Hybrid promoter engineering strategies in *Yarrowia lipolytica*: isoamyl alcohol production as a test study

Yu Zhao
Tianjin University of Science and Technology
Shiqi Liu
Tianjin University of Science and Technology
Zhihui Lu
Tianjin University of Science and Technology
Baixiang Zhao
Tianjin University of Science and Technology
Shuhui Wang
Tianjin University of Science and Technology
Cuiying Zhang
Tianjin University of Science and Technology
Dongguang Xiao
Tianjin University of Science and Technology
Jee Loon Foo
National University of Singapore
Aiqun Yu (yuaiqun@tust.edu.cn)
Tianjin University of Science and Technology https://orcid.org/0000-0002-2488-8636

Research

Keywords: Y. lipolytica, metabolic engineering, hybrid promoter, isoamyl alcohol, synthetic promoter

Posted Date: April 2nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-369447/v1

Abstract

Background

In biological cells, promoters drive gene expression by binding to RNA polymerase specifically. They determine the starting position, timing and level of gene expression. Therefore, rational fine-tuning of promoters to regulate the expression levels of target genes for metabolic engineering applications to optimize biosynthetic pathways has recently become an active area of research.

Results

In this study, we systematically detected and characterized the common promoter elements in the unconventional yeast *Yarrowia lipolytica*, and constructed an artificial hybrid promoter library that covers a wide range of promoter strength. We also report for the first time that upstream activation sequences (UAS) of *Saccharomyces cerevisiae* promoters can be functionally transferred to *Y. lipolytica*. Subsequently, using the production of a versatile platform chemical isoamyl alcohol as a test study, the hybrid promoter library was applied to optimize the biosynthesis pathway expression in *Y. lipolytica*. Under the control of $P_{UAS1B8-LEUm}$, the strongest promoter we constructed, overexpression of a key pathway gene led to 7.7-fold increase in the titer of isoamyl alcohol. Interestingly, a much weaker promoter $P_{UAS1B4-EXPm}$ increase the isoamyl alcohol titer by 30.3-fold. These results suggest that our hybrid promoter library can be a powerful toolkit for identifying optimum promoters for expressing metabolic pathways in *Y. lipolytica*.

Conclusion

We envision that this promoter engineering strategy and the rationally engineered promoters constructed in this study could also be extended to other non-model fungi for strain improvement.

Background

Promoters are one of the most important components of synthetic biology, and well-controlled promoters are very critical for regulating gene expression in eukaryotes. The activity of a promoter is co-regulated by various elements. In yeast, the common promoter elements usually include upstream activation sequences (UAS), TATA box and core promoter [1, 2]. By rational modification of these elements, the activity of promoters can be fine-tuned.

At the beginning of transcription, regulatory signals are transmitted from the UAS to the core promoter, then transcription factors and RNA polymerase assembly combined with the transcription preinitiation complex (PIC) [3]. The core promoter significantly contributes to the regulation of gene expression and is also the key factor determining the promoter strength. Although the core promoters were initially thought

to be invariant, researchers have found that they exhibit great structural and functional diversity [4, 5]. TATA box, the recognition site of the transcription factor TATA binding protein (TBP), is one of the first kind of functional elements identified to regulate the promoter strength of the core promoter and typically located 40–120 bp upstream of the transcription start site [3, 6]. Mutations in the TATA box usually alter the promoter strength [1, 7–10]. The UAS, which is usually located at the 5' of the promoter [3, 11], is also known to affect the strength of the promoter by varying its copy number. By analysing the function of the endogenous alkaline extracellular protease 2 (XPR2) gene promoter P_{XPR2} of *Yarrowia lipolytica*, Madzak et al. identified UAS1B as the most significant functional element that activates the promoter P_{XPR2} [12]. Subsequently, evaluation of a hybrid promoter library consisting a minimal P_{LEU} fragment and different copy numbers of the UAS1B indicates that enhancement in promoter strength is correlated to increased copy number of UAS1B [12]. When present in a promoter, different types of UAS can cooperate to control transcription. For example, by combining different UAS elements (UASTEF and UAS1B) in *Y. lipolytica*, the expression level of a constructed promoter was 7-fold higher than the wild-type promoter [2]. Taken together, by exploring the synergy between various promoter elements and understanding the working mechanism of the promoter, promoters with stronger activity and wider expression range can be constructed.

In this study, in order to explore the mechanism of synergy between various elements in *Y. lipolytica*, the promoter elements were characterized and rearranged. Consequently, a library of hybrid promoters that enables stable expression and covers a wide range of promoter strength was constructed. Subsequently, we employed the hybrid promoters for promoter engineering of the pathway genes in isoamyl alcohol biosynthesis. Isoamyl alcohol, an important platform chemical, is widely applied in the production of biofuels, fragrances, medicines and fine chemicals [13] and has been produced in recent years by metabolic engineering of diverse microbial cells, such as *Escherichia coli*, *Corynebacterium glutamicum* and *Aspergillus oryzae* [14–16]. While there is a report on improving the production of isoamyl alcohol in *Y. lipolytica* by metabolic engineering, a native promoter was employed [17]. Therefore, we used this pathway as a testbed and demonstrated the efficacy of our promoter library for optimizing metabolic pathways by significantly improving the isoamyl alcohol titer (Fig. 1). The outcome of this work shows that promoter engineering is an effective strategy for facilitating metabolic engineering efforts to biosynthesize valuable chemicals and our hybrid promoter library is a powerful toolkit for future metabolic engineering work in *Y. lipolytica*.

Results And Discussion

Screening of the fluorescent reporter gene in Y. lipolytica

Efficient engineering of microbial cell factories relies on optimizing the genetic construct of metabolic pathways to direct the carbon flux toward the desired product of interest. The key to achieving this goal is to eliminate metabolic bottlenecks and tune the expression of target gene precisely. To this end, we aim to construct a hybrid promoter library. For characterization of the promoters, it is critical to develop a

stable, reliable and sensitive reporting system to monitor gene expression. A series of studies have evaluated the effectiveness of some reporters in the unconventional yeast *Y. lipolytica* for examining the strength of promoters, such as green fluorescent protein (GFP) and β-galactosidase [18, 19]. GFP is the most commonly used reporter gene to characterize promoter strength because of its convenience in detection. However, there are many variants of GFP and their applicability to *Y. lipolytica* needs to be assessed. Therefore, in this study, we systematically characterized the expression of different GFPs to investigate which gene may function as an ideal reporter gene in the yeast strain *Y. lipolytica* Po1g *KU70*Δ that was used as a host system for this work. Firstly, expression of different fluorescent reporter genes *GFPuv* (differs from the wild-type GFP by the amino acid replacements Val163Ala, Met153Thr, and Phe99Ser), *hrGFP* (humanized *Renilla reinformis* GFP) and *hrGFPO* (codon optimized *hrGFP* for *Y. lipolytica*) [1, 18, 20] was driven by the hybrid promoter $P_{UAS1B4-LEUm}$, which is the promoter on the commercial integrative vector pYLEX1 for *Y. lipolytica*. Expression of the GFPs were detected by fluorescence microscopy and flow cytometry (Fig. 2, Fig. 3a). The results indicate that fluorescence was not detected in the control strain Po1g *KU70*Δ while the *Y. lipolytica* strains carrying integrated *GFPuv* or *hrGFP* gene fluoresced at different intensities. However, the fluorescence produced in the strain carrying the GFPuv gene was relatively weak, indicating that this is not a good reporter gene for *Y. lipolytica*. In contrast, the detected fluorescence intensity of strains that harbored the *hrGFPO* gene was more stable than strains that possessed the *hrGFP* gene (Fig. 3a). Henceforth, *hrGFPO* was selected as the reporter gene for promoter characterization in this work.

Characterization of native promoters as a basis for the construction of hybrid promoters for Y. lipolytica

The strengths of different native promoters are known to vary greatly in microbes. To form a basis for our hybrid promoter library, we sought to use the *hrGFPO* reporter gene to evaluate the promoter strengths of several commonly used native *Y. lipolytica* promoters: β-isopropylmalate dehydrogenase (LEU2) promoter P_{LEU}, export protein (EXP) promoter P_{EXP} and translation elongation factor-1α (TEF1) promoter P_{TEF}. Based on the results of our experiments (Fig. 3b), the relative fluorescence intensities of the corresponding strains from high to low are $P_{TEF} > P_{EXP} > P_{LEU}$, whereby the strength of P_{TEF} is about an order stronger than both P_{EXP} and P_{LEU}. Subsequently, these promoters were dissected into the various promoter elements, i.e. UAS, TATA box and core promoter, and based on the structures of these native promoters, other known promoter elements were added to build hybrid promoters. In most previous studies on the construction on hybrid promoters, the focus was mainly on the utilization of UAS and there were few studies on varying the other promoter elements. Thus, in this study, we explored the mixing of promoter constituent elements and investigated the influence of the various combinations on the promoter strengths of the resulting hybrid promoters in *Y. lipolytica* (Table 1, Fig. 4).

