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Advancing Yarrowia lipolytica as a superior biomanufacturing platform by tuning gene expression using promoter engineering

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Corresponding Author:	Xiao-Jun Ji, PhD Nanjing Tech University Nanjing, CHINA
First Author:	Mei-Li Sun
Order of Authors:	Mei-Li Sun
	Tian-Qiong Shi, PhD
	Rodrigo Ledesma-Amaro, PhD
	Xiao-Jun Ji, PhD
Abstract:	Yarrowia lipolytica is recognized as an excellent non-conventional yeast in the field of biomanufacturing, where it is used as a host to produce oleochemicals, terpenes, organic acids, polyols and recombinant proteins. Consequently, metabolic engineering of this yeast is becoming increasingly popular to advance it as a superior biomanufacturing platform, of which promoters are the most basic elements for tuning gene expression. We reviewed the endogenous promoters of Yarrowia lipolytica , which are the basis for promoter engineering. The engineering strategies, such as hybrid promoter engineering, intron enhancement promoter engineering, and transcription factor-based inducible promoter engineering are described. Additionally, we review the applications of Yarrowia lipolytica promoter engineering to rationally reconstruct biosynthetic gene clusters and improve the genome-editing efficiency of the CRISPR-Cas system. Finally, research needs and future directions for promoter engineering are also discussed in this review.
Suggested Reviewers:	I-Son Ng, PhD Professor, National Cheng Kung University yswu@mail.ncku.edu.tw Professor Ng is an expert on metabolic engineering and synthetic biology and has published similar papers as this manuscript; she must be familiar with this manuscript.
	Bai-Shan Fang, PhD Professor, Xiamen University fbs@xmu.edu.cn Prof. Fang is an expert on biochemical engineering especially metabolic engineering for chemical production.
	Chang Dou, PhD Assistant Professor, Lawrence Berkeley National Laboratory: E O Lawrence Berkeley National Laboratory cdou@lbl.gov Dr. Dou has many industrial experiences on microbial lipid production using Yarrowia.
	Wenyu Lu, PhD Professor, Tianjin University wenyulu@tju.edu.cn Prof. Lu is an expert on Yarrowia metabolic engineering and has published many related papers.

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Dear Editors:

We would like to submit the enclosed manuscript entitled '**Advancing *Yarrowia lipolytica* as a superior biomanufacturing platform by tuning gene expression using promoter engineering**', which we wish to be considered for publication in '**Bioresource Technology**'. This review belongs to class of **80.040 Metabolic and genetic engineering**. We have previously requested the possibility of publishing this review article in Bioresource Technology, and the **Editor-in-Chief Prof. Pandey** was happy to consider this manuscript since he considered that the topic was certainly relevant to the journal and its readers **(Approved on 26-10-2021).**

To the best of our knowledge, although there are some related review articles in related to the metabolites production using *Yarrowia lipolytica*, there is no specialized review to describe the metabolic engineering tools such as promoter engineering in *Y. lipolytica*, which is developing very fast. Therefore, we have an idea to publish a review article in related to the progress and prospect of advancing *Y. lipolytica* to be a superior biomanufacturing platform by tuning gene expression using promoter engineering.

As known to all, *Y. lipolytica* is recognized as an excellent non-conventional yeast in the field of biomanufacturing, where it is used as a host to produce oleochemicals, terpenes, organic acids, polyols and recombinant proteins. Consequently, metabolic engineering and synthetic biology of this yeast is becoming increasingly popular for reconstructing heterologous metabolic pathways to advance it to be a superior biomanufacturing platform. During the manipulations, promoters, which are the basic elements for transcription initiation, can tune the gene expression and thus pave to the constructed pathway additivity. Here, we reviewed the identified *Y. lipolytica* endogenous promoters, which are the basis of promoter engineering for controlling transcription at the most fundamental level. And the engineering strategies, such as hybrid, intron enhancement, and transcription factor-based inducible promoter engineering are described. Additionally, we review the applications of *Y. lipolytica* promoter engineering in the fields of reconstructing biosynthetic gene clusters rationally and improving genome-editing efficiency of CRISPR-Cas systems. Finally, we will conclude the research needs and future directions of *Y. lipolytica* promoter engineering in this review. **We believed that the theme of the review would be of interest to the readers of Bioresource Technology. And it would become the highly cited paper in the future.**

We have reviewed the final version of the manuscript and approve it for publication. To the best of our knowledge and belief, this manuscript has not been published in whole or in part nor is it being considered for publication elsewhere. Also, the manuscript was not previously submitted to Bioresource Technology.

Please feel free to contact me if you have any questions or need additional information from us concerning this submission.

Sincerely yours,

Xiao-Jun Ji, Ph.D., Professor
Nanjing Tech University
Tel: +86 25 58139342
E-mail: xiaojunji@njtech.edu.cn

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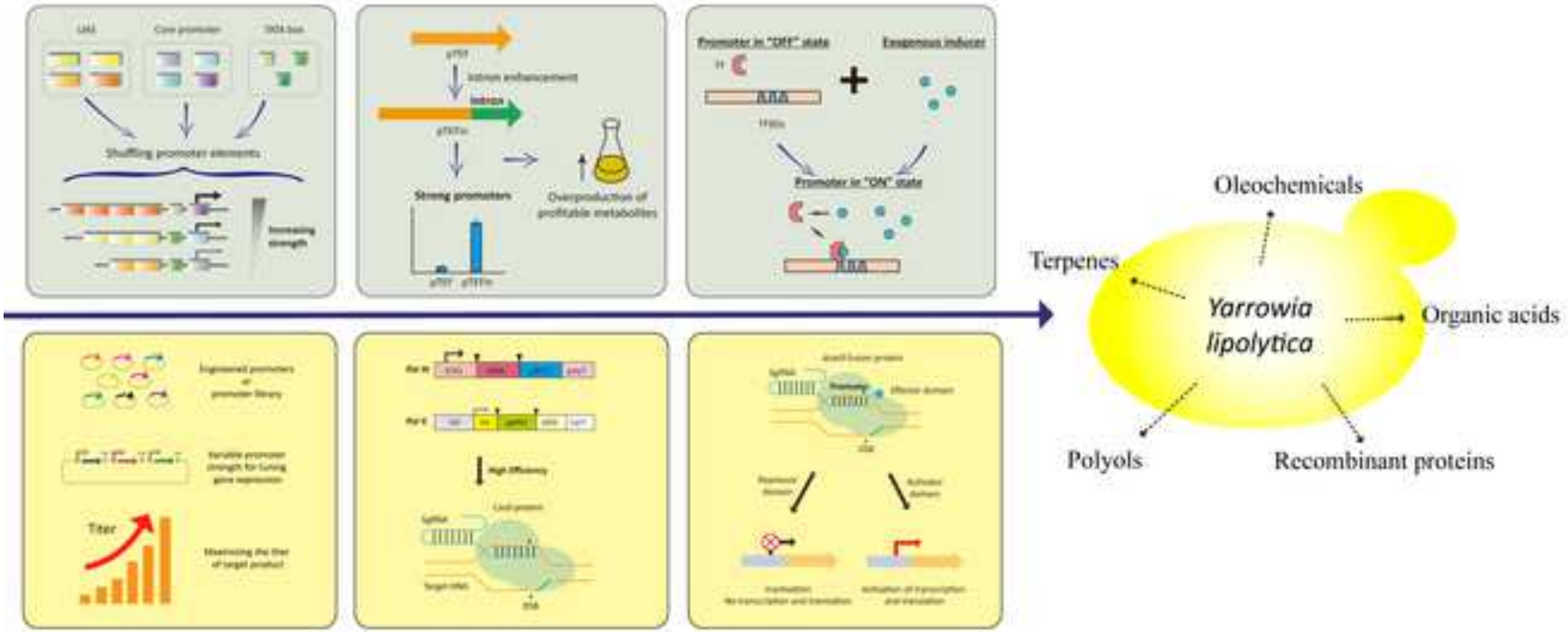

Highlights

- Endogenous promoters of *Y. lipolytica* are summarized and reviewed.
- Engineering strategies enables developing improved promoters for *Y. lipolytica*.
- *Y. lipolytica* promoter engineering helps to reconstruct the biosynthetic gene clusters.
- *Y. lipolytica* promoter engineering is able to improve the CRISPR-Cas efficiency.
- Research needs and future directions of *Y. lipolytica* promoter engineering are highlighted.

