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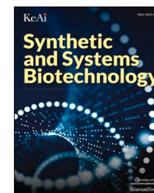

Review Article

Advances and perspectives in genetic expression and operation for the oleaginous yeast *Yarrowia lipolytica*

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ABSTRACT

The utilization of industrial biomanufacturing has emerged as a viable and sustainable alternative to fossil-based resources for producing functional chemicals. Moreover, advancements in synthetic biology have created new opportunities for the development of innovative cell factories. Notably, *Yarrowia lipolytica*, an oleaginous yeast that is generally regarded as safe, possesses several advantageous characteristics, including the ability to utilize inexpensive renewable carbon sources, well-established genetic backgrounds, and mature genetic manipulation methods. Consequently, there is increasing interest in manipulating the metabolism of this yeast to enhance its potential as a biomanufacturing platform. Here, we reviewed the latest developments in genetic expression strategies and manipulation tools related to *Y. lipolytica*, particularly focusing on gene expression, chromosomal operation, CRISPR-based tool, and dynamic biosensors. The purpose of this review is to serve as a valuable reference for those interested in the development of a *Y. lipolytica* microbial factory.

1. Introduction

The study of oleaginous microorganisms has garnered significant interest due to its application to produce valuable fatty acids and derivatives [1]. Moreover, the resulting biodiesel derived from them are particularly important in terms of promoting clean energy while reducing the pollution associated with fossil fuels. Because of these benefits, oleaginous microorganisms are considered a highly promising option for sustainable renewable oil production. Among them, *Yarrowia lipolytica* is the most extensively studied, which possesses desirable qualities, such as high lipid content, robust cell growth, and compatibility with various substrates [2] (see Table 1, Figs. 1–3).

Noticeably, *Y. lipolytica* is a non-conventional oleaginous yeast that holds the generally regarded as safe (GRAS) status [3]. It exhibits unique biochemical and metabolic characteristics, such as efficient acetyl-CoA metabolic pathway, the high flux of TCA cycle, and remarkable lipid accumulation, distinguishing it from *Saccharomyces cerevisiae* [4,5]. *Y. lipolytica* also possesses the ability to utilize a diverse array of low-cost, renewable substrates, including alkanes, fatty acids, organic acids, and proteins [6–8]. These distinguishing features make *Y. lipolytica* an ideal candidate for the biomanufacturing applications. In particular, strains W29 (CLIB89) and its derived strains Po1d, Po1f, Po1g, and Po1h, have been commonly employed as platforms for engineering research and industrial applications [9,10]. Moreover, noteworthy advantages of these strain series include: i) high levels of protein expression and secretion; ii) efficient utilization of inexpensive carbon sources; iii) the elimination of the endogenous alkaline extracellular protease to safeguard the degradation of expressed exogenous proteins.

Currently, with the rapid development of synthetic biology, various innovative methods and strategies have been successfully implemented for gene regulation in *Y. lipolytica*. Moreover, genome editing techniques, such as Cre/loxP and CRISPR, have been effectively developed for use in *Y. lipolytica*. These genetic tools and strategies enable researchers to optimize cellular performance and confer the ability to synthesize novel chemicals. In this review, we emphasized the genetic manipulation tools and strategies developed in *Y. lipolytica*, including gene expression, chromosomal operation, CRISPR-based tools, and dynamic biosensors for metabolic engineering. Additionally, we discussed the limitations and challenges that need to be overcome, and explore emerging opportunities for *Y. lipolytica* in the context of synthetic biology and industrial applications.

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Table 1
Summary of synthetic biology tools in *Yarrowia lipolytica*.

Tools	Characteristics	Application	References
Promoter			
pTEF, pMnDH2, pPHO89	Endogenous promoters; constitutive; strong	/	[13,17]
hp4d, nUAS1$_{XPR2}$-LEU, nUAS1$_{XPR2}$-TEF	Hybrid promoters; derived from pXPR2; carries several tandem copies of UAS1$_{XPR2}$	/	[21,22]
hybrid RNA polymerase III promoters	Hybrid promoters	Improve sgRNA expression and CRISPR-Cas9 function	[71]
pXPR2	Inducible promoters; induced by peptone	/	[23]
pEYK1	Inducible promoters; induced by erythritol and erythrulose	/	[26]
pMT-1 to pMT-6	Inducible promoters; induced by Cu^{2+}	/	[28]
Terminator			
XPR2t, LIP2t, PHO5t	Endogenous terminators	Commonly utilized for the heterologous gene expression	[22]
Synth1t-synth30t	Synthetic terminators; short; easily cloned	Improve expression of heterologous genes	[32]
Multi-gene assembly			
One-step assembly	Obtain multiple expression cassettes by overlap extension PCR (OE-PCR); simple; quick	Integrate the β-carotene biosynthetic pathway into *Y. lipolytica* chromosome	[37]
Golden Gate assembly	Rely on Type IIS restriction endonucleases; high efficiency; stable	Assemble carotenoid pathway genes and improved the efficiency up to 90%	[38]
YaliBricks assembly	Based on BioBrick assembly; rapid multi-component assembly	Construct five-gene violacein pathway	[15]
Gene deletion			
Cre-loxP	Sourced from the P1 phage; composed of cyclized recombinase (Cre) and loxP sites	Integrated a flavonoid pathway into *Y. lipolytica* genome, and obtained different flavonoids	[56]
TALENs	Recombinant restriction enzymes; fusion of the nuclease to the TAL effector DNA binding domains	Generate mutants of the fatty acid synthase (FAS) gene	[84]
CRISPR tools			
CRISPR/Cas9	Composed of a Cas9 protein and the corresponding sgRNA	Multi-gene targeting and marker-free integration	[51,65,85,86]
CRISPRi	Used for gene repression via a catalytically deactivated Cas9	Repress NHEJ to enhance HR efficiency	[70]
CRISPRa	Fusing dCas9 to transcriptional activators	Activate the target genes	[87]
Genetic biosensors			
Fatty acyl-CoA biosensor	Fatty acids as response factors; transcription factor FadR and manipulator fadO were derived from *E. coli*	Regulate the cytochrome P450 enzymes that convert palmitate to ω-hydroxypalmitate	[88]
Naringenin biosensor	Naringenin as response factors; flavonoid-sensing transcriptional activator FdeR; manipulator fdeO	Improved cell fitness and pathway yield	[79]
Xylbiosensor	Xylose as response factors; the activation factor XylR and the operator xylO were derived from *E. coli*	Modulate naringenin synthesis with a yield of (715.3 ± 12.8) mg/L	[80]
Light-controlled biosensor	Light as the response factor; fast response; non-destructive	Application to the dynamic regulation of the biosynthesis and synthetic pathways of coumaric acid and naringenin	[82]