Characterization of features in core promoters that influence promoter strength

The core promoter, first identified in the mammalian gene regulatory region, plays a very important role in the regulatory initiation of genes and is defined as ‘the smallest DNA element for transcription’ [3]. In yeast systems, a large number of studies have shown that the regulation mechanism of the core promoter has a very complex impact on the activity and strength of the promoter, and thus modulate gene expression. For example, in *S. cerevisiae*, the T content in the core promoter upstream of the transcription start site (TSS) has a great influence on the promoter activity. When the gene expression was high, the T content upstream of the TSS was abundant, and the A content downstream of TSS was rich [21]. Thus, we hypothesize that a similar trend exists in *Y. lipolytica*. Therefore, a series of endogenous core promoters of different lengths and contain TATA box, namely LEU, TEF, EXP, POX2 and PAT1, were selected to calculate the content of T upstream of the TSS and verify the functions of the core promoters in *Y. lipolytica*. To confirm the function of core promoters, the UAS1B elements which advance gene transcription were linked to the upstream of the core promoter to express the *hrGFPO* reporter gene for characterizing the promoter strengths by fluorescence. The results indicated that the hybrid promoters we constructed in general followed the trend that the promoter strength increases with the T content upstream of the TSS, with two exceptions, namely LEUm and POX2m (Fig. 3c). We also analysed the length of the TEF core promoter, and found that the shorter the core promoter is, the stronger the hybrid promoter (Fig. 3c). These data suggest that expression level of genes can be regulated largely by both the types and length of core promoters. While there appears to be a relationship between T content and promoter strength in *Y. lipolytica*, further studies are required to elucidate the specific relationship between base content and promoter strength.

Modulating the promoter strength by varying the TATA box

Functional elements of the core promoter including TATA box, initiator element (Inr), downstream promoter element (DPE), TFIIB recognition element (BRE) and motif ten element (MTE) have been identified [22, 23]. The sequence lengths of these functional elements are short, the specificities are low and the combinations in various promoters are different. All these functional elements, except the TATA box, are clearly nonconservative in yeast [24, 25]. The TATA box, which is the binding site of TATA binding protein (TBP), is the first element identified in the core promoter. Previous studies have shown that TATA box has a significant effect on promoter strength [1]. Therefore, a series of TATA boxes (Table 2) were selected to study their specific performance in promoters in *Y. lipolytica*. $P_{UAS1B4+LEU}$, which has the highest activity in the previous section, was selected as the control for engineering. Firstly, we selected several TATA boxes to replace TATA LEU by site-directed mutagenesis. The expression of *hrGFPO* under the promoter variants was evaluated by fluorescence, which showed that strains with different TATA boxes significantly affected the promoter strength. The fluorescence intensity of the strain with the hybrid promoter containing TATA TEF was more than twice that of the control strain with TATA LEU (Fig. 3d). Therefore, the result validates the important role of TATA box in influencing the strength of a promoter and provides a theoretical basis for future promoter engineering studies.

Construction of promoters with various UAS elements from Y. lipolytic and S. cerevisiae

The process of transcriptional regulation begins with the recognition of specific sequences by transcription factors (TFs), such as the recognition of UASs by transcriptional activators and upstream repression sequences (URSs) by repressors. Many studies have shown that UAS has a powerful influence on transcriptional regulation. Several UASs have been identified in *S. cerevisiae*, such as UASTEF [26], UASCLB [27] and UASCIT [28]. However, only a few UASs were identified in *Y. lipolytica*, among which the UAS1B is the most well-studied. In previous studies, it has been shown that the copy number of UAS has significant impact on hybrid promoter strength as well [2, 12]. Four tandem UAS1B from P_{XPR2} and one P_{LEU} core promoter have been combined to construct the strong constitutive promoter $P_{UAS1B4+LEUm}$ [18]. We increased the copy number of UAS and verified that the copy number of UAS is proportional to the hybrid promoter strength (Fig. 3e), which corroborates with published data [18]. In addition, while it has been shown that synthetic terminators can be efficiently transferred in *S. cerevisiae* and *Y. lipolytica* [29], there is no research on the transferability of promoter elements across diverse yeast species. Therefore, different UASs (UASCIT *S.c.*, UASCLB *S.c.*, UASTEF *S.c.* and UASTEF *Y.l.*) [2, 26–28] from *S. cerevisiae* and *Y. lipolytica* with the same copy number as $P_{UAS1B4+LEUm}$ were used to replace UAS1B4 to explore the influence of UAS types and origin on hybrid promoter activity. By expressing the *hrGFPO* gene under the hybrid promoters with different UASs, the activities of promoters were shown to be significantly affected by the variation in UAS. The relative fluorescence intensity from the GFP expressed from the promoters containing various UASs, from high to low, is UAS1B > UASTEF *Y.l.*> UASCIT *S.c.*> UASCLB *S.c.*> UASTEF *S.c.* (Fig. 3f). These results demonstrated for the first time that UAS from *S. cerevisiae* are functional in *Y. lipolytica*.

Taken together, we have constructed a library of hybrid promoters with different promoter strengths using various combination of UASs, TATA boxes and core promoters, as summarized in Fig. 5 and Table 1. To demonstrate the application of our hybrid promoter library, as a testbed, we aimed to optimize a biosynthesis pathway, i.e. isoamyl alcohol production, by promoter engineering using our hybrid promoters to regulate gene expression and improve production level of the target compound.

Construction of the isoamyl alcohol overexpression pathway in Y. lipolytica

As an important platform chemical, isoamyl alcohol is a promising biofuel and biochemical with huge market demand. However, in *Y. lipolytica*, the titer of isoamyl alcohol natively is quite low at a mere 0.37 mg/L (Fig. 6). Thus, the production titer of isoamyl alcohol has much room for improvement and the biosynthesis pathway serves as a good testbed for optimization by promoter engineering using our hybrid promoter library.

In yeast, isoamyl alcohol is generally produced through the Ehrlich pathway, which usually involves three reaction steps: transamination, decarboxylation and reduction. Twelve genes encoding transaminases (*ScBAT1*, *YlBAT1-1* and *YlBAT1-2*), decarboxylases (*ScARO10*, *YlARO10-1* and *YlARO10-2*) and alcohol dehydrogenases (*ScADH2*, *YlADH2-1*, *YlADH2-2*, *YlADH2-3*, *YlADH2-4* and *YlADH2-5*) were selected and individually overexpressed to determine the key genes of isoamyl alcohol biosynthesis in the Ehrlich pathway. For this purpose, twelve strains overexpressing native and heterologous genes in the Ehrlich pathway were constructed. All genes were individually integrated into the genome of *Y. lipolytica* Po1g *KU70*Δ and driven by the constitutive promoter $P_{UAS1B4+LEUm}$. After 3 days of cultivation, individual overexpression of the pathway genes enhanced the isoamyl alcohol titer in the engineered strains compared to that of the control strain Po1g *KU70*Δ (Fig. 6). The results showed that among the three evaluated classes of enzymes in the Ehrlich pathway, the strains overexpressing decarboxylase genes resulted in the most significant increase in isoamyl alcohol production. Among them, the highest isoamyl alcohol production was obtained by the *ScARO10*-overexpressed strain, which reach 1.36 mg/L. The strains which overexpressed transaminase gene *ScBAT1* and dehydrogenase gene *ScADH2* also increased the harvest of isoamyl alcohol moderately. Therefore, to further improve the yield of isoamyl alcohol, the genes *ScBAT1*, *ScARO10* and *ScADH2* were chosen to construct strain Po1g BAA. After 3 days of cultivation, the titer of isoamyl alcohol reached 1.8 mg/L, which was 3.9-fold higher than that of the control strain Po1g *KU70*Δ (Fig. 6). Thus, the strain Po1g BAA was selected for subsequent engineering by promoter replacement with our hybrid promoter library.

Application of the hybrid promoter library to improve the isoamyl alcohol biosynthesis pathway

In metabolic engineering, studies have shown that the yield of the target product can be increased by replacing promoters for pathway genes with stronger ones [19, 30]. Therefore, to demonstrate the application of our promoter library for optimizing metabolic pathways, we employed some of our hybrid promoters in the heterologous isoamyl alcohol pathway of Po1g BAA. We chose from the promoter library nine promoters that cover a range of strengths to express the key gene *ScARO10* in the isoamyl alcohol pathway. These constructed strains were cultured for 3 days, and the titer of the isoamyl alcohol was quantified (Fig. 7). It can be seen from the results that the isoamyl alcohol titer does not correlate to the strength of the promoter used. For example, strain Po1g BA + $P_{UAS1B4+EXPm}$+ARO10 with a low-activity promoter had the highest isoamyl alcohol titer of 11.57 mg/L, which was about 30.3-fold higher than that of Po1g *KU70*Δ and 5.4-fold that of Po1g BAA. This result is consistent with the opinion of Dulermo, *et al.* that stronger promoters do not necessarily increase the expression level and/or function of a protein [31]. In addition, we found that although the activity of P_{EXP} was low, several strains containing P_{EXP} elements (P_{EXP}, $P_{UAS1B4-EXPm}$, $P_{UAS1B4+TATAEXP-LEUm}$) had higher titers of isoamyl alcohol, suggesting that the elements of the P_{EXP} have greater beneficial effects to the expression of the *ARO10* gene, which encodes a key enzyme of the isoamyl alcohol pathway. More studies are needed to better understand the mechanism between the elements of P_{EXP} and gene expression which resulted in the improved

production titer. Nevertheless, we demonstrated successful application of our hybrid promoter for identification of suitable promoters to improve metabolic pathways.

