L in rich media, showing the great potential of *O. polymorpha* as a host for methanol-based biorefinery [49].

In summary, blocking of NHEJ pathway genes increases the ability of NHEJ-dominant strains to undergo HR; at the same time, the DSBs created by the CRISPR proteins create more opportunities for homologous donors to participate in the repair process. Efficient CRISPR systems were developed for use in the aforementioned unconventional yeast strains, which expanded the likelihood of metabolic pathway remodeling of these hosts and application in industrial production [50].

2.5. Optimization of HR-based genome editing without disrupting the NHEJ pathway

Simply knocking out a pathway or stopping pathway activation to enhance another cellular pathway sometimes has unintended consequences. For instance, interruption of NHEJ may negatively affect cellular fitness and growth conditions, potentially impeding microbial factory capacity. Therefore, a CRISPR platform named the lowered indel nuclease system enabling accurate repair (LINEAR) was developed for HR-mediated accurate editing without destroying the NHEJ pathway. Four traditional NHEJ-based yeasts, *Y. lipolytica*, *K. marxianus*, *H. polymorpha* and *S. stipitis* were tested. The targeting of *PEX10*, *XYL2*, *ADE2* and *ARO10* led to an increased deletion efficiency of 67–100%. This system is used to introduce the plant source (S)-norcochlorine pathway into *S. stipitis* by leveraging NHEJ. A 5.7 kb expression cassette with three genes from the (S)-norcochlorine pathway and a 12 kb cassette with three genes from the shikimate pathway were integrated into the *XYL2* locus, with HR efficiencies of 80% and 41%, respectively. The resulting strains were screened, and the highest performing strain could produce 75 µg/l (S)-norcochlorine, which was nearly 15-fold more than the yield from the parent strain [51].

In addition, cell cycle synchronization could also be applied to control the selection of repair methods. When a single copy of chromosomal DNA is present (in the G1 phase, for example), NHEJ is favored. HR occurs only when multiple copies of chromosomes are available (in the S phase and G2 phase, for example) [52] (Fig. 4). The addition of hydroxyurea could block cells in S-phase with higher HR probability and enable higher efficiency genomic modification. By hydroxyurea-mediated cell cycle arrest maintaining cells in the S phase, the DSB repair ability of *ADE2* through HR was enhanced from 0% to 15%, 6%–17%, 1.6%–5.4%, and 79%–97% in *Y. lipolytica*, *S. cerevisiae*, *P. pastoris*, and *K. lactis*, respectively, with 40 bp–50 bp homology arm lengths [53].

Other studies on HR-related proteins in nonyeast organisms have also suggested ideas for HR-enhanced genome editing. The HR protein RAD51AP1 is able to drive the formation of RNA (R)-loops and helps link R-loops with DNA (D)-loops during DSB repair in HEK293T cells, which suggests the ability of this protein to enhance HR repair in NHEJ-dominant cells [54]. Another protein named RecA was characterized in *E. coli* *TB28*. This protein binds single-stranded DNA exposed at a DNA break site and quickly bounces open within 1 min to form a thin layer that expands throughout the nucleus, which is convenient for homologous sequence matching. The 2D search can increase the speed of

matching homologous sequences by more than 100-fold compared with the 3D search [55].

As an accurate DNA repair method, HR is the most widely used method in yeast genome editing. Along with the CRISPR system, we conclude available strategies based on whether or not to edit the DSB repair method (Table 1). When keeping the initial DSB repair method, single gRNA, multiple gRNA array, or tRNA-gRNA arrays can be applied to edit a single site or multiple sites with various CRISPR proteins, including Cas9, Cpf1 and functionally modified nucleases. Attempts have been made to effectively produce mutants in numerous yeast species, such as *S. cerevisiae*, *Y. lipolytica*, *S. pombe*, and *P. pastoris*, which have greatly expanded the choices for metabolic engineering that rely on these cell factories as hosts. To realize genome-scale editing, genome-wide gRNA libraries were constructed. CHANGE is one of the systems that can realize single-nucleotide precision editing within the whole genome of *S. cerevisiae*. Subsequently, the CRISPEY system was published for genome-wide precise parallel editing by enhancing the probability of introducing gRNA and the corresponding donor in the same cell. Prime editing is another precise genome editing system assisted by small insertions. However, the application of this system in yeast cells has not been reported.