2. Gene expression and multi-gene assembly

Y. lipolytica lacks a native plasmid expression system. However, to address this drawback, an artificial plasmid expression system has been developed. Furthermore, a diverse range of gene expression elements has been developed, including promoters and terminators.

2.1. Promoter

Promoter plays a critical role in accurately controlling gene expression, which has changeable transcriptional capacities and required characteristics. In particular, the eukaryotic promoters are structurally complex, spanning thousands of bases from the start site and controlling the intensity and timing of gene transcription. As of now, a variety of promoters, including both inducible and constitutive ones, have been isolated and characterized in *Y. lipolytica* [11]. For instance, the pXPR2 promoter, induced by peptone, was isolated in 1987 [12]. However, its industrial applications were impeded by the complicated regulation and expensive inducer. Currently, the most widely used promoter is the strong constitutive pTEF, which is responsible for the translation elongation factor EF-1α [13]. Notably, it was found that a 122 bp spliceosomal intron was present in the pTEF which greatly affects the corresponding gene expression. Therefore, a platform for high expression was established by Tai et al. [14] using the intron-containing translational extension TEF promoter, and it was demonstrated that this expression system was able to increase gene expression. Furthermore, Wong et al. [15,16] characterized 12 endogenous promoters by means of a sensitive luciferase reporter, and reported that pTEF showed the highest activity. Also, this luciferase reporter system was utilized to screen and characterize 81 other endogenous promoters, ranging in strength from 0.06% to 1.60 times that of pTEF [17]. Besides, 22 lipogenic promoters have been characterized to facilitate the development of structure-based dynamics models, and gain insight into the process of lipogenesis in *Y. lipolytica* [18].

On the other hand, hybrid promoter engineering has been employed to enhance the strength and adjustability of existing promoters in *Y. lipolytica*. The strength of eukaryotic promoters is influenced by different factors, such as the core promoter, the TATA cassette sequence, proximal promoter sequence and upstream activating sequence (UAS) [19]. One particular focus has been on engineering hybrid promoters, which involve combining duplicated UASs with truncated downstream minimal promoters [20–22]. Madzak et al. [23] utilized the distal UAS (UAS1) element to create a hybrid promoter consisting of four consecutive UAS1 copies located upstream of a minimal LEU2 promoter. This recombinant promoter, known as hp4d, exhibits almost no dependence on environmental conditions and maintains an activity similar to that observed under inducing conditions. Furthermore, a tandem duplicate UAS1B (a 105-bp distal UAS fragment, aka UAS1$_{XPR2}$) was made by Blazeck et al. [20] to drive expression of GFP from two minimal constitutive promoters, pTEF and pLEU. This study revealed that the

core promoter and the tandem elements UAS1 function independently, and that the strength of the promoter increases with the number of UAS1 tandem elements. In addition, Dulermo et al. [24] demonstrated that promoter strength is not always correlate with optimal protein expression and activity. Therefore, promoter libraries with different strengths make it easy to identify the best promoter for a particular protein of interest. Zhao et al. [25] constructed a hybrid promoter library to optimize the expression of the biosynthetic pathway of isoamyl alcohol in *Y. lipolytica*. As a result, the isoamyl alcohol titer was increased 1.1–30.3-fold over the control strain *Y. lipolytica* Po1g ΔKU70.