Conclusions

Promoters are one of the most important components of synthetic biology for determining protein expression. Compared to prokaryotes, the regulatory mechanism of the promoter structure in eukaryotes is extremely complex [1, 32]. Increasing the promoter strength is a common method to improve gene transcription and protein expression level. However, recent studies have shown that not all strong promoters can achieve the highest protein expression and activity [31]. We explored the structure and functional characteristics of the promoters of *Y. lipolytica*, and subsequently constructed a series of constitutive promoters which are stable and efficient. Firstly, different variants of green fluorescent protein were screened in *Y. lipolytica* to identify a reporter gene that can be stably expressed. Among different transformants, the codon optimized *hrGFP* (*hrGFPO*) not only expressed at a high level but also expressed stably in *Y. lipolytica*. Therefore, the *hrGFPO* gene was used for subsequent promoter characterization experiments.

Three native promoters of *Y. lipolytica*, P_{LEU}, P_{TEF} and P_{EXP}, were characterized and the results showed that the strengths of these promoters are evidently different. These promoters were dissected into three parts, namely UAS, TATA box and Core promoter, and these elements were combinatorially arranged with other studied promoter elements to construct a constitutive promoter library that contains 21 stable hybrid promoters. It is the first time that the T content upstream of the TSS has been shown to positively correlate with the hybrid promoter strength in *Y. lipolytica*. It is worth noting that some core promoter elements, such as POX2m and LEUm, did not conform to the trend. Therefore, the relationship between the T content upstream of the TSS and the promoter strength in *Y. lipolytica* needs to be further studied. Next, the effects of different UAS elements from *S. cerevisiae* and *Y. lipolytica* on promoter strength were investigated and we discovered for the first time that UAS elements can be transferred between yeast species. These findings lay the groundwork for the development of hybrid promoters which can be efficiently transferred across diverse yeast species.

To demonstrate application of our hybrid promoter library, the isoamyl alcohol production pathway was constructed to serve as a testbed by co-expression of multiple genes from *S. cerevisiae* and *Y. lipolytica*. *ScAOR10*, the key gene of the isoamyl alcohol pathway, was selected as the test gene for expression under various hybrid promoters from our library to optimize the enzyme's expression and activity for enhance isoamyl alcohol production. Consequently, the titer of the isoamyl alcohol increased from 0.37 mg/L to 11.57 mg/L, which was 30.3-fold higher than the control strain Po1g *KU70*Δ. To date, isoamyl alcohol has been successfully produced by metabolic engineering in several studies [17, 33, 34]. Although the titer of isoamyl alcohol from *Y. lipolytica* is lower compared with other studies, it is the first time that the promoter engineering has been applied for the biosynthesis of isoamyl alcohol to provide an advanced solution for the biosynthesis of biofuels and alcohols. Regulation of expression by promoters involves various factors, such as temperature, pH and substrate [1, 35]. In the future, we will further study

the mechanisms of promoters to construct hybrid promoters with stronger activity and wider expression range for optimum expression of biosynthesis pathway genes to achieve high-level production of value-added chemicals.

Materials And Methods

Strains and media

Escherichia coli strain DH5α was used for all cloning and plasmid propagation, and DH5α was grown at 37°C in Luria Bertani (LB), and supplemented with ampicillin to final concentration of 100 μg/mL for plasmid propagation. *Y. lipolytica* strain Po1g *KU70*Δ, a leucine auxotroph devoid of any secreted protease activity, was used as the base strain in this study. *Y. lipolytica* Po1g *KU70*Δ containing plasmid was routinely cultivated at 28°C and 225 rpm with YPD media consisting of 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract. In this study, PCR primers were synthesized by Genewiz (Jiangsu, China) and are listed in Table S1, plasmids are listed in Table S2 and strains used are listed in Table S3.

Chemicals and enzymes

All restriction enzymes were purchased from New England Biolabs (Beijing, China), 2×Phanta® max master mix, 2× Rapid Taq master mix, ClonExpress® II one step cloning kit, FastPure® Plasmid Mini Kit and FastPure® Gel DNA Extraction Mini Kit were purchased from Vazyme Biotech Co., Ltd. (Nanjing, China), peptone and yeast extract were purchased from Thermo Scientific Oxoid Microbiology Products (Basingstoke, England), isoamyl alcohol and n-dodecane were purchased from Aladdin® (Shanghai, China).

Plasmid construction of promoter library

The *GFPuv* gene was preserved in this laboratory, and cloned into pYLEX1 with primers GFPuv-F/GFPuv-R (Table S1) yield plasmid pYLGFPuv (Table S2). The *hrGFP* gene and *hrGFPO* gene were synthesized and cloned into pYLEX1 to yield plasmids pYLhrGFP and pYLhrGFPO (Table S2), respectively, by Genewiz (Jiangsu, China). The UASCIT *S.c.*4, UASCLB *S.c.*4, UASTEF *S.c.*4, UASTEF *Y.l.*4, UAS1B6 and UAS1B8 motifs were synthesized and cloned into plasmids pYLhrGFPO to replace UAS1B4 to yield plasmids $pYLP_{UASCITSC4-LEUm}$+hrGFPO, $pYLP_{UASCLBSC4-LEUm}$+hrGFPO, $pYLP_{UASTEFSC4-LEUm}$+hrGFPO, $pYLP_{UASTEFYL4-LEUm}$+hrGFPO, $pYLP_{UAS1B6-LEUm}$+hrGFPO and $pYLP_{UAS1B8-LEUm}$+hrGFPO (Table S2), respectively, by Genewiz (Jiangsu, China). Three endogenous promoters P_{LEU}, P_{TEF} and P_{EXP} were cloned into vector pYLhrGFPO with primers PLEU-F/LEU-hrGFPO-R, PTEF-F/TEF-hrGFPO-R and PEXP-F/EXP-hrGFPO-R (Table S1) yield plasmids $pYLP_{LEU}$+hrGFPO, $pYLP_{TEF}$+hrGFPO and $pYLP_{EXP}$+hrGFPO (Table S2), respectively. The Core promoters were amplified by primer pairs PAT1m-F/PAT1-hrGFPO-R, POX2m-F/POX2-hrGFPO-R, EXPm-F/EXP-hrGFPO-R, TEFm111-F/TEF-hrGFPO-R, TEF136-F/TEF-hrGFPO-R and TEFm175-F/TEF-hrGFPO-R (Table S1), and then replace the core promoter LEU in $P_{UAS1B4-LEU}$. These promoters were ligated to pYLhrGFPO in place of the $P_{UAS1B4-LEU}$ to yield plasmids $pYLP_{UAS1B4-PAT1m}$+hrGFPO, $pYLP_{UAS1B4-POX2m}$+hrGFPO, $pYLP_{UAS1B4-EXP1m}$+hrGFPO,

pYLP$_{UAS1B4-TEF111}$+hrGFPO, pYLP$_{UAS1B4-TEF136}$+hrGFPO and pYLP$_{UAS1B4-TEF175}$+hrGFPO (Table S2), respectively. The TATA box LEU in P$_{UAS1B4-LEU}$ was replaced by TATA box TEF, EXP, PAT1 and POX2 using primer pairs TATA TEF-F/LEU-hrGFPO-R, TATA EXP-F/LEU-hrGFPO-R, TATA PAT1-F/LEU-hrGFPO-R and TATA POX2-F/LEU-hrGFPO-R (Table S1). These hybrid promoters were ligated to pYLhrGFPO in place of the P$_{UAS1B4-LEU}$ to yield plasmids pYLP$_{UAS1B4-TATATEF-LEU}$+hrGFPO, pYLP$_{UAS1B4-TATAEXP-LEU}$+hrGFPO, pYLP$_{UAS1B4-TATAPAT1-LEU}$+hrGFPO and pYLP$_{UAS1B4-TATAPOX2-LEU}$+hrGFPO (Table S2), respectively.

All plasmids, linearized by Not I or Spe I, were transformed into competent cells of *Y. lipolytica* strains using the lithium acetate method [36].

Plasmid construction of exogenous isoamyl alcohol pathway

The transaminase gene (*BAT1*, GenBank ID: 856615), decarboxylase gene (*ARO10*, GenBank ID: 851987) and alcohol dehydrogenase gene (*ADH2*, GenBank ID: 855349) from *S. cerevisiae* S288C were codon-optimized and synthesized and cloned into pYLEX1 to yield plasmids pYLSCBAT1, pYLSCARO10 and pYLSCADH2 (Table S2), respectively, by Genewiz (Jiangsu, China). In *Y. lipolytica*, the homologous sequences that *YlBAT1-1* and *YlBAT1-2* of *ScBAT1* were cloned into pYLEX1 with primers YLBAT1-1-F/YLBAT1-1-R and YLBAT1-2-F/YLBAT1-2-R (Table S1) to yield plasmids pYLYLBAT1-1 and pYLYLBAT1-2 (Table S2), respectively. The homologous sequences that *YlARO10-1* and *YlARO10-2* of *ScARO10* were cloned into pYLEX1 with primers YLARO10-1-F/YLARO10-1-R and YLARO10-2-F/YLARO10-2-R (Table S1) to yield plasmids pYLYLARO10-1 and pYLYLARO10-2 (Table S2), respectively. The homologous sequences that *YlADH2-1, YlADH2-2, YlADH2-3, YlADH2-4* and *YlADH2-5* of *ScADH2* were cloned into pYLEX1 with primers YLADH2-1-F/YLADH2-1-R, YLADH2-2-F/YLADH2-2-R, YLADH2-3-F/YLADH2-3-R, YLADH2-4-F/YLADH2-4-R and YLADH2-5-F/YLADH2-5-R (Table S1) to yield plasmids pYLYLADH2-1, pYLYLADH2-2, pYLYLADH2-3, pYLYLADH2-4 and pYLYLADH2-5 (Table S2), respectively.