Another commonly used strategy to enhance HR efficiency is knocking out NHEJ pathway-related genes, such as *KU70* and *KU80*. HR efficiency can be significantly enhanced in most unconventional yeast strains including *S. stipitis*, *Y. lipolytica*, *K. lactis*, *K. marxianus*, *O. polymorpha* and *P. pastoris*. The fusion of tRNA and gRNA could further enhance the efficiency. However, knocking out NHEJ can adversely affect the growth rate and industrial production. For this reason, the LINEAR system was constructed to repair DSBs through both HR and NHEJ without disturbing the original repair method. In other cases, enhancing the expression of HR-related proteins such as Rad51 and RecA, cell cycle synchronization, and degrading residual Cas9 in the G1 cell cycle when NHEJ function is robust could also help enhance HR efficiency.

3. CRISPR-mediated genome editing through NHEJ repair

The end-joining pathway has been conserved throughout evolution from bacteria to humans [56]. This repair method can contribute to DSB repair when a homologous donor is not provided. Although NHEJ is a very versatile and competent repair mechanism for both blunt and cohesive ends, the ability to rejoin cohesive overhangs, blunt ends and noncohesive ends can differ significantly among species [57,58]. For instance, in *S. pombe*, both blunt and cohesive DSB ends are proficiently repaired, but in *S. cerevisiae*, only cohesive ends are repaired with high efficiency [59]. Here, we summarize the latest advances in CRISPR-mediated genome editing through NHEJ repair in different yeast species (Table 2).

3.1. Molecular mechanism of NHEJ recombination

In contrast to accurate HR repair, NHEJ normally rearranges the broken strand more generally, which may lead to nucleotide loss or addition and chromosomal translocations [60]. Three core complexes participate in NHEJ repair, including the Ku complex (Yku70 and Yku80) [61], MRX complex (Mre11, Rad50 and Xrs2) [62] and DNA ligase IV complex (Dnl4 and Lif1) [63]. Other proteins, including the DNA ligase IV-associated protein Nej1 and the end-processing enzyme Pol X family member polymerase Pol4 [64], also play essential roles in NHEJ repair (Fig. 3).

During NHEJ repair, the Ku complex is the first factor to be activated due to its superior affinity for DNA ends, which it protects while maintaining the free ends for subsequent end-processing and ligation [65]. Meanwhile, the MRX complex functions as a DNA-binding factor and is immediately recruited to bring both DNA ends together [66]. Then, the Ku complex assists in recruiting the DNA ligase IV complex

Table 2
NHEJ repair of double-strand break for genome editing.

Host Strain	Tools	Editing efficiency	Target	Application	Ref.
<i>S. cerevisiae</i>	CRISPR–Cas9, sgRNA	Mutation rate of up to 140-fold	<i>CAN1</i>	–	[22]
<i>K. marxianus</i>	CRISPR–Cas9, gRNA	Deletion rate of nearly 90%	<i>URA3</i>	Enhancement of lipogenesis production	[77]
<i>K. marxianus</i>	–	–	<i>LEU1, LYS1, and MET17</i> et al.	Integration of linear DNA fragments	[76]
<i>Y. lipolytica</i>	YALICloneNHEJ	Assembly of 4, 7, and 10 fragments: up to 90%, 75%, and 50%	<i>HMG1, ERG8</i> and <i>ERG20</i> et al.	Production of sesquiterpene (–)- α -bisabolol	[78]
<i>Y. lipolytica</i>	genomic library	Enhanced random integration rate by up to 22.74 times	GFP at <i>URA3</i>	Production of lycopene	[79]
<i>Y. lipolytica</i>	–	–	<i>AtoB, HMGR, HMGS</i>	α -farnesene production	[80]
<i>Y. lipolytica</i>	CRISPR–Cas9, gRNA	Random integration: 1.6×10^4 colonies/ μ g DNA	Leu2	Enhance production of lipase and β -carotene	[81]
<i>Xanthophyllomyces dendrorhous</i>	CRISPR–Cas9, gRNA	64.3% of point mutants, 23.8 of deletion, 4.8% of chromosome rearrangement	CrtE and CrtS	–	[89, 90]