Moreover, several inducible promoters have been characterized and developed in *Y. lipolytica*. Marion et al. [26] successfully isolated an inducible promoter called pEYK1, which drives the transcription of the *EYK1* gene encoding erythrulose kinase. It was shown that this promoter is affected in media containing glucose and glycerol, but its induction level is significantly increased when erythritol and erythrulose are present. The upstream activation sequence of the pEYK1 promoter, UAS1 ($UAS1_{EYK1}$), was identified. Subsequently, a hybrid promoter containing tandem repeats of $UAS1_{EYK1}$ or $UAS1_{XPR2}$ was developed, and the expression level is higher compared to the native pEYK1 promoter. Furthermore, Vidal et al. [27] discovered an erythritol-inducible bidirectional promoter (pBDP) situated in the intergenic region between gene *EYK1* and *EYL1*. They utilized this pBDP to co-express RedStarII and YFP fluorescent proteins, demonstrating that its strength was 2.7–3.5-fold higher when oriented towards *EYL1* compared to *EYK1*. Consequently, a hybrid erythritol-inducible bidirectional promoter (pHBDP) containing five copies of $UAS1_{EYK1}$ was developed, increasing expression levels by 8.6–19.2 times. More recently, Xiong et al. [28] isolated 11 copper-inducible promoters with different expression effects. Compared with constitutive promoters, the copper-repressed promoters exhibit higher activity under non-repressing conditions, and their activity can be almost completely inhibited by supplementation with only low concentrations of Cu^{2+}. To expand their dynamic regulation range, these six copper-inducible promoters were engineered with the tandem UAS. This modified approach successfully constructed an efficient pathway for producing wax ester, surpassing the productivity achieved using both the constitutive promoter and copper-inducible promoter.

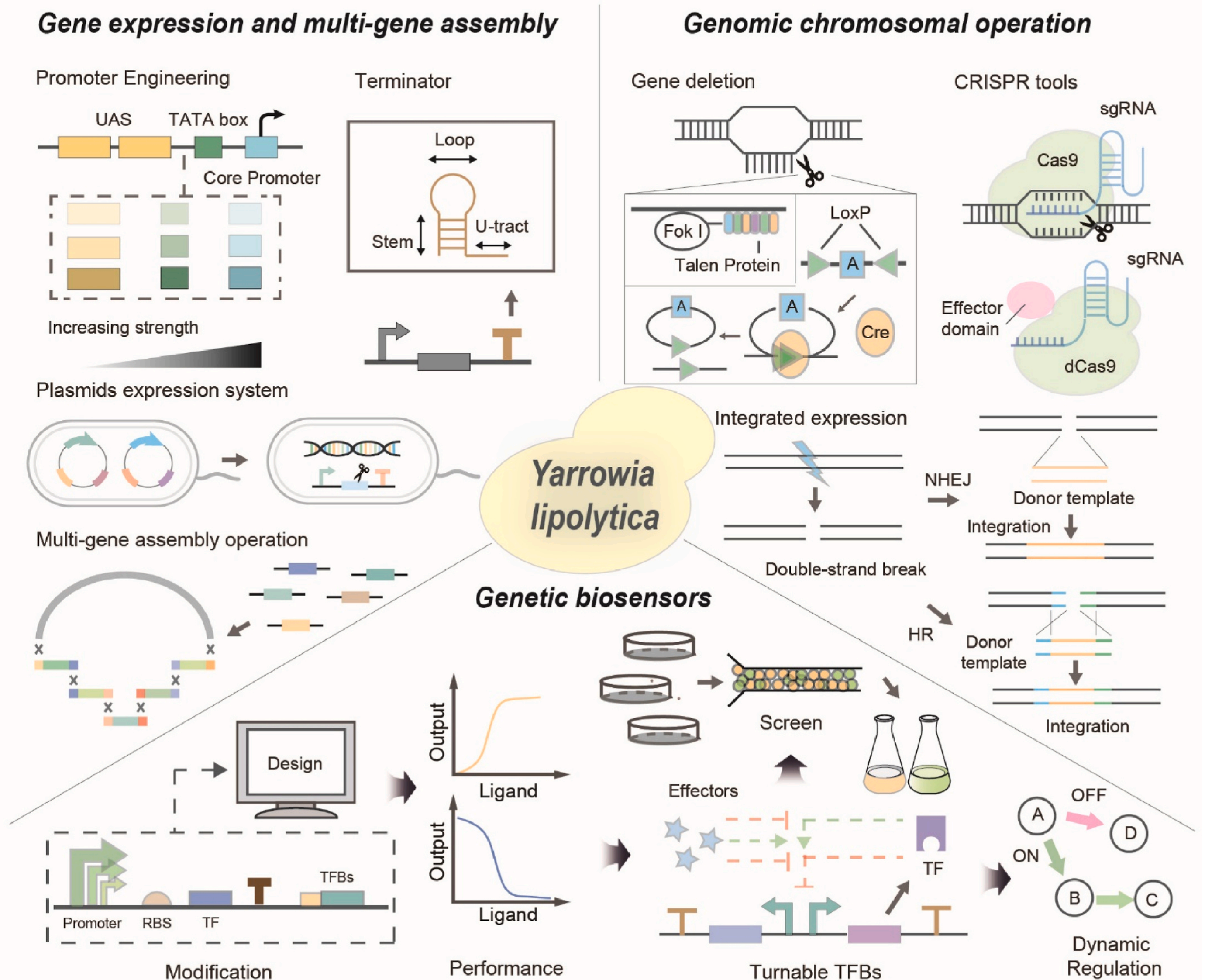

Fig. 1. Genetic technology applicable in *Yarrowia lipolytica*. A. Gene expression and multi-gene assembly strategies, including promoter and terminator engineering, plasmids expression system, and multi-gene assembly operation. **B.** Genomic chromosomal operations, including integrated expression, gene deletion, and CRSPR tools. **C.** The modification, performance, and application genetic biosensors. HR, homologous recombination. NHEJ, the nonhomologous end-joining. TF, transcription factor.