The expression cassettes of *ScARO10* and *ScADH2* were cloned into pYLSCBAT1 with primers BDH-ADH2-F/BDH-ADH2-R and BDH-ARO10-F/ BDH-ARO10-R (Table S1) to yield plasmid pYLBAA (Table S2). All plasmids, linearized by Not I or Spe I, were transformed into competent cells of *Y. lipolytica* strains using the lithium acetate method [36].

Expressing the isoamyl alcohol synthesis pathway using the promoter library

Several promoters from the promoter library were used to express the *ARO10* gene which is the key gene in the isoamyl alcohol pathway. The promoters P$_{EXP}$ and P$_{UAS1B4+EXPm}$ were amplified by primers BDH-ARO10-F/PEXP-ARO10-R (Table S1), and then ligated to *ScARO10* in pYLBAA to yield plasmid pYLBA + P$_{EXP}$+ARO10 and pYLBA + P$_{UAS1B4-EXPm}$+ARO10 (Table S2), respectively. The promoters P$_{UAS1B4-POX2m}$ and P$_{UAS1B4-TEF136}$ were amplified by primers BDH-ARO10-F/POX2-ARO10-R and BDH-ARO10-F/PTEF-ARO10-R (Table S1), and then ligated to *ScARO10* in pYLBAA to yield plasmid pYLBA +

$P_{UAS1B4-POX2m}$+ARO10 and pYLBA + $P_{UAS1B4-TEF136}$+ARO10 (Table S2), respectively. The promoters $P_{UASTEFLY4-LEUm}$, $P_{UAS1B4-TATAEXP-LEU}$, $P_{UAS1B4-TATATEF-LEU}$ and $P_{UAS1B8-LEUm}$ were amplified by primers BDH-ARO10-F/PLEU-ARO10-R (Table S1), and then ligated to *ScARO10* in pYLBAA to yield plasmid pYLBA + $P_{UASTEFLY4-LEUm}$+ARO10, pYLBA + $P_{UAS1B4-TATAEXP-LEU}$+ARO10, pYLBA + $P_{UAS1B4-TATATEF-LEU}$+ARO10 and pYLBA + $P_{UAS1B8-LEUm}$ +ARO10 (Table S2), respectively.

All plasmids, linearized by Not I or Spe I, were transformed into competent cells of *Y. lipolytica* strains using the lithium acetate method [37].

Yeast strain construction

The competent cell scheme and transformation method are referred to Pang, *et al.*[38]. After selection, the following engineered *Y. lipolytica* strains were generated: Po1g $P_{UAS1B4-LEUm}$+GFPuv, Po1g $P_{UAS1B4-LEUm}$+hrGFP, Po1g $P_{UAS1B4-LEUm}$+hrGFPO, Po1g $P_{UAS1B6-LEUm}$+hrGFPO, Po1g $P_{UAS1B8-LEUm}$+hrGFPO, Po1g P_{LEUm}+hrGFPO, Po1g P_{LEU}+hrGFPO, Po1g P_{EXP}+hrGFPO, Po1g P_{TEF}+hrGFPO, Po1g $P_{UAS1B4-EXPm}$+hrGFPO, Po1g $P_{UAS1B4-POX2m}$+hrGFPO, Po1g $P_{UAS1B4-PAT1m}$+hrGFPO, Po1g $P_{UAS1B4-TEF111}$+hrGFPO, Po1g $P_{UAS1B4-TEF136}$+hrGFPO, Po1g $P_{UAS1B4-TEF175}$+hrGFPO, Po1g $P_{UAS1B4-TATAPAT1-LEUm}$+hrGFPO, Po1g $P_{UAS1B4-TATAPOX2-LEUm}$+hrGFPO, Po1g $P_{UAS1B4-TATAEXP-LEUm}$+hrGFPO, Po1g $P_{UAS1B4-TATATEF-LEUm}$+hrGFPO, Po1g $P_{UASTEFSC4-LEUm}$+hrGFPO, Po1g $P_{UASCLBSC4-LEUm}$+hrGFPO, Po1g $P_{UASTEFYL4-LEUm}$+hrGFPO, Po1g ScBAT1, Po1g YlBAT1-1, Po1g YlBAT1-2, Po1g ScARO10, Po1g YlARO10-1, Po1g YlARO10-2, Po1g ScADH2, Po1g YlADH2-1, Po1g YlADH2-2, Po1g YlADH2-3, Po1g YlADH2-4, Po1g YlADH2-5, Po1g BAA, Po1g BA + P_{EXP}+ARO10, Po1g BA + $P_{UAS1B4-EXPm}$+ARO10, Po1g BA + $P_{UAS1B4-POX2m}$+ARO10, Po1g BA + $P_{UASTEFYL4-LEUm}$+ARO10, Po1g BA + $P_{UAS1B4-TEF136}$+ARO10, Po1g BA + $P_{UAS1B4-LEUm}$+ARO10, Po1g BA + $P_{UAS1B4-TATAEXP-LEU}$+ARO10, Po1g BA + $P_{UAS1B4-TATATEF-LEU}$+ARO10, Po1g BA + $P_{UAS1B8-LEUm}$+ARO10 (Table S3).

Flow cytometry

The green fluorescent protein GFPuv, hrGFP and hrGFPO were selected as reporter proteins. The colonies of transformants were selected from plates and grew in 5 mL of fresh YPD medium in tube for 24 h. After that, the seed culture solution was inoculated to 250 mL flasks which contain 40 mL YPD medium, starting from OD_{600} 0.1. Cultures were cultivated at rotary shaker at 225 rpm and 28°C. Before flow cytometry analysis, the cultures were centrifuged at 12000 rpm for 1 min, and washed in 0.1 M phosphate-buffered saline (PBS), then resuspended in PBS. There were 10,000 cell count that were analysed with the BD Accuri C6 flow cytometer (BD Biosciences) using 488-nm excitation wavelength and FL1 channel for fluorescence detection. The CFlow software was used to analysed the data and compute mean fluorescence values.

GC/MS analysis of isoamyl alcohol produced in the engineered Y. lipolytica strains

The engineered *Y. lipolytica* transformants were selected from plate and prepared in 5 mL of fresh YPD medium in tube for 24 h. The seed culture solution was inoculated to 250 mL flasks containing 40 mL of YPD medium, starting from OD_{600} 0.1. The cultures were shaken at 225 rpm and 28°C for 3 days. In order to extract isoamyl alcohol from the cultures, 10% *n*-dodecane was added to the cultures, and the mixture was vortexed for 3 minutes, then centrifuged at 7500 rpm for 5 minutes. The organic phase of 1ul was detected by GC/MS using an Agilent 7890B GC with an 5977B MSD equipped with a HP-5MS column (60 m × 0.25 mm × 0.25 μm, Agilent, Santa Clara, CA, USA). GC oven temperature was initially held at 60°C for 2 min, and then ramped to 140°C at a rate of 5°C/min. It was then subsequently ramped at 10°C/min to 280°C and held for 5 min. The split ratio was 10:1. Helium was used as the carrier gas, with an inlet pressure of 13.8 psi. The injector was maintained at 280°C and the ion source temperature was set to 230°C. Final data analysis was achieved using MassHunter Workstation Software (Agilent, Santa Clara, CA, USA).

Abbreviations

GC/MS
gas chromatography/mass spectrometry;
OD_{600}
optical density at 600 nm;
LB medium
0.5% yeast extract, 1% tryptone and 1% NaCl;
YPD medium
1% yeast extract, 2% peptone and 2% glucose;
YNB plate
2% glucose, 0.67% yeast nitrogen base without amino acids and 2% agar;
PCR
polymerase chain reaction.
YlBAT1-1
YALI0_D01265g
YlBAT1-2
YALI0_F19910g
YlARO10-1
YALI0_D06930g
YlARO10-2
YALI0_E07325g
YlADH2-1
YALI0_A16379g
YlADH2-2
YALI0_D25630g
YlADH2-3

YALI0_E17787g
YlADH2-4
YALI0_A15147g
YlADH2-5
YALI0_E07766g

Declarations

Ethics approval and consent to participate

This manuscript does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All authors give consent to publish the research in Biotechnology for Biofuels.

Availability of data and material

All relevant data generated or analysed during this study were included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

The Natural Science Foundation of Tianjin, China (17JCYBJC40800), the Research Foundation of Tianjin Municipal Education Commission, China (2017ZD03), the Innovative Research Team of Tianjin Municipal Education Commission, China (TD13-5013), Tianjin Municipal Science and Technology Project (18PTSYJC00140, 19PTSYJC00060), Startup Fund for “Haihe Young Scholars” of Tianjin University of Science and Technology, the Thousand Young Talents Program of Tianjin, China.

Authors' contributions

AQY, JLF, DGX and CYZ conceived and designed the study. YZ, SQL, ZHL, BXZ and SHW performed plasmid and strain construction, and fermentation experiments. AQY, JLF, DGX, CYZ revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by the Natural Science Foundation of Tianjin, China (17JCYBJC40800), the Research Foundation of Tianjin Municipal Education Commission, China (2017ZD03), the Innovative Research Team of Tianjin Municipal Education Commission, China (TD13-5013), Tianjin Municipal Science and Technology Project (18PTSYJC00140, 19PTSYJC00060), Startup Fund for ‘Haihe Young

Scholars' of Tianjin University of Science and Technology, the Thousand Young Talents Program of Tianjin, China.