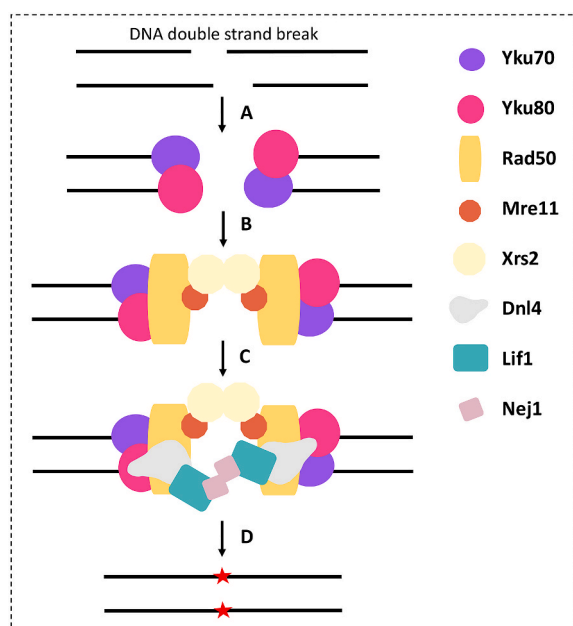


Fig. 3. Model showing a strand restored by NHEJ-related genes. (A) Ku complex (Yku70 and Yku80) binding a DSB end. (B) The Ku complex recruits the MRX complex (Mre11, Rad50, and Xrs2) and facilitates the bridging of the two ends. (C) Lig4, Lif1, and Nej1 are recruited and stimulate gap filling at the DNA ends. (D) The DSB has been repaired.

and the associated protein Nej1 together [67]. During the whole process, the MRX complex interacts with and promotes the intermolecular ligation of the DNA ligase IV complex [58], along with the Ku complex, throughout the process. The lack of the Ku complex can cause a significant reduction in the efficiency and accuracy of NHEJ repair, which is why Ku complex gene knockout is commonly used for NHEJ elimination [68]. Notably, the *NEJ1* gene was not verified as being an essential gene in NHEJ until 2002 [69], when researchers discovered that it supports the function of the DNA ligase IV complex and that yeast cells lacking Nej1 failed to undergo NHEJ repair [70]. Nej1 is inhibited in MAT α /MAT α diploids, which contain the $\alpha 1/\alpha 2$ repressor, but reactivation of Nej1p is sufficient to restore efficient NHEJ in this heterozygote [71]. The final protein involved in NHEJ repair is Pol4, which is the only Pol X family polymerase in yeast; Pol4 always interacts with the DNA ligase IV complex and stimulates its DNA synthesis activity [72].

3.2. NHEJ-based genome editing in NHEJ-dominant yeast strains

Although highly dominant in most unconventional yeast strains, NHEJ is not as frequently used as HR in genome editing due to its imprecision in repair. Although artificially abolishing NHEJ in hosts can lead to enhanced HR efficiency, this process may cause genome instability and affect the cell growth rate and transformation status [73]. Previous research has noted that the structure of DNA ends can influence the precision of NHEJ repair, especially when proceeding with imperfect complementary ends [74]. Thus, NHEJ could serve as a simpler substitute to induce genome-wide mutations. With increasing demand for unconventional yeast strains, including *K. lactis*, *Y. lipolytica*, *H. polymorpha* and *P. pastoris*, for industrial production [75], developing technologies to integrate heterologous genes or expression pathways in these strains has become increasingly important. The application of NHEJ repair with CRISPR proteins is being increasingly applied to these NHEJ-dominant yeasts.

NHEJ-mediated gene integration with DNA fragments was first developed in *K. marxianus* for integrative transformation. By integrating linear DNA fragments from the nutrient biosynthetic pathway originating from *S. cerevisiae*, related mutant genes including *LEU1*, *LYS1*, and *MET17*, were obtained through NHEJ in amino acid-deficient *K. marxianus* [76]. The CRISPR–Cas9 system was subsequently applied to establish the DSB on *K. marxianus* genome. To enhance lipogenesis, two transposases, *ALPHA3* and *KAT1*, were inactivated to prevent mating-type switching; competing pathway-related genes, including *ADH*, *ATF*, *EHT1*, *GPPI1*, *PEX10* and *MFE1*, were also inactivated; and lipid accumulation-related genes, including *DGA1*, *ACCI1*, and the dimer *ACL1/ACL2*, were overexpressed. The engineered strain was bred to isolate high-producing, thermotolerant, and plasmid-accepting strains, and the best-performing strain could produce a similar amount of lipids compared with the parent strain at 45 °C, under which the parent strain could not grow [77].