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Advancing *Yarrowia lipolytica* as a superior biomanufacturing platform by tuning gene expression using promoter engineering

Mei-Li Sun[1], Tian-Qiong Shi[2], Rodrigo Ledesma-Amaro[3], Xiao-Jun Ji*[1]

1. College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, No. 30 South Puzhu Road, Nanjing 211816, People's Republic of China
2. School of Food Science and Pharmaceutical Engineering, Nanjing Normal University, No. 1 Wenyuan Road, Nanjing 210046, People's Republic of China
3. Department of Bioengineering and Imperial College Centre for Synthetic Biology, Imperial College London, London SW7 2AZ, UK

*Corresponding author: xiaojunji@njtech.edu.cn

Abstract

Yarrowia lipolytica is recognized as an excellent non-conventional yeast in the field of biomanufacturing, where it is used as a host to produce oleochemicals, terpenes, organic acids, polyols and recombinant proteins. Consequently, metabolic engineering of this yeast is becoming increasingly popular to advance it as a superior biomanufacturing platform, of which promoters are the most basic elements for tuning gene expression. We reviewed the endogenous promoters of *Yarrowia lipolytica*, which are the basis for promoter engineering. The engineering strategies, such as hybrid promoter engineering, intron enhancement promoter engineering, and transcription factor-based inducible promoter engineering are described. Additionally, we review the applications of *Yarrowia lipolytica* promoter engineering to rationally reconstruct biosynthetic gene clusters and improve the genome-editing efficiency of the CRISPR-Cas system. Finally, research needs and future directions for promoter engineering are also discussed in this review.

Keywords: *Yarrowia lipolytica*, Metabolic engineering, Promoter engineering, Gene expression

1. Introduction

Yarrowia lipolytica has gained popularity in recent years as cell factories for the production of oleochemicals (Dobrowolski et al., 2020; Sun et al., 2017; Wang et al., 2020a), terpenes (Li et al., 2021; Ma et al., 2019), organic acids (Lei et al., 2019; Rzechonek et al., 2019), polyols (Wang et al., 2020b) and recombinant proteins (Madzak et al., 2004; Mou et al., 2021). It also exhibits its robustness and good performance, able to grow on a broad range of substrates (Liu et al., 2019; Sun et al., 2021), which make this yeast a promising candidate for industrial production of various compounds. The traditional strategies for strain improvement, such as natural screening and mutagenesis, are laborious and time-consuming, which makes metabolic engineering of this yeast necessary to elevate the strain performance. Due to the availability of a fully sequenced genome and growing number of synthetic biology tools, metabolic engineering of *Y. lipolytica* can be achieved with relative ease (Lu et al., 2021; Tang et al., 2021), of which promoters are the most fundamental component. Promoters are critical elements to precisely control gene expression. For example, the construction of engineered cell factories and synthetic circuits require transcriptional rewiring of central metabolic pathways and exact control of gene expression. Many studies sought to isolate and characterize endogenous promoters in *Y. lipolytica* to investigate their regulation mechanism and manipulate their effect on transcription, which also greatly facilitated the development of promoter engineering.

Promoter engineering generally refers to the creation of novel promoters, which produce transcripts at levels endogenous promoters are not comparable to, or have regulatory features not found in endogenous promoters. Compared to endogenous promoters, synthetic promoters usually possess (i) stronger expression; (ii) a wide range of transcription levels; (iii) reduced sequence length; (iv) novel inducibility (Rajkumar et al., 2016; Redden and Alper, 2015). Promoters with variable transcriptional capacities or desired properties are an invaluable tool for synthetic biology because fine-tuned gene expression is a prerequisites for ensuring optimal fluxes in pathway engineering and avoiding an excessive metabolic burden.

Furthermore, more ambitious metabolic engineering projects can be envisaged based on emerging genome editing tools, notably the CRISPR-cas systems, which mainly depends on the level of sgRNA transcription.

In this review, we will summarize the identified endogenous promoters of *Y. lipolytica*, which form the basis of promoter engineering for controlling transcription at the most fundamental level. Strategies, such as hybrid promoter engineering, intron enhancement promoter engineering, and transcription factor-based inducible promoter engineering are also discussed. Additionally, we review the applications of *Y. lipolytica* promoter engineering for the rational reconstruction of biosynthetic gene clusters and improvement of the genome-editing efficiency of CRISPR-Cas9 systems. Finally, we will discuss the current research needs in the development of *Y. lipolytica* promoter engineering, as well as the corresponding future directions for promoter engineering.

2. Endogenous promoters in *Yarrowia lipolytica*

Rational design and reconstruction of metabolic pathways via metabolic engineering and synthetic biology require functional promoters that are suitable for *Y. lipolytica*. Eukaryotic promoters, generally hundreds to thousands of base pairs from the transcriptional start point (TSS), are specific noncoding DNA sequences that recruit the cellular transcription machinery to initiate and control transcription (Kanhere and Bansal, 2005). In *Y. lipolytica*, common promoters consist of: a core promoter and enhancer regions in the upstream activating sequences (UAS) (Shabbir Hussain et al., 2016) (Figure 1). The promoter strength is co-regulated by the promoter architecture and can be enhanced by rational engineering of the elements.

It is generally believed that the eukaryotic promoter architecture allows complex regulatory mechanisms, and the transcriptional responses are more difficult to predict than in prokaryotic systems (Kanhere and Bansal, 2005; Shabbir Hussain et al., 2016). Therefore, endogenous promoters are often selected for fine-tuning metabolic pathways in *Y. lipolytica* (Chatzivasileiou et al., 2019; Liu et al., 2017; Ma et al., 2021; Tong et al., 2021). Generally speaking, stronger promoters can increase the

expression level of a protein. Nevertheless, it is important to note that the increase of promoter strength do not always improve the level of protein expression (Bienick et al., 2014; Dulermo et al., 2017; Zhao et al., 2021). Therefore, for the expression of heterologous genes, the strength and either constitutive expression or process-friendly inducibility of the promoter are necessary. Multiple promoters, including constitutive and inducible promoters, have been isolated and/or characterized in *Y. lipolytica* (Table1). The peptone-inducible promoter of the XPR2 gene, which encodes an alkaline extracellular protease, was the first strong promoter cloned from *Y. lipolytica* (Davidow et al., 1987). However, the complex regulation and uneconomical inducer impeded the industrial applications of pXPR2, which inspired the search for new promoters. The strong constitutive TEF promoter, which drives the transcription of the translation elongation factor-1α gene, is also a preferred choice for metabolic engineering applications in *Y. lipolytica* (Müller et al., 1998).

Due to the exceptional ability of *Y. lipolytica* to naturally accumulate lipids, many of the endogenous promoters identified are centering around the lipid-related pathways. Juretzek and colleagues (2000) compare the promoters pG3P, pICL4, pPOT4, pPOX4, pPOX2, and pPOX5 among each other and with the XPR2 promoter in respect to their regulation and activities cultured on different carbon sources. The promoters are inducible by fatty acids, alkanes or glycerol, whereby pICL4, pPOX2 and pPOT4 exhibited the stronger activity. A consortium of laboratories (Wong et al., 2017) selected 11 endogenous lipogenic promoters in addition to the well characterized TEF promoter for characterization of genetic parts. Unsurprisingly, the TEF promoter exhibited the highest activity, while the relative transcriptional activity of the 11 promoters ranged from 0.7 to 29.7% of TEF promoter activity. The expression levels of the promoters were in the order pTEF> pGAP> pACL2> pICL > pIDH2> pFAS1> pDGA1> pFAS2> pZWF1> pPOX4> pACC> pIDP2 (Table 1 for details). Interestingly, four promoters that reduce gene expression at the natural transition from the growth phase to the lipid accumulation phase have also been identified in *Y. lipolytica* (Kamineni et al., 2020). These promoters can be used to

drive genes that are essential or favorable for cell growth and undesirable for lipogenesis without changing the medium during fermentation. Furthermore, the availability of a set of promoters capable of optimizing the lipid yield or changing the cellular lipid profile in the lipid accumulation phase will provide valuable tools for fine-tuning metabolic pathways in *Y. lipolytica* (Kamineni et al., 2020).