Authors' information

[a]State Key Laboratory of Food Nutrition and Safety, Key Laboratory of Industrial Fermentation Microbiology of the Ministry of Education, Tianjin Key Laboratory of Industrial Microbiology, College of Biotechnology, Tianjin University of Science and Technology, No.29 the 13th Street TEDA, Tianjin 300457, PR China

[b]Synthetic Biology Translational Research Programme, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 119228, Singapore

[c]NUS Synthetic Biology for Clinical and Technological Innovation (SynCTI), National University of Singapore, Singapore 117456, Singapore

[d]Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore

References

1. Shabbir Hussain M, Gambill L, Smith S, Blenner MA. Engineering promoter architecture in oleaginous yeast *Yarrowia lipolytica*. ACS Synth Biol. 2016;5(3):213–23.
2. Blazeck J, Reed B, Garg R, Gerstner R, Pan A, Agarwala V, Alper HS. Generalizing a hybrid synthetic promoter approach in *Yarrowia lipolytica*. Appl Microbiol Biotechnol. 2013;97(3):3037–52.
3. Hahn S, Young ET. Transcriptional regulation in *Saccharomyces cerevisiae*: transcription factor regulation and function, mechanisms of initiation, and roles of activators and coactivators. Genetics. 2011;189(3):705–36.
4. Butler JE, Kadonaga JT. The RNA polymerase II core promoter: a key component in the regulation of gene expression. Genes Dev. 2002;16(16):2583–92.
5. Smale ST. Core promoters: active contributors to combinatorial gene regulation. Genes Dev. 2001;15(19):2503–8.
6. Struhl K. Molecular mechanisms of transcriptional regulation in yeast. Annu Rev Biochem. 1989;58:1051–77.
7. Breathnach R, Chambon P. Organization and expression of eucaryotic split genes coding for proteins. Annu Rev Biochem. 1981;50:349–83.
8. Grosveld GC, Shewmaker CK, Jat P, Flavell RA. Localization of DNA sequences necessary for transcription of the rabbit beta-globin gene in vitro. Cell. 1981;25(1):215–26.
9. Hu SL, Manley JL. DNA sequence required for initiation of transcription in vitro from the major late promoter of adenovirus 2. Proc Natl Acad Sci U S A. 1981;78(2):820–4.

10. Wasylyk B, Derbyshire R, Guy A, Molko D, Roget A, Téoule R, Chambon P. Specific in vitro transcription of conalbumin gene is drastically decreased by single-point mutation in T-A-T-A box homology sequence. Proc Natl Acad Sci U S A. 1980;77(12):7024–8.
11. Fantino E, Marguet D, Lauquin GJ. Downstream activating sequence within the coding region of a yeast gene: specific binding in vitro of RAP1 protein. Mol Gen Genet. 1992;236(1):65–75.
12. Madzak C, Tréton B, Blanchin-Roland S. Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*. J Mol Microbiol Biotechnol. 2000;2(2):207–16.
13. Jang YS, Kim B, Shin JH, Choi YJ, Choi S, Song CW, Lee J, Park HG, Lee SY. Bio-based production of C2-C6 platform chemicals. Biotechnol Bioeng. 2012;109(10):2437–59.
14. Connor MR, Liao JC. Engineering of an *Escherichia coli* strain for the production of 3-methyl-1-butanol. Appl Environ Microbiol. 2008;74(18):5769–75.
15. Vogt M, Brüsseler C, Ooyen JV, Bott M, Marienhagen J. Production of 2-methyl-1-butanol and 3-methyl-1-butanol in engineered *Corynebacterium glutamicum*. Metab Eng. 2016;38:436–45.
16. Yamashita N, Motoyoshi T, Nishimura A. Molecular cloning of the isoamyl alcohol oxidase-encoding gene (mreA) from *Aspergillus oryzae*. J Biosci Bioeng. 2000;89(6):522–7.
17. Celinska E, Borkowska M, Bialas W, Kubiak M, Korpys P, Archacka M, Ledesma-Amaro R, Nicaud JM. Genetic engineering of Ehrlich pathway modulates production of higher alcohols in engineered *Yarrowia lipolytica*. FEMS Yeast Res. 2019;19(2).
18. Blazeck J, Liu LQ, Redden H, Alper HS. Tuning gene expression in *Yarrowia lipolytica* by a hybrid promoter approach. Appl Environ Microbiol. 2011;77(22):7905–14.
19. Hong SP, Seip J, Walters-Pollak D, Rupert R, Jackson R, Xue Z, Zhu Q. Engineering *Yarrowia lipolytica* to express secretory invertase with strong FBA1IN promoter. Yeast. 2012;29(2):59–72.
20. Hicks BW. Green fluorescent protein: applications and protocols. Humana Press 2002. p. 35–40.
21. Lubliner S, Keren L, Segal E. Sequence features of yeast and human core promoters that are predictive of maximal promoter activity. Nucleic Acids Res. 2013;41(11):5569–81.
22. Juven-Gershon T, Kadonaga JT. Regulation of gene expression via the core promoter and the basal transcriptional machinery. Dev Biol. 2009;339(2):225–9.
23. Smale ST, Kadonaga JT. The RNA Polymerase II core promoter. Annu Rev Biochem. 2003;72:449–79.
24. Basehoar AD, Zanton SJ, Pugh BF. Identification and distinct regulation of yeast TATA box-containing genes. Cell. 2004;116(5):699–709.
25. Sugihara F, Kasahara K, Kokubo T. Highly redundant function of multiple AT-rich sequences as core promoter elements in the TATA-less RPS5 promoter of *Saccharomyces cerevisiae*. Nucleic Acids Res. 2011;39(1):59–75.
26. Blazeck J, Garg R, Reed B, Alper HS. Controlling promoter strength and regulation in *Saccharomyces cerevisiae* using synthetic hybrid promoters. Biotechnol Bioeng. 2012;109(11):2884–95.

27. Van Slyke C, Grayhack EJ. The essential transcription factor Reb1p interacts with the CLB2 UAS outside of the G2/M control region. Nucleic Acids Res. 2003;31(15):4597–607.
28. Rosenkrantz M, Kell CS, Pennell EA, Webster M, Devenish LJ. Distinct upstream activation regions for glucose-repressed and derepressed expression of the yeast citrate synthase gene CIT1. Curr Genet. 1994;25(3):185–95.
29. Curran KA, Morse NJ, Markham KA, Wagman AM, Gupta A, Alper HS. Short synthetic terminators for improved heterologous gene expression in yeast. ACS Synth Biol. 2015;4(7):824–32.
30. Xiong X, Chen S. Expanding toolbox for genes expression of *Yarrowia lipolytica* to include novel inducible, repressible, and hybrid promoters. ACS Synth Biol. 2020;9(8):2208–13.
31. Dulermo R, Brunel F, Dulermo T, Ledesma-Amaro R, Vion J, Trassaert M, Thomas S, Nicaud JM, Leplat C. Using a vector pool containing variable-strength promoters to optimize protein production in *Yarrowia lipolytica*. Microb Cell Fact. 2017;16(1):31.
32. Portela RM, Vogl T, Kniely C, Fischer JE, Oliveira R, Glieder A. Synthetic core promoters as universal parts for fine-tuningexpression in different yeast species. ACS Synth Biol. 2017;6(3):471–84.
33. Dombrink-Kurtzman MA. A gene having sequence homology to isoamyl alcohol oxidase is transcribed during patulin production in *Penicillium griseofulvum*. Curr Microbiol. 2008;56(3):224–8.
34. Yuan J, Chen X, Mishra P, Ching CB. Metabolically engineered *Saccharomyces cerevisiae* for enhanced isoamyl alcohol production. Appl Microbiol Biotechnol. 2017;101(1):465–74.
35. Madzak C. *Yarrowia lipolytica*: recent achievements in heterologous protein expression and pathway engineering. Appl Microbiol Biotechnol. 2015;99(11):4559–77.
36. Yu AQ, Pratomo N, Ng TK, Ling H, Cho HS, Leong SS, Chang MW. Genetic engineering of an unconventional yeast for renewable biofuel and biochemical production. J Vis Exp. 2016;20(115):54371.
37. Li J, Zhu K, Miao L, Rong L, Zhao Y, Li S, Ma L, Li J, Zhang C, Xiao D, Foo JL, Yu A. Simultaneous improvement of limonene production and tolerance in *Yarrowia lipolytica* through tolerance engineering and evolutionary engineering. ACS Synth Biol. 2021. http://dx.doi.org/10.1021/acssynbio.1c00052.
38. Pang Y, Zhao Y, Li S, Zhao Y, Li J, Hu Z, Zhang C, Xiao D, Yu A. Engineering the oleaginous yeast *Yarrowia lipolytica* to produce limonene from waste cooking oil. Biotechnol Biofuels. 2019;12:241.
39. Xuan JW, Fournier P, Declerck N, Chasles M, Gaillardin C. Overlapping reading frames at the LYS5 locus in the yeast *Yarrowia lipolytica*. Mol Cell Biol. 1990;10(9):4795–806.
40. Damude HG, Gillies PJ, Macool DJ, Picataggio SK, Pollak DWM, Ragghianti JJ, Xue Z. High eicosapentaenoic acid producing strains of *Yarrowia lipolytica*. US. 2013;US8518674:B2.