To realize multigene assembly in *Y. lipolytica*, a Golden Gate Assembly and NHEJ repair-based system named YALICloneNHEJ was developed. This system was applied for the production of sesquiterpene (–)- α -bisabolol by overexpression of related genes in the mevalonate pathway. Genes from this pathway, including *HMG1*, *IDI*, *ERG8*, *ERG10*, *ERG12*, *ERG13*, *ERG19*, and *ERG20*, were selected to enhance the terpene biosynthesis flux. After three rounds of random integration, the concentration of (–)- α -bisabolol increased from 2.5 mg/L to 242.6 mg/L, which is a nearly 100-fold improvement. Fed-batch fermentation experiments in a 5-L bioreactor further increased (–)- α -bisabolol production to 4.4 g/L after 168 h of culture, which is the highest (–)- α -bisabolol production reported in yeast thus far [78].

Previous research has demonstrated that both large DNA fragments and multiple fragments could be randomly integrated into the *Y. lipolytica* genome through NHEJ. The randomly engineered strains resulted

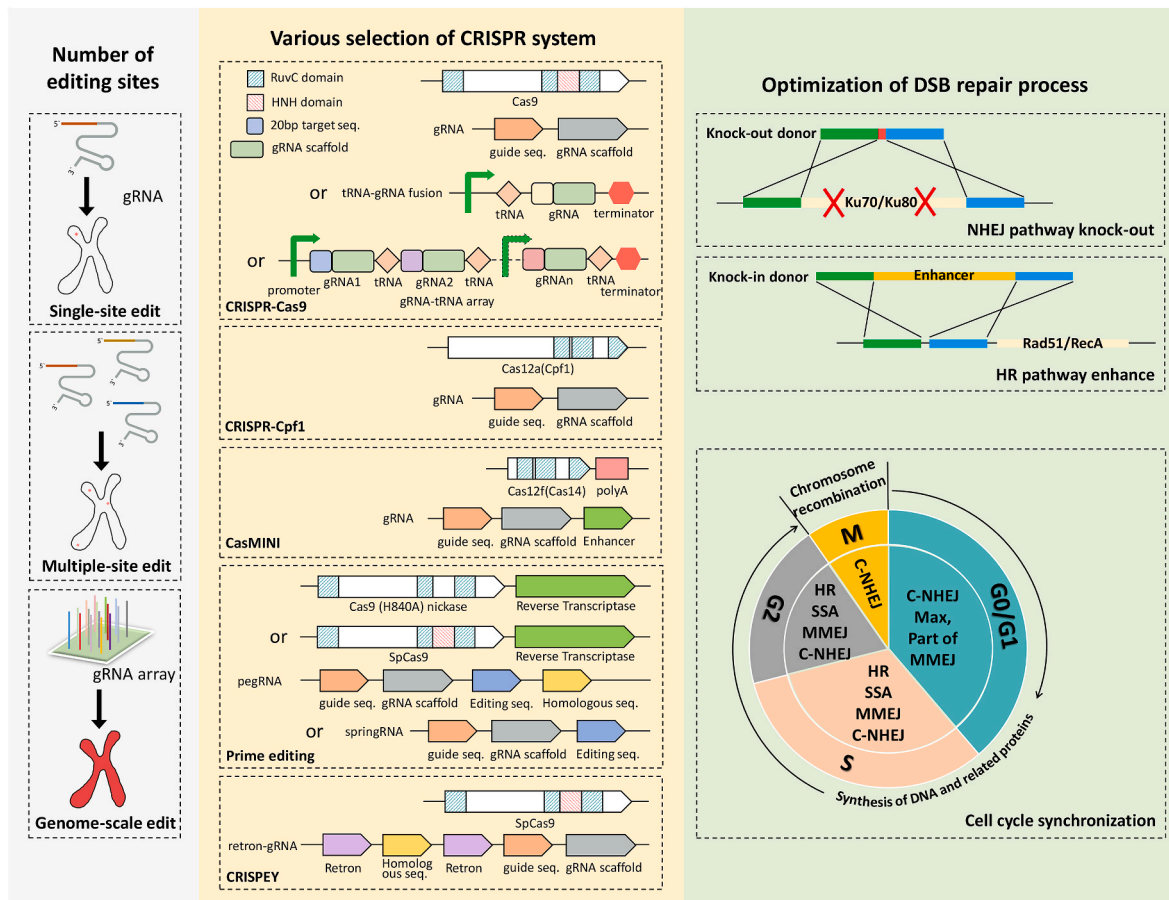


Fig. 4. Strategies for yeast genome editing. Depending on the requirements of genome editing, various cleavage proteins and gRNA designs can be selected. The competing repair pathway can be knocked out, and the required repair pathway can be overexpressed to enhance demand efficiency. The cell cycle phase can be controlled by cell cycle synchronization to enhance possibility of required repair pathways.