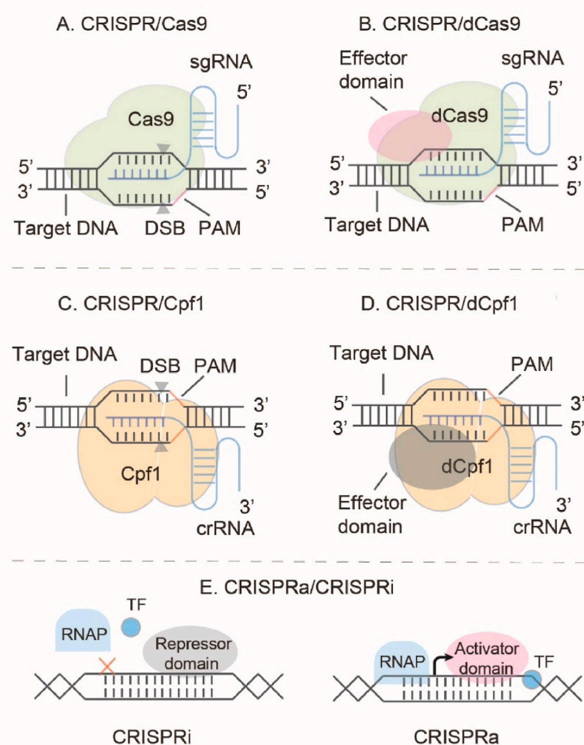

Fig. 2. The CRISPR/Cas genome editing platform for *Yarrowia lipolytica*. **A.** CRISPR/Cas9 method for gene knock-out/knock-in. When the sgRNA recognizes the targeted sequence, which is located before a protospacer adjacent motif (PAM) site, the Cas9 protein will catalyze the formation of a double-strand break (DSB) in the targeted DNA. **B.** CRISPR/dCas9 based gene editing. **C.** CRISPR/Cpf1 based gene editing. **D.** CRISPR/dCpf1 based gene editing. **E.** CRISPRi and CRISPRa methods for gene interference and activation. A catalytically deactivated Cas9 (dCas9), which has no cleavage activity, can be fused with different effector domains to control gene expression. When the targeted region is recognized, the dCas9 fusion protein with the transcriptional repressor domain binds the DNA to repress gene expression. Similarly, the fusion protein of dCas9 and the transcriptional activator domain binds to targeted regions to improve the gene expression level. CRISPRa, CRISPR/Cas based gene activation; CRISPRi, CRISPR/Cas based gene interference; crRNA, CRISPR RNA; DSB, DNA double strand breaks; PAM, protospacer adjacent motif; RNA P, RNA polymerase; sgRNA, single-guide RNA; TF, transcription factor.

2.2. Terminator

Similarly, terminators also play a crucial role in controlling gene expression and can impact protein yield by controlling the half-life of mRNA [29]. Native terminators, such as XPR2t, LIP2t, and PHO5t, are commonly utilized for the heterologous gene expression in *Y. lipolytica* [22]. Nonetheless, synthetic terminators with shorter sequences may offer greater portability compared to native terminators, facilitating vector design, expression cassette construction, and minimizing the risk of homologous recombination [20,30,31]. Moreover, it also has been shown that synthetic terminators have significant effects on various yeast species, exhibiting good interspecific transferability. For instance, Curran et al. [32] demonstrated the functionality of synthetic designs for *S. cerevisiae* in *Y. lipolytica*, resulting in a 60% increase in fluorescent protein production compared to common endogenous terminators. However, terminators in *Y. lipolytica* have not received as much attention as promoters, highlighting the promising future of terminator studies in this organism.

2.3. Plasmids expression system

The episomal plasmids play an essential role in genome editing and recombinant protein production. However, *Y. lipolytica* lacks a natural episomal plasmid. Consequently, artificial plasmids have been developed using *Y. lipolytica*'s chromosomal autonomously replicating sequence/centromere (ARS/CEN) replication origins. Unfortunately, this system is difficult to maintain genetic stability and produces high copy number [33,34]. To overcome these challenges, the origins of mitochondrial DNA (mtDNA) replication in *Y. lipolytica* were characterized by Cui et al. [35]. They confirmed that a 516-bp sequence of mtDNA, known as mtORI, enables the autonomous replication of circular plasmids with high protein expression levels and genetic stability. Moreover, Liu et al. [36] engineered a CEN plasmid by incorporating different promoters upstream of the centromeric region, expanding its regulatory mechanisms and functionality. This modification led to an 80% improvement in gene expression level and copy number, as well as a dynamic range of nearly 2.7 times.

2.4. Multi-gene assembly operation

Various techniques for constructing multigene cassettes are currently available. The first application of a multi-gene assembly method in *Y. lipolytica* was reported in 2014 using the one-step integration method [37]. This method enabled the integration of the β-carotene biosynthesis pathway into the rDNA locus of the *Y. lipolytica* chromosome with a reported maximum efficiency of 21%. Recently, Golden Gate assembly has significantly improved pathway assembly and construction efficiency, considered one of the most robust techniques for multi-gene assembly [38,39]. Therefore, Celinska et al. [40] developed a versatile and robust DNA assembly platform for *Y. lipolytica* using the Golden Gate modular cloning. They constructed a wide range of destination vectors and interchangeable building blocks. Using these elements, the β-carotene pathway was successfully assembled with efficiencies ranging from 67% to 90%, demonstrating the validity of the Golden Gate assembly in *Y. lipolytica*. Furthermore, Larroude et al. [41] presented a new Golden Gate toolkit that includes selective markers and genome integration sequences to one-step assemble three transcription units. This toolkit enables rapid transformation and construction of multiple DNA elements, which were subsequently applied to assemble a three-gene pathway that complemented the availability of xylose. In addition, Tong et al. [42] constructed a library of violacein-producing defatted *Y. lipolytica* strains on the basis of the Golden Gate assembly method. In this library, three promoters of different strength control each gene expression in the violacein pathway.