Tables

Table 1
List of promoters used in this study

Promoters	UAS type	TATA box	Core promoter	Strength	Reference
LEU				+	[39]
EXP				+ +	[40]
TEF				+ + +	[40]
LEUm		LEU	LEU	+	[12]
UASTEFSC4-LEUm	UASTEFSC4	LEU	LEU	+ +	This study
UASCLBSC4-LEUm	UASCLBSC4	LEU	LEU	+ +	This study
UASCITSC4-LEUm	UASCITSC4	LEU	LEU	+ +	This study
UAS1B4-EXPm	UAS1B4	EXP	EXP	+ +	This study
UAS1B4-POX2m	UAS1B4	POX2	POX2	+ + +	This study
UAS1B4-TATAPAT1-LEU	UAS1B4	PAT1	LEU	+ + +	This study
UAS1B4-PAT1m	UAS1B4	PAT1	PAT1	+ + +	This study
UASTEFYL4-LEUm	UASTEFYL4	LEU	LEU	+ + + +	This study
UAS1B4-TEF175	UAS1B4	TEF	TEF175	+ + + +	This study
UAS1B4-TEF136	UAS1B4	TEF	TEF136	+ + + +	This study
UAS1B4-TEF111	UAS1B4	TEF	TEF111	+ + + +	This study
UAS1B4-LEUm	UAS1B4	LEU	LEU	+ + + +	[23]
UAS1B4-TATAPOX2-LEU	UAS1B4	POX2	LEU	+ + + +	This study
UAS1B4-TATAEXP-LEU	UAS1B4	EXP	LEU	+ + + +	This study
UAS1B6-LEUm	UAS1B6	LEU	LEU	+ + + + +	This study
UAS1B4-TATATEF-LEU	UAS1B4	TEF	LEU	+ + + + +	This study
UAS1B8-LEUm	UAS1B8	LEU	LEU	+ + + + +	[18]

Table 2
TATA box tested in this study

TATA box	Sequence
LEU	TATATATA
TEF	TATAAAA
EXP	ATTATATATAA
PAT1	TATATACC
POX2	GTATACTTATATA

Figures

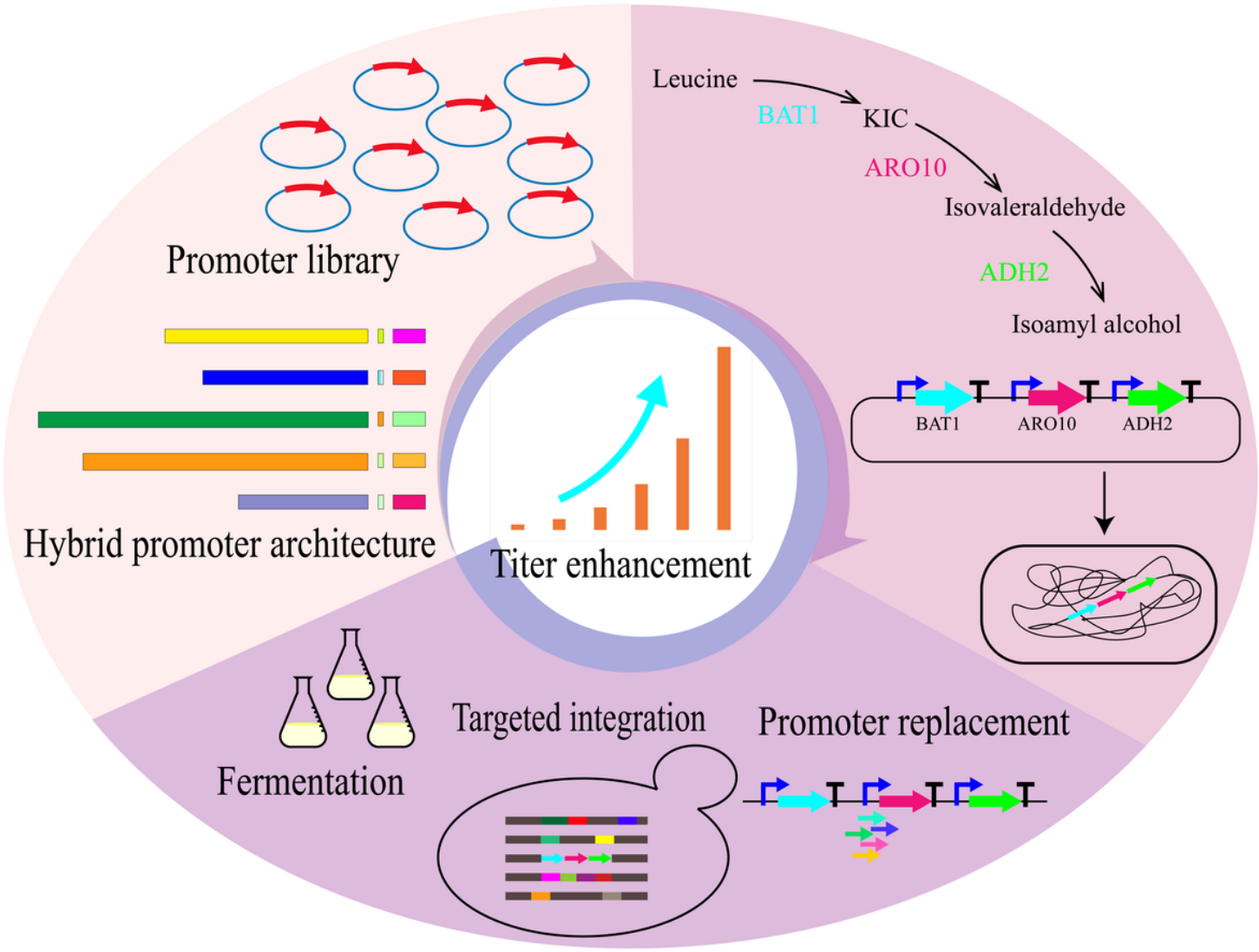

Figure 1

The strategy of promoter engineering using isoamyl alcohol production as the test study An artificial hybrid promoter library that covers a wide range of promoter strength was constructed, and applied to optimize the isoamyl alcohol synthesis pathway in Y. lipolytica.

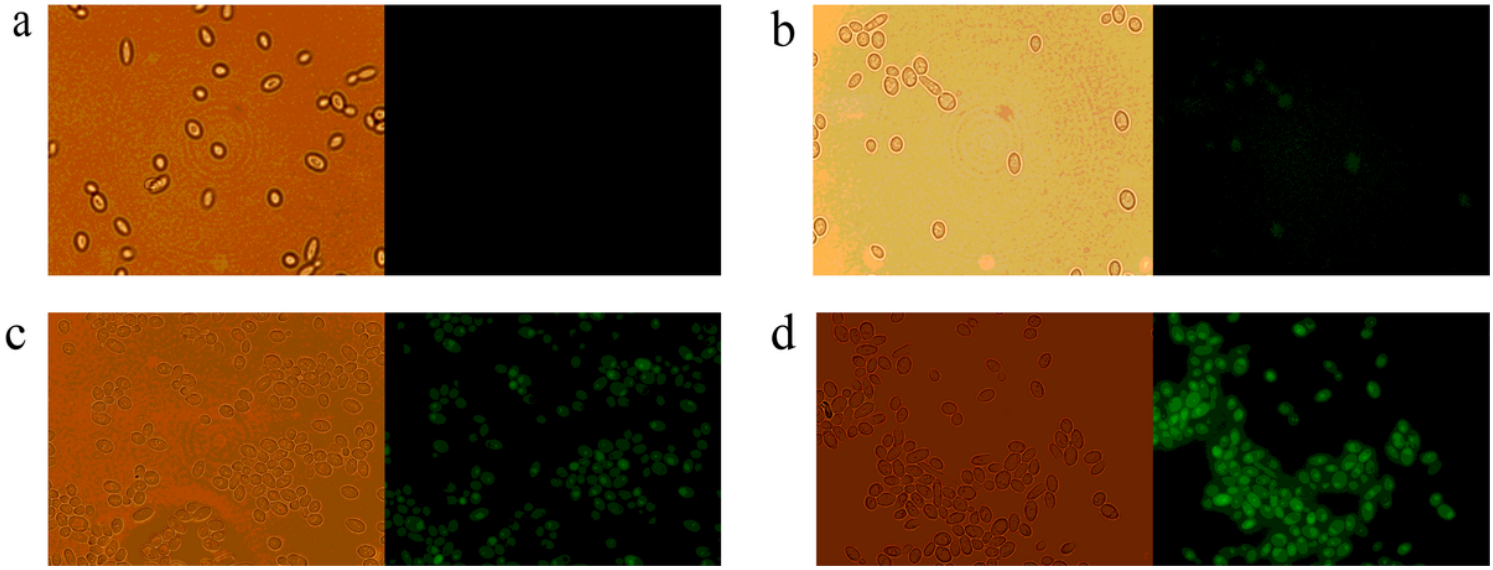

Figure 2

The screening of suitable reporter gene and the characterization of promoter library strength a. The fluorescence image of Y.lipolytica Po1g KU70Δ with the integrative plasmid pYLEX1. b. The fluorescence image of Y.lipolytica Po1g KU70Δ with the integrative plasmid pYLGFPuv. c. The fluorescence image of Y.lipolytica Po1g KU70Δ with the integrative plasmid pYLhrGFP. d. The fluorescence image of Y.lipolytica Po1g KU70Δ with the integrative plasmid pYLhrGFPO.