in a mutant library containing integrates at different loci, which could be used for the screening of better-performing strains. This technology was applied to create a lycopene-producing strain library for screening high lycopene-producing strains. The heterologous genes CRTE, CRTB and CRTI were randomly integrated into the genome and increased production by 23.8-fold compared with HR repair [79]. A similar strategy was employed to introduce the pathway of α -farnesene production in *Y. lipolytica*. The precursor mevalonate pathway and α -farnesene synthase pathway-related genes, including the acetyl-CoA acetyltransferase encoding gene AtoB from *E. coli*, NADH-dependent HMGR from *Bordetella petrii*, and endogenous HMGS from *Y. lipolytica*, were linked to a plasmid and integrated into the genome by NHEJ. The optimized strain could produce as much as 25.55 g/L α -farnesene after 288 h of fed-batch fermentation [80]. In the application for lipase production, an over-expression library was obtained by random integration of the LIP2 gene. The lipase activity reached as high as 1967 U/ml after 96 h of culture, which was 5.3-fold higher than that of the control. To identify the potential speed limit steps in the β -carotene synthetic pathway, related genes were divided into three groups and randomly integrated into the genome. The highest concentration of β -carotene reached 12.1 mg/g DCW, and the corresponding strain showed a higher transcription level of related genes, indicating the potential expression level of these genes within the pathway [81].

The strategy can be expanded to use NHEJ for genome-wide editing with a gRNA library. According to the above applications, the CRISPR system has not been widely applied to NHEJ-related genome editing, which would be a promising tool to define specific integration sites and reduce the proportion of unwanted integration. Some NHEJ-dominant

cell lines have been studied for the possibility of using Cas9 protein and gRNA to introduce DSBs in specific genome sites and then repair these DSBs through NHEJ to cause frameshift mutations or large indels. However, these events always lead to protein errors, such as premature cessation or frameshift translation, leading to purposeful protein inactivation [82].

A more robust NHEJ-based genome editing strategy was constructed with the assistance of CRISPR–Cas9 in mammalian cells [83]. Another application in mammalian cells utilizes protein engineering to generate a small CasMINI system originating from the Cas12f (Cas14) protein, which is less than half the size of Cas9, to realize NHEJ repair-based genome editing. This system is more compact and efficient at generating random mutants near the gRNA target sequence. The most effective variant showed over 120-fold higher gene activation than the nontargeting sgRNA control and was more than 30-fold better than wild-type dCas12f-VPR [84]. Other applications in rice (*Oryza sativa*) have used a genome-scale gRNA library for editing target whole-genome metabolic pathways. The library contained 88541 gRNAs targeting 34234 genes and was constructed to generate a NHEJ repair-based genome-wide mutant library. A total of 182 plants from the resulting mutant library were selected for sequencing. Among these, 139 plants had single correct sgRNAs, 38 plants had mutated sgRNAs, 4 plants had multiple sgRNAs, and one was nontransgenic. Eleven individual plants had increased tiller numbers, reduced heights, and twisted leaves. Sanger sequencing showed that these mutants were *tad1* homozygotes, and their phenotype was consistent with that of previously described mutants [85,86].

3.3. NHEJ-based genome editing in yeast strains with less dominant NHEJ repair

Previous research investigating NHEJ efficiency in *S. cerevisiae* showed that after Cas9 cleavage at a specific site, the survival rate could reach approximately 0.4%, 95% of the yeast cells showed mutations, and the most frequent mutants presented an “add one” insertion, which caused frameshift translation and very likely led to protein inactivation [87]. Knocking out HR pathways to enhance NHEJ efficiency seems to be a valid strategy for enhancing the survival rate, while reverse assays indicate that NHEJ repair efficiency is not enhanced when essential HR components are knocked out in *S. cerevisiae* [88].

Although NHEJ efficiency in some yeast species is relatively low, applying the CRISPR system and NHEJ for genome editing in these yeast species could still be achieved. Researchers have achieved Cas9 cleavage in the yeast *Xanthophyllomyces dendrorhous* with less NHEJ dominance, along with performing Sanger/Illumina sequencing to identify diverse DNA repair patterns, including DNA insertions/deletions, intra-chromosomal translocations, and on-target nucleotide substitutions (point mutations) [89]. Two genes, CrtE and CrtS, encoding the color of *X. dendrorhous*, were selected as target genes, and 283 color-identified mutants from 623,727 colonies were sent for Sanger sequencing. Among these mutants, 64.3% showed point mutations, 23.8% showed deletions, and 4.8% showed chromosome rearrangements [90].