Recently, six bidirectional Cu^{2+}-inducible promoters, P_{MT-1} to P_{MT-6}, were identified in *Y. lipolytica* (Xiong and Chen, 2020). Among these promoters, P_{MT-2} had the highest strength induced by Cu^{2+}, followed by P_{MT-6}, which had a comparable strength to constitutive promoters such as pTEF. The promoters were organized in three bidirectional pairs, including P_{MT-1}/P_{MT-2}, P_{MT-3}/P_{MT-4}, and P_{MT-5}/P_{MT-6}. This is the first report of bidirectional Cu^{2+}-inducible promoters, which constitute valuable tools to rewire genetic circuits with unique dynamics and reprogram genes expression (Yang et al., 2013). Furthermore, the same research team also isolated five repressible promoters, two of which are repressed by Cu^{2+} (Xiong and Chen, 2020). The combinatorial use of the Cu^{2+}-inducible and Cu^{2+}-repressible promoters may provide effective tools for yield optimization without dramatic changes of culture conditions.

Other endogenous promoters that were isolated and characterized in *Y. lipolytica*, including pLEU (Franke et al., 1998), pFBA1, $pFBA1_{IN}$, pTDH1, pGPM1 (Hong et al., 2012), pLIP2 (Sassi et al., 2016), and pEYK (Trassaert et al., 2017) are summarized in Table 1. Expanding the number of available endogenous promoters will provide alternative targets, which is also conducive for further promoter engineering.

3. Promoter engineering in *Yarrowia lipolytica*

For any host organism, it is a prerequisite to have strong and tunable promoters to precisely regulate the gene expression involved in biosynthetic pathways. Improved promoters are always in demand since no 'perfect' promoter exists under all growth and induction conditions. To improve the strength and tunability of available promoters, many strategies have been developed for promoter engineering. For other organisms, such as *Saccharomyces cerevisiae* and *Escherichia coli*, site-directed mutagenesis and error-prone PCR (Ep-PCR) have been used to modify the expression

levels of endogenous promoters (Alper et al., 2005; Nevoigt et al., 2006; Yona et al., 2018). Nevertheless, these methods are quite limited by (i) their dependence on strong and well-defined endogenous promoters and (ii) tendency of changes resulting in activity below the baseline. Beyond point mutations and Ep-PCR, the three strategies: hybrid promoter engineering, intron enhancement promoter engineering and transcription factor-based inducible promoter engineering have enabled the development of improved promoters in a variety of organisms, including *Y. lipolytica*.

3.1 Hybrid promoter engineering

Hybrid promoter engineering treats the promoter elements as "parts", which can be rearranged or replicated to construct a hybrid promoter with stronger or tunable activity. The promoter architecture and elements are the basis for hybrid promoter engineering, which mainly targets the core promoter, TATA box and UAS sequences (Figure 2A).

The core promoter is the minimal DNA sequence, regarded as the key factor determining the promoter strength (Hahn and Young, 2011; Smale and Kadonaga, 2003). The regulation mechanism of the core promoter can be complex in yeast systems. For instance, the content of A/T has a great influence on the promoter activity in *S. cerevisiae*. The promoter has higher activity when the T content upstream of TSS is rich and A content downstream of TSS is abundant (Lubliner et al., 2013). Several studies confirmed this trend in *Y. lipolytica*, but there are exceptions (Shabbir Hussain et al., 2016; Zhao et al., 2021). The TATA box, located in the core promoter, is the first functional element identified to regulate the promoter strength and the most studied component of eukaryotic promoters (Blazeck and Alper, 2013). The sequence of the TATA Box is short and relatively highly conserved. Two well-studied consensus TATA box sequences, TATAAA and TATATA, which have exhibited high activity in *S. cerevisiae*, also found in the endogenous *Y. lipolytica* promoters pTEF and pLEU (Basehoar et al., 2004).

Enhancer elements (UASs) are located several hundreds to thousands of bp upstream of the TSS and act in a distance-dependent manner (Shabbir Hussain et al.,

2016). UASs and their binding TFs are probably required for the expression of all protein-coding genes transcribed by RNA polymerase II (Pol II) (Smale and Kadonaga, 2003). Several studies revealed UAS sequences are involved in the properties of promoter induction (Park et al., 2019; Shabbir Hussain et al., 2016). Blazeck et al. (2011) showed that *Y. lipolytica* promoters are enhancer limited, mainly by the number of UASs. To address this limitation, many researchers have made tandem copies of UAS enhancer sequences to increase transcription. These additional UAS copies have been shown to be genetically stable in *Y. lipolytica* for long-term expression and use (Blazeck et al., 2011). Several enhancers have been identified in *Y. lipolytica* as modular elements that can be used for the construction of new recombinant promoters, including $UAS1_{XPR2}$ (aka UAS1B) (Madzak et al., 1999), UAS_{TEF} (Blazeck et al., 2013), $UAS1_{EYK1}$ (Trassaert et al., 2017) and UAS_{POX2} (Shabbir Hussain et al., 2017). $UAS1_{XPR2}$ is the well-studied sequences and the most extensively used in promoter engineering. Due to the small number of well-defined enhancer elements in *Y. lipolytica*, novel regulatory elements (UAS) remain to be discovered.

Hybrid promoters can be generally constructed by combining a core promoter sequence with one or more copies of UAS elements. This engineering method allows the assembled novel promoters with new properties due to the modularity of UAS elements, for instance, enhanced expression or inducibility. The strength of promoters can often be enhanced based on the number of UAS repeats, such as the representative and most commonly used hybrid hp4d promoter in *Y. lipolytica* (Madzak et al., 2000). Based on the functional dissection of pXPR, the functional DNA sequences were identified, including the distal UAS (UASl) and the proximal UAS (UAS2). The activity of UAS1 was independent of the culture conditions, while UAS2 appeared to be regulated by similar pH and nitrogen availability conditions as the endogenous pXPR2 (Madzak et al., 1999). One to four copies of $UAS1_{XPR}$ and a core minimal LEU2 promoter (reduced to its TATA box) were fused to develop the recombinant promoters, hp1d, hp2d, hp3d and hp4d, whose strength exhibited a linear

increase with the growing number of tandem $UAS1_{XPR}$ repeats (Madzak et al., 2000). Since the recombinant hp4d promoter naturally allows partial dissociation of growth and expression phases to avoid product toxicity (Madzak et al., 2000; Nicaud et al., 2002), it is the most widely used tool for heterologous gene expression in *Y. lipolytica* (Madzak, 2021) and selected for the commercial YLEX™ Expression Kit by Yeastern Biotech Co. (Taiwan). A further reanalysis of hp1d to hp4d, a nearly perfect positive linear correlation was reconfirmed between the numbers of tandem $UAS1_{XPR}$ repeats and promoter strength, which raises the possibility of further improvements. 1 to 32 tandem $UAS1_{XPR}$ were placed upstream of the core promoter sequence from pLEU2. The resulting series of hybrid promoters demonstrate a wide range of promoter strengths, with more than a 400-fold dynamic range of expression between the minimal promoter and the strongest promoters, which was 8-fold stronger than the best endogenous promoters in *Y. lipolytica* (Blazeck et al., 2011).