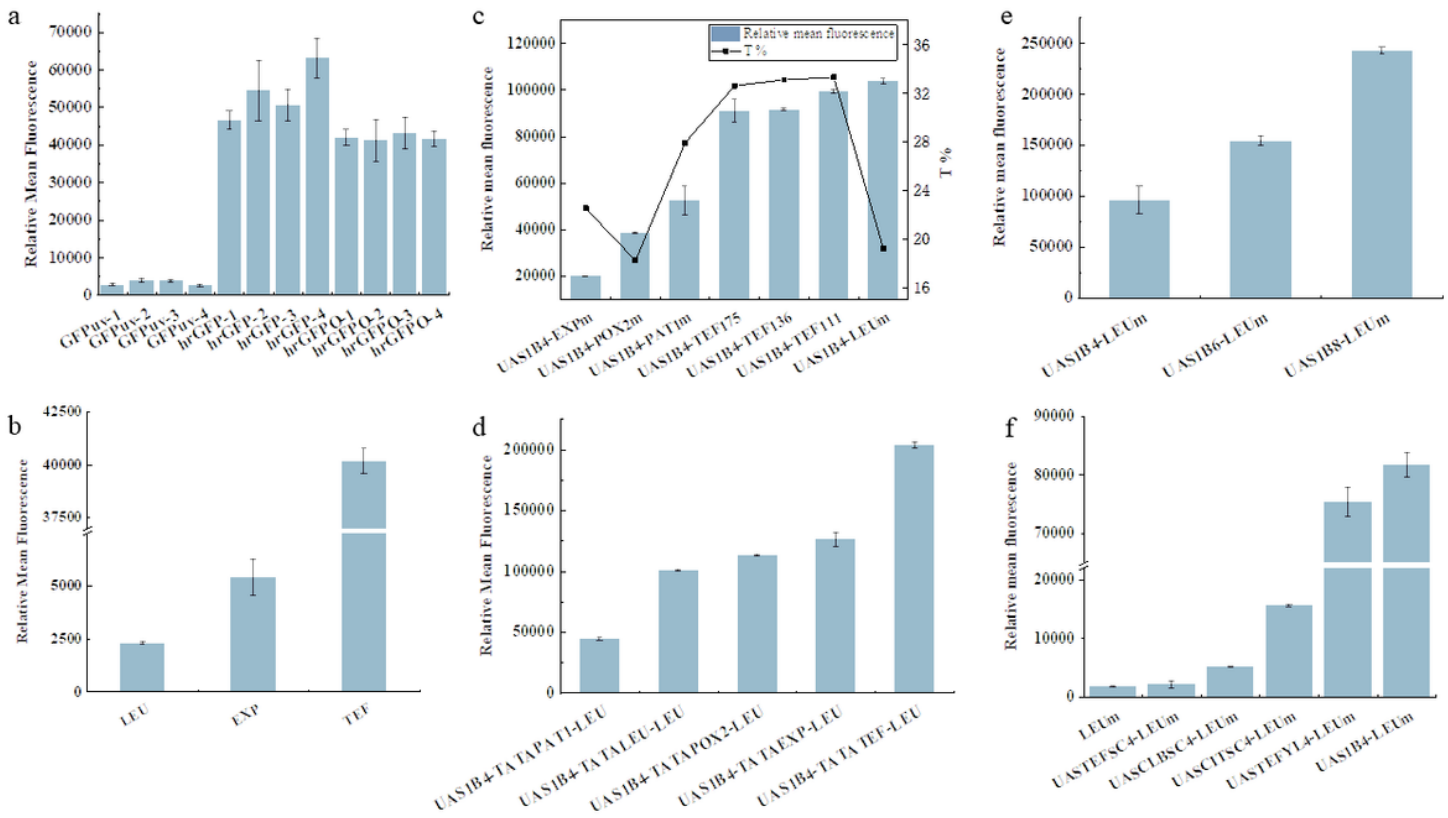

Figure 3

The fluorescence strength of the promoters a. Characterization of different green fluorescent proteins by promoter PUAS1B4-LEUm. b. Characterization of the native promoters PLEU, PEXP and PTEF. c. Characterization of different core promoters, and the relationship between T content upstream of TSS and relative mean fluorescence. Bars represent relative mean fluorescence and lines represent percentage of T content upstream of TSS. d. Characterization of different TATA boxes. e. The relationship between the copy number of UAS and relative mean fluorescence. f. Characterization of different UASs from S. cerevisiae and Y. lipolytica.

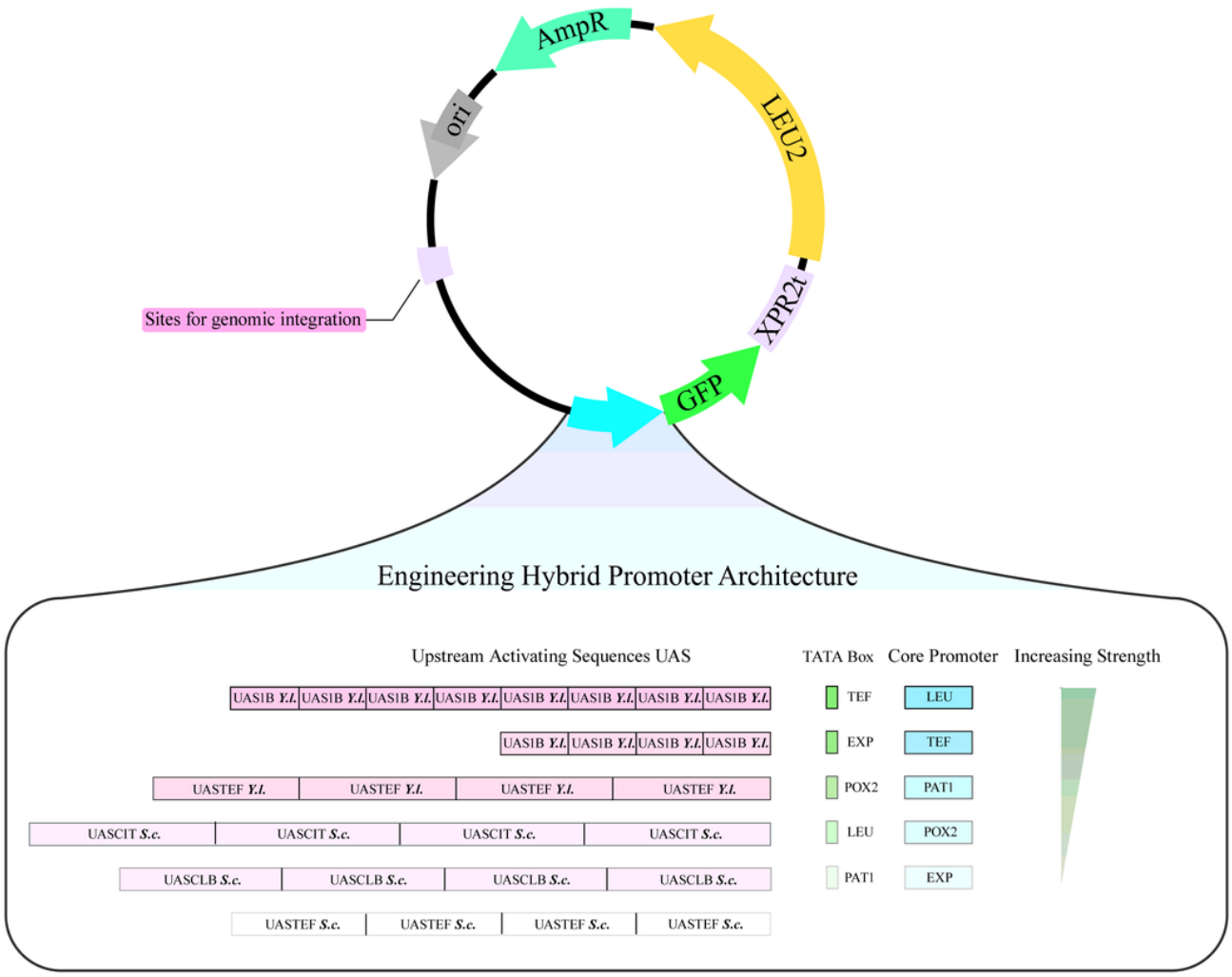

Figure 4

The strategy of engineering hybrid promoter architecture Different promoter elements (UAS, TATA box and core promoter) were tested and ligated to the upstream of the reporter gene hrGFPO to characterize promoter strength in this study.