Due to the complexity of metabolic pathways, the modification of one or several specific genes cannot always be used to comprehensively optimize the related phenotype [91]. Genome-wide random mutation could be a promising strategy. Although genome-scale gRNA libraries for whole-genome editing have been achieved in rice (*O. sativa*), similar reports in yeast species are rare. In the future, a system combining a genome-wide sgRNA library and NHEJ repair for random genome editing in yeast can be considered as a direction for enhancing yeast genome mutations. Several aspects of this strategy could be optimized when applying it to yeast. First, the library could be simplified. Since a considerable portion of genes are housekeeping genes or essential genes, editing of these genes is usually lethal to yeast and ineffective in improving yeast functions. Despite this, the NHEJ editing system can be used to achieve genome-scale editing more efficiently with only metabolic pathway genes targeted. In addition, in species with low NHEJ efficiency, such as *S. cerevisiae*, improvements in the NHEJ pathway are decisive factors affecting editing efficiency by NHEJ repair.

In conclusion, inhibiting NHEJ to enhance HR is not the only feasible method of genome editing. NHEJ can also be a substitute method for genome modification by random editing (Table 2). Applying NHEJ for effective genome editing has been reported in NHEJ-predominant strains, such as *K. marxianus* and *Y. lipolytica*, as well as non-NHEJ-dominant yeasts, such as *X. dendrorhous*. Genome-scale editing through NHEJ in these species could also indicate future research directions for random genome-wide editing in *S. cerevisiae*.

4. CRISPR-mediated genome editing through IR repair

In addition to HR and NHEJ, two other methods, MMEJ and SSA, are also useful for DSB repair in yeast genome editing. However, research investigating these mechanisms is limited because of their low efficiency. Here, we introduce the application of these two types of IR (illegitimate recombination) repair to better understand the less well-studied genome editing mechanisms.

4.1. Molecular mechanisms of MMEJ and SSA repair

MMEJ (microhomology-mediated end-joining), also known as Alt-EJ or A-NHEJ (alternative end-joining), has been found to be entirely dependent on the NHEJ pathway. Similar to NHEJ repair, MMEJ competes with HR to repair DSBs but is not affected by HR abrogation [19]. As an alternative to NHEJ, MMEJ requires a minor homologous

sequence at DNA ends. MMEJ tends to be more error prone than NHEJ repair and may cause chromosome translocation [92,93] (Fig. 1E). In addition, MMEJ generally repairs DNA slowly and inefficiently; thus, MMEJ has always been considered a backup strategy when NHEJ and HR are not applicable [94,95]. However, recent research has indicated that MMEJ plays a critical role in repairing one-ended DSBs after replication fork suspension and endogenous chromosomal DSBs [96,97].

Similar to MMEJ, SSA (single-strand annealing) anneals exposed complementary sequences in the genome with the assistance of RAD52 to complete repair at resected ends [98]. However, although this procedure is a type of homology-based repair, the minimal homologous length of DSB-induced SSA has been proven to be as short as approximately 70 bp in yeast strains. Without competing repair mechanisms, yeasts can perform SSA with 20–30 bp repeats, facilitated by DNA cleavage tools. The intervening sequence between repeats is deleted in the repaired product, resulting in the loss of genetic information [99–101] (Fig. 1D). In addition, this repair procedure is catalyzed and enhanced by topoisomerase I [102,103].

4.2. MMEJ- and SSA-based genome editing

In certain cases, the extra insertion of artificial sequences might affect the function of target genes with a long homologous arm, while MMEJ with a shorter homologous arm of approximately 10–40 bp can modify the genome in less time and with fewer steps. However, due to its extremely low occurrence and possible harmful effects on yeast, unconventional recombination is seldom applied to genome editing despite some special situations (Table 3).

The only application of the CRISPR system and MMEJ repair we found was reported in fission yeast *S. pombe*. A relatively low-homology sequence of approximately 25 bp on both sides showed satisfactory efficiency in introducing point mutations and epitope tags through the CRISPR–Cas9 system. In addition, a single-strand oligo with a 25 bp homologous sequence on both sides can introduce mutations with an efficiency of 90%, rendering them strong tools for inducing mutations without affecting marker genes [104].