Instead of the Golden Gate assembly strategy, multi-component modular assembly suitable with BioBrick standards called YaliBricks has also been developed and tested. Wong et al. [15] developed an effective luciferase reporter and identified 12 native promoters to expand the genetic toolbox for transcriptional regulation in *Y. lipolytica*. Furthermore, Holkenbrink et al. [43] presented the EasyCloneYALI gene toolkit, which simplifies strain construction and improves the efficiency of genome editing in *Y. lipolytica* by using the CRISPR/Cas9. They demonstrated that transformation using non-replicating DNA repair fragments achieved genome editing efficiencies of more than 80%.

3. Genomic chromosomal operation

3.1. Integrated expression

To enable stable expression of heterologous DNA, integrating it into the genome is often preferred [44]. The most commonly used method for this is homologous recombination (HR), which allows for DNA exchange between regions with identical sequences, playing a crucial role in cellular processes like repairing double strand breaks (DSBs) and facilitating horizontal gene transfer [45]. However, when it comes to

Y. lipolytica, the HR efficiency is quite low, which limits its application for targeted integration [46]. To surmount this constraint, certain proteins such as Lig4 or Ku70 should be deleted [47]. Specifically, it was found that knockout of Ku70 resulted in a significant increase in HR-mediated integration 56%, even though the homologous arms of the 5′-and 3′-flankers are short to 500 bp. Moreover, hydroxyurea (HU) has also been observed to induce homologous recombination, which can bring growing cells together into the S phase of cell growth. Researchers such as Jang et al. demonstrated that HU-mediated cell growth synchronization in *Y. lipolytica* lacking Ku70 was highly effective in promoting HR [48]. Recently, the development of CRISPR-Cas9 technology has brought about dramatic changes in gene editing. With this approach, a new HR strategy based on the CRISPR technology has been developed for specific multilocus integration in *Y. lipolytica*, eliminating the need for marker recovery. Schwartz et al. [49] utilized the RNA polymerase III synthetic promoters to enhance the efficiency of CRISPR/Cas9-based genome editing, achieving a markerless HR efficiency of 70% with the donor DNA and 100% in strains with disrupted non-homologous end joining (NHEJ) repair.

Apart from HR-based genomic integration, NHEJ can also be utilized for gene integration in *Y. lipolytica*. Cui et al. [50] developed a modular expression library to optimize biosynthetic pathways in *Y. lipolytica* by employing NHEJ-mediated random integration. Furthermore, they also created a CRISPR/NHEJ-based specific gene integration tool in *Y. lipolytica*, which allows for the DNA fragments integration without the requirement of homologous arms [51]. More recently, Liu et al. [52] employed fluorescence-activated cell sorting (FACS) to construct an NHEJ-based GFP stochastic expression library. By screening the highly-expressed strains and analyzing possible integration sites, they identified new gene integration sites in *Y. lipolytica* [53,54]. These sites exhibited both high gene expression and integration efficiency, thus demonstrating the effectiveness of CRISPR/Cas9 gene editing for achieving successful integration in *Y. lipolytica*. In order to further increase DNA assembly capabilities in *Y. lipolytica*, Li et al. [55] established a Golden Gate modular cloning system called YALIcloneNHEJ.

However, there are some challenges with NHEJ-mediated random genome integration, such as the need for selection markers for gene expression and the potential disruption of important endogenous genes [52]. Therefore, researchers are actively seeking additional genetic tools and integration sites. Lv et al. [56] developed a versatile framework by combining the high recombination efficiency of the Cre-loxP system with the high integration rate of 26s rDNA in *Y. lipolytica*. This framework allows for the iterative integration of multicopy metabolic pathways. In this work, they successfully revealed the efficient genomic integration of multicopy plant flavonoid pathway. Moreover, Guo et al. [57] took a different approach by designing and developing a totally synthetic *Y. lipolytica*-specific artificial chromosome (ylAC). Besides, they introduced an intact xylose and cellobiose co-utilization pathway using this artificial chromosome. These advancements in genetic tools and integration sites are significant in increasing the versatility and