A recent study showed that fusing the $UAS1_{XPR}$ sequence to different minimal promoters can confer higher substrate-specific responsiveness to regulation by oleic acid compared to glucose and glycerol (Shabbir Hussain et al., 2016), in contrast to the notion that they are independent of culture conditions (Madzak et al., 1999). Possible reasons for the contradictory conclusions include (i) the influence of different carbon sources on hp4d, which was not investigated in previous studies, as well as (ii) the different minimal promoter sequences in the hybrid promoters, which may be crucial for the properties of the hybrid promoter. These observations highlighted the incidence of unexpected regulatory behaviors in engineered eukaryotic promoters.

Another general strategy for the development of hybrid promoters is to produce a promoter pool containing variable□strength promoters for customizing gene expression. Trassaert and colleagues (2017) identified the novel upstream activating sequence $UAS1_{EYK1}$ from the erythritol-inducible pEYK1 through promoter characterization and mutagenesis. Subsequently, they constructed new hybrid promoters by combining the core EYK promoter with copies of either $UAS1_{XPR2}$ or

UAS1$_{EYK1,}$ which showed higher expression levels than the endogenous pEYK1 promoter. Furthermore, the strength of the promoter was enhanced in a Δeyk1 strain, allowing the use of cost-effective erythritol and erythrulose as inducers (Trassaert et al., 2017). Park et al. (2019) also identified two novel enhancer elements (UAS1$_{EYD1}$ and UAS2$_{EYD1}$) within erythritol-inducible pEYK1 and pEYKD1 (from two genes involved in erythritol catabolism) via phylogenetic footprinting. Based on this, a set of inducible promoters with variable strengths was successfully developed, ranging from 0.1 to 457.5 SFU/h. Shabbir et al. (2016) developed a new panel of hybrid promoters by shuffling promoter elements (UAS, core promoter, and TATA box) of fungal promoters (TEF, POX2, LEU2, PAT1) in *Y. lipolytica*. They showed that promoter architecture can be engineered to modulate and fine-tune gene expression.

Very recently, the common promoter elements (UAS, TATA box and core promoter) of *Y. lipolytica* were detected and characterized to explore the mechanism of synergy between various elements (Zhao et al., 2021). The researchers subsequently rearranged these elements to construct a library of hybrid promoters with a wide range of transcription strengths, which can be a powerful toolkit for the identification of optimal promoters to improve metabolic pathways in *Y. lipolytica*. Interestingly, three different UASs from *S. cerevisiae* have also been applied to construct hybrid promoters, which is the first report on the transferability of promoter elements from *S. cerevisiae* to *Y. lipolytica* (Zhao et al., 2021).

Hybrid promoter engineering may be a promising and efficient strategy for the construction of individual promoters or promoter pools with enhanced or variable strength. However, the method depends on identified and characterized promoter "parts", which is a limiting factor in the development and application of promoter engineering.

3.2 Intron enhancement promoter engineering

Promoter engineering via intron enhancement requires the cloning of an upstream intron together with a corresponding promoter (Figure 2B). Intron enhancement was first observed in other systems, varying from 2-fold increase in

mammals to more than 1000-fold increase in maize (Furger et al., 2002; Callis et al., 1987). Nevertheless, our understanding of the mechanisms through introns enhance expression remains surprisingly rudimentary (Gallegos and Rose, 2015). A speculative way for introns to increase mRNA levels is by dissipating the torsional strain produced by transcription (Niu and Yang, 2011). Another model with experimental support in yeast is that introns can cause DNA looping that brings the 5' and 3' ends of a gene into proximity, thereby facilitating transcription re-initiation (Moabbi et al., 2012). Introns are also believed to increase expression by favoring mRNA export by coupling splicing to the processes of nuclear export (Le Hir et al., 2003). In *S. cerevisiae*, only 5% of all genes contain introns, but they produce 3.9-fold more RNA on average than non-intronic genes (Juneau et al., 2006). The intron-containing genes of *Y. lipolytica* account for 15% of the total (Mekouar et al., 2010), indicating that introns may play an important role in regulating gene expression.

In order to investigate the effect of introns on gene expression, Tai and Stephanopoulos (2013) created an improved version of the TEF promoter (pTEFin) by including 113bp of the *Y. lipolytica* endogenous intron along with pTEF (Figure 2B). They compared the relative gene expression levels of hp4d, pTEF and pTEFin, which exhibited a 17-fold increase over pTEF and a 5-fold increase over hp4d. The pFBAin containing native introns also showed 5-fold higher expression than pFBA1 without an intron (Hong et al., 2012). The transcriptional enhancement was further confirmed by the construction of the GPM1::FBA1 chimeric promoter by attaching the 5′-region of the FBA1 gene to pGPM1. The chimeric promoter showed a significant increase of transcription activity compared to pGPM1, which confirmed the 5′-region of the FBA1 gene could improve promoter activity (Hong et al., 2012).

Enlisting this endogenous process represents a simple means to increase expression of desired genes for optimizing pathway fluxes, and this approach is applicable in many eukaryotic organisms. While several studies aimed to enhance promoter strength using endogenous introns, the application of introns in biotechnology is a relatively untapped opportunity. More efforts should be devoted to

investigating the function, evolution and roles of introns. The reward for solving this mystery could be a comprehend of an entirely new approach for regulating gene expression.

3.3 Transcription factor-based inducible promoter engineering

In *Y. lipolytica*, native promoters and regulatory elements are often selected for controlled gene expression. Although these regulatory sequences allow genes to be expressed over a broad range of transcription strengths, the control over them is exerted by native TFs. However, employing native promoters or regulatory elements for heterogenous pathways can increase the metabolic burden on the host cells due to competition between product synthesis and cell growth or proliferation and the replication of non-essential genetic elements (Bu et al., 2021; Wu et al., 2016). Furthermore, using promoters with variable strength in the construction of genetic circuits or repeated use of the same regulator in multiple gene expression cassettes can lead to genetic instability (Peng et al., 2015; Sleight et al., 2010). To address these challenges, inducible promoters have been developed to induce expression only under certain conditions, thus making it possible to decouple the growth phase from the product synthesis phase of fermentation.

The inducibility of promoters is driven by the TFs that only bind to their cognate binding site (BS) under specific conditions (Figure 2C). Novel inducible promoters are often constructed by incorporating binding sites of inducible TFs within the UAS. As described above, Park et al. (2019) determined the erythritol-responsive element ($UAS1_{EYK1}$), which imparts inducibility on the EYK1 promoter. Then, they created a set of erythritol-inducible promoters with tandem repeats of $UAS1_{EYK1}$ to increase inducibility, with extensive applications in industrial protein production, metabolic engineering, and synthetic biology. As the example shows, native TF-TFBS pairs are usually employed to engineer promoters in their native host after they are discovered, which can cause a metabolic burden on the cell when multiple-genes of a heterologous pathway are expressed.

Recently, a xylose-inducible biosensor was developed by implementing xylose-

responsive TF (XylR) from *E. coli*, which binds to its cognate binding site only in the presence of xylose (Wei et al., 2020a). XylR was fused to the activation domain VPRH (including VP64, p65, Rta, and HSF1) to form the TF complex VPRHX. Here, four promoters (pTEF, TEF core promoter, Leu core promoter, and pACC1) were selected to construct hybrid synthetic promoters carrying the XylR binding site *xylO* (shortened to Pxo). The biosensor was able to trigger transcription activation of genes of interest in *Y. lipolytica* following the addition of xylose. In addition, the xylose utilization genes were introduced into an engineered *Y. lipolytica* strain and placed under the control of the biosensor, which has great potential for pathway engineering referring to biosynthesis of value-added products form xylose (Wei et al., 2020a). The xylose-inducible system was applied to use xylose as both a substrate and inducer in *Y. lipolytica*, which coupled xylose utilization and naringenin biosynthesis (Wei et al., 2020b). The titer of naringenin under xylose-controlled expression reached 715.3 ± 12.8 mg/L in shake-flask cultivation, which was much higher than the 239.1 ± 5.1 mg/L achieved under constitutive expression of UAS1B8TEF.