Other studies have reported MMEJ- or SSA-based repair with ZFN cleavage, indicating possible application prospects for CRISPR-based editing. One of the attempts in *S. cerevisiae* was to cleave *Lys2* with ZFN for DSB induction. Some repair attempts showed 2 bp inserts, indicating the occurrence of NHEJ repair, and others showed large deletions, indicating the occurrence of MMEJ repair [105].

Another application of SSA with ZFNs in *S. cerevisiae* showed approximately 17.3% repair efficiency with 30 bp repeats. The introduction of ZFNs could increase the SSA efficiency by approximately 10⁴-fold, but this efficiency plateaued when the direct repeat sequence was expanded to approximately 130 bp [101].

Compared to ZFN, which only recognizes 9–18 bp DNA triplets, CRISPR-based DSB cleavage is more flexible and the target selection is easier, which indicating the possibility of applying CRISPR system for MMEJ- and SSA-based yeast genome editing in the future.

Despite limited applications in yeast species, SSA and MMEJ have been used in other species, providing ideas for further research in yeast species. One microorganism in which SSA or MMEJ is applied is the fungus *Aspergillus fumigatus*; in-frame integration in this organism was efficient, with approximately 35 bp homology arms, and showed an accuracy of greater than 95% [106]. In addition, these systems have been applied with the CRISPR–Cas system for editing in *Leishmania*, *Arabidopsis*, HEK293T cells, CHO cells and iPS (induced pluripotent stem) cells [107–111].

MMEJ and SSA repair both have certain connections with NHEJ or HR repair, and their editing efficiencies are low; therefore, they are often considered backup methods for repairing DSBs under certain circumstances. However, some characteristics of these two methods, such as the ability to repair a one-ended DSB after replication fork suspension and deletion of an intervening sequence between repeats, might offer

Table 3
MMEJ and SSA double-strand break repair for genome editing.

Host Strain	Tools	Repair Method	Strategy and efficiency	Target	Ref.
<i>Saccharomyces cerevisiae</i>	ZFNs	SSA	Enhances the SSA efficiency by approximately 10 ⁴ -fold	Gal4	[101]
<i>Schizosaccharomyces pombe</i>	CRISPR–Cas9, single gRNA	MMEJ	Approximately 20 bp homology on both sides can introduce a point mutation or epitope tags through the CRISPR system	Ura4	[104]
<i>S. cerevisiae</i>	ZFNs	MMEJ	Applies ZFNs to induce DSB and detects MMEJ repair through base changes	Lys2	[105]

alternative strategies for yeast cell factory remodeling. Therefore, further research investigating their possible application is warranted.

5. Conclusions and outlook

As a model strain, *S. cerevisiae* is commonly used in industrial production. Unconventional yeast strains, including *Y. lipolytica*, *S. pombe*, *P. pastoris* and *S. stipitis*, have shown varying degrees of superiority in protein, lipid and chemical production [112]. The CRISPR system induces DSBs to allow expansive possibilities for genome editing in different yeast species, offering more opportunities to obtain outstanding strains for industrial production strains. Various strategies for DSB repair can be applied depending on specific demands.

The most frequently used methods involve the HR and NHEJ repair pathways [113]. HR repair is preferred when precise editing or gene deletion/insertion is needed, while error-prone NHEJ repair can be applied when random editing is needed [114]. To realize the application of precise HR repair in NHEJ-dominant strains, HR efficiency can be enhanced either by knocking out NHEJ-associated genes or by enhancing HR-associated genes (Fig. 4). Greatly enhanced HR rates have been achieved in many yeast strains, including *K. maxianus*, *K. lactis*, and *Y. lipolytica*. In most studies, NHEJ serves as a backup function primarily due to its tendency for erroneous repair. However, researchers have developed multiple strategies for precise and imprecise repair through NHEJ in yeast genomes with the help of CRISPR systems to locate and generate DSBs. Other strategies, such as cell cycle synchronization, have also been applied to enhance the HR or NHEJ efficiency (Fig. 4).