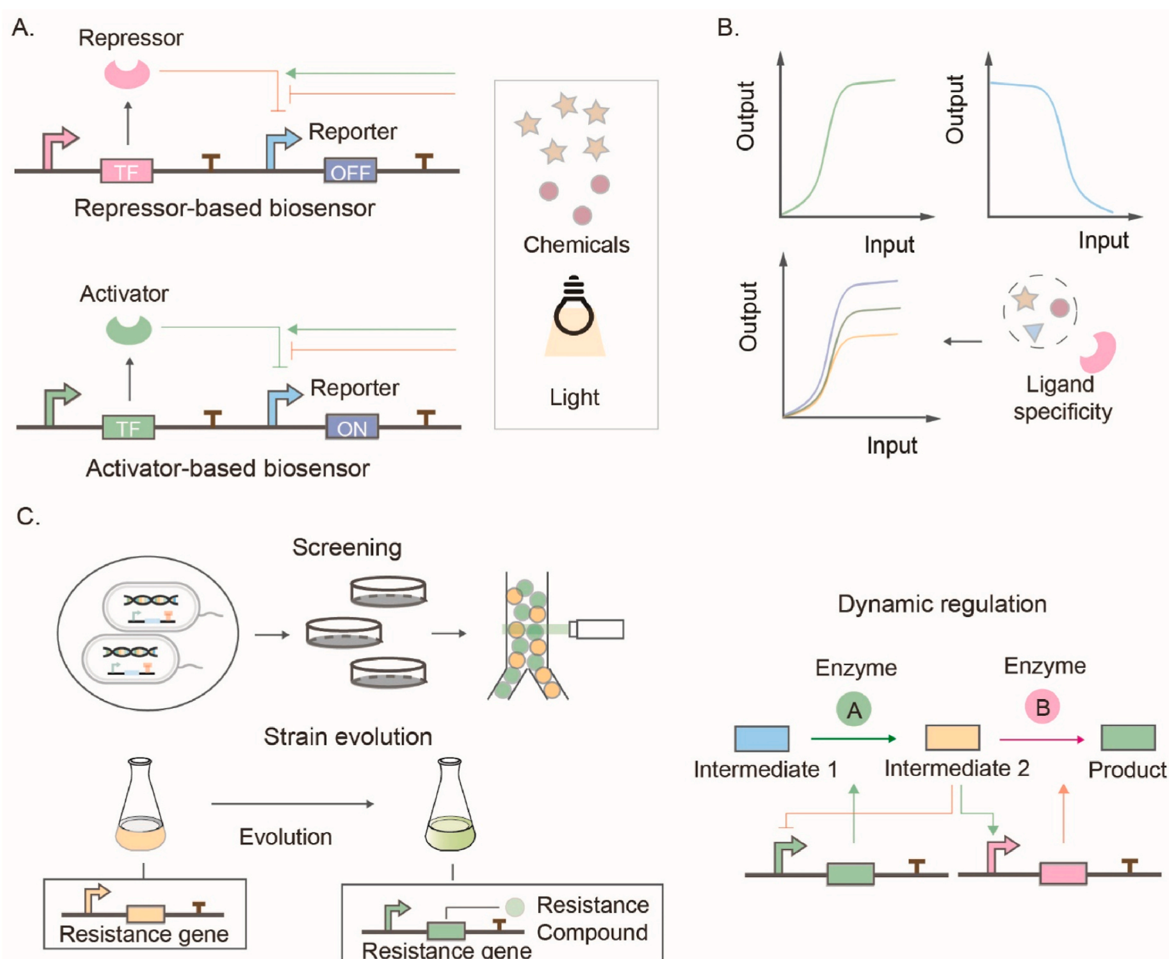

Fig. 3. Dynamic regulation of gene circuits through biosensors in *Yarrowia lipolytica*. A. Transcription factor-based biosensor. Repressor-based biosensors. TF suppresses the expression of target gene expression. Activator-based biosensors. TF activates the expression of a target gene in the presence or absence of the target metabolite. The solid red line indicates inhibition and the green realized arrow indicates activation. **B.** Characterization of changes in the target metabolite concentration. **C.** Application of TF-based biosensor in *Y. lipolytica.*

efficiency of gene expression in *Y. lipolytica*.

3.2. Gene deletion

One such technique is the Cre-loxP system, which is sourced from the P1 phage. Composed of two parts: the cyclization recombination enzyme (Cre) and the loxP site, which facilitate gene deletion at specific genomic sites. This conventional recombination method mediated by Cre-loxP has been efficiently used for marker-free gene integration in *Y. lipolytica*. The disruption cassette is integrated into the targeted locus with an average efficiency of 45% [58]. When the Cre recombinase is expressed, excision of the marker by recombination between the two lox loci at a 98% frequency [58]. Interestingly, Vandermies et al. [59] later improved the construction of the disruption cassette by substituting asymmetric SfiI locus for I-SceI, making the assembly of cassette elements simpler and faster.

In *Y. lipolytica*, the URA3 gene encodes orotidine 5′-phosphate decarboxylase, which is the key enzyme in uracil synthesis. This enzyme catalyzes the conversion of 5-fluoroorotic acid (5-FOA) into a toxic substance. Knockdown of the URA3 gene prevents the formation of the toxic 5-fluorouracil nucleotide from 5-fluoroorotic acid (5-FOA), which is resistant to 5-FOA, and its pyrimidine nutrition can be supplemented by adding uracil to the culture medium through a remedial pathway [56, 58]. Because of the potential it offers to aid in the screening of trophic transformants as well as counter-selection, URA3 is widely used as a routine selection marker for yeasts, including fat-soluble yeasts. Huang et al. [60] blocked the degradation and competition module using URA3 counter-selection. Subsequently, through the expression of functional genes in multi-module combinations, they successfully constructed a non-degradation, non-byproducts *Y. lipolytica* for the production of high titer erythritol from glycerol.