Naseri et al. (2017, 2021) developed an orthogonal and controllable system in *s. cerevisiae* and *Pichia pastoris*, which allows minimal interference with endogenous cellular processes and expression at the desired time. A library of 106 synthetic transcription regulatory units based on plant TFs and their cognate binding sites with up to 2,000-fold IPTG induction level was established. As a result, plant-derived TFs were implemented as independently acting control units in *S. cerevisiae* (Naseri et al., 2017). Then, 17 different combinations of plant-derived artificial TFs and their BSs were tested in *P. pastoris*, and showed a wide range of inducible transcriptional outputs, from 1 to 63-fold induction. The data also indicate that such regulators have high degree of transferability between *S. cerevisiae* and *P. pastoris* (Naseri et al., 2021). The strategy to develop inducible promoters with exogenous TFs for orthogonal control of gene expression is highly desirable for balanced expression of multiple genes. Therefore, more efforts should be devoted to developing novel inducible promoters, which will enhance the capability for controlling gene

expression in *Y. lipolytica*.

4. Applications of promoter engineering in *Yarrowia lipolytica*

4.1 Rational design and reconstruction of biosynthetic gene clusters

Rational design and reconstruction of biosynthetic gene clusters was proven to be effective for achieving stable high-yields of target products (Liu et al., 2020; Wang et al., 2019; Zhang et al., 2018b). Several studies have shown that the production of high-value bioproducts can be promoted by replacing promoters for biosynthetic pathways with stronger ones in *Y. lipolytica* (Tong et al., 2021; Xiong and Chen, 2020). Nevertheless, effective engineering of microorganisms is central for balanced expression of both heterologous and native genes to channel metabolic fluxes toward target products (Table 2). Balanced gene expression relies on rational design and reconstruction of biosynthetic gene clusters via synthetic biology for maximizing the yield of compounds of interest. Biosynthetic pathways often require the expression of multiple genes, which unavoidably interferes with the native metabolism of the host strain and results in an imbalance of the native metabolic fluxes (Raman and Chandra, 2009). However, due to the complexity of eukaryotic gene expression and multiple rate-limiting factors in the target pathways, increasing the expression of single gene or amplification of each gene in biosynthetic pathways often cannot efficiently equalize the gene expression (Otto et al., 2019; Raman and Chandra, 2009; Zhao et al., 2021). Furthermore, sometimes the random overexpression of genes from biosynthetic pathways may even disrupt the intracellular metabolic balance, which will result in the accumulation of intermediates or by-products, thus increasing the metabolic burden to the cell and even lowering the yield of the target product. In order to balance gene expression, promoter engineering has been developed to design and reconstruct biosynthetic pathways using different promoter pairs (Figure 3). Zhao et al. (2021) employed the aforementioned hybrid promoter library with wide expression range to optimize the biosynthetic pathways of isoamyl alcohol, which demonstrated that promoter engineering is an effective strategy for increasing the biosynthesis of valuable chemicals. Wong et al. (2017) employed 12 characterized promoters to

construct pYaliBrick vectors, after which a 12kb natural product biosynthetic pathway for violacein containing five genes was rapidly assembled in *Y. lipolytica*. The YaliBrick vectors provide a unique strategy to facilitate the control of multiple genes expression at optimal levels in *Y. lipolytica.*

In addition, dynamic regulation of gene expression by inducible promoters is also a preferred strategy to reduce the metabolic load on the native cells and increase the yield of target product. In order to convert the intermediates into target products, it is necessary to induce tailoring enzymes' expression at a desired specific state. For example, copper-inducible promoters were engineered to create the hybrid promoter $P_{MT\text{-}2}$-UAS16, whose strength was enhanced and further increased by adding Cu^{2+}. The engineered promoter was successfully employed in the biosynthesis of long-chain wax esters in *Y. lipolytica*, with a titer of up to 149.5 mg/L, which was higher than the titer produced using the constitutive TEF promoter (Xiong and Chen, 2020). While chemical induction may be the easiest method for the synchronization of gene expression to reduce the metabolic burden with rational design and reconstruction of biosynthetic pathway, sometimes it is difficult to find inexpensive proper inducers (Naseri et al., 2021). An alternative strategy is to use a genetic switch. If a simple genetic switch responding to changes in the concentrations of target products or metabolic intermediates exists, dynamic control of heterologous gene expression would be possible without the addition of an inducer. Very recently, Park et al. (2021) designed synthetic promoters containing bacterial FadR binding sequences for CYP expression. These promoters are responsive to the concentration of free fatty acids and activate the ω-hydroxylating pathway to produce ω-hydroxylated long-chain fatty acids. This study presents an excellent example of the synthesis of target product using synthetic promoters as a genetic switch, which is an effective strategy to decouple the growth phase from the production phase to decrease the metabolic burden to cell growth caused by the high expression of heterologous genes.

As these studies illustrate, promoter engineering has shown great potentials and enabled advances in the fields of synthetic biology and metabolic engineering.

However, due to insufficient knowledge surrounding promoters and relevant biosynthetic pathways, as well as the scarcity of advanced technologies, there are still some problems in coordinating gene expression, channeling metabolic fluxes and efficient synthesis of target product at the systemic level. One obvious problem is that most of these strategies are only limited to the manipulation of a few genes, such as those encoding rate-limiting enzymes or regulatory genes, which seriously impedes the industrial applications of *Y. lipolytica* for large-scale production of valuable compounds. In recent years, several strategies for balancing the expression of multi-genes pathway have been developed in *S. cerevisiae*, such as COMbinatorial Pathway ASSembly (COMPASS) (Naseri et al., 2019; Naseri and Mueller-Roeber, 2020) and customized optimization of metabolic pathways by combinatorial transcriptional engineering (COMPACTER) (Yuan et al., 2013). With further development, these strategies could serve as helpful tools for pathway optimization in *Y. lipolytica* in the future.

4.2 Improving the genome-editing efficiency of CRISPR-Cas systems

Due to the growing demand for metabolic engineering in *Y. lipolytica*, a number of innovative genome-editing tools have been developed (as reviewed in Ganesan et al., 2019; Patra et al., 2021; Shi et al., 2018). Promoter engineering has proved to be very efficient in improving the genome editing efficiency of CRISPR/Cas9. The CRISPR-Cas9 system consists of two parts, the Cas9-targeted nuclease and the single guide RNA (sgRNA) (Doudna and Charpentier, 2014). Early on, this strategy was implemented in *E. coli* (Jiang et al., 2013) and *S. cerevisiae* (DiCarlo et al., 2013). Nevertheless, it is a challenge to apply the CRISPR-Cas9 system to a new organism, since the fine-tuned expression of both Cas9 nuclease and sgRNAs is necessary. CRISPR-Cas9 was first implemented in *Y. lipolytica* using a series of promoters, including synthetic RNA polymerase III (Pol III) promoters as well as an RNA polymerase II TEF promoter flanked by hammerhead and hepatitis delta virus (HDV) ribozymes, to drive the expression of sgRNA (Schwartz et al., 2016) (Figure 4A). The highest efficiency of gene knock-out was achieved by combining the expression of the

sgRNA from synthetic hybrid promoters that combine native RNA polymerase III (Pol) promoters with tRNA and the hybrid promoter UAS1B8-TEF driving the expression of Cas9. The resulting replicative vector pCRISPRyl was frequently applied in later studies and deposited at Addgene (Schwartz et al., 2016). Around the same time, the CRISPR-Cas9 system was also developed in *Y. lipolytica* by Gao et al. (2016). They designed a replicative vector for the expression of Cas9 and sgRNA both driven by the strong RNAP II promoter pTEFin, and the editing efficiency reached more than 85%. Subsequently, Wong et al. (2017) modified the type II RNA promoter to drive the expression of sgRNA and expanded a set of vectors to deliver sgRNA for efficient targeted genome editing. Subsequently, Morse et al. (2018) developed an orthogonal T7 polymerase-based CRISPR-Cas system for sgRNA expression in *Y. lipolytica*. Genome editing efficiencies of 60%were achieved using T7 polymerase driven by a strong promoter and sgRNA expression driven by the T7 promoter. Recently, CRISPR/Cas9-based gene editing with efficiency close to 100% was achieved in *Y. lipolytica* by designing a tRNA-sgRNA fusion system to enhance the sgRNA expression level (Abdel-Mawgoud and Stephanopoulos, 2020).