In addition to HR and NHEJ, some illegitimate error-prone DSB repair recombination pathways including MMEJ and SSA, were proven to be able to generate genome rearrangement and oncogenic transformation [2]. MMEJ and SSA have been applied in yeast cells for gene integration with microhomology (Table 3), wherein DSBs were produced by either the CRISPR system or ZFNs. However, to date, most applications have only been conducted in fungal or mammalian cells. Although few efforts have been made to apply MMEJ or SSA to yeast cells, their special capacities for genome rearrangement and oncogenic transformation are still worthy of study, and such studies might shed new light on genome-wide yeast editing.

Various strategies for DSB repair offer extensive opportunities for genome-wide editing across diverse yeast species, however, it is important to note that these strategies are never universal to all demands and the choice depends on careful consideration of their respective advantages and limitations.

Homologous recombination is commonly used in genome editing techniques for precise insertion or deletion of DNA fragments at specific loci. HR repair often results in stable gene modifications with minimal risk of unintended mutations. However, when working with unconventional yeast species where HR proficiency is limited, optimization of HR pathways becomes necessary. Current strategies to improve HR involve suppression of NHEJ pathway-related genes or regulation of the cell cycle phase. Yet, it is important to note that NHEJ pathway suppression can adversely affect yeast growth dynamics in certain species [51]. Furthermore, more challenges are presented when working on gRNA library-level modifications, including the complexity of design and synthesis of donors as well as the synchronized delivery of guide RNA (gRNA) and the corresponding donor molecules into the same

recipient cell.

NHEJ repair also presents advantages in certain contexts. The design for NHEJ repair is straightforward and is independent of exogenous homologous arms, which enables the introduction of random mutations or donor fragments at the target site, thereby increasing the occurrence of random genome editing. This approach is particularly suited for unconventional yeast species which has dominant NHEJ efficiency. However, the drawback lies in the uncontrolled nature of random mutations without donor fragments, often resulting in unwanted mutant traits with detrimental genetic alterations. This also requires more screening efforts and corresponding adaptive evolution strategies [115]. For HR-dominant strains such as *S. cerevisiae*, enhancing NHEJ efficiency presents challenges. Notably, research indicates that the mutation of RAD52 might increase the relative but not the absolute efficiency of NHEJ, and does lead to a lower growth rate [88]. NHEJ-mediated editing offers distinct advantages, yet the mutation efficiency and variability pose challenges. In comparison to traditional mutation introduction methods like UV mutagenesis, the economic and practical feasibility of this approach warrants further optimization.

In previous research on genome-wide random editing, gRNA libraries were constructed to target the whole yeast genome through HR repair mechanism. The construction of donor libraries is another challenge since most of the targeting sites are redundant, which increases the complexity and cost of strain screening. In this context, optimizing the design of gRNA library to reduce redundant gRNAs is important for future applications. A possible resolution is to design a metabolic pathway-specific library that only contains a number of genes of interest. Recent research has laid the foundation for genome-wide editing with a gRNA library that can be customized based on specific species and remodeling requirements. Therefore, an evolution-based strategy targeting specific pathways can be produced to accelerate the directed evolution of a particular metabolic pathway in yeast engineering. Furthermore, in addition to the overexpression or knockout of functional genes, the tuning up or down of gene levels is crucial during metabolic processes in genome editing. Thus, identifying hidden regulatory factors and targeting them through gRNA libraries should also receive increased attention in future research.

Another promising strategy worthy of exploration in the future is random genome-scale editing with a gRNA library through NHEJ repair mechanism. NHEJ repair has advantage to introduce mutations without donors due to its error-prone characteristics. NHEJ repair usually produces small mutagenic base insertions or deletions at the DSB site, and the most common mutation is a 1-bp insertion. Additional mutations such as +2, +3, and –3 mutations, could also be introduced by editing DSB repair-related genes such as Pol4 or MRX complex genes [116]. This strategy has been applied in rice but has not been reported in microbial factories, which would be a new application that would generate a random mutant library with various phenotypes in yeast genome engineering.

Although current strategies for genome editing offer more opportunities to obtain outstanding strains for industrial production, there is still a large gap between laboratory research and industrial production. The current applications mainly focus on laboratory strains and have been validated only at the laboratory scale, while actual industrial production is often more complicated. On the one hand, yeast strains in industrial production tend to face severe stresses due to industrial materials and

fermentation processes. In addition, strains used in industrial production tend to be more robust, which makes lab-engineered strains less competitive and more difficult to maintain. Therefore, maintaining a desired phenotype is a major challenge in industrial production.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

W.B. was a major contributor to writing the manuscript. J.L., C.L. and M.H. revised the manuscript. All authors read and approved the final manuscript.

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