Additionally, nucleases-based tools like CRISPR (see CRISPR tools section) and transcription activator-like effector nucleases (TALENs) have also been employed in *Y. lipolytica*. TALENs are developed by converging the FokI endonuclease catalytic domain and transcription activator-like effectors (TALEs). By formulating the TALE DNA binding domain, targeted DNA double-strand breaks can take place at a specific locus [61]. Rigouin et al. utilized TALEN-based technology to generate mutants of the fatty acid synthase (FAS) gene and demonstrated high efficiency in inducing targeted modifications in the genome [44]. Mutants were generated by repair of error-prone non-homologous end joining at the target sites in 97% of transformants.

3.3. CRISPR tools

CRISPR-Cas systems are categorized into two major classes (Class I and Class II), six types (Type I, Type II and Type III, Type IV, Type V and Type VI) and different sub-types [62]. Particularly, Class II CRISPR/Cas can perform cleavage function through a single multifunctional domain of Cas proteins with high cleavage efficiency [63]. Among them, CRISPR/Cas9, CRISPR/Cas12a (Cpf1) and CRISPR/Cas13a are the most representative and widely applied. As the first Class II CRISPR/Cas system to be discovered and characterized [64], the CRISPR/Cas9 systems are mainly composed of Cas9 proteins and corresponding single guide RNAs (sgRNAs) [65]. The Cas9 is a DNA endonuclease induced by crRNA and *trans*-activating crRNA (tracrRNA) [66]. In contrast, Cas12a has both DNA and RNA endonuclease activity and processes precursor crRNAs into mature crRNAs without relying on tracrRNAs [64]. Furthermore, Cas13a, an RNA-guided nuclease targeting RNA, can be used to editorially manipulate another important genetic material–RNA [67].

Recently, Schwartz et al. successfully adapted the CRISPR/Cas9 system of *Streptococcus pyogenes* to marker-free gene integration and disruption in *Y. lipolytica* [49]. Notably, they achieved the integration of numerous genes at different sites without marker recovery. However, different integration sites influence the efficiency of gene integration. Among the 17 tested sites, 5 demonstrated high frequencies (48–62%) of CRISPR/Cas9-based integration [68]. In addition, an alternative method for CRISPR-Cas9 gene editing in *Y. lipolytica* was developed by Gao et al. [69] to express human codon-optimized Cas9 variants and gRNA flanked by ribozymes through the RNAP II promoter with 86% efficiency after four days of growth.

Furthermore, the CRISPR technique has been further developed for the control of gene expression in *Y. lipolytica*. Schwartz et al. [70] applied CRISPRi and CRISPRa systems in *Y. lipolytica* to modified the sgRNA target loci in the promoter region, which can lead to cascading changes in gene expression levels [71]. Besides, the CRISPRi system can be used to inhibit NHEJ in *Y. lipolytica*. By targeting ku80 and ku70, NHEJ can be repressed, resulting in HR efficiencies of up to 90% [70]. Zhang et al. [72] applied a CRISPRi system to suppress genes using DNase-deactivated Cpf1 (dCpf1), deactivated Cas9 (dCas9), and two fusion proteins (dCpf1-KRAB and dCas9-KRAB). Due to the difficulty of a single gRNA element to achieve a strong level of inhibition and to find an effective target site, a multiplex gRNA strategy based on one-step Golden-brick assembly was employed. When targeting gene *gfp* at three different sites, the gene repression efficiency exceeded 80%. On the other hand, Schwartz et al. [73] discovered that VPR transcriptional activators yielded the highest activation rates. They characterized multiple target locus and four different activation domains in the promoter region. By selecting gRNA target sites upstream from the core promoter and incorporating the VPR activation domain into dCas9, they successfully activated *BGLI* and *BGLII*, two β-glucosidase genes, enabling to grow on cellobiose.

4. Genetic biosensors

Biosensors are biological components capable of converting certain chemical or physical signals into detectable quantities such as fluorescence or gene expression. Depending on the principle, they can be categorized into transcription factor-based, aptamer-based and protein-based biosensors [74,75]. Different types of biosensors have different sensitivities, ranges of action, and response thresholds, and the design of appropriate biosensors can effectively enhance detection efficacy [76]. Recently, genetically encoded biosensors have garnered significant attention in the field of biosynthesis for valuable products. These biosensors are developed and created through rational design, machine learning, or directed evolution, allowing for the effective regulation of gene expression levels in microorganisms in response to specific chemicals or signals. As a result, this regulation leads to a substantial improvement in overall production by altering metabolic flow [77].

One example of such biosensors is the fatty acyl-CoA biosensor constructed by Park et al. in *Y. lipolytica* [78]. In their study, the promoters containing bacterial FadR-binding sequences were developed to activate the ω-hydroxylating pathway in response to increase free fatty acids (FFAs) concentrations. Similarly, Lv et al. developed a naringenin biosensor using the distinctive transcriptional factor FdeR and the specific DNA binding site FdeO [79]. When naringenin acts as the specific effector, FdeR binds to the FdeO site, activating transcription of the reporter gene. Also, Lv et al. demonstrated that this naringenin biosensor could be induced by naringenin within a regulatory range of 0 mg/L to 50 mg/L [79]. Furthermore, Wei et al. established a xylose-inducible biosensor (xylbiosensor) in *Y. lipolytica* [80]. This xylbiosensor comprise the activation factor XylR from *E. coli*, fusion the hybrid promoter with the operator xylO, and activation domain VPRH. Addition of xylose can activate target genes, including xylose and the mcherry reporter genes, in *Y. lipolytica* engineered strain containing xylbiosensor. Besides, Qiu et al. utilized the erythritol-sensitive transcription activator EryD to construct a sensor conditioning system for rapid identification and screening of erythritol overproducers[77]. Its specificity, sensitivity and dynamic response range were improved by further optimizing the structure, and the response to erythritol ranged from 5 to 250 mmol/L [81].