Other CRISPR tools, CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) systems for controlling gene expression, have also been implemented in *Y. lipolytica*. These systems were developed for targeted gene repression or activation by targeting a catalytically deactivated Cas9(dCas9) to the promoter region of gene of interest and fusing dCas9 to transcriptional activators or repressors (Figure 4B). The CRISPRi system was first established in *Y. lipolytica* by Schwartz et al. (2017). The researchers set out to design multiplexed sgRNAs targeting genes of interest at the TSS or the promoter region immediately upstream of the TSS, which demonstrated efficient repression of 8 of 9 targeted genes. Then, the repression was enhanced by fusing dCas9 to the Mxi1 repressor, and 90% homologous recombination efficiency were achieved with multiplexed targeting of KU70 and KU80 (Schwartz et al., 2017). Another similar CRISPRi system for *Y. lipolytica* was designed based on four different repressors, dCpf1, dCas9, dCpf1-KRAB and dCas9-KRAB (Zhang et al.,

2018a). With this strategy, repression efficiencies as high as 85% (dCpf1) and 92% (dCas9) were achieved when three sgRNAs were used to simultaneously target different regions in the promoter of the *gfp* gene. In addition to the CRISPRi system, Schwartz et al. also developed a CRISPRa system to activate gene expression in *Y. lipolytica* (Schwartz et al., 2018). In this work, different activators and target sites in the promoter region were compared. The VPR activator was fused to dCas9 and sequences closest to the core promoter of the gene of interest were chosen as the target site. Two silent native β-glucosidase genes, BGL1 and BGL2, were activated to enable growth on cellobiose as the sole carbon source (Schwartz et al., 2018). Recently, in order to enable finer control of transcription, Misa and Schwartz (2021) used the CRISPRi and CRISPRa systems to repress or increase the expression of any native gene in *Y. lipolytica*. The combined use of CRISPRi and CRISPRa enabled multiplexed activation or repression of more than one gene, which further improves the ability to control the metabolism of *Y. lipolytica.*

Undoubtedly, these CRISPR-Cas systems constitute valuable tools to accelerate metabolic engineering for either genome editing or fine transcriptional control (Schwartz and Wheeldon, 2018). Ongoing efforts in exploring or developing novel promoters for *Y. lipolytica* will enhance the field of CRISPR-Cas-based genome editing in this increasingly important industrial organism.

5. Research needs and future directions

As a Generally Recognized As Safe (GRAS) microbe, *Yarrowia lipolytica* has potential to act as a biotechnological workhorse due to its ability to convert a large range of substrates, including glucose, glycerol, xylose and hydrophobic compounds, into high titers of valuable molecules (Li et al., 2018; Zhang et al., 2018b; Zhou et al., 2021). With the recent advances in synthetic biology, metabolic engineering of *Y. lipolytica* has achieved considerable success. Two commercial products from metabolically engineered *Y. lipolytica* have been developed by DuPont (Xie et al., 2015). Moreover, *Y. lipolytica* is on its way to becoming a model non-conventional organism, which are enhancing the rate of the industrialization of microbes (Lobs et

al., 2017; Wagner and Alper, 2016). It has been a long-term goal to rationally engineer microorganisms for the efficient synthesis of value-added and renewable products. Synthetic biology is a rational approach for the design and construction of genetic parts or modules that allow a predictable and accurate regulation of biological processes. The prospects of synthetic biology are constantly evolving and expanding (Cameron et al., 2014), but this approach fundamentally depends on the premise that biological systems can be constructed from well-characterized parts. Among the modular tools of synthetic biology, promoters are one of the first key synthetic part.

Promoters are ubiquitous genetic elements that enable fine-tuned control of gene expression, and promoter engineering is enabling advances in the fields of pathway engineering and genome editing. By taking advantage of the aforementioned strategies or their combination, it is possible to create strong, constitutive promoters suitable for a variety of different contexts, as well as inducible promoters with synthetic off-switches as the growth conditions change. Promoter strength is one of the major factors controlling the overall metabolic balance and intracellular metabolic load, indicating that promoter engineering is a powerful tool for maximizing the production of compounds of interest. Moreover, recent developments in the design of CRISPR/Cas9 systems for targeted introduction/activation/repression of chromosomal genes are underscoring the significance of promoter engineering.

Although remarkable progress has been made using promoter-based strategies, there are still several problems to be solved in the process of *Y. lipolytica* strain improvement. Firstly, most of endogenous promoters for engineering *Y. lipolytica* have not been characterized rigorously under different growth conditions to meet the standards for predictability and reliability. The strain background also has not been fully taken into consideration when promoters were studied, which may cause unexpected regulatory behaviors. Furthermore, while promoter engineering has proved to be effective for creating constitutive or inducible promoters, such as the strong constitutive $pTEF_{in}$, phase-dependent hp4d and fatty-acid-inducible FadR promoter, current promoter engineering is still limited by a paucity of isolated and

characterized promoter elements, such as UAS, TATA box and core promoter. Finally, inducible promoters with exogenous TFs are needed for orthogonal (minimal interference with the endogenous cellular processes) and controllable (enabling gene expression at the desired time) systems for *Y. lipolytica,* as discussed in section 3.3. This system can fine-tune gene expression to eliminate the metabolic burden caused by the repeated use of native regulatory elements for pathway engineering, especially for large biosynthetic gene clusters.

Although there is a great need for additional advances in the field of promoter engineering to fully exploit this unconventional yeast, significant advances have been made. Therefore, new research is expected to continue improving the biotechnological application potential of *Y. lipolytica.*

6. Conclusions

With the advances of synthetic biology, the field of *Y. lipolytica* promoter engineering has been developing rapidly. Approaches for the construction of promoters using hybrid sequences or intron enhancement have led to valuable advancements in *Y. lipolytica*. However, the constructed promoters are relatively large and depend on native elements. To address some of these limitations, promoters with exogenous orthogonal elements have been developed. Rational design and reconstruction of biosynthetic gene clusters, as well as the genome-editing efficiency of CRISPR-Cas systems, have been greatly promoted by tuning gene expression using promoter engineering, thus advancing *Y. lipolytica* as a superior biomanufacturing platform.