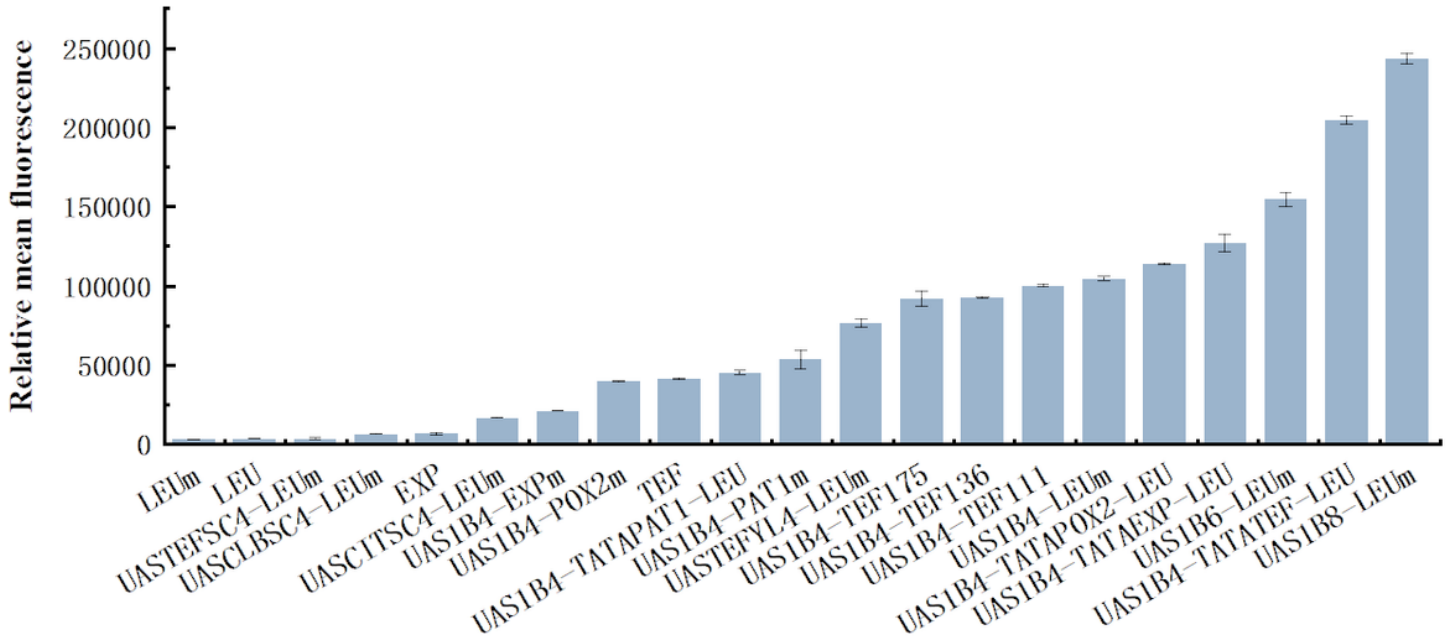

Figure 5

The fluorescence of the promoter library constructed in this study The hrGFPO was used as reporter gene for the promoter library constructed, and the fluorescence were detected by the BD Accuri C6 flow cytometer (BD Biosciences) using 488-nm excitation wavelength and FL1 channel.

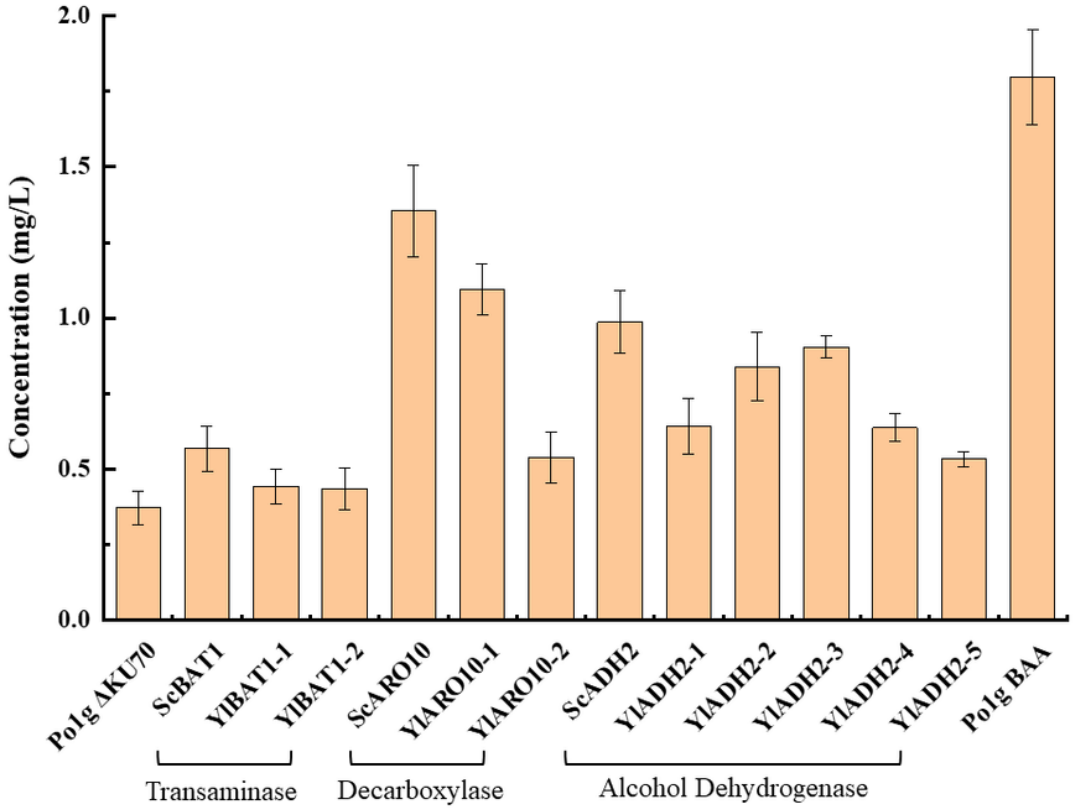

Figure 6

The production of isoamyl alcohol in engineered Y. lipolytica The cultures were grown in 40 mL YPD medium with an initial OD600 of 0.1 and 10% of n-dodecane in a 250 mL shake flask at 225 rpm and 28 °C for 3 days. The organic phase was analysed by GC/MS. ScARO10 is the key gene in the heterologous isoamyl alcohol production pathway. The isoamyl alcohol titer of Po1g ScARO10, which expressed ScARO10 under PUAS1B4-LEUm, was 1.36 mg/L, which was 2.7-fold higher than the control strain Po1g KU70Δ. The strain Po1g BAA, which co-expressed ScBAT1, ScARO10 and ScADH2 under PUAS1B4-LEUm, achieved an isoamyl alcohol titer of 1.8 mg/L, which was 3.9-fold that of control strain Po1g KU70Δ.

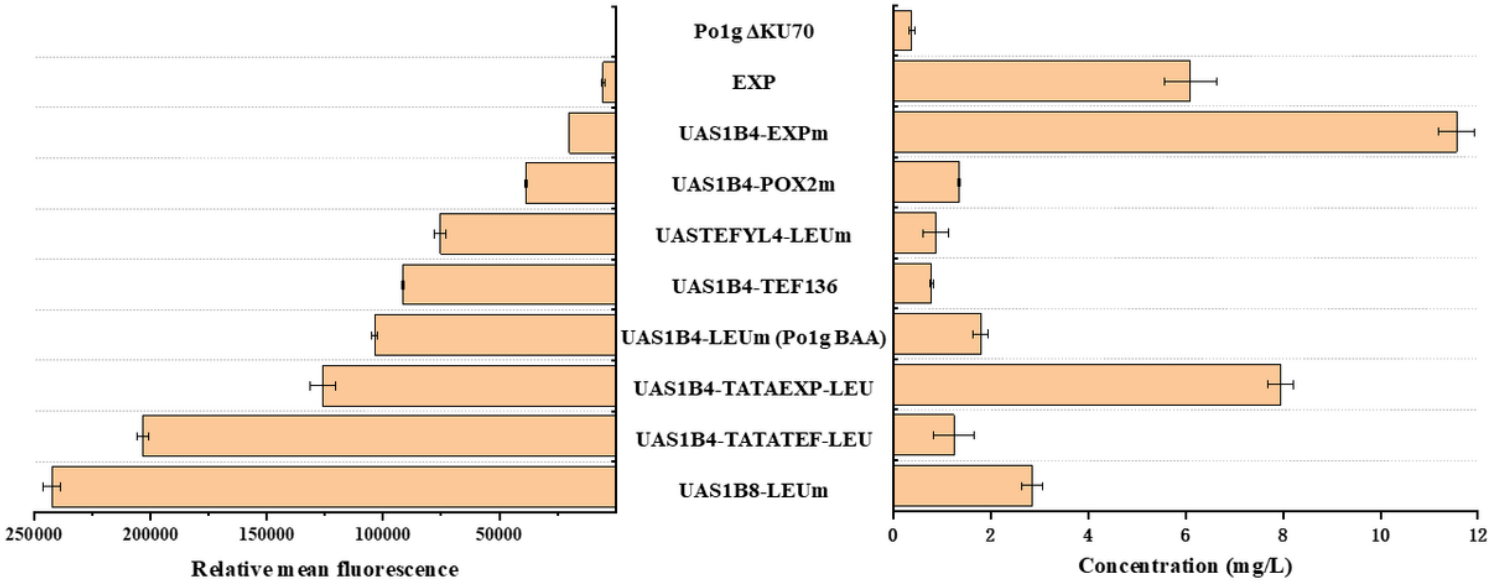

Figure 7

Comparison of partial promoter strength with isoamyl alcohol titer Several promoters were selected to replace PUAS1B4-LEUm for overexpressing ScARO10 in Po1g BAA. The strain with PUAS1B4-EXPm achieved the highest isoamyl alcohol titer of 11.57 mg/L, which was approximately 30.3-fold higher than that of Po1g KU70Δ and 5.4-fold that of Po1g BAA.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalfileforBforB.docx