In addition to using substrates or intermediate metabolites as signals, a light-controlled biosensor has also been established in *Y. lipolytica*. Light as a response factor offers advantages such as high sensitivity, non-destructiveness, reversibility, and spatial specificity. Zhang et al. [82] developed a light-responsive expression system in *Y. lipolytica* that successfully increased the titer and efficiency of naringenin and *p*-coumaric acid. They constructed the light-control complex CarH-VPRH, the core component of the light control system, containing the green light response factor CarH and the transcription activator VPR-HSF1. Under green light irradiation conditions, the CarH-VPRH complex cannot polymerize, thus impeding the regulation of target gene transcription and expression. Besides, another light-controlled biosensor was constructed in *Y. lipolytica* [83]. The light-responsive element constructed from the transcriptional activator VP16 and the blue photosensitive protein EL222 is the core of this blue light-inducible system. Utilizing this light-controlled system, the functional validation and synthesis of the BleoR protein were realized. These results demonstrate the potential of this system for gene regulation, construction of synthetic networks, and large-scale production of desired products [83].

5. Challenges and prospects

The engineering of microbial chassis cells for efficient synthesis of high-value products has garnered broad attention. The unconventional oleaginous yeast, *Y. lipolytica*, have unique biochemical and physiological properties, including an intrinsic mevalonate pathway, a broad substrate range, and a high degree of tolerance to extreme environments [1,2]. These features make *Y. lipolytica* an increasingly popular choice for microbial chassis cells in advanced and sustainable biomanufacturing. With the advancement of synthetic biology technology and the rapid development of gene editing tools, the metabolic engineering modifications of *Y. lipolytica* have been rapidly improved. In recent years, several compounds such as organic acids, proteins, fatty acids, polyketides and flavonoids have been successfully produced using unconventional yeast as chassis cells. However, compared with traditional model microorganisms (e.g., *E. coli*, Saccharo introns myces *cerevisiae*, etc.), *Y. lipolytica* still suffers from the problems of fewer tools, low efficiency, and cumbersome operation, and several challenges remain in systematic modification of the chassis and optimization of complex pathways assembly.

Firstly, new synthetic biology components need to be further explored, such as new promoter and terminators. Although several hybrid and wild-type promoters have been developed, endogenous promoters in *Y. lipolytica* remain incompletely characterized. Different regulatory behaviors of promoters were found under different growth conditions. Therefore, rigorous studies of isolated promoters are needed under the standard conditions, including regulatory behavior and promoter strength using different carbon sources. Moreover, introns widely distributed in biological genomes have been shown to potentially enhance gene expression. For instance, the introns in pTEF, pEXP1, and pTDH1 all lead to an increase in promoter activity [51]. However, the concrete mechanism of intron is still unclear. Therefore, to guide the rational design of effective promoters and accurate regulation of biological processes, more accurate and detailed understanding of the regulatory mechanisms of expression-enhancing introns is needed. Furthermore, inducible promoters that introduce exogenous transcription factors could eliminate the metabolic burden caused by the reuse of natural regulatory elements in pathway engineering. Therefore, there is a need to continue exploring inducible promoters for the introduction of exogenous transcription factors to enable dynamic regulation of *Y. lipolytica*.

Secondly, the development and established of the CRISPR/Cas system has certainly accelerated the pace of genetic engineering for the improvement of *Y. lipolytica*. However, there are still some problems. For example, the efficiency of multi-gene editing is yet to be improved, mutations in the genome need to be localized more precisely, and high-throughput screening techniques after gene editing need to be developed. In addition, compared with the knockout/insertion-oriented CRISPR/Cas9, CRISPRi and CRISPRa are still in their infancy, both of which have been used sparingly in *Y. lipolytica*. Therefore, there is a need to further improve the application of CRISPRa and CRISPRi systems in metabolic engineering research, and the off-target effects remain a challenge to be solved.

Thirdly, constructing diverse biosensors allows for the regulation and detection of intracellular metabolites, resulting in targeted modification of metabolic pathways to improve both yield and quality. Over the past few decades, various chemical induction systems have been employed to regulate gene expression in yeast. However, these systems have limitations such as easy diffusion, difficult removal, and high cost, making them ill-suited for large-scale industrial production. In contrast, light serves as an ideal gene inducer that can be precisely regulated in terms of timing and location. Light-induced sensors have been developed and applied to some extent in the synthesis of natural products, enabling dynamic and real-time regulation of cellular response to green light for the production of *p*-coumaric acid and naringenin. Nevertheless, the current level of shake flask fermentation does not match the yields reported in other studies. Thus, further optimization is required in terms of light intensity, duration, and strategies for zonal compartmentalization of metabolic synthesis to enhance the production of target compounds. Furthermore, the sensor's signal output and strategies for enhancing signal strength warrant further investigation.

In conclusion, *Y. lipolytica* has made important progress in the mining of synthetic biology components and the development of genetic tools. With the further development of synthetic biology, more novel tools and regulatory methods will be developed, which will accelerate the construction of *Y. lipolytica* cell factories with the function of producing high-value products.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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