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Tables:

Table 1. Promoters for heterologous expression in *Yarrowia lipolytica*

Promoters	Corresponding gene	Features	References
pXPR2	Alkaline extracellular protease	Endogenous: induced by peptones	Davidow et al. (1987)
pTEF	Translation elongation factor-1α	Endogenous: Constitutive	Müller et al. (1998)
pRPS7	Ribosomal protein S7	Endogenous: Constitutive	Müller et al. (1998)
pG3P	Glycerol-3-phosphate dehydrogenase	Endogenous: Inducible by glycerol	Juretzek et al. (2000)
pICL1	Isocitrate lyase	Endogenous: Inducible by fatty acids and alkanes or ethanol and acetate	Juretzek et al. (2000)
pPOT1	3-oxo-acyl-CoA thiolase	Endogenous: Inducible by fatty acids and alkanes	Juretzek et al. (2000)
pPOX1	Acyl-CoA oxidase 1	Endogenous: Weakly inducible by alkanes	Juretzek et al. (2000)
pPOX2	Acyl-CoA oxidase 2	Endogenous: Inducible by fatty acids and alkanes	Juretzek et al. (2000)
pPOX5	Acyl-CoA oxidase 5	Endogenous: Weakly inducible by alkanes	Juretzek et al. (2000)
pGAP	Glyceraldehyde 3-phosphate dehydrogenase	Endogenous: The 11 promoters were only used for genetic parts characterization and have not been fully characterized under different carbon, nitrogen and oxygen conditions.	Wong et al. (2017)
pACL2	ATP citrate lyase 2		Wong et al. (2017)
pICL	Isocitrate lyase 1		Wong et al. (2017)
pIDH2	Isocitrate dehydrogenase NAD+ subunit 2 mitochondrial		Wong et al. (2017)
pFAS1	Fatty acid synthase subunit alpha		Wong et al. (2017)
pDGA1	Acyl-CoA: diacylglycerol acyltransferase		Wong et al. (2017)
pFAS2	Fatty acid synthase subunit beta		Wong et al. (2017)
pZWF1	ZWF1 Glucose-6-phosphate dehydrogenase		Wong et al. (2017)
pPOX4	POX4 Fatty-acyl coenzyme A oxidase		Wong et al. (2017)

pACC	Acetyl-CoA-carboxylase 1		Wong et al. (2017)
pIDP2	Cytosolic NADP-specific isocitrate dehydrogenase		Wong et al. (2017)
p1	Similar to gene coding amino acid permeases	Endogenous: The regulation mechanism of the promoters has not been explored in this research.	Kamineni et al. (2020)
p3	Gene of unknown function		Kamineni et al. (2020)
p4	Gene of unknown function		Kamineni et al. (2020)
p5	Similar to gene coding amino acid permeases		Kamineni et al. (2020)
pFBA1	Fructose 1,6-bisphosphate aldolase		Hong et al. (2012)
pTDH1	Glyceraldehyde-3-phosphate dehydrogenase	Endogenous: Constitutive	Hong et al. (2012)
pGPM1	Phosphoglycerate mutase		Hong et al. (2012)
$pFBA1_{IN}$	pFBA1 + FBA intron included		Hong et al. (2012)
pLIP2	Extracellular Lip2p lipase	Endogenous: Inducible by oleic acid	Sassi et al. (2016)
pLEU	β-Isopropylmalate dehydrogenase	Endogenous: Inducible by leucine precursor	Franke et al. (1988)
pEYK1	Erythrulose kinase	Endogenous: Inducible by oleic acid erythritol and erythrulose	Trassaert et al. (2017)
P_{MT-1}- P_{MT-6}	Metallothionein	Endogenous: Inducible by Cu^{2+}	Xiong and Chen (2020)
pTHR1	Homoserine kinase	Endogenous: Repressible by amino acids	Xiong and Chen (2020)
pSER1	3-phosphoserine aminotransferase	Endogenous: Repressible by amino acids	Xiong and Chen (2020)
pMET3	ATP sulfurylase	Endogenous: Repressible by amino acids	Xiong and Chen (2020)
pCTR1	Copper transporter protein	Endogenous: Inducible by Cu^{2+}	Xiong and Chen (2020)
pCTR2	Copper transporter protein	Endogenous: Inducible by Cu^{2+}	Xiong and Chen (2020)
hp4d	/	Hybrid promoter: Growth phase dependent	Madzak et al. (2000)
pTEFin	pTEF1+TEF1 intron included	Constitutive	Tai and Stephanopoulos (2013)

Table 2. Examples of rational design and reconstruction of biosynthetic gene clusters using promoter engineering.

Promoter engineering strategies	Utilized promoters	Product	Titers	Increase compared to the control	References
The five genes encoding the violacein pathway enzymes were controlled by three promoters with varying strengths.	pTEF, pICL1, pZWF1	Violacein	70.04 mg/L	/	Tong et al. (2021)
Codon-optimized *MmWS* gene from mouse was expressed under the control of the engineered promoter $P_{MT\text{-}2}$-UAS16.	$P_{MT\text{-}2}$-UAS16	Wax ester	149.5 mg/L	Higher than the 134.2 mg/L obtained under the control of pTEF	Xiong and Chen (2020)
Hybrid promoter library was used to optimize the expression of the key limiting step gene of *ScARO10*.	$P_{UAS1B4+EXPm}$	Isoamyl alcohol	11.57 mg/L	5.4-fold higher than the 1.8 mg/L obtained under the control of $P_{UAS1B4\text{-}LEUm}$.	Zhao et al. (2021)
The five-gene pathway for violacein was rapidly assembled in the YaliBrick vectors containing TEF promoter.	pTEF	Violacein	31.5 mg/L	/	Wong et al. (2017)
The ω-hydroxy palmitic acid pathway was constructed with synthetic promoters containing bacterial FadR as a genetic switch.	FadR Synthetic Promoter	ω-hydroxy palmitic acid	160 mg/L	/	Park et al. (2021)
A hybrid promoter with the xylO operator was used to couple xylose utilization and naringenin biosynthesis.	Pxo-Ptef	Naringenin	715.3 ± 12.8 mg/L	Higher than the 239.1 ± 5.1 mg/L obtained under the constitutive UAS1B8TEF expression	Wei et al. (2020a, b)

Figure captions

Figure 1. Architecture of eukaryotic promoters. Eukaryotic promoters include a core promoter that may have a TATA box and UASs sequence located further upstream that contains TFBSs.

Figure 2. Hybrid promoter, intron enhancement promoter and transcription factor-based inducible promoter are the three concepts for promoter engineering in *Y. lipolytica*. A: Hybrid promoter engineering treats the promoter elements as "parts", which can be shuffled or replicated to construct hybrid promoters with stronger or tunable activity. B: Promoter engineering via intron enhancement entails retaining an intron with the corresponding promoter to increase expression of desired genes for overproduction of profitable metabolites. C: Promoters with inducible UASs or exogenous TFBSs can be turned-on to promote transcription in the presence of its inducer.

Figure 3. Application of promoter engineering for rational design and reconstruction of biosynthetic gene clusters. Engineered promoters or promoter library that cover a wide range of promoter strengths were applied to tune gene expression in pathway engineering for maximizing the titers of target products.

Figure 4. Promoter engineering to improve the genome editing efficiency of CRISPR-Cas systems. A. Synthetic promoters (Pol III promoter and tRNA) and Pol II TEF promoter flanked by hammerhead and HDV ribozymes drive efficient sgRNA expression and processing, resulting in high disruption efficiency using CRISPR-Cas9. B. dCas9 fusion proteins (dCas9 fused with transcriptional activators or repressors) bind to promoter regions resulting in transcription activation or repression.

Figure 1

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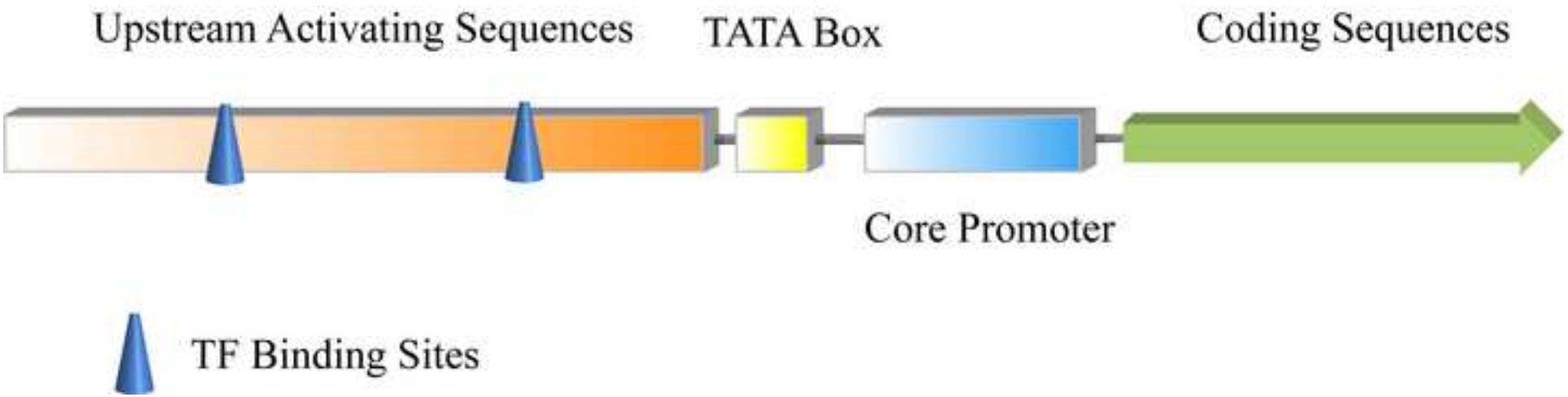

Figure 2

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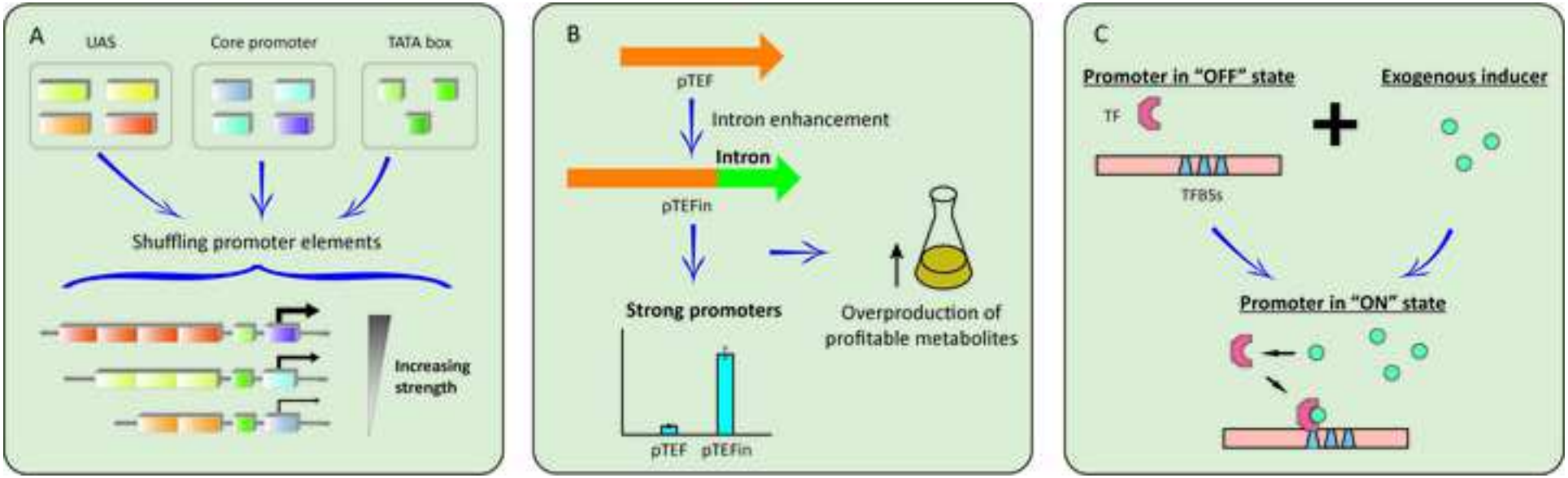

Figure 3

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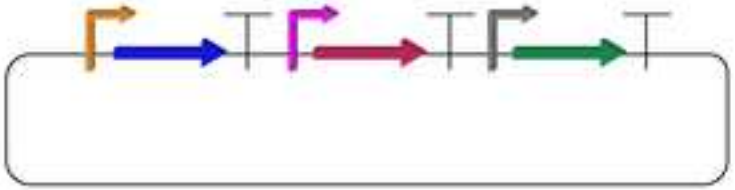
Engineered promoters or promoter library



Variable promoter strength for tuning gene expression

Figure 4

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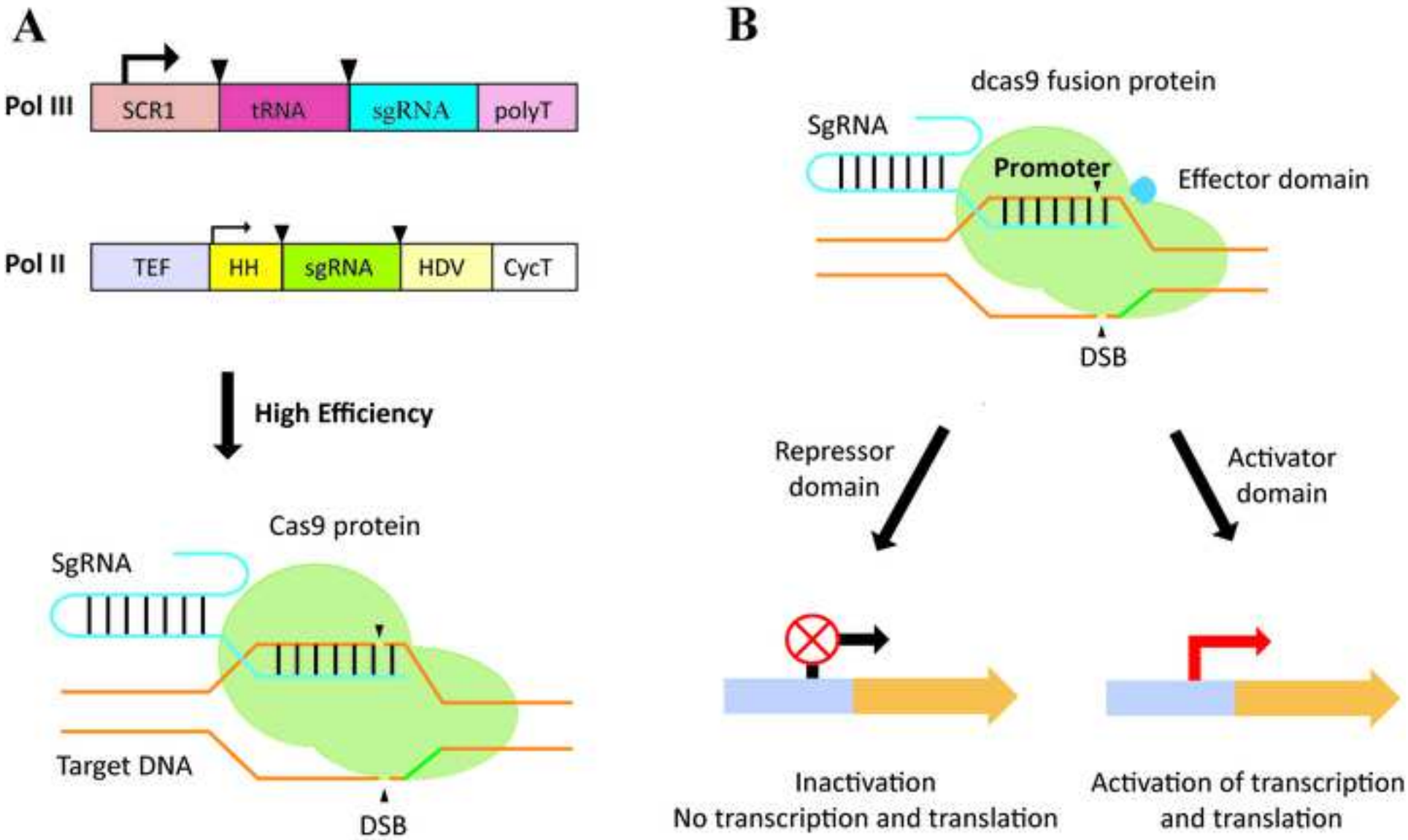

Declaration of Interest Statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, "**Advancing *Yarrowia lipolytica* as a superior biomanufacturing platform by tuning gene expression using promoter engineering**".